EXTENDED REPORT

Characterising the expression and function of CCL28 and its corresponding receptor, CCR10, in RA pathogenesis

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ABSTRACT

Objective This study was conducted to determine the expression pattern, regulation and function of CCL28 and CCR10 in rheumatoid arthritis (RA) pathogenesis.

Methods Expression of CCL28 and CCR10 was assessed in RA compared with other arthritis synovial tissues (STs) or fluids (SFs) by histology or ELISA. The factors modulating CCL28 and CCR10 expression were identified in RA myeloid and endothelial cells by ELISA, FACS and Western blotting. The mechanism by which CCL28 ligation promotes RA angiogenesis was examined in control and CCR10-knockdown endothelial cell chemotaxis and capillary formation.

Results CCL28 and/or CCR10 expression levels were accentuated in STs and SFs of patients with joint disease compared with normal controls and they were predominately coexpressed in RA myeloid and endothelial cells. We show that protein expression of CCL28 and CCR10 was modulated by tumour necrosis factor (TNF)-α and toll-like receptor 4 ligation in RA monocytes and endothelial cells and by interleukin (IL)-6 stimulation in RA macrophages. Neutralisation of CCL28 in RA SF or blockade of CCR10 on human endothelial progenitor cells (EPCs) significantly reduced SF-induced endothelial migration and capillary formation, demonstrating that ligation of joint CCL28 to endothelial CCR10+ cells is involved in RA angiogenesis. We discovered that angiogenesis driven by ligation of CCL28 to CCR10 is linked to the extracellular signal regulated kinase (ERK) cascade, as CCR10-knockdown cells exhibit dysfunctional CCL28-induced ERK signalling, chemotaxis and capillary formation.

Conclusions The overexpression of CCL28 and CCR10 in RA ST and their contribution to EPC migration into RA joints support the CCL28/CCR10 cascade as a potential therapeutic target for RA.

Mucosa-associated epithelial chemokine or CCL28 is a CCR10 ligand, which is typically secreted from epithelial cells in the gut, lung, breast and salivary gland.1,2 CCL28 is also constitutively expressed in epithelial colon cells where its expression level is modulated by proinflammatory cytokines and bacterial products, indicating that it may play a role in attracting CCR10+ cells to the site of colonic inflammation.3–4

Previous studies have shown that CCL28 can recruit CCR10+ IgA or IgE antibody-secreting cells to intestinal and non-intestinal mucosal tissues or respiratory epithelium in asthma.5–7 With the use of CCR10-deficient mice, it has been documented that, while localisation and accumulation of IgA antibody-producing plasma cells were compromised in lactating mammary glands, the presence of these cells were minimally affected in gastrointestinal tract.8 However, despite similar numbers of IgA-antibody producing plasma cells in wild-type and CCR10−/− mice, intestinal IgA response and memory B-cell maintenance were severely dysregulated in the knockout mice, highlighting the importance of CCR10 in IgA antibody-producing plasma-cell and B-cell function.9

Others have shown that trafficking of CCL28-expressing T regulatory cells (Tregs) detected around bile ducts is facilitated by CCL28 secreted from biliary epithelial cells.10 A recent elegant study has also revealed that, in ovarian cancer cells, CCL28 modulated by hypoxia facilitates homing of Tregs, which foster ovarian cancer tumour angiogenesis by increasing vascular endothelial growth factor (VEGF) production.11 These two studies identify dual roles for CCL28-induced Treg extravasation. The study conducted by Eksteen and colleagues10 identifies an anti-inflammatory role for CCL28-recruited Tregs in the mucosal sites, and, in contrast, the latter study reports that the Tregs attracted to CCL28 produced by ovarian cancer cells sustain tumour growth by enhancing tolerance and angiogenesis.11

Rheumatoid arthritis (RA) is a chronic autoimmune disease in which development of new blood vessels facilitates ingress of leucocytes and pannus formation leading to joint deformity and severe disability.12 Angiogenesis is an early and critical event in RA pathogenesis, which is fostered by an imbalance of joint proinflammatory cytokines and chemokines.13,14 Earlier studies have shown that ligation of CCL28 to CCR10 is involved in B- and T-cell trafficking.15 Yet the expression pattern, modulating factors and mechanism by which this cascade mediates its pathogenic effect are completely unknown in RA.

