

Proinflammatory properties of the human S100 protein S100A12

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Abstract: S100 proteins represent a new class of chemoattractants. Here we extend earlier evidence for the proinflammatory properties of human S100A12. A12 induced migration of monocytoid cells, with optimal activity at 10^{-10} M and potency of $>10^{-9}$ M C5a. Neutrophils were poorly responsive, and lymphocyte migration was not affected. Actin polymerization in monocytoid cells was accompanied by a sustained $[Ca^{2+}]_i$ flux of a magnitude comparable with C5a. A12 elicited a transient infiltration of neutrophils (4–8 h) and more delayed recruitment of monocytes (8–24 h) in vivo. A12 (~70 nM) was present in synovial fluid (SF) from rheumatoid arthritis patients, and synovium contained A12-positive neutrophils in the sublining and interstitial region, often surrounding the perivascularity but rarely in the synovial lining layer, although some macrophages were positive. The A12 gene was transiently up-regulated in monocytes by tumor necrosis factor α (6 h); induction by lipopolysaccharide (LPS) was sustained (12–48 h). A12 may contribute to leukocyte migration in chronic inflammatory responses. *J. Leukoc. Biol.* 69: 986–994; 2001.

Key Words: leukocyte recruitment · rheumatoid arthritis · gene induction

INTRODUCTION

S100A12 (calgranulin C) is a member of the S100 multigene family of calcium-binding proteins implicated in the Ca^{2+} -dependent regulation of a variety of intracellular activities, including protein phosphorylation, enzyme activities, cell proliferation and differentiation, the dynamics of cytoskeletal rearrangement and structural organization of membranes, intracellular Ca^{2+} homeostasis, inflammation, and protection from oxidative cell damage [1]. S100A12 (A12) is constitutively expressed in neutrophils [2]. It has been associated with host responses to *Onchocerca volvulus* infection [3] and has filariacidal and filariastatic activities [4]. A12, reported as corneal-associated antigen, has been identified as the target for autoantibodies in Mooren's ulcer [5], and it is up-regulated by proinflammatory cytokines in corneal fibroblasts [6]. Recently, A12 from bovine lung tissue was identified as a ligand for the receptor for advanced glycation end products

(RAGE [7]). A12 is induced in mononuclear cells and the Jurkat T-cell line, and infusion of lipopolysaccharide (LPS) into mice causes time-dependent release of A12-like protein into plasma. Moreover, A12 mediates migration and activation of monocytes through RAGE binding. Footpad injection of bovine A12 into mice results in an influx of leukocytes over 24 h, although the cell types recruited have not been characterized. Anti-A12 antibody or a RAGE analogue substantially blocks leukocyte recruitment in murine models of delayed-type hypersensitivity and colitis [7]. When all of these activities are taken together, A12 appears to play a central role in innate and acquired immune responses.

A12 has highest amino acid sequence homology with S100A9 (47%), which is expressed constitutively together with S100A8 in neutrophils. The S100A8/A9 complex, known as calprotectin [8], is associated with chronic inflammation including rheumatoid arthritis (RA), cystic fibrosis, Crohn's disease, ulcerative colitis, allergic dermatitis, infection, and psoriasis [8]. Since our first demonstration of the potent chemotactic activity of murine S100A8 (mA8, formerly called "CP-10") for neutrophils and monocytes in vitro [9] and in vivo [10, 11], S100A2 and S100A7 were also reported to have chemotactic activity for eosinophils and lymphocytes, respectively [12, 13], suggesting a new class of chemoattractants.

Here we present additional evidence for the chemotactic properties of human A12 (hA12) for human neutrophils and monocytes in vitro and in vivo. A12 initiated actin polymerization accompanied by a calcium flux in monocytoid cells. We show that A12 in monocytes was up-regulated by LPS and tumor necrosis factor (TNF) α , and we report the first evidence of expression of A12 in the synovium and SF from rheumatoid patients, supporting the notion that A12 may contribute to the pathogenesis of acute and chronic inflammatory responses.

MATERIALS AND METHODS

Cytokines and stimulants

LPS (from *Escherichia coli* 055:B5) was purchased from Difco (Detroit, MI). Recombinant TNF α (endotoxin content, <1 EU/mg; specific activity, 6×10^7

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U/mg) was a gift from G. R. Adolf (Ernst-Boehringer Institut für Arzneimittel-forschung, Vienna, Austria). Recombinant interleukin (IL)-1 β (specific activity, 1.87×10^5 U/ μ g) was purchased from R & D Systems (Minneapolis, MN).

Cell culture

THP-1 cells (American Type Culture Collection, Manassas, VA) and Mono Mac 6 cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were maintained in RPMI 1640 as stated below.

Cells were routinely cultured at 37°C in 5% CO₂ in air in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine (Flow Laboratories, Irvine, Scotland), 100 U/mL of penicillin, 100 μ g/mL of streptomycin (Sigma, St Louis, MO), and 10% heat-inactivated bovine calf serum (BCS; Hyclone Laboratories, Logan, UT) unless otherwise stated. The medium was sterilized by filtration through Zetapor 0.2- μ m membrane (Cuno, Meriden, CT) to remove contaminating traces of endotoxin. Endotoxin levels in reagents and medium were routinely monitored and used at <50 pg/mL, tested by the chromogenic *Limulus* amoebocyte lysate assay (Cape Cod Associates, Woods Hole, MA).

Citrated blood from normal donors (Department of Haematology, Prince of Wales Hospital, Sydney, Australia) was used as a source of neutrophils and monocytes in most experiments. Neutrophils were isolated using Mono-Poly resolving medium (Flow Laboratories) as previously described [9].

For isolation of monocytes and lymphocytes, erythrocytes were removed by sedimentation in 0.5% dextran T500 (Pharmacia, Uppsala, Sweden), 0.5% D-glucose (BDH Laboratory Supplies, Poole, England) at room temperature for 45 min. Leukocytes were collected from the plasma by centrifugation at 600 g for 10 min, washed twice in Ca²⁺, Mg²⁺-free Dulbecco's phosphate-buffered saline (PBS, pH 7.3) (Sigma) supplemented with 0.38% sodium citrate, and then suspended in PBS. The cells were then layered on Lymphoprep (Nycomed, Oslo, Norway) and centrifuged at 700 g for 30 min at room temperature. Mononuclear cells were collected from the interface, washed twice with PBS, and suspended in RPMI 1640 containing 2% BCS at 5×10^6 /mL, and monocytes were left to adhere onto tissue culture plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ). Nonadherent cells, which comprise >95% lymphocytes, were removed after incubation at 37°C for 30 min. Adherent monocytes were washed twice with warmed medium and detached from the surface after incubating in RPMI containing 10% BCS overnight.

