

Macrophage inflammatory protein-1 α and C–C chemokine receptor-1 in allergen-induced skin late-phase reactions: relationship to macrophages, neutrophils, basophils, eosinophils and T lymphocytes

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Summary

Background Macrophage inflammatory protein (MIP)-1 α binds to C–C chemokine receptor (CCR)-1 with high affinity. CCR-1 is expressed on neutrophils, eosinophils, monocytes, T lymphocytes and basophils; cells characteristic of atopic allergic inflammation. *In vitro*, MIP-1 α is chemotactic for monocytes, T cells and basophils and is also a potent histamine-releasing factor for basophils and mast cells. Although increased levels of MIP-1 α were shown in atopic allergic disorders, the kinetics of expression of these CC chemokines *in vivo* is largely unknown.

Objective To investigate the kinetics of expression of MIP-1 α and receptor CCR-1 and the relationships between the expression and infiltration of inflammatory cells in allergen-induced cutaneous late-phase reactions in atopic subjects.

Methods Cryostat sections, obtained from skin biopsies from 10 human atopic subjects at 6, 24, 48, 72 h and 7 days after allergen challenge, were processed for immunohistochemistry and *in situ* hybridization using ³⁵S-labelled riboprobes.

Results The peak expression of allergen-induced mRNA for MIP-1 α and CCR-1 was 6 h. This was maintained at 24 h, and gradually returned to base line at 7 days. At 6 h, the number of cells expressing MIP-1 α mRNA significantly correlated with elastase⁺ neutrophils and BB-1⁺ basophils. At 24 h, the MIP-1 α mRNA⁺ cells significantly correlated with CD68⁺ macrophages. There were significant inverse correlations between the numbers of MIP-1 α mRNA cells and the numbers of Tryptase⁺ mast cells at 6 and 24 h after allergen challenge.

Conclusion Allergen-induced cutaneous late-phase reactions in humans were associated with increased expression of MIP-1 α and CCR-1. This may be relevant to the infiltration of neutrophils, eosinophils, basophils and macrophages.

Keywords: allergy, neutrophils, macrophages, basophils, T cells, mast cell, MIP-1 α , CCR-1, skin LPR

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Introduction

Injection of allergen into the dermis of sensitized atopic subjects is associated with an immediate weal and flare reaction followed within hours by an oedematous, red and

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slightly indurated late-phase reaction (LPR) [1,2]. These immediate responses are usually maximal at 15 min and spontaneously resolve within 2–3 h. It is widely accepted that the immediate reaction is dependent on the IgE-mediated release of mast cell-derived mediators such as histamine and leukotrienes. Immunohistochemistry has shown that the LPR was associated with the infiltration of eosinophils, basophils, neutrophils, T lymphocytes and macrophages [1,2]. The precise mechanisms whereby these cell types are recruited from blood vessels into local allergen-injected sites remains to be elucidated.

Increasing evidence *in vitro* and in animal models suggests that C-C chemokines may play an important role in recruitment of inflammatory cells into local tissue *in vivo* [3]. We previously showed that eotaxin expression was associated with the early infiltration, and eotaxin-2 and monocyte chemoattractant protein (MCP)-4 with the later 24 h infiltration, of eosinophils in allergen-induced skin LPR [4]. However, there was no association between basophil and neutrophil numbers and the expression of eotaxin, eotaxin-2, MCP-3, MCP-4 and RANTES. Furthermore, *in vitro* these C-C chemokines have no, or only little, effect on activation and infiltration of other inflammatory cells including neutrophils, macrophages and mast cells. Thus, other C-C chemokines, such as macrophage inflammatory protein (MIP)-1 α , may also be involved in the recruitment and activation of the various cell types associated with allergic inflammation.

