Krüppel-like Factor 4 modulates interleukin-6 release in human dendritic cells after \textit{in vitro} stimulation with \textit{Aspergillus fumigatus} and \textit{Candida albicans}

Kristin Czakai$^{1,*}$, Ines Leonhardt$^{2,*}$, Andreas Dix$^3$, Michael Bonin$^4$, Joerg Linde$^3$, Hermann Einsele$^1$, Oliver Kurzai$^2$ & Jürgen Loeffler$^2$

Invasive fungal infections are associated with high mortality rates and are mostly caused by the opportunistic fungi \textit{Aspergillus fumigatus} and \textit{Candida albicans}. Immune responses against these fungi are still not fully understood. Dendritic cells (DCs) are crucial players in initiating innate and adaptive immune responses against fungal infections. The immunomodulatory effects of fungi were compared to the bacterial stimulus LPS to determine key players in the immune response to fungal infections. A genome wide study of the gene regulation of human monocyte-derived dendritic cells (DCs) confronted with \textit{A. fumigatus}, \textit{C. albicans} or LPS was performed and Krüppel-like factor 4 (KLF4) was identified as the only transcription factor that was down-regulated in DCs by both fungi but induced by stimulation with LPS. Downstream analysis demonstrated the influence of KLF4 on the interleukine-6 expression in human DCs. Furthermore, KLF4 regulation was shown to be dependent on pattern recognition receptor ligation. Therefore KLF4 was identified as a controlling element in the IL-6 immune response with a unique expression pattern comparing fungal and LPS stimulation.

Dendritic cells (DCs) play an important role in pathogen recognition. They recognize pathogens via pattern recognition receptors (PRRs) that are intracellularly localized or expressed on the cell surface\(^1\). Due to biochemically diverse compositions of the pathogen associated molecular patterns (PAMPs) such as LPS, β-glucan, or peptidoglycans, antigen presenting cells (APC) are able to discriminate between a large variety of pathogens\(^2,3\). The Toll-like receptors TLR2, TLR4 and the C-type lectin Dectin-1 are the main receptors involved in the recognition of fungal pathogens\(^4\). Upon pathogen recognition by these receptors, a number of intracellular signalling pathways including NF-kB, PI3K and MAPKs are activated in DCs and characteristic phenotypic and functional changes involved in the process of DC maturation are induced\(^5\). Furthermore, depending on the recognized pathogen, the regulation of transcription factors also influences the expression of specific pro- and anti-inflammatory cytokines. Transcriptional analysis was used to identify key fungus specific transcription factors in the immune response of DCs stimulated with \textit{A. fumigatus} and \textit{C. albicans} compared to the bacterial control antigen LPS. Those fungi were selected as invasive aspergillosis and invasive candidiasis are the most common invasive fungal infections in patients with haematological malignancies\(^6\).

The transcription factor Krüppel-like factor 4 (KLF4) was identified as important player in the immune response to fungal infections. KLF4 has a variety of biological functions and previous work on this transcription factor has demonstrated its roles in both, the development of inflammatory monocytes and in the production of inflammatory cytokines\(^7,8\), thereby KLF4 displayed a pleiotropic nature, as demonstrated by its ability to either

---

\(^1\)University Hospital of Würzburg, Department of Internal Medicine II, WÜ4i, Würzburg, Germany. \(^2\)Septomics Research Centre, Friedrich Schiller University and Leibniz Institute for Natural Product Research and Infection Biology-Hans-Knöll-Institute (HKI), Jena, Germany. \(^3\)Systems Biology/Bioinformatics, Leibniz Institute for Natural Product Research and Infection Biology – Hans-Knöll-Institute, Jena, Germany. \(^4\)Institute of Medical Genetics and Applied Genomics, University of Tübingen, Tübingen, Germany. \(^*\)These authors contributed equally to this work. Correspondence and requests for materials should be addressed to J.L. (email: loeffler_j@ukw.de)
activate or repress transcription, depending on the context. In a murine system, an influence of KLF4 on the Th2 response and on IL-6, a cytokine with pleiotropic functions and plays an important role in the transition from innate to acquired immunity and in promoting Th2 response, was demonstrated. In consequence, we sought to analyse the possible regulatory role of KLF4 in the transition from innate to acquired immunity during fungal infections.

