Inhibition of CXCR1 and CXCR2 blocks Th2/Th17-associated Allergic Lung Inflammation in Mice

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Abstract

Background: IL4, IL5, IL13, and IL17-producing CD4 T helper 2 (Th2)-cells and IL17-producing CD4 T helper 17 (Th17)-cells contribute to chronic eosinophilic and neutrophilic airway inflammation in asthma and allergic airway inflammation. Chemokines and their receptors are upregulated in Th2-Th17-mediated inflammation. However, the ability of CXCR1 and CXCR2 inhibition to suppress Th2 and Th17 cell-mediated allergic lung inflammation has not been reported. **Methods**: Mice sensitized and challenged with cat dander extract (CDE) mount a vigorous Th2-Th17-mediated allergic lung inflammation. Ladarixin is an orally bioavailable allosteric inhibitor of CXCR1 and CXCR2 and was orally administered in this model prior to CDE-challenge. The ability of ladarixin to modulate allergen-challenge induced recruitment of cytokine-secreting CXCR1 and CXCR2-expressing Th2- and Th17-cells and allergic lung inflammation were examined. **Results**: Allergen challenge in sensitized mice increased mRNA expression levels of *Il4, Il5, Il13, Il6, Il1β, Tγφβ1, Il17, Il23, Γατα3,* and *Rorc*, recruited CXCR1- and CXCR2-expressing Th2 cells, Th17-cells, neutrophils, and eosinophils, inducing allergic lung inflammation. Administration of ladarixin vigorously blocked each of these pro-inflammatory effects of allergen challenge. **Conclusions**: Allosteric inhibition of CXCR1 and CXCR2 by oral administration of ladarixin vigorously blocks recruitment of CXCR1- and CXCR2-expressing Th2-cells, Th17-cells, neutrophils, and eosinophils in this mouse model of allergic lung inflammation. We suggest that the ability of oral ladarixin to mitigate Th2 and Th17-mediated allergic inflammation should be investigated in humans.

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Short title

Role of CXCR1 and CXCR2 in Th2/Th17 Allergic Inflammation.

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Methods : Mice sensitized and challenged with cat dander extract (CDE) mount a vigorous Th2-Th17-mediated allergic lung inflammation. Ladarixin is an orally bioavailable allosteric inhibitor of CXCR1 and CXCR2 and was orally administered in this model prior to CDE-challenge. The ability of ladarixin to modulate allergen-challenge induced recruitment of cytokine-secreting CXCR1 and CXCR2-expressing Th2- and Th17-cells and allergic lung inflammation were examined.

Results : Allergen challenge in sensitized mice increased mRNA expression levels of *Il4, Il5, Il13, Il6, Il1β, Tγφβ1, Il17, Il23, Γατα3,* and *Rorc* , recruited CXCR1- and CXCR2-expressing Th2 cells, Th17-cells, neutrophils, and eosinophils, inducing allergic lung inflammation. Administration of ladarixin vigorously blocked each of these pro-inflammatory effects of allergen challenge.

Conclusions : Allosteric inhibition of CXCR1 and CXCR2 by oral administration of ladarixin vigorously blocks recruitment of CXCR1- and CXCR2-expressing Th2-cells, Th17-cells, neutrophils, and eosinophils in this mouse model of allergic lung inflammation. We suggest that the ability of oral ladarixin to mitigate Th2 and Th17-mediated allergic inflammation should be investigated in humans.

Keywords:

Allergic inflammation, CXCR1/2, ladarixin, Th2/Th17

Introduction:

Asthma is a chronic inflammatory disease of the airways characterized by chronic eosinophilic and neutrophilic allergic inflammation. IL4-, IL5-, and IL13-producing CD4 T helper 2 (Th2)-cells[1] and IL17-producing Th17 cells[2,3]contribute to this inflammation. IL4 stimulates Th2 differentiation of CD4+ T-cells by upregulating its master regulator GATA3[4], and these Th2-cells induce allergic lung inflammation[4,5]. IL23, IL1β, and IL6 stimulate CD4+ T-cells to differentiate toward Th17[6-8] by upregulating its master regulator retinoic acid–related orphan receptor- gamma t (RORγt)[9], Th17 cells mediate neutrophilic inflammation[10].

