MECHANISMS UNDERLYING PULMONARY NEUTROPHILIA VERSUS EOSINOPHILIA IN FUNGAL ALLERGY

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Aspergillus fumigatus (*A. fumigatus*) is commonly associated with allergic bronchopulmonary aspergillosis (ABPA), which represents one of the most extreme manifestations of fungal allergy. Eosinophils are considered to be effector cells mediating lung dysfunction, but there is increasing appreciation that pulmonary neutrophilia also contributes to ABPA pathology. In our efforts to recapitulate this fungal allergic condition, we found that persistent exposure to *A. fumigatus* resulted in neutrophil and eosinophil-biased responses in BALB/c and C57BL/6 mice, respectively, and that the former mimicked the inflammation pattern observed in ABPA. By performing a comparative study, we found substantially higher lung TNF-α levels in neutrophil-rich BABL/c mice compared to C57BL/6 mice. TNF-α blockade or deficiency in BALB/c mice switched the response from neutrophilia to eosinophilia, implicating TNF-α as the key mediator. We identified $CD11c^{+}CD11b^{+}Ly6C^{+}$ inflammatory dendritic cells (DCs) and macrophages as the primary sources of TNF-α, and that depletion of these $CD11c^{+}$ cells in CD11c-DTR BALB/c mice dramatically reduced lung TNF-α levels. Importantly, BALB/c DCs demonstrated a stronger TNF-α-producing capacity than C57BL/6 DCs, strongly suggesting that this was the basis for the strain-associated TNF-α difference. As compared to $TNF\text{-}\alpha^{high}$ BALB/c DCs, $TNF\text{-}\alpha^{low}$ C57BL/6 DCs contained more repressive NF-κB p50 homodimers at the TNF-α promoter at early time points following *A. fumigatus* infection, and expressed notably less pattern recognition receptors, in particular TLR2 following persistent fungal exposure. These differences explained

the strain-specific differential TNF-α production by DCs. In addition, TNF-α deficiency itself blunted the accumulation of $Ly6c^{+}CD11b^{+}$ DCs, implicating a positive feedback loop to amplify the cellular sources of TNF-α. Functionally, higher amounts of IL-5 in C57BL/6 and TNF-α-/- mice were associated with higher eosinophil counts, while collaboration between TNF-α and IL-17A triggered significantly higher levels of the neutrophil chemoattractants KC and MIP-2 in the BALB/c mice. In summary, our study identifies that TNF-α, acting as a molecular switch, orchestrates a sequence of events in DCs and $CD4^{+}$ T cells and promotes pulmonary neutrophilia.

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ABBREVIATIONS

A

A. fumigatus: *Aspergillus fumigatus*

ABPA: allergic pulmonary aspergillosis

AF: autofluorescence

AHR: airway hyperresponsiveness

Asf: *Aspergillus*

B

BALF: bronchoalveolar lavage fluid

BM: bone marrow

BMDC: bone marrow-derived dendritic cell

C

CDP: common DC precursor

CF: cystic fibrosis

CFTR: cystic fibrosis transmembrane conductance regulator

CGD: chronic granulomatous disease

CLR: C-type lectin-like receptor

COPD: chronic obstructive pulmonary disease

CRD: curdlan (dectin-1 agonist)

D

d: day(s)

DC: dendritic cells

dLN: draining lymph node

DT: diphtheria toxin

E

ELISPOT: enzyme-linked immunosorbent spot

EMSA: Electrophoretic mobility shift assay

Eos: eosinophil

F

FSC: forward scatter

H

h: hour(s)

HLA: human leukocyte antigen

HSC: hematopoietic stem cell

I

IDO: indoleamine 2,3-dioxygenase

IFA: incomplete Freund's adjuvant

i.n.: intranasally

iNOS: inducible nitric oxide synthase

i.p.: intraperitoneally

IPA: invasive pulmonary aspergillosis

i.t.: intratracheally

ITAM: intracellular tyrosine kinase activation motif

i.v.: intravenously

K

KO: knockout (mice)

L

LP: lymphoid committed precursor

lpDC: lamina propria DCs

Lym: lymphocyte

M

Macs: macrophage

MBL: mannose binding lectin

MDM: monocyte-derived macrophages

MDP: macrophage/dendritic cell progenitors

MFI: mean fluorescence intensity

MMP-9: matrix metallopeptidase-9

MP: myeloid committed precursor

MPO: myeloperoxidase

PAM: Pam3Csk4 (TLR2 agonist)

PAMP: pathogen-associated molecular pattern

N

Neu: neutrophil

P

PBMC: human peripheral blood mononuclear cells

pDC: plasmacytoid DC

Pre-cDC: pre-classical DC

PRR: pattern recognition receptor

PTX3: pentraxin 3

Q

qRT-PCR: quantitative reverse-transcription PCR

R

RC: (*Aspergillus fumigatus*) resting conidia

S

s.c.: subcutaneously

SC: (*Aspergillus fumigatus*) swollen conidia

SP: surfactant protein

SP-A: surfactant protein A

SP-D: surfactant protein D

SSC: side scatter

T

TCR: T cell receptor

Tg: transgenic

Tip-DC: TNF-α/iNOS-producing DC

TLR: toll-like receptor

V

VD3: vitamin D3

W

WT: wild type (mice)

1.0 INTRODUCTION

1.1 *ASPERGILLUS FUMIGATUS* AND HOST DEFENSE

1.1.1 *Aspergillus fumigatus*

Aspergillus fumigatus (A. fumigatus) is a common fungus in the environment. It grows on organic debris in the soil, releasing its spores (conidia) into the air. The small size (2-3 μm) and the hydrophobic surface of the conidia render them airborne, allowing them to easily reach human small airways and alveoli upon inhalation (1). The conidia are masked by immunologically inert melanin and the hydrophobic protein RodA, and thus are incapable of activating immune responses (2, 3). In this stage, they are referred to as resting conidia (RC) (1, 4). In the lung, RC gradually lose the hydrophobic surface, expose their inner cell wall, and germinate into more immunogenic swollen conidia (SC) (2, 4). Glucans, galactomannan, chitin, and cell wall-anchored proteins are the main cell wall components (5). Certain cell wall components, such as β-glucans (6-8), serve as pathogen associated molecular patterns (PAMPs) to activate immune cells through host pattern recognition receptors (PRRs). Under certain circumstances, such as in patients who are immunocompromised, SC further germinate into hyphae, the most invasive form of this fungus (1, 4).

1.1.2 *A. fumigatus*- associated diseases

Healthy individuals inhale hundreds of *A. fumigatus* RC on daily basis, but rarely develop clinical diseases due to the low-pathogenic potential of this organism, and to highly effective host immune mechanisms. Therefore, the acquisition of *A. fumigatus*-associated diseases seems to be related to inadequacies of the host immune system (1, 4). In immunocompetent individuals with underlying lung diseases, such as asthma or cystic fibrosis (CF), *A. fumigatus* can trigger allergic airway responses and cause respiratory disorders, such as a fungal sensitized asthma, and in most extreme cases, a condition termed allergic bronchopulmonary aspergillosis (ABPA) (9-11). In immunocompromised individuals, such as those with leukemia or in patients undergoing allogeneic hematopoietic stem cell transplantation, the uncontrolled growth of hyphae invades and damages host tissues, causing invasive pulmonary aspergillosis (IPA) (1). Experimental IPA is often achieved in murine models utilizing depletion of neutrophils or treatment with immunosuppressive drugs (1).

1.1.3 Host defense against *A. fumigatus*

Innate immunity against *A. fumigatus* is believed to involve a two-tiered attack by alveolar macrophages and neutrophils (12). Alveolar macrophages, as the major resident phagocytic cells in the airway space, ingest both RC and SC, which typically prevents the germination of hyphae (12). A recent report from our group has demonstrated that at early time points after infection, alveolar macrophages mainly adopt a phenotype characterized by expression of Arginase-1, Ym-1, and CD206, the key makers of alternatively activated macrophages, but not of NOS2, the key marker of classically activated macrophages (13). The influx of neutrophils is triggered by the

immunogenic SC that pose an invasive threat but not by dormant, non-immunogenic RC (6). Recruited neutrophils were initially postulated to predominantly attack extracellular hyphae germinating from conidia that escaped macrophage surveillance (12). However, recent studies have highlighted the key role of neutrophils in defense against both conidial and hyphal forms of this fungus. Neutrophils form oxidative-active aggregates around conidia, a structure that is essential in preventing conidia germination (14), while such a "neutrophil extracellular trap" formed around hyphae is more likely to restrain spreading instead of hyphal killing (15). Neutrophils are thought to be even more important than macrophages at early time points following an acute infection by *A. fumigatus*, since the depletion of neutrophils but not macrophages prior to or within 3 hours after inoculation resulted in a marked increase in mortality in mouse models (16).

There are other innate immune cells that have been implicated in antifungal host defense. In vitro and in vivo studies indicate that monocytes are able to phagocytose conidia (17, 18). Platelets have been shown to interact with conidial and hyphal forms of *A. fumigatus*, resulting in their activation, which can then modulate the responses of monocytes. This process helps to prevent conidia germination and serves to damage hyphae (19-21). The role of NK cells in defense against *A. fumigatus* has been explored more extensively. In an invasive aspergillosis model elicited in transiently neutropenic mice, depletion of NK cells compromised antifungal responses, while adoptive transfer of them improved the outcome of the disease (22, 23). The protective role of NK cells in this model has been associated with their potent IFN-γ-producing activity during the early phase. IFN-γ derived from NK cells is able to enhance the hyphal killing activity of macrophages, and to induce chemokines such as CXCL9, CXCL10 that attract other

inflammatory cells (23). Human NK cells were even found to directly kill hyphae in a perforin-dependent pathway (24).

The adaptive immune response to *A. fumigatus* is initiated by pulmonary DCs. Pulmonary DCs recognize both conidia and hyphae, transport them to the draining lymph nodes (dLNs) and initiate the generation of antigen-specific *A. fumigatus*-specific T cell responses, primarily in the CD4 T cell compartment (25). Among multiple pulmonary DC subsets, there is evidence that monocyte-derived inflammatory DCs are critical for T cell priming. In response to a single inoculation of mice with *A. fumigatus*, $Ly6C^{high}$ monocyte-derived $CD11b^{+}$ DCs were found to rapidly accumulate in the lung, take up antigens locally, and migrate into the dLNs. This DC subset was capable of activating naïve T cells when examined ex vivo, implicating their potential role in initiating T cell differentiation in vivo (18). To support this notion, depletion of monocytes and monocyte-derived DCs abolished T cell priming (18).

Regarding Th1/Th2/Th17/Treg differentiation, heterogeneous T cell subsets can coexist after exposure to *A. fumigatus*, but the one that predominates under various conditions seems to determine the outcome of infection in each case. Th1 responses are believed to be protective given that adoptive transfer of fungal specific Th1-type cells into immunocompromised mice conferred resistance to the subsequent fungal infection (26). Furthermore, Th1 cell-associated cytokines, such as IFN-γ, TNF-α and IL-12 elicit beneficial effects in murine invasive aspergillosis models (27). The Th1-associated protection is likely attributable to the ability of Th1 cytokines to improve the phagocytic properties of macrophages and neutrophils (28). In contrast, Th2 cells, and associated cytokines IL-4, IL-10, are deleterious in invasive disease (29, 30), so that mice lacking Th2 cytokines, such as IL-4 KO mice, are less susceptible to infection (30). Th2 cell clones have been isolated from ABPA patients (31) and Th2 cytokines IL-4, IL-5

and IL-13 are known to contribute to allergic pathogenesis (9). Only limited but conflicting data are available regarding the role of Th17 cells in host defense to *A. fumigatus*. There is evidence that following infection by this fungus, IL-23p19-/- mice had a lower fungal burden accompanied by an impaired Th17 response but a higher Th1, implying that the Th17 restrains Th1, serving as a negative regulator of the protective response (32-34). In contrast, experimental neutralization of IL-17A by antibodies 6 h post infection also reportedly resulted in a dramatic increase in fungal burden 24 h or 48 h later, suggesting an immunoprotective role of IL-17A in the early phase (35). In humans, *A. fumigatus* was found to induce relatively weaker IL-17A production from human peripheral blood mononuclear cells (PBMC) compared to *Candida albicans*, a known inducer of IL-17A (36). In a human monocyte-derived macrophage (MDM)/lymphocyte co-culture system, *A. fumigatus* induced a robust IFN-γ response with little observed IL-17A production. Moreover, the addition of *A. fumigatus* or its secreted products into MDM/lymphocyte co-cultures activated by anti-CD3/anti-CD28 beads attenuated IL-17 production (36). These findings therefore argue against the ability of *A. fumigatus* to induce IL-17A production, yet demonstrate the importance of IL-17A in anti-fungal responses in humans. However, genetic association studies have revealed that a non-synonymous mutation of the IL-23 receptor (rs11209026) is associated with decreased risk of *A. fumigatus* infection in the setting of T cell-depleted allogeneic stem cell transplantation (37). This finding is consistent with the negative regulatory role of IL-23/IL-17A signaling in experimental aspergillosis (32). These conflicting data strongly suggest that further studies would be necessary to clarify the role of Th17 cells in *A. fumigatus*-associated conditions.

Regulatory mechanisms have been identified which control the balance between immunoprotection and immunopathology upon *A. fumigatus* infection. Following intranasal (i.n.)

inoculation of mice with 2×10^7 *A. fumigatus* conidia, a population of $CD4^+CD25^+$ T cells with high IL-10 production was observed in the lungs during the early phase (3 d post infection), whereas $CD4^+CD25^+$TGF-β-producers were found in the dLNs during the late phase (10 d post infection) (38). The early IL-10-producing regulatory cells might limit exaggerated inflammation at the expense of fungal clearance, as supported by the observation that B7-2 knockout (KO) mice lacking these early cells had a lower fungal burden but more severe inflammatory pathology. The late $CD4^+CD25^+$TGF-β-producers in the dLNs were potent in inhibiting T cell proliferation and were speculated to prevent fungus-induced allergy (38). The occurrence of these suppressors was accompanied by the induction of the enzyme indoleamine 2,3-dioxygenase (IDO) in lung cells including DCs (38). The important role of IDO is supported by evidence showing that inhibition of IDO activity by 1-methyl-tryptophan treatment or by IDO dysfunction in $p47^{phox-/-}$ mice, resulted in impaired fungal clearance and exaggerated inflammation following *A. fumigatus* infection (33, 38). IDO-producing DCs are known to be involved in Treg development in various settings, and their pivotal role in *A. fumigatus* infection was confirmed by adoptive transfer of IDO-expressing DCs resulting in protection of recipient mice from invasive aspergillosis via induction of balanced Th1/Treg responses (39). However, in vivo protective immunity is likely achieved mainly through IDO in the non-hematopoietic rather than the hematopoietic compartment, as suggested by reciprocal bone marrow (BM) reconstitution experiments (40). In an allergy model induced by repeated *A. fumigatus* antigen inhalation, selective over-expression of IDO in lung epithelial cells significantly reduced $CD4^+$ T cell numbers in the inflamed lung as well as their capacity of to secrete IL-4, IL-5, IL-13 and IFN-γ (41). These findings establish IDO as a key factor in maintaining a desirable balance in immunity to *A. fumigatus*.

In conclusion, innate immune mechanisms mainly mediated by neutrophils and macrophages as well as balanced Th1/Treg adaptive responses confer protection to the host against *A. fumigatus*. Th2 responses are detrimental to host defense and instead can contribute to allergic pathogenesis. Our current understanding of Th17 cells is incomplete and needs to be further explored.

1.1.4 Recognition of pathogen associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs)

A. fumigatus displays its PAMPs in a life cycle stage-dependent fashion, so consequently the host mediates stage-specific recognition by using a variety of PRRs.

1.1.4.1 Toll-like receptors (TLRs)

The involvement of TLRs in *A. fumigatus* recognition has been widely investigated but conflicting results have been reported. Among TLRs, transfection of HEK293 cells with TLR2 and 4, but not TLR1, 3, 5, 6, 7, 8, or 10 activated the NF-κB pathway and induced proinflammatory cytokine production upon conidia stimulation, implying that TLR2 and TLR4 are more important than other TLRs in fungal recognition (42). Therefore, TLR2/TLR4/MyD88 signaling has been explored in the greatest detail. Because of the generally low-pathogenic potential of *A. fumigatus* in the setting of an intact immune system, administration of immunosuppressive drugs or depletion of neutrophils has been commonly used to induce invasive aspergillosis in mice (1), whereas TLRs/MyD88 deficiency is not sufficient to cause invasive disease (43, 44). However, deficiency in TLR2, TLR4 or MyD88 in immunosuppressed mice further impaired pathogen clearance, aggravated invasive disease, and increased the

mortality rate (43, 44). These findings suggest that TLR2/TLR4/MyD88-dependent mechanism is likely involved in host protection but is not absolutely indispensable for fungal control. Conflicting results have been reported in studies using TLR2/TLR4/MyD88 KO mice in the absence of any immunosuppressant treatment. In one study, inoculation of TLR2, TLR4 or MyD88 KO mice three times with 2×10^7 conidia resulted in higher fungal burden compared to wild type (WT) mice at 3 d post the last inoculation. Consistent with the fungal burden data, the protective Th1 response was impaired while the detrimental Th2 response was simultaneously enhanced in deficient mice (43). Analysis of T cell differentiation using an adoptive transfer approach demonstrated that transferred *A. fumigatus*-specific transgenic T cells were activated to express T-bet in the dLNs in WT recipient mice, while this activation was impaired in MyD88-deficient recipients (45). However, the trafficking of these primed T cells into inflamed lung and their acquisition of IFN-γ-producing capacity seemed to be MyD88-independent (45). Nevertheless, optimal development of Th1 responses to *A. fumigatus* does require MyD88 signaling. In contrast to these findings, another study has reported that following i.n. infection with 1×10^7 conidia, MyD88 KO mice did not significantly differ from WT mice in terms of fungal burden or mortality during 5 to 15 days post infection (46). The contradictory results may have been due to the differences in observation time points, thus a time-course study has been performed. Following inhalation of aerosolized *A. fumigatus* conidia (with a mean of 3.4×10^6 CFU recovered per mouse), MyD88 KO mice showed higher fungal burden and more diffused but less severe lung inflammation at early time points (<72h) compared to WT mice, but fungal load rapidly declined thereafter and both WT and MyD88 KO resolved inflammation eventually (47). Therefore, it appears that MyD88 signaling mediates acute fungal clearance and

inflammation early post infection, but the abnormalities due to MyD88 deficiency are eventually normalized over time by redundant antifungal pathways (47).

The contribution of the TLR/TRIF pathway has also been evaluated in *A. fumigatus* infection. TRIF KO mice showed heightened Th2/Th17 responses but reduced protective Th1/Treg responses, and consequently impaired fungal clearance and exacerbated inflammation (40). TLR3 KO but not TLR4 KO mice mimicked the phenotype of TRIF KO mice, suggesting that TLR3/TRIF is the responsible pathway (40). Moreover, reciprocal BM reconstitution experiments identified that TRIF deficiency in the non-hematopoietic compartment, in particular, greatly increased the susceptibility of mice to aspergillosis (40). TRIF deficiency failed to activate the enzyme IDO, the central molecule that controls the balance of anti-*A. fumigatus* defense and immune-mediated pathology as described above. Therefore, the enhancement of IDO has been considered as at least one of the mechanisms by which TRIF limits the unwanted inflammation (40).

The involvement of TLR9 in *A. fumigatus* infection has been recently explored. Analysis of the fungal genome revealed the presence of unmethylated CpG DNA, the natural TLR9 ligand. Consistent with this observation, isolated fungal DNA was found to stimulate cytokine production from BMDCs, at least partially in a TLR9-dependent fashion (48). However, it should be considered that the accessibility of fungal DNA to TLR9 would be the prerequisite step for successful signaling through TLR9. Examination of intracellular trafficking showed that in the Raw264.7 macrophage cell line, TLR9 was specifically redistributed to the phagosomes containing *A. fumigatus* following phagocytosis, allowing TLR9-fungal DNA interaction and potentially TLR9 activation to occur (49). The role of TLR9 has also been analyzed in vivo in different disease settings. Following three i.n. inoculations with 2×10^7 conidia, TLR9 KO mice

had a reduced fungal load (43), indicative of a negative role in fungal clearance. Consistent with this observation, TLR9 KO mice were more resistant to experimental invasive aspergillosis when they were rendered simultaneously neutropenic (50). In a chronic fungal asthma model involving sensitization with soluble *Aspergillus* antigens then challenge with RC (51), TLR9 KO mice had reduced airway hyperresponsiveness (AHR) compared to WT animals (50). When these sensitized mice were challenged with SC, TLR9 KO mice showed higher fungal growth in the lung (50). These findings indicate that TLR9 may modulate immune responses in a context-dependent manner, but further studies are needed to elucidate the mechanism of TLR9 activity in different disease scenarios.

A. fumigatus conidia upregulate TLR5 expression on human monocytes (52), but little is known about any role for TLR5. It seems that TLR5 does not directly interact with conidia, but knockdown of TLR5 improved conidia viability (52), implicating an indirect function of this receptor.

Collectively, the TLR2/TLR4/MyD88 and TLR3/TRIF pathways are protective in *A. fumigatus* infection and are associated with the beneficial Th1/Treg responses. TLR5 and TLR9 appear to have roles, but their functions need to be further explored.

1.1.4.2 C-type lectin-like receptors (CLRs)

CLRs are PRRs that recognize carbohydrate moieties of pathogens. Transmembrane CLRs, in particular dectin-1 and soluble CLRs, such as surfactant protein A and D (SP-A and SP-D) as well as mannose-binding lectin (MBLs), have been implicated in the recognition of *A. fumigatus.*

Dectin-1 was initially identified on DCs (53) and later on many types of cells of the innate immune system, including macrophages, neutrophils and monocytes (54). Dectin-1 is a type II transmembrane protein, with an extracellular domain that recognizes β-glucans, and an

intracellular domain that is responsible for signal transduction (54). Dectin-1 is involved in the recognition of fungi, as well as mycobacteria (55) and influenza (56). The activation of the dectin-1 signaling pathway generally leads to the phagocytosis of organisms and the initiation of inflammatory cascades (54). *A. fumigatus* RC that contain little exposed β-glucan are unable to trigger dectin-1 signaling to mount an appreciable inflammatory response, while SC or hyphae with unmasked β-glucan on the surface are capable of dectin-1 activation. This activation then promotes fungal uptake, cytokine/chemokine production and the release of reactive oxygen species (ROS) (6-8). Studies have shown that dectin-1 deficiency in alveolar macrophages diminished the levels of proinflammatory cytokines such as TNF-α, IL-1β, IL-1α, IL-6, G-CSF and GM-CSF in response to either SC or hyphae (6-8). In vivo studies have shown that mice lacking dectin-1 had impaired pathogen clearance and increased susceptibility to invasive disease (35). In agreement with these experimental findings, clinical studies have indicated that a human dectin-1 early stop codon polymorphism (Y238X) leading to reduced dectin-1 activity was associated with high susceptibility to invasive aspergillosis in hematopoietic transplantation (57). These studies unambiguously demonstrate the protective role of dectin-1 in anti-*A. fumigatus* infection.

Dectin-2 is also a transmembrane CLR, predominantly expressed in tissue macrophages, DCs, Langerhans cells and peripheral blood monocytes. Dectin-2 has an extracellular mannose-binding domain that recognizes numerous pathogens, including fungi, but it appears to preferentially recognize hyphal forms rather than conidial forms of fungi (58). Dectin-2 lacks an intracellular tyrosine kinase activation motif (ITAM) and therefore accomplishes signal transduction through a partner protein FcRγ (59). Although involved in fungal recognition, to our knowledge, recognition of *A. fumigatus* conidia or hyphae by dectin-2 has not been formally

confirmed. However, one group has reported that cysteinyl leukotriene triggered by antigens extracted from *A. fumigatus* in BMDCs was enhanced by overexpression of dectin-2 but inhibited by knockdown of this receptor (60). This finding suggests that dectin-2 may be involved in *A. fumigatus* recognition with the potential for biological consequences.

DC-SIGN, another transmembrane CLR, has been detected on human alveolar macrophages and monocyte-derived DCs. DC-SIGN binds conidia through the recognition of the galactomannan component of fungal cell wall and mediates conidia uptake (61). However the biological significance of this recognition is presently unknown.

Soluble receptors, SP-A and SP-D belong to the collectin subgroup of CLRs. Human SP-A, SP-D and rat SP-D bind to A. *fumigatus* conidia in the presence of calcium, enhancing their uptake by phagocytic cells (62, 63). Both proteins recognize carbohydrate structures, and it has been suggested that SP-D recognizes β 1, 6-glucan on fugal cell walls (64). In immunosuppressed mice, SP-A KO animals were more resistant, whereas SP-D KO mice were more susceptible, to invasive aspergillosis (65). Administration of SP-D to WT or SP-D-deficient mice protected them from otherwise fatal invasive disease, which was associated with an increase in IFN-γ relative to IL-4 (65, 66). In contrast, exogenous SP-A given to SP-A KO mice exacerbated experimental IPA and increased the mortality rate (65). Therefore, in invasive disease model, it seems that SP-D and SP-A are beneficial and detrimental to the host, respectively. However, conflicting data on SP-A also have been reported. Unlike the case of SP-A KO mice, SP-A treatment of WT mice showed protective effects, although this was less effective than administration of SP-D (66).

SP-A and SP-D have also been widely studied in allergy models induced by *A. fumigatus* antigens. SP-D levels in mouse lung were increased following antigen challenges in an IL-4/IL-

13-dependent manner (67, 68). SP-D deficiency resulted in an overall more pronounced allergic inflammation as compared to SP-A deficiency. Treatment of allergic WT or SP KO mice with SP-D, and to a lesser extent SP-A, attenuated allergy-associated pathology through skewing of the Th1/Th2 balance towards the Th1 (69, 70). The effects of SP proteins on the T cell compartment have been further examined ex vivo. When T cells were isolated from allergic mice and restimulated in vitro by *Aspergillus* antigens, both SP-A and SP-D inhibited T cell proliferation and Th2 cytokine production (68, 71). Collectively, data obtained in allergy models suggest that these proteins are induced in a Th2 environment but limit Th2-associted pathology, representing a negative feedback mechanism. But contrary to what would be expected from experimental models, there is clinical evidence that CF patients with or without ABPA did not differ in the SP-D levels in the serum (72), suggesting that serum SP-D level is not a good marker for the diagnosis of allergy caused by this *A. fumigatus*.

MBL is another secreted protein in the colletin subgroup. One isoform of human MBL and two isoforms of mouse MBL, MBL-A and MBL-C have been identified. MBL binds to *A. fumigatus* (73), enhancing fungal uptake by neutrophils, probably through a complement-mediated pathway (74, 75). In an IPA model in immunocompromised mice, administration of MBL greatly improved the disease (74). Human genetic studies have suggested that MBL haplotypes leading to low MBL levels was more common in patients with chronic necrotizing pulmonary aspergillosis (76). However, contradictory to these findings that suggest a protective role of MBL, intravenous (i.v.) inoculation with a lethal dose of *A. fumigatus* conidia resulted in a higher survival rate in MBL-A/D double KO mice in comparison with WT mice, indicating its deleterious role (77). This discrepancy may be related to different infection routes, and whether

or not immunosuppressive drugs have been used, among other possible explanations. Further studies will be required to clarify the role of MBL in anti-*A. fumigatus* immunity.

In a chronic fungal asthma model evoked by the deposition of conidia into mice sensitized with *A. fumigatus* antigens, MBL-A deficiency reduced the levels of Th2 cytokines/pro-allergic chemokines and abolished bronchial hyperresponsiveness. However, no major differences in fungal clearance and airway remodeling were identified between MBL-A KO and WT mice (78). Nevertheless, MBL, to some extent, does appear to contribute to fungal-induced allergy.

Therefore, among the CLR family members, dectin-1 protects the hosts from *A. fumigatus*. The role of soluble receptors (SP-A, SP-D and MBL) has been better addressed in allergic inflammation rather than in invasive disease induced by this fungus. SP-A and SP-D seems to limit, but MBL contributes to, allergic pathology.

