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***Borrelia burgdorferi* infection regulates CD1 expression in human cells and tissues via IL1- β**

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Abstract

The appearance of newly translated group 1 CD1 proteins (CD1a, CD1b, CD1c) on maturing myeloid DC to effective lipid antigen presenting cells. Here we show that *Borrelia burgdorferi*, the causative agent of Lyme disease, triggers appearance of group 1 CD1 proteins at high density on the surface of human myeloid DC during infection. Within human skin, CD1b and CD1c expression was low or absent prior to infection, but increased significantly after experimental infections and in erythema migrans lesions from Lyme Disease patients. The induction of CD1 was initiated by borrelial lipids acting through TLR-2 within minutes, but required 3 days for maximum effect. The delay in CD1 protein appearance involved a multi-step process whereby TLR-2 stimulated cells release soluble factors, which are sufficient to transfer the CD1-inducing effect in trans to other cells. Analysis of these soluble factors identified IL-1 β as a previously unknown pathway leading to group 1 CD1 protein function. These studies establish that upregulation of group 1 CD1 proteins is an early event in *B. burgdorferi* infection and suggest a stepwise mechanism whereby bacterial cell walls, TLR activation and cytokine release cause DC precursors to express group 1 CD1 proteins.

Keywords

T cells; lyme disease; CD1; dendritic cells; interleukin-1

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Introduction

CD1 proteins have structurally diverse antigen grooves that accept self and foreign lipid antigens for display to T cells [1]. The antigenic lipids are amphipathic molecules that include lipids, lipopeptides and glycolipids derived from mammalian cells [2] and microbial sources [3]. The human CD1 gene cluster consists of one lipid transfer protein (CD1e), three group 1 antigen presenting molecules (CD1a, CD1b and CD1c) and one group 2 antigen presenting molecule (CD1d) [4, 5]. For MHC class II, it is well established that the density of peptide-loaded complexes changes greatly during DC maturation and controls the strength and antigenic focus of the resulting MHC-restricted T cell response [6]. New evidence suggests that myeloid APC contribute to the immunologic distinction between uninfected and infected state by actively regulating density of cell surface CD1 proteins in response to pathogen contact [7]. Whereas CD1d is constitutively expressed on monocytes and DCs, group 1 CD1 proteins are absent on circulating monocytes, but are inducibly expressed on myeloid DCs after activation [8].

Although MHC II and group 1 CD1 proteins both increase in cell surface density during the DC maturation program, the timing and cellular mechanisms underlying upregulation are distinct. MHC class II accumulation results from redirected intracellular trafficking in which preformed stores of protein that reside within lysosomal compartments move to the surface [9, 10]. However, the timing and subcellular location of MHC II and CD1 antigen presenting proteins differ when examined in parallel within the same cells [11]. In contrast to MHC II, the appearance of CD1a, CD1b and CD1c on the surface of myeloid DCs during maturation signals results mainly from new protein translation. Recent studies show that myeloid precursors of DCs lack detectable levels of CD1a, CD1b or CD1c, when measured as mRNA transcripts, intracellular proteins or cell surface proteins, but that new protein production starts after exposure of cells to microbial products [12, 13].

If CD1a, CD1b and CD1c protein expression is actively suppressed on blood monocytes and DC precursors, but then released when encountering pathogens in the periphery, this might represent a natural mechanism to limit CD1 autoreactivity and promote T cell responses to foreign antigens [7]. Supporting this hypothesis, IgG and serum lipid agonists of PPAR- γ , which are normally concentrated in the bloodstream, suppress CD1a, CD1b and CD1c expression on monocytes [14–16]. Conversely, events that normally occur while trafficking to the periphery, such as the exposure to *Mycobacterium tuberculosis* or *M. leprae*, lead to upregulation of CD1a, CD1b and CD1c in tissues [13, 17]. Thus, pathogens promote CD1 protein translation, while at the same time releasing lipid antigens that bind in the grooves of newly translated proteins. However, tissue based studies of this phenomenon are limited because mice do not express orthologs of CD1a, CD1b or CD1c [18]. Furthermore, controversy exists as to whether CD1 modulation observed with dispersed monocytes represents an effective model of the more complex events that occur in tissues during infection [17, 18, 19]. Also, nearly all studies on group 1 CD1 upregulation during infection to date focus on mycobacteria, so any role of other pathogens that act as such natural adjuvants for the CD1 system are not understood.

