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Targeting the CCR6/CCL20 axis in entheseal and cutaneous inflammation

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ABSTRACT

Objectives: To assess the involvement of the CCR6/CCL20 axis in psoriatic arthritis (PsA) and psoriasis (PsO) and to evaluate its potential as a therapeutic target.

Methods: First, we quantified CCL20 levels in peripheral blood and synovial fluid of PsA patients and the presence of $CCR6^+$ cells in synovial and tendon tissue. Utilizing an IL-23 minicircle DNA (MC) mouse model exhibiting key features of both PsO and PsA, we investigated CCR6 and CCL20 expression and the preventive and therapeutical effect of CCL20 blockade. Healthy tendon stromal cells were stimulated *in vitro* with IL-1β to assess the production of CCL20 by qPCR and ELISA. The effect of conditioned media from stimulated tenocytes in inducing T cell migration was interrogated with a transwell system.

Results: We observed an upregulation of both CCR6 and CCL20 in the enthesis of IL-23 MC-treated mice, which was confirmed in human biopsies. Specific targeting of the CCR6/CCL20 axis with a CCL20 locked dimer (CCL20LD) blocked entheseal inflammation, leading to profound reductions in clinical and proinflammatory markers in the joints and skin of IL-23 MC-treated mice. The stromal

compartment in the tendon was the main source of CCL20 in this model and accordingly, *in vitro* activated human tendon cells were able to produce this chemokine and to induce CCR6+ T cell migration, the latter of which could be blocked by CCL20LD.

Conclusions: Our studies highlight the pathogenic role of CCR6-CCL20 axis in enthesitis and raise the prospect of a novel therapeutic approach for treating patients with PsO and PsA.

Keywords: Psoriasis; Psoriatic arthritis；enthesitis； CCL20； CCR6

INTRODUCTION

Psoriatic arthritis (PsA) is a chronic inflammatory arthritis affecting up to one third of individuals with psoriasis (PsO) (1). Current treatments aim to control aberrant inflammation in both skin and joints by targeting inflammatory cytokines. Blockade of tumor necrosis factor-α（TNF-α), interleukin (IL)-17A, and IL-23 have been remarkably successful in treating skin psoriasis with PASI 75 (≥75% improvement in Psoriasis Area and Severity Index) scores around 80% (2, 3) while only 60% of patients with PsA achieve a clinically meaningful ACR20 response (1, 4, 5). Furthermore, their use is restricted by their side effect panel, with an increased susceptibility to infections (6), and the development of neutralizing antibodies (7). There is therefore a clear need for novel therapeutics targeting both skin and joint inflammation with greater selectivity, fewer side effects, and longer periods of clinical remission.

Recent findings have underscored the role of IL-23/IL-17 axis as a key player in PsO and PsA (8). In the skin, IL-23 is essential for the differentiation and maintenance of T-helper (Th) 17 cells (9) contributing to the activation of keratinocytes (10), recruitment of neutrophils and perpetuating dysregulated cytokine/chemokine production (11). In PsA, synovial IL-23 expression is associated with higher indexes of disease severity (12) and the neo-vascularity of the tissue correlates with the recruitment of pathogenic IL-23/IL-17-producing CD4+ T cells into the joints (13). Furthermore, the enthesis, an important site of inflammation in PsA that is represented by the connective tissue linking tendons and ligaments with bones, has been shown to contain populations of IL-23R expressing cells capable of producing IL-17, including gamma-delta T cells (γδ T cells) and innate lymphoid cells (ILCs) (14-16).

Chemokines represent a superfamily of immune-modulatory small protein molecules that regulate leukocyte migration to inflammatory sites through their chemoattractant properties. The CC chemokine receptor-6 (CCR6)- C-C motif chemokine ligand 20 (CCL20) axis is a prominent immune modulator in both innate and adaptive immune responses of a wide range of inflammatory disorders (17). CCL20 is produced by epithelial and endothelial cells, peripheral blood mononuclear cells

(PBMCs), Th17 cells and neutrophils whereas CCR6 is expressed in Th17 and Tregs, memory T cells, B cells and dendritic cells (18, 19). Both CCR6 and CCL20 are strongly induced by RORγt (20), a critical transcription factor for Th17 differentiation. Importantly, CCR6 and CCL20 are expressed at significantly higher levels in lesional psoriatic skin and CCR6 expression is increased on circulating PBMCs from psoriasis patients compared to normal donors (21). Whilst CCL20 synovial fluid levels have been shown to correlate with markers of PsA disease activity (12), there remains a paucity of mechanistic investigation into the CCR6/CCL20 axis in PsA and in particularly its role at the enthesis.

We previously manipulated the monomeric wild-type CCL20 sequence such that the resulting protein adopted a locked dimeric structure that is similar to spontaneous dimers of CCL20 that occur in nature (22). The resulting dimeric molecule, called CCL20 locked dimer (CCL20LD), binds CCR6 with physiologic affinity but blocks chemotactic activity of the natural CCL20 and was able to ameliorate psoriasiform dermatitis (PsD) in an IL-23 intradermal injection mouse model.

To further explore the potential for CCR6-CCL20 as a therapeutic target for PsO and PsA, we tested the efficacy of CCL20LD in an IL-23-dependent model of skin and entheseal inflammation in the autoimmune-prone strain of B10.RIII mice (15, 23). We herein demonstrate that CCL20LD not only reduces IL-23-mediated skin inflammation, but also attenuates entheseal and synovial inflammation. Importantly, we show that CCR6-CCL20 axis is highly enriched in tendon tissue and mediates the recruitment of inflammatory cells into the entheseal site.

METHODS

More detailed experimental procedures are described in the supplementary 1 methods and materials.

Human tissue collection and preparation

All procedures and protocols were approved by the NHS West of Scotland Ethics Committee (REC14/WS/1035) and informed consent was obtained from all patients according to standard procedures.

Tendon samples were collected from patients with PsA undergoing shoulder surgery. Tissues were immediately fixed in 4% formalin for a minimum of 24 hours and then embedded in paraffin. Healthy

human tendon stromal cells or tenocytes were explanted from hamstring tendon of patients undergoing anterior cruciate ligament (ACL) reconstruction.

