LETTERS

Surface hydrophobin prevents immune recognition of airborne fungal spores

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The air we breathe is filled with thousands of fungal spores (conidia) per cubic metre, which in certain composting environments can easily exceed 10^9 per cubic metre. They originate from more than a hundred fungal species belonging mainly to the genera *Cladosporium*, *Penicillium*, *Alternaria* and *Aspergillus*[1–4]. Although these conidia contain many antigens and allergens[5–7], it is not known why airborne fungal microflora do not activate the host innate immune cells continuously and do not induce detrimental inflammatory responses following their inhalation. Here we show that the surface layer on the dormant conidia masks their recognition by the immune system and hence prevents immune response. To explore this, we used several fungal members of the airborne microflora, including the human opportunistic fungal pathogen *Aspergillus fumigatus*, in *in vitro* assays with dendritic cells and alveolar macrophages and in *in vivo* murine experiments. In *A. fumigatus*, this surface 'rodlet layer' is composed of hydrophobic RodA protein covalently bound to the conidial cell wall through glycosylphosphatidylinositol-remnants. RodA extracted from conidia of *A. fumigatus* was immunologically inert and did not induce dendritic cell or alveolar macrophage maturation and activation, and failed to activate helper T-cell immune responses *in vivo*. The removal of this surface 'rodlet/hydrophobin layer' either chemically (using hydrofluoric acid), genetically (Δ*rodA* mutant) or biologically (germination) resulted in conidial morphotypes inducing immune activation. All these observations show that the hydrophobic rodlet layer on the conidial cell surface immunologically silences airborne moulds.

Dendritic cells are the sentinels of the immune system controlling fungal immunity[8]. Germinating *A. fumigatus* conidia induced significant expression of co-stimulatory molecules (CD80, CD86, CD40 and CD83) and antigen-presenting molecule human leukocyte antigen DR (HLA-DR) on human dendritic cells, and induced the secretion of inflammatory and anti-inflammatory cytokines, indicating that metabolically active germinating conidia provide maturation-associated signals to dendritic cells. On the other hand, dormant conidia did not modify the expression of surface molecules or the secretion of cytokines (Supplementary Fig. 2). These results, in agreement with previous studies[9–11], suggest that dormant conidia, in contrast to germinated conidia, are immunologically inert. However, dormant conidia contain many immunogenic molecules[12] so that on cell wall disruption, the intracellular material of dormant conidia could activate dendritic cells (Supplementary Fig. 3).

We then attempted to dissect the reasons for the immunologically inert nature of the dormant conidia. *A. fumigatus* dormant conidia are covered by a rodlet layer, a thin coating of regularly arranged RodA hydrophobins[13]. The presence of a glycosylphosphatidylinositol (GPI)-anchoring sequence discovered during analysis of the *rodA* gene (*Afu5g09580*; Supplementary Fig. 4a) indicates that RodA is covalently bound to the cell-wall polysaccharides[14]. Accordingly, hydrofluoric acid treatment that cleaves phosphodiester bonds of GPI anchors/remnants and releases GPI proteins bound to the cell wall was performed[15]. The hydrofluoric acid extract that was completely water soluble accounted for 1.7% of conidial dry weight and resolved into three bands on SDS–PAGE (Fig. 1a). Mass spectrometry (MS) and MS/MS analysis showed that these proteins with an apparent mass of 32, 16 and 14.5 kDa on SDS–PAGE corresponded to the dimeric form of the native RodA, native RodA (consistent with its theoretical mass) and partially degraded or processed RodA (RodA*) (Supplementary Table 1), respectively. We could not observe any RodA released into the culture supernatant when conidia were germinated in various culture media (Fig. 1b, also see ref. 16), indicating the complete degradation of the pre-existing rodlet layer during germination. These observations indicate that the RodA of the outer rodlet layer, covalently bound to the cell wall of the dormant conidia, is degraded during germination, exposing the underlying immunogenic cell wall components usually masked by this rodlet layer.

On the basis of the ability of different morphotypes to induce dendritic cell activation, we surmised that the rodlet layer on the dormant conidia imparts immunological inertness. To examine this, 5×10^5 human dendritic cells were treated with 0.33 μg of RodA (concentration corresponding to 5×10^5 conidia). Interestingly, RodA did not induce maturation of dendritic cells (Fig. 1c, d). Even at higher concentrations of RodA (up to 1 μg), there were no changes in the dendritic cell phenotype. Also, RodA neither induced nor altered the basal level of dendritic cell cytokines (Fig. 1e). Thus, the results are reminiscent of interaction of dendritic cells with dormant conidia (Supplementary Fig. 2). Moreover and in contrast to 18-kDa ribonuclease (Aspf1, encoded by the gene *Afu5g02330*), one of the most immunogenic proteins of *A. fumigatus*, RodA was unable to stimulate lymphoproliferation (Fig. 1f, g) or to activate *Aspergillus*-specific human $CD4^+$ T-cell clones for cytokine production (Fig. 1h). We verified that the lack of activation by RodA was not due to the hydrofluoric acid treatment because this treatment did not alter the immunogenicity of other *Aspergillus* proteins such as Aspf1 (Supplementary Fig. 5). Further, to confirm that RodA does not impart tolerogenic properties and immunological unresponsiveness to dendritic cells on encounter with other immunogenic molecules, we treated dendritic cells with a

