Calcineurin Orchestrates Lateral Transfer of *Aspergillus fumigatus* during Macrophage Cell Death

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Abstract

Rationale: Pulmonary aspergillosis is a lethal mold infection in the immunocompromised host. Understanding initial control of infection and how this is altered in the immunocompromised host are key goals for comprehension of the pathogenesis of pulmonary aspergillosis.

Objectives: To characterize the outcome of human macrophage infection with *Aspergillus fumigatus* and how this is altered in transplant recipients on calcineurin inhibitor immunosuppressants.

Methods: We defined the outcome of human macrophage infection with *A. fumigatus*, as well as the impact of calcineurin inhibitors, through a combination of single-cell fluorescence imaging, transcriptomics, proteomics, and *in vivo* studies.

Measurements and Main Results: Macrophage phagocytosis of *A. fumigatus* enabled control of 90% of fungal germination. However, fungal germination in the late phagosome led to macrophage necrosis. During programmed necroptosis, we observed frequent

cell–cell transfer of *A. fumigatus* between macrophages, which assists subsequent control of germination in recipient macrophages. Lateral transfer occurred through actin-dependent exocytosis of the late endosome in a vasodilator-stimulated phosphoprotein envelope. Its relevance to the control of fungal germination was also shown by direct visualization in our zebrafish aspergillosis model *in vivo*. The calcineurin inhibitor FK506 (tacrolimus) reduced cell death and lateral transfer *in vitro* by 50%. This resulted in uncontrolled fungal germination in macrophages and also resulted in hyphal escape.

Conclusions: These observations identify programmed, necrosis-dependent lateral transfer of *A. fumigatus* between macrophages as an important host strategy for controlling fungal germination. This process is critically dependent on calcineurin. Our studies provide fundamental insights into the pathogenesis of pulmonary aspergillosis in the immunocompromised host.

Keywords: pulmonary fungal diseases; macrophage; necrosis; *Aspergillus*; calcineurin

Aspergillus fumigatus (Af) is a mold with small airborne conidia that are a ubiquitous component of the aerial mycobiota (1). Normally inhaled conidia are cleared by alveolar macrophages to prevent fungal

germination, tissue invasion, and destructive lung disease (2, 3). Our previous work with murine and zebrafish models of invasive aspergillosis showed the importance of macrophages in control of

Af early postinfection, with neutrophil influx as a later event (4, 5). Previous studies have highlighted the importance of inflammatory monocytes in murine invasive aspergillosis (6).

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At a Glance Commentary

Scientific Knowledge on the

Subject: Macrophages are the frontline defense against pulmonary infection with the mold pathogen *Aspergillus fumigatus*. Phagocytosis and generation of reactive oxygen species are known to be crucial for control of infection. However, the cellular outcome from progressive macrophage infection with *A. fumigatus* has not been studied systematically.

What This Study Adds to the

Field: We present a systematic analysis of human macrophage infection with *A. fumigatus*. We show that successful fungal germination in the phagosome leads to necrotic cell death and can result in cell–cell transfer of germinating *A. fumigatus* between macrophages. Ultimately, this process assists control of fungal germination and is orchestrated through the calcium-responsive serine-threonine phosphatase calcineurin. Our systematic analysis of the macrophage response to *A. fumigatus* defines necroptosis as a key early event in the pathogenesis of pulmonary aspergillosis.

A spectrum of immunocompromised states predisposes individuals to high-mortality invasive or chronic forms of pulmonary aspergillosis (PA) (7). Organ transplant recipients are at high risk of PA, with lung transplant recipients being particularly susceptible and having a mortality greater than 40% (8, 9). There are an estimated 3 million individuals with chronic PA and 4.5 million with allergic bronchopulmonary aspergillosis globally (10, 11). *Af* has also been implicated in the pathogenesis of asthma, chronic obstructive airways disease, and bronchiectasis (11–13). Treatment options remain limited, and high mortality rates persist (14). Better understanding of susceptibility to PA in the nonneutropenic host is a key clinical research priority.

Calcineurin–nuclear factor of activated T cells (NFAT) signaling is the target of the calcineurin inhibitor immunosuppressants

used in organ transplantation (15). We have previously shown that calcineurin inhibitor immunosuppressants (FK506 [tacrolimus]) increase susceptibility to murine PA through inhibition of the macrophage Toll-like receptor 9–calcineurin–NFAT pathway (4, 5). This pathway is crucial for activation of inflammatory responses to *Af* and for recruitment of fungicidal neutrophils to the site of infection. Calcineurin–NFAT signaling has also been shown to be critical for the innate immune response to *Candida albicans* and *Escherichia coli* through endocytic mechanisms convergent on phospholipase C- γ -dependent calcium flux (16, 17). These observations underscore the importance of calcineurin–NFAT signaling for myeloid immunity, and they reveal a novel relationship between innate sensing and calcineurin–NFAT signaling in the lung that requires further clinical definition.

Here we show that host programmed necrosis is an important outcome of *Af* germination in the human macrophage. Cell death occurred in response to phagosomal germination, with necroptosis-associated lateral transfer of *Af* between macrophages. Cell death–dependent transfer was calcineurin dependent and enabled control of germination in recipient macrophages. Crucially, inhibition of either calcineurin-dependent cell death or lateral transfer enabled hyphal escape from the macrophage. In this study, we identify programmed necrosis as a key cellular response in PA and show that it is likely to be critically impaired in organ transplant recipients taking calcineurin inhibitors. Our studies reveal cell–cell transfer as a novel and important cell death–associated defense mechanism that enables control of fungal germination in the myeloid compartment.

