A Human Dectin-2 Deficiency Associated With Invasive Aspergillosis

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SUMMARY

We identified a Dectin-2 N170I mutation in an immunocompromised patient who succumbed to invasive aspergillosis. This mutation results in an early stop codon, poor receptor expression and renders Dectin-2 functionally defective.

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ABSTRACT

Immunocompromised patients are highly susceptible to invasive aspergillosis. Herein, we identified a homozygous deletion mutation (507 del C) resulting in a frameshift (N170I) and early stop codon in the fungal binding Dectin-2 receptor, in an immunocompromised patient. The mutated form of Dectin-2 was weakly expressed, did not form clusters at/near the cell surface and was functionally defective. PBMCs from this patient were unable to mount a cytokine (TNF, IL-6) response to *A. fumigatus* and this first identified Dectin-2-deficient patient succumbed to invasive aspergillosis.

KEYWORDS

Dectin-2; CLR; *Aspergillus*; *Candida*; Fungal Immunology; Host-Pathogen Interactions; Innate Immunity; Inflammation.

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BACKGROUND

Invasive fungal infections including invasive aspergillosis (IA) represent a severe disease burden in immunocompromised patients such as acute myeloid leukemia and allogeneic hematopoietic stem cell transplant (HSCT) patients. Absence of a robust anti-fungal immune response permits fungal colonization, invasive growth and disease. IA mortality is unacceptably high, (30-80%) in allogeneic HSCT recipients [1, 2]. Therefore, patients are empirically treated with anti-fungal therapies.

The C-type lectin-like receptors (CLRs) Dectin-1 and Dectin-2 drive anti-fungal immune responses against *Candida* and *Aspergillus* [3-7]. A Dectin-1 single nucleotide polymorphism (SNP) (Y238X) has been associated with mucocutaneous candidiasis [8] and increased susceptibility to IA [9]. SNPs in Dectin-2 have been associated with IA [10]; however, their functional consequences are unknown. Herein, we characterized a novel Dectin-2 exonic deletion mutation identified in an immunocompromised patient who succumbed to IA.

METHODS

Ethics Statement

The study was approved by NISCHR Research Ethics Committee (reference: 14/WA/1119). Written informed consent was obtained from patients in the study. Animal work was performed according to institutional and UK Home Office guidelines. This study was performed in accordance with the Project License. Procedures were approved by Cardiff University Animal Welfare and Ethical Review Body and UK Home Office. The animal care and use protocol adhered to the Animals (Scientific Procedures) Act 1986.

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Genetic Analysis

RNA was extracted from patient blood samples using PAXgene Blood RNA kit (Qiagen) and cDNA was generated using Reverse Transcription Kit (Thermofisher Scientific). Dectin-2 DNA was amplified and sequenced from patient cDNA by PCR using primers (Supplementary Table 1).

Structural Analysis

The structure of wild type (WT) Dectin-2, PDB accession code 5VYB, was used as the starting model. COOT was used to implement mutations and model readjustment. REFMAC5 (CCP4) was used to regularize model geometry.

Cloning and Transfection

WT and mutant *CLEC6A*/Dectin-2-pFB-NEO (Stratagene) and pHR'SIN-cPPT-SXW (pSXW) constructs, with an N-terminus FLAG-tagged Dectin-2, were generated using primers (Supplementary Table 1). FLAG-tagged Dectin-2 WT and mutant PCR products were inserted into pFB-NEO using In-Fusion Cloning kit (Clontech). Stellar Competent Cells (Clontech) were transformed with *CLEC6A*/Dectin-2-FLAG-Tag constructs and grown in LB Broth (Sigma) before plasmid DNA was extracted using DNA Mini/Midiprep Kit (Thermofisher Scientific/Qiagen). FLAG-tagged Dectin-2 WT and mutant PCR products were inserted into SXW lentiviral vector using In-Fusion Cloning kit. Top10 competent *E. coli* (NEB) were transformed with *CLEC6A*/Dectin-2-FLAG-Tag constructs and grown in LB broth before plasmid DNA was extracted using DNA Miniprep Kit. HEK293T cells cultured in DMEM medium (with 10% Fetal bovine serum (FBS), 100U/ml penicillin/streptomycin (Thermofisher Scientific)) were co-transfected with 1.5µg FcγR pMXs-IP, and either pFB-NEO or pFB-NEO containing

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WT or mutant Dectin-2, using Fugene-6 Transfection Reagent (Promega). 48 h later cells were harvested for RNA/protein analysis.

