

Monocyte chemoattractant peptide-1 expression during cutaneous allergic reactions in mice is mast cell dependent and largely mediates the monocyte recruitment response

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Background: Macrophages and infiltrating monocytes comprise the largest population of cells within the airways of patients with allergic asthma. Both become activated and thus contribute to the pathologic features of allergic reactions, but the mechanism by which they are recruited has not been well documented.

Objective: Our purpose was to assess the role of the CC chemokine monocyte chemotactic peptide-1 (MCP-1) in monocyte recruitment during allergic reactions in mice.

Method: We used immunohistochemistry and Northern blotting to assess MCP-1 expression, selective mast cell reconstitution of mast cell-deficient *W/W^v* mice to demonstrate the mast cell dependence of MCP-1 expression and monocyte recruitment and neutralizing anti-MCP-1 antibodies to block monocyte recruitment during cutaneous allergic reactions.

Results: MCP-1 was expressed largely by resident dermal cells within the allergic lesions at 4 hours after challenge, followed within several hours by an influx of monocytes; at 10 hours after challenge monocytes comprised a substantial proportion of the infiltrating cells at the nidus of the response. Mast cell-reconstituted, but not mast cell-deficient *W/W^v* mice expressed MCP-1 transcripts and developed monocyte infiltrates after allergen challenge. Finally, anti-MCP-1 antibody treatments reduced by ~63% the influx of monocytes into the reaction sites.

Conclusions: These data clearly demonstrate the mast cell dependence of the MCP-1 expression and the monocyte influx and establish a substantial, but not exclusive, causal relationship between these 2 events. (*J Allergy Clin Immunol* 2000;106:110-6.)

Key words: Monocyte chemotactic peptide-1, chemokine, allergy, mast cells, monocytes/macrophages

Macrophages and monocytes comprise the largest cell population in the airways of patients with allergic asthma.¹ The alveolar macrophages of asthmatics are constitutively

Abbreviations used

ATTC:	American Type Culture Collection
BMMC:	Bone marrow-derived mast cells
DNP ₃₀₋₄₀ HSA:	Dinitrophenyl-substituted human serum albumin
FcεRI:	High-affinity IgE receptor
MCP-1:	Monocyte chemotactic peptide-1
MIP-1α:	Macrophage inflammatory protein-1α
mRNA:	Messenger RNA
NRS:	Normal rabbit serum
PCA:	Passive cutaneous anaphylaxis
TGF-β:	Transforming growth factor-β
W/W ^v :	Genetically mast cell-deficient WBB6F1-W/W ^v genotype mice

activated,² with basal production of monokines by these cells¹ and monocytes³ being ~4-fold higher than in nonasthmatic subjects. Cytokines from both populations can contribute importantly to the pathogenesis of allergic responses,⁴ exacerbating eosinophil-related pathologic features, increasing IgE antibody production, and reinforcing the T helper type 2 nature of allergen-specific immune responses.⁵⁻⁹ Indeed, high proportions of airway monocytes in patients with extrinsic allergic alveolitis are associated with significantly reduced lung function.¹⁰

The mechanisms by which monocytes are recruited into allergic responses have not been clearly documented. IgE receptor (FcεR)-dependent triggering can activate airway macrophages from asthmatic patients *in vitro*,¹¹ but this would not explain the recruitment of monocytes from the periphery.^{1,10} We have shown that during allergic responses TNF-α and transforming growth factor-β released from FcεRI-stimulated mast cells activate fibroblasts^{12,13} that in turn secrete high levels of monocyte chemoattractant peptide-1 (MCP-1) *in vitro*.¹⁴ Purified MCP-1 chemo-attracts monocytes into the skin of rodents,¹⁵ and MCP-1 is expressed in the affected tissues of patients with allergic rhinitis¹⁶ and asthma.¹⁷ Furthermore, its expression correlates with pulmonary monocyte infiltration in mice with allergic lung disease,¹⁸ and its neutralization reduces bronchial hyperresponsiveness and the accumulation of macrophage-derived inflammatory mediators.¹⁹ However, the precise relationship between this MCP-1 expression and mast cell activation has not been defined.

