The role of IL-17 in inflammatory hyperalgesia

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DECLARATION OF ORGINALITY

I hereby declare that all work described in this thesis is my own, original work unless stated. Where use has been made of the work of others, it has been duly acknowledged in the text.

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease that affects up to 1% of the population. The importance of tumour necrosis factor (TNF) in RA has been established and anti-TNF biologics have proved to be highly effective in reducing inflammation. However, a proportion of RA patients fail to adequately respond to anti-TNF and even those who do respond have residual pain. This has prompted the investigation into other cytokines, such as interleukin-17 (IL-17), as potential new targets.

This study sought to investigate the contribution of IL-17 to acute or chronic hyperalgesia. C57BL/6 mice were injected with recombinant IL-17. This induced a transient hyperalgesia, which was found to be dependent both on neutrophil migration and signalling through TNFR1. Using the air pouch model of cell migration, it was confirmed that the cell infiltration was associated with increased expression of the chemokine keratinocyte attractant (KC). These results suggest that IL-17 induces acute hyperalgesia by inducing TNF from resident cells.

To investigate IL-17 and chronic hyperalgesia, the collagen induced arthritis mouse model (CIA) was used. IL-17RA was found to be up regulated in both the paw and in the dorsal root ganglia in arthritis, suggesting a role for IL-17 in chronic hyperalgesia. IL-17 blockade proved to be anti-arthritic and analgesic during CIA and potently reduced expression of pro-inflammatory cytokines.

This study confirms that IL-17 contributes to acute and chronic hyperalgesia but acts in part via the induction of TNF.

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ABBREVIATIONS

ACPA	**Anti-citrullinated proteins**
AIA	**Antigen-induced arthritis**
ANOVA	**Analysis of variance**
BBB	**Blood-brain barrier**
BSA	**Bovine serum albumin**
CCI	**Chronic constriction injury**
CCP	**Cyclic citrullinated peptide**
cDNA	**Complementary deoxyribonucleic acid**
CeL	**Central nucleus for the amygdala**
CFA	**Complete Freund's Adjuvant**
CGRP	**Calcitonin gene related peptide**
CIA	**Collagen induced arthritis**
CNS	**Central nervous system**
CO_2	**Carbon dioxide**
COX	**Cyclooxygenase**
CRP	**C-reactive protein**
DMARDs	**Disease modifying anti-rheumatic drugs**
DMEM	**Dulbecco's Modified Eagle's Medium**
DNA	**Deoxyribonucleic acid**
dNTP	**deoxy nucleotide triphosphate**
DRG	**Dorsal root ganglia**
DTT	**Dithiothreitol**
EAE	**Experimental autoimmune encephalomyelitis**

ELISA	**Enzyme-linked immunosorbant assay**
ERK	**Extracellular signal regulated kinase**
ESR	**Erythrocyte sedimentation rate**
FCS	**Foetal calf serum**
GFAP	**Glial fibrillary acidic protein**
GM-CSF	**Granulocyte-macrophage-colony stimulating factor**
HLA	**Human leukocyte antigen**
HPRT	**Hypoxanthine-Guanine Phosphoribosyltransferase**
IB4	**Griffonia simplicifola isolectin B4**
IHC	**Immunohistochemistry**
IL-	**Interleukin-**
IL-17	**Interleukin 17 A**
IL-17R	**Interleukin 17 receptor**
IL-17RA	**Interleukin 17 receptor subunit A**
IL-17RC	**Interleukin 17 receptor subunit C**
IL-17RB	**Interleukin 17 receptor subunit B**
KC	**Keratinocyte-derived chemokine**
LRN	**Lateral reticular nucleus**
LABORAS	**Laboratory animal Behaviour Observation Registration and Analysis System**
LOX	**Lipooxygenase**
LPS	**Lipopolysaccharide**
MHC	**Major histocompatibility complex**
MMP	**Matrix metaloprotease**
MPO	**Myeloperoxidase**

mRNA	**Messenger ribonucleic acid**
MTX	**Methotrexate**
NaCl	**Sodium Chloride**
NDGA	**Nordihydroguaretic acid**
NF-κB	**Nuclear factor kappa B**
NGc	**Giantocellular/lateral paragigantocellular reticular nuclei**
NGF	**Nerve growth factor**
NK	**Natural killer cell**
NMDAR	***N*-methyl *D*-aspartate receptor**
NSAIDs	**Non-steroidal anti-inflammatory drugs**
p75NTR	**Common neurotrophin**
PAFs	**Primary afferent fibres**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PCST	**Pain coping skills training**
PI3K	**Phosphoinositide 3-kinase**
PNS	**Peripheral nervous system**
RA	**Rheumatoid arthritis**
RANKL	**Receptor activator of NF-κB ligand**
RF	**Rheumatoid factor**
RNA	**Ribonucleic acid**
rpm	**Revolutions per minute**
RT-PCR	**Reverse transcription polymerase chain reaction**
SCW	**Streptococcal cell wall**

SRD	**Subnucleus reticcularis dorsalis**
Th	**T helper**
TLR	**Toll-like receptor**
TMB	**3,3',5,5'-Tetramethylbenzidine**
TNF-α	**Tumour necrosis factor alpha**
TNFR1	**Tumour necrosis factor alpha receptor 1 (also known as p55)**
TNFR2	**Tumour necrosis factor alpha receptor 2 (also known as p75)**
$TNFR1^{-/-}$	**Tumour necrosis factor alpha receptor 1 knockout mouse**
$TNFR2^{-/-}$	**Tumour necrosis factor alpha receptor 2 knockout mouse**
TrkA	**High affinity tyrosine kinase**
TRPV1	**Transient receptor potential cation channel, sub family V, member 1**
TTX-R	**Tetrodoxin-resistant**
VCAM-1	**Vascular cell adhesion molecule-1**
VPL	**Ventral posterolateral nucleus**
VPM	**Ventral posteromedial nucleus**
ZIA	**Zymosan induced arthritis**

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CHAPTER 1

INTRODUCTION

1.1 Rheumatoid Arthritis

1.1.1 Disease classification and description

Rheumatoid arthritis (RA) is a debilitating and progressive autoimmune condition affecting around 1% of the population. Although the cause of RA is unknown, genetic and environmental factors contribute to disease susceptibility. Symptoms of RA include inflammation of the joints causing difficulty with mobility and chronic pain. This can then develop into severe disability with irreversible cartilage destruction, bone erosion, joint deformity and reduced quality of life (Feldmann and Maini 2001). The diagnosis of RA is based on a number of criteria as described by the American College of Rheumatology as shown in Table 1.1 (Aletaha, Neogi et al. 2010).

Criterion	Score
A. Joint involvement **(Large joints refers to shoulders, elbows, hips, knees and ankles. Small joints refers to the metacarpophalangeal, proximal interphalangeal, 2nd-5th metatarsophangeal , thumb interphalangeal joints and wrists)**	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 **(At least one test result is needed for classification)**	Negative RF (Rheumatoid factor) *and* negative ACPA (anti-citrullinated proteins) = **0** Low-positive RF or low-positive ACPA = **2** High positive RF or high-positive ACPA = **3**
C. Acute-phase reactants **(At least one test result is needed for classification)**	Normal CRP (C-reactive protein) and normal ESR = **0** Abnormal CRP or abnormal ESR = **1**
D. Duration of symptoms	< 6 Weeks = **0** ≥ 6 Weeks = **1**

(Aletaha, Neogi et al. 2010)

Table 1.1 American College of Rheumatology criteria for the classification of RA. A patient is considered to have RA if a score of 6 (out of a possible 10) is achieved. Patients must have at least 1 joint with definite clinical synovitis not explained by another disease *After Aletaha el al 2010*

The most frequent age of onset of RA is 40-60, but it can develop at any time and affects three times as many women as men (Lee and Weinblatt 2001). Drugs targeting the inflammatory response have been successful, but for many the pain relief is unfulfilled (Breivik, Collett et al. 2006). Through a greater understanding of the inflammatory and pain processes involved in RA, more effective drugs to combat this disease and its associated pain can be developed.

1.1.2 Aetiopathogenesis of Rheumatoid Arthritis

RA is characterised by proliferation of the synovial lining layer and infiltration of the joints by a variety of leukocytes including T and B lymphocytes, macrophages and neutrophils (Figure 1.2). The presence of leukocytes leads to release of many factors, including proinflammatory cytokines, prostaglandins and matrix mellatoproteinases (MMPs). The leukocytes together with synoviocytes form pannus, a tissue that grows over the articular cartilage. The articular cartilage is progressively destroyed and the underlying bone eroded (Firestein 2003; Smolen and Steiner 2003).

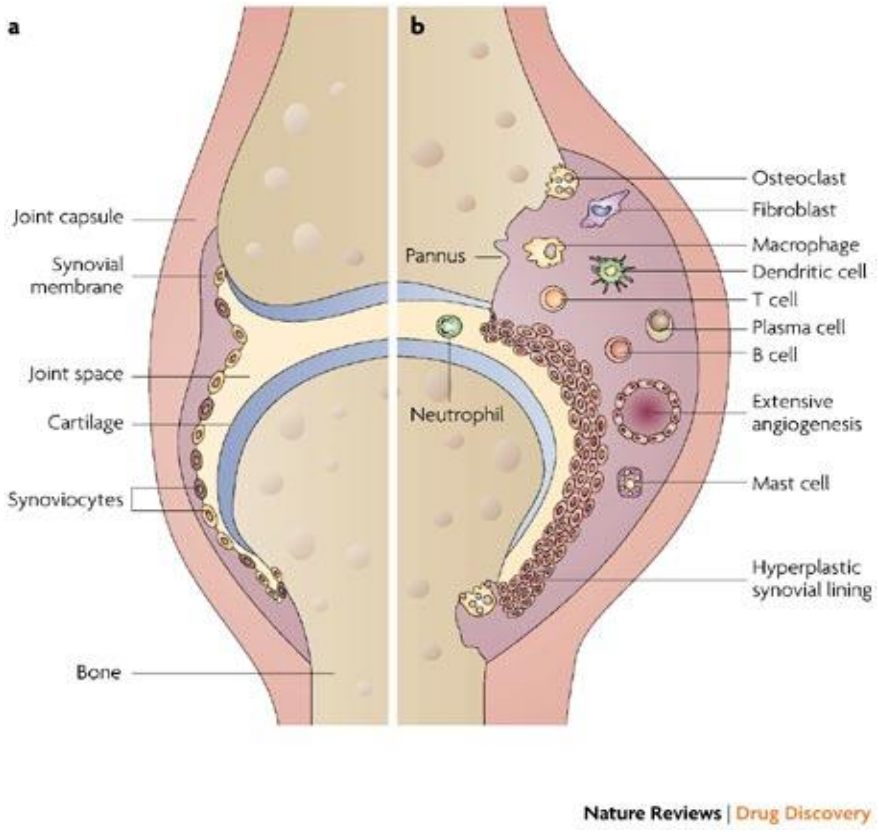

Figure 1.1 The rheumatoid joint. The healthy joint (a) has a synovial membrane <100µm thick consisting of a thin (1-3 cells) layer of synoviocytes. In the RA joint (b) the synoviocytes proliferate, immune cells infiltrate the joint leading to pannus formation, cartilage degradation and bone erosion. *After Smolen and Steiner 2003*

A combination of genetic, infectious, and environmental factors are thought to initiate RA and contribute to its severity. The major histocompatibility complex (MHC) is a gene family separated into 3 classes. Class II molecules are located on antigen presenting cells (APCs) where they express antigens to CD4+ T cells and are thought to be important in the initiation and/or progression of RA. Genetic susceptibility to RA is associated with specific alleles within MHC class II (Newton, Harney et al. 2004). Certain HLA DRB1 alleles are found in increased frequency in patients with RA compared to healthy controls (McInnes and Schett 2007). These alleles share a common sequence, which is termed the shared epitope (SE). Around 90% of RA patients are positive for this SE (Gregersen, Silver et al. 1987), however whether being positive for this shared epitope confers susceptibility or signals a more progressive disease is a matter of some debate (Fugger and Svejgaard 2000; Taneja, Behrens et al. 2008; Taneja and David 2010).

RA diagnosis is also associated with the production of autoantibodies (Table 1.1). The presence of rheumatoid factor (RF) has long been a diagnostic tool for RA (Arnett, Edworthy et al. 1988). RF is detected in 70-90% of RA patients, but is also present in healthy individuals, and patients with other autoimmune diseases (Dorner, Egerer et al. 2004; Dorner and Hansen 2004). Although widely used, RF is not as specific or as sensitive as anti-cyclic citrullinated peptide (anti-CCP) Ab (Klareskog, Ronnelid et al. 2008; Snir, Widhe et al. 2010). Anti-CCP Ab is recognised as having an equal sensitivity but higher specificity (97%) for RA than RF and is now considered a more useful marker (Silveira, Burlingame et al. 2007).

1.1.3 Treatment of RA

RA therapy has traditionally been dominated by use of non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs). NSAIDs target prostaglandin synthesis by inhibiting cyclooxygenase (COX) I and/or II (Vane 1976). Blockade of COX-II is beneficial in treating RA pain and inflammation (Samad, Moore et al. 2001; O'Dell 2004), but does not slow disease progression (Smolen and Steiner 2003). Many NSAIDs also suppress COX-1 which leads to peptic ulceration and gastric haemorrhage (Crofford 1997; Singh 1998; O'Dell 2004). The toxicity of NSAIDs discouraged their long-term use in RA. NSAIDs may be taken in combination with synthetic DMARDs (e.g. methotrexate). Synthetic DMARDs impede RA progression but are slow acting and have a limited efficacy (Smolen and Steiner 2003). To develop a more effective therapy, the pathogenesis of RA was further investigated. The pivotal involvement of proinflammatory cytokines including TNF in RA was then established.

The realisation that TNF was critical in RA followed a series of key studies. Firstly, TNF blockade was found to down regulate production of IL-1 from RA synoviocytes and indicted that TNF was the main inducer of IL-1 (Brennan, Chantry et al. 1989). Granulocyte macrophage colony stimulating factor (GM-CSF), also implicated in RA progression, was also found to be dependent on TNF (Haworth, Brennan et al. 1991). Secondly, TNF was present in rheumatoid synovial tissues but not in normal synovial membrane (Chu, Field et al. 1991). It was then shown that by neutralising TNF in a mouse model of arthritis, inflammation was ameliorated and the joint destruction impeded (Williams, Feldmann et al. 1992).

Blockade of TNF also protected hTNF transgenic mice from cachexia (wasting syndrome) and TNF-induced mortality (Siegel, Shealy et al. 1995). The efficacy of anti-TNF in animal models prompted the successful use of the drug in clinical trials (Elliott, Maini et al. 1994; Elliott, Maini et al. 1994; Elliott, Feldmann et al. 1995). However, as the drug (infliximab) was a chimeric (human/mouse) mAb it was feared that it would lose efficacy over a prolonged period of time because of anti-globulin responses (Feldmann and Maini 2003). This was overcome by combining infliximab with methotrexate, and 60-70% patients responded with a 20% improvement in symptoms, while around 40% patients achieved a 50% improvement (Maini, Breedveld et al. 1998). Similar success was then achieved with new anti-TNF biologics etanercept (Moreland, Schiff et al. 1999; Weinblatt, Kremer et al. 1999) and fully human adalimumab (Weinblatt, Keystone et al. 2003). However, as anti-TNF is not effective in all patients, other proinflammatory cytokines such as IL-17 are being investigated as potential additional targets.

Inadequate pain relief is a common problem in RA patients. For example, it was reported that around 60% of patients with chronic pain visit their doctor about their symptoms an average of 2-9 times in 6 months, (Breivik, Collett et al. 2006). Therefore a decrease in pain score as measured by the American College of Rheumatology (ACR) is a desirable goal in clinical trials. Traditionally, rheumatologists have approached pain relief by targeting local inflammation (Borenstein 2010). However, chronic pain is typified by changes in central sensitisation. This means that chronic pain can persist long after the peripheral damage has healed and can develop in normal tissues away from the original site of inflammation (Woolf 2004; Kidd, Langford et al. 2007). Chronic pain is not just

governed by tissue damage but by gender, environmental, and psychological factors (Kidd, Langford et al. 2007; Keefe and Somers 2010). Pain perception is therefore individual and subjective, and pain relief needs should be addressed on a patient by patient basis.

Current analgesics used in RA pain management are NSAIDs (Kean and Buchanan 2005), often in combination with other analgesic agents such as paracetamol (Graham, Day et al. 1999). RA patients treated with anti-TNF biologics have reported rapid analgesia (Kidd, Langford et al. 2007) . However many remain on additional pain medication, suggesting that any analgesia from anti-TNF is potentially indirect. Further study is needed to understand the relationship between TNF and pain pathways.

Pain management in RA is now also being tackled using pain coping skills training (PCST). PCST provides training in coping strategies in challenging pain situations and RA patients receiving this therapy demonstrate enhanced pain control (Keefe and Somers 2010).

Pain in RA is governed by multiple events, including peripheral inflammation and plasticity of the peripheral and central nervous system. By understanding the mechanisms driving this process and the method of action of analgesics, a more refined, effective pain therapy can be designed.

1.2 Animal models of arthritis

Animal models are used to investigate aspects of RA by identifying potential mediators of the disease and test efficacy of novel therapeutics in vivo (Asquith, Miller et al. 2009). TNF was identified as a key target in RA, with the transfer of the drug to the clinic facilitated by the successful treatment of animal models of arthritis. More recently, these models are being used to assess acute and chronic pain, and to test drugs as analgesics (Inglis, Notley et al. 2007; Boettger, Hensellek et al. 2008). Animal models of arthritis are described in brief in Table 1.2 adapted from (Inglis 2010).

	Model	Induction	Characterisation	Mono/Poly arthritic	Strain-specific	MHC Class II association	Mechanism
Induced disease	CFA-induced arthritis	Inject CFA into the footpad or joint	Infiltration of inflammatory cells, pannus formation, cartilage loss	Mono	No	Yes	Innate inflammation
	Adjuvant-induced arthritis (AIA)	Immunise with CFA at base of tail	Infiltration of inflammatory cells, pannus formation, cartilage loss & bone erosion	Poly	Yes	Yes	APC presentation to self antigen
	Antigen-induced arthritis (mBSA)	Immunisation with mBSA followed by intra-articular injection in the same joint	Synovial hyperplasia, perivascular infiltration with inflammatory cells	Mono	No	No	Targeting inflammation to joint
	Streptococcal cell wall arthritis (SCW)	SCW is injected into the knee then a 2nd i.v. injection is given	Infiltration of inflammatory cells, pannus formation, cartilage loss & bone erosion	Mono	Yes	No	Targeting inflammation to joint
	Collagen-induced arthritis (CIA)	Immunisation with type II collagen in CFA	Synovial hyperplasia, cellular infiltration, pannus formation, cartilage loss & bone erosion	Poly	Yes	Yes	T/B cell response to cartilage antigen
	Zymosan-induced arthritis (ZIA)	Zymosan is injected into the joint	Synovial hyperplasia, cellular infiltration, pannus formation.	Mono	No	Yes	TLR2 ligand
Spontaneous disease	Transgenic cytokine overexpressing mice	Engineered to overexpress pro-inflamatory cytokines	Synovial hyperplasia, cellular infiltration, pannus formation, cartilage loss & bone erosion	Poly	N/A	No	Overexpression of cytokines
	K/BxN arthritis	KRN TCR transgenic mice crossed with NOD mice	Synovial hyperplasia, cellular infiltration, pannus formation, cartilage loss & bone erosion	Poly	Yes	Yes	AutoAb in joint
	Transgenic SKG arthritis	Point mutation in ZAP-70	Synovial hyperplasia, cellular infiltration, pannus formation, cartilage loss & bone erosion	Poly	Yes	Yes	ZAP-70 mutation leads to high selection of arthritogenic T cells

Table 1.2. Animal models of arthritis. Table showing various animal models of arthritis. Adapted from Inglis et al 2010

1.2.1 Adjuvant Induced Arthritis

Adjuvant arthritis (also known as CFA induced arthritis) is induced via a single injection of Complete Freund's adjuvant (Incomplete Freund's adjuvant plus *Mycobacterium tuberculosis*) (Pearson 1956). Injected at the base of the tail in susceptible strains it induces poly arthritis within 10-45 days post-injection. Features of this model include oedema, leukocyte infiltration into the joint, pannus formation, and cartilage and bone erosion. The disease in general subsides after one month, unlike RA (Williams 1998). Although the mechanism of CFA arthritis remains unclear, there is an elevation in the activity of antigen-presenting cells (APCs) following immunisation. This would lead to presentation of the unrecognised endogenous antigen to auto-reactive T cells. Alternatively, CFA is injected into the hind paw where a mono arthritis is rapidly achieved by infiltration of inflammatory cells, pannus formation and cartilage loss. Proinflammatory cytokines such as TNF and IL-6 are increased in the affected paw within 3h post injection. CFA paw injection has been extensively studied in inflammatory pain research (Larson, Brown et al. 1986; Walker, Fox et al. 1999).

1.2.2 Zymosan-induced arthritis

Zymosan is a polysaccharide derived from the cell wall of *Saccharomyces cerevisiae*. When injected into the murine joint, an acute inflammatory arthritis develops within three days, subsides by day 7 and re-activates by day 25 (Keystone, Schorlemmer et al. 1977). Like CFA injection into the paw, it is a mono arthritis.

Zymosan induced arthritis (ZIA) is characterised by synovitis, pannus formation and cellular infiltration. Zymosan is a ligand for toll-like receptor (TLR) 2, which induces proinflammatory cytokines from macrophages, thereby initiating disease (Frasnelli, Tarussio et al. 2005).

1.2.3 Antigen-Induced Arthritis

Antigen-induced arthritis (AIA) develops following intra-peritoneal injection of methylated bovine serum albumin (mBSA) into animals that have previously been immunised with the same antigen (Brackertz, Mitchell et al. 1977). A mono arthritis then develops with leukocyte infiltration into the knee joint, proliferation of the synovial lining layer, pannus formation and cartilage and bone erosion. The disease however, does not spread beyond the injected joint (Williams 1998).

1.2.4 Streptococcal Cell Wall-Induced Arthritis

Sonicated streptococcal cell walls (SCW) are injected intra-articular into the animal knee joint. This is followed by a second dose into the tail vein into susceptible rats or mice resulting in a phasic disease of acute inflammation, remission, and then chronic monoarthritis (Esser, Stimpson et al. 1985; Wilder 1988). The SCW joint bears similarities to RA including infiltration of macrophages, CD4+ T cells and neutrophils, plus pannus, cartilage destruction and bone erosion. Like other forms of monoarthritis, it is limited by a lack of systemic effects.

1.2.5 Spontaneous Arthritis in Transgenic Strains of Mice

1.2.5.1 hTNF and hIL-1α transgenic mice

Mice overexpressing a human TNF transgene develop spontaneous arthritis by 4 weeks of age, which is prevented by continuous treatment with anti-human TNF monoclonal antibody. Although TNF is over expressed systemically, it is the joints that are largely affected, supporting the finding that TNF is a key mediator of RA (Kollias, Douni et al. 1999). In addition, another TNF overexpressing transgenic mouse has been created known as $TNF^{\Delta ARE}$. $TNF^{\Delta ARE}$ mice prevent TNF mRNA destabilisation, effectively removing the brake on TNF production, resulting in TNF overexpression. As well as spontaneous arthritis, these mice also present with inflammatory bowel disease, highlighting the fact that TNF is important in other forms of autoimmunity (Kontoyiannis, Pasparakis et al. 1999).

IL-1 has been shown to be a major mediator of arthritis, and human IL-1 transgenics, like hTNF mice have a severe polyarthritis by 4 weeks of age (Niki, Yamada et al. 2001). In all these models there is synovial lining layer hyperplasia, pannus, degradation of cartilage and loss of function.

1.2.5.2 The K/BxN model

The K/BxN model of arthritis arose from transgenic mice that express a T-cell receptor (TCR) specific for a peptide from bovine pancreatic ribonuclease in the context of I-A^k (Peccoud, Dellabona et al. 1990). These transgenic mice spontaneously exhibit a form of chronic progressive arthritis with many similarities to RA when bred on to the non-obese diabetic (NOD) background (Kouskoff, Korganow et al. 1996). The unusual MHC class II allele derived from the NOD mouse (*H-2*^{g7}) was found to be sufficient to confer susceptibility to spontaneous disease. Further studies revealed that arthritis could be transferred by injecting naive mice with serum IgG from arthritic mice in a complement- and FcγR-dependent manner, indicating the pathological role played by autoantibodies in this model (Korganow, Ji et al. 1999). The molecular target of the autoantibodies was then identified as glucose-6-phosphate isomerase (GPI), a ubiquitous cytoplasmic enzyme found to accumulate on the lining of the articular cavity, particularly on the cartilage surface (Matsumoto, et al 2002). The particular relevance for human RA is that arthritis may arise as a result of an immune response to an antigen that is not confined to the joint. Therefore, the assumption that RA results from an aberrant autoimmune response to an antigen in the joint may not be valid.

1.2.5.3 SKG mice

SKG mice develop a spontaneous inflammatory arthritis at around 8 weeks of age due to a point mutation in ZAP-70. ZAP-70 is a T cell signal transduction molecule and its mutation altered T-cell receptor signalling, leading to selection of

autoimmune T cells due to a failure in thymic deletion. This arthritis is dependent on the murine local environment, as disease does not occur in germ-free conditions. Features of this disease are also similar to aspects of RA pathology, including involvement of many joints, synovitis, pannus formation and loss of function (Sakaguchi, Takahashi et al. 2003).

1.2.6 Collagen-Induced Arthritis

Collagen induced arthritis (CIA) is the most widely-used model for translational RA research due to its pathological similarity to RA (Holmdahl, Andersson et al. 1989). Immunization of primates, rats or mice with foreign type II collagen in adjuvant induces polyarthritis. In mice, an intra dermal injection of bovine or chicken type II collagen in CFA induces onset of arthritis around 14-28 days post immunisation. Like RA there is synovitis, immune cell infiltration, pannus formation, bone erosion, loss of function (Trentham 1982) and chronic pain (Inglis, Notley et al. 2007). Susceptibility to CIA is linked with MHC class II genes and DBA/1 mice classically used bear one of these MHC susceptible haplotypes (I-Aq) making it an ideal strain for this disease (Holmdahl, Karlsson et al. 1989; Holmdahl 2003). Unlike human RA, CIA in DBA/1 mice is relatively acute but like RA features elevated proinflammatory cytokines, including TNF, IL-1, IL-17 and IL-6, in the arthritic joint (Marinova-Mutafchieva, Williams et al. 1997; Lubberts, Joosten et al. 2001; Murphy, Langrish et al. 2003). Blockade of these cytokines reduces clinical score, paw swelling, histological changes and pain (Williams, Feldmann et al. 1992; Alonzi, Fattori et al. 1998; Lubberts, Koenders et al. 2004; Inglis, Notley et al. 2007).

However, investigating the role of poorly characterised mediators is facilitated by the use of transgenic and knockout mice. In general, these animals are bred on a C57BL/6 background. This represents a problem as the C57BL/6 strain is resistant to bovine type II collagen induced arthritis. However, my colleagues and I have now established that C57BL/6 mice are susceptible to CIA when chicken type II collagen is used for immunisation. CIA in C57BL/6 mice is a milder, more chronic disease, but still marked by synovitis, immune cell infiltration, pannus formation, bone erosion and loss of function (Inglis, Criado et al. 2007; Inglis, Simelyte et al. 2008). Anti-TNF biologics are also highly effective in treating C57BL/6 CIA, making this strain a valuable alternative to DBA/1 strain (Inglis, Criado et al. 2007).

1.3 Chronic pain

1.3.1 Pain definitions

The sensation of pain is an unpleasant event associated with actual or ensuing tissue damage (For further classification see Table 1.3). Pain (known as nociception) is normally in response to noxious stimuli, usually localised to the site of injury and diminishes when the stimulus is removed. This pain is managed by high threshold neurons in the peripheral and central nervous system. However, tissue damage can lead to an enhanced pain state due to a lower activation threshold of sensitised nerve fibres (Woolf and Ma 2007). This chronic pain is not useful for tissue protection

and is disabling. Pain (dolor) is one of the hallmarks of inflammation along with heat (calor), redness (rubor) and swelling (tumor).

Rheumatoid arthritis patients present with hyperalgesia (increased pain upon noxious stimuli) and allodynia (an exaggerated pain response caused by a non-painful stimulus) as well spontaneous pain (Schaible, Ebersberger et al. 2002).

Pain type	Duration	Pain duration in relation to cause	Features	Value	Pain response	Example
Acute	Seconds	Instantaneous	Pain proportional to cause	Imperative for tissue damage prevention	Rapid Withdrawal	Hot surface
Prolonged	Hours to days	Resolves on recovery, sometimes prior to complete healing of the tissue	Hyperalgesia, allodynia and spontaneous pain	Useful for protecting healing tissue	Avoiding use of damaged tissue	Inflamed wound
Chronic	Months to years	Long term; Can persist long after initial injury has resolved	Hyperalgesia, allodynia, spontaneous pain and parasthesia	None, disabling	Loss of normal function, psychological effects	Rheumatoid arthritis

Table 1.3 A general classification of pain definitions. *After Millan 1999*

1.3.2 Mechanisms of peripheral and central sensitisation

Peripheral sensitisation occurs due to a reduction in the threshold of nociceptors. After tissue damage, chemical mediators are released at the site by resident cells or by infiltrating immune cells. Some like ATP, protons, or 5-hydroxytryptamine can directly activate the nociceptors, eliciting a pain response. Other mediators released by immune cell such as prostaglandin (PGE2), or bradykinin increase the responsiveness of the nociceptor, thereby reducing the threshold for activity.

