Investigations into inflammation and nerve regeneration in a human model system of neuropathic pain.

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Abstract

Neuropathic pain is a serious and debilitating disease affecting a large proportion of the population. The disease is caused by direct damage to the nervous system and is often chronic in nature. Neuropathic pain is typically poorly treated, with patients often experiencing inadequate relief from symptoms. Part of this issue stems from ineffective treatment options while a lack of understanding of the disease pathology also presents as a major issue.

Recent findings have emerged which implicate the immune system in the initiation and generation of neuropathic pain. These studies have highlighted the role of inflammatory mediators including cytokines and immune cells as being heavily involved in the development of neuropathic pain. The majority of this work has been conducted in pre-clinical animal models where severe nerve injury paradigms are used. These experiments have proven useful in identifying the role of inflammation in neuropathic pain but do not necessarily reflect the nerve injury present in humans. Furthermore, preclinical findings do not always translate to patients in the clinic, which has resulted in a deficit in novel, effective neuropathic pain therapies. The mechanisms of nerve regeneration that occur after nerve injury are also incompletely understood. Similar to neuropathic pain, much work has been done using animal model systems of nerve regeneration, however human nerve regeneration presents with unique challenges and is often incomplete. Further studies are therefore required in humans to elucidate the precise role and action of inflammation in patients with neuropathic pain and to determine the mechanisms that are driving nerve regeneration after injury.

Carpal tunnel syndrome is an entrapment neuropathy commonly occurring in patients which presents as a unique model system with which to study neuropathic pain. Due to the unparalleled opportunity to collect tissues from these patients, both inflammation and nerve regeneration can be explored in the context of neuropathic pain. This thesis will firstly endeavour to describe and

characterise inflammatory associations with neuropathic pain in patients with carpal tunnel syndrome using both genetic and molecular approaches. Using these same approaches, I will also investigate nerve regeneration after injury to uncover mediators stimulating nerve growth and determine associations with inflammation in this process.

Acknowledgements

Declaration

I declare that the work contained in this thesis is my own and was conducted by me with the following exceptions shown below.

All recruitment of participants and the collection of biological samples and phenotypic data was performed by either Annina Schmid or Akira Wiberg

Chapter 2: The preparation of tenosynovial tissue for immunohistochemistry or genetic analysis was performed by Akira Wiberg. The RNA sequencing of the tenosynovial tissue was performed by Georgios Baskozos.

Chapter 3: The preparation of skin biopsies for histological analysis and for genetic analysis was performed by Annina Schmid. Georgios Baskozos performed the differential gene expression analysis of skin biopsy tissue. Cell cultures were prepared and maintained by either Greg Weir or Alex Clark. Supplementary Figure 1 was created by Georgios Baskozos.

Contents

Chapter 4: Molecular markers of nerve regeneration and associations with inflammation

Chapter 1: Introduction

Peripheral neuropathic pain is a persistent and debilitating condition, affecting between 7-10% of the global population.[1] The socioeconomic burden associated with neuropathic pain is comparable to major health conditions such as heart disease or cancer, which have been prioritized as public health concerns.[2] The current treatment options for neuropathic pain are largely inadequate with therapies providing insufficient relief of symptoms and unacceptable side effects (e.g., somnolence, weight gain).[3] The issue of ineffective treatments may partly stem from a lack of understanding of the pathophysiological causes of neuropathic pain, where studies have historically focused on the physiology of damaged neurons and neural regeneration.[4, 5] New insights however suggest a direct role for the immune system in the initiation and maintenance of neuropathic pain[6, 7] as well as the capacity for neural regeneration.[8] The majority of this work has been conducted in pre-clinical animal models which, though have provided useful insight, do not always translate well to humans.[9] There is therefore a critical need for further research to specifically investigate the role of inflammation in generating neuropathic pain in humans. This could help define the molecular and cellular components of neuropathic pain and provide increased understanding into the pathology of the disease, with the ultimate aim of providing improved treatment options to patients. To investigate this, I will endeavour to use patients with carpal tunnel syndrome as a human model system of neuropathic pain to pursue two main aims.

1. To investigate the role that inflammation has in neuropathic pain in humans

2. To characterise nerve regeneration after injury and determine the role of inflammation in this process.

In this introduction, I will firstly introduce entrapment neuropathy, specifically Carpal Tunnel Syndrome and explain its suitability as a human model system for neuropathic pain. Secondly, I will provide an overview of some of the main components of the immune system and explain the

current understanding of how the immune system is involved in the initiation and maintenance of neuropathic pain. Lastly, I will focus on nerve injury and regeneration and describe current findings and molecular mechanisms associated with nerve regeneration.

1.1 Carpal tunnel syndrome as a human model system for focal nerve injury and neuropathic pain

1.1.1 Neuropathic pain and entrapment neuropathy

In humans, entrapment neuropathies are amongst the most common conditions causing neuropathic pain. Entrapment neuropathy is a term describing neuropathies caused by the compression of peripheral nerves at specific anatomical locations within the body.[10] Carpal tunnel syndrome (CTS) affecting the median nerve at the wrist,[11] lumbar radiculopathy caused by compression of the lumbosacral nerve root and Morton's neuroma caused by compression of the plantar digital nerve in the foot[12, 13] are all forms of entrapment neuropathy occurring in humans.[14] Entrapment neuropathies have high incidence rates with CTS being the most common,[14] with a prevalence of 2.5-11% in the general population.[11, 15, 16] Entrapment neuropathies are generally found to have a higher occurrence in middle and old age individuals, with the male to female ratio differing depending on the specific condition.[17, 18, 19] Sensations of pain are a common symptom of entrapment neuropathy which can often radiate outside the innervation territory of the affected nerve/nerve root.[20, 21, 22] The pain can manifest in different ways such as burning and aching pain,[23, 24] and can be of high severity.[25, 26] Entrapment neuropathies can be treated by a number of conservative measures such as non-steroidal anti-inflammatory drugs (NSAIDs),[27] corticosteroid injections,[28-31] and night splinting in the case of CTS.[32] Surgical intervention is also an option however, the therapeutic benefit varies depending on the specific condition being treated. For CTS, surgery serves as the most effective treatment

for achieving complete symptom relief in most cases,[33] but for lumbar radiculopathy, the long term efficacy of surgery remains uncertain.[34] A high socioeconomic burden also is associated with entrapment neuropathy with an estimated cost exceeding 2 billion dollars annually in the US for CTS alone.[35]

1.1.2 Carpal tunnel syndrome as a model system

Although CTS presents as an interesting disease with unmet clinical needs,[36] the main aim of my thesis was not to identify new potential treatments for CTS. Instead, this study sought to use CTS as a human model system with which to investigate the pathomechanisms of neuropathic pain. CTS is well suited to this role for several reasons:

1. CTS is the most common entrapment neuropathy[14] which allows for a relatively high level of patient recruitment, and as neuropathic pain is a common symptom of CTS,[37] it is a good example of the condition in humans.

2. As CTS is a condition affecting the distal extremity, it allows for easy access to take electrophysiological recordings of nerve function and sensory deficits,[38] which is more challenging in more proximal entrapment neuropathies such as radiculopathy. Simultaneously, questionnaires such as the Boston carpal tunnel syndrome questionnaire[39] and the neuropathic pain symptom inventory (NPSI) questionnaire[40] can be used to determine the severity and quality of symptoms. This allows for detailed clinical phenotyping of the patients.

3. A unique feature of CTS as a model system is that surgery is regularly peformed.[11] This provides unparalleled access to human tissue which is not readily available in most neuropathic pain conditions. Access to such tissue allows for detailed analysis of human samples in the context of neuropathic pain.

4. The surgery also provides an exact timepoint at which recovery is initiated with high success

rates in most patients.[33, 36] Such a time-locked recovery initiating event is not available in most

other neuropathic pain conditions, where there is limited effective treatment available or

treatment is provided for a long period of time (e.g., pharmacology).[41, 42] Surgery as a distinct

recovery-inducing event in CTS enables the comparison of phenotypic and biological features in

the active state of the disease, to that of recovery. As such, CTS is a unique model system that

enables unparalleled insights into the pathophysiology of human neuropathic pain.

1.1.3 Pathophysiology of CTS
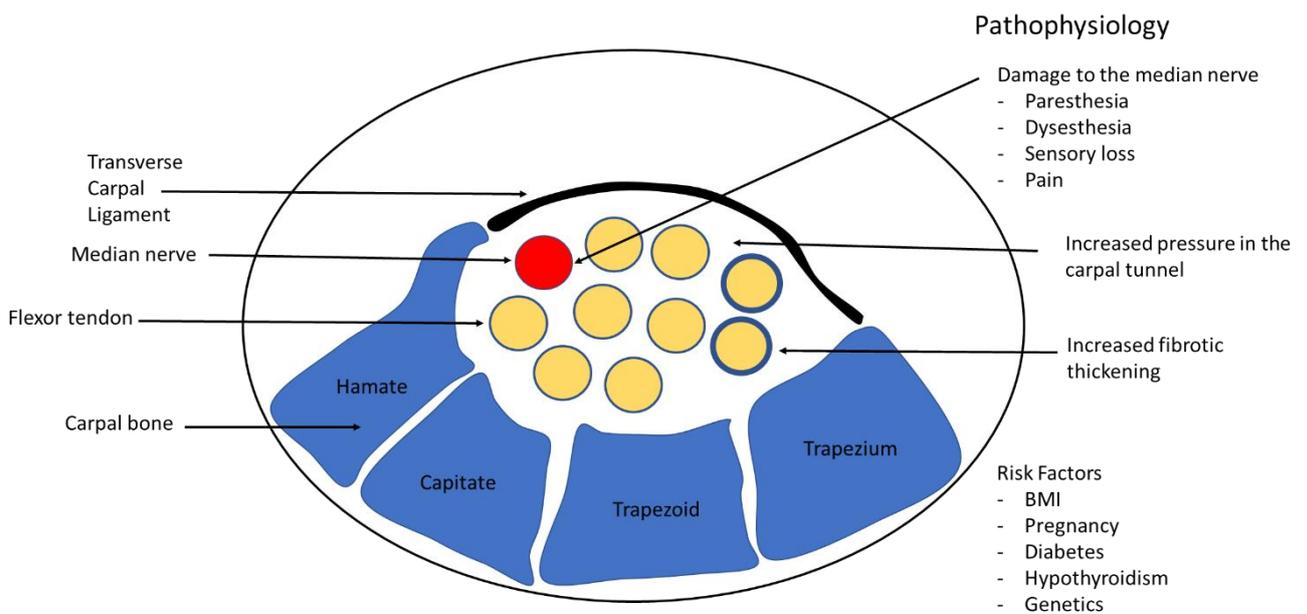
Figure 1.1: Structure of the carpal tunnel and pathophysiology of CTS
The carpal tunnel is formed of four carpal bones (Hamate, Capitate, Trapezoid, Trapezium) and the transverse carpal ligament, which contains nine flexor tendons and the median nerve. Pathophysiological mechanisms occur such as increased pressure in the carpal tunnel and fibrotic thickening that damage the median nerve. Damage to the median nerve can cause symptoms such as paraesthesia, dysesthesia, sensory loss and pain. Risk factors such as increased body mass index, pregnancy, diabetes, hypothyroidism and genetic elements can increase the risk of developing CTS.
CTS= Carpal tunnel syndrome, BMI = Body mass index.

In CTS, the median nerve is compressed within the carpal tunnel.[43] The carpal tunnel itself is an osteofibrous canal formed of the carpal bones and contains 9 flexor tendons along with the median nerve[44] which is enclosed by the transverse carpal ligament (Figure 1.1). CTS is characterised by symptoms such as paraesthesia (tingling or prickling sensation), dyaesthesia (abnormal unpleasant sensation when touched), pain, as well as signs including sensory loss, weakness and muscle atrophy.[45] Symptoms are reported to occur at night as well as in the daytime, with night time symptoms commonly reported as being more severe.[46] The current gold standard for diagnosing CTS is clinical assessment where patient history is collected and a physical examination of the hand is conducted.[32] Two of the most common clinical assessments are the Phalen's test and the Tinel's test, both of which involve evoking symptoms by either maintaining the wrist in flexion or by percussion/tapping over the carpal tunnel respectively.[47] In addition to these, electrodiagnostic testing (EDT) which includes electromyography (EMG) and nerve conduction velocity (NCV) can be done to confirm the diagnosis of CTS.[48]

Increased pressure

The causes of CTS are not well defined with most cases of CTS being described as idiopathic.[32] However the majority of the pathophysiological mechanisms proposed for CTS involve increased pressure within the carpal tunnel which causes damage to structures such as the median nerve that pass through it. Sunderland[49] describes a pressure gradient for different components of the carpal tunnel, which provides functional tunnel homeostasis:

Pa(artery)>Pc(capiliary)>Pf(intrafunicular)>Pv(veins)>Pt(carpal tunnel)

If there is increased pressure in a particular component from an external force, this can result in a reversal of the pressure gradient and induce damage in neighboring components. A related theory for the pathogenesis of CTS involves the presence of a ischemic reperfusion injury,[50] where

repetitive movement, including flexion and extension of the wrist increases the internal pressure within the carpal tunnel.[51, 52] Increased internal pressure is then thought to cause stasis of venous blood flow and eventual endothelial ischemia in the capillaries. This causes increased permeability of the capillaries and allows extravasation of fluid into the carpal tunnel, resulting in oedema. This further increases the pressure in the carpal tunnel which ultimately results in decreased axonal transport and damage, decreased blood flow and fibrotic changes including scar formation, all contributing to the disease.[50] Although this theory cannot be tested directly in human nerves, it is substantiated by findings of oxidative stress in extra neural tissue such as sub-synovial connective tissue from the carpal tunnel of patients with CTS.[53] Nitric oxide synthase (eNOS), nuclear factor (NF)-Ƙβ and transforming growth factor (TGF)-β were found to be increased in patients with CTS compared to controls. The expression of these mediators was also positively correlated with increased symptom severity, suggesting oxidative stress may be related to patient symptoms.[53] Another study investigating the structure and composition of sub-synovial connective tissue identified vascular changes and characteristics indicating hypoxia and degeneration.[54]

Fibrotic changes

Fibrotic thickening of tissue within the carpal tunnel is thought to be another hallmark of CTS and a major contributor in the pathogenesis of the condition.[54-57] Many studies investigating this conducted histological analysis of tenosynovial/sub-synovial connective tissue. Ettema et al, 2004[58] identified altered collagen subtype expression as being involved in the pathogenesis as there was an increased expression of collagen type III fibers present in patients with CTS compared to controls. Oh et al, 2006[59] further found that collagen fibril structure in synovial samples from patients with CTS had a deformed structure with an increased fibril diameter. These studies make a clear distinction that the fibrotic changes identified are non-inflammatory and do

not involve infiltration of immune cells into the tissue. They therefore state that idiopathic CTS is a non-inflammatory condition. However due to basic staining techniques and the manual assessment of staining images, which is subjective and prone to bias, these findings may not be accurate. I explore this in greater detail in Chapter 3. In further support of fibrotic changes, a recent genome wide association study conducted in patients with CTS, found gene variants associated with extracellular matrix architecture to be involved in predisposing individuals to develop CTS.[60] Ultimately it remains unknown whether fibrotic changes occur first and increase the internal pressure causing ischemia or whether ischemic reperfusion injury precludes these events, leading to tissue damage and resulting in altered collagen expression.

Median nerve changes

Regardless of the mechanisms by which pressure or damage occurs in the carpal tunnel, it is well established that the median nerve in patients with CTS is affected. If compression to the nerve is prolonged it can cause demyelination[61] and axonal degeneration in severe cases.[62] Demyelination can cause nerve conduction slowing or nerve conduction block, which can explain the typical loss of sensation and muscle weakness.[63] Large myelinated nerve fibers are thought to be primarily affected after nerve compression[64] and large myelinated fiber dysfunction has been detected in patients with CTS.[65-67] There are however new findings suggesting that small nerve fibers are affected before the onset of large fiber deficits in patients with CTS.[65] Small nerve fiber density determined by intraepidermal nerve fiber counts were found to be significantly decreased in patients with CTS compared to healthy controls. These nerve fiber reductions were not associated with electrodiagnostic test severity. Meisner corpuscle density, which is a marker of myelinated nerve innervation was not found to be different between patients and controls.[65] These findings indicate that small fibers may become damaged and degenerate in CTS before large fibers are affected. These findings build on previous work identifying small nerve fiber dysfunction in CTS,[68]

where one study found that medium diameter myelinated Aδ-fiber damage was significantly correlated with Boston carpal tunnel questionnaire scores and daytime pain in patients with CTS.[69]

1.1.4 Neuropathic pain and inflammation in CTS

CTS has previously been used to explore clinical characteristics of neuropathic pain in humans, with one study finding that patients meeting the neuropathic pain criteria had increased pain scores to those patient whose pain was non-neuropathic.[70] The study also found that there was significantly increased night pain in patients with neuropathic pain.[70] A more recent study by the same group seeking to determine risk factors for developing neuropathic pain, found that a younger age and weaker pain symptoms before surgery were potential risk factors for worse neuropathic pain after surgery.[71] However in both of these studies the phenotyping of the patients was limited and no biological samples were taken. No comparisons could therefore be made between neuropathic pain and biological differences between patients. There is therefore a need for further research to be conducted where deep phenotyping is conducted in patients along with the collection of biological samples to enable characterization of the biological contributions to neuropathic pain.

There is a plethora of pre-clinical research in animal models confirming a role of inflammation in neuropathic pain which can be induced by severe nerve injury models such as nerve transection and chronic constriction injury.[7, 72, 73] This literature is discussed in greater detail below. Such extensive and acute nerve injuries do however not reflect the mild and chronic nature of entrapment neuropathies. Recent work has shown that even mild chronic nerve compression is sufficient to induce intraneural inflammation.[74] Here, neuroinflammation and infiltration of immune cells into the affected nerve area and in the dorsal root ganglion (DRG) was observed after mild nerve compression, which was accompanied by hyperalgesia to blunt pressure and cold

allodynia. This indicated a role for inflammation in the generation of neuropathic pain after mild nerve compression.[74] However, further work is required to determine whether these mechanisms also occur in humans and whether the injury sustained from CTS is sufficient to cause immune cell activation and migration. Some initial work in humans using CTS to investigate inflammation in neuropathic pain found signs of immune dysregulation between patients with CTS and healthy controls.[75] Patients with CTS were found to have increased expression of the cytokines CCL5, CXCL8, CXCL10 and VEGF as well as increased numbers of central memory and effector memory CD4+ T cells in the peripheral blood.[75] However this study used a cross sectional design and so measurements over time were not possible. CTS has also been used as a human model system to investigate nerve regeneration in humans,[76] where after decompression surgery, several neurophysiological parameters and quantitative sensory testing measurements were significantly improved in patients. Partial recovery in intraepidermal nerve fiber density (IENFD) in skin biopsies was also observed, with the extent of recovery correlating with symptom improvement. Another study investigating nerve morphology in skin biopsies from patients with CTS, found Meissner corpuscles to be significantly smaller in patient skin before surgery compared to controls.[77] Myelinated fibers also had a significantly reduced caliber and IENFD counts were significantly reduced.[77] After surgery Meissner corpuscle size significantly increased to similar levels found in controls patients. Myelinated fiber caliber significantly increased in skin samples after surgery compared to before surgery levels. However, IENFD counts remained significantly decreased compared to controls, indicating that nerve regeneration can remain incomplete, even after effective therapy.

Overall little is known of the role of inflammation in the pathogenesis of CTS and how it may contribute to neuropathic pain in these patients, with the majority of studies using basic histology experiments. Further work is therefore required in patients with CTS to unveil inflammatory mechanisms, which could have important implications not only in CTS but for other neuropathic pain conditions in humans.

1.2. Components of the Immune System

1.2.1 Immune cell types and lineages

In adult humans, cells of the immune system are generated by haemopoietic stem cells (HSCs) located within the bone marrow by the process of hematopoiesis[78] (Figure 1.2). HSCs are self-renewing, pluripotent stem cells that are able to produce any cell type of the hematopoietic system.[79] Within the bone marrow HSCs grow and mature on a meshwork of stromal cells, which support the growth and differentiation of HSCs and include fat cells, endothelial cells and mesenchymal stem cells.[80] These non-hematopoietic cells produce a range of factors to create the hematopoietic stem cell niche which promotes the growth and differentiation of HSCs. Two main components of the niche are CXCL12[81] and stem cell factor (SCF)[82] which are involved in the maintenance of HSCs. Hematopoietic cells can also contribute to the HSC niche including regulatory T cells (Tregs), macrophages and megakaryocytes in a feedback mechanism.[83-85] Depending on the specific signals provided by stromal cells, HSCs differentiate into one of two types of progenitor cell that give rise to different cell lineages. Progenitor cells have lost the ability for self-renewal and are committed to producing cells of a certain lineage.[86] IL-7 signaling gives rise to common lymphoid progenitor cells which produce cells of the lymphoid lineage[87] while SCF, thrombopoietin (TPO) and IL-3 signaling produces common myeloid progenitors.[88]

Myeloid progenitors

Common myeloid progenitors further bifurcate into granulocyte-macrophage progenitors (GMPs) through PU.1 expression[89] or, megakaryocyte-erythrocyte progenitors (MEPs) through the expression of GATA-1.[90] GMPs give rise to cells including basophils, eosinophils and neutrophils via the signaling of C/EBPα.[91] Monocytes are derived from GMPs by signaling from monocyte colony stimulating factor (M-CSF) which then give rise to macrophages.[92] Macrophages form an important component of the innate immune system and can be further differentiated into two main types. M1 macrophages (classically activated) and M2 (alternatively activated)

macrophages.[93] MEPs differentiate into megakaryocytes and erythrocytes through TPO and erythropoietin (EPO) signaling respectively.[94] Megakaryocytes produce platelets which are involved in blood clotting processes.[95] Erythrocytes are the main red blood cell responsible for transporting oxygen around the body.[96]

Lymphoid progenitors

Common lymphoid progenitor cells (CLPs) give rise to a more limited range of cell types, including natural killer (NK) cells which are differentiated from common lymphoid progenitors via fms-like tyrosine kinase 3 ligand (FL)[97] and IL-15[98] signaling, producing a cell that has innate cytotoxic activity. CLPs also produce cells that form the adaptive immunity arm of the immune system including T cells and B cells. B lymphocytes or B cells are produced and mature in the bone marrow, where the expression of E2A,[99] EBF[100] and the signaling of PAX5/BSAP,[101] differentiates these cells from CLP precursors. B cells provide an essential role in humoral immunity via the production of antibodies. When B cells become activated they divide at a rapid rate and differentiate into either memory B cells which retain the membrane bound B-cell receptor, or they differentiate into plasma cells, which have short life spans and produce a secreted form of the B-cell receptor (antibodies) in large quantities.[102]

T cells comprise the other cell type in adaptive immunity and originate from CLP cells in the bone marrow. However unlike B cells which mature in the bone marrow, CLP cells migrate to the thymus to become T-cell progenitor cells and begin T-cell differentiation, a process which is dependent on Notch1[103, 104] and IL-7 signalling.[105] T-cell progenitors first arriving in the thymus lack characteristic features of the T cell such the T-cell receptor (TCR), CD3 as well as CD4 or CD8 receptors and are termed double negative. These cells then undergo thymic selection where progenitor cells which start expressing both CD4 and CD8, termed double positive, undergo both positive and negative selection. This removes cells that do not recognise self-antigens or which bind too strongly to self-antigens, leaving cells which are self-tolerant and MHC restricted. These

cells then become either single positive CD4+ or CD8+ mature T cells, for a detailed review on T-cell maturation see Klein, et al.[106] CD8+ T cells are known as cytotoxic T cells and recognise antigens bound to MHC class 1 receptors which are expressed on all cells within the body. CD4+ T cells are termed helper T cells and recognise antigen bound on MHC class 2 receptors, stimulating them to produce a range of specific cytokines which are involved in the activation of other immune cells and help to generate an inflammatory response.[107] Helper T cells (Th) can be further differentiated into specific subpopulations including Th1, Th2, Th17 and Treg cells.[108]
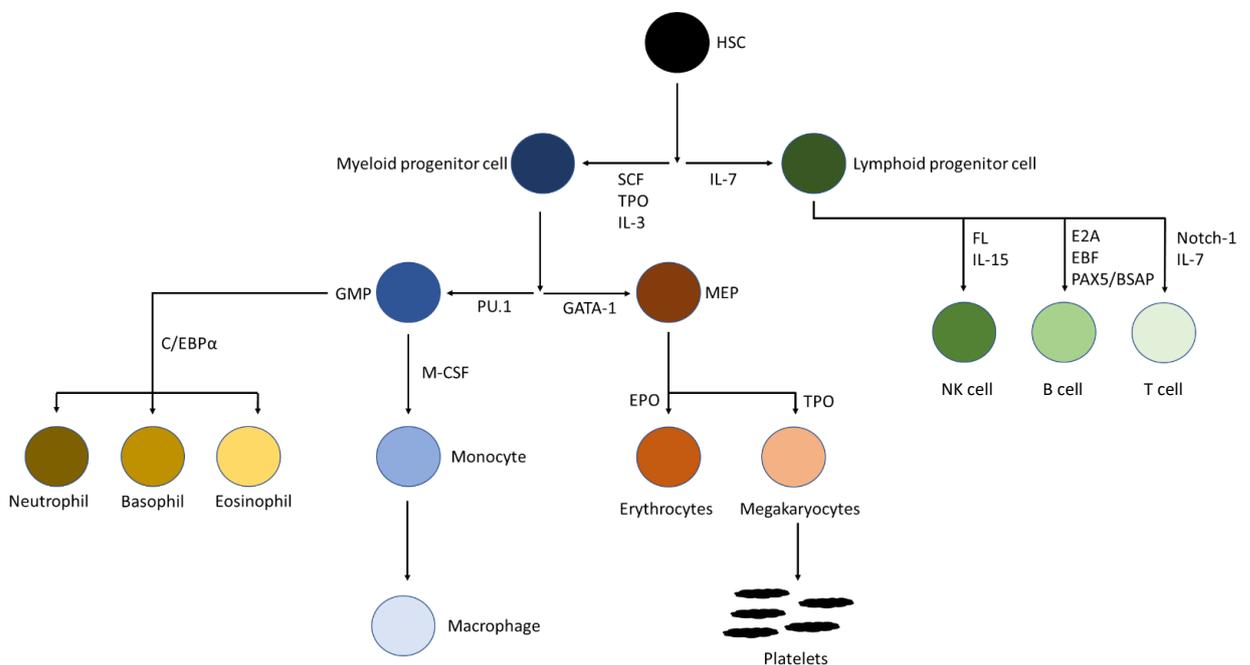
Figure 1.2: Hematopoiesis of Immune cell types
The main components of hematopoiesis are shown for immune cell types. Hematopoiesis starts with the HSC which differentiates into lymphoid progenitor cells, giving give rise to cells such as T cells, B cells and NK cells, or myeloid progenitor cells which produce cells such as macrophages, neutrophils and erythrocytes.
HSC = Hematopoietic stem cell, GMP = Granulocyte-macrophage progenitors, MEP = Megakaryocyte-erythrocyte progenitors.

1.2.2 Cytokines

Cytokines are a highly diverse group of small secreted signalling molecules which primarily affect the function, activity, growth and differentiation of immune cells.[109] Cytokines thereby regulate the immune response by affecting these activities and can function on the same cells that produce them (autocrine), on neighbouring cells (paracrine) as well as cells that are distant from the cytokine producing cell (endocrine).[110] Cytokines generally display 4 distinct characteristics including redundancy,[111, 112] pleiotropy,[111, 112] synergism[113] and antagonism.[110] A cytokine is said to be redundant when at least two cytokines have the same affect or carry out the same function, this can make it difficult in ascribing a specific function to any one cytokine. Pleiotropic cytokines are able to produce different biological affects when signalling on different target cells or in particular inflammatory environments. Synergism occurs when the effect of two cytokines on a particular target cell is greater than the additive effect of either cytokine. Finally, antagonistic actions of cytokines occur when the activity of one cytokine impedes or hinders the function of another cytokine.

Cytokine superfamilies

Within the broad term of cytokines there are several superfamilies of related molecules including interleukins, chemokines, colony stimulating factors, interferons, transforming growth factors and tumour necrosis factor families[114] (Figure 1.3). Members of each superfamily share sequence homology and structural similarities but can have highly diverse functions, with prominent members of each family including, IL-1, IL-6, IL-10 (Interleukins), CXCL5, CCL5, CXCL10 (Chemokines), GM-CSF, M-CSF, G-CSF (Colony Stimulating Factors), IFN-α-γ (Interferons), TGF-β1-3 (Transforming Growth Factors) and TNF-α-γ (Tumor Necrosis Factors).[114]

Cytokine Superfamilies

	Interleukins		Chemokines		Interferons	Colony Stimulating Factors	Transforming Growth Factors	Tumor Necrosis Factors
Examples cytokines	IL-1 IL-6 IL-10		CXCL5 CCL5 CXCL10		IFN-α IFN-β IFN-γ	GM-CSF G-CSF M-CSF	TGF-β1 TGF-β2 TGF-β3	TNF-α TNF-β TNF-γ
Cytokines included in this study	IL-1β IL-1RA IL- 2 IL-4 IL-5 IL-6 IL-7 IL-8 IL-9 IL-10	IL-12 IL-13 IL- 17 IL-18 IL-22 IL-23	CCL2 CCL4 CCL5 CCL11 CCL21 CXCL5 CXCL8 CXCL9 CXCL10 CXCL11	CX3CL1	IFN-γ	G-CSF GM-CSF	TGF-β1	TNF-α

Figure 1.3: Cytokines superfamilies
Cytokines can be grouped into 6 main superfamilies containing many cytokine members. The example cytokines listed above for each superfamily include examples of prominent members and is not a comprehensive list of the members of each family. A list of some of the cytokines that were analyzed as part of investigations in Chapter 2 have also been included.

Cytokine receptor superfamilies

Cytokines exert their functions via the binding of specific receptors, which themselves can be categorised into distinct classes[115] (Figure 1.4). The immunoglobulin superfamily of receptors bind cytokines such as members of the IL-1 superfamily[116] and M-CSF.[117] Another class of cytokine receptors is the type 1 cytokine receptors, which bind many of the interleukins such as IL-2, IL-4 and IL-6.[118] Class 2 cytokine receptors are related to class 1 and bind many members of the interferon family such as IFN-γ as well as other molecules such as IL-10.[119] Both class 1 and class 2 cytokine receptors require JAK kinase activity for their function as they lack intrinsic kinase activity.[120] The JAK kinase activity initiates downstream STAT transcription factors, causing changes in genetic expression of the cell.[121] Class 1 and class 2 cytokine receptors are similar in that they both share four conserved cysteine residues in their N-terminal, however class 1

receptors have a conserved tryptophan-serine-X-tryptophan-serine domain in the C-terminus[115] which type 2 cytokine receptors lack. The TNF receptor family bind members of the TNF family such as TNF-α as well as growth factors including nerve growth factor (NGF) and FAS.[122] Finally the last group of receptors are the chemokine receptors which are G protein coupled receptors (GPCRs) and bind members of the chemokine family such as CCL5, CXCL8 and CXCL10.[123]
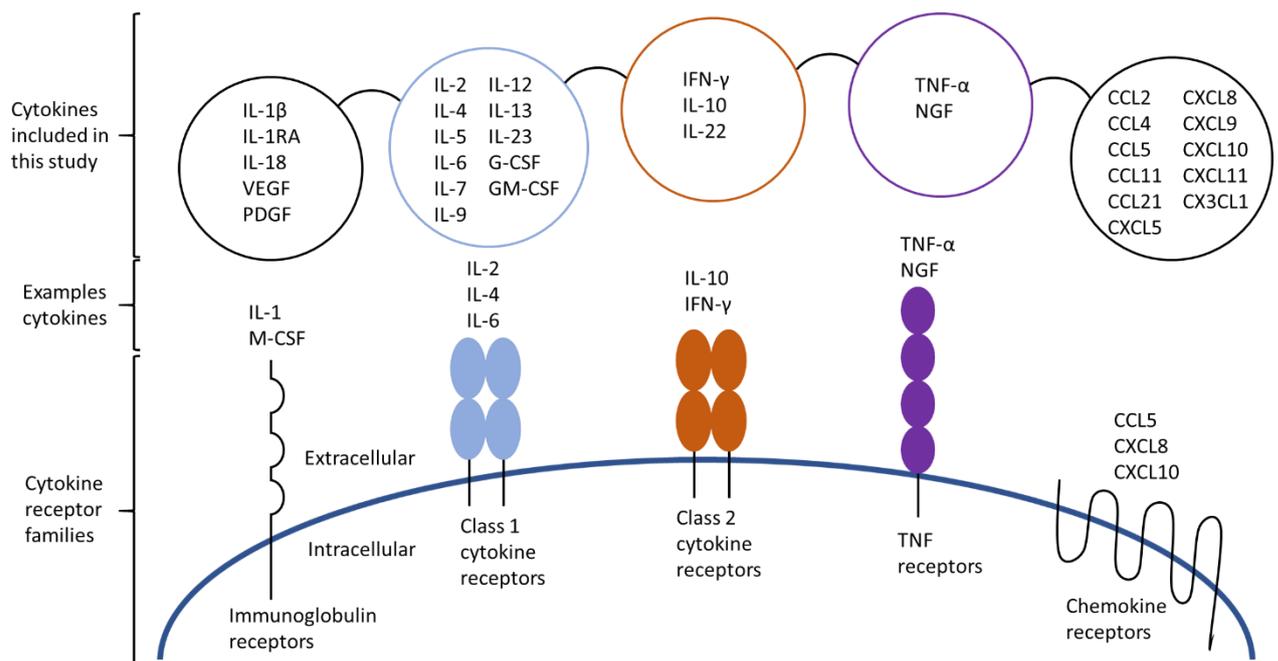
Figure 1.4: Cytokines receptor superfamilies
Cytokine receptors can be grouped into five main receptor families. Each family binds certain cytokine members with example cytokines being shown above. Some of the cytokines involved in the analysis in Chapter 2 are also included, indicating which cytokine receptor family they bind to.

Pro and anti-inflammatory effects of cytokines

Cytokines can be broadly grouped into those with either pro-inflammatory or anti-inflammatory actions.[110] However several cytokines can display pleotropic activity depending on the environment such as IL-4 and IL-13, which display anti-inflammatory actions[124] but can also

contribute to the pathogenesis of allergic asthma.[125, 126] The function of the cytokines in either of these groups is vital to the overall health of an organism as pro-inflammatory cytokines are vital in raising a sufficient immune response against invading pathogens and stopping infection.[127, 128] Anti-inflammatory cytokines are also important as they limit the inflammatory response and ensure that no excessive damage is done to host tissues and organs.[129, 130] The precise interplay between pro and anti-inflammatory cytokines is therefore a complex, balanced process that is essential to the survival of organisms. The role of cytokines has previously been shown in the generation of pain where IL-1β and TNF-α have been found to cause thermal hyperalgesia and mechanical allodynia.[131, 132] IL-6 is another cytokine implicated in the generation of pain.[133] Other cytokines have been found to prevent pain generation and have analgesic effects such as IL-4, where its knockdown in mice causes tactile allodynia.[134] Furthermore the administration of IL-4 after nerve injury has been shown to reduce neuropathic pain symptoms.[135] IL-10, one of the main anti-inflammatory cytokines has also been shown to have antinociceptive effects as IL-10 gene therapy was able to prevent and progressively reverse neuropathic pain cause by chemotherapeutic drugs in rats.[136] IL-10 administration was also found to reduce the writhing response after the initiation of neuropathic pain, potentially through the control of pro-inflammatory cytokines.[137]

1.2.3 Roles of immune cells in inflammation

The innate immune system (Figure 1.5) contains cells from both the myeloid and lymphoid lineages. These cells characteristically do not have somatically recombined antigen-receptors or retain conventional immunological memory.[138] Instead these cells recognise immunological challenge through specialised receptors known as pattern recognition receptors (PRRs). PRRs are germline encoded receptors that are able to recognise molecules associated with pathogens or pathogen associated molecular patterns (PAMPs) as well as molecules produced by damaged or

dying cells, known as damage associated molecular patterns (DAMPs).[138, 139] PRRs are formed of 4

main families, the Toll-like receptors (TLRs), the nucleotide-binding oligomerization domain

leucin-rich repeat containing receptors (NLRs), the retinoic acid-inducible gene 1 like receptors

(RLRs) and the C-type lectin receptors (CLRs).[140] Signalling through these receptors can act as one

of the first lines of defence in eliminating or containing invading pathogens and is one of the

initial features in the immune response.[141]
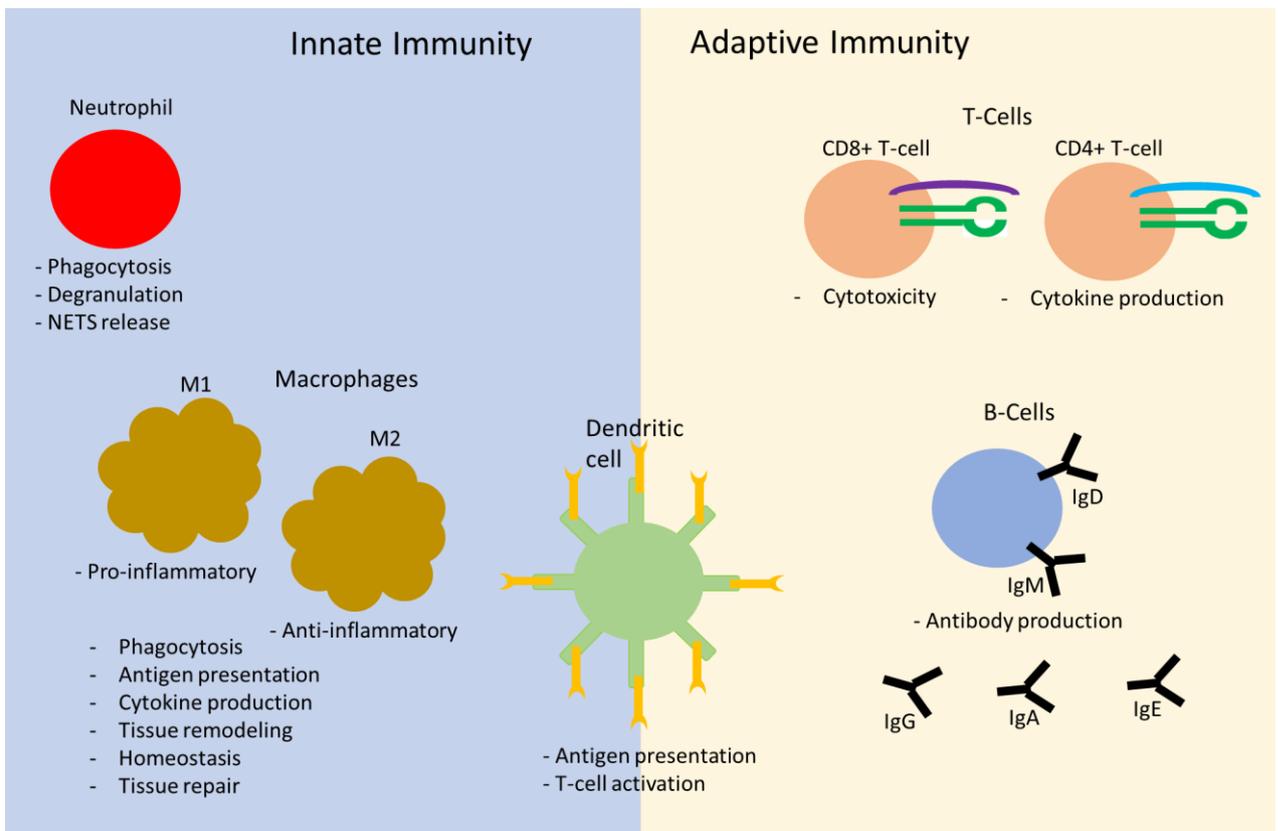
Figure 1.5: Function of immune cell types
The immune system can be broadly separated into the innate and the adaptive immune system. The innate immune system includes cell types such as neutrophils and macrophages. Neutrophils have actions including phagocytosis, degranulation and neutrophil extracellular traps (NETS) release while macrophages have roles in antigen presentation, cytokine production and phagocytosis. Cells of the adaptive immune system include T and B cells. T cells can be subdivided into two main groups including CD4+ T cells and CD8+ T cells which are termed helper and cytotoxic T cells respectively. B cells are involved in humeral immunity and antibody production. B cells can produce different types of antibodies through a process called class switching. Dendritic cells engulf and present antigen fragments to T cells causing their activation, and form a link between the innate and adaptive immune systems.

The Complement system

The complement system is an important part of innate immunity and plays a role in inflammation, which I will briefly outline. The complement system is a complex network of plasma and membrane-associated serum proteins that can elicit inflammatory and cytolytic responses to infectious organisms.[142] The complement system can be activated by three main pathways: the classical pathway, the lectin pathway and the alternative pathway. These are described in detail elsewhere.[142] the main function of the complement system is to defend against microbial attack,[143] however the complement system has also been implicated in several inflammatory diseases including systemic lupus erythematosus,[144] rheumatoid arthritis[145, 146] and allergic asthma.[147]

Neutrophils

Neutrophils are one of the most abundant cells of the immune system with around 10^{11} cells being produced per day[148] and are the first responders to the invasion of pathogens.[149] These innate immune cells circulate through the body surveying for invading pathogens, which once found are quickly trapped by these cells and eliminated. There are 3 main ways in which neutrophils can exert their biological function including phagocytosis, degranulation and the release of nuclear material in the form of neutrophil extracellular traps (NETS).[149] NETs produced by neutrophils are large extracellular web like structures composed of cytosolic and granule proteins and large amounts of DNA as condensed chromatin, originating from both the nucleus and mitochondria.[150, 151] These structures are then able to destroy a range of pathogens including bacteria,[150] viruses[152] and fungi.[153] Neutrophils can also release a number of cytokines which defend against pathogens and signal to other cells of the immune system to activate and translocate to the affected area.[154] These chemokines include CXCL8,[155] CXCL10[156] and CCL2,[157] which can all work to attract other immune cells and amplify the immune response.

