Expression of CD13/Aminopeptidase N by Synovial Fibroblasts: Novel Roles in the Pathogenesis of Rheumatoid Arthritis

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Immunology)
in the University of Michigan
2014

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Dedication

I would like to dedicate this to my mom.

Acknowledgements

I have had the wonderful support of so many people throughout my thesis work. First, thank you David for all of your help and support. You have been the best mentor I could ask for. You are always so patient, kind, and knowledgeable. I also have to thank all the members of the Fox lab past and present. Over the years you have all helped make my experiments and my life better. I would especially like to thank Judy; whether I needed help learning a new technique or I was just having a bad day, you always had the answer.

I also have to thank my thesis committee members and numerous other collaborators at Michigan for giving me their time, knowledge, and reagents. It has been wonderful learning from and working with such generous scientists. Of course I also have to thank Zarinah, Beth, and Keith for making the Immunology Program such a great and supportive learning environment.

Personally I'd like to thank my friends and family for supporting me through the years. Also, my doctors, especially Robert Ike, without whom I don't think I would have been able to finish this work. Finally, a big thank you to all the tissue and blood donors. You are the ones who make work like this possible.

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Abstract

Aminopeptidase N/CD13 is a metallopeptidase that is highly expressed by fibroblast-like synoviocytes (FLS) and may play a role in rheumatoid arthritis (RA). The goal of this study was to define CD13 function in the RA joint by monitoring CD13 expression *in vivo* and *in vitro*, analyzing regulation by pro-inflammatory cytokines of CD13 expression on FLS, describing the mechanisms by which CD13 is released from FLS, and defining potential roles for CD13 in homing of T cells to the RA joint and in regulating the growth and migration of RA FLS. In overview, we find that CD13 is expressed in synovial fluids, sera, FLS lysates, and culture supernatants as measured by ELISA, with a significant increase of CD13 in RA synovial fluids when compared to osteoarthritis. In FLS, pro-inflammatory cytokines (TNFα, IFNγ, IL-17) are able to upregulate CD13 mRNA. However, as surface expression of CD13 did not correlate with CD13 mRNA, and soluble CD13 is present in RA synovial fluid, potential mechanisms for CD13 release from FLS were examined. CD13 was detected in sera, synovial fluids, and FLS culture supernatants, on extracellular vesicles and in soluble form. The release of soluble CD13 from FLS could be blocked by metalloproteinase inhibitors and siRNA directed against MT1-MMP/MMP14. With regard to biological functions, recombinant human CD13 was chemotactic for cytokine activated T-cells (Tck) through a G-protein-coupled receptor and contributed to the chemotactic properties of synovial fluid independently of enzymatic activity. Furthermore, inhibition of CD13 function in FLS, using enzymatic activity inhibitors or anti-CD13 antibodies, resulted in decreased growth and diminished migration of RAFLS. We conclude that CD13 is

upregulated in the RA joint by pro-inflammatory cytokines where it is released on extracellular vesicles and shed as a soluble molecule from the FLS surface by metalloproteinases, including MMP14. Following its release from the FLS surface, CD13 induces chemotaxis of Tck, a T cell population similar to that found in RA synovium. Moreover, CD13 increases FLS growth and migration, thereby contributing to synovial hyperplasia in RA. Our data implicates CD13 in the pathogenesis of RA through enhancement of both T cell infiltration and aggressive FLS outgrowth.

Chapter 1

Introduction

Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is an inflammatory autoimmune disease that primarily targets the synovial tissue (the soft tissue lining of the joint), leading to destruction of the bone and cartilage of multiple affected joints [1]. RA can cause inflammation in other tissues as well, including the lungs and heart [2,3]. RA affects approximately 0.6% of the world's adult population and can lead to significant disability for those affected [1]. Important progress has been made in understanding and treating RA; however, the etiology of RA remains unknown. Theories about the causes of RA involve genetic loci that affect susceptibility to RA (notably MHC and *PTPN22*), environmental factors (such as cigarette smoke), triggering infections (periodontal disease), autoantibodies (rheumatoid factor and anti-citrullinated peptides), and T cell autoreactivity[4–6]. The pathogenesis of RA is a complex process involving many cell types, including cells of the joint (synoviocytes and osteoclasts) and immune cells (T cells, B cells, dendritic cells, and monocytes). This study will focus primarily on interactions between T cells and fibroblast-like synoviocytes (FLS).

T cells

T lymphocytes are key mediators of adaptive immunity, and it is well established that T cells play an important role in RA[7]. They have been identified as crucial in many mechanisms of RA pathology including inflammatory cytokine production, osteoclast differentiation (formation of bone resorbing cells), and stimulation of synovial cells and antigen-presenting cells (APCs) to proliferate and express increased levels of cytokines and chemokines [8–11]. A major question that remains unanswered is how autoreactive T cells in RA become activated. One proposed mechanism involves human leukocyte antigen-DR (HLA-DR), specifically HLA-DR alleles that contain an amino acid sequence that has been termed the shared epitope[12,13]. HLA-DR is the most significant genetic locus that affects susceptibility to RA, with different alleles acting to either confer RA susceptibility or protection. Human leukocyte antigen (HLA) is the genetic locus that encodes the major histocompatibility complex proteins (MHC). MHC proteins present peptides to T cells. The T cells express a T cell receptor protein dimer (TCR) that recognizes MHC and discriminates as to whether the peptide being presented is self or foreign. This TCR/MHC recognition event and subsequent downstream signaling is the primary mechanism by which pathogens are recognized by T cells leading to T cell activation. HLA-DR is an MHC class II molecule. MHC class II molecules are typically found on antigen presenting cells and present peptides from extracellular proteins. The other class of MHC is MHC class I molecules which are present on most cells in the body and express peptides from cytosolic proteins. The shared epitope is a concept that proposes a common amino acid sequence in the peptide-binding cleft of the HLA-DR RA susceptibility alleles. It is unknown how the HLA-DR alleles that contain the shared epitope predispose individuals to RA, but one possibility is that arthritogenic autoantigens, especially modified proteins (such as citrulinated peptides), are presented and

recognized as foreign[8,14,15]. Other lines of evidence support novel mechanisms by which MHC alleles influence autoimmunity in ways unrelated to antigen recognition[8,16,17].

There is also data to suggest that T cells of the joint may become activated in ways other than through the canonical TCR/MHC pathway. Fractalkine, a cytokine strongly expressed in the RA synovium, directly induces activation of senescent RA CD4+ T cells [8,18]. Cytokine activated T cells (Tck) have been shown to closely resemble the T cells found in the RA joint and can potentiate a proinflammatory environment [19]. Tck are an *in vitro* cell population created by treatment of peripheral blood T cells with IL-2, TNFα, and IL-6. Tck are activated without any classical stimulation through the TCR. While this is an *in vitro* cell type, their phenotypic similarity to RA synovial T cells indicates a high probability that a similar pathway may exist *in vivo* [19]. Tck are useful for experiments to mimic RA synovial T cells. It is not possible to isolate the numbers of synovial T cells necessary for many experiments. However, Tck make it possible to estimate functions of RA synovial T cells. It is likely that the extent to which RA T cell activation occurs through classical or nonclassical pathways is influenced by the surrounding inflammatory environment and joint-specific cells.

T cells can be divided into two main subsets: cytotoxic T cells and helper T cells. Cytotoxic T cells (CD8+) recognize and destroy virally infected or tumor cells[20]. Helper T cells (CD4+) assist and regulate other immune cells (cytotoxic T cells, B cells, macrophages) in immunologic processes both through direct cell-cell contact and by secretion of cytokines that govern the immune response[20]. At one time, helper T cells were considered to be comprised of two subsets,

Th1 or Th2, each with distinct patterns of cytokine secretion[21]. The Th1 subset is marked by production of interferon-γ (IFNγ) and aids in defense against intracellular bacteria while, Th2 cells produce predominately interleukin-4 (IL-4) and aid in defense against parasites[20]. RA and other forms of autoimmunity were thought to be mediated primarily by Th1 cells and IFNγ[22]. However, the concept that Th1 cells were key to autoimmunity was later shown to be flawed as anti-IFNγ antibodies exacerbated mouse models of autoimmunity, including collagen-induced arthritis (CIA) a model of RA [23,24].

A primary reason that RA was thought to be a Th1-mediated disease was that Th2 cytokines, such as IL-4, suppress animal models of arthritis [25]. This suppression was thought to act through IL-4 down regulation of IFNγ. However, more recent evidence indicates that IL-4 instead acts by suppressing production of IL-17, a cytokine produced primarily by a third subset of helper T cells known as Th17 cells that were discovered in 2005 [26,27]. Murine dendritic cells (DCs) modified to express IL-4 were shown to suppress CIA in mice, not through an effect on IFNγ, but rather by suppressing production of IL-17[28]. Another cytokine that is part of the Th1/IFNγ pathway is IL-12. IL-12 is a crucial facilitator of Th1 differentiation and is composed of the two subunits IL-12p35 and p40. Anti-IL-12 antibodies suppressed mouse models of arthritis; however, later studies showed that the observed improvement occurred as a consequence of the fact that the treatments targeted the IL-12p40 subunit that is shared with IL-23 (a cytokine known to support Th17 expansion)[23]. Hence, the anti-inflammatory effects attributed to and effect of IL-12 on Th1 differentiation was actually mediated through a suppression of the IL-23-driven up-regulation of Th17 cells. Moreover, IL-17 is essential for multiple mouse models of

RA[8,23,29,30]. For example, CIA was significantly suppressed in IL-17 knockout mice[30]. These data suggests that Th17 cells and IL-17, rather than Th1 cells and IFNγ, play a key role in CIA.

Refinement of our understanding of the roles of T cell subsets in RA animal models has redirected the focus of inquiry toward the Th17/IL-17 pathway in human RA. IL-17 is up-regulated in human RA serum and synovial fluid samples[31–33]. Th17 cells are more numerous in the peripheral blood of early RA patients versus osteoarthritis (OA) or healthy controls and the numbers were even higher in the synovium of RA patients than in the blood. In addition, decreased numbers of Th17 cells were found in patients who respond to treatment versus nonresponders[34].

The mechanism of action for Th17/IL-17 in RA is complex, and multiple relevant functions have been identified that may potentiate RA. IL-17 can stimulate production of other cytokines that are pathogenic in RA, such as tumor necrosis factor α (TNFα) by APCs and IL-6 by FLS[23,35]. TNFα is a cytokine that has been strongly implicated in the pathogenesis of RA, and is significantly elevated in RA synovial fluids[36]. Indeed, the positive response observed in RA patients treated with anti-TNFα therapeutics convincingly supports a role for TNFα in the disease process[37,38]. IL-6, along with transforming growth factor-β (TGFβ), is critical for Th17 differentiation and other pro-inflammatory processes, such as B cell proliferation and antibody production[39].

IL-17 itself can increase joint destruction[32]. It acts directly on chondrocytes (cartilage cells) and osteoblasts (bone producers) to up-regulate RANK ligand which promotes osteoclastogenesis (formation of bone destroying cells) leading to joint destruction [32]. In RA synovial explants the most effective strategy to inhibit inflammation and the capacity for tissue destruction was combination blockade of TNFα, IL-1, and IL-17 [40]. However, even when TNFα or IL-1 are inhibited, IL-17 can act independently in mechanisms relevant to RA pathology [8,41]. This makes IL-17 an attractive therapeutic target in RA, especially for patients in whom the TNFα blockers are ineffective. The newer discoveries concerning Th17 cells also underline the need for a better understanding of the mechanisms underlying joint inflammation, including interactions of T cells with synovial cells such as FLS.

Fibroblast-Like Synoviocytes

RA synovium is primarily comprised of monocyte/macrophage-like synoviocytes (type A) and fibroblast-like synoviocytes (type B)[42]. Fibroblast-like synoviocytes (FLS) are a structural component of the synovium and play several key pathogenic roles in RA[43]. In RA FLS undergo extensive hyperplasia, thereby expanding the synovial tissue. This proliferation of FLS, combined with increased monocyte/macrophage and lymphocyte ingress and angiogenesis, leads to the formation of pannus tissue[44]. This pannus migrates within the joint over cartilage and bone where it actively participates in tissue destruction [44]. An essential part of pannus development is the formation of three-dimensional sheet-like aggregates of FLS. These FLS sheet-like formations are also important to normal joint architecture [44]. Cadherin-11 is one molecule that is crucial to the formation of these sheet-like structures [44,45]. Cadherin-11 plays an

important role in FLS adhesion in both normal synovial lining and RA synovium [44,46,47]. However, Cadherin-11 has also been shown to mediate inflammatory and pathogenic properties of FLS in RA. Cadherin-11 null mice developed a disorganized synovium, and are resistant to development of inflammatory arthritis with decreases in FLS migration, FLS invasion, and destruction of cartilage[47]. Anti-Cadherin-11 also showed therapeutic benefit in a serum-induced inflammatory arthritis mouse model (K/BxN model) [47]. These data support an important role for cadherin-11 in joint destruction in RA. Basic fibroblast growth factor (bFGF) is another molecule linked to FLS proliferation and RA pathogenesis. bFGF increases telomerase activity and decreases Fas-mediated apoptosis in RA FLS leading to aberrant persistence and overgrowth of FLS[48]. A variety of molecules, such as cadherin-11 and bFGF, along with metalloproteinases, adhesion molecules, and growth factors have been linked to FLS growth and migration[49–51]. While our knowledge of FLS is expanding, the understanding of how FLS interact with each other, proliferate to form the pannus, and migrate through the joint, instigating cartilage and bone destruction, is still incomplete. Another question to be asked is: what other molecules can affect FLS growth and migration through either paracrine or autocrine mechanisms?

Initial theories of FLS involvement in RA purported that FLS pannus formation and FLS pathogenic activity were primarily initiated by the surrounding inflammatory milieu from immune cells; however, it has become apparent that FLS play a more active role in inflammation. RA FLS maintain a pro-inflammatory, invasive, and aggressive growth phenotype *in vitro* indicating a more permanent pathogenic program of gene expression in the RA FLS[39,43,52–54]. FLS are even capable of spreading arthritis. In an immunodeficient (SCID)

mouse model, implanted RA FLS migrated from one piece of implanted cartilage to a distant cartilage implant and initiated destruction [55].

A major question that remains is how do the FLS become activated and develop this pathogenic phenotype? One potential mechanism for FLS activation is through pattern recognition receptors (PRRs), such as Toll like receptors (TLRs). FLS express TLRs which can respond to pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) resulting in initiation of an inflammatory process. TLRs are proteins that function as part of the innate immune system, and signaling through TLRs results in activation of cells and production of proinflammatory mediators. Rather than recognizing specific peptide sequences presented by MHC, as the TCR does, TLRs recognize molecular patterns such as double-stranded RNA (viruses) or bacterial components such as lipopolysaccharide (LPS). PAMPs are molecular patterns that are found on pathogens, such as viruses, fungi, or bacteria, while DAMPs are host-derived molecular patterns that occur with cell damage or death. DAMPs often involve the presence of self-molecules where they are not normally located, such as nuclear proteins or DNA outside the nucleus. FLS express the TLRs 2, 3, 4, 5, and 9 [56–58]. Activation of TLRs on the FLS can result in production of proinflammatory cytokines, chemokines, and angiogenic factors. For example, one study showed activation of FLS leading to production of IL-6, IL-8, matrix metalloproteinase-1 (MMP1), and MMP3 in the presence of TLR2,3,4, and 5 ligands [57]. Another study showed that stimulation of FLS with TLR2 or TLR4 ligands followed by co-culture with monocytes induced high levels of receptor activator of nuclear factor κ B (RANK), MMP9, and other factors that lead to osteoclast differentiation[59]. It is uncertain however whether similar activation occurs naturally in RA. While bacteria have been found in some arthritic joints,

DAMPs are a more likely source of TLR activation in the synovium as cellular damage would be found much more frequently. One study proposed necrotic cells in the joint as a possible source of TLR ligands. This study showed that stimulation of TLR3 on FLS by RNA from necrotic cells induced FLS to produce IFNβ, CXCL10, CCL5, and IL-6 [56]. In addition to independent functions for FLS in RA, FLS also interact with other cells in the synovium, both through secreted factors and direct cell-to-cell contact. Co-culture of monocytes with RA synoviocytes resulted in a synergistic increase of IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and leukemia inhibitory factor (LIF). IL-6 production in the co-cultures was further enhanced by stimulation with IL-1β, GM-CSF, IFNγ, or TNFα[60]. The enhancement with T cell-derived cytokines indicates that T cells also likely impact monocyte-synoviocyte interactions in the RA joint. Overall, FLS are central to multiple mechanisms in RA pathology acting both as a distinct pathogenic cell type and in co-operation with other inflammatory cell populations.

FLS/T cell interactions

A central paradigm of immunology involves the interaction between T cells and antigen presenting cells (APCs) resulting in T cell activation and an immune response. While professional APCs (such as monocytes and dendritic cells) are present in the RA joint and undoubtedly play a role in pathogenesis, there is also increasing interest focused on interactions between T cells and cells that are not considered classic immune APCs. FLS are a tissue-specific cell type that can interact with T cells in multiple ways. FLS can act as APCs, expressing MHC and presenting epitopes to the T cell receptor [8,45]. While FLS do not express

the canonical CD80/CD86 (B7-1/B7-2) co-stimulation molecules, they do express many surface structures that can engage corresponding T cell receptors/ligands and induce T cell activation *in vitro*[8,10]. B7-H3, a molecule similar to CD80 and CD86, has been found to be highly expressed on FLS and to localize to the point of T cell-FLS interaction[10]. However, the T cell ligand for B7-H3 remains undefined. CD6 expressed on T cells is another potential co-stimulatory molecule and can bind to CD166/ALCAM and at least one other ligand on FLS[61,62]. Cytokines also play a key role in FLS/T cell interactions. RA FLS also produce IL-15 which up-regulates IL-17 and TNFα production by T cells [63].

T cells can also activate FLS leading to FLS proliferation [45]. Even resting T cells have been shown capable of activating FLS, leading to secretion of IL-6, IL-8, and PGE_2 with further increases in activation seen with addition of IL-17 [64]. FLS activation can often function in a pro-inflammatory cycle through up-regulation of chemokines from the FLS. For example, co-culture of RA FLS and CD4+ T cells resulted in an increase in SDF-1/CXCL12 (a T cell chemokine) production by the FLS. This effect was inhibited by either anti-IL-17 or anti-CD40L with an additive effect when both were used, indicating both a cell contact and cytokine dependent activation [65]. This production of a T cell chemokine results in more T cells being brought to the joint. This leads to further increases in inflammation, stimulation of the FLS by T cells, and increased production of chemokines, reinforcing the cycle. While the initiating step in RA synovial inflammation is unknown, it is clear that FLS and T cell interactions in the joint support a pro-inflammatory cycle leading to RA pathology.

Aminopeptidase N/CD13

CD13 was first defined in 1984 as a marker for myeloid cells and leukemia cell lines in the report on the First International Workshop on Human Leucocyte Differentiation Antigens[66]. Human Aminopeptidase N was first cloned and sequenced in 1988 from intestinal microvilli[67]. In 1989, CD13 was identified as being identical to Aminopeptiase N by Look et al[68]. Aminopeptidase N/CD13 (EC 3.4.11.2) is a metallopeptidase of the M1 family and its enzymatic activity is dependent on zinc binding through a HELAH motif. CD13 is a 130-150 kDa glycoprotein with multiple N and O glycosylation sites. The 130kDa form is believed to be an intracellular precursor that is further modified to its final 150kDa state on the cell surface [68]. Sequential immunodepletion suggests that within a population of CD13 molecules there is variability in the glycosylation states. Different CD13 antibodies were able to recognize distinct isoforms of the metalloenzyme, but following deglycosylation multiple antibodies recognized all of the detectable CD13 [69]. Since CD13 contains multiple glycosylation sites, with 25-30% of the molecular weight contributed by sugar molecules, this variability likely explains the different isoforms of CD13; however, there is no known difference in function for the different glycosylation states. Structurally, CD13 is a type II transmembrane protein that exists primarily as a homodimer. It has a short cytoplasmic tail (9 amino acids) and a helical membrane spanning region[68,70,71]. CD13 is ubiquitously expressed and can be found on the cell membranes of many tissues and cell types, including cells relevant to RA, such as monocytes, fibroblasts, and endothelial cells.

CD13 is an exopeptidase that hydrolyzes the N-terminal amino acid of a substrate preferentially after a neutral amino acid (excluding bonds preceding proline). The mechanism of cleavage suggested for CD13 is similar to that of thermolysin and other metallopeptidases[72]. The two regions most crucial to enzymatic activity are the highly conserved HELAH and GAMEN domains; these two motifs are also commonly found in other metalloproteinases/peptidases. The glutamic acid (E, Glu) in the GAMEN domain is critical for enzymatic activity, and along with the Glu in the HELAH domain and Zn+2 are believed to form a binding cleft for the substrate [72]. The X-ray crystal structure of CD13 bound to one of its substrates, Angiotensin IV, confirmed that the Glu from the GAMEN domain along with a Glu just upstream of the HELAH domain participates in substrate binding, while the histidines (H, His) of the HELAH domain are involved in the binding of Zn+2 to the native conformation of CD13 [73]. Antibodies that inhibit enzymatic activity bind to this area of Zn+2 binding, that is vital to enzymatic activity of CD13[74]. CD13 contains six extracellular cysteines which are also important for CD13 function. Mutation of any of the six cysteines resulted in very low enzymatic activity and retention of the protein in the endoplasmic reticulum, and alteration of the thiol status by protein disulfide isomerase resulted in decreased aminopeptidase activity and altered antibody binding[75]. This suggests that disulfide bonds are critical for the correct folding of CD13. X-ray crystallography has also confirmed that CD13 exists primarily as a homodimer, with the two CD13 molecules surrounding an internal cavity forming the catalytic site [73]. This catalytic cleft, with its key regions HELAH and GAMEN, is a possible target for inhibition of CD13 enzymatic activity.

CD13 expression is known to be regulated through multiple pathways. First, CD13 has two promoter regions. One promoter region controls transcription in epithelial/endothelial cells and a

second promoter controls transcription in myeloid and fibroblast cells [76,77]. While the two promoters result in different length RNA sequences, the resulting protein is the same. Second, CD13 is regulated by a variety of cytokines and other factors in various cell types. One of the first identified roles for CD13 was as a myeloid maker. CD13 was later shown to be upregulated with myeloid activation and differentiation; although, it is also present on resting myeloid cells[78]. It is upregulated on monocytes in response to IFNγ or LPS, in parallel to MHC class II upregulation and increases as monocytes transition to macrophages [78]. TGF-β also increased CD13 on monocytes and monocytic cell lines [79]. Other studies showed that fetal bovine serum (FBS), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) could induce augmented CD13 expression by an endothelial cell line, while dermal fibroblasts increased CD13 expression in response to IL-4 and IFNγ [80,81]. Gingival fibroblasts upregulated CD13 in response to IL-4 as well as IL-13, but CD13 expression was unaffected by IL-2 and IL-15 [82]. In thyroid carcinoma cells CD13 was up-regulated by epidermal growth factor (EGF), IL-6, TNFα, and bFGF [83]. However, differences in regulation between different cell types have been observed. Dermal fibroblasts did not increase surface expression of CD13 with either TNFα or TGFβ [84]. This is in contrast to myeloid cells in which TGFβ up-regulated CD13 and thyroid carcinoma cells in which TNFα up-regulated CD13. This may indicate a difference in regulation on different cell types, a concept which is supported by the presence of two different promoters.

A third possible source of regulation involves cellular localization of CD13. CD13 trafficking to the cell surface and its cellular recycling back into the cell has been proposed as another mechanism of regulation [85]. On FLS and monocytes, CD13 is found on the surface in caveolae

lipid rafts [86,87]. Caveolae lipid rafts are cholesterol rich domains that contain caveolins. The rafts can aid in keeping proteins proximate to each other, and the inclusion of caveolins can lead to cellular internalization of the rafts and associated proteins. However, CD13 localization is not limited to the cell surface. CD13 is also known to exist as in a soluble form (sCD13). sCD13 has been identified by Western blot and by assay of its enzymatic activity in human serum and tumor effusions[88,89]. CD13 identified in human serum is shorter than surface CD13. Two isoforms have been identified in normal serum. The major isoform lacks the amino acids 1-58, and the minor isoform lacks amino acids 1-43 [90,91]. Both isoforms notably lack the intracellular region, amino acids 1-9, and the transmembrane domain, amino acids 9-32[90]. The release of CD13 by cells presents another possible site of CD13 regulation. A study examining the role of IFNγ regulation of CD13 on the monocytic cell line HL-60 suggests that cytokines may have a more complex role in CD13 regulation than initially thought. Surface expression was initially down regulated at 24 and 48 hours and later up-regulated at 72 and 96 hours. A peak mRNA expression was observed at 72hours, and the up-regulation could be blocked by the inhibition of de novo protein synthesis [92]. However, as this study only examined surface CD13 and mRNA for CD13 it doesn't fully explain this observation. One possible explanation is that IFNγ is initially promoting release of CD13 from the cell surface with later replenishment and upregulation of CD13 on the surface by protein synthesis. Multiple questions remain about soluble CD13, including how sCD13 is released from cells and how this process is regulated.

CD13 Functions

CD13 is known to have a wide range of functions. CD13 has many known biological substrates including enkephalins (opioid receptor ligands), angiotensins (hormones that cause vasoconstriction), neurokinins (neurotransmitters), chemokines, and cytokines[70,93–96]. CD13 is ubiquitously expressed in the body on immune cells, skin, intestinal epithelium, brain, joints, and many other tissue and cell types. CD13's biological functions vary depending on where it is expressed. For example, CD13 is involved in antigen processing by trimming peptides for presentation on MHC class I and II [70,76]. In the central and peripheral nervous system, CD13, along with neutral endopeptidase/CD10, can degrade enkephalins, thereby lowering the analgesic effects of these molecules. Therefore, inhibition of CD13 has been targeted in controlling pain[97]. It has been suggested that CD13 can play a role in both cholesterol crystallization and cholesterol uptake [98,99]. CD13 is also a known receptor for NGR peptides (Asparagine-Glycine-Arginine) and for viruses, including coronaviruses and cytomegaloviruses[100–102]. CD13 is expressed in higher amounts on highly phagocytic cells, and it has been demonstrated that CD13 aids in phagocytosis on these cells[103]. One possible mechanism for this observed function is its association with Fcγ receptors, which aid in phagocytosis of particles[104].

Several putative functions for CD13 involve its association with other molecules. CD13 frequently works together with other peptidases, especially CD10 and CD26/dipeptidyl peptidase IV. Cooperation of CD13 with CD10 and/or CD26 aids in the degradation of proteins/peptides, and all three molecules are present in lipid rafts[86]. CD26 is also expressed on FLS and has been linked to RA (and murine RA models) through putative effects on FLS invasion and T cell

proliferation. However, this work has mostly been done with enzymatic inhibitors[105–107]. CD26 removes dipeptides preferentially with proline (and less efficiently alanine) in the penultimate position. When combined with CD13, which cannot degrade proteins with proline in the penultimate position, this allows for degradation of a large number of peptides/proteins[108]. The presence of other peptidases may also be a point of regulation for CD13. While a combination of CD13 and CD26 was shown to cleave substance P (a neurokinin) in mice, substance P was shown to inhibit either purified porcine CD13 or CD13 on a leukemic cell line[109,110]. While this may be due to differences between species, the more likely explanation is the presence of a proline in the penultimate position at the amino end of substance P. When only CD13 is present, the enzymatic activity is inhibited because CD13 cannot degrade past the proline; however, with both CD13 and CD26 substance P is efficiently degraded. Dual inhibition of CD13 and CD26 delays disease onset and decreases severity of experimental autoimmune encephalomyelitis (EAE) and ameliorates disease activity in the sodium dextran sulphate mouse colitis model[111,112]. This dual inhibition likely alters the profile of cleavage targets and the end products. For example, substance P, while primarily a neurotransmitter, can activate immune cells and putatively mediate inflammation[113]. However, while this treatment is useful in mouse models it doesn't address CD13 functions, and possibly CD26 functions, independent of the enzymatic activity.

While CD13 function has been linked primarily to its enzymatic activity, the protease can exert biological effects independently of its enzymatic activity. While CD13 has a very short cytoplasmic tail (9aa), it does have the ability to trigger intracellular signaling[114]. Antibody crosslinking of CD13 on the surface of monocytes leads to the phosphorylation of the

intracellular signaling molecules MPK, extracellular-signal-regulated kinases-1 and 2 (ERK1/2), c-JUN N-terminal kinase (JNK), and p38. Crosslinking can also induce a Ca^{2+} flux and homotypic monocyte aggregation [85,114]. Signal transduction is also important in CD13-mediated monocyte-endothelial cell adhesion which is crucial to monocyte invasion during peritonitis[115,116]. Adhesion and migration of the monocytes is dependent on CD13 expression by both the monocytes and the endothelial cells[116]. This indicates that CD13 may be involved in cellular interactions through self-adhesion/association. These data demonstrate a mechanism of CD13 mediated intracellular signaling where association of multiple CD13 molecules (through cell/cell adhesion or crosslinking) induces signal transduction cascades critical to effective cell function.

Dermal fibroblasts demonstrate another mechanism of CD13 mediated cellular signaling. Knockdown of CD13 on skin fibroblasts blocked p38-dependent induction of expression of MMP1 by stratifin (14-3-3-sigma, a protein released by keratinocytes (cells of the epidermis) which strongly promotes MMP1 on fibroblasts) [117]. Furthermore, dermal fibroblasts treated with keratinocyte conditioned medium showed differences in extra cellular matrix (ECM)-related protein mRNA when CD13 was knocked down. Decreases were seen in MMP1, MMP3, MMP12, tenascin-C, integrin alpha 1, and catenin alpha 1, and increases were seen in fibronectin, SPOCK, and integrin alpha 3 [118]. While knock down of the CD13 protein elicited these effects, treatment of the cells with the enzymatic inhibitor, bestatin, had no effect [118]. These studies suggest another signaling role for CD13 independent of its enzymatic activity. The short cytoplasmic tail does not have any signaling motifs; therefore, it can be hypothesized

that these signaling events occur through CD13 interaction with a secondary protein. The identity of this associated protein remains unknown.

Many suggested functions of CD13 are disease associated, especially in cancer. CD13 is over expressed on tumor cells, where it is known to be involved in angiogenesis and metastasis. Angiogenesis is the formation of new blood vessels from existing blood vessels and can be seen in most types of tissue outgrowth (such as wound healing, tumor growth, or RA hyperplasia) to feed the newly forming tissues. In solid tumors, endothelial cell-associated CD13 was identified on newly forming capillaries. CD13 expression was specific to endothelial cells undergoing angiogenesis (although not just tumor angiogenesis) and was not found on established capillaries and only faintly in arteries [102]. One suggested mechanism for CD13 mediated angiogenesis involves binding of galectin-3 (a pro-angiogenic molecule) to CD13 carbohydrate motifs on endothelial cells[119]. While Petrovic et al implicated enzymatically active CD13 in membrane organization and filopodia formation in bradykinin-mediated angiogenesis[120]. These two studies present different mechanisms by which CD13 may mediate angiogenesis; however, the exact process remains unknown. The first proposed mechanism is independent of enzymatic activity while the second mechanism is dependent on enzymatic activity. It is possible that CD13 is playing multiple roles in angiogenesis which may be dependent on other angiogenic molecules.

CD13 has also been implicated in tumor migration and adhesion. In melanoma cell colonies CD13 relocates to sites of cell-cell contact and associates with ECM components[76]. CD13 has also been shown to aid in the migration of tumors through ECM (using various metastatic tumor

lines)[76]. A third potential role for CD13 in tumors relates to cell proliferation and resistance to apoptosis[121]. Inhibition of CD13 as a strategy to treat cancer has encompassed study of a wide variety of both natural and synthetic enzymatic inhibitors[122]. To summarize, CD13 has been commonly found in tumors and is believed to have a role in cancer pathogenesis with putative functions in angiogenesis, tumor cell migration, invasion into host tissues, aberrant outgrowth of tumor cells, and tumor cell resistance to apopotosis. Some of these processes parallel important components of the behavior of synovial cells in RA.

There are problems however with many CD13 studies involving angiogenesis, proliferation, and migration. One major problem with most of these studies is that they have been done with the chemical inhibitor, bestatin (or occasionally other chemical inhibitors). There is evidence, however, that bestatin is not specific for CD13 and may also inhibit cell proliferation independently of CD13 [123]. It is also possible that the chemical inhibitors may lead to apoptosis, although not necessarily through CD13 targeting. Bestatin and actinonin (a second CD13 inhibitor) prompted an increase in leukemic cell line apoptosis, but the apoptosis was not directly related to CD13 as an anti-CD13 antibody with enzyme inhibitory activity did not exert similar results [124,125]. Studies with anti-CD13 antibodies do support a role for CD13 in cell proliferation and survival, but the mechanisms surrounding the effects of CD13 inhibition (chemical or antibody mediated) on proliferation and resistance to apoptosis remain poorly understood [85]. Another issue is that this work has been done primarily in tumor cell lines, and as such, the biologic significance under *in vivo* conditions is uncertain. However, the data from cancer studies may be suggestive of similar roles in cell types and biologic functions outside of tumors. For example, the RA disease process is also associated with angiogenesis, aberrant cell

migration/invasion, and excessive cell proliferation. As CD13 can be found on both cancer cells and FLS another question is: can CD13 play similar roles in FLS migration and growth as it does in cancer?

While cancer is the most studied disease in connection to CD13, CD13 has reputed roles in several other human diseases, including inflammatory and/or autoimmune processes (e.g. multiple sclerosis, inflammatory bowel disease, psoriasis, and chronic graft-versus-host disease)[126–129]. One study showed increased expression of CD13 on the surface of peripheral blood monocytes from patients with active multiple sclerosis (MS) compared to either MS in remission or non-inflammatory neurological diseases [126]. CD13 has also been linked to inflammatory bowel disease (IBD). CD13 has been suggested as the link between human cytomegalovirus (HCMV) infections and IBD[127]. Many IBD patients have active HCMV infections, and CD13 is a known receptor for cytomegaloviruses [100,130]. Rahbar et al showed the presence of cytotoxic anti-CD13 autoantibodies in the serum of a majority of IBD patients [127]. However, it has not been shown that these antibodies contribute to pathogenicity. Also, the concept that IBD is related to IBD is still controversial[131].

CD13 has also been found in skin cells, especially on dermal fibroblasts and keratinocytes, and it has been implicated in a variety of skin conditions including psoriasis, acne, and wound healing[132]. CD13 inhibitors help slow proliferation and promote accurate differentiation of fibroblasts and keratinocytes in a mouse tail model of psoriasis[128,132]. Another study found that anti-CD13 antibodies inhibited migration of dermal fibroblasts in wound healing[133]. Since

effective wound healing requires keratinocyte migration, while excessive fibroblast migration can lead to ineffectual wound healing and scar formation, this indicates CD13 expression may be important in regulating wound healing. The same group went on to show that topical anti-CD13 antibodies accelerated wound closure[134]. Though not examined, topical CD13 inhibition may also be a potential strategy in psoriasis treatment.

Chronic graft-versus-host disease (cGVHD) is another immune-mediated condition with which CD13 is associated. Soluble CD13 and anti-CD13 antibodies have been found in plasma from patients with cGVHD[129,135–137]. Similar to the IBD studies, a link between HCMV infection and GVHD has been hypothesized. Transplant patients were tested for anti-CD13 antibodies and HCMV. All patients with anti-CD13 antibodies were shown to have cytomegalovirus (CMV) viremia or CMV disease (although not all CMV infected patients had anti-CD13 antibodies), and all of the patients with anti-CD13 antibodies later developed cGVHD [136]. A study by another group showed that sCD13 enzymatic activity was significantly increased in early cGVHD; however, there was no apparent connection between the sCD13 and the anti-CD13 antibody in cGVHD[135,137]. Protein levels of sCD13 were also shown to be significantly elevated in sera of cGVHD patients. It was also noted in this study that higher CD13 levels were found in patients in whom the cGVHD involved the liver[129]. While these data show a strong relationship between CD13 and cGVHD, the function for CD13 in cGVHD remains undetermined. Overall, these data implicate CD13 in autoimmune diseases and inflammatory functions.

