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*J Immunol* 2006; 177:5077-5087; doi: 10.4049/jimmunol.177.8.5077
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RANTES Modulates TLR4-Induced Cytokine Secretion in Human Peripheral Blood Monocytes

Shiva Shahrara,* Christy C. Park,* Vladislav Temkin,* Jared W. Jarvis,* Michael V. Volin,† and Richard M. Pope*

Monocytes are the key regulators of joint inflammation and destruction in rheumatoid arthritis; hence, suppression of their recruitment into the joint may be therapeutically beneficial. Chemokines, including RANTES, are highly expressed in the joints of patient with rheumatoid arthritis, and they promote leukocyte trafficking into the synovial tissue. Because endogenous TLR4 ligands are expressed in the rheumatoid joint, the TLR4 ligand LPS was used to characterize the effects of RANTES on the TLR4-mediated induction of TNF-α and IL-6. Using peripheral blood (PB) monocytes, RANTES decreased LPS-induced IL-6 transcriptionally, whereas TNF-α was suppressed at the posttranscriptional level. RANTES signaled through p38 MAPK, and this signaling was further enhanced by LPS stimulation in PB monocytes, resulting in the earlier and increased secretion of IL-10. Inhibition of p38 by short-interfering RNA or a chemical inhibitor, as well as neutralization of IL-10, reversed the RANTES-mediated suppression of LPS-induced IL-6 and TNF-α. Further, when rheumatoid arthritis synovial fluid was added to PB monocytes, the neutralization of RANTES in fluid reduced the LPS-induced IL-10 and increased TNF-α. In conclusion, the results of this study suggest that RANTES down-regulates TLR4 ligation-induced IL-6 and TNF-α secretion by enhancing IL-10 production in PB monocytes. These observations suggest that the therapeutic neutralization of RANTES, in addition to decreasing the trafficking of leukocytes, may have a proinflammatory effect at the site of established chronic inflammation. The Journal of Immunology, 2006, 177: 5077–5087.

Chemokines, important in leukocyte trafficking, are divided into four subfamilies (CXC, CC, C, and CX3C) based on the arrangement of their amino-terminal cysteine residues (1). Chemokines are important in linking innate and adaptive immune responses by bringing together Ag-loaded dendritic cells and naive T and B cells to generate an adaptive immune response (2). They are also critical for delivering leukocytes to the site of inflammation. RANTES/CCL5 (ligand for CCR1, 3, and 5) is up-regulated in the joints of patients with rheumatoid arthritis (RA) and in experimental arthritis (3–6).

The results of experiments targeting chemokines in experimental arthritis suggest that the effects of chemokines may extend beyond chemotaxis. We have shown that the treatment of adjuvant-induced arthritis before the onset of clinically detectable disease by the inhibition of RANTES with methionylated RANTES (Met-RANTES) decreases joint inflammation, proinflammatory cytokines, bone destruction, and cell recruitment into the ankles (7). In contrast, our unpublished data show that the inhibition of RANTES after disease onset in adjuvant-induced arthritis did not alleviate the disease. Additionally, blockade of CCR2 in the initial phase of collagen-induced arthritis ameliorates the disease, whereas inhibition of CCR2 after disease onset aggravates the arthritis (8). Further, collagen-induced arthritis is increased in CCR2-null mice compared with wild-type controls (9). These findings suggest that chemokine inhibition may differentially affect inflammatory responses in rodent models of RA depending on the time of intervention, suggesting a potential biological role for chemokines beyond their effects on leukocyte trafficking.

Supporting this possibility, RANTES and MCP-1/CCL2 induce RA synovial tissue fibroblasts to produce IL-6 and IL-8 (10). RANTES can also promote IL-2 and IL-5 expression in human T cells (11, 12). MIP-1/CCL3 stimulates the synthesis of IL-1, TNF-α, and IL-6 by murine macrophages (13). Studies examining the effects of RANTES on monocytes are important, because it is possible that the exposure of monocytes to RANTES before entering the site of inflammation may affect subsequent function, which may be important for understanding the pathogenesis of RA as well as the effects of therapeutic intervention aimed at the inhibition of chemokines.

