nature medicine

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells

Stephan R Krutzik[1,2], Belinda Tan[1,2], Huiying Li[3], Maria Teresa Ochoa[1], Philip T Liu[2], Sarah E Sharfstein[1], Thomas G Graeber[3], Peter A Sieling[1], Yong-Jun Liu[4], Thomas H Rea[5], Barry R Bloom[6] & Robert L Modlin[1,2]

Leprosy enables investigation of mechanisms by which the innate immune system contributes to host defense against infection, because in one form, the disease progresses, and in the other, the infection is limited. We report that Toll-like receptor (TLR) activation of human monocytes induces rapid differentiation into two distinct subsets: DC-SIGN$^+$ CD16$^+$ macrophages and CD1b$^+$ DC-SIGN$^-$ dendritic cells. DC-SIGN$^+$ phagocytic macrophages were expanded by TLR-mediated upregulation of interleukin (IL)-15 and IL-15 receptor. CD1b$^+$ dendritic cells were expanded by TLR-mediated upregulation of granulocyte-macrophage colony-stimulating factor (GM-CSF) and its receptor, promoted T cell activation and secreted proinflammatory cytokines. Whereas DC-SIGN$^+$ macrophages were detected in lesions and after TLR activation in all leprosy patients, CD1b$^+$ dendritic cells were not detected in lesions or after TLR activation of peripheral monocytes in individuals with the progressive lepromatous form, except during reversal reactions in which bacilli were cleared by T helper type 1 (T_H1) responses. In tuberculoid lepromatous lesions, DC-SIGN$^+$ cells were positive for macrophage markers, but negative for dendritic cell markers. Thus, TLR-induced differentiation of monocytes into either macrophages or dendritic cells seems to crucially influence effective host defenses in human infectious disease.

The ability of the innate immune system to recognize microbial pathogens is mediated by highly conserved families of pattern recognition receptors that activate host defense pathways. One such family is comprised of the TLRs, which are expressed by a variety of cells of the innate immune system; each TLR family member is endowed with the ability to recognize a distinct class of conserved microbial molecules. The expression and activation of TLRs contributes to host defense against infection in Drosophila, mice and humans[1–5]. There are two different mechanisms by which activation of TLRs can contribute to host defense. First, activation of TLRs can directly mediate innate responses by regulating phagocytosis and triggering antimicrobial activity[6–8]. Second, activation of TLRs can trigger the release of cytokines and the differentiation of immature to mature dendritic cells, enabling the innate immune system to instruct the adaptive immune response[9–11].

Cells of the innate immune system are characterized in normal and diseased tissues according to various cell-surface receptors. The group I CD1 molecules, CD1a and CD1b, are specifically expressed by tissue- and cytokine-derived dendritic cells and facilitate the presentation of nonpeptide antigens to T cells[12–15]. The calcium-dependent (C-type) lectins, dendritic cell–specific ICAM-grabbing nonintegrin (DC-SIGN) and the mannose receptor are expressed on both dendritic cells and macrophages, functioning in phagocytosis and antigen presentation[16,17]. DC-SIGN contains a carbohydrate-recognition domain, enabling cytokine-derived dendritic cells to recognize and mediate phagocytosis or uptake of a broad range of pathogens including HIV and species of *Mycobacteria*, *Leishmania*, *Candida* and *Helicobacter*[17–22].

Leprosy provides an opportunity to investigate mechanisms by which the innate immune system contributes to host defense against infection. The disease presents as a clinical spectrum that correlates with the level of the immune response to the pathogen[23]. At one pole of the disease, individuals with the tuberculoid form (T-lep) are relatively resistant to the pathogen, the infection is localized and the lesions are characterized by expression of T_H1 cytokines characteristic of cell-mediated immunity[24,25]. In contrast, individuals with lepromatous leprosy (L-lep) are relatively susceptible to the pathogen, the infection is systemically disseminated and the lesions characterized by T helper type 2 (T_H2) cytokines characteristic of humoral responses. The spectrum of human leprosy is not static, as those individuals with the L-lep form of the disease can upgrade toward the T-lep pole during a reversal reaction, which is a cell-mediated immune reactions characterized by the reduction of bacilli in lesions and the influx of CD4$^+$ T_H1 cells into disease lesions[26]. We therefore studied leprosy as a model to investigate general mechanisms by which the innate immune system regulates direct and indirect effector pathways, specifically by exploring the differentiation, distribution and function of CD1$^+$ and DC-SIGN$^+$ cells.

[1]Division of Dermatology, [2]Department of Microbiology and Immunology, David Geffen School of Medicine at UCLA and [3]Department of Chemistry and Biochemistry, UCLA-Department of Energy Institute of Genomics and Proteomics, UCLA, 611 Charles Young Drive East, 536 Boyer Hall, Los Angeles, California 90095, USA. [4]Department of Immunology and Center for Cancer Immunology Research, M.D. Anderson Cancer Center, University of Texas, 1515 Holcombe Boulevard, Houston, Texas 77030, USA. [5]Section of Dermatology, University of Southern California School of Medicine, 2020 Zonal Avenue, IRD 620, Los Angeles, California 90033, USA. [6]Office of the Dean, Harvard School of Public Health, 677 Huntington Avenue, Kresge Building 1005, Boston, Massachusetts 02115, USA. Correspondence should be addressed to R.M. (rmodlin@mednet.ucla.edu)