We show for the first time that expression of CCL28 and CCR10 is markedly higher in RA and osteoarthritis (OA) synovial tissue (ST) lining macrophages and sublining endothelial cells than in normal (NL) ST. We found that CCL28 and CCR10 expression is modulated by overlapping proinflammatory factors in RA myeloid and
endothelial cells, where this ligand and receptor pair colocalise. We reveal that CCL28 strongly attracts endothelial cells at the physiological concentration available in RA synovial fluid (SF). Last, we document that knockdown of endothelial CCR10 significantly reduces CCL28-mediated endothelial migration and capillary formation through an extracellular signal-regulated kinase (ERK)-dependent mechanism.

MATERIALS AND METHODS
Antibodies and immunohistochemical analysis
The studies were approved by the institutional review board, and all donors gave informed written consent. STs were deidentified; therefore disease severity and treatment information is unavailable. RA, OA and NL STs were formalin-fixed, paraffin-embedded and sectioned in the pathology core facility. STs were immunoperoxidase-stained using Vector Elite ABC kits (Vector Laboratories, Burlingame, California, USA), with diaminobenzidine (Vector Laboratories) as a chromogen. Briefly, slides were deparaffinised in xylene and then rehydrated by transfer through graded alcohols. Antigens were unmasked by incubating slides in proteinase K digestion buffer (DakoCytomation, Carpinteria, California, USA). Tissues were incubated with antibodies to human CCR10 (1:50; Santa Cruz Biotechnology, Santa Cruz, California, USA) or human CCL28 (1:50; Santa Cruz Biotechnology) or an IgG control antibody. Tissue sections were scored for lining, sublining macrophages and endothelial cell staining on a 0–5 scale by blinded observers (SJK and MVV).16–20 Scored data were pooled, and the mean±SEM was calculated in each data group. To demonstrate colocalisation of CCL28 and CCR10 on macrophages and endothelial cells, RA ST serial sections were stained with antibodies to CCL28, CCR10, von Willebrand factor (VWF; 1:100; Vector Laboratories) and CD68 (1:100; Vector Laboratories). To demonstrate cell purity, endothelial progenitor cells (EPCs) isolated from NL blood were stained for classical endothelial markers, VWF (1:1000), VE-cadherin (1:200) or IgG control, and positive staining was overlapped on 4',6-diamidino-2-phenylindole (DAP)-positive cells (1:1000). Fluorescent images were obtained using a Zeiss LSM 510 confocal microscope.

Cell isolation, culture and treatment
NL and RA peripheral blood (PB) and RA SF mononuclear cells were isolated by Ficoll–Paque gradient centrifugation as described previously.18–22 Monocytes were isolated from NL and RA PB using a negative selection kit, according to the manufacturer’s instructions.18–22 Monocytes were subsequently differentiated to macrophages by culturing in 20% fetal bovine serum (FBS)-containing medium.18–22 EPCs were isolated from NL blood. Briefly, NL PB mononuclear cells (PBMCs) were isolated and cultured on a collagen-coated plate in 10% FBS-containing endothelial basal medium-2 (EBM-2) medium. After 1 day, non-adherent cells were discarded, and medium was replaced daily for 2–4 weeks. Subsequently, endothelial colonies were isolated by a colony ring and transferred to a new plate where only a homogeneous population of endothelial cells was growing.23

RA PB monocytes, RA PB in vitro differentiated macrophages, and human umbilical vein endothelial cells (HUVECs; Lonza, Walkersville, Maryland, USA) were either untreated or treated with 10 ng/mL lipopolysaccharide (LPS; Invivogen, San Diego, California, USA), tumour necrosis factor (TNF)-α, interleukin (IL)-1β or IL-6 or 50 ng/mL IL-17 (R&D Systems, Minneapolis, Minnesota, USA) for 24–72 h before detection of CCL28 production by ELISA or CCR10 expression by FACS analysis (1:1000; Biolegend) or Western blotting (1:1000; Santa Cruz Biotechnology).

