

Chapter

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Carbohydrate-specific signaling through dynamic regulation of the DC-SIGN signalosome directs immunity to mycobacteria, HIV-1 and *Helicobacter pylori*

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Abstract

Cooperation between different innate signaling pathways by pattern recognition receptors on dendritic cells is crucial in tailoring adaptive immunity to pathogens. Here we have identified a mechanism that diversifies adaptive immunity to pathogens by DC-SIGN through carbohydrate-specific signaling. DC-SIGN is constitutively associated with a signalosome complex consisting of the scaffold proteins LSP1, KSR1 and CNK, and the kinase Raf-1. Mannose-expressing mycobacteria and HIV-1 induce recruitment of effector proteins to the DC-SIGN signalosome to activate Raf-1, whereas fucose-expressing pathogens like *Helicobacter pylori* actively dissociate the KSR1-CNK-Raf-1 complex from the DC-SIGN signalosome. This dynamic regulation of the signalosome by mannose- and fucose- expressing pathogens leads to enhancement or suppression of pro-inflammatory responses, respectively. This study has revealed another level of plasticity in tailoring adaptive immunity to pathogens.

Introduction

Pathogen recognition by dendritic cells (DCs) is crucial to the induction of adaptive immunity¹. DCs express a diverse array of pattern recognition receptors (PRRs) that sense invading pathogens and trigger signaling pathways, leading to specific cytokine responses and T helper (T_H) cell differentiation^{2,3}. These PRRs recognize highly conserved structures expressed on micro-organisms, the so-called pathogen-associated molecular patterns (PAMPs) and include Toll-like receptors (TLRs), C-type lectins, nucleotide binding oligomerization domain (NOD) proteins and caspase-recruiting domain (CARD) helicases⁴⁻⁸. Each PRR triggers a distinct innate signaling pathway and the cooperation between different signaling pathways dictates the overall adaptive immune response mounted against the invading pathogen.

TLRs elicit innate signaling pathways via either MyD88 or TRIF adaptor proteins, leading to activation of NF- κ B and other transcription factors^{9,10} to induce adaptive immune responses¹¹. Recent studies show that TLR signaling is modulated by signaling via C-type lectins that bind carbohydrates present on pathogens to tailor immune responses to these pathogens^{7,12,13}. The C-type lectin DC-SIGN is a key player in pathogen recognition and pathogen-tailored adaptive immunity due to its broad carbohydrate specificity. A plethora of pathogens interact with DC-SIGN on DCs through either mannose- or fucose-containing glycans, which are differentially expressed on bacteria, viruses, parasites and fungi¹⁴⁻¹⁶. Remarkably, the immunological outcome of DC-SIGN triggering depends on the pathogen involved. DC-SIGN binding by distinct pathogens can lead to either inhibition or promotion of T_H1 polarization, T_H2 responses as well as the induction of regulatory T cell differentiation¹⁷⁻²⁰. The mechanisms behind these pathogen-specific immune responses of DC-SIGN are unclear but might reflect carbohydrate-specific signaling by DC-SIGN.

We have recently shown that triggering of DC-SIGN by mycobacterial ManLAM induces a distinct innate signaling pathway that leads to the activation of the serine-threonine kinase Raf-1. Upon NF- κ B activation by TLR signaling, Raf-1 activation leads to phosphorylation and subsequent acetylation of p65, one of the key activating subunits of NF- κ B. The acetylation of p65 prolongs transcriptional activity of NF- κ B and enhances the transcription rate from the *IL10* gene, resulting in increased interleukin-10 (IL-10) production²¹. Our data show that Raf-1 activation is central to modulation of TLR-specific immune responses by DC-SIGN in response to several mannose-expressing pathogens, including *Mycobacterium*

tuberculosis, HIV-1 and measles virus²¹. This Raf-1 signaling pathway is distinct from the Raf-1-MEK-ERK pathway²¹ and little is known about the regulation of Raf-1 by DC-SIGN and whether its activation is involved in responses to fucose-carrying pathogens.

Here we demonstrate that DC-SIGN signaling is carbohydrate specific and distinct signaling pathways are induced by mannose- and fucose-expressing pathogens. Intriguingly, carbohydrate-specific ligation of DC-SIGN leads to a switch in the proximal signaling complex (signalosome) which links DC-SIGN to distinct downstream signaling and subsequent modulation of cytokine responses. We demonstrate that DC-SIGN is associated with a scaffolding complex consisting of LSP1, KSR1 and CNK that is required for the constitutive recruitment of Raf-1 to DC-SIGN. Upon binding of DC-SIGN by mannose-expressing pathogens like mycobacteria and HIV-1, Raf-1 becomes activated by recruitment of the upstream effectors LARG and RhoA to the DC-SIGN signalosome. This Raf-1-dependent signaling modulates TLR4 signaling and enhances IL-10, IL-12 and IL-6 expression. In contrast, fucose-expressing pathogens, such as *Helicobacter pylori*, actively dissociate the KSR1-CNK-Raf-1 complex from the signalosome by binding to DC-SIGN and enhance IL-10 while downregulating IL-12 and IL-6 expression in a Raf-1-independent but LSP1-dependent manner. Strikingly, association of LSP1 with DC-SIGN is a prerequisite for cytokine modulation by mannose- as well as fucose-containing ligands. Thus, the carbohydrate moiety of DC-SIGN ligands controls the composition of the DC-SIGN signalosome and dictates modulation of TLR-induced cytokine responses. Our data show that not only the crosstalk between PRRs provides flexibility to adaptive immune responses but that carbohydrate-specific signaling by DC-SIGN adds another level of diversity.

Results

Raf-1 activation is central to mannose- but not fucose-dependent modulation of TLR4-induced signaling by DC-SIGN

We have previously shown that DC-SIGN signaling induced by mycobacterial ManLAM modulates TLR-induced IL-10 expression in DCs through a novel pathway that depends on the activation of Raf-1 and subsequent phosphorylation and acetylation of the NF- κ B subunit p65²¹. Besides IL-10, also the modulation of TLR4-induced expression of IL-12 and IL-6 upon DC-SIGN triggering is dependent on activation of this Raf-1 pathway (Fig. 1a,b). We silenced Raf-1 expression in human primary DCs by RNA interference (RNAi; Supplementary Fig. 1) and treated cells with the TLR4 ligand LPS either alone or together with the DC-