A large volume of literature indicates that CC-chemokine receptors (CCR) are present on eosinophils, Th2-cells, and Th17-cells, and modulate eosinophilic and neutrophilic airway inflammation in asthma by attenuating chemokine-induced migration and activation Th2-associated inflammatory immune cells[11]. CCR3 is expressed on eosinophils and regulates recruitment and degranulation of eosinophils[12-14]. CCR3, CCR4, and CCR8 are expressed on Th2-cells[15], and CCR4 plays a role in recruiting Th2-cells[16,17]. CCR3 expressing eosinophils, and CCR4 and CCR8 expressing Th2-cells can be detected in endobronchial biopsies performed in asthmatic subjects after allergen challenge[18]. Th17-cells express CCR4, CCR5, and CCR6[19-23], and CCR6 facilitates recruitment of Th17-cells[21,23].

Unlike the plethora of studies linking CCR receptors with allergic inflammation, less is known about the role of CXC-chemokine receptors (CXCR) in allergic inflammation. This is somewhat surprising because the levels of CXCL chemokines are elevated in the airways in asthma[24-26], and the expression of CXCL1, CXCL5, and CXCL8 in bronchoalveolar lavage fluid (BALF) and endobronchial biopsies is higher in subjects with asthma than healthy control subjects[2625]. Furthermore, the levels of CXCL5 and CXCL8 are higher in patients with acute severe asthma[24,25]. CXCR1 and CXCR2 are G protein-coupled chemokine receptors for CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8, and are expressed on many immune and

lung structural cells[27-29]. We reported that administration of CXCR2 small molecule inhibitor SB225002 and dual CXCR1 and CXCR2 inhibitor reparixin suppressed allergic airway inflammation and serum IgE levels[30,31]. However, the role of CXCR1 and CXCR2 in modulating Th2 and Th17-associated allergic lung inflammation has not been reported.

Ladarixin is an orally bioavailable allosteric inhibitor of CXCR1 and CXCR2 that binds to an allosteric pocket of the trans-membrane region of both receptors with a 100-fold higher affinity than first-generation CXCR1 and CXCR2 inhibitors[32]. In the present study, we used ladarixin to examine the role of CXCR1 and CXCR2 in Th2 and Th17-associated allergic lung inflammation.

Material and Methods

Allergenic extracts . Lyophilized cat dander extract (CDE) (lots#253320, 351876, and 392583) was purchased from Greer Labs (Lenoir, NC). The level of endotoxin in CDE was measured using a LAL chromogenic endotoxin quantitation kit (Thermo Scientific, Hudson, NH), and was less than 0.1 pg/μg CDE protein, hence unlikely to contribute significantly to inflammation[33].

Protocols used for animal studies. C57Bl/6 mice were anesthetized with an intraperitoneal injection of a low dose of xylazine/ketamine anesthetic mixture for intranasal administration of CDE and sacrificed by a lethal dose of intraperitoneal xylazine/ketamine. The protocol was approved by the IACUC of Baylor College of Medicine.

CDE Multiple Challenge Model (CDE-MCM) to induce allergic sensitization. Naïve wild type (WT) mice were sensitized by five intranasal challenges of CDE (100 μg/60μl) on days 0, 1, 2, 3, and 4. After a rest period of 7 days, these mice were challenged with an intranasal dose of CDE or phosphate-buffered saline (PBS) on day 11 and sacrificed at 2 h, 4 h,16 h, 28 h, 40 h, and 72 h post-CDE challenge. Some mice challenged with CDE on day 11 were orally treated with 15 mg/kg body weight of ladarixin on days 11, 12, and 13 (**Fig 1A**).

CDE Single Challenge Model (CDE-SCM) to induce innate lung inflammation without sensitization . WT mice were intranasally challenged with a single dose of 100 μg/60μl of CDE and administered orally 15 mg/kg body weight of ladarixin simultaneously and sacrificed after 16 h or 28 h post-CDE challenge (**Fig 1B**).

Ladarixin . GMP human-use grade ladarixin was used in all studies performed in this manuscript and was a gift from Dompe pharmaceutical company (Dompé farmaceutici, L'Aquila, Italy).

Processing of mouse BALF and lung tissue samples. The BALF and lung were obtained as described previously[34].

Bone marrow cells isolation . Bone marrow cells were isolated from the femur and tibia bones of the mouse. After red blood cells lysis by red blood cell lysing buffer (Sigma-Aldrich, St. Louis, MO), the bone marrow cells were frozen for subsequent experiments.

qRT-PCR analysis . Total RNA from mouse lungs, bone marrow cells, or BALF cells were extracted with an RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized using a cDNA Synthesis kit (Qiagen). Amplification by real-time PCR was performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using SYBR Green PCR Master Mix Kit (Bio-Rad Laboratories) to examine lung mRNA expression of *Cxcl1, Cxcl 2, Cxcl 3, Cxcl 4, Cxcl 5* , *Cxcl*7, *Cxcr1* , *Cxcr2* . *Gata3, Il1b, Il13, Il17, Il23, Il4, Il5, Il6, Rorc* , and *Tgfb1.* These primers were purchased from Integrated DNA Technologies (Coralville, IA).

