

# Dectin-1 Is Expressed in Human Lung and Mediates the Proinflammatory Immune Response to Nontypeable *Haemophilus influenzae*

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**ABSTRACT** The C-type lectin receptor Dectin-1 is expressed mainly on myeloid cells mediating the immune response targeting respiratory pathogens such as *Aspergillus fumigatus* and *Mycobacterium tuberculosis*. The pulmonary epithelium serves as an important interface for interactions between these pathogens and the respiratory tract. Therefore, we analyzed the expression pattern of Dectin-1 in the human lung. Immunohistochemically stained human lung sections from 17 out of 19 individuals were positive for Dectin-1, which was expressed mainly apically on bronchial and alveolar epithelium. Our results showed no correlation with chronic obstructive pulmonary disease (COPD) or the smoking habits of the patients. Nontypeable *Haemophilus influenzae* (NTHI), an important bacterial pathogen of the respiratory tract with significant importance in COPD, has also been proposed to be recognized by Dectin-1, suggesting a possible impact on the NTHI-dependent immune response in human airways. Therefore, the involvement of Dectin-1 in NTHI-triggered cytokine responses was investigated in primary normal human bronchial epithelial (NHBE) cells and in the A549 cell line stably transfected with Dectin-1. The presence of Dectin-1 significantly increased cytokine release in response to NTHI in NHBE and A549 cells. In addition, phosphorylation of the Dectin-1 hem-immunoreceptor tyrosine-based activation motif (hemITAM) was essential for the Dectin-1-triggered response to NTHI in A549 cells. In conclusion, in human airways, epithelium-expressed Dectin-1 may play a significant role in generating an NTHI-mediated, proinflammatory immune response.

**IMPORTANCE** In this study, we demonstrated, for the first time, the expression of Dectin-1 on human lung tissues and, in particular, pulmonary epithelium by making use of immunohistochemical staining. The epithelial lining of the human airways is an important interface for host-pathogen interactions. Therefore, our data suggest that epithelium-expressed Dectin-1 is of considerable importance for the interaction of the human airways with pathogens detected by this receptor, such as *A. fumigatus* and *M. tuberculosis*. Moreover, we further demonstrated that, in pulmonary epithelial cells, Dectin-1 enhances the proinflammatory immune response to NTHI. In COPD patients, NTHI is a major cause of respiratory tract infections and is associated with proinflammatory immune responses in the lower airways. Therefore, our data suggest that the functional interaction of Dectin-1 with NTHI in human airways may have an important impact on the pathogenesis of COPD.

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Dectin-1, a type II transmembrane receptor, is a member of the group V nonclassical C-type lectin (CTL) family (6, 25). It contains a single extracellular CTL-like domain followed by a cytoplasmic hem-immunoreceptor tyrosine-based activation motif (hemITAM; also called ITAM-like) signaling domain (1). The receptor is expressed on myeloid cells, but few reports have described its inducible expression in pulmonary epithelial cell lines (2, 3). Dectin-1 is a non-Toll-like receptor (TLR) pattern recognition receptor (PRR) of the innate immune system, detecting the  $\beta$ -glucan of fungal cell walls. Consequently, this PRR is of considerable importance in the host response to pathogenic fungi such as

*Candida albicans* and *Aspergillus fumigatus* (4). In addition, Dectin-1 plays a role in the immune response against *Mycobacterium tuberculosis* infections, for which the ligand responsible is currently unknown (5).

Stimulation of Dectin-1 leads to hem-ITAM-mediated signaling cascades in the cell, triggering, for example, phagocytosis, the production of reactive oxygen species (ROS), and the production of proinflammatory chemokines/cytokines (6). The binding of  $\beta$ -(1,3)-glucan to Dectin-1 activates Syk kinase, initiating signaling via the Card9/Bcl10/Malt1 pathway, to induce the activation of NF- $\kappa$ B, as a specific innate activation program distinct from the

TABLE 1 Patient characteristics

Characteristic	Value(s)
No. of males/females	9/10
Mean age ( $\pm$ SD)	63 ( $\pm$ 11)
No. of current smokers/ex-smokers/nonsmokers	5/11/3
Mean no. of pack-years ( $\pm$ SD) for current smokers/ex-smokers/nonsmokers	61 ( $\pm$ 22)/33 ( $\pm$ 22)/0
No. of patients with COPD/without COPD	8/11

response to the TLR ligands (1, 7, 8). In addition, noncanonical NF- $\kappa$ B activation can be controlled by Dectin-1-triggered signaling via Syk and Raf-1 (1, 9, 10). Also, collaborations of Dectin-1 with Toll-like receptors increase proinflammatory signaling and phagocytosis (11–13).

Interestingly, Ahrén et al. found that adherence and phagocytosis of the Gram-negative, noncapsulated bacterium nontypeable *Haemophilus influenzae* (NTHI) by monocytes, eosinophils, and the alveolar epithelial cell line A549 were inhibited by the Dectin-1 antagonist laminarin, indicating that NTHI might also be recognized by Dectin-1 (14, 15).

NTHI is an important pathogen in patients with chronic obstructive pulmonary disease (COPD), where it pathologically colonizes the lower respiratory tract (16). In addition, it is the most common cause of bacterium-induced exacerbations (17) and is associated with increased inflammation during stable COPD (18).

The epithelial lining of the human airways is an important interface for host-pathogen interactions. These cells are equipped for the recognition of pathogens, with several pattern recognition receptors (PRRs) orchestrating proinflammatory immune responses (19). Since NTHI, *A. fumigatus*, and *M. tuberculosis* are important pathogens in the human lung, we hypothesized that Dectin-1 is expressed in the airway epithelium and that it is of particular importance in pulmonary host defense. In addition, we

presumed that a functional interaction of epithelial Dectin-1 with NTHI might enhance proinflammatory responses in COPD.

In this study, we demonstrate that Dectin-1 is expressed in the lungs of 17 out of 19 human donors, in particular, on the bronchial and alveolar epithelium. We also found that Dectin-1 expressed on human pulmonary epithelial cells has a considerable impact on the NTHI-triggered immune response.

(Part of this work is included in the doctoral thesis of K. A. Heyl.)