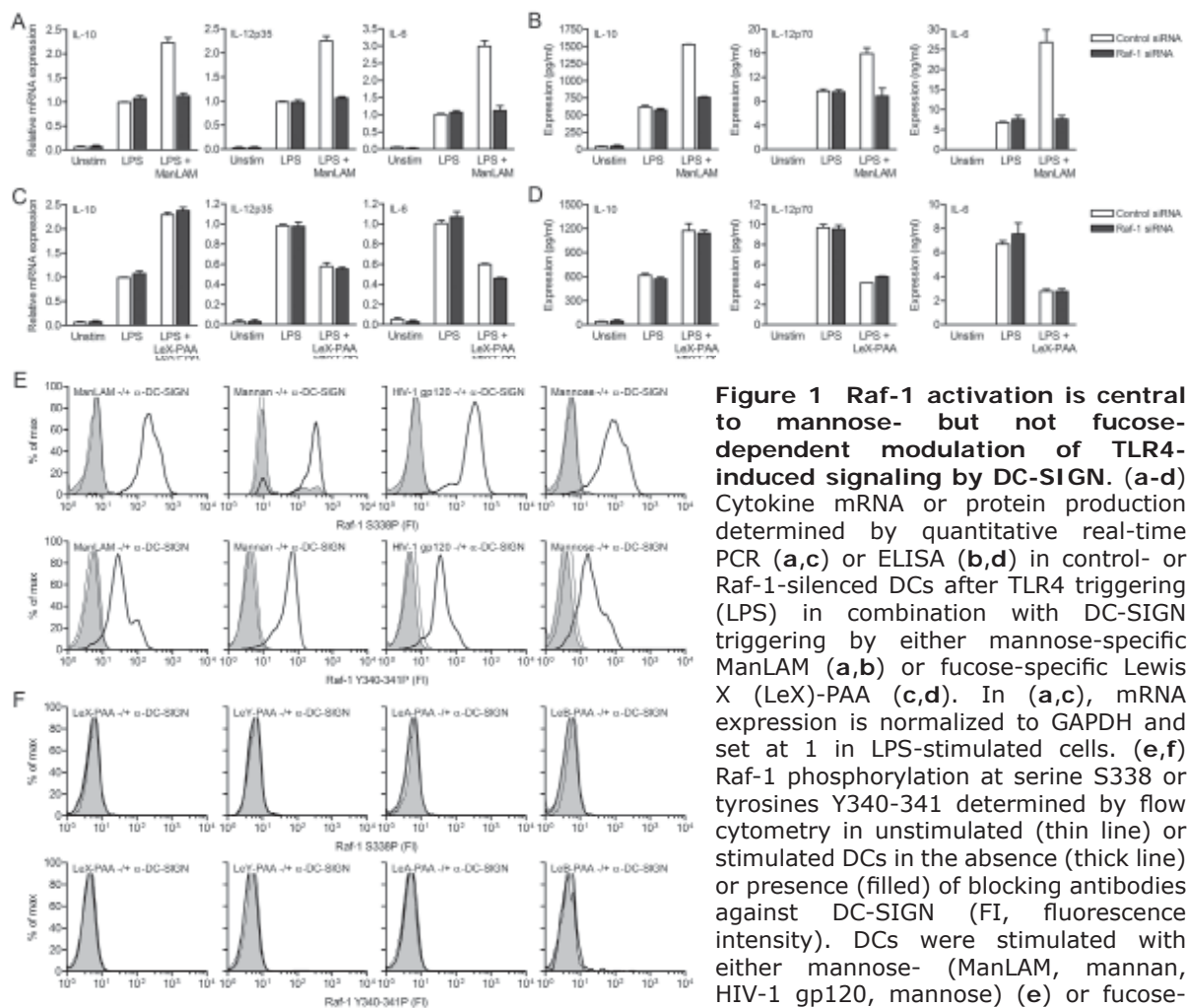


Figure 1 Raf-1 activation is central to mannose- but not fucose-dependent modulation of TLR4-induced signaling by DC-SIGN. (a-d) Cytokine mRNA or protein production determined by quantitative real-time PCR (a,c) or ELISA (b,d) in control- or Raf-1-silenced DCs after TLR4 triggering (LPS) in combination with DC-SIGN triggering by either mannose-specific ManLAM (a,b) or fucose-specific Lewis X (LeX)-PAA (c,d). In (a,c), mRNA expression is normalized to GAPDH and set at 1 in LPS-stimulated cells. (e,f) Raf-1 phosphorylation at serine S338 or tyrosines Y340-341 determined by flow cytometry in unstimulated (thin line) or stimulated DCs in the absence (thick line) or presence (filled) of blocking antibodies against DC-SIGN (FI, fluorescence intensity). DCs were stimulated with either mannose- (ManLAM, mannan, HIV-1 gp120, mannose) (e) or fucose-specific PAMPs (LeX-, LeY-, LeA-, LeB-PAA) (f). Data are presented as mean \pm s.d. of at least five (a,c) independent experiments or presented as mean \pm s.d. of duplicate samples representative of at least two (b,d) independent experiments or representative of at least three (e,f) independent experiments.

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SIGN ligand ManLAM. LPS-induced IL-10, IL-12p35 and IL-6 responses were enhanced several fold by co-stimulation of DC-SIGN with ManLAM (Fig. 1a). Raf-1 silencing abrogated the enhanced mRNA expression by TLR4-DC-SIGN crosstalk completely (Fig. 1a). Similarly, Raf-1 inhibition completely blocked the ManLAM-induced upregulation of LPS-induced IL-10, IL-12p70 (consisting of IL-12 p35 and p40) and IL-6 protein production (Fig. 1b).

DC-SIGN binds bacteria, viruses, fungi and parasites primarily through recognition of mannose- and fucose-containing structures^{14,16,22,23}. We next investigated whether fucose-containing ligands such as Lewis X modulate TLR-induced cytokine expression in a similar manner as mannose-based ligands. Although co-stimulation of DC-SIGN with Lewis X enhanced LPS-induced IL-10 expression (Fig. 1c,d), it impaired expression of IL-12 (IL-12p35 mRNA, Fig. 1c; IL-12p70 protein, Fig. 1d) and IL-6 (Fig. 1c,d). Strikingly, silencing of Raf-1 expression did not affect the Lewis X-mediated modulation of LPS-induced cytokine expression (Fig. 1c,d). These data strongly suggest that DC-SIGN signaling differs depending on the carbohydrate moiety recognized by DC-SIGN, allowing carbohydrate-specific modulation of cytokine responses.

We next investigated whether DC-SIGN triggering by various mannose- or fucose-containing ligands activates Raf-1. Raf-1 kinase activity requires phosphorylation of Raf-1 on serine 338 (Ser338) and tyrosines 340 and 341 (Tyr340-341) by Pak and Src kinases, respectively^{21,24}. Mycobacterial ManLAM, fungal mannan and HIV-1 gp120 are bound by DC-SIGN in a mannose-dependent manner^{12,25,26} and all induced phosphorylation of Ser338 and Tyr340-341 on Raf-1, which was blocked by antibodies against DC-SIGN (Fig. 1e). Monomeric mannose also induced Raf-1 phosphorylation via DC-SIGN (Fig. 1e). In contrast, fucose-containing blood-group antigens Lewis X, Lewis Y, Lewis A and Lewis B, known ligands for DC-SIGN¹⁴, failed to induce Raf-1 phosphorylation (Fig. 1f). These findings show that Raf-1 activation is specifically induced by mannose- but not fucose-containing DC-SIGN ligands, hence accounting for the carbohydrate-specific modulation of cytokine responses via DC-SIGN.

