

AIRWAY EOSINOPHIL ACCUMULATION AND EOTAXIN-2/CCL24 EXPRESSION FOLLOWING ALLERGEN CHALLENGE IN BALB/c MICE

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□ *Eotaxin-1/CCL11 is important for early eosinophil recruitment to the airways of asthmatics. In order to clarify whether eotaxin-2/CCL24 accounts for prolonged airway eosinophilia, the authors determined the expression of CCL11 and CCL24 in lung tissue and bronchoalveolar lavage (BAL) as well as eosinophil infiltration over 14 days in BALB/c mice sensitised (intraperitoneally) and challenged (inhalations) with ovalbumin (OVA). Allergen exposure induced perivascular, peribronchial, and BAL eosinophilia for up to 7 days. CCL11 and CCL24 were highly expressed in lung tissue from 6 and up to 72 hours. Peak expression of CCL11 protein was 1557 ± 109 pg/mL for OVA (mean $\pm$ SEM) versus 404 ± 73 pg/mL in controls (SAL) ($P < .001$) and 1690 ± 54 versus 455 ± 165 pg/mL for CCL24 ($P < .01$). In BAL, only eotaxin-2/CCL24 was significantly increased (1623 ± 85 pg/mL for OVA versus 157 ± 22 pg/mL for SAL, $P < .01$). Peak eosinophilia and CCL24 expression occurred later in BAL than in lung tissue. These data suggest that both CCL11 and CCL24 are important for recruitment of eosinophils to perivascular and peribronchial tissue seen up to 72 hours. This finding implies redundancy between these chemokines rather than differentially regulated expression over time. In contrast, only CCL24 seems important for recruitment of eosinophils into BAL. Specific inhibition of CCL11 alone is therefore unlikely to inhibit eosinophil recruitment to the airways.*

Keywords allergen challenge, asthma, BALB/c mice, eosinophil, eotaxin-1/CCL11, eotaxin-2/CCL24

Asthma is characterized by chronic inflammation of the airways, and the eosinophil is considered to be the major effector of airway damage and

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dysfunction [1]. Endobronchial biopsies and bronchoalveolar lavage from asthmatic patients demonstrate increased numbers of eosinophils [2], and their number correlates with both disease activity and severity [1, 3].

Migration of eosinophils to the tissues results from interaction of several mediators, of which the most important is the CC chemokine eotaxin-1/CCL11 [4–6]. Expression of both CCL11 and its receptor CC-chemokine receptor-3 (CCR3) are increased in bronchial biopsies from asthmatic patients [7]. Levels of CCL11 are increased in the lung and sputum following allergen challenge [8, 9], and serum levels are elevated during acute asthma exacerbations [10].

The chemokines eotaxin-2/CCL24 [11] and eotaxin-3/CCL26 [12] have physiological activity similar to that of CCL11, and also exert their effects via binding CCR-3 [13, 14]. All three eotaxin proteins have been identified in humans, whereas only CCL11 and CCL24 proteins have been found in mice.

Human eotaxin-2/CCL24 shares 39% structural homology, as well as functional similarity, with CCL11, although the latter is a more potent eosinophil chemoattractant and activator than CCL24 [12]. CCL24 also recruits CD34+ eosinophils (progenitor cells) to the airways following allergen challenge [15]. CCL24 is produced primarily by epithelial cells, but also by smooth muscle cells [16] and monocytes/macrophages [17]. It is up-regulated by interleukin (IL)-4 and IL-13, as well as by transforming growth factor beta (TGF β), interferon gamma (IFN γ), and lipopolysaccharide (LPS), and inhibited by glucocorticoids [18]. Expression of CCL24 is up-regulated in mice in response to allergen challenge [19]. It is increased in nasal polyps and allergen-induced cutaneous responses in atopic subjects [20] as well as in atopic and nonatopic asthmatics [7].