Reverse transcriptase (RT)-quantitative PCR
Total cellular RNA was extracted from the different cell types using TRIzol. Subsequently, reverse transcription and real-time RT-PCR were performed to determine CCL28 and CCR10 expression levels as described previously.18–22 24 Relative gene expression levels were determined by the ΔΔCt method based on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels, and results are expressed as fold increase over conditions indicated in the figure legends. CCL28 and CCR10 mRNA expression was quantified in RA PB monocytes treated with disease-modifying antirheumatic drugs (DMARDs) or anti-TNF-α with or without DMARDs.

RA patient population
RA specimens were obtained from patients with RA, diagnosed according to the 1987 revised criteria of the American College of Rheumatology.25 PB was obtained from 30 patients (27 women and 3 men; mean±SD age 51±20.2 years). At the time of evaluation, patients were receiving treatment with non-biological DMARDs (methotrexate, leflunomide, Plaquenil, sulfasalazine, imuran, Minocin or prednisone) alone (n=15; 13 women and 2 men; mean age 46.4±20.0 years), DMARDs plus TNF-α (Humira, Enbrel, Remicade or Cimzia) (n=13; 12 women and 1 man; mean age 53.6±16.4 years) or TNF-α alone (n=2; both women; mean age 61.5 years). These studies were approved by the University of Illinois at Chicago Institutional Ethics Review Board, and all donors gave informed written consent. The total number of patients was 30, but the reader is referred to the figure legends for the exact number of patients in each experiment.

Quantification of human CCL28
A human CCL28 ELISA kit (R&D Systems) was used according to the manufacturer’s instructions to quantify concentrations of CCL28 in STs (RA, OA and NL), SFs (RA, gout, OA and psoriatic arthritis (PsA)), sera (RA, OA and NL) and RA myeloid and endothelial cells treated with inflammatory factors.

Endothelial cell chemotaxis or tube formation
HUVECs were placed in a 48-well Boyden chemotaxis chamber to examine endothelial migration in response to various concentrations of CCL28 (0.001–100 ng/mL; R&D Systems), phosphate-buffered saline (PBS) (negative control) and VEGF (positive control; 10 ng/mL; R&D Systems).17 22 26 The number of migrating cells was counted, and the data represent an average of three high-power fields (HPFs)×40/well, averaged for each triplicate normalised to random migration in the PBS group.

To determine the role of CCL28 and/or CCR10 in RA SF-mediated endothelial migration, HUVEC chemotaxis or tube formation induced by RA SFs was examined after incubation of SFs (1:20) with control IgG or antibody to CCL28 (10 μg/mL) or treatment of HUVECs with antibodies to CCR10 (10 μg/mL; R&D Systems) or IgG control. The total number of branch points/tubes was quantified per well, and the data represent the mean of three wells normalised to the number of tubes in the PBS control well. Next, the contribution of RA SF CCL28 was confirmed in endothelial chemotaxis using EPCs isolated from human blood that expressed classical endothelial markers (VWF and VE-cadherin) and could form tubes in response to VEGF.

To examine which signalling pathway(s) contribute to CCL28-mediated endothelial chemotaxis and tube formation,
HUVECs were incubated with inhibitors (5 μM) to ERK (U0126; with and without CCL28 addition), p38 (SB203580), phosphatidylinositol 3-kinase (PI3K) (LY294002), N-terminal kinase (NKN) (SP600125) or dimethyl sulfoxide (DMSO) for 45 min before addition of CCL28 (50 ng/mL) to the wells. To confirm that CCL28 ligation to CCR10 is inter connected to activation of the ERK pathway and endothelial function, control and CCR10-knockdown cells were examined for endothelial CCR10 reduction (1:1000) and CCL28-induced ERK activation (CCL28; 100 ng/mL) by Western blotting. Control and CCR10-knockdown cells were also assessed for endothelial chemotaxis and capillary formation in response to CCL28 (50 ng/mL).

**CCL28 silencing in endothelial cells**

HUVECs were transfected with CCR10-specific and non-specific control small interfering RNA (Santa Cruz Biotechnologies) at a final concentration of 100 nM using TransIT (Mirus, Madison, Wisconsin, USA) transfection reagent, complying with the manufacturer’s instruction with a few modifications. The transfected cell culture medium was replaced after 24 h, and the endothelial cells were used after 72 h of transfection.