LSP1 is essential for ManLAM-induced Raf-1-mediated DC-SIGN signaling

To understand the mechanisms controlling carbohydrate-specific signaling by DC-SIGN, we set out to elucidate how mannose-containing ligands couple DC-SIGN triggering to Raf-1 activation. A recent study demonstrated that the F-actin binding protein leukocyte-specific protein

1 (LSP1) is associated with the cytoplasmic tail of DC-SIGN, which diverts HIV-1 to the proteasome upon binding to DC-SIGN²⁷. To establish whether LSP1 plays a role in DC-SIGN signaling, we silenced LSP1 expression in DCs by RNAi. Notably, LSP1 silencing completely abrogated the ManLAM-mediated upregulation of LPS-induced IL-10, IL-12p35 and IL-6 mRNA expression (Fig. 2a), to a similar level as observed in Raf-1-silenced DCs (Fig. 1a). Moreover, LSP1 silencing completely abrogated ManLAM-induced Raf-1 phosphorylation at both Ser338 and Tyr340-341 (Fig. 2b). Thus, LSP1 is required for activation of Raf-1 upon DC-SIGN triggering by ManLAM and subsequent modulation of TLR-induced cytokine responses.

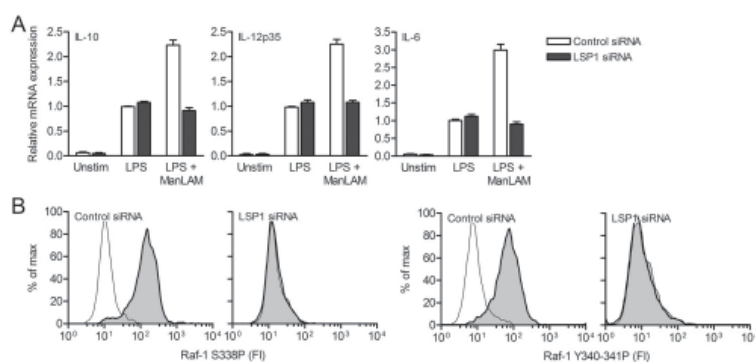


Figure 2 Mannose-specific DC-SIGN signaling requires LSP1. (a) Cytokine mRNA expression determined by quantitative real-time PCR in control- or LSP1-silenced DCs treated with LPS in combination with ManLAM. Expression is normalized to GAPDH and set at 1 in LPS-stimulated cells. (b) Raf-1 phosphorylation at serine S338 or tyrosines Y340-341 determined by flow cytometry in control- or LSP1-silenced DCs that were left unstimulated (thin line) or treated with ManLAM (filled

histogram). Data are presented as mean \pm s.d. of at least four (a) independent experiments or representative of at least three (b) independent experiments.

A signalosome consisting of LSP1 and the trimeric KSR1-CNK-Raf-1 complex is essential for ManLAM-induced DC-SIGN signaling

We next investigated whether DC-SIGN triggering by ManLAM leads to recruitment of Raf-1 to DC-SIGN and LSP1. As expected, LSP1 co-immunoprecipitated with DC-SIGN from whole cell extracts of both untreated DCs and ManLAM-stimulated DCs (Fig. 3a). Strikingly, Raf-1 was not only associated with DC-SIGN in ManLAM-stimulated but also in unstimulated DCs (Fig. 3a). As LSP1 is essential for Raf-1 activation (Fig. 2b), we examined whether prior association of LSP1 with DC-SIGN is a prerequisite for Raf-1 association with DC-SIGN. Raf-1 failed to co-immunoprecipitate with DC-SIGN from extracts of LSP1-silenced cells (Fig. 3b), strongly suggesting the requirement for LSP1 in Raf-1 activation is at the level of Raf-1 recruitment to DC-SIGN.

LSP1 has previously been shown to associate with the scaffold protein kinase suppressor of Ras 1 (KSR1)²⁸. KSR1 is a kinase-dead Raf homolog that can form a trimeric complex with Raf-1 and another scaffold protein, connector enhancer of KSR (CNK) in *Drosophila melanogaster*²⁹. We examined whether KSR1 and CNK are associated with DC-SIGN. Both

KSR1 and CNK co-immunoprecipitated with DC-SIGN from whole cell extracts of both unstimulated and ManLAM-stimulated DCs (Fig. 3a). Furthermore, LSP1 silencing abrogated the association of both KSR1 and CNK with DC-SIGN (Fig. 3b). Also, the association of Raf-1 with DC-SIGN was dependent on the presence of both KSR1 and CNK as we failed to detect Raf-1 bound to DC-SIGN in either KSR1- or CNK-silenced DCs (Fig. 3b). Similarly, association of both KSR1 and CNK with DC-SIGN required expression of Raf-1 (Fig. 3b). Notably, LSP1 remained associated with DC-SIGN in the absence of either KSR1, CNK or Raf-1 (Fig. 3b). In line with these results, silencing of KSR1 or CNK expression by RNAi completely abrogated Raf-1 phosphorylation after ManLAM stimulation (Fig. 3c). These data show that both scaffold proteins KSR1 and CNK are required for Raf-1 association with DC-SIGN through LSP1 and subsequent Raf-1 activation. Furthermore, the upregulation of LPS-induced cytokine mRNA expression by co-stimulation of DC-SIGN with ManLAM was completely attenuated when either KSR1 or CNK were silenced (Fig. 3d), similar to Raf-1 and LSP1 silencing (Fig. 1a and 2a). Thus, a signaling complex (signalosome) consisting of LSP1 and the KSR1-CNK-Raf-1 triad is constitutively bound to DC-SIGN in unstimulated human DCs, which is essential for the activation of Raf-1 by ManLAM and subsequent modulation of TLR-induced cytokine responses.