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We document here the mast cell dependence of MCP-1 expression and monocyte responses during cutaneous allergic responses in mice. We also clearly demonstrate by 2 independent methods that this MCP-1 expression is important to the full development of the monocyte response and provide evidence that the primary sources of this cytokine are resident cells of the skin, likely fibroblasts and endothelial cells.

MATERIAL AND METHODS

Mice

BALB/c mice were from our institutional Animal Resources Centre, and mast cell-deficient WBB6F1-*W/W^v* (*W/W^v*) mice and congenic normal (+/+) littermates from the Jackson Laboratories (Bar Harbor, Me). All animal treatments were in accord with the guidelines of the Canadian Council on Animal Care.

Induction of passive cutaneous anaphylaxis responses and neutralization of MCP-1 activity

Passive cutaneous anaphylaxis (PCA) reactions were induced with use of monoclonal IgE anti-dinitrophenyl²⁰ and dinitrophenyl-substituted human serum albumin (DNP₃₀₋₄₀HSA) (Sigma Chemical, St Louis, Mo), as noted.^{12,21} For neutralization of cytokine activity within the PCA reactions, at the time of antigen challenge we injected the ears with 20 μ L of a 1:100 dilution of either normal rabbit serum (NRS) or rabbit anti-MCP-1 antisera (both kind gifts of Dr R. Strieter).^{12,22} We titrated the anti-MCP-1 antibody (1:20 to 1:1000) for maximal inhibition of the late-phase response in pilot experiments; at the dilution used neither antibody by itself induced detectable responses. The specificity of the MCP-1 antibody for its target has been documented.²³ PCA reactivity was confirmed by extravasation of Evans blue dye.^{12,21} Ear biopsy specimens were fixed¹² for immunohistochemistry or processed for Northern blotting.²⁴

Mast cell reconstitution of genetically mast cell-deficient WBB6F1-*W/W^v* mice

One ear of each *W/W^v* mouse was locally reconstituted with mast cells by the intradermal injection of 5×10^5 bone marrow-derived mast cells (BMMC) from WBB6F1-+/+ mice,^{12,22} generated and maintained as noted.²⁵ The contralateral ear of each mouse was injected with 20 μ L of carrier medium alone. The mice were used 10 weeks later, a period sufficient to permit maturation of the transplanted cells into phenotypically mature cutaneous mast cells.²⁶

Northern blotting of RNA

The messenger RNA (mRNA) was purified from tissues, blotted, and probed as reported,^{21,24} with use of phosphorus 32-labeled murine MCP-1 (American Type Culture Collection No. 37590, ATCC, Rockville, Md) and human γ -actin complementary DNA. The relative MCP-1 mRNA levels in the blots were assessed by densitometry (Alpha Imager 2000, Alpha Innotech, San Leandro, Calif). The data are presented as MCP-1 signal integrated density values.

Immunohistochemical detection of monocytes or MCP-1

Antibodies included monoclonal rat IgG2b anti-mouse monocyte/macrophage antibody F4/80 (No. HB198, ATCC), normal rat IgG (Sigma Chemical), rabbit anti-mouse MCP-1, macrophage inflammatory protein-1 α (MIP-1 α) (Dr R. Strieter) or TNF- α ²⁵ or NRS. Six-micrometer sections of PCA reactions or cell monolayers (fibroblasts or endothelial cells) were blocked with 5% normal goat

serum and then incubated overnight with F4/80 hybridoma supernatants (1:5 dilution) or the normal rat IgG, specific antisera, or NRS (each 1:100 dilution). They were washed with PBS/0.05% Tween 20 (Sigma Chemical), incubated with biotinylated secondary antibodies (Serotec Canada, Toronto, Ontario, Canada), washed again, and then treated first with streptavidin-alkaline phosphatase (Vector Laboratories, Burlingame, Calif) and then with Fast Red RR (Sigma Chemical) and counterstained with hematoxylin. Control treatments (omission of the primary or secondary antibodies) all yielded no specific staining. The staining was specific for infiltrating mononuclear cells (although there was a very low level staining of histiocytes in the deeper dermis), which were enumerated by direct counting at $\times 400$ magnification.

