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Therapeutic targeting of CCR1 attenuates established chronic fungal asthma in mice

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1 CC chemokine receptor 1 (CCR1) represents a promising target in chronic airway inflammation and remodeling due to fungus-associated allergic asthma.

2 The present study addressed the therapeutic effect of a nonpeptide CCR1 antagonist, BX-471, in a model of chronic fungal asthma induced by *Aspergillus fumigatus* conidia.

3 BX-471 treatment of isolated macrophages inhibited CCL22 and TNF-α and promoted IL-10 release. BX-471 also increased toll like receptor-9 (TLR9) and decreased TLR2 and TLR6 expression in these cells.

4 When administered daily by intraperitoneal injection, from days 15 to 30 after the initiation of chronic fungal asthma, BX-471 (3, 10, or 30 mg kg^{-1}) dose-dependently reduced airway inflammation, hyper-responsiveness, and remodeling at day 30 after conidia challenge. The maximal therapeutic effect was observed at the 10 mg kg^{-1} dose.

5 In summary, the therapeutic administration of BX-471 significantly attenuated experimental fungal asthma *via* its effects on both innate and adaptive immune processes.
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Abbreviations: GMS, Gomori methenamine silver; GPCRs, G-protein-coupled receptors; H&E, hematoxylin and eosin; PAS, Periodic Acid Schiff; SPF, specific pathogen free; TLR, toll-like receptor

Introduction

A plethora of CC and CXC chemokine ligands have been identified in the initiation and maintenance of experimental allergic and/or asthmatic responses within the lung (Romagnani, 2002; Lloyd & Rankin, 2003). Unfortunately, this deluge of data have made it difficult to ascertain whether the targeting of a single chemokine will provide therapeutic benefit in clinical asthma (Proudfoot *et al*., 2003). Consequently, recent research attention has focused on the targeting of the specialized G-protein-coupled receptors (GPCRs) that chemokine ligands bind and activate in order to exert their biological effects (Power & Proudfoot, 2001). Attractive chemokine receptor targets have been revealed with the advent of highly specific and selective small molecule antagonists for chemokine GPCRs. Based on experimental work with various allergic airway models, highly anticipated candidate GPCR-targeted therapeutics include CCR3, CCR4, CCR8, and CXCR3. However, other chemokine receptors that show a high degree of ligand promiscuity such as CCR1 deserve further research consideration. CCR1 binds a number of CC chemokines that have been implicated in asthma, including CCL3, CCL5, CCL6, CCL7, and CCL16 (Lloyd & Rankin, 2003).

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We have previously examined the role of chemokine ligands and receptors in a murine model of fungal asthma characterized by elevated IgE, Th2 cytokines, C-C chemokines, eosinophilic inflammation, and persistent airway hyper-responsiveness following pulmonary exposure to the spores or conidia from *Aspergillus fumigatus* fungus (Hogaboam *et al*., 2000). *A. fumigatus* is a ubiquitous, sporulating fungus that complicates cystic fibrosis and asthma in growing number of patients worldwide (Stevens *et al*., 2003). The modeling of fungal asthma through the introduction of live *A. fumigatus* spores or conidia into *A. fumigatus*-sensitized mice revealed that the targeting of chemokine receptors was a far more effective strategy *versus* targeting individual chemokine ligands for preventing the initiation and/or reversing chronic fungal asthma. For example, the genetic deletion of CCR1 did not prevent the initiation of fungal asthma but CCR1−/− mice did not exhibit the airway remodeling (i.e. goblet cell metaplasia and subepithelial fibrosis) observed in their CCR1+/+ counterparts at 30 days after an intrapulmonary challenge with *A. fumigatus* conidia. In addition, whole lung levels of IFN-γ were significantly higher whereas whole lung levels of IL-4, IL-13, and CCL6, CCL11 and CCL22 were significantly lower in CCR1−/− mice compared with CCR1+/+ mice (Blease *et al*., 2000). These data suggested that CCR1 maybe an attractive therapeutic target in established allergic airway disease.

BX-471 (also known as ZK-811752) – *R-N*-[5-chloro-2-[2-[4-[(4-fluorophenyl)methyl]-2-methyl-1-piperazinyl]-2-oxoethoxy]

phenyl]urea hydrochloric acid salt – is the lead compound in a series of nonpeptide CCR1 antagonists originally developed *via* high-capacity screening followed by chemical optimization at Berlex and Schering AG (Hesselgesser *et al*., 1998; Ng *et al*., 1999). BX-471 displaces the CCR1 ligands macrophage inflammatory protein-1alpha (MIP-1α/CCL3), RANTES/CCL5, and monocyte chemotactic protein-3 (MCP-3/CCL7), and blocks CCR1-mediated Ca(2+) mobilization, increase in extracellular acidification rate, CD11b expression, and leukocyte migration (Liang *et al*., 2000a). This antagonist has a 10,000-fold greater selectivity for human CCR1 compared with 28 other human GPCRs. Since BX-471 also exhibits antagonistic activity towards CCR1 in a number of small animal species, it has been tested and shown to be efficacious in a rat experimental allergic encephalomyelitis model of multiple sclerosis (Liang *et al*., 2000a), rat heterotopic heart transplant rejection (Horuk *et al*., 2001a), rabbit renal transplantation (Horuk *et al*., 2001b), murine unilateral ureter obstruction (Anders *et al*., 2002), and progressive immune complex glomerulonephritis in mice (Anders *et al*., 2004). The therapeutic effects of BX-471 were associated with its ability to block CCL5 activation of monocytes (Horuk *et al*., 2001a), prevent CCL3-dependent activation (Horuk *et al*., 2001b), reduce CCR1 and CCR5 mRNA levels, prevent T-cell recruitment (Anders *et al*., 2002; 2004), and reduce renal expression of CC chemokines CCL2, CCL3, CCL4, and CCL5 and the chemokine receptors CCR1, CCR2, and CCR5 (Anders *et al*., 2004).

These previous preclinical findings combined with the demonstration that CCR1 blockade through BX-471 treatment prevented renal fibrotic changes in mice were impetus to examine the therapeutic potential of this antagonist in a murine model of chronic fungal asthma. In the latter model, CCL2 (Blease *et al*., 2001b), CCL5 (Schuh *et al*., 2002a; 2003a), CCL6 (Hogaboam *et al*., 1999), CCR1 (Blease *et al*., 2000), CCR4 (Schuh *et al*., 2002b), and CCR5 (Schuh *et al*., 2002a) have been shown to have prominent roles in the airway hyper-responsiveness and/or airway remodeling that follows the introduction of live *A. fumigatus* spores into *A. fumigatus*-sensitized mice. Herein, we show that systemic CCR1 blockade through daily BX-471 administration signifcantly and markedly attenuated all features of established chronic fungal asthma in mice.

Methods

Macrophage isolation and in vitro *BX-471 experiments*

Specific pathogen free (SPF), female BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.) and housed in the animal care facility (University Laboratory of Animal Medicine) at the University of Michigan. The Animal Use Committee at the University of Michigan approved all experimental procedures involving mice. Naïve, SPF mice were euthanized and subjected to peritoneal lavages with 10 ml sterile saline. Lavages were pooled, red blood cells were lysed in ammonium chloride buffer, and the remaining cells were thoroughly washed with saline. Cells were counted and subjected to Diff-Quik staining to determine the number of peritoneal macrophages. Cells were resuspended in complete DMEM (BioWhittaker, Walkersville, MD, U.S.A.) containing 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were plated in plastic plates and incubated 1–2 h at 37°C in 5% CO_2. Nonadherent cells were removed and adherent cells were washed with complete DMEM. The adherent macrophages were then rested overnight in a CO_2 incubator. Subsequently, vehicle (2.5% DMSO) or BX-471 at 10 μM in fresh DMEM was added to separate tissue culture wells (in triplicate) containing approximately 5×10^5 cells/well of a 24-well tissue culture plate for 24 h. The Department of Medicinal Chemistry at Almirall Prodesfarma (Barcelona, Spain) synthesized the BX-471 compound used in this *in vitro* study and the *in vivo* study that is described below. We selected a dose of 10 μM BX-471 for the *in vitro* studies for the following reasons: (1) this dose was required to a achieve a marked inhibition of the binding of CCL3 to murine CCR1 (Liang *et al*., 2000b), (2) the subcutaneous injection of 20 mg kg^{-1} BX-471 into mice leads to peak plasma levels of 9 μM (Anders *et al*., 2002), and (3) this concentration of BX-471 did not exhibit cellular toxicity (Liang *et al*., 2000b). At the conclusion of the BX-471 treatment, cell-free supernatants were collected for ELISA analysis and the macrophages were prepared for RNA isolation.