1.1.4.3 Inflammasome pathway

Recent work has described that *A. fumigatus* hyphae but not conidia activated the NLPR3 inflammasome pathway in the human THP-1 monocyte cell line, resulting in the release of IL-1β. Activation of the inflammasome was impaired when Syk but not MyD88 was inhibited (79). Since Syk is one of the downstream molecules of dectin-1 signaling (58), implying that dectin-1 might be involved. The function of NLPR3 inflammasome in response to *A. fumigatus* would be worth further investigation.

1.1.4.4 Others

A soluble receptor pentraxin-3 (PTX3) binds to live or heat-killed *A. fumigatus* conidia but not to the hyphal form (80). The binding of PTX3 to conidia was found to be mediated by the N-terminal of this protein (81). PTX3 acts as an opsonin to activate the alternative pathway of

complement and facilitates conidia phagocytosis that involves the actions of FcγRs and CD11b (81). Upon *A. fumigatus* infection, PTX3 deficiency resulted in a skewed Th2 response, impaired fungal clearance and higher susceptibility to invasive disease, and treatment of the KO mice with recombinant PTX3 completely reversed the phenotypic defects (80). In $p47^{phox}$-/- mice that develop chronic granulomatous disease (CGD), excessive inflammation in response to *A. fumigatus* infection has been linked to delayed production of PTX3. Administration of PTX3 at early time points following infection promoted fungal clearance and limited destructive inflammation (82). These observations highlight the protective role of PTX3 in antifungal responses to *A. fumigatus* infection.

In conclusion, the protective role of TLR2/TLR4/MyD88, TLR3/TRIF, dectin-1 and PTX3 in host defense against *A. fumigatus* has been well established. SP proteins and MBL show beneficial and detrimental effects in *A. fumigatus*-induced allergic responses, respectively.

1.1.5 Th1/Th2/Th17/Treg lineage specification in response to *A. fumigatus*

Given the differential role of T cell lineages in determining the outcomes of diseases of *A. fumigatus* infection, it is important to understand T cell differentiation in these settings. The determination of outcomes in the Th1/Th2/Th17/Treg paradigm largely relies on the specific interactions between DC subsets and fungal morphotypes.

A. fumigatus dead RC and hyphae preferentially elicit Th2 responses, while live conidia mainly activate Th1 responses (25, 45, 83). Further examination of different *A. fumigatus* components revealed that several fungal components act as immunodominant antigens to induce specific patterns of Th1/Th2/Th17/Treg differentiation. Certain secreted proteins, such as superoxide dismutase (Sod1p) and ribonuclease (RNUp) promote IL-4-producing cells, while

glycolipids such as the glycosylinositolphosphoceramide (GSL) and the GPI-anchored lipophosphogalactomannan (LGM) mostly result in IL-17-producing clones. Cell wall polysaccharides have variable capabilities to induce IFN-γ, IL-17 and IL-10-producing cells (84). For instance, chitin, as one essential component of *A. fumigatus* cell wall, drives Th2 immunity and allergic responses (85). In contrast, two GPI-anchored proteins, the 1, 3-β glucanosyltransferase (Gel1p) and an ortholog of Crh1p associated in β1,6 glucan-chitin linkages (Crf1p) and a secreted protein, the aspartic protease (Pep1p) preferentially induce Th1/Treg (84). Studies have shown that pretreatment with components that elicit a robust Th1 response but not Th2/Th17 responses protected immunosuppressed mice from a subsequent otherwise fatal challenge with *A. fumigatus* (84), highlighting the protective role of Th1 responses.

Mode and frequency of exposure to *A. fumigatus* seems to contribute to T helper subset specification. It has been established that after an acute infection with *A. fumigatus* conidia, Th1 cells predominate in the CD4+ T cell compartment (25, 45, 83). Recent data from our group and another group have suggested that multiple low-dose exposures to conidia actually reduce the Th1 predominance, and lead to a coexistence of Th1/Th2/Th17 cells (86, 87).

A recent study has investigated the relationship between PRR ligation and Th1/Th17 balance following *A. fumigatus* infection. Adoptive transfer of *A. fumigatus*-specific T cells into MyD88 KO recipients diminished Th1 cells but maintained a normal Th17 response as compared to that in WT recipients. In contrast, adoptive transfer of the same cells into dectin-1 KO recipients diminished Th17 but enhanced Th1 differentiation (88). These findings revealed the important role of MyD88 and dectin-1 signaling in directing Th1 and Th17 responses, respectively. It seems that dectin-1 signaling in DCs induces specific cytokines, which ultimately

downregulate T-bet expression in responding T cells and thereby promote Th17 differentiation (88).

1.2 *A. FUMIGATUS*-INDUCED ALLERGIC DISORDERS

A. fumigatus can induce a spectrum of allergic respiratory diseases, such as an asthma sensitized with *A. fumigatus* (Af-sensitized asthma) and ABPA (9, 11).

1.2.1 Diagnosis

There is evidence that *A. fumigatus* sensitization, defined by skin test and fungal specific serum IgE responses, is more common in patients with severe asthma requiring multiple hospital admissions than those with mild asthma (11). The specific clinical entity ABPA is a complication arising in patients with severe obstructive lung disease, mainly asthma and CF, and may represent one of the most extreme manifestations of fungal allergy. ABPA has been estimated to occur in 1-2% of chronic asthmatics and in up to 10%-15% of patients with CF (9). The disease was first reported in 1952 by Hinson et al, who described patients with allergic symptoms, such as peripheral blood eosinophilia and mucous production, who also produced sputum that grew *A. fumigatus* (89). Later on, the Rosenberg-Patterson criteria became the most used for the diagnosis of ABPA clinically, which requires the patients to have a preexisting asthma or CF, immediate-type skin reactivity to *A. fumigatus* antigens, eosinophilia, precipitating antibodies to *A. fumigatus* antigens, elevated total IgE and elevated *A. fumigatus*-specific IgE and IgG in the serum. Central bronchiectasis is often seen in ABPA patients (ABPA-CB), but ABPA

can also occur with diagnostic serologic markers in the absence of bronchiectasis (Seropositive ABPA or ABPA-S). Colonization of *A. fumigatus* with detectable fungal hyphae can be found in the lung, although tissue invasion is generally not apparent that distinguishes it from invasive disease (90, 91).

1.2.2 Pathogenesis

The pathogenesis of ABPA is complicated and not fully understood. Our current understanding seems to be established based upon human studies and to an even larger extent, on experimental models. Crude *A. fumigatus* extract administered by i.n. or i.t. instillations is commonly used for the induction of experimental diseases in mice (92). The current concept is that ABPA elicits allergic responses in the early phase, and lung injury with airway remodeling in the late phase in response to continuous *A. fumigatus* antigen stimulation. It is believed that the aberrant interactions between the host and the fungus result in the manifestation of ABPA symptoms (93, 94).

T cell subsets that drive immune pathology associated with ABPA have been dissected. T cell clones established from patients with ABPA were characterized as Th2-type cells, with a high IL-4- but little IFN-γ-producing capacity (31, 95). In an experimental model of *A. fumigatus*-induced allergic disease, RAG2 deficient but not B cell deficient mice failed to develop allergic responses. Moreover, adoptive transfer of $CD4^+$ T cells restored AHR, eosinophilia and pulmonary inflammation, highlighting the indispensable role of T cells in disease pathogenesis (96). Among cytokines produced by Th2 cells, depletion or blockade of IL-4 resulted in a significant reduction in AHR, serum IgE levels, and eosinophil numbers, although pulmonary histology was not appreciably affected (97-99). IL-5 has been associated with the

development of eosinophilia and IgE responses but not with AHR (96-98). Depletion of IL-13 attenuated AHR, goblet cell hyperplasia, peribronchial inflammation but not IgE levels (100). Therefore, the individual Th2 cytokines likely have distinct but complementary roles in promoting ABPA pathology.

The central question remaining is why do only certain individuals develop Th2 and allergic responses to *A. fumigatus*. In early studies, particular *A. fumigatus* proteins were described as allergens and called "Asp f" proteins, since these proteins can bind IgE antibody or stimulate $CD4^+$ Th2 clones in people with allergic diseases, such as ABPA (101). But recent studies have demonstrated that many of these allergens actually induced greater Th1 but not Th2 responses in PBMCs isolated from healthy, non-atopic individuals, therefore these proteins are not strictly Th2-directing "allergens". This finding raises the possibility that T helper subsets are greatly influenced by local inflammatory milieu apart form the function of fungal antigens (102). To support this notion, immunological and genetic factors have been identified that predispose individuals to ABPA. Given the essential role of Th2 cells in disease pathogenesis, these factors tend to be directly or indirectly associated with T cell phenotypes as discussed below.

Genetic variability of human leukocyte antigen (HLA) has been linked to ABPA. Epitope mapping studies have defined that the majority of the fungus-responding T cells from ABPA patients were associated with HLA-DR2 and HLA-DR5 haplotypes, while the HLA-DQ2 allele conferred protection in the non-ABPA population (31, 103). This genetic link is likely explained by HLA allele-specific presentation of fungal peptides to T cells and HLA-restricted pathogenic Th2 cell differentiation (31, 103).

As described in 1.1.4, several PRRs have been associated with resistance or susceptibility to ABPA. The protective role of SP-A and SP-D in *A. fumigatus*-induced hypersensitivity has

been documented in experimental models (69, 70). It has been proposed that SP-A and SP-D proteins act in a negative feedback loop, such that SP-A and SP-D proteins induced by Th2 cytokines bias T helper responses towards Th1 but away from Th2, thus limiting Th2-associated pathology (68-70). Consistent with experimental models, polymorphisms in the collagen regions of SP-A1 and SP-A2 in human were identified to be associated with the occurrence and severity of ABPA (104). In contrast to the beneficial role of SP proteins, MBL was found to contribute to production of Th2 cytokines and pro-allergic chemokines as well as to AHR, thereby promoting disease pathogenesis in a mouse model (78). In humans, high MBL activity resulting from homozygous alleles of MBL 1011A is associated with high peripheral blood eosinophilia and low forced expiratory volume (105). These findings consistently suggest an undesirable role of MBL in ABPA.

The persistent presence of *A. fumigatus* antigens in the respiratory system in susceptible individuals seems to be another risk factor associated with the development of ABPA. Despite constant inhalation, healthy individuals usually clear the pathogen efficiently and do not develop a sustained inflammation. In contrast, in individuals susceptible to ABPA, the preexisting abnormal lung condition, typically asthma or CF, may provide a permissive environment for fungal colonization and antigen persistence, which subsequently leads to sustained inflammation (93, 94). As shown by us and another group, at least in experimental models, persistent fungal exposure likely induces aberrant T cell responses by biasing towards Th2 and Th17 (86, 87), thereby contributing to ABPA pathogenesis. The ineffective control of clearance in CF or asthma patients is likely in part attributable to the quality of mucus. Normally, airway mucus traps inhaled pathogens and ciliary movement effectively clears them out of the lungs. However, the dysfunction of cystic fibrosis transmembrane conductance regulator (CFTR) due to variable

mutations in this gene in CF patients leads to reduced ciliary motility, highly concentrated mucus, and ultimately to compromised pathogen clearance (106). This notion is supported by a recent study demonstrating that severe CFTR mutations with minimal protein function were associated with a higher risk of *A. fumigatus* infection, while mutations that permit residual CFTR function had a reduced risk (107). This observation therefore is suggestive of a link between CFTR function and fungal restriction. As described above, ABPA is considered to be a complication primarily occurring in patients with asthma and CF. However recent studies have identified ABPA in patients with chronic obstructive pulmonary disease (COPD) and suggested that this condition could also be a potential risk factor for ABPA (108, 109). Given that COPD patients usually have mucus hypersecretion and impaired mucociliary clearance similar to CF patients, theoretically the pathologic mechanism could be similar. However, a larger scale study may be necessary to confirm this finding since it would be of great interest to determine whether there is an association between fungal persistence and ABPA development in COPD.

Additional evidence suggests that the CFTR protein is not only limited to have influences on fungal clearance. Recent studies in experimental models have identified CFTR as an unexpected T-cell intrinsic factor associated with T helper lineage determination. In a murine model of ABPA, CFTR deficiency promoted exaggerated serum IgE levels and exacerbated allergic pulmonary inflammation, which were alleviated by transferred CFTR or IL-10 genes (110, 111). Surprisingly, further analysis revealed that T cells in CFTR KO mice had intrinsic Th2-biased properties, such that they were capable of producing higher levels of Th2 cytokines upon TCR ligation than comparable cells from WT animals (112). To explore the biological importance of CFTR in the T cell compartment, mice with conditional knockout of CFTR genes in $CD3^+$ T cells were generated and assessed. These mice mimicked the phenotype of CFTR KO

mice, showing a biased Th2 response and hyper IgE-associated inflammation upon challenge with *Aspergillus* antigens. These findings conclusively demonstrate that CFTR deficiency in the T cell compartment independently contributes to the gross phenotypic aberrations observed in CFTR KO mice (113). CFTR had been expected to primarily act in lung epithelial cells but not T cells previously, thus it would be of great value to further assess the relative contribution of CFTR in epithelial cells and T cells.

It is known that not all CF patients colonized with *A. fumigatus* progress to the ABPA disease state, suggesting that although colonization is considered to be important, it is not sufficient to elicit ABPA. To reveal the regulatory mechanisms, a recent elegant study compared *A. fumigatus*-colonized CF patients with or without ABPA. T cells from patients with ABPA were found to exhibit a higher Th2 response but contained a lower frequency of Treg cells compared with those from non-ABPA patients. This reduced Treg response in ABPA patients correlated with a lower level of vitamin D3 (VD3) in their serum, and the supplementation of VD3 was able to enhance the ability of Tregs to suppress Th2 cells (114). Therefore, it seems that ineffective or insufficient control of Th2 responses by Tregs under the condition of VD3 deficiency acts as a predisposing factor for ABPA progression in fungus-colonized CF patients (114). This study reinforces the importance of local environmental cues in modulating susceptibility to ABPA and provides a rationale for VD3 treatment of ABPA.

ABPA is considered as a Th2 disease associated with consequent eosinophilia, but there has been an increasing appreciation of neutrophilia in this disease. Neutrophil infiltration has been observed both in the lung tissue and the sputum of ABPA patients (115-117). As compared to patients with asthma, ABPA patients with bronchiectasis demonstrated an increased intensity of inflammation and significantly higher numbers of neutrophils in the sputum. Moreover, the

neutrophil counts together with eosinophil counts positively correlate with the severity of bronchiectasis and the degree of lung damage (115). High IL-8 levels in the sputum likely provide an explanation for neutrophil influx in ABPA patients, while matrix metalloproteinase-9 (MMP-9) is considered as one of the neutrophil-derived mediators that contribute to lung pathology (116). It has been speculated that IL-8 is derived from epithelial cells in response to the proteases secreted by *A. fumigatus* (116), although this hypothesis has not been tested. Neutrophilia has not been seen in experimental models induced by inhalation of extracts prepared from *A. fumigatus* (92), making it impossible to study neutrophil infiltration in these models. However, in a chronic allergy model elicited in mice sensitized and boosted with *A. fumigatus* extract but challenged with conidia, a rise in neutrophils in the lung has been identified (51). Although lack of neutrophils predisposes mice to invasive aspergillosis following inoculation with a relatively high number of conidia, neutrophil depletion in this allergy model before conidia deposition did not cause invasive disease. Rather, neutrophils in this model contribute to AHR, collagen deposition and lung fibrosis (118). The detrimental role of neutrophils at least partially depends on MMP-9 in this animal model (118), which is consistent with data obtained in human studies (116). Theoretically, factors triggering excessive neutrophil influx might exacerbate the lung pathology and worsen the allergic disease. It would be of great value to explore the mechanisms for neutrophil infiltration and to understand how to regulate it.

In conclusion, it seems that both eosinophilic and neutrophilic inflammation contribute to the pathogenesis of ABPA. The pathogenic role of the Th2-eosinophil axis has been well established, and many predisposing factors have been associated with promoting Th2 responses. However the underlying mechanisms for neutrophil influx are poorly defined.

1.3 MONOCYTE-DERIVED INFLAMMATORY DENDRITIC CELLS

1.3.1 Monocyte subsets and origin

Monocytes circulate in the blood and BM in the steady state, and migrate into the tissues in inflammatory settings. In addition to the BM, spleen has been identified as a site for monocyte storage in homeostasis and as a source of rapid mobilization during injury (119).

Heterogeneity among human monocytes has been recognized for decades (120). Based on the expression of CD14 (part of the receptor for LPS) and CD16 (also known as FcγRIII), human monocytes have been divided into two major subsets: the "classical" $CD14^{+}CD16^{-}$ monocytes and the "non-classical" $CD14^{low}CD16^{+}$ monocytes, and the former is the predominant population, representing up to 95% of the monocytes in healthy individuals. There is also an "intermediate" minor population that is $CD14^{+}$ $CD16^{+}$ (121). Mouse circulating monocytes have been described as consisting of two major populations according to their expression of Ly6C and CX_3CR1, which are $Ly6C^{high}$ $CX3CR1^{low}$ and $Ly6C^{low}$ $CX3CR1^{high}$ monocytes (122). In the steady state, the ratio of $Ly6C^{high}$ /$Ly6C^{low}$ monocyte is around 4:6. $Ly6C^{high}$ monocytes are considered to be "inflammatory monocytes" since infection or inflammation drives their egress from the BM into the peripheral circulation. $Ly6C^{high}$ monocytes express the chemokine receptor CCR2 on their surface and multiple lines of evidence support the indispensable role of CCR2 in mobilizing them out of the BM (123).

Recently studies have shown that in the BM, $CX3CR1^{+}$ $CD115^{+}$ $CD34^{+}$ $CD16^{+}$ Lin^{-} macrophage/dendritic cell progenitors (MDPs) differentiate into monocytes that subsequently egress into the peripheral circulation (124). In the absence of infection, $Ly6C^{high}$ monocytes can traffic back into the BM, or convert into $Ly6C^{low}$ monocytes, which migrate into the blood

vessels (125) (Figure 1). However, it remains unclear whether Ly6C^{low} monocytes exclusively develop from Ly6C^{high} monocytes or can be directly derived from MDPs.

1.3.2 The DC differential potential of monocytes

Monocytes constitute approximately 10% and 4% of peripheral blood leukocytes in humans and in mice, respectively (126, 127). Circulating monocytes have a short life span, with a half-life about 1 day in mice and 3 days in humans, and do not proliferate, but these cells can act as precursors to repopulate macrophages and DCs. It has been known for long time that monocytes can replenish tissue macrophages (128). The ability of monocytes to differentiate into DCs was originally demonstrated by in vitro studies performed in a human system, in which human CD14^{+} monocytes cultured with GM-CSF and IL-4 resulted in the generation of immature DCs (129, 130). Subsequent in vivo studies in a mouse system validated the DC-differentiation potential of monocytes (131).

Recently, accumulating evidence has revealed great complexity in pathways resulting in DC subsets arising from monocytes. In the steady state, monocytes give rise to certain types of mucosal DCs or macrophages that reside in the skin, respiratory or intestinal tract. For example, Ly6C^{-} monocytes can give rise to alveolar macrophages (132) while Ly6C^{+} monocytes contribute to CX3CR1^{+} lamina propria DCs (lpDCs) (125, 133, 134). However, it is clear that DCs and macrophages are not exclusively derived from monocytes, and many DC types have never gone through an intermediate monocytic stage. In the BM, MDPs give rise to monocytes and common DC precursors (CDPs). CDPs lose the potential to give rise to monocytes, instead differentiate into either plasmacytoid DCs (pDCs) or pre-classical DCs (pre-cDCs). Pre-cDCs leave the BM, circulate in blood and enter lymphoid organs, where they give rise to classical

$CD8\alpha^+$ and $CD8\alpha^-$ DCs, or enter non-lymphoid tissues, where they give rise to certain DC subsets, such as $CD103^+$ lpDCs (Figure 1) (135-137). Therefore, it seems that monocytes result in only a minor contribution to the lymphoid DC and pDC network under homeostatic conditions.

During inflammation, $Ly6C^+$ monocytes migrate from the blood or the BM to lymphoid and nonlymphoid tissues in response to signals derived from infection and associated tissue damage. Under these conditions, monocytes produce cytokines, phagocytose other cells and differentiate into inflammatory DCs and macrophages (138). The de novo generation of monocyte-derived DCs has been demonstrated during infection and under other inflammatory settings. For instance, in response to *Leishmania major* infection, monocytes give rise to DCs at the infection sites locally (in the dermis, dermal MoDCs) or in the dLNs (dLN MoDCs). Dermal moDCs migrate into the dLNs and control the induction of a protective Th1 response (139). Another example is the generation of TNF-α/inducible nitric oxide synthase (iNOS)-producing DCs (Tip-DCs). Tip-DCs were initially identified in the spleen at early time points following *Listeria monocytogenes* infection. These DCs were $CD11c^{int}$ $CD11b^{int}$ mac-3^{high} cells, served as major producers of TNF-α/iNOS and mediated bacterial killing (140). Further studies revealed that Tip-DCs were differentiated from $Ly6C^{high}$ $CCR2^+$ inflammatory monocytes. CCR2 signaling mobilizes inflammatory monocytes out of the BM, as a prerequisite step for the accumulation of Tip DCs at inflammatory sites (141) (Figure 1). Therefore, Tip-DCs likely represent a functionally specialized type of monocyte-derived DCs.

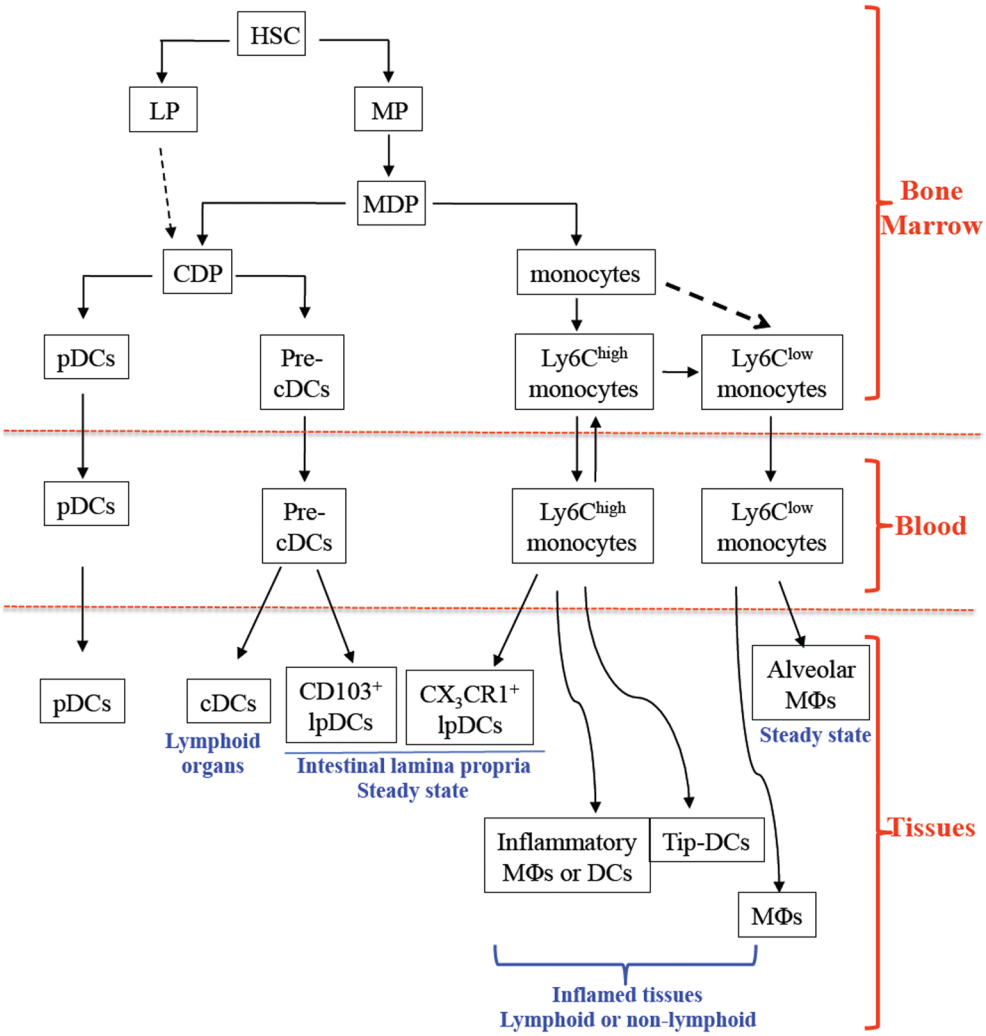

Figure 1. Diagram summarizing the differentiation of DCs and macrophages in mice during homeostasis or in inflammatory settings

HSC: hematopoietic stem cell. LP: lymphoid committed precursor. MP: myeloid committed precursor. MDP: macrophage and DC precursor. CDP: common DC precursor. pDCs: plasmacytoid DCs. Pre-cDCs: pre-classical DCs. cDCs: classical DCs. lpDCs: lamina propria DCs.

In the BM, HSCs produce MP and LP precursors. MDPs give rise to Ly6C^{high} monocytes and CDPs. Ly6C^{high} monocytes in the BM can convert into Ly6C^{low} monocytes, but it remains unclear whether MDPs directly give rise to Ly6C^{low} monocytes (dotted line). CDPs give rise to pre-classical DCs and pDCs. Pre-cDCs circulate in the blood and enter peripheral lymphoid organs, where they give rise to CD8α^{+} and CD8α^{-} cDCs, or enter non-lymphoid tissues, such as intestinal laminar propria, where they may differentiate into CD103^{+} lpDCs. The two monocyte subsets, Ly6C^{high} and Ly6C^{low} monocytes egress out of the BM and enter the peripheral circulation. Under steady state, Ly6C^{low} monocytes might contribute to the tissue macrophage compartment, such as alveolar macrophages, and Ly6C^{high} monocytes give rise to certain DC subsets in non-lymphoid tissues, such as CX3CR1^{+} lpDCs. During

inflammation, Ly6C^{high} monocytes give rise to monocyte-derived inflammatory macrophages and DCs. For instance, TNF-α and iNOS-producing Tip-DCs, a specialized form of monocyte-derived inflammatory DCs, are derived from Ly6C^{high} inflammatory monocytes (142).

1.3.3 Monocyte-derived inflammatory DCs in the lung

In the lung, DCs can be characterized by their respective anatomical locations. Airway mucosal DCs are located within or directly beneath the airway epithelial cells. Alveolar DCs, similar to alveolar macrophages, reside in alveolar space, but only constitute less than 1% of total cells in this location in the steady state. In contrast, interstitial DCs are scattered throughout the lung parenchyma (143).

In the steady state, intraepithelial DCs lining the conducting airways express langerin, α_E (CD103) β_7 integrin and tight junction proteins, such as Claudin-1, Claudin-7 and ZO-2, but do not express CD11b, thus are named CD11b$^-$CD103$^+$ DC (144). Given that lung epithelial cells express E-cadherin, the ligand for α_E (CD103) β_7 integrin, as well as tight junction proteins, it has been proposed that CD11b$^-$CD103$^+$ DCs interact with epithelial cells through protein-protein interactions. These interactions seem to allow DC cell bodies or dendrites to squeeze between the epithelial layer and sample inhaled antigens in the airway lumen (144, 145). The lamina propria immediately below the airway lining and the deeper lung parenchyma primarily contain both CD11b$^+$CD103$^-$ and CD11b$^-$CD103$^+$ DC subsets (144, 146). Alveolar DCs in humans and rats are highly enriched for the CD103+ subset (147). Plasmacytoid DCs (pDCs) express only intermediate levels of CD11c, but express markers characteristic of granulocytes and B cells, therefore distinguishing themselves from CD11b$^+$ or CD103$^+$ DC subsets (147, 148). However, the precise anatomical location of pDCs is not defined. During an ongoing pulmonary inflammation, inflammatory DCs arising from CD11b$^+$ Ly6C$^+$ monocytes, which can still retain

the monocytic marker Ly6C on their surface, can also accumulate in the lung. (18, 149-153). These cells have been shown to be located in both the trachea and the lung interstitium (152).

