PRO-INFLAMMATORY PLATELET FACTOR 4 (CXCL4/PF4) SIGNALING IN RHEUMATOID ARTHRITIS

by

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Pro-inflammatory platelet factor 4 (CXCL4/PF4) signaling in rheumatoid arthritis

submitted by Jeffrey To in partial fulfillment of the requirements for

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in Biochemistry and Molecular Biology

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Abstract

Background: Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the joint tissues caused by activation of locally recruited immune cells. Within the joints, the resident fibroblast-like synoviocytes (FLS) play an important role in RA-associated tissue degradation in part due to their secretion of pro-inflammatory cytokines and tissue-degrading matrix metalloproteinases (MMPs) into the synovium, as well as the cells' ability to invade nearby structures. Platelets also function as immune cells that contain and secrete pro-inflammatory molecules but their role in RA is not understood. Of particular interest is platelet factor 4 (PF4), a major constituent of platelet alpha-granules.

Methods: Cultured SW982 cells were used as a model for FLS. Cells were cultured in the presence or absence of recombinant PF4. The secretion of MMP-1 (interstitial collagenase) was measured by enzyme-linked immunosorbent assay (ELISA). Cell adhesion was determined by wash-off assays.

Results: Cells cultured with recombinant PF4 secreted more MMP-1 relative to controls. PF4 treatment also increased the FLS production of fibronectin, a matrix adhesion molecule. PF4-treated cells also exhibited greater adhesion and spreading.

Conclusion: I conclude that PF4 contributes to an invasive/destructive phenotype in FLS, by promoting MMP-1 release and increased cell adhesion.

Lay Summary

Rheumatoid arthritis (RA) is a crippling disease affecting ~1% of the global population. A hallmark of the disease is chronic inflammation and destruction of the tissues that make up the joints. There is currently no cure for RA. My project studies the potential contribution of platelets, tiny blood cells, in RA. Although primarily known for blood clotting, platelets can also contribute to inflammation and therefore drive RA progression. My results show that platelet factor 4 (PF4), a molecule released by platelets, can alter the characteristics of fibroblast-like synoviocytes (FLS), which are the cells that make up the tissue lining surround the joint. Specifically, I found that PF4 makes cultured FLS more destructive. This research provides a better understanding on how platelets might cause changes in the joint during RA.

Preface

Chapter 2 of this thesis describes the effects on platelet factor 4 (PF4) on cultured fibroblast-like synoviocytes. The experiments were designed by myself, Dr. Hugh Kim and Dr. Hoa Le. All experiments described were performed by me with the exception of the label-free quantitative mass spectrometry. All elements of the mass spectrometry-related experiments, including sample preparation, execution and analysis were performed by Dr. Craig Kerr, under the supervision of Dr. Leonard Foster. Dr. Kerr wrote the corresponding portion of the methods section (Chapter 2, pages 19-22).

Selected elements of Chapter 2 may be incorporated into a future manuscript that will be submitted for publication.

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List of Abbreviations

ACPA	Anti-citrullinated protein antibodies
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CCR	C-C motif chemokine receptor
CXCL	C-X-C motif ligand
CXCR	C-X-C motif chemokine receptor
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothretiol
EBV	Epstein-Barr Virus
ECM	Extracellular matrix
ECL	Enhanced chemiluminescence
Erk 1/2	Extracellular signal-related kinase 1/2
FBS	Fetal bovine serum
FLS	Fibroblast-like synoviocyte
GAG	Glycosaminoglycan
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome wide association studies
HPLC	High performance liquid chromatography
JIA	Juvenile idiopathic arthritis
JNK	c-Jun N-terminal kinase
IL	Interleukin
ILAR	International League of Associations for Rheumatology
LFQ-MS	Label-free quantitative mass spectrometry
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NSAIDS	Nonsteroidal anti-inflammatory drugs
PVDF	Polyvinylidene fluoride
PMP	Platelet microparticle
OD	Optical density
RA	Rheumatoid arthritis
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
PF4	Platelet factor 4
PDGF	Platelet-derived growth factor
PMP	Platelet microparticle
PTM	Post-translational modification
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid Arthritis
RF	Rheumatoid factor
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
TBST	Tris-buffered saline with tween
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha

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I would like to thank my supervisor, Dr. Hugh Kim for bringing me on for this project and continuous support throughout. His guidance and patience have been indispensable and pushed me toward becoming the best scientist I could be. I would also like to thank my committee members: Dr. Calvin Yip and Dr. Jayachandran Kizhakkedathu for their encouragement and insights during this project.

I'd also like to thank my fellow Kim lab members, especially Dr. Hoa Le whose knowledge, patience and guidance went a great way to making this project come together. These members also include: Enoli de Silva, Dr. Manoj Paul, Dr. Kalyan Golla, and Camron Chehroudi for getting me started in the lab, and always being available to provide input and comments.

Lastly, I would like to give thanks to my family and friends for all their love and support. Without them, graduate studies would have been far less manageable.

Chapter 1: Literature Review

Overview

Rheumatoid arthritis (RA) is an autoimmune disease afflicting roughly 0.5-1.0% of the global population, with increased prevalence in certain ethic groups (Rudan et al., 2015). A classic hallmark of RA is the presence of the self-antigen binding anti-citrullinated protein antibodies (ACPA). Citrullination is a post-translational modification (PTM) where the amino acid arginine is converted to citrulline. The presence of citrulline leads to the generation of ACPAs by the immune system, driving a pro-inflammatory response (Alivernini et al., 2017). Females are three times more likely to develop RA as males (Okada et al., 2014; Rudan et al., 2015) . Individuals afflicted typically experience chronic pain and swelling of the joints, ultimately leading to loss of mobility (Smolen et al., 2016). Drug treatment of RA typically includes biologic agents such as methotrexate, which have significantly improved clinical outcomes and in rare cases, remission (Fazal et al., 2018; Okada et al., 2014). However, despite advances in RA treatment, a significant number of patients require lifelong care due to permanent joint damage (Fazal et al., 2018). A better understanding of RA pathology is clearly needed to develop better pre-emptive treatments that may be applied before permanent tissue damage occurs.

Genetic mutations predispose individuals to RA

The exact mechanisms behind RA formation remain elusive, but much has been done to further our understanding of RA progression. Genetics plays an important role in determining individual RA susceptibility. For example, a major breakthrough was the improved ability to detect single nucleotide polymorphisms (SNPs) using genome wide association studies (GWAS). This

allowed for the screening of large amounts of RA patient information to identify SNPs common to RA (Okada et al., 2014; Stahl et al., 2010). In one particular study, over 100,000 RA patients were screened and discovered many novel SNPs as well as validating previously observed ones (Okada et al., 2014). Many of the SNPs discovered have direct links to immune regulation, whether by cytokine signaling or antigen presentation, which help to solidify RA as a disease of immune intolerance (McInnes and Schett, 2011; Okada et al., 2014; Stahl et al., 2010). Surprisingly, some other SNPs have been identified with links to cancer (Okada et al., 2014; Smolen et al., 2016). The presence of SNPs in the individual does not necessarily guarantee RA development but rather, renders them predisposed to developing RA. The identification of SNPs also has major implications for drug discovery. The creation of novel therapeutics, as well as repurposing old drugs is based on what targets of interest appear from screening, allowing for a variety of tailored treatment options (Okada et al., 2014; Plenge et al., 2013). Overall, genetics plays a key role in shaping RA environment, and drug discovery, as well as general knowledge will benefit from further RA linked SNP discovery.

Environmental stresses trigger loss of immune tolerance

As previously mentioned, individuals harboring SNPs for RA are merely at risk to developing RA due to these genetic mutations. Rather, the key driver appears to be the environmental stresses that acquired throughout life. One major reported environmental stressor is smoking (Silman et al., 1996). It is hypothesized that the stress on the mucosa and lungs from smoking may lead to increased PTMs, including citrullination, which can lead to autoantibody production. (McInnes and Schett, 2011; Silman et al., 1996). Obesity and general lack of physical activity have also been associated with RA development (McInnes and Schett, 2011; Symmons et al.,

1997). Links have also been made between RA and other diseases with regards to environmental stressors. Periodontal disease is one such example, which involves chronic inflammation of the gum tissues that surround the teeth. One proposed mechanism behind this link is the presence of PADI4 in *Porphyromonas gingivalis*, which has been shown to citrullinate ECM proteins, making them a target for ACPA binding to drive inflammation (McInnes and Schett, 2011; Wegner et al., 2010). Exposure to Epstein-Barr virus (EBV) has also been thought to be a trigger of RA formation, however, the link between EBV and RA is not clear (Balandraud et al., 2004; Smolen et al., 2016). Recently, there has been increasing implications of intestinal microbiome dysregulation as a potential trigger for RA due to its ability to activate the host immune system (Horta-Baas et al., 2017; Smolen et al., 2016). Regardless of the trigger, the result is the push toward intolerance of the immune system and production of self-antigen binding autoantibodies.

Anatomy of a synovial joint

The synovial joint is an interface between two bones which allows for bending and movement for the organism **(Fig. 1)** (Mc Ardle et al., 2015). Encompassing the cavity is the joint capsule, which contains two layers. The first is the outer (subintima) membrane comprised of fibroblasts which contains ligaments and helps with movement (Catrina et al., 2017). The second is the inner (intima) synovial membrane, a thin layer of specialized cells called fibroblast-like synoviocytes (FLS) which encompass the synovial cavity (Catrina et al., 2017). This cavity is filled with synovial fluid which is produced by the FLS and serves to provide lubrication to the joint to prevent friction during movement (Mc Ardle et al., 2015). Lastly, surrounding the ends of each bone is cartilage, a spongy substance that aids in alleviating joint shock. This cartilage is primary made up of collagens and proteoglycans, major ECM components (Salva and Merrill,

2017). In a healthy joint, the cavity is avascular and free of immune cells, leading to minimal interactions between the resident joint cells (Elshabrawy et al., 2015).

