Genetic deficiency of NOD2 confers resistance to invasive aspergillosis

Mark S. Gresnigt , Cristina Cunha , Martin Jaeger , Samuel M. Gonçalves , R.K. Subbarao Malireddi , Anne Ammerdorffer , Rosalie Lubbers , Marije Oosting , Orhan Rasid , Grégory Jouvion , Catherine Fitting , Dirk J. de Jong , João F. Lacerda , António Campos Jr. , Willem J.G. Melchers , Katrien Lagrou , Johan Maertens , Thirumala-Devi Kanneganti , Agostinho Carvalho , Oumaima Ibrahim-Granet & Frank L. van de Veerdonk

Invasive aspergillosis (IA) is a severe infection that can occur in severely immunocompromised patients. Efficient immune recognition of Aspergillus is crucial to protect against infection, and previous studies suggested a role for NOD2 in this process. However, thorough investigation of the impact of NOD2 on susceptibility to aspergillosis is lacking. Common genetic variations in NOD2 has been associated with Crohn’s disease and here we investigated the influence of these genetic variations on the anti-Aspergillus host response. A NOD2 polymorphism reduced the risk of IA after hematopoietic stem-cell transplantation. Mechanistically, absence of NOD2 in monocytes and macrophages increases phagocytosis leading to enhanced fungal killing, conversely, NOD2 activation reduces the antifungal potential of these cells. Crucially, Nod2 deficiency results in resistance to Aspergillus infection in an in vivo model of pulmonary aspergillosis. Collectively, our data demonstrate that genetic deficiency of NOD2 plays a protective role during Aspergillus infection.
Humans are ubiquitously exposed to airborne spores of *Aspergillus*, but only severe immunocompromised patients are at risk of developing pulmonary invasive aspergillosis (IA)\(^1\). Patients undergoing hematopoietic stem cell transplantation (HSCT) have a distinctive elevated susceptibility to aspergillosis and infections in these patients are associated with a high mortality\(^2\). With increasing knowledge of the antifungal host response, it has become evident that not only the immunocompromised status of patients plays a role in susceptibility to infection, but also the genetic background of both the engrafted bone marrow and the recipient\(^3\). To provide a good risk stratification for the development of IA following HSCT, genetic susceptibility needs to be taken into account. Common polymorphisms in various pattern recognition receptor (PRR) pathways are known to be associated with an increased risk for aspergillosis, which includes dectin-1\(^13,14\), pentraxin-3\(^15\) as well as many other receptors\(^6\). These findings help to predict susceptibility, yet also provide insight into the importance of these pathways in antifungal host defence. These studies, therefore, may also aid in the development of novel immune targeted treatment strategies. Nevertheless, several immune pathways remain unexplored for susceptibility to aspergillosis.

One of these relatively unexplored receptor families are the NACHT-LRR receptors (NLRs), to which the intracellular nucleotide-binding oligomerization domain (NOD) receptors belong. The NLR receptor Nlrlp3 plays a role in IA via regulation of inflammasome activation and subsequently protective IL-1-mediated cytokine responses\(^7\). Two other NLR receptors NOD1 and NOD2 are primarily involved in the recognition of peptidoglycan-derived moieties from bacteria and in the induction of proinflammatory host responses\(^8–12\). Although *Aspergillus* does not contain peptidoglycan, some evidence suggests that these NOD receptors might play a role in host defence against aspergillosis\(^13–16\). In contrast to a previous study that demonstrated a crucial role for NOD1 in activation of corneal epithelial cells by *Aspergillus*\(^16\), we recently reported that NOD1 negatively regulates host defence by reducing cytokine responses and oxidative burst\(^17\). NOD2, another member of the NLR family is highly expressed in lungs of mice infected with *Aspergillus*, and in THP1 cells, RAW macrophages and A549 cells stimulated with *Aspergillus*\(^13,14\). The NOD2 agonist Muramyl-dipeptide (MDP) can synergistically increase *Aspergillus*-induced cytokine levels\(^13,14\). NOD2 also may play a role in host defence against fungal keratitis\(^15,16\). Recently, NOD2 was also suggested to play a role in the recognition of chitin\(^18\), a polysaccharide that is present in the cell wall of all fungi. Polymorphisms in NOD2 have been associated with host defence against infectious diseases. In particular, strong associations between NOD2 polymorphisms and susceptibility to tuberculosis have been identified\(^19–22\). It should, however, be noted that the strongest genetic association with NOD2 is with Crohn’s disease\(^23\). Polymorphisms in NOD2 impact autophagy and antigen presentation in host defence against bacteria\(^24\). Considering recent evidence suggesting a crucial role for the autophagy machinery in host defence against *Aspergillus*\(^25\), NOD2 is a candidate susceptibility gene for aspergillosis.

Although previous studies have revealed enhanced expression of NOD2 during aspergillosis and synergism with *Aspergillus*-induced cytokine responses, these studies did not thoroughly investigate whether NOD2 modulates anti-*Aspergillus* host defence. Additionally, it is unknown whether defective NOD2 signalling influences susceptibility to aspergillosis. Therefore, the present study investigates whether common polymorphisms in NOD2 or its complete deficiency influences susceptibility to IA, and whether it affects the immune response to *Aspergillus*. We demonstrate that genetic variation in NOD2 in humans and complete Nod2 deficiency in mice protects against IA. In line with this, NOD2 deficiency or its neutralization associates with increased antifungal activity of macrophages and monocytes, conversely NOD2 activation neutralizes fungal killing capacity of phagocytes. Our data collectively highlight a detrimental role for NOD2 receptor in anti-*Aspergillus* host defence.