The mechanisms responsible for peripheral sensitisation are governed by post-translational processing (phosphorylation) and changes in gene expression (alterations to the protein made by the nociceptor) of proteins and ion channels that determine the excitability of the nociceptor. These proteins and ion channels are imperative for converting a stimulus into electrical activity, resulting in an action potential (Woolf and Salter 2000). Phosphorylation is the addition of phosphate groups to a protein's amino acids via kinase enzymes and can alter the function of the protein. This alteration can manifest in increasing the excitability or reducing the temperature needed to open an ion channel (e.g. $Na_v1.8$ and TRPV1 respectively) (Gold, Levine et al. 1998; Premkumar and Ahern 2000; Bhave and Gereau 2004). Phosphorylation can also affect how long the ion channel is open for, increasing the effect of the stimulus on the nociceptor. Inflammatory mediators like PGE2 act locally to provoke a rise in cAMP, which activates protein kinase A (PKA), a kinase important in phosphorylation (Taiwo and Levine 1991; Kress, Rodl et al. 1996; Bhave and Gereau 2004). In addition, some proteins are transported along the axon of the nerve fibre to the cell body of the nerve in the dorsal root ganglia (DRG). Once in the cell body, the protein can alter transcription to increase expression of certain genes and affect translation to enhance production of particular proteins. These proteins are shuttled back to the terminal of the nociceptor to assist in enhancing the nociceptor activity (Dubner and Ruda 1992; Woolf and Costigan 1999; Ji and Woolf 2001).

Central sensitization occurs when neurons in the central nervous system have enhanced excitability. This results in an abnormal response from an innocuous input. Increased activity of peripheral nociceptors (in response to tissue injury)

leads to a change in the synaptic connection between the nociceptor and the central neurons of the spinal cord. This is also known as synaptic plasticity. Like peripheral sensitisation, the process is moderated by phosphorylation and changes in gene expression. After a signal has been received from the periphery, the central terminal of the nociceptor releases neurotransmitter (glutamate), neuropeptides (substance P) and synaptic modulator proteins (BDNF). Production of these mediators results in phosphorylation of membrane receptors and channels via kinases, leading to increased excitability of the neurons (Woolf and Thompson 1991; Yashpal and Henry 1993; Woolf and Salter 2000; Kuner 2010). A later phase of central sensitisation is due to increased protein production of inflammatory mediators, which through promoting kinases and therefore phosphorylation targets, maintain excitability, resulting in persistent, chronic pain (Dubner and Ruda 1992; McCarson and Krause 1994; Michael, Averill et al. 1997; Mannion, Costigan et al. 1999; Latremoliere and Woolf 2009).

1.3.3 Methods of pain detection in rodents

Pain detection in humans largely relies on communication. Understanding the location, nature and intensity of the pain and how it affects the patient's activity is important in assessing how effective analgesics are. Rodents cannot communicate information regarding their pain in the same way, but their activity is similarly affected. In humans, facial expressions can also denote pain, and recently the mouse grimace scale (MGS) was developed to measure spontaneous pain using mouse facial changes during pain (Langford, Bailey et al. 2010). Traditionally, pain in

rodents can be quantified by response to a stimulus or by measuring spontaneous activity (Inglis 2010).

Evoked pain response is a simple and rapid way of detecting hyperalgesia. The most common methods involve applying a stimulus to the paw and measuring the time for the rodent to remove the paw from the stimulus. Withdrawal is often accompanied by licking the paw. The Hargreaves test applies an increasing thermal stimulus to the hind paw (Inglis, Notley et al. 2007), and the Von Frey hairs are administered to the paw in increasing thickness, delivering a force stimulus (Inglis, Nissim et al. 2005). When a rodent has hyperalgesia or allodynia, the time taken to elicit a painful response is reduced. In healthy mice for example, the thermal pain response is typically between 8-12 seconds, but in mice with hyperalgesia this is decreased to 1-4 seconds. These tests have the advantage of being applicable to poly arthritis models as well as mono-arthritis and acute hyperalgesia models. If only 1 hind paw is involved, then an additional non-evoked test (Linton incapacitance tester) can be used.

The Linton incapacitance tester is divided into two weighing scales. The animal is placed inside the chamber with one paw on each weighing platform. The weight on each platform is measured and animals with enhanced pain will lean to their unaffected side (Inglis, McNamee et al. 2008; McNamee, Burleigh et al. 2010).

General day-to-day activity is often affected in patients with chronic pain; therefore it is important to investigate general behaviour and activity in rodents. Mice with CIA have reduced climbing, grooming and locomotion, and increased immobility (Inglis, Notley et al. 2007). LABORAS (Laboratory Animal Behaviour Observation

Registration and Analysis System) converts the vibrations created by the mice into activity. With all tests mentioned here, effective analgesics will restore a normal pain response in rodents (Inglis, Notley et al. 2007; McNamee, Burleigh et al. 2010).

1.4 Cells of the Peripheral and Central Nervous System

1.4.1 Cells of the Peripheral Nervous System

The peripheral nervous system (PNS) consists of all nerves and nerve-related cells outside of the central nervous system (CNS) whose function is to provide a link between the CNS and the periphery (Olsson 1990).

Accompanying the peripheral neurons are Schwann cells which wrap around the axon of the neuron. Schwann cells are support cells that provide insulation by producing myelin for myelinated nerves, but are now thought to have additional immune functions. Indeed, Schwann cells are also present in unmyelinated nerves, where they are considered to be vital for the maintenance of these axons and pain sensation (Griffin and Thompson 2008). In mice, when communication between the unmyelinated axons and non-myelinating cells is impaired (via disruption of Neuregulin 1 signalling), they become progressively insensitive to heat and cold stimuli (Chen, Rio et al. 2003). Schwann cells express proinflammatory cytokines and MHC II after injury, with TNF thought to be important for their activation. Although little is understood about the function of Schwann cells besides the

production of myelin, it is suggested that they provide an early defence for the PNS neurons during immune cell infiltration after nerve injury (Armati 2007; Griffin and Thompson 2008).

Satellite cells are glial cells that cover the outside of the neuron cell body and studies regarding their role are limited. Their main function appears to be regulation of the nerve environment, but like Schwann cells, they can express TNF and other pro-inflammatory cytokines when activated (Armati 2007; Takeda, Takahashi et al. 2009). In addition to the nerve-related cells there are reported to be resident macrophages (around 9%) and T cells, which can produce cytokines and chemokines to facilitate immune cell infiltration (Scholz and Woolf 2007).

1.4.2 Cells of the Central Nervous System

The CNS is composed of the brain and the spinal cord and contains two major cell types; the neurons and the glia. Although CNS neurons are the functional cells needed for pain transmission, they are not the dominant cell type in the CNS, accounting for only about 10% of the cell population.

Spinal neurons are separated into three main classes. Sensory neurons take information (such as pain transmission) away from the periphery via the dorsal horn into the CNS but also project to interneurons in the cord where there activity is modified prior to signal relay. Motor neurons follow sensory input and transmit signals back from the CNS via the ventral horn and synapse in the periphery, controlling motor function (including movement away from a pain source).

Interneurons have axons retained entirely in the CNS and are largely inhibitory. Interneurons act as a connector between sensory and motor nerves, relaying information from the sensory nerve to the motor nerve. The white matter contains the axons of the nerves, while the cell body and dendrites are retained in the grey matter (Burke 1977; Brown 1981).

Glia cells in the CNS are made up of astrocytes and microglia. Astrocytes outnumber neurons 5-10 fold, and in common with neurons, originate from the neural crest. Traditionally, these cells were thought of as having a passive function, acting as structural support for the neurons, and maintaining homeostasis. However, it is now known that as well as regulating the neural microenvironment, astrocytes have an active and vital role in healthy and diseased CNS tissue (Sofroniew and Vinters 2010). Astrocytes are star shaped cells with processes radially extending from the cell. The extended branches of the astrocytes are vital for maintaining contact with blood vessels and monitoring CNS blood flow. In addition, it has been suggested that astrocytes are important in blood-brain barrier (BBB) development and integrity (Bush, Puvanachandra et al. 1999; Sofroniew and Vinters 2010). Glial fibrillary acid protein (GFAP) is a classic marker for identification of these cells by immunohistochemistry and is present in the processes. Upon astrocyte activation (astrogliosis), GFAP expression increases as the cell processes extend (Hald 2009). Astrogliosis occurs during CNS trauma or disease, and chronic pain states (Inglis, Notley et al. 2007; Hald 2009). A link between astrocytes and chronic pain was first addressed by demonstrating that increased GFAP correlated with hyperalgesia in a chronic constriction nerve injury (CCI) model (Zhang and De Koninck 2006; Hald, Nedergaard et al. 2009). Since then, astrogliosis has been reported in a variety of

neuropathic and inflammatory pain models, including CIA (Inglis, Notley et al. 2007).

Although there is evidence for astrocytic involvement during chronic pain, there is less to suggest the same for the microglia. Microglia are CNS immune cells with a haematopoietic origin (Perry and Gordon 1988; Flaris, Densmore et al. 1993; Scholz and Woolf 2007). In healthy CNS tissue they maintain a quiescent phenotype, but are not silent. Resting microglia maintain the local environment, including synapses, and survey CNS tissue for pathogens (Kreutzberg 1996). After stimulus from injury or infection, they become active and then phagocytic, and are necessary for CNS protection. However, activated microglia are also cytotoxic, which can result in neuronal cell death (Graeber and Streit 2010). Microglia are implicated in degenerative disorders such as Parkinson's, Alzheimer's and multiple sclerosis (MS) (Graeber and Streit 2010), but there is limited evidence to suggest they are important in arthritis. Microglial activation has been reported in CFA induced arthritis and AIA (Wu, Zhang et al. 2005) (Bao, Zhu et al. 2001), but is not detected in CIA (Inglis, Notley et al. 2007).

1.5 Pain Pathways

1.5.1 Peripheral afferent fibres

Nociceptive information is transmitted from the periphery to the spinal cord (and then onto the brain) by peripheral sensory neurons also known as peripheral afferent fibres, or PAFs (LaMotte and Campbell 1978; Treede 1995; Hunt and Mantyh

2001). PAFs can be broadly divided into three subsets, Aß, Aδ and C fibres depending on their diameter and conduction velocity (Table 1.4). The PAFs cell body is located in the dorsal root ganglia (DRG) but the axons project as far as the dorsal horn of the spinal cord when they synapse to relay the information into the CNS. The dorsal horn is organised into sections called laminae. The lamina that the PAF terminates in differs for each subset (Averill, Davis et al. 2002). Aß PAFs are large in diameter (<10μm), myelinated axons with a low threshold for activation and rapid conduction velocity of 30-100ms^{-1}. In the healthy individual, Aβ-fibres transmit proprioceptive information, such as joint movement, or pressure. Aβ fibres have two projections, one terminating in the inner laminae III/IV and one in lamina V.

Fibre	Diameter	Conduction velocity	Myelin	Laminar location	Normal sensation
Aα/β	Large (6-12μm)	>30-100 m/s	Yes	III-VI	Pressure touch, vibration
Aδ	Medium (2-6μm)	>12-30 m/s	Yes (Thin)	I-II, V	Fast sharp pain
C	Thin (0.4-1.2 μm)	<0.5-2 m/s	No	I-II, V	Slow, dull pain

Table 1.4 Peripheral nerve properties . Properties of sensory neurons in the DRG. *After Millan 1999, Wall and Melzack 2005, Inglis et al 2010*

Aδ-fibres have a medium axonal diameter of 2-6μm, and are thinly myelinated, with a conduction velocity of 12-30ms^{-1} and a medium threshold for activation. The majority of Aδ fibres transmit nociceptive information, specifically inducing a rapid acute pain sensation after a noxious stimulus. Aδ fibres project to lamina I and V. Finally, C-fibres are high threshold unmyelinated neurones with a small diameter of 0.4-1.2μm and a conduction velocity of 0.2-2ms^{-1}. Almost all C fibres are responsible for nociceptive transmissions, but being unmyelinated are slower, eliciting a dull secondary pain. C fibres are further divided into peptidergic or non peptidergic depending on the neurochemical expressed. (Millan 1999; Melzack 2006; Inglis 2010)

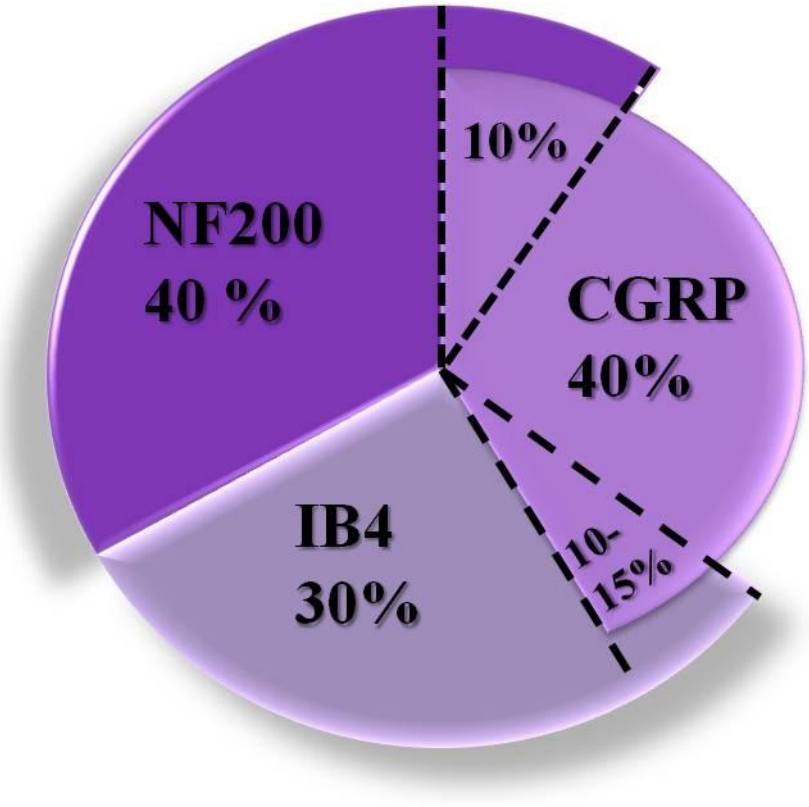

Figure 1.2 A summary of neuronal populations in the rodent DRG. The percentage displayed is the percentage of the total DRG neuronal population bearing that marker. Dashed lines indicate overlapping populations. NF200 neurons are myelinated, and a small proportion (around 10%) are nociceptive (Aδ fibres). The majority of the DRG neurons are nociceptive C fibres. C fibres are differentiated into peptidergic (CGRP) and non-peptidergic (IB4), but around 10-15% display both markers. *Adapted from McMahon and Koltzenburg 2006*

Peptidergic fibres express calcitonin gene related peptide (CGRP), and make up around 40% of the rat L4 and L5 DRG (Millan 1999). Around 92% of these fibres express the NGF receptor TrkA and are NGF-dependent for development and survival (Henderson 1996) (Averill, McMahon et al. 1995). Non-peptidergic fibres bind Griffonia simplicifola isolectin B4 (IB4), and also express the ATP receptor, P2X3. These account for approximately 30% of DRG cells in rodents (Averill,

Davis et al. 2002). There is overlap between these two populations of neurones, with around 10-15% C-fibres expressing both P2X3 and CGRP (Millan 1999) (Figure 1.2). Many sub-populations of nociceptive neurones are contained within peptidergic and non-peptidergic fibres. Furthermore, it is important to note that there are reported differences in C fibres between rats and mice. Over 50% of non-peptidergic neurons in rats also express TRPV1. However, in mice only 6% of non-peptidergic C fibres expressed both IB4 and TRPV1 suggesting that certain pain pathways may be regulated differently depending on the species (Zwick, Davis et al. 2002). Peptidergic C fibres synapse in lamina I and the outer side of lamina II, where as non-peptidergic project further into the inner lamina II (Scholz and Woolf 2007).

1.5.2 Lamina projections

Deep laminae (III-V) (Aß and Aδ fibres) project to lateral reticular nucleus (LRN), subnucleus reticcularis dorsalis (SRD) and the giantocellular/lateral paragigantocellular reticular nuclei (NGc) (all located in the medulla and project to the cerebellum). The cerebellum is 'motor function' area of the brain and this system could be responsible for the physical reaction upon receiving a painful stimulus.

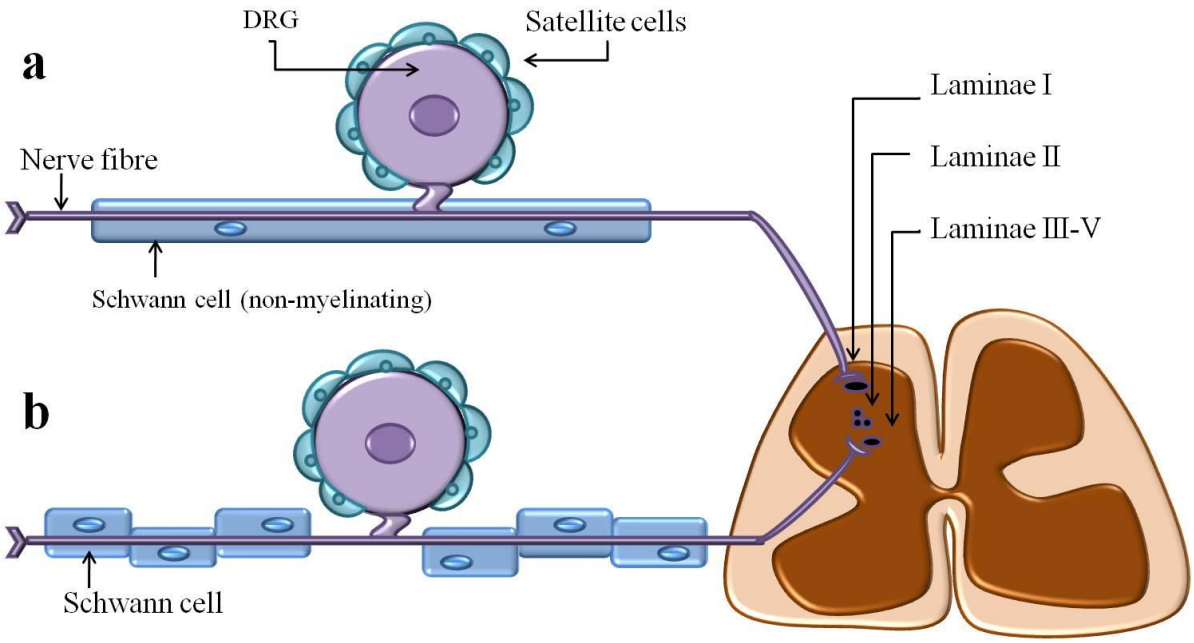

Figure 1.3 Schematic of pain pathways. PAFs (peripheral afferent fibres) relay information to the CNS through nerve fibres which synapse in the dorsal horn of the spinal cord. **A)** Slow unmyelinated C fibres are responsible for nociceptive transmission and project to the outer lamina layers of the dorsal horn. **B)** Fast myelinated fibres relay information about pressure, movement (Aα/β) and nociception (Aδ). A fibres generally project into deeper lamina (III-V) with the exception of some Aδ fibres that project to laminae I. *Adapted from Scholtz and Woolf 2007, Mantyh 2006, Inglis 2010.*

Superficial laminae (I-II) are where Aδ and C fibres terminate (Figure 1.7). Lamina I project to lateral parabrachial area and the caudal area of the lateral thalamus, namely the ventral posterolateral nucleus (VPL), the ventral posteromedial nucleus (VPM) and the central nucleus for the amygdala (CeL). As the superficial laminae are the prime targets for the Aδ and C PAFs, and the amygdala is the centre for emotional reaction, it would follow that these laminae are involved in pain sensation and emotional response. However, C fibres project to different lamina depending on whether they are peptidergic or non peptidergic, suggesting further alternate pathways. Peptidergic fibres project to lamina I suggesting that they are the major fibres for pain sensation and emotional response. Non-peptidergic fibres largely

project to the inner laminae and therefore are likely to regulate motor response after pain sensation (Inglis 2010).

1.6 Inflammatory mediators of nerve sensitisation

Tissue damage results in release of inflammatory mediators from the injured resident cells. Some of these mediators directly sensitise the peripheral nociceptors by lowering their activation threshold. However, other mediators act indirectly through the recruitment of immune cells that release further inflammatory proteins that induce additional directly sensitising agents.

1.6.1 Nerve growth factor and prostaglandins

Nerve growth factor (NGF) is required to promote survival of sensory neurones before birth and growth and neurotransmission after birth. NGF administration induces hyperalgesia in humans (Kidd and Urban 2001) and rodents. It is thought that NGF mainly directly on sensory neurones to induce hyperalgesia, but also requires immune cell infiltration. These infiltrating cells are known to release algesic mediators, including prostaglandins (Cockeran, Steel et al. 2001) and further NGF from the cell or from resident mast cells. Indeed, blockade of neutrophils prevents NGF-induced hyperalgesia in rats (Bennett, al-Rashed et al. 1998).

There are 2 receptors for NGF, high affinity tyrosine kinase (TrkA) and common neurotrophin (p75NTR). Nerve sensitisation by NGF is thought to be largely dependent on TrkA as NGF can still induce thermal hyperalgesia in $p75NTR^{-/-}$

mice. However, neutralising antibodies to p75NTR blocks CFA-induced hyperalgesia (Watanabe, Ito et al. 2008), suggesting further studies are required to fully understand the role of NGF receptors in hyperalgesia. NGF signalling via TrkA results in phosphorylation of TRPV1 and sodium channels such as tetrodoxin-resistant (TTX-R), potentially through PI3K and MEK/ERK intracellular signalling cascades. NGF induced thermal hyperalgesia is thought to be dependent on the phosphorylation of TRPV1, indeed, $TRPV1^{-/-}$ mice are insensitive to NGF induced thermal induced hyperalgesia.

Prostaglandins are lipid compounds that act on prostanoid receptor subtypes in DRG neurons (Huang, Zhang et al. 2006). Prostaglandins are synthesised in response to noxious stimuli and inflammation (Ito, Okuda-Ashitaka et al. 2001) by COX-1 and COX-2 (induced by cytokines and other inflammatory stimuli) (Kidd and Urban 2001) from various cell types including neutrophils (Cockeran, Steel et al. 2001). Prostaglandins are important in hyperalgesia as they sensitise nociceptors by lowering their activation threshold of TRPV1 (Huang, Zhang et al. 2006) and TTX-R via cAMP and kinase induction. (England, Bevan et al. 1996).

1.6.2 Immune cells

There has been an increasing focus in rodent models lately on the importance of cellular infiltration in the genesis of inflammatory hyperalgesia. These cells release key mediators of pain, including prostaglandins and NGF.

Neutrophils are the most abundant immune cells, accounting for around 55-70% of all white blood cells, and are the dominant cell type in sites of inflammation in the first hours following infection or injury. Blockade of neutrophil migration using a polysaccharide selectin inhibitor (fucoidan) abolishes cytokine induced hyperalgesia (Cunha, Verri et al. 2008). Neutrophils have been shown to release NGF and prostaglandins (Cockeran, Steel et al. 2001; Foster, Wicks et al. 2002), which act to sensitise the nociceptive system. Neutrophils also produce lipooxygenase (LOX) metabolites, some of which can directly act as an agonist on TRPV1. Furthermore, inhibition of LOX using nordihydroguaretic acid (NDGA) in murine CFA arthritis diminishes hyperalgesia (Szabo, Helyes et al. 2005). Neutrophils are recruited to sites of inflammation via chemokines (KC, GRO) released at the site of inflammation due to their induction by proinflammatory cytokines. Blockade of these cytokines prevents neutrophil migration (Cunha, Verri et al. 2008).

Macrophages are implicated in the inflammatory hyperalgesia, through the release of proinflammatory cytokines and prostaglandins. Macrophage infiltration into the DRG follows CFA-injection, and also occurs during AIA (Inglis, Nissim et al. 2005; Boettger, Hensellek et al. 2008; Segond von Banchet, Boettger et al. 2009). Anti-TNF therapy in rats following CFA injection reduces macrophage infiltration into the DRG, which correlates with a reduction in mechanical allodynia (Boettger, Hensellek et al. 2008). Macrophage infiltration is dependent of TNF through up-regulation of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (Schaible, von Banchet et al. 2010). Infiltrating cells during neuropathic models are associated with nerve injury. However, this infiltration during AIA is not associated with nerve damage, potentially revealing a novel mechanism for macrophages in

inflammatory pain states in the absence of neuropathy (Segond von Banchet, Boettger et al. 2009; Inglis 2010).

1.6.3 Cytokines

Cytokines are small secreted proteins that regulate cell response over a short range and are vital for maintaining inflammation. Cytokines can be divided into pro-inflammatory (including tumour necrosis factor-α (TNF), interleukin- (IL) -1ß, IL-17 and IL-6) or anti-inflammatory (including IL-10 and IL-4) depending on their action (Sommer and Kress 2004). Cytokines are produced by a number of immune cell types including lymphocytes, macrophages and neutrophils. After tissue damage, pro-inflammatory cytokines are released at the site from injured cells and act to induce a cascade of cytokine and chemokine production, which amplify the inflammatory response. The role of pro-inflammatory cytokines in inflammatory pain has been addressed in several animal models of arthritis. The focus has largely been on TNF due to its well established importance in RA pathology and the clinical success of anti-TNF therapies (Inglis 2010).

Injection of TNF into the footpad produces a dose-dependent, rapid mechanical and thermal hyperalgesia. This acute action of TNF is transient, returning mechanical threshold to normal within 24h (Woolf, Allchorne et al. 1997; Cunha, Verri et al. 2005). TNF induced pain is largely dependent on prostaglandin release (Cunha, Verri et al. 2008) but there is some evidence that TNF can sensitise the sensory fibres directly. Application of TNF will induce nerve discharge in a proportion of C fibres after 2 minutes (Sorkin, Xiao et al. 1997). In injured nerves (neuropathic

pain), TNF is transported retrograde into the dorsal horn and anterograde to the peripheral injury site (Shubayev and Myers 2001). TNF receptors TNFR1 and TNFR2 are present in the DRG (Schafers, Sorkin et al. 2003; Schafers, Sommer et al. 2008), although their exact locations within the DRG cell types are not fully elucidated. To understand any direct mechanism of TNF on sensory nerves, the expression of the receptors must be addressed as there are other non-neuronal cell types in the DRG that TNF could be acting on.

TNF is also important in models of sustained inflammatory pain. TNF is upregulated in the footpad after injection with CFA or carrageenan, and during CIA. Blockade of TNF is analgesic in CFA arthritis (Inglis, Nissim et al. 2005), CIA (Inglis, Notley et al. 2007) and AIA (Boettger, Hensellek et al. 2008), and carrageenan induced hyperalgesia is prevented in TNFR1 deficient mice (Cunha, Verri et al. 2005). TNF is an established mediator of inflammatory pain however the precise mechanisms controlling this are unclear. It is also unknown whether all other pro-inflammatory cytokines are instrumental in inducing hyperalgesia.

1.7 IL-17

1.7.1 Origin , cellular distribution and function

Murine IL-17 was first cloned in 1993 from a T cell hybridoma (Rouvier, Luciani et al. 1993) and the human form was first characterised in 1995 (Yao, Fanslow et al. 1995; Yao, Painter et al. 1995). The structure of IL-17 has been identified as a disulphide linked homodimer consisting of 155 amino acids with a molecular mass

of 35 kDa (Yao, Painter et al. 1995; Fossiez, Djossou et al. 1996). The crystal structure of IL-17 adopts a cystine knot similar to another family member, IL-17F (Gerhardt, Abbott et al. 2009). IL-17R has no homology to any other receptors outside the IL-17 family. Following cloning of IL-17 and its receptor, a further five proteins and four receptors were re-labelled due to sequence homology (Moseley, Haudenschild et al. 2003).

The cellular distribution of IL-17 is primarily in but not exclusive to T cells (CD4+, CD8+ and γδ T cells) (Korn, Bettelli et al. 2009). IL-17 has also been detected in other cell types namely eosinophils (Molet, Hamid et al. 2001), neutrophils (Ferretti, Bonneau et al. 2003), NKT cells (Coquet, Chakravarti et al. 2008), astrocytes (Meeuwsen, Persoon-Deen et al. 2003) and microglia (Kawanokuchi, Shimizu et al. 2007) but the main source of IL-17 remains the memory CD4+ T cell.

IL-17 is a pro-inflammatory cytokine affecting a variety of cell types in inflammatory responses. By inducing and working in synergy with TNF-α and IL-1β, IL-17 increases local inflammation and the expression of other pro-inflammatory cytokines. IL-17 has therefore been implicated in a range of auto-immune diseases governed by cytokine production including inflammatory bowel disease, psoriasis and RA (Fossiez, Djossou et al. 1996; Chabaud, Durand et al. 1999; Kikly, Liu et al. 2006; Ouyang, Kolls et al. 2008; Korn, Bettelli et al. 2009) (Hwang, Kim et al. 2004).

1.7.2 IL-17 Signalling

IL-17 induced signalling is through its receptor IL-17R (Figure 1.8). Initially, IL-17R was thought to be a single receptor for IL-17, but now it is largely agreed that IL-17R is made up of two functional receptors for IL-17 in a heterodimeric complex (Toy, Kugler et al. 2006) (Gaffen 2009). This complex consists of receptor subunits IL-17RA and IL-17RC.

