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Screening of the FDA-approved drug library identifies CCL17 inhibitors that block arthritic pain

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Rheumatoid arthritis (RA) is an inflammatory and destructive autoimmune polyarthritis that causes pain, swelling and deformity in the joints. While clinical trials targeting GM-CSF in RA are showing promise, the potential side effects of anti-GM-CSF therapy highlight the need for identifying downstream mediators of GM-CSF action. CCL17, a downstream inflammatory mediator of GM-CSF in monocytes and macrophages, has been shown to mediate GM-CSF-driven inflammatory arthritis in animal models. CCL17 shares its receptor, CCR4, with CCL22; however, unlike CCL17, CCL22 has been implicated in resolving inflammation. Therefore, drugs that can suppress the formation of CCL17, but not CCL22, may be beneficial in the treatment of inflammatory arthritis. In this study, we screened a panel of 1508 FDA-approved drugs and identified five drugs, namely fluoxetine, ractopamine, ponesimod, terbutaline and etravirine, which potently inhibited CCL17 production without adverse effects on cell viability and CCL22 formation in human monocytes and mouse macrophages. Mechanistically, we demonstrated that these drugs inhibited STAT5 activity and IRF4 expression to suppress CCL17 formation. Significantly, therapeutic administration of these five drugs in an inflammatory arthritis model revealed that fluoxetine, ractopamine, ponesimod and terbutaline could inhibit arthritic pain, correlating with decreased CCL17 expression. Given the need for new and safe anti-inflammatory therapeutics to treat RA and the benefits of repurposing existing drugs for new indications, our findings reported here offer four new promising analgesics for treating inflammatory pain.

Keywords CCL17, CCL22, GM-CSF, Arthritis, Pain, Monocytes, Macrophages, STAT5, IRF4.

Rheumatoid arthritis (RA) is an inflammatory, progressive, and destructive autoimmune disease[1,2]. During RA, intra-articular as well as extra-articular manifestations result in morbidity and, in extreme cases, can lead to mortality. Therapeutic approaches have found that targeting granulocyte/macrophage-colony stimulating factor (GM-CSF) ameliorates inflammation and joint damage associated with RA[3]. While there are several clinical trials targeting either the GM-CSF ligand or its receptor showing promise, their long-term usage may lead to possible side effects, such as infection and lung and gut dysfunction, as evidenced in individuals with autoantibodies against GM-CSF[4].

These potential side effects of anti-GM-CSF therapy highlight the need to elucidate the GM-CSF-activated inflammatory pathway(s) further downstream. Levels of CC chemokine ligand 17 (CCL17), a downstream inflammatory mediator of GM-CSF, are found to be elevated in RA synovial fluid and plasma[5]. Significantly, we have previously demonstrated that CCL17 can induce inflammatory arthritic pain and disease[6,7]. Moreover, GM-CSF-driven and GM-CSF-dependent animal models of arthritis have demonstrated that CCL17 can mediate some of the pro-inflammatory actions of GM-CSF[6]. Therefore, targeting CCL17 to treat inflammatory conditions may be advantageous over targeting its upstream regulator GM-CSF to minimize potential undesirable treatment outcomes.

Despite both CCL17 and CCL22 binding to a common receptor, CCR4, the former is associated with recruiting Th1, Th2 and Th17 cells to maintain inflammation, while the latter is involved in promoting homeostasis through recruiting Tregs[8–10]. Currently, there are no CCL17-targeting drugs approved for use in the clinic. Furthermore,

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there are no therapies available to promote CCL22 expression. Developing new drugs is a time-consuming and cost-intensive process; however, repurposing established drugs for new applications can save time, as well as development costs and risk[11].

In the present study, we screened a panel of 1508 FDA-approved drugs in order to identify compounds that can inhibit GM-CSF-induced CCL17 formation in human monocytes and mouse bone marrow-derived macrophages. We identified five drugs, namely fluoxetine, ractopamine, ponesimod, terbutaline and etravirine, which potently inhibited CCL17 production without adverse effects on cell viability and CCL22 formation. We elucidated the mode of inhibition of these drugs and found that all of them inhibit GM-CSF-induced STAT5 activity and IRF4 expression, which are known to be necessary for CCL17 production. Significantly, therapeutic administration of these five drugs in an inflammatory arthritis model revealed that fluoxetine, ractopamine, ponesimod and terbutaline could inhibit arthritic pain. Given the limitations of currently available drugs to treat RA and the benefits of repurposing existing drugs for new indications, we propose that the four identified CCL17-inhibitng drugs that ameliorate pain would be beneficial in treating inflammatory conditions, such as RA.

Results

Screening of FDA-approved drugs to identify CCL17-inhibiting candidates

GM-CSF was previously shown to induce CCL17 production in human monocytes and mouse bone marrow derived-macrophages (BMDMs) over 16 h[5,6], and therefore we screened 1508 FDA-approved drugs in these cell populations to identify candidates which could inhibit such induction. Human monocytes were pretreated with 10 μM of FDA-approved drugs for 30 min, followed by treatment with GM-CSF (20 ng/mL) or left untreated for 16 h. Monocyte culture supernatants were collected and subjected to CCL17 ELISA. Drugs were grouped according to the percentage of inhibition of CCL17 production, namely 50% and 75%, relative to CCL17 expression in the monocyte culture supernatants that were treated with GM-CSF alone (Supplementary Table 1). Among the 1,508 drugs, 362 (34%) inhibited 50% of CCL17 expression, while 228 (22%) inhibited 75% of CCL17 expression in the presence of GM-CSF. Following culture supernatant removal, the monocytes were subjected to a MTT viability assay to ensure that inhibition of CCL17 secretion was not due to the cytotoxicity of the treated drug. Of the 228 drugs that inhibited CCL17 by more than 75%, 146 drugs had no toxicity as assessed against the PBS value which were selected for further evaluation (Fig. 1A and Supplementary Table 1).

