

TNF- α from inflammatory dendritic cells (DCs) regulates lung IL-17A/IL-5 levels and neutrophilia versus eosinophilia during persistent fungal infection

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Aspergillus fumigatus is commonly associated with allergic bronchopulmonary aspergillosis in patients with severe asthma in which chronic airway neutrophilia predicts a poor outcome. We were able to recapitulate fungus-induced neutrophilic airway inflammation in a mouse model in our efforts to understand the underlying mechanisms. However, neutrophilia occurred in a mouse strain-selective fashion, providing us with an opportunity to perform a comparative study to elucidate the mechanisms involved. Here we show that TNF- α , largely produced by Ly6c⁺CD11b⁺ dendritic cells (DCs), plays a central role in promoting IL-17A from CD4⁺ T cells and collaborating with it to induce airway neutrophilia. Compared with C57BL/6 mice, BALB/c mice displayed significantly more TNF- α -producing DCs and macrophages in the lung. Lung TNF- α levels were drastically reduced in CD11c-DTR BALB/c mice depleted of CD11c⁺ cells, and TNF- α -producing Ly6c⁺CD11b⁺ cells were abolished in Dectin-1^{-/-} and MyD88^{-/-} BALB/c mice. TNF- α deficiency itself blunted accumulation of inflammatory Ly6c⁺CD11b⁺ DCs. Also, lack of TNF- α decreased IL-17A but promoted IL-5 levels, switching inflammation from a neutrophil to eosinophil bias resembling that in C57BL/6 mice. The TNF- α ^{low} DCs in C57BL/6 mice contained more NF- κ B p50 homodimers, which are strong repressors of TNF- α transcription. Functionally, collaboration between TNF- α and IL-17A triggered significantly higher levels of the neutrophil chemoattractants keratinocyte cytokine and macrophage inflammatory protein 2 in BALB/c mice. Our study identifies TNF- α as a molecular switch that orchestrates a sequence of events in DCs and CD4 T cells that promote neutrophilic airway inflammation.

Th17 | tolerance

Asthma is a heterogeneous inflammatory disease of the airways, and accumulating evidence suggests an association between fungal exposures and disease severity (1). *Aspergillus fumigatus*, which induces allergic bronchopulmonary aspergillosis (ABPA) in 25% of severe asthmatics, is one of the most common fungi involved (1, 2). Neutrophilic airway inflammation is now well recognized as being a feature of severe asthma (3–5) and ABPA (6, 7). During acute infection, neutrophils are crucial for antifungal defense (8, 9), and lack of neutrophils is the best appreciated risk factor for invasive aspergillosis (10). However, uncontrolled neutrophil infiltration in *Aspergillus*-induced allergic disorders positively correlates with tissue damage and loss of lung function (6, 11). Because the control of neutrophils is highly desirable in these diseases, there is considerable interest in understanding the mechanisms that promote and perpetuate pulmonary neutrophilia (12).

Individuals inhale *A. fumigatus* conidia, called resting conidia, into the lung on a daily basis, where these resting conidia germinate into immunogenic swollen conidia (SC) that trigger host

immune responses. Under normal circumstances, hosts eradicate them successfully without developing deleterious inflammation because of the highly effective and tightly regulated defense mechanisms. Innate immune cells, mainly macrophages and neutrophils (8, 13), as well as adaptive CD4⁺ Th1 cells (14, 15) mediate antifungal responses. However, both Th2 and Th17 responses are thought to be detrimental, contributing to inflammation (14–17). *A. fumigatus* can trigger aggressive allergic diseases such as ABPA only in individuals with underlying pulmonary disorders in which pathogen clearance is compromised, resulting in a persistent local and systemic inflammatory response that contributes to the disease state (18).

To better understand the mechanisms underlying *Aspergillus*-induced neutrophilic airway inflammation, mice were repeatedly exposed to *A. fumigatus* to mimic the continuous release of fungal antigens in patients who fail to effectively clear the fungus. With this exposure protocol, we observed that two inbred strains of mice, BALB/c and C57BL/6, developed distinct inflammation profiles in the airways, with the former exhibiting neutrophil prominence and the latter an eosinophil bias. We thus probed this strain-associated differential granulocyte influx to better understand the immunological basis for neutrophilic airway infiltration. TNF- α , contributed by inflammatory dendritic cells (DCs) and macrophages, was crucial for promoting IL-17A from CD4⁺ T cells and for collaborating with it to up-regulate expression of the neutrophil chemoattractants keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP-2) as well as airway neutrophilia. TNF- α production and inflammatory DC accumulation were blunted in Dectin-1^{-/-} and MyD88^{-/-} mice. C57BL/6 DCs with a muted TNF- α profile harbored more NF- κ B p50 homodimers, which are repressors of TNF- α transcription. Therefore, our study highlights a central role for TNF- α in promoting neutrophilic airway inflammation in response to persistent fungal exposure.

Results

Repeated Challenges with *A. fumigatus* Induce Distinct Granulocyte Patterns in BALB/c and C57BL/6 Mice. Mice were repeatedly challenged with fungal conidia to mimic persistent local antigenic

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stimulation in individuals incapable of adequate fungal clearance. Multiple challenges reproduced some critical features of allergic disease observed in persistently exposed individuals that were not present after a single exposure and were true for both BALB/c and C57BL/6 mice (Fig. S1 and *SI Results*). However, there was a marked strain-specific difference in the type of granulocytes recruited to the airways. Despite the lower level of cellular infiltrate in the airways, the C57BL/6 mice did not demonstrate a proportionately lower number of cells in every compartment. As shown in Fig. 1*A*, the mice exhibited greater airway eosinophilia as detected in the bronchoalveolar lavage fluid (BALF). In contrast, the BALB/c mice showed a prominent neutrophil-biased response (Fig. 1*A* and Fig. S1*D*). Although inflammatory lesions in C57BL/6 mice spared the conducting airways and were present primarily within the parenchyma adjacent to terminal bronchioles, BALB/c mice harbored lesions involving the large and small airways that obliterated extensive portions of the alveolar spaces. BALB/c bronchioles contained degenerate neutrophils admixed with sloughed epithelial cells, fibrin, and cellular debris. The lungs from BALB/c mice had prominent perivascular infiltrates containing dense sheets of neutrophils admixed with smaller numbers of eosinophils. In contrast, the lungs from challenged C57BL/6 mice showed modest multifocal intraalveolar aggregates of macrophages and occasional multinucleated giant cells intermixed with small clusters of degenerate eosinophils and neutrophils (Fig. 1*B*). This strain-associated difference was only revealed during repeated challenges, with similar neutrophilic influx being observed in both strains soon after administration of 10×10^6 conidia (Fig. S1*E*). These results suggested that mechanisms that promote neutrophilic versus eosinophilic inflammation come into play during persistent antigenic stimulation. Importantly, the accumulated neutrophils in the BALB/c mice after repeated fungal exposure seemed not to be of any added benefit because the C57BL/6 mice with much less neutrophilia did not show compromised fungal clearance in the long term (Fig. S1*F*). It is well known that excessive neutrophils can cause indiscriminate damage to the host. For instance, when released into the extracellular fluid by leakage or by cell lysis, neutrophil myeloperoxidase (MPO) is toxic to the tissue (19). Therefore, the significantly higher MPO in the cell-free BALF of BALB/c mice (Fig. 1*C*) likely contributed to the lung damage

shown in Fig. 1*B*. The inflammatory pattern in BALB/c mice was reminiscent of the neutrophil-rich inflammation in severe asthma and ABPA (6, 20), which persuaded us to investigate the underlying mechanisms.