Published online 8 May 2005; doi:10.1038/nm1246

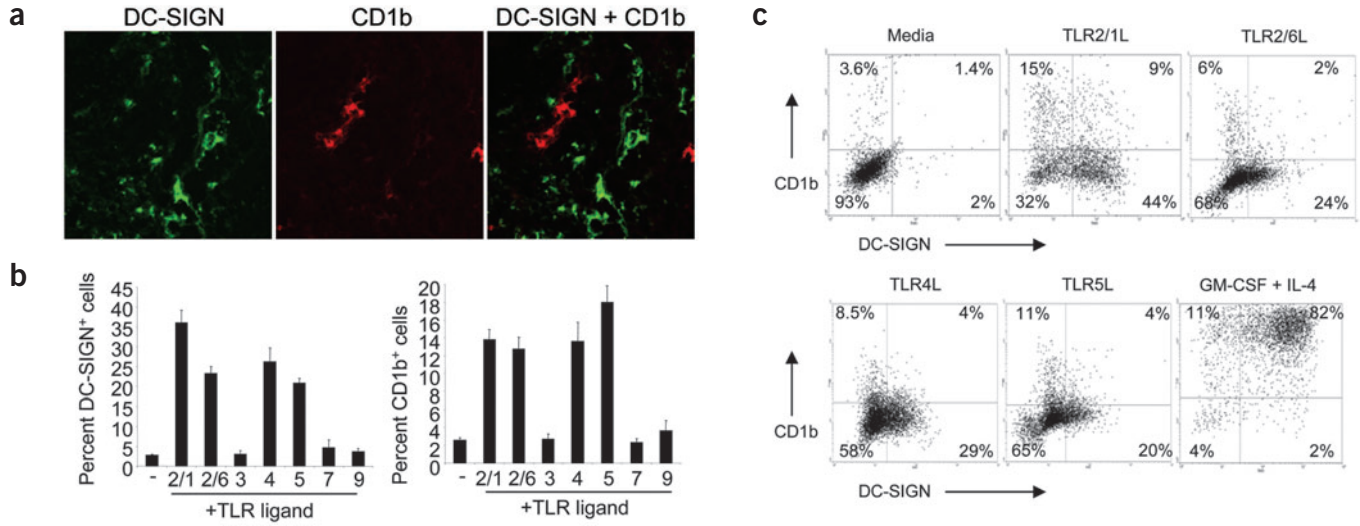

Figure 1 DC-SIGN and CD1b are expressed on distinct subsets of cells and are induced by TLR activation. (**a**) Human tonsil tissue sections were labeled with specific antibodies and visualized using confocal laser microscopy. Original magnification, ×40. (**b**) Human peripheral monocytes were activated with TLR ligands for 48 h and labeled with specific antibodies. Data are shown as the mean ± s.e.m. of between 4 and 14 independent experiments. (**c**) Human peripheral monocytes were stimulated with TLR ligands for 48 h and double labeled. Percent of cells in each quadrant are indicated. Data are representative of between 3 and 14 independent experiments.

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

RESULTS

Expression of DC-SIGN and CD1b *in situ* and *in vitro*

Given the distinct functions of DC-SIGN (antigen uptake) and CD1b (antigen presentation) on cells of the innate immune system, we examined the *in situ* distribution of DC-SIGN and CD1b in human lymphoid tissue. Based on studies of cytokine-derived dendritic cells, we expected to find cells coexpressing DC-SIGN and CD1b. Notably, DC-SIGN and CD1b were expressed on distinct nonoverlapping cell populations (**Fig. 1a**).

This finding raised the question of whether activation of the innate immune system could lead to the expansion of these two distinct cell populations expressing DC-SIGN or CD1b. Given the role of TLRs in recognizing microbial patterns and activating the innate immune system, we focused on this receptor family. Human peripheral monocytes were activated with ligands known to trigger specific TLRs: *Mycobacterium tuberculosis* 19 kDa lipopeptide (TLR2/1)[5,10], MALP-2 (TLR2/6), poly(I:C) (TLR3), lipopolysaccharide (TLR4), flagellin (TLR5), imiquimod (TLR7) and bacterial CpG (TLR9). At time 0, less than 2% of the peripheral monocytes expressed DC-SIGN or CD1b. After 48 h, ligands for TLR2/1, TLR2/6, TLR4 and TLR5 induced expression of both DC-SIGN (18–53%) and CD1b (5–23%; **Fig.1b**). Response to these ligands correlated with the TLR expression pattern of human peripheral monocytes[27].

When TLR-activated monocytes were labeled with DC-SIGN and CD1b, two major and distinct populations of cells were identified: DC-SIGN$^+$ CD1b$^-$ and DC-SIGN$^-$ CD1b$^+$ (**Fig. 1c**). In addition, a third population of DC-SIGN$^+$ CD1b$^+$ were variably identified *in vitro* (1–9%), but never observed in lymphoid tissue. In contrast, after cotreatment with recombinant GM-CSF and recombinant IL-4, 80–90% of cells were DC-SIGN$^+$ CD1b$^+$ (**Fig. 1c**). These data indicate that DC-SIGN$^+$ cells and CD1b$^+$ cells represent two distinct subsets *in vivo*, and can be rapidly induced by activation of TLRs on peripheral monocytes *in vitro*.

Mechanism of TLR-induced differentiation

To identify potential autocrine or paracrine signaling pathways that could mediate the TLR-induced differentiation, we performed DNA microarray experiments on monocytes treated with either media or the mycobacterial 19 kDa lipopeptide TLR2/1 ligand for 0, 3, 6, 12 or 24 h. Using the Database of Ligand-Receptor Partners, which contains 455 known interacting protein ligand-receptor pairs, we searched for those pairs with correlated gene expression in the TLR2/1-treated monocytes using a previously described computational algorithm[28]. Based on this analysis, we discovered 78 ligand-receptor pairs that showed correlated gene expression. We focused on those cytokine genes that showed correlated gene expression and were significantly induced after activation of TLR2/1 ($P < 0.5$). These criteria identified five cytokine ligand-receptor pairs (**Fig. 2a**). We also identified cytokine ligand-receptor pairs that were correlatively downregulated or had an anticorrelated expression pattern (**Supplementary Fig. 1** online).

Monocytes stimulated with recombinant IL-15 expressed DC-SIGN (25–60%) but not CD1b, whereas monocytes stimulated with recombinant GM-CSF expressed CD1b (60–80%) and very little or no DC-SIGN (**Fig. 2b**). Cells stimulated with both recombinant GM-CSF and recombinant IL-15 showed an expression pattern similar to that found after TLR activation (**Fig. 2b**). Recombinant IL-1 receptor antagonist and recombinant IL-5 had no effect on the expression of DC-SIGN or CD1b (data not shown).

Identical to the pattern of DC-SIGN and CD1b induction in **Fig. 1b**, we found that ligands for TLR2/1, TLR2/6, TLR4 and TLR5 induced the expression of *Il15* and *Csf2* mRNA, which encode IL-15 and GM-CSF, respectively (**Fig. 2c**). Furthermore, addition of an IL-15–blocking antibody inhibited TLR-induced DC-SIGN by 60–90% (but not CD54, data not shown) and addition of an antibody specific for GM-CSF blocked TLR-induced CD1b expression by 75–80% (**Fig. 2d**). Therefore, the mechanism by which TLR activation triggers differentiation of monocytes into DC-SIGN$^+$ and CD1b$^+$ populations is through the upregulation of cytokine-receptor pairs: the IL-15–IL-15 receptor pair is responsible for differentiation into DC-SIGN$^+$ cells and the GM-CSF–GM-CSF receptor pair is key for differentiation into CD1b$^+$ cells.

Based on the expression of CD16, two distinct populations of human peripheral monocytes have been identified. TLR2/1-induced

differentiation of monocytes into CD1b$^+$ cells was restricted to the CD16$^+$ versus CD16$^-$ subset, whereas recombinant GM-CSF–induced differentiation into CD1b$^+$ cells was found in both monocyte populations (**Fig. 2e** and **Supplementary Note** online). CD16 does not have a role in the differentiation process, as addition of a neutralizing antibody specific for CD16 had no effect on TLR2/1-induced CD1b expression (**Fig. 2f**). In contrast, the ability of TLR2/1 and recombinant IL-15 to trigger differentiation of monocytes in DC-SIGN$^+$ cells was enhanced in the CD16$^-$ versus the CD16$^+$ subset.