## RESULTS

**Dectin-1 is constitutively expressed in the bronchial epithelium, alveolar epithelium, and pleura.** To investigate the expression pattern of Dectin-1 in human lungs, paraffin sections of human lung tissue from 19 donors (Table 1) were stained with the mouse anti-human Dectin-1 antibody (clone MAB1859). Images of the isotype control antibody are shown in Fig. S1 in the supplemental material. We found that the lung sections from 17 of the 19 donors stained positive for Dectin-1. An overview of the expression in lung tissue is shown in Fig. 1. In particular, the apical sides of the bronchial and the alveolar epithelium showed distinct and constant staining for Dectin-1 in 14 of 16 and 17 of 19 specimens, respectively (Table 2). The pleura stained positive for Dectin-1 in all specimens. In addition, pulmonary macrophages observed in the alveoli and in the submucosa stained positive for Dectin-1 in all specimens. To further evaluate the Dectin-1 expression levels, we assessed the intensity of Dectin-1 staining of specific structures of lung tissues (alveolar epithelium, bronchial epithelium, and pleura) using a scoring system with a range from 0 for no staining to 3 for strong staining. We found that the pleura exhibited more intense staining than alveolar epithelium and bronchial epithelium (Table 2). In general, alveolar epithelium showed marginally weaker staining than bronchial epithelium.

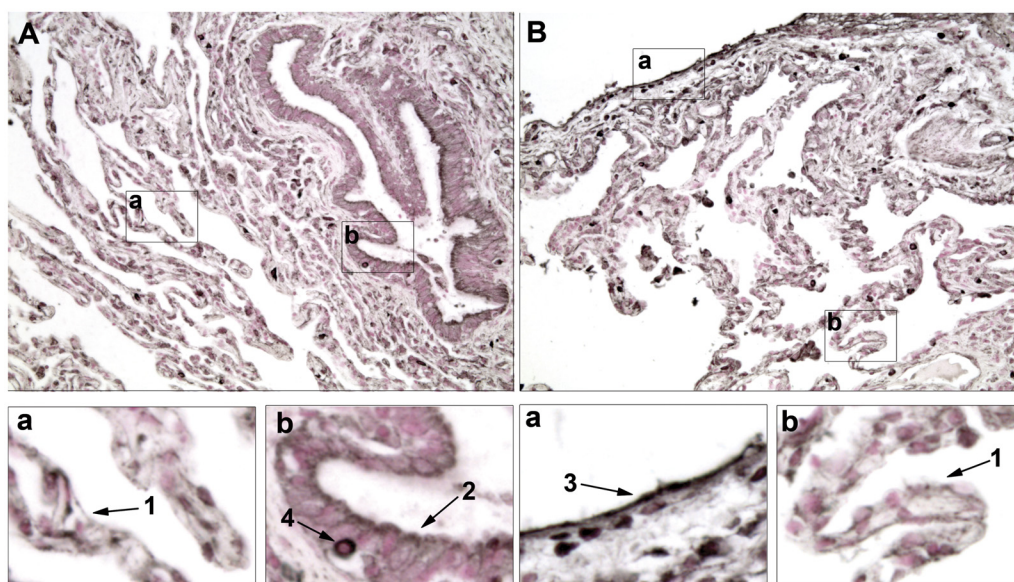


FIG 1 Dectin-1 is expressed in the human lung. (A, B) Representative paraffin sections of human lung tissues from surgical specimens from lung cancer patients were stained for Dectin-1 (clone MAB1859; R&D Systems) and photographed at an optical magnification of  $\times 200$ . Boxes labeled a or b show enlargements of the respective regions in these pictures. Black arrows point to alveolar epithelium (1), bronchial epithelium (2), pleura (3), and alveolar macrophages (4).

TABLE 2 Analysis of the immunohistochemistry of human lung sections ( $n = 19$ )<sup>a</sup>

Lung section	No. of Dectin-1-positive specimens/ total no. of specimens	No. of specimens (/total specimens) with a staining score of:			
		0	1	2	3
Alveolar epithelium	17/19	2/19	7/19	10/19	0/19
Bronchial epithelium	14/16	2/16	1/16	10/16	3/16
Pleura	19/19	0/19	2/19	6/19	11/19

<sup>a</sup> Since not all tissue types were found in all specimens, the number of positive specimens and the number of total specimens, including the respective tissue, are given. The intensity of Dectin-1 staining of the respective tissues was evaluated in the range from 0 (no staining) to 3 (strong staining) relative to the IgG control staining.

**Expression of Dectin-1 is not related to the presence of COPD or the smoking status of patients.** Comparing the intensities of the Dectin-1 staining in the lungs of donors with or without COPD, as well as with different smoking statuses, we found no significant differences (see Fig. S2A and B in the supplemental material). To examine the relationship between smoking and Dectin-1 expression in more detail, the intensity of Dectin-1 staining was compared with the quantity of cigarettes consumed (see Fig. S2C), measured as pack-years (number of packs smoked per day multiplied by number of years of being a smoker). But again no differences were found. The intensity of Dectin-1 staining was further compared to the level of C-reactive protein (CRP) (see Fig. S2D), as a marker of the extent of inflammation. In this case, on bronchial epithelium, a tendency was apparent for an increase in CRP but did not reach statistical significance.

**Dectin-1 expression in airway epithelial cells is upregulated by the TLR3 agonist poly(I·C).** To further study the regulation of Dectin-1 expression, we made use of primary small airway epithelial cells (SAEC) and primary normal human bronchial epithelial (NHBE) cells as an *in vitro* model. Flow cytometry revealed that both cell types stained positive for Dectin-1 (Fig. 2A). In accordance with our investigation of the lung sections, we could not detect any expression of Dectin-1 on the NHBE cells from some of the tested donors (data not shown). Next, we investigated the modulation of Dectin-1 expression in human bronchial epithelial cells in response to different agonists of Toll-like receptors and cytokines, which are important mediators for the innate immune response (20–22). NHBE cells were treated with interferon alpha (IFN- $\alpha$ ), IFN- $\beta$ , IFN- $\gamma$ , tumor necrosis factor alpha (TNF- $\alpha$ ), and the TLR agonists MALP2 (TLR2/6), poly(I·C) (TLR3), and flagellin (TLR5), as well as with NTHI and cigarette smoke extract (CSE) for 24 h. Subsequently, the RNA was isolated and mRNA for Dectin-1 was quantified with reverse transcription-quantitative PCR (RT-qPCR). As shown in Fig. 2B, the treatment with TNF- $\alpha$ , MALP2, flagellin, NTHI, and CSE had no effect on the Dectin-1 upregulation. In contrast, expression of Dectin-1 mRNA was significantly upregulated by the TLR3 agonist poly(I·C), IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$ . In order to confirm this upregulation on the protein level, the surface expression of Dectin-1 was measured via flow cytometry after treatment of the cells with either poly(I·C), IFN- $\alpha$ , IFN- $\beta$ , or IFN- $\gamma$ , for 24 h (Fig. 2C). Interestingly, only poly(I·C), and none of the interferons, increased the expression of Dectin-1 on the cell surface.