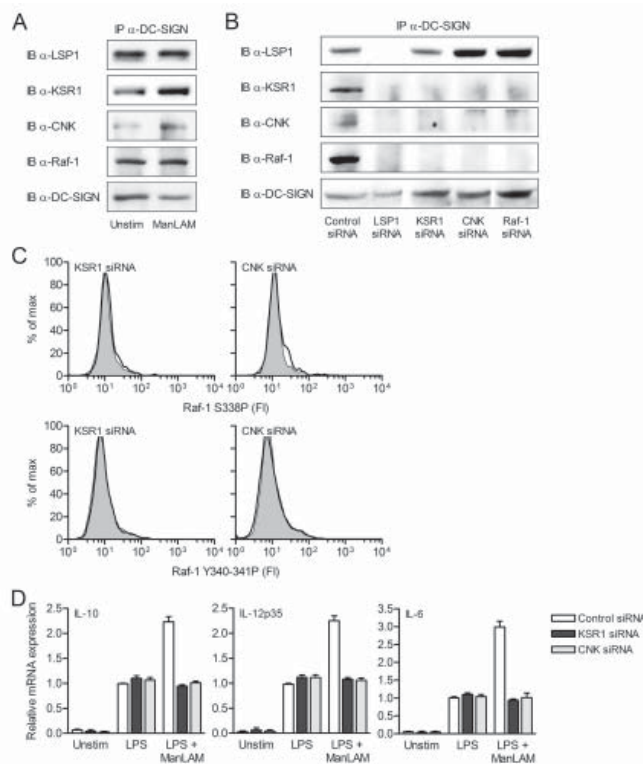


Figure 3 Mannose-specific DC-SIGN signaling requires a signalosome associated with DC-SIGN consisting of LSP1 and the KSR1-CNK-Raf-1 triad complex. (a,b) Association of LSP1, KSR1, CNK and Raf-1 with DC-SIGN was determined by immunoblotting (IB) after immunoprecipitation (IP) with anti-DC-SIGN from whole cell lysates of unstimulated or ManLAM-stimulated DCs (a) or unstimulated control-, LSP1-, KSR1-, CNK- or Raf-1-silenced DCs (b). (c) Raf-1 phosphorylation at serine S338 or tyrosines Y340-341 determined by flow cytometry in KSR1- or CNK-silenced DCs that were left unstimulated (thin line) or treated with ManLAM (filled histogram). (d) Cytokine mRNA expression determined by quantitative real-time PCR in control-, KSR1- or CNK-silenced DCs treated with LPS in combination with ManLAM. Expression is normalized to GAPDH and set at 1 in LPS-stimulated cells. Data are representative of two (b) or three (a,c) independent experiments or presented as mean \pm s.d. of at least five (a) independent experiments.

DC-SIGN triggering by ManLAM recruits LARG and RhoA to the signalosome for Raf-1 activation

Since Raf-1 is present within the DC-SIGN signalosome prior to DC-SIGN triggering, we hypothesized that DC-SIGN triggering leads to the recruitment of immediate upstream effectors that induce Raf-1 activation. A recent study demonstrated that HIV-1 binding to DC-SIGN leads to the successive activation of the Rho guanine nucleotide exchange factor (GEF) LARG and the small GTPase RhoA, which is required for the formation of a viral synapse between HIV-1-infected DCs and T cells³⁰. Therefore, we next investigated whether LARG and RhoA play a role in Raf-1-dependent DC-SIGN signaling. We found that LARG and RhoA were recruited to DC-SIGN after ManLAM stimulation as both proteins co-immunoprecipitated with DC-SIGN from whole cell lysates of ManLAM-stimulated DCs but not from unstimulated cells (Fig. 4a). Although LARG is a known effector of RhoA, silencing of LARG expression inhibited Raf-1 phosphorylation at both Ser338 and Tyr340-341 after ManLAM stimulation (Fig. 4b) whereas silencing of RhoA prevented Raf-1 phosphorylation at Ser338 but not at Tyr340-341 (Fig. 4b). These data suggest that LARG induces Tyr340-341 phosphorylation in a RhoA-independent manner. We found that LARG silencing completely abrogated the upregulation of LPS-induced IL-10,

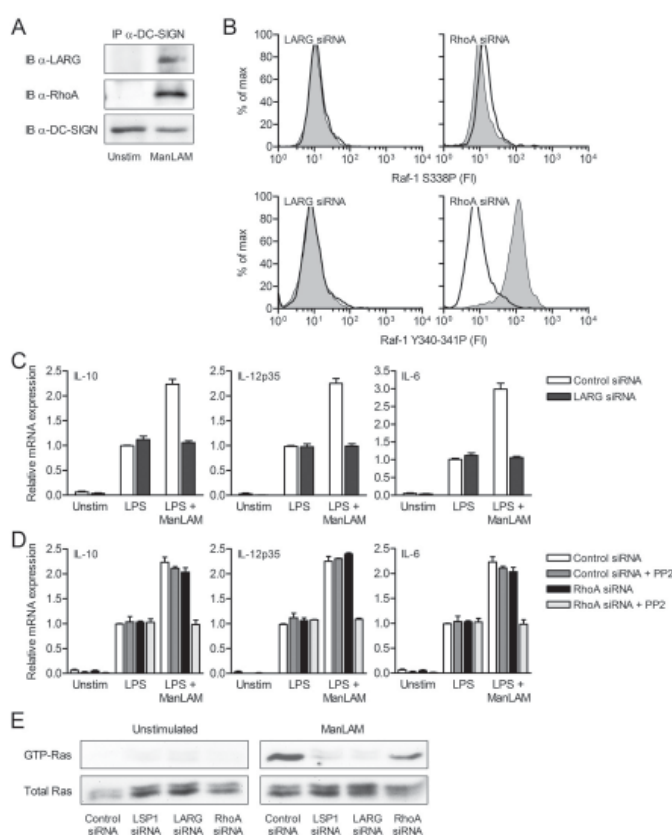


Figure 4 Mannose-specific DC-SIGN triggering recruits LARG and RhoA to the signalosome which is essential to Raf-1 activation via Ras. (a) Recruitment of LARG and RhoA by DC-SIGN was determined by immunoblotting (IB) after immunoprecipitation (IP) with anti-DC-SIGN from whole cell lysates of unstimulated or ManLAM-treated DCs. (b) Raf-1 phosphorylation at serine S338 or tyrosines Y340-341 determined by flow cytometry in LARG- or RhoA-silenced DCs that were left unstimulated (thin line) or treated with ManLAM (filled histogram). (c,d) Cytokine mRNA expression determined by quantitative real-time PCR in control-, LARG- (c) or CNK-silenced (d) DCs treated with LPS in combination with ManLAM. In (d), stimulation occurred in the absence or presence of the Src kinase inhibitor PP2. Expression is normalized to GAPDH and set at 1 in LPS-stimulated cells. (e) Active GTP-bound Ras was determined by immunoblotting after precipitation with GST-Raf-RBD from cell lysates of control-, LSP1-, LARG- or RhoA-silenced DCs that were left unstimulated or treated with ManLAM. Total Ras was detected in lysates to confirm equal amounts. Data are representative of two (a,e) or three (b) independent experiments or presented as mean \pm s.d. of at least three (c,d) independent experiments.

