

Original Article

Recruitment of CCR6-expressing Th17 cells by CCL20 secreted from plasmin-stimulated macrophages

Qun Li^{1,2,3*}, Yves Laumonnier^{3,4}, Tatiana Syrovets³, and Thomas Simmet³

¹Joint Laboratory of Vascular Biology of Ruijin Hospital and Shanghai Institute of Hypertension, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

²Health Science Center, Shanghai Institutes of Biological Sciences (SIBS), Chinese Academy of Sciences, Shanghai 200025, China

³Institute of Pharmacology of Natural Products and Clinical Pharmacology, Ulm University, D-89081 Ulm, Germany

⁴Institute for Systemic Inflammation Research, University of Lübeck, 23562 Lübeck, Germany

*Correspondence address. Tel: +86-21-6384865; Fax: +86-21-64660996; E-mail: liqun@sibs.ac.cn

In the present study, monocyte-derived human macrophages were differentiated from buffy coats. Naïve CD4⁺ T-cells enriched from peripheral blood mononuclear cells using anti-CD4 magnetic beads and the autoMACS separation system were polarized under T-helper 17 (Th17)-promoting conditions for 6 days to get Th17 cells. The frequency of Th17 cell differentiation and the expression of C-C chemokine receptor type 6 (CCR6) on Th17 cells were investigated by flow cytometry. Plasmin-triggered induction of macrophage inflammatory protein-3 α /C-C chemokine ligand 20 (CCL20) genes in macrophages was assessed by reverse transcription-polymerase chain reaction, and secreted protein levels were measured by enzyme-linked immunosorbent assay. Th17 cell migration induced by CCL20 secreted from plasmin-stimulated macrophages was tested *in vitro* by chemotaxis using a transwell system. These results demonstrate that plasmin triggers the expression of chemokine CCL20 messenger RNA and the release of CCL20 protein in human monocyte-derived macrophages, which critically depend on the proteolytic activity of plasmin and activation of p38 mitogen-activated protein kinase and nuclear factor- κ B signaling pathways. Expression of CCR6 was detected on $87.23 \pm 8.6\%$ of Th17 cells *in vitro*. Similar to chemotaxis triggered by recombinant human CCL20, supernatants collected from plasmin-stimulated macrophage-induced chemotactic migration of Th17 cells, which could be inhibited by an anti-CCL20 neutralizing antibody. These results suggest that plasmin generated in inflamed tissues might elicit production of chemokine CCL20 by human macrophages leading to the recruitment of CCR6 positive Th17 cells to the inflammatory sites.

Keywords plasmin; macrophages; signaling; CCL20; Th17; CCR6

Received: January 5, 2013 Accepted: February 18, 2013

Introduction

The serine protease plasmin generated by proteolytic cleavage of its precursor plasminogen represents the key enzyme of the fibrinolytic cascade. Many types of chronic inflammatory conditions including atherosclerosis are invariably associated with the generation of plasmin [1,2]. A large body of evidence indicates that inflammatory mediators and immune responses play crucial roles in the development of atherosclerosis [3]. Plasmin is a potent inflammatory mediator and inducer of immune responses. *In vitro* studies from our lab indicated that plasmin generated at sites of inflammation such as atherosclerotic lesions might be capable of eliciting chemotaxis of human monocytes [4] and dendritic cells (DCs) [5] and stimulating cytokines production from monocytes [4], macrophages [6], and DCs [7], which contribute to inflammation. A recent clinical study revealed that the enhanced plasmin activity is correlated with the degree of lesion complexity in human carotid plaques [8] and a clinical atherosclerosis case report presented that aggravated plasmin generation might indeed lead to cardiovascular complications [9]. In mouse models, atherosclerotic plaque formation is reduced in the plasminogen-deficient mice [10].

Macrophages served as antigen-presenting cells (APCs) are direct effector and cytokine-producing cells. Accordingly, they accumulate at the sites of inflammation and play a central role in immune and inflammatory processes [11]. Macrophages particularly are the main source of cytokines and chemokines in atherosclerotic plaques [12]. In arteriosclerosis-associated inflammatory vascular injury, macrophages play a predominant pathophysiological role [13].

Chemokines are defined by their capability to attract (*in vitro* or *in vivo*) specific cell types to the sites of inflammation. As a result, chemokines play crucial roles in the pathogenesis of diseases that are characterized by controlling

inflammatory cell accumulation and affecting many biological processes, such as atherosclerosis [13–15].

Macrophage inflammatory protein-3 α /C-C chemokine ligand 20 (CCL20) is a CC-type chemokine that is expressed in several tissues, in particular, in inflammatory cells stimulated with cytokines such as interleukin (IL)-1 β or by proinflammatory signals such as lipopolysaccharide (LPS). C-C chemokine receptor type 6 (CCR6) is, so far, known to be the unique receptor for it [16]. One of the main effects of CCL20 is to promote the recruitment of CCR6⁺ lymphocytes to atherosclerotic lesions [17]. In autoimmune pathogenesis of the central nervous system (CNS), sympathetic neuronal activity triggers IL-6-mediated increase of local CCL20 expression and subsequent pathogenic CCR6⁺ T-cell accumulation at the same spinal cord level, which provides evidence for a critical role for CCL20 in CNS pathogenesis [18]. CCR6 is expressed by a variety of cells of the innate and/or adaptive immune system, in particular T-helper 17 (Th17) cells, which exert multiple functions *via* release of cytokine IL-17A in many chronic inflammatory disorders, especially in atherosclerosis [19].

