

RESEARCH LETTER

Attenuation of murine allergic airway inflammation with a CXCR1/CXCR2 chemokine receptor inhibitor

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To the Editor,

More than two decades ago, we were amongst the first to report the presence of neutrophils in the airways in severe asthma.¹ However, the nature of contribution of neutrophils to allergic inflammation remained a scientific enigma until quite recently. Neutrophils have long been viewed as terminally differentiated cells that clear extracellular pathogens. However, a growing body of literature indicates that neutrophils regulate innate and adaptive immune responses, and contribute to allergic diseases.²⁻⁴ Adoptive transfer or recruitment of neutrophils to the skin stimulates allergic skin inflammation.² Likewise, neutrophils are required for both the sensitization and elicitation phase of allergic contact dermatitis.³ Neutrophil activation occurs in vivo during anaphylaxis, and neutrophil depletion inhibits active and passive systemic anaphylaxis.⁵ Importantly, mouse and human neutrophils each restore anaphylaxis in anaphylaxis-resistant mice demonstrating that neutrophils are sufficient to induce anaphylaxis in mice, and suggesting that neutrophils can contribute to anaphylaxis in humans.⁵ *Tlr4*KO mice are unable to mount pollen-induced allergic airway inflammation⁴; adoptive transfer of neutrophils to the lungs of *Tlr4*KO mice increases IL-33 secretion and reconstitutes pollen-induced allergic airway inflammation.⁴ Exposure of the airway epithelium to allergenic extracts recruits reactive oxygen species (ROS)-generating neutrophils to the lungs.⁴ Additionally, inhibiting ragweed pollen extract (RWPE)-induced neutrophil recruitment in mice by administration of CXCR2 small molecule inhibitor SB225002 attenuates allergic airway inflammation and sensitization.⁴ Together, these studies provide compelling evidence for a role of neutrophils in the pathogenesis of allergic inflammation.²⁻⁴

An unexpected development in this story of the role of neutrophils to pathogenesis of allergic inflammation came from a report

that administration of the CXCR2 inhibitor AZD5069 in humans failed to improve severe uncontrolled asthma even though it reduced the levels of neutrophils in sputum and blood.⁶ Neutrophils have CXCR1 and CXCR2 on their surfaces, and these receptors have distinct non-redundant roles in its recruitment and activation. Thus, CXCL8 monomer and dimer differentially activate and regulate function of CXCR1 and CXCR2 on neutrophils.⁷ Inhibition of CXCR1 but not CXCR2 pathway suppresses neutrophil's bacterial killing functions.⁸ We identified the CXC motif in chemokines functions as a conformational switch for high-affinity binding and activation of CXCR1 and CXCR2 receptors. Based on these reports,^{4,7,8} here, we hypothesized that a dual CXCR1 and CXCR2 pharmacologic inhibitor maybe effective in attenuating allergic airway inflammation.

Reparixin is a noncompetitive allosteric inhibitor of both CXCL8 receptors CXCR1 and CXCR2 that is safe for human use. Administration of reparixin in humans in a phase 2 randomized pilot study improved outcome of transplant of allogeneic islets in type 1 diabetes.⁹ To test our hypothesis, we selected reparixin to perform a proof of concept study to elucidate the role of the CXCR1/CXCR2 inhibitor in an animal model of cat dander extract (CDE)-induced innate and allergic lung inflammation. While the functionality of CXCR2 has been extensively reported in mice, it was the recent demonstration of functional CXCR1 receptors in mice¹⁰ that provided the critical rationale for using mice to test our hypothesis. Wild-type (WT) naïve mice were subjected to a CDE single challenge model, CDE-SCM, Figure 1A, by challenging them once with CDE and evaluating markers for innate inflammation were quantified 16 hours later in BALF.⁴ To induce allergic inflammation, WT mice were sensitized to CDE by subjecting them to CDE multiple challenge model, CDE-MCM, Figure 2A, as previously described.⁴ Following a rest period of 1 week, the mice were challenged again on

