Immunopathology of Complex Regional Pain Syndrome

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I declare that this thesis, entitled:

"Immunopathology of Complex Regional Pain Syndrome"

is entirely my own work.

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LIST OF ABBREVIATIONS

ACE	angiotensin-converting-enzyme
APS	Ammonium persulfate
-AR	-adrenergic receptor
ASICs	acid sensing ion channel
ATP	adenosine triphosphate
BSA/FCS	bovine serum albumin/fetal calf serum
CAMP	cyclic AMP
CCL	C-C motif containing ligand
CCR	C-C motif containing ligand receptor
CD	cluster of differentiation
CGRP	calcitonin gene related peptide
CNS	central nervous system
CRPS	complex regional pain syndrome
CSF	cerebro spinal fluid
DAMP	damage associated molecular pattern
DMSO	dimtheyl sulphoxide
DRG	dorsal root ganglion
DTT	dithiothreitol
DUBs	deuquitinating enzyme
EDTA	Ethylenediaminetetraacetic acid
ENaCs	epithelial sodium channel
ERK	extracellular signal-regulated kinase
FMRI	functional magnetic resonance imaging
GMCSF	granulocyte macrophage colony stimulating factor
H&E	haematoxylin and eosin
HCL	hydrochloric acid
HLA	human leukocyte antigen
IASP	international association for the study of pain
IENF	intra-epidermal nerve fibre
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IL-1Ra	interleukin 1 receptor agonist
IVIG	intravenous immunoglobulin
JRA	juvenile rheumatoid arthritis
LC	Langerhans cell
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase

M-CSFR	monocyte-colony stimulating factor receptor
MEG	magneto-encephalography
MHC	major histocompatibility complex
miRNA	micro RNA
MoLCs	monocyte derived Langerhans cell
NA	noradrenaline
NF-KB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NICE	national institute for health and care excellence
NK cell	natural killer cell
NK1R	neurokinin 1 receptor
NMDAR	N-methyl-D-aspartate receptor
NO	nitric oxide
OVA	ovalbumin
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAR	protease activated receptor
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PD	Parkinson's disease
PE	phenylephrine
PGP9.5	protein gene product 9.5
PHA	phytohemagglutinin
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol myristate acetate
RSD	reflex sympathetic dystrophy
SFU	spot forming units
SIL-2R	soluble interleukin 2 receptor
SMP	sympathetically maintained pain
SP	substance P
STNFR	soluble tnf receptor
TEMED	tetrameythlethyelenediamine
TGF	tumor growth factor
TLR	toll like receptor
TNF	tumor necrosis factor
TRP	transient receptor potential channel
UCH-L1	ubiquitin carboxy-terminal hydrolase-L1
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

Acknowledgements

CHAPTER 1: Introduction

1a) *Complex Regional Pain Syndrome.*

  i. *History and Diagnosis -*

Ever since it was first described in the late 19th century the diagnosis of Complex Regional Pain Syndrome (CRPS) has been problematic for clinicians due to the heterogeneity of the symptoms and the lack of reliable and quantifiable diagnostic indicators. Although the term CRPS was only introduced in 1993 the historical description of CRPS is generally traced to a condition resembling CRPS, first described in American civil war soldiers by Silas Weir Mitchell, who used the term "Causalgia" to describe persistent pain symptoms following nerve damage from gunshot wounds[1]. The first clinical documentation of the condition related to inflammation, is credited to Paul Sudeck who, in 1901, published a paper on post-traumatic bone dystrophy in combination with various other clinical phenomena including edema and trophic changes[2]. This condition was described as "Sudecks Atrophy" until the mid-20th century when further research highlighted the possible role of the sympathetic nervous system in the maintenance of peripheral pain, following which term Reflex Sympathetic Dystrophy (RSD) was coined to describe a group of related chronic pain conditions[3]. Finally, in 1993 the International Association for the Study of Pain (IASP) held a conference in Orlando, Florida where the current term Complex Regional Pain Syndrome was agreed as the

new umbrella term for peripheral pain disorders, with varying and complex clinical phenomena[4].

The same meeting also formulated the first standardized and internationally recognized criteria for the diagnosis of CRPS, which included the distinction of conditions based on the presence or absence of an observable nerve injury, designated Type II (causalgia) and Type I (RSD) CRPS, respectively[4]. Despite the advantages of this new framework for diagnosis, implementation of the criteria remained inconsistent and subject to criticism. Despite identifying most cases of CRPS, the IASP criteria had low specificity, resulting in the misdiagnosis of other neuropathic pain conditions as CRPS[5]. As a result of the mounting criticism, new diagnostic criteria were established in 2003 and named the "Budapest critera"[6] (Table 1.1). The Budapest criteria possess a much greater specificity than previous clarifications[7] and are now widely accepted as the international gold standard for CRPS diagnosis with regular updates published[8]. However, by virtue of the complicated pathology of the disease, reliable diagnosis continues to be a problem in CRPS and efforts continue to develop this area, including the proposal of a CRPS severity score[9]. Furthermore, due to the lack of a reliable and quantifiable diagnostic test for CRPS, diagnosis still relies heavily on the experience and knowledge of the practicing clinician, a system which can be exploited by malingerers who falsely report symptoms[10].

Table 1.1 – Criteria for the diagnosis of CRPS according to the Budapest criteria[6]. Patients must fulfil aspects of all four categories before diagnosis is confirmed.

1. Continuing Pain, which is Disproportionate to any Inciting Event.	
2. Must Report at Least One Symptom in 3 of the 4 Following Categories:	
Sensory	Hyperesthesia and/or Allodynia
Vasomotor	Temperature Asymmetry and/or Skin Colour Changes and/or Skin Colour Asymmetry
Sudomotor/Oedema	Oedema and/or Sweating Changes and/or Sweating Asymmetry
Motor/Trophic	Decreased Range of Motion and/or Motor Dysfunction (Weakness, Tremor, Dystonia) and/or Trophic Changes (Hair, Nail, Skin)
3. Must Display at Least 1 Sign at Time of Evaluation in 2 or More of the Following Categories:	
Sensory	Evidence of Hyperalgesia (to pinprick) and/or Allodynia (to light touch and/or Deep Somatic Pressure and/or Joint Movement)
Vasomotor	Evidence of Temperature Asymmetry and/or Skin Colour Changes and/or Asymmetry
Sudomotor/Oedema	Evidence of Decreased Range of Motion and/or Motor Dysfunction (Weakness, Tremor, Dystonia) and/or Trophic Changes (Hair, Nail, Skin)
4. There is no other diagnosis that better explains the signs and symptoms.	

ii. Symptoms -

CRPS is a heterogeneous disease, with a wide range of symptoms occurring across the patient population. Generally, symptoms can be classified into one or more of four basic categories of disturbance: sensory; motor; autonomic and trophic (described in detail in Table 1.2), with the most frequent complaint being pain of some kind in the affected limb[11].

Typically, patients present after some form of minor to moderate injury, such as a small bone fracture, followed by an acute phase characterised by the cardinal signs of inflammation (redness, warmth, swelling and pain)[12]. In particular, an altered skin colouration and temperature between affected and non-affected limbs is apparent in the early stages, usually manifesting as "warm CRPS" i.e. an increased relative skin temperature. After the acute phase, warm CRPS commonly develops into "cold CRPS" i.e. decreased relative skin temperature and cyanotic appearance. Further symptoms also develop within the affected limb, such as hyperalgesia and trophic changes, despite the apparent resolution of inflammation in the affected area. Although these symptoms are confined to the affected limb, they develop independently of any specific nerve(s) that were affected by the inciting event. As the disease progresses, the pain and other clinical symptoms can often spread proximally[13] and even to the contralateral or ipsilateral limb[14]. Disease persistence is also associated with development of sensory loss, and loss of voluntary motor function, with symptoms such as

Table 1.2 – The major physical of symptoms CRPS.

Sensory	Chronic Pain	Spontaneous pain in CRPS is most commonly characterised as burning, pricking, aching or shooting[12].
	Allodynia	Pain in response to a stimulus which does not normally provoke pain such as a brush stroke across the skin.
	Hyperalgesia	A greater intensity of pain than usual in response to a painful stimulus. Mechanical and thermal hyperalgesia are both common in CRPS with a prevalence for cold hyperalgesia opposed to warm[15].
	Hypoesthesia	The loss of sensory perception to pain, touch, temperature or a combination thereof. So called "negative symptoms" are more common in the later stages of CRPS.
Motor	Limb weakness	Muscle weakness is common in CRPS affected limbs. In the acute phase oedema can also contribute to a reduced range of movement. Atrophy can also develop in latter stages.
	Tremor/ Myoclonus	Tremors and muscle twitches occurring in CRPS affected limbs. Thought to be more common in cases with visible nerve damage i.e. Type II CRPS[16].
	Focal Dystonia	Involuntary muscle contractions or adoption of abnormal posture which commonly develops distally in affected limbs, commonly the hands and feet.
Autonomic	Oedema	Most pronounced in the acute phases of disease and associated with inflammatory markers such as vascular leakage
	Vasodilation	Increased blood flow in the affected limb resulting in characteristic "warm" CRPS in which produces a marked difference in both skin colouration and temperature between affected and non-affected limbs.
	Vasoconstriction	Producing the opposite effect to above, resulting in "cold" CRPS and cyanotic appearance in limbs. In most cases, CRPS will progress from the warm to the cold type over time however, but 20% of cases present with only cold CRPS[12].
	Sudomotor	Alteration in sweating
Trophic Changes	Hair/Nail growth	Excessive hair and nail growth are both observed within days of disease onset. However, as with sensory disturbances, these positive symptoms tend to reverse and become negative, resulting in reduced growth and in some cases atrophy.
	Bone density	Reduction in bone density is common in the latter stages of CRPS, but in 40% of cases spotty osteoporosis can be observed as early as 4 weeks from onset[17].

hypoesthesia, hypoalgesia, tremors and twitches appearing[12]. Since utilisation of an affected limb is associated with worsening symptoms, secondary factors such as disuse atrophy, can also develop. If CRPS symptoms do not resolve after continued therapy, the disease is commonly referred to as "longstanding CRPS" and is associated with reduced quality of life and a poorer prognosis[18].

Because the symptoms of CRPS are similar to those found in other conditions, such as fibromyalgia, and awareness of correct diagnostic criteria is generally low among general practitioners, CRPS is frequently diagnosed late. Furthermore, the wide variety of symptoms means that patients will often interact with a huge variety of different healthcare professionals, from neurologists and rheumatologists to psychologists and emergency service staff, which in turn can contribute to delayed diagnoses[18].

iii. Epidemiology -

The first published population-wide review on CRPS incidence was performed by Sandroni *et al*[19] *in* the U.S. in 2003. The findings were regarded as controversial at the time, due to the low reported incidence (5.46 new cases per 100,000 annually) and the fact that the IASP diagnostic criteria had changed over the course of the study period[20]. In 2007 a second population wide review was published with a larger patient cohort (217,653 compared to the earlier 106,470) based in Europe[21] which reported its most conservative estimate as 26.2 new cases of CRPS per 100,000 people annually. Because the second study used the modern diagnostic criteria throughout and had other

advantages, such as incorporating detailed data from pain specialists, it is widely regarded as the most accurate. Despite the significant difference in reported incidence both studies did agree on other aspects of CRPS epidemiology. Both studies reported significantly more cases in females than in males, with ratios of 4:1 and 3.4:1 respectively, the highest levels of incidence in people aged 55 – 75 and both reported fractures and sprains as the most common precipitating events, accounting for over 40% of cases.

No definition of recovery has been defined for CRPS, due mostly to the spontaneous resolution of many diagnostic symptoms but the persistence of chronic pain. From studies of patient outcomes, it is known that 6 years after onset, 54% of patients consider their symptoms to be stable while 30% consider themselves completely recovered[11]. Although CRPS is associated with spontaneous resolution in most cases, the improvement often follows some form of therapy.

iv. Treatment

Since the underlying pathophysiology of CRPS is not well understood treatment and patient manifestation is so wide-ranging treatment regimens remain varied. National guidelines are available including the most recent UK guidelines published by the Royal College of Physicians (summarised in Table – 3)[22,23].

In particular, there is a scarcity of large randomised control trials for the drug treatment of CRPS and evidence in this area is mixed at best. A recent review of evidence based treatment for CRPS evaluated 25 studies of patients receiving oral or topical drug interventions and concluded that in most cases that there was no, or insufficient evidence to support treatment[24]. Furthermore, those treatments shown to have some effect are complicated by a lack of knowledge of drug mechanisms, uncertain dosage, and also by disease duration i.e. not effective in late stage disease. Of the more invasive techniques, only spinal cord stimulation was shown to be effective and is to date still the only CRPS treatment approved by NICE in the UK[25].

One area of treatment regarded as particularly important to a patient's management of pain is through paramedical treatments. Various therapies encompassing physiotherapy, occupational therapy and psychological support have been shown to improve patient outcomes[18,24].

Finally, recent research has also produced promising new avenues of therapy, including application of intravenous immunoglobulin treatment (IVIG), use of NMDA receptor antagonists and various brain training techniques[26,27]. However, all still require long term randomised trials and further refinement to confirm efficacy.

Table 1.3 – The Royal College of Physicians national guidelines for the treatment of complex regional pain syndrome

FOUR PILLARS OF TREATMENT FOR COMPLEX REGIONAL PAIN SYNDROME (CRPS) – an integrated interdisciplinary approach
1. Patient Information and Education.
Patients should be :   • Provided with education about CRPS.   • Reassured of the safety of physical/occupational therapy as a treatment.   • Encouraged to begin a process goal setting and reviewing.
2. Pain Relief (medication and procedures).
Pain specialists should be aware of the current evidence for efficacy of pain interventions in CRPS. No single treatment is recommended at the current time but the following may be considered: • Neuropathic pain medication according to National Institute for Clinical Excellence guidelines *.   • Pamidronate (60mg intravenous single dose) for suitable patients with CRPS < 6 months duration as a one-off treatment.   • Spinal cord stimulator treatment for patients with CRPS who have not responded to appropriate integrated management.
3. Physical and Vocational Rehabilitation.
Should be delivered by therapists competent in treating chronic pain with emphasis on restoration of normal function and activity through acquisition of self-management skills.   This may include elements of chronic pain management: • Body reconditioning through exercise, gait re-education and postural control.   • Restoration of normal activities, including self-care, social, leisure and recreational activities.   • Pacing and relaxation strategies.   • Vocational support.   It may also include specialised techniques to address altered perception of the limb for example: • Self-administered desensitisation with tactile and thermal stimuli.   • Functional movement to improve motor control and limb position awareness.   • Graded motor imagery, mirror visual feedback, mental visualisation.   • Management of CRPS-related dystonia.
4. Psychological Intervention
Based on individual assessment to identify and manage factors that could contribute to perpetuation of pain or disability/dependency including: • Mood evaluation – management of anxiety/depression.   • Internal factors, e.g. counter-productive behavioural patterns.   • External influences or perverse incentives.   This interventional usually follows the principles of cognitive behavioural therapy delivery: • Coping skills and positive thought patterns.   • Support to family/carers to manage needs and maintain relationship.

1b) Peripheral mechanisms of pain and the influence of the immune system.

i. Molecular Basis of Pain

Pain as an evolved mechanism that serves the fundamental purpose of translating potentially noxious environmental stimuli: mechanical, thermal or chemical, into electrochemical activity. This signal transduction occurs when primary sensory pain fibres, known as nociceptors, become sufficiently depolarized to trigger a change in action potential. In the peripheral sensory system, there are two types of nociceptive fibre known as Aδ- and C-fibres, the latter of which is unmyelinated. Both types of fibre are polymodal and thus equipped with various sensor molecules which transduce noxious stimuli into a depolarising sensor potential (Figure 1.1)[28,29]. In most cases, the sensor molecule is a ligand-gated ion channel which facilitates depolarization when open, for example the transient receptor potential (TRP) V1 is open at temperatures higher than $43^{o}C$ and facilitates transport of cations (mostly extracellular Ca^{+}) into the cell. The same receptor can also be opened by chemicals, such as capsaicin, which results in a painful burning sensation, effectively mimicking the sensation of a high temperature[28,30]. Other major receptor types include acid-sensing ion channels (ASICs) which are active at low pH, ATP and purinergic ion channels, activated by the release of ATP from damaged cells, and degerin epithelial sodium channels (ENaCs) which are high-threshold receptors responding to intense mechanical stimulation[31–33].

Neuropeptides

Adrenergic Mediators

Inflammatory Mediators

Cytokines

Neurotrophins

SECOND MESSENGERS

Modulation of sensing potentials

Modulation of action potentials

Signal Transduction

ASICs for protons

PTX receptor for ATP

TRP for thermal transduction

Channel for Mechanical Transduction

K^+ K^+ K^+ K^+

Na^+ Na^+ Na^+ Na^+

Ca^+ Ca^+ Ca^+ Ca^+

Voltage Gated Ion Channels
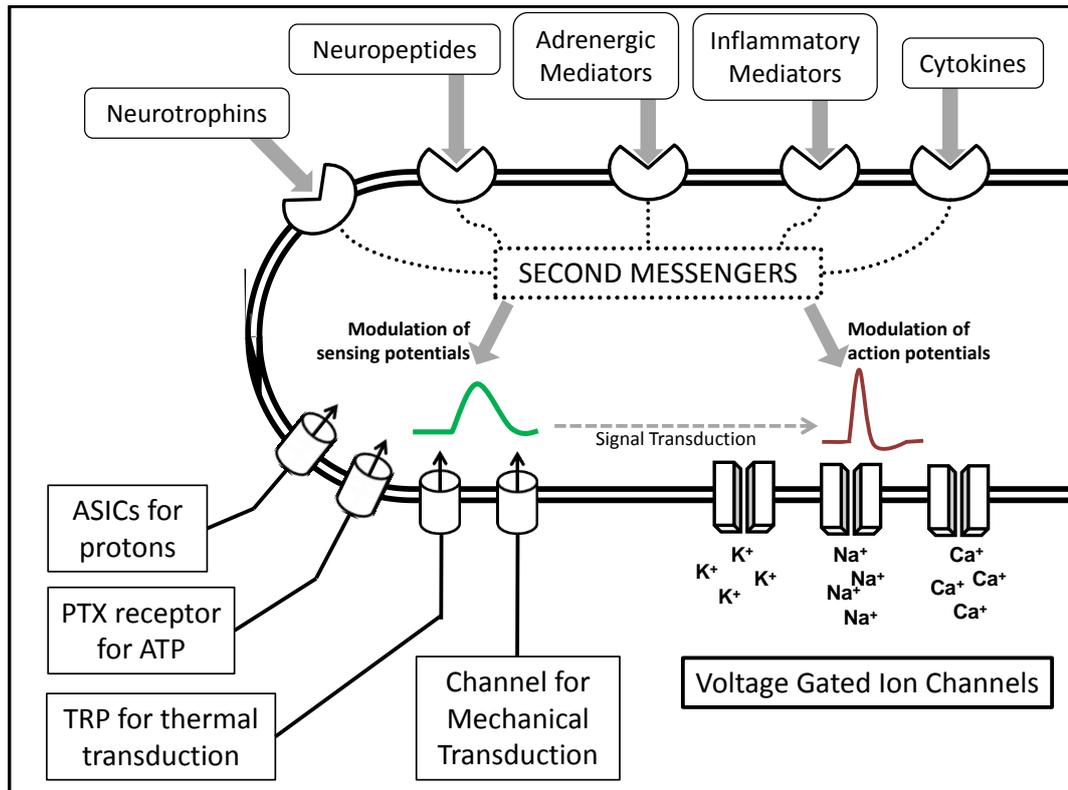
Figure 1.1 – The molecular basis of nociception. Polymodal sensory fibres express a wide variety of basic sensing receptors which, upon binding of a specific ligand, facilitate the induction of a sensing potential. Above a certain sensing potential threshold, signal transduction occurs and an action potential is produced. Intracellular second messenger systems exist which can modulate the thresholds for sensing in response to the binding of cell surface receptors by their specific ligands.

Each receptor is generally classified by the type of stimuli to which it responds e.g. heat or mechano-heat and in combination allow responses to the three main noxious stimuli discussed above.

If the level of depolarisation in the nerve ending is sufficient, then voltage-gated ion channels facilitate the transmission of an action potential along the peripheral nerve fibre to dorsal root ganglion (DRG) cells and eventually on to the cortex and thalamus via the spinothalamic tract[31]. Information regarding

the intensity of a stimulus is rendered by the frequency and duration of the action potential firing[31].

ii. *Inflammatory Pain - Modulation of Pain by the Immune System*

The molecular pain pathway described above is commonly activated as a by-product of inflammation by virtue of the tissue environment generated under inflammatory conditions (altered pH, bystander cell damage, direct damage to neurone etc.). However, active components of the immune system can also signal directly to peripheral nociceptors to alter the transduction thresholds and produce peripheral sensitization[34] (Fig. 1.1). Active inflammation lowers the excitation threshold of nociceptors to noxious stimuli and also sensitizes certain C-fibres, which are otherwise non-excitable by noxious stimuli, contributing to sensitization[28]. This peripheral sensitization produces a state of hyper-excitability within the nociceptors of the central nervous system, thus producing pathophysiological, symptoms such as hyperalgesia and allodynia.

This peripheral sensitization is mediated by a number of different soluble mediators and signalling pathways. Cytokines, such as tumor necrosis factor (TNF-) α[35,36] and interleukin (IL-) 1β[37], have been shown to induce pain through altered nociceptive responses[38]. The pro-inflammatory CC-chemokine ligand (CCL-) 3 has been shown to alter the sensitivity of the TRPV1 receptor[39]. Furthermore, nflammatory proteases, such as mast cell tryptase and trypsin, can also alter the esnitivity of the TRPV1 receptor, through binding of the protease-activated receptor (PAR) 2 expressed on sensory neurones[40]. IL-6 in

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complex with the soluble IL-6 receptor has also been shown to sensitize nociceptors in inflamed joints by binding to glycoprotein 130 expressed on sensory neurones[41]. There is also evidence that various other cytokines including IL-12, -15 and -18 may contribute to nociceptive sensitization when applied exogenously[42].

Other signalling molecules directly related to inflammation can also induce peripheral sensitization. Classical inflammatory mediators, such as prostaglandins and bradykinin, can both activate and sensitize neurones[28,43]. The neurotrophin nerve growth factor (NGF), released in response to inflammatory tissue damage, can directly alter the threshold for thermal excitation by enhancing currents through TRPV1 channels, as well as inducing the release of inflammatory compounds from immune cells[42]. Various neuropeptides including substance P (SP) and calcitonin gene-related peptide (CGRP) are also involved in inflammatory modulation of nociception and are discussed in more detail in the context of CRPS below. Finally, under certain conditions, usually following nerve injury, adrenergic mediators, such as noradrenaline (NA), can have nociceptive effects, a mechanism which may be of relevance to CRPS[44] and is discussed in detail later.

Various kinase families have been implicated as the intracellular signalling systems (second messengers) involved in inflammatory mediated peripheral sensitization. Mitogen activated protein (MAP) kinases, including extracellular signal-regulated kinase (ERK) and p38[45], cyclic adenosine monophosphate

(cAMP) dependent protein kinase A (PKA)[46], calcium dependent protein kinase C (PKC)[47] and c-Jun N-terminal kinase (JNK)[48] have all been implicated. Work in this area is almost exclusively performed using DRG cells, and so extrapolation of these models to primary sensory neurones relies predominantly on the use of pathway specific chemical inhibitors in primary neurones.

iii. Neuropathic Pain – Modulation of Pain by Nerve Damage

Neuropathic pain is distinct from inflammatory pain in that the action potentials responsible for perception of pain are usually ectopic discharges generated in an area affected by nerve damage, though not necessarily directly from a damaged fibre itself[49]. These ectopic discharges can also be produced by inappropriate application of inflammatory mediators, blurring the lines between inflammatory pain and neuropathic pain[28,38,50]. It is clear that direct trauma to a nerve will produce a primary phase of inflammation which is similar to an inflammatory pain response in any damaged or infected tissue. However, in the case of damaged nerve fibres, this inflammatory response can lead to prolonged changes in the peripheral and central nervous systems, which contribute to the development of chronic neuropathic pain[51,52].

If a nerve fibre is completely transected during trauma, then axon degeneration occurs, known as Wallerian degeneration, whereby the nerve fragments distal to the injury bead, swell and then disintegrate, a process which can takes days in humans[53]. Over the course of this process, the products of cellular degeneration, so-called damage associated molecular

patterns (DAMPs), are exposed to the extracellular environment where they can bind Toll like receptors (TLRs) expressed on Schwann cells and on resident immune cells, such as mast cells and tissue resident macrophages[53,54]. These cells then release inflammatory mediators and chemotactic factors, such as TNFα, IL-1β, IL-6 and CCL2 in order to facilitate further immune cell recruitment[55]. This leads to infiltration of neutrophils and monocytes which phagocytose the cellular debris to prevent further immune activation[56]. Monocyte-derived macrophages also secrete neurotophins, such as NGF, and actively promote axon regeneration[57]. Over the course of this process, many of the inflammatory mediators are released in the presence of healthy nerve fibres, resulting in peripheral sensitization by the pathways discussed above.

DRG cells can also become sensitized as a result of neurogenic inflammation in a peripheral sensory fibre. Indeed during peripheral inflammation DRG undergo a similar process to that described above, whereby immune cell infiltration, up regulation of pro-inflammatory cytokines and sensitization all occur[55]. This process may be facilitated by the retrograde transport of inflammatory factors from the damaged axons[52]. If this inflammation is not resolved, the persistent hyper-excitability in DRG cells can result in altered microglial (CNS resident macrophages) behaviour contributing to central sensitization[58]. This process is thought to be an essential step in the development and maintenance of chronic pain in which significant changes in

neuronal plasticity and cerebral processing result in so-called cortical reorganisation[34,59].

It has been suggested that inadequate resolution of neurogenic inflammation contributes to the development of neuropathic pain. Various resolution-associated molecules have been implicated in this process including classical cytokines, such as IL-10 and transforming growth factor (TGF) –β, as well other endogenous mediators, such as resolvins and endocannabinoids, both of which have received significant recent interest as potential therapeutic treatments[55,60,61]. However, it is also been shown that insufficient immune involvement following nerve trauma, decreased debris clearance, axon regeneration and functional recovery, particularly in the absence of CD11b-expressing monocytes and macrophages[62]. It is probable that dysregulation of this delicate balance between axon recovery and excessive inflammation ultimately transforms inflammatory pain into longstanding neuropathic pain, and may be crucial in understanding CRPS.

1c) Complex Regional Pain Syndrome – Disease Etiology and Current Understanding

Due to the heterogeneous symptoms involving multiple body systems and the early confusion in diagnosis and treatment of CRPS, a variety of different hypotheses have developed to explain the pathogenesis and maintenance of CRPS[18]. These hypotheses can generally be categorized by the three related body systems, within which the dysfunction is thought to occur: the immune

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system, the peripheral nervous system and the central nervous system. It has become apparent; however, that no single hypothesis can adequately explain the entire condition and that significant interaction between the suggested mechanisms occurs across different disease subtypes, resulting in an integrative conceptual model of disease pathology, an overview of which is shown on page 46 (Fig. 1.4).

