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Phagocytosis of *Aspergillus fumigatus* conidia by murine macrophages involves recognition by the dectin-1 beta-glucan receptor and Toll-like receptor 2

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Summary

***Aspergillus fumigatus* is a fungal pathogen causing severe infections in immunocompromised patients. For clearance of inhaled conidia, an efficient response of the innate immune system is required. Macrophages represent the first line of defence and ingest and kill conidia. C-type lectins represent a family of receptors, which recognize pathogen-specific carbohydrates. One of them is β1–3 glucan, a major component of the fungal cell wall. Here we provide evidence that β1–3 glucan plays an important role for the elimination of *A. fumigatus* conidia. Laminarin, a soluble β1–3 glucan and antibodies to dectin-1, a well known β1–3 glucan receptor, significantly inhibited conidial phagocytosis. On resting conidia low amounts of surface accessible β1–3 glucan were detected, whereas high amounts were found on small spores that appear early during germination and infection as well as on resting conidia of a *pksP* mutant strain. Swollen conidia also display larger quantities of β1–3 glucan, although in an irregular spotted pattern. Resting *pksP* mutant conidia and swollen wild-type conidia are phagocytosed with high efficiency thereby confirming the relevance of β1–3 glucans for conidial phagocytosis. Additionally we found that TLR2 and the adaptor protein MyD88 are required for efficient conidial phagocytosis, suggesting a link between the TLR2-mediated recognition of *A. fumigatus* and the phagocytic response.**

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Introduction

Aspergillus fumigatus is an opportunistic fungal pathogen responsible for severe, life-threatening infections in immunocompromised patients (Marr *et al*., 2002). Aspergillus conidia are ubiquitously found in the environment and it has been estimated that humans inhale several hundred of them per day (Hospenthal *et al*., 1998). In the lung of immunocompetent hosts, resident macrophages are able to rapidly ingest and kill invading spores, while in immunocompromised patients some conidia may escape the attack of the innate immune system and establish a systemic infection. Although phagocytosis of *A. fumigatus* conidia is crucial to protect the host, we still know little about the mechanisms and molecules involved in this process.

Recognition of conserved structures and molecules on the microbial surface is required for both, activation of macrophages and phagocytosis of microorganisms. These conserved molecules have originally been termed 'pathogen-associated molecular patterns' (PAMPs) (Medzhitov and Janeway, 2000). However, as they are common to pathogenic and non-pathogenic microorganisms it has recently been suggested to rename them to 'microbe-associated molecular patterns' (MAMPs) (Didierlaurent *et al*., 2002). The importance of MAMPs can be traced back to the invertebrates. Organisms like the horseshoe crab *Limulus polyphemus* rely on a primitive innate immune system to combat bacterial and fungal infections. Two lectins found in the haemolymph of Limulus are essential pattern recognition receptors: factor C, which is specific for LPS, and factor G, which recognizes β1–3 glucan (Muta *et al*., 1996). The importance of β1–3 glucan as a general fungal MAMP from Limulus to man, was emphasized by the identification of the corresponding mammalian receptor dectin-1 (Brown and Gordon, 2001). Dectin-1 was shown to be essential for the production of inflammatory molecules in response to fungal pathogens, but it also turned out to be crucial for phagocytosis of yeast zymosan (Brown *et al*., 2002) and

the yeast form of *Candida albicans* (Gantner *et al*., 2005). Gantner *et al*. (2003) furthermore showed that dectin-1 and Toll-like receptor (TLR) 2 act synergistically in mediating a proinflammatory response and the production of reactive oxygen species in macrophages. Very recently, a similar collaboration of dectin-1 and TLR2 was found in the innate immune response to the pathogenic fungi *Coccidioides posadasii* and *A. fumigatus* (Hohl *et al*., 2005; Steele *et al*., 2005; Viriyakosol *et al*., 2005; Gersuk *et al*., 2006).

In this report we provide evidence that laminarin, a soluble β1–3 glucan, partially blocks phagocytosis of *A. fumigatus* conidia by macrophages. We also found that preincubation of conidia with a commercially available *L. polyphemus* preparation, containing the β1–3 glucan-specific lectin factor G, blocks phagocytosis by macrophages. These findings were corroborated by the observed inhibition of phagocytosis by a dectin-1-specific monoclonal antibody (mab). We also found that swollen conidia and conidia of a *pksP* mutant contain higher concentrations of β1–3 glucan on their surface and are phagocytosed about two-times more efficient than resting conidia. Finally, we provide evidence that TLR2, but not TLR4, is essential for efficient phagocytosis of *A. fumigatus* conidia. An impact of TLR2 on conidial phagocytosis was demonstrated using TLR2-deficient macrophages and a TLR2 neutralizing mab. In contrast, TLR2 had no impact on conidial binding to macrophages or transfected HEK 293 cells, suggesting that TLR2 is not a phagocytic receptor, but rather stimulates the phagocytic machinery. Reduced phagocytosis of *A. fumigatus* conidia was also observed for MyD88-deficient macrophages, suggesting that this adaptor protein might be required for the TLR2 signalling leading to enhanced conidial uptake. Taken together, our data suggest that the interaction between dectin-1 and TLR2 is not only important for the immunological recognition of fungal pathogens, but also for their phagocytosis.

Results

β1–3 glucan on the conidial surface plays a major role for phagocytosis of A. fumigatus *by macrophages*

To analyse the potential role of β1–3 glucan during phagocytosis of *A. fumigatus* conidia we infected macrophages in the presence or absence of different concentrations of laminarin, a soluble β1–3 glucan. Laminarin competitively blocked phagocytosis by murine J774 macrophages in a dose-dependent manner (Fig. 1B). The percentage of intracellular conidia was determined using the BCS technique (Luther *et al*., 2006) and corresponding micrographs of J774 cells infected in the presence or absence of laminarin are shown in Fig. 1A. As shown in Fig. 1C, laminarin had no impact on the uptake of biotinylated control beads by J774 macrophages, demonstrating that the presence of this glucan has no general impact on the phagocytic capacity of these macrophages. An inhibition of conidial phagocytosis similar to that observed with J774 macrophages was also found with peritoneal macrophages of C3H/HeN mice (Fig. 1D and data not shown). For both kinds of macrophages a reduction of phagocytosis by approximately 50% was achieved with 100 μg ml^{-1} laminarin. A similar impact of laminarin was also observed for cells of the murine alveolar macrophage cell line MH-S, while 100 μg ml^{-1} laminarin reduced the phagocytosis by primary alveolar macrophages by approximately one third (Fig. 1E).

To provide further evidence that conidial β1–3 glucan molecules are involved in the phagocytic process, we infected macrophages with spores, that had been preincubated with a Limulus extract containing the β1–3 glucan-specific factor G (Muta *et al*., 1996). We hypothesized that binding of this lectin would mask surface exposed β1–3 glucans and thereby inhibit phagocytosis. Preincubation of conidia with a Limulus extract ('Fungitell reagent') and dilutions thereof indeed led to a dose-dependent inhibition of phagocytosis as shown in Fig. 1F. For J774 macrophages maximal inhibition of phagocytosis by factor G reached 62%, a value similar to that obtained with 100 μg ml^{-1} laminarin. The strong, but not complete inhibition by laminarin or the factor G containing Limulus extract suggests that β1–3 glucan is a major, but not the only fungal surface structure recognized by phagocytic receptors of murine macrophages.

Using a specific mab or a soluble recombinant dectin-1 polypeptide, Hohl *et al*. (2005) and Gersuk *et al*. (2006) detected β1–3 glucan on the surface of swollen, but not on resting conidia, which are known to be covered by a characteristic hydrophobic surface layer (Paris *et al*., 2003). Using a soluble dectin-1 protein, Steele *et al*. (2005) detected small amounts of β1–3 glucan on the surface of resting conidia, but found much higher concentrations on swollen conidia and germ tubes. Given that β1–3 glucan is involved in the phagocytic uptake, swollen conidia should be taken up more efficiently than resting spores. As shown in Fig. 2A we indeed observed that swollen conidia were internalized with significantly higher efficiency than resting conidia. This observation prompted us to analyse whether long-term storage of resting conidia had an influence on the measured efficiencies of phagocytosis. We suspected that long-term storage could influence the integrity of the hydrophobic surface layer, thereby unmasking additional β1–3 glucan moieties. However, comparison of freshly isolated conidia and conidia stored in water for 6 months at 4°C (Fig. 2A) revealed no significant difference with respect to phagocytosis by J774 macrophages.