Our previous *in vivo* study has demonstrated that, in a mouse model, plasmin elicits the production of CCL20 at the sites of inflammation, which plays an essential role in the recruitment and activation of pathogenic CCR6⁺ T-cells [20]. Proinflammatory cytokines [IL-1 β or tumor necrosis factor alpha (TNF- α)] contribute to the development of endometriosis *via* up-regulation of CCL20 secretion from endometrial stromal cells to induce the migration of Th17 cells to endometrial tissues [21]. The excessive generation of plasmin, activation of macrophages, and accumulation of Th17 cells play important roles in the innate and acquired immune responses. However, the relationship between these factors, the initiation of the chemokine induction, and the role of active plasmin are still poorly understood.

In this study, we investigated whether plasmin generated in human inflammatory atherosclerotic lesions affect the expression of chemokines by human monocyte-derived macrophages, and whether chemokines secreted from plasmin-stimulated macrophages contribute to the recruitment of CCR6-expressing Th17 cells, a subtype of CD4⁺ T-helper cells mainly producing IL-17.

Materials and Methods

Antibodies and reagents

Antibodies for flow cytometric analysis: anti-human CD4-PE, anti-human CCR6-AlexaFluor[®] 488, mouse IgG1-PE, and mouse IgG2b-AlexaFluor[®] 488 isotype controls were all purchased from BioLegend (San Diego, USA). Anti-human IL-17A-APC, mouse IgG1-APC isotype control, and anti-interferon (IFN)- γ were from eBioscience (San Diego, USA). Anti-human CCL20 polyclonal antibody

(LifeSpan, Seattle, USA) was used for neutralization. Phycoerythrin (PE)-conjugated donkey anti-mouse and donkey anti-rabbit F(ab)₂ (Dianova, Hamburg, Germany) were used as secondary antibodies [6]. Anti-CD28, rhIL-6, and recombinant human transforming growth factor beta (rhTGF- β) were from Pepro-Tech (Rocky Hill, USA). Anti-IL-4 was from BD Pharmingen (San Diego, USA). Phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma (St Louis, USA).

Purified human plasmin was purchased from Athens Research & Technology (Athens, Georgia, USA) and was free of LPS as analyzed by the Pyrogen LAL assay (Lonza, Basel, Switzerland). Plasmin activity is given in Committee on Thrombolytic Agents (CTA) units/ml as previously described [6]. The catalytic inhibitor of plasmin, D-Val-Phe-Lys chloromethyl ketone (VPLCK), the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, and the MAPK or ERK kinase (MEK) inhibitor U0126 were purchased from Calbiochem (San Diego, USA). Chemical pure acetyl-11-keto- β -boswellic acid (AK β BA) isolated as previously described [22] was used as an inhibitor of the nuclear factor-kappaB (NF- κ B) [6]. LPS (*Escherichia coli* serotype 055:B5) was from Sigma. Recombinant human CCL20 (rhCCL20) was purchased from R&D Systems (Minneapolis, USA).

Macrophages preparation and culture

Monocyte-derived human macrophages were differentiated from buffy coats for 7 days with 15 ng/ml macrophage colony-stimulating factor (M-CSF) [23]. The macrophage phenotype was confirmed by flow cytometric analysis of CD14 (BD Biosciences, San Diego, USA), CD68 (DAKO, Hamburg, Germany), and CD71 (BD Pharmingen). Macrophages were cultured in RPMI 1640 (Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum (FCS). The cells were stimulated with plasmin in lysine-free RPMI 1640 (Sigma).

Reverse transcription-polymerase chain reaction analysis

Total RNA from 1×10^6 macrophages stimulated with 0.43 CTA U/ml plasmin, equivalent amounts of catalytically inactivated plasmin (VPLCK-plasmin) [24] or LPS (1 μ g/ml) was isolated by Trizol (Invitrogen), and then reverse transcribed. Complementary DNA was analyzed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Primers for *CCL20* were as follows: forward 5'-ccaagagtgtgctcctggct-3', and reverse 5'-tgcttgctgctctgattcg-3'. Amplification was performed with 25 cycles of amplification (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s). PCR was performed in the linear range of amplification. *GAPDH* served as internal standard [23]. The transcripts were identified by direct

automated sequencing (Genetic Analyzer; Applied Biosystems, Life Technologies, Paisley, UK).

Enzyme-linked immunosorbent assay

The cytokine levels of CCL20 in the medium were quantified by enzyme-linked immunosorbent assay (ELISA) kit for human CCL20 (DuoSet, R&D Systems). The assay was performed following the manufacturer's protocol.

Th17 cell differentiation and purification

Naïve CD4⁺ T-cells were enriched from peripheral blood mononuclear cells using anti-CD4 magnetic beads and the autoMACS separation system according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany), and cultured in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin (Invitrogen).

CD4⁺ T-cells (1×10^5 cells/well) were cultured in a 96-well plate coated with anti-CD3 (5 µg/ml), and stimulated with anti-CD28 (2 µg/ml), rhIL-6 (20 ng/ml), rhTGF-β (2 ng/ml), anti-IL-4 (10 µg/ml), and anti-IFN-γ (10 µg/ml) for 6 days. IL17-A production was triggered by restimulation of the cells with PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of a blocker of secretion (GolgiPlug, BD Biosciences) for 5 h. Isolation of IL-17 secreting cells from above differentiated CD4⁺ T-cells was carried out according to the manufacturer's protocol using the IL-17 Cell Enrichment and Detection Kit (Miltenyi Biotec, human, purity > 95%). CD4⁺ T-cell fraction was always >95% and cell viability was checked using trypan blue.

Flow cytometric analysis

CD4⁺ T-helper cells differentiated to Th17 as described above were stained with anti-CD4-PE and anti-CCR6-AlexaFluor488 antibodies. After fixation and permeabilization (Perm/Fix solution, eBioscience), intracellular staining of IL-17A was performed with anti-IL-17A-APC antibody. Samples were analyzed on a FACScan (Becton-Dickinson, Franklin Lakes, USA) with CellQuest software.