Monocyte-derived inflammatory DCs in the lung have been identified in studies aimed at elucidating the chemokine receptors required for lung DC influx. CCR2 signaling is required for lung DC accumulation in response to a variety of stimuli, such as allergen or particulate antigens, influenza virus or *A. fumigatus* (18, 149-151). Given that CCR2 signaling is essential for the extravasation of $Ly6C^+$ inflammatory monocytes out of the BM, these DCs were hypothesized to arise from monocytes, and this has been subsequently confirmed in many settings (18, 151). In recent years, there has been an increasing appreciation for the functions of this DC population. For instance, following influenza virus infection, it has been reported that at early time points (2 d post infection), $CD103^+$ intraepithelial DCs migrated from the lung tissue into the dLNs, which was accompanied by an influx of monocyte-derived $CD11b^+$ DCs into the trachea and the lung interstitium (152). Interestingly, these monocyte-derived DCs seemed to carry antigens to the dLN as well, but were incapable of antigen presentation and thus T cell priming (152). Considering their strong ability to produce proinflammatory mediators, these cells were proposed to orchestrate local inflammatory responses by recruiting/activating other cells or directly mediating microbial killing (152). This notion was supported by evidence that iNOS production by these DCs is important for host defense against influenza virus (151). In contrast to these findings, their role in initiating adaptive immune responses has been described in some other contexts. In an asthma model induced by inhalation of house dust mite allergens, a population of FcRεRI-expressing monocyte-derived DCs in the dLN was found necessary and sufficient to present antigens to T cells in order to initiate Th2 immunity (153). Similarly, following *A. fumigatus* infection, $Ly6C^{high}$ monocyte-derived $CD11b^+$ lung DCs were found to take up

fungus, migrate into the dLN and initiate T cell responses (18). Collectively, these studies suggest that monocyte-derived DCs exhibit considerable plasticity, and therefore variable function depending on the specific context. Given that Tip-DCs are a functionally specialized monocyte-derived DC subtype, it is possible that other functionally distinct subsets exist, and this would be an interesting avenue for further investigation.

1.4 NEUTROPHIL HOMEOSTASIS AND MOBILIZATION

Neutrophils, eosinophils and basophils constitute a category of white blood cells- known as granulocytes, characterized by the presence of granules in their cytoplasm, multi-lobed nuclei, and specific cell surface markers. The names of these three granulocyte types are derived from their distinct histological patterns under Wright's stain.

Neutrophils are the most abundant type of white blood cells in mammals and usually the first cell type that is recruited to sites of infection or inflammation. The rise in the number of neutrophils under these conditions is mediated by increased neutrophil generation, mobilization and survival (154).

1.4.1 Neutrophil development

In the steady state, neutrophils are generated in the BM by a process called granulopoiesis, where it is estimated that around 10^{11} neutrophils are produced on daily basis. In the BM, HSCs give rise to LPs or MPs (Figure 1), and the latter further differentiate into multiple myeloid lineages including neutrophils through intermediate progenitors (154).

G-CSF is the principle cytokine for neutrophil development in the steady state, and both G-CSF KO and G-CSFR KO mice have compromised basal levels of granulopoiesis (155, 156). Further studies confirmed that G-CSF signaling acts on several stages of granulopoiesis, including stimulating multipotential progenitors cells in the myeloid lineage, inducing the proliferation of granulocytic precursors, and reducing the transit time through the granulocytic compartment (157, 158). G-CSF therefore has been widely used therapeutically for the treatment of neutropenia, which can occur in a large number of clinical settings such as in individuals undergoing chemotherapy for cancer (159). Despite the essential effects of G-CSF on granulopoiesis under normal conditions, its role in regulating granulopoiesis during an "emergency" situation is controversial. For instance, as compared to WT mice, G-CSF KO mice mounted a normal neutrophilic inflammation in response to *Candida albicans* (160) but developed a strikingly diminished neutrophilia following *Listeria monocytogenes* infection (161). Therefore, it seems that G-CSF regulates stress-induced granulopoiesis in a context-dependent fashion.

Apart from G-CSF, other cytokines, such as GM-CSF (162), IL-3 (163, 164) and IL-6 (165) stimulate granulopoiesis in vivo and might contribute to stress-induced granulopoiesis, although mice deficient in any of these cytokines fail to show any defects in basal granulopoiesis in the steady state (166-168).

1.4.2 Neutrophil egress from the bone marrow

Neutrophils are consistently generated in the BM, but in the steady state, only 1-2% of neutrophils are released from the BM into the circulation in order to maintain homeostasis. Upon

infection or inflammation however, neutrophils can be rapidly mobilized into the periphery to fulfill the emergency requirement (154).

Neutrophils are retained within the BM through the retention signals, of which SDF-1-CXCR4 interaction appears to be the most significant. SDF-1 (CXLC12), a CXC chemoattractant for neutrophils, is constitutively produced by the BM stromal cells. CXCR4, the major receptor for SDF-1, is broadly expressed on hematopoietic cells, including neutrophils. CXCR4 deficiency results in the premature release of granulocytic precursors and hence an overabundance of granulocytes in the periphery (169). In myelokathexis syndrome (WHIM), abnormally enhanced SDF-1-CXCR4 signaling due to the mutations on CXCR4 leads to neutrophil retention and peripheral neutropenia (170). Another molecule, CD18 (one subunit of β2 integrin) has been implicated in BM neutrophil retention as well, as supported by evidence that CD18 blockade increased the release of neutrophils in response to MIP-2 (171).

G-CSF can also be involved in regulating neutrophil egress from the BM. G-CSF disrupts the retention SDF-1/CXCR4 signaling by regulating both the ligand and the receptor (172-174). G-CSF also promotes MMP-9, neutrophil elastase and cathepsin G in the BM, raising the possibility that proteases might degrade some key molecules aiding neutrophil release (175). But surprisingly, neutrophil egress is normal in mice deficient in MMP9 KO, or deficient in both elastase and cathepsin G (176), suggesting that these proteases are not absolutely required individually, but rather that there is redundancy in the system.

1.4.3 Neutrophil emigration from the blood to inflamed tissues

Transendothelial migration of leukocytes from the blood to sites of inflammation include several key steps, which are slow rolling, adhesion strengthening, intraluminal crawling, paracellular or

transcellular migration, and migration through the basement membrane. Integrins and selectins are the major adhesion molecules regulating neutrophil emigration (177).

Rolling is reversible, since it is mediated by the relatively weak interaction between selectins (L-, P- or E-selectin) and their carbohydrate ligands. This process enables neutrophils to adhere to inflamed endothelium under conditions of increased blood flow, and also senses chemokines that are immobilized on the endothelial cell membrane. The chemokine signals are essential to convert the low-affinity, selectin-mediated interaction into the high-affinity, integrin-mediated firm adhesion (177). After firm adhesion, neutrophils intraluminally crawl over the endothelial cell surface to the emigration site, which has been shown to rely on Mac-1 (178). Transendothelial migration is primarily mediated in a paracellular manner at the intercellular junctions, and recent studies have shown that it also can be achieved by transcellular migration through the endothelial cells themselves (179).

1.4.4 Neutrophil clearance

In the absence of inflammation, circulating neutrophils are quickly removed with a half-life of 6-8 hours, primarily in the liver, spleen or BM (180). The SDF-1/CXCR4 axis might be involved in this process, since senescent neutrophils upregulate surface CXCR4, which drives their homing to the BM and clearance from the periphery (181). In the steady state, the normal turnover of neutrophils is accomplished through constitutive apoptosis, followed by clearance by macrophages (182). Neutrophil apoptosis is also critical for the resolution of inflammation, but host-derived factors (such as GM-CSF and G-CSF) and microbial products can delay neutrophil apoptosis (183).

1.4.5 Chemokines and neutrophils

The most crucial neutrophil chemoattractants are CXC chemokines including IL-8 (CXCL8) (present in humans but not in mice), MIP-2 (CXCL2) and KC (CXCL1) (present in both humans and mice). Both MIP-2 and KC signal through the receptor CXCR2 (184). These chemokines selectively attract neutrophils and activate them after they arrive at the site of an inflammatory response.

CXC chemokines act on neutrophil release from the BM and at multiple steps of neutrophil migration. CXC chemokines such as KC can desensitize the responsiveness of neutrophils to SDF-1, therefore disrupting the BM SDF-1/CXCR4 retention signaling and thus facilitating neutrophil egress (185). MIP-2 or KC was capable of inducing a dose and time-dependent increase in neutrophil rolling on endothelial cells, which was inhibited by anti-P-selectin antibody, suggesting that these chemokines induce a P-selectin-dependent rolling (186). In vivo studies further confirmed that CXCR2, the receptor for KC and MIP-2, was required for neutrophil tight adhesion to inflamed venules (187). Human chemokine IL-8 has been shown to convert neutrophil rolling into firm adhesion (188). IL-8 can also induce MMP-9, which has been implicated in the degradation of extracellular matrix and facilitation of neutrophil transmigration through the endothelial cells to inflammatory sites (189). In local tissues, IL-8 potentiates the oxidative burst induced by various stimuli, such as fMLP and P-selectin, thus enhancing the killing activity of neutrophils (190).

1.5 EOSINOPHIL HOMEOSTASIS AND MOBILIZATION

Eosinophils, similarly to neutrophils, are a sub-population of granulocytes that develop in the BM and traffic into blood and tissues in response to environment cues. In healthy individuals, circulating eosinophils only account for 1-3% of blood leukocytes. Under homeostatic conditions, eosinophils only reside in the epithelial lining of the gastrointestinal tract, thymus, uterus, spleen and lymph nodes. It is only during inflammatory processes that eosinophils are recruited into other tissues, such as the lungs and skin. Tissue eosinophils have been implicated in the pathogenesis of numerous inflammatory diseases, including adverse reactions to helminth infection and in allergic disorders (191).

1.5.1 Eosinophil development

Eosinophils are produced in the BM from HSC through multiple intermediate precursor stages. Eosinophil lineage differentiation is dictated by at least three families of transcription factors, GATA-1, PU.1, and C/EBP members. Of them, GATA-1 is the most significant, since deletion of the high-affinity GATA-binding site in the GATA-1 promoter, an element presumed to mediate positive autoregulation of GATA-1, resulted in the selective depletion of the eosinophil lineage (192).

Three cytokines, IL-3, IL-5 and GM-CSF are particularly important in directing eosinophil lineage when tested in vitro (193-195). But both IL-3 and GM-CSF can act on other hematopoietic lineages, but IL-5 is the most selective for eosinophil differentiation (196). The central role of IL-5 in eosinophilia is best demonstrated by in vivo studies. Overexpression of IL-5 in transgenic mice was shown to be sufficient for the accumulation of eosinophils but not other

cell types in multiple tissues (197, 198), while depletion of IL-5 resulted in the loss of eosinophilia following allergen challenges or helminth infections (199, 200). In contrast, exogenously administered IL-3 and GM-CSF are far less efficient in eliciting an eosinophilic response (162, 163). These observations suggest that IL-3 and GM-CSF have relatively low effects on eosinophils in vivo, compared to the apparent selective importance of IL-5.

1.5.2 Eosinophil trafficking

In the steady state, basal eosinophilia seems to rely on eotaxin-1 signaling. Eotaxin (or eotaxin-1), the eosinophil-selective chemoattractant, was initially discovered in the bronchoalveolar lavage fluid (BALF) of the guinea pigs following allergen challenges (201) and was subsequently identified in mice (202) and human (203). Two other family members, eotaxin-2 and eotaxin-3 with eosinophil-selective attractant activity have also been identified, although they only share ~30% sequence similarity with eotaxin-1 (204-206). The receptor for these three chemokines is CCR3, a seven-transmembrane G protein-coupled receptor, which is primarily expressed by eosinophils (207, 208). Under normal conditions, eosinophil homing into the gastrointestinal tract occurs early in perinatal development and is independent of bacterial flora (209). This recruitment is regulated by the constitutive expression of eotaxin-1, as supported by evidence that deficiency of eotaxin-1 (209) or its receptor CCR3 (210) but not eotaxin-2 (211) resulted in a marked decrease of this population in the gut. Eosinophil trafficking into other anatomical sites in the steady state is also under the control of eotaxin-1 (212, 213).

In response to inflammatory stimuli, eosinophils are recruited into target sites via a process that involves the action of cytokines (in particular Th2 cytokines IL-4, IL-5 and IL-13), adhesion molecules, chemokines (in particular Rantes and eotaxins) and other molecules. Of the

cytokines and chemokines implicated, only IL-5 and eotaxin are selective for eosinophil trafficking (214).

Given that very few eosinophils are in the circulation under normal conditions, the mobilization of eosinophils from the BM into the blood is the prerequisite for their tissue recruitment upon stimulation, a process that has been shown to rely on IL-5 but not eotaxin (215-217). Following eosinophil egress, tissue recruitment involves several steps, including tethering and rolling along the endothelium mediated by selectins, firm attachment to the endothelium that is triggered by chemoattractant signals and mediated by integrins, and finally transendothelial migration guided by chemoattractant gradients (218). The integrins that are highly expressed by eosinophils and involved in eosinophil trafficking include $\alpha_4\beta_7$ integrin, the CD18 family of molecules, and VLA-4, each of which has variable functions in various inflammatory processes (218).

Notably, the most pronounced eosinophil influx seems to be observed in the presence of both eotaxin and IL-5 but not when eotaxin is present alone. There are several possible explanations for this observation. One possibility is that IL-5 mobilizes eosinophil from the BM pool while eotaxin sequentially recruits eosinophils locally (215, 219). However, it has also been suggested that IL-5 acts cooperatively with eotaxin to promote eosinophil migration into tissues (219). Or alternatively IL-5 primes eosinophils to respond to CCR3 ligands (220). The Th2 cytokines IL-4 and IL-13 upregulate the levels of eotaxin (221-223) and adhesion molecules (224), promoting eosinophil trafficking, while the Th1 cytokine IFN-γ inhibits eotaxin production (225).

1.5.3 Eosinophil priming, survival and apoptosis

Eosinophils isolated from patients with allergic asthma demonstrate an enhanced responsiveness to multiple stimuli, which is generally referred to as eosinophil priming, compared with those derived from healthy controls (191). The priming reaction enhances the function of these cells, including chemotaxis, cytotoxicity, respiratory burst, and the release of toxic cationic proteins and lipid mediators. Eosinophils are primed partially in the circulation but are maximally activated in the tissues (191, 226). Cytokines including platelet-activating factor, GM-CSF, IL-3, IL-5 have been implicated in eosinophil priming (191). For instance, IL-5 was found to enhance eosinophil degranulation and to stimulate the release of eosinophil-derived neurotoxin (227).

Based on in vitro observations, eosinophils were estimated to survive for at least two weeks in tissues. Tissue eosinophil survival can be prolonged by the local cytokine inflammatory milieu, such as IL-3, IL-13, GM-CSF and in particular IL-5 (228). IL-5 has been demonstrated to inhibit Bax translocation to the mitochondria and cytochrome c release, thus preventing cell death (229). The enhanced eosinophil survival contributes to eosinophil-mediated immunoprotection as well as immunopathology under particular circumstances. In contrast, the clearance of eosinophils is critical for maintaining homeostasis and the resolution of inflammation. This process is predominantly mediated through cell apoptosis (230), followed by the phagocytosis by macrophages, epithelial cells, and possibly other cell types (230, 231).

2.0 THE DEVELOPMENT OF AN *ASPERGILLUS*-INDUCED ALLERGY MODEL

2.1 ABSTRACT

A. fumigatus, a ubiquitous environmental fungus, is associated with various allergic pulmonary disorders, of which ABPA represents one of the most extreme manifestations of fungal allergy. ABPA is characterized by a mixed infiltration of eosinophils and neutrophils and both cell types contribute to lung pathology. Existing animal models are characterized by prominent eosinophilia but fail to show appreciable neutrophilia, thus making it impossible to study the neutrophil infiltration that has been observed in human ABPA. In our efforts to develop an animal model, mice were repeatedly exposed to fungal conidia, mimicking a more natural course of disease pathogenesis. This protocol reproduced some critical features of pulmonary allergy, including an evident Th2 response and elevated Th2 cytokines such as IL-13, pulmonary eosinophilia, increased fungus-specific IgG1 and total IgE in the serum as well as upregulated mucus gene expression. More importantly, in addition to pulmonary eosinophilia, this exposure protocol did induce neutrophil infiltration in mice. Interestingly, the two mouse strains studied displayed differential intensities of neutrophilia or eosinophilia, with BALB/c and C57BL/6 strains exhibiting neutrophil-biased or eosinophil-biased inflammatory responses, respectively. The neutrophil-rich response in the BABL/c strain, to some extent, resembled the inflammation pattern observed in human ABPA. Therefore, our animal model with mouse strain-specific

granulocyte infiltration provided us with a useful tool for studying neutrophil and/or eosinophil influx into the lungs under conditions mimicking human ABPA.

2.2 INTRODUCTION

A. fumigatus a ubiquitous fungus that is associated with a spectrum of respiratory allergic diseases. Humans are constantly exposed to airborne *A. fumigatus* spores and it is estimated that individuals inhale many hundreds of conidia every day, a number that could be much higher depending on geographic location and the season of the year (1). Despite this consistent exposure, in the vast majority of individuals there is no detectable ongoing infection or persistent inflammation in the respiratory system due to low-pathogenic potential of this fungus and to effective host immune mechanisms. Therefore *A. fumigatus* is a minor concern to healthy individuals. However in individuals with underlying obstructive lung diseases, such as severe asthma or CF, the pathogen can trigger an aggressive allergic airway response, which potentially progress into ABPA, representing a significant health concern (9-11). However, the mechanisms underlying susceptibility to ABPA are not well defined, but many factors are involved, including the genetic disposition (31, 103), mucus quality, and abnormal host-pathogen interaction (as discussed in section 1.2.2). It seems that susceptible individuals with preexisting lung diseases are compromised in terms of pathogen clearance, thus allowing fungal colonization and antigen persistence. Continuous antigen stimulation might sustain a persistent local inflammatory response, contributing to ABPA pathogenesis (93, 94).

To recapitulate the characteristics of human ABPA in an experimental model, multiple low-dose inoculations of conidia were i.t given to mice, mimicking the continuous release of

fungal antigens in susceptible individuals who inefficiently clear the fungus. With this long-term exposure protocol, we did induce an inflammatory reaction with critical allergic features that were shared by two mouse strains we investigated: the BALB/c and the C57BL/6 strain. Interestingly, despite a granulocyte infiltration consisting of both neutrophils and eosinophils, the BALB/c strain developed a neutrophil-dominated response while the C57BL/6 strain developed an eosinophil-dominated inflammation. The inflammatory pattern in BALB/c mice was reminiscent of the neutrophil-rich inflammation that occurs in ABPA patients (115, 116). Therefore, this animal model provided us with an opportunity to explore eosinophil and/or neutrophil influx in *A. fumigatus*-induced allergy.

2.3 MATERIAL AND METHODS

2.3.1 Mice

BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory. All the mice were housed under pathogen-free conditions and used between 6 and 8 weeks of age. Within experiments, the mice were age and sex matched. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh.

2.3.2 *A. fumigatus* growth and culture

A. fumigatus isolate 13073 (American Type Culture Collection)) was grown on Difco^{TM} potato dextrose agar (BD) for 5 d at 37 ^{O}C. Conidia were harvested by washing the culture flask with 40

ml of sterile PBS supplemented with 0.1% Tween 20. The conidia were then filtered through a sterile 40 μm cell strainer (BD Falcon) to remove hyphal fragments and debris. The conidia harvested in this way were used as RC. To obtain SC, RC were incubated at 37 ^{O}C at a concentration of 1 × 10^7 conidia/ml RPMI 1640 (Gibco) for 6 h. The number of conidia was counted using a hemocytometer.

2.3.3 Animal models

Mice were sensitized i.t. with 1×10^7 *A. fumigatus* conidia in 50 μl PBS on day 0. Unless otherwise indicated, from day 7, mice were i.t. challenged with 1×10^6 conidia in 50 μl PBS daily for 5 days, followed by a 2-day rest period. Mice were then i.t. challenged again with the same dose for another 3 days, which resulted in a total of 8 challenges. 24 h after the final challenge, the left lobe of the lung was tied off and the right lobes were lavaged with 0.7 ml of sterile PBS. BALF was collected, and total as well as differential cell counts were assessed as previously described(232). The left lobe of the lung was homogenized in cytokine buffer for the measurement of different proteins or in Trizol (Invitrogen) for RNA isolation as previous described(233). Cytokines and chemokines in the BALF or the in lung homogenates were detected by ELISA (BioLegend or R&D) or by Milliplex Immunoassay (Millipore). The right lobes of the lung were prepared for hematoxylin/eosin staining as previously described (233). Histological examinations of lung tissues or cellular differential counts in BALF were carried out in a blinded fashion. Control mice were given PBS only (PBS controls).

For the *Aspergillus* antigen extract model, mice were given 6.0 μg of extract i.t. in 50 μ l saline on day 1, 3, 5, 7, 8, 9 and 10. 24 h after the final challenge, inflammation was assessed as described above for the conidia model. *Aspergillus* extract was purchased from GREER

laboratories, and endotoxin in the preparation was removed by passage over EndoTrap red columns (Cambrex). The final endotoxin level in 100 μg of extract ranged from 0.125 endotoxin units (EU) to 0.625 EU. Therefore, the endotoxin level in 6.0 μg of extract that was given to each mouse per day was less than 0.0375 EU.

2.3.4 Myeloperoxidase (MPO) activity assay

MPO activity in cell-free BALF was measured as previously described (234). 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 H_2O_2 (0.05% = 0.4 mM). After 15 min of incubation with shaking at 37^{o}C, the reaction was stopped by adding 100 μl of NaN_3 (1% wt/vol) and results were read at 450 nm. The activity was expressed as units/ml according to comparison with known standards (Sigma).

2.3.5 Fungal burden determination

The left lobe of the lung was homogenized in 1 ml PBS. Lung homogenates were then serially diluted (1:2 and 1:5) and 100 μl of undiluted or diluted samples were plating on potato dextrose agar plates. After overnight culture, colonies were counted and the burden was expressed as colony forming units / lung (CFU/lung).

2.3.6 *A. fumigatus*-specific IgG1 and IgG2a and total serum IgE

Protein was extracted from *A. fumigatus* conidia by using Y-PER Plus dialyzable yeast protein extraction reagents (Thermo scientific). ELISA plates were coated with *A. fumigatus* protein overnight at 4^{o}C and blocked at RT. Sera (1:3 dilution) were added to the plate, and incubated for 2 h at RT, followed by the addition of biotin-conjugated rat anti-mouse IgG2a or goat anti-mouse IgG1 antibody (SouthernBiotech). Streptavidin-labeled horseradish peroxidase and substrate (Sigma) were sequentially added to detect biotin-labeled primary antibodies. The plate was read at 450 nm after the reaction was stopped by the addition of stop solution (R&D). Total IgE in the serum was similarly detected by a commercially-available ELISA kit (BioLegend). .

2.3.7 Antibodies and flow cytometry

The following antibodies were purchased from BD Pharmingen: Purified CD16/CD32 (Fc block, clone 93), 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-17-PE (TC11-18H10), anti-TNF-α-PE (MP6-XT22). The following antibodies were purchased from BioLegend: anti-CD45-FITC or APC (30-F11), anti-TCRγδ-Alexa Fluor 647 (UC7-13D5), anti-IL-4-PE (11B11). Single cell suspensions were stained with antibodies and analyzed on a FACSCalibur flow cytometer (BD Immunocytometry Systems), and data were analyzed by using FlowJo software (Tree Star). For T cell intracellular staining, lung cells were cultured with phorbol 12-myristate 13-acetate (PMA) (25 ng/ml) (Sigma) and ionomycin (500 ng/ml) (Sigma) in the presence of GolgiStop^{TM}

(BD Pharmingen) for 4 hours, labeled with a combination of anti-CD3-PerCP and anti-CD4-APC, followed by fixation, permeabilization and staining for intracellular cytokines according to the manufacturer's protocol (Cytofix/Cytoperm, BD Pharmingen).

2.3.8 Enzyme-linked immunosorbent spot (ELISPOT)

$CD4^+$ T cells from the dLNs were purified by magnetic bead separation using a $CD4^+$ T cell isolation kit following the manufacturer's instructions. IFN-γ, IL-17, IL-5 and IL-13-producing $CD4^+$ T cells were assayed with ELISPOT kits (eBioscience) as previously described (235).

2.3.9 Quantitative reverse-transcription PCR (qRT-PCR)

Tissues or cells were treated with Trizol (Invitrogen) and stored at -80^{o}C prior to RNA extraction. RNA was isolated using an RNeasy kit (Qiagen) and treated with RNase-free DNase (Qiagen). cDNA was synthesized and used for quantitative PCR using Taqman Gene Expression Assays (Applied Biosystems) according to manufacturer's instructions. The level of mRNA was normalized to GUS expression and the results were analyzed by the $2^{-\Delta\Delta Ct}$ method (236).

2.3.10 Statistical analyses

Student's unpaired two-tailed t-tests were used for comparisons between two groups. One-way analysis of variance (ANOVA) with Tukey posttest and two-way ANOVA with Bonferroni posttest were used for comparisons between multiple groups when appropriate. Differences

between two groups were considered significant when $p<0.05$. n.s. indicates not significant. All statistical analyses were performed with GraphPad Prism software.

2.4 RESULTS

2.4.1 Repeated challenges induce distinct granulocyte pattern in BALB/c and C57BL/6 mice

In our study, mice were sensitized with 10×10^6 conidia to induce an adaptive immune response. After 7 days of rest, mice were repeatedly challenged with 1×10^6 conidia for 8 times to mimic the persistent local antigenic stimulation in individuals incapable of adequate fungal clearance. Surprisingly, the same exposure protocol induced markedly different outcomes in two mouse strains. Overall, BALB/c mice demonstrated substantially more severe airway inflammation when examined 24 h after the final challenge, which was characterized by a higher total cell count in the BALF compared to C57BL/6 mice (Figure 2A). Despite the lower level of inflammatory cell infiltration, C57BL/6 mice did not demonstrate a proportionally lower number of cells in every compartment. While the BALB/c mice showed a prominent neutrophil-biased response, the C57BL/6 strain exhibited a high-eosinophilia but a low-neutrophilia pattern (Figure 2A). The preferential accumulation of neutrophils over eosinophils in the BALB/c strain was not only observed in the BALF (Figure 2A) but also in the lung tissue (Figure 2B). 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 MPO is toxic to tissues (237). We found significantly higher MPO levels in the cell-free BALF of BALB/c mice (Figure

2C), which likely contributed to the lung damage. Notably, this marked strain-associated difference was only revealed following repeated challenges, with similar neutrophil influx being observed in the both strains soon after sensitization with 10×10^6 conidia (Figure 2D), raising the possibility that strain-specific regulatory mechanisms come into play during persistent antigenic stimulation.

