Chemokine receptor 2 blockade prevents asthma in a cynomolgus monkey model

Mario Mellado, Ana Martín de Ana, Lucio Gómez, Carlos Martínez-A. and José Miguel Rodríguez-Frade

Department of Immunology and Oncology, Centro Nacional de Biotecnología/CSIC, UAM Campus de Cantoblanco, E-28049 Madrid Spain (MM, AMdA, LG, CMA, JMRF)
Running title: Chemokine receptor 2 blockade prevents asthma in monkeys

Corresponding author: José Miguel Rodríguez-Frade
Department of Immunology and Oncology
Centro Nacional de Biotecnología
Darwin 3, Campus de Cantoblanco
E-28049 Madrid Spain
Tel (+34) 91/585-4852
Fax (+34) 91/372-0493
e-mail jmrfrade@cnb.uam.es

Number of text pages: 28
Number of tables: 1
Number of figures: 5
Number of references: 40
Words (Abstract): 208
Words (Introduction): 567
Words (Discussion): 1270

Abbreviations used: BAL, bronchoalveolar lavage; BHR, bronchial hyperresponsiveness;
BSA, bovine serum albumin; C_{DYN}, dynamic compliance; DMSO, dimethyl sulfoxide;
ELISA, Enzyme-linked immunosorbent assay; FCS, fetal calf serum; HEPES, 4-(2-
hydroxyethyl)-1-piperazineethanesulfonic acid; i.d., intradermal; mAb, monoclonal antibody;
ORD, optimal response dose; OVA, ovalbumin; PBMC, peripheral blood mononuclear cells;
PBS, phosphate-buffered saline; R_L, lung resistance

Section: Inflammation, Immunopharmacology, and Asthma
Abstract

The pathophysiology of asthma is characterized by accumulation and activation of several cell types in the lung, which correlates with coordinated production of specific cytokines and chemokines. To study the effect of selective CCR2 chemokine receptor blockade on leukocyte recruitment to the lung and on bronchial function, we used a non-human primate model of allergic airway disease that closely resembles human asthma. Allergic cynomolgus monkeys were treated with the antagonist anti-CCR2 (CCR2-05) monoclonal antibody, and then challenged with *Ascaris suum* antigen; the effect of antibody treatment on macrophage and eosinophil infiltration was determined. Pulmonary function was calculated by measurement of lung resistance and dynamic compliance. Local inflammatory responses were analyzed after intradermal challenge with *Ascaris suum* antigen. CCL2 upregulation in bronchoalveolar lavage (BAL) was analyzed by ELISA, and *in vitro* CCR2-05 antagonistic activity was tested in monkey peripheral blood mononuclear cells using chemotaxis and calcium mobilization assays. The results show that neutralization of CCR2 reduces antigen-induced bronchial hyperresponsiveness, and attenuates macrophage and eosinophil accumulation in the BAL of asthmatic monkeys. The results confirm that selective blockade of a single chemokine receptor involved in early stages of asthma can condition later disease stages, and suggest the utility of anti-CCR2 neutralizing monoclonal antibodies in the treatment of asthma in man.
Introduction

Atopic asthma is a heterogeneous, chronic inflammatory disease of the airways that leads to peribronchial inflammation and altered lung responsiveness. It is characterized by increased levels of circulating IgE antibodies, positive skin tests to allergens, and large numbers of eosinophils, T lymphocytes and mast cells in airways and lung interstitium (Bousquet et al., 1999). Initial local activation of mast cells and basophils is induced in response to antigenic or environmental stimulus, leading to release of inflammatory mediators; this process is closely related to the severity of the asthmatic reaction (Carroll et al., 2002; Marone et al., 2005). Later phases of asthma are characterized by greater infiltration of lymphocytes, macrophages and eosinophils, responsible for inflammation and airway damage (Bradley et al., 1991; Gonzalo et al., 1998). Control of this infiltration is thus thought to be a main element in asthma management. The prominent role of CD4+ T cells is well established; an increase in these cells tends to correlate with a Th2 type response that includes IL-4, IL-5 and IL-13 secretion (Umetsu et al., 2003). These T cell-derived cytokines work in concert with locally released chemokines and mediators in the airway epithelium to coordinate recruitment and activation of mast and other inflammatory cells (Lloyd et al., 2000; Pease, 2006).