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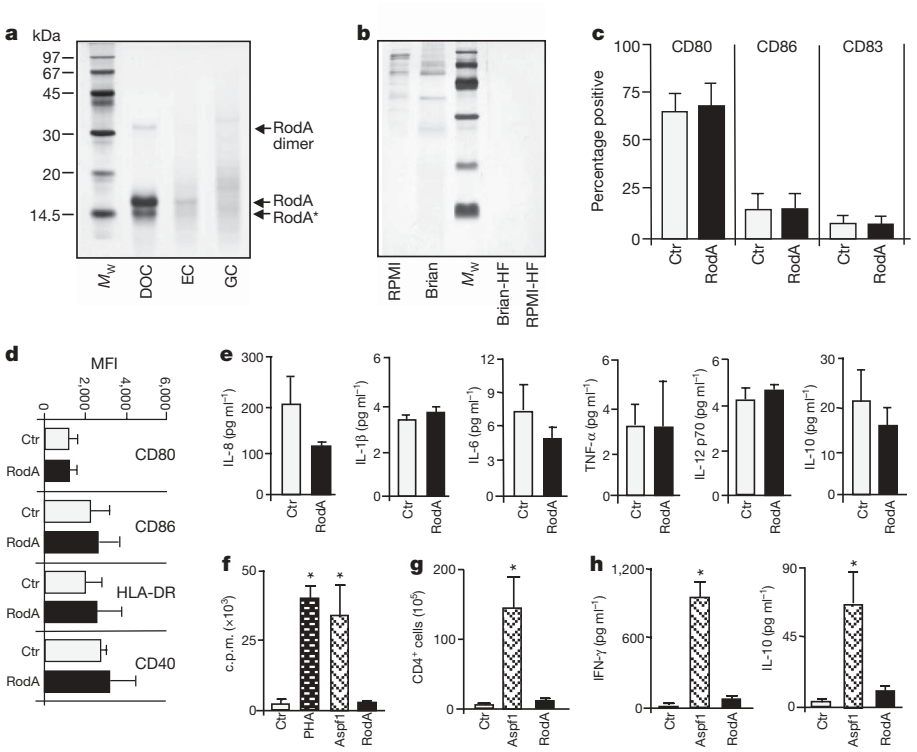

Figure 1 | *Aspergillus fumigatus* conidial surface-hydrophobin, RodA, fails to activate human dendritic cells and CD4$^+$ T lymphocytes. a, **b**, SDS–PAGE profiles of hydrofluoric acid extracts from dormant (DOC), enlarged (EC) and germinating (GC) conidia (**a**) and naive or hydrofluoric acid-treated supernatant of the Brian's (5% glucose, 1% asparagine, 1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, 0.24% NH_4NO_3, 2.6×10^{-3} % $ZnSO_4 \cdot 7H_2O$, 2.6×10^{-4}% $CuSO_4 \cdot 5H_2O$, 1.3×10^{-4}% $Co(NO_3)_2 \cdot 6H_2O$ and 6.5×10^{-3}% $CaCl_2$, pH 5.4) or RPMI media in which conidia were germinated (**b**). **c**–**e**, Phenotype markers of human dendritic cells showing the percentage of positive cells (**c**), the mean fluorescence intensities (MFI; **d**) and the cytokine secretion (**e**). **f**, **g**, **h**, Lack of lymphoproliferation (**f**, c.p.m.; **g**, frequency) and activation (**h**) of *Aspergillus*-specific human CD4$^+$ T-cell clones ($n = 3$–7; means ± s.e.m. are shown; *$P < 0.001$, Mann–Whitney test). Aspf1 and PHA (phytohaemoagglutinin) were used as positive controls. Ctr, negative control.

mixture of RodA and a recombinant Aspf1; RodA did not suppress dendritic cell activation by Aspf1 (Supplementary Fig. 6).

To confirm that the RodA layer on the cell wall masks the immunogenicity of dormant conidia, we used Δ*rodA* conidia that lack RodA and therefore the rodlet layer[13]. In contrast to the wild-type conidia and despite being dormant, Δ*rodA* conidia induced strong upregulation of costimulatory and antigen-presenting molecules on human dendritic cells (Fig. 2a, b). Additionally, Δ*rodA* conidia also induced high amounts of dendritic cell cytokines (Fig. 2c). The hydrofluoric acid-treated dormant wild-type conidia, with exposed immunogenic