1d) The Role of the Immune System in CRPS

Due to the obvious inflammation that occurs in the acute phase of CRPS the role of the immune system in disease pathology has received much attention. However, it is not clear what role the immune system plays beyond the acute phase and if any pathological changes in immune activation contribute to the maintenance of longstanding disease. The role of the immune system throughout CRPS disease is discussed below and also summarised in figure 1.2.

i. Immunology of CRPS - Soluble Mediators

Due to the lack of reliable diagnostic indicators in CRPS, there is considerable effort directed at measuring levels of serum proteins, particularly cytokines, in order to identify a reliable biomarker. However, results in this area have been conflicting. Some groups have shown increases in plasma concentrations of relevant inflammatory cytokines, including IL-8 and soluble TNF receptors (sTNFR)[63]. Üçeyler *et al* showed elevated levels of TNFα and increased levels of TNFα and IL-2 mRNA in patient blood, but the same study showed decreases in IL-8 mRNA[64]. More recently, a large study of 148 CRPS patients (100 type I CRPS

and 48 type II CRPS) showed significant increases in the plasma levels of interferon (IFN) $-\gamma$, IL-1β, IL-4, IL-7, TNF-α, soluble IL-1 receptor I (sIL-1RI), soluble IL-2 receptor (sIL-2R) $-\alpha$, soluble TNF receptors (sTNFR) I&II, IL-1 receptor antagonist (IL-1Ra) and CCL-2 when compared to healthy controls[65]. Cluster analysis of these data showed that a cluster consisting of 36% of the CRPS patients was responsible for these changes, with TNF-α determined to be the most important factor for cluster separation. The remaining 64% of CRPS patients demonstrated plasma analyte levels similar to those found in healthy control individuals. Although no significant, differences were observed in disease type, duration or symptoms, nor in the age, BMI or gender of the two clusters, TNF-α did show a significant positive correlation with disease duration in the plasma analyte-high cluster. Furthermore, sTNFR I&II and IL-1Ra were also positively correlated with pain intensity in this same group of patients. Micro RNA (miRNA) profiling of CRPS patients has also been used to stratify the disease, resulting in a patient cluster, comprising approximately 60% of patients in the study and no controls, in which elevated levels of IL-1Ra, CCl-2 and vascular endothelial growth factor (VEGF) were all positively correlated with levels of pain[66].

The polarisation of plasma cytokines, with little or no concurrent change in clinical manifestation, may explain contrasting results from other groups. Huygen *et al* showed no change in the plasma levels of various inflammatory mediators, including TNFα, IL-1β and IL-6[67]. Similarly, van De Beek *et al*

ACUTE PHASE

Cellular

Radiolabelled leukocyte accumulation in affected limbs

Mast cells:
Shown to be important in animal models

Monocytes:
Increased numbers of circulating inflammatory monocytes

T-cells:
Decreased numbers of CD8+ T-cells

Neutrophils:
Impaired neutrophil function

Soluble

Immune

Peripheral Blood:
TNF-α
IL-2
IL-7

IL-1β
IL-4
IL-8

Cerebrospinal Fluid:
IL-6
IL-1β

IL-4
IL-10

Artificial Blisters:
IL-6
TNF-α
Mast cell Tryptase

Neurogenic

Peripheral Blood:
Substance P
Calcitonin Gene Related Peptide
Bradykinin

LONGSTANDING

Langerhans Cells:
Small study showing increased numbers in epidermis

Resolution of acute inflammation results in reduction of swelling and cell infiltration into tissues.

Levels of inflammatory mediators normalise over time with no concordant improvement in disease symptoms

Figure 1.2 – Evidence for Immune System Involvement in CRPS Pathology

A wide variety of evidence now suggests that the immune system plays a key role in the pathology of acute phase CRPS. Although conflicting reports still occur regarding elevated peripheral cytokine concentrations, measurements taken from artificially induced blisters show more consistent results. In cases of longstanding CRPS there is a much smaller body of evidence. Long term studies have shown decreases in cytokines and inflammation is obviously reduced in affected limbs however symptoms persist. A small study reported increased numbers of epidermal dendritic cells (Langerhans cells) in longstanding CRPS. It is unknown whether some aspect immune dysregulation persists in these cases or if the role of the immune system is limited to an acute inflammatory response.

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recorded no change in cytokine release from peripheral blood cells of CRPS patients after stimulation, when compared to healthy controls[68]. Another explanation for this disparity is that systemic changes in inflammatory mediators between different patients may represent transient cytokine "overspill" from sites of local inflammation[69]. However, beyond the work discussed above, there is little evidence of a correlation between peripheral inflammatory mediators and disease symptoms.

Cytokine measurements in cerebrospinal fluid (CSF) are also inconsistent between studies. Increased levels of IL-6 and IL-1β were observed in the CSF of CRPS patients compared to control[70], while in a subset of CRPS patients, increases in nitric oxide metabolites (relevant to glial cell activation) and decreases in IL-4 and IL-10 were also observed[71]. However, this finding was not supported by a second, similar study specific to patients with dystonia[72]. Due to the differing symptoms of the CRPS patients analysed, it could be that changes in inflammatory markers in the CSF are dynamic, and that variations in levels impact on symptoms.

Quantification of the levels of local inflammation has been predominantly measured through analysis of tissue fluid from artificial blisters and has consistently shown high levels of IL-6, TNFα and mast cell tryptase[67,73]. However, no link between cytokine levels and disease symptoms could be

established[74]. Blister fluid from CRPS affected limbs was also assessed using a multiplex bead array (25 different analytes measured simultaneously) and confirmed previous observations of increased IL-6 and TNF-α, in addition to modest increases in IL-8, IL-1Ra, IL-12, CCL2 and CCL-4[75]. In response to these finding a longitudinal study was implemented to investigate the long-term implications of increased IL-6 and TNFα, which showed a decrease in inflammatory cytokines over time with no concordant improvement in disease symptoms. These data suggest an early and indirect mechanism of action for IL-6 and TNFα in CRPS pathology[76]. Analysis of skin biopsies from CRPS affected limbs also showed increases in TNF-α expression compared to patients with osteoarthritis, despite no difference between the levels of serum TNF-α between the two groups[77]. Interestingly, the same study also showed increased levels of TNF-α in the skin of fracture patients, providing a direct link between a common inciting event and the development of CRPS, an hypothesis supported by data from a tibia fracture model of CRPS[78]. In a small scale study increased levels of TNF-α in the affected limbs of CRPS patients has also been detected following administration of radiolabeled anti-TNF antibodies[79].

The role of TNF-α, and to a lesser extent IL-6, has received considerable interest in CRPS, in part due to the availability of new anti-cytokine therapies. A case report (2 patients) involving treatment with the anti-TNF drug, infliximab, reported a positive treatment outcome[80]. Use of thalidomide (prescribed for multiple myeloma and Behçets disease) also showed some efficacy in the

treatment of CRPS[81–83]. However, the only randomized control study involving the anti-TNF-α and the anti-IL-6 agent lenalidomide, remains unpublished, despite successful completion[84]. The application of glucocorticoids, particularly in the early stages of the disease, has also been shown to have beneficial effects[85].

ii. Immunology of CRPS - Cellular Mediators

As discussed above increased levels of mast cell tryptase have been consistently recorded in artificial blister fluid collected from CRPS affected tissue[86]. This finding has led to the implication that mast cells may play an important role in disease pathology. However, it is not known if this finding reflects increased cell activation/degranulation or physical differences in mast cell density within the affected tissue. Mast cells are potentially interesting cells due to their release of large quantities of nociceptive sensitizing molecules, such as histamine, prostaglandins and proteases. In addition to the expression of neuropeptide receptors, such as neurokinin (NK) 1 receptor, this functionality places mast cells at a crucial juncture in CRPS pathology[86]. Various animal models of neuropathic pain, similar to CRPS, have reported a role for mast cells. Klein *et al* reported an increase in the number of mast cells surrounding damaged nerves in hyperalgesic rats, compared to those with no hyperalgesia, following needle stick nerve injury[87]. Mast cells were also shown to play a key role in pain development in a rat tibia fracture model through interaction with the neuropeptide SP[88]. In this model, inflammatory mediators

released by mast cells, following an injury, sensitize peripheral nociceptors, which in response release SP. This neurogenic SP then binds to the NK1 receptor expressed on mast cells and induces further degranulation and drives a vicious circle maintaining local inflammation around the nerve. Interestingly, it has been shown that mast cell sensitivity to SP varies between different strains of rat, with nanomolar doses being sufficient to cause histamine release in some cases[89]. Although these differences in responsiveness have not been described in humans, it could be a potential explanation for predisposition to the development of CRPS.

There is limited evidence regarding the involvement of other leukocytes in CRPS. Tan *et al* used radioligand binding to investigate leukocyte accumulation in the hands of CRPS I patients, compared to control[90]. Although significantly increased leukocyte accumulation was observed in CRPS patients compared to controls, the technique is limited as it cannot differentiate between leukocyte subsets. CRPS patient neutrophils showed impaired function, including decreased phagocytosis of zymosan in autologous plasma[91]. The same group also demonstrated diminished T-helper 1 response, based largely on decreased numbers of CD8+ T-cells in CRPS patients, compared to healthy controls, suggesting modulation of the adaptive arm of the immune system[92]. Furthermore, CRPS patient monocytes from peripheral blood samples displayed significantly increased levels of Nitric Oxide (NO) release after stimulation with IFN-γ, when compared to healthy controls. More recent work

reported an increase in circulating inflammatory monocytes (CD14+CD16+) in patients with CRPS, but no change in the levels of T-cells (CD4+/CD8+), B-cells (CD19+), natural killer (NK) cells (CD56+) or monocyte/macrophages (CD14+), when compared to healthy control[93].

iii. Immunology of CRPS - Neurogenic Inflammation and Neuropeptides

Interactions between immune cells and the nervous system, particularly via neuropeptides, may be especially important in CRPS. SP has already been discussed in relation to mast cell function, but it has a wide range of effects on immune cells, including chemo-attraction, activation and proliferation of lymphocytes and degranulation and respiratory burst activation in neutrophils[94]. It has similar effects on monocytes, although there are conflicting reports on the increased production of TNF-α and IL-6 in these cells[95,96]. In the acute phase of CRPS, SP is present at significantly higher concentrations in patient serum and correlates inflammatory symptoms[63]. The amount of SP required to induce plasma protein extravasation is also thought to be altered in the affected limbs of CRPS patients, thus facilitating neurogenic inflammation, possibly to due impaired inactivation of this pathway[97,98]. Aside from its direct effects on nerves and immune cells, SP can also induce IL-1β production by keratinocytes in a rat tibia fracture model, a process mediated by the NALP1 containing inflammasome[99,100], and can also activate NK1 receptor expressing osteoclasts, possibly contributing to osteoporosis[101,102].

Other neuropeptides elevated during the acute phase of CRPS include bradykinin and CGRP, which decrease in concentration after the resolution of inflammation[103,104]. CGRP can produce many of the features of acute CRPS, such as edema and vasodilation, as well as increasing both sweating and hair growth[102,105]. CGRP also induces IL-1β production in keratinocytes in a similar manner to SP[100]. Both SP and CGRP are released from small nerve fibres (Aδ and C-fibres), but CGRP is constitutively expressed in certain epidermal nerve fibres which share a close physical relationship to epidermal dendritic cells known as Langerhans cells (LCs)[102,106]. Increased numbers of LCs were reported in skin biopsies from a small case series of CRPS patients[107]. Because the identification of the LCs in this study was based upon non-specific immuno-staining (of the protein S100) and morphological appearance, the accuracy of the observation is questionable. However, the crucial role of LCs at the interface between the immune and nervous systems, and the involvement of CGRP, provides indirect support to this argument and is discussed in more detail below.

Finally, a recent study has also shown that CRPS may be associated with angiotensin-converting-enzyme (ACE) inhibitor treatment[108]. Because ACE is capable of metabolizing both SP and bradykinin to inactive forms, it has been postulated that ACE inhibitor therapy may lead to elevated levels of these neuropeptides and facilitate CRPS onset.

iv. Immunology of CRPS – Auto-Antibodies and Auto-Immunity

One hypothesis receiving much attention recently is the concept of CRPS as a novel auto-immune disease, mediated by circulating auto-antibodies[109]. However, the fact that CRPS pathology is localized to peripheral limbs, specifically to an area affected by trauma, and the apparent absence of meaningful tissue destruction in these areas, argues against this hypothesis. To account for these unusual characteristic, a two-step model of autoimmunity has been proposed, involving the existence of pre-existing auto-antibodies which only become pathogenic after the exposure of their auto-antigen following trauma[109]. There is a small body of evidence to support the role of antecedent infections, including increased seroprevalence of *Campylobacter jejuni,* a causative agent of Guillian-Barré syndrome, and Parvovirus B19 antibodies in CRPS serum[110,111]. Furthermore, "traditional" auto-immune characteristics have also been described in CRPS, such as human leukocyte antigen (HLA) associations and an increased prevalence in women[21,112]. Auto-antibodies directed against neuronal antigens have since been recorded in the serum of CRPS patients, including antibodies directed against differentiated neuroblastoma cells, and antibodies that bind to β2 adrenergic receptors and muscarinic-2 receptors expressed on the surface of Chinese Hamster Ovary cells[113,114]. Passive transfer of CRPS serum into mice has also shown altered rearing behaviour. However, the mice did not develop typical CRPS symptoms, such as sensitivity to temperature or touch[115,116].

Some of the strongest evidence to support this hypothesis comes from the treatment of CRPS patients with IVIG, regarded itself as circumstantial support of an auto-immune etiology. In an open investigation of 130 patients with chronic pain, including 11 patients with CRPS, low dose IVIG achieved dramatic pain relief in 3 of the 11 CRPS patients[117]. Following this initial study, a randomized controlled crossover trial of low dose IVIG was conducted and found significant pain relief and limb improvement was reported in some patients with longstanding (6 months to 2.5 years) CRPS, with effects lasting no longer than 3 months[26]. Although the exact mechanism of action of IVIG is not known, it is thought that saturation of Fc receptors may block pathogenic antibodies from binding. However, alternative mechanisms have also been suggested such as the expression of inhibitory Fc receptors or the induction of anti-inflammatory profiles in dendritic cells[118,119]. Other evidence however, suggests that inflammation only plays an early role in CRPS development and is not involved in disease maintenance. Thus it is thought that these auto-antibodies in CRPS may bind and activate their target receptor directly and thus contribute to aberrant pain signalling, which in turn sustains central sensitization[109].

v. Immunology of CRPS – Limb Ischemia

One possible outcome of exaggerated inflammation in the early phase of CRPS is tissue ischemia due to micro vascular damage caused by oxygen free radicals[120]. This theory is based on observations in an animal model where

induction of ischemia and subsequent reperfusion results in CRPS-like symptoms lasting up to one month in the complete absence of nerve damage[121]. The proposed mechanism involves a cycle of inflammatory and oxidative stress, which both sensitizes nociceptors and causes further micro-vascular damage, leading further bouts of ischemia. Tissue hypoxia has been reported in the affected limbs of CRPS patients in which nuclear factor erythroid 2-related factor (Nrf2) may play a key role[122,123].

The pharmacological scavenging of free radicals has also been suggested as a possible treatment for CRPS. While a variety of treatments including 50% dimethylsulfoxide (DMSO), N-acetylcysteine and mannitol, have ben trailed, none of these methods have led to significant patient improvement[124,125]. Prophylactic treatment with vitamin C, a scavenger of oxygen free radicals, decreased the risk of CRPS development after wrist fracture in a randomized control trial, and another trial using the vasodilator tadalafil, improved pain symptoms in patients with cold CRPS, supporting the hypothesis of hypoxia induced pain[126,127].

1e) The Role of the Peripheral Nervous System in CRPS

As discussed above, there is a large body of evidence suggesting a direct role of the immune system in the development CRPS pathology. However, it is also clear that there is significant cross-talk between systems and that the nervous system plays a key role in disease maintenance. The mechanisms by which the two systems interact within the periphery is summarised in Figure 1.3.

IMMUNE SYSTEM

SOLUBLE INFLAMMATORY MEDIATORS
e.g. cytokines (TNFα, IL-1 etc), tryptase, histamine

Bind to receptors expressed on peripheral nerve fibres and sensitize by lowering the threshold for signal transduction or altering the tissue environment e.g. reducing pH

Bind to adrenergic receptors expressed on immune cells fibres and sensitize and alter immune cells function. The effects of which may be dependent on the specific (subtype) adrenergic receptor repertoire expressed by the cell.

CATECHOLAMINES
e.g. adrenaline & noradrenaline

AUTO-ANTIBODIES
weakly interacting antibodies specific to nervous tissue

May bind to muscarinic and adrenergic receptors on nerve cells altering function and/or resulting in inflammation. Unknown effects on immune cell.

Bind to specific receptors (e.g. neurokin 1 receptor) on immune cells and alter behaviour. Including degranulation of mast cells (substance P) and altered antigen presentation by dendritic cells (CGRP)

NEUROPEPTIDES
e.g. Substance P, Calcitonin Gene Related Peptide

NERVOUS SYSTEM

Figure 1.3 – Mechanisms of Peripheral Neuro-Immune Crosstalk Relevant to CRPS.

The proposed role of the immune system in CRPS is generally limited to classic inflammatory mechanisms within the acute phase of disease. However, significant crosstalk occurs between the immune and nervous systems, dysregulation of which may have a role in late stage disease where classic inflammatory signs have been resolved. The complex interactions between these two systems take a number of different forms and are summarised above. The potential dysregulation of these mechanisms, particularly in late stage disease where central changes in nervous signalling are common, could have a major role in the establishment and maintenance of longstanding disease.

i. Peripheral Nerves - Nerve Damage

One interesting aspect of CRPS pathology is the classical appearance of neuropathic pain symptoms, seemingly in the absence of any nerve injury. CRPS symptoms present in a similar way as small-fibre polyneuropathies, in which the small Aδ- and C-fibres are impaired or lost due to damage[128]. Due to these parallels, it has been suggested that CRPS may represent a regionally-restricted small fibre neuropathy[129]. This hypothesis centres on the idea that a sub-clinical nerve injury can cause partial denervation of key tissue structures, such as sweat glands and microvasculature, resulting in dysfunction; damaged nerves then release neuropeptides (discussed above) resulting in neurogenic inflammation and sensitization of the remaining (healthy) nociceptors.

Studies conducted on skin punch biopsies and amputated limbs have shown decreased axon densities in the affected limbs of CRPS patients, including reductions in epidermal, sweat gland and vascular innervation which was associated with a decrease in CGRP expression[130,131]. It is also possible that CRPS cases in which there is no record of trauma, may represent small fibre neuropathies brought on by a viral infection, such as shingles, usually referred to as post-herpetic neuralgia[132]. However, the only treatment commonly used for neuropathic pain that has been assessed for efficacy in CRPS treatment is gabapentin, producing negative results[133]. This observation suggests that although small fibre neuropathy may play a role in CRPS pathology it's etiology

is distinct from the group of conditions referred to as small fibre polyneuropathies.

ii. Peripheral Nerves - Sympathetically Maintained Pain

The significant changes to skin sweating and blood flow (colour and temperature) in CRPS are, in part, due to dysregulation of the efferent sympathetic nerve pathway. Aberrant coupling of the efferent sympathetic pathway and the afferent nociceptive pathway can then produce so-called sympathetically maintained pain (SMP) [134]. The mechanism by which this coupling occurs is thought to be via altered expression of adrenergic receptors on peripheral sensory fibres, effectively producing hypersensitivity to sympathetic catecholamine, such as NA[135]. In fact, during the acute phase of CRPS there is loss of autonomic cutaneous vasoconstrictor activity and decreased levels of NA, which result in the classic early symptoms of increased limb temperature and redness[136]. The decrease in circulating catecholamines from efferent sympathetic fibres may, in fact, be what induces the expression of adrenergic receptors on nociceptive fibres[137]. There is also a significant body of evidence from type II CRPS disease models that nerve injury can alter the adrenergic profile of peripheral sensory fibres, including Aδ- and C-fibres, resulting in nociceptive sensitization to catecholamines[138–140]. Indeed, the intra-dermal application of NA induces spontaneous pain in individuals where this adrenergic sensitivity is established, but has no effect in control individuals[141]. One interesting aspect of this altered expression is that even uninjured

nociceptive fibres show increases in adrenergic receptor expression and spontaneous activity following nerve injury[142], and that nociceptive fibres can release neuropeptides in response to this stimulation[143]. A similar sensitization can also occur in DRG, providing a direct link to central sensitization via the SMP pathway[143].

This work on SMP has led to the suggestion that depletion of NA from autonomic nerve endings, by the regional application of guanethidine, would lead to resolution of pain. However, this is not the case in CRPS type I[144]. Although a subset of CRPS patients do respond to sympathetic blockade through the application of an anaesthetic to sympathetic ganglia, responders are generally in the early phase of CRPS onset where other treatment options are available[145,146].

iii. Peripheral Nerves - Adrenergic Receptors and Immunity

As discussed above, CRPS patients often present with altered sympathetic regulation including changes in adrenergic receptor expression and circulating levels of catecholamines[135,137]. One potential impact of an altered adrenergic profile, such as that observed in SMP, is the modulation of the immune system. Autonomic influence on immunity via the sympathetic nervous system is well characterised, and involves the secretion of catecholamines both centrally, via the adrenal medulla, and from peripheral nerve fibres[147]. This control is affected via the expression of adrenergic receptors on immune cells and tissues, and contributes to the complex relationship between psychological

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stress and immune responses, often manifesting as disease flares in many inflammatory conditions[148].

Adrenergic receptors themselves are G-protein linked cell surface receptors which are divided into three subgroups: α1, consisting of a, b and d subtypes; α2, consisting of a, b and c subtypes, and the β-receptors consisting of β1, β2 and β3[149]. The prevailing view of adrenergic receptor expression on human immune cells (Table 4) is that the primary receptor involved in sympathetic control is the β2 adrenergic receptor (β2-AR), signalling through which increases intracellular levels of cAMP and PKA[147]. Stimulation of inflammatory cells, including monocytes, mast cells and neutrophils, with pharmacological agonists of the β2-AR inhibits cell activation and release of inflammatory mediators, suggesting this dominant pathway is anti-inflammatory in nature[150]. However, stimulation through the same receptor in conjunction with a co-stimulatory signal, such as antigen or CD-40 ligand (CD-40L) synergistically increases expression of CD86 on B-cells[151]. A similar scenario has been described following α2 adrenergic receptor (α2-AR) stimulation of macrophages in conjunction with lipopolysaccharide (LPS) which results in increases in TNF-α production and suggests that α2-ARs are expressed by peripheral blood cells[149,152,153].

In contrast, α1 adrenergic receptors (α1-ARs) are not expressed on peripheral blood mononuclear cells but can be induced on T- and B- cells following treatment with phytohaemagglutinin (PHA), and on monocytes following

treatment with IL-β, TNF-α or LPS[154–156]. Another interesting aspect of α1-AR regulation on immune cells is that the different subtypes are differentially regulated. Stimulation of monocytes with dexamethasone or with a β2-AR agonist, (both regarded as anti-inflammatory) selectively induces expression of α1b- and α1d-ARs[154,157]. The link between these two subtypes is reinforced by expression studies in which surface expression of the α1d-AR subtypes is dependent on co-expression of the α1b-, but not the α1a-AR[158].

Table 1.4 – Expression of adrenergic receptor on immune cells

Adrenergic Receptor Family	Subtypes	Immune Cell Expression	Immune Modulation	Ref
α1	a	Inducible expression following stimulation with PHA (T-cells) or inflammatory cytokines (monocytes)	Synergistic increase in IL-6 production in the presence of LPS.	150-155
	b	Expression as above but also inducible on monocytes following treatment with dexamethasone or β2-receptor agonists.	As above, but also linked to anti-inflammatory responses during immunosuppression	
	d			
α2	a	Peripheral blood mononuclear cells.	Synergistically increases inflammatory cytokine production in the presence of TLR ligands.	145, 148, 149
	b			
	c			
β2	Non	Peripheral mononuclear cells, Mast cells, Neutrophils,	Predominantly anti-inflammatory but can synergistically enhance cell activation in the presence of co-stimulatory signals	143, 146, 147

Despite the lack of α1-AR expression on healthy human peripheral blood cells, functional α1-ARs have been described in peripheral blood mononuclear cells of patients with the inflammatory condition, juvenile rheumatoid arthritis

(JRA). Furthermore, stimulation of these cells with the α1-AR agonist phenylephrine (PE) resulted in increased production of IL-6[159]. Further work in JRA patients showed enhanced LPS induced IL-6 production by peripheral blood cells following a noradrenergic stressor[160]. More recently a single nucleotide polymorphism in the α1a-AR gene was identified as a risk factor for CRPS development following distal radius fracture[161].

Aside from the possible dysregulation of the sympathetic nervous system in CRPS, adrenergic receptors may also be targets for auto-antibody activation, as discussed above. Although initial work has focussed on the expression of auto-antigens in nervous tissue, the proposed activating anti-adrenergic receptor auto-antibodies in CRPS could also have direct immune-modulatory effects[109,114].

1f) The Role of the Central Nervous System in CRPS

Although the focus of this review is on peripheral systems, it is essential to briefly discuss the involvement of the central nervous system (CNS) to understand how chronic pain may be maintained centrally after the resolution of peripheral symptoms, and how CNS changes can alter efferent nervous signalling.

    i. Central Nerves - Central Sensitization

The term central sensitization refers specifically to the molecular process that occurs following repeated and/or intense stimulation of the ascending pain

pathway resulting in the de-coupling of pain perception and the peripheral perception of noxious stimuli[34]. The reasons for the intense or persistent stimulation of peripheral nociceptors, both inflammatory and neuropathic, have been discussed in detail above. One crucial aspect in the development of central sensitization is the recruitment of non-nociceptive fibres into the nociceptive network, particularly those with low transduction thresholds such as large myelinated Aβ mechanoreceptors[34]. The altered membrane excitability within the CNS effectively translates signals from low threshold, sensory fibres into pain signals, producing allodynia and hyperalgesia. There also appears to be a conversion of nociceptive specific neurons into dynamic neurons capable of responding to innocuous stimuli[162]. This neuronal plasticity combines with, and contributes to, the hyper-excitability of central neurons. This relationship is symptomatically manifest through the progressively increased pain response observed during repeated application of innocuous stimuli, sometimes referred to as temporal wind-up.

The molecular basis for the development of central sensitisation is thought to revolve around intracellular increases in Ca^+ above a certain threshold leading to the activation of multiple signalling pathways within the neuron including PKC, ERK and cAMP binding protein (CREB)[163–165]. These activated kinases then post-transcriptionally modify, via the phosphorylation of C-terminal residues, the function of certain key receptors, resulting in altered activity and membrane trafficking, which directly contributes to the development and

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maintenance of central sensitisation[166,167]. In particular, the activation of the glutamate binding N-methyl-D-Aspartate receptor (NMDAR) is thought to be crucial in this process. Under normal conditions the ion channel present in the NMDAR is blocked by Mg^{2+} ions, but sufficient membrane depolarisation causes Mg^{2+} release and allows glutamate to bind the receptor[168]. The binding of glutamate to the NMDA receptor allows Ca^+ influx into the neuron, contributing to the pathway described above. The depolarisation required to remove the voltage-dependent Mg^{2+} block and thus promote Ca^+ influx, can be induced by the sustained release of glutamate from nociceptors, possibly as a result of peripheral sensitisation. Nociceptive release of the neuropeptides SP and CGRP can also contribute to this membrane depolarisation[168].

Evidence for the role of central sensitization in CRPS pathology derives from two recent randomized control trials, in which low dose intravenous application of the NMDAR antagonist ketamine dramatically reduced CRPS pain[27,169]. In these studies, average pain intensity was decreased for several weeks after treatment, but with no concordant improvement in associated limb function and the effect was shown to be independent of the patient's disease duration. These results have led to much discussion in the field, but due to the poorly-understood side-effects and concerns about possible neurotoxicity, it is not clear how this treatment will translate clinically[170,171].

ii. Central Nerves - Cortical Reorganisation

Some of the least well understood aspects of CRPS pathology are the permanent changes that occur within the brain processing of CRPS patients, usually referred to as cortical reorganisation. Previous work has shown that patients with a variety of pain disorders, such as phantom limb pain or chronic back pain, develop modified processing within the somatosensory cortex[172]. Activity in this area of the brain, responsible for the sensory processing of tactile stimuli, can be mapped using imaging techniques, such as magneto encephalography (MEG) and functional magnetic resonance imaging (fMRI) and represented spatially using a sensory homunculus. The first reports of cortical reorganisation in CRPS showed increased responses to tactile stimulation and altered perception of the hand, particularly the thumb and little finger, in the affected limb[173]. Subsequent studies also showed significant changes in the perception of affected limbs, shown by shrinking and shifting on the Penfield sensory homunculus, and that these changes correlated with pain intensity[173,174]. Furthermore, it was shown that these patterns of cortical reorganisation were corrected after successful recovery from CRPS[175]. These modified sensory maps may manifest symptomatically in patients through peculiar perceptions about an affected limb, including: mismatching between how the limb looks and how the limb feels; misinterpretation of the physical position of the limb in relation to the body; the inability to form mental representations of particular limb areas; and a desire to self-amputate the affected limb[176]. Often these symptoms are not reported to clinicians and can

contribute further to the feeling of alienation and psychological stress experienced by many CRPS patients[18].

