

ROLE OF NEUTROPHIL ELASTASE AND PROTEINASE-ACTIVATED  
RECEPTOR-2 IN THE JOINT INFLAMMATION AND PAIN ASSOCIATED WITH  
EXPERIMENTAL ARTHRITIS

by

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This thesis is dedicated to my parents  
हा प्रबंध माझ्या आई-वडिलांस समर्पित आहे

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## **Abstract**

Arthritis affects many people around the world; it is a leading cause of chronic joint pain, and physical disability. Currently available drug treatments are inadequate and associated with side-effects, so there is a need for new treatments with better efficacy and safety profiles. It has been suggested that synovial inflammation plays an important role in the development of arthritic symptoms. The focus of this thesis was to assess the contribution of neutrophil elastase and proteinase-activated receptor-2 (PAR2) to the development of experimental knee joint arthritis. To this end, we evaluated the effect of local administration of neutrophil elastase on joint inflammation and pain. The role of PAR2 in mediating neutrophil elastase-induced joint inflammation and pain was assessed. Additionally, the effect of endogenous neutrophil elastase inhibition or PAR2 blockade was investigated using preclinical models of knee arthritis. Local administration of neutrophil elastase caused pro-inflammatory (an increase in leukocyte-endothelial interactions and synovial blood flow) and pro-nociceptive (a decrease in the hindpaw withdrawal threshold) effects. These effects could be blocked by the neutrophil elastase inhibitors (sivelestat or serpinA1). Neutrophil elastase-induced joint inflammation and pain can be blocked by PAR2 antagonist and do not develop in PAR2 knockout mice. Inhibition of endogenous neutrophil elastase produced anti-inflammatory effect in the kaolin/carrageenan model of acute synovitis; however, the pain response was not improved. PAR2 knockouts prevented both joint inflammation and pain in the kaolin/carrageenan model. Prophylactic inhibition of endogenous neutrophil elastase reduced Freund's complete adjuvant (FCA)-induced chronic joint inflammation at the end of the study. FCA-induced weight-bearing deficits and withdrawal threshold were ameliorated in the acute phase of the model. In monoiodoacetate (MIA)-induced experimental osteoarthritis, we observed increased proteolytic activity of neutrophil elastase during the acute inflammatory phase of the model. Inhibition of neutrophil elastase during this inflammatory phase prevented the development of joint inflammation, pain and late-stage neuropathy in the MIA model. Modulation of PAR2 prevented MIA-induced joint inflammation, pain and neuropathy. Collectively, our findings highlight the potential of neutrophil elastase and/or PAR2 as a therapeutic target for the treatment of joint inflammation and pain associated with arthritis.

## List of Abbreviations Used

ACL	Anterior cruciate ligament
ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
ARRIVE	Animal Research: Reporting of In Vivo Experiments
ATF-3	Activating transcription factor 3
CACF	Carleton Animal Care Facility
CD99L	CD99 antigen-like protein
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
COX	Cyclooxygenase
CRP	C-reactive protein
DAMPs	Danger-associated molecular patterns
DRG	Dorsal root ganglion
DWB	Dynamic weight-bearing
ESAM	Endothelial cell-selective adhesion molecules
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FCA	Freund's complete adjuvant
g	Grams
GAP 43	Growth associated protein 43
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GFAP	Glial fibrillary acidic protein
GPCR	G protein coupled receptor
GRKs	GPCR kinases
h	Hour
IASP	International Association for the Study of Pain
ICAM	Intercellular adhesion molecules
IFN- $\gamma$	Interferon gamma
IL-1 $\beta$	Interleukin 1 beta
IL-6	Interleukin 6
IP	Intraperitoneal
IVM	Intravital microscope
JAM	Junctional adhesion molecule
kg	Kilograms
LASCA	Laser Speckle Contrast Analysis
LCL	Lateral collateral ligament
LFA1	Lymphocyte function-associated antigen 1
MAC1	Macrophage antigen 1
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MCL	Medial collateral ligament
MHC	Major histocompatibility complex
MIA	Monoiodoacetate
mg	Miligrams

MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
NE 680	Neutrophil Elastase 680 FAST
ng	Nanogram
NGF	Nerve growth factor
NK-1	Neurokinin-1 receptor
NO	Nitric oxide
OA	Osteoarthritis
PAMPs	Pathogen-associated molecular patterns
PAR	Proteinase-activated receptor
PAR2KO	Proteinase-activated receptor-2 knockout
PCL	Posterior cruciate ligament
PECAM	Platelet/ endothelial-cell adhesion molecule
PG	Prostaglandin
PRRs	Pattern-recognition receptors
PSGL1	P-selectin glycoprotein ligand 1
RA	Rheumatoid arthritis
RF	Rheumatoid factor
s	Seconds
SC	Subcutaneous
SEM	Standard error of the mean
SERPIN	Serine proteinase inhibitor
SF OA	Synovial fluid signs of OA

SLCDC	Survey on living with chronic diseases in Canada
SP	Substance P
TC	Thymus chemokines
TGF	Transforming growth factor beta
Th1	Type 1 helper T-cells
TLR	Toll-like receptor
TMPRSS2	Transmembrane protease, serine 2
TNF- $\alpha$	Tumor necrosis factor alpha
TRPV1	Transient receptor potential cation channel 1
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VGSC	Voltage-gated sodium channels
VIP	Vasoactive intestinal peptide
VLA4	Very late antigen 4
WT	Wild type mice

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## **Chapter 1: Introduction**

### **1.1 Arthritis**

The term arthritis means joint inflammation (Greek: *arthron* – joint, *itis* – inflammation) and it collectively represents over 100 different types of joint-related conditions. General symptoms of arthritis include joint swelling, stiffness, reduced mobility and most importantly joint pain that patients experience during arthritis. Arthritis can affect various joints such as hip, knee, hand, spine, ankle, wrist and metatarsophalangeal joints (McInnes and Schett, 2011; Martel-Pelletier et al., 2016). Consequently, patients suffering from arthritis experience a significant reduction in their quality of life. Some of the leading forms of arthritis include osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, gout and infectious arthritis.

### **1.2 Osteoarthritis**

Osteoarthritis (OA) is the most common form of arthritis and can affect men and women at any age; however, it is more common in the older population (Martel-Pelletier et al., 2016). OA affects millions of people around the world and it is one of the highest contributors to global disability (Cross et al., 2014). According to a recent report, the global burden of knee OA is 3.8% and hip OA is 0.85% (Cross et al., 2014). Furthermore, with an ageing population, it has been estimated that the prevalence of OA will increase more rapidly (Cross et al., 2014). The joints that are affected during OA are knee, hip, spine, hand and neck (Litwic et al., 2013). In 2009, the survey on living with chronic

diseases in Canada (SLCDC) conducted by Statistics Canada estimated that 37% of Canadians aged  $\geq 20$  have OA (MacDonald et al., 2014).

### 1.2.1 Diagnosis

In 1986, Altman and colleagues devised the criteria for the classification of knee OA. Before development of these criteria, OA was almost exclusively diagnosed based on radiographic changes occurring in the knee joint and clinical features were not taken into consideration. Given that OA is a heterogeneous condition in which multiple factors can contribute to the development of symptoms, it was important to find the criteria which blend findings from different clinical assessments. Therefore, clinical criteria were developed which utilize data from the medical history, physical examination and laboratory testing (Table 1.1) (Altman et al., 1986).

**Table 1.1 The American College of Rheumatology (ACR) clinical classification criteria for OA of the knee**

When individuals present with knee pain that is persistent on most days in a past month, they are assessed for the presence of at least 5 of the following symptoms,

1. Age - $>50$ years
2. Morning stiffness - $<30$ min
3. Crepitus on active joint motion
4. Bony enlargement of the knee
5. Bony tenderness of the knee
6. No palpable warmth of synovium

7. ESR - <40 mm/hour
8. RF - <1:40
9. SF OA

ESR = erythrocyte sedimentation rate (Westergren); RF = rheumatoid factor; SF OA = synovial fluid signs of OA (clear, viscous, or white blood cell count <2000/mm<sup>3</sup>) (Adapted from Altman et al., 1986).

### **1.2.2 Synovial inflammation and OA**

Synovial joint cavities are lined with a thin cellular layer (synovial membrane) which is semipermeable in nature and responsible for secretion of synovial fluid, nutrients and lubricant factors; for example, hyaluronan and proteoglycan 4 (Martel-Pelletier et al., 2016). These molecules contribute to the viscosity of synovial fluid and provide lubrication to articular cartilage (Kapoor, 2015).

Synovitis is inflammation of the synovial membrane and involves infiltration of various inflammatory cells within the synovial compartment (Berenbaum, 2013). For decades, OA has been believed to be a degenerative form of arthritis and the presence of synovial inflammation and its contribution towards the pathogenesis of the disease was underappreciated by researchers and clinicians (Sokolove and Lepus, 2013). However, studies published in recent years have helped to change this paradigm. A study by Sohn et al. (2012) has shown the upregulation of various inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6), chemokines (monocyte chemoattractant protein-3, macrophage inflammatory protein-1 $\alpha$  and -1 $\beta$ ), vascular endothelial growth factor (VEGF), and granulocyte

macrophage colony-stimulating factor in the synovial fluid and serum samples obtained from OA patients. Moreover, studies have shown that a subset of patients with OA experience systemic low-grade inflammation as indicated by an increase in the levels of C-reactive protein (CRP) (Stürmer et al., 2004; Pearle et al., 2007). Interestingly, it has been found that a good correlation exists between serum CRP and synovial inflammation (Pearle et al., 2007), and the severity of pain in OA patients (Stürmer et al., 2004). Additionally, with the development of sensitive imaging modalities it is now possible to better assess synovitis in osteoarthritic patients. Studies using either contrast-enhanced (gadolinium-based contrast agents) or non-contrast enhanced MRI were able to effectively assess effusion-synovitis, infrapatellar fat pad-synovitis and correlate with cartilage loss (Ayril et al., 2005; Roemer et al., 2011).

The reasons behind the development of synovial inflammation in OA are not yet clear. It has been suggested that during OA a variety of factors cause damage to cartilage which results in the formation of damaged fragments, which remain in the joint and eventually come in contact with the synovium (Figure 1.2). These fragments are recognized as a foreign substance by synovial cells which then mount a response involving the production of inflammatory mediators and matrix metalloproteinase (MMP) enzymes. These mediators contribute to further cartilage degradation (Berenbaum, 2013). It has been suggested that the innate immune system plays an important role in the generation of inflammation at an early stage of the disease. The innate immune system involves different pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) molecules which bind to pattern-recognition receptors (PRRs) to elicit an immune response (Kawai and Akira,

2010). Interestingly, a study has found increased expression of Toll-like receptor (TLR-2 and 4), a type of PRR, in the OA cartilage lesions (Kim et al., 2006). Various components of the extracellular matrix such as biglycan (Melrose et al., 2008), fibronectin (Chevalier et al., 1996), and low-molecular weight hyaluronic acid (Dahl and Husby, 1985) can serve as DAMPs and therefore can activate TLR-2 or TLR-4 (Berenbaum, 2013; Sokolove and Lepus, 2013) to induce the immune response.

### **1.2.3 Pain mechanisms in OA**

Data obtained from preclinical and clinical studies indicate that the presence of synovial inflammation can be nicely correlated to joint pain which means synovitis is a contributor to joint pain (Stürmer et al., 2004; Hill et al., 2007; Orita et al., 2011; Orita et al., 2012). Joint inflammation involves the release of several inflammatory mediators which can cause peripheral sensitization by reducing the firing threshold of primary afferent nociceptors (Mease et al., 2011). Additionally, unveiling of silent nociceptors occur which are unresponsive to noxious stimuli under normal condition, however, becomes responsive due to inflammation (Schmidt et al., 1995; Hunter et al., 2008). Numerous studies indicate that there is an increased production of various inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 in the synovial membrane of OA patients (Smith et al., 1997; Bondeson et al., 2006). These cytokines can contribute to the generation of pathological pain by directly sensitizing nociceptors (Zhang and An, 2007). An interesting investigation has shown that co-culturing dorsal root ganglion (DRGs) from an adult rat with synovial tissues from OA patients results in an increase in the gene expression of tachykinin 1, neuropeptide Y and calcium channel  $\alpha 2\delta 1$  in DRGs. These

molecules are involved in the transmission of pain. Simultaneously, the study also found increased expression of cyclooxygenase 2 and IL-6 (Li et al., 2011). Authors of the paper suggested that cytokines produced in the synovial membrane can sensitize sensory neurons to elicit the pain response (Li et al., 2011; Zhang et al., 2013). Consistent with these findings, a preclinical study has found a positive correlation between the level of cytokines and the pain response in a rat model of OA (Orita et al., 2011).

Additionally, the levels of neuropeptides such as substance P (SP), calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP) are increased within the inflamed synovium of OA patients (Menkes et al., 1993; Sutton et al., 2009; Mcnearney et al., 2016). Preclinical studies support this clinical observation, as it has been shown that all the above-mentioned neuropeptides are upregulated in experimental OA animals resulting in generation of the pain response (Schuelert and McDougall, 2006; Orita et al., 2011). It has been shown that SP and nociceptin when administered locally induce sensitization of joint afferents which results in an increase in neuronal firing (McDougall et al., 2000, 2001; Pawlak et al., 2001). These studies also report that the effect is mediated by neurokinin-1 (NK-1) receptor which is present on joint afferents (McDougall et al., 2000, 2001; Pawlak et al., 2001). Another inflammatory neuropeptide VIP has been shown to modulate OA joint pain as administration of VIP antagonists alleviates OA joint pain (McDougall et al., 2006).

Studies indicate that OA pain can occur due to central sensitization (Mease et al., 2011). A study by Hee-Jeong Im et al. (2011) examined sensory and spinal responses in different experimental models of OA. It was observed that the expression of inflammatory mediators (IL-1 $\beta$  and TNF- $\alpha$ ), and neuropeptides (CGRP, SP, neuropeptide

Y and galanin) were upregulated at L3-L5 DRG levels in monoiodoacetate (MIA), anterior cruciate ligament transection and surgical destabilization of the medial meniscus experimental model of OA. This finding suggest interaction between inflammatory mediators and nociceptors in osteoarthritic animals which could contribute to central sensitization. Furthermore, the study also observed increased expression of IL-1 $\beta$  and  $\alpha$ , IL-17, thymus chemokines (TC), TNF $\alpha$ , L-Selectin and VEGF in the lumbar spinal cord of MIA-injected animals which indicate changes occurring at the central level are contributing to OA pain (Hee-Jeong Im et al., 2011).

### **1.3 Rheumatoid arthritis**

Rheumatoid arthritis (RA) is another leading form of arthritis which can affect multiple joints in the body (McInnes and Schett, 2011). RA symmetrically affects small joints of hands and feet, wrists, ankles and knee joints (Gibofsky, 2012). RA is associated with an immune-driven chronic systemic inflammation which results in synovial inflammation, formation of autoantibodies and bone lesions (McInnes and Schett, 2011). Additionally, patients experience extra-articular manifestations, for example, small vessel vasculitis, keratoconjunctivitis sicca, salivary gland swelling and pulmonary fibrosis (Cojocaru et al., 2010). The global burden of RA is around 1% with a continuously rising prevalence (Gibofsky, 2012). In Canada, about 1% of the total population is suffering from RA with more women affected than men (Wong et al., 2010). Thus, RA poses a huge socioeconomic burden on society.

### 1.3.1 Diagnosis

In 1987, the ACR proposed criteria for the classification and diagnosis of RA that was widely utilized (Arnett et al., 1988). However, the criteria lacked sensitivity for the detection of early RA as it had placed more emphasis on those patients who have advanced RA (i.e. patients with chronic lesions). Thus, there was a need for criteria that could identify early disease in patients who are likely to get more benefit from the treatment because of early detection. To overcome this limitation, in 2010, the ACR and European League Against Rheumatism (EULAR) worked in collaboration and developed criteria that are now widely accepted and utilized (Table 1.2) (Aletaha et al., 2010).

**Table 1.2 The 2010 ACR and EULAR clinical classification criteria for RA**

When an individual is presented with joint inflammation which could not be explained with other disease, they are assessed using following criteria ( $\geq 6/10$  score means patient has RA)

Criteria	Description	Score
<b>Joint involvement</b>	1 large joint	0
	2 - 10 large joints	1
	1 - 3 small joints (with or without involvement of large joints)	2
	4 - 10 small joints (with or without involvement of large joints)	3
	>10 joints (at least 1 small joint)	5
<b>Serology</b>	Negative RF and negative ACPA	0
	Low-positive RF or low-positive ACPA	2
	High-positive RF or high-positive ACPA	3
<b>Acute-phase reactants</b>	Normal CRP and normal ESR	0
	Abnormal CRP or abnormal ESR	1
<b>Duration of symptoms</b>	<6 weeks	0
	$\geq 6$ weeks	1

**Large joints** - shoulders, elbows, hips, knees, and ankles.

**Small joints** - metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists.

**ACPA** – anti-citrullinated protein antibody.

**CRP** - C-reactive protein.

**RF** – Rheumatoid factor.

**ESR** - Erythrocyte sedimentation rate

(Adapted from Aletaha et al., 2010)

### **1.3.2 Immune-mediated synovial inflammation and RA**

Pathogenesis of RA is a complex process in which a person's own immune system starts to attack joints as the immune system loses its ability to distinguish between 'self' versus 'non-self'. Activation of an innate and adaptive immune system occurs which results in recruitment of inflammatory cells within the synovium, formation of autoantibodies and perpetuation of an inflammatory response. These events lead to synovial inflammation, bone lesions and systemic inflammation (Picerno et al., 2015).

Innate immune system activation involves infiltration of inflammatory cells such as neutrophils, macrophages and mast cells (McInnes and Schett, 2011) (Figure 1.3). Neutrophils are found in the synovial fluid where they release cytokine (IL-8), prostaglandins (PGs) and chemokines which contribute in the development of synovitis (Cornish et al., 2009). Additionally, neutrophils release several proteinases including neutrophil elastase, proteinase 3, cathepsin G, MMP-8 and MMP-9 which can initiate degradation of extracellular matrix within cartilage (Wright et al., 2014). Macrophages are accumulated in synovial tissue where they can release a wide range of mediators including cytokines (TNF- $\alpha$  and IL-1, 6, and 12), reactive oxygen intermediates, nitrogen intermediates and prostanoids which can contribute to an inflammatory response (McInnes and Schett, 2011). Mast cells possess receptors for immunoglobulin E (IgE)

and hence it can promote the crosslinking of IgE. The crosslinking of IgE contributes to degranulation of mast cells which results in secretion of histamine, proteoglycan and proteases (e.g. trypsin and tryptase). Histamine release causes an increase in vascular permeability and proteases trigger degradation of matrix proteins (Nigrovic and Lee, 2005). Interestingly, these mediators can also sensitize primary afferent neurons.

Presence of autoantibodies like rheumatoid factor (RF) and antibodies against citrullinated peptides/proteins (ACPA) is one of the features of RA which suggest an important role of the adaptive immune system in the pathogenesis of the disease (Aletaha and Blüml, 2016). Studies indicate that dendritic cells, myeloid cells and B cell are expressed in RA synovium (Schröder et al., 1996; Lebre et al., 2008). These cells play an important role in the detection of an arthritogenic antigen and presenting it to type 1 helper T-cells ( $T_h1$ ) which are then activated. Once  $T_h1$  cells are activated they promote inflammation by enhancing the release of cytokines (TNF- $\alpha$ , IL-1 and 6, and IFN $\gamma$ ) and chemokines (CXCL12) (Picerno et al., 2015). Additionally, there is release of IL-17 which is a cytokine for  $T_h17$  cells (Gaffen, 2009). Recently, it has been suggested that  $T_h17$  cells can contribute to synovial inflammation by inducing the release of pro-inflammatory cytokines (Gaffen, 2009). B cells, in addition to antigen presentation, are involved in the pathogenesis of RA by causing increase in the production of autoantibodies and secretion of pro-inflammatory cytokines (Smolen et al., 2007).

### 1.3.3 Pain mechanisms in RA

Studies indicate that the synovial fluid of RA patients contain increased levels of TNF- $\alpha$ , IL-1, IL-6, IL-23, SP, CGRP and VEGF (Menkes et al., 1993; Fava et al., 1994; Brennan and McInnes, 2008; Wang et al., 2015). These inflammatory mediators present within the articular microenvironment can sensitize neurons and lower their excitation threshold to signal pain (Walsh and McWilliams, 2014). In accordance with clinical observations, preclinical RA studies have also found similar results. It was observed that L1-L5 DRG expression of SP, CGRP, neuropeptide Y and bradykinin receptor was upregulated in an antigen-induced arthritis model of RA (von Banchet et al., 2000; Schaible et al., 2002; Walsh and McWilliams, 2014; Wang et al., 2015). Furthermore, in the collagen-induced arthritis model the expression of activating transcription factor 3 (ATF-3), a nerve injury marker, was increased at L3-L5 DRG level (Inglis et al., 2007) which was correlated with increased levels of TNF- $\alpha$  and IL-1 $\beta$  in joints of arthritic animals (Marinova-Mutafchieva et al., 2006). Additionally, the spontaneous behaviour in mice could be blocked by anti-TNF therapy (Inglis et al., 2007). These findings suggest that persistent joint inflammation could contribute to peripheral sensitization and thereby cause neuropathy in RA animals. Increase in sensory input occurs because of peripheral sensitization which can result in sensitization of neurons at spinal and supraspinal levels (Schaible et al., 2002). Inglis et al. (2007) have also detected astrogliosis in the lumbar spinal cords of collagen-induced arthritic mice and it was assessed using glial fibrillary acidic protein (GFAP) labeling (Inglis et al., 2007). This observation was supported by another study which found that spinal microglial response was increased in arthritic mice and the response was attenuated after inhibition of cathepsin S and fractalkine (Clark et

al., 2012). It should be noted that cathepsin S and fractalkine are required for neuronal-microglial communication and maintenance of neuropathic pain (Clark and Malcangio, 2012). Changes can occur at the supraspinal level during RA which can contribute to generation of the pain response. A study has found time-dependent activation of specific brain regions such as the anterior pretectal nucleus, anterior cingulate cortex and nucleus accumbens in Freund's complete adjuvant injected animals which suggest possible involvement of these supraspinal structures in processing the pain response (Neto et al., 1999). Supraspinal regions can further process pain signals because of the presence of a descending inhibitory system which tries to block incoming nociceptive information at the level of spinal cord (Basbaum and Fields, 1984). Descending pain facilitatory system, on the other hand, promotes nociception (Vanegas and Schaible, 2004). Normally, the activity of descending inhibitory and facilitatory system is balanced; however, during chronic pain conditions, it has been suggested that activity of descending inhibitory system is dysregulated or descending pain facilitatory system activity is enhanced (Vanegas and Schaible, 2004).

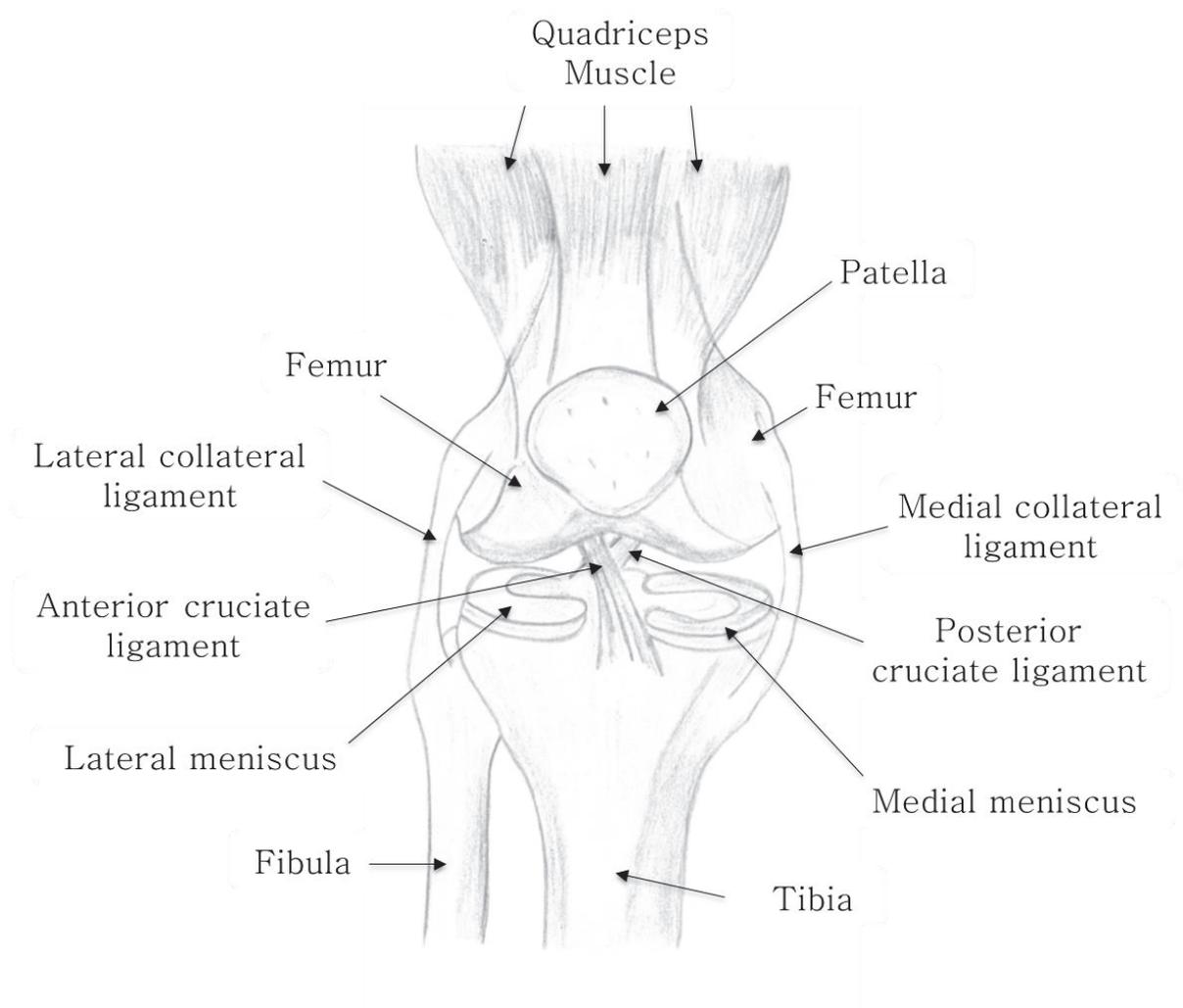
## **1.4 Knee joint**

### **1.4.1 Anatomy**

The knee joint is one of the largest joints in the body, and it is a type of hinge joint (Schuenke et al., 2006). The knee joint consists of three bones: the femur, tibia, and patella (Figure 1.1) (Schuenke et al., 2006). These bones form two different articular surfaces, the tibiofemoral (femur and tibia) and patellofemoral (patella and femur)

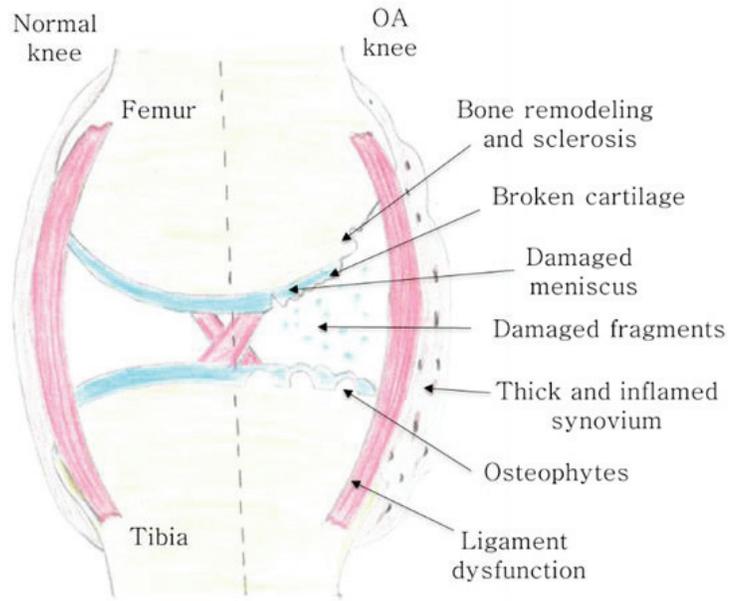
surface which allow flexion and extension (Schuenke et al., 2006). These movements enable us to perform many different activities like walking, running, jumping, standing and sitting. The femur is one of the strongest bones in the body (Schuenke et al., 2006). At the lower extremity of this bone, there are two prominent round surfaces present called the lateral and medial condyles which articulate with the tibial medial and lateral condyles present on the upper surface of the tibia (Makris et al., 2011). At the articular surface, a thin layer of hyaline cartilage is found; it is elastic in nature and can absorb shock during movement to prevent bones from damage (Schuenke et al., 2006). The patella is a small sesamoid bone present on the anterior surface of the knee joint, and it helps during the knee extension by increasing the flexibility for the tendon that connects quadriceps muscle to the tuberosity of the tibia (Makris et al., 2011). The fibula is another bone which occurs parallel to tibia from the lateral side, and it is attached to tibia from top and lower end. The fibula is not a part of the knee joint as it does not articulate with femur or patella. A capsule, which surrounds bones of the knee, is a bilayer structure with the outer layer consisting of a fibrous tissue and the inner layer composed of synovial cells (Makris et al., 2011). The synovial cells secrete synovial fluid which provides lubrication and helps to reduce the friction between the articulating bones during movement. A healthy human knee joint contains 4 ml of synovial fluid within the synovial cavity (Gatter and Schumacher, 1991). Two menisci (lateral and medial) are located between femur and tibia where they serve to reduce friction between the two bones and help to stabilize the joint during loading. It is because of the shape of the menisci (wedge) they can help femoral condyle to stabilize when they articulate with the flat tibial plateau (Makris et al., 2011). There are four different ligaments present in the

knee: the anterior cruciate ligament (ACL), posterior cruciate ligament (PCL), medial collateral ligament (MCL) and lateral collateral ligament (LCL) which surround the joint and provide stability during movement. An additional patellar ligament connects the patella to the tuberosity of the tibia and assists to maintain the position of the patella (Schuenke et al., 2006).



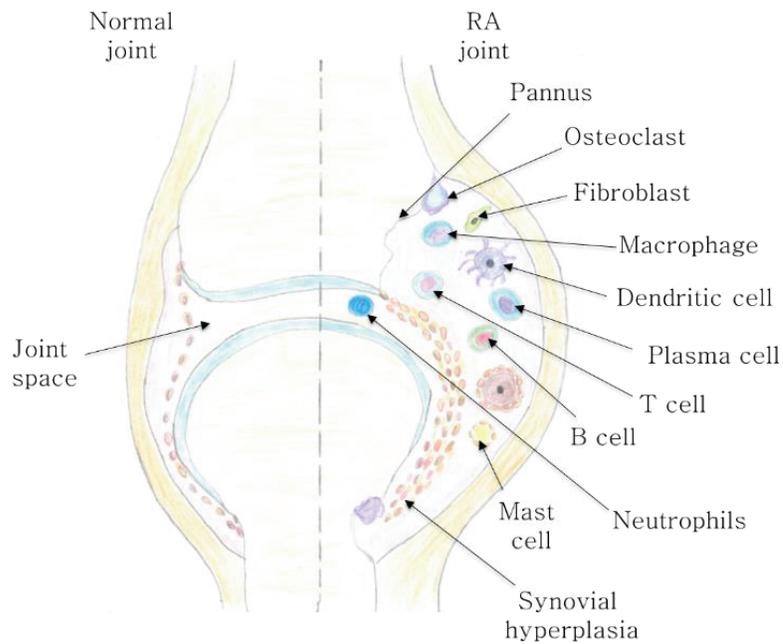
**Figure 1.1 Human knee joint anatomy**

**(Drawing by Ketki M. Muley based on the image from Schuenke et al., 2006 and <https://www.healthpages.org/wp-content/uploads/2010/06/Knee-anatomy-570.jpg>)**



**Figure 1.2 Normal and OA knee**

**(Drawing by Ketki M. Muley based on the image from Hunter, 2011)**



**Figure 1.3 Normal and RA joint**

**(Drawing by Ketki M. Muley based on the image from Strand et al., 2007)**

### 1.4.2 Joint innervation

The knee joint can accomplish co-ordinated joint movements by establishing communication with the central nervous system (CNS). The communication involves transmission of action potential towards (afferent) and away from (efferent) the CNS. Sympathetic division of the autonomous nervous system carries efferent signals (Table 1.3). A study has found that sympathetic nerve fibres, which innervate the medial and posterior region of the knee joint, originate from lumbar paravertebral ganglia (L4-L6) (Heppelmann and Schaible, 1990). These postganglionic nerve fibres terminate near joint blood vessels and due to their ability to control vasomotor tone they can regulate the blood flow in the region (McDougall, 2006). Interestingly, it has been shown that during chronic joint inflammation the sympathetic vasomotor tone is compromised which leads to perfusion changes in arthritic joints (McDougall, 2001). The knee joint is also innervated with sensory nerve fibres which assist in articular proprioception and nociception (Ferrell, 1992). Four different classes of afferent nerve fibres innervate the knee joint (Table 1.3). Class I ( $A\alpha$ ) and class II ( $A\beta$ ) fibres have myelin sheath surrounding their axons which allows them to fast conduct the nerve impulses. These large diameter nerve fibres innervate the ligament and fibrous layer and function to recognize the relative position of the joint during movement. Class III ( $A\delta$ ) nerve fibres are thinly myelinated and innervate the fibrous layer, synovium and subchondral bone (Serre et al., 1999). Class IV (C) nerve fibres do not contain myelin sheath around their axons and they innervate fibrous layer, synovium and subchondral bone (Serre et al., 1999). These small diameter nerve fibres have high threshold and respond to noxious stimuli (McDougall, 2006). Studies indicate that 80% of rat knee joint afferents are small

diameter and unmyelinated (Hildebrand et al., 1991) which suggests their ability to detect pain signals.

**Table 1.3 Afferent and efferent nerve fibres of the knee joint**

Type of fibre	Diameter (µm)	Conduction velocity (m/s)	Myelination	Location	Function
Efferent sympathetic fibre	0.4–2.4	1	unmyelinated	medial and posterior region	sympathetic vasomotor tone
Afferent fibres					
Class I - Aα	10–18	60 - 100	myelinated	ligaments	Proprioceptor
Class II - Aβ	5–12	20 - 70	myelinated	fibrous layer	Proprioceptor
Class III - Aδ	1–5	2.5 - 20	thinly myelinated	fibrous layer, synovium and subchondral bone	Nociceptor
Class IV - C	< 1	< 2.5	unmyelinated	fibrous layer, synovium and subchondral bone	Nociceptor

Adapted from (Krustev et al., 2015) and expanded from (Ferrell, 1992)

### 1.4.3 The joint vascular system

Human knee joint receives constant blood supply from different arterial branches. The popliteal artery, a branch of the femoral artery, further branches into five different genicular arteries which mainly supply blood to the knee joint (Scapinelli, 1968). The five arterial branches include medial superior genicular artery, lateral superior genicular artery, middle genicular artery, lateral inferior genicular artery and medial inferior

genicular artery (Scapinelli, 1968). These arterial branches are connected with each other to form a rich network (anastomosis) around the knee (Scapinelli, 1968). The medial and lateral superior genicular artery travels down along the femur and supplies blood to the respective femoral condyle bones (Shim and Leung, 1985). These arteries form a network around femoral condyles. Additionally, these arteries can supply blood to the adjacent synovium (Shim and Leung, 1985). The middle genicular artery is located behind the femoral condyle and is a small branch originating from the popliteal artery which supplies blood to the synovium, ligaments and menisci. The medial and lateral inferior genicular artery forms a network around tibial condyles and supply blood to this region. Additionally, these arteries supply blood to the capsule, tendons and collateral ligaments (Shim and Leung, 1985). The saphenous artery, an additional branch of the descending genicular artery, travels in the anteromedial portion of the knee and is accompanied by the saphenous nerve (Scapinelli, 1968). It should be noted that all the tissues of the knee joint, except cartilage, are supplied with blood vessels; however, the degree of vascularization in these tissues varies. The synovium is highly vascularized with a dense network of arterioles, capillaries and venules supplying blood to the tissue (Haywood and Walsh, 2001). Cartilage is an avascular tissue which receives nutrients and oxygen supply from synovial fluid or subchondral blood vessels (Haywood and Walsh, 2001). Like humans, rodent knee joints also receive blood supply from the branches of genicular and saphenous arteries (Haywood and Walsh, 2001). In normal joints the vascular network is highly organised, however, during arthritis the network is disturbed due to chronic inflammation leading to altered blood supply to the tissue (Haywood and Walsh, 2001).

## **1.5 Inflammation**

Inflammation is a protective mechanism induced in response to a variety of stimuli including tissue damage or injury, pathogenic infection, foreign bodies, irritants and immune reactions (Lawrence and Gilroy, 2007). Inflammation is a Latin term 'inflammare' which means 'to set on fire' (Scott et al., 2004). The four cardinal signs of an inflammatory response were first described by Celsus (1st century AD) and they are rubor (redness), calor (heat), tumour (swelling) and dolor (pain) (Rocha de Silva M, 1994). In 1871, Virchow added the fifth cardinal sign of inflammation *functio laesa* (loss of function) (Rocha e Silva M, 1994). Inflammation can be described as either acute or chronic. Acute inflammation is short-lasting and mainly attempts to eliminate the inflammatory agent, repair the damaged tissue and then resolve. However, if the acute inflammatory response is not resolved appropriately it could turn into a chronic inflammation which is maladaptive and can produce detrimental effects (e.g. rheumatoid arthritis, osteoarthritis, inflammatory bowel disease and chronic obstructive pulmonary disease).

### **1.5.1 Acute inflammation**

Acute inflammation is an immediate response which can last from several minutes to a few hours or days. The important events that occur during acute inflammation are increase in the blood flow to the site of injury, increase in vascular permeability which leads to leakage of plasma proteins and leukocyte transmigration

from blood circulation to the site of tissue injury which leads to an accumulation of leukocytes within the tissue.

### **1.5.1.1 Leukocyte extravasation**

Leukocyte extravasation is a multistep process and mainly involves five steps such as the capture of leukocytes, rolling, slow rolling, firm adhesion and finally transendothelial migration into inflamed tissue (Figure 1.4). These events are responsible for moving leukocytes from the bloodstream to the site of inflammation. These events are orchestrated by different chemokines and cytokines, which induce the expression of cellular adhesion molecules (e.g. selectins and integrins) leading to an activation of vascular endothelium.

#### **1.5.1.1.1 Capture of leukocytes**

This is the first step of the leukocyte extravasation cascade which involves binding of leukocytes to the activated endothelium and it is accomplished by formation of a weak bond between leukocyte and endothelium. This step is mediated by an adhesion molecule P-selectin which interacts with its leukocyte ligand P-selectin glycoprotein ligand 1 (PSGL1). This step ensures that leukocytes are not washed away by continuously flowing blood (Eriksson et al., 2001).

#### **1.5.1.1.2 Leukocyte rolling**

Leukocytes, after attachment to the activated endothelium, begin to roll along with a velocity less than other free-flowing cells in the same blood vessel. This step is mediated by different selectins such as P-selectin, E-selectin and L-selectin although P-selectin plays the most dominant role in promoting leukocyte rolling (Ley et al., 2007). These adhesion molecules interact with PSGL1 to assist with the leukocytes rolling. It should be noted that PSGL1 is constitutively expressed on various inflammatory cells including neutrophils, eosinophils, lymphocytes and monocytes (Ley et al., 2007). Furthermore, studies show that leukocytes express L-selectin and activated endothelium express P-selectin and E-selectin (Kansas, 1996; Ley et al., 2007). P-selectin is formed in Weibel-Palade bodies which are the storage granules of endothelial cells and it is transferred to the surface after stimulation by pro-inflammatory cytokines such as TNF $\alpha$  or IL-1 $\beta$  (Eriksson et al., 2001).

#### **1.5.1.1.3 Slow rolling of leukocytes**

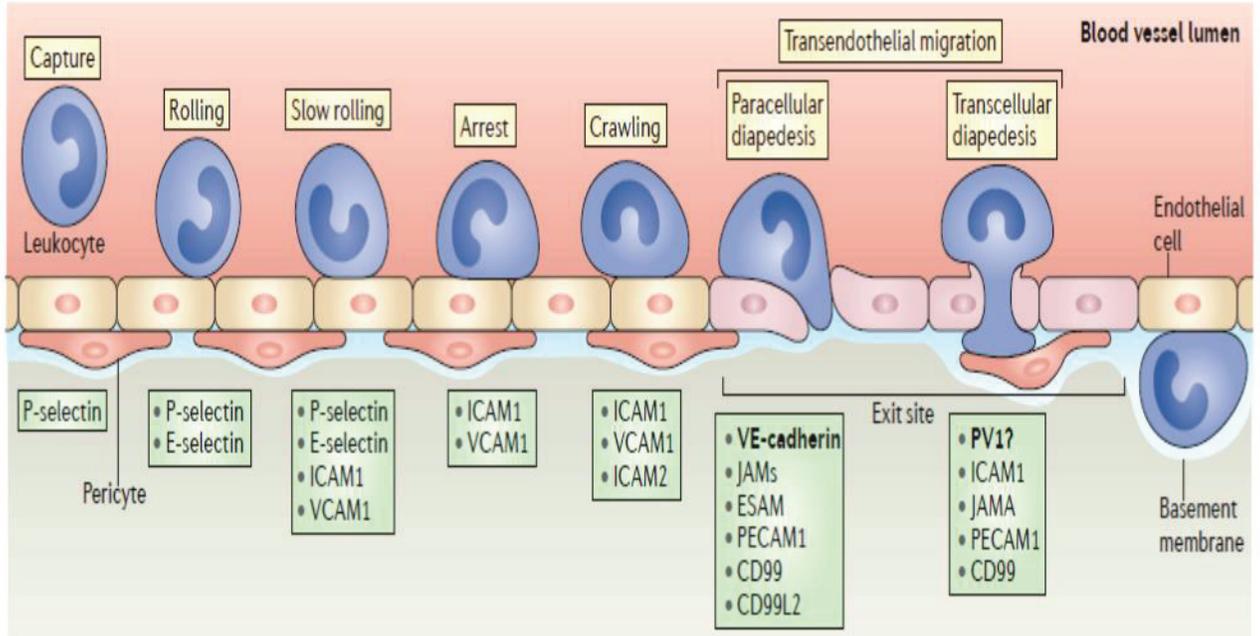
The critical velocity of the leukocytes is decreased gradually before they become stationary and adhere to the endothelium. Studies show that the velocity of the slow rolling leukocytes is 3-5  $\mu\text{m/s}$  and E-selectin plays an important role in mediating slow rolling (Kunkel and Ley, 1996). Additionally, it has been shown that  $\beta$ 2-integrins such as lymphocyte function-associated antigen 1 (LFA1) and macrophage antigen 1 (MAC1) contribute to slowing down rolling leukocytes (Dunne et al., 2002).