Mast cell-dependent induction of MCP-1 expression in fibroblasts or endothelial cells

We used immunostaining to assess the expression of MCP-1 within mast cell-activated mouse embryonic fibroblasts¹³ and endothelial SVEC4-10 cells.²⁷ The cell monolayers, in DMEM/1% FCS (GIBCO/BRL, Mississauga, Ontario, Canada), were incubated for ≥ 4 hours with 2-hour supernatants (10% final concentration) from immunologically activated or quiescent IL-3-dependent BMMC as noted in detail¹²⁻¹⁴ and then stained for MCP-1 as above. We have documented previously that 2 hours is the time of maximal release from BMMC of fibroblast-reactive cytokines.^{12,13}

Statistical analyses

Data were analyzed with the software program Statview 4.1 (Abacus Concepts, Berkeley, Calif) with use of ANOVA and post-hoc Fisher protected least significant difference testing. The data are presented as the means $\pm$ SEM. The immunolocalization experiments were repeated 3 times, the studies in the *W/W^v* mice twice, and the anti-MCP-1 neutralization studies 5 times. In each case the data depicted are from one representative experiment.

RESULTS

Monocytes are an important component of the cellular infiltrates into cutaneous allergic reaction sites in BALB/c mice

We assessed the numbers of monocytes (F4/80 antibody-positive cells) infiltrating PCA reaction sites at 4, 10, and 20 hours after allergen challenge (Fig 1). Few monocytes but many neutrophils were seen in the reaction sites at 4 hours after challenge but, by 10 hours, 24.0% $\pm$ 1.7% of the cells in the nidus of the reactions comprised monocytes. In pilot experiments we also observed substantial monocyte influx at 7 hours after challenge as well (data not shown). Most of the F4/80+ cells were confined to the lower dermis (Fig 1, A). Interestingly, at the periphery of the 10-hour response large numbers of neutrophils were evident but monocytes were scarce. At 20 hours the number of monocytes increased only slightly overall, but the bulk of the newly arrived cells were found in the periphery of the reaction sites.

MCP-1 is expressed at high levels during cutaneous allergic reactions in mice

At varying times after allergen challenge we localized MCP-1 and, as controls, MIP-1 α and TNF- α expression within the PCA reactions. No MCP-1 or MIP-1 α was detected within healthy (ie, 0 hours or vehicle-treated) tis-

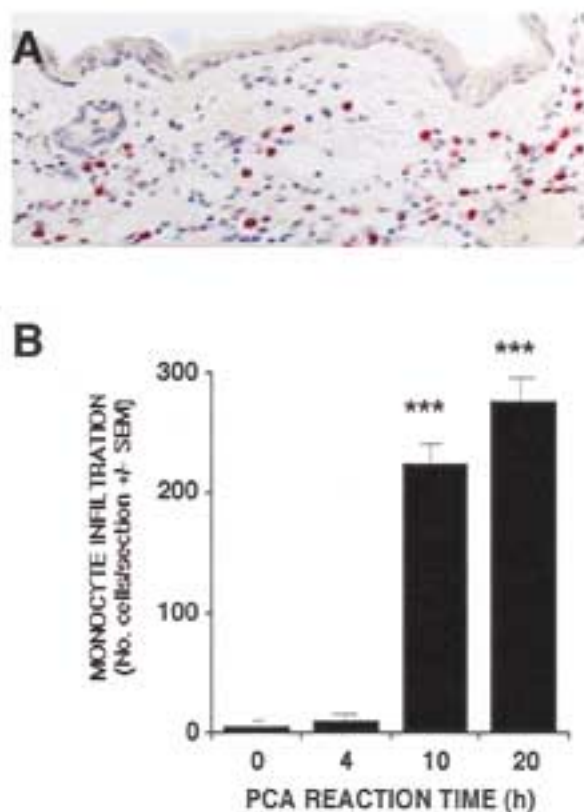


FIG 1. Monocytes are a prominent component of the allergic late-phase response in mice. We induced PCA reactions in the ears of BALB/c mice and used monoclonal F4/80 (or control) antibodies to identify (**A**) and quantify (**B**) the monocytes infiltrating the lesions. No discernible tissue reactions were observed at any time in the ears that had been sensitized with medium alone. At 10 hours after challenge, monocytes (*red-stained cells*) comprised a substantial proportion of the cells infiltrating the PCA reactions. Tissues stained with the normal rat IgG antibodies were uniformly negative. The results are expressed as the mean $\pm$ SEM. Three asterisks, $P \leq .001$ ($n = 4$ mice per time point) versus medium-sensitized tissues.