Eotaxin-1/CCL11 is up-regulated in animal models of allergen-induced airway inflammation [21], and administration of neutralising anti-CCL11 antibodies attenuates this inflammation [22, 23]. Studies in animal models implicate eotaxin-1/CCL11 as the major chemoattractant for airway eosinophils during the early phase following allergen challenge; however, there is considerable evidence to suggest that it does not account for ongoing eosinophil recruitment to the airways [8, 20, 23–25]. In mice, eotaxin-1 mRNA induction occurs within 1 hour following challenge, peaking at 3 hours, followed by a rapid fall-off seen already 6 hours post challenge [26]. The kinetics of eotaxin-1 expression seem, however, to vary between species, and even between strains [20, 24, 25]. In comparison with wild-type mice, eotaxin-1-deficient mice show a 70% reduction in bronchoalveolar lavage (BAL) eosinophil count 18 hours post challenge with OVA, but no difference in eosinophil number 48 hours after challenge [25].

In asthmatic patients, levels of eotaxin-1/CCL11 mRNA and protein in BAL peak within 4 hours after allergen challenge, but are lower by 24 hours [8]. We have previously found that expression of CC11 and CCL24 mRNA

is higher in bronchial biopsies from asthmatic patients than in normal controls, but that only eotaxin-3/CCL26 expression is elevated 24 hours after allergen challenge [27]. Based on these and other studies [20, 23], it seems likely that eosinophil recruitment to the airways results from differentially regulated expression over time of the different eotaxins.

In this study, we determined whether CCL24 may account for prolonged airway eosinophilia following allergen challenge in mice. We demonstrate that CCL24 as well as CCL11 are highly expressed in airway tissue, and that both chemokines are expressed within 6 hours and remain elevated for at least 72 hours after challenge. In contrast, in BAL peak eosinophilia occurs later (48 to 72 hours) and only CCL24 is highly expressed, whereas low levels of CCL11 are found in this compartment.

MATERIALS AND METHODS

Allergen Challenge Protocol

Animal ethics review board approval was obtained for all experiments. Female BALBc mice (Harlan Laboratories, Israel) aged 8 to 10 weeks were housed under specific pathogen-free (SPF) conditions and provided adequate food and water. Animals were sensitized with intraperitoneal ovalbumin (OVA; 10 μ g OVA/1 mg Al (OH)₃ in 0.5 ml 0.9% saline) (Sigma, St. Louis, MO, USA) on days 1 and 10. Mice were then challenged with inhaled OVA (2% weight/volume diluted in 0.9% saline [SAL]: 4 mL/inhalation) on days 15, 16, and 17. Control mice (SAL) received intraperitoneal (IP) saline (0.5 mL) on days 1 and 10, followed by saline inhalations on days 15, 16 and 17. For OVA and saline challenges, mice were transferred into a Perspex 30 $\times$ 40 $\times$ 40-cm box and exposed to OVA administered by Micromist nebuliser (Hudson RCI, Temecula, CA, USA) feeding directly into the box for 20 minutes, with a flow rate of 7 L/min. The box contains additional holes for air circulation. Mice were sacrificed at 6, 24, 48, and 72 hours and 7 and 14 days after the last challenge. Groups contained four treated (OVA), and four control (SAL) animals for each time point. The entire experiment was repeated 3 times.

Lung Pathology

Animals were anaesthetized using 150 μ L pentobarbitone sodium (200 mg/mL) (CTS Chemical, Tel Aviv, Israel) and a plastic cannula was placed in the trachea via a small transverse cut made in the neck. The trachea was exposed by blunt dissection and the cannula tied in position. BAL was performed using 4 aliquots of 1 mL warmed 0.9% saline. BAL was centrifuged at 1000 rpm for 10 minutes and supernatant stored at -20°C for enzyme-linked immunosorbent assay (ELISA). Cell pellets were

resuspended in 1 mL and aliquots of 100 μ L were used for preparing cytospin preparations using a Shandon cytospin (Shandon Scientific, U.K.) and stained using Diffquik stain (Dade diagnostica, Munich, Germany). A total of 200 cells were counted for each slide. The remaining cells were placed in TRIpure reagent for RNA extraction and polymerase chain reaction (PCR) analysis. Animals were then sacrificed by abdominal aortic transection and exsanguination. Thoracotomy was performed and the right main bronchus tied and the right lung removed and snap-frozen in liquid nitrogen for RNA analysis and for tissue ELISA. The left lung was fixed by intrabronchial infusion with 1 mL 4% formaldehyde (Biolab, Jerusalem, Israel). The lung was then immersed in fixative for an additional 24 hours. Transverse sections of the lung were embedded in paraffin and sequential 4- to 6- μ m sections stained with hematoxylin-eosin or used for immunohistochemistry.