Macrophages

Macrophages are one of the main phagocytic cells of the immune system involved in the inflammatory response,[158, 159] they are also involved in a large number of other physiological process such as the homeostasis,[160] tissue repair[161, 162] and resolution of inflammation.[163] Macrophages can be considered to adopt either an M1 (classically activated) or M2 (alternatively activated) phenotype with M1 macrophages typically being pro-inflammatory while M2 macrophages are thought to assume an anti-inflammatory profile and are involved in tissue repair.[164] M1 macrophages are produced via the signalling of IFN-γ[165] while M2 macrophages are produce via the result of IL-4 and IL-13 signalling.[166, 167] However simply assigning macrophages into two discrete groups is an oversimplification of the spectrum of macrophage phenotypes.[168] In the inflammatory response macrophages have several important roles. Firstly, macrophages phagocytose invading pathogens and cellular debris, which is initiated by binding of PRRs[169] and scavenger receptors[170] to certain PAMPs, forming a bridge between the macrophage and the pathogen. Membrane protrusions then surround the pathogen and absorb it into the phagosome, which is formed by the fusion of the cell membranes.[171] Once the pathogens have been absorbed they are exposed to both oxygen dependant and independent attacks where molecules such as myeloperoxidase, various hydrolases and lysosomes such as azurophilic granules fuse to the phagosome and initiate degradation of the contents.[172-174] This is aided by an ATPase pump which lowers the pH of the phagolysosome.[173] Macrophages also present antigens which cause activation of cells such as CD4+ T cells and aid in generating and amplifying the inflammatory response.[175] Lastly, when macrophages are activated they are able to produce a large range of cytokines, which is important in activating and recruiting other immune cells to the site of damage and in generating the inflammatory response.[176] A range of chemokines such as CXCL5,

CCL5 and CXCL8-10 are also produced, which are important in the chemotactic movement of immune cells to the site of inflammation.[177-179]

Dendritic cells

Dendritic cells (DCs) are the professional antigen presenting cells of the immune system and display antigens on their cell surface to activate T cells. DCs circulate through the blood to reach a wide variety of tissues where they stay to survey the local environment for invading pathogens, which they detect through PRRs.[180] Once stimulated, DC intake antigen by phagocytosis[181] and endocytosis[182] and migrate to the lymph node to signal to T cells.[180] Internalised antigens are processed so that they can be mounted on MHC receptors and efficiently signal to T cells. Peptides loaded onto MHC 1 receptors are recognised by CD8+ cytotoxic T cells whereas peptide loaded onto MHC 2 receptors are recognised by CD4+ helper T cells.[183] As MHC 2 receptors are only expressed on specialised immune cells,[184] activation of CD4+ T cells is a characteristic feature of dendritic cells with certain types having a superior ability to process and display antigen on MHC 2 receptors.[185] As such dendritic cells have a unique capacity to differentiate Th cells down distinct pathways to effector profiles including Th1,[186] Th2,[187] Th17[188] and Tregs[189] by the production of specific signalling molecules and cytokines that promote certain T-cell subpopulations.[190] This antigen presentation provides a unique way in which the innate immune system can interact and activate the adaptive immune system.

B cells

Cells of the adaptive immune system (Figure 1.5) such as B and T cells are characterised by the ability to produce antigen dependant and antigen specific immune responses, as well as their capacity for immunological memory.[191] After maturation in the bone marrow, naïve B cells circulate to secondary lymphoid tissues where they can become activated by binding of antigen

with their membrane bound B-cell receptor (BCR).[192] After activation B cells can change the isotype of their BCR through a process called isotype switching. Naïve B cells originally express IgM and or IgD type antibodies but can switch to different classes including IgG, IgA and IgE antibodies.[193] When an antigen is coated with antibody it can be eliminated in several ways,[194] firstly the binding of antibodies can neutralize the pathogen by blocking sites vital for its pathogenic activity such as ligands that interact with cells of the host.[195] Activation of complement is another way in which antibodies can eliminate pathogens[196] as well as antibody mediated destruction through phagocytosis.[197] As B cells were not the main focus of my thesis, I have not described them in detail here.

T cells

T cells have multiple actions in inflammation and the immune response. Naïve CD8+ and CD4+ T cells migrate to secondary lymphoid organs such as the lymph nodes where they await antigen stimulation from antigen presenting cells.[198] T cells can only be stimulated by antigen presented on MHC receptors, with the combination of the peptide and the MHC receptor being required to produce an immune response.[199] Naïve CD8+ T cells recognise antigens and are stimulated to become effector cytotoxic T cells that can destroy virus or bacteria infected cells and tumour cells.[200-204] Stimulation of co-receptors and the action of specific cytokines such as IL-12 and type 1 interferons[205] are also required for effective activation of cytotoxic T cells. Effector cytotoxic T cells destroy target cells via two main mechanisms, exocytosis of lytic proteins such as granzyme B and perforin or receptor ligand binding of Fas/APO molecules.[206, 207] Effector cytotoxic T cells produce a number of cytokines with one of the most predominant being IFN-γ.[208] the release of this cytokine can help eliminate viruses[209] and can further increase the cytotoxic potential of CD8+ T cells.[210] Once the invading pathogens have been eliminated the effector T-cell population reduces by apoptosis to leave only 5-10% of the effector population.[211] The remaining T cells become long lived memory T cells that provide enhanced protection from secondary infection.[211]

Th1 and Th2 T-cell subtypes

CD4+ T cells form the helper T-cell population and are responsible for the production of a large number of cytokines which help control and mediate other cells in the inflammatory response.[212] CD4+ T cells can be subdivided into specific populations based on their cytokine expression and function within the immune system (Figure 1.6). Two of the main Th subsets that were first identified were Th1 and Th2 cells.[213] Th1 cells are differentiated through IL-12[214] and IFN-γ[215] signalling and are involved in immunity against intracellular bacterial and viral infections.[216] Th1 cells are characterised by the production of the cytokine IFN-γ[213] and through the expression of the characteristic gene TBX21/Tbet.[217] Th1 cell cytokine production guides the immune response to destroy pathogens via the activation of other immune system components, increasing their activity.[212] During aberrant Th1 signalling this population can contribute in the formation of autoimmune diseases.[218] Th2 cells are involved in immunity against parasitic infection and extracellular pathogens.[219, 220] Th2 cells characteristically produce the cytokines IL-4, IL-5, IL-13[213, 221] and express the gene GATA3.[222] Th2 cells are differentiated from naïve CD4+ T cells by IL-4 signalling[223] and have been implicated in allergic inflammation and asthma.[125, 224]

Additional Th cell populations

Later studies identified additional Th populations in addition to Th1 and Th2, these groups include Th17, Th9 and Treg cells. Th17 cells are a highly inflammatory subpopulation and characteristically express the cytokine IL-17[225] as well as IL-22.[226] Th17 cells express the gene RORγ encoding the orphan retinoic acid receptor, which directs naïve CD4+ T cells down this lineage[227] along with IL-6, TGF-β[228] and IL-23 signalling.[229] In the healthy immune system Th17 cells are involved in the defence against extracellular bacteria and fungus[230] where IL-17 plays a key role.[231, 232] Th17 cells have also been heavily implicated in autoimmune disease such as asthma[233] and rheumatoid arthritis.[234] Th9 cells have been newly identified and are characterised by the production of IL-9 and IL-10.[235] Th9 cells are induced by TGF-β and IL-4[235] signalling and are

thought to contribute in the inflammatory response.[236] This cell type has also been found to contribute to inflammatory disease such as inflammatory bowel disease.[237, 238] Finally, Tregs present as a unique subset of Th cells and opposed to the other groups of CD4+ T cells, Tregs work to resolve inflammation and bring about homeostasis.[239] Tregs are differentiated by TGF-β[240] and IL-2[241] signalling and are characterised by the expression of the master transcription factor FOXP3.[242, 243] Treg control of the inflammatory response has been suggested to be mediated by several different mechanisms. One such mechanism is the production of cytokines such as the anti-inflammatory cytokine IL-10.[244] Tregs also express high levels of IL-2 receptor and are thought to inhibit inflammatory signalling by competitive uptake of IL-2 in the inflammatory environment, stopping IL-2 from stimulating effector T cells, resulting in their apoptosis.[245] Other mechanisms of action include cytolytic activity by granzymes and perforins[246] and release of adenosine nucleosides.[247]
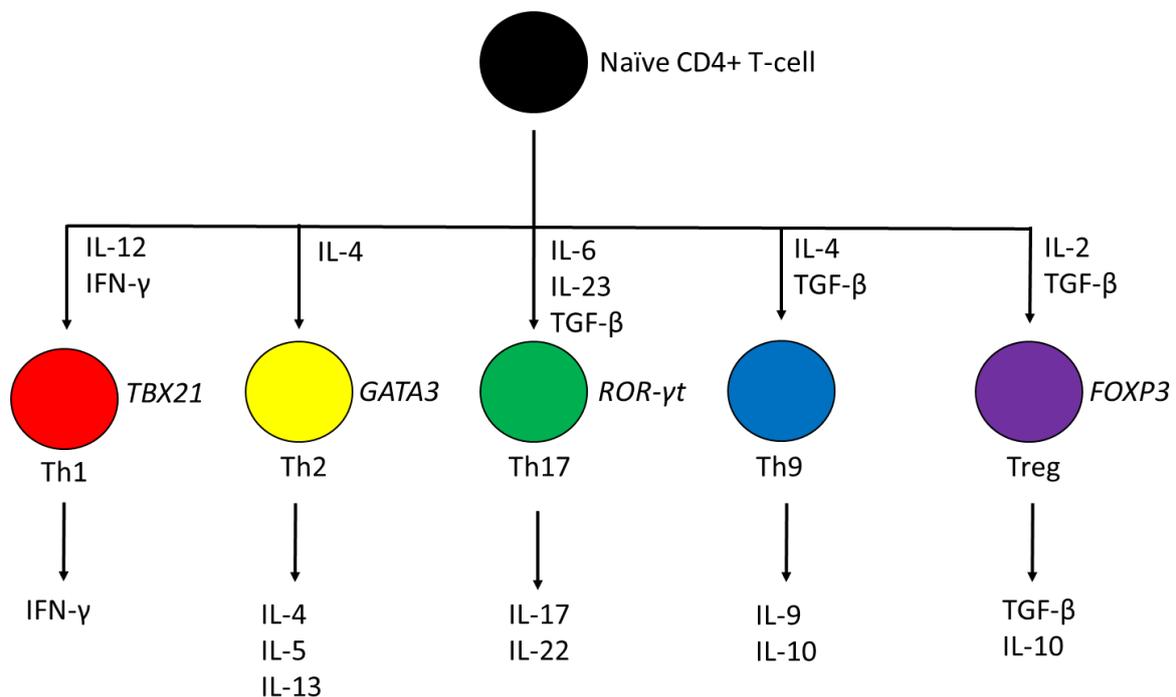
Figure 1.6: Differentiation of helper T-cell subsets
Naïve T helper (Th) cells differentiate into several subgroups including Th1, Th2, Th17, Th9 and Treg cells. Naïve CD4+ T cells are differentiated into subgroups via the action of specific cytokines. T helper subgroups have specific functions within the immune system and express characteristic cytokines and transcription factors. Italics indicate genes.

1.3. Inflammation in neuropathic pain

1.3.1 Wallerian degeneration

After nerve injury, immune cell activity is important for effective repair of the damaged nerves.[248] A good example of this is Wallerian degeneration (Figure 1.7), which describes the highly orchestrated and evolutionary conserved process of nerve degeneration after injury and the clearance of axonal and cellular debris, which paves the way for regeneration of the neuron.[249] This process was first discovered by Augustus Waller in 1850 who characterized morphological changes in sections of frog glossopharyngeal and hypoglossal nerves after transection.[250]

After peripheral nerve injury occurs, such as nerve transection, the nerve is divided into the proximal stump, which is the side of the nerve still connected to the soma and the distal stump, which is the other side of the break not connected to the nerve cell body. Initially after nerve injury there is a lag phase where the distal stump remains intact and can still transmit action potentials.[251, 252] The lag phase varies in length of time depending on the species, with it lasting only 24-48 hours in mice[253] and rats[254] but for up to several days in humans.[255] The initiation of Wallerian degeneration is started by a large influx of Ca^{2+} ions into the axon at both proximal and distal stumps, which can come from both extracellular and intracellular sources.[256, 257] A recently identified axonal protein sterile-α and Toll/interleukin 1 receptor (TIR) motif containing protein 1 (SARM1) has also been found to be involved in the initiation of Wallerian degeneration potentially by the degradation of it substrate nicotinamide adenine dinucleotide (NAD).[258] The breakdown products of NAD have also been found to be potent mobilizers of calcium.[258] The high increase in Ca^{2+} causes activation of calpain proteins. These are proteases involved in the degeneration of the cytoskeleton and axons and are required for the initiation of Wallerian degeneration.[257, 259] After axon degeneration has been initiated, one of the first cells to respond to the injury are Schwann cells, which undergo genomic changes.[260, 261] Initially Schwann cells are thought to sense nerve damage by the detection of endogenous cellular components and fragments that form debris

after axon degeneration.[262] Detection of damage in the nerve causes the Schwann cells to lose

their myelinating capabilities and upregulate genes characteristic of premature Schwann cells, a

process known as dedifferentiation.[263] In this dedifferentiated state, the Schwann cells are able to

phagocytose cellular and myelin debris that accumulates after nerve injury, acting as the main

phagocytosing cell in the initial stages of Wallerian degeneration.[264] As well as phagocytosing

myelin, Schwann cells also produce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and LIF,

which recruit immune cells into the damaged area.[265-267] Initially neutrophils are the first cells to

be recruited and infiltrate the injury site where they help to phagocytose debris and recruit other

immune cells such as macrophages.[268] Macrophages play a prominent role as the main

phagocytosing cells in the later stages of Wallerian degeneration,[269] involving both tissue resident

endoneurial macrophages and infiltrating macrophages from the circulation.[270] Endoneurial

macrophages resident in the nerve begin proliferating and phagocytosing myelin debris within 2

days post axotomy and before the blood derived monocytes arrive.[271, 272] After around 4 days,

blood derived monocytes infiltrate the tissue where they mature into differentiated

macrophages.[270] The blood derived macrophages then provide essential aid in phagocytosing

debris[273] as well as signaling with cytokines to activate Schwann cells[274] and trophic factors to

stimulate axon regrowth.[275] The importance of macrophages in effective debris clearance and

Wallerian degeneration is further shown in studies that have pharmacologically or genetically

ablated the macrophage population[273, 276] These studies found that myelin degradation was

decreased and axon regeneration and locomotor function recovery were severely affected after

macrophage ablation. T cells are the last immune cell type to enter the injury site, with their

numbers peaking between 14-28 days after the injury.[277] Both Th1 and Th2 T cells have been

shown to be important in promoting axon regeneration and functional recovery.[278]
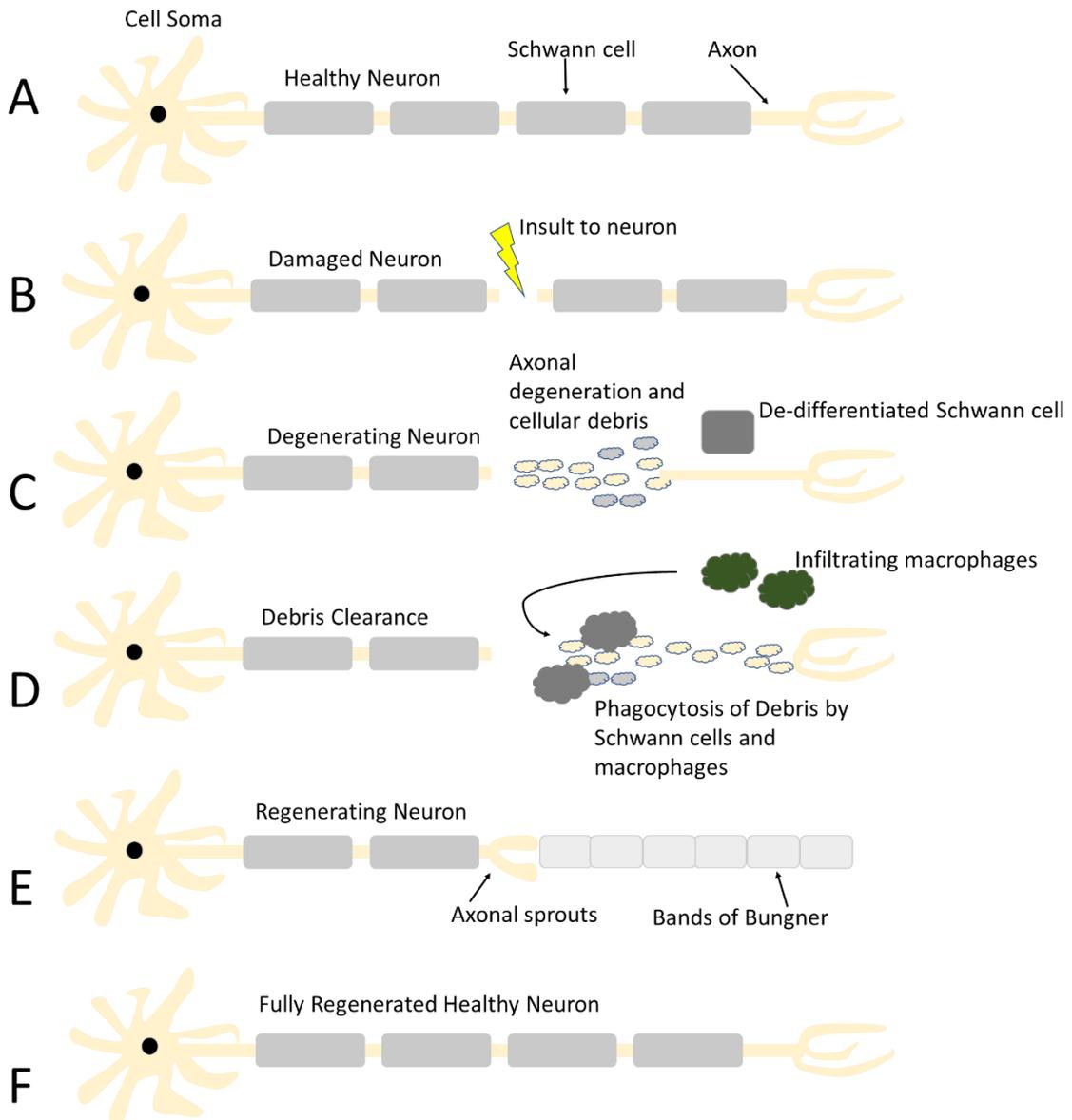
Figure 1.7: Wallerian degeneration
The stages of Wallerian degeneration are outlined above. A. A healthy nerve with contact to peripheral targets and an intact myelin sheath. B. Damage is caused to the neuron causing lacerations or complete transection. C. The distal portion of the nerve begins to break down and myelinating Schwann cells begin to de-differentiate. D. De-differentiated Schwann cells begin to phagocytose the cellular debris. Other immune cells such as macrophages infiltrate into the area to aid with debris clearance. E. Axonal sprouts project from the proximal stump and project towards the peripheral target. De-differentiated Schwann cells form bands of Bungner which guide the axonal sprouts to the correct target. F. A healthy nerve is reformed.

1.3.2 Evidence from preclinical models of immune cells in the generation and maintenance of neuropathic pain

Immune cell infiltration at the site of injury

Immune cell infiltration into the peripheral nerve after nerve injury has been heavily implicated in the generation and maintenance of neuropathic pain[72] (Figure 1.8). However, the majority of these findings have been produced from pre-clinical studies using animal models of nerve injury. After injury, neutrophils and macrophages are the first cells to infiltrate the injured nerve.[268, 279] Neutrophils have been shown to contribute to early hypersensitivity after peripheral nerve injury as the administration of antibodies that ablate the neutrophil population causes a reduction in hyperalgesia.[268]

Macrophages have also been found to contribute to the generation of neuropathic pain after infiltration into the peripheral nerve, as the depletion of this population via liposomes containing clodronate reduces the development of neuropathic hyperalgesia.[280] This finding was replicated in a more recent study where clodronate containing liposome mediated ablation of macrophages was able to inhibit the generation of tactile allodynia after partial nerve injury.[281] Within the injured nerve, the release of inflammatory mediators such as IL-1β, MIP-1α and IL-6 by macrophages has been found to contribute to the generation of neuropathic pain after peripheral nerve damage.[282, 283] As these cytokines are characteristically produced by M1 macrophages, this indicates a pivotal role for the M1 subtype in the initiation of neuropathic pain.[284]

T cells have also been heavily implicated in the generation and maintenance of neuropathic pain following nerve injury, as studies have shown that T cells infiltrate the injured nerve site at characteristically later time points between 7 and 28 days post injury.[277, 285] T cells are thought to arrive from the circulation and penetrate the nerve from the endoneurial vasculature instead of

migrating across the nerve sheath.[286] The contribution of T cells towards neuropathic pain has been shown in a study using athymic rats to determine the generation of neuropathic pain after chronic constriction injury (CCI).[277] It was found that thermal and mechanical hypersensitivity were reduced in these mice compered to wild type controls. Furthermore the administration of Th1 T cells were able to restore the pain hypersensitivity. Experiments using recombination activation gene 1 (RAG1) deficient mice (RAG1-/-),[287] which are unable to produce mature T cells, displayed reduced thermal hypersensitivity after CCI[288] as well as a decrease in mechanical allodynia after spared nerve injury (SNI).[289]

Immune cell infiltration at distant sites

As well as infiltration at the site of injury, immune cells have been found to enter distant sites such as the dorsal root ganglion and the spinal cord (Figure 1.8). Macrophages were found to enter the spinal cord and DRG after nerve injury.[290, 291] However a more recent study has suggested that the increased macrophage numbers in the DRG after nerve injury are the result of proliferation of DRG macrophages as opposed to infiltrating cells, though they do not rule out a contribution from infiltrating macrophages.[292] Several studies have detected T-cell infiltration into the DRG[279, 293, 294] and the spinal cord[289, 295] after nerve injury, where they contribute to the generation of neuropathic pain. One such study determined a specific role for the T-cell cytokine IFN-γ in neuropathic pain as the expression of this cytokine was increased in the spinal dorsal horn after injury.[281] The role of this cytokine was further shown as the knockout of the IFN-γ gene caused a reduction in mechanical allodynia, potentially revealing a role for Th1 cells in neuropathic pain.

Immune cells in the resolution of neuropathic pain

Immune cells can also modulate the generation of neuropathic pain, with Tregs being one of the main immune cell types identified to perform this role. One of the first studies to investigate the function of Tregs in neuropathic pain found that application of the Treg super agonist CD28 was able to significantly reduce mechanical hypersensitivity in two models of neuropathic pain (CCI and experimental autoimmune neuritis).[296] Application of the super agonist also caused an increase in Treg numbers in the sciatic nerve and the DRG, while causing a decrease in infiltrating T cells and macrophages into these same areas. Further study by the same group[297] found that Treg cells infiltrate the injury site, DRG and spinal cord after CCI and that depleting these cells causes significant increases in pain hypersensitivity. After Treg depletion they also found changes in serum expression of cytokines with CCL5, IL-2 and IL-5 all being significantly increased and IL-12 and IFN-γ serum concentrations being significantly decreased, which may indicate that Tregs are mediating their effect through the manipulation of certain cytokines. Other studies have proposed different mechanisms of action for the function of Tregs in neuropathic pain with one study finding that signaling through the TNFR2 receptor was essential for Treg mediated reductions in pain hypersensitivity.[298] This study found that TNFR2 knockout mice developed non-resolving pain that did not recover after CCI. Further, the use of a TNFR2 agonist EHD2-sc-mTNF$_{R2}$ caused significant increased recovery from mechanical and cold allodynia and thermal hyperalgesia compared to control treated mice. A more recent study has found that the analgesic effects of Tregs in neuropathic pain are the result of inhibitory interactions with Th1 T cells. This study found that after Treg depletion the expression of IFN-γ and TBX21 were increased in the injured sciatic nerve. CD4+ IFN-γ+ cells were also increased in the sciatic nerve after injury in Treg depleted mice. This study also suggested that the actions of Tregs could be through the signaling of IL-10.
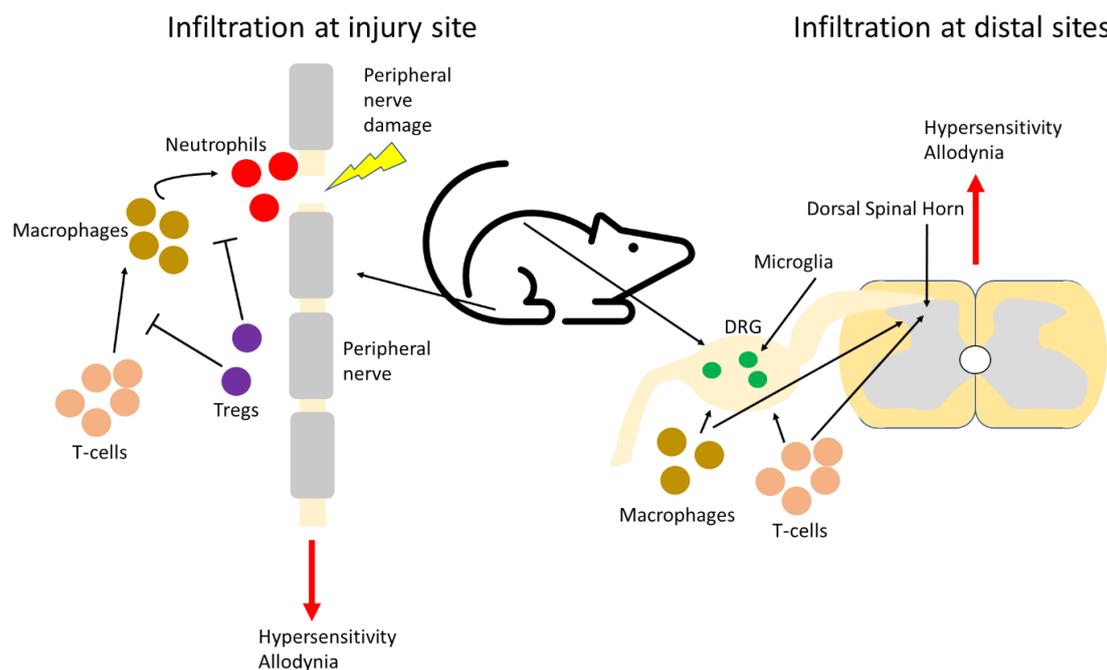
Figure 1.8: Immune cells in neuropathic pain
After peripheral nerve injury neutrophils are the first cells to infiltrate the damaged nerve site. Macrophages arrive shortly afterwards and contribute to hypersensitivity and neuropathic pain by the production of inflammatory mediators. Lastly, T cells infiltrate the damaged nerve where they contribute to neuropathic pain via cytokine production. At sites distant from the nerve injury, such as the DRG and the spinal dorsal horn, immune cells have also been found to infiltrate and contribute to neuropathic pain. Macrophages and T cells have both been identified in these structures. As well as infiltrating immune cells, resident immune cells in the peripheral nervous system such as microglia have also been found to be activated in the DRG and spinal dorsal horn, and may contribute to neuropathic pain.
DRG = Dorsal root ganglion, Tregs = T regulatory cells.

1.3.3 Inflammation in chronic neuropathic pain conditions in humans

As highlighted above, extensive work has been conducted in pre-clinical animal studies to reveal

the involvement of inflammation in neuropathic pain. However, limited investigations have been

conducted in humans to reveal the associations of inflammation and pain due to the limited

access to human tissue. There are however some neuropathic conditions in humans that have a

well-defined inflammatory component such as Complex Regional Pain Syndrome (CRPS) and

Chronic Inflammatory Demyelinating Polyneuropathy (CIDP). For CIDP, studies have made use of nerve biopsies to determine an inflammatory role directly in the nerve tissue. One study found that epineural T cells and endoneural macrophages were increased in sural nerve biopsies of patients with CIDP compared to patients with non-inflammatory neuropathies and healthy controls.[299] However, there was no difference in T-cell numbers in the endoneurium across groups. A further study found increased T-cell numbers in the total sural nerve area in the endoneurium of patients with CIDP compared to patients with non-inflammatory neuropathy (chronic idiopathic axonal polyneuropathy (CIAP)) and healthy controls.[300] However only few patients with CIDP showed elevated T-cell numbers. Lastly, a more recent study found increased endoneurial macrophages in the sural nerve of patients with CIDP compared to patients with hereditary neuropathies and controls. However this study did not find a difference in total T-cell numbers or endoneurial T-cell numbers between the different groups.[301] While these studies show that immune cells can be increased in patients with inflammatory neuropathy compared to those with non-inflammatory neuropathy, this is not consistent and varies depending on the cell type and the area of tissue under investigation. In these studies, no associations between inflammation and pain were investigated, however one study investigating cytokine expression in sural nerve biopsies from patients with painful and painless neuropathies found IL-6 and IL-10 to be higher in patients with painful neuropathy.[302] Another study by the same group found no correlation with pain and T-cell or macrophage infiltration in the sural nerve of patients with neuropathy (including conditions such as CIDP, CIAP and Progressive idiopathic axonal neuropathy).[303] These findings suggest that intraneural inflammation may not necessarily always contribute to pain in systemic neuropathy but also shows that inflammation and immune cell infiltration is not unique to classically inflammatory neuropathies, and so could be a feature of neuropathies more generally. To further this point, the results from a study investigating post-surgery neuropathy in a cohort of patients who had no apparent mechanical nerve damage, found signs of inflammation and immune cell infiltration in nerve biopsies.[304] Patients were also

found to benefit from immunotherapy. However in all of these studies only generic immune cell markers (CD3, CD45, CD68) were used and so provide limited information regarding the specific immune cell populations that could be of interest in peripheral neuropathies. Importantly, nerve biopsies are hard to obtain from patients and healthy controls. Other studies have therefore looked more indirectly such as in blood to determine the presence and role of inflammation in the context of neuropathic pain. For CRPS, a meta-analysis of 15 studies investigating the expression of inflammatory mediators in samples from patients, identified IL-8 and soluble tumor necrosis factor receptors 1 and 2 to be increased in the blood of patients with acute CRPS.[305] In patients with chronic CRPS, multiple inflammatory mediators from different samples were increased such as TNF-α, IL-6 and IL-1β in the blood, blister fluid and CSF respectively.[305] Another study performed immunophenotyping of blood samples from CRPS patients compared to controls.[306] This analysis identified significant increases in central memory T-cell populations such as Th1, Tregs and CD8+ T cells. This directly implicates these populations in the pathogenesis of the disease, however none of the increases in these groups correlated with pain scores.[306] Microglia activation was also identified in the spinal cord of cadaveric tissue from a patient with long term CRPS.[307] However, the precise pathological events occurring in CRPS remain unknown,[305] making it difficult to specifically determine the pathogenesis of pain and how it may relate to other types of peripheral nerve injury.

In human neuropathic pain conditions where the role of inflammation is less well defined, one study has made use of imaging techniques to detect inflammation. Using combined positron emission tomography and magnetic resonance imaging, the authors identified neuroinflammation in the spinal cord and nerve roots of patients with chronic lumbar radicular pain.[308] They found elevated levels of the inflammatory marker 18 kDa translocator protein (TSPO) in patients compared to controls, allowing for the *in vivo* identification of neuroinflammation in humans with neuropathic pain. Another study using MRI imaging of patients with persistent low back pain, found an association between inflammatory pain

characteristics and MRI findings such as erosions and bone marrow edema.[309] Investigations using flow cytometry have also been undertaken to analyze immune cell populations in humans with neuropathic pain. One study identified a Th17/Treg imbalance in the peripheral blood where there was a significant increase in the number of Treg cells in neuropathic pain patients (symmetrical polyneuropathy/peripheral mononeuropathy, post herpetic neuralgia and orofacial pain) compared to healthy controls. This finding was confirmed by the significant increase in FOXP3 mRNA expression in a CD4+ cell fraction extracted from the peripheral blood of these patients.[310] A similar study investigating T-cell subgroups in patients with lumbar disk herniation found increased CD4+ T-cell numbers in the blood of patients compared to controls.[311] The increases in CD4+ T cells also correlated with VAS pain scores.[311] These studies go to show that immune cell populations in patients with neuropathic pain are altered compared to healthy controls, which could have implications in the pathogenesis of the disease. Many studies have investigated the expression of inflammatory mediators in the serum of patients with neuropathic pain.[75, 312-315] In these studies several inflammatory mediators have been found to be increased in patients compared to healthy controls or associated with clinical phenotypes. However in the majority of cases, only a small number of cytokines are investigated, leaving potentially important mediators uncharacterized.

The findings from the studies highlighted above show some evidence for immune cell infiltration directly into the nerve in neuropathic pain conditions. However this was mostly done in severe conditions where an inflammatory pathology had already been described and so may not represent the pathophysiology of milder conditions such as entrapment neuropathies. In neuropathic pain conditions without a clear inflammatory pathology, the findings tended to show indirect signs of inflammation via imaging studies or increased inflammatory mediator and immune cell populations in the peripheral blood. Investigations are therefore required to evaluate the presence of inflammation and immune cell infiltration locally at the nerve injury site of milder nerve injuries and to better characterize the local inflammatory environment.

1.4 Peripheral nerve regeneration

1.4.1 Mechanisms of nerve regeneration

The events of peripheral nerve regeneration contributing to the generation of neuropathic pain are incompletely understood. The surviving nerves must regrow their damaged axons along the distal stump and re-establish connections with their peripheral targets.[316] The process of Wallerian degeneration mentioned above, implicates the immune system in this process, as it is required for debris clearance after injury. One of the main events that occurs after nerve injury, which allows for effective nerve regeneration, is the changing of the neuron from a state of signal transduction and maintenance to a state of axonal growth.[317] This is a complex process involving a milieu of signaling events that ultimately induces changes in gene expression within the neuron. This process can be split into two phases (Figure 1.9) with the first phase of signals involving the influx of calcium and sodium ions into the axoplasm through the damaged axon site.[318] This causes an increase in axon potential firing[319] which propagates retrogradely towards the soma. These action potentials cause an influx of calcium ions through voltage dependent ion channels,[320] and contributes to the activation of signaling (Figure 1.9).[321] The second phase of signals involves the function of activated proteins termed the 'positive injury signals'.[322] These are

endogenous axoplasmic proteins[323, 324] which undergo post translational modifications at the site of injury and are then trafficked back to the cell body,[325] where they induce the activation of transcription factors.[326] The change in activity of the transcription factors causes a large change in the gene expression profile, with cytoskeletal proteins, cytokines and neuropeptides all being significantly increased after injury.[327, 328] This change in expression allows the formation of new growth cones and supports elongation and regeneration. Deactivation of negative injury signals are also required for effective axonal growth. Negative injury signals are thought to be continuously retrogradely transported along the axon to the cell body where they signal to repress axonal growth and help to maintain the correct function of the neuron.[322]

Cytokines have also been shown to aid in nerve regeneration after injury, with IL-6 and IL-1 having implications in stimulating nerve regrowth. The genetic knockout of IL-6 in mice with sciatic nerve crush caused a delay in the restoration of sensory function,[329] while upregulation in the expression of IL-6 and the IL-6 receptor caused increases in nerve regeneration after injury.[330] IL-1β administration before nerve injury showed signs of promoting nerve regeneration compared to vehicle treated controls.[331] Another study investigated the effects of low and high doses of several cytokines on the growth of cultured mouse DRG neurons in the presence of varying combinations of neurotrophic factors (NT-3, NT-4 and NGF).[332] Low doses of IL-6 and high doses of IFN-γ caused an increase in axonal outgrowth. High doses of TNF-α however caused a significant decrease in neurite outgrowth. IL-4 had mixed effects, as low doses caused a decrease in axonal outgrowth while high dose increased neurite growth.[332] Altogether these studies highlight a role for inflammatory mediators and the immune system in nerve regeneration. It also shows the variable effect of cytokines and how different conditions can affect their action in the regeneration process.
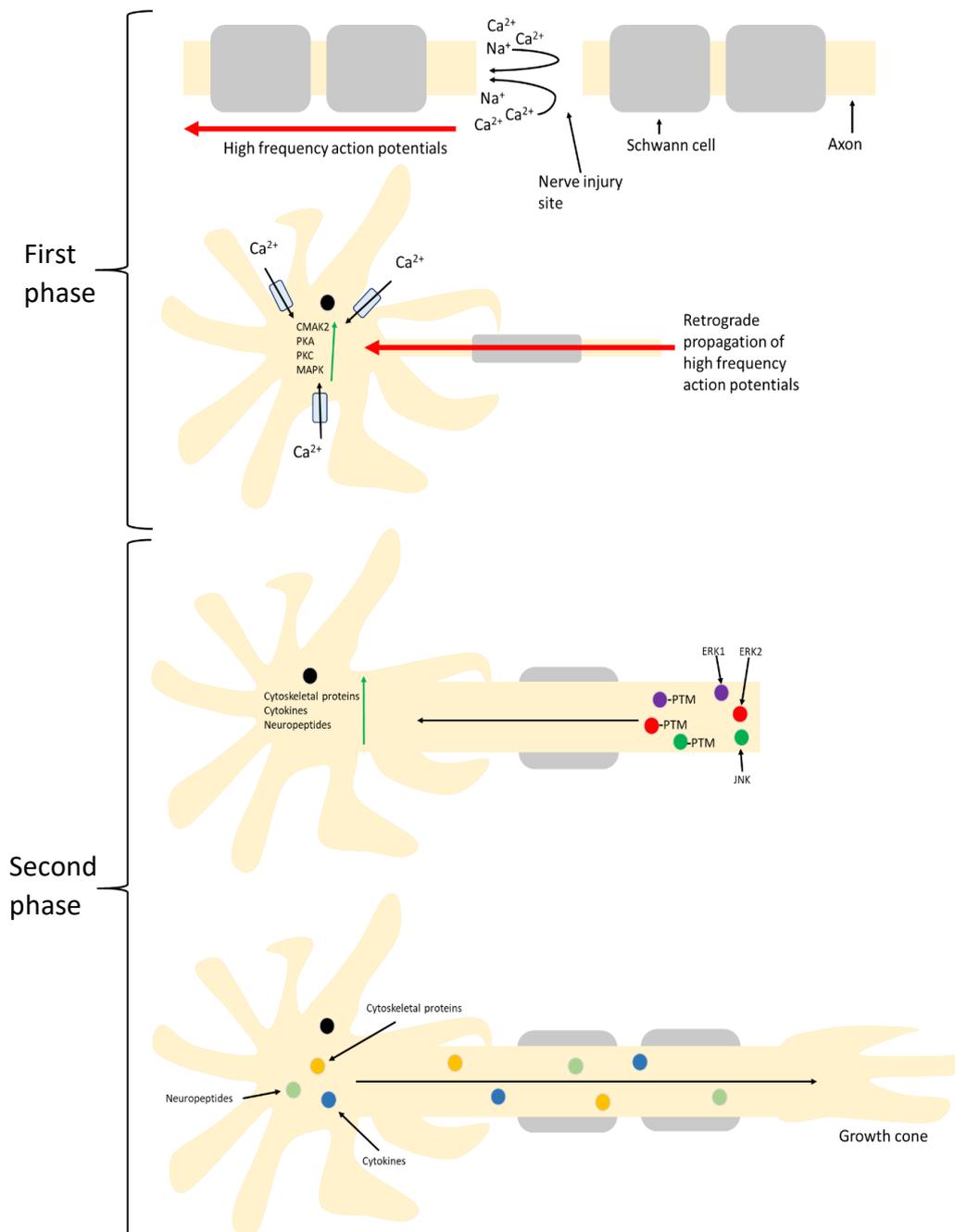
Figure 1.9: Mechanisms of nerve regeneration
The initial processes of nerve regeneration can be split into two phases. The first phase involves an influx of sodium and calcium ions into the axoplasm at the site of nerve injury. This causes the retrograde propagation of high frequency action potentials which are received by the cell soma. The high frequency action potentials cause an influx of calcium ions through voltage dependent ion channels, which contribute to the activation of certain signaling pathways and factors such as CMAK2, PKA, PKC and MAPK. The second phase involves the activation of endogenous axoplasmic proteins such as ERK1, ERK2, JNK and importin, which form the positive injury signals. These proteins are post translationally modified at the site of injury and are trafficked back to the cell body via the retrograde transport system where they increase the activity of transcription factors. These transcription factors induce the expression of cytoskeletal proteins, cytokines and neuropeptides which aid in growth cone formation, regeneration and elongation.
CMAK2 = calcium/calmodulin-dependent kinase 2, PKA = protein kinase A, PKC = protein kinase C, MAPK = mitogen-activated protein kinase, PTM = Post translational modification, ERK1-2 = Extracellular signal-regulated kinase 1-2, JNK = c-Jun N-terminal kinase.

Mechanisms of target re-innervation

For functional recovery to take place, the damaged axons have to re-establish contact with their peripheral targets. Growth cones sprout from the proximal nerve at the lesion site and elongate if conditions are suitable.[320] Axonal growth is guided by other cell types and structures such as the bands of Büngner,[333] which are formed of dedifferentiated Schwann cells that line up with the endoneurial tubes and provide support for regenerating axons.[334] The inflammatory mediator IL-1β has been found to be heavily implicated in the de-differentiation process of Schwann cells after nerve damage.[335] Bands of Büngner aid regeneration by the production of multiple growth factors including nerve growth factor (NGF)[336] and brain-derived nerve growth factor (BDNF)[337] which helps to direct the growing axons to their peripheral targets.[338] The cellular materials required for nerve growth are mainly provided by the nerve soma itself and are transported from the cell body along the axon to the peripheral target.[339] However, local axonal synthesis of proteins have recently been identified whereby a large number of proteins including cytoskeletal proteins, endoplasmic reticulum proteins and metabolic proteins can be produced near to the lesion site and can contribute to the growing axon.[340] This process may be driven by target of rapamycin (TOR), p38 MAPK, and caspase-3 dependent pathways as inhibition of these proteins resulted in reduced growth cone formation.[341] Initially many sprouts can form from the proximal parent axon and can elongate into the distal nerve, allowing the nerve to access many distal pathways.[342] When the elongating nerves reach their peripheral target the surplus sprouts are withdrawn and removed via axonal pruning, this aids in refining the selectivity of axon to target innervation.[320]

Issues with nerve regeneration

Despite the plethora of mechanisms that aid nerve growth, optimal recovery is rarely achieved.[343] Often after nerve regeneration there are both structural and functional abnormalities of the nerve including reductions in the size of the neurons and in their conduction velocity for long periods after nerve regeneration.[344, 345] One of the main factors affecting functional recovery after injury is inappropriate distal reconnection, where elongating axons do not reinnervate the correct peripheral target or the innervation of the target is insufficient.[346] If the correct neurons do not innervate the appropriate target this can lead to a sustained loss of function of the target organ/structure. Regenerating axons that do not reach their target do not receive stimulatory signals that help maintain the growth of the neuron and subsequently die back and degenerate,[347] leaving the structures chronically axotomized.[348] This is less of an issue in nerve crush where the neural tube remains intact, allowing axons to be directed to their original target more effectively than after nerve transection. Optimal nerve growth can also be hindered by damage to the neuronal cell body due to axotomy or retrograde degeneration, making the nerve unable to mount successful regeneration. An underlying disease where neuropathy is present can also hinder regeneration.[320] The complex interplay of nerve regeneration processes and the milieu of factors involved in nerve regeneration, including inflammatory mediators are therefore important in achieving effective nerve repair. Further characterization of these processes and of the regeneration factors in the milieu of repair mediators would prove useful in bettering our understanding of nerve repair, and could uncover new therapeutic opportunities.