Specific chemokine profiles characterize the distinct stages of asthmatic disease. Several aspects of asthma pathology have been linked to high CCL2, CCL3, CCL5 and CCL11 levels in bronchoalveolar lavage (BAL); these chemokines are responsible for eosinophil accumulation around the airways (Gonzalo et al., 1998; Chvatchko et al., 2003). These and many other chemokines and receptors are implicated in cell migration from the vascular compartment to the interstitium and in cell localization around airways, but may also alter cell survival, proliferation or airway remodeling (Murray et al. 2006). Although none of the chemokines appears to be essential individually, reports suggest the relevance of specific
expression patterns and tissue localization (Lukacs et al. 2003). The use of neutralizing anti-CCL2, -5, -11, -12, -17, and -22 antibodies prevents eosinophil migration in mouse models (Gonzalo et al., 1998; Gonzalo et al., 1999; Campbell et al., 1999; Kawasaki et al., 2001; Chvatchko et al., 2003). Furthermore, CCL11−/− and CCR3−/− mice show reduced eosinophil recruitment and increased bronchial hyperresponsiveness (BHR) (Rothemberg et al., 1997; Humbles et al., 2002), highlighting the complexity of the interaction among mediators in the orchestration of the asthmatic phenotype. CCR2−/− mice are resistant to allergen-induced responses, have deficient Th2 responses, and show impaired airway hyperreactivity (Campbell et al., 1999; Kim et al., 2001; Huang et al., 2001). Intratracheal CCL2 injection induces mast cell degranulation and long-term airway hyperreactivity as a consequence of leukotriene C4 release (Campbell et al., 1999). Taken together, these data assign an important role to the CCL2/CCR2 chemokine/chemokine receptor pair in the asthmatic process.

We analyzed inflammatory responses in the lung following allergen challenge in allergic cynomolgus monkeys (Macaca fascicularis), a non-human primate asthma model (Gundel et al., 1990; Turner et al., 1994). The results indicate that treatment with a neutralizing anti-human CCR2 monoclonal antibody (mAb) (Frade et al., 1996) delayed allergic reactions and reduced inflammatory cell numbers in the lung. As a consequence, treatment with this mAb diminished asthma symptoms and improved pulmonary function. Although findings in animal models may not fully reflect the human disease, our data indicate that blockade of the CCL2/CCR2 axis may be an important target in controlling mechanisms involved in human asthma.
Methods

Proteins, antibodies and cells. CCL2 and CXCL12 were from Peprotech (London, UK). Anti-CCR2 and control mIgG2b mAb were generated in our laboratory (Frade et al., 1996) and purified using HiTrap affinity columns (Amersham Pharmacia, Uppsala, Sweden). Endotoxin in purified materials was measured in a QLC-1000 Limulus Amebocyte Lysate assay (Bio-Whittaker, Walkersville, MD).

PBMC were obtained from whole blood of healthy cynomolgus monkeys by centrifugation in Accuspin tubes (Sigma; 700 xg 15 min, room temperature (RT).

Flow cytometry analysis. Cells were centrifuged, plated in V-bottom 96-well plates (2.5 x 10^5 cells/well) and incubated with 50 µl/well biotin-labeled anti-human CCR2 mAb (5 µg/ml, 30 min, 4°C). Cells were washed twice and fluorescein isothiocyanate-labeled streptavidin (Southern Biotechnologies, Birmingham, AL), anti-monkey CD3, CD14 or CD19 (BD Pharmingen, San Diego, CA) was added, incubated (30 min, 4°C) and plates washed twice. Cell-bound fluorescence was measured in a Profile XL flow cytometer (525 nm; Coulter, Miami, FL).

Calcium determination. Cells (2.5 x 10^6 cells/ml) were resuspended in RPMI 1640 medium containing 10% FCS and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) and incubated with Fluo-3AM (Calbiochem, San Diego, CA; 300 mM in DMSO (dimethyl sulfoxide), 10 ml/10^6 cells, 30 min, 37°C). Cells were washed, resuspended in medium with 2 mM CaCl_2 and maintained at a 10-fold mAb excess (4°C, 20 min) before CCL2 or CXCL12 addition. Ca^{2+} flux was measured separately in monocytes and lymphocytes in an EPICS XL flow cytometer (525 nm; Coulter).