MIP-1 α is a C-C chemokine that binds to chemokine receptor CCR-1 with high affinity [5,6]. CCR-1 is expressed on many inflammatory cells, including monocyte/macrophages, T lymphocytes, neutrophils, eosinophils, and basophils [7–11]. MIP-1 α -induced calcium flux was also observed in these cells [12]. This may endow MIP-1 α with leucocyte chemotactic and activating properties. For instance, MIP-1 α is a potent chemotactic and activating factor for monocytes, T lymphocytes, and basophils [13–16]. MIP-1 α is also a chemoattractant for neutrophils [13] and eosinophils [17]. Injection of human MIP-1 α into the footpads of C3H/HeJ mice was shown to induce a localized inflammatory response characterized by the early influx of polymorphonuclear leucocytes (PMNs), followed by a later infiltration of mononuclear cells [18]. Furthermore, intradermal administration of MIP-1 α to humans produced a local neutrophil/monocyte-rich inflammation [19]. When MIP-1 α was inhibited *in vivo* in a mouse model there was substantial attenuation of the accumulation of PMNs after injection of *Mycobacterium bovis* Bacille Calmette Guérin [20]. Additionally, MIP-1 α also induced mouse mast cell degranulation *in vitro* [18] and *in vivo* [18,21].

Increased expression of MIP-1 α was also observed in bronchoalveolar lavage fluid from asthmatics both at

baseline [22] and after allergen challenge [23]. However, the kinetics of expression of MIP-1 α in provoked human allergic tissue reactions has not been elucidated. In this paper, we attempted to investigate expression of mRNA for MIP-1 α and CCR1 in allergen-induced skin LPR.

Methods

Human subjects

Atopic subjects ($n = 10$) were recruited from the Allergy Clinic of the Royal Brompton Hospital and from the staff of the National Heart and Lung Institute (London, UK). 8/10 had been included in a previous study [4]. Inclusion criteria were as previously described [1,4]. Patients taking oral medication in the two weeks before the study began were not included.

Study design and processing specimens

The study was approved by the Ethics Committee of the Royal Brompton & Harefield NHS Trust, London and was performed with all subjects' written informed consent. Lysophilized extracts (Aquagen SQ, ALK, Hørsholm, Denmark) of either timothy grass pollen or house dust mite extract (ALK) were reconstituted at 1500 biological units (BU)/mL in isotonic sterile saline diluent. Thirty biological units of either allergen extract (0.02 mL) were injected intradermally into individual sites on the extensor aspect of the forearms of each subject. A further site was injected with a similar volume of diluent. The size of the late response was measured at 6, 24, 48 and 72 h and 7 days by evaluating skin induration [4]. Biopsies were obtained at each time-point and were processed as previously described [4,24].

In situ hybridization (ISH)

All reagents were from Sigma Chemicals (Poole, UK), unless otherwise indicated. To avoid any possible cross-hybridization, all of the cDNA inserts for generating riboprobes were full lengths encoding human MIP-1 α (300 bp, full encoding region, a kind gift from Dr K. Shimada, Department of Internal Medicine, Kumamoto University Medical School, Japan) [25], and CCR-1 (~1100 bp, full length of encoding region, a kind gift from Dr P. M. Murphy, National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA) [5]. These cDNAs were subcloned into RNA expression vector, Bluescript, respectively. Using the method of *in vitro* transcription, riboprobes (sense and anti-sense) were labelled with ³⁵S-UTP as previously described [4,24]. Briefly, riboprobes (anti-sense or sense) were synthesized

in the presence of adenine triphosphate (ATP), guanosine triphosphate (GTP), cytosine triphosphate (CTP), and ^{35} -S-uridine triphosphate (UTP), and appropriate RNA polymerases (T3: anti-sense; T7: sense, respectively).

Slides were defrosted for the experimental procedure. Permeabilization, prehybridization, and hybridization protocols were as described previously [4,24]. Negative controls employed hybridization with the sense probe and pretreatment of slides with RNase A (Promega, Southampton, UK) prior to hybridization with the anti-sense probe. Autoradiography was performed on the slides and mRNA positive signals were identified and counted as previously described [4,24]. At least two sections from each biopsy were hybridized, from which whole sections (10–19 fields per section, each field = 0.202 mm²) were counted blind in coded random order by two observers.