We previously found that some signaling pathways involved in regulating CCL17 production could also be involved in upregulating CCL22[5,6,12] and therefore CCL22 levels in the GM-CSF-treated monocyte supernatants were also measured to examine the specificity of the drugs in inhibiting CCL17 formation. Among the 146 candidates, 125 (86%) also inhibited GM-CSF-induced CCL22 expression, while the remaining 21 (14%) maintained or even elevated the expression of the CCL22 protein (Fig. 1A and Supplementary Table 1). The inhibitory effects of these 21 drugs on CCL17 formation were further evaluated using monocytes from multiple donors. Among them, 17 consistently inhibited GM-CSF-induced CCL17 expression, while the remaining four drugs did not show significant inhibition (Fig. 1A,B). The 17 drugs that consistently inhibited the CCL17 protein in multiple donors were further analyzed for their effects on regulating CCL22 production to test whether they only inhibit CCL17. Among the 17 drugs, 14 increased the level of the CCL22 protein compared to PBS, while 3 significantly inhibited the level of CCL22 expression compared to cells treated with GM-CSF (Fig. 1A,C). These 14 drugs were further analyzed in mouse BMDMs to identify candidates that could be subsequently tested in mouse arthritis models.

Mouse BMDMs were pretreated for 30 min with the 14 drugs (10 μM) followed by treatment with GM-CSF (20 ng/mL) or left untreated for 16 h. Among them, only five drugs inhibited CCL17 expression (Fig. 2A and Supplementary Fig. 1). The five CCL17-inhibiting drugs were tested for their effects on CCL22 expression in BMDMs. The five drugs were found to maintain CCL22 levels that were comparable to those in supernatants of BMDM treated with either GM-CSF or PBS (Fig. 2B). Consistent with the ELISA data, all five drugs inhibited *Ccl17* mRNA (Fig. 2C), but not *Ccl22* mRNA (Fig. 2D), as ascertained by qPCR. These five candidate CCL17-inhibiting drugs were subjected to titration in human monocytes to determine the optimal concentration for CCL17 inhibition. All five drugs effectively inhibited CCL17 formation at 10 μM concentration (Supplementary Fig. 2). This dosage was chosen to further examine their inhibitory mechanisms.

Fluoxetine, ractopamine, ponesimod, terbutaline and etravirine inhibit GM-CSF-induced STAT5 activity and IRF4 expression in BMDMs

GM-CSF-induced CCL17 expression has been found to be dependent on IRF4 expression in monocytes and macrophages[5,6]. Furthermore, treatment of monocytes and monocyte-derived dendritic cells with GM-CSF activates STAT5, leading to increased expression of CCL17[13,14]. Therefore, we hypothesized that the five identified CCL17-inhibiting drugs could inhibit GM-CSF-induced CCL17 formation by blocking the transcription factors STAT5 and IRF4. The five drugs significantly inhibited *Irf4* mRNA expression (Fig. 3A) but not *Irf5* mRNA (Fig. 3B) in BMDM. Furthermore, Western blot analyses of STAT5 and IRF4 proteins indicated that all five drugs could also inhibit GM-CSF-induced STAT5 phosphorylation as well as IRF4 protein expression in BMDM (Fig. 3C,D and Supplementary Fig. 3).

Fluoxetine, ractopamine, ponesimod, and terbutaline ameliorate arthritic pain

To investigate whether the five identified CCL17-inhibiting drugs could ameliorate arthritic pain and disease, the zymosan-induced arthritis (ZIA) model was used[15]. ZIA is a monoarticular acute arthritis model, and it exhibits key pathological features similar to RA, including synovial hyperplasia, bone erosion and cartilage destruction. C57/BL6 mice were given intra-articular injections of saline or zymosan, followed by intraperitoneal injections of saline or fluoxetine (25 mg/kg)[16], ractopamine (9 mg/kg)[17], ponesimod (30 mg/kg)[18], terbutaline (1.2 mg/

