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Characterizing 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 to other arthritis synovial tissues (ST)s or fluids (SF)s 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 or CCR10 knockdown endothelial cell chemotaxis and capillary formation.

Results—CCL28 and/or CCR10 expression levels were accentuated in STs or SFs of patients with joint disease compared to 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 TNF- α and TLR4 ligation in RA monocytes and endothelial cells and by IL-6 stimulation in RA macrophages. Neutralization of CCL28 in RA SF or blockade of CCR10 on human endothelial progenitor cells (EPC)s significantly reduced SF induced endothelial migration and capillary formation, demonstrating that ligation of joint CCL28 to endothelial CCR10+ cells participates in RA angiogenesis. We uncovered that angiogenesis driven by ligation of CCL28 to CCR10 is linked to the ERK cascade as CCR10 knockdown cells exhibit dysfunctional CCL28 induced ERK signaling, chemotaxis and capillary formation.

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Designed the research: ZC, SJK, SS

Performed the research: ZC, SJK, ABE, MVV

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

Keywords

RA; CCL28; CCR10; angiogenesis; macrophages; endothelial cells

Mucosa associated epithelial chemokine (MEC) 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 pro-inflammatory cytokines and bacterial products indicating that this chemokine may play a role in attracting CCR10+ cells to the site of colonic inflammation^{3, 4}.

Previous studies demonstrate that the CCL28 can recruit CCR10+ IgA or IgE antibody secreting cells to intestinal and non-intestinal mucosal tissues or to respiratory epithelium in asthma⁵⁻⁷. Employing CCR10 deficient mice it was documented that while localization and accumulation of IgA antibody producing plasma cells were compromised in lactating mammary glands the presence of these cells were minimally affected in gastrointestinal tract⁸. However despite similar numbers of IgA antibody producing plasma cells in wild type and CCR10^{-/-} mice, the intestinal IgA response and the 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 function⁹.

Others have shown that trafficking of CCR10 expressing T regulatory cells (Tregs) detected around bile ducts is facilitated by CCL28 secreted from biliary epithelial cells¹⁰. A recent elegant study also uncovers that in ovarian cancer cells, CCL28 modulated by hypoxia facilitates homing of Tregs that foster ovarian cancer tumor angiogenesis by accentuating VEGF production¹¹. These two studies identify dual roles for CCL28 induced Treg extravasation. The study conducted by Eksteen and colleagues identifies an anti-inflammatory role for CCL28 recruited Tregs in the mucosal sites¹⁰, in contrast the latter study reports that the Tregs attracted to CCL28 produced from ovarian cancer cells, sustain tumor growth via enhancing tolerance and angiogenesis¹¹.

RA is a chronic autoimmune disease in which development of new blood vessels facilitate ingress of leukocytes and pannus formation leading to joint deformity and severe disability¹². Angiogenesis is an early and critical event in RA pathogenesis that is fostered by imbalance of joint pro-inflammatory cytokines and chemokines^{13, 14}. Earlier studies have shown that ligation of CCL28 to CCR10 is involved in B and T cell trafficking¹⁵, yet the expression pattern, modulating factors and the mechanism by which this cascade mediates its pathogenic effect is completely unknown in RA.

We show for the first time that expression of CCL28 and CCR10 is markedly higher in RA and osteoarthritis (OA) ST lining macrophages and sublining endothelial cells compared to normal (NL) ST. We found that CCL28 and CCR10 expression are modulated by overlapping proinflammatory factors in RA myeloid and endothelial cells, where this ligand