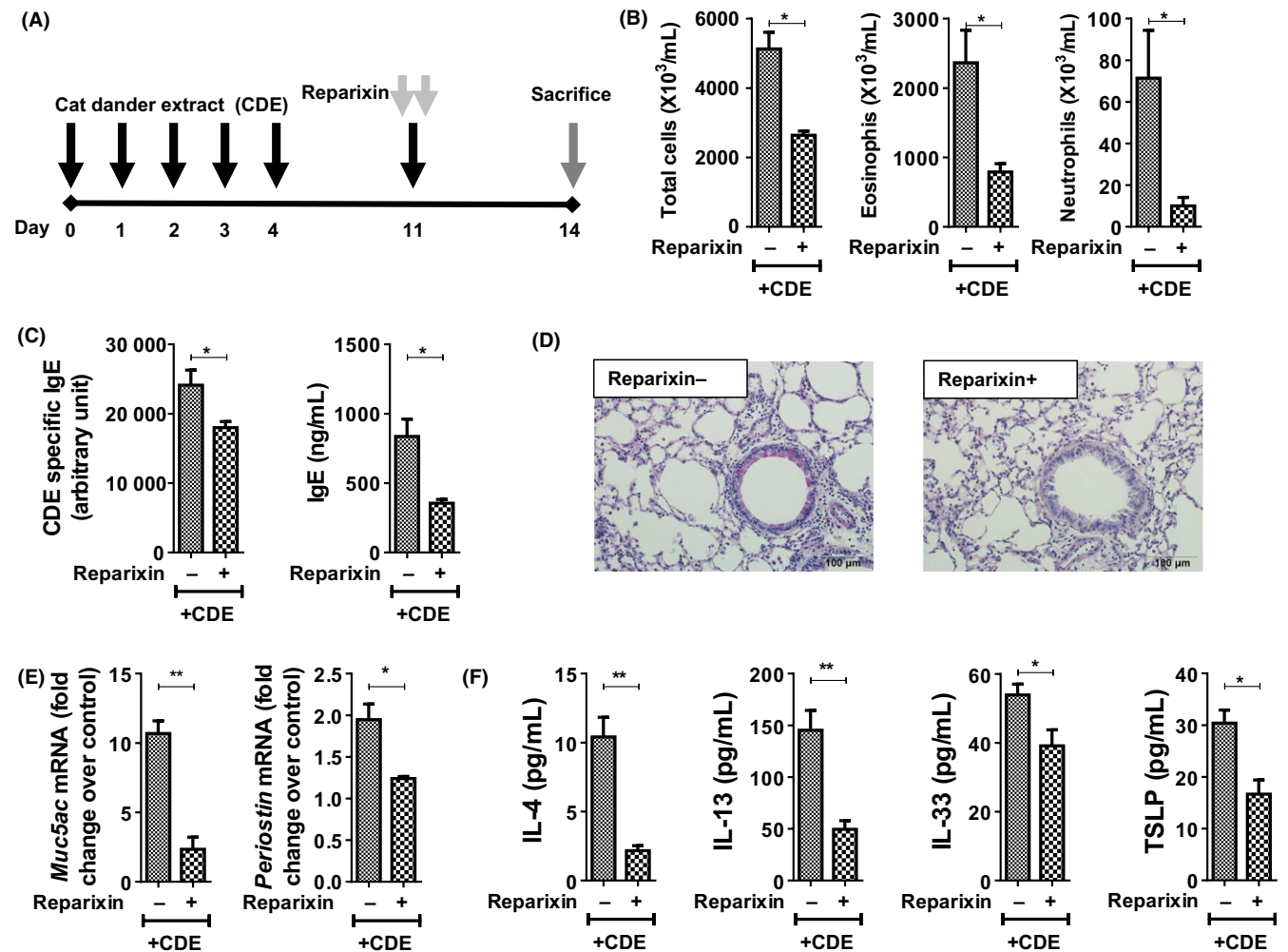
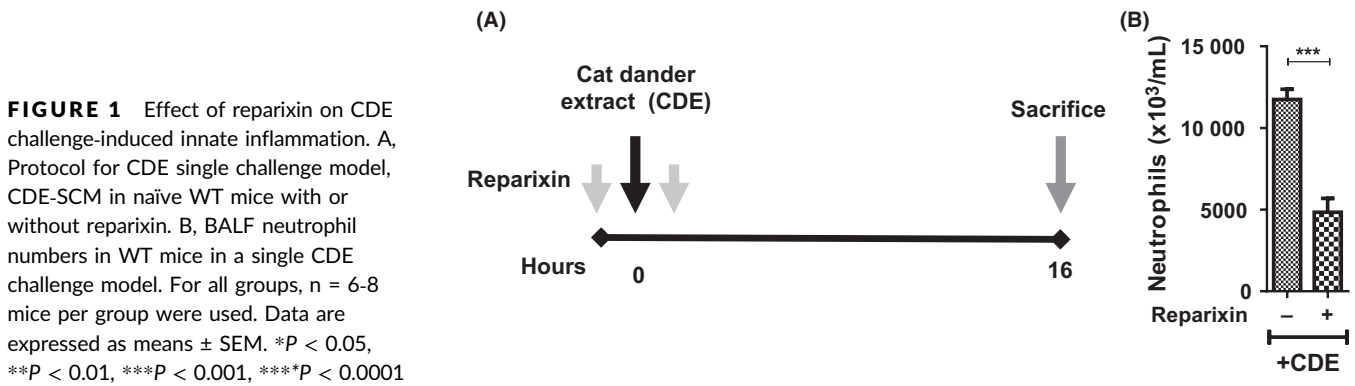