Immunohistochemistry (IHC)

The alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was used to enumerate immunoreactive cells. Monoclonal antibodies used were purchased as follows: EG2 (human activated eosinophils, Pharmacia, Uppsala, Sweden), CD3 (T lymphocytes, Becton Dickinson, Cowley, UK), and anti-tryptase (mast cells, Chemicon, Temecula, CA, USA), anti-neutrophil elastase (human neutrophils, Dakopatts, High Wycombe, UK), and CD68 (macrophages, Dakopatts). BB-1, a novel monoclonal antibody recognizing a human basophil granular protein [26] was a kind gift from Dr A. F. Walls (University of Southampton, UK). The APAAP technique was performed as described previously [1,4,27]. Optimal concentrations of all antibodies used were determined in pilot experiments. Briefly, the sections were incubated with the monoclonal antibodies against cellular markers for 30 min, washed in Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl), then incubated with rabbit anti-mouse immunoglobulin (Dako) for 30 min. After washing in TBS, the sections were incubated with soluble complexes of alkaline phosphatase and mouse anti-alkaline phosphatase (APAAP, Dako) for a further 30 min, and developed with Fast Red (Sigma) as chromogen for signal visualization. For immunostaining of BB1⁺ basophils, the slides were pretreated with 0.1% saponin (Sigma)/PBS for 30 min, incubated BB-1 (1 : 10) overnight at room temperature then followed as above description.

Positive cells were stained red colour after development with Fast Red. Omission or substitution of the primary antibody with an irrelevant antibody of the same species was used as a negative control. The positive cells within the whole sections (see above) were counted and the results were expressed as the total number of positive cells per square millimeter of biopsies.

Statistical analysis

Data were analysed using a statistical package (Minitab Release 7, Minitab Inc., State College, PA, USA). Variability of parameters studied was analysed with Friedman's test, followed by two-by-two comparisons between time-points using the Wilcoxon signed rank test. Correlation coefficients were obtained by Spearman's method with correction for tied values. Bonferroni's correction was also applied to correlation tests. For all tests, *P*-values less than 0.05 were considered significant.

Results

Cutaneous LPR and cell infiltration

All subjects gave a cutaneous LPR after injection of the allergen but not after the diluent saline controls (*P* < 0.002). The peak of the LPR was reached 24 h after challenge (the mean diameters, mm ± SEM = 75.6 ± 10.1), although there were no significant differences between this and the 6 h (61.8 ± 3.9) or 48 h (53.4 ± 10.8) time-points. At 72 h and 7 day the LPR was virtually absent.

Using the technique of IHC, we confirmed that after allergen challenge the numbers of infiltrating elastase⁺ neutrophils, EG2⁺ eosinophils, BB-1⁺ basophils, CD3⁺ total T cells and CD68⁺ macrophages were elevated significantly compared with diluent challenge at all time-points (Friedman's test, *P* < 0.001). The highest infiltration of these cell types was observed at 6, 24 and 72 h, respectively (Table 1), which are consistent with our previous studies [1,4,24,27]. All infiltrating inflammatory cells, but not tryptase⁺ mast cells, persisted for up to 7 days. In contrast with basophils, the number of tryptase⁺ mast cells was reduced after allergen challenge compared with diluent controls and the reduction persisted up to 7 days (Table 1). This suggested mast cell degranulation, which is consistent with our previous findings [27].

mRNA⁺ cells for MIP-1α and CCR1

In diluent challenged sites, very few cells expressed mRNA for MIP-1α. At the allergen-challenged sites there were significant increases in the numbers of mRNA⁺ cells for this chemokine at 6 h, 24 h and 48 h (Friedman's test, *P* < 0.01) (Fig. 1). In general MIP-1α mRNA⁺ cells were mainly located within areas of inflammatory infiltrate in the upper part of the dermis as well as the deep reticular dermis. According to morphology, endothelial cells of blood vessels, and macrophages were the major sources for MIP-1α mRNA⁺ cells. After allergen challenge the peak expression of MIP-1α was 6 h. This gradually declined at