Here we sought to determine whether *Borrelia burgdorferi* infection alters CD1 expression. CD1d proteins present *Borrelia burgdorferi* monogalactosyl diacylglycerols (BbGLII) to mouse NKT cells [20–23], raising the possibility that CD1 might function in the host response in Lyme disease. *B. burgdorferi* infects human skin via injection by tick bite, where organisms spread centripetally within skin as *erythema migrans* (EM) lesions. For many patients, symptoms in the skin, joints and other organs resolve with antibiotic treatment and eradication of borrelia. However, in a subset of genetically susceptible patients, infection of the joint may cause persistent arthritis for months or even several years

after the eradication of spirochetes with antibiotic therapy. The prolonged immune response that exceeds the duration of detectable infection suggests that in some cases, acute infection converts to a self-propagating inflammatory syndrome, called antibiotic-refractory Lyme arthritis [24]. Because the early events occur within skin, this disease potentially offered a new human model whereby skin biopsies could allow direct study of the kinetics of the CD1 induction process in vivo or ex vivo [25, 26].

Here we report that natural and experimental *B. burgdorferi* infection upregulates cell surface expression of CD1a CD1b, CD1c in the dermis of human skin. Whereas CD1d and NK T cells are thought to act at the earliest stages of the innate response, we found that the process of group 1 CD1 induction requires antecedent signaling through TLR-2 and a days long series of events whereby the cell-to-cell spread of cytokines leads to CD1 appearance on maturing DCs. These studies support a role for CD1 in host response in human Lyme disease and demonstrate a previously unknown pathway whereby IL-1 β cleavage leads to selective induction of group 1 CD1 proteins after infection.

Results

CD1 expression in erythema migrans lesions of Lyme patients

Mechanistic studies of group 1 CD1 induction have been carried out using dispersed blood monocytes [12, 13, 19], highlighting the need for studies of infected human tissues. To determine whether group 1 CD1 proteins are induced within skin during natural *B. burgdorferi* infection, we first studied frozen sections of EM skin lesions from 10 patients with Lyme disease. The diagnosis of Lyme Disease was confirmed by culture or serology, or in most instances, by both methods (Table 1). In addition to culture-positivity, 3 patients had evidence of spirochetes in the blood and >6 symptoms, including fever, headache, stiff neck, arthralgias, myalgias and fatigue; and 2 had multiple EM skin lesions. Eight patients were infected with *B. burgdorferi* OspC type A or K strains, the 2 most common *B. burgdorferi* genotypes [27, 28].

Hoechst dye staining viewed at low power showed nuclei clustering in rete patterns that corresponded to the dermal-epidermal junction (Fig. 1A), as confirmed in serial sections stained with hematoxylin and eosin (not shown). In two color immunohistochemistry samples stained with anti-CD1a, many large cells were seen in the epidermis, likely representing Langerhans cells (LC), a DC subtype that constitutively expresses CD1a (Fig. 1A). In contrast, CD1b and CD1c in normal skin were consistently seen at low levels on about 1 percent of dermal cells (Fig. 1B and data not shown). For two patients (Table 1, A and J), CD1b and CD1c could be detected with bright staining on many (~ 5 percent) large cells in the dermis (Table 1, Fig. 1A). One of these 2 patients (A) had severe infection, with a positive PCR test for *B. burgdorferi* DNA in blood, >6 symptoms, and multiple EM lesions. Both patients (A and J) were infected with the OspC type A genotype, a particularly virulent *B. burgdorferi* subtype that grows to high numbers in EM lesions [27, 28]. Thus, group 1 CD1 proteins are expressed at high levels in the skin of certain patients with Lyme disease, but were not seen uniformly among patients with early infection.

Increased CD1b and CD1c expression in infected human keratomes

These findings led to experiments designed to assess infection of human skin in a controlled study of live spirochetes infecting full thickness human skin explants (keratomes). Blinded analysis of 10 $\times$ low power fields assessed the number of CD1 expressing cells within the dermis and epidermis. There were no significant changes in the number, apparent brightness or size of CD1a expressing LCs in the epidermis, when comparing infected or sham treated keratomes (Fig 1B–C). The number of CD1a expressing cells in the dermis (4.1 % of all

cells) increased slightly after infection (6.1 %) but did not reach statistical significance ($p = 0.34$). However, the number of CD1b ($p < 0.0027$) or CD1c ($p < 0.0086$) expressing cells showed a significant increase after infection (Fig. 1C). Also, we observed marked increases in brightness of staining in each of three experiments. Although CD1d could be detected at very low levels in flow cytometry experiments (Fig. 2), CD1d staining was not seen at levels higher than isotype matched staining control samples (Fig. 1C). We conclude that evaluation of CD1a induction was limited by constitutively positive LCs, but increased CD1b and CD1c expression are induced during *B. burgdorferi* infection of human skin.