Murine model of chronic fungal-induced allergic airway disease or fungal asthma

SPF, female CBA/J mice were purchased from the Jackson Laboratories (Bar Harbor, ME, U.S.A.) and were maintained in an SPF facility for the duration of this study. Prior approval for mouse usage was obtained from the University Laboratory of Animal Medicine facility at the University of Michigan Medical School. Systemic sensitization of mice to a commercially available preparation of soluble *A. fumigatus* antigens was performed as previously described in detail (Hogaboam *et al*., 2000). At 7 days after the third intranasal challenge, each mouse received 5.0×10^6 *A. fumigatus* conidia suspended in 30 μl of 0.1% Tween-80 *via* the intratracheal route (Hogaboam *et al*., 2000).

BX-471 therapy during fungal-induced allergic airway disease

BX-471 (Liang *et al*., 2000a) has been used extensively in several animal models (Anders *et al*., 2002; 2004; Horuk *et al*., 2001a, b). Previous studies have shown that BX-471 is 20-fold less potent in inhibiting mouse CCR1 relative to human CCR1 (Anders *et al*., 2002). Prior pharmacokinetic parameter analysis revealed that the subcutaneous injection of 20 mg kg^{-1} BX-471 resulted in peak plasma levels of 9 μM at 30 min and steady-state levels of approximately 0.1 μM at 8 h after injection (Anders *et al*., 2002). Based on these previous studies, we employed single daily intraperitoneal injections of BX-471 at 3, 10 or 30 mg kg^{-1} of body weight in groups of five *A. fumigatus*-sensitized mice. BX-471 treatment was initiated at day 15 after conidia challenge, and this time corresponded with peak peribronchial accumulations of eosinophils and CD4+ T cell, significant airway hyper-responsiveness to methacholine, goblet cell hyperplasia, and subepithelial collagen deposition in *A. fumigatus*-sensitized mice (Hogaboam *et al*., 2000). Another group of five *A. fumigatus*-sensitized mice received 250 μl of BX-471 vehicle comprised of

0.25% methylcellulose and 0.05% Tween-80 *via* the same route beginning at day 15 and concluding on day 30 after conidia challenge.

Measurement of bronchial hyper-responsiveness

At day 30 after the *A. fumigatus* conidia challenge, bronchial hyper-responsiveness in vehicle- and BX-471-treated mice was measured in a Buxco™ plethysmograph (Buxco, Troy, NY, U.S.A.) as previously described (Hogaboam *et al*., 2000). Sodium pentobarbital (Butler Co., Columbus, OH, U.S.A.; $0.04\,mg\,g^{-1}$ of mouse body weight) was used to anesthetize each mouse prior to its intubation for ventilation with a Harvard pump ventilator (Harvard Apparatus, Reno, NV, U.S.A.). The following ventilation parameters were used: tidal volume = 0.25 ml, breathing frequency = $120\,min^{-1}$, and positive end-expiratory pressure $\cong 3$ cm H_2O. Within the sealed plethysmograph mouse chamber, transrespiratory pressure (i.e. Δ tracheal pressure $-\Delta$ mouse chamber pressure) and inspiratory volume or flow were continuously monitored online by an adjacent computer, and airway resistance was calculated by the division of the transpulmonary pressure by the change in inspiratory volume. The data shown in this manuscript are focused on doses of 210 and $420\,\mu g\,kg^{-1}$ methacholine because these methacholine doses elicited prominent airway hyper-responsiveness in *A. fumigatus*-sensitized mice after the conidia challenge but failed to elicit a response in nonsensitized mice. At the conclusion of the assessment of airway responsiveness, a bronchoalveolar lavage (BAL) was performed with 1 ml of normal saline. Approximately 500 μl of blood was then removed from each mouse and transferred to a microcentrifuge tube. Sera were obtained after the sample was centrifuged at 10,000 r.p.m. for 5 min. Whole lungs were finally dissected from each mouse and snap frozen in liquid N_2 or prepared for histological analysis.

Morphometric analysis of leukocyte accumulation in BAL samples

Lymphocytes and macrophages were enumerated in BAL samples cytospun (Shandon Scientific, Runcorn, U.K.) onto coded microscope slides. Each slide was stained with a Wright–Giemsa differential stain, and the average number of each cell type was determined after counting a total of 300 cells in 20 high-powered fields (HPF; $\times 1000$) per slide. A total of approximately 5×10^5 BAL cells were cytospun onto each slide to compensate for differences in cell retrieval.

Whole lung histological analysis

Whole lung samples from all vehicle and BX-471 treatment groups at day 30 after *A. fumigatus* conidia challenge were fully inflated with 10% formalin, dissected and placed in fresh formalin for 24 h. Routine histological techniques were used to paraffin-embed the entire lung, and 5 μm sections of whole lung were stained with Gomori methenamine silver (GMS), hematoxylin and eosin (H&E), or with Periodic Acid Schiff (PAS). Inflammatory infiltrates and structural alterations were examined around small airways and adjacent blood vessels using light microscopy at a magnification of $\times 200$.

SuperArray analysis and quantitative TAQMAN polymerase chain reaction (PCR) analysis

SuperArray analysis of transcript expression was performed according to the directions provided with these kits (GEArray™ Q series KIT nonradioactive; SuperArray Inc.,. Bethesda, MD, U.S.A.). Briefly, from cultured macrophages or homogenized mouse lungs, total RNA was isolated using the Trizol reagent (Invitrogen/Life Technologies, Carlsbad, CA, U.S.A.). Once isolated, 1–2.5 μg of total RNA, pooled from the *in vitro* and *in vivo* treatment groups, was used as the template for Biotin-labeled cDNA probe synthesis. The labeled probes were then hybridized to the mouse inflammatory cytokine/receptor GEArray™ Q series membrane containing 96 genes related to murine cytokine and chemokine ligands and receptors. After an overnight incubation at 60°C, the membranes were washed several times, blocked, and subjected to chemiluminescent detection (alkaline phosphatase-conjugated streptavidin; 1 : 10,000 dilution) with the chemiluminescent substrate for alkaline phosphatase, phenylphosphate substituted 1,2 dioxetane (CDP-star). After exposing the membranes to X-ray film, the developed films were scanned to create raw image files, which were analyzed using an image analysis software program (Scanalyze by Michael Eisen). Each GEArray™ Q series membrane contained a negative control of pUC18 DNA and housekeeping genes such as β-actin, GAPDH, cyclophilin, and ribosomal protein L13a. The relative abundance of a particular transcript was estimated by directly comparing its signal intensity to the signal derived from three or four combined housekeeping genes. The results were expressed as the ratio of the normalized spot intensity in the BX-471-treated macrophages/whole lungs *versus* the vehicle-treated macrophages/whole lung group. Only two-fold or greater changes in gene expression were considered significant and were reported below. The SuperArray analysis was repeated in order to determine whether the pattern of gene expression observed in these samples was reproducible.

For quantitative TAQMAN analysis, total RNA was isolated from cultured macrophages ($\pm 10\,\mu$M BX-471 or vehicle). A total of 0.5 μg of total RNA was reverse transcribed to yield cDNA, and TLR2, TLR6, and TLR9 were analyzed by real-time quantitative RT-PCR procedure using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). GAPDH was analyzed as an internal control. Gene expression was normalized to GAPDH before the fold change in gene expression was calculated. The fold changes in TLR2, TLR6, and TLR9 gene expression were calculated *via* the comparison of gene expression of each TLR in untreated macrophages. TLR levels in naïve macrophage were assigned a value of 1.