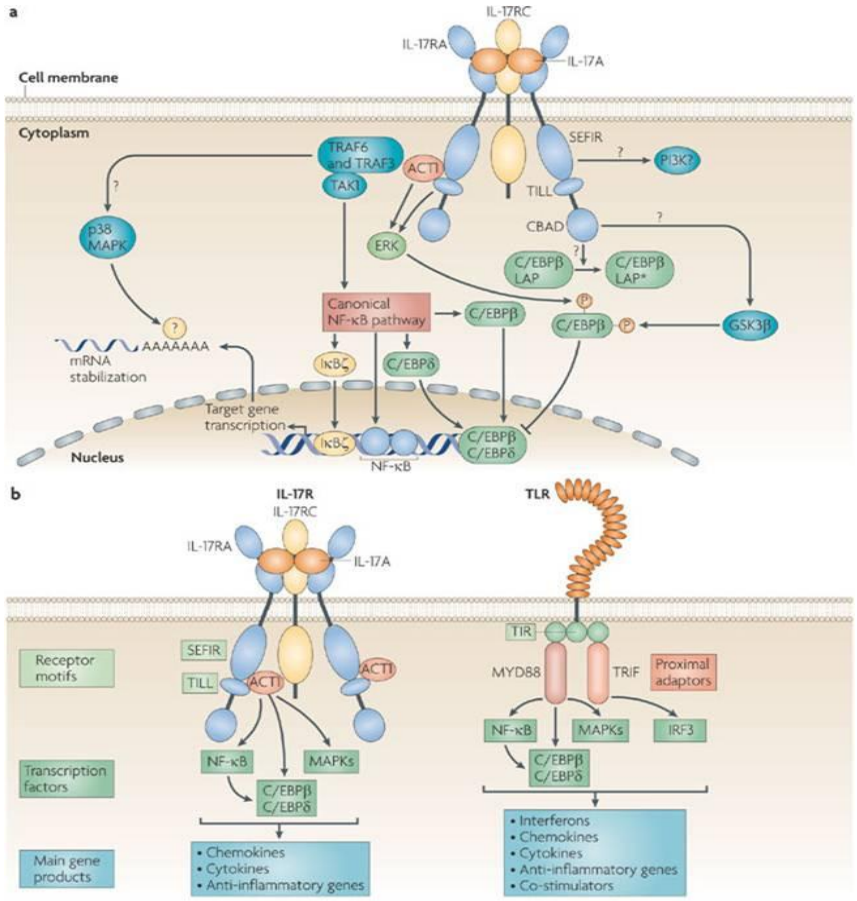

Figure 1.4 IL-17 signalling pathways. IL-17 signals through IL-17R, a complex consisting of IL-17RA and IL-17RC. A) Schematic illustrating IL-17R signalling. B) Comparison of IL-17R signalling with TLR signalling. *Taken from Nature Reviews Immunology Gaffen 2009*

IL-17RC was first described as a receptor for the closely related (≈55%) IL-17 homolog IL-17F (Toy, Kugler et al. 2006), but functional studies labelled IL-17RC as imperative for IL-17 signalling (Zrioual, Toh et al. 2008; Hu, Ota et al. 2010). Interestingly, the affinity for each subunit by IL-17 or IL-17F differs from mice to humans. In humans, IL-17RA binds to IL-17 with high affinity but only weakly to IL-17F. IL-17RC, thought binds IL-17 and IL-17F with equal affinity. Conversely, murine IL-17RA binds IL-17 and IL-17F equally but IL-17RC binds preferentially to IL-17F. As IL-17RC binds IL-17F with high affinity in both humans and mice it has been suggested that IL-17F signalling through this complex could modulate IL-17 signalling (Ho and Gaffen 2010). Although murine IL-17RC is required for IL-17 dependent responses during infection and experimental autoimmune encephalomyelitis (EAE) (Hu, Ota et al. 2010), IL-17 can induce signals in murine T cells that are thought to lack IL-17RC (Ho and Gaffen 2010) . With the precise nature of IL-17 signalling and its receptor unknown, it is difficult to assess how exactly IL-17 exerts its effects.

What is known regarding the IL-17/IL-17R signalling pathway is that both subunits contain SEFIR domains (Maitra, Shen et al. 2007; Ho, Shen et al. 2010). IL-17RA engages this SEFIR domain containing ACT1 to promote downstream signalling. ACT1 is involved in the activation of NF-κB, necessary for induction of most genes by IL-17 (Chang, Park et al. 2006). ACT1 links IL-17R to TRAF6, an adaptor molecule used to transduce the signal to activate NF-κB and kinase pathways

(Gaffen 2009; Iyoda, Shibata et al. 2010; Roussel, Houle et al. 2010). ACT1 is therefore important in IL-17 signalling, but may not act alone. ACT1-/- cells stimulated with IL-17 have activated extracellular signal-related kinases (ERK) (Qian, Liu et al. 2007), suggesting IL-17R has additional novel signalling pathways that require further attention.

1.7.3 Th17 Cell Differentiation and Transcription Factors

Upon antigenic stimulation and cytokine signalling, naive T cell differentiate into T helper cell subsets (Crome, Wang et al. 2010). T helper cells have traditionally been split into either Th1 or Th2 subsets depending on the cytokine profile produced by the cell (Furuzawa-Carballeda, Vargas-Rojas et al. 2007). The mediators driving Th1/Th2 cells are different depending on cell type. Th1 cell differentiation is reliant on IL-12 for differentiation and T-bet transcription factor for linage specific cytokines, whilst Th2 cells require IL-4 for differentiation and GATA-3 transcription factor for linage specific cytokines. Th1 cells are implicated in pathogen clearance and autoimmunity, whilst Th2 cells are associated with host defence against parasitic infections as well as allergy (Bi, Liu et al. 2007; Weaver, Hatton et al. 2007; Korn, Bettelli et al. 2009; Lee, Mukasa et al. 2009). A recent addition to the T helper cell family are Th17 cells which produce cytokines distinct from Th1/2 subsets (Harrington, Hatton et al. 2005) (Bettelli, Carrier et al. 2006). Th17 cells express IL-17, IL-17F, IL-22, IL-21, IL-26, and TNF (Korn, Bettelli et al. 2009; Lubberts 2010). Th17 differentiation requires IL-6 and TGF-β initially, followed by IL-23 for development and survival (Langrish, Chen et al. 2005;

Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006) as depicted in Figure 1.5.

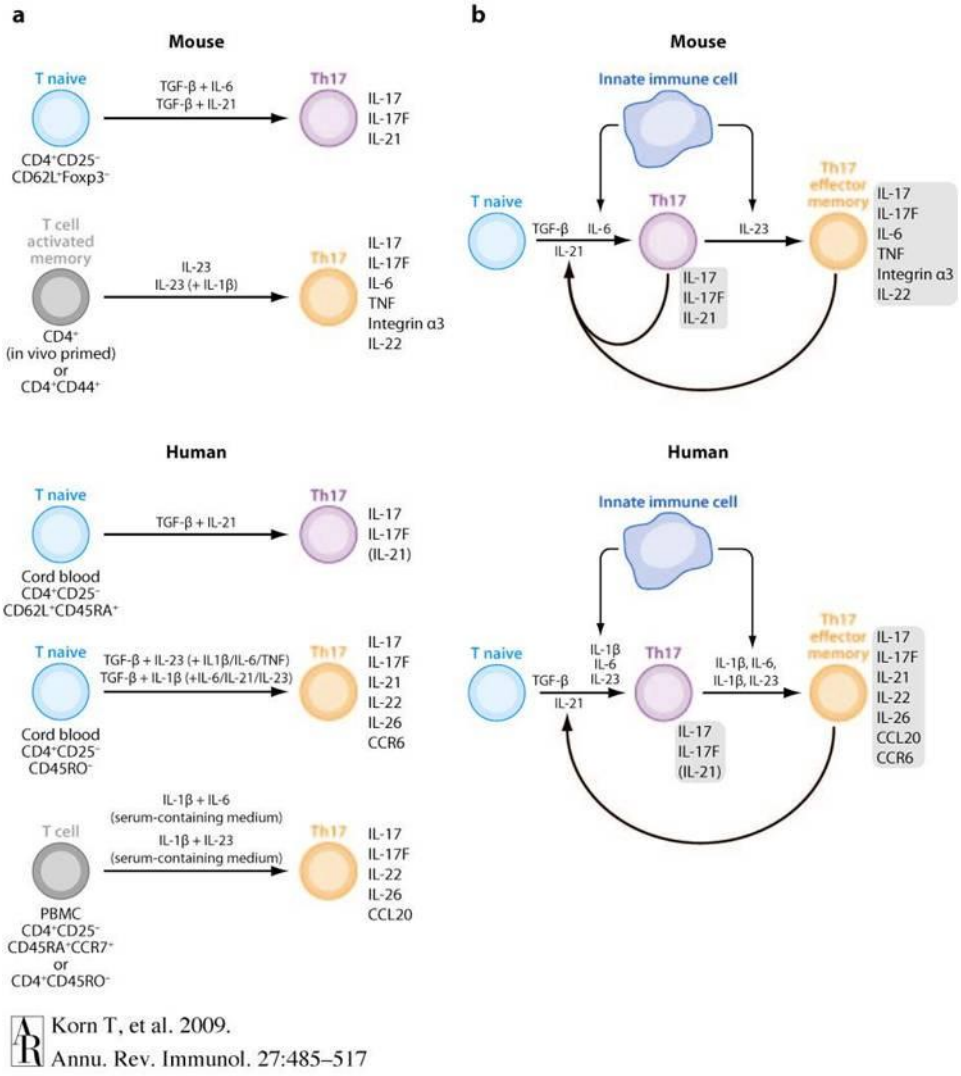

Figure 1.5 Differentiation of Th17 cells in mice and humans. Factors required to induce the development of Th17 cells in mice and humans, from naive cells (blue) or activated CD4+ cells (grey)(*b*) The sources of Th17 differentiation factors in mice and humans

IL-23 has been shown to be critical in mediating Th17 responses in CIA (Murphy, Langrish et al. 2003), thereby making the IL-23/IL-17 axis a proposed therapeutic target (Iwakura and Ishigame 2006; Kikly, Liu et al. 2006; Yago, Nanke et al. 2007). IL-21 has been suggested as an additional factor in murine and human Th17 differentiation in the absence of IL-6, although this is subject to some debate (Deenick and Tangye 2007; Coquet, Chakravarti et al. 2008). The transcription factor RORγt is selectively expressed by murine Th17 cells and IL-17 positive T cells (e.g. γδ T cells) (Ivanov, McKenzie et al. 2006). It has been suggested that IL-17 production is dependent on RORγt as mice deficient in RORγt have much reduced IL-17 producing cells (Korn, Bettelli et al. 2009; Littman and Rudensky). Human Th17 cells express the human orthologue of RORγt, RORC2 (Annunziato, Cosmi et al. 2007; Wilson, Boniface et al. 2007). Overexpression of RORC2 elevates expression of IL-17, IL-17F and IL-26, key cytokines in the Th17 subset (Crome, Wang et al. 2009).

1.7.4 IL-17 in RA

IL-17 was first implicated in RA when its addition to RA synoviocytes was found to elevate IL-6 and IL-8 production (Fossiez, Djossou et al. 1996; Miossec 2007). IL-17 was then shown to be important for neutrophil infiltration via the induction of IL-8 (Laan, Cui et al. 1999). The presence of bioactive IL-17 *in vitro* in RA synovial membrane cultures and synovial fluid further associated it with the disease, although serum levels are minimal (Chabaud, Durand et al. 1999; Kotake, Udagawa

et al. 1999). IL-17 was implicated as a mediator of joint pathology as by administering it *in vitro* osteoclast (bone absorbing cells key for bone erosion) differentiation from bone marrow cells was provoked (Kotake, Udagawa et al. 1999). This was supported by showing that repeated intra-articular injection of IL-17 facilitated joint inflammation, cartilage proteoglycan loss and bone erosion in mice (Lubberts, van den Bersselaar et al. 2003; Lubberts, Koenders et al. 2005). Through the use of animal models of arthritis, blockade of IL-17 was found to be therapeutic and prevent joint erosion (Lubberts, Koenders et al. 2004; Koenders, Lubberts et al. 2005). In addition, mice deficient in IL-17 have reduced severity of CIA (Nakae, Nambu et al. 2003).

Through the upregulation of inflammatory mediators and chemoattractants, IL-17 drives many of the cellular processes that cause the tissue damage and inflammation associated with RA (Figure 1.6). T cells infiltrating into the arthritic joint provide the main source of IL-17, where it acts on synoviocytes and macrophages to produce and synergise with IL-6, IL-1β, and TNF-α to maintain inflammation. IL-17 facilitates cartilage damage by acting on chondrocytes to induce nitric oxide (NO) which prevents matrix synthesis, and on macrophages to release matrix metalloproteinases (MMPs) (Koshy, Henderson et al. 2002). Accumulation of neutrophils increases cartilage loss through release of proteolytic enzymes (Kowanko, Bates et al. 1989) and respiratory burst. The interaction between IL-17 and osteoblasts results in RANKL, a protein necessary for osteoclastogenesis, which leads to bone erosion. IL-17 induced acute joint inflammation and cartilage destruction is dependent on TNF (Lubberts, Joosten et al. 2001), but there is little known about IL-17 being independent of TNF during arthritis (Koenders, Lubberts

et al. 2006). Dissecting the relationship between TNF and IL-17 during acute and arthritic conditions is therefore critical when considering IL-17 as a therapeutic target. IL-17 blockade as a therapy is now being assessed in the clinic, although this is largely based on animal studies and the efficacy of anti-IL-17 treatment in human RA is currently unknown (Genovese, Van den Bosch et al. 2010).

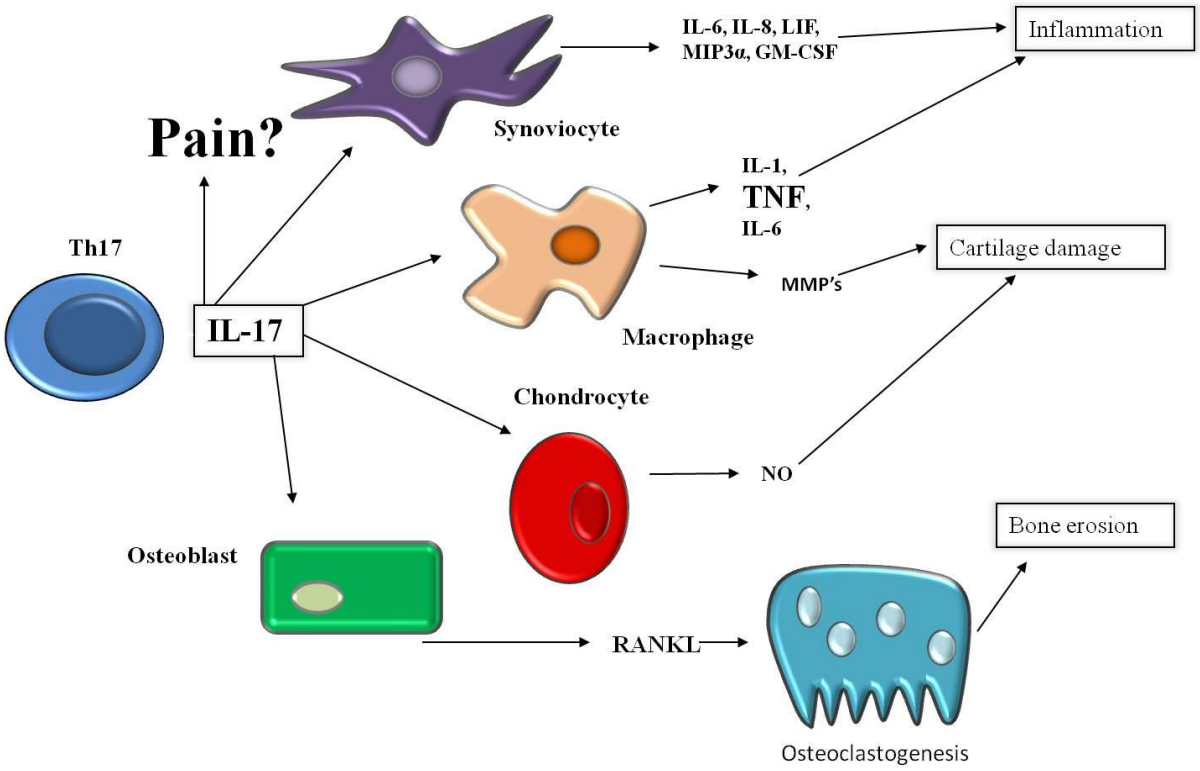

Figure 1.6 IL-17 and RA. IL-17 is implicated in RA pathology by acting on several cells to facilitate inflammation, cartilage degradation and bone erosion. *After Koenders et al 2006.*

1.7.5 IL-17 and Pain

As IL-17 is implicated in RA pathology, new studies are emerging to understand what role IL-17 has in inflammatory pain. Recently, IL-17 was shown to be involved in mBSA AIA hyperalgesia. Blockade of IL-17 in this model reduced mechanical hypernociception. In addition, immune cells also played a role as, with IL-17 induced neutrophil migration into the knee joint, and prevention of this cellular infiltration diminishing hypernociception (Pinto, Cunha et al. 2010). However, this is the only study currently published; therefore this thesis aims to identify further the role of IL-17 in inflammatory hyperalgesia.

1.8 Hypothesis

The hypothesis of this study is that IL-17 through induction of inflammatory proteins, mediates inflammatory hyperalgesia in the periphery. As IL-17 is known to induce TNF, it could be that IL-17 acts upstream of TNF, initiating an inflammatory cascade leading to peripheral sensitisation.

It is established that IL-17 is an important target in CIA, but through the enhancement of peripheral sensitisation, it is hypothesised that IL-17 blockade would be also beneficial in treating chronic pain. By decreasing IL-17 induced

release of pro-inflammatory cytokines at the site of inflammation, there would be a reduction in nerve sensitisation.

1.9 Aims of study

The overall aims of this study are to understand if IL-17 plays a role in mediating inflammatory hyperalgesia both in acute and chronic settings. This will be achieved as follows;

1. IL-17 will be investigated in acute pain through injection into the murine footpad.

2. The relationship between IL-17 and TNF will be examined in the same setting to see if IL-17 is acting independently of TNF.

3. To examine the importance of IL-17 during chronic pain, an anti-IL-17 therapy will be administered to mice with CIA to assess efficacy in both disease modification and hyperalgesia. Gene expression changes are known to be important in the generation of peripheral and central sensitisation and this will be assessed in the periphery (joint and DRG) and the spinal cord.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animal models

Adult male C57BL/6, TNFR1$^{-/-}$ or TNFR2$^{-/-}$ aged 10-12 weeks were used for all acute experiments. C56BL/6 mice have been used previously in studies of IL-17 mediated hyperalgesia, making the strain an appropriate choice. TNFR1-/- and TNFR2-/- mice were maintained on a C57BL/6 background. DBA/1 (classical CIA strain) or C57BL/6 mice ages 10-12 weeks were used for all CIA experiments. Mice were housed in groups of 5 (C57BL/6) or 8 (DBA/1) and maintained at a temperature of 21 ± 2°C (mean ± SEM) on a 12-hour light/dark cycle (7:00 AM to 7:00 PM), with food and water available *ad libitum*. All experimental procedures were approved by the UK Home Office and Ethical Review Process Committee and followed guidelines issued by the International Association for the Study of Pain.

2.1.1 Acute hyperalgesia model

Animals were either given an intra-plantar injection (10µl) of rTNF, rIL-17 (PeproTech EC Ltd, London) or vehicle (PBS +1% mouse plasma) into the right hind paw, the concentration being described in Chapter 3. All injections were performed by Kay McNamee, the exception being the initial pilot study conducted by Kay McNamee and Julia Inglis. Fucoidan at 20mg/kg (as described (Cunha, Verri et al. 2008) in 100µl of PBS (Sigma-Alridge) was administered 2h prior to injection with recombinant cytokines in order to prevent leukocyte infiltration. Anti-TNF polyclonal antibody was raised in rabbits in-house by a previous group and serum IgG purified using Protein G (Melon Gel Pierce), performed by Kay McNamee. In both behaviour examinations, animals were subjected to each test the day prior to study and 1, 3, 6, 18 and 24h post injection.

2.1.2 Air pouch model

The air pouch model of leukocyte infiltration allows for easy retrieval of migrated cells and the exudates can be used to detect proteins secreted in the pouch. Air pouches were raised on the back of mice by subcutaneous injection of 5 mls air on day 0, followed by 1ml on day 4. On day 5, 50 ng of rIL-17, rTNF or PBS vehicle in 100µl was injected into the pouch. 4hrs post injection, the animals were sacrificed and 2ml of PBS was injected into the pouch. After massaging the pouch to ensure efficient cell retrieval, the liquid was re-drawn and the cells pelleted by centrifugation. The cells were then resuspended in fresh PBS to be counted and the supernatant collected for measurement of cytokines by ELISA. All experiments were conducted by Kay McNamee.

2.1.3 Collagen-induced Arthritis model

The CIA model has been previously described as a model of hyperalgesia and so was utilised in investigating analgesic effects of IL-17 blockade in a chronic setting. Mice were immunized by subcutaneous injection at 2 sites at the base of the tail with 100 µl of bovine (DBA/1 mice) or chicken (C57BL/6 require chicken collagen due to bovine collagen being insufficient to induce disease) type II collagen (4 mg/ml) emulsified in Freund's complete adjuvant (CFA) (BD Biosciences, Oxford, UK). For the method of isolating collagen, see 2.4. Arthritis developed in the mice 14–28 days after immunization. The clinical score of the arthritic mice was

determined with a maximum of 3 for each paw, with a maximum score of 12 per mouse. The criteria for each score were as follows; 0= normal, 1= slight swelling and/or erythema, 2= pronounced swelling, 3= ankylosis. The hind paw swelling was measured using calipers. Clinical score and paw swelling measurements were taken for a total of 10 days post onset. Treatment of C57BL/6 mice was carried out with anti-IL-17 (a kind gift from Paula Sardinha at UCB CellTech UK). Anti-IL-17 treatment was by administering 2mg/ml of drug in 100µl sterile PBS intra-peritoneally on the day of disease onset and day 3, 5, 7 and 10 post onset. Immunisation of the mice was conducted by Kay McNamee, Dany Perocheau and Saba Alzabin, and clinical score was largely measured by Kay McNamee, with assistance from Saba Alzabin and Dany Perocheau.

2.2 Behavioural tests

To investigate the involvement of cytokine in the generation of inflammatory hyperalgesia, appropriate tests must be used to detect enhanced pain responses. Animals with hyperalgesia will react more quickly to a heat stimulus, and elicit a withdrawal response with less mechanical force than a naive counterpart. In addition, if there is pain on only one side of the body, the subject will exert more pressure on their unaffected side. Evoked pain and spontaneous behaviour were measured in both the acute and CIA studies. Briefly, thermal hyperalgesia was assessed using the Hargreaves plantar apparatus and mechanical nociception was determined using Von Frey filaments. The Hargreaves and Linton tests were utilised in the acute studies. Both the Hargreaves and Von Frey tests were performed at day

1, 3, 7 and 10 post onset in arthritic, anti-IL-17 treated mice, and age-matched naive controls. LABORAS was only performed day 10 post onset of arthritis.

2.2.1 Hargreaves

Nociceptive thresholds to thermal stimulation were measured using the Hargreaves test (Ugo Basile, Varese, Italy) in both the acute and chronic studies. Animals were placed in a Perspex box and an increasing heat source was applied to the plantar surface of the injected hind paw. The time before the paw was lifted was measured. Care was taken to take only one or two measurements to prevent sensitisation of the paw from repeated tests. Naive animals registered with a time of 8-12 seconds before paw withdrawal, while animals with hyperalgesia recorded times between 1-4 seconds before lifting the paw. All tests conducted by Kay McNamee.

2.2.2 Linton incapacitance tester

Differential distribution of weight was measured using the Linton incapacitance tester in the acute studies. The Linton incapacitance tester is not suitable for CIA measurements as it relies on a change in only one side, where as the CIA model affects multiple limbs. Animals were placed into the Linton with their hind paws on two separate sensors, each measuring weight in grams. The difference between the left (uninjected) and right (injected) was calculated as a percentage. A 100% value indicates equal weight distribution, where as a value of 70% or lower suggests

leaning to the uninjected side. All tests using the Linton were conducted by Kay McNamee with early support from Julia Inglis.

2.2.3 Von Frey

Nociceptive thresholds to mechanical stimuli in the CIA/chronic studies were measured using Von Frey filaments. The filaments are of increasing gauge (0.6-6g) and by depressing the filament under the arthritic paw until bending point it yields a consistent force to the footpad. Using the Dixon up/down method, the lowest gauge of filament consistently eliciting a rapid withdrawal by the mouse (at least 3 times) was considered to be the nociceptive threshold. All use of Von Frey filaments were conducted by Kay McNamee.

2.2.4 LABORAS

LABORAS (Laboratory Animal Behaviour Observation Registration and Analysis System) (Metris B.V. The Netherlands) is a non-invasive technique designed to quantify normal behaviour in rodents (Inglis, Notley et al. 2007; Inglis, McNamee et al. 2008). Each animal is placed into a separate cage linked to a platform that detects motion by vibration. Each vibration is then translated to a particular spontaneous behaviour. C56BL/6 mice from three groups were tested over an 18h period for changes in locomotion, immobility, climbing and grooming. The three groups consisted of mice with CIA at 10 days post onset, mice with CIA at 10 days post onset with anti-IL-17 treatment, and age-matched naive controls. To account

for any cage differences, each group was measured equally between all the LABORAS cages. All use and analysis of LABORAS was done by Kay McNamee.

2.3 Cell culture

2.3.1 NIH 3T3 murine fibroblasts

The murine fibroblast cell line NIH 3T3 was obtained from the ATTC repository. Cells were grown in DMEM containing 10% FBS (PAA) and 100 U/ml penicillin/100µg/ml streptomycin to 60-80% confluency in a 10 cm tissue culture dish. Cells were detached by incubation with prewarmed EDTA/Trypsin (PAA) at 37°C, 5% CO_2 for 5 m, before washing and re-plating 1:4 in fresh medium.

2.3.2 Stimulation of NIH 3T3 cells with TNF or IL-17

Cells were seeded at 5 x 10^3 cells/well in a 96 well flat-bottomed tissue culture plate and left to adhere over-night at 37°C and 5% CO_2. Cells were stimulated in complete medium with 10ng/ml of cytokine. To test TNF blockade, cells were pre-treated overnight with anti-TNF antibody at 10µg/ml. Before stimulation the cells were washed to remove any unbound antibody in the cell supernatant. The cells were then stimulated with cytokine as before. The conditioned medium was harvested over a 24h time course and stored at -70°C prior to chemokine/cytokine determination by ELISA.

2.4 Collagen

Type II collagen was prepared from either chicken carcasses or bovine joints as described previously (Miller 1972; Herbage, Bouillet et al. 1977; Williams, Williams et al. 1992; Inglis, Simelyte et al. 2008).

Cartilage was powdered using a liquid nitrogen freezer mill (Spex, UK). Proteoglycans were then removed by adding 5 volumes of 4M guanidine-HCL in 0.05M Tris (pH 7.5) and left to stir overnight at 4°C. Insoluble material was removed by centrifugation (12,000 rpm) and washed twice in 0.5M acetic acid. The pellet was then resuspended in 20 volumes of 0.5M acetic acid, and pH adjusted to 2.8 using 70% formic acid. 1g of pepsin was added to the solution for every 20g of cartilage (original wet weight) and was left stirring at 4°C for 48h, then centrifuged (12,000 rpm) and the supernatant retained.

NaCl was added to the supernatant remaining from Step 3 resulting in a 0.9M solution to precipitate dissolved type II collagen. The solution was centrifuged (12,000 rpm), then redissolved in 0.05M Tris plus 0.5M NaCl (pH 7.5) and left stirring overnight at 4°C. The solution was then centrifuged and the pellet re-suspended in 100ml 0.1M acetic acid and dialysed again Na_2PO_4 (pH 9.4) to inactivate residual pepsin.

The collagen was spun down (12,000 rpm) and re-suspended in 100ml 0.5M acetic acid. The final product was dialysed extensively against 5l acetic acid (0.5M)

then freeze-dried and stored in a desiccator at 4°C. For immunisation, freeze-dried collagen was dissolved in 0.1M acetic acid at 4mg/ml overnight at 4°C. Collagen was produced by the Richard Williams group, including assistance by Kay McNamee.

2.5 ELISAS

Murine ELISA kits for TNF (BD Biosciences Cat. No 558874), KC (R+D Systems Cat. No. DY453) and IL-6 (BD Biosciences) were utilised according to the manufacturer's instructions.

2.6 Genotyping

DNA was isolated from tail tips or ear clips of transgenic mice using Sigma Genotyping kit (Sigma). Genotyping transgenic mice was carried out using primers for TNFR1 and conducted by Kay McNamee and Dany Perocheau.
(5'-GGATTGTCACGGTGCCGTTGAAG-3'Fwd
5'CCTTTACGGCTTCCCAGAATTACC-3'Rev) (Marshall, Toh et al. 2004)
or TNFR2
(5'-CCTCTCATGCTGTCCCGGAAT-3'Fwd
5'-AGCTCCAGGCACAAGGGCGGG-3' Rev) respectively. (Balasa, Van Gunst et al. 2000)
The PCR conditions were as follows;

Step 1: 95°C for 5 minutes
Step 2: 95°C for 30 seconds
Step 3: 60°C for 45 seconds } **Repeat for 29 cycles**
Step 4: 72°C for 60 seconds

PCR products were visualised on a 1% agarose gel with ethidium bromide at 488 base pairs for TNFR1 and 200 base pairs for TNFR2.

2.7 Histology

Paws were fixed in 10% formalin and sent to the Charing Cross Hospital (London, UK) for processing. Paw sections were decalcified, and then paraffin embedded and cut using a cryostat by David Essex (Histology). Sections were then stained with haematoxylin and eosin (H+E) and visualised at 20 x magnification. Photographs were taken by Kay McNamee and Julia Inglis.

2.8 Myeloperoxidase assay

Neutrophil infiltration into the plantar tissue was assessed by histology and a kinetic-colorimetric assay for myeloperoxidase (MPO). The protocol was adapted from previous studies (Cunha, Verri et al. 2008). Paws were broken up using a bio-pulveriser (Stratech Scientific LTD, UK) and liquid nitrogen. The powder was then homogenised in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) (Sigma-Alridge). The tissue was centrifuged at 13,000 *g* for 10 min and the pellet discarded. To prepare the solution for analysis,

50µl of the supernatant was mixed with 50 µl 50 mM phosphate buffer, pH 6.0, and tetramethylbenzidine (TMB) substrate was added to detect the MPO. MPO activity was calculated by each plate being measured every minute for 5 minutes at 450nm. The change in absorbance each minute was averaged, with a change of 1 in absorbance equal to 1 unit of MPO activity (McNamee, Burleigh et al. 2010).The protocol was optimised by Kay McNamee who performed all experiments and analysis.

