Neutrophils Mediate Maturation and Efflux of Lung Dendritic Cells in Response to *Aspergillus fumigatus* Germ Tubes

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Invasive aspergillosis is a life-threatening complication of neutrophil deficiency or dysfunction. Neutropenia has previously been associated with enhanced influx of CD11b-expressing conventional dendritic cells to the lungs in response to *Aspergillus* species, but whether neutrophils directly modulate the function of dendritic cells in this infection is not known. We hypothesized that, in the setting of intrapulmonary challenge with *Aspergillus*, neutrophils promote the maturation and traffic of lung conventional dendritic cells to draining mediastinal lymph nodes. We report that neutropenia results in a marked accumulation of dendritic cells in the lungs of mice challenged with *Aspergillus* but greatly diminishes their egress to mediastinal lymph nodes independent of neutrophil microbicidal functions. Furthermore, the phenotype of lung dendritic cells was more immature in neutropenic animals than in nonneutropenic mice exposed to the microorganism. Consistent with this, coinubation with neutrophils greatly enhanced the upregulation of costimulatory molecules on dendritic cells exposed to *Aspergillus* *in vitro*, a process that was dependent on cell contact and the dendritic cell receptor DC-SIGN. Taken together, our data support an immunomodulatory cross talk between neutrophils and dendritic cells in the context of host response to *Aspergillus* that promotes the maturation and efflux of lung dendritic cells.

Invasive aspergillosis is a common and life-threatening complication of immunosuppression, with high mortality despite recent advances in prevention, diagnosis, and treatment (19, 24, 31). Better understanding of the defense mechanisms against the infection in these hosts has the potential to lead to novel immune-based therapies.

The host response to *Aspergillus* species differs sharply between healthy and immunocompromised hosts and, also, among immunocompromised hosts, depending on the nature of the immunologic defect (22). The spores of *Aspergillus* molds are ubiquitously distributed in the environment and are inhaled by humans daily. In healthy hosts, even large numbers of spores are efficiently eliminated from the respiratory tract once they become swollen and metabolically active. In immunocompromised hosts, the swollen conidia germinate and produce hyphal forms that penetrate the respiratory epithelium, causing pneumonia (or, less commonly, invasive rhinosinusitis) that can disseminate hematogenously or by direct extension.

Neutrophils have long been recognized as critical to host defense against *Aspergillus* species (2, 19, 24). In the conventional view, neutrophils are thought of as short-lived cells that exclusively execute antimicrobial functions in the initial phase of infection, before acquired immune responses have developed. Emerging evidence, however, points to immunomodulatory functions of neutrophils that are distinct from their antimicrobial effector functions and which extend beyond the initial phase of innate immunity (15). The contribution of these immunomodulatory roles to host defense is highly relevant to infections of immunocompromised hosts but has not been studied extensively.

A substantial body of literature has established a critical role of CD11b-expressing conventional dendritic cells (DCs) in the immune responses to *Aspergillus* (5, 6). Lung dendritic cells recognize, ingest, and kill both swollen conidia and hyphae and initiate protective T cell immunity against the organism. We recently reported a marked accumulation of conventional DCs in the lungs

of neutropenic patients with invasive aspergillosis and mice with experimentally induced neutropenia challenged with intrapulmonary *Aspergillus* (20). This effect was limited to CD11b⁺ conventional DCs (not the CD103⁺ epithelial DCs or plasmacytoid DCs), was independent of direct neutrophil microbicidal effects, and was, in part, attributable to the production of high levels of tumor necrosis factor (TNF) by lung DCs in neutropenic hosts, inducing the local production of chemokine ligands that mediate enhanced further recruitment of dendritic cells or their precursors to the lungs. Prior work in *in vitro* systems has shown that neutrophils and dendritic cells can interact directly, resulting in maturation of DCs (16, 32, 33), but this issue has not been addressed in the context of *in vivo* infections. Given our previous finding of the accumulation of DCs in the lungs of neutropenic animals, we tested the hypothesis that, in the setting of intrapulmonary challenge with *Aspergillus*, neutrophils promote the maturation of lung dendritic cells and their efflux to draining mediastinal lymph nodes.