In vitro migration assay

Migration assay was performed in 24-well plates (Costar, Cambridge, USA) carrying transwell-permeable supports with a 3-µm polycarbonate membrane for T-cells. rhCCL20 and the supernatants collected from macrophages stimulated with or without plasmin for 6 h were placed into the lower chambers. Purified Th17 cells from *in vitro*-differentiated CD4⁺ T-cells [100 µl, 2.0×10^6 cells/ml RPMI 1640 medium containing 0.5% bovine serum albumin (BSA)] were placed onto the upper wells of transwell membranes and incubated for 7 h at 37°C. The cells on the upper surface of the membranes were completely removed by washing with distilled water. Migrated cells on the lower surface of the membranes were fixed with paraformaldehyde, stained

with hematoxylin (Sigma-Aldrich), and the number of cells was counted in six randomly selected non-overlapping fields of the wells under light microscope ($\times 1000$ magnification, oil immersion). To calculate the chemotactic index, the number of cells migrated in response to chemokines was divided by the number of spontaneously migrated cells.

Statistical analysis

Values shown represent mean \pm standard error of mean (SEM) where applicable. Statistical significances were calculated with the Newman-Keuls test. Differences were considered significant when $P < 0.05$.

Results

Plasmin elicits induction of chemokine CCL20 in human macrophages

Monocyte-derived human macrophages phenotype was confirmed by flow cytometry showing reduction of the monocyte-specific surface marker CD14, increased expression of CD68, and exposure of CD71 [Fig. 1(A)], which is characteristic for macrophages [23]. Our previous study showed that plasmin triggered the expression and release of proinflammatory cytokines in macrophages [6]. Chemokines are the major mediators of migration of all leukocytes and are expected to become important targets for pharmacological intervention in a wide variety of human diseases in the near future [25]. We analyzed the ability of plasmin to elicit the expression of chemokine in macrophages. Plasmin (0.43 CTA U/ml) induced a time-dependent increase of *CCL20* messenger RNA (mRNA) [Fig. 1(B)]. As expected, LPS (1 µg/ml) stimulation also increased the amount of *CCL20* mRNA. In both cases, the *CCL20* induction was very fast and already reached a maximum for plasmin after 3 h of stimulation. The expression of *CCL20* mRNA in macrophages stimulated with plasmin for 3 h was also concentration-dependent [Fig. 1(C)]. Although 0.143 CTA U/ml plasmin increased *CCL20* mRNA, the optimum response was reached at 0.43 CTA U/ml [Fig. 1(C)]. Plasmin-VPLCK failed to stimulate the expression of *CCL20* mRNA [Fig. 1(D)] confirming that the activation of *CCL20* expression by plasmin in macrophages requires proteolytically active plasmin. The induction of *CCL20* mRNA in plasmin- and LPS-stimulated human primary macrophages is followed by a strong release of CCL20 protein in the supernatant [Fig. 1(E)]. The chemokine became detectable as early as 1 h after stimulation.

Plasmin induces chemotactic CCL20 release in macrophages *in vitro*, which is dependent on p38 and NF-κB activation

We have previously shown that plasmin triggers various signaling pathways, including janus kinase 1 (JAK1)/signal

