

# Host-derived oxidized phospholipids and HDL regulate innate immunity in human leprosy

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**Intracellular pathogens survive by evading the host immune system and accessing host metabolic pathways to obtain nutrients for their growth. *Mycobacterium leprae*, the causative agent of leprosy, is thought to be the mycobacterium most dependent on host metabolic pathways, including host-derived lipids. Although fatty acids and phospholipids accumulate in the lesions of individuals with the lepromatous (also known as disseminated) form of human leprosy (L-lep), the origin and significance of these lipids remains unclear. Here we show that in human L-lep lesions, there was preferential expression of host lipid metabolism genes, including a group of phospholipases, and that these genes were virtually absent from the mycobacterial genome. Host-derived oxidized phospholipids were detected in macrophages within L-lep lesions, and 1 specific oxidized phospholipid, 1-palmitoyl-2-(5,6-epoxyisopropane E<sub>2</sub>)-sn-glycero-3-phosphorylcholine (PEIPC), accumulated in macrophages infected with live mycobacteria. Mycobacterial infection and host-derived oxidized phospholipids both inhibited innate immune responses, and this inhibition was reversed by the addition of normal HDL, a scavenger of oxidized phospholipids, but not by HDL from patients with L-lep. The accumulation of host-derived oxidized phospholipids in L-lep lesions is strikingly similar to observations in atherosclerosis, which suggests that the link between host lipid metabolism and innate immunity contributes to the pathogenesis of both microbial infection and metabolic disease.**

## Introduction

Intracellular pathogens survive by evading the host immune response while at the same time accessing host metabolic pathways, which for mycobacteria depends on the use of host-derived lipids. For example, mycobacteria are critically dependent on isocitrate lyase genes as part of the glyoxylate shunt to metabolize fatty acids for generation of ATP during *in vivo* growth (1). Furthermore, metabolism of host-derived fatty acids is required for the synthesis of mycobacterial lipids including virulence factors such as phthiocerol dimycocerosate, sulfolipid-1, and polyketide synthase-derived phenolic glycolipid (2, 3). Therefore, host lipids are used both for growth and virulence.

Of all mycobacteria, *Mycobacterium leprae* is likely the most dependent on the host for basic metabolic functions, in part because of its extensive genomic decay (4). *M. leprae* is the causative agent of leprosy, a disease that manifests as a clinical spectrum correlating with the host innate and adaptive immune response (5). At one end of the spectrum, in tuberculoid leprosy (T-lep), the infection is contained and self limited; there are characteristically few lesions, bacilli can rarely be identified, and cell-mediated immunity to the

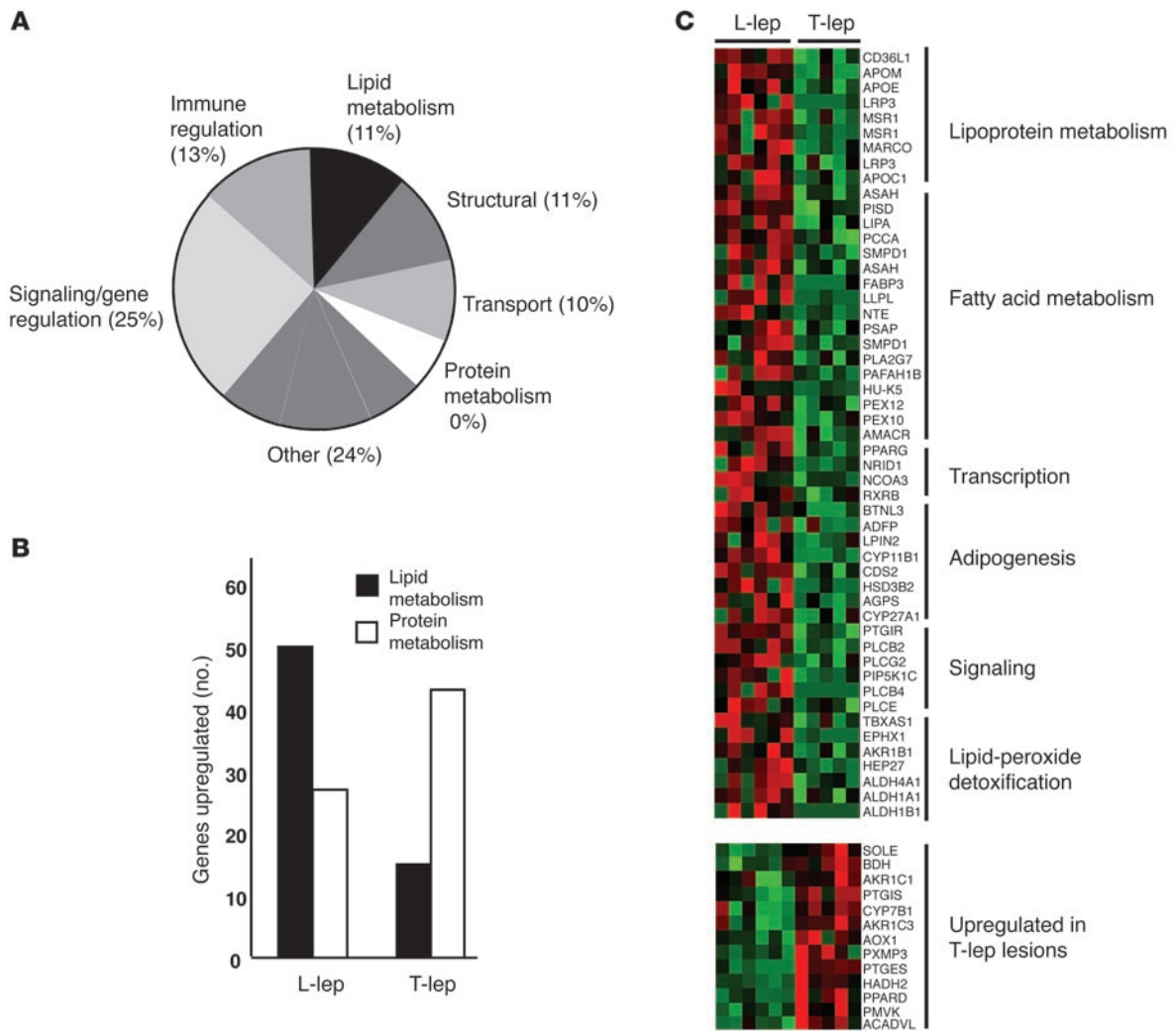
pathogen — as measured by skin reaction to *M. leprae* — is robust. In contrast, in lepromatous leprosy (L-lep), the infection is progressive and disseminates, intracellular bacteria are abundant, and cell-mediated immunity to the pathogen is weak or absent. In terms of the innate immune response, a key correlate is the DC, abundant in T-lep lesions compared with L-lep lesions and expressing the antigen presentation molecules CD1a, CD1b, and CD1c, which present mycobacterial lipids to T cells (6, 7). Furthermore, there is greater expression in T-lep lesions of TLR2/1 (8), pattern recognition receptors that mediate the response to mycobacterial lipoproteins and lead to production of IL-12 and IL-10, key cytokines that instruct the adaptive T cell response. The link between the innate and adaptive response is further apparent in the increased frequency of *M. leprae*-reactive MHC class II- and CD1-restricted T cells — both producing Th1 cytokines — in T-lep compared with L-lep patients (9–11).

One of the classic hallmarks of leprosy, originally described by Virchow in 1863, is the accumulation of lepra cells (also referred to as Virchow cells) in L-lep lesions (12). Histochemical analysis of the lipids in human leprosy revealed the accumulation of both fatty acids and phospholipids in L-lep lesions, but not T-lep lesions; the origin and significance of these lipids is unclear, although they are generally presumed to derive from the bacteria (13). Here, we investigated the nature of these lipids as well as the relationship between host lipid metabolism and innate immunity as it pertains to disease pathogenesis. Our results demonstrate that L-lep lesions were characterized by a host lipid metabolism gene program and the accumulation of host-derived oxidized phospholipids in macrophages. The ability of mycobacterial infection to inhibit innate immune responses was reversed by normal HDL, but not by HDL

**Nonstandard abbreviations used:** AMACR,  $\alpha$ -methylacyl-CoA racemase; BCG, bacillus Calmette–Guérin; CYP27b1, 1-hydroxylase; ESI-MS, electrospray ionization–mass spectrometry; L-lep, lepromatous leprosy; LysoPC, 1-palmitoyl-2-hydroxyl-*sn*-glycero-3-phosphorylcholine; ox-, oxidized; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; PEIPC, 1-palmitoyl-2-(5,6-epoxyisopropane E<sub>2</sub>)-*sn*-glycero-3-phosphorylcholine; POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine; T-lep, tuberculoid leprosy.