It is interesting, though, to note that not all studies involving CD13 point to a pro-inflammatory role. The preponderance of evidence in either mouse models or *in vitro* cell studies indicates a role for CD13 in inflammation, in experiments that employ either anti-CD13 antibodies or CD13 enzymatic inhibitors, as discussed above. However, a study using CD13 knockout (KO) mice showed no difference in four models of inflammation (dextran sodium sulfate colitis, collagen antibody-induced arthritis, thioglycollate-induced peritonitis, and croton oil-induced contact hypersensitivity) with a lack of CD13 [138]. However, these systems tend to focus on the involvement of neutrophils, mast cells, or monocytes and are not necessarily appropriate models for lymphocyte driven diseases, like RA. Despite this caveat, other studies have demonstrated that CD13 may not be entirely pro-inflammatory. The steroid dexamethasone increases surface CD13 enzymatic activity on dermal fibroblasts in a protein synthesis dependent manner [81,139]. As steroids are anti-inflammatory this indicates CD13 may in some way be involved in an anti-inflammatory pathway. Furthermore, the production of pro-inflammatory cytokines (IL-1 and IL-2) were increased in splenocytes when mice were treated with the CD13 inhibitor bestatin, suggesting a potential suppressive role for CD13 in regulating inflammatory responses [140]. These three studies are suggestive of anti-inflammatory CD13 properties. While several processes that involve CD13, such as angiogenesis and monocyte migration, are inflammatory (as previously discussed), the cytokine regulation pattern related to CD13 is mixed. Both pro-inflammatory cytokines, such as TNFα and IFNγ, and anti-inflammatory cytokines, such as IL-4 and TGFβ, can up-regulate CD13. Altogether these data are interesting as they suggest a complicated role for CD13 in inflammatory conditions.

CD13 on T cells

CD13 has been linked to various autoimmune diseases, but the mechanisms by which CD13 may mediate inflammation/pathogenesis are poorly understood. Some proposed mechanisms involve CD13 functions on T cells, but there is debate regarding the presence of CD13 on T cells. Generally, it is accepted that mature lymphocytes (peripheral blood, splenic, tonsillar) are CD13 negative, although there are also reports of aminopeptidase activity on T cells[76,141]. T cells at the early stages of development, such as thymic stem cells and multipotent thymic progenitors, are CD13 positive but T cells lose CD13 expression as they mature[142]. However, CD13 expression has been reported on T cells from disease fluids[143,144]. For example, T cells from the synovial fluid of RA or juvenile chronic arthritis as well as pericardial fluid from cardiac disease patients were positive for CD13 by flow cytometry, thereby supporting the hypothesis that CD13 can be induced on activated T cells[143,144]. The best evidence so far indicates cell-cell contact between T cells and CD13 expressing cells (such as fibroblasts, synoviocytes, monocytes/macrophages) is necessary for this induction of CD13.

One study found CD13 mRNA to be upregulated in tonsillar T cells when incubated with mitogens, but did not see a corresponding increase in cell surface CD13[145]. Only when the cells were incubated with $CD13^+$ cells (including FLS, macrophages, human vascular endothelial cells [HUVECs], or cancer cell lines) was CD13 expression seen; furthermore, addition of mitogens could further increase this expression. This study suggests that CD13 itself may be involved in the induction. Further studies narrowed the mechanism of expression by showing that membrane fractions from FLS could induce CD13 mRNA in T cell cultures, while an aminopeptidase

activity inhibitor, actinonin, inhibited this mRNA induction [145]. Moreover, both membrane fractions and fixed FLS induced CD13 mRNA and protein on T cells, but soluble CD13 or separation of T cells and FLS by a membrane did not induce T cell CD13 [146]. This indicates that cell-cell adhesion signals are necessary for induction of CD13 by T cells, and it is likely that both CD13 and a secondary cell surface molecule are needed. And while the T cells take an active role in this process, the CD13-presenting cells do not. In contrast, another study claims to have found CD13 on resting lymphocytes which was upreglated by concanavalin A, while another paper showed CD13 on T cells after mitogen activation[147,148]. The first paper found CD13 positive T cells by double staining and flow cytometry as well as the use of the CD13 enzymatic inhibitor probestin[147]. However, the antibodies employed in this study are not commonly used and are perhaps inadequately tested. Furthermore, this data was not confirmed by other groups using different antibodies[146]. The antibodies that were used showed only about 60% of monocytes as CD13 positive, while other antibodies typically show all monocytes as CD13 positive[147]. The study also showed that probestin only partially inhibited CD13-like activity in their samples, indicating that either the enzymatic activity assay or the inhibitor or both are not CD13 specific. A second paper, by the same group, showed CD13 on the surface of mitogen, phytohemagglutinin-L (PHA-L)/phorbol myristate acetate (PMA), stimulated T cells using the more commonly used WM15 antibody. In this study they found that the surface expression was inhibited by protein synthesis inhibitors[148]. Another study indicated that the T cell lines HuT78 and H9 have CD13 mRNA and enzymatic activity, but did not show the presence of CD13 protein[149].

Some of the differences between studies could be explained by the use of different antibodies, differences in sensitivity of assays, or differences in how CD13 was measured (protein blots versus mRNA). It is possible that molecules similar to CD13 are present on activated T cells and are detected in the various assays, especially the aminopeptidase activity assays. One possibility is that since CD13 mRNA has been found by some groups in mature lymphocytes, either the protein is not efficiently made in T cells or a form of CD13 not recognized by all antibodies is on T cells (for example a differently glycosylated form). Another possibility could be in the differences in T cell isolation and purity. It is possible that if a higher number of CD13 expressing monocytes remain in the T cell experimental population (in the mitogen only studies) the CD13 seen on the T cells is actually being induced through the combination of stimulus with cell-cell contact with the monocytes. Overall the best evidence seems more in support of T cells only expressing CD13 after cell-to-cell contact with CD13 positive cells.

Chemotaxis

Chemotaxis is the directed movement of cells toward a chemical signal. It is a crucial part of immune function that directs movement of cells into immune tissues, such as lymph nodes, and to sites of infection or damage[20]. Chemotaxis is a key component of inflammatory disease pathology, including RA. It is central in bringing immune cells (monocytes, T cells, B cells, neutrophils) into the joint to mediate disease development[150]. Chemotaxis includes 4 main steps: rolling/tethering, activation, firm adhesion, and transmigration[20]. This migration typically involves a combination of adhesion molecules and chemokines. Chemokines are a group of cytokines that induce directed chemotaxis [20]. In response to a chemokine gradient, responding

leukocytes establish transient interactions with the blood vessel wall and display a rolling behavior mediated by loose coupling between adhesion molecules on leukocytes and endothelial cells. Then, cell interactions with a chemokine activate the cell in a manner that leads to firm adhesion between adhesion molecules. Once cell movement along the vessels is arrested, the responding cell migrates between endothelial cell junctions and moves into the tissue. Chemokines and other chemotactic molecules help direct the cells during rolling and allow them to arrest movement at the site of infections/inflammation; as well as, aiding in migration into and through the tissue[151–153]. Chemokines are a set of small proteins divided into CXC, CC, C, and CX_3C families. They function primarily by binding to members of the chemokine receptor family (CXCR, CCR, CR, and CX_3CR). Several chemokines have been identified in the RA joint including IL-8/CXCL8, ENA-78 (epithelial-derived neutrophil-activating peptide-78)/CXCL5, RANTES (regulated on activation, normal T cell expressed and secreted)/CCL5, MIP-1α (macrophage inflammatory protein-1α)/CCL3, SDF-1 (stromal cell-derived factor-1)/CXCL12, and fractalkine/CX3CL1[152]. Chemokines in the joint are produced primarily by FLS and monocytes, and production is often up-regulated by pro-inflammatory molecules such as TNFα, IL-1, or IFNγ.

The second component of chemokine function is the chemokine receptor, a superfamily of G-protein-coupled receptors. There are 19 known major chemokine receptors, each with a common seven-transmembrane motif coupled to an internal G-protein and a flexible N-terminal region[154]. The identity of the cell types responding to a given chemokine gradient is dictated by the pattern of chemokine receptor expression. While neutrophils, monocytes, and lymphocytes each express distinct patterns of chemokine receptors, chemokine receptor expression can be further fine-

tuned even within subclasses of immune cell populations. For example, Th1 cells express CCR5, CXCR3, and CXCR6; Th2 express CCR3, CCR4, and CCR8; and Th17 express CCR4 and CCR6 [151]. While some receptor-ligand interactions are discrete, most receptors recognize multiple chemokines and many chemokines can bind to multiple receptors[154]. This means that there may not be a direct correlation between specific chemokines and chemokine receptors. Nevertheless, many of the chemokine receptors found in the RA joint match to chemokines linked to RA. Chemokine receptors that have been found on RA T cells include CCR4, CCR5, CXCR3, and CX_3CR1[155]. To complicate matters chemokine receptors can be either constitutive or induced[154]. This may help explain a degree of variability seen in chemokine and receptor studies in RA, with differently activated cell states expressing different sets of receptors. For example, CD4+ cytokine-activated T cells (Tck, produced by culture of resting T cells with a combination of TNFα+IL-2+IL-6) have increased expression of the chemokine receptors CXCR4, CXCR7, CXCR2, CXCR3, CCR5, CCR6, and CCR8 when compared to resting CD4+ T cells[156]. This is one example which suggests that the complex milieu in the RA joint likely modulates the chemokine receptor profile of T cells.

There is debate as to the identity of receptors that are critical to directing T cells into the RA joint. Effector memory T cell clones (CD4+CD28-), generated from RA peripheral blood express CCR7, CCR5, and CXCR4, but while a subset migrated to RA synovium in an RA tissue- SCID mouse chimera the T cells preferentially migrated to the lymph nodes. However, IL-12 treatment of the cells upregulated CCR5 and skewed the migration toward the implanted human synovium [157]. While these findings suggest that CCR5 is central to T cell migration in RA, these results stand in contrast to a study that identified CXCR4 as the most common

chemokine receptor found on memory T cells isolated from RA synovial tissue [158]. The major chemokines for CCR5 and CXCR4, SDF-1 and RANTES respectively, have been detected in the RA joint, supporting a potential role for both receptors in RA[65,159]. Though characterizing chemokine receptor expression in T cell populations harvested from RA synovial tissue or fluids can be informative, it is worth noting that chemokine receptor expression on circulating T cell populations may be different. RA peripheral blood contains significantly higher numbers of T cells expressing CCR2, CCR4, CCR5, and CX_3CR1 than does normal peripheral blood. However, T cells in synovial fluid expressed higher levels of CCR5 alone, while synovial tissue T cells were enriched for CCR4 and CXCR3 [155]. These data may indicate different roles for chemokines in attracting cells to the synovium versus retaining the cells in the synovium. While there are a variety of explanations for the differing results of these studies it is clear that chemotaxis in RA is a complex mechanism involving multiple chemotactic factors.

While chemotaxis is the primary function of chemokines, these molecules can display multiple other functions in RA. For example, IL-8 can induce synovial inflammation, and Groα/CXCL1 can stimulate FLS proliferative responses[152,160]. Multiple chemokines are also able to promote angiogenesis (IL-8, ENA-78, Groα, SDF-1, MCP-1)[152,161]. Interestingly, RA FLS themselves can express chemokine receptors, e.g. CCR2, CCR5, CXCR3, and CXCR4. Depending on the chemokines that bind to these receptors, both general and more specific cell responses can be elicited. For example, MCP-1/CCR2, IP-10/CXCR3, SDF-1/CXCR4 induced FLS migration, proliferation, and collagenase activity, while RANTES/CCR5 only increased the collagenase activity [162]. Chemokines have also been linked to structural joint changes. The chemokine, BCA-1/CXCL13, and its receptor, CXCR5, have been identified as a likely initiator of lymphoid

like germinal center formation in affected joints [163]. Given the range of chemokine-associated effects in RA, from classical functions in cell migration/infiltration to roles in stimulating and organizing cells within the diseased joint, these molecules have considerable potential as therapeutic targets.

Overall current data shows that chemotactic molecules play important roles in perpetuating a pro-inflammatory loop in RA. In this regard, cell-cell interactions between FLS and activated T cells increase chemokine production (IL-8, MCP-1, MIP-1α) by the FLS[164]. Hence, chemokines produced by FLS trigger T cell infiltration into the joint, and T cells in turn stimulate the FLS to produce more chemokines. Ultimately, this can result in a positive feedback loop reinforcing inflammation and RA progression. An additional consideration is that while chemokines are the primary effectors of chemotaxis, other molecules can also display chemotactic properties. For example, the activated complement factor, C5a, as well as lipids and carbohydrates can also direct immune cell chemotaxis[165]. While a variety of chemokines and other molecules have been identified in RA, the key factors responsible for controlling T cell ingress and retention in the RA joint remain subject to conjecture and the possibility that other chemotactic molecules are involved in immune cell trafficking to the joint deserves additional consideration. Could other molecules expressed in the RA synovium be contributing to T cell trafficking to the joint?

CD13 and chemotaxis

In considering the potential identity of other chemotaxins operative in RA, it is interesting to note that CD13 has been implicated in chemotaxis in a variety of ways. First, the chemokines, CXCL8/IL-8 and CXCL11/I-TAC, are known substrates of CD13. In both cases, CD13 is known to degrade the respective chemokine, resulting in loss of chemotactic activity [95,166]. A second mechanism supports a role for CD13 in regulation of chemokine receptors, by down-regulating CXCR4, thereby decreasing responses to CXCL12/SDF-1[167]. While these data demonstrate negative effects of CD13 on chemotactic activity, CD13 may also promote chemotaxis. Crosslinking CD13 on the surface of monocytes induces IL-8 mRNA expression while inhibiting CD13 enzymatic activity represses IL-8 production, although it is uncertain how this affects overall chemotaxis as CD13 can also cleave IL-8 to a less active form[114,168]. In sum, these data point to a variety of indirect roles for CD13 in chemotaxis, but a more direct role for CD13 in chemotaxis has also been suggested. Interestingly, purified human CD13 was shown to be chemotactic for T cells with a preference for CD4+ over CD8+ T cells[169]. CD13 has been proposed as either a chemotatic molecule in-itself or as a regulator of other chemotactic molecules, but there are still many questions about the relevance of these roles *in vivo.* Could CD13 be involved in the chemotaxis of T cells to the RA synovium?

<u>Metalloproteinases: MMPs and ADAMs</u>

Matrix metalloproteinases (MMPs), a family of zinc-dependent metalloenzymes implicated in matrix-remodeling events, were first discovered in 1962 with the characterization of the first mammalian collagenase[170]. With the discovery of twenty or more structurally-related MMPs, it

is recognized that these proteinases can be involved in a wide variety of functions, including cleavage of extracellular matrix (ECM) proteins, the processing of cytokines/chemokines, the activation of downstream targets (including other metalloproteinases), and participating in intracellular signaling [171–175]. It is commonly believed that MMPs play an important role in the joint destruction that characterizes RA. FLS typically express several MMPs [176,177]. MMP14/MT1-MMP is expressed in both the lining and sublining of RA joint synovium and is strongly expressed on RA FLS [178]. MMP16/MT3-MMP has also been identified in RA synovial tissue while MMP15/MT2-MMP and MMP17/MT4-MMP appear to be only marginally expressed in RA tissue [178]. The soluble MMPs MMP1, 2, 3, 8 and 9 were found in higher levels in synovial fluid from RA patients than OA patients, and mRNA of MMP1, 2, 13, 14, and 15 are expressed by stimulated RA synoviocytes [49,179,180]. However, another study did not find MMP15 mRNA in RA, OA, or normal synovial tissue samples [181]. MMPs only represent a small subcategory of metalloproteinases, however, and other metalloproteinases are also found in the joint.

Specifically, a disintegrin and metalloproteinases (ADAMs), another subfamily of metalloproteinases, have also been identified in synovial tissue [182]. ADAMs are a class of predominantly membrane bound metalloproteinases, many of which are known sheddases[183]. Some ADAMs are tissue specific (testis or brain) and/or do not have an active metalloproteinase site but several may be important in the joint including: ADAMs 8,9,10,12,15,17,19, and 33[183]. The mRNA of ADAMs 9, 10, 15, and 17 was found in most RA and OA synovial tissues while other ADAMs were expressed in lower numbers of samples. Only ADAM15 was found to be expressed at significantly higher levels in RA over OA[182]. In another study ADAM8 was found

in the RA pannus specifically next to erosions, and it was linked to osteoclastogenesis[184]. ADAM10 has also been found to be elevated in RA synovial tissue (versus OA or normal) both in the lining and on endothelial cells and has been linked to RA angiogenesis[185]. These studies implicate ADAMs 8, 10, and 15 in RA disease pathology; however, other ADAMs may also be involved.

Of the metalloproteinases, MMPs and ADAMs are the most commonly linked to RA. The main role suggested for MMPs involves the degradation of ECM and the invasion of FLS. Natural inhibitors of MMPs can inhibit FLS proliferation and invasion through either collagen or Matrigel (a mixed ECM substance), while ADAM15 has been directly linked to FLS apoptosis resistance [186,187]. ADAM17 and MMP8 shed lymphotoxin $\alpha_1\beta_2$ (a pro-inflammatory mediator) which in turn binds to receptors on FLS and results in inflammation [188]. When MMPs and ADAMs become upregulated in the RA synovium these various functions may contribute to disease pathology.

MMP14/MT1-MMP

MMP14/MT1-MMP is expressed on a variety of cells including fibroblasts, monocytes/macrophages, dendritic cells, natural killer cells, and mast cells[189]. One of the most studied targets of MMP14 is MMP2/gelatinase A (a soluble, type IV collagenase). However, MMP14 can cleave many substrates, including ECM components (such as collagen, gelatin, and fibrin) as well as a variety of non-ECM targets associated with inflammatory events including, pro-TNFα, CD44, ICAM-1, and IL-8 [189,190]. While MMP14 is best known for cleaving

molecules proximal to the cell membrane, it has also been linked to the shedding of cell surface proteins including MUC1 (a mucin) from epithelial cells and endoglin (TGFβ co-receptor) from HUVEC[191,192]. Interestingly, MMP14 can also display functions independent of its enzymatic activity, by triggering the activation of phosphinositide 3-kinase delta (PI3Kδ) during its trafficking to the nuclear compartment leading to a more inflammatory profile of cytokines[175].

In addition to its ability to hydrolyze a variety of target substrates, MMP14 has also been linked to cell invasion. MMP14 expression is often localized to the invading front and podosomes of migrating cells such as FLS and macrophages [193,194]. While all MMPs, possess collagenolytic activity, membrane bound expression seems essential for degradation leading to tissue-invasion[193]. Transfection and expression of MMP14 or MMP15 was able to confer invasive and angiogenic capabilities on cells while the secreted MMPs 1,2,3,7, or 9 could not [193]. In addition, in MMP14 deficient fibroblasts transfection of a mutant membrane-bound form of MMP1 could confer a collagen-invasive phenotype[177]. Of the membrane bound MMPs MMP14 has been found to be crucial for cell invasion. In fibroblasts MMP14 alone was shown to be essential to type I collagen degradation and the collagen invasive phenotype [177]. These findings are not confined to fibroblasts as mesenchymal stem cells likewise express multiple MMPs (MMPs 1,2,13,14, and 16) and degrade collagen, but only MMP14 was required for their invasion and differentiation [195]. Interestingly, MMP14 has also been linked to CD8+ T cell invasion into pancreatic tissue in type I diabetes [196]. Because of its critical nature in a variety of functions, regulating MMP14 is vital to maintaining homeostasis in the synovium and other tissues. In addition to natural inhibitors (tissue inhibitor of metalloproteinases [TIMPs]) MMP14 can also be regulated by endocytosis and its intracellular trafficking to either the cell surface or lysosomal

network[197]. Similar to CD13, MMP14 can be found on lipid rafts and may be internalized into the cell through caveolae-dependent pathways, although another study indicated that MMP14 inclusion in caveolae within lipid rafts enhanced membrane persistence at the cell surface rather than internalization [190,197]. Regardless, caveolae and lipid rafts may be a point of regulation for both MMP14 and CD13.

MMP14 is of particular interest to this study because it has been strongly linked to RA in multiple aspects of the disease process. MMP14 is highly expressed in the RA pannus especially at the invasion front where the pannus and cartilage meet [198]. Furthermore, RA FLS invasion into a collagen matrix or cartilage was correlated with MMP14 expression[198]. More specifically, Sabeh et al demonstrated that while RA synoviocytes express multiple MMPs (e.g. MMPs1,2,13,14,16), only MMP14 conferred FLS with collagen degradation and invasion activity [49]. These studies suggest a specific role for MMP14 in invasion of RA FLS.

Other specific functions for MMP14 in arthritis include leukocyte migration, bone formation/destruction, and angiogenesis[189,199,200]. One key component of RA is the increased influx of leukocytes into the joint. MMP14 has been linked to leukocyte mobility through cleavage of cell adhesion molecules and ECM components[189]. MMP14 can also activate TGF-β produced by osteoblasts resulting in decreased osteoblast apoptosis and increase in conversion to osteocytes [199]. While it is uncertain whether the conversion of osteoblasts to osteocytes could contribute to RA bone destruction, there exists a delicate balance of osteocytes (bone homeostasis), osteoblasts (bone formation), and osteoclasts (bone absorption) and disruption of

the balance can accelerate bone loss as well as destructive bone remodeling. Functionally, MMP14 may also impact RA bone destruction by regulating monocyte fusion, a process critical to osteoclast formation[200].

In RA, the increased mass of synovial tissue in the RA joint is supported by an increased blood flow through angiogenesis and additional process that can be linked to MMP14 activity. RA synovial tissue can induce a strong angiogenic response in model systems (i.e. the developing chick embryo) and silencing MMP14 not only blocks invasive activity, but angiogenesis as well[49]. While endothelial cell derived MMP14 had been linked directly to angiogenesis (i.e. by controlling the tissue-invasive activity of sprouting endothelial cells), synoviocyte-induced angiogenesis demonstrates that MMP14 can also generate a pro-angiogenic factors that stimulate neovascularization[201]. However, the synoviocyte induced angiogenesis data indicates that MMP14 can cleave a pro-angiogenic factor that may mediate angiogenesis in RA. Currently the effectors responsible for MMP14-induced angiogenic responses remain unknown.

Extracellular vesicles and exosomes

Extracellular vesicles (EVs) of various origins are of increasing interest in immunology and immunologic diseases, including RA[202]. EVs have interesting potential in immunology as they could allow for the interactions of surface proteins apart from direct cell-cell contact, even transporting surface proteins to act in autocrine, paracrine, or endocrine fashions. Classifications of EVs include exosomes, microparticles, microvesicles, and apoptotic bodies. While there is a good deal of debate over categorizing of EVs, the individual groups of EVs are primarily defined

by size, method of generation, and/or cellular orgigin[203,204]. Here extracellular vesicles will refer to the overall group, microvesicles will refer to EVs 50-1,000 nm in size shed by budding from the cell surface, microparticles will refer to microvesicles from platelets or erythrocytes, and exosomes will refer to small EVs that originate intracellularly.

Exosomes are secreted by most cell types and the proteins expressed reflect the cell surface of the cells from which they are released. Exosomes are bi-layer lipid membrane vesicles, 40-100nm in size. In addition to being representative of the secreting cell, exosomes also typically contain some conserved proteins including intracellular proteins (like actin, tubulin, and Rab proteins) and signaling proteins (kinases and heterotrimeric G-proteins)[203]. These proteins likely contribute to basic exosome structure and function. Exosomes are found in culture supernatants of most cells as well as in many bodily fluids including: blood, urine, tumor ascites, and synovial fluid[203,205]. Exosomes are formed intracellularly in multivesicular bodies (MVBs). MVBs then fuse with the plasma membrane, releasing the exosomes; a process dependent on small GTPases in most cells[202]. While the exact mechanism of release is undefined, metalloproteinases appear to be involved. Treatment of human vascular endothelial cells (HUVECs) with a broad spectrum zinc-metalloproteinase inhibitor significantly decreased their release of exosomes[206]. In addition to exosomes having surface proteins, they also contain cytoplasmic proteins and can carry mRNA and microRNA (miRNA)[207].

One main suggested function for exosomes is cell-cell communication. This can occur by interaction of surface proteins, for example, DCs can secrete exosomes with surface MHC

capable of activating T cells through interaction of the exosomal-MHC with the cell surface TCR [208]. Alternatively, exosomes can aid in cellular communication following internalization by target cells and release of their contents inside the cell, such as micro RNA generated in one cell can be transported inside an exosome to a target cell population where it suppresses or activates responses [203]. Exosomes may also function by allowing exchange of proteins or lipids from one cell to another, such as exchange of MHC or cholesterol, or they can simply serve as a means of elimination of cellular proteins[205,207]. Another suggested function of exosomes in the body is in persistence of signaling molecules. Exosomes not only have a lipid bilayer, but have a high concentration of sphingomyelin, cholesterol, GM3 (a glycolipid), and proteins like CD55 that protect against complement[203]. The presence of this lipid and protein profile suggests that exosomes may be a way of increasing stability of proteins outside the cell, compared to soluble proteins. In addition to the biological functions of exosomes, they are of interest in diagnosis as biomarkers for various diseases (such as renal disease or malignancies)[203].

Extracellular vesicles have been found in RA and other forms of arthritis. Microparticles have been identified in RA synovial fluid and other inflammatory arthritides, but not in OA synovial fluid, and were determined to originate primarily from platelets[209,210]. When RA synovial fluid microparticles were isolated and added to FLS cultures, they induced production of monocyte chemotattractant protein 1 (MCP-1), IL-8, IL-6, ICAM-1, VEGF, and RANTES[211]. By contrast, others have reported that microvesicles in the RA joint arise primarily from monocytes or granulocytes but not platelets [212]. Interestingly, microvesicles isolated from either monocyte or T cell line cultures can induce the production of pro-angiogenic chemokines (CXCL-1,2,3,5,6) and matrix metalloproteinases (MMP1,3,9,13) by RA FLS *in vitro*[213,214].

Exosomes have also been found in OA, RA, and reactive arthritis synovial fluids. Of particular interest, it was noted that the synovial fluid exosomes expressed citrullinated proteins[215]. Citrullination is a posttranslational modification, and anti-citrullinated protein antibodies (ACPAs) have been linked to RA[216]. Exosomal citrullinated proteins included: SPα (CD5 antigen-like protein), fibrinogen fragment D, fibrinogen β-chain (and precursor), fibrin fragments, and other unidentified proteins. Non-citrullinated proteins were also identified including fibronectin, IgG1 γ-chain, and α_2-macroglobulin[215]. While ACPAs are specific for RA, exosomal citrullinated proteins were found in the synovial fluids of OA and reactive arthritis patients as well as those of RA patients. This may indicate that exosome-associated citrullinated proteins function in disease-independent mechanisms, or that it is only when ACPAs are produced to the proteins that citrullination is pathogenic.

FLS also produce exosomes *in vitro*. RA FLS-derived exosomes express membrane-bound TNFα while OA FLS derived exosomes are TNFα-negative[217]. The RA FLS exosomes also induced apoptosis resistance in CD3+ T cells[217]. While it was suggested that this apoptosis resistance was related to the TNFα presentation, the mechanism of resistance is uncertain. Overall, the profile of exosomal proteins/molecules in RA and from FLS is not complete. Many proteins associated with RA, and known to be expressed on synovial cells, have not been examined on exosomes in synovial fluid. However, some RA associated proteins have been found on exosomes derived from other cell types. Exosomes can express multiple metalloproteinases, and CD13 has been found on exosomes derived from microglia cells or mast

cells[203,207]. MMP14 has been found on fibrosarcoma- and melanoma-derived exosomes, and ovarian carcinoma-derived exosomes contained ADAM17 and ADAM10[218,219]. Not only do we not know all the possible relevant proteins on exosomes in RA, but the potential functions of these proteins in the context of exosomal presentation are unknown. Is CD13 on exosomes produced by RA FLS, and are CD13 positive exosomes present in RA synovial fluid?

Potential roles of CD13 in rheumatoid arthritis

As discussed previously, CD13 has been linked to a variety of inflammatory mechanisms. CD13 is known to be expressed on multiple cell types involved in the pathogenesis of RA, including macrophages/monocytes, epithelial cells, and fibroblasts. Studies of dermal fibroblasts have shown CD13 can affect fibroblast migration and expression of MMPs[117,118,133,134]. It is possible that these effects could extend to synovial fibroblasts as well. Tumor cells are another cell type where CD13 expression has been linked to cellular migration[220]. CD13 has been identified in synovial tissue via immunostaining, and surface CD13 has been identified on OA and RA FLS [169]. In addition, aminopeptidase activity (attributed to CD13) is reportedly increased in RA synovial fluid as well as FLS (with accompanying increases in CD13 mRNA and protein levels) when compared to OA [169]. Nevertheless, these findings remain controversial as other studies failed to detect differences in CD13 levels in RA versus OA FLS as assessed by flow cytometry [221].

Previously, CD13 protein levels have not been definitively measured on FLS. Typically, CD13 protein amount is inferred based on its enzymatic activity. The conflicting data using Western

blot analysis versus surface staining may reflect differences in cellular localization of CD13 (intracellular versus cell surface). In addition to cellular CD13 on FLS, CD13 aminopeptidase activity is found in the synovial fluid. However, the distribution of this activity between extracellular vesicles and soluble CD13 remains to be determined. It is also unknown whether soluble/EV-associated CD13 is present or potentially pathogenic in RA.

A putative role for sCD13 in T cell chemotaxis has been specifically suggested to play a role in RA by Shimizu et al [169]. This group showed a correlation of N-aminopeptidase activity and lymphocyte counts in RA synovial fluid samples. They also showed inhibition of peripheral blood T cell chemotaxis toward RA synovial fluid by bestatin. While this evidence is suggestive of a role for CD13 in RA, it is inconclusive. CD13 has yet to be measured in RA samples, and the enzymatic activity assays are not specific to CD13. Without a true measure of CD13 it is difficult to determine whether the amounts of purified human CD13 shown to be chemotactic for T cells are biologically relevant [222]. Bestatin is also not specific to CD13 and could potentially be affecting other aminopeptidases in synovial fluid [123]. This study will therefore examine the expression, regulation, and localization of CD13 produced by FLS and asses potential roles for CD13 first as a chemotactic molecule for T cells in RA and as a mediator of RA FLS growth and migration.

Chapter 2

CD13 in Rheumatoid Arthritis

T cells and FLS have been shown to interact in RA in a variety of ways. One prime mechanism is through T cell secreted factors, such as cytokines, activating FLS resulting in regulation of pathogenic proteins. Two main cytokines involved in RA disease pathogenesis are TNFα and IL-17. Both molecules bind to receptors on FLS and induce subsequent effects. IL-17 can upregulate IL-6, various chemokines, MMPs, and growth factors [35]. TNFα can induce FLS proliferation, production of collagenase, and GM-CSF [223]. Because of these and other mechanisms, IL-17 and TNFα are both of interest as therapeutic targets in RA. However, there is still much we don't understand about the pathogenesis of RA and novel pathways on FLS may lead to new therapeutics.

CD13 involvement has been previously suggested in RA, and phenomena that can be functionally linked to CD13 are seen in RA (angiogenesis, cellular migration, monocyte activation)[70,76,85,169]. However, a definitive role for CD13 in RA has not been shown, and the protein has not been directly measured. Enzymatic assays of CD13 activity and chemical inhibition of its enzymatic activity have been employed, but neither the assays nor the inhibitors of CD13 are specific for CD13 versus other aminopeptidases[123]. Our goal in this chapter was to

measure CD13 protein in RA *in vivo* and *in vitro.* This will provide better evidence for CD13 involvement in RA.

Identification of CD13 as an IL-17-induced protein on FLS

TNFα and IL-17 are two cytokines strongly linked to RA. Currently TNFα inhibition is a target for several therapeutic agents used to treat RA. However, many patients either do not respond or have inadequate responses to TNFα therapeutics. Therefore, TNFα independent pathways are of increasing interest in identifying new RA therapeutic targets. This study commenced as a screening experiment to identify a pathway dependent on IL-17, but independent of TNFα. To identify a surface structure on FLS that was regulated by IL-17 independently of TNFα, IL-17 treated FLS were used to immunize BALB/c mice and hybridoma clones were created. The clones were screened on resting FLS, IL-17 treated FLS, and TNFα treated FLS. A hybridoma was selected that produced an antibody (591.1D7.34) that recognized an FLS surface protein upregulated by IL-17 but not TNFα at 48 hours (Figure 2.1A). The antibody 1D7 was screened on a variety of cell types to determine which cells are positive for the protein recognized by 1D7 (Figure 2.2). Of the cell types tested, the monocytic cell line, U937, showed the strongest expression of the molecule identified by 1D7. We therefore used 1D7 to immunoprecipitate a protein from U937 that migrated on SDS-PAGE as a single band of 150kDA, and that was identified as CD13/Aminopeptidase N by sequencing of multiple peptide fragments (Figure 2.1B and C).

Since there is debate over whether CD13 is expressed on activated T cells we did a more extensive screening looking for any CD13 present on various T cell types. We tested: resting

peripheral blood T cells, phytohaemagglutinin (PHA) activated, concanavalin-A (conA) activated, phorbol 12-myristate 13-acetate/ionomycin (PMA/Iono) activated, cytokine activated (Tck), JURKAT, and HUT78. We looked for CD13 by mRNA with quantitative real time PCR and protein using a novel CD13 ELISA, aminopeptidase activity, and flow cytometry. We did not find CD13 by any of our methods on any of the T cell types tested (data not shown).

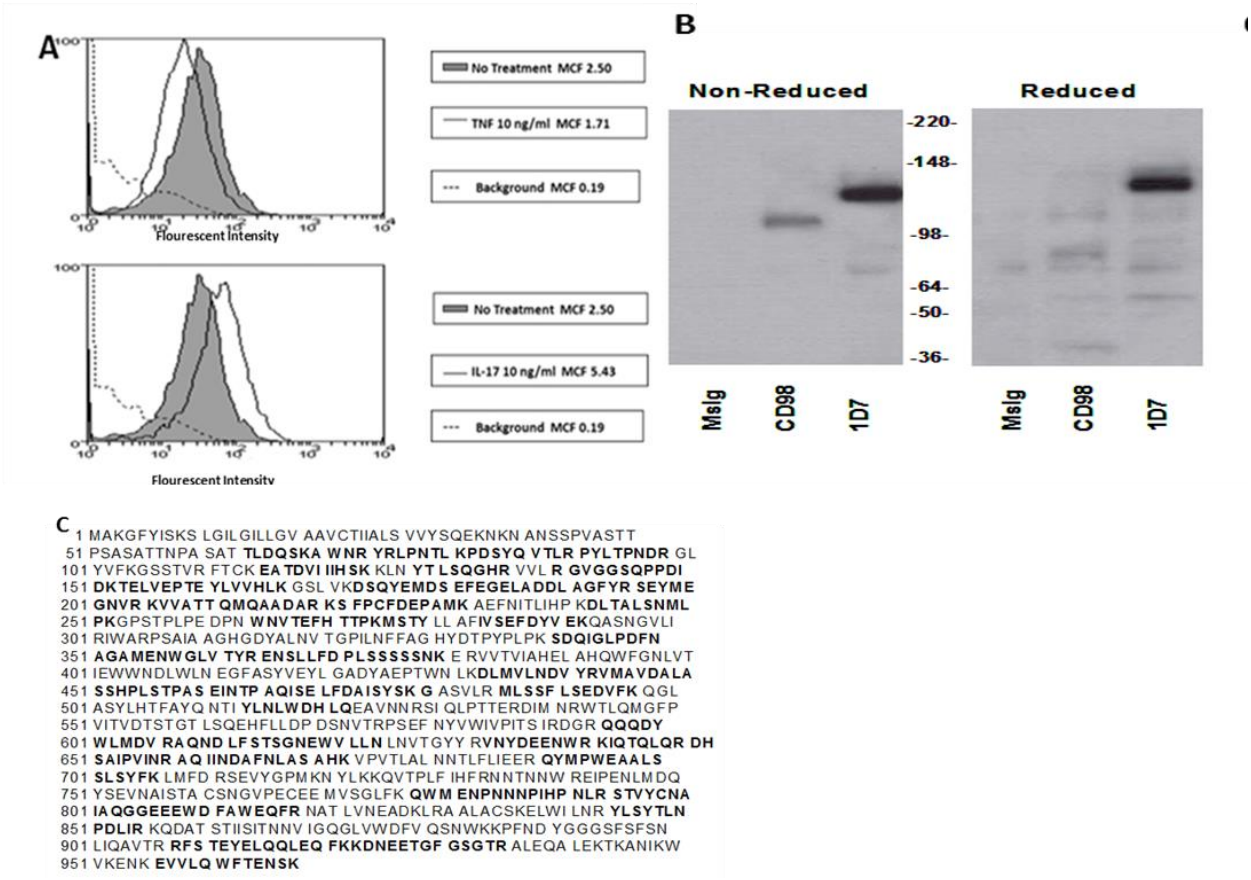

Figure 2.1 Identification of the protein recognized by mAb 591.1D7.34 and upregulated by IL-17 as CD13.