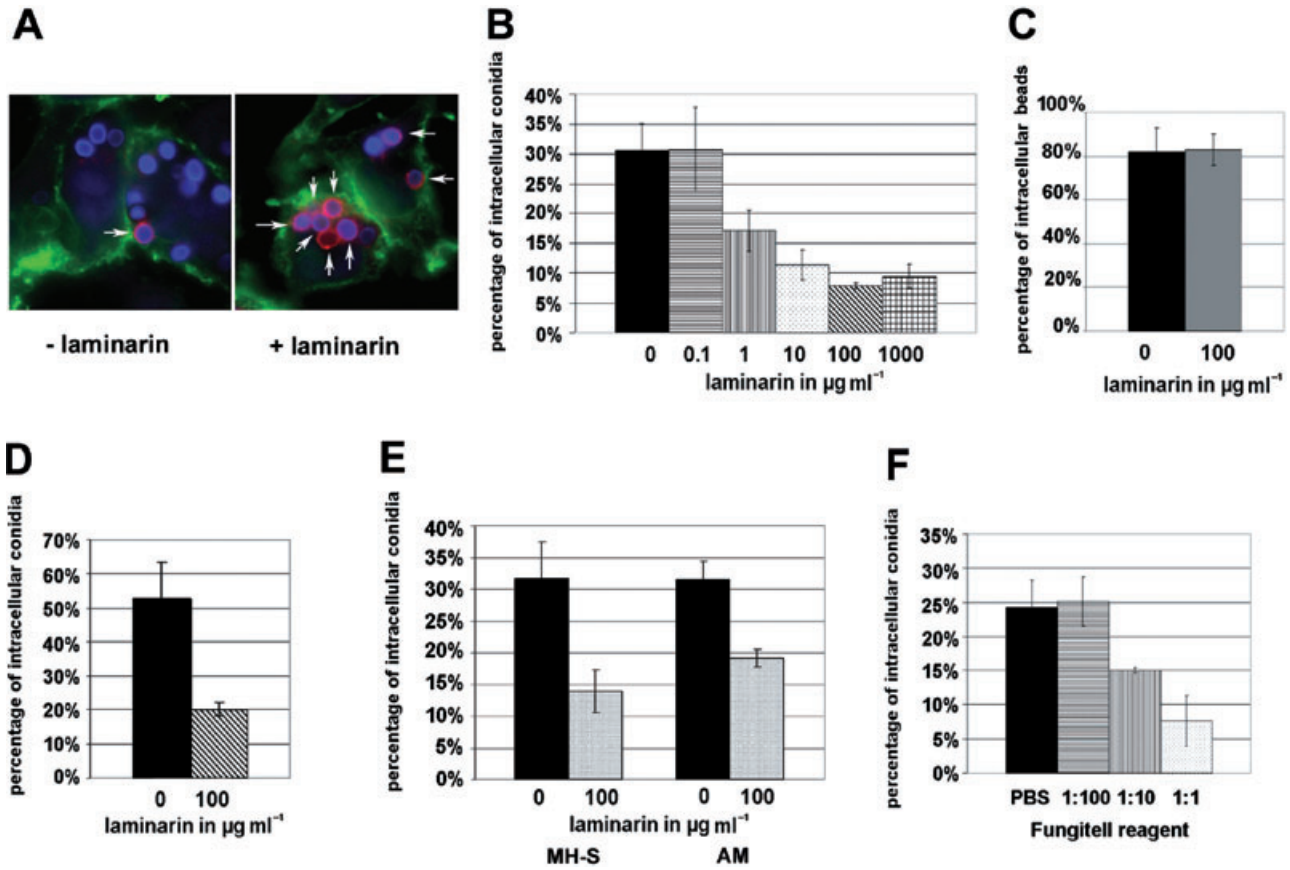

Fig. 1. Involvement of β1–3 glucan in conidial phagocytosis by macrophages.
A. Macrophages were infected with resting *A. fumigatus* conidia for 3 h with an moi of 10. The percentage of intracellular conidia was determined using the BCS technique. Representative micrographs of J774 macrophages infected in the presence or absence of laminarin (100 µg ml^{-1}) are shown in A. Macrophages are stained in green using ConA-FITC, extra- and intracellular conidia are visualized in blue using calcofluor and extracellular conidia are shown in red using streptavidin-Cy3.
B. J774 macrophages were infected in the presence or absence of the indicated concentrations of laminarin.
C. Uptake of biotinylated control beads by peritoneal C3H/HeN macrophages in the presence and absence of laminarin.
D. Peritoneal C3H/HeN macrophages were infected in the presence or absence of laminarin (100 µg ml^{-1}).
E. MH-S murine alveolar macrophages and primary alveolar macrophages (AM) were infected in the presence or absence of laminarin (100 µg ml^{-1}).
F. Resting conidia were incubated with the indicated dilutions of Fungitell reagent in PBS for 30 min at RT immediately before infection of J774 macrophages. All data represent results of three independent experiments.

We furthermore analysed resting spores of a *pksP* mutant, which lacks an essential enzyme for synthesis of dihydroxynaphthalene (DHN)-like melanin, resulting in white conidia with a characteristic smooth surface (Jahn *et al.*, 2000). Most resting spores of the *pksP* mutant showed very strong labelling with the β1–3 glucan-specific mab 2G8 (Torosantucci *et al.*, 2005) (Fig. 3D′), whereas only very low amounts of β1–3 glucan were detectable on wild-type spores by conventional immunofluorescence microscopy (Fig. 3A′). Using FACS analysis 2G8 binding to resting wild-type conidia was detectable, although this binding was weak compared with that obtained for *pksP* mutant conidia (Fig. 3E). Consistently with this difference in the concentration of surface accessible β1–3 glucan, we found that *pksP* mutant conidia were ingested by macrophages as efficient as swollen conidia (Fig. 2B).

Using mab 2G8 we also analysed the distribution of β1–3 glucan on the fungal surface during germination. As described above we observed only a weak staining for resting wild-type conidia (Fig. 3A′ and E). However, after a germination time of 4 h about 10% of the spores were very strongly labelled (Fig. 3B′) indicating a high concentration of surface accessible β1–3 glucan in this conidial subpopulation. These conidia had a small diameter like resting spores (approx. 2 µm) and might represent spores that had already lost their hydrophobic surface layer before entering the spherical growth phase. After another 2 h most conidia were swollen (diameter of approx. 4 µm). If smaller conidia were still found they were strongly

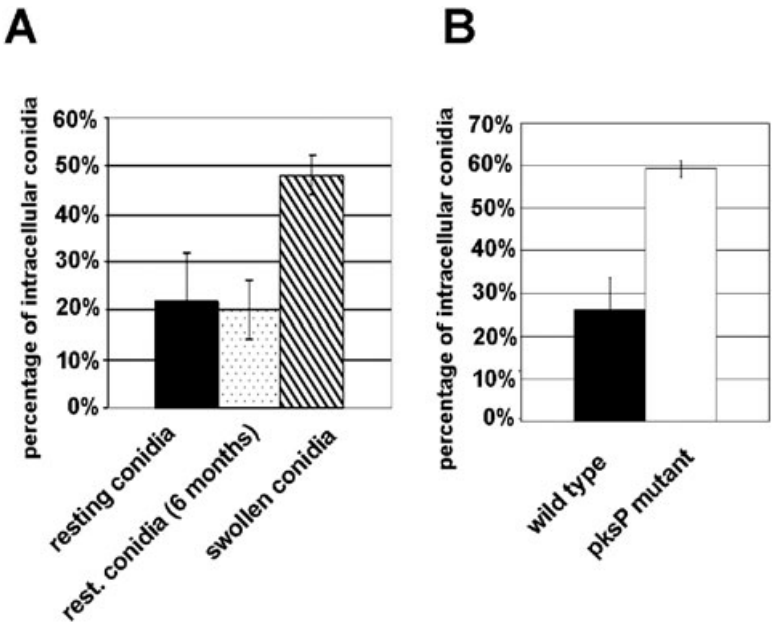

Fig. 2. Enhanced uptake of swollen wild-type and pksP mutant conidia. Macrophages were infected with *A. fumigatus* conidia at an moi of 10 for 3 h. The percentage of intracellular conidia was determined using the BCS technique.
A. Infection of J774 macrophages with freshly isolated resting conidia, resting conidia stored for 6 month at 4°C and freshly isolated swollen conidia.
B. Infection of J774 macrophages with resting conidia of a white *pksP* mutant and the corresponding wild-type strain. All data represent results of three independent experiments.