Isolation of hA12

Erythrocytes remaining in leukocyte suspensions from whole blood by dextran sedimentation were removed by lysis in ice-cold isotonic ammonium chloride (155 mM NH₄Cl, 10 mM NaHCO₃, and 0.1 mM EDTA) for 10 min. Cells were washed with PBS and centrifuged at 600 g for 5 min at 4°C. The neutrophil pellet was lysed in 0.2 mM Tris-HCl–0.1% Triton X-100 (Sigma), pH 8.0, containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablet; Boehringer Mannheim, Mannheim, Germany). Cell debris was removed by centrifugation at 20,000 g for 10 min at 4°C, and the supernatant was dialyzed against 25 mM NaOAc, 25 mM NaCl, pH 4.5, at 4°C, followed by centrifugation at 20,000 g for 10 min.

The dialysate was fractionated by cation-exchange chromatography on a Mono S HR 5/5 column (Pharmacia) equilibrated with 25 mM NaOAc, 25 mM NaCl, pH 4.5. Elution was performed over 25 min at a flow rate of 1 mL/min with a linear concentration gradient of 0.025–1.0 M NaCl in 25 mM NaOAc, pH 4.5. Fractions of 1.0 mL each were collected and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by silver staining and Western blot analysis. A12 was identified using polyclonal anti-A12 rabbit immunoglobulin G (IgG). Fractions containing A12 (eluted in 0.35–0.42 M NaCl) were pooled and further fractionated on a C4-RP-HPLC Vydac 300 Å, 5 μ m, 250 mm by 4.6 mm column (Separations Group, Hesperia, CA) with a gradient of 35–50% acetonitrile–0.1% trifluoroacetic acid at 1 mL/min over 35 min. A12 eluted in 45% acetonitrile; purity was confirmed by SDS-PAGE, silver staining, and Western blot analysis. The protein stored in acetonitrile was lyophilized and resuspended in sterile saline immediately before use. In A12 preparations used for in vitro and in vivo studies, endotoxin levels were <2 pg/10 μ g of A12.

A12 expression plasmid and isolation of recombinant protein

Single-stranded cDNA was synthesized from human bone marrow polyadenylate-RNA using a SuperScript™ Preamplification System (GIBCO). hA12 DNA was amplified from the cDNA mix with two oligonucleotides 5'-CGGGATC-CACAAAACCTTGAAGA GCATCTGGAGGG-3' and 5'-CGGGATCCCTACTCTTTGTGGGTGTGG-3'. PCR-amplified fragments were digested with *Bam*HI and cloned into the Glutagene pGEX2T bacterial expression vector (Pharmacia) to produce the recombinant expression plasmid.

The glutathione-S-transferase–A12 fusion protein was induced and affinity purified essentially as previously described [14]. The fusion protein was extracted from the bacterial lysate using glutathione-agarose (Sigma), and bound fusion protein was eluted with 25 mM glutathione. Recombinant A12 (rA12) was cleaved from the fusion protein with bovine α -thrombin (Sigma) in 25 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 incubated at 37°C for 24 h. Preparative C8-RP-HPLC (Aquapore, 10 mm by 100 mm; Applied Biosystems Inc., Foster City, CA) was used to separate rA12 from cleaved fusion protein in a 20-min gradient of 35–65% acetonitrile–0.1% trifluoroacetic acid at 3 mL/min. rA12 was further purified on C4-RP-HPLC as described above, and purity was confirmed by SDS-PAGE and Western blot analysis. A12, stored in acetonitrile at –80°C, was lyophilized just before use and resuspended in saline for testing. Endotoxin levels were <2 pg/10 μ g of A12.

Production of polyclonal anti-A12 antibodies

New Zealand White rabbits were immunized by multiple intradermal dorsal injections with 50 μ g of pure human native A12 in 0.9% NaCl mixed in complete Freund's adjuvant (Sigma) and boosted 2 and 6 weeks later in incomplete Freund's adjuvant (Sigma), containing the same amount of protein. Antisera were collected at 2-week intervals thereafter and tested for reactivity to S100A8, -A9, and -A12 on Western blots. IgG from prebleed rabbit serum and from anti-A12 sera was purified by protein A affinity chromatography (Pharmacia). The anti-A12 polyclonal IgG cross-reacted weakly with S100A8 and S100A9, and reactivity was removed by affinity absorption with recombinant A8 and A9 (prepared according to protocols described elsewhere [43]) coupled to CNBr-Sepharose (Pharmacia).

RNA extraction and semiquantitative reverse transcriptase-PCR

Total RNA was extracted using the single-step guanidinium thiocyanate-phenol-chloroform method [15]. To analyze expression of A12 mRNA, oligonucleotides corresponding to the N-terminal and C-terminal amino acids of the protein (5'-ACAAAACCTTGAAGAGCATCTGGAGGG-3' and 5'-CTACTCTTTGTGGGTGTGG-3') were used and yielded a 273-bp fragment. Typically, 0.5 μ g of total RNA was reverse-transcribed to the first-strand cDNA using the SuperScript™ preamplification system (GIBCO). The resulting cDNA mix (1/20 of cDNA reaction volume) was amplified using 10 pmol of each A12 primer and 2.5 U of Taq DNA polymerase (Perkin-Elmer, Branchburg, NJ). cDNA concentrations of the samples were monitored by the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the two primers 5'-CCACCCATGGCAAATTCATGCGCA-3' and 5'-TCTAGACGGCAGGT-CAGTCCACC-3'), which yielded an amplification product of 600 bp. PCR was performed in a GeneAmp PCR System 2400 (Perkin-Elmer); cycling parameters of 95°C for 2 min followed by 30 cycles of 95°C for 45 s, 60°C for 1 min, and 72°C for 1 min were used.

Detection of A12 protein

A double-sandwich enzyme-linked immunosorbent assay (ELISA) was developed. Flat-bottom 96-well microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated at 50 μ L/well with 5 μ g/mL of anti-A12 IgG in 0.05 M sodium carbonate buffer, pH 9.6; incubated for 2 h at 37°C; washed four times with 20 mM Tris-HCl, 150 mM NaCl, and 0.01% Tween 20, pH 7.4 (wash buffer) and blocked with 0.1% ovalbumin (Sigma) in wash buffer for 1 h at 37°C. Plates were washed four times before samples were added. Samples diluted in RPMI 1640 containing 0.1% BSA (100 μ L/well) were incubated overnight at 4°C. Recombinant A12 (0.0625–2.5 nM) diluted in RPMI 1640 and 0.1% BSA was included as the standard. After four washes, biotinylated

anti-A12 IgG (4 $\mu\text{g}/\text{mL}$, 100 $\mu\text{L}/\text{well}$) was added, incubated for 1 h at 37°C, and washed four times with wash buffer. Plates were incubated with streptavidin-horseradish peroxidase conjugate (1:1,000 dilution; Amersham, Buckinghamshire, England) for 1 h at 37°C. After washing, plates were incubated with ABTS [2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid)] and H_2O_2 (Kirkegaard & Perry, Gaithersburg, MD) for 1 h at room temperature, the reaction was stopped with 1% SDS, and absorbency at 405 nm was measured (Titertec Multiscan MCC/340; Labsystem, Helsinki, Finland).