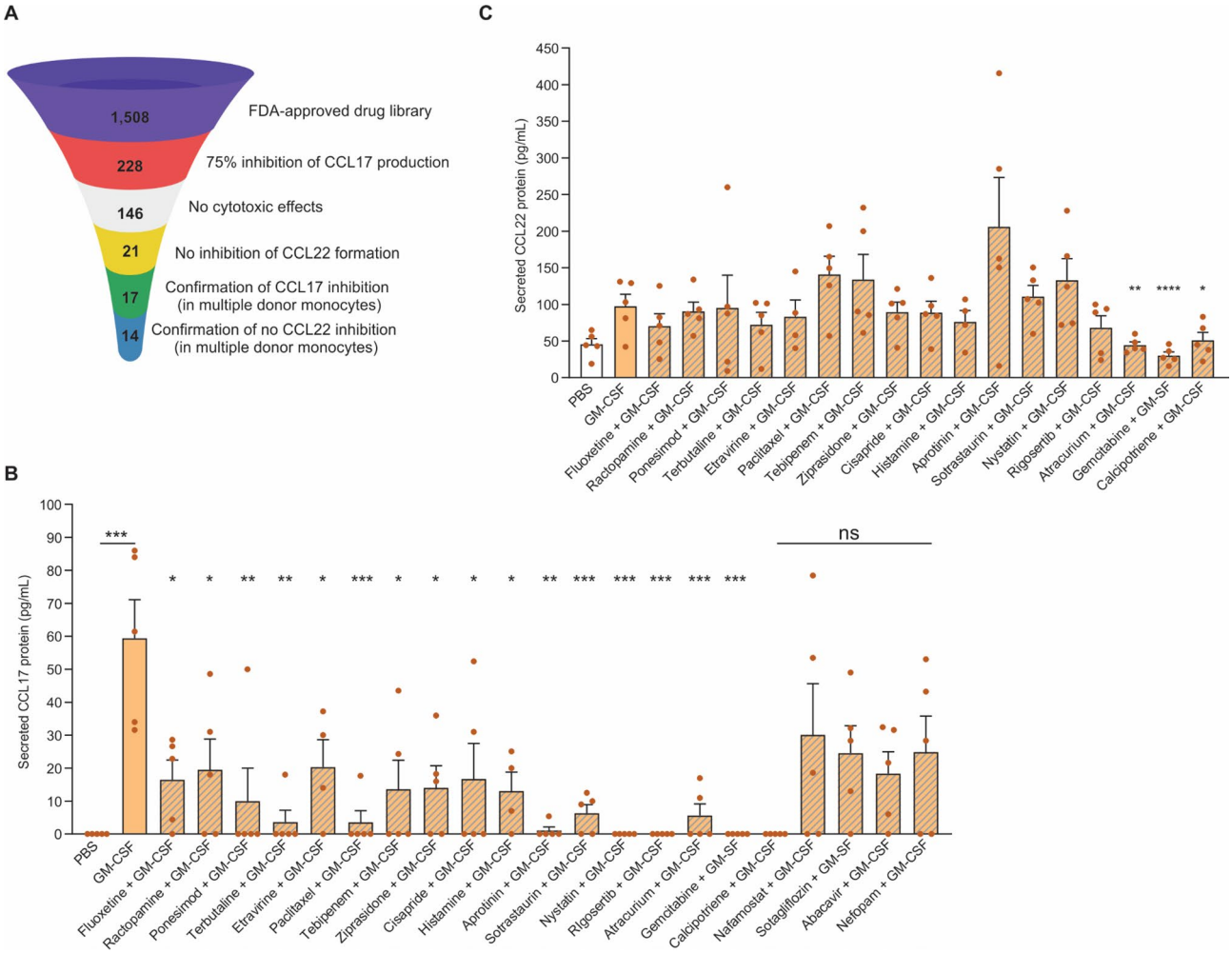

Fig. 1. CCL17-inhibiting candidates from the screening of the FDA-approved drug library. (**A**) Summary of drug screening depicting that among the 1508 FDA-approved drugs, 228 drugs inhibited GM-CSF-induced CCL17 expression by 75%. Following MTT viability assay, 146 of the CCL17-inhibintg drugs showed no toxic effects on human monocytes. These drugs were further analyzed for their effects on CCL22 expression using the same supernatants. Among the 146 drugs, only 21 drugs had no inhibitory effects on basal CCL22 expression in human monocytes. These 21 drugs were further evaluated for their inhibitory effects using monocytes from multiple donors. Among these 21 drugs, 17 drugs consistently inhibited GM-CSF-induced CCL17 expression and 14 of them did not inhibit CCL22 expression. (**B,C**) Human monocytes were pre-treated with either PBS or one of the CCL17-inhibiting drugs (10 μM), identified by the high-throughput screening, for 30 min before being treated with GM-CSF (20 ng/mL) for 16 h. Monocyte culture supernatants were subjected to (**B**) CCL17 and (**C**) CCL22 ELISA. Data are graphed as scatter plots with bars indicating mean ± SEM. P values were obtained using one-way ANOVA with Tukey post-test ($n = 4–5$), where * < 0.05, **< 0.01, *** <0.001. ND, not detected, and NS, not significant. The complete list of FDA-approved drugs and their effects on CCL17 (and CCL22) are provided in Supplementary Table 1.

kg), and etravirine (25 mg/kg)[19] twice a week on days 1 and 4. Zymosan-injected mice developed monoarticular arthritic pain that was monitored over the 7 days. Pain-like behavior, referred to as pain from here on, was measured by a change in weight distribution between the inflamed hindlimb and the non-inflamed hindlimb using an incapacitance meter, as previously described[5]. Following zymosan injection, WT mice developed severe pain on day 1 that became less severe by day 5. However, compared to the saline control group, fluoxetine, ractopamine, ponesimod, and terbutaline, but not etravirine, significantly ameliorated the pain on day 2 which lasted until day 7 (Fig. 4A).

On day 7, the zymosan-treated joints, together with the untreated joints, were histologically evaluated to investigate the level of cell infiltration, bone erosion and proteoglycan loss. The joints injected with zymosan showed significant cellular infiltration, bone erosion and proteoglycan loss (Fig. 4B and Supplementary Fig. 4). Although there were some trends noted toward a decrease in cellular infiltration and proteoglycan loss in drug-injected mice, no significant changes were recorded compared to the mice that received saline. Significantly, decreased levels of joint *Ccl17* mRNA, but not *Ccl22* mRNA, were measured in mice that received fluoxetine, ractopamine, ponesimod and terbutaline, correlating with their decreased pain (Fig. 4C).