Pathological changes within RA joints

Following the onset of RA **(Fig. 2)**, gradual changes begin to take place within the joints. The synovial cavity is normally well regulated and avascular. But, as RA develops, extensive angiogenesis can be seen with the production of new blood vessels in the synovial cavity, thus

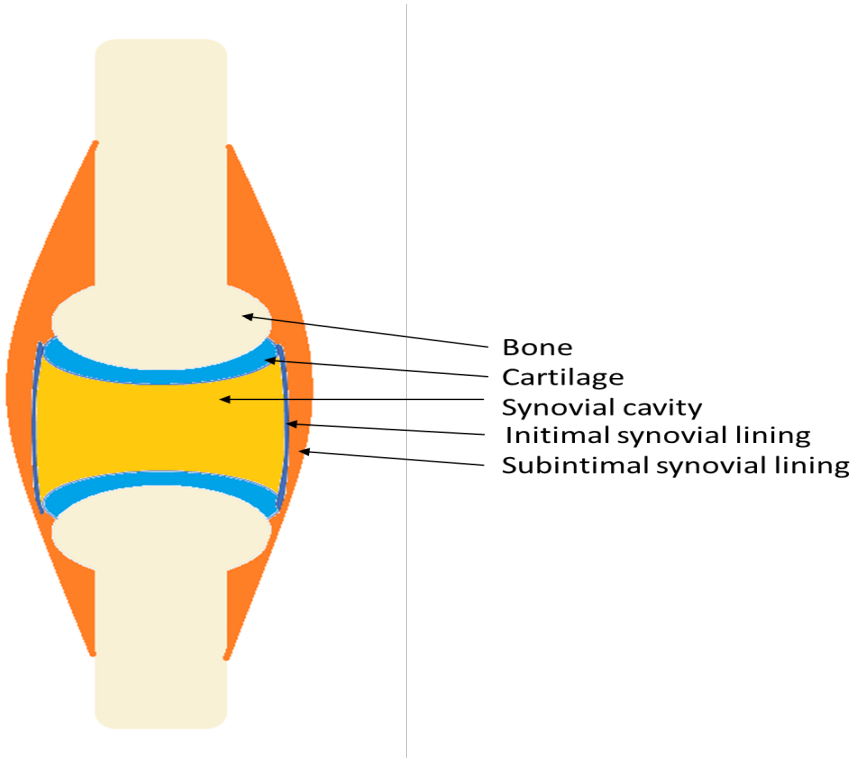

Figure 1. Anatomy of the joint. The structure of the synovial joint is formed to allow for movement and to reduce stress on the bones. Surrounding the joint is the synovium which is comprised of an outer (subintimal) layer and inner (intimal) layer. The intimal layer is comprised on the specialized fibroblast-like synoviocytes that secrete lubricating solution into the cavity. Lastly is the cartilage that acts as a sheath around the bones to absorb shock that occurs from joint movement.

generating new interfaces by which blood components can diffuse into the joint (Elshabrawy et al., 2015). Among which are the immune cells, such as B cells, T cells and macrophages, which are now able to diffuse into the synovium. These cells are a major contributor for cytokines such as tissue necrosis factor-alpha (TNF-α), Il-1 and IL-17, ultimately creating a pro-inflammatory environment. This aids in disrupting the balance between osteoblast/osteoclast formation, driving bone and cartilage destruction (Miossec et al., 2009; Smolen et al., 2016). Lastly, is the dysregulation of the synovial lining. As RA develops, the once uniform tissue begins to hyper-proliferate, forming a tumor-like pannus. This pannus is an overgrowth of FLS cells and is triggered by the influx of signaling molecules in the synovium as well as increased nutrients available due to increased angiogenesis. The pannus is able to continue to grow until it reaches the bone and cartilage where the FLS cells are able to secrete cartilage degrading MMPs, promoting joint destruction (McInnes and Schett, 2011; Smolen et al., 2016).

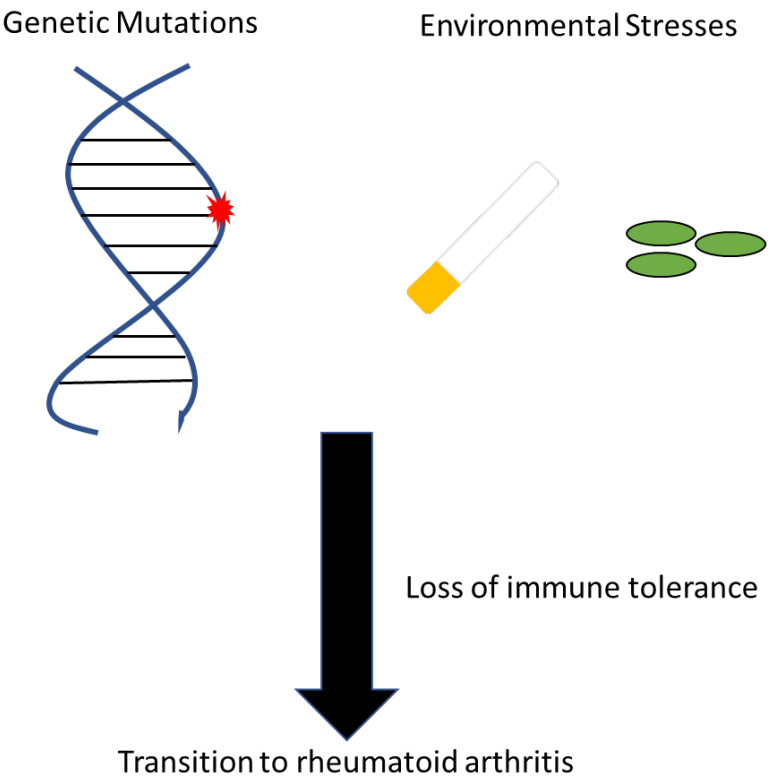

Figure 2. Pathology of rheumatoid arthritis Rheumatoid arthritis is an autoimmune disease that primarily affects the joints. Before symptoms present themselves, formation of the "pre-arthritis" state occurs through a combination of genetic mutations and environmental triggers. This leads to the production of autoantibodies against various proteins and a rampant activation of the immune system, transitioning to arthritis. Joints are affected by chronic inflammation due to the infiltration of immune cells as well as permanent structural damage.

Immune cell recruitment – host-mediated tissue degradation

In a healthy joint, the synovium is typically free of most immune cells. However, this changes as RA develops within the joint. The influx of pro-inflammatory cytokines into the synovium becomes an environment which attracts the blood immune cells. These immune cells are able to enter the synovium by means of the newly formed blood vessels invading into the joint (Elshabrawy et al., 2015). Here, the immune cells come into contact with the inflammatory environment, causing the release of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α into the local environment (Liu et al., 2018). These cytokines then primarily serve to exert their effects on the resident FLS cells and chondrocytes, causing the release of cartilage degrading matrix-degrading metalloproteinases (MMPs) into the synovium, leading to irreversible tissue damage (Garcia-Vicuna et al., 2004; Nakamura et al., 2006; Smolen et al., 2016). Furthering this is the FLS-mediated release of additional pro-inflammatory cytokines, which can then act as a feedback mechanism to propagate the pro-inflammatory environment. For example, it was shown that FLS stimulation of macrophages in a co-culture setting was able to induce a pro-inflammatory macrophage response in a TNF-related manner (Donlin et al., 2014).

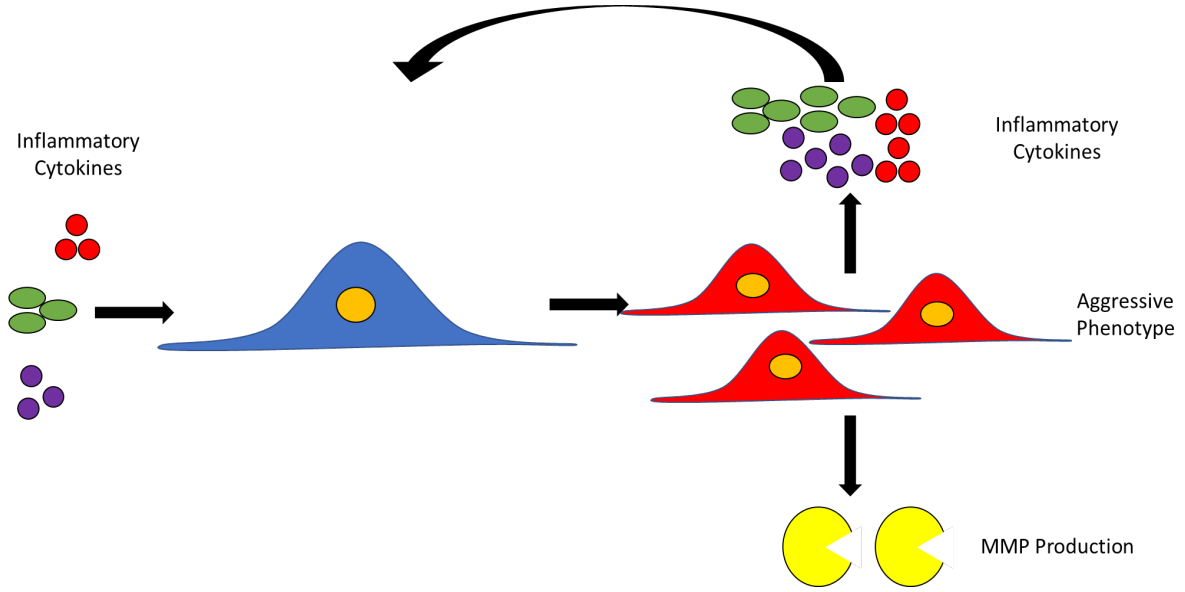

Figure 3. Fibroblast-like synoviocytes are key players in RA progression. Fibroblast-like synoviocytes (FLS) are the major constituent of the synovial lining and play key roles in maintaining joint homeostasis. As RA develops, FLS cells exposed to aggravating triggers adopt a more aggressive phenotype to drive disease. FLS secretion of cytokines is increased thus promoting a pro-inflammatory environment. Cartilage and bone degrading MMP production is upregulated, leading to permanent joint destruction. Lastly, the cells adopt cancer-like properties where they hyper-proliferate to form the tumor-like pannus.