Cell migration. PBMC were placed (0.25 x 10^6 cells/100 µl) in the upper well of 24-well transmigration chambers (5 µm pore; Transwell; Costar, Cambridge, MA) precoated with type VI collagen (Sigma, St. Louis, MO; 20 µg/ml, 2 h, 37°C). CCL2 or CXCL12 (0.1-100
nM in 0.6 ml RPMI 1640 containing 0.25% BSA) was added to the lower well; after incubation (2 h, 37°C), cells that migrated to the lower chamber were counted and the cell migration index calculated as the x-fold increase in migration observed over the negative control (medium). To block ligand-induced chemotaxis, cells were preincubated (30 min, 37°C) with different concentrations of anti-CCR2 or isotype-matched control mAb.

**Acute asthma model in cynomolgus monkeys**

We used eight adult cynomolgus monkeys (*Macaca fascicularis*), previously screened and shown to exhibit a positive bronchoconstrictor response to a specific dose of inhaled *Ascaris suum* antigen. All technical procedures were performed in accordance with the test facility’s Standard Operating Practices, and approved by the internal IACUC.

In Phase I, animals were anesthetized (propofol, 3 mg/Kg, i.v.), intubated and mechanically ventilated. Pulmonary function values were recorded throughout the challenge period with equipment from Buxco Electronics (Wilmington, NC). *A. suum* antigen was administered at the optimal response dose (ORD) for each animal by aerosol inhalation (single dose, 15 breaths). The ORD is the *A. suum* dose that produces an increase in lung resistance (*R*<sub>L</sub>) of at least 40% and a decrease in dynamic compliance (*C*<sub>DYN</sub>) of at least 35%. Maximum percent change from baseline for *R*<sub>L</sub> and *C*<sub>DYN</sub> was calculated after antigen challenge. Following a 3-week washout period, animals underwent Phase II procedures. Immediately prior to *A. suum* challenge, animals were treated with the CCR2-05 mAb or control mIgG2b mAb (2 mg/Kg) by intravenous bolus injection. Challenge procedures were as before. All animals responded to antigen challenge (phase I) and there were no major differences in cell percentages in BAL between phase I and phase II animals (not shown).

BAL was obtained by guiding a pediatric fiber optic bronchoscope past the carina to wedge in a major bronchus. Three aliquots of sterile saline (20 ml each) were instilled and aspirated for collection, and the total fluid volume collected for each animal at each time
point was recorded. BAL was collected prior to aerosol A. suum challenge (0 h), and at 3 h and 24 h after each challenge (Phases I and II).

BAL cells and fluid collected were separated by centrifugation (2700 xg, 10 min, 4°C). Supernatant was concentrated and frozen for analysis. Cell pellets were combined and resuspended in sterile saline (2 ml) for determination of total nucleated cell numbers, manually or using a Sysmex TOA E-2500 hematology analyzer. The cell suspension was diluted in saline (10^3 cells/ml) for cytopsin preparation to determine cell morphology. Two slides were prepared by cytocentrifugation (100 µl aliquots, 80 xg, 5 min; same conditions for all cell types), air-dried, fixed in 100% methanol, and Wright-Giemsa stained; morphology and differential cell count were determined by counting a minimum of 200 nucleated cells. Relative and absolute counts were determined for macrophages, eosinophils, neutrophils, lymphocytes and mast cells.

**Intradermal challenge.** Prior to initiation of intradermal (i.d.) challenge, the lateral thoracic skin was shaved, with care to avoid abrasion. Ten minutes prior to i.d. challenge, animals received an injection of 0.5% Evan’s Blue dye (0.2 ml/Kg, i.v.), followed by an injection (0.1 ml/site) of PBS in one site, histamine (0.275 mg/ml) in one site, and a 1:10,000 dilution of A. suum antigen in two sites. Injection sites were scored 15 min post-challenge for degree of dye migration. By definition, the histamine site receives a score of 2 and the saline site, a score of 0. All A. suum scoring is relative to these controls (0 = no change, color/size is the same as the saline site; 1 = mild color/size change, color/size is between the saline and histamine sites; 2 = marked color/size change, same degree of blue dye influx as histamine site).