2.9 RNA isolation and cDNA generation from murine tissues

Julia Inglis extracted the tissue for the DBA CIA experiments and Kay McNamee isolated all tissue for the C57BL/6 CIA study. Kay McNamee processed all tissue samples from both studies for RNA isolation and conducted all real-time PCR and analysis.

It is often difficult to adequately quantify a range of cytokines and their receptors in a disease model. This is due to several factors; recovery of good quality material can be low and time consuming depending on the tissue type, and the tissue may require additional stimulation to detect cytokines even in an inflammatory environment. Detecting cytokine protein concentration uses a large amount of tissue, limiting the amount of cytokines that can be investigated. Real-time PCR using cDNA can amplify product from a single cell, making it ideal when tissue is limiting as for joint, spinal cord and the DRG.

Total RNA was extracted from various tissues. Mice were sacrificed and perfused with saline. Hind paws from naive mice, arthritic mice (day 10 post onset) and anti-IL-17 treated arthritic mice (day 10 post onset) were snap frozen in liquid nitrogen. The frozen paws were then homogenised using a bio pulveriser and liquid nitrogen, and the resulting powder ground on resin (GE Healthcare) in RLT lysis buffer (QIAGEN) with 2M DTT. The lysate was spun down on the resin and the supernatant added to a QIAshredder column for further homogenisation. The RNA extraction was then continued using the RNeasy RNA isolation kit (QIAGEN) according to the manufacturer's protocols.

DRG's and the lumbar region of the spinal cord were stored in RNA later (QIAGEN) until processing. Samples were taken from the RNA later and pulverised on ceramic beads with RLT lysis buffer in a Precellys™ Control Device for soft tissue homogenisation. The RNA was extracted from supernatant using the RNeasy kit (QIAGEN).

Reverse transcription (RT) was performed on RNA from all tissues using an ABI High Capacity Kit (Applied Biosystems) according to the manufacturer's instructions. The RT master mix for each reaction contained;

4.0µl 10X RT Buffer

1.6µl 25X dNTP Mix (100mM)

4.0µl 10X RT Random Primers

2.0µl MultiScribe™ Reverse Transcriptase

2.0µl RNase Inhibitor

6.4µl Nuclease-free H_20

The total reaction volume was 20µl master mix with an equal volume of RNA. The reaction was then reverse transcribed using a thermocycler under the following conditions;

Step 1: 25°C for 10 mins

Step 2: 37°C for 120 mins

Step 3: 85°C for 5 mins

Step 4: 4°C ∞

The cDNA was diluted to 120 µl with Nuclease-free H_20 prior to real-time PCR.

2.10 Real-time quantitative PCR

Quantitative real-time PCR was performed on a Corbett Lifesciences Rotorgene 6000 thermocycler (Corbett Lifesciences, Sydney) or ABI AB7900HT 384 platform system (Applied Biosystems). PCR was performed using a two-step amplification method as detailed here;

Step 1: 95 °C for 10 mins
Step 2: 95 °C for 2 seconds } **Repeat for 45 cycles**
Step 3: 60 °C for 20 seconds

The concentration of each target gene was calculated using the $\Delta\Delta C_t$ method and expressed as relative units using a naive sample as a calibrator (arbitrary unit 1) after normalizing against HPRT. All primers used were purchased from the ABI Taqman inventory. Details of individual primers used are below.

HPRT: Mm00446968_m1

IL-17: Mm00439619_m1

TNF: Mm99999068_m1

IL-1: Mm00434228_m1

IL-6: Mm00446190_m1

IL-10: Mm00439616_m1

IL-17RA: Mm00434214_m1

IL-17RC: Mm00661861_m1

TNFR1: Mm00441875_m1

TNFR2: Mm00441889_m1

GFAP: Mm00546086_m1

2.11 Statistical analysis

All statistical analysis was performed using Graphpad Prism software (Version 5.02). For all experiments involving more than two groups, a one-way ANOVA test was used with Dunnett's post test to the naive or vehicle controls. For two groups of

animals, a paired t-test was used when assessing differences in opposing paws of the same mice (i.e. comparing contralateral and ipsilateral limbs). For two groups of different mice (i.e. naive vs arthritic) an unpaired t-test was performed.

CHAPTER 3

IL-17 INDUCES HYPERALGESIA VIA TNF DEPENDENT NEUTROPHIL INFILTRATION

3.1 Introduction

Cytokines are known to be important in inflammatory pain as shown by extensive studies using TNF (Cunha, Poole et al. 1992; Woolf, Allchorne et al. 1997; Cunha, Verri et al. 2005). Injection of TNF into the hindpaw of rodents induces hyperalgesia by 1h post injection, persisting for up to 6h but returning to naive levels within 24h (Woolf, Allchorne et al. 1997). This hyperalgesia has been found to coincide with neutrophil migration, and these neutrophils are vital for hyperalgesia to occur (Cunha, Verri et al. 2008). TNF hindpaw injection also triggers release of IL-1 and KC which is attenuated with TNF blockade (Cunha, Verri et al. 2005). Pre-treatment with anti-NGF prevents TNF and IL-1 induced hyperalgesia (Woolf, Allchorne et al. 1997). This finding lead to the hypothesis that TNF provokes enhanced pain through the induction of an inflammatory cascade, ending in the release of NGF and other agents (e.g. PGE_2). These mediators sensitise sensory nerves, resulting in hyperalgesia.

TNF has been widely acknowledged as an important target for RA therapy (Maini, Elliott et al. 1995), but TNF blockade is also analgesic in animal models of arthritis and acute inflammatory pain (Inglis, Notley et al. 2007; Boettger 2008). Although anti-TNF has been very successful in treating RA, not all patients respond, creating a need to investigate other targets. There have also been suggestions of rapid analgesia in anti-TNF responders, provoking further examination of cytokines in pain.

Chapter 3: IL-17 induces hyperalgesia via TNF dependent neutrophil migration

IL-17 is a pro-inflammatory cytokine largely produced by activated T cells as well as neutrophils (Ferretti, Bonneau et al. 2003), NKT cells (Coquet, Chakravarti et al. 2008) and astrocytes (Meeuwsen, Persoon-Deen et al. 2003). IL-17 can induce TNF and synergises with TNF, IL-1, and IL-6 to maintain inflammation (Chabaud, Page et al. 2001; Hwang, Kim et al. 2004). IL-17 is also a potent chemotactic agent for leukocytes through its induction of chemokines (Laan, Cui et al. 1999; Witowski, Pawlaczyk et al. 2000). IL-17 is implicated in RA as it is increased in the synovium and synovial fluid of RA patients (Chabaud, Durand et al. 1999; Kotake, Udagawa et al. 1999). However, like other T cell cytokines, the level of IL-17 in RA synovial culture is very low and its blockade has modest effects in vitro (Hillyer, Larche et al. 2009). Clinical trials of drugs targeting IL-17 are in the early stages. Blockade of IL-17 reduces clinical score, paw swelling and joint destruction in animal models of arthritis (Nakae, Nambu et al. 2003; Lubberts, Koenders et al. 2004; Koenders, Lubberts et al. 2005; Rohn, Jennings et al. 2006). Based on these observations, IL-17 has potentially a major role in the pathogenesis of RA; though any contribution from IL-17 in inflammatory pain has received limited attention (Pinto, Cunha et al. 2009).

The overall objective of this first chapter is therefore to investigate the role of IL-17 in the generation of acute inflammatory pain.

3.2 Aims of chapter

The aims of this chapter were to address the question of whether IL-17 induces hyperalgesia in naive C57BL/6 mice and to investigate the relationship with TNF. These aims were achieved as follows:

1. IL-17 was administered into the hind paw of naive C57BL/6 mice and then thermal hyperalgesia and weight bearing were assessed and compared to the effect of TNF administration.

2. IL-17 dependency on TNF was investigated by a) IL-17 injection into wild type mice treated with anti-TNF antibody and b), IL-17 injection into $TNFR^{-/-}$ or $TNFR2^{-/-}$ mice.

3. The importance of neutrophil migration in cytokine-induced hyperalgesia was assessed by blockade of selectins required for leukocyte infiltration.

3.3 Results

3.3.1 TNF and IL-17 paw swelling response.

Before IL-17 could be investigated in an arthritic model of chronic pain, it was first established whether a single injection of the cytokine could induce acute pain in naïve mice. TNF is known to induce acute pain and so this was used as a positive control. C57BL/6 mice were injected with 10, 20 or 50 ng of rTNF or rIL-17. The negative control was the vehicle (PBS with 1% mouse plasma).

The mice were assessed for paw swelling pre injection and 1h, 3h, 6h and 24h after injection (Figure 3.1). Paw swelling was measured using calipers with only the 50 ng dose in both rTNF and rIL-17 treated mice showing definite paw swelling, although there was clear redness in both groups at the 20 ng dose showing that there was inflammation but it was not enough to cause overt swelling. In all experiments the swelling returned to levels comparable to the vehicle control within 24h. As 20 ng of rTNF or rIL-17 could induce pain and leaning in the mice without significant swelling, this was the dose used for all further experiments.

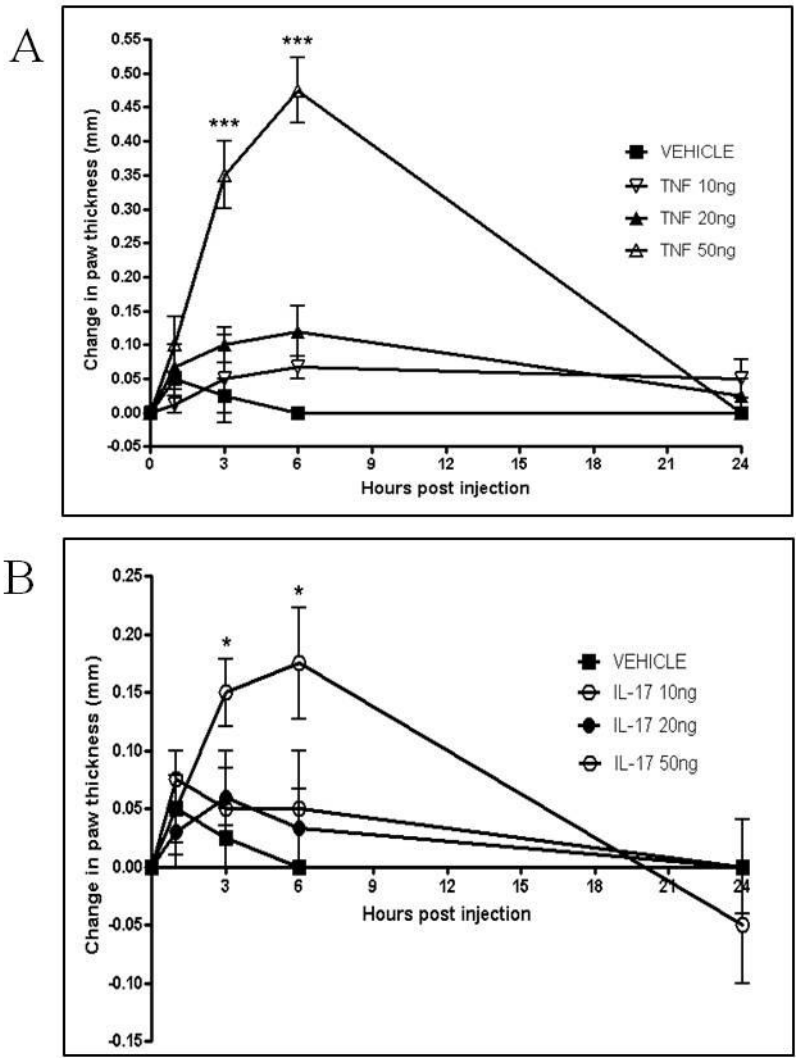

Figure 3.1. TNF and IL-17 paw swelling response. C57BL/6 mice were injected with 10, 20 or 50ng TNF (A) or 10, 20, or 50ng IL-17 (B). Vehicle (PBS with 1% mouse plasma) was injected separately as a control group. All groups were assessed over 24h with calipers to detect changes in paw thickness. N=4 per group. Statistical analysis was undertaken using one-way ANOVA with a Dunnetts multiple comparison post test to compare groups at each time point.

*P=<0.05, **P<0.01 and ***P= <0.001

3.3.2 IL-17 and TNF induce hyperalgesia and neutrophil migration.

Initially, IL-17 was investigated for its ability to induce hyperalgesia and weight distribution change. TNF having previously been shown to induce hyperalgesia was injected as a positive control. Animals received a 10µl intraplantar injection of 20ng rTNF, 20ng rIL-17, or vehicle (PBS with 1% mouse plasma) and were assessed for thermal hyperalgesia and weight bearing prior to injection and 1h, 3h, 6h, and 24h after injection (Figure 3.2a/b).

TNF induced thermal hyperalgesia as early as 1h with IL-17 injected mice showing hyperalgesia 3h post injection. At 6h both TNF and IL-17 treated mice were leaning significantly onto their uninjected paw signifying pain. As IL-17 induced thermal hyperalgesia was delayed in comparison to TNF, this raised the possibility that IL-17 was causing pain indirectly through the generation of another mediator.

Next the question of whether TNF and IL-17 induced hyperalgesia was dependent on the inflammatory response was addressed. Although TNF and IL-17 were injected at doses that did not induce significant paw swelling, the possibility of sub-clinical inflammatory changes could not be discounted. Both TNF and IL-17 have been shown to induce neutrophil migration, and this would be the main population of migrating cells to the site of inflammation in this time period. Hence, MPO activity was assayed throughout the 24h period. MPO is produced by activated neutrophils and is an accepted marker of neutrophil migration (Cunha, Verri et al. 2008). It was found that MPO activity increased in parallel to TNF and IL-17

induced hyperalgesia, both being significant at 3h (Figure 4d). To further assess the neutrophil infiltration, animals were injected with TNF or IL-17 as before and at 3h post injection paws were fixed for histology and stained with haematoxylin and eosin (H+E) (Figure 3.2c). The presence of neutrophils was observed in the TNF and IL-17 injected mice but not vehicle control at 3h post injection. This shows that there is a direct association between neutrophil migration and cytokine induced hyperalgesia.

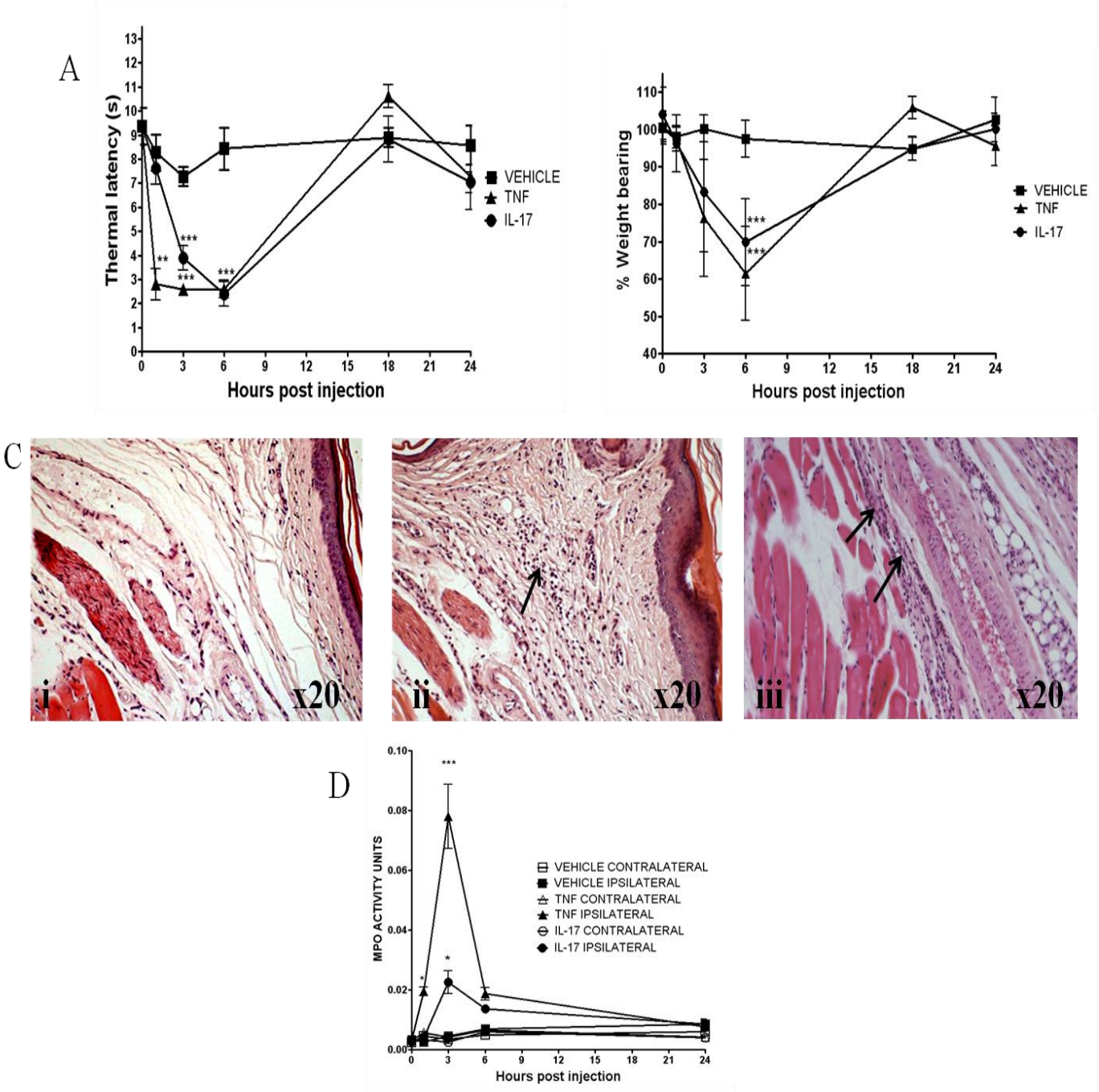

Figure 3.2. TNF and IL-17 induced thermal hyperalgesia and weight distribution change correlates with neutrophil migration . C57BL/6 mice were injected with 20ng rTNF, rIL-17, or vehicle and assessed for thermal hyperalgesia (A) and weight distribution change (B) over a 24h period. Paws were fixed at 3h post injected and sections stained with H+E to detect neutrophils (C). Migrated neutrophils were seen in the TNF **(ii)** and IL-17 **(iii**) treated mice but not vehicle control **(i)**. Paws were then taken over a 24h period and assessed for MPO activity (D) N=6 per group. Statistical analysis of both weight distribution and thermal hyperalgesia were undertaken using one-way ANOVA with a Dunnetts multiple comparison post test to compare groups. MPO activity was quantified using an paired t-test to compare ipsilateral paws to contralateral paws.

*P=<0.05, **P<0.01 and ***P= <0.001

3.3.3 IL-17 induced hyperalgesia and neutrophil migration is dependent on TNF.

IL-17 is a known inducer of TNF, therefore the extent to which IL-17 administration led to hyperalgesia via the production of TNF was investigated. Animals were injected with IL-17, TNF, or vehicle as described above, with or without 2μg of polyclonal anti-TNF IgG purified antibody. Thermal hyperalgesia and weight distribution change were again measured over a 24h period. There was no hyperalgesia or weight bearing changes in anti-TNF treated mice injected with either TNF or IL-17 (Figure 3.3a/b), indicating that IL-17 induced hyperalgesia is dependent on TNF.

As it had been shown that cytokine induced hyperalgesia correlated with MPO activity, MPO activity at 3h post injection was again examined by comparing anti-TNF treated mice with controls. As shown in Figure 3.3c, neither TNF nor IL-17 injected mice treated with anti-TNF had significant MPO activity at 3h post injection. This demonstrates that IL-17 induced neutrophil migration is dependent on TNF.

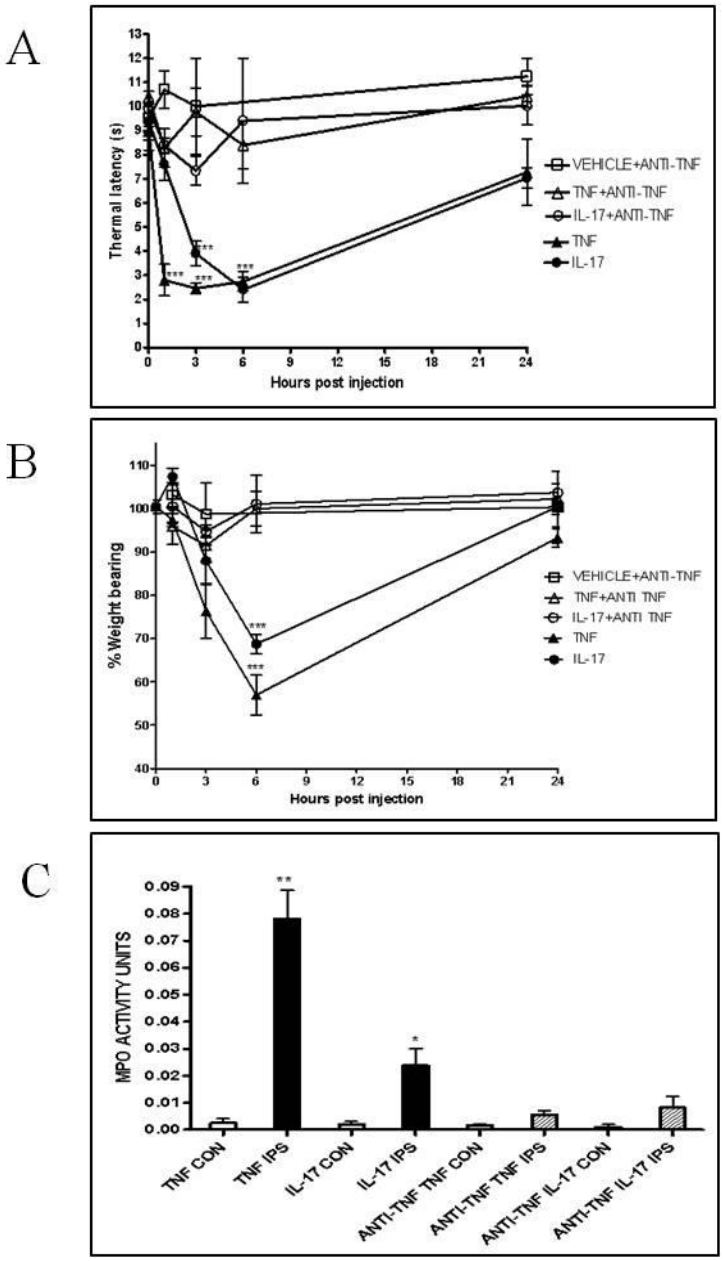

Figure 3.3. TNF and IL-17 induced thermal hyperalgesia and weight distribution change is dependent on TNF. C57BL/6 mice were injected with 20ng rTNF, rIL-17, or vehicle in combination with anti-TNF (2µg/paw) and assessed for thermal hyperalgesia (A) and weight distribution change (B) over a 24h period. N=6 per group. MPO activity at 3h post injection (C) was assessed in TNF or IL-17 injected mice and mice pre-treated with anti-TNF. *P=<0.05, **P<0.01 and ***P= <0.001

3.3.4 TNFR1 but not TNFR2 is imperative for IL-17 induced hyperalgesia and neutrophil migration.

Having established that IL-17 acts via TNF, it was investigated which of the two TNF receptors (TNFR1 or TNFR2) was mediating hyperalgesia.

In order to assess differential receptor effects during hyperalgesia, $TNFR1^{-/-}$ mice and $TNFR2^{-/-}$ mice were injected with IL-17, TNF or vehicle as described above. Thermal hyperalgesia and weight distribution change were again measured over a 24h period. In $TNFR2^{-/-}$ mice, both TNF (Figure 3.4a) and IL-17 (Figure 3.4b) induced hyperalgesia comparable to that observed in wild type mice. However, there was no hyperalgesia or weight bearing change in $TNFR1^{-/-}$ mice after either TNF (Figure 3.4d) or IL-17 administration (Figure 3.4e). This data shows that IL-17 and TNF induced hyperalgesia is dependent on TNF binding to TNFR1 but not to TNFR2.

As TNF and IL-17 induced hyperalgesia were found to be dependent on TNFR1, it was examined whether neutrophil migration was similarly regulated. MPO activity at 3h post injection was assessed, comparing TNF and IL-17 injected wild type mice with TNF and IL-17 injected TNFR1 or TNFR2 mice. $TNFR2^{-/-}$ mice showed increased neutrophil migration following cytokine injection, comparable to that of wild type controls (Figure 3.4c). In contrast, there was no significant neutrophil infiltration in $TNFR1^{-/-}$ mice treated with either TNF or IL-17 (Figure 3.4f), demonstrating that neutrophil migration is also dependent on TNF binding to TNFR1.

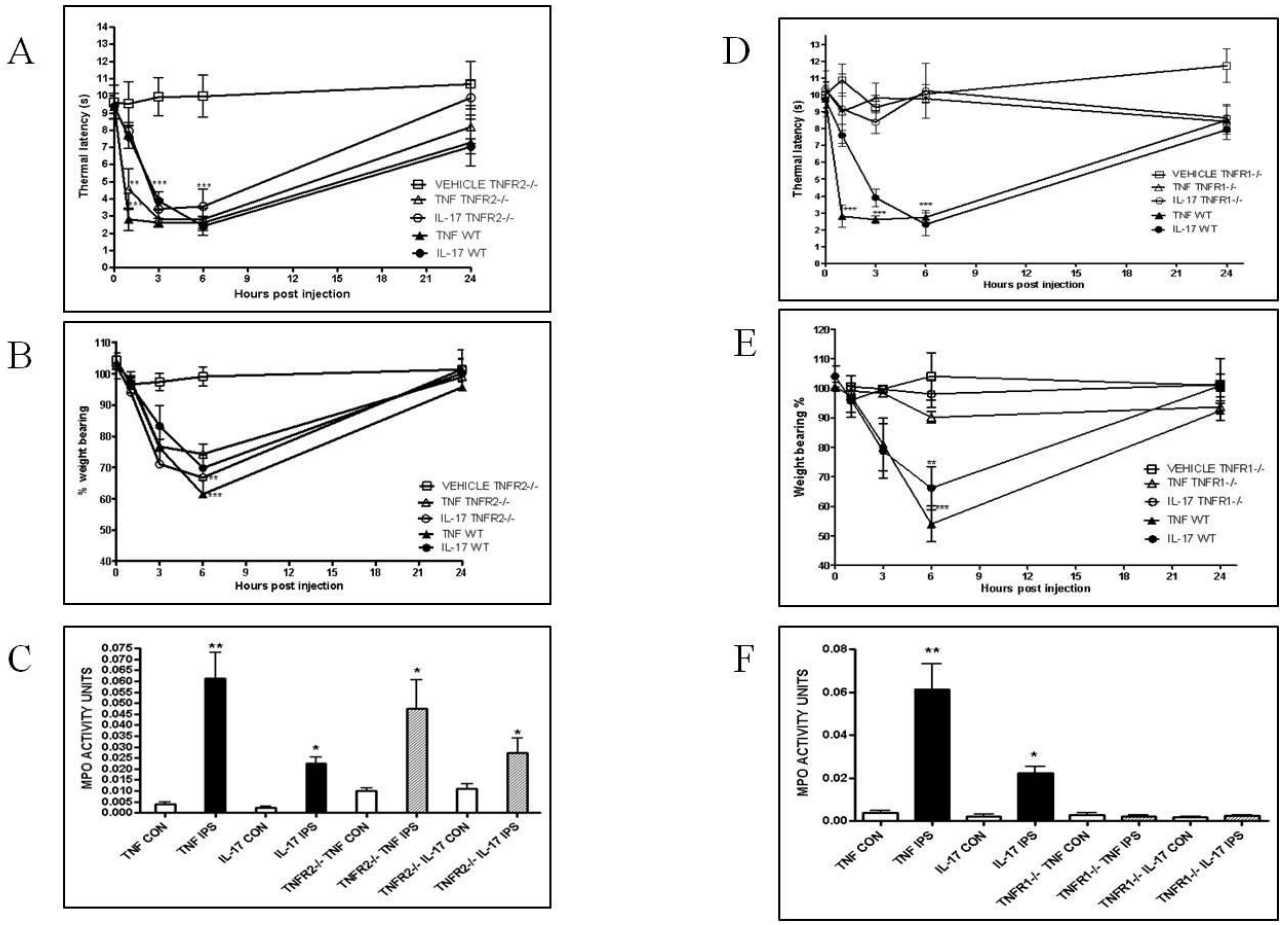

Figure 3.4. TNF and IL-17 induced thermal hyperalgesia and weight distribution change is dependent on TNFR1. TNFR2-/- mice were injected with 20ng rTNF, rIL-17, or vehicle and assessed for thermal hyperalgesia (A) and weight distribution change (B) over a 24h period and MPO (C) at 3h post injection. This was repeated in TNFR1-/- mice (D, E and F respectively). N=6 per group.

*P=<0.05, **P<0.01 and ***P= <0.001

3.3.5 IL-17 induced hyperalgesia is dependent on neutrophil migration

Recent studies have suggested that neutrophils make an important contribution to hyperalgesia, and previous experiments showed that cytokines such as TNF (Cunha, Verri et al. 2008) and NGF (Bennett, al-Rashed et al. 1998) induce hyperalgesia in rodents only when neutrophils are present. Therefore, it was investigated whether blockade of neutrophil migration would prevent hyperalgesia in IL-17 treated mice.

Mice were pre-treated with 20mg/kg fucoidan (a competitive inhibitor of selectins needed for neutrophil migration) i.p. 2h prior to injection with either TNF or IL-17. The hyperalgesia and weight bearing change was examined as described in Chapter 2. A proportion of the mice were killed and their paws taken 3h post injection with either cytokine to confirm neutrophil blockade by MPO activity assay. In TNF or IL-17 mice pre-treated with fucoidan, there was neither hyperalgesia (Figure 3.5a) nor weight distribution changes (Figure 3.5b), nor significant MPO activity (Figure 3.5c). These data confirm that neutrophil migration is required for cytokine induced hyperalgesia.