Fibroblast-like synoviocytes are key drivers of RA

One of the major players in driving RA progression are the fibroblast-like synoviocytes (FLS), the cells that comprise the synovial lining. FLS, also known as type B synoviocytes, are thought to be mesenchymal in origin, and as the name implies, share several features with fibroblasts (Smolen et al., 2016; Tu et al., 2018). In healthy conditions, FLS are responsible for providing joint structure as well as maintaining the synovial fluid through the secretion of lubrication proteins like lubricin (Bartok and Firestein, 2010; Smolen et al., 2016). This changes during RA, as the FLS become "activated" and adopt a more aggressive phenotype (RA FLS). One noticeable shift is the increase in cytokine/chemokine production. This includes IL-6 production, which is the major cytokine secreted by RA FLS, as well as granulocyte-macrophage colony-

stimulating factor (GM-CSF) (Leizer et al., 1990; Perlman et al., 2003). This allows for communication with the now present immune cells in the synovium, which can in turn generate a feedback loop on the RA FLS and perpetuate the pro-inflammatory environment **(Fig. 3)**. In addition, other resident joint cells, such macrophage-like synoviocytes, are affected by the influx of pro-inflammatory cytokines, which manifests as the increased secretion of pro-inflammatory cytokines and the formation of bone-resorbing osteoclasts (Tu et al., 2018). Next, RA conditions promote secretion of MMPs from RA FLS. Some major players include MMP-1, MMP-3 and MMP-13, which serve to irreversibly destroy the bone and cartilage (McInnes and Schett, 2011; Perlman et al., 2003; X.-L. Shui, 2017). Lastly, and perhaps the most unique is the transformation to a cancer-like phenotype. The RA FLS begin hyper-proliferating as cell growth becomes unchecked (Garcia-Vicuna et al., 2004; Smolen et al., 2016). Supporting this is the shutdown of apoptotic pathways, where key apoptotic pathways are shut down (Baier, 2003). Furthermore, autophagy is upregulated to allow help sustain the increased cell growth (Vomero et al., 2018). This continues as the cells become anchorage-independent and lose cell contact inhibition. The unchecked cells can then multiply non-stop, forming a tumor-like structure called a pannus. Finally, this newly formed mass of cells in the pannus are even able to migrate toward the bone and cartilage, allowing for destruction of bone and cartilage as well as interaction with chondrocytes (Bartok and Firestein, 2010; M.M.C. Steenvoorden, 2007). Overall, FLS are key player in driving RA as they adopt the aggressive phenotype to modulate the local environment.

Cell adhesion/spreading and relevance to RA

A hallmark of the activated RA FLS phenotype is their hyper-migration and motility. As these cells activate, they can migrate toward nearby tissues and exert their tissue destroying

capabilities (Garcia-Vicuna et al., 2004; Wang et al., 2017). This is mediated by cellular adhesion and migration mechanisms which form specialized structures that allow the cell to migrate to their destination (Parsons et al., 2010). The process begins with the formation of focal adhesion complexes at the site of integrin receptor-ECM adhesion. Focal adhesion components such as vinculin and myosin are then recruited to form the mature focal adhesion. This helps to stabilize filopodia formation as the cell spreads in the direction of movement, forming the leading edge (Jacquemet et al., 2015). Next, contractile forces from the formed complexes propel the spread cell in the desired direction. Finally, the old adhesion complexes in the rear are disassembled, allowing for cellular retraction and process to repeat (Jacquemet et al., 2015; Parsons et al., 2010). As such, the process of cellular adhesion and spreading is essential for motility. In the context of RA, this increase in adhesion and migration has been well characterized in FLS cells, with pro-inflammatory stimuli inducing an increase in motility (Garcia-Vicuna et al., 2004; You et al., 2014). This allows them to reach and adhere to the cartilage where they can release destructive MMPs to cause irreversible structural damage.

Matrix metalloproteinases (MMPs)

MMPs are a family of zinc-dependent enzymes that play roles in degrading ECM components. The primary function of these MMPs is to assist with the breakdown of key molecules for tissue remodeling (Burrage PS, 2006; Tchetverikov et al., 2005). Some major targets for MMP activity include collagens, gelatins and stromelysins which in turn aids in classifying them into types which includes the collagenases: MMP-1, MMP-8, MMP-13, gelatinases: MMP-2 and MMP-9, and stromelysins: MMP-3, MMP-10, MMP-11 (Burrage PS, 2006; Malemud, 2017). These enzymes are typically tightly regulated due to their destructive capabilities, which is

accomplished through either their direct inhibition, or by preventing the cleavage of the inactive pro-MMP to the active MMP form (Agere et al., 2017). However, during active RA, there is a preponderance of MMP activation (Burrage PS, 2006; Garcia-Vicuna et al., 2004; X.-L. Shui, 2017). The ultimate result is the destruction of tissue and ECM components, leading to impaired function. With regards to RA, many of these MMPs belonging to various families have been implicated in driving RA progression **(Table 1)**.

Table 1. MMPs relevant to the pathogenesis of rheumatoid arthritis

Name	Cell Source	Target(s)	Relevance to RA	Referance
MMP-1	Fibroblast-like synoviocytes, Chondrocytes, Monocytes	Collagen Type I, II,III,VII,X ECM	Plays a pivotal role in joint destruction in RA. Has been shown to directly contribute to cartilage degradation	(Agere et al., 2017; Huang et al., 2017; Hwang et al., 2015; Malemud, 2017; Tchetverikov et al., 2005; Tolboom et al., 2001; Zhu et al., 2005)
MMP-2	Fibroblast-like synoviocytes, Chondrocytes	Gelatin, ECM Collagen Type IV	Enhanced levels in RA patient synovium. Participates in cartilage and basement member destruction	(Choi et al., 2004; Ishiguro et al., 1999; Itoh et al., 2002; Malemud, 2017)
MMP-3	Fibroblast-like synoviocytes, Chondrocytes	Elastin, Fibronectin,laminin, Collagen type III,IV,IX,X, proteoglycans	Participates in the activation of MMP-1 and MMP-9. Participates in destruction of joint structure.	(Hasei et al., 2017; Klein et al., 2016; Lerner et al., 2018; Malemud, 2017; Tolboom et al., 2001)
MMP-8	Fibroblast-like synoviocytes Neutrophils, Chondrocytes, Endothelial cells	Aggrecan, Collagen Type I, II, III	Elevated levels of MMP-8 are associated with higher mortality in RA. Participates in cartilage and ECM degradation.	(Cole et al., 1996; Hanemaaijer et al., 1997; Malemud, 2017; Mattey et al., 2012)
MMP-9	Fibroblast-like synoviocytes, Chondrocytes Macrophages	Collagen Type I, IV, gelatin	Elevated levels of MMP-9 were detected in synovial tissue of RA pateitns. Activated by MMP-3. Target substrates are key	(Ardi et al., 2007; Itoh et al., 2002; Malemud, 2017; Ray et al., 2005; Zhou et al., 2014)

			components of cartilage and basement membrane, suggesting RA driving function.	
MMP-10	Fibroblast-like synoviocytes, Chondrocytes	Proteoglycans, fibronectin	Activator of MMP-1, -8 and -10. Presence of MMP-10 associated with increased invasiveness of FLS.	(Barksby et al., 2006; Malemud, 2017; Tolboom et al., 2001)
MMP-13	Fibroblast-like synoviocytes, Chondrocytes	Collagen Type I, II, III, aggrecan, fibronectin, ECM.	Elevated in synovium of RA patients. Has ability to degrade collagen and ECM components of cartilage.	(Agere et al., 2017; Huh et al., 2015; Malemud, 2017; Moore et al., 2000)

MMP-1 (collagenase) in RA

Interstitial collagenase, or MMP-1, is initially expressed as an inactive pro-MMP-1 that is activated upon catalytic cleavage between the pro-domain and the catalytic domain of MMP-1 (Agere et al., 2017). The primary target for MMP-1 is type I collagen but has been able to cleave other types such as type II and type III (Agere et al., 2017; Burrage PS, 2006). The primary sources of MMP-1 in the joint are the chondrocytes which reside in the cartilage and the FLS cells that comprise the synovial lining (Huang et al., 2017; Hwang et al., 2015; Malemud, 2017). This secretion occurs under physiological conditions to aid in tissue remodeling and is typically counterbalanced through the secretion of MMP inhibitors. However, during RA development, the influx of pro-inflammatory cytokines into the synovium triggers an additional release of MMP-1 from these cells. Indeed, an increased level of MMP-1 can be detected in the synovium of RA patients as compared to healthy individuals (Malemud, 2017; Tchetverikov et al., 2005). This leads to the shift of balance toward rampant MMP-1 activity, leading to cartilage and basement membrane destruction.

MAPK signaling and relevance to RA

The control of expression of various proteins in response to stimuli is tightly regulated by a series of signaling cascades within the target cell. A well-known signaling cascade in eukaryotic cells is the mitogen activated protein kinase (MAPK) pathway (Zhang and Liu, 2002). This intracellular signaling cascade is first started through the binding of a ligand to a cell surface receptor **(Fig. 4)**. This ligand can include a host of signaling molecules, including pro-inflammatory cytokines (Kim et al., 2004). This leads to the initial phosphorylation of a signaling kinases, which in turn phosphorylates another kinase. This process repeats until the final phosphorylation of a transcription factor which can enter the nucleus to induce transcription of the gene of interest (Johnson and Lapadat, 2002). Some common kinases that participate in the MAPK cascade include Ras. Raf. MRK 1/2, Erk1/2 and JNK (Johnson and Lapadat, 2002). Importantly, the MAPK signaling pathway regulates multiple cellular functions, including cell proliferation, differentiation, inflammatory response and apoptosis (Sun et al., 2017; Zhang and Liu, 2002). The upregulation of protein production is also utilized in diseases such as RA. For example, in FLS cells, Erk 1/2 and JNK, well known endpoints of the MAPK cascade, have been shown to be modulated, leading to increased MMP-1 expression (Li et al., 2017; Pillinger et al., 2003).

