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Syk-Dependent Cytokine Induction by Dectin-1 Reveals a Novel Pattern Recognition Pathway for C Type Lectins

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Summary

Pattern-recognition receptors (PRRs) detect molecular signatures of microbes and initiate immune responses to infection. Prototypical PRRs such as Toll-like receptors (TLRs) signal via a conserved pathway to induce innate response genes. In contrast, the signaling pathways engaged by other classes of putative PRRs remain ill defined. Here, we demonstrate that the β-glucan receptor Dectin-1, a yeast binding C type lectin known to synergize with TLR2 to induce TNFα and IL-12, can also promote synthesis of IL-2 and IL-10 through phosphorylation of the membrane proximal tyrosine in the cytoplasmic domain and recruitment of Syk kinase. *syk*$^{-/-}$ dendritic cells (DCs) do not make IL-10 or IL-2 upon yeast stimulation but produce IL-12, indicating that the Dectin-1/Syk and Dectin-1/TLR2 pathways can operate independently. These results identify a novel signaling pathway involved in pattern recognition by C type lectins and suggest a potential role for Syk kinase in regulation of innate immunity.

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Introduction

Early detection of potential pathogens is critical for protection from infection. PRRs play an important role in this process, sensing the presence of conserved molecular signatures of microbes and signaling for leukocyte activation and induction of antimicrobial immunity (Janeway, 1989). The archetypal examples of PRRs are the Toll-like receptors (TLRs), which regulate proinflammatory gene expression via a conserved signaling pathway involving activation of NF-κB, mitogen-activated protein kinases (MAPKs), and interferon regulatory factors (IRFs) (Akira and Takeda, 2004; Beutler, 2004). However, the term PRR is also used more widely to denote receptors that have not been shown to regulate transcription of innate response genes but are involved in microbial binding and clearance by phagocytes (Gordon, 2002). This deviation from the initial meaning is beginning to prove correct, as it is increasingly clear that some of those proteins regulate gene induction and modulate TLR signaling (Underhill, 2003). For example, the β-glucan receptor, Dectin-1, which mediates nonopsonic phagocytosis of yeast by mouse macrophages (MØ) (Brown et al., 2002), synergizes with TLR2 to augment proinflammatory cytokine responses to the microbe (Brown et al., 2003; Gantner et al., 2003). Similarly, human DC-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN) engagement by *Mycobacterium tuberculosis* alters TLR-induced cytokine responses (Geijtenbeek et al., 2003). However, it remains unclear to what extent Dectin-1, DC-SIGN, and other C type lectins signal directly or simply act as TLR coreceptors (Cambi and Figdor, 2003; Geijtenbeek et al., 2004).

In vertebrates, DCs translate PRR signaling into adaptive immunity (Reis e Sousa, 2004). PRR-mediated activation of DCs leads to an increase in T cell stimulatory potential and production of immunomodulatory cytokines, which is essential for T cell priming and effector T cell differentiation (Pasare and Medzhitov, 2004; Spörri and Reis e Sousa, 2005). Notably, the spectrum of cytokines produced by DCs is, to a large extent, dictated by the particular microbial stimulus to which they have been exposed (Kapsenberg, 2003; Reis e Sousa, 2004). Cytokine production by DCs can, therefore, be used as a readout to dissect pathways involved in innate recognition. In this context, we and others have previously reported that exposure to yeasts, as well as yeast cell walls (zymosan), induces production of very high levels of IL-10 by mouse DCs (Edwards et al., 2002; Qi et al., 2003). Although zymosan particles contain a ligand for TLR2 (Underhill et al., 1999), IL-10 production by DCs in response to yeast does not always require TLR2 or its adaptor MyD88 (Edwards et al., 2002; E.C.S. and C.R.S., unpublished data). Zymosan has also recently been shown to induce production of high levels of IL-2 by murine DCs (Feau et al., 2005; Granucci et al., 2003). Here, we show that IL-2 and IL-10 induction by yeasts may be due to β-glucan recognition and Dectin-1 signaling via Syk ki-

nase. Our results indicate that C type lectins can directly signal for gene transcription events, thereby acting as autonomous PRRs that can couple microbe sensing to innate responses.

Results

MyD88-Dependent and -Independent Responses in DCs Stimulated with Zymosan

Although mouse DCs have been reported to produce IL-10, IL-12 p40, and IL-2 upon stimulation with zymosan (Edwards et al., 2002; Feau et al., 2005; Gantner et al., 2003; Granucci et al., 2003; Qi et al., 2003), it is unclear as to what extent all of these cytokines can be produced by the same cell. To address this issue, zymosan-stimulated murine DCs grown in vitro from bone marrow precursors (BM-DCs) were analyzed for cytokine production by intracellular staining. As shown in Figure 1A, over 50% of DCs stained positive for IL-12 p40, and a significant fraction of these cells also stained for IL-2. IL-10-producing cells were found in both the IL-12 p40$^+$ and IL-12 p40$^-$ fractions, and some IL-10$^+$ DCs also produced IL-2 (Figure 1A). Crosscorrelation showed that some DCs can produce all three cytokines (Figure 1A and data not shown). Costaining for TNFα further suggested the presence of some cells that can produce all four cytokines (data not shown). Thus, individual DCs are able to produce multiple cytokines in response to zymosan.

We next determined whether all of these cytokines were induced via the TLR2/MyD88-dependent pathway for yeast recognition (Ozinsky et al., 2000; Underhill et al., 1999). Production of IL-12 p40 by BM-DCs stimulated with zymosan was partially dependent on MyD88 (Figures 1B and 1C), consistent with published results (Gantner et al., 2003). Similarly, IL-2 production was partially decreased in cultures of *MyD88*$^{-/-}$ DCs (Figure 1B). In contrast, the production of IL-10 by BM-DCs stimulated with zymosan was not decreased by MyD88 deficiency (Figure 1B), particularly when CD40L was used as a costimulus to increase the levels of the cytokine (E.C.S. and C.R.S., unpublished data). This was not because the stimulus was used at saturation, as IL-10 production by wild-type and *MyD88*$^{-/-}$ BM-DCs displayed similar dose-response curves (Figure 1C). Thus, in addition to the MyD88-dependent pathway that contributes to IL-2 and IL-12 p40 synthesis, BM-DCs possess a MyD88-independent pathway for yeast recognition that is sufficient for production of IL-10, confirming previous observations with spleen DCs (Edwards et al., 2002).

Cytokine Induction by Zymosan Involves β-Glucan Recognition by DCs

Mannans and β-glucans are the major carbohydrate components of yeast cell walls (Di Carlo and Fiore, 1958). We found that addition of two soluble β-glucans, glucan-phosphate or laminarin, but not mannose-containing glycans such as mannosylated-BSA, could partially inhibit zymosan induction of IL-2, IL-10, and IL-12 p40 in BM-DCs (Figure 2A) and spleen DCs (see Figure S1 in the Supplemental Data available with this article online). In addition, the same β-glucans also decreased zymosan uptake by DCs (Figure S1). However, phagocytosis of the particles was not necessary for IL-12 p40 or IL-10 induction because inhibition of actin polymerization with latrunculin B did not prevent synthesis (Figure 2B), although it blocked zymosan internalization (data not shown). In contrast, IL-2 production was inhibited by latrunculin B (Figure 2B), although it is unclear whether this reflects a requirement for particle phagocytosis or a possible role for G-actin in regulating signaling for IL-2 gene expression (Sotiropoulos et al., 1999). Together, these results suggest that a DC-expressed β-glucan binding receptor could be involved in signaling for cytokine induction and/or zymosan binding.