Mice

The therapeutic effects of CCL20LD were examined *in vivo* with an IL-23 MC model. Female B10.RIII-H2r H2-T18b/(71NS)SnJ (B10. RIII) at 8-10 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were acclimatized for 1 week before any treatments and housed in the same animal vivarium. All animal experiments were performed under protocols (#20960) approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

CCL20LD treatment

CCL20LD (provided by Xlock Biosciences, LLC, Muskego, WI) was expressed and purified as previously described (22). Protein purity was determined by mass spectrometry and biological function was confirmed by the inhibition of wild type CCL20-dependent migration of CCR6-transfected Jurkat cells in a transwell chemotaxis assay.

In the therapeutic model, mice received I.P. injection of 20 µg (~1mg/kg) or 100 µg (~5mg/kg) once daily in 200 µl PBS starting at day 7 after IL-23 MC delivery. To create a preventative model, mice received I.P. injections of 20 ug CCL20LD once daily (~1mg/kg) dissolved in 200 ul PBS starting at day 0.

Statistical analysis

Statistical comparisons were performed with mean ± SEM for continuous variables of experiments. Data were analyzed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA) and SAS® software for Windows® version 9.4 (SAS Institute Inc., Cary, NC). A two-sided unpaired Student's t-test was used to compare two groups, and one-way analysis of variance (ANOVA) with Dunnett post hoc test was used for multiple comparisons unless otherwise indicated. Give the relatively small sample size of mice, Cohen's d value was calculated to assess the effect size before using parametric tests. Clinical associations were analyzed using the Wilcoxon rank sum test or Spearman rank correlation test. A P-value less than 0.05 was considered statistically significant.

RESULTS

CCR6/CCL20 expression is elevated in patients with PsA

In order to assess the contribution of CCR6 and CCL20 to PsA pathogenesis, we first measured CCL20 levels in serum and synovial fluid (SF) from PsA patients compared with healthy controls and individuals with osteoarthritis (OA). Whilst CCL20 was comparable in serum samples between healthy controls and PsA patients, SF levels were significantly increased in PsA when compared with the OA group (figure 1A). We also found that in PsA patients CCL20 levels were higher in SF than in peripheral blood, with a significant correlation observed between CCL20 SF levels and bone erosions or the use of NSAIDs (table S1, 2).

We then screened the presence of multiple chemokines and cytokines in SF. As expected, levels were higher in PsA as compared to OA patients (figure 1B, C, table S3). It was striking, however, that the fold change of CCL20 in PsA *vs.* OA patient samples was the highest among all 17 measured chemokines (figure 1D). Moreover, in PsA patients, the intensity of CCL20 was positively correlated with IL-6 (figure 1E). No significant correlations were observed between the levels of CCL20 with any other cytokines/chemokines listed in figure 1C (data not shown).

Based on the presence of high levels of CCL20 in joint fluids from PsA patients, we next assessed the presence of CCR6+ cells in synovial tissues. As shown in figure 1F, we observed CCR6-positive cells in both healthy synovial membrane and in PsA patients, with staining in vessels, infiltrating

immune cells and stromal cells. Thus, both CCL20 and its receptor CCR6 are locally expressed at high levels in PsA, suggesting a potential role for this pathway in its pathogenesis.

CCL20/CCR6 expression is elevated in IL-23 minicircle DNA (MC)-injected mice

We next investigated if CCR6 and CCL20 expression was similarly increased in the IL-23 MC mouse model that recapitulates the clinical and immunological features of both PsO and PsA (24, 25). IL-23 MC injected mice revealed a significant elevation of IL-23 in serum as early as 24 hours after delivery (figure S1A). Four days following IL-23 MC treatment, skin erythema and hind paw inflammation were visible and by day 7 both hind and front paws were involved. Typical features of PsO (scaling, redness, and edema) and PsA (swollen paws) were observed for over 3 weeks (figure S1B, C).

Our previous study suggested that γδ T cells were the predominant cell type expressing CCR6 in murine models of psoriasis (26). On day 7, IL-23 MC delivery led to increased percentages and absolute numbers of γδ T cells expressing CCR6 into the ear skin, cervical skin draining lymph nodes (cLN), ankle joints and popliteal lymph nodes (pLN) (figure 2A-E, figure S2A). Consistently, γδ T cells from IL-23 MC delivered mice had higher mean fluorescent intensity (MFI) in CCR6 expression (figure S2B). To be noted, there are two distinct subsets of γδ cells in mice skin in terms of the amount of TCR present on their surface, γδ-high expressing T cells (also known as dendritic epidermal T cell, DETC) and γδ-low expressing (γδ-low) T cells (27). We have previously shown that γδ-low T cells account for the majority of IL-17A production and are required for the development of maximal skin inflammation in an IL-23 injection model of psoriasiform dermatitis (28). Consistent with this, our data show that γδ-low T cells, as opposed to γδ-high T cells, were the major cell population expressing CCR6 in ear skin from IL-23 MC-injected mice.

PCR analysis further confirmed significant upregulation of *Ccr6* and *Ccl20* expression in both ear skin and hind paw tissue from IL-23 MC-treated mice (figure 2F). Thus, systemic induction with IL-23 MC resulted in an upregulation of CCR6 and CCL20 in both skin and joints.

CCL20LD attenuates IL-23-mediated skin inflammation

We then tested the efficacy of CCL20LD in preventing psoriatic inflammation in the IL-23 MC model, with daily intraperitoneal (IP) injections of either 20 μg CCL20LD or vehicle being administered for 7 consecutive days (figure S3A). Within 7 days of initiation, vehicle-treated mice developed remarkable erythema, desquamation and induration (figure S3B), whereas CCL20LD-treated mice developed significantly less skin inflammation, as measured clinically by a nearly 50% reduction in ear thickness and psoriasis severity index (PSI) score (figure S3C), as well as histologically by a 54% reduction in epidermal thickness and a 62% reduction in Munro microabscesses (figure S3D). In keratinocytes, CCL20LD treatment resulted in reduced nuclear staining of Ki-67 and phospho-STAT3 (figure S3E). Flow cytometric analysis revealed marked reductions in $CD45^+$ leukocytes, $CCR6^+$ γδ-low T cells, and neutrophils (figure S3F). Gene expression of psoriasis-associated cytokines including *Il17a, Il17f, Il22, Il6,* and *S100a8* was also suppressed by CCL20LD (figure S3G).