Cells expressing DC-SIGN have a macrophage phenotype

The DC-SIGN$^+$ cells had a macrophage-like phenotype, expressing higher levels of CD14 (TLR coreceptor), CD16 (FcγRIII), CD32 (FcγRII) and CD64 (FcγRI), even though they derived from CD16$^-$ precursors (**Fig. 3a**). In contrast, CD1b$^+$ cells had an immature dendritic cell phenotype with higher expression of CD1a (antigen presentation molecule), CD206 (mannose receptor), CD86 (B7.2, costimulatory molecule) and CD40 (T cell costimulation) as well as low expression of CD14 and CD16 (**Fig. 3a**). The level of TLR2 was found to be higher on the DC-SIGN$^+$ cells. Meanwhile, levels of MHCII (antigen presentation molecule), CD54 (ICAM-1, adhesion), CD80 (B7.1, costimulatory molecule) and CD11c (myeloid marker) were similar between the two populations, whereas both populations were negative for the tissue macrophage marker CD163 and the mature dendritic cell markers CD83 and DEC-205. Identical expression patterns were observed upon activation with ligands for TLR2/6, TLR4 and TLR5, although we found that activation through TLR4 induced the expression of CD163 on both cell populations (**Fig. 3b**). Furthermore, activation of monocytes with a second TLR2/1 ligand, the triacylated lipopeptide Pam_3CSK_4, also resulted in distinct DC-SIGN$^+$ and CD1b$^+$ cell populations with a similar expression profile (**Supplementary Fig. 2** online). We observed similar cell-surface expression profiles when we cultured monocytes with recombinant IL-15 or recombinant GM-CSF, allowing us to use recombinant cytokines in subsequent experiments to differentiate monocytes into the distinct populations found following activation with TLR ligands (data not shown). These data indicate that TLR activation induces two distinct cell types; cells expressing DC-SIGN have a macrophage-like phenotype, whereas those expressing CD1b have an immature dendritic cell phenotype.

DC-SIGN$^+$ macrophages bind and phagocytose mycobacteria

DC-SIGN is thought to be involved in the direct binding of mycobacteria[19,18]. We therefore compared the ability of DC-SIGN$^+$ macrophages and CD1b$^+$ dendritic cells to bind Bacille Calmette-Guérin (BCG). Monocytes were differentiated with recombinant GM-CSF or recombinant IL-15 and then cultured with BCG expressing green fluorescent protein (GFP). We found that across a multiplicity of infection (MOI) between 10 and 40, approximately 3–4% of DC-SIGN$^+$ cells bound BCG, whereas only 0.5–1.2% of CD1b$^+$ cells bound BCG (**Fig. 4a**). This binding was blocked by 40–60% in the presence of a neutralizing antibody specific for DC-SIGN (**Fig. 4b**). These data indicate that DC-SIGN facilitates the ability of macrophages to bind mycobacteria.

As binding of the bacteria to the cell is necessary but not sufficient for phagocytosis, the ability of DC-SIGN$^+$ and CD1b$^+$ cells to phagocytose BCG was compared by incubating the different cell populations with mycobacteria for 18 h. Analogous to the binding experiments, DC-SIGN$^+$ cells were more phagocytic than CD1b$^+$ cells, particularly at a MOI of 5 or 10, at which up to 80% of the DC-SIGN$^+$ cells had taken up BCG, compared to 30% of the CD1b$^+$ cells (**Fig. 4c**). Finally,

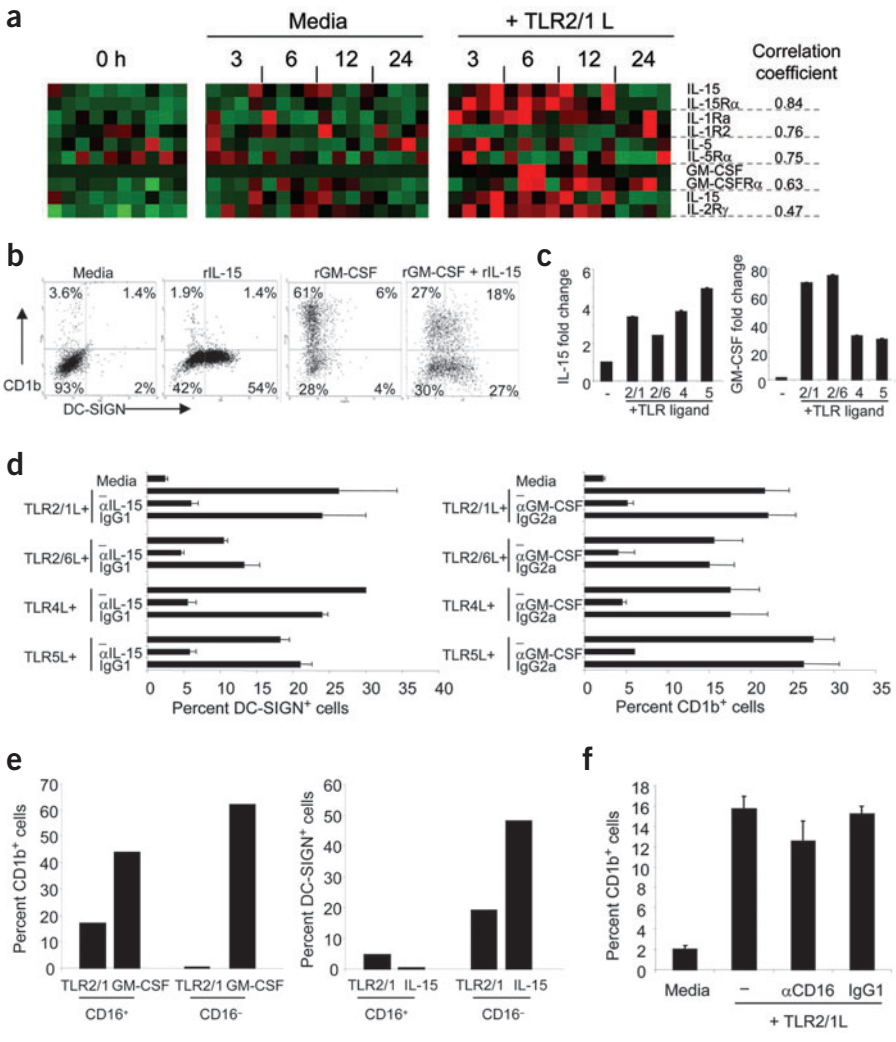

Figure 2 IL-15 and GM-CSF induce monocyte differentiation. (**a**) TLR2/1 activation triggers cytokine ligand-receptor pairs with correlated expression. Each column represents a single donor. (**b**) Monocytes were cultured with recombinant cytokines. Representative data shown ($n = 7$). rIL-15, recombinant IL-15; rGM-CSF, recombinant GM-CSF. (**c**) mRNA levels of TLR-stimulated monocytes (mean of duplicate wells ± s.d. and representative of two donors). (**d**) Monocytes were stimulated with TLR ligands. Data are represented as mean percent positive cells ± s.e.m. from between two and five experiments. (**e**) Monocyte subpopulations were activated with either the TLR2/1 ligand or cytokines and represent data from two independent experiments. (**f**) Monocytes were activated with the 19 kDa TLR2/1 ligand in various conditions. Data represent the mean ($n = 3$) ± s.e.m.

