Facilitation of Allergic Sensitization and Allergic Airway Inflammation by Pollen-Induced Innate Neutrophil Recruitment

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Abstract

Neutrophil recruitment is a hallmark of rapid innate immune responses. Exposure of airways of naive mice to pollens rapidly induces neutrophil recruitment. The innate mechanisms that regulate pollen-induced neutrophil recruitment and the contribution of this neutrophilic response to subsequent induction of allergic sensitization and inflammation need to be elucidated. Here we show that ragweed pollen extract (RWPE) challenge in naive mice induces C-X-C motif ligand (CXCL) chemokine synthesis, which stimulates chemokine (C-X-C motif) receptor 2 (CXCR2)-dependent recruitment of neutrophils into the airways. Deletion of Toll-like receptor 4 (TLR4) abolishes CXCL chemokine secretion and neutrophil recruitment induced by a single RWPE challenge and inhibits induction of allergic sensitization and airway inflammation after repeated exposures to RWPE. Forced induction of CXCL chemokine secretion and neutrophil recruitment in mice lacking TLR4 also reconstitutes the ability of multiple challenges of RWPE to induce allergic airway inflammation. Blocking RWPE-induced neutrophil recruitment in wild-type mice by administration of a CXCR2 inhibitor inhibits the ability of repeated exposures to RWPE to stimulate allergic sensitization and airway inflammation. Administration of neutrophils derived from naive donor mice into the airways of *Tlr4* knockout recipient mice after each repeated RWPE challenge reconstitutes allergic sensitization and inflammation in these

mice. Together these observations indicate that pollen-induced recruitment of neutrophils is TLR4 and CXCR2 dependent and that recruitment of neutrophils is a critical rate-limiting event that stimulates induction of allergic sensitization and airway inflammation. Inhibiting pollen-induced recruitment of neutrophils, such as by administration of CXCR2 antagonists, may be a novel strategy to prevent initiation of pollen-induced allergic airway inflammation.

Keywords: neutrophil; allergic inflammation; CXCR2; reactive oxygen species; Toll-like receptor 4

Clinical Relevance

The observations in this manuscript indicate that pollen-induced recruitment of neutrophils is Toll-like receptor 4 and chemokine (C-X-C motif) receptor 2 (CXCR2) dependent and that recruitment of neutrophils is a critical rate-limiting event that stimulates induction of allergic sensitization and airway inflammation. Inhibiting pollen-induced recruitment of neutrophils, such as by administration of CXCR2 antagonists, may be a novel strategy to prevent initiation of pollen-induced allergic airway inflammation.

Neutrophil recruitment to an inflamed site is a hallmark of early innate immune responses (1). Allergen challenge in subjects with asthma or seasonal allergic rhinitis has been reported to stimulate IL-8 secretion (2–4) and

neutrophil recruitment (5) to the airways. Pollens contain numerous intrinsic factors that can stimulate an innate immune response (6). We and other have reported that intranasal challenge with pollen extract rapidly

recruits neutrophils into the airways of naive mice (5, 7–10). However, the innate immune mechanisms that control and regulate pollen-induced recruitment of neutrophils to the airways have not been critically evaluated.

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There has been growing interest in defining the additional effects of neutrophils outside of their bacteriocidal effects (11, 12). Of considerable interest is their ability to modulate innate and adaptive immune responses (11, 12). An example of this ability was reported in the recent landmark study that demonstrated that neutrophil recruitment to the skin is essential for induction of subsequent allergic cutaneous inflammation (13). However, the contribution of pollen-induced innate recruitment of neutrophils to subsequent allergic sensitization and airway inflammation has not been critically evaluated. The objectives of the present study were to elucidate the innate mechanisms of the rapid recruitment of neutrophils to the airways within hours of exposure to pollen allergenic extract and to examine the contribution of these recruited neutrophils to the induction of allergic sensitization and airway inflammation.

Materials and Methods

Mice

Male wild-type (WT) C57BL/10SNJ mice (8–12 wk old) and *Toll-like receptor 4* (*Tlr4*) knock-out (KO) mice (C57BL/10ScNJ) were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were maintained in a pathogen-free environment at the University of Texas Medical Branch (Galveston, TX). Animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Experimental Animals and were approved by the UTMB Animal Care and Use Committee (approval no. 9708038A).

