Aspergillus-mediated allergic airway inflammation is triggered by dendritic cell recognition of a defined spore morphotype

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Background: Exposure to fungi, especially *Aspergillus fumigatus,* can elicit potent allergic inflammation that triggers and worsens asthmatic disease. Dendritic cells (DCs) initiate allergic inflammatory responses to allergic stimuli. However, it is unclear if *Af* spores during isotropic growth (early spore swelling) can activate DCs to initiate allergic responses or if germination is required. This lack of basic understanding of how *Af* causes disease is a barrier to developing new treatments.
Objective: We sought to show that a precise *Af* morphotype stage during spore swelling can trigger DCs to mediate allergic inflammatory responses and ascertain if antifungal therapeutics can be effective at suppressing this process.
Methods: We used an *Af* strain deficient in pyrimidine biosynthesis *(ΔpyrG)* to generate populations of *Af* spores arrested at different stages of isotropic growth (swelling) via temporal removal of uracil and uridine from growth media. These arrested spore stages were cultured with bone marrow–derived DCs (BMDCs), and their activation was measured via flow cytometry and ELISA to examine which growth stage was able to activate BMDCs. These BMDCs were then adoptively transferred into the airways to assess if they were able to mediate allergic inflammation in naïve recipient mice. Allergic airway inflammation *in vivo* was determined via flow cytometry, ELISA, and real-time quantitative PCR. This system was also used to determine if antifungal drug (itraconazole) treatment could alter early stages of spore swelling and therefore BMDC activation and *in vivo* allergic inflammation upon adoptive transfer.
Results: We found that *Af* isotropic growth is essential to trigger BMDC activation and mediate allergic airway inflammation. Furthermore, using time-arrested *Af* stages, we found that at least 3 hours in growth media enabled spores to swell sufficiently to activate BMDCs to elicit allergic airway inflammation *in vivo.* Incubation of germinating *Af* with itraconazole reduced spore swelling and partially reduced their ability to activate BMDCs to elicit *in vivo* allergic airway inflammation.
Conclusion: Our results have pinpointed the precise stage of *Af* development when germinating spores are able to activate DCs to mediate downstream allergic airway inflammation. Furthermore, we have identified that antifungal therapeutics partially reduced the potential of *Af* spores to stimulate allergic responses, highlighting a potential mechanism by which antifungal treatment might help prevent the development of fungal allergy. (J Allergy Clin Immunol 2025;155:988-1001.)

Key words: Aspergillus fumigatus, *fungal asthma, dendritic cells, asthma, allergic airway inflammation, itraconazole, antifungal*

Fungi are abundant in the environment, with a diverse range of fungal species (including *Aspergillus fumigatus, Candida albicans,* and *Alternaria alternata*) known to be major drivers of asthma.[1-4] At least 10 million people worldwide display significant sensitivity to *Af,* which increases asthma severity[5] and can lead to the development of debilitating chronic fungal infections[6,7] such as allergic bronchopulmonary aspergillosis and severe asthma with fungal sensitization.[8] Despite the significant clinical burden of these diseases, treatment options are limited. Moreover, there is much debate on whether it is preferable to administer therapeutics, such as corticosteroids, that dampen the underlying allergic inflammation but may interfere with antifungal immunity[9] or to utilize antifungal therapies, such as the drug itraconazole, which have been shown to improve health for individuals with chronic allergic fungal conditions.[10,11] Although the precise mechanisms that might explain how such treatments are effective are unknown, these observations suggest that *Af* plays a critical, yet underappreciated, role in mediating responses that underpin allergic airway inflammation.

The immune events that cause individuals to become sensitized to *Af* spores and develop allergic airway inflammatory disease is unclear. In healthy settings, the majority of spores are removed in the upper airways, with their small size (2-3 μm) allowing around 20% of inhaled *Af* spores to reach the alveoli, where they are thought to be cleared by cells of the epithelial barrier, along with innate immune cells such as macrophages and dendritic cells (DCs).[12-15] However, relatively few studies have focused on whether *Af* spores activate these cells to coordinate allergic inflammatory responses. In the lung, DCs sample the airway and

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Abbreviations used
BAL: Bronchoalveolar lavage
BMDC: Bone marrow–derived DC
cDC1/2: Conventional cell type 1/2
DC: Dendritic cell
EDTA: Ethylenediaminetetraacetic acid
FACS: Fluorescence-activated cell sorting
FBS: Fetal bovine serum
GM-CSF: Granulocyte-macrophage colony-stimulating factor
LPS: Lipopolysaccharide
MHC-II: Major histocompatibility complex class II
PBS: Phosphate-buffered saline
PFA: Paraformaldehyde
PMA: Phorbol 12-myristate 13-acetate
qPCR: Real-time quantitative PCR
RELMα: Resistin-like molecule alpha
u/u: Uridine and uracil
WT: Wild type

migrate to the draining lymph nodes to mediate antigen-specific T-cell responses against a range of stimuli, including fungi.[16-18] We have recently identified that DCs orchestrate $CD4^+$ T-cell responses to repeated exposures of *Af* spores to generate a mixed type 2 (eosinophilia with IL-4, IL-5, and IL-13) and type 17 (neutrophilia with IL-17) inflammatory cytokine environment.[19] These immune changes mediate the hallmark symptoms of asthma, including airway hypersensitivity, luminal narrowing, smooth muscle hyperplasia, and mucus overproduction.[20] In contrast to other allergens, such as house dust mite, little is known about whether *Af* spores actively reveal factors that trigger DC-mediated sensitization to promote fungal allergic airway inflammatory disease.

If not rapidly cleared from the airways, within hours, *Af* spores can undergo morphogenetic changes as they swell and then germinate to form hyphae.[21,22] Resting *Af* spores are coated in an immunologically inert dense α-1,3-glucan, melanin, and rodlet hydrophobin layer.[23-28] This layer is shed as spores break dormancy and undergo isotropic growth (swelling),[23] exposing pathogen-associated molecular patterns, such as carbohydrate motifs including β-1,3-glucan, that can be recognized by innate immune cells and trigger potent inflammatory responses.[28-31] Furthermore, numerous *Af* allergens have been identified, with hyphal proteases (Asp f 5, Alp1, and Asp f 13) particularly implicated in mediating allergic inflammatory responses.[32-35] However, whether these allergens are expressed in early germinating spores and mediate initial sensitization via DCs is unknown. Deeper understanding of the potential immunogenicity of molecules exposed on the swollen spores[36] is vital to precisely map when *Af* spores develop allergenic properties and to identify how this process may be therapeutically manipulated.[37]

In this study, we have demonstrated a requirement for *Af* spore swelling to induce immune activation, both *in vitro* and *in vivo,* with a particular focus on DCs because of their crucial role in directing allergic airway inflammation. Further, we have identified the stage in *Af* isotropic growth at which spores are able to activate bone marrow–derived DCs (BMDCs) to initiate allergic airway inflammation in female mice. Finally, we have shown that treatment with the antifungal itraconazole can partially reduce the allergenicity of *Af* spores, and therefore their capacity to activate BMDCs to initiate allergic airway inflammation. These results not only precisely define the development of allergenicity during *Af* spore swelling but also provide mechanistic evidence to explain the therapeutic benefit of antifungal treatment in allergic airway inflammation.

METHODS

Aspergillus strains and culture

Af A1160 *ΔpyrG (pyrG*$^-$*)* was used to generate stage-arrested *Af* spores, while A1160 *pyrG*$^+$ (referred to as A1160 wild type) strain was used as control for comparisons for *in vitro* and *in vivo Af* challenge.[38-40] The A1160 strain was derived from CEA10, an invasive aspergillosis clinical isolate.[41] In experiments involving DC transfer to recipient mice, mice were challenged with spores of the parental CEA10 strain. *Af* was grown on Sabouraud dextrose agar (Oxoid) at 37°C supplemented with 5 mmol uridine and 5 mmol uracil (u/u, both Sigma) for the A1160 *ΔpyrG* strain. Spores were collected and resuspended in RPMI 1640 (Sigma) at 1.6×10^7 spores/mL in the presence or absence of 5 mmol u/u. In some experiments, these were further supplemented with 2 μg/mL itraconazole (Thermo Fisher Scientific). Stage-arrested spores were generated by incubation at 37°C (with shaking) for defined periods and washed in nonsupplemented RPMI 1640 (Gibco; Thermo Fisher Scientific) to remove excess u/u. To aid counting, spores were sonicated for 3 seconds at 20% amplitude to disperse clumps. Swollen spores were stored in nonsupplemented RPMI 1640 at 4°C for up to 24 hours before addition to BMDC culture or assessed directly by flow cytometry.