Chemotaxis assay

Cells ($5 \times 10^6/\text{mL}$) suspended in Hanks balanced salt solution (HBSS; Sigma) and 0.2% BSA were incubated with 5 μM calcein AM (Molecular Probes Inc, Eugene, OR) for 15 min at 37°C in 5% CO_2 in air, washed twice with HBSS, and resuspended at $0.5 \times 10^6/\text{mL}$ in RPMI 1640 and 0.2% BSA. Assays were performed using Neuro Probe MBA 96-well chambers fitted with polycarbonate membranes (Neuro Probe, Inc., Bethesda, MD) with pore sizes of 5 μm for neutrophils and monocytes, or 8 μm for THP-1 cells. Native A12 or rA12 (10^{-13} to 10^{-8} M) diluted in RPMI 1640 and 0.2% BSA (410 μL) were placed in the lower compartment, and cells (300 μL) in the upper compartment. The chamber was incubated at 37°C in 5% CO_2 in air for 90 min for neutrophil chemotaxis or for 2 h for monocytes or lymphocytes. The number of migrated cells collected in the lower chamber was measured by fluorescence at $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 530$ nm using a CytoFluor[®] multiwell plate reader system (PerSeptive Biosystems, Framingham, MA). Cell numbers were extrapolated from a standard curve calibrated based on a fluorescence reading of a known number of cells. C5a (Sigma) or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma; 10^{-9} M) was used as a positive control in all experiments. Data from at least three experiments were analyzed using the Student's *t*-test.

Actin polymerization

Cells ($0.5 \times 10^6/\text{mL}$) suspended in HBSS and 0.2% BSA were prewarmed at 37°C in 5% CO_2 before addition of A12 preparations diluted in the same buffer at 37°C. The reaction was performed in 96-well plates and initiated by mixing 2.5×10^4 cells with 50 μL of A12 preparations to give final concentrations between 10^{-12} to 10^{-9} M. At various times, fixative (3.7% formaldehyde) was added, the plate was immediately placed on ice, and cells were permeabilized and stained for F-actin for 60 min with a mixture of 0.05% (w/v) digitonin (Calbiochem, San Diego, CA) and 0.5 μM fluorescein isothiocyanate (FITC)-phalloidin (Sigma). Cells were then pelleted and washed twice in HBSS. Fluorescence emission was assayed at $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 530$ nm. fMLP (10^{-9} M) was used as positive control in all experiments.

Measurement of cytosolic Ca^{2+} flux

Changes in $[\text{Ca}^{2+}]_i$ were monitored using the fluorescent probe Fluo-3 (Molecular Probes Inc.). Cells ($5 \times 10^6/\text{mL}$) suspended in HBSS containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10 mM glucose, and 0.2% BSA were incubated for 30 min at 37°C with 10 μM Fluo-3 added from a 10 mM dimethyl sulfoxide stock solution. The cell suspension was then diluted with an equal volume of HBSS and 0.2% BSA, incubated for 30 min at 37°C, centrifuged, washed in HBSS, and resuspended in HBSS plus 20 mM HEPES, 10 mM glucose, and 0.2% BSA ($2 \times 10^6/\text{mL}$). Prior to the assay, 5 μL of anti-fluorescein-rabbit IgG (A-889; Molecular Probes Inc.) per mL of cell suspension was added, and 50 μL of cell suspension was then loaded to a 96-well plate containing 50 μL of various dilutions of A12. Fluorescence emission was assayed immediately for 10–20 cycles (10 s/cycle) at $\lambda_{\text{ex}} = 480$ nm and $\lambda_{\text{em}} = 530$ nm. C5a or fMLP (10^{-8} M) was used as positive controls in all experiments.

Leukocyte recruitment in vivo

Specific pathogen-free-BALB/c mice (8–10 weeks old) were injected intraperitoneally with 1.0 mL of HBSS containing 10 μg of rA12 or native A12 (total endotoxin level < 2 pg). Cells were harvested by peritoneal lavage using 6–8 mL of HBSS plus 3.8% (w/v) sodium citrate, and cell numbers were counted. Viability of the washed cell suspensions was >98% as determined by trypan blue exclusion. Differential staining was performed using a modified Wright-Giemsa stain (Diff-Quik Set; Lab-Aids, Sydney, Australia) on cytospin preparations. An average of 300 cells/slide were counted, and mean numbers

of total cells, monocytes, lymphocytes, and neutrophils from at least three animals were recorded.

Isolation of S100 proteins from SF

SF obtained from RA patients was depleted of cells by centrifugation at 600 *g* for 5 min at 4°C, and 3 mL of the fluid was mixed with an equal volume of ice-cold PBS and applied to rabbit anti-A9 or anti-A12 IgG coupled to CNBr-Sepharose (Pharmacia). Samples were mixed for 16 h at 4°C on a rotating wheel, and unbound protein was removed by washing twice in 10 mL PBS plus 10 mL of 1.0 M NaCl and twice with 10 mL of PBS. Bound protein was eluted with two 2-mL washes of 0.1 M glycine, pH 3.0, and separated on a Vydac C4 RP-HPLC column as described above. Eluted proteins were concentrated by lyophilization and analyzed by SDS-10% PAGE followed by silver staining and Western blot analysis.

Immunohistochemical staining of rheumatoid synovium

Formalin-fixed, paraffin-embedded, inflamed synovial tissue ($n = 4$) from patients with RA was serially sectioned (4 μm) and processed immunohistochemically to determine the expression and cellular source of A12. In brief, tissue sections were blocked with goat serum [1:5 (v/v)] diluted with 2% BSA plus 0.05 M Tris-buffered saline, pH 7.6, for 30 min at room temperature, followed by an overnight incubation at 4°C with 2 to 10 $\mu\text{g}/\text{mL}$ of antibody to A12, CD68 (Dako, Sydney, Australia) or isotype control IgG (Dako). Sections were extensively washed in Tris-buffered saline, after which biotin-conjugated antibodies (Dako) were added (goat anti-rabbit for A12 and goat anti-mouse for CD68) for 30 min. Sections were rinsed and incubated with horseradish peroxidase-conjugated streptavidin (Dako), and immunoreactivity was revealed by adding 3-amino-9-ethylcarbazole (Sigma), followed by counterstaining with hematoxylin. Other control reactions included sections incubated in the absence of primary antibody.