ELISA analysis

Murine CCL3, CCL5, CCL6, CCL22, TNF-α, IL-4, IL-5, IL-12, IL-13, IFN-γ, and IL-10 levels were measured in 50-μl samples from cell-free supernatants (from macrophage cultures) and/or whole lung homogenates using a standardized sandwich ELISA technique previously described in detail (Evanoff *et al*., 1992). Each ELISA was screened to ensure antibody specificity and recombinant murine cytokines, and chemokines were used to generate the standard curves from which the concentrations present in the samples were derived.

The limit of ELISA detection for each cytokine was consistently above 5 pg ml^{-1}. The cytokine and chemokine levels in each whole lung sample were normalized to total protein levels measured using the Bradford assay.

Serum levels of IgE, IgG1, and IgG2a at day 30 after conidia challenge in the control and BX-471 treatment groups were analyzed using complementary capture and detection antibody pairs for IgE, IgG1, and IgG2a (PharMingen, San Diego, CA, U.S.A.). Immunoglobulin ELISAs were performed according to the manufacturer's directions. Duplicate sera samples were diluted to 1 : 100 for IgE determination and 1 : 10 for determination of IgG levels. Immunoglobulin levels were then calculated from optical density readings at 492 nm, and immunoglobulin concentrations were calculated from a standard curve generated using recombinant IgE, IgG1, or IgG2a (both standard curves ranged from 5 to 2000 pg ml^{-1}).

Statistical analysis

All results are expressed as mean ± standard error of the mean (s.e.m.). An unpaired Student's *t*-test was used to detect differences between the vehicle and BX-471 treatments *in vitro*. A one-way analysis of variance and a Dunnett's Multiple Comparisons Test were used to reveal statistical differences between the control group and the BX-471 treatment groups at day 30 after the conidia challenge. $P<0.05$ was considered statistically significant.

Results

Pharmacologic targeting of CCR1-modulated chemokine generation, and chemokine and TLR expression by murine macrophages

Our interest in assessing the pharmacological validity of targeting CCR1 during experimental chronic fungal asthma stemmed from our previous study in which mice deficient in CCR1 did not chronically maintain allergic airway responses to *A. fumigatus* (Blease *et al*., 2000). In the present study, cultured macrophages were examined to determine the effect of a CCR1 antagonist BX-471 on the activation of these important innate immune cells. As shown in Figure 1, BX-471 (10 μM) treatment of naïve, cultured murine macrophages for 24 h significantly increased immunoreactive levels of CCL3 and CCL6 and significantly decreased CCL22 levels (top panel). Analysis of the same cell supernatants revealed that significantly less TNF-α was present, whereas IL-10 levels were dramatically increased in these cell free supernatants (bottom panel). SuperArray gene array analysis revealed other effects of BX-471 treatment on cultured murine macrophages (Table 1). Identical results shown were obtained from a repeated SuperArray analysis of the same macrophage samples. The BX-471 treatment increased by $\geqslant$2-fold the transcript expression of several proallergic factors including CCR3, CCR6, IL-1 receptor, CCL24, CCL9, and TGF-β (Zimmermann *et al*., 2000; Romagnani, 2002). Conversely, the BX-471 treatment also increased the expression of potent antifungal or proinflammatory factors including CXCL2, CXCL15, CCL5, IL-20, LTB receptor, and macrophage inflammatory protein (MIF) (Table 1) (Mehrad *et al*., 1999). Together, these data suggested that BX-471 prevented the

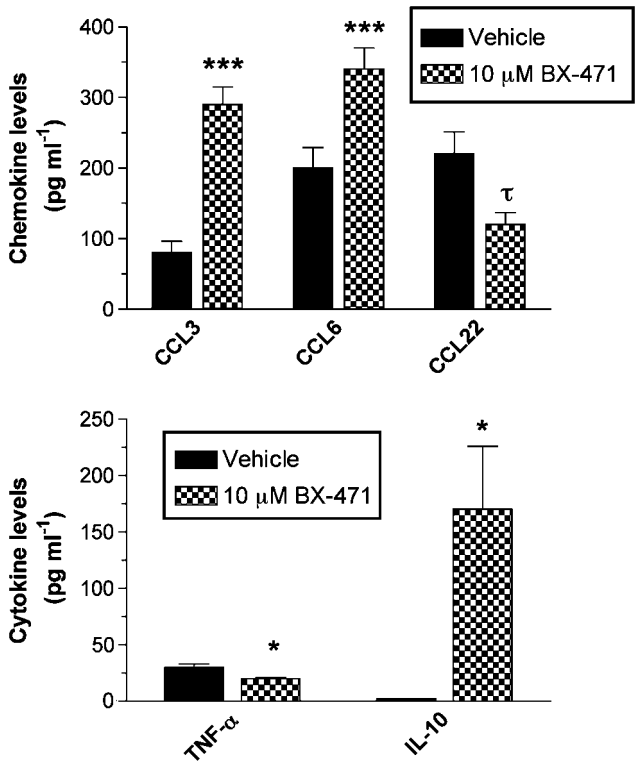

Figure 1 Spontaneous CCL3, CCL6, CCL22 (top panel), TNF-α and IL-10 (bottom panel) generation by isolated macrophages from naïve mice. ELISA was used to determine specific chemokine and cytokine levels in cell-free supernatants removed at 24 h after culture. BX-471 (at 10 μM) or the vehicle (3% DMSO) for this CCR1 antagonist was included in these 24-h cultures. Data are mean ± s.e.m. of triplicate or quadruplicate samples. $^{*}P\leqslant0.05$, $^{***}P\leqslant0.001$ compared with chemokine or cytokine levels detected in culture wells containing vehicle-treated macrophages. $^{\tau}P\leqslant0.05$ compared with CCL22 levels detected in vehicle-treated macrophages.

binding of CCR1 ligands (i.e. CCL3 and CCL6) to CCR1 expressed by macrophages, but reduced the production of proallergic factors such as CCL22 and TNF-α (Barnes, 2001; Romagnani, 2002). In addition, BX-471 increased the expression of IL-10, a potent immunomodulatory cytokine necessary for regulating the antifungal response to Aspergillus (Grunig *et al*., 1997). Finally, BX-471 inhibited the transcript expression of proallergic factors, but enhanced the expression of antifungal factors by cultured murine macrophages.

Further investigation into the *in vitro* effects of BX-471 on naïve macrophages further showed major alterations in TLR receptor transcript expression (Figure 2). Three TLR transcripts were examined: TLR2, TLR6, and TLR9. Interest in TLR2 and TLR9 stems from recent work by Redecke *et al*. (2004), who showed that TLR2 stimulation leads to the enhancement of Th2 responses during allergic airway disease whereas TLR9 activation results in the converse. TLR6 was examined because this TLR is abundantly expressed in the lung (Takeuchi *et al*., 1999) and commonly associates with TLR2 to form a heterodimer (Hallman *et al*., 2001). Although BX-471 (10 μM) did not alter TLR6 transcript levels in isolated macrophages, treatment with this CCR1 antagonist for 24 h reduced TLR2 gene expression by 50% and increased TLR9 gene expression by 50% (Figure 2). Thus, these data suggested that pharmacological antagonism of CCR1 with BX-471 had a

Table 1 SuperArray analysis of cytokine and chemokine ligand and receptor transcript expression in isolated murine macrophages treated with 10 μM BX-471 for 24 h

Gene	*Fold decrease in expression*
CCR3	6
CCR6	5
IL-1R1	3
CCL9	2
CCL24	3
TGF-β	2
	Fold increase in expression
CXCL2	20
CXCL15	10
CCL5	3
LTB receptor	6
IL-20	3
MIF	3

Fold changes are relative to gene expression detected in total RNA from murine macrophages exposed to 2.5% DMSO (the vehicle for BX-471) for 24 h.