and receptor pair colocalize. We uncovered that CCL28 strongly attracts endothelial cells at the physiological concentration available in RA SF. Last, we document that knockdown of endothelial CCR10 significantly reduces CCL28 mediated endothelial migration and capillary formation through an 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 de-identified; therefore disease severity and treatment information is unavailable. RA, OA, and NL ST were formalin fixed, paraffin embedded, and sectioned in the pathology core facility. ST were immunoperoxidase-stained using Vector Elite ABC kits (Vector Laboratories, Burlingame, CA), with diaminobenzidine (Vector Laboratories) as a chromogen. Briefly, slides were deparaffinized in xylene, followed by rehydration by transfer through graded alcohols. Antigens were unmasked by incubating slides in proteinase K digestion buffer (DakoCytomation, Carpinteria, CA). Tissues were incubated with Abs to human CCR10 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), human CCL28 (1:50; Santa Cruz Biotechnology) or an IgG control Ab. 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 $\pm$ SEM was calculated in each data group. To demonstrate colocalization of CCL28 and CCR10 on macrophages and endothelial cells, RA ST serial sections were stained with anti-CCL28, anti-CCR10, anti-VWF (1:1000; Vector Laboratories) and anti-CD68 (1:100; Vector Laboratories) Abs. 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 DAPI+ 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% FBS containing media ^{18–22}. EPCs were isolated from NL blood. Briefly, NL peripheral blood mononuclear cells (PBMC)s were isolated and cultured on a collagen coated plate in 10% FBS containing EBM2 media. After 1 day, nonadherent cells were discarded and media was replaced daily for 2–4 weeks. Subsequently, endothelial colonies were isolated by a colony ring and were transferred to a new plate where only a homogenous 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, MD) were either untreated or treated with 10 ng/ml LPS (Invivogen, San Diego, CA), TNF- α , IL-1 α , IL-6 or 50 ng/ml IL-17 (R&D Systems, Minneapolis, MN) for 24–72h prior to detecting CCL28 production by ELISA or

CCR10 expression via FACS analysis (1:1000; Biolegend) or Western blotting (1:1000; Santa Cruz Biotechnology).

RT-qPCR

Total cellular RNA was extracted using TRIzol from the different cell types. 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 was determined by the C_t method based on the GAPDH levels, and results were expressed as fold increase above conditions indicated in the figure legends. CCL28 and CCR10 mRNA expression was quantified in RA PB monocytes treated with disease-modifying antirheumatic drugs (DMARD) or anti-TNF- α $\pm$ DMARD.

RA patient population

RA specimens were obtained from patients with RA, diagnosed according to the 1987 revised criteria of the American College of Rheumatology²⁵. PB was obtained from 30 patients, 27 women and 3 men (mean age, 51 ± 20.2 y old). At the time of evaluation, patients received treatment with nonbiological DMARDs (Methotrexate, Leflunomide, Plaquenil, Sulfasalazine, Imuran, Minocin, or Prednisone) alone ($n=15$; 13 women and 2 men; mean age, 46.4 ± 20.0 y old); treatment with DMARDs plus α -TNF (Humira, Enbrel, Remicade or Cimzia) ($n=13$; 12 women and 1 men; mean age, 55.6 ± 16.4 y) or treatment with α -TNF alone ($n=2$ both women; mean age, 61.5 y old). These studies were approved by the University of Illinois at Chicago Institutional Ethics Review Board, and all donors gave informed written consent. The number of patients was 30, but refer to the figure legends for the exact number of patients in each experiment.

Quantification of human CCL28

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

Endothelial cell chemotaxis or tube formation

HUVECs were placed in 48-well Boyden chemotaxis chamber to examine endothelial migration in response to varying concentrations of CCL28 (0.001 to 100 ng/ml, R&D Systems), PBS (negative control) and VEGF (positive control, 10 ng/ml)^{17, 22, 26}. The number of migrating cells was counted and the data represent an average of 3 high power fields (HPF)s $\times$ 40 /well, averaged for each triplicate normalized 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 following incubation of SFs (1:20) with control IgG or anti-CCL28 antibody (10 μ g/ml; R&D Systems) or treatment of HUVECs with antibodies to CCR10 (10 μ g/ml; R&D Systems) or IgG control. The total number of branch points/tubes was quantified per well and the data represent average of 3 wells normalized 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 (R&D; 10 ng/ml).

To examine which signaling pathway(s) contribute to CCL28 mediated endothelial chemotaxis and tube formation, HUVECs were incubated with inhibitors (5 μ M) to ERK (U0126; with and without CCL28 addition), p38 (SB203580), PI3K (LY294002), JNK (SP600125) or DMSO for 45 min prior to adding the CCL28 (50 ng/ml) to the wells ¹⁷.

To determine that CCL28 ligation to CCR10 is interconnected to activation of 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).

