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Inhibition of Monocyte Chemoattractant Protein-1 Ameliorates Rat Adjuvant-Induced Arthritis

Shiva Shahrara,†* Amanda E. I. Proudfoot,‡ Christy C. Park,* Michael V. Volin,‡ G. Kenneth Haines,§ James M. Woods,‡ Christopher H. Aiken,¶ Tracy M. Handel,¶ and Richard M. Pope*  

Chemokines, including RANTES/CCL5 and MCP-1/CCL2, are highly expressed in the joints of patients with rheumatoid arthritis, and they promote leukocyte migration into the synovial tissue. This study was conducted to determine whether the inhibition of RANTES and MCP-1 therapeutically was capable of ameliorating rat of adjuvant-induced arthritis (AIA). Postonset treatment of AIA using a novel inhibitor for endogenous MCP-1 (P8A-MCP-1) improved clinical signs of arthritis and histological scores measuring joint destruction, synovial lining, macrophage infiltration, and bone erosion. Using immunohistochemistry, ELISA, real-time RT-PCR, and Western blot analysis, we defined joint inflammation, bony erosion, monocyte migration, proinflammatory cytokines, and bone markers, and p-p38 levels were reduced in rat AIA treated with P8A-MCP-1. In contrast, neither the dominant-negative inhibitor for endogenous RANTES (44AANA47-RANTES) nor the CCR1/CCR5 receptor antagonist, methionylated-RANTES, had an effect on clinical signs of arthritis when administered after disease onset. Additionally, therapy with the combination of 44AANA47-RANTES plus P8A-MCP-1 did not ameliorate AIA beyond the effect observed using P8A-MCP-1 alone. Treatment with P8A-MCP-1 reduced joint TNF-α, IL-1β, and vascular endothelial growth factor levels. P8A-MCP-1 also decreased p38 MAPK activation in the joint. Our results indicate that inhibition of MCP-1 with P8A-MCP-1 after the onset of clinically detectable disease ameliorates AIA and decreases macrophage accumulation, cytokine expression, and p38 MAPK activation within the joint. The Journal of Immunology, 2008, 180: 3447–3456.
Effective when initiated after disease onset (17). These observations suggest that the outcome inhibition of MCP-1 or CCR2 blockade may be different depending on the disease model, the timing, and the mechanism of therapeutic intervention; further studies are needed to define the potential benefit of inhibiting MCP-1 after disease onset.

Similarly, the outcome of blocking RANTES activity in experimental arthritis may depend on the timing of intervention. Therapeutic intervention before disease onset using CCR1 and CCR5 inhibitors reduces joint inflammation, bone erosion, proinflammatory cytokines, and monocye and neutrophil recruitment in experimental arthritis (20, 21). We are not aware of studies that have examined the inhibition of RANTES, initiated after the onset of clinical disease. In this study, modified chemokines, which inhibit MCP-1 and RANTES, were used to treat rats with AIA. We found that the severity of arthritis was reduced when MCP-1 was inhibited after the onset of AIA with a mutated MCP-1 (P8A-MCP-1), which displaces endogenous MCP-1 from the endothelial surface and further prevents monocye infiltration in vivo (A. E. L. Proudfoot, manuscript submitted). In contrast, neither 44AANA47-RANTES, which inhibits RANTES-induced cell rolling and adhesion by disrupting its glycosaminoglycan binding and oligomerization, nor methionylated (Met)-RANTES that blocks CCR1/CCR5 receptor binding had any effect on the disease activity when injected after the onset of AIA (22). The combination of P8A-MCP-1 and 44AANA47-RANTES was not more effective than the P8A-MCP-1 alone. Together with earlier studies, these observations suggest that RANTES may be more important for the initiation of disease, whereas MCP-1 is critical for the progression of AIA.