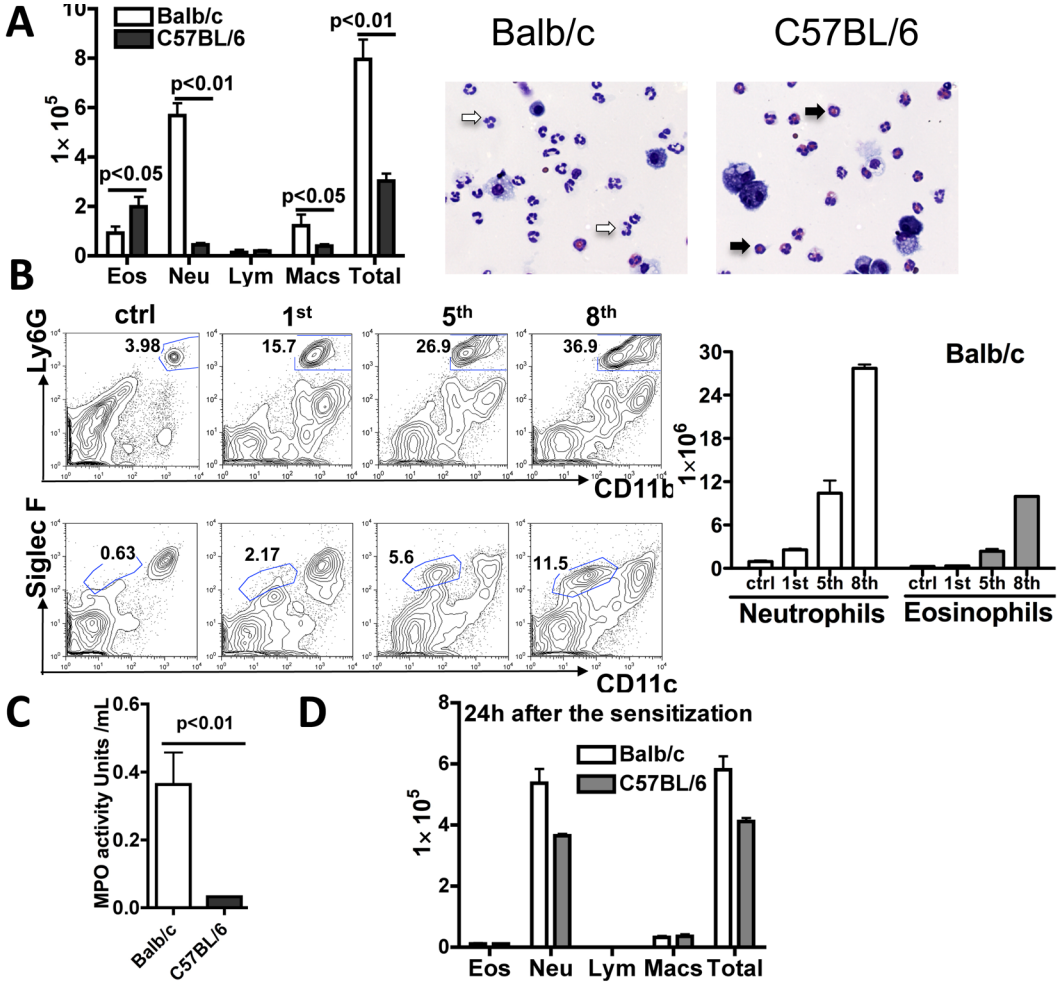

Figure 2. Characterization of pulmonary inflammation induced by repeated challenges

(A) Mice were sensitized with 10×10^6 conidia on day 0. From day 7, mice were challenged with 1×10^6 conidia daily for 8 days. Total as well as differential cell counts were examined 24 hours after the final challenge. Representative images of cytospins are shown, and the white and black arrows indicate typical neutrophils and eosinophils respectively. (B) Neutrophils or eosinophils in the lung tissue from BALB/c mice were examined at different time points by flow cytometry. FACS plots show the percentage of $CD45^+CD11b^{high}$ $Ly6G^{high}$ neutrophils and $CD45^+$ $CD11c^{-/low}$ $Siglec\text{-}F^+$ eosinophils within the lung $CD45^+$ leukocytes in the PBS controls or challenged BALB/c mice

24 h after the 1st, 5th or 8th challenges. Plots show live CD45+ leukocytes and representative plots for three mice per group are shown. The absolute numbers of neutrophils and eosinophils in the lung per mouse were calculated accordingly. (C) Mice were immunized as described in (A) and MPO activity in the cell-free BALF was examined 24 hours after the 8th challenge. (D) Mice were sensitized with 10×10^6 conidia and 24 hours later, total and differential cell counts were examined by cytospins. Eos: eosinophils, Neu: neutrophils, Lym: lymphocytes, Macs: macrophages. Results are expressed as mean± SD (n≥4). Data are representative of three independent experiments.

2.4.2 Repeated challenges induce distinct lung pathology in BALB/c and C57BL/6 mice

Histological examination of lungs from challenged BALB/c and C57BL/6 mice revealed significant differences in both distribution and severity of lesions (Figure 3). While 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 and obliterating extensive portions of the alveolar spaces. BALB/c bronchioles contained degenerate neutrophils admixed with sloughed epithelial cells, fibrin and cellular debris. Bronchiolar epithelium was hyperplastic, exhibiting rare epithelial necrosis and sqamous metaplasia. BALB/c alveolar spaces were entirely filled with dense sheets of foamy macrophages interspersed with large numbers of multinucleated giant cells and intralveolar microhemorrhages. Scattered between the macrophages were clusters of degenerate neutrophils, fibrin and cellular debris surrounded by aggregates of lymphocytes and plasma cells. Lungs from BALB/c mice had prominent perivascular infiltrates containing dense sheets of neutrophils admixed with smaller numbers of eosinophils. In contrast, lungs from challenged C57BL/6 mice showed modest multifocal intralveolar aggregates of macrophages and occasional multinucleated giant cells intermixed with small clusters of degenerate eosinophils and neutrophils. Perivascular and peribronchiolar cuffing in C57BL/6 mice was minor and contained primarily eosinophils, neutrophils and lymphocytes.

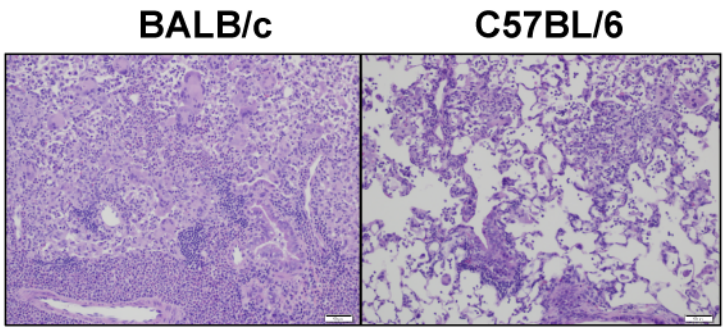

Figure 3. Lung pathology following repeated challenges.

Representative hematoxylin/eosin staining of lung sections from BALB/c or C57BL/6 mice 24h after the 8th challenge. Mice were sensitized and challenged as described in Figure 2A legend. Magnification X200 and size bars = 50 µm.

2.4.3 Repeated challenges induce critical features of pulmonary allergy in both mouse strains

Repeated challenges reproduced some critical features of allergic disorders (238) regardless of genetic background, including upregulated Muc5ac that encodes the major airway mucin, induced IL-13 in the lung, elevated total serum IgE (Figure 4) and eosinophil influx into the airways and lungs (Figures 2A and 2B). Eosinophil influx was barely seen after sensitization (Figure 2D) or after one challenge (Figure 2B), but became evident with multiple challenges (Figures 2A and 2B), suggesting that persistent antigenic stimulation is required for the demonstration of allergy-related pathology in this model.

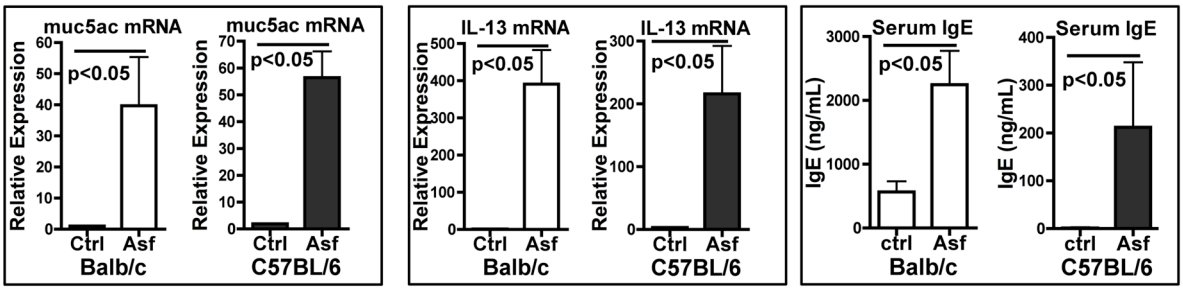

Figure 4. Characterization of allergic features induced by repeated challenges

Mice were sensitized with 10×10^6 conidia on day 0. From day 7, mice were challenged with 1×10^6 conidia for 8 days and examined 24 hours after the final challenge. The expression of Muc5ac or IL-13 in the lung was examined by qRT-PCR and expressed as fold induction compared to their corresponding PBS controls. Total serum IgE was assayed by ELISA. Results are expressed as mean± SD ($n\geq4$). Experiments were repeated at least twice.

2.4.4 Repeated challenges induce mixed Th1/Th2/Th17 responses

Consistent with previous studies, sensitization with 10×10^6 conidia primarily primed Th1 cells in the lung dLNs (25, 45, 83), while repeated challenges preferentially expanded Th2/Th17 cells (Figure 5A) regardless of genetic background. Similarly, at inflammatory sites, effector T cells accumulated during challenges and the end result was a mixed Th1/Th2/Th17 pattern (Figure 5B and 5C). As mentioned, eosinophil influx, a Th2-associated pathology, became pronounced only after multiple challenges (Figures 2A, 2B and 2D). Neutrophil infiltration, potentially associated with Th17 responses, also progressively increased with multiple challenges (Figure 2B). Therefore, persistent stimulation by fungal antigens not only boosted the response, but also altered the nature of immunity by reducing the Th1 predominance and promoting Th2 and Th17 responses and their associated pathology such as eosinophilia and neutrophilia respectively. Of note, despite impressive increases in Th2 cytokine gene expression and IgE levels in the BALB/c mice after 8 challenges, this strain displayed a neutrophil-biased response in the lungs and airways, suggesting that a neutrophil-dominated host response was possible even in an allergic setting.

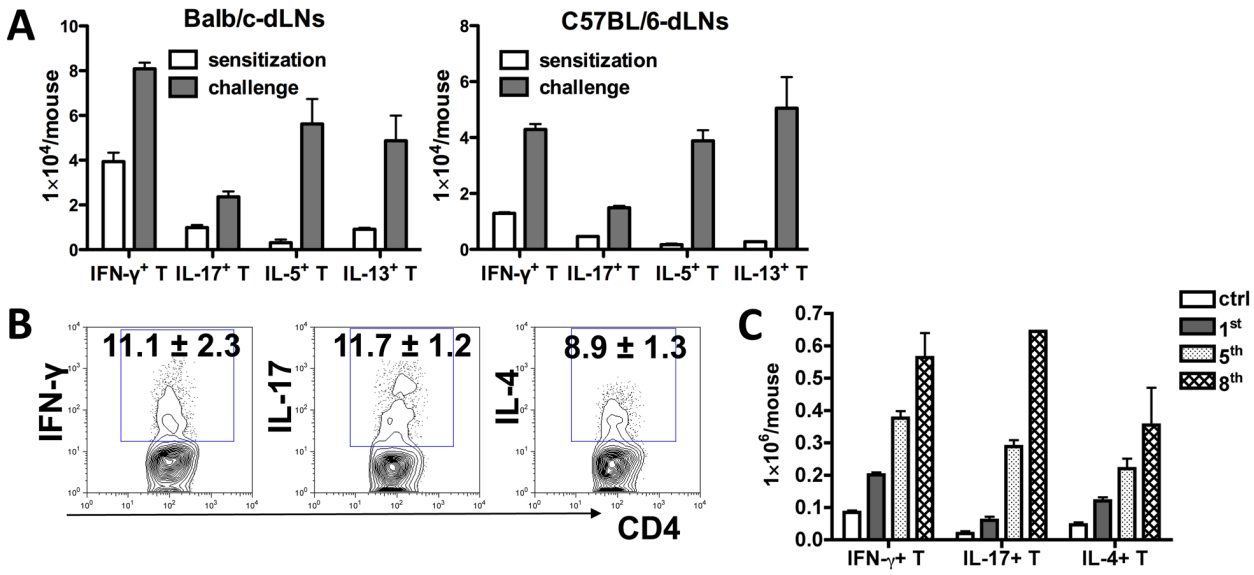

Figure 5. T helper cell differentiation during repeated challenges

(A) BALB/c and C57BL/6 mice were sensitized with 10×10^6 conidia, with or without subsequent challenges. IFN-γ, IL-17, IL-5 and IL-13-producing $CD4^+$ T cells in the lung dLNs were examined 7 days after sensitization (sensitization group) or 24 hours after the 5th challenge (challenge group) by ELISPOT. (B) Representative FACS plots show the intracellular staining for IFN-γ, IL-17 or IL-4 in the $CD3^+CD4^+$ T cells from challenged BALB/c mice at 24 hours post the 8th challenge. Plots show live $CD3^+CD4^+$ leukocytes and representative plots for three mice per group are shown. The numbers indicate the percentages of cytokine-producing $CD4^+$ T cells within the total $CD3^+CD4^+$ T cell compartment, expressed as average ± SD. (C) IFN-γ, IL-17 or IL-4-producing $CD3^+CD4^+$ T cells in the lung at different time points were quantified by intracellular cytokine staining and are shown as cytokine-producing cells per mouse. Lung cells were isolated from PBS control or challenged BALB/c mice 24 hours after 1, 5 or 8 challenges. Results are the average of three mice and expressed as average ± SD. Experiments were repeated at least twice.

2.4.5 Repeated challenges induce IgG1, IgG2a and IgE responses

One of the most important functions of $CD4^+$ T cells is to provide help to B cells for antibody responses. Th1 and Th2 cells have selective functions in inducing IgG2a and IgG1 isotype antibodies, respectively. We detected both *A. fumigatus*-specific IgG1 and IgG2a antibodies in the sera of mice that received repeated challenges (Figure 6A). Total serum IgE, also an indicator of Th2 responses, was elevated by repeated fungal exposures as described in Figure 4.

Consistent with the regulation of Th2 cells, mice after repeated challenges showed much higher IgE levels than mice that were only sensitized (Figure 6B). The data on antibody responses also reflect the mixed Th1/Th2 involvement in this model.

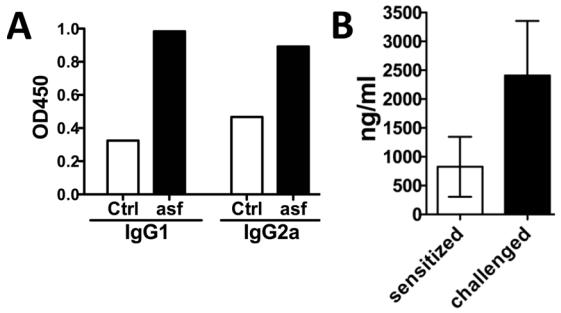

Figure 6. Antibody responses following repeated challenges

(A) BALB/c mice were sensitized and challenged as described in Figure 2A legend and sampled 24 h after the final challenge. *A. fumigatus*-specific IgG1 and IgG2a antibodies in the sera of PBS control mice or challenged mice were detected by ELISA. (B) BALB/c mice were sensitized with or without subsequent challenges. Total serum IgE was detected by ELISA after sensitization (sensitized group) or 24 hours after the 5^{th} challenge (challenged group). Results are the average of 3-4 mice and expressed as average ± SD. Experiments were repeated at least twice.

2.4.6 The C57BL/6 strain has unimpaired fungal clearance

Importantly, accumulated neutrophils in the BALB/c mice during the challenge phase (Figure 2A and 2B) seemed not of any added benefit to the host since the C57BL/6 mice with much less neutrophilia did not exhibit compromised pathogen clearance in the long term (Figure 7) or develop invasive disease (data not shown). Rather, as we described before, excessive numbers of neutrophils and their derivatives such as MPO (Figure 2C) in the BALB/c strain likely caused tissue damage (Figure 3).

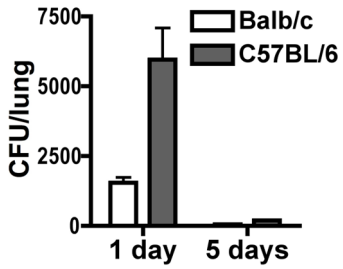

Figure 7. Lung fungal burden following repeated challenges

BALB/c or C57BL/6 mice were sensitized with 10×10^6 conidia, and challenged with 1×10^6 conidia for a total of 8 times. Fungal burden in the lung was determined 1 or 5 days following the 8th challenge. Results are the average of four mice and expressed as average ± SD. Data are representative of at least two independent experiments.

2.4.7 A comparison of inflammation to persistent exposures to *Aspergillus* conidia or to extract

To recapitulate characteristics of *A. fumigatus*-induced allergic disorders in humans, soluble crude antigen extract of this fungus has been most commonly used in mouse models, in which eosinophilia seems to be the most prominent feature that has been observed (92). We therefore compared the inflammation patterns induced by *A. fumigatus* conidia and extract in the BALB/c mice (Figure 8A). In agreement with previous studies (92), *A. fumigatus* extract elicited eosinophil-rich inflammation (Figure 8B). Consistent with this, only Th2 cytokines such as IL-5 and IL-13 were appreciably induced after challenges with extract (Figure 8C). In contrast, as we described above, Th1/Th2/Th17 cytokines were induced following repeated exposures to conidia (Figure 5 and 8C). Interestingly, despite obviously distinct cytokine profiles, lung Muc5ac mRNA levels in the two models did not show significant difference (Figure 8C). Given that muc5ac gene encodes a major component of airway mucus, this observation implicates that mucus hypersecretion in these two models might be comparable.

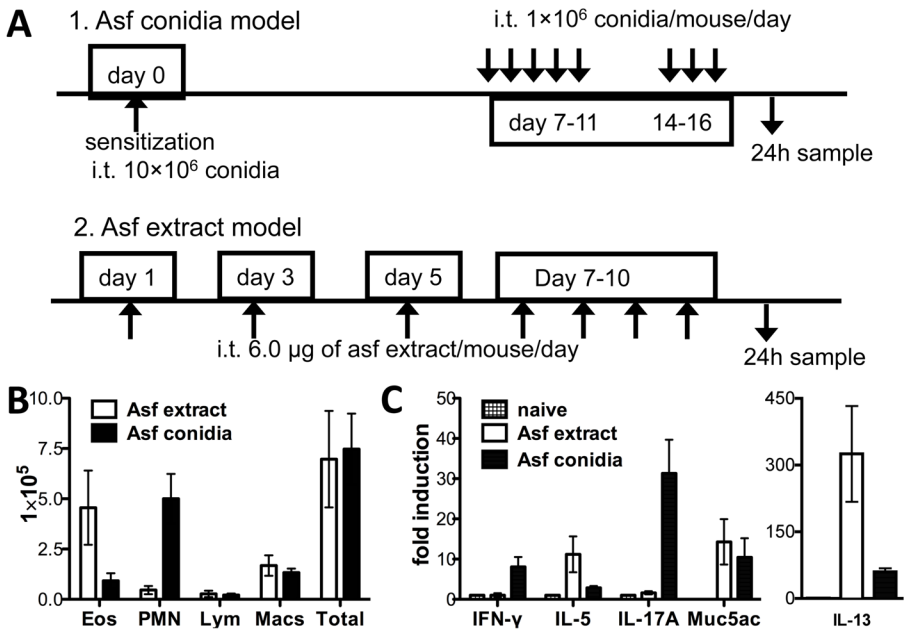

Figure 8. A comparison of inflammation induced by repeated challenges to conidia or to *Aspergillus* extract

(A) Schematic diagram showing the protocols used to induce airway inflammation using *A. fumigatus* conidia (Asf conidia model in upper panel) or extract (Asf extract model in lower panel) in BALB/c mice. In the Asf conidia model, mice were sensitized with 10×10^6 conidia, and challenged with 1×10^6 conidia for 8 times. In the Asf extract model, mice were challenged with 6 μg of *Aspergillus* extract on day 1, 3, 5, 7, 8, 9 and 10. For both models, inflammation was assessed 24 h after the final challenge. (B) BALF total and differential cell counts in the two models (in BALB/c mice). (C) The mRNA levels of different genes were examined by qPCR. PCR results were expressed as fold induction compared to their corresponding controls in naïve mice and expressed as average ± SD (>=4 mice in each group).

2.5 DISCUSSION

A. fumigatus is believed to be the etiologic agent in a number of allergic conditions, of which, ABPA represents one of the most severe disease states. ABPA patients demonstrate eosinophilia, the cardinal feature of allergic diseases as the consequence of Th2 responses (9-11). Concomitant with this Th2-eosinophilia axis, neutrophil infiltration has been observed in the lung and sputum of ABPA patients and was found to contribute to lung injury (115, 116). This neutrophil influx is

presumably induced by neutrophil chemoattractants, such as IL-8, but this has not been formally proven (116). Therefore, there exists an interest in elucidating the mechanisms for neutrophil influx in this fungal allergy.

Animal models are of great value to understand disease pathogenesis. In established murine models of allergic aspergillosis, soluble crude *A. fumigatus* antigen extract has been mostly routinely used. These antigens were derived from culture filtrate and mycelial extract both of which are free of living organisms (92). Different protocols have been employed across various laboratories but they collectively and consistently generate typical allergic responses. For example, when mice were given i.n. extract 5 times at 4-d intervals, they demonstrated allergic inflammation characterized by a massive infiltration of eosinophils and IL-4^+ cells into the lungs, AHR, and elevated serum IgE levels. These responses were $CD4^+$ T cell-dependent, since RAG-1 or RAG-2-deficient mice failed to demonstrate lung pathology while reconstitution of those mice with $CD4^+$ T cells restored the allergic inflammation (96). In contrast, B cells and antibodies were not required because B cell-deficient mice showed no reduction in inflammation (96). IL-4 and IL-5 were found to mediate AHR and eosinophilia, respectively (96). The critical point to note however is that neutrophilia is not present in these types of relatively acute models.

In contrast, Cory M. Hogaboam and colleagues have developed a chronic model of *A. fumigatus*-induced allergy. Basically, mice were systemically sensitized with an intraperitoneal (i.p.) and a subcutaneous (s.c.) injection of extract dissolved in incomplete Freund's adjuvant (IFA). To localize the inflammation in the lung, mice then received three weekly challenges i.n. with extract. One week later, mice were inoculated i.t. with 5.0×10^6 conidia. This protocol induced a chronic allergic inflammation, with persistent AHR, airway eosinophilia and evident Th2 responses even at 30 days after conidia deposition. Goblet cell hyperplasia and airway

fibrosis were also observed during the chronic phase. Notably, neutrophils represented an important cell type in the BALF and in the lung (51). Depletion of these neutrophils did not compromise fungal clearance; rather it alleviated the disease by reducing AHR and lung fibrosis, indicating the detrimental role for neutrophils. The mechanisms underlying neutrophil-mediated disease worsening were associated with neutrophil-derived MMP-9, which substantially contributed to AHR (118). However, the mechanisms for driving this neutrophilia have not been explored. Importantly, consideration must be given to the fact that i.p or s.c. sensitization in the presence of adjuvant is not the route of natural exposure in humans. It is becoming widely appreciated that adjuvant and immunization routes have a significant impact on the quality of immune responses, thereby raising a significant concern that this protocol may result in some artificial effects, which may not faithfully reflect the nature of fungus-induced allergy in humans.

The use of *A. fumigatus* live conidia alone has been reported, but this method is not as common as the use of fungal extract. In one study, BALB/c mice were exposed i.n. either to 50,000 spores or extract daily for 15 days, followed by 10-days of rest and then a second phase of identical challenges for an additional 5 days. Serum total IgE, eosinophil counts and eosinophil peroxidase were elevated in response to either conidia or extract at 24 h after the last exposure (239). This study clearly indicates that long-term exposure to conidia is capable of inducing allergic features. However once again, neutrophilia was not reported in this study.

Based on the analysis of the current literature as discussed above, it seems that there is a need to develop an experimental model that can reproduce the mixed neutrophil/eosinophil infiltration in the settings of fungal allergy such as human ABPA. Our exposure protocol evoked cardinal features of allergic responses, including an appreciable Th2 response, pulmonary eosinophilia, mucus hyperproduction and elevated levels of serum total IgE, fungus-specific

IgG1 and Th2 cytokines in the two mouse strains that we examined. AHR as an indicator of lung function has not been tested in our model. However, at least theoretically it is very likely that AHR can be induced, since we observed the upregulation of IL-13 and IL-17, two cytokines known to drive AHR (238, 240). More importantly, we found pulmonary neutrophilia in both BALB/c and C57BL/6 mouse strains, although this occurred to differing degrees in each. The prominent neutrophilic inflammation in the BALB/c strain, to some extent, is reminiscent of the inflammation pattern in ABPA and severe asthma. Neutrophils have been increasingly appreciated as being important in human ABPA, and this model presents an excellent opportunity to study them experimentally.

Repeated i.t. challenges with conidia resulted in a coexistence of Th1/Th2/Th17 cells, and similar results have been reported in a recent study (86). In our hands, in agreement with reports by others (96), *Aspergillus* extract elicited a potent Th2 response accompanied by very poor Th17 or Th1 responses. It is not clear why fungal extract and live conidia induced such distinct inflammation patterns upon repeated challenge. Given that fungal antigens may contribute to the differentiation of specific T cell lineages (as discussed in section 1.1.5), this difference in T cell development might be related to unique antigens that conidia and extract each present to the host. When live conidia are present in the lung, there is an ongoing pathogen-host interaction, which not only modifies the responses of host, but also in turn necessitates adaptation on the part of the fungus in order to evade the host defense system. For example, conidia can actively participate in host-pathogen interaction by secreting soluble mediators, and changing their genotype or phenotypes, such that new fungal antigens can be synthesized. In contrast to this dynamic process, *Aspergillus* extract contains a fixed mixture of antigens derived from culture filtrate and mycelia. Therefore it is very likely that antigens delivered by live fungus

and extract are different. We speculate that this active fungus-host interaction, which also occurs in humans, might be required for driving Th17/neutrophilic responses.

There is evidence that one inoculation with live *A. fumigatus* conidia induces a Th1-predominant response and no signs of Th2-associated allergic inflammation (25, 45, 83). In our model, multiple conidia challenges were required for the development of pronounced Th2/Th17 responses and associated lung pathology, suggesting T helper subsets are influenced by the exposure frequency. It is not clear whether this difference is related to specific fungus-host interactions under different exposure modes.

Following an acute *A. fumigatus* infection, depletion of neutrophils predisposes mice to invasive diseases (1). However, in a chronic allergy model, depletion of neutrophils did not significantly impair fungal clearance but alleviated lung pathology (118). In our study, in response to persistent exposure to conidia, the low intensity of neutrophilia was sufficient for fungal containment in the C57BL/6 strain (Figure 7) and the exaggerated neutrophilic inflammation in the BALB/c strain seemed unnecessary for defense against this fungus that does not pose a serious threat in immunocompetent hosts. Clearly, immunocompetent mice are able to handle as many as 10^8 conidia without developing apparent disease (1). Rather, excessive neutrophilia in the BALB/c mice tended to damage the lung tissue. These findings highlight the importance for adequate control of neutrophils. Notably, strain-dependent differential neutrophilia was only induced by repeated exposures but not by one inoculation, suggesting that the regulatory mechanisms for neutrophil recruitment differ depending on specific inflammatory settings. Therefore, it would be interesting to explore this context-dependent neutrophil regulation.

In conclusion, our animal model recapitulates the critical characteristics of *A. fumigatus*-induced allergic pulmonary diseases in humans. In particular, with mouse strain-specific granulocyte infiltration, this model provides us with a great opportunity to dissect the pathways responsible for the recruitment of neutrophils and/or eosinophils.

2.6 ACKNOWLEDGEMENTS

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3.0 MECHANISMS UNDERLYING STRAIN-ASSOCIATED DIFFERENTIAL GRANULOCYTIC RESPONSES

3.1 ABSTRACT

ABPA caused by a common fungus, *A. fumigatus* may represent one of the most extreme manifestations of fungal allergy. In our efforts to develop an animal model to recapitulate human ABPA, we found a mouse strain-associated differential eosinophilia and neutrophilia following persistent exposure to fungal conidia in BALB/c compared to C57BL/6 mice. Based on comparison of the two mouse strains, we found that lung TNF-α levels were substantially higher in the neutrophil-rich BALB/c strain than those in the eosinophil-biased C57BL/6 strain. We identified TNF-α as the key mediator in determining the strain-specific granulocyte pattern, since TNF-α blockade or deficiency biased the response towards eosinophilia but away from neutrophilia. $CD11c^+$ $CD11b^+$ $Ly6C^+$ inflammatory DCs and macrophages were identified to be the primary sources of TNF-α, and depletion of the $CD11c^+$ cells in DTR-CD11c mice dramatically reduced lung TNF-α levels. Moreover, DCs but not macrophages demonstrated a strain-specific TNF-α-producing capacity during fungal exposure, thus suggesting that DCs are of greater importance in determining strain-associated the lung TNF-α difference. Functionally, TNF-α not only favored Th17 over Th2 responses but also in collaboration with IL-17A elevated neutrophil chemoattractants, consequently increasing the neutrophil/eosinophil ratio. Therefore,

the adequate control of TNF-α or TNF-α-producing DCs may achieve a balance between host protection and immune-mediated pathology.