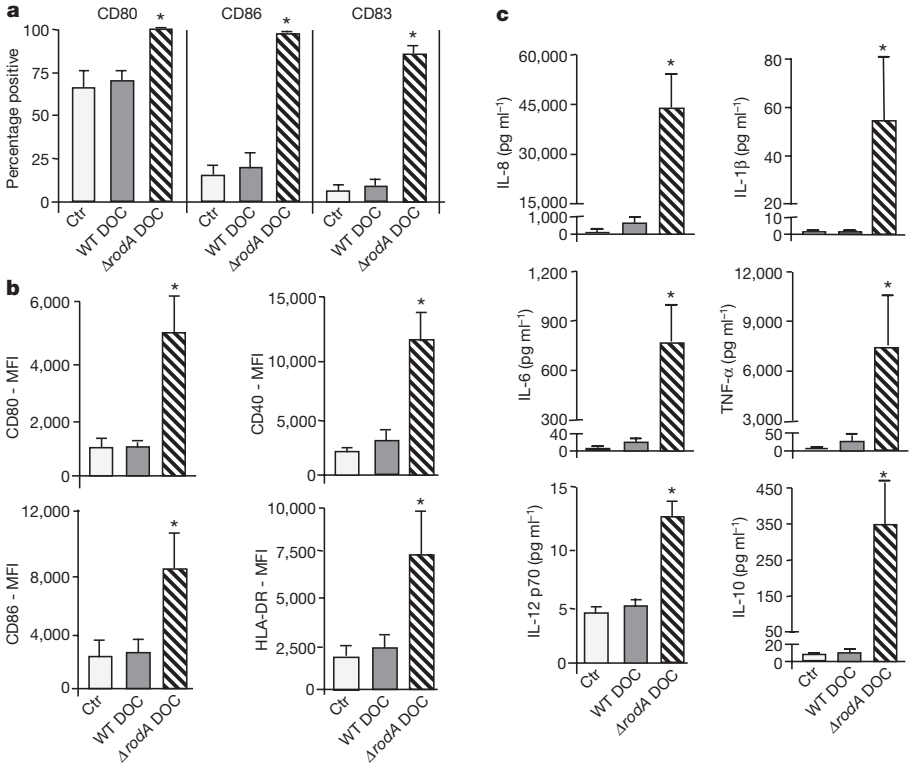

Figure 2 | Dormant conidia of Δ*rodA* mutant in contrast to wild-type conidia induce maturation and activation of human dendritic cells. a–**c**, Six-day-old human dendritic cells were cultured with GM-CSF and IL-4 alone (Ctr) or with wild-type dormant conidia (WT DOC) or Δ*rodA* dormant conidia (Δ*rodA* DOC). **a**, Percentage of cells expressing the indicated markers; **b**, mean fluorescence intensities (MFI) and **c**, cytokine secretion ($n = 4$; mean ± s.e.m. and statistical significance (*$P < 0.05$ in **a**; $P < 0.001$ in **b** and **c**) determined by the Mann–Whitney test).

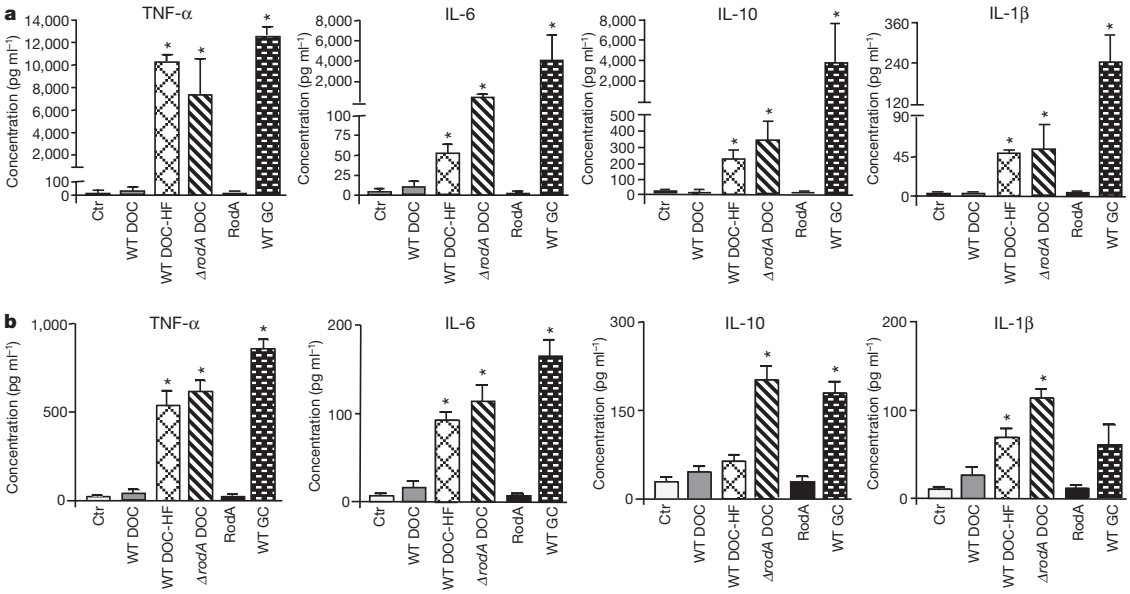

Figure 3 | Biological (conidial germination), chemical (hydrofluoric acid-treatment) or genetic (Δ*rodA* mutant) removal of RodA from dormant conidia induces activation of human dendritic cells and murine alveolar macrophages *in vitro*. **a**, **b**, Human dendritic cells (**a**) and murine alveolar macrophages (**b**) were incubated with wild-type naive (WT DOC) or hydrofluoric acid-treated dormant conidia (WT DOC-HF), Δ*rodA* dormant conidia, RodA and germinated conidia (WT GC). Concentrations (mean ± s.e.m.) of secreted tumour necrosis factor α (TNF-α), IL-6, IL-10 and IL-1β are shown. Statistical significance (*$P < 0.05$) determined by Mann–Whitney test (**a**; $n = 4$) and paired *t*-test (**b**; $n = 3$) compared to unstimulated cells (Ctr) is shown.