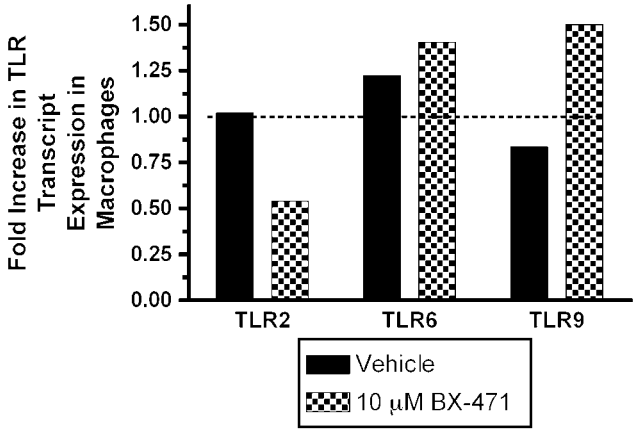

Figure 2 Quantitative TAQMAN PCR analysis of TLR2, TLR6, and TLR9 transcript expression in vehicle- and BX-471-treated macrophages. The fold-increase in TLR transcript expression above transcript levels in untreated mouse macrophages is shown. Data are mean $\pm$ s.e.m. of triplicate or quadruplicate samples.

marked effect on the transcript expression of important immunomodulatory TLRs in macrophages.

BX-471 enhances fungal clearance from the lungs of mice with chronic fungal asthma

Our previous examination of *A. fumigatus*-sensitized CCR1$-/-$ mice revealed that fungal material was cleared more rapidly and efficiently in these mice relative to their wild-type controls (Blease *et al*., 2000). A number of recent studies have shown that TLRs exert prominent roles in the clearance of *A. fumigatus* from nonsensitized mice (Blease *et al*., 2001a; Mambula *et al*., 2002; Meier *et al*., 2003). In contrast to TLR4 and MyD88, TLR2 has a minor role in the innate antifungal response directed against *A. fumigatus* conidia in mice (Bellocchio *et al*., 2004), but it appears that the exuberant expression of TLR2 may markedly enhance allergic responses in the lung (Redecke *et al*., 2004). Interestingly, the absence of TLR9 had no effect on the development of invasive Aspergillosis in mice (Bellocchio *et al*., 2004). In the context of BX-471 therapy during chronic fungal asthma, it was clear that the presence of fungal material was markedly diminished in the lungs of mice treated with 10 mg kg^{-1} (Figure 3c) of BX-471 relative to the control group (Figure 3a) and the other BX-471 treatment groups (Figure 3b and d).

BX-471 therapy dose-dependently attenuated allergic hallmarks of chronic fungal asthma including inflammatory leukocyte accumulation

Our previous studies in CCR1$-/-$ mice showed that serum IgE levels did not differ in these mice compared with serum IgE levels measured in CCR1$+/+$ mice at any time after conidia challenge (Blease *et al*., 2000). The therapeutic effects of vehicle, 3, 10, and 30 mg kg^{-1} BX-471 were examined at day 30 after conidia challenge of *A. fumigatus*-sensitized mice and no changes in serum levels of IgE, IgG1, and IgG2a were observed. ELISA analysis results for the vehicle, 3, 10, and 30 mg kg^{-1} BX-471 groups, respectively, were as follows: (1) IgE $= 4 \pm 2$, 6 ± 1, 5 ± 1, and 7 ± 1 ng ml^{-1}; (2) IgG1 $= 15 \pm 5$, 16 ± 6, 14 ± 5, and 17 ± 6 ng ml^{-1}; (3) IgG2a $= 20 \pm 5$, 21 ± 5 and 25 ± 5, 21 ± 5 ng ml^{-1}.

A BX-471 dose-dependent reduction in the presence of inflammatory leukocytes was observed in BAL samples from each group of mice (Figure 4). Significantly fewer macrophages were detected in all BX-471 treatment groups compared with the vehicle-treated group. Few neutrophils and eosinophils were observed in BAL samples from *A. fumigatus*-sensitized mice challenged 30 days previously with *A. fumigatus* conidia; however, significantly fewer numbers of both granulocyte subtypes were detected in BAL samples from the 10 mg kg^{-1} BX-471 treatment group compared with the vehicle control BAL samples (Figure 4). We also observed that BAL samples from the 30 mg kg^{-1} BX-471 treatment group contained approximately eight-fold greater numbers of eosinophils compared with vehicle-treated groups. At all doses tested in the present study, lymphocytes (predominantly CD4+ T cells) were significantly lower compared with the control group (Figure 4).

The diminished numbers of inflammatory cells in BAL samples from the BX-471 treatment groups was confirmed in histological samples from these groups. As shown in Figure 5, the intensity of peribronchial inflammation was reduced by BX-471 therapy in a dose-dependent manner at day 30 after conidia challenge. The greatest reduction in inflammation was observed in the group of allergic mice that received 10 mg kg^{-1} BX-471 (Figure 5c). Together, these data demonstrated that the pharmacological targeting of CCR1 with BX-471 markedly attenuated the recruitment of inflammatory leukocytes into the airways and the accumulation of these leukocytes around the airways of mice with chronic fungal asthma.

BX-471 therapy dose-dependently reduced airway hyper-responsiveness at day 30 after conidia challenge in A. fumigatus*-sensitized mice*

Our previous examination of the role of CCR1 during chronic fungal asthma did not reveal a role for this chemokine receptor in the regulation of methacholine-induced airway

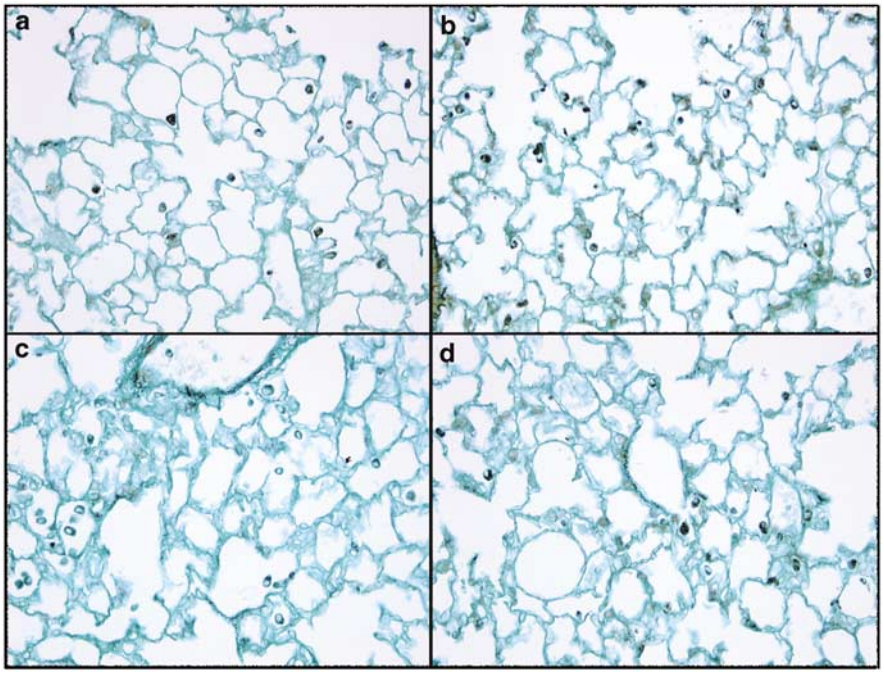

Figure 3 Gomori methenamine silver (GMS) staining of whole lung samples from vehicle (a)- and BX-471-treated (3 mg kg^{-1}) (b), 10 mg kg^{-1} (c) and 30 mg kg^{-1} (d) groups at day 30 after conidia challenge. *A. fumigatus*-sensitized mice were challenged intratracheally with live *A. fumigatus* conidia and 15 days later groups of five mice received vehicle alone or BX-471 at one of the three doses indicated above on a daily basis until day 30. Whole lungs samples were processed used routine histological techniques. Mice that received 10 mg kg^{-1} of BX-471 showed no evidence of fungal material (highlighted by black stained material in mononuclear cells) retention in contrast to the control and other BX-471 treatment groups. Original magnification was $\times 200$.