We next assessed if CCL20LD could block established skin disease in a therapeutic manner. On day 7 following IL-23 MC injection mice were treated daily with vehicle alone, 20 or 100 μg of CCL20LD daily for 7 additional days (figure 3A). Mice treated with the higher dose developed significantly less scaling and erythema (figure 3B) and had a nearly 50% reduction in ear thickness and clinical PSI score (figure 3C). Histologically, both CCL20LD treatment groups exhibited reductions in epidermal thickness and profoundly decreased numbers of Munro microabscesses (figure 3D). CCL20LD treatment dampened the increased nuclear staining of Ki-67 and phospho-STAT3 in a dose-dependent manner (figure 3E). Flow cytometric analysis showed a reduction in the infiltration of $CD45^+$ leukocytes, $CCR6^+$ γδ T cells, and neutrophils (figure 3F). Although not statistically significant, mice receiving high-dose treatment exhibited downregulation of *Ccr6* and *Ccl20* gene expression (figure S4A). Similar trends were seen in the expression profiles of cytokine transcripts such as *Il17a*, *Cxcl1*, *Cxcl2, Il6* and *S100a9*. A significant reduction was observed in the gene expression of *Il1b* and *S100a8* in the group with high-dose treatment (figure S4B). We further performed IHC staining of CD4, CD8 and F4/80. In contrast to CCR6+ γδ T cells and neutrophils, the infiltration of CD4+ T cell, CD8+ T cells and F4/80 macrophage was not reduced by CCL20LD treatment, which may contribute to the sustained levels of some cytokines (figure S5).

Thus, CCL20LD suppresses both the clinical, histologic, and molecular features of IL-23-mediated dermatitis in both a preventative and therapeutic manner in a dose-dependent manner.

CCL20LD attenuates IL-23-mediated joint inflammation

Earlier, we demonstrated that IL-23 MC delivery resulted in significantly swollen hind paws by day 7 post-delivery of IL-23 MC (figure S1). When treated with preventative administration of CCL20LD, we observed a 60% reduction in the incidence of arthritis and a 95% reduction in severity (figure S6A, B). Synovial hyperplasia and inflammation were profoundly attenuated, with a reduction in synovial neutrophilic infiltration (figure S6C, D). The increased expression of *Tnfsf11* (*RANKL*), but not *Tnfrsf11b* (*Opg*), in paw tissue from IL-23 MC mice was also suppressed by CCL20LD (figure S6E). In contrast to the partial reduction of proinflammatory markers in skin, CCL20LD treatment resulted in a greater than 75% reduction in all measured proinflammatory markers in the paw tissues, including *Il17a, Tnf, Il6* and *Il1b* (figure S6F).

In the therapeutic model, mice treated with CCL20LD had clinically ameliorated joint inflammation, with greater improvement seen in the high-dose group than in the low-dose group (60% vs. 50% reduction in paw swelling score, respectively, figure 4A, B). Histologically, IL-23-induced joint swelling was characterized by pannus formation with destruction of articular surfaces, which was milder in the low-dose group and nearly absent in the high-dose group (figure 4C). Accordingly, a significant reduction in histological score was observed in the CCL20LD-treated group (figure 4D). In the paw tissue, the reduced inflammation was corroborated by a reduction in synovial neutrophilic infiltration (figure S7) and decreased metabolic activity as demonstrated by lower [^{18}F] fluorodeoxyglucose (FDG) uptake on positron emission tomography (PET)/ computed tomography (CT) scans (figure 4E). Gene expression of *Ccr6* and *Ccl20* was downregulated in mice treated with CCL20LD (figure S8) and a similar trend was observed in the expression of other genes involved in bone and tissue erosion (*RANKL*, *Mmp9,* and *Ctsk*) (29) and inflammation (*Il17a, Il1b*, *Il6* and *Tnf*) (figure 4F).

We then compared the therapeutic effect between CCL20LD and anti-17A antibody, a biological agent that has previously demonstrated a high level of efficacy in treating psoriasis and PsA (2, 30).

Mice treated with CCL20LD at a daily dosage of 100 ug exhibited a better therapeutic effect on ear inflammation compared to those received 100 μg anti-17A antibodies every other day, as measured by lower ear thickness, PSI score and decreased infiltration of neutrophils and CCR6+γδ-low T cells (figure S9A-D). Treatment of CCL20LD and anti-17A antibody both resulted in significant improvement of IL-23-mediated joint inflammation as evidenced by decreased score of inflammation, less cellular infiltrates in ankle joint and suppressed transcripts of IL-17A, IL-1β, IL-6 and TNF-α (figure S9E-H).

Collectively, CCL20LD treatment led to profound reductions in all measured clinical and proinflammatory markers in the joints of IL-23 MC-treated mice in both prevention and therapeutic settings.

CCL20LD attenuates IL-23-mediated enthesitis

To understand the potential role of the CCR6-CCL20 axis in enthesitis, we first assessed CCR6 expression in human tendon tissue. In healthy tissue, CCR6 staining localized on vessels in the para-tendon tissue, with some tenocytes showing CCR6 cytoplasmic staining. In tendon tissue from PsA patients, CCR6 staining increased with the cell infiltrate and numerous $CCR6^+$ cells were observed throughout the tendon (figure 5A).