In this study, LPS was used as a surrogate for endogenous TLR4 ligands such as heat shock proteins and extra domain A fibronectin, which are highly expressed in the joints of patients with RA (14–16). Pretreatment of peripheral blood (PB) monocytes with RANTES decreased the LPS-induced expression of IL-6 mRNA and protein, whereas RANTES reduced LPS-induced TNF-α at the posttranscriptional level. Inhibition of p38 MAPK signaling or neutralization of IL-10 reversed the effects of RANTES on LPS-induced IL-6 and TNF-α production. Using RA synovial fibroblasts, RANTES failed to suppress LPS-induced IL-6, and IL-10 was not secreted. Further, when PB monocytes were cultured in rheumatoid synovial fluid, neutralization of RANTES increased LPS-induced TNF-α and reduced IL-10. These results indicate that

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*Department of Medicine, Division of Rheumatology, Northwestern University, Feinberg School of Medicine, Chicago, IL 60611; and †Department of Microbiology and Immunology, Midwestern University, Chicago College of Osteopathic Medicine, Downers Grove, IL 60515

Received for publication April 10, 2006. Accepted for publication July 28, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institutes Health Grants AR049353 (to S.S.) and AR049217 and AR048269 (to R.M.P.);

2 Address correspondence and reprint requests to Dr. Shiva Shahrara, Northwestern University, Feinberg School of Medicine, Department of Medicine, Section of Rheumatology, McGaw Pavilion, 240 East Huron, Suite 2220, Chicago, IL 60611. E-mail address: s-shahrara@northwestern.edu

3 Abbreviations used in this paper: RA, rheumatoid arthritis; PB, peripheral blood; siRNA, short-interfering RNA; Met-RANTES, methionylated RANTES; MK2, MAPK-activated protein kinase.

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RANTES may be capable of modulating the inflammatory response via the induction of IL-10 in a cell type-specific manner. These effects may be critical for understanding the therapeutic consequences of chemokine inhibition for chronic inflammation.

Materials and Methods

Isolation of PB monocytes

Monocytes were separated from theuffy coat (Lifesource), which was obtained from healthy donors. Mononuclear cells, isolated by Histopaque (Sigma-Aldrich) gradient centrifugation, were separated by countercurrent centrifugal elutriation (JE-6B; Beckman Coulter) in the presence of 10 mg/ml polymyxin B sulfate as previously described (17–20). Monocyte purity was >90% as determined by morphology and CD14 staining. Monocytes were cultured in RPMI 1640 containing 10% heat-inactivated FBS plus 1 μg/ml polymyxin B sulfate (Sigma-Aldrich).

RA synovial tissue fibroblasts and synovial fluid

Synovial tissue fibroblasts were isolated from fresh synovial tissues by mincing and digestion in a solution of dispase, collagenase, and DNase (21, 22). Cells were used between passages 3 and 9. Synovial fluids from the patients with RA were collected and immediately centrifuged at 800 x g at room temperature for 10 min and then stored frozen until use. These studies have been reviewed and approved by an appropriate institutional review committee.

Reagents

Actinomycin was obtained from Sigma-Aldrich. Anti-CCR1 was from Santa Cruz Biotechnology, anti-CCR5 (2D7) from BD Pharmingen, Met-RANTES from Serono, SB203580 (10 μM) from Calbiochem, LPS from Sigma-Aldrich, and RANTES and monoclonal anti-human IL-10 from R&D Systems.

Inhibition of p38 MAPK by short-interfering RNA (siRNA)

Human p38 MAPK (MAPK14; siGenome SMARTpool) specific and non-specific control (nontargeting siRNA pool) silence RNA oligonucleotides (siRNA) (Dharmacon) were transfected into PB monocytes (cultured in 6-well plates) by Lipofectamine 2000 (4 μl/well; Invitrogen Life Technologies) following the manufacturer’s protocol with several modifications. Immediately before transfection, the monocytes were replaced by 2 ml/well fresh RPMI 1640 (no additives). The p38 MAPK or control siRNA was transfected at a final concentration of 100 nM. The transfection reactions were supplemented with 250 μl of FBS after 2 h and 2250 μl of 10% FBS in RPMI 1640 after 5 h. After 48 h, the cells were treated with control medium or RANTES at the indicated time points and used in the experiments described in Results.