Allergenic Extracts

Lyophilized ragweed pollen extract (RWPE) was purchased from Greer Labs (Lenoir, NC). Endotoxin levels in RWPE were quantified using an LAL chromogenic endotoxin quantitation kit (Thermo Scientific, Hudson, NH). The levels were exceedingly low (<0.1 pg/ μ g RWPE protein).

Protocols Used for Animal Studies

Mice were sedated with low-dose intraperitoneal xylazine-ketamine anesthetic mixture for intranasal sensitization or challenge and killed by lethal intraperitoneal xylazine/ketamine overdose (7, 14).

Single-challenge model. WT mice and *Tlr4* KO mice were intranasally challenged

with a single dose of 100 μ g/60 μ l RWPE reconstituted from lyophilized RWPE (Greer Laboratories, Lenoir, NC) and killed after 0.5, 1, 4, 16, or 72 hours. In some experiments, to generate superoxide (7, 15), *Tlr4* KO mice were intranasally challenged with 0.32 mM xanthine (X) (Sigma-Aldrich, St. Louis, MO) with 50 mU xanthine oxidase (XO) (Sigma-Aldrich) in the presence or absence of RWPE. In some experiments, 1 hour before RWPE challenge, WT mice were treated with intranasal administration of 4 mg/kg body weight chemokine (C-X-C motif) receptor 2 (CXCR2) inhibitor SB225002 (Calbiochem, San Diego, CA) and challenged with RWPE and killed as described above.

Repeated-challenge model. WT mice or *Tlr4* KO mice were sensitized by five intranasal doses of RWPE (100 μ g/60 μ l) on Days 0, 1, 2, 3, and 4. These mice were challenged with intranasal RWPE or PBS on Day 11 and killed on Day 14 (14). In addition, WT mice were sensitized by five intranasal doses of RWPE on Days 0, 1, 2, 3, and 4 with RWPE with or without the intranasal administration of 4 mg/kg body weight SB225002 (16) 1 hour before each intranasal dose of RWPE. The mice were challenged with intranasal RWPE on Day 11 and killed on Day 14 (14). In some experiments, *Tlr4* KO mice were sensitized by five intranasal doses of RWPE on Days 0, 1, 2, 3, and 4 in the presence or absence of X+XO and challenged with intranasal RWPE in the presence or absence of X+XO on Day 11 and killed on Day 14 as described above (14). In some experiments, RWPE and negatively selected neutrophils derived from bone marrow of donor mice were repeatedly intranasally instilled in *Tlr4* KO mice. The purity of neutrophils was assessed to be 99% by FACS. *Tlr4* KO mice were intranasally administered neutrophils (4×10^6 neutrophils per mouse) 8 hours after intranasal instillation of RWPE on Days 0, 1, 2, 3, 4, and 11 and killed on Day 14 as described previously to evaluate allergic inflammation.

Results

A Single RWPE Challenge in Naive Mice Induces Chemokine (C-X-C Motif) Ligand 2 Chemokine Secretion and Neutrophil Recruitment

We used RWPE as a model system to define rapid innate events because ragweed pollen

allergy is a common cause of human atopic disease (17). All experiments were performed using RWPE with extremely low levels of endotoxin (i.e., <0.1 pg/ μ g RWPE protein). A single challenge of RWPE (Figure 1A) in naive C57BL WT mice recruited neutrophils in BALF, peaking (kinetic data not shown) 16 hours after challenge (Figure 1B). At 72 hours after RWPE challenge, there was no detectable increase in BALF neutrophils (Figure 1B). At both 16 hours and 72 hours after challenge, there was no detectable increase of eosinophils, lymphocytes, macrophages, or mucin in the airway epithelium (data not shown).