IL-17A Contributes to Neutrophil Influx in both Strains. IL-17A is known to promote neutrophil-rich responses (21). In our model, IL-17A⁺ cells were detectable after the first challenge and expanded during multiple challenges (Fig. 2*A*). IL-17A is not exclusively produced by CD4⁺ T cells, and a recent study showed that, by 6 d postinoculation with 5×10^6 *A. fumigatus* conidia, $\gamma\delta$ rather than $\alpha\beta$ T cells are a crucial source of IL-17A in the lung (17). In our model, at an early time point (24 h after the first challenge), CD4⁺ T and $\gamma\delta$ T cells represented $\sim 40\%$ and $\sim 13\%$ of IL-17A-producing cells, respectively (Fig. 2*A*). CD4⁺ T cells became a predominant source of IL-17A after eight challenges, responsible for $> 60\%$ of IL-17A⁺ cells, whereas $\gamma\delta$ T cells remained $\sim 10\%$ of IL-17A⁺ cells in both strains (Fig. 2*A*). Systemic blockade of IL-17A in BALB/c mice by anti-IL-17A antibody or IL-17RA deficiency on the C57BL/6 background selectively reduced neutrophil counts in the BALF by $\sim 60\%$ (Fig. 2*B*), suggesting that IL-17A was functional in both strains. We further asked whether differences in IL-17A between the two strains could explain the differential neutrophilia. Because CD4⁺ T cells were the main IL-17A-producers (Fig. 2*A*), we evaluated Th17 development. Interestingly, the C57BL/6 mice mounted a normal, if not even better, Th17 response, with a slightly higher percentage of Th17 cells within the CD4⁺ T population, a stronger cytokine-secreting capacity (higher mean fluorescence intensity of the positive cells) (Fig. 2*C*), and equivalent mRNA expression in the CD4⁺ T cells (Fig. 2*D*). There was a slightly lower IL-17A in the lung homogenate of C57BL/6 mice (Fig. 2*E*), which might be because of the lower number of lung CD4⁺ T cells in this strain. Thus, IL-17A was important in promoting neutrophilia, regardless of the genetic background. However, the difference in IL-17A production in the two strains did not seem large enough to explain the strain-associated drastic difference in airway neutrophilia. Given that *in vitro* studies have suggested synergism between IL-17A and TNF- α in the induction of factors that support development and recruitment of neutrophils (22–24), we investigated whether TNF- α contributed to the neutrophil-biased airway inflammation in the BALB/c strain.

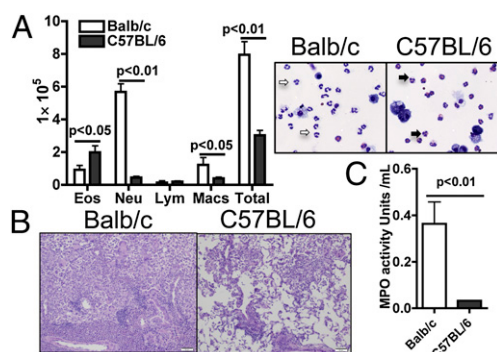
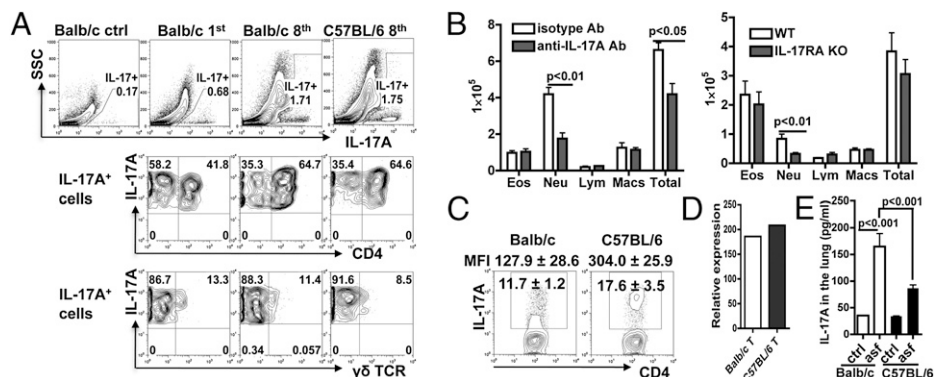


Fig. 1. Characterization of the inflammatory responses induced by repeated *A. fumigatus* challenges in BALB/c and C57BL/6 mice. Mice were sensitized with 10×10^6 conidia, challenged with 1×10^6 conidia eight times, and sampled at 24 h after the last challenge. (*A Right*) Differential cell counts in the BALF. Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes; Macs, macrophages. (*Left*) Representative images of cytospin are shown, and the white and black arrows indicate neutrophils and eosinophils, respectively. (*B*) Representative hematoxylin/eosin staining of lung sections. (Magnification: $\times 200$; scale bars: 50 μ m.) (*C*) MPO activity in the cell-free BALF. Results are represented as mean $\pm$ SD ($n = 4$ mice per group). Shown is an experiment representative of at least two experiments.

TNF- α , Mainly Derived from CD11c⁺ DCs and Macrophages, Is Important in Determining Strain-Specific Pulmonary Neutrophilia.

In response to repeated challenges, TNF- α was appreciably induced in BALB/c mice but only marginally elevated in C57BL/6 mice compared with their corresponding controls (Fig. 3*A*). The TNF- α difference was much greater than the difference in IL-17A (Fig. 2*E*), suggesting that TNF- α could be the determinant in strain-specific neutrophilia. BALB/c mice treated with anti-TNF- α antibody (Fig. 3*B*) or TNF- α KO BALB/c mice (Fig. S2*A*) showed decreased neutrophil counts. Interestingly, the decrease in neutrophils was accompanied by a tendency toward higher eosinophil counts (Fig. 3*B* and Fig. S2*A*), resembling the granulocyte pattern in the airways of the C57BL/6 strain. The IL-17A level in the lung was decreased by TNF- α neutralization, whereas the level of IL-5, which was higher in C57BL/6 than in BALB/c mice, was increased when TNF- α was absent (Fig. 3*C*), in agreement with the shift from neutrophilia to eosinophilia. This finding suggested that TNF- α acted as a molecular switch to regulate the neutrophil/eosinophil ratio. The drastic strain-associated TNF- α difference required multiple challenges (Fig. S2*B*), consistent with the finding that distinct granulocyte patterns only occurred after repeated challenges (Fig. 1*A* and Fig. S1*E*). Together, these results suggested that strain-specific regulatory mechanisms triggered as a result of persistent stimulation by fungal antigens de-

Fig. 2. IL-17A is involved in driving neutrophilia in both strains. (A) Representative FACS plots show intracellular IL-17A staining in the lung cells from PBS control and challenged mice at 24 h after the first and eighth challenges. IL-17A⁺ gates were drawn, and CD4 or $\gamma\delta$ T-cell receptor ($\gamma\delta$ TCR) expression is shown within the IL-17A⁺ cells. The numbers indicate the percentages of cells in the gated populations. (B) BALB/c mice treated with isotype or anti-IL-17A antibody (Ab), IL-17A^{-/-} (on the C57BL/6 background), or WT C57BL/6 mice were challenged, and cell differential counts in the BALF were assessed at 24 h after the eighth challenge. (C) Representative FACS plots show intracellular IL-17A staining in the lung CD4⁺ T cells at 24 h after the eighth challenge. The percentage of IL-17A⁺ cells within lung CD4⁺ T cells and mean fluorescence intensity (MFI) of IL-17A⁺ cells are indicated. (D) IL-17A mRNA in lung CD4⁺ T cells at 24 h after the eighth challenge was expressed as fold increase compared with that in lung CD4⁺ T cells of naïve mice. (E) ELISA of IL-17A in the lung homogenates prepared from mice after the eighth challenge or from PBS controls. Results are expressed as mean $\pm$ SD ($n = 4$ mice per group). Shown is an experiment representative of at least two experiments.



terminated the differential TNF- α outcome, which, in turn, contributed to strain-associated differential neutrophilia.