RNA extraction, reverse transcription, and real-time RT-PCR

Total RNA was isolated from PB monocytes using the TRIzol reagent according to the manufacturer’s protocol (Invitrogen Life Technologies). One microgram of total RNA was reverse transcribed as previously described (23) according to the manufacturer’s specifications (Promega). For the TaqMan assay, the TaqMan Universal PCR Master Mix kit (Applied Biosystems) was used. The human IL-6, TNF-α, and IL-10 primer and probe sets were labeled with the 5’ reporter dye FAM and the 3’ quencher TAMRA (Applied Biosystems). The GAPDH primer and probe set was labeled with the 5’ reporter dye VIC and the 3’ quencher TAMRA (Applied Biosystems). The thermocycling reaction contained 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 50°C for 2 min and 95°C for 10 min followed by 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 of target cDNA ranging from 10⁷ to 10¹. With standard analysis parameters of the baseline set between cycle thresholds of 3 and 15 and the ΔΔt, threshold set at 0.1, a standard curve of mean cycle threshold for three replicates at each dilution vs the log₂ amount of cDNA was determined (R² = 0.9985) (24). The relative concentration of the mRNA was based on three triplicates normalized to their GAPDH values. Finally, the data is shown as fold increase compared with the control.

CHEMOKINES CAN REGULATE CYTOKINE PRODUCTION

Cytokine quantification

Commercial kits for human IL-6, TNF-α, and IL-10 (DuoSet ELISA; R&D Systems), were used for cytokine quantification as per the manufacturer’s instructions.

Flow cytometric analysis of TLR expression

Cell surface expression of TLR2 and TLR4 from PB monocytes was analyzed by Ab cell surface staining and two-color flow cytometry as described previously (25). PB monocytes were blocked for 1 h in 50% human serum at room temperature. After blocking, cells were stained with the following FITC-conjugated Abs: anti-TLR2 mAb (eBioscience), anti-TLR4 mAb (Imagenex), and isotype-matched control IgG (eBioscience). After a second washing, cells were labeled with PE-conjugated anti-CD14 mAb (BD Pharmingen) for 30 min to recognize CD14 markers on monocytes. After staining, cells were fixed for flow cytometric analysis performed at the Northwestern University’s Flow Cytometry Core Facility (Chicago, IL) using Beckman Coulter Epics XL-MCL System II. Cells were gated by the combination of forward and side scatters, and the fluorescence intensity of CD14-positive cells (PE staining) was normalized to that of unstained cells. The level of monocyte surface TLR expression was indicated by the mean fluorescence intensity (MFI) of the FITC-positive cells in the PE-positive cell population (double-positive cells).

Western blot analysis

As shown in Fig. 3A, PB monocytes were pretreated for 1 h with either PBS or Met-RANTES, and thereafter cells were stimulated with RANTES from 0 to 30 min. In Fig. 3C, monocytes were untreated or treated with RANTES for 0.25–24 h. In Fig. 7B, RA fibroblasts were either untreated or treated with RANTES for 0.25–5 h. In Fig. 3C, PB monocytes were either untreated or treated with RANTES from 0.25 to 24 h. In Fig. 7C, RA fibroblasts were treated with PBS or RANTES for 24 h and thereafter cells were treated with LPS for 0–3 h. Western blot analysis was conducted as previously described (23). Blots were probed with phosphorylated p38 MAPK (Cell Signaling Technology) at 1/1000 overnight and, after stripping, were reprobed with either p38 MAPK (Cell Signaling Technology) at 1/1000 and/or tubulin (Sigma-Aldrich) at 1/3000 for 60 min. Blots were scanned and analyzed for measurement of the band intensities with UNSCAN-IT version 5.1 software. Band intensity corresponded to the sum of all pixel values in the segment selected minus the background pixel value in that segment.

Statistical analysis

The data were analyzed using Student’s t tests for paired samples. A value of p < 0.05 was considered significant.