Next we focused our efforts on identifying the mechanism of RWPE-induced neutrophil recruitment into the airways of naive mice. Neutrophil recruitment from blood to a sterile extravascular site is a hallmark of the innate immune response and has been reported to be dependent specifically on leukotriene B₄ (LTB₄) (1). Secretion of LTB₄ from skin tissue has been reported to recruit neutrophils that drive subsequent cutaneous allergic inflammation (13). Based on these earlier reports (1, 13), we initially hypothesized that neutrophil recruitment induced by RWPE challenge was induced by LTB₄ secretion. Unexpectedly, RWPE challenge did not induce a measurable increase in BALF LTB₄ levels at any time point (data not shown), implying an alternative mechanism of RWPE-induced neutrophil recruitment to the airways. Because neutrophil recruitment is mediated by C-X-C motif ligand (CXCL) chemokines (18), we next investigated whether RWPE induced secretion of CXCL chemokines. A single RWPE challenge (Figure 1A) in naive WT mice induced secretion of CXCL1 and CXCL2 in BALF in 4 hours (Figure 1C).

Because deficiency of gp91phox, the major NADPH oxidase in neutrophils, has been reported to decrease reactive oxygen species (ROS) generation in lungs and to induce allergic airway inflammation (19), we next hypothesized that pollen-recruited neutrophils are likely “activated” and generate superoxide. To test this hypothesis, we performed *ex vivo* analysis of BALF cells for superoxide generation at 30 minutes after challenge when no neutrophils are recruited and at 16 hours after challenge, the peak of neutrophil recruitment, with no other cell types increasing significantly. RWPE challenge in

Figure 1. Ragweed pollen extract (RWPE) challenge induces an innate immune response in the airways. (A) Single-challenge model protocol. (B) Bronchoalveolar lavage fluid (BALF) neutrophil number. RWPE challenge in naive wild-type (WT) mice recruited neutrophils into airways 16 and 72 hours after challenge ($n = 5-7$ per group). (C) BALF levels of chemokine (C-X-C motif) ligand (CXCL) 1 and CXCL2 4 hours after challenge. RWPE challenge in naive WT mice increases CXCL1 and CXCL2 in airways 4 hours after challenge. (D) *Ex vivo* superoxide generation from BALF cells of WT mice. WT mice were killed at 30 minutes and 16 hours after RWPE challenge ($n = 3-5$ per group), and *ex vivo* superoxide generation from BALF cells was quantified. There was no difference in superoxide generation in any treatment group 30 minutes after challenge. However, at 16 hours after challenge, the RWPE challenge group produced more superoxides. Data are expressed as means $\pm$ SEM. * $P < 0.05$. ** $P < 0.01$. **** $P < 0.0001$. NS, not significant.

WT mice increased superoxide generation from BALF cells 16 hours after challenge but not at 30 minutes after challenge (Figure 1D). Together these observations indicate that a single RWPE challenge induces an innate immune response characterized by CXCL chemokine secretion and recruitment of neutrophils and suggest that these recruited neutrophils are likely activated and produce superoxides.

Toll-Like Receptor 4 Mediates RWPE-Induced CXCL1/2 Secretion and Recruitment of Neutrophils to the Airways

Building on our observations that RWPE induces an innate immune response characterized by CXCL1/2 secretion and recruitment of neutrophils, we attempted to identify the innate mechanism of RWPE-

induced CXCL chemokine synthesis. Because stimulation of Toll-like receptor 4 (TLR4) has been shown to induce CXCL chemokines (20), we hypothesized that TLR4-mediated RWPE induces chemokine synthesis. A single RWPE challenge (Figure 1A) in WT mice increased lung mRNA expression of neutrophil-recruiting CXC chemokines *Cxcl1* and *Cxcl2* at 1 hour (Figure 2A), induced secretion of CXCL1 and CXCL2 in BALF at 4 hours (Figure 2B), and stimulated recruitment of neutrophils at 16 hours (Figure 2C). Consistent with our hypothesis, deletion of TLR4 reduced CXCL1 and CXCL2 mRNA expression by 95%, secretion of CXCL1 and CXCL2 by 80 to 90%, and recruitment of neutrophils by 97%. Furthermore, compared with superoxide generation from BAL cells at 16 hours in naive WT mice (Figure 2D), deletion of TLR4 reduced RWPE-induced

superoxide generation from BALF cells by 82% (Figure 2D). These observations indicate that TLR4 regulates RWPE-induced innate immune response consisting of CXCL chemokine secretion and recruitment of neutrophils and provide additional evidence that neutrophils are the likely cell source of superoxide generation.