**Conflict of interest:** M. Navab and A.M. Fogelman are principals in Bruin Pharma, and A.M. Fogelman is an officer in Bruin Pharma. The remaining authors have declared that no conflict of interest exists.

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**Figure 1** Differential expression of host lipid metabolism genes in polar forms of leprosy. (A) Genes preferentially expressed in L-lep lesions were categorized according to metabolic function. (B) L-lep lesions preferentially expressed a greater number of lipid metabolism genes, but fewer genes involved in protein metabolism, compared with T-lep lesions. (C) Lipid metabolism genes preferentially expressed in L-lep lesions ( $n = 6$  lesions) and T-lep lesions ( $n = 5$  lesions) were subcategorized according to function and listed by ascending  $P$  value. All genes had at least 1.5-fold relative expression and  $P < 0.05$ .

from L-lep patients. These data indicate a link between host lipid metabolism and immune function in human infectious disease.

**Results**

*Host lipid metabolism gene profiles in leprosy lesions.* We have previously established that host gene expression profiles of leprosy lesions accurately correlate with the 2 polar forms of the disease, with statistical verification by both unsupervised and supervised analysis (14). Examination of the gene expression profiles revealed a number of differentially expressed gene families correlating with the type of immune response of the different patient groups. To determine the role of host lipid metabolism genes in leprosy, we examined the differential host gene expression profiles across the spectrum of disease according to major biologic systems. Genes that met differential expression criteria between groups ( $P < 0.05$ ; fold change  $> 1.5$ ) were organized according to biologic processes and pathways.

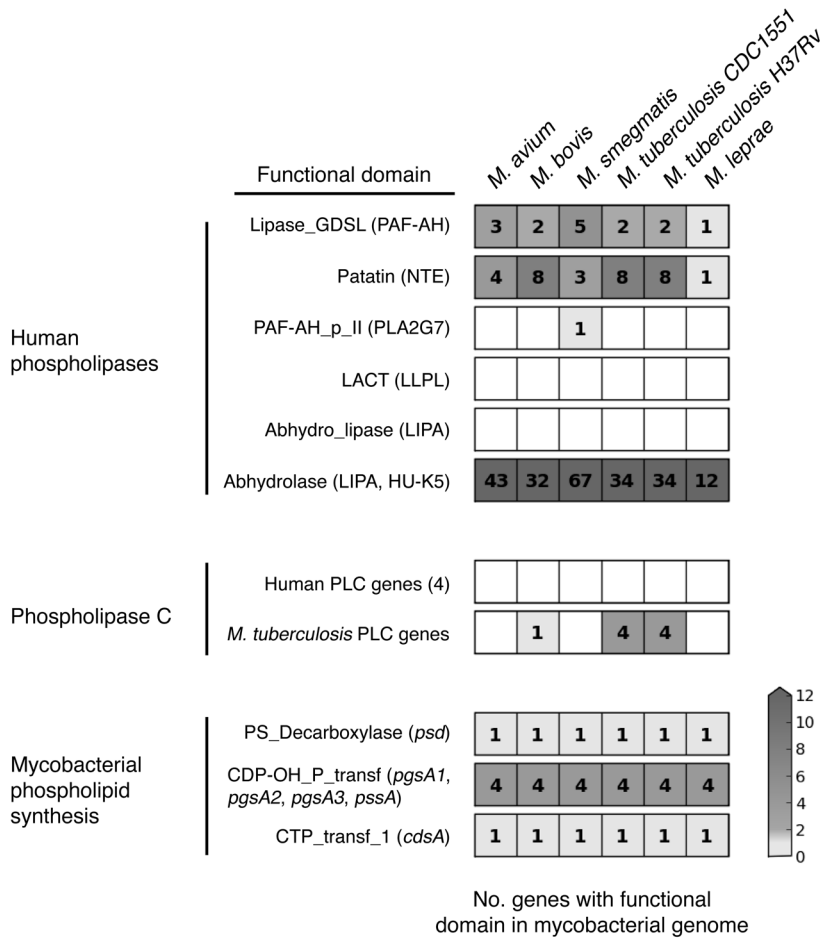
Although interindividual variation was noted, perhaps in part as a result of heterogeneity of disease lesions and genetic background, a striking pattern emerged. In L-lep lesions, host lipid metabolism genes accounted for 11% of the differentially upregulated genes, making it the third largest category of genes more highly expressed in L-lep than in T-lep lesions (Figure 1A). The categories that contained greater percentages of upregulated genes were signaling/gene regulation members and immune response genes, at 25% and 13%, respectively. In comparing the absolute number of genes upregulated in L-lep versus T-lep lesions, the number of host lipid metabolism genes was approximately 4-fold greater in L-lep lesions than in T-lep lesions (Figure 1B). This was not the case for all metabolism genes; in fact, the number of protein metabolism genes upregulated in L-lep lesions was approximately half that of T-lep lesions.

The host lipid metabolism genes upregulated in L-lep lesions were further divided into functional subcategories according to



**Figure 2**

Comparative domain profiling between leprosy lesions and mycobacterial genomes. Top: Number of mycobacterial genes containing functional lipase domains similar to those of the human genes upregulated in L-lep lesion transcriptome. The PFAM domain containing the lipase activity is shown in parentheses. Middle: Phospholipase C genes were absent in *M. leprae*, but induced in the L-lep transcriptome. Bottom: Number of mycobacterial genes containing selected domains involved in phospholipid biosynthesis. Gene names are listed in parentheses for each domain.



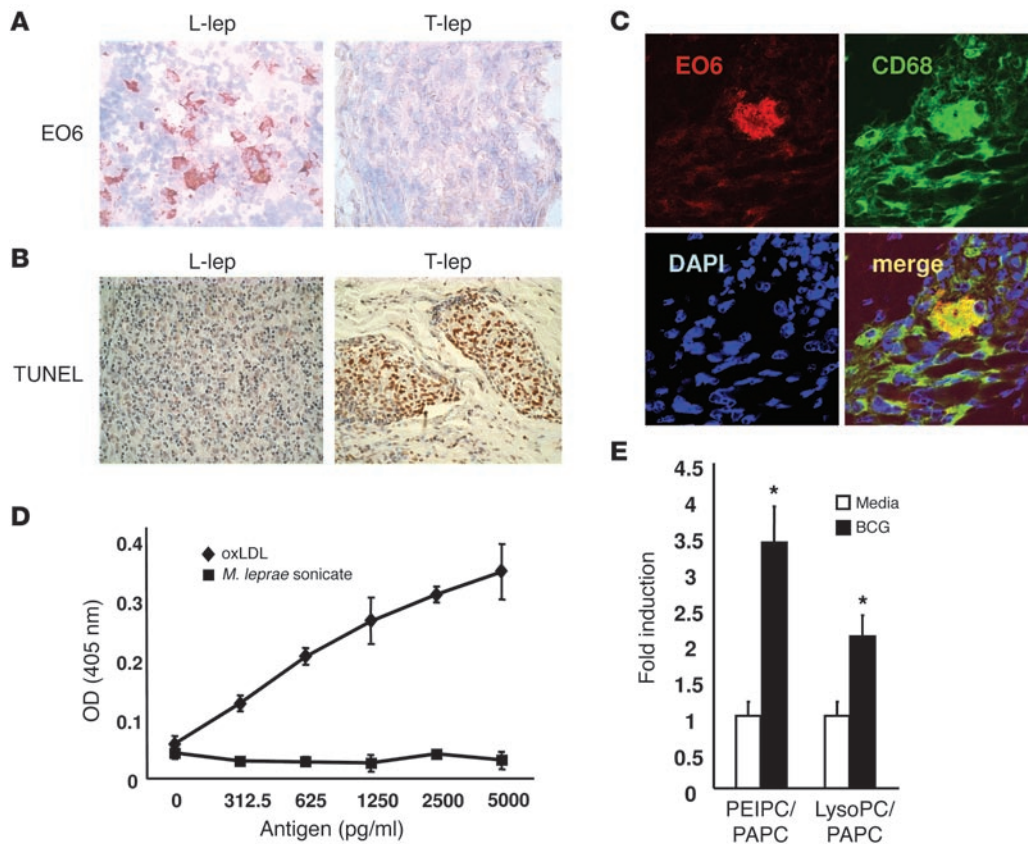
metabolic function (Figure 1C). In L-lep lesions, the most frequent groups of upregulated host lipid metabolism genes were those involved in lipoprotein metabolism and fatty acid metabolism, including lipases involved in lipid catabolism. These categories accounted for more than one-third of the upregulated lipid metabolism genes. Several genes associated with the recognition, metabolism, and degradation of oxidized phospholipids were among the most highly induced; these included scavenger receptors that promote uptake of oxidatively modified lipoproteins (e.g., MSR, MARCO, and CD36L receptor), enzymes important for degrading oxidized fatty acid moieties on phospholipid oxidation products (e.g., epoxide hydrolase and aldehyde dehydrogenase), and phospholipases that hydrolyze phospholipids (e.g., platelet activating factor acetyl hydrolase, neuropathy target esterase, and 4 phospholipase C genes). Together, 9 different host lipases with the capacity to metabolize phospholipids were upregulated in L-lep lesions. There were 13 host lipid metabolism genes upregulated in T-lep lesions (Figure 1C), with no more than 2 belonging to a particular subclass.