**CCL28-activated signalling pathway(s) in the endothelial cells**

HUVECs were either untreated or treated with CCL28 (10 or 100 ng/mL) for 0–60 min. Cell lysates were examined by Western blot analysis, and blots were probed with phospho(p) ERK, p-p38 mitogen-activated protein kinase (MAP), p-protein kinase B (AKT)1 or pJNK (1:1000) overnight and then reprobed with ERK, p38, AKT, JNK or actin (1:3000). CCR10-knockdown and control cells untreated or stimulated by CCL28 (100 ng/mL) were probed for CCR10 and pERK (1:1000) as well as ERK and actin (1:3000). Cell lysates from EPCs or HUVECs were probed for CCR10 or actin expression. Densitometric analysis of the Western blot bands was performed using Image J software.

**Statistical analysis**

One-way analysis of variance was used for comparisons among multiple groups, followed by Student’s post hoc two-tailed t test. Student’s paired and unpaired two-tailed tests were used for comparisons between two groups. p<0.05 was considered significant.

**RESULTS**

**Colocalisation of CCL28 and CCR10 in RA ST myeloid and endothelial cells**

When expression of CCL28 and CCR10 was examined in RA compared with OA and NL ST, we found that individuals with RA and OA have markedly higher levels of CCL28 and CCR10 expression on ST macrophages and endothelial cells (figures 1A, B, 2A,B and online supplementary figure S1). Next, colocalisation of CCL28 and CCR10 on RA ST myeloid cells and blood vessels was validated by serial section staining of CCL28 and CCR10 to CD68- and VWF-positive cells (figure 2C,D). Consistent with our histological studies, we document that patients with arthropathies express elevated levels of CCL28 in their ST (RA and OA) and SF (RA, OA, gout, PsA) compared with NL individuals (assessed using ST or serum) (figure 1C,D). These results suggest that, in patients with joint-related disease, the inflammatory response may contribute to production of CCL28 from myeloid and endothelial cells relative to normal controls. Interestingly, we found that transcription levels of both CCL28 and CCR10 are increased in RA compared with NL myeloid cells. While RA macrophages differentiated from monocytes exhibit a greater trend for CCL28 expression, CCR10 expression trend is higher in RA monocytes than differentiated macrophages (figures 1E and 2E). On the basis of these results, the effect of RA inflammatory mediators on myeloid and endothelial CCL28 and CCR10 expression was evaluated.

**CCL28 and CCR10 are similarly regulated in RA monocytes and endothelial cells**

Since both CCL28 and CCR10 are highly coexpressed in RA ST myeloid and endothelial cells, we asked whether their expression levels are regulated in a similar manner. We found that, in RA monocytes and endothelial cells, CCL28 secretion was very responsive to stimulation and was similarly enhanced by toll-like receptor (TLR)4 ligand, TNF-α, IL-1β, IL-17 and IL-6 treatment (figure 3A,C), whereas LPS and TNF-α were the common proinflammatory factors that increased CCR10 protein levels in RA monocytes and endothelial cells (figure 3D,F). Our results further demonstrate that, while stimulation with TLR4 ligand and TNF-α can enhance CCL28 and CCR10 protein levels in RA monocytes and endothelial cells (figure 3A,C,D,F), levels of this ligand and receptor pair are regulated by IL-6 in RA macrophages (figure 3B,E). Consistent with the stimulatory effect of TNF-α on myeloid CCL28 and CCR10 expression, anti-TNF-α therapy shows an insignificant lower trend of CCL28 and CCR10 expression in RA monocytes (see online supplementary figure S2). Taken together, these results suggest that CCL28 and CCR10 protein levels are modulated by an overlapping mechanism of function in RA myeloid and endothelial cells.

**Characterisation of EPCs extracted from NL human blood**

Although RA ST endothelial cells express CCR10, obtaining sufficient quantities of RA endothelial cells from STs is not possible. Furthermore, these cells are not commercially available. Therefore we tested EPCs as possible substitutes for RA endothelial cells. We show that EPCs purified from normal PBMCs were >95% pure based on cells being positive for both VWF and VE-cadherin, while no fluorescence staining was observed in the IgG control group (figure 4G,H). In addition, we document that, similarly to HUVECs, EPCs are capable of forming capillary tubes in response to a proangiogenic stimulus, such as VEGF, and express high levels of CCR10 (figure 4E,F). Hence in this study, human EPCs were used as surrogates for RA endothelial cells.