**Dectin-1 increases the proinflammatory response against nontypeable *Haemophilus influenzae* in human airway epithelial cells.** To explore the impact of Dectin-1 on the cellular activation of bronchial epithelial cells in response to NTHI, Dectin-1-expressing NHBE cells were stimulated with two different strains of NTHI (2019 and 86-028) or the Dectin-1 ligand zymosan

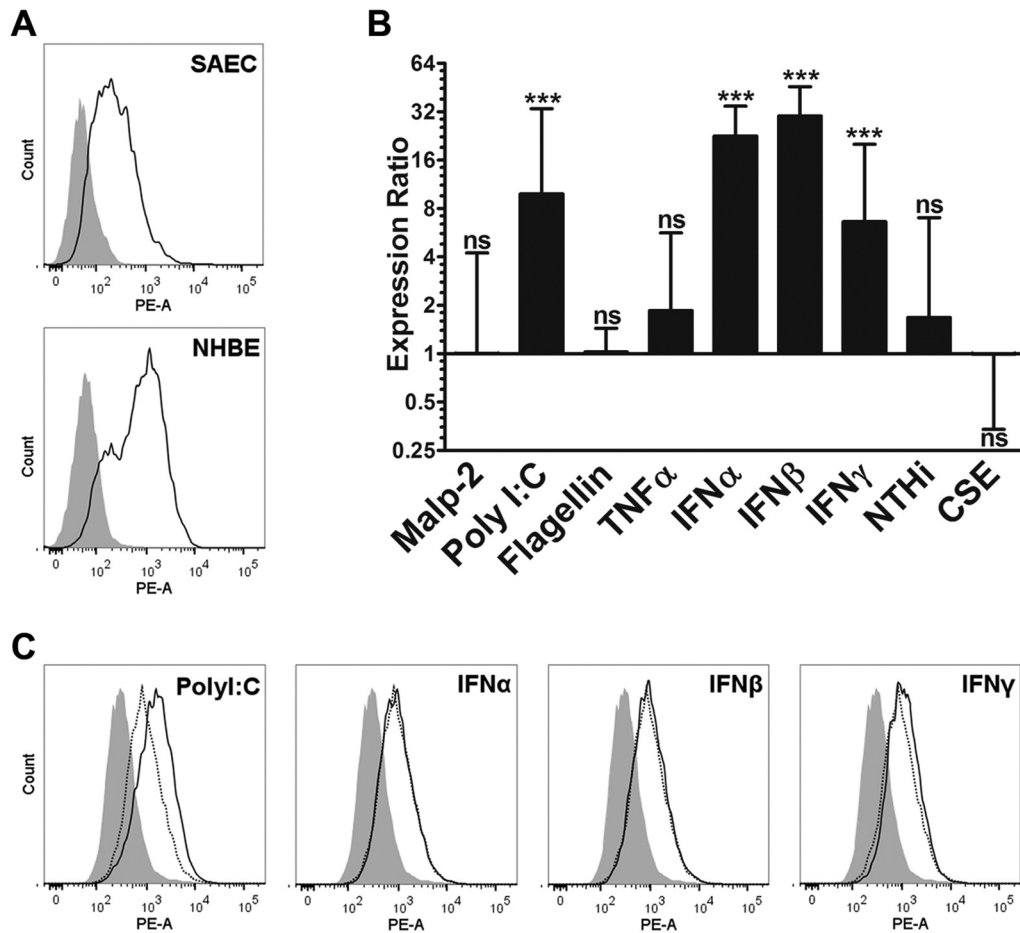
(Fig. 3). Stimulations were carried out in the presence or absence of anti-human Dectin-1 antibody, clone MAB1859, that is known to neutralize Dectin-1 (23, 24) or in the presence of the Dectin-1 antagonist laminarin. Subsequently, interleukin 8 (IL-8) and IL-6 were measured in the supernatants of the cells by enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 3, inhibition of Dectin-1 was able to significantly reduce the IL-8 and IL-6 secretion in response to NTHI or zymosan. In accordance with this finding, similarly treated NHBE cells lacking Dectin-1 expression, measured by flow cytometry, showed no reduction in the NTHI and zymosan-induced IL-8 response (see Fig. S3 in the supplemental material).

To confirm the regulatory function of Dectin-1 during the infection of the NHBE cell monolayer by NTHI, we performed “gain-of-function experiments” by transfecting a Dectin-1-expressing vector in Dectin-1-negative A549 cells. Flow cytometry showed that the human alveolar basal epithelial cell line A549 did not express Dectin-1 (data not shown). After stable transfection of the A549 cells with plasmids encoding Dectin-1 and subsequent sorting, we confirmed expression of the protein on the cell surface by flow cytometry (Fig. 4A). To analyze the importance of the hemITAM domain for the NTHI-induced Dectin-1 signaling, we used a mutated nonfunctional form of the cytoplasmic domain of Dectin-1, with the tyrosine residues within the hemITAM at positions 3 and 15 replaced by phenylalanines (Dectin-1\_Y3:15F). Unlike wild-type Dectin-1 transfectants, cells expressing Dectin-1\_Y3:15F failed to increase the Dectin-1-mediated IL-8 and IL-6 release (Fig. 4B) as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF- $\alpha$  release (data not shown). Our results show that Dectin-1 enhances the cytokine secretion in response to NTHI and zymosan in pulmonary epithelial cells and indicate that this effect is based on a functional hemITAM.