BMDC culture

BMDCs were generated with granulocyte-macrophage colony-stimulating factor (GM-CSF) as previously described.[40] In brief, 2×10^5 bone marrow cells were seeded in complete medium (RPMI 1640 [Sigma] containing 20 ng/mL GM-CSF [PeproTech], 10% fetal calf serum (Sigma), 2 mmol L-glutamine (Gibco), 50 U/mL penicillin, and 50 μg/mL streptomycin (Life Technologies; Thermo Fisher Scientific). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. On day 3, 10 mL of complete medium was added, and on days 6 and 8, 9 mL of media was gently aspirated and replaced with 10 mL of fresh complete medium. After 10 days of culture, BMDCs were collected and replated at 2×10^6 cells/mL for further assays. *Af* spores were added to BMDCs at multiplicity of infection 5:1 and incubated for 5 hours at 37°C in a humidified atmosphere of 5% CO_2 in complete medium with 5 ng/mL GM-CSF. Media or 250 ng/mL lipopolysaccharide (LPS; Sigma) was added as negative and positive controls, respectively.

Murine models

C57BL/6 (Envigo) mice were maintained under specific pathogen-free conditions at the University of Manchester (licence P44492AC9) or the University of Exeter (license P6A6F95B5 or PP6094315). All animal studies were ethically reviewed and carried out in accordance with the Animals (Scientific

Procedures) Act of 1986 and the GSK Policy on the Care, Welfare, and Treatment of Animals. Female mice aged 6 to 17 weeks were used for analysis. No *a priori* sample size calculations were performed to determine number of mice used per group. All mice were included in the analysis, with no exclusions. For statistical calculations, individual mice were regarded as the experimental unit. Blinding and randomization of treatment groups was not performed. To account for confounding cage effects, mice from different treatment groups were cohoused.

In repeat *Af* exposure models, experimental mice were exposed intranasally to 4×10^5 *Af* spores (A1160) in 30 μL phosphate-buffered saline (PBS) 0.05% Tween or vehicle control on days 0, 2, 4 7, 9, and 11. Mice were humanely killed at day 12 after the first *Af* dose. In DC transfer models, mice were sensitized via intranasal transfer of 5×10^4 BMDCs (day 0), challenged with *Af* (CEA10) on days 12 and 13 after transfer (4×10^5 each day), and humanely killed on day 14.

Cell isolation

For analysis of cellular changes *in vivo,* cells were isolated from the bronchiolar lavage (BAL) sample, lung tissue, and mediastinal lymph nodes. BAL samples were collected by washing the lungs with PBS containing 2% fetal bovine serum (FBS) and 2 mmol ethylenediaminetetraacetic acid (EDTA; Sigma). Lungs were processed via incubation at 37°C with 0.8 U/mL Liberase TL (Sigma) and 80 U/mL DNase I type IV (Sigma) in Hanks balanced salt solution (Gibco), as previously described.[40] After 40 minutes, digestion was halted with PBS containing 2% FBS and 2 mmol EDTA, and the suspension was passed through a 70 μm cell strainer. Red blood cells were lysed using a red blood cell lysis buffer (Sigma). To assess cytokine secretion potential, lung cells were stimulated *ex vivo* for 3 hours at 37°C with 20 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) and 1 μL/mL GolgiStop (Becton Dickinson [BD]) in X-vivo-15 (Lonza) supplemented with 1% L-glutamine (Gibco) and 0.1% β-mercaptoethanol (Sigma). Cultures were carried out in 96-well round-bottomed plates, with 4×10^5 cells per well in 200 μL final volume.

Flow cytometry

Isolated cell suspensions were washed with PBS and stained for viability with ZombieUV (1:2000; BioLegend). Samples were then blocked with 5 μg/mL αCD16/CD32 (2.4G2; BioLegend) in fluorescence-activated cell sorting (FACS) buffer (PBS containing 2% FBS and 2 mmol EDTA) before staining for surface markers at 4°C for 30 minutes. After staining, cells were washed twice in FACS buffer and then fixed in 1% paraformaldehyde (PFA) in PBS for 10 minutes at room temperature. For detection of intracellular antigens, cells were fixed with BD Cytofix/Cytoperm (BD) for 1 hour. Cells were then washed 3 times with eBioscience (Thermo Fisher Scientific) permeabilization buffer before overnight staining with intracellular cytokine antibodies in eBioscience permeabilization buffer. Samples were acquired on a BD Fortessa device with FACSDiva (BD) software and analyzed by FlowJo v10 (Treestar). Gating was informed by the use of fluorescence-minus-1 controls. Flow cytometry gating schemes are shown in Figs E1-E3 in this article's Online Repository available at www.jacionline.org. Antibodies used are listed in Table E1, also in the Online Repository.

ELISA

Enzyme-linked immunosorbent assays (ELISAs) were performed on BAL and BMDC culture supernatants using paired monoclonal antibodies and recombinant cytokine standards, or DuoSets following the manufacturer's instructions (BioLegend, R&D Systems, and PeproTech). A list of the antibodies used is provided in Table E2 in the Online Repository available at www.jacionline.org.

RNA extraction and qPCR gene expression

Lung tissue was collected in RNAlater (Thermo Fisher Scientific) for storage at −80°C before subsequent processing. Once thawed, tissues were homogenized in RLT lysis buffer in a Geno/Grinder (SPEX) and RNA isolated using RNeasy columns (Qiagen) following the manufacturer's instructions. Complementary DNA was generated from extracted RNA using SuperScript-III and Oligo-dT (Thermo Fisher Scientific). Relative quantification of genes of interest was performed by real-time quantitative PCR (qPCR) analysis using the QuantStudio Pro 7 system (Thermo Fisher Scientific) and SYBR Green Master Mix (Thermo Fisher Scientific), compared to a serially diluted standard of pooled cDNA. Expression was normalized to GAPDH.[19] Primers used are listed in Table E3 in the Online Repository available at www.jacionline.org.

Histologic staining

Lungs were collected into 10% neutral buffered formalin (Sigma), fixed overnight, and washed and stored in 70% ethanol. Lungs were then processed in a Leica ASP 300 Tissue Processor (Leica Biosystems) before embedding in paraffin wax, and 5 μm thick sections were cut with a Leica RM2255 Rotary Microtome (Leica Biosystems) and mounted on Superfrost slides (Thermo Fisher Scientific).

To assess airway mucus, Alcian blue–periodic acid–Schiff staining was used. Slides were rehydrated using a Leica autostainer. Slides were then stained with Alcian blue (Sigma) for 15 minutes, 1% periodic acid (Sigma) for 5 minutes, Schiff reagent (Sigma) for 10 minutes, and hematoxylin (Sigma) and 5% acetic acid (Sigma) for 30 seconds. Between each step, slides were washed in distilled water. After staining, all slides were dehydrated and mounted using a Leica autostainer and Leica CV5030 coverslipper. A Panoramic250 slide scanner (3D Histec) was used to obtain images.

Mucus area and epithelial thickness were assessed by an in-house macro. Briefly, a manual line was drawn around the epithelial basement membrane to demarcate an airway. Color thresholding was then used to distinguish between epithelia and mucus (darker purple), with both the area of mucus and the epithelia extracted. The area of mucus was normalized by dividing by the total area of the measured airway. The average thickness of the epithelia per airway was calculated. A median (range) of 9 (5-17) airways per individual mouse was used, with the median mucus area and epithelial thickness for each mouse plotted.