Results

RANTES suppresses LPS-induced IL-6 and TNF-α by monocytes

To determine the effect of RANTES on LPS-induced cytokine production, human PB monocytes were incubated with PBS or RANTES (10 ng/ml) for 24 h. Thereafter, cells were treated with LPS (10 ng/ml) for 24 h. The supernatants were harvested, and the levels of IL-6 and TNF-α were measured. RANTES suppressed the LPS-induced IL-6 and TNF-α secretion by 40% (p < 0.05; Fig. 1, A and B). The LPS-induced expression of IL-6 and TNF-α mRNA peaked at 4 h, and RANTES suppressed (p < 0.01) IL-6 mRNA levels at each time point examined (Fig. 1C). In contrast, RANTES had no effect on LPS-induced TNF-α mRNA expression (Fig. 1D). To determine whether RANTES affected mRNA stability, PB monocytes were pretreated with RANTES and then with LPS for 2 h, treated next with actinomycin D, and the cells were harvested from 0 to 120 min. RANTES had no effect on the decay of LPS-induced IL-6 and TNF-α (Fig. 1, E and F). These observations demonstrate that, with monocytes, RANTES decreased LPS-induced IL-6 at the transcriptional level and TNF-α at the posttranscriptional level.
Both CCR1 and CCR5, but not changes in TLR4 expression, contribute to suppression of LPS-induced cytokine production by RANTES.

Studies were performed to determine whether the effects of RANTES were mediated through CCR1 or CCR5. The addition of Abs to CCR1 (p < 0.01) or CCR5 (p < 0.05) each partially reversed the effects of RANTES on the LPS induction of IL-6 (Fig. 2A) and TNF-α (Fig. 2B). Blockade with Abs to both CCR1 and CCR5 or with Met-RANTES completely reversed (p < 0.01) the effects of RANTES (Fig. 2, A and B). These results demonstrate that the effect of RANTES on LPS-induced cytokine production in monocytes is mediated through ligation to CCR1 and CCR5.

Because LPS-induced MIP-1α was shown to reduce CCR1 on monocytic cells (26), experiments were performed to determine whether the effects of RANTES were mediated through the expression of TLR4. The treatment of PB monocytes with RANTES had no effect on the cell surface expression of either TLR4 or TLR2 (Fig. 2C). Therefore, the effects of RANTES were not mediated by the effects of TLR4 expression.

The effect of RANTES is mediated through p38 MAPK

Because monocyte chemotaxis induced by RANTES is dependent on activation of p38 MAPK (27), experiments were performed to determine whether the suppression of cytokine production was mediated through this pathway. RANTES-induced activation of p38 MAPK was determined by examining the phosphorylation of p38 (Fig. 3, A and B). The RANTES-mediated activation of p38 was suppressed (p < 0.01) by preincubation of the monocytes with Met-RANTES (Fig. 3, A and B). Our results show that RANTES signals through p38 and that the effects are still apparent after 24 h
of stimulation (Fig. 3C). The effect of RANTES on LPS-induced p38 activation was also examined. Pretreatment of monocytes with RANTES increased \( p < 0.05 \) LPS-induced p38 activation (Fig. 3, C and D). These observations demonstrate that RANTES induced the activation of p38 and that the pretreatment of monocytes with RANTES resulted in increased LPS-induced p38 activation.

**IL-10 mediates the effects of RANTES**

Because p38 regulates IL-10 in monocytic cells (28) following the treatment of monocytes with RANTES, control IgG or Abs specific for IL-10 were added together with LPS. Anti-IL-10 Ab dose-dependently reversed the effects of RANTES on LPS-induced IL-6 secretion (Fig. 4A) and the expression of IL-6 mRNA (Fig. 4B). The addition of Abs to IL-10 also abrogated the effects of RANTES on LPS-induced TNF-\( \alpha \) secretion (Fig. 4C), whereas no effect was observed on the level of LPS-induced TNF-\( \alpha \) mRNA (Fig. 4D). These observations suggest that the effects of RANTES were mediated through the expression of IL-10.