***B. burgdorferi* lipids induce CD1 expression via TLR-2**

To study the cellular mechanisms of CD1 induction by *B. burgdorferi*, we measured CD1 expression on human monocytes in culture. To determine whether the events seen *ex vivo* could be modeled *in vitro*, we first measured CD1 expression on monocytes after infection with live bacteria or by treatment of cells with lipids extracted from bacteria with chloroform and methanol. Fresh monocytes and control monocytes sham treated with medium for three days did not detectably express CD1a, CD1b or CD1c proteins at the surface, but CD1d was detected at low density on some cells (Fig. 2A and data not shown). *Ex vivo* infection with live spirochetes (data not shown) or cell wall lipids (Fig. 2A) increased cell surface expression of CD1a, CD1b and CD1c proteins to high levels. CD1a surface density increased in a dose-dependent fashion (Fig. 2B). The resultant CD1a cell surface expression was sufficient to activate a CD1a autoreactive T cell line (Fig. 2C). The low levels of baseline expression of CD1d were unaltered or slightly decreased, so that they were undetectable (Fig. 2A). These results confirm that *B. burgdorferi* potentially activates group 1 CD1 expression on monocyte-derived DCs in a model that mimics many aspects of the *in vivo* observations. In particular, these data show selective upregulation of group 1 CD1 proteins over 3 days.

Activation of myeloid cells by *B. burgdorferi* lipoproteins is mediated through TLR-2 [29]. Also, a synthetic TLR-2 agonist triacyl-CSK₄, which mimics the structure of the N-terminus of a borrelial lipoprotein, can induce CD1 expression [30]. Therefore, among all activating receptors on monocytes, we focused on the possibility of that CD1 induction by *B. burgdorferi* might involve TLR-2, keeping in mind that the intact bacterium can activate immune responses by TLR-independent mechanisms [31]. For example, MyD88 deletion in mice affects immune mediated pathogen clearance, while allowing many inflammatory processes to proceed [32, 33].

We pre-treated monocytes with a neutralizing monoclonal antibody against TLR-2 (T2.5) and pulsed them with borrelial lipids, leaving blocking antibody in culture [34]. As noted previously in cytokine-activated monocytes [12], the range of CD1a expression on borrelia-activated cells is broad and the histogram is bimodal in nature. T2.5 reduces the number and mean level of CD1a expression as compared to isotype matched antibody treated controls, but some cells retain detectable staining (Fig. 2B, D). For CD1c, the histogram of activated cells shows a single population with a normal Gaussian distribution, and treatment with anti-TLR-2 blocked expression to levels seen in unactivated cells (Fig. 2D). Thus, live *B. burgdorferi* and its hydrophobic components selectively increased group 1 but not CD1d protein expression using TLR-2.

CD1 induction involves transfer of factors from cell to cell

CD1 cell surface expression might be induced through the NF- κ B signaling pathway within a single cell that expresses both TLR-2 and CD1. Alternatively, CD1 might appear through a multi-cell mechanism in which the TLR-2 expressing cells secrete transferable factors. The single cell model is plausible because we found that TLR-2 and group 1 CD1 are co-

expressed on myeloid cells (data not shown). On the other hand, a prior study of cellular infection showed that CD1 appeared on individual myeloid cells harboring fluorescent mycobacteria as well as uninfected bystander cells [13]. The natural TLR-2 agonists in *B. burgdorferi* are chemically diverse, but mechanistic studies could more reliably be carried out using a single compound of defined molecular structure. Therefore we used a synthetic CSK4 (triacyl-CSK₄) [34]. Validation of this TLR-2 agonist showed its ability to stimulate group 1 CD1 protein expression on monocytes in a dose-dependent manner (data not shown). Because this and other preliminary studies found concordant upregulation of CD1a, CD1b and CD1c by TLR agonists [13, 17], we measured CD1a as a surrogate for group 1 CD1 proteins [4].