We next assessed the involvement of the enthesis in IL-23 MC-treated mice, observing an increased percentage and absolute number of CCR6-positive γδ T cells in the Achilles tendon from IL-23 MC mice (figure 5B). Strikingly, 50- and 1000-fold increases in *Ccr6* and *Ccl20* gene expression were found in the tendons of IL-23 MC mice, respectively (figure 5C), significantly higher than those observed in the paw tissue overall. Of note, expression of *Ccl20* in CD45-negative cells was 6-fold higher than in CD45-positive cells (figure 5D). The upregulation of *Ccl20* was accompanied by dramatic elevations in several inflammatory mediators in the Achilles tendon such as *Il23r, Il17a, Il22, Il6, Il1b,* and *Tnf* (figure S10). In line with these transcriptional changes, IL-23 MC mice also exhibited histological findings of enthesitis, such as infiltration of leukocytes in and adjacent to the Achilles tendon and synovio–entheseal complex (figure 5E, figure S11). No obvious calcaneal erosion was found in IL-23MC-injected mice treated with vehicle or CCL20LD (figure S12). These

histological changes, together with accumulation of Gr-1-positive neutrophils, were markedly attenuated by CCL20LD treatment (figure S13). Consistent with these findings, expression of *Ccr6* and *Ccl20* as well as other proinflammatory markers was suppressed in the tendon of mice therapeutically treated with CCL20LD following establishment of enthesitis (figure 5F). These results suggest an important role of the stromal compartment in the tendon in the production of CCL20 and show that CCL20LD blockade dampens this inflammatory response in the entheses following IL-23 MC induction.

Human tenocytes produce CCL20 upon activation with IL-1β

Because our data in the IL-23 MC mice model suggested that tendon stromal cells were the main producers of CCL20 in this model, we wondered if activated human tendon stromal cells, also known as tenocytes, could have the capability to produce CCL20. For that, we stimulated healthy tenocytes with IL-1β, an inflammatory cytokine that has been involved in sterile inflammation (31) and shown to be increased in animal models of tendon injury and exercise (32). We observed a striking upregulation of *CCL20* gene expression (fold change 680.5+/-215.2; $p<0.0001$, n=5) and significant increases of CCL20 protein in supernatants of activated tenocytes (481.8+/- 84.26 pg/ml; $p<0.0001$, n=13) (figure 6A).

We next assessed the capacity of conditioned media from tenocytes activated with IL-1ß to induce the migration of T cells using a Transwell assay. After 4 hours we observed increased migration of CCR6+ (mean percentage CCR6+ cells +/- SEM, media 20.39+/-2.9, unstimulated tenocytes 20.56± 2.6, activated tenocytes 24.77+/-3.1) and CCR6+CD161+ cells (mean percentage CCR6+CD161+ cells +/- SEM, media 11.1+/-1.99, unstimulated tenocytes 11.59+/-1.89, activated tenocytes 14.66+/-2.48) towards conditioned media from tenocytes, particularly if they had been previously activated (figures 6B-C). While migration was not vigorous, it should be noted that ~30% of the unsorted T cells used in these assays (data not shown) expressed CCR6, thus limiting the migration response that we could be detected. We thus performed chemotaxis assays with a human Jurkat T cell line that does not overexpress CCR6 and a retrovirally transduced CCR6-overexpressing (CCR6+) Jurkat T cell line (figure S14A) (22). Only CCR6+ Jurkat cells but not the non-transduced Jurkat cells

responded to CCL20 in a dose-dependent way (figure S14B). Compared to media from unstimulated tenocytes, media from stimulated tenocytes attracted more migration of CCR6+ Jurkat cells, which was totally blocked by 100ng/ml CCL20LD (figure 6D). Such increased chemotaxis by media from stimulated tenocytes and further inhibition by CCL20LD was not observed when using CCR6-negative Jurkat cells (figure S14C). Together, these results demonstrate that stromal cells in human tendon are able to produce chemotactically active CCL20 in response to inflammatory stimuli and damage, resulting in enhanced recruitment of $CCR6^+$ cells *in vitro* and, possibly, into the tendon *in vivo*.

DISCUSSION

Strategies to target chemokines and their respective receptors are extremely attractive to reduce the burden of chronic inflammatory diseases. Among the ~20 known chemokine receptors, the CCL20-CCR6 pair particularly has been associated with Th17-dominant immune activity (33). We have previously shown in an experimental model of human psoriasis involving local intradermal injection of IL-23 that CCR6 is essential for the epidermal trafficking of IL-17/22-producing cells (28) and that CCR6-deficient and anti-CCL20 monoclonal antibody (mAb)-treated mice are resistant to the development of psoriasiform dermatitis (PsD) (26, 34). In accordance with this, treatment with CCL20LD led to a partial reduction in IL-23-mediated skin inflammation in both the preventative and therapeutic model in the current work.

In addition to PsO, where CCR6 has long been proposed as a target for therapy (33), emerging data also suggest that CCL20-CCR6 signaling is involved in the pathology of other inflammatory rheumatic diseases such as rheumatoid arthritis (RA). In patients with RA, increased numbers of CCR6+ T cells have been found in the circulation as well as in inflamed joints (35). Synoviocytes from arthritic joints of both mice and humans were found to produce large quantities of CCL20 (20). Furthermore, administration of blocking antibodies to CCL20 inhibited experimental joint inflammation in a collagen-induced arthritis (CIA) model (20). Indeed, a CCL20 neutralizing antibody (GSK3050002) with a high binding affinity to human CCL20 has been used in a first-in-humans study to show selective blockade of $CCR6^{+}$ cells in a suction blister model without significant safety concerns (36), suggesting that a blocking antibody-based approach may be possible in humans. In contrast to this approach to CCL20-CCR6 blockade, CCL20LD only differs by one internal amino acid residue from the natural chemokine, and thus is likely to be resistant to the development of anti-drug antibodies that lead to loss of therapeutic efficacy with many antibody-based therapeutics (37).

In the current study, we showed that CCL20LD and anti-IL-17A antibodies had similar therapeutic effect on IL-23-mediated skin and joint inflammation. Th17 cells are important mediators of immune responses against extracellular bacteria and fungi, and, as such, play critical regulatory roles in maintaining mucosal homeostasis (38). For example, mice deficient in IL-17A or IL-17RA are more vulnerable to local infection of *C. albicans*, however, CCR6-deficient mice exhibit similar resistance

to *C. albicans* compared with WT mice (39). Further studies are warranted to evaluate CCR6-targeted therapy in terms of risk for infection.