**The RANTES-enhanced expression of IL-10 is regulated by p38**

Because Abs to IL-10 reversed the effects of RANTES, studies were performed to determine the effect of RANTES on the expression of IL-10. The treatment of monocytes with RANTES increased IL-10 mRNA observed after 24 h and before the addition of LPS (Fig. 5A). In contrast, in response to RANTES alone no IL-10 was detected in the monocyte culture supernatants (Fig. 5B). The addition of LPS to RANTES-pretreated monocytes resulted in up to 4-fold increase of IL-10 mRNA (Fig. 5A). Additionally, RANTES resulted in a 3–4-fold increase of LPS-induced secretion of IL-10 (\( p < 0.01 \); Fig. 5B). When examined 1 h after the addition of LPS, IL-10 was detected in the culture supernatants of monocytes preincubated with RANTES, whereas no IL-10 was detectable in the supernatants of PBS control-treated cells (data not shown). To determine whether the effects of RANTES on IL-10 were mediated by p38, the expression of p38 was suppressed. The transfection of monocytes with p38 MAPK, but not with nonspecific control siRNA, resulted in the reduction of total and activated p38 (Fig. 5D). Inhibition of p38 MAPK by SB20380 (data not shown) or siRNA abrogated \( p < 0.01 \) the effect of RANTES on LPS-induced IL-10 secretion (Fig. 5E) and mRNA expression (Fig. 5E). These observations indicate that RANTES augments LPS-induced IL-10 production through activation of the p38 MAPK pathway.
Experiments were performed to determine whether the effects of RANTES on IL-6 and TNF-α were mediated by p38. The forced reduction of p38 MAPK reversed the RANTES-mediated suppression of the secretion of IL-6 (p < 0.05; Fig. 6A) and the expression of IL-6 mRNA (p < 0.05; Fig. 6B). Further, although the knockdown of p38 suppressed TNF-α secretion (p < 0.05; Fig. 6C), no effect on TNF-α mRNA levels was observed (Fig. 6D). At the outset of our study, p38 was inhibited by two different doses of SB203580, namely 5 and 10 μM. However, because neither concentration may be specific for p38 inhibition, the data presented focused primarily on the use of siRNA knockdown of p38. The inhibition of p38 with the inhibitor SB203580 had the same effect on IL-6 and TNF-α as did the forced reduction of p38 (data not shown). These observations indicate that p38 MAPK mediates the effects of RANTES on the LPS-induced expression of IL-6 and TNF-α.

RANTES does not decrease LPS-induced IL-6 secretion in RA synovial fibroblasts

Experiments were performed to determine whether RANTES suppressed the LPS-induced cytokine production by another cell type. LPS induced the secretion of IL-6 ∼3-fold (p < 0.01) using RA synovial fibroblasts (Fig. 7A). In contrast to monocytes, pretreatment of the synovial fibroblasts with RANTES failed to suppress the secretion of IL-6. Therefore, experiments were performed to examine the effects of RANTES and LPS on the expression of IL-10 and the activation of p38. RA synovial fibroblasts did not secrete IL-10 in response to LPS in the presence or absence of RANTES. Nonetheless, RANTES induced p38 MAPK phosphorylation (Fig. 7B) and increased (p < 0.05) LPS-induced p38 MAPK phosphorylation in RA synovial fibroblasts (Fig. 7, C and D). These observations demonstrate that RANTES does not suppress LPS-induced IL-6 secretion in RA synovial fibroblasts and suggest that this was not due to the inability to activate p38, but to the inability to secrete IL-10.

Neutralization of RANTES in RA synovial fluid increases LPS-induced TNF-α secretion from PB monocytes