3.2 INTRODUCTION

ABPA is a hypersensitivity reaction to *A. fumigatus* and exhibits pulmonary infiltration with both eosinophils and neutrophils. Eosinophils are primarily implicated in allergic cascades, as well as anti-helminth and anti-fungal infections (214). Eosinophils are capable of fungal killing demonstrated using the fungus *Alernaria alternata* (241), although their *A. fumigatus*-killing ability has not been examined. However, eosinophils are also considered to be effector cells in *A. fumigatus*-associated allergic disorders, resulting in tissue damage and dysfunction (9). This seems to occur through an array of mechanisms, such as the release of toxic proteins or lipid mediators (214). Neutrophilic inflammation in the lung is being increasingly appreciated in multiple disease states including cystic fibrosis (CF), severe asthma, chronic obstructive pulmonary diseases (COPD) (242), cancer (243) and ABPA (115, 116). Upon *A. fumigatus* infection, neutrophils are crucial for antifungal defense (16) and the lack of neutrophils is the best-appreciated risk factor for fungal outgrowth and the development of IPA (1). However, uncontrolled neutrophil infiltration under allergic disease conditions triggered by this fungus predicts a poor outcome. In human patients, the neutrophil burden positively correlates with the degree of central bronchiectasis, a clinical manifestation that is often seen in the late phase of ABPA (115). In experimental models, neutrophils have been reported to contribute to AHR through their production of MMP-9 (118). Therefore, it is crucial to adequately regulate the

influx of eosinophils and neutrophils in order to achieve a balance between the host protection and immune-associated pathology.

Granulocyte influx into tissues is mediated by a complicated cascade of events. Certain cytokines or chemokines directly act on granulocytes or their progenitors to promote their development, trafficking, activation or survival. For neutrophils, G-CSF is a key cytokine for their generation, while G-CSF, KC and MIP-2 recruit neutrophils to inflammatory sites (154) (as discussed in section 1.4). GM-CSF, IL-3 and IL-5 promote eosinophil generation, of which IL-5 is the only one that selectively acts on eosinophils. In addition, IL-5 and eotaxin are of particular importance in selectively attracting eosinophils into inflamed tissues (214) (as discussed in section 1.5). Other regulators may act upstream of these cytokines and/or chemokines in this cascade. For instance, IL-17A promotes the generation of neutrophils from progenitor cells through triggering of G-CSF (244, 245). IL-17A also selectively recruits neutrophils in various settings, mostly through the induction of KC and MIP-2 (245-247). TNF-α is another cytokine that can act upstream, and it has been implicated in the induction of neutrophil chemoattractants KC and MIP-2 as well as adhesion molecules that facilitate neutrophil trafficking (248). In response to an acute *A. fumigatus* infection, depletion of TNF-α resulted in reduced lung neutrophil counts (249). However, the effects of IL-17A or TNF-α on neutrophils in the context of *A. fumigatus*-induced allergic inflammation remain undefined.

As described in the previous chapter, in response to persistent exposure to *A. fumigatus* conidia, BALB/c and C57BL/6 mice developed a neutrophil-biased and an eosinophil-biased inflammation, respectively. We therefore performed a comparative study to explore the regulatory mechanisms underlying this differential infiltration of granulocytes. We identified that TNF-α primarily derived from inflammatory DCs and macrophages acted as the key molecular

switch to bias the inflammation towards neutrophilia but away from eosinophilia. Particularly, DCs but not macrophages of different strain origins exhibited distinct TNF-α-producing capacity, seeming to account more for the lung TNF-α difference between the two strains. Functionally, TNF-α not only tipped the Th2/Th17 balance but also in collaboration with IL-17A promoted neutrophil chemoattractants. Therefore, the adequate control of TNF-α may achieve host protection by avoiding unwanted tissue damage caused by excessive inflammation.

3.3 MATERIAL AND METHODS

3.3.1 Mice

BALB/c, C57BL/6, TNF-α KO (Stock number 007082), CD1d KO (Stock number 003814) and CD11c-DTR-EGFP transgenic (Tg) mice (Stock number 004512) were purchased from The Jackson Laboratory. All three of the KO mice were on the BALB/c background. IL-17RA KO mice on the C57BL/6 background were bred at Taconic Farms, Inc. All mice were housed under pathogen-free conditions and used between 6 and 8 weeks of age. Within experiments, the mice were age and sex matched. All animal experiments were approved by the IACUC at the University of Pittsburgh.

3.3.2 Animal models

For the conidia model, as described in section 2.3.3, mice were sensitized i.t. with 1×10^7 conidia and challenged with 1×10^6 conidia daily for total 8 times. For neutralization experiments, mice

were administered anti-IL-17A or isotype control antibody (300 μg per mouse), or anti-TNF-α (MP6-XT22, BioLegend) or isotype antibody (250 μg per mouse) i.p. 24 h before sensitization and then every 3 days during the entire challenge phase. For in vivo depletion of $CD11c^+$ cells, 50 ng of diphtheria toxin (DT) was injected i.t. into the DTR-CD11c EGFP Tg mice right after the 8^{th} challenge. 24 h after the final challenge, mice were sacrificed and the inflammation was assessed as described in section 2.3.3. For the *Aspergillus* extract model, inflammation was induced and assessed as described in section 2.3.3.

3.3.3 Antibodies and flow cytometry

For antibody information, please refer to section 2.3.7. T cell intracellular staining was performed as described in section 2.3.7. 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. Cells were then stained for surface markers and intracellular TNF-α according to the manufacturer's protocol (Cytofix/Cytoperm, BD Pharmingen).

3.3.4 Cell isolation and culture

The lungs were perfused, removed and digested as described (250). Tissues were then dissociated on gentleMACS Dissociator (Miltenyi Biotech), and single cell suspensions were obtained. For isolation of lung DCs and macrophages, $CD11c^+$ cells were purified using anti-CD11c microbeads (Miltenyi Biotech), stained with anti-CD11c-APC antibody, and sorted on a FACSAria sorter (BD Immunocytometry Systems) based on their side scatter, differential expression of CD11c and autofluorescence. Basically, $CD11c^{low}$ SSC^{high} eosinophils were first

excluded, and low autofluorescent DCs and high autofluorescent macrophages were sorted for further experiments. For isolation of lung T cells, lung cells were stained with anti-CD4-APC and anti-CD3-PE antibodies and $CD3^+CD4^+$ T cells were sorted on a FACSAria.

DCs or macrophages were seeded at 1×10^5 per well in a 96-well plate in triplicate, and stimulated with SC (2×10^5 per well) for 14-15 h. For ex vivo DC-T cell cocultures, lung T cells (2×10^5 /well) were stimulated with sorted lung DCs (2×10^4 /well) in the presence of SC (1×10^5 /well). Isotype control or anti-TNF-α antibody (10 μg/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 or BioLegend).

3.3.5 qRT-PCR

As described in section 2.3.9.

3.3.6 In situ hybridization

Mouse CXCL1 (KC) cDNA was amplified using RT-PCR with primers KDG_mCXCL1_F1 (5'-CATGATCCCAGCCACCCGCT-3') and KDG_mCXCL1_R1 (5'-CTCCGTTACTTGGGGACACCT-3'). Mouse CXCL2 (MIP-2) cDNA was RT-PCR amplified with primers KDG_mCXCL2_F1 (5'-TAGCGCCATGGCCCCT-3') and KDG_mCXCL2_R1 (5'-CAGGTCAGTTAGCCTTGCCT-3'). PCR products were ligated to the pGEM-T vector (Promega) and DNA sequenced. The cDNA-containing plasmids were linearized by restriction digest. Gene-specific riboprobes were synthesized by in vitro transcription using a Maxiscript SP6/T7 kit (Ambion) and unincorporated nucleotides were removed using RNA Mini Quick spin

columns (Roche). Paraffin-embedded tissue specimens were pretreated as described (251), following deparaffinization in xylene and rinsing in ethanol. In situ hybridization with ^{35}S-labeled riboprobes was performed at 50^{o}C overnight as described (251, 252), with 0.1M dithiothreitol included in the hybridization mix. Tissue sections were coated with NTB-2 emulsion (Kodak) and exposed at 10^{o}C for 10 d. The sections were counterstained with hematoxylin (Vector) and mounted with Permount (Fisher).

3.3.7 Statistical analyses

As described in section 2.3.10.

3.4 RESULTS

3.4.1 IL-17A contributes to neutrophilia in both mouse strains

IL-17A is a well-recognized cytokine that promotes neutrophil-rich responses (247). Therefore, we questioned whether IL-17A played a role in the differential airway neutrophilia in our model. IL-17A-secreting cells were detectable after the 1st challenge and expanded during multiple challenges (Figure 9A). IL-17A is not exclusively produced by CD4 T cells, and there is accumulating evidence that other types of cells provide IL-17A thus promoting neutrophil recruitment upon infection (253, 254). A recent study showed that by 6 days post inoculation with 5 × 10^{6} *A. fumigatus* conidia, γδ T cells rather than αβ T cells are a crucial source of IL-17A in the lung (33). In our model, at an early time point (24 h after the 1st challenge), CD4^{+}T cells

and γδ T cells represented ~ 40% and ~13% of IL-17A-producing cells respectively. $CD4^+$ T cells became predominant sources of IL-17A after eight challenges, representing more than 60% of IL-$17A^+$ cells, whereas γδ T cells remained as ~10% of IL-$17A^+$ cells in both strains (Figure 9A). 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 ~60% (Figure 9B), 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 (Figure 9A), we evaluated Th17 development in both strains. 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 of those cells (higher mean fluorescence intensity of the positive cells) (Figure 9C), and equivalent mRNA expression in the $CD4^+$ T cells (Figure 9D). By comparing IL-17A protein at the whole lung level between the two strains, we found slightly lower IL-17A levels in the lung homogenates of C57BL/6 mice (Figure 9E), which might be because of the lower number of lung $CD4^+$ T cells in this strain (data not shown). Taken together, IL-17A was important in promoting neutrophil infiltration regardless of the genetic background, but the difference in IL-17A production in the two strains did not seem large enough to explain strain-associated the appreciable difference in airway neutrophilia. In addition, the blockade of IL-17A failed to affect eosinophil counts, suggesting that IL-17A was not responsible for strain-associated differential eosinophilia.

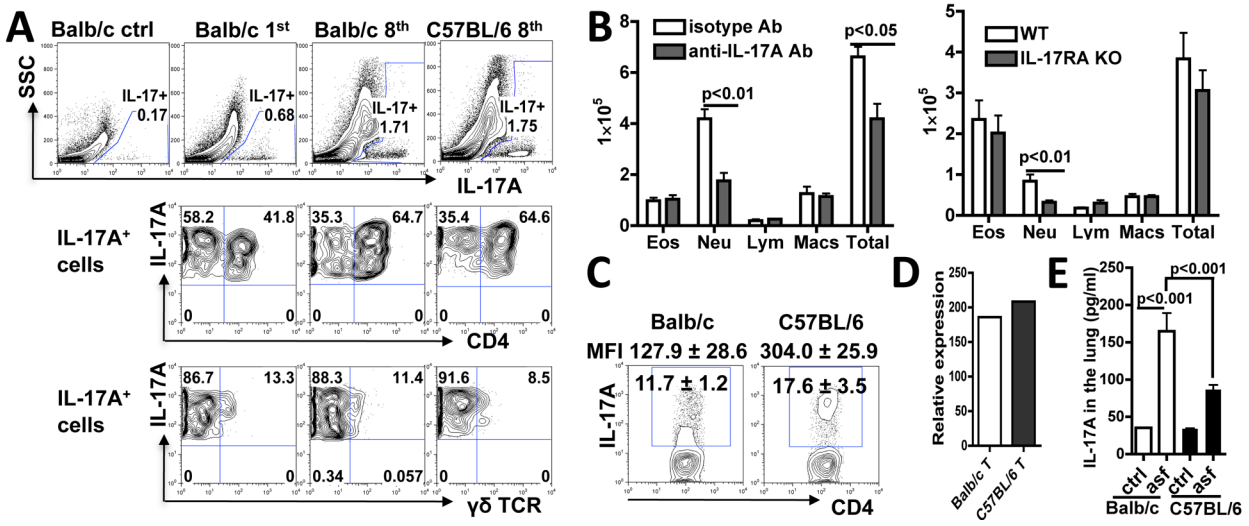

Figure 9. Characterization of cellular sources and role of IL-17A in airway neutrophilia

(A) Representative FACS plots for four mice per group show intracellular IL-17A staining in the lung cells from PBS control or challenged mice 24 h after the 1st and 8th challenges. IL-17A$^+$ gates were drawn, and CD4 or γδTCR expression is shown within the IL-17A$^+$ cells. The numbers represent the percentage of cells in the gate. (B) BALB/c mice treated with isotype or anti-IL-17A antibody (Ab), IL-17RA$^{-/-}$ (on the C57BL/6 background) or WT C57BL/6 mice were repeatedly challenged and cell differential counts in the BALF were assessed 24 h after the 8th challenge. (C) Representative FACS plots for four mice per group show intracellular IL-17A staining in the lung CD3$^+$CD4$^+$ T cells from challenged BALB/c or C57BL/6 mice at 24 h after the 8th challenge. Plots show live CD3$^+$ CD4$^+$ T cells. The percentages of cytokine-positive cells within the lung CD3$^+$CD4$^+$ T cells and mean fluorescence intensity (MFI) of IL-17A$^+$ cells are indicated. Results are expressed as mean ± SD (n=4 mice per group). (D) IL-17A mRNA in lung CD4+ T cells at 24 h after the 8th challenge was examined by qRT-PCR and expressed as fold increase compared with that in lung CD4$^+$ T cells isolated from naïve mice. (E) ELISA of IL-17A in the lung homogenates prepared from PBS control mice or challenged mice 24 h after the 8th challenges. Data are expressed as average ± SD (n=4 mice per group). Shown is an experimental representative of at least two independent experiments.

3.4.2 Myeloid cells make a minor contribution to lung IL-17A levels

We further investigated whether lung CD11b$^+$ or CD11c$^+$ cells contributed to IL-17A levels in the lung following repeated challenges. At an earlier time point (24 hour after the 1st challenge) or a later time point (24 hours after the 5th challenge), only around 5% of IL-17A-producing cells

were CD11b$^+$ or CD11c$^+$ cells in the both strains (Figure 10A). When total lung cells, CD11c$^-$ cells, CD11c$^+$ DCs or CD11c$^+$ macrophages were isolated after 5 challenges and restimulated in vitro with SC, neither lung DCs nor lung macrophages produced an appreciable amount of IL-17A (Figure 10B). In contrast, total lung cells or CD11c$^-$ cells secreted a significant amount of IL-17A in response to SC restimulation. These observations suggest that myeloid cells in our model make a minor contribution to lung IL-17A levels.

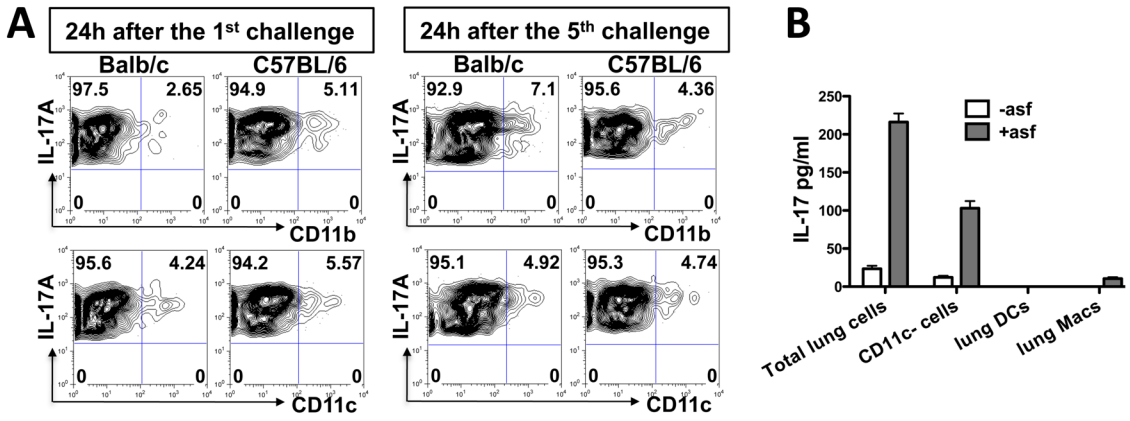

Figure 10. Characterization of contribution of lung CD11b$^+$ or CD11c$^+$ cells to lung IL-17A levels

(A) Representative FACS plots for four mice per group show intracellular IL-17A staining in the lung cells from mice 24 h after the 1st and 5th challenges. The IL-17A$^+$ gates were drawn, and CD11b or CD11c expression is shown within the IL-17A$^+$ cells. Numbers represent the percentage of cells in the gate. (B) Total lung cells, CD11c$^-$ cells or CD11c$^+$ lung DCs, or CD11c$^+$ lung macrophages were isolated from BALB/c mice at 24 h after the 5th challenge. Cells were cultured in vitro with or without restimulation by SC (cells: SC=1:2) in triplicates. After overnight culture, IL-17A in the culture supernatants was assayed by ELISA. Data are expressed as average ± SD and are representative of at least two independent experiments.

3.4.3 NKT cells are not involved in pulmonary inflammation following persistent exposure

In addition to γδT cells, NKT is another type of cells that have been involved in immune responses to diverse infectious agents. Although a minor population, upon activation, NKT cells

rapidly secrete a large amount of cytokines within few hours (255). Depending on different stimuli, NKT cells can be activated to produce specific cytokines, such as IL-17A (256). In order to examine the role of NKT cells in our model, we used CD1d KO mice, which lack type I and type II NKT cells. WT and KO mice did not differ in the cellular composition of BALF (Figure 11), indicating that NKT cells were not required for inflammatory cell infiltration in this model.

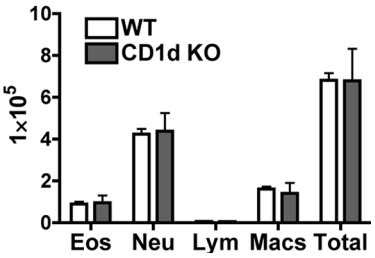

Figure 11. Characterization of contribution of NKT cells to airway inflammation

WT BALB/c mice or CD1d KO mice were sensitized and challenged as described in Figure 2A legend. Total and differential cell counts in the BALF were assessed at 24 h after the 8th challenge. Data are expressed as average ± SD (n=4 mice per group). Data are representative of at least two independent experiments.

3.4.4 TNF-α is important in determining strain-dependent pulmonary neutrophilia

Given that in vitro studies have suggested synergy between IL-17A and TNF-α in the induction of factors that support development and recruitment of neutrophils (257-259), we investigated whether TNF-α contributed to the neutrophil-biased airway inflammation in the BALB/c strain. In response to repeated challenges, TNF-α was highly induced in BALB/c mice at both mRNA and protein levels (Figure 12A), but only marginally elevated in C57BL/6 mice compared with their corresponding PBS controls, highlighting a remarkable difference in this aspect between the two strains (Figure 12A). The TNF-α difference was much greater than the difference in IL-17A, suggesting that TNF-α could be the determinant in strain-specific neutrophilia. TNF-α KO mice on the BALB/c background (Figure 12B) or BALB/c mice treated with neutralizing antibody

against TNF-α (Figure 12C) showed decreased neutrophil counts. Interestingly, the decrease in neutrophils was accompanied by a tendency toward higher eosinophil counts (Figures 12B and 12C), which resembled the granulocyte pattern in the airways of the C57BL/6 strain we observed (Figure 2A). By investigating the kinetics of TNF-α production, we found that a single challenge with 1×10^6 conidia did not trigger a pronounced TNF-α response in either strain. However, two subsequent challenges were sufficient to establish an exaggerated TNF-α response in the BALB/c strain, but maintained a low-TNF-α state in the C57BL/6 mice (Figures 12D). Therefore the drastic strain-associated TNF-α difference required multiple challenges, consistent with the finding that distinct granulocyte patterns only occurred after repeated challenges (Figure 2). Taken together, these results suggested that strain-specific regulatory mechanisms triggered due to persistent fungal stimulation determined the differential TNF-α outcome, which in turn, contributed to strain-associated differential neutrophilia.

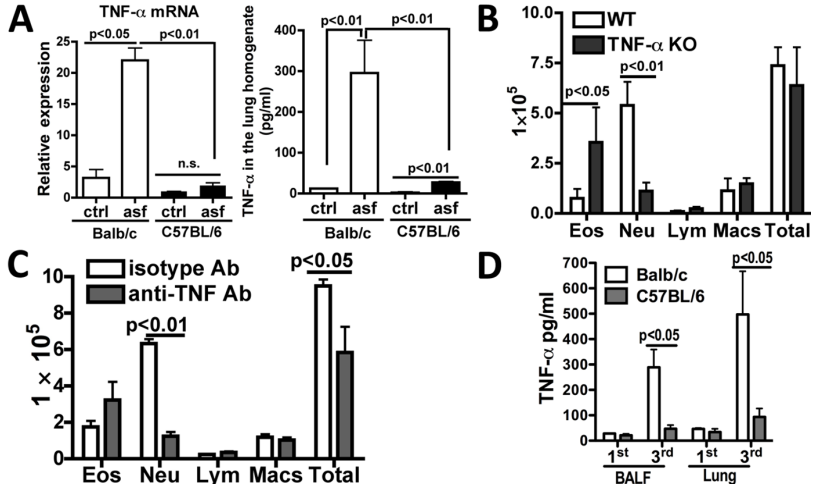

Figure 12. Role of TNF-α in determining neutrophilic inflammation

(A) TNF-α mRNA and protein levels in the lung tissue were examined by qRT-PCR and ELISA, respectively. Mice were sensitized and challenged as described in Figure 2A legend, and sampled 24h after the 8th challenge. (B) WT BALB/c and TNF-α KO mice or (C) or BALB/c mice treated with isotype or anti-TNF-α antibody were repeatedly challenged and cell differential counts were assessed 24 h post the 8th challenge. (D) TNF-α in the BALF or lung

homogenate was assayed 24 hours after the 1st or 3rd challenge. Data are expressed as average ± SD (n=4 mice per group). The results are representative of at least two independent experiments.

3.4.5 CD11c$^+$ cells are the predominant cellular sources of TNF-α

Next, we investigated the cellular sources of TNF-α. In BALB/c mice, >95% of TNF-α$^+$ cells detected in the lung were CD45$^+$ leukocytes, and ~50% were CD11c$^+$ cells following repeated challenges (Figure 13A). We then examined the importance of CD11c$^+$ cells in TNF-α production by depleting these cells in vivo using CD11c-DTR-EGFP Tg mice (on the BALB/c background). Administration of DT after challenges depleted more than 60% of CD11c$^+$ cells (Figure 13B) by 24 h, which caused a 50%-70% reduction in TNF-α levels in both the BALF and the lung homogenate (Figure 13C), confirming that the CD11c$^+$ cells were major sources of TNF-α. We also cultured lung cells isolated from challenged mice in vitro in the presence of SC to assess their TNF-α-secreting ability. Consistent with the results described above (Figure 12A), lung cells from BALB/c mice produced a much larger amount of TNF-α than those from C57BL/6 mice (Figure 13D), which was mainly derived from the CD11c$^+$ but not CD11c$^-$ cells (Figure 13D). Furthermore, the strain-associated TNF-α difference seemed to be attributable to the CD11c$^+$ fraction, because CD11c$^+$ but not CD11c$^-$ cells demonstrated strain-associated difference in TNF-α production (Figure 13D).

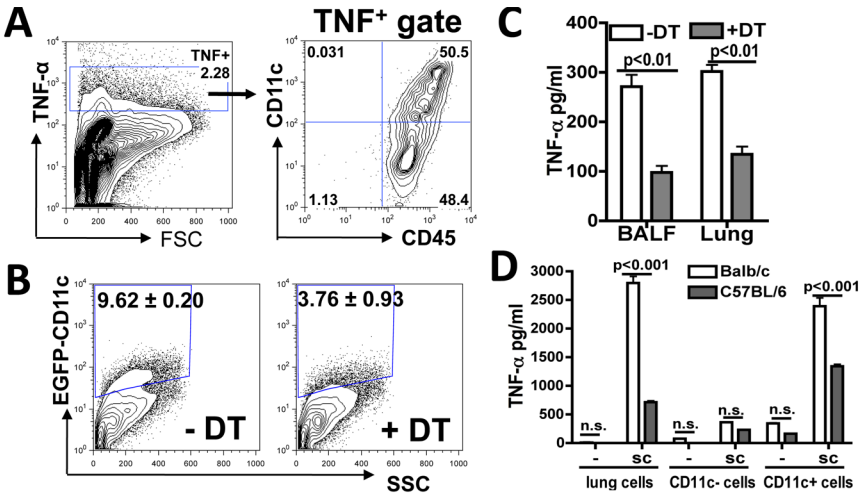

Figure 13. Cellular sources of TNF-α in response to repeated challenges

(A) Representative FACS plots show intracellular TNF-α staining in the lung cells from BALB/c mice at 24 h after the 5th challenge. The TNF-α^{+} gate was drawn, and CD45 vs CD11c expression is shown within the TNF-α^{+} gate. (B) (C) CD11c-DTR-EGFP Tg mice were challenged for 8 times. At the same time of the final challenge, mice were maintained without DT (-DT) or treated with DT (+DT). The percentages of CD11c^{+} cells within the total lung cells are shown in (B). TNF-α levels in the BALF or lung homogenate was assayed 24 h after the final challenge, and are shown in (C). Data are expressed as average ± SD (n=4 mice per group). (D) ELISA of TNF-α in the culture supernatants. Total lung cells, CD11c^{-} and CD11c^{+} cells were prepared from mice 24 h after the 8th challenge and equal numbers were cultured in vitro with/without SC restimulation (cells: SC = 1:2). Data are expressed as average ± SD (at least in triplicates in culture). The results are representative of at least two independent experiments.

3.4.6 TNF-α is mainly produced by CD11c^{+} macrophages and DCs

In challenged BALB/c mice, the CD11c^{+} fraction could be further divided into three cell types, CD11c^{low} high side scatter (SSC^{high}) Siglec F^{+} MHCII^{-} eosinophils, CD11c^{+} Siglec F^{-} low autofluorescent (AF^{low}) DCs and CD11c^{+} Siglec F$^{+/-}$ high autofluorescent (AF^{high}) macrophages (macs, Figure 14A). DCs were confirmed to express MHC class II, and were roughly consisted of two subsets: CD11b^{+} CD103^{-} and CD11b^{-} CD103^{+} DCs. Macrophages partially expressed MHC class II, and did not express CD103. No expression of Siglec F or Ly6G was detected in the DC population (Figure 14A), excluding the possibility that DCs were contaminated with

eosinophils or neutrophils in our analyses. The cytospins of FACS-sorted DCs or macrophages further confirmed the typical morphology of each type of cells (Figure 14B**)**. Intracellular staining revealed that in our model, DCs and macrophages but not eosinophils were able to produce TNF-α (Figure 14C).

We further examined the different DCs subsets and found that TNF-α production was restricted to the $CD11b^+$ DC subset and preferential to the $Ly6C^+$ DC subset (Figure 14C). The expression of Ly6C reflected their monocytic origin, suggesting that these TNF-α^+ DCs might be newly derived from monocytes. Repeated challenges also dramatically induced iNOS expression in DCs or macrophages derived from BALB/c strain, while the expression was much less in both types of cells isolated from C57BL/6 mice (Figure 14D). Therefore, the phenotype of the TNF-α-producing DCs in our model was reminiscent of Tip-DCs, which are also $Ly6C^+$ cells and differentiated from inflammatory monocytes (140, 141). However, surprisingly, in our model, the TNF-α-producing DCs did not coexpress iNOS (Figure 14E**)**. Rather it seemed that TNF-α^+ DCs and $iNOS^+$ DCs were different DC subsets (Figure 14E**)**, suggesting that the TNF-α^+ DCs induced in response to repeated fungal challenges are distinct from Tip-DCs.