Various techniques have been used to restore a normal sensory map in an effort to decrease or resolve pain in CRPS. Therapeutic application of computer-based training exercises, which involve improving recognition of the affected limb, known as graded motor imagery (GMI), can decrease pain and swelling in CRPS patients[177]. Mirror therapy is another "brain training" exercise which uses a perpendicular mirror to create a virtual limb (a reflection of the opposite, non-affected limb) in place of the affected limb. By performing bi-lateral exercises whilst looking into the mirror, the affected limb thus has normal appearance and function. This technique has been shown to improve CRPS symptoms in early and intermediate CRPS patients, as well as patients who developed CRPS following a stroke[178,179]. Due to the low cost and relative ease with which these techniques can be applied they are now widely-practiced by clinicians in the treatment of CRPS[18]. While some patients do not respond to this type of therapy, the resolution of physical symptoms, such as oedema and inflammation, implicates central efferent signalling in the maintenance of CRPS pain i.e. central responses to altered somatosensory maps may lead to inappropriate signalling via the efferent nerve pathway, possibly producing changes in the peripheral nerve environment.

1g) The Integrative Conceptual Model of CRPS[18]

A new integrative conceptual model for CRPS pathology has now been proposed in light of the evidence discussed above and is summarised in Figure 1.4. This model includes most aspects of observed CRPS pathology and represents a combination of different mechanisms which may or may not be active in different individual patients. By identifying the dominant interacting mechanisms in the different phases and types of CRPS disease, it is hoped that distinct subsets will emerge with corresponding pathways for intervention.

Figure 1.4 – The integrative conceptual model of complex regional pain syndrome pathology.

In an attempt to combine existing theories on the etiology of CRPS an integrative conceptual model has been suggesting. **1**. Onset is triggered by some form of trauma in the affected limb. **2**. Sub clinical nerve damage contributes to immune activation. **3**. Immune infiltration and activation leads to acute symptoms such as swelling and redness. **4**. Neurogenic elements and neuro-immune crosstalk contributes to the maintenance of an inflammatory response. **5**. High levels of cell activation can lead to compartment like syndrome and limb ischemia. **6**. Persistent activation of peripheral nociceptors alters neuronal plasticity in the CNS. **7**. Changes in the CNS facilitate hyper excitable pain transmission pathways via NMDARs. **8**. Chronic hyperexcitablity in the CNS leads to cortical reorganisation and modifications in sensory maps. **9**. Following reorganisation efferent responses are altered based on modified sensory maps. **10-11.** positive feedback may then occur through changes in sympathetic signalling producing nociceptive-sympathetic coupling and altered adrenergic receptor profiles. **13**. The presence of auto-antibodies may drive these processes by linking the consequences of altered sympathetic signalling to immune cell activation/modulation.

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1h) Skin: At the Axis of Neuro-Immune Interaction

As described in detail above, the interaction between the nervous and immune systems is integral in CRPS pathology. Human skin, as the single largest interface between "self" and the external environment, is constantly exposed to exogenous stimuli which require responses from both the immune and nervous systems. The continuous application of exogenous and endogenous signals produces a dynamic environment in which cells of both systems are exposed to a wide range of immuno- and neuro-genic signalling molecules. This shared interaction with, and response to, the external environment produces an intricate connection sometimes referred to as the "brain-skin axis"[180,181].

Human skin is broadly divided into the epidermal (outer) and dermal (inner) layers (Fig. 1.5). In CRPS, many of the symptoms are linked to structures in the dermal layer, such as eccrine sweat glands, hair follicles and vascular endothelium, all of which are densely innervated by efferent autonomic nerve fibres[182,183]. The dermis also contains a wide variety of polymodal, unmyelinated nerve fibres, including nociceptors, used for sensing. The discovery of a protein ,subsequently termed protein gene product (PGP) 9.5, which is ubiquitously expressed in all nerve fibres, has allowed for the staining of epidermal nerves fibres which extend from the papillary dermis, through to the lower layers of the epidermis[184] (Fig. 1.5). PGP 9.5 has since been identified as ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), although use of the term PGP 9.5 is still commonplace in histology.

As the most peripheral sensory fibres, intra-epidermal nerve fibres (IENFs) are particularly interesting in pain research. Due to the small diameter and unmyelinated structure, IENF are only detectable using immuno-staining techniques on skin biopsy samples. Indeed, quantification of IENF density in skin has now become a standardised method for the assessment of some peripheral neuropathies[185,186]. As discussed above, it has been suggested that
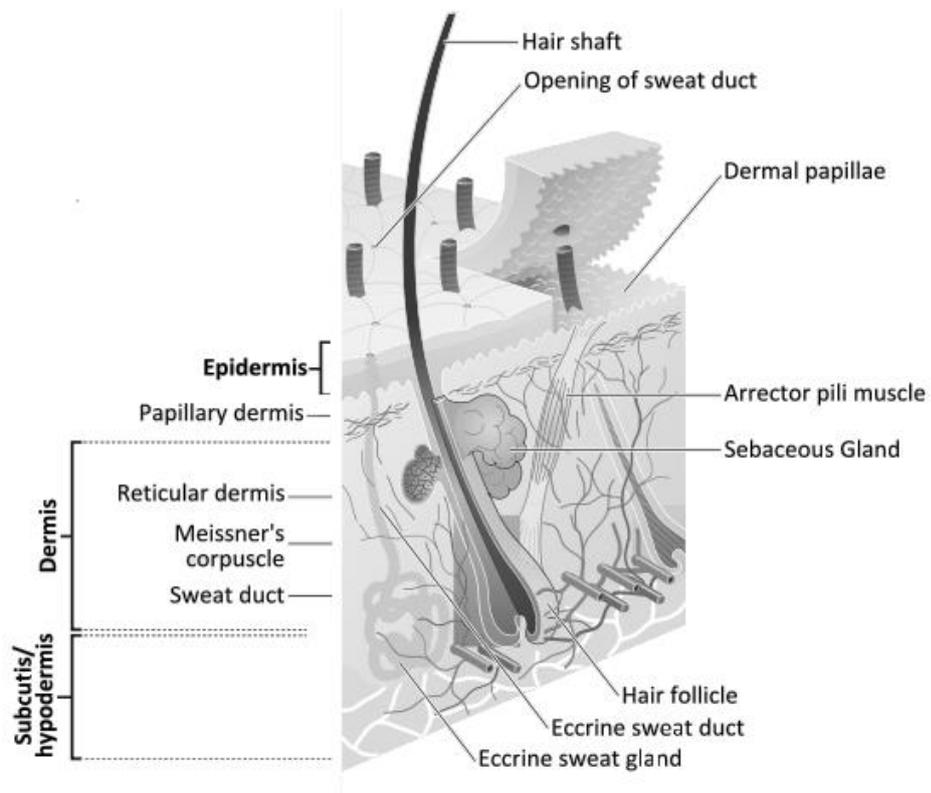
Figure 1.5 – Cutaneous structure of hairy skin.

Hairy human skin is characterised by densely innervated hair follicles and sweat glands. The Epidermis is distinctly separated from the dermis by the lowest layer of the epidermis, the stratum basale which contains basal keratinocytes, melanocytes, Langerhans cells and also the distal end of intra-epidermal nerve fibres.

CRPS may be a form of small fibre neuropathy affecting IENF, although negative data regarding CRPS symptoms and IENF density has been published, a recent review of studies in this area concluded that the commercial methods used to assess IENF as may be insufficient to detect ultra-microscopic changes and/or changes in IENF function[129,187].

Mast cells, which have been discussed above in the context or CRPS, are crucial in neuro-immune crosstalk, particularly in stress responses. Due to the ability to rapidly respond to neurogenic signalling and facilitate neurogenic inflammation, mast cells are now regarded as key mediators between efferent nerve signalling and immune responses in the skin, and also as the critical mediators of stress-induced disease flares apparent in many inflammatory skin conditions, such as psoriasis[181,188]. Although there is now a growing population of identified dermal dendritic cells thought to be important in skin immune responses, the epidermal LC is still regarded as the other dominant immune cell in skin, particularly in the context of neuro-immunology[106,189].

1i) Langerhans Cells – Origin and Function

In 1868, Paul Langerhans described a type of nerve cell which formed a contiguous network within the epidermis of human skin[190]. Over intervening years, many theories were proposed regarding LC function. We now know that LCs are epidermal dendritic cells of the myeloid lineage which constitute approximately 5% of all cells in the epidermis. By forming a continuous

network throughout the epidermis, LCs are able, by sampling and processing antigen from the environment, to act as the body's first line of defence.

As the only cell of a myeloid lineage within the epidermis, LCs are easily identified *in situ* by the expression of CD45 and/or Major Histocompatibility Complex (MHC)-II. However, the definitive feature is the expression of distinctive "tennis racket" intracytoplasmic organelles, described in 1961 and named Birbeck granules after their discoverer[191]. Although the role of these granules is still poorly understood they appear to be involved in endosomal recycling via a cell surface expressed C-type lectin receptor known as Langerin (CD207)[192,193]. Langerin is growing in prominence as a marker of LCs due to its interaction with Birbeck granules. However, it has since been described on a variety of other dendritic cells, including a newly characterised population of dermal dendritic cells[194,195]. In humans, but not in mice, LCs also express high levels CD1a, a member of the lipid antigen presenting CD1 protein family that can present microbial lipid antigens to T-cells[196]. CD1a is well described as a marker of LCs in humans although the lack of expression in mice precludes its use in many important murine models[197]. A thorough list of markers expressed on LCs can be found in the review paper by Merad, Ginhoux and Collin (see Table 1)[194].

In contrast to other hematopoietic dendritic cells, LCs precursors are seeded in the developing epidermis prior to birth[198]. In mice, this process is initiated by primitive myeloid progenitor cells known as yolk sac macrophages, which are

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also responsible for the development of embryonic glial cells, followed by an influx of fetal liver monocytes which replace the yolk sac cells[199]. Although embryonic studies are much more difficult in humans, the same paradigm has been suggested, with LCs found to be present in human epidermis at 9 weeks estimated gestational age[200,201]. Although the exact lineage of LCs in human is still not clear, murine models have shown that LC development is fms-like tyrosine kinase 3 (Flt3) and Flt3 ligand (Flt3L)-independent, but colony-stimulating factor-1 receptor (CSF-1R) dependant[202,203]. In contrast FLt3, and Flt3L are essential in the development of lymphoid tissue dendritic cells and the CSF-1R is not[202,204]. Interestingly IL-34, a CSF-1R ligand expressed by keratinocytes and neurons, was recently shown to be crucial for the maintenance of both LCs and microglia in a knockout mice model, further enforcing the links between these cell types[205]. TGF-β has also been shown to be essential in the differentiation and survival of LCs and is expressed by both keratinocytes and LCs[206,207]. TGF-β, in combination with IL-4 and GM-CSF is also essential for the *in vitro* differentiation of monocyte-derived LCs (MoLCs)[208].

Under steady state conditions most dendritic cells are continually replaced by a pool of circulating, bone marrow derived precursor cells. However, LCs are maintained by a radio-resistant precursor cell within the skin itself, a paradigm proven by the chimeric and parabiotic mice models of Miriam Merad [194,209]. A similar scenario to the persistence of host LCs in the skin of bone marrow transplanted mice is also apparent in humans, as shown by the striking

longevity of donor LCs in hand transplant patients, where donor cells survived in the graft for over 4 years[210]. Only in the presence of inflammation, such as that induced by UV radiation, are cells recruited from the circulation to repopulate the pool of epidermal LCs, a process found to be dependent on CCR2, CCR6 and macrophage colony-stimulating factor receptor (M-CSFR) expression[194,203,209].

The principle role of LCs in the skin is to sample antigens that break the skin barrier and, when stimulated by an appropriate pathogen associated molecular pattern or inflammatory cytokine signal, migrate to the draining lymph node. During migration, the LC functionally matures, by increasing co-stimulatory molecule expression and inflammatory cytokine production, into an activating dendritic cell capable of providing the second and third signals required to activate and direct naive T-cell maturation, thus tailoring the adaptive immune response to the type of antigen encountered[194]. More recently the paradigm of LC function has shifted to include a possible role in establishing peripheral tolerance. It has been suggested that by presenting self-antigen during the steady state, i.e. in the absence of an activating signal, LCs induce anergy in any self-reactive T-cells residing in the draining lymph node[211]. The novel immunoregulatory functions of LCs are still not well understood and, coupled with the increasingly complex network of dermal dendritic cells it may be some time before the intricate interactions of skin dendritic cells, and the wider immune system are fully understood[212].

1j) Langerhans Cells –Interaction with Neurogenic Factors

As described above, LCs share both a close morphological relationship to epidermal nerve fibres and a similar developmental pathway to glial cells[106,205]. Given these facts, it is perhaps unsurprising that LC function can be significantly modulated by IENFs through the release of neuropeptides and catecholamines such as NA[213]. As with mast cells in the skin, much of this research has centred on the role of stress and its modulation of cell function. A striking example of this relationship described by Kleyn *et al* is the significantly decreased LC density observed in human skin following an acute psycho-social stressor, namely the Trier public speaking test, a phenomenon that was associated with increases in epidermal CGRP and PGP9.5[214].

i. *Langerhans Cells - Modulation by Neuropeptides*

CGRP in particular has received much attention as a modulator of LC function. Treatment of LCs with CGRP increases intracellular levels of cAMP in mice and inhibits the presentation of ovalbumin (OVA) to a OVA-specific T-cell clones[106,215]. Inhibition of antigen presentation is possibly mediated by decreased expression of CD86 co-stimulatory molecules as observed in the LC-like cell line XS52 following CGRP stimulation in the presence of LPS and GM-CSF[216]. The same cell line also exhibited increased levels of LPS and GM-CSF induced IL-10, when treated with CGRP, whilst secretion of IL-1β and transcription of IL-12 mRNA were both decreased. It has since been shown that CGRP inhibits the LPS-induced production of TNF-α in both XS52 cells and

enriched LCs, a mechanism mediated, in part, through the inhibition of IκB kinase-β (IKK-β) phosphorylation and subsequent prevention of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation[217].

Despite previous observations of its effects on antigen presentation, CGRP has been shown to enhance LC antigen presentation to a Th-2 type T-cell clone[218]. Pre-treatment of primary murine LCs with CGRP dose dependently increased IL-4 production in a conalbumin specific Th-2 clone while concomitantly decreasing IFN-γ production in a keyhole limpet hemocyanin specific Th-1 clone. Furthermore CGRP inhibited the production of the Th-1 chemokines CXCL-9 and CXCL-10 in primary murine LCs and the LC-like XS106 cell line (a cell line derived from A/J mice epidermal cells to model mature LC behaviour[219]) whilst increasing the production of Th-2 chemokines CCL-17 and CCL-22, although this interaction appears to be independent of the primary CGRP receptor[218].

Similar modulation of LC function has been described for the neuropeptides, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating peptide (PACAP), including suppression of antigen presentation and inhibition of inflammatory cytokine secretion[220,221]. In contrast to the Th-2 polarising effect of CGRP on LCs, VIP and PACAP produce a Th-17 bias in LCs when presenting antigen to T-cells[222].

Despite its important role in neurogenic inflammation and the observed expression of NK1 receptors on LCs, SP has only been shown to suppress LC

function at very high concentrations ($10^{-4} - 10^{-5}$ M) in a mixed cell system, where indirect effects are likely [223].

ii. Langerhans Cells - Modulation by Catecholamines

LCs isolated from mouse epidermis, XS52 cells (a cell line similar to xs106 but derived from fetal BALB mice epidermis[219]) and XS106 cells have been shown to express the α1a- and β2-ARs mRNA and *in vitro* stimulation of unseparated epidermal cell suspensions with adrenaline and NA decreased the level of antigen presentation to a specific T-cell clone[224]. Furthermore isolation and *ex vivo* stimulation of murine epidermal cells suspensions with NA suppressed delayed type hypersensitivity in pre-immunized mice, an effect blocked by the β2-AR specific antagonist ICI 188,551 but not by the α-AR specific antagonist phentolamine[224]. The immunosuppressive effects of β2-AR stimulation on LCs was further expounded by the observation that locally, and even distally, administered adrenaline supressed contact hypersensitivity to an epicutaneously administered hapten[224]. Pharmacological blockade of NA release, via injection with the ganglionic blocker pentolinium, increased levels of IFN-γ in draining lymph nodes and increased contact hypersensitivity to fluorescein isothiocyanate (FITC) in mice[225]. ICI 188,551-mediated blockade of β2-ARs during the induction of sensitisation to FITC in mice also decreases LC migration and increases hypersensitivity[226]. Inhibition of LC migration and contact hypersensitivity was also reported following α1b-AR blockade; however, this affect was later attributed to local release of NA acting on β2-

ARs[226,227]. β2-AR agonists also influence LC chemotaxis by increasing responsiveness to CCL-19 and CCL-21, a process mediated by IL-10 secretion[213].

Since LCs have now been shown to be functionally redundant in contact hypersensitivity responses, and many of the above studies did not use a marker specific for epidermal dendritic cells it cannot be ruled out that some of these effects may be mediated through a population of dermal dendritic cells as opposed to LCs[228,229].

1k) Langerhans cells – Relevance to a pain mechanisms

In the absence of an activating signal, LCs routinely sample the epidermal environment and migrate to draining lymph nodes in a process thought to induce peripheral tolerance[212]. Although it is well established that LCs are capable of secreting inflammatory cytokines with the potential to sensitise peripheral nociceptors, in most cases this induction requires some form of stimulus, such as the recognition of a pathogen associated molecular pattern[230–232]. As established above, neurogenic signalling can significantly influence the behaviour of LCs and so this presents an interesting area of study in CRPS and other chronic pain conditions, where dysregulated nerve signalling is suspected as a key contributor to pathology. This relation is revealed by the loss or interruption of nerve signalling in the epidermis, which can significantly alter normal LC function, to the extent that artificially induced denervation of the epidermis (destruction of IENF) has been reported to both abolish, and enhance contact hypersensitivity in mice[233,234].

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As previously mentioned, a small case study has suggested that there may be elevated levels of LCs in the skin of CRPS patients[107]. Oaklander *et al* reported no difference in LC density in patients with post-herpetic neuralgia, when compared to healthy controls, but patients with painful diabetic small fibre neuropathy were shown to exhibit increased LC densities in affected tissues, a finding which negatively correlated with IENF density[235,236]. A striking observation from studies involving the denervation of epidermis, particularly in models based on nerve injury as opposed to denervation using neurotoxin, was the expression of UCH-L1 by LCs as measured by immunofluorescent staining with anti-PGP9.5 antibodies[237]. Of particular relevance to this research is that epidermal denervation, and subsequent UCH-L1 expression by LCs is often seen in models of neuropathic pain, including nerve transection and painful neuropathy induced by the chemo-therapeutic treatment paclitaxel[237–239].

Expression of UCH-L1 in LCs and mature dendritic cells has been related to cell maturation in the absence of nerve fibres and could possibly represent altered processing of internalized antigen or more generally altered protein synthesis within the cell[240–242]. Interestingly, a related ubiquitin C-terminal hydrolase, UCH37, has been shown to regulate TGF-β signalling, an important pathway in LC development *in vivo*[243]. One possible reason for the observed expression of UCH-L1 in LCs is through the phagocytosis of products of neuronal degradation as opposed to *de novo* synthesis, a prospect which might suggest a potential auto-immune element to LC function in these models and which would support

a role for UCH-L1 in altered antigenic processing. However, increased levels of UCH-L1 transcripts in denervated skin extracts, where LC were the only cells that stained positive for a PGP9.5 antibody, suggests that UCH-L1 expression was a result of *de novo* synthesis from within the cells themselves[238]. It has also been established that isolation of LCs from healthy epidermal tissues, effectively denervating the cells, induces UCH-L1 expression whereas, LCs differentiated from $CD34^+$ cord blood precursor cells in the absence of nerves, do not express UCH-L1[241].

An interesting aspect of LC-nerve interaction is that during the growth of new nerve fibres into the epidermis following epidermal denervation, the extension of fresh IENF appeared to be directed towards LCs[238]. Furthermore supernatants from LPS-stimulated LCs and XS52 cells induced the differentiation of PC12 pheochromocytoma cells into sympathetic neuron-likes cells, a process mediated by IL-6, NGF and basic fibroblast growth factor (bFGF)[244]. Both XS52 and XS106 cells were shown to express mRNA for the neurotrophins ciliary neurotrophic factor and neurotrophic factor 4/5, both of which contribute to nerve cell growth and survival[245]. In response to these findings, a hypothesis has been suggested whereby denervated LCs respond to the absence of nerve fibres by secreting neurotrophic factors in a process which requires the *de novo* production of UCH-L1. However, this hypothesis remains untested and the importance of UCH-L1 expression, and the signal (or lack thereof), which induces its expression in LCs is yet to be described.

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1l) UCH-L1

As described briefly above UCH-L1 is an ubiquitin C-terminal hydrolase and a member of a family of enzymes generally involved in the recycling of free ubiquitin from the ubiquitin protein degradation system. Identified as a "brain specific protein" in 1981, the newly discovered protein, designated PGP9.5, received significant attention due to its constitutive expression in neuronal cells[246]. Despite being labelled as "neuron specific" UCH-L1 is merely expressed in much greater concentrations in neuronal tissue, constituting an estimated 1-2% of total soluble brain protein, but can be detected in a variety of other tissues[240]. Of note, UCH-L1 expression has been described in Schwann cells, following nerve transection injuries, and in fibroblasts during wound healing[247,248].

The ubiquitin system to which UCH-L1 is related, is a post-translational modification system responsible for the ubiquitination, and thus altered cellular processing, of protein substrates. Generally regarded as a proteolytic system, protein ubiquitination can result in a huge variety of different outcomes including functional modifications or altered intracellular transport[249]. Deubiquitinating enzymes (DUBs), to which UCH-L1 belongs, are responsible for removing bound ubiquitin from ubiquitinated substrates. UCH-L1 is thought to remove ubiquitin by hydrolysing ubiquitin-lysine bonds present in ubiquitin-isopeptides thought to be the end products of proteolysis, thus recycling ubiquitin after protein degradation[240]. However, the low hydrolytic

activity of UCH-L1 compared to other ubiquitin hydrolases has led to a variety of different theories regarding its primary function, including the stabilisation of mono-ubiquitin, the processing of poly-ubiquitin and ubiquitin ligase activity[250–252].

UCH-L1 received significant attention following the identification of a missense mutation in the UCH-L1 gene in a German family with Parkinson's disease (PD)[253]. The mutation resulted in a shortage of free ubiquitin and since PD is associated with the accumulation of ubiquitin-conjugated proteins, this suggested UCH-L1 might play an active role in disease pathology. The discovery of UCH-L1 inhibitors such as LDN-57444, also allowed for the specific blocking of UCH-L1 function in order to understand its role in pathology, work performed mostly in the context of PD[254]. Treatment of UCH-L1 expressing neuroblastoma SK-N-SH cells with the inhibitor LDN-57444 induced cell apoptosis by triggering endoplasmic reticulum stress, possibly through the accumulation of incorrectly folded proteins[255]. However, treatment of UCH-L1 transfected cell lines with the same inhibitor at a lower dose only inhibited UCH-L1 function with no induction of apoptosis[256].

1m) Hypothesis

The evidence discussed above implicates the immune system in CRPS disease pathology in the acute phase of the disease, but evidence suggests that inflammation resolves during disease progression. It is unknown if immune dysregulation persists in CRPS affected tissue, or if other immune-mediated mechanisms contribute to long term disease maintenance.

Given the documented changes in central nervous processing in longstanding CRPS, understanding the relationships between immune cells and the nervous system in longstanding disease may assist in the development of more effective therapeutic strategies, or identify new biomarkers for disease diagnosis

The hypothesis to be tested in the thesis, therefore, is that "**Immune cell dysfunction and interaction with the nervous system contributes to the pathology of long-standing CRPS**".

1n) Thesis Aims

This thesis aims to interrogate the above hypothesis by defining the interactions between immune cells and the nervous system in longstanding CRPS patients. To achieve this aim the project was divided in to several specific thesis objectives:

- Quantify and analyse the immune cell population within CRPS affected and non-affected skin in patients with long standing disease, with specific focus on epidermal LCs and dermal mast cells.

- Determine the expression and function of UCH-L1 in MoLCs in order to explain the relevance of this protein to chronic pain development and/or maintenance.

- Define the role of immune cell adrenergic receptor expression in CRPS disease and the potential of auto-antibody mediated adrenergic receptor binding on immune cells.