Blocking RWPE-Induced Recruitment of Activated Neutrophils to the Airways by Deletion of TLR4 also Blocks Induction of Allergic Airway Inflammation

Building on our observation that TLR4 mediates RWPE-induced CXCL chemokine synthesis and recruitment of neutrophils, we used *Tlr4* KO mice to test its ability to block allergic airway inflammation. Repeated intrapulmonary exposure of naive mice to RWPE without intraperitoneal injections of alum and allergen mimics chronic exposure of human airways to pollen allergen and facilitates induction of allergic inflammatory response to a later challenge with RWPE (14). To examine the contribution of RWPE-induced innately recruited neutrophils to the development of allergic inflammation, we performed a repeated-challenge model in WT mice and *Tlr4* KO mice (Figure 3A). As expected, in WT mice repeated RWPE challenge increased BALF eosinophils (Figure 3B), BALF total inflammatory cells (Figure 3B), airway epithelial mucin secretion (Figures 3C and 3D), RWPE-specific IgE in serum (Figure 3E), and BALF levels of IL-5, IL-13, thymic stromal lymphopoietin (TSLP), and IL-33 (Figure 3F). By contrast, repeated RWPE challenge in *Tlr4* KO mice (Figures 3B–3F) failed to increase any of these parameters. These observations indicate that the intensity of allergic inflammation 72 hours after RWPE challenge mirrors the intensity of RWPE-induced innate immune response 1 to 16 hours after challenge, and both are TLR4 regulated. Based on these “mirroring data,” we hypothesized that RWPE-induced innate neutrophil recruitment stimulates allergic sensitization and inflammation.

Forcing Recruitment of Innate Immune Response in *Tlr4* KO Mice Reconstitutes RWPE-Induced Allergic Inflammation

To test this hypothesis, we attempted to force neutrophil recruitment in *Tlr4* KO mice to determine if this approach would

Figure 2. Deletion of Toll-like receptor 4 inhibits RWPE challenge-induced innate immune response. (A) CXCL1 and CXCL2 mRNA expression in lungs. RWPE challenge increased the expression of CXCL1 and CXCL2 mRNA 1 hour after challenge in naive WT mice but not in *Tlr4* knockout (KO) mice ($n = 3$ per group). (B) BALF levels of CXCL1 and CXCL2. RWPE challenge increased CXCL1 and CXCL2 levels 4 hours after RWPE challenge in naive WT mice but not in *Tlr4* KO mice ($n = 3$ –5 per group). (C) BALF neutrophil numbers. RWPE challenge increased the number of neutrophils in BALF at 16 hours after challenge in naive WT mice but not in *Tlr4* KO mice ($n = 5$ –7 per group). (D) *Ex vivo* superoxide generation from BALF cells. After PBS or RWPE challenge, *ex vivo* superoxide generation from BALF was quantified. *Ex vivo* superoxide generation from BALF cells increased 16 hours after RWPE challenge in naive WT mice but not in *Tlr4* KO mice ($n = 3$ –5 per group). Data are expressed as means $\pm$ SEM. * $P < 0.05$. ** $P < 0.01$. **** $P < 0.0001$.

also reconstitute development of allergic airway inflammation. We initially attempted to force neutrophil recruitment in *Tlr4* KO mice by intranasal administration of a superoxide generator, xanthine + xanthine oxidase (X+XO). However, challenge with X+XO failed to stimulate CXCL1 chemokine secretion (Figure 4A) or to induce neutrophil recruitment 16 hours after challenge (Figure 4B). Next, we intranasally coadministered a cocktail of RWPE and X+XO. This cocktail successfully stimulated CXCL1 chemokine secretion (Figure 4A) and restored RWPE-induced neutrophil recruitment in the airways of *Tlr4* KO mice (Figure 4B). We used this ability of RWPE+X+XO to reconstitute neutrophil recruitment in *Tlr4* KO mice to test our hypothesis that RWPE challenge-induced repeated recruitment of