To investigate the role of NF- $\kappa$ B in NTHI-induced and Dectin-1-dependent pulmonary epithelial cell IL-8 secretion, Dectin-1-transfected and vector-transfected A549 cells were infected with NTHI. Subsequently, chromatin immunoprecipitation (ChIP) of the IL-8 promoter sequence was performed, in order to investigate the binding of the NF- $\kappa$ B subunit p65 (Fig. 4C). In response to NTHI stimulation, p65 was recruited to the IL-8 promoter at considerably higher levels in the Dectin-1-expressing cells than in the vector-transfected cells. In addition, the concordant recruitment of polymerase II to the IL-8 promoter indicated activation of gene expression, suggesting an important role for Dectin-1-mediated NF- $\kappa$ B activation for the NTHI-induced IL-8 gene expression.

**Dectin-1 directly interacts with NTHI and increases bacterial internalization but not adhesion.** In order to address the question of whether NTHI directly interacts with Dectin-1, pull-down experiments with NTHI and the extracellular domain of





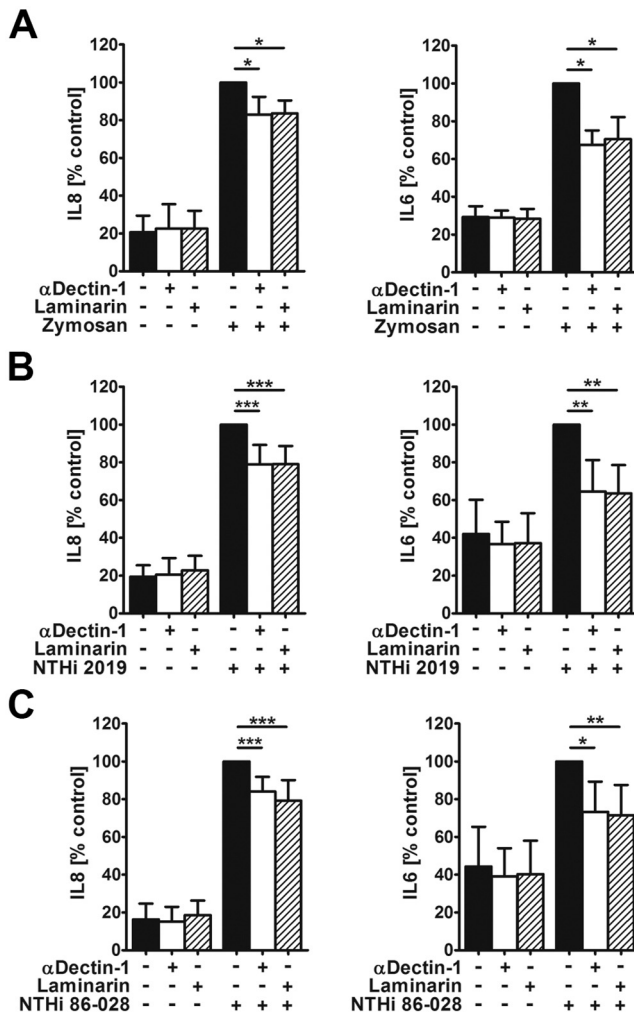
**FIG 2** Dectin-1 is expressed in primary pulmonary epithelial cells. Flow cytometry of the expression of Dectin-1 (open histograms, GE2 antibody) on the surface of small airway epithelial cells (SAEC) and normal human bronchial epithelial (NHBE) cells. Filled histograms, control staining with PE-labeled secondary antibody. (B) Relative Dectin-1 mRNA expression levels in NHBE cells after treatment with different TLR agonists, chemokines, or cigarette smoke extract (CSE) for 24 h, measured by qPCR normalized to untreated cells. Column bar graphs show mean expressions from three independent experiments with standard errors. ns, not significant; \*\*\*,  $P < 0.001$ . (C) Regulation of Dectin-1 protein expression on NHBE cells after treatment with poly(I-C) or interferons for 24 h, measured by flow cytometry. Filled histograms, control staining; open histograms, Dectin-1 staining (GE2 antibody); dotted line, Dectin-1 expression of untreated cells; black line, Dectin-1 expression after the respective treatment.

human Dectin-1 fused to the C terminus of human IgG1-Fc (Fc-hDectin-1a; InvivoGen) were performed (Fig. 5). NTHI 2019 was cocultured with the recombinant extracellular domain of human Dectin-1 (Fc-hDectin-1a) in the presence or absence of the Dectin-1 inhibitor laminarin or the Dectin-1-inhibiting antibody MAB1859. The Fc portion of human IgG (IgG-Fc) was used as a control. After intensive washing, the bacteria were lysed and the Fc domain was detected via Western blotting. As shown in Fig. 5A, Fc-Dectin-1a, but not the control protein, was detected in the bacterial lysate, indicating a specific binding of the extracellular Dectin-1 domain to the bacterium. The specificity of the binding was further supported by the finding that laminarin completely inhibited and the inhibitory anti-Dectin-1 antibody (MAB1859) partly inhibited the binding of Fc-Dectin-1 to NTHI. Although the Dectin-1 antibody only partly inhibited the binding of NTHI, it has been demonstrated to inhibit Dectin-1 dependent signaling (23, 24). It is likely that the antibody impedes the binding of NTHI to Dectin-1 and possibly inhibits the activation of Dectin-1 via a blockade of conformational changes instead of complete prevention of binding.

In order to investigate the role of Dectin-1 for NTHI adherence to or invasion of respiratory epithelial cells, the A549 “gain-of-function” cell model was used. As shown in Fig. 5B, we found no significant difference in NTHI adhesion to A549 cells dependent on Dectin-1 expression on the cell surface. In contrast, the internalization of the bacteria significantly increases in cells expressing wild-type Dectin-1 compared to that in cells expressing Dectin-1 with the mutated hemITAM signaling domain (Fig. 5C).