1.4.2 Treatments for peripheral nerve regeneration

Nerve regeneration in humans is often less successful than that observed in animal models of regeneration.[343] One of the main factors affecting the success of nerve growth in humans is the long distance that the nerve has to regrow to reach peripheral targets.[349] This causes increased

times of axotomy and chronic denervation, where the distal nerve segments remain without any axonal contact,[343] causing a reduction in successful regeneration.[350] Schwann cells lining the endoneurium, which provide trophic support to growing axons, can suffer from atrophy over time which decreases their ability to support nerve growth.[351, 352] The slow rate of nerve regrowth in humans may also contribute to these factors, as the nerve growth rate in humans of 1.5-2mm/day has been found to be slower than that observed in rodents.[349]

Several treatments to facilitate peripheral nerve regeneration have been trialed including medications and electrical nerve stimulation, which induces nerve growth and inhibits the denervation associated atrophy of the target tissue. This involves the electrical stimulation of nerve or muscle via the application of an electrical current.[353, 354] However alternative methods have been employed, such as the use of molecular therapies to aid in nerve regeneration, which is discussed below.

Molecular therapy in animal models

Due to their implications in nerve regeneration, neurotrophic factors have been used as molecular therapy in an attempt to increase the rate of nerve regeneration. Much work has been done using pre-clinical animal models of nerve injury where the neurotrophic factor NGF has been frequently used. One such study conducting experiments in a rat model of sciatic nerve crush investigated the application of NGF on nerve regeneration.[355] In cultured Schwann cells, NGF application was able to activate autophagy and accelerate myelin debris clearance. NGF administration was also found to be able to promote axon regeneration and myelination in the early stages of injury in rats.[355] A similar study using sciatic nerve crush in rats found significant improvements in electrophysiological parameters as well as significantly more regenerated myelinated nerve fibers after NGF treatment compared to controls.[356] In a different injury model using optic nerve crush (ONC), the administration of NGF was again found to reduce the loss of

retinal ganglion cells and stimulate axonal regrowth,[357] highlighting the therapeutic potential of

NGF as a treatment in nerve recovery. Other neurotrophic/growth factors such as Vascular

Endothelial Growth Factor (VEGF),[358] Insulin like Growth Factor (IGF)[359] and Glial Derived

Neurotrophic Factor (GDNF)[360] have been used as therapy in animal models of nerve injury to

stimulate nerve regeneration, which have produced positive findings. However, as these findings

have been produced from animal models they would likely suffer from the lack of transferability

to the human condition, as previously stated. As a result, experiments in humans would be

needed to fully investigate the potential of these molecules as effective treatment in nerve

regeneration and to confirm their potential in regenerative therapy.

Molecular therapy in humans

In humans there is limited information as to the use of molecular therapy, including the use of

neurotrophic factors, to treat nerve injury. However, NGF has previously been used in clinical

trials to treat patients with diabetic neuropathy. Initially in a phase 2 trial the use of recombinant

human NGF caused improvement in a composite score including measurements of thermal

detection, small nerve fiber function and nerve impairment scores.[361] However in a subsequent,

larger phase 3 clinical trial both primary outcomes (neuropathy impairment score for the lower

limbs) and the secondary outcome scores (quantitative sensory testing, neuropathy symptoms

and change score and patient benefit questionnaires) were not met and significant hyperalgesia

was produced at the injection site.[361] Clinical trials using neurotrophic factors have also been

conducted to treat patients with entrapment neuropathy. Here, multiple doses of the

neurotrophic factor BG00010, a member of the GDNF family, were used to treat patients with

sciatica. However no significant trends were observed for the different doses of BG00010 and

clinical parameters including IENFD, Quantitative Sensory Testing (QST) or pain.[362] Similarly in a

follow on study, no dose response effect of BG00010 on patient pain scores was seen.[363] These

studies highlight the lack of viable molecular therapies to facilitate nerve regeneration in humans, and showcases the poor efficacy and adverse side effects as major hurdles to overcome. The identification of new molecular mediators that can effectively aid in the regeneration of nerves after injury without serious adverse side effects are critical to achieve effective new therapies.

Thesis aims and objectives

Due to the limited knowledge of mediators involved in nerve regeneration in humans, and in light of the poor efficacy of molecular therapies, I will seek to identify molecular markers associated with nerve regeneration and assess their contribution to nerve regrowth in a human model system. The association of nerve regeneration with inflammatory mediators will also be investigated to determine the role of inflammation in the regeneration process. The findings from these investigations will provide answers to the second main aim of the thesis: To characterize nerve regeneration after injury and determine the role of inflammation in this process.

Summary

Neuropathic pain is a debilitating disease that has a high prevalence, is poorly treated, and is associated with a large socioeconomic burden. Through this introduction I have highlighted the challenges with neuropathic pain and have introduced the role that inflammation has in its generation and maintenance. From the review of the literature it is apparent that the overwhelming majority of research investigating the role of inflammation in neuropathic pain has come from pre-clinical animal models. The few studies conducted in humans are often indirect or are done using severe or systemic neuropathies, which do not reflect the pathology of entrapment neuropathy. I have also introduced peripheral nerve injury and the complications

surrounding effective nerve regeneration. Similarly to inflammation in neuropathic pain, many studies for nerve regeneration have been conducted in animals models with poor translatability to the condition in humans. Current molecular therapies for nerve regeneration in humans are also lacking and so the identification of new molecular targets are required. There is therefore a great need for experiments investigating inflammation, neuropathic pain and nerve regeneration after peripheral nerve injury in humans to effectively characterize the cellular and molecular components that are involved in these processes. I have introduced CTS as a viable human model system in investigate these processes with the main aims being as follows:

Aims

1. To investigate the relationship of inflammation with neuropathic pain in humans

2. To characterise the molecular environment of nerve regeneration after injury and determine associations with inflammation in this process.

Chapter 2: Markers of systemic inflammation in patients with Carpal tunnel syndrome

2.1 Introduction

Idiopathic CTS has long been considered to be a non-inflammatory condition. This has primarily been based on the conclusions from histological studies using tissue from the carpal tunnel of patients.[364-366] Other studies have investigated the expression of inflammatory mediators in the blood of patients with CTS to determine an immune component, with mixed findings. Some studies analysing the expression of inflammatory markers in serum have found no significant difference in inflammatory mediator levels between patients with CTS and controls.[50, 367] Another study however has detected a significant increase in the serum levels of IL-6 in patients with CTS compared to control patients, although the concentrations of IL-1β or TNF-α were not significantly different.[368] Increased inflammatory mediator levels have also been observed in tenosynovial lysates for IL-6 and Prostoglandin-E2 (PGE2).[50, 369] The role that inflammation plays in the pathogenesis of CTS therefore remains contentious. However in the majority of studies investigating the expression of inflammatory mediators, very few cytokines were analysed and the phenotypic information available for the patients, detailing the severity of their symptoms, was lacking. These limited investigations leave the actions of the majority of inflammatory mediators and their associations with clinical phenotypes largely unknown in CTS. As such, important interactions and functions of inflammatory mediators in the pathogenesis of CTS may have been missed, highlighting the need of detailed further investigation into the role that inflammatory mediators may play in CTS. A recent study has attempted to answer several of these questions by conducting analysis on a larger range of inflammatory mediators in the serum of patients with CTS.[75] Detailed phenotypic information was also collected for these patients and associations between inflammatory mediator levels and phenotype characteristics were

investigated. Serum concentrations of CCL5, VEGF, CXCL8 and CXCL10 were all significantly increased compared to healthy controls, indicating a level of systemic inflammation in patients with CTS. Interestingly the chemokine CCL5 was found to be negatively associated with patients scores for the questionnaires douleur neuropathique 4 (DN4) and the neuropathic pain symptom inventory (NPSI) composite score, which determine the probability of neuropathic pain and the severity of neuropathic pain respectively.[40, 370] These findings provide evidence that the activity of inflammatory mediators could be having an effect on the symptoms experienced by patients with CTS. In addition to this central memory (Tcm) and effector memory (Tem) CD4+ T-cell populations were found to be increased in the peripheral blood of patients with CTS compared to controls, providing further evidence of immune dysregulation in patients with CTS. Though this study has furthered the understanding of the role of inflammation in CTS, the number of cytokines analysed was not comprehensive and no analysis was conducted on patients with CTS before and after surgery. Analysing inflammatory mediator expression before and after surgery would provide insight into how the inflammatory environment changes within patients from before surgery to after treatment and could identify subtle changes occurring within patients that contribute to pathogenesis. Further investigations analysing the activity of a greater number of inflammatory mediators in patients before and after surgery are therefore required to fully interrogate the action of inflammation in CTS. I will seek to determine this by analysing the expression of 44 genes and 20 proteins of inflammatory mediators in blood of patients with CTS and healthy age and sex matched controls.

2.1.1 Aims

The aim of this chapter will be to further determine the role of inflammatory mediators in neuropathic pain by investigating systemic immune dysregulation in patients with CTS and relate these findings to clinical phenotypes. Inflammatory mediators will be analysed at both the genetic

and protein level and comparisons will primarily be made (1) between patients with CTS pre-surgery and healthy controls and (2) between patients with CTS before and six months after surgery. The expression data will then be correlated with patients' symptoms to determine potential associations between markers of inflammation and symptomatology.

2.2 Methods

2.2.1 Participants

In this study I made use of an existing prospective longitudinal cohort study collected by Annina Schmid, including 60 patients with electrodiagnostically confirmed CTS. Patients were recruited from surgical waiting lists at Oxford University Hospitals NHS Foundation Trust. Patients were excluded from the study if electrodiagnostic testing revealed a nerve dysfunction other than CTS, if there was another medical condition affecting the upper limb/neck (e.g. hand osteoarthritis, cervical radiculopathy) or if there was a history of trauma to the upper limb or neck, if patients already had coexisting systemic disease (e.g. rheumatoid arthritis, diabetes) or were pregnant. Patients with CTS were assessed at baseline (before surgery) and six months following surgery. 21 healthy controls (proportionally age and sex matched) were included in the study and did not present with any systemic disease or have a history of medical conditions relating to the upper limb or neck. Healthy participants were recruited via public notice boards and media advertisement. All healthy controls attended one assessment. Informed written consent was obtained from all participants and ethical approval was given for the project (Riverside London ethics committee Ref 10/H0706/35).

2.2.2 Phenotypic data

A detailed description of the phenotypic data collected is available elsewhere.[76] In brief, age, sex and duration of symptoms were recorded for each individual. Symptom severity was evaluated with the Boston carpal tunnel questionnaire[39], which contains a symptom and function subscore (0 no symptoms/functional deficit, 5 severe symptoms /functional deficit,). Only the symptom questionnaire was used in this analysis. Neuropathic pain severity was evaluated with the Neuropathic Pain Symptom Inventory[40] (NPSI), which includes numerical rating scales (0 no pain to 10 worst pain imaginable) for burning, deep pressure pain, paraesthesia, paroxysmal symptoms, evoked pain as well as a composite total score (0-100). Severity of pain was also recorded over the past 24 hours on a visual analogue scale (VAS) (0 no pain, 10 worst pain imaginable). Surgical outcome was recorded on the Global Rating Of Change Scale, which ranges from -7 (a very great deal worse) to +7 (a very great deal better).[371] Standard electrodiagnostic testing (EDT) of the median nerve was performed with an ADVANCE^{TM} system (Neurometrix, USA). Electrodiagnostic test severity was graded on the scale derived by Bland et al, 2000[372] as follows: normal (grade 0), very mild (grade 1), mild (grade 2), moderate (grade 3), severe (grade 4), very severe (grade 5), extremely severe (grade 6). All patient clinical phenotype scores were collected by Annina Schmid.

2.2.3 Blood sampling and processing

Three ml of blood was sampled into RNA stabilising tubes (TempusTM blood RNA tube, Fisher Scientific, UK) and stored at -20° for batch processing. Blood serum was extracted from whole blood collected into a BD Vacutainer® SST™ tube for Serum collection (BD, UK). The blood was left to clot in the tube before being centrifuged at 3000 rpm for 10 minutes at 4^0c to separate the serum fraction. The serum fraction was aliquoted into cryotubes and stored at -80^0c for batch processing.

2.2.4 Gene expression

RNA was extracted from blood of 55 patients with CTS and 21 healthy controls via the Thermo-Fisher user protocol (Tempus™ Blood RNA Tube and Tempus™ Spin RNA Isolation Kit, publication number: 4379232). Briefly, samples were defrosted and PBS added to each sample, which was then vortexed and centrifuged. The RNA pellet was re-suspended and purified via column filtration. RNA was converted into cDNA using the EvoScript Universal cDNA Master kit (EvoScript Universal cDNA Master, 4th version, Cat. No. 07 912 455 001). All samples were used at 1200ng cDNA per reaction. If the initial RNA concentration was too low (n=26), samples and reaction mixes were doubled or tripled during cDNA synthesis to achieve the concentration of 1200 ng total cDNA. In three samples, there was not enough starting RNA. In these instances, the maximum concentration attainable was used (1134, 774 and 630 ng). Since data were normalised to housekeeping genes, the lower concentration in these samples would not affect the quantification of the target genes.

Custom made TAQMAN array microfluidic cards (ThermoFisher, UK) were designed containing 44 markers implicated with inflammation and neuropathic pain as well as 4 housekeeping genes. The gene list contained cytokines and chemokines, both anti and pro-inflammatory and immune cell markers such as CD3D, CD16 and CD14 to detect the presence of T cells, neutrophils and monocytes respectively. The full list of genes can be found in Table 2.1. The cards were run as instructed in the TaqMan® Gene Expression Assays—TaqMan® Array Cards user manual. In brief, 60ul of patient cDNA was mixed with 60ul of TAQMAN Fast Advanced Master Mix to achieve a final volume of 120µl and cDNA concentration of 10ng/µl. Paired patient samples from before and after surgery were processed on the same card with each assay being run in singlet. The cards were run on a Quantstudio 7 Flex Real-Time PCR System (Applied Biosystems, USA). Cycle times (Ct) for each gene in each sample were recorded and used in future analyses. Several studies have found that the expression of routinely used housekeeping genes such as GAPDH, B2M and HPRT1 can have variable expression depending on the tissue type and experimental

condition,[373-375] making them unsuitable for the use as housekeeping genes. Other genes such as

TRAP1, DECR1 and PPIB have been suggested to be stably expressed in blood[376, 377] and so where

chosen as housekeeping genes in my study.

Table 2.1: Gene and protein list of inflammatory mediators
The gene and proteins included in the analysis from patient blood is shown below. Genes shaded in grey indicate housekeeping genes.

Gene	Protein
IL-1β	IL-1β
IL-1RN/IL-1RA	
IL-2	IL-2
IL-4	IL-4
IL-5	
IL-6	IL-6
IL-7	
IL-8	IL-8
IL-9	IL-9
IL-10	IL-10
IL-12b	IL-12
IL-13	
IL-17a	IL-17A-F
IL-18	
IL-22	
IL-23	
IFN-γ	IFN-γ
TNF-α	TNF-α
CCL2	CCL2
CCL4	
CCL5	CCL5
CCL11	
CCL21	
CXCL5	CXCL5

CXCL9	
CXCL10	CXCL10
CXCL11	
CX3CL1/Fractalkine	CX3CL1
CSF3/G-CSF	GM-CSF
VEGF-a	VEGF
NGF	
TGF-β1	TGF-β
PDGFA	
CRP	CRP
TAC1/Substance P	
PTGES2	
MMP9	
CD3D	
CD14	
FCGR3B/CD16	
CD80	
CHI3L1	
NOS2	
TLR4	
TRAP1	
DECR1	
PPIB	
18S	

2.2.5 Protein expression

To determine if expression at the genomic level was translated to a change in protein production,

analysis of serum proteins from 55 patients with CTS and 20 healthy controls was conducted.

MSD U-PLEX plate custom biomarker multiplex assay kits (Meso Scale Diagnostics LLC, USA) were

custom designed to detect 18 selected cytokines/chemokines from the list of inflammatory genes

(Table 2.1). All patient samples and an 8 point standard curve were run in duplicate as per

standard protocol (UPLEX Human Multiplex Assays 18158-v5-2018May). Paired patient samples

(before and after surgery) were always processed on the same plate. Standards were composed

of known concentrations of each cytokine/chemokine being analysed and were used to calculate

the protein concentrations in each patient sample. Briefly, the method was as follows: each

capture antibody was combined to a specific linker molecule and incubated at room temperature

for 30 minutes. The linking reaction was inhibited with the addition of stop solution. Linked

capture antibodies were pooled together and 50µl was added to each well of the assay plate and

incubated at 4°C overnight on a shaker. The next day, the capture antibody solution was removed

and the plate was washed three times (PBS +0.05% tween20). The plates were tapped dry on

absorbent paper and 25µl assay buffer was added along with either 25µl patient/healthy control

serum or assay standards. The plates were incubated on a shaker at room temperature for 1

hour. Patient samples and standards were removed and the wells were washed 3x with wash

buffer. The plates were tapped dry and 50µl of detection solution was added to each well and

incubated for 1 hour at room temperature on a shaker. The plates were washed 3x with wash

buffer and 150µl of read buffer was added before the plates were read on a MESO QuickPlex SQ

120 plate reader (Meso Scale Diagnostics LLC, USA). For detection of TGF-β in serum, the samples

were acidified. This was needed as TGF-β is secreted as a pro-protein which is linked to a latency

associated protein (LAG),[378] this first needs to be removed before quantification. To achieve this, I

first treated with 20µl 1M HCl per 100ml and incubated at room temperature for 10 minutes. The

samples were then neutralised by adding 14µl of 1.2M NaOH in 0.5M HEPES buffer per 100µl of

sample.

As CCL5 was not available in the U-plex panel, the detection of CCL5 (RANTES) in serum was done

using MSD R-PLEX plates (Meso Scale Diagnostics LLC, USA). The standard protocol (R-PLEX

Antibody Sets Singleplex Assays, 18183-v6-2018Oct) was used. In brief the procedure was the same as described above with the exceptions that the serum samples were diluted 1:50. No specific linker molecules were used, instead, biotinylated anti CCL5 capture antibodies were used to bind the chemokine.

To detect Human C Reactive Protein (CRP) in serum, I used the R&D Systems CRP Quantikine ELISA kit (R&D Systems, US, Cat. No. DCRP00) and followed the standard protocol. Briefly, 100µl of assay diluent was added to each well, followed by 50µl of either standard or sample (serum samples were diluted 100 fold in calibrator diluent). I ran samples along with an eight-point standard curve in duplicate on all plates. The plate was sealed and incubated at room temperature for 2 hours. Sample or standards were then aspirated off and wells were washed 4x with 400µl wash buffer. 200 µl of human CRP conjugate (secondary antibody with horseradish peroxidase activity) was added to each well. I then sealed the plates and incubated them at room temperature for 2 hours. The plates were then washed 4x times with 400µl buffer and 200µl of substrate solution was added. The plates were sealed and incubated at room temperature for 30 minutes in the dark. 50µl of stop solution was added and the plates were read on a BMG FLUOstar Omega (BMG Labtech Ltd, UK) with the wavelength set to 450 nm.

2.2.6 Statistical Analysis

Molecular quantitative Polymerase Chain Reaction data were analysed in R[379], using the software package limma[380] to determine differential gene expression. Briefly, data were normalised to the average expression of housekeeper genes and batch effect correction was conducted on the data set. An empirical Bayesian method was used to shrink sample variances within the data. Data were then fit into a mixed linear model to determine differential gene expression using patient ID as blocking factor. Differential expression was firstly investigated between patients before

surgery and healthy controls, and secondly between patients before and after surgery. Results were corrected for false discovery rate (FDR) with Benjamini-Hochberg correction where an adjusted p value of <0.05 was considered significant. Only those genes that were significant after FDR correction and had a Log 2 fold change (Log2FC) greater than 1 were used in further analyses. IL-6 was significantly dysregulated and had a Log2FC just below 1 and so was included in further analysis.

Serum protein concentrations were determined by normalisation to assay standards of known concentrations. Mann-Whitney tests were used to compare serum protein levels between healthy participants and patients with CTS before surgery. Wilcoxon rank-sum tests were conducted to compare serum protein levels between patients with CTS before and after surgery. Results were corrected for false discovery rate using Benjamini-Hochberg correction where an adjusted p value of <0.05 was considered significant. Any protein remaining significant after FDR correction was included in further analyses.

To determine associations between gene expression and protein levels, gene expression and clinical phenotype and protein levels and clinical phenotype, Spearmans' rank correlation analyses were conducted with p values <0.05 considered significant. In these analyses both before and after surgery data were used. For duration of symptoms and GROC scores, only before and after surgery data was used respectively. P values from this analysis were not FDR corrected as these investigations were exploratory.

A secondary analysis was conducted for the genetic expression and protein data to determine if differential expression could be determined based on phenotypic data. For these analyses phenotypic data were binned (age: 0-59,60-85 years, sex: males and females, duration: 0-24, 25-500 months, Boston symptom score: 0-2.9, 3-5, NPSI composite score: 0-29, 30-100, VAS Pain: 0-2.9, 3-10, GROC: -7-5, 6-7, EDT grade, 0-3, 4-6). A mixed linear model was fit for gene expression,

for the protein analysis Mann-Whitney tests were used. Only one cytokine was differentially expressed based on age or sex from the mixed linear model and had a low Log2FC below 1 (Supplementary Table 1 in Appendix). Very few age and sex associations were determined by a two way ANOVA using protein data (Supplementary Table 2 in Appendix). As such, age and sex were not used as covariates in further analysis.

2.3 Results

2.3.1 Patient demographics and clinical data

The demographic and clinical data of patients with CTS and healthy controls involved in this study can be found in Table 2.2

Table 2.2: Demographic and Clinical Data
Demographic and clinical data are shown for patients with CTS and healthy controls. Healthy controls were age and gender matched to patients with CTS as determined by Kolmogorov-Smirnov independent samples test and Fisher's exact test respectively. Paired T tests were used to determine statistical differences in clinical phenotypes from before to after surgery.

	Healthy Controls	CTS Patients Pre	CTS Patients Post	P value
Number of Participants	21	55	55	
Mean Age (SD) [years]	59 (13.8)	63 (11.68)		0.57
Male:Female	7:14	18:37		1
	(66.7 % Female)	(67.3 % Female)		
Mean Duration of symptoms (SD) [Months]		68.36 (99.82)		
Median EDT grade [IQR]		3 [2]	2 [2]	<0.001
Boston symptom Score (SD)		2.68 (0.69)	1.44 (0.48)	<0.001

VAS Pain (SD)	2.51 (2.75)	0.44 (1.26)	<0.001
NPSI composite pain score (SD)	24.77 (17.01)	5.72 (10.75)	<0.001
NPSI burning pain (SD)	1.95 (2.91)	0.26 (0.87)	<0.001
NPSI deep pain (SD)	2.11 (2.73)	0.30 (0.97)	<0.001
NPSI evoked pain (SD)	1.45 (1.91)	0.75 (1.40)	0.025
NPSI Paraesthesia sensation (SD)	5.52 (2.87)	0.64 (1.66)	<0.001
NPSI Parsoxymal pain (SD)	1.68 (2.40)	0.59 (1.46)	0.009
Median GROC Score [IQR]		7 [1]	

SD = Standard Deviation, IQR = Inter-Quartile Range, EDT = Electrodiagnostic testing, VAS = Visual analogue scale. NPSI = Neuropathic pain symptom inventory, GROC = Global rating of change.

2.3.2 Inflammatory mediators are dysregulated at the genetic level

Table 2.3 contains the results of the gene expression analyses. Differential expression analysis between healthy controls and patients with CTS pre-surgery revealed a single gene (PTGES2) to be significantly dysregulated (p=0.0132). The PTGES2 gene encodes prostaglandin-E2 and was decreased in patients compared to healthy controls, however the Log2FC was below 1 (Log2FC=0.7). In the paired analysis (before vs after surgery), a total of 12 genes were found to be differentially expressed (all p <0.05). This included the genes IL-9, CCL5, PDGFA, IL-1β, CXCL5, TGFB1, VEGFA, IL-4, TLR4, FCGR3B, IL-6 and CD3D. Of these genes, IL-9 was increased post-surgery and was the only cytokine to have a Log2 fold change greater than 1 (Log2FC=-1.099). IL-6 was decreased after surgery and had a Log2 fold change just below 1 (Log2FC=0.92) and so was included in further analysis.

Table 2.3: Gene expression in patients with CTS pre-surgery compared to healthy controls and within patients with CTS from before to after surgery

A. Differential expression of genes between patients with CTS pre-surgery and healthy controls**.** B. Differential expression of genes between patients with CTS before and after surgery. Genes have been ranked in descending order based on adjusted p values. Significant dysregulation is indicated with grey shading where the adjusted P<0.05.

A

Gene	Log2FC	AveExpr	P.Value	Adj.p.Val
PTGES2	0.7172	7.3508	0.0003	0.0132
FCGR3B	-0.9934	6.7129	0.0022	0.0516
IL4	-0.6443	11.0769	0.0041	0.0551
CXCL5	-0.8798	6.2100	0.0046	0.0551
IL23A	0.3648	9.2615	0.0114	0.0761
CCL5	-0.4250	1.0649	0.0127	0.0761
CXCL8	-0.7565	6.5907	0.0120	0.0761
IL12B	-0.9411	16.5997	0.0084	0.0761
DECR1	-0.2576	4.1709	0.0203	0.0950
IL1B	-0.4282	5.9645	0.0218	0.0950
TLR4	-0.4309	5.1541	0.0186	0.0950
TGFB1	-0.2175	2.5946	0.0577	0.2239
PDGFA	-0.3876	9.6482	0.0606	0.2239
CXCL10	0.5005	10.6873	0.0714	0.2447
IL9	-0.6090	16.8580	0.0795	0.2544
IFNG	-0.3273	9.7282	0.1136	0.3027
MMP9	-0.3619	4.8195	0.1028	0.3027
IL6	-0.9653	11.8264	0.1158	0.3027
CX3CL1	-2.5594	15.6411	0.1234	0.3027
IL22	-4.5496	14.2196	0.1261	0.3027
CRP	2.0356	15.7354	0.1564	0.3574
18S	0.2072	-9.7609	0.1723	0.3760
CCL2	0.4044	13.0409	0.1807	0.3772
CCL21	5.5908	15.3015	0.2120	0.4240
CHI3L1	-0.4129	6.0407	0.2405	0.4617

	Log2FC	AveExpr	P.Value	Adj.p.Val
NOS2	-0.6876	15.8343	0.2529	0.4670
IL10	0.2794	13.8695	0.2783	0.4797
CD80	-0.2246	11.5727	0.2798	0.4797
IL7	-0.1585	9.5757	0.4228	0.6998
TNF	-0.2001	6.4277	0.4433	0.7094
CD14	0.1081	8.2658	0.4705	0.7286
VEGFA	0.0888	9.7152	0.5118	0.7444
CCL4	-0.1478	6.9467	0.4998	0.7444
CXCL11	0.1315	11.7226	0.5282	0.7457
CD3D	0.0837	4.3878	0.5461	0.7460
PPIB	-0.1530	3.9356	0.5664	0.7460
IL13	-0.1817	16.1530	0.6062	0.7460
IL17A	-1.1482	15.8197	0.6054	0.7460
CCL11	-8.9839	7.2127	0.5936	0.7460
TRAP1	0.0504	5.5901	0.6239	0.7487
TAC1	4.4387	-1.0813	0.6395	0.7487
NGF	3.8337	8.1875	0.6737	0.7699
IL18	-0.1516	6.8301	0.7160	0.7992
IL1RN	1.0467	11.7885	0.8076	0.8810
IL2	-0.0423	13.3034	0.8485	0.8915
CXCL9	-0.0463	11.2973	0.8543	0.8915
CSF3	0.3872	14.0298	0.9127	0.9322
IL5	0.0917	13.6599	0.9737	0.9737

B	Gene	Log2FC	AveExpr	P.Value	Adj.p.Val
	IL9	-1.0990	16.2636	0.0003	0.0143
	CCL5	-0.2410	0.8270	0.0025	0.0269
	PDGFA	-0.2691	9.4066	0.0028	0.0269
	IL1B	-0.3332	5.6795	0.0021	0.0269
	CXCL5	-0.5454	5.6942	0.0015	0.0269

TGFB1	-0.1555	2.4568	0.0053	0.0312
VEGFA	-0.2004	9.6396	0.0065	0.0312
IL4	-0.3055	10.7461	0.0059	0.0312
TLR4	-0.3102	4.8800	0.0056	0.0312
FCGR3B	-0.4466	6.2151	0.0059	0.0312
IL6	0.9219	12.0206	0.0077	0.0335
CD3D	0.1714	4.4966	0.0097	0.0387
IL10	-0.2891	13.8021	0.0256	0.0946
IL13	-0.3828	15.8937	0.0291	0.0998
MMP9	-0.2526	4.5932	0.0315	0.1008
CHI3L1	-0.2778	5.7877	0.0582	0.1746
CXCL10	-0.2401	10.7056	0.0642	0.1812
TRAP1	-0.0824	5.5628	0.0942	0.2511
NOS2	-0.8622	15.2322	0.1090	0.2753
CXCL8	-0.1767	6.2933	0.1488	0.3570
CX3CL1	1.4831	15.6233	0.1810	0.4138
IL18	-0.3195	6.6284	0.2269	0.4950
CSF3	2.0166	15.5522	0.2602	0.5429
IL22	2.2109	13.8817	0.3059	0.6118
PPIB	-0.1649	3.8109	0.3603	0.6918
CCL11	15.4281	9.3608	0.3937	0.7268
TNF	0.1036	6.4242	0.4493	0.7987
DECR1	0.0378	4.1186	0.4875	0.8358
TAC1	6.5492	0.4854	0.5445	0.8366
18S	0.0447	-9.6813	0.5444	0.8366
IL2	-0.0681	13.2577	0.5403	0.8366
CCL2	-0.0800	13.1126	0.5577	0.8366
IL12B	-0.1835	16.1009	0.5869	0.8537
NGF	1.0493	10.7297	0.7727	0.8626
IL1RN	0.9726	12.9838	0.6938	0.8626
CRP	0.1334	16.4570	0.7164	0.8626

CXCL11	0.0303	11.7740	0.7693	0.8626
CD14	0.0262	8.3087	0.7289	0.8626
IL7	-0.0304	9.5167	0.7393	0.8626
CD80	-0.0355	11.4929	0.7166	0.8626
CXCL9	-0.0461	11.2615	0.7081	0.8626
PTGES2	-0.0473	7.5253	0.6376	0.8626
IL17A	-0.7184	14.8202	0.6417	0.8626
IL5	0.1944	13.7847	0.9033	0.9752
CCL21	0.0928	16.5193	0.9549	0.9752
CCL4	0.0065	6.9091	0.9468	0.9752
IFNG	-0.0096	9.6330	0.9211	0.9752
IL23A	0.0021	9.3633	0.9776	0.9776

Log2FC = Log2 Fold Change, AveExpr = Average Expression, P.Value = p value, Adj.p.Val = Adjusted P value.

2.3.3 Mediators of inflammation are dysregulated in the serum of patients with CTS from before to after surgery and between patients with CTS before surgery and healthy controls

The serum concentrations of immune mediators between healthy participants and patients with CTS before surgery revealed two mediators that were significantly dysregulated. Both TGF-β (p=0.016) and CCL5 (p=0.047) were increased in patients with CTS pre-surgery compared to healthy controls (Figure 2.1). Paired analysis of inflammatory mediator expression in patients with CTS from before to after surgery identified IL-4 as being increased pre-surgery (P=0.002) (Figure 2.2). The cytokine IL-9, previously identified as being significantly dysregulated at the genetic level showed a comparable trend at protein level, although this failed to reach significance after stringent FDR correction (p=0.09). IL-6 was not found to be significantly dysregulated (p=0.24, Figure 2.2).
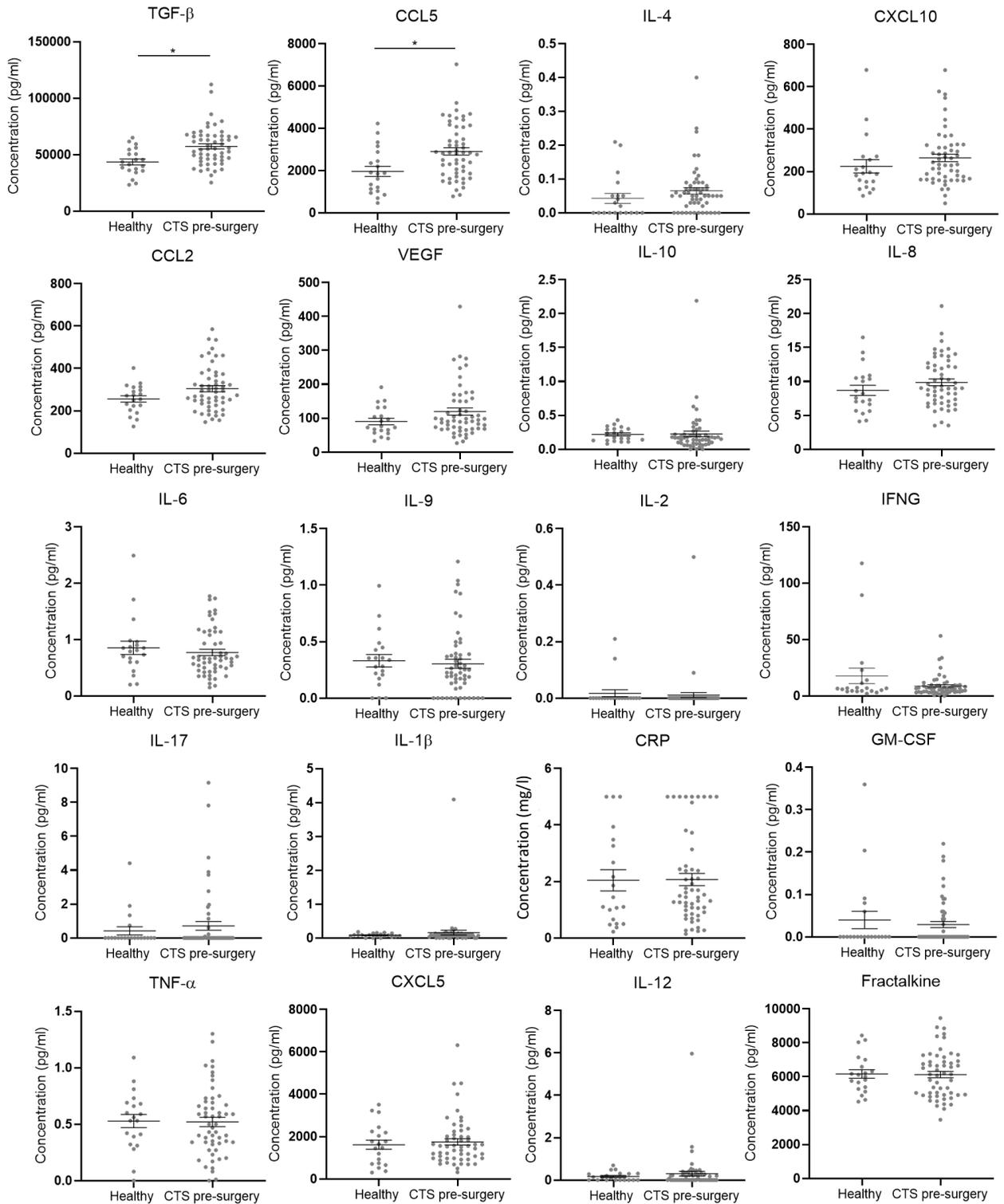
Figure 2.1: Serum inflammatory mediator expression between patients with CTS pre-surgery and healthy controls

TGF-β and CCL5 concentrations were increased in patients with CTS. Protein concentration is given in pg/ml except for CRP which is given in mg/l. Mann-Whitney U tests were used to determine statistical differences between patients with CTS and healthy controls. Data are shown as mean +/- SEM and single datapoints. Significant dysregulation is indicated with *P<0.05.
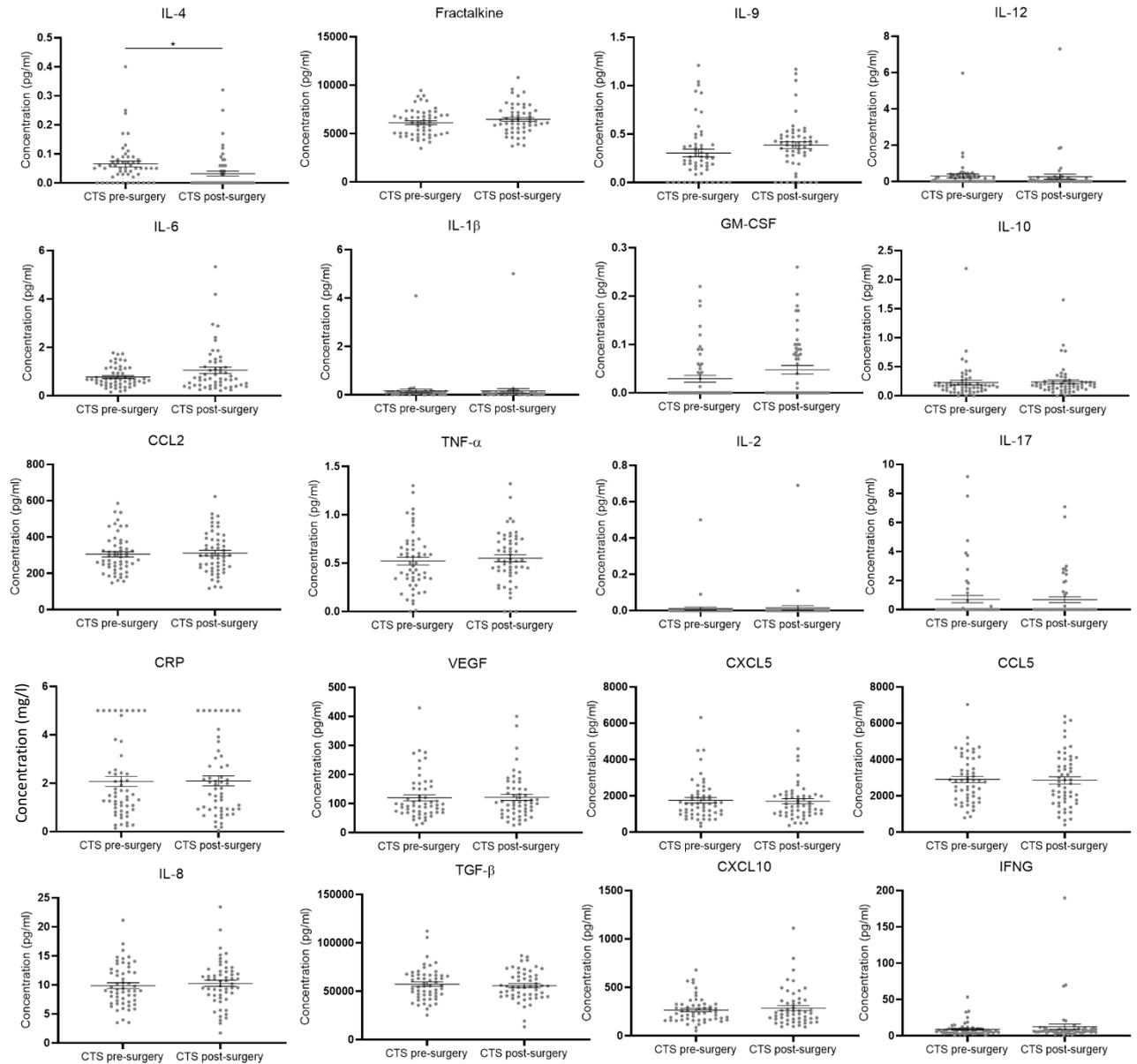
Figure 2.2: Serum inflammatory mediator expression in patients with CTS from pre to post-surgery

IL-4 was significantly increased pre-surgery compared to post. Protein concentration is given in pg/ml except for CRP which is given in mg/l. Wilcoxon tests were used to determine statistical differences between groups. Data are shown as mean +/- SEM and single data points. Significant dysregulation is indicated with *P<0.05.

2.3.4 Gene expression of inflammatory mediators do not correlate with serum protein concentration

No significant correlations were found between the gene and protein expression of any of the differentially expressed cytokines (IL-4, IL-6, IL-9, TGF-β and CCL5), indicating that the level of RNA expression of these genes does not necessarily translate to protein production (Figure 2.3, Table 2.4).
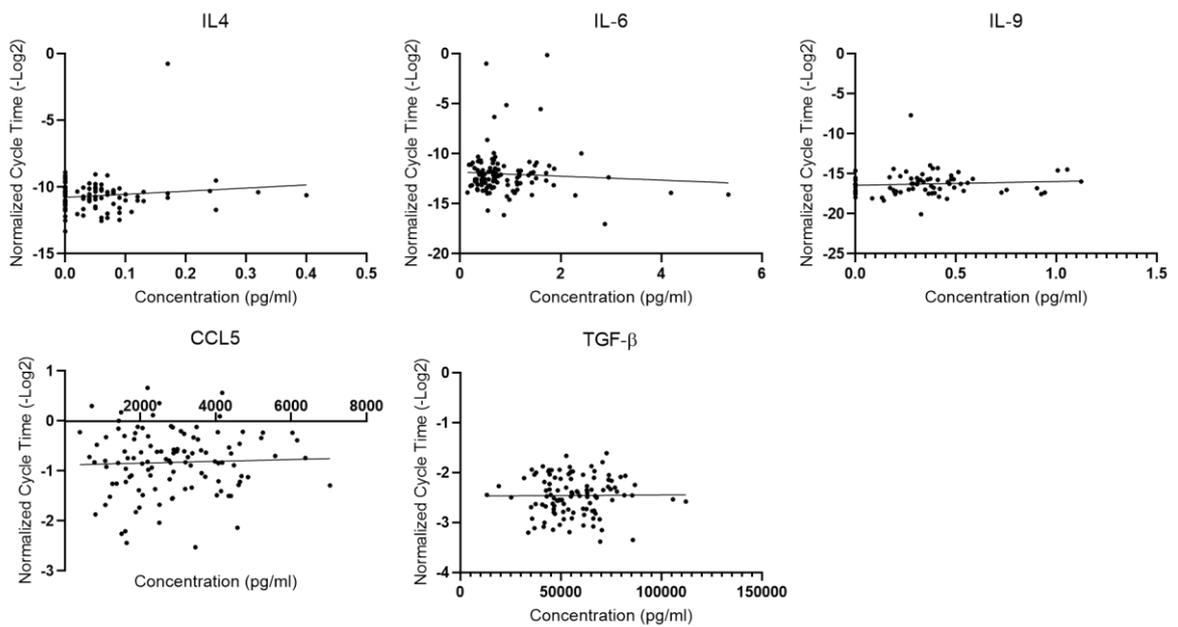
Figure 2.3: Correlation analysis for gene expression and serum protein concentration
Correlation analysis was conducted on the gene expression and protein concentration of the inflammatory mediators IL-4, IL-6, IL-9, CCL5 and TGF-β. No significant correlations were identified for any of the inflammatory mediators. Both pre and post-surgery data were used in the analysis. Spearman correlation was used with $p<0.05$ being considered significant.