Materials and Methods

**AIA induction and treatment with P8A-MCP-1 and 44AANA47-RANTES**

Five-week-old female Lewis rats (100 g) were injected s.c. with 300 μl (9 mg/ml) of lyophilized *Mycobacterium butyricum* (Difco Laboratories) in sterile mineral oil at the base of the tail on day 0. On day 14 postadjuvant, rats were injected with either P8A-MCP-1 (1 mg/kg), 44AANA47-RANTES (1 mg/kg), P8A-MCP-1 (1 mg/kg), and 44AANA47-RANTES (1 mg/kg), or a placebo (0.9% NaCl). Another group of rats was treated either with Met-RANTES (1 mg/kg) or with vehicle (0.9% NaCl) beginning on day 14 postadjuvant injection. Rats were injected i.p. daily until day 21 (8 or 9 rats per treatment group; 7-day treatment), or day 28 postadjuvant (8 or 9 rats per treatment group; 14-day treatment). The dosage of 44AANA47-RANTES was chosen based on the in vivo dose response performed for cell recruitment in the peritoneum and the dosage of Met-RANTES was chosen based on the in vivo dose response performed for cell recruitment in experimental arthritis (A. E. I. Proudfoot, manuscript submitted). In our previous study, which demonstrated amelioration of AIA when used preventively (20). Tissues were harvested from each group on days 0, 21, and 28 postadjuvant injection. Rats were injected i.p. daily until day 21 (8 or 9 rats per treatment group; 14-day treatment), or day 28 postadjuvant (8 or 9 rats per treatment group; 14-day treatment). The dosage of 44AANA47-RANTES was chosen based on our previous study, which demonstrated amelioration of AIA when used preventively (20). Tissues were harvested from each group on days 0, 21, and 28 postadjuvant for ELISA, Western blot, mRNA, and immunohistochemical studies.

**Clinical measurements**

Clinical parameters measured included articular index (AI) score and ankle circumference, as previously described (20). AI scores were recorded for each ankle joint by a consistent observer blinded to the treatment received by the animals. Scoring was performed on a 0–4 scale, in which: 0 = no swelling or erythema, 1 = slight swelling and/or erythema, 2 = low to moderate edema, 3 = pronounced edema with limited joint usage, 4 = excess edema with joint rigidity. Ankle circumference determinations were performed by measurement of two perpendicular diameters, including the lateral-lateral diameter and the antero-posterior diameter, as measured with a caliper (Lange Caliper; Cambridge Scientific Industries). Circumference was determined using the following formula: circumference = 2π(r + b²/2), where a and b represent the diameters. AI score and ankle circumference evaluations were performed on days 0, 7, 10, 14, 18, 21, 23, 25, and 28. Rats were sacrificed on days 0, 21, and 28, and the serum was saved for laboratory tests.

**Abs and immunohistochemistry**

Rat ankles were decalcified, embedded in formaldehyde, and sectioned in the pathology core facility of Northwestern University. Inflammatory, joint destruction, and synovial lining (based on a 0–4 score) were determined using H&E-stained sections. Rat ankles were immunoperoxidase stained using Vector Elite ABC Kits (Vector Laboratories), with dianamobenzidine (Vector Laboratories) as a chromogen. Slides were deparaffinized in xylene for 20 min at room temperature, followed by rehydration by transfer through graded alcohols. Ags were unmasked by first incubating slides in boiling citrate buffer for 15 min, followed by type II trypsin digestion for 30 min at 37°C. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ for 5 min. Nonspecific binding of antibody was blocked using an avidin/biotin blocking kit (Vector Laboratories). Nonspecific binding of Ab to the tissues was blocked by pretreatment of tissues with diluted normal horse serum. Tissues were incubated with rabbit polyclonal Ab to CD68 (1/100 dilution; Serotec), or phosphorylated (p)-p38 (1/100 dilution; Cell Signaling Technology) or a rabbit IgG control Ab (Beckman Coulter). Slides were counterstained with Harris’ hematoxylin and treated with lithium carbonate for bluing.

**Microscopic analysis**

Macrophages were distinguished based on CD68 immunostaining in the lining and the sublining. The mean percentage of reactivity was determined for three high power fields in the STs of five separate animals in each treatment group for each cell type and Ab analyzed. Each slide was evaluated by a single blinded pathologist (G. Haines) (13, 20). Inflammation (1, normal; 2, increased number of inflammatory cells, and 3, marked diffuse infiltrate of inflammatory cells, including distinct clusters (aggregates); 4, marked diffuse infiltrate of inflammatory cells) was scored on a 1–4 scale, as described (20). Bone destruction was graded from 0 to 4 (0, normal; 1, minimal; 2, mild; 3, severe; 4, extreme bone destruction with no bone integrity), as described (20). For CD68 and p-p38 immunostaining, each of the ST components was graded by a frequency of attaining scale, scored 0–100%, in which 0% indicates no staining and 100% indicates that all cells were immunoreactive. Score data were pooled, and the mean ± SEM was calculated in each data group.