CHAPTER 2: Materials and Methods

2A) Materials

Table 2.1 Materials used - media and chemicals

Media & chemicals	Supplier
Glycerol Sodium dodecyl sulphate Tris	Fischer Scientific (Loughborough, UK)
RPMI 1640 (2mM L-gluatamine)	Gibco (Paisley, UK)
Phosphate Buffered Saline (PBS) tablets	Promega (Southampton, UK)
Acetone Ammonium chloride (NH_4Cl) Ammonium persulphate (APS) Bovine Serum Albumin (BSA) Dithiothreitol (DTT) Dimethyl sulfoxide (DMSO) Ethanol (lab grade) Ethylenediaminetetracetic acid (EDTA) Non-enzymatic cell dissociation solution Paraformaldehyde Saponin Tetramethylethylenediamine (TEMED) Tween-20	Sigma (Poole, UK)
Hydrochloric acid (HCl)	VWR International (Leicestershire, UK)
Polyacrylamide	Severn Biotech (Kiddiminster, UK)

Table 2.2 Materials used – cytokines, stimulants and inhibitors

Cytokines, cell stimulants & chemical inhibitors	Supplier
Recombinant human IL-1β	Invitrogen (Paisley, UK)
Phorbol 12-myristate 13-acetate (PMA) Recombinant human IL-6 Bacterial lipopolysaccharide (LPS) Substance P acetate salt hydrate (SP) Norepinephrine bitartrate salt (NA) Human calcitonin gene related peptide (CGRP) Phenylephrine hydrochloride (PE) LDN-57444 UCH-L1 inhibitor	Sigma (Poole, UK)
Recombinant human CCL-19 Recombinant human GM-CSF Recombinant human IL-4 Recombinant human TGFβ Recombinant human TNFα	R & D Systems Europe (Abingdon, UK)

Table 2.3 Materials used – antibodies and chemical dyes

Antibodies and dyes	Supplier
Mouse anti-human CD1a (ab24055) Rat anti-human HLA-DR (ab10544) Rabbit anti-human α1a-adrenergic receptor (ab54730) Rabbit anti-human β2-adrenergic receptor (ab61778) FITC Goat anti-mouse IgG (Fab2) Mouse anti-human actin	Abcam (Cambridge, UK)
Rabbit anti-human PGP9.5 (7863-0504)	AbD Serotec (Oxford, UK)

HRP donkey anti-rabbit IgG	Amersham Life Sciences (Bucks, UK)
Citifluor anti-fadent mountant AF-1	citifluor (London, UK)
R-PE goat anti-rat IgG R-PE goat anti-rabbit IgG Mouse IgG2a isotype control AF-488 mouse anti-human CD1a (CD1a20) Rabbit primary antibody isotype control	Invitrogen (Paisley, UK)
1% toluidine blue solution	American Mastertech (Lodi, CA, USA)
Mouse anti-human CD86 (discontinued)	PharMingen (Cowely, UK)
Per CP Mouse anti-human CD14 (FAB3832C) Mouse anti-human IL-1β (MAB6964)	R & D Systems Europe (Abingdon, UK)
Mouse anti human pan-α1-adrenergic receptor (A270) Ponceau Stain	Sigma (Poole, UK)

Table 2.4 Materials used – Pre-optimized laboratory kits

Self-contained laboratory kits	Supplier
TaqMan® Gene expression assays: Hs00171263_m1 - α 1 b Hs00169124_m1 - α 1 a Hs00169865_m1 - α 1 d Hs00265090_s1 - α 2 b Hs03044628_s1 - α 2 c Hs00265081_s1 - α 2 a Hs00240532_s1 - β 2 Hs99999903_m1 - β actin control TaqMan® Gene expression mastermix	Applied Biosystems (Paisley, UK)

Dynabeads® Untouched™ human monocytes Dynabeads® sheep anti-mouse IgG Luminex® Cytokine Human 10-plex panel Luminex® IL-17 human singleplex bead kit Luminex® MIP-1α human singleplex bead kit Luminex® IFNα human singleplex bead kit Superscript III first strand cDNA synthesis kit	Invitrogen (Paisley, UK)
Immoblion western chemiluminescence HRP-substrate	Millipore (Herts, UK)
RNeasy mini kit	Qiagen (Crawley, UK)

Table 2.5 Materials used – other miscellaneous

Other materials	Supplier
Enhanced chemiluminescence hyperfilm	Amersham Life Sciences (Buckinghamshire, UK)
Polymorphprep® Lymphoprep®	AxisShield (Cambridge, Uk)
dNTPs	Bioline (London, UK)
Biotinylated protein ladder detection pack	Cell Signalling Technology (Massachusetts, USA)
Custom Primers	Eurofins MWG Operon (Ebersberg, Germany)
Lithium heparin green top vacutainers Z-serum clot activator gold top vacutainers	Grenier Bio-one (Gloucestershire, Uk)

6mm punch biopsy pack Optimo suture pack sliver	Healthcare Equipment Supplies (Surrey, UK)
RNase OUT	Invitrogen (Paisley, UK)
Polyethylene Terephthalate (PET) millicell hanging well inserts 5 µm Polyvinylidene fluoride (PVDF) membrane	Millipore (Hertfordshires, UK)
Q5™ High-Fidelity DNA Polymerase	New England Biolabs (Hertfordshire, UK)
Human Heart cDNA (Biobanked)	Primer Design (Hants, UK)
Random primers	Promega (Southampton, UK)
Marvel non-fact milk powder	Tesco

2B) Methods

i. Patient Numbers

10 CRPS patients, as defined by the Budapest criteria[6] (table 1.1), were recruited to this study. Controls recruited were 5 Fibromyalgia patients, as defined by the American College of Rheumatology, and 9 healthy controls. Patients were selected from those seen in the clinics of the principal investigator, Dr Andreas Goebel, and other consultants at The Walton Centre. Ethical approval for the study was obtained from the North West 8 Regional Ethic committee - Greater Manchester East (09/H1013/56), and written informed consent was obtained from all participants.

ii. Biopsy Sampling and Processing

Two 6 mm epidermal punch biopsies were taken from each patient and volunteer at the Jefferson Day Ward within The Walton Centre NHS Hospital. The biopsies were taken from the affected limb of CRPS patients and disease controls, whereas those from healthy volunteers were taken from matched limbs. A further two biopsies were taken from each CRPS patient from a non-affected limb, as a contra-lateral control to facilitate comparison between affected and non-affected limbs in the same patient (Fig 2.1). One biopsy from each pair was sent to the Buxton Histopathology Ward within the hospital for formalin fixation and batch processing for histochemical analysis. The remaining biopsy was taken to The School of Biological Sciences, at the University of Liverpool for epidermal sheets processing (see below). In 4 cases, biopsies designated for processing for epidermal sheets were also bisected, and half the tissue used for T-cell isolation (see below)
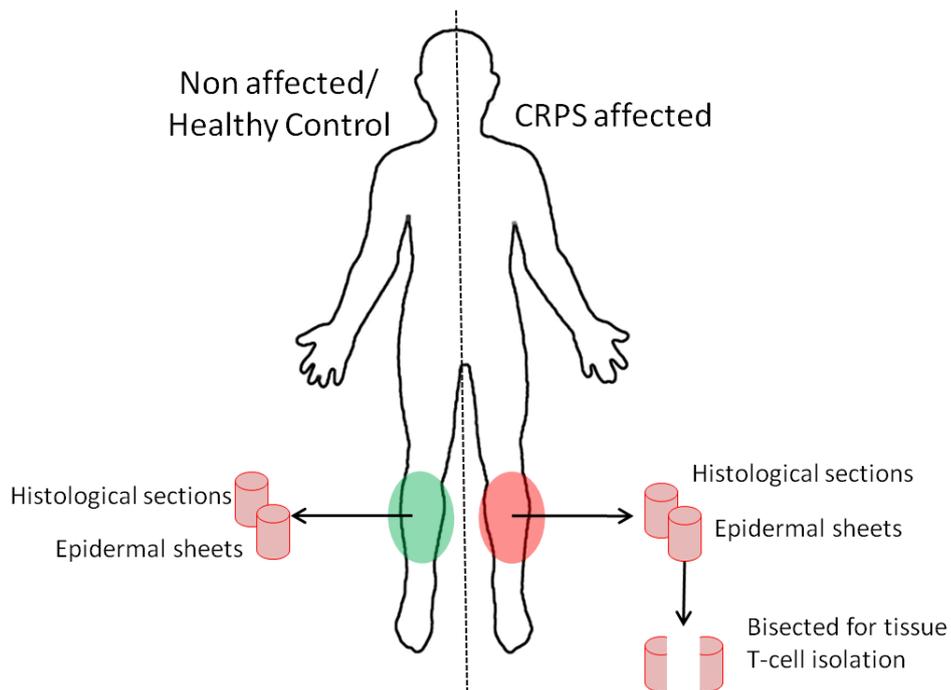
Figure 2.1 – Schematic of Biopsy material and usage

Biopsies were taken from the affected limb of CRPS patients and disease controls. Biopsies in healthy controls were taken from matched limbs. Two further biopsies were taken from each CRPS patient from contra-lateral locations on non-affected limbs to facilitate comparison within each individual. Following biopsy taking tissue was fixed for histological analysis or immediately processed for epidermal sheet staining (see protocols below). In 4 cases biopsies from CRPS affected were bisected with half of the material used for tissue T-cell isolation in addition to normal epidermal sheet processing

iii. Blood Sampling and Processing

30 ml of blood was taken from each patient and volunteer (during the biopsy taking at the Jefferson Day Ward) using Z-serum clot activator vacutainers (gold top) for serum isolation. A further 30 ml of blood was taken from 3 CRPS patients and used for peripheral blood mononuclear cell (PBMC) isolation using lithium heparin vacutainers (green top). Blood taken from healthy donors taken at the University of Liverpool, was approved by the University Committee for Research Ethics and written informed consent was obtained in all cases.

iv. Serum Isolation

Blood taken for serum isolation was drawn into gold top vacutainers containing a z-serum clot activator and a separation gel specifically designed for serum isolation. After allowing the blood to clot for approximately 10 min the tubes were centrifuged at 1300 g for 15 min and the serum aspirated from the top of the tube. Isolated serum was then stored at -80°C for investigation of serum antibodies at a later date.

v. Peripheral Blood Cell Isolation

Blood was collected in heparinized tubes and cells isolated by density-dependent centrifugation. Whole blood was diluted 1:1 with 0.9% NaCl and layered onto Lymphoprep media for purification of lymphocytes according to

the manufacturer's guidelines. Following isolation, cells were counted using an automated cell counter (Beckman-coulter) and resuspended in RPMI 1640 media at a cell concentration of 1×10^6 ml^{-1}. Mixed cell populations were also isolated by extracting the mononuclear layer of Polymorphprep preparations (a one-step density-dependent centrifugation procedure for neutrophil isolation). Mononuclear cells from polymorph preparations were then washed and resuspended in RPMI 1640 media and subject to a second density-dependent centrifugation using Lymphoprep, as above. Mixed mononuclear cell populations were then used in protocols as described below or subject to the monocyte purification protocol detailed below.

vi. Isolation of untouched™ monocytes by negative selection using Dynabead® magnetic beads

Purified blood monocytes were isolated by negative selection using a pre-formulated untouched™ human monocytes magnetic bead isolation kit according to the manufacturer's guideline (Invitrogen). Briefly, peripheral blood mononuclear cells (minimum 5×10^7 ml^{-1}) were incubated with a premixed antibody cocktail and blocking solution for 20 min at 4^0C. Cells were then washed and resuspended in buffer containing magnetic Dynabeads® and incubated for 15 min at 4^0C with tilting and rotation. Finally, bead-bound cells were vigorously resuspended by repeated pipetting and separated by a magnet field. Unbound cells were then removed from the solution and used as

negatively selected monocytes for further applications and the bead bound were cells discarded.

vii. Differentiation of Monocyte Derived Langerhans Cells (MoLCs)

MoLCs were generated using previously described methods[208]. Negatively selected monocytes were resuspended at 1×10^6 cell ml^{-1} in RPMI 1640 supplemented with 10% FCS and 1% Penicillin-Streptomycin (complete media) and incubated with TGF-β2 at 10 ng ml^{-1}, IL-4 at 20 ng ml^{-1}, and GM-CSF at 100 ng ml^{-1}. They were then plated in 1 ml aliquots in a 24 well plate before 7 d incubation at 37^0C in a humidified incubator with 5% CO_2. Cells were fed on day 4 by removing 50% of media and replacing with cytokine-supplemented media as above.

viii. Cell incubations

Cells were incubated as detailed in Table 2.6. In order to investigate modulation of UCH-L1 expression in MoLCs, day 7 cultures were incubated with a variety of neurogenic factors with known effects on LC function. The functional effect of UCH-L1 expression was explored through incubation with a UCH-L1 inhibitor followed by cell activation using cytokines and LPS and a variety of functional tests, described below. PBMCs were stimulated with LPS and cytokines to induce changes in adrenergic receptor subtype expression, assessed by western blotting. Finally, negatively selected monocytes were

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stimulated with LPS and/or PE to examine the known synergistic effect on IL-1β production. Patient or healthy IgG fractions were then used in place of PE to study potential adrenergic receptor binding by auto-antibodies present in IgG.

Table 2.6 - Experimental protocols for cell incubations

Cell type	Stimulant	Diluent	Concentration	Conditions	Ref.
MoLC (day 7)	CGRP	Pure H_2O	100 nM	24 h ; 37^oC; 5% CO_2	218
	NA		100 nM		257
	SP		10 µM		258
MoLC (day 6)	LDN-57444	DMSO	10 µM		255,256
MoLC (day 7)	TNFα	PBS	100 ng ml^{-1}	18 h ; 37^oC; 5% CO_2	259
	LPS		50 ng ml^{-1}		257
	IL-1β		100 ng ml^{-1}		259
PBMCs	LPS	PBS	100 ng ml^{-1}	18 h ; 37^oC; 5% CO_2	
	TNFα		10 ng ml^{-1}		
	IL-1β		10 ng ml^{-1}		
	IL-6		10 ng ml^{-1}		
Negatively selected monocytes	LPS		25 ng ml^{-1}	3-6 h 37^oC; 5% CO_2	260
	PE	Pure H_2O	10 µM		
	IgG	RPMI 1640	7-16 mgml^{-1}		

ix. Flow Cytometry

Preparation and staining of cells for flow cytometric analysis is described in Table 2.7. Cells were stained in 100µl aliquots using sterile PBS with 2% BSA (staining buffer). Each staining protocol was followed by a 10 min wash step in staining buffer. Cells were resuspended in 250 µl (at approximately 0.4x10^{-6} ml^{-1}) prior to analysis using a Guava Easycyte bench top flow cytometer (Millipore). Unstained cells were used to define baseline fluorescence and

forwards and side scatter gates were used to exclude extracellular debris. CD1a+ cells were defined as MoLCs when recording co-expression of PGP 9.5, HLA-DR and CD86 proteins. The isotype controls were also carried using indirect staining protocols with mouse IgG and rabbit IgG in place of specific primary antibodies at matched concentrations. Analysis of flow cytometric data was completed using Express Pro that is provided within the Cytosoft software package suite.

Table 2.7 – Experimental protocols for flow cytometry

Protocol	Preparation	1° Antibodies	Incubation	2° Antibodies	Incubation
MoLC PGP 9.5 expression	Fixed in 2% PFA on ice 10 min. Permeabilized using 0.5% saponin for 10 min.	Mouse anti-human CD1a at 40 µg ml^{-1}	1 h in staining buffer +0.1% saponin	anti-mouse AF-488 1:200	1 h in staining buffer +0.1% saponin
		Rabbit anti-human PGP 9.5 at 1:200		anti-rabbit R-PE at 1:200	
MoLC activation marker expression	live cells	AF-488 conjugated mouse anti-human CD1a	1 h in staining buffer	Directly conjugated	
		rat anti-human HLA-DR at 10 µgml^{-1}	1 h in staining buffer	R-PE goat anti-rat IgG at 1:200	1 h in staining buffer + AF-488 CD1a (see
		rabbit anti human		R-PE goat anti-rabbit	

		CD86 at 1:100		IgG at 1:200	above)
PBMCs/ Pure monocytes	live cells	Mouse anti human pan-α1-adrenergic at 1:100	1 h in staining buffer	anti-mouse AF-488 1:200	1 h in staining buffer
		PerCP conjugated mouse anti-human CD14	1 h in staining buffer	Directly conjugated	

x. Chemotaxis Assay

MoLCs at day 6 of culture were pre-treated with either with LDN-57444 or a vehicle control for 24 h before an 18 h incubation with TNFα or IL-1β (table 2.6). Following stimulation cells were counted and resuspended in complete media at 2×10^{-6} cells ml^{-1}. In parallel, transwell chambers were prepared in duplicate by aliquoting 600 μl of CCL-19, prepared in 0.2% BSA at 200 ng ml^{-1}, or vehicle control into a 12 well plate. 5 μm PET well inserts were placed into each well and incubated at 37^0C and 5% CO_2 to equilibrate for 30 min. 100 μl of the pre-stimulated cells were then aliquoted, in duplicate, into each well chamber as appropriate and the plate returned to the incubator for 3 h. Following incubation, the number of migrated cells was counted using an automated cell counter (Beckman-coulter).

xi. Western Blotting

Protein lysates were generated using boiling Laemmli buffer (containing dithiothreitol). Lysates were then loaded onto SDS-polyacrylamide gels alongside biotinylated protein markers, and run for 60-75 min at 200 V. Proteins were transferred onto PVDF membranes for 1 h at 100 V. After transfer, membranes were incubated in blocking buffer (TBS (10mM Tris, 150mM NaCl, pH 8.0) 5% Marvel (w/v), 0.075% Tween20) for 1 h at room temperature to block non-specific protein binding. Membranes were then washed 3x for 5 min in wash buffer (TBS (10 mM Tris, 150 mM NaCl, pH 8.0), 0.075% Tween20) and incubated in antibody buffer (TBS (10 mM Tris, 150 mM NaCl, pH 8.0) 5% BSA, 0.075% Tween20) with primary antibodies, as indicated in Table 2.8, overnight at 4°C. Membranes were then washed 2x 30 s, 2x 5 min and 1x 15 min in wash buffer before incubating with antibody buffer containing secondary antibody (HRP conjugated donkey anti-rabbit) and a marker antibody (HRP conjugated anti-biotin) for 1 h at room temperature. Membranes were then washed 2x 30 s, 2x 5 min and 1x 15 min in wash buffer. Protein detection was performed using Amersham ECL detection reagents and hyperfilm.

Table 2.8 - Experimental protocols used for western blotting

Cell lysate	Gel %	1° Antibodies	Concentration	Protein size on product insert
PBMCs	9%	Rabbit anti-human α1a-AR	1:5000	80kDa
Pure monocytes	12%	Rabbit anti-human IL-1β	2 µg ml^{-1}	32kDa
CD1a+/- selected	15%	Rabbit anti-human PGP 9.5	1:5000	27kDa

xii. RNA isolation & Reverse Transcription PCR

RNA was isolated using the RNeasy® mini kit from Qiagen and cells lysed by repeat pipetting using a 20 gauge needle and syringe. cDNA was synthesised using the Superscript® III first strand synthesis kit from Invitrogen and random primers. A minimum of 5ng of RNA template, measured using a NanoDrop (Thermo-Fischer), was used in each reverse transcription reaction with 1µl of dNTPs (10 mM) and sterile water to a total of 13µl. Reaction mixtures were incubated at 65°C for 5 min followed by 1 min on ice before addition of 5x first strand buffer, 0.1M DTT, RNase OUT RNase inhibitor and the Superscript III reverse transcriptase, as per the manufacturer's instructions. Following a 5 min incubation at room temperature cDNA was then synthesised by incubating reaction mixes in a Thermo PX2 thermal cycler at 50°C for 60 min. Reactions were inactivated by heating to 70°C for 15 min and cDNA fractions were stored at -80°C.

xiii. Standard PCR

Standard PCR was performed using Q5® high fidelity DNA polymerase according to the manufacturer's instructions. Briefly, 5x Q5 reaction buffer, 10mM dNTPs, and Q5 polymerase were mixed with 10µM forward and reverse primers (detailed in Table 2.9) and a minimum of 5ng of template DNA before adjusting to 50µl with nuclease free water. Thermocycling conditions used for standard PCR were:

Stage 1 - denaturation - 98°C for 30 sec
Stage 2 - denaturation - 98°C for 10 sec
                annealing   - 60°C for 30 sec   } 30 cycles
                extension   - 72°C for 30 sec
Stage 3 – extension   - 72°C for 2 min
Stage 4 – Hold   -4°C

Table 2.9 – Primer pairs used for standard PCR

Gene	Sequence	Product	Ref.
UCH-L1 –Forward *UCH-L1* -Reverse	5'-CTGTGGCACAATCGGACTTA-3' 5'-CCATCCACGTTGTTAAACAGAA-3'	243	[261]
CD207 –Forward *CD207* -Reverse	5'-CAACAATGCTGGGAACAATG-3' 5'-GGGGAAGAAAGAGGCATTTC-3'	203	[262]
GAPDH –Forward *GAPDH* -Reverse	5'-CTCAACGACCACTTTGTCAAGCTCA-3' 5'-GGTCTTACTCCTTGGAGGCCATGTG-3'	106	

xiv. Quantitative PCR

qPCR was performed using pre-optimised TaqMan® gene expression assays (applied biosystems) and mastermix, according to the manufacturer's instructions. Briefly, reactions were made up in opaque 96-well plates using up to 4 μl of cDNA template (minimum 1ng) combined in with 1 μl of TaqMan® gene expression assay mix and 10 μl of the TaqMan® gene expression master mix. RNase-free water added up to a total volume of 20 μl. Each reaction was performed in triplicate using the pre-optimized assays described above (Table 2.4). Following setup, plates were sealed and centrifuged for 1 min before loading into a LightCycler 480® qPCR instrument (Roche) for real-time analysis. CT values were generated automatically using Light Cycler 480 software (Roche) and used to calculate relative gene expression using the mean normalised expression method ($\mu(2\wedge Target)/\mu(2\wedge Ref)$) with β-Actin as the internal control[263]. Thermocycling conditions were defined by the manufacturer and were as follows:

Stage 1 - Hold   - 50°C for 2 min

Stage 2 - Hold   - 95°C for 10 min

Stage 3 - Cycle   - 95°C for 15 s

            - Cycle - -60°C for 1 min   } 40 cycles

xv. Epidermal Sheet Separation and Langerhans Cell Staining

Epidermal sheets were prepared as previously described [214]. Briefly, whole biopsies were washed twice in PBS to remove excess blood, and then placed into 2 mmol.L^{-1} EDTA and incubated at 37°C for a minimum of 2 h. Forceps were then used to gently separate the epidermis from the dermis. The epidermal sheet was then washed in phosphate buffered saline (PBS) and fixed in acetone for 20 min at −20°C. After fixing, the sheet was washed in PBS and incubated for 1 h at room temperature with an anti-CD1a monoclonal antibody diluted to 10 μgml^{-1} in PBS containing 0.1% bovine serum albumin (BSA). The epidermal sheet was then washed in PBS and incubated for 1 h at room temperature with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse polyclonal antibody diluted 1:100 in 0.1% BSA/PBS. Finally, sheets were washed in PBS, mounted on microscope slides using Citifluor anti-fadent mounting solution and sealed with nail varnish. The same method was used but instead using an unconjugated rat anti-human HLA-DR primary at 10 μgml^{-1} in PBS containing 0.1% bovine serum albumin, and a R-phycoerythrin-conjugated goat anti-rat secondary diluted 1:100 in 0.1% BSA/PBS to achieve dual staining to confirm the professional antigen presenting cell phenotype. A mouse IgG isotype control was also used, as above. LCs were visualised using an LSM710 confocal microscope (Zeiss) with a 40x fluor lens. Imaging was performed using a 488nm laser excitation with a 488nm dichroic mirror and 500-550nm band pass filter for emission detection. Multiple composite images (tilescans) were used to match the surface area covered in previous studies

(constituting approximately 28 fields at 350µm^2 per field)[264]. Vertical sections through the sheet (Z-stacking, 20 sections at 3 µm intervals) were then compiled to allow for 3D image construction and quantification of cells throughout the epidermis. Cell density was then calculated as LC per mm^2 of epidermis using the Volocity 3D (Perkin Elmer, USA) imaging software package (counting objects greater than 10µm^3 with a fluorescence value greater than background), and manual counting (acetate overlays on PC monitor to mark off counted cell) was performed to validate cell densities. Cells were also visually assessed for their size and the number of dendritic processes. Increases in cell size and dendrite retraction can be indicative of cell activation[265].

xvi. Immunohistochemistry

Formalin biopsy tissues were bisected and paraffin-embedded. Three non-consecutive 5 µm sections were then cut from the fixed sections and adhered on to glass slides. Prior to staining, the tissue sections were de-waxed in xylene and rehydrated through a series of ethanol solutions (2X 100% ethanol, 2X 95% ethanol). Haematoxylin and eosin (H&E), and a 1% toluidine blue solution were used to stain cellular infiltration. Sections were then dehydrated through increasing ethanol concentrations (as above) before submersion in xylene and mounted using DPX. H&E sections were then blinded and randomized before scoring/counting. Slides were assigned a score based on the level of cell infiltration into the tissue compared to normal skin ('histological assessment score', see Appendix Table 1). Scoring was performed by two independent

assessors, including a qualified dermatologist, and the mean taken, where scores did not concur. Kappa values were calculated using GraphPad. Inter-observer weighted kappa was "moderate" (κ=0.525). Intra-observer weighted kappa values were both "good" (κ=0.765, and κ=0.685). Mast cells were quantified by counting metachromatic granular cells in toluidine blue-stained sections. Toluidine blue is a chemical stain used to identify mast cells, based predominantly on the presence of heparin within mast cell granules. 10 random fields from the papillary dermis of each section were assessed at x400 magnification (0.2mm x 0.2mm field size) using an Axiovert100 microscope and data recorded as mean mast cell density per mm^2 (Zeiss).

   xvii. Tissue T-cell Isolation and analysis

Tissue T-cell isolation and phenotyping was performed by Dr. J. Farrell and Dr. D. Naisbitt of the University of Liverpool Department of Molecular and Clinical Pharmacology. In 3 cases, biopsy tissue taken from affected and non-affected skin for the isolation of epidermal sheets, were bisected and used for T-cell isolation also. Tissue was minced with a scalpel, and cultured in a single well of a 24 well tissue culture plate for 2-3 d in complete media (RPMI 1640 media supplemented with pooled heat-inactivated human AB serum (10%, v/v), HEPES (25 mM), L-glutamine (2 mM), transferrin (25 μg/ml), streptomycin (100 μg/ml) and penicillin (100 U/ml), supplemented with 100U/ml IL-2. The content of the wells was then aspirated and passed through a 50 μm filter to remove debris. The resultant cell solution was centrifuged to pellet the cells,

resuspended and cultured for 14 d with complete medium supplemented with 5µg/ml phytohemaglutinin (PHA), IL-2 and irradiated (45Gy) allogeneic peripheral blood mononuclear cells (PBMC) cells to expand T-cell numbers. Cells were then collected for cloning by limiting dilution (equivalent to 0.5 cells per well) and repetitive mitogen stimulation. The single cells were stimulated on day 0 and 14 with 5 µg/ml PHA, irradiated (45Gy) allogeneic PBMC $5x10^4$/well and IL-2. Twenty four clones from each biopsy were expanded for subsequent CD4/CD8 phenotyping by flow cytometry, and the analysis of cytokine secretion. To measure cytokine secretion profiles, individual clones were incubated in the presence and absence of PHA (5 µg/ml) and subjected to IFN-γ and IL-13 ELIspots (Mabtech, Sweden). In total, 72 clones from affected and non-affected skin were assessed. Cytokine secretion was recorded as spot-forming units (SFU) where one spot represents a single clonal cell secreting the specific analyte i.e. IL-13 or IFN-γ. The mean number of SFU per clone was then used to extrapolate the cytokine profile of the original tissue resident T-cell, and thus determine the T-cell phenotype within the biopsied tissue. IL-13 and IFN-γ were selected as key markers for Th2 and Th1 T-cell polarisation, respectively, following a well-established paradigm [266].

xviii. IgG Samples

Patient IgG samples were kindly provided by Dr. V. Thompson of the University Of Liverpool Institute Of Translational Medicine. IgG samples were isolated using protein G columns and suspending in RPMI 1640 medium for use in cell cultures.

xix. Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 4.04 for Windows, GraphPad Software, and La Jolla California USA). Data were tested for normality (kolmogorov-smirnov) before application of the appropriate test. Data which were not significantly different from normal were assessed using parametric tests (Independent or paired Student's t-tests as appropriate). Data which was significantly different from normal were analysed using non-parametric tests equivalent to those used for normal data (Mann Whitney U test and Wilcoxon Signed rank test respectively). Pearson's correlation and Spearman's rank sum test were used to assess correlation. Data are expressed as mean ±SEM, with differences considered significant if $p < 0.05$.

CHAPTER 3: The cutaneous immune cell population in CRPS.

3A) Introduction

There is now a substantial body of evidence to support the involvement of immune cells in the pathology of CRPS. Various studies have found systemic changes in immune cells in CRPS, including altered responses in lymphocytes, neutrophils and monocytes [91–93]. In experiments to characterise the local tissue environment in CRPS, recent studies have focussed on the cytokine analysis from artificially induced blister fluid, showing consistently elevated levels of IL-6, TNF-α and mast cell tryptase in CRPS skin compared to controls[67,73,77]. However, there is still relatively little information regarding cellular involvement CRPS-affected skin tissue. Radioligand binding showed leukocyte accumulation in the hands of CRPS I patients compared to controls and increased levels of mast cell tryptase implicate mast cells in local tissue pathology [86,90].

One small case report using skin biopsies from amputated CRPS limbs, showed increased Langerhans cell (LC) densities in the skin of CRPS patients. However, the non-specific nature of the immuno-staining and the accuracy of the quantification of cells in transverse section is subject to criticism[107]. Patients with painful diabetic small fibre neuropathy also exhibit increased LC densities in affected tissues, although no difference was found in patients with post herpetic neuralgia[235,236]. LCs are particularly interesting in the study of nerve:immune system interactions as they are the only myeloid-derived cell in the epidermis,

and have close morphological contact with the most peripheral sensory fibres[106].
Furthermore, the intricate modulation of LC behaviour through neurogenic
signalling, and the reciprocal alterations in sensory nerve fibre behaviour, place
LCs at a crucial axis in neuro-immune interaction, a key relationship in the onset
and maintenance of CRPS pathology[244,267].

Aberrant interaction between immune cells, such as mast cells and LCs, and
peripheral nociceptive fibres in the skin can produce painful sensitisations, such
as hyperalgesia and allodynia and may also contribute to mechanisms of central
sensitisation thought to be crucial for the maintenance of disease[28,50]. The
cortical reorganisation believed to occur in CRPS may then produce aberrant
efferent signalling which perpetuates immune cell dysfunction in affected tissues
and effectively maintains disease. Thus, the immune cell population within CRPS
affected tissues could be a key effector mechanism for the development and
maintenance of disease, but may also be a crucial indicator for centrally-
mediated disease processes.

i. Hypothesis

It is possible that despite the resolution of active inflammation in acute CRPS
disease, on-going dysregulation of the immune system occurs through aberrant
interactions between immune cells and disrupted nerve fibre networks.