Methods

Ethics Statement

The present study was approved by the Biomedical Research Unit (National Research Ethics Service reference 10/H0504/9), Royal Brompton and Harefield NHS Trust (AS1). All experiments conformed to the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all participants.

Zebrafish Care and Maintenance

Our experiments were approved by the U.K. Home Office in accordance with project license PPL 70/7446. Full details are given in the EXPERIMENTAL PROCEDURES section of the online supplement.

Fungal Strains and Culture

A. fumigatus strain CEA10 was used for Western blotting, Luminex Multiplex (Luminex, Austin, TX), fungal burden, ImageStream (EMD Millipore, Billerica, MA), phosphoproteomic, and RNA sequencing experiments. ATCC 46645 enhanced green fluorescent protein (American Type Culture Collection, Manassas, VA), a gift from Frank Ebel, was used for microscopy experiments. See EXPERIMENTAL PROCEDURES section in the online supplement for details.

Isolation of Human Macrophages

Human alveolar macrophages (hAMs) were isolated from bronchoalveolar lavage by adherence. Purified hAMs were rested for 3 days. Monocytes were isolated from peripheral blood mononuclear cells from healthy volunteers by Ficoll–Paque gradient centrifugation and negative magnetic bead isolation (Pan Monocyte Isolation Kit; Miltenyi Biotec, Auburn, CA). Human monocyte-derived macrophages (hMDMs) were differentiated using 5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10% human serum for 7 days. See EXPERIMENTAL PROCEDURES section in the online supplement for details.

RNA-seq Analysis

Illumina RNA-seq libraries (Illumina, San Diego, CA) were prepared from hMDMs ($n = 6$ healthy donors) pretreated with 10 ng/ml FK506 for 1 hour or vehicle and stimulated with live *Af* swollen conidia (SC) (multiplicity of infection, 1) for 1 hour and 6 hours. Illumina paired-end reads were mapped to the human reference genome (hg19) using TopHat2 (18) (Gene Expression Omnibus database accession number GSE74924), and differential expression analyses were performed using Cufflinks v2.2.1 (19). Gene analyses were performed using DAVID v6.7 (20). Human protein–protein interaction network modules were obtained using MCODE (21) analysis in Cytoscape. See EXPERIMENTAL

PROCEDURES section in the online supplement for details.

Phosphoproteomic Analysis

Analysis with the Phospho Explorer Antibody Microarray was conducted by Full Moon BioSystems Inc. (Sunnyvale, CA).

hMDMs were differentiated at 1×10^7 cells in 75-mm² tissue culture flasks. At Day 7, cells were stimulated with live *Af* SC (multiplicity of infection, 1) following pretreatment for 1 hour with FK506 (10 ng/ml; Calbiochem, San Diego, CA) or vehicle (dimethyl sulfoxide diluted in RPMI

1640 medium). At 1 hour postinfection, cells were washed five times with 10 ml of ice-cold phosphate-buffered saline containing protease inhibitors (Cell Signaling Technology, Danvers, MA) and collected by centrifugation ($250 \times g$ for 10 min at 4°C). Cells were frozen at -80°C

Figure 1. *Aspergillus fumigatus* (*Af*) activates calcineurin-nuclear factor of activated T cells (NFAT)-dependent inflammatory responses in human macrophages. (A) Human monocyte-derived macrophages (hMDMs) were stimulated with live *Af* swollen conidia (SC) (multiplicity of infection [MOI], 1). hMDM nuclear extracts were collected at 30, 60, and 120 minutes postinfection and probed by Western blotting for NFAT, cytoplasmic, calcineurin-dependent 2 (NFATc2). The maximum translocation of NFATc2 to the nucleus was observed at 60 minutes postinfection. Western blots show representative data from four experiments. (B and C) hMDMs were pretreated with FK506 (10 ng/ml) and stimulated with live *Af* SC (MOI, 1). Nuclear extracts were probed by Western blotting for NFATc2 and whole-cell extracts for regulator of calcineurin 1 (RCAN-1). NFAT translocation was assessed by confocal microscopy. NFAT is shown in red, nuclei in blue, and *Af* conidia in green. Representative images of live conidia stimulating NFAT nuclear translocation are shown. Western blots show representative data from four experiments. (D) hMDMs were pretreated with FK506 (10 ng/ml) and stimulated with live *Af* SC (MOI, 1). FK506 completely inhibited RCAN-1 production. Western blot shows representative data from four experiments. (E) hMDMs were pretreated with FK506 (10 ng/ml) and stimulated with live *Af* SC (MOI, 1). Culture supernatant cytokines were measured at 6 hours postinfection by Luminex assay ($n = 7$ biological replicates). Scale bars = 10 μm . BF = brightfield; GM-CSF = granulocyte-macrophage colony-stimulating factor; HDAC1 = histone deacetylase 1; MCP-1 = monocyte chemoattractant protein 1; MIP-1 = macrophage inflammatory protein 1; NF-κB = nuclear factor-κB; TNF-α = tumor necrosis factor-α.

and transferred to Full Moon BioSystems on dry ice. The array consisted of 1,318 phospho-specific antibodies. Proteins were labeled with biotin and adhered to preblocked microarray slides. After a washing step, detection of total and phosphorylated proteins was conducted using cyanine 3-conjugated streptavidin. Expression of phosphorylated proteins was normalized to the corresponding total protein abundance. Fold change was calculated as the phosphoprotein-to-nonphosphoprotein ratio of FK506-treated cells divided by the phosphoprotein-to-nonphosphoprotein ratio of untreated cells.

