

Original article

Skin reactivity and local cell recruitment in human atopic and nonatopic subjects by CCL2/MCP-1 and CCL3/MIP-1 α

Background: Monocyte chemotactic protein (MCP-1/CCL2), the ligand for CCR2 and CCR5, and macrophage inflammatory protein-1 α (MIP-1 α /CCL3), the ligand for CCR1 and CCR5, are potent chemo-attractants *in vitro* and produce lesions in experimental animals, which resemble immediate and delayed-type hypersensitivity (DTH) reactions. CCL3 induces mononuclear cell and granulocyte infiltration in human atopic and nonatopic skin. Whether CCL2 (MCP-1) has comparable activity in man is uncertain as is the capacity of both the chemokines to elicit immediate- and DTH-like reactions in humans.

Methods: Inflammatory cells were counted by immunohistochemistry in 24 and 48-h skin biopsies from atopics and nonatopics after intradermal injection of CCL2 and CCL3. Immediate (15 min) wheals-and-flares and delayed (24 and 48 h) indurations were also recorded.

Results: Both chemokines induced immediate- (15 min) and delayed (24 and 48 h) reactions, which were associated with significant infiltrations of CD68+ macrophages, CD3+, CD4+ (but not CD8+) T cells, neutrophils, and eosinophils in biopsies from injection sites. CCL2, but not CCL3, also induced infiltration of basophils. Neither chemokine produced significant changes in the numbers of tryptase+ cutaneous mast cells. There were no differences in the pattern of skin reactivity or the numbers of infiltrating leukocytes in response to CCL2 and CCL3 between atopic and nonatopic subjects. In general, maximal infiltration of inflammatory cells was observed at the 24-h, rather than the 48-h, time point.

Conclusions: CCL2 and CCL3 induce both immediate and delayed skin reactions in atopics and nonatopics, and evoke a similar profile of local T cell/macrophage and granulocyte recruitment which, in general, confirm previous *in vitro* findings and *in vivo* experimental animal data.

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Chemokines (chemotactic cytokines) are low molecular weight proteins, which induce directional locomotion and activation of several cell types *in vitro*, promote cellular accumulation when administered to experimental animals *in vivo* and are highly expressed in many human pathological processes, including asthma and atopic allergic diseases (1–4). The CC (β -family) chemokines are predominantly monocyte- and lymphocyte-specific, although they also have granulocyte chemotactic and activating activity (5). Monocyte chemotactic protein-1 (MCP-1/CCL2) and macrophage inflammatory protein-1 α (MIP-1 α /CCL3) are particularly well studied. CCL2 acts predominantly on cells expressing CCR2 and CCR5 whereas CCL3 is a ligand for CCR1 and CCR5 (5). Both chemokines induce lesions, which resemble immediate and delayed-type hypersensitivity (DTH) reactions after cutaneous injection in rodents (6) and promote cell locomotion *in vitro* and accumulation of monocytes and lymphocytes, and to a lesser extent, neutrophils (7, 8) eosinophils (9), and basophils (10) *in vivo*.

Because of the limitations of studies in rodents and other animal species, it is imperative that human *in vivo* responses to bioactive agents, such as chemokines and cytokines are assessed. Also, atopic and nonatopic individuals may differ in their response to chemokines administered *in vivo* as was previously shown for another CC chemokine, RANTES (11). Lee et al. (12) found that recombinant CCL3-induced local neutrophil and monocyte recruitment, as well as a small eosinophil response, after cutaneous injection into human subjects. Surprisingly, no immediate or delayed (up to 24 h) reactions were observed even though CCL3 had previously been shown to produce early and late skin responses in mice (6).

We have had the opportunity to perform a similar human study to Lee et al. (12) using CCL2 and have been able to compare the effect of cutaneous injection of CCL2 and CCL3 in the same subject. Thus, the objectives of the present work were to study the immediate and late macroscopic appearances of the skin as well as the

pattern of local cellular infiltration, following intradermal injection of these two chemokines in human atopic and nonatopic volunteers. We have been able to demonstrate that both CCL2 and CCL3 induced lesions, which resembled immediate- and delayed-type skin hypersensitivity reactions both in atopics and nonatopics. The pattern of local T cell/macrophage and granulocyte recruitment, in general, confirmed previous *in vitro* and experimental animal data.

Materials and methods

Twenty-four subjects with seasonal or perennial rhinitis were recruited by advertisement. Of these, 13 (six male and seven female) were atopic rhinitics without asthma and 11 (six male and five female) were normal, nonatopic, and healthy subjects (Table 1). Seasonal rhinitics were challenged out of season. Individuals with significant disease or abnormal hematology or biochemistry tests had been excluded at screening. The age range was between 19 and 45 years for atopics (mean 27 years) and between 19 and 35 years for nonatopics (mean 26 years). Atopy was defined by positive history for allergic rhinitis and positive skin prick tests, and/or radioallergosorbent tests (RAST) to common aeroallergens. Nonatopic normal human volunteers had no history of atopic disease and negative skin prick tests, and/or RAST tests to a panel of common aeroallergens. All subjects provided voluntary, written informed consent with comprehension of all aspects of the Informed Consent Form. The study was approved by the Royal Brompton NHS Trust Ethics committee. Those with current or recent wheezy

breathlessness were excluded (<1 month) as were those who had used oral or injected corticosteroids, leukotriene receptor antagonists or antihistamines within the previous last 48 h before intradermal injection of chemokines. Only two patients had received antihistamines 48 h prior to the study and both had a positive reaction to histamine.