determinants after removal of rodlet protein, also stimulated human dendritic cells similarly to germinating conidia (Fig. 3a, Supplementary Figs 2 and 7). Furthermore, when murine alveolar macrophages were exposed *in vitro* to naive or hydrofluoric acid-treated dormant conidia, Δ*rodA* dormant conidia, RodA and germinated conidia, all fungal samples except naive dormant conidia and RodA induced inflammatory cytokines, chemokines and reactive oxygen intermediates (ROI) (Fig. 3b and Supplementary Fig. 8). Together, these data show that removal of the surface rodlet layer from conidia either chemically (hydrofluoric acid treatment),

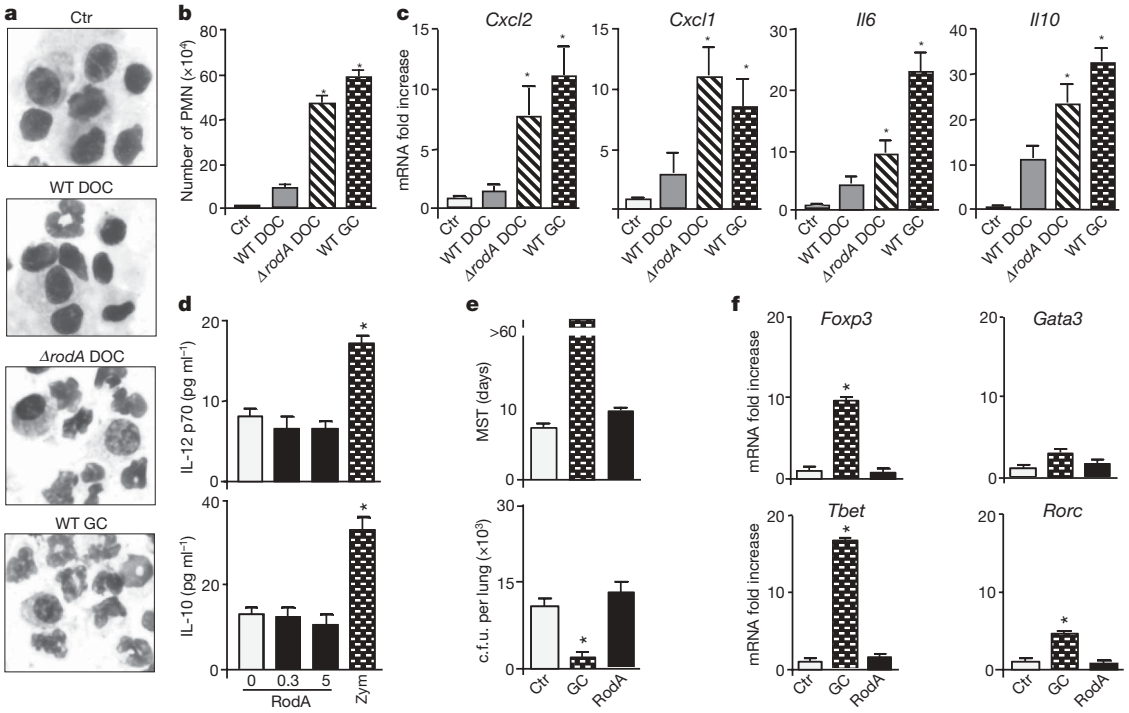

Figure 4 | RodA and wild-type dormant *A. fumigatus* conidia do not activate murine immune system *in vivo*, contrary to Δ*rodA*-dormant or wild-type-germinated conidia. **a**, Lung cytospin preparations; **b**, neutrophils (polymorphonuclear cells, PMN) in cytospins and **c**, *Cxcl2*, *Cxcl1*, *Il6* and *Il10* mRNA in lungs 24 h after conidial inoculation ($n = 3$; mean ± s.e.m.; *$P < 0.05$, paired *t*-test). WT DOC, wild-type dormant conidia; WT GC, wild-type germinated conidia; Δ*rodA* DOC, mutant Δ*rodA* dormant conidia. **d**, Cytokine secretion by RodA- and zymosan (Zym.)-stimulated lung $CD11c^+$ dendritic cells. **e**, Median survival time (MST) and colony forming units (c.f.u.); **f**, helper T-cell-responses in mice injected with RodA-dendritic cells or germinated conidia-dendritic cells (GC), followed by intranasal inoculation of live conidia ($n = 3$; mean ± s.e.m.; *$P < 0.001$, Mann–Whitney test).

biologically (germinated conidia) or genetically (Δ*rodA* mutant) results in an activation of the human or murine innate immune cells.