Statistical Analysis

The results are presented as mean $\pm$ SEM and were analyzed using Prism software (version 6.0; GraphPad Software, La Jolla, CA). Significance was determined using Student's *t* test for unpaired

observations. When three or more groups were compared, we used one-way analysis of variance with the Bonferroni correction. Full details of the experimental procedures are available in the online supplement.

Results

A. fumigatus Activates Calcineurin-NFAT-Dependent Inflammatory Responses in Human Macrophages

Af phagocytosis activates NFAT nuclear translocation in murine macrophages (4). We therefore characterized the role of NFAT in hMDMs. hMDMs were infected with live *Af* SC or resting conidia (RC), and NFAT translocation was determined by Western blot analysis and confocal microscopy. SC phagocytosis led to sustained NFAT translocation after 30 minutes (Figures 1A and 1B; see also Figure E1A in the

online supplement). RC induced a weak, transient NFAT activation response (Figures E1B and E1C). NFAT translocation in response to SC was confirmed in hAMs from lung transplant recipients (Figure E1D). Maximum translocation of NFATc2 to the nucleus was seen in response to *Af* at 60 minutes postinfection. NFAT translocation was blocked by the calcineurin inhibitor FK506 (Figures 1B, 1C, and E1E), but nuclear factor- κ B Rel65 translocation was unaffected (Figure 1B).

NFAT transcriptional activity was defined by measuring expression of the NFAT-specific target regulator of calcineurin 1 (*RCAN1*). SC infection increased *RCAN1* expression, which was abrogated by FK506 (Figures 1D and E1F). To determine the role of the calcineurin-NFAT pathway in inflammatory responses, we quantified chemokine and cytokine production in hMDMs during SC

Figure 2. Human macrophage phagocytosis, reactive oxygen species (ROS) production, and control of *Aspergillus fumigatus* (*Af*) growth are calcineurin dependent. (A) Human monocyte-derived macrophages (hMDMs) pretreated with FK506 (10 ng/ml) or vehicle were stimulated with live *Af* swollen conidia (SC) (multiplicity of infection [MOI], 1), and total RNA was isolated at 1 and 6 hours postinfection. Fungal growth was assayed by reverse transcription-polymerase chain reaction of fungal RNA (*n* = 5 biological replicates). (B) hMDMs pretreated with FK506 (10 ng/ml) or vehicle were infected with live enhanced green fluorescent protein-expressing *Af* SC (MOI, 1), and hyphal transition was assessed using time-lapse video microscopy over a 10-hour period (*n* = 4 biological replicates). (C) hMDMs pretreated with FK506 (10 ng/ml) or cytochalasin D (5 nM) or vehicle were stimulated with live *Af* SC (MOI, 1) prestained with calcofluor white. Phagocytosis was quantified by using an ImageStream flow cytometer postinfection (*n* = 4 biological replicates). (D) hMDMs pretreated with FK506 (10 ng/ml) or vehicle were stimulated with live *Af* SC (MOI, 1), and ROS production was assayed by luminescence (*n* = 4 biological replicates). (E) hMDMs pretreated with FK506 (10 ng/ml) or vehicle were stimulated with live *Af* SC (MOI, 1), and cathepsin B activation was assayed by live confocal time-lapse microscopy. Phagosomal cathepsin B activation was quantified by using ImageJ software (National Institutes of Health, Bethesda, MD) (*n* = 4 biological replicates). (F) Representative confocal microscopic image showing cathepsin B activation in *Af* conidia-containing phagosomes in hMDMs. Activated cathepsin B is shown in red, nuclei in blue, and *Af* in green. Data are presented as mean $\pm$ SEM. Scale bars = 10 μ m. BF = brightfield; RFU = relative fluorescence units.

Figure 3. Macrophages infected with swollen *Aspergillus fumigatus* (Af) undergo calcineurin-dependent lateral transfer. (A) Representative time-lapse widefield microscopic images of Af lateral transfer in human monocyte-derived macrophages (hMDMs) infected with live Af swollen conidia (SC) (multiplicity of infection [MOI], 1), shown in green. Transferring macrophage is labeled a, and receiving macrophage is labeled b. Images were taken at 10-minute intervals. (B) hMDMs were stimulated with live enhanced green fluorescent protein (eGFP)-Af SC (MOI, 1), and time-lapse widefield imaging was performed at 10-minute

Video 1. Human monocyte-derived macrophages infected with swollen *Aspergillus fumigatus* (Af) undergo lateral transfer. Representative time-lapse widefield microscopy images show Af lateral transfer in human monocyte-derived macrophages infected with live Af swollen conidia (multiplicity of infection, 1; shown in green). Lateral transfer is highlighted with a black arrow. The images were taken at 10-minute intervals.