**Determination of CCL2 levels.** Chemokine levels were determined in BAL fluids from A. suum-challenged monkeys by ELISA (R&D Systems, Minneapolis, MN).
Statistical analyses. Data were analyzed using one-way ANOVA and Tukey’s multiple comparison tests to evaluate the differences among the experimental conditions. All statistical analysis was performed using GraphPad Prism Software (GraphPad, San Diego, CA). Unless otherwise indicated, data are expressed as mean ± SEM.
Results

**CCL2 is upregulated in cynomolgus monkey BAL after A. suum challenge**

We first determined CCL2 levels in BAL from adult cynomolgus monkeys that had been challenged with *A. suum* antigen (2 mg/Kg, i.v.). As in the case of ovalbumin (OVA)-induced lung inflammation in mice (Gonzalo et al., 1998), CCL2 was strongly expressed shortly (3 h) after antigen challenge (Fig. 1A). This increase correlated with cell recruitment into the airways (Fig. 1B). The infiltrate consisted mainly of macrophages (76.00 ± 4.63% at 0 h, 79.38 ± 3.06% at 3 h, 51 ± 3.88% at 24 h) and eosinophils (4.50 ± 0.85% at 0 h, 11.88 ± 1.80% at 3 h, 40.25 ± 3.54% at 24 h). Other cell types, such as mast cells (17.13 ± 3.59% at 0 h, 4.86 ± 1.13% at 3 h, 3.50 ± 0.91% at 24 h), neutrophils (0.50 ± 0.19% at 0 h, 1.50 ± 0.50% at 3 h, 6.34 ± 0.82% at 24 h) and lymphocytes (<1% at all times measured) were also detected in BAL. Infiltration kinetics varied among cell types, with macrophage numbers peaking at 3 h, and eosinophil numbers increasing and reaching a peak at 24 h post-challenge (Fig. 1B). Neutrophils also showed a modest but significant increase after antigen challenge, although their contribution to the total cell infiltrate diminished (Fig. 1B). No accumulation of mast cells or T lymphocytes was observed at these early time points (Fig. 1B).

**Anti-human CCR2 neutralizing mAb blocks *in vitro* CCL2-mediated responses in cynomolgus monkey cells**

We developed several mAb against the human CCL2 receptor, CCR2. Two of these, CCR2-04 and -05, were selected based on their ability to neutralize responses to human CCL2 in migration and Ca²⁺ mobilization assays (Frade et al., 1996). CCR2-05 is a mIgG2b that recognizes the third extracellular loop of human CCR2 (amino acids 273-292), a sequence that is identical in the monkey counterpart, with an affinity constant of 0.35 nM for CCR2. The level of endotoxins in the purified mAb preparations was <0.005 endotoxin units (EU)/ml.
To evaluate the feasibility of using these mAb to block cell infiltration in monkey models of asthma, we tested whether the mAb reacted with the monkey CCR2 homologue. Peripheral blood mononuclear cells (PBMC) from healthy cynomolgus monkeys were co-stained with anti-CD3, -CD14 or -CD19 and specific anti-human CCR2 mAb. High CCR2 expression (mean intensity of fluorescence 32.6 ± 6.3 vs 3.55 ± 2.25 in mIgG2b controls) was observed in most CD14+ cells (69.07% ± 5.57), but not in CD3+ cells (6.30% ± 2.65) (Fig. 2A). The elevated CCR2 expression in the monocyte/macrophage population, together with increased CCL2 expression and macrophage infiltration in BAL from antigen-challenged monkeys (Fig. 1), suggested the utility of blocking monkey CCR2 to ameliorate disease symptoms.

We tested the ability of the anti-CCR2 mAb to block in vitro CCL2 responses in PBMC from these monkeys. Cells were preincubated with CCR2-05 or mIgG2b (50 µg/ml, 30 min, 37°C) as control, and their ability to mobilize Ca2+ in response to CCL2 was evaluated (Fig. 2B). CCL2-induced Ca2+ mobilization was blocked specifically by CCR2-05, which did not affect the mobilization induced by another chemokine/receptor pair, CXCL12/CXCR4 (Fig. 2B). CCR2-05 stimulation did not trigger Ca2+ mobilization, indicating that the mAb does not activate CCR2.