sues, but substantial numbers of highly granulated cells within the dermis were strongly positive for TNF- α . At 4 hours after challenge, well before monocytes began to accumulate in significant numbers, small numbers of basal epidermal cells and substantial numbers of dermal cells with no observable cytoplasmic granules were strongly positive for MCP-1 (Fig 2). That the anti-MCP-1 antibody staining was cytokine specific was reconfirmed²³ by the demonstration that MIP-1 α and TNF- α were expressed by distinctly different populations of cells (Fig 2, *C* and *D*). MIP-1 α , another CC chemokine, was confined to a very few cells in the epidermis, whereas TNF- α expression was confined still to the now largely degranulated mast cells. At 10 hours MCP-1 was no longer detectable within the resident cells, but weak signals could be detected within the infiltrating inflammatory cells.

MCP-1 expression in these lesions occurred in the upper dermis before any substantial monocyte influx and was not associated with the heavily granulated mast cells. Alternate resident sources of MCP-1 in these lesions could include endothelial cells and fibroblasts, both of which are readily activated by mast cell-derived transforming growth factor- β (TGF- β) or TNF- α .^{12-14,28}

Although none of the MCP-1-positive cells in the dermis of our mice were discernibly associated with the vasculature, we could not by light microscopy unequivocally rule out endothelial sources for this cytokine and did not have available suitable cell-specific antibodies for double immunolocalization. Thus we next used an *in vitro* approach to compare the effects on MCP-1 expression of adding 2-hour supernatants from Fc ϵ RI-stimulated or quiescent mast cells to monolayers of endothelial cells or dermal fibroblasts. When unstimulated or after the addition of supernatants from unstimulated mast cells, both populations stained at only background levels (*insets*, Fig 2, *E* and *F*). However, secretion products from activated mast cells induced both populations to express MCP-1 at ~ 4 hours or more after challenge, the fibroblasts strongly and the endothelial cells more moderately (Fig 2, *E* and *F*). These *in vitro* data suggest that the cells that expressed MCP-1 in our PCA reactions could be fibroblasts and endothelial cells. We know that mast cell TNF- α and TGF- β strongly activate fibroblasts during cutaneous allergic responses¹² and now also that these mast cell cytokines similarly up-regulate MCP-1 and proliferative responses by mouse dermal fibroblasts *in vitro*.^{13,14}

