CCL15/Leukotactin-1: A novel mediator of rheumatoid arthritis fibroblast-like synoviocyte migration via CCR3 and MAP kinases

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ABSTRACT

Purpose: Rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS) play a key role in mediating inflammation and joint destruction. RA FLS aggressively invade bone and cartilage, and their activation increases expression of matrix-degrading enzymes and inflammatory cytokines. CCL15 has previously been shown to be expressed in RA synovial tissue and synovial fluids. CCL15 has been characterized to interact with its receptors, CCR1 and CCR3, where the former serves as a weak agonist and the latter serves more potent functions. We hypothesized that CCL15 induces RA FLS migration and proliferation, acting through CCR1 and/or CCR3, and signaling through mitogen-activated protein (MAP) kinases.

Methods: FLS were derived from hip or knee synovial membrane at the time of joint replacement due to RA, and were subsequently analyzed using Alexa Fluor 488 Phalloidin staining of F-actin, proliferation assays, chemotaxis assays, and flow cytometry. In addition, we used MAP kinase inhibitors in combination with chemotaxis assays to determine whether MAP kinase signaling was required for CCL15-induced RA FLS migration.

Results: Evaluation of F-actin staining subsequent to CCL15 stimulation demonstrates significant reorganization of the cytoskeletal structure. When stimulated with 1 or 10 nM CCL15, maximal cytoskeletal rearrangement occurs after 2 to 3 hrs, consistent with the slow migration associated with fibroblasts. Chemotaxis assays demonstrate that CCL15 is a novel chemoattractant for RA FLS, effectively inducing migration of all 4 RA patients that we tested ($p<0.05$). Concentrations of 1 to 50 nM CCL15 induced migration that was significantly higher than background, however, similar concentrations of CCL15 had no effect on RA FLS proliferation. Flow cytometry demonstrates that a significant number of RA FLS express CCR3

but not CCR1. On average, 73% of RA FLS express CCR3. Consistent with CCR3 being a G protein coupled receptor, pertussis toxin (PTX) completely abolishes CCL15-induced migration. Finally, pre-incubation of FLS with a MEK 1/2 inhibitor (U0126), the kinase which activates ERK 1/2, significantly decreased chemotaxis induced by CCL15 in RA FLS derived from 2 of 3 patients. Similarly, pre-incubation of cells with a JNK inhibitor (SP600125) likewise decreased CCL15-induced migration in FLS from 2 of 3 patients.

Conclusions: Our results suggest that CCL15 regulates the migration and cytoskeletal structure of RA FLS. Migration is likely induced by a G protein (Gi/Go) coupled receptor, since this event is inhibited by PTX. To our knowledge, these are the first results to suggest that RA FLS express CCR3. Finally, migration of RA FLS can be significantly decreased by inhibiting the ERK and JNK pathways. A more thorough understanding of the RA FLS/CCL15 relationship is needed because of the key role that these cells play in arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease causing pain, swelling, stiffening, inflammation, and eventual destruction of the joints if left untreated. It affects more than two million adults in this country alone and epidemiologists suggest that women are at a two to three times greater risk of developing RA (1). In fact, there are two to three times as many women that are inflicted with the disease than men (4). Women are at the greatest risk between the ages of 40-50. RA is uncommon under the age of 15 but its incidence continually rises until about 80 (4). In addition, smokers are four times more likely to develop the disease than non-smokers, probably due to a compromised immune system (1).

Autoimmune disorders are diseases in which inherent protection systems mount an attack against self-antigens. In a normally functioning immune system foreign invaders are attacked, destroyed, and eliminated. To do this, the immune system must recognize self from non-self and limit the response to foreign antigens. In diseases such as RA, the immune system fails to make this distinction and launches an attack against the synovium, the interior lining of the joint. One likely hypothesis is that it mistakenly identifies cells in the synovium as foreign because of their apparent similarity to an outside organism, a process called molecular mimicry. The FLS of the joint is responsible for secreting synovial fluid, which provides lubrication and cushion between the articulating surfaces of the joint. A damaged synovium leads to a loss of fluid production and an eventual loss of protection between the moving parts of the joint. The different surfaces begin to abrade each another causing eventual destruction of the joint and severe pain.

The disease progresses in three main stages beginning within the synovium. The first stage is characterized by the swelling of the synovial lining, which leads to an increase in heat and stiffness due to increased constriction (5). FLS act as a protective agent against inflammation but in the second stage display a much faster rate of division, apparently in response to the autoimmune attack. Proliferation of these cells causes the lining to thicken causing further constriction, stiffness, and more pain. The final stage is associated with the release of enzymes to digest this extra mass. However this also results in digestion and loss of surrounding bone and joint tissue, which progresses to loss of function and complete disability (1). In severe RA, even the most minor tasks become difficult or impossible due to severe pain and loss of mobility.

RA has its largest effects on smaller joints in the hands and feet, but larger joints such as the shoulder and knee are not spared (1). RA typically progresses in a symmetrical pattern such that onset of RA in the left hand means that there is a high likelihood that the right hand will also

be affected. Moreover, although RA mostly manifests itself in joints, its damaging effects can be far reaching. Patients often complain of severe fatigue, lassitude, and wide-spread inflammation can lead to anemia. In rare and more severe cases, RA has led to inflammation of the vasculature, the visceral and pleural cavities of the lung, and the pericardium (1).

The current cause of RA is unknown but many factors have been suggested to play a role, including environment and genetics (6). However, there is some evidence that a microbial agent, either a virus or bacterium, could be the instigator of this disease (5). While this remains a matter of some conjecture, it is clear that the massive inflammatory response is due to an autoimmune attack. This inflammation is characterized by the infiltration of immune cells recruited by cytokines.

Cytokines are soluble mediators released from cells that initiate a chemical reaction and a subsequent biological reaction. They are key players in the development of the chronic inflammation found in RA. The inflammatory process begins with the migration of leukocytes towards the site of injury and the adherence to endothelial cells located within the synovium (7). This adherence causes leukocytes to release cytokines, thereby initiating the inflammatory cascade. They activate two major arms of the immune system, the Th1 and Th2 (T-Helper cells) responses (6).

The Th1 response is linked to cell-mediated immunity and the Th2 response initiates humoral or antibody-mediated immunity. Both responses are found in RA, but in an antagonistic manner. Thus, the Th1 response is pro-inflammatory and is associated with the release of the cytokines tumor necrosis factor (TNF-α), interferon, and interleukin-1 (IL-1). The Th2 response is considered anti-inflammatory in RA, and is associated with the release of a variety of other

interleukin molecules and the subsequent activation of antibodies. The Th2 response also inhibits the Th1 response observed in arthritis patients. Therefore, the degree of inflammation seen in RA is related to the extent of imbalance between pro-inflammatory and anti-inflammatory cytokines.

TNF-α and IL-1 are major contributors to inflammation and joint destruction in RA. TNF-α acts as a positive feedback regulator for other cytokines, exacerbating the inflammation, and its effects are potentiated by IL-1, which stimulates the release of proteolytic enzymes involved in tissue and joint destruction (6). These concerted actions are responsible for a substantial portion of the symptoms characteristic of RA. Nevertheless, there are other chemokines that are commonly found in patients with RA, namely IL-8, GRO-α MCP-1, MIP-1α, and CCL5 (6). Typically, the release of these cytokines occurs secondary to the release of IL-1 and TNF-α (6). Collectively, these pro-inflammatory cytokines contribute to the pain, swelling, and dysfunction found in RA. However, their effects are constantly being challenged by the Th2 response. This response releases cytokines that prevent IL-1 from binding to its receptor, thereby reducing inflammation. Moreover, additional cytokines are secreted that inhibit the release of proteolytic enzymes, which prevents tissue and joint destruction.