MATERIALS AND METHODS

Animals and *in vivo* procedures. C57BL/6 or *Itgax*-DTR mice (12) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained under pathogen-free conditions and in compliance with institutional animal care regulations; experiments were performed in age- and gender-

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matched 6- to 10-week-old animals. Neutrophil depletion was achieved with a single intraperitoneal injection of 80 μ g of a monoclonal antibody (Ab) (Gr-1, clone RB6-8C5) 1 day before an intratracheal challenge with *Aspergillus fumigatus*, resulting in peripheral blood neutropenia for 3 days, as previously described (21, 23). We have previously reported that this protocol does not influence the number of lung or spleen lymphocytes or DC subsets (20, 25). For depletion of DCs, heterozygous *Irgax-DTR* mice were intraperitoneally injected with diphtheria toxin (Sigma-Aldrich, St. Louis, MO), as described previously (12), 1 day prior to challenge with *Aspergillus* germ tubes. To track the movement of cells from the lungs to lymph nodes, we labeled lung phagocytes with 0.5- μ m yellow-green latex microspheres (Ploysciences, Warrington, PA), delivered as a 1:25 dilution in 30 μ l of saline intratracheally together with *Aspergillus* germ tubes.

Preparation and administration of *A. fumigatus*. In order to compare the responses of neutropenic and nonneutropenic hosts, we used a previously characterized mouse model of intrapulmonary challenge with ethanol-killed *A. fumigatus* germ tubes (20). Briefly, *A. fumigatus* (strain 13073, American Type Culture Collection) conidia were collected in 0.1% Tween in phosphate-buffered saline (PBS) from 7- to 14-day-old cultures on Sabouraud's dextrose agar plates, filtered through sterile gauze, and counted under a hemacytometer. The resulting resting conidia were then grown in RPMI 1640 in a shaking 37°C incubator for 12 h to obtain germ tubes; the resulting fungal forms were then killed by resuspension in 70% ethanol in sterile water for 48 h. The viability of the resulting suspension was determined to be <1 in 2.6×10^7 CFU by serial dilution and culture. Fungal forms were administered intratracheally in inocula ranging from 6×10^5 to 9×10^5 in 30 μ l of saline per mouse.

Identification of leukocyte subsets. Animals were euthanized by CO₂ asphyxiation, the pulmonary vasculature was perfused via the right ventricle with PBS containing 5 mM EDTA, whole lung and mediastinal lymph nodes were removed, and single cell suspensions were prepared as previously described (20, 21, 25). The following antibodies were used to label cells for flow cytometry (from BD Biosciences, San Jose, CA, or eBiosciences, San Diego, CA): anti-CD11b-allophycocyanin-Cy7 (clone M1/70), anti-CD11c-phycoerythrin (PE)-Cy7 (clone HL3), anti-CD45-peridinin chlorophyll protein (clone 30-F11), anti-CD40-PE (clone 3/23), anti-CD86-PE (clone GL1), and anti-I-A/I-E (clone M5/114.15.2) conjugated to fluorescein isocyanate, biotin, allophycocyanin, or Pacific Blue. Samples were analyzed on a Canto II fluorescence-activated cell sorter (FACS) instrument using Diva software (BD Biosciences). The absolute number of each leukocyte subset was determined as the product of the percentage of the cell type and the total number of cells in the sample, as determined using an automated cell counter (Countess; Invitrogen, Carlsbad, CA).