IL-12p35 and IL-6 mRNA production by ManLAM (Fig. 4c). In contrast, silencing of RhoA had no effect on cytokine expression upon LPS and ManLAM co-stimulation (Fig. 4d). This is consistent with our previous findings that inhibition of Raf-1 kinase activity upon TLR4-DC-SIGN co-stimulation requires inhibition of phosphorylation at both Ser338 and Tyr340-341²¹. However, the upregulation of LPS-induced cytokine expression by ManLAM was completely abolished when we combined RhoA silencing, which blocks Pak-mediated Raf-1 phosphorylation at Ser338 (Fig. 4b), with the Src kinase inhibitor, PP2 that prevents Raf-1 Tyr340-341 phosphorylation²¹ (Fig. 4d). These data demonstrate that DC-SIGN binding by ManLAM leads to the recruitment of both LARG and RhoA to the DC-SIGN signalosome to induce Raf-1 activation. Furthermore, these findings strongly suggest that LARG controls Raf-1 activation in two ways: Raf-1 phosphorylation at Ser338 involves LARG-dependent activation of RhoA, whereas Tyr340-341 phosphorylation depends on a LARG-induced, but RhoA-independent mechanism.

LARG is essential for ManLAM-induced activation of Ras

Raf-1 phosphorylation is essential for its kinase activity^{21,24}, however Raf-1 activation involves a highly complex sequence of events in which release of autoinhibition, transformational changes and dephosphorylation critically precede Raf-1 phosphorylation. The small GTPase Ras plays an essential role in this process as binding of the active GTP-bound form of Ras to Raf-1 is the crucial first step^{24,31}. Therefore, we investigated whether LARG was required for Ras activation. Strikingly, we found that silencing of LARG expression abolished the activation of Ras by ManLAM as determined by GTP-Ras pull-down (Fig. 4e). In addition, LSP1 silencing also prevented Ras activation by ManLAM (Fig. 4e), indicative of its role as main scaffolding protein in the DC-SIGN signalosome. In contrast, silencing of RhoA did not block ManLAM-induced Ras activation (Fig. 4e), supporting our data that RhoA is only involved in Raf-1 activation through activation of Pak kinases that phosphorylate Ser338. These data indicate that the recruitment of LARG to the DC-SIGN signalosome after ManLAM stimulation is essential to Raf-1 activation via both Ras activation as well as RhoA-mediated Raf-1 phosphorylation.

Fucose-containing ligands dissociate KSR1-CNK-Raf-1 from the DC-SIGN signalosome, but modulate cytokine expression via a LSP1-dependent pathway

We next evaluated the role of the LSP1-KSR1-CNK-Raf-1 signalosome in the Raf-1-independent signaling pathway induced by fucose-containing

DC-SIGN ligands. Silencing of LSP1 expression completely abrogated Lewis X-mediated upregulation of IL-10 and inhibition of IL-12p35 and IL-6 mRNA expression after LPS stimulation (Fig. 5a). Similar effects were observed when LSP1-silenced DCs were co-stimulated with LPS and Lewis Y (Fig. 5a). Thus, fucose-containing DC-SIGN ligands induce a LSP1-dependent but Raf-1-independent signaling pathway that modulates TLR-induced cytokine responses.

We next investigated whether the other scaffold proteins KSR1 and CNK are similarly important to fucose-mediated DC-SIGN signaling as LSP1. Remarkably, we found that neither KSR1 nor CNK were involved in fucose-mediated DC-SIGN signaling as silencing of neither KSR1 nor CNK expression affected the modulation of LPS-induced cytokine expression by Lewis X (Fig. 5b) or Lewis Y (data not shown). These data strongly indicate that KSR1 and CNK, like Raf-1, are dispensable for DC-SIGN signaling upon fucose-directed ligand binding. Strikingly, neither KSR1, CNK nor Raf-1 co-immunoprecipitated with DC-SIGN from whole cell lysates of Lewis X-treated DCs (Fig. 5c). Since all three proteins were associated with DC-SIGN in unstimulated cells (Fig. 3a and 5c), these data strongly suggest that the triad complex is actively excluded from the DC-SIGN signalosome upon binding of fucose-containing structures to DC-SIGN. Thus, DC-SIGN binding by Lewis X results in dissociation of the KSR1-CNK-Raf-1 complex from DC-SIGN without disrupting association of LSP1 with DC-SIGN, which is essential to fucose-induced modulation of TLR-induced cytokine responses.

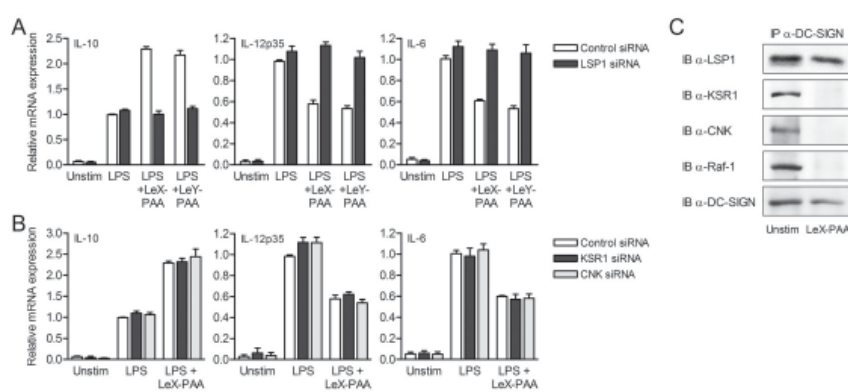


Figure 5 Fucose-specific DC-SIGN triggering dissociates KSR1-CNK-Raf-1 but not LSP1 from the signalosome. (a,b) Cytokine mRNA expression determined by quantitative real-time PCR in control-, LSP1- (a), KSR- or CNK-silenced (b) DCs treated with LPS in combination with the fucose-specific DC-SIGN ligands Lewis X (LeX-PAA) (a,b) or Lewis Y (LeY-PAA) (a). Expression is normalized to GAPDH and set at 1 in LPS-stimulated cells. (c) Association of LSP1, KSR1, CNK and Raf-1 with DC-SIGN was determined by immunoblotting (IB) after immunoprecipitation (IP) with anti-DC-SIGN from whole cell lysates of unstimulated or Lewis X (LeX-PAA)-stimulated DCs. Data are presented as mean \pm s.d. of at least three (a,b) independent experiments or representative of two (c) independent experiments.