Results

Transcriptome analysis of human DCs identifies fungal specific regulation of KLF4. Human DCs were confronted with A. fumigatus, C. albicans and LPS, which was used as a standard control stimulus. Whole transcriptome analysis was performed at 6 h post infection of four independent biological replicates. Hierarchical clustering analysis was used to examine differences in the response of DCs to LPS and the fungi and revealed a clustering of the A. fumigatus and C. albicans stimulated DCs (Supplementary Figure S1). Stimulation with LPS induced a differential regulation of 2793 genes after 6 h, while confrontation with A. fumigatus and C. albicans resulted in 743 and 1729 differentially regulated genes, respectively. 528 genes were regulated in all tested conditions after 6 h of co-cultivation (Fig. 1A).

These 528 genes were used for an analysis of regulated transcription factors to get a better insight in cellular responses of DCs facing either fungi or LPS and to determine differences in the regulation pattern between fungi and LPS. A total of 1896 genes are listed as transcription factors in the TRANSFAC database, which is a knowledge-based database of published eukaryotic transcription factors (www.biobase-international.com) and 60 transcription factors were differentially regulated among the 528 genes (Fig. 1B). 59 Transcription factors (Fig. 2) were either induced or reduced by all three stimuli. KLF4 was identified as the only transcription factor that showed a different regulation pattern comparing bacterial and fungal stimuli (Fig. 3A). Expression of KLF4 in DCs was strongly reduced during contact with A. fumigatus (log fold change: −2.6, p-value = 2.73E-08) and C. albicans (log fold change: −2.9, p-value = 1.48E-09) whereas LPS stimulation induced a 1.7 log fold up-regulation (p-value = 4.38E-05). This differential regulation was validated by RT-qPCR on RNA isolated from DCs stimulated with A. fumigatus, C. albicans and LPS for 6 and 12 h which showed a strong down-regulation of KLF4 in fungal treated DCs (not shown). In contrast, LPS stimulation induced KLF4 gene expression after 6 h; this induction was time-dependent and not stable over 12 h. Furthermore, we analyzed the regulation of KLF4 by fungi and the bacterial control stimulation at the protein level after stimulation for 6, 12 and 24 h (Fig. 3B). A time-dependent reduction of KLF4 protein in DCs stimulated with either A. fumigatus or C. albicans was observed and significant after 12 and 24 h co-cultivation. The control stimulus LPS induced up-regulation of KLF4, which was confirmed for the time points 6 and 12 h but was not stable over 24 h.

KLF4 is involved in the IL-6 response. One of the main functions of DCs is to act as a bridge between the innate and adaptive immune system. KLF4 was identified by GOrilla analysis to be a member of Gene Ontology categories such as response to stress (GO: 0006950), regulation of programmed cell death (GO: 0043067), regulation of signal transduction (GO: 0009966) and regulation of cytokine production (GO: 0001817). To determine the biological consequence of KLF4 reduction by fungi, the culture condition with induced KLF4 expression was taken for further downstream analysis using RNA interference gene knock down. For these experiments DCs were electroporated with KLF4 siRNA and random, non-silencing (ns) siRNA treated cells served
Figure 2. Regulation of transcription factors. DCs were either stimulated with *A. fumigatus* (Af), *C. albicans* (Ca) (both MOI 1) or LPS (1 μg/ml) in four independent experiments for 6 h. Affymetrix whole genome expression arrays (HGU-219) were performed. Displayed are the fold changes of the 59 of 60 differentially regulated transcription factors identified in Fig. 1B that were commonly regulated by *A. fumigatus*, *C. albicans* and LPS. (A) displays up- and (B) down-regulated transcription factors.

Figure 3. KLF4 was the only transcription factor down-regulated by *A. fumigatus* and *C. albicans* but induced by LPS. DCs were either stimulated with *A. fumigatus* (Af, light shaded), *C. albicans* (Ca, dark shaded) (MOI 1) or LPS (1 μg/ml, black). (A) KLF4 mRNA regulation after 6 h was determined by Affymetrix whole genome expression arrays (HGU-219) of four independent experiments. Displayed are the logfold changes with added FDR adjusted p-Values of the differentially regulated gene KLF4. (B) DC total cellular extracts of three independent experiments, co-cultivated for 6 h (white bars), 12 h (black bars) and 24 h (squared bars) were analyzed by western blotting. The time dependent KLF4 protein regulation was analyzed by ImageJ, relative to untreated control (striped bars) and loading control β-Actin. Significant changes in protein expression are labeled with asterisks (*p* < 0.05, **p** < 0.01 Student's paired t-test). Data is illustrated as mean plus standard error of the mean (SEM).
as controls. siRNA transfection resulted in a >75% KLF4 transcript reduction compared to the non-silencing control (supplementary Figure S2A). In addition, knock-down of KLF4 by siRNA was also confirmed at protein level (supplementary Figure S2B).