Statistical analysis

For all figures, to compare between groups, mixed linear models were fitted by RStudio (R Core Team) and the 'lme4' package.[42] To account for random variation, experimental day

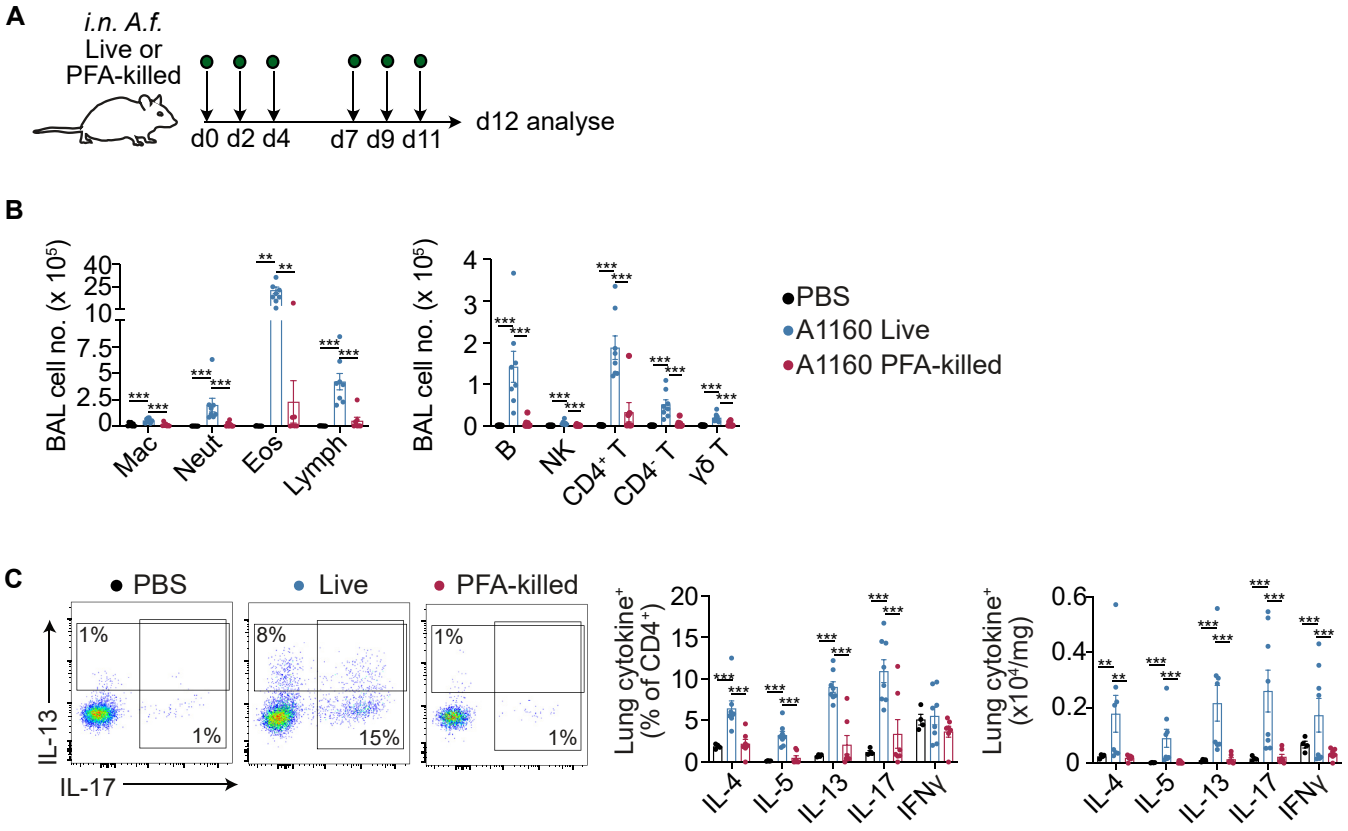

FIG 1. PFA-killed spores are unable to induce fungal allergic airway inflammation. **A,** C57BL/6 mice were exposed to 6 doses of live spores (A1160), PFA-killed spores (A1160) (0.4×10^6 per dose), or PBS intranasally on indicated days and tissues collected 24 hours after sixth dose (day 12). **B,** Numbers of different cell populations isolated from BAL fluid of *Af* or PBS exposed mice were assessed by flow cytometry. **C,** Flow cytometry plots show $CD4^+$ T-cell intracellular cytokine production after *ex vivo* stimulation with PMA/ionomycin of lung cells from *Af* or PBS exposed mice. Data were combined from 2 independent experiments (n = 7-8 per group). Each data point is 1 individual mouse. Linear mixed effect modeling applied, with experimental repeat as random effect variable; to compare multiple groups, a *post hoc* Tukey HSD test was used. $*P < .05$, $**P < .01$, $***P < .001$.

was designated a random effect variable, with time point or genotype as fixed effect variables. To compare multiple groups, a *post hoc* Tukey HSD test conducted by the 'multcomp' package.[43] For Fig E5, available in the Online Repository available at www.jacionline.org, GraphPad Prism v8.0 (GraphPad Software) was used, with multiple comparisons testing applied after fitting a mixed model using a compound symmetry covariance matrix and restricted maximum likelihood. Bar charts show means and standard errors of the mean.

RESULTS

Af spore viability is required for induction of fungal allergic airway inflammation

To determine the requirement for *Af* viability in mediating early immune sensitization events, we used a murine model of fungal allergic airway inflammation[19,44] that involved administering repeated doses (4×10^5) of live or PFA-killed spores via intranasal transfer (Fig 1, *A*). Our prior work has demonstrated that this fungal allergic response is dependent on DCs, with repeat spore dosing triggering expansion and activation of certain DC subsets in the lung and draining lymph nodes.[19] As expected, 24 hours after 6× *Af* doses (day 12), we found that mice that received live spores had a significant influx of granulocytes and lymphoid cells in the lung airway (via analysis of cells isolated from BAL fluid) (Fig 1, *B*). This was also accompanied by markedly increased type 2 (expressing IL-4, IL-5, and IL-13) and type 17 (expressing IL-17) $CD4^+$ T cells in the lung (Fig 1, *C*), confirming previous reports that repeat intranasally dosing with live *Af* spores induces hallmarks of allergic airway inflammation.[44] Strikingly, we found that the response in mice that received PFA-killed spores was significantly reduced, and similar to PBS controls (Fig 1, *B* and *C*), confirming and extending previous observations[44] that spore viability is crucial to induce allergic airway inflammation.

A major drawback when using PFA to kill spores is that it can dramatically alter cell wall surface epitopes[45] as well as halt spore metabolism, both of which could influence the ability of spores to interact with and activate DCs during allergic airway inflammation. Therefore, we utilized the Δ*pyrG Af* strain (lacking orotidine-5′-monophosphate decarboxylase, an essential enzyme in the pyrimidine biosynthetic pathway), which is viable but unable to develop as a result of disrupted uridine synthesis to investigate whether spore swelling is crucial for *Af* induction of allergic