To confirm that hydrophobins regulate the immune response to conidia *in vivo*, intranasal inoculation of mice was performed with the fungal samples mentioned earlier. First, Δ*rodA* dormant conidia or wild-type germinated conidia were highly inflammatory as shown by the massive influx of polymorphonuclear cells in the lungs (Fig. 4a, b) and induction of high levels of chemokines *Cxcl1*, *Cxcl2*, inflammatory *Il6* and anti-inflammatory *Il10* cytokines (Fig. 4c). In contrast, wild-type dormant conidia covered by RodA failed to activate the mouse immune system *in vivo* (Fig. 4a–c). In addition, germinated, hydrofluoric acid-treated dormant and Δ*rodA* dormant conidia stimulated alveolar macrophages *in vivo*, but wild-type dormant conidia and RodA did not (data not shown). Second, to assess the biological activity of RodA in a mouse model, we exposed murine lung dendritic cells to RodA, and evaluated their cytokine production and their ability to activate helper T-cell (Th) responses on adoptive transfer *in vivo*. Confirming the human dendritic cell results, RodA neither induced maturation of dendritic cells, even at high concentrations (up to 5 $\mu g\,ml^{-1}$, data not shown), nor induced secretion of cytokines, in contrast to the positive control (zymosan; Fig. 4d). On adoptive transfer *in vivo* in mice with aspergillosis[17], RodA-pulsed dendritic cells neither increased survival nor decreased fungal growth in the lungs, as opposed to germinated conidia-pulsed dendritic cells (Fig. 4e). This was associated with the failure to activate antigen-specific *Tbet*$^+$/Th1, *Gata3*/Th2, *Rorc*$^+$/Th17 and *Foxp3*$^+$/regulatory T-cell responses (Fig. 4f). In addition, RodA failed to induce antibody responses in mice, despite repeated injections in the presence of Freund's complete/incomplete adjuvants. These results show that the inability of RodA to induce activation of antigen-presenting cells (Figs 1, 3 and 4) is translated into failure to activate antigen-specific adaptive T- and B-cell immune responses *in vivo* in mice. This lack of immune response to RodA may also be due to its resistance to lysosomal proteolytic degradation (Supplementary Fig. 9), resulting in the lack of generation of antigenic peptides and their presentation by dendritic cells.

Rodlet proteins were also extracted using hydrofluoric acid from the conidia of three other moulds of the airborne fungal flora: the food-borne *Penicillium camemberti*, the toxinogenic *Penicillium verrucosum* and the allergenic *Cladosporium cladosporioides* that are known to have rodlets on their aerial conidial surface[18,19]. The hydrofluoric acid-extracted protein from *P. camemberti*, on single MS and MS/MS analyses, matched exactly with the Q1I187 sequence of the *P. camemberti* hydrophobin (data not shown). In addition, sequence analysis of the *P. camemberti* hydrophobin showed an organization similar to RodA of *A. fumigatus* (Supplementary Fig. 4b). We found that dormant conidia or hydrophobins from these three fungal species were also immunologically inert, whereas hydrofluoric acid-treated dormant conidia induced a very strong immune reaction in human dendritic cells (Supplementary Figs 10 and 11), indicating that the lack of recognition of rodlet protein by the immune system is a universal phenomenon.

Taken together, these results demonstrate that the surface rodlet layer of the conidial cell wall makes airborne conidia of filamentous fungi inert to both innate and adaptive immunity. The cell wall of filamentous fungi is a complex structure, rich in many immunologically active components, including either constitutive cell-wall polysaccharides such as β-glucans and galactomannan or secreted (glyco)proteins in transit in the cell wall before being secreted[14,20]. Activation of dendritic cells and macrophages by these cell-wall immunogenic motifs requires their exposure to the immune cells mentioned earlier[14,20,21]. *In vivo*, intraphagosomal germination of thermotolerant live conidia or autolysis of fungal species that are sensitive to temperatures above 37 °C results in a slow exposure or release of immunologically active molecules after the removal of the surface rodlet layer. Recognition of these exposed molecules by pathogen-recognition-receptors, including dectin-1 (see Supplementary Fig. 12, the lack of rodlet layer in Δ*rodA* dormant conidia exposes β-(1,3)-glucans that are recognized, in a punctuated pattern, by dectin-1), then leads to the activation of a controlled immune response[9–11,22]. From a pathogen point of view, immunological inertness of the rodlet layer can help dormant conidia to escape the host defence mechanisms and stay dormant in a hidden niche until conditions are suitable for germination. Moreover, considering the fact that airborne conidia are ubiquitous in the atmosphere, the inert nature of hydrophobin rodlet layer makes teleological sense as it prevents undue and exacerbated host response by innate immune cells and hence prevents inflammation and host damage. From a therapeutic point of view, the ability of rodlet proteins to resist degradation could be used to generate rodlet-protein-based nanoparticles containing embedded therapeutic proteins and molecules that have to be slowly released within the host or transported to a specific body location without being recognized by the host immune system[23].