Synthetic CCL2 and CCL3 ligands were supplied by Millenium Inc. (Cambridge, MA, USA) and produced according to Good Manufacturing Practice (GMP) standards. The sterility and endotoxin levels of these materials were verified prior to injection. Chemokines were stored at -70°C and final aliquoting was performed under aseptic conditions. The ligands and vehicle, saline/diluent control were injected in volumes of 120 μl , and the sites inspected at 15 min, 24 h, and 48 h. As the 24 and 48-h reactions had the characteristics of classical DTH, we adopted the standard method used for measuring in Mantoux tests (a pencil is moved towards the injection site pressing the skin until it stops at the point of induration). We repeated this 10–15 times from various directions all around the reaction until we had the outline. We copied the area of reaction on clear adhesive tape. We outlined the border of the 15 min flare, again using a pen. Volunteers remained under observation for 90–120 min at each visit.

Each volunteer was given a total of six intradermal injections on the extensor aspect of the forearm. Group 1 ($n = 8$) received 1 μg and 10 μg of CCL2, 1 μg and 10 μg of CCL3, normal saline as a negative control, and the relevant positive control. For the atopics, the positive control was 30 biological units of whole allergen and for the nonatopics, 10 tuberculin units of purified protein derivative (PPD) were used. Group 1 had skin biopsies taken at 24 h. Group 2 ($n = 12$) received duplicate injections of 10 μg of CCL2, so that a 24-h biopsy was taken from the first and a 48-h biopsy from the other site), duplicate injections of 10 μg of CCL3 (again to have 24 and 48-h biopsies) as well as normal saline and the positive control. In atopic subjects, allergen was used as positive control and biopsies were taken at 24 h from both the saline and the positive control sites. In nonatopic subjects, PPD was used as positive control and biopsies were taken at 48 h from both the saline and the positive control sites. Group 3 ($n = 4$) received 1 μg of CCL2 (24-h biopsy), 1 μg of CCL2 (48-h biopsy), 1 μg of CCL3 (24-h biopsy), 1 μg of CCL3 (48-h biopsy), and the positive and negative controls as in group 2. Data from groups 1, 2, and 3 were pooled.

Four-millimeter punch biopsies were taken under 2% plain lignocaine anesthesia, immediately fixed in 4% paraformaldehyde, washed in 15% phosphate buffered solution (PBS)-buffered sucrose (Sigma, Poole, UK), embedded in OCT medium and then snap-frozen in isopentane, precooled in liquid nitrogen. Cryostat sections ($<8\text{ }\mu\text{m}$) were cut, mounted onto Superfrost Plus slides, dried overnight at 37°C , and stored with silica gel at -80°C until use (all VWR, Dagenham, UK, unless stated).

The alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was used [as previously described (13)] to enumerate cells immunoreactive to monoclonal antibodies against CD68, CD3, CD4, CD8, neutrophil elastase, eosinophil major basic protein (MBP) and tryptase (all Dako), and basophil BB1 (a generous gift from Dr Andrew Walls, University of Southampton, UK). Briefly, the sections were incubated with the monoclonal antibodies against phenotypic markers for 30 min, then washed in PBS. The slides were incubated for 30 min with the DAKO EnVisionTM System (Dako), which consisted of an alkaline phosphatase (AP)-labeled polymer pre-conjugated to secondary antibodies, thus eliminating the need for separate applications of link antibody and AP-conjugated reagents. Tissue sections were developed with Fast Red (Sigma) as chromogen for signal visualization (Dako). Cells were counter-stained with Harris' Hematoxylin (VWR) and mounted in glycerol. For immunostaining of BB1 + basophils, the slides were

Table 1. Characteristics of the allergic-rhinitic, nonasthmatic subjects studied

Patient no.	Age	Sex	Atopic status (skin test positivity)
1	22	M	Atopic (HDM)
2	22	F	Atopic (G,T,D)
3	41	M	Atopic (G, HDM)
4	27	M	Atopic (HDM, G,C,D)
5	45	M	Atopic (HDM)
6	21	M	Atopic (G)
7	21	F	Atopic (G)
8	19	F	Atopic (G,T,D)
9	24	F	Atopic (G, HDM)
10	19	M	Atopic (HDM)
11	35	F	Atopic (HDM)
12	28	F	Atopic (T)
13	26	F	Atopic (HDM)
14	24	F	Nonatopic
15	21	M	Nonatopic
16	33	M	Nonatopic
17	19	M	Nonatopic
18	19	F	Nonatopic
19	27	M	Nonatopic
20	26	M	Nonatopic
21	25	M	Nonatopic
22	19	F	Nonatopic
23	34	F	Nonatopic
24	31	F	Nonatopic

HDM, house dust mite; G, grass; T, tree; C, cat; D, dog.

pretreated with 0.1% saponin (Sigma)-PBS for 30 min, incubated overnight with BB1, and then processed as above using 0.1% saponin-PBS as the buffer for all stages.