Next, we investigated the cellular source of TNF- α . Administration of diphtheria toxin (DT) to CD11c-DTR-EGFP transgenic (Tg) mice (on the BALB/c background) after challenges depleted >60% of the CD11c⁺ cells (Fig. S2C) by 24 h, which caused a 50–70% reduction of TNF- α levels in both the BALF and the lung homogenate (Fig. 3D), confirming that CD11c⁺ cells were the major source of TNF- α . Consistent with the results described above (Fig. 3A and D), in response to in vitro SC restimulation, lung cells from BALB/c mice produced a much larger amount of TNF- α than those from C57BL/6 mice, which was mainly derived from CD11c⁺ but not CD11c⁻ cells (Fig. S2D). In challenged BALB/c mice, the CD11c⁺ fraction could be further divided into eosinophils, DCs, and macrophages (Fig. S3A and B and SI Results), and both DCs and macrophages were able to produce TNF- α (Fig. 3E). In the DC compartment, TNF- α production was largely restricted to CD11b⁺ DCs and preferentially to Ly6C⁺ DCs (Fig. 3E). The expression of Ly6C reflected their monocytic origin, suggesting that these TNF- α ⁺ DCs were newly derived from monocytes, which was reminiscent of DCs producing TNF and inducible nitric oxide synthase (iNOS) (called “Tip-DCs”) (25, 26). However, surprisingly, in our model, the TNF- α -producing DCs did not coexpress iNOS, rather TNF- α ⁺ DCs and iNOS⁺ DCs were different DC subsets (Fig. S3C), implying that the TNF- α ⁺ DC in our model is a distinct cell type.

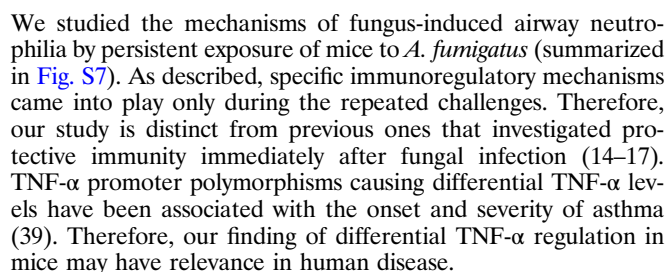
TNF- α -Producing DCs Contribute the Most to Strain-Associated TNF- α Difference. Numerically, BALB/c mice had more DCs and macrophages (Fig. S4A). In the BALB/c strain, the frequency of CD11b⁺Ly6C⁺ DCs, the subset that was enriched for TNF- α (Fig. 3D), progressively increased with repeated challenges (Fig. S4B) but, interestingly, C57BL/6 DCs had a much smaller fraction of this subset, a difference that occurred as early as 24 h after the first challenge (Fig. S4B). The phenotypic difference did reflect a functional difference because purified BALB/c DCs isolated after five challenges secreted more than threefold more TNF- α than did C57BL/6 DCs on a per-cell basis, whereas purified macrophages from both strains produced equal amount of TNF- α in response to SC in vitro (Fig. 3F). Thus, macrophages from the two strains possessed comparable TNF- α -secreting capacity on a per-cell basis, although the recovered numbers were different (Fig. S4A). However, comparatively, DCs derived from the two strains not only differed in their numbers but also in their capacity to produce TNF- α , thereby accounting more for the strain-associated TNF- α difference.

Differential Activation of NF- κ B and Toll-Like Receptor 2 (TLR2) Pathway Results in DCs with Differential TNF- α -Producing Capacities.

Next, we sought to dissect the signaling pathways responsible for the strain-based neutrophilic response. TLR2, TLR4, and dectin-1 are the most critical pattern recognition receptors (PRRs) for the recognition of *A. fumigatus* (27–29). In our BALB/c model, the absence of MyD88 or dectin-1, but not TLR4, significantly reduced the infiltration of inflammatory cells, including neutrophils (Fig. S5A), which suggested that, besides dectin-1, TLR2 rather than TLR4 might be more important. We then more closely examined the generation of TNF- α -producing DCs. MyD88 and dectin-1 signaling were essential because lack of either profoundly diminished TNF- α ⁺ DCs (Fig. 4A). Furthermore, lack of dectin-1 signaling reduced the abundance of lung DCs, particularly CD11b⁺ Ly6C⁺ DCs, indicating the importance of dectin-1 in the development of this DC type (Fig. 4B).

We next questioned whether DCs from the two strains differentially expressed critical PRRs, which subsequently led to differential TNF- α production in DCs and in the lungs. Based on the data generated (Fig. S5A), we focused on dectin-1 and TLR2. Both were expressed on CD11b⁺ Ly6C⁺ DCs (Fig. 4C), the subset that was enriched for TNF- α . Notably, TLR2 was largely colocalized with dectin-1 on the same cells (Fig. 4C). Between the strains, there was no apparent difference in the baseline expression of these molecules. One challenge with fungal conidia only produced a subtle difference between the two strains. However, repeated challenges resulted in modestly higher dectin-1 but substantially higher TLR2 on BALB/c DCs, collectively resulting in a higher frequency of TLR2⁺ Dectin-1⁺ DCs in the BALB/c strain compared with the C57BL/6 strain (Fig. 4C). Although both MyD88 and dectin-1 pathways were required for TNF- α production by DCs, dectin-1 signaling alone was not sufficient to induce functionally different DCs because DCs from both strains after repeated challenges produced similar levels of TNF- α in response to the dectin-1 agonist curdlan (Fig. 4D). With evidence of crosstalk between dectin-1 and TLRs (30), our data suggest that higher levels of TLR2 on BALB/c DCs ultimately cause the remarkable difference in TNF- α output between the strains because BALB/c DCs secreted significantly more TNF- α upon stimulation with the TLR2 agonist Pam3Cysk4 (Fig. 4D). Notably, macrophages from both strains mounted a comparable TNF- α response to both ligands (Fig. 4D), consistent with the observation that macrophages expressed comparable levels of the receptors regardless of the strain.

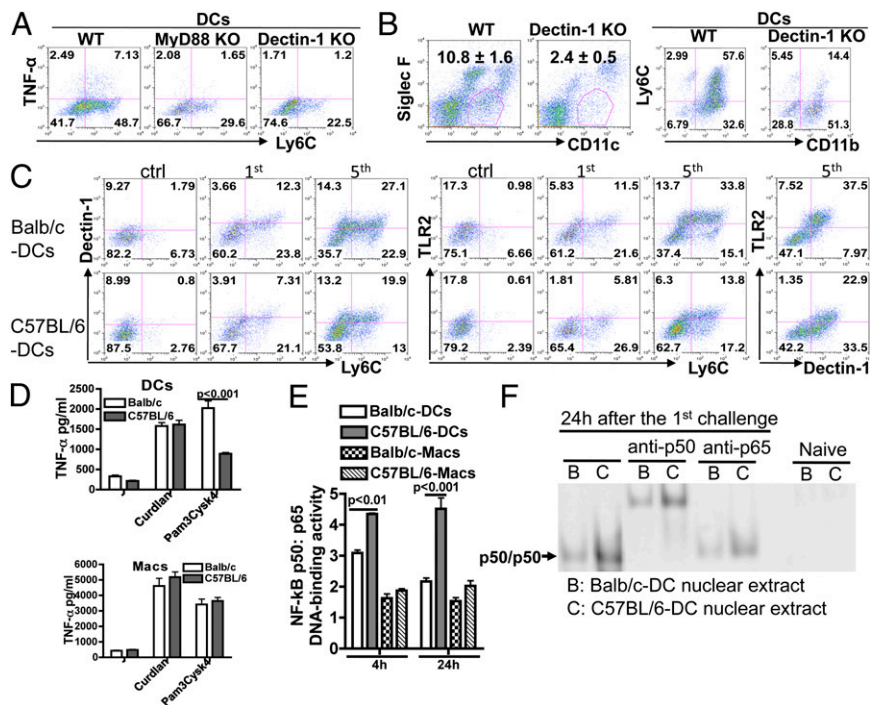
To further understand the differential TNF- α response in the DCs, we investigated more upstream mechanisms. Given that multiple signaling pathways converge on NF- κ B in inflammation (31), we investigated whether there was an inherent difference in



NF- κ B composition in the nuclei of the DCs in the two strains. We chose to examine early time points (4 h and 24 h) after the first challenge, at which time the expression of PRRs was similar but a modest strain-associated difference in DC-produced TNF- α was observed when DCs were restimulated *in vitro* (Fig. S5B). A higher p50/p65 DNA-binding activity was detected in the nuclei of C57BL/6 DCs compared with that in BALB/c DCs (Fig.