#### **1.5.1.1.4 Firm adhesion**

Leukocytes, after gradually slowing down, are arrested and attached firmly to the activated endothelium for at least 30 seconds. It has been shown that interactions between integrins such as vascular cell adhesion molecule 1 (VCAM1), intercellular adhesion molecules (ICAM1 and ICAM2) expressed on leukocytes and their ligands present on the activated endothelium are important for leukocyte adhesion (Ley et al., 2007). Additionally, a study has shown E-selectin being able to cause firm leukocyte adhesion *in vivo* (Ley et al., 1998).

#### **1.5.1.1.5 Transendothelial migration**

Transendothelial migration is a final step in the leukocyte trafficking cascade involving migration of leukocyte into inflamed tissue. It should be noted that leukocytes first crawl out of the blood vessel lumen and cross three different barriers such as endothelial cells, the endothelial-cell basement membrane, and pericytes before reaching the inflamed tissue and this process can take approximately  $\geq 15$  min (Ley et al., 2007). A study has shown that neutrophils and monocytes use the aforementioned process to emigrate to the inflamed tissue in a MAC1- and ICAM1-dependent manner (Phillipson et al., 2006). Additionally, adhesion molecules like platelet/ endothelial-cell adhesion molecule 1 (PECAM1), LFA-1, CD99 and very late antigen 4 (VLA4) have been implicated in transendothelial migration (Nourshargh and Alon, 2014).



**Figure 1.4 Multistep cascade of leukocyte extravasation with cell adhesion molecules**

**involved in each step.** ICAM, intercellular adhesion molecule; VCAM1, vascular cell adhesion molecule 1; JAM, junctional adhesion molecule; ESAM, endothelial cell-selective adhesion molecule; PECAM1, platelet endothelial cell adhesion molecule 1; CD99L2, CD99 antigen-like protein 2 (Appendix II - reproduced with permission from Macmillan Publisher Ltd: Nature Reviews Immunology, 15(11): 692-704; Vestweber, 2015).

### 1.5.1.2 Hyperaemia

Increase in blood flow to the site of injury (or alternatively termed hyperaemia) is one of the features of the inflammatory response. A variety of mediators including vasoactive amine, prostaglandin (PG), bradykinin, nitric oxide and neuropeptide are involved in mediating hyperaemic response (Egan et al., 2002). Granules of the mast cell,

basophil and platelet contain preformed vasoactive amines such as histamine and serotonin, and they are released through degranulation, in response to a stimulation by IL-1 or IL-8 during inflammation (Medzhitov, 2008). Upon release, these amines induce vasodilation and alter the vascular permeability to facilitate leakage of plasma proteins. Membrane phospholipids are converted into arachidonic acid and lysophosphatidylcholine by phospholipase enzyme A<sub>2</sub>. Cyclooxygenase (COX) enzymes catalyze conversion of arachidonic acid into PGH<sub>2</sub> which is further catalyzed by PGI and PGE synthase into PGI<sub>2</sub> and PGE<sub>2</sub> respectively which can cause vasodilation (Ricciotti and FitzGerald, 2011). Interestingly, PGE<sub>2</sub> has been shown to be capable of mediating hyperalgesic response and fever (Medzhitov, 2008). Bradykinin, a peptide mediator, is produced during inflammation, can elicit a variety of effects such as vasodilation in the vessels of gut, aorta, uterus and urethra (Golias et al., 2007), and hyperalgesia by causing sensitization of nociceptors (e.g. in the knee joint) (Neugebauer et al., 1989). Nitric oxide (NO), a potent mediator of inflammation, produced and released by endothelial cells causes vasodilation. Additionally, the NO can mediate vasodilation induced by bradykinin and histamine (Wallace, 2005). Lastly, the peptides released by afferent nerves (e.g. substance P) can also cause vasodilation and increase in the vascular permeability. Furthermore, substance P can also induce vasodilation by causing mast-cell degranulation (Medzhitov, 2008).

### **1.5.2 Chronic inflammation**

Chronic inflammation can occur due to persistent exposure to irritants or toxic material, chronic pathogenic infection and autoimmune disease. The presence of chronic

inflammation also suggests that body is unable to resolve an acute inflammatory response and hence this type of inflammation can last for months or years. Chronic inflammation is mainly characterized by an accumulation of inflammatory cells (monocytes which mature to become macrophages, lymphocytes, plasma cells, neutrophils and eosinophils), fibrosis and tissue destruction.

#### **1.5.2.1 Role of inflammatory cells in chronic inflammation**

Macrophages, by performing a variety of functions, can contribute to chronic inflammation and they are the dominant cells in this type of inflammation. During inflammation, bone marrow tissue starts to produce monocytes from monoblasts, which are then released into blood circulation. Monocytes emigrate to the site of inflammation and differentiate into macrophages (M1 or M2). Macrophages (M1 type) are activated in response to a microbial endotoxin leading to multiple actions such as activation of a toll-like receptor, release of IFN- $\gamma$ , nitric oxide, reactive oxygen species and proteolytic enzymes. Moreover, pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, chemokines and eicosanoids are released resulting in induction and maintenance of an inflammatory response (Nathan and Ding, 2010). Furthermore, M1 macrophages can activate T cell lymphocytes by presenting an antigen to them which then triggers a cell-mediated immune response. On the other hand, M2 macrophages, which are activated by anti-inflammatory cytokines such as IL-4 and IL-13, are involved in phagocytosis of parasites (Sica and Mantovani, 2012). M2 macrophages also contribute to collagen synthesis and formation of new blood vessels through secretion of growth factors (Sica and Mantovani, 2012).

The adaptive immune system consists of specialized cells such as T and B lymphocytes which function to remove pathogens or foreign antigens from the body. However, the adaptive immune system fails during an autoimmune condition (e.g. rheumatoid arthritis) which can result in the amplification and propagation of chronic inflammation. Increase in the expression of cell adhesion molecules occur which helps lymphocytes to transmigrate to the inflamed site. Furthermore, upon exposure of an antigen, naïve CD4+T cells differentiate and mature which results in the release of pro-inflammatory mediators, such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6, which can further promote inflammation. B lymphocytes and plasma cells secrete antibodies to contribute to chronic inflammation (Nathan and Ding, 2010).

Neutrophils have always been considered to play a major role in an acute inflammation and their contribution in chronic inflammation was underappreciated. However, recent evidence suggests that neutrophils can contribute to a chronic inflammation through multiple actions. Neutrophils can communicate with other immune cells by releasing various cytokines and chemokines, presenting antigens to T cells by coordinating with MHC class II antigens. Moreover, neutrophils release proteinases which can promote the release of other inflammatory mediators such as cytokines and chemokines (Wright et al., 2014). Some of the proteinases include neutrophil elastase, cathepsin G and proteinase 3. It has also been suggested that released proteinases activate a variety of adhesion molecules which helps promote leukocyte extravasation.

During allergic reactions, IgE antibodies promote chronic inflammation by recruiting inflammatory cells such as mast cells, eosinophils and basophils (Stone et al., 2010). Degranulation of eosinophils causes release of major basic protein which is

capable of inflicting damage to epithelial cells. Likewise, degranulation of mast cells and basophils causes release of vasoactive amines which induce vasodilation and alter vascular permeability (Stone et al., 2010).

## **1.6 Pain**

For arthritis patients, pain is the most important concern that makes them visit a physician and seek medication. The international association for the study of pain (IASP) defines pain as ‘an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage’. Acute pain response is protective in nature and usually serves to warn us about actual or prospective damage; however, chronic pain which is long-lasting serves no such benefit, is difficult to treat and causes a drastic reduction in the quality of life of a patient.

### **1.6.1 Inflammatory pain**

Inflammation is triggered in response to tissue damage or injury which entails the release of a cocktail of inflammatory mediators such as histamine, bradykinin, prostaglandins, cytokines, nerve growth factors and neuropeptides. These mediators sensitize and activate nociceptors to generate pain signals. Many acute and chronic diseases involve inflammation which can contribute to underlying pain associated with the disease.

Histamine released by mast cells during inflammation can contribute to neurogenic inflammation by sensitizing small-diameter A $\delta$  and C fibres which results in the release of neuropeptides such as SP and CGRP, and these mediators contribute in the transmission of pain (Rosa and Fantozzi, 2013). A study has shown that histamine H1 receptor knockout mice prevented a behavioural pain response which suggests the involvement of this receptor in nociceptive transmission (Mobarakeh et al., 2000).

Pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 are not only involved in mediating inflammation but they have also been implicated in pain (Kidd and Urban, 2001). These cytokines can either sensitize neurons directly or induce the release of neurosensitizing mediators such as PGs and neuropeptides (Oprea et al., 2000). Studies indicate that administration of these cytokines produces mechanical and thermal hyperalgesia which can be reversed by modulating the release of these cytokines (Woolf et al., 1997; Oprea et al., 2000; Kidd and Urban, 2001).

Nerve growth factor (NGF) is a neurotrophic factor required for the growth of primary neurons, however, studies indicate that it is capable of inducing mechanical and thermal hyperalgesia when administered exogenously (Lewin et al., 1993). In a study, administration of NGF to TRPV1-knockout mice prevented the thermal hyperalgesia, which suggests the involvement of TRPV1 in mediating NGF-induced thermal hyperalgesia (Chuang et al., 2001). It has been shown that the mechanical and thermal hyperalgesia induced by TNF- $\alpha$  and IL-1 $\beta$  can be attenuated by blocking NGF (Woolf et al., 1997) indicating the role of the neurotrophic factor in mediating inflammatory pain.

Mediators of neurogenic inflammation such as SP and CGRP are known to cause vasodilation and increase in vascular permeability. Additionally, they can cause

peripheral sensitization by sensitizing afferent neurons, stimulating the degranulation of mast cells to cause the release of histamine and promoting formation of PGE<sub>2</sub> (Kidd and Urban, 2001). Furthermore, these neuropeptides can also induce excitability in spinal neurons and glial cells (Dray, 1996).

### **1.6.2 Neuropathic pain**

Neuropathic pain arises as a consequence of direct injury to the somatosensory nervous system and it is characterized by shooting, electric shock-like or stabbing pain (Cohen and Mao, 2014). Many chronic conditions are associated with neuropathic pain, for example, studies indicate that 29-34% of OA patients and 31-36% of RA patients experience joint pain with neuropathic qualities (Hochman et al., 2010; Perrot et al., 2013; Oteo-Álvarez et al., 2015; Akkar et al., 2016). Several mechanisms have been proposed to be responsible for the development of neuropathic pain such as peripheral sensitization due to persistent inflammation, neuronal plasticity changes, upregulation of ion channel expression and central sensitization.

As mentioned above, various inflammatory mediators possess the capacity to directly sensitize and activate the nociceptive neurons which can then generate a bombardment of nerve impulses. However, in diseases that are associated with chronic inflammation (e.g. rheumatoid arthritis, osteoarthritis and diabetes) persistent stimulation of nociceptive neurons is possible by inflammatory mediators producing a direct injury to the nerve (Milligan et al., 2003; Schafer et al., 2003; Cohen and Mao, 2014). Additionally, it is possible that increased input from the periphery due to persistent

inflammation induces plasticity changes in spinal neurons (Bridges et al., 2001). Changes in neuronal plasticity involve hypersensitization of neurons that are present in central pain pathways which results in increased responsiveness of neurons; these changes contribute to the maintenance of the pain response (Woolf and Salter, 2000).

Voltage-gated sodium channels (VGSCs) play a crucial role in the conduction of action potentials from the axons of primary sensory neurons (Xie, 2007). Some of the recent studies have indicated a role of  $\text{Na}_v1.7$  and 1.8 in the development of neuropathic pain (Gold et al., 2003; Jarvis et al., 2007; Hoyt et al., 2007). These studies show that blockade of VGSCs result in the attenuation of spontaneous and electrically evoked action potentials (Jarvis et al., 2007; Hoyt et al., 2007). This means that  $\text{Na}_v1.7$  and 1.8 channels can contribute to the ectopic discharge and spontaneous pain which are important characteristics of neuropathic pain (Levinson et al., 2007). Interestingly, it has been shown that  $\text{Na}_v1.8$  are involved in OA pain transmission (Schuelert and McDougall, 2012). Additionally, voltage-gated  $\text{Ca}^{2+}$  Channels (VGCCs) have been implicated in neuropathic pain (Levinson et al., 2007). VGCCs upon activation promote the release of SP, CGRP and glutamate which act on their respective receptors present on postsynaptic membrane resulting in excitation of sensory neurons in the dorsal horn (Xie, 2007). It has been observed that the expression of VGCCs (N-type) is increased in primary afferent neurons and dorsal horn neurons due to nerve injury which can lead to increased neuronal hyperexcitability due to increased intracellular calcium (Yaksh, 2006).

Sprouting of nerve fibres has been suggested to be another mechanism for the development of neuropathic pain (Bridges et al., 2001). Anatomically, nerve fibres have their central terminals at specific locations in the dorsal horn; specifically, laminae I and

II contain terminals for A $\delta$  and C-fibres, and laminae III and IV contain terminals for A $\beta$ -fibres (Woolf et al., 1992). It has been shown that after peripheral nerve injury, A $\beta$ -fibres sprout into laminae II and this anatomical re-organization could result in generation of abnormal pain signals (Woolf et al., 1992).

Microglia are the macrophages of the central nervous system and they have been shown to play an important role in the development of neuropathic pain (Mika et al., 2013). Activation of glial cells in dorsal root ganglia and the spinal cord results in the generation of a neuro-immune response which involves the release of a variety of pro-inflammatory cytokines such as TNF- $\alpha$ , IFN- $\gamma$ , TGF- $\beta$ , IL-1 $\alpha$  and IL-1 $\beta$  which can induce hyperexcitability in neurons (Mika et al., 2013).

## **1.7 Proteinases**

Proteinases (or alternatively called proteases) are a large group of proteolytic enzymes which possess the ability to hydrolyze the peptide bond (proteolysis) present in polypeptides and protein structures (Puente et al., 2003). Proteinases are classified into five different classes based on catalytic mechanism including aspartic-, cysteine-, serine-, threonine-proteases and metalloproteases (Puente et al., 2003). The cysteine-, serine- and metalloproteases represent the major class of proteinases because each class has more than 140 human encoding genes. The aspartic and threonine-proteases represent the minor class with each class having a little more than 20 genes (Puente et al., 2003). Human genome studies have shown that proteinase and proteinase inhibitor related genes collectively constitute more than 2% of the total human genome which implies their

ability to contribute to a variety of physiological processes (Puente et al., 2005). Indeed, studies have confirmed involvement of proteinases in different physiological processes (e.g. blood coagulation, bone formation, cell growth and fertilization) and pathophysiological conditions (e.g. inflammation, arthritis, immune-related, cardiovascular, pain and neurodegenerative) as well (Barrett et al., 2004; Puente et al., 2003; Muley et al., 2016; Ramachandran et al., 2016). It should be noted that under normal homeostatic condition the activity of proteinases is tightly regulated by a variety of proteinase inhibitors (Table 1.4). Proteinases, due to their ability to cause proteolysis, were initially thought to be involved in the breakdown of dietary proteins. Therefore, some of the early research work done using proteinases has placed more emphasis on their catalytic abilities. However, a study published in 1954 by German scientists had identified a hypotensive peptide substance in human urine samples which possessed the ability to cause contractions in uterine smooth muscles; this finding indicated the ability of proteinases to generate kinins from precursor molecules by proteolysis which can then contribute to an inflammatory cascade (Werle and Erdos, 1954). Thereafter, many studies have shown important contribution of proteinases in activation of coagulation, kinin and the complement system (Hollenberg et al., 2011). Following activation of these systems, the inflammatory cells arrive at the site and release more proteinases which further amplifies the inflammatory response (Ramachandran et al., 2016). Additionally, studies published by Rieser showed insulin-like actions of the proteinase trypsin which involved stimulation of glucose oxidation, glycogen formation and lipid breakdown in a rat diaphragm preparation and isolated fat cells (Rieser and Rieser, 1964; Rieser, 1967). Around the same time, other groups showed the ability of thrombin, a serine proteinase,

to regulate the function of platelets (Davey and Lüscher, 1967; Ganguly, 1974). The mechanism of action for these ‘hormone-like’ effects by proteinases is still not clear. However, it was assumed that proteinases may have been engaging some type of ‘receptor’ on target tissue to elicit these pharmacological effects. In 1991, two different laboratories discovered the proteinase-activated receptor (PAR) which was shown to be involved in mediating actions of thrombin proteinase on platelets (Rasmussen et al., 1991; Vu et al., 1991).

**Table 1.4 Proteinases and their inhibitors**

<b>Proteinase class</b>	<b>Proteinase inhibitor</b>
Serine proteinases	SERPINS (serine proteinase inhibitors)
Metalloproteinases (MMPs)	Tissue inhibitor of metalloproteinase (TIMPs 1, 2, 3, and 4)
Cysteine proteinases	Cystatins, thyropins
General	Alpha 2-macroglobulin

Adapted from (Ramachandran et al., 2016)

### **1.7.1 Serine proteinase**

Serine proteinases constitute one-third of all proteolytic enzymes (Hedstrom, 2002). Hence, these abundantly found proteolytic enzymes have been implicated in different physiological and pathophysiological processes (Cera, 2009). These proteolytic

enzymes are named ‘serine proteinases’ because they contain a nucleophilic serine in the enzyme active site, which can donate an electron to carbonyl moiety of the substrate peptide bond to form an acyl-enzyme intermediate which is subsequently hydrolyzed. The ability of serine to donate an electron depends on a three-amino acid residue (Asp-His-Ser) which is commonly referred to as ‘charge relay station’ or ‘catalytic triad’ (Cera, 2009). A variety of inflammatory cells secrete serine proteinases; for example, neutrophils secrete neutrophil elastase, proteinase 3 and cathepsin G, and mast cells secrete trypsin and tryptase (Korkmaz et al., 2010).

#### **1.7.1.1 Neutrophil elastase**

In 1906, Eugene Opie in his seminal paper studied an enzyme which existed in bone marrow; the enzyme was active in a neutral or alkaline medium and it could digest protein. He then proposed that since the polymorphonuclear neutrophils and the enzyme are formed in bone marrow, the enzyme should possibly be referred to as ‘leukocyte-protease’ (Opie, 1906). In 1968, Janoff and Scherer investigated the activity of the same neutral proteinase which is released by human neutrophilic neutrophils against elastin. They observed that the proteinase possessed elastinolytic activity and hence they started to refer to the proteinase as ‘leukocyte-elastase’ (Janoff and Scherer, 1968). The serine proteinase neutrophil elastase exists in azurophilic granules of neutrophils and participates in a non-oxidative phagocytosis of pathogens (Korkmaz et al., 2010). It should be noted that pancreatic acinar cells also produce a type of elastase which is involved in protein digestion and used as a fecal biomarker for assessing pancreatic exocrine insufficiency (Lindkvist, 2013). Interestingly, human neutrophil elastase which

consists of 218 amino acid sequence shares 43% homology with pancreatic elastase (Sinha et al., 1987). Other inflammatory cells such as eosinophils, monocytes and macrophages could release small quantities of elastase which is similar to neutrophil elastase (Lungarella et al., 1992; Owen et al., 1994; Dollery et al., 2003). Studies indicate that neutrophil elastase is involved in a variety of chronic inflammatory diseases such as arthritis, acute respiratory distress syndrome, chronic obstructive pulmonary disease, and inflammatory bowel disease (Gouni-Berthold et al., 1999; Ohbayashi, 2002; Korkmaz et al., 2010; Wright et al., 2014).

#### **1.7.1.2 Role of neutrophil elastase in arthritis**

Studies indicate that neutrophils (source of neutrophil elastase) are increased in the synovial fluid of patients with arthritis (Bender et al., 1986; McInnes and Schett, 2011, Abd-El-Hafez et al., 2014; Wright et al., 2014). A study published in 1997 assessed the levels of neutrophil elastase in the synovial fluid of patients with RA and OA. The study found that the levels of neutrophil elastase were increased in both types of patients, however, RA patients showed higher levels than OA patients (Momohara et al., 1997). Neutrophil elastase, upon release by neutrophils, can contribute to the development of arthritis due to its proteolytic and pro-inflammatory properties. Neutrophil elastase has been shown to possess substrate specificity towards various components of the basement membrane and connective tissue such as chondromucoprotein, elastin, types I-IV collagen, laminin, fibronectin and sulfated proteoglycan (Oronsky and Perper, 1975; Pipoly and Crouch, 1987; Watanabe et al., 1990). These properties allow neutrophil elastase to cause articular oedema, initiate degradation of the extracellular matrix and

cause articular damage. Furthermore, neutrophil elastase can contribute to some of the important pathophysiological events that take place during arthritis including leukocyte transmigration, increase in the cytokine, and chemokine expression. Neutrophil elastase plays an important role in vascular injury and leukocyte transmigration due to its ability to cause damage to the endothelium (Smedly et al., 1986) and cleave cell adhesion molecules (Champagne et al., 1998; Levesque et al., 2001). Additionally, there is evidence which suggests that neutrophil elastase can modulate the expression of cytokines TNF- $\alpha$ , MIP-2, IL-6 and IL-36 and this effect is mediated, in part, by Toll-like receptor 4 (Benabid et al., 2012; Henry et al., 2016). Annexin A1 (or also known as lipocortin 1) due to its anti-inflammatory and pro-resolution properties has been suggested to be useful for the treatment of various inflammatory conditions including arthritis (Perretti and D'Acquisto, 2009). A study has shown that neutrophil elastase has the capacity to cause cleavage at N-terminal of Annexin A1 and render the protein inactive (Vago et al., 2016). Therefore, it is possible that neutrophil elastase inhibition would be beneficial as it would promote pro-resolution of inflammation in the context of arthritis. In addition to the above-mentioned actions, neutrophil elastase has been shown to activate a cell surface receptor PAR2 (Ramachandran et al., 2011). The activation of this G-protein coupled receptor elicits a range of pro-inflammatory effects and has been shown to be involved in the development of experimental models of arthritis (Russell and McDougall, 2009).

### **1.7.1.3 Role of neutrophil elastase in pain**

The studies available looking at the role of neutrophil elastase in the development of pain are scarce. A study by Zhao et al. (2015) has assessed proinflammatory and pronociceptive effects of neutrophil elastase in mice. The study observed that intraplantar injection of neutrophil elastase produced inflammation and mechanical hyperalgesia in wild-type mice but not in PAR2<sup>-/-</sup> or TRPV4<sup>-/-</sup> mice. The finding suggests that neutrophil elastase produces its effects in a PAR2 and TRPV4-dependent manner (Zhao et al., 2015). Another study looked at involvement of neutrophil elastase in the development of neuropathic pain. It was observed that mice lacking serpinA3N, a neutrophil elastase inhibitor, developed more neuropathic pain than their wild-type counterparts. The pain response in wild-type animals could be reduced by administration of serpinA3N. Further experiments within the same study showed that infiltrating T cells into the DRGs release neutrophil elastase in nerve injured animals. Moreover, the pain response due to nerve injury in neutrophil elastase knockout mice or in wild-type mice treated with neutrophil elastase inhibitor (sivelestat) was prevented suggesting a contribution of neutrophil elastase to the development of neuropathic pain (Vicuna et al., 2015).

### **1.7.1.4 Neutrophil elastase inhibitors**

Although there are many neutrophil elastase inhibitors available including endogenous and synthetic inhibitors, for our experiments two different inhibitors, sivelestat and serpinA1, were used.

Sivelestat is a potent synthetic inhibitor of neutrophil elastase with an IC<sub>50</sub> of 0.044 μM (Kawabata et al., 1991). Sivelestat has a short half-life of 2 hours (Yoshikawa et al., 2013). It has been suggested that sivelestat inhibits neutrophil elastase by a reversible ‘acylation–deacylation’ mechanism (Nakayama et al., 2002). Several studies published in the literature which use sivelestat have shown that the drug possesses an anti-inflammatory and anti-arthritic activity (Kakimoto et al., 1995; Hagiwara et al., 2009). The drug is produced by Ono pharmaceuticals (ONO 5046) and has been approved for the treatment of acute respiratory distress syndrome in Japan (Iwata et al., 2010).

SerpinA1 (also known as alpha-1 antitrypsin or alpha-1 proteinase inhibitor) is a member of the serpin (serine proteinase inhibitor) family of proteinase inhibitors (Janciauskiene et al., 2011). It is a plasma protein which inhibits neutrophil elastase irreversibly by forming an enzyme/inhibitor complex (Korkmaz et al., 2010). It can inhibit some other serine proteinases including trypsin, chymotrypsin, proteinase 3, cathepsin G (Korkmaz et al., 2010). SerpinA1 has a half-life of 15.5 hours in mice (Lamontagne et al., 1981). SerpinA1, by directly inhibiting neutrophil elastase and other proteinases, can reduce inflammation associated with different conditions such as pulmonary emphysema, RA and gut ischemia reperfusion (Janoff, 1972; Gadek et al., 1981; Korkmaz et al., 2010; Janciauskiene et al., 2011; Bergin et al., 2012).

## **1.8 Proteinase-activated receptors**

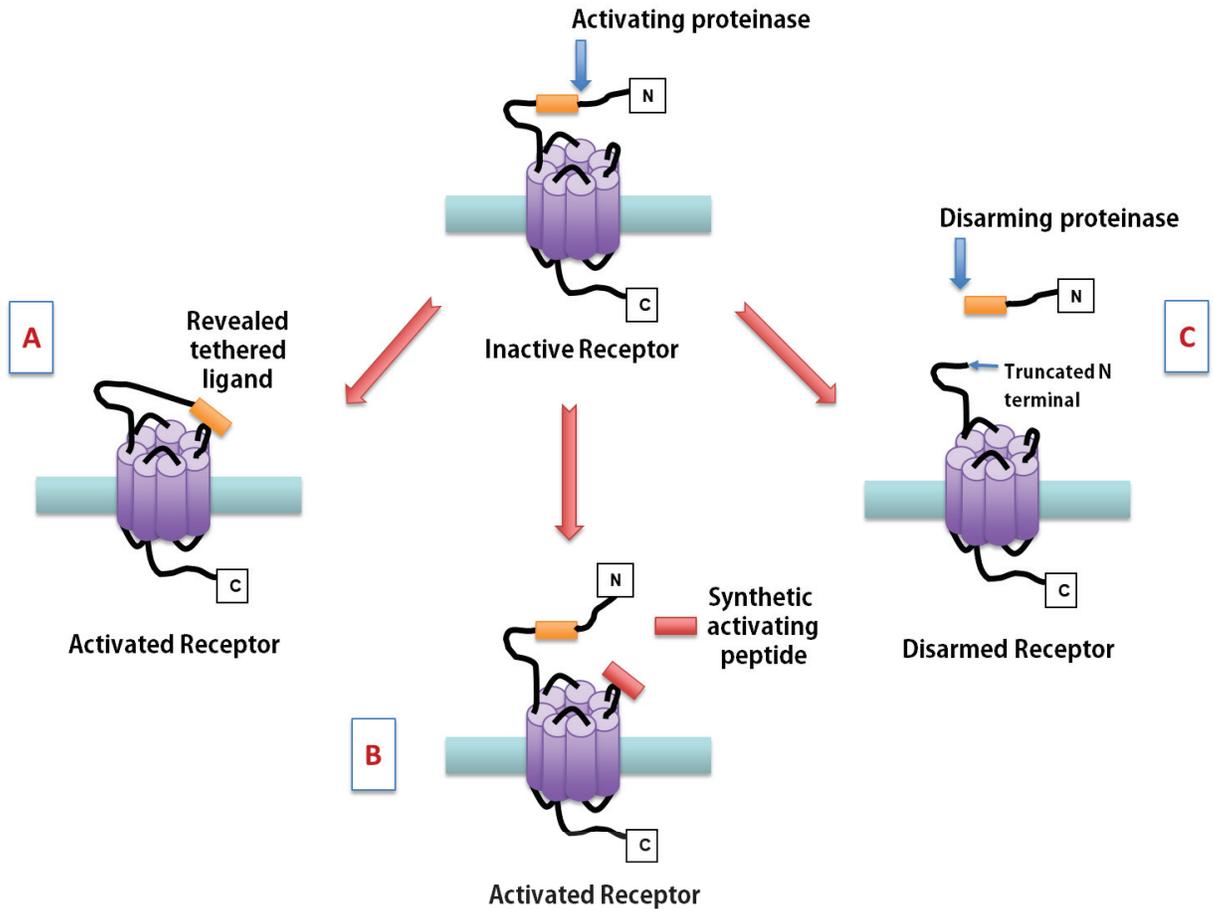
Proteinase-activated receptors are a family of G-protein coupled receptors consisting of four members (PAR1-PAR4) (Macfarlane et al., 2001). In 1991, two different groups cloned and identified PAR1, a receptor that is involved in mediating thrombin-induced platelet aggregation (Rasmussen et al., 1991; Vu et al., 1991). Subsequently, the second member of the family PAR2 was cloned and identified (Nystedt et al., 1994; Bohm et al., 1996). PAR2 could be activated by different serine proteinases such as trypsin but not thrombin. Finally, the last two members of the family (PAR3 and PAR4) which could also be activated by thrombin were cloned and identified (Ishihara et al., 1997; Xu et al., 1998). The study published by Vu et al. (1991) first described the unique mechanism by which PARs are being activated. These receptors can be activated by proteinases leading to downstream signalling.

### **1.8.1 Activation of PARs**

The activation mechanism for PARs is unique in nature as the activating ligand is present within the extracellular domain of the receptor. Proteinases cleave an amino-terminal in the extracellular domain of the receptor which leads to unmasking of the new amino-terminal, referred to as a tethered ligand. The tethered ligand folds and intramolecularly binds to the second extracellular loop leading to the activation of the receptor (Vu et al., 1991) (Figure 1.5A). For research purposes, scientists have designed synthetic ligands using the sequence of newly exposed amino-terminal (Figure 1.5B) and specific antagonists for each receptor which helped understand the role of PARs in

different physiological and pathophysiological responses (Table 1.5). It is interesting to note that the composition of N-terminal present on the extracellular domain of the receptor could impact the ability of proteinases to activate the receptor. This observation is exemplified by the experiments performed by Bouton et al. (1995) in which it was observed that PAR1 contains a thrombin cleavage site in the extracellular domain; at the carboxy-terminal of the cleavage site a sequence DKYEPFWEDEE is present which resembles the sequence found in the leech anticoagulant hirudin; this sequence binds to thrombin's fibrinogen-binding exosite and this interaction allows thrombin to activate PAR1 at a low concentration (Coughlin, 1998). Such hirudin-like domain is also observed in the case of PAR3. PAR4, however, does not contain a hirudin-like domain which makes them a low affinity receptor for thrombin and therefore relatively higher concentrations are required for thrombin to activate PAR4 (Coughlin, 1998). Another interesting observation to note about PARs is that PAR3 can act as a co-factor and facilitate thrombin-mediated PAR4 activation (Nakanishi-Matsui et al., 2000). In a series of experiments, it was observed that deletion of the PAR3 gene or inhibition of thrombin binding to PAR3 was sufficient to inhibit the activation of mouse platelets only at a low concentration of thrombin, however, at a high concentration platelets were activated. It should be noted that PAR4 is also expressed on mouse platelets and is activated in response to a high concentration of thrombin which means PAR4 mediates thrombin-induced activation of mouse platelets in the absence of PAR3. Furthermore, overexpression of PAR3 did not induce thrombin signaling. However, the coexpression of thrombin and PAR3 resulted in an increase in the potency of thrombin signaling to PAR4 by 6-15-fold (Nakanishi-Matsui et al., 2000). Experimental observations from this

study instituted the concept of cofactor assisted PAR activation in which one GPCR serves as an accessory molecule for a protease to facilitate the activation of another GPCR that is present within the same cell.



**Figure 1.5 Schematic representation of proteinase-activated receptor (PAR) activation and disarming.** (A) Activating proteinases cleave and unmask tethered ligand (orange box) which binds to the extracellular loop leading to activation of PAR signaling. (B) Synthetic activating peptide (red box) causes activation of PAR by binding to the extracellular loop. (C) Disarming proteinases cleave downstream to the activation site leading to disarming of PAR signaling. (Adapted from McDougall and Muley, 2015; Appendix III – copyright permission).

**Table 1.5 PAR-activating proteinases, synthetic peptides and antagonists**

<b>Proteinase-activated receptor (PAR)</b>	<b>Activating proteinase</b>	<b>Activating peptide</b>	<b>PAR antagonist</b>
PAR1	Thrombin FXa Trypsin Granzyme A MMP-1 Gingipains-R	TFLLR SFLLR	FR-171113 RWJ-56110 RWJ-58259 BMS-200261 SCH-79797
PAR2	Trypsin Tryptase Factor VIIa Factor Xa Neutrophil elastase Granzyme A Matriptase Acrosien Gingipains-R Proteinase 3 Thermolysin Serralysin Duodenase TMPRSS2	SLIGKV SLIGRL FLIGRL	FSLRLY-NH2 GB-83 GB-88 ENMD-1068 AZ8838 AZ3451
PAR3	Thrombin Factor Xa	None	None
PAR4	Thrombin Trypsin Plasmin Bacterial gingipains Cathepsin G Factor VIIa Factor X	GYPGQV AYPGKF	Pepducin P4pal-10 tcY-NH2 YD-3

Adapted from (McDougall and Muley, 2015) and expanded from (Vergnolle, 2009) and (Cheng et al., 2017)

### **1.8.2 Disarming of PARs**

A study published in 1997 noted that preincubation of endothelial cells with neutrophils released proteinases such as human leukocyte elastase, cathepsin G, and proteinase 3 prevented thrombin-induced cytosolic calcium mobilization and prostacyclin synthesis. Interestingly, this effect was not observed when a synthetic ligand which corresponded to a thrombin cleaved sequence was applied (Renesto et al., 1997). These findings were hinting at the possibility of disarming the receptor which would lead to removal of a cleavage site from the extracellular domain making thrombin unable to activate the receptor (Figure 1.5C). A similar observation was published by Chignard and Pidard, (2006) where they observed that neutrophil-released proteinases disarmed PAR2 on respiratory epithelial cells. In 2011, a study showed that neutrophil elastase, cathepsin G, and proteinase 3 cleave PAR2 at a site downstream to the cleavage site for trypsin leading to inhibition of trypsin-induced Gq-coupled PAR2 calcium signaling. Interestingly, elastase alone triggered PAR2-mediated activation of the p44/42 MAPK pathway in a biased manner without causing the release of intracellular calcium (Ramachandran et al., 2011).

### **1.8.3 Downregulation and internalization of PARs**

The activation of PARs by proteinases is irreversible given that proteinases expose a tethered ligand by causing proteolytic cleavage in the exodomain of the receptor. Two events mainly occur following PAR signaling. First, downregulation of PARs which involves phosphorylation of the receptor by GPCR kinases (GRKs),

$\beta$ -arrestin interaction, uncoupling of GPCR and the G protein, and endocytosis. Second, it has been suggested that once these receptors are internalized they are dephosphorylated by protein phosphatases and either sorted for recycling and reinsertion into the membrane or subjected to complete degradation by lysosomal enzymes (Vergnolle, 2009; Ramachandran et al., 2012). For PAR1, it has been suggested that  $\beta$ -arrestin-1 can regulate desensitization, however, it is not essential for internalization and downregulation of the receptor (Paing et al., 2001). Other studies indicate that PAR1 is internalized in a dynamin- and clathrin-dependent manner (Hoxie et al., 1993; Trejo et al., 2000). Studies suggest that for PAR2, interaction with both  $\beta$ -arrestin 1 and  $\beta$ -arrestin 2 is required for desensitization, internalization and transfer to lysosomes (Déry et al., 1999; Kumar et al., 2007). Some studies have shown that  $\beta$ -arrestin 2 can regulate PAR4 signaling in platelets (Li et al., 2011) and this signaling terminates slowly as compared to PAR1 signaling (Shapiro et al., 2000). More studies are required to address how PAR3 and PAR4 signaling are regulated.

#### **1.8.4 Role of PAR2 in arthritis**

The role of PAR2 in the development of arthritis has been investigated using a variety of preclinical models. In 2003, William Ferrell and colleagues carried out a detailed investigation in which they observed an increase in the expression of PAR2 in synovium and other periarticular tissues of adjuvant arthritic animals (Ferrell et al., 2003). PAR2 knockout mice prevented joint swelling and histopathological damage due to arthritis compared to their wild-type counterparts. Administration of PAR2 activating peptide produced a robust inflammatory response (Ferrell et al., 2003). These findings

were supported by other investigations where it was found that joint inflammation associated with acute monoarthritis could be prevented by blocking PAR2 using small interfering RNA, anti-PAR2 antibody or a PAR2 antagonist (ENMD-1068) (Kelso et al., 2006). Later, Lohman et al. (2012) showed that blockade of PAR2 has a disease modifying effect in a collagen-induced RA model. The study observed inhibition of inflammatory cell infiltration, mast cell degranulation and an improvement in histopathological changes after treatment with a potent PAR2 antagonist (GB88) (Lohman et al., 2012). These studies firmly suggested that PAR2 is expressed on different joint structures and has the capacity to mediate joint inflammation associated with arthritis. Studies involving the use of human tissue showed that PAR2 is expressed on synovium and chondrocytes of patients with OA and this expression is upregulated or down-regulated in response to pro- or anti-inflammatory cytokines respectively (Xiang et al., 2006). Another study found that the release of pro-inflammatory cytokines from RA synovium could be blocked by a PAR2 antagonist (Kelso et al., 2007). These studies corroborated preclinical observations and suggested prevention of cytokine release as one of the mechanism for an anti-inflammatory effect observed after blockade of PAR2. When a study compared the expression of PAR2 in the synovium obtained from RA and OA patients, it was observed that the expression was higher in RA than in OA patients (Nakano et al., 2007), however, another study has found that even though the expression of PAR2 is differential in both type of arthritic cohorts it can still be correlated with the degree of synovitis. Blockade of PAR2 reduced levels of pro-inflammatory cytokine in both cohorts (Tindell et al., 2012). A set of recent studies addressing the role of PAR2 in surgery models of posttraumatic OA has found that the deficiency of PAR2 results in

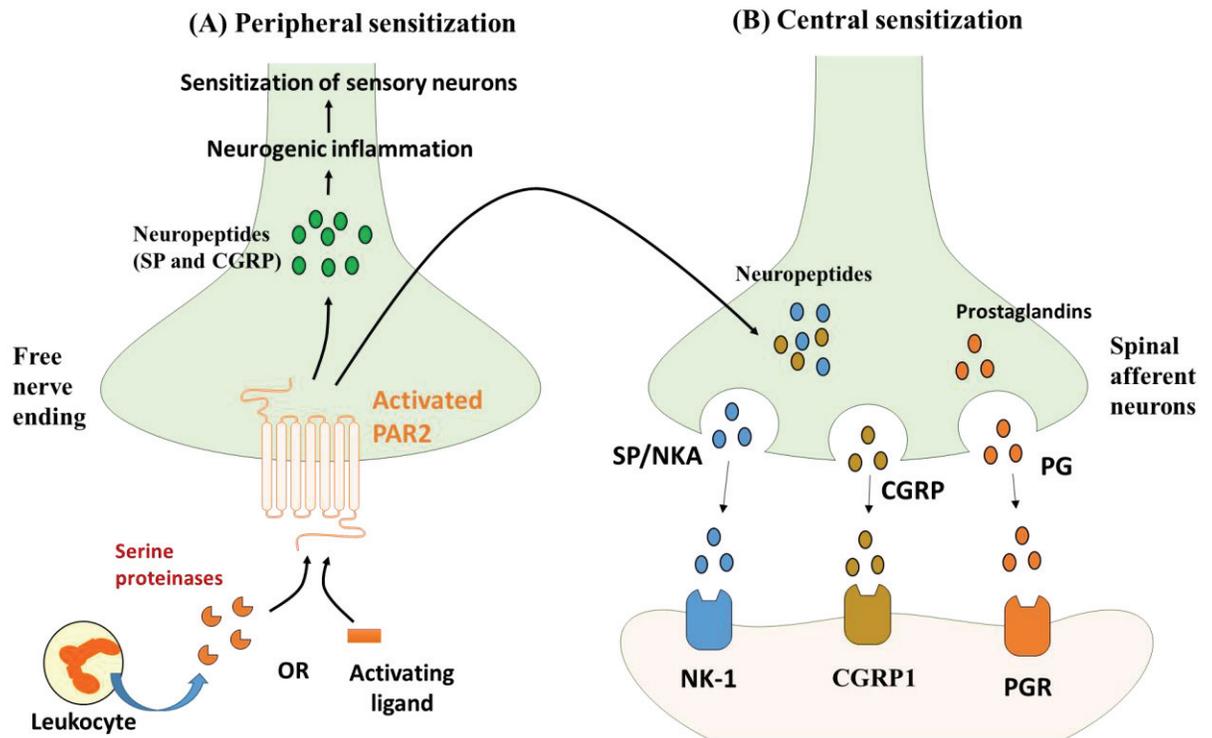
prevention of chondrocyte differentiation, focal cartilage lesions, erosion of cartilage, osteophyte formation, synovial macrophage activation and the pain response associated with OA (Ferrell et al., 2010; Jackson et al., 2014; Huesa et al., 2016).