CCR10 silencing in endothelial cells

HUVECs were transfected with CCR10 specific and nonspecific control siRNA (Santa Cruz Biotechnologies) at a final concentration of 100 nM using TransIT (Mirus, Madison, WI) transfection reagent complying with the manufacturer's instruction with few modifications. The transfected cell culture media was replaced after 24h and the endothelial cells were utilized after 72h of transfection.

CCL28 activated signaling 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 p-ERK, p-p38 MAPK, p-AKT1, or p-JNK (1:1000) overnight then reprobed with ERK, p38, AKT, JNK or actin (1:3000). Knockdown CCR10 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 were 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 2-tailed T-test. Students paired and unpaired 2-tailed test were used for comparisons between 2 groups. *P* values less than 0.05 were considered significant.

RESULTS

Colocalization of CCL28 and CCR10 in RA synovial tissue myeloid and endothelial cells

When expression of CCL28 and CCR10 was examined in RA compared to OA and NL ST, we found that individuals with RA and OA have markedly higher CCL28 and CCR10 expression levels on ST macrophages and endothelial cells (Figs. 1A-B, 2A-B, S1). Next, colocalization 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 (Figs. 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, psoriatic arthritis (PsA)] compared to NL individuals (assessed using ST or sera) (Figs. 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 both CCL28 and CCR10 transcription levels are increased in RA compared to NL myeloid cells. While RA macrophages differentiated from monocytes exhibit a greater trend for CCL28 expression, CCR10 expression trend is higher in RA monocytes compared to differentiated macrophages (Figs. 1E, 2E). Based on these results, the impact of RA inflammatory mediators was evaluated on myeloid and endothelial CCL28 and CCR10 expression levels.

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 therefore 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 TLR4 ligand, TNF- α , IL-1 α , IL-17 or IL-6 treatment (Figs. 3A, 3C). Whereas, LPS and TNF- α were the common proinflammatory factors that accentuated CCR10 protein levels in RA monocytes and endothelial cells (Figs. 3D, 3F). 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 (Figs. 3A, 3C, 3D, 3F), levels of this ligand and receptor pair is regulated by IL-6 in RA macrophages (Figs. 3B, 3E). Consistent with the stimulatory effect of TNF- α on myeloid CCL28 and CCR10 expression, anti-TNF- α therapy demonstrates an insignificant lower trend of CCL28 and CCR10 expression in RA monocytes (Fig. S2). Taken together these results conclude that CCL28 and CCR10 protein levels are modulated by overlapping mechanism of function in RA myeloid and endothelial cells.

Characterization 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 and 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 IgG control group (Figs. 4G-H). Additionally, we document that similar to HUVECs, EPCs are capable of forming capillary tubes in response to proangiogenic stimulus such as VEGF and express high levels of CCR10 (Figs. 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 similar to RA, CCR10 was highly expressed in EPCs and HUVECs (Fig. 4F), these cells were used as substitutes for RA endothelial cells and their role was investigated in CCL28 mediated RA angiogenesis. We uncovered that CCL28 can strongly attract endothelial cells starting at 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 (Fig. 4A). We further document that ligation of SF CCL28 to endothelial

CCR10 is involved in RA angiogenesis, as neutralization of CCL28 in RA SF or blockade of CCR10 on HUVECs or EPCs can significantly reduce RA SF driven endothelial chemotaxis or tube formation by 30-40% (Figs. 4B-C, 4I). These results suggest that CCL28 at concentrations present in the RA joint can foster endothelial infiltration and capillary formation through CCR10 ligation; hence supporting the importance of CCL28/CCR10 cascade in RA angiogenesis.

CCL28 driven angiogenesis is interconnected to ERK signaling

To determine which endothelial signaling pathways are activated by CCL28, phosphorylation of the MAPK and AKT pathways was determined by immunoblot analysis. Our data demonstrate that CCL28 strongly phosphorylates ERK (at 10 and 100 ng/ml) however AKT1, p38 and JNK pathways were not affected by this process (Figs. 5A, S3-S4). To determine which signaling pathway contributes to CCL28 induced endothelial migration and capillary formation, chemical inhibitors at concentrations of 5 μ M were utilized, as 10 μ M 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 ERK cascade markedly reduced both functions by 30-40% (Fig. 5B-D). These results suggest that joint CCL28 promotes RA angiogenesis through activation of ERK pathway.