A, FLS were treated for 48 hours with medium alone, TNF-α (10ng/ml), or IL-17 (10ng/ml) before staining with 1D7 and Alexa Fluor 488-conjugated goat anti-mouse IgG . Cell surface CD13 was measured by flow cytometry. (method p138) **B,** An OA FLS cell line was biotinylated and lysed. Immunopreciptiation was used to isolate protein recognized by 1D7, MsIg (isotype control), or anti-CD98. The proteins from the 1D7, MsIg, and anti-CD98 beads were resolved by SDS-PAGE under reducing or non-reducing conditions. Protein from the same number of cells was loaded in each lane. **C,** Myeloid line U937 was lysed and 1D7 was used to immunoprecipitate the recognized protein, which was run on two gels. One was stained with Coomassie blue. The second gel was excised at a spot corresponding to the primary band in the stained gel. The protein was extracted and multiple peptide fragments sequenced, each of which matched portions of Aminopeptidase N/CD13 (bolded sequences). (method p136)

Cell Type	Pos	Neg
Jurkat		X
HSB2		X
Hut 78		X
Molt 13		X
HBL-100		X
PBMC		X
T cells, resting		X
T cells, cytokine activated		X
PHA blasts		X
PHA blasts + IL-17		X
EBV- DC		X
EBV – WC		X
EBV- VH		X
SaOS-2 (osteoblast)		X
Mel-SK1 (melanoma)		X
CaCO-2 (epithelial)	(X)	
hFOB 1.19 (fetal osteoblast)	XXX	
Synovial Fibroblast RA	XX	
Synovial Fibroblast OA	XX	
Skin Fibroblast	XX	
Lung Fibroblast	X	
Dendritic Cells	XX	
Monocytes	XX	
U937	XXX	

Figure 2.2 Staining of different cell types with 591.1D7.34.
Various cell types were stained with the antibody produced by the hybridoma 591.1D7.34 and secondary Alexa Fluor 488-conjugated goat anti-mouse IgG. Cells were screened by flow cytometry. Positive and negative staining is as indicated, with degree of positivity indicated by the number of Xs. The highest degree of staining was observed on the myeloid cell line U937. (method p138)

CD13 *in vivo*

Having identified FLS CD13 as a potential part of an IL-17 induced pathway we next sought to determine whether the concentration of a putative soluble form of CD13 is elevated in the RA joint. This was previously suggested by determination of levels of aminopeptidase activity in synovial fluid (SF) samples, but without direct measurement of CD13 protein [169,222]. We developed a novel ELISA to measure CD13 in healthy control (HC) serum (n=23), RA serum (n=33), and both RA and OA SFs (n=98). RA SFs (n=39) had an average CD13 concentration of 1191.09±121.58 ng/ml with an activity of 3402.45±239.58 μM/hr, and OA SFs (n=59) had an average CD13 concentration of 646.11±45.64 ng/ml and an activity of 2250.28±93.18 μM/hr (Figure 2.3A). The RA SFs were significantly higher in both amount ($p<0.00001$) and activity ($p<0.00001$). RA SF CD13 concentration was also significantly higher than in RA sera, 397.95±72.18 (n=33, $p<0.00001$), or HC sera, 683.34±203.37 (n=23, $p=0.0023$) (Figure 2.3B). CD13 was significantly higher in HC sera than in RA sera, $p=0.0040$. The results are consistent with the observation that IL-17 upregulates CD13 expression by FLS and leads to the hypothesis that generation of sCD13 in the RA joint may play a role in a pro-inflammatory pathway.

CD13 was also examined on tissue sections from RA, OA, and normal synovium. CD13 was observed on the synovium in all three with higher expression in RA and OA. CD13 also co-localized with cadherin-11 (a FLS cell marker) in RA and OA, as shown by yellow (Figure 2.4). Both overall CD13 expression and CD13/cadherin-11 co-localization were higher in RA over OA. In RA synovium infiltrating myeloid cells were also observed as indicated by CD11c (a myeloid/monocyte marker). Only low levels of CD11c were seen in OA and normal. In RA there was co-localization between CD11c and CD13 on myeloid cells indicating CD13 is present

not only on FLS but myeloid cells as well in the RA joint (Figure 2.4). CD13 was also expressed on vasculature in RA (Figure 2.5). These data are consistent with previous publications showing CD13 on FLS, myeloid lineage cells, and neovasculature [66,86,224].

We also examined CD13 concentrations in the serum from inflammatory conditions other than RA. CD13 and CD13 aminopeptidase activity were measured in chronic graft-versus-host disease (cGvHD, n=19), myositis (n=4), Sjogrens syndrome (n=6), scleroderma (n=51), and systemic lupus erythematosus (n=3), as well as RA and HC serum as discussed above (Figure 2.6). CD13 and its enzymatic activity were found to be significantly elevated in cGvHD over healthy controls. Serum from cGvHD patients contained 2431.49±2192.31 ng/ml with an activity of 5977.87±1995.00, p=0.0026 and 0.0051 respectively. Similar to RA, myositis serum was significantly lower in both CD13 amount (p=0.00025) and enzymatic activity (p=0.046) than HC serum, 236.93±89.00 ng/ml with activity of 3400.30±1053.22 μM/hr. Lupus, while significantly lower in CD13 amount (231.78±5.97 ng/ml, p=0.00092), was not significantly different in its aminopeptidase activity (3678.44±1273.99 μM/hr, p=0.26). Scleroderma samples had a significant decrease in serum aminopeptidase activity from normal serum (3649.41±1436.14 μM/hr, p=0.00021) but no significant difference in CD13 amount (565.65±1120.84 ng/ml, p=0.62). Scleroderma samples were also analyzed after subdividing the samples into limited and diffuse groups. This did not change the overall results, with both groups showing a significant decrease in enzymatic activity and no significant difference in CD13 protein concentration; however, there was one new observation. When separated into limited (n=33) and diffuse (n=18) the limited scleroderma samples contained lower levels of CD13 (435.27±720.72 ng/ml) than normal samples while the diffuse samples contained higher

levels of CD13 (804.68±1619.77 ng/ml) than normal samples. Sjogren's syndrome samples were not significantly different than HC samples, 505.23±632.62 ng/ml with an activity of 3626.94±908.73 μM/hr.

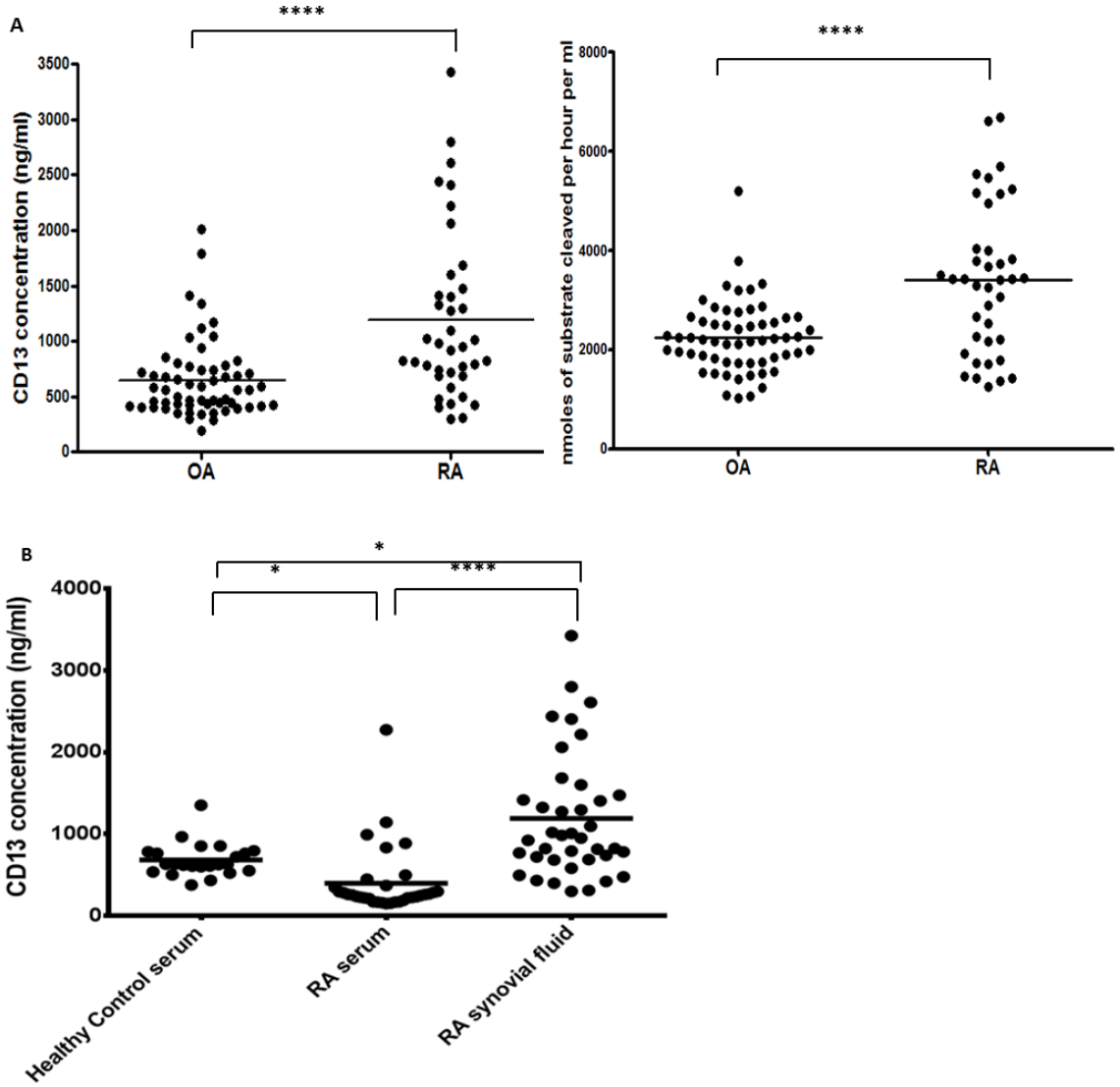

Figure 2.3 CD13 is found *in vivo* and is higher in RA than in OA synovial fluid.
A, CD13 was measured by enzyme-linked immunosorbent assay (ELISA, method p 139) and enzymatic activity (method p138) in RA (n=39) and OA (n=59) synovial fluids. **B,** CD13 was measured by ELISA in serum from healthy controls (n=23), serum from RA patients (n=33) and in synovial fluids from RA patients (n=39, not matched to serum). * $p<0.05$; ****$p<0.00001$, by One-way ANOVA

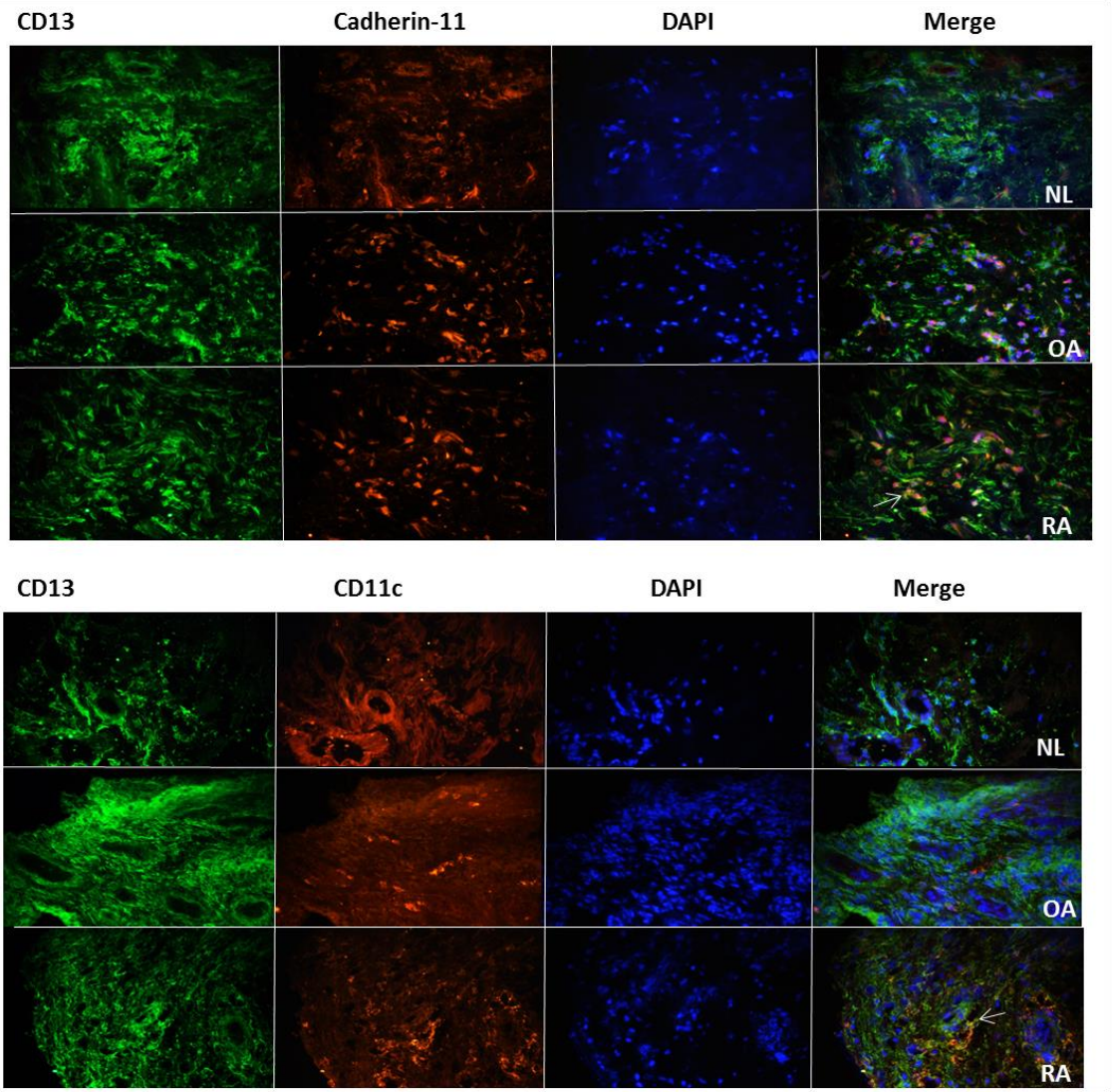

Figure 2.4 CD13 staining is present in synovial tissue from normal, OA, and RA patients. CD13 staining more strongly co-localizes with FLS (cadherin-11) and monocytic lineage cells (CD11c) in RA tissue.

Tissues were harvested form normal, OA, and RA patients during surgery. Tissue sections were frozen with liquid nitrogen and 7 µm frozen sections were made using a cryostat. The slides were fixed in Acetone at 4^0C for 30 minutes, washed, and blocked in 20% Fetal Bovine Serum and 5% goat serum in PBS. Slides were incubated with the primary antibodies anti-human CD13 (1D7, 10µg/mL) and rabbit anti-human CD11c (Abcam, 1:100 dilution) or rabbit anti-human Cadherin 11 (Zymed 10µg/mL). Controls were rabbit and mouse IgG at equivalent concentrations. Secondary antibodies for both sets of slides were Alexa fluor goat anti-mouse 488 and goat anti-rabbit 555 (Life Technologies 10µg/mL), and DAPI (Invitrogen, 1:5000 in PBS) was used on all slides. Slides were mounted with Fluoromount-G (Southern Biotech). Pictures were taken on an Olympus BX microscope at 40x magnification. Arrows point to examples of co-localization. (method p139)

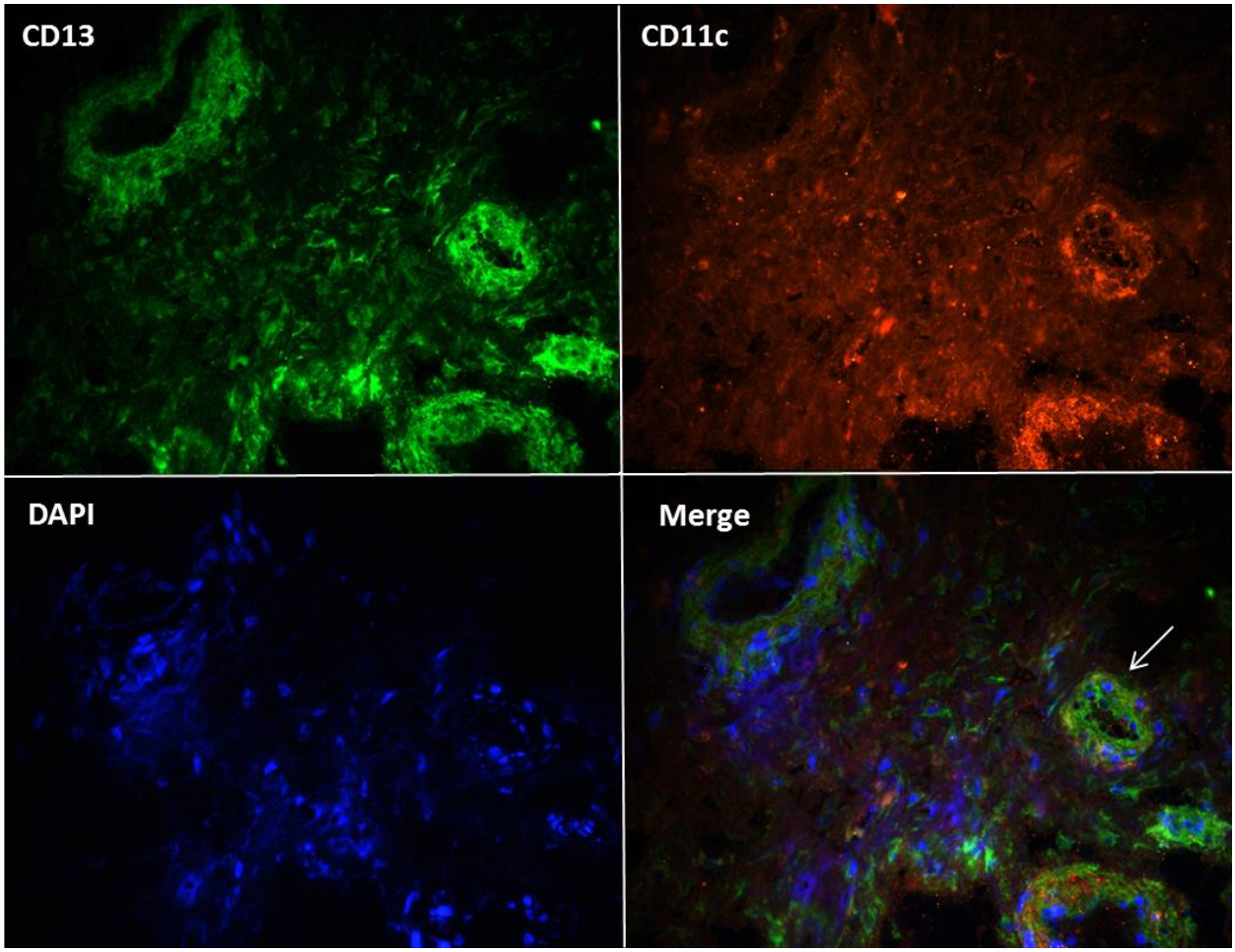

Figure 2.5 CD13 staining is present on neovasculature in RA synovium.
Tissues were harvested form normal, OA, and RA patients during surgery. Tissue sections were frozen with liquid nitrogen and 7 µm frozen sections were made using a cryostat. The slides were fixed in Acetone at 4^0C for 30 minutes, washed, and blocked in 20% Fetal Bovine Serum and 5% goat serum in PBS. Slides were incubated with the primary antibodies anti-human CD13 (1D7, 10µg/mL) and rabbit anti-human CD11c (Abcam, 1:100 dilution). Controls were rabbit and mouse IgG at equivalent concentrations. Secondary antibodies for both sets of slides were Alexa fluor goat anti-mouse 488 and goat anti-rabbit 555 (Life Technologies 10µg/mL), and DAPI (Invitrogen, 1:5000 in PBS) was used on all slides. Slides were mounted with Fluoromount-G (Southern Biotech). Pictures were taken on an Olympus BX microscope at 40x magnification. Arrow points to an example of vasculature. (method p139)

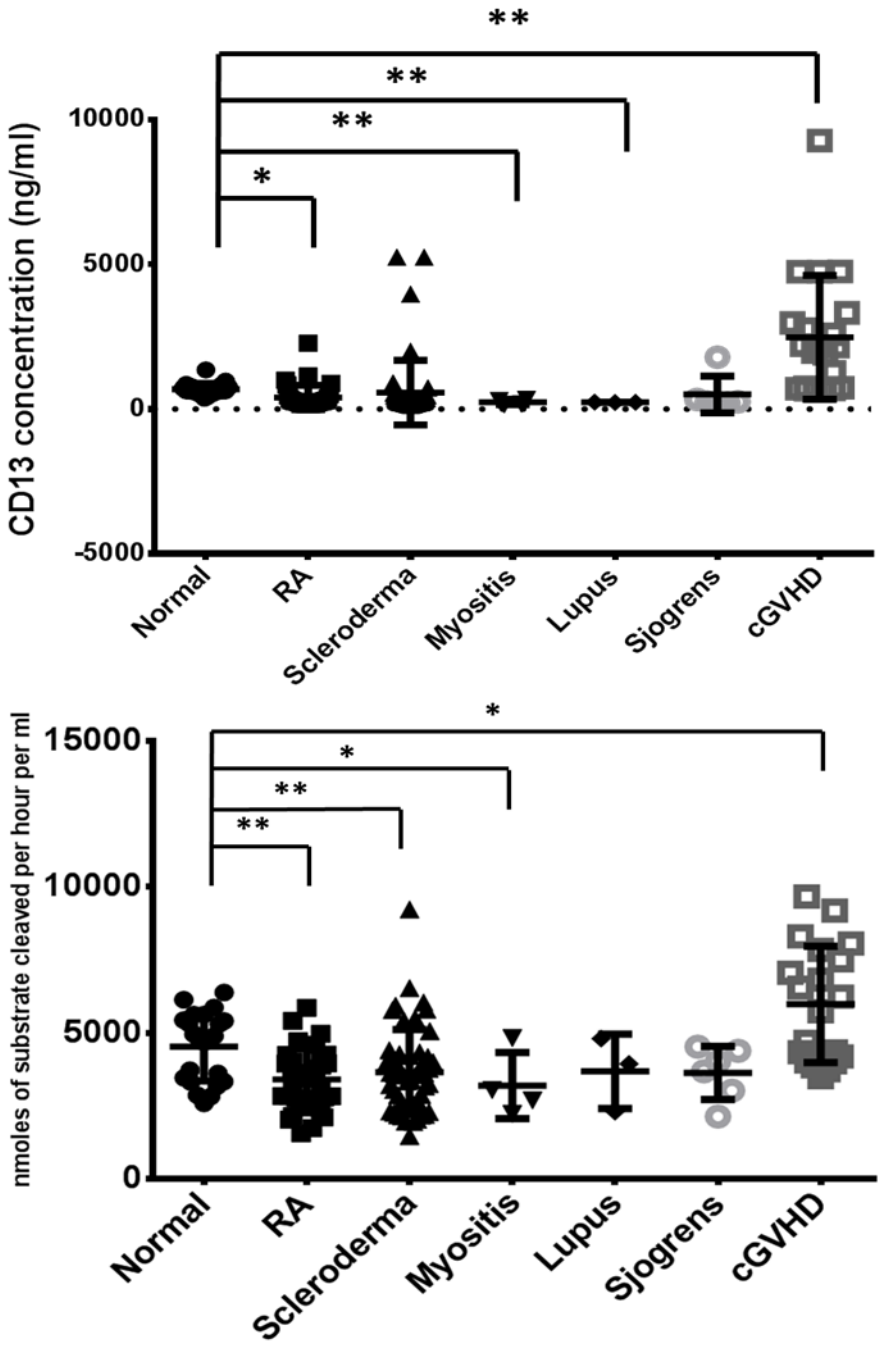

Figure 2.6 CD13 is significantly elevated in chronic graft-versus-host disease serum over CD13 in healthy control serum, and is significantly decreased in RA, myositis and lupus serum compared to healthy control serum.
Serum was collected from patients with various inflammatory immune mediated conditions and CD13 was measured by ELISA and aminopeptidase activity assay. *p<0.05; **p<0.001 (methods p138-139)

CD13 on FLS from RA and OA

Having identified CD13 on FLS and sCD13 in synovial fluid, we asked whether the difference between RA and OA SFs corresponded to an increase in CD13 expression by RA FLS. We used antibodies WM15 and SJ1D1 in addition to 1D7 to measure CD13 by flow cytometry (Figure 2.7A). Each antibody detected CD13 on FLS, but with no significant difference in surface CD13 between resting RA (n=7) and OA (n=5) FLS. Moreover, measurement of total cell CD13 in lysates of RA and OA FLS by ELISA (p=0.74) and enzymatic activity assay (p=0.15) showed no significant differences (Figure 2.7B). We found 4867.39±1196.81ng/ml of CD13 with an activity of 2993.65±743.40 μM/hr in RA FLS and 4256.74±1306.71ng/ml of CD13 with 2123.05±1203.81 μM/hr of enzymatic activity for OA FLS. To determine whether FLS could release sCD13, we measured CD13 in FLS culture supernatants. FLS were cultured in serum free media to eliminate interference from bovine CD13. sCD13 was detected by ELISA in the supernatants and was enzymatically active, with almost identical results from RA (51.28±5.15 ng/ml, 957.69±819.02 μM/hr) and OA (50.78±7.16ng/ml, 962.69±552.26 μM/hr) FLS (Figure 2.7C).

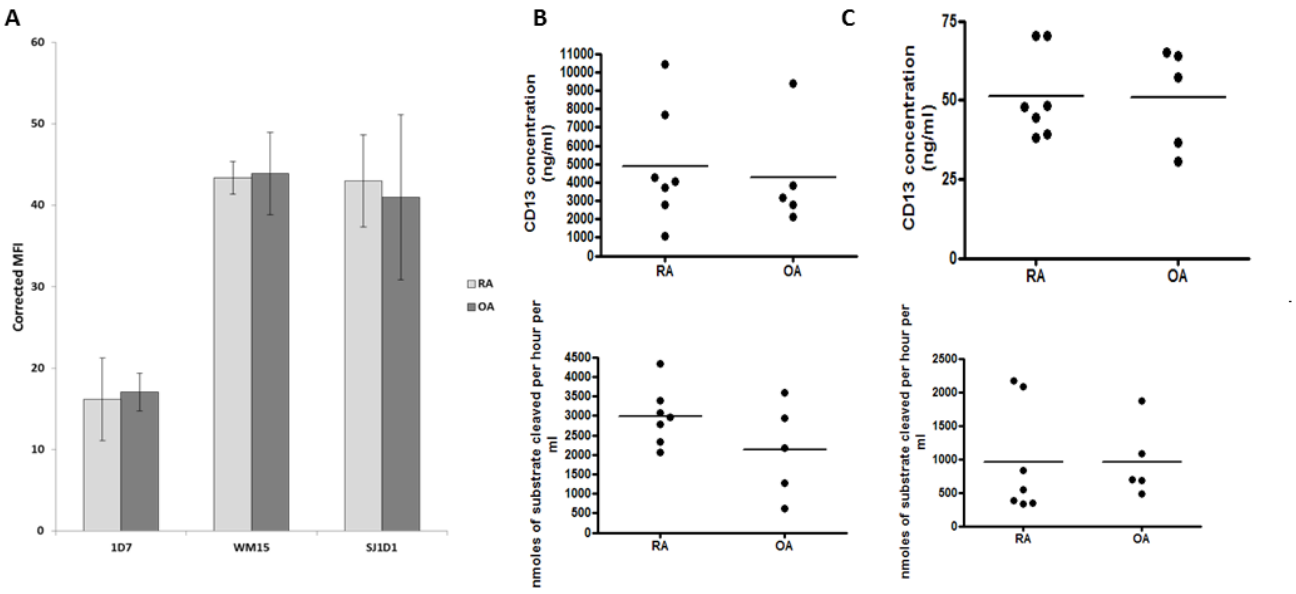

Figure 2.7 Equivalent expression of CD13 by RA and OA FLS.

A, FLS were immunostained for CD13 using three antibodies which recognize different epitopes 1D7, WM15, SJ1D1. All three showed high expression as measured by flow cytometry. Mean fluorescent intensity (MFI) was adjusted for the isotype control as follows MFI CD13 – MFI MsIg = corrected MFI. **B**, RA (n=7) and OA (n=5) FLS were grown to confluence in 20% CMRL and switched to serum free media for 48 hours prior to harvesting. Cells were lysed and CD13 was measured by ELISA and enzymatic activity assay. **C,** The 48 hour FLS culture supernatants were concentrated from 25mls to 1ml through at 30KDa centrifugal filter and measured by ELISA and enzymatic activity assay. (methods p138-139)

CD13 and aminopeptidase activity in the joint

Although we found elevated levels of CD13 protein and enzymatic activity in RA *in vivo*, we did not find any differences *in vitro* between RA and OA FLS. We next sought to confirm that the N-aminopeptidase activity attributed to CD13 in SF samples was really due to CD13. The correlation between the concentration of CD13 and the aminopeptidase activity was R^2=0.1365, p=4.24x10^{-9} by ANOVA in SF samples. Considering the OA samples separately, this correlation remained highly significant, R^2=0.418, p=3.17x10^{-8}. However, in the RA samples the correlation was non-significant, R^2=0.0947, ANOVA p=0.057 (Figure 2.8A). Several outliers could indicate the variable presence of other proteins with aminopeptidase activity.

To further assess the issue, we decided to perform immunodepletions on SFs. To determine which antibody to use for the immunodepletion we tested three antibodies (1D7, WM15, and SJ1D1) for inhibition of CD13 enzymatic activity and degree of CD13 protein depletion. First, we tested for inhibition of enzymatic activity to ensure that the depletion did not also affect the aminopeptidase activity. We found as previously reported that WM15 did inhibit aminopeptidase activity and SJ1D1 did not[74]. We also found that 1D7 is not an inhibitor of aminopeptidase activity (Figure 2.9A). Since immunodepletion did not always result in complete removal of CD13 we tested one synovial fluid with either each antibody alone or all three combined to determine if there was a difference in degree of depletion. We found that there was no difference with any of the depletions in amount of CD13 remaining (Figure 2.9B). Even serial depletion with all three antibodies did not significantly reduce the remaining CD13 over any antibody alone. From this data we decided to use 1D7 for the immunodepletion since it was the closest to MsIg in effect on enzymatic activity. We immunodepleted RA (n=6) and OA

(n=3) SFs using 1D7 and assayed the depleted fractions for CD13 and aminopeptidase activity. Successful immunodepletion of CD13 (partial or complete) was verified by ELISA. We showed significant depletion of both the CD13 protein amount (11.50%±0.26% remaining, p=0.00055) and enzymatic activity (55.74%±0.31% remaining, p=0.0027) (Figure 2.8B). In most SFs the remaining percentage of aminopeptidase activity was higher than the remaining percentage of CD13 protein. Moreover, 100% depletion of CD13 protein failed to remove all of the aminopeptidase activity. An example in Figure 2.8C shows one RA SF with complete depletion of CD13 as measured by ELISA in which the enzymatic activity in the sample went from 876.61±36.06 μM/hr in the control depleted down to 356.00±17.47 μM/hr ($p=2.3x10^{-5}$) in the CD13 depleted sample. Much of the enzymatic activity in this sample appeared in the eluate from the 1D7 immunopreciptation (3888.00±39.97 μM/hr). However, this only accounted for approximately 60% of the starting enzymatic activity.

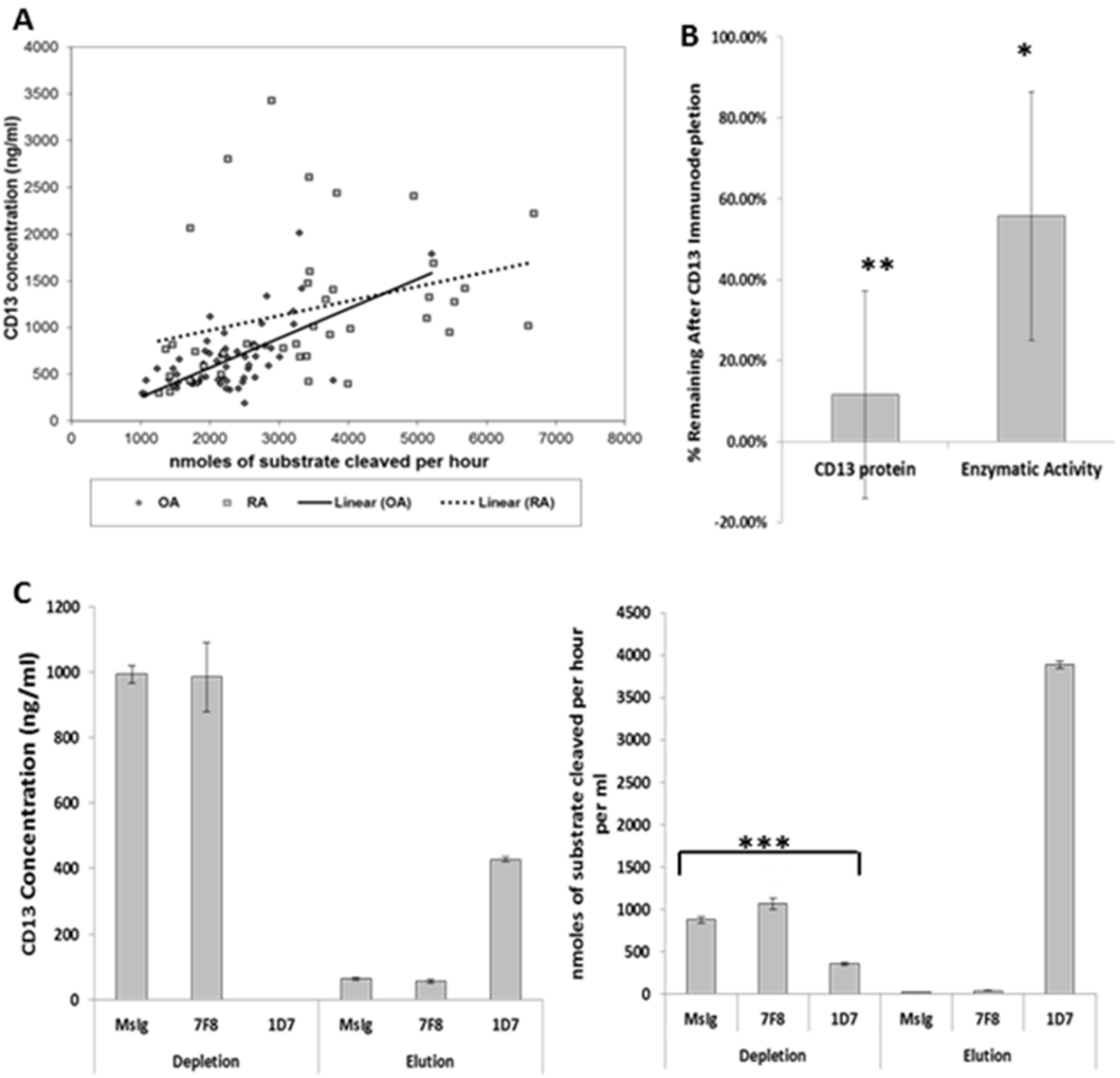

Figure 2.8 CD13 accounts for most but not all of the aminopeptidase activity in synovial fluid.