### **1.8.5 Role of PAR2 in pain**

Sensory nerves, by releasing neuropeptides such as SP and CGRP, mediate inflammation and the pain response. Interestingly, an investigation by Steinhoff et al. (2000) has identified the expression of PAR2 at the plasma membrane and within intracellular locations of L1–L6 DRG neurons. Furthermore, the study observed co-localisation of PAR2 with SP and CGRP in approximately 60% of DRG neurons. PAR2 activating serine proteinases (trypsin and tryptase) and synthetic peptide agonists were able to directly signal the DRG neurons promoting the release of SP and CGRP from nerve fibres in peripheral tissues and in the spinal cord (Steinhoff et al. 2000). Also, the oedema that was produced in response to a PAR2 agonist could be blocked by pharmacological antagonists of SP and CGRP, and by removal of spinal C-fibres using capsaicin treatment (Steinhoff et al. 2000). These findings instituted a role of PAR2 in mediating neurogenic inflammation. The next logical query was to see whether PAR2 is capable of mediating a pain response. A study by Vergnolle et al. (2001) addressed this query and through a series of experiments showed that activation of PAR2 on nociceptive primary afferent neurons can result in a hyperalgesic response and involves modulation of central neurokinin-1 receptor (NK-1) and the release of PGs (Figure 1.6). The study used sub-inflammatory doses of PAR2 agonists which after intraplantar injection produced mechanical and thermal hyperalgesia. The sub-inflammatory doses of agonists

were used to ensure that the hyperalgesic response observed was due to administration of PAR2 agonist and not a secondary effect associated with inflammation.

Immunohistochemistry data showed increased spinal Fos protein expression at the L4 & L5 level (Vergnolle et al., 2001). Interestingly, peripheral blockade of NK-1 did not reverse the changes, however, administration of centrally acting NK-1 receptor antagonists blocked the pain response. Likewise, NK-1 knockout mice also prevented PAR2 agonist-induced hyperalgesia. Subcutaneous, but not intraplantar, injection of COX inhibitor indomethacin blocked hyperalgesic response (Vergnolle et al., 2001). In joints, PAR2 is expressed on knee DRG L3-L5 neuronal cell bodies (Russell et al., 2012). Activation of PAR2 results in neuronal sensitization, increased leukocyte trafficking, cytokine release, joint pain and secondary allodynia which appear to be mediated by activation of TRPV1 and NK-1 receptors (Helyes et al., 2010; Russell et al., 2012). A few other studies have shown a functional link between PAR2 and TRPV4 which can result in sustained inflammation and pain (Grant et al., 2007; Poole et al., 2013). It appears, therefore, that PAR2 contributes to peripheral and central sensitization which could result in pain hypersensitivity. The role of PAR2 has also been investigated in the development of neuropathic pain. PAR2 is involved in the development of chemotherapeutic agent-induced neuropathy, for example, paclitaxel and oxaliplatin (Chen et al., 2011; Chen et al., 2015). It has been shown that the activation of PAR2, which is activated by proteases that are released in response to administration of chemotherapeutic agent, causes the sensitization of TRPV1, TRPV4 and TRPA1, and involves the release of neuropeptides. These events lead to mechanical and thermal hyperalgesia in animals (Chen et al., 2011; Chen et al., 2015).



**Figure 1.6 PAR2 activation on afferent neurons contributes to peripheral and central sensitization. (A)** Serine proteinases released by leukocytes or synthetic ligands activate PAR2 and sensitizes sensory nerve endings causing release of neuropeptides SP and CGRP resulting in neurogenic inflammation. **(B)** Increased input from periphery triggers release of tachykinins SP, neurokinin A (NKA), CGRP and prostaglandins (PGs) from spinal afferent neurons contributing to central sensitization.

## **1.9 Models of experimental knee arthritis**

### **1.9.1 Kaolin-carrageenan-induced acute monoarthritis**

This model involves an intra-articular injection of two substances (kaolin and carrageenan) into the knee joint and is routinely used to study acute joint inflammation and pain in animals. Kaolin, a type of china clay consisting of hydrated aluminum silicate ( $\text{H}_2\text{Al}_2\text{Si}_2\text{O}_8 \cdot \text{H}_2\text{O}$ ), mainly causes mechanical damage to the cartilage and irritation to the synovial membrane after injection. Carrageenan (or  $\lambda$ -carrageenan type IV) is a sulfated polysaccharide and induces inflammation post-injection (Gardner, 1960). It has been shown that carrageenan causes activation of Toll-like receptor 4, which in turn, induces activation of NF- $\kappa$ B and IL-8 to promote inflammation (Bhattacharyya et al., 2008). The inflammatory response observed in this model appears within 1–3 h of injection, it peaks and plateaus around 5–6 h and resolves within 3 days (Neugebauer, 2007). The pain response also appears within 1-2 h, peaks around 24 h and within 2-3 days it resolves (Ren and Dubner, 1999). Inflammatory and pain related changes include formation of oedema, cellular infiltration, guarding of the leg, limping and decreased withdrawal threshold to von Frey filaments (Neugebauer, 2013). A variety of neurochemical changes observed in this model could potentially contribute to inflammation and pain. These neurochemical changes include an accumulation of prostaglandins ( $\text{E}_2$  and  $\text{I}_2$ ), neuropeptides (SP and CGRP), excitatory amino acid (glutamate), nitric oxide metabolites both peripherally and centrally to cause sensitization of neurons (Sluka and Westlund, 1993; Schaible and Grubb, 1993; Neugebauer and Li, 2003).

### **1.9.2 Freund's complete adjuvant (FCA)-induced chronic inflammatory monoarthritis**

This model involves an intra-articular injection of an emulsion which contains an antigen (*Mycobacterium tuberculosis* H37Ra strain, heat killed and dried) into the knee joint. Injection of FCA produces a robust inflammatory response which involves oedema, infiltration of inflammatory cells, and histopathological changes including cartilage damage, bone resorption and periosteal bone proliferation (Bendele, 2001). Interestingly, it has been shown that injection of FCA causes chronic articular hypoaemia, which is also a feature of RA (McDougall et al., 1995). A wide variety of inflammatory mediators have been implicated in adjuvant arthritic animals including nitric oxide, PGE<sub>2</sub>, LTB<sub>4</sub>, IFN $\gamma$ , IL-2, IL-12, TNF- $\alpha$ , MMP3 and substance P (Uematsu et al., 2011; Nisar et al., 2015). These mediators can contribute to the development of arthritic symptoms. Additionally, studies have shown that FCA injection inflicts joint inflammation and mechanical hyperalgesia that is mediated by TRPV1 and TRPA1 receptor (Keeble et al., 2005; Fernandes et al., 2011).

### **1.9.3 Monoiodoacetate (MIA)-induced experimental osteoarthritis**

MIA disrupts the glycolytic pathway by inhibiting an enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which is required for the breakdown of glucose for energy. Injection of MIA into the knee joint causes inhibition of the enzyme in chondrocytes leading to cell death (Kalbhen, 1987). Inflammatory changes occurring in this model include infiltration of inflammatory cells such as neutrophils, macrophages,

lymphocytes and plasma cells (Bove et al., 2003). It should be noted that inflammatory response observed in this model is transient and resolves within a week. Additionally, pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  are released by synovial cells and chondrocytes which promote the release of NGF in MIA injected animals (Orita et al., 2011). Furthermore, it has been suggested that injection of MIA triggers release of neuropeptides such as SP and CGRP from sensory fibres (Ferland et al., 2011). These inflammatory changes can induce joint inflammation, structural damage which is evident in histological analysis of MIA treated joints and include changes such as chondrocyte degeneration, collapse of bony trabeculae, increased osteoclastic activity and cartilage loss (Guzman et al., 2003). Additionally, MIA animals show neuropathy as indicated by an increase in the expression of nerve injury markers ATF-3 and growth associated protein 43 (GAP 43) in DRGs and the spinal cord (Ivanavicius et al., 2007; Orita et al., 2011; Thakur et al., 2012). Collectively, this model nicely represents many important aspects of human OA.

## **1.10 Assessment of joint inflammation and pain**

### **1.10.1 Joint inflammation**

#### **1.10.1.1 Joint oedema**

During inflammation, tissues become oedematous due to an accumulation of fluid in the interstitial or serous cavities. Typically, experimental knee joint arthritis is associated with oedema which essentially indicates joint inflammation. Using digital calipers the distance between the medial and lateral femoral condyles can be measured

which allows the experimenter to assess joint swelling. This measurement enables the experimenter to capture the severity of the disease over a time course with or without treatment. One of the limitations of this technique is that readings could differ between experimenters due to the amount of squeezing by the calipers applied to the knee joint and the ability to maintain a constant distance across multiple measurements.

#### **1.10.1.2 Leukocyte trafficking**

During inflammation leukocytes move from the blood circulation to the site of injury by engaging in transitory interactions with vascular endothelium. One technique that can visualise joint microcirculation and allow us to measure these interactions is intravital microscopy (IVM). A fluorescent dye, rhodamine 6G, is used to label leukocytes which are then visualised under intravital microscope. This dye is lipophilic in nature and stains leukocytes by accumulating within the mitochondria of the cells (Wang et al., 2005). The absorption spectrum for rhodamine 6G is between 440 nm and 570 nm with a peak observed at 530 nm (Brackmann, 1986). Leukocytes go through multiple steps before reaching the site of injury. Rolling and adhesion are important steps of the leukocyte extravasation cascade. Therefore, assessment of these properties can be used as a measure of inflammation.

#### **1.10.1.3 Synovial blood flow**

Inflammation can involve an increase in blood perfusion at the affected site. Measurement of synovial blood flow in arthritic animals can provide one measure for

assessing the degree of joint inflammation. Laser speckle contrast analysis (LASCA) technique can capture blood perfusion changes occurring in synovial microcirculation (Briers and Webster, 1996). When a coherent laser light is shone on an object, a portion of light is backscattered as a consequence of reflection. The backscattered light forms a ‘speckle pattern’ which consists of dark and bright areas (Briers and Webster, 1996). Speckle pattern can change based on the degree of movement that is present in an object. This means that shining a stable laser light on an object which is static (does not contain moving particles) produces an interference pattern that will not change temporally; however, if an object is dynamic (e.g. blood which contain cells that move constantly), the resulting interference pattern changes over time (Draijer et al., 2009). Therefore, the level of blurring obtained for dynamic objects differ and it can be quantified by the speckle contrast. The LASCA imaging system contains a CCD camera which captures speckle pattern and using a built-in software an image is processed and the speckle contrast can be calculated. The speckle contrast obtained from tissues *in vivo* can be correlated with blood flow and assigned an arbitrary perfusion unit (PU) (Briers and Webster, 1996; Draijer et al., 2009).

## **1.10.2 Joint pain**

### **1.10.2.1 von Frey hair algometry**

This technique utilizes von Frey hairs which are graded monofilaments with constant length and varying thickness. Each of these filaments exerts a specific bending force when applied to the plantar surface of a hindpaw of an animal. Naïve animals

typically respond to a filament with a higher bending force, however, inflamed or arthritic animals show a response to a lower bending force. This change in the hindpaw withdrawal threshold can be inferred as referred or secondary pain as it is experienced at a location other than site of injury (knee). Interestingly, the presence of referred pain has been noted in arthritis patients (Khan et al., 2004). Various changes occurring peripherally and centrally could contribute to an alteration in neuronal sensitivity that leads to an increase in sensitivity to von Frey filaments. Inflammation can cause a decrease in the excitation threshold of polymodal nociceptors which makes them excite on application of a non-noxious stimulus (Schaible and Grubb, 1993). Furthermore, inflammatory mediators can cause up-regulation of ion channel expression (e.g. TRPV1, VGSCs and VGCCs) and activation of silent nociceptors (Hunter et al., 2008) which can assist in the maintenance of the pain response (McCleskey and Gold, 1999; Caterina et al., 2000). Additional contributing factors include nerve injury which leads to ectopic firing, spinal hyperexcitability, inadequate descending inhibitory signals and plasticity changes resulting in broadening of the receptive field (Schaible, 2006). This technique allowed us to measure mechanical nociceptive threshold in arthritic animals with or without treatment.

#### **1.10.2.2 Dynamic weight bearing**

It has been noted that arthritic patients experience joint pain at rest (Schaible et al., 2002) and therefore it is important to study this ‘spontaneous pain’ in a preclinical setting as well. Dynamic weight bearing can measure spontaneous-like pain in freely-moving arthritic animals (Griffioen et al., 2015). This technique involves measurement of

changes in hindpaw weight distribution via a pressure sensitive mat inside a chamber. It should be noted that naïve animals distribute their weight uniformly on both hindlimbs, however, post-induction of monoarthritis animals prefer to put more weight on their uninflamed or non-injured hindlimb. This shift in weight distribution can be inferred as non-evoked or spontaneous-like pain. This technique has been shown to be effective in predicting pain in different animal models such as osteoarthritis, inflammatory, neuropathic and cancer pain (Tétreault et al., 2011; Quadros et al., 2015).

## **1.11 Objectives and hypotheses**

The objectives of this thesis were:

- 1) To study the effect of local administration of neutrophil elastase on joint inflammation and pain,
- 2) To assess the involvement of PAR2 in mediating neutrophil elastase-induced joint inflammation and pain, and
- 3) To study the role of endogenous neutrophil elastase and PAR2 in the development of knee joint arthritis using different animal models.

Sub-objectives and sub-hypotheses were later identified and have been explicitly stated in the individual results chapters.

**Our central hypothesis was that neutrophil elastase contributes to the development of joint inflammation and pain associated with experimental knee joint arthritis via activation of PAR2, and that blockade of neutrophil elastase or PAR2 would decrease joint inflammation and pain associated with experimental knee joint arthritis.**

## Chapter 2: Methods and Materials

### 2.1 Animals

All the experimental procedures described here were approved by the Dalhousie University Committee on Laboratory Animals, which complies with the guidelines drafted by the Canadian Council for Animal Care ([http:// www.ccac.ca/](http://www.ccac.ca/)). Approved Experimental Protocols: #14-056, #15-117 and #16-077.

Experiments were performed on male or female C57BL/6 mice (20-42g; 8–16 weeks old; Charles River Laboratories Inc., QC, Canada) or wild-type (PAR2<sup>+/+</sup>) and PAR2-deficient (PAR2<sup>-/-</sup>) mice raised in-house (original breeders developed on a C57BL/6 background from Jackson Laboratories, Bar Harbor, ME, USA).

Male Wistar rats (250-450g; 8–15 weeks old; Charles River Laboratories Inc., QC, Canada) were used for some of the experiments. Animals were housed in the Carleton Animal Care Facility (CACF) at Dalhousie University, Halifax, Canada. Following arrival at the facility, animals acclimated for at least 7 days. Cages were lined with woodchip bedding and animals were provided with environmental enrichment. Animals were maintained in a controlled environment with constant temperature (22°C), humidity (55–65%) and 12 h light:dark cycle (light-on from 7:00-19:00) and were provided with standard lab chow (Prolab RMH 3000, LabDiet, MO, USA) and water *ad libitum*. For reporting of the experiments ARRIVE guidelines were followed.

## **2.2 Animal models of arthritis**

### **2.2.1 Kaolin-carrageenan-induced acute monoarthritis**

Mice were anesthetized using isoflurane (2-4%; 100% oxygen at 1 L/min), and deep anesthesia was confirmed by the absence of a hindpaw withdrawal reflex before any procedures were performed. The right knee joint was shaved, swabbed with 70% alcohol, allowed to dry and the baseline knee joint diameter was measured using digital calipers (Control Company, Friendswood, TX, USA). Acute monoarthritis was induced by injecting kaolin (2%, 10  $\mu$ l) using a 30-gauge, 0.5-inch needle into the intra-articular space of the joint and the limb was flexed and extended for 10 min to disperse the substance throughout the joint. Injection of kaolin into the joint mainly causes irritation of the synovium and debridement of the cartilage. Next, carrageenan (2%, 10  $\mu$ l) was injected in the same manner and was followed by 30 s of hindlimb flexion and extension. Post-injection mice were returned to their home cages for 24 h, after which joint inflammation and pain were assessed.

### **2.2.2 Freund's complete adjuvant-induced chronic inflammatory monoarthritis**

This experiment was performed in male Wistar rats. Briefly, rats were anesthetized using isoflurane (2-4%; 100% oxygen at 1 L/min), and deep anesthesia was confirmed by failure to elicit hindpaw withdrawal reflex. Before injection, the right knee joint was shaved, swabbed with 70% alcohol, allowed to dry and the baseline knee joint diameter was measured using digital calipers (Control Company, Friendswood, TX, USA). Chronic inflammatory monoarthritis was induced by injection of Freund's complete adjuvant (50  $\mu$ l) using a 30-gauge, 0.5-inch needle into the intra-articular space

of the right knee joint. The hindlimb was flexed and extended for 30 s to ensure complete distribution of the substance within the joint space. The emulsion was vortexed just before injection into each rat. Post-injection, rats were returned to their home cages and joint inflammation and pain were assessed at several time points over a three-week time course.

### **2.2.3 Monoiodoacetate-induced experimental osteoarthritis**

Animals were deeply anesthetized using isoflurane (2-4%; 100% oxygen at 1 L/min), and a surgical plane of anaesthesia was confirmed by the absence of hindpaw withdrawal reflex. The right knee joint was shaved, swabbed with 70% alcohol, allowed to dry and the baseline knee joint diameter was measured using digital calipers (Control Company, Friendswood, TX, USA). Experimental OA was induced by injection of sodium monoiodoacetate (0.3 mg/10  $\mu$ l) using a 30-gauge, 0.5-inch needle directly into the right knee joint. Post-injection, mice were returned to their home cages, and joint inflammation and pain were assessed at several time points over a two-week time course.

## **2.3 Assessment of joint inflammation**

### **2.3.1 Knee diameter**

Joint swelling indicates presence of inflammation or distension by an inert chemical substance. Joint swelling was assessed by measuring knee diameter. For measurement of knee diameters, digital calipers (Control Company, Friendswood, TX, USA) were used. They were placed along the joint line in a horizontal plane of the knee

between the medial and lateral femoral condyles. Three readings were taken, and an average value was calculated.

## **2.3.2 Vascular assessments**

### **2.3.2.1 Surgical preparation**

Animals were anesthetized by an intraperitoneal injection of urethane (25% stock solution; 2 g/kg), and deep anesthesia was confirmed by the absence of the corneal reflex or the hindpaw withdrawal reflex before any surgical procedures were performed. Rats or mice were placed in a supine position on a heated blanket (SoftHeat HP710-24-3P-S Electric Heating Pad, Kaz Inc., Southborough, MA, USA) to maintain core body temperature at 37°C. An ocular lubricant (Lacri-Lube, Allergan, Inc., NJ, USA) was applied to protect the cornea of an anesthetized animal. Mineral oil was used to the neck region to lacquer the hair. A small longitudinal incision was made in the skin, and the trachea was exposed. The trachea was then cannulated using polyethylene tubing [(mouse - 0.76 mm internal diameter, 1.22 mm outer diameter; rat – 1.57 mm internal diameter, 2.08 mm outer diameter); Clay Adams, Parsippany, NJ, USA] to allow unrestricted breathing. Next, the mouse carotid artery and jugular vein were carefully isolated and cannulated with polyethylene tubing (0.28 mm internal diameter, 0.61 mm outer diameter; Clay Adams, Parsippany, NJ, USA) filled with heparinized saline (1 U/ml). Rat carotid artery (0.5 mm internal diameter, 1.0 mm outer diameter; Clay Adams, Parsippany, NJ, USA) and jugular vein (0.4 mm internal diameter, 0.8 mm outer diameter; Clay Adams, Parsippany, NJ, USA) were similarly cannulated. A pressure

transducer (Kent Scientific Corporation, Torrington, CT, USA) was connected in series to the carotid artery cannula to measure mean arterial pressure which was recorded on a differentially amplified blood pressure monitor (BP-1; World Precision Instruments, Sarasota, FL, USA). Lastly, a small piece of the skin (~1 cm long × ~0.5 cm wide) covering the knee joints and all superficial fasciae were removed to get a clear view of the joint microvasculature. To prevent tissue desiccation, the surface of the knee was perfused intermittently with warm (37°C) physiological buffer was prepared in-house (composition - 135 mM NaCl, 20 mM NaHCO<sub>3</sub>, 5 mM KCl, 1 mM MgSO<sub>4</sub>\*7H<sub>2</sub>O, pH =7.4).

### **2.3.2.2 Assessment of leukocyte trafficking using intra-vital microscopy (IVM)**

Leukocyte trafficking within the knee joint microvasculature was captured using intravital microscopy (IVM), as previously described (Andruski et al., 2008; Krustev et al., 2014; Muley et al., 2016) (Figure 2.1). After surgical preparation, the animal was placed under a Leica DM2500 microscope with a HCX APO L 20X objective and a HC Plan 10X eyepiece (Leica Microsystems Inc., Richmond Hill, ON, Canada; final magnification 200×) and the knee joint microvasculature was visualised under fluorescent light. For the staining of leukocytes, a fluorescent dye, rhodamine 6G (0.05%; mice - 0.06ml, rats – 0.12ml), was injected slowly through the jugular vein cannula immediately before measurement of leukocyte trafficking. After scanning the knee joint microvasculature, a straight, unbranched, postcapillary venule with a diameter between 20–50 µm was selected for analysis. A video camera (DFC3000G camera, Leica Microsystems Inc., Richmond Hill, ON, Canada) attached to the microscope was used to

record leukocyte activity over a 1-min duration. The video recordings were used for the assessment of two important leukocyte properties: (a) a rolling leukocyte refers to cells which begins to roll along after attaching to the activated venular endothelium, these leukocytes have a velocity less than other free flowing cells in the same vessel. (b) an adherent leukocyte refers to the leukocytes which hold tightly to the endothelial cell wall for 30 s or longer. Their number was assessed within a 100  $\mu\text{m}$  length of venule (Figure 2.2). Three different venules per knee joint were used and three videos were captured. The values over these three videos were averaged to give a single measurement per animal.

### **2.3.2.3 Assessment of blood perfusion using laser speckle contrast analysis (LASCA)**

Changes in the microvascular perfusion of rodent knee joints were studied using a laser speckle contrast analyser (LASCA – PeriCam PSI System, Perimed Inc., Ardmore, PA, USA), as previously described (Krustev et al., 2014; Muley et al., 2016) (Figure 2.3). This system uses a solid-state (70mW) laser source which directs laser light (wavelength: 785 nm) onto the knee joint microvasculature. A CCD camera is used to capture the speckle pattern which is converted into a measure of blood perfusion and assigned an arbitrary perfusion unit (PU). After placing an animal under the imager, a one-minute recording was taken at a working distance of 10 cm with a frame rate of 25 images per second (Figure 2.4 and 2.5). Lastly, the animal was sacrificed by anesthetic overdose (sodium pentobarbital; 1000 mg/kg i.p.) and a final scan was captured. This ‘dead scan’ was performed to record tissue optical noise and it serves as a ‘biological zero’ measurement. The dead scan value (typically 2-10% of mean perfusion) was subtracted

from all previous measurements for that animal to get a final mean perfusion value. Studies in which the resting mean arterial pressure differed between animals, vascular conductance was calculated using following equation,

$$\text{Vascular conductance} = \frac{\text{Mean blood perfusion}}{\text{Mean arterial pressure}}$$

#### **2.4 Assessment of proteolytic activity of neutrophil elastase in inflamed joints**

The proteolytic activity of serine proteinase neutrophil elastase was assessed in inflamed joints using the substrate Neutrophil Elastase 680 FAST (NE 680). The substrate NE680 is designed using a highly specific neutrophil elastase peptide sequence (PMAVVQSVP) which has two near-infrared fluorochromes attached to either end of the peptide sequence (Kossodo et al., 2011). The substrate is optically silent initially, but starts to fluoresce immediately upon proteolytic cleavage by neutrophil elastase (Figure 2.6) (Kossodo et al., 2011; Muley et al., 2016). Mice were anaesthetized (2–4% isoflurane; 100% oxygen at 1 L/min) and the hair was completely removed from both hind limbs. Animals were placed in a prone position in the imaging chamber of an In-Vivo Xtreme imaging system (Bruker Corporation, Billerica, MA, United States) and a baseline scan was recorded. Next, the substrate NE 680 (1nmol/25  $\mu$ l) was injected subcutaneously over both knee joints and animals were placed again in the imaging chamber. The substrate was then excited at 650 nm wavelength and the emitted fluorescence was captured at 700 nm wavelength with an exposure of 2.5s. During the scan, the field of view was maintained at 10 cm and the lens aperture (fSTOP) kept at 2.

Lastly, the resulting fluorescence images were analyzed using proprietary Bruker molecular imaging software (version 7.5.2.22464). Identical regions of interest were drawn around the inflamed (ipsilateral) and control (contralateral) knees and the fluorescence intensity was calculated. The fluorescence intensity of the control contralateral knee joint was subtracted from that of the inflamed ipsilateral knee joint to account for any release of neutrophil elastase caused by the injection of the substrate itself.

## **2.5 Assessment of joint pain**

### **2.5.1 von Frey hair algometry**

von Frey hair algometry was used to assess secondary allodynia in animals. The assessment involves an application of a set of calibrated von Frey hair filaments (Figure 2.7) to the plantar surface of the ipsilateral mouse or rat hindpaw using a modification of the Dixon's up-down method (Chaplan et al., 1994). Each of these filaments exerts a specific bending force when applied to the plantar surface. An elevated Plexiglas chamber was used (dimensions: 30cm long x 9cm wide x 24cm tall) which was positioned on a metal mesh flooring (Figure 2.8). Before measurement, animals were allowed to acclimate to the chamber for 15-20 min. until their exploratory or grooming behaviour ceased. A von Frey hair filament was inserted through the wire mesh and applied perpendicular to the plantar surface of the hindpaw of an animal (avoiding the toe pads) until the filament started to bend; the hair was held in this position for 3 s. Animal responses to filament application were carefully monitored. A positive response was

considered when a mouse showed prominent withdrawal, shaking or licking of the hindpaw after application of the von Frey hair filament. The next, lower filament was then applied after a positive response. However, if there was no response to the filament application, the next higher strength hair was applied until it hit a maximum cut-off level which in the case of a mouse is 4 g and rat is 15 g bending force. Four more measurements were made after the first difference in response was observed. The pattern of responses was converted to a 50% withdrawal threshold calculated using the following formula:  $10[X_f + k\delta]/10\ 000$ ; where  $X_f$  = value (in log units) of the final von Frey hair used,  $\delta$  = mean difference (in log units) between stimuli, and  $k$  = tabular value for the pattern of the last six positive/negative responses (shown below).

<b>von Frey filament Markings</b>	<b>Force (g)</b>	<b>Response</b>					
2.44	0.04						
2.83	0.07						
3.23	0.16	0					
3.61	0.4	0					
3.84	0.6	0					
4.08	1	0					
4.17	1.4	0					
4.31	2	0	X		X		X
4.56	4	0		0		0	

X= positive response  
0= no response

### 2.5.2 Dynamic incapacitance

This test essentially measures spontaneous pain in arthritic rats or mice based on changes in hindpaw weight-bearing. The dynamic weight-bearing apparatus (Bioseb, Vitrolles, France) consists of a Plexiglas chamber (mouse - 12cm long x 12cm wide x 20cm tall; rat – 22cm long x 22cm wide x 38cm tall) with a video camera

(DFK22AUC03 camera, ImagingSource, Charlotte, NC, USA) attached on the top and a pressure sensitive mat (Bioseb DWB Mouse or Rat Sensor Pad) placed at the bottom (Figure 2.9). The animals are kept in the chamber where they can move freely. Over a 4-5 min test period, hindlimb weight bearing was continuously monitored via the sensor pad and a video recording was made using camera which was used during analysis to recognize the orientation of the animal. Hindpaw weight distribution was calculated using Bioseb software (Version 1.4.2.92) and a percentage weight borne by the ipsilateral (arthritic) paw was calculated using following formula,

$$\% \text{ weight on ipsilateral paw} = \left( \frac{\text{Weight borne by the ipsilateral paw}}{\text{Weight borne by the ipsilateral paw} + \text{Weight borne by the contralateral paw}} \right) \times 100$$

## 2.6 Preparation of saphenous nerve

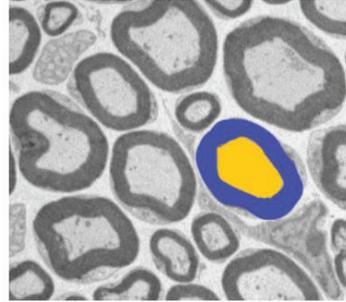
The collection of saphenous nerves and the calculation of the G-ratio for multiple nerve fibres was carried out as previously described (McDougall et al., 2017, 2017a). Before collecting saphenous nerves from the mice, they were euthanized by anesthetic overdose (sodium pentobarbital; 1000 mg/kg i.p.). The collected saphenous nerve samples were fixed using freshly diluted 2.5% glutaraldehyde in 0.1M sodium cacodylate for at least 24 hours. The fixed nerves were rinsed with 0.1M sodium cacodylate buffer for 3 times. The nerve samples were fixed again with 1% osmium tetroxide for 2 hours, washed with distilled water and kept in 0.25% uranyl acetate at 4<sup>0</sup>C overnight. Next, the nerve samples were dehydrated using a graduated series of acetone (50%, 70%, 95%,

100% acetone and lastly by 100% acetone beads). Nerve samples were subjected to infiltration with epon araldite resin (3:1 ratio of dried 100% acetone to resin for 3 hours, 1:3 ratio of dried 100% acetone to resin overnight, then 100% epon araldite resin 2 times for 3 hours). Samples were finally embedded in 100% epon araldite resin and placed in 60°C oven for 48 hours to cure. A Reichert – Jung Ultracut E Ultramicrotome with a diamond knife was used to cut thin (100nm) sections which were placed on copper grids. These samples were stained with 2% aqueous uranyl acetate for 10 minutes followed by two rinses with distilled water for 5 minutes. These rinsed samples were kept in lead citrate for 4 min and rinsed again with distilled water and left to air dry. The samples were viewed using a JEOL JEM 1230 Transmission Electron Microscope (JEOL, Japan) at 80 kV and one good quality image was captured per animal using a Hamamatsu ORCA-HR digital camera at 2500X magnification.

## **2.7 Assessment of G-ratio**

G-ratio, is a measure of myelin thickness and it was calculated using a plug-in from the ImageJ software (<https://imagej.nih.gov/ij/plugins/index.html>). First, the image was processed by applying a median filter to remove noise which allows better edge detection on an image. Next, for the measurement, the inner and outer part of the nerve fibre was outlined and the area was calculated. Finally, the G-ratio value was calculated using the following equation:

$$g = \sqrt{\frac{a}{A}}$$



where  $g$  is the G-ratio,  $a$  is the internal axon area, and  $A$  is the area of the whole fibre. All nerve fibres (36 to 113 per section) present in a captured image were measured and were averaged to give a mean G-ratio value for each animal.

## 2.8 Statistical analysis

All data are presented as means  $\pm$  SEM and were analysed with the statistical software package GraphPad Prism v.7.00 (Graph-Pad Software Inc., San Diego, CA, USA). The data were first tested for normal distribution using the Kolmogorov–Smirnov test. If all the groups under comparison passed the normality test, appropriate parametric statistical tests were used. However, if one or more groups failed to pass the normality test, then an appropriate non-parametric statistical test was used. As the statistical tests differed between each study, the precise test is described in the corresponding figure legend. For each comparison,  $n$  is equal to the number of animals in each experimental group.

## 2.9 Materials

**Table 2.1 List of Drugs**

Sr. No.	Drug	General Description	Source	Vehicle Used
1	GB83	<ul style="list-style-type: none"> <li>• PAR2 antagonist</li> <li>• N-((S)-3-cyclohexyl- 1-((2S,3S)-1-(2,3-dihydrospiro[indene-1,4'-piperidine]-1'-yl)- 3-methyl-1-oxopentan-2-ylamino)-1-oxopropan-2-yl) isoxazole-5-carboxamide)</li> </ul>	Axon Medchem (Groningen, The Netherlands)	DMSO: cremophor: saline (1:1:8)
2	Isoflurane	<ul style="list-style-type: none"> <li>• Inhalation Anesthetic</li> </ul>	Piramal Critical Care Inc (Bethlehem, PA, USA)	-
3	Neutrophil elastase	<ul style="list-style-type: none"> <li>• Serine proteinase</li> </ul>	Elastin Products (Owensville, MO, USA)	Saline
4	SerpinA1	<ul style="list-style-type: none"> <li>• Serine proteinase inhibitor</li> </ul>	Abcam, Inc. (Toronto, ON, Canada)	Saline
5	Sivelestat	<ul style="list-style-type: none"> <li>• Neutrophil elastase inhibitor</li> <li>• 4-[[[2-[[[(carboxymethyl)amino] carbonyl]phenyl]amino]sulfonyl] phenyl ester 2,2-dimethyl-propanoic acid, monosodium salt, tetrahydrate)</li> </ul>	Caymen Chemicals (Ann Arbor, MI, USA)	Saline
6	U0126	<ul style="list-style-type: none"> <li>• MAPK inhibitor</li> <li>• 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene)</li> </ul>	Caymen Chemicals (Ann Arbor, MI, USA)	DMSO: cremophor: saline (1:1:8)
7	Urethane	<ul style="list-style-type: none"> <li>• Anaesthetic</li> <li>• Ethyl carbamate</li> </ul>	Sigma Aldrich (St. Louis, Missouri, USA)	Saline

**Table 2.2 List of Reagents**

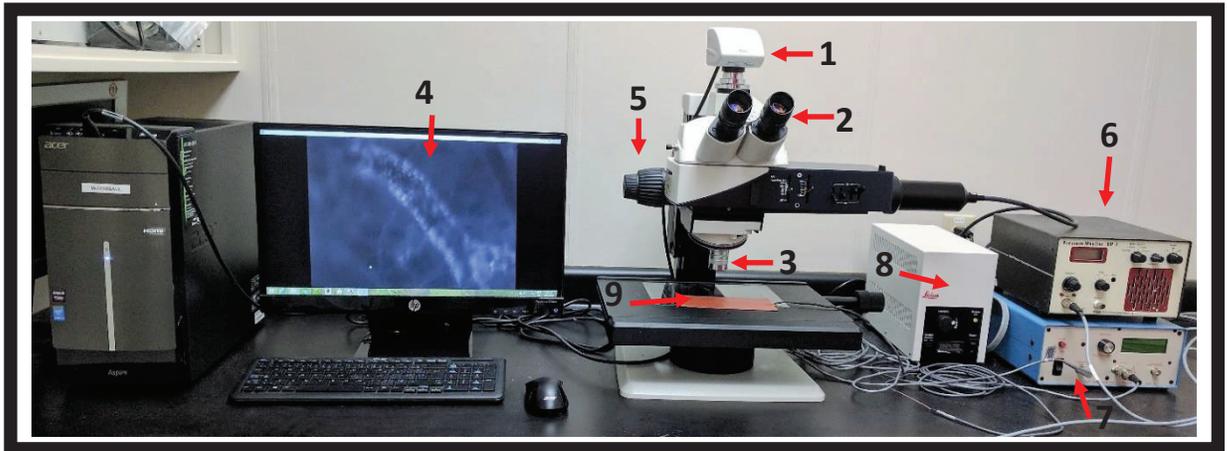
Sr. No.	Reagent	General Description	Source	Vehicle Used
1	Carrageenan	<ul style="list-style-type: none"> <li>• <math>\lambda</math>-Carrageenan type IV</li> <li>• Sulfated polysaccharide</li> <li>• Used to induce acute monoarthritis</li> </ul>	Sigma-Aldrich (St. Louis, MO, USA)	Saline
2	Cremophor	<ul style="list-style-type: none"> <li>• Formulation vehicle</li> </ul>	Sigma-Aldrich (St. Louis, MO, USA)	-
3	Dimethyl sulfoxide	<ul style="list-style-type: none"> <li>• Polar aprotic solvent</li> </ul>	Sigma-Aldrich (St. Louis, MO, USA)	-
4	Freund's complete adjuvant	<ul style="list-style-type: none"> <li>• <i>Mycobacterium tuberculosis</i> (H37Ra), heat killed and dried emulsified in paraffin oil</li> <li>• Used to induce arthritis</li> </ul>	Sigma-Aldrich (St. Louis, MO, USA)	Paraffin oil
5	Kaolin	<ul style="list-style-type: none"> <li>• Hydrated aluminum silicate (<math>H_2Al_2Si_2O_8 \cdot H_2O</math>)</li> <li>• Used to induce acute monoarthritis</li> </ul>	Sigma-Aldrich (St. Louis, MO, USA)	Saline
6	Magnesium sulfate	<ul style="list-style-type: none"> <li>• Inorganic salt</li> </ul>	EMD Chemicals (Gibbstown, NJ, USA)	Saline
7	Neutrophil Elastase 680	<ul style="list-style-type: none"> <li>• Fluorescent Imaging Agent</li> </ul>	PerkinElmer (Massachusetts, USA)	PBS
8	Potassium chloride	<ul style="list-style-type: none"> <li>• Salt</li> </ul>	EMD Chemicals (Gibbstown, NJ, USA)	Saline
9	Rhodamine 6G	<ul style="list-style-type: none"> <li>• Fluorescent dye used to stain leukocytes</li> </ul>	Sigma-Aldrich (St. Louis, MO, USA)	Saline
10	Sodium bicarbonate	<ul style="list-style-type: none"> <li>• Salt composed of sodium and bicarbonate ions</li> </ul>	EMD Chemicals (Gibbstown, NJ, USA)	Saline
11	Sodium chloride	<ul style="list-style-type: none"> <li>• Table salt</li> </ul>	EMD Chemicals (Gibbstown, NJ, USA)	Saline
12	Sodium monoiodoacetate	<ul style="list-style-type: none"> <li>• Chondrocyte glycolysis inhibitor</li> <li>• Used to induce experimental OA</li> </ul>	Sigma-Aldrich (St. Louis, MO, USA)	Saline

**Table 2.3 List of Equipment**

Sr. No.	Device	Model	Manufacturer
1	Advanced Dynamic Weight Bearing System	BIO-DWB-AUTO-M - For mice with DFK22AUC03 camera	DWB - Bioseb, (Vitrolles, France)  Camera - ImagingSource, (Gernlinden, Germany)
2	Blood Perfusion Imager	PeriCam PSI Normal Resolution with PimSoft Software	Perimed Inc., (Ardmore, Pennsylvania, USA)
3	Blood Pressure Monitor	BP-1	World Precision Instruments (Sarasota, Florida, USA)
4	Clamps	Schwartz Micro Serrefines  Micro Clamps	Fine Science Tools Inc. British Columbia, Canada
5	Digital Balance	AL54	Mettler Toledo (Mississauga, Ontario, Canada)
6	Digital Caliper	62379-531	VWR (Friendswood, Texas, USA)
7	Dissecting Microscope #1	SZ40	Olympus Canada Inc. Richmond Hill, Ontario, Canada
8	Dissecting Microscope #2	MZ 125	Leica Microsystems (Wetzlar, Germany)
9	Electron Microscope	JEOL JEM 1230 Transmission Electron Microscope  Hamamatsu ORCA-HR digital camera C4742-97-12HR	JEOL, Japan  Hamamatsu, Japan
10	Forceps	Adson Forceps  Dumont #5/45 - Cover Slip Forceps	Fine Science Tools Inc. British Columbia, Canada

<b>11</b>	Heated Blanket	SoftHeat HP710-24-3P-S	Electric Heating Pad, Kaz Inc., Southborough, MA, USA
<b>12</b>	Hemostats	Ultra Fine Hemostats	Fine Science Tools Inc. British Columbia, Canada
<b>13</b>	Intravital Microscope	Leica DM2500 microscope with a HCX APO L 20X objective and an HC Plan 10X eyepiece (final magnification 200X), with a DFC3000G camera	Microscope - Leica Microsystems Inc. (Richmond Hill, Ontario, Canada)
<b>14</b>	Micropipettes (set #1)	20, 100, 200 and 1000µl	Eppendorf Research (Mississauga, Ontario, Canada)
<b>15</b>	Micropipettes (set #2)	20, 100, 200 and 1000µl	VWR International (Mississauga, Ontario, Canada)
<b>16</b>	Microprocessor Controlled Waterbath	280 Series	Precision (Winchester, Virginia, USA)
<b>17</b>	Pressure Transducer	BLPR	Kent Scientific Corporation, Torrington, CT, USA
<b>18</b>	Scissors	Vannas Spring Scissors - 2mm and 4mm Cutting Edge  Hardened Fine Scissors	Fine Science Tools Inc. British Columbia, Canada
<b>19</b>	Temperature Control Monitor and Heating Pad	TC1000	CWE, Inc. (Ardmore, Pennsylvania, USA)
<b>20</b>	Von Frey Hair Filaments	Semmes Weinstein Microfilaments	North Coast Medical, (Gilroy, California, USA)
<b>21</b>	Vortex Analog Mixer	10153-838	VWR International (Mississauga, Ontario, Canada)