**Protein extracts**

To perform ELISA and Western blot, protein was extracted from ankles, as previously described (13).

**ELISA**

Cytokine levels in ankle homogenates were determined using commercially available ELISA kits that specifically recognize the rat cytokines TNF-α and IL-1β, and vascular endothelial growth factor (VEGF) (BioSource International), according to the manufacturer’s procedure (23, 24).

**Ankle x-rays and radiographic scoring**

Upon sacrifice on day 28 postadjuvant, ankles were removed on ice, and x-rays were taken. Radiographs were scored for the degree of bony destruction/erosions (from 0 to 4), assigning a point for any erosion present in the tibia, calcaneus, talus, and metatarsals (considered together). The maximum score that an ankle could receive was 4 if erosion was present in the tibia, calcaneus, talus, and any one or more of the metatarsals. Joint space abnormality (or narrowing) was graded on a scale of 0–3 (0, none; 1, mild; 2, moderate; 3, severe). X-rays were scored by two observers blinded to the experimental groups (C. Park and C. Aikens) (23, 24).

**TagMan real-time PCR**

Total RNA was isolated from AIA ankles using the TRizol reagent, according to the manufacturer’s protocol (Invitrogen Life Technologies). A total of 20 μg of total RNA was reverse transcribed, as previously described (25), according to the manufacturer’s specifications (Promega). For the TaqMan assay, the TaqMan Universal PCR Master Mix Kit (Applied Biosystems) was used. The rat receptor activator of NF-κB ligand (RANKL), matrix metalloproteinase-9 (MMP-9), osteoprotegerin, and GAPDH primer and probe sets were labeled with the 5’ reporter dye FAM and the 3’ quencher TAMRA (Applied Biosystems). The thermocycling reaction contained the following: 6 μl of H₂O, 10 μl of TaqMan Universal PCR Master Mix, 1 μl of primer and probe set, and 3 μl of cDNA. The reactions were run on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The amplification program was as follows: 50°C for 2 min, 95°C for 10 min, followed by

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40 cycles of 95°C for 15 s, and 60°C for 1 min. The efficiency of the TaqMan assays was determined by assaying serial 10-fold dilutions, ranging from 10⁰ to 10⁶, of target cDNA. With standard analysis parameters of baseline set between cycle threshold 3 and 15 and the $R^2$ set at 0.1, a standard curve of mean cycle threshold for three replicates at each dilution vs log₁₀ amount of cDNA was determined ($R^2 = 0.9985$) (26). The relative concentration of the mRNA was based on three triplicates normalized to their GAPDH value (20). Finally, the data are shown as fold increase compared with the control.

Western blot analysis

Equal amounts of each protein were loaded on a 10% SDS-PAGE and transferred to nitrocellulose membranes using a semidry transblotting apparatus (Bio-Rad). Nitrocellulose membranes were blocked with 5% nonfat milk in TBST buffer-20 mM Tris, 137 mM NaCl (pH 7.6), with 0.1% Tween 20 for 60 min at room temperature. Blots were incubated with anti-p-p38 MAPK and anti-p38 MAPK Abs (Cell Signaling Technology) at 1:1000, in TBST containing 5% nonfat milk overnight (13). Blots were scanned and band intensities were determined using UN-SCAN-IT version 5.1 software (Silk Scientific). Band intensities correspond to the sum of all pixel values in the segment selected minus the background pixel value in that segment.

Statistical analysis

The clinical parameters were analyzed using ANOVA and Student’s t tests. The remainder of the data was analyzed using Student’s t tests. Values of $p < 0.05$ were considered significant.