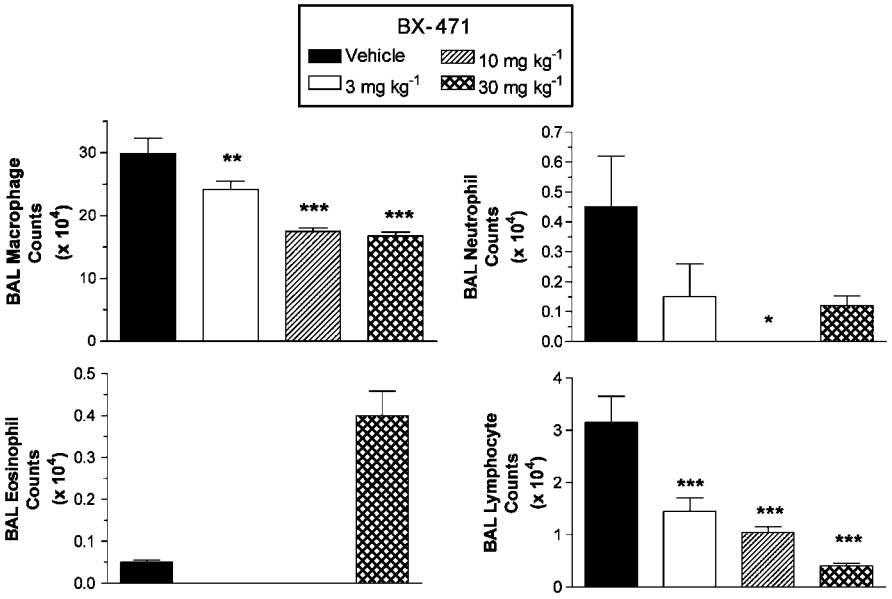

Figure 4 Leukocyte counts in bronchoalveolar lavage (BAL) samples from vehicle- and BX-471-treated groups at day 30 after *A. fumigatus* conidia challenge into *A. fumigatus*-sensitized mice. *A. fumigatus*-sensitized mice were challenged intratracheally with live *A. fumigatus* conidia and 15 days later groups of five mice received vehicle alone or on a daily basis until day 30. BAL cells were dispersed onto microscope slides using a cytospin, and eosinophils, lymphocytes, neutrophils, and macrophages were differentially stained with Wright–Giesma stain. Values are expressed as mean$\pm$s.e.m. $^*P\leqslant 0.05$, $^{***}P\leqslant 0.001$ compared with vehicle-treated group.

hyper-responsiveness (Blease *et al*., 2000). However, in the present study, it was apparent that pharmacological targeting of CCR1 had a prominent effect on airway hyper-responsiveness in mice with chronic fungal asthma. As shown in Figure 6 (top panel), BX-471 dose-dependently inhibited methacholine-induced (at 420 μg kg^{-1}) airway hyper-responsiveness in anesthetized and ventilated mice. The greatest inhibition of airway hyper-responsiveness was observed in the 10 mg kg^{-1}

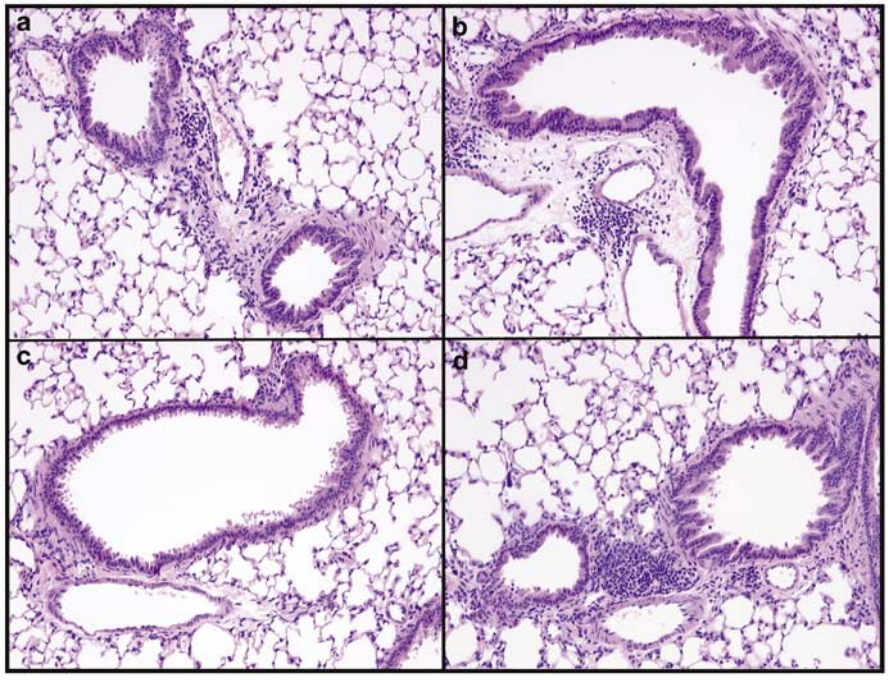

Figure 5 Hematoxylin and eosin (H/E) staining of whole lung samples from vehicle (a)- and BX-471-treated (3 mg kg^{-1}) (b), 10 mg kg^{-1} (c) and 30 mg kg^{-1} (d) groups at day 30 after conidia challenge. *A. fumigatus*-sensitized mice were challenged intratracheally with live *A. fumigatus* conidia and 15 days later groups of five mice received vehicle alone or BX-471 at one of the three doses indicated above on a daily basis until day 30. Whole lungs samples were processed using routine histological techniques. Mice that received 10 mg kg^{-1} of BX-471 showed the greatest reduction in peribronchial inflammation compared the control and other BX-471 treatment groups. Original magnification was $\times$ 200.

treatment group in which airway resistance was reduced by $75 \pm 5\%$ relative to the methacholine response invoked in the vehicle treatment group (Figure 6, bottom panel). Thus, the administration of BX-471 to mice with established fungal asthma markedly and significantly reduced methacholine-induced airway resistance.

Whole lung chemokine receptor transcript and CCR1 ligand protein levels were altered following BX-471 therapy in chronic fungal asthma

Table 2 summarizes the fold changes in chemokine and chemokine receptor transcript expression in whole lung samples from BX-471-treated mice at day 30 after conidia challenge. SuperArray analysis was performed on whole lung total RNA from the vehicle and all three BX-471 treatment groups. In whole lung samples from the 3 and 10 mg kg^{-1} BX-471 treatment groups, transcript expression for CCR1, CCR3, CCR4, CCR8, CCR9, CXCR3, CXCR4, CXCR5, and XCR1 was decreased by two-fold or greater compared with the vehicle control group (Table 2). Similar changes in chemokine receptor expression were not observed in the fungal asthma group that received 30 mg kg^{-1} BX-471. Interestingly, only one CCR1 ligand, CCL6, and no other chemokine ligands exhibited decreased transcript expression following 3, 10, or 30 mg kg^{-1} BX-471 treatment (Table 2). Identical results shown were obtained from a repeated SuperArray analysis of the same whole lung samples. Thus, these data showed that BX-471 treatment modulated chemokine transcript expression in whole lung samples at day 30 after conidia challenge.

Whole lung ELISA analysis of CCR1 ligand levels at day 30 after conidia challenge is shown in Figure 7. Significantly higher levels of CCL3 and CCL5 were detected in whole lung samples from the group that received 30 mg kg^{-1} of BX-471 from day 15 to day 30 after conidia challenge. In contrast, the levels of CCL6 were significantly decreased in this treatment group, and lower in the other two treatment groups relative to the vehicle treatment group. Whole lung ELISA analysis of Th2-type (i.e. IL-4, IL-5, and IL-13), Th1-type (IL-12 and IFN-γ) and IL-10 levels did not reveal any differences between the vehicle- and BX-471-treated groups (data not shown). Together, these data showed that CCR1 antagonism modulates whole lung levels of CCR1 ligands.