At the site of injury cytokines act as a chemical attractant for pro-inflammatory complexes through a concentration gradient. This gradient attracts a variety of cells that play an integral part in the symptoms associated with RA. CCL15 is such a chemoattractant, but it has unique properties. Chemokines typically have four cysteine residues and are classified based on whether these cysteine residues are adjacent (cc-subfamily) or separated by an intervening residue (cxc-subfamily) (2). The cc-subfamily genes are located on the chromosome 4q12-21 and the cxc-subfamily is located on human chromosome 17q11-32 (2). There are an increasing number of cc-chemokines being identified in each subfamily and some contain two additional

cysteine residues that are linked by a third disulfide bond (2). These unique cytokines are classified as C6 β-chemokines because they contain six conserved cysteine residues instead of the normal four (2).

CCL15 was serendipitously identified in 1998 while searching for novel cc-chemokines in the C6 β-chemokine subfamily. This cytokine goes by a number of different names, such as hemofilitrate, CC chemokine (HCC)-2, macrophage inflammatory protein (MIP)-1 delta, MIP-5, leukotactin (LKN), Lkn-1 or CCL15. CCL15 is a potent chemoattractant for blood neutrophils, lymphocytes, and monocytes (2), although the cellular mechanism of this later effect is unknown. CCL15 is expressed in the liver, the small intestine and colon, and in certain leukocytes that are involved in RA (11). Most relevant to our work, is the previous determination by Haringman and co-workers that CCL15 is specifically increased in RA synovial tissue (14). The genes regulating its expression are located on human chromosome 17 (11). CCL15 is an agonist for two different receptors, CCR1 and CCR3, that function via G-protein coupled receptors (12) and whose genes are located on chromosome 3p (12).

The mechanism by which tissue and joint destruction is accomplished is not completely understood but it has been observed that patients with RA have increased secretion of matrix metalloproteinases (MMP) within the synovium that digest proteins of the synovium (4). Cytokines facilitate the release of MMPs and also stimulate the production of other inflammatory mediators such as prostaglandins, which suppress the production of molecules that directly block MMP (6). Noticeably, the inflammatory cytokines induce tissue damage through the release of MMPs and the indirect effects of inhibiting the production of molecules that block MMPs. MMP gene expression is regulated by MAP kinases (3).

MAP kinases are serine/threonine kinases that respond to some external stimuli, typically a mitogen. They are components of signaling cascades that lead to the initiation of gene expression, cell division, and a host of post-translational responses. Three main MAP kinase pathways have been identified including c-Jun N-terminal kinases (JNK), the p38 MAP kinases (p38), and the extra-cellular signal-regulated kinases (ERK) (3). These pathways are ubiquitous and regulate a variety of functions from cell differentiation and proliferation to coordinating inflammation and cellular responses to stress (3), (8).

Current treatment for RA is mainly designed to alleviate the symptoms of pain and inflammation and to slow the destruction of tissue (9). Thus, current treatment is limited to a symptomatic approach rather than a preventative or curative approach (8). NSAIDs are typically used to reduce inflammation and newer drugs called disease-modifying anti-rheumatic drugs are being incorporated to slow the progression of the disease (8). The combination of these two classes of drugs can reduce symptoms and slow progression.

Emerging therapies include monoclonal antibody therapy, and recombinant cytokine and cytokine receptor development that both mimic anti-inflammatory agents such as IL-1, which act as cytokine receptor antagonists (10). The increase in anti-inflammatory agents coupled with blocking pro-inflammatory agents from binding to their corresponding receptor is a current focus of research, since inflammation results from an imbalance between pro-inflammatory and anti-inflammatory cytokines. In patients with RA, the pro-inflammatory agents outweigh the anti-inflammatory agents. By recombination methods, scientists are able to increase the number of cytokines fighting against inflammation within the tissue and thus lowering the amount of inflammation present. Another useful approach is to prevent the pro-inflammatory agents from binding to their receptor. If these cytokines cannot bind to their receptor inflammation will not

develop. Scientists are working to develop both methods to work in concert and CCL15 is one of the recombinant cytokines currently being studied. By examining the interaction between CCL15 and RA FLS and their potential signaling pathways, a better understanding of the pathogenesis of RA will be established. In this study, we hypothesized that CCL15 induces RA FLS migration and proliferation, acting through CCR1 and/or CCR3, and signaling through mitogen-activated protein (MAP) kinases.

METHODS

RA FLS: All synovium was obtained from 4 RA patients undergoing total knee replacement and had a mean age of 65. The synovial tissue was digested and minced in an enzymatic mixture for 2 hours at 37°C and washed vigorously to remove any nonadherent cells. The cell lines were cultured in RMPI 1640 with 10% fetal bovine serum (FBS) and antibiotics. After passage 3, all the cells appeared spindled shaped with a characteristic fibroblast-like morphology.

F-actin staining:

RA FLS were plated on gelatin coated round glass coverslips in a 24-well tissue culture plate at 50,000 cells/well in RPMI + 10% FBS. The following day the cells were approximately 50-60% confluent. The cells were then washed with PBS and the media replaced with serum free RPMI. After one hour at 37°C, 10 or 1 nM recombinant human CCL15 (rhCCL15; R&D Systems, Minneapolis, MN) was added to the wells for the times indicated (10 min, 30 min, 1 hr, 2 hr, or 3 hr). The cells were fixed in 1 ml of 3.7% formaldehyde in PBS at room temperature for 10 min and washed twice with PBS. The glass coverslips were removed from the plate and the cells permeabilized with acetone at -20°C for 3 min and then immediately washed in PBS. The

coverslips were blocked in 100 µl PBS + 1% BSA for 25 minutes. The block was replaced with Alexa Fluor® 488 Phalloidin (Molecular Probes, Carlsbad, CA) in PBS + 1% BSA + DAPI (10 µg/ml) for 20 min at room temperature. The coverslips were washed twice in PBS, air dried, and mounted on a microscope slide, and then stored in the dark at 4ºC until observed. Representative photographs were taken using a Nikon Eclipse E400 microscope fitted with a Spot Digital camera.

Flow Cytometery:

RA FLS were cultured until roughly 80% confluent. Once ready, the cells were then detached from the flask using Accutase for 5 minutes. Cells were collected and centrifuged at 1,000g for 10 minutes. The cell pellet was resuspended in 1 mL of RPMI 1640 containing 10% FBS, and counted with a hemacytometer. Samples were removed from the culture stock at numbers between $5.0x10^4$ to $1.0x10^5$ per sample tube. The cells were then washed with 0.5 mL PBS and centrifuged 1,000 g for 10 min. The cells were then fixed in 0.25% formaldehyde solution for at least 30 min at 4ºC. Following fixation, the cells were washed and centrifuged twice with PBS. The cell pellet was then re-suspended using 25 µL of donkey serum to block. To this solution, either 25 µL of rat anti-human CCR3 (50 µg/mL, R&D Systems) or 25 µL of rat IgG (1:56 dilution, Vector labs) was added and then incubated at 4ºC for 30 min. After two PBS washes, the cells were again re-suspended in 25 µL of donkey serum, followed by the addition of 25 µL of donkey anti-rat IgG, conjugated to allophycocyanin (APC) (1:200 dilution, Jackson Immunologicals), and incubated 30 min in the dark at 4ºC. The cells were then washed and centrifuged twice with PBS and re-suspended in PBS with 2% FBS and 0.05% NaN3. Samples were analyzed on a Becton-Dickinson FACSCalibur flow cytometer, collecting 10,000 gated events of the population of interest using the FSC vs. SSC scatter plot.