Eosinophil numbers were determined using hematoxylin-eosin (H&E)-stained lung sections at a magnification of $\times 1000$. The number of eosinophils was counted in 5 separate peribronchial and perivascular high-power fields and averaged for each animal.

ELISA for Eotaxin-1/CCL11 and Eotaxin-2/CCL24

ELISA for CCL11 and CCL24 was performed on BAL and lung tissue using DuoSet ELISA kits (R&D Systems, Minneapolis, MN, US). For BAL, 100 μ L antibody (anti-eotaxin-1: 0.8 μ g/mL goat anti-mouse; or anti-eotaxin-2: 2 μ g/mL rat anti-mouse) was placed in 96-well plates and left to incubate overnight at room temperature. Wells were then washed 3 times with 300 μ L wash buffer (0.05% Tween 20 in phosphate-buffered saline [PBS]) and blocking agent was added (300 μ L block buffer for eotaxin-1, 300 μ L reagent diluent for eotaxin-2) for 1 hour at room temperature. Washing was repeated and 100 μ L of BAL or tissue homogenate was added to each well. Samples were performed in triplicate together with standards (recombinant protein) for construction of a standard curve. Incubation was performed at room temperature for 2 hours followed by addition of 100 μ L of secondary biotinylated detection antibody (200 ng/mL goat anti-mouse eotaxin-1 or 50 ng/mL goat anti-mouse eotaxin-2) for a further 2 hours. Wells were rewashed, 100 μ L streptavidin-horseradish peroxidase (HRP) was added for a further 20 minutes, they were washed again, and a substrate solution was added (1:1 mixture of H_2O_2 and tetramethylbenzidine [TMB]), followed by a final wash and addition of stop solution. Samples were read at a wavelength of 450 nm.

For tissue ELISA, lung tissue stored at -80°C was placed in 1% bovine serum albumin (BSA) ELISA buffer (Sigma) (100 mg lung tissue/0.5 mL), phenol red 0.005% (Sigma), Tween 20 0.05% (JT Baker, USA), and sodium azide (NaN_3) 0.1% (BDH, Poole, UK), pH 7.4. Tissue was homogenised for

20 seconds using a polytron and the homogenate was then transferred to an eppendorf and centrifuged at 14,000 rpm for 10 minutes. Supernatants were used for ELISA [28].

Real-Time PCR

Total RNA from murine lung tissue and BAL cells was extracted using TRI reagent (Sigma). Reverse transcription was performed on 1 μ g of total RNA using random primers in a volume of 20 μ l according to the manufacturer's protocol (Promega, Madison, WI, USA). cDNA was diluted in a final volume of 100 μ l. Real-time PCR analysis was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, USA). The PCR mixture contained 4 μ L cDNA, 10 μ L of 2 $\times$ Taqman Universal PCR master mix (Roche, USA), and 1 μ L of 20 $\times$ mix of unlabeled primers and 5' Carboxyfluorescein (FAM)-labeled fluorescent probe in a total volume of 20 μ L. Primer pairs and probe were assay-on-demand gene expression products designed by Applied Biosystems. The housekeeping 18s RNA was used as the internal control for normalisation of the samples and DEPC water and total untranscribed RNA served as negative controls to verify the specificity of the reaction. Probe sequences for the genes analyzed are as follows:

Eotaxin-1: TGGCTCACCCAGGCTCCATCCCAAC

Eotaxin-2: GCAGGGGTCATCTTCATCACCAAGA

18S: TGGAGGGCAAGTCTGGTGCCAGCAG

For each primer pair a dilution curve of a positive cDNA sample was included to calculate the efficiency of the amplification. Universal thermal cycling parameters were used for all reactions with an initial denaturing step at 95°C for 15 seconds followed by annealing/ extension at 60°C for 1 minute. Forty cycles were performed and each sample was amplified in duplicate. The threshold cycle (C_t) was recorded for each sample to reflect the mRNA expression level and relative quantification was determined using ΔC_t calculated as $C_{t(\text{target gene})} - C_{t(18s \text{ gene})}$. Product is expressed as fold change of gene expression according to the formula $2^{-\Delta C_t}$.

Data Analysis

Mean and standard error of mean (SEM) values are given for each group. Data were analyzed using the nonparametric analysis of variance (ANOVA) statistics for multiple comparisons (Kruskal-Wallis), followed by Dunn post-test analysis for comparison of paired groups (such as OVA versus SAL at 6 hours). $P \leq .05$ was considered significant.

RESULTS

Eosinophilic Inflammation

The number of eosinophils in perivascular and peribronchial regions are shown in Figure 1A, B. Eosinophil counts were elevated within 6 hours following the last allergen challenge and remained relatively unchanged until 7 days before falling to baseline by day 14 ($P < .05$ for OVA versus SAL at all time points except 14 days and $P < .05$ for comparisons of OVA-treated animals at all time points versus day 14).

Percentage eosinophils from BAL in OVA and SAL mice are shown in Figure 1C. Eosinophil counts in BAL were elevated within 6 hours, but in contrast to tissue, continued to rise up to 72 hours, were still high at 7 days, and dropped by 14 days. As expected, no eosinophils were present in the airways of SAL-treated mice. ($P < .05$ for comparisons of OVA-treated animals at all time points versus day 14; $P < .05$ for OVA-treated mice at 72 hours versus 6 hours).

Expression of Eotaxin-1/CCL11 and Eotaxin-2/CCL24 in Lung Tissue

Both CCL11 and CCL24 protein were readily detectable in lung tissue of control animals at all time points examined.

For CCL11, peak protein expression in OVA-treated mice was observed at 6 hours (1557 ± 109 versus 404 ± 73 pg/mL for SAL; $P < .001$ (Figure 2A). Expression of CCL11 in lung tissue from OVA-treated animals was significantly greater than SAL at 6 hours and remained so until 72 hours. No difference between OVA and SAL was observed at 7 and 14 days (505 ± 48 versus 284 ± 44 pg/mL at 7 days and 331 ± 35 versus 273 ± 4 pg/mL at 14 days). For comparisons within the OVA groups, levels at 6, 24, 48, and 72 hours were significantly greater than those observed at 7 days and 14 days ($P < .05$).

mRNA expression of CCL11 is shown in Figure 2B. A similar pattern to protein expression was observed with a significant difference between SAL and OVA up to 72 hours and low mRNA levels at 7 and 14 days.

Expression of CCL24 protein is demonstrated in Figure 2C. Tissue levels and trends over time were similar to those found for CCL11. High levels of protein were already present at 6 hours (1581 ± 425 versus 458 ± 57 pg/mL; $P < .01$) and remained high until 72 hours (1690 ± 54 versus 455 ± 165 pg/mL; $P < .01$). Values were similar to controls at 7 days (641 ± 129 versus 523 ± 192 pg/mL) and at 14 days (373 ± 36 versus 290 ± 21 pg/mL). For comparisons within the OVA groups, levels at 6, 24, 48, and 72 hours were significantly greater than those observed at 7 and 14 days ($P < .05$).