Fig. 4. Differential activation of NF- κ B and TLR2 results in DCs with differential TNF- α -producing capacities. (A) Representative FACS plots show intracellular TNF- α staining in DCs from WT, MyD88 $^{-/-}$, or dectin-1 $^{-/-}$ mice at 24 h after the fifth challenge.

(B) Representative FACS plots show lung DC abundance and DC subsets in WT BALB/c or dectin-1 $^{-/-}$ mice at 24 h after the eighth challenge. For DC abundance, plots were gated on CD45 $^{+}$ cells, and lung DCs are CD45 $^{+}$ CD11c $^{+}$ Siglec-F $^{-}$ AF ^{low} cells. The numbers indicate the percentage of DCs within the live cells. (C) Representative FACS plots show Dectin-1 vs. Ly6C, TLR2 vs. Ly6C, and TLR2 vs. Dectin-1 expression on lung DCs at different time points. Lung CD11c $^{+}$ cells were isolated from PBS control or challenged mice at 24 h after the first and fifth challenges using microbeads. Unless otherwise indicated, lung DCs were gated as described in the legend to Fig. 3E, and the numbers in the FACS plots are the percentages of cells in the gated populations. (D) ELISA of TNF- α in the culture supernatant. Macrophages or DCs sorted 24 h after the fifth challenge were cultured without stimulation (–), with curdlan (dectin-1 agonist), or with Pam3Cysk4 (TLR2 agonist). (E) Plots show the ratio of p50/p65 DNA-binding activity in the nuclei of lung DCs or macrophages at 4 h and 24 h after the first challenge. Results shown are mean $\pm$ SD ($n = 4$ mice per group). (F) The binding complex at the κ B3 site using nuclear extracts from DCs prepared 24 h after the first challenge or from DCs of naïve mice was examined by EMSA (excess probe was run off the gel). The letters B and C represent complexes formed with nuclear extracts from BALB/c and C57BL/6 mice, respectively. Data shown are representative of at least two independent experiments.

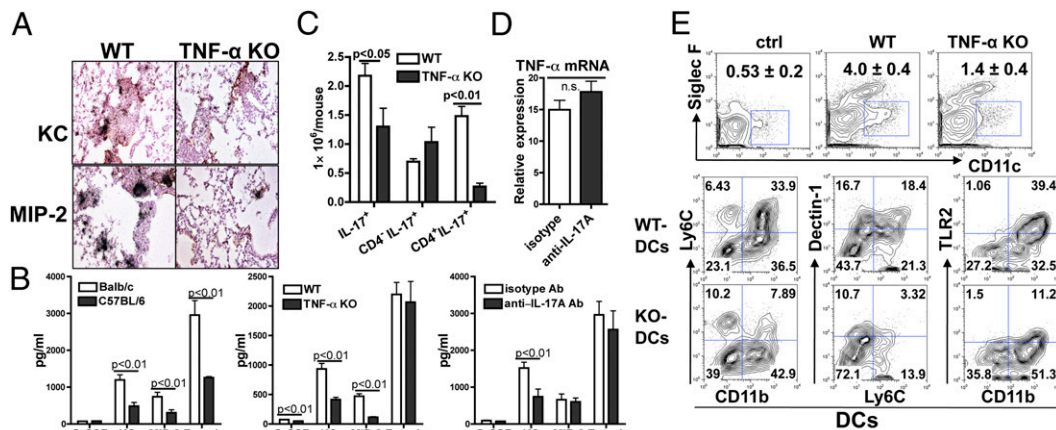


A recent study has identified monocyte-derived inflammatory DCs that participate in fungal phagocytosis and T-cell activation early after *A. fumigatus* infection (40). Here we show that inflammatory DCs producing TNF- α play an important role in shaping the immune response to persistent fungal exposure. Interestingly, in our model, TNF- α -producing DCs did not coexpress iNOS, which distinguishes them from Tip-DCs (25, 26). TNF- α , derived from DCs and macrophages, also promoted the expansion of the pathogen-sensing and TNF- α -producing inflammatory DCs, which might have been through indirect mechanisms, such as the up-regulation of GM-CSF, the critical cytokine that controls the differentiation of DCs from monocytes (41). Alternatively, TNF- α produced by DCs may act as an autocrine

factor to promote cell survival, as was shown in the case of macrophages (42), or to induce DC-recruiting chemokines (43). The induction of dectin-1 on DCs by TNF- α in vivo could be through GM-CSF as well, because GM-CSF directly induces dectin-1 expression on macrophages in vitro (44).

TNF- α acts as a molecular switch in our model to regulate the neutrophil/eosinophil balance. The target of the inhibitory effect of TNF- α on eosinophilia seems to be IL-5. It has been reported that, only in the presence of IL-5, intranasal administration of eotaxin induces eosinophil influx because IL-5 primes eosinophil to be responsive to eotaxin (45). This observation might explain why the BALB/c strain, despite a higher eotaxin level, did not

Fig. 5. TNF- α and IL-17A collaborate to induce airway neutrophilia. (A) In situ hybridization images show lung KC and MIP-2 in WT or TNF- α $^{-/-}$ mice. (B) ELISA of KC, G-CSF, MIP-2, and eotaxin in the lung at 24 h after the eighth challenge. (C) The numbers of lung IL-17A $^{+}$, CD4 $^{+}$ IL-17A $^{+}$, and CD4 $^{+}$ IL-17A $^{+}$ cells per mouse were determined by intracellular cytokine staining methods at 24 h after the eighth challenge. (D) Lung TNF- α mRNA in mice at 24 h after the eighth challenge. Results are expressed as fold increase over PBS controls. (E) Representative FACS plots show lung DC abundance, DC subsets, and PRR expression on DCs in PBS control or WT BALB/c and TNF- α $^{-/-}$ mice at 24 h after the eighth challenge. For DC abundance, plots were gated on CD45 $^{+}$ AF ^{low} cells (AF ^{high} macrophages were excluded). Lung DCs are CD45 $^{+}$ AF ^{low} CD11c $^{+}$ Siglec-F $^{-}$, and the numbers indicate the percentage of DCs within the live cells. The other plots were gated on lung DCs as described in the legend to Fig. 3E, and the numbers indicate the percentages of cells in the gated populations. Results are expressed as mean $\pm$ SD ($n = 4$ mice per group). n.s., not significant. Shown is an experiment representative of at least two experiments.



