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CCR4 blockade does not inhibit allergic airways inflammation

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Abstract

The CC chemokine receptor 4 (CCR4) shows selectivity for the recruitment of memory T cell subsets, including those of the T helper cell type 2 (Th2) phenotype. In humans, $CCR4^+$ T cells are recruited to the asthmatic lung in response to allergen challenge; however, the contribution of this pathway to allergic disease remains uncertain. We therefore investigated the role of CCR4 in allergic airways inflammation in the guinea pig. Blockade of CCR4 with a specific antibody resulted in only minor changes in numbers of $CCR4^+$ Th cells in the bronchoalveolar lavage fluid of allergen-challenged guinea pigs and failed to inhibit the generation of eotaxin/CC chemokine ligand (CCL)11 or macrophage-derived chemokine/CCL22 or the recruitment of inflammatory leukocytes to the lung. These data suggest that although CCR4 was originally proposed as a marker of Th2 status, antigen-specific Th2 cells are recruited to the lung predominantly by other pathways. This study casts doubts on the validity of CCR4 as a therapeutic target in the treatment of asthma.

Keywords

T lymphocytes; chemokines; allergy

INTRODUCTION

T helper cell type 2 (Th2) T cells are principal regulators of the inflammatory responses seen in asthma. The regulation of T cell trafficking is largely through the chemokine network, and CC chemokine receptor (CCR)3, CCR4, and CCR8 reported to show selective expression on Th2 cells [1–3]. In humans, $CCR3^+$ T cells are rare at sites of allergic inflammation [4], and significant numbers of $CCR4^+$ T cells and expression of CCR4 ligands have been reported in the asthmatic lung [5, 6]. Neutralization of CCR4 ligands [thymus and activation-regulated chemokine/CC chemokine ligand (CCL)17 and macrophage-derived chemokine (MDC)/CCL22] in mice has suggested a role for CCR4 in allergic airways inflammation [7, 8], although the existence of an alternative receptor for CCL22 has been postulated [9], and a truncated form of CCL22 stimulates monocytes, although it has no effect on CCR4 transfectants [10]. Results from CCR4 knockout mice have been confusing, with evidence that $CCR4^{-/-}$ mice develop unimpaired allergic inflammation [9] and that they show reduced airways hyper-reactivity in an alternative allergic model [11]. Studies of these mice are further complicated by a role for CCR4 in thymic maturation of T cells [12]. Similarly, studies of $CCR8^{-/-}$ mice mostly fail to

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demonstrate a significant contribution of this receptor to allergic pulmonary inflammation [13, 14], although one study demonstrated a role for CCR8 in allergic inflammation [15], and another has shown a role for the CCR8 ligand, CCL1, in eosinophil (but not Th2 T cell) trafficking [16]. We have previously identified guinea pig CCR4, showed it responded to human and murine CCR4 ligands, and described a monoclonal antibody (mAb) that blocked CCR4 signaling on guinea pig T cells in vitro [17]. Here, we investigated the actions of this mAb on allergic inflammatory responses in the guinea pig lung.

MATERIALS AND METHODS

General reagents were from Sigma-Aldrich (Poole, UK). The immunoglobulin G (IgG)2a isotype anti-CCR4 mAb 10E4 [17] was prepared in-house at Millennium Pharmaceuticals Inc. (Cambridge, MA), and endotoxin < 1 EU/ml. Sterile mouse IgG was from Jackson Laboratories (West Grove, PA); R-phycoerythrin (RPE)–rabbit anti-mouse IgG, from Dako (Ely, UK); fluorescein isothiocyanate (FITC)–anti-guinea pig Th and control Ab, from Serotec (Oxford, UK). Dunkin-Hartley guinea pigs (300–400 g) were from Harlan (Oxford, UK). Work was performed in accordance with the Animal (Scientific Procedures) Act, 1986.