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

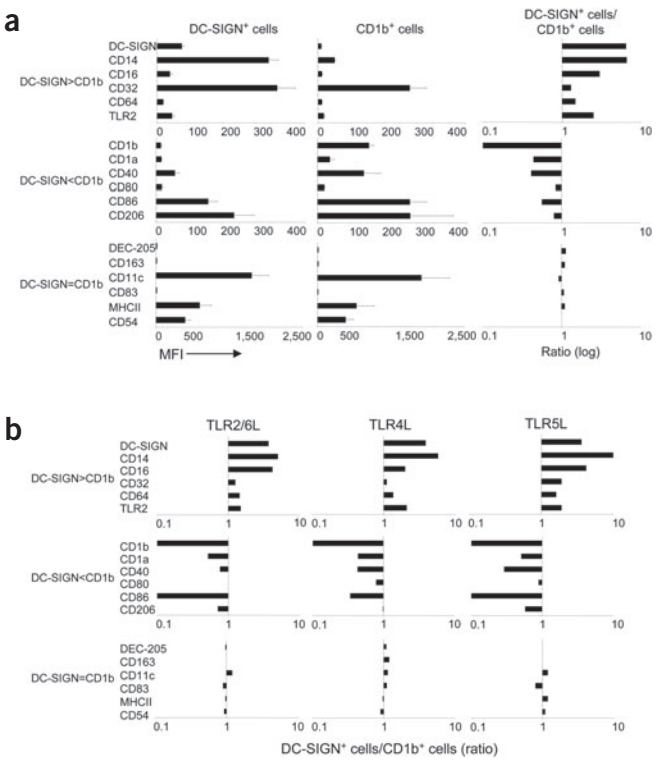

Figure 3 DC-SIGN+ cells have a macrophage phenotype whereas CD1b+ cells have a dendritic cell phenotype. (**a**) Monocytes stimulated with the 19 kDa TLR2/1 ligand were labeled with DC-SIGN or CD1b together with the indicated markers. Data are shown as mean fluorescence intensity (MFI) of at least four independent experiments ± s.e.m. (left and center). Data are also represented as a ratio of expression (right) on DC-SIGN+ cells versus CD1b+ cells. (**b**) Monocytes were stimulated with TLR ligands, labeled as in **a** and are represented as a ratio of expression based on two to three independent experiments.

by confocal laser microscopy, BCG was shown to be intracellular, and not bound simply to the cell membrane (**Fig. 4d**). Together, these data suggest that DC-SIGN+ macrophages are more efficient at the binding and phagocytosis of mycobacteria than immature dendritic cells.

CD1b+ dendritic cells produce cytokine and activate T cells

After TLR activation, CD1b+ cells were more potent producers of cytokine as compared to DC-SIGN+ cells, secreting 14-fold more IL-12p40 and 6-fold more TNF-α (**Fig. 5a**). Neither cell subset produced measurable levels of IL-10 (data not shown).

In a mixed-lymphocyte reaction, CD1b+ cells were more potent than DC-SIGN+ cells, inducing between 10- and 700-fold greater T cell proliferation across a range of T cell/antigen-presenting cell ratios (**Fig. 5b**). The ability of CD1b+ dendritic cells and DC-SIGN+ macrophages to present antigen to an major histocompatibility complex class II (MHCII)-restricted T cell clone, D103.5, which recognizes a peptide from the *Mycobacterium leprae* 10 kDa GroES, was also tested. Despite having similar levels of MHCII on the cell surface, CD1b+ cells triggered approximately 20–50-fold more T cell proliferation and 10-fold more production of interferon (IFN)-γ compared to DC-SIGN+ cells (**Fig. 5c**). These results suggest that CD1b+ dendritic cells are substantially more potent activators of adaptive T cell responses than DC-SIGN+ macrophages.

Expression of DC-SIGN and CD1b in human leprosy

To understand the significance of TLR-induced monocyte differentiation in a disease model we investigated the capacity of peripheral monocytes from leprosy patients to differentiate after activation with the mycobacterial TLR2/1 ligand. Similar to healthy donors, monocytes from T-lep patients were able to differentiate into both DC-SIGN+ macrophages (28–61%) and CD1b+ dendritic cells (8–10%; **Fig. 6a**). Notably, althogh peripheral monocytes from L-lep patients differentiated into DC-SIGN+ macrophages (33–66%), they did not differentiate into CD1b+ dendritic cells (1–3%; **Fig. 6a**). In those L-lep patients undergoing reversal reaction, both DC-SIGN+ macrophages (38–61%) and CD1b+ dendritic cells (6–25%) were detected at frequencies similar to those seen in T-lep patients. In all patients, cytokine-stimulated monocytes were able to differentiate into CD1b+ dendritic cells (**Fig. 6b** and **Supplementary Fig. 3** online). Clearly, peripheral monocytes from L-lep patients activated through TLR receptors are able to differentiate into DC-SIGN+ macrophages but not CD1b+ dendritic cells.

The *in vivo* relevance of these differentiation pathways was tested according to the expression of DC-SIGN and CD1b in leprosy skin lesions. Both DC-SIGN+ and CD1b+ cells were present in T-lep lesions

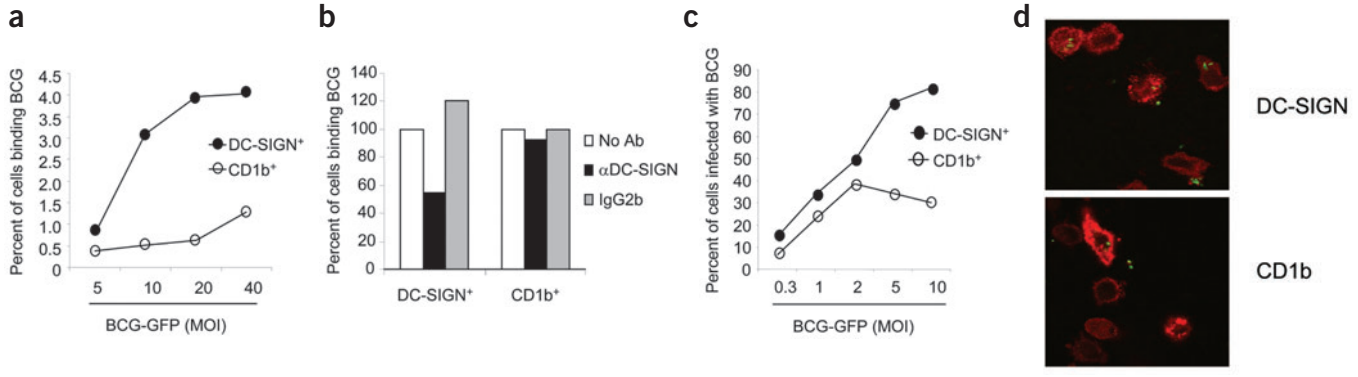

Figure 4 DC-SIGN+ macrophages bind and phagocytose mycobacteria. (**a**) Cytokine-differentiated monocytes were cultured with *Mycobacterium bovis* BCG. Data represent percent of cells that are double positive for either DC-SIGN and BCG-GFP (solid circle) or CD1b and BCG-GFP (open circle) from two independent experiments. (**b**) Binding was measured in the presence of a DC-SIGN blocking antibody or controls. Data are represented as percent of binding relative to media control. (**c**) DC-SIGN+ and CD1b+ cells were incubated with BCG-GFP and uptake was measured by double labeling. Data is representative from two independent experiments. (**d**) Confocal images of cells cultured and labeled as in **c** (DC-SIGN or CD1b, red; BCG-GFP, green).