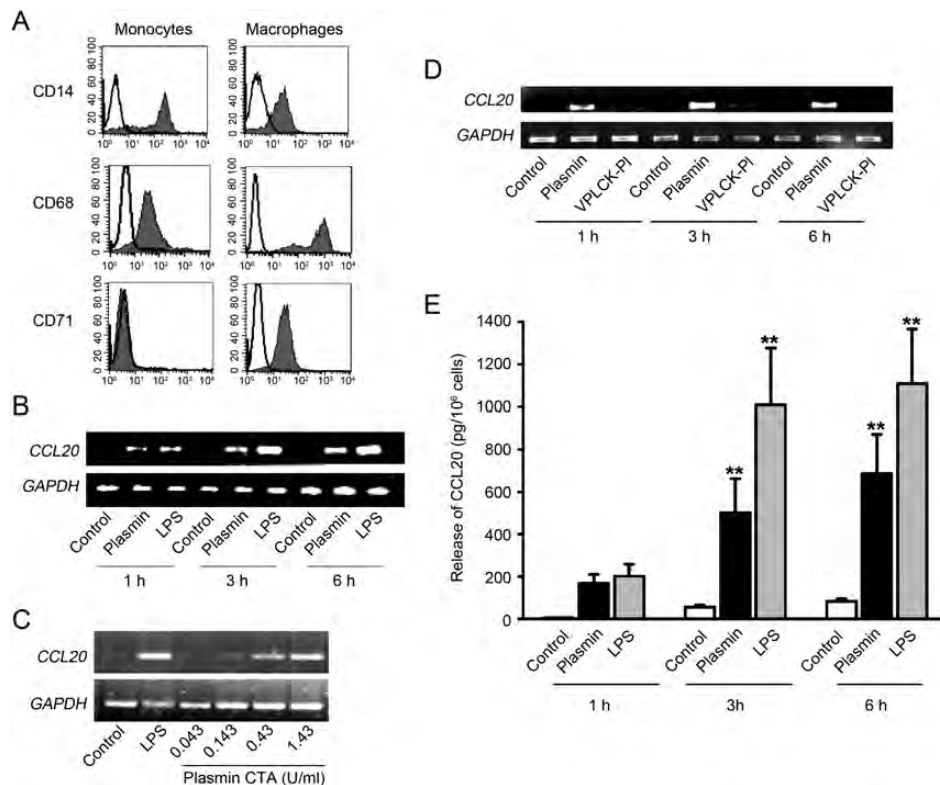


Figure 1 Plasmin triggers the time- and dose-dependent expression of the *CCL20* gene in human macrophages (A) Monocyte-derived human macrophages differentiated from buffy coats for 7 days with 15 ng/ml M-CSF, which was confirmed by flow cytometry of the cell surface marker CD14 (monocytes), CD68, and CD71 (macrophages). Empty peaks are isotype controls. (B–D) Time- or dose-dependent stimulation of the *CCL20* mRNA expression depends on the proteolytic activity of plasmin. Macrophages were stimulated with plasmin (0.43 CTA U/ml) or LPS (1 μ g/ml) for the indicated times (B) or plasmin (for the indicated concentration) or LPS (1 μ g/ml) for 3 h (C), or equivalent amounts of catalytically inactivated plasmin (VPLCK-plasmin) (D) for the indicated times, the mRNA levels were analyzed by RT-PCR. In each case, one representative out of three experiments is shown. *GAPDH* is the loading control. (E) Macrophages were stimulated with plasmin (0.43 CTA U/ml) or LPS (1 μ g/ml) for the indicated time. The supernatants were recovered and the concentration of CCL20 was measured by ELISA. Data are presented as the mean \pm SEM of four independent experiments. ** $P < 0.01$ vs. control.

transducer and activator of transcription 3 (STAT3), p38 and extracellular signal-regulated kinases 1/2 (ERK1/2) MAPK, and NF- κ B in macrophages [6]. To address the role of these signaling pathways in the plasmin-induced release of CCL20, we pretreated macrophages with inhibitors for JAK1 (AG490), p38 MAPK (SB203580), MAPK/ERK kinase pathway (U0126), and an inhibitor of NF- κ B (AK β BA) [26] for 30 min before stimulation with plasmin (0.43 CTA U/ml) for 3 h (for mRNA test by RT-PCR) or 6 h (for protein test by ELISA). Pretreatment of macrophages with SB203580 (1 μ mol/l), or AK β BA (3 or 10 μ mol/l), not with AG490 (50 μ mol/l) or U0126 (1 μ mol/l), showed that p38 and NF- κ B activation is essential for the plasmin-mediated expression of *CCL20* mRNA in human macrophages [Fig. 2(A)]. Pretreatment of macrophages with SB203580 (1 μ mol/l) or AK β BA (3 or 10 μ mol/l) inhibited the plasmin-induced CCL20 release by 74.2 ± 6.1 , 57.9 ± 7.4 , and $71.8 \pm 4.8\%$ ($P < 0.01$), compared with the cells with only plasmin treatment, respectively [Fig. 2(B)]. In contrast, inhibition of JAK1 using AG490 (50 μ mol/l) or ERK1/2 using the MEK inhibitor U0126

(1 μ mol/l) had no impact on plasmin-mediated expression of CCL20, compared with the cells with only plasmin treatment [Fig. 2(B)], although these inhibitors affected significantly the release of TNF- α and IL-6 [6]. These data imply that plasmin-induced macrophages CCL20 expression through the MAPK p38 and NF- κ B signaling pathways, followed by the increase of CCL20 secretion.

Preparation of Th17 cells *in vitro*

To analyze whether plasmin-stimulated macrophages release the biologically active CCL20, first, we prepared Th17 cells *in vitro* from the CD4⁺ T-cells differentiated under the Th17-promoting conditions, then checked the CCR6 protein expression on Th17 cells surface by flow cytometry. The data showed that $14.26 \pm 1.98\%$ of the differentiated CD4⁺ T-cells were IL-17A⁺ [Fig. 3(A)]. Sequential gating revealed that $87.25 \pm 8.61\%$ of CD4⁺/IL-17A⁺ Th17 cells expressed the CCL20 receptor, CCR6. [Fig. 3(B)]. CCR6 appears to be expressed on the majority of human Th17 cells, which is consistent with the previous study [27].

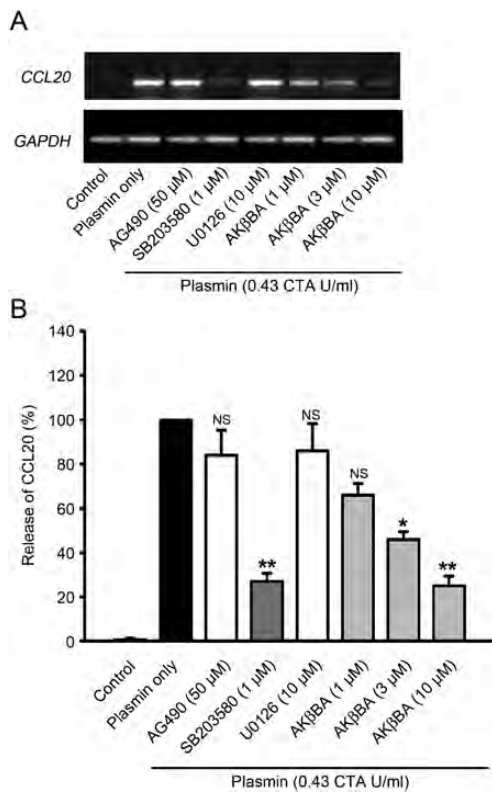


Figure 2 Effects of pharmacological inhibitors on the plasmin-induced CCL20 expression and release (A) Macrophages were pre-incubated with AG490 50 μmol/l (JAK1 inhibitor), SB203580 1 μmol/l (p38 MAPK inhibitor), U0126 10 μmol/l ([MAPK or ERK kinase (MEK) inhibitor], or AKβBA 1, 3, 10 μmol/l (NF-κB inhibitor) for 30 min, respectively, and then stimulated with plasmin (0.43 CTA U/ml) for 3 h. Expression of CCL20 and GAPDH (control) was analyzed by RT-PCR. (B) Macrophages which were pre-incubated with the indicated inhibitors for 30 min were stimulated with plasmin (0.43 CTA U/ml) for 6 h. CCL20 was analyzed by ELISA. The data represent the mean ± SEM of six independent experiments. ** $P < 0.01$ vs. plasmin only. * $P < 0.05$ vs. plasmin only. NS, no significant difference.