Statistical analysis

Data are presented as the means \pm SE of the specified number of experiments. Statistical comparisons were performed using Student's *t*-test for unpaired data.

RESULTS

Chemotactic response of human leukocytes to A12

Human monocytic cells, neutrophils, and lymphocytes were tested for chemotactic responsiveness to hA12 in vitro. rA12 (10^{-13} to 10^{-8} M) consistently induced dose-dependent migration of THP-1 cells (**Fig. 1A**) with optimal activity at 10^{-10} M ($P < 0.01$ compared with control). The native and recombinant proteins induced similar responses with blood monocytes (**Fig. 1B**, **Fig. 2**), with maximal activity at 10^{-10} M ($P < 0.01$) which was generally more potent than responses provoked by 10^{-9} M C5a ($P < 0.05$ compared with C5a; **Fig. 2**). Addition of equivalent concentrations of A12 in the upper and lower chambers abolished migration (**Fig. 1A**; $P < 0.01$ relative to maximal chemotaxis at 10^{-10} M A12 in the lower chamber), confirming A12's ability to induce directional migration. In contrast, neutrophils responded only weakly to 10^{-10} M A12 with activity consistently fourfold less than that provoked by C5a ($P < 0.01$; **Fig. 2**), and responses did not increase with higher or lower amounts of A12. Lymphocytes were unresponsive to A12 over the concentration range.

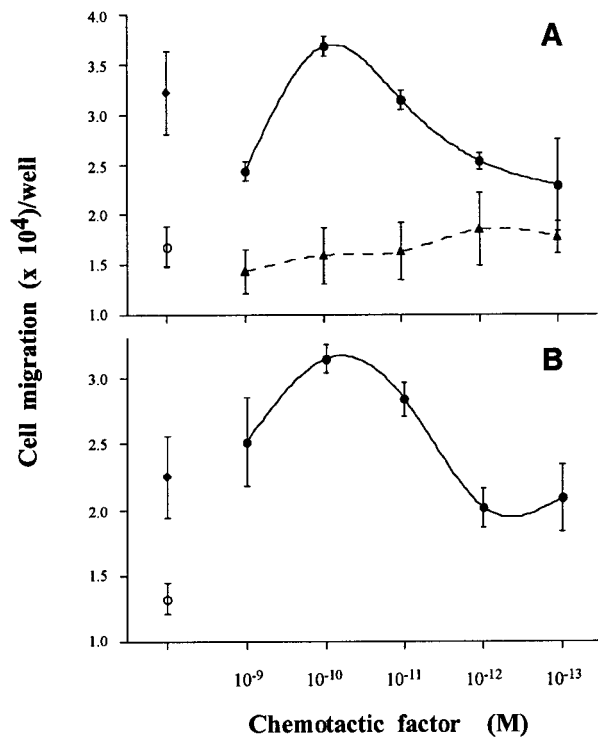


Fig. 1. Chemotactic response of THP-1 cells (A) and monocytes (B) to increasing doses of hA12 (A), rA12, or (B) native A12 (●). Responses were compared to the basal migration in RPMI 1640-0.2% BSA (○) and 10⁻⁹ M C5a (◆). The ability of A12 to induce directional versus random migration was confirmed by adding equivalent concentrations of A12 in the upper and lower chambers (▲). Data, expressed as mean numbers of migrated cells in six wells ± SD of a single experiment, are given and are representative of three experiments.

hA12 induces actin polymerization and calcium-influx in monocytoid cells

Polymerization of F-actin is a central event in cell migration. The ability of A12 to cause F-actin polymerization was determined by quantitating FITC-phalloidin-stained polymerized F-actin in THP-1 cells. Changes were obvious with 10⁻¹² to 10⁻⁹ M rA12. **Figure 3A** shows rapid elevation (≤30 s) in polymerization provoked by fMLP and rA12 compared with unstimulated cells. Increases in actin polymerization were followed by characteristic oscillations of depolymerization and polymerization. The initial response to rA12 at between 10 and 30 s was followed by stronger polymerization between 45 and 60 s.

Changes in [Ca²⁺]_i by A12 (10⁻¹¹ M) are shown in Fig. 3B. Increases were of a magnitude comparable to that of C5a (10⁻⁸ M). Effects were evident with between 10⁻¹³ to 10⁻⁸ M A12 (data not shown).

A12 recruits leukocytes in vivo

To determine whether A12 activities in vitro reflect its ability to recruit leukocytes in vivo (**Fig. 4**), A12 (*n* = 9) was injected intraperitoneally into mice and was found to increase peritoneal leukocyte numbers from 1.8 ± 1.0 × 10⁶ in animals (*n* = 12) injected with vehicle control to 4.2 ± 1.9 × 10⁶ after 8 h (*P* < 0.01). Neutrophils increased from 1.59 ± 0.718 × 10⁵ in

control mice to 18.27 ± 7.42 × 10⁵ cells in mice injected with A12 (*P* < 0.05) and declined to normal levels by 24 h (*P* < 0.05 compared with 8 h). At 8 h, macrophage numbers increased above those of control mice [from 13.86 ± 0.42 × 10⁵ to 18.97 ± 1.29 × 10⁵ cells (*P* < 0.01)] and were even higher at 24 h [23.1 ± 1.852 × 10⁵ (*P* < 0.05) compared with levels at 8 h]. In contrast, lymphocyte numbers at 8 and 24 h remained relatively unchanged compared with numbers in control-injected mice. Total leukocyte numbers in A12-injected mice declined by 24 h although levels were still somewhat higher compared with controls, but differences were not significant [3 ± 2.1 × 10⁶ (*n* = 6; *P* > 0.05)].

Isolation of S100A8, -A9, and A12 from SF

S100A8 and -A9 are associated with inflammatory processes, particularly RA. Using anti-S100A9 IgG Sepharose (which recognizes the A8-A9 complex), approximately 2 and 1.35 μmol/L of A8 and A9 were detected, eluting with retention times of 18.7–19.2 and 19.5–19.9 min, respectively. **Figure 5** shows the RP-HPLC elution profile of A12 (retention time, 17.7–17.9 min) eluted from an anti-A12-IgG. Approximately 70 nmol/L of A12 was extracted in this manner. Identities of the proteins were confirmed by Western blot analysis with anti-A8, -A9, and -A12 IgG. No A12 was extracted using anti-A9 IgG Sepharose.