**Ligation of joint CCL28 to endothelial CCR10 facilitates RA SF-mediated angiogenesis**

Since similarly to RA, CCR10 was highly expressed in EPCs and HUVECs (figure 4F), these cells were used as substitutes for RA endothelial cells, and their role was investigated in CCL28-mediated RA angiogenesis. We found that CCL28 strongly attracts endothelial cells starting at a concentration of 0.1 ng/mL, indicating that CCL28 (up to 3300 pg/mL expressed in RA SF) can contribute to endothelial cell migration at a physiologically relevant concentration (figure 4A). We further document that ligation of SF CCL28 to endothelial CCR10 is involved in RA angiogenesis, as neutralisation of CCL28 in RA SF or blockade of CCR10 on HUVECs or EPCs significantly reduces RA SF-driven endothelial chemotaxis or tube formation by 30–40% (figure 4B,C,I). These results suggest that CCL28 at concentrations present in the RA joint can foster endothelial
**Figure 1**  CCL28 is elevated on rheumatoid arthritis (RA) synovial tissue (ST) myeloid and endothelial cells as well as in RA synovial fluid (SF) and serum. (A) Normal (NL), osteoarthritis (OA) and RA STs were stained with antibody to human CCL28 (1:50) (original magnification ×200). (B) Positive immunostaining was scored on a 0–5 scale in ST lining, sublining macrophages (Mac) and endothelial cells (Endo); staining is shown as mean±SEM (n=10). (C) CCL28 protein levels were quantified in NL, OA and RA STs by ELISA (n=10). (D) Protein concentration of CCL28 was assessed in serum obtained from RA (n=22), OA (n=10) and NL (n=19) and SF from patients with RA (n=22), OA (n=10) or psoriatic arthritis (PsA) (n=10). (E) Expression levels of CCL28 were quantified in NL and RA monocytes (Mono) and peripheral blood (PB) differentiated macrophages (Mac) by real-time reverse transcriptase-PCR and normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); the values are shown as fold increase vs NL PB monocytes (n=12–15). Values are mean±SE. *p<0.05.

**Figure 2**  Rheumatoid arthritis (RA) synovial tissue (ST) myeloid and endothelial cells show accentuated CCR10 immunostaining. (A) Normal (NL), osteoarthritis (OA) and RA STs were stained with antibody to human CCL10 (1:50; original magnification ×200; n=10. (B) Positive immunostaining was scored on a 0–5 scale in ST lining, sublining macrophages (Mac) and endothelial cells (Endo); staining is shown as mean±SEM (n=10). Colocalisation of CCL28 and CCR10 on macrophages (CD68+) (C) and endothelial cells (VWF+) (D) was examined when RA serial sections were stained with antibodies to CCL28, CCR10, CD68 and VWF (original magnification ×200). (E) Expression levels of CCR10 were quantified in NL and RA monocytes (Mono) and peripheral blood (PB) differentiated macrophages (Mac) by real-time reverse transcriptase-PCR and normalised to GAPDH; the values are shown as fold increase vs NL PB monocytes and are normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (n=12–15). Values are mean±SE. *p<0.05.
infiltration and capillary formation through CCR10 ligation, supporting the importance of the CCL28/CCR10 cascade in RA angiogenesis.

CCL28-driven angiogenesis is interconnected to ERK signalling

To determine which endothelial signalling pathways are activated by CCL28, phosphorylation of the MAPK and AKT pathways was determined by immunoblot analysis. Our data demonstrate that CCL28 strongly phosphorlates ERK (at 10 and 100 ng/mL), but AKT1, p38 and JNK pathways were not affected by this process (figure 5A, online supplementary figures S3 and S4). To determine which signalling pathway contributes to CCL28-induced endothelial migration and capillary formation, chemical inhibitors at a concentration of 5 μM were used, as 10 μM was toxic and resulted in cell death. As expected, inhibition of PI3K/AKT, JNK and p38 was ineffective in suppressing CCL28-induced endothelial chemotaxis and tube formation, while inhibition of the ERK cascade markedly reduced both functions by 30–40% (figures 5B–D). These results suggest that joint CCL28 promotes RA angiogenesis through activation of the ERK pathway.