Lentivirus infection

HEK293T cells cultured in DMEM medium with 10% FBS and 100U/ml penicillin/streptomycin were co-transfected with 1.5µg pR8.91, 1µg pMD2G and either pSXW empty vector, Dectin-2 WT pSXW or Dectin-2 N107I pSXW, using Effectene Transfection Reagent (Qiagen). 48 h and 72 h later supernatant was collected, filtered using 0.45µm sterile millexGP filter (Millipore Ireland Ltd.) and overlaid on 20% sucrose (Sigma) gradient in ultracentrifuge conical tubes (Beckman Coulter). The gradient was centrifuged at 120,000 g at 4°C for 90 min and virus pellet re-suspended in AIM V medium (Thermofisher Scientific). Bone-marrow (BM) cells were isolated from Dectin-1-Dectin-2 (D1D2) DKO mice. BM derived macrophages (BMDMs) were generated as previously described [7]. For lentiviral infection, BMDMs were harvested and lentivirus was added in the presence of fresh media containing M-CSF.

Determination of Dectin-2 expression

48 h after transfection of HEK293T cells and 72 h after infection of D1D2 DKO BMDMs, RNA was extracted using TRIZOL (Thermofisher Scientific) and purified using RNeasy Mini Kit (Qiagen). cDNA was synthesized using TaqMan Reverse Transcription Kit (Invitrogen). *CLEC6A*/Dectin-2 mRNA was quantified by qPCR using ABI Taqman Primer/Probe Sets (Thermofisher Scientific) and normalized against *HPRT1*. Dectin-2 protein expression was measured by intracellular FACS staining with anti-FLAG (L5 Biolegend) or by surface FACS staining with anti-Dectin-2 (545943 R&D).

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Cytospin

72 h after lentiviral infection, D1D2 DKO BMDMs were collected and centrifuged. The pellet was fixed with BD Cytofix/Cytoperm solution (BD Biosciences), washed with BD Perm-Wash solution (BD Biosciences) and blocked with FACS block solution (DPBS, 5mM EDTA, 2mM NaN3, 0.5% BSA, 5% Rabbit serum) containing 4µg/ml 2.4G2. Cells were stained with anti-CD11b (M1/70 Biolegend) and anti-FLAG (L5 Biolegend) and washed with BD Perm-Wash. 4',6-diamidino-2-phenylindole (DAPI; Thermofisher Scientific) was added before cells were washed and resuspended in DPBS. 10^4 cells per sample were cytospun and mounted with Prolong Gold Antifade (Fisher Scientific). Cells were imaged using a Zeiss Cell Observer Spinning Disk confocal microscope with a 63x objective to obtain Z-stacks of the whole cell thickness. Images were then analyzed using Imaris 9.3.1 to obtain 3D reconstruction of cell structure.

Fungal Cultures

A. fumigatus 13073 (ATCC) was cultured on potato dextrose agar for 7 days at 37°C. Conidia were harvested and passed through a 40µM filter to remove hyphal fragments. Resting conidia were washed and resuspended in DPBS [7]. *C. albicans* SC5314 (ATCC) was cultured on YPD agar plates overnight at 30°C, then cultured in YPD broth for 16 h at 30°C with shaking, washed with DPBS and resuspended in DPBS [5].

Generation of Bone-Marrow Derived Dendritic Cells (BMDCs)

BMDCs were generated by culturing BM cells for 8-10 days in RPMI 1640 medium containing 10% FBS, 2mM L-glutamine (Thermofisher Scientific), 100U/ml penicillin/streptomycin, 10mM HEPES (Life Technologies), 1% NEAA (Life Technologies), 1mM Sodium pyruvate (Thermofisher Scientific), 50µM β-mercaptoethanol (Fisher) and 10ng/ml GM-CSF (Peprotech).