Localization of A12 in RA synovium

To immunolocalize cells expressing A12, synovial tissue from RA patients (*n* = 3) was examined immunohistochemically using anti-A12 IgG. In all cases, A12 staining was associated with neutrophils in the sublining and interstitial region, often within the perivascular region (**Fig. 6A**). A12 was rarely detected in the synovial-lining layer (Fig. 6B), although occasional macrophages were A12 positive (Fig. 6C), confirmed by sequential sections stained with CD68 (Fig. 6D). Positive staining of A12 in macrophages was always less intense than in neutrophils. Specificity of anti-A12 reactivity was confirmed by the use of preimmune IgG and with anti-A12 IgG preadsorbed with 10-fold excess rA12 (data not shown).

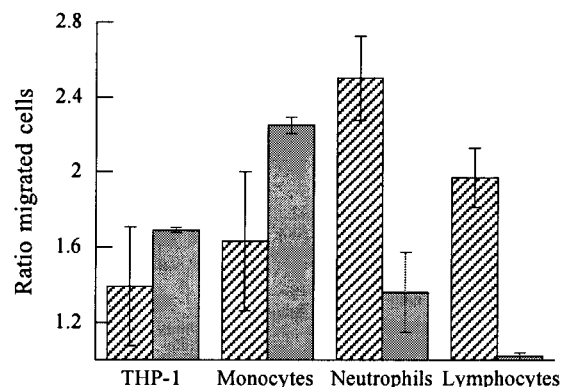


Fig. 2. Chemotactic response of THP-1 cells, monocytes, neutrophils and lymphocytes towards C5a (10⁻⁹ M) (hatched bars) or recombinant A12 (10⁻¹⁰ M) (filled bars). Data shown are means ± SD of results of three experiments of six wells, in which the ratio of migration compared with control is given.

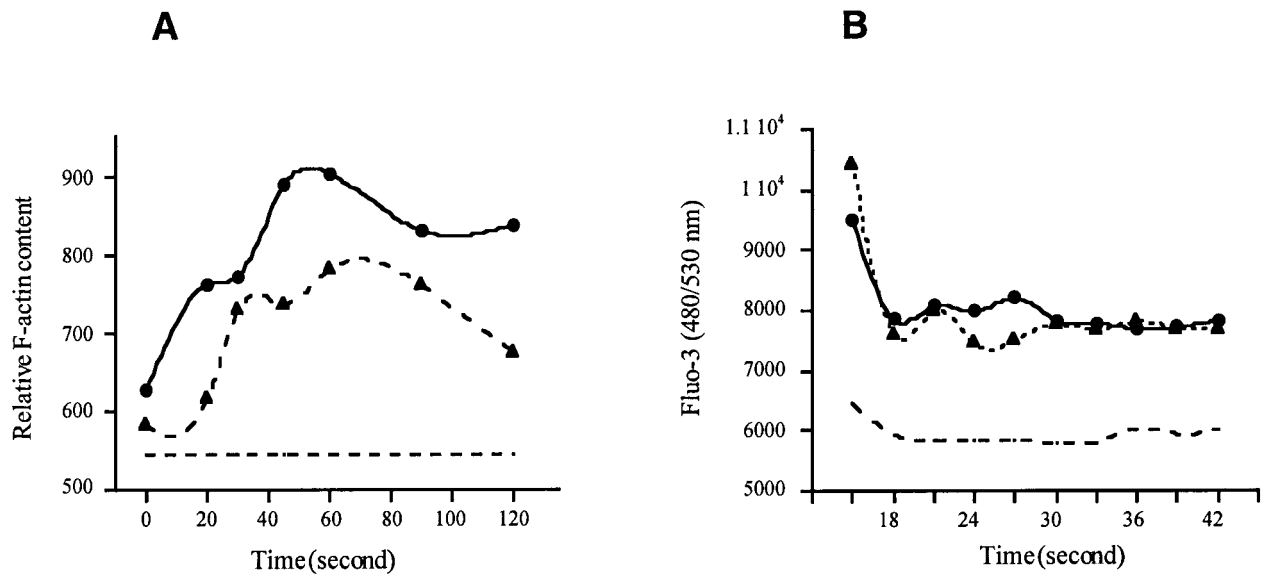


Fig. 3. (A) Relative F-actin content in THP-1 cells stimulated with fMLP (10^{-9} M) (▲) or rA12 (10^{-11} M) (●) over 120 s. Basal F-actin content of cells in RPMI 1640/0.2% BSA is shown as the dashed line. Samples were analyzed by fluorescence emission of FITC-phalloidin-labeled cells as described in Materials and Methods, and data are representative of three experiments. (B) Effect of C5a (10^{-8} M) (▲) or rA12 (10^{-11} M) (●) on $[Ca^{2+}]_i$ in THP-1 cells. Basal $[Ca^{2+}]_i$ of cells in RPMI 1640/0.2% BSA is shown as the dashed line. Samples were analyzed by fluorescence emission of Fluo-3-labeled cells as described in Materials and Methods. The data are representative of three experiments.

A12 is inducible in monocytoid cells

Expression of A12 was initially investigated in Mono Mac 6 cells. A12 mRNA was barely detected in unstimulated cells by RT-PCR (Fig. 7). Substantial transcripts were evident after

exposure to LPS, with approximately threefold induction with 1 ng/mL of LPS. Maximal stimulation occurred with 10 ng/mL of LPS (Fig. 7A), whereas higher amounts, particularly 500 ng/mL, yielded approximately half the mRNA induced by 10

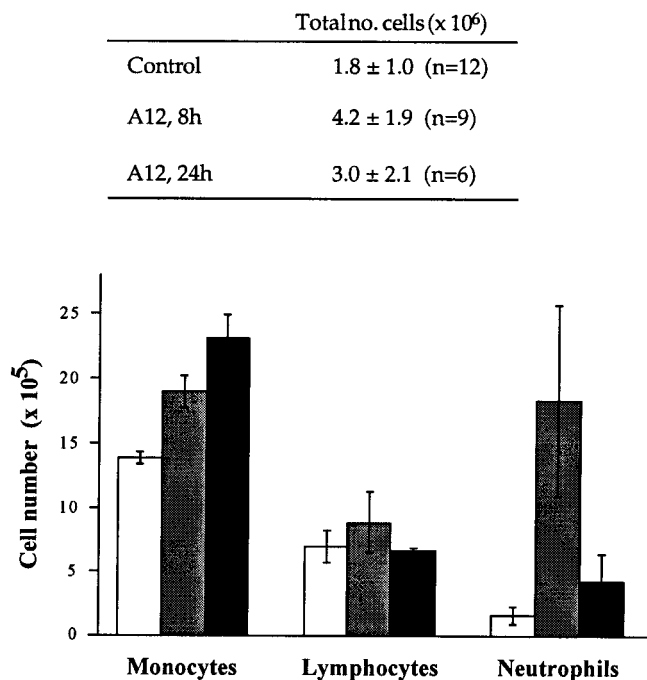


Fig. 4. Accumulation of leukocytes elicited by intraperitoneal injection of 10 μ g A12. Insert table represents the total number of peritoneal leukocytes accumulated 8 and 24 h after injection with A12 or vehicle control. Monocytes, neutrophils, and lymphocytes recruited at 8 (gray bars) and 24 h (black bars) postinjection with A12 or with vehicle control (white bars) were determined by differential staining. Results are the means \pm SD from at least three animals.