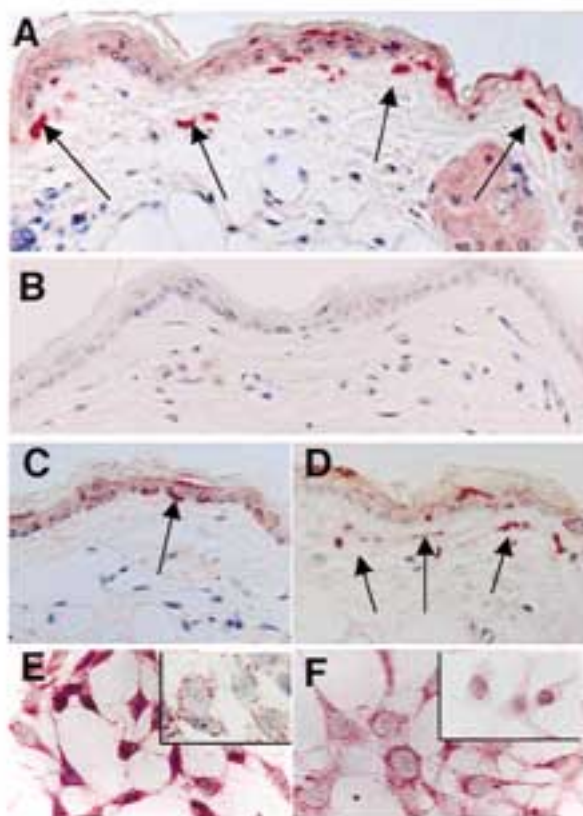


FIG 2. MCP-1 expression by dermal and epidermal cells within allergic reactions. **A to D**, PCA reactions were elicited as in Fig 1 and MCP-1 expression was assessed by immunohistochemistry. By 4 hours after challenge, well before monocytes began to appear in the tissues, cells within the dermis (arrows) and epidermis of the allergic lesions were strongly positive for MCP-1 (**A**). Tissues incubated with NRS as the first antibody were uniformly negative (**B**). Unlike MCP-1, MIP-1 α was detected within a very few cells that were confined to the epidermis in the 4-hour biopsy specimens (arrow, **C**), whereas TNF- α remained localized to the now largely degranulated dermal cells (arrows, **D**). We could not determine unequivocally the identities of the MCP-1-positive dermal cells in our tissues, so we also examined the abilities of 2-hour activated or quiescent mast cell secretion products to induce MCP-1 expression in vitro in mouse dermal fibroblasts (**E**) or endothelial cells (**F**). After stimulation with supernatants from activated (**E**, **F**) but not quiescent (insets, **E**, **F**) mast cells, both populations expressed MCP-1.

MCP-1 expression and the monocyte response during cutaneous allergic responses in mice are mast cell dependent

To critically address the issue of whether this monocyte recruitment and MCP-1 induction were mast cell dependent, as opposed to a nonspecific consequence of the inflammatory responses, we used BALB/c and genetically mast cell-deficient W/W^v and locally mast cell-reconstituted W/W^v mice. As above, BALB/c mice had marked monocyte responses at 10 hours after allergen challenge, and the mast cell-deficient tissues of the W/W^v mice did not, but local mast cell reconstitution of the W/W^v mice also locally reconstituted the monocyte response to allergen challenge ($P > .05$ vs 10-hour BALB/c tissues, Fig 3, A). High steady-state levels of MCP-1 mRNA were observed in the IgE-sensitized, but not medium-sensitized, tissues of BALB/c mice at 4 hours after allergen challenge (Fig 3, B). Only very low levels of MCP-1 transcripts were found in allergen-challenged mast cell-deficient tissues of the W/W^v mice, but

local mast cell reconstitution also locally reconstituted the 4-hour allergen-dependent MCP-1 response (Fig 3, B). As expected, the timing of the MCP-1 protein and mRNA expression was consistent. These results demonstrate unequivocally that mast cells are essential for MCP-1 expression within, and the monocyte component of, cutaneous allergic responses in mice.

MCP-1 expression is important to the recruitment of monocytes into cutaneous allergic responses in mice

It has been shown recently that anti-MCP-1 antibody treatments of mice with allergic lung disease down-regulate the appearance of monocyte/macrophage-derived inflammatory mediators in the airways,¹⁹ but because the alveolar macrophages are also sources of these molecules these studies did not directly address the issue of monocyte recruitment. We therefore assessed the effects on monocyte recruitment of treating BALB/c mice with anti-MCP-1 or control antibodies at the time of allergen