To identify down-stream molecules of the transcription factor KLF4, we conducted a literature research and identified CCL2, RANTES, CXCL10, TNFα, IL-10, IL-1 and IL-6 as potential immune relevant targets of KLF414–20. In our experiments, we did not detect any KLF4-dependent regulation of IL10, RANTES, CXCL10, IL1B, and CCL2 but a significant influence on TNFα (supplementary Figure S3). However, IL6 regulation was highly affected by the KLF4 knock-down. While KLF4 silencing in fungal-stimulated DCs weakly reduced IL6 expression, IL6 levels were significantly reduced in LPS treated control DCs, compared to the non-silencing control (51.8%, p-Value = 0.009) (Fig. 4C). Furthermore, the LPS-stimulated and KLF4-siRNA transfected DCs secreted significantly reduced amounts of IL-6 compared to control (42.8%, p-Value 0.0175) (Fig. 4D). This confirmed the gene expression data and showed a KLF4 dependent regulation of IL-6 in DCs at the level of both RNA and protein.

KLF4 regulation is dependent on PRR activation. Activation of human DC is mediated via PRRs. To further investigate which PRRs are involved in differential KLF4 regulation, we stimulated DCs with defined ligands of several PRRs. We used LPS, a known TLR4 and TLR2 ligand and ultra-pure LPS, which exclusively interacts with TLR4. The synthetic triacylated lipoprotein Pam3CSK4 was used for the activation of TLR2/TLR1 signalling and depleted zymosan, a β-glucan, for Dectin-1 activation. All of these PRRs have been shown to be involved in fungal – DC recognition and activation. Additionally, DCs were co-cultivated with E.coli and A. fumigatus conidia to analyse if phagocytic mechanisms might affect regulation of KLF4. Stimulation of DCs with ultrapure LPS and LPS led to increased expression of KLF4. However, stimulation with the TLR2/TLR1 and Dectin-1 ligands Pam3CSK4, depleted zymosan significantly decreased KLF4-expression in DCs (Fig. 5A). These transcription data were further confirmed by western blot analysis (Fig. 5B). At the protein level only depleted zymosan and Pam3CSK4 reduced KLF4 protein. This indicates a PRR-dependent distinction in KLF4 signalling

Figure 4. KLF4 dependent IL-6 regulation. DCs were stimulated with LPS (1μg/ml) A. fumigatus (Af) C. albicans (Ca) (both MOI 1) or left untreated for 6h. (A) IL6 mRNA regulation was determined by microarray analysis. (B) IL-6 secretion was quantified by enzyme linked immunosorbent assay. (C,D) DCs were transfected by electroporation with either non-silencing siRNA (white bars) or with siRNA targeting KLF4 (black bars). 24h after electroporation, DCs were stimulated with A. fumigatus, C. albicans (MO1) and LPS (1μg/ml) for 6h. (C) IL6 mRNA level were quantified by real-time PCR relative to non-silencing control. ALAS1 served as reference gene. (D) Release of IL-6 of LPS stimulated DCs was quantified by enzyme linked immunosorbent assay. Data is illustrated as FC or mean plus SEM of 3 to 4 independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001; Student’s paired t-test).
indicating TLR4 activation to be responsible for KLF4 accumulation and TLR2 and Dectin-1 for reduction of KLF4. Stimulation with E coli significantly reduced KLF4 expression in DCs. However, no KLF4 regulation was observed in DCs stimulated with A. fumigatus conidia that are also internalized by DCs (data not shown).

To further assess the role of the PRRs in KLF4 regulation, RNA interference using siRNA targeting CLEC7A (Dectin-1) mRNA was performed. The siCLEC7A treatment achieved a reduction of CLEC7A mRNA over 90% and on protein level over 80% (not shown). An induction of KLF4 of DCs stimulated with fungi was observed,

Figure 5. KLF4 regulation is dependent on PRR activation. DCs had been co-cultivated with LPS, LPS ultra-pure (LPS up) (1 μg/ml), depleted zymosan (100 μg/ml), Pam3CSK4 (100 ng/ml) or E.coli (MOI1). (A) After 6 h of co-cultivation mRNA was isolated and KLF4 mRNA was quantified by quantitative real-time PCR relative to untreated control (Ctrl). ALAS1 served as reference gene. (B) Western blot of KLF4 was performed of DCs cell lysates co-stimulated for 12h. Data was analyzed relative to untreated Control (Ctrl). β-Actin served as loading control (*p < 0.05, ***p < 0.001 Student’s paired t-test). Data of five independent experiments is illustrated as mean plus SEM. C) Exemplary Western Blot.