*Comparative analysis of the mycobacterial genome and host transcriptome.* The induction of host phospholipases in L-lep lesions led us to hypothesize that host metabolic pathways were being recruited to complement deficiencies in the *M. leprae* genome. The *M. leprae* genome has a paucity of lipase genes, with only 1 potential gene, *lipE*, whose product can act as a scavenging lipase to help break down host lipids (4). In contrast, more than 25 potential lipases are

annotated in the *Mycobacterium tuberculosis* genome (15). We used comparative functional domain profiling to analyze differences in lipid metabolism genes between the mycobacterial genome and the local host inflammatory response to the pathogen.

For each human phospholipase upregulated in L-lep lesions, we determined whether the presumed lipolytic domain (as defined in the PFAM database; ref. 16) was encoded in any mycobacterial proteins (Figure 2, top). For some domains, no homolog was found in mycobacteria. For other lipolytic domains, we could not be certain of a lipid substrate, as was the case for the AB\_hydrolase\_1 domain (PF00561), which occurred frequently in all mycobacterial genomes. However, for 2 of the lipase domains, Lipase\_GDSL (PF00657) and Patatin (PF01734), we found potential phospholipases in *M. leprae* and other mycobacteria. Notably, while there were 10 such potential phospholipases in *M. tuberculosis*, the *M. leprae* genome contained only 2 proteins with these phospholipase domains, consistent with the pronounced genome decay documented for this organism. Phospholipase C genes and sphingomyelinases, also upregulated in the L-lep lesions, were conspicuously absent in the *M. leprae* genome (Figure 2, middle). Four genes capable of phospholipase C and sphingomyelinase activity are present in the *M. tuberculosis* genome, and mutant strains show impaired virulence (17). These data indicate that the host inflammatory response to *M. leprae* includes a phospholipase gene program, perhaps to complement the absence of these genes in the pathogen.





**Figure 3**

Oxidized phospholipids accumulate in L-lep lesions and during mycobacterial infection. (A) Human leprosy lesions were labeled with the monoclonal antibody EO6. Shown are representative sections from L-lep ( $n = 5$ ) and T-lep ( $n = 4$ ) lesions. Original magnification,  $\times 40$ . (B) TUNEL in leprosy lesions ( $n = 4$  per group). Original magnification,  $\times 20$ . (C) Immunofluorescence 3-color confocal microscopy of L-lep lesions. Red stain, EO6; green stain, CD68; blue stain, DAPI. Original magnification,  $\times 63$ . (D) oxLDL, but not *M. leprae* sonicate, reacted with EO6, as determined by ELISA. Data (mean  $\pm$  SEM) are representative of 4 independent experiments. (E) Primary human macrophages accumulated PEIPC during BCG infection. Phospholipids were analyzed by ESI-MS, and relative abundance was determined by comparison with levels of native PAPC. Data are mean  $\pm$  SEM of 3 independent experiments. \* $P < 0.001$  versus media.

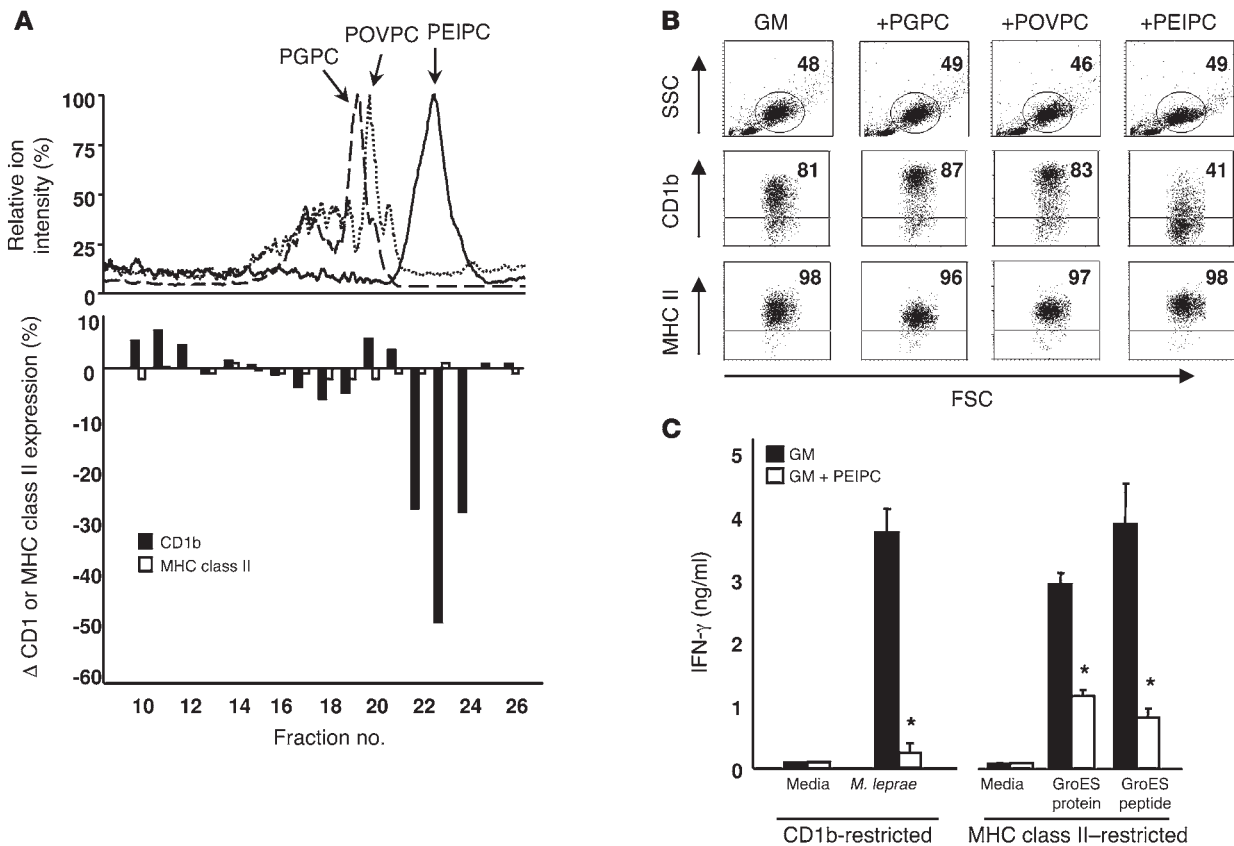
In addition to phospholipases, there were additional host lipid metabolism genes expressed in leprosy lesions that were also absent in the mycobacterial genome. For example,  $\alpha$ -methylacyl-CoA racemase (AMACR) is critical for  $\beta$ -oxidation of branched fatty acids in both mycobacteria and humans, and there is remarkable 43% identity between human and mycobacterial AMACR (18). Yet, while *M. tuberculosis* was found to harbor 3 functional AMACR homologs, *M. leprae* had 3 pseudogenes lacking the catalytic site. As is the case with host phospholipases, there was induction of the host AMACR gene in multibacillary L-lep lesions.

In contrast to the loss of phospholipases, the *M. leprae* genome encoded for enzymes that synthesize the major mycobacterial phospholipids (Figure 2, bottom). Conversion of phosphatidic acid to CDP-diglyceride occurred via a conserved CDP-diglyceride synthetase (*cdsA*; PF01148). The CDP-alcohol phosphatidyltransferase domain (PF01066) was conserved in 4 genes in all mycobacteria and provided for synthesis of phosphatidylinositol (*pgsA1*), cardiolipin (*pgsA2*), phosphatidylglycerol (*pgsA3*), and phosphatidylserine (*ppsA*). All mycobacteria, including *M. leprae*, also contained a conserved gene (*psd*) adjacent to *ppsA* that catalyzed the synthesis of phosphatidylethanolamine from phosphatidylserine. This comparative func-

tional domain analysis revealed that the *M. leprae* genome encodes few enzymes that catabolize phospholipids, but that host phospholipases were represented in the host inflammatory response.