labelled by mab 2G8 (Fig. 3F, arrow). Interestingly, all swollen spores showed a dotted labelling, which became especially apparent using confocal microscopy (Fig. 3F and F′). This irregular staining was not as strong as that observed for small 2G8-positive conidia (Fig. 3F, arrow) suggesting that the density of surface exposed β1–3 glucan moieties decreases during spherical growth of originally small and 2G8-positive conidia. However, polar growth led to a very strong and homogenous labelling of those surface areas where germ tube formation takes place (Fig. 3C′ and G, arrows) and a similar strong labelling of the hyphal surface has already been described by Torosantucci *et al*. (2005). During infection of J774 macrophages 2G8-positive conidia were not found after 30 min, but appeared after 1 h (data not shown). A representative micrograph showing β1–3 glucan-positive and -negative conidia in infected macrophages after 3 h is shown in Fig. 3H. Note that the β1–3 glucan-positive conidia, like resting conidia, still have a diameter of approx. 2 μm. We next analysed whether these 2G8-positive conidia are taken up with higher efficiency. As shown in Fig. 3I, strongly 2G8-positive conidia were taken up with a twofold higher efficiency than all the rest of the conidia.

Dectin-1 is required for efficient phagocytosis of A. fumigatus *conidia*

Recognition of β1–3 glucan is mediated by dectin-1, a receptor found on dendritic cells and macrophages (Brown *et al*., 2002). Binding of β1–3 glucan by dectin-1 has been shown to trigger a proinflammatory response in macrophages (Brown *et al*., 2003; Gantner *et al*., 2003) and also turned out to be essential for phagocytosis of yeast zymosan (Herre *et al*., 2004) and *C. albicans* (Gantner *et al*., 2005). These findings prompted us to analyse whether dectin-1 is involved in the phagocytosis of *A. fumigatus* conidia. For these experiments we used a specific antibody that recognizes the extracellular domain of dectin-1 and is therefore suitable to block its activity (Brown *et al*., 2002). The presence of mab 2A11 blocked phagocytosis of *A. fumigatus* conidia by J774 (Fig. 4A) and peritoneal C3H/HeN macrophages (Fig. 4B) in a dose-dependent manner. At a concentration of 10 μg ml^{-1} the antibody reduced the phagocytic uptake of conidia by approximately 80% for J774 and 60% for primary C3H/HeN macrophages, while a corresponding isotype control had no impact (data not shown). In control experiments mab 2A11 (10 μg ml^{-1}) had no impact on the phagocytosis of beads by J774 and peritoneal macrophages (Fig. 4C and data not shown). We also determined the impact of mab 2A11 on the conidial binding to macrophages. As shown in Fig. 4D we observed a reduction of the conidial binding that was consistently observed in several experiments (data not shown), suggesting a role for dectin-1 in the initial interaction between conidia and the macrophage surface. However, the diminished conidial binding is not responsible for the reduced phagocytosis, because efficiency of phagocytosis was calculated as the percentage of internalized conidia in relation to the entirety of all cell-associated conidia.

Localization of dectin-1 in infected macrophages

Dectin-1 is functionally implicated in recognition and phagocytosis of β1–3 glucan containing particles, and

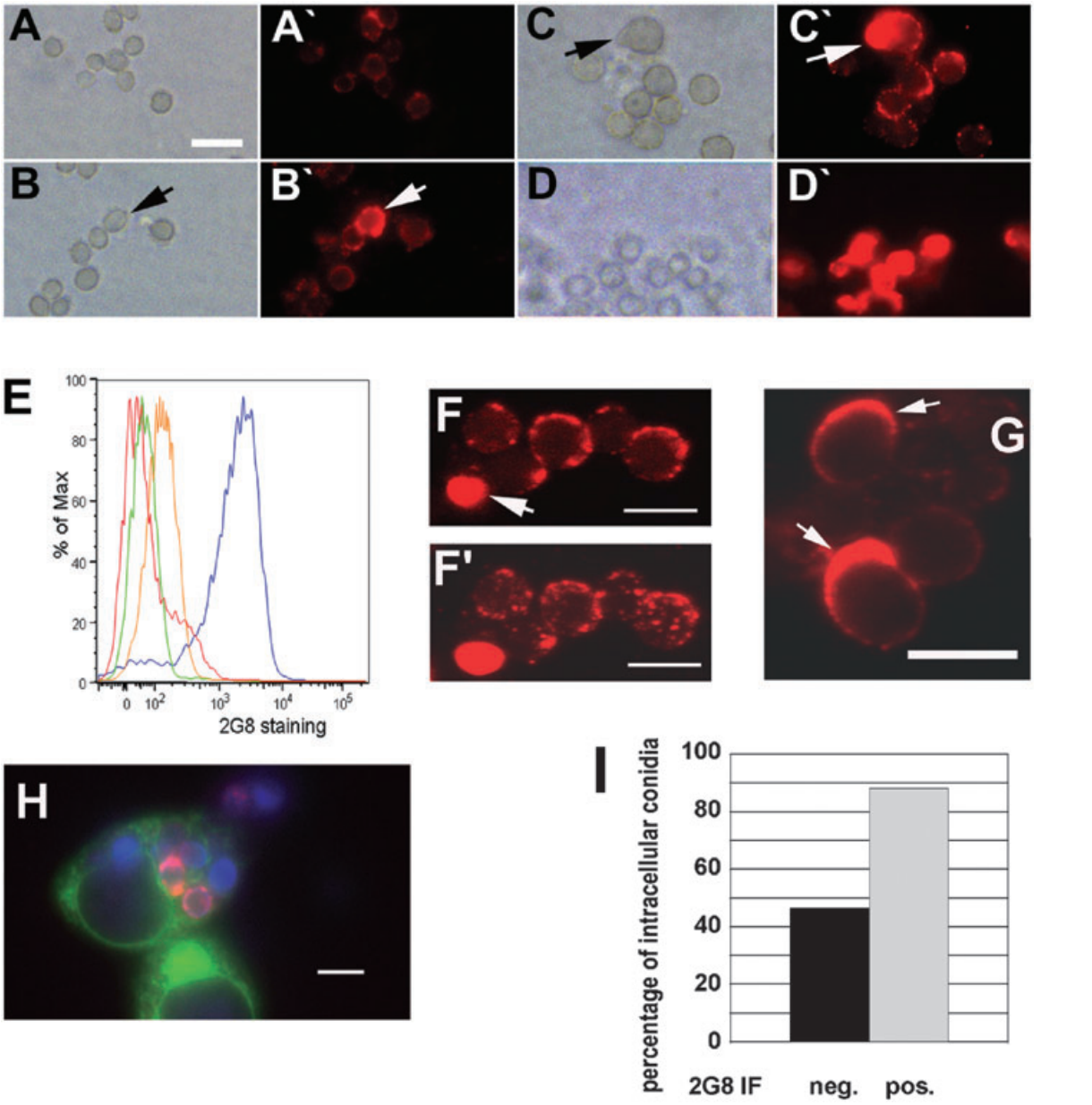

Fig. 3. Localization of β1–3 glucan on the surface of *A. fumigatus* conidia. Surface accessible β1–3 glucan was detected using mab 2G8. A/A′–D/D′. A–D represent micrographs of conidia visualized by light microcopy, while the corresponding 2G8 stainings are depicted in A′–D′. Resting wild-type conidia are shown in A and A′, conidia after 4 h germination in YG medium in B and B′ and conidia after 6 h germination in YG medium in C and C′. Resting conidia of a *pksP* mutant are shown in D and D′. A small but strongly 2G8A-positive conidium is indicated by an arrow in B and B′. A swollen conidium with a short germ tube is indicated by an arrow in C and C′. Bar in A represents 5 μm and is valid for A to D′.

E. FACS analysis of resting wild-type and *pksP* mutant conidia is shown. Wild-type conidia stained with mab 2G8 and the corresponding control staining with the secondary antibody are shown as a orange and green line respectively; *pksP* mutant conidia stained with mab 2G8 and the corresponding control staining are shown as a blue and red line respectively.