Because RANTES is highly expressed in the rheumatoid joint (5, 29, 30) experiments were performed to determine whether neutralization of RANTES in RA synovial fluids could induce TNF-α secretion by normal monocytes. RA synovial fluids were incubated with mouse anti-RANTES (10 μg/ml) or control IgG for 1 h and then added to monocytes, which were then incubated with control medium or LPS. When control IgG was added, the RA synovial fluids did not induce the secretion of TNF-α after 24 h. In contrast, when RANTES was neutralized the RA synovial fluids could induce TNF-α secretion by normal monocytes. RA synovial fluids were incubated with mouse anti-RANTES (10 μg/ml) or control IgG for 1 h and then added to monocytes, which were then incubated with control medium or LPS. When control IgG was added, the RA synovial fluids did not induce the secretion of TNF-α after 24 h. In contrast, when RANTES was neutralized the RA synovial fluids could induce TNF-α secretion in the absence of LPS (p < 0.01; Fig. 8A). Human anti-RANTES Ab alone did not induce TNF-α secretion in the absence of RA synovial fluid (data not shown). Consistent with our observations using recombinant RANTES, neutralization of RANTES increased the LPS-induced TNF-α (p < 0.05; Fig. 8B) and suppressed LPS-induced IL-10 (p < 0.05; Fig. 8C). These
FIGURE 4. IL-10 mediates the effects of RANTES on LPS-induced cytokine expression. A and C, Monocytes were either treated with PBS or RANTES for 24 h and then with control IgG or anti-IL-10 (1, 10, and 20 ng/ml) together with LPS. Supernatants were harvested after 24 h and examined for IL-6 (A) and TNF-α (C). B and D, monocytes were either treated with PBS or RANTES for 24 h and then with control IgG or anti-IL-10 (20 ng/ml) together with LPS for 2 or 4 h. The cells were harvested and examined for IL-6 (B) and TNF-α (D) mRNA expression. The values were normalized by cytokine gene expression after LPS treatment for 2 h. The values represent the mean ± SE of three independent experiments. * p < 0.05.

FIGURE 5. The regulation of IL-10 by RANTES is mediated by p38 MAPK. A, Monocytes were pretreated with PBS or RANTES for 24 h and then stimulated with LPS for 0–5 h. Cells were harvested and examined for IL-10 mRNA, which was normalized with GAPDH. B, Monocytes were treated with PBS or RANTES (R) for 24 h. Cells were further stimulated with LPS for 24 h. C, Monocytes were transfected with nonspecific control (NC) or p38 MAPK siRNA for 48 h. The cells were treated with RANTES for 0–1 h, and the cell lysates were examined for total and phosphorylated p38 MAPK (p-p38 MAPK) as well as tubulin. D, PB monocytes were transfected with nonspecific control (NC) or p38 MAPK siRNA (p38 MAPKi) for 48 h and then treated with PBS or RANTES (R) for 24 h and then LPS for 24 h. Supernatants were examined for IL-10. E, Monocytes were transfected with NC or p38 and treated with RANTES as in D, and then LPS was added for 4 h and the cells were examined for IL-10 mRNA. The values in each panel represent the mean ± SE of three independent experiments. * p < 0.05; ** p < 0.01.
observations suggest that RANTES in RA synovial fluid is capable of suppressing activation mediated through TLR4 activation. Low concentrations of TNF-α and no IL-10 were detected in the synovial fluids (data not shown).

Discussion

RANTES is highly expressed in the joints of patients with RA and is important in the trafficking of leukocytes, including PB monocytes, to the site of inflammation (5, 7, 30, 31). Further, activation of RA fibroblasts with the TLR2 ligand peptidoglycan induces RANTES production through NF-κB (32). These studies were performed to determine whether RANTES or the therapeutic neutralization of RANTES might result in unanticipated consequences to the inflammatory response. We demonstrate with normal monocytes that, although RANTES did not directly induce TNF-α or IL-6, it did activate p38 MAPK (Fig. 9). Subsequent TLR4 ligation with LPS resulted in enhanced p38 activation and an earlier and increased expression of IL-10. The enhanced secretion of IL-10 suppressed the LPS-induced expression of IL-6 transcriptionally and that of TNF-α at the posttranscriptional level. Further, using RA synovial fluids we demonstrate that neutralization of RANTES resulted in reduced LPS-induced IL-10 and increased TNF-α.

Our results demonstrate that the effects of RANTES were mediated through both CCR1 and CCR5, because blocking either receptor suppressed the effects of RANTES. However, the inhibition of CCR1 was more effective than that of CCR5, perhaps due to the fact that CCR1 is more highly expressed on monocytes (31, 33). An earlier study demonstrated that the LPS-induced reduction of CCR1 and CCR2 was mediated by MIP-1α (26). RANTES did not work by affecting TLR expression, because RANTES had no effect on surface TLR4 expression in monocytes.