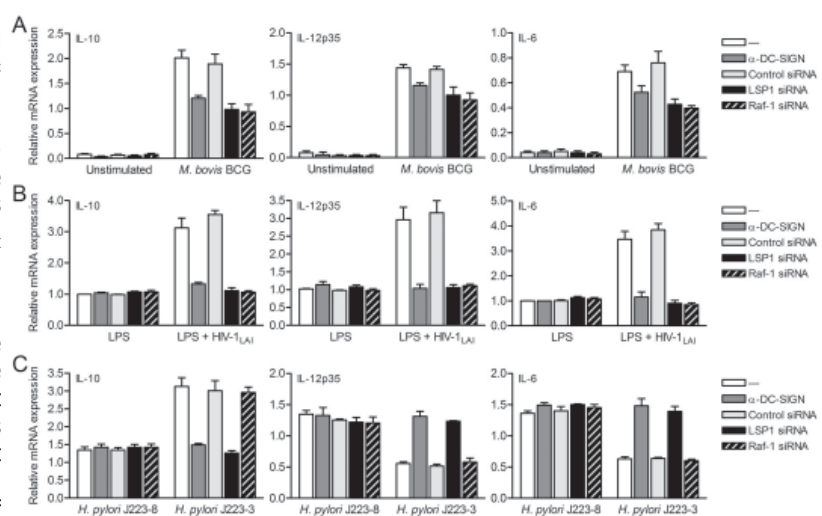
Carbohydrate-specific DC-SIGN signaling leads to pathogen-tailored cytokine responses

We next investigated whether intact pathogens expressing mannose- or fucose-structures induce carbohydrate-specific immune responses as we observed with the single mannose- and fucose-containing DC-SIGN ligands. *M. bovis* bacillus Calmette-Guérin (BCG) interacts with TLRs and also C-type lectins including DC-SIGN and dectin-1^{12,32,33}. Binding to DC-SIGN is mediated by mannose-containing glycoproteins and glycolipids, including ManLAM³⁴. We found that *M. bovis* BCG induced IL-10, IL-12p35 and IL-6 mRNA expression which was down-modulated by blocking antibodies against DC-SIGN as well as LSP1 or Raf-1 silencing (Fig. 6a).

Binding of HIV-1 to DC-SIGN is mediated by mannose-structures abundantly present on its envelope glycoprotein gp120^{25,26}. Co-stimulation of DC-SIGN with HIV-1 enhanced LPS-induced cytokine expression several fold, which was completely abrogated by blocking antibodies against DC-SIGN as well as LSP1 and Raf-1 silencing (Fig. 6b). Thus, mannose-expressing pathogens modulate immune responses via DC-SIGN-mediated LSP1-Raf-1-dependent signaling, similar to single mannose-containing DC-SIGN ligands.

The humangastric pathogen *Helicobacter pylori* spontaneously switches LPS Lewis antigens on and off through phase-variable expression¹⁷. *H. pylori* strain J223-3 interacts with DC-SIGN through Lewis Y to block T_H1 and promote T_H2 induction, while the Lewis Y-negative phase-variant J223-8 does not bind DC-SIGN¹⁷. We found that Lewis Y-positive *H. pylori* J223-3 enhanced IL-10 and decreased IL-12p35 and IL-6 mRNA expression compared to the Lewis Y-negative strain, which could be blocked

Figure 6 Carbohydrate-specific DC-SIGN signaling leads to pathogen-specific cytokine responses. (a-c) Cytokine mRNA expression determined by quantitative real-time PCR in DCs treated with the mannose-expressing pathogens *M. bovis* BCG (a) and HIV-1_{LAI} (b) or the Lewis Y-expressing pathogen *H. pylori* J223-3 (c). In (b), DCs were co-stimulated with LPS. In (c), DCs were stimulated as a control with the Lewis Y-negative phase-variant *H. pylori* J223-8. Expression is normalized to GAPDH and set at 1 in LPS-stimulated cells. Data are presented as mean ± s.d. of at least three (a-c) independent experiments.



with DC-SIGN antibodies (Fig. 6c). Notably, LSP1 silencing completely abrogated the *H. pylori* J223-3-mediated upregulation of IL-10 mRNA, whereas decreased IL-12p35 and IL-6 expression levels were restored to the level observed with *H. pylori* J223-8 (Fig. 6c), demonstrating that Lewis Y-mediated signaling by *H. pylori* via DC-SIGN is LSP1-dependent. In contrast, Raf-1 silencing did not affect cytokine expression induced by either *H. pylori* phase-variant (Fig. 6b). Thus, the fucose-expressing pathogen *H. pylori* J223-3 signal via DC-SIGN in an LSP1-dependent but Raf-1-independent manner similar to fucose-containing glycoconjugates. Our findings strongly suggest that DC-SIGN tailors immune responses to specific pathogens: the recognition of distinct carbohydrate structures expressed by pathogens dynamically regulate signalosome assembly, which is essential to DC-SIGN signaling.

Discussion

The crosstalk between different signaling pathways induced by various PRRs is crucial to the induction of pathogen-specific immune responses^{2,3}. Whereas known PRRs induce a specific signaling pathway in response to different ligands, here we have identified a novel mechanism of PAMP-specific signaling by one PRR that further diversifies adaptive immunity to pathogens. Here we show that DC-SIGN induces pathogen-specific immune responses by differential assembly of a signaling complex (signalosome) associated with DC-SIGN upon recognition of carbohydrate-specific PAMPs. In quiescent DCs, DC-SIGN is constitutively associated with a signalosome consisting of the scaffolding proteins LSP1, KSR1 and CNK and the kinase Raf-1. Binding of mannose-expressing pathogens, such as mycobacteria and HIV-1, to DC-SIGN induces the recruitment of upstream effectors of Raf-1, such as LARG and RhoA, to the DC-SIGN signalosome, which leads to activation of Raf-1 and enhanced IL-10, IL-12 and IL-6 expression. In contrast, binding of fucose-specific pathogens like *H. pylori* leads to the dissociation of the KSR1-CNK-Raf-1 triad from the DC-SIGN signalosome and leads to upregulation of IL-10 but downregulation of IL-12 and IL-6 in a Raf-1-independent but LSP1-dependent manner. Thus, DC-SIGN induces carbohydrate-specific signaling through dynamic regulation of its signalosome to direct adaptive immunity to specific pathogens.

We have previously shown that DC-SIGN triggering by various mannose-expressing pathogens such as mycobacteria (*M. bovis* BCG, *M. tuberculosis*, *M. leprae*), fungi (*Candida albicans*) and viruses (HIV-1,

measles virus) modulate TLR-induced cytokine responses via a signaling pathway that was dependent on the activation of Raf-1²¹. Here we found that DC-SIGN is constitutively associated with Raf-1 in unstimulated DCs. The association of Raf-1 with DC-SIGN is dependent on the presence of three scaffolding proteins, i.e. LSP1, KSR1 and CNK. Our data strongly suggest that LSP1 is directly bound to DC-SIGN which is in line with a previous report²⁷. The prior association of LSP1 is crucial for the recruitment of the triad complex of KSR1, CNK and Raf-1 to DC-SIGN as silencing of LSP1 completely abrogated the recruitment of the KSR1-CNK-Raf-1 complex. However, LSP1 binding to DC-SIGN is independent of the triad complex, since LSP1 remained bound to DC-SIGN upon silencing of either KSR1, CNK or Raf-1. Notably, preassembly of the KSR1-CNK-Raf-1 triad seems to be a prerequisite before association with DC-SIGN-LSP1 as silencing of expression of either KSR1, CNK or Raf-1 attenuated recruitment of the other proteins into the DC-SIGN signalosome without interfering with LSP1 recruitment. This is further substantiated by our data showing that fucose-expressing pathogens actively induced the dissociation of the KSR1-CNK-Raf-1 triad but not LSP1 from the signalosome (see below).