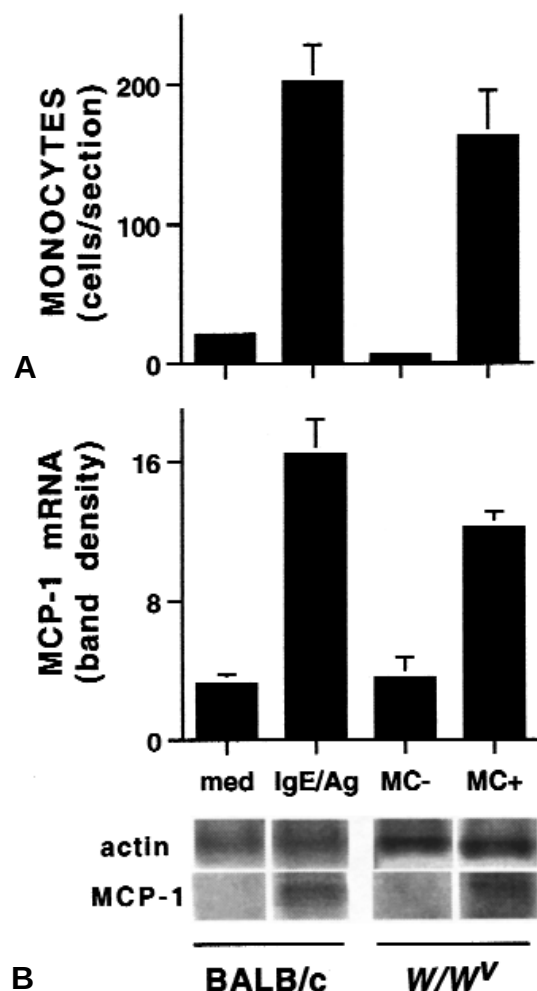


FIG 3. Monocyte infiltration into and MCP-1 expression within cutaneous allergic responses in mice are dependent on FcεRI signaling and mast cells. We sensitized BALB/c mice with either medium alone or IgE and then induced PCA reactions, as in Fig 1. In addition, we injected IgE antibody into both ears of genetically mast cell-deficient *W/W^v* mice that had been locally reconstituted with mast cells (MC+) in one ear; the contralateral ear of each mouse remained mast cell deficient (MC-). **A**, The monocyte responses in the IgE- (IgE/Ag) but not medium- (med) sensitized tissues of the BALB/c mice at 10 hours after allergen challenge were essentially as in Fig 1. Few if any monocytes were detected in the mast cell-deficient tissues of the allergen-challenged *W/W^v* mice, whereas the mast cell-reconstituted tissues of these mice contained large numbers of monocytes. There were no significant differences in the monocyte responses in BALB/c mice and the mast cell-reconstituted tissues of the *W/W^v* mice ($P > .05$). **B**, Graphic and autoradiographic Northern blotting results with mRNA obtained from ears of mice treated as above. At 4 hours after allergen challenge of the BALB/c mice, essentially no MCP-1 mRNA was detectable in the tissues sensitized with medium alone, whereas high steady-state levels of MCP-1 mRNA were found in the tissues challenged via the FcεRI. Very little MCP-1 mRNA was detectable in the mast cell-deficient tissues of the *W/W^v* mice, but local mast cell reconstitution also locally reconstituted the expression of MCP-1 mRNA. The data are presented as the MCP-1 signal integrated density values ($\pm$ SEM). The data clearly indicate that the monocyte infiltration response and MCP-1 expression are both critically dependent on mast cells and FcεRI signaling within these cells ($n = 4$ mice/treatment).

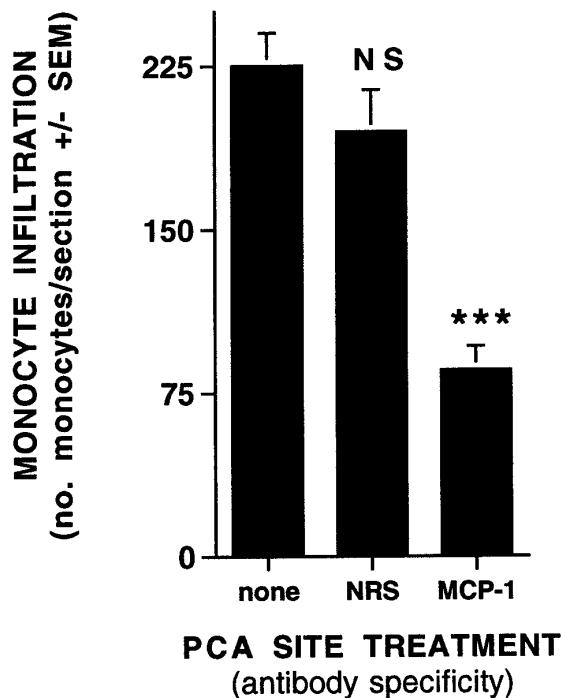


FIG 4. Anti-MCP-1 antibodies down-regulate monocyte influx into the skin of mice undergoing passive cutaneous anaphylaxis responses. PCA reactions were induced and the monocytes enumerated at 10 hours as in Fig 1, except that immediately after allergen challenge we injected either NRS or rabbit anti-MCP-1 antisera into the ears of each animal. The NRS had no statistically significant effect on monocyte infiltration, whereas the anti-MCP-1 treatments reduced the numbers of monocytes infiltrating the reaction sites by ~63%. The results are expressed as the numbers of monocytes per reaction site tissue section ($\pm$ SEM). Three asterisks, $P \leq .001$ versus the NRS-treated ear ($n = 4$ mice/treatment).

challenge. NRS treatments had no significant effect on the monocyte response (Fig 4), but anti-MCP-1 antibodies reduced monocyte infiltration by ~63% relative to NRS ($F_{[6, 25]} = 92.95$, $P \leq .0001$, Fig 4). The immediate (ie, 30 minutes) hypersensitivity responses, as determined by Evans blue dye extravasation, and the neutrophilic component of the late-phase responses were unaffected by the anti-MCP-1 antibody treatments. Thus these data confirm that the MCP-1 expressed in the PCA reaction site is indeed important to the full expression of the monocyte response.