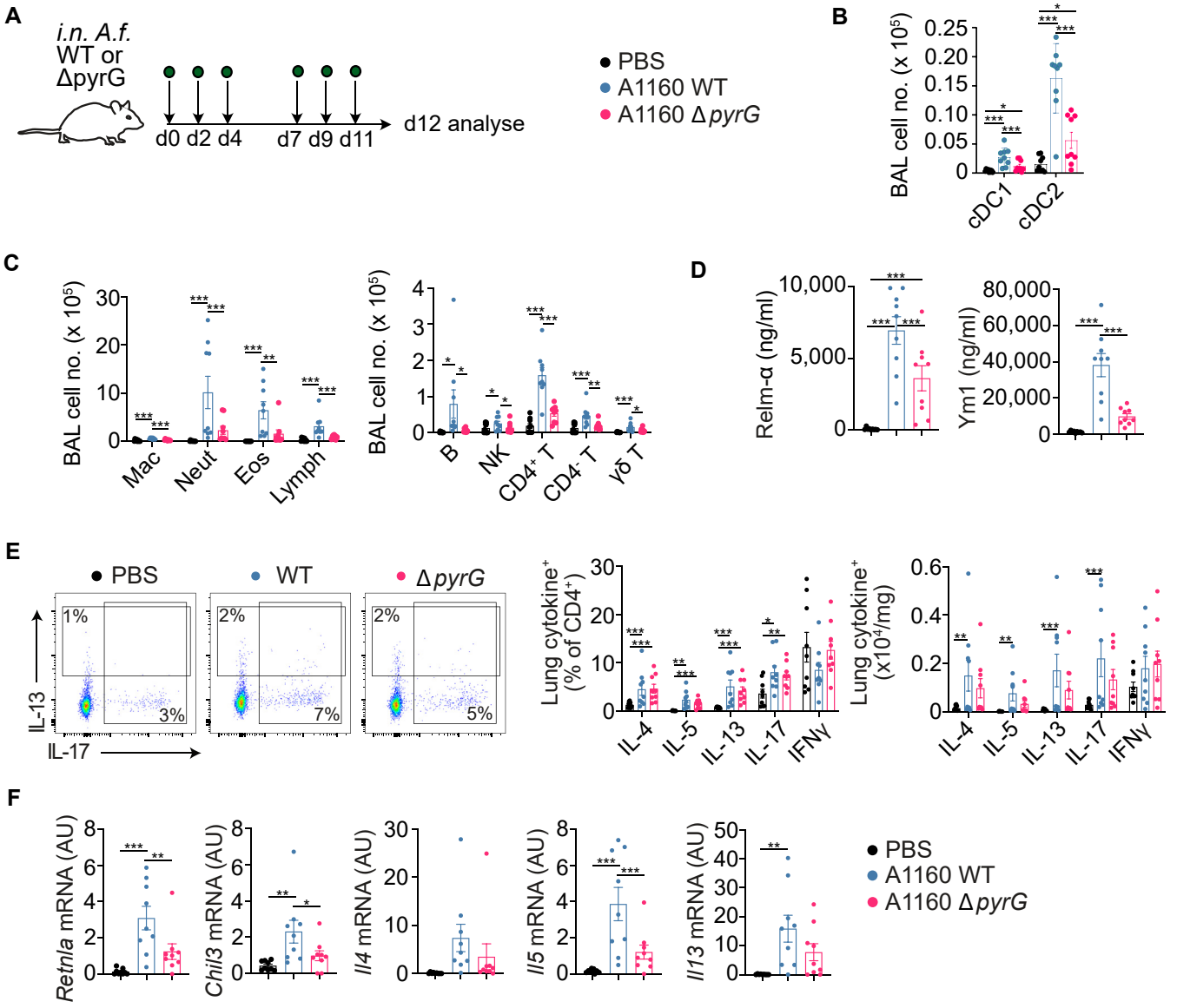

FIG 2. Fungal allergic airway inflammation in response to *Af* is dramatically reduced when spore swelling is arrested. **A,** C57BL/6 mice were exposed to 6 doses of *Af* spores (A1160), nongerminating *ΔpyrG Af* spores (0.4×10^6 per dose), or PBS intranasally on indicated days and tissues were collected 24 hours after sixth dose (day 12). **B,** Conventional DC subsets 1 and 2 (cDC1, $XCR1^+$) and (cDC2, $CD11b^+$) in airway (BAL fluid), assessed by flow cytometry. **C,** Immune cell populations in BAL fluid were assessed by flow cytometry. **D,** Secretory factors in BAL fluid were quantified by ELISA. **E,** Representative flow cytometry plots and graphs show lung $CD4^+$ T-cell intracellular cytokine staining after *ex vivo* stimulation with PMA/ionomycin. **F,** Lung tissue mRNA expression was assessed by qPCR (normalized against *Gapdh,* a.u.). Data are combined from 2 independent experiments (n = 8-9 per group). Each data point is 1 individual mouse. Linear mixed effect modeling applied, with experimental repeat as random effect variable; to compare multiple groups, a *post hoc* Tukey HSD test was used. $*P < .05$, $**P < .01$, $***P < .001$.

airway inflammation *in vivo*.[38-40] Mice that were repeatedly exposed to nonswollen *ΔpyrG* spores showed significantly reduced DC subsets (cDC1 and cDC2), macrophage, granulocyte, and lymphoid cell populations in BAL fluid compared to mice exposed to wild type (WT) ($pyrG^+$) spores (Fig 2, *A-C*). Furthermore, nonswollen Δ*pyrG* spores triggered reduced secretion of mediators associated with allergic inflammation in the airway (measuring resistin-like molecule alpha [RELMα] and chitinase-like protein [Chi3l3] in BAL fluid) compared to WT spores, which can germinate in the host (Fig 2, *D*). Surprisingly, despite these differences in inflammatory cell types, nonswelling Δ*pyrG* spores induced a similar proportion of $CD4^+$ T cells to produce type 2 and type 17 cytokines (Fig 2, *E*), although there was a trend for reduced numbers of these cells. Expression of genes associated with type 2 inflammation *(Retnla, Chi3l3, Il5)* were significantly reduced in the lung tissue of mice that received nonswelling Δ*pyrG* versus WT spores (Fig 2, *F*), further supporting that allergic immune responses were impaired.

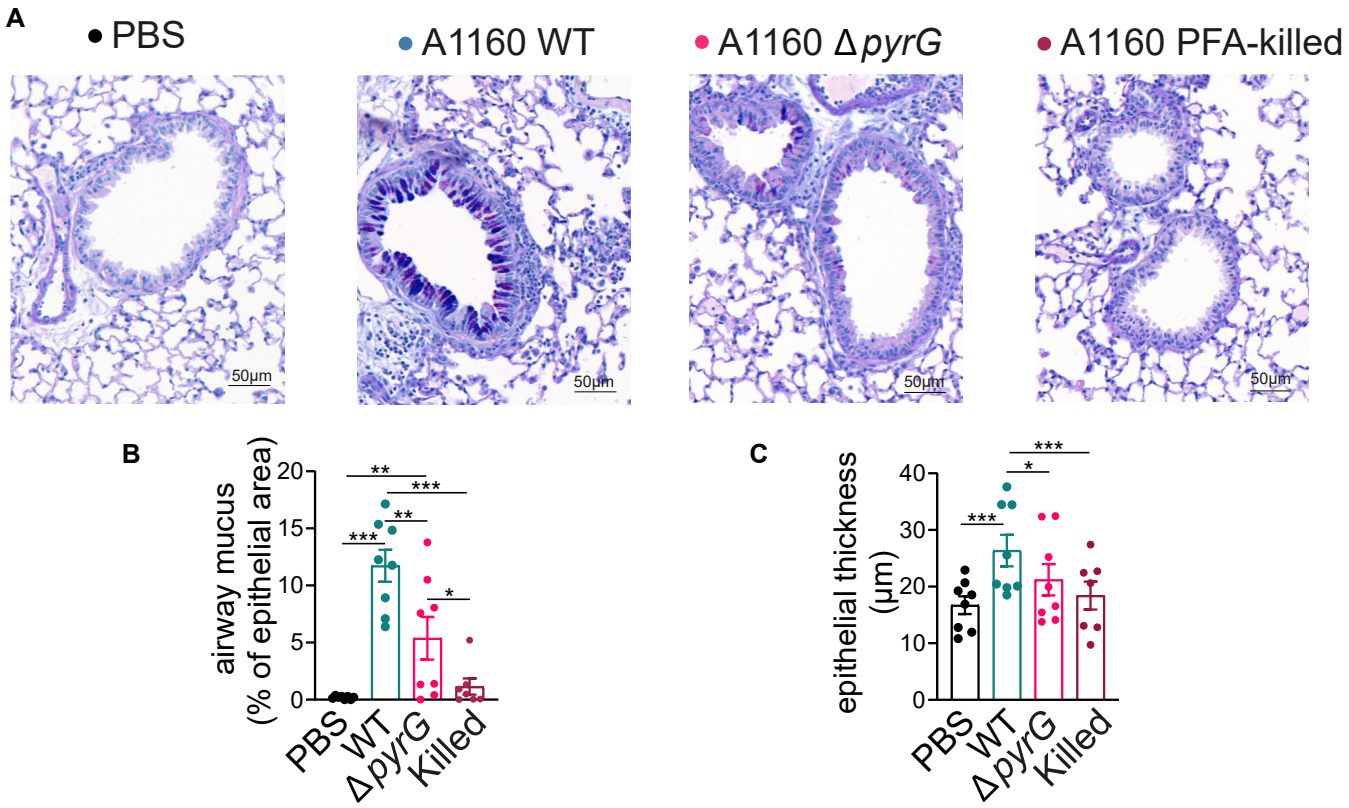

FIG 3. Fungal allergic airway inflammation–induced lung pathology is reduced when spores are killed or if spore swelling is arrested. C57BL/6 mice were exposed to 6 doses of *Af* spores (A1160), nongerminating *ΔpyrG Af* spores, PFA-killed spores (A1160) (0.4×10^6 per dose), or PBS intranasally on days 0, 2, 4, 7, 9, and 11, and tissues were collected 24 hours after sixth dose (day 12). **A,** Representative images showing lung sections stained with AB-PAS to assess expansion of mucus-producing goblet cells. **B,** Mucus area in airway epithelia quantified as proportion of overall epithelial area. **C,** Epithelial thickness, measured from basement membrane to lumen, is quantified. Data are combined from 2 independent experiments (n = 7-8) per group. Each data point is 1 individual mouse. Linear mixed effect modeling applied, with experimental repeat as random effect variable; to compare multiple groups, a *post hoc* Tukey HSD test was used. $*P < .05$, $**P < .01$, $***P < .001$. *AB-PAS,* Alcian blue–periodic acid–Schiff.

To address whether reduced fungal allergic immunity altered lung tissue inflammation, histologic analysis revealed that WT live spores induced epithelial thickness and goblet cell hyperplasia, as expected (Fig 3). In contrast, both PFA-killed spores and *ΔpyrG* spores induced less airway mucus and airway epithelial thickening of the lung tissue (Fig 3, *A* and *B*). In summary, these results show that live spores that are incapable of isotropic growth have a much-reduced capacity to trigger fungal allergic airway inflammation.