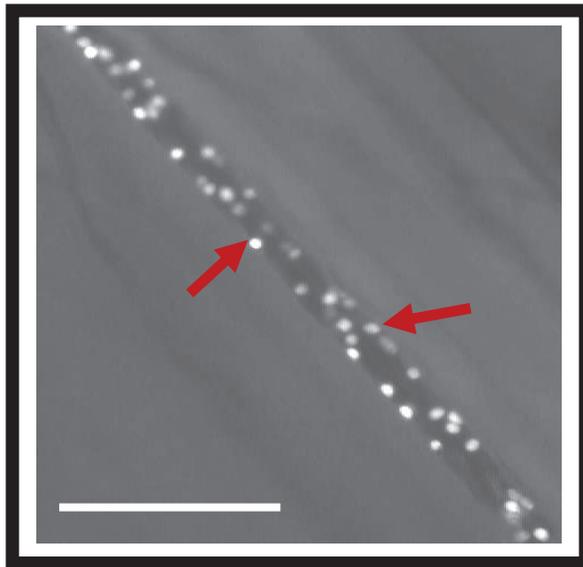
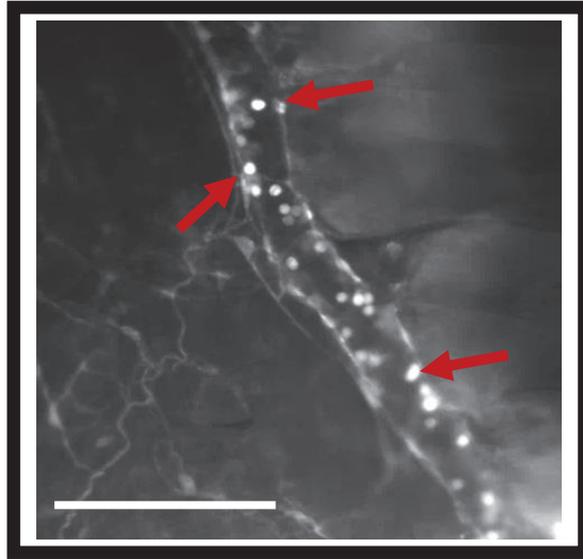
## 2.10 Figures



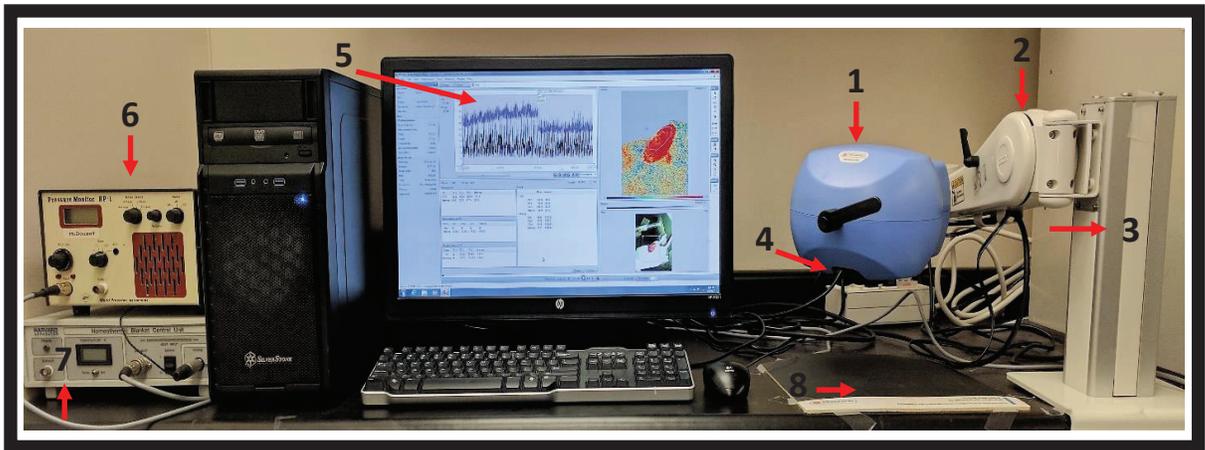
**Figure 2.1 Intravital Microscope**

### **Components:**

1. DFC3000G camera
2. HC Plan 10X eyepiece
3. HCX APO L 20X objective
4. Acquired recording using Leica Acquisition Suite (Version 4.5)
5. Coarse and fine focus
6. Blood pressure monitor
7. Temperature control
8. External light source (Leica EL6000)
9. Heating pad



**Figure 2.2 Micrographs of Knee Joint Microvasculature  
(Leukocytes Denoted by Arrows, Scale bar = 50 $\mu$ m)**



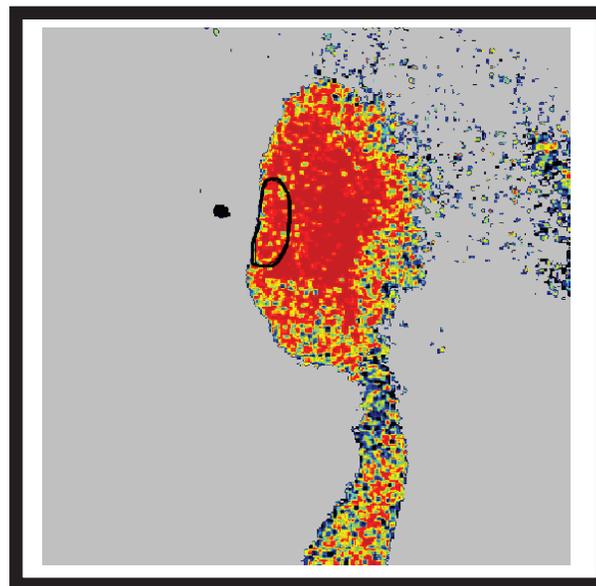
**Figure 2.3 Laser Speckle Contrast Imager**

**Components:**

1. PeriCam PSI HR head
2. Adjustable arm
3. Table mount
4. Laser aperture
5. Acquired recording using PIMSoft (Version 1.5.4.8078)
6. Blood pressure monitor
7. Temperature control monitor
8. Heating pad



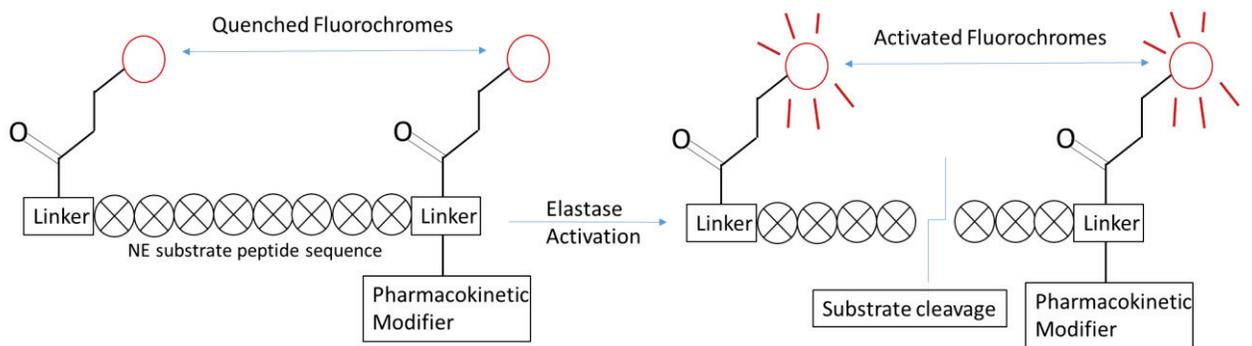
**Figure 2.4 Mouse Lower Body with Exposed Knee Joint (Denoted with Arrow)**



**Low perfusion**

**High perfusion**

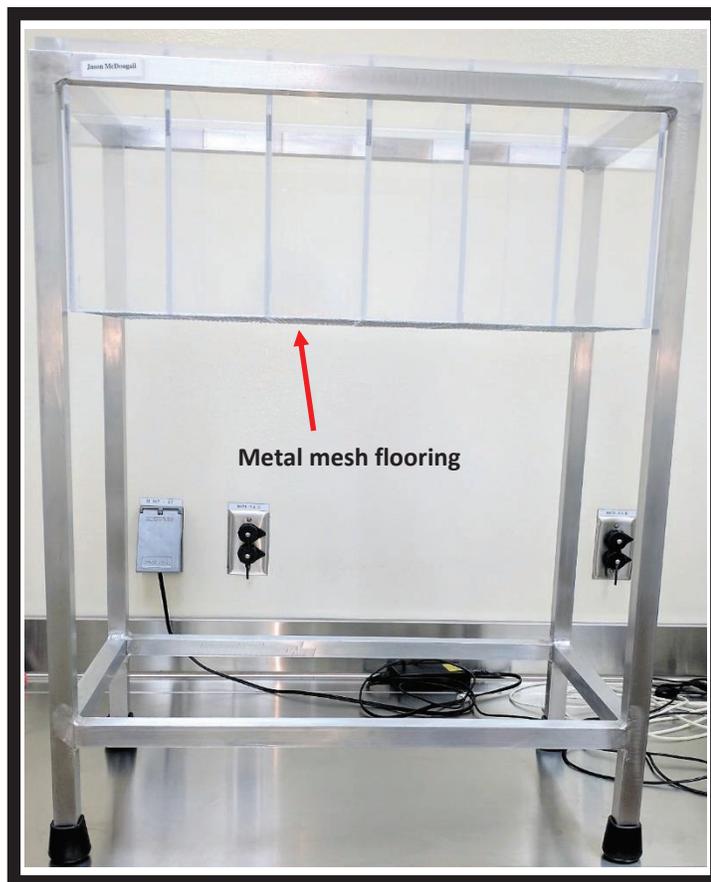
**Figure 2.5 Mouse Knee Joint Blood Flow Map (Circled)**



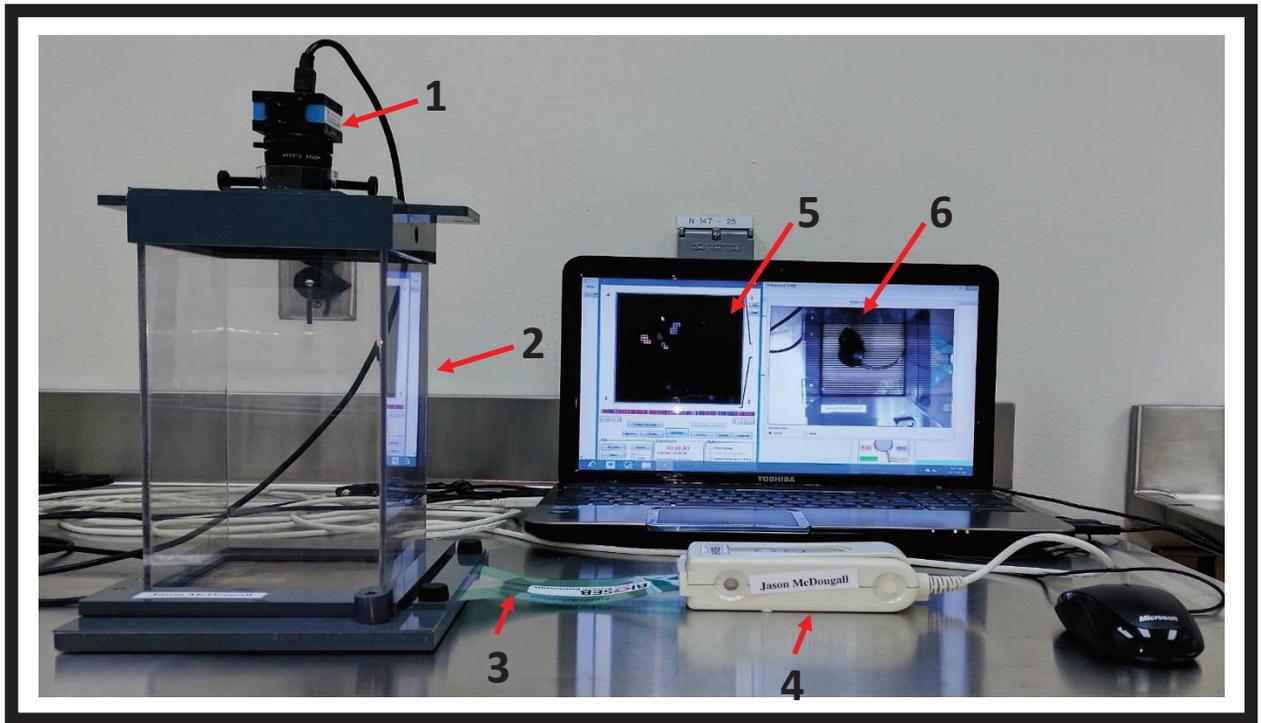
**Figure 2.6 Activation of the substrate NE 680 by Neutrophil Elastase  
(Adapted from Kossodo et al., 2011)**



**Figure 2.7 von Frey Hair Filaments (Left to Right – Increasing Bending Force)**



**Figure 2.8 Plexiglas Chamber for von Frey Hair Assessment**



**Figure 2.9 Dynamic Incapitance**

**Components:**

1. DFK22AUC03 camera
2. Plexiglass chamber (mouse)
3. Mouse sensor mat
4. USB electronic interface
5. Acquired recording (paw prints)
6. Acquired video using DWB software (Version 1.4.2.92)

## **Chapter 3: The Effect of Local Administration of Neutrophil Elastase on Mouse Knee Joint Inflammation and Pain and Involvement of Proteinase-Activated Receptor-2**

### **3.1 Disclosures**

- Most results presented in this chapter have been published in the British Journal of Pharmacology 2016; 173(4):766-77. (Appendix I – copyright permission to reproduce the content from the research article).
- The experiments involving neutrophil elastase-induced joint pain in mice and the involvement of PAR2 and p44/42 MAPK in the pain response were performed by my colleague Ms. Allison Reid.

### **3.2 Background and hypotheses**

Acute joint inflammation can result in response to trauma, injury and pathogenic infection (McDougall, 2006). When the body fails to resolve the acute response, inflammation persists and turns into a chronic inflammation which can contribute to the development of arthritis. Joint inflammation involves an accumulation of inflammatory cells, the release of mediators like cytokines, prostaglandins, and histamine (McDougall, 2006). Additionally, mediators like SP and CGRP are released from afferent neurons which contribute to the inflammatory response (Scott et al., 1994; McDougall, 2006). The net result is increased vascular permeability, increased blood flow, and leukocyte extravasation. Neutrophils arrive first at the site of the inflammation, where they release a cocktail of proteolytic enzymes from azurophilic granules; for example, neutrophil elastase, cathepsin G, and proteinase-3 (Korkmaz et al., 2010). Neutrophil elastase can contribute to erosive destruction of the joints due to its broad substrate specificity towards components of the basement membrane and connective tissue (Watanabe et al., 1990; Racine and Aaron, 2013).

PAR2 is expressed on different inflammatory cells, for example, T and B cells, neutrophils and macrophages (Russell and McDougall, 2009). Activation of PAR2 by serine proteinases or synthetic ligands results in a pro-inflammatory effect. Studies have shown pro-inflammatory effects of PAR2 activation in the lungs, gut and joints (Vergnolle, 1999; Cenac et al., 2002; Dulon et al., 2003; Zhao et al., 2015). The DRG neurons co-express PAR2 and SP and CGRP (Steinhoff et al., 2000). Activation of neuronal PAR2 causes release of these neuropeptides to induce neurogenic inflammation and pain (Steinhoff et al., 2000; Vergnolle et al., 2001).

Typically, PARs are cleaved at an established site in the extracellular domain by different serine proteinases to unmask the tethered ligand sequence that then initiates G protein-coupled receptor signaling. However, recent studies show that PARs can be cleaved at alternative sites to unmask a specific tethered ligand sequence that initiates biased signaling (Hollenberg et al., 2014). PAR2, for example, can engage biased signaling based on its activation by either synthetic peptides or serine proteinases (Ramachandran et al., 2009). Activation of PAR2 by neutrophil elastase results in selective activation of the intracellular p44/42 MAPK pathway (Ramachandran et al., 2011). In this study, the effect of exogenous neutrophil elastase on mouse knee joint inflammation and pain was assessed. Also, the involvement of PAR2 and p44/42 MAP kinase in mediating neutrophil elastase-induced joint inflammation and pain was investigated.

**The following hypotheses were tested in this study:**

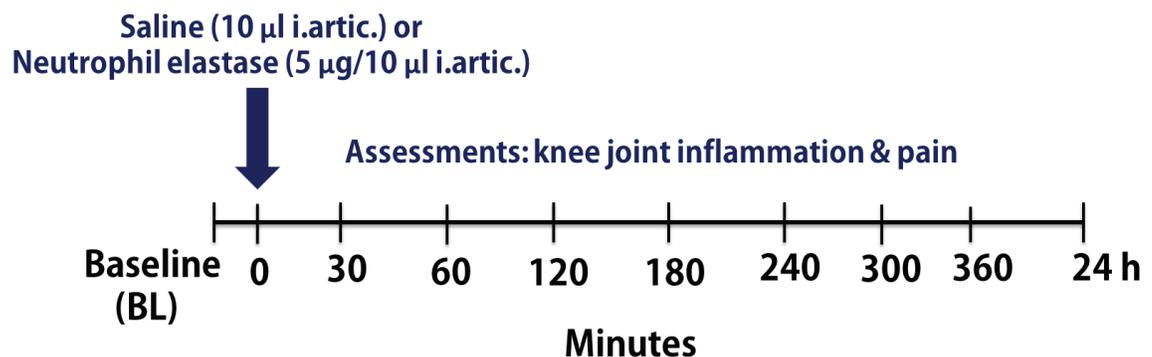
- I. Local administration of neutrophil elastase increases leukocyte trafficking, synovial blood flow and pain in the mouse knee joint.**
- II. Inhibition of neutrophil elastase or blockade of proteinase-activated receptor-2 attenuates leukocyte trafficking, synovial blood flow and pain responses.**

### 3.3 Neutrophil elastase-induced joint inflammation and pain

#### 3.3.1 Method

Mice were deeply anesthetized (2–4% isoflurane; 100% oxygen at 1 L/min) and 5  $\mu\text{g}$  (4.4 U) of neutrophil elastase (10  $\mu\text{l}$ ) was injected into the intra-articular space of the right knee joint and the limb was flexed and extended for 30 s to disperse the neutrophil elastase throughout the joint. Leukocyte kinetics and blood perfusion were measured in the knee joint microvasculature using IVM (refer to section 2.3.2.2) and LASCA (refer to section 2.3.2.3), respectively, at several time points over 24 h post-injection. A surgical preparation was required before carrying out inflammatory assessments which involved cannulations of the trachea, carotid artery and jugular vein (refer to section 2.3.2.1). For inflammation experiments, mice were injected with physiological saline (10  $\mu\text{l}$ ) into the intra-articular space of the left (contralateral) knee joint and the values obtained were subtracted from readings taken from the neutrophil elastase injected knee.

A separate cohort of mice was injected with saline (10  $\mu\text{l}$ ) or neutrophil elastase (5  $\mu\text{g}/10 \mu\text{l}$ ) into the intra-articular space of the right knee joint and behavioural pain was assessed using von Frey hair algometry (refer to section 2.5.1) at several time points over 24 h post-injection.



### **3.3.2 Results**

Intra-articular injection of neutrophil elastase caused a progressive increase in the number of rolling and adherent leukocytes. Compared to baseline, the increase in the number of rolling (Figure 3.1A,  $P < 0.05$ ,  $n=5-14$  per time point) and adherent (Figure 3.1B,  $P < 0.01$ ,  $n=5-14$  per time point) leukocytes was significant at 4 h post-injection of neutrophil elastase. Likewise, blood perfusion was increased significantly at 4 h post-injection of neutrophil elastase (Figure 3.1C,  $P < 0.05$ ,  $n=5-15$  per time point). However, these inflammatory changes resolved by the end of 24 h.

Local injection of neutrophil elastase into the knee joint caused a progressive decrease in the withdrawal threshold which was statistically significant at 2 h, 4 h, 5 h, and 6 h (Figure 3.1D,  $P < 0.05$ ,  $n=11$  per time point) compared to baseline. However, the withdrawal threshold returned to baseline by the end of 24 h.

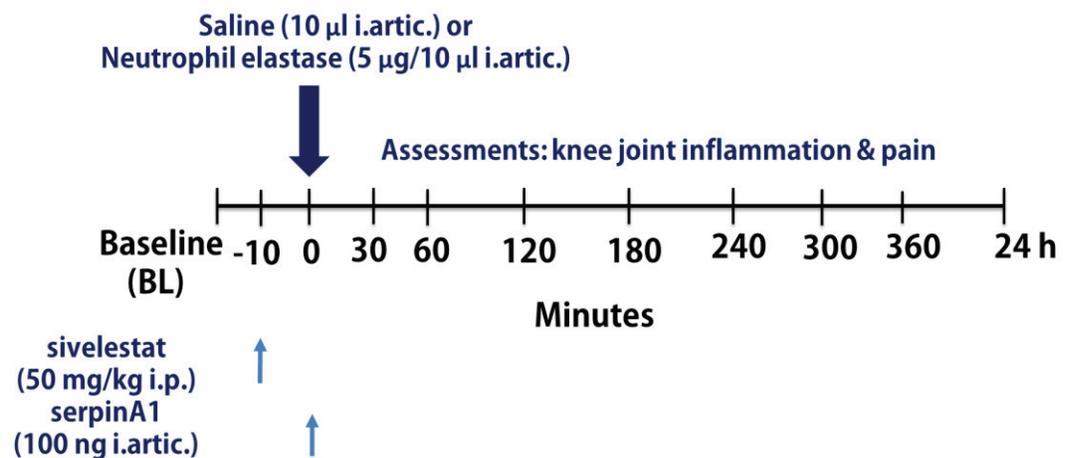
## **3.4 Effect of sivelestat or serpinA1 on neutrophil elastase-induced joint inflammation and pain**

### **3.4.1 Method**

As the local administration of neutrophil elastase produced an acute joint inflammation and pain, our next aim was to study if these changes could be reversed by inhibiting neutrophil elastase. A synthetic inhibitor (sivelestat) and an endogenous inhibitor (serpinA1) of neutrophil elastase were used for these experiments.

The following protocol was used:

- Mice were treated with sivelestat (50 mg/kg i.p.) 10 min prior to a local injection of neutrophil elastase (5  $\mu$ g/10  $\mu$ l) into the right knee joint and saline (10  $\mu$ l) into the left knee joint. The inflammation was assessed at several time points over 24 h.
- A separate cohort of mice was treated with sivelestat (50 mg/kg i.p.) 10 min prior to a local injection of neutrophil elastase (5  $\mu$ g/10  $\mu$ l) into the right knee joint. The pain was assessed at several time points over 24 h.
- Mice were treated with neutrophil elastase (5  $\mu$ g/10  $\mu$ l) or a combination of neutrophil elastase (5  $\mu$ g) and serpinA1 (100 ng) (10  $\mu$ l) into the right knee joint. The inflammation was assessed at several time points over 24 h.
- A separate cohort of mice was treated with neutrophil elastase (5  $\mu$ g/10  $\mu$ l) or a combination of neutrophil elastase (5  $\mu$ g) and serpinA1 (100 ng) (10  $\mu$ l) into the right knee joint and pain was assessed at several time points over 24 h.



### **3.4.2 Results**

Injection of neutrophil elastase caused an increase in leukocyte trafficking and blood perfusion. Treatment with a synthetic inhibitor of neutrophil elastase, sivelestat, blocked the increase in the number of rolling leukocytes (Figure 3.2A,  $P < 0.01$ ,  $n=5-9$  per time point), adherent leukocytes (Figure 3.2B,  $P < 0.01$ ,  $n=5-9$  per time point) and knee joint blood perfusion (Figure 3.2C,  $P < 0.0001$ ,  $n=5-9$  per time point) across the time course. Likewise, treatment with an endogenous inhibitor of neutrophil elastase, serpinA1, also blocked the increase in the number of rolling leukocytes (Figure 3.2A,  $P < 0.01$ ,  $n=5-9$  per time point), adherent leukocytes (Figure 3.2B,  $P < 0.01$ ,  $n=5-9$  per time point) and knee joint blood perfusion (Figure 3.2C,  $P < 0.001$ ,  $n=5-9$  per time point) throughout the time course.

Intra-articular injection of neutrophil elastase caused a decrease in the withdrawal threshold. Treatment with the neutrophil elastase inhibitors sivelestat (Figure 3.2D,  $P < 0.0001$ ,  $n=6-11$  per time point) or serpinA1 (Figure 3.2D,  $P < 0.001$ ,  $n=6-11$  per time point) prevented the decrease in the withdrawal threshold throughout the time course.

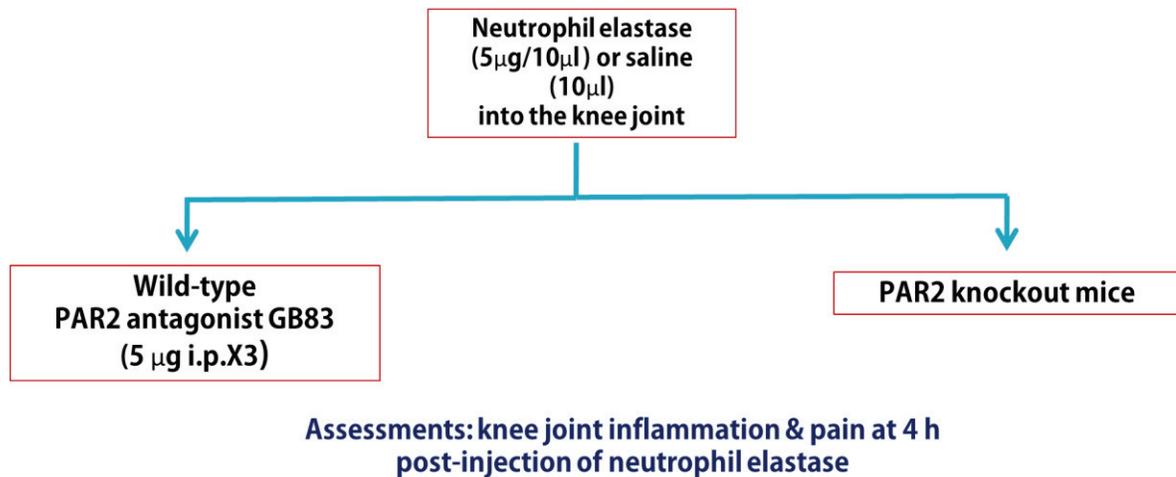
## **3.5 Involvement of PAR2 in mediating neutrophil elastase-induced joint inflammation and pain**

### **3.5.1 Method**

Studies indicate that neutrophil elastase has the capacity to activate PAR2 (Zhou et al., 2013; Zhao et al., 2015); therefore, we wanted to characterize the involvement of PAR2 in mediating neutrophil elastase-induced joint inflammation and pain. To do this,

we tested the effect of neutrophil elastase in the presence of the PAR2 antagonist (GB83) and in PAR2 knockout mice. The experiment focused on the 4 h time point as neutrophil elastase produced its peak effect at this time point. The following protocol was used:

- Mice were treated with the PAR2 antagonist GB83 (5 µg i.p.) 10 mins prior to a local injection of neutrophil elastase (5 µg/10 µl) into the right knee joint and saline (10 µl) into the left knee joint. The PAR2 antagonist GB83 (5 µg i.p.) was administered again at 110 and 230 min after neutrophil elastase injection. The inflammation was assessed at 4 h post-injection of neutrophil elastase.
- A separate cohort of mice was treated with the PAR2 antagonist GB83 (5 µg i.p.) 10 mins prior to a local injection of neutrophil elastase (5 µg/10 µl) into the right knee joint. The PAR2 antagonist GB83 (5 µg i.p.) was administered again at 110 and 230 min after neutrophil elastase injection. The pain was assessed at 4 h post-injection of neutrophil elastase.
- An intra-articular injection of neutrophil elastase (5 µg/10 µl) was made into the right knee joint and saline (10 µl) into the left knee joint of PAR2 knockout mice. The inflammation was assessed at 4 h post-injection of neutrophil elastase.
- An intra-articular injection of neutrophil elastase (5 µg/10 µl) was made into the right knee joint of a separate cohort of PAR2 knockout mice. The pain was assessed at 4 h post-injection of neutrophil elastase.



### 3.5.2 Results

Systemic treatment with the PAR2 antagonist GB83 blocked an increase in knee joint diameter (Figure 3.3A,  $P < 0.05$ ,  $n=9$ ), the number of rolling (Figure 3.3B,  $P < 0.05$ ,  $n=8$ ) and adherent (Figure 3.3C,  $P < 0.05$ ,  $n=8$ ) leukocytes, and blood perfusion (Figure 3.3D,  $P < 0.05$ ,  $n=9$ ) as compared to neutrophil elastase control ( $n=15$ ) at 4 hour. Intra-articular injection of neutrophil elastase into the knee joints of PAR2 knockout mice failed to cause an increase in the knee joint diameter (Figure 3.3A,  $P < 0.05$ ,  $n=9$ ), the number of rolling (Figure 3.3B,  $P < 0.05$ ,  $n=9$ ) and adherent (Figure 3.3C,  $P < 0.05$ ,  $n=9$ ) leukocytes, and blood perfusion (Figure 3.3D,  $P < 0.01$ ,  $n=11$ ) compared to neutrophil elastase control at 4 hour.

Intra-articular injection of neutrophil elastase caused a significant decrease in the withdrawal threshold at 4 hours as compared to saline control (Figure 3.3E,  $P < 0.01$ ,  $n=9$ ). This effect was blocked by treatment with the PAR2 antagonist GB83 (Figure 3.3E,  $P < 0.01$ ,  $n=9$ ) and was absent in PAR2 knockout mice (Figure 3.3E,  $P < 0.05$ ,  $n=9$ ).

## **3.6 Activation of the intracellular p44/42 MAPK pathway following PAR2 activation**

### **3.6.1 Method**

A study by Ramachandran et al. (2011) has shown that activation of PAR2 by neutrophil elastase causes activation of a p44/42 MAPK pathway; therefore, we investigated involvement of this pathway in eliciting joint inflammation and pain. To do this, we used U0126, which is a non-competitive inhibitor of p44/42 MAP kinase. The experiment focused on the 4 h time point, the peak effect of neutrophil elastase. The following protocol was used:

- A local injection of neutrophil elastase (5 µg/10 µl) was made into the right knee joint and saline (10 µl) into the left knee joint, then animals were treated with the p44/42 MAPK inhibitor U0126 (30 mg/kg, i.p.) 2 h post-injection of neutrophil elastase. Inflammation was assessed at 4 h post-injection of neutrophil elastase.
- A separate cohort of mice received a local injection of neutrophil elastase (5 µg/10 µl) into the right knee joint, then animals were treated with the p44/42 MAPK inhibitor U0126 (30 mg/kg, i.p.) 2 h post-injection of neutrophil elastase. Pain was assessed at 4 h post-injection of neutrophil elastase.

### **3.6.2 Results**

Systemic treatment with the p44/42 MAPK inhibitor U0126 significantly blocked an increase in knee joint diameter (Figure 3.3A,  $P < 0.05$ ,  $n=12$ ), the number of rolling leukocytes (Figure 3.3B,  $P < 0.01$ ,  $n=12$ ), and joint perfusion (Figure 3.3D,  $P < 0.05$ ,  $n=14$ ) compared to neutrophil elastase control.

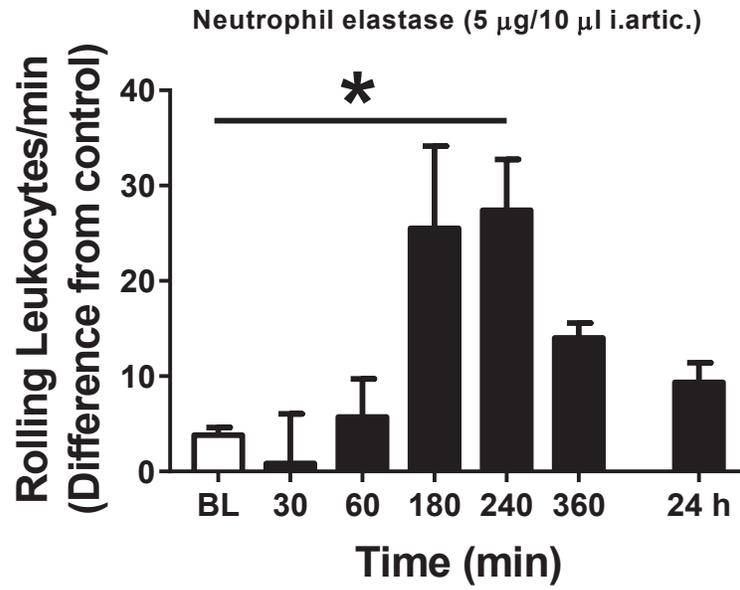
Treatment with the p44/42 MAPK inhibitor U0126 significantly prevented the decrease in joint mechanosensitivity caused by an intra-articular injection of neutrophil elastase (Figure 3.3E,  $P < 0.01$ ,  $n=11$ ).

### **3.7 Chapter summary**

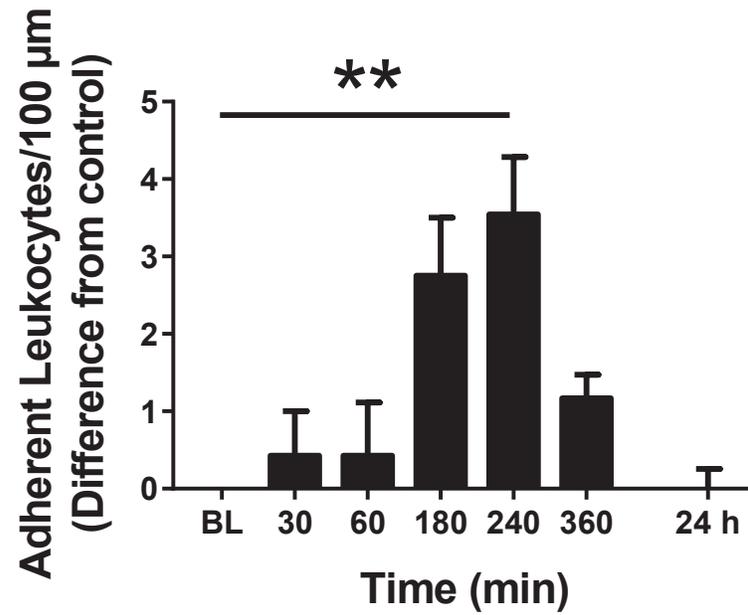
The results of this study suggest that administration of neutrophil elastase into the knee joints of mice causes joint oedema, synovial hyperaemia, and an increase in leukocyte trafficking and pain. These changes are blocked by neutrophil elastase inhibitors, a PAR2 antagonist, and a p44/42 MAPK inhibitor, and do not develop in the absence of PAR2. Thus, joint inflammation triggered by neutrophil elastase is mediated by PAR2 and appears to involve activation of the p44/42 MAPK pathway.

3.8 Figures

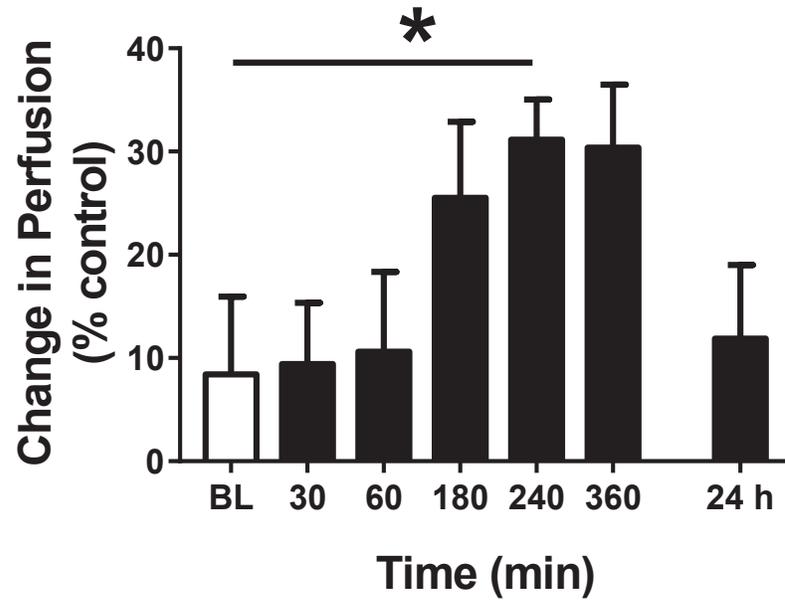
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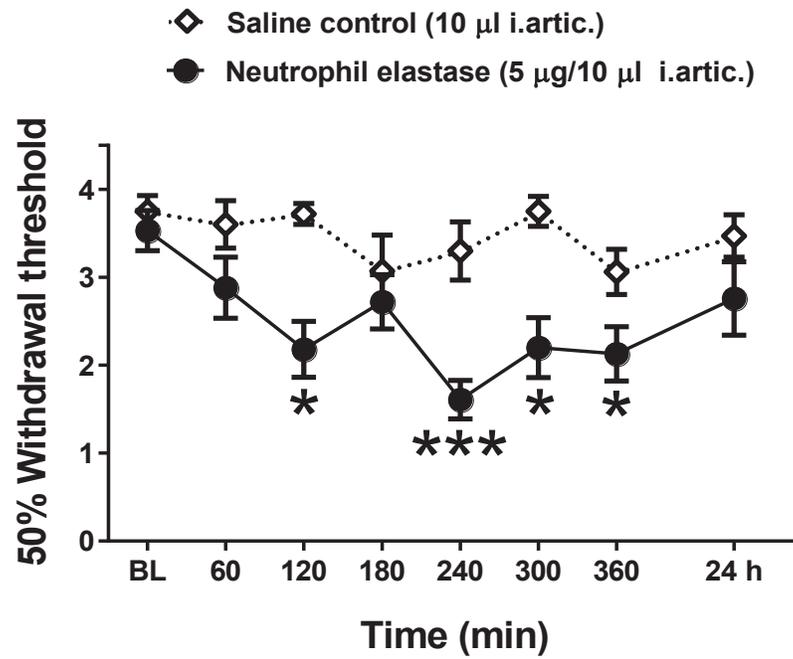
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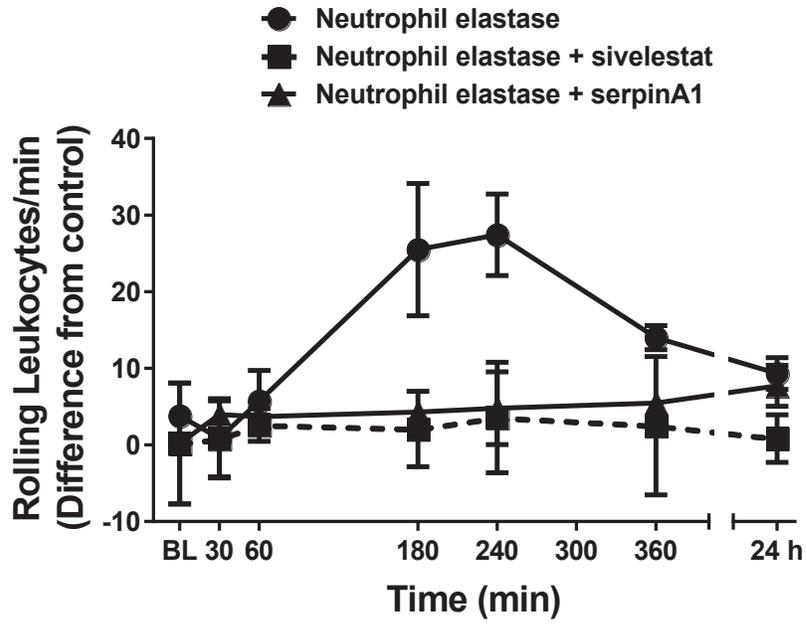
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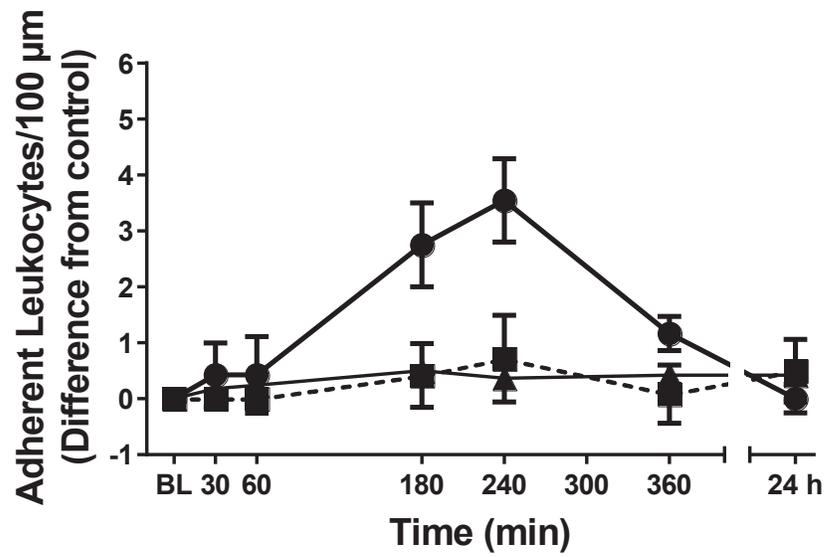
### Figure 3.1

**Neutrophil elastase-induced joint inflammation and pain.** Time course of the effect of an intra-articular injection of neutrophil elastase which caused a significant increase in the number of **(A)** rolling and **(B)** adherent leukocytes at 4 h post-injection, compared to baseline. **(C)** Time course of the effect of an intra-articular injection of neutrophil elastase which results in a significant increase in the knee joint blood perfusion at 4 h post-injection, compared to baseline. **(D)** Time course of the effect of an intra-articular injection of neutrophil elastase which caused a significant decrease in the withdrawal threshold at 2, 4, 5 and 6 h post-injection, compared to baseline. Withdrawal threshold did not change due to intra-articular injection of saline. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  one-way ANOVA with Dunnett's *post hoc* test,  $n=5-15$  per time point. (Previously published, Muley et al., 2016).