Measurement of IL23 protein in the lung tissue . Mouse lungs were lysed with Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) and sonicated, then the content of IL23 in the lung lysate was measured using a DuoSet ELISA development kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Lung single cells isolation . Lung tissues from mice were cut into small pieces and incubated with liberase-thermolysin medium (Sigma-Aldrich) in 42 μg/mL and 20% heat-inactivated fetal bovine serum in Hanks balanced salt solution at 37°C for 20 mins. After passing the digested lung through a 70 μm cell strainer, ice-cold PBS was added to neutralize the enzyme activity. After several wash steps with PBS, the lung single cells were used for flow cytometry analysis or culture system.

Flow cytometry analysis with lung single cells. Lung single cells were pre-incubated with TruStain FcX solution (BioLegend, San Diego, CA) for 10 mins at 4°C, and stained with fluorophore-conjugated antibodies Fixable Viability Dye eFluor 780 (eBioscience, San Diego, CA), CD45 monoclonal antibody-pacific orange (Thermo Fisher Scientific, MA, USA, #MCD4530, clone 30-F11), anti-mouse CD3-Alexa Fluor 700 (Biolegend, #100216, clone 17A2), rat anti-mouse CD4-Brilliant Violet 520 (BD Biosciences, #563106, clone RM4.5), PE rat anti-mouse CD181 (CXCR1) (BD Biosciences, San Jose, CA, #566383, clone U45-632), BV711 Rat Anti-Mouse CD182 (CXCR2), (BD Biosciences, #747812, clone V48-2310), BV786 rat anti-mouse Siglec-F (BD Biosciences, #740956, clone E50-2440), Spark YG 593 anti-mouse Ly-6G Antibody (Biolegend, #127668, clone 1A8), Spark NIR 685 anti-mouse/human CD11b Antibody (Biolegend, #101278, clone M1/17) at 1:100 dilution in flow stain buffer containing FBS for 30 mins at 4degC, then cells were permeabilized with Fixation/Permeabilization kit (BD Biosciences) according to the protocol from vendor and stained with intracellular cytokine specific antibodies Rat anti-mouse Rorγt- Brilliant Violet-650 (BD Biosciences, #564722, clone Q31-378), mouse anti-GATA3 Alexa Fluor F488 (BD Bioscience #560163, clone L50-823,) Rat anti-mouse IL4-PE/Cyanine7 (BD Biosciences, #560699, clone 11B11), anti-mouse/anti-human IL5-Allophycocyanin (APC) (BD Biosciences, #554396, clone TRFK5), IL13 monoclonal antibody ((eBio13A), Brilliant Ultra Violet 805, eBioscience), Rat Anti-Mouse IL17A-PE (BD Biosciences, # 559502, clone TC11-18H10), and rat anti-mouse IL23 p19 Alexa Fluor 647 (BD Biosciences, # 565317, clone N71-1183) for 45 mins at 4°C. After washing, a flow cytometer was performed using a high-parameter Cytek®Aurora Flow Cytometer. Staining specificity was determined by fluorescence minus one (FMO) control to enhance the reliability of the gating analysis. Absolute cell numbers were quantified using Precision true count beads (BioLegend). The flow cytometry data was analyzed using FlowJo 10.8.1 Software. FC plots showing gating strategies and FMO are shown in **sup Fig 1** . Neutrophils were identified as live CD45+ CD11b+ Ly6G+ Siglec F- cells. Eosinophils were identified as live CD45+ CD11b+ Siglec F+ Ly6G- cells. CD4+ T-cells were identified as live CD45+ CD3+ CD4+ cells.

Measurement of serum total IgE and CDE-specific IgE. The methods have been described previously[34]. Briefly, the plates were coated with CDE overnight or rat anti-mouse IgE (BD Biosciences) for 2 h. After blocking with sea block buffer for 2 h, the serum from the mice were added onto the plate. After washing, biotin-conjugated rat anti-mouse IgE (BD Biosciences) were plated onto the plate and incubated with avidin-conjugated alkaline phosphatase for 45 mins at 4°C (Sigma-Aldrich). Fluorescence intensities were measured with AttoPhos Substrate Solution (Promega, Madison, WI) using the Varioskan LUX reader (Thermo Fisher Scientific).