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

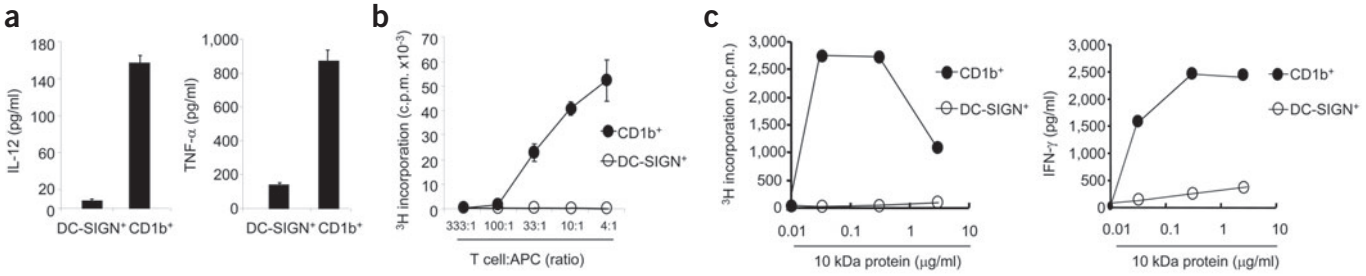

Figure 5 CD1b$^+$ dendritic cells produce cytokines and are potent T-cell activators. (**a**) Cytokine-differentiated monocytes were enriched for cells expressing DC-SIGN or CD1 and stimulated with the TLR2/1 ligand. Data represent the average of triplicate wells of two independent experiments ± s.e.m. (**b**) We obtained cells as in **a**, plated them with unmatched T cells and measured proliferation (CD1b$^+$ cells, solid circle; DC-SIGN$^+$ cells, open circle). Data are representative of two independent experiments. APC, antigen-presenting cell. (**c**) Antigen-presenting cells added together with various concentrations of peptide and a MHCII-restricted T cell line, D103.5. Data are representative of two independent experiments.

(**Fig. 6c**). In contrast, DC-SIGN$^+$ but not CD1b$^+$ cells were found in L-lep lesions. In those L-lep patients undergoing reversal reaction, both DC-SIGN$^+$ and CD1b$^+$ cells were detected at frequencies similar to T-lep lesions, consistent with the observed gain of cell-mediated immunity and local CD4$^+$ T_H1 responses.

Further examination of leprosy skin lesions using confocal laser microscopy showed that within T-lep lesions, DC-SIGN and CD1b are expressed on distinct, nonoverlapping cell populations (**Fig. 6d**). The DC-SIGN$^+$ cells were found to express the monocyte and macrophage markers CD14, CD16, CD64 and CD68 (**Fig. 6e**), but did not express the dendritic cells markers CD1a, CD1b, CD63, CD83 or DC-LAMP (**Fig. 6f**). Our analysis of T-lep lesions showed only a small number (<5%) of DC-SIGN$^+$ cells that were positive for the dendritic cell markers examined. Identical results were obtained during our analysis of human tonsil (**Supplementary Fig. 4** online). These data indicate that the majority of DC-SIGN$^+$ cells express cell surface markers typically found on monocytes and macrophages.

To ascertain whether DC-SIGN$^+$ macrophages identified in L-lep lesions contained *M. leprae*, lesions were labeled using monoclonal antibodies against DC-SIGN and the *M. leprae* 22 kDa bacterioferritin major membrane protein II (MMPII). Confocal laser microscopy showed that indeed DC-SIGN$^+$ macrophages containing *M. leprae* were found in L-lep lesions (**Fig. 6g**). *M. leprae* was also found in CD16$^+$ DC-SIGN$^-$ cells, indicating that there are different subsets of macrophages in leprosy lesions. As expected, mycobacteria were not detected in T-lep lesions. These data indicate important differences between the forms of leprosy: T-lep lesions contain DC-SIGN$^+$ macrophages and CD1b$^+$ dendritic cells, whereas L-lep lesions contain *M. leprae*-infected DC-SIGN$^+$ macrophages but lack CD1b$^+$ dendritic cells.

DISCUSSION

The innate immune system triggers both direct and indirect effector pathways to combat microbial pathogens. First, the innate immune response acts directly to localize the invading pathogen so it can be destroyed by antimicrobial mechanisms. Second, the innate immune response acts indirectly, through cytokine release and upregulation of costimulatory molecules, to induce adaptive T and B cell response. Here we show that activation of Toll-like receptors causes the rapid differentiation of distinct precursors of human peripheral monocytes into DC-SIGN$^+$ and CD1b$^+$ cells. The DC-SIGN$^+$ cells have a macrophage-like phenotype, are phagocytic, and use DC-SIGN to facilitate the uptake of bacteria. In contrast, CD1b$^+$ cells have an immature dendritic cell phenotype, release proinflammatory cytokines and function as efficient antigen-presenting cells. These data provide evidence that the dual host defense roles of the innate immune system are mediated by the induction of two distinct phenotypic and functional populations, DC-SIGN$^+$ macrophages and CD1b$^+$ dendritic cells (**Supplementary Fig. 5** online). Furthermore, the DC-SIGN$^+$ macrophages derived *in vitro* were identical in phenotype to the DC-SIGN$^+$ cells found *in situ* in human disease lesions, expressing CD16, CD64, but not expressing CD1a, CD1b, CD83, providing *in vivo* relevance for these distinct subsets in innate immunity.

As we previously showed that *M. leprae* predominantly activates TLR2/1, we investigated activation of the heterodimer and whether the regulated expression of TLR2/1 in lesions correlated with the clinical form of the disease[5]. Notably, TLR2/1-activation of peripheral monocytes from L-lep patients triggered differentiation into DC-SIGN$^+$ macrophages but not CD1b$^+$ dendritic cells, whereas activation of monocytes from T-lep patients triggered differentiation into both effector populations (**Supplementary Fig. 5** online). Correspondingly, DC-SIGN$^+$ macrophages but not CD1b$^+$ dendritic cells were detected in skin lesions from L-lep patients, whereas both cell types were detected in T-lep lesions. Because *M. leprae* was found to be abundant in DC-SIGN$^+$ macrophages in L-lep lesions, we infer that the innate immune system can mediate its direct effect in these patients, phagocytosing the bacteria into macrophages, but is unable to mediate its indirect effect, inducing dendritic cells to stimulate the adaptive T cell response required to kill intracellular pathogens[29]. When L-lep patients upgraded their clinical presentation during 'reversal reactions' characterized by a clearance of bacilli, CD1b$^+$ cells were generated after TLR activation and found to be present in the cutaneous lesions. Given that these lesions show an influx of CD4$^+$ T_H1 cells[26] and dendritic cells are required to activate antigen-specific CD4$^+$ T_H1 cells, the ability of TLR activation to regulate differentiation of monocytes into CD1b$^+$ dendritic cells is likely to be a key immunologic event for host defense.