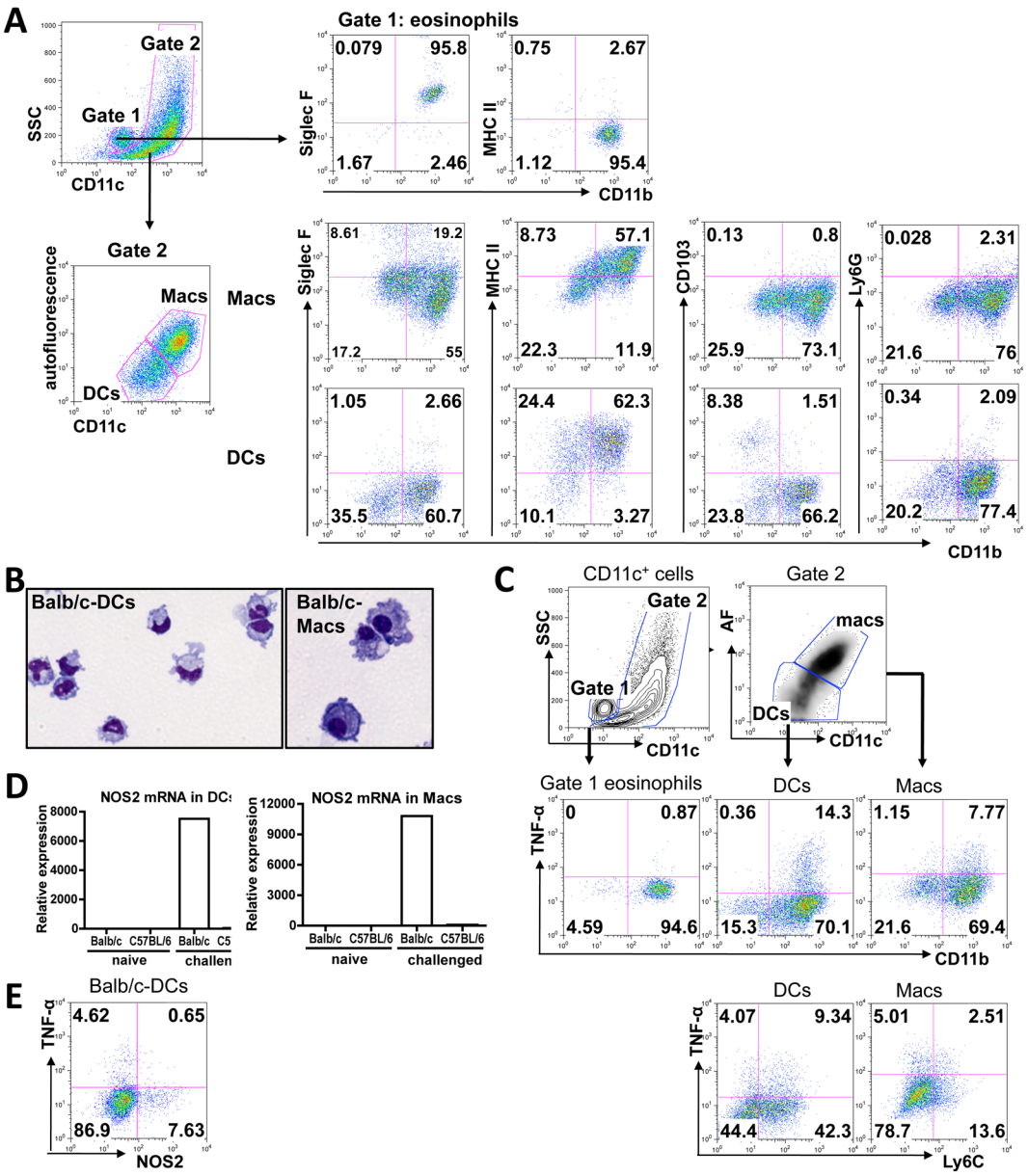

Figure 14. Characterization of contributions of different CD11c$^+$ cell types to TNF-α production

(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 the gate 2 were further divided into CD11c$^+$ low autofluorescent (AF^{low}) DCs and CD11c$^+$ high autofluorescent (AF^{high}) cells. Cells in the gate 1 are CD11c^{low} CD11b$^+$ Siglec-F$^+$ MHC II$^-$ eosinophils, which was confirmed by staining of cytospins (data not shown). CD11c$^+$ AF^{low} cells in the 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 express CD11b, Siglec-F and MHC II. Macrophages and DCs do not express Ly6G. (B) Representative cytospin images of FACS-sorted DCs and macrophages from BALB/c mice after the 5th challenge. (C) Representative FACS plots show intracellular TNF-α staining in cells from BALB/c mice at 24 h after the 8th challenge. The CD11c$^+$ cells were first divided into eosinophils, DCs and Macs

based on the description in (A). CD11b vs TNF-α or Ly6C vs TNF-α expression is shown in different cell types. (D) iNOS expression in DC or macs isolated after 8th challenges was examined by qPCR and expressed as fold increase compared to those in DC or macs isolated from naïve mice. (E) Representative FACS plot shows intracellular TNF-α and iNOS dual staining in BALB/c DCs isolated after the 5th challenge. Plot was gated on DCs as described in (A). For FACS plots, numbers represent the percentage of cells in the gate. Data are representative of three independent experiments.

3.4.7 TNF-α producing DCs are the primary contributors to strain-associated TNF-α difference

Since DCs and macrophages were the primary sources of TNF-α in our model (Figure 14), we further investigated their relative contribution to the strain-associated TNF-α difference in the lung. As expected, in naïve mice, within the CD11c^{+} population, there were very few DCs and around 90% of the cells were macrophages (eosinophils were excluded if there were any in the analyses). Although both DCs and macrophages were expanded upon challenges, CD11c^{+} DCs, in particular, were preferentially expanded and the DC/macrophage ratio was increased during repeated challenges (Figure 15A and 15B). Both strains shared this preferential expansion of DCs especially in the case of CD11b^{+} DCs, except that longer-term exposure revealed fewer overall DCs and macrophages in the C57BL/6 strain (Figure 15B and 15C). The frequency of CD11b^{+}Ly6C^{+} DCs, the subset most robust in terms of TNF-α production, progressively increased among the total DC population over time (Figure 15C). But strikingly, C57BL/6 DCs had a smaller fraction of the CD11b^{+}Ly6C^{+} subset, a difference that occurred as early as 24 h after the 1st challenge (Figure 15C). Despite the difference in Ly6C expression, DCs from the both strains did not show major differences in the expression of MHC class II and costimulatory markers (Figure 15D). To assess whether the phenotypic difference reflected a functional difference, we examined TNF-α production. Purified macrophages from both strains isolated

after five challenges responded similarly in vitro to SC, producing equal amount of TNF-α. In contrast, BALB/c DCs secreted >3-fold more TNF-α than did C57BL/6-DCs on a per-cell basis (Figure 15E). Taken together, macrophages from the two strains possessed comparable TNF-α-secreting capacity on a per cell basis although the recovered numbers were different (Figure 15B and 15E). However, comparatively, DCs derived from the two strains not only differed in their numbers, but also in their ability to produce TNF-α, thereby accounting for the majority of the strain-associated TNF-α difference.

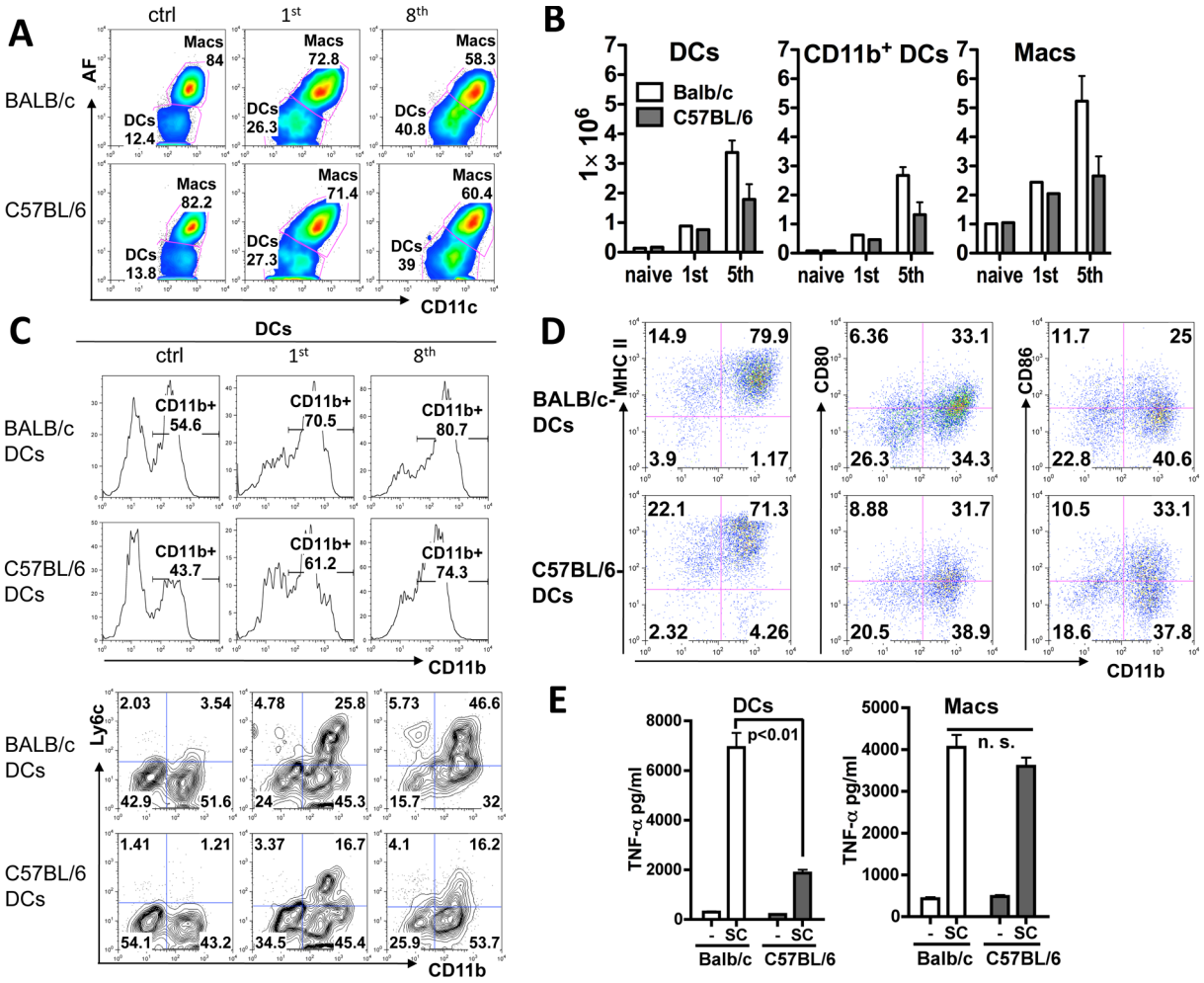

Figure 15. TNF-α-producing DCs are of greater importance in strain-associated lung TNF-α difference

(A) Representative FACS plots show the relative abundance of DCs versus Macs in PBS controls or challenged mice at 24 h post the 1st and 8th challenges. Plots were gated on $CD11c^{+}$ cells and $CD11c^{low}$ SSC^{high} eosinophils (if there were any) were excluded from the analyses. DC and Macs are $CD11c^{+}$ AF^{low} and $CD11c^{+}$ AF^{high} cells

respectively as described in Figure 14A. AF: autofluorescence. (B) The absolute numbers of DCs, $CD11b^+$ DCs and macrophage from naïve control, challenged mice after the 1^{st} or 5^{th} challenge were calculated based on the frequencies of cells and the total lung cell counts. (C) Representative FACS plots show the abundance of $CD11b^+$ and $CD11b^+Ly6C^+$ DCs in the total lung DCs from PBS control or challenged mice at 24 h after the 1^{st} and 8^{th} challenge. Plots were gated on $CD11c^+$ AF^{low} lung DCs. (D) Representative FACS plots show the surface markers on DCs isolated from challenged BALB/c or C57BL/6 mice at 24 h after the 8^{th} challenge. Plots were gated on $CD11c^+$ AF^{low} lung DCs as described in Figure 14A. (E) ELISA of TNF-α in the culture supernatants. Lung DCs or Macs were isolated from mice 24 h after the 5^{th} challenge and cultured in vitro (0.1 million cells/well) with/without SC restimulation (cells: SC = 1:2). Data are expressed as average ± SD (at least in triplicates in culture). For FACS plots, numbers indicate the percentage of cells in the gate. Experiments were repeated at least three times.

3.4.8 TNF-α regulates the balance of Th17 and Th2 responses

To better understand the IL-17A and TNF-α collaboration, we examined how they regulated each other. DC-derived TNF-α is believed to synergize with IL-23 to promote an IL-17A response (260, 261). Given that the TNF-α-producing DCs in our model showed a preferential and continuous expansion during repeated challenges (Figure 15A and 15B), it was hypothesized that they might have an impact on Th17 responses. Blockade of TNF-α or TNF-α deficiency diminished lung IL-17A levels, but promoted IL-5 levels (Figure 16A), consistent with the shift from neutrophilia to eosinophilia in the airways we observed (Figure 12B and 12C). The IL-5 level was also higher in the C57BL/6 strain than that in the BALB/c strain (Figure 16A), possibly explaining the eosinophil-biased response in the C57BL/6 strain. Analysis of $CD4^+$ T subsets revealed that in the absence of TNF-α, there was a higher percentage of IL-4-producing Th2 cells, but a lower frequency of IL-17A-producing Th17 cells (Figure 16B). Furthermore, it appeared that lack of TNF-α selectively reduced the number of $CD4^+$IL-$17A^+$ cells, but not that of $CD4^-$IL-$17A^+$ cells (Figure 16C). Moreover, neutralization of TNF-α in lung DC-T cocultures ex vivo also reduced IFN-γ and IL-17A but promoted IL-5 production (Figure 16D).

Collectively, these results demonstrated the important role of TNF-α in regulating the Th1/Th2/Th17 profiles and, in turn, the neutrophil/eosinophil ratio. In contrast, IL-17A showed no regulatory effects on TNF-α (Figure 16E).

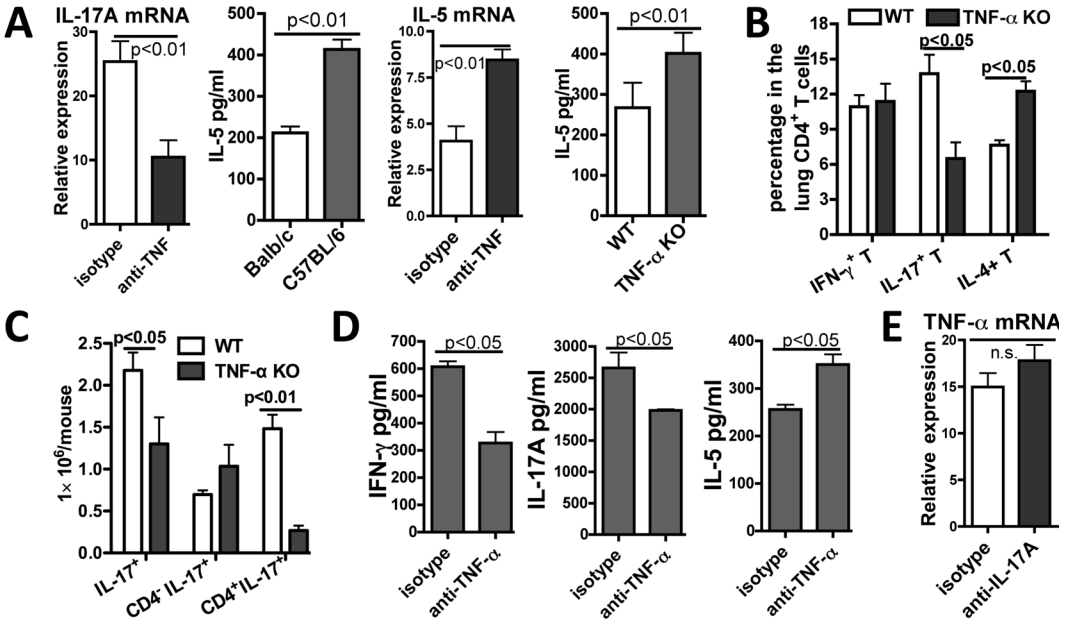

Figure 16. TNF-α regulates the Th17/Th2 balance

(A) ELISA of TNF-α in the lung homogenates 24 h after the 8th challenge. BALB/c and C57BL/6, or BALB/c mice treated with isotype or anti-TNF-α antibody, or WT BALB/c and TNF-α KO mice (on the BALB/c background) were sensitized and challenged as described in Figure 2A legend. (B)WT or TNF-α KO mice were repeatedly challenged, and the percentages of IFN-γ-, IL-17A- and IL-4-producing $CD4^+$ T cells in the lung $CD4^+$ T cell compartment were determined by intracellular staining at 24 h after the 8th challenge. (C) The numbers of lung IL-$17A^+$, $CD4^-$IL-$17A^+$ and $CD4^+$ IL-$17A^+$ cells per mouse were determined by intracellular cytokine staining at 24 h after the 8th challenge. Results are expressed as mean ± SD (n≥4 mice). (D) Lung DCs and T cells were isolated from BALB/c mice after 8 challenges, and were cocultured 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 ± SD (at least in triplicates in culture). (E) Lung TNF-α mRNA in mice 24 h after the 8th challenge. Mice treated with isotype or anti-IL-17A antibody were sensitized and challenged as described in Figure 2A legend. Results are expressed as fold increase over PBS controls. In A, B, C and E, results are expressed as mean ± SD (n≥4 mice). Data are representative of at least two independent experiments.

3.4.9 TNF-α and IL-17 collaborate to induce neutrophilic inflammation

We then investigated whether IL-17A and TNF-α in our model have an additive or synergistic effect on mediators that drive neutrophil mobilization as suggested by previous in vitro studies (257-259). As compared to WT mice, the levels of KC, MIP-2 and, to a lesser extent, G-CSF were lower in TNF-α KO mice (Figure 17A and 17B). However, only KC was significantly reduced by IL-17A neutralization (Figure 17B). KC and MIP-2, but not G-CSF levels were also lower in the lungs of C57BL/6 mice compared with that in BALB/c mice (Figure 17B). These data highlighted the role of both IL-17A and TNF-α in inducing chemoattractants to augment neutrophil recruitment. In contrast, neither TNF-α nor IL-17A influenced eotaxin levels in the lung. Interestingly, despite a lower eosinophilia, BALB/c mice had a higher eotaxin level (Figure 17B), but a lower IL-5 level (Figure 16A).

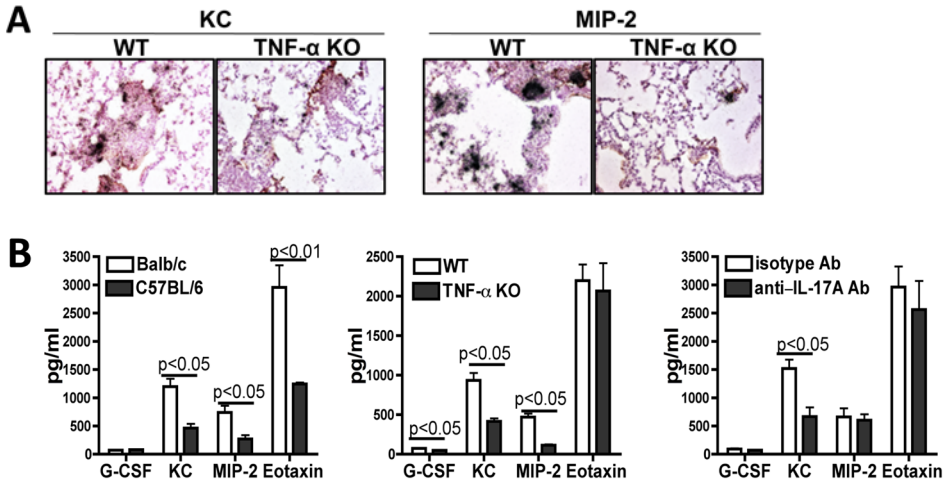

Figure 17. TNF-α and IL-17A collaborate to induce airway neutrophilia

(A) In situ hybridization images show lung KC and MIP-2 in WT or TNF-α KO mice. (B) ELISA of KC, G-CSF, MIP-2 and eotaxin in the lung homogenates 24 h after the 8th challenge. Mice were sensitized and challenged for 8 times as described in Figure 2A legend. Results are expressed as mean ± SD (n=4 mice per group). Shown is an experiment representative of at least two.

3.4.10 A comparison of TNF-α responses following repeated exposures to *A. fumigatus* conidia or extract

We demonstrated that following repeated challenges with conidia, high levels of TNF-α were observed in neutrophil-rich BALB/c mice (Figure 12 and 18), while low TNF-α levels were associated with diminished neutrophilia in the C57BL/6 mice (Figure 12). As opposed to *A. fumigatus* conidia, fungal extract failed to induce prominent neutrophilia in the BALB/c strain (Figure 8). Interestingly, this was also associated with minimally induced TNF-α expression in the lungs (Figure 18). This finding further highlighted the importance of TNF-α in promoting neutrophilic inflammation in fungus-induced allergy.

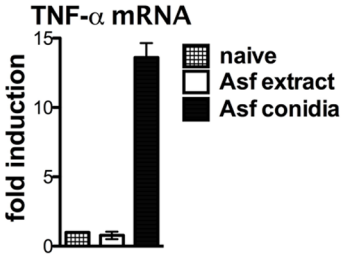

Figure 18. A comparison of TNF-α levels following repeated challenges to conidia or to *Aspergillus* extract

BALB/c mice were sensitized and challenged with conidia (Asf conidia group) or challenged with *Aspergillus* extract (Asf extract group) as described in Figure 8A legend. TNF-α mRNA levels in the lungs were examined by RT-PCR. The results were expressed as fold induction as compared to that in naïve mice. Results shown are mean ± SD (>=4 mice per group).

3.4.11 A proposed model for the control of pulmonary neutrophilia versus eosinophilia by TNF-α during persistent fungal exposure

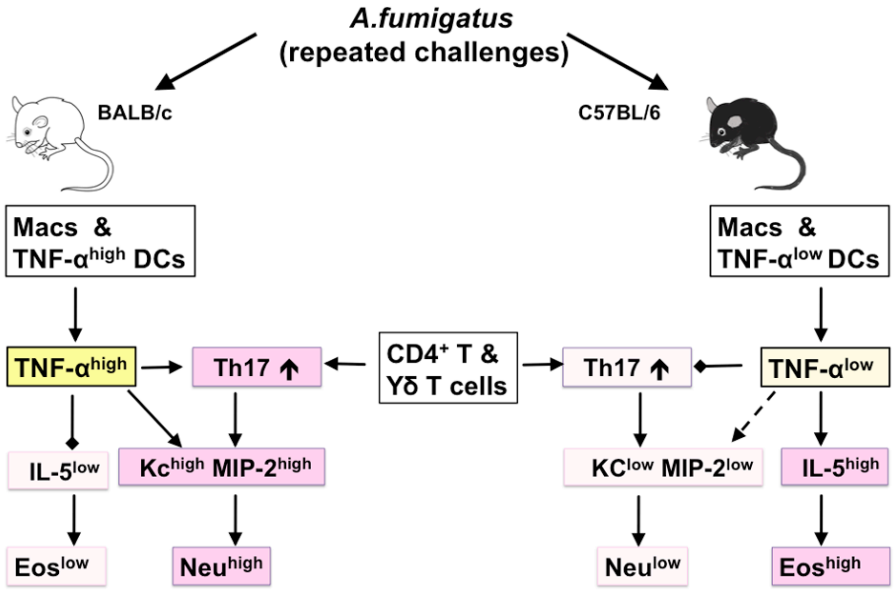

Figure 19. A proposed model showing how TNF-α regulates granulocyte infiltration in the lungs of BALB/c and C57BL/6 mice during persistent fungal exposure

In the allergic pulmonary inflammation elicited by persistent exposure to *A. fumigatus* conidia, neutrophils and eosinophils predominate in the BALB/c and C57BL/6 mice, respectively. BALB/c mice have more TNF-α in the lung, which is mainly derived from inflammatory DCs and macrophages. Moreover, BALB/C DCs exhibit a stronger TNF-α-producing capacity than C57BL/6 DCs do, whereas macrophages of the two strains demonstrate a comparable capacity in producing TNF-α. Therefore it seems that DC-derived TNF-α contributes more to strain-associated TNF-α differences at the lung tissue level. Functionally, TNF-α favors Th17 responses over Th2 responses, and consequently promotes IL-17A but inhibits Th2 cytokine IL-5. TNF-α also in collaboration with IL-17 primarily derived from Th17 cells induces neutrophil chemoattractants, in particular KC. The levels of IL-5 and neutrophil chemoattractants ultimately determine the intensity of eosinophilia and neutrophilia, respectively. This model highlights the key role of TNF-α as a molecular switch to regulate tissue neutrophilia versus eosinophilia. (Macs: macrophages, Neu: neutrophils, Eos: eosinophils).

3.5 DISCUSSION

As described in the chapter 2, mouse strain-specific neutrophil or eosinophil infiltration was observed following repeated challenges with *A. fumigatus* conidia but not after a single inoculation, implicating a context-specific regulation of granulocyte influx into the lungs. Therefore, distinct from previous studies that have investigated neutrophil recruitment as a protective mechanism immediately following fungal infection (12, 14-16), our present work has been more focused on elucidating the mechanisms underlying excessive neutrophilic inflammation in pulmonary allergy induced by persistent fungal exposure. We found that TNF-α was the key factor to regulate the Th17/Th2 balance and the neutrophil/eosinophil ratio, thus responsible for the mouse strain-associated differential granulocyte infiltration (as summarized in Figure 19).

Exogenously administered IL-17A and TNF-α can act independently to induce neutrophil infiltration into the airways (262, 263), but the relative contributions of endogenous IL-17A and TNF-α may vary in different settings. For example, IL-17A (264) but not TNF-α (265) is essential for LPS-triggered pulmonary neutrophilia. However on many occasions, functional cooperation between the two induce the most pronounced effects. There is evidence that TNF-α deficiency attenuated recombinant IL-17A-induced pulmonary neutrophilia (266), raising the possibility that IL-17A provokes a TNF-α response to amplify neutrophil recruitment. TNF-α can act upstream of IL-17A, which is supported by the evidence that TNF-α in synergy with IL-23 promotes IL-17A production from $CD4^+$ T cells (260, 261). In addition, in vitro TNF-α and IL-17A synergistically stimulate airway epithelial and endothelial cells to produce chemokines/cytokines for neutrophil development, recruitment and survival (257-259). In our study, despite an active Th17 response in the C57BL/6 strain, the absence of a robust TNF-α

response was associated with a low degree of neutrophil infiltration, indicating the importance of the cooperation between these two cytokines. The high levels of TNF-α in the BALB/c strain favored Th17 responses, and importantly, in collaboration with IL-17A, promoted the production of neutrophil chemoattractants, thereby causing a neutrophil-dominated response (summarized in Figure 19). Our model did not support an appreciable role for IL-17A in regulating lung TNF-α levels, inconsistent with the notion suggested by previous reports (266).

The absence of TNF-α in our study not only diminished neutrophilia but also promoted eosinophilia, implying a role for TNF-α in suppressing eosinophil influx. TNF-α biased the response away from Th2 and consequently diminished Th2 cytokines, such as IL-5 (summarized in Figure 19). IL-5 is considered to be the key regulator for eosinophils and it modulates eosinophil generation and function at multiple stages, such as differentiation, mobilization, tissue accumulation, activation and survival (as discussed in section 1.5). The low TNF-α profile in the C57BL/6 strain or TNF-α deficiency was associated with higher IL-5 levels, which might explain why there were more eosinophils in each of these situations. In contrast, the BALB/c mice with a low IL-5 profile, despite higher eotaxin levels, did not demonstrate a prominent eosinophilia (summarized in Figure 19). Although eotaxin is a well-known eosinophil chemoattractant, it has been reported that only in the presence of IL-5, does intranasal administration of eotaxin induce eosinophil influx into the lung (267). One possible explanation is that IL-5 is required to prime eosinophils to respond to eotaxin (220). Or alternatively, it may be that IL-5 but not eotaxin is necessary to mobilize eosinophil from the BM pool, which is the prerequisite for the subsequent eotaxin-mediated tissue recruitment (215, 219) (also see discussion in section 1.5.2). Therefore, it is likely that the limited amount of IL-5 in the BALB/c mice is not sufficient to prepare neutrophils for subsequent eotaxin-mediated mobilization. In

contrast to this dual role of TNF-α in regulating both neutrophilia and eosinophilia, we did not observe obvious effects of IL-17A on eosinophilia.