The effect of CCR2-05 was evaluated on the migration of monkey PBMC in response to stimulation with various CCL2 or CXCL12 concentrations (0.1 to 100 nM). Migration towards CCL2 (50 nM) was completely blocked by CCR2-05 treatment, with maximum inhibition at 37.5 µg/ml. A control isotype-matched mAb (mIgG2b) had no effect (Fig. 2C).

All together, these data indicate that the anti-human CCR2 mAb recognizes the primate homologue, does not trigger signals, and retains its antagonistic activity. As CCL2 is upregulated rapidly in a primate model of asthma, we evaluated disease severity in monkeys treated with CCR2-05 mAb.
Neutralizing anti-CCR2 mAb prevents eosinophil and macrophage infiltration to asthmatic cynomolgus monkey lung and restores pulmonary function

To characterize the type of leukocyte recruited to lung airways after antigen challenge, BAL fluid was collected from monkeys pretreated with CCR2-05 or control mIgG2b, prior to challenge with aerosolized A. suum (0 h), and at 3 and 24 h after each challenge (see Methods). Pretreatment with control mIgG2b had no effect on the total cell number in lung airways. In CCR2-05-pretreated animals, however, we observed a significant reduction (p<0.01) in the total cell number in BAL, especially at 24 h post-challenge (Fig. 3A). Characterization of BAL cell populations from CCR2-05-pretreated monkeys showed diminished numbers of macrophages (Fig. 3B, top) and eosinophils (Fig. 3C, top) compared to BAL from mIgG2b mAb-pretreated animals (Fig. 3B, C, bottom, and Table 1).

In this asthma model, monkeys develop a dose-dependent reduction in dynamic lung compliance (C_DYN) and a variable increase in lung resistance (R_L) after A. suum antigen challenge (Rothenberg et al., 1997; Humbles et al., 2002). We thus tested the effect of pretreatment with CCR2-05 mAb or control mAb (2 mg/Kg, i.v) on R_L and C_DYN following challenge. In the absence of pretreatment, all animals showed a post-challenge R_L increase of at least 40% over pre-challenge baseline values (mean 107.4 ± 8.61%) and a C_DYN decrease of at least 46% (mean -60.43 ± 3.14%) (Fig. 4). CCR2-05 pretreatment markedly reduced antigen-induced R_L levels (61.00 ± 11%), whereas no effect was observed in monkeys treated with mIgG2b (88.25 ± 7.50%) (Fig. 4A). Similarly, animals pretreated with CCR2-05, but not with control mAb, showed a modest but representative restoration of normal C_DYN values (Fig. 4B). The results illustrate that CCR2-05 mAb-treated monkeys showed improved pulmonary function and reduced asthma symptoms.

CCR2 blockade prevents local inflammatory responses following A. suum antigen challenge in cynomolgus monkeys
To evaluate whether the mAb had an effect on the acute phase of the allergic response, CCR2-05- and mIgG2b-treated animals were challenged i.d. (0.1 ml/site) with PBS, histamine (0.275 mg/ml) as positive control, or *A. suum* antigen (1:10,000 dilution). Allergic inflammation was evaluated and quantified (see Methods) 15 min after i.d. challenge in both groups. CCR2-05 treatment reduced both size and color of the dermal response compared to that in control monkeys (Fig. 5). CCR2-05- and mIgG2b-treated animals showed equal dermal response to histamine (Fig. 5). The results indicate that this mAb reduces not only chronic but also acute phases of the allergic response.


Discussion

Experimental animal models have been extremely useful in delineating the roles of cell types, cytokines, and chemokines in the pathogenesis of asthma (Campbell et al., 1999; Gutierrez-Ramos et al., 2000; Humbles et al., 2002). Asthma is characterized by airway inflammation, bronchial hyperresponsiveness (BHR), excessive mucus production and airway remodeling (Kay, 2005). Disease is a consequence of the coordinated action of several factors including chemokines and adhesion receptors, cytokines, lipid mediators and growth factors (Gonzalo et al., 1998; Gutierrez-Ramos et al., 2000; Lukacs et al., 2003) that can be produced by T cells and eosinophils (Pascual and Peters, 2005). The principal cell type(s) that mediate airway changes nonetheless continue to be debated. Use of a bispecific antibody that linked CCR3 to CD300a, which inhibits mast cell and eosinophil activation in CCR3+ cells, reduced eosinophil and mast cell numbers, and consequently reduced mucus production and diminished airway remodeling (Munitz et al., 2006). Although CCR3 blockade diminishes eosinophilia and airway remodeling, eosinophil trafficking into the lung does not depend exclusively on CCR3 (Wegmann et al., 2007).