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Cytokine Assays

Human peripheral blood mononuclear cells (PBMCs) were isolated from patient blood using FicollPLUS (Sigma), washed with PBS^{Mg+Ca+} (Thermofisher Scientific) and washed three times with RPMI 1640. Cells were resuspended in PBMC media (RPMI 1640 with 10% FBS, 2% human serum (Sigma), 10mM L-glutamine (Thermofisher Scientific), 10mM Sodium pyruvate and 100µg/ml Gentamicin (Thermofisher Scientific)). PBMCs were rested for 4 h before 100µl of $5x10^6$ PBMCs/ml were challenged with 100µl of 1µg/ml LPS or 100µl of $5x10^6$/ml *A. fumigatus* swollen conidia. Swollen conidia were cultured in RPMI at 37°C for 6 h. After 24 h supernatant was collected, and cytokines measured by ELISA (R&D). WT and Dectin-2 KO BMDCs were resuspended in RPMI containing 10% FBS and 100U/ml penicillin/streptomycin. 100µl of $1x10^6$ BMDCs/ml were challenged with 100µl of $1x10^6$/ml *A. fumigatus* conidia, 100µl of $1x10^6$/ml *C. albicans or* 100µl of 2ng/ml LPS. Amphotericin B (2.5µg/ml) was added to *C. albicans* 2 h after stimulation. After 24 h supernatant was collected, and cytokines measured by ELISA (R&D).

Statistical Methods

Data were analyzed using GraphPad Prism. Data are presented as means ± SEM. One-way ANOVA followed by Tukey's post-test or Two-way ANOVA followed by Bonferroni's post-test was used for statistical analysis for multiple groups. When data were not normally distributed, it was transformed by Y=sqrt(Y+0.5) and ANOVA. *p* values less than 0.05 were considered statistically significant: *p<0.05, **p<0.005, ***p<0.001.

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RESULTS

CLEC6A (Dectin-2) Mutation

The patient possessed a homozygous base pair deletion (507delC) in exon 6 of Dectin-2 *(CLEC6A)* (Supplementary Figure 1A), which causes a frame shift (N170I) and premature termination of Dectin-2 (Figure 1A). Loss of Cys176 removes a disulphide bridge, while loss of Asp191 removes a Ca^{2+} and Na^{+} stabilizing bridge (Figure 1B) and loss of the final β-strand would leave a large hole at the core of the protein (Figure 1C), resulting in failure of the protein to fold. Furthermore, mutant Dectin-2 could not bind its ligand (Supplementary Figure 1B-C). Therefore, this Dectin-2 mutation likely has serious functional and clinical consequences.

Based on computational modelling, we hypothesized that mutant Dectin-2 would not produce a stable protein product. HEK293T cells expressing mutant Dectin-2 displayed increased RNA levels but minimal protein levels compared to WT Dectin-2 (Supplementary Figure 1D-F). Similarly, Dectin-1-Dectin-2 DKO BMDMs expressing mutant Dectin-2 displayed normal RNA levels (Figure 1D) but minimal protein levels compared to BMDMs expressing WT Dectin-2 (Figure 1E-F). Furthermore, while WT Dectin-2 clustered at/near the cell surface, mutant Dectin-2 was expressed at low levels throughout the cytosol and did not form clusters (Figure 1G, Supplementary Figure 1G). Together, these data indicate that Dectin-2 N170I does not form a stable protein product, is minimally expressed and therefore functionally defective.

Functional Consequences of *CLEC6A* Mutation

To determine the functional consequences of Dectin-2 N170I, we tested whether the patients in our study were able to mount an effective immune response against *A. fumigatus*. Most WT PBMCs produced LPS- and *A. fumigatus*- induced cytokines. However, mutant Dectin-2 PBMCs only produced LPS-induced cytokines and not *A. fumigatus*-induced cytokines (Figure 2A). To confirm a

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role for Dectin-2 in *A. fumigatus*-induced cytokine production we utilized Dectin-2 KO cells. Dectin-2 KO BMDCs displayed reduced *A. fumigatus*- (Figure 2B) and *C. albicans*-induced cytokine production compared to WT controls whilst they displayed normal LPS-induced cytokine production (Figure 2C). However, Dectin-2 did not contribute to fungal killing (Supplementary Figure 2A).

We next investigated whether Dectin-2 mediated binding to *A. fumigatus*. Dectin-1-Dectin-2 DKO BMDMs expressing mutant Dectin-2 displayed a modest reduction in binding *A. fumigatus* compared to DKO BMDMs expressing WT Dectin-2 (Supplementary Figure 2B-C). *A. fumigatus*-induced trained immunity could be significantly reduced by blocking Dectin-2 in human monocytes (Supplementary Figure 2D). Together, these data indicate Dectin-2 N170I has detrimental consequences for anti-fungal immunity. In agreement with this, the patient was diagnosed with probable IA and lung abnormalities consistent with fungal infection, which progressively worsened until death (Supplementary Table 2).