A, ELISA and enzymatic activity assay results for RA and OA synovial fluids were correlated separately and together. Correlations were analyzed by ANOVA, all points together R^2=0.1365, p=4.24x10^{-9}, OA alone R^2=0.418, p=3.17x10^{-8}, RA alone R^2=0.0947, p=0.057. (methods p138-139) **B,** Synovial fluids (n=9) were immunodepleted with anti-CD13 (1D7), Ig isotype control (MsIgG), or an isotype matched non-relevant antibody (7F8 [CD98]) using an immunoprecipitation kit. The removed protein was eluted off the beads by low pH. Values are the mean±SEM. * p<0.05; ** p<0.001 (method p140) **C,** Example of complete depletion of an RA synovial fluid as measured by enzyme-linked immunosorbent assay (ELISA) and the corresponding enzymatic activity (1 synovial fluid 3 replicates). Left, values are the mean±SEM. Right, values are mean±SD ***p≤0.0001 unpaired two tail t-test (methods p139-140)

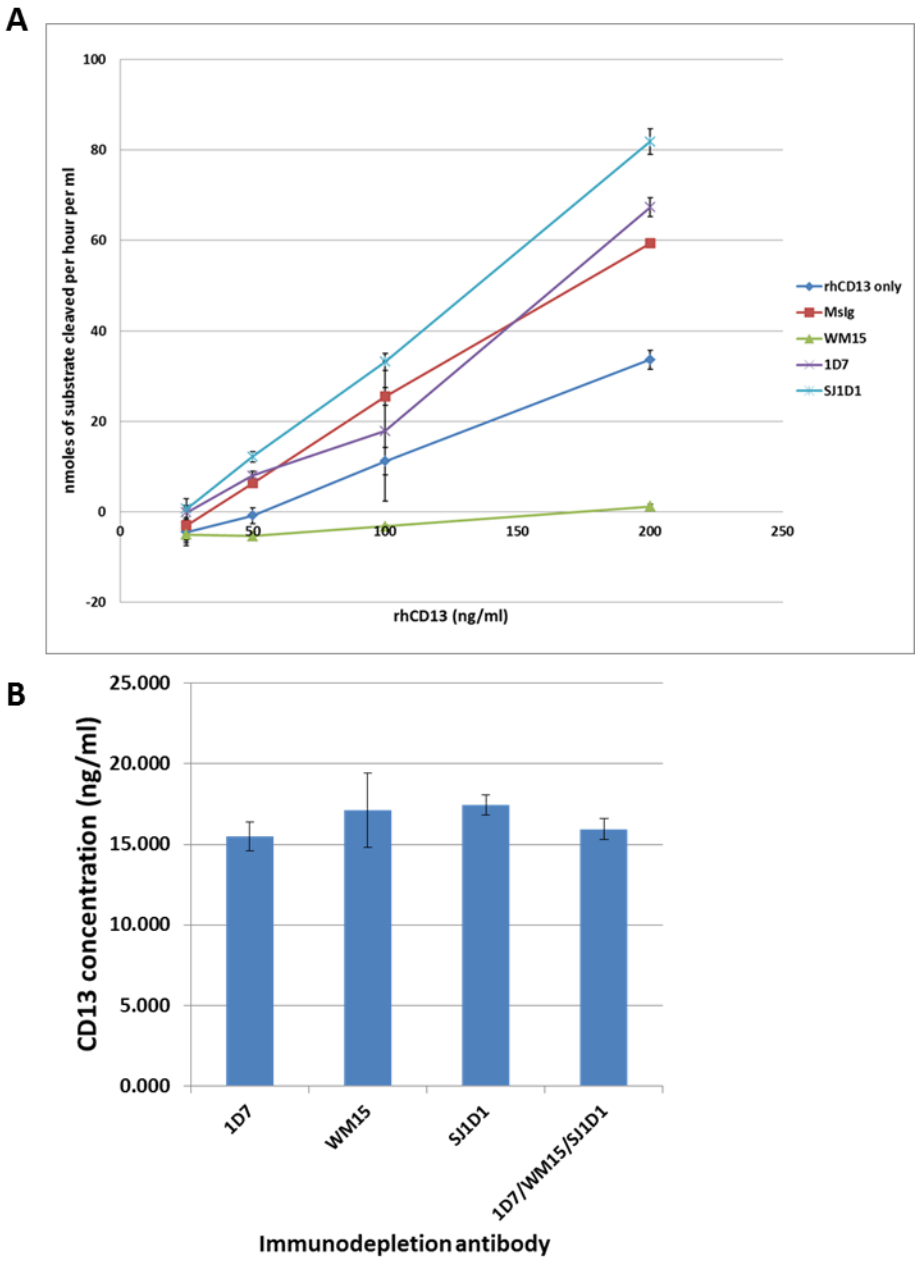

Figure 2.9 1D7 efficiently depletes CD13 from synovial fluid and does not inhibit CD13 enzymatic activity.

A, Recombinant human CD13 (rhCD13) at various concentrations (25ng/ml-200ng/ml) was incubated with the anti-CD13 antibodies 1D7, WM15, and SJ1D1 and with an isotype control MsIgG for 1hour at 37°C. An aminopeptidase activity assay was then run. (method p138) **B,** One synovial fluid was immunodepleted with 1D7, WM15, SJ1D1, or all three. Depleted fluids were then assayed for remaining CD13 by ELISA. mean±SD (method p139)

Discussion

It has become clear that RA is not mediated by a single cell type but rather by interactions between the various cells in the RA joint: including FLS and T cells. RA FLS express matrix metalloproteinases (MMPs), secrete pro-inflammatory mediators, and produce chemotactic agents to attract inflammatory cells to the joint. T cells are prime producers of multiple pro-inflammatory cytokines (such as IL-17, TNFα, IFNγ). In this chapter we examined CD13 on FLS as another potential mediator of FLS/T cell interactions in the RA joint.

Although CD13 has been identified in synovial fluids by Western blot and through N-aminopeptidase enzymatic activity assays, it has never been directly measured. We developed a novel CD13 ELISA and showed that CD13 is significantly higher in RA SFs than in OA SFs, HC serum, or RA serum. We also noted a significantly higher amount of CD13 in HC serum when compared to RA serum (Figure 2.3B). We observed higher co-staining of CD13 and a monocytic linage marker (CD11c) in RA synovial tissues than in OA or normal (Figure 2.5). These data suggest that activated, CD13 expressing monocytes migrate into the synovium in RA. We were specifically interested in whether FLS could contribute to the sCD13 we found in the SF. FLS highly express surface CD13 and are therefore a logical choice for shedding of CD13. We were able to show that sCD13 does come from FLS (Figure 2.7C), and can thereby infer that FLS contribute to the sCD13 in SF. We expected that CD13 would be elevated on RA FLS when compared to OA FLS, as previously published [169]. However, our data showed no noticeable difference (Figure 2.7). We noted a large variation in the intensity of CD13 expression on our cell lines and in SF and serum samples. We believe that this variation may account for the differences between our results and the previous publication [169].

There are at least four possible explanations for the higher amount of sCD13 in RA versus OA SFs. First, the RA joint environment includes cytokines and growth factors that could upregulate CD13 expression or release from the FLS *in vivo* in a manner that might not be maintained *in vitro* [8]. This is supported by our finding that IL-17 increases surface expression of CD13 (Figure 2.1A). Second, synovial tissue undergoes hyperplasia in RA resulting in much higher numbers of FLS that could release CD13. Third, the increase could be due to other cell types. Endothelial cells and cells of the monocytic cell lineage express surface CD13 [70,76,85]. These cells are often found in higher numbers in the RA joint and so could account for the increased sCD13. Lastly, there is increased expression of proteases in the RA synovium which could increase shedding of CD13 from the FLS [225]. The most likely explanation for the higher sCD13 in RA synovial fluid combines all four possibilities. The complex milieu present in the RA joint contributes to the higher number of FLS and other cells as well as regulation and shedding of CD13 from those cells, although monocytic shedding of CD13 has only been observed previously in apoptotic cells [226].

We wanted to ensure that the aminopeptidase activity we were measuring could be attributed to CD13. 1D7 was used for the immunodepletion since it does not block enzymatic activity (Figure 2.9). We found that CD13 contributes the majority of the N-aminopeptidase activity to the synovial fluids; however, it appears that at least one other protein present has similar activity (Figure 2.8)[88]. The correlation analysis (Figure 2.8A) showed a few synovial fluid samples that also appear to have a high concentration of CD13 but lower enzymatic activity. This could indicate the presence of natural inhibitors and/or intrinsic differences in the CD13 protein from

post-translational modifications resulting in differing degrees of enzymatic activity. Natural inhibitors may be higher in some people leading to the pattern of high CD13 protein low aminopeptidase activity. Natural inhibitors of metalloproteinases are often found in higher levels in RA than OA[180]. Specifically, one suggested inhibitor of CD13, substance P, has been found in RA synovial fluid in higher concentrations than in normal synovial fluid [110,227]. The higher levels of these types of inhibitors may explain the RA patients with CD13 protein but low aminopeptidase activity. It is also possible that these individuals have lower amounts of other aminopeptidases than average leading to lower overall aminopeptidase activity.

In this chapter we also examined the CD13 in serum from various inflammatory conditions (Figure 2.6). It is interesting to note that the only disease with significantly increased amounts of serum CD13 over healthy controls was cGVHD. CD13 has been identified in cGVHD as a possible component of a biomarker panel [129]. In general the other rheumatologic conditions (myositis, lupus, and scleroderma) more closely matched the pattern seen in RA with a decrease in serum CD13 or enzymatic activity. In general due to the high degree of variability of CD13 between individuals, higher numbers of samples would be needed to reach a definitive conclusion about CD13 in these diseases. However, one interesting observation could be made. When scleroderma samples were separated into limited versus diffuse disease types a difference was observed. The limited scleroderma samples contained lower levels of CD13 than HC sera, while the diffuse scleroderma sera contained higher levels of CD13 than HC sera. This may implicate systemic CD13, as represented by high serum levels of CD13, in more systemic diseases. This could include both cGVHD and diffuse scleroderma. While in diseases with more specific sites of inflammation (such as RA, myositis, and limited scleroderma), a decrease

is seen in systemic CD13 while CD13 increases at the sites of inflammation. We saw this pattern in RA, low serum CD13 and high synovial fluid CD13. Further studies would be needed to determine if this pattern is present in diseases other than RA.

In total, in this chapter we demonstrated that CD13 is elevated in the RA joint. It is highly expressed by FLS and can be released in soluble form from FLS into the synovium. CD13 is the major, but not only, contributor to N-aminopeptidase activity in the RA synovium. These data are suggestive of a role for CD13 in RA pathogenesis.

Chapter 3

CD13 on Fibroblast Like Synoviocytes: Expression, Shedding, and Regulation

In the previous chapter we demonstrated that CD13 is elevated in RA *in vivo*. We first identified CD13 through a screening to identify a protein on the surface of RA FLS that was differentially regulated by IL-17 and TNFα. However, it became apparent that we had only observed a small picture of the regulation of CD13. The identification was made based on cell surface CD13 at 48 hours; however, there are other possible locations for CD13 identified *in vitro* and *in vivo*. CD13 is primarily a cell surface molecule; however, it has also been identified in soluble fractions of biological fluids. We showed that CD13 is upregulated in RA synovial fluid compared to osteoarthritis (OA) synovial fluid, normal human serum, or RA serum. CD13 was also found in fibroblast like synoviocytes (FLS) culture supernatants demonstrating that CD13 is released from FLS. CD13 has been identified as a truncated soluble protein in human serum by Western blot; however, because CD13 is highly expressed on the cell surface, extracellular vesicles, which can reflect the protein composition of the cell surface, are another potential source of CD13 in cell free fractions [90,91].

Extracellular vesicles (EVs) are composed of a variety of small vesicles including exosomes, microparticles, and apoptotic bodies. Apoptotic bodies are released by dying cells and

microparticles are released primarily from platelets, but exosomes can be released from a wide variety of cell types including FLS [202]. Exosomes are small (40-120 nm diameter) lipid bilayer vesicles that typically express a surface profile similar to that of the cells from which they are released [202]. CD13 has been previously demonstrated to be on exosomes from microglial cells and mast cells [203,207].

In this chapter we sought to further understand the expression and function of CD13 on human RA FLS. We examined the effect of three pro-inflammatory cytokines linked to RA on CD13 expression by RA FLS, and determined how CD13 is released from FLS. We also examined the possibility that CD13 is present on exosomes or other extracellular vesicles derived from FLS, and measured soluble versus vesicle bound CD13 in sera, synovial fluids, and FLS culture supernatants.

CD13 is present on extracellular vesicles including exosomes

In the last chapter we identified CD13 protein in cell free portions of human synovial fluid, serum, and FLS culture supernatants. While CD13 has been suggested to be present in soluble form in serum, the possibility of extracellular vesicle-associated CD13 has not been examined in these fluids [90,91]. However, CD13 is predominately a cell surface molecule, and as such it may be present on the surface of vesicles as opposed to being a true soluble protein. To test the possibility that EVs also contain CD13, we isolated EVs from fluids identified to have CD13 in the cell depleted fractions (FLS culture supernatants, normal human plasma, and RA synovial fluids) and measured CD13 in the EV and soluble protein fractions. We used differential ultracentrifugation to isolate EVs specifically around the size of exosomes. We identified CD13

in both soluble protein and exosome fractions in plasma, RA FLS culture supernatant, and RA synovial fluid (Figure 3.1). Plasma contained an average of 109.96±48.63 ng/ml of CD13 on EVs with an activity of 2753.38±531.20μM/hr and 235.85±164.52 ng/ml of soluble CD13 with an activity of 3484.21±657.93 μM/hr. FLS culture supernatant contained an average of 58.79±16.36 ng/ml of CD13 on EVs with an activity of 253.37±63.92 μM/hr and 59.56±32.76 ng/ml of soluble CD13 with an activity of 435.47±225.64 μM/hr. RA synovial fluid contained an average of 56.24±9.14 ng/ml of CD13 on EVs with an activity of 681.06±156.26 μM/hr and 141.48±21.98 ng/ml of soluble CD13 with an activity of 1852.39±200.68 μM/hr.

Although differential centrifugation is a suitable protocol for isolation of exosomes, there may be other contaminants of similar density (including apoptotic bodies and protein aggregates). To further define where CD13 is located we divided the EV fraction over a discontinuous Optiprep density gradient with seven fractions from 1.268g/ml to 1.031 g/ml. We visually confirmed a band at the density gradient between fractions 4 and 5 around where exosomes would be expected (density between 1.084g/ml and 1.163 g/ml fractions 3-5). We also found CD13 present by ELISA in all seven gradients plus originally isolated soluble protein fractions (Figure 3.2). The three fluids run (RA FLS culture supernatant, healthy control plasma, and RA synovial fluid) each contained a distinct pattern of CD13 localization (Figure 3.3). FLS culture supernatant was the most balanced with 48.67% soluble CD13, 29.76% exosomal CD13, and 21.58% other EV CD13. Healthy control plasma was predominantly soluble with 85.60% soluble CD13, 7.09% exosomal, and 7.31% CD13 on other EVs. The RA synovial fluid contained 67.55% soluble CD13, 11.92% exosomal CD13, and 20.53% CD13 from other EVs.

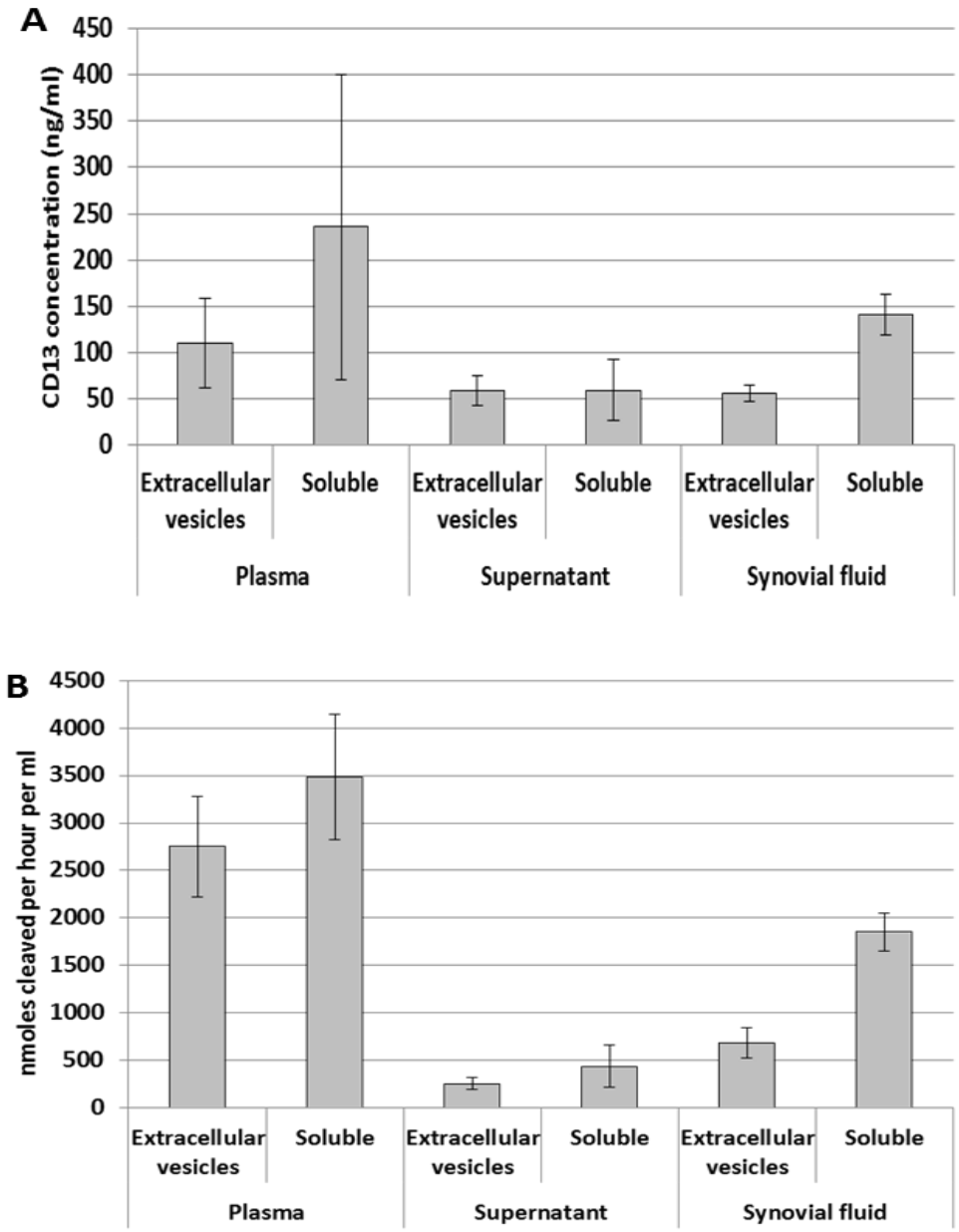

Figure 3.1 CD13 was found as a soluble protein and on extracellular vesicles in FLS culture supernatant, normal human plasma, and RA synovial fluid. Supernatants from 3 flasks of RA fibroblasts were concentrated through a 30K centrifugal filter. RA synovial fluid was diluted with PBS (4:1), and 10mls of plasma was obtained from a healthy individual. Vesicle fractions were isolated from the samples by serial centrifugation. Supernatants from the final centrifugation were collected as the soluble protein fraction. The vesicle pellet was resuspended in 1ml PBS. mean±SEM $n \geq 3$ (method p142)

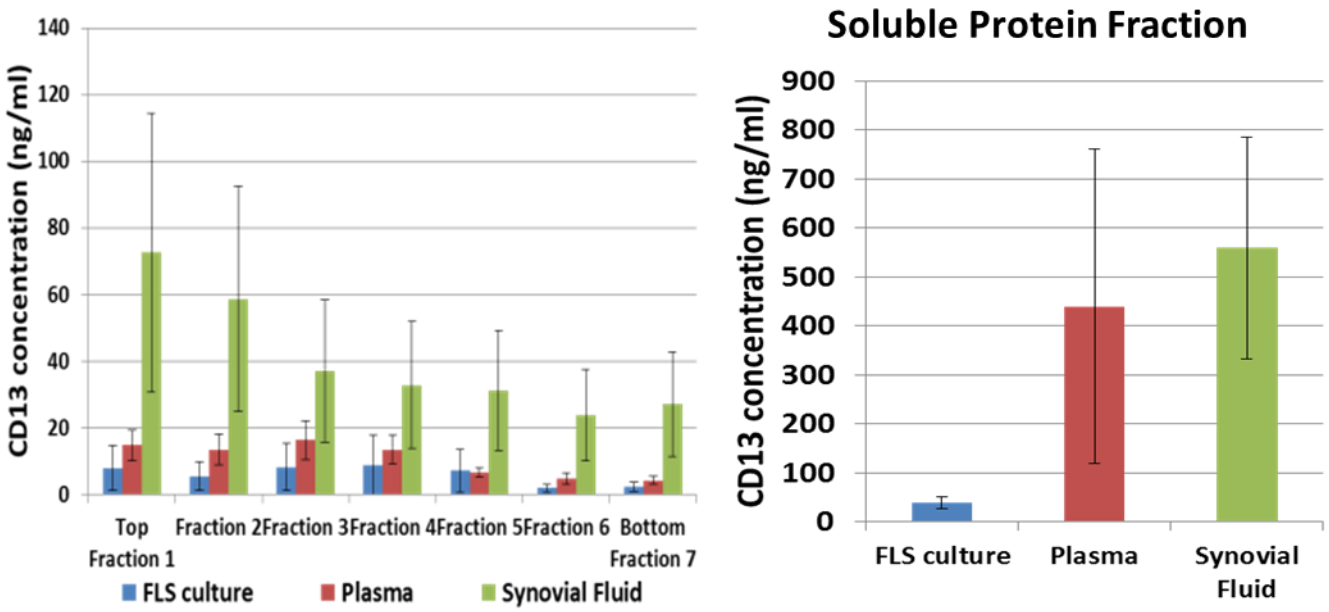

Figure 3.2 CD13 was found as a soluble protein and in multiple fractions of a density gradient separation of extracellular vesicles of FLS culture supernatant, normal human plasma, and RA synovial fluid.

A discontinuous optiprep gradient was created in seven fractions from 1.268g/ml to 1.031g/ml. 500ul of the resuspended vesicles was layered onto the top of the gradient. The loaded gradients were centrifuged at 100kg for one hour. Fractions were collected in reverse. Fractions were washed in PBS at 110kg for 2hr and the pellets were resuspended in 500ul PBS. mean±SEM $n \geq 3$ (method p142)

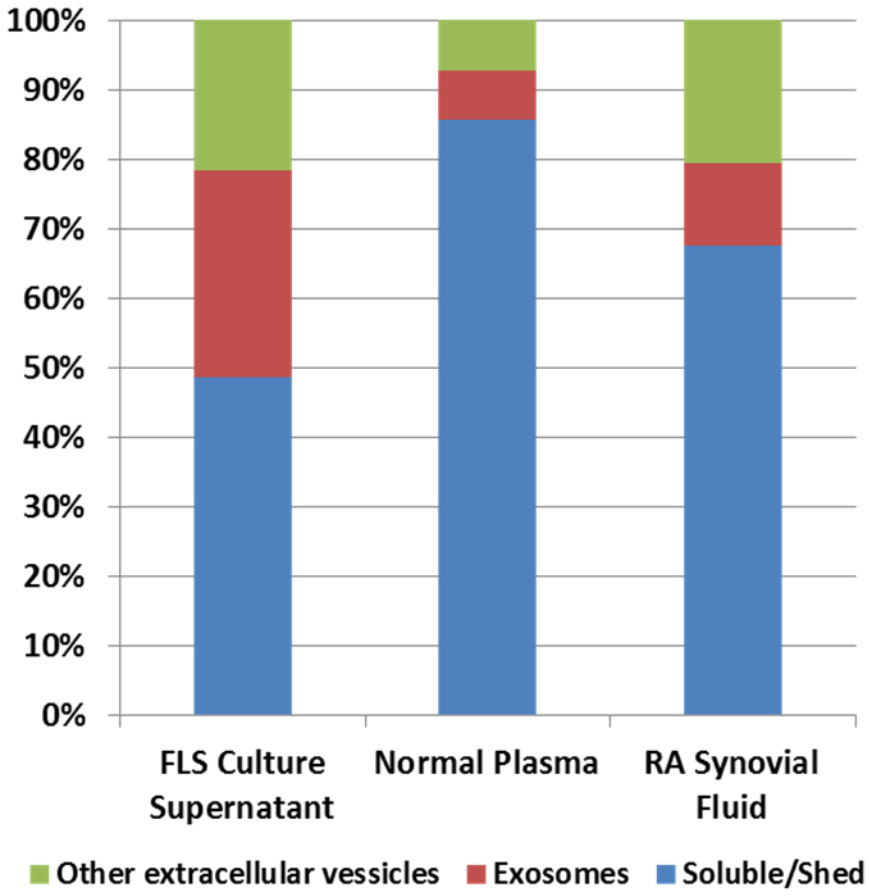

Figure 3.3 CD13 was found as a soluble protein and in multiple fractions of a density gradient separation of extracellular vesicles with distinct localization for FLS culture supernatant, normal human plasma, and RA synovial fluid.
Supernatants from 3 flasks of RA fibroblasts were concentrated through a 30K centrifugal filter. RA synovial fluid was diluted with PBS (4:1), and 10mls of plasma was obtained from a healthy individual. Vesicle fractions were isolated from the samples by serial centrifugation. Supernatants from the final centrifugation were collected as the soluble protein fraction. The vesicle pellet was resuspended in 1ml PBS. A discontinuous optiprep gradient was created in seven fractions from 1.268g/ml to 1.031g/ml. 500ul of the resuspended vesicles was layered onto the top of the gradient. The loaded gradients were centrifuged at 100kg for one hour. Fractions were collected in reverse. Fractions were washed in PBS at 110kg for 2hr and the pellets were resuspended in 500ul PBS. Data is expressed as percentage of total fluid. n≥3 (method p142)

Metalloproteinases cleave CD13 from FLS

Since CD13 exists as a soluble molecule in cell-free portions of biological fluids separate from vesicle-associated CD13, soluble CD13 must be released from cells. Since soluble CD13 was also found in FLS culture supernatants, we decided to explore how CD13 was being released from FLS. CD13 is highly expressed on the cell surface of FLS and as such the most likely hypothesis is that CD13 is shed from the cell surface. To test this mechanism we added protease inhibitors to FLS cultures covering all classes of proteases. We tested five protease inhibitors: pepstatin A (aspartic), aprotinin (serine), leupeptin (serine/cysteine), GM6001 (metalloproteinase), and E-64 (cysteine), and found that only one, GM6001, blocked CD13 release from FLS. GM6001significantly reduced CD13 protein found in the supernatant by 93.62±4.78%, $p \leq 0.0001$ (Figure 3.4A). Leupeptin led to a significant ($p \leq 0.05$) increase (48.40±14.29%) in CD13 released. To confirm that we were affecting cleavage and not expression, CD13 was also measured in the FLS cell lysates. In the cell lysates no significant difference was observed with GM6001; however, aprotinin did induce a significant increase (22.17±6.18%, $p \leq 0.05$) in CD13 expression (Figure 3.4B). These results indicate that CD13 is cleaved from FLS by metalloproteinases.

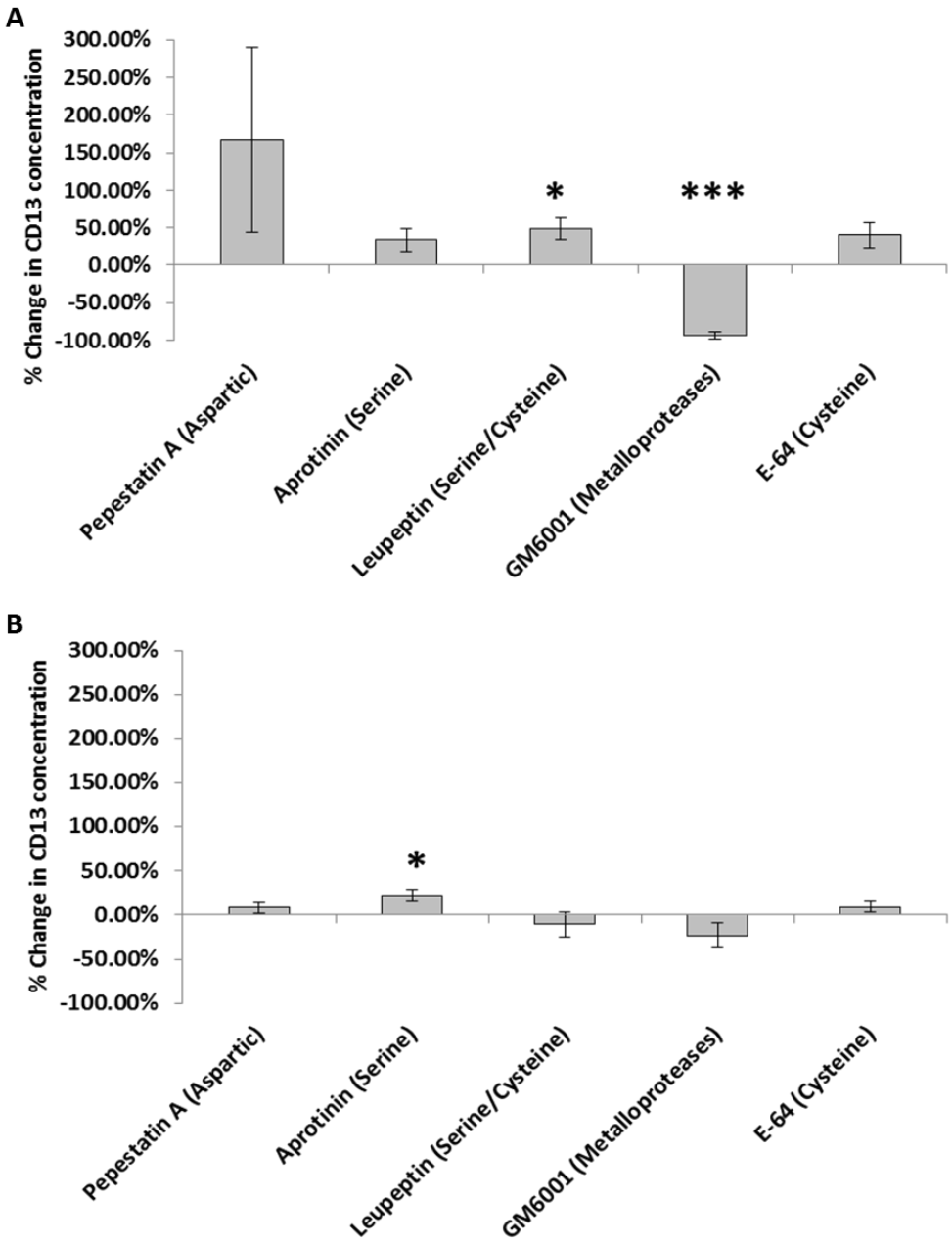

Figure 3.4 Metalloproteinases cleave CD13 from the surface of FLS.
Five different protease inhibitors were added to FLS cultures covering all classes of proteases. Data is expressed as % change from untreated FLS (untreated FLS at line 0). **(A)** The only inhibitor to decrease shedding of CD13 into the supernatant of the cultures was GM6001. **(B)** No significant decreases were seen in cell lysate CD13 concentrations. Cultures were incubated with serum free media containing protease inhibitors for 48 hours: Pepstatin A (aspartic) 10μM, Aprotinin (serine) 100nM, Leupeptin (serine/cysteine) 10μM, GM6001 (metalloproteinase) 25μM, and E-64 (cysteine) 10μM. n=3 mean of % change ± SEM *$p<0.05$; ***$p<0.0001$ (methods p138-139)

MMP14/MT1-MMP cleaves CD13 from FLS

MMP14/MT1-MMP is a cell surface metalloproteinase on FLS that is critical to FLS invasion of collagenous structures[49,177]. To test the hypothesis that MMP14 also releases CD13, we knocked down this protease on RA FLS using siRNA. Figure 3.5 shows an example of successful knockdown of MMP14. Fold change of MMP14 mRNA over GAPDH (ΔCt) of FLS was 20.52 and was decreased to 0.50 with addition of an MMP14 siRNA cocktail ($p=5.63x10^{-7}$, Figure 3.5A). GFP plasmid transfection was used to determine transfection efficiency. Higher fluorescence was observed with both flow cytometry and fluorescent microscopy over mock transfection controls. Green fluorescence measured by flow cytometry went from 6.77 mean fluorescent intensity (MFI) in the negative control (mock transfected) to 111.23 MFI in the transfected FLS (Figure 3.5B). Knockdowns (KDs) were done with siRNA cocktails of 3 individual siRNAs. KD was confirmed with each siRNA separately, and mRNA and protein results were measured in the same group of cells harvested on the same day

KD of MMP14 significantly decreased the CD13 released from FLS (Figure 3.6A). Samples were normalized to mock transfection in order to compare between experiments ($n\geq5$) and mock transfection levels are shown on the graph as a line at one. The only significant difference in the siRNA KD was seen with MMP14, in which case the KD cells released CD13 at 68%±7% of control levels, p=0.00045 (Figure 3.6A). To confirm we were measuring cleavage and not a decrease in CD13 expression, CD13 protein was also measured in cell lysates. KD of MMP14 did not significantly alter cellular CD13; MMP14 KD ratio over mock was 1.25±0.53 (Figure 3.6B). Because MMP14 KD resulted in an average decrease of only 32% in supernatant CD13, this indicates that more than one protease cleaves CD13. To confirm that the other CD13-

releasing protease(s) are also metalloproteinases, GM6001 was added to MMP14 KD cultures (Figure 3.6A, n=2). Similar to previous results, GM6001 prevented the release of CD13 into the culture supernatant (mock to mock+GM6001, 1 to 0.10±0.05 $p=1.42x10^{-9}$). Addition of GM6001 to MMP14 KD cultures decreased supernatant CD13 to a similar level, 0.11±0.081, significantly lower than MMP14 KD without GM6001, $p=3.21x10^{-9}$. GM6001 treatment significantly increased CD13 in the cell lysate of mock transfected cells, mock+GM6001 ratio 1.38±0.20 p=0.0070.

To confirm MMP14 as a cleaver of CD13, confocal microscopy was used to look for co-localization on the surface of RA FLS (Figure 3.7). Cells were stained with DAPI for nuclei (blue), anti-CD13-FITC (green), and anti-MMP14-PE (red). Anti-CD90-PE was used as a positive control for FLS and negative control for co-localization. We observed co-localization of CD13 and MMP14 on all tested FLS. The co-localization was predominant on all cell lines tested with minor areas of individual staining. Images shown in Figure 3.7 are representative of n=5.