Our *in situ* studies of leprosy lesions and human tonsil together with our *in vitro* studies of TLR-activated peripheral monocytes suggest that DC-SIGN$^+$ cells have a macrophage phenotype (CD16$^+$, CD64$^+$) but not a dendritic cell phenotype (CD1a$^-$, CD1b$^-$, CD83$^-$). This is consistent with other studies indicating that DC-SIGN is not expressed on human dendritic cells, but is found on monocytes differentiated in the presence of cyclic nucleotides and macrophages in the lymph nodes and at sites of inflammation[30–33]. Our data cannot exclude the possibility that the DC-SIGN$^+$ cells may represent a subset of dendritic cells with a macrophage-like phenotype and function. Nevertheless, the role of DC-SIGN in the phagocytosis of microbes by macrophage-like cells is relevant for the studies of other infectious diseases such as HIV

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

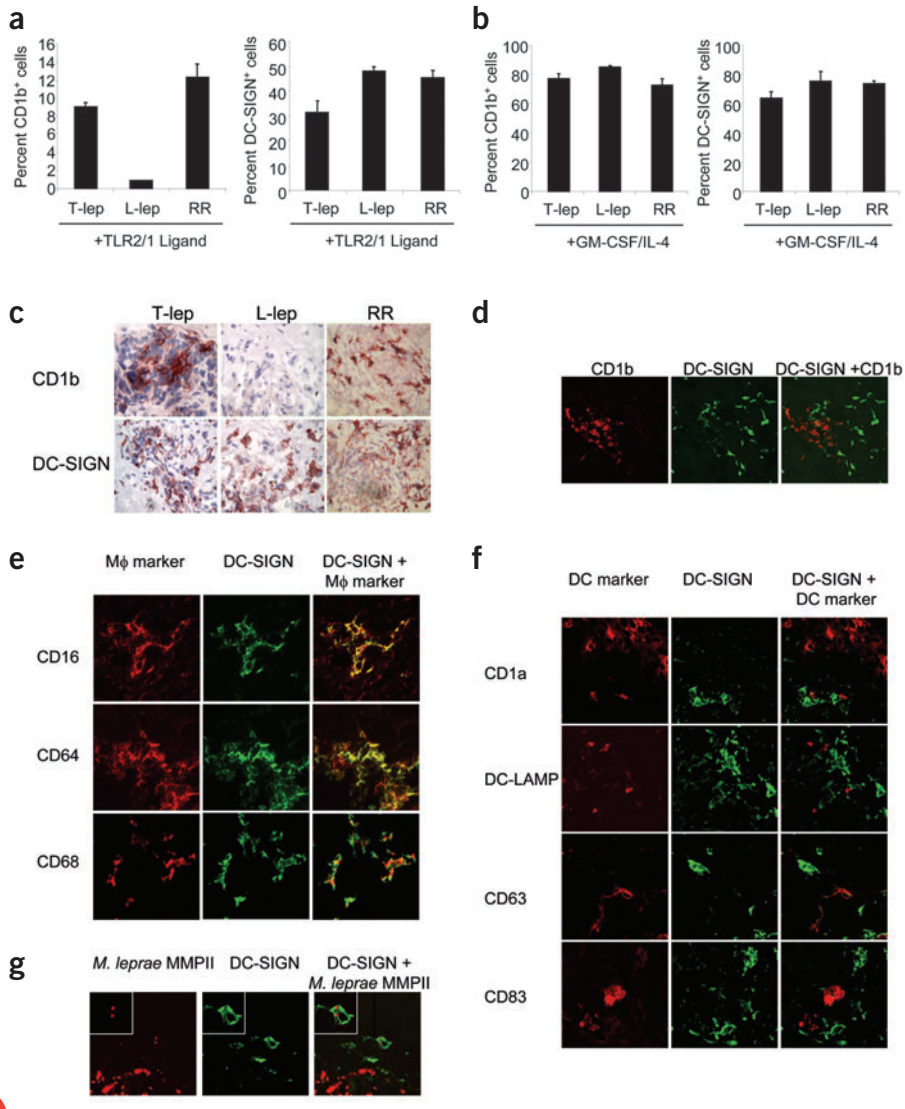

Figure 6 Macrophage and dendritic cell subsets in leprosy. We stimulated monocytes with either (**a**) the 19 kDa TLR2/1 ligand or (**b**) recombinant GM-CSF and recombinant IL-4. Data is represented as cells expressing either DC-SIGN or CD1b relative to unactivated control ± s.e.m. (L-lep, $n = 6$; T-lep, $n = 4$; reversal reaction (RR), $n = 6$). (**c**) We labeled skin biopsy sections from leprosy lesions by the immunoperoxidase method. (**d**) Immunofluorescence confocal images from T-lep lesions. (**e**) Two color confocal images with macrophage markers. (**f**) Confocal images with dendritic cell makers. (**g**) Two color confocal images from L-lep lesions for the *M. leprae* bacterioferritin major membrane protein II (MMPII) and DC-SIGN.

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

infection in which the function of DC-SIGN–mediated uptake is part of the host response[34].

The mechanism by which TLR2/1 activation triggered monocyte differentiation was elucidated using gene expression data and a computational algorithm that identifies potential autocrine and paracrine loops of known receptor and ligand pairs. Together with functional studies, the data showed two distinct regulatory mechanisms responsible for the rapid expansion of DC-SIGN$^+$ cells and CD1b$^+$ cells. The upregulation of GM-CSF and GM-CSF receptor was responsible for the induction of CD1b. Additionally, the upregulation IL-15 and two of its receptor components, IL-15Rα and IL-2Rγ, leads to the induction of DC-SIGN, providing new information about the regulation of this C-type lectin[30,35–37]. Simultaneous addition of both recombinant GM-CSF and recombinant IL-15 led to the differentiation of monocytes into distinct DC-SIGN$^+$ and CD1b$^+$ cells, similar to the pattern we observed following TLR activation[38]. Our investigation of the expression and function of cytokines triggered by TLR2/1 activation shows the powerful utility of using ligand-receptor gene expression–based analysis to identify new autocrine and paracrine pathways of immune regulation.

In conclusion, we show that the dual immunological functions of the innate immune system—capturing and inhibiting pathogens, and presenting antigens to adaptive T and B cell responses—are mediated by distinct phenotypic and functional subsets of cells. DC-SIGN$^+$ macrophages carry out the phagocytic and presumably microbicidal or static activity, and CD1b$^+$ immature dendritic cells serve as are potent cytokine-producing antigen-presenting cells. The study of human leprosy provides evidence that the regulation of these DC-SIGN$^+$ macrophages and CD1b$^+$ dendritic cells correlate and probably contributes to the resistant or susceptible outcome of human intracellular infection.