### Results

**NOD2 genetic variation decreases the risk of IA after HSCT.** To investigate the relationship between genetic variability in NOD2 and susceptibility to IA, four nonsynonymous SNPs in the NOD2 coding sequence were analysed (Table 1). The probability of IA was assessed according to recipient or donor genotypes by estimating the cumulative incidence of infection among transplant recipients at 24 months after HSCT. Among the SNPs tested, the donor, but not recipient, P268S (rs2066842) SNP in NOD2 was associated with an increased risk of IA (Fig. 1a). Other polymorphisms did not allow accurate risk estimations due to low (<0.05) allele frequencies in our study population. The cumulative incidence of IA for donor P268S was 32.7% for the CC genotype, 21.6% for CT and 20.0% for TT genotypes, respectively (Fig. 1a). The key contribution of the CC genotype to the risk of infection was further illustrated upon modelling a dominant mode of inheritance (cumulative incidence of IA, 32.7% for CC vs. 21.3% for CT and TT genotypes combined) (Fig. 1b). In a multivariate model accounting for age, gender, post-transplant neutropenia and acute graft-versus-host disease (GVHD)\(^26\), the donor CC genotype at P268S conferred a 2.1-fold increased risk of developing IA after transplantation (95% CI, 1.15–4.47; \(p = 0.021; p\)-values calculated using Gray’s test). Collectively, these results highlight genetic variation at the NOD2 locus as a critical risk factor regulating susceptibility to IA after HSCT.

**The NOD2 P268S SNP alters pulmonary cytokine levels in IA.** To assess whether the genotypes at P268S in NOD2 differentially regulate pulmonary inflammation in aspergillosis, cytokine levels in bronchoalveolar lavage (BAL) samples from patients with IA were assessed. Genotype-specific differences were observed, with patients transplanted with bone marrow carrying the TT genotype displaying lower median concentrations of IL-10 and IL-8 than

### Table 1 Description of the SNPs in the NOD2 gene evaluated in our study

| RefSNP    | Genome coordinates | aa change | Alleles  | CEU MAF   | MAF in our study | HWE       |
|-----------|---------------------|-----------|----------|-----------|-----------------|-----------|
| rs2066842 | chr16:50710713      | P268S     | C > T    | 0.102     | 0.278           | 0.72      |
| rs2066844 | chr16:50712015      | R702W     | C > T    | 0.014     | 0.027           | 0.77      |
| rs2066845 | chr16:50722629      | G908R     | G > C    | 0.005     | 0.002           | 1.00      |
| rs2066847 | chr16:50729867      | 1007fs    | – > C    | 0.006     | 0.022           | 0.98      |

Publically available sequencing data from Pilot 1 of the 1000 Genomes Project (www.1000genomes.org) was used to determine MAF. Genome coordinates were extracted from the hg18 build. SNP single-nucleotide polymorphism, aa amino acid, P proline, S serine, R arginine, W tryptophan, G Glycine, fs frameshift, CEU Utah Residents (CEPH) with Northern and Western Ancestry, MAF minor allele frequency, HWE Hardy Weinberg Equilibrium.
CC + CT carriers (0.41 vs. 2.6 pg/mL; and 1125 vs. 2560 pg/mL). In addition, a trend toward decreased IL-6 and TNF levels was also observed among patients transplanted with bonemarrow from TT carriers (Fig. 1c). Collectively, these findings point to a NOD2 genotype-determined alteration in cytokine production in response to Aspergillus infection.

**NOD2 variants alter Aspergillus-induced cytokine responses.**

To examine the impact of NOD2 variation on host defence against Aspergillus, the impact of the NOD2 genetic variants on Aspergillus immune recognition and cytokine production was investigated. In vitro Aspergillus-induced cytokine responses of primary human PBMCs were stratified based on P268S (rs2066842), G908R (rs2066845), and R702W (rs2066844) genotypes, to investigate their influence on cytokines responses to A. fumigatus.

Individuals carrying the T-allele at P268S, which was associated with a reduced susceptibility to aspergillosis in patients (Fig. 1), induced significantly lower IL-1β and demonstrated a trend toward lower TNF production in response to Aspergillus stimulation (Fig. 2a). Additionally, the TT-genotype was associated with significantly lower IL-17A responses compared with individuals carrying the CC or CT genotypes (Fig. 2b). Heterozygous carriers of the G908R and R702W polymorphisms did not show significantly altered cytokine responses to Aspergillus (Supplementary Fig. 1), and homozygous carriers of these polymorphisms were not represented within our cohort.

Insertion of a cysteine at position 1007 (1007insC) (rs2066847) induces a frameshift, which results in a defective NOD2 receptor, and homozygous carriage of this mutation results in complete NOD2 deficiency and is highly associated with Crohn’s disease27. Healthy individuals heterozygous for this mutation demonstrated significantly lower IL-1β and a trend toward lower TNF, but not IL-6 responses to Aspergillus (Fig. 2c). The decreased IL-1β correlated with significantly lower IL-17A responses in individuals carrying the Cysteine-insertion on one allele (Fig. 2d). Interestingly, Aspergillus-induced IFNγ or IL-22 production was not affected by this genotype (Fig. 2d).