Table 2.4: Correlation data for gene and protein expression
Correlation analysis was done for the gene and protein expression of inflammatory mediators. Only those which were differentially expressed (and or had a Log2FC>1 from the genetic analysis) were included in the analysis. The Spearman correlation coefficients (r) and p values (p) for correlations between the gene and protein expression of IL-4, IL-6, IL-9, CCL5 and TGF-β are shown.

	r	p
IL-4	-0.02	0.80
IL-6	-0.03	0.76
IL-9	0.13	0.28
CCL5	0.03	0.74
TGF-β	0.036	0.71

2.3.5 Systemic inflammatory mediators correlate with clinical pain phenotypes

Correlation analyses, using both before and after surgery data for mRNA expression and clinical phenotype scores revealed a significant correlation of IL-6 gene expression and duration of symptoms (r=0.36, p=0.0072, Figure 2.4). No significant correlations were observed between IL-6 expression and phenotype scores reflecting pain severity, such as the Boston symptom questionnaire, VAS Pain and NPSI scores (Table 2.5). In contrast, IL-9 expression was associated with several pain scores (Figure 2.4, Table 2.5). There was a negative correlation with the Boston symptom score, VAS Pain and several NPSI scores including the composite and subdomains for burning and deep pain as well as paraesthesia. IL-9 also negatively correlated with EDT grade, indicating higher IL-9 expression in patients with less severe CTS. Differential gene expression analysis of inflammatory mediators was also conducted based on the phenotypes listed in Table 2.2. In this analysis only CCL5 showed significant differential gene expression but had a low Log2FC of below 1 (Supplementary Table 1, Appendix).
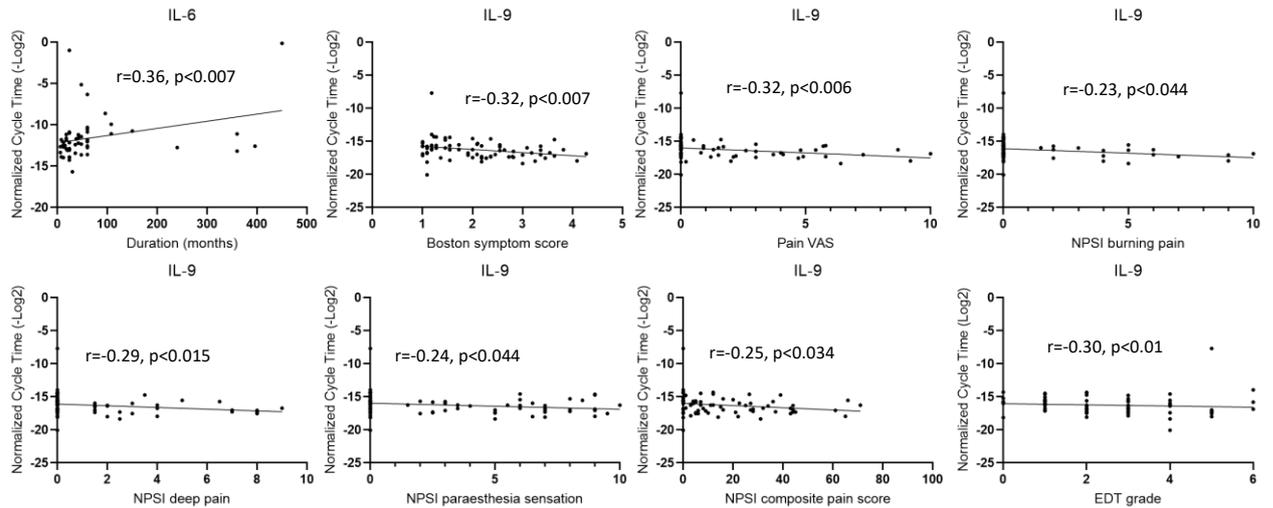
Figure 2.4: Correlation analysis for IL-6 and IL-9 gene expression and clinical phenotypes
Correlation analyses for IL-6 and IL-9 gene expression with patients' clinical phenotypes. IL-6 positively correlated with duration of symptoms only. IL-9 negatively correlated with EDT grade, Boston symptom score, VAS Pain, NPSI burning, deep pain, paraesthesia and the NPSI composite score. Both pre and post data were used in the analysis. Spearman rank correlation was used with a $p < 0.05$ being considered significant. VAS = Visual analogue scale, NPSI = Neuropathic pain symptom inventory, EDT = Electrodiagnostic testing.

I next conducted correlation analysis using before and after surgery data for serum concentrations of IL-4, IL-9, TGF-β and CCL5 with clinical phenotypes. Correlation analysis was done on these cytokines as they were either significantly dysregulated or indicated a trend for dysregulation by being significant before FDR correction. Correlation coefficients and p values are shown in Table 2.5. No significant correlations were found between protein expression and clinical phenotype scores for CCL5 or TGF-β. IL-9 serum concentration negatively correlated with three components of the NPSI questionnaire (NPSI paraesthesia sensation, NPSI paroxysmal pain and NPSI composite score) (Figure 2.5, Table 2.5). In addition, IL-4 was found to positively correlate with pain specific phenotype scores including the Boston Symptom questionnaire, the NPSI composite score and NPSI deep pain (Figure 2.5, Table 2.5). IL-4 also correlated with EDT

grade (Figure 2.5, Table 2.5). Correlation analysis was also done using only the pre-surgery values

for patients with CTS and phenotype scores. However, from these analyses only IL-6 was found to

weakly correlate with NPSI evoked scores. The data for this analysis is shown in Supplementary

Table 3 in the Appendix. Differential expression based on the phenotypes scores was also

conducted for inflammatory mediators in the serum but no significant dysregulation was found

(Supplementary Table 4, Appendix).
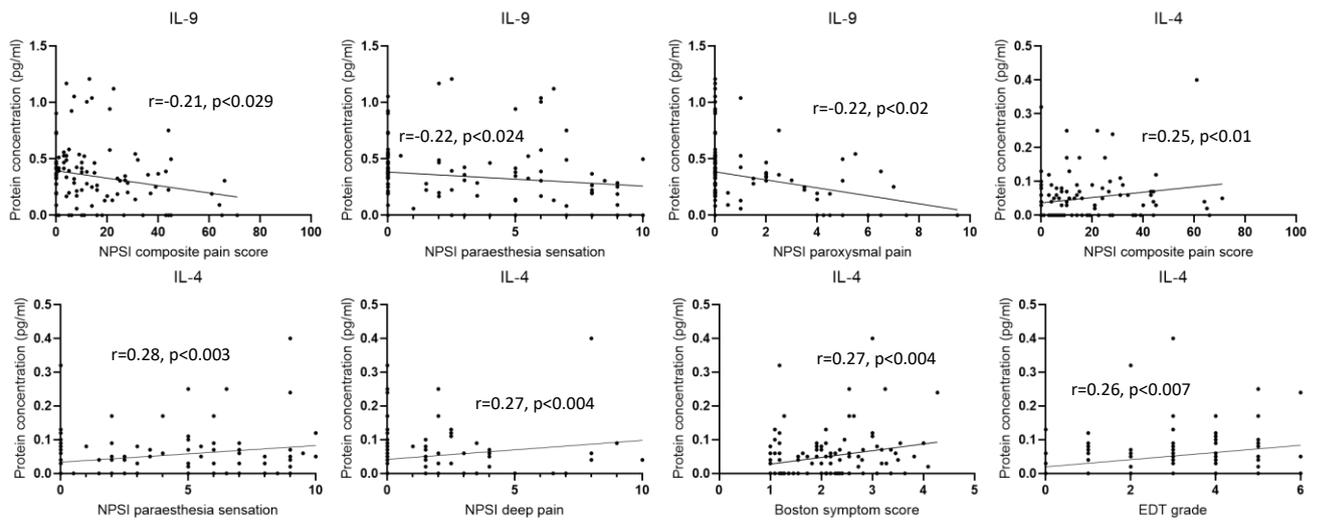
Figure 2.5: Correlation analysis for IL-4 and IL-9 protein expression and clinical phenotypes
Significant correlations are shown for IL-4 and IL-9 protein concentration with clinical phenotype scores. IL-9 negatively correlated with the NPSI composite score as well as the subdomains NPSI paraesthesia and NPSI paroxysmal pain. IL-4 positively correlated with the NPSI composite score, the subdomains NPSI paraesthesia and NPSI deep pain as well as the Boston symptom score and EDT grade. Both pre and post data were used in the analysis. A Spearman correlation was used with a p<0.05 being considered significant. NPSI = Neuropathic pain symptom inventory, EDT = Electrodiagnostic testing.

Table 2.5: Correlation data for gene and protein expression with phenotype scores

Spearman correlation coefficients (r) and p values (p) are shown for the correlation analyses of inflammatory mediator expression and phenotype scores. Both pre and post data were used in this analysis, for duration of symptoms and GROC scores only post and pre data was used respectively. Significant correlations are indicated with grey shading.

	IL-6 (Gene)		IL-9 (Gene)		IL-9 (Protein)		IL-4 (Protein)		CCL5 (Protein)		TGF-β (Protein)	
	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value
Boston symptom score	0.045	0.64	-0.32	0.007	-0.17	0.08	0.27	0.0042	0.054	0.57	0.073	0.45
VAS Pain	-0.0024	0.98	-0.32	0.0064	-0.15	0.12	0.16	0.093	0.018	0.85	0.14	0.15
NPSI (burning)	-0.048	0.62	-0.24	0.045	-0.12	0.20	0.17	0.07	-0.032	0.74	0.017	0.86
NPSI (deep)	0.035	0.72	-0.29	0.015	-0.16	0.11	0.27	0.0044	-0.0059	0.95	0.069	0.48
NPSI (evoked)	-0.12	0.20	-0.11	0.37	-0.13	0.19	-0.0096	0.92	0.057	0.56	0.094	0.33
NPSI (paraesthesia)	0.088	0.36	-0.24	0.045	-0.22	0.024	0.28	0.0031	-0.036	0.71	-0.084	0.38
NPSI (paroxysmal)	-0.087	0.37	-0.12	0.32	-0.22	0.02	0.11	0.24	-0.084	0.38	-0.044	0.65
NPSI (composite)	0.022	0.82	-0.25	0.034	-0.21	0.029	0.25	0.01	-0.10	0.28	-0.031	0.75
EDT grade	-0.0069	0.94	-0.30	0.01	-0.16	0.10	0.26	0.0073	0.11	0.26	0.06	0.54
GROC score	0.14	0.30	-0.20	0.27	-0.55	0.69	0.14	0.32	0.26	0.051	0.22	0.11
Duration	0.36	0.0072	-0.013	0.94	-0.12	0.38	0.27	0.05	0.17	0.21	-0.05	0.71

VAS = Visual analogue scale, NPSI = Neuropathic pain symptom inventory, EDT = Electrodiagnostic testing, GROC = Global rating of change.

2.4 Discussion

This chapter sought to investigate systemic markers of inflammation in patients with focal nerve injury and neuropathic pain and relate these findings to healthy controls and patients' phenotypes. IL-6 and IL-9 were the two main cytokines identified as dysregulated at the gene level in patients with CTS from before to after surgery, where IL-6 was increased pre-surgery and IL-9 was increased post-surgery. Analysis of inflammatory serum mediators revealed both CCL5 and TGF-β to be significantly increased in patients with CTS before surgery compared to healthy controls. Serum IL-4 was found to be increased in patients with CTS before surgery compared to after surgery. Correlation analyses identified IL-6 gene expression to be positively correlated with duration of symptoms, while IL-9 was negatively correlated with several patients' phenotype scores representing pain and electrodiagnostic test severity at both the genetic and protein level. Similarly, serum concentrations of IL-4 positively correlated with patients' phenotype scores representing pain and electrodiagnostic test severity.

2.4.1 IL-9 expression indicates a pro-resolution role in patients with CTS

The most striking findings were that of IL-9, which showed a significant increase in gene expression post-surgery, a similar trend at protein level and negative associations with a range of pain severity scores. The post-operative condition of CTS is reflective of a state of recovery, where CTS has resolved and the affected nerve is in the process of repair.[76] The post-operative increase in IL-9 may therefore highlight a role for this cytokine in resolution and the repair process. This notion is supported by the identification of several negative correlations between IL-9 expression from both the genetic and protein analyses and patients' phenotype scores. As IL-9 expression was found to negatively correlate with several pain specific phenotypes, it may have an impact on the generation or maintenance of neuropathic pain in these patients. IL-9 may therefore have an

anti-nociceptive role in neuropathic pain and work to decrease pain signalling, potentially through

mediation of the immune system. Overall for the majority of the correlations with IL-9 and clinical

phenotypes, the level of association was generally weak and so further validation is required to

confirm the associations of IL-9 and clinical phenotypes. IL-9 is a relatively understudied cytokine

with limited literature describing its function and implication in disease. However a recent study

has identified IL-9 as being increased in the serum of patients with intervertebral disk herniation

and chronic radicular back pain compared to healthy controls.[381] They also state that IL-9

concentrations were significantly increased in patients with severe disk herniation compared to

patients with mild disk herniation. However on close inspection of their data it is apparent that IL-

9 levels were actually significantly increased in patients with mild disk herniation compared to

those with severe disk herniation, indicating instead that IL-9 may have a protective role,

preventing a more severe condition. They also did not find a significant association of IL-9 levels

and pain, further indicating that IL-9 was not contributing to symptom severity.[381] This may

provide further evidence of a pro-resolution role for IL-9 in entrapment neuropathy. Other

available literature on IL-9 showcases its pleotropic nature, with the cytokine having involvement

in inflammatory conditions both in a pro and anti-inflammatory capacity.[237, 382-384] This is

highlighted in studies of patients with inflammatory bowel disease, where increased IL-9 serum

concentration correlated with a less favourable prognosis and increased disease severity[385, 386].

These studies show the pro-inflammatory actions of IL-9 in autoimmune and inflammatory

conditions. However, IL-9 has also been shown to have pro-resolution effects. Compelling data is

now emerging implicating IL-9 in the activation and activity of regulatory T cells (Tregs).[384, 387] In a

mouse model of rheumatoid arthritis, IL-9 enhances the resolution of inflammation by activating

innate type two lymphoid cells (ILC2s), which in turn increase the activity of Tregs. In line with

this, ILC2 cell numbers were increased in the synovial tissue of patients with rheumatoid arthritis

in clinical remission and correlated with disease activity in these patients. This highlights an

interesting mechanism by which IL-9 may indirectly increase the potency of pro-resolution Tregs

to combat inflammation. The pro-resolution effects of IL-9 with regards to the activity of Treg cells has been shown in another study using cultured Treg cells from mice where the application of IL-9 enhanced the suppressive capabilities of Treg cells.[388] The anti-inflammatory cytokines that I investigated in this study, such as IL-10, IL-13 and IL-1 receptor antagonist, did not show any dysregulation between patients and controls or have any association with clinical phenotypes in the genetic or protein analysis. Mediators of immune resolution have not readily been investigated in CTS, however IL-10 has been shown to be increased in patients with mild sciatica compared to those with severe sciatica or healthy controls.[315] IL-10 also negatively correlated with Oswestry Disability Index (ODI) scores in these patients.[315] Overall IL-9 presents itself as an interesting cytokine in the resolution of CTS. In future work, it would be useful to determine the cellular source of IL-9 and the mechanism by which IL-9 is acting, and whether this is a Treg related mechanism. Considering the negative correlation of IL-9 with pain severity, further work may seek to determine whether IL-9 has any therapeutic utility to treat patients with neuropathic pain clinically.

2.4.2 Signs of a pro-inflammatory phenotype before surgery

I found IL-6 to have differential gene expression where it was increased before compared to after CTS surgery in this cohort. IL-6 is an important pro-inflammatory cytokine that is involved in a range of inflammatory and autoimmune conditions and signals to generate and maintain inflammatory environments.[389-392]As IL-6 was increased during the active state of the disease, it could thus be maintaining an inflammatory environment contributing to the pathogenesis of CTS, while it reduces during postoperative recovery. IL-6 expression in the serum of patients with CTS has previously been investigated, with mixed findings. Some reported IL-6 to be increased in patients with CTS compared to patients without CTS[368] while others found no increase in serum IL-6 in their CTS cohorts compared to healthy controls.[50, 75, 367] Similarly, I also did not find any

difference in IL-6 serum levels between healthy controls and patients with CTS. Indeed the majority of my findings were generally observed when comparing between patients with CTS before and after surgery, I will discuss this phenomenon in more detail later. In my study, IL-6 was not found to be associated with pain symptoms, which is in agreement with previous findings.[75, 367] However in lumbar radiculopathy IL-6 has been frequently found to be associated with worse symptoms[315] and more intense pain,[314, 393] implicating a role for IL-6 in the development of neuropathic pain in entrapment neuropathy. The reason for the discrepancy in the action of IL-6 in two related entrapment neuropathies is unclear. However, it could be down to the severity of impact on the affected nerve in each condition. As the severity of symptoms in patients with lumbar radiculopathy is generally high[25, 26] this could indicate increased damage to the compressed nerve. These nerves could therefore be more exposed to inflammatory mediators, resulting in increased symptom severity. *In-vitro* and *in-vivo* experiments have shown that IL-6 is able to sensitise nociceptive neurons to noxious stimuli.[133, 394, 395] The association with IL-6 and increased pain in patients with lumbar radiculopathy could therefore be the result of direct IL-6 action on the compressed nerve. As the injury to the nerve in CTS is mild[396] IL-6 may not be directly acting on the damaged median nerve and so the pain experienced by patients with CTS could be generated through other mechanisms. Another explanation is that the IL-6 gene is not being translated into an active protein, indicated by the low expression in the serum analysis. If limited functional protein is being produced, the involvement in the pathogenesis of CTS and the resulting phenotypes may be limited. Interrupting IL-6 signalling has previously been used in humans to treat neuropathic pain. Studies using Tocilizumab, a humanised antibody that binds the IL-6 receptor (IL-6R), to treat patients with sciatica and discogenic low back pain, found significant reductions in pain sensations compared to control therapy.[397, 398]

Serum IL-4 was also increased before compared to after surgery and so may be contributing to the pathogenesis of CTS. IL-4 has many actions within the immune system, playing a major role in the differentiation of naïve CD4+ T cells towards the Th2 lineage[223, 399, 400]. This shows IL-4 as

having major implications in allergic responses and in mediating immune defence against parasitic infection[219, 220, 224]. However from these investigations it cannot be known whether the IL-4 identified here exerts it effects through a Th2 mechanism during the active phase of CTS. Serum IL-4 concentrations positively correlated with EDT grade and several pain scores. These correlations indicate an association between IL-4 and a more severe disease phenotype and pain sensations. This is somewhat contradictory to previously published data identifying IL-4 as a key mediator in reducing neuropathic pain behaviours in models of peripheral nerve injury.[134, 401] However as the expression of IL-4 in the serum was low (<0.5 pg/ml) the clinical relevance of IL-4 to CTS remains limited. Taken together my findings of increased IL-4 and IL-6 expression before surgery suggests an increase in immune activity and cytokine production that could be contributing towards the pathogenesis of the disease.

2.4.3 Immune dysregulation in CTS compared to healthy controls

The majority of my findings were observed when investigating differences from before to after surgery in patients with CTS. This may be down to the paired analysis, where differences in cytokine expression were measured within the same patients in different conditions. As repeated measurements from the same individual are related, the variability between subjects can be isolated, allowing for the detection of subtle changes within a patient over the course of treatment.[402] When comparing between patients and controls, the biological measurements are independent and due to the high variability in cytokine expression in humans,[403] larger effects may be needed for a significant change to be detected. However differences were observed in cytokine expression between patients with CTS before surgery and healthy controls. CCL5 and TGF-β levels both increased in patients with CTS before surgery. CCL5 is a potent activator of T-cell activity[404, 405] and is involved in inflammatory disease conditions[406, 407, 408, 409]. In line with my findings, CCL5 has previously been identified as being increased in the serum of patients with CTS

compared to healthy controls, where CCL5 alone was able to discriminate patients with CTS using discriminant function analysis.[75] CCL5 is a strong chemotactic molecule for T cells[410, 411] and so could be functioning to recruit T cells to the site of nerve injury in CTS, which suggests a role for T-cell mediated inflammation in the pathogenesis of CTS. I was not able to detect any significant correlations for CCL5 with any clinical phenotypes. CCL5 has however previously been implicated in the generation of neuropathic pain in experiments treating mice with the CCL5 antagonist met-RANTES[412] or by using CCL5 knockout mice[413]. Investigators were able to reduce behavioural sensitivity to nociceptive stimuli after nerve injury, reduce infiltrating immune cells and decrease the secretion of pro-inflammatory cytokines.[412, 413] Interestingly however a previous study has found that serum CCL5 expression in patients with CTS negatively correlated with DN4 and NPSI composite scores,[75] and previous animal models have shown that CCL5 can be involved in the resolution of inflammation by encourage a pro-resolution phase in macrophages in a mouse model of peritonitis.[414] the exact role that CCL5 plays in the pathogenesis of CTS therefore remains uncertain.

TGF-β has previously been implicated in the pathogenesis of CTS, though mainly as a driver of fibrotic change due to increases in the local tissue expression of TGF-β.[58, 415-417] TGF-β has been shown to cause increased expression of collagen sub-types which drive fibrotic thickening in the carpal tunnel, and is thought to be a major component in the pathogenesis of CTS.[58] The fibrotic action of TGF-β is also well established in other conditions such as lung fibrosis[418] and arteriosclerosis[419], though it has not been reported previously in other entrapment neuropathies. In previous CTS studies, increases in TGF-β expression were identified in tissue closely surrounding the affective nerve such as sub-synovial connective tissue. In my study I detected systemic differences in TGF-β expression, which is distant from the site of injury, taken together this provides strong evidence for the involvement of TGF-β in the pathogenesis of CTS. As it was not the main focus of my study, the direct actions of TGF-β on fibrotic changes were not investigated here. TGF-β also has many immunomodulatory effects and can function as an

immunoregulatory cytokine,[420, 421] producing pro-inflammatory effects,[422, 423] and can act as a chemoattractant for immune cells including monocytes[424] and neutrophils.[425] TGF-β may then also have a role in modulating the immune system in patients with CTS, driving inflammation in these patients. Ultimately, my findings highlight the potential role of immune dysregulation in CTS as increased inflammatory mediator expression was found in patients with CTS compared to healthy controls. These results are in agreement with previous studies highlighting both CCL5 and TGF-β to be important mediators involved in the pathogenesis of CTS, and show that there are biological differences between patients with CTS and healthy controls that can be detected systemically.

2.4.4 Differentially expressed cytokines are involved in naïve CD4+ T-cell differentiation

An interesting feature of several of the dysregulated inflammatory mediators identified here is that they are known to be involved in naïve CD4+ helper T-cell (Th) differentiation (See Figure 1.6 in the general introduction). As discussed previously, IL-4 is known to be involved in differentiating naïve CD4+ T cells towards the Th2 lineage[223, 400]. IL-4 in combination with TGF-β directs naive CD4+ T cells into Th9 cells and TGF-β in combination with IL-6 induces naïve CD4+ T cells into Th17 cells, which are heavily implicated in autoimmunity and inflammation[228, 426-428]. TGF-β alone or with IL-2 directs naïve CD4+ T cells into Tregs[240, 429, 430]. The cytokines identified here may therefore be working in combination to orchestrate specific sub-populations of CD4+ Th cells that could be involved in the pathogenesis of CTS. Previous flow cytometric analysis of peripheral blood in patients with CTS identified CD4+ T-cell effector memory and central memory populations to be increased in patients with CTS compared to healthy controls[75]. These changes in the populations of CD4+ T cells further highlight the potential role that CD4+ subgroups might play in CTS. As IL-9 was differentially expressed and increases in IL-4 and TGF-β were observed, this could indicate the presence of Th9 cells in patients with CTS. However, as IL-9 was increased

post-surgery while IL-4 and TGF-β were increased pre-surgery, it seems unlikely that Th9 is the cellular source of IL-9 due to the discrepancy in the time of expression of the cytokines. Further, Th9 cells are implicated as being pro-inflammatory[237, 382] and as IL-9 negatively correlated with clinical phenotypes, it seems doubtful that IL-9 is being produced by Th9 cells. To further elucidate this, flow cytometry could be conducted using peripheral blood to determine whether Th9 cells are present in patients and whether the population of Th9 cells changes before/after surgery or is different from healthy controls. Investigations into other Th subgroups might provide information as to the source of other dysregulated cytokines. The involvement of CD4+ sub-groups in the pathogenesis of CTS nevertheless presents itself as an interesting area of study, which is addressed in subsequent chapters.

2.4.5 Discrepancies between mRNA and protein expression

In this study, dysregulated inflammatory mediators in the genetic analyses did not show corresponding dysregulation in the serum. The discrepancy between gene expression and protein concentration is a phenomena that has been commonly noted[431, 432]. There are many molecular mechanisms involved in mRNA and protein regulation that can affect their expression[433-435]. One of the greatest factors affecting protein regulation is the rate of protein degradation and the protein half-life.[436, 437] It has previously been found that the protein half-life accounts for a large amount of variability in the correlation between mRNA abundance and protein concentration.[436] Cytokines are known to have short half-lives, particularly IL-6[438, 439], and so this may account for some of the discrepancies observed between mRNA and protein concentrations, and explain why genes dysregulated in the genetic analysis are not found to be dysregulated at the protein level.

2.4.6 Limitations

A limitation of this study if that the cellular sources of the cytokines and the target cells with which the cytokines act upon are unknown. Knowing which cells are expressing the dysregulated cytokines would provide valuable insight into the pathogenesis of the condition, if immune cells

such as T cells were the main cytokine producing cell in CTS this would then implicate inflammation as a main factor in the pathogenesis of the condition and could have implications into how the disease is treated. If cells from within the carpal tunnel such as Schwann cells or those making up the tenosynovium were the main source of cytokine production, this could indicate that cytokines are being produced and working in an autocrine or paracrine fashion to produce effects locally within the carpal tunnel, which may not involve the influx of immune cells. Determining the cellular source of the cytokines would therefore prove useful in fully defining the role of inflammation in CTS. Another issue with measuring cytokine expression in the blood of patients with CTS, is that it is removed from the site of injury and so may not accurately reflect the local environment of the carpal tunnel. As such, cytokines that are heavily involved in the pathogenesis of CTS and act locally within the carpal tunnel, but do not circulate at high levels in the blood, would be missed from this analysis. Finally the list of inflammatory mediators investigated in my analysis was not exhaustive and were limited by feasibility and cost, so only a selection of cytokines were investigated. Important mediators in CTS may be missing from my analysis and future experiments could seek to investigate a larger array of mediators to achieve a more comprehensive coverage of cytokines.

2.4.7 Conclusions

Investigating the systemic expression of inflammatory mediators revealed an increase in IL-9 post-surgery at the genetic level, with a similar trend at protein level. IL-9 also negatively correlated with several pain specific phenotypes indicating IL-9 to be associated with decreased pain and less severe symptoms. This suggested a pro-resolution role for IL-9 in CTS. A pro-inflammatory phenotype was detected in patients with CTS pre-surgery as genetic and protein levels of IL-6 and IL-4 were detected respectively. IL-4 was also found to positively correlate with increased pain and symptom scores. Immune dysregulation was also observed between patients with CTS and healthy controls as both TGF-β and CCL5 were increased in patients with CTS. The

use of CTS as a model system highlights an increased inflammatory presence in focal nerve injury. All together these findings provided evidence for an inflammatory component in the pathogenesis of CTS and implicates certain cytokines which may have specific roles in the context of neuropathic pain.

Chapter 3: Genetic and histological indications of inflammation in tenosynovial tissue

3.1 Introduction

The consensus that CTS is a condition where the median nerve in the wrist is compressed as a result of a non-inflammatory fibrotic thickening, is primarily based on immunohistochemistry (IHC) analysis of tenosynovial tissue from patients with CTS. One such study by Schuind, et al[57] found hyperplasia, increased collagen and necrotic regions in the tenosynovium, but no evidence of inflammation. They hypothesized that the development of CTS was the cause of mechanical stresses on the flexor tendons in the carpal tunnel, which caused synovial scaring. They proposed that the synovial scaring causes friction and leads to a self-propagating cycle of increased damage leading to CTS. Similar IHC analysis by Gross, et al[366]; Jafari et al[56] and Fuchs et al[364] found necrobiosis of collagen, fibrous thickening of the vasculature and vascular sclerosis respectively in the tenosynovium. A further study by Oh et al[54] produced similar findings where altered vascular morphology and aberrant extra cellular matrix (ECM) structures were observed. Importantly in all studies these changes were determined to be non-inflammatory. These studies mention evidence of inflammation in only a small number of cases, 10-11%[56, 364, 366] and state that due to the relative infrequency, inflammation is not the cause of or a defining factor of CTS. However, only Fuchs provides an explanation of how they defined inflammation in the tissue, as they specified the presence of immune cells in their study as an indication of inflammation. Jafari, Gross and Oh did not state their inflammation criteria, and so their findings may be subject to interpretation and not accurately describe tissue inflammation.

Importantly, the majority of these studies used a basic staining protocol of haematoxylin and eosin (H&E) to determine the presence of inflammation or immune cells, which is based on the morphology of the cells instead of antigenicity. As H&E staining is non-discriminate for different cell types, the determination of immune cell presence in the tissue is subjective to the operator. The study from Oh suffers from the same shortcomings in the staining method, as they used Verhoeff Van-Gieson staining which can only distinguish between components of the vasculature and ECM, and cannot specifically differentiate immune cells. The investigation of immune cell infiltrates into the tissues using specific immune cell markers has not readily been investigated. To my knowledge, there is currently only one study by Yesil et al[55] using specific immune cell markers (anti CD3 and anti CD20 antibodies for T cells and B cells respectively) to determine the presence of inflammation in tenosynovium of patients with CTS. This study found no significant difference in immune cell presence between CTS and controls. However, the control samples used were from patients undergoing surgery due to either flexor tendon injury or distal radius fracture. As a result, the samples from these controls could have had an increased immune cell infiltration in response to the trauma, concealing a potential difference in immune cell density between groups. Collectively, all but two of the studies mentioned here were conducted over a decade ago when the staining techniques were comparatively basic. Due to the improvement in the histological field, investigating the presence of immune cells and inflammatory markers within tissue samples using antibodies against unique cell identification markers is now possible. Furthermore, none of the mentioned studies had age and sex matched controls. Due to these identified shortcomings, further study is required to fully investigate the role of inflammation and immune cells in CTS, and that methods specific for immune cell detection are required to determine their role in this disease. The suggestion that CTS symptoms arise from alterations in connective tissue, rather than the damaged nerve itself, provides further impetus to investigate tenosynovial tissue.[365]

A potential role of inflammation in CTS is apparent by the beneficial (albeit short-term) benefit of corticosteroid injections (Marshall, et al, 2007).[440] This systematic review analyzed 12 studies covering a total of 671 participants where the use of corticosteroids was used to treat CTS. The main findings suggest that local corticosteroid injections improved CTS symptoms over placebo, and that local injection was superior to oral administration or systemic injection in terms of symptom improvement. Interestingly they also found that the local administration of corticosteroids did not significantly improve CTS symptoms more than when anti-inflammatory treatment was given. The review did state however that the effectiveness of corticosteroid use was only shown in the short term and that further work was required to demonstrate long term effectiveness of this treatment. These findings are of particular interest as they demonstrate the effectiveness of a medication with anti-inflammatory properties as well as direct anti-inflammatory treatment on CTS symptoms. These findings provide strong rational for the current investigation of the involvement of inflammation in the tenosynovium of patients with CTS.

3.1.1 Aims

Through the use of immunohistochemistry, using antibodies against specific immune cell markers and genetic analysis, I will seek to determine immune cell infiltration in the tenosynovium and characterize immune cell phenotype association with clinical characteristics.

3.2 Methods

3.2.1 Participants

I made use of the same cohort of patients introduced in Chapter 2. Briefly, I included patients with clinically and electrodiagnostically confirmed CTS from an existing prospective longitudinal cohort study collected by Annina Schmid and Akira Wiberg.

3.2.2 Phenotypic data

A detailed description of the phenotypic data collected is available elsewhere[76]. In brief, age, sex and duration of symptoms were recorded for each individual. Symptom severity was evaluated with the Boston carpal tunnel questionnaire[39], which contains a symptom and function sub-score (0 no symptoms/functional deficit, 5 severe symptoms /functional deficit). Only the symptom score was used in this analysis. Neuropathic pain severity was evaluated with the Neuropathic Pain Symptom Inventory[40] (NPSI), which includes numerical rating scales (0 no pain to 10 worst pain imaginable) for burning pain, deep pressure pain, paraesthesia, paroxysmal symptoms, evoked pain and a composite score (0-100). Severity of pain was also recorded over the past 24 hours on a visual analogue scale (VAS) (0 no pain, 10 worst pain imaginable). Surgical outcome was recorded on the Global Rating Of Change Scale (GROC), which ranges from -7 (a very great deal worse) to +7 (a very great deal better).[371] Standard electrodiagnostic testing (EDT) of the median nerve was performed with an ADVANCE[TM] system (Neurometrix, USA). Electrodiagnostic test severity was graded on the scale derived by Bland et al, 2000[372] as follows: normal (grade 0), very mild (grade 1), mild (grade 2), moderate (grade 3), severe (grade 4), very severe (grade 5), extremely severe (grade 6). Phenotypic data was collected by Annina Schmid.

3.2.3 Tissue collection and preparation

Tenosynovial tissue was collected during CTS decompression surgery. Samples were cut into two pieces, with one being used in histological analysis and the other for RNA sequencing. Samples for histological analysis were placed into fresh periodate-lysine-paraformaldehyde (PLP) fixative. Samples remained in PLP fixative at room temperature for 6hrs before being washed 3x with 0.1M phosphate buffer. Washed samples were placed in 4.4M sucrose solution and stored at 4°C

for 42-74 hrs. Samples were then frozen in optimal cutting temperature (OCT) in base moulds and stored at -80°c.

The other half of the tenosynovium specimen, used for RNAseq, was frozen in RNAlater solution (Thermo Fisher Scientific, UK) at -80°c. RNA was extracted from the tissue using a combination of phenol extraction and column purification. Briefly, samples were defrosted and homogenized in TRIzol (Invitrogen, USA), along with chloroform. Samples were centrifuged for phase separation and the supernatant was transferred to filter columns of the High Pure RNA Isolation kit (Roche, Germany). RNA was purified by repeated wash cycles and DNAse treatment. RNA was eluted from the filter and collected in RNAse free Eppendorf tubes. The concentration of RNA was determined by UV absorbance at 260nm on a NanoDrop spectrophotometer. RNA samples were frozen at -80°c until further use. Tenosynovial tissue for IHC and RNAseq was collected and processed by Akira Wiberg.

3.2.4 RNAseq

RNA samples from the tenosynovium of 41 patients with CTS were sequenced at the Oxford Genomics Centre at the Wellcome Trust Centre for Human Genetics.

The RNAseq library was poly(A)-enriched and directional. Library preparation was performed using the Illumina TruSeq Stranded mRNA Library Prep Kit and standard universal Illumina multiplexing adaptors. The poly(A)-selected RNA was converted to cDNA using the strand specific dUTP strand-marking protocol[441], with the amplification using unique dual indexing. The Illumina HiSeq4000 system was used to perform the sequencing with a 75 bp read length and paired-end reads. Quality metrics were encoded by the Phred score in the resulting FastQ sequencing files, which followed the Sanger standard[442]. Samples from individuals were multiplexed in lanes and all

sequencing lanes gave a high yield, with consistent guanine and cytosine (GC) and high quality base content.

Raw RNAseq data was mapped to the hg19 (GRCh37) human genome using the splice-site aware STAR aligner programme[443]. The genome was indexed using –sjbdOverhang (Read mate length 1) option, with the gene counts being determined with HTSeq[444] against the GRCh37.87 ENSEMBL reference gene set. The data was processed in R, and normalised for effective library size using the DESeq2 package[445] for differential expression analysis. For visualisation and downstream analysis purposes read counts were Log2-transformed using the regularised log transformation[445] or were transformed in gene level Transcript Per Million (TPM) counts. This made the data scaled and centred so as to be directly comparable across samples. Effect sizes were moderated to reduce overestimation, for lowly expressed genes. RNAseq analysis was conducted by Georgios Baskozos and I received the data already processed.

3.2.5 Immunohistochemistry

I conducted immunohistochemistry on 20 tenosynovium samples to detect specific cells of the immune system, 18 of these samples were from patients also included in the RNAseq analysis. As no control tissue was available to compare patient tenosynovium to, I selected patients samples based on their EDT grade allowing the comparison of tissue inflammation between patients with mild/moderate or severe CTS. Initially I attempted double staining for combinations of immune cell markers, however this produced poor quality staining. I instead decided to do single stains for immune cell markers on serial tissue sections. For each patient, 3 serial 14um thin sections were cut using a cryostat (Leica, Germany) and adhered to a gelatinised glass microscope slide (Thermo Fisher Scientific, UK). Briefly, I gelatinised the slides by dissolving 1.5g Gelatine type A (Sigma Aldrich, UK) in 500ml of dH$_2$O. Once the gelatine had dissolved fully, 0.25g of chromium

potassium sulphate was added. Slides were dipped into warm gelatine solution (40°C – 50°C) two times and then left to drain overnight. Once sections were adhered to gelatinised slides I baked them for 1 hr at 60°C to ensure sample adherence to the slide. I then used Mild Heat Induced Epitope Retrieval (HIER) by incubating slides in EDTA buffer (10mM tris Base, 1mM EDTA, 0.05% Tween, pH9.0) for 4-5hrs, a method adapted from Dawes et al[446]. After HIER treatment, I washed the slides for 3 minutes in PBS. Samples were incubated in blocking solution (PBS + 0.2% TritonX + 10% goat serum) for 1hr before I applied the primary antibody, which was either a rabbit anti-human CD3 (Abcam, 1:100, cat no: ab16669), rabbit anti-human CD4 (Abcam, 1:200, cat no: ab133616) or rabbit anti-human CD68 (Abcam, 1:200, cat no: ab213363) overnight at 4°C in a humidified chamber. The next day slides were washed 3x for 10 minutes each in PBS + 0.2% Triton X. An Alexa Fluor 546 anti-rabbit secondary antibody (Life Technologies, 1:500, cat no: A10040) was applied to each slide and incubated at room temperature in the dark for 2 hours in a humidified chamber. After incubation, slides were washed 3x for 10 minutes each in PBS + 0.2% Triton X before mounting media containing DAPI was applied and sections were mounted and imaged on an Observer Z1 Confocal imaging system (Zeiss, Germany). For one patient the CD3 stain did not work and so is absent from analysis.

3.2.6 Cell Counting

Full sections were imaged unless the samples were larger than 9mm^2, in which case 6 randomly selected images of 1.5mm^2 were taken to cover a significant proportion of the section. In each image, positively stained cells were manually counted using the counting tool in ImageJ. The tissue area was calculated by tracing the tissue outline in ImageJ. The number of cells was then divided by the tissue area for each image to give a cell count in cells/mm^2. The cells/mm^2 counts for the 2-6 images of each section were then averaged to give a cells/mm^2 count for each cell type in each patient. For CD8 staining, I tried two separate primary antibodies, both of which

produced poor staining that was not quantifiable. As a result I decided to indirectly obtain CD8 cell numbers from CD3 and CD4 cell counts. For this, the CD4 cell count for a particular patient was subtracted from the CD3 cell count of the same patient to give an indirect measurement of CD8 cell numbers.

3.2.7 Troubleshooting immunohistochemistry staining in tenosynovium

Using a simple protocol of immunohistochemistry staining for immune cells where only a primary and fluorescent secondary antibody were used produced images of poor quality. These sections suffered from low fluorescent signal and so methods to amplify the signal were investigated. Biotin-streptavidin amplification was initially used to increase the fluorescent signal for the immune cell populations. At first, this technique greatly amplified the fluorescent signal making all immune cell populations of interest visible within the tissue (Figure 3.1).

However, using this technique often produced staining with high background, which occurred when using any of the four cell markers. This made it difficult to consistently determine specific staining of immune cells in all samples (Figure 3.2). In addition to this, when no primary antibody was used in a blank control, high background was observed which appeared to be surrounding the cell nuclei (Figure 3.2). This mimicked the appearance of genuine specific cell staining that could lead to false positives when quantifying. Based on these staining results, it became clear that biotin-streptavidin amplification was not suitable to generate images of sufficient quality to quantify.