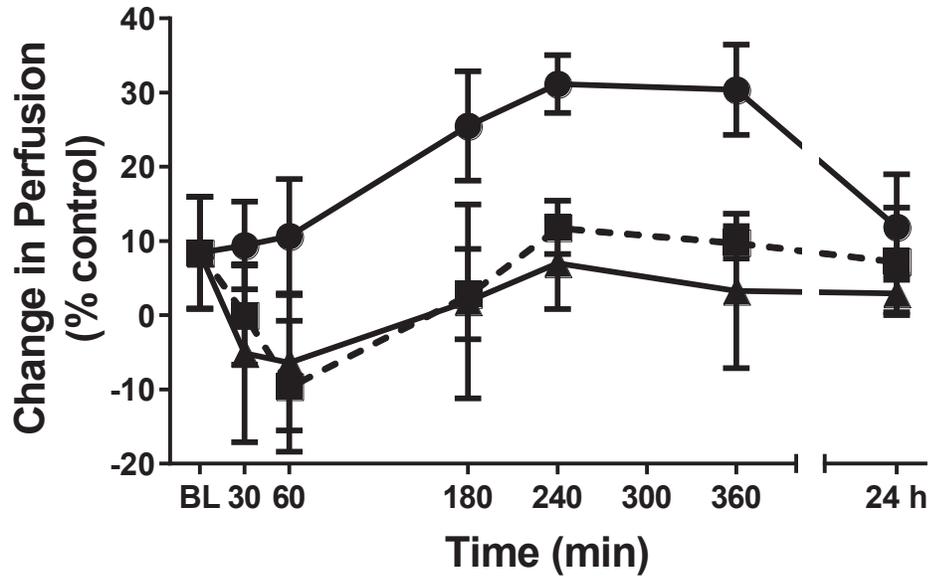
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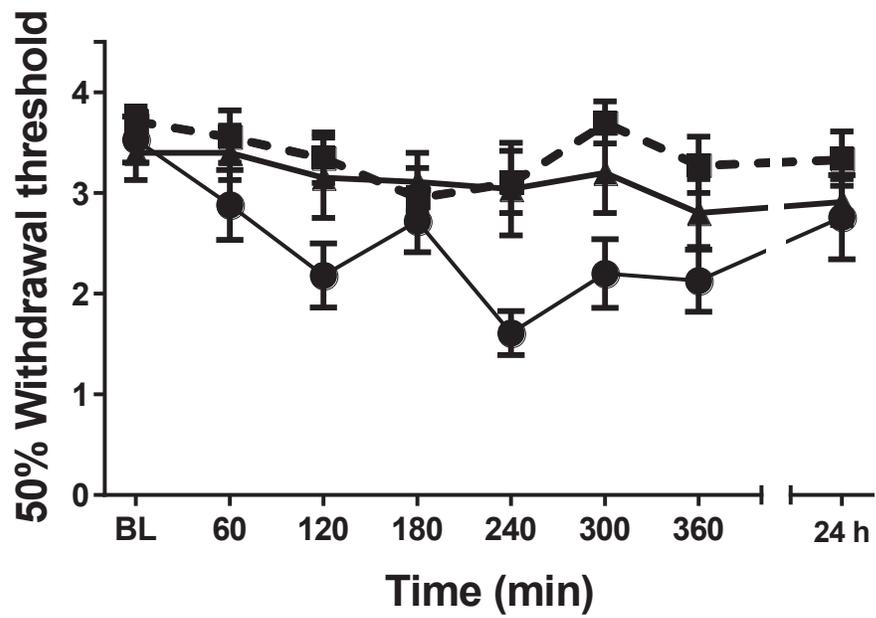
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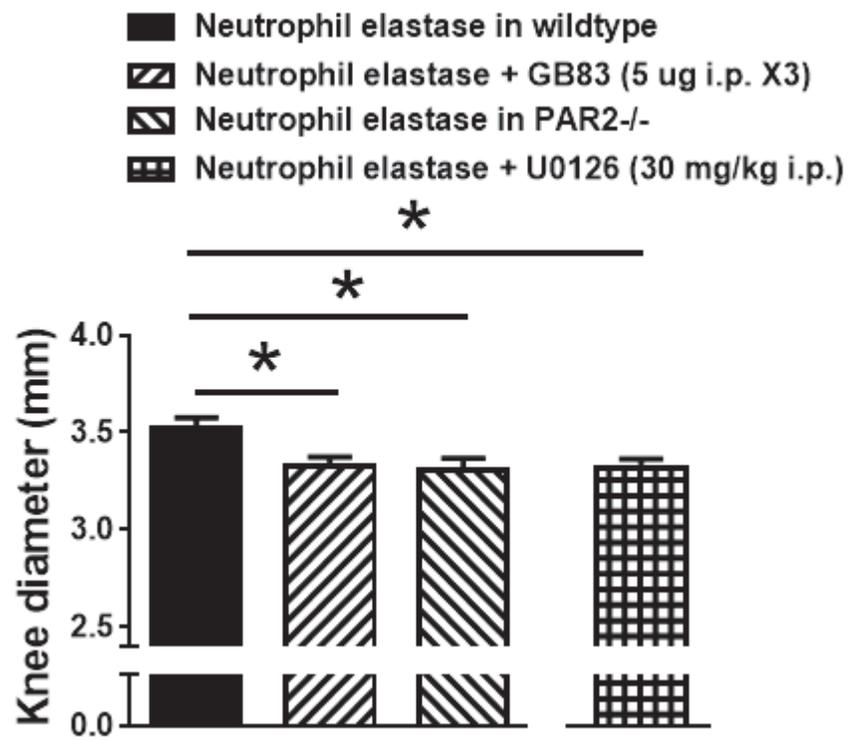


### **Figure 3.2**

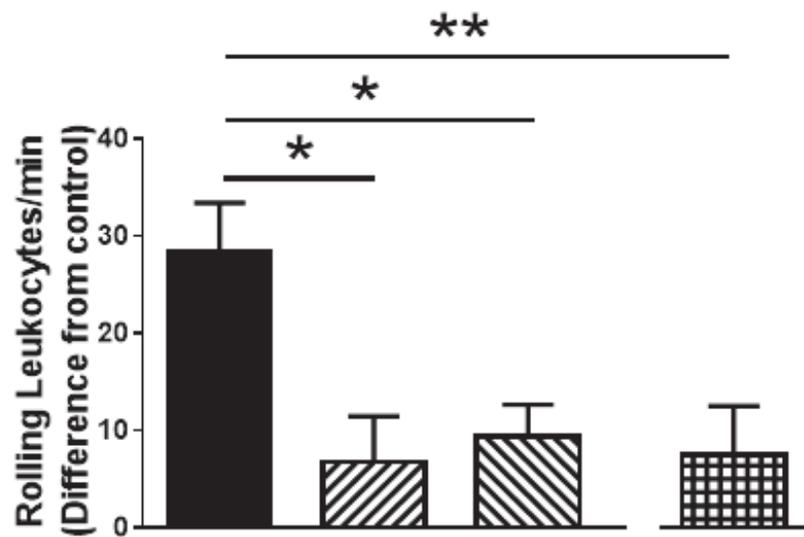
#### **Time course of the effect of neutrophil elastase inhibition with sivelestat or**

**serpinA1.** Treatment with the neutrophil elastase inhibitors sivelestat (50 mg/kg i.p.) or serpinA1 (100 ng i.artic.) significantly blocked the neutrophil elastase-induced increase in the number of **(A)** rolling (\*\*P < 0.01, two-way ANOVA, n=5-9 per time point) and **(B)** adherent leukocytes (\*\*P < 0.01, two-way ANOVA, n=5-9 per time point) throughout the 24 h time course. **(C)** An increase in blood perfusion due to intra-articular injection of neutrophil elastase was blocked by treatment with sivelestat or serpinA1 (\*\*\*P < 0.001, two-way ANOVA, n=5-9 per time point) throughout the 24 h time course. **(D)** Treatment with sivelestat or serpinA1 caused an improvement in tolerance to tactile stimulation throughout the 24 h time course (\*\*\*P < 0.001, two-way ANOVA, n=6-11 per time point). (Previously published, Muley et al., 2016).

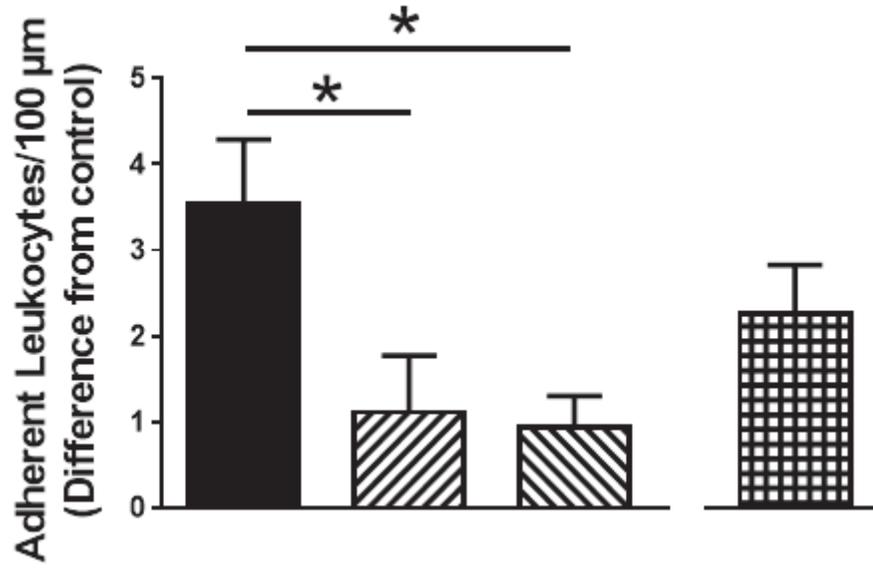
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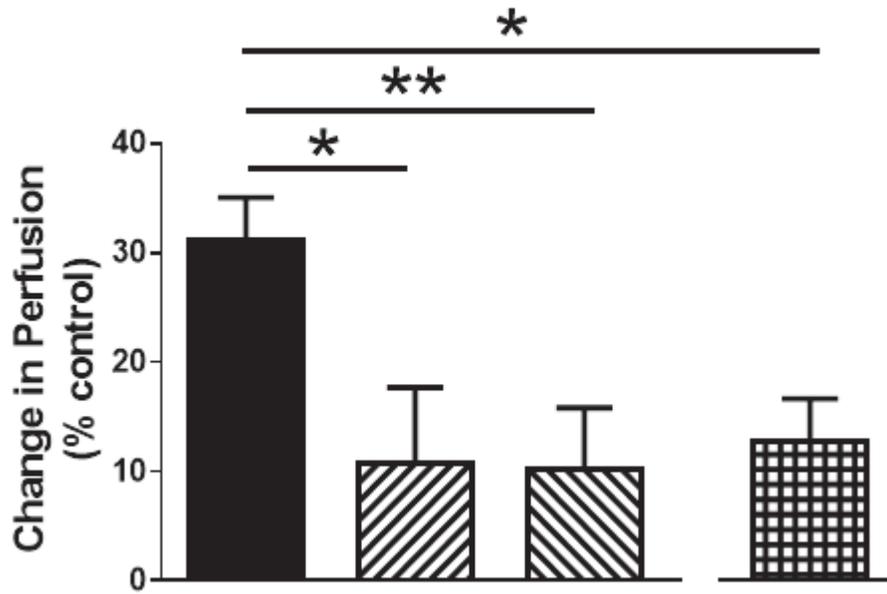
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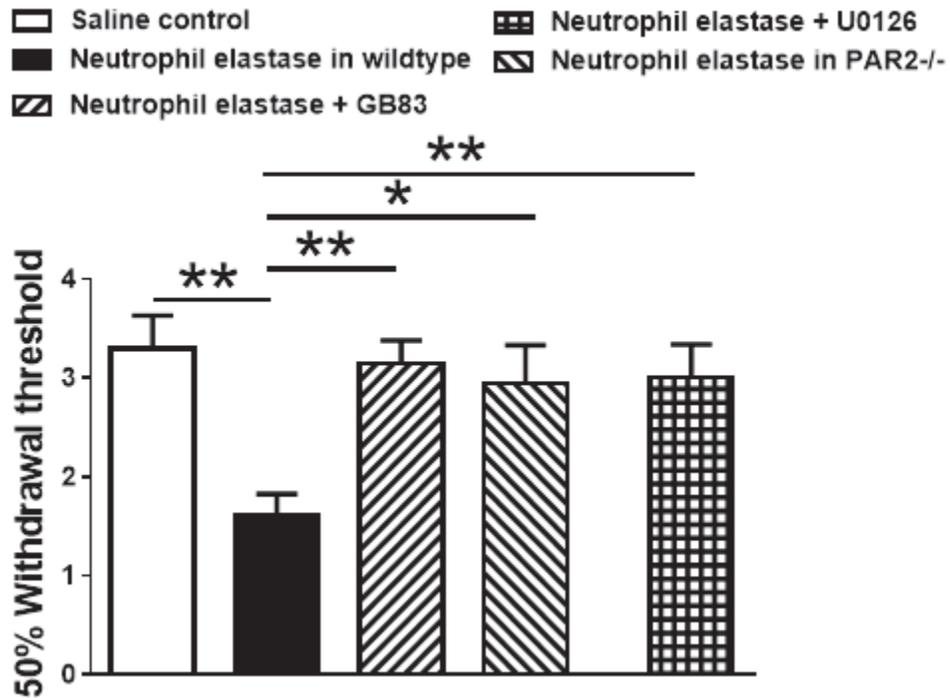
C



D



**E**



**Figure 3.3**

**Activation of PAR2 and the intracellular p44/42 MAPK pathway and their involvement in mediating neutrophil elastase-induced joint inflammation and pain.**

(A) Neutrophil elastase caused joint oedema at 4 h post-injection which was blocked by treatment with the PAR2 antagonist GB83 and was absent in PAR2 knockout mice, and was significantly blocked by the p44/42 MAPK inhibitor U0126. Intra-articular injection of neutrophil elastase caused a significant increase in the number of (B) rolling and (C) adherent leukocytes at 4 h post-injection which was blocked by treatment with the PAR2 antagonist GB83 and was absent in PAR2 knockout mice. The MAPK inhibitor U0126

significantly blocked the increase in the number rolling leukocytes. **(D)** Knee joint blood perfusion was significantly increased at 4 h after neutrophil elastase injection. This effect was blocked by treatment with the PAR2 antagonist GB83 and was absent in PAR2 knockout mice. The p44/42 MAPK inhibitor U0126 significantly blocked the neutrophil elastase-induced effect **(E)** Withdrawal threshold was significantly decreased at 4 h post intra-articular injection of neutrophil elastase and was blocked by treatment with the PAR2 antagonist GB83 and it was improved in PAR2 knockout mice. The p44/42 MAPK inhibitor U0126 blocked the decrease in the withdrawal threshold. \*P < 0.05, \*\*P < 0.01, compared with neutrophil elastase alone, one-way ANOVA with Dunnett's *post hoc* test, n=8-15 per group. (Previously published, Muley et al., 2016).

## **Chapter 4: The Effect of Blockade of Neutrophil Elastase and Proteinase-Activated Receptor-2 on the Knee Joint Inflammation and Pain Associated with Inflammatory Monoarthritis**

### **4.1 Disclosures**

- The experiments involving the assessment of the effect of sivelestat on kaolin/carrageenan-induced joint inflammation and pain included in the manuscript were published in the British Journal of Pharmacology 2016; 173(4):766-77. (Appendix I – copyright permission to reproduce the content from the research article).

## 4.2 Background and hypotheses

Rheumatoid arthritis is one of the leading forms of arthritis (Gibofsky, 2012). In RA, most patients experience episodes of inflammatory flares and pain (McInnes and Schett, 2011). Synovitis is a prominent clinical feature of the RA joints which mainly involves infiltration of inflammatory cells into the synovial compartment. Studies indicate that synovitis can contribute to cartilage and bone damage, and the development of pain in RA patients (Hitchon and El-Gabalawy, 2011).

Various inflammatory cells that migrate to the synovial compartment include neutrophils, macrophages, mast cells, T and B cells. These cells are essential for driving inflammatory processes within the joint (McInnes and Schett, 2011). Neutrophils are an important component of the innate immune system and can contribute to the development of synovitis by releasing a variety of proteinases, reactive oxygen species and prostaglandins (Cascao et al., 2010). Interestingly, the synovial fluid obtained from RA patients show increased levels of neutrophil elastase which suggest their potential in the development of RA (Momohara et al., 1997). Furthermore, neutrophil elastase can produce pro-inflammatory effect by promoting leukocyte adhesion and extravasation, and by activating pro-inflammatory cytokines (Champagne et al., 1998; Levesque et al., 2001; Benabid et al., 2012; Muller, 2013). As shown in the previous chapter, exogenous neutrophil elastase can cause an increase in joint oedema, leukocyte trafficking and synovial blood flow, and these effects are mediated by PAR2. Several studies in the literature suggest that activation of PAR2 using serine proteinases or synthetic ligands cause joint inflammation pain (Ferrell et al., 2003; Steinhoff et al., 2005; Russell and McDougall, 2009; Russell et al., 2012; Zhao et al., 2015).

This study was undertaken to investigate the role of endogenous neutrophil elastase and PAR2 in the joint inflammation and pain associated with inflammatory monoarthritis. Two different animal models of inflammatory monoarthritis were used. First, an acute inflammatory monoarthritis was induced by a local injection of kaolin/carrageenan into the knee joint of mice. This is a routinely used model which produces robust joint inflammation, pain and degeneration of hyaline cartilage in rodents (Gardner, 1960; Neugebauer et al., 2007). Second, chronic inflammatory monoarthritis was induced by a local injection of Freund's complete adjuvant into the knee joint of rats. Injection of FCA produces persistent joint inflammation and pain (Muley et al., 2016a).

**The following hypotheses were tested in this study:**

- I. Local administration of kaolin/carrageenan increases leukocyte trafficking, synovial blood flow and pain in the mouse knee joint.**
- II. Inhibition of endogenous neutrophil elastase or blockade of proteinase-activated receptor-2 attenuates kaolin/carrageenan-induced leukocyte trafficking, synovial blood flow and pain responses.**
- III. Local administration of Freund's complete adjuvant increases leukocyte trafficking, synovial blood flow and pain in the rat knee joint.**

#### **IV. Inhibition of endogenous neutrophil elastase attenuates Freund's complete adjuvant-induced leukocyte trafficking, synovial blood flow and pain responses.**

### **4.3 Effect of neutrophil elastase inhibition on kaolin/carrageenan-induced joint inflammation and pain**

#### **4.3.1 Method**

After anesthetizing mice with isoflurane (2–4%; 100% oxygen at 1 L/min), 2% kaolin (10  $\mu$ l) was injected into the intra-articular space of the right knee joint and the limb was flexed and extended for 10 min to ensure kaolin is properly dispersed throughout the joint. Next, 2% carrageenan (10  $\mu$ l) was injected into the intra-articular space of the same knee joint and the limb was again flexed and extended for 30 s to disperse the carrageenan throughout the joint. A separate cohort of mice was injected with sterile saline (10  $\mu$ l) into the intra-articular space of the right knee joint. Knee joint diameter was measured using Vernier calipers (refer to section 2.3.1). Animals were surgically prepared which involved cannulations of the trachea to allow unrestricted breathing, carotid artery for measuring mean arterial pressure and jugular vein for injection of rhodamine 6G (refer to section 2.3.2.1). Leukocyte-endothelial interactions were captured using IVM (refer to section 2.3.2.2) and blood perfusion using LASCA (refer to section 2.3.2.3) at 24 h post-injection of kaolin/carrageenan. Vascular conductance was calculated by normalising mean blood perfusion value against mean arterial pressure value of that mouse (for equation refer 2.3.2.3).

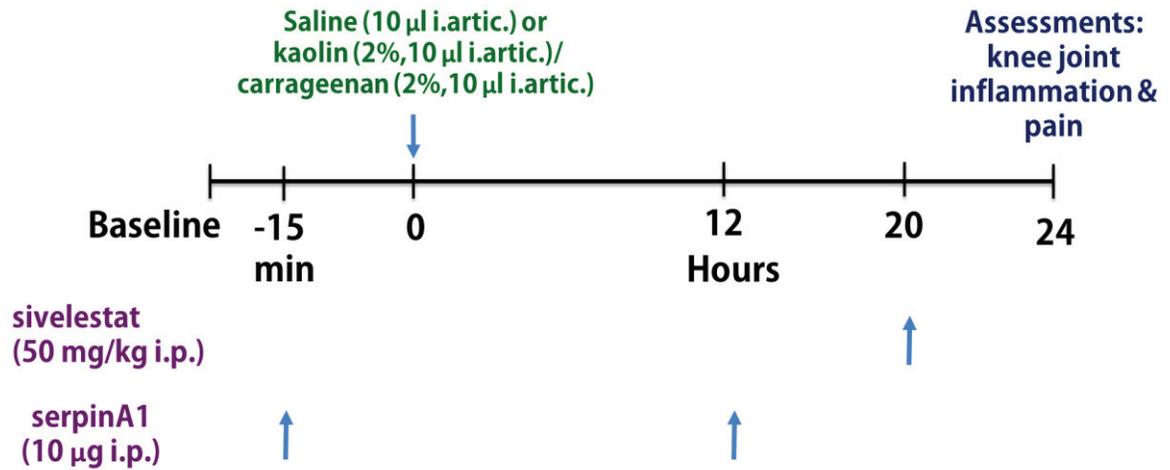
Behavioural pain was assessed using von Frey hair algometry (refer to section 2.5.1) in a separate group of mice that was injected with saline or kaolin/carrageenan into the right knee joint at 24 h post-injection.

For the assessment of involvement of endogenous neutrophil elastase in the kaolin/carrageenan model, animals were treated with the neutrophil elastase inhibitors sivelestat and serpinA1.

The following protocol was used:

- Mice were treated with sivelestat (50 mg/kg i.p.) 20 h after injection of kaolin (2%, 10  $\mu$ l)/carrageenan (2%, 10  $\mu$ l) (i.e. 4 h before measurements). Inflammation was assessed 24 h post-injection of kaolin/carrageenan.
- A separate cohort of mice was treated with sivelestat (50 mg/kg i.p.) 20 h after injection of kaolin (2%, 10  $\mu$ l)/carrageenan (2%, 10  $\mu$ l) (i.e. 4 h before measurements). Pain was assessed 24 h post-injection of kaolin/carrageenan.
- A separate group of kaolin/carrageenan-injected animals received treatment with an endogenous inhibitor of neutrophil elastase (serpinA1, 10  $\mu$ g i.p.) administered 15 min before and 12 h after kaolin/carrageenan injection and inflammation was assessed 24 h after injection of kaolin/carrageenan (The different dosing schedule was used for serpinA1 because it has a longer half-life than sivelestat).
- A separate group of kaolin/carrageenan-injected animals received treatment with an endogenous inhibitor of neutrophil elastase (serpinA1, 10  $\mu$ g i.p.)

administered 15 min before and 12 h after kaolin/carrageenan injection and pain was assessed 24 h after injection of kaolin/carrageenan.



#### 4.3.2 Results

Local injection of kaolin/carrageenan caused an increase in knee joint diameter (Figure 4.1A,  $P < 0.0001$ ,  $n=9$ ), the number of rolling (Figure 4.1B,  $P < 0.001$ ,  $n=9$ ) and adherent (Figure 4.1C,  $P < 0.0001$ ,  $n=9$ ) leukocytes, and vascular conductance (Figure 4.1D,  $P < 0.01$ ,  $n=8$ ) at 24 h post-injection, as compared to saline control ( $n=8-10$ ).

Treatment with the synthetic neutrophil elastase inhibitor, sivelestat, blocked the increase in knee joint diameter (Figure 4.1A,  $P < 0.01$ ,  $n=9$ ), the number of rolling (Figure 4.1B,  $P < 0.05$ ,  $n=10$ ) and adherent (Figure 4.1C,  $P < 0.01$ ,  $n=10$ ) leukocytes, and vascular conductance (Figure 4.1D,  $P < 0.01$ ,  $n=9$ ), as compared to kaolin/carrageenan alone.

Likewise, treatment with an endogenous neutrophil elastase inhibitor, serpinA1, blocked the increase in knee joint diameter (Figure 4.1A,  $P < 0.05$ ,  $n=8$ ), the number of rolling (Figure 4.1B,  $P < 0.001$ ,  $n=8$ ) and adherent (Figure 4.1C,  $P < 0.0001$ ,  $n=8$ ) leukocytes,

and vascular conductance (Figure 4.1D,  $P < 0.05$ ,  $n=8$ ), as compared to kaolin/carrageenan alone.

Intra-articular injection of kaolin/carrageenan produced a decrease in hindpaw withdrawal threshold (Figure 4.1E,  $P < 0.01$ ,  $n=6$ ) 24 h post-injection, as compared to saline control ( $n=17$ ). Interestingly, treatment with the neutrophil elastase inhibitors did not prevent the decrease in the withdrawal threshold (Figure 4.1E,  $P = 0.85$  &  $P = 0.23$ ,  $n=6-8$ ) in kaolin/carrageenan-injected animals.

#### **4.4 Involvement of PAR2 in mediating kaolin-carrageenan-induced joint inflammation and pain**

##### **4.4.1 Method**

Administration of neutrophil elastase inhibitors caused a decrease in kaolin/carrageenan-induced joint inflammation suggesting that injection of kaolin/carrageenan causes the release of endogenous neutrophil elastase which contributes to the joint inflammation. Our previous findings suggest that neutrophil elastase activates PAR2 to elicit joint inflammation and pain in mice (refer to Chapter 3). Therefore, our next aim was to assess the involvement of PAR2 in mediating kaolin/carrageenan-induced joint inflammation and pain. To do this, kaolin/carrageenan was injected into the knee joints of PAR2 knockout mice. The following protocol was used:

- An intra-articular injection of kaolin (2%, 10  $\mu$ l) and carrageenan (2%, 10  $\mu$ l) was made into the right knee joint of PAR2 knockout mice. Inflammation was assessed 24 h post-kaolin/carrageenan injection.
- A separate cohort of PAR2 knockout mice was injected with kaolin (2%, 10  $\mu$ l) and carrageenan (2%, 10  $\mu$ l) into the right knee joint and pain was assessed at 24 h post-injection.

#### 4.4.2 Results

Injection of kaolin/carrageenan into the knee joints of PAR2 knockout mice failed to produce an increase in knee diameter (Figure 4.2A,  $P < 0.001$ ,  $n=6-9$ ), the number of rolling (Figure 4.2B,  $P < 0.01$ ,  $n=6-9$ ) and adherent (Figure 4.2C,  $P < 0.01$ ,  $n=6-9$ ) leukocytes, and vascular conductance (Figure 4.2D,  $P < 0.05$ ,  $n=6-8$ ) as compared to injections in the wild-type counterparts at 24 h post-injection of kaolin/carrageenan.

Intra-articular injection of kaolin/carrageenan into the knee joints of wild-type mice produced a decrease in hindpaw withdrawal threshold (Figure 4.2E,  $P < 0.01$ ,  $n=6$ ) 24 h post-injection, compared to baseline. However, PAR2 knockouts failed to produce a decrease in hindpaw withdrawal threshold (Figure 4.2E,  $P = 0.99$ ,  $n=6$ ) 24 h post-kaolin/carrageenan injection.

## **4.5 Characterization of Freund's complete adjuvant (FCA)-induced joint inflammation and pain in rats**

### **4.5.1 Method**

For the assessment of the role of endogenous neutrophil elastase in the FCA model Wistar rats were used. It has been suggested that rats produce more robust inflammation and pain than mice in the FCA model (Bolon et al., 2011). FCA (50  $\mu$ l) was injected into the intra-articular space of the right knee joint of deeply anesthetized (2–4% isoflurane; 100% oxygen at 1 L/min) rats and the limb was flexed and extended for 30 s to disperse FCA throughout the joint. Joint inflammation was assessed by measuring knee joint diameter using Vernier calipers (refer to section 2.3.1), leukocyte trafficking using IVM (refer to section 2.3.2.2), and mean blood perfusion using LASCA (refer to section 2.3.2.3) in separate cohorts at baseline and on days 3, 7, 14 and 21 post-injection. Animals were surgically prepared before assessing leukocyte trafficking and mean blood perfusion (refer to section 2.3.2.1).

A separate cohort of rats was injected with saline (50  $\mu$ l) or FCA (50  $\mu$ l) into the intra-articular space of the right knee joint and behavioural pain was assessed using von Frey hair algometry (refer to section 2.5.1) and dynamic incapacity (refer to section 2.5.2) at baseline and on days 1, 3, 7, 10, 14, 17 and 21 post-injection.

### **4.5.2 Results**

Intra-articular injection of FCA caused a significant increase in knee joint diameter on day 3 (Figure 4.3A,  $P < 0.0001$ ,  $n=6$ ), day 7 (Figure 4.3A,  $P < 0.0001$ ,  $n=6$ ),

day 14 (Figure 4.3A,  $P < 0.0001$ ,  $n=6$ ) and day 21 (Figure 4.3A,  $P < 0.0001$ ,  $n=6$ ) post-injection, as compared to baseline. FCA caused an increase in the number of rolling leukocytes on day 3 (Figure 4.3B,  $P < 0.05$ ,  $n=6$ ), day 7 (Figure 4.3B,  $P < 0.01$ ,  $n=6$ ), day 14 (Figure 4.3B,  $P < 0.0001$ ,  $n=6$ ) and day 21 (Figure 4.3B,  $P < 0.0001$ ,  $n=6$ ) post-injection, as compared to baseline. Similarly, the number of adherent leukocytes were increased significantly on day 3 (Figure 4.3C,  $P < 0.0001$ ,  $n=6$ ), day 7 (Figure 4.3C,  $P < 0.0001$ ,  $n=6$ ), day 14 (Figure 4.3C,  $P < 0.001$ ,  $n=6$ ) and day 21 (Figure 4.3C,  $P < 0.05$ ,  $n=6$ ) post-injection, as compared to baseline. However, blood perfusion (Figure 4.3D,  $P = 0.97$ ,  $n=6$ ) did not change over the three-week time course post-injection of FCA.

Intra-articular injection of FCA produced a decrease in hindpaw withdrawal threshold on day 3 (Figure 4.3E,  $P < 0.05$ ,  $n=5$ ), day 7 (Figure 4.3E,  $P < 0.0001$ ,  $n=5$ ), day 10 (Figure 4.3E,  $P < 0.0001$ ,  $n=5$ ), day 14 (Figure 4.3E,  $P < 0.0001$ ,  $n=5$ ), day 17 (Figure 4.3E,  $P < 0.0001$ ,  $n=5$ ) and day 21 (Figure 4.3E,  $P < 0.0001$ ,  $n=5$ ) post-injection, as compared to saline control. FCA-injection also induced weight-bearing deficits in animals with a significant difference observed on day 1 (Figure 4.3F,  $P < 0.0001$ ,  $n=6$ ), day 3 (Figure 4.3F,  $P < 0.0001$ ,  $n=5$ ), day 7 (Figure 4.3F,  $P < 0.0001$ ,  $n=5$ ), day 10 (Figure 4.3F,  $P < 0.0001$ ,  $n=5$ ), day 14 (Figure 4.3F,  $P < 0.0001$ ,  $n=5$ ), day 17 (Figure 4.3F,  $P < 0.0001$ ,  $n=5$ ) and day 21 (Figure 4.3F,  $P < 0.0001$ ,  $n=5$ ) post-injection, as compared to saline-injected animals ( $n=10$ ).

## **4.6 Effect of neutrophil elastase inhibition on FCA-induced joint inflammation and pain**

### **4.6.1 Method**

Injection of FCA caused joint inflammation which appeared on day 3 and persisted to day 21. Similarly, mechanosensitivity and weight-bearing deficits appeared immediately and lasted to three-weeks post-injection of FCA. Our aim was to assess the effect of prophylactic treatment with the neutrophil elastase inhibitors on FCA-induced joint inflammation and pain. Inflammatory changes were assessed on day 21 post-injection of FCA. The pain response was assessed throughout the three-week time course.

The following protocol was used:

- Rats were treated with serpinA1 (100 µg i.p.) on day -1 and, 15 min before FCA injection (50 µl) into the right knee joint on day 0, and once on days 2, 4 and 6 post-FCA injection. Inflammation was assessed at day 21 post-injection of FCA
- Rats were treated with sivelestat (50 mg/kg i.p.) 15 min before and 240 min after FCA injection (50 µl) into the right knee joint on day 0, and once on days 1 to 5 post-FCA injection. Inflammation was assessed at day 21 post-injection of FCA.
- Rats were treated with sivelestat (50 mg/kg i.p.) 15 min before and 240 min after FCA injection (50 µl) into the right knee joint on day 0, and once on days 1 to 5 post-FCA injection. Pain was assessed on days 1, 3, 7, 10, 14, 17 and 21 post-injection of FCA.

#### 4.6.2 Results

Prophylactic treatment with an endogenous inhibitor of neutrophil elastase, serpinA1, blocked the increase in the number of rolling (Figure 4.4B,  $P < 0.05$ ,  $n=5$ ) and adherent (Figure 4.4C,  $P < 0.05$ ,  $n=5$ ) leukocytes on day 21 post-injection, compared with FCA alone. Treatment with a synthetic inhibitor of neutrophil elastase, sivelestat, blocked the increase in the number of adherent leukocytes (Figure 4.4C,  $P < 0.01$ ,  $n=6$ ) on day 21 post-injection, compared with FCA control. Knee joint diameter (Figure 4.4A,  $P = 0.67$ ,  $n=5$ ) and mean blood perfusion (Figure 4.4D,  $P = 0.07$ ,  $n=5$ ) did not change after treatment with the neutrophil elastase inhibitors.

Prophylactic treatment with a synthetic inhibitor of neutrophil elastase, sivelestat, prevented the decrease in the withdrawal threshold on day 7 (Figure 4.4E,  $P < 0.05$ ,  $n=6$ ) post-injection, compared with FCA alone. However, at later time points no improvement in the withdrawal threshold was observed. Likewise, weight-bearing deficits were prevented in sivelestat treated animals only on day 3 (Figure 4.4F,  $P < 0.05$ ,  $n=6$ ), compared with FCA alone.

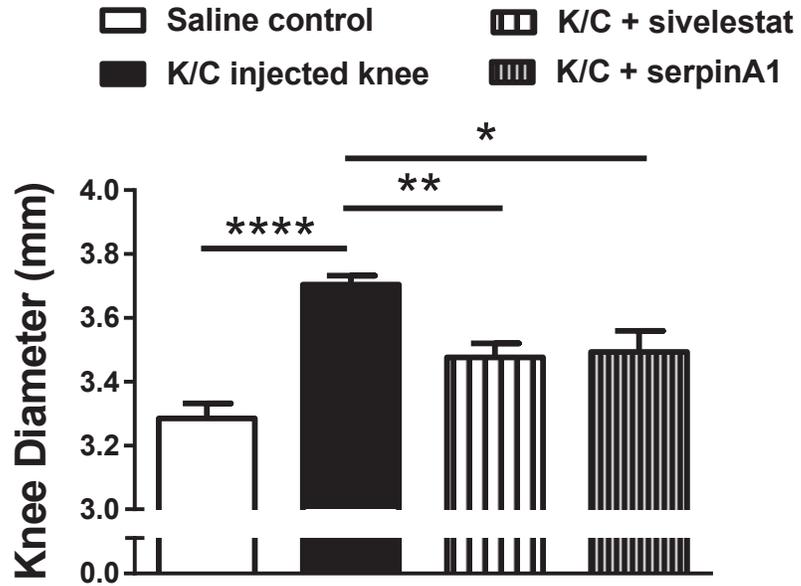
#### **4.7 Chapter Summary**

The results of this study suggest that local administration of kaolin/carrageenan into the knee joints of mice caused an acute joint inflammation and pain. Inhibition of endogenous neutrophil elastase in the kaolin/carrageenan model prevented the joint inflammation. However, neutrophil elastase inhibitors were unable to prevent a decrease in the hindpaw withdrawal threshold. Lack of the PAR2 gene prevented both joint inflammation and pain due to injection of kaolin/carrageenan in mice.

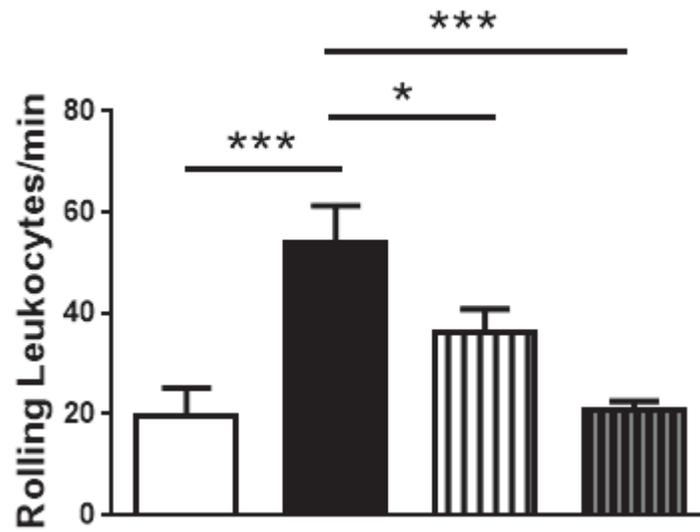
Injecting rats with FCA triggered joint inflammation, mechanosensitivity and weight-bearing deficits which appeared immediately and persisted throughout the three-week time course. Prophylactic blockade of endogenous neutrophil elastase produced mild anti-inflammatory effects on day 21 in adjuvant-injected joints. Mechanosensitivity and weight-bearing deficits were only prevented acutely after treatment with neutrophil elastase inhibitors.