**The 1007insC frameshift mutation enhances fungal killing.** For the P268S and 1007insC polymorphisms the impact on fungal killing capacity was evaluated. Although, the P268S polymorphism did not significantly impact Aspergillus killing (Fig. 2e), PBMCs of healthy individuals carrying the Cysteine-insertion (rs2066847) on one allele had a significantly increased capacity to neutralize Aspergillus-conidia (Fig. 2f). Production of reactive oxygen species (ROS) is highly important for host defence against aspergillosis, especially when considering that CGD patients that cannot produce ROS are highly susceptible to aspergillosis28. However, oxidative burst in response to Aspergillus was not influenced by the different NOD2 genotypes (Fig. 2g, h).
Human NOD2 deficiency reduces Aspergillus-induced cytokines. To further investigate the importance of NOD2 in Aspergillus-induced cytokine response, we analysed responses of primary human PBMCs within a background of complete NOD2 deficiency. PBMCs from patients with Crohn’s disease, homozygous for the 1007insC polymorphism and thus deficient for the NOD2 receptor, were stimulated with A. fumigatus. NOD2-deficient PBMCs demonstrated significantly lower IL-1β and TNF responses compared to controls (Fig. 3a). NOD2 deficient PBMCs also showed a significant reduction in production of the T-helper cytokines IL-22 and Interferon(IFN)γ and a trend toward decreased IL-17A induced by conidia (Fig. 3b). These reduced cytokine responses correlated with a reduced capacity to expand populations of IL-17A+, IL-22+, and IFNγ−/−CD4 T-cells (Fig. 3c). Similar to individuals with heterozygous 1007insC mutations, the homozygous individuals demonstrated a trend toward improved fungal killing (Fig. 3d). However, no change in the capacity to induce ROS by zymosan or Aspergillus was observed (Fig. 3e).

*Nod2*−/− mice are less susceptible to IA. Since NOD2 genetic variation was associated with a reduced risk of IA, the impact of full Nod2 deficiency on susceptibility to aspergillosis was validated in an experimental model of IA. Wild-type (WT) C57BL/6 and Nod2-deficient (Nod2−/−) C57BL/6 mice were immunosuppressed using cyclophosphamide and subsequently subjected to lethal *Aspergillus* infection89. Nod2−/− mice demonstrated a significantly improved 14-day survival, compared to WT mice (Fig. 4a). During infection, WT mice decline in bodyweight and seven out of eleven mice did not survive the infection whereas eight out of nine Nod2−/− mice survived the infection, despite having similar weight loss as WT mice during the first 3 days of infection (Fig. 4b). Although Nod2−/− mice demonstrated severe symptoms such as hunching, head tilting, and circling, symptoms that have been described in the in vivo aspergillosis mouse model89, they survived the infection, in contrast to WT mice. Bioluminescence imaging revealed that Nod2−/− mice rapidly cleared the luciferase-expressing *Aspergillus*, whereas WT mice developed a fungal infection as indicated by a significantly higher luminescence signal on day 3 post infection (pi) (Fig. 4c, d). After day 3 pi the luminescence could not be reliably compared between groups due to mice dropping out of the experiment (Supplementary Fig. 2), and severe hypoxia in critically ill mice that influences bioluminescence readout. For assessment of histopathological damage in the lungs, inflammation and fungal burden, mice were sacrificed on day 3 pi. The decreased bioluminescence signal in the lungs of Nod2−/− mice correlated with the fact that almost no *Aspergillus* DNA could be detected in the lung homogenates of Nod2−/− mice (3 out of 8 mice were PCR positive with low values). However, in the lung homogenates of WT mice, *Aspergillus* PCR was positive for 5 out of 8 mice (Fig. 4e).

*Nod2*−/− mice show reduced pulmonary histopathological damage. Using histopathological analysis differences inflammatory and pathological damage to the lungs and sinuses of WT and Nod2−/− mice were assessed. Within the lungs, WT mice displayed multifocal large areas of ischaemic necrosis (Fig. 5a I, circles and arrowheads), with fibrinous thrombi and destruction of blood vessels (Fig. 5a II, arrow). In contrast, Nod2−/− mice

![Fig. 2 Human NOD2 polymorphisms influence Aspergillus-induced cytokine responses and fungal killing.

a–d IL-1β, TNF, IL-6, IL-17A, IL-22, and IFNγ levels measured in culture supernatants of PBMCs stimulated with (a, c) live *Aspergillus* conidia for 24 h or (b, d) heat-inactivated (HI) *Aspergillus* conidia for 7 days. The PBMCs of individuals with various genotypes of the NOD2 gene were compared. These genotypes included (a, b) the P2685 mutation (rs2066842; reference: CC n = 36, heterozygous: CT n = 28 and homozygous: TT n = 4) and (c, d) the 1007insC mutation (rs2066847; reference n = 62 and heterozygous: insC n = 4). e, f Fungal killing capacity of human PBMCs assessed as CFU remaining of *A. fumigatus* (2 × 10⁵) following exposure for 24 h to (5 × 10⁵) PBMCs results are stratified based on the e P2685 (rs2066842; ref: CC n = 49, heterozygous: n = 45 and homozygous: TT n = 7) and (f) 1007insC (rs2066847; ref n = 98 insC n = 7) genotypes. g, h Area under the curve (AUC) of relative light units (RLU) induced by luminol oxidation by reactive oxygen species (ROS) released by PBMCs, results are stratified based on the (g) P2685 (rs2066842; reference: CC n = 47, heterozygous: n = 50 and homozygous: TT n = 9) and (h) 1007insC (rs2066847; ref n = 112 insC n = 5) genotypes. Data are a represented scatter dot plot with the median. Each dot represents an individual patient, with (a, b, e, g) black filled dots representing carriers of the ancestral (reference) CC genotype, half-filled black/gray dots representing carriers of the heterozygous CT genotype, and gray dots representing carriers of the homozygous TT genotype, and (c, d, f, h) black filled dots representing carriers of the reference (ref) genotype without insertion and half-filled black/gray dots representing carriers of one Cysteine insertion (insC). The means were compared using the Mann–Whitney U test, p-values of statistical tests are shown within the graphs.
rarely displayed inflammatory infiltrates and when present small (Fig. 5a I, circle and arrowhead; II, arrow). Severe fungal invasion was observed in WT mice (Fig. 5a III) with a high density of hyphae that invaded blood vessels (Fig. 5a III arrow). In contrast, fungi were rarely observed in lungs of Nod2−/− mice, only conidia (Fig. 5a III arrow), without invasion of the parenchyma or blood vessels. Macrophages were observed in the lesions, either randomly distributed (Fig. 5a IV, wild-type mice) or gathered in the small infiltrates (Fig. 5a IV, Nod2−/− mice). Although the immune suppression drastically decreased the number of F4/80+ cells no differences were observed between the groups (Fig. 5a IV).