infection. Expression of tumor necrosis factor- α (TNF- α), GM-CSF, monocyte chemoattractant protein 1 (MCP-1), MCP-1 α , and MCP-1 β was attenuated by calcineurin inhibition ($P = 0.02$, $P = 0.02$, $P = 0.03$, $P = 0.0003$, and $P = 0.009$, respectively) (Figures 1E and 1G). Flow cytometric characterization of hMDMs and lung transplant hAMs revealed similar inflammatory activation status with CD11c⁺/major histocompatibility complex class II⁺/CD206⁺ surface expression. There were significant differences in surface CD11b ($P \leq 0.001$) and CD86 ($P \leq 0.001$) expression (Figure 1H). Blockade of the fungal C-type lectin receptor Dectin-1 ($P = 0.001$) or inhibition of downstream Syk signaling ($P = 0.001$) impaired TNF- α

responses, but it had no effect on NFAT translocation (Figures E1I and E1J). These results indicate that the calcineurin-NFAT signaling pathway is critical for early macrophage inflammatory responses to Af, independent of Dectin-1 and Syk, in humans.

Human Macrophage Phagocytosis, Reactive Oxygen Species Production, and Killing of *A. fumigatus* Are Calcineurin Dependent

Next, we characterized the role of calcineurin-NFAT in killing of Af. hMDMs were infected with SC following treatment with FK506 or vehicle, and fungal growth was analyzed at 1 and 6 hours postinfection. Both hMDMs and hAMs pretreated with FK506 exhibited impaired control of fungal growth ($P < 0.001$ and $P < 0.0001$, respectively) (Figures 2A and E2A). Time-lapse video microscopy demonstrated increased Af hyphal transition in FK506-treated macrophages ($P = 0.02$) (Figure 2B). To identify the calcineurin-dependent mechanism of fungal growth inhibition, we assessed the efficiency of Af phagocytosis. Calcineurin inhibition delayed phagocytosis of SC in hMDMs ($P < 0.01$) and hAMs ($P = 0.04$) early postinfection, but phagocytosis of RC was not affected (Figures 2C, E2B, and E2C). We measured reactive oxygen species (ROS) production and cathepsin B (a lysosomal cysteine protease) activation, which are critical for Af killing (22, 23). ROS production was partially calcineurin dependent ($P < 0.0001$) but had no effect on mitochondrial ROS (Figures 2D, E2D, and E2E) or activated cathepsin B production (Figures 2E and 2F). Calcineurin-dependent effects on phagosomal maturation were analyzed by phagosomal acidification and recruitment of the late phagosomal maturation marker lysosomal-associated membrane protein 1 (LAMP-1) to Af-containing

Video 2. Human alveolar macrophages (AMs) infected with swollen *Aspergillus fumigatus* (Af) undergo lateral transfer. Representative time-lapse widefield microscopy images show Af lateral transfer in human AMs infected with live Af swollen conidia (multiplicity of infection, 1; shown in green). Lateral transfer is highlighted with a black arrow. The images were taken at 10-minute intervals. Scale bar = 10 μ m.

phagosomes. Calcineurin inhibition increased LAMP-1 recruitment at early time points after infection ($P < 0.05$) (Figures E2F and E2G). Phagosomal acidification was not calcineurin dependent (Figures E2H and E2I). These results indicate that inhibition of the calcineurin-NFAT pathway impairs the ability of human macrophages to control fungal hyphal transition, delays fungal phagocytosis, and reduces ROS production.

Macrophages Traffic Af-Containing Endosomes to Neighboring Cells through Calcineurin-Dependent Lateral Transfer

During live time-lapse confocal imaging, we observed lateral transfer of germinating Af between hMDMs (Figure 3A and

Figure 3. (Continued). intervals for 12 hours postinfection. The timing of lateral transfer events per hour in macrophages infected with live Af SC is shown ($n = 3$ biological replicates). (C) hMDMs pretreated with FK506 (10 ng/ml) or vehicle were stimulated with live eGFP-Af SC (MOI, 1), and lateral transfer events were quantified by time-lapse widefield imaging at 10-minute intervals for 12 hours postinfection ($n = 5$ biological replicates). Total lateral transfer events over the 12-hour period were quantified for each biological replicate. (D) hMDMs were stimulated with live or fixed Af SC or resting conidia (MOI, 1), and lateral transfer events were quantified by time-lapse widefield microscopy. Cytochalasin D (5 nM) and dynasore (100 μ M) were added at 90 minutes postinfection, and unbound conidia were washed away 45 minutes postinfection ($n = 4$ biological replicates). (E) Representative confocal microscopic image of dynamin-dependent lateral transfer in hMDMs stimulated with live Af SC. Dynamin is shown in red, nuclei in blue, and Af in green. (F) hMDMs were stimulated with live Af SC (MOI, 1), and nonphagocytosed conidia were washed away at 45 minutes postinfection. Lateral transfer events were captured by fixing cells every 10 minutes from 120 to 180 minutes postinfection. Characterization of Af lateral transfer was performed by staining for Ras-related protein 5 (Rab 5), Rab 7, and lysosomal-associated membrane protein 1 (LAMP-1) (shown in red) alongside actin (shown in cyan). Nuclei are shown in blue and live Af SC in green. Representative images show positive Rab 7 localization to Af-containing endosomes during lateral transfer, with little Rab 5 localization and no LAMP-1 localization. (G) mpeg: mCherry zebrafish larvae were infected with approximately 50 eGFP-expressing conidia of Af, and time-lapse confocal microscopy was performed. Representative images show Af lateral transfer between macrophages. The transferring macrophage is labeled a, and the receiving macrophage is labeled b. Images shown were taken at 7-minute intervals. Data are represented as mean $\pm$ SEM. Scale bars = 10 μ m. BF = brightfield.