Kinetic studies showed that CD1a expression was transiently detected at high densities after 48 to 72 hours after stimulation (Fig. 3A). When triacyl-CSK₄ was pulsed onto cells and then washed off, there was a delay of more than two days before CD1a proteins appeared at the surface, even though only 10 to 60 min of exposure to the initial stimulus was sufficient to trigger CD1a expression (Fig. 3B and data not shown). Prior studies have shown that the proximal signaling events involving MyD88, IRAK4, IRAK1, TRAF6, TAK1, IKK and I κ B leading to NF κ B activation are complete within hours [35–40]. Therefore, the delay in appearance of CD1 was suggestive of a multi-step pathway that exists downstream of these rapidly acting signaling molecules, including that secreted factors that act in trans to induce CD1 on nearby cells. Therefore, we carried out supernatant transfer experiments under conditions in which the synthetic TLR-2 agonist was washed from cells prior to supernatant conditioning. Supernatants conditioned for 6 hours were sufficient to induce CD1a expression on fresh monocytes (Fig. 3C), although the percentage of cells expressing CD1 was lower than the percentage of CD1-positive cells treated directly with the TLR agonists. This decrement is expected because factors may be consumed during conditioning and were diluted during transfer. Thus, TLR-2 agonists work via mechanism that requires only minutes of TLR stimulation but plays out over three days in a process that involves cell to cell transfer of host factors.

GM-CSF and IL-1 β regulate CD1-induction

To identify the host factors, we first screened conditioned supernatants using a multiplex bead-based cytokine array. Consistent with known patterns of TLR-2 dependent cytokine secretion [26, 41], we detected increased levels of IL-1 β , IL-6, IL-8, TNF α , and we also found GM-CSF in monocyte supernatants. Using recombinant cytokines, we found that GM-CSF or IL-1 β were sufficient to induce CD1a, CD1b and CD1c expression (Fig. 4C and data not shown). Quantitative ELISA detection showed that both GM-CSF and IL-1 β were detected in conditioned supernatants within the dose range at which recombinant cytokines activate CD1a expression (~100 – 500 pg/ml), consistent with the conclusion that both contribute to CD1 induction (Fig. 4A–C, S1 and data not shown). The role of GM-CSF in CD1 induction has been previously observed with recombinant cytokines [12] or mycobacterial infection [17], so we considered this a confirmatory result, while extending the range of pathogens that work via this mechanism.

IL-1 β differentially affects group 1 and group 2 CD1 proteins

We undertook more detailed studies of IL-1 β because it is a key mediator of innate immunity that occurs downstream of TLRs, potentially providing insight in the pathways that connect TLR ligation to CD1 induction. Also, the potential role of IL-1 β in CD1 gene regulation was not previously known and therefore represented a new adjuvant for activating the CD1 system. In our study, the CD1a induction was seen in response to two preparations of recombinant mature IL-1 β (17Kd) that were free of detectable lipopolysaccharide (data not shown). Also, anti-IL-1 β blocked CD1a induction, demonstrating that IL-1 β was the

only active component in the recombinant cytokine preparation (Fig. S1). Measurement of surface expression of all three group 1 were upregulated from trace to high levels in a dose-dependent fashion by IL-1 β (Fig. 4C), whereas the group 2 CD1 protein (CD1d) was unaffected (Fig. 4C). Further, IL-1 β induction of group 1 proteins increased activation of CD1a autoreactive T cells (Fig. S2).

Classically, production of bioactive IL-1 β is mediated by a dual signal involving TLR signals for pro-IL-1 β production and caspase-dependent IL-1 cleavage events [42–44]. Recent studies have shown that separate, exogenous activation of inflammasome pathways is not always stringently required for IL-1 β cleavage, especially in monocytes or in situations in which strong cellular activation leads to ATP release and autoinduction of the inflammasome [45–47]. Western blotting showed that monocytes treated with ATP alone did not produce detectable cleaved IL-1 β , but triacyl-CSK₄ with or without added ATP produced detectable cleaved IL-1 β (Fig. 4D). CD1 induction correlated with IL-1 β cleavage, as flow cytometric measurement of surface CD1a induction showed that triacyl-CSK₄, but not ATP was sufficient to induce CD1 (Fig. 4D). Thus, TLR-2 activation is necessary and sufficient, and so can be considered the main driver of CD1 induction under these conditions. Separate, pharmacologic activation by ATP contributes quantitatively to the response.