At least 3 hours of isotropic growth is essential for spores to elicit BMDCs to mediate fungal allergic airway inflammation

After identifying that spore swelling was essential for *in vivo Af* induction of allergic airway inflammation (Figs 2 and 3), we wanted to define the precise time point at which spores were able to activate BMDCs to mediate allergic inflammation. Because *Af* spores are rapidly cleared after inhalation, we suggest that immune responses to early stages of spore swelling may be critical. To identify at which point after breaking dormancy *Af* spores were able to elicit an immune response, we further utilized *ΔpyrG* spores with timed addition and then removal of u/u-supplemented media, to arrest isotropic growth at defined time points (Fig 4, *A*). We confirmed this was achieved by measuring spore morphogenesis via flow cytometry and microscopy, observing that spore size began to increase at 3 hours of culture, with a significant increase in the proportion of swollen spores at 4 hours (Fig 4, *B,* and Fig E4). Arrested spores remained viable after the removal of u/u, and they were able to germinate when u/u was reintroduced 24 hours later (Fig E4). These stages of growth-arrested *ΔpyrG* spores were then each separately cultured with BMDCs to determine their ability to trigger DC activation (Fig 4, *C*). We consistently found that 3 hours of swelling was the earliest isotropic growth stage that triggered significant BMDC activation, indicated by increased surface expression of costimulatory molecules (CD86, CD80, and major histocompatibility complex class II [MHC-II]) alongside secretion of proinflammatory cytokines (IL-6, IL-12p40, and TNF) (Fig 4, *D* and *E*). As expected, later growth stages (4 and 6 hours) induced the most marked BMDC activation (Fig 4, *D* and *E*). In comparison, all spores grown in the absence of u/u, 0-, 1-, or 2-hour spore stages did not trigger BMDC activation (Fig 4, *D* and *E*). Our findings using the arrested *ΔpyrG* system were later confirmed using PFA-killed spores, preswollen for 4 hours, which were able to induce immune activation in BMDCs (Fig E5).

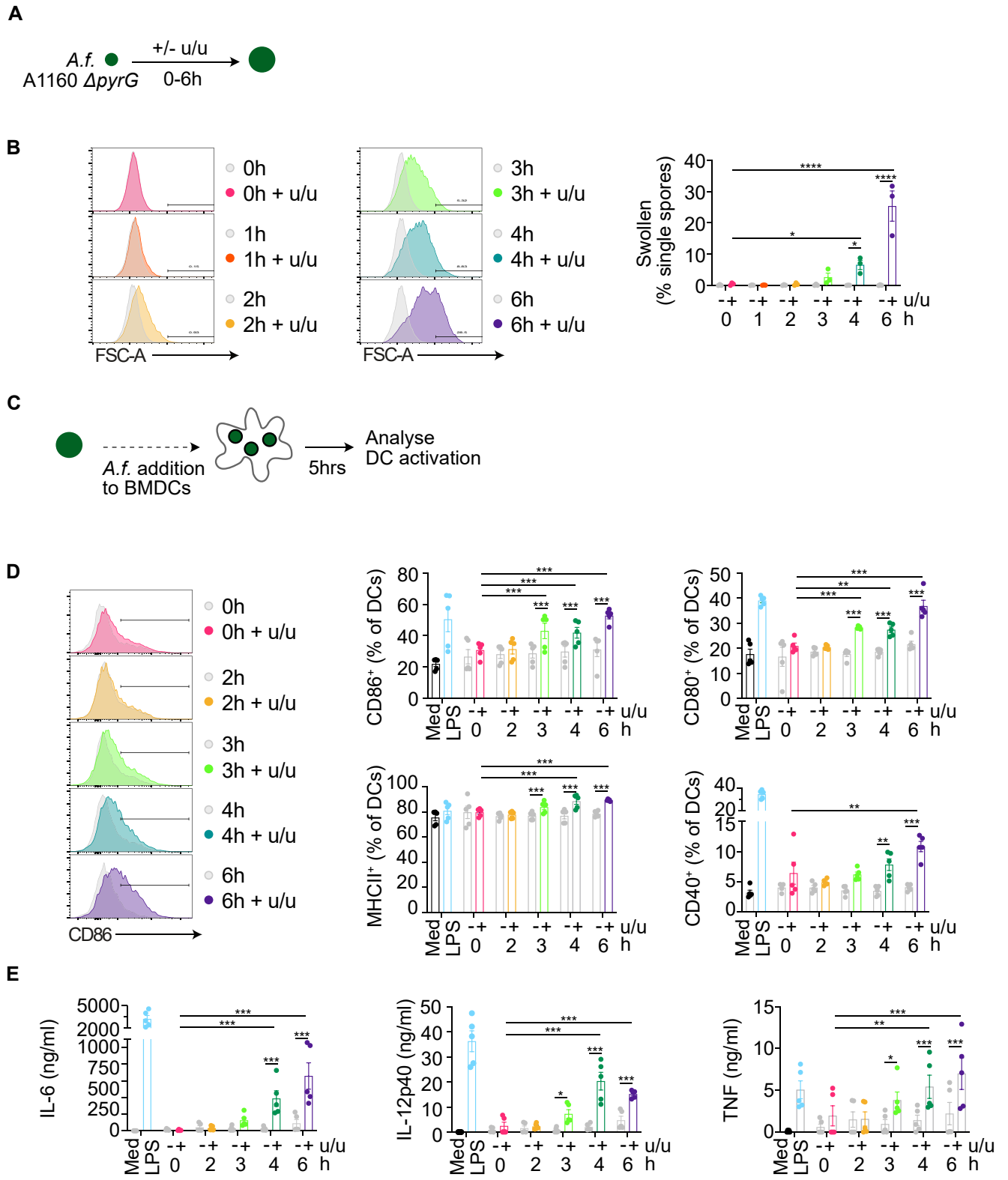

FIG 4. *Af* activation of DCs requires spores to undergo at least 3 hours of isotropic growth. **A,** *ΔpyrG* spores were incubated in presence or absence of u/u with growth arrested by removal of u/u media at indicated timings (between 0 and 6 hours). **B,** Spore size was determined by flow cytometry. **C,** Growth-arrested *ΔpyrG* spores were incubated with DCs for 5 hours (MOI 5:1) and DC activation analyzed. **D,** DC expression of costimulatory molecules and MHC-II was analyzed via flow cytometry. **E,** DC cytokine secretion was measured via ELISA. Data were combined from 2 independent experiments (n = 5 per group). Each data point is 1 individual DC culture. Linear mixed effect modeling applied, with experimental repeat as random effect variable; to compare multiple groups, *post hoc* Tukey HSD test was used. Statistical comparisons to LPS positive control are not shown. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001. *MOI,* Multiplicity of infection.