Positive cells stained red after development with Fast Red. Substitution of the primary antibody with an irrelevant isotype-matched antibody of the same species was used as a negative control. One biopsy specimen from each time point was evaluated for each patient.

Each section was approximately 2.5–3.5 mm² in area. The total number of positive cells/mm² was determined by counting whole sections in a blinded fashion at 40 magnification with an eyepiece graticule using an Olympus BH-2 microscope (Olympus, New York, NY, USA).

Statistics

The data were tested for normality using a Shapiro-Wilks statistic and either a parametric paired *t*-test or a nonparametric Wilcoxon signed rank test used depending on the results. A *P*-value, <0.05 was considered significant and *P* < 0.01 highly significant. Data was analyzed using SAS version 8.2 (SAS Institute, Cary, NC, USA) for a PC running Windows XP.

Results

CCL2 and CCL3 produce immediate and delayed type skin reactions

The macroscopic responses of the skin to intradermal injection of 1 and 10 μ g of CCL2, and CCL3 are shown in Fig. 1. Both chemokines produced an immediate wheal-and-flare reaction in most patients, when given at the 10 μ g dose. Macroscopically, the reaction was similar to that observed after intradermal injection of whole allergen to atopic subjects; however, they were less pronounced than the ones induced by allergen. The size of the reactions were significantly larger than the diluent control (*P* = 0.001 for CCL3 and *P* < 0.001 for CCL2). The lower, 1 μ g dose of CCL3, but not CCL2, was also increased significantly (*P* = 0.005). Some whealing was observed with the diluent control, as expected, as it was necessary to administer the chemokines in a relatively large volume (120 μ l). However, the reaction at the diluent site waned over time whereas at the chemokine-injected sites it persisted, and in some instances increased in time to 24 h, and then decreased slightly at the 48-h time point. The difference between the diluent control and either CCL2 or CCL3 were most marked at 24 h at which time CCL2, and CCL3 produced red, raised indurated lesions, which were typical of DTH reactions (i.e. as in a tuberculin reaction). The reactions were clinically small but, at 24 h, CCL2, and CCL3-injected sites had statistically significantly larger lesions which, compared with controls, gave *P*-values of <0.001 for the 1 μ g and *P* < 0.01 and *P* < 0.001, respectively, for the 10 μ g dose. At 48 h, the mean diameters of the lesions were still indurated and although not marked, they were very