FIGURE 2 Effect of reparixin on CDE challenge-induced allergic inflammation. A, Protocol for CDE Multiple Challenge Model, CDE-MCM in naïve WT mice with or without reparixin. B-F, Effect of multiple CDE challenge in the presence or absence of reparixin in WT mice. B, BALF total inflammatory cell, eosinophil, and neutrophil numbers. C, Serum total IgE and CDE-specific IgE. D, Mucin secretion in airway epithelial cells. E, *Muc5ac* and *Periostin* mRNA expression in lungs. F, Th2 cytokines in BALF. For all groups, $n = 4$ mice per group were used. Data are expressed as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

Day 11, and allergic inflammation was quantified in the lungs on Day 14. Two doses of reparixin or vehicle were administered one hour before and after CDE challenge in a subset of the CDE-SCM group or CDE-MCM group on Day 11, and the effects of the inhibitor on innate or allergic lung inflammation were elucidated.

In the CDE-SCM model (Figure 1A), administration of reparixin (15 mg/kg body weight) suppressed neutrophil recruitment into the lungs (Figure 1B). Building on this observation, we used the same dose of reparixin in the CDE-MCM model (Figure 2A). As expected, CDE challenge in the CDE-MCM model stimulated eosinophil

recruitment, allergic inflammation and sensitization (Figure 2B-F). Administration of reparixin inhibited eosinophil, neutrophils, and total cell numbers in BALF (Figure 2B), serum levels of total IgE and CDE-specific IgE (Figure 2C), airway epithelial mucin secretion (Figure 2D), levels of Th2 inflammation-associated genes *periostin* and *muc5ac* (Figure 2E), and the BALF levels of IL-4, IL-13, IL-33, and TSLP in BALF (Figure 2F). These results indicate that pharmacological inhibition of CXCR1/2-axis by administration of reparixin inhibits allergen-induced innate and allergic airway inflammation in mice. Taken together with the earlier report demonstrating that neutrophils recruited and activated by the CXCR1/2 axis contribute to allergic sensitization and inflammation,⁴ our data from the present study suggest that reparixin attenuates allergic airway inflammation by inhibiting allergen-induced recruitment and activation of neutrophils.

To our knowledge, this is the first report showing that administration of dual CXCR1/2 inhibitor suppresses allergen challenge-induced neutrophilic inflammation and allergic airway inflammation. As second generation CXCR1/2 dual inhibitors such as ladarixin are developed and tested, some of these inhibitors may prove to be even more effective than reparixin in inhibiting allergic inflammation. Since asthma is a heterogeneous disease, administration of these new generation CXCR1/2 inhibitors may prove to be effective as controller therapy in neutrophil-dominant forms of asthma, such as the one described by us earlier.¹

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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1 **Materials and Methods (Supplemental information)**

2 **Mice.** Eight- to 12-wk-old male wild type (WT) C57BL/6J mice were obtained from Jackson Laboratory
3 (Bar Harbor, ME). All mice were maintained in a pathogen-free environment at the University of Texas
4 Medical Branch (Galveston, TX). Animal studies were approved by UTMB IACUC (Institutional Animal
5 Care and Use Committee) (approval no. 9708038B) and were performed according to the NIH Guide
6 for Care.