Video 1). This was confirmed in alveolar macrophages from lung transplant recipients (Video 2). Lateral transfer events occurred in $3.0 \pm 0.4\%$ of cells infected with SC, peaking at 4–6 hours postinfection (Figure 3B). Calcineurin inhibition markedly reduced lateral transfer ($53.9 \pm 10.3\%$) ($P = 0.002$) (Figure 3C).

We assessed the role of fungal germination in lateral transfer of *Af* by time-lapse microscopy of metabolically inactive RC or dead, fixed SC. Lateral transfer was dependent on both live and swollen *Af* ($P < 0.001$) (Figure 3D). We observed colocalization of actin and dynamin to conidia undergoing lateral transfer (Figures 3E and 3F), suggesting an actin- and dynamin-dependent process. This was confirmed by treating hMDMs with actin polymerization or dynamin inhibitors (cytochalasin D or dynasore, respectively) following phagocytosis ($P < 0.001$) (Figure 3D). Fungal burden was increased after inhibition of lateral transfer post-phagocytosis using dynasore, suggesting that transfer enables recipient macrophage control of hyphal germination ($P < 0.01$) (Figure E3A). Transmission electron microscopy showed that transfer of *Af*-containing cargo between hMDMs occurred in membrane-bound compartments (Figure E3B). The *Af*-containing cargo transferred between hMDMs was further characterized by staining for endolysosomal markers (Ras-related protein 5 [Rab 5], Rab 7, and LAMP-1). This showed that *Af* was transferred between hMDMs in Rab 7-positive and Rab 5- and LAMP-1-negative compartments, suggesting late endosomal trafficking (Figures 3F and E3C).

To determine whether this phenomenon occurred *in vivo*, we exploited our zebrafish invasive aspergillosis model (4). Using an mpeg:mCherry transgenic zebrafish line and enhanced green fluorescent protein-expressing *Af*, we examined the macrophage-*Af* interaction *in vivo* using time-lapse high-resolution confocal microscopy. We observed similar macrophage *Af* lateral transfer in zebrafish larvae, consistent with an evolutionarily conserved macrophage response to *Af* infection that occurs *in vivo* (Figure 3G and Video 3). Taken together, these observations indicate that successful fungal germination in the late endosome triggers exocytic transfer of *Af* between macrophages.

Macrophages Undergoing Calcineurin-Dependent Programmed Necrosis Transfer *Af* Conidia to Neighboring Cells to Control Fungal Germination

hMDMs transferring *Af* had increasingly translucent cytoplasm, organelle swelling, and increased cell volume, suggesting programmed cell death during transfer. This was confirmed by confocal microscopy using propidium iodide (Figure 4A and Video 4). Around 90% of macrophages successfully control fungal germination and do not die (Figure 4B). Propidium iodide staining revealed that, in dying macrophages ($13 \pm 2\%$), lateral transfer occurred in $37 \pm 5\%$ (Figure 4D). This represents approximately 3–4% of total macrophages, as previously described for those infected with *Af* SC. Transfer of *Af* conidia from dying hMDMs enhanced control of germination as compared with dying hMDMs, where conidia were not transferred ($P = 0.003$) (Figure 4B). Germination of *Af* in live hMDMs was well controlled compared with cells undergoing programmed necrosis (Figure E3D), further supporting a model where programmed cell death and lateral transfer occur in response to successful fungal germination in macrophages to enable containment of infection in recipient macrophages (Figure 4B).

We observed massive vacuole formation adjacent to the *Af*-containing compartment consistent with programmed cell death (Figure E3E and Video 5) (24). Transmission electron microscopy also showed conidia residing within large vacuoles (Figure E3F). Vacuole formation was inhibited by pretreatment with the vacuolar H^+ -ATPase inhibitor bafilomycin ($P = 0.003$), with a trend toward decreased transfer ($P = 0.06$) (Figure E3G).

To define the relationship between cell death and transfer, induction of necroptosis with the pan-caspase inhibitor Z-VAD-FMK (an inhibitor of caspase-dependent apoptosis that increases necroptosis) was performed (25, 26). This increased lateral transfer ($P = 0.003$) (Figure 4C). Addition of necrostatin-1, an inhibitor of receptor-interacting serine/threonine-protein kinase 1-dependent necroptosis, to Z-VAD-FMK inhibited both cell death ($P = 0.078$) and lateral transfer ($P = 0.028$) induced by Z-VAD-FMK alone (Figure 4C). However, induction of necroptosis with Z-VAD-FMK

Video 3. *In vivo* confirmation of macrophage lateral transfer in zebrafish macrophages infected with swollen *Aspergillus fumigatus* (*Af*). MPEG:mCherry Zebrafish larvae were infected with ~50 enhanced green fluorescent protein-expressing conidia of *Af*, and time-lapse confocal microscopy was performed. Representative three-dimensional reconstructed images show *Af* lateral transfer between macrophages. The transfer process is highlighted with a yellow arrow. The images shown are at 7-minute intervals. Scale bar = 10 μ m.

led to increased fungal burden due to recipient macrophage death ($P < 0.01$) (Figure E3H). Necrostatin-1 alone or necrosulfonamide could not inhibit macrophage programmed cell death or lateral transfer (Figure 4C). As pyroptosis following macrophage infection with *C. albicans* was recently shown, we blocked pyroptosis with an irreversible cell-permeable caspase 1 inhibitor (Z-YVAD-FMK), which did not affect cell death or lateral transfer (Figure 4C) (27). These observations are consistent with transfer of *Af* between macrophages during a programmed cell death process with hallmarks of necroptosis.