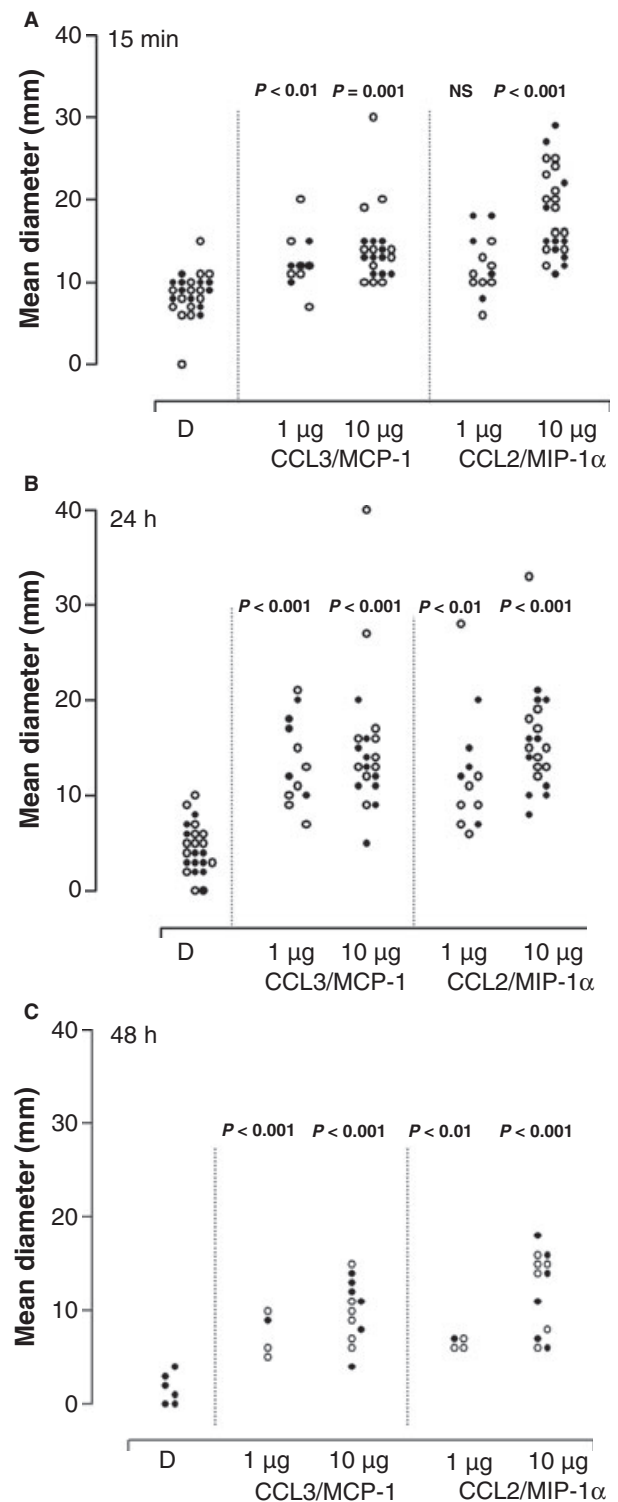


Figure 1. Immediate and delayed skin reactions elicited in human volunteers following the intradermal injection of various concentrations of MIP-1 α /CCL3 and MCP-1/CCL2 in atopics (closed circles) and nonatopics (open circles). Immediate (15-min reactions) are shown in Fig. 1A and delayed (24 and 28-h reactions) are shown in Fig. 1B,C respectively. D, diluent.

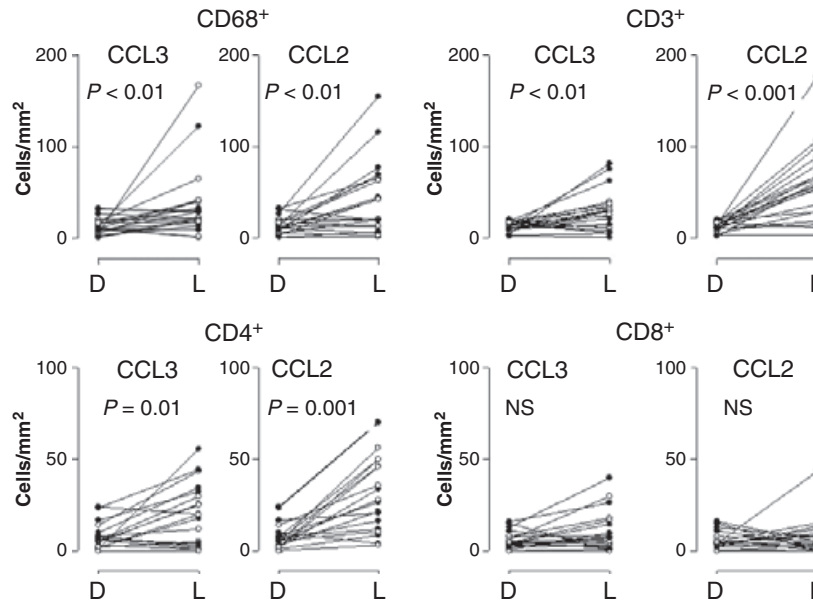


Figure 2. The effect of intradermal MIP-1 α /CCL3 and MCP-1/CCL2 on the infiltration of CD68 $^{+}$ macrophages, CD3 $^{+}$, CD4 $^{+}$, and CD8 $^{+}$ T cells 24 h after administration. D, diluent; L, ligand. 10 μ g of each ligand was injected. Atopic subjects are shown in the open circles and nonatopics in the closed circles.

significantly larger than the control sites. Thus CCL2 and CCL3 gave P values of <0.001 and <0.01 , respectively, for the 1 μ g and $P < 0.0001$ for the 10 μ g dose.

CCL2 and CCL3 recruit macrophages, T cells, and granulocytes

The numbers of CD68 $^{+}$ macrophages and CD3 $^{+}$, CD4 $^{+}$, and CD8 $^{+}$ T cells migrating to the sites of injection of 10 μ g MCP-1 or MIP-1 α for all subjects at 24 h is shown in Fig. 2. Macrophage infiltration was more marked with CCL2 injection than CCL3. Thus the P -values (diluent vs ligand) were $P = 0.006$ and $P = 0.002$ for CCL2 and CCL3, respectively. In some subjects, especially with CCL2, there was little increase or even a decrease in the macrophage response. Recruitment of CD3 $^{+}$ cells was similar in that CCL2 produced a slightly more vigorous response than CCL3, although both were highly significant (diluent vs ligand, $P < 0.01$ and $P = 0.0001$, respectively). Both chemokines provoked marked recruitment of CD4 $^{+}$ cells but again the effect of CCL2 was slightly more marked ($P = 0.001$) than CCL3 ($P = 0.01$). Neither chemokine evoked a significant CD8 response.

The response of eosinophils, neutrophils, mast cells, and basophils to the ligands is shown in Fig. 3. CCL2 and CCL3 produced marked eosinophil and neutrophil accumulation. In both instances, CCL2 gave a more significant response than CCL3 ($P < 0.001$ vs $P < 0.01$, respectively, for eosinophils and $P = 0.001$ and $P < 0.01$ for neutrophils). Neither ligand produced a significant change in numbers of tryptase $^{+}$ cells, whereas

CCL2, but not CCL3, induced basophil recruitment, which was just significant ($P = 0.05$).