Using morphometric analysis, the average number of lesions per section and the affected area was quantified. WT mice had a trend towards a higher average number of lesions per section (Fig. 5b). Moreover, the affected area of the lesions was significantly larger in WT mice (Fig. 5b).

Additional histology slides confirmed our morphometric analysis, as WT mice displayed marked lung lesions characterized by large foci of ischaemic necrosis (Fig. 5c I: left of the black line) with destruction of the bronchi/bronchiolar epithelium (Fig. 5c I, black arrowheads), fungal invasion of lung parenchyma (Fig. 5c II top row), destruction of alveoli (Fig. 5c III top row) and invasion of blood vessels (Fig. 5c IV top row). Similar lesions were observed in other WT mice (Fig. 5c second row) with invasion of blood vessels (Fig. 5c third row) and hyphae crossing the bronchiolar epithelium lining (Fig. 5c IV second row, black arrowhead). In contrast, Nod2−/− mice displayed no or minimal lesions (Fig. 5c third and fourth row). At a low magnification, no lesions could be observed (Fig. 5c I, II fourth row), whereas at a high magnification, few hyphae could be detected in the alveoli/alveolar walls (Fig. 5c III, IV fourth row, black arrowheads).

WT mice displayed invasive sinusitis, whereas nasal sinus lesions were absent in Nod2−/− mice (Fig. 6). Arrowheads indicate destruction of nasal mucosa (Fig. 6a, enlarged in Fig. 6b), and invasion of fungi (Fig. 6c). Nasal sinuses of Nod2−/− mice did not demonstrate any signs of destruction (Fig. 6d, e) or presence of fungi (Fig. 6f).

**NOD2 augments Aspergillus-induced cytokine responses.** Since NOD2 genetic variation and its complete deficiency correlated with a decreased cytokine release, the capacity of NOD2 signalling to boost Aspergillus-induced cytokine responses was investigated. Co-stimulation of NOD2 by MDP augmented Aspergillus-induced IL-1β and TNF responses (Fig. 7a). This could, however, not be achieved in cells of Crohn’s disease patients carrying the 1007insC mutation (Fig. 7b). Similarly, cytokine responses to A. fumigatus by cells of Nod2−/− mice were investigated. Although BMDMs of Nod2−/− mice did not demonstrate altered IL-6, KC and TNF responses (Fig. 7c), splenocytes of Nod2−/− mice showed a reduced capacity to mount IL-6, KC, and TNF responses (Fig. 7d).

**NOD2 inhibits phagocytosis and killing of A. fumigatus.** The reduced susceptibility of Nod2−/− mice and patients with NOD2 genetic variants may be explained by enhanced killing capacity of myeloid cells due to their NOD2 deficiency, as monocytes from NOD2-deficient individuals demonstrated a trend toward Aspergillus killing (Fig. 3d). BMDMs from WT and Nod2−/− mice were compared for their fungal killing capacity, and Nod2−/− BMDMs proved to be more efficient at eradicating live Aspergillus conidia (Fig. 8a). Subsequently, NOD2 gene expression was silenced in human monocyte-derived macrophages (MDMs) to validate that the absence of NOD2 also positively influences fungal killing in human cells. Treatment of MDMs with NOD2 targeting siRNA augmented fungal killing capacity (Fig. 8b).
Curves were compared by repeated measurements two-way ANOVA.

The signal at day 1 to 3 post infection from the luminescent immunosuppressed WT (Nod2−/−) was silenced showed a trend towards an increased phagocytosis in monocytes and macrophages. Phagocytosis and ROS production are well-described fungal killing capacity of Aspergillus in monocytes and macrophages. Phagocytosis and ROS production are well-described fungal killing capacity of Aspergillus spores (Fig. 8c).

Several antifungal mechanisms could account for the observed increased killing capacity of Aspergillus in monocytes and macrophages. Phagocytosis and ROS production are well-established factors that influence the fungal killing capacity. Therefore, these two possible mechanisms were systematically addressed to explain increased killing. Nod2−/− BMDMs demonstrated an enhanced capacity to engulf FITC-labelled A. fumigatus conidia, illustrated by a higher percentage FITC-positive macrophages and an overall higher mean fluorescence intensity (MFI) of the macrophages (Fig. 8d), indicating that more conidia were engulfed and more cells were actively engulfing conidia. Similarly, human MDMs in which NOD2 was silenced showed a trend towards an increased phagocytosis (Fig. 8e). Conversely, MDP-stimulated MDMs demonstrate a reduced phagocytosis of FITC-labelled conidia (Fig. 8f).

Although no influence of human NOD2 deficiency on ROS production was found (Fig. 8g), we wanted to validate that ROS production was indeed not influenced by NOD2 deficiency and NOD2 stimulation. BMDMs of WT and Nod2−/− mice stimulated with zymosan demonstrated a similar capacity to produce ROS (Fig. 8h). NOD2 stimulation of human MDMs also did not influence ROS production in response to zymosan stimulation (Fig. 8i). These data suggest that the observed increased killing in the setting of NOD2 deficiency is due to enhanced phagocytosis and not via increased ROS production in contrast to NOD1 deficiency.