## DISCUSSION

Here, we have presented the first comprehensive study based on immunohistochemistry demonstrating that Dectin-1 is commonly expressed in several tissues of the human lung, including the epithelia of the airways and alveoli. Although epithelial expression of Dectin-1 was not correlated with COPD and smoking, interactions with pathogens might be facilitated by impaired mucociliary clearance, which is associated with later phases of COPD (23). Under these conditions, the apical expression of Dectin-1 allows pathogens to interact with this receptor once they reach the normally sterile lower airways (19). Through recognition of



**FIG 3** Dectin-1 enhances the NTHI-induced proinflammatory immune response of NHBE cells. NHBE cells were pretreated for 1 h with two different Dectin-1 inhibitors (anti-Dectin-1 antibody MAB1859 [20  $\mu$ g/ml] or laminarin from *Laminaria digitata* [1 mg/ml]) and stimulated with 100  $\mu$ g/ml zymosan, NTHI strain 2019 (MOI of 100), or NTHI strain 86-028 (MOI of 100) for 18 h. Subsequently, IL-8 (A) and IL-6 (B) cytokine levels were determined in the supernatants by ELISA. The actual level of IL-8 response of NHBE cells was in the range of 2.3 to 36.0 ng/ml for NTHI 2019, in the range of 3.0 to 49.6 ng/ml for NTHI 86-028, and in the range of 1.0 to 3.7 ng/ml for zymosan. The actual level of IL-6 induced in NHBE cells in response was in the range of 11.4 to 390.34 pg/ml for NTHI 2019, in the range of 8.9 to 513.4 pg/ml for NTHI 86-028, and in the range of 87.3 to 396.2 pg/ml for zymosan. Because of donor-specific variance in absolute cytokine levels, mean data derived from at least four independent experiments were normalized to the stimulated control (set as 100%). The error bars show the standard deviations of the normalized data. Significances were analyzed using the nonnormalized data by one-way ANOVA repeated-measures test with Dunnett's *post hoc* test: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

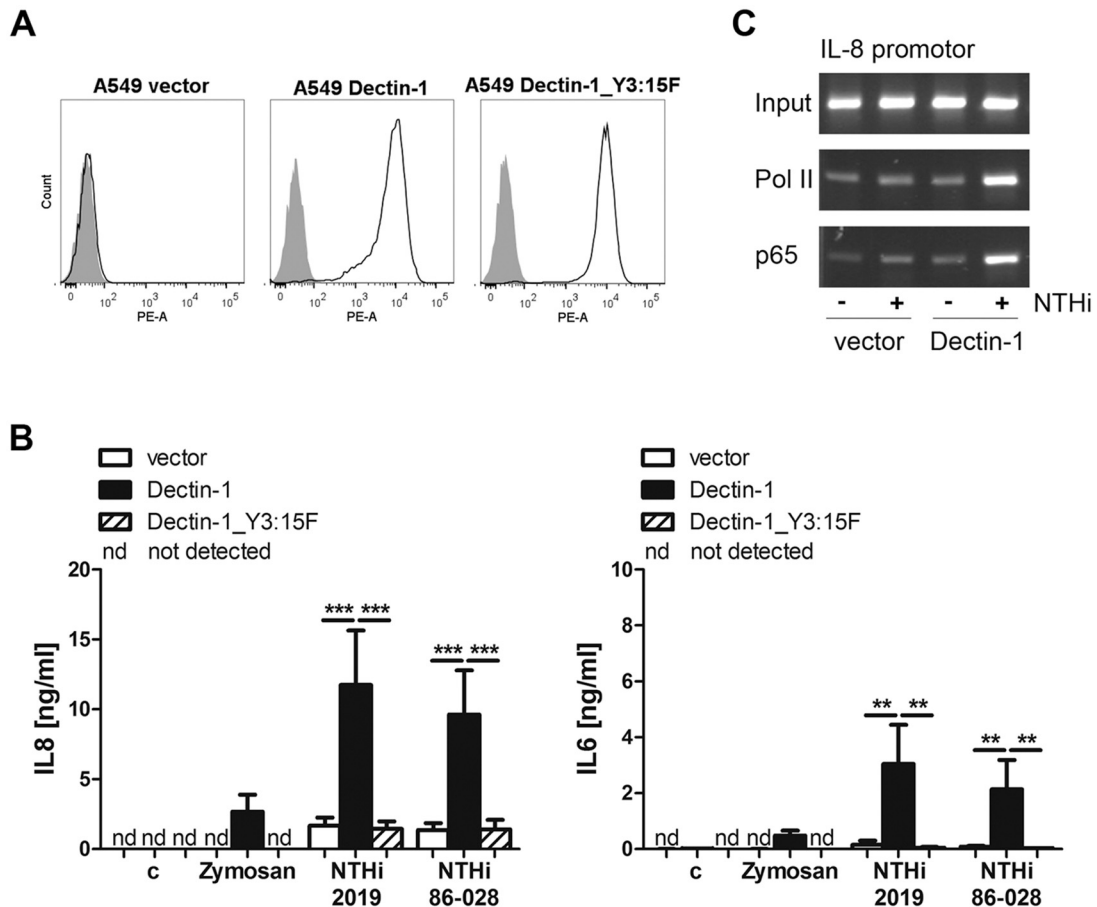
$\beta$ -glucans, Dectin-1 binds several fungal species that affect the respiratory system, such as *Aspergillus*, *Coccidioides*, *Penicillium*, and *Pneumocystis*. Therefore, the focus of research on the immunological role for Dectin-1 has been directed at fungal immunity (25). In patients with hematopoietic stem cell transplantations, a high risk of invasive pulmonary infection (IPA) with *A. fumigatus* has been reported by Cunha et al. under conditions of recipient Dectin-1 deficiency (26). Therefore, it can be suggested that

Dectin-1 expressed on nonhematopoietic cells, such as bronchial epithelial cells, plays a crucial role in the induction of immune protection to the fungus. Thus, it is likely that pathogen recognition of epithelium-expressed Dectin-1 also plays an important role in inducing and maintaining the inflammatory immune response in these patients.