Activation of the ERK pathway is linked to ligation of CCL28 to endothelial CCR10 and RA angiogenesis

Since phosphorylation of ERK is pivotal for CCL28-induced chemotaxis and tube formation, we next asked whether ligation of CCR10 is interconnected to CCL28 activation of the ERK pathway. Using CCR10-knockdown cells, which had 80% lower CCR10 expression, we found that, while CCL28-driven ERK phosphorylation was abolished compared with control cells, total ERK signalling was unaffected in this process (figure 6A–C). Consistent with this notion, CCR10-knockdown cells showed significantly reduced chemotaxis (40%) and tube formation (70%) in response to CCL28 compared with the control cells, suggesting that the proangiogenic effect of CCL28 is attributable to CCR10 ligation (figures 6D–F). Collectively, these results suggest that ERK is indispensable for CCR10 ligation of CCL28 and angiogenesis mediated by this factor.

DISCUSSION

The distribution pattern, regulation and mechanistic function of CCL28 and CCR10 in RA have not been defined. We found that in RA ST, CCL28 and CCR10 are uniquely expressed on myeloid and endothelial cells. Notably, we show that expression levels of this ligand and receptor is similarly modulated by IL-6 in RA macrophages and by TNF-α and TLR4 ligation in endothelial blood vessels and RA monocytes. We reveal that ligation of joint CCL28 to endothelial CCR10 plays an integral role in RA angiogenesis, supporting a novel mechanism of action for the CCL28/CCR10 cascade in RA pathology.

We show for the first time to our knowledge that CCL28 and CCR10 expression is elevated in RA and OA ST compared with NL ST myeloid and endothelial cells. Yet, upregulation of CCL28 has been reported in epithelial cells of inflamed mucosal tissue in the colon, duodenal mucosa and ileum of patients with Crohn’s disease and lungs, liver bile ducts and skin of patients with psoriasis and atopic dermatitis. Under normal physiological conditions, CCL28 is predominately produced by epithelial cells present in small and large intestine, reproductive tract, lung, lactating mammary gland and salivary gland as well as by pulmonary dendritic cells. Moreover, the CCL28 receptor, CCR10, is expressed in T cells, IgA antibody-secreting plasma cells, Langerhans cells and melanocytes. Thus the expression profile of CCL28 and CCR10 suggests that they are primarily responsible for regulating immune responses in epithelial-rich tissues.
Increased levels of CCL28 were detected in ST or SF of arthritic patients (RA, OA, gout and PsA) compared with normal individuals, indicating the importance of the CCL28/CCR10 cascade in inflammation. Although comparable levels of CCL28 were detected in RA and OA ST and SF, CCL28 concentrations were markedly lower in OA than RA sera. Therefore studies were conducted to examine the mechanism by which CCL28 and CCR10 promote pathogenesis in RA myeloid and endothelial cells. Interestingly, we show that differentiated macrophages show a greater trend of CCL28 expression than monocytes, highlighting the significance of CCL28 function in RA joints rather than in circulating blood cells. In contrast, the differentiation process reduces CCR10 expression trend in macrophages. These findings suggest that circulating monocytes may require higher levels of CCR10 to respond to the CCL28 produced by joint cells; however, once these CCR10-positive monocytes reach their destination, the differentiation process results in marginal CCR10 downregulation. In contrast with our observations with CCL28, others have shown that RA monocyte differentiation to macrophages does not alter CXCL16 secretion.