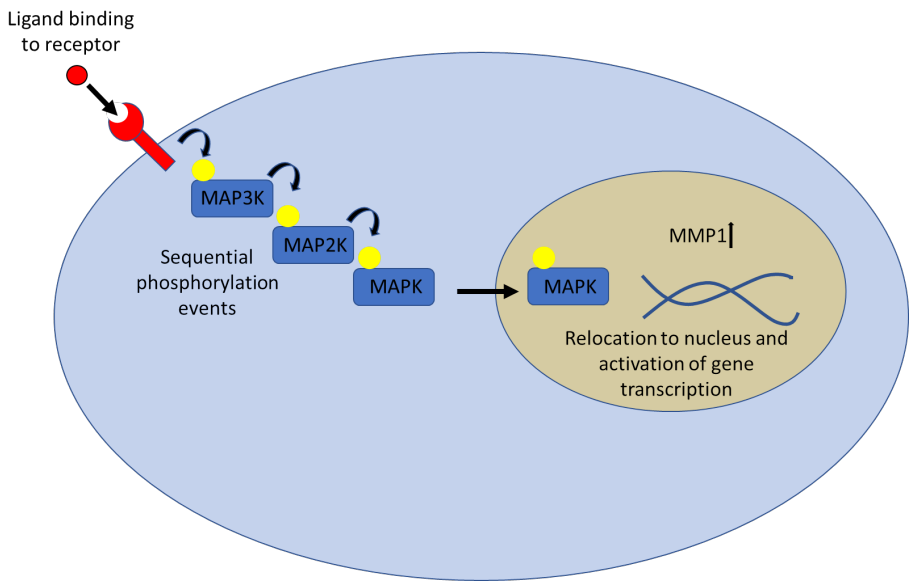

Figure 4. Regulation of MMP-1 expression by the MAPK signaling cascade. Binding of an extracellular molecule such as cytokine to a cell surface receptor initiates the signaling process. This triggers the phosphorylation of an upstream kinase, which in turn goes on to phosphorylate another kinase in the chain. This phosphorylation cascade continues until the final kinase is phosphorylated and translocates into the nucleus to induce gene transcription.

Platelets

Platelets are small (2-3 μm in diameter) anucleate cells that circulate in the bloodstream. Platelets are formed by blebbing from bone marrow megakaryocytes (Gremmel et al., 2016). However, platelets do contain many cellular organelles such as mitochondria and have some capacity to translate mRNA into proteins when stimulated (Gremmel et al., 2016; Mills et al., 2017). The primary role of platelets is maintaining hemostasis through clot formation to seal wounds (Clemetson, 2012). This is accomplished following platelet activation, adhesion, spreading and secretion that ultimately leads to thrombus formation (Clemetson, 2012). The process leading to activation is typically tightly regulated, allowing most platelets to circulate in their quiescent states under normal physiologic conditions. However, there is an increasing body of evidence suggesting that dysregulation of platelet activation may be a key factor in driving

various chronic inflammatory diseases including RA (Golebiewska and Poole, 2015; Jenne and Kubes, 2015).

Platelet granules

Platelets are loaded with a wide variety of cargo molecules upon formation from megakaryocytes as well as through internalization (Golebiewska and Poole, 2015). Upon platelet activation, the granules can fuse with the platelet membrane and release their cargo, modulating the local environment. Cargo molecules are stored within 3 types of granules within the platelet. The first type is the alpha (α)-granule, which are the most abundant type of granules within platelets (Blair and Flaumenhaft, 2009). These granules are also the largest and have been shown to contain over 300 different molecules (Coppinger et al., 2004). These include chemokines such as C-X-C motif ligand 1 (CXCL1), a member of the CXC family of cytokines which are named after their first two cysteine residues separated by an amino acid (Garcia-Vicuna et al., 2004). Additionally, α-granules have also been shown to secrete major cytokine family members such as IL-1, TNF-α and transforming growth factor-beta (TGF-β). (Blair and Flaumenhaft, 2009; Coppinger et al., 2004; Gremmel et al., 2016). In addition, key clotting proteins such as von Willebrand factor and fibrinogen are stored in α-granules, which highlights the role of platelets in hemostasis (Blair and Flaumenhaft, 2009; Sehgal and Storrie, 2007). Furthermore, there are also membrane components, such as P-selectin which are translocated to the platelet surface upon granule fusion, promoting cellular interactions (Versteeg et al., 2013). Next are the dense granules, which are the second-most abundant granules in platelets and are named due to their electron-dense appearance under transmission electron microscopy observation (Fukami, 1997). The primary cargo of dense granules are nucleotides such as adenosine diphosphate (ADP) and adenosine

triphosphate (ATP) in addition to cations such as calcium (Chen et al., 2018; Holmsen and Weiss, 1979). Many of these molecules have been shown to play roles in platelet stimulation to promote thrombosis (Savage et al., 2013). Furthermore, dense granules are also host to platelet seronotin, which can promote inflammation by stimulating lymphocytes and neutrophils (Mauler et al., 2016). The third class of granule is the lysosome, which contains many degrading enzymes such as collagenases and acid phosphatase. As is observed in other cell types, the key role of platelet lysosomes is to orchestrate the catalytic breakdown of proteins. (Heijnen and van der Sluijs, 2015). Proper formation of these granules is essential for normal platelet function (Nurden and Nurden, 2007).

Platelets and their role in chronic inflammation and disease

Although platelets are primarily known for their role in hemostasis, an increasing body of evidence associates platelet activation and platelet-derived molecules with various pathological states. Platelet-derived cargo molecules, including major cytokine family members such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), been shown to be key pro-inflammatory factors in diseases such as arthritis, periodontal disease and inflammatory bowel disease (Giancane et al., 2016; Leizer et al., 1990; Levin et al., 2016; McDevitt et al., 2000). The release of platelet cargo molecules is partly due to the presence of platelet surface receptors that detect pathogenic molecules and ultimately mediate interactions with host immune cells(Estevez and Du, 2017; Kapur et al., 2015). Among these are the toll-like receptors (TLRs), which sense pathogenic molecules from foreign sources (Hamzeh-Cognasse et al., 2018). For example, platelets express toll-like receptor 4 (TLR), which sense bacterial lipopolysaccharide (LPS), causing platelet activation and the secretion of pro-inflammatory cytokines to attract leukocytes

to the site of bacterial infection (Andonegui et al., 2005). They also have roles in sensing viral infections through toll-like receptor 7 (TLR7), allowing platelets to mount an antiviral response through the release of α-granule content (Koupenova et al., 2014). Furthermore, these triggers support direct interactions between platelets and neutrophils, leading to the latter's activation and secretion of tissue damaging reactive oxygen species (ROS) (Hidalgo et al., 2009). Conversely, platelets have also been shown to be modulated by various cancers to further disease progression. Specifically, the activation of platelet TLR4 and the secretion of platelet-derived anti-inflammatory cytokines can serve to drive metastasis and immune evasion (Amo et al., 2014; Yu et al., 2014). In addition, metastasized cancer cells are able to directly adhere to platelets via CD97 and signal the release ATP which disrupts endothelial junctions, thus allowing cancer cell invasion through the disrupted barrier (Ward et al., 2018).

Platelets in RA

Indirect evidence suggest a role for platelets in rheumatoid arthritis (Olumuyiwa-Akeredolu et al., 2019). For example, RA patients have elevated platelet counts and mean platelet volume compared to healthy individuals (Tecer et al., 2016). Furthermore, in a mouse model of RA, mice that had their platelets depleted prior to inducing arthritis showed reduced clinical symptoms such as bone and cartilage erosion (Boilard et al., 2010). Platelets have also been shown to play a key role in RA progression within the joint. It has been shown that platelets utilize the newly formed joint vasculature to drive FLS-mediated RA progression (Boilard et al., 2011). However, the ability for platelets to actually enter the joint is not defined. Rather than actual platelets, platelet microparticles (PMPs) which are released by activated platelets, have been detected within RA joints. This suggests that PMPs provide an avenue by which platelet-derived

molecules can enter the joint (Boilard et al., 2010). These microparticles have been shown to contain a host of platelet-derived molecules that have been implicated in driving RA progression (Boilard et al., 2015). PMPs have also been shown to be able to interact with the cells residing within the joint. For example, the incubation of FLS cells with PMPs was enough to increase FLS invasion and motility (Wang et al., 2017). As previously mentioned, a hallmark of RA progression is the rampant invasion of immune cells, including neutrophils, into the synovium (Smolen et al., 2016). In addition, neutrophils incorporate the pro-inflammatory content of PMPs thus potentially exacerbating joint inflammation (Duchez et al., 2015). Overall, the mounting body of evidence suggests a pathogenic role of platelets and PMPs in driving RA.

Platelet factor 4 (CXCL4/PF4)

Platelet factor 4 (PF4), also known as CXCL4, is a chemokine primary found in platelet α-granules (Kowalska et al., 2010). However, recent studies have suggested that other cells such as monocytes, are able to secrete PF4 (Schaffner et al., 2005). PF4 has is synthesized as a 7.8 kDa protein that's 70 amino acids in length. In addition, a splice variant of PF4 exists (PF4L1) that differs by amino C-terminal amino acids, potentially suggesting separate function (Vandercappellen et al., 2011). Under physiological conditions, four monomers of PF4 assemble into two heterodimers that collectively form an active tetramer (Mayo and Chen, 1989; Vandercappellen et al., 2011). This active tetramer contains a positively charged ring which allows for interaction with negatively charged regions of proteins such as heparin (Zhang et al., 1994). Canonically, CXCL4 has been shown to signal through the CXCR3 receptors as well as surface proteoglycans. Recently, the C-C chemokine receptor 1 (CCR1) receptor was demonstrated to be a target for PF4 signaling and induced monocyte migration (Fox et al., 2018).

Recent evidence indicates that PF4 plays a role in mediating inflammation by modulating the immune system. For example, PF4 was reported to be able to attract and stimulate NK cells and T cells (Struyf et al., 2011). Moreover, PF4 was recently shown to form complexes with DNA to signal via dendritic cell TLR9 receptors in systemic sclerosis (Lande et al., 2019).