METHODS SUMMARY

A. fumigatus strains were the clinical isolate CBS144-89 (wild type) and the mutant Δ*rodA-47* (ref. 13). *P. camemberti*[18], *P. verrucosum* and *C. cladosporioides* were wild-type strains. Dormant conidia were obtained by growth on 2% malt extract agar. Enlarged and germinating conidia were produced after 4 and 8 h incubation in 2% glucose + 1% mycopeptone medium at 37 °C, respectively. Fungal conidia were fixed in 2% *p*-formaldehyde. The rodlet layer was extracted by incubating dry conidia with 48% hydrofluoric acid for 72 h at 4 °C. For sequence analysis, protein bands on SDS–PAGE gels were excised manually, trypsin-digested and the peptides obtained[24] were subjected to proteomic analyses by matrix-assisted laser desorption/ionization–time of flight/time of flight (MALDI-TOF/TOF; see ref. 25). Immature human dendritic cells were generated by culturing monocytes of healthy donors in the presence of interleukin 4 (IL-4) and granulocyte–macrophage colony-stimulating factor (GM-CSF; see ref. 26). They were incubated for 48 h in the presence of cytokines alone or with RodA or Aspf1[27] or with naive or hydrofluoric acid-treated dormant, enlarged and germinating conidia of wild-type *A. fumigatus*, or with Δ*rodA* dormant conidia. The surface markers on dendritic cells were analysed by flow cytometry. Cytokines were quantified in cell-free culture supernatants by cytometric bead array assay. Murine alveolar macrophages and dendritic cells from C57BL6 (*H-2*^{b}) mice were purified from lungs and stimulated *in vitro* with the same fungal samples as those used for human dendritic cells. Alternatively, mice received 2×10^7 conidia intranasally or the equivalent treated samples. Expression of murine cytokine and chemokines was analysed by polymerase chain reaction with reverse transcription (RT–PCR) and/or enzyme-linked immunosorbent assay (ELISA)[28] and helper T-cell-specific transcription factors in CD4$^+$ T cells were analysed by RT–PCR[28].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions J.-P.L. initiated the study; V.A., J.B., L.R. and J.-P.L. designed the research; V.A., C.C., S.P. and J.-P.L. prepared and characterized conidia and fungal materials; J.B., S.R.E. and S.V.K. performed experiments with human dendritic cells; S.B., K.P. and L.R. performed experiments with T-cell clones, murine dendritic cells and macrophages; O.K. and A.A.B. performed protein sequence analysis; V.A., J.B., S.B., O.K., K.P., L.R. and J.-P.L. analysed the results; and V.A., J.B., O.K., L.R. and J.-P.L. wrote the paper.

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METHODS

Mice. Female, 8- to 10-week-old inbred C57BL6 (*H-2*^{b}) mice were purchased from Charles River Breeding Laboratories. Experiments were performed according to the Italian Approved Animal Welfare Assurance A-3143-01.

Generation of dendritic cells: human dendritic cells. Peripheral blood mononuclear cells were isolated from buffy bags of blood from healthy donors by Ficoll-Hypaque density gradient centrifugation. Monocytes were positively isolated using CD14 beads (Miltenyi Biotech). Immature dendritic cells were generated by culturing monocytes for 6 days in RPMI 1640 containing 10% fetal calf serum, 50 U ml^{-1} penicillin, 50 U ml^{-1} streptomycin, IL-4 (500 IU per 10^6 cells) and GM-CSF (1000 IU per 10^6 cells)[26]. Recombinant human GM-CSF and IL-4 were from ImmunoTools. The immature dendritic cells express high levels of CD1a, intermediate levels of HLA-DR, CD80 and CD86, and were negative or low-positive for CD83.

Generation of dendritic cells: murine dendritic cells. Dendritic cells (between 5 and 7% positive for CD8α and between 30 and 35% positive for Gr-1) from naive mice were purified from collagenase D (Sigma)-treated lungs by magnetic cell sorting with MicroBeads (Miltenyi Biotech) conjugated to hamster anti-mouse CD11c monoclonal antibodies (clone N-418)[29].

Isolation of murine alveolar macrophages. Alveolar macrophages were purified from bronchoalveolar lavage fluid of naive or infected mice 6 h after the infection. Alveolar macrophages were collected using a total of 10 ml RPMI-1640 medium containing 0.5 mM EDTA.

Preparation of fungal material. The *A. fumigatus* strains used for this study were CBS144-89, a clinical isolate used as wild-type strain, and a *rodA* minus mutant Δ*rodA-47* (ref. 13). Conidia were harvested from a 1-week-old culture grown at 25 °C on 2% malt extract agar (dormant conidia, DOC). The dormant conidia were germinated for up to 8 h in Sabouraud liquid medium (2% glucose, 1% mycopeptone (Biokar)) at 37 °C. After 4 h, there was iso-diametrical growth and the conidia appeared swollen (enlarged conidia, EC) and after 8 h, growth becomes polarized, leading to the emergence of germ tubes of length <10 μm (germinating conidia, GC). Conidia at appropriate stage of development were fixed with *p*-formaldehyde (2.5% w/v in phosphate buffered saline (pH 7.4) at 4 °C overnight), neutralized with 0.1 M NH_4Cl, washed and reconstituted in PBS.

The rodlet layer was extracted from the spore surface by incubating dry conidia with 48% hydrofluoric acid for 72 h at 4 °C. The contents were centrifuged (9,000*g*, 10 min) and the supernatant obtained was dried under N_2. The dried material was reconstituted in H_2O and an aliquot was subjected to SDS–PAGE (15% gel) and visualized by silver nitrate staining following standard protocols. Preliminary assays have shown that the optimum amount of RodA without any contaminants (as proven by SDS–PAGE) could be obtained after 3 days of incubation (0.8, 1.5 and 1.7% RodA was released per dormant conidial dry weight after 1, 2 and 3 days of incubation, respectively). An ultracentrifugation step (100,000*g*, 40 min) showed that the rodlet hydrophobins were completely soluble in water. The rodlet hydrophobins were also extracted from dry conidia of *Penicillium camemberti* (strain PCENS1, a gift from J.-F. Cavin), *P. verrucosum* (strain IP 1231-80, Institut Pasteur) and *Cladosporium cladosporioides* (strain IP1232-80, Institut Pasteur) using hydrofluoric acid under conditions identical to those used for RodA extraction from *A. fumigatus*. Besides being a food-borne fungus, *P. camemberti* was selected because, similar to *A. fumigatus*, it is one of the few fungal species from which surface hydrophobins were both isolated biochemically and sequenced[18].