Signals through Dectin-1 Couple Yeast Recognition to IL-10 and IL-2 Production

Dectin-1 is a type II transmembrane protein that contains a single β-glucan binding C type lectin motif in its extracellular domain (Herre et al., 2004a) and is expressed by several leukocytes, including spleen DCs (Ariizumi et al., 2000; Reid et al., 2004; Taylor et al., 2002) and BM-DCs (Gantner et al., 2003). We found that Dectin-1 expression is independent of MyD88 (Figure S2), suggesting that this lectin could be involved in both the TLR-dependent and -independent effects of zymosan in DCs. In the absence of a Dectin-1-deficient mouse, we tried a "gain-of-function" experiment and examined the ability of Dectin-1 to confer zymosan responsiveness to heterologous cells. We chose to use the LK35.2 cell line, a mouse B cell hybridoma (Kappler et al., 1982), because it does not bind zymosan but produces IL-2 and IL-10 (although not IL-12 p40) in response to B cell receptor crosslinking (data not shown). LK cells transduced with a GFP-reporter retrovirus carrying an HA-tagged version of full-length Dectin-1 displayed unimodal surface HA staining on all GFP$^+$ cells, indicating uniform receptor expression at the cell surface (Figure S3A). Dectin-1-transduced LK cells, but not cells transduced with empty virus, were able to bind fluorescent zymosan (Figure S3B) and displayed accumulation of the receptor at sites of zymosan apposition with the membrane (Figure 3A). Remarkably, the same cells now produced both IL-2 and IL-10 in response to zymosan (Figure 3B), and this was completely inhibitable by glucan phosphate (Figure S3C). Notably, LK cells transduced with a truncated version of Dectin-1 that lacks the cytoplasmic domain were able to bind zymosan more avidly than cells expressing full-length Dectin-1 (Figure S3B), likely because they expressed higher levels of the receptor (Figure S3A). However, they were unable to produce IL-2 or IL-10 in response to the yeast particles (Figure 3B). We conclude that full-length, but not truncated, Dectin-1 can couple yeast recognition to IL-2 and IL-10 production.

The Intracellular Portion of Dectin-1 Can Recruit Syk Kinase upon Tyrosine Phosphorylation

The inability of truncated Dectin-1 to mediate IL-10 and IL-2 induction suggested that the cytoplasmic domain of the lectin was able to mediate downstream signaling. In MØ, the cytoplasmic tail of Dectin-1 is known to be tyrosine phosphorylated upon zymosan binding (Gantner

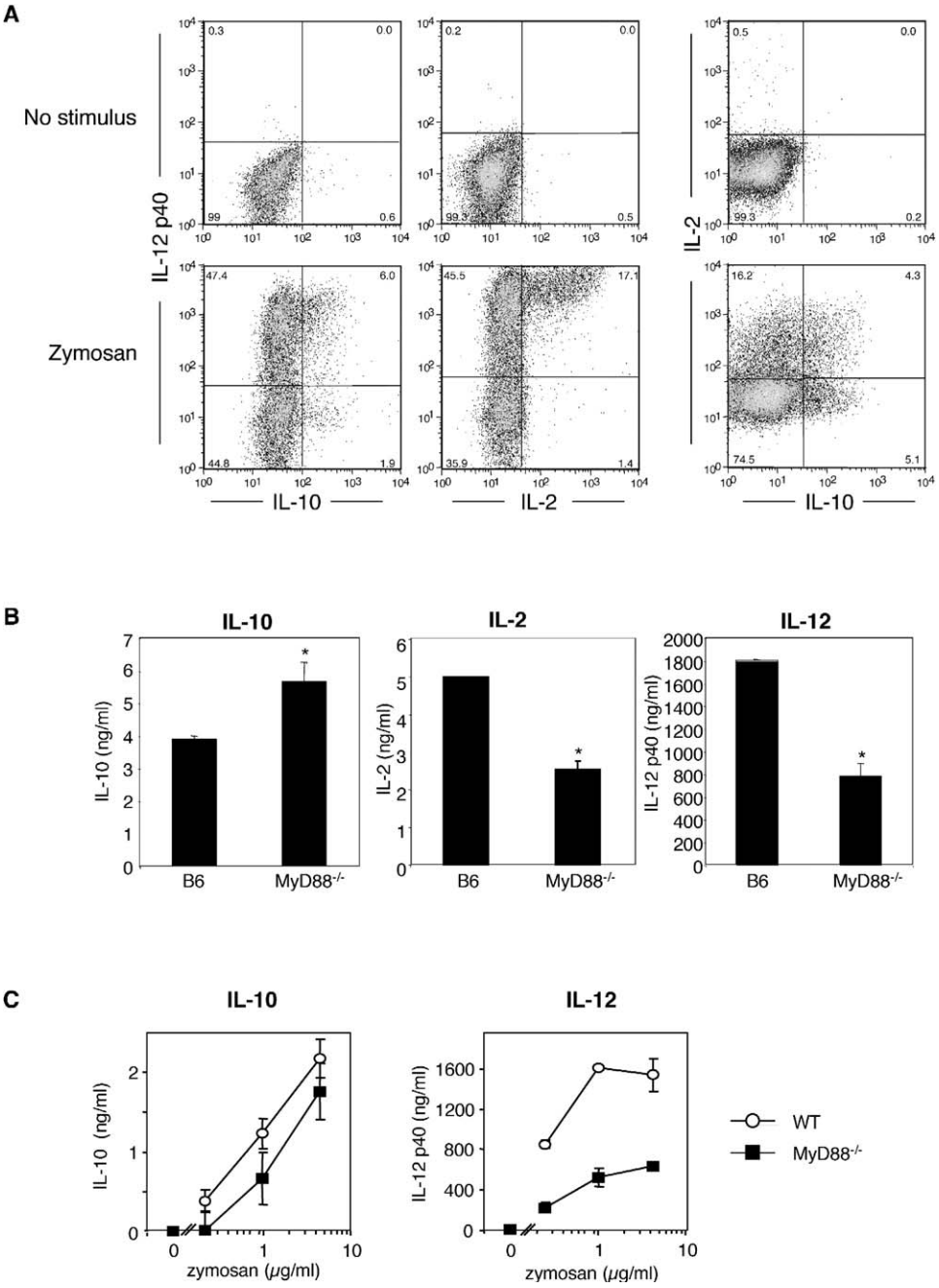

Figure 1. Production of Cytokines by BM-DCs upon Zymosan Stimulation

(A) C57BL/6 BM-DCs were cultured for 3 hr in the presence or absence of 50 μg/ml of zymosan before adding brefeldin A (final concentration 5 μg/ml) and culturing for a further 6 hr. Cells were recovered, fixed, and stained for the indicated intracellular cytokines. Plots represent gated $CD11c^+$ DCs, and numbers indicate the percentage of cells in each quadrant.