Long amplicon quantitative-PCR (LA-qPCR). DNA damage in the lung tissue was quantified by LA-qPCR as previously described[35,36]. Genomic DNA extraction from the mouse lung was performed using the genomic-tip 20/G kit (Qiagen) per the manufacturer's protocol. After precise quantitation of the DNA by Pico Green (Invitrogen, Carlsbad, CA), the genomic DNA was digested with the *E. coli* enzymes Fpg and Nei to induce strand breaks at the sites of the unrepaired oxidized base lesion. Gene-specific LA-qPCR analyses for measuring DNA damage were performed using Long Amp Taq DNA polymerase (New England Biolabs) for long amplicon (LA) and Green mix (New England Biolabs) for short amplicon (SA). The PCR condition for LA was optimized at 94 °C for 30 s (94 °C for 30 s, 60 °C for 30 s depending on the oligo annealing temperature, 65 °C for 10 min) for 25 cycles and 65 °C for 10 min. The PCR conditions for SA were 94 °C for 30 s (94 °C for 30 s, 58°C for 20 s, and 68 °C for 30 s) for 25 cycles and 68 °C for 5 min. The amplified products were then visualized on agarose gels and quantitated with an ImageJ automated digitizing system (National Institutes of Health). In this assay, we amplified a long amplicon (6.5 kb of *ΔNA πολψμερασε β (πολ β)* and 8.7 kb of *β-γλοβιν*) vs. the short amplicons (250 and 200 bp, respectively).

The sequence of the oligos:

LA-qPCR forward primer for mouse $\pi o\lambda\ \beta$ · TATCTCTCTTCCTCTTCACTTCTCCCCTGG

LA-qPCR reverse primer for mouse $\pi o\lambda\ \beta$ · CGTGATGCCGCCGTTGAGGGTCTCCTG

LA-qPCR forward primer for mouse β-$\gamma\lambda o\beta\iota\nu$; TTGAGACTGTGATTGGCAATGCCT

LA-qPCR reverse primer for mouse β-$\gamma\lambda o\beta\iota\nu$; CCTTTAATGCCCATCCCGGACT

SA-qPCR forward primer for mouse $\pi o\lambda\ \beta$ · TATGGACCCCCATGAGGAACA

SA-qPCR reverse primer for mouse $\pi o\lambda\ \beta$ · AACCGTCGGCTAAAGACGTG

SA-qPCR forward primer for mouse β-$\gamma\lambda o\beta\iota\nu$; ACACTACTCAGAGTGAGACCCA

SA-qPCR reverse primer for mouse β-$\gamma\lambda o\beta\iota\nu$; ATACCCAATGCTGGCTCCT

Statistical Analysis. The statistical analysis was performed by unpaired t-test for comparison of two groups or ANOVA for three or more groups using the software package GraphPad Prism 6 (GraphPad Software, San Diego, CA). The results are shown as mean ± SEM. All statistical analyses indicated data as significant at $p < 0.05$. *=P< .05, **=P< .01, ***=P< .001, ****=P< .0001.

Results

CDE challenge increases CXCL mRNA expression in the lung and CXCR1/2 in bone marrow of sensitized mice

Mice were sensitized to CDE by subjecting them to CDE-MCM (**Fig 1A**)[31,34]. Compared to PBS challenge (**Fig 1A** left panel), CDE challenge (**Fig 1A** middle panel) in these sensitized mice induced much greater lung expression levels of *Cxcl1* , *Cxcl2* ,*Cxcl3* , and *Cxcl5* at 2 h and/or 4 h, but not *Cxcl4* and *Cxcl7* (**sup Fig2A**). A shared feature of these upregulated *Cxcl1* , *Cxcl2* , *Cxcl3* , and *Cxcl5* chemokines is that they bind CXCR1 and CXCR2[37,38]. We reasoned that expression and secretion of these *Cxcl* chemokines in the lungs should increase in the number of progenitor cells expressing CXCR1 and CXCR2[37,38] in the bone marrow to prepare them for migration to the lungs. To test this hypothesis, we examined the time kinetics of CDE challenge-induced upregulation of *Cxcr1* and *Cxcr2* mRNA expression in bone marrow of sensitized mice. CDE challenge increased the mRNA expression of *Cxcr1* and *Cxcr2* in bone marrow cells at 16 h post-CDE challenge (**sup Fig2B**), suggesting that these receptors play a role in recruiting inflammatory cells from bone marrow to the lungs.