4.8 Figures

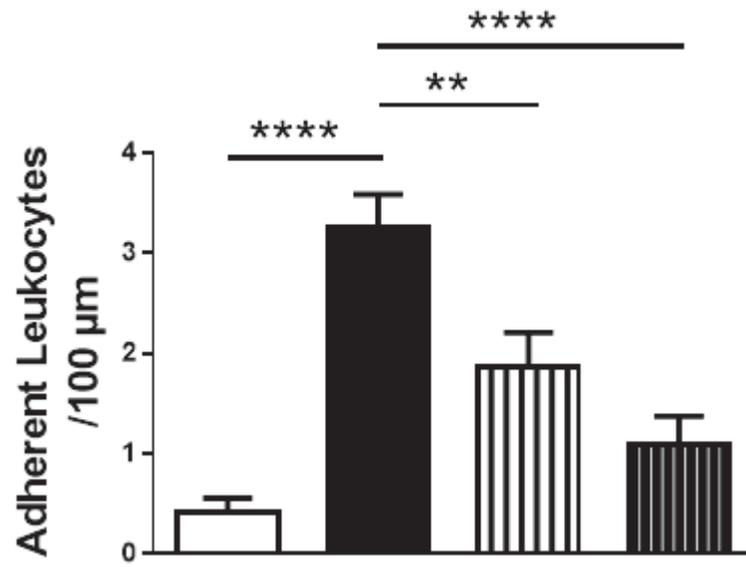
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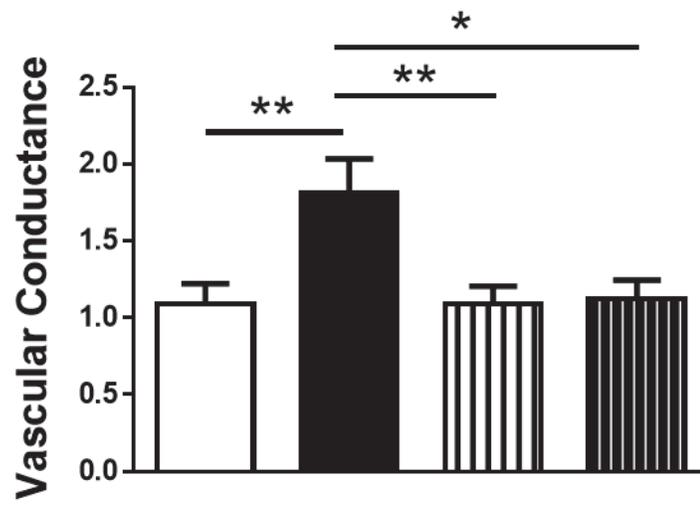
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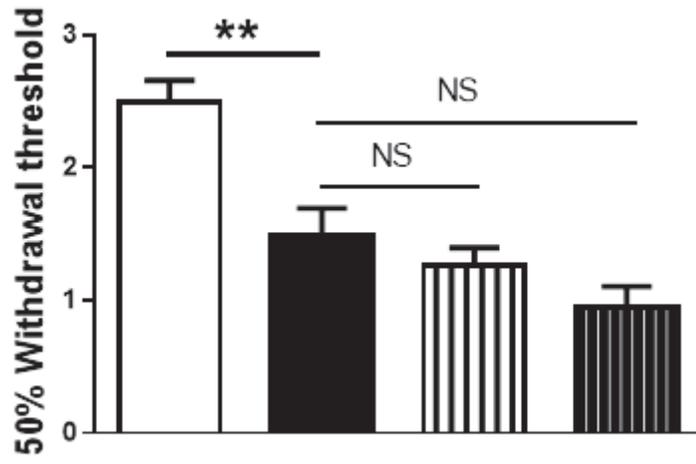
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**E**

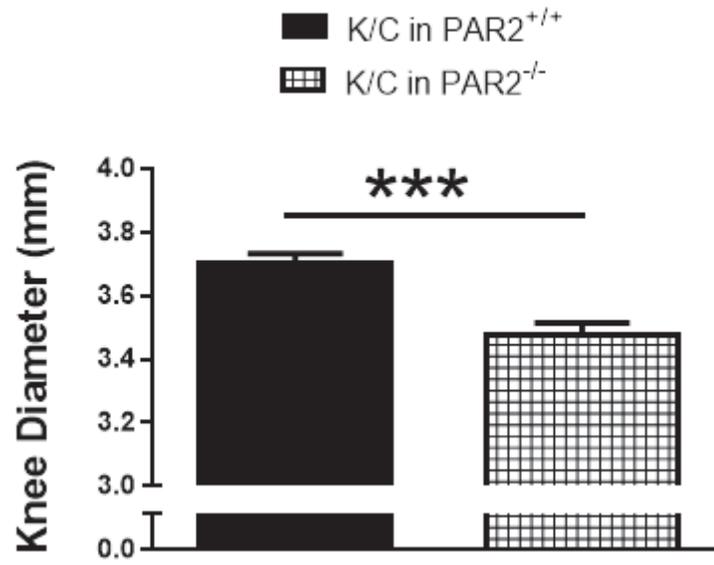


## Figure 4.1

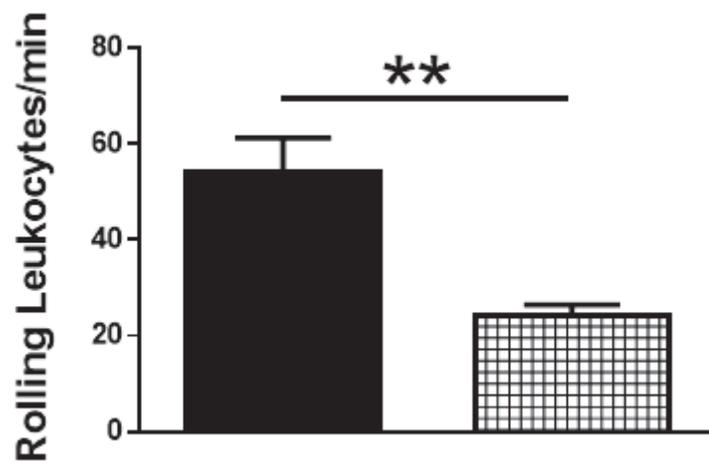
### **Effect of neutrophil elastase inhibition on kaolin-carrageenan-induced joint**

**inflammation and pain.** Injection of kaolin/carrageenan (K/C) caused an increase in (A) knee diameter, the number of (B) rolling and (C) adherent leukocytes, (D) vascular conductance, and caused (E) a decrease in the withdrawal threshold at 24 h post-injection. Treatment with the neutrophil elastase inhibitors sivelestat (50 mg/kg i.p.) or serpinA1 (10 µg i.p.) significantly blocked the increase in knee diameter, and the number of rolling and adherent leukocytes, and vascular conductance. Withdrawal threshold did not improve after treatment with the neutrophil elastase inhibitors. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001, compared with kaolin/carrageenan alone, one-way ANOVA with Dunnett's *post hoc* test, n=6-17 per group. (Previously published, Muley et al., 2016).

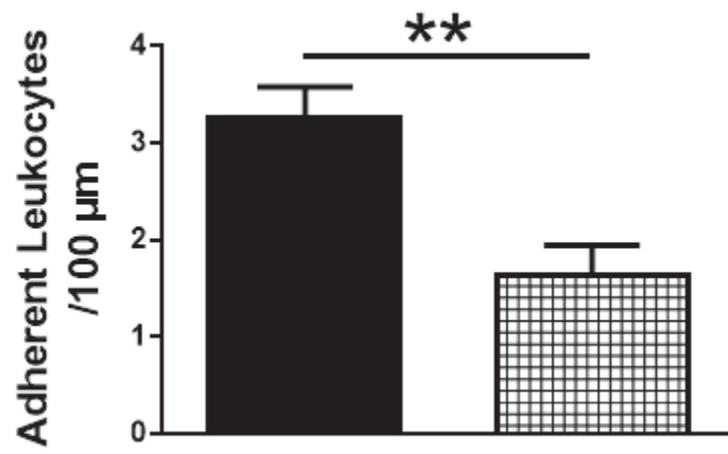
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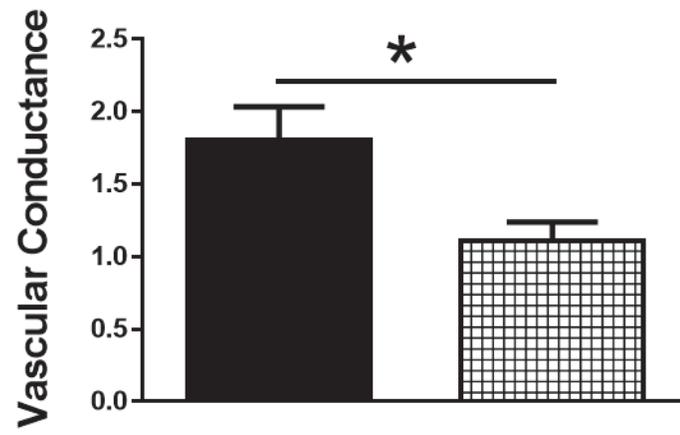
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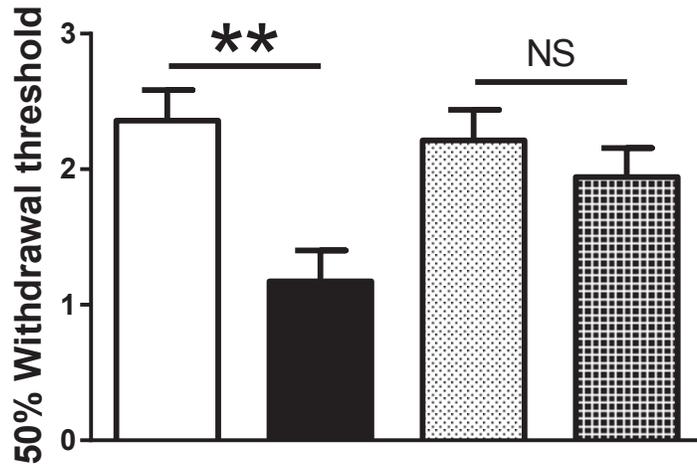


D



**E**

□ PAR2<sup>+/+</sup> - baseline      ▨ PAR2<sup>-/-</sup> - baseline  
■ PAR2<sup>+/+</sup> - day 1 (K/C i.artic.)      ▩ PAR2<sup>-/-</sup> - day 1 (K/C i. artic.)



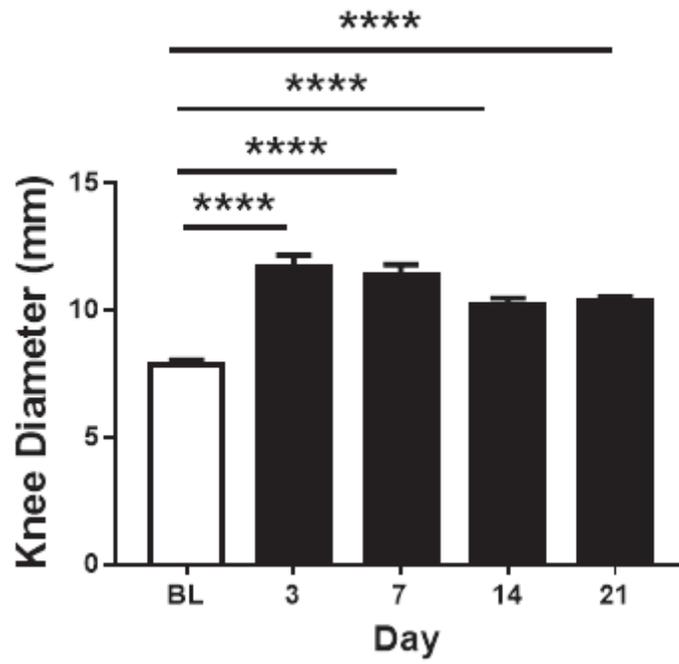
## Figure 4.2

### **Involvement of PAR2 in mediating kaolin-carrageenan-induced joint inflammation**

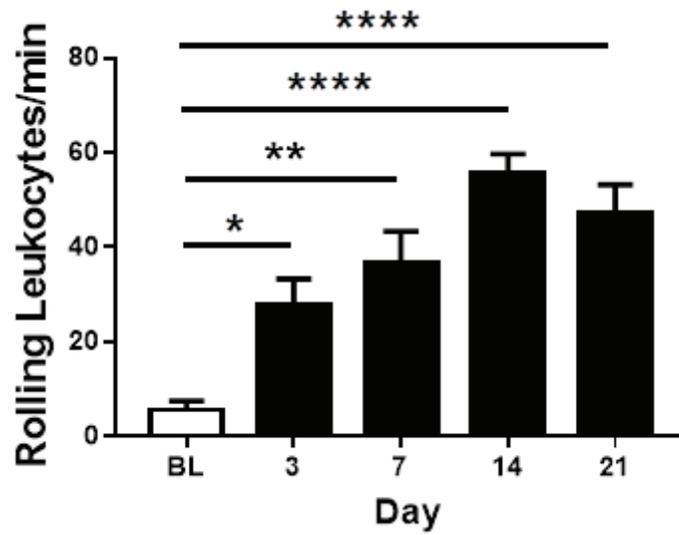
**and pain.** Injection of kaolin/carrageenan (K/C) into the knee joint of PAR2 knockout mice failed to cause an increase in **(A)** knee diameter, the number of **(B)** rolling and **(C)** adherent leukocytes, and **(D)** vascular conductance, 24 h post-injection, compared to wild-type mice. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, Student's unpaired t-test, n=6-9 per group.

**(E)** Injection of kaolin/carrageenan into the knee joint of wild-type mice caused a decrease in the withdrawal threshold at 24 h post-injection. PAR2 knockouts did not exhibit this effect. \*\*P < 0.01, one-way ANOVA with Bonferroni's multiple comparisons *post hoc* test, n=6 per group.

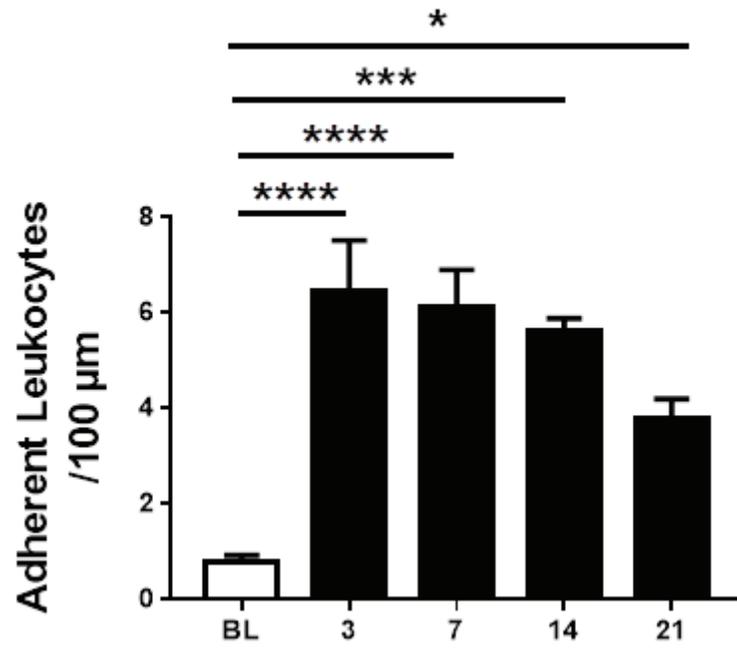
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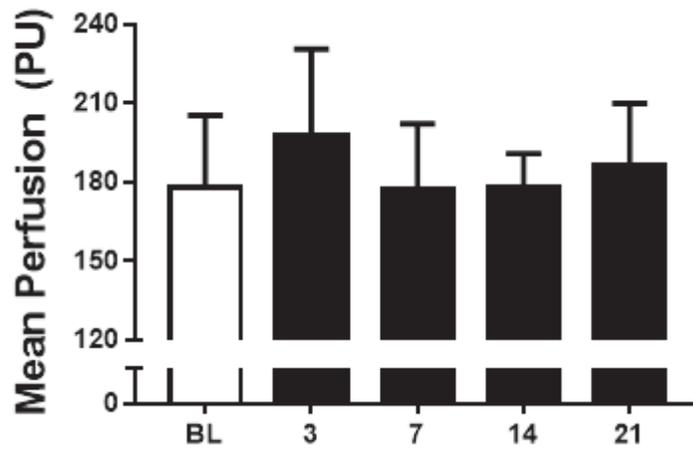
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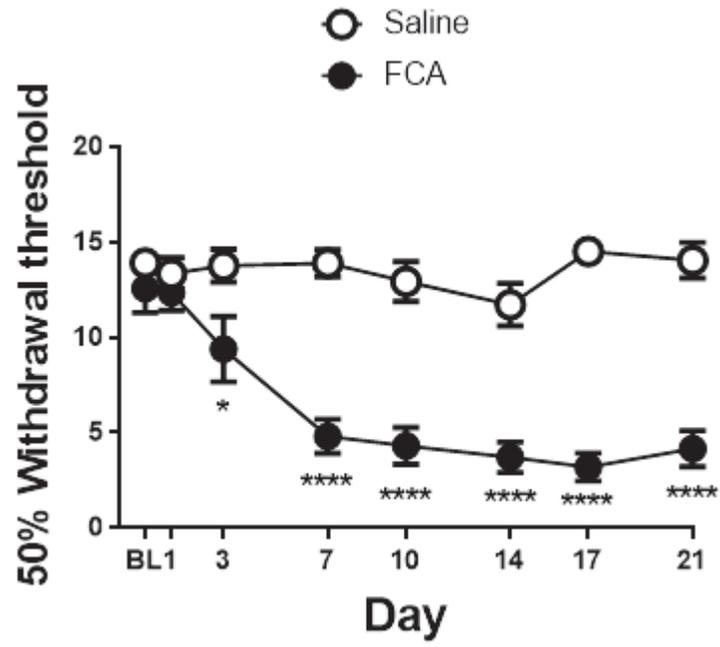
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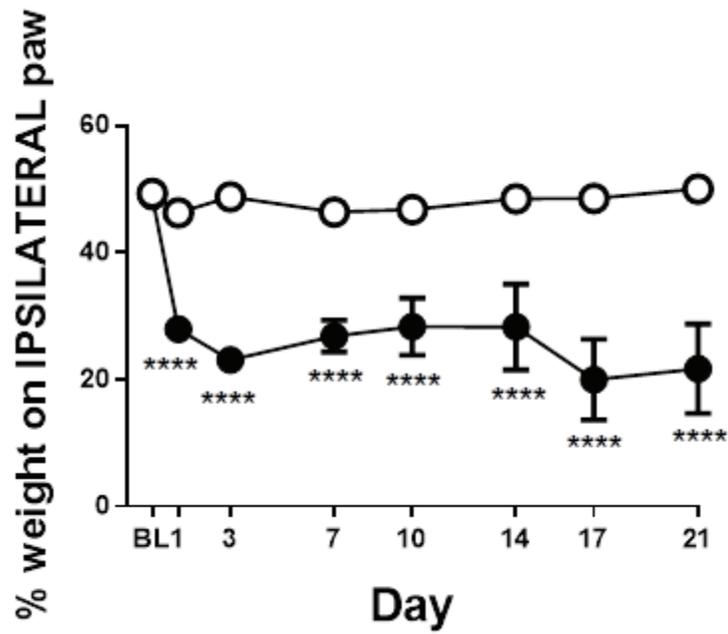
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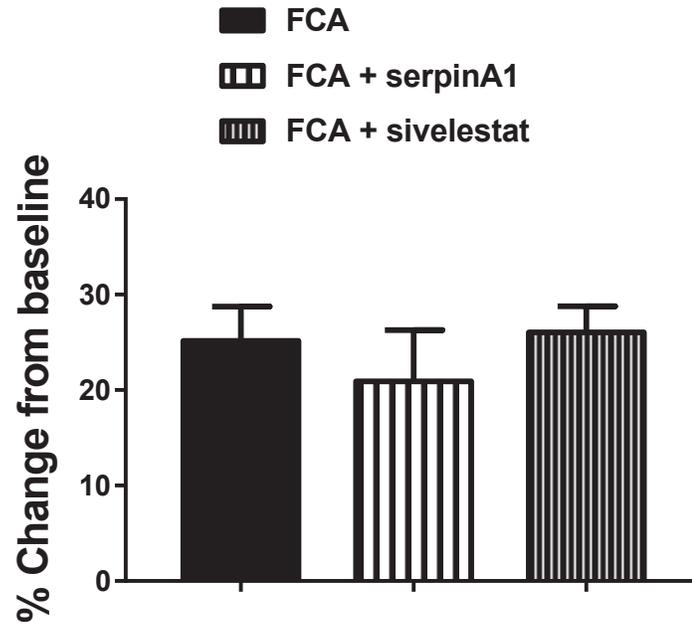


### Figure 4.3

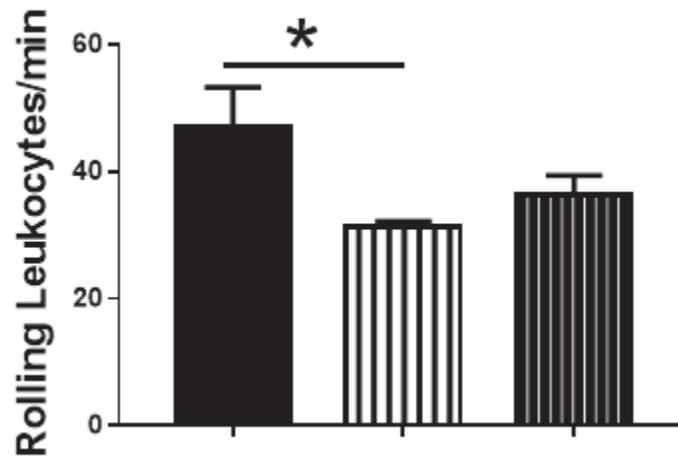
**FCA-induced knee joint inflammation and pain.** Intra-articular injection of FCA caused a significant increase in **(A)** knee diameter, the number of **(B)** rolling and **(C)** adherent leukocytes on days 3, 7, 14, 21 post-injection. **(D)** Mean perfusion did not change after injection of FCA. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001, compared with baseline, one-way ANOVA with Dunnett's *post hoc* test, n=6 per time point.

**(E)** Intra-articular injection of FCA caused a significant decrease in the withdrawal threshold on days 3, 7, 10, 14, 17 and 21 post-injection. **(F)** FCA caused weight-bearing deficits on days 1, 3, 7, 10, 14, 17 and 21 post-injection. \*P < 0.05, \*\*\*\*P < 0.0001, compared with saline control, two-way ANOVA with Bonferroni's multiple comparisons *post hoc* test, n=5-10 per time point.

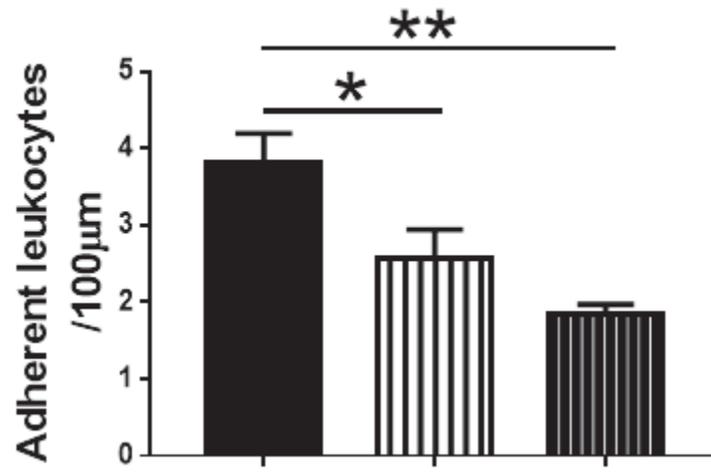
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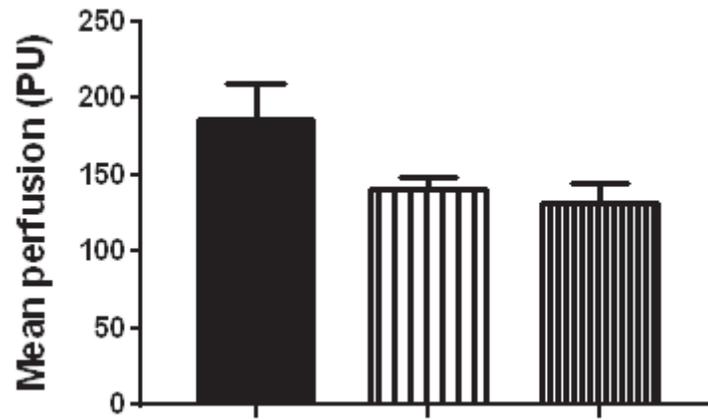
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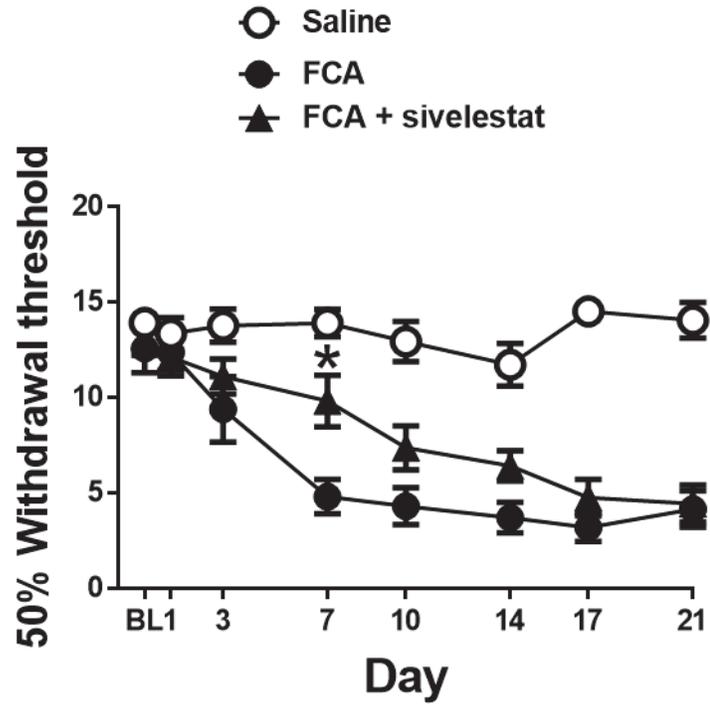
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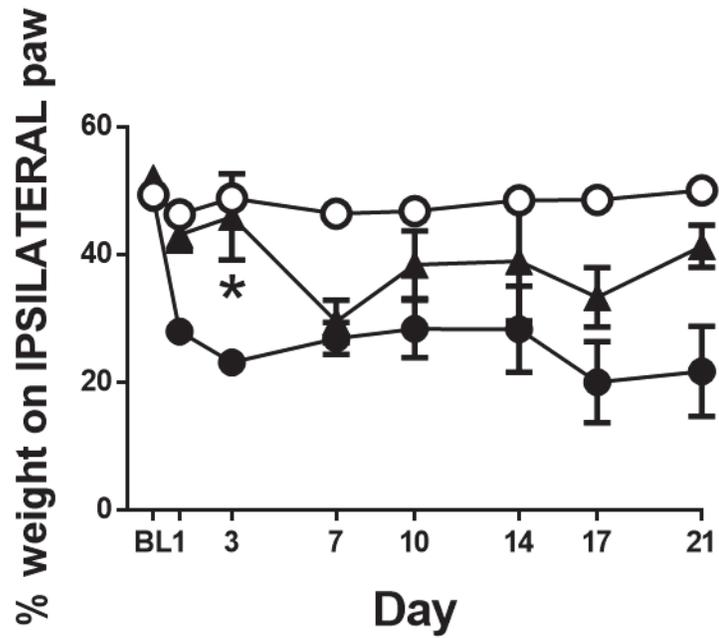
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**E**



**F**



#### **Figure 4.4**

##### **Effect of neutrophil elastase inhibition on FCA-induced joint inflammation and**

**pain.** Prophylactic treatment with the neutrophil elastase inhibitors serpinA1 (10 µg i.p.) or sivelestat (50 mg/kg i.p.) blocked the increase in the number of **(B)** rolling and **(C)** adherent leukocytes at day 21 post-injection of FCA. **(A)** Knee diameter and **(D)** mean perfusion did not improve after treatment with the neutrophil elastase inhibitors on day 21 post-injection of FCA. \*P < 0.05, \*\*P < 0.01, compared with FCA control, one-way ANOVA with Dunnett's *post hoc* test, n=5-9 per group.

**(E)** Prophylactic treatment with the neutrophil elastase inhibitor sivelestat improved hindpaw withdrawal threshold on day 7 post-injection of FCA. **(F)** Weight-bearing deficits were improved after treatment with sivelestat on day 3 post-injection of FCA. \*P < 0.05, two-way ANOVA with Bonferroni's multiple comparisons *post hoc* test, n=5-6 time point.

## **Chapter 5: The Effect of Early Blockade of Neutrophil Elastase and Proteinase-Activated Receptor-2 on the Development of Joint Inflammation, Pain and Neuropathy Associated with Monoiodoacetate-Induced Osteoarthritis**

### **5.1 Disclosures**

- Most results presented in this chapter have been included in the manuscript which was recently revised and submitted to the Journal of Neuroinflammation.
- Data from the characterization of the monoiodoacetate model of joint inflammation experiment (Figure 5.1) has been included in a research article published in the journal of Arthritis Research & Therapy (McDougall et al., 2017a; Original publishers - BioMed Central).

## 5.2 Background and hypotheses

Osteoarthritis is one of the leading cause of chronic pain and disability worldwide (Berenbaum, 2013). It has been suggested that synovial inflammation could contribute to the development of arthritic symptoms (Berenbaum, 2013). Experimental evidence suggests that synovitis is correlated with knee joint pain and the structural progression of the disease (Ayril et al., 2005; Baker et al., 2010). Hence it has been proposed that, for a subset of OA patients, synovitis could be used as a marker for assessing the severity of the disease (Ayril et al., 2005; Baker et al., 2010).

A variety of factors such as trauma, old age, and obesity can increase the risk of OA joint degeneration (Nuki, 1999). A cocktail of inflammatory mediators is released into the joint space following injury, which contributes to further damage and the development of pain. Cytokines, for example, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 play a significant role in driving inflammatory processes within joints (Saklatvala, 1986; Goldring et al., 1988; Kaneko et al., 2000). These inflammatory cytokines also possess the capacity to initiate neuropathic pain by causing neuronal damage (Milligan et al., 2003; Schafers et al., 2003). It should be noted that the peripheral nervous system can also contribute to the development of joint inflammation (Levine et al., 1985). Additionally, neuropeptides such as SP and CGRP which are released from the afferent neurons can contribute to the inflammatory response associated with OA (McDougall et al., 1999; Ferland et al., 2011). These neuropeptides are capable of inducing peripheral neuropathy by causing direct damage to the nerves (Tatsushima et al., 2011; Malon et al., 2011).

Data from our previous studies indicate that blockade of endogenous neutrophil and PAR2 reduces joint inflammation and pain associated with inflammatory

monoarthritis. A recent study has shown that activation of PAR2 by neutrophil elastase further results in functional activation of TRPV4 to elicit neuronal sensitization, inflammation and pain (Zhao et al., 2015). PAR2 has been identified on synovium, bone, and articular chondrocytes which implies their potential involvement in the structural damage associated with arthritis (Abraham et al., 2000; Xiang et al., 2006; Kelso et al., 2006). Interestingly, deletion of the PAR2 gene prevents the structural damage associated with post-traumatic OA models (Ferrell et al., 2010; Jackson et al., 2014; Huesa et al., 2016). PAR2 activation on knee joint afferents leads to joint inflammation and neuronal sensitization (Russell et al., 2012) and this response is mediated by TRPV1 and NK-1 receptors (Helyes et al., 2010; Russell et al., 2012).

A growing body of evidence suggests that 60-74% of patients suffering from OA experience low-grade inflammation within the joint (Guermazi et al., 2014; Neogi et al., 2016; Mathiessen and Conaghan, 2017) which can contribute to neurodegeneration. Therefore, intervention that could alleviate the development of inflammation presents a strategy to impede the neurodegeneration process and improve joint neuropathic pain. Intra-articular injection of MIA, a GAPDH inhibitor, causes death of chondrocytes by disrupting the glycolytic pathway (Kalbhen, 1987). This is a routinely used model for studying OA pain (Kalbhen, 1987). Studies indicate that injection of MIA into the joint produces an acute inflammatory response which usually resolves within a week (Bove et al., 2003; Fernihough et al., 2004; Beyreuther et al., 2007). Several studies report for the presence of a peripheral neuropathic component in the MIA model (Ivanavicius et al., 2007; Orita et al., 2011; Thakur et al., 2012). Therefore, it is possible that the early inflammation observed in this model could contribute to the development of late-stage

neuropathy and neuropathic pain. The first goal of this study was to characterize the inflammatory component of the MIA model by assessing joint oedema, leukocyte trafficking, and synovial blood flow. The involvement of neutrophil elastase and PAR2 in mediating MIA-induced joint inflammation, pain and neuropathy were then investigated.

**The following hypotheses were tested in this study:**

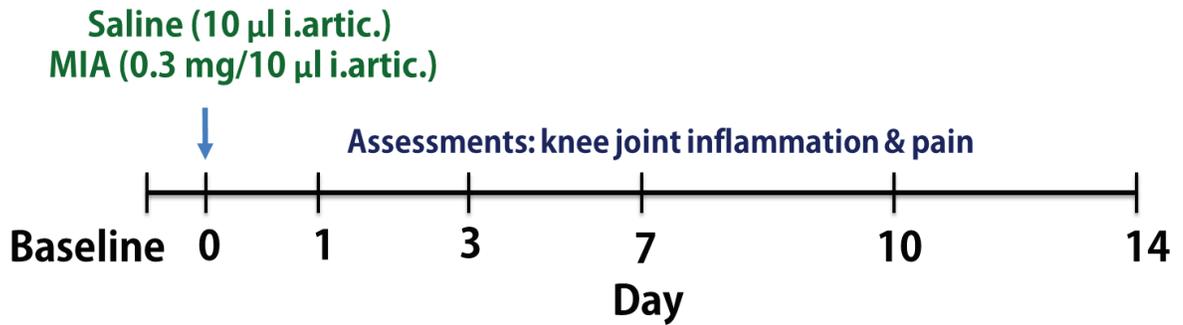
- I. Local administration of MIA increases the proteolytic activity of neutrophil elastase during the acute inflammatory phase of the MIA model.**
- II. Inhibition of neutrophil elastase decreases the increased proteolytic activity of neutrophil elastase during the acute inflammatory phase of the MIA model.**
- III. Prophylactic inhibition of neutrophil elastase during the inflammatory phase of MIA-induced arthritis attenuates the development of MIA-induced joint inflammation, pain and neuropathy.**
- IV. Prophylactic modulation of PAR2 during the inflammatory phase of MIA-induced arthritis attenuates the development of MIA-induced joint inflammation, pain and neuropathy.**

## **5.3 Characterization of MIA-induced joint inflammation and pain in mice**

### **5.3.1 Method**

Experimental OA was induced by injecting MIA (0.3 mg/10  $\mu$ l) into the right knee joint of deeply anesthetized mice (2–4% isoflurane; 100% oxygen at 1 L/min). A separate group of mice was injected with physiological saline (10  $\mu$ l) into the right knee joint and the limb was flexed and extended for 30 s to disperse the solutions throughout the joint. Inflammatory assessments included measuring joint oedema using Vernier calipers (refer to section 2.3.1), leukocyte-endothelial interactions using IVM (refer to section 2.3.2.2), and synovial blood perfusion using LASCA (refer to section 2.3.2.3). These inflammation parameters were measured in separate cohorts at baseline and on days 1, 3, 7, 10 and 14 post-injection. Before vascular assessments, a surgical preparation was required which involved cannulations of the trachea, carotid artery and jugular vein (refer to section 2.3.2.1). The mean blood perfusion values obtained using LASCA were normalised against the mean arterial pressure of that mouse to give a vascular conductance value (for equation refer to section 2.3.2.3).

A separate group of mice was injected with saline (10  $\mu$ l) or MIA (0.3 mg/10  $\mu$ l) into the right knee joint and behavioural pain was assessed using von Frey hair algometry (refer to section 2.5.1) and dynamic incapacitance (refer to section 2.5.2) at baseline and on days 1, 3, 7, 10 and 14 post-injection.



### 5.3.2 Results

Intra-articular injection of MIA caused a significant increase in knee joint diameter on day 1 (Figure 5.1A,  $P < 0.0001$ ,  $n=14-28$ ), day 3 (Figure 5.1A,  $P < 0.0001$ ,  $n=6-11$ ) and day 10 (Figure 5.1A,  $P < 0.05$ ,  $n=4-11$ ) post-injection, compared to saline-injected animals. However, the knee joint diameter returned to baseline by the end of two weeks. Likewise, injection of MIA produced an increase in the number of rolling leukocytes (Figure 5.1B,  $P < 0.05$ ,  $n=13-27$ ), adherent leukocytes (Figure 5.1C,  $P < 0.0001$ ,  $n=15-27$ ) and vascular conductance (Figure 5.1D,  $P < 0.05$ ,  $n=15-27$ ) on day 1 as compared to saline control. However, at later time points there was no statistical difference observed between saline and MIA-injected animals.

Intra-articular injection of MIA produced a decrease in hindpaw withdrawal threshold on day 1 which persisted to day 14 (Figure 5.1E,  $P < 0.0001$ ,  $n=10-15$ ) post-injection, as compared to saline control. MIA induced weight-bearing deficits with a significant difference observed on day 3 (Figure 5.1F,  $P < 0.01$ ,  $n=19-20$ ) post-injection, as compared to saline-injected animals.

## **5.4 Assessment of neutrophil elastase proteolytic activity in MIA-inflamed joints**

### **5.4.1 Method**

As injection of MIA caused an inflammatory response which peaked on day 1 and subsided by day 14, the initial aim was to test the proteolytic activity of neutrophil elastase at these two-time points post-MIA injection.

The following protocol was used:

- Mice were deeply anesthetized (2–4% isoflurane; 100% oxygen at 1 L/min) and MIA (0.3 mg/10 µl) was injected into the right knee joint on day 0. The proteolytic activity of neutrophil elastase in the inflamed knee joints was determined (refer to section 2.4) using the substrate NE 680 FAST (1 nmol/25 µl s.c. over the knee joint) on days 1 and 14 post-MIA injection.
- A separate cohort of MIA-injected animals was treated on day 1 with a synthetic inhibitor of neutrophil elastase (sivelestat, 50 mg/kg i.p.) and proteolytic activity was assessed 4 hours later.
- A separate group of MIA-injected animals received treatment with an endogenous inhibitor of neutrophil elastase (serpinA1, 10 µg i.p.) administered 15 min before and 12 hours after MIA injection and the proteolytic activity was assessed 24 hours after injection of MIA (the dosing schedule used for serpinA1 was based on its half-life).

### **5.4.2 Results**

Intra-articular injection of MIA caused an increase in the proteolytic activity of neutrophil elastase within the inflamed knee joints which was significantly reduced by

treatment with the neutrophil elastase inhibitors sivelestat (Figure 5.2B,  $P < 0.001$ ,  $n = 5$ ) and serpinA1 (Figure 5.2B,  $P < 0.001$ ,  $n = 5$ ) on day 1 post-injection. Also, the proteolytic activity of neutrophil elastase was found to be low on day 14 compared to day 1 (Figure 5.2B,  $P < 0.0001$ ,  $n = 7$ ).

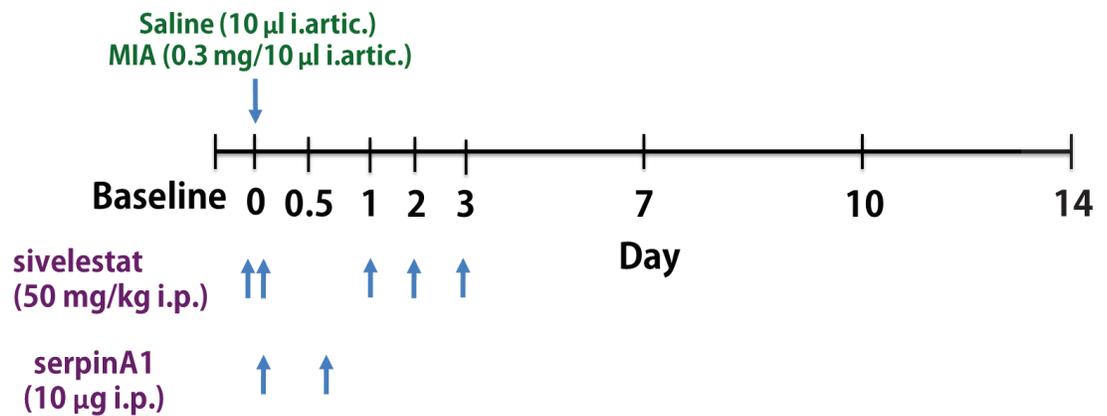
## **5.5 Effect of prophylactic inhibition of neutrophil elastase on MIA-induced knee joint inflammation and pain**

### **5.5.1 Method**

After confirming the proteolytic activity of neutrophil elastase in MIA-inflamed joints, the next aim was to assess the functional relevance of neutrophil elastase in the MIA model; therefore, the effects of prophylactic inhibition of neutrophil elastase on the development of MIA-induced joint inflammation and pain were assessed. The following protocol was used:

- Mice were treated with sivelestat (50 mg/kg i.p.) administered 10 min before and 240 min after MIA injection (0.3 mg/10  $\mu$ l) into the right knee joint on day 0 and once on days 1 to 3 post-MIA injection. Inflammation was assessed on days 1, 3, 7, 10 and 14 post-injection.
- A separate cohort of mice received treatment with serpinA1 (10  $\mu$ g i.p.) administered 15 min before and 12 hours after MIA injection (0.3 mg/10  $\mu$ l) into the right knee joint. Inflammation was assessed on days 1, 3, 7, 10 and 14 post-injection.

- Mice were treated with sivelestat (50 mg/kg i.p.) administered 10 min before and 240 min after MIA injection (0.3 mg/10  $\mu$ l) into the right knee joint on day 0 and once on days 1 to 3 post-MIA injection. Pain was assessed on days 1, 3, 7, 10 and 14 post-injection.
- A separate cohort of mice received treatment with serpinA1 (10  $\mu$ g i.p.) administered 15 min before and 12 hours after MIA injection (0.3 mg/10  $\mu$ l) into the right knee joint. Pain was assessed on days 1, 3, 7, 10 and 14 post-injection.



### 5.5.2 Results

Intra-articular injection of MIA caused acute joint inflammation which peaked on day 1, reduced by day 3 and remained low for the remaining two-week time course.

Treatment with the neutrophil elastase inhibitor sivelestat blocked the increase in knee joint diameter (Figure 5.3A,  $P < 0.0001$ ,  $n = 5-28$  per time point), the number of rolling leukocytes (Figure 5.3B,  $P < 0.01$ ,  $n = 5-28$  per time point) and adherent leukocytes (Figure 5.3C,  $P < 0.001$ ,  $n = 5-28$  per time point) throughout the time course. Likewise, treatment with serpinA1 blocked an increase in the knee joint diameter (Figure 5.3A,  $P < 0.0001$ ,

n=5-28 per time point), the number of rolling leukocytes (Figure 5.3B,  $P < 0.05$ , n=5-28 per time point) and adherent leukocytes (Figure 5.3C,  $P < 0.001$ , n=5-28 per time point) throughout the time course. However, the neutrophil elastase inhibitors did not reduce the increased vascular conductance (Figure 5.3D,  $P = 0.32$  &  $P = 0.09$ , n=5-28 per time point).

Intra-articular injection of MIA induced a decrease in hindpaw withdrawal threshold and weight-bearing. Treatment with the neutrophil elastase inhibitors sivelestat or serpinA1 prevented the decrease in the withdrawal threshold (Figure 5.3E,  $P < 0.0001$ , n=8-9 per time point) throughout the time course. However, weight bearing deficits were not improved after treatment with the neutrophil elastase inhibitors (Figure 5.3F,  $P = 0.99$  &  $P = 0.55$ , n=8-9 per time point) at any time point in the two-week time course.

## **5.6 Involvement of PAR2 in mediating MIA-induced knee joint inflammation and pain**

### **5.6.1 Method**

Inhibition of neutrophil elastase blocked joint inflammation and pain associated with the experimental OA. Our previous findings suggest that neutrophil elastase activates PAR2 to elicit joint inflammation and pain in mice (refer to Chapter 3). Therefore, we wanted to assess the involvement of PAR2 in mediating MIA-induced joint inflammation and pain. To do this, we tested the effect of MIA in the presence of the PAR2 antagonist GB83 and in PAR2 knockout mice. Inflammatory assessments focused on the 24 h time point, as MIA produced its peak effect at this time point.