Figure 6. Influence of CLEC7A knock down on KLF4. DCs were transfected by electroporation with either non-silencing siRNA (white bars) or with siRNA targeting CLEC7A (black bars). 24h after electroporation, DCs were stimulated with A. fumigatus, C. albicans (MOI1) and LPS (1 μg/ml). (A) KLF4 mRNA level were quantified after 6h by real-time PCR relative to non-silencing control. ALAS1 served as reference gene. (B) Western blot of KLF4 was performed of DCs cell lysates co-stimulated for 12 h. Data was analyzed relative to non-silenced control relative to β-Actin as loading control (*p < 0.05 Student’s paired t-test). Data of four independent experiments is illustrated as mean plus SEM. ( ) Exemplary Western Blot.
while KLF4 was reduced in siCLEC7A transfected DCs stimulated with LPS (Fig. 6A). On protein level an induction of KLF4 in siCLEC7A treated DCs stimulated with either *A. fumigatus*, *C. albicans* or LPS was determined (Fig. 6B).

**Discussion**

*C. albicans* and *A. fumigatus* are opportunistic fungal pathogens that cause potentially life-threatening diseases in individuals with an immunodeficiency or immunosuppression. The incidence of fungal diseases has increased over the past decades; in hematopoietic stem cell transplant recipients invasive aspergillosis and invasive candidiasis are the most common invasive fungal infections.

To further characterize the immune response and immunopathology and to identify distinct marker molecules regulated by fungi a genome wide transcriptome analysis was performed. In this study KLF4 was identified as the only transcription factor that showed inverse expression in DCs if co-cultivated with either *A. fumigatus*, *C. albicans*, *E. coli* or the control stimulus LPS.

Our results confirm data from murine macrophages in demonstrating KLF4 induction in human DCs after LPS stimulation. Interestingly, co-cultivation of DCs with *A. fumigatus* or *C. albicans* led to specific KLF4 down-regulation. This specific expression pattern was time-dependent as shown at RNA and protein levels. In human macrophages, KLF4 was induced after six hours of co-cultivation with LPS, while a prolonged stimulation of 16 h lead to down-regulation in murine macrophages. This implies a role of KLF4 as a mediator of early cellular responses. In a murine model LPS is able to induce KLF4 expression, which in consequence leads to activation of inducible nitric-oxide synthase (iNOS) and nitric oxide, a key mediator in the innate immune defence. This iNOS activation is cooperatively induced with NF-κB p65 (RelA) activation. Thus, besides iNOS activation, KLF4 regulates inflammatory signals via TGF-β1/Smad3 expression, demonstrating its prominent role as a regulator of key signalling pathways. In murine DCs, KLF4 was found to bind to and activate the IL-6 promotor. Moreover, in mouse microglial cells, KLF4 knock-down decreased levels of the pro-inflammatory cytokine IL-6. Consequently, we analysed KLF4-dependent regulation of IL-6 in human DCs by RNAi knock-down of KLF4. This knock-down resulted in reduced IL6 expression levels and reduced IL-6 secretion by DCs. Interestingly, if human DCs were stimulated with fungus, low-level induction of IL-6 was observed, which was influenced by KLF4 knock-down. Expression of other inflammatory molecules such as RANTES, IL10 and CXCL10 were not targeted by KLF4 silencing, indicating specificity for IL6 regulation. Also no influence of KLF4 knockdown on cytokine levels of IL-1α/β, IL-10, IP-10 (CXCL10) and MIP-3α, MCP-1 (CCL2), MIP-1α and RANTES was detected. Relevance of silenced KLF4 on TNF alpha levels was only detectable on mRNA but not on protein level.

DCs and other mononuclear phagocytes express PRRs on their surface to enable the recognition of pathogens. TLR2, TLR4 and Dectin-1 are the major receptors on the surface of DCs involved in fungal recognition. TLR2 is mainly known for the recognition of peptidoglycan, the major components of gram-positive bacterial cell wall; whereas TLR4 is activated by LPS, derived from gram-negative bacteria. TLR deficient mice provided evidence for the involvement of both, TLR2 and TLR4 receptors in the recognition of *Aspergillus spp.* and *Candida spp.* depending on the fungal morphology and cell wall composition, though the main receptor for *A. fumigatus* and *C. albicans* is Dectin-1. Type II transmembrane protein binds to β-glucan, a major structural fungal cell wall component. We dissected which of these receptors is involved in KLF4 regulation. LPS and LPS ultra-pure are ligands for TLR4/TLR2 and TLR4, respectively. Both ligands induced KLF4 expression. On the other hand, activation of TLR2/TLR1 by Pam3CSK4 or Dectin-1 activation by depleted zymosan reduced KLF4 expression (Fig. 7).