Discussion

A now widely used nomenclature system was originally developed in which the 5 human CD1 APCs were divided into two groups based on amino acid sequence homology [48]. New data, including the responses to *B. burgdorferi* reported here, show that group 1 protein (CD1a, CD1b, CD1c) and group 2 (CD1d) protein expression response are dichotomously different. *B. burgdorferi* infection strongly and selectively upregulated CD1a, CD1b and CD1c gene products with no discernable effects on constitutively expressed CD1d. The constitutive expression of CD1d at all stages is consistent with its proposed function in activating NK T cells during the earliest stages of innate immunity. In contrast, the group 1 CD1 isoforms are not commonly expressed on circulating monocytes or at high levels or on uninflamed dermal skin, and so require some antecedent stimulus of the innate immune system before APCs become competent to activate T cells.

We found evidence for group 1 CD1 upregulation as an early event in Lyme disease pathogenesis and developed a new clinical model to study of human CD1 proteins in situ. Results obtained on dermal DCs in vivo, ex vivo (Fig. 1) or with dispersed myeloid cells in vitro generally agree with one another and show marked upregulation of group 1 CD1 proteins. However, some differences were seen based on the route of the infection, the types of cells or the particular CD1 isoform analyzed. Bright staining for group 1 CD1 proteins was seen at margin of certain EM lesions, providing clear evidence can CD1 can be expressed at the site of the spread of spirochetes early in the disease. Many patient samples did not show CD1 expression present above baseline levels (Table 1, Fig. 1A), but CD1b and CD1c upregulation was seen in all cases when the infection was carried out under controlled experimental conditions that avoid sampling bias. In no case did we see strong expression of group 1 CD1 in the dermis of uninfected skin (Fig. 1), indicating that the default condition is lack of abundant expression of group 1 CD1 proteins. In vitro, kinetic analysis of CD1 expression shows that proteins are detectable over a fairly narrow time range between 2 and 4 days, rather than a highly durable effect (Fig. 3A).

Conclusions relating to CD1 expression in the dermis of infected skin can be formally stated for CD1b and CD1c. We also noted an upward trend in CD1a expression, but it did not reach statistical significance (Fig. 1). However, large numbers of CD1a expressing LCs in

the nearby epidermal compartment provide a higher baseline of staining that complicates interpretation of CD1a expressing cells in the dermis (Figs 2, 4). Collectively, the results show that CD1b and CD1c proteins are rare or absent on cells in the dermis under normal conditions, but are locally upregulated on DCs in the dermis after coming into the proximity with the infecting borrelial pathogen. Whereas CD1a induction is linked to CD1b and CD1c in myeloid cells, only CD1a is constitutively expressed at high levels on epidermal LCs. Previous ex vivo studies showed that human dermal DCs and epidermal LCs play distinct roles in response to borrelia infection, with dermal DCs having more efficient mechanisms of internalization and processing of *B. burgdorferi* [25], so it is of interest that the new CD1 appears on the same type of cell that may be most directly exposed to foreign lipid antigens.

Triacyl-CSK₄ and natural triacylated lipoproteins present in mycobacteria and borrelia bind to hydrophobic pockets in the TLR1-2 heterodimer and signal through Myd88 and NF κ B to stimulate secretion of diverse cytokines [49]. The cellular signaling pathway leading to increased CD1 gene translation might result from cell autonomous signals within TLR-2-expressing cells. However, direct connections between NF κ B signaling and CD1 promoters are not known, and our data show that secreted factors are sufficient to transfer CD1-inducing activity from cell to cell under conditions in which TLR-2 is not activated. These results suggest that the pathogen sets up a local field whereby cytokines stimulate CD1 expression in many cells near the site of infection, even if individual CD1 expressing cells themselves are not infected or in direct contact with the pathogen.

Whereas the effects of GM-CSF were known [12, 17–50], the identification of IL-1 β as a regulator of CD1 protein expression provides a new downstream function of this innate cytokine [51]. IL-1 β has been implicated as an in vitro factor for inducing CD1a expression on Langerhans cell precursors [52], but identification of mature IL-1 β as a group 1 CD1 inducing factor on myeloid cells is a new finding with several implications. First, IL-1 β can be used therapeutically as an adjuvant to stimulate CD1 antigen processing function in human monocytes. Second, because the role of TLR-2 in regulating IL-1 β is well known, the mechanisms by which TLR-2 signals through and NF κ B pathways to regulate IL-1 provides a more clearly defined pathway that could explain the observed ability of TLRs to regulate CD1 expression [35]. Recent studies show that both *B. burgdorferi* and *M. tuberculosis* normally activate the inflammasome and caspase production in ways that lead to cleavage of pro-IL-1 β to its active form, paving the way for future studies of the inflammasome in CD1 function [53–56].