Representative FACS plots show lung DC abundance, DC subsets, and PRR expression on DCs in PBS control or WT BALB/c and TNF- α $^{-/-}$ mice at 24 h after the eighth challenge. For DC abundance, plots were gated on CD45 $^{+}$ AF ^{low} cells (AF ^{high} macrophages were excluded). Lung DCs are CD45 $^{+}$ AF ^{low} CD11c $^{+}$ Siglec-F $^{-}$, and the numbers indicate the percentage of DCs within the live cells. The other plots were gated on lung DCs as described in the legend to Fig. 3E, and the numbers indicate the percentages of cells in the gated populations. Results are expressed as mean $\pm$ SD ($n = 4$ mice per group). n.s., not significant. Shown is an experiment representative of at least two experiments.

show prominent eosinophilia, which might be because of insufficient lung IL-5 levels.

p50 homodimers in LPS-induced tolerance limit deleterious inflammation (32, 33). The generation of “less inflammatory” C57BL/6 DCs with enriched p50/p50 at the κ B3 site could also be a self-control strategy, responding to repeated PRR stimulation to avoid deleterious outcomes. Interestingly, it is cell context-specific and not present in macrophages, which has not been previously appreciated. Our findings provide a framework for the clinical heterogeneity in the response of patients to *Aspergillus*.

The TNF- α producing DC may act as a double-edged sword, such that tight regulation of this cell type is required to achieve an acceptable balance between host protection and immune-mediated pathology. Our findings, therefore, lend a cautionary note to the potential use of injected TNF- α for DC maturation and trafficking to lymph nodes in the context of DC-mediated cancer vaccines. In specific contexts, the risks may outweigh the benefits. Thus, TNF- α production and neutrophilic inflammation may be causally linked in various disorders, which is not yet

adequately appreciated in the literature. Our study suggests that controlling the expansion of TNF- α -producing DCs by targeting TNF- α levels may help ameliorate these disease states.

Materials and Methods

BALB/c, C57BL/6, TNF- $\alpha^{-/-}$, TLR4^{LPS-d}, and CD11c-DTR-EGFP Tg mice on the BALB/c background were purchased from The Jackson Laboratory. MyD88^{-/-} (46) and dectin-1^{-/-} (47) mice on the BALB/c background were bred at the animal facility at the University of Pittsburgh, and the IL-17RA^{-/-} mice (21) were bred at Taconic Farms. All mice were housed under pathogen-free conditions and used between 6 and 8 wk of age. All animal experiments were approved by the Animal Care and Use Committee at the University of Pittsburgh. Information on the animal model and all experimental procedures is provided in *SI Materials and Methods*.

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Supporting Information

Fei et al. 10.1073/pnas.1015476108

SI Materials and Methods

***Aspergillus fumigatus* Growth and Culture.** Resting conidia of *A. fumigatus* were harvested as previously described (1). To obtain swollen conidia (SC), conidia were incubated at 37 °C at a concentration of 1×10^7 conidia per mL in RPMI medium 1640 (Gibco) for 6 h.

Animal Model. Mice received intratracheal (i.t.) 1×10^7 *A. fumigatus* conidia in 50 μ L of PBS on day 0. Unless otherwise indicated, from day 7, mice were i.t. challenged with 1×10^6 conidia in 50 μ L of PBS daily for 5 d, rested for 2 d, and challenged for another 3 d, resulting in a total of eight challenges. At 24 h after the final challenge, inflammation was assessed as described below. Control mice were given PBS only (PBS controls). For neutralization experiments, anti-IL-17A or isotype antibody (300 μ g per mouse) or anti-TNF- α (MP6-XT22; BioLegend) or isotype antibody (250 μ g per mouse) was administered i.p. at 24 h before fungal exposure and then every 3 d during the challenge phase. For in vivo depletion of CD11c⁺ cells, 50 ng of diphtheria toxin (DT) was injected i.t. into CD11c-DTR-EGFP transgenic (Tg) mice after the eighth challenge, and the animals were sampled after 24 h.

Assessment of Airway Inflammation. At 24 h after the final challenge, the left side of the lung lobe was tied off, and the right lobes were lavaged with 0.7 mL of sterile PBS. Bronchoalveolar lavage fluid (BALF) was collected, and total as well as differential cell counts were assessed as previously described (2). The left lobe of the lung was homogenized in cytokine buffer for the measurement of different proteins as previously described (3) or in TRIzol (Invitrogen) for RNA isolation. Cytokines and chemokines in the BALF or the lung homogenate as well as total serum IgE were detected by ELISA (BioLegend or R&D Systems) or Milliplex Immunoassay (Millipore). The right lobes of the lung were prepared for hematoxylin/eosin staining as previously described (3) or for in situ hybridization. Histological examinations of lung tissues or cellular differential counts in the BALF were carried out in a blinded fashion. For quantitative RT-PCR, tissues or cells were treated with TRIzol (Invitrogen). RNA was isolated with the RNeasy kit (Qiagen) and treated with RNase-free DNase (Qiagen). cDNA was synthesized and used for quantitative PCR with TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. The level of mRNA was normalized to β -glucuronidase expression, and the results were analyzed by the $2^{-\Delta\Delta C_t}$ method.

Fungal Burden Determination. The left lobe of the lung was homogenized in 1 mL of PBS. Lung homogenates were then serially diluted (1:2 and 1:5), and 100 μ L of undiluted or diluted samples were plated on potato dextrose agar plates. After overnight culture, colonies were counted, and the burden was expressed as cfu per lung.

Cell Isolation, Sorting, and Culture. The lungs were perfused, removed, and digested as described (4). Tissues were then dissociated on a gentleMACS Dissociator (Miltenyi Biotec), and single-cell suspensions were obtained. For isolation of lung dendritic cells (DCs) and macrophages, CD11c⁺ cells were purified by using anti-CD11c microbeads (Miltenyi Biotec), stained with anti-CD11c allophycocyanin (APC) antibody, and sorted on a FACSARIA sorter (BD Immunocytometry Systems) based on their side scatter (SSC), differential expression of CD11c, and autofluorescence.

Basically, CD11c^{low} SSC^{high} eosinophils were first excluded, and low-autofluorescent (AF^{low}) DCs and high-autofluorescent (AF^{high}) macrophages were sorted for further experiments. For isolation of lung T cells, lung cells were stained with anti-CD4 APC and anti-CD3 phycoerythrin (PE) antibodies, and CD3⁺CD4⁺ T cells were sorted on a FACSARIA sorter.

DCs or macrophages were seeded at 1×10^5 cells per well in a 96-well plate in triplicate and stimulated with SC (2×10^5 per well), curdlan (100 μ g/mL; Wako Pure Chemicals), or Pam3-Cysk4 (5 μ g/mL; InvivoGen) for 14–15 h. For ex vivo DC/T-cell cocultures, lung T cells (2×10^5 per well) were stimulated with sorted lung DCs (2×10^4 per well) in the presence of SC (1×10^5 per well). Isotype control or anti-TNF- α antibody (10 μ g/mL, MP6-XT22; BioLegend) was added to the coculture. Supernatants were collected after 72 h, and cytokines were assayed by ELISA kits (R&D Systems).

Antibodies and Flow Cytometry. The following antibodies were purchased from BD Pharmingen: purified CD16/CD32 (Fc block, clone 93), purified polyclonal rabbit anti-mouse nitric oxide synthase 2 (NOS2) or inducible nitric oxide synthase (iNOS), anti-CD3 PE or PerCP (145-2C11), anti-CD4 APC (L3T4), anti-CD11b PE or APC (M1/70), anti-CD11c FITC or APC (HL3), anti-Ly6C FITC (AL-21), anti-Ly6G PE (1A8), anti-Siglec-F PE (E50-2440), anti-MHC class II PE (NIMR4), anti-CD80 PE (16-10A1), anti-CD86 PE (GL1), anti-IFN- γ PE (XMG1.2), anti-IL-17A PE (TC11-18H10), and anti-TNF- α PE (MP6-XT22). The following antibodies were purchased from BioLegend: anti-CD45 FITC or APC (30-F11), anti- $\gamma\delta$ T-cell receptor Alexa Fluor 647 (UC7-13D5), and anti-IL-4 PE (11B11). Purified anti-Dectin-1 or anti-dectin-1 FITC or PE (2A11) antibodies were obtained from AbD Serotec, anti-Toll-like receptor 2 (TLR2) PE (T2.5) antibody was from eBioscience, and secondary antibody for iNOS staining (goat anti-rabbit IgG Alexa Fluor 647) was from Invitrogen.