*Accumulation of oxidized phospholipids in leprosy.* Given the upregulation of genes in L-lep lesions related to phospholipid and fatty acid metabolism, as well as the accumulation of fatty acids and phospholipids in L-lep lesions, we hypothesized that the lipid-laden macrophages contain host oxidized phospholipids, accumulated during the course of *M. leprae* infection. Oxidized phospholipids can be localized in tissue sections using the monoclonal antibody EO6, which recognizes oxidized phosphatidylcholines, such as those found in oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (oxPAPC), but not unoxidized phospholipids. More specifically, 2 oxidation products of PAPC, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphorylcholine (POVPC) and 1-palmitoyl-2-(5,6-epoxyisoprostane  $E_2$ )-*sn*-glycero-3-phosphorylcholine (PEIPC) have been shown to react with EO6 (19). EO6 has therefore proven useful in identifying such lipids within foam cells in atherosclerosis lesions (20).

In L-lep lesions, labeling with EO6 revealed large ovoid cells with cytoplasmic staining, similar to those seen in atherosclerosis



**Figure 4**

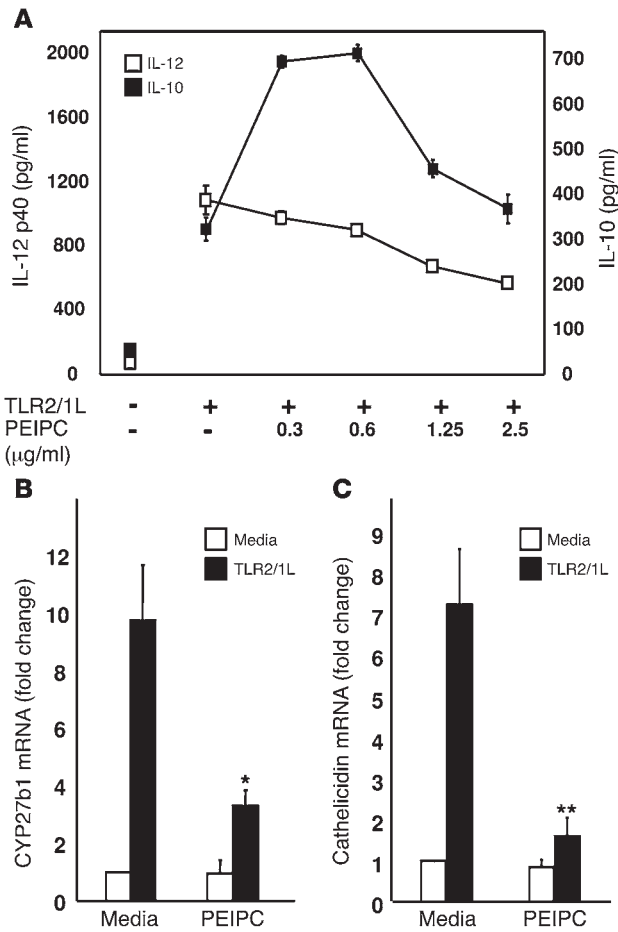
Oxidized phospholipids alter DC differentiation and function. (A) oxPAPC was separated by HPLC, and 1-minute fractions were collected and individually tested for their effects on CD1b and MHC class II expression on differentiating DCs. Chromatograms demonstrating which fractions contained PGPC, POVPC, and PEIPC have been superimposed with biological activity. (B) Representative fractions enriched in PEIPC (fraction 23), POVPC (fraction 20), and PGPC (fraction 19) differentially affected CD1b expression on differentiating DCs. GM, GM-CSF; FSC, forward scatter; SSC, side scatter. Numbers within plots denote percent cells within gate or positive for CD1b. (C) PEIPC altered DC function. After 48 h differentiation in the presence or absence of 500 ng/ml PEIPC, cells were washed, irradiated, pulsed with antigen, and cocultured with T cell lines. CD1b- or MHC class II–restricted T cell activation was assessed by IFN- $\gamma$  production. Data (mean  $\pm$  SEM of triplicate wells) are representative of 3 experiments for CD1b-restricted T cells, 3 experiments for MHC class II–restricted T cells with GroES protein, and 2 experiments using GroES peptide. \* $P < 0.03$  versus GM alone.

lesions (Figure 3A). Surprisingly, EO6 labeling in T-lep lesions was weak or absent. Because EO6 also reacts with apoptotic cells in vitro, we assessed whether the pattern of reactivity could be due to cellular apoptosis. However, consistent with prior work (21), we found that there was increased apoptosis in T-lep lesions rather than in L-lep lesions (Figure 3B); the percentage of apoptotic cells in T-lep and L-lep lesions was  $57.5\% \pm 6.8\%$  and  $5.9\% \pm 1.4\%$ , respectively ( $P = 0.007$ ;  $n = 4$  per group). Confocal microscopy demonstrated that the EO6-reactive ovoid cells were CD68<sup>+</sup>, confirming that these were macrophage-derived foam cells (Figure 3C).

In addition to oxPAPC, EO6 also reacts with *Streptococcus pneumoniae* and other bacteria with cell walls containing phosphorylcholine moieties (22, 23). Although mycobacterial species are not known to contain phosphorylcholine, we used an ELISA to determine whether EO6 antibody reacted with *M. leprae*. The results clearly showed that EO6 recognized oxLDL but not *M. leprae* (Figure 3D). L-lep lesions also preferentially labeled with the monoclonal antibody NA59, which recognizes proteins modified by the lipid oxidation product, 4-hydroxynonenal (ref. 24 and data not shown). These data indicate that L-lep lesions,

but not T-lep lesions, accumulate oxidized host phospholipids and oxidatively modified proteins. The presence of these compounds in both atherosclerotic lesions and disseminated leprosy suggests that lipid oxidation may contribute to the pathogenesis of both diseases.

We have previously described bioactive oxidation products of PAPC, including POVPC and PEIPC, that have distinct biological activities (25, 26). Because the EO6-reactive phospholipids accumulate in L-lep lesions, we sought to determine whether mycobacterial infection of macrophages leads to the intracellular production of host-derived oxidized phospholipids. Because *M. leprae* is not viable in human macrophages in vitro, alternate mycobacterial species are often used in its place for in vitro studies. An attractive surrogate for these experiments was *Mycobacterium bovis* bacillus Calmette–Guérin (BCG), because it is viable in human macrophages, has previously been shown to induce lipid body formation in human macrophages (27), and can be extensively studied with low biosafety precautions. To determine the relative abundance of oxidized phospholipids in the setting of a mycobacterial infection, we decided to use primary human macrophages, because they are



**Figure 5**

Oxidized phospholipids alter TLR2/1 activation. **(A)** Monocytes activated with 10 µg/ml TLR2/1 ligand (TLR2/1L) 19 kDa in the presence of PEIPC produced less IL-12 p40, but more IL-10, than those activated with TLR2/1 ligand alone, as determined by ELISA performed in triplicate wells. Data (mean ± SEM) are representative of 2 experiments. **(B)** Effect of PEIPC on TLR2/1-mediated induction of CYP27b1 mRNA. Monocytes were activated by TLR2/1 ligand in 5% FCS in the presence or absence of PEIPC for 24 h, and CYP27b1 mRNA levels were determined by quantitative PCR. Data are mean ± SEM of 4 separate experiments. \**P* < 0.03 versus media. **(C)** Effect of PEIPC on TLR2/1-mediated induction of cathelicidin mRNA. Monocytes were activated by TLR2/1 ligand in 10% human serum (vitamin D sufficient) in the presence or absence of oxPAPC for 24 h, and cathelicidin mRNA levels were determined by quantitative PCR. Data are mean ± SEM of 3 separate experiments. \*\**P* = 0.02 versus media.