A great number of chemokines (CXCL1, CXCL5, CXCL8, CXCL16 and CCL2, CCL3, CCL5) and chemokine receptors (CXCR1, CXCR2, CXCR4, CXCR5, CCR1, CCR2, CCR5, CCR7, CCR8 and CX3CR1) have been found to be secreted, or are expressed, on RA myeloid cells. Macrophages play a central role in RA pathogenesis, as proinflammatory factors produced by these cells foster monocyte recruitment, neovascularisation and bone destruction, and hence the number of joint myeloid cells correlates closely with inflammation, joint pain and radiological damage. Earlier studies have shown that CCL28 production is modulated by IL-1β and TNF-α in human keratinocytes and by IL-1β, flagellin and/or LPS in the epithelial cells of human colon or bile duct. Others have shown that IL-17 drives CCL28 production in human airway epithelium. Consistent with these observations, our results show that the same proinflammatory factors, namely LPS, TNF-α, IL-1β, IL-17 and IL-6, provoke CCL28 production in RA monocytes and endothelial cells. However, CCL28 secretion is exclusively modulated by IL-17 and IL-6 in RA macrophages. Like CCL28, expression levels of CCL28 strongly promote endothelial progenitor cell (EPC) migration and capillary tube formation through CCR10 binding. (A) A dose–response curve of CCL28-induced human umbilical vein endothelial cell (HUVEC) chemotaxis was constructed in a Boyden chemotaxis chamber with varying concentration (0.001–100 ng/mL; n=3). Anti-CCL28 (10 μg/mL) or control IgG was added to rheumatoid arthritis (RA) synovial fluid (SF) (1:20) or HUVECs were incubated with antibodies to CCR10 (10 μg/mL) or isotype control for 1 h before chemotaxis (B) or tube formation (C) in response to RA SFs (n=8). (D) Photomicrographs were taken of representative wells treated with phosphate-buffered saline (PBS), vascular endothelial growth factor (VEGF; 10 ng/mL), IgG or anti-CCL28 (10 μg/mL) plus RA SFs (1:20) and IgG or anti-CCR10 (10 μg/mL) plus HUVECs. (E) The ability of EPCs to form capillary tubes was tested in response to PBS and VEGF (10 ng/mL) (n=3). (F) Expression levels of CCR10 and equal actin loading was assessed in EPCs and HUVECs by Western blot analysis (n=3). (G) To validate EPC purity, cells were stained with endothelial markers, von Willebrand factor (VWF; 1:1000), VE-cadherin (1:200) or IgG control, and positive staining was overlapped on 4',6-diamidino-2-phenylindole (DAPI)+ cells (1:1000) (original magnification ×400) (n=3). (H) Anti-CCL28 (10 μg/mL) or control IgG was added to RA SFs (1:20), and EPCs were incubated with antibodies to CCR10 (10 μg/mL) or isotype control for 1 h before chemotaxis (n=3). Endothelial cell chemotaxis or tube formation is shown as a relative value and is normalised to PBS values. Values are mean±SE. *p<0.05. HPF, high-power field.
Figure 5  CCL28-driven endothelial migration and tube formation is facilitated by extracellular signal regulated kinase (ERK) activation. (A) Cells were stimulated with CCL28 (100 ng/mL) for 0–60 min and thereafter cell lysates were probed for phospho (p) protein kinase B (AKT1), pJNK (Jun N-terminal kinase), pERK, p-p38 or equal loading controls (n=4–6). Human umbilical vein endothelial cells (HUVECs) were incubated with dimethyl sulfoxide (DMSO) or inhibitors (5 μM) to ERK (U0126; with or without CCL28 addition), p38 (SB203580 (SB)), phosphatidylinositol 3-kinase (PI3K) (LY294002 (LY)) or JNK (SP600125 (SP)) for 45 min before endothelial chemotaxis (B) or tube formation (C) in response to CCL28 (50 ng/mL) (n=3). (D) Photomicrographs were taken of representative wells treated with phosphate-buffered saline (PBS), vascular endothelial growth factor (VEGF; 10 ng/mL), U0126, DMSO (D) plus CCL28 (100 ng/mL), U0126 plus CCL28, SB203580 (SB) plus CCL28, LY294002 (LY) plus CCL28 or SP600125 (SP) plus CCL28. HPF, high-power field.