More generally, dissection of the stepwise mechanisms by which *B. burgdorferi* leads to CD1 induction over a period of days suggest two separate models for CD1-restricted T cell activation. CD1d and NK T cells act within minutes of infection and are considered to represent an intrinsic part of the innate response to infection [57–59]. In contrast, myeloid cells from the dermis and blood generally lack constitutive expression of CD1a, CD1b or CD1c, which appear only after recognition of TLR activation by pathogens. The delay in appearance of group 1 CD1 proteins is consistent with a model that the diverse T cells recognizing CD1a, CD1b and CD1c act just after, rather than during, the earliest phases of innate immunity.

Prior studies of Lyme disease have focused on TLR-2, MHC-restricted T cells and peptide antigens, but the discovery of a borrelial modulation of CD1 suggests a new hypothesis whereby microbially induced CD1 proteins might be available to present both self or foreign lipids to T cells after infection triggers their expression. Although symptoms in most Lyme disease patients resolve with antibiotic treatment, a subset of patients shows long-acting immune response. This model of infection as a gateway to prolonged inflammation fits with certain observations seen here in which borrelia triggers CD1 expression, which participates

in the acute host response but could in theory be available for presentation of any self or foreign antigen thereafter. The identity of any borrelial lipid ligands for CD1a, CD1b or CD1c are not known, but borrelia-induced IFN γ secretion by cells in patients with Lyme disease is mediated by CD1b and CD1c [60], suggesting that antigens for these CD1 proteins await discovery. Serological responses to known CD1d-presented borrelial glycolipids (BbGLI and BbGLII) are weak during the subacute infection, but after a period of months, nearly all human patients have high titre responses [61]. Thus, there is overlap in the borrelial lipids presented by CD1 and the downstream events involving B cells in the evolution of the chronic phase of the syndrome. Our studies provide a potential link between these early and late events by showing how *B. burgdorferi* actively modulates CD1 expression.

Materials and Methods

Bacteria

B. burgdorferi strain N40 or green fluorescent protein (GFP) expressing bacteria (Justin D. Radolf, University of Connecticut) [62] were cultured in Barbour-Stoenner-Kelly medium at 37°C in 18×150 mm borosilicate culture tubes (Fisher Scientific) with MicroAero packs (Mitsubishi). Total lipids were obtained by extracting spirochetes at 1 mg/ml in chloroform and methanol (1:1, V:V) for 1 hour at 20° C and drying lipids under nitrogen and sonicating in RPMI complete medium as described [63].

Cell preparation and flow cytometry

Monocytes were isolated from peripheral blood by centrifugation over Ficoll-Hypaque followed by adherence to plastic flasks. To detect CD1 induction, fresh monocytes were treated with lipids (1 μ g/ml) and stained with 10 μ g/ml of Abs binding to CD1a (OKT6), CD1b (BCD1b3.1), CD1c (F10/21A3.1) or CD1d (CD1d42) isotype control (P3), followed by a FITC-labeled goat anti-mouse IgG (BioSource International) and analyzed by a FACScalibur flow cytometer (BD Biosciences) with CellQuest and FlowJo software (Tree Star). Monocytes were pretreated with an anti-TLR-2 mAb (T2.5) or isotype control (T2.13) [34] in serum-free medium for 30 min at room temperature and for 20 min at 37°C before adding lipids (1 μ g/ml). After 2 hours, monocytes were washed 3 times and resuspended in fresh RPMI medium with 10% FBS containing 10 μ g/ml of the anti-TLR-2 mAb or control mAb, and cultured for 72 hours prior to by flow cytometric analysis using fluorescein-labeled CD1a (CBT6, Ancell) or CD1c (M241, Ancell) or isotype control Abs (MOPC-21, BD Pharmingen). To measure CD1a induced T cell activation, activated monocytes were incubated with 50,000 CD1a autoreactive T cells (BC2) in 96 well plates for 24 hours, followed by measurement of interferon- γ by capture ELISA (Invitrogen).