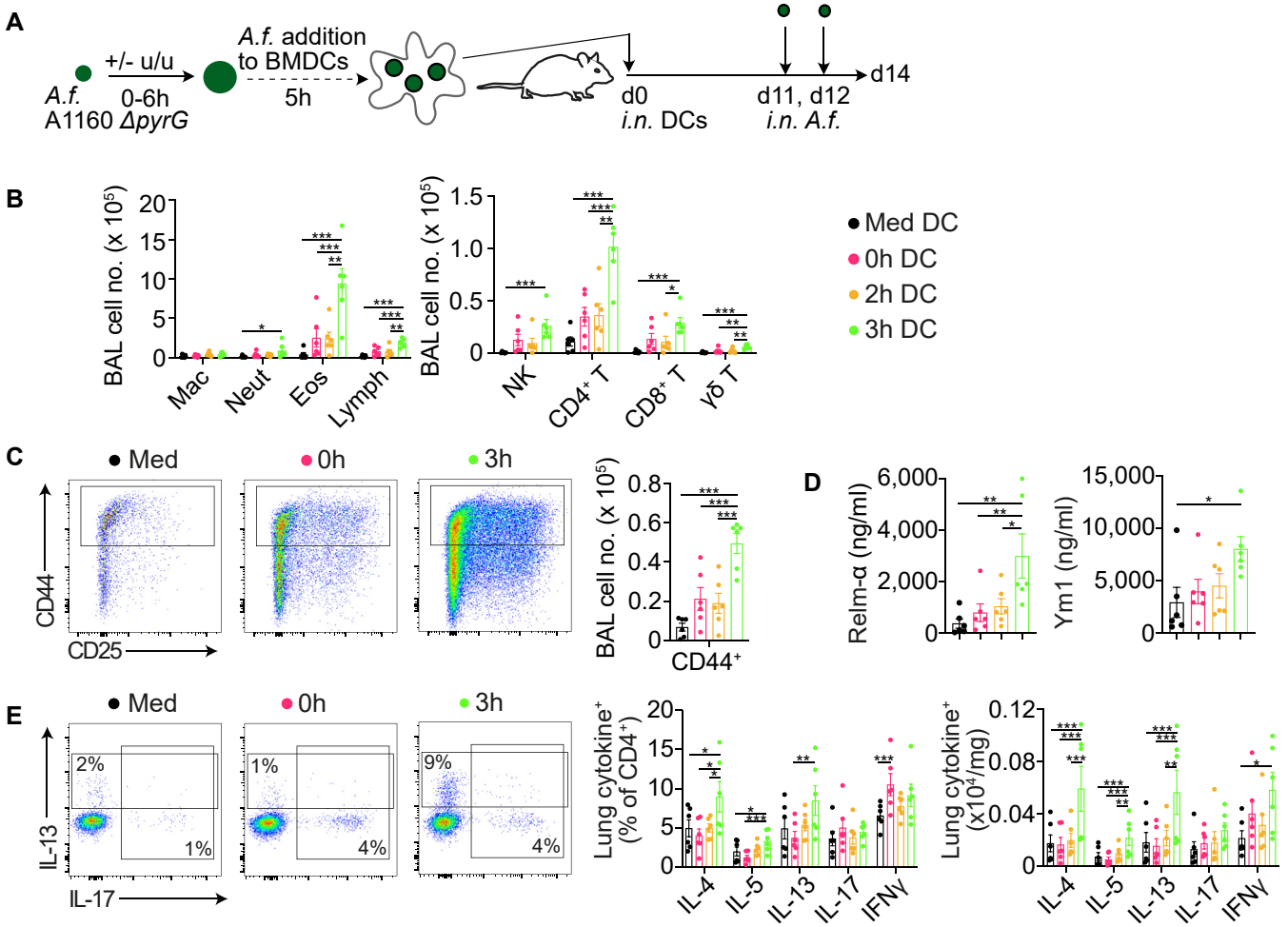

FIG 5. Three hours of isotropic growth is essential for spores to elicit DC ability to mediate fungal allergic airway inflammation. **A,** Arrested *ΔpyrG* spore stages were generated by incubating in presence of u/u, then removed and washed after 0, 2, or 3 hours. These were then cultured with BMDCs for 5 hours before intranasal transfer of 50,000 DCs into C57BL/6 mice. Mice were challenged intranasally at days 11 and 12 with 0.4×10^6 live CEA10 *Af* spores. **B,** Immune cell populations in BAL fluid were assessed by flow cytometry. Number of different cell populations isolated from BAL fluid of DC-sensitized mice was assessed by flow cytometry. **C,** Representative flow cytometry plots of $CD44^+CD4^+$ T cells in BAL fluid. **D,** Secretory factors in BAL fluid were quantified by ELISA. **E,** Representative flow cytometry plots and graphs show lung $CD4^+$ T-cell intracellular cytokine staining after *ex vivo* stimulation with PMA/ionomycin. Data were combined from 2 independent experiments (n = 6 per group). Each data point is 1 individual mouse. Linear mixed effect modeling applied, with experimental repeat as random effect variable; to compare multiple groups, *post hoc* Tukey HSD test was used. $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

Importantly, incubation of BMDCs with live spores for 5 hours only induced minor BMDC immune activation, likely because more immunogenic stages (3+ hours) had reduced time to interact with the BMDCs (Fig E5). This highlights the importance of using growth-arrested *Af* to allow sufficient time for immune activation by each spore morphotype. Taken together, by utilizing the A1160 *ΔpyrG* strain and a novel approach of timed addition and removal of u/u, we have pinpointed the earliest morphotype spore stage that is able to trigger BMDC activation.

Having shown that 3 hours of *Af* isotropic growth was sufficient to induce BMDC activation *in vitro,* we wanted to ascertain if these were able to mediate allergic airway inflammation *in vivo.* To test this, we adapted a BMDC adoptive transfer model that we have used previously,[40] whereby BMDCs previously exposed for 0, 2, or 3 hours to arrested *ΔpyrG* spores were administered intranasally to naïve recipient mice, followed by 2 further intranasal challenge doses of live *Af* spores (days 11 and 12 after BMDC sensitization) (Fig 5, *A*). Only mice that were sensitized to BMDCs pulsed with 3-hour spores displayed increased cell recruitment to the airways, with significantly increased granulocytes (neutrophils and eosinophils) and lymphocyte (natural killer cells, $CD4^+$ and $CD8^+$ T cells, and γδ T cells) populations in BAL fluid compared to mice sensitized with BMDCs that had been cultured in media alone (Fig 5, *B*). In addition, there was also a significant increase in the number of activated $CD4^+$ T cells ($CD44^+$) (Fig 5, *C*), as well as secretory factors associated with allergic inflammation (RELMα and Ym1) in BAL fluid (Fig 5, *D*). Adoptively transferred BMDCs that had been cultured with 3-hour spores were able to induce significant expansion of type 2 cytokine–expressing $CD4^+$ T cells (Fig 5, *E*). In contrast, mice that received BMDCs that had been exposed to 0- or 2-hour spores showed no evidence of fungal allergic

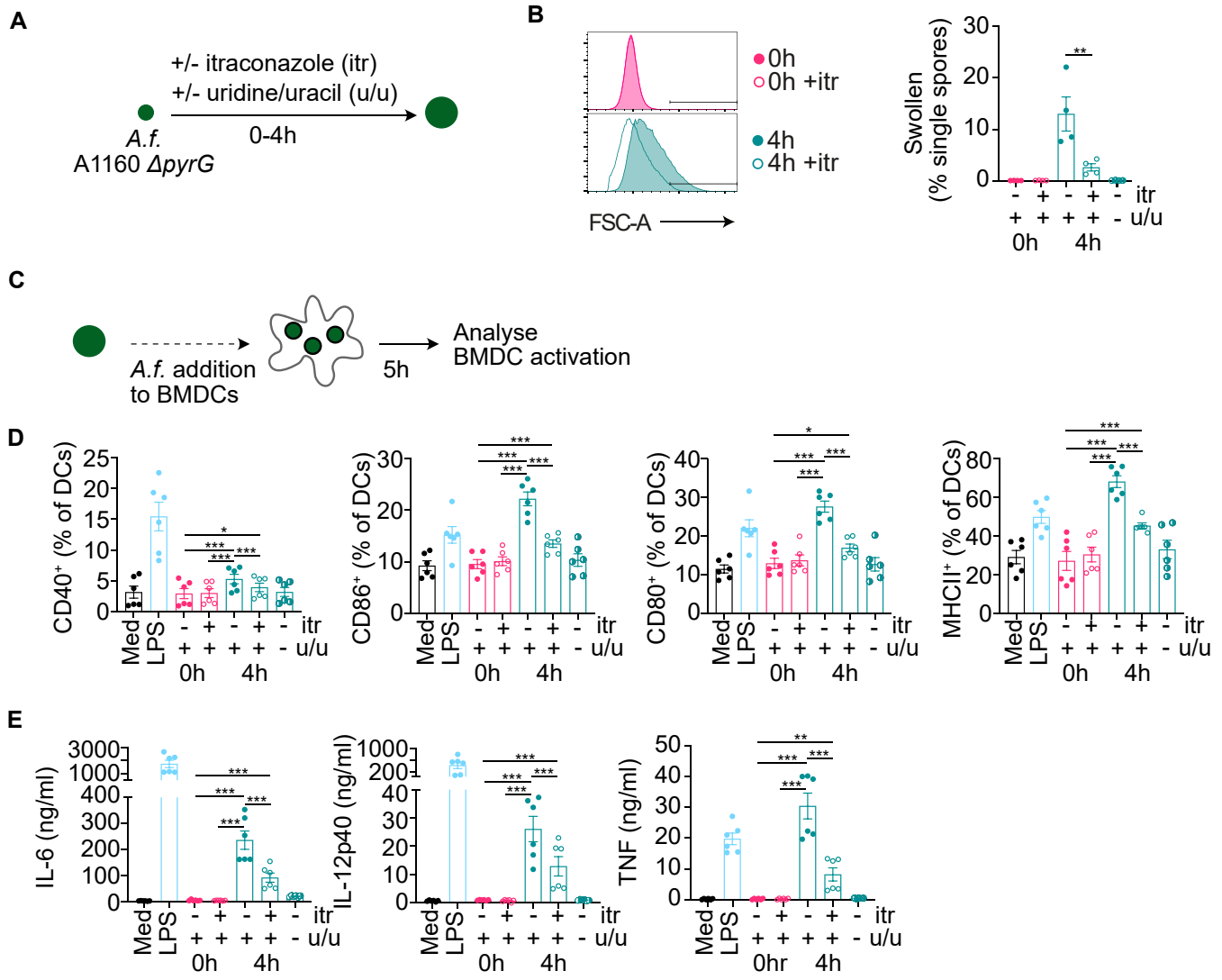

FIG 6. Itraconazole reduces swelling of spores and ability to activate DCs. **A,** *ΔpyrG* spores were incubated with u/u-containing media in presence or absence of itraconazole (itr); then growth was arrested by removal of u/u media at 0 or 4 hours. **B,** Spore size was determined by flow cytometry. **C,** Growth-arrested and itraconazole-treated *ΔpyrG* spores were incubated with DCs for 5 hours (MOI 5:1) and DC activation analyzed. **D,** DC expression of costimulatory molecules and MHC-II was analyzed via flow cytometry. **E,** DC cytokine secretion was measured via ELISA. Data were combined from 2 independent experiments (n = 6 per group). Each data point is 1 individual DC culture. Linear mixed effect modeling applied, with experimental repeat as random effect variable; to compare multiple groups, *post hoc* Tukey HSD test was used. Statistical comparisons to LPS-positive control are not shown. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001. *MOI,* Multiplicity of infection.