Interestingly, our results also suggest that Dectin-1 directly interacts with NTHI and is involved in the NTHI-induced proinflammatory immune response of bronchial epithelial cells, although we did not identify the ligand of NTHI that induces Dectin-1 signaling. NTHI colonizes the lower respiratory tract of ~30% of individuals with COPD, and the acquisition of new strains of NTHI is an important cause of lower respiratory tract infection, resulting in exacerbations of COPD (18, 27). Therefore, it is suggested that persistent or repetitive exposure of the airways to NTHI products contribute to airway inflammation in COPD. In accordance with the findings of other groups (28–30), we also found that alveolar macrophages stained positive for Dectin-1, suggesting that these cells have an additional, important impact on the pulmonary immune response targeting NTHI. Interestingly, we demonstrated that Dectin-1 expressed on bronchial epithelial cells increased the secretion of IL-8 and IL-6 in response to infection with NTHI. Therefore, our study provides evidence for the first time that, in addition to its role for the recognition of fungal pathogens, Dectin-1 contributes to NTHI-induced inflammation in the human airways. In addition, we found that Dectin-1 was able to increase endocytosis of NTHI by pulmonary epithelial cells, although adhesion of the bacteria to the cells was not affected. This is in agreement with the findings of Ahrén et al. (14, 15), who reported that receptor-mediated endocytosis of NTHI by pulmonary epithelial cells and monocytes was facilitated via a  $\beta$ -glucan receptor which could be blocked by laminarin, an inhibitor commonly used to block Dectin-1-dependent immune responses. In contrast to our results, in the studies by Ahrén et al., binding of NTHI to eosinophils was blocked by the Dectin-1 inhibitor laminarin (14, 15). Apparently, pulmonary epithelial cells provide other molecules which NTHI can adhere to, so that on epithelial cells Dectin-1 is important for signaling but not for adhesion. Taking our observation into account, it is likely that the  $\beta$ -glucan receptor Dectin-1 is able to act as a PRR of NTHI. Our results are also in agreement with a report by Sancho et al. demonstrating that Dectin-1 also recognizes an unidentified ligand on mycobacteria and thereby implicates the ability of Dectin-1 to detect ligands of a broader spectrum of pathogens than just those of fungal origin (5). As shown in Fig. S4 in the supplemental material, NTHI did not express  $\beta$ -glucan in a detectable manner. It is therefore tempting to speculate that Dectin-1 detects a broader spectrum of ligands than has been described up until now. Another possibility may be that the bacteria express  $\beta$ -(1,3)-glucan at a very low level, below the detection limit of this assay. Further studies are needed in order to elucidate the nature of these bacterial ligands recognized by Dectin-1.

Our results from the A549–Dectin-1 overexpression model also demonstrated that the enhancement of the NTHI-induced IL-8 response was mediated via the Dectin-1 hemITAM signaling domain and resulted in the subsequent activation of NF- $\kappa$ B, a well-documented common feature of the Dectin-1-dependent signaling in hematopoietic cells (1, 10, 25, 31).

Dectin-1 expression varied between different donors. This applied to the surgical lung specimens and the primary bronchial

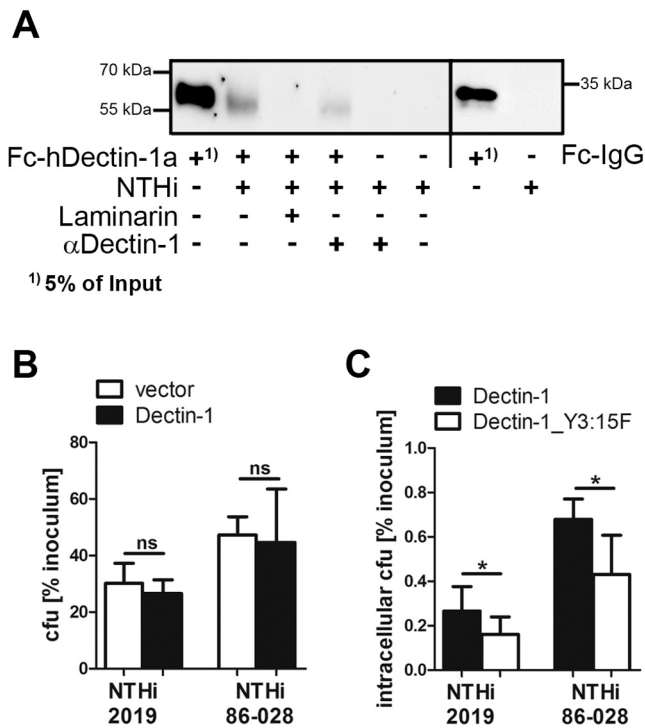


**FIG 4** The Dectin-1-mediated IL-8 response in NTHI-infected pulmonary epithelial cells depends on the Dectin-1 hemITAM domain and NF- $\kappa$ B. A549 cells were stably transfected with Dectin-1, a Dectin-1 hemITAM mutant (Dectin-1\_Y3:15F), or the vector without Dectin-1. (A) Flow cytometry of the transfected cells after sorting. Filled histograms, control staining with PE-conjugated secondary antibody; open histograms, Dectin-1 staining (GE2 antibody). (B) Transfected cells were stimulated with NTHI strains 2019 or 86-028 for 18 h, and IL-8 and IL-6 levels were measured in the supernatants of the cells. Column bar graphs are mean intensities and standard deviations from four independent experiments. Significances were determined by one-way ANOVA test (ns, not significant; \*\*\*,  $P < 0.001$ ). (C) Chromatin immune precipitation (ChIP) of polymerase II and NF- $\kappa$ B subunit p65 and subsequent amplification of IL-8 gene promoter. Dectin-1-expressing A549 cells and vector control were stimulated with NTHI 2019 for 60 min and subjected to ChIP and IL-8 promoter amplification. Amplicons were visualized using agarose gel electrophoresis and ethidium bromide staining. One representative experiment from three independent experiments is shown.

epithelial cells, suggesting a specific regulation of epithelial Dectin-1 expression. Low levels of Dectin-1 expression in primary bronchial epithelial cells were increased after treatment of the cells with the TLR3 agonist poly(I-C). This is consistent with the findings of Melkamu et al., demonstrating an upregulation of Dectin-1 on immortalized NHBE cells in response to poly(I-C) (32). Type I interferons are known to be induced in response to the recognition of poly(I-C) by TLR3 in dendritic cells (33). In addition, our group recently reported that IFN- $\beta$  was strongly induced in NHBE cells 4 h after poly(I-C) treatment (34). Nevertheless, we found that treatment of the NHBE cells with IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$  upregulated expression levels of Dectin-1 mRNA but not Dectin-1 protein on the cell surface of NHBE cells. Therefore, the poly(I-C)-induced secretion of interferons is most likely not associated with the observed upregulation of Dectin-1 protein. Our data suggest that a TLR3-specific but interferon-independent effect is responsible for this observation. Interestingly, Weck et al. (35), in contrast, found in monocyte-derived, dendritic cells that poly(I-C) downregulated Dectin-1. Thus,

Dectin-1 expression seems to be differently regulated in different cell types. An activation of innate immune response by TLR3 via detection of viral double-stranded RNA (dsRNA) is described for a wide range of respiratory viral infections, such as the respiratory viruses influenza A virus, respiratory syncytial virus, and rhinovirus type 1b (36). Therefore, it is possible that an upregulation of Dectin-1 expression in the airways is associated with the occurrence of viral infections, and it is tempting to speculate that this upregulation then influences the immune response to a secondary NTHI infection.