F and G. Showing laser scanning confocal microscopic micrographs of swollen conidia after 6 h in YG medium using mab 2G8. A single confocal plain is shown in F, and the corresponding projection of all confocal plains is shown in F′. A strongly stained small conidium is indicated by an arrow (F). The bars in F and F′ represent 5 μm. Another confocal plain of swollen conidia is shown in G. Two of these spores are at the beginning of the polar growth phase and areas of germ tube formation are indicated by arrows. Note that the signal intensity in G has been reduced in comparison to that of F to improve the visualization of the polarized distribution of the 2G8 staining. Bar in G represents 5 μm.

H. J774 infected with *A. fumigatus* wild-type conidia are shown. Macrophages are visualized by ConA staining (green), while conidia are stained with calcofluor (blue). Staining of β1–3 glucan using mab 2G8 is shown in red. The bar represents 5 μm.

I. Percentage of internalized 2G8-positive and -negative conidia after 3 h of infection in J774 macrophages.

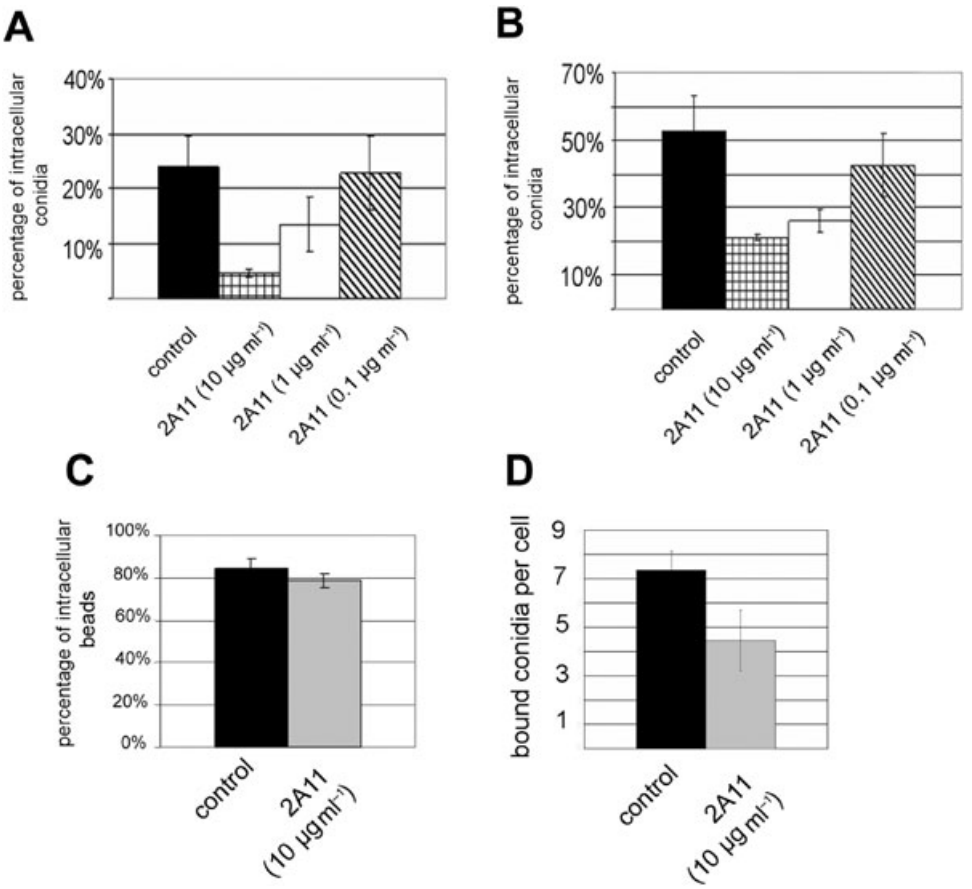

Fig. 4. Inhibition of phagocytosis of *A. fumigatus* conidia by an antidectin-1 antibody.
A and B. J774 macrophages (A) and peritoneal C3H/HeN macrophages (B) were infected with *A. fumigatus* conidia at an moi of 10 in the presence of the indicated concentrations of the antidectin-1 mab 2A11. The percentage of intracellular conidia was determined using the BCS technique.
C. Uptake of biotinylated control beads by peritoneal C3H/HeN macrophages in the presence and absence of mab 2A11.
D. Quantification of the average number of cell-associated conidia per macrophages in the presence and absence of the dectin-1-specific mab 2A11. All data represent results of three independent experiments.

recruitment of this receptor to phagocytic vacuoles has been demonstrated in transfected cells and primary macrophages (Herre *et al*., 2004; Gantner *et al*., 2005; Hohl *et al*., 2005). Using mab 2A11 and a polyclonal dectin 1-specific antibody we analysed the localization of dectin-1 in J774 and peritoneal macrophages before and after phagocytosis of resting *A. fumigatus* conidia. After permeabilization of non-infected cells dectin-1 was detectable in distinct intracellular structures (Fig. 5A). As mab 2A11 has been shown to recognize an extracellular epitope (Brown *et al*., 2002), it should be suitable to stain surface exposed dectin-1 molecules in non-permeabilized macrophages. However, hardly any dectin-1-specific labelling was found on intact cells (data not shown) suggesting that only a small percentage of the cellular dectin-1 is localized on the macrophages surface. In infected cells colocalization of dectin-1 and some, but not all, intracellular conidia became apart after 1 h of infection (Fig. 5B and B′). Similar ring-shaped antidectin-1 staining patterns were found after 3 h of infection (Fig. 5C and C′) suggesting that dectin-1 is recruited to a certain subset of phagosomes containing *A. fumigatus* conidia.

TLR2, but not TLR4, is required for efficient phagocytosis of A. fumigatus *conidia*

A synergistic interaction between dectin-1 and TLR2 has been reported in the proinflammatory response of macrophages to zymosan (Gantner *et al*., 2003) and to the pathogenic fungi *A. fumigatus* and *C. posadasii* (Hohl *et al*., 2005; Steele *et al*., 2005; Viriyakosol *et al*., 2005; Gersuk *et al*., 2006). TLR2 and TLR4 have been implicated in the immunological recognition of *A. fumigatus*, and we therefore analysed their potential impact on phagocytosis of *A. fumigatus* conidia. Infection of peritoneal macrophages from wild-type mice (C3H/HeN) and mice lacking either functional TLR4 (C3H/HeJ), TLR2 (C3H/HeN TLR2$^{-/-}$) or both (C3H/HeJ TLR2$^{-/-}$) revealed

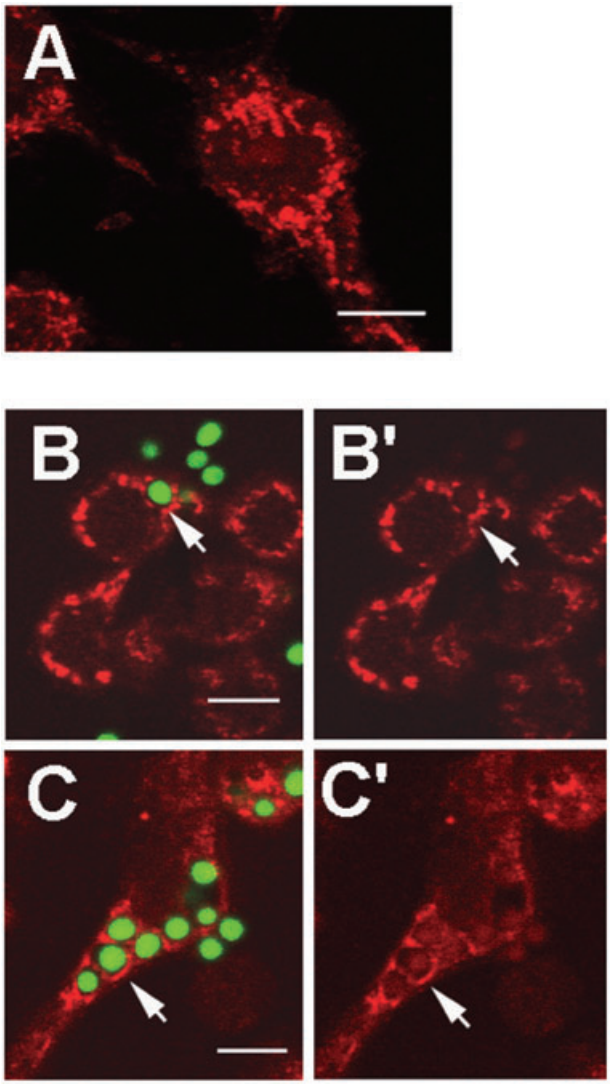

Fig. 5. Localization of dectin-1 in infected and non-infected macrophages. Localization of dectin-1 was analysed using mab 2A11. Infected or non-infected J774 macrophages were analysed after fixation and permeabilization. Dectin-1 staining of non-infected J774 macrophages is shown in A. The other panels show J774 cells infected with GFP-expressing *A. fumigatus* conidia at an moi of 10. Cells infected for 1 h or 3 h are shown in B/B′ and C/C′ respectively. In B′ and C′ the dectin-1-specific staining is shown in red. B and C show additionally GFP-expressing conidia in green. Recruitment of dectin-1 to a phagosome containing an *A. fumigatus* conidium is indicated by arrows. All bars represent 10 μm.