Generation of allergic lung inflammation

Guinea pigs were sensitized by intraperitoneal injection of 10 μg ovalbumin (OVA) in 2 mg alum on days 1 and 14 [18]. On day 21, animals were treated with mouse IgG or 10E4 (1 mg/kg) intravenously (i.v.), a dose shown in pilot studies to saturate binding on circulating $CCR4^+$ Th cells. Thirty minutes later, the guinea pigs were exposed to aerosolised saline or OVA (1% in saline, 20 min) and were humanely killed at the indicated time points. Blood was taken by cardiac puncture. Bronchoalveolar lavage was performed as described previously [18]. Bronchoalveolar lavage fluid (BALF) cells were counted, differential counts performed on hematoxylin and eosin-stained cytospins [18], and CCR4 expression analyzed by fluorescence-activated cell sorter (FACS). The lungs were removed and perfused, a small sample disaggregated using a MediMachine (Dako), according to the manufacturer's protocol, and CCR4 expression determined by FACS. Lung eosinophil numbers were measured by eosinophil peroxidase (EPO) content [18].

Flow cytometric analysis

Blood was washed in Ca^{2+}-free phosphate-buffered saline/0.25% bovine serum albumin (staining buffer). Aliquots were sequentially incubated at 4°C with buffer, 10E4, or isotype control (10 μg/ml) for 30 min, RPE secondary Ab (preabsorbed with 10% guinea pig serum) for 20 min, mouse IgG (50 μg/ml) for 10 min, and FITC anti-guinea pig Th Ab (1:25 dilution) for 30 min as described [17]. Red cells were lysed with FACSLyse (Becton Dickinson, Cowley, UK) and analyzed by FACS. Cells (5×10^5 per point) isolated from lung and BALF were stained as above, omitting the lysis. Analysis gates were consistent between animals.

Chemokine detection by enzyme-linked immunosorbent assay (ELISA)

BALF and lung homogenates (5% wt/vol suspension in Hanks' balanced saline solution) were prepared as described [18]. CCL22 was measured using a matched anti-human CCL22 Ab pair (R&D Systems, Abingdon, UK). CCL11 was measured using a mouse anti-guinea pig CCL11 mAb (clone 72D, a kind gift from Dr Timothy Wells, Serono, Geneva, Switzerland), a rabbit anti-guinea pig CCL11 detector antibody (B3) generated in-house, and donkey anti-rabbit IgG-horseradish peroxidase, which was developed by incubation with K-blue substrate (Neogen, Lexington, KY).

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Statistics

Data were analyzed by ANOVA followed by Tukey's post-test.

RESULTS

CCR4$^+$ T cell trafficking in a model of allergic airways inflammation

In preliminary experiments, 28.2% (±1.9% SEM, n=8) of circulating Th$^+$ cells in naïve guinea pigs were found to express CCR4. Sensitization of guinea pigs with OVA did not alter the percentage of Th$^+$ cells expressing CCR4 in the circulation (30.5±2.4%, n=8).

We next investigated the trafficking of CCR4$^+$Th$^+$ cells in a guinea pig model of allergic airways inflammation [18] and studied the effects of CCR4 blockade using an anti-CCR4 mAb. OVA-sensitized guinea pigs were treated i.v. with IgG or anti-CCR4 and were challenged with saline or OVA. CCR4 expression on Th$^+$ cells in the blood, lung, and BALF was measured at 6, 24, and 48 h after challenge.