Chemotaxis Assays:

RA FLS were fed the night before the assay with RPMI containing 10% FBS. The cells (3.9 x 10^4 cells in 26 µl of RPMI containing 0.1% FBS) were added to the bottom wells of 48-well chemotaxis chambers (Neuroprobe, Gaithersburg, MD) with an 8 µm gelatin-coated polycarbonate membranes. The chambers were inverted and incubated for 2 hrs in a 5% CO2 atmosphere at 37°C, allowing attachment of the cells to the membrane. A phosphate-buffered saline (PBS; negative control), 3% fetal bovine serum (FBS; positive control), and dilutions of rhCCL15 were added to the top wells and further incubated overnight at 37°C. The membranes were removed and fixed in methanol and stained with Diff-Quik (Dade Behring, Deerfield, IL). Each sample was assayed in quadruplicate and the migrated cells were counted in three randomly selected high power fields. Statistical analysis was conducted using a Student's *t*-test.

Cell Proliferation Assays:

RA fibroblasts were fed the night before the assay with RPMI and 10% FBS. The following day cells were washed, trypsinized and resuspended at 5.4 x 10^4 cells/ml in full growth media. Cell suspension (50 µl) was added to wells of a 96-well tissue culture plate which was later incubated at 37°C for 4 hrs to allow cells time to adhere to the bottom of the wells. After incubation cells were washed with PBS and the media was changed to serum free RPMI for 48 hrs. After this initial serum starvation, incubation media was changed to serum free RPMI with or without 1 or 10 nM rhCCL15. Media alone served as negative control and 3% FBS was used as a positive control. The plate was further incubated at 37°C for 72 hours. A CellTiter® 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI) was used to determine the

number of viable cells. To each well, 20 µl of MTS/PMS solution was added and the plate was further incubated at 37°C for 4 hrs. Absorbance was read at 490 nm wavelength using a Beckman Coulter DTX 880 ELISA plate reader with Multimode detection software.

Statistical Analysis:

The statistics used for CCL15 induced migration of RA FLS (figure 1) was a one-way ANOVA, and a post-hoc Newman-Keuls test,. Other migration assays were analyzed by Student's t test. Inhibition of JNK mediated migration of RA FLS (figure 4 B) was analyzed by both the Student's t-test and the one-way ANOVA with a post-hoc Student Newman-Keuls test.

RESULTS

CCL15 induces migration of RA FLS. We isolated RA FLS from 4 different patients and performed chemotaxis assays with various concentrations of CCL15 as the chemoattractant. A representative assay is shown in figure 1 where CCL15 induces significant levels of migration in all cases over the negative control, PBS ($P < 0.05$; $n = 4$ patients; one-way ANOVA, and a post-hoc Newman-Keuls test). Fetal bovine serum (3%; a known fibroblast chemoattractant) was used as the positive control. As shown in figure 1 the amount of migration is concentration dependent and increases from 1 nM CCL15, to a maximum migration observed at 25 nM, followed by a decline at 50 nM. This is characteristic of a traditional bell-shaped curve observed when studying the migration of leukocytes induced by chemokines.

CCL15 stimulation of F-actin cytoskeletal rearrangement. To further confirm that CCL15 did indeed induce migration of RA FLS, we examined whether there was F-actin cytoskeletal

rearrangement in response to CCL15 stimulation. In all three patients tested, CCL15 induced significant rearrangement of the F-actin filaments at 1 nM and 10 nM CCL15 concentrations, with each sample showing nearly identical results. As shown in figure 2 the reorganization of the cytoskeletal structure begins at 30 minutes and is maximal at 2-3 hours of CCL15 stimulation.

Regulation of CCL15 migration. To elucidate a potential signaling mechanism of this reorganization, we pretreated the RA FLS with pertussis toxin, a known inhibitor of G protein coupled receptors, and assessed the migration of RA FLS to CCL15. This is shown in figure 3 where migration to 10 nM CCL15 was compared with background migration, in the presence or absence of pertussis toxin. The presence of pertussis toxin completely abolished CCL15-induced migration indicating G protein (Gi/Go) coupled receptor activity (*$p<0.05$; $n=3$; Student's t test). Since MAP kinases are often involved in chemotaxis signaling, we next examined the JNK and ERK pathways. To study these proposed signaling mechanisms, migration of RA FLS to CCL15 (Figure 4) was compared with background migration in the presence or absence of an ERK 1/2 inhibitor (U0126) at 10 nM or a JNK inhibitor (SP600125) at 10 nM or 30 nM. Inhibition of ERK significantly reduced chemotaxis with RA FLS from 2 out of 3 patients (*$p<0.05$ in 2 of 3 patients by Student's t test) while inhibition of JNK yielded similar results (*$p<0.05$ in 2 of 3 patients by Student's t test but not by a post-hoc Newman-Keuls test). This data suggests the JNK and ERK pathways as potential signaling mediators through which CCL15 signals to promote RA FLS migration.

CCL15 does not induce RA FLS proliferation. A number of chemokines induce proliferation of RA FLS and may do so through the same signaling pathway as migration. Therefore, we

decided to determine whether CCL15 acts as an RA FLS mitogen. Even though CCL15-induced migration is mediated through JNK and ERK signaling, our results indicate that CCL15 does not induce significant proliferation of RA FLS as shown in figure 5 ($p>0.05$). The fact that CCL15 does not, indicates a different mechanism other than CCL15-induced proliferation of RA FLS. One possibility is the TNF-α mediated expression of SYN via the ERK pathway, which does lead to hyper-proliferation of RA FLS (8).

RA FLS CCR1 and CCR3 expression. Since CCL15 is known to bind to CCR1 and CCR3, we examined expression of these two receptors on RA FLS. Receptor expression was determined by flow cytometry. We found little or no expression of CCR1 by RA FLS, however, we did find significant expression of CCR3 on the same cells (Figure 6). To our knowledge, this is the first evidence showing expression of CCR3 on RA FLS. On average, 73% of RA FLS express CCR3, whereas <5% of these cells express CCR1 indicating CCR3 as the main receptor (n = 3).

DISCUSSION

As we search for a better understanding of the pathogenesis of RA, the essential role played by fibroblast-like synoviocytes in regulating inflammation and joint destruction is becoming more profound. RA FLS aggressively invade bone and cartilage, and their activation increases expression of matrix-degrading enzymes and inflammatory cytokines. Moreover, FLS are key effectors in the destruction of connective tissue and the facilitation of bone erosion. Investigation of chemokines such as CCL15, which may drive the disease process and the physiological invasion of RA FLS through cartilage and into bone, is an important step towards gaining a clearer picture of what directs the progression of RA. In this study, we identified CCL15 as a novel chemoattractant of RA FLS via CCR3 and MAP kinase signaling.

To gain a better understanding of the relationship between CCL15 and RA FLS we initially studied the migration of RA FLS to CCL15, since CCL15 expression is significantly increased in RA. We found that CCL15 did induce significant levels of migration in the cells from 4 patients tested, and when inhibitors such as pertusiss toxin were added to the chemotaxis assay, migration was completely abolished. Moreover, migration was dramatically decreased when JNK and ERK inhibitors were added to the assays. Pertusiss toxin is a known uncoupler of G protein (Gi/Go) couple receptors and so the complete loss of migration strongly indicates the likelihood that signaling is through a G protein coupled receptor, such as CCR1 or CCR3. In addition, we have further insight into the CCL15 mechanism, since both JNK and ERK inhibitors significantly decreased migration as well. It is important to validate the potential CCL15-induced activation of the JNK and ERK pathways because they are a known problem involved in RA.