An alternative staining approach using heat induced epitope retrieval (HIER), described above, was done to increase the fluorescent signal. This technique greatly increased the signal intensity for all cell markers tested apart from CD8, where no staining of sufficient quality could be

generated. The use of this technique did not produce high background staining as was observed

with biotin-streptavidin or produce non-specific staining when a no primary antibody control

(blank) was used (Figure 3.3, A).
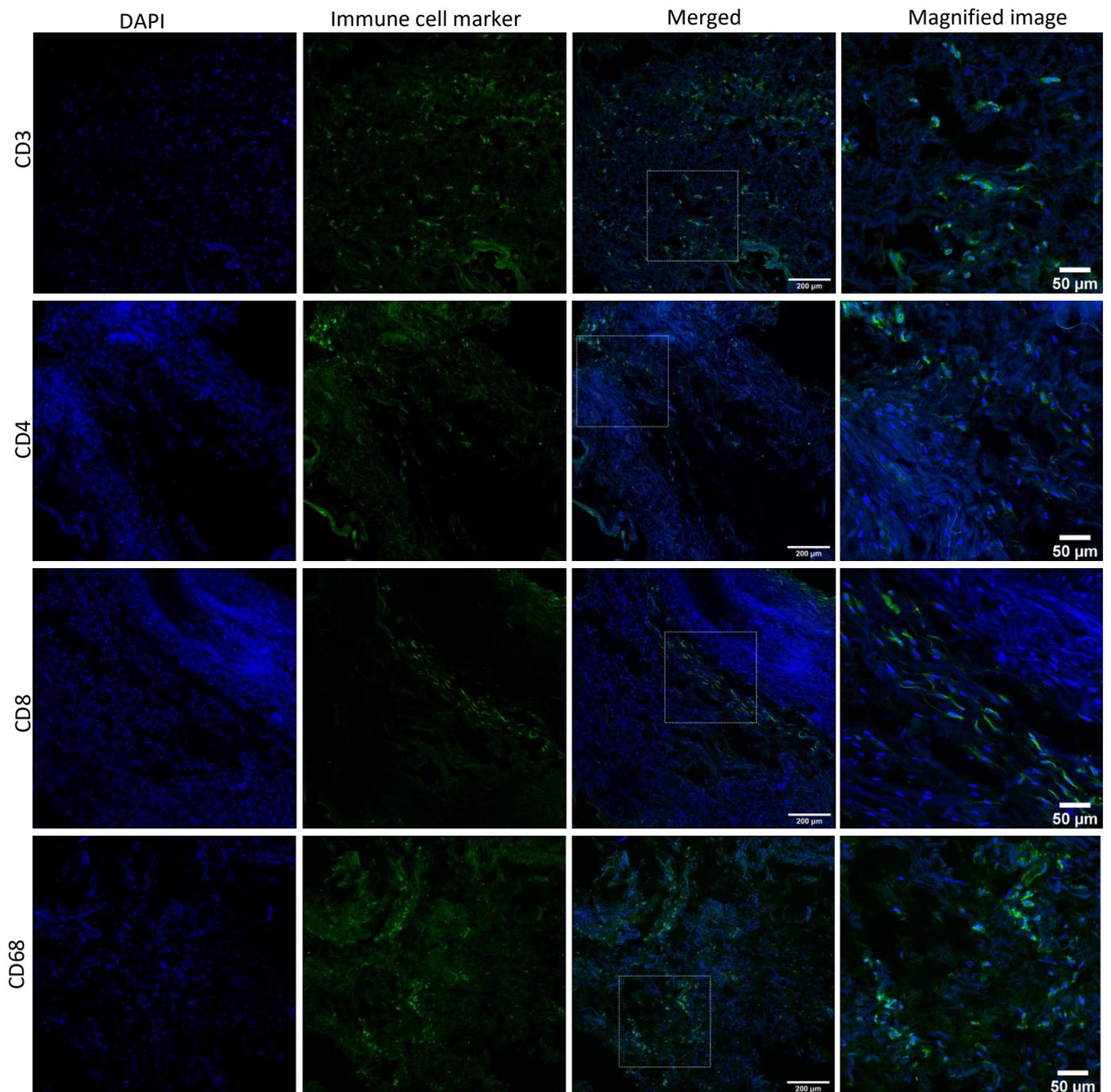
Figure 3.1: Immune cell visualization in tenosynovium tissue after biotin-streptavidin amplification
Immunohistochemistry staining for immune cell types using the markers CD3, CD4, CD8 and CD68 are
shown where T-cell populations and macrophages can be seen in the tissue. In all cases immune cell
markers are shown in green and are counterstained by DAPI in blue. White boxes indicate regions of
interest. Panels on the far right show magnified images of the regions of interest. Images in the first three
columns were taken at 20x magnification.
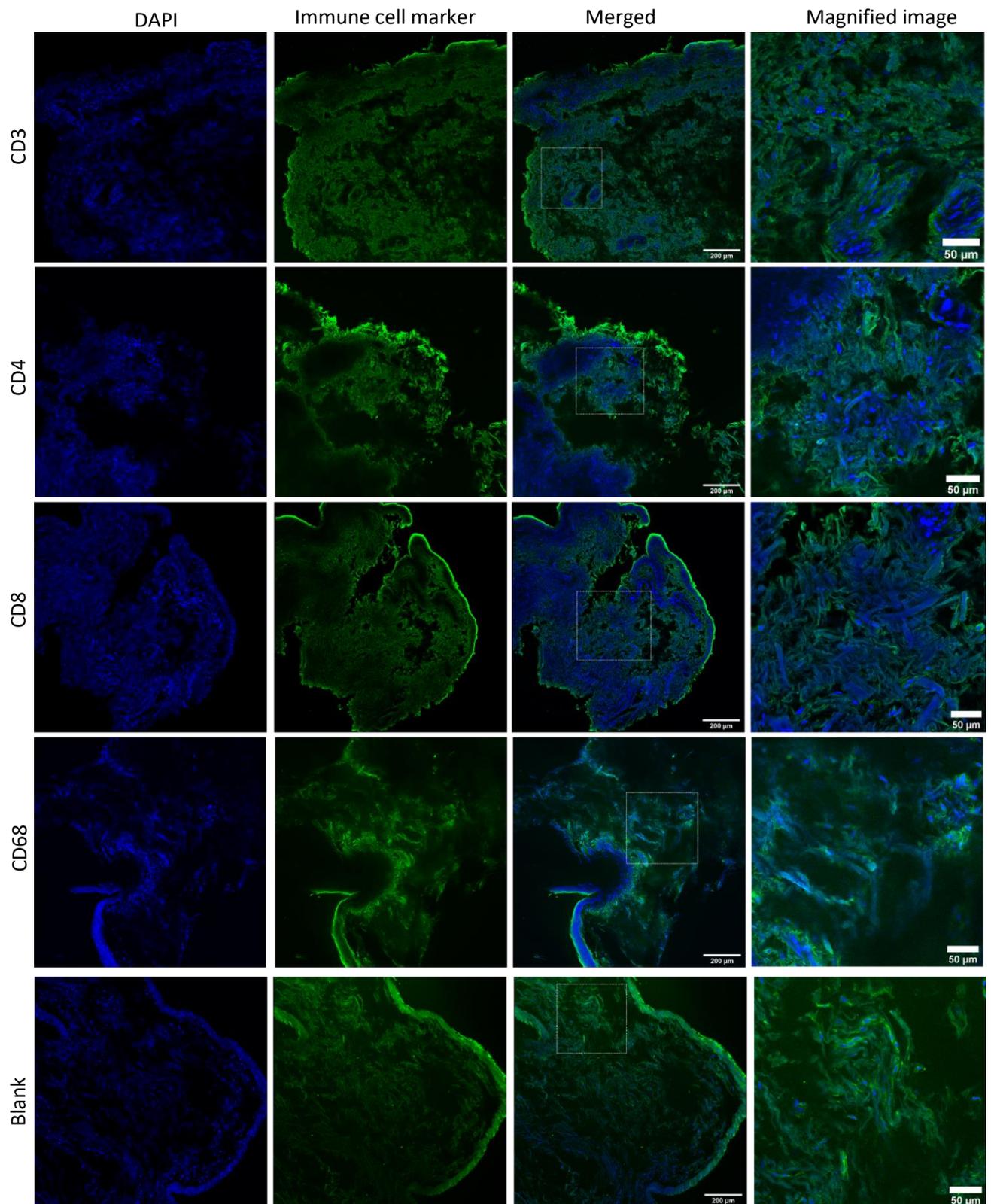
Figure 3.2: Biotin-streptavidin amplification caused high background and non-specific cell staining
The use of biotin-streptavidin amplification to increase signal intensity produced high background staining for each cell marker (CD3, CD4, CD8 and CD68) shown in green. Even in the absence of a primary antibody, using this technique produced non-specific staining that appeared to surround cell nuclei. All sections were counterstained with DAPI (blue). White boxes indicate regions of interest. Panels on the far right show magnified images of the regions of interest. Images in the first three columns were taken at 20x magnification.

3.2.8 Serum cytokines

Serum cytokines identified in Chapter 2 were used to compare to tenosynovium immunohistochemistry results, see methods of Chapter 2.

3.2.9 Statistical Analysis

To determine statistical differences between multiple groups (3 or more), one way ANOVAs were conducted with Tukey's post hoc test where P<0.05 was considered significant. For differences between two groups an independent T test was conducted with P<0.05 being significant. All correlations were conducted using the Spearman's rank correlation where P<0.05 was significant. Correlations were not corrected for multiple testing as these investigations were exploratory. The Gene Set Enrichment and Deconvolution analysis contained their own statistical procedures and are discussed in the relevant sections.

3.2.10 Gene Set Enrichment Analysis (GSEA)

To determine the interaction of unique immune cell gene signatures with clinical phenotypes listed in Table 3.1, I conducted a Gene Set Enrichment Analysis.[447] The procedure was followed as set out in the Bioconductor vignette.[448] Briefly, normalised RNAseq data obtained from the tenosynovium were used in this analysis. After a review of the literature gene signatures for immune cells subtypes including Naïve CD4+ T cells, Th1, Th2, Th17, Treg, CD8+ T cells, M1 and M2 macrophages were obtained from four separate studies.[177, 449-451] Six of the eight signatures were derived from immune cells solely of the peripheral blood. These signatures were used as no gene expression profiling has been done for immune cells in the tenosynovium and so no gene signatures were available for immune cells in this tissue. These gene sets along with phenotype

scores were input into the analysis. For the Th1 subgroup, an initial gene signature (from cultured Th1 cells derived from PBMCs) was used[449] followed by the use of a second signature[450] (derived from a meta-analysis of studies covering a range of tissues and blood) to confirm the associations with clinical phenotypes. The Log2 fold changes in gene expression for a particular phenotype of interest were ranked and the enrichment score was determined as a running sum statistic going down the gene rank. The primary output of the GSEA was the Enrichment Score (ES), which determined the extent of gene over-representation at the top or bottom of a ranked list of genes. This ES was normalised based on the size of the gene set under investigation to give the Normalised Enrichment Score (NES). The NES then provided a measure of association between gene signatures and phenotypes scores. A positive NES indicated an increased expression of signature genes in patients with more severe symptoms, in males, older participants or those with a longer duration of symptoms. A negative enrichment indicated the contrary. Data were corrected for multiple comparisons by Benjamini-Hochberg (FDR) correction with a cut-off of 25% where a corrected (FDR) p value of $p < 0.05$ was considered significant.

3.2.11 Deconvolution analysis

Deconvolution of 10 immune cell types from bulk RNAseq data from tenosynovial tissue was done using the quanTIseq deconvolution package as previously described[452]. Normalised RNAseq reads (Transcript per Million TPM) were input to the quanTIseq programme where deconvolution was performed. The deconvolution algorithm models the expression of bulk RNAseq data (M) as a linear combination of the expression of genes in different cell types, which are summarised in a signature matrix (S) and weighted by relative cell fractions (f). Given the input data (M) and reference matrix (S), the unknown cell fractions (f) are estimated using least square regression to solve the system of linear equations (M = S x f). Constrained least square regression forces the estimated cell fractions to be non-negative and their sum to be lower or equal to one. This

produced cell fractions for 10 immune cell types consisting of B cells, M1 macrophages, M2 macrophages, monocytes, neutrophils, natural killer cells, CD4+ T cells, CD8+ T cells, Tregs and dendritic cells. The remainder of the total cell fraction is made up of cells termed 'other', which are cells in the sample not belonging to one of the prespecified 10 immune cell groups. By estimating the 'other' cell fraction, the algorithm refers all immune cell fractions to the total cellular content, allowing inter and intra-sample comparison. The quanTIseq algorithm uses the TIL10 signature matrix consisting of 153 genes derived from 51 RNAseq datasets containing information on the 10 immune cell types stated. For Tregs and CD4+ cells, the signatures used to determine each cell types were separate and so Tregs cells form a group discrete from CD4+ T cells. The quanTIseq software was chosen as it was specially designed to be used for RNAseq data instead of microarray-based deconvolution packages.[452]

3.3 Results

3.3.1 Patient demographics and clinical phenotypes

Demographic and clinical data for patients involved is shown in Table 3.1.

Table 3.1: Patient demographic data and clinical phenotypes
Demographic data are shown for patients involved in IHC and RNAseq analysis.

	Patients with CTS IHC	Patients with CTS RNAseq
Number of participants	20	41
Mean Age (SD) [years]	61 (13)	64 (12)
Male:Female	6:14	14:27
Duration of symptoms (SD) [Months]	94 (134)	63 (100)
Median EDT grade [IQR]	3 [3]	3 [1]
Boston Symptom score (SD)	2.88 (0.67	2.74 (0.68)

Boston Pain sub-score (SD)	2.84 (0.92)	2.45 (0.98)
Boston Weakness sub-score (SD)	2.5 (1.2)	2.48 (1.08)
Boston Paraesthesia sub-score (SD)	3.13 (0.77)	3.25 (0.78)
VAS Pain (SD)	3.24 (2.37)	2.74 (2.53)
NPSI composite score (SD)	13.33 (9.51)	13.37 (8.95)
NPSI burning (SD)	2.05 (2.85)	2.10 (2.83)
NPSI deep pressure (SD)	2.58 (2.68)	2.32 (2.57)
NPSI evoked (SD)	1.75 (2.09)	1.81 (2.14)
NPSI paraesthesia (SD)	5.25 (2.81)	5.34 (2.82)
NPSI paroxysmal (SD)	1.60 (2.00)	1.80 (2.28)

SD = Standard Deviation, IQR = Inter-Quartile Range, VAS = visual analogue scale, NPSI = Neuropathic Pain Symptom Inventory, EDT = Electrodiagnostic test.

3.3.2 Immune cells are present in tenosynovial tissue

Using IHC with a HIER method, I stained tenosynovial tissue for immune cell markers and was able to detect T cells (CD3), the CD4+ Th subgroup (CD4) and macrophages (CD68) within this tissue (Figure 3.3, A). No staining was observed when the primary antibody was not applied (Blank control). The different morphologies of the immune cell types can be seen with typical cell appearances being highlighted by white arrows. T cells appear with a flattened disk shape while macrophages have a more diffuse, foamy appearance. Immune cell staining for each cell marker was quantified in each patient (Figure 3.3, B). When comparing group means for cell counts (Figure 3.3, C), there were significant differences between all cell types apart from CD4+ T cells (CD4) and macrophages (CD68). Overall as expected, CD3 cell numbers were greater than CD4. CD3 cell numbers were also greater than CD68 in all patients, indicating a higher number of T cells in the tissue than macrophages.
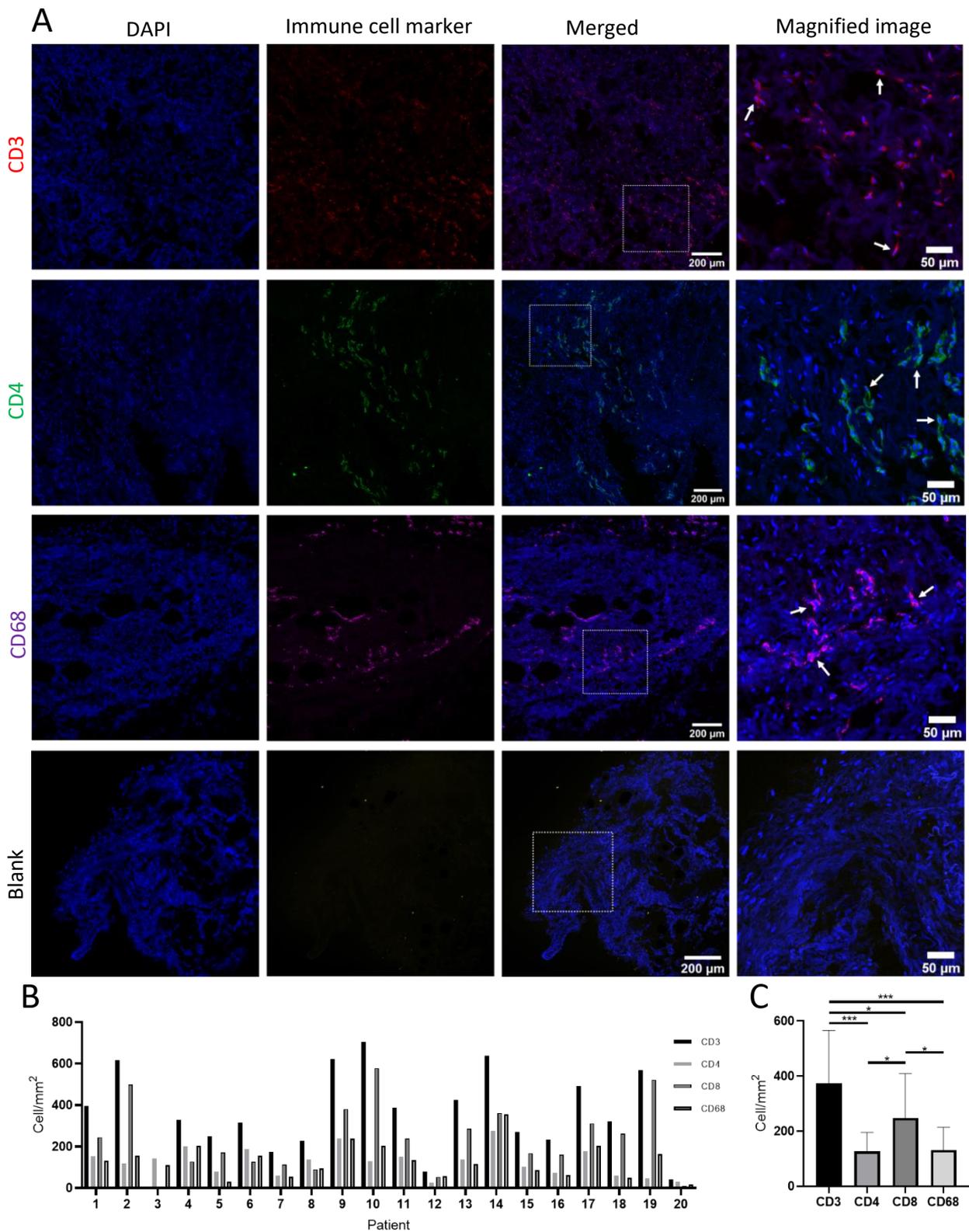
Figure 3.3: Detection of immune cells in the tenosynovium using HIER

A. Immune cell populations were detected in tenosynovium based on cellular markers (CD3, CD4, and CD68), shown in red, green and magenta respectively. All sections were counterstained with DAPI (blue). The use of a no primary antibody control (blank) did not produce any specific staining. White boxes indicate regions of interest. Panels on the far right show magnified images of the regions of interest. White arrows indicate cells displaying typical immune cell type morphology. Images in the first three columns were taken at 20x magnification. B. Immune cell counts were quantified for CD3, CD4 and CD68 for each patient. C. Group means for cell counts show that T cells (CD3) were the most abundant cell type. CD8 numbers were determined by subtracting the CD4 cell counts from the CD3 counts, the remainder being determined as the CD8+ T-cell fraction. Cells counts are displayed as cells/mm^2. *P<0.05, **P<0.01, ***P<0.001.

3.3.3 CD4+ T cells are associated with more severe symptoms

No difference in immune cell counts based on EDT grade was found (Figure 3.4, A), indicating that immune cell numbers are not increased in patients with more severe CTS. Correlation analysis (Table 3.2), for CD4 cell counts and clinical phenotypes showed a moderate significant correlation with the Boston symptom score (Figure 3.4, B) implicating increased CD4+ cell numbers in patients with more severe symptoms. Further investigation of the Boston symptom score sub-domains revealed a significant correlation between paraesthesia and CD4+ cell counts (Figure 3.4, C) suggesting that the Boston questionnaire associations may be driven by paraesthesia symptoms. For CD3 and CD8, a significant correlation was observed for duration of symptoms (Figure 3.4, D-E). However, from inspection of the graphs, the correlations appear to be driven by the same 3 patients for each cell type and so may not reflect a real association. No significant correlations were found between CD68 cell counts and phenotype scores.
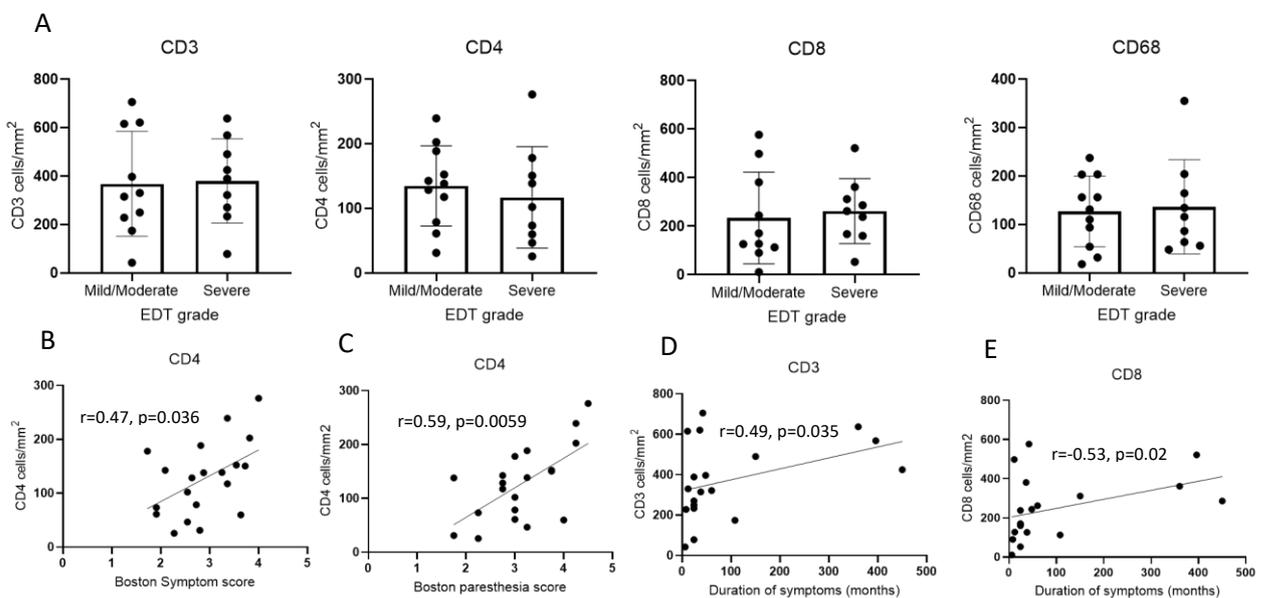
Figure 3.4: Associations between immune cell counts and clinical phenotypes
A. Immune cell counts for CD3, CD4, CD8 and CD68 did not significantly differ based on the EDT grade of patients. B. CD4+ cell counts positively correlated with Boston symptom scores. C. CD4+cell counts positively correlated with Boston paraesthesia sub-score. D-E. CD3+ and CD8+ cell counts positively correlated with duration of symptoms. Independent T-tests were used to determine differences between groups where p<0.05 was considered significant. Spearman's correlation was used where p<0.05 was significant.

3.3.4 Immune cell counts do not correlate with inflammatory markers in the serum

I next correlated immune cell counts from tenosynovial tissue with inflammatory mediator expression in the serum, to determine if systemic inflammation was associated with local inflammation. However no significant associations were determined (Table 3.2) apart from IL-10, which showed a negative correlation with CD4+ cell counts (Figure 3.5, Table 3.2).
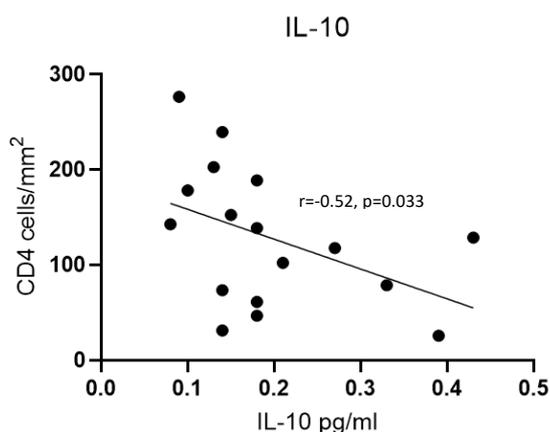
Figure 3.5: CD4 cell counts correlate with IL-10 serum concentration
CD4 cell counts from tenosynovium tissue were found to negatively correlate with serum concentrations of IL-10. Spearman's correlation was used where p<0.05 was significant.

Table 3.2: Correlations for immune cell counts and clinical phenotypes
The correlation coefficients and p values from Spearman's rank correlation are shown for each immune cell count and clinical phenotype. Significant correlations are shaded in grey.

	CD3 cells/mm^2		CD4 cell/mm^2		CD8 cells/mm^2		CD68 cells/mm^2	
	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value
Boston symptom score	0.37	0.12	0.47	0.036	0.20	0.42	0.32	0.17
Boston pain sub-score	N/A	N/A	0.24	0.32	-0.10	0.68	N/A	N/A
Boston weakness sub-score	N/A	N/A	-0.0016	0.99	0.43	0.068	N/A	N/A
Boston paraesthesia sub-score	N/A	N/A	0.59	0.0059	0.41	0.082	N/A	N/A
VAS Pain	0.29	0.26	0.18	0.45	0.16	0.53	0.29	0.25
NPSI (burning)	0.037	0.88	-0.025	0.92	0.018	0.94	-0.058	0.81
NPSI (deep)	0.20	0.41	0.18	0.45	0.10	0.67	0.039	0.87

NPSI (evoked)	0.15	0.55	0.25	0.30	0.10	0.70	0.049	0.84
NPSI (paraesthesia)	0.073	0.77	0.041	0.86	-0.90	0.71	-0.049	0.84
NPSI (paroxysmal)	0.26	0.29	0.31	0.19	0.072	0.77	0.35	0.14
NPSI (composite)	0.12	0.63	0.13	0.59	0.054	0.82	-0.040	0.86
EDT grade	0.019	0.94	-0.33	0.15	0.16	0.52	-0.042	0.86
Duration of symptoms	0.49	0.035	0.17	0.47	0.53	0.02	0.31	0.18
IL-4 pg/ml	0.38	0.14	0.42	0.098	0.30	0.26	0.26	0.32
IL-6 pg/ml	-0.062	0.82	-0.050	0.85	-0.12	0.65	-0.26	0.99
IL-9 pg/ml	-0.21	0.41	-0.23	0.37	-0.14	0.58	-0.33	0.18
IL-10 pg/ml	-0.11	0.69	-0.52	0.033	0.071	0.79	-0.34	0.18
IFN-γ pg/ml	0.26	0.32	-0.17	0.51	0.35	0.19	0.012	0.97
TNF-α	-0.053	0.85	-0.36	0.15	0.08	0.77	-0.24	0.36
CCL5	0.0088	0.98	-0.022	0.94	0.026	0.93	0.020	0.94
TGF-β	-0.38	0.15	-0.022	0.94	-0.35	0.18	-0.33	0.20

VAS = visual analogue scale, NPSI = neuropathic pain symptom inventory, EDT = electrodiagnostic testing.

3.3.5 Immune cell gene signatures have variable associations with clinical phenotypes

The positive correlation between CD4+ cell counts and Boston symptom scores prompted the further investigation of CD4+ Th subsets including Th1, Th2, Th17 and Tregs and their association with clinical phenotypes. Gene set enrichment analysis (GSEA) of Th subsets and naïve CD4+ gene signatures with clinical phenotypes revealed several significant enrichments (Supplementary Table 5 in Appendix). The majority of significant enrichments were found with the Th1 gene set (Figure 3.6), where signature genes were positively enriched for age and sex and negatively enriched for the Boston symptom score, the Boston pain sub-score, the NPSI deep, paroxysmal, paraesthesia and composite pain scores and duration of symptoms. Th2 and Treg signature genes only showed significant enrichment for the Boston weakness sub-score and age and NPSI burning pain respectively (Supplementary Table 5 in Appendix). Th17 signature genes were not significantly enriched in any clinical phenotype. Naïve CD4 T-cell signature genes showed significant positive enrichment for age and significant negative enrichments for the NPSI paraesthesia sub-score and duration of symptoms (Supplementary Table 5 in Appendix).
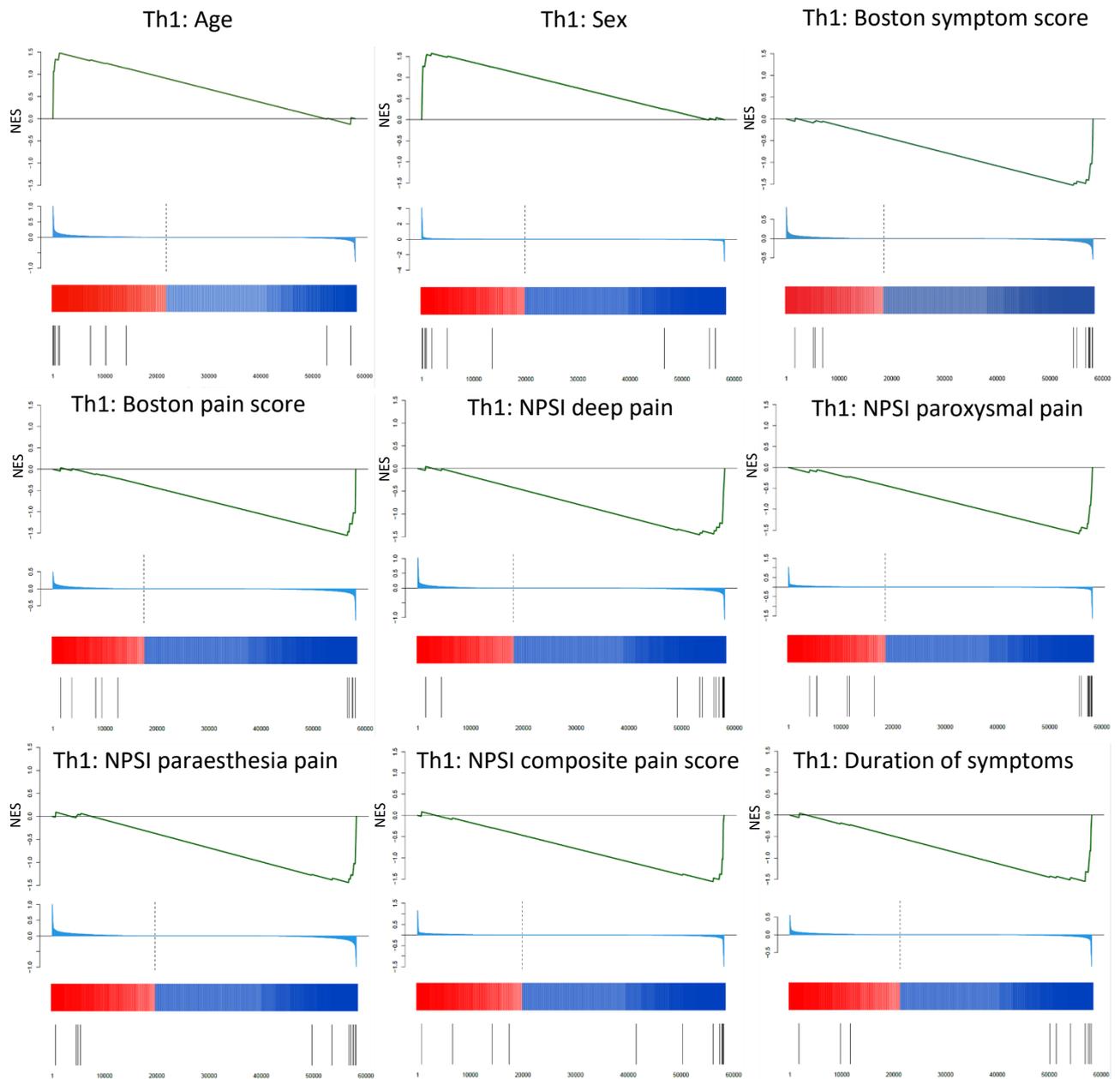
Figure 3.6: Enrichments of Th1 specific genes with clinical phenotypes

The plots from the GSEA primarily provide the enrichment score. The graph in the top portion of the plot provides the enrichment score which can be both positive or negative. The green line shows the enrichment score as you travel down the list of ranked genes with the peak of the green line indicating the enrichment score for that analysis. Positive enrichment scores indicate there is an increased expression of signature genes in patients with more severe symptoms, patients who are older or who are males. Negative enrichments indicate the contrary. Genes specific for the Th1 T-cell sub-population were positively enriched for age and sex, indicating that older participants and males have higher expression of the Th1 specific genes. Th1 genes were negatively enriched for the Boston symptom score, Boston pain sub-score, NPSI deep, paroxysmal, paraesthesia, and composite pain scores and duration of symptoms, indicating that Th1 specific genes have higher expression in patients with lower neuropathic pain symptom severity and shorter duration of symptoms. NES=Normalized enrichment score.

As the Th1 signature genes showed the most enrichments with clinical phenotypes, I sought to confirm these associations by conducting a further GSEA using a different set of Th1 specific genes (described previously in the methods).[450] Using the secondary Th1 gene signature produced similar enrichments as were seen with the first signature (Supplementary Table 6 in Appendix), further corroborating my findings. Combining these two gene sets together into one signature caused increased significant enrichments with clinical phenotypes (Supplementary Table 6 in Appendix). Several of the Th subset gene signatures showed significant enrichment for age and sex, which could be influencing the associations between gene signatures and other clinical phenotypes. To remove this effect, age and sex were added as covariates in the GSEA analysis, which changed the results substantially (Table 3.3). The combined Th1 signature genes were found to be positively enriched for NPSI evoked and composite pain scores while being negatively enriched for the Boston symptom score, the Boston pain sub-score and EDT severity (Figure 3.7, A). GSEA analysis for other Th T-cell subtypes (Naïve CD4+ T cells, Th2, Th17 and Tregs) using age and sex corrected data identified Treg signature genes to be positively enriched for the NPSI composite pain score. This positive enrichment indicates increased Treg gene expression in patients with more severe neuropathic pain sensations. Treg genes were also negatively enriched for The Boston symptom score (Figure 3.7, B). Th17 signature genes were positively enriched for NPSI evoked pain but negatively enriched for the Boston pain sub-score (Figure 3.7, C). Th2 gene signatures did not show any significant enrichments (Table 3.3). Naïve CD4+ T-cell signature genes were positively enriched for NPSI evoked and composite pain and negatively enriched for the Boston symptom score, Boston pain sub-score, NPSI paraesthesia and duration of symptoms (Table 3.3).

Table 3.3: Gene set enrichment analysis of immune cell gene signatures with clinical phenotypes

The normalized enrichment scores, p values and adjusted p values are shown for immune cell gene signatures and each phenotype score. The number of genes in each set are shown in gene set size. Significant enrichments are shaded in grey.

Variable	Gene set	Gene set size	NES	P value	FDR p value
Boston Symptom score	Th1 genes (combined)	34	-1.67079	0.000856559	0.000856559
Boston Weakness score			1.271176	0.1481485	0.1481485
Boston Paraesthesia score			-1.30433	0.1078163	0.1078163
Boston Pain score			-1.71219	1.03747E-05	1.03747E-05
Pain VAS			0.881963	0.660891	0.660891
EDT Severity			-1.453104	0.036002	0.036002
NPSI (burning pain)			-1.02376	0.4030412	0.4030412
NPSI (deep pain)			1.404759	0.07007203	0.07007203
NPSI (evoked pain)			1.587131	0.006434651	0.006434651
NPSI (paroxysmal pain)			1.33359	0.09576718	0.09576718
NPSI (paraesthesia pain)			-1.36391	0.0787495	0.0787495
NPSI (composite pain score)			1.582861	0.00634544	0.00634544
Symptom duration			-1.38183	0.07041604	0.07041604
Boston Symptom score	Th2 genes	38	-1.34029	0.190535	0.190535
Boston Weakness score			1.366911	0.089313	0.161055
Boston Paraesthesia score			1.115196	0.617782	0.310724
Boston Pain score			-1.28957	0.260723	0.260723
Pain VAS			-1.31452	0.219092	0.219092
EDT Severity			-1.13447	0.592228	0.592228
NPSI (burning pain)			-1.33498	0.157046	0.078523
NPSI (deep pain)			1.511093	0.053401	0.05644
NPSI (evoked pain)			1.317533	0.251659	0.251659
NPSI (paroxysmal pain)			1.179527	0.511634	0.255817
NPSI (paraesthesia pain)			-1.27732	0.286369	0.286369
NPSI (composite pain score)			0.80374	0.751971	0.751971
Symptom duration			-1.1618	0.492499	0.492499
Boston Symptom score	Th17 genes	18	-1.40322	0.053906	0.053906
Boston Weakness score			-0.78604	0.757745	0.757745
Boston Paraesthesia score			-0.74533	0.804415	0.804415
Boston Pain score			-1.43613	0.032919	0.032919
Pain VAS			-0.75887	0.782804	0.782804
EDT Severity			-1.19310	0.26684	0.26684
NPSI (burning pain)			-0.92579	0.561132	0.561132
NPSI (deep pain)			0.941218	0.52521	0.52521
NPSI (evoked pain)			1.47476	0.031925	0.031925
NPSI (paroxysmal pain)			1.241126	0.2075	0.2075
NPSI (paraesthesia pain)			-1.24392	0.203905	0.203905
NPSI (composite pain score)			1.16354	0.305544	0.305544

Score	Gene set	N	Value	p	p adj
Symptom duration			-0.97202	0.506716	0.506716
Boston Symptom score	Treg genes	47	-1.55483	0.02275	0.02275
Boston Weakness score			1.244377	0.31796	0.31796
Boston Paraesthesia score			-0.88506	0.619665	0.619665
Boston Pain score			-1.44665	0.097522	0.097522
Pain VAS			1.15928	0.489127	0.489127
EDT Severity			-1.04615	0.36049	0.36049
NPSI (burning pain)			1.342404	0.083811	0.082021
NPSI (deep pain)			1.100836	0.458698	0.621408
NPSI (evoked pain)			1.416816	0.105512	0.115045
NPSI (paroxysmal pain)			1.40485	0.137316	0.137316
NPSI (paraesthesia pain)			-1.38098	0.186842	0.186842
NPSI (composite pain score)			1.660152	0.007434	0.007434
Symptom duration			0.893236	0.862487	1
Boston Symptom score	Naïve CD4+ T cells	164	-1.62867	0.001791	0.000896
Boston Weakness score			1.00034	0.440156	0.440156
Boston Paraesthesia score			-0.99434	0.438724	0.438724
Boston Pain score			-1.45387	0.023858	0.011929
Pain VAS			0.88761	0.692677	0.692677
EDT Severity			-1.21963	0.218042	0.218042
NPSI (burning pain)			1.034863	0.374402	0.374402
NPSI (deep pain)			1.129798	0.193047	0.193047
NPSI (evoked pain)			1.518881	0.014291	0.007145
NPSI (paroxysmal pain)			1.319209	0.114734	0.057367
NPSI (paraesthesia pain)			-1.58676	0.004861	0.004861
NPSI (composite pain score)			1.605432	0.004934	0.002467
Symptom duration			-1.44893	0.031198	0.015599
Boston Symptom score	CD8+ T cells	69	-1.71427	0.000105	0.000105
Boston Weakness score			1.40707	0.045696	0.045696
Boston Paraesthesia score			-1.53165	0.018362	0.018362
Boston Pain score			-1.74038	6.34E-05	6.34E-05
Pain VAS			-0.91754	0.602869	0.602869
EDT Severity			-1.55203	0.01442	0.01442
NPSI (burning pain)			-0.92088	0.601509	0.601509
NPSI (deep pain)			1.244242	0.12204	0.12204
NPSI (evoked pain)			1.660045	0.001847	0.001847
NPSI (paroxysmal pain)			1.547424	0.005639	0.005639
NPSI (paraesthesia pain)			-1.37646	0.056229	0.056229
NPSI (composite pain score)			1.530712	0.015213	0.015213
Symptom duration			-1.3346	0.080209	0.080209
Boston Symptom score	M1 macrophage genes	51	-1.83933	5.93E-06	5.93E-06
Boston Weakness score			1.452495	0.0487	0.0487
Boston Paraesthesia score			0.928592	0.591029	0.591029
Boston Pain score			-1.86857	0.00017	0.00017

Pain VAS			-1.36668	0.083962	0.063247
EDT Severity			-1.159937	0.451586	0.451586
NPSI (burning pain)			1.341525	0.173562	0.173562
NPSI (deep pain)			1.65788	0.003105	0.003105
NPSI (evoked pain)			1.804959	0.001181	0.001181
NPSI (paroxysmal pain)			1.757558	2.9E-05	2.9E-05
NPSI (paraesthesia pain)			-1.8587	2.52E-06	2.52E-06
NPSI (composite pain score)			1.896539	9.96E-09	9.96E-09
Symptom duration			-1.78855	0.000803	0.000803
Boston Symptom score	M2 macrophage genes	40	-1.09887	0.311808	0.311808
Boston Weakness score			1.344754	0.058744	0.058744
Boston Paraesthesia score			-1.27628	0.284118	0.142059
Boston Pain score			-0.85662	0.684008	0.684008
Pain VAS			1.350617	0.083962	0.083962
EDT Severity			-0.96728	0.522837	0.522837
NPSI (burning pain)			1.231991	0.178181	0.178181
NPSI (deep pain)			0.904512	0.612437	0.612437
NPSI (evoked pain)			1.148156	0.253847	0.253847
NPSI (paroxysmal pain)			1.289049	0.117055	0.117055
NPSI (paraesthesia pain)			-0.93494	0.556	0.556
NPSI (composite pain score)			1.388938	0.04862	0.04862
Symptom duration			-1.11206	0.289456	0.289456

VAS = visual analogue scale, NPSI = neuropathic pain symptom inventory, EDT = electrodiagnostic testing.
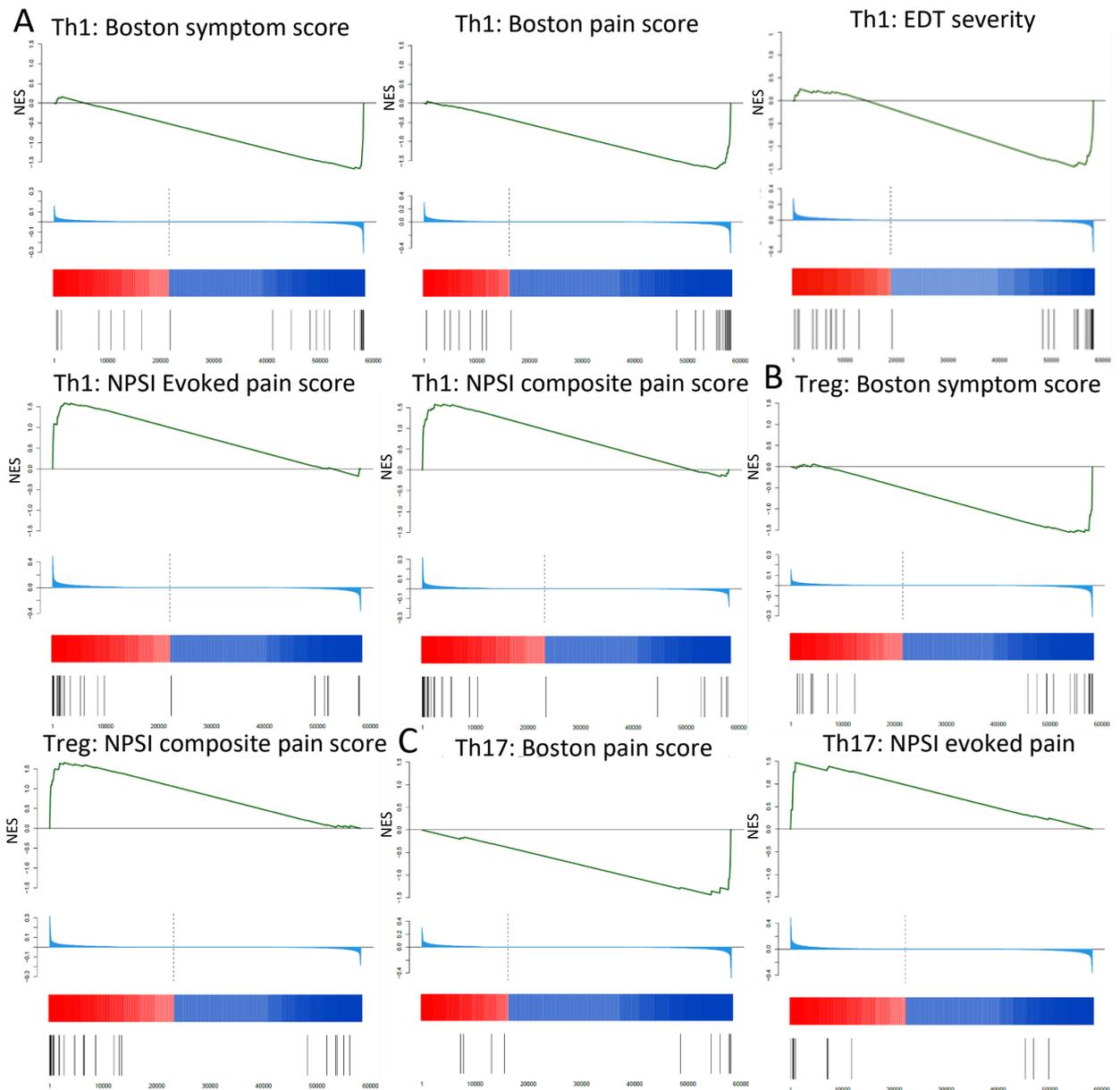
Figure 3.7: GSEA analysis of Th1 and Treg gene signatures with clinical phenotype scores
A. Genes specific for the Th1 T-cell subpopulation were negatively enriched for Boston symptom score, Boston pain sub-score and EDT severity and positively enriched for the NPSI evoked and composite pain scores. B. Treg gene signatures were negatively enriched for Boston symptom score and positively enriched for NPSI composite pain. C. Th17 genes were negatively enriched for the Boston pain sub-score and positively enriched for NPSI evoked pain.