LCs are a particularly good candidate with which to investigate this possibility
due to their close relationship to peripheral nerve fibres. Any observed

differences in these tissues may be directly relevant to chronic disease mechanisms in longstanding CRPS tissues. Thus the hypothesis to be tested in this chapter is "**Dysregulation of immune cells in CRPS affected tissue, mediated by altered nerve fibre networks, contributes to the maintenance of long-standing CRPS disease**"

3B) Aims

Although a key role for the immune system in the development of CRPS pathology is now widely accepted, there still remain significant gaps in knowledge regarding the cellular population in affected tissues. Defining the immune cell population within CRPS affected tissues in longstanding disease may help to develop a better understanding of the aberrant process underpinning CRPS pathology, with particular emphasis on LCs which, positioned at the axis of neuro-immune crosstalk, are seen as key indicators of neuro-immune function in the skin. The specific aims of this chapter are to:

- Measure immune cell numbers in CRPS affected tissue with a focus on dermal mast cells and epidermal Langerhans cells.

- Determine functional differences in immune cells in affected tissues through phenotyping of tissue resident T-cells.

- Investigate the relationship between immune cell populations and disease symptoms by correlating observed differences with relevant clinical data e.g. Pain intensity, disease duration etc.

3C) Results:

i. Patient Demographics

To investigate the local immune cell populations within CRPS-affected skin, 6mm punch biopsies were taken from affected areas and from non-affected areas on contra-lateral limbs. Biopsies were also taken from fibromyalgia patients (as a non-CRPS pain control) and from healthy individuals. Patient and control characteristics are summarised in Table 3.1 and Table 3.2. The age-range of CRPS participants varied from 24 to 50 years, with the mean age being 38 ± 8. The age of non-CRPS pain volunteers and healthy volunteers ranged from 24 to 67 years with a mean age of 45 ± 12. The length of disease duration in CRPS patients ranged from 1 to 12 years with a mean duration of 5.5 ± 1.4 years. Pain NRS scores ranged from 5 to 8 with a mean of 6.6 ± 0.3 in CRPS patients, and from 2 to 7 with a mean of 2.8 ± 3 in non-CRPS pain controls.

ii. Tissue histology

Haematoxylin- and eosin-stained sections are commonly used to assess tissue integrity and cellular infiltration by histology. Tissue samples were blinded and evaluated by two independent assessors, including a qualified dermatologist, based on histological assessment table (Appendix Table 1). No significant difference in histological assessment of immune cell infiltration was observed

Table 3.1 - Patient disease characteristics and demographics.

Study no.	Age	Sex	Affected Limb(s) (upper/lower)	Control limb (upper/lower)	Disease duration (years)	NRS	Injury	CRPS type	Medication at time of Biopsy	Biopsy location	Epidermal Sheets	Tissue T-cells
1	50	F	LL	U	6	5	spontaneous	I	nil	Inside right thigh	Y	N
2	37	F	LL	U	10	8	Minor trauma	I	Nifedipine 5mg; Amitriptyline 50mg; Morphine 10mg	upper left calf	Y	N
3	39	F	UU	L	12	8	spontaneous	I	nil	right forearm	Y	N
4	45	M	L	L	10	5	Ankle fracture	II	Tramadol 600mg; Clonazepam 2mg; Daktacort; Domperidone 10mg; Lansoprazole 15mg	upper left calf	Y	N
5	29	F	L	L	1.2	8	Left common peroneal nerve neuropathy of unknown genesis	II	Morphine 10mg; Diclofenac 50mg; Gabapentin 1200mg; nortriptyline 80mg	upper left calf	Y	N
6	24	F	L	L	1.2	7	Knee arthroscopy	I	Morphine 100mg; Gabapentin 1200mg; Clonidine 50mg; Fluoxetine 60mg	upper right calf	Y	N
7	46	F	L	L	2	6	Ankle fracture	I	nil	upper left calf	Y	Y
8	40	F	L	L	1	6	Ankle fracture	I	Salbutamol 5mg; Gabapentin 900mg; nortriptyline 80mg	upper right calf	Y	Y
9	38	F	L	L	2.5	6	Ankle fracture	I	Morphine 10mg; Gabapentin 1200mg; cotrimoxazole 300mg; Cerovite supplement	upper left calf	Y	Y
10*	35	M	L(U)	L	1(10)	7	(crush injury), Trivial injury	I	Pregabalin 150mg; Amitriptyline 50mg	upper right calf	Y	N

*upper limb CRPS now well controlled with spinal cord stimulator in situ, no biopsy taken from upper limb. Developed lower limb CRPS one year before biopsy procedure.

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Table 3.2 – Control patient characteristics and demographics.

Study no.	Age	Sex	Biopsy location	Fibromyalgia patient	Epidermal Sheets
1	25	M	left calf	N	N
2	51	M	left calf	N	Y
3-p	55	F	left calf	Y	Y
4	68	F	right calf	N	Y
5-p	50	F	right forearm	Y	Y
6-p	50	F	left calf	Y	Y
7-p	67	M	left calf	Y	Y
8-p	41	F	left calf	Y	Y
9	50	M	left calf	N	Y
10	48	F	right calf	N	Y
11	37	F	left calf	N	Y
12	37	F	right calf	N	Y
13	35	F	left calf	N	Y
14	32	F	left calf	N	Y

in line with previous studies in CRPS showing a general decline in immune activity (measured by cytokine release) following the acute phase of CRPS[76]. This may suggest that mast cells are directly involved in the early inflammatory stages of CRPS disease onset but their role is diminished during disease progression

iii. Langerhans Cell (LC) Density in CRPS affected Tissue

LCs are the only myeloid cell present in the epidermis and, in humans, uniquely express CD1a on their cell surface[194]. Separation of epidermal sheets from skin punch biopsies facilitates the direct *in situ* staining of LCs using fluorescently-labelled antibodies and provides a unique perspective for LC cell quantification

with minimal disruption of tissue. CD1a staining of epidermal sheets showed positive cells with dendritic morphology indicative of LCs (Fig. 3.2A). Dual staining with MHC-II was used to confirm cell lineage (Fig. 3.2B & Fig. 3.2C). Protocols in which the primary antibody was omitted or an isotype control antibody was used showed negative staining. CD1a stained epidermal sheets from a patient with a history of blistering manifestations exhibited a clearly altered LC morphology (Fig. 3.2D), possibly as a result of underlying dermal tissue disruption, as recorded in the histological assessment score. For this reason, this sample was excluded from the summary LC density counts. Two further samples exhibited bilateral CRPS distribution which necessitated the sampling of biopsies from non-contralateral control tissues. Following analysis, these samples (P1 & P3) introduced outliers in the data set and so were excluded on the basis of biopsy locations. CD1a+ LC quantification was performed using composite confocal images and 3D software assisted manual counting, and was calculated as LC/mm^2 of epidermis (Fig. 3.3A).
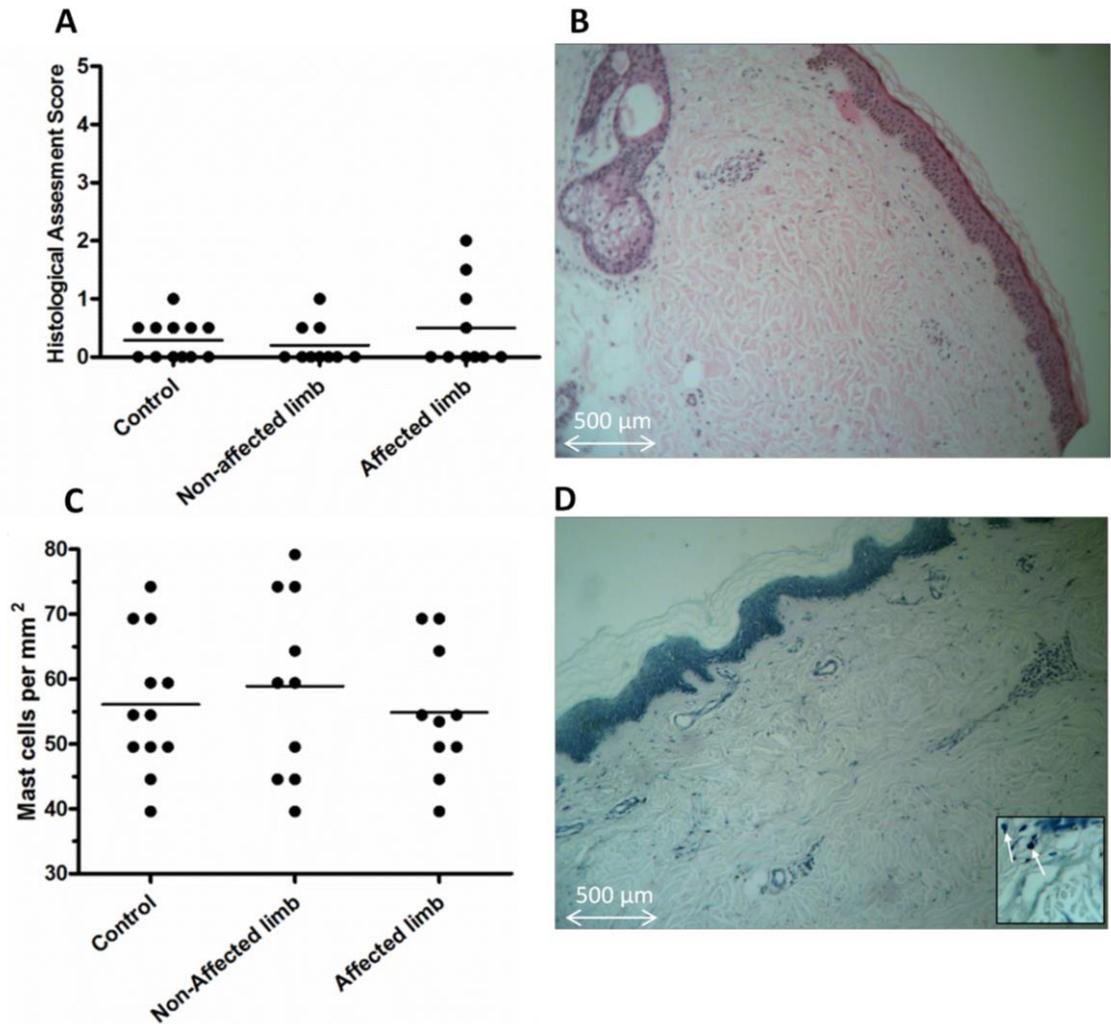
Figure 3.1 – Immune Cell Infiltration and Mast Cell Densities in CRPS affected Tissue.

Formalin fixed skin sections were stained (as described in the text) to assess immune cell infiltration and mast cell density in CRPS affected tissues. **A&B**) Double-blind histological assessment scoring of haematoxylin and eosin stained was used to evaluate inflammation and tissue damage. **C&D**) Mast cell densities were calculated by visual counting of metachromatic granular cells (white arrows) within 10 random fields of the papillary dermis per tissue sections.

Mean LC cell densities ranged from 328 LCs/mm^2 (CRPS affected) to 807 LCs/mm^2 (CRPS non-affected). Mean values for CRPS affected, CRPS non-affected, healthy and non-CRPS pain were 480 ± 37 LCs/mm^2, 609 ± 43 LCs/mm^2, 457 ± 53 LCs/mm^2 and 421 ± 48 LCs/mm^2, respectively. Comparative analysis of cell densities throughout the epidermis (Fig. 3.3B) revealed a significant difference only between non-CRPS pain control tissue and CRPS non-affected tissue ($U_{(6)}$ = 4.00, $p<$ 0.05) as measured by a Mann-Whitney U test. No further significant differences in the means of the groups were observed, possibly due to high levels of variation in LC density between donors, the reasons for which are discussed later.

However, a paired t-test of CRPS-affected and non-affected limbs (i.e. comparing samples from different locations across each individual donor) showed a significant difference between LC densities ($t_{(6)}$ = 4.75, p <0.01) (Fig. 3.3C) with a mean percentage difference in LC density of 24% ± 5.57 between affected and non-affected limbs (Fig. 3.3D).
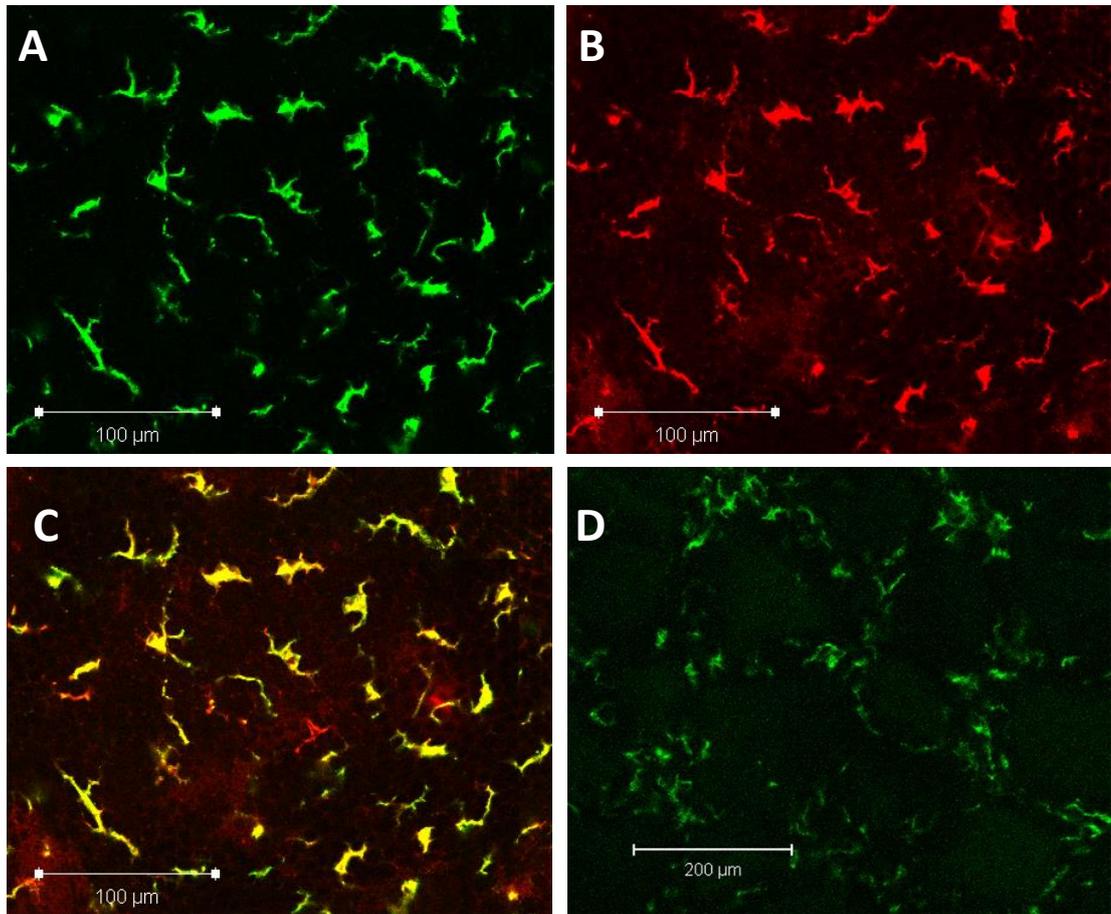
Figure 3.2 – Immuno-fluorescent Staining of Langerhans Cells in Epidermal Sheets

Epidermal sheets isolated from skin punch biopsies were fixed in acetone and dual-stained for LC specific markers. **A**) CD1a was used to specifically label LCs within the epidermal sheet. **B**) Single staining with HLA-DR was also performed to label professional antigen presenting cells **C**) Dual staining with both HLA-DR and CD1a shows co-localisation confirming the professional antigen-presenting capacity of CD1a^{+} epidermal LCs. **D**) In a single skin biopsy from a CRPS affected site with a previous history of blistering disease LCs showed a non-uniform distribution pattern that was distinct from the pattern seen in other samples.

Statistical analysis, including those patients with non-contralateral controls (P1 & P3), also demonstrated a significant difference between LC densities in affected and non-affected limbs (p <0.05) (Appendix Figure 1).

This finding shows that there is a significant observable difference in LC densities in individual CRPS patients when comparing affected and non-affected limbs. Since the differences are independent of healthy control samples, it is not clear if the difference represents a relative increase or decrease in cell number in affected tissues, the implications of which are discussed in more detail below. However, this striking difference does indicate that there is some form of differential regulation of LCs in CRPS affected tissue when compared to healthy tissue from the same individual. As LCs share a close functional relationship with distal nerve fibres in the epidermis, and other evidence indicates that conventional inflammatory processes are resolved in longstanding CRPS, it is possible this difference is as a result of efferent nerve signalling as opposed to immune mediated (e.g. by cytokines). This could have important implications for our understanding of disease mechanisms in longstanding patients and could also constitute a useful biomarker for CRPS disease in these patients.
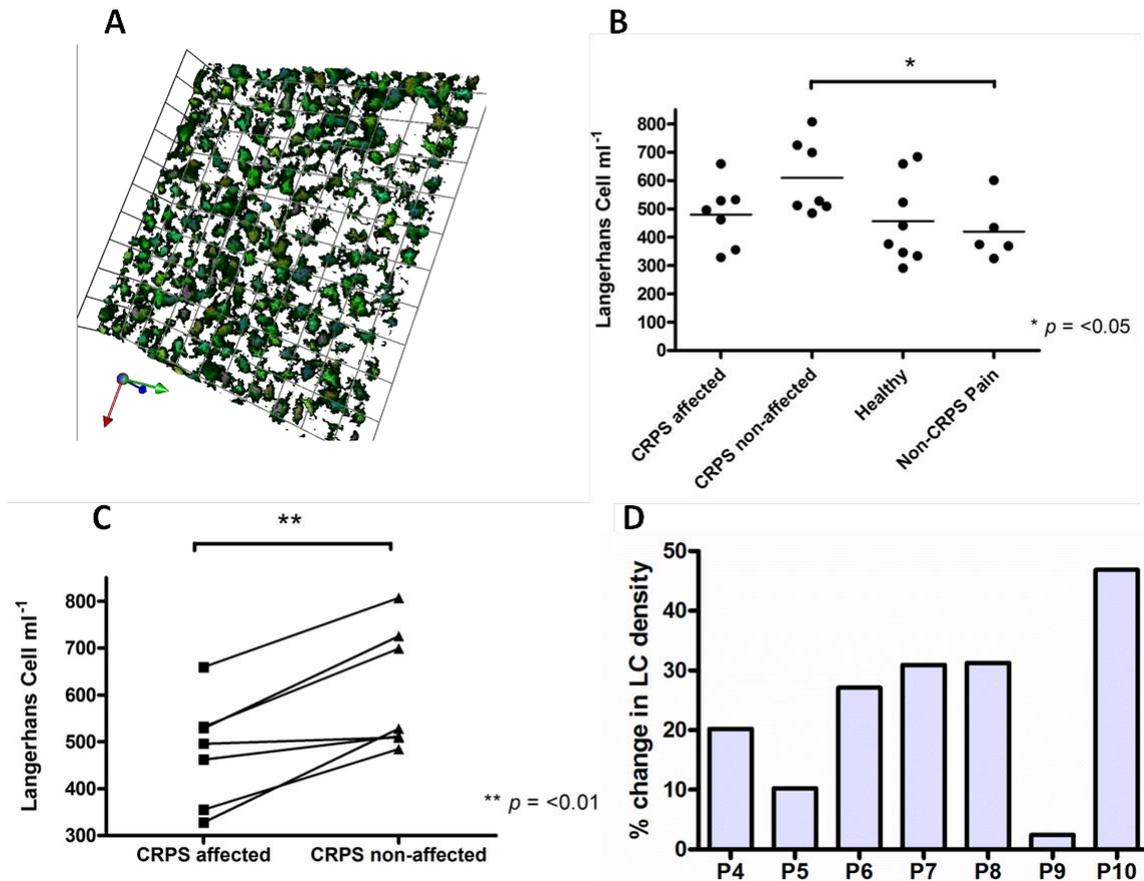
Figure 3.3 – Langerhans Cell Densities in CRPS Affected tissues.

CD1a stained epidermal sheets imaged by confocal microscopy and a 3 dimensional picture constructed of the epidermal sheet following which LC were quantified and cell density recorded as cells per mm^2 of epidermis. **A**) A combination of Volocity 3D imaging software and manual counting was used to quantify cells numbers within the epidermal sheet. **B**) Mean LC densities observed in CRPS non-affected and non-CRPS control tissue were significantly different ($p < 0.05$). **C**) Paired analysis between LC densities in CRPS affected and non-CRPS affected tissue from the same individual showed a significant difference ($p < 0.01$) but due to variation within the control sample populations it is not clear if this difference represents a significant *increase* or *decrease* when comparing CRPS-affected and non-affected tissues. **D**) In order to analyse differences in LC density in relation to other data sets the % difference between CRPS affected and CRPS non-affected sites within each individual was calculated.

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iv. Tissue Resident T-cell Isolation and Phenotyping

Although existing data and the data presented above, suggest that inflammation and cell infiltration into affected tissue is resolved in cases of longstanding CRPS it is not known if functional differences in immune cells persist. Functional differences in immune cells in the skin could occur as a result of alterations in the tissue environment which could be relevant to disease mechanisms. As T-cells are present in dermal tissue under steady state conditions and have well characterised developmental and phenotypic pathways, they represent a useful reporter cell for the tissue environment. Differential regulation of the functional phenotype of these cells in affected and non-affected tissue could be indicative of aberrant disease processes, the manner of which may be inferred from the specific way in which the T-cells are differentiated.

To test this hypothesis resident T-cells were isolated from CRPS patient bisected skin punch biopsies and cloned over a number of weeks. T-cells were then phenotyped based on the cytokine expression profile following mitogen stimulation in ELIspot assays. Release of IFN-γ and IL-13 was used to assess Th-1 and Th-2 T-cells respectively, and granzyme B (a serine protease) release was used to assess polarisation towards a cytotoxic cell profile. Individual results from each biopsy are summarised in Figure 3.4. Donor-dependent variation in T-cell responsiveness to mitogen stimulation resulted in different levels of Spot Forming Units (SFU) between the different assays and thus arbitrary gates are

106
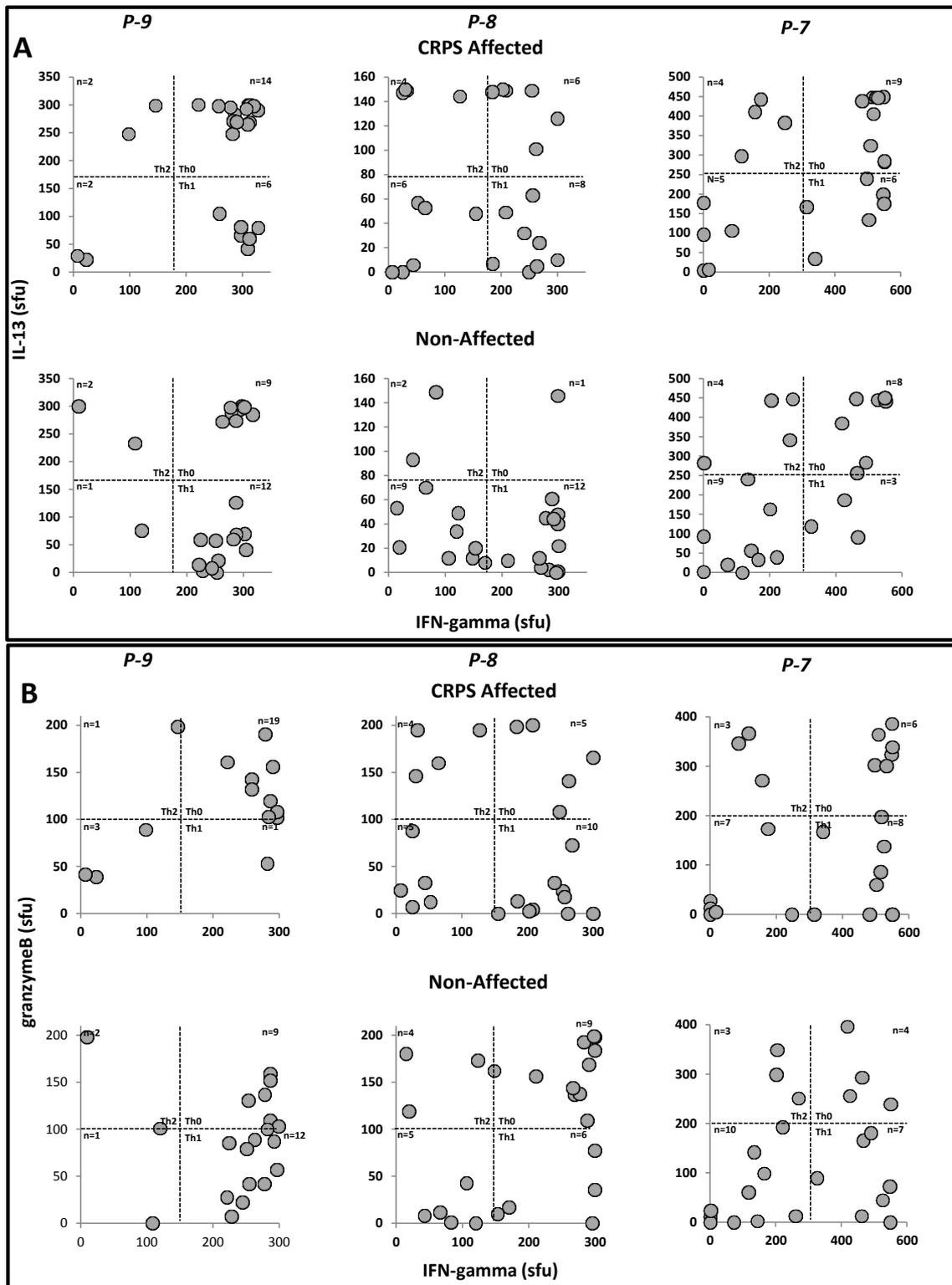
Figure 3.4 – Phenotyping of Tissue Resident T-cells Isolated from CRPS-affected and Non-affected Skin.

Biopsy tissue from 3 patients was bisected and half the material minced and subject to tissue T-cell isolation. T-cells were serially diluted to produce single clones and these clones were expanded over 2 weeks before phenotyping. **A&B**) 24 T-cell clones isolated from either CRPS-affected or CRPS non-affected tissue were subject to ELIspot assays for IFN-γ, IL-13 or Granzyme B following mitogen stimulation. Cytokine secretion is recorded as spot forming units (SFU) where one spot represents a single clonal cell secreting the specific analyte.

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shown to differentiate cell polarisation across each donor. A general trend towards increased numbers of IL-13 producing cells in CRPS-affected tissues was observed, particularly in patient 8 (Fig. 3.4A). Patient 9 also displayed a distinctive polarisation towards IFN-γ producing T-cells, in both affected and non-affected tissues and an increased number of granzyme B producing T-cells in affected tissue relative to non-affected tissue (Fig. 3.4 A & B).