(B and C) $MyD88^{-/-}$ or control C57BL/6 BM-DCs ([B], 10^5 cells per well; [C], 1.4×10^5 cells per well) were cultured overnight with 50 μg/ml (B) or the indicated doses (C) of zymosan. Cytokine levels in culture supernatants were determined by ELISA. Error bars represent 1 SD from the mean of triplicates. Asterisks in (B) indicate statistically different ($p < 0.05$) levels of cytokine production by $MyD88^{-/-}$ DCs compared to C57BL/6 controls. Data are representative of three independent experiments.

et al., 2003). The only tyrosines in the intracellular portion of Dectin-1 are within the sequence MK**Y**HS HI ENLDEDG**YTQL**DFSTQ, which resembles the immunoreceptor tyrosine-based activation motif (ITAM; bolded above) found in the signaling chains of lymphocyte antigen receptors (Reth, 1989). Dually phosphorylated ITAMs recruit tandem SH2-containing kinases of the Syk family (Syk and ZAP-70), which undergo autophosphorylation and initiate a signaling cascade (Turner et al., 2000). Although Syk is not necessary for Dectin-1/

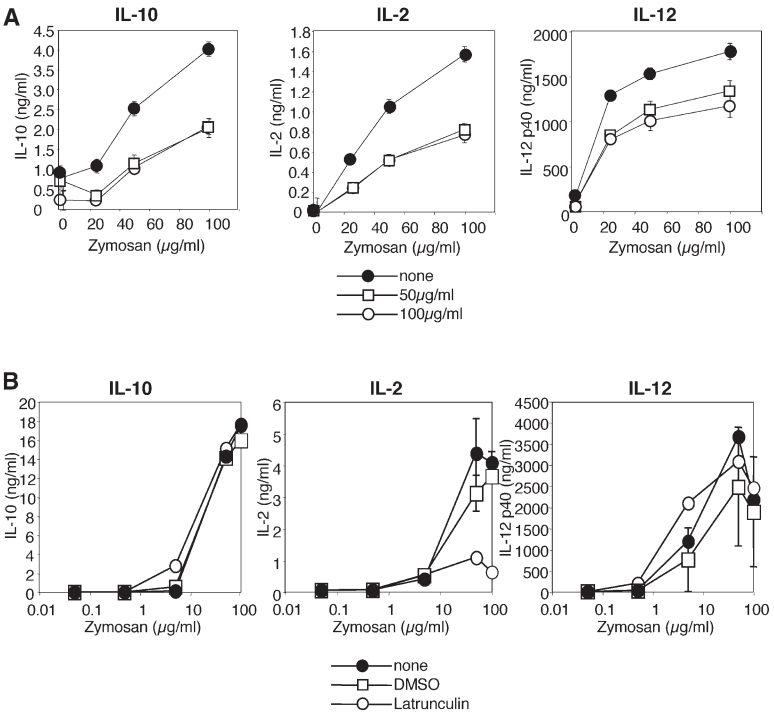

Figure 2. Zymosan Stimulation Is Partially Blocked by Soluble β-Glucans and Does Not Require Particle Internalization

(A) C57BL/6 BM-DCs (10^5 cells per well) were cultured with the indicated concentrations of zymosan in the presence or absence of 50 or 100 μg/ml glucan phosphate. Cytokine production was measured after overnight culture.

(B) Bulk C57BL/6 BM-DC populations (unenriched by magnetic sorting; 2×10^5 cells per well) were cultured with the indicated concentrations of zymosan in the presence or absence of 10 μM latrunculin B or vehicle (DMSO) alone. Cytokine production was measured after overnight culture. Error bars represent 1 SD from the mean of triplicates. Data are representative of three separate experiments.

TLR2-mediated TNFα production by MØ (G.D.B., unpublished data), it was possible that Dectin-1 could signal through this kinase in other responses. In line with this notion, we were able to visualize phosphorylated Syk at phagocytic cups formed during the interaction of zymosan particles with Dectin-1-transduced LK cells (Figure 4A). Importantly, phospho-Syk enrichment at these sites was only observed in cells expressing the intact receptor and not the truncated mutant (Figure 4A), consistent with the fact that the tail of Dectin-1 is critical for signaling.

To determine if Dectin-1 has the potential to recruit Syk directly, we examined whether the Dectin-1 tail can associate with Syk kinase. A peptide comprising the Dectin-1 tail sequence specifically precipitated recombinant Syk when dually phosphorylated on tyrosines (pY-pY; Figure 4B). In contrast, a control peptide with the same sequence but containing unphosphorylated tyrosines (Y-Y) did not bind Syk (Figure 4B). Surprisingly, dual phosphorylation was not required, as Syk could be precipitated by a peptide containing a single phosphotyrosine at position 15 (membrane-proximal site; Y-pY) although not by an equivalent peptide with a single phosphotyrosine at position 3 (membrane-distal site; pY-Y) (Figure 4B). Indeed, peptides containing a single phosphotyrosine at position 15 and a phenyalanine or alanine at position 3 precipitated recombinant Syk, formally demonstrating that the membrane-distal tyrosine is dispensable for Syk binding (Figure 4B). Endogenous Syk could similarly be precipitated by the pY-pY or Y-pY peptide from cell lysates (Figure 4C). Precipitation of Syk with either peptide required equivalent peptide concentrations, suggesting that both bind Syk with similar affinities (Figure 4C).

The above results suggested that single phosphorylation of the membrane-proximal tyrosine in the Dectin-1 tail might be sufficient to permit association with Syk kinase and couple to downstream responses. To test this prediction, LK cells were transfected with point mutants of Dectin-1 in which either or both of the tyrosines were mutated to phenylalanine. As shown in Figure 5A, all three mutants were able to bind zymosan, yet phospho-Syk recruitment was only observed in the Y3F mutant, in which the membrane-proximal tyrosine is preserved, and was lost in the other two mutants, Y15F and the double mutant, in which the membrane-proximal tyrosine is eliminated. Consistent with these data, cells transfected with the Y3F Dectin-1 mutant showed unaltered IL-2 and IL-10 responses to zymosan, whereas cytokine production could not be induced in cells transfected with either the Y15F or double mutant versions (Figure 5B). We conclude that the membrane-proximal tyrosine of Dectin-1 (position 15) is sufficient to mediate Syk recruitment and to couple to downstream IL-2 and IL-10 responses.