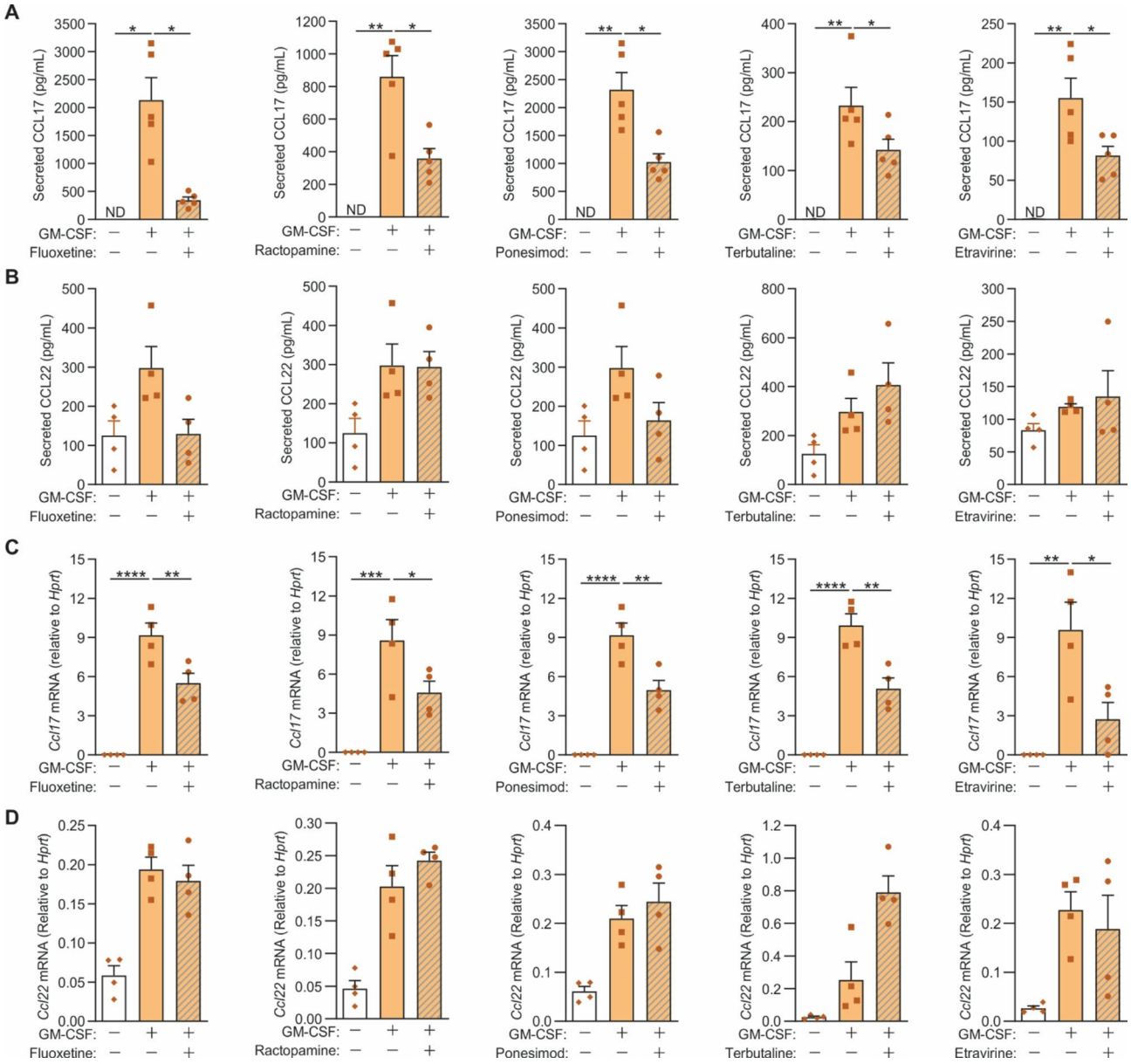

Fig. 2. The effects of CCL17-inhibiting drugs from monocytes on GM-CSF-induced CCL17 and CCL22 expression in mouse macrophages. BMDMs were pretreated with either PBS or one of the drugs (10 μM) for 30 min before being treated with GM-CSF (20 ng/mL) for 16 h. Macrophage culture supernatants were subjected to (**A**) CCL17 and (**B**) CCL22 ELISA. (**C**) *Ccl17* and (**D**) *Ccl22* mRNA expression were by qPCR. Data are graphed as scatter plots with bars indicating mean ± SEM. P values were obtained using one-way ANOVA with Tukey post-test ($n = 4–5$), where * < 0.05, **< 0.01. ND, not detected. Secreted CCL17 protein data for the remaining nine drugs that inhibited GM-CSF-induced CCL17 expression in human monocytes but not in BMDMs are presented in Supplementary Fig. 1.

Discussion

Treatment options for RA have advanced significantly in recent decades; however, a substantial number of patients do not respond to prescribed drugs and develop resistance to disease modifying anti-rheumatic drugs and biologics over time[20,21]. These drugs can be associated with high financial costs and can cause adverse reactions, including hyperlipidemia and renal failure. These challenges highlight the need to identify new therapeutic targets and minimize the cost of manufacturing the drugs. By screening a panel of FDA-approved drugs, we identified five CCL17-inhibiting drugs, namely fluoxetine, ractopamine, ponesimod, terbutaline, and etravirine, that can potentially be repurposed for the treatment of RA.

We have previously shown that GM-CSF-induced CCL17 expression is dependent on the IRF4 transcription factor in both human monocytes and BMDM[5,6,12]. Binding of GM-CSF to its receptor activates several downstream signaling pathways, including PI3K/AKT, MAPK, NF-κB and JAK2/STAT5[6,22–24]. Significantly,