However, behavioural pain was assessed at several time points over a two-week time course. The following protocol was used:

- Mice were treated with the PAR2 antagonist GB83 (5 µg i.p.), 10 min before and 120 and 240 min after MIA (0.3 mg/10 µl) injection into the right knee joint on day 0 and once on day 1 (60 min before assessment). Inflammation was assessed 24 h post-injection of MIA.
- An intra-articular injection of MIA (0.3 mg/10 µl) was made into the right knee joint of PAR2 knockout mice. Inflammation was assessed 24 h post-injection of MIA.
- Mice were treated with the PAR2 antagonist GB83 (5 µg i.p.), 10 min before and 120 and 240 min after MIA (0.3 mg/10 µl) injection into the right knee joint on day 0 and once on days 1 – 3 post-MIA injection. Pain was assessed at baseline and on days 1, 3, 7, and 14 post-MIA injection.
- An intra-articular injection of saline (10 µl) or MIA (0.3 mg/10 µl) was made into the right knee joint of wild-type or PAR2 knockout mice. Pain was assessed at baseline and on days 1, 3, 7, 10 and 14 post-MIA injection.

### **5.6.2 Results**

Intra-articular injection of MIA caused joint oedema and leukocyte trafficking on day 1 post-injection. Treatment with the PAR2 antagonist GB83 blocked the increase in knee joint diameter (Figure 5.4A,  $P < 0.05$ ,  $n=9$ ) and the number of adherent leukocytes (Figure 5.4C,  $P < 0.001$ ,  $n=9$ ), compared to MIA control. In PAR2 knockouts, injection

of MIA failed to increase knee joint diameter (Figure 5.4A,  $P < 0.05$ ,  $n=9$ ), the number of rolling (Figure 5.4B,  $P < 0.05$ ,  $n=9$ ) and adherent (Figure 5.4C,  $P < 0.01$ ,  $n=9$ ) leukocytes on day 1 post-injection. There was no difference observed for vascular conductance between any of the treatment groups on day 1 post-injection (Figure 5.4D,  $P = 0.08$ ,  $n=9$ ).

Intra-articular injection of MIA caused a decrease in the withdrawal threshold which appeared on day 1 and persisted to day 14. Prophylactic treatment with the PAR2 antagonist prevented the decrease in the hindpaw withdrawal threshold (Figure 5.5A,  $P < 0.001$ ,  $n=8$ ) throughout the two-week time course. Treatment with the PAR2 antagonist did not improve weight-bearing deficits caused by injection of MIA (Figure 5.5B,  $P = 0.09$ ,  $n=5$ ).

Intra-articular injection of saline had no effect on the withdrawal threshold in both wild-type and PAR2 knockouts (Figure 5.5C) throughout the two-week time course. Injection of MIA produced a decrease in the withdrawal threshold in both wild-type (Figure 5.5C,  $P < 0.0001$ ,  $n=10$ ) and PAR2 knockouts (Figure 5.5C,  $P < 0.001$ ,  $n=10$ ) as compared to their respective saline controls. Interestingly, the withdrawal threshold observed in PAR2 knockouts was significantly less severe compared to wild-type mice (Figure 5.5C,  $P < 0.0001$ ,  $n=10$ ). Intra-articular injection of saline did not induce weight bearing deficits in wild-type or PAR2 knockouts (Figure 5.5D) throughout the two-week time course. Injection of MIA induced weight-bearing deficits in both wild-type (Figure 5.5D,  $P < 0.01$ ,  $n=10$ ) and PAR2 knockouts (Figure 5.5D,  $P < 0.001$ ,  $n=10$ ) compared to their respective saline controls.

## **5.7 Effect of neutrophil elastase inhibition on MIA-induced saphenous nerve demyelination**

### **5.7.1 Methods**

There is evidence for the presence of a peripheral neuropathic component in the MIA model (Ivanavicius et al., 2007; Thakur et al., 2012); therefore, we wanted to assess the involvement of neutrophil elastase and PAR2 in MIA-induced saphenous nerve demyelination. To do this, we isolated saphenous nerves at day 14 post-injection of MIA from pain experiment animals (refer to section 5.5 and 5.6). The nerves were processed (refer to section 2.6) and G-ratio (measure of myelin thickness) was calculated for a sample of saphenous nerve fibres (refer to section 2.7).

### **5.7.2 Results**

Intra-articular injection of MIA caused an increase in the G-ratio value compared to saline-injected animals (Figure 5.6,  $P < 0.01$ ,  $n=8$ ) indicating demyelination of the saphenous nerve fibres. Treatment with the neutrophil elastase inhibitors sivelestat (Figure 5.6,  $P < 0.05$ ,  $n=6$ ) and serpinA1 (Figure 5.6,  $P < 0.01$ ,  $n=5$ ) prevented the demyelination due to injection of MIA.

MIA caused an increase in the G-ratio value compared to saline-injected wild-type mice (5.7A,  $P < 0.01$ ,  $n=8$ ) indicating nerve demyelination. Interestingly, there was no difference observed between G-ratio values of saline and MIA-injected PAR2

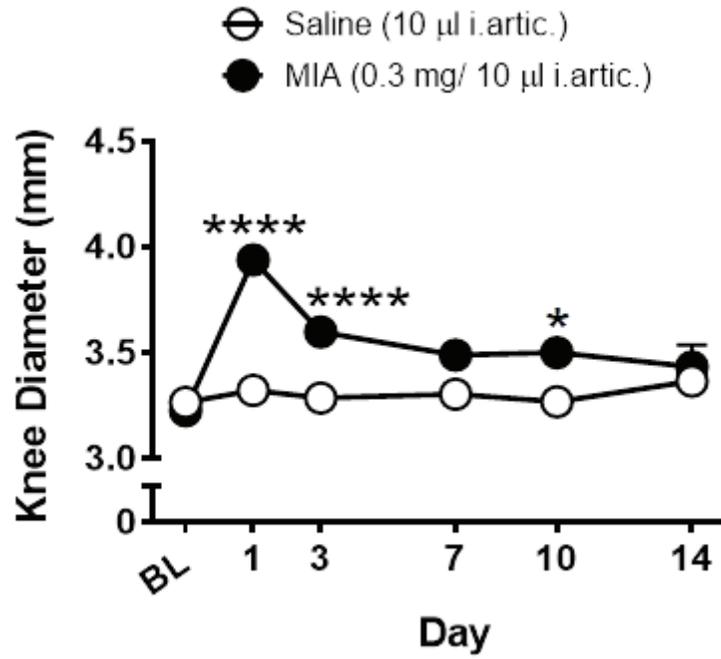
knockout mice which suggests that PAR2 is necessary for MIA-induced demyelination (5.7B,  $P = 0.81$ ,  $n = 5-10$ ).

## 5.8 Chapter Summary

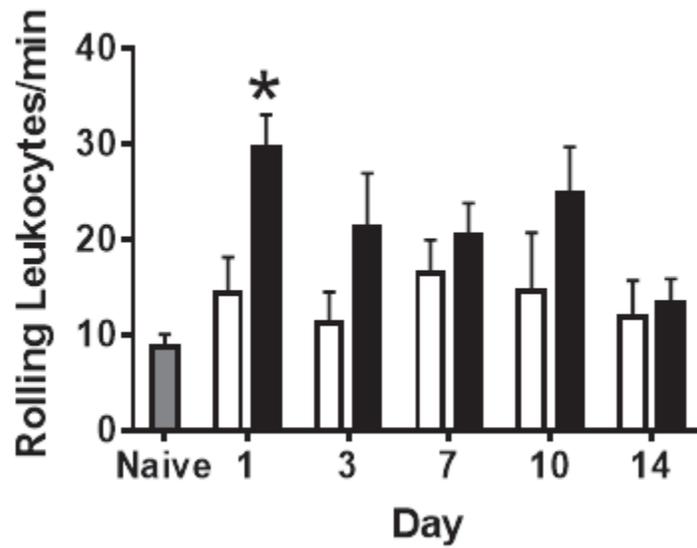
The results of this study suggest that local administration of MIA into the knee joints of mice causes an acute inflammatory response which appears on day 1, declines by day 3 and remains low for the remaining two-week time course. The pain response due to MIA appears on day 1 and persists to day 14 post-injection. The proteolytic activity of neutrophil elastase is increased on day 1 but not on day 14 post-MIA injection. Saphenous nerve fibre demyelination is observed in the late, chronic phase of the MIA model. Prophylactic inhibition of neutrophil elastase in MIA mice improves joint inflammation during the early phase of the model. This anti-inflammatory effect translates into an improvement in hindpaw withdrawal threshold and peripheral neuropathy during the late-stage; however, weight-bearing deficits are not improved. Modulation of PAR2 (using a pharmacological antagonist or knockouts) also improves joint inflammation, withdrawal threshold and peripheral neuropathy but not weight-bearing deficits in the MIA model.

5.9 Figures

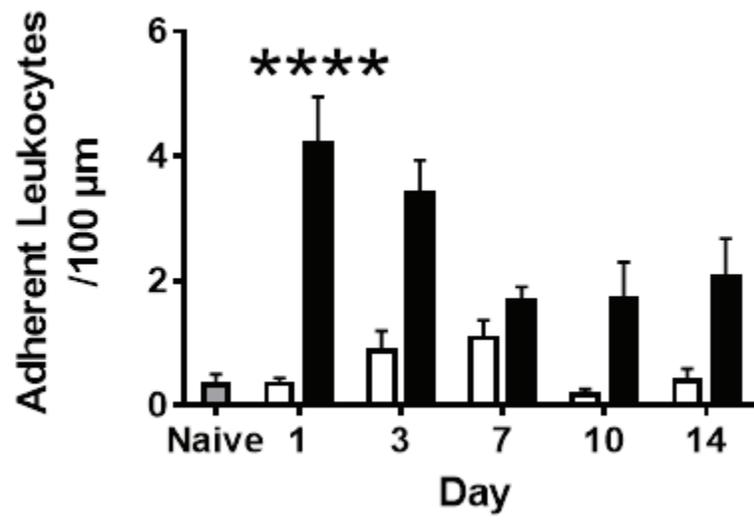
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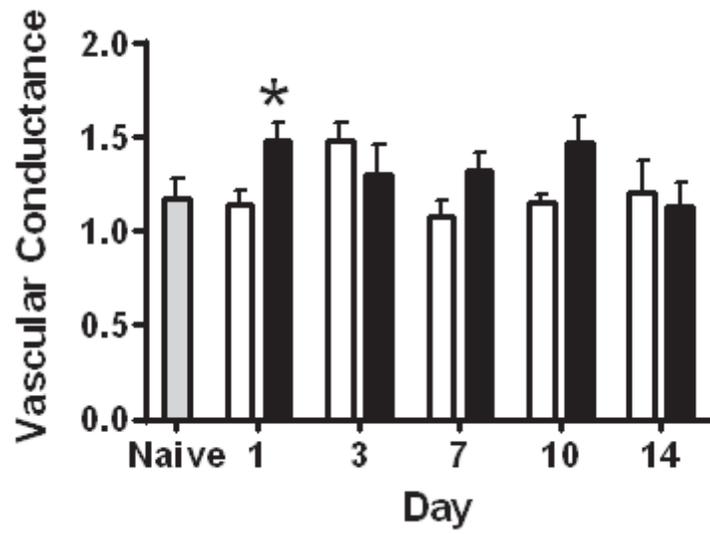
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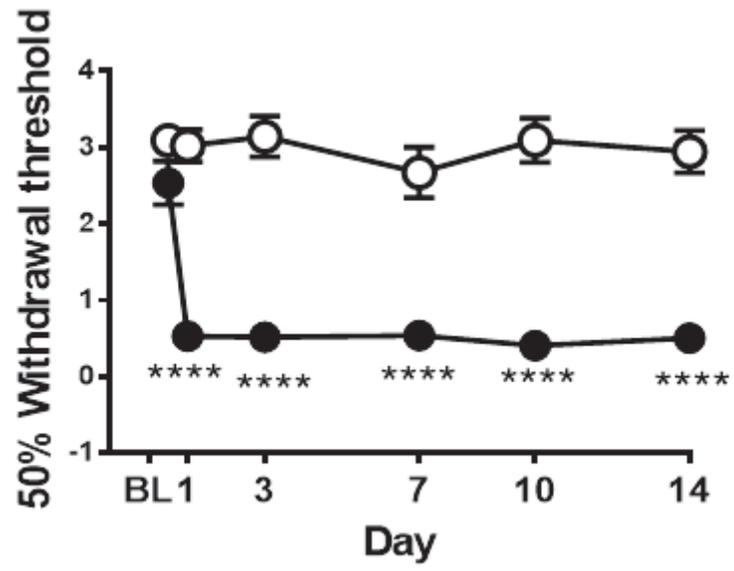
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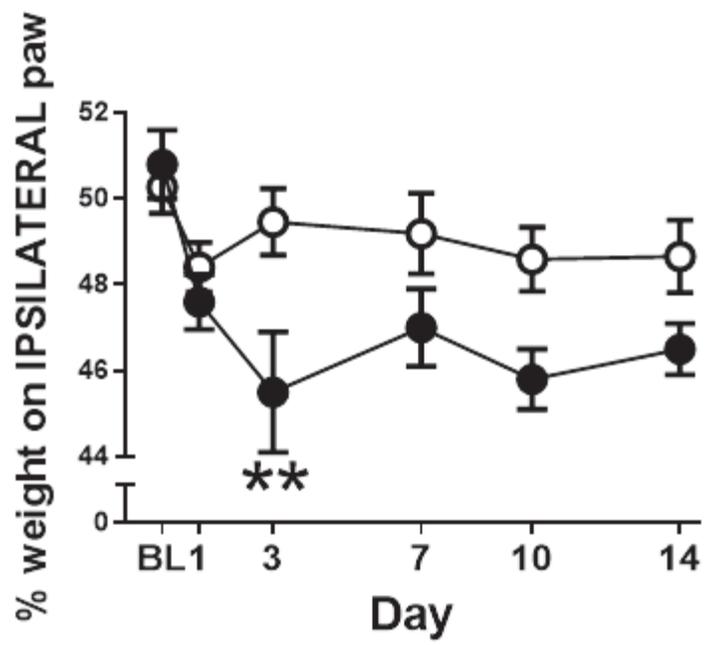
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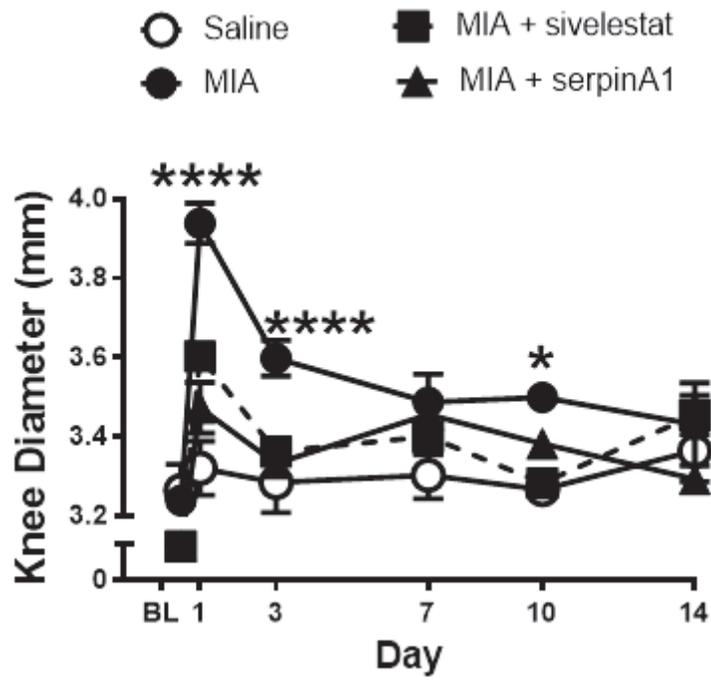


## Figure 5.1

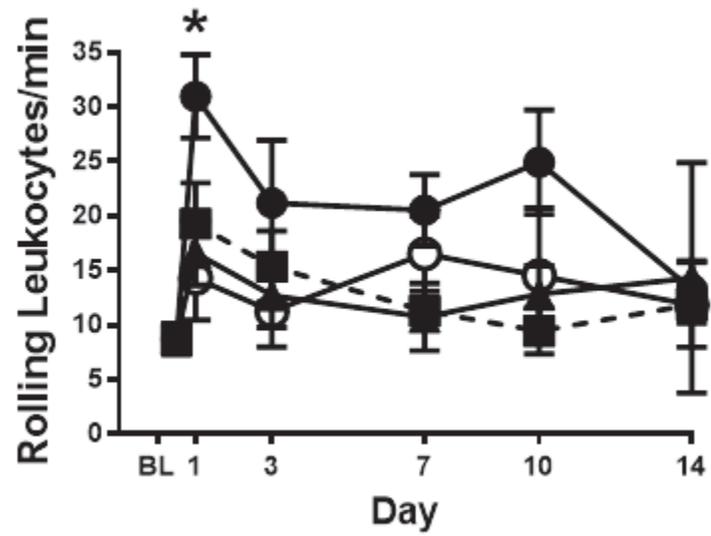
**MIA-induced knee joint inflammation and pain.** Intra-articular injection of MIA caused an increase in **(A)** knee joint diameter on days 1, 3 and 10 post-injection, compared to saline control. The increase in the number of **(B)** rolling leukocytes, **(C)** adherent leukocytes and in **(D)** synovial hyperaemia was significant on day 1 post-injection, compared to saline control. Injection of MIA resulted in a significant decrease in the **(E)** withdrawal threshold on day 1 that persisted to day 14 post-injection, compared to saline control. A significant decrease in **(F)** weight borne on the ipsilateral paw was observed on day 3 post-injection, compared to saline control. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ , compared with saline control, two-way ANOVA with Bonferroni's *post hoc* test, n=6-28. (Previously published, McDougall et al., 2017a).



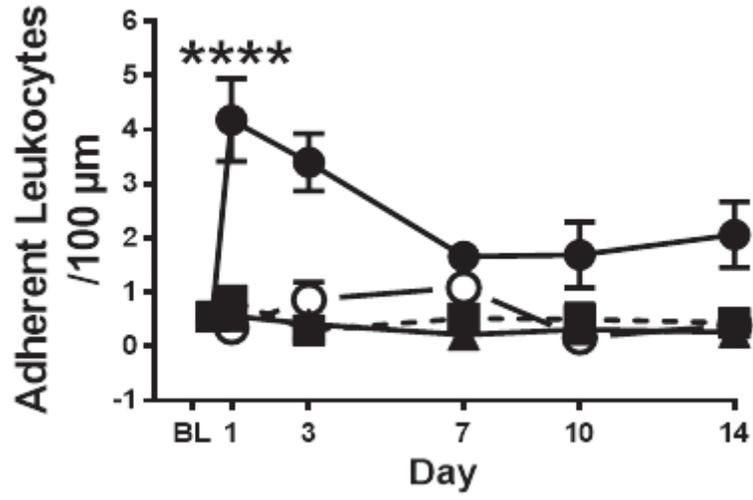
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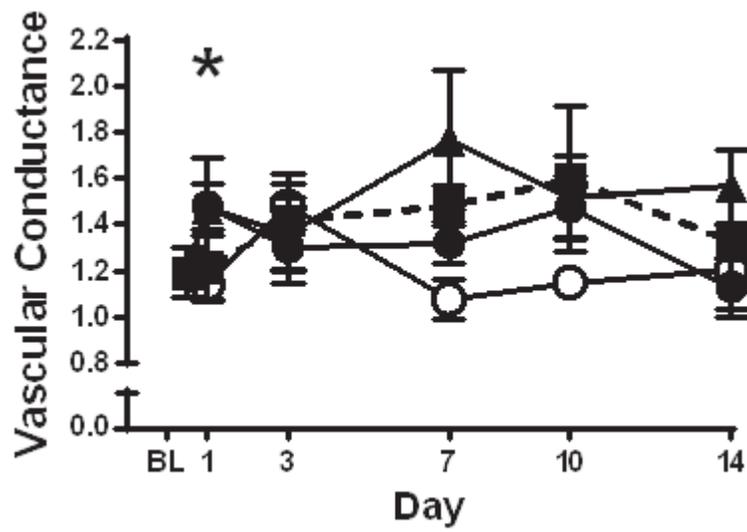
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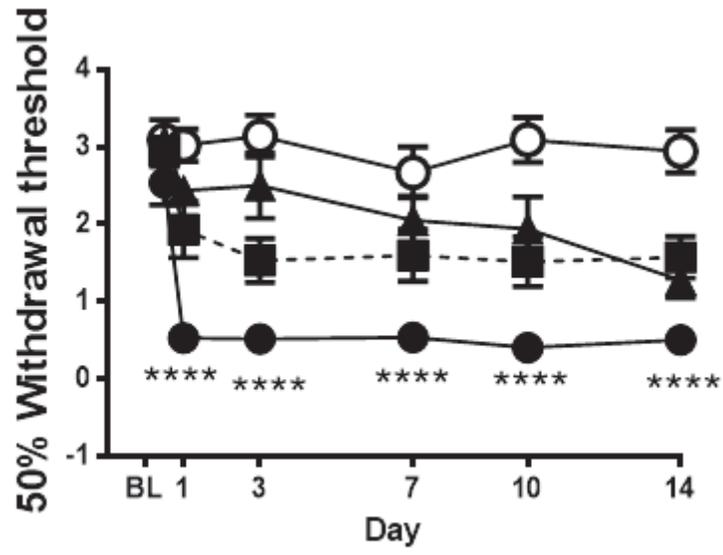
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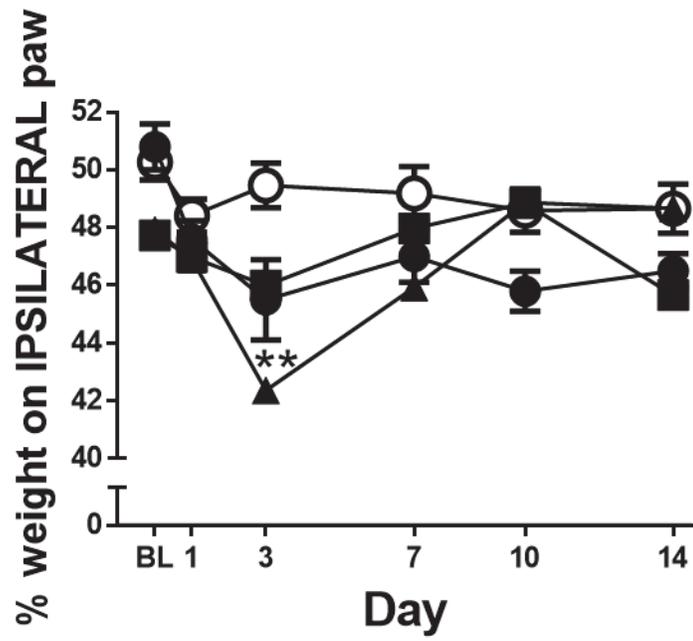
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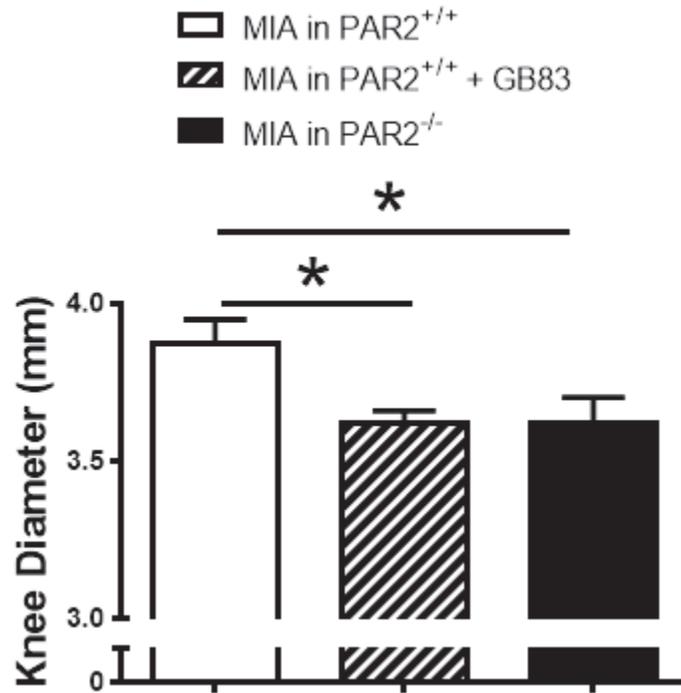


### Figure 5.3

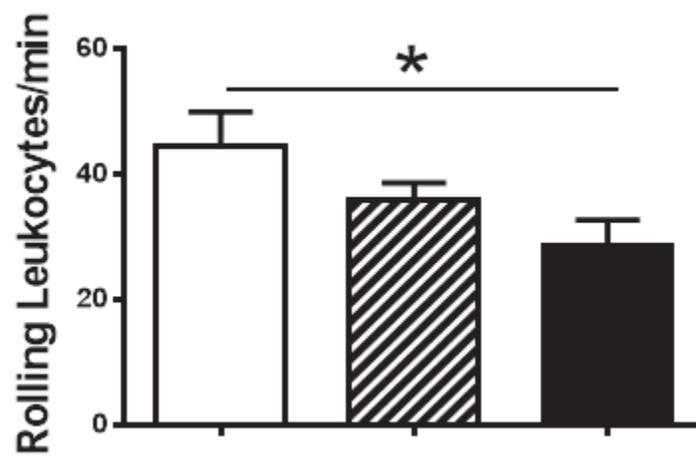
#### **Effect of neutrophil elastase inhibition on MIA-induced joint inflammation and**

**pain.** (A) Injection of MIA caused an increase in knee joint diameter which was blocked by systemic treatment with the neutrophil elastase inhibitors sivelestat ( $P < 0.0001$ , two-way ANOVA) or serpinA1 ( $P < 0.0001$ , two-way ANOVA). The MIA-induced increase in the number of (B) rolling and (C) adherent leukocytes was prevented by treatment with sivelestat ( $P < 0.01$ ,  $P < 0.001$ , respectively, two-way ANOVA) or serpinA1 ( $P < 0.05$ ,  $P < 0.001$ , respectively, two-way ANOVA). (D) The increase in vascular conductance was not improved at any time point after treatment with sivelestat or serpinA1 ( $P=0.32$  &  $P=0.09$  respectively, two-way ANOVA). (E) Prophylactic inhibition of neutrophil elastase prevented a decrease in the withdrawal threshold due to MIA ( $P < 0.0001$ , two-way ANOVA). (F) Weight-bearing deficits induced by injection of MIA were not improved after treatment with sivelestat or serpinA1 ( $P=0.99$  &  $P=0.55$  respectively, two-way ANOVA). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ , compared with saline control, two-way ANOVA with Bonferroni's *post hoc* test,  $n=5-28$ .

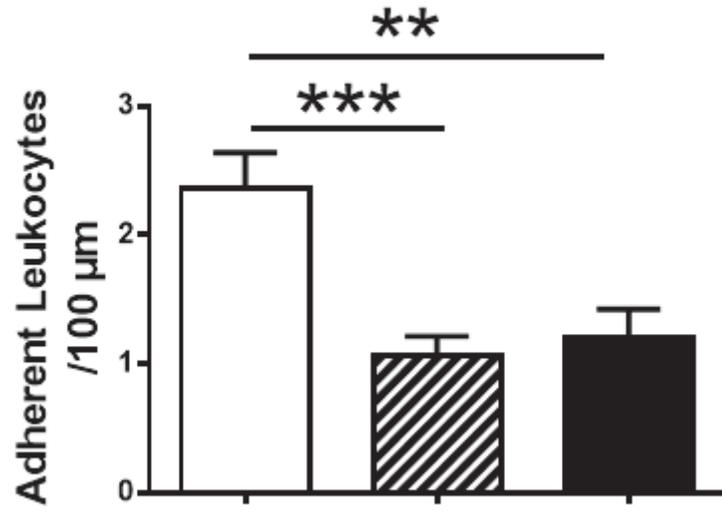
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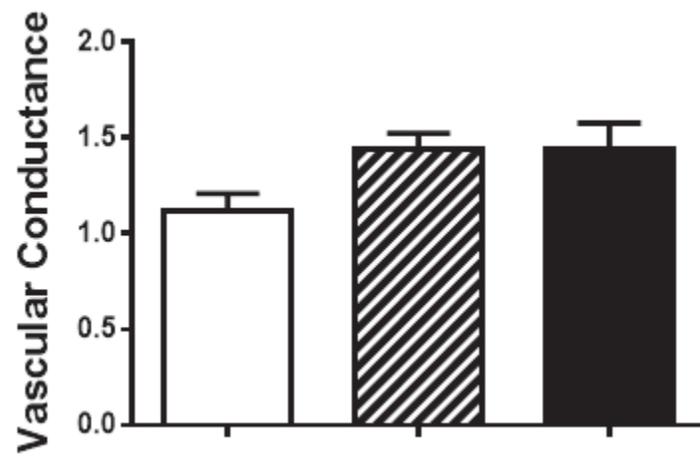
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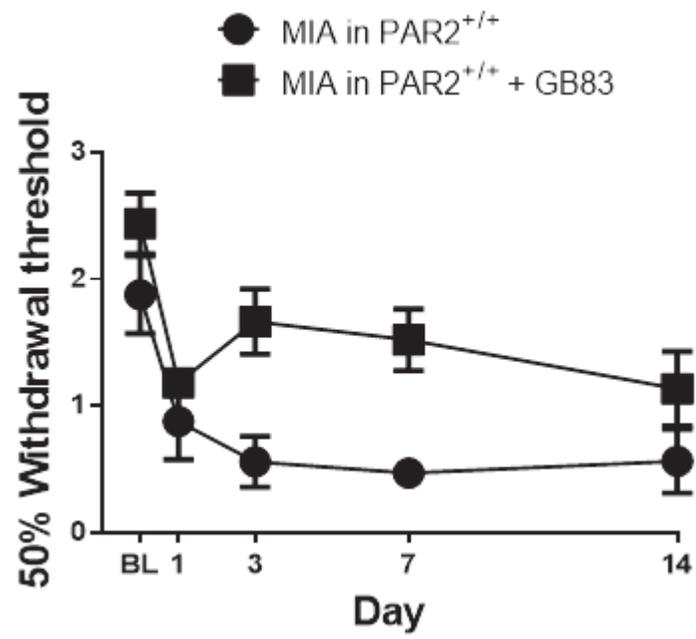
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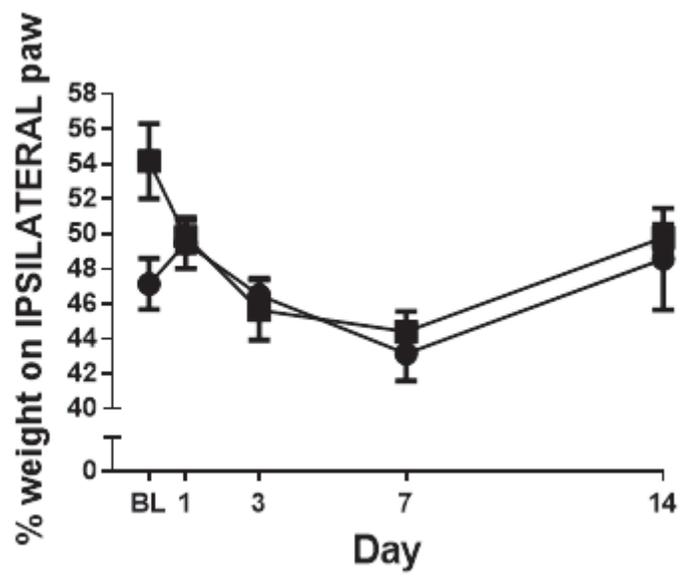
#### Figure 5.4

**Involvement of PAR2 in mediating MIA-induced joint inflammation.** Injection of MIA caused an increase in knee joint diameter and leukocyte trafficking on day 1 post-injection. **(A)** Systemic treatment with the PAR2 antagonist GB83 or knockout of PAR2 significantly blocked the increase in knee joint diameter. **(B)** PAR2 knockout prevented an increase in the number of rolling leukocytes on day 1 post MIA injection. **(C)** Systemic treatment with the PAR2 antagonist GB83 or knockout of PAR2 significantly prevented the increase in the number of adherent leukocytes on day 1 post MIA injection. **(D)** No differences were observed in vascular conductance between any of the treatment groups. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, compared to MIA control, one-way ANOVA with Dunnett's *post hoc* test, n=9.

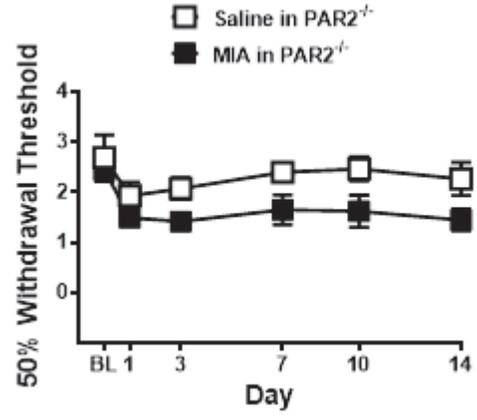
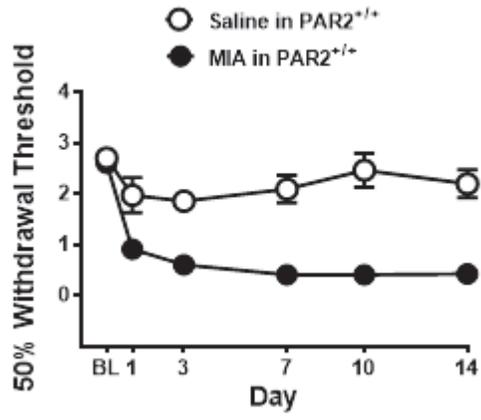
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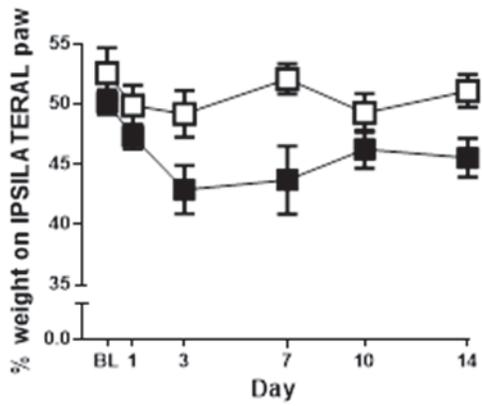
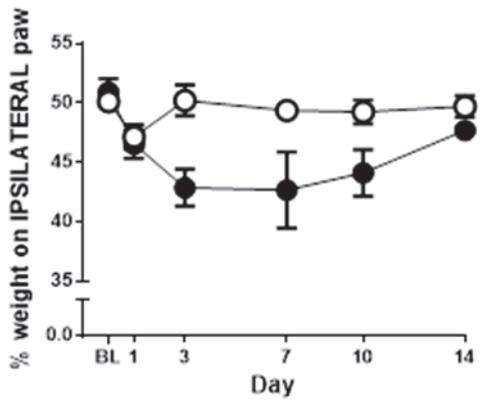
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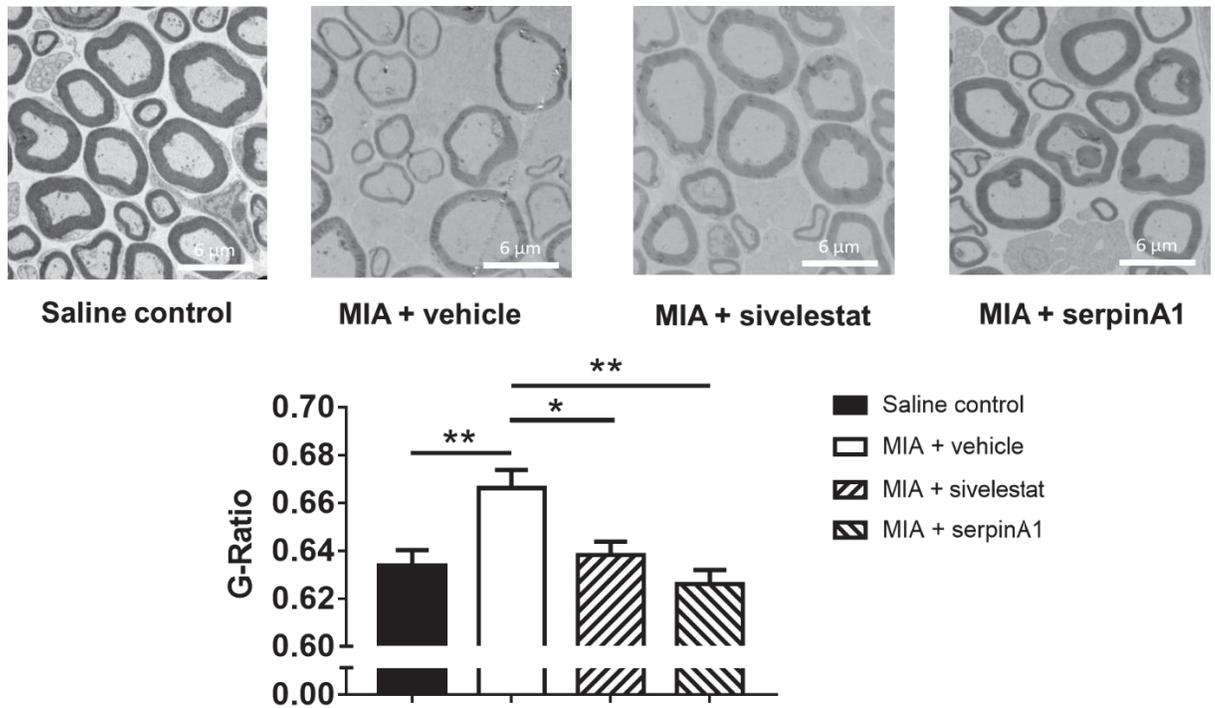


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## Figure 5.5

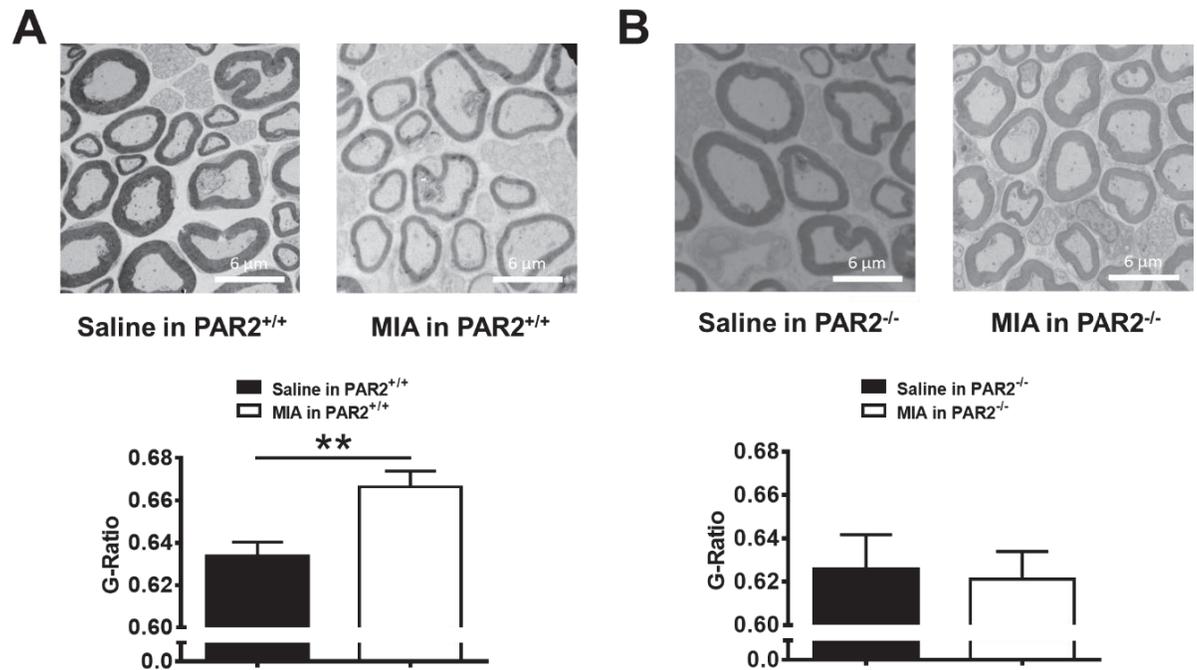
**Involvement of PAR2 in mediating MIA-induced joint pain.** (A) Systemic treatment with the PAR2 antagonist GB83 prevented the decrease in the withdrawal threshold due to injection of MIA ( $P < 0.001$ , two-way ANOVA) throughout the two-week time course. (B) Weight-bearing deficits were not improved in PAR2 antagonist GB83 treated animals ( $P = 0.09$ , two-way ANOVA). (C) MIA produced a decrease in the withdrawal threshold in wild-type ( $P < 0.0001$ , two-way ANOVA) and PAR2 knockout ( $P < 0.001$ , two-way ANOVA) mice, compared to their respective saline controls. (D) MIA produced weight-bearing deficits in wild-type ( $P < 0.01$ , two-way ANOVA) and PAR2 knockout ( $P < 0.001$ , two-way ANOVA) mice, compared to their respective saline controls.  $n=5-10$ .



**Figure 5.6**

**Effect of neutrophil elastase inhibition on MIA-induced saphenous nerve**

**demyelination.** Representative electron micrographs of saphenous nerve fibres are shown in upper panel. Lower panel shows assessment of myelin thickness of saphenous nerve fibres on day 14 post intra-articular injection of MIA or saline. Injection of MIA caused a significant increase in G-ratio values compared to injection of saline. Systemic treatment with the neutrophil elastase inhibitors sivelestat or serpinA1 prevented MIA-induced demyelination. \*P < 0.05, \*\*P < 0.01, compared to vehicle control, one-way ANOVA with Dunnett's *post hoc* test. n= 5-8 per group.