inflammation (Fig 5, *E*). However, these did not trigger IL-17 immune responses (Fig 5, *E*). In summary, these data show that BMDC induction of fungal allergic airway inflammation against *Af* is dependent on their recognition of a defined spore morphotype that requires at least 3 hours of growth to become immunogenic.

Treatment of *Af* with antifungal agents partially reduces spore capacity to activate ability of BMDC to induce allergic airway inflammation

Although it has been reported that antifungal therapies (including azole drugs such as itraconazole) can be a successful treatment strategy for patients with severe fungal asthma,[46-48] the mechanisms that underpin this are poorly understood. Therefore, we reasoned that one partial explanation may be that antifungal treatment could interfere with early spore swelling and thus reduce their capability to activate BMDCs to induce allergic airway inflammation. To our knowledge, few studies have assessed how antifungal drugs affect early stages of *Af* spore swelling. We found that incubating *ΔpyrG Af* in the presence of itraconazole (and u/u) for 4 hours significantly reduced spore swelling (Fig 6, *A* and *B*). To test whether this affected *Af* immunogenicity, spores were grown in the presence or absence of itraconazole, washed extensively to remove itraconazole, and then cultured with BMDC (Fig 6, *A-C*). Itraconazole-treated 4-hour

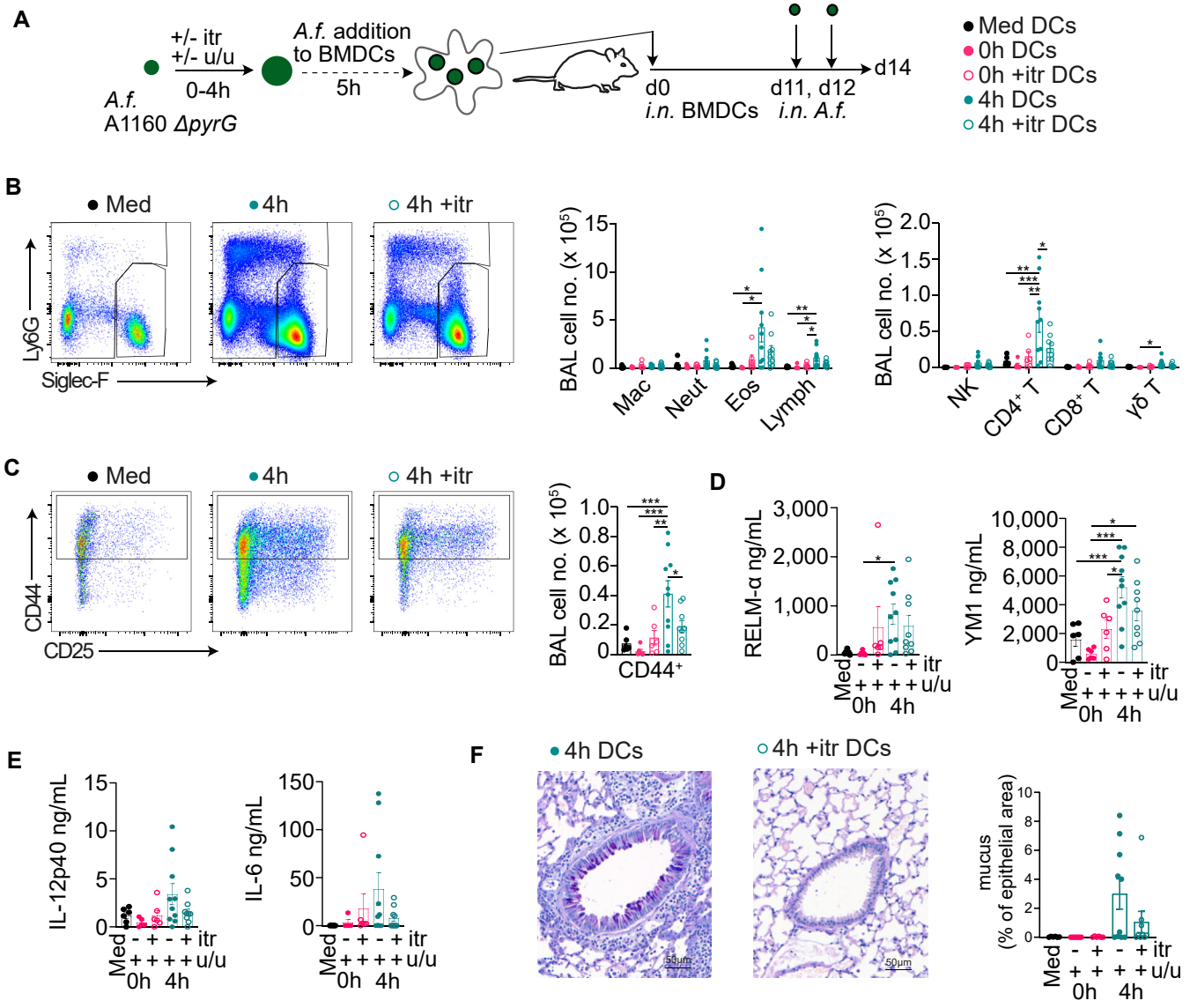

FIG 7. Spores treated with itraconazole partially reduced their capacity to activate DC ability to induce fungal allergic airway inflammation. **A,** *ΔpyrG* spores were incubated with u/u-containing media in presence or absence of itraconazole (itr); then growth was arrested by removal of u/u media at 0 or 4 hours. These were then incubated with DCs for 5 hours before intranasal transfer of 50,000 DCs into C57BL/6 mice. Mice were challenged intranasally at days 11 and 12 with 0.4×10^6 live CEA10 *Af* spores. **B,** Immune cell populations in BAL fluid were assessed by flow cytometry. Number of different cell populations isolated from BAL fluid of DC-sensitized mice was assessed by flow cytometry. **C,** Representative flow cytometry plots of $CD44^+CD4^+$ T cells in BAL fluid. **D** and **E,** Secretory factors in BAL fluid were quantified by ELISA. **F,** Lung sections were stained with AB-PAS to assess expansion of mucus-producing goblet cells, with mucus area in airway epithelia quantified as proportion of overall epithelial area. Data are combined from 2 independent experiments (n = 6-10 per group). Each data point is 1 individual mouse. Linear mixed effect modeling was applied, with experimental repeat as random effect variable; to compare multiple groups, *post hoc* Tukey HSD test was used. $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$. *AB-PAS,* Alcian blue–periodic acid–Schiff.

spores showed a significantly impaired ability to activate BMDCs compared to 4-hour spores that had not been treated with itraconazole in terms of induction of surface expression of costimulatory molecules (CD40, CD80, CD86, and MHC-II) and production of proinflammatory cytokines (IL-6, IL-12p40, and TNF) (Fig 6, *D* and *E*). However, while itraconazole-treated 4-hour arrested spores were less proficient at activating BMDCs than untreated 4-hour spores, the response detected was still significantly above the activation state of BMDCs cultured with 0-hour spores. Together, these data indicate that *Af* treatment with azole-based drugs can reduce, but not eliminate, the ability of early swollen spore stages to activate BMDCs *in vitro.*

To test if itraconazole treatment also affected the ability of early swollen *Af* spores to influence BMDC induction of fungal allergic airway inflammation, we transferred BMDCs that had been cultured with 0- or 4-hour arrested spores germinated in the presence or absence of itraconazole into the airways of naïve recipient mice (Fig 7, *A*). BMDCs that had been activated with

either 4-hour spores or itraconazole-treated 4-hour spores promoted similar levels of cellular inflammation in the airways of recipient mice (Fig 7, *B*), with a trend that BMDCs activated with itraconazole-treated spores induced less marked eosinophilia. Notably, BMDCs that had been cultured with itraconazole-treated spores did display significantly reduced ability to activate $CD4^+$ T cells *in vivo,* particularly in terms of number of activated $CD44^+CD4^+$ T cells recruited to the airways (Fig 7, *B* and *C*). Analysis of mediators in BAL fluid revealed that while 4-hour spore-treated BMDCs could induce *in vivo* secretion of RELMα and Ym1 (likely from resident macrophages), this was not significantly reduced with itraconazole treatment (Fig 7, *D*). When specifically focusing on IL-12p40 and IL-6, in line with our *in vitro* results (Fig 6, *E*), we saw a tendency for increased airway IL-6 and IL-12p40 in the 4-hour DC group, which tended to reduce in the 4-hour plus itraconazole group (Fig 7, *E*). A similar pattern was observed with airway mucus, with a tendency for the most mucus area in the 4-hour DC group (Fig 7, *F*). Taken together, these data show that itraconazole treatment of *Af* spores disrupts their morphotype transition while tending to make them less effective at activating BMDCs to induce allergic airway inflammation.