The results of our study did not reveal a direct correlation between Dectin-1 expression and COPD or smoking status. We also ruled out a direct influence of cigarette smoke extract for Dectin-1 expression in NHBE cells. However, the Dectin-1 upregulation via viral dsRNA implies that Dectin-1 might also be involved in the spatial and temporal association between viral and bacterial infections in COPD patients (37). The COPD-associated increasing impairment of mucociliary clearance during the course of the disease (38) may additionally augment the access to



**FIG 5** Dectin-1 interacts with NTHI. (A) NTHI–Dectin-1 pull-down: Western blot analysis targeting human Fc after coincubation of NTHI 2019 with recombinant Fc–hDectin-1a in the presence or absence of the Dectin-1 inhibitors laminarin (1 mg/ml) or anti-Dectin-1 antibody MAB1859 (20  $\mu$ g/ml). IgG1-Fc was used as a control. The image shows one representative experiment of three. (B) Adhesion of NTHI to A549 cells without Dectin-1 (vector; white bars) or A549 cells overexpressing Dectin-1 (black bars). (C) Internalization of NTHI into A549 cells overexpressing wild-type Dectin-1 (black bars) or Dectin-1 with a mutated hemITAM signaling motif (Dectin-1\_Y3:15F; white bars). Bar graphs are mean CFU, given as percentages of inoculum and standard deviations from at least three independent experiments. Significant differences were calculated using a two-tailed Student *t* test. ns, not significant; \*, *P* < 0.05.

Dectin-1, resulting in an increased interaction of NTHI and Dectin-1-expressing pulmonary epithelial cells. Therefore, it is likely that COPD patients may be predisposed to sustained inflammatory responses to NTHI, due to Dectin-1 expression and accessibility. This might also play a role in relation to infections with *A. fumigatus* as a well-known ligand of Dectin-1 in the lower airways, in later stages of the disease (39).

In conclusion, we found evidence that Dectin-1 is epithelially expressed in several tissues of the human lung, where it contributes as a PRR to the recognition of NTHI. Further studies will be needed to shed light on this new aspect of Dectin-1–bacterium interactions and its role in the pathogenesis of COPD.

## MATERIALS AND METHODS

**Materials and antibodies.** All materials were purchased from Sigma or Merck, unless otherwise stated. Primary antibodies were mouse anti-human Dectin-1 clone GE-2 (kindly provided by Gordon Brown, Institute of Medical Sciences, University of Aberdeen, Aberdeen, United Kingdom), mouse anti-human Dectin-1 clone MAB1859 (R&D Systems), monoclonal antibody (MAb) Be9.2 (as the isotype control; kindly provided by W. Reutter, Charite-Universitätsmedizin Berlin, Germany), NF- $\kappa$ B p65 (C-20; Santa Cruz Biotechnology), polymerase II (N-20; Santa Cruz Biotechnology), goat anti-human IgG-Fc, and horseradish peroxi-

dase (HRP)-conjugated antibody (Thermo Scientific). Secondary antibodies were phycoerythrin (PE)-labeled goat anti-mouse antibody (antibodies-online GmbH) and HRP-coupled goat anti-mouse antibody (Dianova).

**Acquisition and processing of human lung specimens.** Tissue was obtained from surgical specimens from patients who underwent surgery for lung resection to treat lung cancer. A positive vote of the ethics committee of the University of Heidelberg was obtained, and informed consents were received from patients. The resected tissue was fixed in formalin and embedded in paraffin using a standard procedure (40).

**Immunohistochemical analysis.** Immunohistochemical staining with MAb anti-Dectin-1 (25  $\mu$ g/ml, clone MAB1859) was carried out on paraffin wax sections obtained from 19 different human lung sections. Sections were treated as described (34). An isotype-matched MAb was used as a negative control.

**Preparation of aqueous-phase cigarette smoke extract.** CSE was prepared as described (34, 41). CSE was used within 30 min of preparation at a final concentration of 4%. Lau et al. showed that NHBE cells were induced to secrete IL-8 and remained viable after treatment with 4% CSE for 24 h (41).

**Bacterial strains.** NTHI clinical isolate strains 2019 and 86-028 (kindly provided by Edward Swords, Department of Microbiology, University of Iowa, United States) were freshly grown overnight at 37°C in 5% CO<sub>2</sub> on chocolate agar (BD Biosciences). For infection experiments, NTHI from overnight cultures were grown in brain heart infusion broth (BD Biosciences), supplemented with 10  $\mu$ g/ml hemin (Sigma-Aldrich) and 10  $\mu$ g/ml of NAD (MP Biomedicals) at 37°C to mid-log phase. Subsequently, bacteria were harvested by centrifugation and resuspended in Dulbecco's phosphate-buffered saline (PBS). Bacterial concentrations were determined by measurement of the optical density (optical density at 600 nm [OD<sub>600</sub>] of 0.1 correlates with 1  $\times$  10<sup>8</sup> CFU/ml), and a multiplicity of infection (MOI) of 100 was used as the stimulation dose.

**Cells.** Normal human bronchial epithelial (NHBE) cells were purchased from Lonza. The cells were cultured with bronchial epithelial cell basal medium (BEBM; Lonza) supplemented with a BEGM bullet kit (Lonza) in collagen I-coated flasks or plates (BD Biosciences). For stimulation, NHBE cells were grown until confluence. For inhibition of Dectin-1, NHBE cells were treated with 1 mg/ml laminarin from *Laminaria digitata* (Sigma-Aldrich) or 20  $\mu$ g/ml mouse anti-human Dectin-1 antibody (clone MAB1859) 1 h prior to overnight stimulation.

Cells were stimulated with 100  $\mu$ g/ml zymosan (InvivoGen), 100 ng/ml MALP-2 (Enzo Life Sciences), 100 ng/ml poly(I:C) (high molecular weight; InvivoGen), 100 ng/ml flagellin (invivoGene Biotech), 100 ng/ml IFN- $\alpha$  (recombinant human interferon alpha 1A; ImmunoTools), 100 ng/ml IFN- $\beta$  (recombinant human interferon beta 1a; ImmunoTools), 100 ng/ml IFN- $\gamma$  (recombinant human interferon gamma; Promokine), 100 ng/ml TNF- $\alpha$  (recombinant human tumor necrosis factor alpha; R&D Systems), 4% CSE, or NTHI (MOI of 100). Small airway epithelial cells (SAEC; Lonza) were cultured with small airway epithelial cell basal medium (SABM; Lonza) supplemented with an SAGM bullet kit (Lonza).