CCL20 secreted from plasmin-stimulated macrophages induces migration of Th17 cells

To elucidate the role of CCL20 secreted from plasmin-stimulated macrophages for the regulating function of Th17 cells-expressing CCR6, we performed the following migration assays *in vitro*. First, we analyzed if rhCCL20 induces migration of Th17 cells. As shown in Fig. 4(A), rhCCL20 triggered Th17 cell migration in a dose-dependent manner, already at 0.1 μg/ml of concentration. At 0.1 and 1 μg/ml, it significantly increased the chemotactic index of Th17 cells by 3.78 ± 0.65 and 10.91 ± 2.03 -fold compared with control (RPMI 1640–0.5% BSA medium), respectively. Only with plasmin (0.43 CTA U/ml) stimulation served as negative control. To prove that the migration is indeed induced by CCL20 released from the plasmin-stimulated macrophages. The supernatants collected from plasmin-stimulated macrophages for 6 h induced migration of Th17 cells by about 3.64-fold, the above supernatants treated with

anti-CCL20 neutralizing antibody (CCL20 Neu-Ab) (1 and 3 μg/ml) induced migration of Th17 cells only about 1.43 ± 0.15 and 1.23 ± 0.019 -fold. All folds compared with the supernatants collected from macrophages without plasmin stimulation [Fig. 4(B)]. Next, we found that the supernatants collected from the plasmin-stimulated macrophages pre-incubated with the pathway inhibitors (SB203580, inhibitor of p38, 1 μmol/l; AKβBA, inhibitor of the NF-κB, 10 μmol/l) for 30 min significantly decreased chemotactic index to 1.81 ± 0.74 and 1.59 ± 0.04 , compared with the supernatant from the plasmin-stimulated macrophages used as control (chemotactic index is 4.23 ± 1.03), respectively [Fig. 4(C)]. These results showed that plasmin-induced CCL20 secretion through the p38 and NF-κB-dependent pathways, and that CCL20 could contribute to the recruitment of CCR6⁺ Th17 cells to the sites of plasmin generation.

Discussion

The inactive pro-enzyme plasminogen is transformed into the proteolytically active plasmin mainly by two plasminogen activators, tissue plasminogen activator and urokinase plasminogen activator (uPA). The uPA/plasminogen system has been causally related to atherosclerosis [2]. Experimental data have demonstrated that macrophage-expressed uPA contributes to the progression and complications of atherosclerosis [28]. In atherosclerotic plaques, macrophages are particularly important since they are recruited in the largest numbers. Because of contact activation, generation of plasmin occurs in inflammatory sites [2] where they produce inflammatory mediators and play a critical role during atherosclerosis [29].

The release of CCL20 is a key feature in chronic inflammatory diseases. CCL20 is the chemokine for the cells bearing CCR6 receptor. Thus, it is expected to play a critical role in trafficking and homing many CCR6 positive cells identified in atherosclerosis, including memory T-cells and immature dendritic cells to inflammatory sites [30]. Under inflammatory conditions, epithelial cells [31], endothelial cells, fibroblasts, and monocytes are thought to be important sources of CCL20 production [16,32]. Similarly, some studies have shown that high expression of CCL20 is induced in differentiated macrophages in intestinal epithelium [33] and lung [32]. In human inflamed pulp sections, CCL20 is mostly expressed by macrophages accumulated in the area adjacent to carious lesions and CCR6 expression is also elevated in inflammatory infiltrating lymphocytes, which suggest that CCL20 may play a major role in the advancement of pulpal inflammation *via* the recruitment of CCR6-expressing lymphocytes [34]. CCR6/CCL20 interactions critically play a role in organ selective liver metastasis of colorectal tumor and several types of cancer [35]. The