Single-cell suspensions were stained with antibodies and analyzed on a FACSCalibur flow cytometer (BD Immunocytometry Systems), and data were analyzed with FlowJo (Tree Star). For intracellular staining for TNF- α , cells were cultured in vitro with SC for 4 h, and GolgiStop (BD Pharmingen) was added 3 h before harvesting. For T-cell intracellular staining, lung cells were cultured with phorbol 12-myristate 13-acetate (25 ng/mL; Sigma) and ionomycin (500 ng/mL; Sigma) in the presence of GolgiStop for 4 h. Cells were then stained for surface markers and intracellular cytokines (Cytofix/Cytoperm; BD Pharmingen).

Nuclear Extract Preparation, NF- κ B DNA Binding Activity, and EMSA. Nuclear extracts were prepared with a nuclear extract kit (Panomics Quantitative Biology). NF- κ B DNA binding activity was determined with a TransAM NF- κ B family kit (Active Motif).

EMSA was performed as previously described by us and others (5–7). Basically, 5 μ g of nuclear extract was gently mixed with 5 $\times$ complete binding buffer at room temperature for 10 min, incubated with antibody for supershift at room temperature for 10 min, and finally incubated with 200 fmol of 5' biotin-labeled 20-bp double-stranded oligonucleotide at room temperature for 20 min. Complexes were then resolved on a 4.5% nondenaturing PAGE gel in 0.5 $\times$ Tris-borate/EDTA (TBE) buffer and transferred to a positively charged nylon membrane (Pierce). Transferred DNA was cross-linked to the membrane by using a Stratilinker UV Crosslinker (Stratagene). Finally, biotin-labeled

DNA on the membrane was detected with the LightShift Chemiluminescent EMSA Kit (Pierce).

The 1× complete binding buffer used in the binding reaction contained 10 mM Tris-HCl (pH = 7.5), 50 mM NaCl, 5% glycerol, 1% IGEPAL CA-630 (Sigma), 1 mM EDTA, 0.1 μg/μL poly (dIdC), and 0.05 μg/μL BSA. The oligonucleotide was the κB3 (−510) site from the murine TNF-α promoter, and the sequence was 5′-AACAGGGGGCTTCCCTCCT-3′ (Invitrogen) (6). The antibody used in the supershift assay was sc-114X (anti-p50) or sc-109X (anti-p65) (Santa Cruz Biotechnology).

Neutrophil Myeloperoxidase (MPO) Activity Assay. MPO activity in the cell-free BALF was measured as previously described (8). Basically, 50 μL of BALF was mixed with 200 μL of *O*-dianisidine dihydrochloride (1.25 mg/mL in PBS) supplemented with BSA (0.1% wt/vol) and H₂O₂ (0.05% = 0.4 mM). After 15 min of incubation with shaking at 37 °C, the reaction was stopped by adding 100 μL of NaN₃ (1% wt/vol) and read at 450 nm. The activity was expressed as units/mL according to standards.

Enzyme-Linked Immunosorbent Spot (ELISPOT). CD4⁺ T cells from the lung-draining lymph nodes were purified by CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) following the manufacturer's instructions. CD4⁺ T cells producing IFN-γ, IL-17A, IL-5, and IL-13 were assayed with ELISPOT kits (eBioscience) as previously described (9).

In Situ Hybridization Studies. Mouse CXCL1 (KC) cDNA was amplified by using RT-PCR with primers KDG_mCXCL1_F1 (5′-CATGATCCCAGCCACCCGCT-3′) and KDG_mCXCL1_R1 (5′-CTCCGTTACTTGGGGACACCT-3′). Mouse CXCL2 (MIP-2) cDNA was amplified by using RT-PCR with primers KDG_mCXCL2_F1 (5′-TAGCGCCATGGCCCCCT-3′) and KDG_mCXCL2_R1 (5′-CAGGTCAGTTAGCCTTGCCCT-3′). PCR products were ligated to the pGEM-T vector (Promega), and DNA was sequenced. The cDNA-containing plasmids were linearized by restriction digestion. Gene-specific riboprobes were synthesized by in vitro transcription using a MAXIscript SP6/T7 kit (Ambion), and unincorporated nucleotides were removed by RNA Mini Quick Spin Columns (Roche). Paraffin-embedded tissue specimens were pretreated as described (10), after deparaffinization in xylenes and rinsing in ethanol. In situ hybridization with ³⁵S-labeled riboprobes was performed at 50 °C overnight as described (10, 11) with 0.1 M DTT included in the hybridization mix. Tissue sections were coated with NTB-2 emulsion (Kodak) and exposed at 10 °C for 10 d. The sections were counterstained

with hematoxylin (Vector) and mounted with Permount (Fisher Scientific).

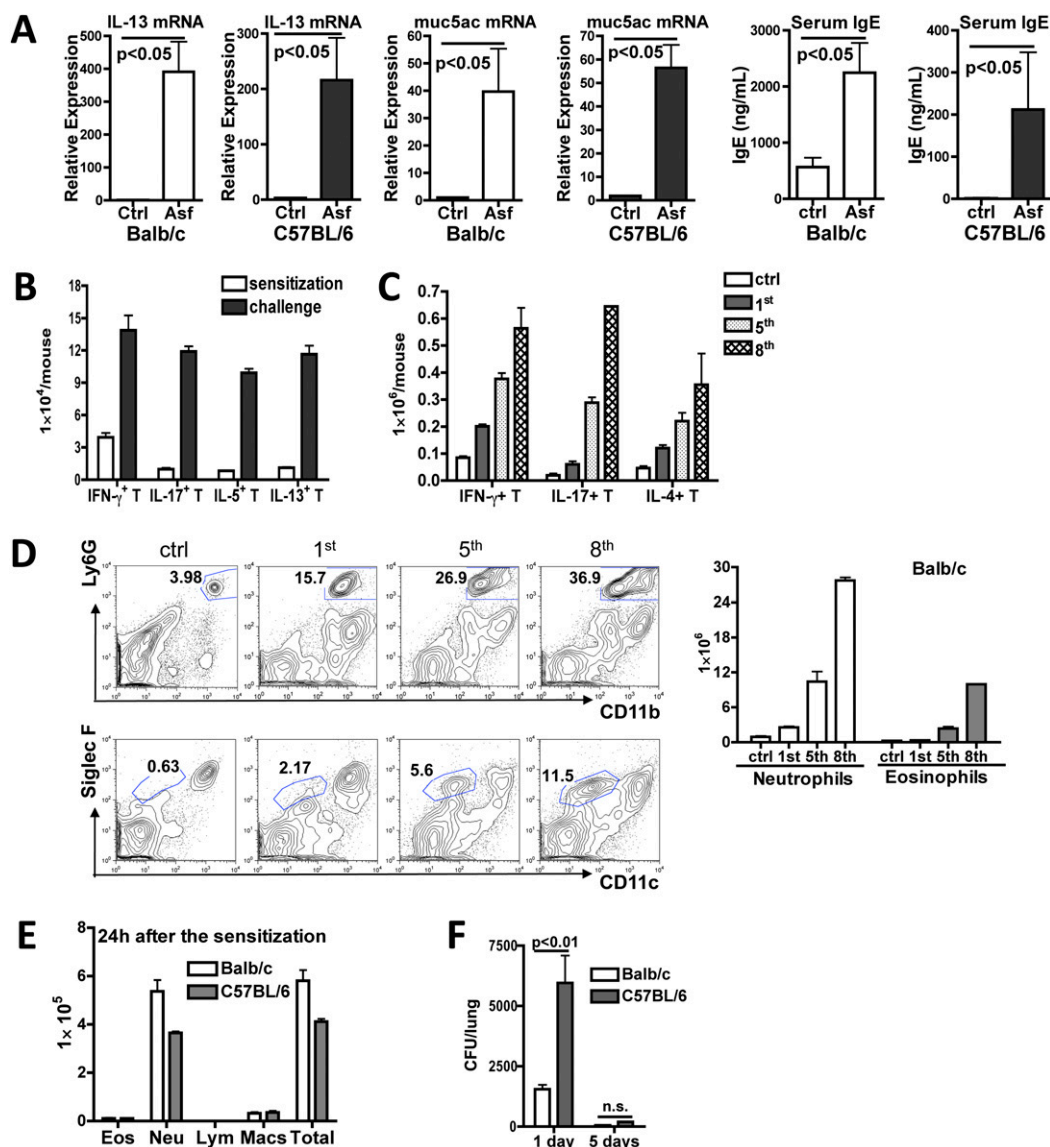
Statistical Analyses. Student's unpaired two-tailed *t* test was used for comparisons between two groups. One-way ANOVA with Tukey's post hoc test and two-way ANOVA with Bonferroni's post hoc test were used for comparisons between multiple groups, when appropriate. Differences between two groups were considered significant when *P* < 0.05. All statistical analyses were performed with GraphPad Prism software.