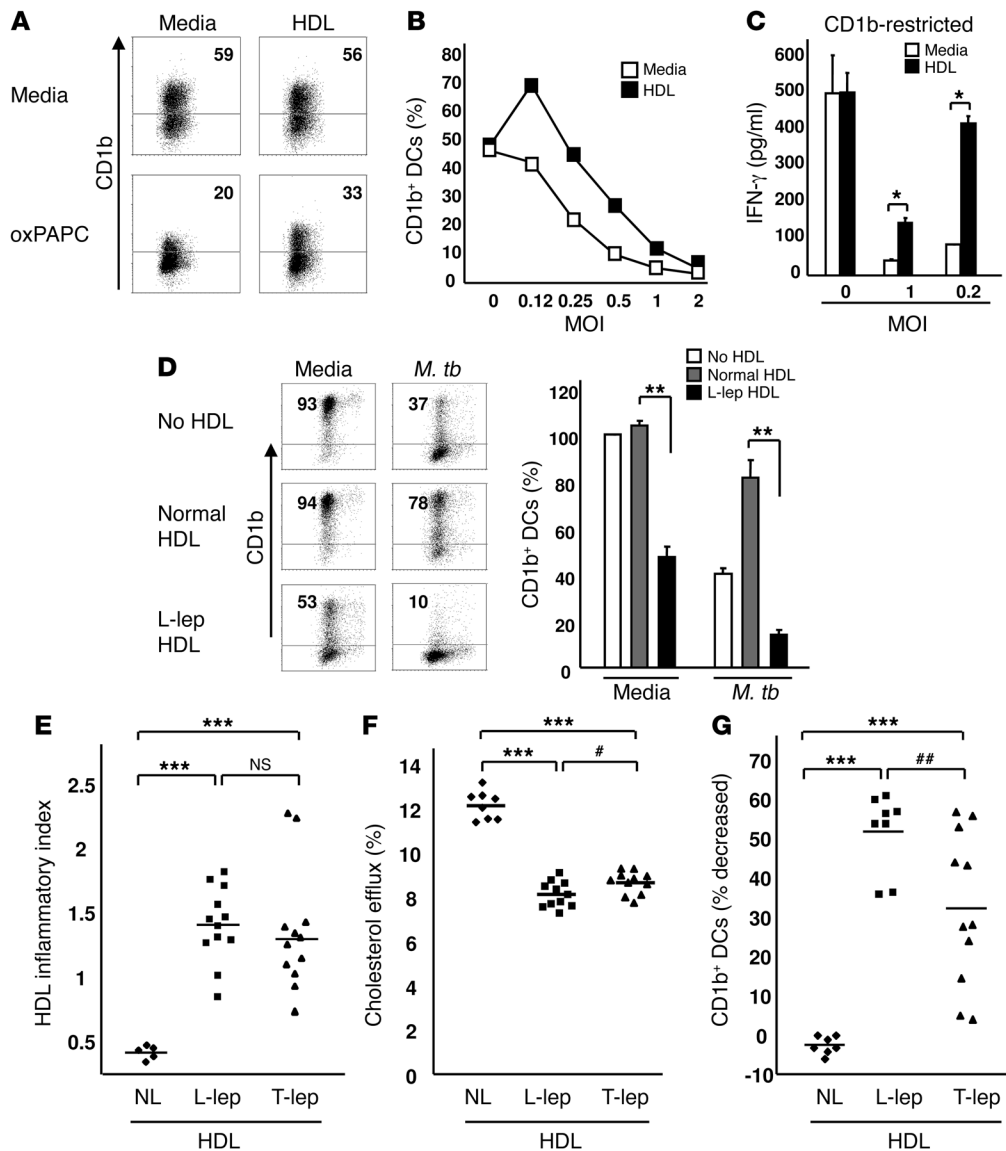
known to most frequently harbor the pathogen in the lesion. After infecting primary human macrophages with BCG, phospholipids were isolated from a total lipid extraction and analyzed by multiple reaction monitoring electrospray ionization-mass spectrometry (ESI-MS). The phosphocholine product ion (*m/z* 184.1) derived from collision-induced dissociation of PEIPC (*m/z* 828.6), 1-palmitoyl-2-hydroxyl-*sn*-glycero-3-phosphorylcholine (LysoPC; *m/z* 496.3), and PAPC (*m/z* 782.5) at retention times identical to authentic standards was used for relative quantitation (see Methods). Mycobacterial infection of macrophages resulted in an increase of PAPC-derived oxidized phospholipids, specifically an approximately 3.5-fold increase of PEIPC, compared with uninfected macrophages (Figure 3E). In addition, there was a concomitant increase in LysoPC, the breakdown product of oxidized phosphorylcholine. Relatively minor changes of other PAPC oxidation products were observed (data not shown). The presence of EO6-reactive oxidized phospholipids in leprosy lesions together with the increased abundance of PEIPC in macrophages infected with mycobacteria suggest that host-derived EO6-reactive oxidized phospholipids are induced and accumulate at the site of mycobacterial infection.

*Oxidized phospholipids induced by mycobacterial infection alter DC CD1 expression and antigen presentation.* Given the rapid accumulation of intracellular oxidized phospholipids by mycobacterial infection and the known immunomodulatory effects of oxPAPC (28, 29), we hypothesized that the induction of these host lipids alters host innate immune responses to the pathogen. A key cell of the innate

immune system is the DC, whose differentiation can be followed by the expression of CD1 molecules, which are themselves antigen-presenting elements for lipid antigens to T cells. Previously, independent groups have shown that oxLDL (30) and mycobacterial infection (31, 32) cause suppression of CD1 molecules on DCs. Furthermore, oxPAPC has been demonstrated to prevent the maturation of immature DCs (29). Because PEIPC is an important early product of LDL oxidation and was found to be increased upon mycobacterial infection of macrophages, we sought to determine whether PEIPC affects CD1 expression on developing DCs.

Components of oxPAPC were separated by HPLC (Figure 4A), and each fraction was tested for its ability to prevent the upregulation of CD1b on monocytes treated with GM-CSF for 48 h. PEIPC eluted from the column in fractions 22–24, the same fractions that most potently decreased CD1b expression (up to 50%). Fractions containing other major components of oxPAPC had no inhibitory effect (Figure 4, A and B). In a separate preparation analyzing effects on CD1 expression, ESI-MS analysis revealed that only fractions enriched in PEIPC (*m/z* 828), but not the dehydration product (*m/z* 810), maintained inhibitory activity (Supplemental Figure 1, A–C; supplemental material available online with this article; doi:10.1172/JCI34189DS1). Moreover, PEIPC isolated from either infected macrophages or oxPAPC had chromatographic and mass spectrometric properties identical to those described above. Purified PEIPC, but not POVPC, recapitulated the inhibitory effects of oxPAPC on CD1 expression (Supplemental Figure 1D). Therefore, as with mycobacterial infection (31, 32), PEIPC inhibited expression of CD1 molecules on differentiating DCs. In contrast, fractions enriched in PEIPC did not inhibit expression of MHC class II on monocyte-derived DCs (Figure 4B). Importantly, the dysregulation of DC differentiation by PEIPC was functionally significant, because the activation of *M. leprae*-reactive CD1b-restricted and MHC class II-restricted T cells was inhibited, as measured by production of IFN-γ (Figure 4C). For MHC class II-restricted T cells, PEIPC inhibited antigen presentation of both protein and peptide antigens without altering MHC class II expression. The mechanism by which PEIPC inhibits MHC class II presentation to T cells requires further investigation. Taken together, these data show that PEIPC alters DC differentiation, resulting in reduced antigen presentation to T cells.

*Oxidized phospholipids induced by mycobacterial infection inhibit TLR2/1 activation.* A key aspect of the innate immune system is the ability of TLRs to recognize and respond to microbial ligands (33). The recognition of mycobacterial triacylated lipoproteins by

**Figure 6**

HDL preserves DC differentiation and function during mycobacterial infection. (A) Effect of HDL on oxPAPC-mediated inhibition of CD1b<sup>+</sup> DC differentiation. Data are representative of 2 experiments. Numbers within plots denote percent CD1b<sup>+</sup> cells. (B) Effect of HDL on mycobacteria-mediated inhibition of CD1b<sup>+</sup> DC differentiation. Monocytes were infected with BCG alone or with 150  $\mu$ g/ml HDL. Data are representative of more than 6 experiments. (C) Effect of HDL on CD1b-restricted mycobacterial antigen presentation by *M. tuberculosis* H37Ra-infected DCs. T cell activation was assessed by IFN- $\gamma$  release. Data (mean  $\pm$  SEM of triplicate wells) are representative of 3 experiments. \* $P < 0.001$ . (D) Effect of HDL from L-lep patients on CD1b<sup>+</sup> DC differentiation. Left: Representative cell surface staining comparing normal and L-lep HDL (50  $\mu$ g/ml) with and without *M. tuberculosis* H37Ra (*M. tb*) infection (MOI, 0.1). Numbers within plots denote percent CD1b<sup>+</sup> cells. Right: Percent CD1b<sup>+</sup> DCs relative to uninfected. Data represent mean  $\pm$  SEM of 3 experiments using HDL from normal (NL;  $n = 3$ ) and L-lep ( $n = 5$ ) donors. \*\* $P \leq 0.02$ . (E) Functional assessment of HDL from leprosy patients. Monocyte chemotactic activity in normal (NL;  $n = 5$ ) and leprosy HDL (L-lep and T-lep,  $n = 12$  per group). \*\*\* $P = 0.001$ . L-lep versus T-lep HDL was not significant ( $P = 0.23$ ). (F) Reverse cholesterol transport with normal and leprosy HDL. Cholesterol efflux with normal ( $n = 8$ ), L-lep ( $n = 11$ ), and T-lep ( $n = 11$ ) HDL (25  $\mu$ g/ml). \*\*\* $P = 0.001$ ; # $P = 0.049$ . (G) Effect of normal and leprosy HDL on CD1b<sup>+</sup> DC differentiation. Normal ( $n = 7$ ), L-lep ( $n = 8$ ), and T-lep ( $n = 11$ ) HDL (50  $\mu$ g/ml) was added to differentiating monocytes, and CD1b<sup>+</sup> DCs were quantified by flow cytometry. \*\*\* $P = 0.001$ ; ## $P = 0.02$ .