7 **Allergenic extracts.** Lyophilized cat dander extract (CDE) (lot# 253320) was obtained from Greer
8 Labs (Lenoir, NC). The level of endotoxin in CDE were measured using LAL chromogenic endotoxin
9 quantitation kit (Thermo Scientific, Hudson, NH): the level of endotoxin were exceedingly low, less
10 than 0.1 pg/μg CDE protein¹⁻².

11 **Protocols used for animal studies.**

12 Mice were anesthetized with intraperitoneal injection of low dose xylazine/ketamine anesthetic mixture
13 for intranasal sensitization or challenge and sacrificed by lethal intraperitoneal xylazine/ketamine
14 overdose.

15 **CDE Single Challenge Model, CDE-SCM.** One hour before and after CDE challenge, WT mice were
16 treated with intraperitoneal administration of 15 mg/kg body weight of reparixin (Cayman Chemical,
17 Ann Arbor, MI) or PBS, and intranasally challenged with a single dose of 100 μg/60μl of CDE and
18 sacrificed after 16 hours.

19 **CDE Multiple Challenge Model, CDE-MCM.** WT mice were sensitized by five intranasal doses of
20 CDE (100 μg/60μl) on days 0, 1, 2, 3, and 4. Then, these mice were injected with intraperitoneal
21 administration of 15 mg/kg body weight of Reparixin or PBS one hour before and after each intranasal
22 dose of CDE on day 11 and sacrificed on day 14.

23 **Processing of mouse BALF and lung tissue samples.**

The collection of bronchoalveolar lavage fluid (BALF) and histological examinations by PAS staining for mucin were performed as described previously¹⁻².

Measurement of IL-4, IL-13, IL-33, and TSLP in BALF. The levels of IL-4, IL-13, IL-33, and TSLP in BALF were measured using a DuoSet ELISA development kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

qRT-PCR of mouse lung mRNA. RNA from mouse lung tissue was extracted with RNeasy kit (Qiagen, Valencia, Calif.) according to the manufacturer's protocol and reverse-transcribed, and amplified using real-time PCR in ABI 7000 (Applied Biosystems, Foster City, CA).

The primer sequences were as follows:

mouse Hprt, forward, 5'-AACAAAGTCTGGCCTGTATCC-3', reverse,
5'-CCCCAAAATGGTTAAGGTTGC-3';

mouse Muc5ac, forward: 5'-CTGGTTGAGTGGTTGTGTGT -3', reverse,
5'-CCCATGTGTATTCCTCTCCCA -3';

mouse Periostin, forward, 5'-CCTTTCATCCCTTCCATTCTCA -3', reverse,
5'-CCTGTAAGAACTGGTATCAAGGT -3'.

These primers were purchased from Integrated DNA Technologies (Coralville, IA).

Measurement of total IgE and cat dander-specific IgE in serum. Plates were coated with 100 µg/ml of cat dander protein overnight at room temperature or rat anti-mouse IgE (BD Biosciences, San Jose, CA) for 2 hours at room temperature. After blocking with sea block buffer for 2 hours, the plate was applied with serum from mice overnight at room temperature. After washing, the plates were

applied with biotin-conjugated rat anti-mouse I IgE (BD Biosciences, San Jose, CA), incubated with
avidin-conjugated alkaline phosphatase for 45 minutes at 4°C (Sigma-Aldrich, St. Louis, MO).
Fluorescence intensities were measured using AttoPhos Substrate Solution (Promega, Madison, WI).

Statistical Analysis. Results of the study are shown as means $\pm$ SEM. Paired data were analyzed by
unpaired *t*-test. The software package GraphPad Prism 6 (GraphPad Software, San Diego, CA) was
used for all data analyses and preparation of graphs. All statistical analyses indicated data as
significant at $p < 0.05$.

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