Table 1. Infiltration of inflammatory cells in allergen-induced skin LPR

	Diluent	6 h	24 h	48 h	72 h	7 day
Elastase ⁺ neutrophils	4.2 (0.0–38.2)	101.4 (11.8–153.4)	59.3 (14.7–122.9)	48.5 (1.5–116.6)	41.6 (30.4–80.1)	7.1 (1.9–11.4)
EG2 ⁺ eosinophils	0.0 (0.0–2.5)	78.9 (44.8–161.7)	66.1 (19.6–160.2)	42.1 (10.5–94.2)	40.3 (21.5–64.9)	7.8 (4.3–45.9)
BB-1 ⁺ basophils	0.4 (0.0–2.9)	17.7 (0.1–40.4)	33.1 (12.1–61.9)	5.6 (0.8–36.9)	3.5 (0.3–3.8)	1.7 (0.0–3.7)
Tryptase ⁺ mast cells	83.3 (35.8–100.4)	37.7 (14.7–58.8)	53.2 (4.9–85.3)	49.5 (19.6–84.3)	53.9 (38.2–71.8)	58.8 (46.7–60.9)
CD3 ⁺ T cells	74.2 (46.2–107.9)	92.6 (59.9–130.8)	181.1 (86.4–290.1)	172.7 (47.0–214.6)	69.4 (45.1–71.9)	67.1 (39.6–75.6)
CD68 ⁺ macrophages	52.3 (11.8–151.9)	54.2 (14.7–214.5)	117.9 (15.7–328.3)	116.9 (18.6–301.4)	163.3 (158.3–208.7)	102.3 (95.6–130.9)

The results are expressed as median and range of positive cells per mm² of skin biopsies.

24 h and 48 h and returned to baseline by 7 days (Fig. 1). At 72 h, but not at 7 days, the numbers of cells expressing MIP-1 α mRNA were still higher than those in diluent sites (Fig. 1). The numbers of CCR-1 mRNA⁺ cells at the diluent site were negligible. After allergen challenge there were increases at all time-points which were significant at 6, 24 and 48 h (Friedman's test, $P < 0.01$) with peak expression at 6 h. Based on their morphology, granulocytes were the majority of cells expressing CCR-1 mRNA.

Relationships between the infiltration of inflammatory cells and the expression of MIP-1 α and CCR-1

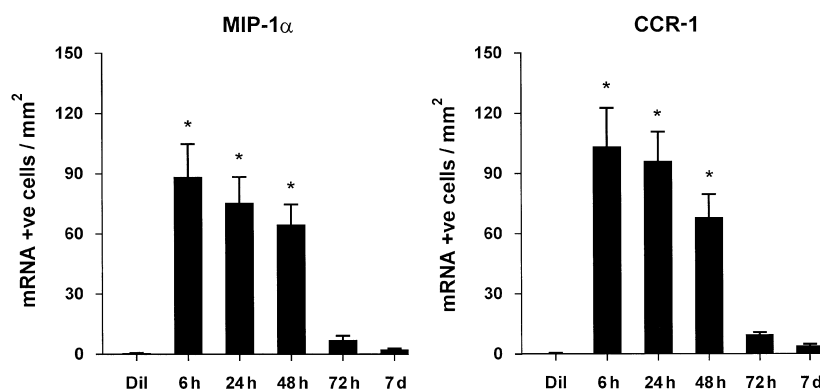
At the 6 h time-point, the numbers of elastase⁺ neutrophils and BB-1⁺ basophils significantly correlated with the numbers of MIP-1 α and CCR-1 mRNA⁺ cells at 6 h after allergen challenge (Fig. 2). There was also a weak but significant correlation between EG2⁺ eosinophils and the numbers of MIP-1 α cells ($r = 0.593$, $P = 0.041$) (data not shown). At the 24 h time point, the numbers of MIP-1 α ⁺

cells significantly correlated with the late phase of infiltration of CD68 macrophages (Fig. 3). Interestingly, there were highly significantly inverse correlations between the numbers of MIP-1 α mRNA⁺ cells and tryptase⁺ mast cells at 6 and 24 h, respectively (Fig. 4). The numbers of MIP-1 α mRNA⁺ cells also significantly correlated with the numbers of CCR-1 mRNA⁺ cells at 6 h after allergen challenge ($r = 0.81$, $P < 0.001$). There were no correlations between MIP-1 α mRNA⁺ cells and CD3⁺ T lymphocytes at any time points (data not shown).