JNK is important for MMP expression because it phosphorylates c-JUN, a protein that is required for the expression of MMP (3). JNK activity is regulated by a particular MAP kinase kinase (MKK-7) and requires cytokine stimulation, specifically IL-1β (3). Once stimulated, a signal is sent to initiate gene expression of MMP leading to the degradation of tissue proteins. This leads to a variety of options as potential drug targets that might be effective in treating RA. A selective JNK inhibitor is one of these options to prevent the release of MMPs (3). An alternative approach would be to target kinases that are upstream and regulate JNK, namely the MAP kinase kinase MMK-7; a molecular switch that would permit activation of JNK under certain circumstances but would prevent its activity by cytokine-activated synoviocytes (3). Han et al showed that systemic administration of the same JNK inhibitor used in this study (SP600125) significantly decreased rat-paw swelling in rats with adjuvant-induced arthritis. Moreover, a histological assessment of inflammation within the synovium showed a significantly

beneficial effect in the animals treated with the JNK inhibitor. And arguably the most important finding of their study was that JNK inhibitor-treated animals showed, by radiographic analysis, a marked decrease in bone and cartilage damage (18).

ERK is significant in RA because it is the pathway by which the expression of synovilin (SYN), a protein that triggers hyper-proliferation of FLS is carried out (8). This pathway is also stimulated by cytokines, namely IL-1β and TNF-α, initiating the inflammatory cascade (7). A distinguishing feature of the ERK pathway is that it appears to be the only MAP kinase whose activation is involved in the transcription of SYN (8). This was discovered by noting that a specific inhibitor against ERK, but not JNK, prevented the expression of SYN (8). Moreover, stimulation of the ERK pathway leads to an increased release of SYN, which, as stated previously triggers the hyper-proliferation of FLS cells (8). Consequently, this increases the release of IL-1β and TNF-α, which induces more SYN transcription (8). This positive feedback loop has implications in RA development and could provide multiple targets for pharmacological intervention.

In previous studies, CCL15 has been evaluated by its chemoattractant properties in comparison with other known chemokines that facilitate lymphocyte chemotaxis, namely IL-8 for neutrophils, CCL5 for lymphocytes, and MIP-1α for monocytes (2). The chemotaxic strength was comparable to MIP-1α for monocytes and to IL-8 for neutrophils, but did not exhibit that same chemical attractiveness as CCL5 did for lymphocytes (2). Initial exposure of CCL15 to specific receptors desensitized both CCL5 and MIP-1α from lymphocytes and monocytes respectively (2). However, initial exposure of CCL5 and MIP-1α were not able to desensitize CCL15 from its receptor (2). This suggests that not only does CCL15 compete with CCL5 and MIP-1α for the same receptors, but CCL15 has a higher affinity for these receptors, CCR3 and

CCR1 respectively. An analysis was also conducted between CCL15 and IL-8. There was no desensitizing from either one by initial or subsequent exposure indicating these two cytokines bind to completely different receptors (2). However, even though CCL15 does not bind to the same receptor as IL-8, its chemoattractant properties for neutrophils were just as strong (2). This is noteworthy because this implies that CCL15 signals neutrophils through a possibly unidentified receptor and could be a potential area for future research.

An interesting question is what receptors does CCL15 utilize to mediate its physiological effect. It turns out CCL15 binds to CCR1 and CCR3, the latter being expressed on RA FLS (Figure 6). To our knowledge, this is the first evidence showing expression of CCR3 on RA FLS. In fact, Garcia-Vicuna et al showed RA FLS did not express CCR3, conflicting with our results (15). They found surface expression of a variety of cytokine receptors but their results did not show cell surface expression of CCR3 on un-stimulated FLS in 6 patients. Interestingly, they also show that a 24-hour stimulation of FLS with TNF-α, a cytokine known to cause the secretion of chemokines and potentially their corresponding cell surface receptors, did not cause expression CCR3 as well. Obviously these conflicting results show the need for further investigation on whether or not RA FLS express the CCR3 receptor on its cell surface.

CCL15 binds with a higher affinity to CCR3 but is generally considered a weak agonist for CCR1 (13). However, certain physiological processes exist that can increase CCL15's potency for CCR1 greater than a 1000-fold (13). When a foreign pathogen is detected by local tissue macrophages or mast cells, these cells recruit leukocytes which release high levels of proteases that have the ability to truncate the N-terminal domain of CCL15 if present in the area (13). A truncated version of CCL15 has a much increased potency for CCR1-mediated signaling. This implies that the N-terminus is a regulatory domain and when removed causes CCL15 to

have a significant increase in pro-inflammatory signaling through CCR1 (13). In addition, N-terminally truncated CCL15 was detected at relatively high levels in synovial fluid of RA patients. Synovial fluid consists of approximately eighty percent polymorphonuclear leukocytes (PMN), which Richter et al show that proteins released from PMN's at the site of inflammation proteolyticially cleave circulating CCL15 and facilitate the infiltration of monocytes into the site of inflammation (16). This suggests that the inflammatory response from leukocytes converts CCL15 to a truncated version, which acts as a significantly more potent chemoattractant during the inflammatory response causing enhanced tissue and joint deterioration (13). Moreover, experimental observations by immunohistological assay confirm an abundant expression of CCL15 in synovial tissue of patients with RA (14). This confirmation suggests that fibroblast migration to CCL15 is regulated by CCL15 expression in the synovium rather than at the level of CCR3 expression on the surface of FLS. Consistent with this notion, is that CCL15 is expressed at lower levels in normal synovium (14).

CCL15 has also been found to have angiogenic activity. Hwang et al showed that CCL15 induced neovascularization via a chick CAM assay, and, moreover, can also induce endothelial cell sprouting from rat aortic rings. This was mediated in the absence of inflammatory infiltrates, which indicates CCL15's direct effect in promoting angiogenesis (17). Another major contributor to the development of angiogenesis is matrix metalloproteinases (MMP's), which assist in degrading the proteins that keep the vessel walls intact. When hyper-proliferation of FLS in RA occurs, there is an abundant release of MMP's as a countermeasure to reduce the developing mass within the joint. However, the MMP's released also cause proteolysis of the vessel wall and allows the endothelial cells to escape into the interstitial matrix and induce angiogenesis leading to a greater degree of inflammation seen in RA.

In this study, we have identified CCL15 as a novel chemoattractant for RA FLS. Our results suggest that CCL15 regulates the migration and cytoskeletal structure of RA FLS, and that these actions are likely induced by a G protein (Gi/Go) coupled receptor. We also found that RA FLS express the CCR3 receptor, which, to our knowledge, are the first results suggesting this type of receptor expression on FLS. Finally, migration of RA FLS can be significantly decreased by inhibiting the ERK and JNK pathways. A more thorough understanding of the RA FLS/CCL15 relationship is needed because of the key role that these cells play in arthritis in conjunction with the high level of CCL15 that is expressed.