The assembly of a scaffolding module is a key theme in signaling and allows kinases to come into close proximity of both upstream and downstream effectors^{35,36}. Raf-1 activation upon DC-SIGN ligation is dependent on the activation of the small GTPase Ras²¹, as binding of GTP-bound Ras is the essential first step in the complex activation of Raf-1^{24,31}. Subsequent phosphorylation of Raf-1 at key residues Ser338 and Tyr340-341 by Pak and Src kinases, respectively, is then required to activate the kinase activity of Raf-1^{21,24}. Here we demonstrate that the recruitment of the guanine nucleotide exchange factor LARG to the DC-SIGN signalosome upon recognition of mannose structures is essential for the activation of Ras as well as RhoA. LARG is a known GEF for RhoA³⁷, and further studies will show whether LARG functions as a direct GEF for Ras or whether other signaling proteins are involved in the activation of Ras as a result of LARG recruitment. RhoA was also recruited into the signalosome upon DC-SIGN binding by mannose-specific ligands. Activation of LARG and RhoA has previously been reported in response to HIV-1 binding to DC-SIGN³⁰. Recruitment of RhoA to the signalosome is most likely mediated via binding to CNK, an association which has been described before³⁸ and would serve to bring it into proximity of LARG for activation. The activation of RhoA was required for the phosphorylation of Raf-1 at one of the key residues, Ser338. It remains to be determined how RhoA activation results in the activation of Pak kinases, the direct mediators of Ser338 phosphorylation. CNK might also be involved in

the recruitment of other upstream effectors to induce Raf-1 activation: the phosphorylation of Raf-1 at Tyr340-341 is dependent on Src family kinases^{21,24} and CNK has been implicated previously in Src-mediated Raf-1 activation³⁹. Thus, binding of DC-SIGN by mannose-specific ligands results in the recruitment of upstream effectors of Raf-1 to the signalosome; LARG recruitment leads to both Ras and RhoA activation which is essential to the activation of Raf-1 and the subsequent modulation of cytokine responses.

The DC-SIGN-induced Raf-1 signaling pathway is distinct from the classical Raf-1-MEK-ERK cascade^{24,40}, as we have previously shown that ERK is not activated after binding of mannose-expressing ligands to DC-SIGN²¹. ERK activation is mediated by recruitment of ERK and subsequent phosphorylation to a KSR1-MEK-Raf-1 scaffolding module after mitogenic or antigenic signals^{36,40,41}. Our data strongly suggest that the KSR1-CNK-Raf-1 complex associated with DC-SIGN represents another example of a scaffolding module which does not link Raf-1 to activation of ERK but to a yet to be identified kinase, resulting in phosphorylation of p65 at Ser276 and hence modulation of adaptive immune responses²¹.

Our data suggest that the LSP1-KSR1-CNK-Raf-1 signalosome is not just important for modulation of cytokine expression upon DC-SIGN ligation by HIV-1, but also for HIV-1 degradation as well as viral transmission to T cells, since LSP1 is essential for DC-SIGN-mediated trafficking of HIV-1 to the proteasome²⁷ and LARG and RhoA are required for formation of the viral synapse³⁰, respectively.

Although Raf-1 is a constitutive component of the signalosome, the crosstalk between TLR and DC-SIGN signaling in the modulation of TLR-induced cytokine responses by pathogens with fucose-specific ligands was completely independent from Raf-1. Importantly, we found that the binding of fucose-containing ligands by DC-SIGN led to the active dissociation of the KSR1-CNK-Raf-1 triad from the signalosome. Notably, LSP1 remained associated with DC-SIGN and its presence was also essential to the DC-SIGN signaling pathways induced by fucose-containing ligands. The dynamic regulation of the composition of its signalosome provides the molecular basis for the plasticity that DC-SIGN exhibits in directing pathogen-tailored immunity. Effective immunity to pathogens requires the induction and maintenance of T helper cell differentiation which is determined by specific cytokine profiles induced upon recognition of PAMPs by combinations of PRRs. Here we demonstrated that the binding of carbohydrate-specific PAMPs to DC-SIGN resulted in differential cytokine expression profiles. The mannose-dependent binding of pathogens like mycobacteria and HIV-1 by DC-SIGN led to the Raf-1-dependent upregulation of IL-10, IL-12 and IL-6 production.

We have previously shown that the upregulation of IL-10 as a result of DC-SIGN binding by mannose-expressing pathogens is mediated by the phosphorylation and acetylation of the NF- κ B subunit p65, which greatly enhances the transcriptional activity of NF- κ B. The upregulation of IL-12 and IL-6 by TLR4-DC-SIGN crosstalk is similarly dependent on p65 acetylation (data not shown), suggesting that the DC-SIGN-induced Raf-1-dependent pathway leading to acetylation of p65 is responsible for upregulation of all three cytokines. Recently it has been reported that mycobacteria prime naive T cells into becoming IL-10-producing T cells without a bias towards either T_H1 or T_H2 differentiation⁴². Our data show that both the T_H1-promoting cytokine IL-12 as well as the T_H1-repressing cytokine IL-10 are upregulated upon DC-SIGN triggering, which might explain the unbiased T helper cell differentiation.

In contrast to mannose-expressing pathogens, binding of the Lewis X-expressing pathogen *H. pylori* by DC-SIGN induces IL-10 expression, but down-modulates both IL-12 and IL-6 expression. The upregulation of IL-10 as well as the decrease in IL-12 and IL-6 was LSP1-dependent but Raf-1-independent, which corroborates our data that fucose-containing ligands of DC-SIGN disengage KSR1-CNK-Raf-1 but not LSP1 from the signalosome. The binding of DC-SIGN by *H. pylori* has previously been shown to inhibit T_H1 polarization¹⁷, which is supported by our data that fucose-induced signaling enhances IL-10 but decreases IL-12 expression. Furthermore, the soluble egg antigens of the parasite *Schistosoma mansoni* also express Lewis X, which is recognized by DC-SIGN^{14,23}. Exposure of DCs to *S. mansoni* eggs in the presence of TLR4 ligand results in T_H2 differentiation, which is required for the expulsion of intestinal helminths²⁰. The immune responses induced by these two pathogens through binding of their fucose-specific PAMPs are consistent with the DC-SIGN-LSP1-dependent downregulation of IL-12 expression.