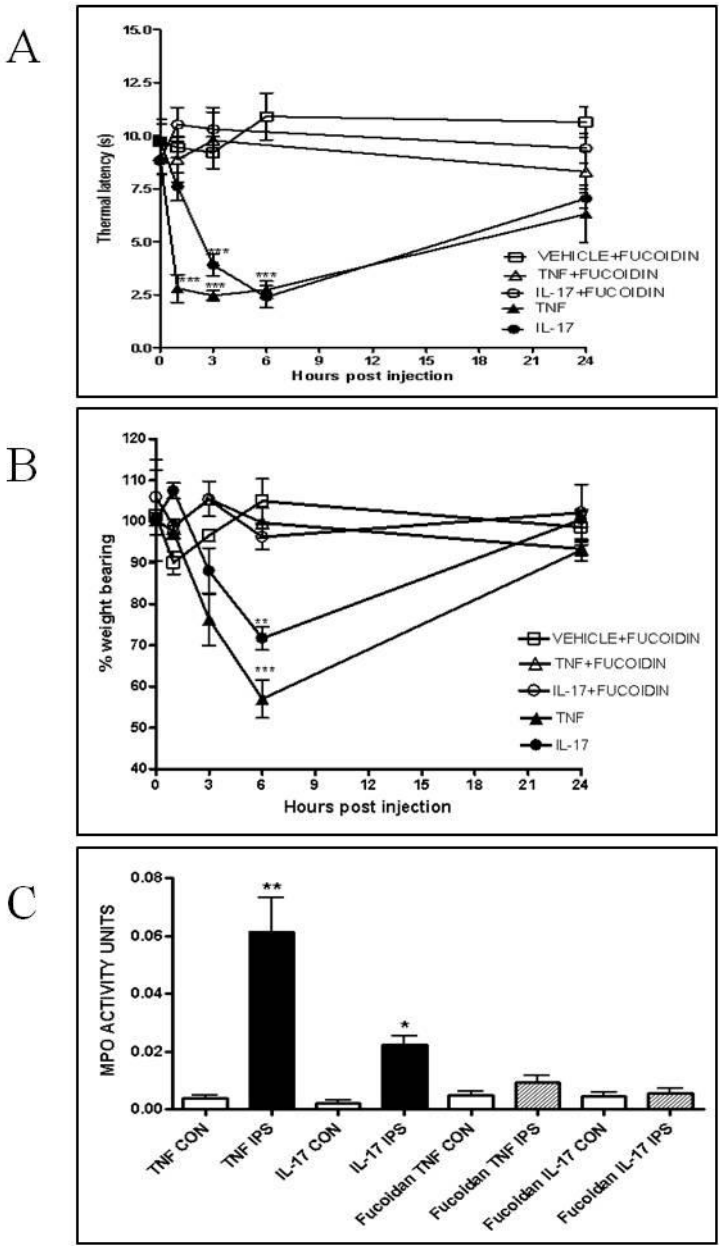

Figure 3.5. Neutrophil migration is required in cytokine-induced pain. C56BL/6 mice were pre-treated with 20mg/kg fucoidan then 2h later injected with 20ng rTNF, rIL-17, or vehicle and assessed for thermal hyperalgesia (A) and weight distribution change (B) over a 24h period and MPO (C) at 3h post injection. Paws were taken at 3h post injection to assess MPO activity. N=6 per group. *P=<0.05, **P<0.01 and ***P= <0.001

3.4 Discussion

In this chapter, it has been shown that IL-17 induces hyperalgesia. IL-17 induced hyperalgesia is however indirect as it is dependent on TNF binding to TNFR1. In addition it is clear that neutrophil recruitment is required for cytokine induced hyperalgesia and is also dependent on TNF.

In agreement with previous studies, TNF induced transient changes in weight bearing and thermal hyperalgesia, peaking between 3-6h after injection and returning to normal responses by 24h. IL-17 also induced hyperalgesia and weight bearing changes. However, there was a delay in IL-17 induced hyperalgesia relative to TNF induced hyperalgesia. This delay in the IL-17 treated mice suggested that this cytokine was inducing hyperalgesia indirectly through the induction of another mediator (e.g. TNF). It has been shown previously that macrophages stimulated with IL-17 produce TNF after a delay of 3h (Jovanovic, Di Battista et al. 1998), which is comparable to the delay in IL-17 induced pain in this initial study.

IL-17R is expressed on resident TNF producing cell types such as endothelial cells, resident macrophages and fibroblasts, therefore IL-17 could potentially induce TNF from any of these cells. IL-17 is known to synergise with TNF in the production of other cytokines and chemokines such as IL-6 and IL-8 (Fossiez, Djossou et al. 1996; Hartupee, Liu et al. 2007). Despite this synergistic relationship between IL-17 and TNF, the dependency of IL-17 on TNF is largely unknown. However, a previous study showed that IL-17 induced joint inflammation in naive mice but was

dependent on TNF as cell infiltration into the joint was prevented in IL-17 injected TNF deficient mice (Koenders, Lubberts et al. 2006). TNF signals through two receptors, TNFR1 and TNFR2. Previous studies suggest that TNF induced hyperalgesia is dependent on binding TNFR1 in a chronic constriction injury (CCI) model of neuropathic pain. Thus, neutralisation of TNFR1 reduces thermal hyperalgesia and mechanical allodynia whereas neutralisation of TNFR2 has no effect. In addition, in a methylated BSA (mBSA) mouse model, IL-17 mediated hyperalgesia is reduced in $TNFR1^{-/-}$ mice and with TNF blockade (Pinto, Cunha et al. 2009). In this study, even a single injection of IL-17 in a healthy mouse elicits hyperalgesia for up to 6h. Both IL-17 and TNF induced hyperalgesia is absent in $TNFR1^{-/-}$ mice but present in $TNFR2^{-/-}$ mice. These findings clearly show that both TNF and IL-17 induced hyperalgesia is dependent on TNF signalling via TNFR1. These data are the first to show divergent roles for TNF receptors in inflammatory hyperalgesia.

Recently there has been an increasing focus on the importance of cellular infiltration in the genesis of cytokine induced hyperalgesia. Neutrophils are the most abundant immune cells, accounting for 55-70% of all white blood cells, and are the dominant cell type in sites of inflammation in the first hours following infection or tissue damage. Neutrophils account for 90% of the cells in the arthritic synovial fluid (Wright, Moots et al. ; Hilliquin, Dulioust et al. 1995) and release proteases and cytokines leading to further tissue damage. After TNF injection there is a rapid accumulation of neutrophils in to the hind paw, and these neutrophils are required for hyperalgesia to occur (Cunha, Verri et al. 2008; Pinto, Cunha et al. 2009). As

IL-17 is chemotactic for neutrophils via the induction of chemokines, the contribution of neutrophils to IL-17 induced hyperalgesia was investigated. Firstly, TNF and IL-17 induced neutrophil migration to the hind paw by histology and MPO activity assay was confirmed. Both IL-17 and TNF injected mice had a significant increase in MPO activity which correlated with hyperalgesia. The elevated pain response and elevated MPO activity returned to naive control levels concurrently. These parallel time courses suggested that neutrophil migration was linked to TNF and IL-17 induced hyperalgesia.

This link was further investigated to show that neutrophil migration was prevented in mice treated with anti-TNF and $TNFR1^{-/-}$ mice but not $TNFR2^{-/-}$ mice, revealing a novel finding that IL-17 is dependent on TNF binding to TNFR1 to induce neutrophil migration as well as hyperalgesia.

This data shows a positive correlation between neutrophil recruitment and hyperalgesia. Indeed, neither IL-17 nor TNF injected mice pre-treated with fucoidan (to block neutrophil migration) developed hyperalgesia, confirming the necessity of neutrophil migration for cytokine induced pain. This experiment supports recent studies showing that fucoidan prevents TNF induced hyperalgesia and IL-17 mediated mechanical hypernociception respectively (Cunha, Verri et al. 2008; Pinto, Cunha et al. 2009). In the TNF study, fucoidan was also found to only prevent neutrophil rolling and not to reduce the level of TNF induced pro-inflammatory cytokines in the plantar tissue. This suggests that neutrophil induced hyperalgesia is not achieved through the further production of TNF. Neutrophils have been shown to release NGF and prostaglandins, both of which directly

sensitise nociceptors. Further studies are needed to identify the exact mechanisms by which neutrophils cause hyperalgesia in this system.

CHAPTER 4

IL-17 MEDIATED NEUTROPHIL INFILTRATION IS DEPENDENT ON TNF INDUCED KC

4.1 Introduction

As demonstrated in the previous chapter, neutrophil migration is required for TNF and IL-17 induced inflammatory hyperalgesia to occur in mice. Neutrophils are generated in the bone marrow and on maturing are released into the circulation (Bainton, Ullyot et al. 1971; Berkow and Dodson 1986). Neutrophils then remain in the blood unless they are mobilised and recruited to a site of inflammation by following a chemical gradient in a process known as chemotaxis. This gradient is governed by chemokines locally produced at the site of inflammation (Zigmond 1974; Wilkinson 1990; Baggiolini 1998).

IL-17 has long been established as a mediator of neutrophil infiltration. In one study, conditioned media from IL-17 stimulated human endothelial cells added to a transwell migration assay significantly increased neutrophil chemotaxis in an IL-8 dependent manner (Laan, Cui et al. 1999). In a peritoneal cavity model, IL-17 administration was found to recruit neutrophils via production of the chemokine GROα (Witowski, Pawlaczyk et al. 2000). IL-17 has been shown to elicit airway neutrophil infiltration *in vitro* by increasing adhesion molecules on endothelial cells, thereby enhancing leukocyte progression to sites of inflammation (Roussel, Houle et al. 2010). Injection of IL-17 into the knee joint of mice resulted in neutrophil migration and this was dependent on TNF (Lemos, Grespan et al. 2009). However, assessing the production of chemokines in this model can be difficult as the knee joint will only yield a small amount of exudate (in the range 1-3µl) and cells.

Chapter 4: IL-17 mediated neutrophil migration is dependent on TNF induced KC

The air pouch model of leukocyte infiltration allows for easy retrieval of migrated cells and the exudates can be used to detect proteins secreted in the pouch. Traditionally zymosan is used to stimulate chemokines and provoke cellular infiltration but recently cytokines have been used for more specific studies. TNF injection into the air pouch model facilitates neutrophil infiltration by increasing expression of chemokines KC and MIP-2 (Tessier, Naccache et al. 1997; Clish, O'Brien et al. 1999). In addition, blockade of MIP-2 and KC significantly reduces leukocyte recruitment induced by TNF (Tessier, Naccache et al. 1997).

The previous chapter has suggested that IL-17 induced hyperalgesia is dependent on neutrophil infiltration mediated by TNF/TNFR1 signalling. Therefore, it was necessary to validate and further explore IL-17 dependency on TNF in the context of neutrophil migration.

4.2 Aims of chapter

The aims of this chapter were to confirm IL-17 dependency on TNF for neutrophil migration, and understand further the mechanism by which this occurs. This aim was achieved as follows;

1. IL-17 was injected into air pouches in wild type mice to ascertain that it was sufficient to induce neutrophil migration
2. IL-17 was injected into air pouches in TNFR1$^{-/-}$ or TNFR2$^{-/-}$ mice to assess TNF dependency
3. Air pouch exudates were analysed for secretion of KC and levels compared with numbers of neutrophils found in each experiment
4. A fibroblast cell line was stimulated with IL-17 to investigate if IL-17 could induce KC and whether this production was dependent on TNF

4.3 Results

4.3.1 IL-17 and TNF induce neutrophil migration

Having demonstrated that IL-17 and TNF induced hyperalgesia was dependent on neutrophil migration the mechanism needed to be examined more thoroughly. It was hypothesised that IL-17 was inducing TNF from resident cells and in turn TNF was inducing chemokines from the same cells to attract neutrophils. To investigate this, an air-pouch animal model was used. Firstly, it was determined that administration of IL-17 or TNF (50 ng) into the pouch would elicit neutrophil infiltration. After 4 hours, the mice were sacrificed and the cells in the pouch washed out with PBS (Figure 4.1a). The cells were then pelleted, counted using a haemocytometer and trypan blue exclusion, and the supernatant from the pelleted cells collected for chemokine/cytokine detection by ELISA. Injection of IL-17 or TNF into the air pouch induced significantly higher numbers of cells when compared with vehicle injected mice at 4h post injection.

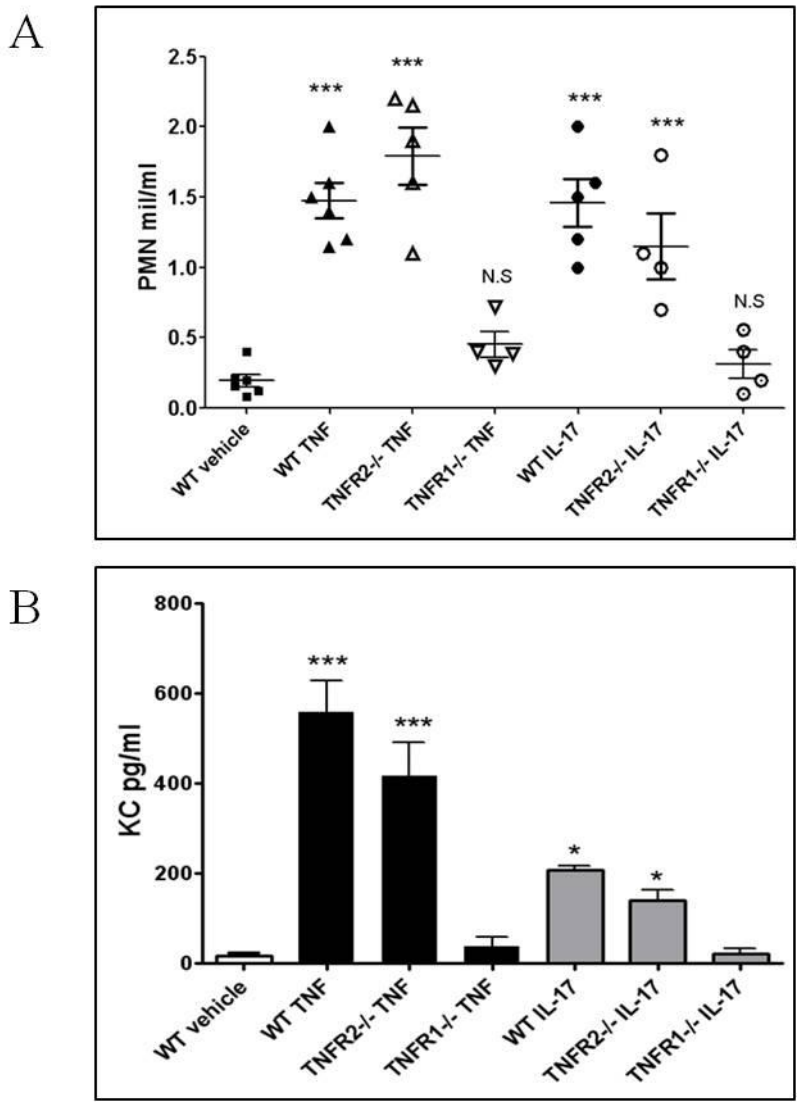

Figure 4.1 Neutrophil infiltration in the air-pouch model requires TNFR1 but not TNFR2. C57BL/6, TNFR2-/-, or TNFR1-/- mice had an air pouch administered on the dorsum, then injected with 50ng TNF, IL-17 or vehicle. 4h post cell numbers were counted (A) and exudates were measured in a KC ELISA (B) N=4 per group. Statistical analysis was performed using a 1 way ANOVA with Dunnetts multiple comparison post test to compare groups to WT vehicle control.
*P=<0.05, **P<0.01 and ***P= <0.001

4.3.2 IL-17 induced neutrophil migration is dependent on TNFR1 but not TNFR2

To validate the divergent roles for TNF receptors and IL-17 dependency on TNF, $TNFR1^{-/-}$ and $TNFR2^{-/-}$ mice were injected with IL-17 or TNF (Figure 4.1a). In the $TNFR2^{-/-}$ mice neutrophil migration was increased compared to WT mice, suggesting an inhibitory role for TNFR2. IL-17 induced neutrophil migration was dependent on TNF binding to TNFR1, which is in agreement with the acute hyperalgesia study in Chapter 3.

4.3.3 IL-17 induced KC is dependent on TNFR1 but not TNFR2 in a model of leukocyte migration

As neutrophils migrate to the site of inflammation by following a chemokine gradient, we next investigated whether TNF induced chemokines from resident cells in the pouch. After migrated cells were washed out of the pouch and spun down, the supernatant was removed and used in an ELISA for KC, the murine homolog of IL-8 (Figure 4.1b). Both IL-17 and TNF injected pouches had significantly elevated KC expression compared to the $TNFR1^{-/-}$ mice, in which KC secretion had largely been prevented. In the $TNFR2^{-/-}$ mice there was a reduction in the amount of KC when compared to the wild type controls but KC production in $TNFR2^{-/-}$ was still significant when compared to the $TNFR1^{-/-}$ mice.

4.3.4 IL-17 induced KC from a fibroblast cell line is dependent on TNF

To investigate the role of IL-17 in neutrophil migration, the cells producing TNF and KC must be considered. Fibroblasts along with macrophages are major resident cells in the air-pouch (and the footpad), but assaying cytokines produced by these cells requires extensive *ex vivo* tissue culture. Alternatively, the tissue can be paraffin embedded and sectioned for IHC, but many antibodies are ineffective in paraffin fixed tissue.

To rapidly assess induction of inflammatory mediators by cytokines, a 3T3 fibroblast murine cell line was cultured and stimulated with either IL-17 or TNF. To determine the amount of cytokine to be used, fibroblasts were stimulated with a range of concentrations from 5-100ng/ml (Figure 4.2). IL-6 had been shown to be elevated previously with either TNF or IL-17 stimulation, therefore detection of this cytokine by ELISA was used as a control to determine the concentration of cytokine to be used in further experiments. At 24h post-stimulation, both TNF and IL-17 at concentrations between 10-100ng/ml had induced IL-6 in the supernatant. As cytokine expression is generally low in vivo, it was decided that the lowest dose with clear IL-6 up-regulation would be used. At 5ng/ml TNF had increased IL-6 but IL-17 did not, therefore a dose of 10ng/ml were both cytokines had clear up-regulated IL-6 was used for all experiments.

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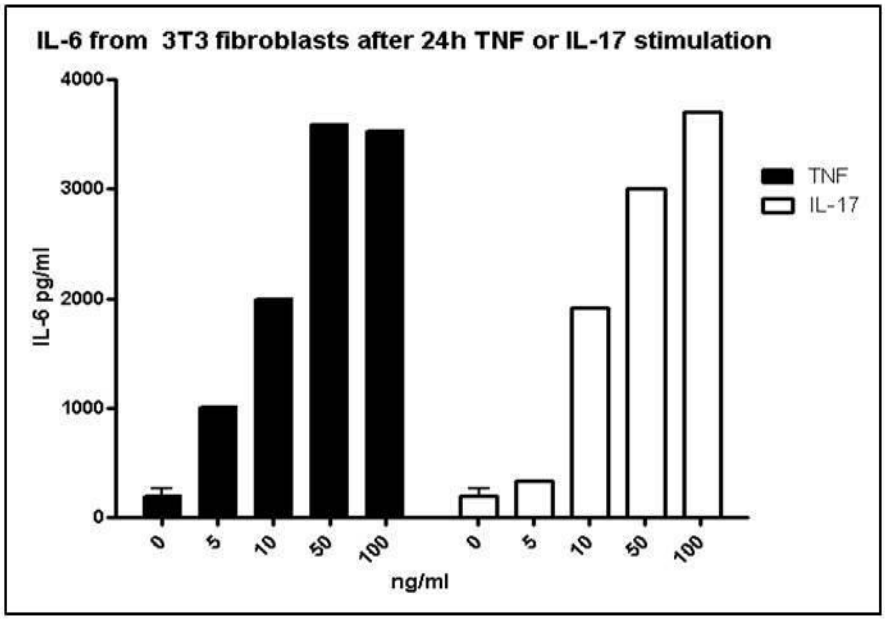

Figure 4.2 IL-17 induces IL-6 from a fibroblast cell line . (A) 3T3 fibroblasts were cultured and stimulated with either TNF or IL-17 in a range of doses between 5 and 100ng/ml . Dose response was determined by measuring IL-6 at 24h post stimulation.

To determine if KC was produced by fibroblasts, 3T3 fibroblasts were stimulated with TNF or IL-17 at 10ng/ml and KC in the supernatant was measured by ELISA over a 24h period (Figure 4.3). TNF induced significant KC levels by 1h post stimulation, while KC elevation significant in IL-17 treated cells at 3h post stimulation. IL-17 KC production was significantly reduced when the fibroblasts were pre-treated with anti-TNF, supporting previous data in this thesis that IL-17 actions in an acute setting are dependent on TNF.

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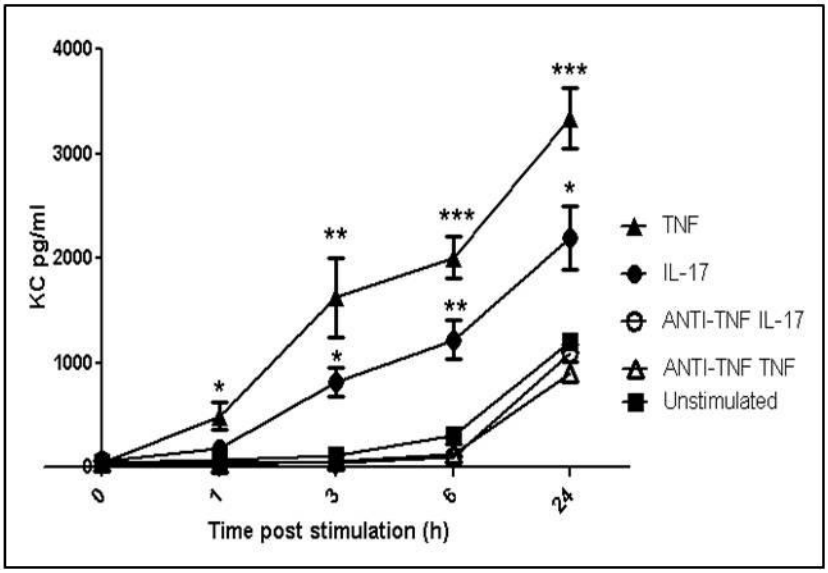

Figure 4.3 IL-17 induced KC from fibroblast cell line is dependent on TNF. (A) 3T3 fibroblasts were then stimulated with TNF or IL-17 AT 10ng/ml after cell were pre-treated with anti-TNF antibody (10μg/ml) or vehicle. Supernatants were taken at 24h for use in a KC ELISA . *P=<0.05, **P<0.01 and ***P= <0.001

4.4 Discussion

Data shown previously has suggested that IL-17 induced neutrophil migration is dependent on TNF. This finding was verified here in the air pouch model of cell migration whereby TNF and IL-17 induced neutrophil migration after 4h in wild type mice. In addition, in TNFR1$^{-/-}$ mice, neutrophil migration was abolished supporting the earlier investigation.

It is well established that IL-17 is important for neutrophil infiltration into sites of inflammation through induction of chemokines. Human neutrophils migrate towards 16HBE cells stimulated with IL-17 within 20 min, but this infiltration is abolished with anti-IL-8 treatment (Laan, Lotvall et al. 2001). IL-17 increased neutrophil numbers in rat bronchoalveolar lavage (BAL) fluid is prevented by anti-MIP-2 therapy. In a peritoneal cavity cell infiltration model, IL-17 stimulation yields a significant number of neutrophils by 2h post stimulation, peaking at 4h. This is preceded by highly increased levels of KC, and reduced with KC neutralisation (Laan, Cui et al. 1999). IL-17 stimulates chemokine expression from a variety of cell types. These include human IL-8 from transformed human bronchial epithelial cells (Laan, Cui et al. 1999) and rheumatoid synovial fibroblasts (Hwang, Kim et al. 2004), MIP-2α from murine mesothelial cells (Witowski, Pawlaczyk et al. 2000), and KC from mouse embryo fibroblasts (Hartupee, Liu et al. 2007). In addition, IL-17RA$^{-/-}$ mice have a reduced basal level of MIP-2α and impaired neutrophil recruitment after challenge with *K. pneumonia* (Ye, Rodriguez et al. 2001). It has been suggested previously that IL-17 mediated neutrophil migration is dependent on

TNF (Koenders, Lubberts et al. 2006; Lemos, Grespan et al. 2009). The latter study showed that IL-17 induced chemotaxis depended on CXC chemokines. However, it was unknown whether IL-17 is dependent on TNF to stimulate the initial release of these chemokines.

As neutrophil migration requires chemokine secretion, KC expression in the air pouch was quantified here. A KC ELISA using the pouch exudates revealed that IL-17 and TNF treated wild type and $TNFR2^{-/-}$ mice had significantly higher KC expression than the $TNFR1^{-/-}$ mice where KC expression was prevented. Thus, it was concluded that TNFR2 is not required for neutrophil migration. It has been shown previously that IL-17 mediated neutrophil infiltration is dependent on TNF (Lemos, Grespan et al. 2009). However, this data is the first to suggest that IL-17 acts specifically via TNF/TNFR1 to induce KC for neutrophil migration.

In both the acute hyperalgesia study and the air pouch study the major resident cells were fibroblasts and macrophages. KC is expressed in a number of cell types including fibroblasts; therefore TNF and IL-17 induction of KC from fibroblasts was investigated using a 3T3 cell line. By 3h, both TNF and IL-17 had induced a significant amount of KC. However, when the cells were pre treated with anti-TNF, KC expression was reduced in both the TNF and IL-17 stimulated cells. This suggests that IL-17 induces neutrophil migration by inducing TNF, which in turn induces KC from the fibroblasts to allow neutrophil migration. To support this hypothesis, IL-17 is known to induce release of TNF from macrophages and these cells are also resident in the footpad and the airpouch. Therefore this chapter suggests that IL-17 induces TNF from resident cells (macrophages/fibroblasts)

which results in KC production. Neutrophils then infiltrate the site of inflammation, expressing other algesic mediators that more directly sensitise the nerve, leading to hyperalgesia (Figure 4.4). Indeed, NGF is known to be produced by neutrophils and blockade of neutrophils prevents NGF induced hyperalgesia (Bennett, al-Rashed et al. 1998).

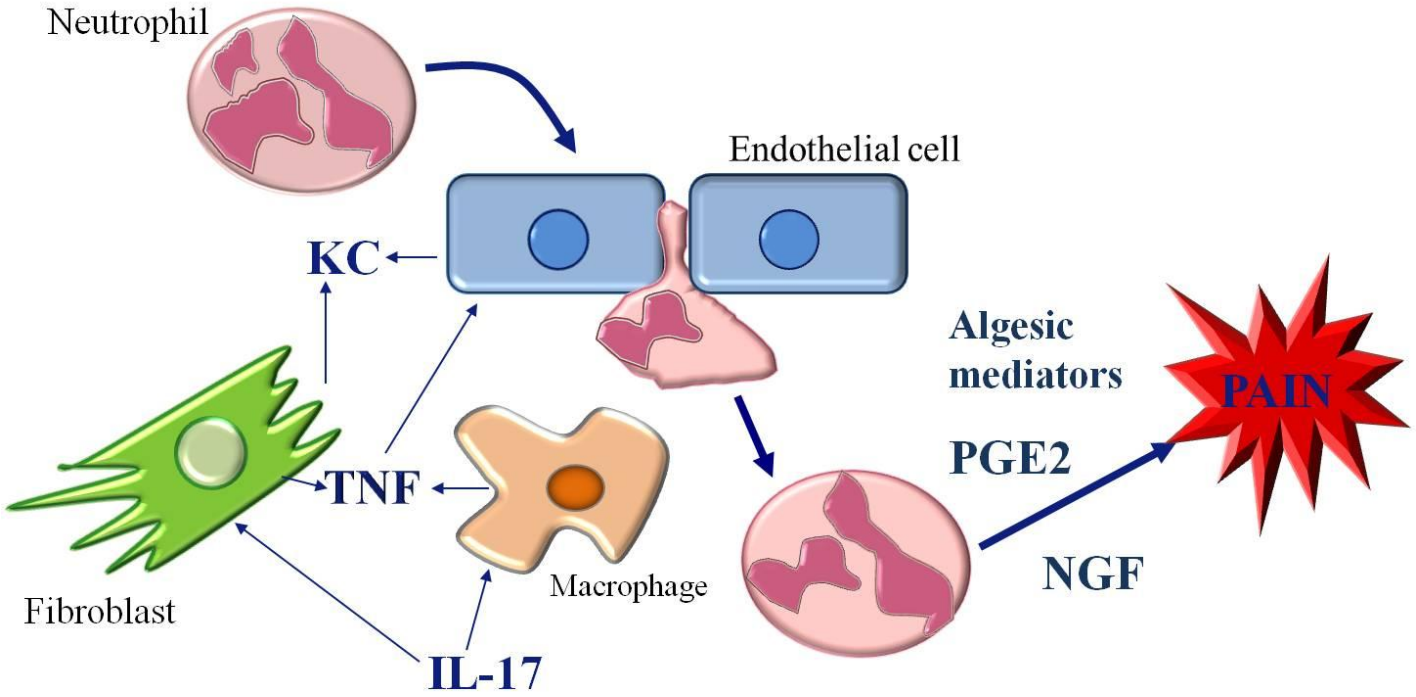

Figure 4.4 IL-17 induced hyperalgesia hypothesis. IL-17 induces acute hyperalgesia indirectly. Firstly IL-17 at the site of inflammation induces TNF from resident cells (macrophages). TNF then induces KC from fibroblasts or endothelial cells to facilitate neutrophil infiltration. Finally neutrophils migrate to the site , releasing further proinflammatory cytokines and algesic mediators to sensitise the peripheral nerve.