Inhibition of CXCR1 and CXCR2 blocks allergen challenge-induced allergic lung inflammation

Ladaraxin is an orally bio-available allosteric inhibitor of CXCR1 and CXCR2. Ladarixin treatment of naïve mice subjected to CDE-SCM (**Fig 1B**) reduced CDE challenge-induced innate neutrophil recruitment into the lung by 30% (**Fig 1C**). Next, we compared to PBS challenge, CDE challenge in CDE-MCM (**Fig 1A**) increased the number of total cells, macrophages, neutrophils, and eosinophils in BALF (**Fig 1D**) and lung tissues quantified by flow cytometry at 72 h after the final CDE challenge (**Fig 1E**), as well as total IgE and CDE-specific IgE in serum (**Fig 1F**). Oral administration of ladarixin in CDE-MCM model (**Fig 1A** , right panel) inhibited each of these parameters (**Fig 1D-F**).

Inhibition of CXCR1 and CXCR2 blocks allergen challenge-induced recruitment of Th2-cells

Next, we examined the role of CXCR1 and CXCR2 on Th2 gene expression and recruitment of Th2 cells. GATA3 is a key transcription factor associated with Th2-mediated allergic lung inflammation[4,5]. We examined *Gata3* mRNA expressions in BALF cells at 28 h post-CDE challenge in CDE-SCM (**Fig 1B**) or 28h post-final CDE challenge in CDE-MCM (**Fig 1A**). Compared to naïve mice, CDE-challenge in CDE-SCM failed to increase expression levels of Gata3. By contrast, CDE challenge in CDE-MCM sensitized mice induced higher levels of *Gata3* mRNA expression compared to naïve mice and mice subjected to CDE-SCM

(**Fig 2A**). Oral administration of ladarixin in mice subjected to CDE-MCM (**Fig 1A right panel**) inhibited CDE challenge-induced increase in *Gata3* mRNA expression (**Fig 2B**). CDE challenge in CDE-MCM model upregulated expression levels of*Il4, Il5* , *Il13* , and *Tgfb1* in BALF cells (**Fig 2C**), expression levels of *Il4* and *Il13* in lung tissue (**Fig 2D**), and the numbers of IL4, IL5, IL13-secreting and GATA3-expressing Th2-cells in the lungs (**Fig 2E**). Oral administration of ladarixin vigorously blocked each of these effects of CDE challenge (**Fig 2C-E**), including a remarkable 90% suppression of *Il4* mRNA expression (**Fig 2C**).

Inhibition of CXCR1 and CXCR2 blocks allergen challenge-induced Th17-associated mRNA expression and recruitment of Th17-cells

IL23 is a crucial cytokine that regulates the survival of Th17-cells and mediates Th17 inflammation[39-41]. Prior studies have shown that IL23, IL1β, and IL6 are elevated in the BALF obtained from the subjects with asthma[42,43]and these cytokines stimulate Th17 differentiation[6-8] by upregulating its master regulator RORγt[9]. Building on our observation that inhibiting CXCR1 and CXCR2 vigorously reduced the Th2-associated mRNA expression levels and recruitment of Th2 cells, we examined the effects of CDE challenge in sensitized mice (**Fig 1A)** on the expression levels of IL23, IL1β, and IL6. CDE challenge upregulated*Il23, Il1b,* and *Il6* mRNA expression in BALF cells (**Fig 3A**) and lung tissues (**Fig 3B**). CDE challenge increased IL23 protein level in lung tissues (**Fig 3C**), and the numbers of IL23 positive T-cells, neutrophils, eosinophils, and lung epithelial cells in single cells obtained from lung tissues (**Fig 3D**). Oral administration of ladarixin blocked each of these effects of CDE challenge (**Fig 3A-D**).

Because inhibiting CXCR1 and CXCR2 reduced Th17-promoting cytokines, we next evaluated the ability of CDE challenge in recruiting Th17-cells to the lungs. Compared to naïve mice, CDE-challenge in CDE-SCM failed to increase expression levels of *Rorc* mRNA (**Fig 4A**). By contrast, CDE challenge in CDE-MCM sensitized mice induced higher levels of *Rorc* mRNA expression compared to naïve mice and mice subjected to CDE-SCM (**Fig 4A**). CDE challenge in CDE-MCM upregulated*Il17* and *Rorc* mRNA expression in BALF cells (**Fig 4B**), upregulated *Il17* mRNA in lung tissues (**Fig 4C**), increased the number of RORγt-expressing T-cells (**Fig 4D**), and IL17-expressing Th17-cells (**Fig 4E**). Oral administration of ladarixin blocked each of these effects of CDE challenge (**Fig 4B-E**), including a remarkable 90% suppression of *Il17* mRNA expression (**Fig 4B**).