Airway remodeling is attenuated by BX-471 therapy in chronic fungal asthma

The most dramatic consequence of CCR1 deficiency in mice sensitized and challenged with *A. fumigatus* was the marked absence of asthma-associated airway remodeling features such as goblet cell metaplasia and peri-bronchial fibrosis (Blease *et al*., 2000). Goblet cell metaplasia (revealed by PAS staining in Figure 8) and peri-bronchial fibrosis (revealed by Trichrome Masson staining in Figure 9) were significantly reduced by BX-471 treatment. Specifically, the 10 mg kg^{-1} treatment group did not show evidence of goblet cell metaplasia (Figure 8c) nor peri-bronchial fibrosis (Figure 9c) at day 30 after conidia treatment in *A. fumigatus*-sensitized mice. These findings contrasted sharply with the vehicle-treated group that showed profound goblet cell metaplasia (Figure 8a) and peri-bronchial fibrosis (Figure 9a). The 3 mg kg^{-1} BX-471 treatment group

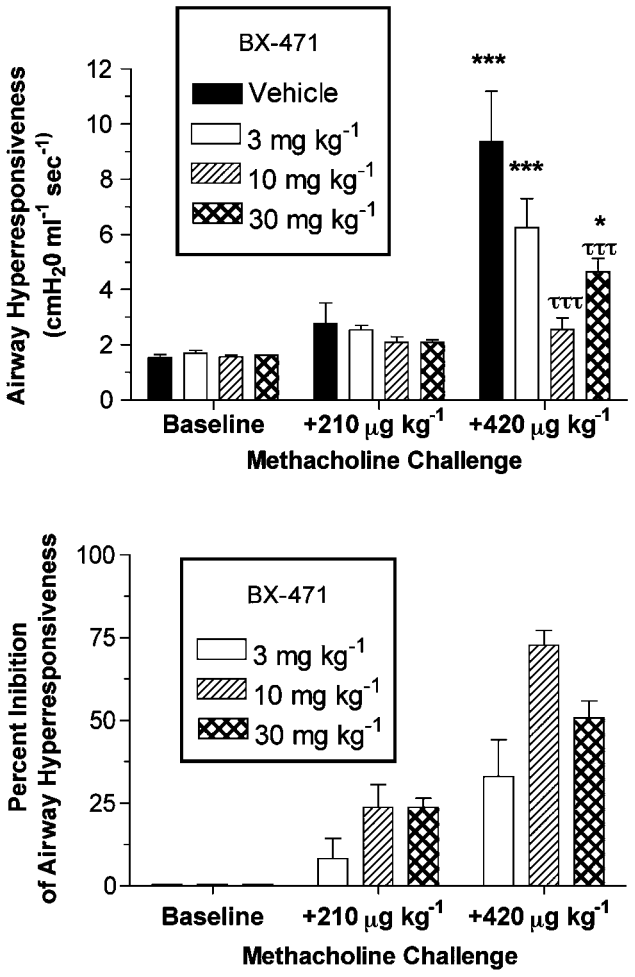

Figure 6 Airway hyper-responsiveness in vehicle- and BX-471-treated groups at day 30 after *A. fumigatus* conidia challenge into *A. fumigatus*-sensitized mice (top panel). *A. fumigatus*-sensitized mice were challenged intratracheally with live *A. fumigatus* conidia and 15 days later groups of five mice received vehicle alone or on a daily basis until day 30. Peak increases in airway resistance or hyper-responsiveness (units = cm H_2O^{-1} ml^{-1} s^{-1}) were determined at each time point after the intravenous injection of methacholine. Values are expressed as mean ± s.e.m.; $n = 5$/group/time point. $^{*}P \leqslant 0.05$, $^{***}P \leqslant 0.001$ compared with baseline airway responses measured in the appropriate vehicle- or BX-471-treated group. $^{\tau\tau\tau}P \leqslant 0.001$ compared with the airway responses evoked by the 420 $\mu g\,kg^{-1}$ methacholine challenge in the vehicle-treated group. The percent inhibition of airway hyper-responsiveness in the BX-471 treatment groups relative to the vehicle control group is shown in the bottom panel.

showed similar airway remodeling (Figures 8b and 9b) to that observed in the vehicle-treated group, whereas airway remodeling was diminished in the 30 mg kg^{-1} (Figures 8d and 9d) group compared with the vehicle group. Thus, a histological survey of whole lung samples from mice with chronic fungal asthma confirmed that BX-471 dose-dependently attenuated the airway remodeling typically associated with this model.

Discussion and conclusions

The pharmacological targeting of chemokine receptors has been long recognized as a viable treatment approach in allergy and asthma (Lloyd & Rankin, 2003; Proudfoot *et al*., 2003), but a quandary persists as to which targeted receptor will provide the greatest therapeutic effect in these diseases (Owen, 2001). This quandary is further deepened by the lack of animal models that exhibit the chronicity typically observed in clinical versions of allergy and asthma. Our development of a model of chronic fungal asthma that exhibits persistent features of airway hyper-responsiveness and remodeling following a single intratracheal bolus challenge of *A. fumigatus* conidia into *A. fumigatus*-sensitized mice (Hogaboam *et al*., 2000) has allowed us to examine the utility of targeting chemokines and their G-protein-coupled receptors in this disease (Hogaboam *et al*., 2003). One of our previous studies highlighted the importance of CCR1 in the persistence of airway remodeling during chronic fungal asthma (Blease *et al*., 2000), and these findings were the impetus to explore further whether the pharmacological targeting of this chemokine receptor was as effective in attenuating this and other features of chronic allergic airway disease due to Aspergillus. It was observed that the systemic administration of the CCR1 antagonist BX-471 beginning at day 15 after conidia challenge in *A. fumigatus*-sensitized mice attenuated all the features of airway disease typically observed in this model at day 30 including peribronchial inflammation, airway hyper-responsiveness, and airway remodeling. In addition, we observed effects of BX-471 on the transcript expression of TLR receptors in isolated macrophages and on the transcript expression of various proallergic chemokine receptors in whole lung samples from BX-471-treated mice. The ability of BX-471 to decrease TLR2 and increase TLR9 may have profound effects on the clearance of Aspergillus conidia from the lungs of allergic mice and hence the persistence of airway disease in these mice. The effect of BX-471 on CC and CXC chemokine receptor transcript expression, all of which have been implicated in allergic airway disease and asthma (Nagase *et al*., 2001; Chantry & Burgess, 2002), increases the therapeutic potential of CCR1 antagonism in clinical disease.

Considerable research attention now surrounds the relative contribution of TLR receptors to the chronic Th2-cytokine-

Table 2 SuperArray analysis of chemokine ligand and receptor transcript expression in pooled whole lung samples from the BX-471 treatment groups at day 30 after conidia challenge

Gene	*Fold decrease in expression*		
	3 mg kg^{-1} BX-471	*10 mg kg^{-1} BX-471*	*30 mg kg^{-1} BX-471*
CCR1	4	7	–
CCR3	3	Absent	–
CCR4	50	98	–
CCR8	4	9	–
CCR9	Absent	7	–
CXCR3	3	6	–
CXCR4	2	6	–
CXCR5	11	3	–
XCR1	Absent	6	–
CCL6	6	3	3

Fold changes are relative to gene expression detected in whole lung samples from the vehicle-treated group at the same time after conidia challenge. (–) indicates that transcript expression was not changed relative to transcript expression in the vehicle-treated group.