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Figure 1. CCL15 induces migration of RA FLS

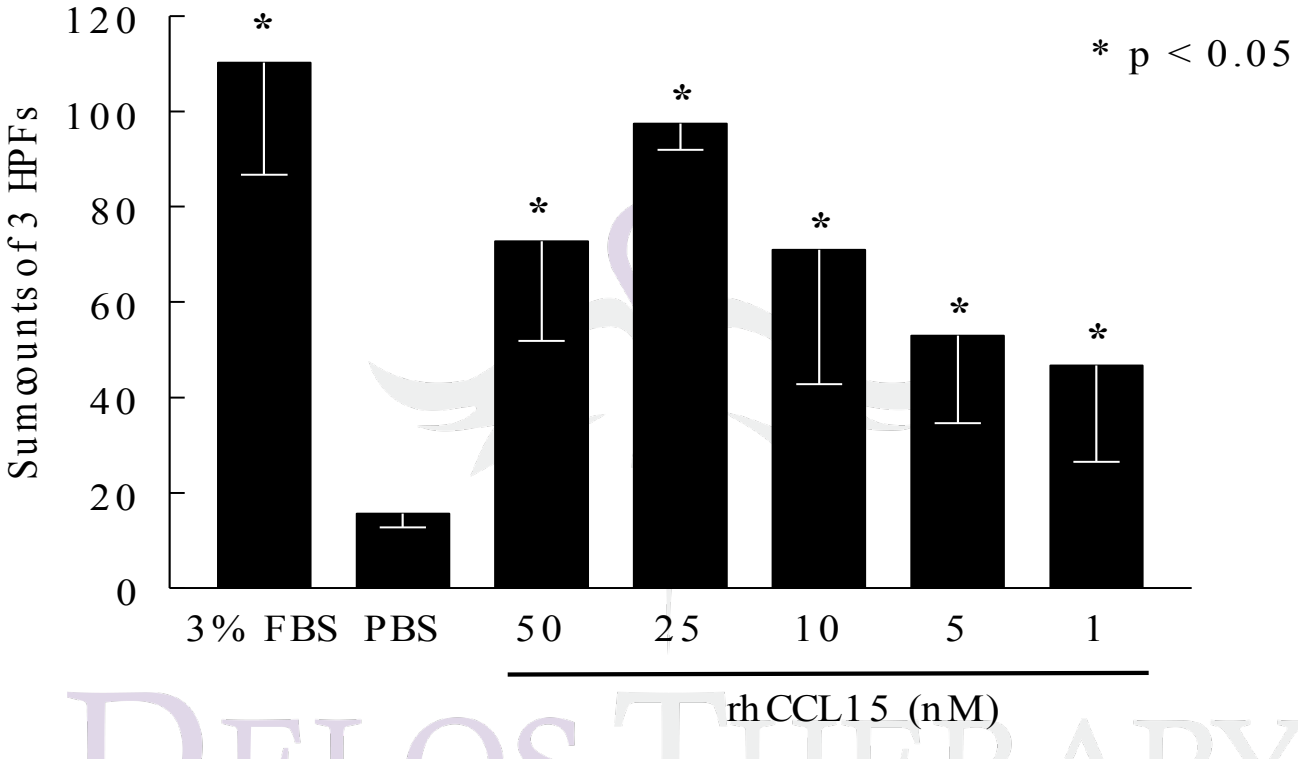


Fig 1. The positive control on the left indicates RA FLS migrating towards 3% fetal bovine serum (a fibroblast chemoattractant). Background migration is indicated by the negative control, phosphate buffered saline (PBS). Migration towards several concentrations of CCL15 are shown. This graph is representative of assays performed on RA FLS obtained from 4 different patients, where CCL15 induced significant migration. Bars and error bars represent the mean and the SEM of counts summed from 3 high powered fields (HPFs).

Figure 2. CCL15 stimulates RA FLS cytoskeletal F-actin rearrangement

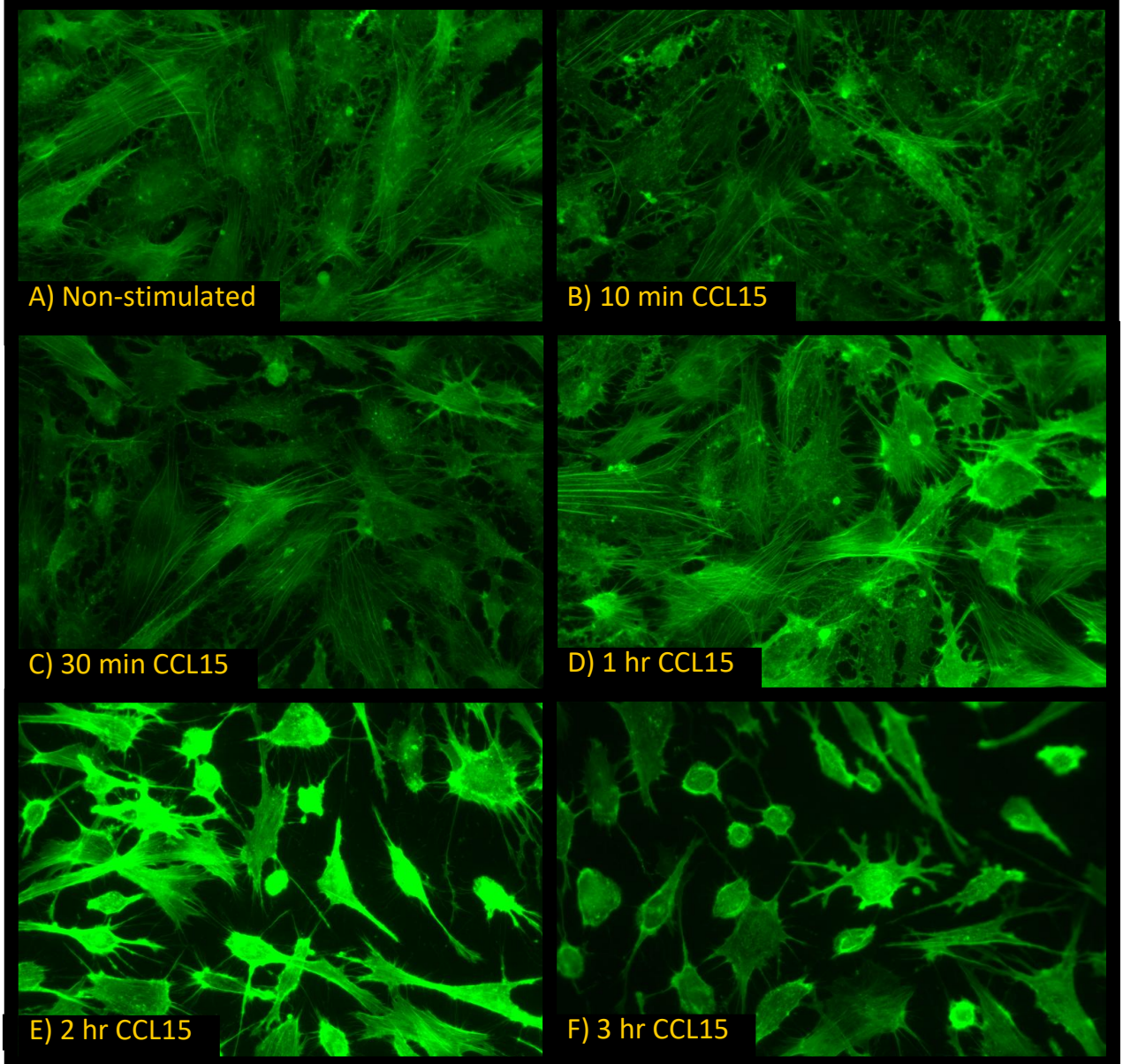


Figure 2. CCL15-stimulated and non-stimulated RA FLS were stained for F-actin and compared. Fig. 1A shows background staining of F-actin in non-stimulated RA FLS. Cells stimulated with 10 nM CCL15 and stained for F-actin after 10 min (B), 30 min (C), 1 hr (D), 2 hrs (E), and 3 hrs (F) are shown for comparison. (n = 3)