Inhibition of CXCR1 and CXCR2 attenuates the recruitment of allergic inflammatory cells to the lungs

Neutrophils express CXCR1 and CXCR2[44-46]. Based on our observations showing the remarkable efficacy of ladarixin in blocking CDE-challenge-induced recruitment of inflammatory cells (**Fig 2-4)** , we hypothesized that various inflammatory cells recruited by CDE challenge also express CXCR1 and CXCR2. To test this hypothesis, we quantified the number of CXCR1 and CXCR2 expressing inflammatory cells in the lung in CDE-MCM that secrete cytokines or expression key transcription factors**.** CDE challenge in mice subjected to CDE-MCM increased the recruitment of CXCR1-expressing (**Fig 5A**) and CXCR2-expressing (**Fig 5B**) GATA3-, IL4-, IL5-, IL13-, RORγt-, and IL17-expressing T-cells. Furthermore, CDE challenge increased the recruitment of CXCR1-expressing (**Fig 5A**) and CXCR2-expressing (**Fig 5B**) neutrophils, eosinophils, IL23-expressing neutrophils, and IL23-expressing eosinophils. Oral administration of ladarixin blocked CDE challenge-induced recruitment of CXCR1-expressing (**Fig 5A**) and CXCR2-expressing (**Fig 5B**) GATA3-, IL4-, IL5-, IL13-, RORγt-, and IL17-expressing T-cells, neutrophils, eosinophils, IL23-expressing neutrophils, and IL23-expressing eosinophils. Together these results indicate that CXCR1 and CXCR2 are key regulators of Th2 and Th17-mediated allergic inflammation.

Inhibition of CXCR1 and CXCR2 blocks allergen challenge-induced DNA damage and restores genome integrity

We have previously reported that allergen challenge including CDE-challenge induces oxidative stress and DNA damage in the lung, and damaged DNA contributes to allergic lung inflammation[30,34,47-49]. Building on our observation that CXCR1 and CXCR2 are key regulators of Th2 and Th17-mediated allergic inflammation, we hypothesized that ladarixin may inhibit CDE challenge-induced lung DNA damage by suppressing the

recruitment of ROS-generating inflammatory cells. To determine if treatment with ladarixin rescues CDE-mediated oxidative genome damage, we performed gene-specific LA-qPCR and analyzed DNA damage in two representative genes, $\pi o\lambda\ \beta$ and $\beta\ \gamma\lambda o\beta\iota\nu$. In the long amplicons of $\pi o\lambda\ \beta$ and β-$\gamma\lambda o\beta\iota\nu$, there is a higher probability of oxidative genome damage that can block the elongating Taq DNA polymerase and consequently, there will be less efficient PCR amplification[35,36]. In a short amplicon, the probability of genome damage is much lower and comparable PCR amplification efficiency is expected. Compared to CDE challenge in sensitized mice without ladarixin, oral treatment of ladarixin along with and after CDE challenge in CDE-MCM reduced long amplicon PCR product, indicating less DNA damage and restoration of genome integrity (**Fig 6A and B**). Together these data indicate that ladarixin rescues mice from CDE challenge-induced lung DNA damage.

Discussion

Our findings indicate that CXCR1 and CXCR2 play a major role in Th2 and Th17- mediated allergic lung inflammation. These receptors play a critical role in the recruitment of CXCR1- and CXCR2-expressing Th2 cells, Th17 cells, eosinophils, and neutrophils. We show for the first time that allosteric inhibition of CXCR1 and CXCR2 by oral administration of ladarixin potently inhibits Th2 and Th17-mediated allergic lung inflammation.

A growing number of monoclonal antibodies that target either a single Th2 cytokine or its cognate receptor are currently used to treat patients with severe asthma. Thus monoclonal antibodies block IL5 (mepolizumab[50] and reslizumab[51]), IL5 receptor alpha-chain (benralizumab[52,53]), and IL4 receptor alpha (dupilumab[54]) are currently used in the management of severe asthma. IL17-secreting Th17 cells also contribute to airway remodeling and disease severity in severe endotype asthma[2,3]. Biologics that block IL17 have been or are being tried in this asthma phenotype[55,56]. Unexpectedly, administration of the anti-IL17 receptor antibody brodalumab to the subjects with moderate to severe asthma failed to improve the clinical symptoms[55]. A number of studies indicate a crucial role of IL23 in maintaining Th17 cells[39], and regulating Th2 and Th17 cells[57,58]. Thus lack of IL23 suppressed naïve Th cell differentiation toward Th2 cells[57], and treatment with anti-IL23 antibody suppressed ovalbumin-induced IL5 and IL13 production and eosinophil recruitment[58]. In the Th2/Th17 dominant subgroup of severe asthma[42,43], BALF levels of IL23 were elevated[43]. These studies suggest that inhibiting IL23 with a monoclonal antibody should be beneficial in asthma. However, a clinical trial of human anti–IL23p19 monoclonal antibody risankizumab demonstrated that it had no beneficial effect in patients with severe asthma[59]. Thus, at this time, there is no effective treatment for Th17-associated allergic lung inflammation. Our results suggest that allosteric inhibition of CXCR1 and CXCR2 may suppress Th17-associated allergic inflammation.