DISCUSSION

We demonstrated unequivocally that mast cells and, more specifically, FcεRI triggering are critical to monocyte recruitment into and MCP-1 expression within cutaneous allergic lesions in mice. We previously reported such MCP-1 mRNA data in preliminary form.²⁹ Monocytes similarly infiltrate the lung tissues of patients with allergic asthma^{1,10} and mice with allergic lung disease¹⁸ after allergen challenge and become markedly activated.

ed,^{1-3,30} so that monocyte/macrophage-derived inflammatory mediators are readily detectable in the airways.¹⁹ Similarly, MCP-1 is expressed in the lungs or nasal secretions of patients with allergic asthma¹⁷ or rhinitis,¹⁶ and this further substantiates the relevance of our observations to allergic disease in humans.

The MCP-1-positive populations in the skin of our mice comprised both epidermal and dermal cells, with fibroblasts and endothelial cells being the likely dermal sources and mast cell TNF- α and TGF- β the likely inducers. We have shown previously that these 2 mast cell cytokines drive the fibroblast activation seen during cutaneous allergic response in mice¹² as well as the high-level induction of MCP-1 secretion¹⁴ and proliferative responses¹³ by mast cell-activated fibroblasts in vitro. Keratinocytes have been reported previously to secrete MCP-1,³¹ but the epidermal MCP-1 expression in our mice represented only a small fraction of the total cytokine production. Although we reported some 10 years ago that mast cells themselves express MCP-1,³² the cells that strongly expressed MCP-1 in the dermal tissues of our mice were not obviously mast cells (ie, granulated). The fact that we did not observe MCP-1 or monocyte responses in IgE-sensitized mast cell-deficient tissues that were challenged with allergen further indicates that non-mast cell Fc ϵ R⁺ cells (eg, Langerhans cells, monocytes) did not contribute greatly to these cutaneous responses.

Irrespective of the cellular sources of MCP-1, this cytokine was important to monocyte recruitment. Neither MCP-1 nor monocyte responses were detected in the skin of mast cell-deficient mice, but mast cell reconstitution also reconstituted these responses. Furthermore, MCP-1 expression in our mice preceded monocyte influx by several hours, and this is the time frame for monocyte responses to intradermally injected MCP-1.³³ Finally, MCP-1 neutralization reduced much of the monocyte response. These data confirm an important but not exclusive causal association between MCP-1 expression and the monocyte response. That MCP-1 is also relevant to the activation of monocytes/macrophages once they have been recruited into the tissues has been confirmed. Anti-MCP-1 antibodies down-regulate the expression of macrophage inflammatory mediators in the airways and dramatically dampen the bronchial hyperresponsiveness of mice with allergic lung disease.¹⁹ MCP-1 could also affect basophils³⁴ and mast cells¹⁵ themselves, for it potently activates both populations. Thus it is quite apparent that MCP-1 has multiple roles, direct and indirect, in allergic responses.

Anti-MCP-1 antisera only blocked the monocyte responses by ~63%. The residual anti-MCP-1-resistant response could be attributable to other chemokines such as MIP-1 α , which was also expressed in our allergic lesions. MIP-1 α has both monocyte/macrophage and neutrophil chemoattractant activities,³⁵ as do an impressive array of other chemokine and nonchemokine agonists. We have previously hypothesized that cytokines released by mast cells would act to recruit leukocytes and

other cells, which in turn would release their own profiles of novel and redundant cytokines.³⁶ We and others have previously documented the "first generation" (ie, granulocyte, fibroblast) recruitment responses in allergic disease,^{12,13,22,28} but here we document one mechanism within a second wave of cellular recruitment (eg, mast cell-activated fibroblast recruitment of monocytes). There is little doubt, however, that multiple analogous mechanisms could be or eventually will be proved operative in the pathogenesis of allergic responses.

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