T cells and eosinophils are thought to be fundamental in asthma, and participate in inflammation and tissue remodeling; the role of monocytes in human asthma is nonetheless much less clear. Monocyte chemoattractants have a critical function in mediating inflammation in murine models of allergic airway disease (Gonzalo et al., 1998). CCL2 is produced by a wide variety of cells, including monocyte/macrophages, lymphocytes, fibroblasts, endothelial and epithelial cells, neutrophils, mast cells and dendritic cells, all of which are involved in airway remodeling, and can contribute to the recruitment of CCL2-sensitive cells (monocytes, T cells, dendritic cells and neutrophils) both to and within the lung airways (Palmqvist et al., 2007).

Using a specific anti-CCR2 mAb in a primate model of asthma, we observed that CCR2
blockade attenuates the pathophysiological consequences of asthma, although further studies are needed to determine whether increased numbers and activation of inflammatory cells are also reduced by CCR2 blockade.

Disease development in mouse models involves the action of eosinophilic (CCL3, CCL5, CCL11, CCL12) and non-eosinophilic chemokines (CCL2), whose levels are modulated during disease progression. Each of these chemokines appears to govern a distinct stage and pathway in the development of OVA-induced lung eosinophilia and BHR (Gutierrez-Ramos et al., 2000; Lukacs et al., 2003). An initial increase was shown in the BAL monocyte/macrophage population in challenged mice, which correlated with CCL2 expression in lung (Gonzalo et al., 1998). Although transient, this early increase determines disease outcome, as it regulates other cell types (eosinophils and T lymphocytes) in the lung.

In cynomolgus monkeys, inhalation of A. suum antigen causes bronchoconstriction and airway inflammation with eosinophilia, symptoms also characteristic of human asthma (Bousquet et. al., 1999; Umetsu and DeKruyff, 2006). This model has been used effectively in the pharmaceutical industry to screen candidate asthma drugs, although this allergen is not normally associated with human asthma (Mauser et al., 1995; Turner et al., 1996). In the cynomolgus monkey asthma model, post-challenge BAL cell populations parallel those described in mice (Gonzalo et al., 1998), with an initial increase in macrophage/monocytes, probably as a consequence of elevated circulating CCL2 levels. A recent large-scale profile of gene expression in a monkey model of allergic asthma showed that a cluster of mostly IL-4-dependent genes was induced after allergen challenge, including chemokines CCL2 (14-fold increase), CCL7 (400-fold) and CCL11 (13-fold) (Zou et al., 2002). These data suggest that the CCL2/CCR2 pair may have a major role in asthma, representing a potential target for therapeutic intervention. Pulmonary function improved in monkeys in which CCR2-mediated responses were abrogated, as indicated by the trend towards normalization of $R_L$.
and $C_{DYN}$ values. This attenuation correlated with reduced monocyte and eosinophil numbers in BAL. Suppression of eosinophils in circulation and BAL fluid was previously reported to have no effect on allergen-induced airway hyperresponsiveness (Flood-Page et al., 2003). These differences were attributed to a lack of suppression of eosinophils in airway tissue and residual eosinophil degranulation in the airways, but could also involve other cell types, since airway hyperresponsiveness is the result of various eosinophil-dependent and -independent mechanisms (Flood-Page et al., 2003). The improved pulmonary function in the CCR2-05-treated monkeys suggests that CCR2 may also be implicated in airway eosinophil depletion and in prevention of the degranulation required to diminish allergen-induced asthma symptoms.