The gene signatures of other immune cell types including CD8+ T cells and M1 and M2 macrophages were analysed by GSEA (Table 3.3). CD8+ T-cell genes were significantly associated with several clinical phenotypes. These include positive enrichments for NPSI evoked, paroxysmal and composite pain scores and the Boston weakness sub-score. Negative enrichments were observed for Boston symptom score, the Boston pain and paraesthesia sub-scores and EDT severity. This indicates that CD8+ T-cell genes have increased expression in patients with higher neuropathic pain scores, but are lower in patients with higher Boston scores (which may or may not be driven by neuropathic pain) and EDT grade. M1 specific genes were positively enriched for NPSI deep, evoked, paroxysmal and composite pain scores, but were negatively enriched for Boston symptom, Boston pain, NPSI paraesthesia scores and duration of symptoms. As with CD8+ T cells, This indicates increased expression of M1 genes in patients with more severe neuropathic pain and lower expression in patients with increased Boston symptoms. M2 macrophage genes only showed a significant enrichment for the NPSI composite pain score. A selection of significant CD8+ T-cell and macrophage enrichments are shown in Figure 3.8.

A

CD8 T cells: Boston symptom score

CD8 T cells: Boston pain score

CD8 T cells: Boston Paraesthesia score

CD8 T cells: EDT severity

CD8 T cells: NPSI composite pain score

CD8 T cells: NPSI evoked pain score

CD8 T cells: NPSI paroxysmal pain

B

M1: Boston symptom score

M1: Boston pain score

M1: NPSI composite pain score

M1: NPSI deep pain

M1: NPSI evoked pain

M1: NPSI parsoxysmal pain

C

M2: NPSI composite pain score

Figure 3.8: GSEA analysis of CD8+ T cells and macrophage gene signatures with phenotype scores
A. CD8+ T-cell genes showed variable enrichments among clinical phenotypes with negative enrichments for the Boston symptom score, the Boston pain and paraesthesia sub-scores and EDT severity. Positive enrichments were observed for NPSI composite, evoked and paroxysmal pain. B. M1 macrophage signature genes had significant enrichments for several phenotypes including negative enrichments Boston symptom scores as well as the Boston pain sub-score. M1 genes were positively enriched NPSI composite, deep, evoked and paroxysmal pain. C. M2 genes were positively enriched for NPSI composite pain scores. NES = Normalized enrichment score.

3.3.6 Deconvolution analysis reveals immune cell proportions

Deconvolution analysis using RNAseq data from the tenosynovium revealed the proportions of ten immune cell types within the tissue (Figure 3.9, A, Supplementary Table 7 in Appendix). Of these ten cell types only B cells, M1 macrophages, Neutrophils, NK cells and Tregs were consistently identified in patients (Supplementary Table 7, in Appendix). Correlation analysis was then done to determine associations between clinical phenotypes, however only Treg proportions and B-cell proportions weakly correlated with the Boston paraesthesia sub-score and NPSI evoked pain respectively (Table 3.4). Immune cell counts from IHC staining were correlated with immune cell proportions from the deconvolution analysis (Figure 3.9, B). Specifically, CD68+ cell counts were compared to the combined cell proportions of M1 and M2 macrophages while CD4+ cell counts were compared to Treg and NK-cell proportions separately. CD68+ cell counts did not correlate with macrophage proportions however both Treg and NK-cell proportions significantly correlated with CD4+ cell counts (Figure 3.9, B).
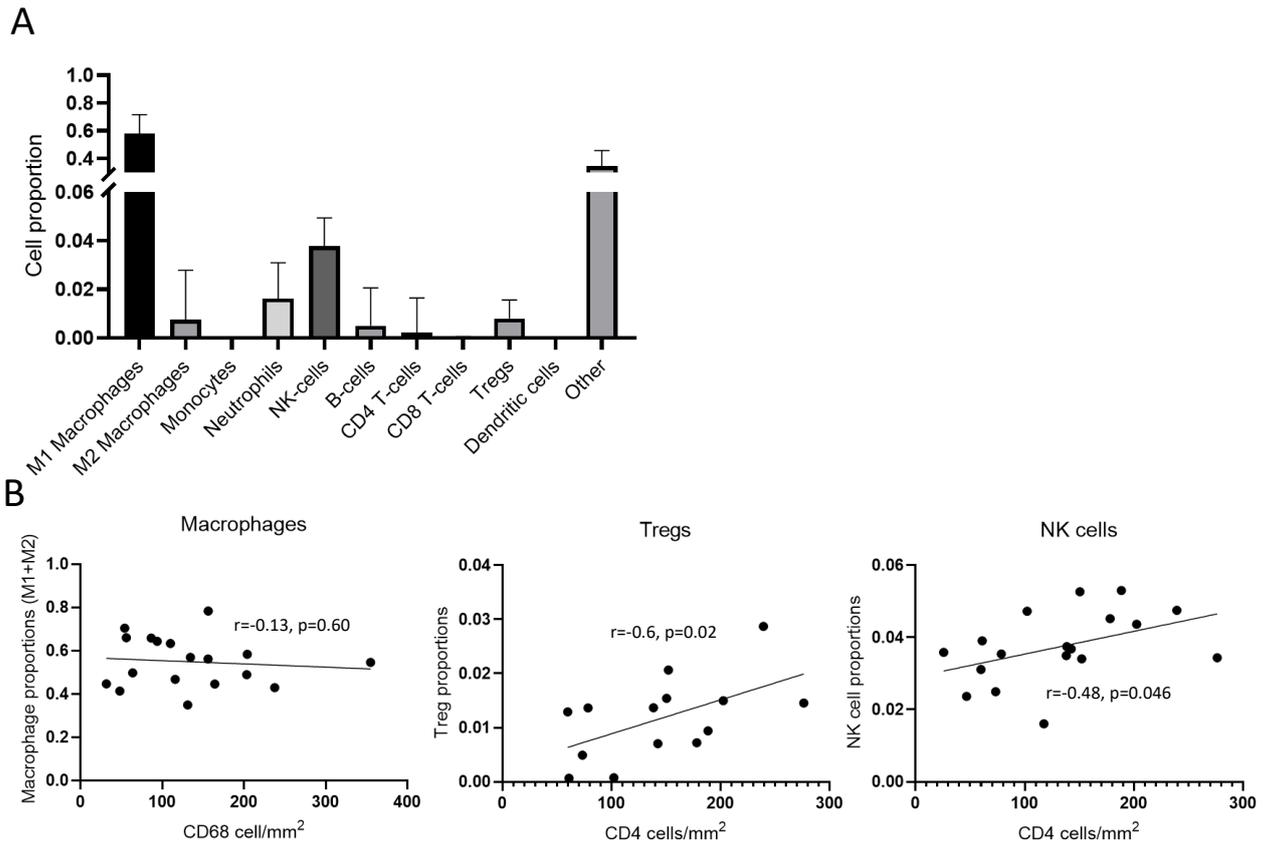
Figure 3.9: Immune cell proportions from deconvolution analysis and correlations with IHC immune cell counts

A. Cell proportions were determined from deconvolution analysis where only five cell types (M1 macrophages, NK cells, Neutrophils, B-cell and Tregs) were consistently identified in patient samples. Overall M1 macrophages had the greatest immune cell proportion in these patients, followed by NK cells, neutrophils, Tregs and B cells. A large proportion of 'other' cells (non-immune cells) were also detected. B. Correlations between cell proportions derived from deconvolution of RNAseq data and IHC immune cell counts were conducted. Macrophages as determined by CD68 staining did not correlate with M1+M2 macrophage proportions. Treg and NK-cell proportions however were significantly correlated with CD4+ T-cell counts. Spearman's correlation was used where $p < 0.05$ was significant.

Table 3.4: Correlation of deconvolution derived cell proportions with clinical phenotypes

For the five cell types where proportions could be consistently determined in patients, correlations were conducted with clinical phenotypes. The correlation coefficients (r) and p values are shown. Significant correlations are shaded in grey.

	Treg cell proportion		M1 macrophage cell proportion		NK-cell proportion		B-cell proportion		Neutrophil proportions	
	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value	Correlation coefficient (r)	P Value
Boston symptom score	0.228	0.151	-0.238	0.134	0.014	0.933	0.041	0.800	0.106	0.511
Boston pain score	0.169	0.291	-0.151	0.347	-0.006	0.971	0.015	0.928	0.069	0.667
Boston weakness score	0.116	0.471	-0.194	0.224	-0.175	0.273	0.055	0.732	0.067	0.677
Boston paraesthesia	0..337	0.031	-0.278	0.079	0.124	0.440	0.228	0.152	0.206	0.195
VAS Pain	0.039	0.812	0.047	0.777	-0.010	0.952	0.140	0.394	0.027	0.869
NPSI (burning)	-0.059	0.715	0.052	0.745	-0.215	0.177	-0.142	0.375	-0.023	0.886
NPSI (deep)	0.190	0.233	-0.182	0.254	-0.070	0.663	0.075	0.639	0.012	0.939
NPSI (evoked)	0.301	0.056	-0.242	0.128	0.241	0.128	0.493	0.001	0.122	0.446
NPSI (paraesthesia)	0.203	0.204	-0.101	0.529	0.096	0.552	0.302	0.055	0.022	0.891
NPSI (paroxysmal)	0.077	0.633	0.006	0.971	0.128	0.424	0.108	0.500	-0.088	0.584
NPSI (composite)	0.192	0.228	-0.102	0.527	0.028	0.864	0.234	0.142	-0.046	0.774
EDT grade	-0.070	0.203	-0.143	0.373	-0.037	0.818	-0.027	0.865	0.099	0.537
Duration	0.203	0.665	-0.221	0.165	0.039	0.808	0.220	0.167	0.186	0.244

VAS = visual analogue scale, NPSI = neuropathic pain symptom inventory, EDT = electrodiagnostic testing.

3.4 Discussion

IHC analysis of tenosynovial tissue revealed the presence of both macrophages and T cells. The pan T-cell marker CD3 was more abundant than the subgroup marker CD4 and T cells in total were more abundant than macrophages. Correlation analyses between immune cell counts and clinical phenotypes revealed a moderate correlation of CD4+ cell numbers and Boston symptom scores. Associations of CD4+ Th subtypes with clinical phenotypes was determined by GSEA analysis where Th1 signature genes showed several associations with clinical phenotypes. After correcting for age and sex, GSEA revealed Th1, Th17 and Treg genes to have both negative and positive associations with clinical phenotypes. Other cell signatures for M1 macrophages and CD8+ T cells showed similar associations. Following deconvolution of RNAseq data, the most consistent immune cell types identified were M1 macrophages, B cells, NK cells, neutrophils and Tregs. The cell proportions of Tregs and NK cells were found to significantly correlate with CD4+ cell counts established with immunohistochemistry.

3.4.1 Immune cells are present in the tenosynovium

I was able to detect the presence of immune cells in the tenosynovial tissue from patients with CTS. CD3, CD4 and CD68 cell markers were detectable, with T cells (CD3) being the most abundant cell type. The presence of immune cells indicates that there may be inflammation within the tissue, which has implications for immune cells in contributing to the pathogenesis of the disease. This is contrary with several immunohistochemical studies conducted in the tenosynovium of patients with CTS, which found inflammation to be either not present[54, 57] or present in very few cases[56, 364, 366]. One of the most prominent of these studies was by Fuchs et al[364], who directly investigated the presence of inflammation in tenosynovial tissue and found it only to be present in 10% of patient samples. They considered the presence of immune cells to be fundamental in assigning inflammation to a patient specimen. Using their definition,

inflammation was detected in 100% of the samples tested in my study, which is remarkably

higher than the Fuchs study and several others previously mentioned. This discrepancy is likely

due to my use of antibodies specific for immune cell types rather than unspecific H&E staining. By

using these antibodies, I have been able to confirm the presence of immune cells by specific

molecular markers as opposed to morphological features which are subjective and can be open to

investigator bias. To my knowledge only one other study by Yeşil et al[55] has used specific immune

cell markers when investigating inflammation in tenosynovial tissue, where CD3 and CD20 were

used to detect T and B cells respectively in patients with CTS. Though both markers were used in

their study only the T-cell marker CD3 was used to designate an inflammatory phenotype, which

was found in only 4 (16%) study subjects and in 1 (7.1%) control patient. For B cells only 2 (14.3%)

control patients were positive for the CD20 stain. Inflammation detected in the Yeşil study was

again far lower than those detected here. A reason for this discrepancy could be due to

differences in staining protocol. In my analysis, generating IHC stains of sufficient quality took

several rounds of optimization, and included the essential use of HIER to unmask antigens.

Indeed, if I had not persevered with antibody optimization, I may have greatly underestimated

the number of immune cells present in tissues due to poor signal to noise ratio. Unfortunately it is

difficult to compare my staining techniques to the Yeşil study as they provide no detail on how

they conducted their immunohistochemistry and so the techniques cannot be directly compared.

In the studies mentioned previously, all but one made use of control tenosynovial tissue. In my

study I was limited by the fact that I was unable to obtain control tissue with which to compare

my findings with. As a result no conclusions can be made as to the level of immune cell presence

in tenosynovium as I cannot compare cell counts from normal tissue. However it is clear from my

findings that immune cells are present in the tenosynovium of patients with CTS, and when

compared to the staining in control tissue from the study by Yesil,[55] who did stain for T cells, my

results suggest that there is immune cell infiltration and inflammation in patients with CTS.

As I did not have access to control tissue I had to select patient samples based on the severity of their clinical phenotype from EDT scores. This allowed the comparison between patients with mild/moderate and severe CTS to determine whether there was a difference in immune cell presence based on CTS severity. Immune cell counts were not significantly different in patients based on EDT scores. This indicates that tenosynovial immune cell infiltration is independent of neurophysiological neuropathy severity. Tenosynovial immune cells may therefore not be mediating their pathological effects entirely through interactions with the median nerve, but instead could be eliciting pain through other mechanisms. This is in agreement with the suggestion by Hirata et al[365] who suggested that symptoms of CTS are caused by alterations to connective tissue instead of nerve pathology, however they made the clear distinction that these changes were not inflammatory. A study using a mouse model of arthritis investigated neuronal sprouting after injury and found increased density of both sympathetic and peptidergic neurons in the synovial membrane compared to controls[453]. The synovium of a joint is likely very different to synovium surrounding the flexor tendons and to my knowledge there is no previous information regarding axonal sprouting into the tenosynovium after nerve compression. However, it would be of interest to determine potential changes in innervation of the tenosynovium during CTS and investigate whether there is axon sprouting in this damaged tissue. Infiltrating immune cells could then be causing pain sensations via interactions with these tissue innervating nociceptors. Co-staining of immune cell and neuronal markers could determine whether these cells are interacting with nociceptive nerve endings, potentially contributing to symptoms.

3.4.2 CD4+ T cells correlate with symptom severity

The most convincing findings from the IHC experiments were observed in correlations between immune cell counts and clinical phenotypes, where CD4+ cell counts showed a moderate

correlation with Boston symptom scores. This correlation indicated that patients with higher numbers of CD4+ cells in their tenosynovium had increased symptom severity. This implicates CD4+ cells as potential drivers of pathogenesis in CTS by immune cell mediated mechanisms. I am currently not aware of any study that has investigated the expression of CD4 in CTS tenosynovial tissue using IHC and so comparisons to previous findings are not possible. However the study by Fuchs found that the immune cell infiltrates in their tenosynovial tissue consisted almost exclusively of lymphocytes.[364] When they correlated severity of symptoms including numbness, tingling, burning and soreness with inflammation, they found that there were no significant correlations. However, due to the limitations previously mentioned regarding their basic staining techniques, they may have greatly underestimated the immune cell presence in their patient tissues, and so were not accurately comparing inflammation with clinical phenotypes . In other entrapment neuropathies, such as lumbar radiculopathy, histological analysis of herniated disks has found T cells to be present within the tissue.[454, 455] In these studies T-cell numbers were not found to correlate with disc degeneration or pain duration, indicating that T cells were not driving symptom severity in these patients. However, neither of these studies used CD4 as their T-cell marker and so may not have identified the same population of T cells as I have in the tenosynovium. As CD3 cell counts, representing all T cells, did not correlate with clinical phenotypes in my study, this further suggests that the actions of a specific T-cell population such as CD4 are involved in the disease, which are not highlighted in pan T-cell staining. In line with my findings, one study investigating the expression of T-cell subsets in the blood of lumbar radiculopathy patients found a significant increase in CD4+ T cells in patients compared to controls[311]. Intriguingly, CD4+ cell counts strongly correlated with increased pain severity, suggesting that CD4+ T cells may be involved in the pathogenesis and symptomatology of entrapment neuropathies.

CD4 is characteristically expressed on a subgroup of T cells, however, its expression has been detected on other immune cells such as macrophages and dendritic cells.[456, 457] In this study, to

confirm that the CD4 signal was coming from T cells, a CD3 and CD4 co-stain would ideally have

been used to confirm the cellular source of CD4. This was not possible in my study as both CD3

and CD4 primary antibodies were derived from rabbits and other antibodies from different

species did not provide sufficient staining quality. However the morphology of the staining for

CD4 can be seen to be quite different in appearance from the CD68 stain. The cells stained by T-

cell markers display a disk shape while the cells stained by macrophage markers have a foamy,

bubbly appearance. This indicates that the CD4+ staining is coming from a cell type distinct from

macrophages. The morphology of the CD4 staining also resembles CD4+ T-cell staining from the

literature,[458] providing confidence that most of the CD4 signal seen here is coming from CD4+ T

cells.

3.4.3 GSEA analysis showed variable enrichment of immune cell signatures with clinical phenotypes

GSEA analysis of immune cell signatures indicated variable associations with clinical phenotypes.

This was most evident for Th1, CD8 and M1 signatures where multiple negative and positive

enrichments were found with phenotype scores. Gene sets tended to show negative enrichments

with phenotypes such as the Boston symptom score, Boston questionnaire sub-domains, EDT

severity and duration of symptoms while components of the NPSI questionnaire tended to have

positive gene enrichments. Negative enrichments indicate that the gene set has higher expression

in patients with lower symptom severity, which in the case of the Boston symptom questionnaire,

EDT severity and duration of symptoms, indicates that patients with lower scores on these scales

had higher expression of the immune cell genes. Positive enrichments on the other hand indicate

higher gene expression in patients with more severe symptoms, and so in the case of NPSI scores

my findings indicate that patients with higher neuropathic pain phenotypes have increased

expression of immune signature genes. Parts of these findings are in good agreement with the

reported functions of Th1 cells and M1 macrophages in neuropathic pain, where both cell types are thought to contribute.[459, 460, 461] Th1 cells have been found to cause neuropathic pain in mice undergoing CCI,[277] with another study identifying Th1 T cells as the main T-cell subtype that infiltrates the spinal dorsal horn after nerve injury and is associated with neuropathic pain.[462] My findings therefore contribute to the growing evidence indicating Th1 cells as being important in the generation of neuropathic pain. Immunohistochemical staining of Th1 T cells and M1 macrophages in the tenosynovium would be useful in confirming the presence of these immune cells in the tenosynovium. It would also be of great interest to investigate the affected nerve of CTS patients both genetically, using RNAseq and histologically, using IHC, to determine if there is an immune cell presence directly in the nerve tissue. This would however be difficult to achieve as the median nerve is not excised as part of decompression surgery and so alternative entrapment neuropathies where the nerve is excised could be an option (e.g., Morton's neuroma).

However the Th1 gene subset, which is a CD4+ population, was also negatively enriched for Boston symptom and pain scores, this is contrary to the findings from the IHC experiments. In those findings there was a positive correlation with CD4+ cell counts and Boston symptom scores. Previous studies have however noted a decrease in Th1 cells in neuropathic pain conditions, with one group finding a reduction in the Th1/Th2 ratio in peripheral blood of patients with neuropathic pain including postherpetic neuralgia, orofacial pain and peripheral poly and mononeuropathy compared to controls, though this difference was not significant.[310] In addition, a study investigating T-cell populations in patients with complex regional pain syndrome (CRPS) and fibromyalgia, found a reduction in the Th1/Th2 ratio in the peripheral blood of patients with CRPS.[463] These studies indicate that patients with neuropathic pain may indeed have decreased pro-inflammatory cells and that during neuropathic pain, cells with anti-inflammatory actions such as Th2 are increased in an attempt to control the inflammation and reduce neuropathic pain. This may partially explain the negative enrichments observed for Th1 genes and the Boston

symptom scores. In addition, as I was investigating tenosynovial tissue in my study, as opposed to neuronal structures such as the affected nerve, DRG or spinal dorsal horn, this could account for the discrepancy in the negative enrichments for Th1 and its association with pain reported in the literature.

Treg signature genes displayed negative enrichments with the Boston symptom scores but had positive enrichment for NPSI composite pain scores. This is contrary to multiple studies showing that Tregs are associated with reducing neuropathic pain.[296, 297, 459] However, gene sets for most immune cell types showed positive enrichments for NPSI components. This could indicate a generally higher immune cell activation in those patients with increased neuropathic pain symptoms. Increased Treg activation may be present in these patients as well, in an increased attempt to reduce neuropathic pain.

As with Th1 genes, the CD8+ T-cell signature was found to be positively enriched for components of the NPSI questionnaire, but negatively enriched for Boston questionnaire scores and EDT severity. One study has however identified CD8+ cells to be involved in resolving neuropathic pain in a CIPN model in mice,[464] as CD8+ T cells were the main T-cell type found in the DRG after injury and administration of CD8+ T cells to Rag1$^{-/-}$ mice caused increased recovery to mechanical allodynia.[464] CD8+ T cells have also been implicated in reducing pain in a model of arthritis, where CD8+ cell depletion increased pro-inflammatory cytokines and reduced endogenous opioid levels.[465] The negative enrichment of CD8+ genes with clinical phenotypes such as the Boston score may then be a reflection of its actions to inhibit (neuropathic) pain.

Alternatively, the discrepancy between the negative enrichments and the reported functions of pro-inflammatory cells could be due to the variable behavior of the genes within each set when ranked according to clinical phenotypes. From the GSEA, genes were present both at the top and bottom of the gene ranks, indicating that some signature genes had increased and others decreased expression in patients with higher symptom scores. However as the genes at the

bottom of the ranks were usually more abundant, the overall behavior of the gene set produced a negative enrichment. A good example of this can be seen in the GSEA for CD8+ T-cell signatures and the Boston paraesthesia sub-score (Figure 3.8). The variable behavior of the genes within the set could be due to the fact that the majority of the gene signatures were derived from immune cells that were extracted from the peripheral blood. These signatures were used as no immune cell signatures were available for immune cells derived from the tenosynovium of humans. Immune cells from the peripheral blood can have different gene expression profiles to tissue resident immune cells.[451, 466] The genes within the set may then not accurately represent immune cells from the tissue and so in the GSEA, these gene sets cause negative enrichments.

3.4.4 CD4+ cell counts correlate with Treg and NK-cell proportions

Correlations found between CD4+ cell counts from IHC and cell proportions of Tregs and NK cells derived from gene expression, may provide detail as to the identity of the CD4+ cells found in the tenosynovium staining. The association with Tregs may indicate that these cells are contributing to the CD4+ cells in the tenosynovium, as they display CD4 as part of their lineage[239]. Tregs are known to infiltrate the site of injury after damage to the nerve[459] and so may equally infiltrate the tenosynovium as a result of injury. For NK cells, the positive correlation could indicate two possible scenarios. One is that a proportion of the CD4+ cells stained in the tenosynovium could be NK cells instead of T cells. This is supported by the finding that NK cells are able to express CD4 on their cell surface, which has been found on NK cells present in the tissue.[467] The same study also found that CD4 expression on NK cells led to increased IFN-γ and TNF-α production and chemotaxis, providing functional relevance to CD4 expression on these cells. It could therefore be possible that NK cells, activated by tissue damage express CD4 in the tenosynovium and contribute to inflammatory responses. An alternative possibility is that the positive correlation between CD4+ cells and NK-cell proportions is indicating an increased infiltration of NK cells along

with CD4+ T cells. NK cells have been shown to infiltrate the peripheral nerve after injury in a mouse model of neuropathic pain[468] and so could be preforming a similar role here. It may also be possible that a combination of the two is occurring, whereby CD4+ and negative NK cells are infiltrating the tissue in conjunction with CD4+ T cells. To clarify Treg and NK-cell infiltration in the tenosynovium, further IHC experiments would be required. Co-staining of the Treg marker FOXP3 and CD4 would indicate the proportion of CD4+ cells that are Tregs, while co-staining using an NK-cell marker along with CD4 would determine whether NK cells are infiltrating the tenosynovium and whether they are contributing to the CD4+ cell numbers.

3.4.5 Limitations

IHC and RNAseq analysis of tenosynovial tissue was only conducted in samples from CTS patients as I did not have access to tenosynovium from healthy controls. This limits the interpretation of the results as no findings can be compared to 'normal tissue', as such it is difficult to say whether the immune cells or the gene signatures detected within the tenosynovium reflect true immune cell infiltration related to injury rather than populations present even in healthy tissues. I am also unable to determine whether the current results indicate an activation of tissue resident immune cells, as opposed to immune cell infiltration as normal immune cell presence in the tissue cannot be determined. Control tissue would also provide information as to whether the immune cell composition changes during CTS, as though the total immune cell numbers may stay the same, the composition of immune cells may reflect the activity of certain cell types in the pathogenesis of the disease. Potential options to collect healthy tenosynovial tissue include cadaveric sources as well as patients undergoing wrist surgery for conditions unrelated to CTS[364]. Indeed the acquisition of healthy tissue from such sources was currently being setup within my research group, however tissue was not yet available to be used in my study.

For the IHC staining, only 3 immune cell markers were used to identify cells, overall providing a limited phenotype of immune cells in this tissue. In light of the findings from the RNAseq data it would be of interest to conduct further immunophenotyping to either confirm or further reveal immune cell populations in tenosynovium. Also, as the stain for CD8 did not work in these experiments, the numbers for CD8 were inferred from CD3 and CD4 cell counts, which may not provide an accurate count of CD8+ T cells. Further work would look to produce a reliable CD8 stain to better determine one of the main populations of T cells. However as it can be difficult to obtain good staining in human samples, as evident in my study, the successful identification of these immune cell populations is encouraging for future staining of additional cell types.

Apart from the GSEA analysis, no corrections for multiple testing was conducted for associations between biological measurements and clinical phenotypes. This was due to the analysis being exploratory in nature and so not necessitating the need for such tests. However this may mean that there is a risk of false positives, and therefore validation studies are required to follow up on my findings. Nevertheless my results provide an important first step toward the detailed characterization of immune cells in neuropathic pain.

3.4.6 Conclusions

To conclude, immune cells were found to be present in tenosynovial tissue in all patients with CTS, challenging the common belief that the tenosynovium is devoid of immune cells.[54, 57] CD4+ T-cell counts were positively correlated with Boston symptom scores indicating that higher numbers of CD4+ T cells in the tenosynovium were associated with more severe symptoms. However, when investigating the association of immune cell gene signatures with clinical phenotypes, it was found that there was a variable enrichment pattern, indicating increased gene expression with certain symptoms and decreased gene expression with others. This conundrum could be partially explained by the variable behavior of the genes within the signatures, as well as

114

the cellular sources of the gene signatures themselves. Finally, Treg and NK-cell proportions were detected through deconvolution analysis and correlated with CD4+ cell counts in the tenosynovium, highlighting the possibility that both Tregs and NK cells could be present within the tissue and that they may be forming part of the CD4+ cells identified. Overall my findings indicate immune cells are present in the tenosynovium with some being significantly associated with clinical phenotypes.

Chapter 4: Molecular markers of nerve regeneration and associations with inflammation

4.1 Introduction

Peripheral nerve injury due to trauma[469] or disease[470, 471] is a common occurrence in humans. Surgical interventions such as direct nerve repair to restore the function of the nerve are commonly conducted.[472] However, surgical treatment is often inadequate and full functional recovery is not often achieved.[473] As outlined in my introduction (section 1.4.2), there are currently no effective treatments using neurotrophins in patients to facilitate nerve regeneration. Other treatment options for nerve injury are therefore needed to aid the regeneration of the peripheral nerves to achieve long lasting and complete recovery. Pre-clinical studies to determine the molecular and cellular processes of nerve regeneration have identified a myriad of processes and interactions important for nerve regeneration including successful Wallerian degeneration after injury,[474] activation and activity of Schwann cells[475] and the prevention of chronic denervation.[476] Much of the work done to study peripheral nerve regeneration has made use of pre-clinical animal models,[316] however these findings often poorly translate to nerve regeneration in humans.[477] One of the main factors affecting the success of nerve growth in humans and a reason why pre-clinical models poorly translate, is the longer distance that the nerve has to regrow to reach peripheral targets.[349] This causes increased times of axotomy and chronic denervation[343] resulting in a reduction of successful regeneration.[350] As a result, more work is needed specifically in human systems to elucidate the mechanisms of nerve regeneration with the ultimate goal to identify therapeutic targets. To achieve this, I have conducted experiments using skin biopsies from patients with CTS. This has served as a human model system to investigate chronic nerve denervation. Notably, the recovery after surgical intervention has

enabled the prospective characterization of cellular and molecular determinants of nerve regeneration and their association with clinical phenotypes.

Skin biopsies from patients with CTS were collected before and six months following surgery. In experiments performed before the start of my DPhil, both molecular and immunohistochemical analyses were conducted. Molecular analysis identified the gene ADCYAP1 to be significantly dysregulated with an overall increased expression in the cohort after surgery (analyses performed by Annina Schmid and Georgios Baskozos). ADCYAP1 encodes the protein Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) which is a pleotropic protein with diverse functions within the nervous system, acting as a neurotrophic factor[478, 479] and neuromodulator.[480, 481] PACAP is produced in two forms, PACAP-27 and PACAP-38[482] and binds with high affinity to its main receptor PAC1 (PAC1R).[483] PACAP shares sequence homology with the vasoactive intestinal peptide (VIP) and can bind to receptors VPAC1 and VPAC2, which have equal affinity for PACAP and VIP.[484] PACAP has previously been found to aid in the regeneration of sensory neurons in animal studies,[485, 486] and motor neurons in humans.[487] However whether it can increase the regenerative capacity of sensory neurons in humans remains unknown.

In addition to neuronal associated pathways and factors, inflammation has also been implicated in affecting nerve regeneration.[8] However the consensus as to the exact role that inflammation plays in nerve regeneration is uncertain. This is due to conflicting findings, where some studies have identified inflammation to be required for effective nerve repair,[276, 468] while others have found it to hinder successful nerve regeneration.[488] Further investigation is therefore required to understand the role that inflammation plays in nerve regeneration.

4.1.1 Aims

I will seek to characterize the expression of ADCYAP1/PACAP in the target innervation territory and look specifically at the effects it has on human sensory neuron regeneration. Building on findings from Chapters 2 and 3, where several signs of inflammation have been detected, I will

also endeavor to explore the presence of inflammation in the target tissue, and discover how this correlates with indicators of nerve regeneration and clinical phenotypes.

4.2 Methods

4.2.1 Participants

In this study I made use of an existing prospective longitudinal cohort study collected by Annina Schmid, including patients with electrodiagnostically confirmed CTS. This cohort has been described previously in Chapters 2 and 3.

4.2.2 Phenotypic data

A detailed description of the phenotypic data collected is available elsewhere[76] and the phenotypic data used in this chapter has been described in Chapter 3, 2.2.2 and Chapter 3, 3.2.2 phenotypic data.

4.2.3 Tissue collection and preparation

To determine markers of nerve regeneration in target tissue innervated by the median nerve, a 3mm in diameter skin biopsy was taken at two assessment periods (before and 6 months following surgery). The first skin biopsy was taken from the ventrolateral aspect of the proximal phalanx of the index finger. The second skin biopsy was taken several millimeters more proximal to the first to try and avoid the first biopsy site. Skin biopsies were performed under sterile conditions following administration of 1% lidocaine (1–1.8 ml). Half of each biopsy was snap-frozen in RNAlater solution (Thermo Fisher Scientific, UK) at -80°C for RNA extraction. For details of RNA purification see Chapter 3, 3.2.3. The concentration of RNA was determined by UV

absorbance at 260nm on a NanoDrop spectrophotometer. Samples were frozen at -80^0c until further use.

The other half was fixed in fresh periodate-lysine-paraformaldehyde (PLP) fixative for 30 min at room temperature. After fixation, samples were washed 3x in 0.1 M phosphate buffer and cryoprotected in 15% sucrose in 0.1 M phosphate buffer at 4^oC for 3-4 days. Tissue samples were then embedded in OCT in plastic molds, snap frozen and stored at -80^oc.

Markers of nerve degeneration and regeneration such as the intraepidermal nerve fibre density (IENFD) and Meissner corpuscle density were quantified. IENFD counts were determined from skin biopsies by counting 3 sections from each patient and taking the average count, which was expressed in fibres/mm. Meissner corpuscles were counted per mm epidermis. The classification of patients being either regenerators or non-regenerators, defined by IENFD fold change (post-surgery – pre-surgery)/pre-surgery where >0 = regenerator and <0 = non-regenerator was also determined. Along with the phenotypic data outlined above, these markers of nerve degeneration and regeneration were used in further analyses including GSEA to determine an association with inflammation. The collection and preparation of tissue and the quantification of markers of degeneration/regeneration was conducted by Annina Schmid.

4.2.4 RNA Sequencing

The extracted RNA of 47 patients with CTS was sequenced at the Wellcome Trust Centre for Human Genetics in Oxford, UK.

Prior to preparation of the library all RNA samples were normalized to 650ng using the Illumina TruSeq Stranded mRNA Library Prep Kit and standard universal Illumina multiplexing adaptors. The RNA sequencing library was poly(A)-enriched and directional with the poly(A)-selected RNA being converted to cDNA using the strand specific dUTP strand-marking protocol,[441] with the

amplification using unique dual indexing. The Illumina HiSeq4000 platform was used for paired-end sequencing with a read length of 75 bp. Quality metrics were encoded by the Phred score in the resulting FastQ sequencing files, further quality controls was done using Samtools.[489] Samples from individuals were multiplexed in lanes and all sequencing lanes gave a high yield. Reads were mapped to the GRC.h.38 Human genome using the splice-site aware STAR aligner programme[443] with standard ENCODE options. Gene counts were generated using HTSeq[444] against the GRC.h.38.88 ENSEMBL reference gene set.

Differential gene expression (DEG) analysis was preformed using DESeq2. The raw gene count data was processed in R, and normalized for effective library size using the DESeq2 package.[445] Normalized gene counts were fitted to the negative binomial distribution where hypothesis testing was done using the Wald test. P values were FDR corrected using the BH method as well as independent hypothesis weighting (IHW).[490] Both moderated (values had been shrunk towards zero for lowly expressed genes) and non-moderated Log2 fold changes were used in hypothesis testing. A gene was considered to be significantly dysregulated if it had an adjusted p value <0.05 in two out of three hypothesis tests that were done such as moderated Log2 fold changes with FDR correction, un-moderated Log2 fold changes with FDR correction and un-moderated Log2 fold changes with IHW correction.

Gene ontology (GO) enrichment for biological processes for the DEGs was carried out using topGO[491] and GSEA.[492] The hypothesis testing was done with the weighted Fisher test where the significance cut-off was 0.01.

ADCYAP1 gene counts

Log2 normalised counts for ADCYAP1 expression, obtained from RNA sequencing were included in analyses such as GSEA to determine an association with inflammation. RNA sequencing was performed by Annina Schmid and Georgios Baskozos.

4.2.5 ddPCR

To validate the RNAseq findings, I used the alternative gene expression quantification method of digital droplet PCR (ddPCR). Before and after surgery samples from N = 39 patients were used in the validation experiment. Complementary DNA was constructed using the Evoscript Universal cDNA Master kit (Roche Diagnostics, UK). TaqMan™ assays (Thermo Fisher Scientific, UK) for the gene of interest ADCYAP1 (Hs00174950_m1) and the housekeeping gene HPRT1 (Hs03929098_m1) were run in a duplicate reaction where 1 µl of FAM labelled target probe was added with 1 µl of VIC labelled reference probe in the same reaction using the ddPCR Supermix for Probes (Bio-Rad, UK). QuantaSoft v1.7.4.0917 software (Bio-Rad, UK) was used to determine the concentration of genes (copies/µl), which were reported as normalized gene expression values (ratios of target over reference data).

To compare the gene expression of ADCYAP1 from RNAseq and ddPCR analysis, graphed results were inspected by eye to determine the similarity of expression of ADCYAP1 by these two techniques. To statistically confirm the similarity of expression of ADCYAP1 between RNAseq and ddPCR, a Pearson's correlation was conducted on before and after surgery expression values for the two techniques where a $p < 0.05$ was considered significant.

4.2.6 Immunohistochemistry

To determine the localization of PACAP within the skin biopsies, I used immunofluorescent staining that was adapted from a previous study.[493] briefly, 50 mm skin sections were cut on a cryostat (Leica, Germany) and placed into the well of a 96 well plate filled with PBS + 0.2% Triton-

X, where they were blocked with 10% goat serum in PBS + 0.2% Triton-X for 30 min. I removed the blocking solution and primary antibodies for PACAP (gift from Prof Jan Fahrenkrug, 1:5) and PGP 9.5 (Zytomed, 1:200, cat no: 516-3344) were added and incubated overnight at 4°C on a shaker. Primary antibodies were removed and a biotinylated goat anti-mouse antibody (Vector laboratories, 1:200) along with an Alexa Fluor 546 anti-rabbit antibody (Life technologies, 1:500) was then applied for 2 h at room temperature on a shaker. After 3 washes with PBS + 0.2% Triton-X and 2 washes with PBS, I incubated sections for 30 min with an avidin-biotin-horseradish peroxidase (VECTASTAIN Elite ABC Kit, Vector laboratories, UK) before washing 2x with PBS and 2x with 0.1M borate buffer. Sections were incubated in FITC conjugated tyramide (Perkin Elmer, 1:100) diluted in 0.1 M borate buffer containing 0.0003% hydrogen peroxide for 10 minutes. After 3 washes with PBS + 0.2% Triton-X for 10 minutes each, I applied mounting media and sealed the sample with a coverslip for imaging on an Observer Z1 confocal imaging system (Zeiss, Germany). The same settings were used within each patient, where microscope settings were always configured using the pre-surgery sample, this was then used when imaging the post-surgery sample. Average PACAP staining intensity in samples from ten patients was quantified before and after surgery using ImageJ (NIH, USA). Staining intensity was quantified firstly in an area overlying the epidermis and subepidermal plexus including both neuronal and non-neuronal structures and secondly, specifically within PGP+ nerve fibers using thresholding. Wilcoxon tests were used to determine differences in the PACAP fluorescence intensity from before to after surgery. Pearson's correlations were used to determine associations between the Log2 fold changes of ADCYAP1 expression and PACAP fluorescence intensity. In each case a P<0.05 was considered significant.

I also determined the localization of the PAC1 receptor in human skin by using double immunostaining with PAC1 (Cambridge Bioscience, 1:100) and PGP 9.5 (Bio-Rad, 1:200) primary antibodies. Briefly, I cut 50 mm skin sections on a cryostat (Leica, Germany) and placed them into a well of a 96 well plate filled with PBS + 0.2% Triton-X before boiling them in 10 mM citric acid with 0.05% Tween-20 for 5 min. Samples were then incubated for 30 minutes in 10% goat serum

in PBS and 0.2% Triton-X at room temperature. I added primary antibodies to the samples and incubated them overnight at 4°C on a shaker. The next day, sections were washed 3x with PBS + 0.2% Triton-X and incubated with biotinylated goat anti-rabbit antibody (Vector laboratories, 1:200) for 2h at room temperature. I then added secondary antibodies Alexa Fluor 546 anti-mouse (Life technologies, 1:1000) and Streptavidin 488 (Life technologies, 1:500) for 2h at room temperature in the dark. Sections were washed, mounted and imaged on an Observer Z1 confocal imaging system (Zeiss, Germany). No statistical tests were conducted here as the presence of PAC1 in the skin was reported qualitatively.

4.2.7 Cell culture

To investigate the regenerative capacity of PACAP on human sensory neurons, I used human induced pluripotent stem cell derived (hiPSCd) sensory neurons. Two control iPSC lines were used that were derived from the fibroblasts of a healthy 44-year-old female (NHDF)[494] and a healthy 51-year-old male (AD2) (NRES Committee South Central – Berkshire UK, REC 10/H0505/ 71). These cell lines were differentiated to sensory neurons as previously described.[495-497] Briefly, cells from each line were plated out at high density following Versene EDTA (Thermo Fisher Scientific, UK) passaging. Neural induction was initiated in KSR medium containing Knockout-DMEM, 15% knockout-serum replacement, 100µM β-mercaptoethanol, 1% nonessential amino acids 1% and Glutamax (Thermo Fisher Scientific, UK), by the dual inhibition of SMAD using SB431542 (Sigma, 10µM) and LDN193189 (Sigma, 100nM). On day three, 3 small molecules CHIR99021 (Sigma, 3µM), SU5402 (R&D Systems, 10µM) and DAPT (Sigma, 10µM) were introduced and on day 5 the dual SMAD inhibitors were withdrawn. KSR medium was transitioned in quarter increments to neural medium containing N2/B27- Neurobasal medium, 2% B27 supplement, 1% N2 supplement and 1% Glutamax, (Thermo Fisher Scientific, UK) over a period of 11 days. Cells were then dissociated and replated out onto coverslips in neural medium that was supplemented with

growth factors BDNF (Thermo Fisher Scientific, UK) NT3, NGF, GDNF (PeproTech, USA).

CHIR90221 was included for an extra 4 days. Phenol-free Matrigel (Corning, 1:300) was used from

25 days onward and cell medium was changed twice a week. Young neurons (8 weeks old) and

neurons matured for up to 27±3 weeks were used in neurite outgrowth assays.

To determine the regenerative capabilities of PACAP on human primary sensory neurons, I made

use of a humanized *in vitro* model of nerve injury previously described.[498] Mature hiPSCd sensory

neurons were first treated with 0.1% Trypsin (Thermo Fisher Scientific, UK) for 30 min before

being mechanically dissociated with a glass pipette. Single cells were replated onto Matrigel™

treated coverslips at low density to detect individual nerve growth in the presence of varying

concentrations of the PACAP protein (10 nM - 10 µM, AnaSpec and Bachem), vehicle (0.01%

DMSO, Sigma) or a selective PAC1 agonist maxadilan (1 µM, Bachem). Cells were then fixed 18

hours later and analyzed by immunocytochemistry. This method was designed to mimic axonal

injury and subsequently work as a model for nerve regeneration in a dish. Cell culture

experiments were performed by Greg Weir and Alex Clark.