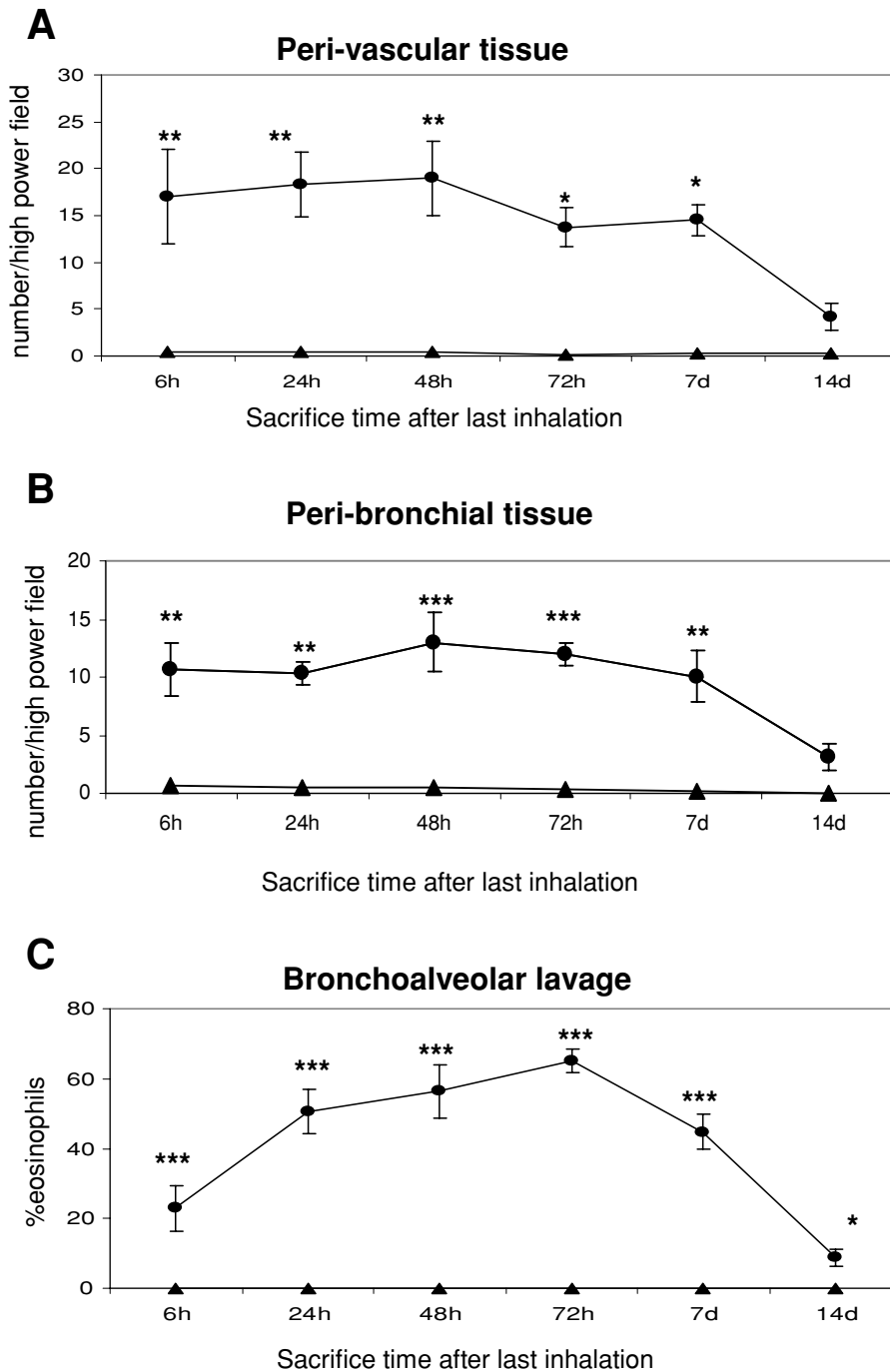


FIGURE 1 Eosinophil numbers (mean $\pm$ SEM) in perivascular (A) and peribronchial (B) lung tissue and in bronchoalveolar lavage fluid (C) in ovalbumin- (OVA) (●) and saline- (SAL) (▲) treated mice. Numbers are per high power field ($\times 1000$) or as percentage of total cells. * $P < .05$; ** $P < .01$; *** $P < .001$ versus SAL. $n = 4$ for each group.