Fig 3. PTX inhibits RA FLS migration towards CCL15

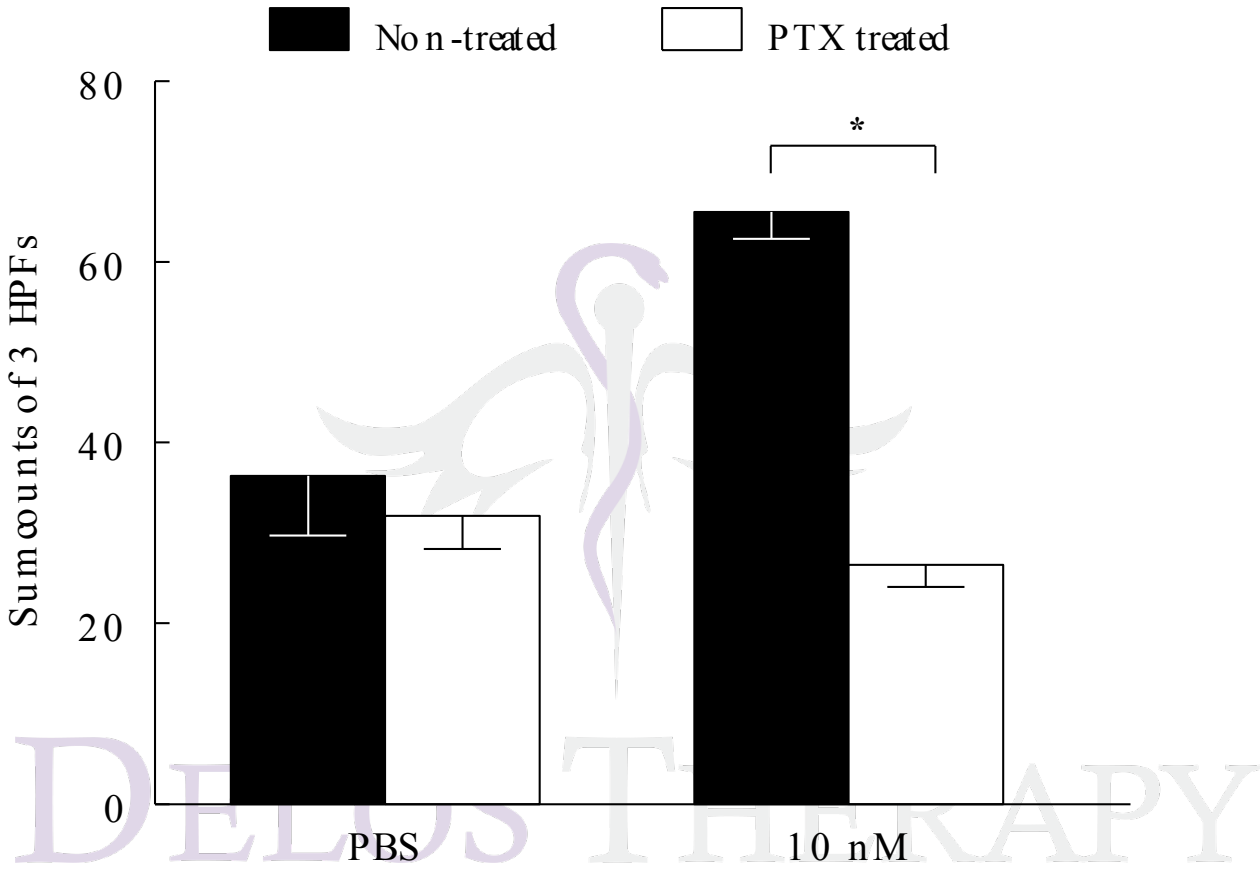
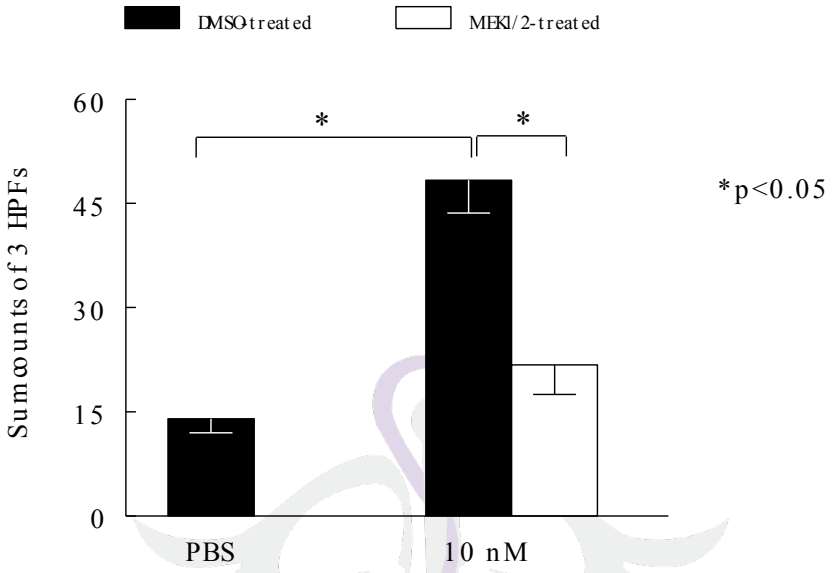


Fig 3. Migration to 10 nM CCL15 was compared with background migration, in the presence or absence of pertussis toxin (PTX). Results are representative of assays performed on RA FLS obtained from 3 different patients. Bars show the mean and SEM of counts summed from 3 high-power fields (HPF).

Figure 4. Inhibitors of JNK or MEK1/2 reduce CCL15-induced RA FLS migration

A



B.

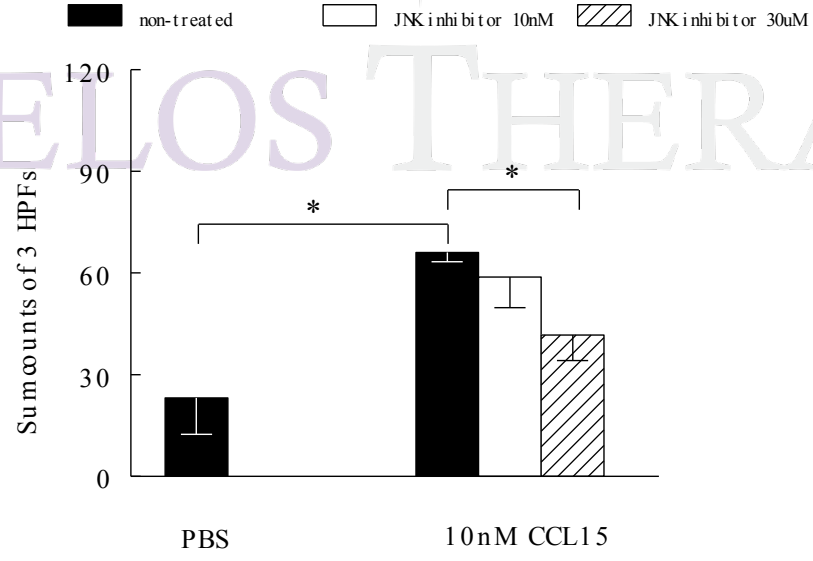


Fig 4. Migration to CCL15 was compared with background migration in the presence or absence of A) a MEK (and ERK 1/2) inhibitor (U0126) at 10 nM or B) a JNK inhibitor (SP600125) at 10 or 30 nM. MEK 1/2 significantly reduced chemotaxis with RA FLS from 2 out of 3 patients. Similarly, 30 nM JNK inhibitor also significantly reduced RA FLS migration from 2 of 3 patients. Bars show the mean and SEM of counts summed from 3 high-power fields (HPF).