Mean SFU were calculated for IL-13, IFN-γ and granzyme B from each of the 24 T-cell clones per biopsy. These data are then used to represent the relative polarisation towards each specific T-cell phenotype (Fig. 3.5A). Mean SFUs in T-cell clones isolated from CRPS-affected tissues were 183 ± 57 SFU, 255 ± 52 SFU and 118 ± 24 SFU for IL-13, IFN-γ and granzyme B, respectively. Mean SFUs in clones isolated from CRPS non-affected tissues were 144 ± 57 SFU, 246 ± 27 SFU and 107 ± 13. Analysis using a non-parametric paired t-test (Wilcoxon signed rank), as a direct comparison between affected and non-affected T-cell clones within in each individual donor (n=72), showed a significant increase in mean IL-13 SFU from T-cell clones isolated from CRPS-affected tissue (Fig. 3.5B), suggesting an increase in IL-13 producing cells in these tissues relative to non-affected tissue. However, there was no significant difference in the mean SFU of any of the three analytes when comparing CRPS-affected and non-affected tissue in the absence of pairing, possibly due to the aforementioned donor dependent variation in T-cell responsiveness.
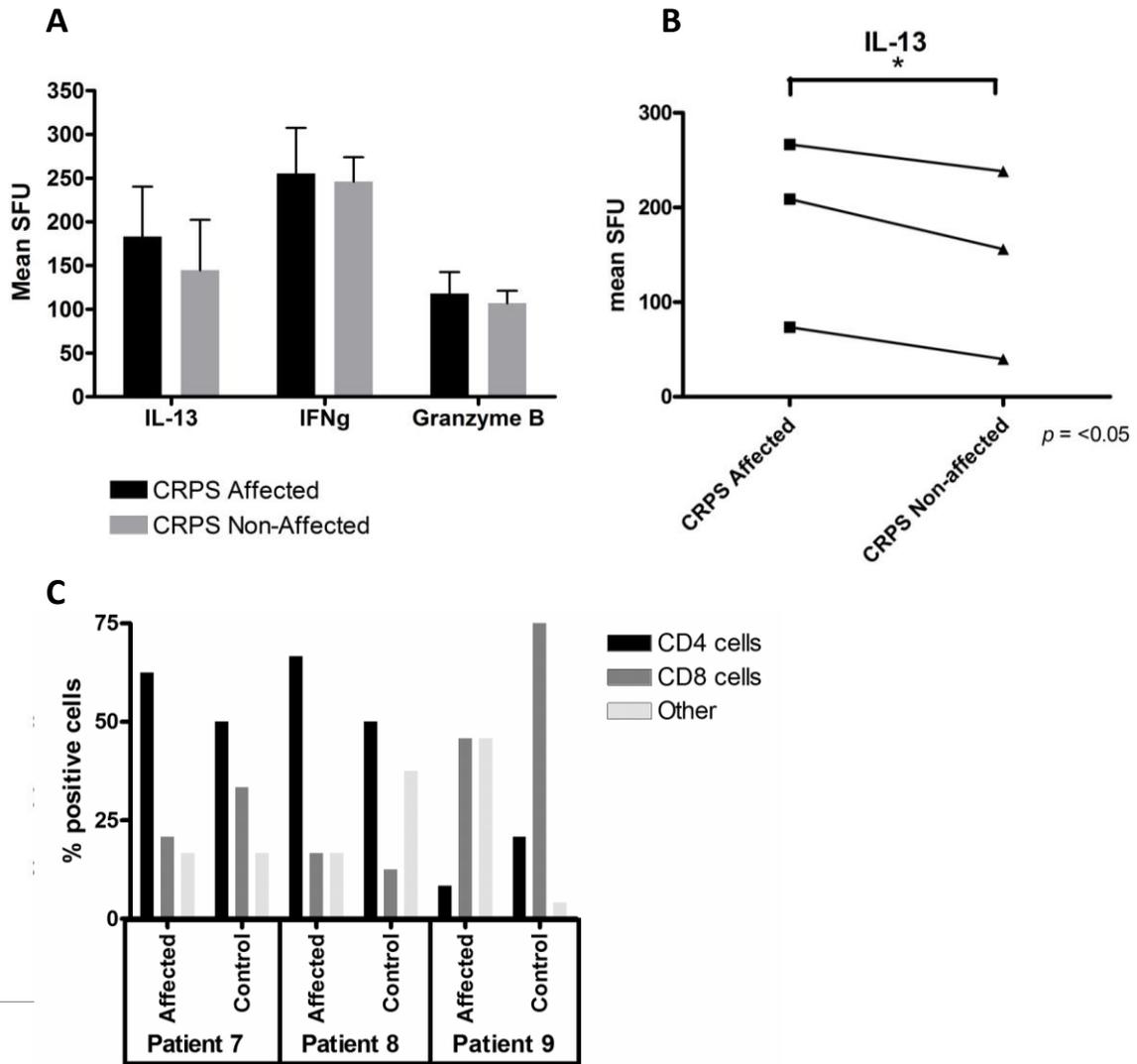
Figure 3.5 – Tissue Resident T-cell Polarisation

Following isolation from tissue, T-cells were phenotyped using ELIspot assays and mean SFU for each analyte was calculated as a measure of T-cell polarisation. **A**) Despite a trend for reduced SFU for each analyte in CRPS non-affected tissue compared to CRPS affected tissue, there were no significant differences between data sets. **B**) Paired analysis, i.e. comparing T-cell polarisation in affected and non-affected tissues for each individual, showed a significant reduction in mean IL-13 SFUs produced by T-cells from non-affected tissues compared to those from affected tissue, suggesting a relative increase in IL-13 producing cells in CRPS affected tissues compared to non-affected tissues. Data shown are means of 24 clones per biopsy. Paired analysis was performed on all clones i.e. n=72 **C**) T-cell clones were also stained for CD4 and CD8 to differentiate T-helper and cytotoxic subtypes respectively; un-stained or dual-stained cells were recorded as "other". Despite patient 9 displaying a distinctly different profile, no significant differences were observed between groups. Error bars show the mean ±SEM.

As IL-13 is considered a Th-2, closely related to IL-4, this finding may indicate a Th-2 bias within CRPS affected tissues[266]. This finding may indicate that although inflammation and immune activation is resolved in longstanding CRPS, there is on-going dysregulation of the immune system in these patients. A view further supported by the LC density data described above. The type of polarisation could suggest that Th-2 cytokines may be prevalent within affected tissues in longstanding disease, the further implications of which are discussed below.

Surface marker expression was also recorded for each T-cell clone to assess differences in the expression of CD4 (T-helper) and CD8 (cytotoxic T-cell). Positive staining for each marker was calculated as a percentage of T-cell clones isolated (Fig. 3.5C). Unstained or dual positive cells were recorded as "other". No difference was found in the percentage of CD4$^+$ or CD8$^+$ T-cell clones when comparing CRPS affected and non-affected tissue. Patient 9 expressed distinctly altered marker expression compared to the two patients with a greater proportion of CD8+ T-cells. Since this patient was receiving anti-biotic medication (Table 3.1) which may indicate some form of active infection, one possibility is that this infection is responsible for skewing the T-cell profile. However, further repeats would be necessary to confirm this.

v. Measurement of Serum Cytokines

There is conflicting evidence regarding serum cytokine concentrations in CRPS with the general consensus forming that increased levels observed in acute

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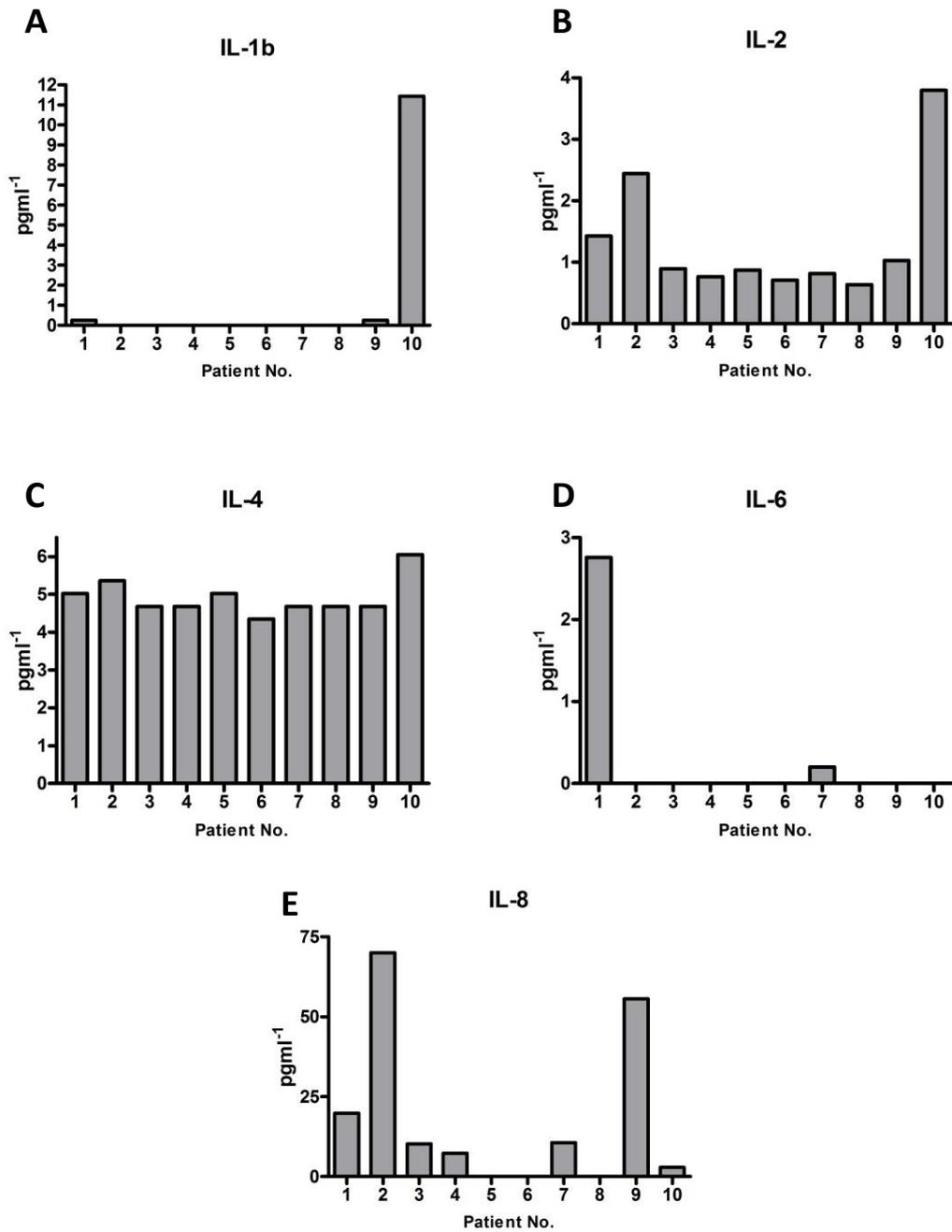
Figure 3.6 – Serum Cytokine Concentrations in CRPS

Multiplex ELISA was used to simultaneously measure 13 different analytes in CRPS patient serum. **A-E)** Of the 13-analytes measured 5 were consistently detected at reliable levels. Cytokine levels varied across all patients with only IL-4 displaying a consistent concentration in across the sample population.

phase disease are resolved over the course of disease progression[64,74]. However, since significant differences in tissue regulation had been observed in our patients (Fig. 3.3 & 3.5) serum cytokines were investigated as a possible co-factor in the dysregulation of either LCs or tissue resident T-cells. For this reason healthy controls were excluded as only comparisons between cytokine concentrations and existing significant data would be analysed. Cytokine concentrations were measured for each biopsy donor using multiplex ELISA to simultaneously measure 13 different analytes: IL -1β, -2, -4, -5, -6, -8, -10, 17a, GM-CSF, IFNγ, TNFα, IFNα and MIP1α, (a comprehensive overview of results can be found in Appendix Table 2). Of those analytes assayed only 5 were detected within the reliable range of the assay and are shown in Figure 3.6. Low levels of IL-2 and IL-4 were detected in all samples, in contrast to IL-1β, IL-6 and IL-8 which were detected over a range of concentrations in the different donors, and in some cases were not detectable. This data is consistent with previous reports showing high levels of variability in serum cytokine in longstanding CRPS patients[65].

vi. Correlation between Data Sets

To identify putative relationships between the different aspects of the immune system measured in this study, correlation analyses between the multiple data sets was performed. By calculating percentage difference in LC density in each patient (Fig. 3.3D), direct analysis of correlation between LCs and the other clinically relevant data could be undertaken. This method was then used assess if
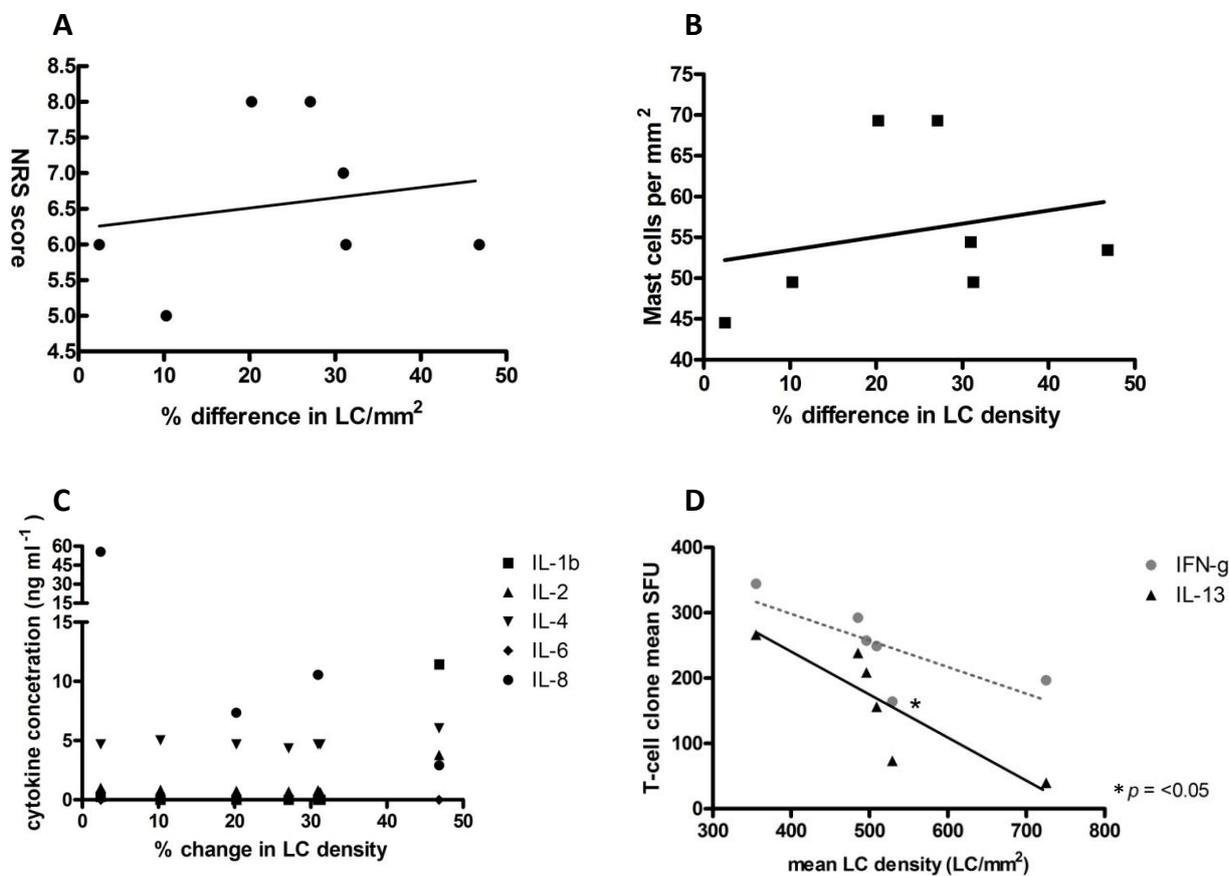
Figure 3.7 Langerhans Cell Density and Clinical Correlates

Following the observation of significantly altered LC density between CRPS affected and non-affected limbs, % difference in LC density between limbs was used to explore the relationship between LC density and other clinically relevant data. **A&B**) Despite weak positive trends in both cases, no significant correlation was observed between the % difference in LC density and either pain, as recorded on an NRS, or mast cell density in dermal tissue. **C**) Patient serum cytokine concentrations were assayed using a 13-analyte multiplex ELISA, and of the 5 cytokines reliably detected by the assay none were significantly correlated with the % difference in LC density between affected and non-affected limbs. **D**) Mean IL-13 SFU produced by tissue resident T-cells showed a significant negative correlation ($p < 0.05$) with LC density recorded in the same biopsy. Mean IFN-γ SFU showed the same negative trend but did not reach statistical significance.

there is a relationship between significantly altered LC density and other aspects

of CRPS disease. A summary of these finding is shown in Figure 3.8. No significant

correlation was observed between percentage change in LC density and pain NRS

scores or mast cell density (Fig. 3.7 A&B). There was also no correlation between

percentage change in LC density and detectable serum cytokine concentrations

(Fig. 3.7C). Since only 3 biopsy samples were used for the tissue resident T-cell

isolation protocol, any comparisons between CRPS affected and non-affected

limbs were limited. However, examining each individual biopsy as a single data

point, irrespective of the CRPS-affected or non-affected status of the tissue,

produced a significant negative correlation between LC cell density in tissue and

the mean IL-13 SFU produced by tissue resident T-cells (r_6 = -0.859 p = 0.029)

(Fig. 3.7D) which would suggest a link between increased LC density and Th-2

polarization within the tissue. However, since there was a similar, but non-

significant, trend in the production of IFNγ by T-cells, it appears that this

relationship is linked with T-cell responsiveness, as opposed to polarisation

towards a T-helper subtype. There was no correlation between the percentage

change in LC density and the percentage change in SFU of IL-13, IFNγ or

granzyme B produced by tissue resident T-cells (Appendix Fig. 2).

3D) Discussion

In summary the key findings from this chapter are:

- There is no significant difference in the number of mast cells in CRPS-affected skin tissue, compared to control. Indicating any role for mast cell involvement may be restricted to early stage CRPS.

- There is a significant difference in LC cell density between CRPS-affected and non-affected limbs within individual patients. This demonstrates differential regulation of CRPS affected tissue which could have implications for understanding disease mechanisms and improving diagnosis.

- Tissue resident T-cells from CRPS-affected skin are polarised towards a Th2 phenotype, compared to cells from non-affected tissue in the same patient. This finding supports differential regulation of CRPS affected tissue and indicates that classical immune cells may still be involved in disease processes.

i. Mast Cells in CRPS

Increased levels of mast cell tryptase have been reported in blister fluid from CRPS affected tissues[73]. Mast cells are distributed throughout the dermis and are potent activators of inflammation, capable of secreting CRPS-relevant cytokines, such as TNF-α, and also respond to and secrete neuropeptides such as SP [88,270]. Recent research in CRPS has focused on the possible role of mast

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cells in disease pathogenesis, particularly in the early stage of disease where neurogenic inflammation and neuroimmune crosstalk are apparent[86]. Furthermore, therapeutic interventions linked to an inhibition of mast cell function are being suggested for the treatment of CRPS and the efficacy of existing immuno-modulating drugs may be attributed, at least in part, to their effects on mast cell activation[85,271]. In the above results, I have shown that there is no significant difference in the number of mast cells present in CRPS tissue, compared to non-affected or control tissue (figure 3.2C). Previous data which have implicated mast cells in CRPS disease is based on markers of mast cells activation, such as the presence of mast cell tryptase without any consideration of cell number. Similarly the techniques used in this study cannot account for the activation status of the mast cells in the tissue, although decreased staining can be observed if mast cells are de-granulated. A further consideration is that mast cell involvement may be more important in the early stages of disease onset, where neurogenic inflammation is thought to be a key aspect in peripheral and central sensitisation[88]. Given that the CRPS patients in this study are all in the latter stages of disease, it is possible that any change in mast cell numbers, which could facilitate inflammation in affected tissues, may have normalised following the resolution of active inflammation.

ii. Langerhans Cells in CRPS

As previously established LCs are the only immune cell present in the human epidermis under steady-state conditions, and are involved in the maintenance

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of peripheral tolerance making them functionally distinct from the newly-characterised mixed population of dendritic cells found in the dermis [189,195]. LCs *in situ* also share a close morphological relationship with peripheral nerve endings and have been shown to display altered behaviour following stimulation with a variety of neurogenic signalling components, including neuropeptides and catecholamines, and also exhibit altered behaviour in models of neuropathic pain [213,217,224,237,238]. In the context of CRPS disease, LCs are capable of producing the relevant inflammatory cytokines and are uniquely positioned at a crucial interface between immune and nervous systems in the periphery, and thus represent a key cell type in context of CRPS pathology [272,273].

In the above data, I have shown a significant difference in CD1a+ LC density in the epidermis of CRPS affected limbs when compared to non-affected tissue from the same patient (Fig. 3.4C). Previously, a small case-series using skin punch biopsies from the amputated limbs from CRPS patients reported increased numbers of an epidermal cell with dendritic morphology following staining with a LC non-specific (S100) antibody [107]. More recently, Casanova-Molla *et al* showed increased numbers of LCs in the epidermis of patients with painful diabetic small fibre neuropathy, compared to control tissue, a finding which negatively-correlated with intra-epidermal nerve fibre density [236]. However, Oaklander has previously reported no difference in LC numbers in skin affected by post herpetic neuralgia, compared to control, including no

change in LC numbers following the loss of cutaneous innervations [235]. It is likely that differences in experimental protocols, such as the use of non-cell specific markers and different biopsy locations, could explain some of these differences, in addition to any underlying differences in disease pathology. It is well established within the literature that exposure of skin surfaces to environmental stimuli, particularly UV light, can result in reduced LC numbers when compared to UV- protected tissue sites [274]. In addition to physical stimuli, even moderate psychosocial stress has been shown to significantly alter LC density [214]. These factors introduce significant sources of error when attempting to compare epidermal LC densities in different individuals, as the different behavioural patterns and states of mind of participants is difficult to control for. This is of particular importance in the field of pain, where patients are usually less mobile, and thus less likely to encounter environmental stress such as UV, and sometimes also present with altered mental states, particularly in the context of a potentially painful surgical procedure such as a biopsy. In the present study, we have shown a relative difference between CRPS-affected and non-affected limbs within individual patients and thus eliminated potential bias from prior exposure of tissues to environmental stress and also any psychological effects. By comparing CRPS-affected and non-affected tissue alone, it is not clear if the observed difference represents a relative increase in non-affected tissues or relative decrease in affected tissues, nor were there any consistent changes in LC cell morphology that could be indicative of cell activation status. However, there is also a significant increase in the mean LC

density in CRPS non-affected tissue, compared to the non-CRPS pain control group (Fig. 3.4B). Since this control group was included to mimic the behavioural and/or psychological effects of pain suffering, and thus reduce potential error, these data might suggest that LC densities in CRPS non-affected limbs are increased relative to CRPS-affected limbs. However, since by the same measure i.e. mean LC density within the sample group, there is no significant difference in LC density between CRPS affected and non-affected tissues, this conclusion cannot be reliably established without further work.

If the difference in LC density between CRPS-affected and non-affected tissue represents a relative increase in LC density in non-affected limbs, this could be indicative of altered efferent nervous signalling. In this scenario, the regulation of LC migration and/or recruitment to and from the epidermis has been altered only in healthy tissues as a result of central re-organisation of nerve signalling[59]. As described earlier, LC behaviour is regulated in many different ways by a variety of neurogenic elements. If central re-organisation is established following persistent aberrant signalling from damaged nerves in CRPS affected tissues, the resulting changes in efferent nerve signalling may be most apparent in healthy tissues with functioning small fibre networks, as opposed to tissues where small fibre signalling is disrupted. If this were the case, the resulting effect on LC behaviour would manifest only in healthy tissue.

The alternative hypothesis is that LC densities are reduced in CRPS-affected tissues, relative to non-affected tissues. A reduction in LC density is often observed in the context of cell migration away from the epidermis under both inflammatory and non-inflammatory conditions [212]. However, decreased LC densities could also be a result of diminished migration and/or development of precursor cells. LCs are repopulated from a precursor cell population present in the epidermis under steady state conditions and recent work has shown that hair follicles are crucial to LC precursor development and migration to the epidermis [275,276]. This finding is of increased relevance given the well-established changes to hair growth in CRPS.

iii. Tissue Resident T-cells in CRPS

As with the LCs, it is not clear if the observed difference in the number IL-13 producing cells in CRPS-affected skin, compared to non-affected tissues, is a relative increase or decrease. It has previously been suggested there may be a Th2 bias in CRPS, but the observation was inferred from diminished Th1 responses as a result of reduced numbers of circulating CD8$^+$ T-cells [92]. Interestingly, it has been reported that certain neuropeptides, in particular CGRP, can skew LC-mediated activation of T-cells towards a Th2 phenotype[218]. The negative correlation between LC density and the number of IL-13 producing T-cells could be interpreted as an increase in LC migration away from the tissue and subsequent activation and recruitment of IL-13 producing T-cells into the tissue. However, if this were the case one might expect to observe

increased infiltration of T-cells into CRPS affected tissue, a finding not supported by the histological sections. Similarly, the same trend was observed with IFN-γ producing T-cells and so the effects appears to be independent of skewness and more related to T-cell responsiveness in the ELIspot assays.

iv. Conclusions

The data presented in this chapter support existing ideas within the field of CRPS disease that inflammation and immune cell activation apparent in the acute phase of the disease is resolved in patients with longstanding CRPS. However, the above data have also shown that on-going, disease specific, dysregulation is still apparent in the immune cells of these patients, as demonstrated by altered LC densities within tissue and the possible phenotypic skewing of tissue T-cells, a finding which is important to our understanding of how tissue is regulated in late stage disease.

As discussed above, it is possible that central changes within the nervous system could produce changes in LC behaviour. This change could be indicative of disease progression from an acute phase, mediated by inflammation within affected tissue, to a longstanding phase in which peripheral inflammation and immune involvement is resolved and any on-going immune dysregulation is mediated by central nervous signalling. In addition to its potential as a biomarker for CRPS disease this finding also supports the hypothesis that therapeutic intervention directed at the immune system may have a limited window of effectiveness during the acute phase.

Furthermore the observation of a potential Th-2 bias in tissue resident T-cells in affected tissue suggests the acute phase inflammation results in a T-cell bias in longstanding disease despite the resolution of inflammation. This finding will need further exploration but could also have potential for understanding CRPS disease mechanisms and progression for acute to longstanding disease.

CHAPTER 4: Effects of UCH-L1 Expression on Monocyte-Derived Langerhans Cell Function.

4A) Introduction

Previous research conducted in animal models of chronic pain has shown a link between the onset of pain and the expression of the deubiquitinating enzyme UCH-L1, also known as PGP9.5, which is constitutively expressed in peripheral nerve fibres[237–239]. The expression of UCH-L1 by LCs in these models of pain is often concomitant with denervation of the epidermis. Increased levels of UCH-L1 transcription within denervated epidermis containing UCH-L1+ LCs suggests that this expression is a product of *de novo* synthesis as opposed to the phagocytosis of UCH-L1 expressing peripheral nerve material[238]. LCs isolated from healthy epidermis also express UCH-L1 suggesting a direct link between epidermal nerve contact and suppression of UCH-L1 expression[241]. Interestingly, various studies have reported neurotrophic functions for LCs, including directing the extension of growing nerve fibres into the epidermis and also the functional differentiation of developing nerves[238,244]. Taken together these data suggest that LCs respond to a loss of contact with peripheral nerve fibres by expressing UCH-L1, a process which is associated with the onset of chronic pain.

However, LCs differentiated from CD34^{+} cord blood precursor cells do not express UCH-L1 despite differentiation occurring in the absence of any

neurogenic signal[241]. Since in the steady state LCs are maintained by precursor cells within the epidermis, this may suggest that maturation within the tissue is a key aspect of UCH-L1 expression in LCs[277]. Under inflammatory conditions, such as those observed in the acute phase of CRPS disease, peripheral blood monocytes are recruited to the epidermis where they differentiate into mature LCs to help maintain and restore the LC population[277]. These MoLCs are predisposed to an inflammatory phenotype compared to LCs differentiated from CD34$^+$ cells, as might be expected as monocytes are recruited to tissue only under inflammatory conditions *in vivo*. However, the expression of UCH-L1 has not yet been described in MoLCs nor have there been any reports on the functional effects of UCH-L1 expression on LC function.

UCH-L1 is a ubiquitin C-terminal hydrolase and a member of family of enzymes involved in the recycling of free ubiquitin from the ubiquitin protein degradation system[240]. Although the exact recognised role of UCH-L1 in the ubiquitin system is yet to be fully elucidated, it is understood that UCH-L1-mediated recycling of ubiquitin is an important factor in relieving endoplasmic reticulum stress through the "unfolded protein response", a process which prevents the build-up of misfolded protein in cells with very high levels of protein translation[240,255]. Inhibition of UCH-L1 function by the specific inhibitor LDN-57444 results in cell death in a neuroblastoma cell line which constitutively expresses UCH-L1, but has no effect on apoptosis in UCH-L1 transfected cells[254–256].

i. Hypothesis

UCH-L1 expression in LCs has been observed in animal models of chronic pain similar to CRPS. Defining the role UCH-L1 in LCs could identify new mechanisms by which chronic pain develops and elucidate the role of LCs in this process. Thus the hypothesis to be tested in this chapter is "**UCH-L1 expression in LCs is required for cell activation including the regulation of processes relevant to chronic pain development**".