CHAPTER 5

REAL-TIME PCR PROFILE OF NOCICEPTIVE TISSUES DURING CIA

5.1 Introduction

The previous chapters have shown that IL-17 can induce hyperalgesia via induction of neutrophil migration but that this is dependent on TNF. However, these studies have been in healthy mice in short-term, acute experiments where the enhanced pain desists within 24h. Cytokine injection alone therefore, does not represent the complexity of chronic pain and inflammation during arthritis.

RA is characterised by inflammation of the joints, proliferation of the synovial lining layer, leukocyte infiltration, pannus formation, and joint destruction. A number of animal models are used to mimic aspects of RA pathology to understand the disease and develop novel targeted therapies. The most widely used model is CIA which elicits a polyarthritis 14-28 days post-immunisation with bovine or chicken collagen. CIA involves synovitis, immune cell infiltration into the joint, pannus formation, cartilage erosion, joint destruction and loss of function. Indeed, TNF blockade was shown to be a highly effective treatment for CIA, facilitating its transfer to the clinic. In addition, both RA patients and CIA mice have underlying chronic pain, making this model ideal to investigate new drugs for disease modification and analgesia.

Pro-inflammatory cytokines are accepted as being important in the pathogenesis of RA and CIA. Expression levels of these proteins in the arthritic joint are determined to ascertain whether their kinetics change during arthritis. Increased amounts of pro-inflammatory cytokines during CIA can indicate novel targets for therapy.

The central nervous system (CNS) is known to produce proinflammatory cytokines during adjuvant-induced arthritis (AIA) (Bao, Zhu et al. 2001). Astrocytes in the spinal cord are known to be activated during CIA, potentially driving central sensitisation, thereby maintaining chronic hyperalgesia (Inglis, Notley et al. 2007). Activated astrocytes and microglia are known to produce pro-inflammatory cytokines, including TNF and IL-17 in vitro (Meeuwsen, Persoon-Deen et al. 2003; Kawanokuchi, Shimizu et al. 2007) but it is unknown whether these cells express cytokines during CIA. IL-17 is present in RA synovium and synovial fluid and IL-17 blockade ameliorates CIA. However, any potential role IL-17 and its receptor have in the PNS and CNS (DRG and spinal cord/brain respectively) during CIA is unknown. This chapter addresses the kinetics of IL-17/IL-17RA expression in key tissues in the nociceptive pathway (joint, DRG, spinal cord) during CIA in DBA/1 mice.

5.2 Aims of chapter

The aim of this chapter was to understand kinetics of cytokine expression during CIA in key tissues of the nociceptive pathway. This aim was achieved as follows;

Tissues from DBA/1 mice with CIA were taken and expression of cytokine and their receptor mRNA was determined by quantitative real-time PCR to examine whether changes in mRNA were apparent during arthritis.

5.3 Results

5.3.1 IL-17, TNF, IL-1 and IL-6 are upregulated in the joints of mice with CIA.

In order to investigate IL-17 kinetics during CIA, it was first established whether IL-17 message was expressed in the joint and whether this expression changed during arthritis. DBA/1 mice with CIA were sacrificed day 10 post onset of disease and arthritic paws analysed for IL-17 mRNA using quantitative real-time PCR. When compared to naive age-matched controls, IL-17 mRNA was significantly increased in the joints of mice with CIA. TNF, IL-1 and IL-6 expression were also compared as these cytokines had been shown to be up regulated in previous investigations. TNF was significantly upregulated and IL-6 and IL-1 were also highly increased in the CIA joint.

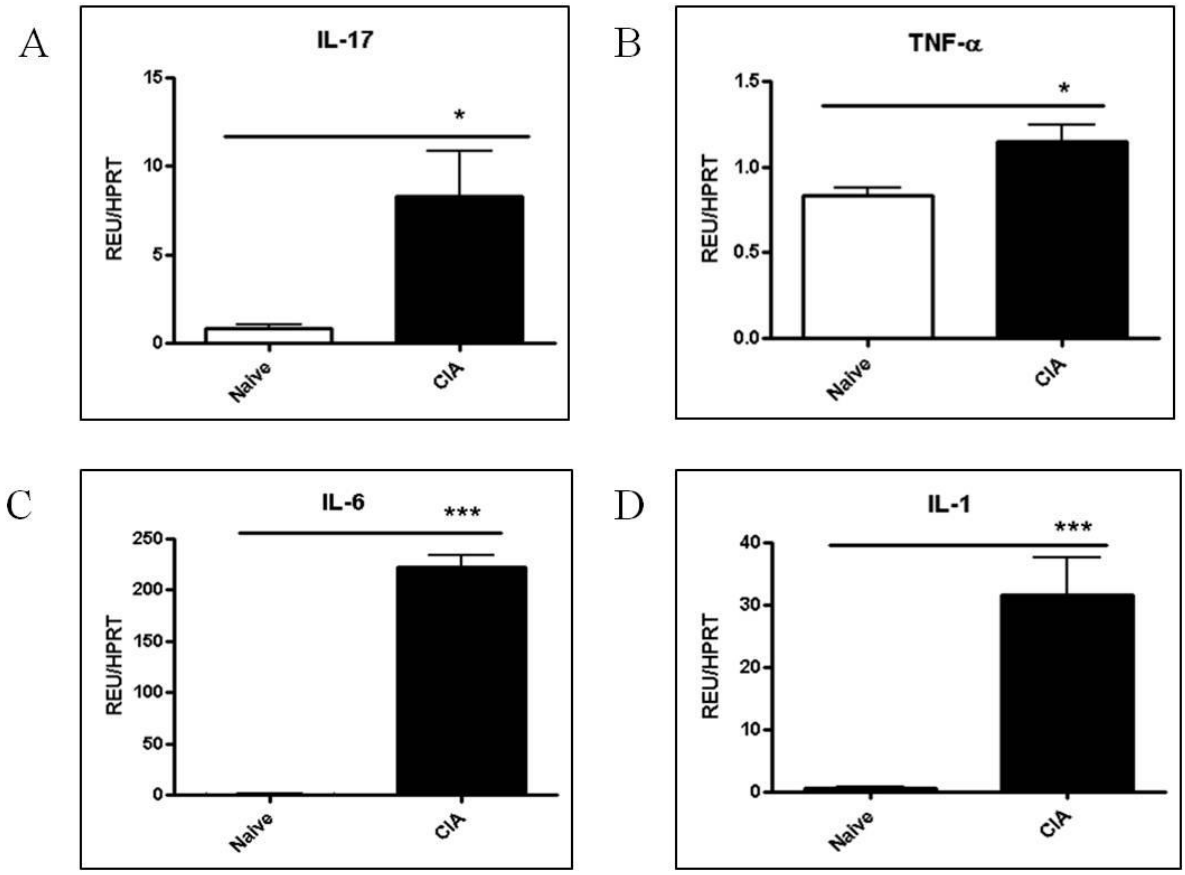

Figure 5.1 Pro-inflammatory cytokines are upregulated during CIA.
Real-time PCR showing increased message expression of IL-17 (A), TNF (B), IL-6 (C) and IL-1 (D) in the CIA paw compared to naive paw controls.
*p <0.05, **p <0.01 and ***p <0.001.

5.3.2 TNF receptors and IL-17 receptors are differently expressed in the joints of mice with CIA. Both IL-17 and TNF mRNA were significantly upregulated in the CIA joint, and as previous investigations had shown divergent roles for TNFR1 and TNFR2 during acute conditions, it was important to assess the kinetics of the receptors for each cytokine receptor in the CIA joint. TNFR1 message was not significantly different from the naive group but TNFR2 was upregulated. IL-17RA message also increased in the CIA joint.

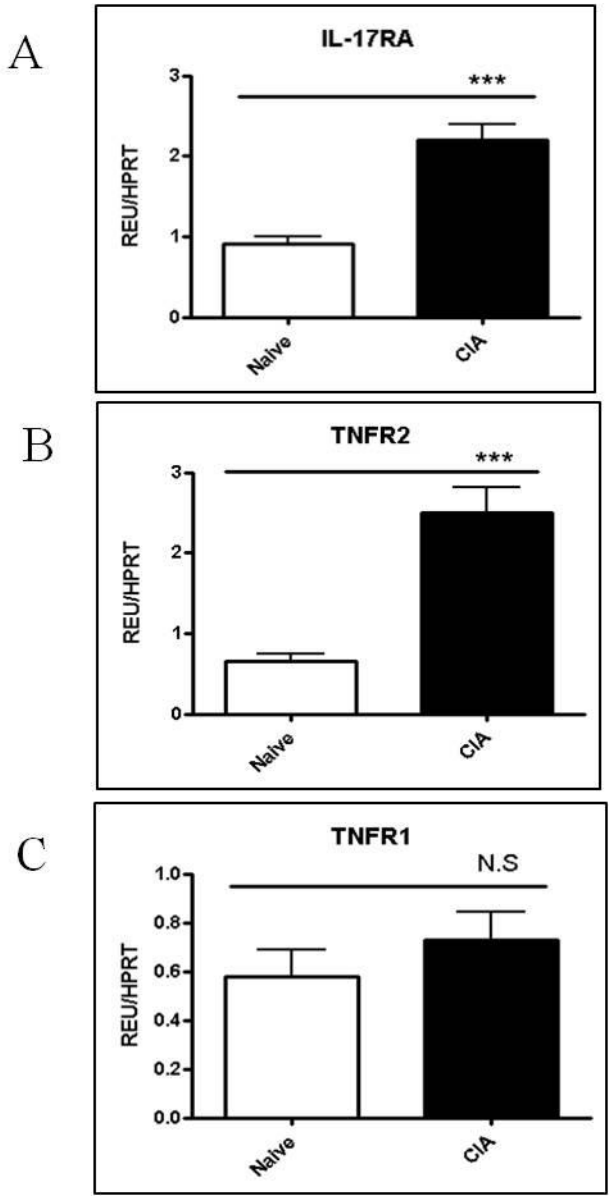

Figure 5.2 IL-17RA and TNFR2 are upregulated in the CIA joint. Real-time PCR showing increased message expression of IL-17 (A) and TNFR2 (B) but not TNFR1 (C) in the CIA joint when compared to naive controls. *p <0.05, **p <0.01 and ***p <0.001.

5.3.3 IL-17RA, TNFR1 and 2 are up regulated in the L4/L5 DRGs of mice with CIA. As there was increased expression in the CIA joint of both IL-17 and TNF, it was considered whether either cytokine could have a more direct influence on the nervous system during CIA. Therefore the DRGs (L4/L5) innervating the joint were investigated for expression of their respective receptors IL-17RA, TNFR1 and TNFR2. All three receptors had significantly elevated expression during CIA.

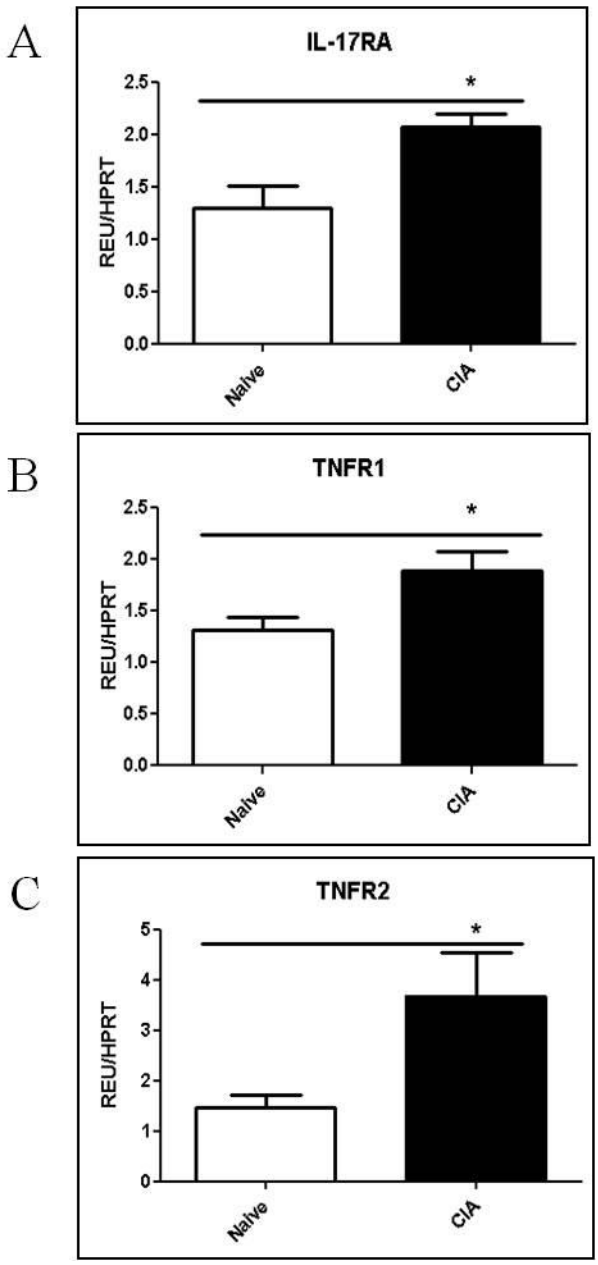

Figure 5.3. IL-17RA ,TNFR1 and TNFR2 are upregulated in the DRG. Real-time PCR showing increased levels of IL-17RA (A), TNFR2 (B) and TNFR1 (C) in the CIA DRG when compared to naive controls. *p <0.05, **p <0.01 and ***p <0.001.

5.3.4 TNF and IL-1 but not IL-6 or IL-17 is upregulated in the spinal cords of mice with CIA. Glia and microglial cells are known to produce TNF, IL-1, IL-6 and IL-17 when activated, however expression of these cytokines in the CNS have never been investigated during murine CIA. To address this, the lumbar region of the spinal cords were analysed for TNF, IL-1, IL-17 and IL-6 message and expression compared to levels in naive controls. TNF (Figure 5.4a) and IL-1 (Figure 5.4b) were significantly up-regulated. IL-6, although present was comparable to the naive control (Figure 5.4c). IL-17 message was detectable but too poorly expressed in the tissue to be quantified.

5.3.5 GFAP is upregulated in the spinal cords of mice with CIA. Earlier investigations have shown that astrocytes produce pro-inflammatory cytokines when activated, so as TNF and IL-1 were upregulated in CIA, it was necessary to look for markers of astrocyte activation. GFAP is a marker for astrocyte activation and it was observed that GFAP was significantly increased in the spinal cord during CIA (Figure 5.4d), indicating astrocyte activation, which is in agreement with previous findings using an IHC approach (Inglis, Notley et al. 2007).

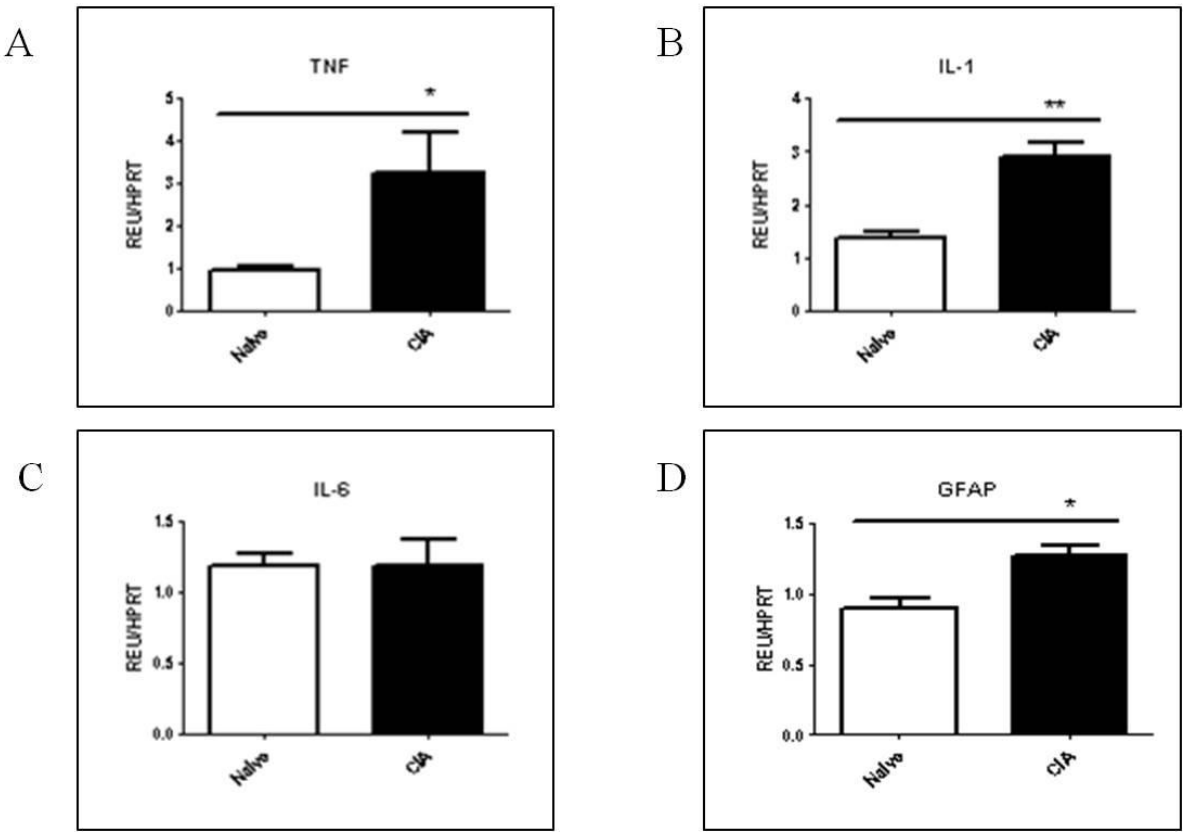

Figure 5.4. Proinflammatory cytokine and GFAP expression in the spinal cords of mice with CIA. Real-time PCR showing no difference in expression of IL-6 message (A) but increased levels of TNF (B) , IL-1 (C) and GFAP (D) in the CIA spinal cord when compared to naive controls. *p <0.05, **p <0.01 and ***p <0.001.

5.3.6 TNFR1/2 and IL-17RA are upregulated in the spinal cords of mice with CIA. Expression of TNFR1 and 2 was analysed in spinal cords of CIA mice and found to be significantly increased over the naive control receptor levels (Figure 5.5a and b). IL-17RA was also upregulated (Figure 5.5c), despite unquantifiable expression of IL-17A.

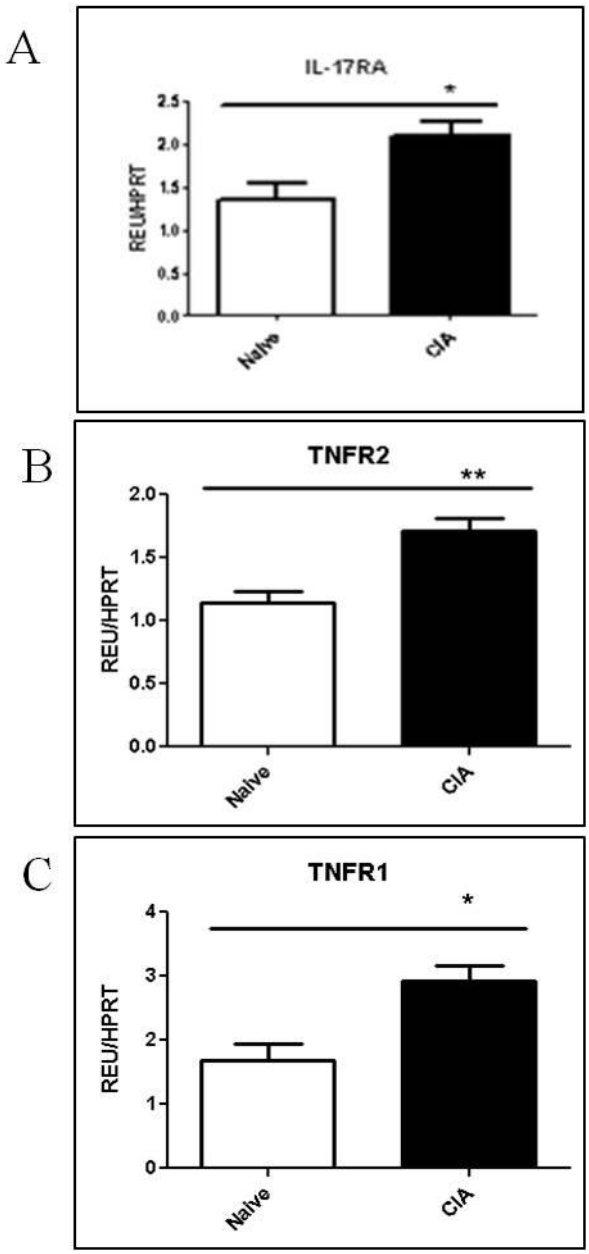

Figure 5.5. IL-17RA ,TNFR1 and TNFR2 are upregulated in the spinal cords of mice with CIA. Real-time PCR showing increased levels of IL-17RA (A), TNFR2 (B) and TNFR1 (C) in the CIA spinal cord when compared to naive controls. *p <0.05, **p <0.01 and ***p <0.001.

Discussion

This chapter has shown that cytokines and their receptors expressed in nociceptive tissues undergo distinct changes during arthritis. By day 10 post onset of arthritis, pro-inflammatory cytokines are elevated in the joint, and TNF and IL-17 receptors are upregulated in the DRG innervating the arthritic joint. For the first time it has been shown that TNF and IL-1 are elevated in the spinal cord during CIA and that the expression of TNF and IL-17 receptors also increases. A potential role for astrocytes in CIA has also been indicated by increased expression of their activation marker during arthritis.

It is often difficult to adequately quantify a range of cytokines and their receptors in a disease model. This is due to several factors; recovery of good quality material can be low and time consuming depending on the tissue type, and the tissue may require additional stimulation to detect cytokines even in an inflammatory environment. Detecting cytokine protein concentration uses a large amount of tissue, limiting the amount of cytokines that can be investigated. By extracting RNA from the tissue, mRNA can be transcribed into complementary DNA (cDNA) and the cytokine gene detected using specific primers and real-time PCR. Real-time PCR can amplify product from a single cell, making it ideal when tissue is limiting as for joint, spinal cord and the DRG.

Enhanced production of pro-inflammatory cytokines has long been associated with RA. This elevation has also been shown in the joints of rodents during experimental

arthritis with protein levels correlating with mRNA expression levels (Rioja, Bush et al. 2004; Pohlers, Siegling et al. 2005). TNF, IL-1 and IL-6 expressing cells progressively increase between day 1 and 10 post onset of disease (Marinova-Mutafchieva, Williams et al. 1997). TNF, IL-1, IL-6 and IL-17 increases in message correlate with increased disease severity (Ferrari-Lacraz, Zanelli et al. 2004). The significantly enhanced message for TNF, IL-17, IL-6 and IL-1 demonstrated here therefore concurs with earlier investigations.

As previous chapters focussed on TNF and IL-17, receptors for each cytokine were examined in the CIA joint. IL-17RA and TNFR2 were significantly upregulated; however, TNFR1 expression was not different from naive controls. TNFR1 is expressed on most cell types (Kollias, Douni et al. 1999) and considered to be imperative for most inflammatory actions of TNF (MacEwan 2002). Indeed, it has been reported that TNF acting through TNFR1 is critical for arthritis to occur in animal models (Mori, Iselin et al. 1996; Arntz, Geurts et al. 2010), although there is opposing literature which suggests that arthritis incidence is unaffected by TNFR1 deficiency (Tada, Ho et al. 2001; Williams-Skipp, Raman et al. 2009). In addition, a novel immunoregulatory role for TNF has been described which appears to be dependent on TNFR1. TNF inhibits p40 which regulates Th1/Th17 populations in the lymph nodes during CIA. However, this inhibition of p40 is lost in $TNFR1^{-/-}$ mice, resulting in expansion if Th1/Th17 cells (Notley, Inglis et al. 2008). Here expression of TNFR1 was not significantly increased, potentially due to the ubiquitous expression of TNFR1. Alternatively, TNFR1 is down regulated in late arthritis possibly to limit duration of tissue inflammation. To answer this, kinetics of the receptor must be assessed in a time course of CIA. Unlike TNFR1, the

expression of TNFR2 is limited to cells of haemopoetic origin endothelial origin (Kollias, Douni et al. 1999). During CIA, immune cells infiltrate the affected joints which could account for the increased expression of this receptor. TNFR2 has been linked with a more protective role for TNF, due to its expression on highly suppressive Tregs (Chen, Subleski et al. 2008) and exacerbation of experimental colitis in mice lacking TNFR2+ T cells (Dayer Schneider, Seibold et al. 2009). To investigate any protective role for TNFR2 during CIA, $TNFR2^{-/-}$ mice should be compared to wild-type mice for disease susceptibility and progression. IL-17RA, like TNFR1 is ubiquitously expressed, and its enhanced expression here could be due to infiltrating cells. However, IL-17RA is a subunit of the IL-17R signalling complex, therefore expression of IL-17RC in the CIA joint must be considered to fully understand the action of IL-17 during late arthritis.

As discussed in Chapter 1, it has been reported that cytokines are present in the DRG but expression in this tissue for cytokine receptors is poorly understood. In this study, mRNA levels for TNFR1, TNFR2 and IL-17RA were significantly increased. Upregulation of TNFR1 and TNFR2 has previously been shown in CFA arthritis (Inglis, Nissim et al. 2005) which suggested that TNFR1 was predominately expressed in neuronal cells, whilst TNFR2 was expressed by small non-neuronal cells in the DRG. IL-17RA in the DRG has not previously been shown, and it is unknown where in the PNS it is expressed. Greater expression of the receptors could mean a more direct action of the ligands on the DRG during CIA, but additional work is required to identify the precise cells involved.

Recently, there has been a focus on investigating the role of the CNS in maintaining chronic pain states and the central production of cytokines. Astrogliosis is associated with chronic pain in CIA detected by an increase in GFAP (Inglis, Notley et al. 2007). Here, GFAP is significantly increased, indicating that astrocytes are active and central involvement is important in CIA. Microglia are activated in some forms of arthritis, but have previously been shown not to be activated during CIA (Inglis, Notley et al. 2007). However, as it is unknown whether other cell types infiltrate the CNS during CIA, increased expression could also be due to infiltrating cells and therefore must be investigated.

Activated astrocytes and microglia are also known to produce TNF, IL-1, IL-6 and IL-17 (Benveniste, Sparacio et al. 1990; Chung and Benveniste 1990; Van Wagoner, Oh et al. 1999; Meeuwsen, Persoon-Deen et al. 2003). As it has been suggested that microglia are not active during CIA, any elevation seen here could be localised to astrocytes, depending on CNS infiltrates. TNF, IL-1 and IL-6 are known to be upregulated in the AIA spinal cord (Bao, Zhu et al. 2001), but this chapter is the first study to show elevation of TNF and IL-1 in the spinal cord during CIA. Surprisingly, IL-6 expression did not differ from naive controls. IL-17 although present, was too low to be quantified. This indicates that IL-6 and IL-17 are not required at elevated levels in CIA CNS inflammation. TNFR1, TNFR2 and IL-17RA were all found to be upregulated in the spinal cord during CIA. It has been suggested that TNF receptors have divergent roles in the CNS. TNFR1 is thought to aggravate neuronal cell death and induce upregulation of molecules known to be important in CNS infiltration by leukocytes (Gimenez, Sim et al. 2004; Marchetti, Klein et al. 2004). Conversely, TNFR2 is considered to be neuroprotective

(Marchetti, Klein et al. 2004), but interestingly is thought to be expressed only by microglia and not astrocytes (Dopp, Mackenzie-Graham et al. 1997; McCoy and Tansey 2008) (although this is subject to controversy (Kuno, Yoshida et al. 2006)). As microglia are reported not to be activated during CIA (Inglis, Notley et al. 2007), TNFR2 upregulation may indicate an additional route to maintain quiescence via TNF, thereby preventing further inflammation and neuronal cell death. Further investigations utilising $TNFR1^{-/-}$ or $TNFR^{-/-}$ mice are needed to further elucidate the potential paradoxical role of TNF in the CNS during CIA. As IL-17 is weakly expressed here, elevation of the IL-17RA could be due to cellular infiltration. However, as IL-17RA is one subunit of the IL-17 receptor, IL-17RC expression must therefore be assessed. Further work is required to understand the role of IL-17RA and IL-17RC during CIA.

This chapter has shown that IL-17 is a potential target in CIA due to its upregulation in the joint. The increased expression of IL-17RA in the DRG may point to a more direct action for IL-17 in the PNS during CIA. The influence of IL-17 on other proinflammatory cytokines in all these tissues can be examined by IL-17 blockade during CIA.

CHAPTER 6

IL-17 BLOCKADE IN CIA IS ANTI-ARTHRITIC AND ANALGESIC

6.1 Introduction

Anti-IL-17 treatment has been shown to be effective in several animal models of arthritis. Neutralising antibodies to IL-17 prevented joint inflammation and bone erosion in DBA/1 mice with CIA (Lubberts, Koenders et al. 2004). Furthermore, IL-17 blockade during AIA suppressed joint inflammation and bone erosion by potently reducing expression of TNF and IL-1 and RANKL (Koenders, Lubberts et al. 2005). However, although the anti-arthritic effects of IL-17 blockade have been extensively described, it has never been investigated whether anti-IL-17 therapy can also be analgesic.

The CIA model of arthritis has previously been described as a valid model for chronic hyperalgesia (Inglis, Notley et al. 2007). Hyperalgesia was tested in CIA using the Hargreaves and Von Frey assessments, with both tests revealing a reduction in thermal and mechanical thresholds on the day of disease onset. Reduced thresholds were maintained up to 18 days (thermal) and 28 days (mechanical) post onset of arthritis. Astrogliosis, known to occur during chronic pain states, was evident by day 10 post onset. After administration of anti-TNF on the day of onset, thermal latency was significantly increased in comparison to vehicle treated mice by day 3, with mechanical latency significantly increasing by day 7 of arthritis. In addition, astrogliosis was suppressed at day 10 post onset after anti-TNF administration. In addition, AIA in the rat knee also results in lowered pain thresholds and reduced locomotion. Both etanercept and infliximab attenuated hyperalgesia and restored locomotion (Boettger, Hensellek et al. 2008). These studies show that anti-TNF treatment is analgesic in animal models of arthritis, as

IL-17 is also implicated in RA it is important to address whether IL-17 blockade is analgesic as well as therapeutic. As previous chapters have shown that IL-17 is dependent on TNF in acute conditions, future experiments need to consider dependency of IL-17 on TNF in arthritic conditions, facilitating the need to use TNFR1/TNFR2-/- mice. These mice are bred on a C57BL/6 background; therefore the initial experiments use C56BL/6 mice for CIA as a more suitable strain comparison.