Activation of 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 ERK pathway. Employing CCR10 knockdown cells which had 80% lower CCR10 expression, we found that while CCL28 driven ERK phosphorylation was abolished compared to control cells, total ERK signaling was unaffected in this process (Figs. 6A-C). Consistent with this notion, CCR10 knockdown cells showed significantly reduced chemotaxis (40%) and tube formation (70%) in response to CCL28 compared to the control cells, suggesting that the proangiogenic effect of CCL28 is attributable to CCR10 ligation (Figs. 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 the mechanistic function of CCL28 and CCR10 is undefined in RA. 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 uncovered that ligation of joint CCL28 to endothelial CCR10 plays an integral role in RA angiogenesis, thus supporting a novel mechanism of action for CCL28/CCR10 cascade in RA pathology.

To our knowledge, we show for the first time that CCL28 and CCR10 expression is elevated in RA and OA ST compared to NL ST myeloid and endothelial cells. Yet, upregulation of

CCL28 has been reported in the epithelial cells of inflamed mucosal tissue detected in colons, duodenal mucosa, ileum of patients with Cohn's disease, lungs and liver bile ducts as well as in the skin epithelial cells of patients with psoriasis and atopic dermatitis^{2, 10, 27-29}. In normal physiological conditions, CCL28 is predominately produced from epithelial cells present in small and large intestines, reproductive tracts, lungs, lactating mammary glands and salivary glands as well as from pulmonary dendritic cells^{1, 2, 4}. Moreover the CCL28 corresponding receptor CCR10 is expressed in T cells, IgA antibody secreting plasma cells, Langerhans cells and melanocytes^{30, 31}. Thus expression profile of CCL28 and CCR10 suggests that they are primarily responsible for regulating the immune responses in epithelial rich tissues.

Accentuated levels of CCL28 were detected in ST or SF of arthritis patients (RA, OA, gout and PsA) compared to normal individuals, indicating the importance of 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 compared to 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 demonstrate a greater trend of CCL28 expression compared to monocytes, highlighting the significance of CCL28 function in the RA joints rather than in the circulating blood cells. On the contrary, the differentiation process reduces CCR10 expression trend in macrophages. These findings suggest that the circulating monocytes may require higher levels of CCR10 to respond to the CCL28 produced by joint cells, however once these CCR10+ monocytes reach their destination the differentiation process results in a marginal CCR10 downregulation. In contrast to our observation with CCL28, others have shown that RA monocyte differentiation to macrophages does not alter CXCL16 secretion³². A great number of chemokines (CXCL1, 5, 8, 16 and CCL2, 3, 5) and chemokine receptors (CXCR1, 2, 4, 5 and CCR1, 2, 5, 7, 8 and CX3CR1) have been found to be secreted or are expressed on RA myeloid cells³³. Macrophages play a central role in RA pathogenesis as pro-inflammatory factors produced from these cells foster monocyte recruitment, neovascularization and bone destruction hence the number of joint myeloid cells closely correlates with inflammation, joint pain and radiological damage^{34, 35}.

Earlier studies demonstrate that CCL28 production is modulated by IL-1 α and TNF- α in human keratinocytes and by IL-1 α , flagellin and/or LPS in the epithelial cells of human colon or bile duct^{3, 10, 29}. Others have shown that IL-17 drives CCL28 production in human airway epithelium⁷. Consistent with these observations, our results demonstrate that the same pro-inflammatory factors namely LPS, TNF- α , IL-1 α , 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 CCR10 are influenced by IL-1 α , IL-17 and TNF- α stimulation in stromal cells; however CCR10 is uniquely enhanced by retinoid acids in plasma cells that produce IgA+ antibody^{36, 37}. 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 demonstrate an insignificant lower trend of CCL28 and CCR10 expression compared to DMARDs. The fact that CCL28 and CCR10 are modulated by overlapping inflammatory

factors in the cell types where they colocalize, makes RA myeloid and endothelial cells very responsive for CCL28 signaling.