Syk Is Essential for Zymosan-Induced IL-2 and IL-10 Production by DCs

As Syk is also expressed by DCs (Sedlik et al., 2003), we assessed if the kinase is recruited to sites of DC contact with zymosan, as seen with Dectin-1-transfected LK cells. BM-DCs phagocytose zymosan very avidly, and most particles are found within phagosomes minutes after cell contact. Notably, most of these phagosomes showed clear phospho-Syk accumulation (Figure 6A), which was unaltered in MyD88$^{-/-}$ cells (data not shown). Therefore, we determined whether Syk is necessary for yeast induction of IL-10 and IL-2 in DCs. *syk*$^{-/-}$ DCs were purified from spleens or grown from bone marrow of chimeric mice that had been reconsti-

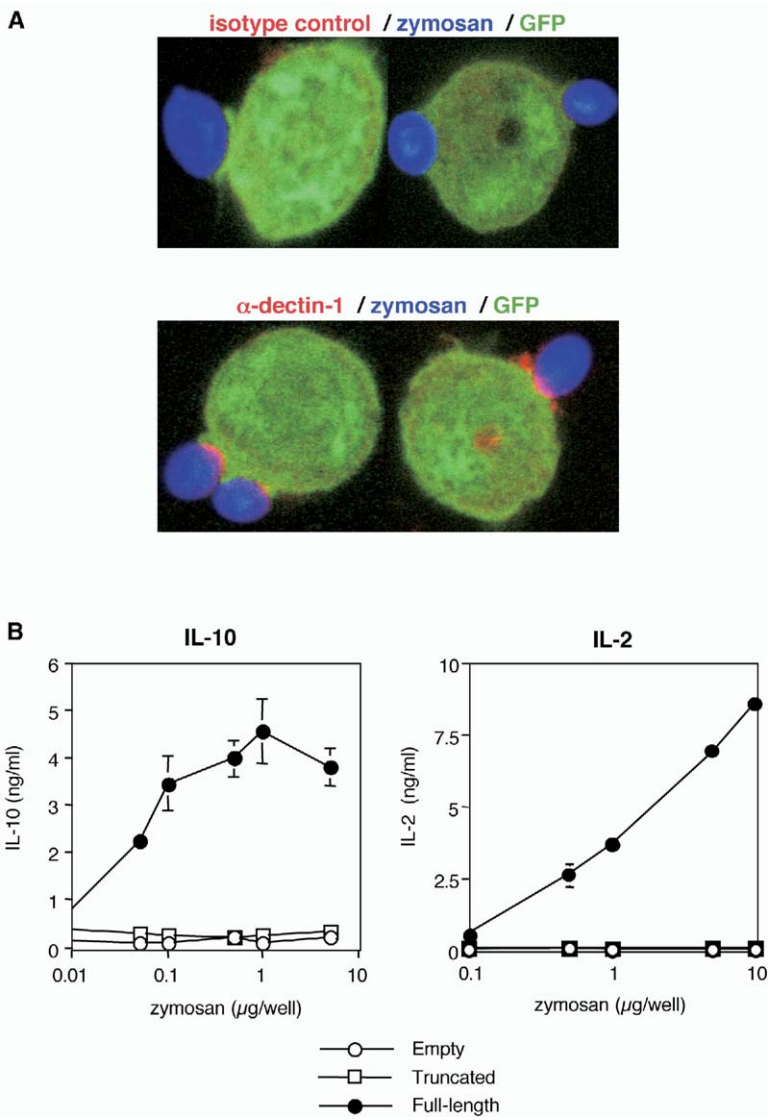

Figure 3. Full-Length Dectin-1 Is Sufficient to Mediate Yeast Induction of IL-10

(A) LK cells expressing Dectin-1 bind zymosan and show receptor accumulation in phagocytic cups. LK cells transduced with full-length Dectin-1 (see Figure S3) were co-cultured with Cy5-labelled zymosan for 5 min before fixation and staining with anti-Dectin-1 or an isotype-matched control antibody, as indicated. Images show an optical section through GFP$^+$ cells (green). Receptor staining (bottom; red) can be seen in close apposition with bound zymosan particles (blue).

(B) LK cells (10^5 cells per well) transduced with empty vector, full-length Dectin-1, or a truncated version of the receptor were stimulated with the indicated doses of zymosan. IL-10 (left) or IL-2 (right) levels in supernatants were measured after overnight culture. Error bars represent 1 SD from the mean of triplicates. Data are representative of three independent experiments.

tuted with *syk*$^{+/-}$ or *syk*$^{-/-}$ fetal liver (Sedlik et al., 2003). *syk*$^{-/-}$ BM-DCs pulsed with zymosan no longer stained for phospho-Syk, as expected (Figure S4). Strikingly, Syk-deficient BM-DCs were also completely unable to produce IL-2 or IL-10 in response to zymosan but produced normal levels of IL-12 (Figure 6B) and IL-6 (data not shown), demonstrating that they could still bind the particles and signal via the Dectin-1/TLR2 pathway (Brown et al., 2003; Gantner et al., 2003). However, Syk-deficient DCs also showed a defect in zymosan phagocytosis (Figure S5), in contrast to previous observations with MØ (Herre et al., 2004b). Therefore, in some experiments, we included latrunculin B to prevent particle internalization and to normalize for the differences in zymosan uptake between wild-type and *syk*$^{-/-}$ DCs. Independently of uptake, there was a profound difference between *syk*$^{-/-}$ and Syk-sufficient DCs at the level of IL-10, but not IL-12 production (Figure 6C). IL-2 synthesis could not be assessed in these experiments, as it is inhibited by latrunculin B (see above). Syk-deficient DCs also responded normally to CpG-containing oligonucleotides, which signal via TLR9 (Figure 6C). DCs doubly deficient for Syk and ZAP-70 behaved identically to the *syk*$^{-/-}$ singly deficient cells (data not shown), consistent with reports that DCs do not express ZAP-70 (Sedlik et al., 2003). In line with the DC data, treatment of Dectin-1-transfected LK cells with piceatannol, a Syk inhibitor, also abrogated IL-2 and IL-10 induction by zymosan (data not shown). We conclude that Syk is required for zymosan-induced IL-2 and IL-10 production, but not for IL-12 synthesis.