A549 cell lines (type II alveolar cells), obtained from DSMZ, were subcultured in Dulbecco's modified Eagle's medium (DMEM; high glucose) with Glutamax (Invitrogen), supplemented with 10% fetal bovine serum (FBS). For overexpression of Dectin-1, cells were transfected with the plasmids pUNO1 (control vector; InvivoGen), pUNO1-hDectin1a (bearing the human Dectin-1 gene [*clac7A*] isoform a; InvivoGen), or pUNO1-hDectin1a\_Y3:15F (bearing *clac7a* isoform a, with the tyrosines on sites 3 and 15 replaced by a phenylalanine; constructed via gene synthesis and subcloned by Entelechon GmbH) by using GeneCellin transfection reagent (BioCellChallenge). Transfected cells were selected with 30  $\mu$ g/ml Blastidicin S (InvivoGen), and cells positive for Dectin-1 expression were sorted by BD FACSAria II. For infection, cells were grown until confluence, and 2 h before stimulation, the medium was changed to serum-free medium without antibiotics.



**Flow cytometry.** Cells were cultured as described above until confluence, detached with trypsin-EDTA, and fixed on ice in 2% paraformaldehyde-PBS for 20 min. After being blocked in PBS containing 10% FBS for 1 h, cells were stained with anti-Dectin-1 antibody clone GE2 (10  $\mu\text{g}/\text{ml}$ , 45 min) and with PE-conjugated goat-anti-mouse IgG (5  $\mu\text{g}/\text{ml}$ , 30 min). FACS analysis of Dectin-1 expression on A549 cells was performed on nonfixed cells under sterile conditions, because cells positive for Dectin-1 staining were subsequently sorted and subcultured. Samples were measured using a FACSCalibur (BD Biosciences) or FACSAria II (BD Biosciences), and data were analyzed using the FLOWJO7.6.4 software.

**Pulldown and Western blotting.** NTHI 2019 was coincubated with 10  $\mu\text{g}/\text{ml}$  Fc-Dectin-1a (InvivoGen) with or without 1 mg/ml laminarin (Sigma-Aldrich) or 20  $\mu\text{g}/\text{ml}$  mouse anti-human Dectin-1 antibody (clone MAB1859; R&D Systems) or with normal human IgG-Fc control protein (Millipore) for 1 h at room temperature. The bacteria were washed four times with PBS and afterward lysed with 0.5% SDS. After DNA digestion, proteins of the lysates were separated by SDS gel electrophoresis under reducing conditions and transferred to a nitrocellulose membrane. Subsequently, the human Fc was detected using HRP-conjugated anti-human Fc antibody (goat anti-human IgG-Fc; Thermo Scientific).

**Adhesion and internalization assay.** For the adhesion assay, A549-vector cells and A549-Dectin-1 cells were treated with NTHI (MOI of 100) for 1 h (37°C, 5% CO<sub>2</sub>). After cells were washed five times with PBS, trypsin-EDTA solution (Lonza) was added for 5 min. Cells that were detached were suspended in 20% fetal calf serum (FCS)-PBS and vortexed for 1 min, and serial dilutions were plated out on chocolate agar. For the internalization assay, A549-Dectin-1 cells and A549-Dectin-1\_Y3:15F cells were treated with NTHI (MOI of 100) for 2 h. Next, cells were washed twice with PBS and incubated for 2 h in DMEM and 1 $\times$  penicillin-streptomycin solution (Invitrogen) and 100  $\mu\text{g}/\text{ml}$  gentamicin (Life Technologies) to kill extracellular bacteria. Afterward, cells were washed two times with PBS, scraped, and mechanically lysed in lysing matrix tubes containing 1.4-mm ceramic spheres (MP Biomedicals) using a Fast-Prep-24 homogenizer (MP Biomedicals). Subsequently, probes were serially diluted and plated on chocolate agar. For both assays, CFU were determined the next day using a ProtoCOL 2 colony counter (Symbiosis).

**RT-qPCR analysis.** To quantify relative gene expression of Dectin-1, total RNA was extracted and reverse transcription (RT)-qPCR and real-time qPCR were performed as previously described (34). The relative expression of target genes was analyzed with the Pfaffl method (42). The expression levels were normalized to the geometric mean of two housekeeping genes: the hypoxanthine phosphoribosyltransferase1 (HPRT1) and peptidylpropyl isomerase B (PPIB) genes. The stability of the housekeeping genes was assessed using the BestKeeper algorithm (43). Relative differences in mRNA expression between different experimental conditions were analyzed by pairwise, fixed randomization tests using REST 2009 (44). Dectin-1 primers were as follows: forward, TCTTCCAGCCCTTGTCTC; reverse, CCAGTTGCCAGCATTGTCTT.

**ELISA.** Cytokine secretion by NHBE and A549 cells was analyzed with commercially available ELISA kits (human IL-8 ELISA set [BD Biosciences]; human IL-6 ELISA Ready-Set-Go [eBioscience]), according to the manufacturer's protocols.

**Chromatin immunoprecipitation.** The chromatin immunoprecipitation was performed as described previously (45). A549 cells were stimulated 1 h prior to fixation. After immunoprecipitation of p65 or polymerase II, IL-8 promoter DNA was amplified by semiquantitative PCR using *Taq* polymerase (5 Prime). The resulting PCR products were separated on an ethidium bromide-stained agarose gel and visualized under a UV transilluminator.

**Statistical analysis.** Data are shown as means  $\pm$  standard deviations (SD) from at least three independent experiments. Unless stated otherwise, statistical differences between means were assessed by one-way analysis of variance (ANOVA) with Bonferroni *post hoc* correction. All tests

were two-sided, and a *P* value of  $<0.05$  was considered statistically significant (\*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001). GraphPad Prism5 software was used for data analysis.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01492-14/-/DCSupplemental>.

Figure S1, TIF file, 7 MB.

Figure S2, TIF file, 4.1 MB.

Figure S3, TIF file, 1.6 MB.

Figure S4, TIF file, 1.3 MB.

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