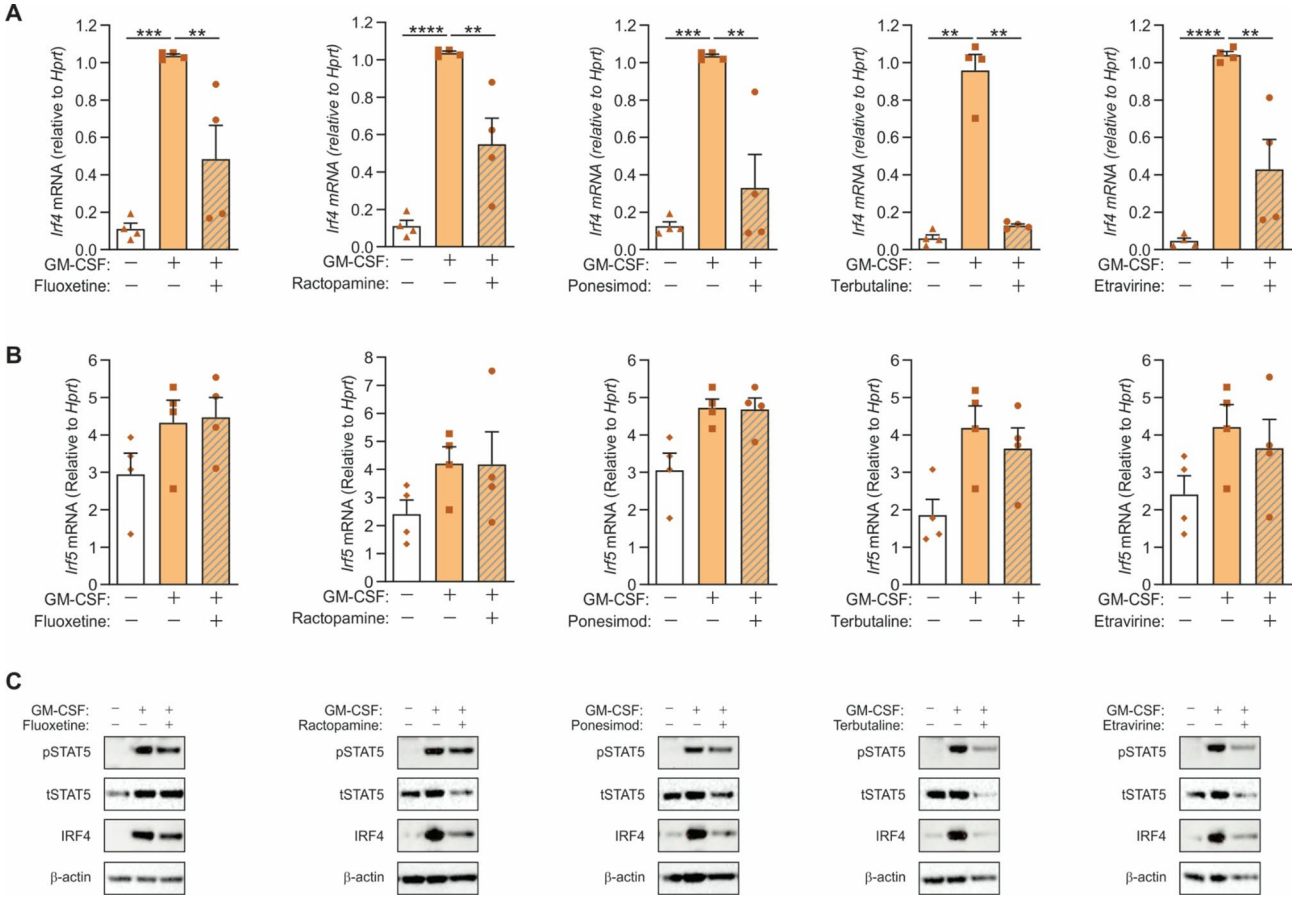

Fig. 3. Fluoxetine, ractopamine, ponesimod, terbutaline and etravirine down-regulate GM-CSF-induced STAT5 activity and IRF4 expression in BMDMs. BMDMs were pre-treated with fluoxetine, ractopamine, ponesimod, terbutaline and etravirine (10 μM) for 30 min and treated with GM-CSF (20 ng/mL) for 16 h. (**A**) *Irf4* and (**B**) *Irf5* mRNA expression (qPCR) were determined. (**C,D**) Whole cell lysates were subjected to Western blotting with anti-pSTAT5, anti-tSTAT5, anti-IRF4, and anti-β-actin antibodies. (**C**) A representative blot is shown. (**D**) The quantified data are graphed as scatterplots with bars, indicating mean ± SEM. P values were obtained using one-way ANOVA with Tukey post-test ($n = 4$), where $^{*}p < 0.05$, $^{**} < 0.01$. ND, not detected. Original Western blots are presented in Supplementary Fig. 3.

the presence of STAT5 and IRF4 transcription factor binding sites in the promoter region of the CCL17 gene suggests that GM-CSF can transcriptionally regulate CCL17 expression by activating and/or regulating these two transcription factors[12,13,25]. We found above that the five CCL17-inhibiting drugs can suppress both STAT5 activity and IRF4 expression, suggesting that the mode of inhibition of these drugs in monocytes/macrophages can be through suppression of the transcriptional regulation of the CCL17 gene. Although the function of other GM-CSF-activated signaling pathways, in addition to JAK2/STAT5, in regulating CCL17 formation was not explored, the identified CCL17-inhibiting drugs have been shown to inhibit some of these pathways in other cell types. For example, fluoxetine, ponesimod and terbutaline have been shown to inhibit NF-kB, PI3K, and ERK pathways, respectively[26–28] and therefore, the mode of inhibition of CCL17 by these drugs might also be through the suppression of other signaling pathways.

The expression of CCL17 and CCL22 can be controlled by some common regulatory factors in GM-CSF-treated monocytes and in other cell types, including DCs, macrophages, and myeloid cells[6,29–31]. For example, robust induction of CCL22 is observed in DCs treated with IL-7 and GM-CSF[29]. Furthermore, CCL17, CCL22 and IRF5 are among the most highly expressed transcripts in GM-CSF-treated eosinophils[32]. In GM-CSF-treated mouse bone marrow cultures, it has been reported that both the CCL17 and CCL22 genes can be regulated by IRF5[33]. However, while fluoxetine, ractopamine, ponesimod, terbutaline, and etravirine inhibit GM-CSF-induced CCL17 formation, they suppress the expression of neither *Irf5* nor *Ccl22*. These findings suggest that there may be differences between the pathway involved in GM-CSF-induced CCL17 and CCL22 expression.

Therapeutic administration of these five drugs in the ZIA inflammatory arthritis model revealed that fluoxetine, ractopamine, ponesimod and terbutaline could inhibit arthritic pain, correlating with decreased CCL17 expression. While these inhibitory studies indicate a role for CCL17 in inflammatory arthritic pain, how it may directly contribute to pain development remains largely unknown. It has been suggested that CCL17 may mediate pain signaling by acting directly on sensory neurons through its receptor[34]; however, another study