6.2 Aims of chapter

The aims of this chapter were to assess CIA in the C57BL/6 mice as a model for hyperalgesia and to investigate the efficacy of anti-IL-17 treatment as both therapeutic and analgesic. These aims were achieved as follows;

1. C57BL/6 mice were immunised with type II chicken collagen in CFA and on the day of disease onset were either treated with anti-IL-17 or left untreated. Disease progression and hyperalgesia were monitored in each group over a 10 day period. Spontaneous behaviour was assessed on day 10 post onset.

2. On day 10 post onset of disease, animals were culled and tissues were taken and examined for changes in cytokine and cytokine receptor expression by real-time PCR.

6.3 Results

6.3.1 IL-17 blockade suppresses clinical score and paw swelling in CIA. Firstly it was necessary to test whether administration of anti-murine IL-17 could suppress disease in C57BL/6 mice with CIA. C57BL/6 mice were immunised with type II chicken collagen in CFA as described previously (Inglis, Simelyte et al. 2008). On the day of disease onset, 200µg anti-IL-17 polyclonal antibody was injected intra-peritoneally in 100µl sterile PBS and then every other day until day 10 post onset (Figure 6.1). Anti-IL-17 treatment markedly reduced clinical severity and paw swelling by day 4 post onset of disease when compared to control mice with CIA. This suppression of disease was maintained for the duration of the treatment period.

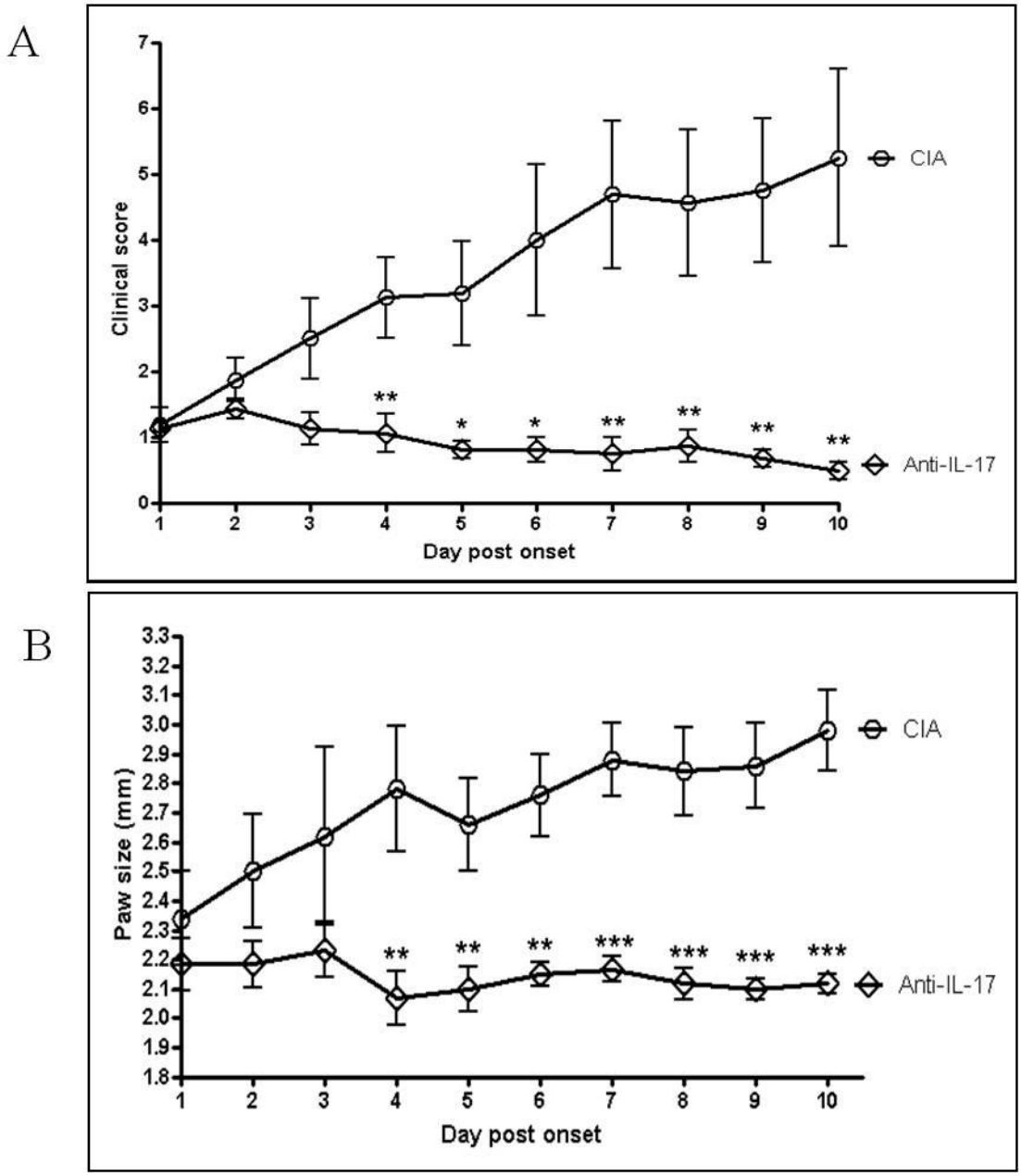

Figure 6.1 IL-17 blockade reduces clinical score and paw swelling in mice with CIA. C57BL/6 mice were immunised with Type II chicken collagen. After onset of arthritis, mice were treated with 200µg anti-IL-17 , and the clinical score (A) and paw swelling (B) assessed over a 10 day period. Values are the SEM of 6 individual mice and are representative of 2 independent experiments. *p <0.05, **p <0.01 and ***p <0.001.

6.3.2 IL-17 blockade reduces thermal and mechanical hyperalgesia in CIA. As CIA had been shown to be a model for chronic hyperalgesia in DBA/1 mice, it was important to assess hyperalgesia in the milder form of arthritis observed in C57BL/6 mice. Arthritic and anti-IL-17 treated arthritic mice were examined for thermal and mechanical nociception using the Hargreaves and Von Frey evoked pain tests (Figure 6.2). On the day of disease onset, and day 3, 7 and day 10 post onset, each treated animal had their thermal and mechanical thresholds assessed alongside control mice with CIA. By day 7 post onset, both thermal and mechanical thresholds had significantly increased when compared with arthritic mice, which demonstrated hyperalgesia throughout the course of disease. By day 10 post onset, nociception in anti-IL-17 treated mice was comparable to naive mice.

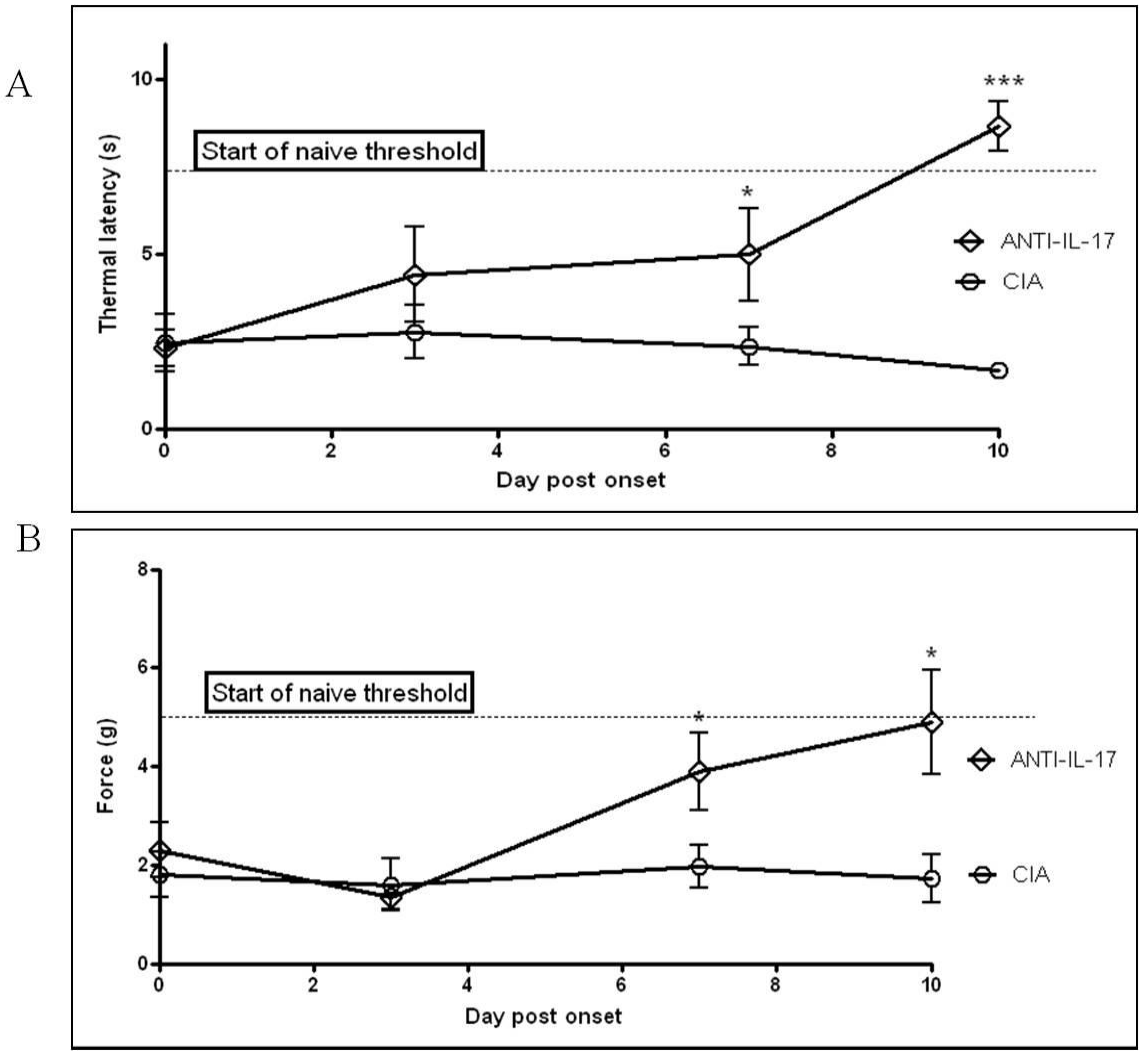

Figure 6.2 IL-17 blockade is analgesic in mice with CIA. C57BL/6 mice were immunised with Type II chicken collagen. After onset of arthritis, mice were treated with 200µg anti-IL-17 , and pain assessed by Hargreaves (A) and Von Frey(B) over a 10 day period. Values are the SEM of 6 individual mice and are representative of 2 independent experiments. *p <0.05, **p <0.01 and ***p <0.001.

6.3.3 IL-17 blockade normalises spontaneous behaviour to that of naive mice.

Arthritis effects spontaneous behaviour in mice with CIA (Inglis, Notley et al. 2007). In order to investigate whether IL-17 blockade restored change in spontaneous behaviour, mice were placed in a LABORAS monitor at day 10 post onset (Figure 6.3). Naive and IL-17 treated groups were compared with the CIA group. Mice with CIA had significantly reduced climbing and locomotion and increased immobility when compared to age-matched naive mice. Grooming appeared to be less in mice with CIA, but this was not significant. With anti-IL-17 administration, locomotion and immobility in the treated arthritic mice had returned to naive levels. Climbing in the treated mice increased compared to the CIA group but was still significantly less than the naive mice, indicating a partial recovery.

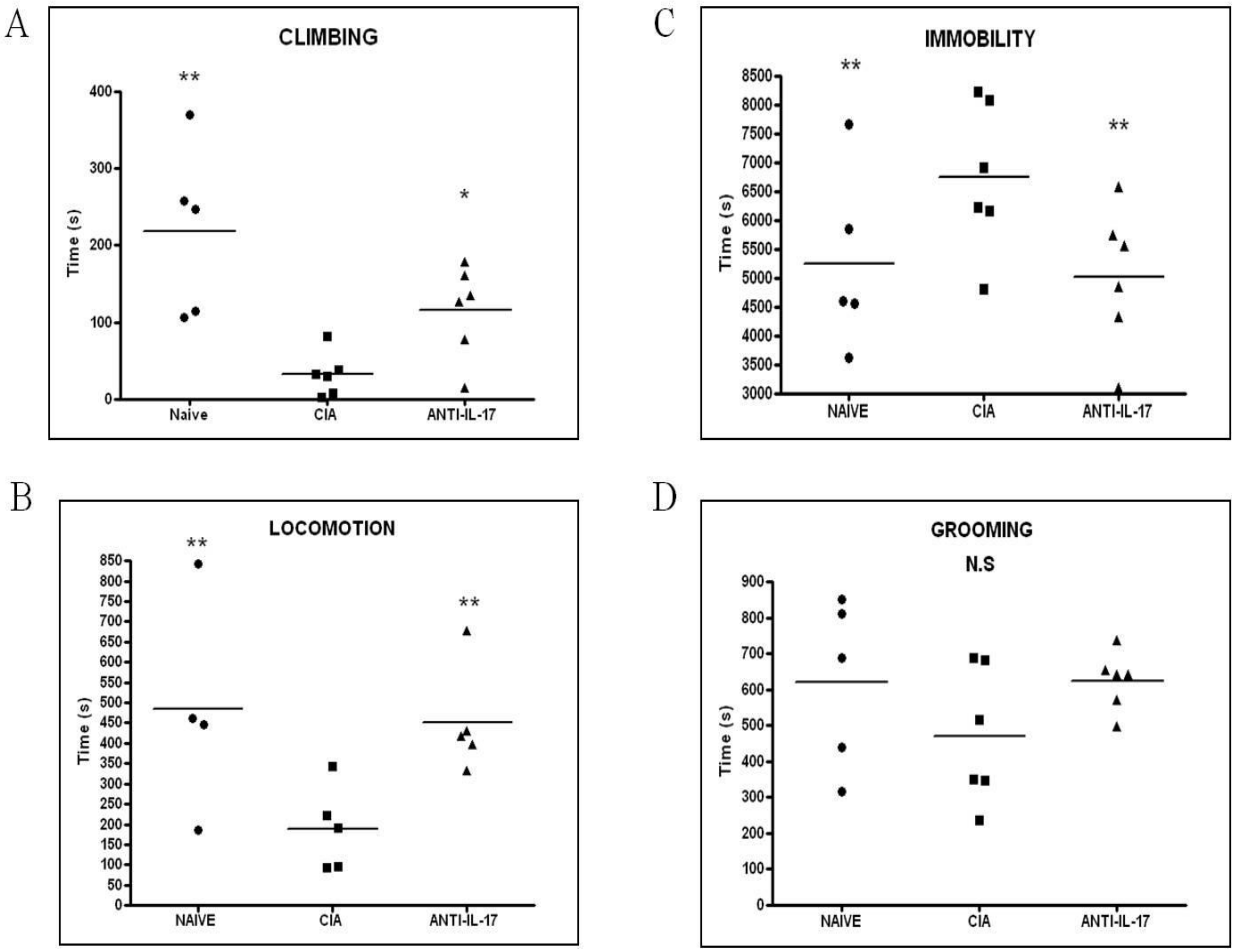

Figure 6.3 IL-17 blockade restores spontaneous behaviour in mice with CIA. C57BL/6 mice were immunised with Type II chicken collagen. After onset of arthritis, mice were treated with 200μg anti-IL-17 , and spontaneous behaviour assessed by LABORAS at day 10 post onset. Changes in climbing (A), locomotion (B), immobility (C) and grooming (D) were used as markers of behaviour. Values are the SEM of 4-6 individual mice from 1 experiment. *p <0.05, **p <0.01 and ***p <0.001.

6.3.4 Real-time PCR profile

6.3.4.1 Real-time PCR profile of joints from anti-IL-17 treated and control mice with CIA

In order to investigate the effect of anti-IL-17 treatment on other pro-inflammatory cytokines in the joints of mice with CIA, hind paws were removed at day 10 post onset, snap frozen and RNA isolated for real-time PCR. Real-time PCR was used to quantify message expression levels in all tissues due to low tissue yield. Message levels of IL-17 (Figure 6.4a), TNF (Figure 6.4b), IL-1 (Figure 6.4c) and IL-6 (Figure 6.4d) all increased during CIA but were down regulated in anti-IL-17 treated mice.

IL-17RA was upregulated in mice with CIA (Figure 6.5a) , but reduced with anti-IL-17 treatment. IL-17RC (Figure 6.5b) in contrast, was down regulated in CIA and this reduction was maintained after therapy. TNFR1 (6.5c) was also reduced in comparison to naive joints and again TNFR1 message continued to be significantly reduced in anti-IL-17 treated mice versus controls. TNFR2 (Figure 6.5d) was upregulated during CIA, which is consistent with the results obtained in DBA/1 mice in Chapter 5. TNFR2 expression was reduced with anti-IL-17 therapy and was not significantly different from naive joints.

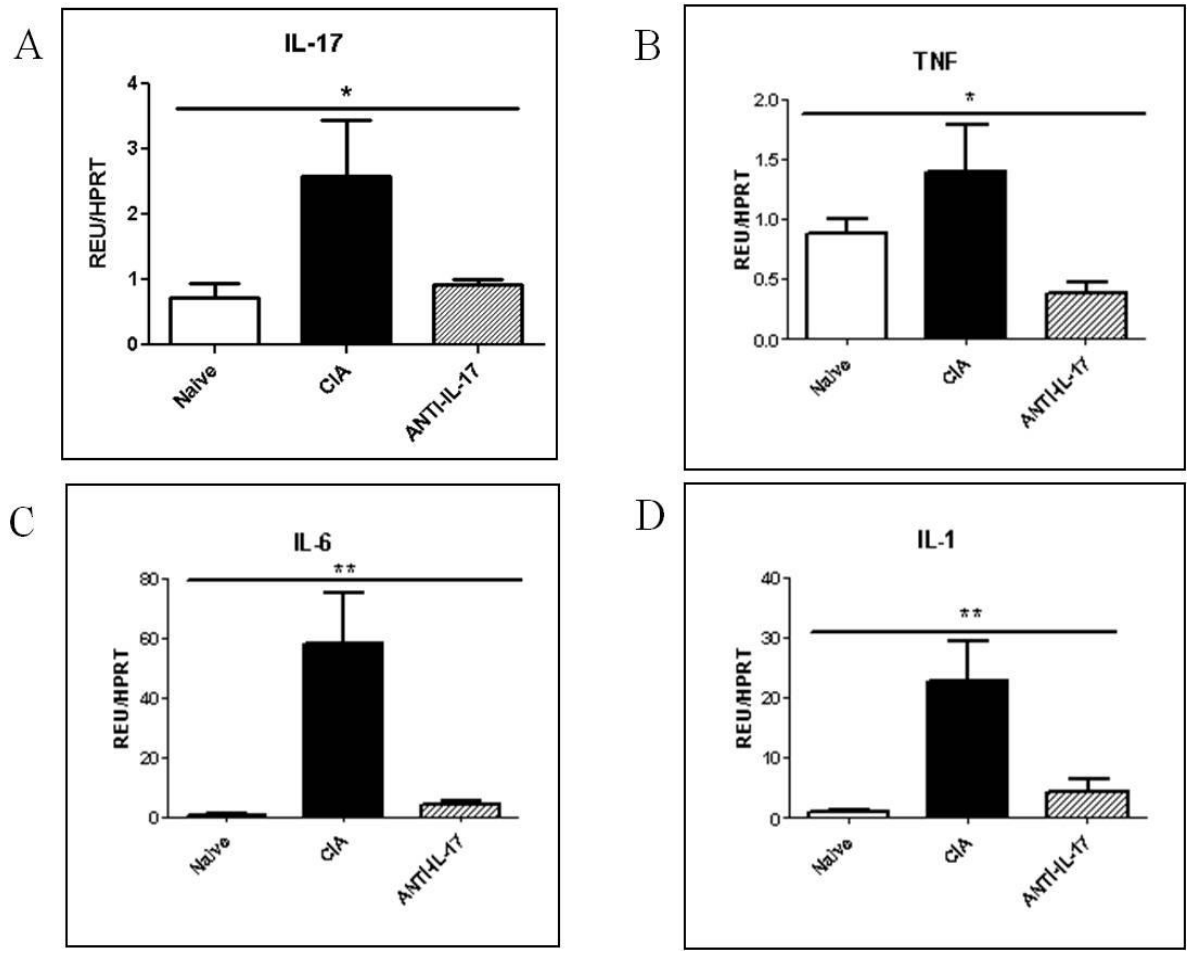

Figure 6.4 IL-17 blockade reduces pro-inflammatory cytokines in the joints of mice with CIA
C57BL/6 mice were immunised with Type II chicken collagen. After onset of arthritis, mice were treated with 200μg anti-IL-17 and expression of IL-17 (A), TNF (B), IL-6 (C) and IL-1 (D) assessed by real-time PCR at day 10 post onset and each group compared to a naive control. Values are the SEM of 5 individual mice. *p <0.05, **p <0.01 and ***p <0.001.

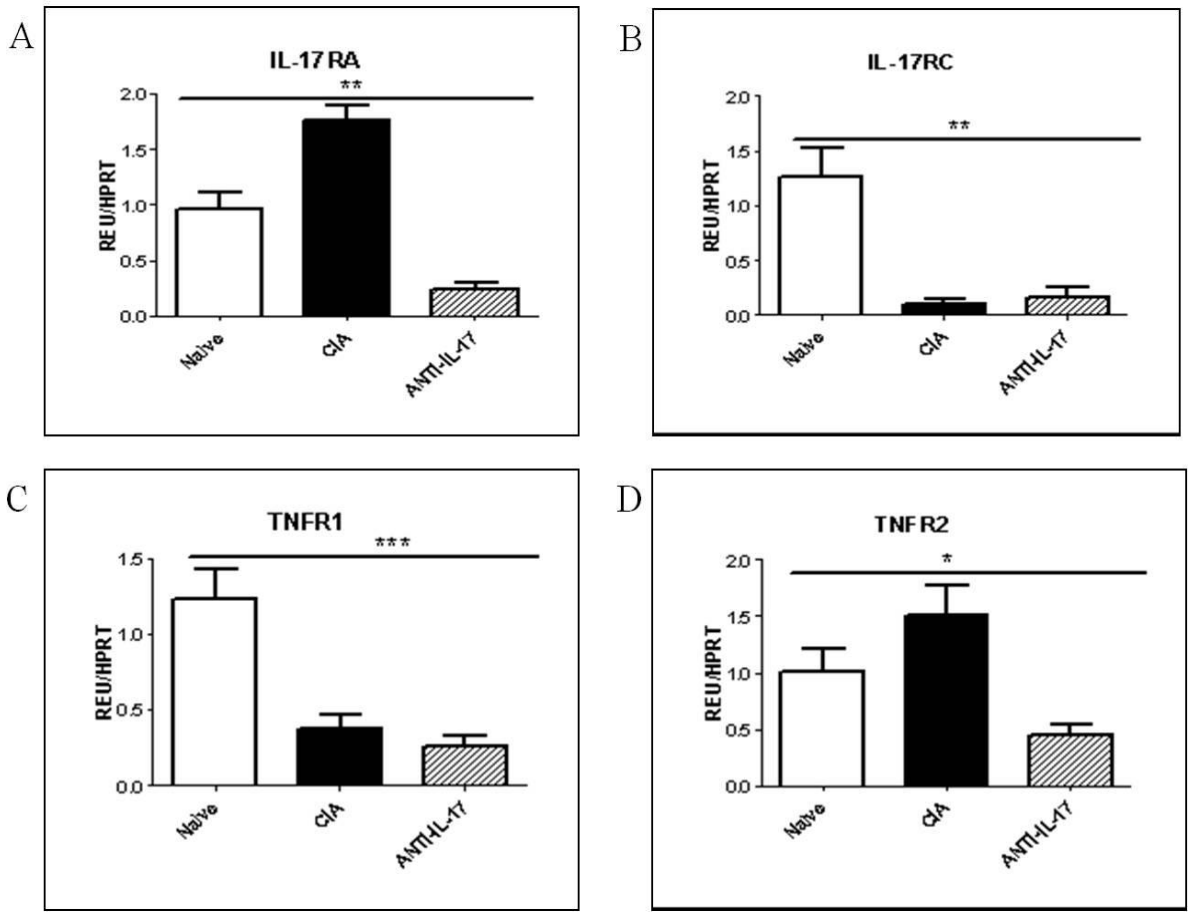

Figure 6.5 IL-17 and TNF receptor expression after anti-IL-17 treatment in the joints of mice with CIA. C57BL/6 mice were immunised with Type II chicken collagen. After onset of arthritis, mice were treated with 200μg anti-IL-17 and expression of IL-17RA (A), IL-17RC (B), TNFR1 (C) AND TNFR2 (D) were assessed by real-time PCR at day 10 post onset and each group compared to a naive control. Values are the SEM of 5 individual mice. *p <0.05, **p <0.01 and ***p <0.001.

6.3.4.2 Real-time PCR profile of L4/L5 DRGs from anti-IL-17 treated and control mice with CIA

As the previous chapter had shown changes in the kinetics of IL-17RA and the TNF receptors in DBA/1 mice, it was necessary to validate these findings in the C57BL/6 mice. L4/L5 DRGs from arthritic and treated mice were removed at day 10 post onset and RNA was isolated. All samples were then examined for changes in message expression by real-time PCR. As seen in the DBA/1 mice in Chapter 5, IL-17RA (Figure 6.6a), TNFR1 (Figure 6.6c) and TNFR2 (Figure 6.6d) were upregulated in CIA. A novel finding was that IL-17RC (Figure 6.6b) also increased during CIA. All these receptors were reduced to naive levels with anti-IL-17 treatment.

Although it is known that the DRG can produce proinflammatory cytokines, it was unknown whether there was expression during CIA. TNF (Figure 6.7a) and IL-1 (Figure 6.7b) message was significantly increased by day 10 post onset but with IL-17 blockade were comparable to the expression level in naive mice. IL-6 message (Figure 6.7c) did not significantly differ between the groups. IL-17 was only detectable in a proportion of the CIA group and therefore could not be quantified.

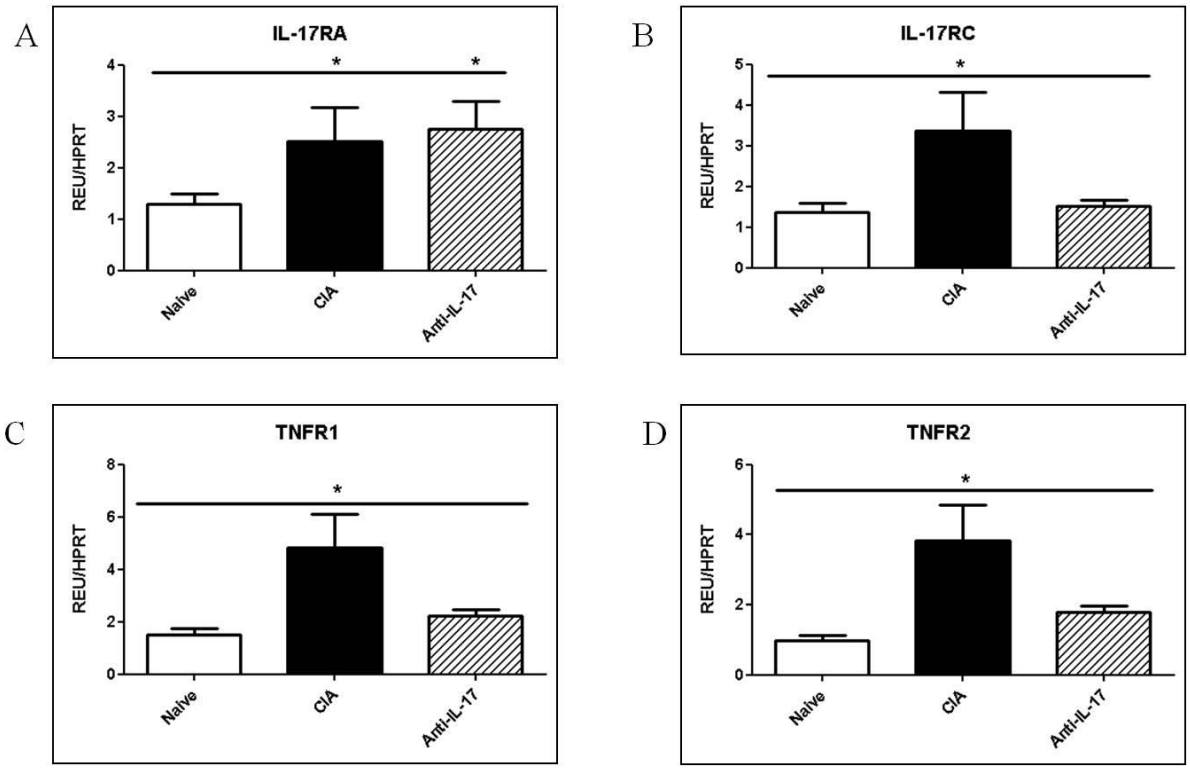

Figure 6.6 IL-17 and TNF receptor expression after anti-IL-17 treatment in the L4/L5 DRGs of mice with CIA. C57BL/6 mice were immunised with Type II chicken collagen. After onset of arthritis, mice were treated with 200µg anti-IL-17 and expression of IL-17RA (A), IL-17RC (B) TNFR1(C) and TNFR2 were assessed by real-time PCR and each group compared to a naive control. Values are the SEM of 5 individual mice. *p <0.05, **p <0.01 and ***p <0.001 .