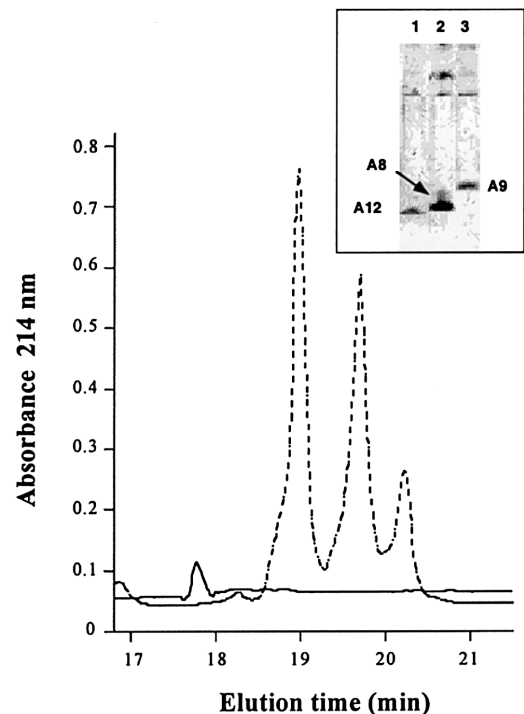


Fig. 5. S100 proteins in SF from RA patients. RP-HPLC elution profiles of S100 proteins extracted by anti-A12 IgG Sepharose (—) and anti-A9 IgG Sepharose (---) from 2 mL of fluid. Insert shows silver-stained image of the SDS-PAGE; lane 1, A12 bound by anti-A12 IgG Sepharose (retention time 17.7–17.9 min), lane 2, A8 (18.7–19.2 min), and lane 3, A9 (19.5–19.9 min) bound by anti-A9 IgG Sepharose. Samples from three patients were analyzed with similar results.

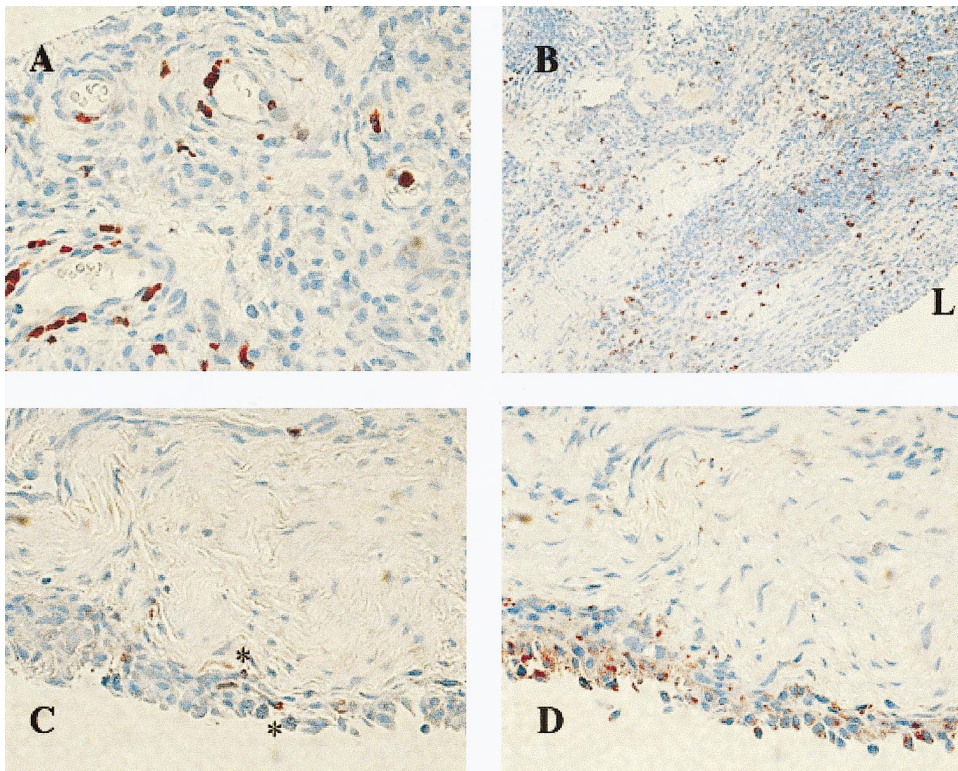


Fig. 6. Localization of A12 in RA synovium. (A) Positive staining of perivascular neutrophils ($\times 60$); (B) almost total absence of A12 in the lining layer (L; $\times 20$); (C) A12-positive macrophages (asterisk) at the lining layer ($\times 60$); (D) sequential section to panel C, stained with macrophage marker CD68 ($\times 60$). Digital images were on the same synovium, representative of three patients.

ng/mL. A12 mRNA was evident 6 h after addition of LPS, increased approximately sixfold after 24 h, and was sustained over 48 h (Fig. 7B). Fig. 7C indicates low levels of A12 transcripts in unstimulated blood monocytes, and levels increased by approximately twofold after 24 h of stimulation with LPS (100 ng/ml).

Induction of A12 mRNA by $\text{TNF}\alpha$ and $\text{IL-1}\beta$ was investigated. Induction by $\text{TNF}\alpha$ in Mono Mac 6 cells and monocytes was transient, with optimal expression of more than twofold above baseline levels after 12 and 6 h, respectively, and declined to control values by 24 h (Fig. 8). $\text{IL-1}\beta$ failed to induce A12 mRNA (data not shown).

Using ELISA, 50 pmol/L of A12 was detected in LPS-stimulated cell lysates harvested at 24 h (detection level up to 50 pmol/L). No A12 was detected in supernatants of LPS- or $\text{TNF}\alpha$ -stimulated cells with this technique.