The recombinant Aspf1 protein (18-kDa RNase) from *A. fumigatus* was produced as described previously[27]. Aspf1 and wild-type conidia also treated with hydrofluoric acid were used as controls to monitor the putative negative effect of the hydrofluoric acid treatment.

Analysis of rodlet hydrophobins by mass spectrometry. Proteins bands of the hydrofluoric acid extract on SDS–PAGE (%T-15, revealed by amido black 10B) were excised manually, followed by tryptic digestion according to an earlier protocol[24]. Peptides thus obtained were extracted for 1 h with acetonitrile (ACN): trifluoracetic acid (TFA) 0.1% (1:1 v/v), mixed with saturated α-cyano-4-hydroxycinnamic acid in ACN: TFA 0.1% (1:2 v/v) and allowed to dry on a stainless steel anchor chip target (dry droplet preparation). The samples were measured on an Ultraflex I MALDI-TOF/TOF device using flexControl 3.0 for data collection and flexAnalysis 3.0 for spectra analysis/peak list generation (Bruker Daltonics)[25]. Up to five peptides of the PMF spectra were chosen for post source decay MS/MS analyses. For identification, peptide mass fingerprint (PMF) and peptide fragmentation fingerprint (PFF) spectra were submitted to the MASCOT server (MASCOT 2.1.02, Matrix Science), searching the NCBI database. Sequences of the rodlet proteins of *A. fumigatus* and *P. camemberti* were already available in databases with reference numbers 70997898 and 94982475, respectively. With respect to the sample preparation, fixed modification of cysteines to *S*-carbamidomethyl derivatives and variable methionine oxidation was defined for the database search. Further, no missed cleavage and a peptide mass tolerance of 50 p.p.m. was allowed. Results were regarded as significant with an allowed likelihood for a random hit of $P \leq 0.05$, according to the MASCOT score. Database searches were triggered and archived on a ProteinScape 1.3 database server (Protagen). Accuracy of raw peak lists was improved by automated internal recalibration using known contaminants (Coomassie, trypsin and keratin fragments) and application of the peak rejection filter of the Score Booster tool, implemented into the ProteinScape 1.3 database software.

Assessing the reactivity of conidia to dectin1–Fc by fluorescence light microscopy. Conidia from wild-type and Δ*rodA* mutant were fixed as described above. After extensive washing with 0.2 M glycine and 1 h post coating in 1% bovine serum albumin buffer, the samples were incubated with dectin-1–human IgGFc chimaeric protein (provided by G. Brown[30]) at 5 μg ml^{-1} in PBS containing 1% bovine serum albumin (BSA) and 0.1% Tween 20. After extensive washing and 1 h incubation in a 1% mock goat serum, the conidia were incubated with a goat anti-human Fc immunoglobulin coupled to fluorescein isothiocyanate (Sigma, diluted 1:100 in PBS containing 1% BSA). Control was either the secondary antibody alone or the dectin–Fc incubated for 1 h in the presence of 10 mg ml^{-1} laminarin.

Susceptibility of RodA to cathepsins. RodA (2.5 μg) was treated with 0.125 μg cathepsin S or L (Sigma) in acetate buffer (100 mM, pH 4.5) containing 0.2 mM dithiothreitol and 0.2 mM EDTA in a final volume of 25 μl at 25 °C for 30 h. The reaction mixtures were subjected to SDS–PAGE (15% gel) and the protein bands were revealed by silver staining. The recombinant Aspf1 protein was taken as a positive control to check the proteolytic actions of cathepsin S and L.

Stimulation of dendritic cells: human dendritic cells. Six-day-old immature human dendritic cells were cultured in medium containing GM-CSF and IL-4 alone (5×10^5 cells ml^{-1} per well) or with *p*-formaldehyde-inactivated fungal materials for 48 h. Dendritic cells were cultured with dormant (naive or hydrofluoric acid-treated), enlarged and germinated conidia of wild type *A. fumigatus* and Δ*rodA* dormant conidia at a 1:1 ratio (5×10^5 conidia ml^{-1} per well), 0.33 μg of RodA (the concentration equivalent to 5×10^5 conidia), 0.6 μg of intracellular material of dormant conidia or 0.33 μg of 18-kDa RNase (Aspf1), 5×10^5 *Penicillium* and *Cladosporium* dormant conidia (naive or hydrofluoric acid-treated) or 0.33 μg of *Penicillium* and *Cladosporium* hydrofluoric acid-extracts. It was verified by inverted microscopy that all fungal morphotypes were engulfed by dendritic cells following culture. The surface staining of dendritic cells was performed with fluorochrome-conjugated antibodies to CD83, HLA-DR, CD80 and CD86 (BD Biosciences) and CD40 (Beckman Coulter) and proceeded for flow-cytometry (LSR II, BD Biosciences). Data were analysed by BD FACS DIVA software (BD Biosciences). Cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12 p70 and TNF-α) were quantified in cell-free culture supernatants using a BD cytometric bead array kit (BD Biosciences).