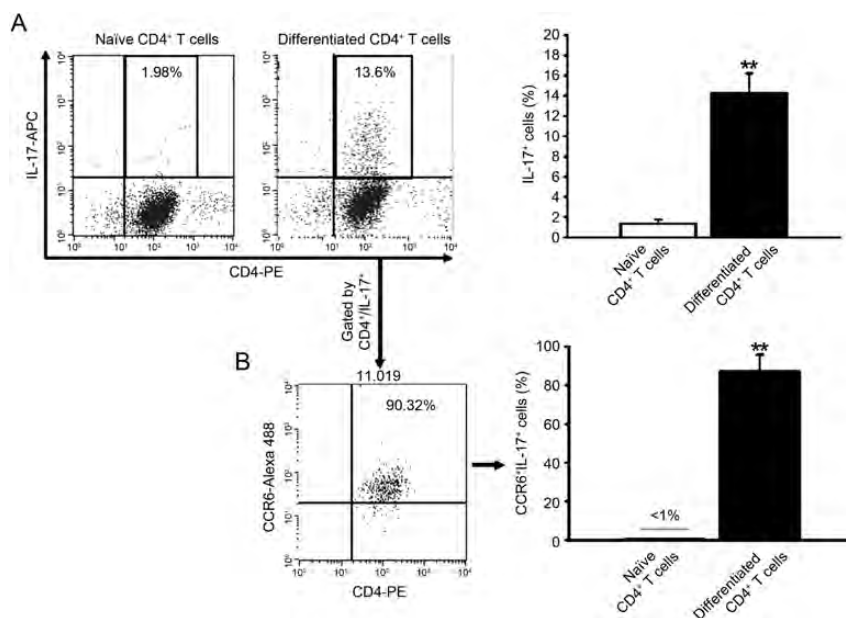


Figure 3 Expression of CCR6 in Th17 cells differentiated from CD4⁺ T-cells under the Th17 cells differentiation conditions Th17 cells differentiated from Naive CD4⁺ T-cells were first stained extracellularly with anti-CD4-PE and anti-CCR6-AlexaFluor488 antibodies, then fixed and permeabilized with Perm/Fix solution, and stained intracellularly with anti-IL-17A-APC antibody. Samples were analyzed by multicolor flow cytometry. (A) Percentage of Th17 cells in naïve CD4⁺ T-cells or the differentiated CD4⁺ T-cells. (B) IL-17A⁺ Th17 cells are mostly positive for CCR6⁺. The graphs are mean ± SEM of four independent experiments. ***P* < 0.01 vs. naïve CD4⁺ T-cells.

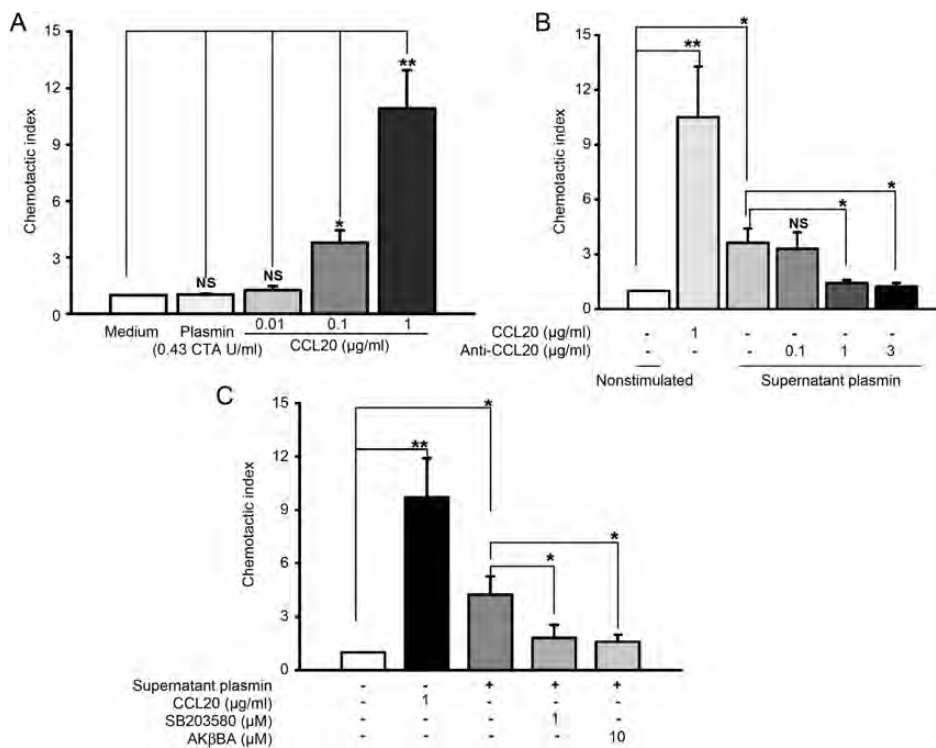


Figure 4 CCL20 derived from the plasmin-stimulated macrophages induces the Th17 cell migration *in vitro* (A) The Th17 cell migration was induced by rhCCL20 (for the indicated concentration) for 7 h. Plasmin (0.43 CTA U/ml) stimulation was used as a negative control (*n* = 4). The chemotactic index was calculated. (B) The Th17 cell migration induced by the supernatants collected from the macrophages which were stimulated with plasmin (0.43 CTA U/ml) for 6 h. Non-stimulated or rhCCL20 (1 µg/ml) was used as a negative or positive control. The addition of CCL20 Neu-Ab (for the indicated concentration) to the supernatants inhibited the Th17 cell migration (**P* < 0.01). (C) Addition of the p38 inhibitor (SB203580, 1 µmol/l) or NF-κB inhibitor (AKβBA, 10 µmol/l) to the macrophages prior stimulation with plasmin inhibited the Th17 cell migration. The data are expressed as the mean ± SEM of four independent experiments. **P* < 0.05, ***P* < 0.01, NS, no significant difference.

functional role of CCL20/CCR6 positive feedback loop has been described in Th17 cells, which are both CCR6 positive and able to produce CCL20 [27], and in atherogenesis, in *ApoE*-deficient mice [36], but not in humans [37].

Our study provided a possible explanation for the role of plasmin in chronic inflammation through macrophage activation, CCL20 release, and recruitment of Th17 cells to inflammatory sites, which may play critical role in the progression of chronic diseases, such as atherosclerosis. Here we showed for the first time that plasmin elicits the production of CCL20 in human macrophages, and may have an essential role in the recruitment of Th17 cells to the inflammatory sites.