Results

**P8A-MCP-1 reduces clinical features of rat AIA, whereas **$^{44}$AANA$^{47}$-RANTES and Met-RANTES do not**

To determine the effect of inhibiting MCP-1 and RANTES on active disease, treatments were administered beginning day 14 postadjuvant, when 50% of the rats demonstrated clinically active arthritis. P8A-MCP-1, $^{44}$AANA$^{47}$-RANTES, and the combination of both antagonists were administered i.p. from day 14 until day 21 (for 7 days) or 28 (for 14 days). In the control saline-injected rats, disease activity determined by ankle circumference (Fig. 1A) or by AI (Fig. 1B) began on day 14 and progressed through day 21, plateauing thereafter until the termination of the experiments on day 28. There was no difference from the controls, by either measure of disease activity, in the animals treated with $^{44}$AANA$^{47}$-RANTES.

Because $^{44}$AANA$^{47}$-RANTES was not effective, additional experiments were performed with another inhibitor of RANTES, Met-RANTES, an antagonist to RANTES and MIP-1α binding to CCR1 and CCR5, which was shown to ameliorate AIA when administered preventively (20). Consistent with the results obtained with $^{44}$AANA$^{47}$-RANTES, Met-RANTES was ineffective at suppressing AIA when therapy was initiated after the onset of disease (Fig. 1, C and D). In contrast, the animals treated with P8A-MCP-1 alone or the combination of P8A-MCP-1 plus $^{44}$AANA$^{47}$-RANTES demonstrated significantly ($p < 0.05$) reduced (30–40%) joint
circumferences and AI scores compared with the controls or those treated with **44AANA**$^{47}$-RANTES alone, on days 18 –28. Furthermore, the mean body weight of the animals treated with P8A-MCP-1 and the combination were significantly higher than those treated with **44AANA**$^{47}$-RANTES or the saline-injected controls (data not shown). These results demonstrate that P8A-MCP-1, but not **44AANA**$^{47}$-RANTES or Met-RANTES, was effective at reducing the progression of AIA when administered after the onset of disease.

**P8A-MCP-1 decreases joint inflammation, destruction, and synovial lining**

Histologic examination of the joints was performed to determine the effects of treatment on inflammation and joint destruction. In the control group, the synovial lining scores for days 21 (data not shown) and 28 (Fig. 2E) were identical; however, the inflammatory score was lower and the joint destruction score was higher in day 28 rat AIA ankles compared with day 21. Consistent with the clinical data, the histological studies demonstrate that the animals treated with P8A-MCP-1 (Fig. 2, C and E), or with the combination of P8A-MCP-1 plus **44AANA**$^{47}$-RANTES (Fig. 2, D and E), demonstrated significantly ($p < 0.05$) reduced scores for inflammation, joint destruction, and synovial lining thickness on days 21 (data not shown) and 28 postadjuvant (Fig. 2E), compared with the animals treated with **44AANA**$^{47}$-RANTES alone (Fig. 2, B and E) or with saline (Fig. 2, A and E). There was no difference of the histological scores between the P8A-MCP-1 and P8A-MCP-1 plus **44AANA**$^{47}$-RANTES-treated animals, and treatment with **44AANA**$^{47}$-RANTES alone provided no significant protection of joint inflammation or destruction, or synovial lining thickness. These results suggest the P8A-MCP-1, but not **44AANA**$^{47}$-RANTES, resulted in reduced inflammation and joint destruction in AIA.

**P8A-MCP-1 suppresses radiographic damage and its associated markers**

To determine the effect of the different treatments on the bone destruction in AIA, x-rays from all ankles harvested on day 28 were scored for joint space abnormality or narrowing (0–3 scale) and bone erosion (0–4 scale), and the results are presented as sum of both parameters (23, 24). Consistent with the physical examination, P8A-MCP-1 and combination therapy resulted in reduced radiographic joint damage by 30–40% compared with the control and **44AANA**$^{47}$-RANTES treatment groups (Fig. 3A).