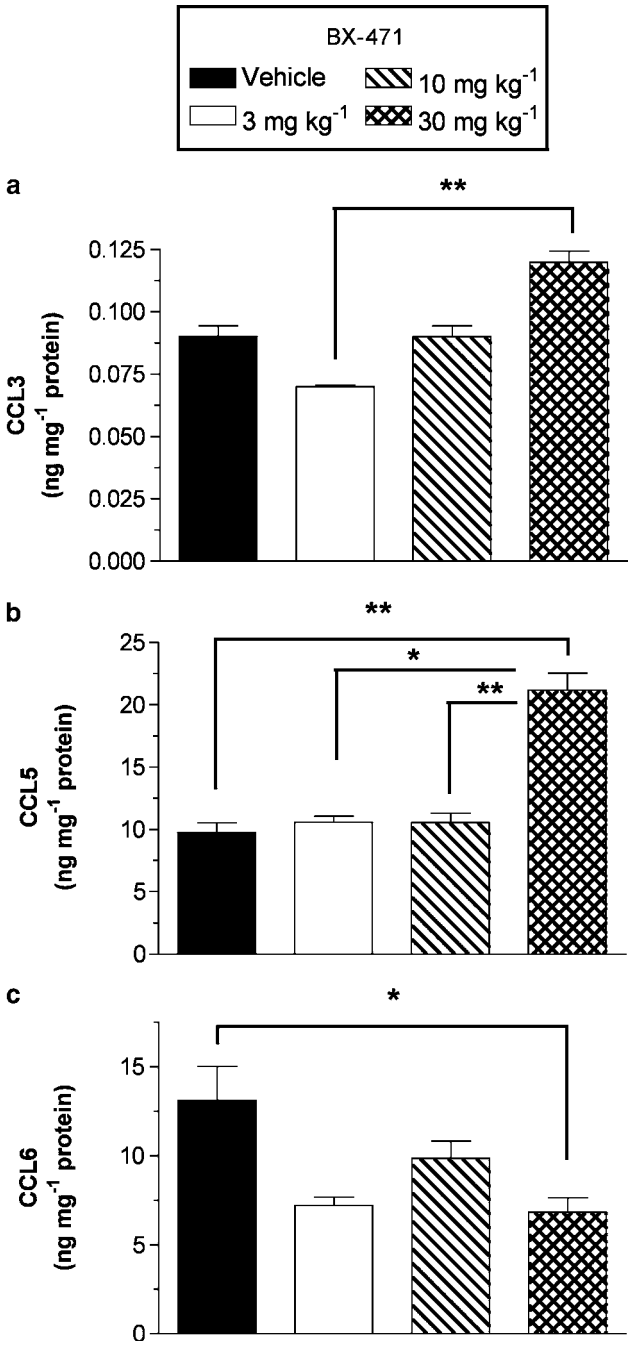

Figure 7 Whole lung levels of C-C chemokine receptor-1 (CCR1) agonists CCL3 (a), CCL5 (b), and CCL6 (c) from vehicle (a)- and BX-471-treated ($3\,mg\,kg^{-1}$) (b), $10\,mg\,kg^{-1}$ (c), and $30\,mg\,kg^{-1}$ (d) groups at day 30 after conidia challenge. *A. fumigatus*-sensitized mice were challenged intratracheally with live *A. fumigatus* conidia and 15 days later groups of five mice received vehicle alone or BX-471 at one of the three doses indicated above on a daily basis until day 30. CCL3, CCL5, and CCL6 were measured using a specific ELISA as described in the Methods section. Data are expressed as mean$\pm$s.e.m.; $n=5$ group^{-1}. *$P\leqslant0.05$, **$P\leqslant0.01$ compared with whole lung chemokine levels in the vehicle-treated group.

dominated diseases such as allergy and asthma. Growing evidence points to a proallergic role for TLR2 in the allergic host, whereas TLR9 activation appears to downregulate allergic responses by promoting Th1 cytokine generation (Eder *et al*., 2004; Hayashi *et al*., 2004; Redecke *et al*., 2004). In addition, TLR2, TLR3, and TLR4 have been found to be expressed in the mouse tracheal smooth muscle and activation of these receptors leads to a number of signaling events that culminate in hyper-responsiveness to bradykinin (Bachar *et al*., 2004). Not surprisingly, TLRs also play a role in the innate immune responses to Aspergillus but only TLR4 and the common TLR adaptor protein MyD88 have been shown to be necessary for the effective clearance of Aspergillus conidia and the prevention of invasive Aspergillosis (Bellocchio *et al*., 2004). CpG-ODN is the major ligand for TLR9 and it has been shown to induce CCL2 and CCL5 and the chemokine receptors CCR1 and CCR5 in murine macrophages (Anders *et al*., 2003). In the present study, it was observed that the *in vitro* treatment of BALB/c mouse macrophages with BX-471 diminished the expression of TLR2 and increased the expression of TLR9. Although we did not specifically examine whether isolated CBA/J macrophages were similarly altered by BX-471 treatment, the shift in the balance of the expression of these TLRs may have also accounted for the diminished retention of fungal material in the lungs of *A. fumigatus*-challenged CBA/J mice treated with BX-471 *in vivo*. We provide this speculation due to our unpublished findings and published findings from other investigators (Philippe *et al*., 2003) that demonstrate the conserved innate immune response of murine macrophages from various mouse strains, particularly during exposure to *A. fumigatus* conidia. It was also interesting to note that the transcript expression of antifungal chemokine ligands such as CXLC2 (Mehrad *et al*., 1999; 2002) and CXCL15 (Mehrad *et al*., 1999) were also increased, thereby possibly contributing to the enhanced clearance of conidia observed in BX-471-treated mice (particularly in mice that received $10\,mg\,kg^{-1}$). Recently, we have observed that CXCR2 ligands have regulatory effects on mouse macrophage activation thereby possibly further contributing to the innate immune response against fungus (Ness *et al*., 2003). Further examination of the effect of BX-471 on the antifungal response during fungal asthma is currently underway.

In contrast to the responses reported in CCR1−/− mice (Blease *et al*., 2000), the pharmacological targeting of CCR1 with BX-471 in genetically intact mice revealed a major role for CCR1 ligands in the recruitment of inflammatory leukocytes including macrophages, eosinophils, and T cells to the airways and into the BAL of mice with fungal asthma (see Table 3 for a detailed comparison of the findings from CCR1−/− *versus* BX-471-treated mice). Previously, we observed that the lack of CCR1 was associated with a significant increase in the number of macrophages in the lungs of mice at day 30 after the conidia challenge. Another contrasting finding between our study of CCR1−/− mice and the present study pertains to the cytokine profile in whole lung samples. Previously, we observed that levels of Th2-type cytokine such as IL-4 and IL-13 were significantly lower in the gene-deficient group compared with the wild-type group at day 30 after conidia (Blease *et al*., 2000). A similar shift in the whole lung cytokine pattern was not observed in whole lung samples from BX-471-treated mice. A satisfactory explanation for this discrepancy is not immediately forthcoming, but it is worth noting that the CCR1−/− were of a mixed genetic background of C57BL/6 and 129Sv whereas the present BX-471 study was performed in CBA/J mice. Clear differences in the levels and

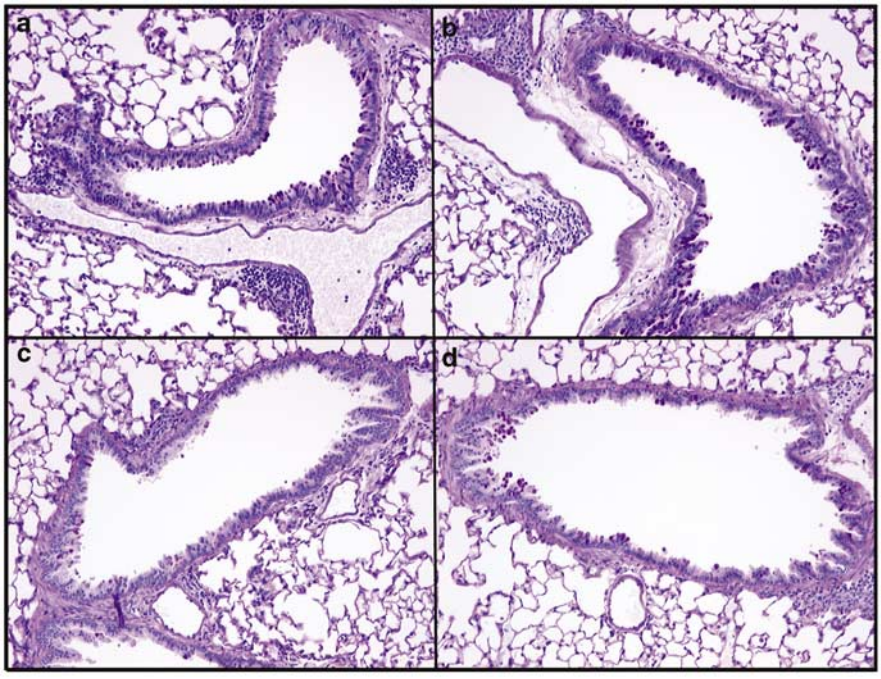

Figure 8 Periodic Acid Schiff (PAS) staining of whole lung samples from vehicle (a)- and BX-471-treated ($3\,mg\,kg^{-1}$) (b), $10\,mg\,kg^{-1}$ (c), and $30\,mg\,kg^{-1}$ (d) groups at day 30 after conidia challenge. *A. fumigatus*-sensitized mice were challenged intratracheally with live *A. fumigatus* conidia and 15 days later groups of five mice received vehicle alone or BX-471 at one of the three doses indicated above on a daily basis until day 30. Whole lungs samples were processed using routine histological techniques. Mice that received $10\,mg\,kg^{-1}$ of BX-471 showed the greatest reduction in goblet cell metaplasia compared the control and other BX-471 treatment groups. Original magnification was $\times 200$.