**NOD2 negatively regulates dectin-1 expression.** One of the most crucial receptors for A. fumigatus recognition and engulfment is dectin-1. Therefore, we investigated whether NOD2 influenced the expression of dectin-1. Nod2−/− BMDMs showed an increased expression of Clec7a, the gene encoding dectin-1 (Fig. 8i). Similarly, silencing NOD2 in human MDMs slightly
enhanced CLEC7A mRNA expression (Fig. 8j). Conversely, MDP stimulation reduced surface dectin-1 expression on human MDMs (Fig. 8k).

**MDP inhibits antifungal immunity in WT cells.** To verify that MDP did not have off-target effects negatively influencing fungal killing, phagocytosis, and dectin-1 expression, monocytes of healthy volunteers that were wild type for the investigated NOD2 SNPs were compared with three NOD2-deficient patients. Similarly, fungal killing was assessed in murine BMDMs. MDP significantly reduced in murine BMDMs (Fig. 9a) and in human monocytes fungal killing (Fig. 9b), phagocytosis (Fig. 9c), and dectin-1 expression (Fig. 9d), whereas in the cells of Nod2−/− mice or NOD2-deficient patients no effect of MDP could be detected.

![Fig. 5 Nod2−/− mice show reduced histological damage and fungal burden following Aspergillus infection. a] histology of lung sections of wild-type and Nod2−/− mice at day 3 pi, stained in HE (I, II), Grocott’s Methenamine Silver (III) or labelled using anti-F4/80 antibody (specific for macrophages), counterstained with Haematoxylin staining. Scale bars represent 1 mm (I) and 200 μm (II-IV). b Morphometric analysis of the lesions in the whole lung sections using Image J software to quantify the lesions in number and size. c Representative lung sections of two additional WT and Nod2−/− mice, stained in HE (I) and Grocott’s Methenamine Silver (II-IV). Scale bars represent 200 μm (I, II) and 50 μm (III, IV), means were compared for significance using the Mann-Whitney U test.
Discussion

PRRs are key players in activating the antifungal host response during invasive aspergillosis (IA) by inducing cytokine responses and facilitating phagocytosis with subsequent fungal killing. PRRs on the cell surface, such as Toll-like receptors and C-type lectin receptors, have been extensively described in inducing these responses in host defence against *Aspergillus*31. Genetic variation in PRRs is common in the general population, however, in hematopoietic stem cell transplant patients (HSCT), such variations can drastically impact susceptibility to IA6. The only intracellular PRRs explored to date, NLRP3 and NOD1 belonging to the NLRs, provide evidence that this class of receptors can modulate host responses against *A. fumigatus*7,17. However, one of the most well known NLRs that is directly linked with...
immunodysregulation that leads to disease, namely NOD2, remains largely unexplored in the context of anti-Aspergillus host defence. Here, we systematically addressed the role of NOD2 in susceptibility to Aspergillus infection.

We report an association between NOD2 genetic variation, Nod2 deficiency and decreased susceptibility to IA. Specifically, the TT-genotype at P268S confers resistance to IA after HSCT, a finding highlighting a potential NOD2-dependent detrimental effect on antifungal immunity. A potential limitation of our study is the lack of association for other NOD2 polymorphisms eventually with more noticeable loss-of-function phenotypes. This may, however, be explained by the low allele frequency of such variants, which do not allow accurate risk estimations. A previous study also investigated NOD2 polymorphisms in association with aspergillosis in HSCT patients. Although in this study a lack of association due to the low frequency of the variants was observed
for 1007insC and G908R polymorphisms, a strong trend towards a reduced presence of the mutated R702W allele was observed in IA patients.

Functionally, we demonstrate that in particular the 1007insC polymorphism impacts the response of primary immune cells to Aspergillus, namely in cytokine signalling and fungal killing, whereas we only observed an effect of the P268S polymorphism on cytokine responses.

Immunosuppression and cytostatic drugs needed for the treatment of cancer and autoimmune disorders makes patients highly susceptible to invasive fungal infections such as IA. Cyclophosphamide is a drug used to treat hematological malignancies or to suppress the immune system to preventgraft rejection and renders mice highly susceptible to develop infections with Aspergillus fumigatus. This immunosuppression allows a low dose of intranasally administered conidia to cause an invasive infection that is lethal within days, which in immunocompetent mice would have been efficiently cleared. Nod2-deficient mice were resistant against aspergillosis despite being immunosuppressed and showing severe symptoms of aspergillosis such as weight loss, hunching, head tilting, and circling. The protection observed in Nod2−/− mice was associated with reduced fungal burden and reduced histopathological damage to the lungs. A deficiency in PRRs being protective against lethal aspergillosis is a striking observation. Especially since it is challenging to protect immunosuppressed mice even with available potent antifungal therapies, which often requires combinational therapies to achieve survival of these mice.

The fact that we observe protection of Nod2 deficiency in an immunocompromised mouse model raises the question which cells are responsible for the protection. We observed resident macrophages, that remain in the lung even after immunosuppressive therapy, which could potentially mediate fungal killing. In the HSCT patients, the NOD2 P268S polymorphism was only associated with a reduced incidence of aspergillosis in the donor genotype. The donor genotype will represent the genotype of the patient’s myeloid cells following transplantation suggesting that the protective effect of NOD2 genetic variation lies within the myeloid compartment. Furthermore, we observe that NOD2 negatively affects the antifungal capacity of various types of myeloid cells, including murine BMDMs, human MDMs and human monocytes.