Additional studies were performed to determine the effect of therapy on markers of bone destruction. For this purpose, the expression of mRNA for RANKL and MMP-9 was examined. In the control saline- and **44AANA**$^{47}$-RANTES-treated rats, the levels of
RANKL mRNA increased from day 0 to day 21 and remained elevated at day 28. The RANKL mRNA was 3-fold higher in the control- and 44AANA47-RANTES-treated ankles compared with those from animals treated with P8A-MCP-1 or P8A-MCP-1 plus 44AANA47-RANTES (Fig. 3B). Similarly, the control- and the 44AANA47-RANTES-treated rats demonstrated 5-fold greater MMP-9 mRNA compared with those treated with P8A-MCP-1 or the combination (Fig. 3C). These results demonstrate that P8A-MCP-1 resulted in a significant reduction of RANKL and MMP-9 mRNA at both 21 and 28 days.

FIGURE 3. P8A-MCP-1 ameliorates markers of bone destruction in the AIA ankles. A. Ankles were radiographed on day 28 and scored for joint space abnormality and bony erosion. RANKL mRNA (B) and MMP-9 mRNA (C) quantified by real-time RT-PCR and normalized by their GAPDH content in ankles of AIA in all the experimental groups. The results are shown as fold increase compared with mRNA levels detected on day 0. Values are the mean ± SE. *, Represents p < 0.05, and **, denotes p < 0.01.

RANKL mRNA increased from day 0 to day 21 and remained elevated at day 28. The RANKL mRNA was 3-fold higher in the control- and 44AANA47-RANTES-treated ankles compared with those from animals treated with P8A-MCP-1 or P8A-MCP-1 plus 44AANA47-RANTES (Fig. 3B). Similarly, the control- and the 44AANA47-RANTES-treated rats demonstrated 5-fold greater MMP-9 mRNA compared with those treated with P8A-MCP-1 or the combination (Fig. 3C). These results demonstrate that P8A-MCP-1 resulted in a significant reduction of RANKL and MMP-9 mRNA at both 21 and 28 days.

FIGURE 4. Decrease in CD68+ macrophage immunostaining in rats with AIA-treated P8A-MCP-1. A. ST from control rats; B, ST from rats treated with 44AANA47-RANTES. C, ST from rats treated with P8A-MCP-1. D, ST from rats treated with 44AANA47-RANTES and P8A-MCP-1 harvested on day 21 postadjuvant, immunostained with CD68+. E, Quantification of CD68+ immunostaining in AIA ST from all experimental groups harvested on day 21 postadjuvant. Values are the mean ± SE. *, Represents p < 0.05.
P8A-MCP-1 reduces CD68⁺ macrophages in the ST

Macrophages in the ST are derived from circulating monocytes. Therefore, studies were performed to characterize the presence of CD68⁺ macrophages in the ST (Fig. 4, A–D). In line with the clinical data, P8A-MCP-1 and the combination therapy resulted in significantly (p < 0.05) reduced numbers of CD68⁺ macrophages in the lining (P8A-MCP-1 alone, 54% ; combination therapy, 57%) and sublining (P8A-MCP-1 alone, 48%; combination therapy, 40%) at day 21 (Fig. 4E) and day 28 (data not shown). No significant difference in staining for CD68 was observed between the 44AANA47-RANTES- and the control saline-treated animals. Because inflammatory ST macrophages are derived from peripheral blood monocytes, these observations suggest decreased monocyte recruitment into the joints of rats with AIA treated with P8A-MCP-1, when therapy was initiated after disease onset.

P8A-MCP-1 reduces proinflammatory mediators in AIA ankle

Because the expression of proinflammatory mediators is characteristic of joint inflammation, the effect of therapy on joint TNF-α, IL-1β, and VEGF expression was examined. In the control saline- and 44AANA47-RANTES-treated groups, the levels of TNF-α and IL-1β within the joints increased 17-fold, from day 0 to 21, and these values were reduced by ~50% on day 28 compared with day 21 (Fig. 5, A and B). TNF-α and IL-1β were significantly (p < 0.05) reduced in the rats treated with P8A-MCP-1 or the combination therapy on days 21 and 28 (Fig. 5, A and B). Similar changes for circulating IL-1β were observed, with significant (p < 0.05) reductions in those treated with P8A-MCP-1 or the combination compared with those treated with saline or 44AANA47-RANTES (Fig. 5C). P8A-MCP-1 and the combination therapy also resulted in reduced levels of VEGF on days 21 and 28 compared with the control group or those treated with 44AANA47-RANTES alone (p < 0.05) (Fig. 5D). These results indicate that treatment with P8A-MCP-1 decreased the levels of TNF-α, IL-1β, and VEGF in the joints, and circulating IL-1β, in rats with AIA.