that TLR2 deficiency significantly reduced conidial phagocytosis after 1 and 3 h of infection (Fig. 6A). In contrast, no significant inhibition was observed for TLR4-deficient macrophages. Against a role of TLR4 during phagocytosis also argues, that similar rates of phagocytosis were observed for TLR2-deficient and TLR2/4-double-deficient macrophages (Fig. 6A). The impact of TLR2 on phagocytosis of *A. fumigatus* conidia was highly reproducible with macrophages from mice with a C3H/HeN- and C57/Bl6-genetic background (data not shown and Fig. 7B). With respect to conidial binding to macrophages we observed no effect due to the lack of TLR2 and/or TLR4 (Fig. 6B) suggesting that both receptors are not engaged in the initial interactions between conidia and macrophage surface receptors.

To exclude that the reduced phagocytosis of *A. fumigatus* conidia observed for TLR2-deficient macrophages reflects a general defect in the phagocytic machinery of these cells, we compared the ability of these macrophages to internalize biotinylated control beads. Wild-type and TLR2-deficient C3H/HeN macrophages internalized these beads with similar efficiency (Fig. 6C), demonstrating that $TLR2^{-/-}$ macrophages are not generally impaired in phagocytosis.

To provide further evidence for the relevance of TLR2 for conidial phagocytosis, we analysed cells in which TLR2 was blocked using the specific mab T2.5 (Meng *et al*., 2004). Antibody-treated cells showed a reduced conidial uptake, whereas an isotype control antibody had no effect (Fig. 6D). Inhibition of conidial phagocytosis by mab T2.5 was also observed for cells of the alveolar macrophage cell line MH-S, as well as primary alveolar macrophages derived form C57/Bl6 mice (Fig. 6B). Taken together, these data independently confirm the results obtained with TLR2-deficient macrophages. Pre-incubation of peritoneal or J774 macrophages with a combination of 2A11 and T2.5 had no synergistic effect (data not shown). However, we found that the presence of mab 2A11 (10 μg ml^{-1}) and laminarin (100 μg ml^{-1}) further reduced the residual conidial phagocytosis of TLR2-deficient macrophages from C57/Bl6 mice by 71% and 60% respectively. Our data therefore suggest that a TLR2-independent dectin-1-mediated phagocytosis of *A. fumigatus* spores may exist under certain conditions, but additional experimental evidence is clearly required to proof this.

The constant rates of conidial binding to wild-type and TLR2-deficient macrophages (Fig. 6B) suggested that TLR2 is not engaged in the initial binding of spores to the macrophage surface. To confirm this, HEK293 cells were transfected with a plasmid encoding GFP-actin alone or in combination with a plasmid encoding either TLR2 or dectin-1. Expression of these proteins was verified using specific antibodies (data not shown). Quantification of conidial binding revealed no impact for TLR2, whereas expression of dectin-1 led to a significant increase in the number of cell-associated conidia (Fig. 7A). These data suggest that dectin-1, but not TLR2 is directly engaged in conidial binding.

We next analysed whether the impact of TLR2 on conidial uptake requires signalling via MyD88, as this adaptor protein is required for an inflammatory response triggered by TLR2. As shown in Fig. 7B, MyD88-deficient

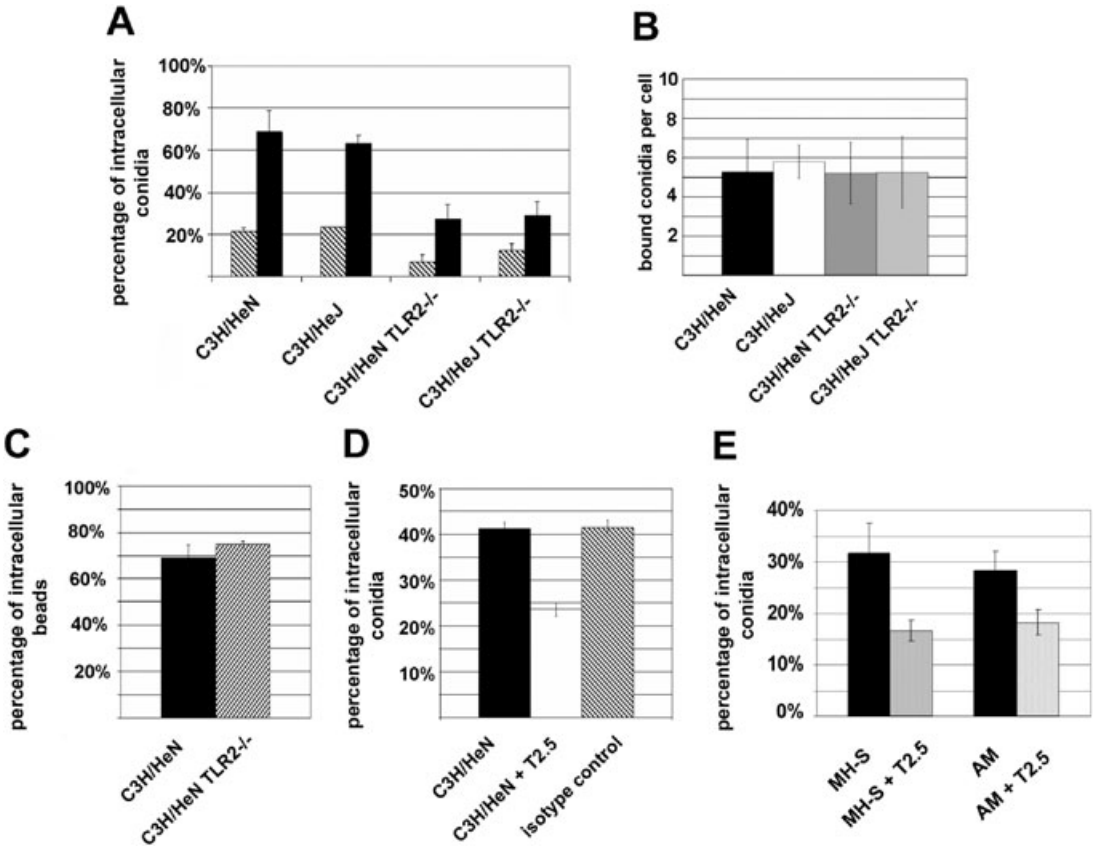

Fig. 6. TLR2, but not TLR4, is required for efficient phagocytosis. Peritoneal macrophages of C3H/HeN wild-type mice, C3H/HeJ mice lacking a functional TLR4, C3H/HeN TLR2$^{-/-}$ mice and C3H/HeJ TLR2$^{-/-}$ mice, lacking TLR2 and a functional TLR4, were infected with *A. fumigatus* conidia for 1 h (hatched bars) and 3 h (solid bars).
A and B. The percentage of intracellular conidia was determined using the BCS technique (A). The average numbers of cell-associated conidia per macrophage are shown in B for cells infected for 3 h.
C. Uptake of BSA-coupled control beads by wild-type (C3H/HeN) and the corresponding TLR2-deficient macrophages is shown.
D. The impact of the TLR2-specific mab T2.5 and a corresponding isotype control was analysed by preincubation of peritoneal C3H/HeN macrophages with 35 μg ml^{-1} of the respective antibody for 30 min. Subsequent infections were performed in the absence (black bar) or presence (white and hatched bar) of the indicated antibodies.
E. The presence of the TLR2 neutralizing mab T2.5 (35 μg ml^{-1}) partially blocks phagocytosis of *A. fumigatus* conidia by cells of the murine alveolar macrophage cell line MH-S, as well as primary murine alveolar macrophages derived from C57/Bl6 mice. Infections were performed using an moi of 10. All data represent results of three independent experiments.

macrophages showed a reduced rate of phagocytosis similar to that of the corresponding TLR2-deficient macrophages, suggesting that MyD88-dependent signalling of TLR2 might enhance the phagocytic response to *A. fumigatus* conidia.