DISCUSSION

Herein, we characterized a novel Dectin-2 N170I mutation identified in a HSCT patient who succumbed to IA. This mutation results in truncation of Dectin-2 and radically alters the receptor's tertiary structure, leading to significantly reduced expression. Dectin-2 is important for fungal binding, trained immunity, and fungal-induced cytokine production.

Multiple polymorphisms in CLRs and their signaling component CARD9 increase susceptibility to fungal infection [9, 11], some even without immune suppression [8]. Two patients with reduced CARD9 protein expression developed IA [12] and HSCT patients with the Dectin-1 Y238X SNP, which results in a truncated CLR, displayed increased susceptibility to IA [9]. Here, we demonstrate the Dectin-2 N170I mutation also results in a truncated CLR. The Dectin-2 mutation was identified in the

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recipient prior to SCT; however, up to 70% of tissue resident cells remain from host origin and may persist for up to one year [13-15]. The patient displayed signs of *A. fumigatus* infection <1-year post-SCT, when host cells expressing mutant Dectin-2 were likely present in the lung. While the patient succumbed to IA, further patients would be required to confirm a direct link between Dectin-2 N170I and IA.

Structural modelling of Dectin-2 N170I predicted incorrect folding of the protein. Consistent with this, we showed reduced expression of Dectin-2 N170I at the cell surface despite the presence of RNA and low level of dispersed intracellular protein. These results suggest Dectin-2 N170I forms an unstable structure, is poorly transported to the cell membrane and is minimally expressed, similar to Dectin-1 Y238X [8, 9]. Furthermore, Dectin-2 predominantly recognizes mannose, and hence *Aspergillus*, through its EPN motif, a structure lost in Dectin-2 N170I [4].

Dectin-2 generates robust cytokine and chemokine responses against *A. fumigatus* [4] and mice deficient in Dectin-2 are susceptible to *C. albicans* infection [5]. Here, we found a significant role for Dectin-2 mediating *A. fumigatus*- and *C. albicans*-induced TNF and IL-6 secretion. Similarly, PBMCs from CARD9-deficient patients display impaired fungal-induced cytokine production [16] and mice with TNF blockade or IL-6 deficiency are highly susceptible to IA [17, 18]. Dectin-2 has previously been shown to bind to *A. fumigatus* hyphae [4], and we observed a modest reduction in binding of mutant Dectin-2 to *A. fumigatus* compared to WT Dectin-2. Importantly, we observed Dectin-2 mediated *A. fumigatus* -induced trained immunity, further supporting the importance of Dectin-2 during IA.

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Our research is the first to functionally characterize a Dectin-2 mutation associated with decreased anti-fungal responses. Furthermore, the Dectin-2 mutant patient developed, and succumbed to IA. The Dectin-2 N170I mutation renders the CLR functionally null and loss of Dectin-2 results in defective anti-fungal responses. Identifying mutations that increase patient's fungal susceptibility may permit a personalized approach and enable targeted prophylaxis of patients at high-risk of fungal disease [11].

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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Figure 1: Dectin-2 mutation results in minimal protein expression. (A) Partial amino acid sequence of wild-type (WT) and mutant (507delC) Dectin-2 with key residues and EPN mannan-binding motif highlighted. (B) The fold of WT Dectin-2 5VYB (cartoon) in complex with mannan (stick model). Blue = present in the mutant protein, orange = absent in the mutant protein. Two Ca^{2+} atoms (green spheres) and a Na^{+} atom (purple) are also displayed. (C) Predicted surface model of Dectin-2 covering the mutant structural elements only. The final β-strand at the core of the structure is missing in the mutant, resulting in a collapse of the motif, corruption of ligand interface and inability to bind mannan. (D-G) BMDMs from Dectin-1-Dectin-2 DKO mice were infected with constructs expressing FLAG-tagged Dectin-2 WT, mutant or empty vector and harvested 72 h later. (D) RNA was isolated, cDNA was prepared, and *CLEC6A* mRNA transcript was detected by RTqPCR. mRNA levels were normalized to *HPRT1*. Graph displays mean +/- SEM from 3 independent experiments. One-way ANOVA with Tukey's post-test on transformed data. (E-F) Cells were permeabilized, stained with anti-FLAG and analyzed by flow cytometry. (E) Dashed black line = empty vector, solid black line = Dectin-2, solid grey line = Dectin-2 mutant. Histogram representative of 3 independent experiments. (F) Graph displays mean +/- SEM mean fluorescent intensity (MFI) from 3 independent experiments. One-way ANOVA with Tukey's post-test. (G) BMDMs were stained with anti-CD11b (magenta) and anti-FLAG (green), nuclei were stained with DAPI (blue). Images are representative of 2 independent experiments.