The time course (24 and 48 h) and dose-responses (1 and 10 μ g) data are shown in Fig. 4. For CD3 $^{+}$ (Fig. 4A) and CD4 $^{+}$ (Fig. 4B) cells the response to CCL2 was generally greater than that to CCL3, and gave a clear dose response. The apparent greater potency of CCL2 over CCL3 at 10 μ g in terms of CD3 $^{+}$ cell recruitment between 24 and 48 h time points was not significantly different. Unlike CCL2, the CD3 $^{+}$ and CD4 $^{+}$ responses to CCL3 were similar with either doses of the ligand. No significant CD8 responses were observed with either dose at 24 or 48 h. CCL2 and CCL3 attracted eosinophils (Fig. 5A), neutrophils (Fig. 5B), and macrophages (Fig. 5C) in a clear dose-response fashion but there was no appreciable difference between 24 and 48-h time points for either ligand. The response to CCL2 was similar to CCL3 in terms of the magnitude of the response of eosinophils, neutrophils, and macrophages. In general, CCL2 and CCL3 gave comparable data at the two time points although the response to highest dose of the two ligands was higher at 48 h than 24 h.

Examples of photomicrographs of the CD68, CD3, and CD4 responses to CCL2 and CCL3 are shown in Fig 6A,B. There was very little staining with diluent but vigorous cell responses, particularly in the superficial layers of the dermis, were observed in response to CCL2 (CD68 and CD4 – Fig 6A) and CCL3 (CD68 and CD3 – Fig 6b) at both the 24 and 48-h time points after injection.

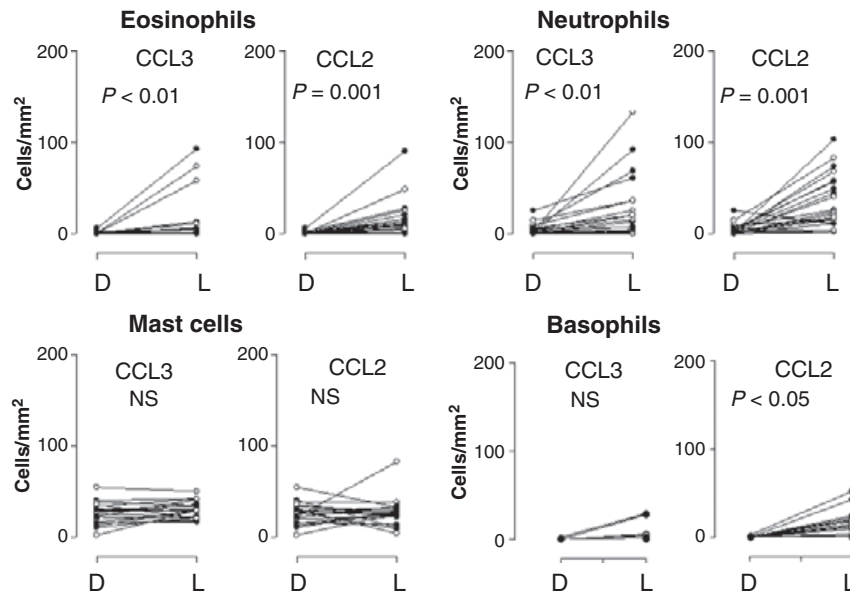


Figure 3. The effect of intradermal MIP-1 α /CCL3 and MCP-1/CCL2 on the infiltration of MBP+ eosinophils, neutrophil + elastase, tryptase + mast cells and BB1 + basophils 24 h after administration. D, diluent; L, ligand. 10 μ g of each ligand was injected. Atopic subjects are shown in the open circles and nonatopics in the closed circles.

Discussion

As far as we are aware, this is the first report on the administration of CCL2 to human subjects. It is also unique in that it directly compares the biological effect of CCL2 with CCL3 in the same subject. Our first novel observation was that CCL2 and CCL3 both elicited a small, but statistically significant, immediate wheal-and-flare reaction suggesting that these ligands activate mast cells in human skin either directly or indirectly. The finding is in agreement with several murine studies. For example, CCL2 and CCL3 both induced histamine release and mast cell degranulation in the skin of mice (6). Furthermore, CCL3 released histamine from isolated mouse peritoneal mast cells (14), and CCL2 provoked activation of rat mast cells (15). Also of relevance is the finding of Miyazaki et al. (16) who reported, in a mouse model, that CCL3 constitutes an important second signal for IgE-mediated mast cell degranulation. In contrast, several *in vitro* studies using isolated human mast cells (17), and *ex vivo* experiments with microdialysis of human skin (18) have shown that CCL2 and CCL3 were active on basophils but not on mast cells. This suggests that isolated human mast cells may lose reactivity to these chemokines possibly as a result of treatment with digestive enzymes. Lee et al. (12) were unable to show an immediate effect of CCL3 (up to 1000 pmols) in human skin. However in the present study, we used somewhat higher doses (up to 1300 pmols) which may explain the difference in findings. We were unable to show a decrease in the number of tryptase + mast cells after injection of CCL2 or CCL3, i.e. we have no direct evidence that these ligands produce mast cell degranulation. However, our previous human studies

(13) have shown that mast cell degranulation (as assessed by the numbers of tryptase + cells) is usually minimal even after cutaneous allergen challenge suggesting that more sensitive techniques may be necessary to demonstrate mast cell activation in tissue biopsies.