Role of CCL28 and CCR10 has been extensively studied in B and T cell infiltration, maintenance and immune response ¹⁵. 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 demonstrate an impaired IgA memory response to pathogen re-infection ⁹. Further CCL28 secreted from biliary epithelial cells attracts CCR10+ Tregs leading to accumulation of Tregs in the mucosal surfaces ¹⁰.

We uncovered that EPCs similar to RA joint endothelial cells express high levels of CCR10 and can form capillaries in response to proangiogenic factors. Corroborating with these observations, other have shown that EPCs have a greater ability to migrate into RA ST compared to NL ST implants and the number of infiltrated EPCs is markedly elevated in collagen antibody induced arthritis (CAIA) synovium ³⁸; 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 signaling as CCR10 knockdown cells demonstrate dysfunctional CCL28 induced ERK signaling and chemotaxis. To our knowledge this is the first evidence that shows a direct role for CCL28 and CCR10 in angiogenesis. A recent study found that in ovarian tumor cells, hypoxia triggered CCL28 production, promotes homing of CCR10+ Tregs into the ovarian tumor ¹¹. Interestingly the authors further report that secretion of VEGF from CCR10+ Tregs contributes to ovarian tumor angiogenesis. In this model, Tregs are integral for VEGF production as anti-CCR10 or anti-CD25 (markers for Tregs) treatment greatly suppresses tumor vascularity and VEGF levels ¹¹. Although both we and others demonstrate that angiogenesis can be fostered by ignition of CCL28/CCR10 cascade, there are a number of dissimilarities between these two studies. While we document that CCL28 can directly mediate neovascularization by attracting CCR10+ endothelial cells, the study by Facciabene and colleagues show that CCL28 ligation to Tregs is indirectly responsible for tumor angiogenesis.

We uncovered a novel ligand and receptor pair whose expression was neither described in the cell types identified in this study nor in RA. The current study unravels the regulation and unique mechanism of function of CCL28/CCR10 in RA, supporting CCL28/CCR10 as a potential therapeutic target in RA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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LITERATURE CITED