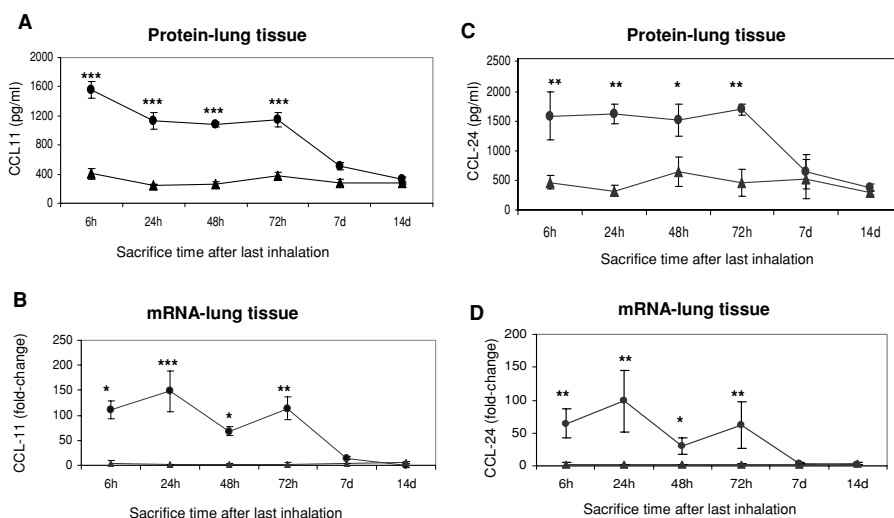


FIGURE 2 Eotaxin-1/CCL11 and eotaxin-2/CCL24 protein expression in lung tissue as determined by tissue ELISA (A, C) and mRNA expression as determined by real-time PCR analysis (B, D) (mean $\pm$ SEM). PCR data are expressed as fold increase in gene expression. $\bullet$ = OVA; $\blacktriangle$ = saline. * P < .05; ** P < .01; *** P < .001 versus SAL. n = 4 for each group.

CCL24 mRNA expression in lung tissue is shown in Figure 2D. A significant difference in mRNA expression in OVA versus SAL was observed from 6 hours up to and including 72 hours; however, at the 7- and 14-day time points, no difference from SAL was apparent.

Expression of Eotaxin-1/CCL11 and Eotaxin-2/CCL24 in BAL

Levels of CCL11 protein in BAL are shown in Figure 3A. Although levels were higher in OVA than SAL, differences were small and did not reach significance at any of the time points examined. Peak eotaxin-1 levels in BAL were 56.0 ± 19.7 pg/mL at 48 hours. Protein values in BAL were 20 to 30 times *lower* than those seen in lung tissue. mRNA expression of CCL11 was very low (virtually undetectable) at all time points examined (Figure 3B).

For CCL24, protein levels measured in BAL from OVA-treated mice were more than 20 times higher than those for CCL11 and were of similar magnitude to those observed in lung tissue. Significantly increased levels were observed at 48 hours and peaked at 72 hours (2171 ± 623 versus 289 ± 85 pg/mL; P < .01), with a return to levels similar to saline by 7 days (Figure 3C). Comparison of levels within the OVA group show that levels at 48 hours and 7 days were significantly greater than those at 7 days and 14 days (P < .05). The time course of CCL24 expression in BAL mirrors that of BAL eosinophilia, with gradual increase to peak levels at 72 hours. This contrasts with the rapid rise (seen at 6 hours) and sustained eosinophilia and CCL11 and CCL24 expression seen in lung tissue.