Discussion

The study of innate recognition offers much promise for understanding and manipulating immunity. Here, we describe a novel pattern recognition pathway involving the β-glucan receptor Dectin-1 and a kinase, Syk, not previously implicated in innate responses. We show that soluble β-glucans inhibit zymosan-induced cyto-

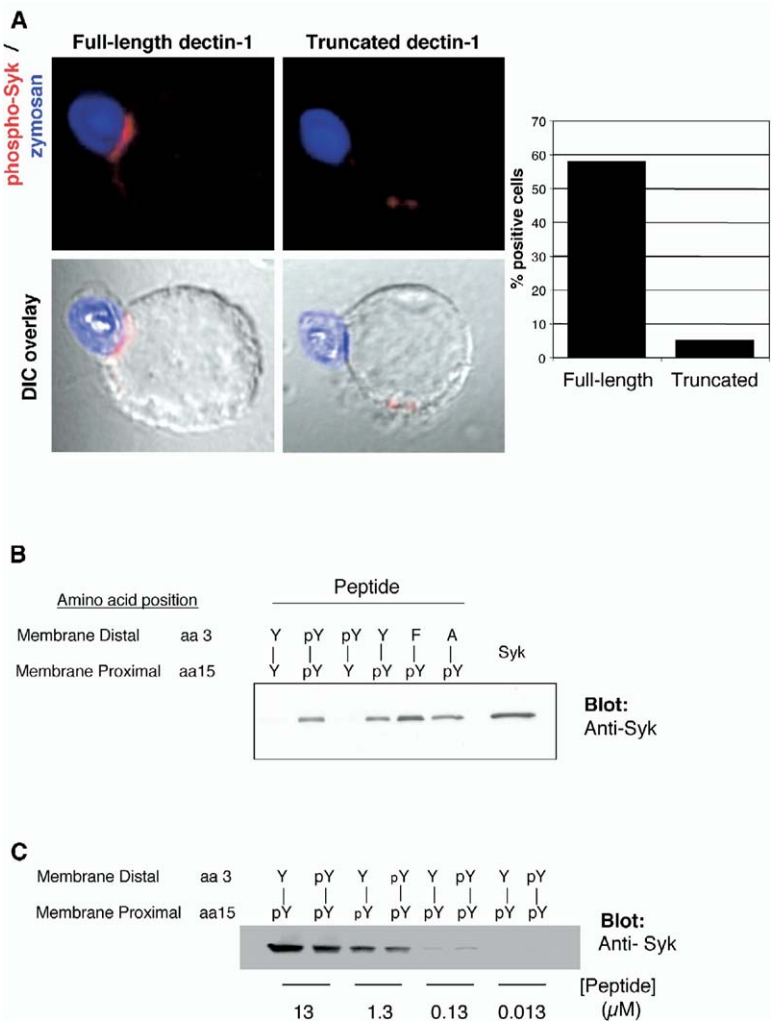

Figure 4. The Intracellular Tail of Dectin-1 Can Directly Mediate Syk Recruitment

(A) LK cells expressing full-length or truncated Dectin-1 were stimulated with Cy5-zymosan for 5 min at 37°C before fixation and staining for phospho-Syk. Graph represents the percentage of cells in which red staining was seen adjacent to bound zymosan particles (n = 161 cells expressing full-length Dectin-1 and n = 212 cells expressing the truncated receptor). GFP fluorescence has been omitted for clarity.

(B) 50 ng of recombinant full-length human Syk was incubated with biotinylated peptides corresponding to the N terminus of murine Dectin-1 and precipitated with streptavidin-sepharose. Western blots were probed with anti-Syk. Right lane shows a control immunoprecipitation with anti-Syk.

(C) Lysates from LK cells were used for precipitation with the indicated concentrations of Y-pY or pY-pY peptide as in (B). Western blots were probed with anti-Syk.

kine production by DCs, and that Syk-deficient DCs are unable to produce IL-2 and IL-10 in response to the stimulus but still produce IL-12. We further show that Dectin-1 is sufficient to confer zymosan responsiveness to a B cell line and that in such cells, as well as in DCs, phosphorylated Syk is found associated with zymosan-containing phagosomes and phagocytic cups. In addition, we present evidence suggestive of a direct interaction between the phosphorylated membrane-proximal YxxL motif of the Dectin-1 tail and Syk kinase. Therefore, we believe that Dectin-1 phosphorylation upon ligand binding (Gantner et al., 2003) leads to recruitment of Syk and to initiation of a signaling cascade that among other things, permits IL-2 and IL-10 gene transcription. Maximal IL-2 synthesis by DCs requires not only Syk but also signaling via MyD88 (Figure 1B), although it is unclear at this stage whether this is due to a requirement for TLR signaling in induction of IL-15, which is important for IL-2 production by DCs (Feau et al., 2005), or whether it reflects cooperation between Dectin-1 and TLR2, as reported for IL-12 p40 and TNFα (Brown et al., 2003; Gantner et al., 2003). In contrast, IL-10 induction can occur independently of TLR signaling, especially when the cells receive a costimulus through CD40, indicating that the Dectin-1/Syk axis can operate as a TLR-independent pattern recognition-pathway. IL-10 induction involves coupling of Syk to the ERK pathway (E.C.S. and C.R.S., unpublished data), which is known to regulate DC production of the cytokine in other systems (Agrawal et al., 2003; Dillon et al., 2004).

Dectin-1 contains an ITAM-like motif in its intracellular tail. Peptides corresponding to this motif are able to bind Syk in vitro, but this interaction is atypical in that it only requires a single phosphotyrosine residue (Figures 4B and 4C). Notably, mutation of the same tyrosine in the intact receptor abrogates Syk recruitment in transfected cells (Figure 5A). Although these data cannot completely rule out an indirect interaction between Syk and Dectin-1 in vivo, they suggest that the interaction between the two molecules could occur via direct binding of a single SH2 domain of the kinase to the membrane-proximal phosphotyrosine of the receptor. This would be consistent with the fact that the interaction displays affinities in the μM range (Figure 4C), which are too low for binding via the tandem SH2 domains (Ottinger et al., 1998). Nevertheless, in the context of the plasma membrane, Syk might form a bridge across two Dectin-1 molecules that are in close