Our previous study has shown that in human macrophages plasmin triggers several signaling pathways, including the JAK1/STAT3, MAPK p38 and ERK1/2, and NF- κ B [6]. In the present study, we demonstrated that CCL20 was released by plasmin-stimulated human macrophages *via* the p38 and NF- κ B signaling pathways. Chemokines are regulated primarily at the level of gene transcription in a manner dependent on cell types and different activators. The CCL20 promoter region contains binding sites for different transcription factors such as activator protein-1 (AP-1), CAAT/enhancer-binding protein (C-EBP), and stimulating protein 1 (SP1) [30]. AP-1 [38], C-EBP [39], and SP1 transcription factors have been known to be direct targets of p38 activation. The p38 MAPK is also indispensable for the expression of CCL20 in human gingival fibroblasts stimulated with either IL-1 β , TNF- α , or *E. coli* LPS [34]. Similarly, airway epithelial cells stimulated with IL-1 β or TNF- α secrete CCL20 in a p38 MAPK-dependent manner [40], and a functional NF- κ B site between -82 and -91 is responsible for the CCL20 expression [41]. Consistently, we demonstrated that the p38 and NF- κ B pathways are indispensable for plasmin-induced CCL20 expression in macrophages. Furthermore, the CCL20 secreted from the plasmin-stimulated macrophages induces chemotaxis of Th17 cells.

In summary, we showed that plasmin may be a potent activator of macrophages triggering CCL20 expression by the activation of p38 and NF- κ B signaling pathways and consequent chemotaxis of Th17 cells. The inflammatory milieu of the tissue may up-regulate CCL20 expression through plasmin, subsequently augmenting the Th17 cell migration and progression of human diseases. Understanding the mechanism of chemokines and chemokine expression promoting the Th17 cell recruitment might reveal new therapeutic targets interfering with the Th17 cell migration in chronic inflammatory diseases, such as atherosclerosis.

Funding

This work was supported by the grants from the Deutsche Forschungs gemeinschaft (to T.S. and T.S.), the Shanghai

Scientific Research Innovation Program (11YZ59 to Q.L.), the Shanghai Pujiang Program (10PJ1407300 to Q.L.), the New Scholar Programs Foundation of Ministry of Education of China (20110073120103 to Q.L.), and the National Natural Science Foundation of China (81202298 to Q.L.).

References

- Syrovets T, Lunov O and Simmet T. Plasmin as a proinflammatory cell activator. *J Leukoc Biol* 2012, 92: 509–519.
- Syrovets T and Simmet T. Novel aspects and new roles for the serine protease plasmin. *Cell Mol Life Sci* 2004, 61: 873–885.
- Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005, 352: 1685–1695.
- Burysek L, Syrovets T and Simmet T. The serine protease plasmin triggers expression of MCP-1 and CD40 in human primary monocytes via activation of p38 MAPK and janus kinase (JAK)/STAT signaling pathways. *J Biol Chem* 2002, 277: 33509–33517.
- Li X, Syrovets T, Genze F, Pitterle K, Oberhuber A, Orend KH and Simmet T. Plasmin triggers chemotaxis of monocyte-derived dendritic cells through an Akt2-dependent pathway and promotes a T-helper type-1 response. *Arterioscler Thromb Vasc Biol* 2010, 30: 582–590.
- Li Q, Laumonier Y, Syrovets T and Simmet T. Plasmin triggers cytokine induction in human monocyte-derived macrophages. *Arterioscler Thromb Vasc Biol* 2007, 27: 1383–1389.
- Li X, Syrovets T and Simmet T. The serine protease plasmin triggers expression of the CC-chemokine ligand 20 in dendritic cells via Akt/NF- κ B-dependent pathways. *J Biomed Biotechnol* 2012, 2012: 186710.
- Leclercq A, Houard X, Loyau S, Philippe M, Sebbag U, Meilhac O and Michel JB. Topology of protease activities reflects atherothrombotic plaque complexity. *Atherosclerosis* 2007, 191: 1–10.
- Brands-Nijenhuis AV, van Geel PP and Meijer K. Acute myocardial ischemia in a patient with heterozygous alpha-2-plasmin inhibitor deficiency. *Blood Coagul Fibrinolysis* 2009, 20: 599–600.
- Ploplis VA, French EL, Carmeliet P, Collen D and Plow EF. Plasminogen deficiency differentially affects recruitment of inflammatory cell populations in mice. *Blood* 1998, 91: 2005–2009.
- Shi C and Pamer EG. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* 2011, 11: 762–774.
- Tedgui A and Mallat Z. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev* 2006, 86: 515–581.
- Weber C and Noels H. Atherosclerosis: current pathogenesis and therapeutic options. *Nat Med* 2011, 17: 1410–1422.
- Zimmerman NP, Vongsa RA, Wendt MK and Dwinell MB. Chemokines and chemokine receptors in mucosal homeostasis at the intestinal epithelial barrier in inflammatory bowel disease. *Inflamm Bowel Dis* 2008, 14: 1000–1011.
- Bonecchi R, Galliera E, Borroni EM, Corsi MM, Locati M and Mantovani A. Chemokines and chemokine receptors: an overview. *Front Biosci* 2009, 14: 540–551.
- Schuttyser E, Struyf S, Menten P, Lenaerts JP, Conings R, Put W and Wuyts A, *et al.* Regulated production and molecular diversity of human liver and activation-regulated chemokine/macrophage inflammatory protein-3 alpha from normal and transformed cells. *J Immunol* 2000, 165: 4470–4477.
- Liao F, Rabin RL, Smith CS, Sharma G, Nutman TB and Farber JM. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3alpha. *J Immunol* 1999, 162: 186–194.