Discussion

Although it is well documented that C-C chemokines are chemotactic for neutrophils, basophils, eosinophils, macrophages and T lymphocytes *in vitro*, the mechanisms of the cell accumulation *in vivo* in humans are unclear since most of the studies, including those on MIP-1 α , have been performed either *in vitro* or in animal models [3,28]. This is the first comprehensive time course study on MIP-1 α

Fig. 1. The time course of appearance of mRNA⁺ cells for MIP-1 α and CCR-1 into allergen-challenged skin sites in atopic subjects. Diluent-challenged sites (Dil) were used as controls. The results are expressed as the numbers of positive cells (mean $\pm$ SEM) per mm² of skin biopsies. Significant differences (diluent and 6, 24 and 48 h allergen-challenge time-points, Friedman's test) were observed in the cells expressing MIP-1 α ($P < 0.01$) and CCR-1 ($P < 0.01$).



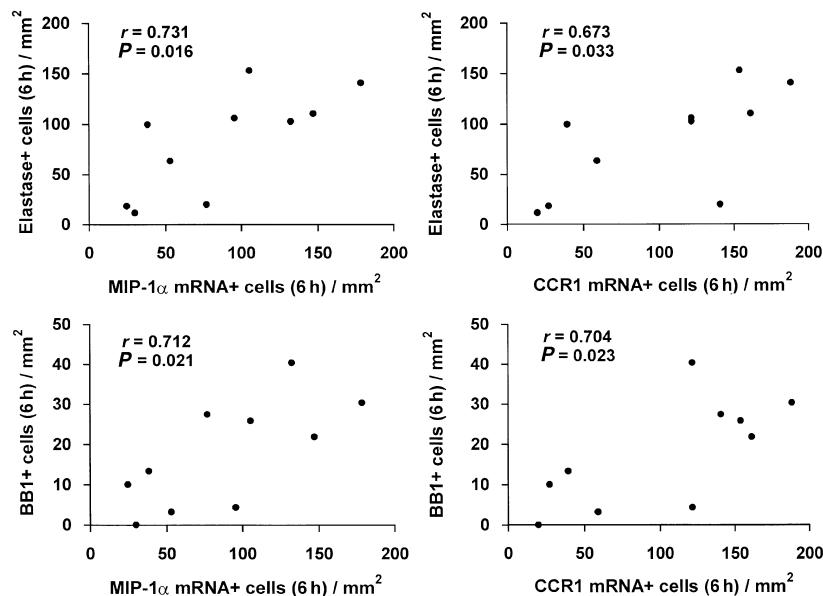


Fig. 2. Correlations between the numbers of elastase⁺ neutrophils, BB-1⁺ basophils, and mRNA⁺ cells for MIP-1 α and CCR-1 at 6 h after allergen challenge. Correlations were obtained by Spearman's method with correction for tied values.

expression in human allergic tissue reactions. Furthermore MIP-1 α has been compared with CCR-1 and the infiltration of CCR-1-bearing inflammatory cells.

The expression of MIP-1 α and CCR-1 mRNA significantly correlated with the numbers of infiltrating elastase⁺ neutrophils at an early time-point (i.e. 6 h) after allergen challenge (Fig. 2). Although MIP-1 α has no direct effect on neutrophil chemotaxis *in vitro* [29], our data suggest that MIP-1 α may participate in neutrophil infiltration *in vivo*. This point of view is supported by a numbers of observations. For example, injection of MIP-1 α into mouse induced a neutrophil-rich severe inflammatory reaction [18]. Administration of anti-MIP-1 α antibody also significantly inhibited ovalbumin-induced infiltration of neutrophils in a murine model of allergic peritonitis [30]. Similar results were also obtained in lung inflammatory cell recruitment and lung injury of murine endotoxemia [31]. Targeting CCR-1 reduced infiltration of leucocytes and suppressed development of acute and chronic cardiac

allograft rejection in a mouse model [32]. Lee *et al.* [19] showed that intradermal injection, to human subjects, of MIP-1 α induced up-regulation of E-selectin, and infiltration of neutrophils, monocytes, lymphocytes and, to a lesser extent, eosinophils. The neutrophil infiltration occurred as early as 2 h, preceding the appearance of other cells, and peaked at 10 h. The infiltration of other cell types peaked at 10–24 h [19]. *In vitro*, MIP-1 α also up-regulated CD11b expression on neutrophils [19]. Taken together, these studies indicate that MIP-1 α , together with IL-8 [33], play a role in the recruitment of neutrophils into local tissue. A weak, but significant correlation was also observed between the numbers of BB-1⁺ basophils and mRNA⁺ cells for MIP-1 α and CCR-1 at 6 h after allergen challenge (Fig. 2). This suggests that MIP-1 α may also contribute to basophil infiltration through binding CCR-1. However, the infiltration of basophils peaked at 24 h after allergen challenge, when MIP-1 α was declining (Fig. 1). Because basophils also express other receptors such as CCR-2,