Several investigators have shown that CCL3 (19, 20) and CCL2 (21, 22) induce lesions resembling DTH reactions in murine skin. Our human study shows a substantial 24 and 48-h skin response with both ligands. For practical reasons, we were unable to measure the 6- to 9-h time point (optimal for late-phase reactions). However, the late-phase reaction persists at 24 h and this may represent either the waning of a late-phase reaction or establishment of a lesion resembling classical DTH. The clear indurated swelling we observed at 48 h is compatible with the hypothesis that mononuclear cell infiltration induced by these two ligands confers delayed-type reactions. Our time course studies show comparable cell infiltration by either CCL2 or CCL3 for macrophages, CD3+ and CD4+ cells at 24 and 48 h. This is in keeping with the typical 24 and 48-h macroscopic delayed-type reaction and is in agreement with previous animal studies, which show either CCL2- or CCL3-induced DTH with local mononuclear cell infiltration (23–27).

Our demonstration of a vigorous macrophage response to CCL2 and CCL3 was also in keeping with previous studies. Thus, CCL2 was shown to be a potent chemo-attractant for monocytes (28) and induced macrophage accumulation in human skin (12). We have confirmed this finding but show that CCL2 has marginally more potent activity than CCL3 in human skin. Monocytes are well known to express CCR2 (present on monocytes and

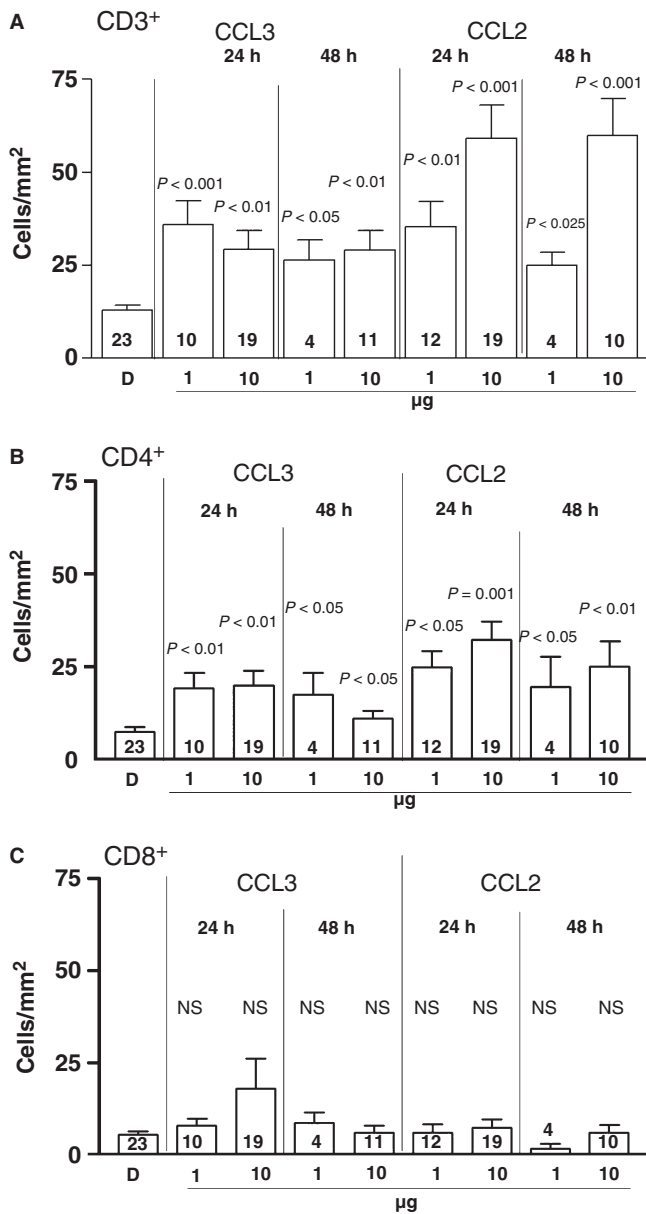


Figure 4. The effect of intradermal MIP-1 α /CCL3 and MCP-1/CCL2 in human volunteers at doses of 1 and 10 μ g at 24 and 48 h after administration. Data are shown for (A) CD3+ T cells, (B) CD4+ T cells, and (C) CD8+ T cells. The values in the boxes represent the number of subjects studied whereas the bars represent the mean $\pm$ SD.

macrophages), and it was previously shown that CCL2-deficient mice could not mount a local tissue monocyte/macrophage response (24). We were also able to establish that the two chemokines elicited a vigorous infiltration of CD3+ and CD4+, but not CD8+ cells. This is in keeping with the findings of Kunstfeld et al. (25) who showed that CCL2 and CCL3 recruited T cells into the skin *in vivo* in a system using severe-combined immunodeficient (SCID) mice grafted with human skin.