Interestingly, stimulation with *E. coli* also reduced KLF4 gene expression, whereas in contrast, no regulation of KLF4 by stimulation with inactivated *A. fumigatus* conidia was observed. Conidia are covered with a hydrophobic rodlet layer that prevents recognition by PRRs. This gives hint on the involvement of the phagocytic machinery on KLF4 regulation. However, phagocytic events alone are obviously not sufficient to impact KLF4 regulation. In consequence, recognition via PRRs seems to be essential for KLF4 regulation.
To determine downstream effects of the transcription factor KLF4, a large variety of cytokine levels was quantified. Cytokine secretion is a main DC function in the transition from innate to acquired immunity. IL-6 was significantly reduced after KLF4 knockdown and subsequent LPS stimulation; these effects were less prominent after fungal stimulation. Thus, specific binding of β-glucan to Dectin-1 activated at least partly alternative pathways, such as CD37 co-localization. The tetraspanin CD37 is important for dectin-1 stabilization in membranes of DCs and controls dectin-1-mediated IL-6 production. Furthermore, both, TLR2 and Dectin-1 collaborate in inflammatory responses and Dectin-1 was shown to amplify TLR2 dependent cytokine induction in mice. Thus, IL-6 production and secretion might depend on the individual stimulus and thereby KLF4 is a regulator of IL-6 levels. A TLR4 dependent attenuation of IL-6 release by α- and β-glucan and galactomannan and an α-glucan dependent IL-6 response via TLR2 signalling was shown. The TLR signalling effects are dependent on the morphological state as hyphae but not conidia selectively down-regulated the TLR4 response. This may indicate an immune evasion strategy of the fungus by reducing the immune system activation.

In summary, we describe for the first time KLF4 as an important regulatory factor in the host response to fungi and its PRR-dependent regulation. Furthermore, an influence of KLF4 on IL-6 secretion was shown in human DCs. IL-6 is a pleiotropic cytokine that has been implicated in a number of diseases and inflammatory settings and plays an important role in the transition from innate to acquired immunity. At the transcriptional level, it is similarly regulated in response to fungal and bacterial infection, both in DCs and human whole blood. A major function of IL-6 is T-cell recruitment, but IL-6 also takes part in naïve CD4+ T-cell differentiation, thereby promoting Th2 and Th17 responses. The Th1/Th2 balance is known to be a critical factor which determines the outcome of invasive fungal infections. KLF4 may influence the Th1/Th2 balancing via IL6 and thereby be a key regulator in fungal infections.

**Methods**

**Fungal and bacterial strains and growth conditions.** The fungal strain *A. fumigatus* ATCC 46645 (American Type Culture Collection, LGC Standards) was used for all experiments. Conidia and germ tubes were prepared as previously described. Germ tubes were inactivated by incubation in 100% ethanol for 45 min. Wild-type *C. albicans* SC5314 was maintained as previously described. For experiments, colonies were transferred to M199 medium (PAA), pH 4 and cultured at 37°C to stationary phase. Germ tubes were induced by culturing in M199 medium, pH 8 for one hour at 37°C. *E. coli* ATCC25922 was cultured in LB (1% tryptone, 0.5% yeast extract, 1% sodium chloride, Carl Roth) medium. *C. albicans* and *E. coli* cells were inactivated by washing them in phosphate-buffered saline (PBS) followed by incubation in PBS containing 0.1% thimerosal (Sigma-Aldrich) for one hour at 37°C with shaking. Afterwards, cells were washed 5 times and then re-suspended in RPMI-1640. Killing of *C. albicans* and *E. coli* cells was confirmed by plate counts on YPD (2% D-glucose, 1% peptone, 5% yeast extract, Roth) or LB agar plates, respectively.

**Generation of Monocyte-derived DCs.** DCs were generated from PBMCs as previously described. Briefly, PBMCs were isolated from healthy volunteers by ficoll (Bicoll Separation, Biochrom AG) density gradient centrifugation. Magnetic activated cell sorting with paramagnetic CD14-beads (Miltenyi Biotec) was used to further separate monocytes. Monocyte-derived dendritic cells (DCs) were generated in RPMI-1640 supplemented with 10% FBS (Sigma-Aldrich), 120 mg/l Refobacin (Merck), 10 ng/ml IL-4 (Miltenyi Biotec) and 100 ng/ml GM-CSF (Bayer Healthcare) for 5 to 6 days.

Co-cultivation experiments of DCs with *A. fumigatus* or *C. albicans* and *E. coli* were performed on day 6 with a multiplicity of infection (MOI) of 1 in a concentration of 10^{10}/ml (LPS 1 μg/ml, Sigma-Aldrich), LPS ultra-pure (1 μg/ml), zymosan depleted (100 μg/ml), Pam3CSK4 (100 ng/ml) (Invivogen) were used in the indicated concentrations.