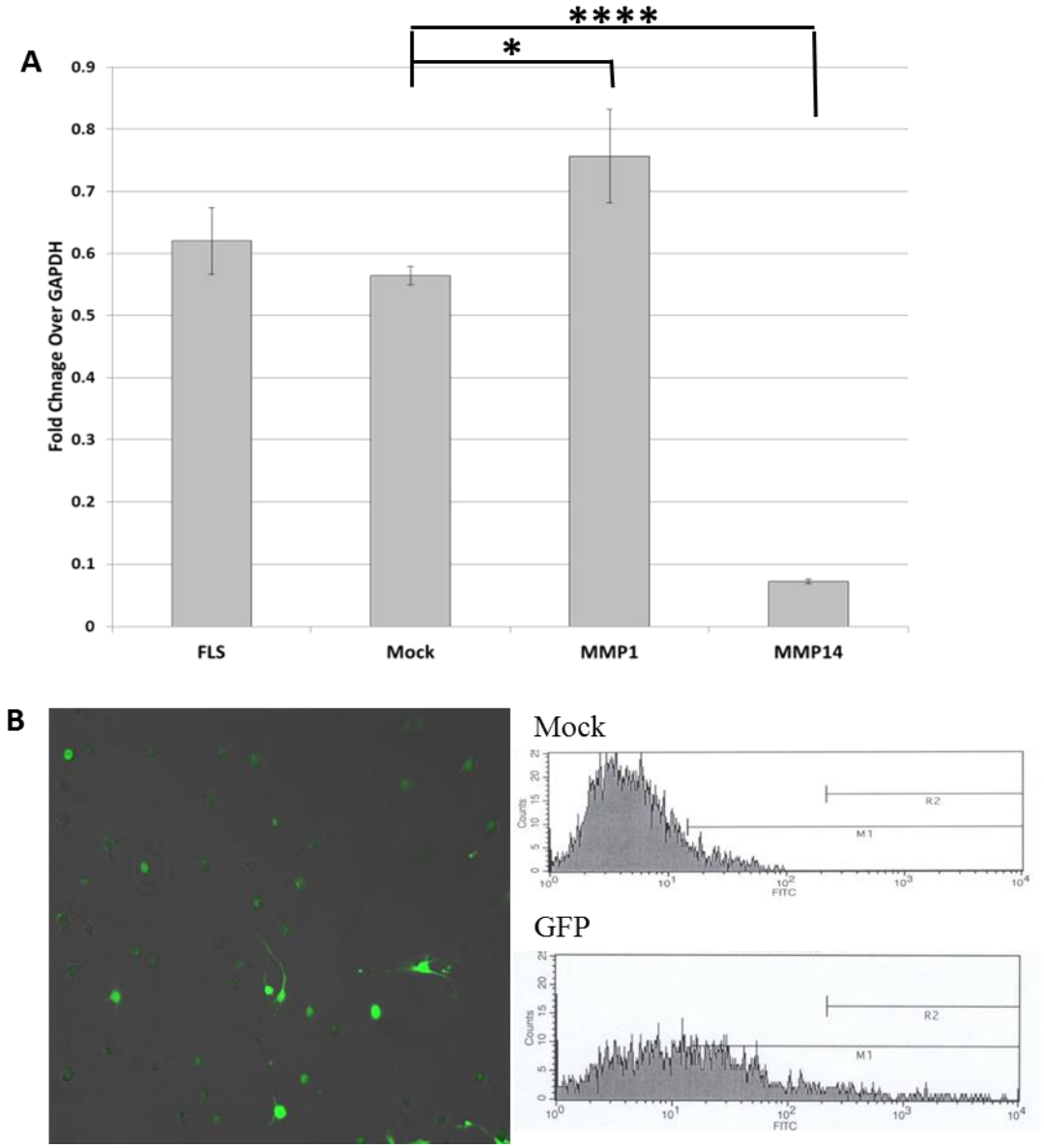

Figure 3.5 MMP14 siRNA knocks down MMP14 in RA FLS.
A, MMP14 mRNA was almost completely removed by transfection with MMP14 siRNA. n=3 representative of 7 transfections mean±SD * $p<0.05$, **** $p<0.00001$
B, Transfection efficiency was determined by increase in GFP by flow cytometry and flourescent microscopy with transfection of a GFP vector. The flourescent microscopy image shows green above mock control background. n=1, representative of 7 transfections (methods p141)

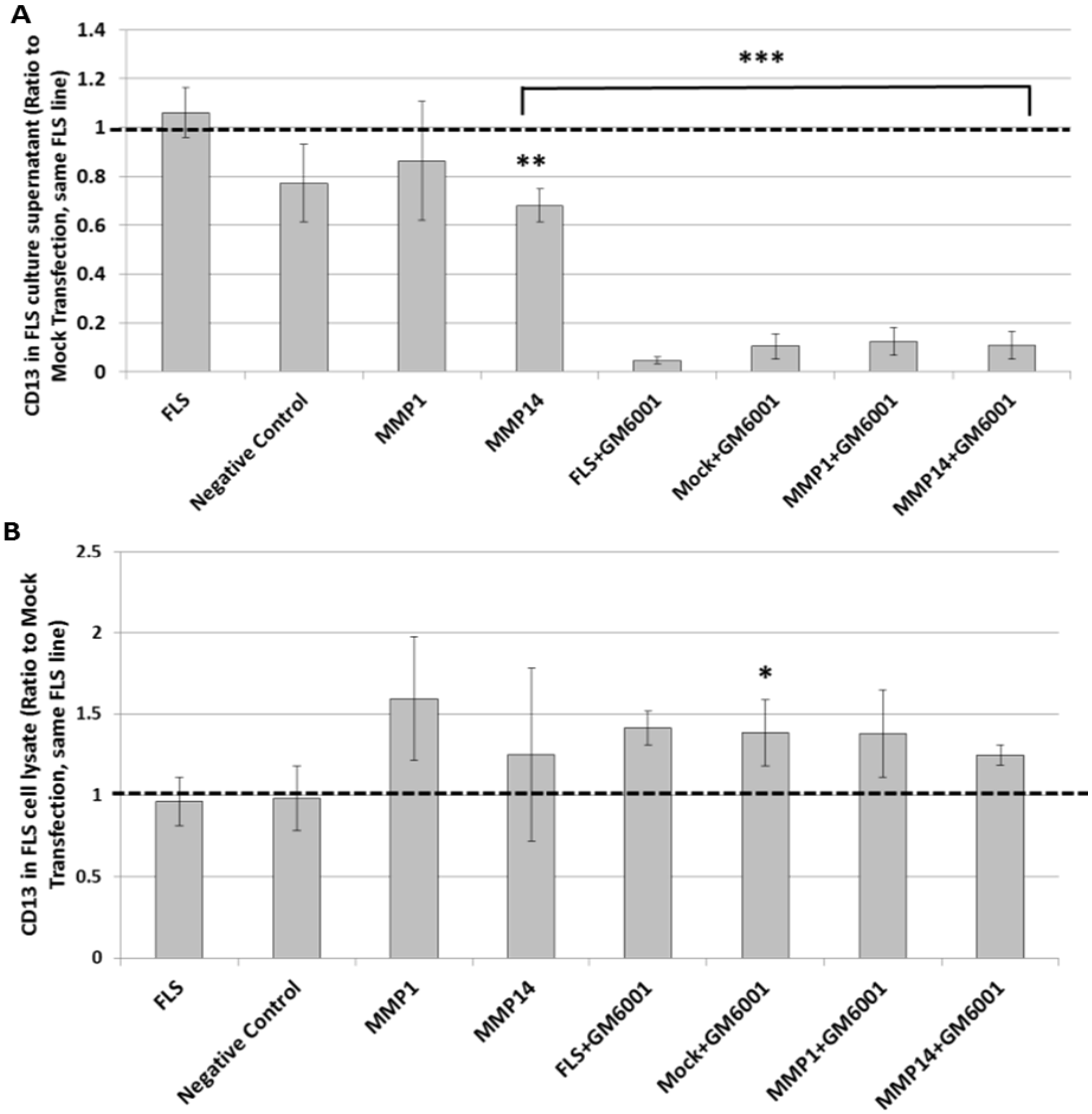

Figure 3.6 MMP14 knockdown partially inhibits the shedding of CD13 from FLS. **A**, Shedding of CD13 into FLS culture supernatant was inhibited by knockdown of MMP14. Further inhibition is seen with GM6001. **B**, CD13 in FLS lysate was not changed by knockdown of MMP14. FLS were grown to confluence then transfected with siRNA using an Amaxon nucleofector kit. Cells were grown to 75% confluence then switched to serum free growth media (Peprogrow) supplemented with 1ng/ml IL-1 and 10ng/ml TNFα for 48 hours with or without 25μM GM6001 . * $p<0.05$; ** $p<0.0005$; *** $p<0.0001$ mean±SEM ($n\geq2$) (methods p141 and 138)

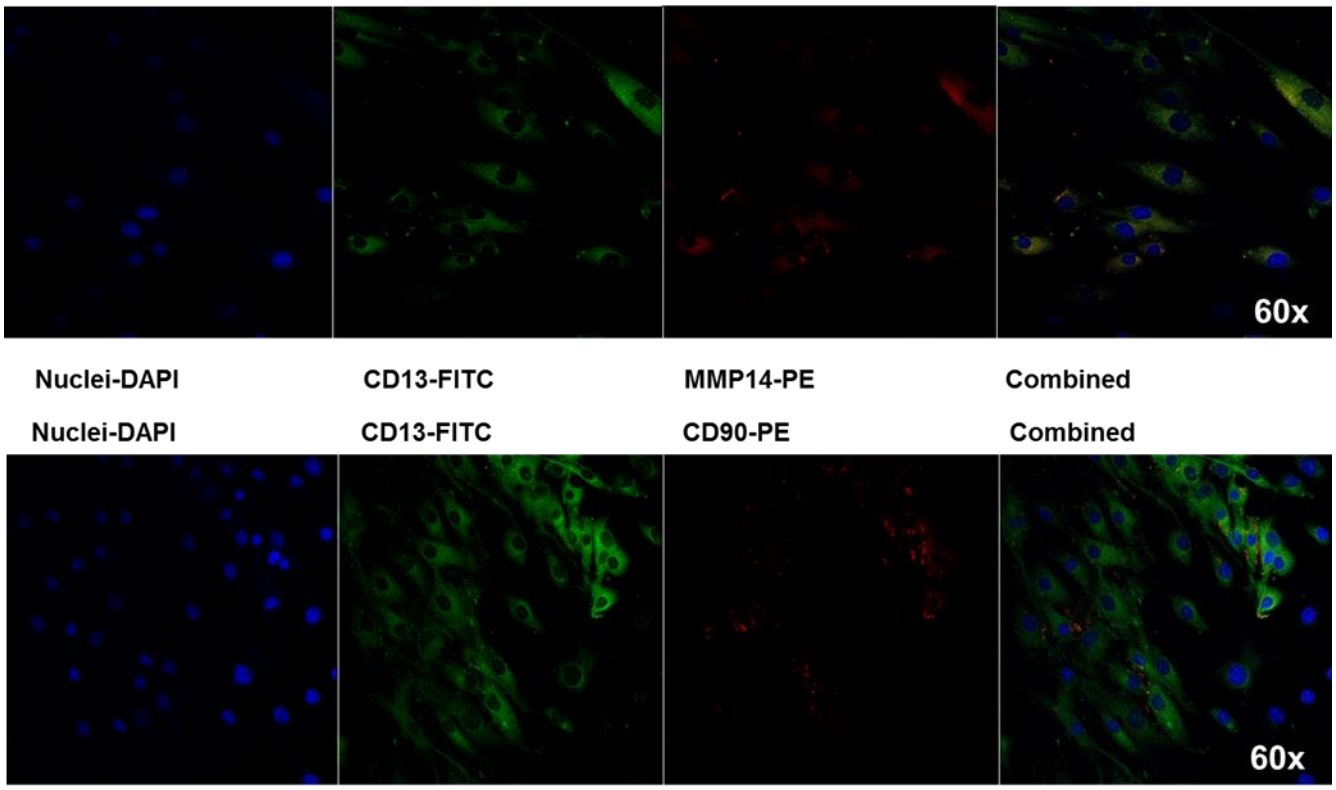

Figure 3.7 Fluorescent staining of FLS shows co-localization of CD13 and MMP14.
RA FLS were grown to 90% confluence on 8-well glass chamber slides. Cells were fixed with 1% Formalin and blocked with Fc block (10% human serum/10% mouse serum in PBS). Cells were incubated for 1hour at room temperature with anti-CD13-FITC (1D7) 1μg/100μl and anti-MMP14-PE (128527) at 1.67μg/100μl (appropriate isotype controls and single staining were also done, not shown). The nuclei were counter stained with DAPI at 1μg/ml. Cells were mounted using anti-fade media. Confocal microscopy was done using an Olympus microscope. All images corrected for background – thresholds determined by DAPI alone, MsIg-FITC alone, and MsIg-PE alone. Representative of n=5 (method p142)

Regulation of CD13

CD13 is strongly upregulated in RA SF over OA. However, *in vitro* RA and OA FLS cultures expressed similar amounts of CD13. One possibility to explain this observation is that the pro-inflammatory cytokines in the RA joint could contribute to the upregulation of CD13. Our initial screening experiments identified CD13 as being upregulated by IL-17 and downregulated by TNFα (Figure 2.1); however, as these studies progressed we realized that CD13 has multiple cellular locations (surface, soluble, EV-associated, intracellular). Our initial study had only examined CD13 cell surface expression at 48 hours. We decided to further examine the effect of pro-inflammatory cytokines on FLS CD13 expression. Cultured RA FLS were treated with IFNγ, TNFα, or IL-17 over a time course from 0 to 72 hours. CD13 mRNA was measured by quantitative PCR, and was upregulated by all three cytokines with a peak expression around 48 hours (Figure 3.8A). Data shown are a ratio of cytokine treated FLS CD13 mRNA to untreated FLS CD13 mRNA at the same time point. Figure 3.1 shows one FLS cell line and is representative of three FLS cell lines. Peak mRNA following exposure to IFNγ was at 48 hours and was 3.46±0.76 fold higher than in untreated FLS ($p=0.0066$). The peak IL-17 effect on CD13 mRNA was also at 48 hours and was 2.59±0.077 fold higher ($p=0.0011$). The effect of TNFα peaked at 24 hours with a fold increase of 3.43±0.67 ($p=0.0034$). Overall IFNγ and TNFα significantly upregulated CD13 mRNA ($p\leq0.05$) at 12, 24, 48 and 72 hours, and the effect of IL-17 was significant at 12, 24, and 48 hours. However, cell surface expression of CD13 on the three cell lines exhibited variability and fluctuations that did not match the change in CD13 mRNA. Figure 3.8B shows the results of one cell line examined by flow cytometry with staining by anti-CD13 (1D7).

We also examined the correlation between the cytokines (IFNγ, TNFα, and IL-17) and CD13 *in vivo*. We measured CD13 and the cytokines in serum from a variety of immune-mediated rheumatologic conditions including: RA, scleroderma, lupus, Sjogren's syndrome, and myositis. Cytokines in serum can be low and difficult to accurately measure so the data shown here is only for the samples that had measureable amounts of the cytokine. IFNγ levels did not significantly correlate to CD13 amounts in the serum, R^2=0.036 ANOVA p=0.45 (Figure 3.9A). TNFα levels were significantly correlated to CD13 levels, ANOVA p=0.026, but the correlation was not strong, R^2=0.22 (Figure 3.9B). The strongest correlation was between IL-17 and CD13, R^2=0.61, and the correlation was also highly significant, ANOVA p=1.33×10^{-14} (Figure 3.9C).

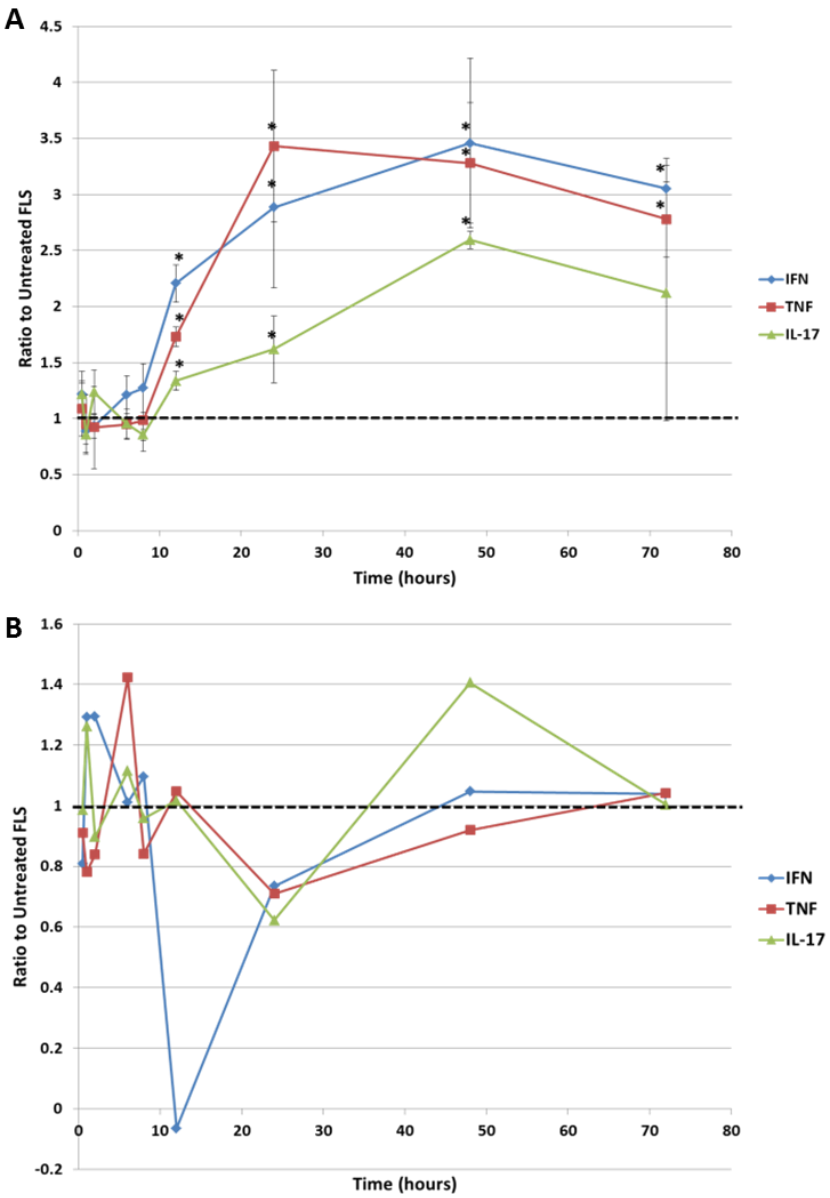

Figure 3.8 CD13 is upregulated on FLS by IFNγ, TNFα, and IL-17 at the mRNA level but the effects on surface CD13 are varied.

FLS were stimulated over a time course of 0-72 hours with IFNγ (1U/ml), TNFα (10ng/ml), or IL-17 (10ng/ml) (method p137). Cells were harvested and processed for either mRNA (A) or surface expression (B). mRNA was measured by qRT-PCR (method p140). CD13 was measured on the surface with anti-CD13 (1D7) and flow cytometry (method p138). Gating was done to isolate the major cell population and exclude debris and dead cells. Data is expressed as a ratio to unstimulated FLS at the same time point in either ΔΔCt normalized to GAPD (mRNA) or mean fluorescent intensity corrected for MsIg (surface) mRNA n=3 flow n=1 Figures are for 1 cell line and representative of 3 *p<0.05

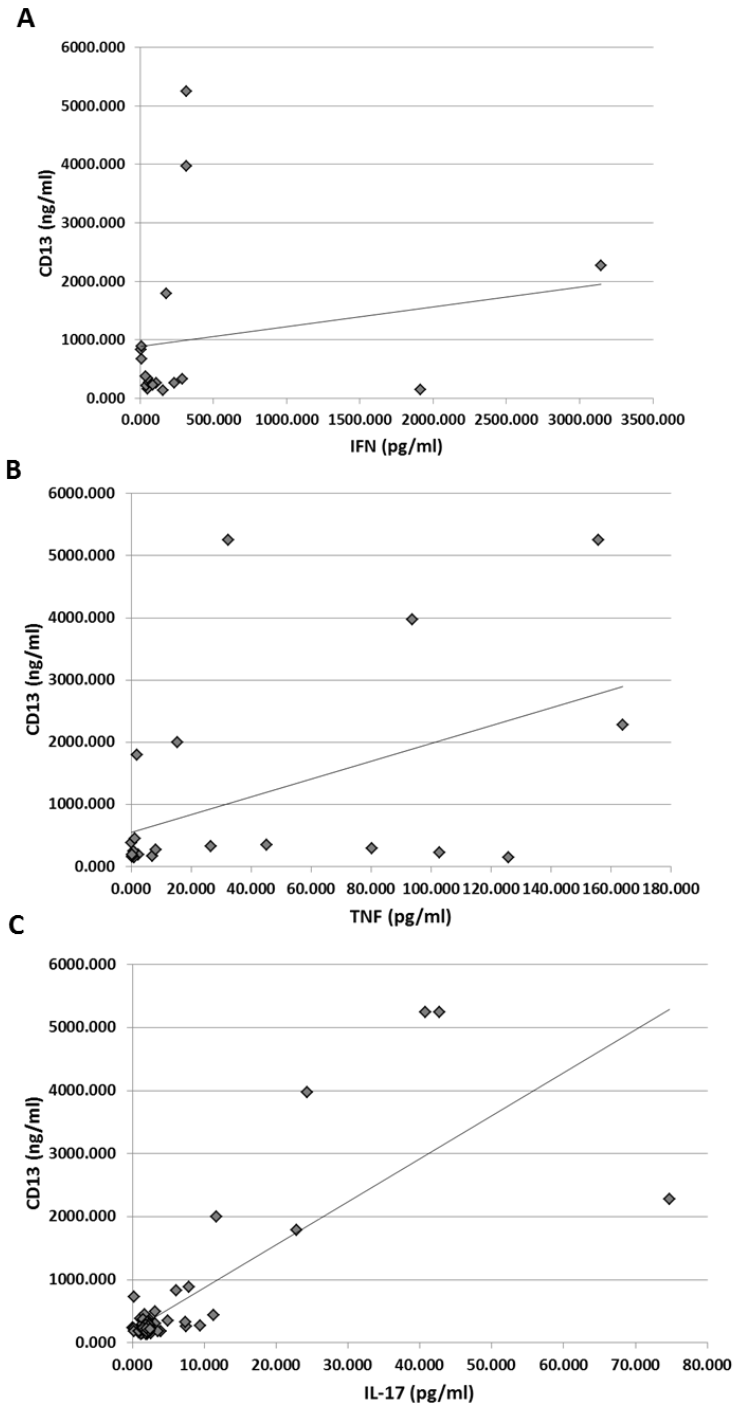

Figure 3.9 CD13 correlates with TNFα and IL-17 in the serum of patients with inflammatory, rheumatologic diseases.

Serum was collected from patients with a variety of autoimmune diseases. Levels of CD13, IFNγ (A), TNFα (B), and IL-17 (C) were measured by ELISA. Data shown are samples with measureable levels of the cytokines. Correlation significance was determined by ANOVA. (methods p137 and 141)

Discussion

CD13 has been identified as a protein highly expressed by RA FLS, and may contribute to T cell-FLS interactions. In the joint, T cells are known to activate RA FLS through cell-cell interactions and the release of pro-inflammatory cytokines. This activation can result in greater production of chemokines and other pathogenic proteins by the FLS resulting in a self-perpetuating, pro-inflammatory cycle [65]. To determine whether CD13 was part of this loop, we examined the effect of three pro-inflammatory cytokines on CD13 expression by FLS. CD13 mRNA was upregulated by IFNγ, TNFα, and IL-17 in FLS. However, the primary location for CD13 expression by FLS (the cell surface) did not match this regulation pattern. Even before the mRNA was upregulated (peak around 48 hours) the cytokines were inducing fluctuations in cell surface CD13 (Figure 3.8). This led us to infer that the cytokines were also likely leading to changes in the localization of CD13 protein. CD13 has also been found in the cell free portions of various biological fluids, raising the possibility that CD13 could be released by the FLS from the cell surface [88,89].

We also examined the correlation of this putative soluble CD13 in serum with the pro-inflammatory cytokines present in serum. Interestingly despite the increase in CD13 mRNA in FLS with treatment by IFNγ, we did not see a correlation between CD13 and IFNγ in serum from patients with inflammatory conditions. This may indicate a difference in protein production versus mRNA; however, there were increases in surface CD13 at some time points on FLS with IFNγ treatment. This could also represent a difference in biological localization. While IFNγ can increase CD13 in the joint by an effect on FLS, this effect may not carry over to other cell types and sera concentrations. Alternatively, this could indicate difference in the strength of

regulation when in a complex biological environment. In our experiments we treated FLS with one cytokine at a time. In the body, represented by the serum, there are multiple cytokines with possible regulatory effects on CD13. The lack of correlation with IFNγ in sera could indicate that in an IFNγ dominated disease other cytokines are also upreguated that downregulate CD13. The effect of these hypothetical down-regulators could be stronger than the upregulation of IFNγ. We did see correlations between CD13 amounts and both TNFα and IL-17 sera concentrations. These data corroborate what we saw with RA FLS CD13 mRNA regulation. This could indicate that in IL-17 or TNFα dominated disease states, the upregulation of CD13 by these cytokines is the dominate effect on CD13 expression. A third possibility is that the three cytokines differentially regulate release of CD13. The correlation of sCD13 in serum with TNFα and IL-17 could indicate that these cytokines aid in the release of CD13 as well as upregulate the mRNA, while IFNγ upregulates the mRNA but more specifically leads to cellular localization of CD13.

There are three possible mechanisms by which FLS may release CD13. First, as a cell surface molecule, CD13 may be cleaved by a protease. Second, CD13 could be secreted, a process which involves the golgi apparatus forming secretory vesicles (containing the soluble proteins) that undergo exocytosis. Third, CD13 could be secreted on the surface of extracellular vesicles such as exosomes. First we examined the possibility of CD13 as a shed molecule. Soluble CD13 identified in serum is truncated and lacks the intracellular and transmembrane domains, suggesting cleavage from the cell membrane [90]. We examined this mechanism using inhibitors specific for different classes of proteases: pepstatin A (aspartic), aprotinin (serine), leupeptin (serine/cysteine), GM6001 (metalloproteinases), and E64 (cysteine). Our data indicate that

CD13 is cleaved from FLS by metalloproteinases (Figure 3.4A). Metalloproteinases fall primarily into one of two groups, either matrix metalloproteinases (MMPs) or a disintegrin and metalloproteinase (ADAMs). Transmembrane proteases which are known to participate in cleavage and release of proteins anchored in the membrane are most likely responsible for release of CD13. Several of these proteases are members of the metalloproteinase family (MMPs14,15,16,17 and ADAMs).

Of the membrane-type metalloproteinases, MMP14 is found in the highest amount on RA FLS [178,181]. In RA, MMP14 has already been linked to matrix degradation by FLS and osteoclast-mediated bone resorption [178]. One study showed that of the MMPs found by RT-PCR on RA synoviocytes (MMP1,2,13,14,15) only MMP14 was shown to function as a type I and type II collagenase under biological conditions [49]. The same study also demonstrated that RA synoviocytes can induce angiogenesis at tissue sites proximate to the cells, a function that can be blocked by silencing MMP14. This data indicate that MMP14 can cleave a pro-angiogenic factor that may mediate angiogenesis in RA. Because CD13 is strongly linked to angiogenesis in tumors we hypothesized that MMP14 could cleave CD13 leading to subsequent angiogenesis [102,228,229]. Indeed, siRNA inhibition of MMP14 resulted in a significant decrease in CD13 cleaved from FLS (Figure 3.6A). Inhibition of all metalloproteinases by GM001 further reduced the CD13 released into the supernatant. However, MMP14 may be more involved than indicated in our results. While KD did result in most of the MMP14 mRNA being removed, the MMP14 protein was not successfully measured. As such there may be MMP14 protein remaining which cleaves the CD13, but even at the lowest CD13 in the supernatant was only reduced to around

35% (in one cell line). While some MMP14 protein may still remain, it seems likely that at least one other metalloproteinase, besides MMP14, sheds CD13 from FLS.

Looking at overall MMP expression in RA, stimulated RA synoviocytes have been shown to express mRNA for MMP1, 2, 13, 14, and 15 [49]. Although it is possible that the soluble MMPs (MMP1-13) may cleave CD13, cell surface enzymes are much more common in the role of sheddases. In addition, CD13 can be cleaved *in vitro* under serum free conditions. Soluble MMP activity is dependent on zymogen conversion, typically by plasmin [49]. Though MMP zymogen conversion can occur under serum free culture conditions, we did not see a decrease in CD13 shedding with aprotinin. Aprotinin inhibits plasmin activity and would therefore decrease the active forms of soluble MMPs in the culture supernatants. This is biologically relevant as serum and synovial fluid contain anti-proteinases that have been shown to limit collagenolytic activity of soluble MMPs from RA synoviocytes [49].

It is widely known that members of the metalloproteinase family are redundant, with multiple proteins performing the same functions. Collagenolytic activity is the most predominant, with most MMPs demonstrating this function. However, the similarity of cleavage sites and activity may also carry over to other substrates [177]. It is possible that other membrane bound MMPs (MMP15, 16, or 17) are also involved in CD13 shedding. MMP15 mRNA has previously been found in RA synoviocytes, and MMP16 has been found on synovial tissue biopsies [49,178]. The other possible group of CD13 sheddases is the ADAMs. In particular the two related ADAMs, ADAM10 and ADAM17, have been linked to shedding of various cytokines and chemokines [230]. In general ADAM10 is linked to constitutive cleavage while ADAM17 cleavage is in response to

a stimulus [231]. ADAM17 is particularly of interest as a recent publication has shown that CD13 and ADAM17 can associate on the cell surface. This study examined acute monocytic leukeumia cells and showed that when anti-CD13 antibodies were used to target CD13 it resulted in a downregulation of both surface CD13 and surface ADAM17[232]. This indicates that CD13 and ADAM17 co-localize on the cell surface and thus can be internalized together. Therefore the most likely molecules on FLS that could cleave CD13, other than MMP14, are MMP15/MT2-MMP, MMP16/MT3-MMP, and/or the ADAMs [181]. Attempts at single knockdown of MMPs15 or 16 or various ADAMs (ADAM10, 15, and 17) did not result in any significant reduction in CD13 released (data not shown). Based on our observations the most probable explanation of our data is that MMP14 is the primary cleaver of CD13 from FLS with multiple other metalloproteinases acting in a similar but redundant fashion.

To confirm MMP14 as a cleaver of CD13, we also looked for co-localization of CD13 and MMP14 on FLS. CD13 and MMP14 have previously been found in similar cell surface domains, but their proximity has not been determined. Both CD13 (FLS) and MMP14 (breast carcinoma and glioma cells) have been found in caveolae-enriched lipid rafts [86,197]. We show that on the surface of FLS CD13 and MMP do co-localize and in some cells we have observed a punctate pattern which may be indicative of inclusion into lipid raft structures. Overall these data are supportive of the conclusion that MMP14 can cleave CD13.

Because cleavage of CD13 did not appear to account for all of the CD13 in the supernatant we also looked at the possibility of CD13 as a vesicle-bound molecule. We used differential ultracentrifugation to isolate soluble proteins and exosomes/extracellular vesicles. We identified

CD13 both in the soluble fraction and around the density of exosomes (Figure 3.1). Further density separation identified CD13 at all densities (1.268-1.031 ng/ml), indicating its presence on exosomes as well as other extracellular vesicles of similar density. One problem with the differential centrifugation method is that it can isolate other EVs or large protein aggregates of similar density to exosomes. One group that can fall into this category is apoptotic blebs [233]. However, the additional separation by density gradient can distinguish between exosomes, other EVs, and protein aggregates. Apoptotic blebs float at about a >1.23g/ml density while exosomes float at 1.10-1.21g/ml [233]. We found CD13 on both exosomes and other EVs. Our results demonstrate that CD13 is present as both a soluble molecule and on extracellular vesicles derived from FLS. However, the proportion of CD13 on EVs in FLS supernatant exceeded the residual CD13 in supernatants of GM6001 treated cultures. This may be because metalloproteinase inhibitors, such as GM6001, are able to inhibit exosome release. Even though it is uncertain what role metalloproteinases assume in exosome release from cells, GM6001 inhibited the release of exosomes from endothelial cells [206]. Thus, the addition of GM6001 may be blocking both MMP-mediated cleavage of CD13 and some of the release of exosomes from FLS, leaving only a small portion of exosomes and other EVs expressing CD13 in the supernatant.

Overall, our results point to roles for CD13 in the pro-inflammatory milieu of the RA synovium. Previous data showed CD13 to be elevated in RA synovial fluid and present on RA FLS. In this chapter we have shown that CD13 is shed from RA FLS by metalloproteinases and is also released on extracellular vesicles. The CD13 in synovial fluid is predominately soluble, with CD13 also present on extracellular vesicles. While MMP14 has been identified as one molecule

that cleaves CD13, future studies will be needed to define other CD13 sheddases. The current results identify both CD13 and MMP14 as potential targets in amelioration of RA synovial pathology.

Chapter 4

Functions of CD13 in Rheumatoid Arthritis

We have demonstrated that CD13 is elevated in RA synovial fluid, expressed in RA synovial tissue, and expressed on RA FLS. In the previous chapter we showed that CD13 is not only expressed on the surface of FLS but is cleaved by metalloproteinases including MMP14 to become a soluble molecule. It is also found on extracellular vesicles including exosomes. We also demonstrated that it is upregulated by the pro-inflammatory cytokines IL-17, TNFα, and IFNγ with differing patterns of localization and kinetics. Having defined CD13 in the RA joint and on RA FLS the next question was: what is the function of CD13 in the RA joint? We examined the effect of CD13 on two cell types important to RA, T cells and FLS. First, we examined the putative function for CD13 as a T cell chemoattractant. Second, we sought to determine if CD13 is involved in FLS growth and migration. Both functions are important parts of RA pathogenesis. T cell chemotaxis brings T cells to the RA synovium, where they help mediate several pathogenic functions. RA FLS have an aggressive growth and migration phenotype which contributes to RA hyperplasia and eventual joint destruction.

Recombinant human CD13 aids in the migration of cytokine activated T cells

Two previous publications have suggested that CD13 is chemotactic for T cells [169,234]. We sought to verify this observation and to determine whether CD13 could be chemotactic for cytokine activated T cells (Tck), an *in vitro* generated cell population that phenotypically and functionally resembles T cells found in RA synovium [19,156]. We used a modified under agarose chemotaxis system with SDF-1/CXCL12 and TARC/CCL17 as positive controls. SDF-1 and TARC, had chemotactic indices (CI) of 0.45±0.23 (p=0.068) and 0.42±0.12 (p=0.0012) respectively without fibronectin and 0.88±0.29 (p=0.0046) and 0.46±0.42 (p=0.28) with fibronectin (Figure 4.1). Recombinant human CD13 (rhCD13) was used over a range of concentrations, from 1000ng/ml to 50ng/ml, and was chemotactic for Tcks between 700ng/ml and 50ng/ml. Peak chemotaxis for CD13 was from 200ng/ml (0.34±0.29) to 500ng/ml (0.50±0.18) and was significant over medium alone (medium alone CI=0) at both concentrations, p=0.029 and 0.0079 respectively. RhCD13 was also significant at 200ng/ml with fibronectin coating (0.72±0.29, p=0.018) (Figure 4.1). Representative images of the chemotaxis assays are shown in Figure 4.2.