METHODS

TLR ligands. Lipopolysaccharide (Sigma) and MALP-2 were used as described[39,40]. Flagellin was a gift of K. Smith, University of Washington. We purchased TLR ligands: 19 kDa lipopeptide (EMC Microcollections), imiquimod (Sequoia Research), CpG (Invitrogen), poly(I:C) (Amersham) and Pam_3CSK_4 (EMC Microcollections).

Microarrays. We isolated RNA using TriZol reagent (Invitrogen) and prepared the probe according to the Affymetrix protocol. The UCLA Microarray Core Facility performed the hybridization to Affymetrix U133A Genechip. A total of 10 donors were used with time 0 h samples prepared for each. For time points, we used cells from 4 of the 10 donors to prepare media and TLR2/1-stimulated samples.

Ligand-receptor pair algorithm. The algorithm for identifying autocrine and paracrine signaling from gene expression data has been described[28]. Briefly, for each known cognate ligand-receptor pair from the Database of Ligand-Receptor Partners measured on the gene microarry (434 of the 455 pairs), we determined the Pearson correlation between the ligand and receptor expression profiles.

Differentially expressed genes. We ranked each gene by the probability that the means of its expression values are statistically distinct between media- and TLR2/1 ligand–treated populations using the Student *t*-test. We generally focused on genes meeting our criteria: $P < 0.05$ and fold change >2.0. Diagrams were generated using the Cluster and TreeView software.

Antibodies and cytokines. Antibodies for cell surface molecules were as follows: CD14 (Zymed); CD1a, CD1b (OKT6, Bcd3.1, ATCC); CD1a, CD68 (Dako); C11c, CD15, CD16, CD32, CD54, CD63, CD64, CD80, CD86, CD163, DC-SIGN, CD206 (BD Pharmingen); DEC-205, TLR2 (eBioscience); DC-LAMP (Immunotech); CD83, CD40, MHCII and IgG controls (Sigma). Cytokines used

were as follows: IL-4 (Peprotech), IL-5 (R&D Systems), IL-1Ra (R&D Systems), IL-15 (R&D Systems) and GM-CSF (Immunex).

Monocyte differentiation and enrichment. We obtained whole blood from healthy donors (UCLA Institutional Review Board #92-10-591-31) with informed consent. We stimulated adherent monocytes[5] with media, *Mycobacterium tuberculosis* 19 kDa (10 μg/ml), Pam_3CSK_4 (0.1 μg/ml), poly(I:C) (1 μg/ml), lipopolysaccharide (10 ng/ml), MALP-2 (10 ng/ml), flagellin (1–10 ng/ml), imiquimod (5 μg/ml), CpG DNA (1 μg/ml), recombinant GM-CSF (1–10 U/ml), recombinant IL-15 (200 ng/ml), recombinant IL-1Ra (200 ng/ml) or recombinant IL-5 (200 ng/ml) for 48 h. To enrich for DC-SIGN$^+$ or CD1b$^+$ cells, we cultured monocytes with recombinant IL-15 or recombinant GM-CSF and labeled with a DC-SIGN or CD1 antibody. A MACS microbead-secondary antibody (Miltenyi Biotec) was added for 30 min at 4 °C. Flow-through was designated as the negative population. Remaining cells were designated as the positive population.

To study precursor populations, we enriched monocytes from blood using Rosette Sep monocyte enrichment cocktail (StemCell Technologies). CD16$^+$ monocytes were isolated with an antibody specific for CD16 (BD Pharmingen) and α-mouse IgG1-MACS beads (Miltenyi Biotech).

Patients and clinical specimens. We classified patients with leprosy according to the criteria of Ridley and Jopling[41] and obtained as previously described[5] (I.R.B., University of Southern California School of Medicine).

Cell surface labeling. Surface expression was determined using specific antibodies. A phycoerythrin-conjugated secondary antibody was used for CD1b. Cells were acquired and analyzed as described[5]. For blocking, we stimulated monocytes with TLR ligands in the presence of media, an IL-15-specific (R&D Systems) or GM-CSF–specific antibody (BD Pharmingen) or an isotype control.

Cytokine secretion. We cultured monocytes with either recombinant IL-15 or GM-CSF for 48 h and enriched as above. Cells were activated with the TLR2/1 ligand for 24 h. We measured IL-12 and TNF-α levels by ELISA (Pharmingen).

T cell assays. For the mixed-lymphocyte reaction, DC-SIGN$^+$ and CD1b$^+$ cells were obtained as above, irradiated and plated (3.3×10^2 to 2.5×10^5 cells/well). We obtained T cells from an unmatched donor with Rosette Sep T cell enrichment cocktail (StemCell Technologies). Media or 2×10^5 T cells were added. We measured IFN-γ levels by ELISA (BD Pharmingen). Proliferation was measured as described[14].

For MHCII studies, monocytes were differentiated, enriched and irradiated as above. Cells were cultured with MHCII (D103.5) restricted T-cells (1×10^5) and the 10 kDa antigen derived from *M. leprae* as previously described[42]. We then measured IFN-γ and proliferation.

Binding and phagocytosis. DC-SIGN$^+$ and CD1b$^+$ cells were cytokine derived as above. For binding, BCG-GFP was added at 4 °C for 4 h in antibiotic-free media. Cells were harvested, washed and labeled with specific antibody. For blocking, we pretreated cells with media, an IgG2b isotype or a DC-SIGN–specific antibody (BD Pharmingen) for 1 h at 4 °C. For phagocytosis, we cultured cells with BCG for 16 h and performed double labeling.

Real time qPCR. Monocytes were stimulated with either media or 19 kDa for 3 h. We isolated RNA and synthesized cDNA as described[5]. GM-CSF primers were as follows: forward, 5′-GCCTCACCAAGCTCAAGGG-3′; reverse 5′-GGAGGGCAGTGCTGTTTGTAG-3′. IL-15 primers were as follows: forward, 5′-TGTAGGAGGCATCGTGGATG-3′; reverse 5′-CCTTAAGTATTGAAGAAG AGCTGGCT-3′. Reactions used Sybr Green PCR Master Mix (BioRad). The relative quantities of GM-CSF and IL-15 per sample and normalization were calculated as described[5].

Immunoperoxidase and immunofluorescence. Immunoperoxidase labeling was performed as described[5]. Double immunofluorescence was performed by serially incubating sections with antibodies against DC-SIGN followed by an isotype-specific fluorochrome (Caltag). Sections were washed, incubated with antibodies for CD1a, CD1b, CD16, CD63, CD64, CD68, CD83, DC-LAMP or *M. leprae* 22 kDa for 1 h followed by a TRITC-conjugated secondary (Southern Biotechnology) and examined as described[43].

URLs. Database of Ligand-Receptor Partners, http://dip.doe-mbi.ucla.edu/dip/DLRP.cgi
Cluster and TreeView software, http://rana.lbl.gov/

Statistical analyses. For analysis of gene microarrays, *P* values were calculated using Student *t*-test.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS
We would like to thank P. Brennan (Colorado State University and NIAID Contract NO1 AI-75320) for the gift of the *M. leprae* bacterioferritin major membrane protein II (MMPII) monoclonal antibody. We would also like to thank K. Grossheider for her technical help. This work was supported in part by grants from the US National Institutes of Health (AI07126, AI22553, AI47866).