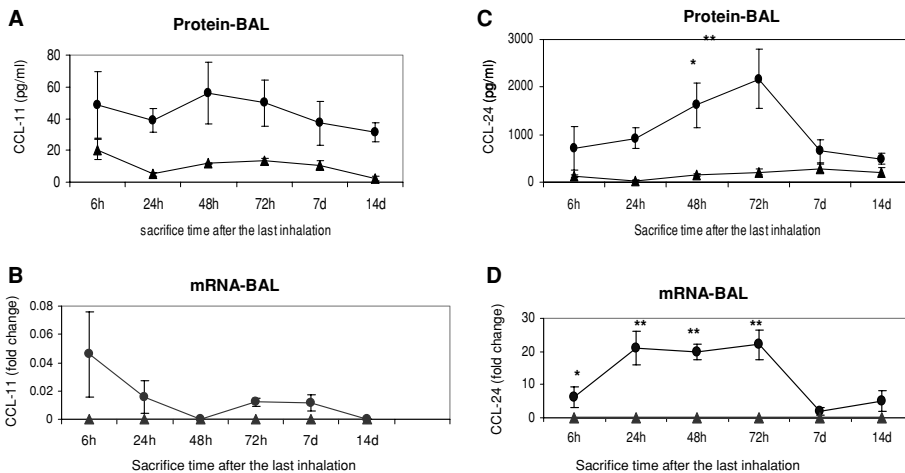


FIGURE 3 Eotaxin-1/CCL11 and eotaxin-2/CCL24 protein expression in bronchoalveolar lavage fluid as determined by ELISA (A, C) and mRNA expression as determined by real-time PCR analysis (B, D) (mean $\pm$ SEM). PCR data are expressed as fold increase in gene expression. $\bullet$ = OVA; $\blacktriangle$ = saline. * P < .05; ** P < .01; *** P < .001 versus SAL. n = 4 for each group.

A similar trend was observed for CCL24 mRNA expression in BAL (Figure 3D).

DISCUSSION

We determined the expression of the CC chemokines eotaxin-1/CCL11 and eotaxin-2/CCL24 in perivascular and peribronchial lung tissue and BAL of BALB/c mice over a 14-day period following ovalbumin challenge. We also determined the extent and kinetics of tissue and BAL eosinophilia. Both CCL11 and CCL24 are highly expressed in airway tissue. In contrast, only CCL24 is present at high levels in BAL following allergen challenge. Peak eosinophilia and peak chemokine expression occur at a later time point in BAL as compared to perivascular and peribronchial tissue.

Previous data suggest that CCL11 accounts for early (up to 6 hours) but not sustained (beyond 24 hours) recruitment of eosinophils to the lung in response to allergen challenge in human asthma as well as in animal models of allergen challenge [8, 23–26]. This suggests that alternative mediators account for late phase or persistent eosinophilic airway inflammation. We embarked on this study in an attempt to elucidate whether CCL24 accounts for persistent eosinophil recruitment to the airways in mice following allergen challenge.

Ying and colleagues [20] found a correlation between 24-hour tissue eosinophilia and eotaxin-2/CCL24 expression in human allergen-induced cutaneous responses, suggesting that this cytokine may be important in late-stage allergen-induced inflammation. Increased eotaxin-2 expression has

also been shown in atopic and nonatopic asthmatics [7]. We have previously found that expression of eotaxin-3/CCL26 but not eotaxin-1 or -2 mRNA is increased 24 hours after allergen challenge in asthmatic patients, suggesting that this chemokine may account for ongoing eosinophil recruitment in humans [27].

In this study, we show in a murine model that CCL11 as well as CCL24 are highly expressed for up to 72 hours, suggesting that in the mouse, both chemokines are likely to be important for initial as well as for ongoing eosinophil recruitment to the airway. The temporal pattern of expression of CCL11 and CCL24 in lung tissue was concordant rather than sequential, suggesting that there is redundancy rather than differentially regulated expression of these chemokines in this compartment. This is in contrast to the generally held view that cytokine and chemokine expression is synchronized in response to allergen challenge [20, 23, 29]. Our findings are in line with those of Pope and colleagues [30] who found that eotaxin-1- and eotaxin-2-deficient mice had no reduction in tissue eosinophilia following allergen exposure, whereas double-deficient mice and CCR3-deficient mice had markedly reduced eosinophil numbers. Our data do not allow us to exclude the possibility that CCL11 and CCL24 expression are "synchronized" prior to 6 hours (for example, CCL11 may peak at 1 to 3 hours whereas CCL24 is low at this early time point); however, we can conclude that these chemokines overlap from 6 to 72 hours.