- 18 Arima Y, Harada M, Kamimura D, Park JH, Kawano F, Yull FE and Kawamoto T, *et al.* Regional neural activation defines a gateway for auto-reactive T cells to cross the blood-brain barrier. *Cell* 2012, 148: 447–457.
- 19 Tesmer LA, Lundy SK, Sarkar S and Fox DA. Th17 cells in human disease. *Immunol Rev* 2008, 223: 87–113.
- 20 Li Q, Ke F, Zhang W, Shen X, Xu Q, Wang H and Yu XZ, *et al.* Plasmin plays an essential role in amplification of psoriasiform skin inflammation in mice. *PLoS ONE* 2011, 6: e16483.
- 21 Hirata T, Osuga Y, Takamura M, Kodama A, Hirota Y, Koga K and Yoshino O, *et al.* Recruitment of CCR6-expressing Th17 cells by CCL 20 secreted from IL-1 beta-, TNF-alpha-, and IL-17A-stimulated endometrial stromal cells. *Endocrinology* 2010, 151: 5468–5476.
- 22 Buchele B and Simmet T. Analysis of 12 different pentacyclic triterpenic acids from frankincense in human plasma by high-performance liquid chromatography and photodiode array detection. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003, 795: 355–362.
- 23 Colognato R, Slupsky JR, Jendrach M, Burysek L, Syrovets T and Simmet T. Differential expression and regulation of protease-activated receptors in human peripheral monocytes and monocyte-derived antigen-presenting cells. *Blood* 2003, 102: 2645–2652.
- 24 Laumonier Y, Syrovets T, Burysek L and Simmet T. Identification of the annexin A2 heterotetramer as a receptor for the plasmin-induced signaling in human peripheral monocytes. *Blood* 2006, 107: 3342–3349.
- 25 Christopherson K and Hromas R, 2nd. Chemokine regulation of normal and pathologic immune responses. *Stem Cells* 2001, 19: 388–396.
- 26 Syrovets T, Buchele B, Krauss C, Laumonier Y and Simmet T. Acetyl-boswellic acids inhibit lipopolysaccharide-mediated TNF-alpha induction in monocytes by direct interaction with IkappaB kinases. *J Immunol* 2005, 174: 498–506.
- 27 Yamazaki T, Yang XO, Chung Y, Fukunaga A, Nurieva R, Pappu B and Martin-Orozco N, *et al.* CCR6 regulates the migration of inflammatory and regulatory T cells. *J Immunol* 2008, 181: 8391–8401.
- 28 Cozen AE, Moriwaki H, Kremen M, DeYoung MB, Dichek HL, Slezicki KI and Young SG, *et al.* Macrophage-targeted overexpression of urokinase causes accelerated atherosclerosis, coronary artery occlusions, and premature death. *Circulation* 2004, 109: 2129–2135.
- 29 Stoneman V, Braganza D, Figg N, Mercer J, Lang R, Goddard M and Bennett M. Monocyte/macrophage suppression in CD11b diphtheria toxin receptor transgenic mice differentially affects atherogenesis and established plaques. *Circ Res* 2007, 100: 884–893.
- 30 Schutyser E, Struyf S and Van Damme J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev* 2003, 14: 409–426.
- 31 Hromas R, Gray PW, Chantry D, Godiska R, Krathwohl M, Fife K and Bell GI, *et al.* Cloning and characterization of exodus, a novel beta-chemokine. *Blood* 1997, 89: 3315–3322.
- 32 Power CA, Church DJ, Meyer A, Alouani S, Proudfoot AE, Clark-Lewis I and Sozzani S, *et al.* Cloning and characterization of a specific receptor for the novel CC chemokine MIP-3alpha from lung dendritic cells. *J Exp Med* 1997, 186: 825–835.
- 33 Hausmann M, Bataille F, Spoettl T, Schreiter K, Falk W, Schoelmerich J and Herfarth H, *et al.* Physiological role of macrophage inflammatory protein-3 alpha induction during maturation of intestinal macrophages. *J Immunol* 2005, 175: 1389–1398.
- 34 Nakanishi T, Takahashi K, Hosokawa Y, Adachi T, Nakae H and Matsuo T. Expression of macrophage inflammatory protein 3a in human inflamed dental pulp tissue. *J Endod* 2005, 31: 84–87.
- 35 Ghadjar P, Rubie C, Aebbersold DM and Keilholz U. The chemokine CCL20 and its receptor CCR6 in human malignancy with focus on colorectal cancer. *Int J Cancer* 2009, 125: 741–745.
- 36 Wan W, Lim JK, Lionakis MS, Rivollier A, McDermott DH, Kelsall BL and Farber JM, *et al.* Genetic deletion of chemokine receptor Ccr6 decreases atherogenesis in ApoE-deficient mice. *Circ Res* 2011, 109: 374–381.
- 37 Yilmaz A, Lipfert B, Cicha I, Schubert K, Klein M, Raithel D and Daniel WG, *et al.* Accumulation of immune cells and high expression of chemokines/chemokine receptors in the upstream shoulder of atherosclerotic carotid plaques. *Exp Mol Pathol* 2007, 82: 245–255.
- 38 Pramanik R, Qi X, Borowicz S, Choubey D, Schultz RM, Han J and Chen G. p38 isoforms have opposite effects on AP-1-dependent transcription through regulation of c-Jun. The determinant roles of the isoforms in the p38 MAPK signal specificity. *J Biol Chem* 2003, 278: 4831–4839.
- 39 Ambrosino C, Iwata T, Scafoglio C, Mallardo M, Klein R and Nebreda AR. TEF-1 and C/EBPbeta are major p38alpha MAPK-regulated transcription factors in proliferating cardiomyocytes. *Biochem J* 2006, 396: 163–172.
- 40 Reibman J, Hsu Y, Chen LC, Bleck B and Gordon T. Airway epithelial cells release MIP-3a/CCL20 in response to cytokines and ambient particulate matter. *Am J Respir Cell Mol Biol* 2003, 28: 648–654.
- 41 Sugita S, Kohno T, Yamamoto K, Imaizumi Y, Nakajima H, Ishimaru T and Matsuyama T. Induction of macrophage-inflammatory protein-3a gene expression by TNF-dependent NF-kB activation. *J Immunol* 2002, 168: 5621–5628.