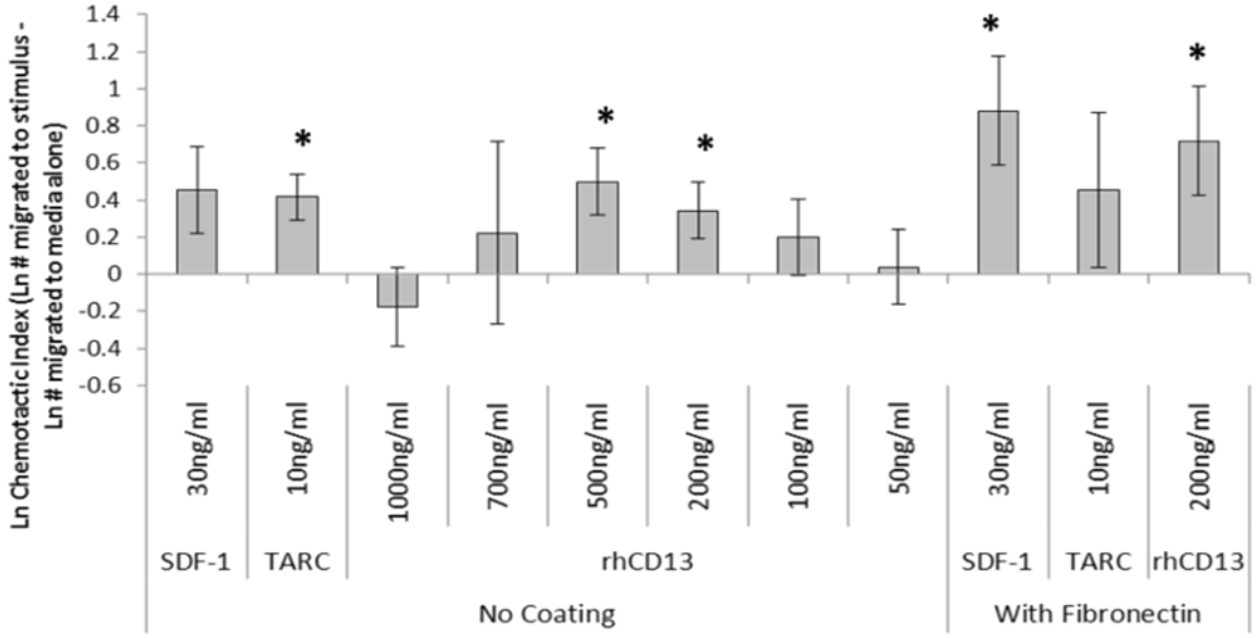

Figure 4.1 CD13 is chemotactic for cytokine activated T cells.
Tck were placed in the center well of the under agarose chemotaxis system with a medium only control in one of the side wells and a chemotactic agent in the opposite well. Some plates were coated with fibronectin. Positive wells were loaded with chemotactic controls SDF-1/CXCL12 (30ng/ml) or TARC/CCL17 (10ng/ml) or rhCD13 (1000 to 50ng/ml) ($n \geq 12$). (CI= Ln # of cells migrated to stimulus – Ln # of cells migrated to media alone)±SEM. *$p<0.05$ paired two tailed T-test between media alone and test wells (method p144)

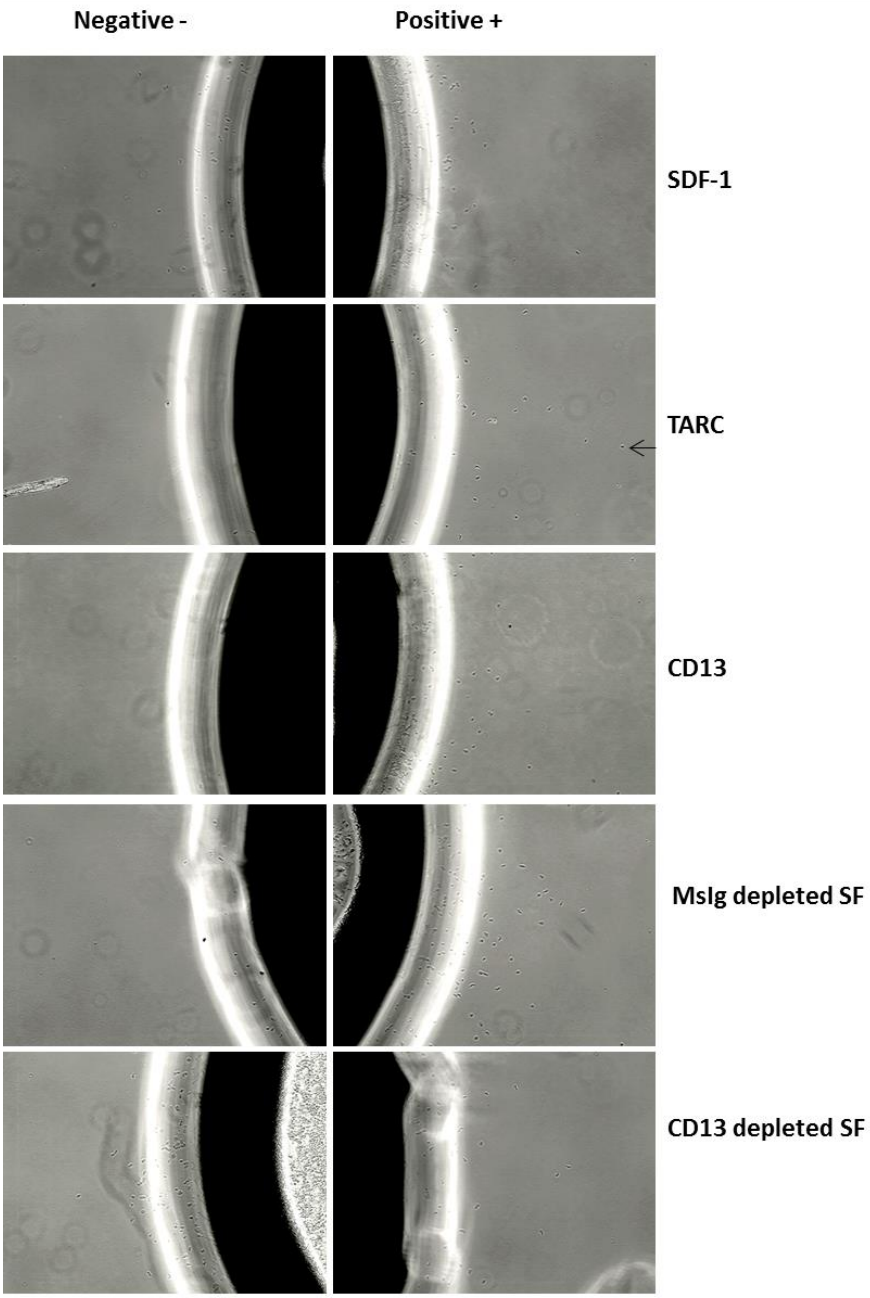

Figure 4.2 Representative illustrations of under agarose chemotaxis.
Tck were placed in the center well of the under agarose chemotaxis system with a medium only control in one of the side wells and a chemotactic agent in the opposite well. Some plates were coated with fibronectin. Positive wells were loaded with chemotactic controls SDF-1/CXCL12 (30ng/ml) or TARC/CCL17 (10ng/ml) or rhCD13 (1000 to 50ng/ml) Pictures are representative of images obtained for counting from under agaorse chemotaxis ($n \geq 19$). Positive pictures show cells migrating toward the indicated chemotactic solution. Negative wells show the migration toward the medium only control for the same well. Arrow shows one migrated cell as an example. (method p144)

CD13 contributes to the chemotactic activity of SF independent of its enzymatic activity

We next asked whether CD13 contributes to the chemotactic potential of RA SF. To test for a potential role of CD13 in Tck chemotaxis, we immunodepleted SFs with anti-CD13 (1D7) or a mock (MsIg) depleted control (n=3). The mock depleted SFs had a CI of 1.37±0.44 which was significantly chemotactic, p=0.0065. Immunodepleting CD13 from the SFs resulted in a significant decrease in chemotaxis p=0.041, CI of 0.35±0.21 (Figure 4.3A).

We next examined whether CD13's chemotactic ability required its enzymatic activity. COS-1 cells were used to express plasmids containing either wild type (WT) CD13 or an enzymatically inactive mutant CD13 (E355Q)[72]. The CD13 ELISA was used to measure CD13 concentration, and loss of enzymatic activity in the mutant was confirmed by the N-aminopeptidase assay (Figure 4.3B). We supplemented CD13 depleted synovial fluids with 200ng/ml of either WT or mutant CD13 from the COS-1 supernatants, both of which partially restored the chemotactic activity of the depleted SFs to a similar level. WT CD13 brought the CI from 0.35±0.21 to 1.02±0.31 and the mutant brought the CI to 1.10±0.30, each representing significant chemotaxis above medium alone, p=0.0031 and p=0.0018 respectively (Figure 4.3A).

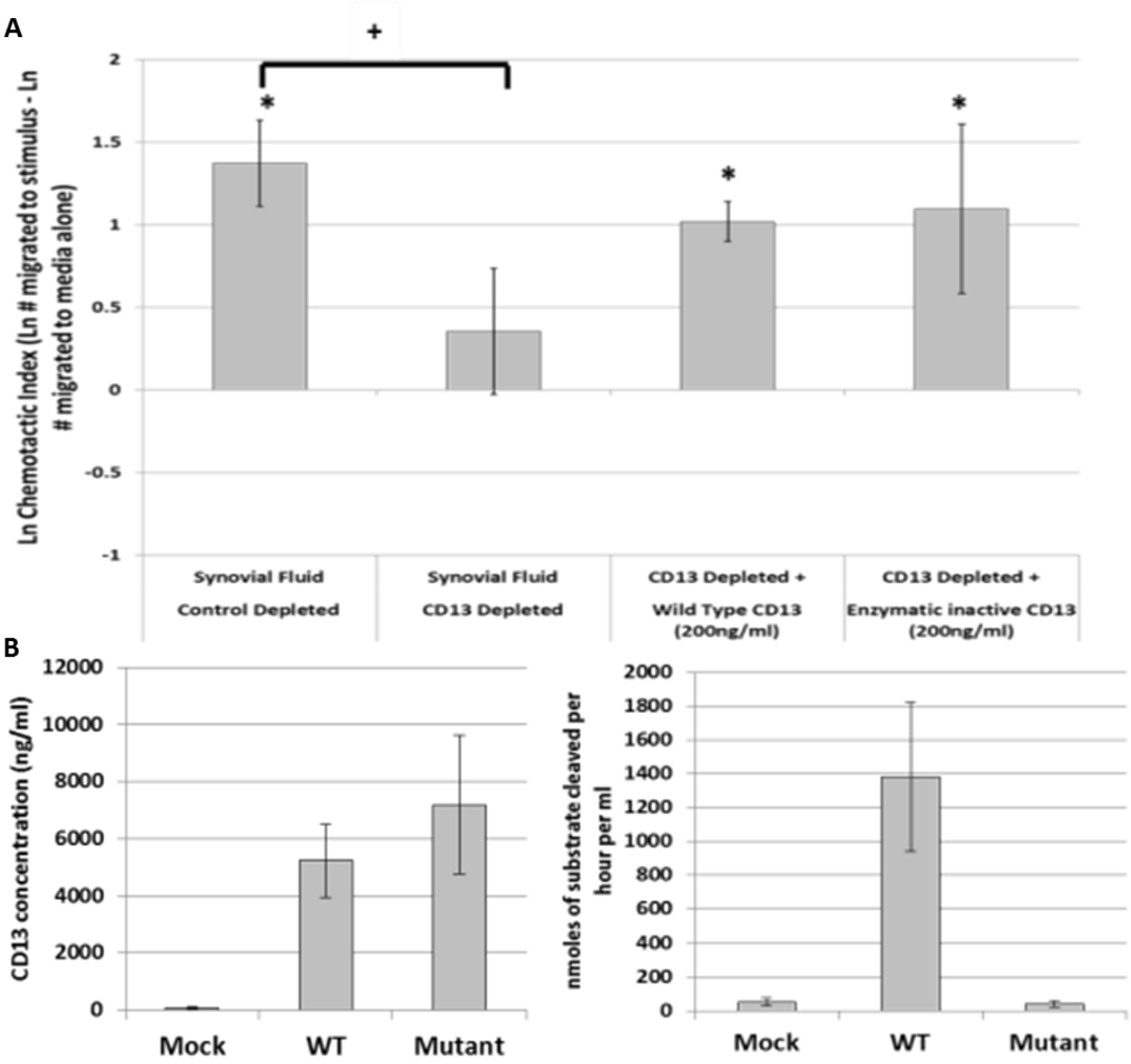

Figure 4.3 CD13 is a significant portion of RA synovial fluid T cell chemotaxis, independent of its enzymatic activity.

Tcks were placed in the center well of the under agarose chemotaxis system with a medium only control in one of the side wells and a chemotactic agent in the opposite well. **A**, Positive wells were loaded with synovial fluid (3 different random donors), MsIgG control depleted or CD13 depleted (1D7), at a 1:10 dilution, or with CD13 depleted synovial fluid supplemented with supernatant from transfected COS-1 cells containing wild type or enzymatic inactive mutant CD13 ($n \geq 19$). CI= Ln # of cells migrated to stimulus – Ln # of cells migrated to media alone±SEM. *$p<0.05$ paired two tailed T-test between media alone and test wells; +$p<0.05$ unpaired two tailed T-test **B,** The CD13 amount in the COS-1 supernatants was determined by ELISA and was used at 200ng/ml in assays shown in panel A. Values expressed as mean±SEM. Enzymatic activity was measured by N-aminopeptidase activity assay. Values expressed as mean±SD. (method p144-146)

CD13 initiates chemotaxis of T cells through a G-protein coupled receptor

Most known chemokines function through a G-protein coupled receptor (GPCR)[235]. We used Pertussis toxin (PTX) to determine whether the mechanism for CD13-induced chemotaxis functions similarly. PTX is an ADP-ribosylating toxin produced by the bacteria *Bordetella pertussis*. It catalyzes the ribosylation of α subunits of $G_{i/o}$ proteins meaning that they cannot interact with GPCRs[236,237]. This keeps the $G\alpha_{i/o}$ proteins in an inactive state and prevents the function of associated GPCRs. Through this mechanism PTX treatment of cells can be used to inhibit chemotaxis that functions through $G_{i/o}$ associated GPCRs. PTX treatment of Tcks significantly reduced chemotaxis toward 200ng/ml rhCD13 either with a fibronectin coating (0.42±0.20 to -0.50±0.50, p=0.047) or without (0.44±0.15 to -0.84±0.44, p=0.00096), and also inhibited migration toward SDF-1 and TARC positive controls (significant for TARC, uncoated, at p=0.05) (Figure 4.4). Background cellular movement, indicated by migration toward medium alone control, was not affected by PTX treatment. We examined average cell counts in both PTX treated and untreated Tcks and saw no decrease in cellular migration except for the directed chemotaxis (data not shown).

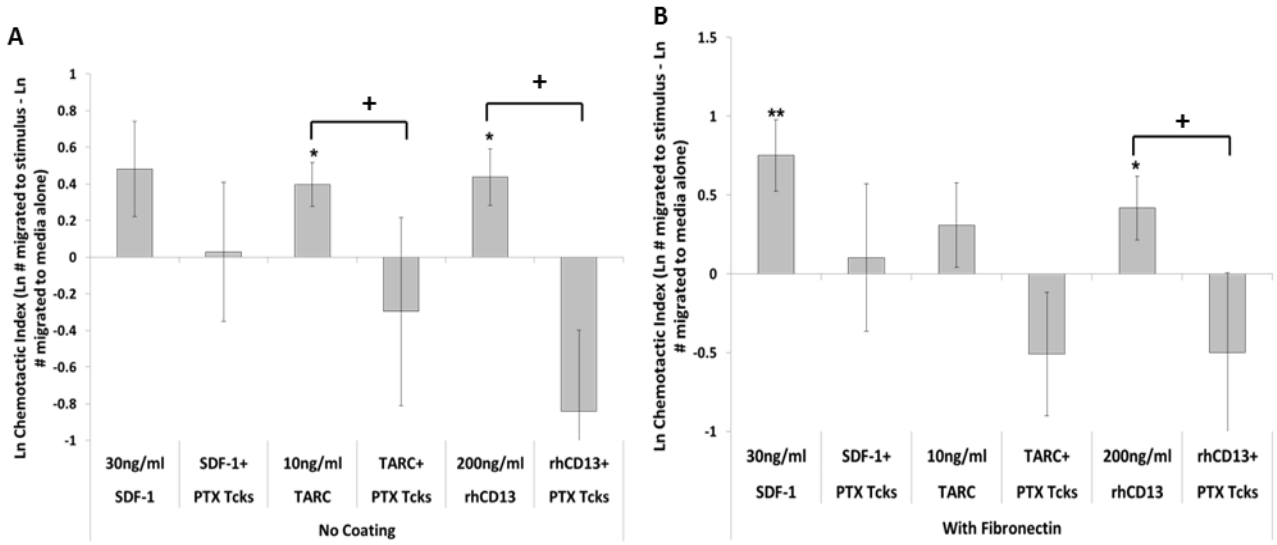

Figure 4.4 CD13 chemotaxis functions through a pertussis toxin sensitive G-protein coupled receptor.

Tcks were placed in the center well of the under agarose chemotaxis system with a media only negative control in one of the side wells and a chemotactic agent (SDF-1, TARC or rhCD13) at indicated concentration in the opposite well. Plates were left untreated (**A**) or fibronectin (10μg/ml) coated overnight (**B**). Some Tcks were treated with 100ng/ml of pertussis toxin for 2hr at 37°C. ($n \geq 20$) Cell counts were Ln transformed and data is expressed as a chemotactic index (CI= Ln # of cells migrated to stimulus – Ln # of cells migrated to media alone) with error expressed as SEM. *$p<0.05$; *$p<0.001$ paired two tailed T-test between media alone and test wells; + $p \leq 0.05$ un-paired two tailed T-test between two groups of chemotactic indices (method p144)

CD13 aids in growth and migration of RA FLS

We identified CD13 as a soluble molecule and on extracellular vesicles released by FLS. Because we did not observe a correlation between the upregulation seen at the mRNA level and cell surface CD13, we looked at the effect of IFNγ, TNFα, and IL-17 on CD13 in FLS culture supernatants. We found results similar to what was observed on the cell surface with variable, fluctuating regulation from 0 to 72 hours (Figure 4.5). This indicates that CD13 may be taken up by the RA FLS in an autocrine manner. To determine possible functions for CD13 on FLS we looked at the effect of anti-CD13 antibodies (WM15 or 1D7) or CD13 chemical inhibitors (bestatin or actinonin) on RA FLS growth and migration. Anti-CD3 was used as a negative control. We observed a significant slowing of cell growth with both CD13 inhibitors and both anti-CD13 antibodies (Figure 4.6A). Data are expressed as the change from 0hr as a ratio to untreated FLS of the same cell line at the same time point. Actinonin was the strongest inhibitor of FLS cell proliferation and significant decreases were seen with actinonin treatment at time points between 24 and 120 hours. Significant decreases ($p \leq 0.05$) in FLS proliferation were also seen with bestatin from 24-60 hours and at 120 hours, with 1D7 at 36-120 hours, and with WM15 24-60 hours and at 120 hours. Significant decreased ($p \leq 0.05$) were also seen in RA FLS migration in a wound healing assay with actinonin, WM15 and 1D7 primarily from 36 hours to 72 hours (Figure 4.6B). Figure 4.7 shows example images of control anti-CD3, actinonin, and 1D7 treated scratch wounds. Antibodies were tested at both 25ng/ml and 50ng/ml. A significant decrease in migration was seen with both concentrations and the data for the lower concentration is shown. While a decrease was seen in growth with 25ng/ml, the significance was borderline. The data shown are for the 50ng/ml concentration in which the decrease was more definitively significant.

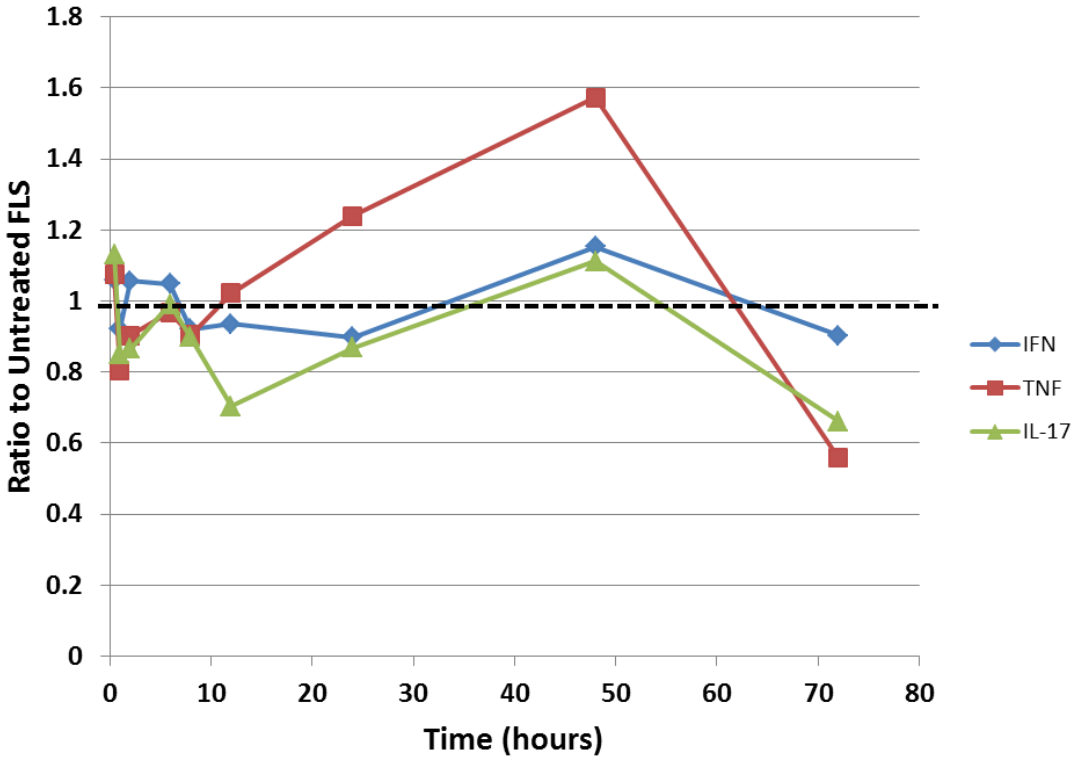

Figure 4.5 CD13 released by FLS fluctuates in response to IFNγ, TNFα, and IL-17. FLS were stimulated over a time course of 0-72 hours with IFNγ (1U/ml), TNFα (10ng/ml), or IL-17 (10ng/ml). Culture supernatants were collected and concentrated through at 30KDa centrifugation filter. Data are expressed as a ratio of cytokine treated to untreated FLS at the same time point. n=1 representative of n=3 (methods p138 and 140)

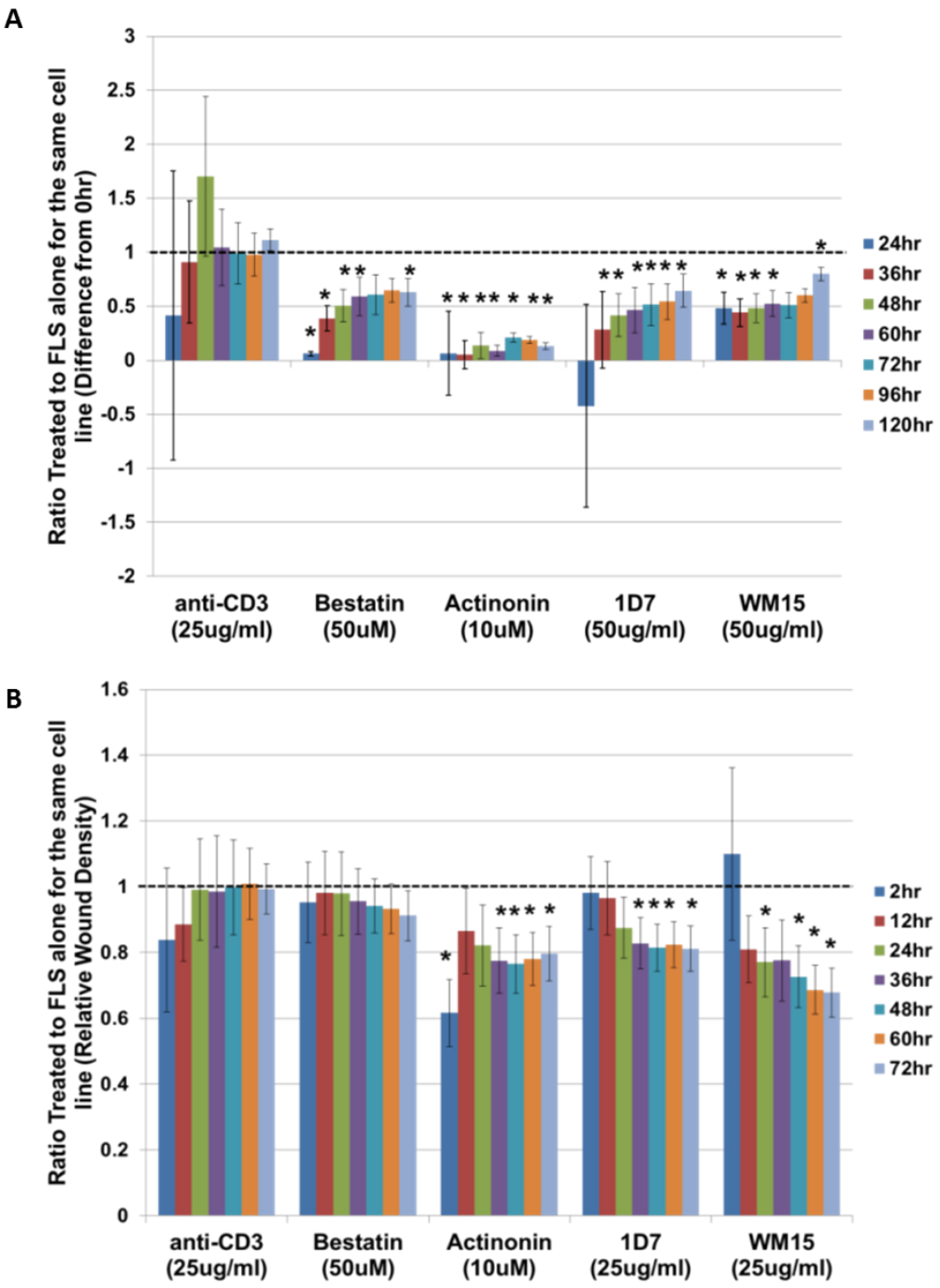

Figure 4.6 CD13 chemical inhibitors or antibodies significantly slow growth and migration of RA FLS *in vitro.*

FLS were seeded on 96-well plates overnight. An Essen Incucyte system was used for both growth **(A)** and migration **(B)**. Growth was calculated as the difference in percent confluence from time 0. Migration was measured in a scratch wound assay using relative wound density. All data is expressed as a ratio to untreated FLS of the same cell line. mean±SEM n≥4 *p<0.05 (method p147)

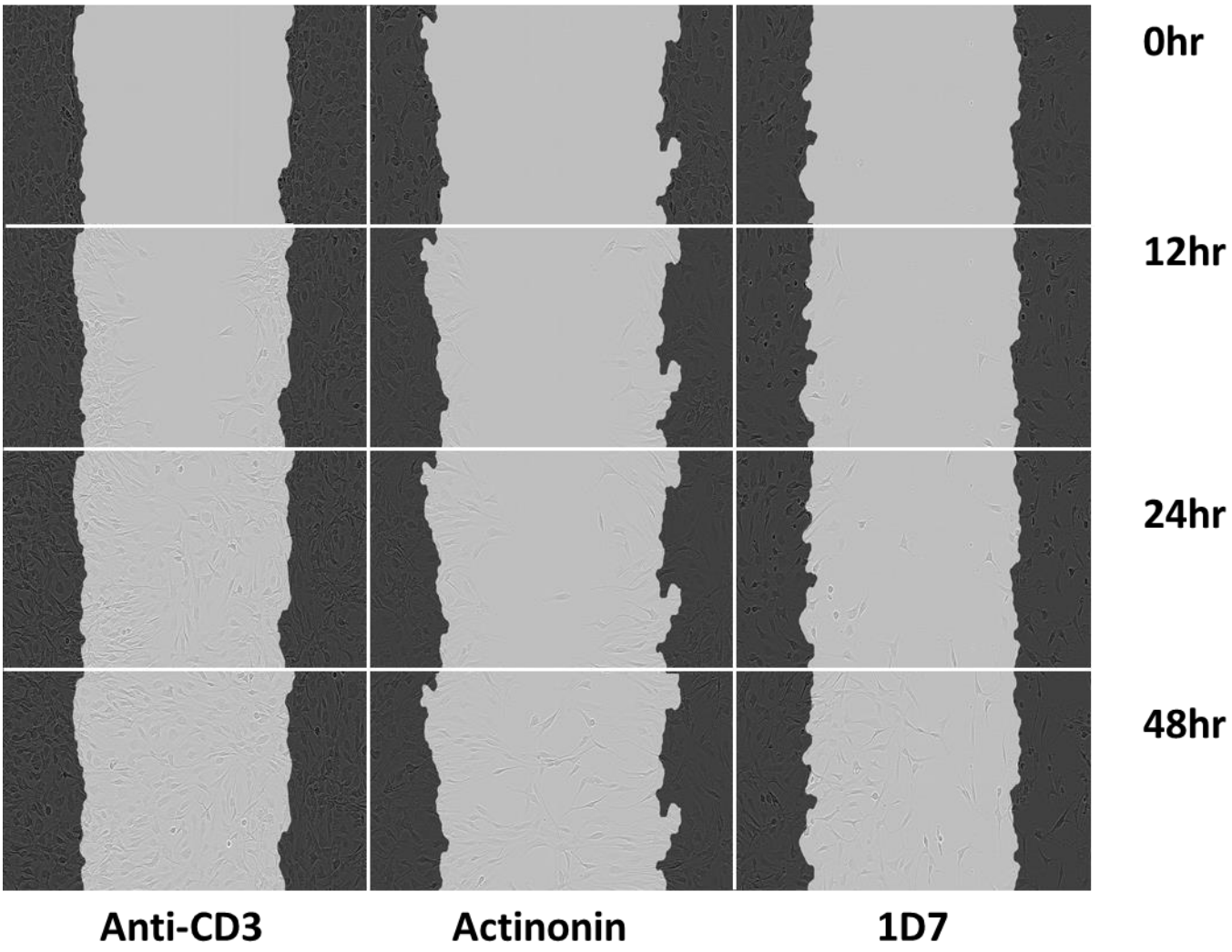

Figure 4.7 Sample images from scratch wound assay.
Actinonin and 1D7 suppress migration of RA FLS into a scratch wound. Anti-CD3 is used as an irrelevant negative control. Dark areas show the edges of the initial wound. Images are representative of n≥6 for n≥4 experiments (method p147)

Discussion

Two publications have suggested that CD13 may act as a chemokine for T cells [169,234]. We wanted to further explore this function as one possible aspect of the interaction between FLS and T cells in the RA joint. We tested for chemotaxis with SDF-1, TARC, or rhCD13 using not only Tcks, but also resting T cells, anti-CD3/CD28 activated, Jurkat, HUT78, and mitogen activated T cells (data not shown). We chose Tck because we achieved the most consistent and significant chemotaxis with both positive control chemokines and rhCD13 with this cell type. This is of particular interest as Tck phenotypically resemble T cells found in the RA joint [19]. Chemotaxis assays with rhCD13 were performed both over a fibronectin coated and uncoated plate, to explore both a basic chemotaxis (uncoated) and a matrix invasion type function (fibronectin coating). Even though rhCD13 and synovial fluid were significantly chemotactic in both systems, the two known T cell chemokines (TARC and SDF-1) were each significantly chemotactic in only one of the systems. Fibronectin improved adhesion of the Tck to the plate, magnifying both specific and non-specific movement. This could be primarily noted by the difference in time to optimally measure chemotaxis. With an uncoated plate, we found the optimal time to distinguish chemotaxis was around 20-22 hours. When plates were coated with fibronectin cells migrated faster, and the optimal time to count cells was at around 5 hours.

We selected two chemokines as positive controls for chemotaxis. SDF-1/CXCL12 was chosen because the receptor (CXCR4) is upregulated on Tcks, and SDF-1 is the only chemokine that has been previously shown to be chemotactic for Tcks specifically [156]. TARC/CCL17 specifically binds to the CCR4 receptor. CCR4 is specific for T cells and has been found on high numbers of T cells in RA synovial tissue [155,238]. We did note some differences between the two known

chemokines, TARC and SDF-1. While we expected that both would be chemotactic for T cells, we found SDF-1 was significant only across a fibronectin coated surface while TARC was significant only over an untreated plate. As the chemotactic indices are similar this may be a reflection of the high background. However, observation of the chemotactic assays and the total cell counts indicate that this may be a real phenomenon. The difference is the fibronectin, and one possible explanation for the difference in the two chemokines is proposed by Patel et al. This group found that chemokines bind to ECM components with different degrees of strength [239]. Solid phase versus soluble chemokines may have different functions in the different stages of cell migration with solid phase chemokines being needed for firm adhesion [239,240]. While it is unknown if TARC binds to fibronectin, SDF-1 does and this association with fibronectin aids in cell migration [241]. Our data could indicate a difference in the ability of SDF-1 and TARC to bind to fibronectin, and this could explain the difference in significance. Related data is also supportive of an *in vivo* role for CD13 chemotaxis. It has been suggested that for *in vivo* chemotaxis, chemokines need to bind to cell surface glycosaminoglycans [239,242]. CD13 is already expressed as a cell surface protein on endothelial cells, a placement linked to monocyte migration/invasion [85]. The cell surface CD13 could act to aid in cellular migration/invasion without the extra step of binding to a glycosaminoglycan. Thus, both soluble CD13 and cell surface CD13 may aid in chemotaxis.

It is important to note that the optimal levels of CD13 in the chemotaxis assays correspond to sCD13 levels found *in vivo* indicating this process is likely biologically relevant (Figure 4.1). Although the average CD13 concentration in RA synovial fluid is higher than the peak chemotactic range, it is possible that this serves to create a gradient between the joint and the

serum with the high concentration in the joint aiding in T cell retention. Consistent with this hypothesis, the average concentration of CD13 in OA SF is similar to normal serum concentrations (Figure 2.3).

It is known that there are multiple chemokines that contribute to the migration of T cells to the RA joint[243,244]. Notwithstanding the effects of other chemokines, CD13 appears to significantly contribute to the chemotactic activity of RA SF as Tck chemotaxis decreases when CD13 is depleted from such samples (Figure 4.3A). CD13 is capable of regulating other chemokines, but *in vitro* studies with rhCD13 indicate that CD13 can initiate chemotaxis directly [60,95,166,167]. To determine whether the CD13 dependent chemotaxis in SF was direct or through enzymatic effects on other molecules, we supplemented rhCD13 back into the depleted SFs using either WT or an enzymatically inactive mutant (E355Q). The glutamic acid at amino acid 355 is part of the highly conserved GAMEN motif that is involved in substrate binding[73,74]. Both the WT and mutant CD13 proteins were able to partially restore chemotactic activity to a similar degree demonstrating that the mechanism of chemotaxis is independent of CD13's enzymatic activity (Figure 4.3). This also indicates that CD13 likely binds directly to a receptor on the T cells.

To confirm this we used PTX treated Tck. PTX is known to inhibit cellular responses mediated through $G_{i/o}$ proteins including most chemokine receptors[235,245]. Here we were able to show that CD13 also acts through a PTX-sensitive GPCR (Figure 4.4). We also saw similar results with a fibronectin coating indicating a similar mechanism for basic chemotaxis or migration involving ECM. PTX has been shown to function through both G protein dependent and independent pathways. The G protein dependent pathways involve the A subunit; while, the G protein

independent pathways involve the B subunit [245]. The B subunit is capable of activating T cells [246–248]. The two mechanisms can be distinguished by how the PTX is used. The A subunit uses low concentrations of the PTX and the effect is slow but long lasting [245,249]. The B subunit requires a high concentration and the effect is rapid but transient [246–248]. For our studies we used a low concentration of PTX. We also washed off the PTX before examining the effects over a long range of time. The effect we observed was also specific to the chemotactic effect. This supports the conclusion that CD13 is acting through a PTX sensitive GPCR.

Although we have defined one mechanism for CD13 mediated T cell chemotaxis in RA, CD13 in the RA joint is also likely involved in cleaving chemokines, extracellular matrix proteins, and other molecules that regulate migration of T cells and interaction of those T cells with FLS. Future investigation of these aspects of CD13 function and definition of the T cell receptor for CD13 will yield additional insights into the roles of CD13 in RA.

Another component of disease pathology in RA is aggressive outgrowth and migration of FLS, manifested clinically as synovial hyperplasia. Our data indicate a role for CD13 in the growth and migration of RA FLS. It is uncertain whether this is dependent on soluble CD13 or cell surface CD13. We found that while inhibitors of CD13 enzymatic activity and anti-CD13 antibodies each inhibited FLS proliferation and migration, additional rhCD13 did not consistently affect the RA FLS (data not shown). This may reflect the large amount of CD13 produced by the FLS that can act in an autocrine fashion. To help determine if the effects of FLS growth and migration were dependent on CD13 enzymatic activity we used two chemical inhibitors of enzymatic activity, one anti-CD13 antibody that inhibits enzymatic activity

(WM15), and one anti-CD13 antibody that does not inhibit enzymatic activity (1D7). The fact that a chemical inhibitor could block growth and migration indicates that the enzymatic activity is likely necessary for these functions of CD13. Actinonin was the strongest inhibitor of FLS proliferation, indicating that FLS growth in particular is dependent on enzymatic activity. Though it is uncertain why an antibody that does not inhibit CD13 enzymatic activity can block CD13 mediated FLS cell growth similarly to chemical or antibody inhibitors of CD13 enzymatic activity, the most likely explanation is steric hindrance. 1D7 does not block cleavage of the small molecule L-leu-AMC in the CD13 aminopeptidase activity assay; however, it may block the ability of CD13 to associate with larger substrates on FLS, thereby indirectly but effectively blocking the enzymatic activity and cell growth or migration. It is also uncertain why bestatin blocked FLS growth but not migration. It is possible that FLS migration is not dependent on the enzymatic activity, but that actinonin is binding to CD13 in such a way to block the area that is responsible for mediating migration. Alternatively, while the antibodies are mediating a CD13 dependent decrease in FLS migration, actinonin could be acting in a separate CD13 independent mechanism. Actinonin is not specific for CD13 and has been shown to negatively affect tumor cells in a CD13 independent fashion [250]. Neither bestatin nor actinonin are specific for CD13, and as discussed in chapter 1 are not the best measures for CD13 activity. However, the antibody inhibition of RA FLS proliferation and migration supports a specific role for CD13 in RA FLS outgrowth. We included the chemical inhibitors to relate our data to most of the previous work done with CD13, which uses bestatin or actinonin. The conclusion can be drawn because it is supported by the specific antibodies.