4.2.8 Quantification of neurite outgrowth

hiPSCd sensory neurons derived from four separate differentiations were used for outgrowth

quantification. Neuronal cells cultured on a 24-well plate were taken and dissociated onto

individual coverslips to form each experimental unit (n). Six dissociation experiments were

performed. I calculated the average neurite outgrowth from 3–10 technical replicates (number of

coverslips of re-plated neurons for a particular condition). hiPSCd sensory neurons that were

treated with either vehicle or PACAP were immunostained with the neuronal marker NF200 using

immunocytochemistry. I chose NF200 as it is expressed in almost all human DRG neurons.[499]

I applied the primary NF200 antibody (Sigma, 1:500) to sensory neurons fixed on coverslips and incubated them overnight at room temperature. Cells were then washed 3x with PBS + 0.01% Triton-X. The secondary anti-mouse antibody Alexa Flour 488 (Life Technologies: 1:1000) was added for 2h at room temperature in the dark. Neurons were washed 3x with PBS + 0.01% Triton-X and the coverslips were then mounted on slides and sealed. 20x magnified images of individual sensory neurons from each coverslip were taken on an Observer Z1 imaging system (Zeiss, Germany), neurite length was analyzed using WIS-Neuromath software.[500] Only neurons with a neurite length of at least half the diameter of the cell body were included. I measured neurite length in μm per cell and the neurite lengths from individual neurons were averaged per coverslip. Independent t-tests or one-way ANOVA using Fisher's Least Significant Difference (LSD) post hoc tests were done to compare neurite lengths between conditions. Neuronal binding of PACAP (biotinylated protein, AnaSpec) on sensory neurons was analyzed using double-labelling with the protein of interest and NF200 (Abcam, 1:2000) to determine neuronal structures. The immunohistochemistry protocol outlined above was used.

4.2.9 Gene set enrichment analysis

GSEA for immune cell signatures and clinical phenotypes using RNAseq data derived from patients' skin biopsies was conducted as set out in Chapter 3, 3.2.9. For these analyses, RNAseq data were corrected for age and sex and the phenotypic and clinical data included in the analyses are shown in Table 4.1 and Table 4.2. Associations were not corrected for multiple testing as these investigations were exploratory.

4.2.10 Deconvolution analysis

Deconvolution analysis was conducted with RNAseq data derived from patient skin biopsies as described in Chapter 3, 3.2.10.

4.3 Results

4.3.1 Patient demographics and clinical phenotypes

Patient demographic and clinical data are shown below in Table 4.1. Patient markers of nerve regeneration are shown in Table 4.2.

Table 4.1: Patient demographic data and clinical phenotypes
Data are shown for patients with CTS before (pre) and six months following surgery (post). Paired T-tests were conducted to determine differences between clinical phenotype scores before and after surgery. Unless otherwise stated data are shown as mean ± standard deviation.

	Patients with CTS Pre-Surgery	Patients with CTS Post-Surgery	P values
Number of participants	47	47	
Mean Age (SD) [years]	63 (12)		
Male:Female	18:29		
Duration of symptoms (SD) [Months]	69.23 (106.19)		
Median EDT grade [IQR]	3 [1]	2 [2]	<0.001
Boston Symptom score (SD)	2.83 (0.72)	1.48 (0.51)	<0.001
Boston Pain sub-score (SD)	2.67 (1.06)	1.48 (0.51)	<0.001
Boston Weakness sub-score (SD)	2.64 (1.06)	2.06 (0.78)	0.001
Boston Paresthesia sub-score (SD)	3.19 (0.84)	1.28 (0.49)	<0.001
VAS Pain (SD)	3.17 (3.05)	0.33 (0.75)	<0.001
Median GROC Score [IQR]		7[2]	
NPSI composite score (SD)	13.84 (9.03)	2.16 (3.62)	<0.001
NPSI burning (SD)	2.53 (3.27)	0.23 (0.94)	<0.001
NPSI deep pressure (SD)	2.15 (2.55)	0.22 (0.71)	<0.001
NPSI evoked (SD)	1.37 (1.94)	0.62 (0.99)	0.027
NPSI paresthesia (SD)	5.79 (3)	0.59 (1.37)	<0.001
NPSI paroxysmal (SD)	2.01 (2.62)	0.51 (1.27)	0.001

SD: Standard Deviation, IQR: Inter-Quartile Range, VAS = visual analogue scale, NPSI = Neuropathic Pain Symptom Inventory, EDT = Electrodiagnostic test, GROC = Global rating of change.

Table 4.2: Patient markers of nerve regeneration

Molecular markers indicating nerve regeneration are shown for patients with CTS before (pre) and six months following surgery (post) with paired T-tests identifying statistical difference between groups. Data are shown as mean ± standard deviation.

	Patients with CTS Pre-Surgery	Patients with CTS Post-Surgery	P values
IENFD counts (SD) [fibres/mm]	4.21 (2.85)	5.13 (2.83)	0.006
Meissner corpuscles (SD) [per mm epidermis]	0.39 (0.32)	0.43 (0.36)	0.53
ADCYAP1 expression [Log2 normalized counts]	2.42 (0.13)	2.59 (0.41)	
Regenerators:Non-regenerators		29:18	

IENFD = Intraepidermal Nerve Fiber Density.

4.3.2 ADCYAP1 expression is increased post-surgery

The RNAseq of patients' skin biopsies from before and after surgery revealed 34 dysregulated genes, where 23 genes were up regulated after surgery and 11 genes were downregulated (Figure 4.1, A). The gene showing the greatest increase in expression after surgery was ADCYAP1, which was identified to be involved in neuronal growth and survival.[485, 487, 501] As ADCYAP1 was an interesting candidate for further study based on its biological relevance and as it was found to correlate with IENFD regeneration,[76] the expression of this gene was confirmed using ddPCR (Figure 4.1, B). The expression pattern of ADCYAP1 in patient samples both before and after surgery were very similar between the two techniques, which was confirmed by Pearson's correlation (Figure 4.2, C).
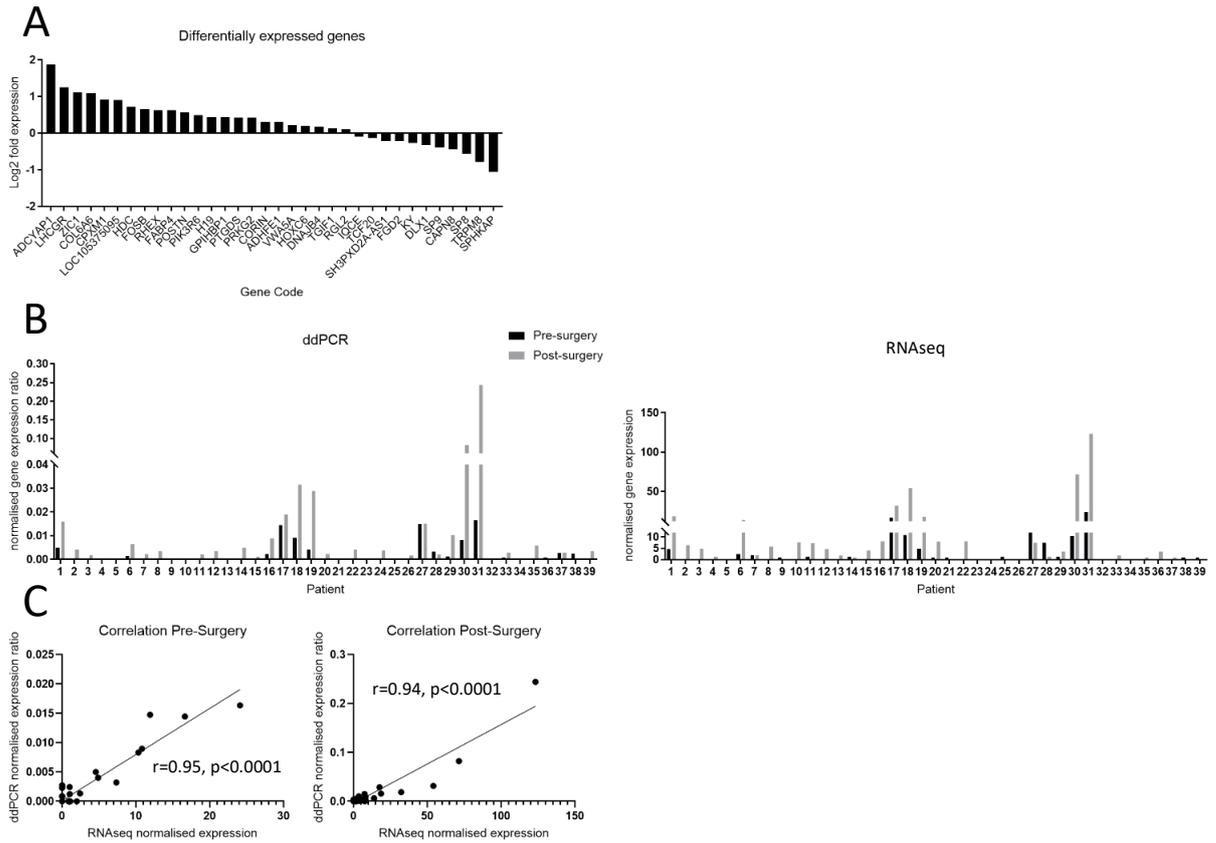
Figure 4.1: ADCYAP1 gene expression dysregulation was identified by RNAseq and confirmed by ddPCR

A. A list of 34 significantly dysregulated genes from the RNAseq analysis of patient skin biopsies. ADCYAP1 gene expression showed the greatest fold change in expression post-surgery. B. ADCYAP1 expression was confirmed using ddPCR. Pre and post-surgery expression patterns for ADCYAP1 were similar to the expression patterns from the RNAseq analysis. RNAseq data is presented as Log2 normalised gene counts while ddPCR data are presented as a normalised gene expression ratio (target expression/reference expression). C. Correlation analysis for pre and post-surgery expression values of ADCYAP1 between ddPCR and RNAseq showed a strong correlation. Pearson's correlation was used where p<0.05 was considered significant.

4.3.3 PACAP is expressed in nerves innervating the skin

As ADCYAP1 was found to be dysregulated in the genetic analysis, I next investigated the

expression and localization of the ADCYAP1 encoded protein PACAP in patient skin biopsies

(Figure 4.2, A). PACAP staining co-localized with neuronal markers indicating that PACAP is

located within sensory nerves innervating the skin (Figure 4.2, A). PACAP staining also appeared

to be present in the basement membrane of the skin, potentially indicating PACAP is also being

expressed by skin cells such as keratinocytes. However as no co-staining was done for

keratinocytes and PACAP, the expression of PACAP by these cells cannot be determined here. The

fluorescence intensity of the PACAP staining in before and after surgery samples was quantified,

both within neuronal structures (Figure 4.2, B-D) and within a specific section including neuronal

and non-neuronal structures (Figure 4.2, E-G). However, the fluorescence intensity of the PACAP

staining before and after surgery did not match with the fold change in ADCYAP1 expression

when patients were grouped on this characteristic (Figure 4.2, B and E). Correlation analysis of

the Log2 fold change in fluorescence intensity with the Log2 fold change for ADCYAP1 expression

showed no significant correlation when looking either within neuronal structures or within a

region of interest (r=0.23, p=0.5 and r=0.3, p=0.4 respectively) (Figure 4.2, C and F). However

when comparing fluorescence intensity from before to after surgery at a group level, PACAP

staining intensity was significantly increased after surgery both within neuronal structures and in

a region of interest (Figure 4.2, D and G). The expression of ADCYAP1 in the skin may therefore

not directly translate to protein production in every patient however PACAP expression appears

to be generally increased after surgery. Challenges associated with quantification of fluorescent

signal have also to be considered for the discrepancy between genetic expression and

fluorescence intensity.
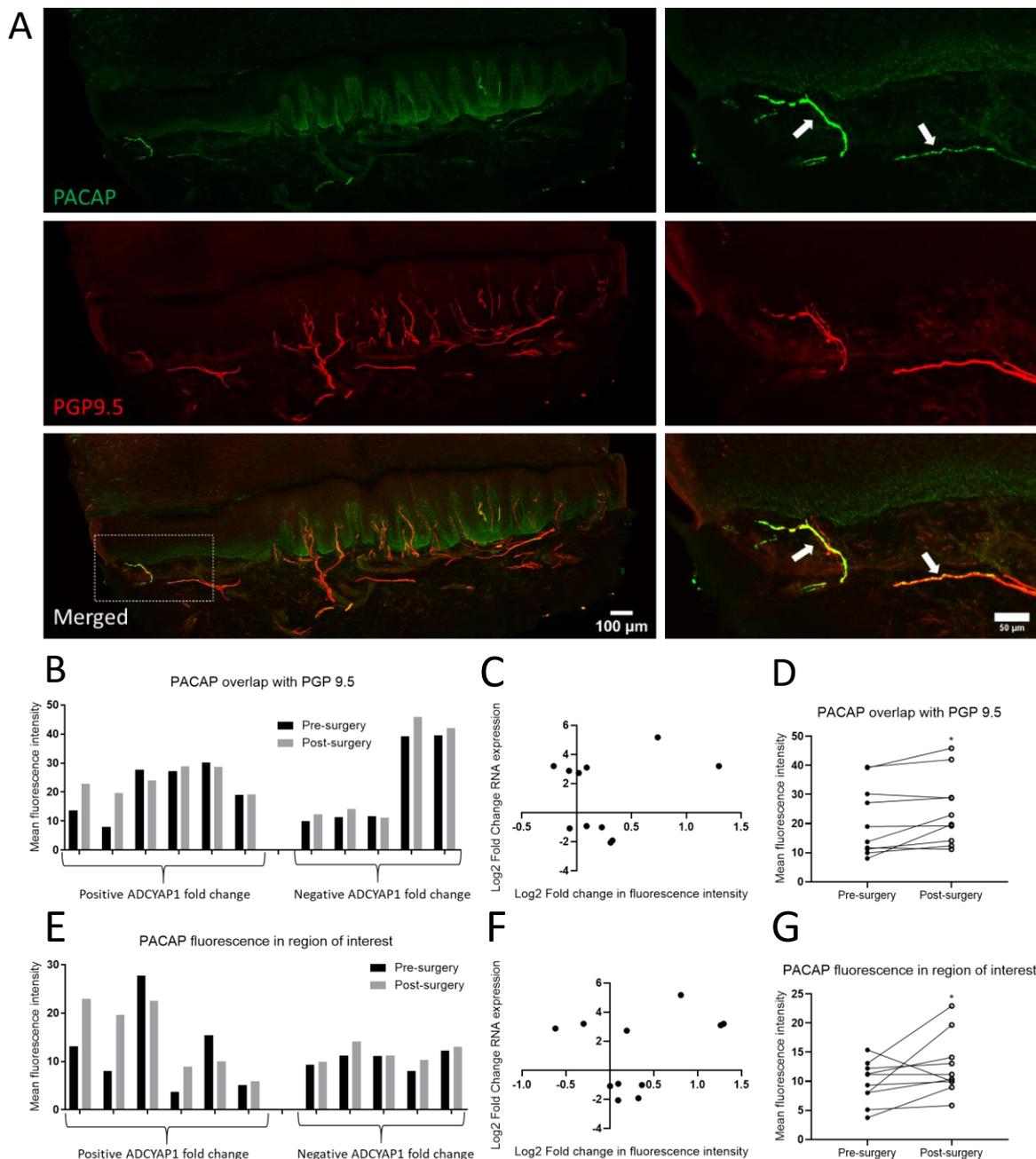
Figure 4.2: PACAP localised with sensory afferents in the skin but did not correlate with ADCYAP1 expression

A. Using IHC, PACAP co-localised in the skin with PGP 9.5 indicating PACAP is located within neurons. The white box indicates a region of interest which is shown enlarged in panels on the right. White arrows indicate PACAP staining which co-localises with PGP 9.5. B & E. Quantification of the mean fluorescence intensity of PACAP within neuronal structures and within a region of interest respectively, from skin biopsies both pre and post-surgery. Patients have been grouped based on their ADCYAP1 fold change from the RNAseq analysis. Patients with a positive ADCYAP1 fold were expected to display an increase in PACAP fluorescence post-surgery while patients with a negative ADCYAP1 fold change were expected to have decreased PACAP fluorescence post-surgery. However this was not the case for the majority of patients when looking within neuronal structures of in a region of interest. C & F. Log2 fold changes for fluorescence intensity from IHC and ADCYAP1 expression from RNAseq are shown, however no significant correlations were found, indicating that changes in ADCYAP1 expression may not translate to PACAP production. D & G. When comparing mean fluorescence intensity from pre to post-surgery the PACAP fluorescence intensity was found to generally increase post-surgery. This was the case when looking within neuronal structures and in a region of interest. Data are presented as single data points. *P<0.05.

130

4.3.4 PAC1 colocalizes with sensory afferents innervating the skin

The PACAP specific receptor PAC1 was found to co-localize with neuronal markers in the skin, indicating PAC1 expression in sensory afferents (Figure 4.3). This was not found to be the case when a no primary antibody control (Blank) was used.
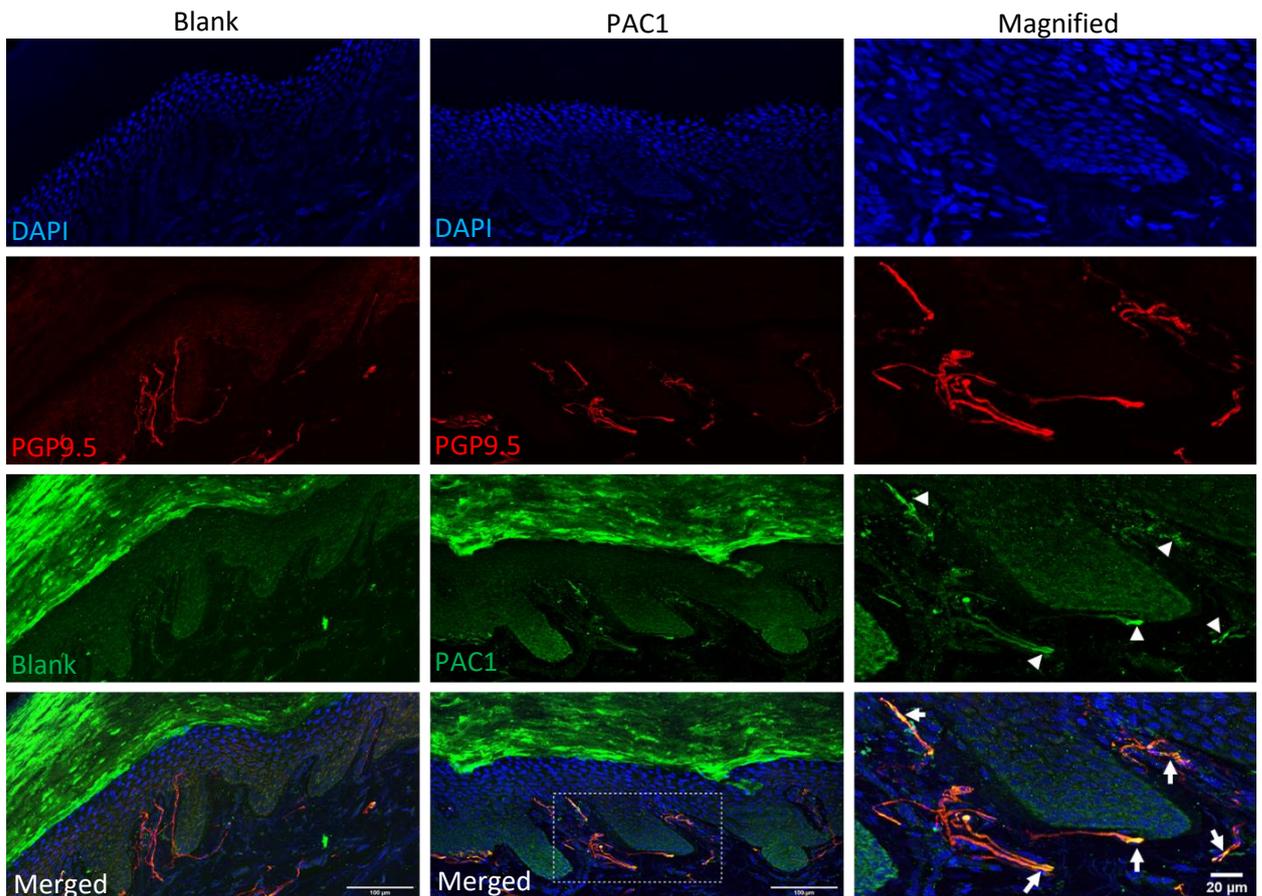
Figure 4.3: PAC1R staining in the skin
The PACAP receptor PAC1 colocalised with the neuronal marker PGP9.5 indicating expression within sensory afferents innervating the skin. PAC1 staining was not present in a blank control where no primary antibody was used. The white box indicates a region of interest which is magnified in panels on the far right. Arrow heads indicate PAC1 staining and arrows indicate PAC1 staining colocalised with PGP9.5

4.3.5 Exogenous PACAP increases neurite outgrowth after injury

As ADCYAP1 was increased after surgery and PACAP was found to be expressed in sensory afferents in the skin and upregulated after surgery, I then investigated the effect of PACAP on

regeneration of hiPSCd sensory neurons after nerve injury. Initially, the application of 10nM PACAP on hiPSCd sensory neurons (NHDF cell line) was able to cause a significant increase in neurite outgrowth (Figure 4.4, A). Representative neurons showed characteristic neuron morphology of arborising neurons growing in a web-like structure. I then sought to replicate this finding using another cell line (AD2), however on conduction of the experiment no significant increase in neurite outgrowth could be detected in PACAP treated neurons (Figure 4.4, B). The morphology of these cells was however unusual as they produced long singular projections as opposed to characteristic web-like structures (Figure 4.4, B). Due to the atypical structure of the neurons, I repeated the experiment (Figure 4.4, C), however these neurites again displayed atypical morphology (singular long projections) and neurite outgrowth was again no different between PACAP and vehicle treated cells (Figure 4.4, C). Upon strategic comparison of the experimental parameters I discovered, that one main difference between the neurons used in the original experiment to those used later, apart from the difference in cell line, was that the original neurons were much older (27±3 weeks compared to 8 weeks). I hypothesized that the different ages may cause different regenerative phenotypes in the hiPSCd sensory neurons, as the younger neurons may have a predisposition for increased growth anyway and could express higher levels of factors aiding in neurite growth.

I therefore compared gene expression data previously obtained in our lab of young hiPSCd sensory neurons (8 weeks) and mature hiPSCd sensory neurons (27 ± 3 weeks). This revealed that the endogenous expression of ADCYAP1 was indeed significantly higher in younger neurons compared to mature neurons (Supplementary Figure 1 in Appendix). This increase in endogenous ADCYAP1 expression could have made the availability of PACAP in the vehicle treated cells equal to that of the PACAP treated cells. As a result this may have reduced any difference in neurite outgrowth after PACAP treatment as endogenous PACAP was also readily available to vehicle treated cells.

To test this hypothesis, I repeated the initial experiment using the same cell line (NHDF) and made sure that to prevent a potential pro-regenerative phenotype of young neurons, I used mature neurons (27 ± 3 weeks). The administration of PACAP on neurons of the same maturity as used in the original experiment, indeed replicated my original findings (Figure 4.4, D). Of note, the morphology of these cells were again characteristic of sensory neurons. Two different doses of PACAP were used in this assay (100nm and 10μm) and I discovered a clear dose dependent significant increase in neurite outgrowth (Figure 4.4, D).

To confirm that PACAP was binding to hiPSCd sensory neurons, the application of biotinylated PACAP showed specific staining around the cell soma by immunocytochemistry (Figure 4.4, E). This was not observed when using a no primary antibody control (Figure 4.4, F).

I also endeavored to determine whether modulation of the PAC1 receptors with an exogenous agonist could cause similar effects on neurite outgrowth, and so I applied the PAC1R agonist maxadilan to hiPSCd sensory neurons after injury (Figure 4.4, G). Maxadilan was able to significantly increase neurite outgrowth compared to vehicle treatment. The increase in neurite outgrowth was comparable to that of PACAP treatment (Figure 4.4, G).
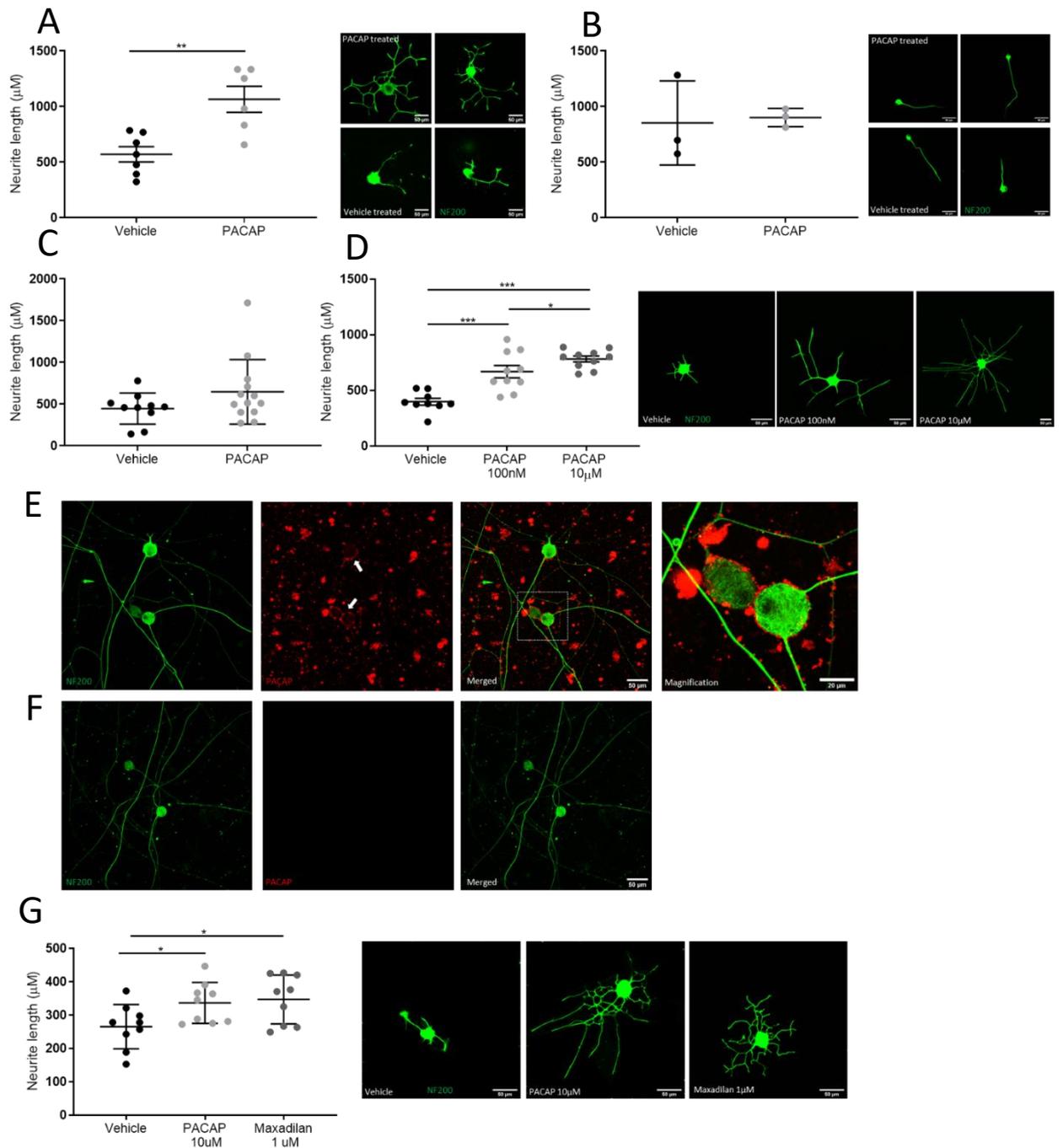
Figure 4.4: Exogenous PACAP causes increase neurite outgrowth after injury in mature neurons
A. The application of PACAP caused an significant increase in the outgrowth of mature (NHDF cell line of 27±3 weeks old) hIPSCd sensory neurons after injury. Representative images show that neurons treated with PACAP had longer and more complex neurite projections. B. PACAP did not have an effect on the outgrowth of younger (AD2 cell line of 8 weeks old) neurons. Neuron images show an irregular cell morphology where cells sprout singular, elongated neurites. C. The repeat experiment using younger neurons found that PACAP administration did not have an effect on neurite outgrowth. D. The use of more mature neurons (NHDF cell line of 27±3 weeks old) re-established the effect of PACAP on neurite outgrowth as cells treated with PACAP had a significantly increased outgrowth compared to controls. This effect was found to be dose dependent. Images demonstrate increased growth at higher concentrations. E. PACAP bound to the cell soma of neurites. White arrows indicate PACAP binding and the white dotted box marks a region of interest. Panels on the far right show a magnified region of interest. F. Using a no-primary antibody control did not produce any specific staining. G. 1µM maxadilan and 10µM PACAP caused significant increases in neurite outgrowth compared to vehicle (AD2 cell line of 27±3 weeks old). No difference was observed between PACAP and maxadilan treated cells. Data are shown as mean ± SEM and as single data points. *P<0.05, **P<0.01, ***P<0.001.

4.3.6 T-cell genes are enriched with patient symptoms pre-surgery which is abolished following de-compression surgery

I next sought to determine whether signs of inflammation/immune cells could be identified in the target innervation territory of the median nerve and whether they are associated with nerve regeneration. Using GSEA, enrichments of signature immune cell genes were determined from patients' skin biopsies, both before and after surgery (Supplementary Table 8 in Appendix). Pre-surgery, no significant enrichments were found for genes specific to Th1, Th2, Th17, Treg, CD8+ T cells or M1 macrophages. However, several significant enrichments were observed for genes specific to naïve CD4+ T cells. These genes were positively enriched for the Boston symptom score as well as pain and weakness subdomains, the NPSI composite score and Meissner corpuscles number (Figure 4.5, A). This indicated that the expression of naïve CD4+ T-cell genes were increased in patients who had more severe symptoms or increased Meissner corpuscles numbers before surgery. Genes for M2 macrophages also showed a significant positive enrichment for the Boston pain sub-score pre-surgery (Figure 4.5, A).

However, in the GSEA using post-surgery samples, the positive enrichments for naïve CD4+ T-cell genes were abolished and only a negative enrichment for Meissner corpuscle numbers could be detected (Figure 4.5, B). There were however several significant enrichments for macrophage related genes where M1 specific genes were positively enriched for VAS pain and negatively enriched for the NPSI paroxysmal sub-score. M2 specific genes were negatively enriched for Meissner corpuscle numbers, Boston paresthesia sub-scores and ADCYAP1 expression, but were positively enriched for VAS pain (Figure 4.5, B). No other significant enrichments were observed for immune cell gene signatures using post-surgery data.

GSEA carried out to determine whether the expression of immune genes changed from before to after surgery did not show any significant enrichments, indicating that immune cell gene expression is not significantly different when looking directly between pre-and post-surgery

samples (Supplementary Table 8, in Appendix). Apart from some significant enrichments for

Meissner corpuscle numbers and PACAP expression, no other markers of regeneration such as

IENFD counts or patient regeneration status were associated with immune cell genes.

A

CD4+ T cells: Meissner corpuscles CD4+ T cells: Boston symptoms CD4+ T cells: Boston pain sub-score

CD4+ T cells: Boston weak sub-score CD4+ T cells: NPSI composite score M1: Boston pain sub-score

B

CD4+ T cells: Meissner corpuscles M1: VAS pain M1: NPSI paroxysmal sub-score

M2: Meissner corpuscles M2: Boston paresthesia sub-score M2: VAS pain

M2: ADCYAP1 Expression
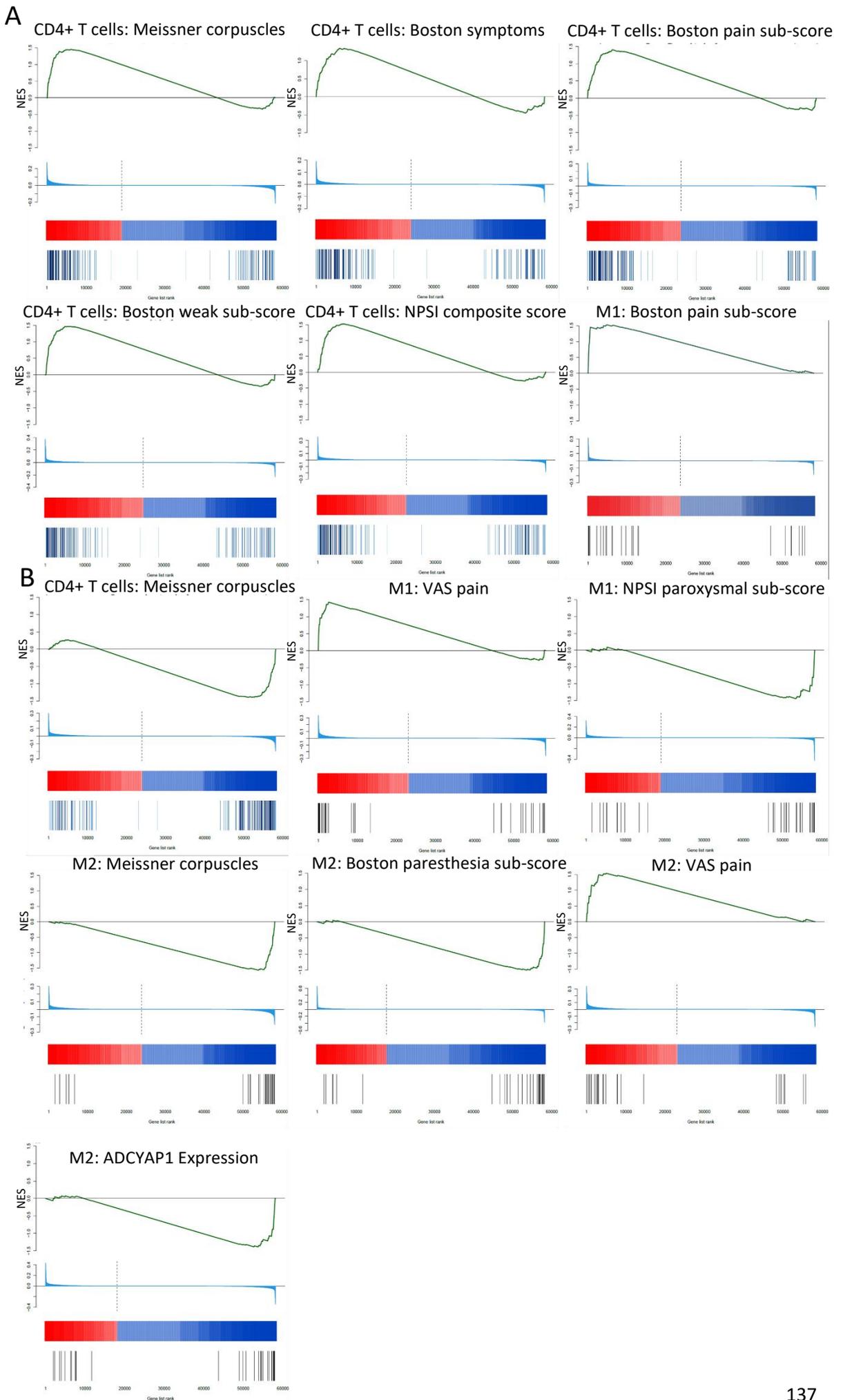
Figure 4.5: GSEA analysis of RNA from pre and post-surgery skin biopsies

A. GSEA using RNA from pre-surgery skin samples revealed significant positive enrichments of genes specific for naïve CD4+ T cells with Meissner corpuscle counts, Boston symptom scores as well as pain and weakness sub-domains and the NPSI composite score. M2 macrophage specific genes were enriched for Meissner corpuscle counts. Positive enrichments indicate increased gene expression in patients with more severe symptoms or increased signs of regeneration (e.g. higher Meissner corpuscle numbers). B. GSEA using RNA from post-surgery skin samples identified CD4+ T-cell genes to be negatively enriched for Meissner corpuscle counts. M1 macrophage genes were positively enriched with VAS pain and negatively enriched for NPSI paroxysmal sub-score values. M2 genes were negatively enriched for Meissner corpuscle counts and Boston paresthesia sub-scores, but were positively enriched for VAS pain. Negative enrichments indicate increased gene expression of specific immune cell genes in patients with less severe symptoms or decreased markers of regeneration.

4.3.7 Deconvolution analysis revealed macrophages, neutrophils and NK cells as the most prominent immune cells in the skin.

To determine the proportions of common immune cell types in the skin biopsies, deconvolution analysis was conducted using RNAseq data from before and after surgery samples. M1 and M2 macrophages as well as neutrophils and NK cells were the only cell types consistently expressed in the samples, with B cells being expressed in only a few patients (Supplementary Table 9, in Appendix). Of the immune cell types, NK cells had the greatest proportion, followed by neutrophils, M1 and M2 macrophages (Figure 4.6, A). The proportion for cells termed 'other' had the largest proportion, which provides reassurance in the findings as the skin is mainly composed of non-immune cells such as keratinocytes, melanocytes and Merkel cells.[502] The proportions of macrophages, neutrophils and NK cells did not change significantly from before to after surgery (Figure 4.6, B). When correlating the proportions of macrophages, neutrophils and NK cells with clinical phenotypes, significant correlations were observed predominantly with post-surgery scores where M1 macrophages negatively correlated with the Boston symptom score, the Boston pain and weakness subdomains, neutrophils negatively correlated with GROC scores and NK cells positively correlated with IENFD values (Supplementary Table 10, in Appendix). M2 macrophage proportions before surgery positively correlated with duration of symptoms. A selection of these correlations are shown in Figure 4.6, C. The negative association of M1 macrophages with Boston

scores indicates that patients with lower numbers of macrophages after surgery have decreased symptom severity. The positive association with NK cells and IENFD suggests that patients with increased NK-cell numbers in the skin after surgery have increased intraepidermal nerve fibres in the skin.
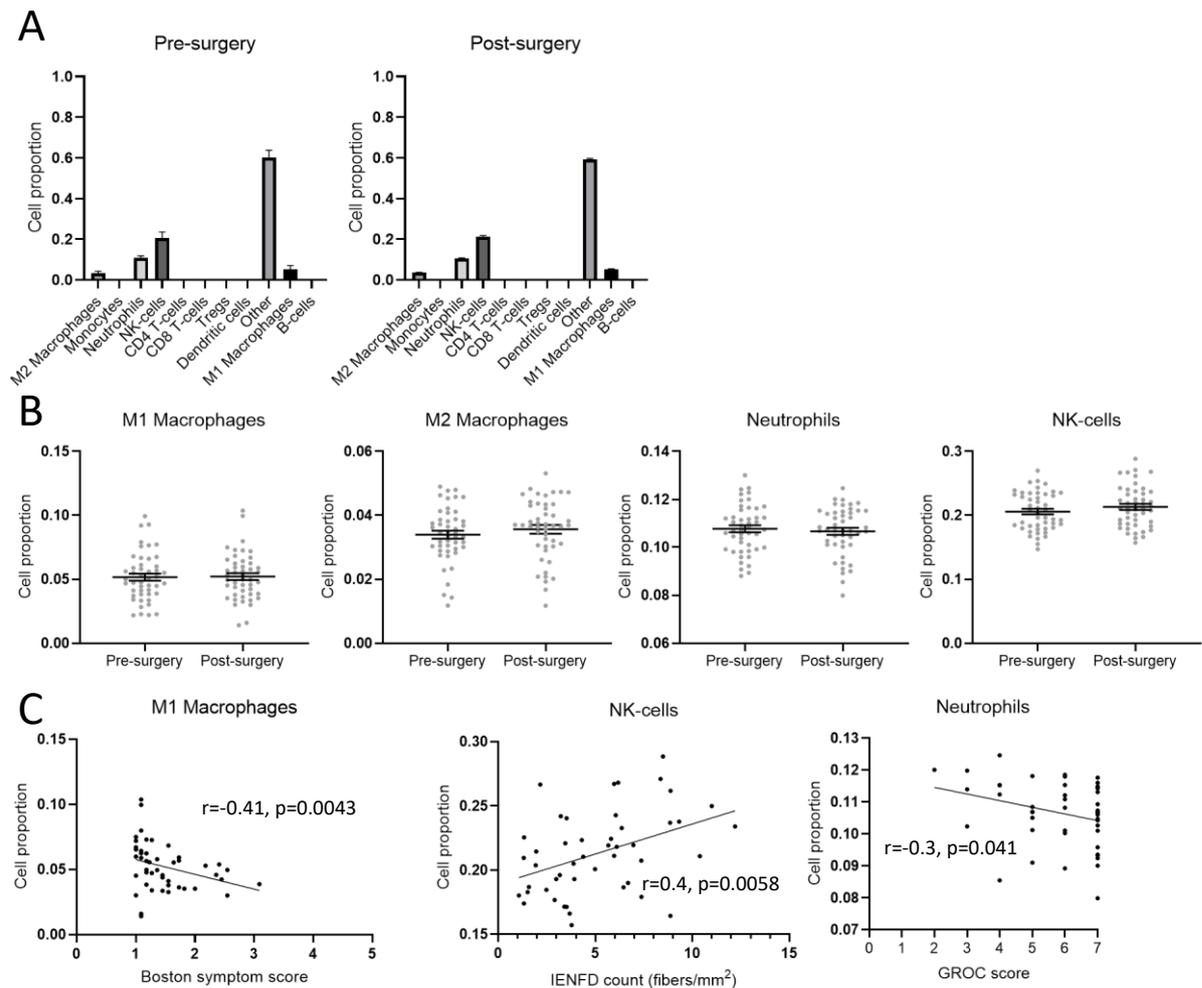
Figure 4.6: Deconvolution analysis of RNA from pre and post-surgery skin biopsies
A. The average immune cell proportions in the skin from deconvolution analysis are shown for pre and post-surgery skin samples. B. The cellular proportions of the four immune cell types present in the tissue do not change from pre to post-surgery. C. Correlation analysis revealed M1 proportions were negatively correlated with the Boston symptom score. NK cells were positively correlated with IENFD counts. Data are presented as Mean ± SEM. Spearman's correlation analysis was used where P<0.05 was significant. IENFD = Intraepidermal nerve fibre density.