It is possible that CCL11 and CCL24 have other nonoverlapping functions that are unrelated to eosinophil recruitment. These chemokines may even have a mutual inhibitory or quenching effect on each other by inducing down-regulation or internalization of CCR3 receptors on target cells, as has been shown for CCL26 in A549 cells [31].

Expression of both CCL11 and CCL24 in lung tissue remained high for 72 hours after the last allergen challenge. This was longer than expected or previously reported for CCL11 [23–26], although marked variation in the duration of CCL11 expression has been observed and may be related to the use of different allergen challenge protocols across studies.

Both CCL11 and CCL24 expression had returned to low levels 7 days following challenge, although tissue and BAL eosinophilia were still present at this time. This raises the question as to what accounts for the sustained eosinophilia seen in tissue and BAL at 7 days. One possibility is that these eosinophils were recruited by CCL11 and CCL24 several days earlier and remain in the tissue. Under normal circumstances, up to 90% of eosinophils die within 4 days [32]; however, survival may be prolonged in asthma. Several survival factors for eosinophils are present in the airways following allergen exposure, including granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-5 and eotaxins themselves [33, 34]. Eosinophil viability in tissue may thus be sustained for up to 6 days. An alternative explanation for the persistent eosinophilia seen at 7 days, despite the low CCL11

and CCL24 levels at this time point, is that an alternative chemokine may account for the persistence of the eosinophilia. This seems somewhat unlikely based on the virtual abolishment of eosinophil recruitment to the airways in CCL11/ CCL24 double knockout mice [35]. Eotaxin-3 may account for this role in humans but functional CCL26 protein is not present in mice [36].

We were surprised to find that although CCL11 and CCL24 expression (mRNA and protein) were similar in lung tissue, only very low levels of CCL11 were detected in BAL. CCL24 mRNA and protein were substantially higher at all stages following challenge. It is possible that CCL11 in BAL peaked early (3 hours), as has been found by others [8, 14], and was back to control levels by 6 hours. We think it extremely unlikely that this is the case, but even if this were so, CCL11 clearly does not contribute to BAL eosinophilia seen after 6 hours. Thus although both CCL11 and CCL24 may both play a role in the recruitment of eosinophils to the perivascular and peribronchial mucosa, only CCL24 is likely to be important for the development of BAL eosinophilia. Yang and colleagues found that CCL24, in coordination with IL-5, induces bronchial airway eosinophilia and bronchial hyperreactivity via induction of IL-13. These events were not inhibited when eotaxin-1/CCL11 knockout mice were used [29]. In another study, CCL24-deficient mice had a profound reduction in airway eosinophilia following intratracheal IL-13 administration, with no change in peribronchial eosinophil infiltration, suggesting that CCL24 is the major chemokine accounting for BAL eosinophilia [36]. Our results are concordant with Pope and colleagues who found that allergen-exposed eotaxin-2-deficient mice have a 4-fold reduction in BAL eosinophil numbers, whereas eosinophil number in eotaxin-1-deficient mice are similar to wild-type mice. In allergen-exposed wild-type mice, they also found highly elevated eotaxin-2 protein in BAL fluid, whereas eotaxin-1 levels were only mildly increased. Alveolar lavage macrophages are the likely source of CCL24 production [30].

In conclusion, we found that both eotaxin-1/CCL11 and eotaxin-2/CCL24 are highly expressed in airway tissue of allergen exposed BALB/c mice, and that expression is prolonged for up to 72 hours. This finding suggests that there is redundancy rather than synchronized expression of these chemokines in this compartment. In contrast, only CCL24 seems to be relevant to eosinophil recruitment present in bronchoalveolar lavage. Thus strategies aimed at reducing persistent eosinophil recruitment to the airways in asthma are unlikely to succeed if they are CCL11 specific and inhibition of CCL24 or of the CCR3 receptor is likely to be a more successful therapeutic approach.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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