We have demonstrated two mechanisms by which CD13 may contribute to RA pathogenecity. First, CD13 is a chemokine for T cells, similar to those in the RA joint. Second, CD13 aids in the proliferation and migration of RA FLS. Both of these functions could contribute to the inflammatory pathogenesis of RA. Combined with data from previous chapters we can conclude that CD13 is upregulated in the RA synovium. It is found in high levels on RA FLS, and is shed by RA FLS into the synovial fluid. This soluble CD13 contributes to the chemotaxis of T cells to the RA joint. Those T cells can then further upregulate CD13 expression through the secretion of pro-inflammatory cytokines. CD13 also contributes to FLS proliferation and migration, further increasing the CD13 in the synovium. In total, CD13 could play an important role as part of a positive feedback loop in the RA joint, in which CD13 has important pro-inflammatory effects on both T cells and FLS.

Chapter 5

Conclusions and Future Directions

The experiments described here indicate that CD13 may contribute to RA disease pathology. Previous work that measured CD13 in RA and proposed that is was chemotactic for T cells relied primarily on CD13 aminopeptidase activity measurements. We developed a novel CD13 ELISA to conclusively measure CD13. Our data is in part supportive of previous conclusions reached by measurement of CD13 enzymatic activity. CD13 is significantly elevated in RA synovial fluid compared to OA (Figure 2.3). However, we also inferred that in synovial fluid, and especially in RA synovial fluid, there are other proteins with similar aminopeptidase activity (Figure 2.8). Therefore aminopeptidase activity assays alone do not provide a complete or significantly accurate picture of the expression and function of CD13. This is also likely true of other biological fluids such as serum. We did find that CD13 appears to be the major contributor to aminopeptidase activity in synovial fluid; however, this may not be the case for all biological fluids.

Our finding that cultured RA and OA FLS express similar levels of CD13 *in vitro* is contradictory to a prior publication (Figure 2.7)[169]. However, this study analyzed just three cell lines per group and was done with enzymatic activity and Western blot only. Our data represents

a higher n and a quantitative measure of CD13 by ELISA. We found a high degree of variation in CD13 levels that likely explains the differences between the two conclusions. This leaves the conclusion that while CD13 is significantly elevated in RA over OA *in vivo* there is no significant difference *in vitro* between RA and OA FLS. We discussed four possible explanations for the differences: the cytokine milieu in the RA joint, increased numbers of monocytes and endothelial cells in the RA joint, hyperplasia of RA FLS, and increased expression of proteases in RA. All of these mechanisms are likely to be relevant and our data directly support the first two. We have shown that pro-inflammatory cytokines (IL-17, TNFα, and IFNγ) upregulate CD13 mRNA in cultured RA FLS (Figure 3.8). These effects may further be magnified when combined in the RA joint along with other stimulatory cytokines. We observed fluctuating kinetics of protein localization and regulation on RA FLS cell lines in response to the three different cytokines. Differing levels of cytokines in the joints of different RA patients may help to explain the wide degree of variation in CD13 concentrations in synovial fluid. We also observed higher numbers of monocytes/macrophages expressing CD13 in RA synovium sections than in OA or normal synovium. Evidence of CD13 on neovascular structures was also observed in RA synovium (Figure 2.4). These data demonstrate the presence of other CD13 expressing cells in the RA joint.

While we did not specifically examine the latter two possibilities, evidence is supportive of both. It is known that RA FLS undergo hyperplasia resulting in more cells present in the joint. While in cultures we examined similar numbers of RA and OA FLS, we also demonstrated that CD13 itself may play a role in RA FLS proliferation. We also showed that CD13 is cleaved by metalloproteinases and specifically MMP14. Metalloproteinases and particularly MMP14 are

elevated in RA. In addition pro-inflammatory cytokines such as TNFα increase metalloproteinase expression[251]. In conclusion, our data indicates that a combination of increased numbers of CD13 expressing cells (FLS, monoctyes/macrophages, endothelium) and upregulation of CD13 and CD13 sheddases (metalloproteinases) on these cells by the pro-inflammatory milieu results in the higher CD13 levels in the RA synovial fluid.

Another interesting observation was that CD13 is lower in RA sera than in normal sera. This may be explained by the migration of CD13 expressing cells into the RA synovium. This is consistent with our observation of increased CD13 in RA synovial fluid and increased numbers of CD13/CD11c co-expressing cells (monocytes/macrophages) in RA synovial tissue. However, circulating monocyte numbers do not go down in RA[252]. But, CD13 is known to be upregulated on activated monocytes. This may imply that the highly activated, inflammatory monocytes that migrate into the synovium express higher levels of CD13 than those that remain in circulation. Alternatively, serum CD13 may be taken up and utilized in RA at an accelerated rate over normal serum CD13. This utilization could represent the putative functions in RA, such as chemotaxis. It is also suggestive that the CD13 receptor may be upregulated on cells in RA. This could be useful in identification of the CD13 receptor. It is interesting to note that CD13 is not the first metalloenzyme to be found with this phenotype. CD26, a related protein, has also been found in lower amounts in RA sera than in normal sera[253]. While the mechanism of this decreases is also unknown, in the case of CD26 it has been suggested that the decrease may function to lower degradation of SDF-1 and increase chemotaxis in RA[106]. From the conclusions that CD13 is increased in RA synovial fluid and decreased in RA serum, we can infer the possibility that CD13 is acting as a chemoattractant in RA. There is no difference in CD13

amount between normal sera CD13 and OA synovial fluid; however, the differences in RA indicate a potential function for CD13 in the RA joint. The higher amount of CD13 inside the joint and the lower amount in the serum results in a gradient that could bring CD13-responsive cells, specifically T cells, into the joint. Our data confirms that CD13 acts as a T cell chemoattractant at biologically significant levels, and that CD13 represents a significant portion of the T cell chemotactic ability of RA synovial fluid.

T cell chemotaxis is not the only function for CD13 in the RA joint. In RA multiple cell types contribute to inflammation and pathogenicity. We found, when examining the effects of cytokines on FLS expression of CD13, a fluctuating pattern at both the cell surface and in the culture supernatant. These fluctuations included increases in soluble CD13 followed by decreases. While we could not definitively demonstrate that soluble CD13 is being taken up by FLS, these data are suggestive of that possibility. CD13 has also been implicated in migration of dermal fibroblasts [133]. Based on these observations we examined the function of CD13 on FLS growth and migration by the use of chemical inhibitors or anti-CD13 antibodies. From the results we can conclude that CD13 is likely involved in RA FLS pathogenicity. We have shown that CD13, through its enzymatic activity, can contribute to RA FLS growth and migration, processes that are relevant to the FLS hyperplasia that is a clinical and pathological hallmark of RA.

Overall we can conclude that CD13 is likely playing a role in the pathogenicity of RA by contributing to a pro-inflammatory loop in the joint. CD13 is expressed on FLS, and during RA

CD13 is upregulated on FLS by pro-inflammatory cytokines (IFNγ, TNFα, IL-17) and cleaved by metalloproteinases (which are also upregulated in RA). Soluble and vesicle bound CD13 accumulates in the intra-articular space. CD13 from the synovium forms a gradient into the surrounding serum where it can bind a GPCR on the surface of T cells bringing the T cells into the joint. Once in the joint the T cells become further activated leading to production of more pro-inflammatory cytokines. This leads the FLS to further upregulate CD13. The CD13 in the joint also aids in proliferation and migration of the FLS, also ultimately leading to more CD13 production. Ultimately our data suggests that CD13 participates in a self-perpetuating state of inflammation in RA as diagramed in Figure 5.1.

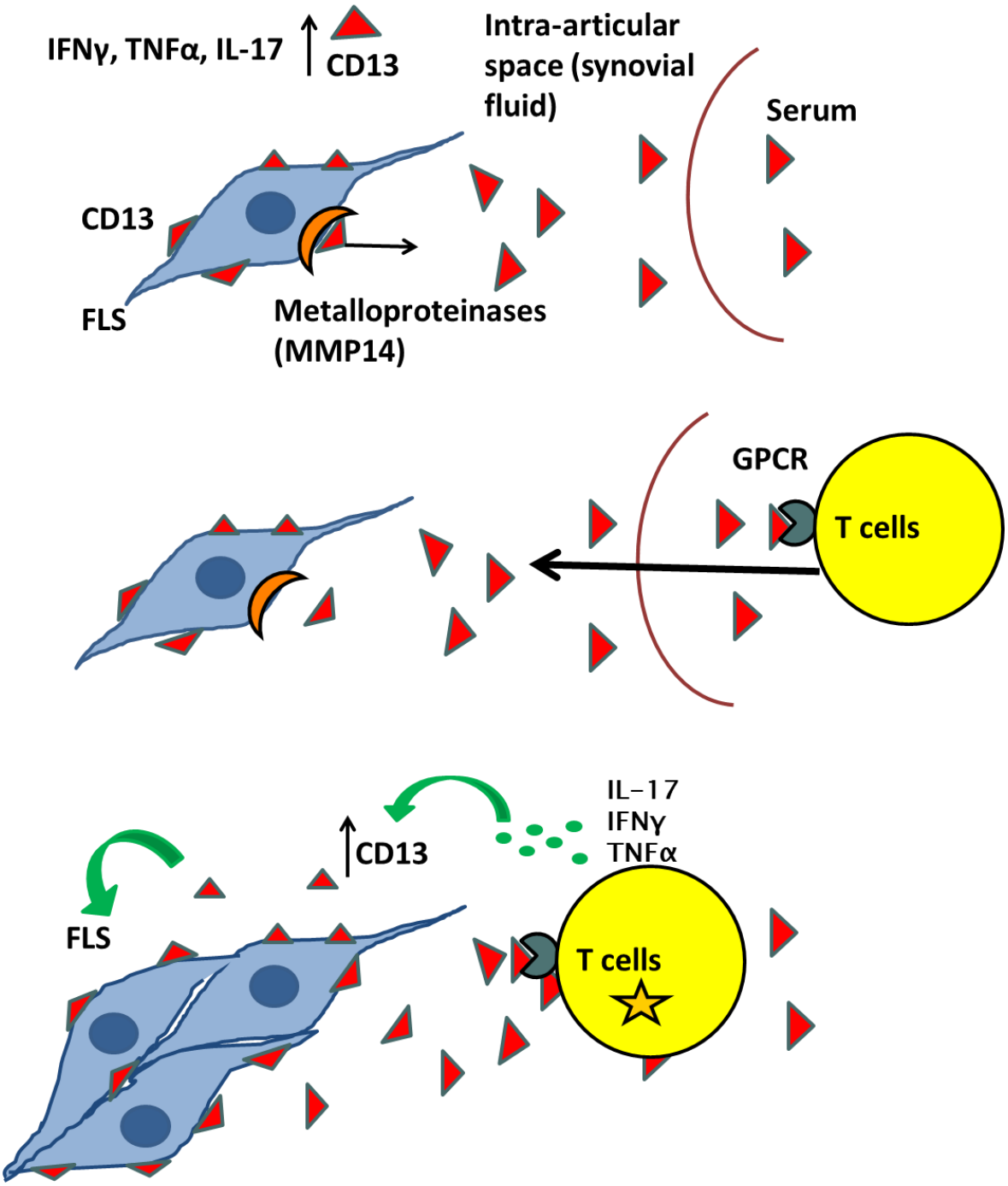

Figure 5.1 Diagram of a model for CD13 in a pro-inflammatory loop contributing to RA.

FLS express CD13 that is upregulated by pro-inflammatory cytokines. CD13 is cleaved by metalloproteinases, and the soluble form can migrate into the serum forming a chemotactic gradient. The CD13 binds to a GPCR on T cells initiating chemotaxis into the joint. In the joint T cells become activated and produce more pro-inflammatory cytokines, which in turn leads to increased CD13 expression. CD13 also contributes to proliferation of the FLS increasing CD13 levels further, thus restarting the loop.

CD13 in RA: future directions

We have shown that CD13 is found in high levels in the RA joint and is upregulated in RA versus OA synovial fluid. We also established roles for CD13 in RA in the growth and migration of FLS and the migration/invasion of T cells. However, there are still many other possible mechanisms through which CD13 could act in RA. First, CD13 may also be involved in other T cell processes. Expression of CD13 on T cells has been linked to activation. This activation and CD13 expression require cell-cell adhesion with CD13 expressing cells, including FLS [145,146]. This may imply that CD13 is involved in FLS-T cell interactions and subsequent T cell activation. While we did not find CD13 expressed on any T cells, we did not examine T cell/FLS co-cultures. Further experiments should be done to examine other effects of CD13 on T cells. Assays could include proliferation (thymidine incorporation), cytokine production (IFNγ, IL-17, TNFα, IL-4), and signaling molecule phosphorylation. First steps would be to do these assays with a range of soluble rhCD13 concentrations, from low levels of CD13 up to the high concentrations found in RA synovial fluid (10ng/ml-2000ng/ml). The enzymatically inactive mutant could be used to determine whether enzymatic activity is necessary for any effects. Our results demonstrate an effect of CD13 on Tcks; however, the effect of CD13 may not be limited to just Tcks. These assays should also be run on resting T cells, CD3/CD28 activated T cells, and mitogen activated T cells. Subsets of T cells could also be used including naïve T cells versus memory T cells, CD4 versus CD8, regulatory T cells, and Th1/Th2/Th17 skewed T cells. This could help determine if CD13 has a general effect on T cells or specifically affects different subsets or activation states of T cells. This may provide additional information about the specific role for CD13 in RA.

Our data demonstrates a chemotactic response of Tcks toward soluble CD13. Once in the synovium, T cells interact directly with FLS and other synovial cells. CD13 may also participate in these cell-cell interactions. Therefore experiments to assess the role of cell surface CD13 on T cells should also be done, through T cell co-culture with CD13 expressing cells. FLS and DCs/monocytes should be tested as potential sources of cell surface CD13 in the joint. Similar experiments to those discussed above with resting or previously activated T cells and irradiated FLS/DCs could look at T cell proliferation and cytokine secretion. In this case CD13 would need to either be knocked down by siRNA or inhibited with antibodies on the FLS or DCs. Alternatively knock-in of CD13 into a CD13 negative cell line (chinese hamster ovary cells [CHO]) could be used. This could also help determine if CD13 alone is capable of activating T cells or if it works in context with other molecules on the FLS/DCs. Knock-in of the wild type versus the enzymatically inactive mutant could help define if effects on T cells are dependent on the enzymatic activity. Wild type versus enzymatically inactive mutant CD13 could also be added back to FLS subsequent to CD13 knockdown. This could help determine a role for soluble versus membrane bound CD13. We have shown that CD13 in the joint is important in T cell chemotaxis but further experiments may reveal other roles for CD13 in FLS/T cell interactions.

CD13 is expressed on multiple cell types that are involved in RA pathogenesis besides FLS, including endothelial cells, monocytes/macrophages, and mesenchymal stem cells. Functions of CD13 on these cell types may also be relevant for RA. CD13 is involved in macrophage/monocyte aggregation and migration in a mouse model of peritonitis [85,114–116,254]. As migration of monoctyes is also important in RA pathogenesis it can be hypothesized that

CD13 may play a similar role in RA. The data presented here is also supportive of a putative role for monocytic derived CD13. We showed an increase in CD11c/CD13 co-expressing cells in RA synovial tissue. This demonstrates the presence of higher numbers of CD13 expressing monocytes in RA. We also found a significantly lower amount of CD13 in serum of RA patients compared to healthy controls. Combined with the elevated levels in the RA synovial fluid this could prompt the question: is this chemotactic gradient important for just T cell migration or could it also contribute to monocyte chemotaxis?

To address the question of CD13 induced monocyte chemotaxis, chemotaxis experiments similar to those done with T cells could be done with monocytes. A possibility that wasn't explored in the previous studies is whether soluble CD13 is chemotactic for monocytes. This could be done in a standard chemotaxis assay with rhCD13. To examine a specific role for CD13 associated monocyte migration in RA, RA synovial fluids could be depleted of CD13 by immunodepletion and used in the chemotaxis assays. These experiments could also be done with the enzymatically inactive mutant compared to the wild type CD13. Again it would be best to do this with the rhCD13 supplemented back into depleted synovial fluid as done with the Tck. To address the question of other cell types shedding CD13 in the synovium, experiments would need to be done to examine shedding of CD13 from monocytes, DCs, macrophages, and epithelial cells. This could be done by examining culture supernatants for CD13 by ELISA. All culture experiments would need to be done in serum free conditions to eliminate bovine serum CD13. Previously monocyte shedding of CD13 has only been observed during apopotosis[226]. Therefore, it may also be necessary to examine monoctyes under various stimulation conditions. Specific

conditions that could be tried should include proinflammatory stimuli (such as TNFα) which would be elevated in RA and biological conditions (human sera depleted of CD13).

However, basic monocyte chemotaxis may not reveal a complete picture of putative CD13 mediated monocyte migration. As previous studies demonstrate a role for endothelial cell/monocyte adhesion in monocyte migration, additional chemotaxis studies could be performed in a transendothelial system where endothelial cells are cultured on top of the membrane [116,255]. The transendothelial system should be tested as above with both rhCD13 and CD13 depleted or whole RA synovial fluid. Additionally since both the monocytes and endothelial cells express CD13 knockdown experiments should also be done. These experiments would involve knocking down CD13 on the monocytes, the endothelial cells, or both cell types before using the cells in the migration assays. These experiments would further define the role for CD13 in monocyte migration while distinguishing its role on the different involved cell types.

Human mesenchymal stem cells (MSCs) also express CD13 [256]. It has not been explored though how this may relate to RA. Interestingly MSCs can be beneficial in RA, even repairing bone and cartilage damage. In fact in a mouse model of RA injection of murine MSCs was shown to decrease inflammation and joint destruction[257]. This may relate back to putative anti-inflammatory roles for CD13 (as discussed in Chapter 1). However, MSCs also have the potential to differentiate into osteoclasts which can potentiate RA joint destruction. Another future direction would be to examine the effect of CD13 on MSC differentiation. This could be

done by either knocking down CD13 in the MSCs or by addition of exogenous CD13 at biologically relevant concentrations and examination of MSC differentiation [258,259]. Outcomes that could be examined are efficiency of MSC differentiation into osteoclasts versus osteoblasts, and the function of those osteoclasts and osteoblasts. These experiments could help determine whether CD13 on MSCs can contribute to the pathogenesis of and/or be beneficial for RA.

Several known substrates of CD13 could impact RA in ways that are governed by the enzymatic activity of CD13. One group has shown that CD13 in conjunction with CD10 on synovial fibroblasts can degrade enkephalins, and they propose this as a mechanism for pain and inflammation in the RA joint[221]. This could mean that targeting CD13 or CD13 pathways therapeutically in RA could help with pain as well as inflammation. Further studies examining CD13's ability to degrade enkephalins and the ability of these shortened proteins to bind to the opioid receptors would be needed. Kinins are another group of molecules cleaved by CD13 that may be involved in RA pain and inflammation [260]. Kinins have been suggested to trigger the release of both neurotransmitters (substance P) and inflammatory mediators (IL-1 and TNFα) in the joint [261]. This may suggest another source of CD13-mediated regulation of pain and inflammation in the RA joint. Neurotransmitters have also been linked to joint processes other than pain. Substance P has been found in high levels in the RA joint and has been linked to FLS proliferation and expression of vascular adhesion molecule-1 (VCAM-1) [262]. CD13 has also been implicated to cleave substance P in cooperation with CD26 [109]. Further studies would need to be done to determine the effects of CD13 cleavage on these various substrates. The large amounts of CD13 in the RA synovium may thus help to contribute to the pathogenicity of enkephalins, kinins, and neurotransmitters.

CD13 has also been linked to trimming of peptides for presentation on MHC. This has been demonstrated by showing that CD13-mediated degradation has an impact on T cell recognition of an artificial peptide [263]. Autoantigen presentation is one of the key mechanisms of disease pathogenesis in RA. A possible future study could examine the relationship between known RA pathogenic peptides and CD13 degradation. It would be of particular interest to examine the relationships between CD13, pathogenic peptides, and the RA linked MHC allele, HLA-DR4. Using various artificial peptides it could be examined whether CD13 degradation leads to changes in presentation on HLA-DR alleles linked to RA susceptibility. Also, it could be determined if CD13 preferentially cleaves pathogenic peptides for presentation, such as citrulinated proteins.

Yet another possible role for CD13's enzymatic activity in RA is through an effect on extra cellular matrix (ECM). While it is frequently suggested that CD13 may cleave ECM components during cellular migration (usually tumor cell) through matrigel or specific ECM components, there is little data about CD13's actual role in cleavage of ECM. CD13 has been suggested to cleave entactin and type IV collagen but further studies are needed to be conclusive [264]. Future studies could examine both these and other ECM components (such as fibronectin and type I collagen). However, these studies are likely to be difficult as CD13 is doubtful as a solo degrader. ECM molecules are very large and CD13 is a peptidase. Removal of a single amino acid at a time is an inefficient degradation mechanism. However, CD13 may work with proteases and/or other peptidases to help in ECM degradation, such as CD26. ECM degradation

assays typically involve looking for invasion and podosome formation of cells into various matrixes, often using fluorescent microscopy. To examine a specific role in joint ECM destruction, these studies could be done with RA FLS and siRNA knockdown of CD13. This could be done by culturing FLS on top of a type I collagen matrix, fibronectin, or Matrigel (a complex protein system of a basement membrane matrix that is used to mimic *in vivo* ECM), looking for either specific degradation or degradation of complex ECM. SiRNA knockdown of CD13 on fibroblasts would be expected to show a decrease in migration into the gels if CD13 is involved in FLS migration into ECM matrices [49]. This could indicate the involvement of CD13 in the degradation of ECM, and this type of study would not be limited by the involvement of other proteases/peptidases.

A function for CD13 in FLS invasion could also be examined using a chick chorioallantoic membrane (CAM) assay. In this assay articular cartilage fragments from rabbits are cultured with RA or OA FLS and then implanted on top of the CAM of chick embryos. Invasion of the FLS into the cartilage can be then viewed by immunohistochemistry [49]. SiRNA knockdown of CD13 could be performed on the FLS before implantation which would show whether CD13 is important in FLS invasion into cartilage. While the data shown in this report demonstrates that CD13 is important for migration of RA FLS, it is in an *in vitro* system and only examines basic migration. The CAM assay allows for examination of FLS invasion into cartilage. It would show whether CD13 was involved in cartilage degradation and/or FLS migration into cartilage in a more biologically relevant system.

One complication in studying CD13 mediated functions of FLS in the joint is that CD13 is present as both a cell surface molecule and a shed factor. A question that remains from this work is whether CD13 involvement in FLS migration is dependent on its localization. Our studies did not find a noticeable difference when additional soluble CD13 was added to FLS growth and migration experiments (data not shown). This may however be due to saturation of the system by the large amounts of CD13 already expressed and shed by the FLS. A possible future experiment to examine this would be to look at normal human FLS with addition of soluble rhCD13. We did examine OA FLS in growth and migration assays, with results similar to those of RA FLS (data not shown). However, since we did not find a difference in CD13 expression in RA versus OA on cultured FLS this is not unexpected. Our staining of synovial tissues however indicates that normal FLS may express lower levels of CD13 than RA or OA FLS, which may carry over to culture conditions. Normal FLS do not easily grow in cultures, and our data indicates that one possible difference that may assist in FLS growth is the amount of CD13. Therefore, a possible experiment would be to add rhCD13 to normal FLS cultures and measure cell proliferation. This would help to define whether soluble CD13 aids in FLS cell growth. Another possibility is that the surface CD13 is needed for FLS growth and migration. Knockdown (KD) experiments of CD13 in RA or normal FLS would help in determining whether surface CD13 is needed. CD13 could be knocked down on RA FLS and soluble rhCD13 added back to the culture. If CD13 KD slows FLS cell growth and addition of soluble CD13 does not restore proliferation this would indicate that the cell surface localization is important in CD13 mediated cell growth. Alternatively, if sCD13 does not help in increasing proliferation of normal FLS, knock-in of CD13 could increase the expression of CD13. This could examine a role for cell associated CD13 in FLS growth.

Angiogenesis, the growth of new blood vessels, is also a key part of synovitis and RA pathogenesis. The increased angiogenesis in RA also increases leukocyte invasion into the RA joint making it another target to reduce T cell invasion and inflammation. In RA VEGF has been identified as a mediator of angiogenesis [48,265]. While VEGF is a potent angiogenic mediator, other angiogenic molecules have also been identified in RA such as the family of molecules termed Le^y/H, cytokines (TNFα), MMPs, adhesion molecules, and other additional factors [45,152]. CD13 has been linked to angiogenesis in cancer and may play a similar role in RA. We identified CD13 on the vasculature in RA tissue sections (Figure 2.5). This implicates CD13 in RA angiogenesis. Data from literature is also suggestive of an angiogneic role for CD13 in RA. Angiogenic factors, VEGF and bFGF, have been shown to up-regulate CD13 through the endothelial promoter[266]. VEGF is increased in RA synovium compared to OA, and RA FLS production of VEGF is increased in response to pro-inflammatory factors (such as IL-17 and TNFα) [48]. This points to a complex up-regulation of CD13 during inflammation that may contribute to angiogenesis. Pro-inflammatory cytokines upregulate VEGF and CD13, and VEGF further upregulates CD13. Then both CD13 and VEGF can act to induce angiogenesis.

Angiogenesis is a multistep process. Pro-angiogenic molecules activate endothelial cells which produce proteolytic enzymes, like MMPs, which degrade the ECM around the existing blood vessels. The endothelial cells then proliferate and migrate forming new blood vessels. CD13 has the potential to be involved in any step of this angiogenesis process. CD13 is expressed on the surface of endothelial cells [267]. It is also expressed by the FLS and is present in high amounts

in the joint as a soluble protein. Some cell surface proteins can be pro-angiogenic as soluble molecules, such as E-selectin[268]. CD13 mediated angiogenesis could be a function of its cell surface expression, its soluble expression, or both. One basic system that could be used to examine the putative function for CD13 in angiogenesis would be an *in vitro* endothelial culture system. Endothelial cells are grown on Matrigel and new tube formation is counted [152,185]. A second standard assay would be to examine chemotaxis of endothelial cells (HUVECS or HMVECS) in a chamber assay system [268]. The matrigel assay could help determine whether CD13 is involved in matrix degradation and subsequent invasion by endothelial cells. The chemotaxis assay would help determine if it is more involved in the migration step of angiogenesis.

Because CD13 has multiple sources in the joint, the potential for CD13 to be involved in angiogenesis in the RA joint should be examined with both soluble and endothelial cell associated CD13. Both the matrigel invasion assay and the HMVEC chemotaxis assay should be done with addition of soluble rhCD13. However, both assays should also be done with either siRNA knockdown of CD13 or inhibition of CD13 by antibodies or chemical inhibitors on the endothelial cells. With knockdown experiments it could be necessary to use known inducers of angiogenesis (VEGF, RANTES) to stimulate a response and then look for a decrease in matrigel invasion or HMVEC chemotaxis. The results of using soluble CD13 versus inhibition of endothelial CD13 could help in determining what function CD13 has in angiogenesis. The CD13 expressed on FLS could also impact the vasculature in the joint. A tube formation assay in matrigel using co-culture of endothelial cells and FLS should be done with siRNA knockdown of CD13 in endothelial cells and/or FLS to examine this possibility [185]. The chemotaxis assays

of HUVECs or HMVECs should also be performed with synovial fluid either whole or depleted of CD13 (similar to the Tck assays shown in this report). This could help determine if CD13 significantly contributes to angiogenesis in RA.

Angiogenesis could also be further examined using a CAM system. This system has been used to demonstrate that MMP14 is able to induce angiogenesis at sites distal to the FLS[49]. In this work we identified CD13 as a cleavage target of MMP14, and hence one possible mediator of this angiogenesis. Repeating the CAM assays with CD13 siRNA knockdown in place of MMP14 knockdown would show whether or not the neovascularization response could be attributed to CD13. The advantage of the CAM assay system is that it is biologically relevant to RA. There are also two *in vivo* assays for angiogenesis. The first is the rat corneal implant assay[268]. In this assay a small bead containing test or control substances are implanted into a rat cornea and an increase in vasculature can be visualized. Test substances would include rhCD13 as well as RA SF, either whole or depleted of CD13. The second assay is a matrigel plug assay. In this assay matrigel is injected below the skin of a mouse. The matrigel would contain either controls or rhCD13. After several days the mice are sacrificed and the hemoglobin content of the plug is measured [269]. These two assays would look at CD13 induced angiogenesis under biological conditions. All of the assays could also be performed with both the wild type CD13 and the enzymatically inactive mutant to determine whether the putative CD13 mediated angiogenesis is dependent or independent of its enzymatic activity.

While in this study we have demonstrated that CD13 is highly upregulated in the RA joint over OA, there is still much work to be done in understanding the impact of this on RA pathology. CD13 has been identified to be important in T cell chemotaxis; however, it may also impact other T cell functions in the joint. CD13 is expressed on RA FLS but may also be important when expressed on other cell types in joint. Also, yet to be examined is what effect the enzymatic activity of CD13 has on various potential substrates in the synovium, and how this relates to RA.

Regulation of CD13

We have demonstrated that CD13 is upregulated in RA synovial fluid over OA. However, this was not reflected on FLS *in vitro*. Cultured RA and OA FLS had similar expression levels of CD13. We also did not find any differences in CD13 released from FLS in culture. This indicates that something in the RA joint is affecting CD13 expression. As discussed above further experiments are needed to define the expression, release, and function of CD13 on other cell types in RA. Another possibility though is that molecules such as cytokines in the RA joint affect expression of CD13 on FLS. Removal of the FLS from the joint and subsequent culture in medium on tissue culture plastic could however reduce the expression to levels similar to that of OA. The RA joint is a complex system involving multiple cell types and numerous soluble molecules. One way to further examine CD13 in the joint would be to look at freshly isolated joint synovium samples. Small pieces of RA and OA synovium could be cultured in serum free medium and the CD13 released into the medium could be measured. This would determine whether RA tissue releases CD13 at a higher rate than OA; however, it would not isolate the role

of FLS versus other cell types. Another option would be to sort the cells from a fresh piece of synovial tissue to isolate the FLS, and then examine the FLS by ELISA for cellular CD13 levels.

The initial screening that identified CD13 as of interest on RA FLS was to look for a TNFα independent pathway. However, when we expanded the cytokine regulation studies we found that CD13 regulation is more complicated than initially thought. We examined the influence of three pro-inflammatory cytokines on the expression of CD13 on FLS. We chose to look at IFNγ, TNFα, and IL-17 because these three cytokines are implicated as being pathogenic in RA[32,223,270,271]. Due to high levels of variability between cells lines and fluctuations within cell lines, few conclusions can be reached. The primary observation is that at the mRNA level all three cytokines upregulate CD13. Considerable complexity occurs at the protein level. All three cytokines appear to affect not only the expression of CD13 but the localization of CD13. We did observe the initial pattern of a CD13 increase with IL-17 and a decrease with TNFα at 48 hours on the cell surface for most of the cell lines examined. However, this pattern only appeared true for that specific location and time. Over a time course the regulation and localization fluctuate. We have shown that CD13 can be expressed on the cell surface of FLS, as a soluble molecule released from FLS, and on extracellular vesicles from FLS. We also noted that the cytokine initiated fluctuations were not consistent between cell surface CD13 and total cell lysate CD13 (data not shown). This may imply that CD13 can also be found intracellularly. These multiple sites for CD13 complicate the matter of determining overall CD13 regulation by cytokines. We can infer that cytokine stimulation of FLS results in changes in the localization and expression of CD13 with distinct kinetics for each cytokine. These changes show as both up and downregulation compared to untreated FLS over a time course.

The natural high variability in CD13 expression by different FLS cell lines further complicates these experiments. To determine overall patterns these experiments would need to be repeated with many different cell lines. The data outlined in this study may help to identify the time points to be the focus of examination. There appear to be differences between early regulation (0.5-12 hours) and long term regulation (48-72 hours). In addition to further studies of IFNγ, TNFα, and IL-17, future studies could look at other cytokines found in the RA joint. CD13 expression on other cell types has been examined in response to various cytokines. Cytokines that have been shown to have an effect on CD13 include: TGF-β, bFGF, VEGF, IL-4, IL-13, EGF, and IL-6[79–82,272]. Other pro-inflammatory cytokines, like IL-1 and IL-22, or potentially regulatory cytokines, like IL-10, should also be investigated. We did a screening using one RA cell line and one OA cell line treated with various cytokines (data not shown). This study implicated TGF-β, IL-1b, GMCSF, IL-4, IL-13, IL-10, IL-12, and IL-15 as potential targets for future studies. Future studies could also examine CD13 regulation on other cell types relevant to RA, such as monocytes or endothelial cells.

To determine biological significance it may also be necessary to treat FLS, or other cell types, with multiple cytokines. The correlations between cytokine levels and sCD13 in serum indicate that single cytokine treatment may not provide a complete picture of what is going on *in vivo* (Figure 3.9). Specifically the fact that IFNγ does not correlate with sera sCD13 but does increase CD13 mRNA in FLS could indicate that multiple cytokines are affecting biological expression levels and localization. Future studies could include treating cells with gradients of two or more

cytokines at a time. This would be especially relevant if opposite effects are observed for different chemokines. For example, if one cytokine is identified as downregulating CD13 on FLS and another upregulates CD13 on FLS, then FLS should be treated with both to identify to dominate effect. Or, if one cytokine promotes cleavage of CD13 and expression in the supernatant and another promotes localization to the cell surface and decreases soluble CD13, both cytokines could administered to determine the dominate effect.

CD13 receptor

Our results imply that CD13 has a receptor on T cells. The fact that rhCD13 has a direct effect on T cells inducing chemotactic movement in a buffer containing only BSA protein demonstrates that CD13 is either directly interacting with the T cell or with something the T cells are producing. Using pertussis toxin we were able to demonstrate that the first is the most likely hypothesis. Pertussis toxin is known to inhibit function of g-protein coupled receptors (GPCR)[273]. However, one main issue with determining the CD13 receptor is the wide variety of possibilities. We identified CD13 aminopeptidase activity as being irrelevant to CD13 initiated Tck chemotaxis. This eliminates receptors dependent on enzymatic activity, such as the protease activated receptors (PARs). However, GPCRs are one of the largest groups of proteins. While the exact number is unknown one estimate was over 800 [274]. PTX does help to narrow the field a little as it only inhibits Gi/o proteins and their associated receptors, although this still leaves a large number of possibilities [273]. Another complicating factor was found when we attempted to confirm the involvement of a GPCR. We observed a biphasic effect on cyclic adenosine monophosphate (cAMP) when Tcks were stimulated with CD13 (data not shown). At 200ng/ml CD13 increased cAMP levels in Tcks, and at 500ng/ml CD13 decreased cAMP levels in Tcks.

With a PTX sensitive receptor a decrease would be expected. This data may indicate that CD13 can act through more than one mechanism on T cells, and this could be dependent on the concentration of CD13 present.