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Figure 2: Dectin-2 mediates cytokine response to *A. fumigatus* and *C. albicans*. (A) Patient PBMCs were stimulated with *A. fumigatus* swollen conidia at a ratio of 1:1 or with LPS for 24 h. Cytokine levels in supernatants were measured by ELISA. Graphs show mean +/- SEM from 41 patients with WT Dectin-2 and 1 patient homozygous for mutant Dectin-2. Patient results are additionally stratified by their IA status. (B) BMDCs from WT and Dectin-2 KO mice were stimulated with *A. fumigatus* conidia at a ratio of 1:1 for 24 h. Cytokine levels in supernatants were measured by ELISA. Graphs show mean +/- SEM from 4 independent experiments, 2-way ANOVA on transformed data with Bonferroni's post-test. (C) BMDCs from WT and Dectin-2 KO mice were stimulated with *C. albicans* at a ratio of 1:1 or LPS for 24 h. Amphotericin B was added after 2 h and supernatants were harvested after 24 h. Graphs show mean +/- SEM from 3 independent experiments, 2-way ANOVA on transformed data with Bonferroni's post-test.

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Figure 1

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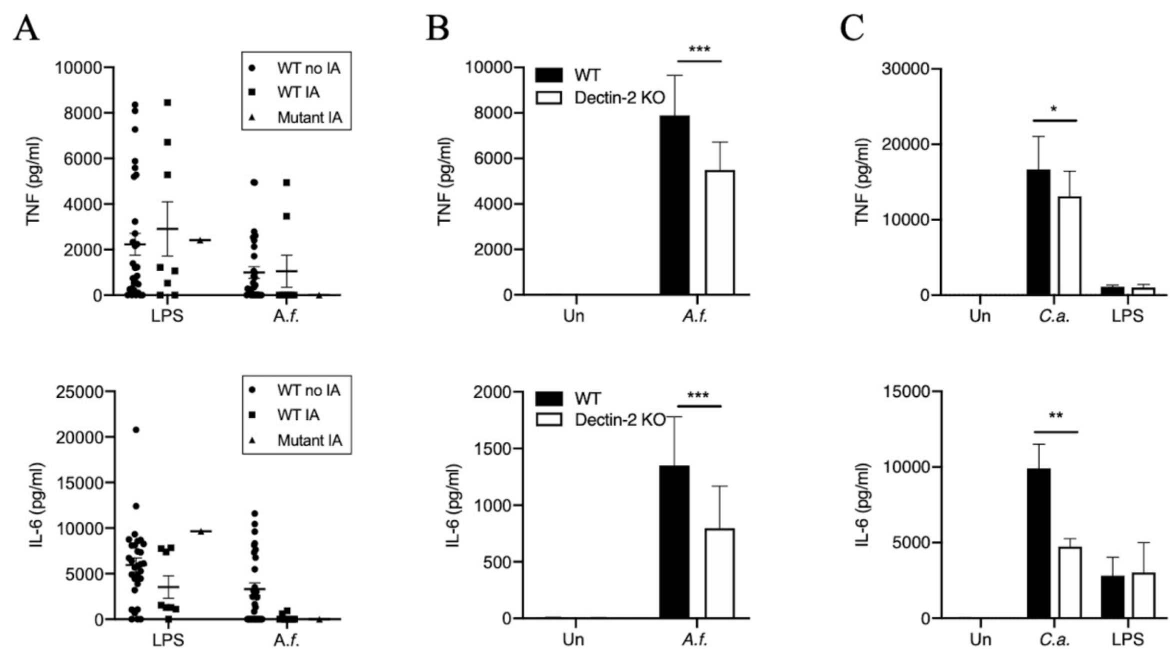
Figure 2



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