Interestingly, Staphylococcus aureus pneumonia in Nod2-deficient mice was less severe than in wild-type animals due to reduced pulmonary inflammation. We observed that some cytokines have lower levels in the BAL of aspergillosis patients having the TT-genotype at P268S (rs2066842). This might indicate a less severe infection that may be related to enhanced fungal killing. Nevertheless, we observed that NOD2 polymorphisms, as well as the complete deficiency of the receptor, were associated with decreased Aspergillus-induced pro-inflammatory cytokine responses. NOD2 stimulation augments Aspergillus-induced cytokine responses. It has been widely described that excessive inflammation, and in particular IL-17-mediated inflammation, can result in detrimental immunopathology during Aspergillus infections in mice, but this is primarily observed in situations where the immune system is largely functional such as cystic fibrosis, allergic bronchopulmonary aspergillosis, corticosteroid, and fully immunocompetent models. Although reduced cytokine-driven inflammation can contribute to less damage in certain aspergillosis models, a lower capacity to mount early cytokine response is also known to be a primary risk factor for susceptibility. Based on our data, we can only conclude that NOD2 has a potential to modify Aspergillus-induced cytokines in vitro, but it needs to be elucidated whether this in any way contributes to the observed protection in HSCT patients with NOD2 variants and Nod2−/− mice.

What could then be the mechanism of protection in the setting of genetic NOD2 deficiency? Carriage of the 1007insC polymorphism correlates with an increased fungal killing capacity. In addition, Nod2-deficient mice demonstrated improved fungal clearance compared to WT mice, which was associated with an absence of histological damage and fungal outgrowth within the lungs. In addition Nod2 deficiency in murine BMDMs or NOD2 silencing in human MDMs augments fungal killing, whereas NOD2 stimulation by MDP in human MDMs or monocytes suppresses fungal killing. Subsequently, we systematically addressed whether phagocytosis capacity and/or ROS production, which are well-established mechanisms needed for the killing of Aspergillus, would be altered in NOD2-deficient cells. We observed no effect on the ROS production. However, observed that NOD2 negatively regulates phagocytosis. Silencing of NOD2 gene expression slightly enhanced engulfment of A. fumigatus conidia, whereas NOD2 stimulation suppressed the phagocytic capacity of human MDMs and monocytes. Nod2−/− BMDMs were more efficient at engulfing A. fumigatus conidia than their WT counterparts. This is in line with a previous report showing that NOD2 polymorphisms improve phagocytosis of the...
gram-negative bacterium *Escherichia coli*31. However, in NOD2-deficient patients, we did not observe augmented phagocytosis compared to healthy donor cells. At first sight this might argue against a role for NOD2 in phagocytosis, however, it needs to be taken into account that the rate of phagocytosis is already variable between humans. This is most likely explained by a different genetic background and, in the case of our patients, maybe even immunosuppressive medication. Comparing WT and Nod2-deficient murine cells and silencing of the same human cells making them NOD2 deficient practically eliminates these donor factors that contribute to variability of phagocytosis. To prove that NOD2 influences phagocytosis in human cells we made use of the following knowledge. NOD2 is a receptor for derivatives of bacterial peptidoglycan, such as MDP, which is present in the peptidoglycan of both gram positive and negative bacteria4,10,11. When we studied killing and phagocytosis of *Aspergillus* in the presence or absence of MDP we observed that NOD2 stimulation indeed decreases phagocytosis and killing. By performing these experiments in cells isolated from NOD2-deficient patients we show that MDP in these cells did not influence phagocytosis and killing. These data strengthen the conclusion that NOD2 negatively influences phagocytosis and killing of *Aspergillus* and supports the concept that genetic NOD2 deficiency could confer protection against invasive aspergillosis by an increased capacity of NOD2-deficient cells to control fungal burden in the host.

One of the crucial PRRs for phagocytosis of *A. fumigatus* is the c-type lectin receptor dctin-142,43. On the one hand, when we studied the expression of dctin-1 in the setting of Nod2 deficiency or silencing we observed that when phagocytosis was increased this correlated with increased dctin-1 expression. On the other hand NOD2 stimulation with MDP decreased surface dctin-1 expression. The observed correlation between increased dctin-1 expression and increased phagocytosis and killing within the setting of NOD2 deficiency, may argue for a role for dctin-1, but does not exclude that other mechanisms are still playing a role in the observed protection.

Although it has previously been shown that other fungi such as *Candida albicans* are not recognized by NOD44,45, the fungal cell wall component chitin/chitosan that is present in both *Aspergillus* and *Candida* has been suggested to be a ligand for NLRs18,46. Chitosan activates NLRP3 and thereby activates the inflammasome and induces IL-1β production, whereas chitin did not activate NLRP346. Chitin induces IL-10 dependent on TLR9, mannose receptor and NOD218. These data suggest that NOD2 plays a role in the recognition of fungal molecules such as chitin. However, a different study demonstrated that chitin-induced IL-1Ra production in human PBMCs is independent of NOD247. In addition, chitin can synergize with the NOD2 ligand MDP to augment IL-1β and TNF response47, similar to our current observation that *Aspergillus* synergizes with MDP stimulation. Although this underlines that chitin and possibly other fungal cell wall molecules synergize with NOD2 signalling to augment cytokine responses, further studies are required to identify the PAMPs in *Aspergillus* that are recognized by NOD2. Moreover, NOD2 may not be directly involved in recognizing *Aspergillus*, but rather coordinate the responses that are induced by other (membrane-bound) PRRs, for example, the orchestration of phagosome composition. NOD2 synergizes with TLR signalling to yield more potent inflammatory responses18–52. Selective modulation of signals from PRRs that recognize *Aspergillus* is a possible mechanism by which the NOD receptors regulate the host response to *A. fumigatus*.