A focus of earlier studies of CXCR1/2 inhibitors in mitigating neutrophil recruitment in allergic inflammation[30,31]. In addition to our novel finding that allosteric inhibition of CXCR1 and CXCR2 blocks the recruitment of Th2 and Th17 cells, we show that ladarixin suppresses the recruitment of neutrophils, including IL23-secreting neutrophils. These data build on our earlier report that neutrophils can augment allergic airway inflammation and sensitization[30]. Other studies have shown that neutrophil recruitment to the site is closely associated with local allergic inflammation in atopic dermatitis[60], allergic contact dermatitis[61,62], anaphylaxis[63], and asthma[30,64]by regulating innate and adaptive immunity. Since neutrophils express abundant CXCR1 and CXCR2, inhibition of their recruitment likely also contributes to ladarixin-induced mitigation of allergic lung inflammation.

Allergenic extracts stimulate Toll-like receptor 4/Myd88-dependent ROS generation and oxidative DNA damage[34,47]. Oxidative stress stimulates ROS-generating neutrophils recruitment via CXCL1/2-CXCR1/2 axis[30,31]which in turn facilitates allergic airway inflammation[30]. Administration of ladarixin suppressed CDE challenge-induced lung DNA damage likely via two mechanisms. First, since stimulation of CXCR2 induces ROS generation[65], ladarixin may directly suppress ROS generation from CXCR2-expressing cells. Second, ladarixin inhibits the recruitment of CXCR1/2-expressing ROS-generating inflammatory cells, such as neutrophils and eosinophils. Since oxidative DNA damage in the lung facilitates allergic inflammation[30,34], ladarixin might suppress allergic inflammation by inhibiting DNA damage and augmenting genome integrity.

Ladarixin is a dual CXCR1/2 antagonist that demonstrated beneficial effects on tumors and endocrine disorders. Treatment with ladarixin inhibited the melanoma xenografts *in vivo* [32], delayed and prevented spontaneous diabetes onset in NOD mice[66], and when administered with an immune checkpoint inhibitor like anti-PD-1 inhibited tumor growth and improved the survival in tumor xenograft mouse model[67]. Based on the clinical trial recently performed in patients with new-onset type 1 diabetes, the use of ladarixin is safe and well tolerated[68]. We suggest that future human studies should carefully investigate the role of allosteric inhibition of CXCR1 and CXCR2 are a novel approach to mitigating allergic inflammation involving activation of Th2-and Th17-cells and pathways.

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Figure Legends

Fig 1. CXCR1 and CXCR2 stimulate allergen challenge-induced allergic lung inflammation

(**A**) Protocol for CDE-MCM with or without ladarixn treatment. (**B**) Protocol for CDE-SCM with or without ladarixin treatment. (**C**) The levels of BALF neutrophils at 16 h post-CDE challenge in CDE-SCM. (**D**) The numbers of inflammatory cells in BALF at 72 h post-CDE challenge in CDE-MCM. (**E**) FCM analysis of the numbers of inflammatory immune cells in the lungs at 72 h post-CDE challenge in CDE-MCM. (**F**) The levels of serum total IgE and CDE-specific IgE at 72 h post-CDE challenge in CDE-MCM.

BALF; bronchoalveolar lavage fluid

CDE; cat dander extract

CDE-MCM; CDE-multiple challenge model

CDE-SCM; CDE-single challenge model

Fig 2. CXCR1 and CXCR2 stimulate CDE-challenge induced Th2 gene expression and recruitment of GATA3 and Th2 cytokine expressing T-cells

(**A**) *Gata3* mRNA expressions in the BALF cells after CDE challenge in CDE-SCM and CDE-MCM mice at 28 h post-CDE challenge. (**B-E**) at 28 h post-CDE challenge in CDE-MCM. (**B**)*Gata3* mRNA expressions in BALF cells. (**C**) *Il4, Il5, Il13* , and *Tgfb1* cells mRNA expressions in BALF cells at 28h after CDE challenge (**D**) The lung mRNA expressions of *Il4* and *Il13* at 28h post-CDE challenge. (**E**) The number of IL4, IL5, IL13, and GATA3 expressing lung Th2-cells at 72h post-CDE challenge.