1. Pan J, Kunkel EJ, Gossler U, et al. A novel chemokine ligand for CCR10 and CCR3 expressed by epithelial cells in mucosal tissues. *J Immunol.* 2000; 165:2943–2949. [PubMed: 10975800]
2. Wang W, Soto H, Oldham ER, et al. Identification of a novel chemokine (CCL28), which binds CCR10 (GPR2). *J Biol Chem.* 2000; 275:22313–22323. [PubMed: 10781587]
3. Ogawa H, Iimura M, Eckmann L, et al. Regulated production of the chemokine CCL28 in human colon epithelium. *American Journal of Physiology Gastrointestinal and Liver Physiology.* 2004; 287:G1062–1069. [PubMed: 15246961]
4. Hieshima K, Ohtani H, Shibano M, et al. CCL28 has dual roles in mucosal immunity as a chemokine with broad-spectrum antimicrobial activity. *J Immunol.* 2003; 170:1452–1461. [PubMed: 12538707]
5. Kunkel EJ, Kim CH, Lazarus NH, et al. CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. *J Clin Invest.* 2003; 111:1001–1010. [PubMed: 12671049]
6. Lazarus NH, Kunkel EJ, Johnston B, et al. A common mucosal chemokine (mucosae-associated epithelial chemokine/CCL28) selectively attracts IgA plasmablasts. *J Immunol.* 2003; 170:3799–3805. [PubMed: 12646646]
7. Scanlon KM, Hawksworth RJ, Lane SJ, et al. IL-17A induces CCL28, supporting the chemotaxis of IgE-secreting B cells. *Int Arch Allergy Immunol.* 2011; 156:51–61. [PubMed: 21447959]
8. Morteau O, Gerard C, Lu B, et al. An indispensable role for the chemokine receptor CCR10 in IgA antibody-secreting cell accumulation. *J Immunol.* 2008; 181:6309–6315. [PubMed: 18941222]
9. Hu S, Yang K, Yang J, et al. Critical roles of chemokine receptor CCR10 in regulating memory IgA responses in intestines. *Proc Natl Acad Sci U S A.* 2011; 108:E1035–1044. [PubMed: 21969568]
10. Eksteen B, Miles A, Curbishley SM, et al. Epithelial inflammation is associated with CCL28 production and the recruitment of regulatory T cells expressing CCR10. *J Immunol.* 2006; 177:593–603. [PubMed: 16785557]
11. Facciabene A, Peng X, Hagemann IS, et al. Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. *Nature.* 2011; 475:226–230. [PubMed: 21753853]
12. Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature.* 2003; 423:356–361. [PubMed: 12748655]
13. Szekanecz Z, Pakozdi A, Szentpetery A, et al. Chemokines and angiogenesis in rheumatoid arthritis. *Front Biosci (Elite Ed).* 2009; 1:44–51. [PubMed: 19482623]
14. Szekanecz Z, Besenyei T, Szentpetery A, et al. Angiogenesis and vasculogenesis in rheumatoid arthritis. *Curr Opin Rheumatol.* 2010; 22:299–306. [PubMed: 20305562]
15. Xiong N, Fu Y, Hu S, et al. CCR10 and its ligands in regulation of epithelial immunity and diseases. *Protein Cell.* 2012; 3:571–580. [PubMed: 22684736]
16. Pickens SR, Chamberlain ND, Volin MV, et al. Local expression of interleukin-27 ameliorates collagen-induced arthritis. *Arthritis Rheum.* 2011; 63:2289–2298. [PubMed: 21384333]
17. Pickens SR, Chamberlain ND, Volin MV, et al. Role of the CCL21 and CCR7 pathways in rheumatoid arthritis angiogenesis. *Arthritis Rheum.* 2012; 64:2471–2481. [PubMed: 22392503]
18. Chen Z, Kim SJ, Chamberlain ND, et al. The Novel Role of IL-7 Ligation to IL-7 Receptor in Myeloid Cells of Rheumatoid Arthritis and Collagen-Induced Arthritis. *J Immunol.* 2013
19. Chamberlain ND, Kim SJ, Vila OM, et al. Ligation of TLR7 by rheumatoid arthritis synovial fluid single strand RNA induces transcription of TNFalpha in monocytes. *Annals of the Rheumatic Diseases.* 2013; 72:418–426. [PubMed: 22730373]
20. Chamberlain ND, Vila OM, Volin MV, et al. TLR5, a novel and unidentified inflammatory mediator in rheumatoid arthritis that correlates with disease activity score and joint TNF-alpha levels. *J Immunol.* 2012; 189:475–483. [PubMed: 22661088]