neutrophils facilitates induction of allergic inflammation. We performed repeated instillation of X+XO or RWPE with or without X+XO into the lungs of *Tlr4* KO mice. Repeated challenge with X+XO in isolation failed to induce allergic inflammation (data not shown). By contrast, repeated challenge with a cocktail of RWPE and X+XO also reconstituted allergic inflammation in *Tlr4* KO mice (Figures 4C–4E). This allergic inflammation was characterized by recruitment of 300% higher eosinophils (Figure 4C), 160% higher total inflammatory cells (Figure 4C), and 1,250% higher mucin secretion in airway epithelial cells (Figures 4D and 4E). Together these observations give further credibility to our hypothesis that RWPE-induced recruitment of neutrophils is a critical rate-limiting event that stimulates induction of

pollen-induced allergic airway inflammation.

CXCR2 Mediates RWPE-Induced Recruitment of ROS-Generating Activated Neutrophils

We next focused our efforts on validating our hypothesis that RWPE-induced innate recruitment of neutrophils facilitates induction of allergic airway inflammation. CXCR2, the shared receptor for CXCL1 and CXCL2, has been shown to regulate neutrophilic inflammation (18). SB225002 is a small molecule inhibitor of ligand binding to CXCR2 that has been used by investigators to inhibit CXCR2-driven neutrophilic inflammation (18). To define the role of CXCL1 and CXCL2 in recruitment of neutrophils in our study, we tested the ability of SB225002 to inhibit RWPE-mediated neutrophil recruitment. Administering SB225002 before RWPE challenge in the single-challenge model (Figure 5A) suppressed neutrophil recruitment into the lungs by 66% (Figure 5B). Administration of this inhibitor before RWPE challenge did not alter the number of other cell types in BALF (data not shown), indicating specificity of the inhibitor for blocking neutrophil recruitment. To test the contribution of recruited neutrophils to RWPE-induced superoxide generation from BAL cells *ex vivo*, WT mice were treated with the same dose of SB225002 that inhibited RWPE challenge-induced neutrophil recruitment in Figure 5B, and *ex vivo* superoxide generation of BALF cells was quantified 16 hours after RWPE challenge.

Administration of CXCR2 inhibitor blocked RWPE-induced recruitment of superoxide-generating cells (Figure 5C). Together these observations indicate that RWPE challenge induces a CXCR2-dependent recruitment of superoxide generating “activated” neutrophils.

Repeated CXCR2-Dependent Recruitment of Activated Neutrophils by RWPE Facilitates Induction of Allergic Airway Inflammation

Building on our observation in the present study that a single RWPE challenge induces a CXCR2-dependent recruitment of activated neutrophils to the airways, we hypothesized that repeated recruitment of activated neutrophils after repeated RWPE challenge shifts the immune response to an allergic phenotype. To test this hypothesis,

Figure 3. Effect of Toll-like receptor 4 on RWPE-induced allergic airway inflammation. (A) Repeated-challenge model protocol. (B–F) RWPE repeated-challenge model in WT mice and *Tlr4* KO mice. (B) BALF eosinophil and total inflammatory cell numbers. Multiple challenges with RWPE induced a greater increase in the number of eosinophils and total inflammatory cells in WT mice compared with *Tlr4* KO mice. (C and D) Mucin secretion in airway epithelial cells. Multiple challenges with RWPE induced a greater increase in mucin secretion in WT mice compared with *Tlr4* KO mice. (C) Mucin secretion in airway epithelial cells. Original magnification: $\times 400$. (D) Epithelial mucin score. (E) Serum ragweed-specific IgE. Multiple challenge with RWPE induced an increase in serum ragweed-specific IgE in WT mice but not in *Tlr4* KO mice. (F) T helper type 2 (Th2) cytokines in BALF. Multiple challenges with RWPE induced an increase in BALF levels of IL-5, IL-13, thymic stromal lymphopoietin (TSLP), and IL-33 in WT mice but not in *Tlr4* KO mice. For all groups, 5 to 21 mice per group were used. Data are expressed as means $\pm$ SEM. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

we administered a CXCR2 inhibitor before each of five intranasal RWPE administrations to block repeated recruitment of activated neutrophils. Compared with administering vehicle, administering SB225002 before each of the RWPE instillations in the repeated-challenge model (Figure 5D) attenuated RWPE-induced allergic inflammation. This attenuation consisted of a 50 to 80%