TLR2/1 leads to cytokine production and upregulation of antimicrobial pathways (34, 35). We determined whether oxidized phospholipids affect TLR2/1-induced production of the proinflammatory cytokine IL-12 and the antiinflammatory cytokine IL-10. We have previously demonstrated that oxPAPC inhibits TLR2/1 cytokine production, possibly through the disruption of

lipid rafts (28). Notably, PEIPC selectively inhibited IL-12 while enhancing IL-10 production in primary human monocytes (Figure 5A). Therefore, PEIPC decreased the TLR2/1-induced IL-12/IL-10 ratio of cytokine production, consistent with the pattern previously observed in L-lep lesions: Th1 cytokines predominate in T-lep lesions, whereas Th2 cytokine predominate in L-lep lesions (36).





TLR2/1 activation in human monocytes and macrophages induces a vitamin D–mediated antimicrobial pathway, depending upon upregulation of the 1-hydroxylase (CYP27b1), which results in the conversion of inactive to active vitamin D (1,25D3). The intracellular increase in 1,25D3 subsequently induces the antimicrobial peptide cathelicidin (37). We therefore assessed the effect of oxidized phospholipids on this vitamin D–dependent antimicrobial pathway. PEIPC inhibited the TLR-mediated induction of CYP27b1 mRNA (Figure 5B) as well as cathelicidin mRNA (Figure 5C), a specific vitamin D receptor downstream gene necessary for vitamin D receptor–mediated antimicrobial activity against mycobacteria (38). These data suggest that the generation of oxidized phospholipids during mycobacterial infection can result in alteration of the innate immune antimicrobial response by abrogating the TLR-induced vitamin D/cathelicidin pathway.

**HDL preserves DC function during mycobacterial infection.** One of the mechanisms by which oxidized lipids are removed from the peripheral tissues is via the function of HDL. In addition to reverse cholesterol transport (39), HDL hydrolyzes oxidized phospholipids, in part through associated enzymes such as platelet-activating factor acetylhydrolase and paraoxanase-1 (40–42). Because both oxLDL and mycobacterial infection contain or induce oxidized phospholipids that alter DC differentiation and function, we hypothesized that HDL might preserve DC differentiation and function. HDL diminished the effect of oxPAPC on CD1b expression (Figure 6A). Likewise, HDL preserved CD1b expression on differentiating DCs infected with BCG, although at high MOIs, CD1 expression was less effectively preserved (Figure 6B). These findings were not the result of effects of HDL on BCG uptake, as determined using GFP-labeled BCG, and the HDL did not directly kill mycobacteria (data not shown). Importantly, HDL treatment of infected DCs rescued antigen presentation of mycobacterial lipid antigens to *M. leprae*-reactive CD1b-restricted T cells (Figure 6C).

HDL functions, including reverse cholesterol transport and removal of oxidized phospholipids, can be lost through incompletely defined mechanisms, resulting in dysfunctional HDL (43). Therefore, it is possible that insufficient or dysfunctional HDL could be associated with deposition of oxidized phospholipids (44). Paradoxically, L-lep patients have previously been reported to have higher levels of HDL than either T-lep patients or normal controls (45, 46). In testing the function of HDL from leprosy patients, we infected differentiating DCs with *M. tuberculosis* H37Ra as an attenuated surrogate for the human pathogen. *M. tuberculosis* downregulated CD1b expression on differentiating DCs, similar to that observed with BCG (Figure 6D). In addition, normal HDL preserved CD1b expression in the context of *M. tuberculosis* infection. However, unlike normal HDL, which preserved CD1b expression during differentiation and rescued CD1b expressed during *M. tuberculosis* infection, HDL isolated from L-lep patients inhibited CD1b expression during differentiation and further inhibited CD1b expression during infection with *M. tuberculosis* (Figure 6D). Accordingly, unlike normal HDL, which preserved DC function, HDL from L-lep patients inhibited both CD1b-mediated and MHC class II–restricted antigen presentation to T cells (Supplemental Figure 2).

To compare HDL function across the spectrum of leprosy, HDL was isolated from both L-lep and T-lep patients and was tested in an HDL assay system that has been well characterized to differentiate functional from dysfunctional HDL (41). In contrast to normal HDL, HDL from L-lep patients was unable to prevent human LDL from becoming oxidized and inducing monocyte chemotactic activ-

ity in human aortic endothelial cell cultures (Figure 6E). HDL from T-lep patients behaved similarly to HDL from L-lep patients, unable to inhibit LDL-induced monocyte chemotactic activity. Similarly, reverse cholesterol transport was markedly decreased in both L-lep and T-lep HDL, with L-lep HDL significantly less effective than T-lep HDL (Figure 6F). In contrast, a more striking difference in HDL function was observed using the DC differentiation model. Again, HDL from L-lep patients had the greatest inhibition on CD1b<sup>+</sup> DC differentiation, while HDL from T-lep patients had a significantly less inhibitory effect, yet still greater than that of HDL from normal individuals (Figure 6G). These data indicate a dysregulation in host lipid metabolism and HDL function across the spectrum of leprosy, with the greatest HDL dysfunction in the progressive form of the disease, and provide a framework for investigating the mechanism by which HDL alters DC differentiation and function.

## Discussion

The ability of an intracellular pathogen to establish a productive infection is dependent on its capacity to evade the host immune response and simultaneously access host metabolic pathways to facilitate growth. To understand the interplay between mycobacterial infection and host metabolic pathways, we compared the host transcriptome in disease lesions across the spectrum of leprosy. In the disseminated form of leprosy, there was marked upregulation of host lipid metabolism genes, including those encoding 9 phospholipases, raising the possibility that complementation of the pathogen genome by host genes contributes to the survival of this obligate intracellular bacterium. Furthermore, mycobacterial infection of macrophages triggered the induction and accumulation of host-derived oxidized phospholipids, both in disseminated leprosy lesions and in vitro. Oxidized phospholipids impaired host innate immune responses to mycobacterial infection, including the dysregulation of DC differentiation and function and TLR-induced cytokine and antimicrobial responses. Therefore, the accumulation of host-derived lipids at the site of infection may provide mycobacteria with essential metabolic substrates for growth and virulence (1–3) while dampening innate immune responses to the pathogen.

The major host-derived oxidized phospholipid generated during mycobacterial infection, PEIPC, had specific regulatory effects on the innate immune response. PEIPC, but not other major components of oxPAPC, inhibited CD1b expression on DCs and inhibited TLR2/1-mediated induction of IL-12 while promoting TLR2/1 induction of IL-10. PEIPC also inhibited the TLR-induced vitamin D receptor/cathelicidin antimicrobial pathway that defends against mycobacteria in human monocytes and macrophages (37). In addition, other PAPC-derived oxidized phospholipids are known to inhibit both proteolytic degradation and phagolysosomal fusion (47), 2 important host defense pathways. Together, these findings indicate that oxidized phospholipids inhibit innate immune responses in monocytes. Yet previous studies have also demonstrated that oxPAPC-derived phospholipids activate endothelial cells to induce monocyte recruitment (25). Therefore, the induction of host-derived oxidized phospholipids may provide a mechanism by which mycobacteria can trigger accumulation of macrophages with impaired innate immune responses at the site of infection, thereby favoring the growth of the intracellular pathogen and determining the outcome of the infection. It is of interest that PEIPC, but not other components of oxPAPC, regulated DC differentiation and function; whether this is attributable to its uptake, receptor-mediated signaling, and/or modification of extra- or intracellular targets remains to be determined.





Mycobacterial growth is critically dependent on the catabolism of host fatty acids. Sixty years ago, Middlebrook demonstrated that mycobacterial growth in vitro was enhanced by supplementation with oleic acid (48), and shortly thereafter it was discovered that mycobacteria replicating in vivo selectively catabolize fatty acids (49). More recently, it has been shown that mycobacterial growth in vivo depends on the use of fatty acids by the glyoxylate shunt (1) and that the metabolism of host fatty acids to methyl malonyl CoA is coupled with *M. tuberculosis* pathogenesis through its incorporation into lipid-containing virulence factors (2). The data presented here indicate that multibacillary *M. leprae* infection is associated with a dramatic induction of host lipid metabolism genes, including multiple lipases with functional domains that were predicted by comparative domain analysis to complement the pathogen's deficiencies. At the same time, the mycobacterial genome preserves genes involved in phospholipid synthesis. The local accumulation of host-derived oxidized phospholipids coupled with the induction of host phospholipid catabolism genes provides one potential mechanism by which *M. leprae* can use host-derived lipids and host genes for growth and virulence.