SI Results

Continuous Antigenic Stimulation by Fungal Antigens Elicits Critical Features of Allergic Disorders. Repeated challenges reproduced critical features of allergic disorders (12) regardless of the genetic background, including up-regulated Muc5ac (which encodes the major airway mucin), increased IL-13 levels in the lung, and elevated total serum IgE (Fig. S1A). Consistent with previous studies, sensitization with 10 × 10⁶ conidia predominantly primed Th1 cells in the lung-draining lymph nodes (13–15), whereas repeated challenges preferentially expanded Th2/Th17 cells (Fig. S1B). Similarly, at inflammatory sites, effector T cells accumulated during challenges and demonstrated a mixed Th1/Th2/Th17 pattern (Fig. S1C). Eosinophil influx, a Th2 response-associated pathology (12), was barely detectable after sensitization (Fig. S1E) or after one challenge (Fig. S1D) but became evident after multiple challenges with fungal conidia (Fig. S1D). Neutrophil infiltration, most likely associated with a Th17 response, progressively increased with the multiple challenges (Fig. S1D). Therefore, persistent stimulation by fungal antigens not only boosted the response but also altered the nature of immunity by reducing Th1 dominance and promoting Th2/Th17 responses and their associated pathology, such as eosinophilia and neutrophilia, respectively.

Characterization of CD11c⁺ Lung DCs and Macrophages. In repeatedly challenged mice, the CD11c⁺ fraction could be further divided into three cell types: CD11c^{low} SSC^{high} Siglec-F⁺ MHC II[−] eosinophils, CD11c⁺ Siglec-F[−] AF^{low} DCs, and CD11c⁺ Siglec-F^{+/−} AF^{high} macrophages (Fig. S3A). No expression of Siglec-F or Ly6G was detected in the DC population (Fig. S3A), excluding the possibility that DCs were contaminated with eosinophils or neutrophils in our analyses. The cytoplasts of FACS-sorted DCs or macrophages further confirmed the typical morphology of each type of cells (Fig. S3B).

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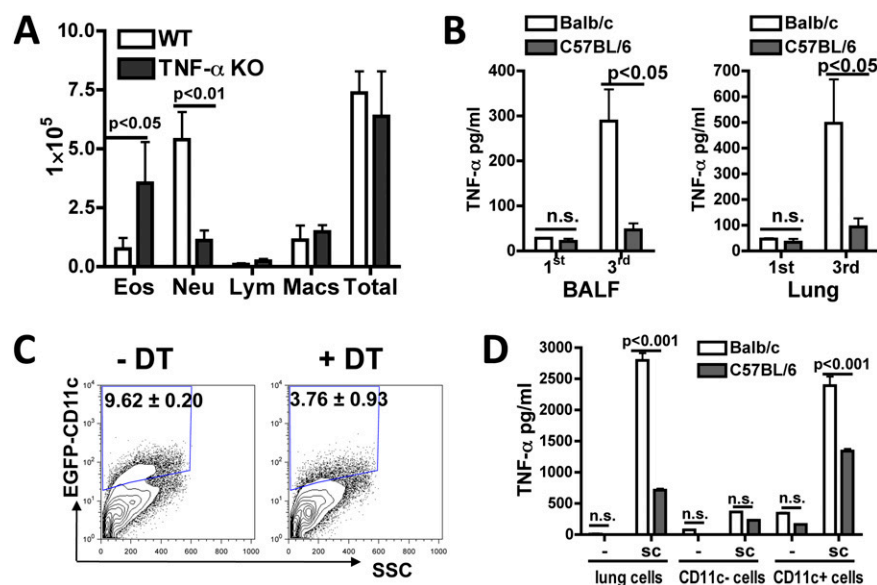


Fig. S2. Characterization of the function and the cellular source of TNF- α . (A) Total and differential cell counts in the BALF from WT BALB/c and BALB/c TNF- α KO mice were assessed at 24 h after the eighth challenge. (B) TNF- α in the BALF or lung homogenates was assayed at 24 h after the first or third challenge. (C) Representative FACS plots show the efficiency of EGFP-CD11c $^+$ cell depletion without DT (-DT) or with DT (+DT) treatment in CD11c-DTR-EGFP Tg mice. (D) TNF- α levels in different culture supernatants. Total lung, CD11c $^-$, and CD11c $^+$ cells were prepared from mice at 24 h after the eighth challenge, and equal numbers were cultured in vitro with and without SC (ratio of cells:SC = 1:2). Results are expressed as mean $\pm$ SD ($n \geq 3$ mice). n.s., not significant. Experiments were repeated at least twice.