Collectively our data highlight a detrimental effect for NOD2 on antifungal host defence against *A. fumigatus*. This places NOD2 in a unique position in anti-Aspergillus host defence. It has the capacity to increase phagocytosis and killing in a ROS independent way. This could provide a rationale for treating patients that are immunosuppressed, either due to primary immunodeficiency such as chronic granulomatous disease that lack ROS or in patients that receive corticosteroids that suppress immune cells to produce ROS. Moreover, the effects of NOD2 deficiency are in sharp contrast with NOD1 deficiency. NOD1-deficient cells show increased cytokine production in response to *Aspergillus*. This might be beneficial, but could also be detrimental during the natural course of aspergillosis. Moreover, the oxidative burst is significantly higher under NOD1 deficient conditions and is decreased by NOD1 stimulation48, whereas in NOD2 we do not find an association with altered ROS production. NOD1 and NOD2 are closely related and interact with each other, therefore one would expect they behave similar in anti-Aspergillus host defence, but here we demonstrate clear different roles for NOD2 than the previous effects described for NOD117. A potential explanation for the different phenotypes observed with NOD1 and NOD2 deficiency is that *Aspergillus* PAMPs may have different affinities for the two different receptors or that different PAMPs bind and/or activate the receptors. Binding of the receptors by different PAMPs could lead to the fact that both receptors compete for the downstream adapter RICK, which was previously proposed to explain differential regulation of inflammation by NOD1 and NOD2 in arthritis53. An alternative explanation for the different phenotypes observed with NOD1 and NOD2 is that one, or both, of these receptors, can, in addition to RICK, induces an alternative-signalling cascade. Of note, it has previously been demonstrated that NOD2 can signal through the intracellular adaptor CARD954, which has a strong association with antifungal host response55,56. It is tempting to speculate that the detrimental effect of NOD2 may be due to sequestering CARD9 from other receptors requiring CARD9 as a signalling adaptor, such as dcutin-157, dcutin-158. Further studies using NOD1/NOD2, RICK, and NOD2/CARD9 knockout mice and co-precipitations would be required to investigate how the molecular pathways of NOD1 and NOD2 intertwine to mediate detrimental effects on the antifungal host response against *Aspergillus*.

NOD2 deficiency mediates protection against *Aspergillus* in mice, and polymorphisms in NOD2 alter the susceptibility of HSCT patients to develop aspergillosis. These effects are in the context where NOD2 seems to play a role in the induction of innate and adaptive cytokine responses against *Aspergillus* in humans. The absence of NOD2, however, strongly correlates with an enhancement of fungal killing and phagocytosis, which is independent of ROS. This makes NOD2 an attractive therapeutic target in the treatment of invasive aspergillosis.

**Methods**

**Study design.** A total of 310 consecutive haematological patients of European descent undergoing allogeneic HSCT at Instituto Português de Oncologia, Porto, and at the Hospital de Santa Maria, Lisbon, between 2010 and 2014, and respective matched healthy donors, were included in the genetic association study. The demographic and clinical characteristics of the patients were as previously described39 and are presented in Supplementary Table 2. Exclusion criteria were the development of fungal infection other than that caused by *Aspergillus spp.*, and pre-transplant fungal infection. It should be noted that this cohort was previously successfully used for identification of genes conferring increased susceptibility to aspergillosis43. The sample size was estimated to provide a power of 80% (1 − β = 0.80) with a type I error below 5% (α = 0.05) for genetic variants with minor allele frequencies between 10 and 20% conferring a relative risk of 2.0.

For the functional genomics study, similarly, the cohort was previously successfully used for identification of polymorphisms that lead to reduced cytokine responses39. The functional genomics cohort consisted of 200 healthy volunteers, of which approximately 80 (variable per genotype) were included in the current study. Individuals of which the genotype could not reliably be determined using SNP assays, and individuals that were not assessed for cytokine production were excluded from the analysis. The sample size of 80 healthy individuals was estimated to provide a power of 70% (1 − β = 0.70) with a type I error below 5% (α = 0.05) for genetic variants with a minor allele frequencies of 20% conferring an odds ratio
of 2.5. No patients were excluded in these studies. All cytokine and killing assays were performed by a researcher blinded for the genotype.

Ethics statement. For the genetic association study, approval was obtained from the Ethics Subcommittee for Life and Health Sciences of the University of Minho, Portugal (125/014 and 014/015), the Ethics Committee for Health of the Instituto Português de Oncologia—Porto, Portugal (26/015), the Ethics Committee of the Lisbon Academic Medical Center, Portugal (632/014), and the National Commission for the Protection of Data, Portugal (1950/015).

For the functional genomic study and pathology studies, drawing of blood samples from patients and healthy volunteers was approved by the local ethical board at the Radboud University Nijmegen (Arnhem-Nijmegen Medical Ethical Committee). For assessment of BAL cytokine levels approval was obtained from the Ethics Subcommittee for Life and Health Sciences of the University of Minho, Portugal (126/014), and the Ethics Committee of the University Hospitals of Leuven, Belgium.

All patients and healthy volunteers provided written informed consent. Mice were cared for in accordance with Institut Pasteur guidelines, in compliance with European animal welfare regulation. This study was approved by the ethical committee for animal experimentation CETEA (Comité d’éthique en experimentation animale, Project license number 2013-0020). Animal studies were conducted under protocols approved by St. Jude Children’s Research Hospital Committee on Use and Care of Animals (protocol no 482-100265-1-13).