P8A-MCP-1 reduces p-p38 MAPK activation

Because RANTES and MCP-1 induce monocyte chemotaxis through activation of p38 (2), the effect of the therapies on p38 activation in AIA ankles was examined. Activated or p-p38 was strongly expressed in the AIA lining, as well as the sublining and the endothelium in the control- and 44AANA47-RANTES-treated animals (Fig. 6, A and B). P8A-MCP-1 and the combination therapy significantly (p < 0.05) reduced the expression of p-p38, determined by immunohistochemistry, in the synovial lining and sublining and in endothelial cells on days 21 and 28 postadjuvant (Fig. 6, C–E). These results demonstrate that therapy with P8A-MCP-1...
resulted in decreased p38 MAPK activation in the joints of rats with AIA.

To confirm these findings, we also examined the phosphorylation of p38 by immunoblot analysis using joint lysates (Fig. 6F). The activation of p38 was reduced in those treated with P8A-MCP-1, and the combination therapy, by 60–70% compared with the controls, whereas there was no effect of the 44AANA47-RANTES when used alone (Fig. 6G). These results indicate that treatment with P8A-MCP-1, initiated after the onset of AIA, reduced p38 MAPK activation in the joints.

Discussion
In this study, we examined the effects of the inhibition of the function of RANTES and MCP-1, when therapy was initiated following the onset of rat AIA. 44AANA47-RANTES is a dominant-negative inhibitor for endogenous RANTES that has been shown to functionally inhibit RANTES in vivo (22). When administered i.p., 44AANA47-RANTES accesses the circulation rapidly due to its reduced glycosaminoglycan binding, and recruits the endogenous RANTES to form nonfunctional heterodimers, thereby preventing RANTES oligomerization and RANTES-induced cell recruitment in vivo (22). Furthermore, 44AANA47-RANTES has been shown to reduce the clinical score of experimental autoimmune encephalomyelitis in mice, as well as inhibiting RANTES-induced leukocyte-endothelial cell interactions in rats (22). The mechanism through which P8A-MCP-1 inhibits MCP-1 function is more complex. The P8A-MCP-1 mutation renders the variant an obligate monomer (27), which is unable to recruit cells in vivo (28). However, it retains glycosaminoglycan binding and displaces endogenous MCP-1 from the endothelial surface, thereby preventing further cell recruitment (A. E. I. Proudfoot, manuscript submitted).

In these studies, when therapeutic intervention was initiated after disease onset, inhibition of MCP-1, using P8A-MCP-1, was highly effective at suppressing disease determined by clinical examination, by histology, and radiographically. In contrast, inhibition of RANTES, with 44AANA47-RANTES, provided no benefit when used alone or combined with P8A-MCP-1. This difference was not due to lack of function of 44AANA47-RANTES, because this compound has been shown to suppress RANTES-induced chemotaxis in vivo in rats and ameliorate experimental autoimmune