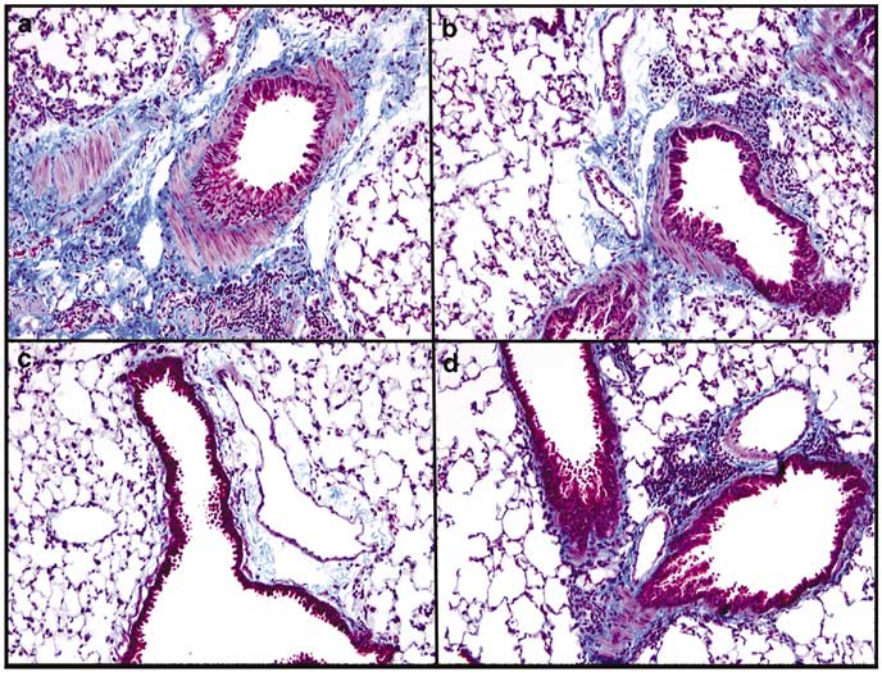

Figure 9 Trichrome Masson staining of whole lung samples from vehicle (a)- and BX-471-treated ($3\,mg\,kg^{-1}$) (b), $10\,mg\,kg^{-1}$ (c), and $30\,mg\,kg^{-1}$ (d) groups at day 30 after conidia challenge. *A. fumigatus*-sensitized mice were challenged intratracheally with live *A. fumigatus* conidia and 15 days later groups of five mice received vehicle alone or BX-471 at one of the three doses indicated above on a daily basis until day 30. Whole lungs samples were processed using routine histological techniques. Mice that received $10\,mg\,kg^{-1}$ of BX-471 showed the greatest reduction in peribronchial fibrosis compared the control and other BX-471 treatment groups. Original magnification was $\times 200$.

Table 3 Effect of CCR1 gene deletion (Blease *et al*., 2000) or CCR1 antagonism (present CCR1 antagonism study with BX-471) on various features of fungal asthma at day 30 after conidia challenge of *A. fumigatus*-sensitized mice

	CCR1−/−	*BX-471* ($mg\,kg^{-1}$)	*BX-471* ($mg\,kg^{-1}$)	*BX-471* ($mg\,kg^{-1}$)
		3	10	30
GMS-staining	⇓	⇔	⇓	⇔
IgE, IgG1, IgG2a	⇔,ND,ND	⇔,⇔,⇔	⇔,⇔,⇔	⇔,⇔,⇔
Whole Lung				
IL-4, IL-13	⇓,⇓	⇔,⇔	⇔,⇔	⇔,⇔
IFN-γ	⇑	⇔	⇔	⇔
CCL3	⇔	⇔	⇔	⇔
CCL5	⇔	⇔	⇔	⇑
CCL6	⇓	⇔	⇔	⇓
CCL11	⇓	⇔	⇔	⇔
CCL22	⇓	⇔	⇔	⇔
BAL				
Eosinophils	⇔	⇓	⇓	⇓
Neutrophils	⇔	⇔	⇓	⇔
Monocytes/macrophages	⇑	⇓	⇓	⇓
T lymphocytes	⇑	⇓	⇓	⇓
Airway hyper-responsiveness	⇔	⇓	⇓	⇓
Goblet cell metaplasia	⇓	⇔	⇓	⇔
Peribronchial fibrosis	⇓	⇔	⇓	⇓

⇑ = significantly increased compared with appropriate control group; ⇓ = significantly decreased compared with appropriate control group; ⇔ = no different from appropriate control group; ND = not determined in study.

timing of expression for Th1- and Th2-associated cytokines has been observed in all three strains of mice during the course of chronic fungal asthma (Schuh *et al*., 2003b). Another major difference between CCR1−/− and BX-471-treated mice related to airway hyper-responsiveness, which was not affected by gene deletion of CCR1. Again, a suitable explanation for this major difference between our two studies is not immediately apparent.

It was interesting to observe that BX-471 treatment decreased chemokine receptor expression in cultured mouse macrophages and in whole lung samples. The *in vitro* treatment of macrophages with BX-471 markedly reduced CCR3 (another chemokine receptor that shares many of the same ligands with CCR1 (Sabroe *et al*., 2000)) and CCR6 transcript expression. Both lower doses of BX-471 abolished or markedly reduced the transcript expression of CCR1, CCR3, CCR4, CCR8, CCR9, CXCR3, CXCR4 and CXCR5 and XCR1 in whole lung samples removed at day 30 after the conidia challenge. Thus, the reduction in leukocyte recruitment observed in the groups that received 3 or $10\,mg\,kg^{-1}$ BX-471 may have been the direct result of this drug's ability to modulate chemokine receptor expression on inflammatory leukocytes and possibly tissue resident cells such as epithelial cells, smooth muscle cells, and fibroblasts.

The maximal therapeutic effect of this CCR1 antagonist was observed when it was administered at $10\,mg\,kg^{-1}$ rather than the $30\,mg\,kg^{-1}$. While the $10\,mg\,kg^{-1}$ BX-471 treatment markedly and significantly diminished allergic airway inflammation, hyper-responsiveness and remodeling, the same was not observed with the highest dose of BX-471 at $30\,mg\,kg^{-1}$, which partially decreased airway inflammation and significantly attenuated airway responsiveness. The partial therapeutic effect of BX-471 at this dose may be explained by the marked reduction in transcript and protein expression for CCL6 in the present study. We have previously reported that CCL6 is a major chemokine contributor to airway inflammation and hyper-responsiveness during airway responses to Aspergillus antigen (Hogaboam *et al*., 1999). However, the increased presence of eosinophils (observed in the BAL), and the increased levels of CCL3 and CCL5, which can also bind CCR5, may have accounted for the goblet cell metaplasia observed in the $30\,mg\,kg^{-1}$ BX-471 treatment group. A higher dose of $50\,mg\,kg^{-1}$ BX-471 delivered multiple times over a period of weeks was previously used to attenuate renal injury due to unilateral ureter ligation (Anders *et al*., 2002) and lupus nephritis (Anders *et al*., 2004), so it is unlikely that the partial airway inflammation and intact remodeling were adverse responses to the highest dose of BX-471 employed in the present study.

Fungus-induced asthmatic disease is characterized by persistent airway hyper-reactivity and remodeling and the present study demonstrates that pharmacological targeting of CCR1 attenuates both major features of this disease. The present study points to a major role for CCR1 in the recruitment of inflammatory cells that contribute to allergic airway disease including macrophages, eosinophils, and T cells. CCR1 antagonism with BX-471 was also observed to alter the transcript expression of TLRs and/or chemokine ligands and receptors in cultured macrophages and whole lung samples. Alterations in TLR and antifungal chemokine expression lend credence to the importance of CCR1 in the link between the innate and acquired immune responses, particularly in the context of allergy and asthma. Thus, the targeting of CCR1 may restore the appropriate innate immune response needed to rid the lung of proallergic substances such as fungus, as well as attenuate the cell recruitment and activation events that characterize chronic fungal asthma.

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