Limited indirect evidence suggests that PF4 may be associated with RA. For example, PF4 is detectable in the synovial tissue at the early stages of RA, which forms the basis of its potential as a biomarker for RA development (Nguyen et al., 2019; Yeo et al., 2016). While other platelet-derived cytokines such as platelet-derived growth factor (PDGF) and CCL5 have also been well documented with regards to their ability to drive RA pathogenicity (Agere et al., 2017; Zhao et al., 2016), there is little direct knowledge with regards to the role of PF4 in the context of RA pathogenesis. The lack of information of PF4 in RA, combined with the established role of platelet secretion in chronic inflammation, provides a compelling rationale for studying this chemokine in more detail, especially given that PF4 is a predominant component of platelet α-granules.

STATEMENT OF THE PROBLEM:

The role of platelets in the pathogenesis of chronic inflammatory diseases such as RA is not well understood. The presence of PF4 in early RA has been suggested to be a potential biomarker for RA progression as well as a marker for positive response to anti-TNF-α (Nguyen et al., 2019; Trocme et al., 2009). Furthermore, PF4 levels were found to be elevated in the joints of RA patients (Yeo et al., 2016). However, there exists a large gap in the knowledge of how PF4 signals to the resident joint tissues at a cellular level.

HYPOTHESIS:

I hypothesized that platelet activation (and by extension, PF4) would have a role in driving joint inflammation by its actions on fibroblast-like synoviocytes. Specifically, I hypothesized that PF4 would enhance cell adhesion and MMP-1 secretion by FLS as these are some of the characteristics of the invasive/destructive FLS phenotype.

Chapter 2: The role of platelet factor 4 (PF4/CXCL4) on the behavior of fibroblast-like synoviocytes

Materials and Methods

Reagents

Human fibronectin protein, poly-L-lysine and goat serum were purchased from Sigma (Oakville, ON, Canada). Recombinant human platelet factor 4 (PF4) protein, and antibodies against fibronectin and vinculin were obtained from Abcam (Cambridge, MA). Primary antibodies against phospho-p44/42 MAP kinase (phospho-Erk1/2), total Erk1/2, phospho-SAPK/JNK, and total JNK, as well as horseradish peroxidase-conjugated secondary antibodies, were obtained from Cell Signaling Technologies (Danvers, MA). The Alexa Fluor 488-conjugated secondary antibody, as well as all reagents for cell culture (Gibco) were obtained from Thermo Fisher Scientific (Grand Island, NY).

Cell culture

SW982 human fibroblast-like synoviocytes (FLS), from synovial sarcoma, were purchased from ATCC (Manassas, VA). Cells were cultured in Dulbecco's minimal essential medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. All experiments were conducted on cells between passages 5 and 13.

MMP-1 secretion and cell viability assays

To quantify MMP-1 secretion, SW982 cells were seeded on to tissue culture-treated 96-well plates at a density of $3x10^5$ cells/mL in complete media and allowed to reach confluency. Cells

were then stimulated with recombinant PF4 in serum-free media for 24 hours. Cell culture supernatants were harvested, and the concentration of MMP-1 was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam) as per the manufacturer's instructions. The viability of the cells was tested using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) as per the manufacturer's instructions.

Immunoblotting

For immunoblotting of intracellular proteins, cells were grown on 35 mm plates were serum-starved for 24 hours prior to the addition of serum free medium containing PF4. Cells were washed with ice-cold phosphate buffered saline (PBS) and scraped into RIPA buffer supplemented with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Cell lysates were clarified by centrifugation at 4oC and protein concentrations were determined using the DC protein assay (Bio-Rad, Hercules, CA) as per the manufacturer's instructions. Cell lysates were resolved by SDS-PAGE and transferred on to PVDF membranes (Millipore, Burlington, MA). The membranes were blocked in a Tris buffered saline-Tween solution (TBS-T) containing 5% bovine serum albumin (BSA) for 1 hour, then incubated with the primary antibody solution overnight at 4^{o}C, followed by the HRP-conjugated secondary antibody for 1 hour. Detection was performed by enhanced chemiluminescence (ECL).

Mass spectrometry

For shotgun proteomic analysis, ~7.0×10^{6} cells were harvested from control and 1, 6 and 24-hour PF4 (0.1 μg/mL) stimulated cells grown on 10 cm plates. Cells were lysed in Lysis Buffer) and heated at 95°C for 5 min. Samples were then centrifuged for 10 min at 16,000 rcf. at 4°C and the

supernatant was collected. Protein concentrations were then measured via BCA assay (Thermo Fisher). 30 µg of protein from each sample was subjected to acetone precipitation. Protein pellets were resuspended in a 6 M/2 M urea/thiourea mixture. Samples were then reduced and alkylated by adding 6 µg of DTT and 15 µg of iodoacetamide and incubating at room temperature in the dark for 30 min and 20 min, respectively. 3 µg of LysC was added to each sample and incubated for 3 h at room temperature. Subsequently, samples were diluted with 4 volumes of Digestion Buffer (50 mM NH_4HCO_3) and trypsin (Promega) was added at a ratio of 1:50. Samples were incubated shaking overnight at room temperature. The resulting peptide supernatant was acidified to pH < 2.5 and purified using homemade Stop-and-go-extraction tips (StageTips) composed of C18 Empore material (3 M) packed in to 200 µl pipette tips (Rappsilber et al., 2003). StageTips were conditioned with methanol and equilibrated with 1% trifluoroacetic acid (TFA; loading buffer). Peptide supernatants were loaded onto the columns and washed with two bed volumes of Buffer A (0.5% formic acid). Peptides were eluted with Buffer B (0.5% formic acid) supplemented with 80% MeCN, dried down. Dried peptides were resuspended in Buffer A for mass spectrometry analysis.

Tandem liquid chromatography mass spectrometry of LFQ samples

Purified peptides were analyzed using an Impact II quadrupole time of flight mass spectrometer Bruker Daltonics, Billerica, MA) on-line coupled to an Easy nano LC 1000 HPLC (Thermo Fisher) using nanoBooster with methanol and a Captive spray nanospray ionization source (Bruker Daltonics) including a 2 cm long, 100 µm inner diameter fused silica fritted trap column, and 40 cm long, 75 µm inner diameter fused silica analytical column with an integrated spray tip [6-8 µm diameter opening, pulled on a P-2000 laser puller (Sutter Instruments, Novato, CA)].

The trap column was packed with 5 μm Aqua C-18 beads (Phenomenex, Torrance, CA) while the analytical column was packed with 1.9 μm diameter Reprosil-Pur C-18-AQ beads (Dr. Maisch, Ammerbuch, DE). Samples were resuspended in buffer A and loaded with the same buffer. Standard 90 min gradients were from 0% B to 35% B over 90 min, then to 100% B over 2 min, held at 100% B for 15 min. Before each run, the trap column was conditioned with 20 μl buffer A, the analytical with 4 μl of the same buffer, and the sample loading was set at 20 μl (for samples up to 13 μl volume). The LC thermostat temperature was set at 7°C. The Captive Spray Tip holder was modified similarly to an already described procedure (Beck et al., 2015). The fused silica spray capillary was removed (together with the tubing which holds it) to reduce the dead volume, and the analytical column tip was fitted in the Bruker spray tip holder using a piece of 1/16 in × 0.015 PEEK tubing (IDEX), an 1/16 in metal two-way connector and a 16-004 Vespel ferrule. The sample was loaded on the trap column at 850 Bar and the analysis was performed at 0.25 μl/min flow rate. The Impact II was set to acquire in a data-dependent auto-MS/MS mode with inactive focus fragmenting the 20 most abundant ions (one at a time at 18 Hz rate) after each full-range scan from m/z 200 Th to m/z 2000 Th (at 5 Hz rate). The isolation window for MS/MS was 2 to 3 Th depending on parent ion mass to charge ratio and the collision energy ranged from 23 to 65 eV depending on ion mass and charge (Beck et al., 2015). Parent ions were then excluded from MS/MS for the next 0.4 min and reconsidered if their intensity increased more than 5 times. Singly charged ions were excluded since in ESI mode peptides usually carry multiple charges. Strict active exclusion was applied. Error of mass measurement is typically within 5 ppm and was not allowed to exceed 10 ppm. The nano ESI source was operated at 1900 V capillary voltage, 0.20 Bar nanoBooster pressure, 3 L/min drying gas and 150°C drying temperature. The cross connector between the trap column, waste out capillary and

analytical column was grounded via a 0.4 mm platinum wire to prevent electrical corrosion of the LC S valve.

Protein identification and quantification

Protein identification and quantification was performed using MaxQuant version 1.5.3.30 (Cox and Mann, 2008). The data were searched against the *Homo sapiens* UniProt database (UniProt Consortium, 2018). For shotgun experiments, the following parameters were used: peptide mass accuracy, 10 parts per million (ppm) for first search, 10 ppm for second search; trypsin enzyme specificity, fixed modifications, carbamidomethyl; variable modifications, methionine oxidation, deamidation (NQ), and N-acetylation (protein N-terminus); and all other parameters as preset. Label-free quantitation (LFQ) was enabled, with a minimum ratio count of two. Only those peptides exceeding the individually calculated 99% confidence limit (as opposed to the average limit for the whole experiment) were considered as accurately identified. In all analyses, contaminants and reverse hits were removed using Perseus version 1.6.1.1 (Tyanova et al., 2016).

Immunocytochemistry and cell spreading assay

For immunofluorescence staining of fibronectin and vinculin, PF4-treated cells were grown on 8-well chamber slides, fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton-X-100, and blocked with 5% goat serum. Cells were incubated overnight at 4^{o}C with a solution of anti-fibronectin or anti-vincuin primary antibody, followed by an Alexa-488-conjugated secondary antibody for 1 hour. Imaging was performed using a Zeiss Axiovert spinning disk confocal microscope and SlideBook software (Intelligent Imaging Innovations, Denver, CO). Cell

spreading was determined from visual assessments of the cell morphology (spread/not spread) and quantified based on the mean percentage (%) of spread cells per field of view.

Cell adhesion assay

8-well chambered culture slides were pre-coated with fibronectin (0.01 mg/mL) or poly-L-lysine (0.01%) and allowed to dry. SW982 cells were suspended in serum-free media containing PF4 and 0.1% bovine serum albumin for one hour prior to plating on to the chamber slides and allowed to adhere for 20 minutes prior to 3 consecutive jet-washes with PBS. After the last wash, cells were incubated for 1 hour with 100 µl of complete media prior to the addition of 20 µl of MTS viability reagent (Promega) for 2 hours. Cell adhesion was determined based on the colorimetric readout from the viability assay.