21. Pickens SR, Chamberlain ND, Volin MV, et al. Characterization of interleukin-7 and interleukin-7 receptor in the pathogenesis of rheumatoid arthritis. *Arthritis Rheum.* 2011; 63:2884–2893. [PubMed: 21647866]
22. Kim SJ, Chen Z, Chamberlain ND, et al. Angiogenesis in Rheumatoid Arthritis Is Fostered Directly by Toll-like Receptor 5 Ligation and Indirectly Through Interleukin-17 Induction. *Arthritis Rheum.* 2013; 65:2024–2036. [PubMed: 23666857]
23. Zhang Y, Ingram DA, Murphy MP, et al. Release of proinflammatory mediators and expression of proinflammatory adhesion molecules by endothelial progenitor cells. *Am J Physiol Heart Circ Physiol.* 2009; 296:H1675–1682. [PubMed: 19252096]
24. Pickens SR, Chamberlain ND, Volin MV, et al. Characterization of CCL19 and CCL21 in rheumatoid arthritis. *Arthritis Rheum.* 2011; 63:914–922. [PubMed: 21225692]
25. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis and Rheumatism.* 1988; 31:315–324. [PubMed: 3358796]
26. Pickens SR, Volin MV, Mandelin AM 2nd, et al. IL-17 contributes to angiogenesis in rheumatoid arthritis. *J Immunol.* 2010; 184:3233–3241. [PubMed: 20173024]
27. Maeda S, Ohno K, Nakamura K, et al. Quantification of chemokine and chemokine receptor gene expression in duodenal mucosa of dogs with inflammatory bowel disease. *Vet Immunol Immunopathol.* 2011; 144:290–298. [PubMed: 21996024]
28. John AE, Thomas MS, Berlin AA, et al. Temporal production of CCL28 corresponds to eosinophil accumulation and airway hyperreactivity in allergic airway inflammation. *Am J Pathol.* 2005; 166:345–353. [PubMed: 15681819]
29. Kagami S, Saeki H, Tsunemi Y, et al. CCL27-transgenic mice show enhanced contact hypersensitivity to Th2, but not Th1 stimuli. *Eur J Immunol.* 2008; 38:647–657. [PubMed: 18266301]
30. Homey B, Wang W, Soto H, et al. Cutting edge: the orphan chemokine receptor G protein-coupled receptor-2 (GPR-2, CCR10) binds the skin-associated chemokine CCL27 (CTACK/ALP/ILC). *J Immunol.* 2000; 164:3465–3470. [PubMed: 10725697]
31. Jarmin DI, Rits M, Bota D, et al. Cutting edge: identification of the orphan receptor G-protein-coupled receptor 2 as CCR10, a specific receptor for the chemokine ESkin. *J Immunol.* 2000; 164:3460–3464. [PubMed: 10725696]
32. van Lieshout AW, van der Voort R, Toonen LW, et al. Regulation of CXCL16 expression and secretion by myeloid cells is not altered in rheumatoid arthritis. *Annals of the Rheumatic Diseases.* 2009; 68:1036–1043. [PubMed: 18628285]
33. Szekanecz Z, Koch AE. Macrophages and their products in rheumatoid arthritis. *Curr Opin Rheumatol.* 2007; 19:289–295. [PubMed: 17414958]
34. de Hair MJ, Harty LC, Gerlag DM, et al. Synovial tissue analysis for the discovery of diagnostic and prognostic biomarkers in patients with early arthritis. *J Rheumatol.* 2011; 38:2068–2072. [PubMed: 21885519]
35. Kennedy A, Fearon U, Veale DJ, et al. Macrophages in synovial inflammation. *Front Immunol.* 2011; 2:52. [PubMed: 22566842]
36. Sun C, Zhang YY, Tang CL, et al. Chemokine CCL28 induces apoptosis of decidual stromal cells via binding CCR3/CCR10 in human spontaneous abortion. *Mol Hum Reprod.* 2013
37. Shirakawa AK, Nagakubo D, Hieshima K, et al. 1,25-dihydroxyvitamin D3 induces CCR10 expression in terminally differentiating human B cells. *J Immunol.* 2008; 180:2786–2795. [PubMed: 18292499]
38. Silverman MD, Haas CS, Rad AM, et al. The role of vascular cell adhesion molecule 1/ very late activation antigen 4 in endothelial progenitor cell recruitment to rheumatoid arthritis synovium. *Arthritis Rheum.* 2007; 56:1817–1826. [PubMed: 17530710]

Figure 1. CCL28 is elevated on RA ST myeloid and endothelial cells as well as in RA SF and sera
A. NL, OA and RA ST were stained with anti-human CCL28 (1:50) (original magnification $\times 200$). **B.** Positive immunostaining was scored on a 0-5 scale in ST lining, sublining macrophages (Mac) and endothelial cells (Endo) and staining is shown as mean $\pm$ 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 sera obtained from RA ($n=22$), OA ($n=7$) and NL ($n=19$) and SF procured from RA ($n=22$), OA ($n=10$), gout ($n=10$) and PsA ($n=10$) patients. **E.** Expression levels of CCL28 were quantified in NL and RA monocytes (Mono) and PB differentiated macrophages (Mac) by real time RT-PCR which were normalized to GAPDH and the values are shown as fold increase above NL PB monocytes, $n=12-15$. Values are the mean $\pm$ SE. * represents $p < 0.05$.

Figure 2. RA ST myeloid and endothelial cells demonstrate accentuated CCR10 immunostaining
A. NL, OA and RA ST were stained with anti-human CCR10 (1:50) (original magnification $\times 200$), $n=10$. **B.** Positive immunostaining was scored on a 0-5 scale in ST lining, sublining macrophages and endothelial cells and staining is shown as mean $\pm$ SEM, $n=10$. Colocalization of CCL28 and CCR10 on macrophages (CD68+) (**C**) and endothelial cells (VWF+) (**D**) was examined when RA serial sections were stained with anti-CCL28, anti-CCR10, anti-CD68 and anti-VWF antibodies (original magnification $\times 200$). **E.** Expression levels of CCR10 were quantified in NL and RA monocytes and PB differentiated macrophages by real time RT-PCR which were normalized to GAPDH and the values are shown as fold increase above NL PB monocytes and are normalized to GAPDH, $n=12-15$. Values are the mean $\pm$ SE. * represents $p < 0.05$.