FIGURE 6. Reduced p-p38 MAPK immunostaining in rats with AIA-treated P8A-MCP-1. Shown in staining of the following: A, ST from control rats; B, ST from rats treated with 44AANA47-RANTES; C, ST from rats treated with P8A-MCP-1; and D, ST from rats treated with P8A-MCP-1 and 44AANA47-RANTES harvested on days 21 postadjuvant, and immunostained with p-p38 MAPK. E, Quantification of p-p38 immunostaining in AIA ST from all experimental groups harvested on day 21 postadjuvant. F, AIA ankle homogenates harvested from day 28 were probed for p-p38 by Western blot analysis. C = control-treated rats; R = 44AANA47-RANTES; M = P8A-MCP-1; M&R = rats treated with the combination therapy. G, Quantification of Western blot analysis from day 21 and 28 (F) ankle rat homogenates; the intensity of p-p38 was normalized by p38. Values are the mean ± SE. *, Represents p < 0.05.
encephalomyelitis in mice (22). Furthermore, the ineffectiveness of RANTES as a therapeutic target was validated when rats with AIA were treated therapeutically with Met-RANTES. Consistent with the lack of effect of 44AANA47-RANTES, Met-RANTES, which we previously demonstrated was effective when administered before AIA onset in rats (20), failed to suppress disease when administered after disease onset. Whole blood RANTES is up-regulated on day 8, before the onset of clinically detectable AIA, and is decreased at the time of peak inflammatory response, about day 18 (14). CCR1 and CCR5, the receptors for RANTES, are increased in the AIA joint progressively from day 14 postadjuvant with maximum up-regulation on day 18 (13). Furthermore, CCR1 and CCR5 are expressed on the ST macrophages of rats with AIA (29). Together these observations suggest that RANTES may be important in the initiation of AIA, attracting monocytes into the joint. Supporting this possibility, treatment before disease onset with a polyclonal anti-RANTES Ab markedly ameliorated the development of AIA (14, 20). In contrast, in the current study, therapeutic intervention with 44AANA47-RANTES, which is capable of inhibiting leukocyte migration in rats in vivo, was ineffective, either alone or in combination with P8A-MCP-1 at suppressing AIA. This lack of effect may be related to the possibility that the expression of RANTES peaks before disease onset (14). It is also possible that the CCR1- and CCR5-expressing cells in the circulation had already migrated to the joint, before the onset of therapy.

In contrast to the effects of inhibition of RANTES, in this study, inhibition of MCP-1 was highly effective when therapy was initiated after disease onset. In rat AIA, MCP-1 was significantly increased in the joints at the time of peak arthritis, on day 18, and subsequently (15). Supporting functional role for MCP-1, CCR2-positive macrophages are significantly increased in the ST of rats with AIA beginning on day 14 postadjuvant and thereafter (29). These observations support the possibility that inhibition of MCP-1 may be effective when initiated after the onset of arthritis in AIA. Consistent with this interpretation, P8A-MCP-1 was effective at ameliorating disease activity, clinically, histologically, and radiographically. These observations support the role of MCP-1 as a target for therapeutic intervention in established disease.

Our observations concerning the inhibition of MCP-1 contrast with others that have examined the role of MCP-1 and its receptor, CCR2, in experimental models of arthritis. CIA was more severe in CCR2−/− mice compared with the controls (30). Increased disease activity was associated with increased anti-collagen Ab levels, the production of rheumatoid factor, and the increased accumulation of macrophages within the joints, suggesting that the lack of CCR2 affected the mechanisms contributing to the development of autoimmunity, and that in the absence of CCR2, chemokines other than MCP-1 were important for the recruitment of monocytes to the joint. However, in wild-type animals, CCR2 blockade with an Ab or with a small molecule antagonist of MCP-1, before the onset of arthritis in CIA or AIA, results in disease amelioration (16–18). Furthermore, a recent study demonstrates that daily postonset administration of low dose of anti-CCR2 Ab (10 μg) ameliorates CIA by markedly decreasing the monocyte infiltration (31). In contrast, when initiated after disease onset, anti-CCR2 Ab blockade at high concentrations (500 μg) markedly aggravated the clinical and histological signs of arthritis by increasing the IL-6 secretion from basophils (16). Consistent with this observation, results from a randomized small clinical trial involving the administration of neutralizing anti-MCP-1 Abs to patients with active rheumatoid arthritis showed that although the treatment did not change the clinical outcome of the disease, it was associated with increased levels of C-reactive protein, and increased CD68+ macrophages to rheumatoid arthritis ST sublining (32).