Screening and confirmatory cytokine assays

Human monocytes were treated with triacyl-CSK₄ for 2 hours, 50 μ l of each supernatant were screened for IL-1 β , IL-6, IL-8, IL-10, IL-12p70, IL-18, IFN α , TNF α and GM-CSF by a multiplexed sandwich-ELISA system (Pierce Endogen). To confirm the presence of cytokines detected in the initial screen, IL-1 β was measured by sandwich ELISA using the M421B capture mAb (2 μ g/ml) and the biotin-labeled mAb M421BB, with a streptavidinhorseradish peroxidase (Pierce Endogen). GM-CSF was detected in sandwich ELISA after capture (Pierce Endogen M500A-E) and development with biotinylated anti-GM-CSF (M501B) and avidin AKP.

Conditioning and transfer of supernatants

To measure secreted factors, experiments were carried out in a 3 step protocol whereby monocytes: 1) were pulsed with TLR agonists and washed 2) cultivated in media to obtain conditioned supernatants enriched with soluble factors 3) and conditioned supernatants were transferred to fresh cells for measurement of CD1 in the presence or absence of reagents that block cytokine function. For pulsing, fresh monocytes were treated with synthetic 3-bis(palmitoyloxy)-(2-RS)-propyl-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys(4)-OH, triacyl-CSK₄, 100 ng/ml, EMC Microcollections, Germany) in 24-well plates (10⁶ cells and 1 ml of media per well), for 10 minutes to 6 hours followed by 3 washes. For conditioning supernatants, fresh media (1 ml per well) was added and the cells (10⁶ well) were cultured for an additional 3 days. For the measurement phase, fresh monocytes (10⁶/well) were cultured (0.9 ml/well) with previously conditioned media for 3 days before flow cytometric analysis.

Monocyte stimulation

Fresh monocytes were treated with triacyl-CSK₄ (500 ng/ml) in the presence or absence of ATP (1 mM). IL-1 β production was analyzed after 24 hours of stimulation by immunoblotting and CD1 induction was analyzed after 72 hours of stimulation. For immunoblot analysis, monocytes were lysed in 50 mM Tris, pH 7.5, 1% (vol/vol) Triton X-100, 150 mM NaCl, 10% (vol/vol) glycerol, 1 mM EDTA and a protease inhibitor 'cocktail'. Proteins were separated by electrophoresis through NuPAGE gels and were transferred onto nitrocellulose membranes.

Membranes were blocked for 1 hour with 5% (wt/vol) milk proteins in 1 $\times$ PBS and 0.5% (vol/vol) Tween-20, then were blocked overnight with 5% (wt/vol) BSA in Tris-buffered saline with Tween and stained with a mouse polyclonal antibody to human IL-1 β (Santa Cruz Biotechnology) and a horseradish peroxidase-conjugated goat antibody to mouse immunoglobulin (Jackson ImmunoResearch) followed by ECL detection (Pierce).

Infection and analysis of human skin explants

Normal discarded skin from plastic surgery under the Partners Institutional Review Board oversight was aseptically trimmed into 6 mm² pieces into which 5 $\times$ 10⁴ of live *B. burgdorferi* GFP in 50 μ l was injected and incubated in complete RPMI medium at concentration of 10⁶ spirochetes/ml in 4ml per well for 72 hours [25]. Skin samples were frozen in Optimal Cutting Temperature Compound cut into sections (5 microns), plated on glass slides, fixed in 3% paraformaldehyde for 2 minutes followed by 70% ethanol for 2 minutes at 4 $^{\circ}$ C, washed with PBS and blocked with goat serum for 1 hour before incubation with primary antibodies, followed by an Alexa Fluor 546 F(ab')₂ fragment of goat anti-mouse IgG (1:500 dilution) (Invitrogen). Slides were treated Hoechst 33342 dye (Invitrogen) prior to acquiring images with a Nikon Eclipse 800 confocal microscope, digitally captured using a SPOT RT digital camera, and compiled using Adobe Photoshop software. Digital images of 10 non-overlapping fields from epidermal layer and 10 non-overlapping fields from dermal layer were randomly taken from each skin section and examined at 200 $\times$ magnification. Total numbers of cells in each field were obtained by counting Hoechst 33342-positive nuclei. CD1-positive cells were defined as having distinct visible surface pattern and punctate red staining. Numbers of CD1-positive cells were evaluated in the dermis and epidermis in a blinded manner by two experienced researchers. Four hundred cells were evaluated for each CD1 molecule dermal expression for each study condition. The χ^2 test was used to evaluate statistical significance of the differences in CD1 expression between infected and non-infected skin samples. *P*-values of <0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. CD1 expression on human skin infected with *B. burgdorferi*

(A–B) Sections of frozen skin taken from a Lyme disease patient (A) or normal donors with ex vivo *B. burgdorferi* infection (*B. burgdorferi*) and without (Medium) (B) were analyzed by indirect fluorescence with Ab to CD1a, CD1b, CD1c or CD1d. Samples were stained with blue Hoechst dye to image nuclei and rete patterns that distinguish dermis from epidermis. (C) The numbers of CD1a, CD1b and CD1c positive cells (B) were counted in a blinded fashion by two observers at 200× magnification and expressed as a percentage of total cells based on 1000 or more cells. Results are representative of three independent infection experiments. Statistical differences were evaluated using the Student's *t* test.