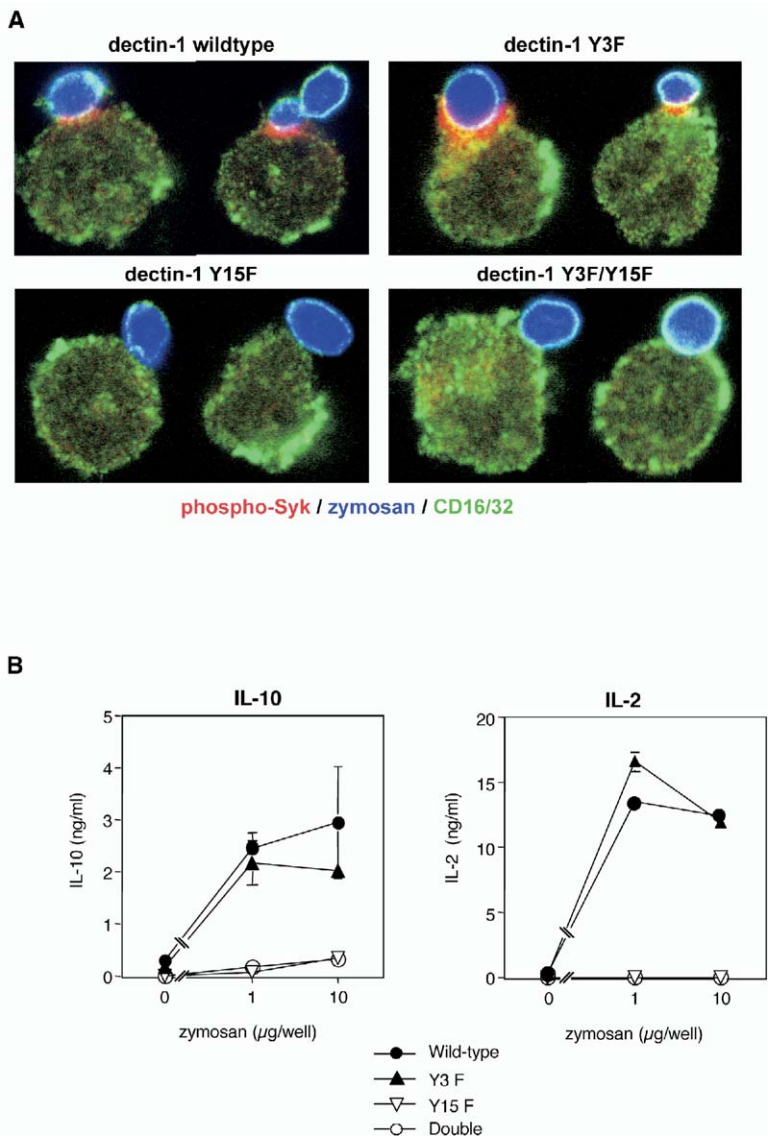

Figure 5. The Membrane Proximal Tyrosine in the Intracellular Tail of Dectin-1 Is Sufficient to Mediate Syk Recruitment and Induce IL-2 and IL-10 Responses

(A) LK cells expressing either wild-type or the indicated Dectin-1 mutants and stimulated with Cy5-zymosan (blue) for 5 min were fixed and stained for phospho-Syk (red) and CD16/32 (green). Images are optical sections through representative cells.

(B) The same mutants as in (A) (10^5 cells per well) were stimulated overnight with the indicated concentrations of zymosan. IL-2 and IL-10 levels in supernatants were determined by ELISA. Error bars represent 1 SD from the mean of triplicates. Data are representative of two independent experiments.

proximity, binding to each via a single SH2 domain. Notably, several C type lectins display an YxxL motif in the intracellular domain (Cambi and Figdor, 2003) and may also recruit Syk. In human DCs, engagement of DC-SIGN suppresses Th1 responses, subverts TLR signaling, and elicits production of IL-10 (Bergman et al., 2004; Geijtenbeek et al., 2003), although it is not known if this is Syk dependent. DC-SIGN-related (Takahara et al., 2004) and other lectins in mouse DCs may also signal via Syk and could contribute to zymosan recognition and induction of IL-2/IL-10, consistent with the fact that Syk deficiency has a more profound effect than β-glucan inhibition on the zymosan response (compare Figure 2A with Figures 6B and 6C). Thus, Dectin-1 may be one of a family of innate receptors that signals via Syk and participates in responses to yeasts and other microbial organisms.

Zymosan phagocytosis by DCs is partially Syk dependent (Figure S5), as previously suggested for Dectin-1-transfected fibroblasts (Herre et al., 2004b). In contrast, in MØ, zymosan phagocytosis is Syk independent, although it requires the membrane-proximal tyrosine of Dectin-1 (Herre et al., 2004b). Thus, it would appear that Syk recruitment to the membrane-proximal tyrosine of Dectin-1 may take place in DCs and LK cells, but not in MØ, perhaps because of competition by another SH2 domain-containing protein. Consistent with this notion, we have observed that zymosan induces only low levels of IL-10 from MØ (A.D.E. and C.R.S., unpublished data). The cell-type dependence of these responses may reflect the large number of innate receptors putatively involved in yeast recognition, as well as the multitude of intracellular signals initiated from the cytoplasmic tail of C type lectins, which can involve direct or indirect coupling to Syk as well as TLR-dependent pathways (Brown et al., 2003; Gantner et al., 2003; Herre et al., 2004b). Further work in this area will require comparison of responses to zymosan versus agonists that selectively trigger Dectin-1 signaling.