**Figure 5.7**

**Involvement of PAR2 in MIA-induced saphenous nerve demyelination.**

Representative electron micrographs of saphenous nerve fibres are shown in upper panel.

Lower panel shows assessment of myelin thickness of saphenous nerve fibres on day 14 post intra-articular injection of MIA or saline from wild-type or PAR2 knockout mice.

**(A)** Compared to saline-injected wild-type mice, injection of MIA caused a significant increase in the G-ratio values indicating nerve demyelination. **(B)** Knockout of PAR2 prevented demyelination, as there was no difference observed between saline and MIA injected mice. \*\*P < 0.01, Student's unpaired t-test. n= 5-10 per group.

## Chapter 6: Discussion

### 6.1 General summary of results

The results presented here demonstrate that local administration of neutrophil elastase into the knee joint of mice leads to an increase in the leukocyte trafficking, blood perfusion, and a decrease in the hindpaw withdrawal threshold. These effects are mediated by activation of PAR2 and possibly involve activation of the p44/42 MAPK pathway. Inhibition of endogenous neutrophil elastase in a model of acute synovitis blocked joint inflammation; however, the pain response was not prevented in this model after inhibition of neutrophil elastase. Modulation of PAR2, however, blocked both joint inflammation and pain in acute synovitis model. Prophylactic inhibition of endogenous neutrophil elastase in a chronic inflammatory monoarthritis model produced a mild anti-inflammatory effect at the end of the study period. Also, an early improvement in the weight-bearing deficit and hindpaw withdrawal threshold was observed during the three-week time course. The neutrophil elastase was found to be proteolytically active during the inflammatory phase of the MIA model of experimental OA. Prophylactic inhibition of neutrophil elastase prevented the development of joint inflammation, pain and neuropathy in the MIA model. These changes appear to be mediated by PAR2 as the modulation of the receptor blocked the development of joint inflammation, pain and neuropathy associated with experimental OA (Table 6.1).

## 6.2 Neutrophil elastase-induced joint inflammation

Neutrophils, after recruitment to the site of inflammation, release neutrophil elastase. Studies suggest that the serine proteinase might be involved in the development of various chronic inflammatory diseases such as rheumatoid arthritis, chronic obstructive pulmonary disease, and inflammatory bowel disease (Gouni-Berthold et al., 1999; Ohbayashi, 2002; Ohbayashi, 2002a; Wright et al., 2014). The objective of this study was to assess the effect of local administration of neutrophil elastase on joint inflammation. The results presented in Chapter 3 indicate that local injection of neutrophil elastase caused an increase in the number of rolling and adherent leukocytes, and blood perfusion within the knee joint microvasculature. Leukocyte extravasation is a multi-step process resulting in recruitment of the cells to the site of tissue injury and this process is orchestrated by various adhesion molecules (VCAM-1, ICAM-1, P-selectin and E-selectin), chemokines, and cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6) (Nourshargh and Alon, 2014). Studies indicate that neutrophil elastase has the capacity to cleave important adhesion molecules (ICAM-1, VCAM-1, P-selectin) and activate pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6); therefore, it is possible that through these actions neutrophil elastase is contributing to the increase in leukocyte trafficking observed in this study (Champagne et al., 1998; Levesque et al., 2001; Benabid et al., 2012; Muller, 2013). In contrast, leukocyte trafficking can be inhibited by reducing the levels of various pro-inflammatory cytokines and chemokines which then inhibit the expression of adhesion molecules to prevent leukocyte migration. In this study, two different neutrophil elastase inhibitors (sivelestat and serpinA1) were tested for their efficacy against joint inflammation induced by neutrophil elastase. It was observed that treatment with the

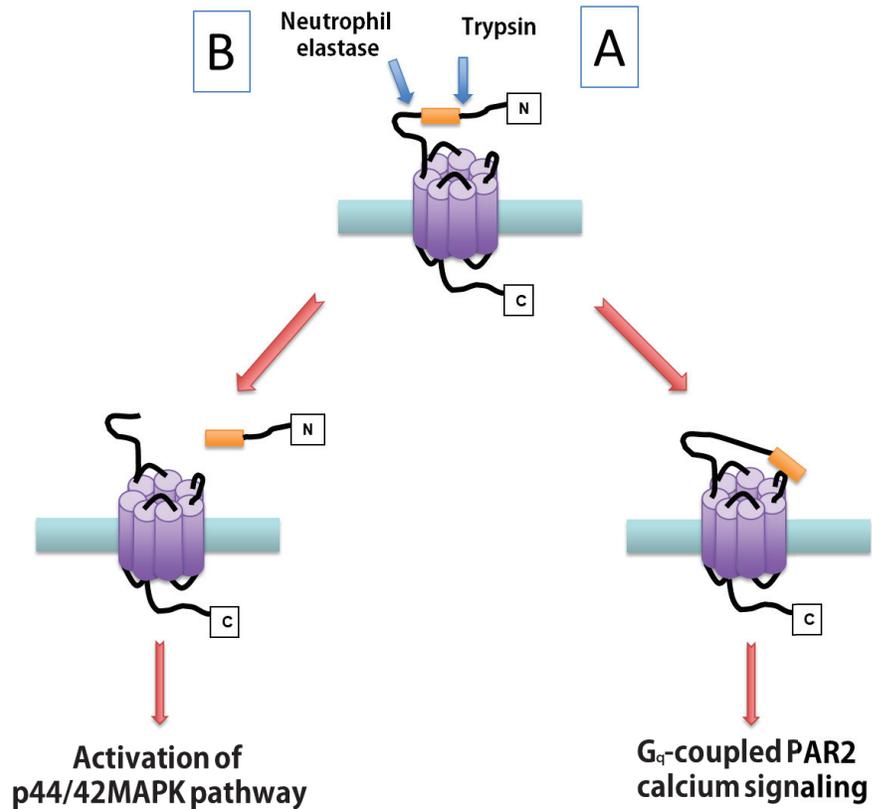
neutrophil elastase inhibitors prevented an increase in the number of rolling and adherent leukocytes, and prevented the hyperaemic response within the knee joint microvasculature. It appears that these inhibitors are eliciting anti-inflammatory effects by inhibiting activity of exogenously administered neutrophil elastase and potentially endogenous neutrophil elastase which is released during inflammation. Correspondingly, several studies in the literature suggest that sivelestat and serpinA1 can reduce inflammation by directly inhibiting the activity of neutrophil elastase in emphysema, RA, cystic fibrosis and gut ischemia reperfusion-induced impairment of gut immunity (Janoff, 1972; Gadek et al., 1981; Kakimoto et al., 1995; Nakayama et al., 2002; Hagiwara et al., 2009; Fukatsu et al., 2010; Bergin et al., 2010).

The present study investigated the involvement of PAR2 in mediating neutrophil elastase-induced joint inflammation. It was observed that treatment with the PAR2 antagonist GB83 blocked neutrophil elastase-induced increase in leukocyte trafficking and blood perfusion. Injection of neutrophil elastase into the knee joints of PAR2 knockout mice failed to induce joint inflammation which further confirms the effect that was observed with the pharmacological antagonist treatment in wild-type mice. It has been shown that activation of PAR2 using serine proteinase or synthetic peptides mediate inflammatory response in different tissues such as gut, lung and joint (Cenac et al., 2002; Dulon et al., 2003; Zhao et al., 2015). Various inflammatory changes following activation of PAR2 include an increase in the production of pro-inflammatory cytokines, upregulation of intercellular adhesion molecules, leukocyte-endothelial interactions, joint oedema and synovial hyperaemia (Vergnolle, 1999; Ferrell et al., 2003; Buddenkotte et al., 2005; Russell et al., 2012; Maeda et al., 2013; Muley et al., 2016). Therefore, it is

possible that the joint inflammation that was observed following injection of neutrophil elastase is mediated by PAR2.

Studies suggest that canonical activation of PAR2 by serine proteinase (e.g. trypsin) induces the release of intracellular calcium (Macfarlane et al., 2001; Hollenberg et al., 2014) (Figure 6.1A). However, it has been shown that elastase that is released by human leukocytes or *Pseudomonas aeruginosa* disarms PAR2 by cleaving downstream to the trypsin cleavage site and thereby prevents intracellular calcium signaling mediated by PAR2 (Dulon et al., 2003, 2005). Interestingly, a study published in 2011 revealed that this non-canonical (i.e. cleavage at a different site) activation of PAR2 by neutrophil elastase triggers different signaling pathway in a biased manner (Ramachandran et al., 2011). The study showed that neutrophil elastase could selectively activate the p44/42 MAPK pathway following activation of PAR2 (Ramachandran et al., 2011) (Figure 6.1B). The data presented in Chapter 3 indicates that upon treating neutrophil elastase-injected animals with the p44/42 MAPK inhibitor U0126, we observed prevention of an increase in the number of rolling leukocytes and blood perfusion. This finding suggests involvement of the p44/42 MAPK pathway in neutrophil elastase-induced joint inflammation. It can be assumed that during inflammation there is increased release of neutrophil elastase from infiltrating neutrophils which could potentially initiate PAR2 biased signaling which involves activation of the p44/42 MAPK pathway. Of note, there is evidence which suggests a role of the p44/42 MAPK pathway in joint inflammation (Luo et al., 2010). Additionally, it has been shown that biased activation of PAR2 by neutrophil elastase causes sensitization of TRPV4 which can contribute to neurogenic

inflammation and pain in the hindpaws of animals (Grant et al., 2007; Poole et al., 2013; Zhao et al., 2015).



**Figure 6.1 PAR2 activation by trypsin and neutrophil elastase (A)** Canonical activation of PAR2 by trypsin activates PAR2 and triggers Gq-coupled PAR2 calcium signaling. **(B)** Neutrophil elastase removes trypsin cleavage site and prevents PAR2 calcium signaling. However, it can still activate PAR2 (non-canonical) and induce p44/42MAPK signaling in a biased manner.

### **6.3 Neutrophil elastase-induced joint pain**

Pain is the most important concern for patients suffering from arthritis. The present investigation assessed the effect of neutrophil elastase injection on joint pain. The data presented in Chapter 3 indicate that in response to a local administration of neutrophil elastase, the hindpaw withdrawal threshold (secondary allodynia) was significantly decreased in mice. Tissue injury or inflammation leads to the release of different inflammatory mediators which sensitize the nerve terminal and lower their excitation threshold (Bingham et al., 2009). It appears that inflammatory changes induced by neutrophil elastase are contributing to the pain response observed here.

PAR2 has been shown to be involved in the transmission of the nociceptive signals from the periphery to the CNS (Vergnolle et al., 2001). Activation of PAR2 by serine proteinases (e.g., trypsin or mast cell tryptase) leads to neurogenic inflammation and pain (Steinhoff et al., 2000; Vergnolle et al., 2001). Furthermore, activation of articular PAR2 by synthetic activating peptides causes functional activation of TRPV1 channel which results in the release of SP and CGRP (Helyes et al., 2010; Russell et al., 2012). These neuropeptides can mediate the pain response. In our experiments, it was observed that treating neutrophil elastase-injected animals with the pharmacological antagonist of PAR2 or deletion of the PAR2 gene from animals prevented a decrease in the hindpaw withdrawal threshold. This finding implies that PAR2 is mediating neutrophil elastase-induced joint pain.

Next, the involvement of the p44/42 MAPK in neutrophil elastase-induced joint pain was assessed. The experimental data showed that treatment with the p44/42 MAPK inhibitor prevented the pain response in neutrophil elastase injected animals. It should be

noted that the MAPK-ERK pathway plays an important role in mediating inflammatory pain and thermal hyperalgesia (Hu and Gereau, 2003; Lever et al., 2003; Karim et al., 2006). Thus, it is possible that the p44/42 MAPK pathway is involved in mediating neutrophil elastase-induced joint pain.

#### **6.4 Kaolin-carrageenan-induced joint inflammation and pain**

Numerous studies indicate that injection of kaolin and carrageenan causes a robust pro-inflammatory and pro-nociceptive response (Sluka and Westlund, 1993; Kelso et al., 2006; McDougall et al., 2009). In the present investigation, we assessed the effect of intra-articular injection of kaolin/carrageenan on joint diameter, leukocyte-endothelial interactions and synovial perfusion. Results presented in Chapter 4 show that kaolin/carrageenan injection caused an acute inflammatory response as indicated by joint oedema, increased leukocyte-endothelial interactions and increased synovial perfusion. The joint inflammation is accompanied by the pain response in kaolin/carrageenan injected animals as indicated by a decrease in the hindpaw withdrawal threshold. Injection of kaolin/carrageenan triggers release of a variety of inflammatory cells which in turn release a variety of mediators such as prostaglandins, neuropeptides, and excitatory amino acids in the inflamed tissues and spinal cord which results in joint inflammation and pain (Sluka and Westlund, 1993; Kelso et al., 2006; Schaible and Grubb, 1993; Neugebauer, 2013).

#### **6.4.1 Contribution of neutrophil elastase and PAR2 in kaolin/carrageenan model**

It has been shown that intra-articular injection of carrageenan causes infiltration of neutrophils into the synovial tissue (Santer et al., 1983); these infiltrated neutrophils could potentially release neutrophil elastase. Indeed, a study has identified increased proteolytic activity of neutrophil elastase in kaolin/carrageenan-injected inflamed knee joints (Muley et al., 2016). The present study assessed the contribution of endogenous neutrophil elastase to the joint inflammation and pain in kaolin/carrageenan-injected animals using inhibitors of the serine proteinase. Data presented in Chapter 4 indicate that treatment with sivelestat or serpinA1 reduced joint oedema, leukocyte trafficking and hyperaemia within the knee joint microvasculature which implies neutrophil elastase is present and contributes to the joint inflammation. These findings are in accordance with the results presented in Chapter 3, where inhibition of exogenously administered neutrophil elastase blocked leukocyte-endothelial interactions and hyperaemic response in mice. Furthermore, a study has found that inhibition of neutrophil elastase caused a decrease in the number of rolling and adherent leukocytes, and reduced the levels of different cytokines in various *in vivo* models including carrageenan-induced paw oedema and carrageenan-induced pleurisy model (Oliveira et al., 2010). Interestingly, the pain response was not improved in kaolin/carrageenan animals after treatment with the neutrophil elastase inhibitors. There are a few possibilities why neutrophil elastase inhibition did not improve the pain response in kaolin/carrageenan injected animals. The first possibility is that injection of kaolin/carrageenan causes the release of neutrophil elastase within the synovial tissue; the local concentrations of neutrophil elastase are enough to trigger joint oedema and leukocyte-endothelial interactions. However, these

concentrations are not high enough to induce the pain response. The second possibility is that neutrophil elastase is simply not a contributor to the generation of pain in kaolin/carrageenan model.

Injection of kaolin/carrageenan into the knee joints of PAR2-deficient mice prevented joint oedema, an increase in the number of rolling and adherent leukocytes, and mean blood perfusion. These findings concur with a previous study where using multiple approaches authors had shown that PAR2 can contribute to joint inflammation associated with an acute inflammatory monoarthritis (Kelso et al., 2006). Our experimental results also indicate that PAR2-deficient mice prevent a decrease in the hindpaw threshold due to administration of kaolin/carrageenan. These results suggest that activation of PAR2 is mediating kaolin/carrageenan-induced joint inflammation and pain. It is likely that injection of kaolin/carrageenan induces the release of different PAR2-activating proteinases from infiltrating inflammatory cells which activate PAR2 and promote joint inflammation and pain in this model. Additionally, several lines of evidence suggest presence of neurogenic component in the kaolin/carrageenan model (Sluka and Westlund, 1993; Neugebauer, 2013) which involves release of peptides from sensitized articular nerve endings, and these mediators can mediate joint inflammation and pain. PAR2 is co-expressed with neuropeptides SP and CGRP in DRG neurons and activation of PAR2 leads to release of these inflammatory neuropeptides (Steinhoff et al., 2000; Vergnolle et al., 2001). It is, therefore, possible that PAR2 is contributing to acute inflammation and pain by a neurogenic mechanism in this model. An immunohistochemical examination attempting to identify the expression of PAR2 and whether it is colocalized with neuropeptides SP and CGRP in knee joint DRGs of

kaolin/carrageenan inflamed animals would help to test this hypothesis. Additionally, assessing the modulation of PAR2 and its impact on the expression of these neuropeptides in kaolin/carrageenan-injected animals could also be useful.

### **6.5 Freund's complete adjuvant-induced chronic inflammatory monoarthritis**

Injection of FCA into the knee joint of rat produces joint inflammation and pain that lasts for several weeks. Previous studies have shown that in response to FCA injection a robust inflammatory response is generated which involves extravasation of inflammatory cells including neutrophils, macrophages and T cells within inflamed joints (Nisar et al., 2015). Additionally, there is release of mediators such as proteinases, prostaglandins, cytokines, nitric oxide and leukotrienes (Nisar et al., 2015). These events contribute to the development of arthritic symptoms observed in this model. In our experiments, we observed that injection of FCA caused a persistent joint oedema and increased leukocyte trafficking that lasted for three weeks. Blood perfusion, however, was not changed during the three-week time course. FCA caused considerable weight-bearing deficits that appeared immediately and lasted for the entire study duration. However, animals only developed sensitivity to von Frey filaments from day 3 onwards which was indicated by a decrease in the withdrawal threshold and it persisted up to three weeks post-injection. On day 3, the response observed was less intense, however, by the end of first week the response became more intense and it stayed that way for the rest of the three-week time course. Slight delays in the appearance of mechanosensitivity hints at the possibility of initiation of central sensitization in FCA-injected animals.

### **6.5.1 Contribution of neutrophil elastase to the joint inflammation and pain associated with Freund's complete adjuvant-induced chronic inflammatory monoarthritis**

Data presented in Chapter 4 show that prophylactic treatment with the neutrophil elastase inhibitors sivelestat or serpinA1 caused a decrease in the number of rolling and adherent leukocytes on day 21 post-injection of FCA. In this investigation, we did not assess joint inflammation at early time points in neutrophil elastase treated animals, however, it is possible that early blockade of neutrophil elastase using its inhibitors has altered the development of inflammation due to FCA which has resulted in a mild anti-inflammatory effect on day 21 of the study.

Treatment with the neutrophil elastase inhibitor sivelestat showed an improvement in weight-bearing deficits and mechanosensitivity in FCA-injected animals. These findings are in accordance with a previous report where administration of a potent neutrophil elastase inhibitor (EL-17) in adjuvant arthritic animals (FCA was injected into the tibiotarsal joint) prevented weight-bearing deficits, mechanosensitivity and histopathological damage (Mannelli et al., 2016). Based on the results presented here, it appears that prophylactic inhibition of endogenous neutrophil elastase reduces FCA-induced inflammation and therefore attenuates peripheral sensitization in this model (Quadros et al., 2015). It is interesting to note that in our study sivelestat treatment improved mechanosensitivity changes acutely, however, the effect did not last throughout the study duration as the hindpaw withdrawal threshold kept decreasing gradually until the end of three weeks. It appears that central sensitization develops in this model which could be a result of increased sensory bombardment from the periphery due to persistent

joint inflammation (Chen et al., 2015a). Hence, it can be assumed that prophylactic inhibition of neutrophil elastase was not able to prevent the development of central sensitization in the FCA model and hence we did not see a persistent improvement in the hindpaw withdrawal threshold during end-stage arthritis. Perhaps, a study with a longer dosing regimen or treatment with higher doses of the neutrophil elastase inhibitors in the FCA model would help to address this query. With this approach, it is likely that there would be a sustained inhibition of neutrophil elastase activity which, in turn, would reduce inflammation effectively and possibly hinder central sensitization in FCA-injected animals.

#### **6.6 Monoiodoacetate-induced experimental osteoarthritis**

In this study, we characterized the MIA model for joint inflammation and pain. Data presented in Chapter 5 indicate that local injection of MIA into the knee joint of mice caused an acute inflammatory response which peaked on day 1, decreased by day 3 and remained at a low level over the remaining two-week study period. These findings are in accordance with previous studies where authors observed joint oedema which lasted up to day 5 post-injection of MIA (Fernihough et al., 2004). Furthermore, two different studies assessed histological changes in MIA-injected animals at early timepoints and they also noted a similar trend in acute inflammatory changes (Bove et al., 2003; Guzman et al., 2003). These studies observed that on day 1 the degree of synovial inflammation was higher and it was characterized by infiltration of inflammatory cells such as neutrophils, macrophages, plasma cells and lymphocytes within the synovial

membrane. These changes were decreased by day 3 and subsided by day 7 (Bove et al., 2003; Guzman et al., 2003).

Our experimental data suggest that injection of MIA induces a pain response in mice immediately and it persists for the subsequent two-week time course. Other studies corroborate this finding as it has been noted that MIA injection produces a persistent pain response as compared to intra-articular saline (Bove et al., 2003; Fernihough et al., 2004).

### **6.6.1 Contribution of neutrophil elastase and PAR2 to the joint inflammation and pain associated with monoiodoacetate-induced experimental OA**

The initial aim of these experiments was to assess the proteolytic activity of neutrophil elastase using the substrate NE680 FAST on day 1 and day 14 post-MIA injection. Before performing this experiment, we conducted several *in vitro* trials that involved recording fluorescence from the substrate with or without neutrophil elastase. We detected fluorescence from the substrate only in the presence of neutrophil elastase. Substrate or neutrophil elastase alone did not produce any fluorescence. These trials confirmed that the substrate NE680 FAST is specific and is activated immediately only in the presence of neutrophil elastase. Results obtained using *in vivo* imaging studies show for the first time, to our knowledge, that the proteolytic activity of neutrophil elastase is increased on day 1 and this activity subsides by day 14 in the MIA model. The increased proteolytic activity of neutrophil elastase was significantly reduced after systemic treatment with the neutrophil elastase inhibitors sivelestat or serpinA1 on day 1 post-MIA injection. This finding confirms that these drugs can block MIA-induced increase in the proteolytic activity of neutrophil elastase. The data also indicate that neutrophil elastase

is present, and can potentially contribute to the development of acute joint inflammation associated with the MIA model.

Systemic treatment with the neutrophil elastase inhibitors sivelestat or serpinA1 blocked the activity of neutrophil elastase during the acute inflammatory phase of the MIA model and resulted in an anti-inflammatory effect as indicated by a decrease in the knee joint diameter, and the number of rolling and adherent leukocytes. The data suggest that neutrophil elastase is contributing to the leukocyte extravasation in the MIA inflamed knee joints. This finding is in agreement with the results presented in Chapter 4, where it was observed that inhibition of endogenous neutrophil elastase reduces joint oedema and leukocyte trafficking in the kaolin/carrageenan and FCA models of inflammatory joint disease. However, the blood perfusion was not reduced after treatment with the neutrophil elastase inhibitors which indicates that the MIA-induced synovial hyperaemic response does not involve contribution from neutrophil elastase.

Prophylactic treatment with the neutrophil elastase inhibitors prevented a decrease in the withdrawal threshold in the MIA model throughout the time course. This means that early blockade of the neutrophil elastase reduces inflammation, which not only helps prevent the pain response during the treatment phase, but also prevents it from becoming chronic. Several lines of investigation have shown the presence of a neuropathic component in the MIA model which could contribute to the pain response observed at the late-stage in this model (Ivanavicius et al., 2007; Orita et al., 2011; Thakur et al., 2012). Furthermore, these findings correlate nicely with a clinical study in which it was observed that OA patients show neuropathy in the knee joint (Grönblad et al., 1988). Interestingly, in our experiments we observed that injection of MIA caused

demyelination of the saphenous nerve fibres on day 14 post-injection. Intriguingly, those animals which had received prophylactic treatment with the neutrophil elastase inhibitors prevented this demyelinating effect. These results suggest that early presence of neutrophil elastase is at least, in part, responsible for the neuropathy and pain observed in the chronic stage of the MIA model. Thus, blockade of neutrophil elastase reduces joint axonal damage in osteoarthritic animals which results in attenuation of chronic pain in these animals. A study has found that post-nerve injury there is secretion of neutrophil elastase by infiltrating T cells within the DRGs which can contribute to the neuropathic pain. The study noted that administration of serpinA3N (serine proteinase inhibitor) or sivelestat (neutrophil elastase inhibitor) attenuates mechanical allodynia (Vicuna et al., 2015).

Systemic treatment with the PAR2 antagonist GB83 prevented joint oedema and increase in leukocyte trafficking in MIA-injected animals on day 1 post-injection. Also, these inflammatory changes were absent in PAR2 knockout mice. These findings corroborate the results presented in Chapter 3 and 4 where modulation of PAR2 blocked exogenous neutrophil elastase- and kaolin/carrageenan-induced joint inflammation. Interestingly, modulation of PAR2 did not change blood perfusion in the MIA model which implies that activation of PAR2 does not mediate MIA-induced synovial hyperaemia.

Prophylactic treatment with the PAR2 antagonist prevented the development of secondary allodynia in MIA-injected animals throughout the two-week time course. Additionally, PAR2 knockout mice were able to partially prevent the development of secondary allodynia due to injection of MIA. Lastly, g-ratio analysis revealed that MIA

injection caused demyelination of saphenous nerve fibres in wild-type mice. Interestingly, absence of the PAR2 gene prevented myelin loss due to injection of MIA. Although this investigation does not provide any direct evidence that links neutrophil elastase and PAR2 in the MIA model, results presented in Chapter 3 suggest that neutrophil elastase-induced inflammation and pain can be blocked by treatment with the pharmacological antagonist of PAR2, or these changes are absent in the PAR2 knockout mice. Therefore, it is feasible that endogenous neutrophil elastase is causing proteolytic activation of PAR2, which mediates MIA-induced inflammation, referred pain and neuropathy.

In our experiments, we did not notice any improvement in weight-bearing deficits in MIA-injected animals after treatment with the neutrophil elastase inhibitors or after modulation of PAR2. However, both interventions improved mechanosensitivity that was assessed using von Frey filaments. This difference of effect observed in two different articular pain assessment techniques is intriguing. It should be noted that these techniques assess different types of pain in animals. Dynamic weight-bearing measures spontaneous nociception (non-evoked) whereas von Frey algometry measures secondary allodynia (evoked). It appears, therefore, that the neutrophil elastase/PAR2 pathway is not involved in mediating spontaneous pain associated with experimental osteoarthritis.

Synovial inflammation plays a critical role in the development of symptoms associated with RA and OA. A range of soluble inflammatory mediators that are released within articular microenvironment contributes to the pathophysiology of these arthritic conditions. In the present study, we used different agents such as neutrophil elastase, kaolin/carrageenan, Freund's complete adjuvant and sodium monoiodoacetate to induce

experimental knee arthritis. Intra-articular injection of these agent induces the release of various inflammatory mediators which promote peripheral and central sensitization. The findings presented in the thesis reveal that inhibition of neutrophil elastase or modulation of proteinase-activated receptor-2 results in attenuation of joint inflammation and pain. These findings suggest an important role of neutrophil elastase and proteinase-activated receptor-2 in the development of joint inflammation and pain associated with different experimental models of knee arthritis. Our data also suggest the potential utility of neutrophil elastase and proteinase-activated receptor-2 as drug targets for the treatment of joint inflammation and pain associated with arthritis. The drugs that we used for our experiments, sivelestat and serpinA1, are potent inhibitors of neutrophil elastase. In fact, sivelestat is a clinically approved drug for the treatment of acute respiratory distress syndrome (Aikawa and Kawasaki, 2014) which means that clinical development of this drug for the treatment of arthritis could be fast-tracked. The clinical development of candidates acting on PARs has been challenging because the receptor has an atypical activation and signaling mechanism. Despite years of research we only have one approved drug that can target PAR (Vorapaxar or SCH 530348, a PAR1 antagonist which possess antiplatelet activity and has been used for the prevention of acute coronary syndrome) (Chackalamannil et al., 2008). Several compounds have been designed that can target PAR2 (Kelso et al., 2006; Kanke et al., 2009), however, it has been suggested that these compounds are weak antagonists of PAR2 (Suen et al., 2012). Recently, Barry et al. (2010) synthesized a series of PAR2 antagonists which are considered more potent than the existing PAR2 antagonists. Furthermore, an investigation published by Cheng et al. (2017) proposed the crystal structure of PAR2 along with two new antagonists

(AZ8838 and AZ3451) and a blocking antibody for the PAR2. The study suggests that these novel antagonists possess slow binding kinetics and can prevent structural rearrangement which is a prerequisite for the activation of the receptor and signaling (Cheng et al., 2017). Therefore, more studies are required to facilitate the development of these PAR2 compounds in for the treatment of different conditions.

## **6.7 Limitations**

### **6.7.1 Related to techniques**

In the experiments described here, all the agents (neutrophil elastase, kaolin, carrageenan, Freund's complete adjuvant and monoiodoacetate) were injected directly into the knee joint of rodents to elicit joint inflammation and pain. The direct administration of the substances into the knee joint was accomplished by making an intra-articular injection, which involves insertion of a fine needle through the patella ligament into the synovial cavity of the knee joint. While this is a minimally invasive and routinely used technique to induce experimental knee arthritis, it could still induce a mild injury in animals due to the injection. Additionally, before assessment of inflammation, an invasive surgical procedure was performed on animals which involved cannulations of the trachea, carotid artery and jugular vein. These procedures can cause a mild increase in the leukocyte trafficking and blood perfusion within the knee joint microvasculature. Therefore, we control for these limitations by testing vehicle (saline) in our experimental animals.

For the assessment of leukocyte trafficking within the knee joint microvasculature, intra-vital microscopy was used. Before assessment, a fluorescent dye, rhodamine 6G, was injected through the jugular vein to stain leukocytes. Rhodamine 6G staining is non-specific in nature; therefore, it is not possible to differentiate between the type of leukocytes that are present within inflamed knee joint microvasculature.

For the assessment of proteolytic activity of neutrophil elastase in the MIA animals, we used NE680 FAST substrate which was injected subcutaneously over the knee joint. Injection of the substrate over the inflamed knee could trigger further injury

which can potentially cause additional release of neutrophil elastase. To overcome this limitation, we injected the substrate subcutaneously over contralateral naïve knee joint. The data were presented as the difference in fluorescence activity between the ipsilateral and contralateral knee joint.

Behavioural pain was assessed using von Frey hair algometry which involves application of hair filaments to the plantar surface of the hindpaw of an animal. Since the technique involves noting a withdrawal response exhibited by an animal to a monofilament application, experimenter bias is possible (i.e. differentiating between nocifensive or startle response). Also, the response to monofilament may differ between experimental animals and for that reason we applied multiple filaments (a standard range) and calculated a withdrawal threshold based on several responses obtained. Another behavioural pain assessment technique used was dynamic weight bearing which involves capturing videos of a freely moving animal inside a chamber on a pressure sensitive mat. Video analysis is labor intensive particularly for studies involving recording at multiple time points. While the animals are allowed to move freely, they could still experience anxiety because of being in a confined space which could potentially lead to variability between animals.

We obtained saphenous nerves from MIA-injected animals with or without treatment and calculated area based G-ratio to assess myelin thickness in saphenous nerve fibres. Using this approach, we were able to measure myelin thickness in all the nerve fibres present in an electron micrograph. However, we could not express the change in myelin thickness in different types of nerve fibres present in the same electron micrograph as we did not differentiate between the different types of nerve fibres.

### **6.7.2 Related to drugs**

The dose of different drugs (sivelestat, serpinA1, GB83 and U0126) used in our experiments were selected based on the documented evidence and the dosing regimen was designed based on the half-life of the drug. Therefore, the experiments where these treatments did not produce significant effects could be a result of using a dose or dosing regimen which is not suitable for the experimental parameters we were testing. For example, prophylactic treatment with the neutrophil elastase inhibitor sivelestat (day 0 – 5) in the FCA model produced an acute improvement in the hindpaw threshold but the effect was not lasting.

### **6.7.3 Related to animal model**

One of the models utilized for the assessment of the role of endogenous neutrophil elastase and PAR2 was kaolin/carrageenan-induced acute synovitis. This is a well-established model which can produce robust joint inflammation, pain and damage to hyaline cartilage in animals (Neugebauer, 2013) in a short period, however, in humans, full development of arthritis take years. Therefore, this animal model is unable to recapitulate all the aspects of human disease (e.g. subchondral bone lesions).

Freund's adjuvant-induced inflammatory monoarthritis model involves pronounced joint inflammation and pain which is persistent throughout the study duration (3 weeks). While these and many other features of this model resemble human RA, the cartilage damage observed in this model is mild as compared to human RA (Bendele, 2001).

MIA-induced experimental OA is a widely-used animal model which produces robust joint pain and neuropathy. However, it has been suggested that histopathological changes observed in this model lacks correlation with human OA (Little and Zaki, 2012).

## **6.8 Future directions**

### **6.8.1 Mechanism for pro-inflammatory and pro-nociceptive effects by neutrophil elastase and PAR2**

Further studies are required to delineate exact mechanisms for the generation of inflammation and pain by neutrophil elastase and PAR2. Additionally, we noted that blockade of endogenous neutrophil elastase and modulation of PAR2 prevented MIA-induced neuropathy in mice, further studies are required to identify mechanism for this effect as well. Documented studies suggest that there is functional activation of TRPV1, TRPV4 and TRPA1 following activation of PAR2 by serine proteinases or synthetic ligands (Dai et al., 2007; Helyes et al., 2010; Poole et al., 2013; Zhao et al., 2015). It has been shown that these TRP receptors can play a role in the development of both inflammatory and neuropathic pain.

### **6.8.2 Inhibition of neutrophil elastase in the FCA model**

In our studies, we noticed a mild anti-inflammatory effect due to prophylactic treatment with the neutrophil elastase inhibitor sivelestat (day 0 - 5) at day 21 post-injection of FCA. However, we did not assess inflammatory changes during the treatment phase in the FCA-injected animals. It would be interesting to assess inflammatory changes at several time points during the three-week time course. Assessment of

inflammation during these early time points would give a better idea of the exact contribution of neutrophil elastase towards the development of joint inflammation and pain in the FCA model. Additionally, studies should attempt a longer treatment regimen using the neutrophil elastase inhibitors in the FCA model and assess its impact on weight-bearing capacity and secondary allodynia.

### **6.8.3 Assessment of joint pathology in the OA models**

In the experiments described here, we observed that prophylactic blockade of neutrophil elastase or PAR2 in the MIA model prevents chronic joint pain and peripheral neuropathy. It is possible that these prophylactically treated animals did not develop arthritis which has resulted in an analgesic effect. Therefore, it could be interesting to assess the impact of our treatments on joint pathology. These pathological assessments should be carried out in other models (e.g. posttraumatic OA models) which recapitulates histopathological damage more closely to human OA. Furthermore, these histopathological evaluations could give us some insights into disease-modifying effects of these drugs. It is likely that, in these assessments, due to early inhibition of neutrophil elastase or modulation of PAR2, we would see prevention of infiltration of inflammatory cells within the synovial compartment. Furthermore, due to inhibition of neutrophil elastase proteolysis we could expect prevention of proteoglycan damage, reduced chondrocyte degeneration and cartilage lesions.

#### **6.8.4 Role of other serine proteinases in the development of arthritis**

Our findings highlight the important role of the serine proteinase neutrophil elastase in the development of joint inflammation and pain associated with experimental knee arthritis. However, there are many other serine proteinases such as trypsin, tryptase, and matriptase (for detailed list refer Table 1.5) that are released by infiltrating inflammatory cells such as mast cells, macrophages and neutrophils during arthritis and can contribute to disease pathogenesis. Future studies should examine their contribution to the development of arthritic symptoms. Interestingly, a latest study has shown efficacy of inhibiting tryptase, a serine proteinase released by mast cells, in reducing joint inflammation associated with experimental arthritis (Denadai-Souza et al., 2017). Additionally, studies are required to identify the role of other types of PARs in the development of arthritis; for example, PAR4 which has been shown to produce proinflammatory effects (increased joint blood flow and oedema) and pronociceptive effects after its activation by selective agonists (McDougall et al., 2009). Furthermore, blockade of PAR4 reduced joint inflammation associated with acute synovitis (McDougall et al., 2009).

**Table 6.1 Summary of findings**

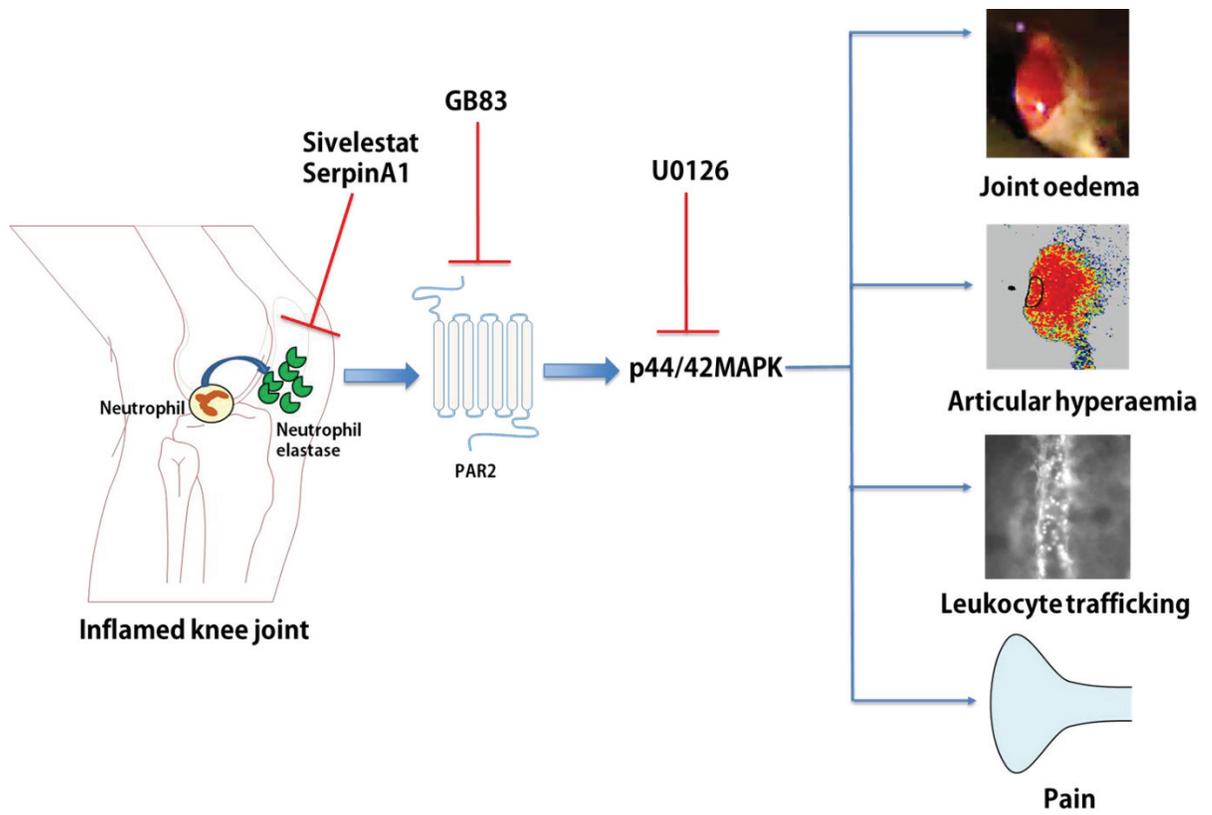
Model	Parameter	Treatment/ Modulation				
		sivelestat	serpinA1	GB83	PAR2 <sup>-/-</sup>	U0126
Neutrophil elastase	Inflammation	↓	↓	↓	↓	↓
	Pain	↓	↓	↓	↓	↓
Kaolin/carrageenan	Inflammation	↓	↓	Not evaluated	↓	Not evaluated
	Pain	No improvement	No improvement	Not evaluated	↓	Not evaluated
Freund's complete adjuvant	Inflammation	Mild reduction	Mild reduction	Not evaluated	Not evaluated	Not evaluated
	Pain	Acutely improved	No improvement	Not evaluated	Not evaluated	Not evaluated
Monoiodoacetate	Inflammation	↓	↓	↓	↓	Not evaluated
	Pain	↓	↓	↓	↓	Not evaluated
	Neuropathy	↓	↓	Not evaluated	↓	Not evaluated

## 6.9 Conclusions

The experimental data presented here show that local administration of neutrophil elastase produces an acute pro-inflammatory and a pro-nociceptive effect in the knee joints of mice (Figure 6.2). Blockade of PAR2 prevents neutrophil elastase-induced inflammation and pain which suggest proteolytic activation of PAR2 by neutrophil elastase to elicit these changes (Figure 6.2). The data also suggest involvement of the p44/42 MAPK pathway in mediating joint inflammation and pain (Figure 6.2). Neutrophil elastase contributes to the development of joint inflammation, but not pain

associated with acute inflammatory monoarthritis model. PAR2, however, mediates both joint inflammation and pain induced by injection of kaolin/carrageenan. Prophylactic inhibition of neutrophil elastase in a chronic inflammatory monoarthritis model produces a mild anti-inflammatory and anti-nociceptive effect. Furthermore, our results showed that neutrophil elastase is proteolytically active in the early inflammatory phase of the MIA model. Early presence of neutrophil elastase in MIA inflamed knee joint contributes to the development of joint inflammation, late-stage pain and neuropathy.

Pharmacological blockade of PAR2 or removal of the PAR2 gene from mice also prevents the development of MIA-induced joint inflammation, pain and neuropathy which suggests activation of PAR2 by locally released neutrophil elastase in the MIA model. Our data provide further evidence for the role of neutrophil elastase and PAR2 in the development of experimental knee joint arthritis which highlights the potential utility of treatment with neutrophil elastase inhibitors and/or PAR2 blockers to reduce the development of arthritic inflammation and pain.



**Figure 6.2** Involvement of PAR2 and p44/42 MAPK in neutrophil elastase-induced joint inflammation and pain

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