The effects of RANTES were mediated through p38. Reduction of p38 by siRNA or inhibition with SB203580 (data not shown) before the addition of RANTES abrogated the suppression of LPS-induced IL-6 and TNF-α. Additionally, pretreatment with RANTES increased the activation of p38 induced by LPS. However, it is unlikely that the increased p38 activation directly suppressed cytokine production, because p38 activation promotes the expression of IL-6 and TNF-α (34, 35). Numerous studies have documented the role of p38 in stabilizing IL-6 mRNA (36, 37) via
FIGURE 8. RA synovial fluid (SF) RANTES modulates LPS-induced cytokine expression. RA synovial fluids were diluted 1/20 and incubated with mouse anti-human RANTES (anti-RANTES) or control IgG (Cont. Ig) (10 mg/ml) for an hour. The fluids were incubated with control IgG or anti-RANTES for 1 h and then added to PB monocytes; the supernatants were collected after 24 h (A) or cells were stimulated with 4 h of LPS (B and C). The supernatants were examined for TNF-α (A and B) or IL-10 (C). The values represent the mean ± SE of three independent experiments. *, p < 0.05; **, p < 0.01.
the activation of its downstream target MAPK-activated protein kinase-2 (MK2) that is mediated through AU-rich elements of IL-6 (36). The activation of p38 may also regulate the transcription of IL-6 by increasing transcription (38). The activation of p38 promotes LPS-induced IL-6 transcription by enhancing the accessibility of the cryptic NF-κB binding site of the IL-6 promoter, but not the TNF-α promoter (38). In contrast, p38 promotes the translation of LPS-induced TNF-α with little or no effect on mRNA stability, and this effect is mediated through the AU-rich elements of TNF-α (37, 39, 40). In our study the activation of p38 by RANTES resulted in the reduction of both TNF-α and IL-6, suggesting that p38 was not directly involved.

The effects of RANTES on LPS-induced IL-6 and TNF-α were mediated by IL-10, which was secreted by the monocytes following the addition of LPS. Consistent with earlier results (41), RANTES alone did not induce IL-6 and TNF-α in monocytes. Additionally, RANTES alone did not result in the secretion of IL-10, although IL-10 mRNA was readily detected in monocytes following incubation with RANTES. The discrepancy between the effects of RANTES on the transcription and secretion of IL-10 may be due to a number of factors. Our assay may not have detected low levels of IL-10 that may have been secreted, even though our assay was sensitive to 15 pg/ml. It is possible that the signal induced by RANTES was not strong enough to induce IL-10 secretion or that a signal in addition to p38 was required to promote IL-10 translation or secretion. When LPS was added to monocytes that had been preincubated with RANTES, the expression of IL-10 mRNA was increased at all of the time points examined. Additionally, in monocytes preincubated with RANTES, IL-10 was detected in the culture supernatants 1 h after LPS stimulation, whereas, in the case of cells preincubated with the control PBS, IL-10 was first detected 2 h after stimulation (data not shown). The LPS-induced secretion of IL-10 was increased up to 4-fold following incubation with RANTES. The activation of p38 was necessary for the expression of IL-10, because p38 siRNA or chemical inhibition with SB203580 (data not shown) prevented the RANTES-induced expression of IL-10. This observation supports the data demonstrating that the inhibition of p38 suppresses LPS-induced IL-10 production in THP-1 cells (28). The same study demonstrated that p38 activates transcription of the human IL-10 gene through the transcription factor SP1 (28). In summary, pre-treatment with RANTES induced the expression of IL-10 mRNA and resulted in the earlier and enhanced LPS-induced IL-10 secretion by monocytes.

The knockdown of p38 in monocytes resulted in reduced LPS-induced TNF-α and IL-6. When the cells were treated with non-specific control siRNA plus RANTES, LPS-mediated TNF-α and IL-6 secretion were also reduced. However, when p38 was knocked down and RANTES was added, the suppressive effects of each treatment were reversed. There are a number of potential explanations for these observations. For example, p38 siRNA likely did not totally deplete the monocytes of p38. It is possible that because the expression of IL-10 was reduced following the addition of LPS to the RANTES, sufficient p38 may have been activated to permit the full expression of TNF-α and IL-6. Another potential explanation may be related to the observation that RANTES activated other pathways, including NF-κB and JNK (data not shown). It is possible that activation of these pathways by both RANTES and LPS may have contributed to the reduced suppression of TNF-α and IL-6 expression when p38 was knocked down (Fig. 9).