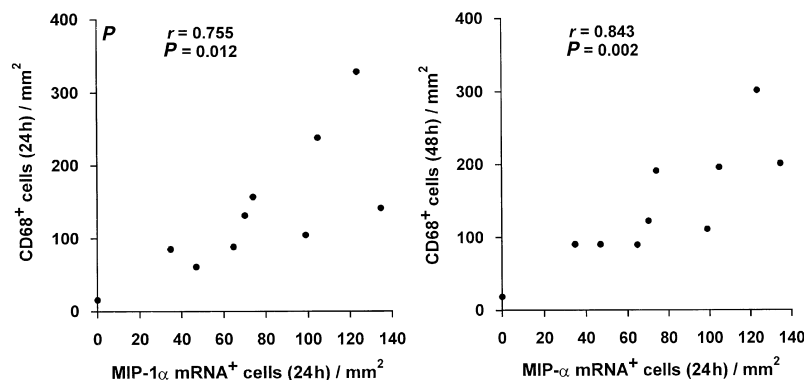
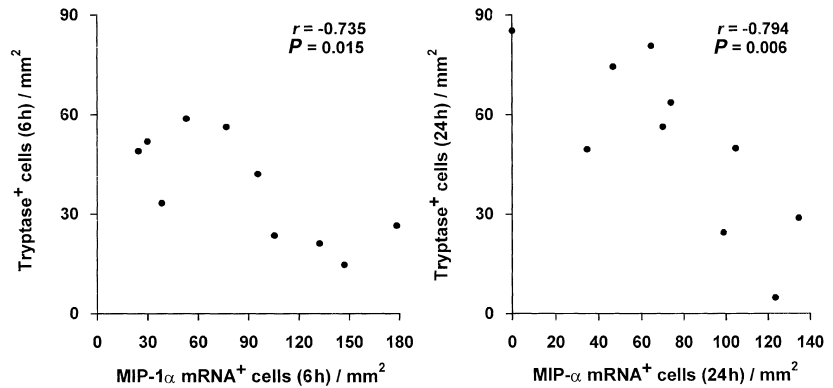


Fig. 3. Correlations between the numbers of CD68⁺ macrophages (at 24 and 48 h) and the numbers of MIP-1 α positive cells (at 24 h). Correlations were obtained by Spearman's method with correction for tied values.

Fig. 4. Correlations between the numbers of tryptase⁺ mast cells (at 6 and 24 h) and the numbers of MIP-1 mRNA positive cells (at 6 and 24 h). Correlations were obtained by Spearman's method with correction for tied values.



CCR-3 and CCR-4 [34], other C-C chemokines and their receptors may also contribute to their accumulation in allergen-induced skin LPR. This explanation may also apply to eosinophils. Eosinophils strongly express CCR-3 but weakly express CCR-1 [34]. Although the role of MIP-1 α on eosinophil chemotaxis is not as effective as that of eotaxin and other eosinophil chemotactic factors (e.g. eotaxin-2 and RANTES), MIP-1 α -mediated eosinophil infiltration has been observed in mice [35] and humans [19]. Thus, apart from these factors, MIP-1 α may also be involved in eosinophil accumulation via binding CCR-1.

MIP-1 α gene-targeted mice have a reduced mononuclear cell content in virus-infected tissue compared with that seen in wild-type mice [36]. There were significant correlations between MIP-1 α mRNA⁺ cells and CD68⁺ macrophages in allergen-induced skin LPR at 24 h (Fig. 3). However, the expression of MIP-1 α was at early-phase (i.e. 6 h), while the infiltration of macrophages was relatively late (peaked at 72 h). This suggests that early expression of MIP-1 α may have resulted in the consequent influx of monocytes from blood into local tissue. Injection of MIP-1 α by subcutaneous or intravenous routes significantly increased peripheral blood monocyte counts in cancer patients and normal healthy volunteers, indicating that MIP-1 α may not only attract monocytes but also induce release of monocytes from bone marrow pool [37]. MIP-1 α also induced CD11b and CD11c expression by monocytes, which may further enhance infiltration of these cells [19]. Additionally, MIP-1 α may also induce monocyte infiltration via CCR-5 [38].