Statistical analysis

Using GraphPad Prism software (Version 7.0), a one-way analysis of variance (ANOVA) and Dunnett's post-hoc multiple comparison tests were used to determine the effect of PF4 treatment on MMP-1 release, cell adhesion and cell spreading. Statistical significance was set at $p<0.05$.

Results

Platelet factor 4 (PF4) induces the secretion of MMP-1 from fibroblast-like synoviocytes (FLS)

To better characterize the role of platelet-derived cytokines in the pathogenesis of rheumatoid arthritis (RA), we used SW982 cells as a model for fibroblast-like synoviocytes. Cells were cultured in the presence or absence of recombinant PF4. ELISA analysis of the cell culture supernatants showed a ~10-20% increase in MMP-1 release following PF4 treatment, in a dose-dependent manner **(Fig. 5A)**. The increase in MMP-1 release was statistically significant ($p<0.05$) at a PF4 concentration of 1 μg/mL. Data from the MTS viability assay confirmed that PF4 treatment did not adversely affect cell survival **(Fig. 5B)**.

PF4 induces a modest activation of Erk 1/2 and JNK signaling

Since MMP-1 gene transcription is reportedly linked to the Erk1/2 and JNK signaling pathways, we set out to determine whether PF4 treatment activates phosphorylation of Erk1/2 and JNK. Immunoblotting of SW982 lysates showed a slight and non-significant ($p>0.05$) increase in Erk1/2 phosphorylation after 10 minutes of PF4 treatment; phospho-Erk levels returned to baseline within 60 minutes post-treatment **(Figs. 6A & 6B)**. Similarly, PF4 induced a short-term and non-significant ($p>0.05$) increase in JNK phosphorylation that was attenuated within 60 minutes post-treatment **(Figs. 6A & 6C)**.

PF4 upregulates fibronectin expression in FLS

To determine whether PF4 treatment might other trigger differences in gene/protein expression by FLS, we employed a broad screening approach using label-free quantitative mass spectrometry to compare the proteome of SW982 cells in the presence or absence of recombinant

PF4. From this screen, the expression of 6 proteins was found to be differentially and significantly altered as a result of PF4 treatment. Of these, the adhesion protein fibronectin was elevated by the greatest magnitude following PF4 treatment **(Fig. 7)**. I then used 2 separate approaches to validate this finding: immunofluorescence and immunoblotting. The initial experiment revealed similar levels of fluorescence intensity in SW982 cells stained with an anti-fibronectin antibody **(Fig. 8A)**. However, these data are difficult to interpret due to the absence of control cells stained with an isotype control antibody that would show the levels of non-specific background staining. In contrast, immunoblot analysis confirmed that protein levels of fibronectin are increased in SW982 cells following PF4 treatment **(Fig. 8B)**. These data support the finding of the mass spectrometry screen, in that fibronectin is upregulated in FLS by PF4 treatment.

PF4 increases FLS adhesion and spreading

We then used a 2-pronged approach to assess the effects of PF4-induced fibronectin in the context of cell function. Firstly, we performed a cell adhesion assay in which cell adhesion was compared in PF4-treated cells and controls following jet-washing with PBS. Cells treated with PF4 showed significantly ($p<0.05$) greater adhesion to fibronectin following jet-washing, relative to untreated controls **(Fig. 9A)**. No differences attributable to PF4 treatment were observed for cells plated on a poly-L-lysine-coated control surface **(Fig. 9B)**. Secondly, we used confocal microscopy to evaluate focal adhesion formation in SW982 cells in the presence or absence of PF4. Cells were stained with vinculin, a classical marker of focal adhesions **(Fig. 10A)**, and the cellular morphology of cells was classified as being “rounded” or “spread.” Cells treated with PF4 exhibited significantly ($p<0.05$) greater proportions of spread cells **(Fig. 10B)**. Collectively,

these data indicate that PF4 treatment induces a pro-adhesive cellular phenotype in fibroblast-like synoviocytes.

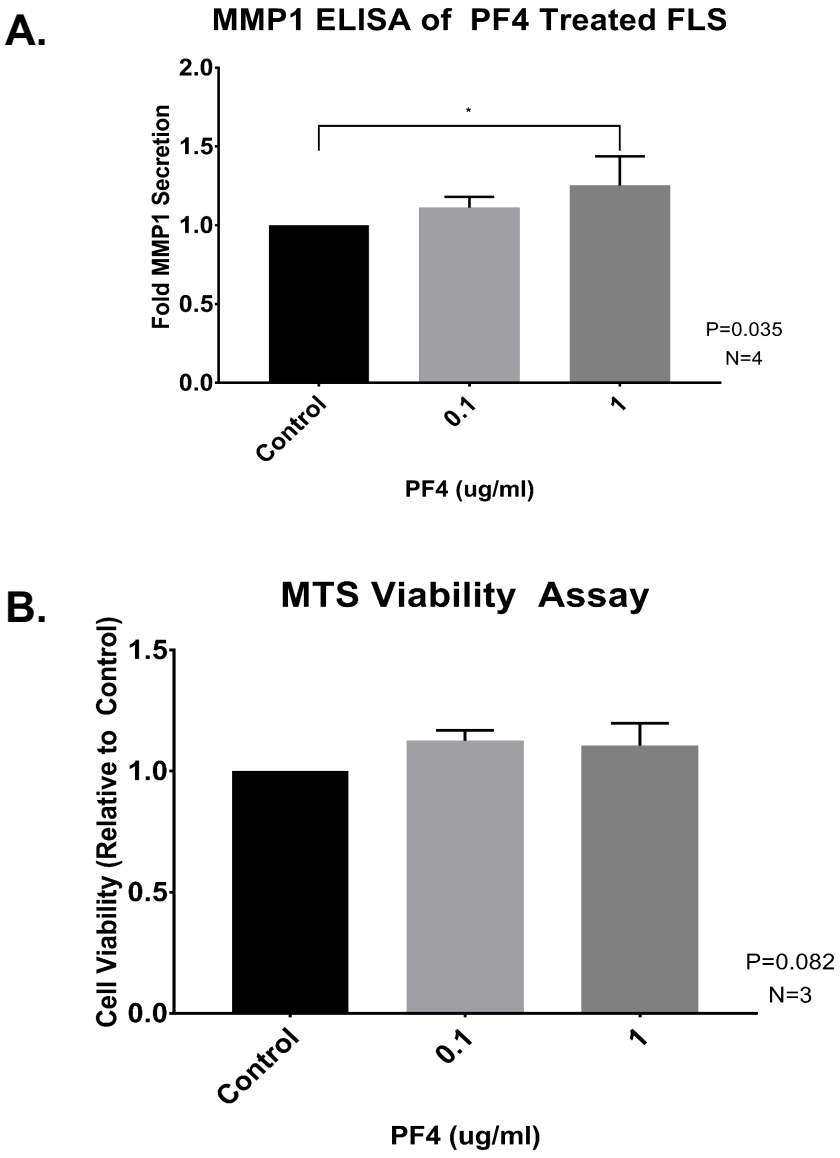

Figure 5. PF4 induces MMP-1 secretion from fibroblast-like synoviocytes. **A.** SW982 cells were stimulated with PF4 at the indicated concentrations in serum-free media for 24 hours. Cell supernatants were then collected and the levels of MMP-1 were measured using ELISA. Data are shown as mean ± SD, with the control group set at 1. *P<0.05. **B.** SW982 cells were stimulated with PF4 at the indicated concentration in serum-free media prior to the addition of MTS reagent. Cell viability (relative to the control group) was determined by a colorometric assay. Data are shown as mean ± SD, and represent cell viability relative to the control group, set at 1.

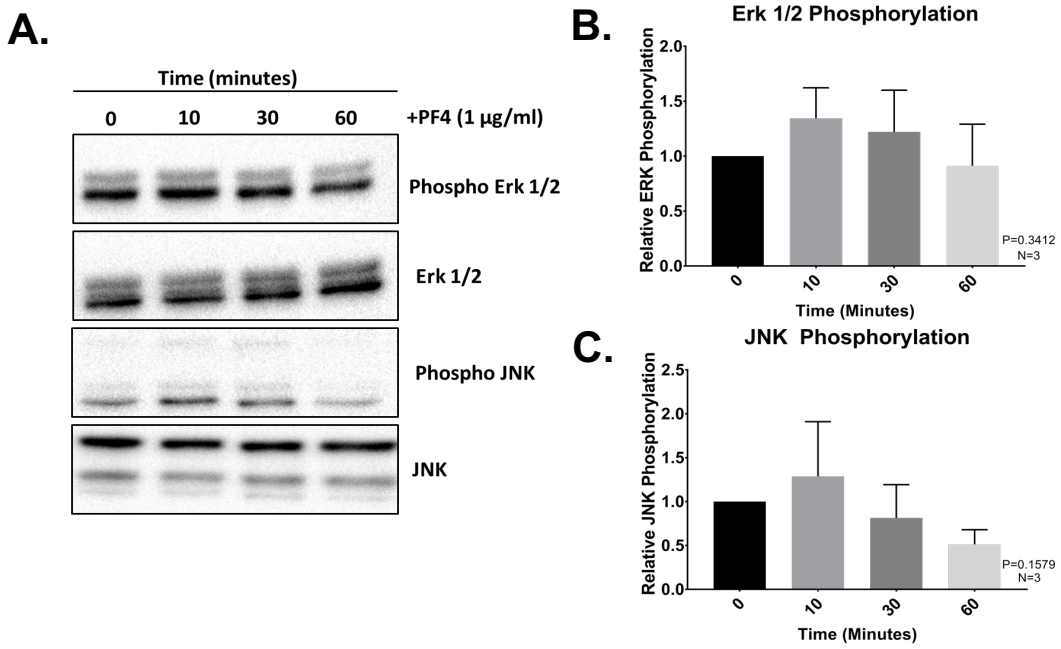

Figure 6. PF4 stimulation produces a modest increase in Erk 1/2 and JNK phosphorylation in fibroblast-like synoviocytes. **A.** SW982 cells were starved for 24 hrs prior to PF4 stimulation for the indicated times. Equal amounts of protein lysate were resolved by SDS-PAGE and probed for antibodies against phospho-Erk1/2 and phospho-JNK, as indicated. Blots were then stripped and re-probed for antibodies against total Erk1/2 and total JNK, as loading controls. Images are representative of three experiments. **B.** Densitometric analysis of Erk 1/2 phosphorylation of PF4 treated cells. Data are shown as the phospho-Erk:total Erk ratio, relative to the control group set at 1. Data are mean ± SD and represent 3 independent experiments. **C.** Densitometric analysis of JNK phosphorylation of PF4 treated cells. Data are shown as the phospho-JNK:total JNK ratio, relative to the control group set at 1. Data are mean ± SD and represent 3 independent experiments.