The presence of macrophages, which have been characterized as foamy or lipid laden, is a cardinal histopathologic feature of L-lep as first described by Virchow in 1863: "In the fresh state they have one characteristic that is especially noteworthy, i.e., their tendency to form a sort of vacuole, apparently from taking up water, so that under the circumstances they acquire a wholly physaliferous appearance" (12). It is generally thought that the lipids in these cells are derived from mycobacterial lipids, including phospholipids and fatty acids (13). Similarly, foamy macrophages have previously been identified in advanced tuberculosis lesions (50, 51). Our present data indicate that the lipids in Virchow's foamy macrophages represent, at least in part, the accumulation of host-derived oxidized phospholipids. First, analysis of the host transcriptome in L-lep lesions indicated induction of host genes important for the metabolism of host lipid oxidation products. Second, we noted the presence of abundant EO6<sup>+</sup> cells that coexpressed the monocyte/macrophage marker CD68 in L-lep lesions. Finally, we observed the induction of PEIPC in BCG-infected macrophages in vitro. These findings — although some were derived from studying *M. tuberculosis* and BCG in vitro — point to striking similarities between the type of inflammation in leprosy and atherosclerosis. For instance, both are characterized by the presence of foam cells, EO6<sup>+</sup> macrophages, and oxidatively modified proteins as well as deposition of apolipoprotein B (52), the major apolipoprotein of oxLDL. It might at first seem surprising that oxidized phospholipids accumulate in lesions where there is an increase in transcripts of genes that lead to the breakdown of oxidized phospholipids. However, in atherosclerosis, the amount of lipid accumulation is determined by a balance between synthesis and uptake of lipids against the metabolism of these lipids.

The similar pattern of oxidized phospholipids in atherosclerosis and L-lep raises the possibility for a therapeutic link. HDL, through associated enzymes and reverse cholesterol transport, is known to reverse foam cell formation and reduce plaque volume in atherosclerosis (53). Remarkably, although HDL from normal subjects prevented mycobacterial downregulation of CD1b<sup>+</sup> DC differentiation, HDL from L-lep patients was found to be dysfunctional and by itself significantly inhibited the CD1b<sup>+</sup> DC differentiation. HDL from T-lep patients was also dysfunctional, but to a lesser degree than HDL from L-lep patients. In conjunction with

the differences in deposition of oxidized phospholipids at the site of infection, the extent of HDL dysfunction indicates a disturbance of host lipid metabolic function that differs across the spectrum of leprosy. However, because a diverse array of proteins and phospholipids are known to be associated with HDL, and composition can be altered in disease (54), the mechanism by which HDL loses function remains elusive. Accordingly, alterations in either the protein or the phospholipid components of HDL from leprosy patients could contribute to its dysfunction. Nevertheless, while the present work does not directly demonstrate a causative role for the deposited lipids or dysfunctional HDL in mycobacterial persistence and dissemination, it does provide a rationale for further investigation of host lipid metabolism in mycobacterial pathogenesis, potentially providing a basis for developing a clinical intervention strategy.

It is becoming increasingly evident that the regulation of host lipid metabolism and its impact on host immunity is central to the pathogenesis and outcome of infectious disease. Examples of this include *H. pylori* glucosylation of cholesterol (55) and oxidation of host phospholipids associated with influenza infection (56). Our investigation provides evidence that the host-derived oxidized phospholipids, which accumulate at the site of mycobacterial infection, alter innate immunity. The accumulation of host-derived oxidized phospholipids in leprosy is strikingly similar to atherosclerosis, which suggests that the link between host lipid metabolism and innate immunity contributes to the pathogenesis of both microbial infection and metabolic disease. Alteration of host lipid transport has previously been shown to regulate inflammation in mouse models of viral pneumonia, atherosclerosis, and diabetes (57, 58). Therefore, pharmacologic agents that target lipid homeostasis (58–60) may be useful to regulate host immune responses in human diseases in which lipid metabolism and inflammation intersect.

## Methods

**Reagents.** TLR2/1 ligand is a synthetic 19-kDa *M. tuberculosis*-derived lipopeptide obtained from EMC Microcollections. PAPC and POVPC were purchased from Avanti Polar Lipids. PGPC was purchased from Cayman Inc. Chloroform, methanol, and water (Optima Grade) were obtained from Fisher Scientific.

**DNA microarray data analysis.** Microarrays comparing 10 leprosy patients (L-lep,  $n = 6$ ; T-lep,  $n = 4$ ) have previously been described (14), and raw gene expression data are available through the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>; series entity GSE443). Genes that met differential expression criteria between groups ( $P < 0.05$ ; fold change  $> 1.5$ ) were organized into categories based on function as described in the OMIM (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim>) and Locus Link (<http://www.ncbi.nlm.nih.gov/projects/LocusLink/>) databases. In some cases, expressed sequence tags were identified using the Celera Discovery System.

**Comparative domain profiling.** Because of fundamental differences in the complexity and structure of full-length human and mycobacterial proteins, we found it more informative to compare functional domains rather than proteins. Mycobacterial genomes and predicted proteomes were downloaded from GenBank. Predicted human protein sequences corresponding to upregulated probe sets were downloaded via matches to the UniGene database (61). We chose to define potential functional domains via the PFAM database (16). When available, proteins were matched to functional domains via the swisspfam file provided by PFAM. For genomes not covered by swisspfam, we defined domains using the genome annotation and/or by querying the genome locally with the trusted cutoffs defined for each



hidden Markov model in PFAM. For phospholipase C genes, we did not find *M. leprae* homologs by using BLAST on full-length protein sequences with default parameters.

**Immunohistochemistry and confocal microscopy.** Immunoperoxidase labeling was performed with EO6 (1–2 µg/ml) and NA59 (diluted 1:50) or isotype controls as previously described (24). Double immunofluorescence was performed by serially incubating sections with EO6 followed by an isotype-specific fluorochrome (Caltag). Sections were washed, incubated with antibodies for CD68 for 1 h followed by a TRITC-conjugated secondary antibody (Southern Biotechnology), and examined as described previously (62).

**TUNEL assay.** Apoptotic cells in leprosy lesions were identified by using the TUNEL method in accordance with supplier instructions (Boehringer Mannheim). Paraffin skin sections were dewaxed by heating at 60°C for 1 h, washed in xylene, and rehydrated through a graded series of ethanol. Slides were incubated in permeabilization solution (20 µg/ml proteinase K in 10 mM Tris-HCl) for 20 min at 37°C, followed by incubation with TUNEL reaction mixture for 60 min at 37°C. After labeling, samples were incubated with Converter-POD (Roche Diagnostics) for 30 min at 37°C, followed by the addition of the substrate 3-amino-9-ethylcarbazole and incubation for 10 min. Slides were counterstained with hematoxylin and mounted in aqueous dry mounting medium. Negative controls included incubation with label solution instead of TUNEL reaction mixture. The percentage of TUNEL-positive cells in leprosy biopsies was estimated by 2 independent observers; their reading invariably agreed within 10%.

**Mycobacterial infection.** *M. tuberculosis* H37Ra (kindly provided by J.D. Ernst, New York University School of Medicine, New York, New York, USA), *M. bovis* BCG, and *M. bovis* GFP-labeled BCG (kindly provided by B. Bloom, Harvard University, Boston, Massachusetts, USA) were used in these experiments. All mycobacterial strains cause similar inhibition of CD1b expression on differentiating monocytes. BCG was used to infect human macrophages (MOI, 3–5) to quantify changes in phospholipid composition by ESI-MS. To measure mycobacterial uptake, monocytes were infected overnight with GFP-labeled BCG in the presence or absence of HDL. Cells were washed and analyzed by flow cytometry to determine the extent of mycobacterial uptake.

**Liquid chromatography and mass spectrometry.** oxPAPC was applied to a semi-preparative reverse-phase column (Biosil C8, 10 mm × 250 mm; Thermo Electron Inc.) and eluted with a mobile phase starting at 65% methanol in water with a linear gradient over 20 min to 100% methanol at a flow rate of 2 ml/min. The eluent was passed through a flow splitter, which directed one-twentieth of the flow into the ion source of an LCQ Advantage Max ion trap mass spectrometer (Thermo Electron Inc.) and the rest to a fraction collector programmed to collect fractions every 30 s. Individual oxidation products of oxPAPC eluting from the column were detected as positive charged ions [M + H<sup>+</sup>] between *m/z* 450 and *m/z* 950. Reconstructed selected ion chromatograms were constructed for each ion of interest using Xcalibur software (version 1.4; Thermo Electron Inc.).