Figure 6  Angiogenesis fostered through ligation of CCL28 to CCR10 is interconnected to ERK signalling. (A) Human umbilical vein endothelial cells (HUVECs) transfected with CCR10-specific and nonspecific control small interfering (si)RNA (100 nM) were either untreated or treated with CCL28 (100 ng/mL) for 10 min. Thereafter cell lysates were examined for CCR10, pERK (extracellular signal regulated kinase), ERK and actin. Densitometric analysis of the CCR10/actin (B) and pERK/ERK (C) was performed using Image J software (n=3). CCL28 (50 ng/mL)-mediated HUVEC chemotaxis (D) and tube formation (E) was assessed in control and CCR10-knockdown cells (n=3). (F) Photomicrographs were taken of representative wells treated with phosphate-buffered saline (PBS), vascular endothelial growth factor (VEGF; 10 ng/mL), control (Ctl) knockdown cells plus CCL28 (50 ng/mL) and CCR10-knockdown cells plus CCL28. Values are mean±SE. *p<0.05.
CCR10 are influenced by IL-1β, IL-17 and TNF-α stimulation in stromal cells; however, CCR10 is uniquely enhanced by retinoic acids in plasma cells that produce IgA-positive antibody. We notably show that TNF-α is the common factor that drives CCL28 and CCR10 expression in blood vessels and RA monocytes, as such RA patients treated with anti-TNF blockers show an insignificant lower trend of CCL28 and CCR10 expression compared with those treated with DMARDs. The fact that CCL28 and CCR10 are modulated by overlapping inflammatory factors in the cell types in which they colocalise makes RA myeloid and endothelial cells very responsive to CCL28 signalling.

The role of CCL28 and CCR10 has been extensively studied in B- and T-cell infiltration, maintenance and immune response. CCL28 plays a highly significant role in homing and preservation of the CCR10+ IgA antibody-secreting plasma cells to mucosal epithelium, since CCR10-deficient mice have an impaired IgA memory response to pathogen reinfection. Further, CCL28 secreted from biliary epithelial cells attracts CCR10+ Tregs, leading to accumulation of Tregs on the mucosal surfaces.

We found that EPCs, similarly to RA joint endothelial cells, express high levels of CCR10 and can form capillaries in response to proangiogenic factors. Corroborating these observations, others have shown that EPCs have a greater ability to migrate into RA ST than NL ST implants, and the number of infiltrated EPCs is markedly elevated in collagen antibody-induced arthritis synovium; therefore EPCs were used as surrogates for RA endothelial cells. Our research team has pioneered the field by demonstrating that the production of CCL28 from joint myeloid and endothelial cells is a strong promoter of angiogenesis in EPCs at a physiologically relevant concentration. We further show that both CCL28 and CCR10 are involved in RA SF-mediated EPC chemotaxis. Moreover, our data document that angiogenesis facilitated through ligation of CCL28 to CCR10 is interconnected to ERK signalling, as CCR10-knockdown cells show dysfunctional CCL28-induced ERK signalling and chemotaxis. To our knowledge, this is the first evidence of a direct role for CCL28 and CCR10 in angiogenesis. A recent study found that, in ovarian tumour cells, hypoxia-triggered CCL28 production promotes homing of CCR10+ Tregs to ovarian tumour. Interestingly, the authors further report that secretion of VEGF from CCR10+ Tregs contributes to ovarian tumour angiogenesis. In this model, Tregs are integral to VEGF production, as anti-CCR10 or anti-CD25 (markers for Tregs) treatment greatly suppresses tumour vascularity and VEGF levels. Although we and others have shown that angiogenesis can be fostered by ignition of the CCL28/ CCR10 cascade, there are a number of dissimilarities between these two studies. While we document that CCL28 can directly mediate neovascularisation by attracting CCR10+ endothelial cells, the study by Facciabene and colleagues shows that CCL28 ligation to Tregs is indirectly responsible for tumour angiogenesis.

We have uncovered a novel ligand and receptor pair whose expression has not previously been reported in the cell types identified in this study or in RA. This study unravels the regulation and unique functional mechanism of CCL28/CCR10 in RA, supporting CCL28/CCR10 as a potential therapeutic target in this disease.

Contributors Designed the research: ZC, SJK, SS. Performed the research: ZC, SJK, ABE, MVV. Analysed the data: ZC, SJK, MVV, ABE, SS. Provided essential reagents: OMV, WS, SA, SV, NS, LVS. Wrote the paper: all the authors contributed.

Funding This work was supported in part by awards from the National Institutes of Health AR055240, a grant from Within Our Reach from The American College of Rheumatology, and funding provided by Department of Defense (PR093477) and Arthritis Foundation Innovative Research Grants.

Competing interests None.

Patient consent Obtained.

Ethics approval The studies were approved by the Institutional Review Board of University of Illinois at Chicago.

Provenance and peer review Not commissioned; externally peer reviewed.

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Ann Rheum Dis published online May 15, 2014

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Published online May 15, 2014 in advance of the print journal.

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