Fig 5. CCL15 does not induce RA FLS proliferation

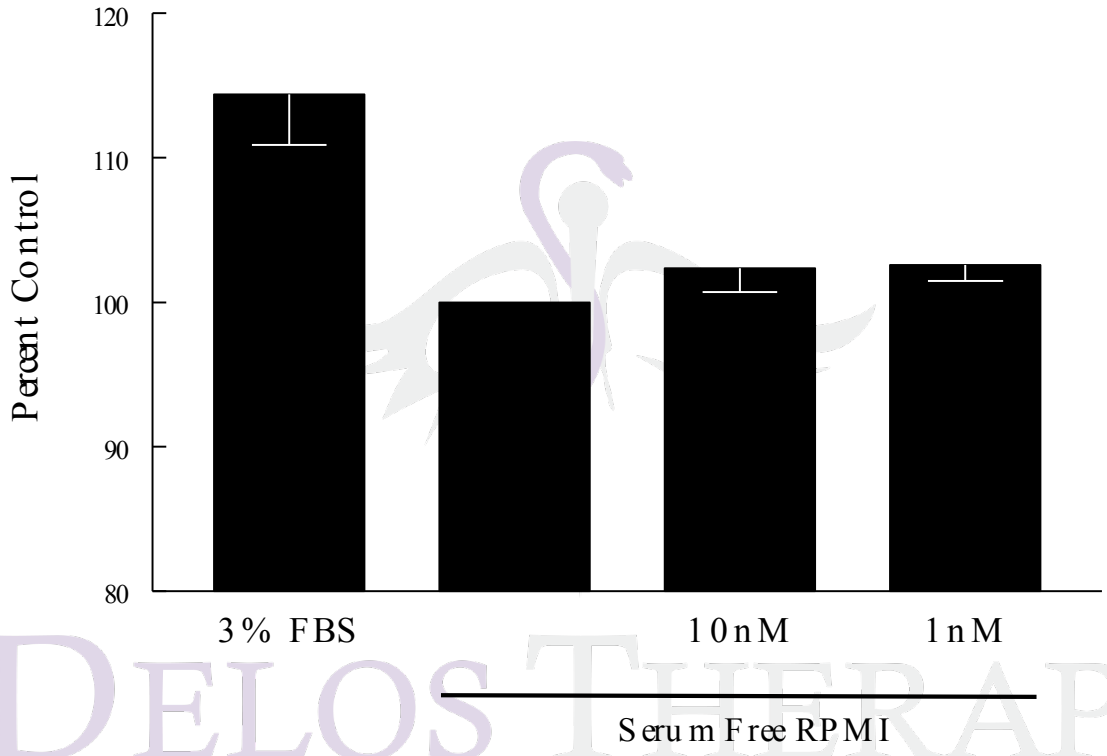


Fig 5.Cells plated in a 96-well plate were incubated with 3% FBS (positive control) or CCL15 at 1 and 10 nM for 72 hrs in serum free RPMI. At the end of the incubation 20 µl of MTS/PMS solution was added to each well and the absorbance was read at 490 nm wavelength to check for viable cells. The graph represents combined data from three different patients, where bars and error bars represent the mean +/- the SEM. (n = 3)

Fig 6. RA FLS express the CCL15 receptor CCR3 but not CCR1

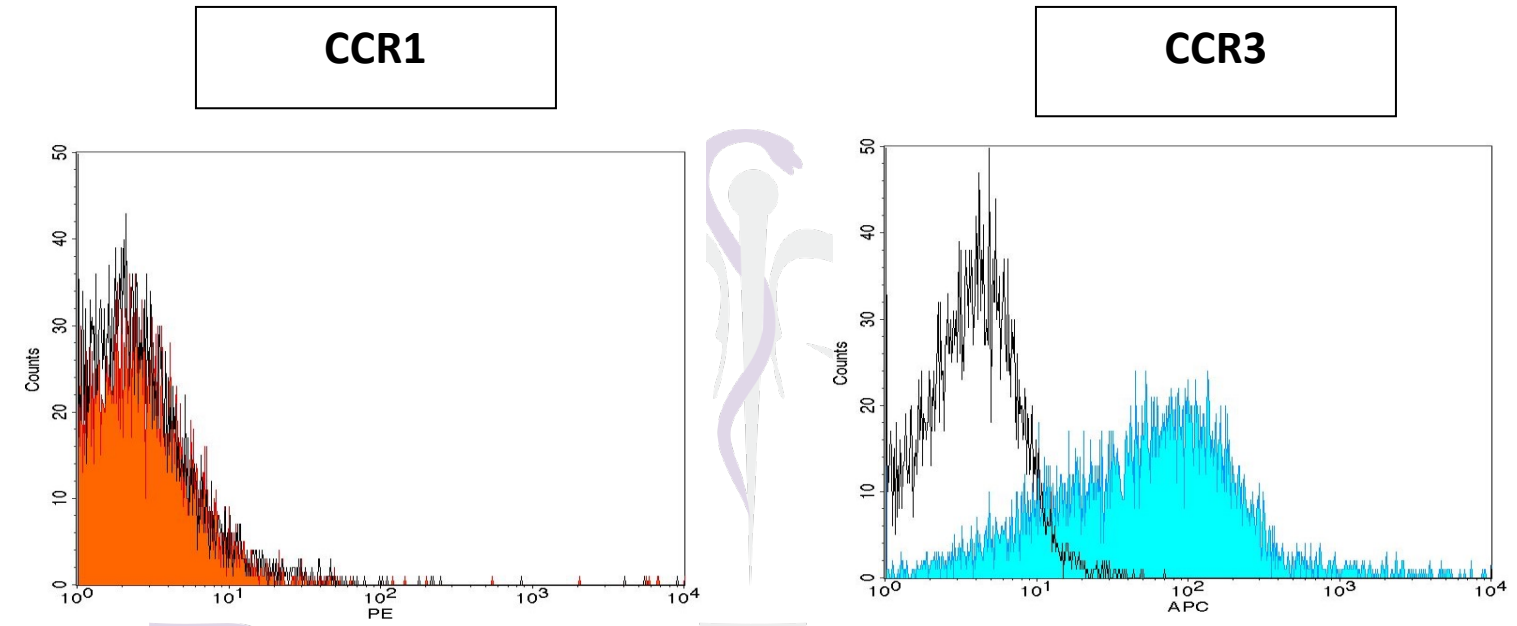
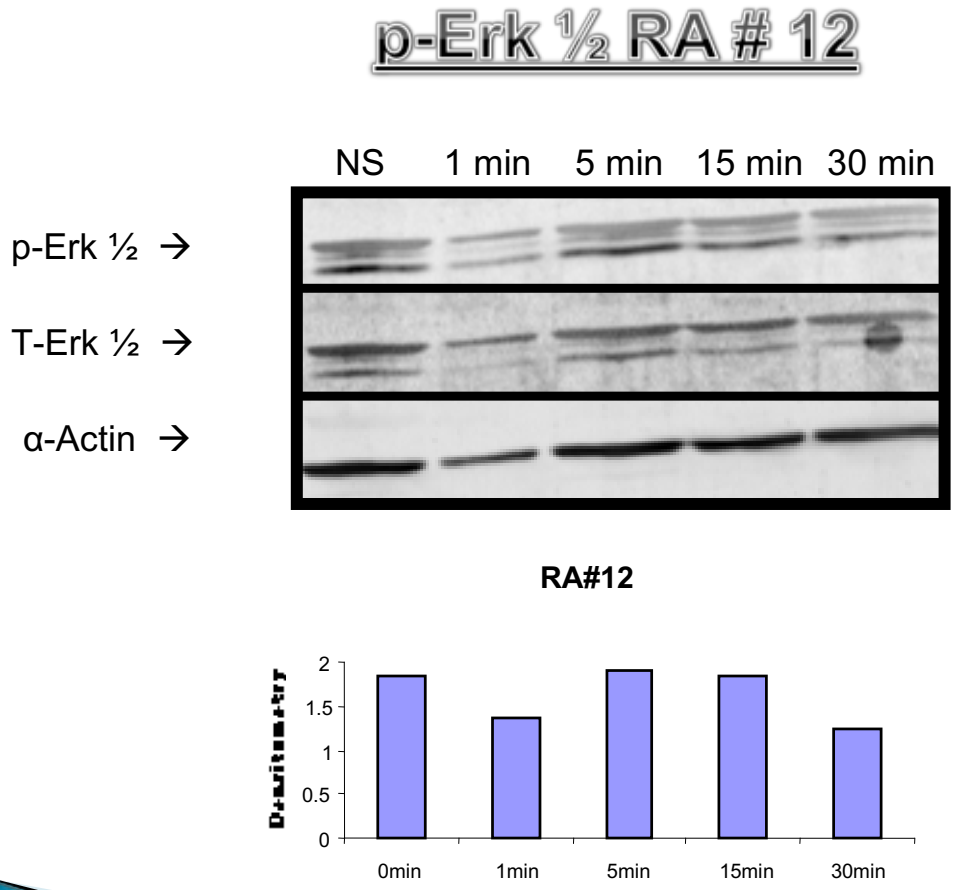