Though the significance of Th17 cells in PsA was identified a decade ago, the cellular and molecular mechanisms of enthesitis in spondyloarthritis, including PsA, remains largely unknown (40). Recent studies have provided evidence for the role of mechanical stress in the pathogenesis of arthritis (41). Mechanical stress in the enthesis, areas where tendons and ligaments attach to bones and therefore responsible for the transmission of mechanical forces from the muscle to the bone (42), could trigger the development of a chronic immune response in individuals with a certain genetic background. This fact, together with the observation of increased local levels of CCL20, prompted us to interrogate if the stromal compartment could be the source of CCL20. In the IL-23 MC model, we found that that $CD45^-$ cells exhibited higher CCL20 gene expression and that healthy human tenocytes were able to produce CCL20 in response to activation with IL-1β, as previously reported for synovial fibroblasts (20). As our *in vitro* experiments suggest, tenocytes can then promote the migration of CCR6+ cells, such as γδ T cells or ILC, that populate healthy entheses (14, 16, 43). In sum, we propose that tenocytes likely play a critical role both in initiation and perpetuation of enthesitis by locally regulating interactions of CCL20 and CCR6.

We have previously showed that CCR6-deficient mice on the C57BL/6J strain background were resistant to IL-23-induced skin inflammation but exhibited no observed changes in the incidence or severity of relatively mild joint inflammation compared to wild-type (WT) mice (44). It is, however, well known that the standard C57BL/6J strain of mice is relatively resistant to experimental autoimmune arthritis (45) whereas others, such as the closely related B10.RIII strain are much more prone to and have greater severity of induced arthritis (15, 23). The susceptibility is associated with the major histocompatibility complex (MHC) allele, H2r, the T cell receptor V β8 chain, and other non-MHC loci (23, 34). These differences in the propensity of arthritis between different mouse strains led us to conduct our follow up studies using the B10.RIII strain and CCL20LD to more conclusively evaluate the role of the CCL20-CCR6 axis in psoriatic joint inflammation. We found that the fold change of transcripts of CCR6 and CCL20 in paw tissue was higher in B10.RIII mice than that in C57BL/6J mice after delivery the same dose of IL-23 MC (fold change, mean±SD,

C57BL/6J *vs* B10.RIII: *Ccr6*：1.59±0.59 *vs* 3.14±1.03; *Ccl20*: 10.69±6.07 *vs* 22.40±8.12) (44), which may contribute to the discrepancy between our studies. Given the fact that the levels of CCL20 were highly elevated in the SF from PsA patients, we suggest that the results from B10.RIII mice will probably more closely mirror the response to CCL20-CCR6 blocking treatment in human patients.

The present study had some limitations such as small sample size and heterogeneity of the recruited patients. Further studies with an enlarged sample size are necessary to comprehensively evaluate the profile of CCL20 in SF as well as its correlation with the clinical phenotype. Also, considering the higher dosage of IL-23 MC we used (25), it is possible that the high levels of IL-23 made it difficult to reverse or improve some of the outcomes, mice models with different disease severity by titering IL-23MC, optimal dosing and route of administration of CCL20LD treatment need to be explored and warrant further optimization along with determination of the ability of this therapy to reverse or ameliorate bone destruction.

In summary, these data confirm the role of CCR6/CCL20 axis in IL-23/IL-17 mediated skin and joint disease, but, perhaps most importantly, we could also bridge the role of CCR6 and CCL20 in entheseal disease as suggested by both human and murine studies. In IL-23 MC-driven entheseal and joint inflammation, an engineered variant of CCL20 reverses the histologic and molecular signs of inflammation, offering hope that targeting this pathway may be a new avenue for treating an unmet need in treating PsA.

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Author contributions: Z.S. and E Garcia-Melchor performed, analyzed the experiments and drafted the manuscript. N.L.M and S.T.H designed the experiments and reviewed the manuscript. X.W., M.H. and T.L. assisted in processing the sample. D.J.R. performed PET/CT and assisted in analyzing the data. A.E.G. and B.F.V. provided the CCL20LD, contributed conceptually to the project and assisted in manuscript preparation. M.W. assisted in analyzing clinical data and provide statistical suggestion. F.Z gathered and processed serum samples and provided clinical data from patients. M.A. contributed to execution of *in vitro* experiments. M.N. assisted in manuscript preparation. S.K. R. and S.P.R performed the cytokine array and assisted in analyzing joint inflammation.

Competing interests: AEG, BFV, and STH have intellectual property interest in the CCL20LD molecule and ownership interest in Xlock Biosciences.

Data and materials availability: No datasets were generated during the current study, but some or all data are available from the corresponding author by request.

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Figure and figures legends

Figure 1. CCL20/CCR6 expression in patients with psoriatic arthritis (PsA)

(A) Quantification of CCL20 by ELISA in serum from healthy controls (HC) (n=19) and patients with PsA (n=19), and in synovial fluid (SF) from patients with osteoarthritis (OA) (n=14) or PsA (n=31). (B-D) Representative image of cytokine array showing the profile of 80 chemokines/cytokines (B), heatmap of chemokines and cytokines (normalized by Z-score for each protein) (C) and quantification of normalized signal intensity of chemokines (D) in SF from patients with OA or PsA (n=6). (E) Normalized signal intensity results with correlation analysis of CCL20 with IL-6. (F) Immunohistochemical (IHC) staining of CCR6 in PsA synovial and healthy synovial membrane, 10X magnification and detail of 40X. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, by using Mann-Whitney U test

for serum samples in (A), Kruskal-Wallis test followed by Dunn multiple comparison for SF samples in (A), two-tailed Student's t-test in (D) and Spearman's correlation coefficients in (E).