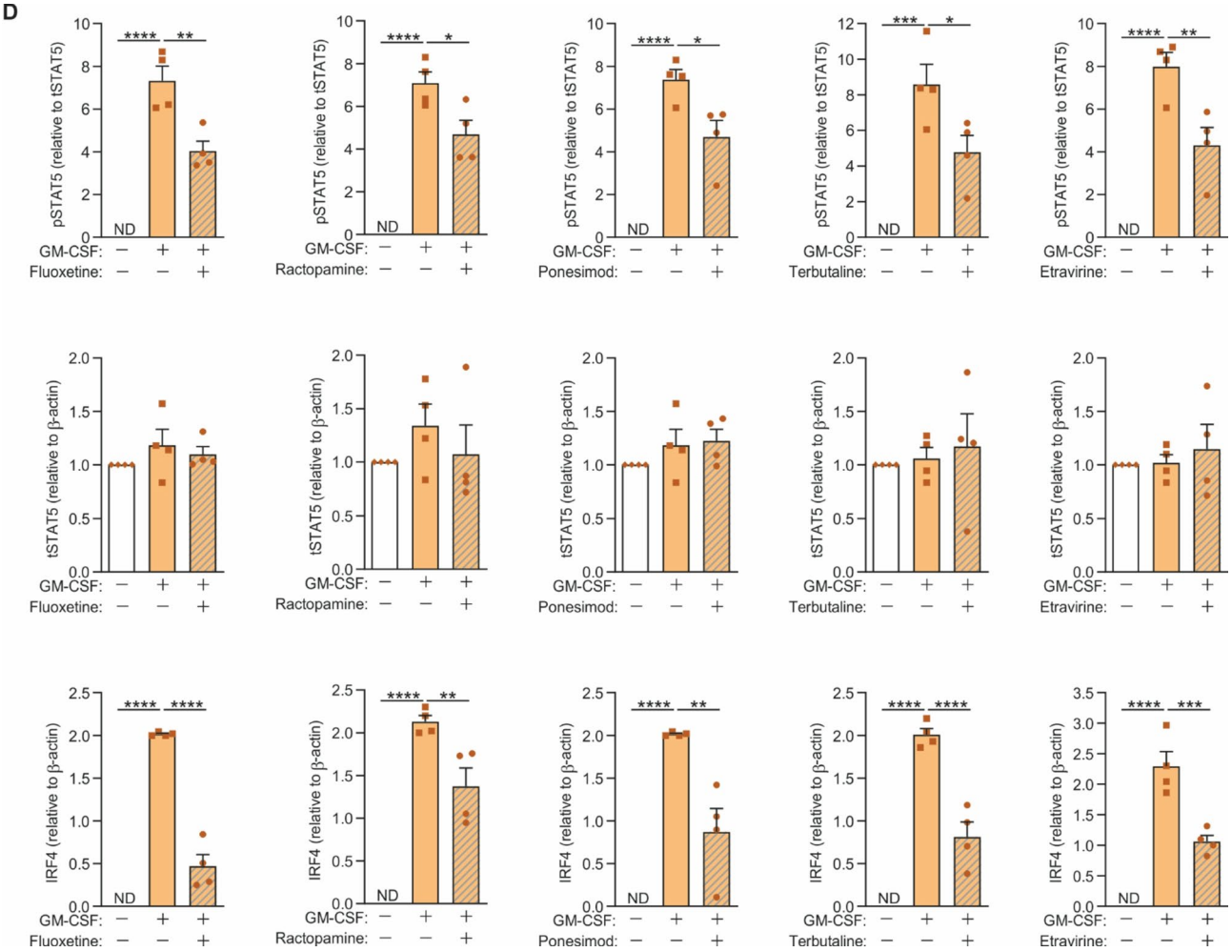

Fig. 3. (continued)

found no CCR4 expression by these cells[35]. These observations warrant further research into the molecular functions of CCL17 upon binding to CCR4 receptor. In addition, the identified CCL17 drugs and their effects on acute arthritic pain and development in ZIA model need to be validate in alternative models of arthritis that can better mimic chronic pain, which is often experienced by patients with arthritis.

Fluoxetine, known as a selective serotonin reuptake inhibitor, is the most prescribed drug for the treatment of severe depression. Interestingly, CCL17 has been shown to be highly elevated in patients with asthma experiencing high comorbid anxiety and depression[36]. Fluoxetine treatment leads to a decrease in IL-1β, IL-6 and TNF in macrophages 24 h post-exposure to lipopolysaccharide (LPS) by inhibiting the JAK/STAT3 and TLR4/JNK pathways[37]. Recently, fluoxetine has been suggested as an anti-inflammatory therapy in SARS-CoV-2 infection through a mechanism related to suppression of inflammatory genes, such as IL-6 signal transduction protein (gp130) and NF-kB[38]. These inflammatory cytokines are known to enhance pain sensation by promoting peripheral sensitization of joint nociceptors[39,40]. Notably, a randomized double-blind clinical trial in patients with persistent somatoform pain disorder has found that fluoxetine has analgesic effects[41]. Furthermore, its administration significantly reduced the expression of cyclooxygenase 2 (COX2), which is involved in the formation of several pain-inducing eicosanoids[42]. We have previously shown that inflammatory and arthritic pain driven by CCL17 are COX2 dependent, and CCL17 neutralization leads to a decrease in COX2 levels in arthritic joints[6,7]. Given our finding that fluoxetine can inhibit CCL17 formation, it is tempting to speculate that abrogation of the CCL17-dependent COX2 pathway may have also contributed to its favorable outcomes in treating pain.

Both ractopamine and terbutaline are β adrenergic agonists; however, the former acts as both a β1- and a β2-adrenergic receptor agonist, while the latter is specific for the β2-adrenergic receptor[28]. Ractopamine has been used as a food additive to promote leanness and to reduce food consumption[43]. Terbutaline, on the other hand, has been shown to have anti-inflammatory effects in lungs[44]. Adrenergic receptors are known to play a key role in modulating pain and targeting adrenergic signaling has been explored in many inflammatory conditions, including RA and OA[45,46]. β adrenergic agonists stimulate adenylyl cyclase activity to produce cAMP which is a second messenger that regulates inflammation and immune responses[47]. For example, an increased level of intracellular cAMP in LPS-treated monocytes leads to suppression of proinflammatory cytokines, such as