BALF; bronchoalveolar lavage fluid

CDE; cat dander extract

CDE-MCM; CDE-multiple challenge model

GATA3; GATA binding protein 3

Th2; T helper 2

Fig 3. CXCR1 and CXCR2 mediated cytokines expressions which induce Th17 cells differentiation and maintenance

(**A-B**) *Il23, Il1b,* and *Il6* mRNA expressions in the BALF cells (**A**) and in the lung (**B**) in CDE-MCM at 28 h post-CDE challenge. (**C**) The protein levels of Il23 in the lung in CDE-MCM at 28 h post-CDE challenge. (**D**) The numbers of IL23 producing T-cells, neutrophils, eosinophils, and epithelial cells in the lung in CDE-MCM at 72 h post-CDE challenge.

BALF; bronchoalveolar lavage fluid

CDE; cat dander extract

CDE-MCM; CDE-multiple challenge model

Th17; T helper 17

Fig 4. CXCR1 and CXCR2 mediate Th17 gene expression and recruitment of Th17 cells

(**A**) *Rorc* mRNA expressions in the BALF cells after CDE challenge in CDE-SCM and CDE-MCM mice at 28 h post-CDE challenge. (**B**) *Il17* and *Rorc* mRNA expressions in the BALF cells in CDE-MCM at 28 h post-CDE challenge. (**C**) *Il17*mRNA expressions in the lung in CDE-MCM at 28 h post-CDE challenge. (**D-E**) The number of RorγT T-cells (**D**) and IL17 secreting Th17 cells (**E**) in the lung in CDE-MCM at 72 h post-CDE challenge.

BALF; bronchoalveolar lavage fluid

CDE; cat dander extract

CDE-MCM; CDE-multiple challenge model

RorγT; retinoic acid–related orphan receptor- gamma t

Th17; T helper 17

Fig 5. Ladarixin inhibits CDE-challenge induced recruitment of CXCR1 and CXCR2 expressing inflammatory cells

(**A-B**) CDE-MCM at 72 h post-CDE challenge. (**A**) The numbers of CXCR1 expressing cells in the lung. (**B**) The numbers of CXCR2 expressing cells in the lung.

CDE; cat dander extract

CDE-MCM; CDE-multiple challenge model

Fig 6. Ladarixin inhibits CDE-challenge induced lung DNA damage

(**A-B**) CDE-MCM at 72 h post-CDE challenge. (**A**) Representative gels showing PCR-amplified fragments encompassing mouse$\pi o\lambda$ β and β-$\gamma\lambda o\beta\iota\nu$ in the lung, respectively. Amplification of a long fragment (LA, upper panels) was normalized to the corresponding short fragment of the same gene (SA, lower panels). Columns 1-7; mice challenged with CDE without the treatment of ladarixin. Column 8-13; mice challenged with CDE treated with ladarixin. (**B**) The bar diagram represents the ratio of LA to SA amplification of each gene from two replicate gels.

CDE-MCM; CDE-multiple challenge model

LA; long amplicon

SA; short amplicon

Supplemental Fig 1. Representative FC plot gating strategy to identify live CD45+ CD3+ CD4+ CXCR1+ IL4+ Th2-cells in the lungs

The forward and side scatter (FSC vs SSC) density plots were generated to separate cell populations of interest from debris. Next, doublets and debris were excluded by forward scatter height versus forward scatter area density plot (FSC-H vs FSC-A). Next, dead cells were excluded by gating only live cells using fluorophore-conjugated antibodies Fixable Viability Dye eFluor 780. In all subsequent steps, side scatter area (SSC-A) was plotted against fluorescence-tagged antibodies to gate positive cells by excluding cells in the corresponding FMO gate. Sequentially, live cells were gated using CD45+ cells to gate hematopoietic cells, CD3+, CD4+ double positive cells to gate for CD4+ T-cells, CXCR1+ cells to gate CXCR1-expressing CD4+ T-cells, then intracellular IL4+ to gate for CXCR1+ IL4+ Th2-cells in the lungs.

FMO; fluorescence minus one

Supplemental Fig 2. CXCL expressions in the lung and CXCR1/CXCR2 expressions in the bone marrow cells and BALF cells in CDE-multiple challenge model

(**A**) *Cxcl* expressions in the lungs at 2 h and 4 h post-CDE challenge in CDE-MCM. (**B**) Time kinetics of*Cxcr1* and *Cxcr2* expressions in the bone marrow cells in CDE-MCM.

CDE; cat dander extract

CDE-MCM; CDE-multiple challenge model

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