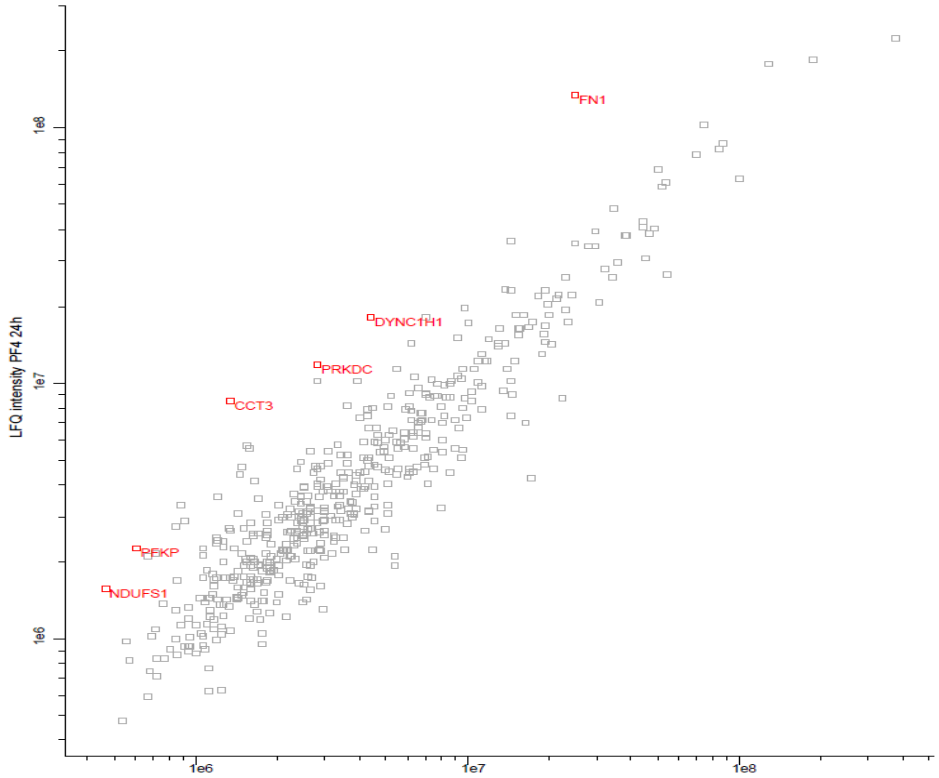

Figure 7. Label-free quantitative mass spectrometry identifies a PF4-induced upregulation of fibronectin. PF4 stimulated cells (0.1 ug/ml) as well as control cells were lysed and processed before analysis using label-free quantitative mass spectrometry. Unique spectra were identified, and their intensities quantified. The intensities of each unique protein were then plotted as a relation between the treatment and control group values. Protein intensities that were found to be statistically significant between treatment and control groups are highlighted in red.

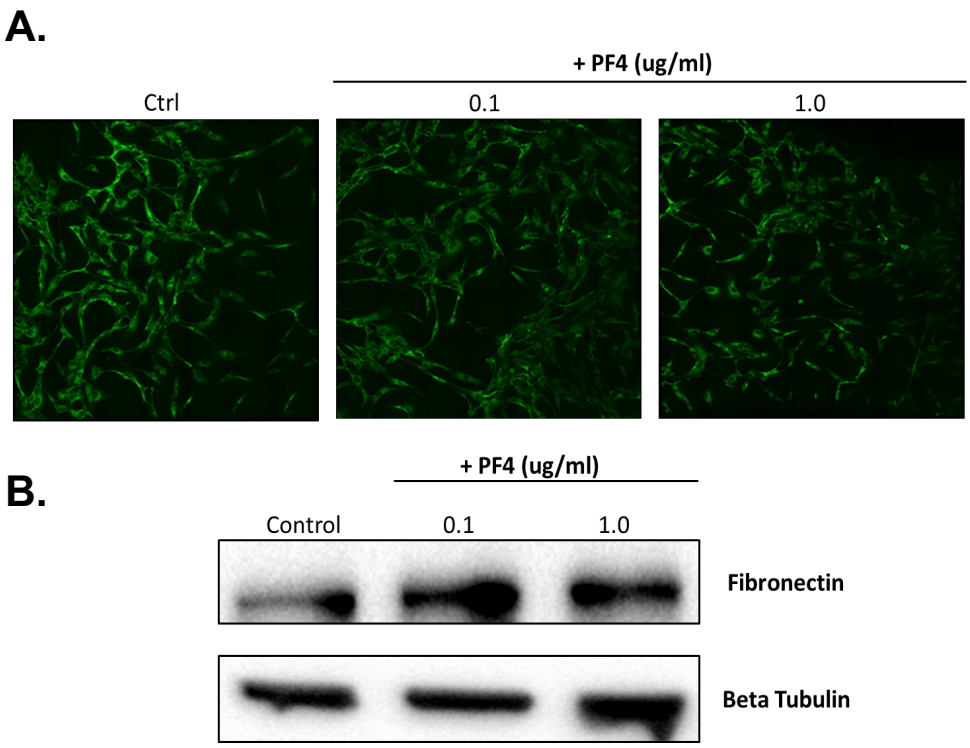

Figure 8. Validation of mass spectrometry of PF4-induced upregulation of fibronectin. A. SW982 cells were stimulated with the indicated concentrations of PF4 in serum-free media for 24 hrs prior to staining for fibronectin. **B.** SW982 cells were stimulated with PF4 at the indicated concentrations for 24 hours. Equal amounts of protein lysate were resolved by SDS-PAGE and immunoblotted with an anti-fibronectin antibody. Beta tubulin is shown as a loading control.

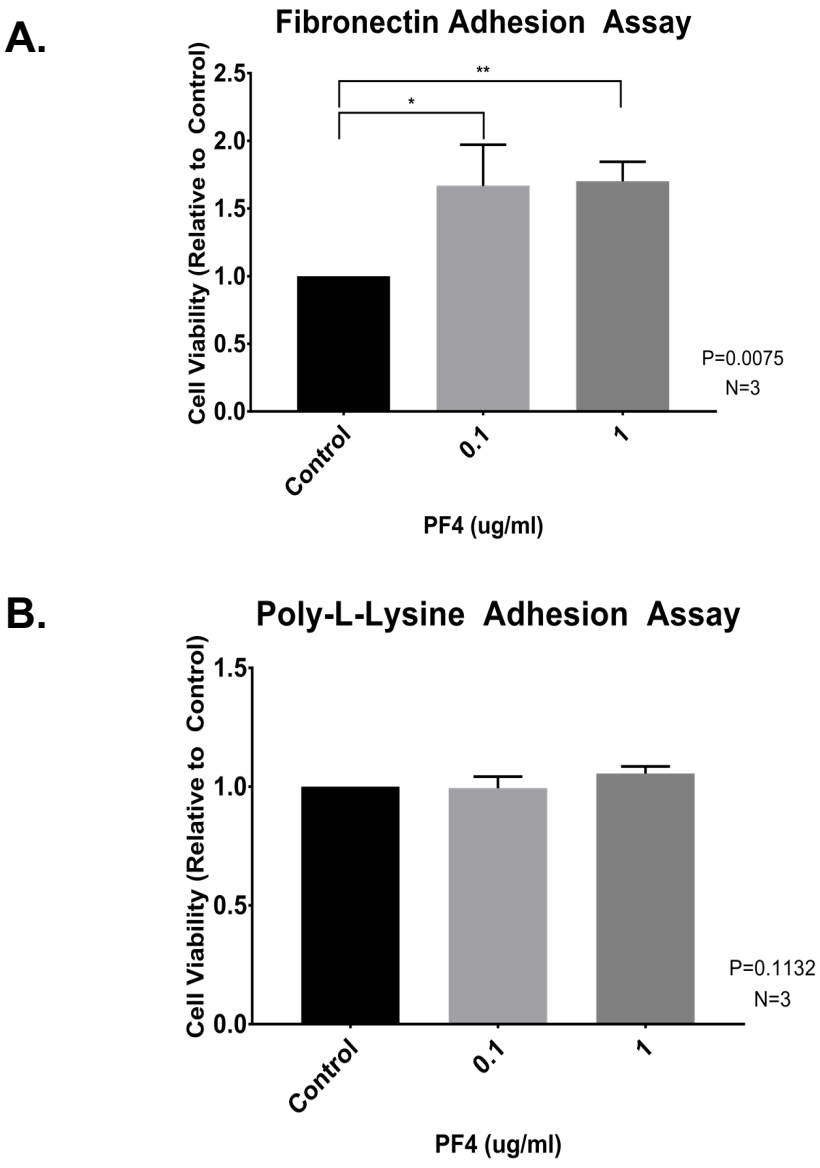

Figure 9. PF4-stimulated fibroblast-like synoviocytes exhibit increased adhesion to fibronectin. A. FLS cells were stimulated in suspension with the indicated concentration of PF4 in serum free media for 1 hr before being plating on to fibronectin **(A)** or poly-L-lysine **(B)**. The cells were then allowed to partially adhere before being subjected to PBS jet-washing, followed by re-incubation in complete media. MTS viability reagent was then added to quantify the number of cells remaining. Bar graphs depict cell viability, represented by the relative numbers of adherent cells post-jet-washing, normalized to the control group (set at 1). Data are mean ± SD and represent 3 independent experiments.