In vitro studies. Immature bone marrow-derived myeloid DCs were prepared as described previously (25). In brief, bone marrow cells were cultured in 20 ng/ml murine granulocyte-macrophage colony-stimulating factor (mGM-CSF) for 5 days and positively enriched by immunomagnetic selection of CD11c⁺ cells according to the manufacturer's instructions (Miltenyi, Auburn, CA), resulting in $>95\%$ purity. Recovered cells were $>95\%$ viable by trypan blue exclusion and consistent with an immature phenotype, had low expression (mean fluorescence intensity) of CD86, CD40, and major histocompatibility complex class II (MHC-II) molecules by flow cytometry. For coculture experiments, resting bone marrow neutrophils were obtained from naïve animals as described previously (18) and incubated alone or with germ tubes in RPMI 1640 with 5% fetal bovine serum (FBS) in 24-well plates (Corning, Corning, NY) at 37°C in 5% CO₂ for 5 h before the addition of DCs; cells were then cultured for an additional 16 h. The ratio of DCs to germ tubes to neutrophils was 1:1:10. For cultures performed in transwell plates, DCs were placed in the lower chambers, while both ethanol-killed hyphae and neutrophils were added to the upper chambers. In blocking antibody experiments, 20 μ g/ml final concentration of affinity-purified goat anti-mouse anti-

CD209b/SIGNR1 or control goat IgG (R&D Systems) was added to the medium.

Statistical analysis. Data were analyzed on a Macintosh Powerbook G4 computer using the Prism statistical package (version 4.0a; Graphpad Software, San Diego, CA). Values between 2 groups over multiple times were compared with 2-way analysis of variance (ANOVA), comparisons between 2 groups at a single time were performed with an unpaired two-tailed Mann-Whitney (nonparametric) test, and comparisons between multiple groups at a single time were performed using the Kruskal-Wallis test with Bonferroni posttest. Probability values were considered statistically significant if they were less than 0.05.

RESULTS

Neutropenia is associated with a reduced number of DCs in mediastinal lymph nodes after challenge with *Aspergillus*. Given our previous findings of the accumulation of CD11b⁺ conventional DCs in the lungs of neutropenic animals with experimental invasive aspergillosis (20, 25), we began by simultaneously comparing the number of CD11b⁺ conventional DCs in the lungs and mediastinal lymph nodes of neutropenic and nonneutropenic animals challenged with *Aspergillus* germ tubes. Since neutropenia in this model results in failure of the host to control the growth of *Aspergillus*, we challenged the animals with ethanol-killed *Aspergillus* germ tubes, as previously described (20). As expected, there were increased numbers of CD11b⁺ conventional DCs in the lungs of both neutropenic and nonneutropenic mice after challenge, which was more pronounced in neutropenic hosts, resulting in 2- to 4-fold greater numbers of DCs in the lungs of neutropenic mice (Fig. 1A). The numbers of CD11b⁺ DCs in the draining mediastinal lymph nodes, however, showed the opposite trend: while the number of lymph node DCs increased in nonneutropenic mice after challenge with *Aspergillus*, consistent with migration of DCs from the lungs to the lymph nodes, there was no detectable change in the number of draining lymph node DCs in neutropenic animals, despite the accumulation of a large number of these cells in the lungs in neutropenic mice (Fig. 1B).

Neutropenia results in impaired migration of DCs from lungs to lymph nodes after challenge with *Aspergillus*. Given the accumulation of DCs in the lungs of neutropenic animals with experimental invasive aspergillosis, we reasoned that the number of leukocytes in the lung represents a dynamic balance between the arrival or local differentiation of cells on the one hand and departure or death on the other. Thus, in addition to the previously observed increase in influx of DCs to the lung in neutropenic hosts challenged with *Aspergillus*, the mismatch between the large numbers of DCs in the lungs of neutropenic mice with the small numbers of draining lymph node DCs suggested failure of migration to the draining lymph nodes as a mechanism.