DISCUSSION

S100 calcium-binding proteins belong to a highly conserved family, several of which have chemotactic properties, generally acting within the subnanomolar to picomolar range in vitro. mA8 recruits neutrophils and monocytes in vitro and elicits a sustained recruitment of neutrophils and monocytes in vivo

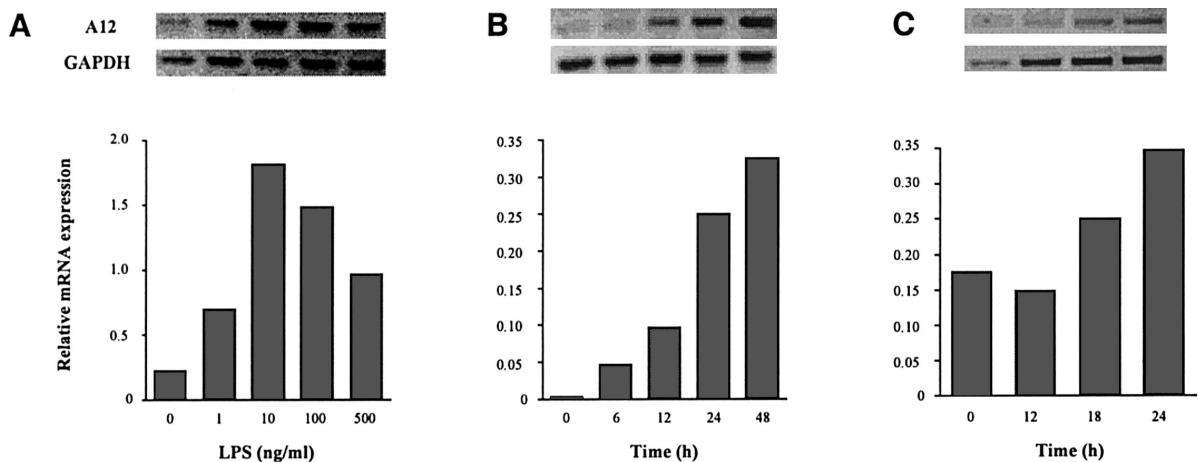


Fig. 7. LPS induces A12 mRNA expression in monocytes. Dose-dependent induction of A12 mRNA in Mono Mac 6 cells by LPS for 24 h (A). Time course of A12 mRNA induction by LPS (100 ng/mL) in Mono Mac 6 cells (B) and peripheral blood monocytes (C). mRNA was analyzed by semiquantitative RT-PCR as described in Materials and Methods; quantitation densitometry of A12 and GAPDH mRNA is given. Results are representative of two experiments.

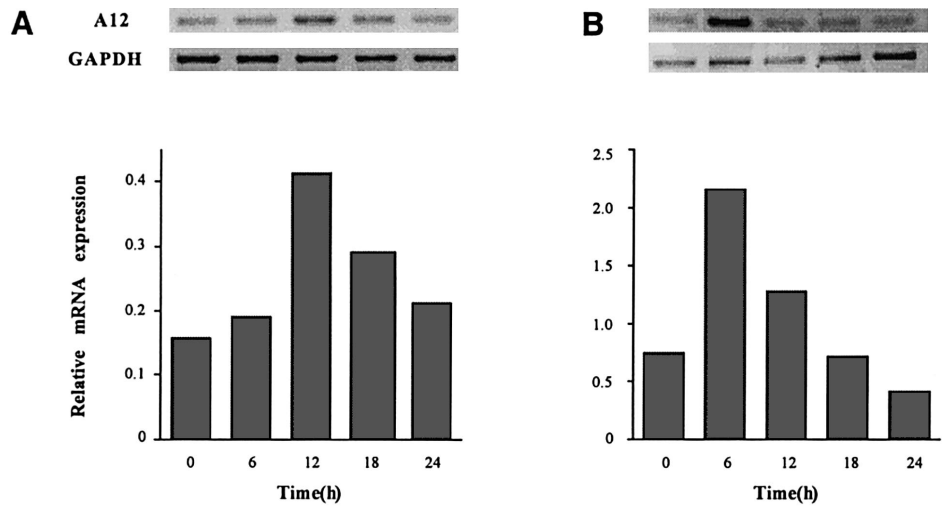


Fig. 8. Kinetics of TNF α -induced A12 mRNA expression in Mono Mac 6 cells (A) and monocytes (B). Cells were stimulated with 100 U/mL of TNF α for the indicated times. mRNA was analyzed by semiquantitative RT-PCR and is representative of two experiments.

[9–11]. Bovine S100A2 (S100L) selectively recruits eosinophils [12], and human S100A7 (psoriasin) recruits CD4⁺ lymphocytes and neutrophils [13], *in vitro*. We show here that monocytes were most responsive to hA12 *in vitro*. The activity of native and recombinant A12 yielded a bell-shaped dose-dependent response (10^{-13} to 10^{-8} M) typical of most chemoattractants, with optimal activity for THP1-cells or peripheral blood monocytes at 10^{-10} M (Fig. 1A, B). A12 affected directional rather than random migration (Fig. 1A), confirming results reported for bovine A12 [7]. A12 activity was more potent than the classical chemoattractants C5a or fMLP but within a range similar to some chemokines including monocyte chemoattractant protein-1 [16], macrophage-inflammatory protein-1 [17, 18], and RANTES (regulated on activation, normal T expressed and secreted) [19] as well as to other chemotactic S100 proteins. Chemotaxis was not caused by contaminating LPS because endotoxin levels in A12 preparations (<2 pg) were negligible and this amount did not affect migration in the absence of A12. Furthermore, activity of the same magnitude as reported here for A12 has been demonstrated with chemically synthesized A12 [20], which did not contain any endotoxin. Hofmann et al. (1999) recently showed that bovine A12 (called “EN-RAGE” for extracellular newly identified RAGE-binding protein) provoked human monocyte migration in a dose-dependent manner, although the effective concentrations (1×10^{-3} to 5×10^{-7} M) were several logs higher than reported here, possibly due to the cross-species use of bovine A12 on human monocytes. Although A12 was also maximally chemotactic for neutrophils at 10^{-10} M, its potency was lower than for monocytes and significantly less than that of C5a, and lymphocytes did not respond to any preparation of A12 (Fig. 2).