decrease in BALF eosinophils (Figure 5E); BALF total inflammatory cells (Figure 5E); accumulation of mucin in epithelial cells (Figures 5F and 5G); serum RWPE-specific IgE (Figure 5H); and BALF levels of IL-5, IL-13, TSLP, and IL-33 in BALF (Figure 5I). These observations provide further evidence supporting our hypothesis that repeated RWPE-induced recruitment of activated neutrophils contributes to

induction of allergic sensitization and airway inflammation in naive mice.

Administration of Neutrophils from Donor Mice into *Tlr4* KO Recipient Mice Reconstitutes RWPE-Induced Allergic Sensitization and Airway Inflammation

To directly test the role of neutrophils in induction of allergic sensitization and

Figure 4. Effect of forced neutrophil recruitment in *Tlr4* KO mice on RWPE-induced allergic airway inflammation. (A) BALF CXCL1 levels in *Tlr4* KO mice. Intranasal challenge with RWPE alone or xanthine with xanthine oxidase (X+XO) alone failed to induce secretion of CXCL1. Administration of a cocktail of RWPE and X+XO induced secretion of CXCL1. (B) BALF neutrophil numbers in *Tlr4* KO mice. Intranasal challenge with RWPE alone or X+XO alone failed to increase neutrophil recruitment in BALF 16 hours after challenge. Administration of a cocktail of RWPE and X+XO increased recruitment of neutrophils. (C–E) Effect of repeated RWPE challenge in the presence or absence of X+XO in *Tlr4* KO mice. (C) BALF eosinophil and total inflammatory cell numbers in *Tlr4* KO mice. RWPE+X+XO challenge increased the number of eosinophils and total inflammatory cells in BALF. (D and E) Mucin secretion in airway epithelial cells of *Tlr4* KO mice. RWPE multiple challenges induced a greater increase in mucin secretion in mice challenged with RWPE+X+XO compared with RWPE. (D) Mucin secretion in airway epithelial cells. Original magnification: $\times 400$. (E) Epithelial mucin score. For all groups, five to eight mice per group were used. Data are expressed as means $\pm$ SEM. $**P < 0.01$. $***P < 0.001$. $****P < 0.0001$.

inflammation, we performed a “reconstitution experiment” with repeated intranasal administration of neutrophils derived from naive donor mice to *Tlr4* KO recipient mice 8 hours after each RWPE challenge (Figure 6A). As expected from our data shown in Figure 3, repeated intranasal administration of RWPE in *Tlr4* KO mice without subsequent administration of neutrophils failed to induce allergic sensitization or late-phase allergic airway inflammation (Figures 6B–6F). By contrast, repeated intranasal administration of RWPE in *Tlr4* KO mice followed by neutrophils reconstituted allergic sensitization and late-phase allergic airway inflammation. This was characterized by an increase in BALF eosinophils (Figure 6B); BALF total inflammatory cells (Figure 6B); mucin

secretion in airway epithelial cells (Figures 6C and 6D); serum levels of RWPE-specific IgE (Figure 6E); and BALF levels of IL-5, IL-13, TSLP, and IL-33 (Figure 6F). Together these observations indicate that RWPE-induced innate recruitment of neutrophils is a critical rate-limiting step that is required for induction of allergic sensitization and stimulation of allergic airway inflammation.

Discussion

Our data indicate that pollen-initiated repeated recruitment of activated neutrophils plays a critical role in pollen-induced induction of allergic sensitization and airway inflammation in a naive host. A recent study demonstrated that LTB4

mediates OVA-induced neutrophil recruitment to the skin, and these recruited neutrophils facilitate induction of subsequent allergic skin inflammation (13). Another study reported that LTB4 specifically mediates recruitment of neutrophils after infection with *Pseudomonas aeruginosa* to lymph nodes (1). However, in the present study, intranasal challenge of mice with RWPE failed to increase LTB4 levels in the airways. Instead, recruitment of neutrophils by RWPE was regulated by the TLR4–CXCR2 pathway. The difference in mechanism of neutrophil recruitment between the earlier studies and the present study could be due to differences in which organ system was exposed to allergen (skin or lymph nodes in earlier studies [1, 13] versus lung in the present study) or to the type of allergen (OVA or *P. aeruginosa* in earlier studies [1, 13] versus RWPE in the present study).