As calcineurin regulates cell death, we assessed its role in macrophage necrosis following infection (28). Calcineurin inhibition reduced macrophage programmed necrosis by $57 \pm 13.7\%$ ($P = 0.01$) (Figure 4D) and was associated with accelerated fungal germination at late time points in macrophages that failed to undergo necrosis-dependent transfer ($P < 0.001$) (Figures E3I and E3J). This is consistent with a calcineurin-dependent checkpoint for programmed necrosis during *Af* germination.

Together, these results indicate that hMDMs infected with live *Af* SC undergo calcineurin-dependent cell death with hallmarks of programmed necroptosis and transfer *Af*-containing endosomes to neighboring cells to facilitate control of fungal germination.

Figure 4. Macrophages undergoing programmed necrosis laterally transfer germinating *Aspergillus fumigatus* (Af) conidia in endosomes through an actin-dependent process. (A) Representative time-lapse confocal microscopic images of lateral transfer of live Af swollen conidia (SC) (shown in green) between human monocyte-derived macrophages (hMDMs). Propidium iodide (PI) staining (red) shows cell death of macrophage transferring Af conidia (labeled a) to a neighboring macrophage (labeled b). Images were taken at 10-minute intervals. (B) Time-lapse confocal microscopy was used to quantify the fate of transferred versus nontransferred conidia from dying hMDMs and to conidia within live macrophages. Germination was assessed over a 12-hour period. Control of fungal germination was significantly increased in dying hMDMs that transferred conidia to neighboring cells compared with those that did not

Video 4. Macrophages infected with germinating *Aspergillus fumigatus* (Af) conidia undergo lateral transfer during programmed cell death. Representative time-lapse confocal microscopy images show lateral transfer of live Af swollen conidia (shown in green) between monocyte-derived macrophages. Propidium iodide staining (red) shows cell death of a macrophage transferring Af conidia to a neighboring macrophage. The transfer process is highlighted by a black arrow. The images were taken at 10-minute intervals.

Calcineurin Regulates Cell Death and the Mitogen-activated Protein Kinase Pathway during Af Infection

To further define the macrophage response to Af, we undertook next-generation transcript profiling of hMDMs during phagocytosis. hMDMs pretreated with FK506 were infected with SC for 1 hour or 6 hours, and genes differentially expressed compared with baseline and untreated infection controls were identified (Figure E4A). The major gene sets regulated by calcineurin at 6 hours postinfection were associated with cell death, inflammatory responses, and transcriptional regulation (Figure E4B). Fifty-one genes associated with the cell death pathway were calcineurin dependent at 6 hours postinfection (Table E1).

In overrepresentation analysis using ConsensusPathDB, we identified calcineurin-dependent control of the programmed cell death, mitogen-activated protein kinase (MAPK) signaling, and cytokine–cytokine interaction pathways at 6 hours postinfection (Figure E4C). To assess the interaction between the pathways regulated by calcineurin, a protein–protein interaction network was created. By use of this method, we identified significant interactions and clustering of transcriptional regulation pathways, immune response pathways, apoptosis, and the activator protein 1 (AP-1) transcription factor network (Figure 4E). Other studies have suggested a critical role for the MAPK–AP-1 pathway and dynamin-related protein 1 (Drp1)-mediated mitochondrial fission in necroptosis (25, 29). As mitochondrial translocation of Drp1 and MAPK–AP-1 pathway regulation are thought to be calcineurin dependent, this was explored in Af-infected hMDMs (30–32). Significant modulation of MAPK–AP-1 pathway activation and Ser637 P-Drp1 phosphorylation was observed (Figure E4D). Thus, RNA- and protein-level analyses further support a role for calcineurin in the macrophage programmed cell death response to Af.

Af-Containing Endosomes Transferred from Macrophages to Macrophage Are Enveloped within Actin–Vasodilator-stimulated Phosphoprotein-Rich Cargo

Calcineurin phosphatase activity has multiple NFAT-independent effects on cytoskeletal reorganization and regulation of MAPK pathways (31, 33, 34). To identify calcineurin phosphatase targets, we performed a comparative phosphoproteomic array on FK506- versus vehicle-pretreated hMDMs infected with SC (Figure 5A). There were differences in phosphorylation of

Video 5. Massive vacuole formation occurs during *Aspergillus fumigatus* (Af) germination in human monocyte-derived macrophages (hMDMs). Representative time-lapse widefield microscopy images show vacuole formation in hMDMs infected with live Af swollen conidia (multiplicity of infection, 1; shown in green). The images were taken at 10-minute intervals.

proteins within the MAPK pathway, including c-Jun and the cell-cycle regulatory protein M-phase inducer phosphatase 1. This is consistent with our transcriptomic findings of cross-control by calcineurin of the MAPK pathways and cell death pathways.