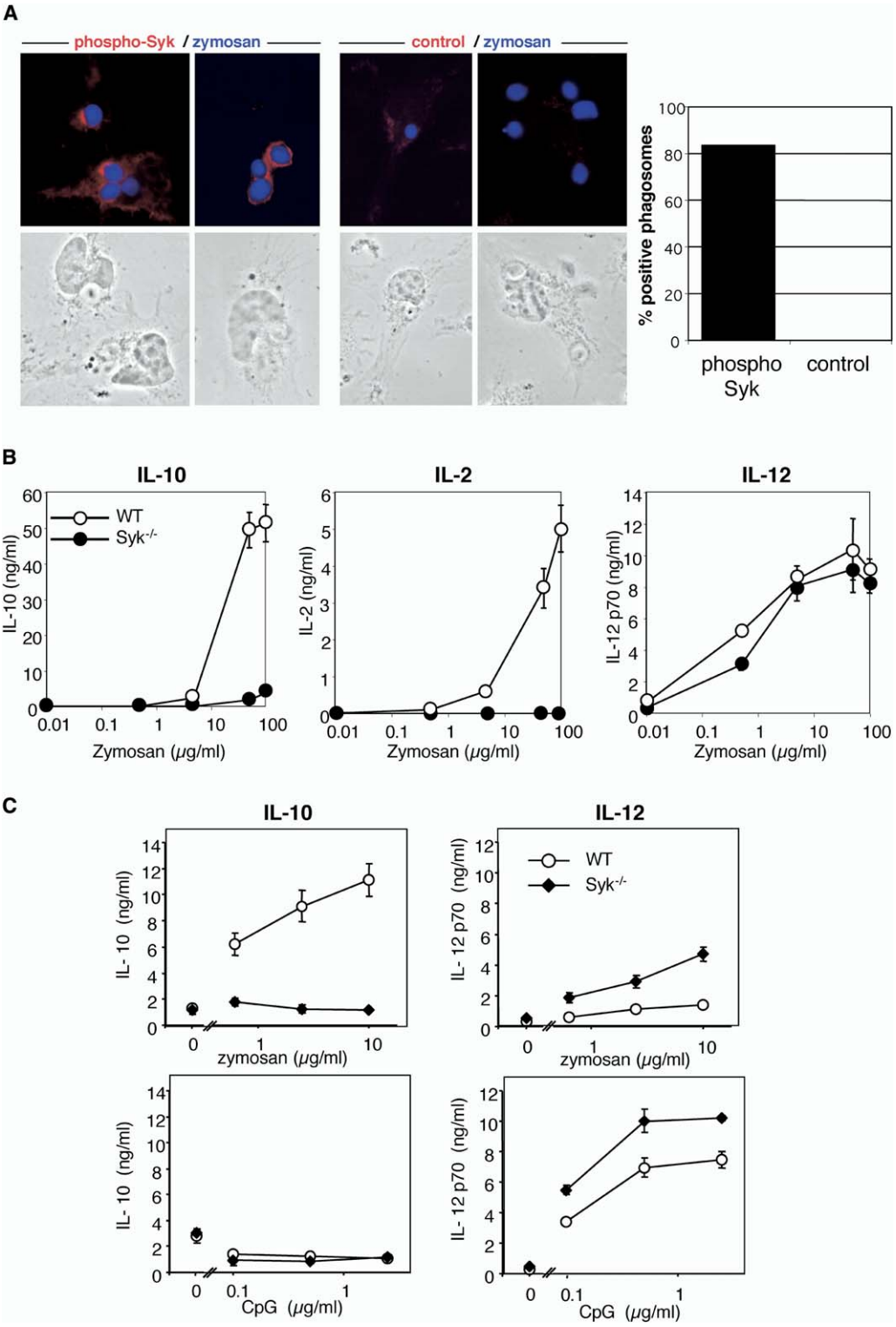

Figure 6. Zymosan-Induced IL-2 and IL-10 Production by DCs Requires Syk

(A) Phospho-Syk (red) is recruited to DC phagosomes containing zymosan (blue). B6 BM-DCs were incubated with Cy5-zymosan for 5 min at 37°C before fixation and staining with anti-phospho-Syk (left) or secondary antibody alone (right). Fluorescence and corresponding phase-contrast images are shown in the top and bottom panels, respectively. All cells also stained with anti-CD11c (not shown for clarity). Graph indicates the percentage of zymosan-containing phagosomes in B6 BM-DCs that stained for phospho-Syk (n = 30). Staining was regarded positive when the mean red fluorescent intensity of 5 × 5 pixels around the phagosome was 2-fold greater than the average intensity in the rest of the cell. The control was the signal with the secondary antibody alone.

Many yeasts are commensal organisms, yet they can cause opportunistic infections in immunocompromised individuals (Romani, 2004). Resistance to yeast infection has been linked to strong Th1-biased responses in which IL-12 plays a critical role (Romani, 2004). In this context, it seems paradoxical that yeast cell walls should also induce significant levels of IL-10, an anti-inflammatory cytokine that counteracts the effects of IL-12 and is associated with suppressing rather than promoting Th1 immunity. It could be envisaged that this represents an immune escape mechanism by yeasts, as in the case of pathogens that target DC-SIGN (Bergman et al., 2004; Geijtenbeek et al., 2003). Indeed, it has been argued that TLR2-mediated IL-10 induction by *Candida albicans* is primarily of benefit to the pathogen (Netea et al., 2004). However, it is difficult to see why an immune escape mechanism would be conserved in nonpathogenic yeasts such as the *Saccharomyces cerevisiae* used here as a zymosan source. A more likely explanation is that the induction of IL-10 is of benefit to the host (Romani, 2004). Indeed, recent evidence suggests that IL-10 plays an important role in the development of regulatory T cells (Tregs) during infection with *C. albicans* (Montagnoli et al., 2002; Netea et al., 2004). Similarly, IL-2 is a well-known growth factor for Tregs (Malek and Bayer, 2004). Although Tregs may be deleterious in the case of disseminated candidiasis (Montagnoli et al., 2002; Netea et al., 2004), it is thought that they serve to limit immunopathology in the context of a local infection (Romani, 2004) and that they prevent complete elimination of the fungus, allowing persistence of immunity and resistance to reinfection (Montagnoli et al., 2002). In line with the latter, Tregs also prevent sterile immunity in a *Leishmania major* infection model, thereby maintaining immunological memory to the parasite (Belkaid et al., 2002). The results presented here suggest that the development of Th1 effectors versus Tregs in infection might be controlled by separate innate recognition pathways. If so, pathways preferentially leading to regulation, such as the Dectin-1/Syk axis, could be exploited therapeutically in allergy, autoimmunity, and graft rejection.

Experimental Procedures

Mice and Cells

BM-DCs were generated as described (Inaba et al., 1992) from bone marrow of MyD88-deficient (Adachi et al., 1998) (kind gift from Shizuo Akira) or control C57BL/6 mice (both strains bred at Cancer Research UK, Clare Hall). Unless stated otherwise, DCs were first purified from bulk BM-DC cultures by magnetic enrichment with anti-CD11c beads. Splenic DCs (from C3H/HeJ mice, Charles River UK, unless otherwise stated) were purified by magnetic enrichment and cell sorting as described (Edwards et al., 2002). Radiation chimeras were used to analyze DCs genetically deficient for Syk. Fetal livers from $syk^{+/-}$ × $syk^{+/-}$ matings (B10.D2 background or B10.D2 backcrossed twice to B10 [Turner et al., 1995]) were removed from d16.5 embryos, and $syk^{-/-}$ fetuses were identified by a hemorrhaging phenotype upon removal (Turner et al., 1995). Fetal liver cell suspensions from $syk^{-/-}$ and Syk-sufficient womb mates were then used to reconstitute irradiated recipients (BALB/c or B6.SJL). DCs were purified by cell sorting from spleens or were grown from bone marrow of radiation chimeras 5–8 weeks after reconstitution. All DCs from the chimeric mice were of donor origin (data not shown).

Reagents

Culture medium was RPMI 1640 or Dulbecco's modified Eagle's medium (both from Gibco) supplemented with glutamine (Gibco), penicillin, streptomycin (Gibco), and 10% heat-inactivated fetal calf serum (Autogen Bioclear, Wiltshire). CpG-containing DNA was the phosphorothioate-linked oligonucleotide 1668 with the sequence 5′-TCCATGACGTTCCTGATGCT-3′ (Krieg et al., 1995). Zymosan (Sigma, Poole, UK) was boiled for 30 min and washed twice in phosphate-buffered saline (PBS) before storage in aliquots. Fluorescent zymosan was prepared by adding zymosan to fluorescein-isothiocyanate (FITC; Sigma, Poole, UK) dissolved in water or by adding zymosan to Cy5 maleimide (Amersham Pharmacia, Amersham, UK) dissolved in 100 mM HEPES. After 5–30 mins at room temperature, the particles were washed five times in PBS. Glucan phosphate was prepared as described (Muller et al., 1996). Brefeldin A, laminarin, dextran, and mannosylated bovine serum albumin (Man-BSA) were from Sigma (Poole, UK). Latrunculin B was from Calbiochem (San Diego, CA). GM-CSF was made by the Cancer Research UK protein purification service, and batches were titrated to give optimal growth conditions for BM-DCs. Full-length, truncated, and point mutant Dectin-1 in pFB-Neo (Brown et al., 2003) or subcloned into pMSCV-EGFP ([Tsujimura et al., 2003]; kind gift from Hideki Tsujimura, National Institutes of Health) were used for transfection of LK35.2 cells (Kappler et al., 1982) (LK cells, American Type Culture Collection HB-98) or for retroviral production in an ecotropic packaging cell line (Pear et al., 1993), followed by LK cell transduction. LK cells expressing Dectin-1 were selected on the basis of EGFP expression (pMSCV-EGFP vector) or neomycin resistance (pFB-Neo vector).