Activation of TLR2 by the specific and synthetic ligand P_3CSK_4 triggers activation of NF-κB in a MyD88-dependent manner. We therefore hypothesized that pretreatment of macrophages with P_3CSK_4 would result in an enhanced uptake of *A. fumigatus* spores. However, with respect to conidial phagocytosis no difference was detectable between P_3CSK_4-activated and untreated J774 cells (Fig. 7C). Similar results were obtained for C3H/HeN peritoneal macrophages (data not shown), suggesting specific requirements for the activation of TLR2 to impact on conidial phagocytosis.

Discussion

Macrophages, as a first line of defence, have to be able to discriminate between self and foreign structures. In this process, so-called 'pattern recognition receptors' play an essential role and in recent years especially members of the Toll-like receptor family and their contribution to recognition and defeat of various microorganisms have gained much interest. We and others showed that TLR2 and TLR4 are involved in the immunological recognition of Aspergillus *in vitro*, leading to an activation of macrophages, production and release of proinflammatory molecules and recruitment of neutrophils to the site of infection (Wang *et al*., 2001; Mambula *et al*., 2002; Meier *et al*., 2003; Braedel *et al*., 2004). Further studies confirmed the importance of TLR2 (Balloy *et al*., 2005) and

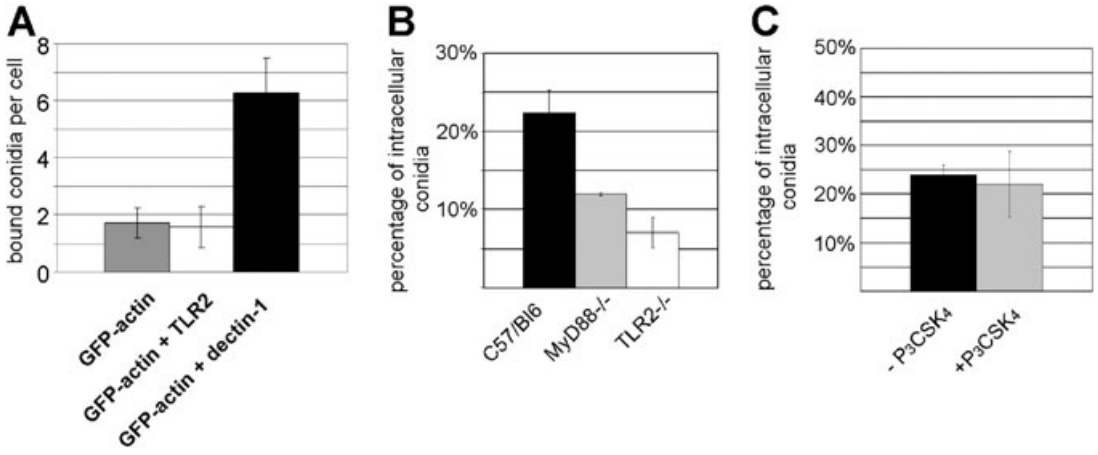

Fig. 7. Relevance of TLR2 and TLR2-mediated signalling during conidial binding and phagocytosis.
A. To analyse the impact of TLR2 and dectin-1 on binding of *A. fumigatus* conidia HEK293 cells were transfected with a plasmid encoding GFP-actin alone or in combination with plasmids conferring expression of either human TLR2 or murine dectin-1. Transfected cells were infected with *A. fumigatus* wild-type conidia at an moi of 10 for 3 h. Conidia bound to transfected green fluorescent cells were quantified and the average number of cell-associated spores per macrophage was calculated. Conidial binding to non-transfected cells (data not shown), control cells expressing GFP (grey bar) and cells additionally transfected with TLR2 (white bar) were indistinguishable, while a significantly higher number of bound conidia were observed for dectin-1 expressing cells (black bar).
B. Peritoneal macrophages of C57/Bl6 mice (black bar) and the corresponding MyD88- and TLR2-deficient mice (grey bar and white bar respectively) were infected with *A. fumigatus* wild-type conidia at an moi of 10 for 3 h. The percentage of intracellular conidia was determined using the BCS technique.
C. J774 macrophages pretreated with the TLR2-specific ligand P_3CSK_4 (grey bar) and control cells without pretreatment (black bar) were infected with *A. fumigatus* wild-type conidia at an moi of 10 for 3 h. The percentage of intracellular conidia was determined using the BCS technique. All data represent results of three independent experiments.

TLR4 (Belloccio *et al*., 2004) in murine infection models of invasive aspergillosis.

Apart from TLRs, other pattern recognition receptors have been described. The C-type lectin surfactant proteins D binds to β1–6 glucan moieties on the surface of *A. fumigatus* conidia (Allen *et al*., 2001) and mediates their enhanced phagocytosis by macrophages (Madan *et al*., 1997). Other carbohydrates, like β1–4 glucans and galactomannan, have been implicated in binding of *A. fumigatus* conidia to macrophages, dendritic cells or Langerhans cells (Kan and Bennett, 1991; Persat *et al*., 2003; Serrano-Gómez *et al*., 2004), providing evidence that carbohydrates are present on the conidial surface and are targeted by pattern recognition receptors.

β1–3 glucan is an abundant and characteristic component of the fungal cell wall and Kataoka *et al*. (2002) demonstrated its ability to activate macrophages, leading to the production and release of proinflammatory molecules. Later on recognition of β1–3 glucans by macrophages was shown to be mediated by dectin-1, a C-type lectin-like surface receptor (Brown *et al*., 2002). In the present study we provide several lines of evidence that interaction between β1–3 glucan and dectin-1 is crucial for phagocytosis of resting and swollen *A. fumigatus* conidia: (i) laminarin, a soluble β1–3 glucan, blocks conidial phagocytosis in a dose-dependent manner; (ii) preincubation of conidia with the β1–3 glucan-specific lectin factor G from Limulus inhibits conidial phagocytosis in a dose-dependent manner; (iii) swollen conidia and conidia of a *pksP* mutant are phagocytosed with high efficiency and both display large amounts of β1–3 glucans on their surface; and (iv) a dectin-1-specific mab blocks conidial phagocytosis in a dose-dependent manner. In line with these data Gersuk *et al*. (2006) very recently provided evidence that phagocytosis of *A. fumigatus* germ tubes can be inhibited by laminarin or dectin-1-specific antibodies. The residual phagocytosis of about 60% that could not be blocked by laminarin or antidectin-1 antibodies suggests that β1–3 glucan-independent ways of phagocytosis exist. Opsonization by complement is unlikely to account for this residual phagocytosis, because heat-inactivated fetal calf serum was used in all experiments. Interactions between macrophage receptors and conidial carbohydrates other than β1–3 glucan have been reported and may mediate the observed laminarin-resistant uptake (Kan and Bennett, 1991; Persat *et al*., 2003; Serrano-Gómez *et al*., 2004).

According to our immunofluorescence data only very low amounts of dectin-1 are found on the surface of mouse macrophages, whereas we clearly detected dectin-1-positive intracellular structures. Colocalization of dectin-1 with a subset of intracellular conidia started 1 h after infection, when also β1–3 glucan-positive conidia became apparent. However, prominent ring-shaped dectin-1 staining was not detected for all phagosomes containing conidia suggesting that although dectin-1 is

engaged in the uptake process, its presence in the phagocytic vacuole might be only transient. Interestingly we have observed a similar distribution for TLR4 (A. Meier and F. Ebel, unpubl. data), which is required for the inflammatory response to *A. fumigatus* conidia.

Evidence underlining the importance of β1–3 glucan in the immune response to *A. fumigatus* came from a very recent study by Torosantucci *et al*. (2005). The authors showed that immunization of mice with laminarin triggered a humoral response that protected them from systemic *A. fumigatus* infections. It appears likely that increased phagocytosis due to opsonization of conidia with β1–3 glucan-specific antibodies results in an enhanced elimination of spores and thereby contributes to the observed protective effect.