For quantitation of oxidized phospholipids in macrophages, lipids were extracted from cells by the addition of 500 µl of chloroform/methanol (2:1, v/v) followed by 300 µl water (63). The solution was mixed and centrifuged, and the chloroform phase was collected. Chloroform (300 µl) was added to the residual aqueous phase. The solution was mixed and centrifuged, and the chloroform phase was pooled with that previously collected. The phospholipids were isolated by solid phase chromatography (64), and then the total lipid extract was applied to a reverse phase column and analyzed with a triple quadrupole mass spectrometer (Sciex ABI III; PerkinElmer) in multiple reaction monitoring mode. Quantification was achieved by determining the area under the peak of the product ion, phosphocholine (*m/z* 184.1), derived from the parent ion of interest at the retention time (RT) determined by authentic standards produced by total organic synthesis

under identical chromatographic conditions (65, 66). PEIPC was quantified by *m/z* 828.6→184.1 (RT, 26–31 min), LysoPC by *m/z* 496.3→184.1 (RT, 13–15 min), and PAPC by *m/z* 782.5→184.1 (RT, 50–53 min). Phosphatidylcholines with a parent mass similar to that of PEIPC (–828.6→184.1) eluted beyond 40 min and were excluded from quantitative analysis.

**Lipid oxidation.** Phospholipids were oxidized by transferring 1 mg, in chloroform, to a 13 mm × 100 mm glass test tube, evaporating the solvent under argon, and leaving the lipid residue to autoxidize in air (67). The extent of oxidation was monitored by ESI-MS daily. After adequate oxidation (usually 24–72 h), the lipid residue was resuspended in chloroform and stored at –80°C. All oxidized phospholipid preparations were tested for LPS contamination and found to be below the level of detection (1 pg/ml) as determined by Limulus amoebocyte lysate assay (Cambrex). This amount of LPS (1 pg/ml), when added to monocytes, does not induce or inhibit immune responses.

**DC and macrophage differentiation.** Monocytes were isolated from healthy human donors, after informed consent was obtained (UCLA Institutional Review Board protocol no. 92-10-591-31), as previously described (62). To derive DCs, GM-CSF (10 U/ml; Immunex) was added for 48–72 h in the presence or absence of mycobacteria, oxidized phospholipids, or HDL at the indicated concentrations in 5% FCS (Omega). In some experiments, DCs were derived with GM-CSF (100 U/ml) and IL-4 (100 U/ml), and similar suppression of CD1 was observed with either mycobacterial infection or oxidized phospholipids. Macrophages were derived by culturing human monocytes with 10% autologous serum for 5–6 d as previously described (28).

To test the ability of HDL to rescue CD1b<sup>+</sup> DC differentiation during mycobacterial infection, GM-CSF (10 U/ml), BCG, or *M. tuberculosis* H37Ra (MOI, 0.05–1.0) and HDL was added at the same time, followed by 48–72 h of incubation. Differentiation was defined as percent cells expressing CD1b (relative to isotype control) by flow cytometry. When comparing normal and L-lep HDL, all HDL preparations were used at 50 µg/ml, and *M. tuberculosis* H37Ra was added at a MOI of approximately 0.1. Mycobacteria, oxidized phospholipids, and HDL at the indicated concentrations did not exhibit cytotoxicity as determined by trypan blue exclusion and flow cytometry.

**Flow cytometry.** DCs were labeled with the following antibodies: CD1b (2.5 µg/ml; clone BCD1b3.1) or CD1a (2.5 µg/ml; OKT6) followed by a phycoerythrin-conjugated goat anti-mouse IgG1 secondary antibody (BD Biosciences – Pharmingen). HLA-DR and isotype controls (Sigma-Aldrich) were directly conjugated. Cells were fixed with 2% paraformaldehyde and analyzed as previously described (68).

**T cell assays.** Human monocytes were differentiated to DCs as described above. After 48–72 h, DCs were harvested, washed twice in fresh RPMI media, and counted by trypan blue exclusion. For MHC class II-restricted T cells, HLA-DR-specific DCs were used. CD1b-restricted (CD4.CD1b-LAM3) and HLA-DR-restricted (CD4.DR-GroES2) T cell lines were used. DCs (1 × 10<sup>4</sup>) were pulsed with either *M. leprae* sonicate (5 µg/ml for CD1b-restricted clone) or GroES protein/peptide (3 µg/ml for protein, 0.03 µM for peptide, for HLA-DR-restricted clone) in a 96-well plate, followed by the addition of T cells (1 × 10<sup>4</sup>) in a final volume of 200 µl in triplicate wells. After 16 h, we measured IFN-γ levels by ELISA (BD Biosciences – Pharmingen).

**Cytokine secretion.** Monocytes were cultured in the presence or absence of oxidized phospholipids in serum-free RPMI for 5 min prior to the addition of TLR2/1 ligand (19 kDa at 10 µg/ml) and 5% FCS. IL-10 and IL-12 p40 were measured in triplicate and measured by ELISA (BD Biosciences – Pharmingen).

**EO6 ELISA.** EO6 antibody was coated at 2.5 µg/ml overnight at 4°C. After blocking for 1 h, oxLDL or *M. leprae* sonicate were added for 2 h. Plates were washed 5 times in PBS containing 0.2% Tween to remove nonspecific hydrophobic interactions. Biotinylated EO6 (0.2 µg/ml) was then added for 1 h. We used streptavidin-horseradish peroxidase at 1:1,000 dilution



(BD Biosciences – Pharmingen) followed by substrate according to the manufacturer's protocol (BD Biosciences – Pharmingen).

**Patients and clinical specimens.** We classified patients with leprosy according to the criteria of Ridley and Jopling (68), and skin biopsy specimens (6 mm in diameter) containing both epidermis and dermis were collected by standard punch technique after informed consent was obtained. Leprosy patients were recruited on a volunteer basis from the ambulatory population seen at the Los Angeles Hansen's Disease Clinic at the University of Southern California Medical Center, with approval from the University of Southern California School of Medicine Institutional Review Board. Plasma was isolated within 6 h of isolation and immediately cryopreserved in sucrose prior to use.

**Lipoproteins.** All plasma samples were cryopreserved in sucrose prior to use, and HDL was isolated by ultracentrifugation or by fast performance liquid chromatography (FPLC) as previously described (40, 41). After FPLC, HDL was concentrated in Ultra-4 10K centrifugal filters (Amicon). Lipoproteins were tested for LPS by Limulus amoebocyte lysate assay (Cambrex) and were below the level of detection (0.05 pg/ $\mu$ g protein).

**Monocyte chemotaxis.** The ability of the HDL from each subject to inhibit LDL-induced monocyte chemotactic activity in human aortic cell cultures was determined by techniques previously described (40, 41). The LDL used was prepared from a normal donor and was aliquoted and cryopreserved in sucrose as previously described (40, 41). The number of migrated monocytes was determined microscopically and expressed as the mean  $\pm$  SD of 9 standardized high-power fields counted in triplicate wells. The inflammatory index was calculated as the ratio of monocytes adhered in the presence of LDL and specific HDL to the number of monocytes adhered in the presence of LDL.

**Cholesterol efflux.** Cellular cholesterol efflux was performed as described previously (69), with minor modifications. Mouse macrophage RAW264.7 cells (ATCC) were cultured on 24-well tissue culture plates and grown in DMEM media (Gibco; Invitrogen) with 10% FBS overnight. Cells were

washed with serum-free media and loaded with 3H-cholesterol (1  $\mu$ Ci/ml) and acetylated LDL (50  $\mu$ g/ml) in media with 0.2% fatty acid-free BSA (Sigma-Aldrich) overnight. Labeled cells were washed, resuspended in DMEM with 0.2% BSA, and incubated with HDL (25  $\mu$ g/ml) for 6 h at 37°C. Radioactivity in the supernatants and total cell extracts were measured and expressed as the percentage of total radioactive counts accumulated in the supernatants during the efflux period.

**Real-time quantitative PCR.** Monocytes were stimulated with either media or TLR2/1 ligand 19 kDa for 24 h. We isolated RNA and synthesized cDNA as described previously (37). Reactions used Syber Green PCR master Mix (Bio-Rad) and primers for cathelicidin and CYP27b1 as described previously (37).

**Statistics.** Statistical significance was determined using 2-tailed paired Student's *t* tests. A *P* value less than 0.05 was considered significant.

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