To determine if systemic antibody administration resulted in levels likely to be effective in vivo, we examined the labeling of Th cells over the course of the experiment. Blood samples were taken from each guinea pig, washed to remove plasma and unbound antibodies, and then stained with the RPE-conjugated secondary antibody alone, which identified any antibody bound to Th cells, or by the addition of more anti-CCR4 mAb in vitro before secondary antibody staining, which identified cell-surface CCR4 that had not been occupied by antibody in vivo. Figure 1 shows that in the early samples taken 6 h post-challenge, there was some antibody bound nonspecifically to Th cells in the IgG-pretreated group, but this nonspecific binding declined in the later samples. Allergen challenge only caused small changes in the percentage of Th$^+$ cells expressing CCR4 in the circulation in IgG-treated animals (Fig. 1). In those animals that had received anti-CCR4 treatment, staining with secondary antibodies alone before Th- dual staining revealed that a significant proportion of Th cells was labeled with anti-CCR4. It is important that staining blood from the same animals with anti-CCR4 ex vivo did not result in an increase in the detected proportion of CCR4$^+$Th$^+$ cells, suggesting effective labeling of these cells in vivo. Blockade of CCR4 did not alter the relative proportion of circulating Th$^+$ cells expressing CCR4 over the course of the experiment.

CCR4 expression was detected on a greater proportion of lung versus blood Th$^+$ cells (mean ±SEM: 57±13% vs. 25±13%, respectively, n=8), suggesting some enrichment of this T cell subset in the lungs of these sensitized animals. However, allergen challenge of IgG-treated animals was associated with minimal changes in the proportion of intrapulmonary Th$^+$ cells expressing CCR4, and treatment with anti-CCR4 did not have any impact on proportional CCR4 expression after challenge (Fig. 2).

Th$^+$ cells recovered from BALF showed similar expression of CCR4 to Th$^+$ cells isolated from lung tissue (Fig. 2B). Allergen challenge of IgG-treated animals resulted in an initial decrease and then a return to baseline values in the proportion of BALF Th$^+$ cells expressing CCR4. There were no differences between IgG-treated and anti-CCR4-treated animals with respect to BALF Th$^+$ cell expression of CCR4. However, we also calculated the total numbers of CCR4$^+$Th$^+$ cells in the BALF and found that in IgG- and anti-CCR4-treated animals, CCR4$^+$Th$^+$ cell numbers increased after allergen challenge (Fig. 2C) and after 48 h, were higher in the anti-CCR4-treated animals.

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Recruitment of leukocytes to the lung and the airways

Allergen challenge of IgG-treated guinea pigs induced eosinophil recruitment to the lung and subsequently to the BALF (Fig. 3). Mononuclear cells and to a lesser extent, neutrophils were also increased in the BALF. Recruitment of mononuclear cells, eosinophils, and neutrophils to the lungs and airways was not inhibited by anti-CCR4 treatment, and indeed at 6 h after challenge, blockade of CCR4 was associated with an increase in allergen-induced lung eosinophilia.

Chemokine generation in allergen-challenged guinea pigs

The CCR3 ligand, CCL11, was detected in the BALF early after allergen challenge (Fig. 4A), in keeping with previous data [18]. In addition, the CCR4 ligand, CCL22, was also significantly increased at 6 h in the lung and BALF in response to allergen challenge (Fig. 4). Only low levels of CCL22 were observed using a cross-reacting anti-human CCL22 ELISA, and this ELISA may be less sensitive in detecting guinea pig than human CCL22. The increase in CCL11 and CCL22 following allergen challenge was not modulated by CCR4 blockade.

DISCUSSION

Many studies have supported a role for CCR4 in the trafficking of T cells to the skin [6, 19–23], but the contribution of CCR4 to allergic inflammation in the lung is more uncertain. Conflicting reports regarding the role of CCR4 in allergic lung inflammation in mice [9, 11] and humans [5, 6, 24] led us to study a guinea pig model of allergic lung disease in the first dissection of CCR4 actions in vivo by receptor blockade.

In IgG-treated, saline-challenged animals, CCR4 was expressed on blood Th^+ cells at similar levels to those seen in humans [25], and a greater proportion of Th^+ cells in the lung and BALF expressed CCR4, showing enrichment of this subset in the lung.