**RNA interference.** All RNA interference experiments were performed as previously described. Briefly, DCs were electroporated (EPI 2500, Dr. L. Fischer) with either short interfering double stranded KLF4-siRNA, CLEC7A-siRNA or non-silencing, random RNA (Qiagen) at 340 V for 10 ms on day 5 after isolation and then incubated at 37°C and 5% CO₂ for 24 h in culture medium.

**RNA isolation.** RNA was isolated by using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA quantity was determined using a Nanodrop 1000 (Thermo Scientific).

**Real Time RT-PCR.** cDNA was generated from 100 ng template mRNA by First Strand cDNA synthesis Kit (Thermo Fisher Scientific). Quantitative real-time PCR was performed on a StepOne Plus (Applied Biosystem) with iTaq Universal SYBR Green Supermix (BioRad) and the primers for *KLF4* (NM_004235.4) fw: TACCAAGAGCTGTTGGCCACC, rv: CGGTTAATCACAAGTGTGGG, *IL6* (NM_000600.3) fw: AAGAAGGCAGCTGGCAGAAA, rv:TTGTCACAGGACATTCTC, *CCL2* (NM_002982.3) fw: CACCTCTTGTTAT, rv: AGATCTCTTTGGCCACATG, *TNF* (NM_000594.2) fw TGCTTGGTTCCCTCACGCTTC, *CXCL10* (NM_001565.3) fw: GCCAGTGCACCTGCTGTAT, rv: ATGGTGTCTGGGACGAGATG, *CXCL11* (NM_000572.2) fw: TACCTGGAGGAGGTGATGC, rv: GGCCCTGGCTTTGCTTCCTCC, *RANTES* (NM_002985.2) fw: GAGGAGGTTCTGGACCAATG, *ALAS1* (NM_000688.5) fw: GGCAGCAGACATGGATCAGA, rv: CTTCCATCGGTGTTCACACT (Sigma-Aldrich) were used in a concentration of 0.5 μM at a total reaction volume of 20 μl.

**Transcriptome Analysis.** DCs of four independent donors were harvested after six hours co-culture with *A. fumigatus*, *C. albicans* and LPS and analysed with Affymetrix whole genome expression arrays. RNA samples had a RNA integrity number (RIN) above 8 determined by Agilent Bioanalyzer. RNA samples were hybridized to an
Affymetrix HG-U219 array plate. Scanned images were subjected to visual inspection to control for hybridization artefacts and proper grid alignment; these were analysed with AGCC 3.0 (Affymetrix) to generate CEL files. The resulting files, containing a single intensity value for each probe region delineated by a grid on each array image, were imported into Partek Genomics Suite (version 6.6, Partek, St. Louis, MO) for probe set summarization and statistical analysis. Model based Robust Multichip Analysis was performed for probe set summarization to obtain a single intensity value representing transcript abundance for each probe set and thus enable comparisons between arrays, by normalizing and logarithmically transforming array data and stabilizing variance across the arrays. Microarray data has been deposited in the GEO NCBI database (accession code GSE69723).

The R package limma was used to compare the expression of the probe sets between the conditions30,31. A probe set was regarded as significantly differentially expressed; if it has a False-Discovery-Rate (FDR) adjusted p-value < 0.01 a fold-change |FC| ≥ 2 of the mean transcript abundance32. Arrays were validated by RT-PCR of IL6, KLF4, SYK, CCL5 and IL1B. ALAS1 served as reference gene (not shown). Hierarchical clustering was based on the Spearman correlation of the samples, considering only the differentially expressed genes. The hclust function of R was used with “average linkage” as agglomeration method.

For the Gene Ontology (GO) analysis, the tool GOrilla33 was used to find overrepresented GO terms. Terms with an FDR adjusted p-value < 0.001 were considered as significantly enriched.

Quantification of secreted proteins. The concentrations of TNF-α, IL-1α, IL-1β, IL-10, IP-10 (CXCL10), MIP-3α (CCL20), IFN-gamma, IL-12p70, IL-15, IL-18, IL-2 [by Milliplex MAP, Millipore] and MCP-1 (CCL2), MIP-1α (CCL3), RANTES (CCL5) [by ProcartaPlex, Ebioscience] in cell-free supernatants were determined by performing multiplex ELISA assays according to the instructions from the manufacturers.