To distinguish between dendritic cells arriving in the lymph node after challenge from those that are resident in the lymph node, we made use of a transgenic mouse in which CD11c-expressing populations, including lung conventional DCs, can be conditionally ablated (12). Systemic administration of diphtheria toxin to these animals has previously been shown to result in depletion of CD11c-expressing populations, which in otherwise unmanipulated mice is followed by their homeostatic reconstitution over several days (12, 13). We reasoned that the number of DCs in a given compartment represents the dynamic relationship between their recruitment and local differentiation on the one hand and their efflux and death on the other. We have previously reported that the rate of apoptosis of lung conventional DCs in mice

FIG 1 Effect of neutrophil depletion on the number of conventional DCs in lungs and mediastinal lymph nodes after challenge with *Aspergillus*. Numbers of lung (A) and mediastinal lymph node (B) conventional DCs are shown at various times after challenge with *Aspergillus* germ tubes in animals with Ab-mediated neutrophil depletion and mice treated with isotype control Ab. Data shown represent means $\pm$ standard errors of the means (SEM); $n = 4$ to 6 mice per group per time point pooled from 2 independent experiments. Time zero represents unchallenged animals. *, $P < 0.05$ comparing trend between the two groups over time.

challenged with *Aspergillus* is not influenced by neutropenia (20). To address the issue of DC efflux from the lungs to draining lymph nodes, we sought to deplete DCs before *Aspergillus* challenge and compare the reappearance of DCs in the mediastinal lymph nodes as a measure of DCs that have transited through the lungs, using repopulation of a distant lymphoid organ, the spleen, as an internal control for DC repopulation independent of traffic through the lungs. We administered diphtheria toxin to mice 1 day prior to challenge with germ tubes. This resulted in nearly total ablation of lung conventional DCs on the day of challenge, followed by a gradual increase in the number of lung DCs (Fig. 2A). The repopulation of lung DCs was not influenced by neutropenia (Fig. 2A), consistent with our prior finding that the enhanced recruitment of DCs to the lungs in neutropenic hosts is driven by a positive feedback loop that requires DC-derived inflammatory mediators (20). Despite similar numbers of lung DCs between neutropenic and nonneutropenic animals, we found reduced numbers of mediastinal lymph node DCs in neutropenic mice compared to the numbers in nonneutropenic mice (Fig. 2B); in contrast, the repopulation of DCs in the spleen was unaffected by neutropenia (Fig. 2C), suggesting a selective defect in the migration of lung DCs to lung draining lymph nodes.

To use another method to test the hypothesis that an impairment of efflux is the mechanism underlying DC accumulation in the lungs of neutropenic mice challenged with *A. fumigatus*, we tracked the migration of DCs from the lungs to the draining mediastinal lymph nodes. To achieve this, lung phagocytes were labeled with fluorescent latex beads coadministered intratracheally with *Aspergillus* germ tubes. Analysis of lung cell suspensions from animals with and without neutrophil depletion revealed comparable numbers of bead-labeled resident lung macrophages (data not shown). Examination of the mediastinal lymph nodes showed that, compared to animals challenged with latex beads alone, challenge with *A. fumigatus* resulted in migration of bead-associated MHC-II⁺ CD11b⁺ CD11c⁺ conventional DCs from the lungs to the lymph nodes (Fig. 3). In contrast, the efflux of conventional DCs from the lungs to draining lymph nodes was greatly diminished in neutropenic hosts challenged with *A. fumigatus* (Fig. 3). Taken together, these data suggest that the efflux of lung DCs to draining lymph nodes in response to *A. fumigatus* is dependent on the presence of neutrophils.

Neutrophils mediate the maturation of lung DCs in response to *Aspergillus* germ tubes. Conventional DCs that are resident in nonlymphoid tissues, including the lungs, have a relatively low

FIG 2 Role of neutrophils in repopulation of conventional DCs after ablation of CD11c⁺ cells in mice challenged with *Aspergillus*. Itgax-DTR mice with Ab-mediated neutrophil depletion and mice treated with isotype control Ab were treated with diphtheria toxin. Number of lung (A), mediastinal lymph node (B), and spleen (C) conventional DCs are shown at various times after challenge with *Aspergillus* germ tubes in animals with Ab-mediated neutrophil depletion and mice treated with isotype control Ab. Data represent means $\pm$ SEM; $n = 4$ mice per group per time point pooled from 3 independent experiments. Time zero represents unchallenged animals. *, $P < 0.05$ compared with neutropenic wild-type mice.