Secreted IL-10 was responsible for the effects of RANTES on LPS-induced cytokine production in monocytes. Abs to IL-10 completely reversed the effects of RANTES, demonstrating that the suppression of LPS-induced IL-6 and TNF-α by RANTES was mediated through IL-10. Although RANTES signaled through p38 MAPK and increased LPS-induced p38 MAPK phosphorylation in RA synovial fibroblasts, RANTES failed to reduce LPS-induced IL-6 secretion in this cell type. The fact that IL-10 was not induced by RANTES in the presence or absence of LPS by RA synovial fibroblasts supports the importance of IL-10 in mediating the effects of RANTES. Therefore, p38 MAPK activation by RANTES was not sufficient to decrease LPS-induced cytokine expression.

The mechanisms contributing to the regulation of cytokine gene expression by enhanced production of IL-10 were not characterized in this study other than to document that the effects on IL-6 were transcriptional, whereas those on TNF-α were posttranscriptional. Previous studies have demonstrated that IL-10 enhances IL-6 mRNA degradation in PB mononuclear cells (42, 43). In contrast, we saw no effect on the decay of IL-6 mRNA. These differences may be due to the concentrations of IL-10, because the concentration secreted by our monocytes was much lower than that added exogenously in the earlier studies (43, 44). It is possible that STAT3 or NF-κB may be responsible for the effects of IL-10. In STAT3-null mice or in human macrophages transfected with a dominant negative STAT3, IL-10 is unable to reduce LPS-induced IL-6 production (45, 46). However, others have shown that IL-10 inhibits LPS-induced IL-6 transcription by inhibiting NF-κB activation in human monocytes (44). These observations suggest that the effects of IL-10 on the expression of IL-6 may be mediated through STAT3 or NF-κB.

In murine macrophages, IL-10 inhibits TNF-α posttranscriptionally by blocking LPS-induced p38 activation (47). However, in human monocytes IL-10 did not suppress LPS-mediated phosphorylation of p38 MAPK (48). Similarly, with human macrophages the suppression of TNF-α by IL-10 was not through p38 but was mediated through the STAT3 pathway (46, 49). Consistent with these observations, we did not see a reduction of p38 activation at any time point examined. It is possible that the activation of STAT3 may interfere with events downstream of p38, perhaps interfering with the action of MK2 or producing other effects independently of p38 and MK2. It is possible that there are differences between species and cell types in the way that IL-10 exerts its anti-inflammatory effect.
The biological relevance of our observations is suggested by studies using synovial fluids from patients with RA. The neutralization of RANTES in RA synovial fluid resulted in the significantly increased induction of LPS-mediated TNF-α and reduced IL-10 level in PB monocytes. These results suggest that synovial fluids contain concentrations of RANTES sufficient to modulate activation through TLR4. Potential TLR4 ligands such as heat shock protein and extra domain A fibronectin are abundant in the synovial fluids. Consistent with this possibility, following the neutralization of RANTES, RA synovial fluid-induced monocyte secretion of TNF-α. These observations suggest that therapeutic intervention of chronic inflammation may not result in the expected effect because of the potential effects of RANTES in suppressing the inflammatory response.

This possibility is supported by our observations in adjuvant-induced arthritis. We previously reported that treatment with the RANTES antagonist Met-RANTES ameliorates adjuvant-induced arthritis (7). In contrast, unpublished data from our laboratory demonstrate that the inhibition of RANTES after disease onset in adjuvant-induced arthritis did not alleviate the disease (data not shown). Although these results may indicate that the inhibition of RANTES may simply not be sufficient to treat established disease, it also possible that, in addition to suppressing leukocyte traffic into the joint, the inhibition of RANTES may actually enhance the ongoing inflammatory process. Although further studies will be required to discern these possibilities, these observations suggest that RANTES, and possibly other chemokines such as MCP-1 (52, 53), may have previously unrecognized mechanisms that are important in modulating chronic inflammation.

Acknowledgments

We acknowledge Dr. Amanda E. I. Proudfoot for providing us with Met-RANTES.

Disclosures

The authors have no financial conflict of interest.

References


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