Stimulation Assays

Stimulation assays were performed as described (Edwards et al., 2002). Briefly, 10^5 LK cells, 4–20 × 10^4 BM-DCs, or 2–5 × 10^4 sorted spleen DCs per well (numbers indicated in figure legends) were cultured overnight in 100–200 μl culture medium with or without GM-CSF in 96-well flat-bottomed plates in the presence of zymosan or control stimuli. In experiments with splenic DCs, CD40L-expressing fibroblasts (plated on the eve of the experiment at 10^4 cells per well) were included in order to amplify IL-10 levels via CD40 triggering (Edwards et al., 2002). Supernatants were removed, and cytokine levels were measured by sandwich ELISA. For inhibition studies, cells were preincubated for 30 min with glycans and/or latrunculin B before addition of zymosan and subsequently were cultured in the continued presence of the inhibitors. In some cases, DCs remaining in the wells were analyzed for zymosan uptake by flow cytometry as described below.

Flow Cytometry

For analysis of binding and/or uptake of zymosan, cells were cultured with fluorescent zymosan for 20 min to overnight and then analyzed by flow cytometry. In some cases, cells were preincubated for 10–30 min with the indicated carbohydrate preparations and/or latrunculin B. In experiments with spleen DCs, the cells were stained for CD8α, CD4, and CD11c either before or after culture with the particles. For analysis of Dectin-1 expression, cells were

(B) $syk^{-/-}$ or wild-type (wt) $CD11c^+$ BM-DCs (10^5 cells per well) were stimulated with the indicated doses of zymosan for 24 hr. Cytokine levels in supernatant were determined by ELISA.
(C) $syk^{-/-}$ or wt $CD11c^+$ spleen DCs (9 × 10^4 cells per well) were stimulated with the indicated doses of zymosan or CpG. CD40L-expressing fibroblasts were included in the cultures to amplify cytokine production, as described (Edwards et al., 2002). Latrunculin B was added to prevent zymosan internalization. Levels of IL-12 p70 and IL-10 in overnight supernatants were determined by ELISA. Error bars (B and C) represent 1 SD from the mean of triplicates. Data are representative of three independent experiments.

incubated with 10 μg/ml anti-Fc-receptor (clone 2.4G2), followed by biotinylated anti-Dectin-1 (clone 2A11 [Brown et al., 2002]) or biotinylated rat IgG2b isotype control (Pharmingen, San Diego, CA). After washing, cells were stained with PE-labeled streptavidin and, where appropriate, APC-labeled anti-CD11c and FITC-labeled anti-MHC-II (all Pharmingen). For cytokine intracellular staining, BM-DCs were stained with anti-CD11c, fixed with Fix and Perm Reagent A (Caltag Laboratories, Burlingame, CA), and then resuspended in Fix and Perm Reagent B (Caltag Laboratories) containing anti-IL-2 (JES6-5H4), anti-IL-10 (JES5-16E3), or anti-IL-12 p40/70 (C17.8) (all Pharmingen) all directly conjugated to different fluorophores. Data acquisition was performed on a FACSCalibur (Becton Dickinson, San Jose, CA); analysis was performed with FlowJo Software (Tree Star, Inc., San Carlos, CA).

Immunoprecipitations, Peptide Pulldowns, and Western Blotting

Cell extracts were prepared in lysis buffer (50 mM HEPES [pH 7.4], 150 mM sodium chloride, 100 mM sodium fluoride, 10 mM tetrasodium pyrophosphate, 1 mM sodium orthovanadate [pH 10.0], 1 mM EDTA [pH 8.0], 1.5 mM magnesium chloride, 10% glycerol, 1% Triton X-100, 1 mM PMSF, and "Complete" protease inhibitor cocktail tablets [Roche]); insoluble material was discarded. Biotin-conjugated peptides were dissolved in 40% DMSO before dilution in lysis buffer. Recombinant human Syk (Upstate) diluted in lysis buffer or cell extracts were incubated with the indicated biotinylated peptides corresponding to the Dectin-1 intracellular tail (Cancer Research UK Peptide Synthesis Laboratory) or with anti-Syk rabbit serum ("2131" serum raised against a synthetic peptide corresponding to amino acids 318–330 of murine Syk [Turner et al., 1995]) and streptavidin- or GammaBind Plus-sepharose (Sigma Biosciences AB, Uppsala, Sweden). After affinity purification, Sepharose beads were washed once in lysis buffer and boiled in SDS gel-loading buffer containing 10% β-mercaptoethanol. Proteins were separated by sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto Immobilon PVDF membranes (Millipore Corporation, Bedford, MA), and probed with rabbit anti-Syk (a combination of 2131 serum and anti-Syk from Cell Signaling Technology, Inc., catalog number 2712, raised against a synthetic peptide corresponding to the C terminus of human Syk) followed by chemiluminescent detection.

Confocal Microscopy

LK cells or BM-derived DCs were allowed to adhere to poly-L-lysine-coated coverslips. Cy5-labeled zymosan was added at 15 μg per well and pelleted onto the cells by brief centrifugation, and the cultures were incubated at 37°C for 5 min. Cells were fixed in 2% paraformaldehyde/PBS, and free aldehyde groups were quenched with 0.1 M glycine/PBS. In some instances, cells were subsequently permeabilized in 0.1% Triton-X100/PBS for 4 min. After washing with PBS, cells were blocked with 5% FCS/PBS and anti-Fcγ receptor (clone 2.4G2). Dectin staining was performed with biotinylated anti-2A11 or biotinylated rat IgG2b isotype control. Phosphorylated Syk was detected with rabbit polyclonal anti-phospho-ZAP70(Tyr319)/Syk(Tyr352) (Cell Signaling Technology, catalog number 2701, raised against residues surrounding Tyr319 of human Zap-70; crossreacts with Syk phosphorylated at tyrosine 352) followed by biotinylated mouse anti-rabbit-IgG (Jackson Immunoresearch, West Grove, PA). Biotinylated antibodies were detected with HRP-conjugated streptavidin followed by amplification with Alexa Fluor 546 tyramide according to the manufacturer's protocol (Molecular Probes, Oregon). In the case of BM-DCs, cells were further stained with FITC-labeled anti-CD11c followed by Alexa488-labeled goat anti-fluorescein (Molecular Probes). A confocal series of differential interference contrast and fluorescence images was obtained simultaneously with a laser scanning confocal microscope (Axioplan 2, Zeiss, Germany) with a 63× Plan-Apochromat NA 1.4 oil objective. Image analysis was performed with LSM 510 software (Zeiss, Germany). Deconvolution was performed with Huygens software (SVI, Hilversum, The Netherlands), and 3D reconstruction was performed with Imaris (Bitplane AG, Zurich, Switzerland).

Transmission Electron Microscopy

Cells were fixed in 4% glutaraldehyde for 1 hr before being processed for routine sectioning on a Leica ultra-cryotome. Sections were placed on grids and embedded in 1.8% methyl cellulose/0.4% uranyl acetate before examination with a JEOL 1010 (JEOL USA, Peabody, MA) electron microscope.

Statistical Analysis

A one-tailed, two-sample t test was performed with Excel (Microsoft, WA) on triplicate ELISA measurements of cytokine levels to determine significance.

Supplemental Data

Supplemental Data including five figures are available online with this article at http://www.immunity.com/cgi/content/full/22/4/507/DC1/.

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