The cellular sources of TNF-α vary in different settings. For instance, in ovalbumin-challenged OTII mice, mast cell-derived TNF-α is essential for driving airway inflammation (266). Following an acute *A. fumigatus* infection, macrophages were believed to be the sources of TNF-α. But recent work demonstrated that depletion of neutrophils greatly enhanced lung DC influx in response to killed *A. fumigatus* antigens. In this setting, lung DCs and monocytic cells contributed to lung TNF-α production, whereas macrophages only made a minor contribution (268). However, it seems that the accumulation of TNF-α^+ DCs in this neutropenia condition is a compensatory mechanism triggered by the loss of neutrophils. In an acute infection model in immunocompetent mice, monocyte-derived DCs have been shown to phagocytose conidia in the lung, migrate into the dLNs, and potentially mediate T cell activation (18). However, the proinflammatory properties of these cells in the local tissue have not been fully addressed. In our study, we identified that in addition to macrophages, TNF-α-producing DCs were of crucial importance in the allergic setting elicited by repeated fungal exposures. Moreover, the presence of DCs with distinct TNF-α-secreting capacity likely determined the low- or high- TNF-α phenotype at the lung tissue level, subsequently influencing neutrophil versus eosinophil influx (summarized in Figure 19). Therefore, distinct from previous reports, our study elucidated the role of TNF-α-producing DCs in orchestrating local inflammatory cascade in response to persistent fungal stimulation.

As previously shown, an acute *A. fumigatus* infection triggers the generation of $CD11b^+$ lung DCs from $Ly6C^+$ monocytes (18). In our model, almost all TNF-α-producing DCs were $CD11b^+$, and the majority of them retained the monocytic marker Ly6C, suggesting that these

TNF-α^+ DCs are highly likely to have been derived from monocytes. Some lung DCs in this model also expressed iNOS, but interestingly the iNOS$^+$ DCs and TNF-α^+ DCs were two distinct DC subsets, reflecting a sophisticated division of labor. Little is known regarding the role of iNOS-expressing DCs, which would be an interesting issue that needs to be pursued.

Studies using infection models or non-infectious inflammation have shown de novo induction of inflammatory DCs from Ly6C$^+$ monocytes in tissues or secondary lymphoid organs (269, 270). These DCs usually display proinflammatory properties, and under certain circumstances, TNF-α represents one of the most significant functional mechanisms (269, 270). These TNF-producing DCs are not always beneficial. There is evidence that in the lamina propria, the balance between Ly6^{high} monocyte-derived CD11b$^+$ DCs versus CD11b$^-$ DCs is important in maintaining gut homeostasis. Furthermore, skewing towards CD11b$^+$ DCs exacerbated colitis, which was attributable to TNF-α secreted by these CD11b$^+$ DCs (271). Although Tip-DCs, as a special type of monocyte-derived and TNF-α-producing DCs are beneficial to the host in some models (140, 272), excessive numbers of Tip-DCs have been associated with immune-mediated pathology in infectious models utilizing *Trypanosome brucei* (273) or influenza virus (274), and in other non-infectious diseases such as human psoriasis (275) or multiple sclerosis (276). In our study, in response to repeated exposures to conidia, uncontrolled accumulation of TNF-α-producing DCs promoted a strong neutrophilic response that can release a large amount of oxidants and proteases, such as MPO (Figure 2), causing lung injury. In this regard, the TNF-α-producing DCs may act as a double-edged sword, such that tight regulation of this cell type is required to achieve an acceptable balance between host defense and immune-mediated pathology.

3.6 ACKNOWLEDGEMENTS

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4.0 SIGNALING PATHWAYS INVOLVED IN TNF REGULATION IN DCS

4.1 ABSTRACT

In an A. *fumigatus*- induced pulmonary allergy model, DCs but not macrophages demonstrated mouse strain-specific TNF-α-producing ability, which subsequently influenced granulocyte infiltration and disease outcome. In the present study, we elucidated mouse strain-specific mechanisms for TNF-α regulation in DCs in response to repeated exposure to fungal conidia. We found that after the initial challenge with conidia, as compare to the BALB/c DCs, C57BL/6 DCs contained more NF-κB p50 homodimers acting as gene repressors at the κB3 site of the TNF-α promoter, which was associated with less TNF-α production by C57BL/6 DCs at early time points. During repeated challenges, BALB/C DCs expressed substantially higher TLR2 than C57BL/6 DCs. This expression difference led to the function difference in TNF-α production as tested by TLR2 agonist in vitro, which was associated with the TNF-α^{high} DCs in BALB/c mice but TNF-α^{low} DCs in C57BL/6 during persistent fungal exposure. Moreover, TNF-α deficiency greatly reduced the accumulation of inflammatory $CD11b^{+}$ $Ly6C^{+}$ DCs, the main DC subset that contained TNF-α-producers in the WT mice. This finding suggests that TNF-α sets up a positive feedback loop to amplify the inflammatory cascade. In contrast, macrophages from the two mouse strains failed to show these differences, indicating a cell-context specific regulation.

4.2 INTRODUCTION

Neutrophilia in the disease ABPA contributes to lung injury and predicts a poor outcome. We developed an experimental model mimicking human ABPA to study the mechanisms underlying this neutrophilia and found that DC-derived TNF-α was of great importance in promoting neutrophil infiltration. Interestingly, DCs but not macrophages from the two mouse strains showed differential TNF-α-producing capacity, suggestive of a mouse-strain specific and cell-specific TNF-α regulation. Given the essential role of TNF-α in disease pathogenesis, an increasing understanding of context-specific TNF-α regulation would be of great value.

The secretion of cytokines including TNF-α is one of the end results of engagement of PRRs on the host cell surface by PAMPs expressed by *A. fumigatus*. Based on animal models and cell culture systems, a variety of PRRs have been implicated in *A. fumigatus* recognition, such as TLRs, dectin-1 and soluble receptors, SP-A, SP-D, PTX3 and MBL. Among the TLRs, transfection of HEK293 cells with various TLRs revealed that TLR2 and 4 were more important than other TLRs (TLR1, 3, 5, 6, 7, 8 and 10) in fungal recognition (42) (also see discussion in section 1.4.4). The specific requirement of PRRs for TNF-α production has also been documented, but this has been studied in greater detail in macrophages than in DCs. One caveat of some previous studies that needs to be mentioned is that peritoneal instead of alveolar macrophages have typically been used. Given that peritoneal cavity is not a natural anatomic site of *A. fumigatus* infection, and that these two types of macrophages bear distinct PRR profiles, concepts built on the studies of peritoneal macrophages might be misleading. Therefore, we considered data obtained with alveolar macrophage to be more relevant. Studies with alveolar macrophages have highlighted the role of TLR2 (7, 44) and dectin-1(6, 7) in promoting TNF-α secretion in response to *A. fumigatus*. In regard to BMDCs, both TLR2 and TLR4 were found

essential to be for fungus-induced cytokine responses, although IL-6 and IL-12 but not TNF-α were used as readouts in this particular study (277). In the case of human immature DCs, blockade of dectn-1 signaling but not TLR2 or TLR4 appreciably diminished TNF-α in culture (278). Collectively, TLR2, TLR4 and dectin-1 have been most often associated with TNF-α production. However, the relative contribution of individual PRRs may vary in different cell contexts.

A growing body of literature suggests that there is crosstalk between TLR2 and dectin-1. Simultaneously activation of TLR2 and dectin-1 signaling by their respective agonists shows synergistic effects, in particular, on TNF-α production. This synergy seems to be a general mechanism, as it has been observed in a variety type cell types, such as alveolar macrophages (279), peritoneal macrophages (280), BMDCs (281, 282), as well as human monocytes and macrophages (280, 283). Therefore, under a scenario TLR2 and dectin-1 are co-activated by *A. fumigatus*, this synergy could possibly be achieved as well.

Binding of *A. fumigatus* to particular PRRs transduces signals inside the cells, resulting in biological consequences. Multiple signaling pathways including those of TLRs and dectin-1 converge at the level of NF-κB in inflammatory responses. Functional NF-κB is composed of homodimers or heterodimers made up of five molecular family members, which are p50, p52, p65, RelB and c-Rel. Of them, p50 and p52 do not contain a transactivation domain. It is widely accepted that p50 homodimers are strong gene repressors under most circumstances (284). Although the NF-κB pathway has been extensively studied, it seems that the role of NF-κB in the control of TNF-α gene in lung DCs in response to *A. fumigatus* has not been well characterized.

In the current study, we explored mouse strain and cell context-specific TNF-α regulation in response to persistent fungal exposure. We found that after the initial challenge with conidia,

the relatively weaker TNF-α-producing capacity of C57BL/6 DCs was associated with the presence of more NF-κB p50 homodimers acting as gene repressors at the TNF-α promoter in these cells in comparison with BALB/c DCs. Following repeated challenges, substantially higher TLR2 levels were found on BALB/c DCs, further contributing to the TNF-α difference between these two types of DCs. Importantly, TNF-α deficiency greatly reduced the generation of $CD11b^{+}$ $Ly6C^{+}$ inflammatory DCs, the DC subset that contained a high number of TNF-α producers in WT mice. Therefore, TNF-α is possibly involved in a positive feedback loop to amplify the inflammatory cascade. This regulation in DCs has not been observed in macrophages, suggesting that it is a cell context-specific event.

4.3 MATERIAL AND METHODS

4.3.1 Mice

BALB/c, C57BL/6, TNF-α knockout mice (Stock number 007082) and $TLR4^{LPS\text{-}d}$ mice (stock number 002930) on the BALB/c background were purchased from The Jackson Laboratory. MyD88 KO and dectin-1 KO mice on the BALB/c background were bred at the animal facility at the University of Pittsburgh. All mice were housed under pathogen-free conditions and used between 6 and 8 weeks of age. Within experiments, the mice were age and sex matched. All animal experiments were approved by the IACUC at the University of Pittsburgh.

4.3.2 Animal models

As described in section 2.3.3.

4.3.3 Cell isolation and culture

For the isolation of lung DCs or macrophage, please refer to the section 3.3.4. For examining lung DC profiles, lung DCs were isolated from naïve BALB/c mice. 1×10^5/well of DCs were seeded in 48-well plate in the presence of 5 ng/ml GM-CSF. 1×10^5/well of SC (cells: SC=1:1) or 1 μg/ml of LPS were used to simulate cells for 24 h. Cytokines in the supernatants were examined by Multiplex assay (BioRad). For experiments using dectin-1 or TLR2 agonists, lung macrophages or DCs were isolated 24 h after the 5^{th} challenge. Cells were then cultured without stimulation, or were stimulated with dectin-1 agonist curdlan (100 μg/ml) (Wako Pure Chemicals) or TLR2 agonist Pam3Cysk4 (5 μg/ml) (InvivoGen) for about 15-16 h. For assessing TNF-α-producing capacity of cells in response to SC, DCs or macrophages were seeded at 1×10^5 per well in a 96-well plate in at least triplicates, and were stimulated with SC (2×10^5 per well) for 15-16 h. TNF-α in the supernatants was assayed by ELISA (BioLegend).

4.3.4 Antibodies and flow cytometry

For antibody information and T cell intracellular staining, please refer to section 2.3.7. For intracellular staining for TNF-α, please refer to section 3.3.3.

4.3.5 Active Motif assay

Nuclear extracts were prepared using a nuclear extract kit (Panomics Quantitative Biology). NF-κB DNA binding activity was determined with a TransAM^{TM} NF-κB family kit (Active Motif).

4.3.6 Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described by us and others (285-287). Basically, 5 μg of nuclear extract was gently mixed with 5× complete binding buffer at RT for 10 min, incubated with antibody for supershift at RT for 10 min, and finally incubated with 200 fmole of 5'-biotin-labeled 20 bp double-stranded oligonucleotides at room temperature for 20 min. Complexes were then resolved on a 4.5% non-denaturing PAGE gel in 0.5 × TBE buffer, and transferred to a positively charged nylon membrane (Pierce). Transferred DNA was cross-linked to the membrane using a Stratalinker UV crosslinker (Stratagene). Finally biotin-labeled DNA on the membrane was detected using the Lightshift Chemiluminescent EMSA kit (Pierce).

The 1× complete binding buffer used in the binding reaction contained 10mM Tris-HCl PH=7.5, 50mM NaCl, 5% glycerol, 1% IGEPAL CA-630 (Sigma), 1mM EDTA, 0.1μg/μl polydIdC and 0.05 μg/μl BSA. The probe consisted of the κB3 (-510) site from the murine TNF-α promoter and the sequence is AACAGGGGGCTTTCCCTCCT (Invitrogen) (286). Antibody used in super-shift assays was sc-114X (anti-p50) or sc-109X (anti-p65) (Santa Cruz Biotechnology).

4.3.7 Statistical analyses

As described in section 2.3.10.

4.4 RESULTS

4.4.1 Lung DCs respond differently to LPS or conidia stimulation in vitro

In the previous chapter, we identified that TNF-α-producing DCs were of great importance in determining strain-associated TNF-α differences. To reveal the nature of *A. fumigatus*-induced inflammatory response in lung DCs, we compared the cytokine/chemokine profiles of lung DCs isolated from naïve BALB/c mice in response to either SC or LPS. LPS triggered modestly higher or comparable levels of IL-1α, IL-1β, IL-10, IL-12p40, IL-12p70, KC, MIP-1α, MIP-1β, MCP-1 and eotaxin compared to SC under the culture condition we used. But notably, SC stimulated strikingly more TNF-α while LPS promoted a greater amount of IL-6 (Figure 20). Therefore, lung DCs seem to have an intrinsic tendency to mount a high TNF-α response to SC.

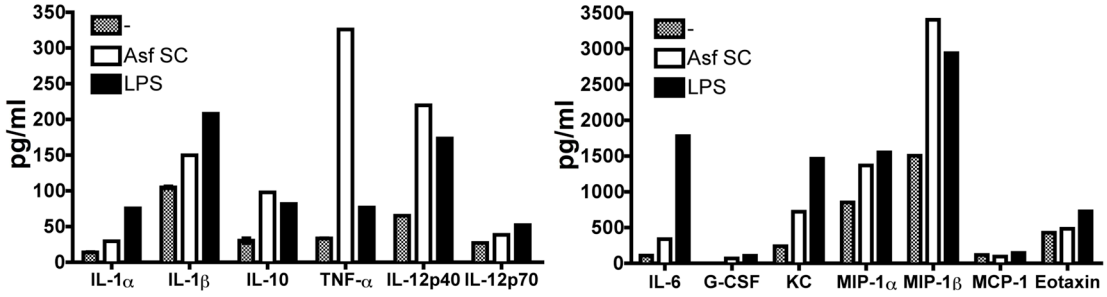

Figure 20. Cytokine/chemokine profile of BALB/c lung DCs stimulated with SC or LPS in vitro

Lung DCs were isolated from naïve BALB/c mice. 1×10^5/well of DCs were seeded in 48-well plate in the presence of 5 ng/ml GM-CSF. 1×10^5/well of SC (cells: SC=1:1) or 1 μg/ml of LPS were used to stimulate cells for 24 h. Cytokines in the supernatants were examined by Multiplex assay.

4.4.2 TLRs/MyD88 and dectin-1 are responsible for *A. fumigatus* recognition

We sought to dissect the signaling pathways responsible for the generation TNF-α-producing DCs. Before that, we first examined which PRRs participated in fungal recognition and drove inflammation at the macroscopic level. TLR2, TLR4 and dectin-1 are the most critical PRRs for the recognition of *A. fumigatus* (35, 42, 277)(also see discussion in section 1.1.4). We confirmed the importance of both the MyD88 and dectin-1 pathways in sensing the organism and in orchestrating the overall lung inflammation on the BALB/c background. Lack of either significantly reduced the infiltration of neutrophils as well as other inflammatory cells (eosinophils and macrophages) in the BALF (Figure 21A and 21B). We did not observe a substantive role for TLR4, since $TLR4^{LPS\text{-}d}$ mice yielded approximately equal numbers of $CD45^+$ immune cells and neutrophils in the lung as compared to WT mice (Figure 21C). These observations suggest that in addition to dectin-1, TLR2 rather than TLR4 is likely involved in the elicitation of neutrophilic inflammation.

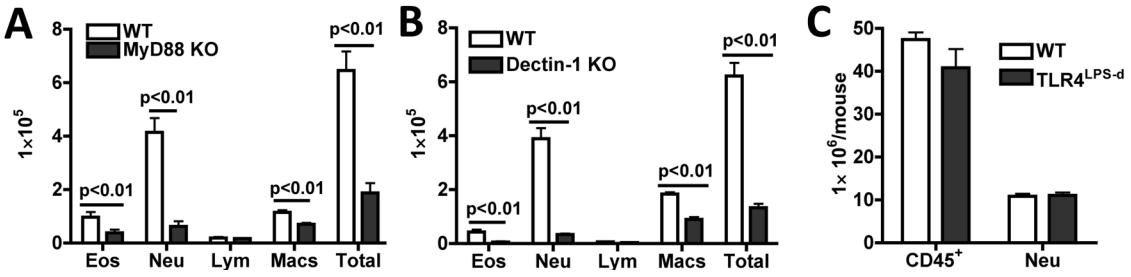

Figure 21. MyD88 and dectin-1 but not TLR4 pathways are involved in airway inflammation

(A) (B) MyD88 KO, Dectin-1 KO or WT BALB/c mice were sensitized and repeatedly challenged as described in Figure 2A legend. Total and differential cell counts in the BALF were assessed at 24 hours post the 8^{th} challenge. (C) $TLR^{Lps\text{-}d}$ or WT BLAB/c mice were sensitized and challenged as described in Figure 2A legend. $CD45^+$ leukocytes and $CD45^+$ $CD11b^{high}$ $Ly6G^{high}$ neutrophils were quantified by surface staining at 24 hours post the 8^{th} challenge. Results are mean ± SD (n=4 mice per group).

4.4.3 TLRs/MyD88 and dectin-1 pathways are required for the generation of TNF-α-producing DCs

Considering the data obtained from the whole animal models, we then closely investigated the mechanisms underlying the generation of TNF-α-producing DCs. Both MyD88 and dectin-1 signaling were essential since lack of either profoundly blocked TNF-α production from DCs (Figure 22A). Lack of dectin-1 signaling also reduced the abundance of lung DCs (Figure 22B), particularly that of $CD11b^+$ $Ly6C^+$ DCs (Figure 22C), indicating its importance in the development of the DC subset that was shown to contain a high number of TNF-α producers in WT mice (Figure 22A).

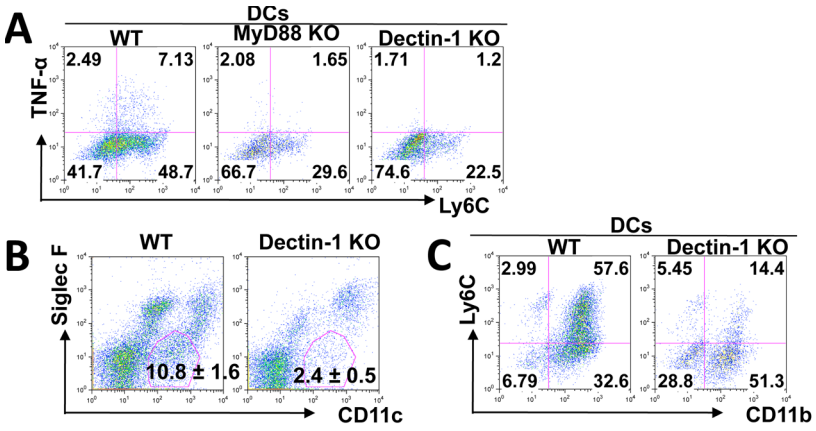

Figure 22. TLRs/MyD88 and dectin-1 are required for the generation of TNF-α-producing DCs

(A) Representative FACS plots show intracellular TNF-α staining in DC from WT, MyD88 or dectin-1 KO mice 24 h after the 5^{th} challenge. The plots show TNF-α vs Ly6C expression in DCs. (B) (C) Representative FACS plots show lung DC abundance in (B), and lung DC subsets in (C) in WT BALB/c or dectin-1 KO mice at 24 h after the final challenge. For DC abundance in B, plots show $CD45^+$ cells and $CD45^+$ $CD11c^+$ Siglec F^- lung DCs are shown in the gates. DCs were also confirmed as AF^{low} cells. The numbers indicate the percentage of DCs within the live cells. Results are mean ± SD (n=4 mice per group). For DC subsets in C, plots show Ly6C versus CD11b expression on DCs. The numbers indicate the percentage of cells in the gated population. Shown is an experiment representative of at least two.

4.4.4 Strain-specific differential expression of TLR2/dectin-1 on DCs during persistent fungal exposure

We next questioned whether DCs from the two strains differentially expressed critical PRRs, which subsequently lead to differential TNF-α production by DCs in the lungs of the two strains (Figure 15E and 12A). Based on the data generated (Figure 21 and 22), we focused on dectin-1 and TLR2. The expression of both receptors on DCs from the two strains was increasingly enhanced by repeated challenges (Figure 23A and summarized in 23B). By further examining the DC subsets, we found that both receptors were preferentially expressed on $CD11b^+$ $Ly6C^+$ DCs (Figure 23A). Notably, TLR2 was largely colocalized with dectin-1 on the same cells, generating dectin-1^+TLR2^+ DCs (Figure 23A). Comparing the two strains, there was no apparent difference in the baseline expression of TLR2 and dectin-1 in PBS control mice. One challenge produced a subtle difference in expression of the receptors. However, continuous challenges resulted in substantially higher TLR2 and modestly higher dectin-1 expression on BALB/c DCs relative to that on C57BL/6 DCs (Figure 23A and summarized in 23B). Consequently, a higher frequency of $TLR2^+$ Dectin-1^+ DCs was noted in the BALB/c mice (Figure 23A). 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, since DCs from both strains after repeated challenges produced similar levels of TNF-α in response to the dectin-1 agonist, curdlan (Figure 23C). With evidence of crosstalk between dectin-1 and TLR2 in promoting TNF-α in multiple types of cells (288), we proposed that higher levels of TLR2 on BALB/c DCs ultimately cause the remarkable difference in TNF-α output between these two types of DCs. This is supported by the evidence that BALB/c DCs secreted significantly more TNF-α upon stimulation with the TLR2 agonist, Pam3Cysk4 (Figure 23C). Notably, consistent with the observation that

macrophages expressed comparable levels of TLR2 or dectin-1 regardless of the strain origins (Figure 23D), macrophages failed to show strain-associated TNF-α production differences in response to both agonists (Figure 23C).

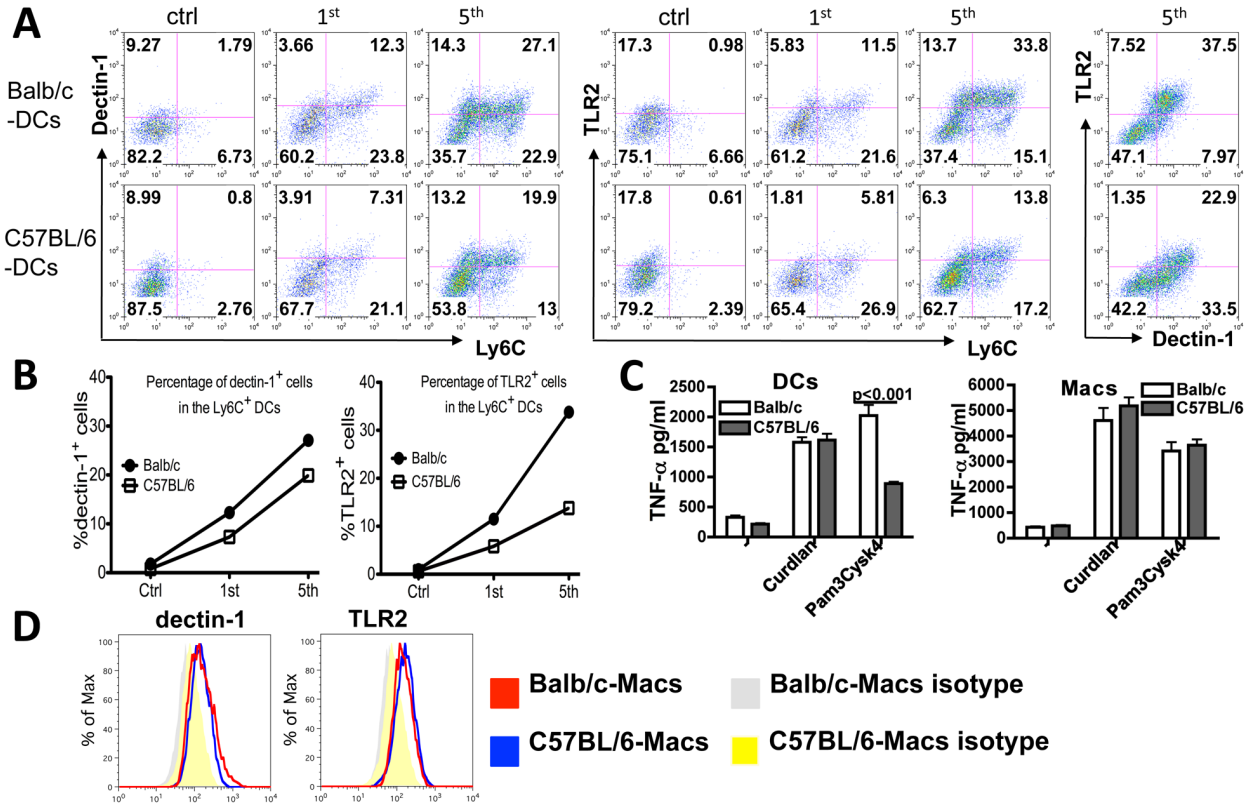

Figure 23. Strain-specific expression of TLR2/dectin-1 on DCs during repeated fungal challenges

(A) 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 cells were isolated from PBS control mice or challenged mice at 24 h after the 1st and 5th challenges. Unless otherwise indicated, lung DCs were gated as described in Figure 14A legend and the numbers in the FACS plots indicate the percentage of cells in the gated population. (B) The summary of FACS plots in (A). (C) FACS plots show dectin-1 or TLR2 expression on lung macrophages at 24 h after the 5th challenge. Lung macrophages were gated as described in Figure 14A legend. (D) ELISA of TNF-α in the culture supernatant. Macs or DCs were isolated 24 h after the 5th challenge, and cultured in vitro without stimulation (-) or stimulated with curdlan (dectin-1 agonist, CRD), Pam3Csk4 (TLR2 agonist, PAM), Results are mean ± SD. Experiments were repeated at least twice.

4.4.5 Strain-dependent activation of NF-κB pathway at early time points during fungal exposure

To further understand the mouse strain-specific TNF-α response in DCs, we decided to investigate more upstream mechanisms. We chose to assess TNF-α production at 4 h and 24 h after the 1st challenge, at which time there was no appreciable difference in TLR2 expression between DCs from the two mouse strains, as opposed to an obvious difference after the 5th challenge (Figure 24A). However, a modest strain-associated difference in DC-produced TNF-α was observed at an early time point (Figure 24B), although the difference was not as great as that observed after longer-term exposure (Figure 15E). These observations indicate that at early time points, some intrinsic events rather than PRR signaling likely initiate the TNF-α difference.