The analysis revealed a 26-fold increase in Ser238 phosphorylated vasodilator-stimulated phosphoprotein (VASP) levels in FK506-treated hMDMs (Figure 5A). VASP is an actin polymerase that promotes assembly of actin networks, and it is important for directional motility and phagocytosis (35–37). Its function is tightly regulated, with Ser238 dephosphorylation required for actin filament formation. We therefore postulated that VASP is a direct calcineurin target. As calcineurin is the only Ca^{2+} -activated serine-threonine phosphatase, we measured Ser238 P-VASP

Figure 4. (Continued). (n = 4 biological replicates). (C) hMDMs pretreated with a pan-caspase inhibitor (50 μM Z-VAD-FMK), a receptor-interacting serine/threonine-protein kinase (RIPK-1) inhibitor (10 μM necrostatin-1), a caspase 1 inhibitor (50 μM Z-YVAD-FMK), 10 ng/ml FK506, or vehicle were stimulated with live Af SC (multiplicity of infection [MOI], 1), and lateral transfer events and cell death were quantified by PI fluorescence-based time-lapse confocal microscopy. Treatment with Z-VAD-FMK induced necroptotic cell death coupled to increased Af lateral transfer, which was inhibited by the addition of a RIPK-1 inhibitor (necrostatin-1) (n = 3 biological replicates). (D) hMDMs pretreated FK506 (10 ng/ml) or vehicle were stimulated with live Af SC (MOI, 1), and cell death was assayed by PI fluorescence and quantified by time-lapse confocal microscopy over a 6-hour period. The graph also shows the proportion of lateral transfer events in dying cells (approximately 30% of dying cells) (n = 4 biological replicates). (E) The network modules of the significantly expressed genes in FK506-pretreated macrophages stimulated with live Af SC (MOI, 1) for 6 hours. Significantly expressed genes were mapped onto a protein–protein interaction network, and network modules were identified using the MCODE plugin application of Cytoscape. Overrepresented pathways involving these network modules were identified using the Kyoto Encyclopedia of Genes and Genomes (“KEGG”) and WikiPathways databases (n = 6 biological replicates). Data are presented as mean $\pm$ SEM. Scale bars = 10 μm . AP-1 = activator protein 1; NOD = nucleotide-binding oligomerization domain.

Figure 5. Lateral transfer of *Aspergillus fumigatus* (Af) occurs through vasodilator-stimulated phosphoprotein (VASP) tunnel-like structures. (A) A phosphoprotein array analysis was performed with human monocyte-derived macrophages (hMDMs) pretreated with FK506 and stimulated with live Af swollen conidia (SC) (multiplicity of infection [MOI], 1) for 1 hour postinfection. Proteins with significant fold change differences in the phosphoprotein-to-nonphosphoprotein ratio between FK506 and control macrophages stimulated with live Af SC are shown. (B) hMDMs were pretreated with a cell-permeable cyclic guanosine monophosphate analog (25 mM 8-[4-chlorophenylthio]-guanosine 3',5'-cyclic monophosphate [8-pCPT-cGMP]) to induce Ser238 VASP phosphorylation and with FK506 (10 ng/ml) or vehicle. They were then stimulated with a calcium ionophore (2 μg/ml ionomycin) to activate

Video 6. Vasodilator-stimulated phosphoprotein (VASP) colocalizes to the human monocyte-derived macrophage (hMDM) *Aspergillus fumigatus* (Af) phagocytic cup. Three-dimensional reconstruction of confocal microscopy image shows phagocytosis of live Af swollen conidia (multiplicity of infection, 1) by hMDMs. The image is of Af phagocytosis at 30 minutes after infection with VASP shown in red, Af in green, and nuclei in blue. Scale bar = 10 μ m.

following the addition of the calcium ionophore ionomycin (to selectively activate calcineurin) to hMDMs with phosphorylated VASP (induced using a cell-permeable cyclic guanosine monophosphate analog) in the presence or absence of FK506 (Figure 5B). This confirmed that VASP is a major dephosphorylation target of calcineurin.

As VASP is an important actin cytoskeletal regulator and calcineurin is required for optimal phagocytosis and transfer of Af, we hypothesized that VASP is involved in actin-dependent phagocytosis and lateral transfer of Af. Using high-resolution confocal microscopy, we observed colocalization of VASP to Af at phagocytic cups, which disappeared following internalization (Figures 5C and 5D and Video 6). Localization of VASP to Af was not calcineurin dependent. During Af lateral transfer at late time points, high-intensity colocalization of VASP and actin staining to Af-containing endosomes was observed (Figure 5E). High-resolution three-dimensional reconstruction revealed that Af-containing endosomes

trafficked between macrophages are enveloped within actin-VASP-rich cargo (Figures 5C–5G and Video 7).

To determine the role of VASP in macrophage Af phagocytosis, small interfering RNA knockdown of VASP was performed in human macrophages, followed by analysis of uptake of calcofluor white-stained live Af SC compared with control. This showed that phagocytosis of live Af SC at early time points is VASP dependent ($P < 0.01$) (Figure 5H), consistent with the defect seen for calcineurin inhibition (Figure 2B). Taken together, these results indicate that calcineurin, by direct dephosphorylation and activation of the actin polymerase VASP, has a key role in macrophage actin cytoskeleton reorganization during fungal infection. This enables optimal actin-dependent Af phagocytosis and lateral transfer of Af-containing endosomes during programmed necrosis, which are critical to achieving control of germinating conidia.