Figure 2. CCL20/CCR6 expression in IL-23 MC-treated mice

(A-D) Representative gating strategy of flow cytometry showing γδ-low T cells expressing CCR6 in ear skin (A), γδ T cells expressing CCR6 in cervical skin draining lymph node (cLN) (B), ankle joint (C) and popliteal lymph node (pLN) (D). (E) Absolute numbers of CCR6+ γδ T cells per ear skin, cLN, ankle joint and pLN. (F) Gene expression of *Ccr6* and *Ccl20* in ear skin or hind paw tissue. All of the data are presented as mean± SEM. Four animals per group. Data are representative of two independent experiments. ** $p<0.01$. *** $p<0.001$, by using two-tailed Student's t-test.

Figure 3. CCL20LD ameliorates IL-23-mediated psoriasiform dermatitis in a therapeutic manner

(A) Schematic illustration of experimental protocols. B10.RIII mice were treated with PBS vehicle or CCL20LD at a dose of 20 μg or 100 μg for 7 consecutive days beginning at day 7 after MC was delivered. (B) Representative photographs and (C) time course of ear thickness and PSI (Psoriasis severity index) score, (D) image of H&E section (scale bars, 50 μm), and histological analysis of epidermal thickness and number of microabscesses, (E) representative images of immunochemical staining of Ki-67 and p-Stat3 (scale bars, 50 μm), (F) absolute numbers of CD45+ leukocytes, γδ -low T cells expressing CCR6 and neutrophils by flow cytometry. All of the data are presented as mean± SEM. Four mice per group. Data are representative of two independent experiments. * $p<0.05$, ** $p<0.01$. *** $p<0.001$, by using two-way ANOVA with Bonferroni's test compared to IL-23 MC+vehicle group in (C) and one-way ANOVA with Dunnett's test compared to IL-23 MC+vehicle group in (D,F).

Figure 4. CCL20LD ameliorates IL-23-mediated joint inflammation in a therapeutic manner

(A) Representative photographs showing the front paws and hind paws of B10.RIII mice treated with PBS vehicle or CCL20LD at a dose of 20μg or 100μg for 7 consecutive days beginning at day 7 after

MC was delivered. (B) Time course of paw inflammation score. (C) Representative images of H&E section (scale bars, 200 μm). (D) Histological score of ankle joints. (E) Representative maximum-intensity projection of 18F-FDG PET and quantification of joint disease by SUV max value in paw area. Enlarged image showing the hind paw (upper left). The pseudo-color in PET indicates higher glucose metabolism, hence increased cellular metabolic activity. (F) Gene expression of osteoclastogenesis-related markers and proinflammatory cytokines in paw tissue. All of the data are presented as mean± SEM. Four animals per group. * $p<0.05$, ** $p<0.01$. *** $p<0.001$, by using two-way ANOVA with Bonferroni's test compared to IL-23 MC+vehicle group in (C), two-tailed Student's t-test in (E) and one-way ANOVA with Dunnett's test compared to IL-23 MC+vehicle group in (F).

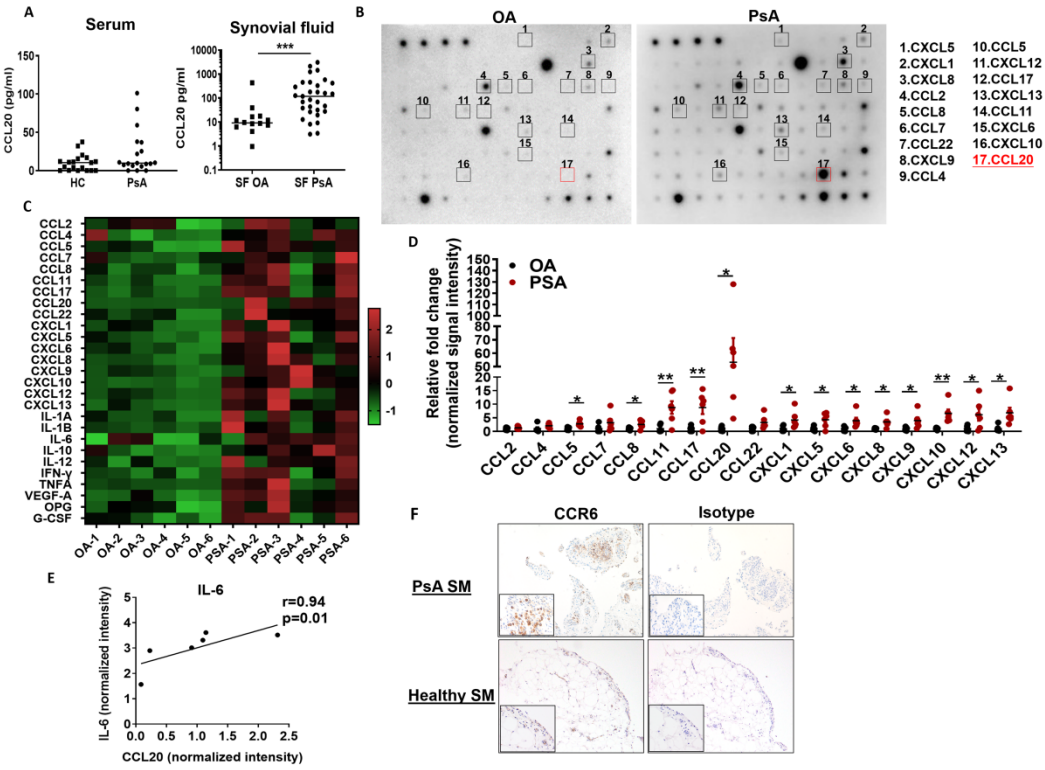
Figure 5. CCL20LD attenuates IL-23-mediated enthesitis

(A) IHC staining of CCR6 in tendon tissue from PsA patients and healthy donors, 40X magnification. (B) Representative intracellular staining, percentage, mean fluorescent intensity (MFI) and absolute counts of CCR6 positive γδ T cells by flow cytometry, (C) gene expression of *Ccr6* and *Ccl20* in Achilles tendon from mice delivered with GFP MC or IL-23 MC at day 7 after gene delivery. (D) Gene expression of *Ccl20* in CD45-positive and CD45-negative cells in Achilles tendon from mice delivered with IL-23 MC. (E) H&E images showing enthesis from mice treated with GFP MC+ vehicle, IL-23 MC+vehicle and IL-23 MC+100μg CCL20LD (scale bars, 100 μm) as protocol shown in Figure 3A. Enthesitis scores were lower in the IL-23 MC group treated with CCL20LD than in the group treated with vehicle. (F) Gene expression of proinflammatory marker in Achilles tendon from IL-23 MC injected mice treated with vehicle or CCL20LD. All of the data are presented as mean± SEM. Three to six mice per group. * $p<0.05$, ** $p<0.01$, by using two-tailed student's T test.