Stimulation of dendritic cells: murine dendritic cells. Murine dendritic cells were pulsed with live unopsonized *Aspergillus* conidia or RodA before adoptive transfer as described[17]. IL-12 p70 (<2.5 pg ml^{-1}, detection limit) and IL-10 (<12 pg ml^{-1}, detection limit) were quantified by ELISA in cell-free culture supernatants of dendritic cells resuspended in serum-free Iscove's medium and pulsed with RodA (0.3–5 μg ml^{-1}) or 10 μg ml^{-1} zymosan from *Saccharomyces cerevisiae* (Sigma) for 24 h.

Stimulation of alveolar macrophages. Alveolar macrophages from naive mice (10^6) were co-cultured with 10^6 *A. fumigatus* conidia, 10 μg RodA or lipopolysaccharide (as positive control) for 2 h at 37 °C, 5% CO_2. Alveolar macrophages from infected mice were immediately used without further stimulation *in vitro*. Secreted cytokines (IL-1β, IL-6, IL-10 and TNF-α) were measured by ELISA. Transcripts for genes involved in the biosynthesis of chemokines, cytokines and reactive oxygen intermediates (ROI) were measured by RT–PCR.

***In vivo* mouse experiments.** Female, 8- to 10-week-old inbred C57BL6 (*H-2*^{b}) mice were inoculated intranasally with a homogenous suspension of *p*-formaldehyde-fixed dormant or germinated conidia of wild type conidia and Δ*rodA* or hydrofluoric acid-treated dormant conidia. Anaesthetized mice received 2×10^7 conidia in 20 μl saline twice intranasally, 6 h apart, before asphyxiation using CO_2 a day after *Aspergillus* conidial inoculation. After perfusion, lungs were collected and neutrophils were counted on May–Grünwald–Giemsa-stained cytospin preparations. At least 200 cells per cytospin preparation were counted. Chemokines and cytokines were quantified by real time polymerase chain reaction with reverse transcription. Increased mouse survival was estimated as median survival time (days) and fungal growth in the lung estimated as c.f.u. during vaccination experiments were performed as described earlier[17,29].

Real time RT–PCR. Real time RT–PCR was performed using the iCycler iQ detection system (Bio-Rad) and SYBR Green chemistry (Finnzymes) to analyse chemokines and cytokines. Total lung cells or *in vitro* cultured cells were lysed and total RNA was extracted using an RNeasy Mini Kit (Qiagen). RNA was

reverse transcribed with Sensiscript Reverse Transcriptase (Qiagen) according to the manufacturer's protocol. The PCR primers used were as follows: forward primer 5′-CCGCTCGCTTCTCTGTGC-3′ and reverse primer 5′-CTCTGGATG TTCTTGAGGTGAATC-3′ for *Cxcl1*; forward primer 5′-CCAACCACCAGGCT ACAG-3′ and reverse primer 5′-CTTCAGGGTCAAGGCAAAC-3′ for *Cxcl2*; forward primer 5′-CCCTTTGCTATGGTGTCCTT-3′ and reverse primer 5′-TGGTTTCTCTTCCCAAGACC-3′ for *Il10*; forward primer 5′-CCGGAGAGG AGACTTCACAG-3′ and reverse primer 5′-TCCACGATTTCCCAGAGAAC-3′ for *Il6*; forward primer 5′-TGACGGACCCCAAAAGATGAAGG-3′ and reverse primer 5′-CCACGGGAAAGACACAGGTAGC-3′for *Il1β*; forward primer 5′-CGAGTGACAAGCCTGTAGCC-3′ and reverse primer 5′-GAAGAGAACCT GGGAGTAGACAAG-3′ for the TNF-α gene (*Tnf*); forward primer 5′-TAGAGACT CCTCCCATGCCT-3′ and reverse primer 5′-CACTGCCTCCTCTCATGCTA-3′ for p47^{phox}. The thermal profile for SYBR Green real-time PCR was at 95 °C for 3 min, followed by 40 cycles of denaturation for 30 s at 95 °C and an annealing/extension step of 30 s at 60 °C. Amplification efficiencies were validated and normalized against *Gapdh*.

Adoptive transfer of dendritic cells. Conidia- or RodA-pulsed dendritic cells (10^5) were administered into recipient mice subcutaneously, 2 and 1 week before the intranasal injection of 2×10^8 *A. fumigatus* conidia. CD4$^+$ T cells were purified (Miltenyi Biotech) from thoracic lymph nodes 3 days post-infection. Total RNA from CD4$^+$ T cells was extracted using RNeasy Mini Kit (Qiagen) and reverse transcribed with Sensiscript Reverse Transcriptase (Qiagen) to monitor the expression of transcription factors. The PCR primers for *Tbet*, *Rorc*, *Gata3* and *Foxp3* were as described[28]. Amplification efficiencies were validated and normalized against *Gapdh*.

Generation of *Aspergillus*-specific human T-cell clones and lymphoproliferation. *Aspergillus*-specific human CD4$^+$ T-cell clones were generated on stimulation by conidia-pulsed dendritic cells as described[17]. The T-cell clones were assessed for specificity against Aspf1- or RodA-pulsed dendritic cells and 0.5% phytohaemoagglutinin (PHA, Biochrom) (as a positive control) by [H^3]-thymidine (Amersham Biosciences) labelling or by measuring cytokine content in supernatants.

Statistical analysis. Statistical significance was determined by using the Mann–Whitney test or paired *t*-test.

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30. Graham, L. M. *et al.* Soluble Dectin-1 as a tool to detect β-glucans. *J. Immunol. Methods* **314**, 164–169 (2006).