In mouse asthma models, monocyte infiltration is followed by eosinophil accumulation in BAL of challenged mice. This may be due to monocyte/macrophage-induced CCL11 expression (Gonzalo et al., 1998), or may be indirect, through macrophage-dependent activation of parenchymal (i.e., epithelial) cells, which would then express CCL11. The use of a CCR2 antagonist in the asthmatic monkey reduces monocytic/macrophage as well as the more abundant eosinophil infiltrate in BAL, without altering the relative contribution of each cell population to the pulmonary infiltrate. Although comparison between individual monkeys indicated diminished overall numbers of macrophages and eosinophils in all CCR2-05-treated animals compared with controls, only eosinophil numbers were statistically significant. These data might reflect the variability in individual responses, both in terms of cell numbers and time points at which the maximum response is observed. In the mouse model, T cells are also found in BAL of challenged mice, and T cell numbers are reduced in mice pretreated with neutralizing anti-CCL2 antibodies (Gonzalo et al., 1998). We nonetheless detected no significant T cell presence in BAL of treated or control monkeys at the time points studied. The contribution of T cells to disease outcome must be interpreted
with caution, however, as the lack of T cells in BAL could be due to the use of non-optimized T cell cytocentrifugation conditions.

In CCR2-05-pretreated monkeys, we observed inhibition of the cutaneous responses linked to the early, local activation of mast cells and basophils. This decreased dermal response is probably due to inhibition of inflammatory mediators that increase vascular permeability and/or activation of resident cells (Carrol et al., 2002; Marone et al., 2005), coinciding with diminished mast cell numbers in CCR2-05-pretreated animals. CCL2 induces leukotriene B4, prostaglandin E2 and thromboxane B2 release, and increases IgE levels in response to OVA (Gonzalo et al., 1998); this potentiates subsequent priming of mast cells and histamine release by basophils. Neutralization of CCR2 during allergen-induced airway responses might thus alter the early phase pathophysiological events.

The use of mAb in therapy has several advantages, including their specificity and predictable biological effects and pharmacokinetics (Glennie and Johnson, 2000; Breedveld, 2000; Li et al., 2002). Therapeutic use of compounds directed against G protein-coupled receptors has previously been successful (William et al., 2001), and targeting a chemokine receptor also presents advantages over ligand targeting. Action on CCR2 influences several levels of the asthmatic process, including mast cell/basophil activation, eosinophil chemotaxis and activation, as well as T lymphocyte recruitment in some models. An unprecedented number of mAb now in clinical development or in the market have potential utility not only for asthma, but also for other inflammatory and pulmonary diseases. Among these are mAb against adhesion molecules (ICAM-1, VCAM-1, VLA-4 CD18, E-selectin) (Ulbrich et al., 2003), pro-inflammatory (TNF-α, IL-1, IL-6) and Th2 cytokines (IL-4, IL-5, IL-9, IL-13) (Hart et al., 2002; Flood-Page et al., 2003), proteases, anti-IgE, anti-CD23, anti-CD4, chemokines (CCL2, 5, 7, 8 and 11) and their receptors (CCR3) (Glennie and Johnson, 2000; Torphy et al., 2001).
In summary, our data indicate that CCR2 blockade attenuates the pathophysiological consequences of asthma at several levels, including inflammatory cell influx, BHR and local release of inflammatory mediators. These findings reinforce the importance of the CCR2/CCL2 axis in the progress of asthma, and show the feasibility of anti-CCR2 mAb use as an effective blocking agent for asthma treatment.
Acknowledgements

We are indebted to Dr. José Lora for critical discussion of the manuscript. We thank Drs. E. Rausell and J. Flores for support with animal samples, M.C. Moreno for help with flow cytometry, and C. Bastos and C. Mark for secretarial and editorial assistance, respectively.
References


Huang D, Wang J, Kivisakk P, Rollins BJ and Ransohoff RM (2001) Absence of monocyte chemoattractant protein 1 in mice leads to decreases local macrophage recruitment and


Footnotes

Financial support

This work was partially supported by grants from the Spanish Ministry of Education and Science (SAF 2005-03388), and the European union (Innochem UE-518167; Molecular Imaging LSHG-CT-2003-503259). The Department of Immunology and Oncology was founded and is supported by the Spanish Council for Scientific Research (CSIC) and by Pfizer.
Legends for Figures

Figure 1. CCL2 levels and cell accumulation increase in BAL after *A. suum* antigen treatment.  
*A*, Increased CCL2 expression in BAL at 3 and 24 h post-challenge, showing CCL2 levels (mean ± SD; *n* = 8).  
*B*, Total cells, eosinophils, macrophages, neutrophils and mast cells in BAL fluid before and after antigen challenge. Total cells were counted for each population and animal, and expressed as mean cell number ± SEM. Significant differences are indicated for 3 h vs 0 h; 24 h vs 3 h (*p* < 0.05, **p* < 0.001, ***p* < 0.0001).