Figure 2. *B. burgdorferi* lipids induce group 1 CD1 cell surface protein expression on human monocytes

(A–B). Human monocytes were treated for 3 days with *B. burgdorferi* total lipid extract (1 µg/ml or indicated concentration) prior to FACS analysis. Monocytes were isolated by adherence to plastic and gated by forward and side scatter and for cells that exclude propidium iodide (Fig. S3). Data shown are representative of more than 3 experiments. (C) Human monocytic antigen presenting cells (APC) with no treatment or exposed to *B. burgdorferi* lipids for 3 days were incubated with the CD1a autoreactive T cell line (BC2) and γ -interferon release was measured. (D) Human monocytes were pre-incubated with an anti-TLR-2 mAb (T2.5) or isotype control Ab (T2.13) for 30 min then treated for 3 days with either borrelia cell wall lipids (1 µg/ml) or medium alone, prior to staining with antibodies specific for CD1a and CD1b and conjugated to fluorescent markers (CB-T6, Ancell; M241, Ancell). Data shown are representative of 2 or more experiments.

Figure 3. Human monocytes produce soluble CD1-inducing factors in response to TLR-2 agonists

(A) Fresh monocytes were treated with control medium only or with synthetic TLR-2 agonist (triacyl-CSK₄; 100 ng/ml) for the indicated time-periods, and CD1a-expressing cells were measured by flow cytometry. (B) Triacyl-CSK₄ (100 ng/ml) was pulsed onto monocytes for time periods ranging from 10 to 360 minutes, then washed, and the cells cultured in media for 3 days before measurement of CD1a- positive cells by flow cytometry. This was compared with cells exposed to triacyl-SK₄ for the entire 72 hour (4320 min) time period. (C) Conditioned supernatants from monocytes treated with triacyl CSK₄ were labeled according to the duration of conditioning and transferred onto fresh monocytes. Fresh monocytes were incubated with conditioned supernatants or triacyl-CSK₄ for 72 hours and CD1a-positive cells were assessed by FACS analysis. As a control to see if residual triacyl-SK₄ was carried over in conditioned supernatants, we treated cells with triacyl CSK₄ and immediately washed them three times, collected buffer comprising the supernatant from third wash (wash buffer) and transferred it to fresh cells. Data shown are representative of more than 3 experiments.

Figure 4. Cleaved IL-1β selectively induces group 1 CD1 protein expression

(A–B) Monocytes were treated with tri-acyl-CSK4 at the indicated concentrations for 24h and the amount of GM-CSF (A) and IL-1β (B) in the supernatant measured by capture ELISA. (C) Monocytes were treated with the recombinant mature IL-1β at the concentrations indicated and the percentage of cells expressing CD1a, CD1b, CD1c, or CD1d, assessed by FACS analysis. The data are expressed in a normalized form so that the channel with the most cells is designated 100 % of maximal (MAX). (D) Monocytes were cultured for 24h with the indicated dose of triacyl-SK₄ and ATP (1 mM), either alone or in combination. Total lysates were prepared and western blotted for IL-β. (Left panel) or CD1a-positive cells were assessed by FACS (right panel). Data shown are representative of more than 3 experiments.

Table 1

Clinical and laboratory findings in patients with EM*

Patient	EM culture for <i>Bb</i>	OspC type of isolate	Sero-positive	<i>Bb</i> DNA in blood	Multiple EM lesions	>6 assoc. symp	High CD1b, c expression in EM lesion
A	+	A	+	+	+	+	+
B	-	N/A	+	-	-	-	-
C	+	K	+	-	-	-	-
D	+	K	+	-	-	-	-
E	+	A	+	+	-	+	-
F	+	K	+	+	+	+	-
G	+	A	+	-	-	-	-
H	+	B	-	-	-	-	-
I	+	K	+	-	-	-	-
J	+	A	+	-	-	-	+

* + = positive, - = negative, N/A = not available