For *C. albicans* it was shown that recognition via dectin-1 is restricted to the yeast form. Surface labelling using a soluble form of dectin-1 revealed β1–3 glucans only in the birth scars of yeasts, but not on the surface of hyphae (Gantner *et al*., 2005). By using a specific mab Hohl *et al*. (2005) demonstrated β1–3 glucan on the surface of swollen *A. fumigatus* conidia, whereas no label was detectable on resting conidia. Steele *et al*. (2005) came to similar results using a soluble form of dectin-1, but in addition detected small amounts of β1–3 glucan on resting conidia. Both studies found that swollen, but not heat-killed resting conidia, are able to trigger a proinflammatory response. Using the β1–3 glucan-specific mab 2G8 (Torosantucci *et al*., 2005) we detected a weak labelling of resting conidia, which confirms that only low concentrations of β1–3 glucan are accessible on the surface of resting spores. However, at early time points during germination we observed a conidial subset with a small diameter comparable to resting conidia, but unexpectedly these spores showed a strongly positive label with the β1–3 glucan-specific mab. At later time points when most spores were already swollen many of the conidia that remained small showed similar properties. High concentrations of β1–3 glucan were also detected on resting spores of a *pksP* mutant, which lacks the characteristic echinulate morphology of resting wild-type conidia. Early during germination *A. fumigatus* wild-type conidia can release their surface layer resulting in a smooth surface (Rohde *et al*., 2002) that resembles that of the *pksP* mutant. Small but strongly β1–3 glucan-positive conidia may therefore have lost surface structures that mask underlying β1–3 glucan moieties. Interestingly, the 2G8 staining on swollen conidia was irregular. Strongly labelled spot-like parts were separated by areas of very weak staining. The β1–3 glucan-positive parts may represent the original conidial surface, whereas novel surface areas that appeared during spherical growth might display only small amounts of β1–3 glucan. Polarized growth of swollen conidia led to the appearance of strongly and homogenously labelled areas at the site of germ tube formation indicating that this newly formed surface, like that of hyphae (Torosantucci *et al*., 2005), displays high concentrations of β1–3 glucan. Taken together, these data suggest that β1–3 glucans are usually present in high density on the surface of the *A. fumigatus* cell wall. However they seem to be masked by the hydrophobic surface layer of resting conidia and may additionally be rare in those parts of the cell wall of swollen spores that are newly exposed during spherical growth. After as early as 1 h during infection, we interestingly observed the appearance of small β1–3 glucan-positive conidia. This finding indicates a fast and substantial re-organization of the conidial cell wall during infection, suggesting that infection experiments using killed resting conidia might not adequately reflect the situation that occurs *in vivo* after the inhalation of spores. Conidia displaying large amounts of β1–3 glucan were internalized with about twofold efficiency as compared with the remaining spores, which confirms the relevance of β1–3 glucan for the phagocytosis of *A. fumigatus* conidia.

All current data indicate that in resting *A. fumigatus* conidia only small amounts of β1–3 glucan are surface accessible. However, the results provided in this study suggest that recognition of β1–3 glucan by dectin-1 nevertheless plays an important role during conidial phagocytosis. One possible explanation would be a change in the conidial surface layer early in the infectious process and we indeed found increased amounts of β1–3 glucan on conidia after 1 h of infection, suggesting that a disintegration of conidial surface structures unmasks underlying β1–3 glucan moieties. However, as we already observed laminarin-sensitive conidial uptake at earlier time points, the quantity of accessible β1–3 glucan on resting spores might be sufficient to trigger phagocytosis, while larger amounts might result in a more efficient uptake that is accompanied by an inflammatory response. Because humans inhale several hundred *A. fumigatus* conidia a day, mechanisms must exist that restrict the production of inflammatory stimuli to situations of real infection. A potential way to achieve this is a modulation of the immune response by the amount of accessible β1–3 glucan. Exposure to resting conidia would support phagocytosis, but not inflammation and the latter has been recently observed with isolated macrophages (Hohl *et al*., 2005; Steele *et al*., 2005; Gersuk *et al*., 2006). Swollen conidia, which indicate activation of fungal cells, not only trigger a stronger activation of the phagocytic machinery, but additionally mount an inflammatory response. The high amounts of β1–3 glucan on the surface of *pksP* mutant conidia, which leads to an enhanced phagocytic uptake by macrophages, might in part be responsible for the reduced virulence of this mutant in a mouse model of infection (Jahn *et al*., 1997). As dectin-1-mediated recog-

nition of β1–3 glucan can trigger a respiratory burst (Gantner *et al*., 2003), it might as well explain the high amounts of reactive oxygen species produced in response to *pksP* mutant conidia (Langfelder *et al*., 1998).

Gantner *et al*. (2003) found that TLR2 and dectin-1 cooperate in the inflammatory response to zymosan, whereas TLR2 is not required for the phagocytic uptake of these fungal particles. However, several studies provided evidence that TLRs can interact with phagocytosis on the levels of phagosome formation, phagosome maturation and expression of proteins directly engaged in the phagocytic process (for review see Underhill and Gantner, 2004). The data presented in this study now implicate TLR2, but not TLR4, in the process of phagocytosis of *A. fumigatus* conidia. Lack of TLR2 or blocking TLR2 using a suitable antibody led to a reduced conidial phagocytosis, but had no impact on the uptake of control beads, demonstrating that the phagocytic machinery of TLR2-deficient macrophages is not generally impaired. With respect to conidial binding to the macrophage surface we observed no impact of TLR2 (or TLR4). This is corroborated by the finding that overexpression of TLR2 in HEK293 cells did not result in enhanced conidial binding, whereas overexpression of dectin-1 did. Because several reasons argue against TLR2 itself being a phagocytic receptor (Underhill and Gantner, 2004), it seems likely that activation via TLR2 might stimulate phagocytosis triggered by dectin-1 or alternative receptors. Activation of NF-κB through TLR2 requires the adaptor protein MyD88. Our observation that MyD-88-negative macrophages are also impaired in conidial uptake suggests that MyD88-dependent signalling is required for both, TLR2-triggered NF-κB activation and TLR2-triggered stimulation of conidial phagocytosis. In the latter process recognition of a specific fungal ligand or recognition in a specific microenvironment seems to be required because activation of macrophages by the generally used TLR2-specific ligand P_3CSK_4 had no impact. Further studies focusing on the role of TLR2 and its ligands in the process of conidial phagocytosis are required to unravel the links between TLR2, dectin-1 and the phagocytic machinery at the molecular level.

Experimental procedures

Materials

Laminarin was obtained from Sigma, Fungitell reagent from Cape Cod Incorporated (East Falmouth, USA), the dectin-1-specific mab 2A11 from HyCult Biotechnology B.V. (Uden, the Netherlands), a dectin-1-specific polyclonal goat antibody from R and D Systems (Wiesbaden, Germany) and synthetic tripalmytoyl-cysteinyl-seryl-$(lysyl)_3$-lysine (P_3CSK_4) from EMC microcollections (Tübingen, Germany). Monoclonal antibody 2G8 has been described recently (Torosantucci *et al*., 2005) and mab T2.5 was kindly provided by C. J. Kirschning (Technical University of Munich, Germany). Isotype control antibodies (mouse IgG_1 and rat IgG_{2B}) (Becton Dickinson, Heidelberg, Germany) were dialysed against PBS using a Slide-A-Lyzer cassette (cut-off 3.5 kDa) (Pierce Biotechnology, Rockford, IL, USA) and used at the indicated concentrations.

Isolation of fungal cells

The *A. fumigatus* strain ATCC46645 (NCTC2109), an isolate from the lung of a patient suffering from invasive aspergillosis, and the corresponding *pks*P mutant (Jahn *et al*., 2000) were grown on potato dextrose agar (BD Difco, Heidelberg, Germany) in 600 ml tissue culture bottles (Nunc, Wiesbaden, Germany) for 4–5 days at 37°C. Conidia were collected by vigorous shaking using sterile 5–7 glass beads (diameter: 4 mm) and 20 ml of 0.1% Tween 20 per bottle. The resulting suspension was filtered through two layers of gauze (Miracloth, Calbiochem), concentrated using a syringe with an attached disposable 0.45 μm filter, washed three times with sterile water, and finally stored in water at 4°C. Conidial concentration was determined using a Neubauer chamber. Generation of a GFP-expressing *A. fumigatus* strain has been described previously (Meier *et al*., 2003). Swollen conidia were obtained by incubating 10^8 resting conidia in 5 ml of yeast-glucose medium (0.5% yeast extract, 2% glucose filtered with a cut-off of 6.–8.000 Da) with shaking (150 r.p.m.) at 37°C for 6 h.