Allergen challenge was only associated with minor transient changes in the proportion of Th^+ cells expressing CCR4 in the blood, lung, and BALF, although there was an increase in total numbers of $CCR4^+$ cells in the BALF. Allergen challenge also caused an increase in CCL22 levels in the lung, detected using an ELISA for human CCL22, consistent with observations in humans [5, 6]. Guinea pig homologues of CCL22 and CCL17 have not yet been identified; thus, analysis of mRNA expression and development of specific ELISAs for guinea pig CCR4 ligands are not yet possible. We tried to inhibit the recruitment of $CCR4^+$ cells by treatment with an anti-CCR4 mAb at a dose predicted to block the receptor in vivo, based on our previous studies showing effective blockade of CCR4 at concentrations of 20 μg/ml in vitro [17]. Our data show that administration of the anti-CCR4 mAb caused persistent labeling of CCR4 in vivo over 48 h and that further staining of blood from these animals with anti-CCR4 in vitro failed to identify a population of cells that was not already bound by antibody, suggesting effective blockade was likely to be maintained during the course of study.

CCR4 blockade did not affect the proportional recruitment of $CCR4^+Th^+$ cells to the lung or their appearance in the BALF. There was, however, an increase in the total number of Th cells expressing CCR4 in the BALF 48 h after allergen challenge. This was in the absence of significant changes in recruitment of other cell types to the lung and BALF at this time point, suggesting that inflammation was not worse in this group and raising the possibility that at later time points, CCR4 might play a role in regulating the clearance of T cells from the BALF, but further experiments are required to test this hypothesis. This suggests that CCR4 expression marked a T cell subset enriched in the lung but that this receptor did not provide the mechanism for their recruitment. This generates an important alternative

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hypothesis explaining the enrichment of $CCR4^+$ T cells in human disease, namely that they coexpress other receptors that are involved in recruitment in inflammation, and this may suggest that CCR4 has a more important role in homeostasis rather than inflammatory disease. Consistent with this, most Th2 cells express CCR4 [25, 26], but these cells are divided into subsets that express a range of other receptors, which may mediate their recruitment in allergic inflammation [25].

Similarly to the lack of effect on T cell trafficking, CCR4 blockade did not reduce other parameters of allergic airways inflammation, including the generation of chemokines (CCL11 and CCL22), and the recruitment of leukocytes, including eosinophils to the lung tissue and airway lumen. Indeed, at one time point (6 h after challenge), anti-CCR4 treatment was associated with an increase in lung eosinophil numbers. The reason for this remains unclear: As the anti-CCR4 antibody did not appear to affect T cell trafficking and as it was endotoxin-free and controlled for by IgG administration in parallel animals, a nonspecific effect seems unlikely. It is possible that minor changes in the activation state of recruited T cells underlie this observation, but the changes seen were small, and the primary observation remained that CCR4 blockade failed to ameliorate allergic inflammation.

We observed moderately higher proportions of Th cells expressing CCR4 in the blood than in our previous work [17] and found CCR4 on a higher proportion of lung Th^+ cells. The increased proportion of Th^+ cells that were $CCR4^+$ in the lungs in these experiments may be a consequence of sensitization or perhaps reflects increased sensitivity of staining in these experiments.

Other possibilities may explain the inability of anti-CCR4 to block allergic inflammation in this model. It is conceivable, although unlikely, given the high interspecies homologies of CCR4 and known ligands, that this antibody was less effective at blocking the actions on CCR4 of endogenous guinea pig ligands than the murine and human ligands against which it has been tested in vitro. To date, guinea pig CCR4 ligands have not been cloned or characterized, so this possibility cannot be tested. Additionally, studies that have demonstrated a positive role for CCR4 in T cell recruitment in human disease have examined subjects in whom disease has been established for many years. It is therefore possible that CCR4 may play a role in more established disease rather than the acute phase of allergic inflammation studied here. However, these studies, together with data obtained by others using CCR4 knockout mice, suggest caution when considering CCR4 as a drug target for the treatment of asthma.