Healthy controls and NOD2 deficient patients. Venous blood samples from healthy controls and patients were obtained and were analysed for polymorphisms in NOD2 gene (P268S rs2066842, 909IR rs2066845, R702W rs2066844 and 1077insC, rs2066847). DNA was isolated from whole blood by using the isolation Gentra Pure Gene Blood kit (Qiagen), according to the manufacturer’s protocol. Gene fragments were amplified and genotyped using commercially available TaqMan SNP Genotyping Assays (Applied Biosystems) according to the manufacturer’s protocol on the StepOnePlus system (Applied Biosystems). Quality control was performed by the incorporation of positive and negative controls and duplication of random samples across different plates.

Nine patients with Crohn’s disease that were homozygous for the 1007insC polymorphism were included for studying NOD2 deficiency. Most patients received anti-inflammatory therapy for treatment of their Crohn’s disease; Patient 1 Mesalazin 1 dd 1000 mg. Patient 2 No immunomodulation, Patient 3 Adalimumab 1 × 2 weeks 40 mg sub cutaneous, Patient 4 mesalazine 3dd 1 g and azathioprine 1dd 200 mg, Patient 5 in NDMP – 9 unknown.

Genotyping. DNA was isolated using the Gentra Pure Gene Blood kit (Qiagen), in accordance with the manufacturer’s protocol. Genotyping was performed using KASPar assays (LGC Genomics, Hertfordshire, UK) in an Applied Biosystems 7500 Fast Real-Time PCR system for the patient cohort. Mean call rate was >97% for all genotyped SNPs. Quality control for the genotyping results was achieved with negative controls, common and rare homoygous controls (whenever available), and retesting of samples with indeterminate results. Details of the MAF of the negative controls, common and rare homozygous controls (whenever available), and linkage disequilibrium was provided in Table 1 and linkage disequilibrium for all genotyped SNPs is shown in Supplementary Table 1.

Aspergillus fumigatus strains. A clinical isolate of Aspergillus fumigatus V05–27 was used for all ex vivo and in vitro stimulations. Aspergillus was grown for 7 days on Sabouraud dextrose agar slants stored in T150 cell culture flasks (Corning). Abundant conidia were produced under these conditions. To harvest conidia phosphate-buffered saline (PBS) with 0.05% Tween 80 was used to suspend the conidia and the surface was gently scraped using a cell scraper. To remove hyphae and debris, the conidial suspension was poured on the conidial suspension was poured on Sabouraud plates. CFUs were counted after 24 h at 37 °C.

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Quantitative reverse transcriptase PCR for CLEC7A expression. RNA was isolated according to the protocol supplied with the TRIzol reagent. Isolated mRNA (1 µg) was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Biorad). Quantitative real-time PCR (qPCR) was performed using Power SYBR Green PCR master mix (Applied Biosystems) and following primers (all manufactured by Biologeo) for human samples hNOD2 Fwd 5’-CCCTGCGACCG TGTAAGGTCGTCG-3' and Rev 5’-AGATGGCAGACAGCATCAG-3'. Freshly isolated PBMCs were differentiated to macrophages using 6-day differentiation in 10% human serum supplemented with 5 ng/mL GM-CSF. NOD2 silencing was performed by transfecting 25 nM NOD2-targeting siRNA (on target) or scrambled (non-target) control siRNA (smart pool, Thermo Scientific) for 24 h at 37 °C (Dharmafect, Thermo Scientific). Subsequently, the culture medium was refreshed and cells were used for killing and phagocytosis assays and PCR analysis.

In vivo experiments. Mice for in vivo experiments were supplied by the breeding center R. Janvier (Le Genest Saint-Ise, France). All mice were housed under specific pathogen-free conditions in IVC cages, and fed standard chow and water ad libitum. For the survival experiment in an immunosuppressed background C57BL/6 wild-type (male/female), and C57BL/6 Nod2−/− mice (28 to 31 g, 10 weeks old) were used. An estimated power of 80% (1-β = 0.80) with a type I error below 5% (p = 0.05) for a variance of 5% were determined. Healthy individuals whose genotype could not accurately be determined were excluded from the studies. Unless otherwise indicated the univariate analysis were entered one by one in a pairwise model together and for competing events. The clinical and genetic variables achieving a significance level p = 0.05 were entered into the final model if they remained significant (p ≤ 0.05). Multivariable analysis was performed using the sub-distribution regression model of Fine and Gray with the cmpsMex package for R version 2.10.167, with censoring of data at the date of last follow-up visit and relapse and death as competing events. The clinical and genetic variables achieving a 0.15 in the univariate analysis were entered one by one in a pairwise model together and kept in the final model if they remained significant (p ≤ 0.05). The Mann-Whitney U test was used to determine statistical significant differences between experimental groups and for paired analysis such as with MDP stimulation or siRNA treatment the paired Wilcoxon signed-rank test was used with *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. All data were analysed using GraphPad Prism v6.0.

Data availability. The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information Files. All relevant data are available by request from the authors, with the restriction of data that would compromise patient confidentiality.

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Author contributions

M.S.G., A.C., O.I.-G., and F.L.v.d.V. conceived and designed the study. M.S.G., C.C., M.J., R.K.S.M., S.M.G., A.A., R.L., M.O., O.R., G.J., C.F., W.M., and O.I.-G. performed experiments. M.S.G., C.C., M.J., R.K.S.M., A.A., G.J., W.M., A.C., O.I.-G., and F.L.v.d.V. analysed the data. D.J.d.J., I.F.L., A.C.J., K.L., and J.M. included patients. T.-D.K. provided valuable reagents and cell lines. M.S.G., M.J., and O.I.-G. wrote the first draft of the manuscript. M.S.G., W.M., T.-D.K., A.C., O.I.-G., and F.L.v.d.V. revised the manuscript.

Additional information

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