Figure 3. Overlapping joint pro-inflammatory factors can modulate protein levels of CCL28 and CCR10 in RA myeloid and endothelial cells

RA monocytes (**A, D**), RA PB in vitro differentiated macrophages (**B, E**) and endothelial cells (**C, F**) were either untreated or stimulated by 10 ng/ml LPS, TNF- α , IL-1 α , IL-6 or 50ng/ml IL-17 for 24-72h prior to determining protein levels of CCL28 by ELISA (**A-C**) or CCR10 by FACS analysis or Western blotting (**D-F**), n=4-5. **F.** Densitometric analysis of CCR10 Western blot bands were normalized to actin using Image J software, n=4. Values are the mean $\pm$ SE. * represents $p < 0.05$.

Figure 4. CCL28 strongly promotes EPC migration and capillary tube formation through CCR10 binding

A. Dose-response curve of CCL28-induced HUVEC chemotaxis was performed in a Boyden chemotaxis chamber with varying concentrations (0.001-100 ng/ml), $n=3$. Anti-CCL28 (10 $\mu\text{g/ml}$) or control IgG was added to RA SFs (1:20) or HUVECs were incubated with antibodies to CCR10 (10 $\mu\text{g/ml}$) or isotype control for 1h prior to performing chemotaxis (**B**) or tube formation (**C**) in response to RA SFs, $n=8$. **D.** Photomicrographs were taken of representative wells treated with PBS, VEGF (10 ng/ml), IgG or anti-CCL28 (10 $\mu\text{g/ml}$) plus RA SFs (1:20) and IgG or anti-CCR10 (10 $\mu\text{g/ml}$) 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 and H.** To validate EPC purity, cells were stained with endothelial markers VWF (1:1000), VE-cadherin (1:200) or IgG control and positive staining was overlapped on DAPI+ cells (1:1000) (original magnification $\times 400$), $n=3$. **I.** Anti-CCL28 (10 $\mu\text{g/ml}$) or control IgG was added to RA SFs (1:20) additionally EPCs were incubated with antibodies to CCR10 (10 $\mu\text{g/ml}$) or isotype control for 1h prior to performing chemotaxis, $n=3$. Endothelial cell chemotaxis or tube formation is shown as a relative value and is normalized to PBS values. Values are the mean $\pm$ SE. * represents $p < 0.05$.

Figure 5. CCL28 driven endothelial migration and tube formation is facilitated by ERK activation

A. Cells were stimulated with CCL28 (100 ng/ml) for 0-60 minutes and thereafter cell lysates were probed for pAKT1, pJNK, pERK, p-p38 or equal loading controls, n=4-6. HUVECs were incubated with DMSO or inhibitors (5 μ M) to ERK (U0126; with or without CCL28 addition), p38 (SB203580), PI3K (LY294002), JNK (SP600125) for 45 min prior to performing 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 PBS, VEGF (10 ng/ml), U0126, DMSO plus CCL28 (100 ng/ml), U0126 plus CCL28, SB203580 (SB) plus CCL28, LY294002 (LY) plus CCL28, SP600125 (SP) plus CCL28.

Figure 6. Angiogenesis fostered through ligation of CCL28 to CCR10 is interconnected to ERK signaling

A. HUVECs transfected with CCR10 specific and nonspecific control siRNA (100 nM) were either untreated or treated with CCL28 (100 ng/ml) for 10 min. Thereafter cell lysates were examined for CCR10, pERK, ERK and actin. Densitometric analysis of the CCR10/actin (**B**) and pERK/ERK (**C**) was performed using Image J software, n=3. CCL28 (50ng/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 PBS, VEGF (10 ng/ml), control (Ctl) knockdown cells plus CCL28 (50 ng/ml) and CCR10 knockdown cells plus CCL28.