Discussion

PA has emerged as a serious infectious complication of a range of immunocompromised states and chronic respiratory diseases. Overt infection is typically characterized by hyperinflammatory tissue destruction, with more complex relationships between airway colonization and progression of chronic respiratory diseases (38). Here we report human macrophage programmed necrosis as an important response to germination of Af. While necrosis has been recognized as a form of cell death since the mid-19th century, it has recently become clear that programmed necrosis may occur through receptor-interacting serine/threonine-protein kinase 3 necroptotic cell death (39). Necroptosis occurs as a firstline defense against intracellular pathogens and is

Video 7. Lateral transfer of germinating *Aspergillus fumigatus* (Af) conidia between human monocyte-derived macrophages (hMDMs) occurs within vasodilator-stimulated phosphoprotein (VASP)-enveloped endosomes. Three-dimensional reconstruction of confocal microscopy image shows hMDM Af lateral transfer with VASP shown in green and Af in magenta. Scale bar = 10 μ m.

central to the pathogenesis of a number of chronic inflammatory conditions, including emphysematous change (40, 41).

Remarkably, we observed frequent lateral transfer of Af during necroptosis, which ultimately enabled control of hyphal escape from the macrophage. Transfer occurred through a late endosomal compartment, suggesting that this process may have similarities to exocytosis (42). While lateral transfer has previously been observed for *Cryptococcus neoformans* at low frequency, the molecular basis for transfer and its relationship to cell death or control of infection have not previously been defined (43). The pathogen escape and transfer processes described to date are pathogen mediated and enhance either tissue dissemination or evasion from immune attack. However, our observations are consistent with host-dependent lateral transfer of Af to limit hyphal escape during macrophage death. The observation of

Figure 5. (Continued). calcineurin. Macrophage whole-cell extracts were probed by Western blotting for Ser238 VASP and total VASP. (C) Representative confocal microscopic images of hMDM phagocytosis and lateral transfer of live Af SC (MOI, 1). VASP is shown in red, nuclei in blue, and Af in green. (1) Uninfected hMDMs. (2) Initial phagocytosis of Af by hMDMs. (3) Late phagocytosis of Af by hMDMs. (4) Lateral transfer of Af between hMDMs. (D) Three-dimensional reconstruction of confocal microscopic images of phagocytosis and lateral transfer of live Af SC (MOI, 1) between hMDMs. Af phagocytosis at 30 minutes postinfection with VASP is shown in red, Af in green, and nuclei in blue. (E) Three-dimensional reconstruction of confocal microscopic image of Af lateral transfer with VASP shown in yellow, actin in magenta, Af in green, and nuclei in blue. (F and G) Representative z-slice confocal microscopic image (F) and three-dimensional reconstruction (G) of Af lateral transfer. VASP is shown in red, and Af is shown in green. (H) THP-1 macrophages treated with either control or VASP small interfering RNA (siRNA) (50 μ M) were infected with live Af SC and stained with Calcofluor White (Sigma-Aldrich, St. Louis, MO) (MOI, 1). Phagocytosis was quantified by flow cytometry ($n = 3$ biological replicates). Data are presented as mean $\pm$ SEM. Scale bars = 10 μ m. AKT1 = RAC- α serine/threonine protein kinase; CDC25A = M-phase inducer phosphatase 1; HDAC1 = histone deacetylase 1; MAPK = mitogen-activated protein kinase; MEK1 = mitogen-activated protein kinase kinase 1; NFAT = nuclear factor of activated T cells; P-VASP = phospho-VASP; SMC1 = structural maintenance of chromosomes protein 1; UT = untreated; VEGFR2 = vascular endothelial growth factor receptor 2.

cell–cell transfer of *Af* supports a model whereby inhaled conidia may persist for prolonged periods in the myeloid compartment as spores. This has potentially important implications for individuals who subsequently undergo immunosuppression.

Notably, we observed that both fungal germination–driven programmed necrosis and lateral transfer were calcineurin dependent, indicating that necroptotic control of infection is likely to be impaired in organ transplant patients. Calcineurin has been postulated to have a role in the regulation of programmed cell death through interaction with Bcl-2 family proteins (25, 26). Consistent with this, we found significant calcineurin-dependent expression of myeloid cell leukemia 1, a Bcl-2 protein that inhibits apoptosis, and Bcl2-modifying factor, a member of the prodeath BH3-only subgroup of Bcl-2 family proteins required for TNF- α -induced necroptosis (27). In transcriptomic and phosphoproteomic

analysis, we defined calcineurin as a key phosphatase mediating cross-control of cell death, the MAPK–AP-1 innate pathway, and VASP-dependent cytoskeletal remodeling during macrophage infection. Calcineurin inhibition impaired these cellular processes, leading to enhanced fungal germination in the late phagosome, impaired lateral transfer, and ultimately hyphal escape. Interestingly, virulent *Burkholderia* spp. have recently been shown to mimic VASP actin polymerases to enable cell fusion and spread (44). Whether fungi are able to directly manipulate host actin polymerases remains to be determined.

In summary, we have identified macrophage programmed necrosis as an important response to progressive germination of *Af* in the human macrophage, and we report a previously undescribed phenomenon of cell death–dependent lateral transfer as a cooperative macrophage behavior that assists in limiting hyphal escape. We show

that calcineurin is a key orchestrator of this process, further defining the importance of this pathway in innate immunity. Our findings yield novel insights into host innate immunity to *Af* in the lung, extend current understanding of the pathogenesis of PA in organ transplantation, and have broader implications for transplant immunity and chronic lung disease. ■

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