Figure 2. CCR2-05 mAb blocks CCL2-mediated responses in cynomolgus monkey PBMC.  
*A*, CCR2 expression in T cells (CD3*) and monocyte/macrophages (CD14*) was determined by flow cytometry using anti-human CCR2 mAb. A representative experiment is shown of five performed.  
*B*, CCL2- or CXCL12-induced Ca²⁺ mobilization in CCR2-05- or mIgG2b-pretreated PBMC was analyzed by flow cytometry. A representative experiment is shown of five.  
*C*, Effect of CCR2-05 on CCL2-induced PBMC migration. Cells that migrated to the lower chamber were counted and expressed as a migration index. Data show mean ± SD of triplicates.

Figure 3. Effect of anti-CCR2 mAb on eosinophil/macrophage recruitment to lung airways after *A. suum* antigen challenge.  
*A*, *A. suum*-induced cell accumulation in BAL fluid obtained post-challenge from untreated or mAb-treated monkeys, showing increase (mean ± SD) in total cell number over cell number before challenge.  
*B,C*, Increase in macrophages (*B*) and eosinophils (*C*) in BAL fluid at 0, 3 and 24 h after challenge. The figure expresses the value of each cell population for each animal as the *x*-fold increase in cell number compared to cell number pre-challenge.

Figure 4. Effect of CCR2-05 mAb on pulmonary function in asthmatic monkeys.  
*A,B*, Changes in $R_L$ (*A*) and $C_{DYN}$ (*B*) following *A. suum* antigen challenge in monkeys, untreated or pretreated as indicated. Data represent the percent change from baseline (mean ± SEM, *n*
= 4). Significant differences between CCR2-05 and mIgG2b treatment are indicated (* p<0.05).

**Figure 5. Effect of anti-CCR2 mAb after intradermal challenge with *A. suum* antigen.**

A, Individual response at 15 min post-i.d. challenge as determined by Evan’s Blue migration to injection sites. Histamine site received a score of 2 and the saline site, a score of 0. All *A. suum* scoring is relative to these controls (0 = no change, color/size is between the saline and histamine sites; 1 = mild color/size change, color/size is between the saline and histamine sites; 2 = marked color/size change, same degree of blue dye influx as histamine site). The mean is also indicated. Significant differences between the treatments (CCR2-05 vs. mIgG2b) are indicated (* p<0.05).
Table 1. Inhibitory effect of anti-CCR2 antibody on macrophages and eosinophils in BAL fluid from *Ascaris suum*-challenged monkeys

<table>
<thead>
<tr>
<th>Cells in BAL</th>
<th>Treatment</th>
<th>3 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>macrophages</td>
<td>control</td>
<td>1.738 ± 0.306</td>
<td>2.588 ± 0.568</td>
</tr>
<tr>
<td></td>
<td>mIgG2b</td>
<td>1.650 ± 0.536</td>
<td>1.625 ± 0.189</td>
</tr>
<tr>
<td></td>
<td>CCR2-05</td>
<td>1.275 ± 0.155</td>
<td>1.150 ± 0.343</td>
</tr>
<tr>
<td>eosinophils</td>
<td>control</td>
<td>2.550 ± 0.495</td>
<td>11.830 ± 1.719</td>
</tr>
<tr>
<td></td>
<td>mIgG2b</td>
<td>2.325 ± 0.340</td>
<td>12.500 ± 1.783</td>
</tr>
<tr>
<td></td>
<td>CCR2-05</td>
<td>1.500 ± 0.071</td>
<td>3.600 ± 0.537 (**)</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SEM of the x-fold increase in total cell numbers. Significant difference between CCR2-05-treated monkeys and control or mIgG2b-treated monkeys is indicated (**, p<0.01, one-way ANOVA test).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5