We also found inverse correlations between the numbers of tryptase⁺ mast cells and the numbers of cells expressing MIP-1 α mRNA at 6 h and 24 h (Fig. 4). Although electron microscope examination of the tissue revealed features of extensive mast cell degranulation, accompanied with local tissue swelling within 30 min after mouse footpad injection of human MIP-1 α [18], MIP-1 α does not directly induce immediate mast cell degranulation *in vitro* in humans [39]. Furthermore, intradermal injection of MIP-1 α did not induce immediate reactions. However, some mild late

reactions occurred 10 h after injection [19]. Allergen challenge in skin induced both immediate and late-phase reactions [1,2,4,27]. Thus, MIP-1 α , as well as allergen, may contribute to the ongoing mast cell degranulation observed at the 6 and 24 h time-points.

Using severe-combined immunodeficient (SCID) mice grafted with human skin and given human T cells into their peritoneal cavities, Kunstfeld and colleagues [40] have shown that recombinant human MIP-1 α attracted significant numbers of human T cells into the skin grafts. Intradermal injection of MIP-1 α in humans also induced the accumulation of CD3⁺ cells in local tissues [19]. However, in the present study there were no correlations between the infiltration of T lymphocytes and the cells expressing MIP-1 α mRNA at any time-points though both cells significantly increased after allergen challenge. This suggests that multiple chemokines and cytokines may be required for the infiltration of T lymphocytes in allergen-induced skin LPR. Additionally, Karpus *et al.* [41] showed that in the presence of MIP-1 α mice T cells preferentially produced IFN- γ , while in the presence of MCP-1, the cells produced more IL-4. Thus, chemokines, including MIP-1 α and MCP-1, may also contribute to the regulation of Th1 and Th2 cells.

Like other C-C chemokines, MIP-1 α is widely expressed by many cell types, including epithelial, endothelial cells, fibroblasts and inflammatory cells [42,43]. In the present study, endothelial cells of blood vessels, and macrophages in the dermis were the major sources for MIP-1 α mRNA. The mechanisms of expression of MIP-1 α are incompletely understood. A recent study showed that activation by IgE, or anti-IgE antibodies, significantly increased the production of MIP-1 α by macrophages from both asthmatics and normal controls, whereas anti-CD23 antibody inhibited IgE- or anti-IgE-induced MIP-1 α production [44]. This may explain, at least in part, the increased expression of MIP-1 α in allergen-induced skin LPR. Additionally, a number of cytokines and mediators are also likely to be involved in the regulation of MIP-1 α expression. For instance, GM-CSF, IL-3, TNF- α , and lipopolysaccharide

(LPS) up-regulate MIP-1 α production by inflammatory cells and structural cells [7,45]. Furthermore, interaction between inflammatory cells and structural cells can also trigger expression MIP-1 α . Co-culturing of monocytes with epithelial [43] or endothelial cells [46] induced expression of MIP-1 α . Blockage of cellular interaction by anti-adhesion molecules or soluble matrix protein could significantly decrease this expression [46].

It is also of interest that kinetic expression of CCR-1 paralleled MIP-1 α expression in the skin LPR (Fig. 1). CCR-1 is constitutively expressed on monocytes, neutrophils, lymphocytes, basophils, eosinophils and dendritic cells [7–12,47]. In a mouse model of lung fibrosis, the treatment of anti-CCR1 antibody significantly reduced the accumulation of inflammatory cells and collagen deposition, resulting in dramatic improvement of injury [48]. Anti-MIP-1 α treatment also had similar results in mice [49]. These observations suggest that CCR-1 and MIP-1 α may be involved in fibrosis. Thus, it is possible that inflammatory cells and their chemoattractants participate in the pathogenesis of inflammation as well as in remodelling through fibrosis. Targeting CCR-1 or the ligand could be a useful therapeutic tool in chronic inflammation.

In summary, our data are compatible with the conclusion that allergen challenge induces enhanced expression of MIP-1 α and CCR-1, which is associated with infiltration of inflammatory cells in skin LPRs.

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