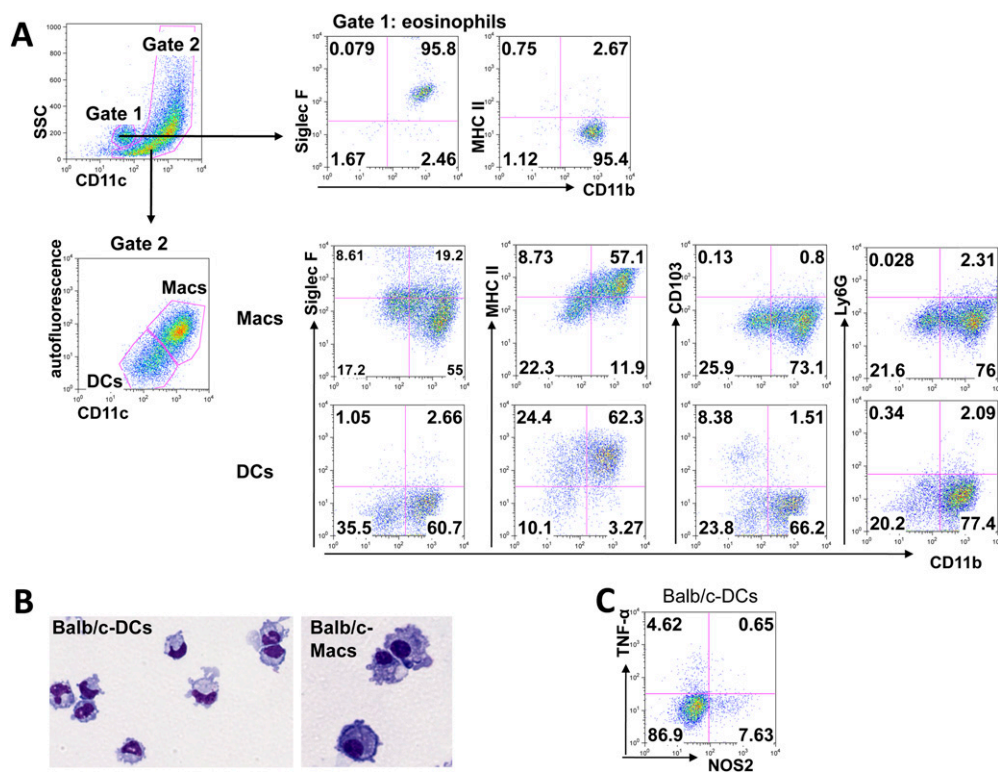


Fig. S3. Characterization of lung DCs and macrophages (Macs) after repeated challenges. (A) Representative FACS plots show the gating strategy. The CD11c $^+$ cells were first divided into gate 1 (CD11c ^{low} SSC ^{high} cells) and gate 2 based on SSC and differential CD11c expression. Cells in gate 2 were further divided into CD11c $^+$ AF ^{low} and CD11c $^+$ AF ^{high} cells. Cells in gate 1 are CD11c ^{low} CD11b $^+$ Siglec-F $^+$ MHC II $^-$ eosinophils, which was confirmed by staining of cytopsin. CD11c $^+$ AF ^{low} cells in gate 2 are CD11c $^+$ AF ^{low} Siglec-F $^-$ MHC II $^+$ DCs, including a CD11b $^+$ and a CD103 $^+$ subset. CD11c $^+$ AF ^{high} cells are macrophages, partially expressing CD11b, Siglec-F, and MHC II. Macrophages and DCs do not express Ly6G. (B) Representative cytopsin images of FACS-sorted DCs and macrophages from BALB/c mice after the fifth challenge. (C) Representative FACS plot shows intracellular TNF- α and iNOS2 dual staining in BALB/c DCs isolated after the fifth challenge. Plot is gated on DCs as described in A. For FACS plots, numbers represent the percentages of cells in the gated populations. Data are representative of three independent experiments.

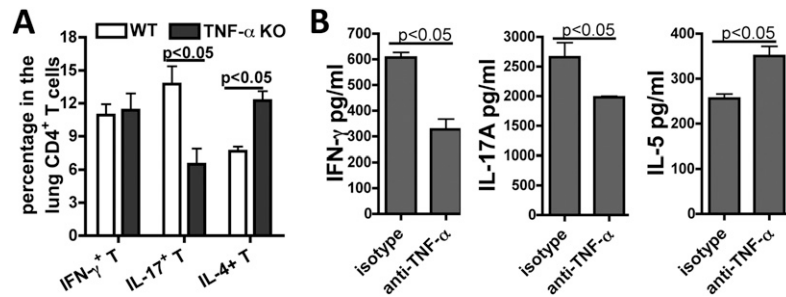


Fig. S6. The effects of TNF- α on Th1/Th2/Th17 differentiation after repeated challenges. (A) WT or TNF- α KO mice were repeatedly challenged, and the percentages of CD4⁺ T cells producing IFN- γ , IL-17A, and IL-4 in the lung CD4⁺ T-cell compartment were determined by intracellular staining at 24 h after the eighth challenge. Results are expressed as mean $\pm$ SD ($n \geq 4$ mice). (B) Lung DCs and T cells were isolated from BALB/c mice after eight challenges and co-cultured with SC in vitro in the presence of anti-TNF- α or isotype antibody. After 72 h, IFN- γ , IL-17A, and IL-5 levels in the culture supernatants were assayed by ELISA. Results are expressed as mean $\pm$ SD. Data are representative of two independent experiments.

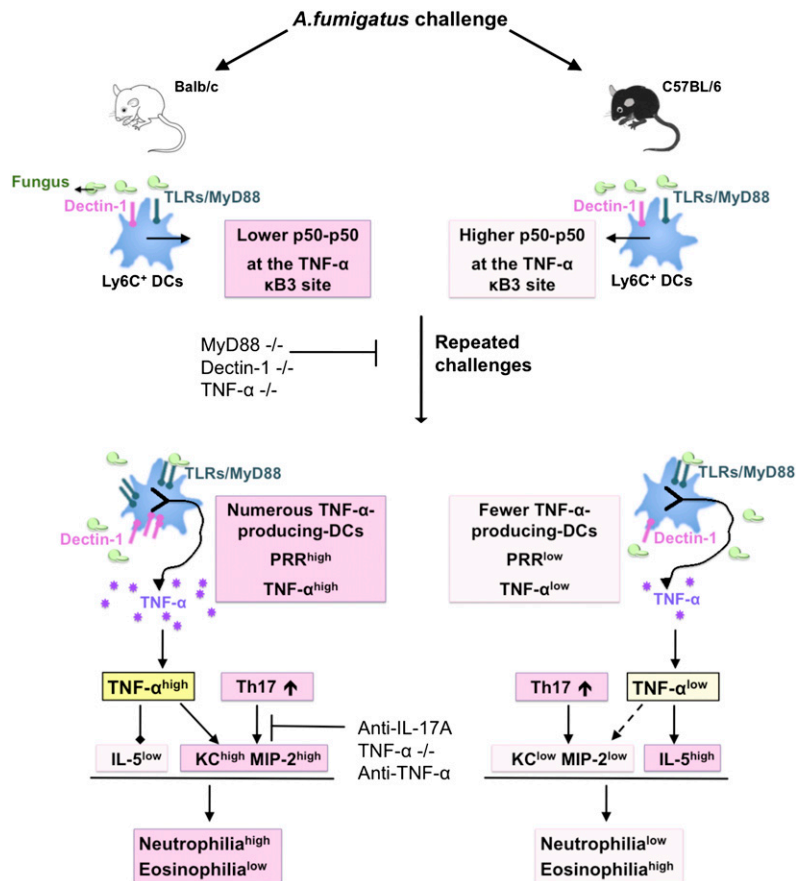


Fig. S7. The illustration depicts the sequence of events that elicits differential granulocyte infiltration in the lungs of BALB/c and C57BL/6 mice. In the BALB/c mice, both TNF- α and IL-17A are required for intense airway neutrophilia in response to persistent fungal exposure. TNF- α is mainly derived from inflammatory DCs and macrophages through collaboration between the TLR2/MyD88 and dectin-1 pathways. TNF- α not only enhances the Th17 response but also regulates its own expression through the promotion of PRR-expressing, TNF- α -producing DCs. Functionally, the high level of TNF- α suppresses IL-5 but, in collaboration with IL-17A, induces neutrophil chemoattractants, leading to a neutrophil-dominated airway inflammation. In the C57BL/6 strain, after the first challenge, the nuclei of DCs in C57BL/6 mice have a higher content of p50 homodimers that can bind to the κ B3 site of the TNF- α promoter, which is known to inhibit TNF- α transcription and may indirectly influence PRR expression. Therefore, during repeated challenges, the poor TNF- α -producing capacity of C57BL/6 DCs is attributable to the suppressive p50 homodimers, potentially causing a relatively lower level of TLR2 expression on the cell surface. Because TNF- α promotes the development of TNF- α -producing DCs, C57BL/6 mice have fewer TNF- α -producing DCs and macrophages compared with the BALB/c strain, which collectively results in the low TNF- α profile at the lung tissue level. Despite a competent IL-17A response, the absence of high TNF- α leads to lower levels of neutrophil chemoattractants but a higher level of IL-5 and consequently an eosinophil-biased response.