4.4 Discussion

The use of CTS as a model system to study molecular determinants of nerve regeneration revealed ADCYAP1 to be the most dysregulated gene showing the greatest fold change increase in expression after surgery, a finding that I confirmed by ddPCR. The PACAP protein, encoded by ADCYAP1 co-localized with neuronal markers and so was located within nerves innervating the skin. Although the fold change in fluorescence intensity of PACAP staining in the skin did not match the fold change of ADCYAP1 expression, there was a clear PACAP upregulation after surgery, in line with RNAseq data. The PACAP receptor PAC1 was also found to be co-localised with sensory afferents in the skin. The exogenous application of PACAP onto hiPSCd sensory neurons after injury caused significantly increased neurite outgrowth compared to sensory neurons treated with vehicle, and was found to be dose dependent. However, this was only observed in mature neurons. The use of maxadilan, a PAC1R agonist, caused comparable neurite outgrowth to that of PACAP, suggesting a therapeutic potential for the modulation of the PAC1 receptor. GSEA analysis, to determine signs of inflammation in the skin and how they might be associated with clinical phenotypes and nerve regeneration, revealed positive enrichments for naïve CD4+ T-cell signature genes. The expression of these genes was increased in patients with more severe symptoms before surgery. After surgery these enrichments were diminished and instead, increased enrichments were observed for macrophage signature genes, indicating a swing in the immune cell type associated with recovery. Deconvolution analysis of the RNA from skin biopsies identified M1 and M2 macrophages, neutrophils and NK cells to be present in the patient samples. Proportions of these cell types did not change from before to after surgery, however M1 macrophages and NK cells showed significant associations with clinical phenotypes.

4.4.1 Increased expression of ADCYAP1 after surgery and PACAP/PAC1R localisation in skin afferents present as molecular markers of nerve regeneration

Increased ADCYAP1 expression after surgery and the localisation of PACAP and PAC1R on sensory afferents in the skin indicate that PACAP could be exerting neurotrophic functions directly on damaged peripheral nerves and so could be aiding in the regeneration and survival of neurons in the skin after injury. The neurotrophic actions of PACAP are well documented[503] and PACAP has been found to be increased in the sciatic nerve and DRG in pre-clinical models after nerve injury, during periods of nerve regeneration.[504-506] In these studies, PACAP expression in the DRG was found to be localised to small and medium diameter neurons before injury, indicating basal PACAP expression in primary sensory neurons. After nerve injury, PACAP expression was found to be increased both in small and large diameter neurons indicating that PACAP is expressed in nerves after injury regardless of neuron cell size and is likely important in nerve repair. Interestingly, the expression of PAC1R was found to be unchanged in the DRG and the spinal cord after injury, suggesting that the increased PACAP is acting on receptors already present.[504]

In humans, there is limited data describing PACAP expression and action after peripheral nerve injury. However, PACAP was found to be decreased in patients with Alzheimer's disease, where PACAP concentrations in the cerebral spinal fluid (CSF) were progressively decreased from controls to patients with mild cognitive impairment and those with more severe cognitive impairment[507]. PACAP expression in the CSF also correlated with several cognitive tests, indicating that PACAP may have a protective effect against neurodegeneration and cognitive impairment. PACAP expression from human cadaveric tissue has indicated that PACAP is expressed in the trigeminal ganglia[508], the DRG and in the nerve fibres of the superficial dorsal horn.[509] The expression of PACAP in humans suggests that PACAP is expressed in primary sensory neurons and matches the expression pattern of PACAP in animals. In my study, PACAP was located in sensory nerve fibres innervating the skin, which was increased after surgery, suggesting that PACAP is

being produced by these neurons after injury during the regeneration phase. From the human

cadaveric studies no information was provided as to the expression of PAC1R in human spinal

cord or DRG, however PAC1R expression was observed in human trigeminal ganglia,[508] though no

indication was given as to the size of the PAC1R+ cell bodies. As PAC1R was found to be located

on skin afferents in the biopsies, it suggests that PAC1R is expressed by sensory neurons

innervating the skin and that PACAP may exert its function directly on these nerves.

The pre-clinical studies that describe increased PACAP expression in the DRG or in the nerve after

injury, suggest an autocrine function for PACAP. However one study has suggested that PACAP

may also have paracrine functions as the application of exogenous PACAP to cultured rat

Schwann cells was able to induce increased expression of Krox20, Mpz and Mbp genes, which are

key markers of myelinating Schwann cells.[510] This indicates that PACAP could increase

regeneration by activating nearby Schwann cells to start myelination. In my study, the source of

PACAP in the skin is unknown, however keratinocytes could be a potential source as PACAP

staining appeared to be present in the basement membrane and PACAP mRNA has been detected

in these cells.[511] It may then be the case that PACAP is acting in a paracrine function on sensory

nerves in the skin and that keratinocytes are providing trophic support to regenerating nerves. *In-situ* hybridisation studies to detect ADCYAP1 mRNA in skin biopsy sections could be useful to

confirm which cell type in the skin is responsible for the production of PACAP. This would help to

further define the role that PACAP plays in nerve regeneration in humans.

PACAP has been suggested to be nociceptive, as high doses of intrathecally injected PACAP were

able to induce pain like syndrome in mice.[512] The nociceptive effects of PACAP are further

demonstrated by knockout mice deficient in PACAP that do not develop neuropathic pain after

nerve injury or mechanical allodynia after NMDA injection.[513] The increase in ADCYAP1 expression

after surgery and the presence of PACAP on nerves in the skin could then be serving two

purposes. One, to provide neurotrophic support to the damaged nerve allowing regeneration and two, to sensitise the nerve to alert the individual to rest the damaged area.

4.4.2 Therapeutic potential of modulating the PACAP/PAC1R pathway in nerve injury

Exogenous PACAP application onto mature cultured hiPSCd sensory neurons increased neurite outgrowth after injury compared to vehicle treated cells. This shows direct neurotrophic and regenerative effects of PACAP on human sensory neurons, which has previously not been demonstrated. PACAP has been shown to increase neurite outgrowth in cultured central and peripheral sensory neurons from animals where trigeminal ganglia and DRG neurons showed a significant increase in neurite growth after PACAP administration.[485, 486, 501, 514] In cultured human cells, PACAP was found to help prevent apoptosis of hiPSCd motor neurons and aid in their survival after neurodegenerative stimuli. PACAP treatment was also found to inhibit the expression of the pro-apoptotic protein BAX in these cultured cells.[487] Due to this large body of work illustrating the neurotrophic potential of PACAP and along with the findings of my study, PACAP therefore could be used for therapeutic benefit in facilitating nerve regeneration. However, more work is needed to define the therapeutic use of PACAP for nerve injury, particularly due to the nociceptive effects observed after PACAP administration.[512]

PACAP has been shown to be able to influence the direction of the growth cones of developing neurons as cultured *Xenopus* cortical neurons were found to grow in the direction of a PACAP gradient.[515] this effect was found to be mediated by PACAP binding to PAC1R at the growth cones of these neurons. It would be of interest to determine whether hiPSCd sensory neurons behave in such a manner and could grow along a PACAP concentration gradient. This may provide information as to the exact function of PACAP in peripheral tissue, and in the case of PACAP expression in the skin, could determine whether PACAP is merely providing trophic support or

actively guiding neurons to their target destination. Future studies may seek to culture hiPSCd

sensory neurons in the presence of a PACAP gradient to determine this.

Interestingly, the addition of maxadilan to hiPSCd sensory neurons caused a similar effect on

neurite outgrowth as PACAP. Maxadilan is a selective PAC1R agonist[516, 517] and so highlights the

therapeutic potential of modulating the PAC1R to initiate nerve regeneration. The therapeutic

potential of maxadilan has already been investigated by treating retinal degeneration in rats

caused by bilateral common carotid artery occlusion (BCCAO).[518] Intravitreal administration of

maxadilan was able to cause significant protection in the thickness of retinal layers compared to

vehicle treatment, with higher concentrations of maxadilan demonstrating an increased effect.

Maxadilan has been further shown to have neuroprotective effects as it can protect mice

olfactory placode cell lines OP6 and OP27 from TNF-α induced damage through the activation of

the PAC1R.[519] Signalling through the PAC1R may therefore prove to be therapeutically useful.

Additionally as maxadilan is selective only for PAC1R, it may have reduced off target effects

compared to PACAP, which can also bind the receptors VPAC1 and VPAC2.[520] Future work to

investigate the therapeutic potential of maxadilan could make use of pre-clinical models of

sensory nerve damage and repair, however as maxadilan has been shown to cause nociceptive

behaviours[521] and mechanical allodynia[522] when administered intrathecally, careful consideration

must be taken to reveal therapeutic benefit without adverse effects. Interestingly, PACAP

antagonists have been trialled as a treatment for migraine in humans,[523] and so inhibition of the

PACAP/PACR1 pathway may also provide therapeutic benefit depending on the specific disease

being treated.

4.4.3 GSEA indicates increased inflammation before surgery which decreases after surgery

GSEA revealed that genes specific to naïve CD4+ T cells were positively enriched with clinical

phenotypes including the Boston symptom score and the NPSI composite score before surgery.

This indicates an increased immune cell inflammation in those patients with more severe symptoms. The increased expression of naïve CD4+ T-cell genes may be somewhat unexpected as naïve CD4+ T cells are usually located in secondary lymphoid organs where they await stimulation through antigen presentation.[198] It may therefore be expected that genes specific for Th effector subgroups such as Th1, Th2 or Th17 would be increased instead, though this was not found to be the case. However one study has shown that a significant number of naïve CD4+ T cells can migrate to non-lymphoid tissues such as the skin as part of normal cell migration patterns.[524] it was found that 18% of the T-cell population in the skin displayed a naive phenotype and were functionally naïve as well. Other work goes further to suggest that the skin may act as a secondary lymphoid organ[525] where memory T cells migrate to become stimulated by antigen presentation on tissue resident antigen presenting cells.[526] However whether naïve CD4+ T cells can actually become activated by antigen presentation in the skin is currently unknown. It may therefore be possible that the enriched naïve CD4+ T-cell genes from the GSEA are a result of increased migratory naïve T cells in patients with more severe symptoms, where increased inflammation could be present. This is supported by a finding in mice showing that naïve T cells can migrate into skin air pouches after treatment with the cytokine CCL21, which is expressed in tissues during inflammation.[527] However as memory T cells have been found to masquerade as naïve T cells[528] and can share gene expression similarities,[451] it may be possible that some of the genes found to be enriched in the naïve CD4+ T-cell signature are in fact coming from an increased memory T-cell population present in the tissue.

Using post-surgery data in the GSEA revealed that naïve CD4+ T-cell genes were no longer enriched with clinical phenotypes and instead showed that genes specific for macrophages were enriched. As the post-surgery time point is reflective of a state of repair, the lack of enrichment for T-cell genes could indicate a cessation of inflammatory action after surgery, which is supported by the finding that genes specific for M2 macrophages, involved in tissue repair and homeostasis,[164] were significantly enriched after surgery. The enrichments of macrophage genes

with clinical phenotypes does however appear to be variable. Negative enrichments of M1 genes for the NPSI paroxysmal sub-domain indicate that patients with lower expression of M1 genes have lower paroxysmal pain symptoms after surgery. This is in agreement with pre-clinical studies finding that M1 macrophages are involved in generating neuropathic pain.[461, 529, 530] However the negative enrichments of M2 genes for the Boston paresthesia sub-score indicate that patients with lower expression of M2 genes have less severe paresthesia symptoms, which may be unexpected as M2 macrophages have been found to be involved in nerve repair.[531] Both M1 and M2 macrophage genes were positively enriched for VAS pain scores, indicating that patients experiencing higher pain after surgery have increased expression of M1 and M2 genes. As both M1 and M2 macrophages infiltrate the injury site after nerve damage,[529] their positive enrichment for VAS pain could indicate prolonged damage even after surgery, which would require M1 macrophages for inflammation and M2 macrophages for continued tissue repair.

4.4.4 Immune cell signatures do not affect nerve regeneration

Overall the effect of immune cell presence/inflammation in target tissue appeared to have a limited effect on nerve regeneration. Immune cell signatures were not enriched in patients who were determined to be regenerators or for ADCYAP1 expression except in one case where M2 genes were negatively enriched for ADCYAP1 expression post-surgery. PACAP and its receptors can be expressed by cells of the immune system[532-534] and can modulate several functions including phagocytosis,[535] differentiation[536] and survival.[537] In this study only one cell type showed a significant enrichment for ADCYAP1 expression and so the interaction between ADCYAP1 and immune cell function in this instance appears minimal. The precise role of inflammation in nerve regeneration is currently not fully understood. Studies have produced conflicting results which suggest that inflammation can both enhance and hinder nerve regeneration. Increased neuroinflammation was identified as the cause of reduced nerve regeneration in older mice after

peripheral nerve injury[488] and depletion of macrophages after nerve injury in rats protected nerve fibres against degeneration and inhibited the generation of pain associated with nerve injury.[280] Conversely, another group found that the ablation of the monocyte/macrophage population after nerve injury significantly decreased axonal regeneration.[276] The cytotoxic action of NK cells after nerve injury in mice has been found to contribute to successful nerve repair by removing damaged axons after injury.[468] Indeed in my study I found NK-cell proportions from the deconvolution analysis to be significantly associated with IENFD counts after surgery. This may suggest that NK cells in the skin are aiding in the regeneration of small nerve fibres, perhaps by the mechanism of clearing damaged axons and allowing for efficient nerve-regrowth. As no significant enrichments were observed for immune cell genes in target tissues of patients who were regenerators, it is difficult to determine the role of immune cells on nerve regeneration in this cohort. However as the target tissue was investigated here, the changes may be less pronounced in this tissue compared to the affected nerve or surrounding tissue. Future experiments may seek to interrogate the nerve tissue directly, where greater changes are likely to occur. Significant enrichments were observed for Meissner corpuscle density, which are used as a marker for myelinated nerve fibres. Naïve CD4+ T-cell genes were positively enriched for Meissner corpuscle density pre-surgery and naïve CD4+ T-cell genes and M2 genes were negatively enriched post-surgery. This indicated that patients with increased naïve CD4+ T-cell genes before surgery had higher numbers of Meissner corpuscles and that after surgery patients with a lower expression of naïve CD4+ T-cell and M2 genes had lower numbers of Meissner corpuscles. This may suggest a protective role for immune cells in the skin to preserve neural structures such as the Meissner corpuscle during and after injury. It would be of interest to conduct immunohistochemistry experiments in skin biopsies from patients with CTS to determine the extent to which immune cells are indeed present in the skin and define their interactions with neuronal structures.

4.4.5 Deconvolution analysis revealed four prominent immune cell types in the skin

Deconvolution analysis of skin RNA revealed M1 and M2 macrophages, neutrophils and NK-cell proportions to be present in the skin. However none of the proportions of these cells changed from before to after surgery indicating that these cells may not be inflammatory cell infiltrates and so are not contributing to any potential inflammation in the skin. However as M1 macrophages were found to negatively correlate with post-surgery Boston symptom and pain and weakness sub-scores, it indicates that this macrophage population may be influencing the symptom severity in these patients after surgery. As the data suggests that macrophage infiltration appears unlikely, these significant correlations could reflect the activation of resident macrophages in the tissue, which then contribute to the disease symptoms. The negative correlations indicate that those patients with less M1 macrophages in the skin have less severe symptoms. This is in agreement with previous findings that M1 macrophages can contribute to symptoms such as neuropathic pain.[460, 538] Ultimately the immunohistochemical profiling of immune cells in this tissue would be useful to confirm their presence. IHC images can also be used in the deconvolution analysis to help validate and refine the immune cell fractions generated.[452] This work may aid in more accurate identification of immune cell presence in the skin of patients with CTS.

4.4.6 Limitations

As Bulk RNAseq was conducted with the skin biopsies, the contribution of gene expression from a particular cell type cannot be defined. Though ADCYAP1 was found to be the gene most increased after surgery, due to the bulk RNAseq the cell types contributing the most expression of ADCYAP1 remain unknown. This could have implications as to the function of PACAP in the skin and even to the therapeutic potential of modulating the PACAP/PAC1R pathway, as defining whether PACAP is produced and functions in an autocrine or paracrine manner could affect the cell type targeted

for therapy. As single cell RNAseq is becoming more widely available, future studies could look to use this technique to fully define which cell types in the skin are producing PACAP. It would also be of interest to further characterise the downstream mechanism by which exogenous PACAP causes increased neurite outgrowth of hiPSCd sensory neurons. Pre-clinical studies have identified cAMP as being a prominent driver of PACAP mediated neurite outgrowth.[515, 539, 540] The precise determination of the full complement of mediators in this pathway could further our knowledge of nerve regeneration in humans, which could eventually be of therapeutic benefit.

The finding that naïve CD4+ T-cell genes were enriched in patient skin biopsies pre-surgery was of interest, however as this analysis used gene expression data, the actual presence of these immune cells in this tissue cannot be known with certainty. This is especially evident after the analysis in Chapters 2, where the gene and protein expression of inflammatory mediators in blood were analysed, and Chapter 3, where a similar GSEA analysis was carried out. Both chapters showed discrepancies between gene expression and protein production. Immunohistochemistry staining for immune cells in the skin could be done to confirm immune cell presence and determine whether there is a correlation with the genetic analysis. Future studies are therefore required to fully elucidate the action of PACAP in nerve regeneration in humans and the role that inflammation may play. As with the analysis in Chapter 3, limited corrections for multiple testing was conducted for associations between biological measurements and clinical phenotypes. However as stated previously this is justified by the analyses being exploratory in nature, and so although the same caveats still apply, my findings highlight interesting associations, which require further study.

4.4.7 Conclusions

To conclude, I have used RNAseq to investigate nerve regeneration after injury in patients with CTS as a human model system. This identified ADCYAP1 as the gene with the greatest fold

increase post-surgery. After confirming the ADCYAP1 expression with ddPCR, I found that the ADCYAP1 encoded protein PACAP was located within sensory afferents innervating the skin of patients with CTS. The PAC1R was also found to be located within nerves in the skin suggesting that PACAP may be mediating its action on skin sensory afferents. Excitingly, the application of PACAP on cultures of mature hiPSCd sensory neurons caused significantly increased neurite outgrowth after injury compared to a vehicle control. The PAC1R agonist maxadilan was found to have a similar effect. Taken together, these data suggest that PACAP could be an important factor driving nerve regeneration in humans and that modulation of the PAC1R pathways could have interesting implications for future therapy in treating nerve damage.

To determine whether immune cell infiltration/inflammation was present in the innervation territory of the median nerve and whether it might be impacting nerve regeneration in the skin, GSEA analysis was conducted. Genes specific for naïve CD4+ T cells were found to be enriched for clinical phenotypes including the Boston symptom score and the NPSI composite score before surgery, which was abolished after surgery. After surgery analysis revealed significant enrichments for macrophage specific genes, indicating a shift in the predominant immune cell type associated with symptoms in before and after surgery skin. These findings indicate a level of inflammation present in target tissue before surgery, which could be contributing to patient symptoms. However immune cell gene expression appeared to have little impact on nerve regeneration in the target tissue as few markers of nerve regeneration were correlated with immune cell genes. Deconvolution analysis identified four immune cell types in the skin that do not change from before to after surgery, indicating that these immune cell types are not inflammatory infiltrates. However significant correlations with clinical phenotypes suggests the association of resident immune cells with symptom severity. These findings indicate an immune cell presence in the target tissue of patients with CTS however the precise role that inflammation plays in nerve regeneration remains to be further elucidated.

Chapter 5: General Discussion.

5.1 Summary of key findings

Throughout my studies I have intended to answer two main questions:

1. What is the role that inflammation plays in the generation, maintenance and resolution of neuropathic pain in humans

2. What are the mechanisms of nerve regeneration, including the molecular processes which occur after injury in humans, and to what extent is inflammation associated in this process.

The sections below detail how I have attempted to answer these specific questions by the experiments undertaken in my study.

5.1.1 Inflammation is present in patients with CTS and is associated with clinical phenotypes

I have answered these questions using CTS as a human model system with unparalleled access to human tissue in the context of neuropathic pain. In answer to the first question I have found evidence of inflammation in several different biosamples from patients with CTS, which have also been associated with clinical phenotypes. Firstly I identified the expression of inflammatory mediators in the blood of patients with CTS. Here there was a dysregulation in cytokine expression both at the genetic and protein level, as well as between patients with CTS and healthy controls and between patients with CTS before and after surgery. The patterns of cytokine expression implicated inflammatory mediators in both the active and resolution phases of the disease. In the latter, IL-9 was the prominent cytokine which was increased in patients after surgery. These dysregulated inflammatory mediators showed significant correlations with clinical phenotypes, suggesting their involvement with disease

151

pathogenesis and symptom severity. Several of these associations were with pain specific clinical phenotypes, highlighting important interactions between inflammation and pain, as well as functions involved in immune resolution. Secondly, analysis using the tenosynovial tissue, which is in close proximity of the nerve at the site of injury, led to the identification of both macrophages and T-cell populations, with CD4+ T cells being associated with disease severity. Gene set enrichment analysis of CD4+ T-cell subsets, CD8+ T cells and macrophage specific genes revealed several associations between immune cell signature genes and clinical phenotypes. The associations were varied and highlighted the complex role that inflammation may play in neuropathic pain and CTS.

5.1.2 Molecular mediators of nerve repair are increased after injury and stimulate nerve outgrowth *in vitro*.

To answer the second question, I conducted genomic and histological analysis of the target innervation territory, which revealed the increased expression of the neurotrophic molecule ADCYAP1/PACAP after surgery. Further, to explore it's regenerative potential *in vitro*, the exogenous application of PACAP was able to stimulate outgrowth of hiPSCd sensory neurons. These experiments helped to characterize the molecular events occurring after nerve injury, which may contribute to nerve regeneration. Specifically this revealed the role that PACAP may have in the regeneration process after peripheral nerve damage in humans. Inflammation however was found to have a limited association with nerve regeneration in the skin, as few significant enrichments were observed between immune cell gene expression and markers of nerve regeneration after gene set enrichment analysis. However, naïve CD4+ immune cell genes in the skin were significantly enriched with clinical phenotypes before surgery, but were abolished following surgery, suggesting a level of inflammation present in the innervation territory during the active state of the disease.

5.1.3 General summary

All together my findings indicate that there is a level of inflammation present in patients with CTS, which is associated with clinical phenotypes, and can be detected at multiple levels from different bio-samples. In addition, specific regenerative factors are produced following nerve injury that could help to facilitate regeneration of nerves in the skin, and while inflammation may be present before surgery, there is limited evidence indicating an association in the regeneration process from the samples I have analyzed.

Summary of Findings

2. Tenosynovium tissue

Main findings

1. Immune cells are present in tenosynovium tissue and are associated with clinical phenotypes

2. Several immune cell type gene signatures including Th1, CD8+ T-cells and M1 macrophages display variable associations with clinical phenotypes

3. Five immune cell types were identified by deconvolution analysis to be present in the patient samples

4. Treg and NK-cell proportions positively correlated with CD4+ cell staining

3. Innervation territory

Main findings

1. ADCYAP1 expression is increased post-surgery

2. PACAP and PAC1R are expressed in the skin with PACAP staining intensity being increased post-surgery

3. The application of PACAP on injured hiPSCd sensory neurons stimulates nerve outgrowth

4. Naïve CD4+ gene signatures are associated with clinical phenotypes pre-surgery which is abolished post-surgery

5. Deconvolution analysis identified the proportions of four immune cell types which do not change from before to after surgery

1. Systemic analysis in blood

Main findings

1. The genetic expression of inflammatory mediators was differentially expressed from before to after surgery

2. The protein expression of inflammatory mediators was significantly dysregulated both from patients before surgery to healthy controls and between patients from before to after surgery

3. Genetic and protein expression of inflammatory mediators significantly correlated with clinical phenotypes.
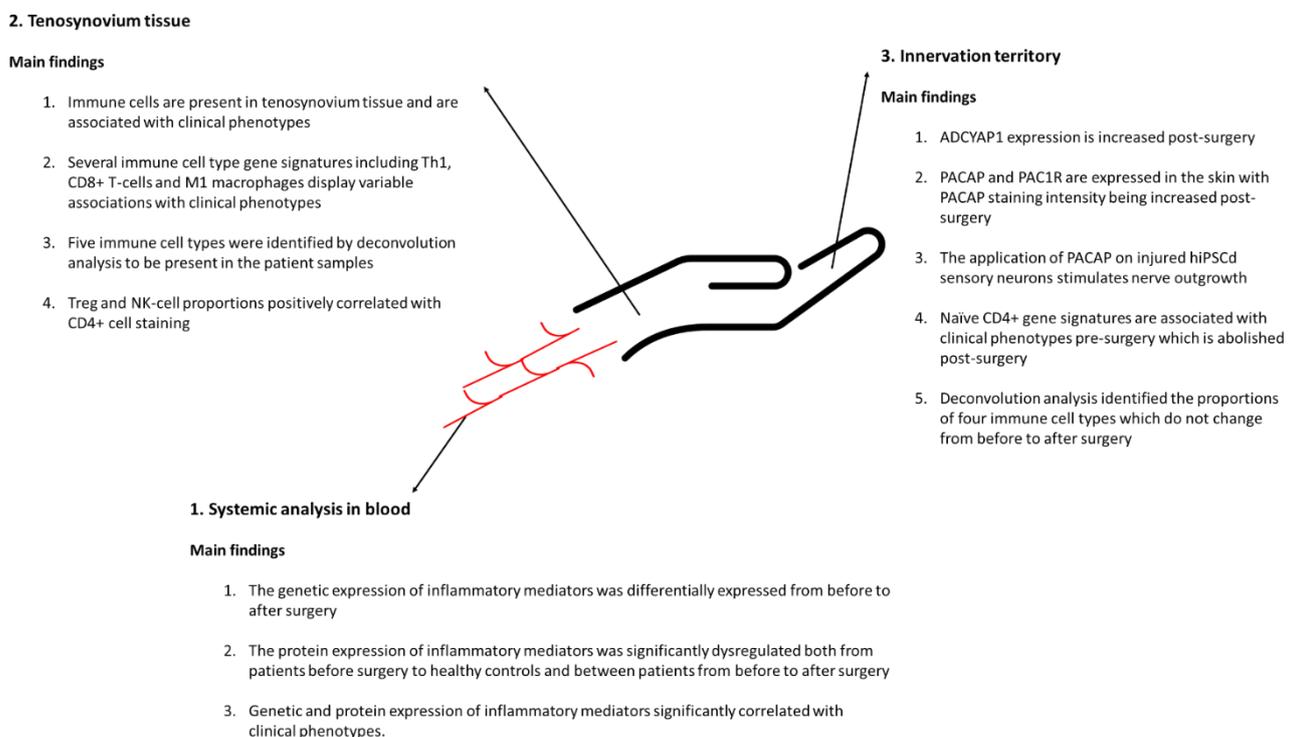
Figure 5.1: Summary of key findings
The key findings from my studies are shown. These findings are separated based on the biological sample from which they were derived from or are associated with.

5.2 Broader implications for neuropathic pain

5.2.1 CD4+ T cells likely play an important role in the generation of neuropathic pain in humans

The work in this study builds on a large body of pre-clinical evidence showing a role for inflammation in neuropathic pain.[7, 72, 73] This has driven my investigations looking at the actions of inflammation on neuropathic pain in a human model system. From my studies I have identified T cells as being heavily implicated in neuropathic pain, as it was apparent that these cells were the main cell type that was associated with neuropathic pain phenotypes. In particular I found CD4+ T-cell populations and the Th1 subpopulation to be associated with clinical phenotypes relating to pain and symptom severity. There have been several studies which have identified the important role that T cells play in neuropathic pain,[277, 285, 289] and as with my studies, pre-clinical animal models have specifically identified CD4+ T cells[295, 541] and Th1 cells[277, 462, 542] as major contributors to neuropathic pain. In these studies CD4+ T cells were specifically found to infiltrate the dorsal leptomeninges[541] and lumbar spinal cord[295] after nerve injury and contribute to neuropathic pain. Th1 cells have also been found to infiltrate the spinal cord after injury,[462, 542] while the adoptive transfer of Th1 cells into athymic rats enhanced mechanical and thermal pain hypersensitivity.[277] My results therefore place these T-cell populations, highlighted from preclinical studies, in a human context of neuropathic pain. The broader implication of my findings may therefore be to show that CD4+ T-cell populations are important in neuropathic pain in humans, which could then implicate the importance of T cells more generally in other human neuropathic pain conditions. My findings therefore also justify the increased need for further investigation into the action of these T-cell populations in neuropathic pain conditions in humans. In addition, as CD4+ and Th1 T cells were identified from pre-clinical studies and my experiments as being important in neuropathic pain, it provides confidence that the pre-clinical findings are accurately depicting inflammatory changes occurring in human conditions of neuropathic pain. When looking more generally at inflammation in neuropathic pain, my findings suggest a level of inflammation

present even after mild nerve damage, which implies that other more serious nerve injuries could

also feature immune activation, and potentially to a greater extent. Overall my findings implicate

inflammatory processes to be present in human patients with neuropathic pain, which has

implications for future research in humans as it highlights the likely role that inflammation plays

in the condition. My findings could therefore encourage other to focus on the inflammatory

aspect of neuropathic pain in humans.

5.2.2 The benefits of a three-pronged attack

The findings from my study may also have implications for the approaches in which future

neuropathic pain research in humans is undertaken. Previous studies have investigated

inflammation in human patients at several different levels, including the blood[75, 305, 314] and

affected nerve tissue[299, 301] to reveal signs of inflammation. In my study I have investigated

changes in the expression and presence of inflammatory mediators and immune cells at multiple

levels within the same patients. This included genetic and protein analysis of inflammatory

mediators in the blood, in tissue surrounding the affected nerve and in the innervation territory

of the affected nerve itself. In all three instances I was able to detect inflammation/immune

dysregulation, which was often associated with clinical symptoms. The identification of immune

dysregulation in blood was somewhat surprising as I initially would not have expected to have

observed a measurable change in cytokine expression systemically after focal damage to a

relatively small nerve. These findings highlight the use of investigating a neuropathic pain

condition from multiple angles to obtain a more comprehensive insight into the role that

inflammation is performing in a human condition of neuropathic pain. Using a three-pronged

approach has produced findings that strongly indicate inflammation to be contributing to the

pathogenesis of CTS. These findings fit well with previous studies that have investigated

inflammation and neuropathic pain from different tissues within the same patients. These studies

have investigated inflammation in samples such as the blood, skin and sural nerve of patients

with peripheral neuropathies, where infiltrating immune cells,[303] cytokines[302] or micro RNA expression[543] have been detected in the various samples. However, the patients included in these studies were suffering from serious or systemic neuropathies where other factors involved in the disease pathogenesis could be impacting the manifestation of inflammation or pain. As my results were derived using patients with a focal nerve injury, they show that markers of inflammation can be found even after mild nerve injury in humans, which relates specifically to a single point of nerve damage. These findings further substantiate the hypothesis that inflammation is playing a major role in neuropathic pain.

5.3 CTS as a viable model system to study neuropathic pain in humans

The results from my study may also contribute in showing that CTS is a viable and useful model system with which to study neuropathic pain in humans. The availability to collect blood, tenosynovial tissue, and skin samples within the same patient has enabled the characterization of inflammation at different levels. In addition, as a relatively large amount of tissue was able to be collected in each instance, this allowed me to conduct both genetic and protein analysis of the tissue, which not only increased my capacity to interrogate the tissue, but also enabled the findings from the genetic analysis to be validated with experiments analyzing proteins. This has proven to be particularly useful in this study as in many cases throughout the project the genetic data did not match with the protein findings. If less tissue was available and only genetic analyses of the samples were conducted, this could have caused inaccurate conclusions. The tissue availability and the flexibility to conduct different analyses opens up the opportunity for researchers to investigate neuropathic pain in many ways, enabling more options to better target their research aims. In addition, the use of surgery in CTS as a distinct recovery inducing event has enabled the investigation of subtle changes within patients from an active disease state to a state of recovery. Indeed the majority of my findings were obtained when comparing changes before to after surgery instead of before surgery to healthy controls. This could enable other scientists

investigating small but important changes to accurately detect and measure specific aspects of neuropathic pain, which may not be obtainable when looking for differences between healthy controls and patients. Finally the ease of access and the good availability to collect clinical phenotype data has proven useful as in my study several biological findings have been found to be associated with clinical parameters.

5.4 Clinical implications

5.4.1 Inflammation in the pathogenesis of CTS and IL-9 as a therapeutic candidate

With regards to clinical implications, the findings from my study may have some implications when specifically treating pain in patients with CTS, as all of my experiments were conducted using samples from these patients. However, there may also be some general implications for treating neuropathic pain in humans and I will address each of these in turn. Firstly for patients with CTS, the findings from my study suggest that inflammation is playing a role in the pathogenesis of the disease, which was previously thought not to be the case, and so could have an impact on the way in which the condition is treated. As such, one may advocate for the increased use of anti-inflammatory treatments such as NSAIDS or corticosteroid injections, which are already prescribed to treat CTS and have shown some benefit.[544] However, particularly for corticosteroids the long term benefit remains unclear.[440] This may be due to the broad anti-inflammatory effect of corticosteroids, which may not specifically target the inflammatory pathway that is contributing to the pathogenesis of the disease. A more nuanced approach could therefore be needed to treat neuropathic pain using immunomodulatory therapies in patients with CTS. Based on the findings from my study, IL-9 could be considered as a potential candidate that could eventually be used to treat patients with CTS. IL-9 was increased in patients after surgery and was negatively associated with several pain specific clinical phenotypes. Pre-clinical work has found IL-9 to contribute to anti-inflammatory processes through the indirect activation

of Treg cells,[387] however there are also studies in human patients identifying IL-9 as contributing to inflammation and disease severity.[385, 386] Due to the pleiotropic nature of IL-9, it is clear that careful consideration and further investigation into the action of IL-9 in the disease is required before justifying its use therapeutically to treat patients with CTS. One potential next step to determine the therapeutic potential of IL-9 would be to observe its action in an animal model of neuropathic pain, where its effect on neuropathic pain could be fully interrogated. Ultimately, IL-9 presents as an interesting candidate with which to explore further.

5.4.2 The importance of CD4+ T cells in neuropathic pain

For more general clinical implications into neuropathic pain in humans, I believe my findings may indicate inflammation as having a greater role in neuropathic pain in humans than first thought. Even if a neuropathy is not caused by an overt inflammatory event, it does not necessarily mean that inflammation is not contributing significantly to the pain experienced in these individuals. As mentioned previously, CD4+ T cells have been identified in this study and by previous research to be involved in neuropathic pain. More attention could therefore be placed on the activity of these cells in patients with neuropathic pain, where their presence or activation in a particular tissue could be used as a biomarker for neuropathic pain presence or severity. In addition, the modulation of this population could have implications on reducing pain sensation, and so medication reducing the number of cells in this population could have therapeutic potential. However as CD4+ T cells comprise a number of different cell subpopulations, extra care may need to be taken to target only the specific population contributing to the disease. As Treg cells form part of the CD4+ T-cell population and have frequently been found to decrease neuropathic pain,[298, 459] the general reduction in the CD4+ T-cell population could therefore reduce Treg numbers and so potentially increase neuropathic pain sensations. The careful modulation of specific cell types would therefore be required.

5.5 Future directions

From the findings in my study, several avenues of future research could be pursued. Firstly I have

investigated tissues remote from the affected nerve site including blood and the target skin as

well as tissues closer to the injury such as the tenosynovium, where I found signs of inflammation

in all cases. The next step would be to investigate the inflammatory presence directly in the

affected nerve tissue to determine the extent of neuro-immune interactions. This would provide

information on how exactly the immune system is interacting with the nerve during neuropathic

pain and could shed light on the identity of the specific components of the immune system that

are the main drivers of neuropathic pain. However, this may be difficult to achieve in patients

with carpal tunnel syndrome as nerve tissue is not excised as part of routine decompression

surgery. Cadaveric tissue could be one possible way in which to obtain nerve samples from

patients with CTS. However the majority of these individuals may have already had

decompression surgery at some point as part of their treatment, and so the nerve sample

retrieved would most likely reflect a nerve that has recovered from the initial injury. An

alternative option would be to use nerve samples from a related entrapment neuropathy where

nerve tissue is removed as part of the surgical procedure to relieve the condition. Morton's

neuroma is such a neuropathy, where the digital plantar nerve is removed during surgery and

could be used to directly investigate inflammation intraneurally. There may however be inherent

differences in the pathology of Morton's neuroma and CTS, which means that inflammation may

not play the same role in both conditions. Ultimately however this would remain as one of the

only ways in which to acquire fresh nerve tissue from an entrapment neuropathy, allowing the

study of nerve compression in a human condition of neuropathic pain.

The second main area of future investigation would look into increased immunophenotyping of

the tenosynovium. In my studies, T cells and macrophages were both found to be present in the

tenosynovium. As CD4+ T-cell subgroup gene signatures such as Th1 and Treg cells were associated with clinical symptoms, I believe it would be interesting to histologically stain for Th subgroups in the tenosynovium to identify specific T-cell subpopulations present in the tissue. This would help characterize the T-cell subpopulations contributing to the disease. In addition to T-cell populations, I could also stain for NK cells, as NK-cell proportions were found to be present in the deconvolution analysis of the tenosynovium and correlated to cell counts from immunohistochemistry. It would therefore be interesting to stain for NK cells in the tenosynovium to determine whether this cell type is indeed present in the tissue. Along with immune cell types that could be of further interest, several cytokines identified in the blood analysis such as IL-9, IL-6 and CCL5 could also be stained for in the tenosynovium. This would be useful in determining which cell types the cytokines are acting on and also which cells are producing them. Alternatively, *in situ* hybridization could be used to detect the cells that are expressing certain cytokines and further to this, single cell sequencing of tenosynovial tissue would give a full expression profile of all the different cell types within the sample. This would greatly help in elucidating the inflammatory environment in the tenosynovium. The staining of neuronal structures in the tenosynovium would be of interest and could help in determining whether immune cells present within the tenosynovium are interacting with nociceptive neuronal sprouts, contributing to the disease. Lastly, as no healthy tenosynovial tissue could be used in my study, future studies would look to obtain healthy tissue with which to compare my current findings to. This would provide information as to whether the immune cells detected in the tenosynovium represent true immune cell infiltration and inflammation.

Thirdly and similar to several of the points raised in the previous section, immune phenotyping in the skin using immunohistochemistry would help in further defining the role of inflammation in nerve regeneration. As naive CD4+ T-cell signatures were associated with clinical phenotypes in the gene set enrichment analysis of the skin RNA, the staining of CD4+ T cells in the skin would

provide insight into the presence of these cells in the tissue, and could help to confirm the findings from the genetic analysis. Along with CD4, other markers of CD4+ T-cell subsets would be of interest as they have been implicated throughout my study. The co-staining of neuronal structures in the skin along with immune cell markers may also provide details as to what extent immune cells are interacting with neurons innervating the skin, and influencing their regeneration after injury. As skin biopsies can be taken with relative ease from patients as well as healthy controls, the level of immune cell infiltration in patients can then be compared to that present in healthy controls.

To further examine nerve regeneration in these patients, it may be useful to determine which cells in the skin are producing PACAP and which cells the PACAP protein is acting upon. From the immunohistochemistry in the skin shown in Chapter 4, PACAP staining appeared to be present in the basement membrane in the skin where cells such as keratinocytes are present. Future work could therefore seek to stain for PACAP along with keratinocyte markers to see if there is an overlap in expression. *In situ* hybridization could also be used to determine which cells are expressing PACAP, and could indicate whether PACAP is being produced by skin cells or nerves innervating the skin. These results would aid in increasing our understanding of the molecular events that occur in nerve regeneration. To further explore the therapeutic potential of modulating the PACAP/PAC1R pathway, maxadilan or PACAP could be administered in animal models of nerve injury to explore the impact it has on nerve regeneration. Maxadilan was shown to produce a similar increase in neurite outgrowth to PACAP in my study, and so maxadilan administration may affect sensory and nociceptive sensitivity after injury. However it would be important to remember that though these factors may have the adaptive effect of contributing to nerve regeneration, they may also have the unwanted side effects of sensitizing primary afferents, causing pain. The findings from these studies could provide insight into the use of maxadilan in facilitating nerve regeneration after injury in humans.

5.6 Concluding remarks

Neuropathic pain is a serious and highly debilitating disease that affects a large proportion of the global population[1] and is associated with a high economic cost.[545] However, as has been discussed, despite the seriousness of the condition and the high socio-economic burden associated with neuropathic pain, patients often do not receive adequate treatment or sufficient relief from symptoms. Effective nerve regeneration in humans is also a major concern as optimal recovery is rarely achieved in patients.[343] The challenge of producing effective therapy for neuropathic pain is not helped by it being a highly complex condition where multiple factors are involved in its manifestation. The difficulty in not knowing at what level to interrupt the pain signaling cascade, or whether to target peripheral or central mechanisms, may have contributed to difficulties in producing therapies that are effective in treating neuropathic pain. A similar situation exists for nerve regeneration, as a myriad of factors are involved in the regeneration process, which presents difficulties when selecting the optimum point with which to target treatment. Ultimately a lack in the understanding of pain mechanisms and nerve regeneration as well as ineffective treatment for either condition present as major hurdles that need to be overcome to provide effective therapy. These two obstacles are of course linked as without an increased knowledge of the biology, better therapies cannot be produced, and so one cannot be solved without the other.

The deficit in our understanding of pain mechanisms is certainly not through a lack of effort in the pain research field as a huge body of work has been generated from analyzing a plethora of different aspects of the neuropathic pain axis. However, as I have previously pointed out, a large amount of this research has been conducted in animal models of neuropathic pain, which have unfortunately not translated well into viable treatments for humans. There are several reasons for this which have been described elsewhere.[9] A similar narrative is true for nerve regeneration, where many studies have been conducted in animal models that have not translated well into

humans, due to reasons such as increased distances for nerves to regrow.[349] Through my study I intended to highlight the need for more research to be conducted in humans to further characterize nerve regeneration and define the mechanisms of neuropathic pain in the organism that will ultimately receive the treatment. My study aimed to showcase that neuropathic pain research in humans can provide fruitful insights into these processes and encourage others to conduct neuropathic pain research using human samples. I also intended to characterize the mechanisms of nerve regeneration in a human system and show that by using such a system, molecular mediators could be identified that can be applied generally to nerve regeneration in humans.

In addition to the majority of research being conducted in animals, another reason for why effective treatment for neuropathic pain has not been developed could be due to the lack of knowledge regarding inflammation in pain, which has only relatively recently generated an interest in the field. A large amount of new research has now been conducted which strongly implicates the immune system as having a role in how neuropathic pain is generated, maintained and also resolved. Immune modulation for therapeutic use had been implemented in treating a range of diseases[546] where it has been found to produce benefit to patients. Immune modulating drugs have also been effective in treating rheumatoid arthritis where pain is a primary symptom of the disease.[547] Based on the strong pre-clinical data for inflammation in neuropathic pain and due to the efficacy of immunomodulating strategies in other conditions, it is my belief that neuropathic pain could also eventually be treated using immuno-therapies, targeting specific inflammatory mechanisms involved in neuropathic pain. It is my hope that future research will continue to explore the role of inflammation in neuropathic pain to achieve the goal of producing new, effective medicine to treat patients. In addition, after having highlighted molecules that can facilitate nerve regeneration after injury in humans, I would also hope that more future research will be conducted in human systems of nerve injury where mechanisms important in nerve

regeneration can be identified. I have great optimism that the work that I have carried out here

will provide a contribution in achieving these aims and form the basis for future study.

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