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

Received 1 March; accepted 8 April 2005
Published online at http://www.nature.com/naturemedicine/

1. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., & Hoffmann, J.A. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86**, 973–983 (1996).
2. Takeuchi, O., Hoshino, K., & Akira, S. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to staphylococcus aureus infection. *J. Immunol.* **165**, 5392–5396 (2000).
3. O'Brien, A.D. *et al.* Genetic control of susceptibility to *Salmonella typhimurium* in mice: role of the LPS gene. *J. Immunol.* **124**, 20–24 (1980).
4. Alexopoulou, L. *et al.* Hyporesponsiveness to vaccination with *Borrelia burgdorferi* OspA in humans and in. *Nat. Med.* **8**, 878–884 (2002).
5. Krutzik, S.R. *et al.* Activation and regulation of Toll-like receptors 2 and 1 in human leprosy. *Nat. Med.* **9**, 525–532 (2003).
6. Blander, J.M. & Medzhitov, R. Regulation of phagosome maturation by signals from toll-like receptors. *Science* **304**, 1014–1018 (2004).
7. Doyle, S.E. *et al.* Toll-like receptors induce a phagocytic gene program through p38. *J. Exp. Med.* **199**, 81–90 (2004).
8. Thoma-Uszynski, S. *et al.* Induction of direct antimicrobial activity through mammalian toll-like receptors. *Science* **291**, 1544–1547 (2001).
9. Medzhitov, R., Preston-Hurlburt, P. & Janeway, C.A.J. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**, 394–397 (1997).
10. Brightbill, H.D. *et al.* Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science* **285**, 732–736 (1999).
11. Hertz, C.J. *et al.* Microbial lipopeptides stimulate dendritic cell maturation via TLR2. *J. Immunol.* **166**, 2444–2450 (2000).
12. Porcelli, S.A. & Modlin, R.L. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu. Rev. Immunol.* **17**, 297–329 (1999).
13. Beckman, E.M. *et al.* Recognition of a lipid antigen by CD1-restricted αβ+ T cells. *Nature* **372**, 691–694 (1994).
14. Sieling, P.A. *et al.* CD1-restricted T cell recognition of microbial lipoglycans. *Science* **269**, 227–230 (1995).
15. Moody, D.B. *et al.* Structural requirements for glycolipid antigen recognition by CD1b-restricted T cells. *Science* **278**, 283–286 (1997).
16. Engering, A. *et al.* The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. *J. Immunol.* **168**, 2118–2126 (2002).
17. Geijtenbeek, T.B. *et al.* Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100**, 575–585 (2000).
18. Geijtenbeek, T.B. *et al.* Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* **197**, 7–17 (2003).
19. Tailleux, L. *et al.* DC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. *J. Exp. Med.* **197**, 121–127 (2003).
20. Appelmelk, B.J. *et al.* Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells. *J. Immunol.* **170**, 1635–1639 (2003).
21. Cambi, A. *et al.* The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for *Candida albicans* on dendritic cells. *Eur. J. Immunol.* **33**, 532–538 (2003).
22. Colmenares, M., Puig-Kroger, A., Pello, O.M., Corbi, A.L. & Rivas, L. Dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN, CD209), a C-type surface lectin in human DCs, is a receptor for *Leishmania amastigotes*. *J. Biol. Chem.* **277**, 36766–36769 (2002).
23. Wemambu, S.N.C., Turk, J.L., Waters, M.F.R. & Rees, R.J.W. Erythema nodosum leprosum: a clinical manifestation of the Arthus phenomenon. *Lancet* **2**, 933–935 (1969).

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine

24. Salgame, P. *et al.* Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science* **254**, 279–282 (1991).
25. Yamamura, M. *et al.* Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* **254**, 277–279 (1991).
26. Yamamura, M. *et al.* Cytokine patterns of immunologically mediated tissue damage. *J. Immunol.* **149**, 1470–1475 (1992).
27. Kadowaki, N. *et al.* Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* **194**, 863–869 (2001).
28. Graeber, T.G. & Eisenberg, D. Bioinformatic identification of potential autocrine signaling loops in cancers from gene expression profiles. *Nat. Genet.* **29**, 295–300 (2001).
29. Ochoa, M.T. *et al.* T-cell release of granulysin contributes to host defense in leprosy. *Nat. Med.* **7**, 174–179 (2001).
30. Soilleux, E.J. *et al.* Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations *in situ* and *in vitro*. *J. Leukoc. Biol* **71**, 445–457 (2002).
31. Chehimi, J. *et al.* HIV-1 transmission and cytokine-induced expression of DC-SIGN in human monocyte-derived macrophages. *J. Leukoc. Biol.***74**, 757–763 (2003).
32. van Lent, P.L. *et al.* Expression of the dendritic cell-associated C-type lectin DC-SIGN by inflammatory matrix metalloproteinase-producing macrophages in rheumatoid arthritis synovium and interaction with intercellular adhesion molecule 3-positive T cells. *Arthritis Rheum.* **48**, 360–369 (2003).
33. Giordano, D., Magaletti, D.M., Clark, E.A., & Beavo, J.A. Cyclic nucleotides promote monocyte differentiation toward a DC-SIGN+ (CD209) intermediate cell and impair differentiation into dendritic cells. *J. Immunol.* **171**, 6421–6430 (2003).
34. Geijtenbeek, T.B. *et al.* DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**, 587–597 (2000).
35. Relloso, M. *et al.* DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-beta, and anti-inflammatory agents. *J. Immunol.* **168**, 2634–2643 (2002).
36. Judge, A.D., Zhang, X., Fujii, H., Surh, C.D. & Sprent, J. Interleukin 15 controls both proliferation and survival of a subset of memory-phenotype CD8(+) T cells. *J. Exp. Med.* **196**, 935–946 (2002).
37. Schluns, K.S., Williams, K., Ma, A., Zheng, X.X. & Lefrancois, L. Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. *J. Immunol.* **168**, 4827–4831 (2002).
38. Pulendran, B. *et al.* Dendritic cells generated in the presence of GM-CSF plus IL-15 prime potent CD8+ Tc1 responses *in vivo*. *Eur. J. Immunol.* **34**, 66–73 (2004).
39. Hirschfeld, M., Ma, Y., Weis, J.H., Vogel, S.N., & Weis, J.J. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. *J. Immunol.* **165**, 618–622 (2000).
40. Takeuchi, O. *et al.* Cutting edge: Preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. *J. Immunol.* **164**, 554–557 (2000).
41. Ridley, D.S. & Jopling, W.H. Classification of leprosy according to immunity. A five-group system. *Int. J. Lepr.* **34**, 255–273 (1966).
42. Kim, J. *et al.* Determinants of T cell reactivity to the *Mycobacterium leprae* GroES homologue. *J. Immunol.* **159**, 335–343 (1997).
43. Stenger, S. *et al.* An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* **282**, 121–125 (1998).

© 2005 Nature Publishing Group http://www.nature.com/naturemedicine