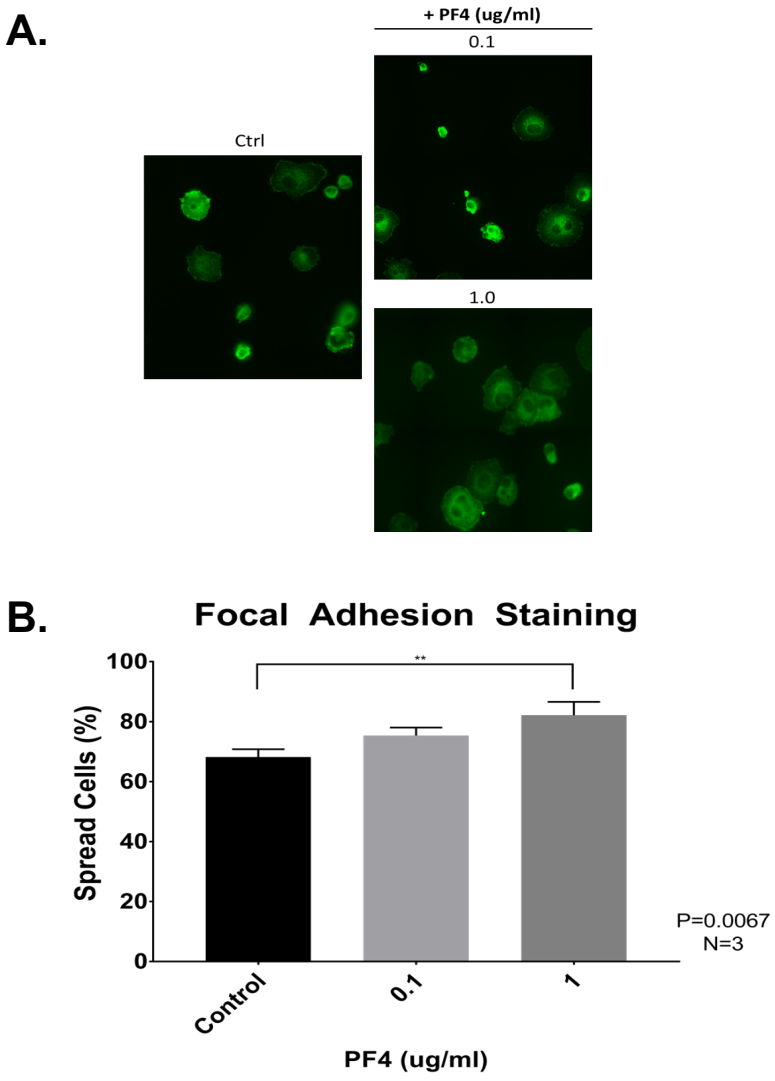

Figure 10. PF4-stimulated fibroblast-like synoviocytes exhibit increased cell spreading. **A.** SW982 cells were stimulated for 24 hrs with PF4 at the indicated concentrations and allowed to adhere to fibronectin-coated plates. Cells were fixed and stained with an antibody against the focal adhesion marker vinculin. Images are representative of 48 images per group from 3 independent experiments. **B.** Bar graph represents the percentage of cells classified as being "spread" based on a visual assessment of cell morphology. Data are mean ± SD and represent 3 independent experiments. **P<0.01.

Discussion

Considerable evidence now suggests that platelets exhibit pro-inflammatory properties and can thus modulate the RA disease process. For example, the production of pro-inflammatory prostacyclin by fibroblast-like synoviocytes is reportedly platelet-dependent (Boilard et al., 2011). Furthermore, PMPs, which shed off of platelets, have been found in the synovial fluid of RA patients and contribute to the joint pro-inflammatory environment (Boilard et al., 2010). In addition, PF4, the major constituent of platelet alpha-granules, is reportedly elevated in the joint tissue of RA patients (Yeo et al., 2016). Notably, PF4 also has a documented role in the chemotaxis of immune cells and in promoting the release of MMP-1 from connective tissue cells (Javaid et al., 2017; Kowalska et al., 2010).

Fibroblast-like synoviocytes (FLS) are central to the pathogenesis of RA, in part due to their release of other pro-inflammatory cytokines; their production of matrix-degrading MMPs; and their potential to adopt an aggressive and invasive phenotype (Smolen et al., 2016). However, the role of PF4 in modulating FLS function is undefined. The results of our study suggest that the pro-inflammatory role of platelets in RA is partly attributable to PF4 signaling to FLS.

PF4 promotes MMP-1 secretion and Erk1/2 and JNK signaling in FLS

A major finding from our study is that MMP-1 secretion was upregulated in SW982 cells following PF4 treatment. Importantly, MMP-1-driven degradation of cartilage, through the targeting of type I, II, III and X collagen, is a key element of joint destruction in RA (Agere et al., 2017; Garcia-Vicuna et al., 2004). Notably, ours are the first data to implicate PF4 in the production of tissue-degrading MMPs by synovial fibroblasts. Our data also indicate that PF4

activates Erk1/2 and JNK signaling, albeit not to a statistically significant degree. The relatively modest response could be explained in part by the dose of PF4 used in our study. Although 1 μg/mL of PF4 is reportedly sufficient to induce Erk 1/2 phosphorylation in mast cells (Willox et al., 2010), concentrations as high as 32 μg/mL are required to induce Erk 1/2 phosphorylation in monocytes (Kasper et al., 2007). Alternatively, it possible that peak phosphorylation is only observed at later time points (~6 hours post-treatment) (Kasper et al., 2007; Willox et al., 2010). Expanded dose-response and time-course studies may better characterize the PF4-driven effects on FLS signaling.

PF4 promotes fibronectin expression in FLS

Data from mass spectrometry and Western blotting indicate that PF4 induces an increase in fibronectin expression in FLS. This is notable since increased fibronectin in joint tissues are associated with RA disease progression (Sen et al., 2002). Importantly, it was previously shown that fibronectin is a target for protein citrullination, allowing for targeting by the immune system for the production of autoantibodies(Chang et al., 2005). Elevated levels of fibronectin in the synovial fluid also coincides with an increase in release of pro-inflammatory cytokines as well as a decrease in apoptosis in FLS cells (Hwang et al., 2015; Mehta et al., 2018). Furthermore, FLS fibronectin has been shown to be cross-linked to osteopontin in RA, promoting FLS interaction with B cells and causing the release of pro-inflammatory cytokines from both cell types (Mehta et al., 2018). Conceivably, PF4-mediated secretion of fibronectin would drive RA progression by further developing the RA-FLS phenotype as by promoting the increased production of autoantibodies through fibronectin citrullination.

PF4 promotes FLS adhesion and spreading

Another major aspect of the aggressive FLS phenotype is the increase in cell motility, which facilitates the invasion of adjacent joint tissues (Garcia-Vicuna et al., 2004; McInnes and Schett, 2011; Smolen et al., 2016). Focal adhesion formation is of particular importance as they serve as anchors for cell adhesion, spreading and migration (Parsons et al., 2010). This is notable in the context of RA pathogenesis since the motility of FLS would facilitate their reaching and adhering to cartilage, and secretion of matrix-degrading MMPs. Our finding that PF4 treatment increases the adhesion and spreading of FLS is therefore consistent with the notion that PF4 induces a destructive phenotype in FLS. Taken together, our data indicate that increased degranulation of platelets in the joint tissue contributes to cell adhesion, pannus formation, MMP release and tissue degradation.

A limitation of our study is that the PF4 receptor was not identified. PF4 is known to signal through the CXCR3 receptor (Struyf et al., 2011), the CCR1 receptor (Fox et al., 2018) and also through cell surface proteoglycans (Vandercappellen et al., 2011). However, the function of these receptors has not been validated in the context of PF4-FLS signaling. In addition, the use of a synovial sarcoma cell line as the culture model may pose a limitation since the cells are by their nature genetically altered from normal FLS. It would therefore be of interest to evaluate, in future studies, the effects of PF4 on cultured primary synovial fibroblasts. This would provide insight into the role of PF4 under more physiological conditions.

Chapter 3: Conclusions and future directions

Conclusions

At the center of RA pathogenesis are the FLS, whose phenotype is strongly implicated in driving RA progression. The contribution of platelets, and specifically PF4, to RA is not defined. The results of my experiments suggest that PF4 triggering an increase in MMP-1 secretion by FLS and also promotes their adhesion to the extracellular matrix. This finding is consistent with my hypothesis that PF4 promotes the destructive phenotype of FLS. This is important in the context of RA since it was shown that PF4 is present in the early stages of RA symptom onset (Yeo et al., 2016). Based on my data, it is therefore possible that PF4 drives the initial development of the activated RA FLS phenotype, thus promoting FLS adhesion, spreading and migration, and the onset of MMP-1 mediated cartilage destruction. This could be followed by the later stages of the disease, where other FLS agonists, such as TNF-α and IL-1, would propagate the destructive FLS phenotype and drive RA disease. Overall, these data are consistent with a pro-inflammatory role for platelets and PF4 in RA, by promoting a destructive and invasive FLS phenotype.

Future directions

This area of research can be expanded as follows:

1. The observed PF4-induced increases in MMP-1 secretion, MAPK activation and cell adhesion were relatively modest. This may be explained by the PF4 doses and treatment times employed. Future studies could include expanded dose-response and time-course experiments to determine if the PF4-induced effects are amplified under those conditions.

2. A limitation with the cell model employed (synovial sarcoma cells) is that these cells may not faithfully replicate the physiologic joint environment. Consequently, a clearer effect of PF4 may be observed if the experiments were to be conducted in primary (non-cancerous and non-immortalized) FLS cell lines.
3. It may be of interest to determine whether inhibition of Erk1/2 and JNK signaling would abrogate PF4-induced secretion of MMP-1 by FLS.
4. Since the cytokine profile of FLS cells (e.g. FLS secretion of interleukin-6) is also altered in RA (Tu et al., 2018), it would be of considerable interest to determine whether PF4 alters the secretion of pro-inflammatory cytokines by the FLS themselves.

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