Neisseria meningitidis mutants have been shown to bind DC-SIGN through GlcNAc structures and induce T_H1 differentiation in a DC-SIGN-dependent manner¹⁹, which differs from the immune responses induced by pathogens expressing either mannose- or fucose-specific PAMPs. This seems to suggest a further plasticity in DC-SIGN signaling.

Thus, our data show that the carbohydrate specificity of PAMPs recognized by DC-SIGN controls the composition of the DC-SIGN signalosome and as such directs the immune responses against distinct pathogens. The combinatorial signaling of all PRRs triggered by an invading pathogen defines the overall adaptive immune response. Whereas different TLRs have evolved to recognize distinct PAMPs expressed by pathogens to induce specific immunity⁴³, DC-SIGN uses its broad carbohydrate specificity to recognize multiple PAMPs and has

adapted its signaling pathways by switching signalosome complexes in a carbohydrate-specific manner to direct pathogen-tailored immunity. Thus, a hallmark of DC-SIGN signaling is its functional specificity as a result of ligand recognition. Our data provide new insights into adaptive immune responses to specific pathogens and will help to target infectious diseases more specifically in therapeutic strategies.

Materials and Methods

Cells, stimulation and inhibition

Immature DCs were cultured as described before¹². In brief, human blood monocytes were isolated from buffy coats (Sanquin bloodbank Amsterdam, the Netherlands) by use of a Ficoll gradient and a subsequent CD14 selection step using a MACS system (Miltenyi Biotec). Purified monocytes were differentiated into immature DCs in the presence of 500 U/ml interleukin-4 and 800 U/ml granulocyte-macrophage colony-stimulating factor (Schering-Plough). DCs were used for experiments at day 6 and 7. This study was performed in accordance with the ethical guidelines of the VU University Medical Center.

DCs were stimulated with 10 ng/ml LPS from *Salmonella typhosa* (Sigma), 10 µg/ml ManLAM from *Mycobacterium tuberculosis* (provided by J. Belisle, Colorado State University), 100 µg/ml mannan from *Saccharomyces cerevisiae* (Sigma), 10 µg/ml recombinant HIV-1_{BaL} gp120 (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), 50 mM mannose, 10 µg/ml Lewis X-PAA, 10 µg/ml Lewis Y-PAA, 10 µg/ml Lewis A-PAA, 10 µg/ml Lewis B-PAA (all from Lectinity), *M. bovis* BCG (at multiplicity of infection (MOI) 8) (provided by B. Appelmelk, VUmc), or *Helicobacter pylori* phase variants J223-3 and J223-8 (MOI 10) (provided by B. Appelmelk, VUmc). Cells were preincubated for 2 hrs with 20 µg/ml DC-SIGN antibody AZN-D1²² or 10 µM Src inhibitor PP2 (LC Laboratories)⁴⁴.

RNA interference

DCs were transfected with 50 nM siRNA using transfection reagents DF4 (Dharmacon), according to the manufacturer's protocol. The siRNAs used were: Raf-1 SMARTpool (M-003601-02); LSP1 SMARTpool (M-012640-00); KSR1 SMARTpool (M-003570-00); CNK SMARTpool (M-012217-00); LARG SMARTpool (M-008480-00); RhoA SMARTpool (M-003860-00); non-targeting siRNA pool (D-001206-13), as a control

(all from Dharmacon). This protocol resulted in nearly 100% transfection efficiency as determined by flow cytometry of cells transfected with siGLO-RISC free-siRNA (D-001600-01). At 72 hrs after transfection, cells were used for experiments. Silenced expression was verified at the mRNA and protein level by quantitative real-time PCR and FACS stainings, respectively, for each experiment (Supplementary Fig. 1).

Quantitative real-time PCR

mRNA isolation, cDNA synthesis and PCR amplification with the SYBR green method in an ABI 7900HT sequence detection system (Applied Biosystems) were performed as described⁴⁵. Specific primers were designed using Primer Express 2.0 (Applied Biosystems). The Ct value is defined as the number of PCR cycles where the fluorescence signal exceeds the detection threshold value. For each sample, the normalized amount of target mRNA was calculated from the obtained Ct values for both target and GAPDH mRNA with $Nt = 2^{Ct(GAPDH)-Ct(target)}$. The relative mRNA expression was obtained by setting Nt in LPS-stimulated samples at 1 within one experiment and for each donor.

Cytokine production

Cell culture supernatants were harvested after 28 hrs of stimulation and concentrations of IL-10, IL-6 (both from Invitrogen) and IL-12p70 (eBioscience) were determined by ELISA.

Raf-1 phosphorylation

For the detection of phosphorylated Raf-1, cells were stimulated for 10 min and then fixed in 3% *para*-formaldehyde for 10 min, followed by permeabilization in 90% methanol at 4°C for 10 min. Phosphorylated Raf-1 was detected using anti-phospho-c-Raf (Ser338) (9427, Cell Signaling) or anti-c-Raf (pTyr340, Tyr341) (553009, Calbiochem), followed by incubation with PE-conjugated donkey anti-rabbit (Jackson ImmunoResearch). Levels of phosphorylated Raf-1 were analyzed on a FACS Calibur™ (Becton Dickinson).

Detection of DC-SIGN signalosome by immunoblotting

DC were stimulated for 5 min and whole cell extracts were prepared using Cell Lysis Buffer (Cell Signaling). Detection of DC-SIGN-associated proteins was performed by immunoprecipitating DC-SIGN from 40 µg of extract with anti-DC-SIGN (AZN-D1) coated on protein A/G-PLUS agarose beads (Santa Cruz), resolved by SDS-PAGE, and detected by

immunoblotting with anti-LSP1 (3812; Cell Signaling), anti-KSR1 (sc-25416; Santa Cruz), anti-CNK (611734, BD Biosciences), anti-Raf-1 (07-396; BD Upstate), anti-LARG (sc-25638, Santa Cruz), anti-RhoA (2117, Cell Signaling), or anti-DC-SIGN (sc-11038, Santa Cruz) to confirm equal precipitation, followed by incubation with the appropriate HRP-conjugated secondary antibody (DAKO) and ECL detection (Pierce).

Ras pull down assay

Cells were stimulated for 5 min and cell lysates were prepared. Active GTP-bound Ras was precipitated with GST-Raf-RBD-agarose (BD Upstate) as previously described⁴⁶, resolved by SDS-PAGE, and detected by immunoblotting with anti-Ras (BD Transduction Laboratories) as described above. Total Ras was detected in lysates to confirm equal amounts.

Statistical analysis

Data are presented as the means \pm standard deviations (s.d.) derived from at least 3 independent experiments unless otherwise stated. Statistical analyses were performed on the data using the Student's *t*-test for paired observations. Statistical significance of the data was set at $P < 0.05$.

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