There are several potential avenues that may help identify the GPCR for CD13. Identifying the intracellular signaling molecules involved may be one direction. Different GPCRs signal through different intracellular pathways including p38, ERK1 and 2, NFκB, JNK, and MAPK [275–277]. Stimulating Tcks with CD13 and then looking for subsequent phosphorylation of these molecules would identify which molecules are involved in CD13 receptor signaling. While this will not directly identify the GPCR, comparing the signaling pathways activated by CD13 to those of known GPCRs may help narrow the field down. Another way to narrow down the alternatives would be to look for receptors associated with known functions of CD13. Many GPCRs have already been associated with RA. The chemokine receptors in particular have been implicated in RA including all CXCRs and several CCRs (1-7)[153]. CCR5 specifically is interesting as it has been linked to T cell chemotaxis in RA as well as activation of RA FLS fulfilling two identified roles of CD13 in RA[155,162]. CXCR4 is also of particular interest as it has been identified as being the most upregulated cytokine receptor on Tck [156]. Another group of GPCRs that are of interest in RA are the sphingosine-I-phosphate receptors ($S1P_{1-5}$). Binding of $S1P_1$ is known to be involved in T cell migration and FLS survival and migration [275]. $S1P_1$ is also highly expressed in the RA synovium [275]. The angiotensin receptors (AT1 and AT2) are another possibility. AT1 is present on FLS and is linked to FLS survival and growth, while both AT1 and AT2 are linked to T cell mediated cardiovascular inflammation in mice [276,278]. The AT1 receptor in particular has been linked to RA and mouse models of RA, and it has even been

proposed as a therapeutic target for RA[279]. However, there are some problems with looking at known GPCRs for the CD13 receptor. In general CD13 is not structurally typical of the ligands for these GPCRs. CD13 is a large, enzymatically active, glycoprotein. The ligands for these receptors tend to be much smaller proteins or non-protein molecules.

The type of cells that respond to CD13 may also help in identifying the CD13 receptor. To this end, a panel of various different cell types could be run through an assay looking for a response to CD13. This response could be chemotaxis, increase in cAMP, or phosphorylation of intracellular signaling pathways. Cells to be tested could include variously activated T cells, monocytes, FLS, and endothelial cells. Based on our assays we can already identify that T cell activation is necessary for chemotaxis, so receptors that are only expressed on activated T cells or go up with activation are possibilities. This offers support for receptors such as AT1, cannabinoid receptor 1, and opioid μ receptor, which are upregulated with T cell activation [280,281]. A microarray comparing mRNA from resting T cells and Tck has been run. While it did not cover all of the potential GPCRs, this data or similar assays could help identify potential receptors. Since we did not observe chemotaxis of resting T cells but did see chemotaxis with Tck, GPCRs that are upregulated on the Tck over resting T cells are potential candidates for the CD13 receptor. In this microarray (covering approximately 2600 sites) receptors that were upregulated on Tck over resting T cells included: CCR1 (1.864 fold increase), CCR5 (1.355), CXCR3 (1.84), CXCR6 (2.75), prostanoid receptor EP_4 (1.648), orphan receptor GPR18 (1.76), orphan receptor GPF35 (1.381), orphan receptor GPR44 (1.291), orphan receptor GPR68 (2.073), and orphan receptor GPR92 (1.216). The greatest increase was seen in CXCR6 which is interesting as this receptor is present on T cells, monocytes, endothelial cells, and FLS, and it is

known to be strongly involved in a mouse model of RA [282]. This is suggestive of CXCR6 as a potential receptor for CD13. Part of what may complicate the process of looking for a CD13 receptor is the number of orphan GPCRs with no known ligand. Since CD13 is not typical of most GPCR ligands, an orphan receptor being CD13 appears even more likely. However, this may make identification of the CD13 receptor even more difficult.

One possible direct way of identifying the CD13 receptor is co-immunoprecipitation. We have attempted to identify CD13 bound to T cells (Tck as well as other T cell types) by incubation of the cells with rhCD13 and flow cytometry. We used both rhCD13 followed by antibodies to CD13 and biotin-conjugated CD13, but could not detect CD13 bound to the T cells (data not shown). This indicates that the binding of CD13 is either weak or transient. For co-immunoprecipitation it would therefore likely be necessary to use a crosslinker. So, a future experiment could entail incubating Tck with rhCD13 in the presence of a crosslinker (such as bis-sulfosuccinimidyl suberate) [117]. Then immunopreciptation of CD13 could be used to pull down CD13 and any associated molecules. Different anti-bodies may need to be tried to find one that binds the CD13-receptor complex. Gel separation followed by sequencing could then be used to identify the protein(s) bound to CD13 (which should include the T cell CD13 receptor). Another possibility would be the use of a broad GPCR screening assay. There are now commercial screening assays available for both known GPCRs and orphan GPCRs that look for a response (such as internalization or phosphorylation of an intracellular signaling molecule) over a wide number of GPCRs [283]. While these assays do not cover all the possibilities it may be one of the best places to start. A future direction would be to perform an assay of this type

which would at the very least eliminate some possibilities and potentially help identify the CD13 receptor.

Once potential candidates have been identified, antibody blocking of the receptor would help identify and confirm its identity. Tck could be incubated with antibodies to the candidate receptors, and then used in the chemotaxis assay. Inhibition of Tck chemotaxis toward CD13 would indicate the correct receptor. Using the blocking antibody in the FLS growth and migration assays could identify whether CD13 acts through the same receptor on both T cells and FLS.

Cleavage of CD13

We have conclusively shown that soluble CD13 is shed by FLS, and that molecules from the metalloproteinase family are responsible. Both main subgroups of metalloproteinases (MMPs and ADAMs) are possible candidates for cleavage of CD13. Membrane bound MMPs (MT-MMPs) and ADAMs are more commonly identified as sheddases than the soluble MMPs. Based on previous studies with RA FLS, we identified MT1-MMP/MMP14 as a possible CD13 sheddase [49]. And indeed, knockdown of MMP14 in FLS showed that MMP14 was involved in the cleavage of CD13. However, the inhibition of cleavage with MMP14 KD was only partial indicating multiple sheddases of CD13. We attempted knockdowns of some of the other potential metalloproteinases (including double knockdowns with MMP14); however, since no other knockdown resulted in a significant decrease we can conclude that MMP14 is the major contributor to CD13 cleavage (data not shown). Other possibilities include the transmembrane

MMPs, MMP15 and MMP16, or ADAMs. ADAMs that have been identified in RA (either mRNA or protein) include ADAMs 8,9,10,15, and 17 [182,184,185].

We examined FLS by qRT-PCR following MMP14 knockdown to determine whether other metalloproteinases were upregulated following knockdown of MMP14. This could indicate compensation. However, we did not see any significant changes in MMP15, MMP16, ADAM10, ADAM15, or ADAM17 mRNA with MMP14 KD (data not shown). We did note in our cells only very low levels of MMP15 and MMP16 at the mRNA level (data not shown). Although this may not reflect protein levels, it is supportive of a hypothesis that ADAMs are the other shedders of CD13. We also attempted to use COS-1 versus CHO shedding of transfected recombinant human CD13. COS-1 (African green monkey kidney epithelial) cells are known to have ADAM molecules but no MMPs; while CHO (Chinese hamster ovary) cells have both ADAMs and MMPs[183,284,285]. While CD13 was found as a soluble molecule in culture supernatants from both cell types, the data was inconclusive. Attempts to block shedding with GM6001 only yielded small decreases in the release of CD13 from both cell types (data not shown). This indicates that release of the transfected CD13 from COS-1 or CHO cells occurs primarily through a metalloproteinase-independent mechanism. Therefore, this data does not help in determining a role for MMPs versus ADAMs in CD13 shedding.

All of our data points towards a complex mechanism involving MMP14 along with other MMPs or ADAMs cleaving CD13 from the surface of FLS. Another potential direction for this research could involve determining which other metalloproteinases are involved. However, one major

problem with determining which MMPs or ADAMs are contributors is that ADAMs can be redundant and frequently compensate for each other[183]. It is possible that to completely abrogate CD13 shedding three or more metalloproteinases would need to be suppressed at the same time. Transient knockdown experiments would be difficult to use, since creating a knockdown involving more than two proteins is difficult. One possible way to explore this would be to attempt to determine which other cells shed CD13 and what stimuli can induce CD13 shedding. After determining which cells shed CD13 it could then be determined which metalloproteinases are expressed on those cells and a better profile of involved metalloproteinases could be developed.

Stimuli resulting in either upregulation or downregulation of shedding could also be tied back to creating a profile of which metalloproteinases are involved in the shedding. Another alternative would be to attempt double knock-in of CD13 and each of the potential cleavers into a cell line looking for an increase in CD13 release. However, this would only give information on which molecules are capable of cleaving CD13 and would not aid in determining how this occurs naturally. In conclusion MMP14 is the major cleaver of CD13 on FLS, and while at least one other metalloproteinase also cleaves CD13, determining which one will likely prove difficult.

CD13 in murine models

We have conclusively shown a role for CD13 in RA in human samples *in vitro*. One of the next steps in looking at the function of CD13 in arthritis is examining CD13 in a mouse model. We have shown that CD13 may be involved in multiple processes in the RA joint. As such, looking

at CD13 *in vivo* will help define its broader impact on disease processes. CD13 knockout mice have been created and appear to be generally healthy [138,228]. The main difference discovered in the CD13 KO mice was a deficiency in pathogenic angiogenesis (in response to hypoxic conditions, VEGF, bFGF, or TGFα) but not in normal development angiogenesis or neovascularization [228]. CD13-KO mice develop collagen antibody induced arthritis (CAIA) normally, and this was suggested to show that CD13 is not necessary for inflammatory infiltration into the synovium [138]. However, CAIA is characterized by polymorphonuclear leukocyte and macrophage infiltrate not a T cell response [286]. Our results indicate that CAIA is not a good model for CD13 in RA, and that instead studies should be run in a T cell dependent model such as collagen induced arthritis (CIA).

Development of a CIA model in CD13-KO mice would be a better model. One difficulty here is that the current CD13-KO mouse is in a C57BL/6 background [138]. CIA does not develop well in C57BL/6 mice, and the preferred background is the DBA/1 strain [287]. There are three possible ways this could be handled. First, a new CD13-KO could be created using DBA/1 mice. Second, extensive backcrossing could be done to transfer the current CD13-KO model from the C57BL/6 background to the DBA/1 background. Third, the current C57BL/6 CD13-KO mice could be used. However, since the ability of these mice to develop arthritis is variable higher numbers of mice would be needed. Measurements of general disease activity (joint counts and diameter of joints) in the CIA CD13-KO model would provide insight into how important CD13 is in disease progression. Of particular interest would be T cell infiltrate into the joints (observable by histopathology) and level of angiogenesis (immunohistochemistry). Another possible mouse model of RA is the K/BxN serum transfer model. While the K/BxN model is

similar to the CAIA model in that it is not T cell dependent, this model could be used to examine angiogenesis. The K/BxN serum transfer model has high levels of angiogenesis in the joints [288]. This model could be used with the CD13-KO mice on either the DBA/1 or C57BL/6 backgrounds. While it may yield similar results to the CAIA when examining the overall disease severity in CD13-KO, it would be interesting to determine if there are differences in the angiogenesis in the joints. This could be examined by histopathology, and while T cells are not as important in K/BxN mice, overall leucocyte counts in the joints could be made.

CD13 should also be measured in a WT CIA model in DBA mice. CD13 aminopeptidase activity is elevated in the synovial fluid, plasma, and the soluble fraction of synovial tissue homogenates in CIA rats [289]. However, our results indicate that at least in human samples CD13 activity is not a completely accurate measure of CD13. A mouse CD13 ELISA should be created and CD13 measured in the joint tissue homogenates and serum of CIA mice. Histopathology could also be used to examine CD13 on murine joint synovium sections. Another way to look at a role for CD13 would involve the use of anti-CD13 antibodies or enzymatic inhibitors in prevention or treatment of CIA in DBA mice. This would help to determine whether CD13 has potential as a therapeutic target.

To further examine the T cell chemotaxis specifically, an RA ST-SCID (rheumatoid arthritis synovial tissue – severe combined immunodeficiency) mouse model could be used. In this model a piece of synovial tissue is engrafted under the skin of a SCID mouse. After the graft heals CD13 would be injected into the graft and labeled human T cells would be injected

intravenously. The graft is harvested and the number of T cells migrated into the tissue can be counted under a microscope [290]. This model has the advantage of being able to examine chemotaxis and tissue invasion specifically but in an *in vivo* system which could provide additional factors that may influence the mechanism.

CD13 and other diseases

This research focused on CD13 in RA; however, CD13 is likely also involved in other diseases. CD13's variety of functions and presence throughout the body indicates that it may have an important role in multiple diseases. CD13 is already a target for drug discovery in cancer, primarily due to its role in angiogenesis. However, our research specifically points to involvement in more immune mediated diseases. Since CD13 has been linked to FLS growth, T cell migration, and inflammation it is logical that it may be involved in inflammatory joint conditions other than RA. It may also implicate CD13 in systemic inflammation. We did examine some serum samples from lupus, scleroderma, myositis, and Sjogren's syndrome patients (Figure 2.6). Similar to RA we found lower levels of serum CD13 by ELISA, in myositis and lupus, or by enzymatic activity assay, in scleroderma and myositis. Our data shows that aminopeptidase levels were decreased in scleroderma samples compared to healthy controls. However, there was no significant difference in CD13 amounts. This may indicate that another aminopeptidase is decreased in the sera of scleroderma patients, that scleroderma sera CD13 has a lower specific activity, or that there are increased levels of a natural inhibitor of CD13 in scleroderma sera. Further experiments would need to be done to clarify this result. Similar to what we did with RA SF, the CD13 should be immunoprecipitated out from scleroderma and healthy sera. Then both the immunoprecipitated CD13 and the depleted fractions could be

examined for aminopeptidase activity. The sample numbers for lupus, myositis, and Sjogren's syndrome were limited and higher numbers would be needed to confirm the results.

Studies involving scleroderma, lupus, and other inflammatory diseases could measure CD13 in fluids and cells from various disease sites. As we noted in RA, CD13 may be elevated in one location (synovial fluid) but decreased in another (serum). Therefore CD13 should be measured not only in serum but at all accessible sites of disease activity. For example, in scleroderma CD13 levels could be measured in serum (systemic), bronchoalvelolar lavage (lung), and skin homogenates. Histopathology could also be used to look at disease tissues to identify which cells are expressing CD13. A previous paper found higher amounts of CD13 enzymatic activity in bronchoalveolar lavage fluid from patients with scleroderma, RA, Sjogren's syndrome, and myositis compared to healthy controls [222]. However, this group also found elevated levels of CD13 enzymatic activity in the sera of patients with RA, lupus, scleroderma, and myositis compared to healthy controls [222]. This is contrary to what we observed; that RA, scleroderma, and myositis have decreased levels of aminopeptdiase activity in sera compared to healthy controls. There is some uncertainty here as these are measurements of enzymatic activity and not directly of CD13. Larger sample numbers of sera, synovial fluid, and bronchoalveloar lavage fluid should be run on the CD13 ELISA, as this would be the best way to determine CD13 levels from specific sites in the various inflammatory diseases.

The presence of CD13 in specific tissue sites or fluids may help to identify possible functions for CD13 in each disease. Lower amounts of CD13 at a particular site could indicate more

uptake/usage of CD13 at those sites. For example, a lower amount in serum may indicate an uptake by the endothelial cells or circulating T cells. Combined with high expression at a disease specific site, joint or lung, this could indicate CD13 acting as a chemoattractant. High expression by a specific cell type may indicate a role in growth and migration of those cells, similar to RA FLS. CD13 involvement should also be examined in other immune mediated conditions. Our data demonstrates that CD13 can act as a T cell chemokine. This could be important in diseases such as psoriasis, asthma, and multiple sclerosis. All of these are diseases linked to T cell involvement and have CD13 present on cells are disease sites (skin, lung, nervous system). Overall we have demonstrated that CD13 can act as a pro-inflammatory agonist, and this may implicate it more broadly in a range of autoimmune processes.

Appendix

Materials and Methods

Development of 591.1D7.34, a novel anti-CD13 monoclonal antibody

The investigations summarized in this report commenced as an approach to defining novel inflammatory pathways in RA involving FLS, trigged preferentially by IL-17 rather than TNF. BALB/C mice were serially immunized with 10^6 FLS treated with IL-17, 10ng/ml, for 48 hours prior to immunization. A spleen from the most reactive mouse (assessed by flow cytometry of FLS using mouse sera) was fused to a non-secreting myeloma cell line. The resulting hybridoma clones were screened on resting FLS, TNFα treated FLS, and IL-17 treated FLS, then selected for subcloning. The clone producing the antibody 591.1D7.34 was identified as being of interest. An OA FLS cell line was biotinylated and lysed. Immunopreciptiation was used to isolate the protein recognized by mAb 1D7. Controls included mouse IgG (MsIgG, isotype control) and anti-CD98 (an FLS positive control). The proteins eluted from the immunoprecipitation beads were electrophoresed under reducing or non-reducing conditions on a 4-20% Tris-glycine polyacrylamide gel. Protein from the same number of cells was loaded in each lane. The gel was transferred to an immobilon-P membrane and incubated with streptavidin-HRP then ECL. U937 myeloid cells were also lysed and 1D7 was used to immunoprecipitate the recognized protein which was resolved by electrophoresis on two identical polyacrylamide gels. One was stained with Coomassie blue, and the other was excised at a spot corresponding to the primary

band in the stained gel. The protein was subjected to trypsin digestion in-gel and the resulting peptide mixture was analyzed by LC-MS/MS on a Qtof premier instrument (Waters Inc). Protein Lynx Global Server and Mascot search engines were used to search the Uniprot and NCBI databases. The protein identified matched Aminopeptidase N/CD13.

Cell Culture

All procedures involving specimens obtained from human subjects were performed under a protocol approved by the University of Michigan Institutional Review Board. FLS were cultured from human synovial tissue obtained at arthroplasty or synovectomy from RA or OA joints following digestion with 1% collagenase and separation through a 70 micron cell strainer. The diagnosis of RA was based upon the presence of at least four of the seven 1987 American College of Rheumatology criteria[291]. The diagnosis of OA was based upon characteristic clinical and radiographic features, and confirmed by pathological findings at joint surgery. U937 cells were cultured in RPMI 1640 (10% fetal bovine serum [FBS], 2% L-Glutamine, 1% HEPES, 1% Sodium Pyruvate, 0.5% glucose). FLS were maintained in Connaught Medical Research Lab (CMRL) medium supplemented with 20% FBS, 2mM L-glutamine, and 1% penicillin/streptomycin and were used between passages 4 and 10. Cultures were moved to serum free Dulbecco's modified Eagle's medium [DMEM]/F-12 with Peprogrow serum replacement (Peprotech) for 24-72 hours before harvesting. Some cultures were treated with protease inhibitors for 72 hours in serum-free medium: pepstatin A (Sigma-Aldrich), aprotinin (Sigma-Aldrich), leupeptin (Sigma-Aldrich), GM6001 InSolution (EMD), or E-64 (Thermo Scientific). Other cultures were treated with cytokines: recombinant human IFNγ (rhIFNγ) (1U/ml), rhTNFα (10ng/ml), or rhIL-17 (10ng/ml) (Peprotech) for 0, 0.5, 1, 2, 6, 8, 12, 24, 48, or 72 hours in serum free medium. T cells were isolated from peripheral blood of healthy subjects

by negative selection using RosetteSep Human T cell enrichment cocktail (Stemcell) and Histopaque (Sigma-Aldrich). Cytokine activated T cells were prepared by culture of T cells in IL-6 (100ng/ml), IL-2 (25ng/ml), and TNFα (25ng/ml) (all Peprotech) for 7-10 days in 10% FBS RPMI 1640 medium[19,156]. The COS-1 cell line (ATCC) was grown in 10% FBS/DMEM.

Sample Preparation

Synovial fluid samples were treated first with 0.05% Hyaluronidase (bovine testis, Sigma-Aldrich) one drop per 1 mL fluid for 5 min. Cells were lysed in cell lysis buffer (10% Nonidet P40, 10% phenylmethylsulfonyl fluoride, 1% Iodoacetinimide, and 0.1% E-64 in TSA) for one hour on ice. FLS culture supernatants were concentrated by centrifugation through an Amicon Ultracel 30K filter (Milliepore). Plasma was isolated from whole blood using heparin vacutainer tubes (BD biosciences). Serum was isolated from whole blood using serum separation vacutainer tubes (BD biosciences).

Flow cytometry

Fibroblasts were removed from flasks by 3mM EDTA in PBS. Cells were stained with MsIgG (negative control) or anti-CD13 (1D7, SJ1D1 [Abcam], or WM15 [Biolegend]), then goat anti-mouse IgG-Alexa fluor 488 (Molecular Probes). Cytometry was performed on a BD Biosciences FACSCalibur.

Aminopeptidase enzymatic activity

CD13 aminopeptidase activity was measured by cleavage of L-Leucine-7-amido-4-methyl coumarin (L-leu-AMC, Sigma-Aldrich) to release the fluorescent molecule AMC. A standard curve was constructed using AMC (Sigma-Aldrich). The assay was run in 0.1 M Tris-HCl

buffer (pH 8.0). Samples were incubated with the substrate at 37°C for one hour then read using a fluorescent plate reader at emission 450, excitation 365. Results were calculated as the amount of substrate cleaved in μM/hr.

CD13 Enzyme-Linked Immunosorbent Assay (ELISA)

High binding ELISA plates were coated with the anti-CD13 monoclonal antibody WM15 in 0.1M carbonate buffer pH9.5 overnight, and blocked with 1x Animal Free Block (Vector Laboratories) overnight. Between steps the plates were washed 5 times with wash buffer (PBS plus 0.05% Tween) and washed 3 times before addition of samples. Samples were then applied to the plates either whole or diluted in block with 10mM EDTA. The standard curve was prepared using recombinant human CD13 (R&D Systems) in block with 10mM EDTA. Plates were incubated overnight then washed 7 times. 1D7 was biotinylated (Biotin-XX Microscale Protein Labeling Kit, Molecular Probes) and applied overnight. Plates were washed 7 times. Streptavidin-horseradish peroxidase (Biolegend) was then added. The plates were visualized with tetramethylbenzidine substrate (TMB, BD Biosciences), stopped with 2M H_2SO_4, and analyzed on a colorimetric plate reader.

Tissue Staining

Tissues were harvested form normal, OA, and RA patients during surgery. Tissue sections were frozen with liquid nitrogen and 7 μm frozen sections were made using a cryostat. The slides were fixed in Acetone at 4^0C for 30 minutes, washed, and blocked in 20% Fetal Bovine Serum and 5% goat serum in PBS. Slides were incubated with the primary antibodies anti-human CD13 (1D7, 10μg/mL) and rabbit anti-human CD11c (Abcam, 1:100 dilution) or rabbit anti-human Cadherin 11 (Zymed 10μg/mL). Controls were rabbit and mouse IgG at equivalent

concentrations. Secondary antibodies for both sets of slides were Alexa fluor goat anti-mouse 488 and goat anti-rabbit 555 (Life Technologies 10µg/mL), and DAPI (Invitrogen, 1:5000 in PBS) was used on all slides. Slides were mounted with Fluoromount-G (Southern Biotech). Pictures were taken on an Olympus BX microscope at 40x magnification. Arrows point to examples of co-localization.

Immunoprecipitation

Immunoprecipitation was performed using the Pierce Direct IP Kit (Thermo Scientific). In brief antibodies were bound to AminoLink Plus beads using cyanoborohydride, either anti-CD13 (1D7), anti-CD98 (7F8), or isotype control (MsIgG)[292]. Samples were diluted 1:1 with IP lysis/wash buffer and incubated overnight with the beads. The depleted portion was removed by centrifugation. Protein was eluted off the beads by low pH (2.8) which was neutralized with 1 M Tris (pH 9.5).

Quantitative RT-PCR

mRNA was isolated from FLS (3 wells of a 6-well plate) using the RNAeasy Kit and Qiacube (Qiagen). cDNA was prepared using a High Capacity cDNA Kit (Life Technologies). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was done using TaqMan Gene Expression Assays on a 7500 Real Time PCR System (Applied Biosystems).

Cytokine ELISAs

Cytokines were measured in human serum samples using ELISA kits. TNFα and IFNγ were measured using the respective OptEIA human cytokine ELISA kits from BD. IL-17 was measured by the human IL-17A ELISA kit from eBioscience. All three cytokines as well as CD13 protein and enzymatic activity were measured on the same day.

siRNA Knockdown

FLS were transfected by electroporation using an Amaxa Nucleofector and the nucleofector kit for dermal fibroblasts (NHDF, Lonza). In brief, FLS were released by trypsin and $5x10^5$ were transfected per condition. Cells were resuspended in transfection solution and either 300nM MMP14 siRNA cocktail (stealth RNAi, Invitrogen), MMP1 siRNA cocktail (stealth RNAi, Invitrogen), negative control cocktail (stealth RNAi medium control, Invitrogen), or 2μg pmaxGFP (Lonza) was added to each transfection cuvette. Each cocktail contains three siRNAs specific for the target. Cells were electroporated and transferred to flasks containing 20% CMRL. Transfected cells were grown for 5-7 days then transferred to serum free medium for 2 days before harvesting. Transfection of GFP control plasmid was measured by fluorescent microscopy (EVOSfl, AME) and flow cytometry (BD Bisoceinces FACSCalibur).

Confocal Microscopy

RA FLS were grown to 90% confluence on 8-well glass chamber slides. Cells were fixed with 1% Formalin and blocked with Fc block (10% human serum/10% mouse serum in PBS). Cells were incubated for 1hour at room temperature with anti-CD13-FITC (1D7) 1μg/100μl and anti-

MMP14-PE (clone128527, R&D) 1.67μg/100μl or anti-CD90-PE 1μg/100μl (3E10, Biolegend). All experiments also included staining with MsIgG isotype controls (MsIg-FITC [eBioceinces], MsIg-PE [Biolegend]) at the same concentrations. The nuclei were counter stained with DAPI at 1μg/ml. Cells were mounted using Pro-gold anti-fade media (Life Technologies). Images were taken with an Olympus Fluo-View 500 confocal microscope system (University of Michigan Microscopy and Image Analysis Core). All images were corrected for background, thresholds were determined by DAPI alone, MsIg-FITC alone, and MsIg-PE alone.

Exosome Isolation

Exosomes were isolated by serial ultracentrifugation [205]. Exosomes were isolated from either the supernatants of 3 flasks of confluent RA FLS, 10mls of plasma, or 1 ml of RA synovial fluid diluted 1:4 with PBS. Cells were spun out at 1500rpm for 5 minutes. Then the supernatants were cleared of heavier debris by spins at 10,000xg for 30 minutes and 30,000xg for 1 hour. Exosomes were then spun down at 110,000xg for 4-20hours. Exosome pellets were washed in PBS at 110,000xg for 1.5 hours – overnight and resuspended in 1ml of PBS. Some exosomes were further purified using a density gradient, Optiprep (Sigma). Optiprep was diluted with PBS to produce the following layers: 5%, 10%, 15%, 20%, 30%, 40%, and 50% w/v (densities of 1.031, 1.050, 1.084, 1.110, 1.163, 1.215, and 1.268 g/ml). 500μl of extra cellular vesicle fractions were floated on the top of the density gradient and the gradients were spun at 100,000xg for 1hour. Fractions were carefully pipetted off, washed with PBS, and spun at 110,000xg for 2 hours. Pellets from the fractions were resuspended in 500μl PBS.

Chemotaxis Assay

The chemotaxis assay was a modified under agarose chemotaxis system (Figure 6.1)[293,294]. Some plates were coated with fibronectin (10μg/ml) overnight at 4°C then washed 2x with PBS. A 2.4% agarose (UltraPure, Invitrogen) solution in 1x HBSS was boiled and combined with 2 parts 1% BSA in RPMI and 1 part 1x HBSS. 3mls of this solution was added to each well of a 6-well plate and allowed to cool to room temperature. A 3-hole punch (Figure 6.1C, University of Michigan Instrument Shop) was used to create three 4 mm wells, with edges 3 mm apart, and the plates were equilibrated to 37°C. Chemoattractants and T cells were adjusted to appropriate concentrations in chemotaxis medium (0.1% BSA, 2% L-glutamine, 1% pen/strep, in RPMI without phenol red). Some Tck samples were treated with 100ng/ml of pertussis toxin for 2hr at 37°C in 10% RPMI, then washed 1x with 0.1% BSA RPMI. $2.5x10^5$ T cells were added to the center well. A medium only control was added to one of the outside wells while the chemoattractant solution was added to the second. Plates were incubated at 37°C, 5% CO_2 overnight (uncoated) or for 5 hours (fibronectin). Pictures were taken of the area directly between the outside and center wells using an EVOS inverted microscope at 100x magnification. Cell counts were Ln transformed and data is expressed as a chemotactic index (CI= Ln cells migrated to stimulus – Ln cells migrated to medium alone).

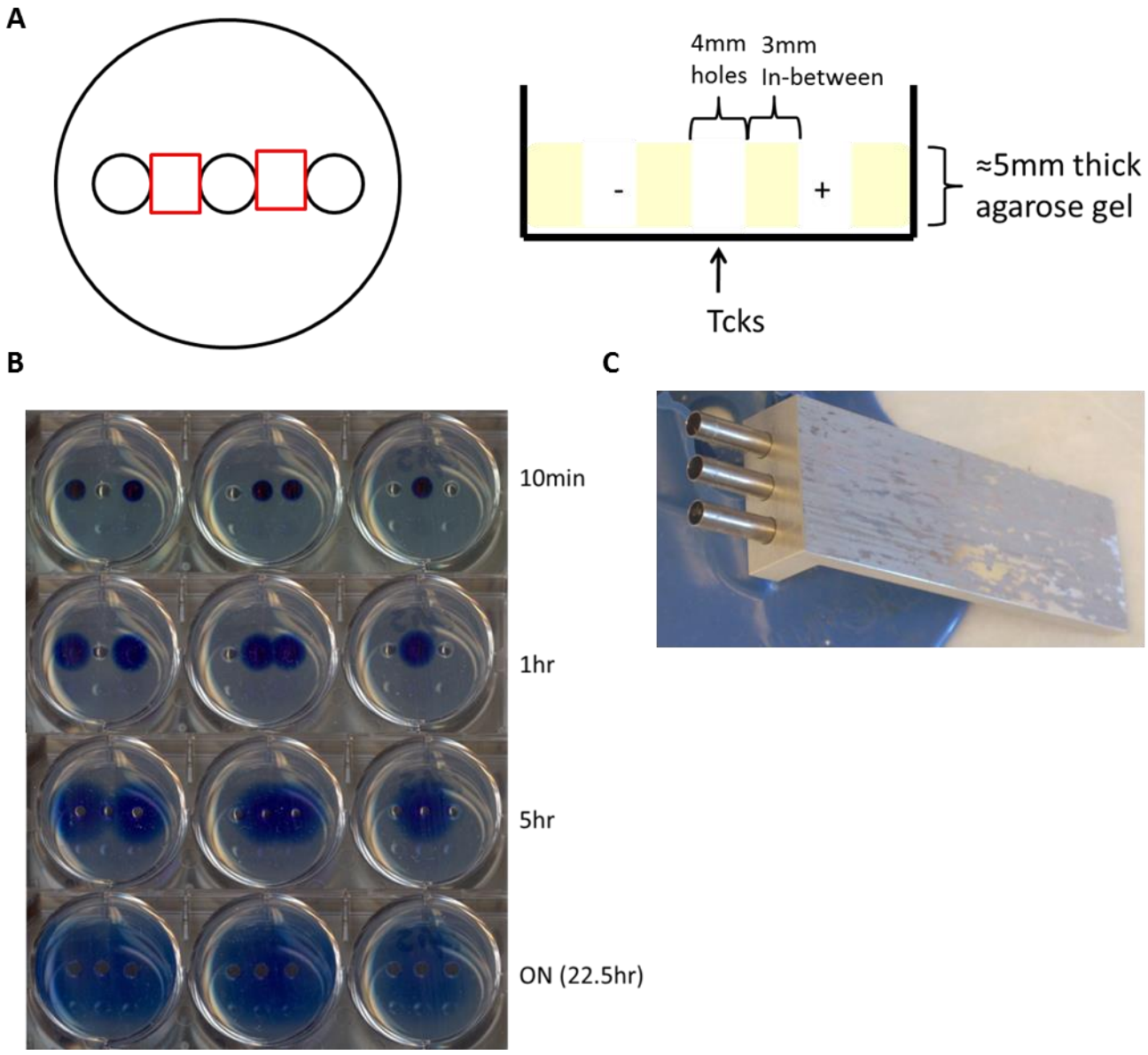

Figure 6.1 Setup of modified under agarose chemotaxis system for Tck chemotaxis.
A, Model of the setup of an under agarose chemotaxis plate. Red boxes indicate the area where pictures were taken to count chemotaxis (toward positive) or background movement (toward negative). **B,** Images demonstrating gradient formation under the agarose. Blue dye was placed in wells and pictures taken at intervals (10 minutes to overnight) to monitor the spread under the agarose. **C,** Picture of the three hole punch tool used to create the holes in the agarose.

CD13 enzymatic mutation

A CD13 clone (MGC Human ANPEP Sequence-Verified cDNA, GenBank accession no. BC058928) was obtained from Open Biosystems. An E355Q enzymatic inactive mutant was created. The plasmid (1µg DNA) was linearized by double digestion with SgrAI (2U) and SbfI (10U) (New England Biolabs). The 7.3kb fragment was purified from a 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen). The plasmid fragment and two gene blocks, (IDT): 5'-

TGGCCTGCCAGACTTCAACGCCGGCGCCATGCAGAACTGGGGACTGGTGACCTACC
GGGAGAACTCCCTGCTGTTCGACCCCCTGTCCTCCTCCAGCAGCAACAAGGAGCGG
GTGGTCACTGTGATTGCTCATGAGCTGGCCCACCAGTGGTTCGGGAACCTGGTGACC
ATAGAGTGGTGGAATGACCTGTGGCTGAACGAGGGCTTCGCCTCCTACGTGGAGTA
CCTGGGTGCTGACTATGCGGAGCCCACCTGGAACTTGAAAGACCTCATGGTGCTGAA
TGA-3' and 5'-

GACCTCATGGTGCTGAATGATGTGTACCGCGTGATGGCAGTGGATGCACTGGCCTCC
TCCCACCCGCTGTCCACACCCGCCTCGGAGATCAACACGCCGGCCCAGATCAGTGA
GCTGTTTGACGCCATCTCCTACAGCAAGGGCGCCTCAGTCCTCAGGATGCTCTCCAG
CTTCCTGTCCGAGGACGTATTCAAGCAGGGCCTGGCGTCCTACCTCCACACCTTTGC
CTACCAGAACACCATCTACCTGAACCTGTGGGACCACCTGCAGGAGGCTGTGAACA
ACCGGT-3', were assembled and transformed into NEB 5-alpha E. Coli using the Gibson Assembly Cloning Kit (New England Biolabs). Clones were selected by Ampicillin resistance and several colonies were sequenced (University of Michigan Sequencing Core) to identify correct clones. Plasmids for wild-type CD13 or enzymatic inactive CD13 were grown up and isolated using an EndoFree Plasmid Maxi Kit (Qiagen). Plasmids were transfected into COS-1

cells (ATCC) using Lipofectamine LTX with Plus Reagant (Life Technologies). Mock transfection with Lipofectamine only was used as a control. Cultures were switched to serum free SFM4CHO media (Thermo Scientific) 48 hours after transfection and cultures were harvested 24 hours after.

FLS Growth and Migration Assays

RA FLS were seeded on Essen Image Lock 96-well plates overnight at either 3,000 cells/well for growth or 30,000 cells/well for migration. For growth the 20% media was removed and cells washed 1x with PBS. 100µl of medium alone (control) or medium containing anti-CD3 (25 or 50ng/ml, OKT3, used as a non-reactive control antibody in this experiment), anti-CD13 1D7 or WM15 (Biolegend) (25 or 50 ng/ml), or CD13 chemical inhibitors actinonin (Sigma-Aldrich) or bestatin (Sigma-Aldrich) (10µM and 50µM respectively) was added to the wells. Images and confluence data were collected using an Essen IncuCyte. For the scratch wound migration assay wounds were made via the Essen scratch wound tool in the seeded 96-well plate. Plates were then washed 2x with PBS and medium was added similar to the growth plates. Data were collected and the confluence or relative wound density calculated by the Essen IncuCyte.

Statistics

Concentrations, percent remaining, percent change in concentration, ratio of relative wound density, ratio of confluence, and chemotactic index are expressed as mean±SEM. Enzymatic activity and fold change over GAPDH are expressed as mean±SD. Statistically significant chemotaxis was assessed by paired student t-test between the Ln stimulus count to Ln medium

alone count; an unpaired T-test was used between groups. Correlations were analyzed by ANOVA. Otherwise significance was determined by unpaired student t-test, unless otherwise noted.

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