FIG 3 Role of neutrophils in migration of lung myeloid DCs to mediastinal lymph nodes in response to *Aspergillus* challenge. Neutropenic and nonneutropenic mice were challenged intratracheally with *Aspergillus* germ tubes mixed with FITC-labeled beads; uninfected mice were challenged with intratracheal FITC-labeled beads only. (A) Representative flow cytometry plots and gating strategy of lymph node cell suspensions. All plots were initially gated on MHC-II⁺ cells. (B) Summary data represented as means $\pm$ SEM of bead-associated cells (bead+) in mediastinal lymph nodes; $n = 8$ per group per time point pooled from 2 independent experiments. *, $P < 0.05$ comparing trend between neutropenic and nonneutropenic challenged mice.

capacity to migrate to draining lymph nodes. Upon encountering microbial molecular patterns, these cells undergo profound phenotypic changes, collectively referred to as maturation, rapidly migrate to draining lymphoid tissues, and efficiently present antigen to naïve T cells. Given the reduced capacity of lung DCs to migrate to the mediastinal lymph nodes in neutropenic mice challenged with *Aspergillus*, we assessed the effect of neutropenia on the maturation phenotype of lung DCs after challenge with *Aspergillus* germ tubes. DCs in lungs of neutropenic hosts were found to express lower levels of the costimulatory molecules CD86 and CD40, as well as MHC-II, than the DCs from nonneutropenic mice, consistent with a more immature phenotype (Fig. 4).

We next sought to assess whether neutrophils directly mediate DC maturation in response to *Aspergillus*. We reasoned that neutrophils in the lungs of animals (or humans) challenged with *Aspergillus* have been activated by having encountered the organism (4); monocyte-derived DCs recruited to the lungs thus presumably encounter the microorganism and activated neutrophils

FIG 4 Effect of neutrophils on maturation of lung conventional DCs *in vivo*. Panels show representative flow cytometry histograms and mean fluorescence intensities of CD40 (A), CD86 (B), and MHC class II (C) gated on CD45⁺ CD11b⁺ CD11c⁺ lung DCs at various times after challenge with *Aspergillus* germ tubes in neutropenic and nonneutropenic mice. Shaded histograms represent isotype control Ab, gray dotted lines denote neutropenic mice, and solid black lines denote nonneutropenic mice. Data shown in bar graphs represent means $\pm$ SEM; $n = 4$ or 5 per group per time point, representative data from 3 experiments. *, $P < 0.05$ comparing trend between neutropenic and nonneutropenic challenged mice. au, arbitrary units.

together. To model this, we isolated resting bone marrow neutrophils and exposed them to *Aspergillus* germ tubes *in vitro* before the addition of dendritic cells; as controls, we used germ tubes alone or neutrophils alone before the addition of DCs (Fig. 5). Cultured immature DCs expressed low levels of CD86 and CD40, and this phenotype was not affected when immature DCs were coincubated with *Aspergillus* germ tubes. Incubation of immature DCs with resting neutrophils in the absence of germ tubes resulted in a modest increase in surface expression of costimulatory molecules; in contrast, maturation of DCs was greatly enhanced when DCs were incubated with both germ tubes and neutrophils and was further enhanced by increasing the number of activated neutrophils added to the coculture (Fig. 5). These data suggest that, in the presence of *Aspergillus* fungal forms, neutrophils directly induce DC maturation in a dose-dependent manner.

Last, we sought to assess mechanisms involved in neutrophil-mediated maturation of DCs coincubated with hyphae. We found that prevention of contact between DCs and neutrophils/hyphae using a transwell system abrogated the upregulation of DC maturation markers (Fig. 6), suggesting that DCs require cell-cell contact with neutrophils or hyphae (or both) in order to mature.