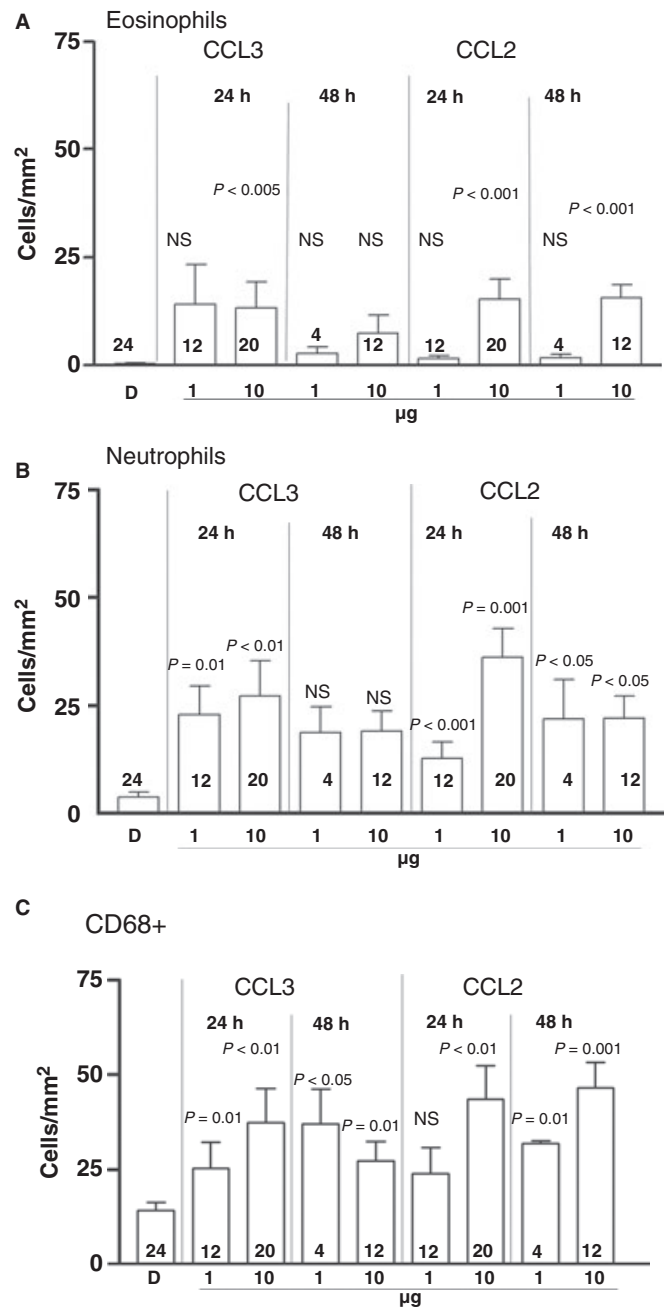


Figure 5. The effect of intradermal MIP-1 α /CCL3 and MCP-1/CCL2 in human volunteers at doses of 1 and 10 μ g at 24 and 48 h after administration. Data are shown for (A) MBP+ eosinophils, (B) neutrophil+ elastase, and (C) CD68+ macrophages. The values in the boxes represent the number of subjects studied whereas the bars represent the mean $\pm$ SD.

CCL2 is also chemotactic for human T cells *in vitro* (26) and expresses CCR2, at least on polarized cells (27).

Neutrophils and eosinophils are generally regarded as being less responsive to the chemotactic effects of CCL2 and CCL3 *in vitro* (5). It was previously shown that CCL3 had some chemotactic activity for normal human