4B) Aims

Despite compelling evidence of a link between epidermal denervation, onset of chronic pain and the expression of UCH-L1 in LCs, there remains little or no evidence of the functional effects of UCH-L1 expression in LCs. Investigation of the expression of UCH-L1 in LCs differentiated from peripheral blood monocytes, a population of cells with a direct bearing on inflammatory skin reactions, may lead to better understand the consequences of UCH-L1 expression in LCs. By characterising the role of UCH-L1 in MoLCs this chapter aims to identify a possible role for LCs in the onset of chronic pain in peripheral tissue and thus develop news targets for therapeutic intervention in this field. The specific aims of this chapter are:

- To investigate the expression and chemical inhibition of UCH-L1 in MoLCs

- Determine the role of UCH-L1 on activation, cytokine secretion and chemotaxis of MolCs.

i. UCH-L1 Expression in MoLCs

To generate MoLCs, peripheral blood monocytes were isolated from mononuclear cell preparations using negative magnetic selection. Negatively selected monocytes were then cultured in the presence of GM-CSF, IL-4 and TGF-β to stimulate cell differentiation into CD1a+ MoLCs. Following differentiation, cells were fixed and permeabilized to facilitate indirect dual staining of CD1a and UCH-L1 (Fig. 4.1). UCH-L1 is commonly referred to as PGP 9.5, in reference to the isolation of the original protein from brain tissue. As commercial antibodies still use the PGP 9.5 nomenclature, throughout this chapter UCH-L1 will be used to refer to gene expression whereas PGP 9.5 refers specifically to protein expression measured by antibody staining. Cytometric gating, based on forward and side scatter to exclude cell contaminants and/or cell debris, showed the percentage of CD1a+ cells following differentiation ranged from 64% - 92% with a mean of 87% (Fig. 4.1 B&E). PGP 9.5 expression was observed predominantly in CD1a+ MoLCs following differentiation, but CD1a- cells (either undifferentiated monocytes or conventional monocyte-derived dendritic cells) also expressed PGP 9.5 (Fig. 4.1C). The number of CD1a+ cells that also stained positive for PGP 9.5, ranged from 48% - 89% with a mean value of 70%. Staining protocols, in which the primary antibody was excluded or an isotype control antibody was used, were negative, and PGP 9.5 staining prior to cell permeabilization resulted in
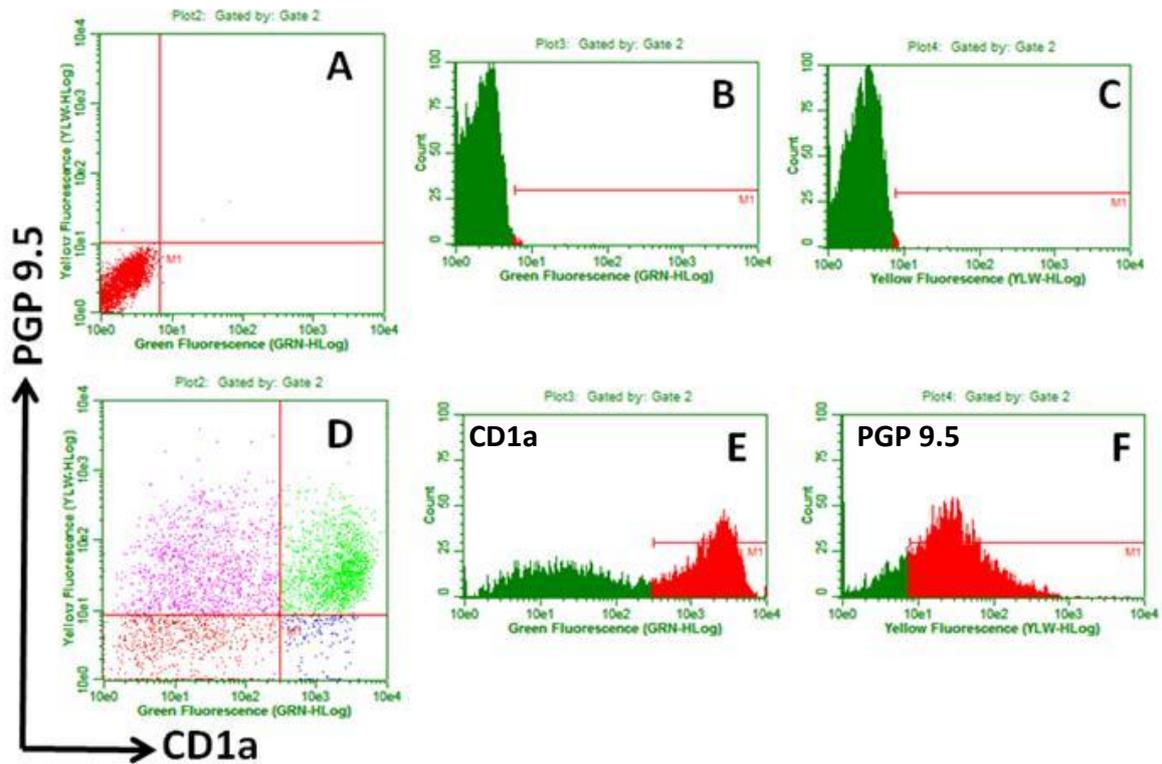
Figure 4.1 – Dual expression of CD1a and PGP 9.5 (UCH-L1) in Monocyte Derived Langerhans Cells

Following negative magnetic isolation negatively selected peripheral blood monocytes were differentiated into MoLCs over 7 d before fixation and permeablization. Cells were dual stained for CD1a and PGP 9.5 and analysed by flow cytometry. **A-C)** Cells were gated using forward and side scatter to eliminate any contaminating cells and cell debris from analysis and fluorescent gates determined by un-stained cells. **D-F)** the majority of cells were labelled dual positive for both CD1a and PGP 9.5 with a smaller population of CD1a-, PGP 9.5+ cells also apparent. The above graphs are representative examples of multiple experiments (n = 7).

decreased levels of staining suggesting an intra-cellular localisation (Appendix Figure 3).

Following the observation of UCH-L1 expression via flow cytometry, live CD1a+ cells were isolated from day 7 MoLC cultures via a two-step positive magnetic selection process, utilizing the same CD1a primary antibody that was used during the flow cytometry protocols. CD1a-positive cells were then lysed in Laemmli buffer for protein isolation or subject to an RNeasy (Qiagen) RNA purification protocol. Cell protein lysates were separated by SDS-PAGE and blotted onto nitrocellulose membranes prior to antibody staining for PGP 9.5 and actin. CD1a+ cell protein extracts were positive for PGP 9.5, as were some CD1a- protein extracts (Fig. 4.2A). Amplification of purified RNA by standard PCR and analysis by agarose gel electrophoresis showed that CD1a+ cells also contain RNA transcripts for UCH-L1 and CD207 (langerin)(Fig. 4.2B).The housekeeping gene GAPDH was also included as an assay control (data not shown) however as no relative gene expression calculations were necessary no further analysis was necessary. These data show that MoLCs constitutively express UCH-L1 and thus these cells are a useful model for exploring the functional effects of UCH-L1. Improving our understanding of this relationship could have direct bearing on the role of LCs in the development of chronic pain.
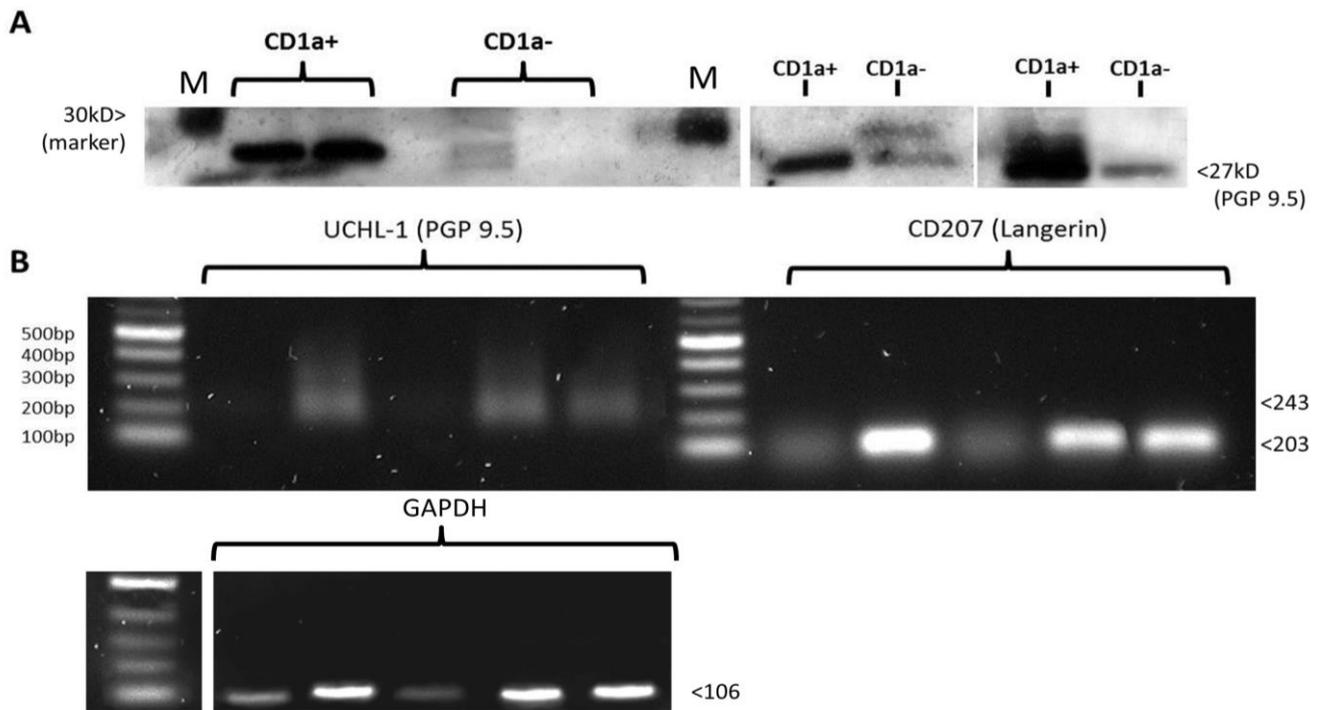
Figure 4.2 – Expression of UCH-L1 protein and RNA in CD1a+ Monocyte Derived Langerhans Cells

Following differentiation CD1a+ MoLCs were positively selected using magnetic beads subject protein or RNA isolation. **A)** Protein lysates from CD1a+ MoLCs were analysed by SDS-PAGE and western blotting using anti-human PGP 9.5 antibodies. CD1a+ protein lysates were positive for PGP 9.5 proteins. PGP 9.5 was also detected in CD1a negative lysates but to a lesser extent. **B)** RNA isolated form CD1a+ MoLCs was analysed using standard PCR and primers specific for the CD207 and UCH-L1 genes. Transcripts were detected for both of the genes probed with the exception UCH-L1 in samples 1 & 3 with low levels of isolated RNA. (M = Molecular Weight Marker)

ii. Suppression of UCH-L1 expression

Previously it has been shown that LCs differentiated from CD34+ cord blood precursor cells do not express UCH-L1 and that LCs isolated from epidermis only become UCH-L1 positive following separation from tissue[241]. Since the above data suggest that CD1a+ MoLCs constitutively express UCH-L1 following differentiation from monocytes, it was hypothesized that UCH-L1 expression *in vivo* may be regulated by neurogenic factors. To investigate this hypothesis, the neuropeptides CGRP and SP and also the catecholamine NA, were added to MoLC cultures for 24 h following cell differentiation. The change in PGP 9.5 expression, measure as immunofluorescence, was calculated relative to the

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fluorescence observed in unstimulated cells, and data are presented as mean percentage expression relative to controls (Fig. 4.3). Mean expression of PGP 9.5 was 111% ±25.52, 87% ±2.29, and 96% ±2.53 following CGRP, NA and SP addition, respectively. There was a significant reduction in the level of PGP 9.5 expression in MoLCs following stimulation with NA ($p<$ 0.05) but no change following stimulation with either of the neuropeptides despite, SP exhibiting a similar trend to NA. In all but one experiment, PGP 9.5 expression was decreased. The exception was one experiment in which CGRP stimulation increased PGP 9.5 expression to a much greater extent than other repeats, and thus introduced significant error into the datasets. Cells from the same donor responded normally to stimulation with NA and SP, and so without further repeats it cannot reasonably be concluded that this data point represents an outlier and so is included in the above data.

iii. Effects of the UCH-L1 Inhibitor LDN-57444 on MoLCs

To determine if UCH-L1 expression in MoLCs has any functional effect which could alter cell behaviour and contribute to immune mediated pain or aberrant pain signalling, the UCH-L1 specific inhibitor LDN-57444 was used to inhibit UCH-L1 activity. LDN-57444 is a specific UCH-L1 inhibitor and has no reported effects on other UCH family members[252]. It has been shown previously that *in vitro* treatment of neuronal cells with 10μM LDN-57444 significantly decreased levels
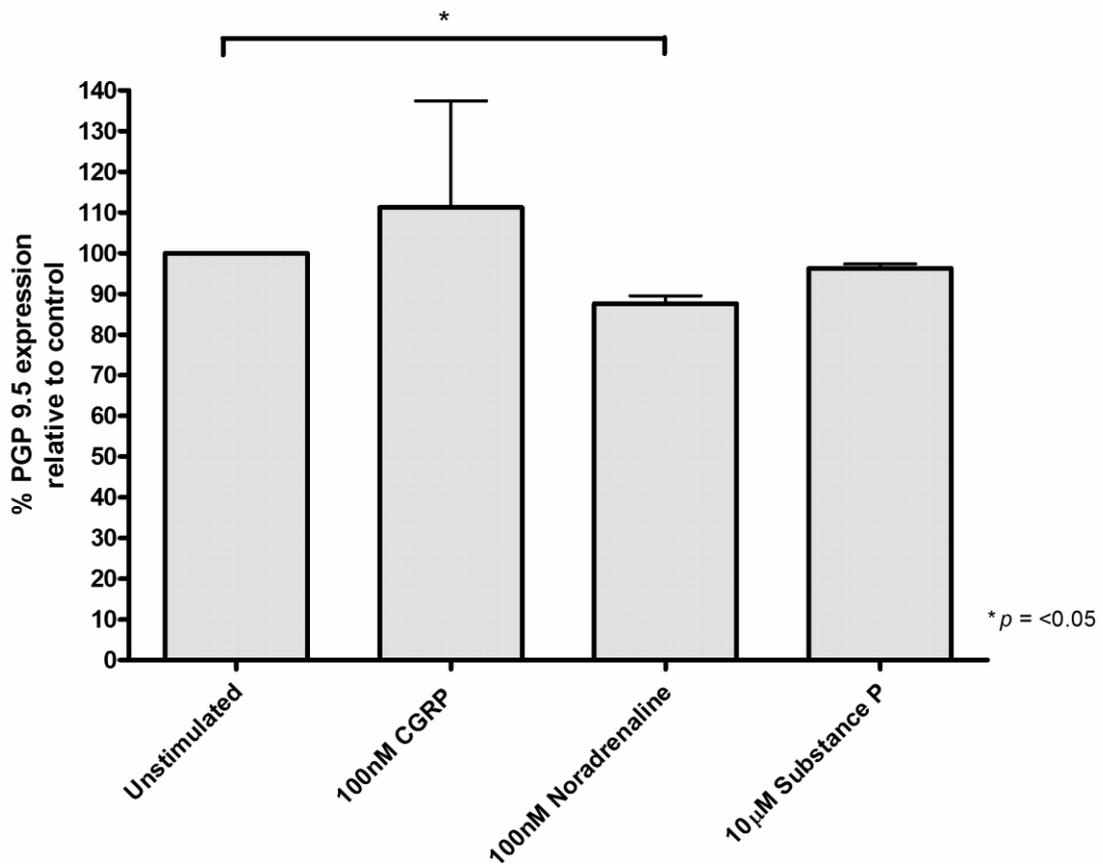
Figure 4.3 – Suppression of PGP 9.5 (UCH-L1) in Monocyte-Derived Langerhans Cells

To assess the effects of neurogenic signalling on UCH-L1 expression in MoLCs cell cultures were stimulated with 100nM CGRP, 100nM NA or 10μM SP and incubated for 24hrs prior to analysis. Cells were then fixed and permeabilized prior to dual staining with CD1a and PGP 9.5 and analysis by flow cytometry. PGP 9.5 expression was normalised to unstimulated cells (taken as 100%) and changes expressed as percentage expression relative to controls. 100nM NA significantly decreased levels of PGP 9.5 expression in MoLCs ($p < 0.05$). Error bars show the mean ±SEM.

Abbreviations: CGRP = Calcitonin Gene Related Peptide; NA = Noradrenaline; SP = Substance P;

of UCH-L1[278]. Higher concentrations of LDN-57444 were also shown to induce apoptosis in a neuronal cell line which constitutively expresses UCH-L1, but not in a cell line transfected with the UCH-L1 gene[255,256]. Since this established published values for efficacy and lethality, and due to the limited numbers of cells available following differentiation, it was decided to use LDN at the single concentration of 10μM. To investigate the effects of UCH-L1 inhibition by LDN-57444 in MoLCs cells were treated with 10uM for 24 h on day 6 of cell differentiation. Changes in PGP 9.5 expression were measured as above, and cell concentrations relative to unstimulated control cells, were used as a measure of cell death (Fig. 4.4). Despite a trend towards increasing PGP 9.5 expression, there was no significant difference between untreated and LDN-57444 treated cells (p = 0.07)(Fig.4.4A). There was also no change in cell number between untreated and LDN-57444 treated cells, suggesting that there was no apoptotic effect in MoLCs (Fig. 4.4B).

iv. UCH-L1 Inhibition in MoLCs – Effect on Markers of Cell Activation

To assess the effects of UCH-L1 inhibition on cell activation, MoLCs were pre-treated with the UCH-L1 inhibitor LDN-57444 or a vehicle control for 24 h prior to overnight stimulation with TNFα, LPS or IL-1β. Cell surface expression of activation markers was then assessed by flow cytometry using cells dual stained for CD1a and either the MHC-II antigen presenting molecule HLA-DR or the co-
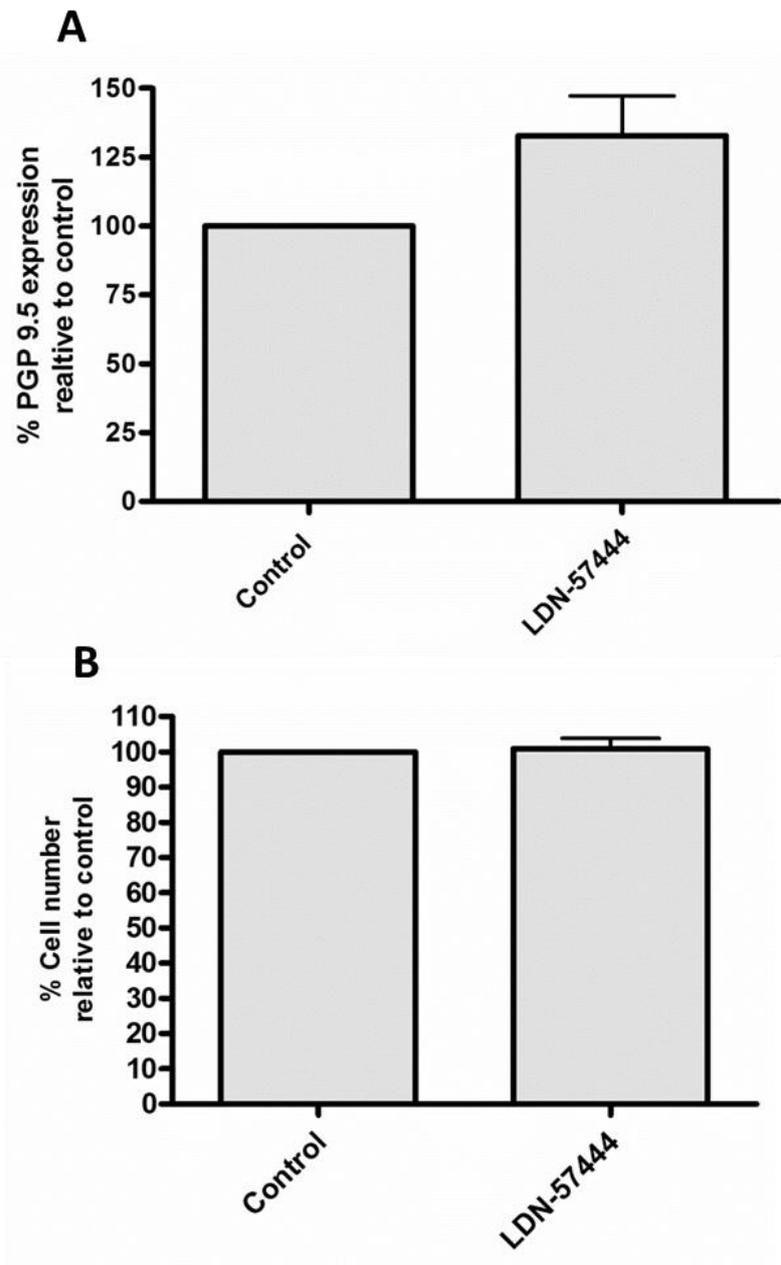
Figure 4.4 – Direct effects of UCH-L1 inhibition in Monocyte Derived Langerhans Cells

On day 6 of cell differentiation, MoLCs were treated with the UCH-L1 specific inhibitor LDN-57444 at a concentration of 10μM for 24 h. Following incubation, cells were analysed for changes in PGP 9.5 expression. **A)** Despite a trend towards increased levels of PGP 9.5 expression in MoLCs treated with LDN-57444 compared to control, the difference was not statistically significant. **B)** To assess for any possible toxic affects following LDN-57444 treatment, the number of MoLCs were counted and normalised to untreated cells. There was no change in cell numbers between the two treatments, suggesting that LDN-5744 treatment is not toxic to MoLCs at a concentration of 10μM. Data shown are the mean values ±SEM (n = 6).

stimulatory molecule CD86. Changes in mean fluorescent intensity were normalised to unstimulated controls and the relative change in expression was recorded as percentage change in expression normalised to control. Pre-incubation with LDN-57444 alone had no effect on either CD86 or HLA-DR expression with mean values of 116% ±30.82 and 72% ±12.36, respectively (Fig. 4.5). Consistent with their role as innate danger signals, both TNFα and LPS increased CD86 expression (Fig. 4.5A). Despite normalised mean expression values of 218% ± 87.22 and 185% ±54.86, respectively variations in donor cell response to stimulation introduced variation and thus the observed trend was not statistically significant. Similarly, despite a trend towards inhibition of CD86 expression in MoLCs pre-treated with LDN-57444, there was no significant difference in CD86 expression following stimulation with or without inhibitor pre-treatment.

HLA-DR expression followed a similar pattern to CD86 expression. Stimulation with either TNFα or LPS significantly increased HLA-DR expression ($p< 0.05$) and LDN-57444 pre-treated MoLCs stimulated with TNFα also expressed significantly increased levels of HLA-DR ($p< 0.05$) (Fig. 4.5B). Despite a trend towards decreased HLA-DR expression in MoLCs pre-treated with LDN-57444, there was no significant difference between different stimulations with or without inhibitor pre-treatment, suggesting that inhibition of UCH-L1 has no effect on MoLC activation marker expression.

A

CD86

B

HLA-DR
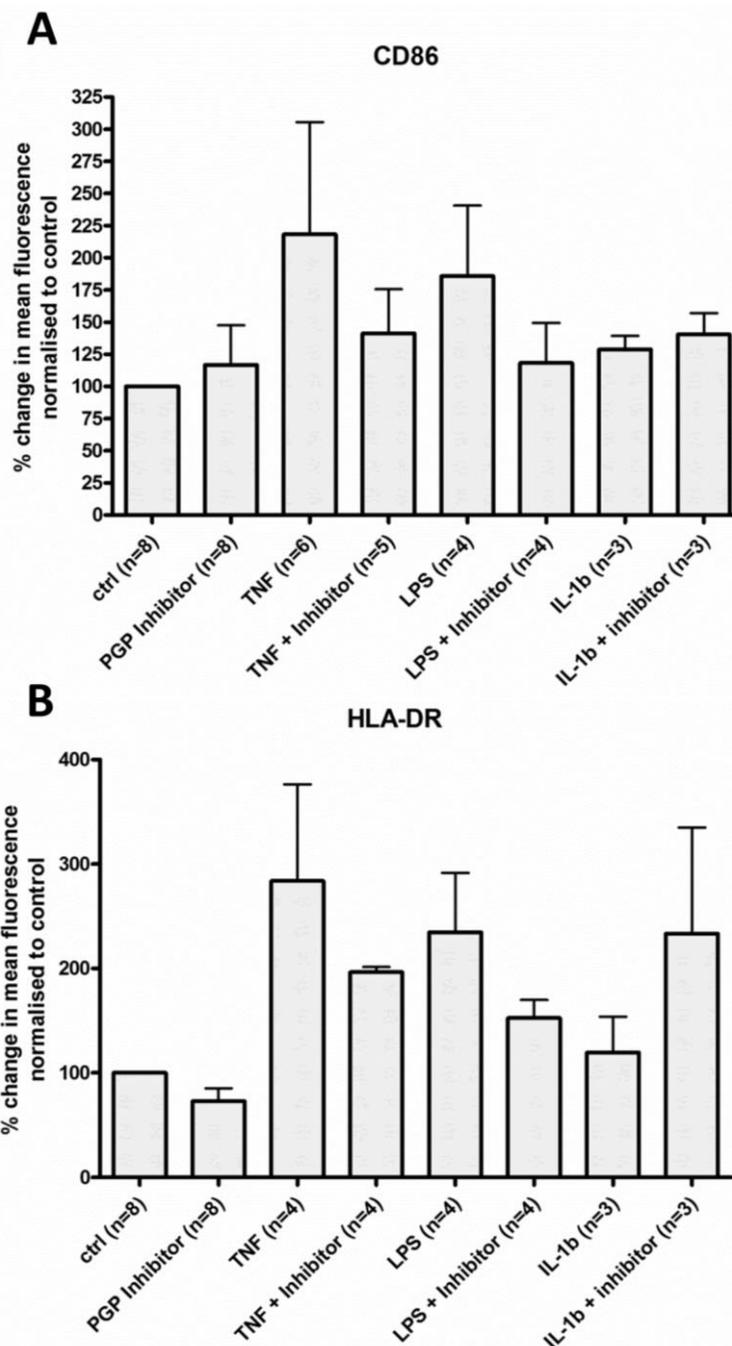
Figure 4.5 – Effects of UCH-L1 Inhibition on Cell Activation Marker Expression

Following differentiation, MoLCs were pre-treated with LDN-57444 or vehicle control for 24 h prior to overnight stimulation with 100ng ml^{-1} TNFα, 50ng ml^{-}1 LPS or 100ng ml^{-1} IL-1β. Live cells were dual stained for CD1a and either CD86 or HLA-DR, and analysed by flow cytometry. **A)** There were no significant changes in CD86 expression despite a trend towards increased expression in stimulated cells and an associated decrease in expression in stimulated cells which had received inhibitor pre-treatment. **B)** Both TNFα and LPS stimulation significantly increased HLA-DR expression (p <0.05). Despite a similar trend towards decreased levels of expression in stimulated cells which had received inhibitor pre-treatment, there were no statistically significant effects following LDN-57444 treatment. Error bars show the mean ±SEM.

Abbreviations: TNF = Tumor Necrosis Factor; LPS = Lipopolysaccharide, IL = interleukin; HLA = Human leukocyte antigen

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IL-1B had no effect on either CD86 or HLA-DR expression. Mean normalised expression values for CD86 and HLA-DR were 128.8% ±10.42 and 119.6% ±34.12 respectively. However, these values ranged from 108.1% - 141.2% for CD86 expression and 63.8% - 181.5% for HLA-DR expression. Since only three repeats were performed for this treatment group, because of time constraints, it is not possible to exclude potential outliers.