Figure 6. Human tenocytes produce CCL20 upon stimulation with IL-1β.

(A) Healthy tendon stromal cells (Ten) were stimulated with IL-1β (1 ng/ml) for 4 hours for analysis of CCL20 gene expression by qPCR or for 18 hours to quantify levels of CCL20 in supernatant by ELISA. (B-C) Migration of CD3+ cells from peripheral blood from healthy donors pre-activated overnight with aCD3 and aCD28 was assessed using a transwell system and conditioned media from

tenocytes with/without activation with IL-1β. After 4 hours, cells that migrated to the lower chamber were analysed by flow cytometry. (B) Representative FACS plots for CCR6+ or CCR6+CD161+ cells from total CD3+ live cells. (C) Percentage and cell counts of CCR6+ or CCR6+CD161+ cells.(D) Migration of CCR6+ Jurkat cells toward blank media, media cultured with tenocytes, IL-1β-stimulated tenocytes or IL-1β-stimulated tenocytes in the presence of CCL20LD (100ng/ml). Results from 3 independent experiments. Data presented as mean +/- SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ by using paired t test and Wilcoxon's signed rank test in (A) , one-way ANOVA with Dunnett's test to compare to media group in (C) and to compare to activated Ten group in (D).



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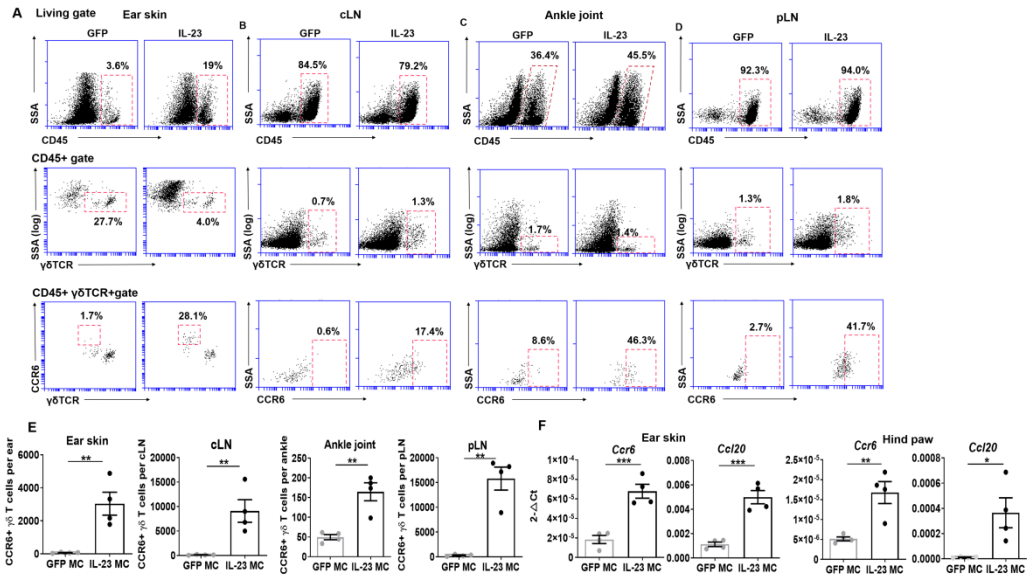

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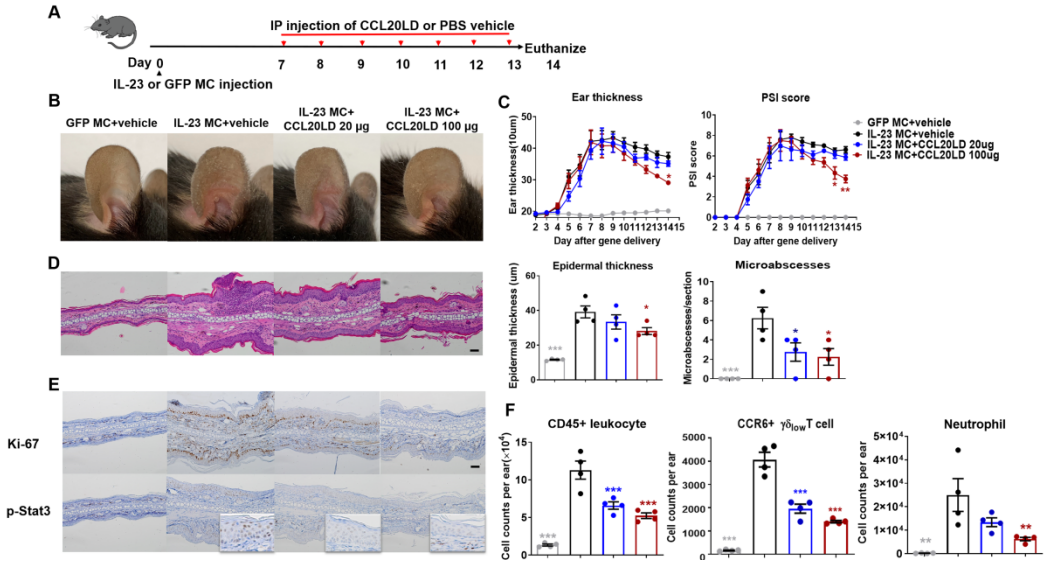