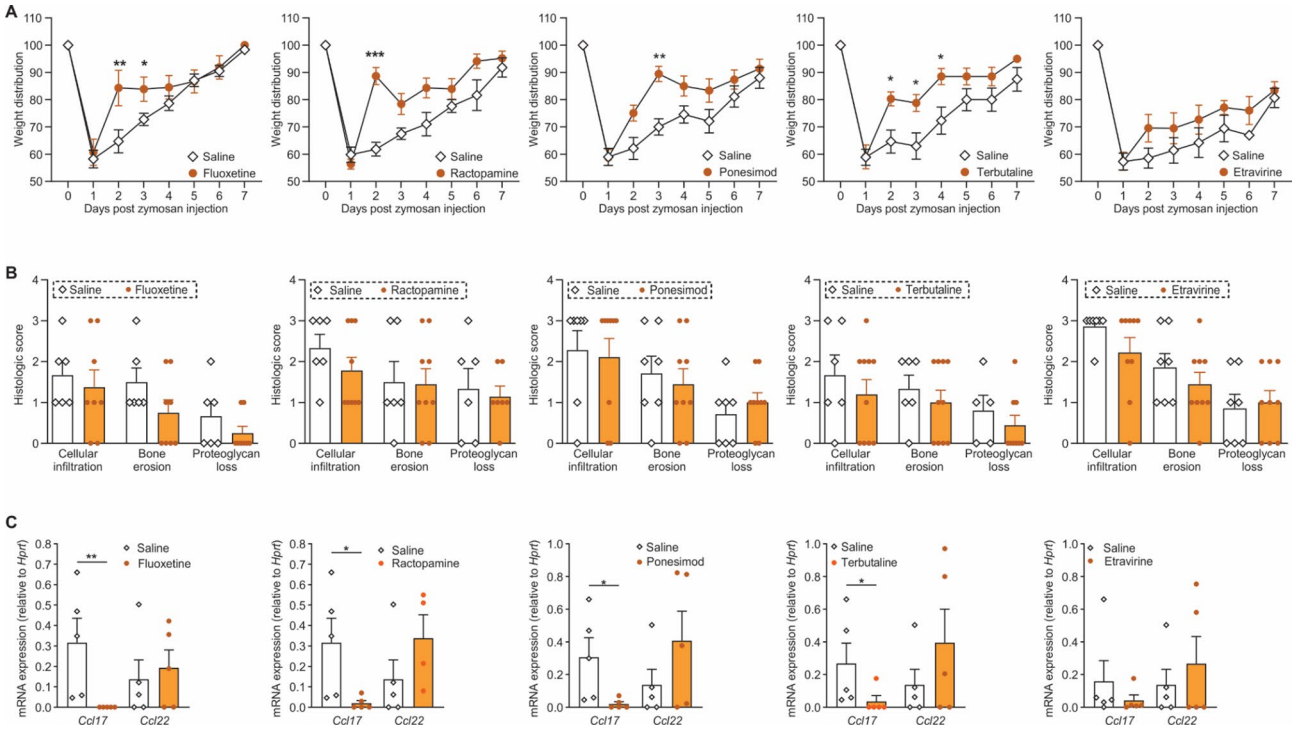

Fig. 4. Fluoxetine, ractopamine, ponesimod and terbutaline ameliorate arthritic pain-like behavior. C57BL/6 mice were intraarticularly injected with zymosan on day 0 and treated (intraperitoneal) with saline or fluoxetine, ractopamine, ponesimod, terbutaline and etravirine therapeutically on days 1 and 4. (**A**) Change in weight distribution (arthritic pain-like behavior) using incapacitance meter ($n = 8$/group), over time. (**B**) Disease (day 7, histologic score, $n = 6$–10 mice/group) and (**C**) joint *Ccl17* and *Ccl22* mRNA expression (day 7, qPCR; $n = 5$/group) were graphed as scatterplots with lines indicating mean ± SEM measured. P values were obtained using two-way ANOVA with Sidak post-test (**A**) or unpaired t test (**B**,**C**), where $^{*}p < 0.05$, $^{**} < 0.01$, $^{***} < 0.001$. Histological images are presented in are presented in Supplementary Fig. 4.

TNF and IL-1β[48]. Anti-inflammatory mechanisms of β2-receptor agonists are reported in the suppression of TNF release due to the inhibition of p38 MAPK activity[49,50]. Furthermore, in LPS-treated macrophages, β2-receptor agonists attenuated TNF production by inhibiting ERK phosphorylation through a cAMP-dependent mechanism[28]. Although several MAPKs have been shown to be activated by GM-CSF, their role in controlling CCL17 expression remains unknown. Intriguingly, treatment of keratinocytes with adenylyl cyclase has suppressed the formation of CCL17 and CCL22 through inhibition of the p38 MAPK pathway[51], suggesting a complex and context-dependent regulation of these chemokines and warranting further investigation of their governing mechanisms.

Ponesimod is a modulator of sphingosine 1 phosphate (S1P), which is a bioactive lysophospholipid whose effects are mediated by a family of G protein-coupled receptors (GPCR)[52,53]. Five S1P receptors have been identified which are targets for immune cell trafficking inhibitors to treat relapsing forms of demyelinating central nervous system disease and multiple sclerosis[52,54]. Ponesimod, with its high affinity for the S1P receptor, can modulate the receptor to enhance cAMP production[54]. Significantly, ponesimod treatment has been shown to modulate the Th1/Th17/Treg cell balance and ameliorate disease in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis[27]. Th17/Treg imbalance is a critical pathological phenomenon in the progression of RA. Significantly, elevated levels of CCL17 and decreased levels of CCL22 are found in RA patients[55,56]. Although both CCL17 and CCL22 share a common receptor CCR4, unlike CCL17, which is associated with recruiting Th1, Th2 and Th17 cells to maintain inflammation, CCL22 has been proposed to promote homeostasis by recruiting Tregs[8,9]. Based on these concepts, drugs that selectively suppress CCL17 levels while maintaining CCL22 levels could be desirable for treating RA.

Of the drugs selected for in vivo testing only etravirine failed to show promising effects in inhibiting ZIA pain when administered therapeutically on days 1 and 4. Etravirine is a non-nucleoside reverse transcriptase inhibitor that is being used in the treatment of HIV infection[57]. This FDA-approved drug showed efficacy in inhibiting proliferation, migration, and invasion in ovarian cancer cells[19] and activates the Wnt pathway in osteosarcoma cells[58]. Although a crosstalk between the Wnt and GM-CSF signaling pathways has been speculated, etravirine-triggered molecular mechanisms resulting in inhibition of GM-CSF-induced CCL17 formation require further investigation. However, accumulating evidence highlights the benefits of inhibiting CCL17 or its upstream regulators to inhibit arthritic pain. A neutralizing anti-CCL17 monoclonal antibody has been shown to ameliorate arthritic pain in several mouse models of arthritis[6,7]. Significantly, phase 2 clinical trials are currently underway

to determine the clinical benefit of targeting CCL17 in patients with knee osteoarthritis pain (ClinicalTrials.gov ID: NCT05838742) and with chronic diabetic peripheral neuropathic pain (NCT05838755).

In summary, we have identified five CCL17-inhibiting drugs (fluoxetine, ractopamine, ponesimod, terbutaline and etravirine) from screening a panel of 1,508 FDA-approved drugs. Mechanistically, we demonstrate that these five drugs inhibit GM-CSF-induced CCL17 formation by inhibiting STAT5 activity and IRF4 expression without suppressive effects on CCL22 or IRF5 expression. We have provided evidence for the first time that fluoxetine, ractopamine, ponesimod and terbutaline can modulate the GM-CSF signaling pathway *in vitro* and therapeutic administration of these drugs lead to the amelioration of arthritic pain, correlating with decreased levels of joint *Ccl17* mRNA. The findings reported here offer four new promising analgesic mediators for treating inflammatory pain.

Methods

Isolation and culture of human monocytes

Human monocytes were isolated from buffy coats[5,12,59], as approved by the University of Melbourne Human Research Ethics Committee (2021–20542), and their research use was in accord with the terms of the informed consents obtained by the Australian Red Cross Lifeblood. All experiments involving human samples were performed in accordance with the relevant guidelines and regulations, as well as adhering to the Declaration of Helsinki. Briefly, monocytes were purified from buffy coats by negative selection of CD14+ monocytes, using the RosetteSep Ab cocktail (Stem Cell Technologies, Vancouver, BC, Canada). They were cultured in RPMI 1640, supplemented with 10% heat inactivated fetal bovine serum, 2 mM GlutaMax-1 (Life Technologies, Carlsbad, CA), 100 U/ml penicillin, and 100 mg/ml streptomycin. Isolated monocytes were seeded in 96-well tissue culture plates (7.5×10^4/well) and pretreated with FDA-approved drugs (10 μM, Selleckchem, Houston, TX) for 30 min before treated with human GM-CSF (20 ng/ml, R&D Systems, Minneapolis, MN) or left untreated for 16 h.