IL-6 was analyzed using an in-house ELISA assay. In brief, 96-well medium-binding microplates were coated with 2 &μg/ml IL-6 capture antibody (R&D Systems) in PBS overnight. All washing steps were performed with PBS + 0.05% Tween-20. Blocking of non-specific binding was achieved by incubation with PBS containing 1% BSA, 5% Sucrose and 0.05% Sodium trinitride. Recombinant human IL-6 (R&D Systems) in PBS with 0.1% BSA served as standard. The biotinylated IL-6 detection antibody (0.1 μg/ml, R&D Systems) was diluted in 20 mM Trizma base, 150 mM sodium chloride and 0.1% BSA. HRP Streptavidin (Biologend) was used in 1:1000 dilutions in PBS + 1% BSA. TMB solution was ordered at Biologend. 1M hydrochloric acid served as stopping solution. Absorbance was measured at 450 nm at a spectrophotometer (Tecan).

Statistics. All experiments were performed in sets of at least three independent experiments and the generated data was statistically analyzed by paired, two-tailed Student’s t-test (GraphPad Prism 5, GraphPad Software Inc.). Statistical values of p < 0.05 (*), 0.01 (**) and 0.001 (***) were considered significant.

Ethical Approval. All methods were carried out in accordance with the approved guidelines #302/12 of the Ethical Committee of the University of Wuerzburg. Informed consent was written and provided by all study participants. Data analysis was conducted anonymously.

References
1. Wuthrich, M., Deepe, G. S. Jr. & Klein, B. Adaptive immunity to fungi. Annual review of immunology 30, 115–148, doi:10.1146/annurev-immunol-020711-074958 (2012).
2. Romani, L. Immunity to fungal infections. Nature reviews. Immunology 11, 275–288, doi:10.1038/nri2939 (2011).
3. Mogensen, T. H. Pathogen recognition and inflammatory signaling in innate immune defenses. Clinical microbiology reviews 22, 240–273, Table of Contents, doi:10.1111/j.1556-9562.2008.00046.x (2009).
4. Ferwerda, G., Meyer-Wentrup, F., Kulberg, B. J., Neta, M. G. & Adema, G. J. Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. Cellular microbiology 10, 2058–2066, doi:10.1111/j.1462-5822.2008.01188.x (2008).
5. Fang, F. et al. Transcription factor E2F1 suppresses dendritic cell maturation. Journal of immunology 184, 6084–6091, doi:10.4049/jimmunol.0902561 (2010).
6. Kontoyiannis, D. P. et al. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001-2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 50, 1091–1110, doi:10.1086/651263 (2010).
7. Feinberg, M. W. et al. Kruppel-like factor 4 is a mediator of proinflammatory signaling in macrophages. The journal of biological chemistry 280, 38247–38258, doi:10.1074/jbc.M509378200 (2005).
8. Alder, J. K. et al. Kruppel-like factor 4 is essential for inflammatory monocyte differentiation in vivo. Journal of immunology 180, 5645–5652 (2008).
9. Evans, P. M. & Liu, C. Role of Krüppel-like factor 4 in normal homeostasis, cancer, and stem cells. Acta biochimica et biophysica Sinica 40, 554–564 (2008).
10. Schell, J., Chalaris, A., Schmidt-Arras, D. & Rose-John, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research 1813, 878–888, http://dx.doi.org/10.1016/j.bbamcr.2011.01.034 (2011).
11. Tussiwand, R. et al. KLF4 expression in conventional dendritic cells is required for T helper 2 cell responses. *Immunity* **42**, 916–928, doi: 10.1016/j.immuni.2014.09.017 (2015).

12. Scheller, J., Chalaris, A., Schmidt-Arras, D. & Rose-John, S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et biophysica acta* **1813**, 878–888, doi: 10.1016/j.bbamcr.2011.01.034 (2011).

13. Wingender, E., Dutzé, P., Karas, H. & Knüppel, R. TRANSFAC: a database on transcription factors and their DNA binding sites. *Nucleic acids research* **24**, 238–241 (1996).

14. Liu, J. et al. KLF4 regulates the expression of interleukin-10 in RAW264.7 macrophages. *Biochemical and Biophysical Research Communications* **362**, 575–581, doi: http://dx.doi.org/10.1016/j.bbrc.2007.07.157 (2007).

15. Hamik, A. et al. Krüppel-like factor 4 regulates endothelial inflammation. *The Journal of biological chemistry* **282**, 13769–13779, doi: 10.1074/jbc.M700782200 (2007).

16. Liao, X. et al. Krüppel-like factor 4 regulates macrophage polarization. *The Journal of clinical investigation* **121**, 2736–2749, doi: 10.1172/JCI45444 (2011).

17. Guan, H. et al. KLF4 is a tumor suppressor in B-cell non-Hodgkin lymphoma and in classic Hodgkin lymphoma. Vol. 116 (2010).