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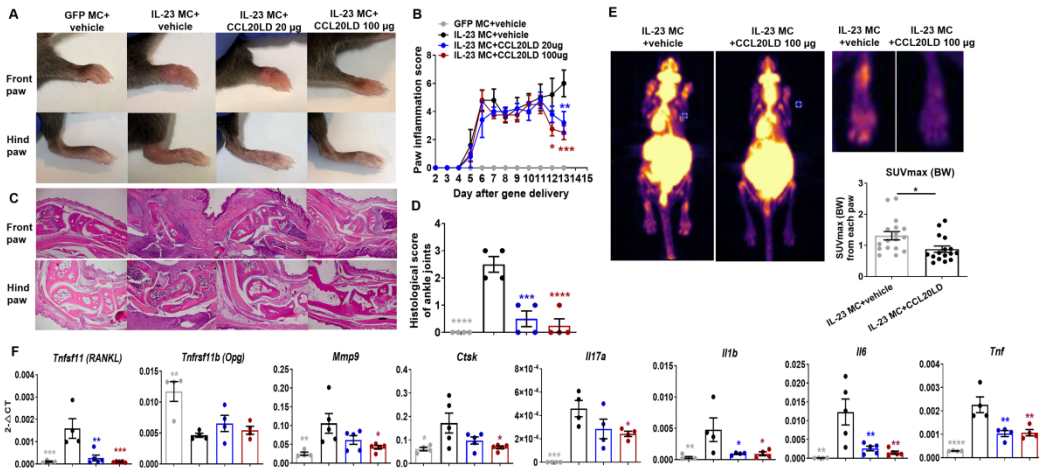

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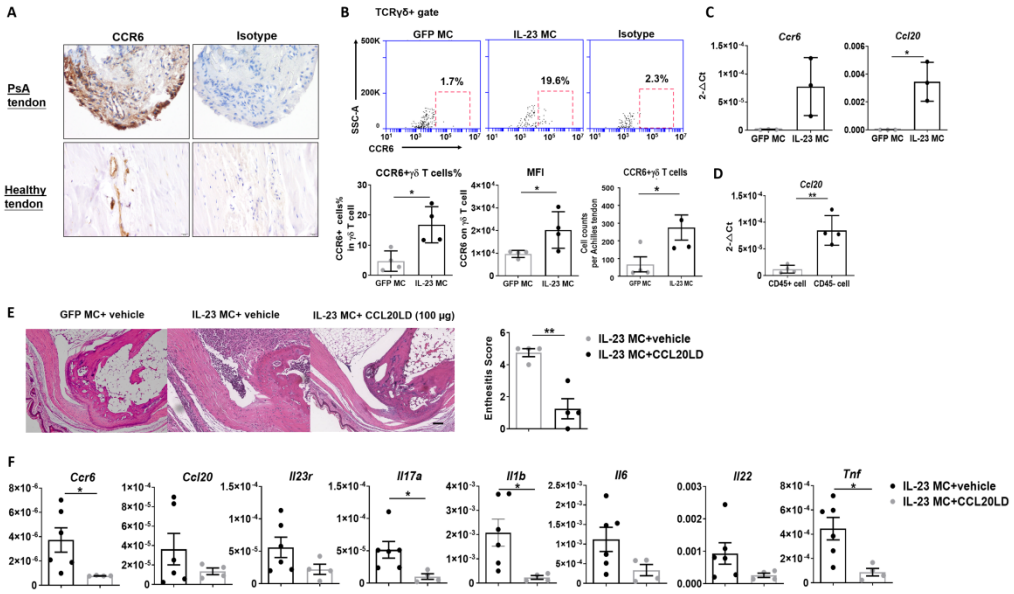

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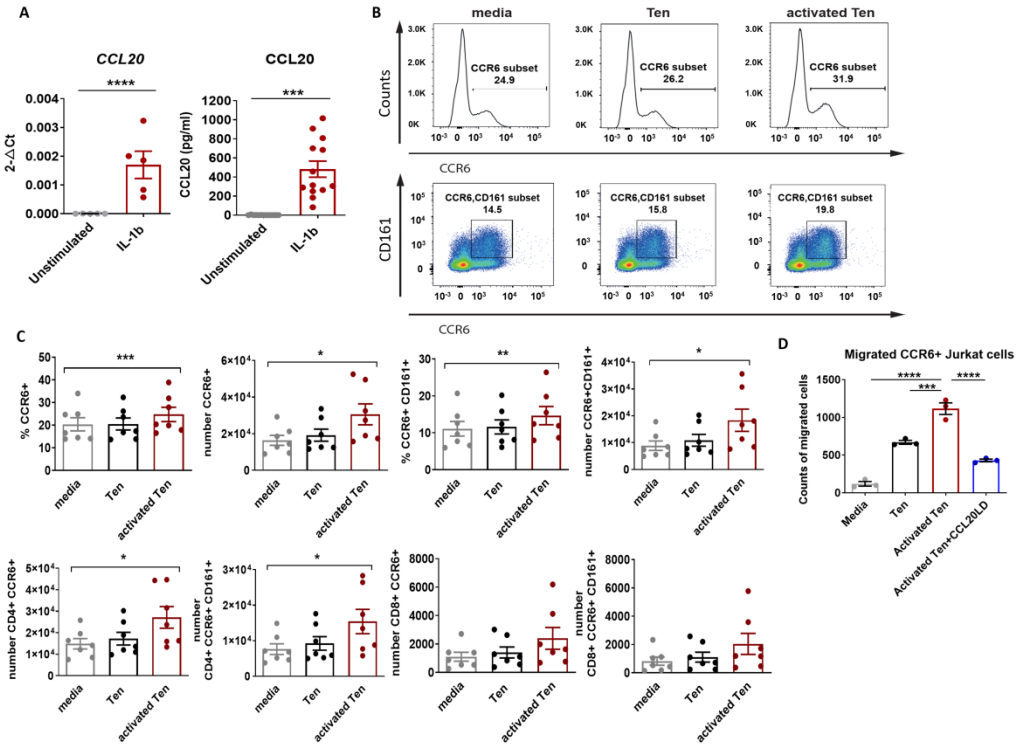

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