Given that multiple signaling pathways converge at the level of NF-κB in inflammatory responses (284), we assayed NF-κB activation. At 4 h or 24 h after the 1st challenge, the DNA-binding activity of NF-κB subunits in the nuclear fraction of DCs or macrophages isolated from the two strains was measured using the Active Motif assay. No significant binding activity of RelB or c-Rel was detected and no consistent and appreciable difference in the p52 binding activity between the two strains was identified (data not shown). Therefore we focused more on the p50 and p65 subunits of NF-κB. There was a considerably higher ratio of p50 versus p65 in the nuclei of the C57BL/6 DCs compared to that in the nuclei of BALB/c DCs (Figure 24C). This observation was reminiscent of LPS-induced hyporesponsiveness. In this context, the binding of p50 homodimers, acting as transcriptional repressors, to the κB3 site of murine TNF-α promoter blocks TNF-α transcription in response to prolonged treatment with LPS (286, 289). We therefore performed EMSA assays to analyze the binding of NF-κB subunits to the κB3 site of the TNF-α gene promoter in DCs from our model. We were not able to detect any protein

complexes at this site using the nuclear extracts prepared from lung DCs of naïve mice (Figure 24D). However, a protein complex was detected using the nuclear extracts prepared at 24h after the 1st challenge (Figure 24D). The supershift assays using anti-p50 and anti-p65 antibodies revealed that this binding complex contained p50 but not p65 subunits of NF-κB (Figure 24D). In line with the lower TNF-α profile of C57BL/6 DCs, the nuclei of C57BL/6 DCs showed greater p50 homodimer binding activity at this site (Figure 24D). This higher level of p50/p50 repression seemed to explain the relatively weaker ability of C57BL/6 DCs to produce TNF-α at early time points (Figure 24B). The repression of TNF-α transcription at this early time might have an impact on later events, such as TLR2 expression, since TNF-α has been reported to trigger TLR2 expression in many cell types (290-293).

Interestingly, macrophages from these two strains did not show this NF-κB difference (Figure 24C), consistent with their comparable TNF-α-producing abilities (Figure 15E). These results revealed an inherent cell-specific difference between macrophages and DCs in the DNA-binding activity of NF-κB subunits.

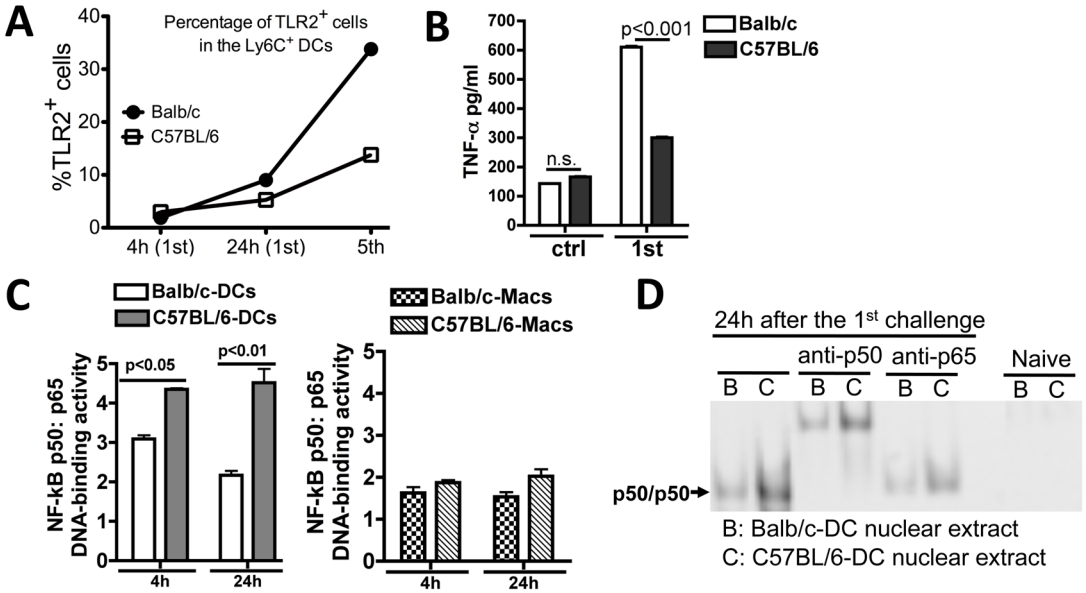

Figure 24. Differential NF-κB activation in the nuclei of DCs isolated from the two strains at early time points

(A) Lung DCs were gated as described in Figure 14A legend. TLR2 versus Ly6C expression on DCs were examined by FACS and the percentages of $TLR2^+$ DCs in the $Ly6C^+$ DC subset were calculated at 4 h, 24 h after the 1^{st} challenge or 24 h after the 5^{th} challenge. (B) ELISA of TNF-α in the culture supernatants. Lung DCs were isolated from PBS control mice or mice after the 1^{st} challenge and cultured in vitro (0.1 million cells/well) with/without SC restimulation (cells: SC = 1:2). Data are expressed as average ± SD (at least in triplicates in culture). (C) Plots show DNA-binding activity of NF-κB family members p50 and p65 in lung DCs and Macs at 4 h and 24 h after the 1^{st} challenge. Results shown are mean ± SD. (D) The binding complex at the κB3 site of the TNF-α gene promoter using nuclear extracts from DCs prepared 24 h after the 1^{st} challenge or from DCs of naïve mice was examined by EMSA (excess probe was running off the gel). B and C represent nuclear extracts from BALB/c and C57BL/6 mice respectively. Data shown are representative of at least two independent experiments.

4.4.6 TNF-α promotes the accumulation of inflammatory DCs

TNF-α not only regulated Th2/Th17 balance as described in section 3.4.9, but also seemed to regulate its own levels in our model. TNF-α deficiency resulted in a lower percentage of lung DCs (Figure 25A), especially that of the $CD11b^+Ly6C^+$ subset (Figure 25B) that was shown to contain a high number of TNF-α-producers in WT mice (Figure 14C and 22A). In addition, lack of TNF-α also caused less TLR2 and dectin-1 expression on DCs, rendering them less responsive to fungal stimulation (Figure 25B). These data demonstrate that TNF-α as an end product also promotes the generation of inflammatory lung DCs capable of pathogen recognition and TNF-α production, seemingly setting up a positive feedback loop to amplify the inflammatory cascade.

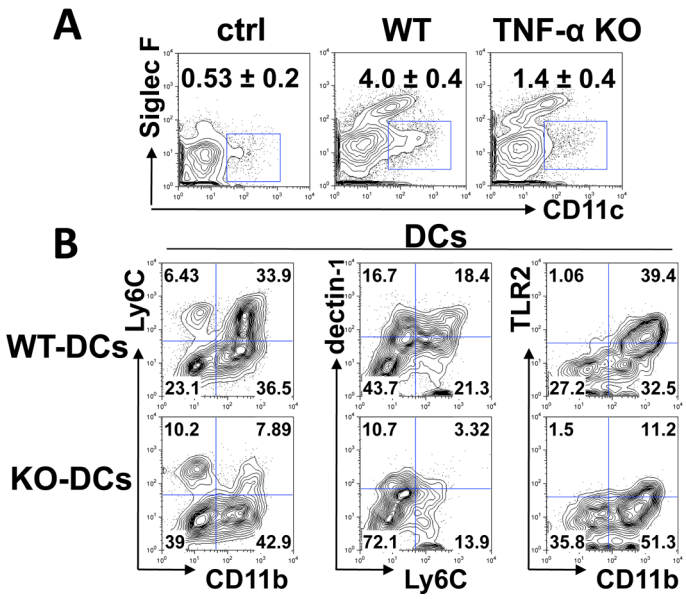

Figure 25. TNF-α promotes DC generation and PRRs expression in vivo

(A) (B) Representative FACS plots show lung DC abundance, DC subsets and PRR expression on DCs isolated from PBS control, challenged WT BALB/c and TNF-α KO mice at 24 h after the 8th challenge. For DC abundance shown in A, plots were gated on CD45^{+} AF^{low} cells (AF^{high} Macs were excluded from the analyses). Lung DCs are CD45^{+} AF^{low} CD11c^{+} Siglec-F^{-}, and the numbers indicate the percentage of DCs within the live cells. Results are expressed as mean ± SD (n=4 mice per group). (B) The plots were gated on lung DCs as described in Figure 14A legend, and the numbers indicate the percentage of cells in the gate. Experiments were repeated at least twice.

4.4.7 A proposed model of TNF-α regulation in DCs during persistent fungal exposure

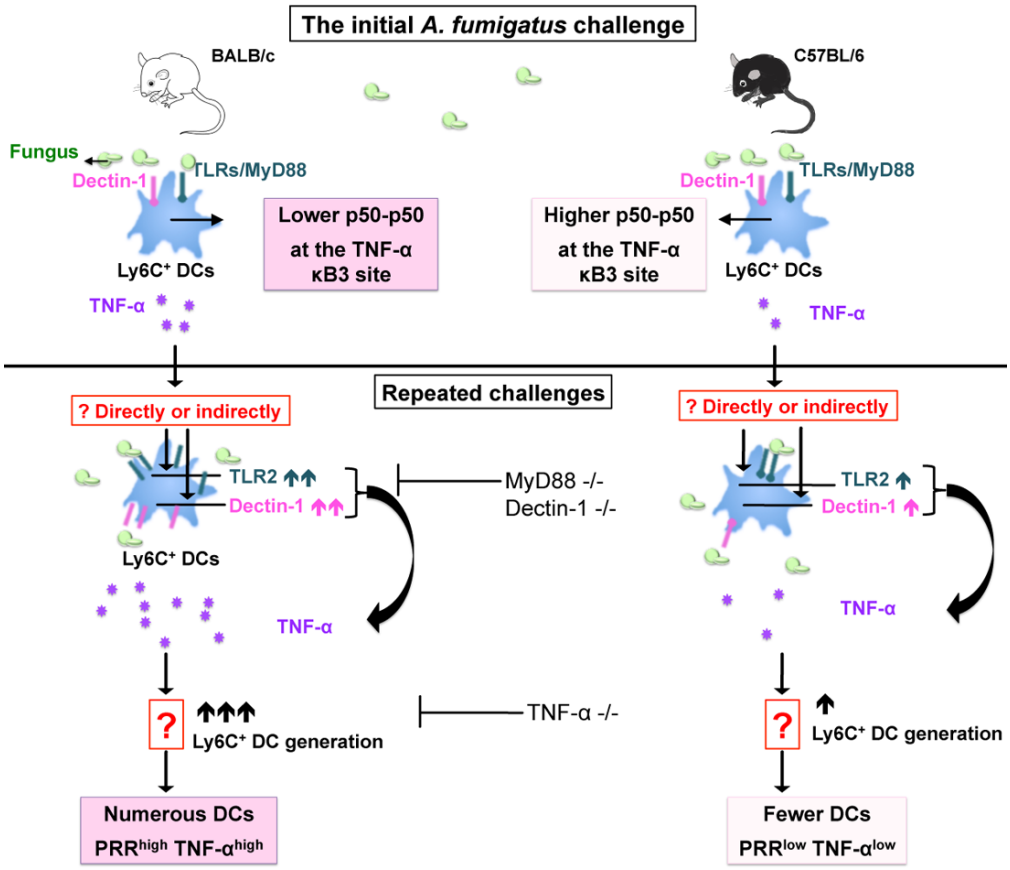

Figure 26. A proposed model of strain-specific TNF-α regulation in DCs during persistent exposure to conidia

Seven days after sensitization, there is comparable expression of PRRs on BALB/c DCs and C57BL/6 DCs. In response to the first challenge with conidia, these PRRs expressed at basal levels recognize fungus and initiate inflammatory cascade in DCs. At early time points (following this first challenge), as compared to BALB/c DCs, C57BL/6 DCs have a higher content of p50 homodimers at the κB3 site of the TNF-α gene. Therefore, the TNF-α gene repression mediated by p50 homodimers leads to less TNF-α produced from C57BL/6 DCs than that from BALB/c DCs, establishing the initial strain-associated TNF-α difference. Given that TNF-α upregulates TLR2 and dectin-1 expression on DCs through direct or indirect mechanisms, this early TNF-α difference results in a difference in the expression of PRRs (e.g. TLR2 and dectin-1). These PRRs continue to mediate fungal recognition and TNF-α responses during persistent fungal exposure, therefore amplifying the strain-associated TNF-α difference. In addition, TNF-α as an end product of DCs promotes the generation of TNF-α-producing DCs through direct or indirect mechanisms, setting up a positive feedback loop. As a result, there is massive accumulation of TNF^{high} DCs in the BALB/c mice whereas only a limited accumulation of TNF-α^{low} DCs in the C57BL/6 mice, thus leading to the drastic difference in lung TNF-α levels between these two mouse strains.

4.5 DISCUSSION

Using the animal model we developed, we demonstrated that lung TNF-α level was critical in determining mouse strain-specific intensity of neutrophilia and eosinophilia. In humans, TNF-α promoter polymorphisms causing differential TNF-α levels have been associated with the onset and severity of asthma (294), substantiating the relevance of our animal model to human disease. Therefore, increasing our understating of TNF-α regulation in mice may shed light on human disease processes. Here, we elucidated the mouse strain-dependent regulation of TNF-α by NF-κB, PRRs and TNF-α itself in DCs, which mechanistically explained the strain-specific TNF-α-producing capacity of DCs. Notably, this regulation was not observed in macrophages (see proposed model in Figure 26).

The mouse strain-associated differences in dectin-1 expression or in the usage of dectin-1 isoforms have been documented in several published reports. Dectin-1 levels were found to be 5 fold less on thioglycollate-elicited peritoneal macrophages from BALB/c mice compared to C57BL/6 mice. Interestingly, BALB/c peritoneal macrophages express similar levels of full and truncated isoforms of dectin-1, while C57BL/6 macrophages predominantly express the truncated isoform (295). Subsequent studies have indicated that the expression of this truncated isoform in C57BL/6 mice is a general phenomenon, and not organ-specific or altered by fungal infections (296). Functional studies of these isoforms have yielded contradictory results. Cell lines transduced with truncated dectin-1 have been shown to either produce more (295) or less (296) TNF-α than cells overexpressing full-length dectin-1 in response to dectin-1 activation. The reasons for such discrepancy are not known. However, it is possible that the reagents used to activate dectin-1 in these studies engage other PRRs simultaneously, therefore TNF-α levels might reflect integrated input from multiple PRRs instead of overexpressed dectin-1 isoforms

alone. BALB/c BMDCs seemed to produce less TNF-α despite higher dectin-1 levels on these cells in comparison with C57BL/6 BMDCs. However, the usage of dectin-1 isoforms has not been assessed in this study (297). In our model, DCs of different strain origins demonstrated a difference in dectin-1 expression but failed to show any difference in TNF-α production upon dectin-1 ligation, raising the possibility that these lung DCs might bear stain-specific dectin-1 isoforms with variable biological activities, which overrides the expression difference.

The regulation of dectin-1 expression has been explored, particularly in macrophages. IL-4/IL-13 and to a lesser extent GM-CSF were found to upregulate dectin-1. In contrast, dexamethasone, IL-10 and LPS were identified as negative regulators. Notably, at least in the case of macrophages, TNF-α and M-CSF had no effects on dectin-1 expression (298). Unlike previous studies, we found that TNF-α deficiency did result in a reduction in dectin-1 expression on DCs in vivo. One possible explanation is that TNF-α modulates dectin-1 expression through an indirect mechanism, for instance, through GM-CSF induction. This is quite possible since TNF-α, as a potent proinflammatory molecule, promotes cytokine/chemokine production, characteristic of an inflammatory microenvironment.

The initial evidence implicating TLR2 regulation seems to have been established in macrophages and T cells. Two murine macrophage cell lines, RAW264.7 and J774, were found to elevate TLR2 but not TLR4 mRNA levels rapidly in response to LPS and cytokines IL-2, IL-15, IL-1β, IFN-γ and TNF-α, which seemed to rely on NF-κB signaling (299). T cells were shown to upregulate TLR2 expression by TCR engagement or IL-2 and IL-15 stimulation (300). Further analysis of the TLR2 promoter region revealed that two NF-κB binding sites in the 5' region of the gene mediated TNF-α responsiveness in macrophages (290). Later, TNF-α was implicated in TLR2 induction in many cell types, such as primary cultured murine hepatocytes

(291), microglia (292) and astrocytes (293). Certain pathogens, such as *Staphylococcus aureus*, augment TLR2 levels through TNF-α (292). Considering the direct and critical role for TNF-α in regulating TLR2, the TLR2 downregulation that we observed in the absence of TNF-α is likely to be a primary effect.

As discussed in section 3.5, the inflammatory $CD11b^+$ $Ly6C^+$ DCs in our model are highly likely to be derived from monocytes, but the mechanisms associated with their development have not been well studied. Recently, adoptive transfer experiments revealed that in *A. fumigatus*-infected mice, there was a rapid accumulation of $CD11b^+$ DCs derived from monocytes following transfer of BM cells. In contrast, in naïve mice, fewer transferred BM monocytes migrated to the lung and they were instead maintained as $CD11c^-MHCII^-$ monocytes but not as DCs. These observations indicate that fungus-induced signals are required for monocyte-to-DC conversion (18). It is possible that monocytes differentiate into DCs directly in response to fungal stimulation through interactions between fungal PAMPs and PRRs on monocytes, or in response to cytokines triggered by the fungus and produced by monocytes themselves or by bystander cells. With regard to cytokines involved in controlling monocyte-derived DCs differentiation, multiple lines of evidence have highlighted the critical role of GM-CSF (271). TNF-α was found to skew human monocytes towards DCs but away from a macrophage lineage in an in vitro culture system (301), however in vivo data are lacking. In general, TNF-α is more often considered as a cytokine associated with DC maturation rather than one promoting monocyte-to-DC conversion. In our system, TNF-α deficiency greatly reduced the number of the $CD11b^+$ $Ly6C^+$ DCs. This raises the possibility that TNF-α may enhance DC differentiation from monocytes through direct mechanisms or indirectly through the action of other factors, such as GM-CSF. Alternatively, TNF-α could possibly recruit DCs by way of

chemokine induction (268) or TNF-α may act as an autrocrine factor to promote cell survival as was shown in the case of macrophages (302). Further studies are needed to elucidate the mechanism of TNF-α activity.

It has been long established in animal models as well as in humans that cells or organisms following prior exposure to a sublethal dose of endotoxin (such as LPS) demonstrate a transient hyporesponsiveness to subsequent endotoxin challenge, which is referred to as endotoxin tolerance. Many proinflammatory genes are downregulated in tolerant cells, of which TNF-α appears to the one that has been described in most detail (303). Endotoxin tolerance has been widely studied and a number of mechanisms have been suggested. In tolerance induced by prolonged LPS treatment, a predominance of p50 homodimers over the active p50/p65 heterodimers was observed in the nuclei of various cell types (289, 304, 305). The binding of p50 homodimers to the κB3 site of the TNF-α gene promoter has been suggested as a mechanism leading to TNF-α gene repression (286). The biological significance of endotoxin tolerance seems to involve limiting deleterious inflammation and maintaining immune homeostasis (303). We found the generation of a "less inflammatory" type of DCs with enriched p50 homodimers at the κB3 site of the TNF-α promoter in the C57BL/6 strain, which to some extent resemble the cells with prolonged LPS treatment. Therefore, this p50 predominance in C57BL/6 DCs could also be the consequence of elaborate self-control strategies in response to repeated TLR and dectin-1 stimulation in order to avoid deleterious outcomes. The precise mechanism that leads to this stain-specific NF-κB pattern is not known, however it very well may be related to genetic variability.

4.6 ACKNOWLEDGEMENTS

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5.0 CONCLUSIONS, SIGNIFICANCE AND FUTURE DIRECTIONS

A. fumigatus, a ubiquitous fungus in the environment, is associated with a spectrum of allergic pulmonary disorders, including one form of asthma and ABPA. ABPA is a complication in patients with severe obstructive lung disease, mainly asthma and CF, and it has been estimated to occur in 1-2% of asthmatics (9) but has been suggested to be even more common in severe asthmatics (11). ABPA is characterized by allergic responses in the early phase, lung injury and remodeling in the late phase, and may progress into central bronchiectasis, therefore representing one of the most extreme manifestations of fungal allergy. The Th2-eosinophil axis has long been recognized as a mechanism leading to lung pathology (9), and in recent years there has been an increasing appreciation for the detrimental contribution of neutrophils to tissue injury (115, 116, 118). However, regulatory mechanisms underlying this neutrophil infiltration are poorly understood. Our incomplete knowledge might in part be due to lack of adequate experimental models that can faithfully recapitulate the neutrophilic component of conditions such as ABPA.

Given that persistent presence of *A. fumigatus* antigens in the respiratory system is likely associated with susceptibility to ABPA in humans, repeated challenge of mice with this fungus has been exploited for the establishment of a novel experimental model in our study. This exposure protocol resulted in coexistence of Th1/Th2/Th17 and pathologic manifestation of pulmonary allergy in both BALB/c and C57BL/6 mice. Importantly, repeated exposures to *A. fumigatus* elicited a prominent neutrophil infiltration into airways as well as lung tissue in

BALB/c mice but an eosinophil-biased inflammation in C57BL/6 mice. The intense neutrophilic inflammation that occurs in the allergic setting in BALB/c mice to some extent resembles the inflammation pattern in patients with ABPA, thus providing a tool to explore the mechanisms for neutrophil infiltration. Subsequent mechanistic studies have suggested a key role for TNF-α, in particular, TNF-α derived from inflammatory DCs in driving pulmonary neutrophilia. We also have revealed that genetically distinct mouse strains differentially regulate TNF-α gene in DCs and thus generate DCs with strain-specific TNF-α-producing capability, which consequently contribute to strain-associated differential neutrophil and eosinophil influx (as summarized in Figure 27).

Our study highlights the dramatic distinctions in many aspects between acute infection with *A. fumigatus* and persistent exposure to this fungus, such as T helper subset differentiation and consequent neutrophil/eosinophil regulation. Considering that ABPA commonly occurs in pulmonary diseases characterized by impaired pathogen clearance, our findings provide evidence in support of the association between fungal persistence and disease predisposition.

The precise mechanism leading to mouse strain-specific regulation in response to the same fungal stimulation is incompletely understood, and could fundamentally be linked to underlying genetic factors. In humans, individuals have variable susceptibility to ABPA, an issue that has not been well addressed although genetic variation has been suggested as a predisposing factor (as discussed in 1.2.2). To our knowledge, correlation between TNF-α levels and disease severity in human ABPA has not been reported, and our study provides a rationale to assess TNF-α levels in human ABPA. However, there is evidence that TNF-α promoter polymorphisms causing differential TNF-α levels is associated with the onset and severity of asthma (294). Therefore, our finding of differential TNF-α regulation in mice may have broader relevance in

human disease. Development of a deeper understanding of this experimental model might shed additional light on the pathogenesis of human ABPA and other diseases.

Our findings reinforce the necessity to maintain a delicate and adequate balance between immunoprotection and immune-mediated injury. Although TNF-α and neutrophilia are considered to be protective mechanisms in host defense against *A. fumigatus*, dysregulated, exaggerated inflammation that develops in BALB/c mice following persistent exposure to this fungus tends to cause tissue damage and thus is not beneficial to the host. Our study suggests that adequate control of the excessive inflammation by targeting TNF-α may help ameliorate the disease symptoms associated with human ABPA.

The future directions of this study will involve investigating the mechanism underlying IL-17A and TNF-α cooperation. We found that lack of either reduced neutrophil chemoattractants in our animal model, but it is not known if these two cytokines exert their functions additively or synergistically in vivo. Moreover, the cellular sources of the chemokines have not been characterized in this study. It has been well established that in vitro, IL-17 and TNF-α have synergistic effects on expression of various chemokines by airway epithelial cells or endothelial cells. However, it was interesting to note that in our model, infiltrated inflammatory mononuclear cells, such as macrophages or DCs, but not epithelial cells, appeared to be the sources of KC and MIP-2 based on in situ hybridization data. Further studies are needed to verify cell types that produce these neutrophil chemoattractants. Following that, we can explore how IL-17A and TNF-α signaling pathways cross-talk with each other in these cell types to promote chemokine responses.

Our data suggested that TNF-α-producing DCs were likely derived from monocytes, but further experiments, such as adoptive transfer experiments, are needed for confirmation. In our model, TNF-α appears to be involved in the development of TNF-α-producing DCs and we have proposed several possible mechanisms (as discussed in section 4.5). It would be important to test these hypotheses.

Finally, our data suggest that the differential regulation of NF-κB at early time points most likely initiates the mouse strain-associated TNF-α difference in the DCs. However, the exact mechanisms leading to this differential NF-κB regulation has yet to be defined. To gain further insight, we can take advantage of gene expression microarrays to define specific transcriptional signatures of DCs from these two mouse strains following persistent fungal exposure. This approach may lead to unbiased identification of genes or pathways involved in the strain-specific NF-κB regulation that would open up a new area for future investigation.

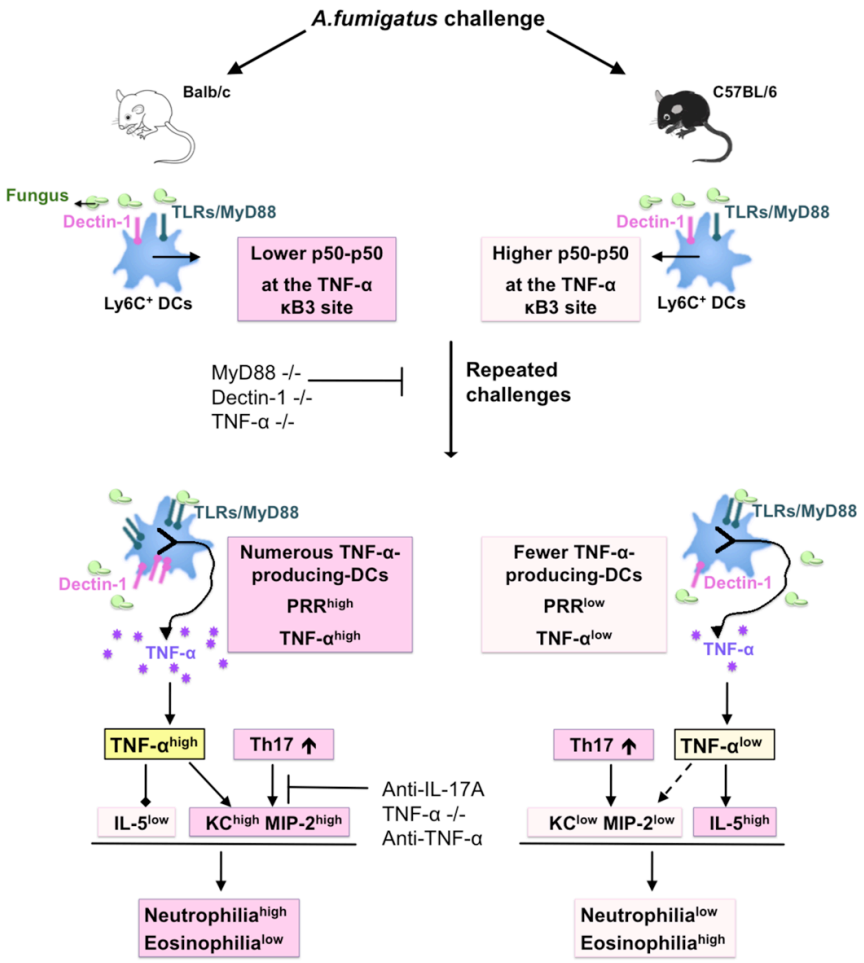

Figure 27. The illustration depicts the sequence of events that elicits differential granulocyte infiltration in the lungs of BALB/c and C57BL/6 mice during persistent exposure to *A. fumigatus* conidia

In the BALB/c mice, both TNF-α and IL-17A are required for the intense airway neutrophilia in response to persistent fungal exposure. TNF-α is mainly derived from inflammatory DCs and macrophages through collaboration between 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 1st challenge, the nuclei of DCs in C57BL/6 mice have a higher content of p50 homodimers that can bind to the κB3 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 and a relatively lower level of TLR2 expression on the cell surface. Since TNF-α promotes the development of TNF-α-producing DCs, C57BL/6 mice have fewer TNF-α-producing DCs and macrophages compared to 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.

6.0 RELEVANT PUBLICATIONS

1. Fei, M., Bhatia, S., Oriss, T.B., Yarlagadda, M., Khare, A., Akira, S., Saijo, S., Iwakura, Y., Fallert Junecko, B.A., Reinhart, T.A., Foreman, O., Ray, P., Kolls, J., Ray, A. 2011. TNF-α from inflammatory dendritic cells (DCs) regulates lung IL-17A/IL-5 levels and neutrophilia versus eosinophilia during persistent fungal infection. *Proc Natl Acad Sci U S A* 108:5360.
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