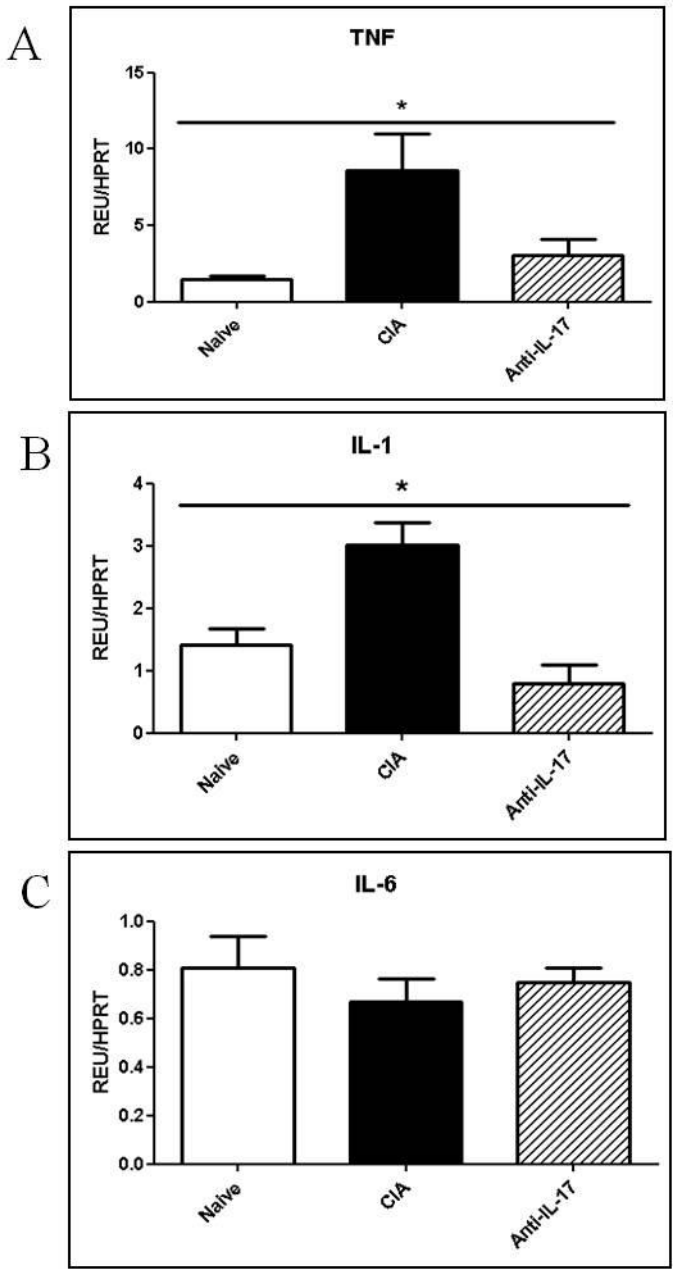

Figure 6.7Pro-inflammatory cytokine expression after anti-IL-17 treatment in the L4/L5 DRGs of mice with CIA. C57BL/6 mice were immunised with Type II chicken collagen. After onset of arthritis, mice were treated with 200µg anti-IL-17 and expression of TNF (A), IL-1 (B) and IL-6 (C) assessed by real-time PCR and each group compared to a naive control. Values are the SEM of 5 individual mice *p <0.05, **p <0.01 and ***p <0.001 .

6.3.4.3 Real-time PCR profile of spinal cords from anti-IL-17 treated and control mice with CIA

As significant differences in message expression had been shown in cytokine and cytokine receptors in the lumbar spinal cord during CIA in DBA/1 mice, any changes also needed to be validated in C57BL/6 mice with CIA.

IL-17 was only present in arthritic mice and therefore unquantifiable, a similar finding to DBA/1 mice with CIA. TNF (Figure 6.8a), IL-1 (Figure 6.8b) and GFAP (Figure 6.8d) message correlated with DBA/1 mice by being upregulated during CIA. However, with anti-IL-17 therapy, TNF, IL-1 and GFAP were all comparable to naive spinal cords levels. IL-6 expression was also similar to DBA/1 findings, with no significant differences in any of the groups (Figure 6.8c).

IL-17RA (Figure 6.9a) and IL-17RC (Figure 6.9b) expression remained unchanged in all groups, but TNFR1 (Figure 6.9c) and TNFR2 (Figure 6.9d) expression was consistent with DBA/1 mice, as both were upregulated. Anti-IL-17 therapy reduced both TNFR1 and TNFR2 to naive levels.

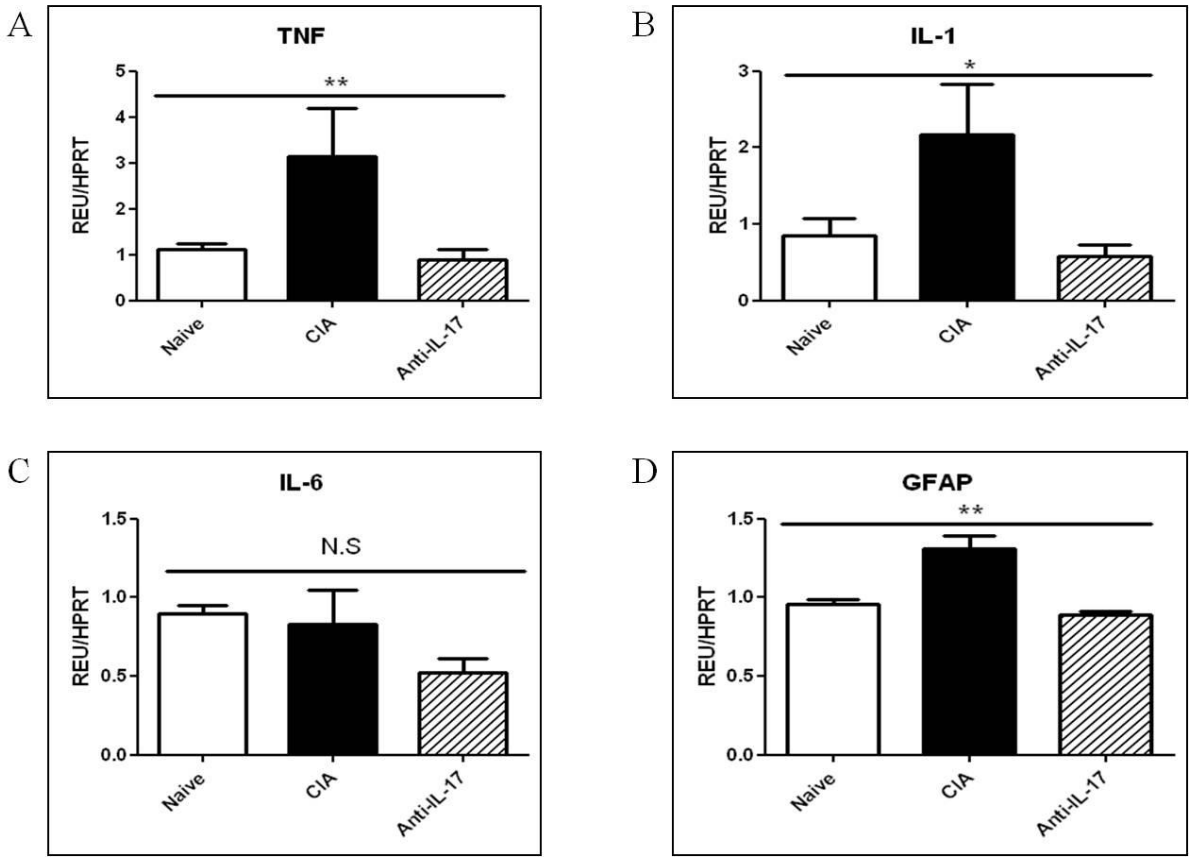

Figure 6.8 Pro-inflammatory and GFAP expression after anti-IL-17 treatment in the spinal cords of mice with CIA. C57BL/6 mice were immunised with Type II chicken collagen. After onset of arthritis, mice were treated with 200µg anti-IL-17 and expression of TNF (A), IL-1 (B) and IL-6 (C) and GFAP were assessed by real-time PCR and each group compared to a naive control. Values are the SEM of 5 individual mice. *p <0.05, **p <0.01 and ***p <0.001 .

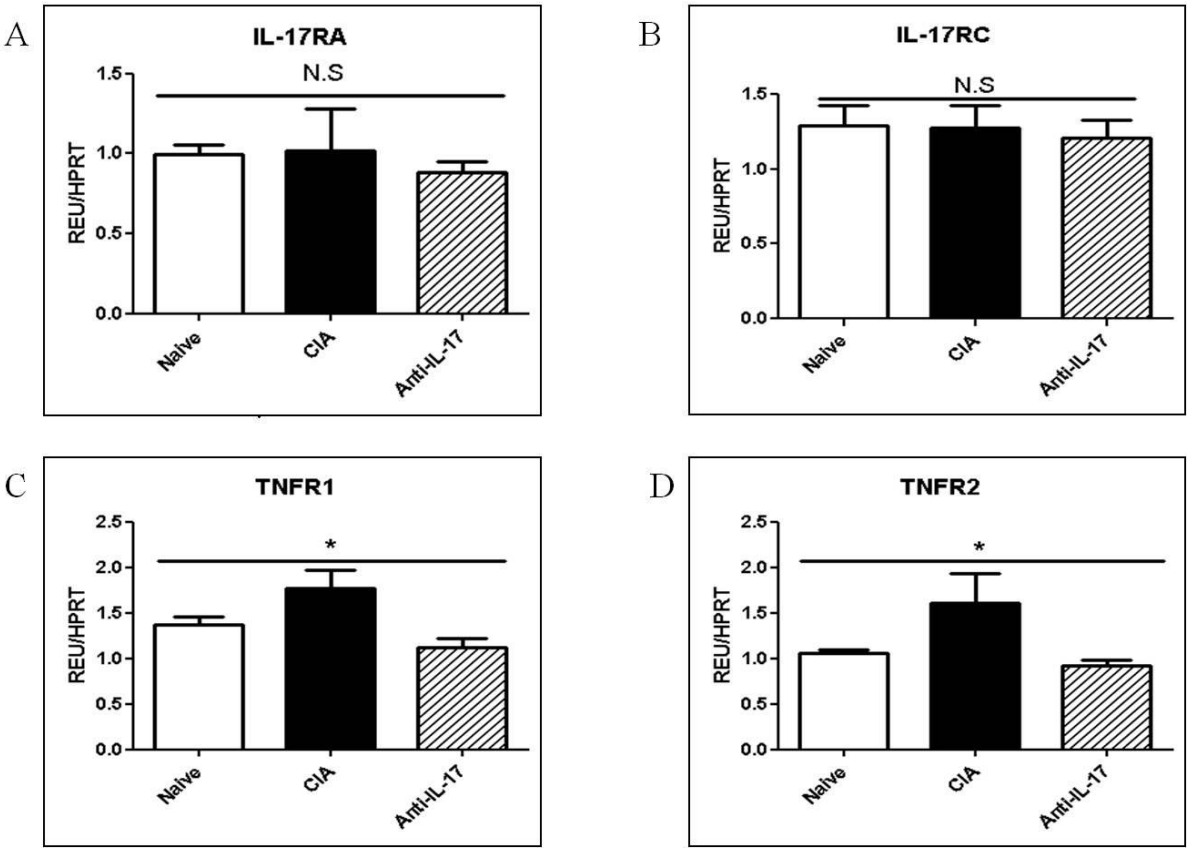

Figure 6.9 IL-17 and TNF receptor expression after anti-IL-17 treatment in the spinal cords of mice with CIA. C57BL/6 mice were immunised with Type II chicken collagen. After onset of arthritis, mice were treated with 200μg anti-IL-17 and expression of IL-17RA (A), IL-17RC (B) TNFR1(C) and TNFR2 were assessed by real-time PCR and each group compared to a naive control. Values are the SEM of 5 individual mice. *p <0.05, **p <0.01 and ***p <0.001.

6.4 Discussion

This chapter has shown that treating CIA in C57BL/6 mice with neutralising antibodies to IL-17 reduces clinical score, paw swelling and hyperalgesia. In addition, proinflammatory cytokines increased during CIA in the joint, DRG and spinal cord and were potently reduced with anti-IL-17 therapy.

Anti-TNF therapy has been highly successful in treating RA patients. However, not all patients respond adequately. This has lead to new investigations into other proinflammatory cytokines targets in RA. IL-17 is present in the RA joint and blockade of IL-17 is therapeutic in animal models of arthritis (Lubberts, Koenders et al. 2004). Here, administration of anti-IL-17 antibodies reduced clinical score and paw swelling significantly by day 4 post onset. This is comparable to TNF blockade in CIA where paw swelling was significantly reduced in comparison to vehicle controls by day 2 post onset and clinical score was reduced by day 5 post onset of disease (Williams, Feldmann et al. 1992). The rapid disease reduction and correlation with an established successful biologic show that anti-IL-17 is a valid target in RA.

Amelioration of disease following TNF blockade in animal models coincides with rapid analgesia (Inglis, Notley et al. 2007; Boettger, Hensellek et al. 2008). In this study, mice with CIA had reduced pain thresholds to thermal and mechanical hyperalgesia. With anti-IL-17 treatment, thermal and mechanical withdrawal latency significantly increased by day 7 post onset of disease and continued to rise

to naive levels by the end of the study period. Significantly reduced inflammation in the joint preceded the elevation in pain thresholds. This suggests that anti-IL-17 is not instantly analgesic, but acts indirectly by down regulating proinflammatory cytokines in the joint and reducing nerve sensitisation, allowing the activation threshold of nociceptors to rise. Indeed, in a previous study, anti-TNF significantly raised mechanical withdrawal latency by day 7 post onset of CIA (Inglis, Notley et al. 2007). However, in the same investigation, thermal withdrawal latency was significantly increased after anti-TNF by day 3 post onset of arthritis where paw swelling was reduced only by day 4, suggesting a more direct action. In this study, there was a small rise in thermal latency by day 3 post onset, but this was not significant.

Changes in spontaneous behaviour have been regarded as an alternative non-invasive measure of chronic pain. Anti-TNF therapy has been shown previously to restore spontaneous behaviour in animal models of arthritis (Inglis, Notley et al. 2007; Boettger, Hensellek et al. 2008). Here, LABORAS was used at day 10 post onset of arthritis to address whether changes in behaviour occurred during CIA. By day 10, climbing and locomotion were significantly reduced in comparison to the naive C57BL/6 group, and immobility increased in mice with CIA, correlating with a previous study using DBA/1 mice. Grooming, although decreased in the DBA/1 model, was not significantly different in C57BL/6 mice with CIA. This could be due to the C57BL/6 disease being less severe than the DBA/1 (Inglis, Criado et al. 2007), resulting in less pronounced systemic effects. Anti-IL-17 therapy elicited a partial restoration of climbing function, as although significantly increased from untreated mice with CIA, it was still decreased when compared to naive controls.

As locomotion and immobility were both restored to comparable naive levels, it was concluded that minor front paw swelling could hamper climbing but not effect general movement.

The results of the previous chapter indicated that the expression of proinflammatory cytokines and some cytokine receptors increased in joints of DBA/1 mice with CIA. In this study, arthritic paws were again removed at day 10 post onset of arthritis for assessment of these cytokines and receptors by qPCR. Consistent with previous results, IL-17, TNF, IL-6 and IL-1 were all increased in joints of C57BL/6 mice with CIA. When treated with anti-IL-17, all elevated cytokines were decreased to naive levels, demonstrating that anti-IL-17 is potently anti-inflammatory in this model.

IL-17RA was again found to be upregulated as in Chapter 5, and was down regulated with anti-IL-17 treatment. IL-17RC expression was assessed due to its importance in IL-17 signalling, but contrastingly was reduced during CIA, and did not recover with anti-IL-17 therapy. IL-17 signals through IL-17RA and IL-17RC in a heterodimeric complex, but the differing expressions suggest that IL-17RA and RC also form complexes with other IL-17R homologs in the IL-17R family. Indeed, IL-17RA has recently been considered to also bind with IL-17RB (Gaffen 2009). IL-17E is thought to signal through the IL-17RA/RB (Gaffen 2009). IL-17E is associated with Th2 responses (Pan, French et al. 2001), and induces expression of IL-4 (Fort, Cheung et al. 2001). Furthermore, IL-17E has been implicated in regulation of Th17 function during autoimmunity (Kleinschek, Owyang et al. 2007). It is therefore speculative, but there is a potential paradoxical role for IL-17RA. In

short, by forming a complex with IL-17RC, IL-17 can enhance the inflammatory response through induction of other proinflammatory cytokines (Ho and Gaffen 2010; Ho, Shen et al. 2010; Hu, Ota et al. 2010), but by IL-17RA forming a complex with IL-17RB, IL-17E can generate an anti-inflammatory response, to regulate inflammation. With IL-17 and its homologs signalling so poorly understood, it is impossible to know whether the upregulation of IL-17RA enhances IL-17E signalling or whether reduced IL-17RC denotes regulation of inflammation. Further work is required to comprehend the role of these receptors in CIA.

TNFR1 message, like IL-17RC was significantly reduced during CIA and was similarly reduced compared to naive mice and arthritic mice with anti-IL-17 therapy, possibly to limit tissue damage as discussed in Chapter 5. TNFR2 expression significantly increased during CIA, again this was consistent with the DBA/1 profile. TNFR2 expression was down regulated with anti-IL-17 therapy, probably due to reduced cellular infiltration.

The production of proinflammatory cytokines in the DRG during arthritis has received limited attention. In this study, TNF and IL-1 message were upregulated during CIA, and then reduced after anti-IL-17 therapy. IL-6 message was present but unchanged in either the CIA or anti-IL-17 groups. IL-17 expression was undetermined in any group in the DRG. The DRG can produce proinflammatory cytokines from satellite cells and Schwann cells (Armati 2007; Takeda, Takahashi et al. 2009), but any infiltration of immune cells into the DRG during CIA is unknown. Macrophages express TNF and IL-1 , and infiltrate the DRG during CFA induced arthritis (Inglis, Nissim et al. 2005). IL-17RA and IL-17RC were both

upregulated during CIA. TNFR1 and TNFR2 also increased during CIA, and expression was reduced with anti-IL-17 therapy. As discussed in chapter 5, elevated message could be due to cell infiltration or point to a more direct action for these cytokines during CIA. To address any potential role for proinflammatory cytokines and their receptors in the PNS during arthritis, the cells expressing these cytokines must first be identified (e.g. by IHC).

Like the DBA/1 model, TNF and IL-1 were again upregulated in the lumbar spinal cord in C57BL/6 mice with CIA and IL-6 was unchanged, and IL-17 only expressed in arthritic mice. GFAP message was also elevated; indicating astrogliosis occurs in C57BL/6 CIA as well as DBA/1. IL-17RA and IL-17RC message were unchanged in all groups, but TNFR1 and TNFR2 were significantly increased, correlating with the DBA/1 model. Anti-IL-17 therapy reduced expression of all genes to naive levels (with the exception of IL-6, IL-17RA and IL-17RC which remained at naive levels throughout). This suggests that anti-IL-17 treatment can mediate central changes although whether this is a direct effect or indirect effect via reduction in peripheral inflammation needs to be investigated.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

7.1 Summary of thesis

This project has investigated the role of IL-17 in inflammation and hyperalgesia. Firstly, IL-17 induced hyperalgesia was examined by injection of the cytokine into the footpads of naive mice. Enhanced pain was measured by the Hargreaves thermal hyperalgesia test and Linton weight distribution change test. Neutrophil infiltration was assessed using histology with H+E staining and the MPO assay. The influence of anti-TNF treatment and TNF receptors on IL-17 induced hyperalgesia were explored in this model, along with the importance of neutrophil migration. Chronic hyperalgesia was investigated using the CIA model of arthritis. Firstly, the kinetics of key cytokines and their receptors were examined in the joints, DRG, and spinal cords of mice with CIA. This was repeated in the C57BL/6 model and compared with anti-IL-17 treated mice to understand the effects of IL-17 blockade during CIA. Hyperalgesia was monitored throughout the disease course using Hargreaves for thermal hyperalgesia and Von Frey filaments for mechanical allodynia. Spontaneous behaviour was measured using LABORAS. This study has shown that IL-17 induces acute hyperalgesia, but this is dependent on TNFR1 and neutrophil infiltration. Furthermore, blockade of IL-17 is anti-arthritic and analgesic in CIA.

7.2 General Discussion and Future Perspectives

IL-17 is a proinflammatory cytokine that has been implicated in a variety of autoimmune diseases, including RA. This thesis has focussed on the role of IL-17 in inflammation and hyperalgesia.

Hyperalgesia induced by proinflammatory cytokines is thought to be primarily governed by TNF. TNF induces a cascade of cytokines including IL-1, IL-8, IL-6 and KC, resulting in neutrophil migration and release of mediators (NGF, prostaglandins) which act on the peripheral neurons. Blockade of IL-1 and NGF prevents TNF induced hyperalgesia (Woolf, Allchorne et al. 1997; Cunha, Verri et al. 2005) , but IL-1 induced hyperalgesia is not attenuated in $TNFR1^{-/-}$ mice (Cunha, Verri et al. 2005). In addition, administration of soluble IL-1Ra does not significantly reduce NGF induced hyperalgesia (Woolf, Allchorne et al. 1997), leading to the hypothesis that nerve sensitisation is preceded by this sequence; TNF-IL-1-NGF (Cunha, Verri et al. 2005). Indeed, with carrageenan induced hyperalgesia, TNF is upregulated before IL-1 (Cunha, Verri et al. 2005).

As IL-17 induced hyperalgesia has been shown in this thesis to be dependent on TNF and TNFR1 (**Chapter 3**), it must be considered whether IL-17 has a place in the cascade hypothesis. **Chapter 4** hypothesised IL-17 induce TNF from resident cells to initiate hyperalgesia as it is known that IL-17 induces TNF by 3h post stimulation in macrophages. Therefore it is possible that IL-17 triggers the cascade through induction of TNF. To investigate this, CFA or carrageenan would be injected into the hind paw as described previously (Woolf, Allchorne et al. 1997;

Cunha, Verri et al. 2005) and levels of cytokines measured over a 24h period. If significant IL-17 levels precede TNF upregulation, then IL-17 could be the instigator of the inflammatory cascade.

Further study would investigate whether IL-17 blockade in TNF or IL-1 injected mice attenuated hyperalgesia. If anti-IL-17 does not prevent hyperalgesia by these cytokines, it would suggest that IL-17's role in inflammatory hyperalgesia is to induce TNF. However, IL-17 would have to induce TNF rapidly as TNF is present in a significant amount by 1h post carrageenan administration and 3h post CFA injection (Woolf, Allchorne et al. 1997; Cunha, Verri et al. 2005). Consequently, IL-17 could potentially have a purely additive role in maintaining inflammation rather than instigating it.

A previous study showed that IL-17 acts in synergy with TNF to enhance chemokine expression through mRNA stabilisation. In the investigation, IL-17 treatment was shown to elicit a post-transcription effect on TNF released KC to extend the half-life of the KC message (Hartupee, Liu et al. 2007). Thus, to further understand the role of IL-17 in inflammation and acute pain, additional experiments with a combination injection of TNF and IL-17 would ascertain if neutrophil migration was increased due to higher KC mRNA stability, potentially extending hyperalgesia in this model.

As acute inflammation does not represent the complexity of the arthritic joint the role of IL-17 was investigated in CIA. Results discussed in **Chapter 5** indicated

that proinflammatory cytokines including IL-17 are upregulated during CIA and their receptors are elevated in the DRG. Enhanced expression of TNF and IL-17 receptors could mean that the DRG is more sensitive to TNF and IL-17 during CIA. However, whether IL-17 could act more directly on neurones during CIA is unknown. TNF added to C fibres results in nerve discharge after 2 mins (Sorkin, Xiao et al. 1997), but the distribution of TNF receptors is not fully understood, thus the exact action of TNF here is unknown. To investigate the potential direct actions of IL-17 on C fibres, IL-17 could be added directly to C fibres and the action measured using whole cell voltage-clamp recordings. It would also be informative to determine the location of TNF and IL-17 receptors using IHC on sections from the DRG. The use of IHC would also determine whether peripheral immune cells infiltrate the DRG during CIA, and therefore increasing receptor levels by infiltration of cells, rather that increased expression on resident cells. An analysis of infiltrating cells would answer how TNF and IL-1 message levels increase during CIA and are reduced with anti-IL-17 therapy (**Chapter 6**). Although TNF and IL-1 are known to be produced in the DRG (Armati 2007; Takeda, Takahashi et al. 2009), if the increase was due to cell infiltration, it would point to an indirect mechanism for anti-IL-17 therapy, reducing inflammation in the periphery and thereby lowering the amount of immune cells migrating to the DRG. Both **Chapter 5 and 6** indicated a role for astrocytes in CIA with an increase in GFAP. To confirm this, further work would assess GFAP protein using immunohistochemistry as previously described (Inglis, Notley et al. 2007). As with the DRG, immunohistochemistry could establish if elevated cytokine production is *de novo*, or due to infiltration of immune cells.

How astrocytes are activated during chronic pain is still an unanswered question, but it appears that astrogliosis occurs mainly in the superficial laminae of the dorsal horn (Hald 2009). These outer layers is where peripheral sensory neurons, largely responsible for nociception project to, releasing neurotransmitters such as glutamate at their central terminal. Elevated release of glutamate leads to phosphorylation of NMDA receptor, allowing calcium influx into the neuron, enhancing their activity (Woolf and Costigan 1999; Jones 2003). An increase in phosphorylated NMDAR therefore indicates central sensitisation and can be investigated using Western blot techniques using spinal cord protein. Although CIA is now additionally described as a model of chronic pain, NMDAR expression has never been investigated in this model.

As the BBB only allows very small molecules to pass through the barrier, anti-IL-17 antibodies are unlikely to pass directly into the central nervous system. As GFAP and pro-inflammatory cytokines are reduced by anti-IL-17 therapy, it is therefore assumed that the lowering of inflammation in the periphery diminishes the sensitisation of the sensory neurons. This restores the nerve threshold, reducing the frequency of nerve firing, and therefore lowers glutamate release.

This hypothesis is supported by data presented in **Chapter 6** which shows that analgesia occurs after inflammation is attenuated with anti-IL-17 therapy. Indeed, the upregulation of pro-inflammatory cytokines was significantly reduced in mice treated with anti-IL-17 therapy, indicating a reduction in paw inflammation. To ensure that any therapeutic effects are due to IL-17 blockade, further investigations

must compare this treatment alongside the IgG isotope control for this drug and the vehicle in which it is administered.

Finally, as this thesis has demonstrated that IL-17 induced acute hyperalgesia is dependent on TNFR1, it must be considered whether this is true during chronic hyperalgesia. Previous studies has indicated that IL-17 acts independently of TNF during arthritic conditions (Koenders, Lubberts et al. 2006), but has not been investigated in CIA. To answer this, anti-IL-17 would be administered to $TNFR1^{-/-}$ mice with CIA. Reduction in clinical score, paw swelling and hyperalgesia in the $TNFR1^{-/-}$ mice with CIA would therefore mean IL-17 is driving inflammation independently of TNF in arthritic conditions.

In conclusion, this thesis has shown that IL-17 does have a role in inflammatory hyperalgesia but further work is required to dissect the mechanisms. During acute inflammation, IL-17 induced hyperalgesia is dependent on TNF mediated neutrophil migration, and IL-17 blockade is analgesic and anti-arthritic during chronic hyperalgesia.

CHAPTER 8

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APPENDIX

LIST OF PRESENTATIONS AND PUBLICATIONS

In press;

Notley, C. A., J. J. Inglis, et al. (2008). "Blockade of tumor necrosis factor in collagen-induced arthritis reveals a novel immunoregulatory pathway for Th1 and Th17 cells." J Exp Med **205**(11): 2491-7.

Inglis, J. J., **K. E. McNamee**, et al. (2008). "Regulation of pain sensitivity in experimental osteoarthritis by the endogenous peripheral opioid system." Arthritis Rheum **58**(10): 3110-9.

McNamee, K. E., A. Burleigh, et al. (2010). "Treatment of murine osteoarthritis withTrkAd5 reveals a pivotal role for nerve growth factor in non-inflammatory joint pain." Pain **149**(2): 386-92.

Inglis, J. J., Schutze, M.U., **McNamee, K.E** (2010). "What we have learned about Pain from Rodent Models of Arthritis?" Current Rheumatology Reviews **6**.

Under review/revision;

McNamee, K.E, Anand P, Hughes, J, Williams, R.O, and Inglis, J.J *IL-17 induces hyperalgesia via TNF-dependent neutrophil infiltration.* (Chapters 3 and 4 of this study)

Clark, J.M, Aleksiyadis, K, Martin, A, **McNamee, K.E**, Williams R.O, Mémet, S and Cope, A.P *Inhibitor of Kappa B Epsilon (IκBε) is a non-redundant regulator of c-Rel-dependent gene expression in murine T and B cells.*

Saba Alzabin, Sonya M Abraham, **Kay E McNamee**, Andrew Palfreeman, Dobrina Hull, Ejaz Pathan, Michael G Tovey, Christophe Lallemand, Erin Paterson, Anne Kinderlerer, Peter J Charles, Marc Feldmann, Peter C Taylor and Richard O Williams *TNF blockade increases Th17 cells in rheumatoid arthritis*

Anna K. Andersson, Percy F. Sumariwalla, Fiona E. McCann, Parisa Amjadi, Chiwen Chang, **Kay E McNamee**, Ditte Tornehave, Claus Haase, Henrik Agersø , Vibeke W Stennicke , Birgitte Ursø, John Trowsdale, Marc Feldmann and Fionula M. Brennan *Blockade of NKG2D ameliorates disease in collagen-induced arthritic mice.*

Key Presentations

Invited speaker at Th17-derived cytokines: New kids on the block of inflammation, Pasteur Institute, Paris **'The role of IL-17 in inflammation and hyperalgesia'** December 2008

Institute Seminar at the Kennedy Institute **'The role of IL-17 in inflammation and hyperalgesia'** June 2009

Guest seminar speaker at the London School of Medicine and Dentistry (Bone and Joint Research unit) **'The role of IL-17 in inflammation and hyperalgesia'** April 2010