DISCUSSION

Using novel approaches to track and arrest differing stages of *Af* germination, we have identified a defined timepoint during isotropic spore growth which activates DCs to elicit robust allergic inflammation after *Af* exposure. We have further identified that itraconazole, a crucial antifungal drug, tends to limit this process, thereby reducing the ability of *Af* to elicit DC induction of allergic airway inflammation.

Sensitization to *Af* triggers and exacerbates the allergic airway inflammation that underpins asthma.[19,31,44] Few studies, however, have dissected the relative importance of early spore growth in triggering innate immune cells to elicit allergic inflammation. Previous studies have proposed that germination, with the breakdown of the hydrophobic rodlet/melanin layer that masks *Af* cell wall pathogen-associated molecular patterns—for example, β-glucan—is crucial to trigger innate cell activation and allergic inflammation.[28,31] These findings were largely achieved using spore-killing approaches (eg, PFA), and we confirm similar results, showing that PFA-killed *Af* is less able to elicit allergic airway inflammation. However, PFA killing alters surface epitopes, disrupts spore metabolism, and significantly changes dispersal patterns in the airways, all of which may have contributed to the observed defect independent of disruption of spore germination.[45,49] Our study overcomes these limitations; by utilizing a mutant strain *(ΔpyrG)*[38,39] that requires exogenous u/u to grow, we demonstrated a requirement for spore development to promote allergic airway inflammation. Surprisingly, *ΔpyrG* spores did induce minor lung inflammation and goblet cell expansion greater than the response elicited by PFA-killed spores, albeit to a much lower extent than responses caused by WT spores. Previous reports have shown that viable ungerminated spores are partially active, with low-level transcriptional activity.[50] The mechanism or mechanisms by which viable ungerminated spores can trigger low levels of inflammation is an important question for future work. In summary, these results highlight that breaking of dormancy and isotropic growth is crucial for promotion of allergic airway inflammation.

We have recently elucidated the role of DCs in mediating allergic inflammation in response to *Af* spores.[19] Therefore, having established a requirement for spore swelling, we wanted to pinpoint if there was a precise *Af* morphotype that triggers DCs to induce allergic inflammation. Via novel use of the *ΔpyrG Af* strain with timed removal of u/u (Fig 3), we discovered that the 3-hour viable early swollen *Af* spore stage is the earliest stage able to induce DC activation and allergic priming (Figs 3 and 4). To our knowledge, we are the first to demonstrate that an early-stage spore morphotype is crucial to generate allergic inflammation—earlier than previously thought, as it is before the development of germlings and hyphal extension. Indeed, prior research on fungal allergy has focused predominantly on *Af* extracts and/or components revealed/secreted at the germling or hyphal stages that are not exposed at the spore stage. For example, research into β-glucan, a key mediator of antifungal responses and allergic inflammation, has focused on its exposure at the germling stage at ~7 hours, but recent work has suggested potential earlier expression.[31,51] Furthermore, many of the previously characterized *Af* allergens are expressed/secreted predominantly at the hyphal stage, such as Asp f 5 and Asp f 13,[34,35,52,53] suggesting these may not be involved in initial spore mediated sensitization. Therefore, we are unaware of crucial mediators expressed by spores that trigger allergic disease. Recent evidence has emerged that the spore wall is more dynamic than previously thought, with both resting and swollen spores (at 5 hours) expressing over 140 surface proteins, including low levels of β-glucan, and earlier (2 hours) swollen *Af* spores able to secrete immunomodulatory proteases.[36,51,54] Our novel approach of timed removal of u/u to arrest *ΔpyrG* spore growth could be crucial to elucidate immunogenic factors expressed at early stages of spore development, which have the potential to interact with host immune cells.

To ascertain the potential of *Af* spores to elicit allergy, we primarily focused on their interactions with DCs because of their well-established role bridging innate and adaptive immunity to initiate allergic airway responses.[14,16] Determining the ability of DCs to mediate type 2 inflammation *in vitro* is challenging because the mechanism(s) they utilize to mediate these responses are unclear.[55] However, 3-hour spores increased DC expression of costimulatory molecules (CD80 and CD86) and proinflammatory cytokines prevalent during allergic inflammation.[56-59] To confirm that these changes resulted in allergic inflammation, we utilized a DC transfer approach, which we have used previously with other stimuli.[60,61] This showed 3-hour-spore pulsed DCs induced a type 2 inflammatory response (including influxes of eosinophils and type 2 cytokine–expressing $CD4^+$ T cells) (Fig 4) similar to responses observed after repeated intranasal *Af* exposure and demonstrates the importance of *in vivo* testing when confirming capability of DCs to induce type 2 polarization. We did not observe induction of IL-17 by 3-hour-spore pulsed DCs, supporting a hypothesis that IL-17 induction occurs via different pathways that may include other DC subsets[19] and/or signals triggered by fungal mediated damage of airway (eg, epithelium).[33] The precise process that DCs utilize to recognize spores and trigger downstream inflammation is unclear. However, *Af* and allergens such as house dust mite have been shown to signal via both Toll-like receptor and C-type lectin receptors on innate immune cells.[62-64] Defining the precise spore morphotype that elicits DCs to mediate

allergic inflammation provides an important platform to address this crucial question.

Despite evidence that antifungal drugs can be utilized as effective treatment strategies for severe asthma with fungal sensitization and allergic bronchopulmonary aspergillosis patients, the fungal morphotypes targeted in this intervention are unclear.[46-48] Here we identify a possible mechanism, one in which antifungal treatment partially inhibits the early stages of spore swelling, which we have shown to be critical to activate DCs to prime fungal airway allergy (Fig 6). A limitation of our work is reduced applicability to males, as only female mice were used. Nonetheless, our results could explain the efficacy observed in clinical trials of itraconazole in severe asthma independent of fungal colonization status, as our data indicate that spores would only need to remain in the airways for 3 hours to induce allergic inflammation.[65] Furthermore, our data highlight that itraconazole treatment alone is not sufficient to completely arrest spore swelling and inhibit their ability to induce DC-mediated allergic airway inflammation or goblet cell expansion. Recent work has identified that combination therapy of antifungal agents can be more effective in treatment of disease and reduce the threat of drug resistance emerging.[66] Therefore, with more therapeutics needed, high-throughput screening of compound libraries to identify candidates able to inhibit early spore swelling may be an effective strategy for discovery of novel drugs to treat fungal allergy.[67]

Taken together, our work has highlighted the critical importance of the early stages of spore swelling in promoting allergic airway inflammation, with understanding the allergenic factors in 3-hour spores a key angle for future research. Additionally, our work extends understanding of the potential mechanism of action of a currently used drug (itraconazole) in fungal asthma while also recommending that future therapeutics should be assessed for their ability to inhibit isotropic growth. Although our focus has been on *Af,* our findings are relevant for other allergenic filamentous fungi, including other *Aspergillus* species such as *A flavus* and *A niger,* with *Aspergillus* sensitization associated with increased asthma severity.[68-71] Whether our findings are translatable to other fungal species associated with allergic disease is unknown and is a key topic for future research.

DISCLOSURE STATEMENT

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Key messages

- **We have pinpointed the precise stage of *Af* development when germinating spores are capable of activating DCs to mediate downstream allergic airway inflammation.**
- **Antifungals (itraconazole) tended to reduce the potential of *Af* spores to stimulate allergic responses, highlighting a potential mechanism by which antifungal treatment might help to prevent the development of fungal allergy.**

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