Isolation and culture of bone marrow-derived mouse macrophages

Experiments involving mouse bone marrow-derived macrophages (BMDM) were approved by the University of Melbourne Animal Ethics Committee (2021-20398). All experiments involving mice were performed in accordance with the relevant guidelines and regulations. Mouse macrophages were prepared as previously described[6]. Briefly, bone marrow cells were isolated from the femurs of C57/BL6 mice and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM GlutaMax-1, 100 U/ml penicillin, and 100 mg/ml streptomycin in the presence of human M-CSF (5,000 U/ml, Chiron, Emeryville, CA). On day 4, non-adherent cells were collected and cultured for another 3 days again in M-CSF (5,000 U/ml) to derive BMDM. Differentiated BMDM (in the absence of M-CSF) were pretreated with FDA-approved drugs (10 μM, Selleckchem, Houston, TX) for 30 min before being treated with mouse recombinant GM-CSF (20 ng/ml, R&D Systems) for 16 h.

Cell viability assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Promega, Madison, WI). The tetrazolium compound is reduced by cells into a colored formazan product that is soluble in tissue culture medium and is evaluated for the cytotoxic potential of the drugs screened. Human monocytes (7.5×10^4/well) were seeded in 96-well tissue culture plates and pretreated with 10 μM of drugs for 30 min and then treated with GM-CSF. Untreated cells served as controls. After 16 h of incubation, cells were centrifuged at 300 × g for 5 min at 4° C. The supernatant was aspirated, and the pellet was resuspended in 100 μl complete culture medium and 20 μl tetrazolium compound. Cells were incubated in the dark for 4 h at 37 °C with 5% CO_2. Absorbance was measured at 490 nm using a Thermo Scientific™ Varioskan™ LUX multimode microplate reader. Cell viability in response to drug/GM-CSF treatments was expressed as a percentage of that of untreated cells.

Quantitative PCR

Total RNA was extracted using the ISOLATE II RNA Mini Kit (Bioline, London, UK) and reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed using the QuantStudio 5 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and pre-developed TaqMan probe/primer combinations for mouse *Ccl17*, *Ccl22*, *Irf4*, *Irf5* and *Hprt* (Applied Biosystems), as before[5,6,12]. The threshold cycle numbers were transformed to cycle threshold values and the results were plotted using GraphPad Prism version 10.1.2.

Western blotting

Whole cell extracts were lysed and Western blotted as described previously[12,60,61]. Protein concentrations of the samples were determined with a Bio-Rad protein assay kit. Equal amounts of protein were loaded on 10% NuPAGE gels (Invitrogen). The separated proteins were transferred onto a polyvinylidene fluoride membrane and then Western blotted with appropriate antibodies. The antibodies were against pSTAT5 (#9351), tSTAT5 (3H7)), IRF4 (D9P5H) (Cell Signaling Technologies, Danvers, MA), and β-actin (A5316) (Sigma-Aldrich, St. Louis, MO).

ELISA

Secreted human and mouse CCL17 and CCL22 (R&D Systems) were measured by ELISA according to the manufacturer's instructions on a Varioskan Lux Plate Reader (Thermo Fisher).

Zymosan-induced arthritis model

The zymosan-induced arthritis (ZIA) model experiments involving mice were approved by the University of Melbourne Animal Ethics Committee (2022-23414) and all experiments involving live animals were performed according to the relevant regulations and as described by the ARRIVE guidelines. For induction of the ZIA model[6,15], C57/BL6 female mice (8–12 weeks) were injected with 300 mg of sonicated zymosan (Sigma-Aldrich) in a 10 µL volume into the left knee joint, while the contralateral knee received saline as a control. Mice with ZIA were treated therapeutically by intraperitoneal injection, beginning once the pain was evident (days 1 and 4), with either the drugs, namely fluoxetine (25 mg/kg)[16], ponesimod (30 mg/kg)[18], terbutaline (1200 µg/kg)[62], ractopamine (9 mg/kg)[17] and etravirine (25 mg/kg)[19], or saline as vehicle control.

Behavioural pain assessment

Pain-like behaviour (referred to as pain throughout) was measured using an incapacitance metre (IITC Life Science Inc, USA) meter over 7 days, as previously described[1,2]. As an indicator of pain-like behaviour, a ratio between two knees (left vs. right) was used as a measure of static weight-bearing joint pain using an incapacitance meter and expressed as percentage weight on the contralateral hindlimb. Values between 90 and 100 for the percentage (%) weight on the contralateral hindlimb are within a normal range of variation (i.e. no pain); a value below 90 indicates pain.

Histopathological assessment of arthritis

On day 7, arthritic knee joints were collected for histological analysis. For ZIA, cell infiltration (H&E stain), bone erosion (H&E stain) and proteoglycan loss (Safranin O/fast green stain) were scored separately from 0 (normal) to 3 (severe), as before[5,6,63–65].

Statistics

Statistical analyses were performed using two-way ANOVA with Dunnett/Sidak post-test to compare two different groups over a constant period of time, one-way ANOVA with Tukey post-test for more than two different groups or unpaired t-test to compare mean of two independent groups, as indicated. A P value < 0.05 indicates significance. Data were graphed as scatter plots with bars indicating mean ± SEM from at least four independent experiments using GraphPad Prism version 10.1.2.

Data availability

All generated or analyzed data during this study are included in this paper.

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Author contributions

Conceptualization was by A.A.A.. Investigation was by M.E., T.J.L. and K.M.C.L. Resources were provided by A.A.A., K.L. and J.A.H. Formal analysis was by M.E., K.M.C.L. and A.A.A. M.E. wrote the original draft. Writing-review and editing were by A.A.A., J.A.H. and K.M.C.L. Supervision by A.A.A., K.M.C.L. and J.A.H.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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