FIG 5 Effect of neutrophils on maturation of myeloid DCs in response to *Aspergillus* *in vitro*. Expression of CD40 (A) and CD86 (B) on immature bone marrow-derived DCs was assessed by flow cytometry after overnight culture with resting neutrophils and *A. fumigatus* germ tubes. Each of the culture condition experiments was performed in triplicate; representative data from 2 independent studies are shown. Zero on the x axis indicates DCs incubated without neutrophils. *, $P < 0.05$ comparing trend between DCs incubated with and without *Aspergillus*. In addition, there was a statistically significant increase in mean fluorescence intensity (MFI) with an increasing number of neutrophils.

Based on prior literature on neutrophil-DC interactions (14), we examined the contribution of DC-SIGN (DC-specific intercellular adhesion molecule-3-grabbing nonintegrin, CD109) to neutrophil-mediated DC maturation. We found that Ab-mediated neutralization of DC-SIGN but not incubation with control IgG inhibited the expression of maturation markers CD86 and CD40 by DCs (Fig. 6). These results provide evidence that, within the limits of an *in vitro* coculture system, activated neutrophils can induce the maturation of conventional DCs via cell-to-cell contact and DC-SIGN during responses to *Aspergillus* hyphae.

DISCUSSION

The lung contains at least three populations of DCs in the steady state: CD11b⁺ conventional DCs, CD103⁺ airway-associated DCs, and the least well-studied, plasmacytoid DCs (10). Upon antigen challenge, all are capable of maturation and mobilization to draining mediastinal lymph nodes and antigen presentation. In immunocompetent hosts challenged with intrapulmonary *Aspergillus* conidia, circulating Ly6C^{hi} monocytes are recruited to the lung via a CCR2-dependent mechanism and differentiate into conventional DCs (11); these cells phagocytose and process fungal elements and transfer them to lymphoid tissues where they initiate T cell-mediated immunity (3, 8). The response in immunocompromised hosts differs from this scenario in several key respects: in the setting of transient Ab-mediated neutropenia, a large number of conventional DCs accumulate in the lung, a process that is partially dependent on CCR6 and its ligand CCL20, and their efflux from the lungs is mediated by CCR7 (9, 25).

Cross talk with neutrophils appears to be critical in determining the recruitment of lung conventional DCs, a process that is independent of the microbicidal functions of these leukocytes: in the absence of neutrophils, the accumulated lung DCs become the major cellular source of TNF in the lungs, which induces local CCL2 and CCL20 production, in turn leading to greater recruitment of DCs or their precursors (20). The importance of this process in host defense in neutropenic hosts is underlined by the observation that inhibition of the influx of DCs, their depletion, or

FIG 6 Mechanism of neutrophil-mediated maturation of myeloid DCs in response to *Aspergillus* *in vitro*. Expression of CD40 (A) and CD86 (B) on immature bone marrow-derived DCs was assessed by flow cytometry after overnight culture with resting neutrophils and *A. fumigatus* hyphae. Each culture condition was performed in triplicate; representative data from 2 independent studies are shown. TW, transwell system with DC in the lower chamber and hyphae and neutrophils or hyphae alone in the upper chamber; aDC-SIGN, blocking Ab against DC-SIGN; IgG, control Ab; *, $P < 0.05$ compared to DC incubated alone, DC plus neutrophils, or DC plus hyphae; **, $P < 0.05$ compared to DC plus neutrophils plus hyphae or DC plus neutrophils plus hyphae plus control Ig; ***, $P < 0.05$ compared to DC plus neutrophils plus hyphae.