18. Kaushik, D. K., Gupta, M., Das, S. & Basu, A. Krüppel-like factor 4, a novel transcription factor regulates microglial activation and subsequent neuroinflammation. *Journal of neuroinflammation* **7**, 68, doi: 10.1186/1742-704X-7-68 (2010).

19. Liu, J. et al. Krüppel-like factor 4 inhibits the expression of interleukin-1 beta in lipopolysaccharide-induced RAW264.7 macrophages. *FEBS Letters* **586**, 834–840, doi: http://dx.doi.org/10.1016/j.febslet.2012.02.003 (2012).

20. Rosenzweig, J. M., Glenn, J. D., Calabresi, P. A. & Whartenby, K. A. KLF4 modulates expression of IL-6 in dendritic cells via both promoter activation and epigenetic modification. *The Journal of biological chemistry* **288**, 23868–23874, doi: 10.1074/jbc.M113.479576 (2013).

21. Chai, L. Y. A. et al. Aspergillus fumigatus cell wall components differentially modulate host TLR2 and TLR4 responses. *Microbes and Infection* **13**, 151–159, doi: http://dx.doi.org/10.1016/j.micinf.2010.10.005 (2011).

22. Liu, J. et al. KLF4 promotes the expression, translocation, and release of HMGB1 in RAW264.7 macrophages in response to LPS. *Shock* **30**, 260–266, doi: 10.1097/shk.0b013e318162ef77 (2008).

23. Chai, L. Y. et al. Aspergillus fumigatus cell wall components differentially modulate host TLR2 and TLR4 responses. *Microbes and infection*/*Institut Pasteur* **13**, 151–159, doi: 10.1016/j.micinf.2010.10.005 (2011).

24. Meeger, M. et al. Proinflammatory response of immature human dendritic cells mediated by dectin-1 after exposure to Aspergillus fumigatus germ tubes. *The Journal of infectious diseases* **197**, 924–931, doi: 10.1086/528694 (2008).

25. Chai, L. Y. et al. Modulation of Toll-like receptor 2 (TLR2) and TLR4 responses by Aspergillus fumigatus. *Infection and immunity* **77**, 2184–2192, doi: 10.1128/IAI.01455-08 (2009).

26. Gantner, B. N., Simmons, R. M. & Underhill, D. M. Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments. *The EMBO journal* **24**, 1277–1286, doi: 10.1038/sj.emboj.7600594 (2005).

27. Aimanianda, V. et al. Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* **460**, 1117–1121, doi: http://www.nature.com/nature/journal/v460/n7259/suppinfo/nature08264_S1.html (2009).

28. Dix, A. et al. Biomarker-based classification of bacterial and fungal whole-blood infections in a genome-wide expression study. *Frontiers in Microbiology* **6**, 171, doi: 10.3389/fmicb.2015.00171 (2015).

29. Leonhardt, L. et al. The Fungal Quorum-Sensing Molecule Farnesol Activates Innate Immune Cells but Suppresses Cellular Adaptive Immunity. *mbio* **6**, e00143–00115, doi: 10.1128/mBio.00143-15 (2015).

30. G., S. Llinas: Linear models for microarray data, in *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. 397–420, doi: 10.1007/0-387-29362-0/23 (2005).

31. Gentleman, R. C. et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* **5**, R80 (2004).

32. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Roy Stat Soc B Methodol*. 289–300 (1995).

33. Eden, E., Navon, R., Steinfeld, I., Lipson, D. & Yakini, Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* **10**, 48–48, doi: 10.1186/1471-2105-10-48 (2009).

Acknowledgements

The authors thank Oliver Morton, University of Western Sydney, Australia, for linguistic corrections and Mirjam Fliesser, University Hospital of Würzburg, Department of Internal Medicine II, WU4i, Germany, for critical reading of the manuscript. This work was supported by IZKF Würzburg, project A127 and the Deutsche Forschungsgemeinschaft (DFG) CRC/Transregio 124 “Pathogenic fungi and their human host: Networks of interaction”, subprojects A2, B3, C3 and INF.

Author Contributions

K.C. and J.L. contributed equally to this work. K.C. wrote the main manuscript text and prepared Figures 1–7. A.D. and J.L. were responsible for Microarray analysis and prepared Figure S1. Microarrays were performed in the laboratory of M.B. H.E., O.K. and J.L. equally supervised the work. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Czakai, K. et al. Krüppel-like Factor 4 modulates interleukin-6 release in human dendritic cells after in vitro stimulation with Aspergillus fumigatus and Candida albicans. *Sci. Rep.* **6**, 27990; doi: 10.1038/srep27990 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/