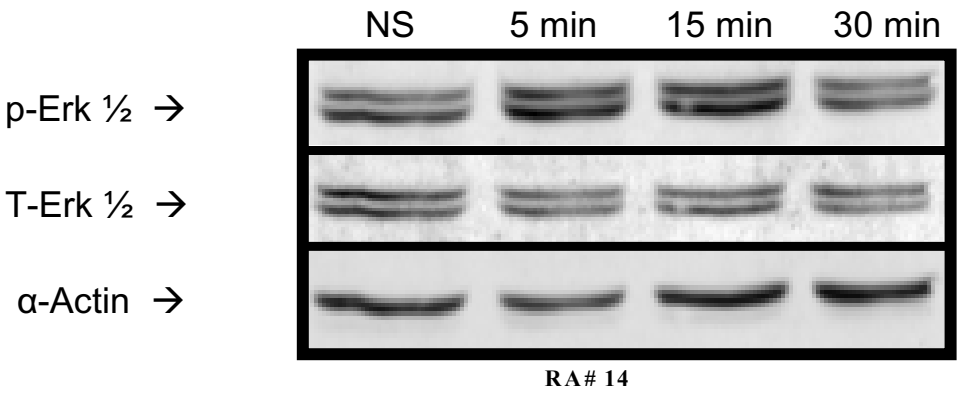
Fig 6. RA FLS indirectly stained for CCR1 (figure A) and CCR3 (figure B). Cells were analyzed via flow cytometry with 10,000 gated events collected. Colored region denotes positive staining for receptor, whereas black line denotes control sample with mouse or rat IgG. On average, 73% of RA FLS express CCR3, whereas <5% of these cells express CCR1. (n = 3)

APPENDIX

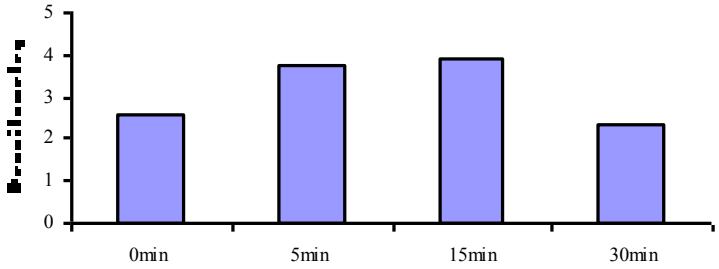
Western Blotting



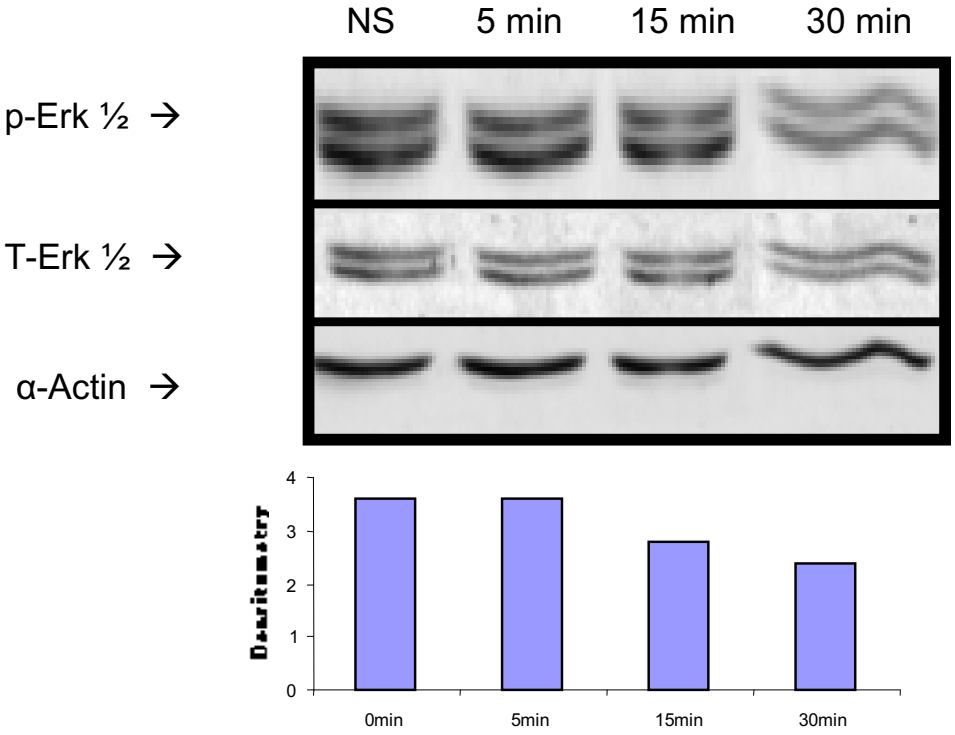
p-Erk ½ RA # 14



RA# 14



p-Erk ½ RA # 16



Summary of Western Blotting

- 2 Lysate Conditions:
  - 1 hour serum free incubation
  - 24 hour serum free incubation
- 5 months were spent running and probing Western blots in an attempt to address this specific aim
- Assessing JNK phosphorylation was problematic, since a single band was obtained at ~38 kD, rather than the expected doublet at 46 kD and 54 kD
- Novel 37 kD stress-activated kinase?
  (De Silva et al., Biochemical and Biophysical Research Comunications 250:647-652, 1998)
- In addition, the band was strongest when NS samples were studied

Summary of Western Blotting Cont…

- Assessing ERK phosphorylation was a challenge, since the non-stimulated lysate often gave the strongest phosphorylation
- Assessing JNK phosphorylation was problematic, since a single band was obtained at ~38 kD, rather than the expected doublet at 46 kD and 54 kD
- The take home message is that this is still a work in progress, but as you will see JNK and ERK inhibitors decrease CCL15-induced migration, strongly suggesting their involvement in signaling.