The suggested ability of hA12 to regulate monocyte migration was supported by its positive effects on actin polymerization; an event immediately following receptor binding and signal transduction and associated with cell shape change during migration [21, 22]. Actin in monocytes underwent typical reversible polymerization within 20–30 s of incubation with A12, in a manner similar to that induced by fMLP (Fig. 3A). The strong response provoked by 10^{-11} M A12 increased over 40 s and, unlike fMLP, was sustained over 120 s. A12-stimulated actin reorganization was accompanied by almost

instantaneous increases in $[Ca^{2+}]_i$ similar to that initiated by C5a (Fig. 3B), fMLP, and IL-8 [23], in which directional cell motility is accompanied by activation. These *in vitro* results indicate differences in the chemotactic mechanisms initiated by A12 compared with mA8, which provokes myeloid cell migration and actin polymerization but does not alter $[Ca^{2+}]_i$ [10, 24]. On the other hand, S100B promotes changes in glial cell morphology, neurite extension, and possibly growth cone motility, and it also rapidly increases $[Ca^{2+}]_i$ in glial and neuronal cells [25].

Intraperitoneal injection of A12 (1 μ M) into mice elicited a mild but sustained recruitment of leukocytes that reached more than twofold control levels after 8 h. Although A12 only weakly induced neutrophil migration *in vitro*, neutrophil numbers increased >10-fold during this time and declined to normal levels by 24 h (Fig. 4). Small but significant increases in monocyte numbers were also evident after 8 h but were greatest after 24 h and declined thereafter. The discrepancy between *in vitro* and *in vivo* studies may be due to the more complex cellular environment in which A12 ligation with RAGE on monocytes/macrophages induces TNF and IL-1 β [7] which, in turn, could induce chemokines responsible for the early recruitment of neutrophils. The kinetics of leukocyte recruitment elicited by hA12 described here were remarkably similar to responses provoked by mA8 *in vivo* [10, 11]. It is interesting that the chemotactic activity of mA8 is not shared by its human counterpart [26]. mA8 and human A8 (hA8) have only 56% amino acid identity [9, 27] which is atypical of the highly conserved interspecies similarities of other members of the S100 family [28, 29]. Screening of cDNA and genomic DNA libraries for a human homologue of murine CP-10 (mA8) failed to yield isolates with greater sequence similarity than hA8, and the murine protein was designated mA8. Despite the relatively low structural homology between mA8 and hA12 (33% identity), similarities in target cell specificities [26] and kinetics of leukocyte recruitment *in vivo* [10, 11] suggest that they may be functional homologues.

Elevated levels of hA8 and hA9 are associated with a number of inflammatory diseases, particularly RA, in which high levels in serum and SF have been reported [30], as well as expression in the synovial membrane [31], but their functions

in this disease are unclear. Here we demonstrate the presence of A12, together with A8 and A9, in SF of RA patients (Fig. 5) and expression of A12 by inflammatory cells in RA synovium (Fig. 6). Approximately 70 nM A12, within the effective chemotactic concentration range (10^{-13} to 10^{-8} M) *in vitro*, was extracted from SF using anti-A12 IgG affinity chromatography. A12 in RA synovium was located in neutrophils at the sublining and interstitial region, often within the perivascular region (Fig. 6A). A12-positive neutrophils at this site might be strategically poised to facilitate leukocyte recruitment. In contrast to S100A8 and -A9 [32], A12-positive cells were rare within the synovial-lining layer (Fig. 6B) although occasional macrophages in the sublining and lining layers were A12 positive (Fig. 6C).

A12 is constitutive in neutrophils, but its expression in monocytes remains in question. Small amounts of A12 were found in lysates of monocytes [33] and weak immunostaining in monocytes but not in unstimulated monocytoid cell lines [34]. However, a recent report indicates no A12 in vitamin D₃-differentiated HL60 cells or monocytes [35]. We show that TNF α , a proinflammatory mediator that plays a pivotal role in RA [36], induced A12 mRNA in Mono Mac 6 cells and monocytes, with maximal expression after 6–12 h (Fig. 8). Although neutrophil-derived A12 may contribute significantly to leukocyte recruitment in RA (Fig. 6), A12 from TNF α -stimulated monocytes/macrophages may be important in the early phases of leukocyte recruitment, in particular when there are acute inflammatory flares. Secretion of the protein, or the transient effect of TNF α may explain the low numbers of A12-positive macrophages detected in the synovium, in which A12 expression may have subsided. Induction of A12 by TNF α is interesting, given that A12 induces TNF α and IL-1 β expression in macrophages [7], suggesting a positive feedback mechanism between TNF α and A12, perhaps in a manner similar to that between macrophage-inflammatory protein- α and TNF α /IL-1 α [37, 38].

Low basal levels of A12 mRNA were observed in monocytes, and stimulation with LPS increased transcript levels in blood monocytes and in Mono Mac 6 cells over a time course of 6–48 h (Fig. 7B), similar to that observed for induction of mA8 in monocytoid cell lines and in elicited macrophages [39]. A12 gene induction was exquisitely sensitive to low levels of LPS (Fig. 7A), typical of those found in the circulation during bacteremia. Increased A12 in plasma 2–24 h after infusion of mice with LPS [7] has been reported, clearly implicating A12 involvement in responses to gram-negative infection. It is interesting that Mono Mac 6 cells stimulated with high amounts of LPS (500 ng/mL) expressed less A12 mRNA than that induced by lower amounts of LPS (Fig. 7A), suggesting production of monocyte-derived mediators that regulate A12 gene expression. Although cell-associated A12 was detected by ELISA, levels in supernatants of stimulated monocytes were below the level of detection by this method.

Despite the similar patterns of gene induction/expression [39, 40] and similar chemotactic properties of mA8 and hA12, there are distinct structural differences between these proteins which may regulate other functions. mA8 and hA8 are exquisitely sensitive to oxidation by hypochlorite, the major oxidant generated by activated neutrophils [41], and hA8, injected as

a complex with A9, suppresses adjuvant-induced arthritis [42]. Furthermore, A8 constitutes 20% of the cytosolic protein of neutrophils and potentially large amounts, released during phagocytosis [43] or as a result of cell necrosis [44] may play a protective role in inflammation. Moreover, oxidation of mA8 to the covalent homodimer negates chemotactic activity, possibly by sterically hindering and/or structurally altering exposure of the chemotactic hinge domain, restricting cellular target recognition. Taken together with hypochlorite's ability to reduce the chemotactic activity of C5a and fMLP by formation of methionine sulfoxides [45], oxidative mechanisms may regulate excess leukocyte accumulation and contribute to terminating progression of acute inflammation. In marked contrast, hA12 is not prone to covalent dimerization or oxidation because it lacks Cys and Met residues, suggesting that its chemotactic properties may be more stable. Moreover, A12 up-regulates vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in endothelial cells and TNF in monocytes, and blockade of A12/RAGE interactions inhibit delayed-type hypersensitivity responses and inflammatory colitis in murine models [7]. Together with the properties reported here, a proinflammatory role is indicated. The levels of this protein found in the SF of patients with RA may contribute to sustained neutrophil and monocyte recruitment during chronic inflammatory episodes.

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