Our study demonstrates an interesting novel finding: exposure to pollens induces an innate recruitment of neutrophils to the airways, and this recruitment facilitates allergic sensitization. In the present study, one possible mechanism by which innate recruitment of neutrophils by pollens shifts the immune response to an allergic phenotype upon repeated challenge is induction of a state of sustained oxidative stress in the airways by repeated recruitment of activated ROS-generating neutrophils. This is suggested by reports that mice deficient in gp91phox, the dominant superoxide-generating enzyme in neutrophils, have decreased ROS generation and mount an attenuated allergic inflammatory response to allergen challenge (19). Additionally, chronic oxidative stress can worsen allergic asthma (21–24), alter dendritic cell function, and modify the Th1/Th2 balance (25, 26). Some publications indicate that neutrophils have numerous additional effects that could modulate allergic inflammation, as evidenced by their ability to promote extracellular infection clearance in a regulation of innate and adaptive immune responses (11, 12). Thus, neutrophils may have contributed to allergic sensitization and inflammation in our study by increasing microvascular permeability (27), inducing proinflammatory cytokines (28), matrix metalloproteinase 9 (29), and MUC5AC (30). However, the precise molecular pathways by which neutrophils initiated

Figure 5. Effect of chemokine (C-X-C motif) receptor 2 (CXCR2) inhibitor (INH) on RWPE challenge-induced innate and allergic inflammation. (A) Protocol for single-challenge model after intranasal administration of CXCR2 inhibitor. (B) BALF neutrophil numbers in WT mice. Administration of CXCR2 inhibitor before RWPE challenge inhibited neutrophil recruitment 16 hours after challenge ($n = 5-9$ per group). (C) *Ex vivo* superoxide generation from BALF cells. Intranasal administration of CXCR2 inhibitor before RWPE challenge inhibited *ex vivo* superoxide generation. $*P < 0.05$ compared with all other groups ($n = 3-4$ per group). (D) Protocol for repeated-challenge model in naive WT mice with or without CXCR2 inhibitor. (E-I) Effect of repeated RWPE challenge in the presence or absence of CXCR2 inhibitor in WT mice ($n = 5-9$ per group). (E) BALF eosinophil and total inflammatory cell numbers. Administration of CXCR2 inhibitor before RWPE challenge inhibited the number of eosinophils and total inflammatory cells. (F and G) Mucin secretion in airway epithelial cells. Administration of CXCR2 inhibitor before RWPE challenge inhibited the increase in mucin secretion. (F) Mucin secretion in airway epithelial cells. Original magnification: $\times 400$. (G) Epithelial mucin score. (H) Serum ragweed-specific IgE. Administration of CXCR2 inhibitor before RWPE challenge inhibited serum ragweed-specific IgE levels. (I) Th2 cytokines in BALF. Administration of CXCR2 inhibitor before RWPE challenge inhibited secretion of IL-5, IL-13, TSLP, and IL-33 in BALF. Data are expressed as means $\pm$ SEM. $*P < 0.05$. $**P < 0.01$.

allergic sensitization in the present study have to be elucidated in future studies.

The role of TLRs in pollen-induced allergic inflammation has been evaluated recently (31, 32). In mice sensitized to RWPE, conjunctival challenge with ragweed pollen extract has been reported to stimulate TLR4-dependent allergic inflammation in murine and human

corneal epithelia (32). Likewise, another group reported that the adaptive immune response to birch pollen extract was inhibited in mice lacking TLR4 (31). Our data indicate that the TLR4-CXCR2 pathway may have contributed to allergic inflammation in those studies by controlling pollen-induced recruitment of activated neutrophils. Activation of the

TLR4-CXCR2 pathway upon exposure to pollens in our study may explain pollen-induced induction of IL-8 (2-4), recruitment of neutrophils (5), and oxidative stress markers (33-36) reported in earlier studies. Additional studies are needed to determine whether the dominance of neutrophils in the airways in severe asthma (37) and sudden-onset fatal