neutralization of TNF all result in worsened survival and increased lung fungal content (17, 20, 25), whereas inhibition of DC efflux from the lungs in CCR7-deficient animals results in improved outcomes (9). Importantly, this mechanism appears to be operational in humans also, since neutropenia is associated with increased lung DCs in human invasive aspergillosis and TNF neutralization is an important predisposing factor in human invasive aspergillosis (20, 30). It has recently been shown that DCs are also the major source of lung TNF in immunocompetent mice challenged with *Aspergillus* conidia, where they are critical to the initiation of Th17 immunity (8), presumably after efflux to draining lymph nodes. This raises a key question: how is the cycle of recruitment and retention of TNF-producing DCs established in neutropenic hosts if the interaction of DCs with *Aspergillus* in the lungs results in their rapid maturation and efflux to lymphoid tissues? In this context, the present study shows that in immunocompetent hosts, neutrophils provide a critical signal for DC maturation and efflux. Neutropenia, on the other hand, results in retention of immature TNF-producing DCs in the lungs, initiating the amplification loop. This concept is supported by the observation that, if the cycle is interrupted by ablation of lung DCs before challenge with germ tubes, neutropenia no longer influences the arrival of DCs in the lungs (Fig. 2).

Several DC and neutrophil receptors and secreted mediators have been implicated in the cross talk between these cells in *in vitro* experimental systems (14). Among these, DC-SIGN is a type II C-type lectin receptor expressed on immature dendritic cells that recognizes mannose and fructose carbohydrate moieties on diverse targets; DC-SIGN acts as a microbial pattern recognition receptor for multiple classes of microorganisms, in addition to several endogenous ligands, and can influence several cellular processes, including adhesion, phagocytosis, and regulation of the development of adaptive immunity (7). Of particular relevance to the present work, DC-SIGN can bind both *Aspergillus* (27, 28) and the sialyl-Lewis-X antigen associated with neutrophil CD11b/CD18 complex (32, 33). Whether the observed effect of DC-SIGN neutralization is related to its interaction with one or both of these ligands is a potential area for further investigation.

A limitation of the present work relates to technical aspects of neutrophil depletion. The evaluation of the role of neutrophils *in vivo* can currently be achieved with drug- or radiation-induced neutropenia, which closely resemble human neutropenia but are hampered by broad off-target effects, or Ab-mediated neutrophil depletion. Ab-induced neutropenia is more selective but still limited by the potential for depletion of nonneutrophil populations, immune-complex-mediated effects, and Fc receptor-dependent Ab effects. In particular, the administration of RB6-8C5 at doses 6- to 12-fold higher than used in the current study has been associated with the depletion of multiple nonneutrophil Ly6C-expressing cells, such as Ly6C^{hi} monocytes and plasmacytoid DCs (1, 29). We previously reported that the current protocol does not deplete lung DCs or macrophage populations and have reported similar lung DC accumulation when neutrophil depletion was achieved with an alternative Ly6G-specific monoclonal Ab (23, 25); Ab-independent methods of neutrophil depletion are nevertheless necessary both to definitively exclude off-target effects of Abs and for applications such as the induction of long-term neutropenia.

The present work has several implications for future investigation. First, the role of neutrophils in the development of T cell-mediated immunity to *Aspergillus* needs to be established. In view of the current study, one may postulate that reduced maturation of DCs in neutropenic hosts may inhibit the development of T cell-mediated immunity. On the other hand, DC-derived interleukin-23 (IL-23), neutrophil-derived IL-17 (34), and DC-derived TNF (8) have been shown to contribute to the development of Th17 immunity in response to *Aspergillus* via dectin-1; thus, neutropenia may influence the polarization of T cell-mediated immunity to *Aspergillus*, similar to recent observations in dectin-1 deficiency (26). Another potential avenue of investigation pertains to the mechanism by which neutrophils interact with dendritic cells in the context of host response to *Aspergillus*: while our data suggest that contact and DC-SIGN signaling are necessary for DC maturation in the *in vitro* system, the role of other dendritic cell receptors, as well as neutrophil functions (such as apoptosis or the production of neutrophil extracellular traps [NETosis]), in DC maturation may represent novel therapeutic targets in manipulating the host response in invasive aspergillosis.

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