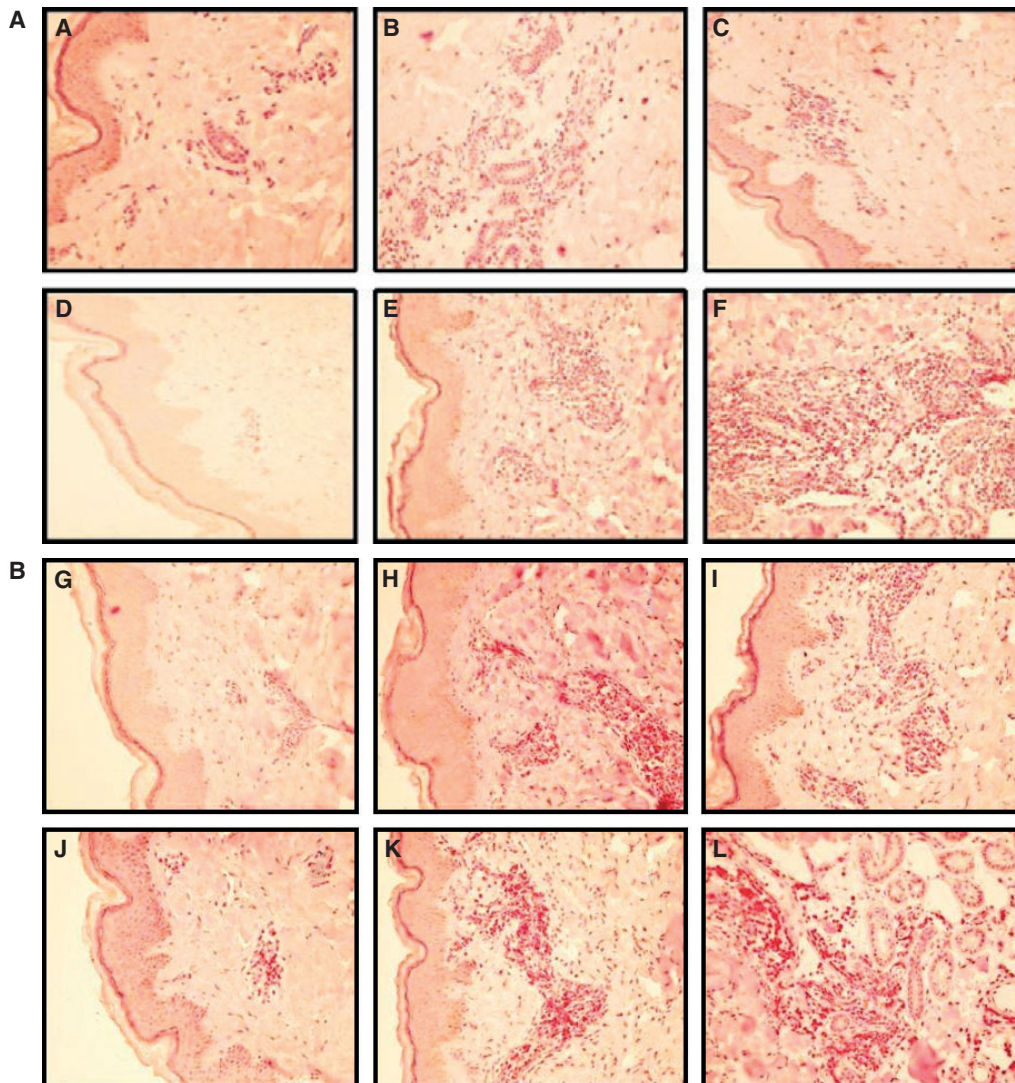


Figure 6. Microphotographs of the cellular responses to intradermal injection of CCL2 and CCL3. Fig. 6A [A, CD68 (diluent site); B, CD68 (CCL2 at 24 h); C, CD68 (CCL2 at 48 h); D, CD4 (diluent); E, CD4 (CCL2 at 24 h); F, CD4 (CCL2 at 48 h)]. Fig. 6B [G, CD68 (diluent); H, CD68 (CCL3 at 24 h); I, CD68 (CCL3 at 48 h); J, CD3 (diluent); K, CD3 (CCL3 at 24 h); L, CD3 (CCL3 at 48 h)] (magnification $\times 40$).

eosinophils (29). More recently, it was shown that eosinophils from a subgroup of individuals responded equipotently to eotaxin and CCL3 in assays of eosinophil shape change (30). Thus, eosinophils from these highly CCL3-responsive donors expressed CCR1 and may represent an important subgroup with regard to future anti-eosinophil therapies. Only three of our subjects gave a vigorous eosinophil response (Fig. 3). These may have represented highly CCL3-responsive donors but we did not have the opportunity to study CCR1 expression on peripheral blood from these subjects. The CCL2 response of eosinophils was more uniform than that found with CCL3. Normal human blood eosinophils were previously shown to express functional cell surface CCR2 (31). Our present *in vivo* data supports this finding.

We were unable to show any difference between the number of tryptase+ mast cells before and after injection of either dose of CCL2 or CCL3. Histamine release may occur from mast cells without there being an overall difference in the number of tryptase+ cells. However it does indicate that, although immediate-type reactions were observed, this was not associated with substantial mast cell degranulation. On the other hand, CCL2 but not CCL3-induced basophil infiltration. Basophils were previously shown to express CCR2 in mice but not to express CCR1 or CCR5 (32).

Although CCL2 and CCL3 are associated with many disease processes, their role in atopy, allergy, and asthma has received relatively little attention. Previous studies have shown that the concentrations of both chemokines

were elevated in bronchoalveolar lavage fluid in asthmatics (5, 15), and also that there was an association of polymorphism of the CCL2 gene with asthma in children (33). The situation is more complex in that whereas MCP-1 has been shown to stimulate Th2 polarization, CCR2 activation stimulates Th1 polarization (34). However, Ying et al. (4) demonstrated expression of CCL3 mRNA in allergen-induced late-phase, skin, and bronchial reactions. In the present study, we show that CCL3 and CCL2 promote the migration in human skin *in vivo*, of cells relevant to allergic inflammation and the asthma process, i.e. T cells, macrophages, eosinophils, neutrophils, and (for CCL2) basophils. However, it is clear that CCL2 and CCL3 also play a major role in Th1-associated DTH reactions so pointing to the considerable pleomorphic properties of these two CC chemokines (9).

In summary, we show that both CCL2 and CCL3 induce lesions resembling immediate and delayed-type skin hypersensitivity reactions following injection in the skins of both atopics and nonatopics. They also produce a pattern of local T cell/macrophage and granulocyte recruitment which, in general, confirm previous *in vitro* and experimental animal data. It is likely that targeting CCL2 and/or CCL3 will have long range biological effects.

Acknowledgments

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