Figure 6. Effect of repeated intranasal administration of neutrophils from donor mice into *Tlr4* KO recipient mice after RWPE challenge. (A) Protocol for the repeated-challenge model in *Tlr4* KO mice with or without neutrophil replacement 8 hours prior to each instillation of RWPE. (B–F) Effect of repeated RWPE challenge with or without replacement of activated neutrophils in *Tlr4* KO mice, assessed on Day 14, 72 hours after the final RWPE challenge on Day 11. (B) BALF eosinophil and total inflammatory cell numbers in *Tlr4* KO mice. Intranasal administration of neutrophils after RWPE challenge increased the number of late-phase (72 h) BALF eosinophils and total inflammatory cells. (C and D) Mucin secretion in airway epithelial cells of *Tlr4* KO mice. Intranasal administration of neutrophils after RWPE challenge stimulated mucin secretion in *Tlr4* KO mice. (C) Mucin secretion in airway epithelial cells. Original magnification: $\times 400$. (D) Epithelial mucin score. (E) Serum ragweed-specific IgE. Intranasal administration of neutrophils after RWPE challenge increased levels of serum ragweed-specific IgE. (F) Th2 cytokines in BALF. Intranasal administration of neutrophils after RWPE challenge increased secretion of IL-5, IL-13, TSLP, and IL-33 in BALF. For all groups, 6 to 18 mice per group were used. Data are expressed as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

asthma (38) are a consequence of chronic activation of the TLR4–CXCR pathways by pollen allergens. Prior epidemiologic studies have suggested a role of TLR4 in human allergic diseases. Several TLR4 SNPs have been identified that facilitate

allergic sensitization and prevalence of asthma (39, 40). Future studies would have to determine whether there are differences in stimulation of pollen-induced TLR4–CXCR2 pathway in individuals with specific TLR4 SNPs,

thereby modulating recruitment of activated neutrophils and induction of allergic sensitization and airway inflammation.

CXCR2 has been shown to play a significant role in infection by regulating

neutrophil trafficking (41–44). Thus, the ability of CXCR2 inhibitors to inhibit RWPE-mediated allergic inflammation in our study is somewhat surprising because it is not conventionally considered relevant to pollen-induced allergic airway inflammation. However, evidence from murine and human studies suggests an importance of CXCR2 signaling in the induction of asthma and allergic inflammation. Thus, *Cxcr2* KO mice sensitized with fungus demonstrate reduced secretion of Th2 cytokines in the airway compared with WT mice (45). Blockade of CXCR2 signaling by neutralizing antibody inhibits OVA-induced airway remodeling in a murine mouse model (46). Treatment of mice with anti-CXCR2 mAb inhibits IL-33-induced late-phase airway obstruction, airway hyperresponsiveness, eosinophilic inflammation, and goblet cell hyperplasia (47). Additionally, treatment of human subjects with severe asthma with CXCR2

inhibitor SCH 527123 reduced asthma exacerbations (48). Because none of these studies focused on the importance of pollen-induced recruitment of neutrophils in the induction of allergic sensitization and airway inflammation (46–48), our study provides crucial mechanistic data that might explain some of the observations reported in the earlier studies. Because TSLP and IL-33 can induce Th2 polarization (49, 50) and because our study indicates that repeated administration of RWPE and neutrophils in *Tr4* KO mice increases BALF levels of these cytokines, it is likely that either neutrophils, a factor derived from neutrophils, or oxidative damage to airway structural cells by neutrophils induces secretion of these cytokines, which in turn stimulate allergic sensitization and inflammation.

Our observations indicate that pollen-induced repeated recruitment of neutrophils

is a critical rate-limiting event that stimulates allergic sensitization and induction of pollen-induced allergic airway inflammation. Furthermore, our study provides a mechanistic link between TLR4 and CXCR2 in pollen-induced recruitment of neutrophils. We suggest that inhibiting recruitment of activated neutrophils by administration of CXCR2 or other antagonists may be a unique strategy for preventing allergic sensitization and pollen-induced allergic disorders. ■

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