Acknowledgments

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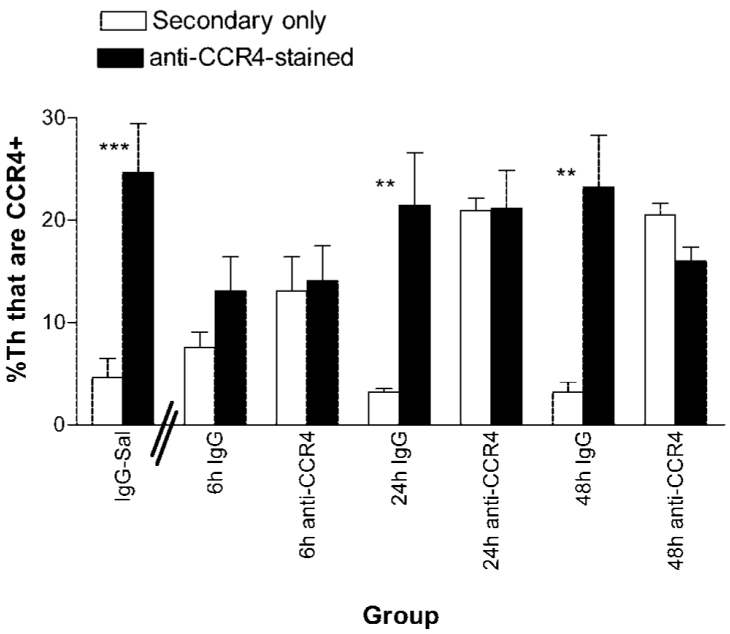

Fig. 1.

Persistence of anti-CCR4 labeling in vivo. Sensitized guinea pigs were treated with IgG or anti-CCR4 mAb 10E4 and were challenged with saline (IgG-Sal group only) or OVA (all other guinea pigs). After challenge (48 h for IgG-Sal, as indicated for other groups), lymphocytes in blood were dual-stained for the Th marker and secondary antibody alone (open bars) or with a further in vitro treatment with anti-CCR4 (solid bars), and expression quantified by FACS. The data show the proportions of Th cells with bound surface antibody. Data are n = 6–8 per point ± SEM. Significant differences between staining with secondary alone versus further anti-CCR4 are indicated: **, $P < 0.01$, or ***, $P < 0.001$.

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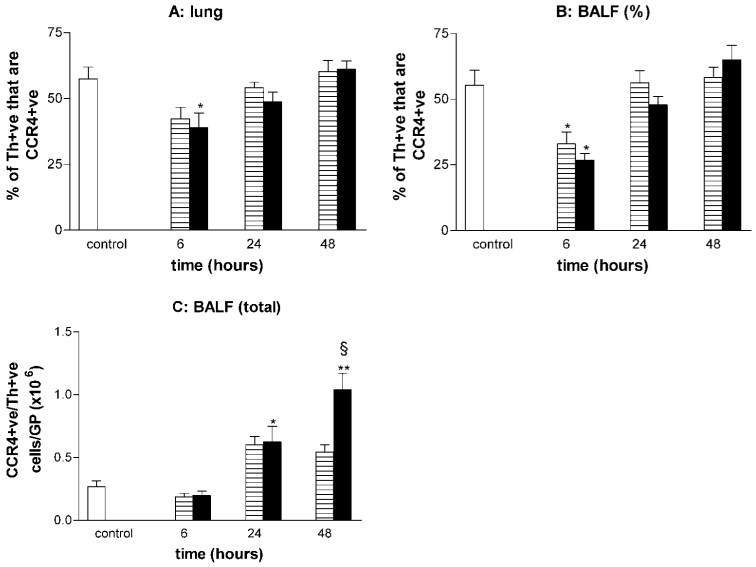

Fig. 2.