Mice and cell lines

Murine J774A.1 macrophages (DSMZ ACC 170), murine alveolar MH-S macrophages (ECACC 95090612) and human embryonic kidney 293 cells (HEK293) (ATCC CRL-1573) were cultivated in RPMI1640 medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine and penicillin-streptomycin. C3H/HeN ($TLR2^{+/+}TLR4^{+/+}$) and C3H/HeJ ($TLR2^{+/+}TLR4^{d/d}$) mice were obtained from Charles River Laboratories (Sulzfeld, Germany). C57/BL6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Mice bearing a targeted disruption of the TLR2 gene (Werts *et al*., 2001) were kindly provided by Tularik (South San Francisco, CA, USA) and were generated by Deltagen (Menlo Park, CA, USA) on Tularik's behalf. Mice lacking both, TLR2 and functional TLR4 (C3H/HeJ $TLR2^{-/-}$), were generated by appropriate crossing with the C3H/HeJ strain (Vabulas *et al*., 2002). MyD88-deficient mice were provided by Shizuo Akira (Research Institute for Microbial Diseases, Osaka University, Japan) (Adachi *et al*., 1998) and used after backcrossing for 10 generations to C57BL/6. All mice were bred under specific pathogen-free conditions.

Isolation of macrophages

Peritoneal macrophages were isolated from 6 to 10 weeks old mice by lavage with sterile PBS according to standard procedures. For isolation of alveolar macrophages C57/Bl6 mice were sacrificed. After opening the Vena clava, 5 ml of PBS was injected into the heart to replace the blood. Thereafter, lungs were lavaged through an intratracheal catheder with calcium- and magnesium-free PBS supplemented with 0.6 mM EDTA. A total of 10 ml was used in each mouse in 0.5 ml increments with a

dwell-time of 30 s. The cells from the lavage fluids were collected by centrifugation. Isolated cells were washed with PBS, counted using a Neubauer chamber and cultivated on glass cover slips in RPMI1640 medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine and penicillin-streptomycin. Histological staining and immunofluorescence analysis using mab F4/80 revealed that the vast majority of the adherent cells derived from the alveolar lavages were macrophages.

Infection experiments and quantification of phagocytosis

The biotin-calcofluor staining was performed as described recently (Luther *et al*., 2006). Briefly, resting or swollen conidia were biotinylated using 10 mg ml^{-1} sulfo-NHS-LC-biotin (Molecular Probes, Karlsruhe, Germany) in 50 mM $NaHCO_3$, pH 8.5 for 2 h at 4°C. Remaining reactive biotin molecules were inactivated by incubation in 100 mM Tris-HCl, pH 8.0 for 40 min at 4°C.

For infection experiments macrophages were seeded on glass cover slips in 24 well plates at a density of 5×10^5 cells per well for J774 macrophages and 1×10^6 cells per well for peritoneal macrophages. On the next day macrophages were washed with prewarmed medium and subsequently infected with biotinylated conidia at a multiplicity of infection (moi) of 10. After the times indicated, infected cells were fixed in 3.7% formaldehyde in PBS for 10 min. Extracellular conidia were detected using Cy3-labelled streptavidin (Dianova, Hamburg, Germany) (diluted 1 : 100 in PBS, 30 min at 37°C). Extra- and intracellular conidia were stained using 0.4 mg ml^{-1} calcofluor white (Sigma) in PBS for 30 min. HOURSost cells were visualized using 0.1 μg ml^{-1} FITC-labelled concanavalin A (Molecular Probes) in PBS for 30 min at room temperature (RT). Micrographs taken with a Leitz RBE microscope (Leica Microsystems, Wetzlar, Germany) and digitally recorded using the MetaMorph software (Visitron Systems, Puchheim, Germany) were used for quantitative analysis. Efficiency of phagocytosis was calculated as the percentage of intracellular spores from the entirety of all cell-associated conidia.

BSA was coupled to latex beads (diameter: 4 μm, Molecular Probes) according to standard procedures and biotinylated as described above. Homogenous labelling was verified using Cy3-labelled streptavidin. Macrophages were incubated with these microspheres at a ration of 10 beads per cell for 3 h. Internalized beads were quantified using the BCS technique (Luther *et al*., 2006). Efficiency of uptake was calculated as the percentage of internalized microspheres from the entirety of all cell-associated beads.

Inhibition of conidial phagocytosis

Laminarin is a soluble β1–3 glucan polymer with β1–6 interstrand linkages from the brown algae *Laminaria digitata*. To analyse a potential competitive inhibition of phagocytosis by soluble β1–3 glucan, we performed infection experiments as described above, but in the presence of the indicated concentrations of laminarin (0.1 μg ml^{-1} to 1000 μg ml^{-1}). To study the impact of the β1–3 glucan-binding factor G on conidial phagocytosis *A. fumigatus* conidia were preincubated with the Fungitell reagent (a Limulus Amebocyte Lysate depleted for the LPS-specific Factor C) and dilutions thereof in PBS for 30 min at RT and 400 r.p.m. Conidia were then washed in PBS and used to infect macrophages as described above. To study the role of dectin-1 and TLR2 in conidial phagocytosis, macrophages cultivated on cover slips were preincubated with medium containing the indicated concentrations of the dectin-1-specific mab 2A11 (Brown *et al*., 2002) or the TLR2-specific mab T2.5 (Meng *et al*., 2004) for 30 min. Infection experiments were performed as described above, but in the presence of the respective mabs. Isotype control antibodies were used at 10 μg ml^{-1} for rat IgG_{2B} and 35 μg ml^{-1} for mouse IgG_1.

Immunofluorescence

Macrophages grown on glass cover slips in 24 well plates were infected with *A. fumigatus* conidia (moi = 10) for the times indicated. Infected cells were fixed using 3.7% formaldehyde in PBS for 5 min at RT. For permeabilization of host cell membranes samples were incubated in 0.2% Triton X-100/PBS for 1 min at RT. To avoid unspecific binding cover slips were preincubated with 2% goat serum in PBS for 30 min at RT. To detect dectin-1, samples were incubated in a humid chamber with 25 μl of a 1 : 50 dilution of either mab 2A11 or a polyclonal goat-antidectin-1 antibody. After 30 min the antibody solution was aspirated and the sample was washed three times with PBS. Bound primary antibodies were visualized using an appropriate Cy3-labelled secondary antibody (Dianova). After a final washing step samples were mounted with FluoroGuard Antifade Reagent (Bio-Rad, Munich, Germany) and analysed using a Leitz RBE microscope and the MetaMorph software or a Leica TCS-NT confocal laser scanning microscope.

For detection of surface exposed β1–3 glucans conidia were fixed with 3.7% formaldehyde in PBS for 10 min at RT. To avoid unspecific binding samples were preincubated with 2% goat serum in PBS for 30 min at RT. Spores were then reacted with mab mAb 2G8 (Torosantucci *et al*., 2005) and bound immunoglobulins were visualized using a Cy3-labelled secondary antibody. Immunofluorescence micrographs were taken using Leitz RBE microscope and the MetaMorph software or a Leica TCS-NT confocal laser scanning microscope. FACS analysis was performed using a FACS Canto benchtop analyzer (Becton-Dickinson).

Transfection of HEK293 cells and quantification of conidial binding

The plasmid for constitutive expression of murine TLR2 was kindly provided by C. Kirschning (Technical University of Munich, Germany). Plasmid dectin-1-V5-His conferring expression of murine dectin-1 was kindly provided by D. M. Underhill (Cedars-Sinai Medical Center, Immunobiology Research Institute, Los Angeles, USA). Plasmid pEGFP-Actin (Clontech, Saint-Germain-en-Laye, France) conferred constitutive expression of GFP-actin. All plasmids were isolated using the EndoFree Plasmid Maxi Kit (Qiagen). Transfection was performed using the Effectene Transfection Reagent according to the instructions of the vendor (Qiagen). Transfected cells expressing GFP-actin were identified after 18 h at 37°C by microscopic inspection. Cells were infected with *A. fumigatus* wild-type conidia at an moi of 10 for 3 h. For quantification of bound conidia light microscopic micrographs were recorded and the average number of cell-associated conidia per cell was calculated.

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