Postonset inhibition of MCP-1 binding to CCR2 using MCP-1(9–76) in MRL-lpr mice reduced the incidence of swelling and the histopathology after 6 days of treatment, although it was not as effective as when combined with an inhibitor of CXCL3/growth-related oncogene-α (17). Nonetheless, this improvement with MCP-1 is consistent with our observations that the postonset inhibition of MCP-1 by P8A-MCP-1 ameliorated AIA disease activity. The variation between these studies may be due to differences in the mechanisms contributing to the pathogenesis, because they studied spontaneous arthritis in MRL-lpr mice exacerbated by CFA, whereas we examined rat AIA. The difference in the effect of MCP-1 inhibition on established disease may also be responsible because P8A-MCP-1 displaces endogenous MCP-1 from endothelial cell surfaces, preventing chemotaxis, whereas MCP-1(9–76) inhibits the binding of MCP-1 to CCR2 (19). Additionally, the outcome of the inhibition of MCP-1 and CCR2 blockade may be different, because CCR2 also serves as a receptor of other MCPs (MCP-2, -3, -4, and -5) (33). Although MCPs are functionally related, different MCPs (MCP-2, -3, and -4) can bind to multiple receptors and activate differential signaling pathways, thereby attracting different cell types (34). Interestingly, MCP-2 and MCP-3 can induce both monocyte and neutrophil migration, whereas MCP-1 specifically attracts monocytes (35).

In addition to contributing to the chemotaxis of monocytes, MCP-1 may also contribute to the activation of monocytes or macrophages. With murine peritoneal macrophages, MCP-1 induces production of TNF-α and IL-1β (36), CCR2−/− and MCP-1−/− mice exhibit reduced expression of IFN-γ production in draining lymph nodes and demonstrate an abnormal skewing toward Th2 cytokine production, as well as impaired macrophage recruitment (3, 37). Interestingly, we detected the reduction of joint TNF-α, as well as joint and serum IL-1β, in AIA rats treated with P8A-MCP-1. This may be directly due to inhibition of macrophage activation by MCP-1 or indirectly due to reduced infiltration of monocytes into the joints, resulting in reduced numbers of differentiated and activated macrophages.

VEGF, RANKL, and MMP-9 are important mediators of the progression of arthritis and joint destruction (38–45). Our studies showed that VEGF levels were increased in the joints of rats with AIA joints, and that P8A-MCP-1 significantly reduced the expression of VEGF. There may be several explanations for this observation. First, in arthritic joints, VEGF is produced by synovial macrophages and fibroblasts (46, 47), and VEGF levels may decrease due to reduced monocyte recruitment. Second, MCP-1 induces VEGF production in human macrophages and aortic endothelial cells (48, 49), and therefore, inhibition of MCP-1 function may reduce VEGF produced by ST macrophages. Third, TNF-α increases VEGF secretion (50), and decreased levels of TNF-α may explain the reduced levels of joint VEGF. Monocytes activated by MCP-1 and RANTES secrete TNF-α (51), and TNF-α modulates both RANKL and MMP-9 (52, 53). It has been shown that TNF-α is necessary for MCP-1-induced MMP-9 production by monocytes (51). Therefore, the down-regulation of joint RANKL and MMP-9 by P8A-MCP-1 may also be due to reduced ankle TNF-α.

RANTES and MCP-1-induced monocyte chemotaxis are mediated through p38 (2). Additionally, both TNF-α and IL-1β signal through p38 MAPK (52). In agreement with previous findings, p-p38 MAPK was predominantly expressed on synovial lining as well as cells in the sublining and endothelial cells (54). In our
studies, whereas the inhibition of RANTES after the onset of disease failed to reduce the p38 activation, treatment with PBA-MCP-1 was associated with reduced p38 activation in the joints. There may be several explanations for the decreased p38 phosphorylation. PBA-MCP-1 suppressed monocyte chemotaxis, resulting in fewer macrophages to become activated within the joint. Additionally, once within the joint it is possible that MCP-1 contributes directly to the activation of p38 in the macrophages localized to the joint. Finally, the reduction of TNF-α and IL-1β that resulted from treatment with the PBA-MCP-1 may have contributed to the reduction of activated p38. Regardless of the mechanism, the reduction of p38 activation is consistent with the prediction by the inhibition of MCP-1.

Taken together, this study demonstrates that inhibition of MCP-1 with PBA-MCP-1 was effective at suppressing AIA, when therapy was initiated before the onset of disease. Although our earlier study observed that inhibition of RANTES was effective when therapy was initiated after the onset of disease, it was not effective when initiated after disease onset. Furthermore, the mechanism by which the function of MCP-1 is suppressed may have profound effects on the clinical outcome.

Disclosures
Dr. Amanda Proudfoot is an employee of Merck Serono and all the other authors have no conflict of interest to disclose.

References