$CCR4^+Th^+$ cell trafficking in allergic airways inflammation. Sensitized guinea pigs (GP) were treated with IgG or anti-CCR4 mAb 10E4 and were challenged with saline (open bars) or OVA (striped bars, IgG treatment; solid bars, 10E4 treatment). At the indicated times after challenge, lymphocytes in lung and BALF were dual-stained for the Th marker and CCR4, and expression was quantified by FACS. (A and B) Proportions of Th cells expressing CCR4 in the indicated site; (C) the total number of $CCR4^+Th^+$ cells in the BALF. Data are n = 8 (control) or n = 7 per point ± SEM. *, $P < 0.05$, versus IgG/saline; **, $P < 0.01$, versus IgG/saline; §, $P < 0.05$, for 10E4 versus IgG pretreatment at the indicated time point.

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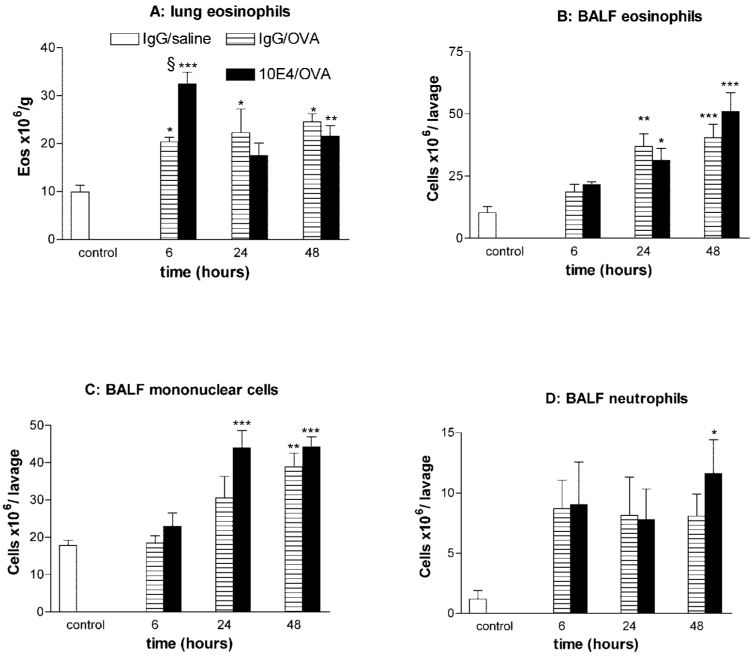

Fig. 3.

Inflammatory leukocyte recruitment in lung and airways. Sensitized guinea pigs were pretreated with IgG or anti-CCR4 mAb 10E4 and were challenged with saline (open bars) or OVA (striped bars, IgG treatment; solid bars, 10E4 treatment). At the indicated times after challenge, lung eosinophil numbers were determined by EPO assay, and BALF leukocytes were quantified as described. (A) Eosinophils (Eos)/ gram of lung tissue; (B–D) total numbers of the indicated leukocyte subset in the BALF. Data are n = 6–8 per point ± SEM. *, **, and ***, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, versus IgG/saline; §, $P < 0.05$, for 10E4 versus IgG pretreatment at the indicated point.

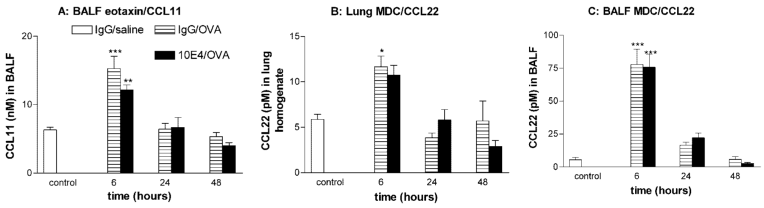

Fig. 4.

Chemokine generation in lung and BALF. Sensitized guinea pigs were pretreated with IgG or anti-CCR4 mAb 10E4 and were challenged with saline (open bars) or OVA (striped bars, IgG treatment; solid bars, 10E4 treatment). CCL11 and CCL22 in lung homogenates and BALF were measured by ELISA. Data are n = 8 (control) or n = 7 per point ± SEM. *, **, and ***, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, versus IgG/saline.

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