These data suggest that UCH-L1 has no role in cell activation via expression of co-receptors for T-cell activation. This is relevant to the *in vivo* models of chronic pain as it suggests that UCH-L1 expression in LCs is not a marker of activation as inhibition of this molecule has no effect on activation marker expression.

v. UCH-L1 Inhibition in MoLCs – Effect on Cytokine Secretion

Following differentiation from negatively selected monocytes and pre-treatment with the UCH-L1 inhibitor LDN-57444 or vehicle control MoLCs were stimulated with TNFα, LPS and IL-1β overnight and cell culture supernatants collected for cytokine analysis. Supernatants were assayed for 12 different analytes (IL's -1β, -2, -4, -5, -6, -8, -10, -17, GM-CSF, IFNγ, TNFα, and MIP1α) using a multiplex ELISA bead assay. Cytokine concentrations were then interpolated from standard curves of known concentration and expressed as pg ml^{-1} of cell culture supernatant. Of the 12 analytes assayed, 10 were consistently and reliably detected in all samples. Although consistently high levels of GM-CSF and IL-4 were detected in all samples, this was almost certainly carry over from the
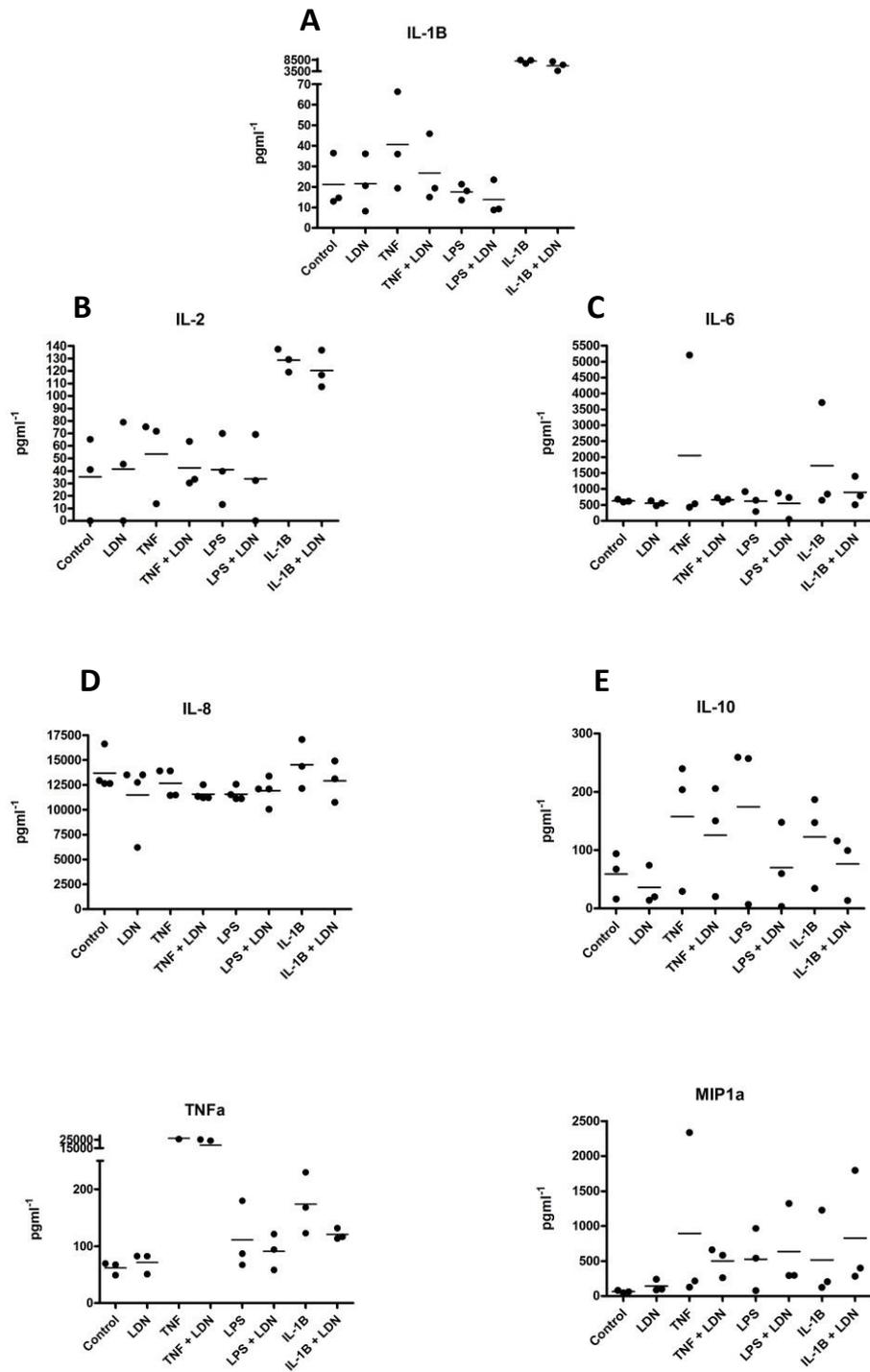
Figure 4.6 – Effects of UCH-L1 Inhibition of Cell Cytokine Secretion

Following differentiation, MoLCs were pre-treated with LDN-57444 or vehicle control for 24 h prior to overnight stimulation with 100ng TNFα, or 100ng IL-1β. Cell supernatants were collected and analysed by multi-plex ELISA for the above analytes. IL-1β was found to significantly increase IL-2 secretion (p <0.05) but there were no significant effects on cytokine secretion following pre-treatment with LDN-57444. Each dot represents a single cell supernatant sample.

Abbreviations: TNF = Tumor Necrosis Factor; IL = interleukin; MIP = Macrophage Inflammatory Protein; LDN = LDN-57444

incubation medium and thus these data are excluded. A summary of the remaining detected 8 cytokines is shown below in Figure 4.6. As with previous experiments, there is significant donor cell variation in response to stimulation. There was no observable difference in the levels of IL-6 or IL-8 secreted in response to stimulation when compared to unstimulated cells despite values being increased in all samples (Fig. 4.6 C&D). Stimulation with TNFα had no effect on IL-2 or MIP1α secretion, but there was a non-significant trend towards increases in IL-10 and IL-1β secretion (Fig. 4.6 A&E). LPS stimulation also resulted in a small increase in IL-10 secretion, but had no effect on any other cytokines (Fig. 4.6E). Stimulation with IL-1β produced a significant increase in IL-2 secretion (t_2 = 4.001, p< 0.05) and non-significant increases in IL-10 and TNFα secretion (Fig. 4.6B,E&F). Pre-treatment with LDN-57444 had no effect on cytokine secretion when compared to vehicle controls. Despite trends towards decreased IL-10 and IL-1β secretion in response to TNFα stimulation, decreased IL-10 secretion in response to LPS stimulation, and diminished IL-2, IL-10 and TNFα secretion in response to IL-1β stimulation there were no significant differences between cells pre-treated with LDN-57444 prior to stimulation and vehicle controls. LDN-57444 pre-treatment did abrogate the significant increase IL-2 secretion following IL-1β stimulation, but since there was no significant difference between IL-1β stimulated and IL-1β + LDN, further repeats would be necessary to explore this relationship. Together, these data suggest that inhibition of UCH-L1 function in MoLCs has no effect on cytokine secretion in response to cell activation.

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vi. UCH-L1 Inhibition in MoLCs – Effect on Chemotaxis

Cell migration is a key aspect of LC function. Detachment from the epidermal matrix and chemotaxis towards the draining lymph node occurs regularly as part of LC sampling of the epidermal environment and also in response to inflammatory stimuli. Under both types of condition, migration is dependent on CCL19 signalling through CCR7 expressed on LCs[279]. Furthermore, the process of LC replenishment under inflammatory conditions is dependent on CCR2 specific recruitment of monocytes from the circulation [209]. To assess the effects of UCH-L1 inhibition on MoLC chemotaxis, cells were pre-treated with LDN-57444 or a vehicle control and primed with a either IL-1β or TNFα. Primed cells were then placed in transwell chambers containing either CCL19 or a vehicle control to assess migratory ability. For each treatment, the number of migrated cells was counted and normalised to the number of migrated cells in untreated controls. Data are presented as percentage chemotaxis normalised to control (Fig. 4.6). Consistent with previous findings, CCL19 significantly increased chemotaxis[259] ($p<$ 0.05). However, donor-dependent variation in response to stimulation is also apparent in these data and was particularly apparent in cytokine-stimulated cells exposed to CCL19, where the mean normalised percentage chemotaxis was 167% ±79.50 and 159% ±89.32 for TNFα and IL-1β treated cells, respectively. In all cases, pre-treatment with LDN-57444 decreased the level of chemotaxis compared to vehicle controls. However, this decrease was only statistically significant in the unprimed cells ($p<$ 0.01) and IL-1β-treated cells ($p<$ 0.05) but

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MoLC Chemotaxis

Figure 4.7 – Effects of UCH-L1 Inhibition on MoLC chemotaxis
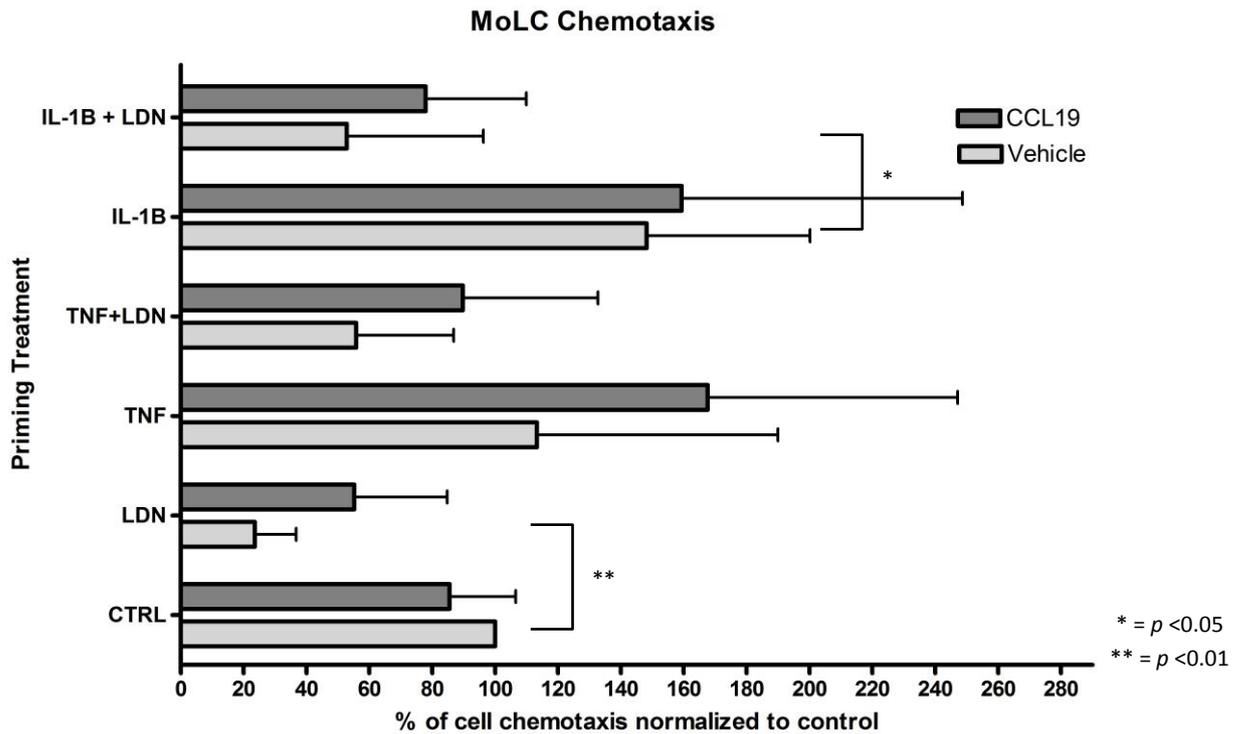
Following differentiation, MoLCs were pre-treated with LDN-57444 or vehicle control for 24 h prior to overnight stimulation with 100 ngml^{-1} TNFα, or 100 ngml^{-1} IL-1β. Cell migration was then analysed by a transwell migration assay in response to CCL19, or a vehicle control. Pre-treatment with LDN significantly decreased cell migration following IL-1β stimulation (p <0.05) and in control cells (p <0.01) in the absence of CCL19. Error bars show the mean ±SEM.

Abbreviations: TNF = Tumor Necrosis Factor; LPS = Lipopolysaccharide, IL = interleukin; LDN − LDN-57444; CCL = CC motif chemokine ligand

not in TNFα treated cells in the absence of CCL19. This statistical significance is abolished, however, in the presence of CCL19, despite the same trend toward reduced chemotaxis in LDN-57444 pre-treated cells. Paired t-test analysis of the whole data set, based solely on the absence or presence of LDN-57444 pre-treatment, showed significant inhibition of chemotaxis in LDN-57444 pre-treated cells, compared to the relevant control ($p < 0.05$), suggesting a role for UCH-L1 in MoLC motility/random migration.

Since the above data has shown inhibition of UCH-L1 has no effect on MoLC activation marker expression or cytokine secretion it would appear the primary role for UCH-L1 in these cells is related to cell motility. In the animal models of chronic pain discussed above, UCH-L1 expression appears in response to epidermal denervation. Thus it is possible that LCs expressing UCH-L1 *in vivo* may be responding to a chemotactic signal or migrating in response to the absence of IENF signalling. A further understanding of this relationship may enhance our understanding of the role LCs play in the onset of chronic pain in these models.

4D) Discussion

In summary, the key finding of this chapter are:

- MoLCs constitutively express UCH-L1 at both the protein and transcript levels.

- Treatment with the UCH-L1 inhibitor LDN-57444 has no effect on UCH-L1 expression or survival in MoLCs

- Chemical Inhibition of UCH-L1 function in MoLCs has no effect on cell activation, as measured by expression of activation markers or cytokine secretion.

- Chemical inhibition of UCH-L1 function in MoLCs significantly decreases MoLC chemotaxis

i. UCH-L1 expression in MoLCs

Various animal models of chronic pain have shown the appearance UCH-L1+ LCs in the epidermis of affected tissues, expression of which parallels the loss of epidermal innervation and the onset of pain sensation [237–239]. Since RNA transcripts for UCH-L1 are elevated in denervated epidermis and the only remaining UCH-L1 immunoreactive cells are LCs, it has been suggested that UCH-L1 expression in these models is as a result of *de novo* synthesis, as opposed to the phagocytosis of material derived from nerves[238]. This has led to speculation

that epidermal denervation, followed by the *de novo* synthesis of UCH-L1 by LCs, has a role to play in chronic pain[239]. LCs derived from CD34+ cord blood precursors do not express UCH-L1 following differentiation[241]. The same group also showed that, despite no UCH-L1 expression by LCs under normal conditions, epidermal disruption and removal of LCs induced UCH-L1 expression[241]. In contrast to the results in cord blood derived LCs I have shown that MoLCs constitutively express UCH-L1 (Fig.4.1). Given that Hamzeh *et al* did not permeabilize differentiated LCs prior to staining, and so only measured cell surface proteins, it is possible this may account for the observed difference in UCH-L1 expression[241]. However, given that both protocols utilized PFA fixation and UCH-L1 staining was decreased in the absence of permeablization as opposed to abolished, it is likely that the apparent differences are not due to protocol dissimilarities. Since MoLCs are derived from peripheral blood monocytes, they have undergone a different differentiation pathway than LCs derived from precursor cells *in vivo,* or directly from CD34+ precursor cells [280]. Furthermore, *in vivo* monocytes are only recruited into the epidermis to replenish the LC population under inflammatory conditions and thus represent a distinct population from LCs generated from an *in situ* precursor cell[277]. Since UCH-L1 expression appears in both LCs isolated directly from disrupted epidermal tissue and also in MoLCs which represent a more inflammatory phenotype, it is possible that UCH-L1 expression is a marker of LC activation. The implications for this in models of chronic pain are not clear although they suggest two possible mechanisms:

a) Following nerve damage and denervation of the epidermis homeostatic interaction between LCs and epidermal nerves are disrupted, leading to cell activation and UCH-L1 expression.

b) Nerve damage and epidermal denervation leads to the recruitment of UCH-L1+ MoLCs, as is seen under inflammatory conditions in which the resident LC population has been eliminated.

It has been suggested that UCH-L1 expression by skin-resident LCs requires both maturation in the presence of neurogenic signalling and the subsequent loss of this or another type of nerve signal[241]. In this chapter the data show that MoLCs do not require culture in the presence of a neurogenic element in order to express UCH-L1, but culture with NA did significantly decrease UCH-L1 expression in MoLCs (Fig. 4.3). Although the decrease was slight compared to the absence of UCH-L1 observed in LCs under steady state conditions, it is possible that repeated application of NA or treatment during the early stages of MoLC differentiation, may have a greater effect. This finding may reflect the close relationship between LCs and IENF *in vivo* and suggests that neurogenic signalling, or absence thereof, may play a major role in LC behaviour. Since many chronic pain models display denervation of the epidermis, understanding the consequences of the removal of this connection between IENF and LCs may have an important bearing on our understanding of chronic pain development.

ii. UCH-L1 Inhibition and MoLC Activation

Consistent with other reports of UCH-L1 inhibition in non-neuronal cell line, the use of LDN-57444 at 10μM did not induce cell death in MoLCs, nor did it have any effect on the expression of UCH-L1 protein as measured by flow cytometry (Fig.4.4). Despite a trend towards decreased levels of activation marker expression in MoLCs pre-treated with LDN-57444, there was no significant difference in the level of either CD86 or HLA-DR expression following stimulation with TNFα, LPS or IL-1β after inhibitor pre-treatment, compared to controls (Fig.4.5). This observation is complicated by donor variation to cell stimulation. Despite an obvious trend towards increased CD86 expression following stimulation, there was no significant difference when compared to unstimulated controls. Similar patterns were observed upon analysis of cell culture supernatant (Fig.4.6). In those treatments where elevated cytokines were detected, such as TNFα induced (Fig.4.6A) IL-1β secretion and IL-1- induced IL-2 secretion (Fig.4.6B), pre-treatment with LDN-75444 decreased the amount of cytokine released, however, this decrease did not reach statistical significance. It is also interesting that the significant increase in IL-2 secretion induced by IL-1β stimulation is abolished in LDN-57444 pre-treated cells, again suggesting that LDN-57444 has an inhibitory effect on cytokine secretion. However given the inherent variation in the data set and the relatively small differences between IL-1β treatments, with and without inhibitor, further work would be required to draw any firm conclusions.

In the context of chronic pain, activation of LCs and secretion of cytokines could contribute to peripheral nerve sensitisation[38]. If UCH-L1 expression was indicative of activation, it may have a role in cytokine production and thus be linked to sensitisation. Since UCH-L1 is thought to be involved in ubiquitin recycling, it is possible that inhibition of this pathway could affect protein translation in general. This hypothesis is supported by the observation that UCH-L1 inhibition by LDN-57444 can cause cell death through endoplasmic reticulum stress and the unfolded protein response[255]. However, data in this chapter suggest that there is no significant effect of UCH-L1 inhibition on cell activation, although a general disruption in protein translation due to the accumulation of misfolded protein could account for the trend towards decreased levels of marker expression and cytokine secretion in LDN-57444-treated MoLCs.

iii. UCH-L1 Inhibition and MoLC Chemotaxis

As discussed above LC migration is a key aspect of skin immunity. The data presented in this chapter show that there is a significant decrease in MoLC motility following pre-treatment with LDN-57444 ($p < 0.05$) (Fig. 4.7). Although decreased levels of CCL19-induced chemotaxis are seen in MoLCs following pre-treatment with LDN-57444, these differences are not significant. However, in the absence of a chemokine gradient, the difference in random cell migration is significant ($p < 0.05$). These data together suggest that UCH-L1 inhibition decreases cell migration but that chemokine sensing remains intact, as evidenced by the increased chemotaxis in response to CCL19.

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A role for UCH-L1 in MoLC migration is consistent with the theory that UCH-L1 expression *in vivo* is associated with activated LC, as cell activation is commonly followed by migration away from the epidermis. Alternatively, *in vivo* recruitment of monocytes to the epidermis to replenish the LC population also requires two stages of chemokine-specific migration, dependent on CCR2 and CCR6 respectively, and thus one might also expect UCH-L1 expression in newly differentiated MoLCs *in vivo*[280].

iv. Conclusions

The data presented in this chapter provides new insights into the role of LCs in models of chronic pain. By investigating the role of UCH-L1 in MoLCs it would now appear that expression of this molecule is primarily related to LC chemotaxis and motility. There was no effect on activation marker expression or cytokine secretion following inhibition of UCH-L1 which suggests that expression *in vivo* is not linked to inflammatory processes in these cells e.g. active cytokine secretion and/or induction of adaptive immunity. Since chemotaxis was significantly reduced following inhibition it seems likely that UCH-L1 expressing LCs *in vivo* are responding to chemotactic stimuli or, given the relationship between IENF and LCs, the removal of a neurogenic signal such as NA. Further work is needed to fully understand this relationship but these data indicate UCH-L1 expression in LCs is not linked to cell activation, and thus it is unlikely these cells are responsible for generating inflammation or sensitising nerves via cytokines.

CHAPTER 5: Expression and Function of Immune Cell Adrenergic Receptors in CRPS

5A) Introduction

Adrenergic receptors are widely-expressed throughout the peripheral nervous system and mediate responses to the catecholamines, such adrenaline and noradrenaline (NA), the principle neurotransmitters of the sympathetic nervous system. The wide-ranging implications of adrenergic receptor signalling in different tissues is typified by the classical "fight or flight" response which results in a variety of physical responses, including a short-lived augmentation of immune cell function[281]. In CRPS pathology dysregulation of sympathetic signalling is demonstrated by changes to skin sweating and blood flow[136]. Furthermore, the aberrant coupling of efferent sympathetic signalling to afferent nociceptive sensing can produce so-called sympathetically-maintained pain (SMP), whereby nociceptors may become hypersensitive to NA via altered adrenergic receptor expression[135]. This theory is supported by data from animal models of type II CRPS, in which nerve injury alters the adrenergic receptor profile of peripheral sensory fibres which then develop hypersensitivity to NA[138–140]. This relationship may occur *in vivo,* as intra-dermal injection of NA induced pain in patients with suspected SMP[141]. Altered levels of circulating catecholamines in affected and non-affected limbs of CRPS patients has been reported, a scenario that could promote differential adrenergic receptor

expression on peripheral nerves[135,137]. However, more recent work has shown no difference in the amount of NA released in response to dermal sympathetic activation in chronic CRPS patients, compared to controls[282].

Because sympathetic dysfunction is suspected in CRPS, abnormal immune activation, through adrenergic receptor signalling mechanisms, may also play a role in disease pathology. The expression of adrenergic receptors on immune cells has not been fully defined, but current ideas support the view (see Chapter 1 – Table 4) that β2-adrenergic receptors (-ARs) are the most widely-expressed, and serve an anti-inflammatory role following ligand binding, but can be pro-inflammatory in the presence of other activating signals[147,151,283]. A similar pro-inflammatory synergy has been described following the stimulation of peripheral blood mononuclear cells with an α2-AR specific agonist and LPS[149,152,153]. Interestingly, the α1-ARs do not appear to be expressed in mononuclear cells but can be up-regulated by inflammatory stimuli[154–156]. This expression under inflammatory conditions has also been described in the peripheral blood cells of patients with juvenile idiopathic arthritis, a finding that was linked to increased IL-6 production by immune cells following a noradrenergic stressor[159,160]. Furthermore, a single nucleotide polymorphism in the α1a-AR gene was recently identified as a risk factor for CRPS development[161]. Thus, dysregulated expression of adrenergic receptors on immune cells in CRPS could influence the progression or maintenance of disease. This effect may also be enhanced by activating auto-antibodies which have recently been identified in CRPS, and could directly

modulate of immune cell behaviour by functionally binding to or blocking ARs[109,114]. This finding is of particular interest given recent work suggesting that a greater number of peripheral blood monocytes in CRPS patients express a pro-inflammatory marker when compared to healthy controls[93].

i. Hypothesis

Since it is believed longstanding CRPS is mediated by central changes to nervous signalling, alterations in efferent sympathetic pathways may influence immune cell behaviour. Elucidating potential differences in this signalling through examining altered adrenergic receptor expression by immune cells could elucidate new mechanisms of disease and pathways for therapeutic intervention. Thus the hypothesis to be tested in this chapter is "**Altered adrenergic receptor signalling in immune cells in longstanding CRPS contributes to disease pathology, a process possibly mediated by adrenergic receptor auto-antibodies**".

5B) Aims

Adrenergic receptors represent an interesting mechanism in CRPS disease due to the established sympathetic dysfunction in the disease. Furthermore, differential expression of adrenergic receptor subtypes by immune cells could alter immune cell behaviour in response to efferent nerve signalling, providing an important link between these two systems. However, the adrenergic receptor profile of

immune cells in CRPS, and the potential effects of auto-antibody activation through these receptors, has not yet been described. The specific aims of this chapter are to:

- Determine the adrenergic receptor expression profile on peripheral blood monocytes isolated from CRPS patients

- Measure the effects of auto-antibody stimulation on monocyte activation

5C) Results

i. Adrenergic Receptor Expression in Peripheral Blood Mononuclear Cells

As the expression of α1-ARs has previously been linked to inflammatory disease, initial work focused on the characterisation of these receptors in peripheral blood cells. In the first set of experiments α1a-AR expression in PBMCs was assessed following stimulation with LPS (100ng/ml), TNFα, IL-1β and IL-6 (all at 10ng/ml) (Fig. 5.1). Although no changes in expression were observed during these treatments, interpretation of data was hampered by the detection of non-specific proteins. Given the structural similarity of receptor subtypes, the possible glycosylation of the receptors, and the similar predicted masses (α1a = 52kDa; α1b = 56kDa; α1d = 60kDa) it seems likely that the antibody was incapable of differentiating between the three receptor subtypes.

Due to the apparent staining of other receptor subtypes by western blot, it was decided to abandon subtype specific staining and use a pan-α1-AR antibody. This
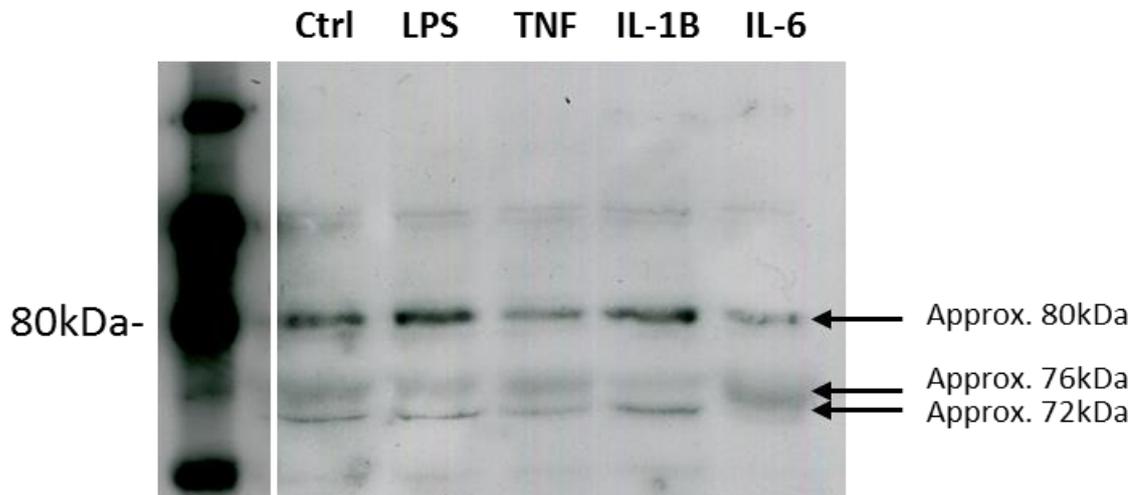
Figure 5.1 – Detection of alpha adrenergic receptor subtypes in PBMC protein lysates

Peripheral blood cells isolated by density dependent centrifugation were stimulated with LPS (100ng/ml), TNFα, IL-1 or IL-6 (all 10ng/ml) for 18 h before protein extraction and western blotting using an α1a-adrenergic receptor antibody. Non-specific staining was apparent in all samples, possibly indicative of expression of other α1-adrenergic receptor subtypes.

Abbreviations: LPS = lipopolysaccharide; TNF = tumor necrosis factor alpha; IL- = interleukin

approach assesses expression of all three α1-AR subtypes using flow cytometry.

Although this method cannot differentiate between subtype receptors it allows

for combination with other cell marker antibodies thus defining which immune

cell populations are positive for α1-ARs. Peripheral blood monocytes had

previously been shown to express α1-AR and so CD14, a monocyte-specific cell

marker, was used in combination with the pan-α1-AR antibody to assess

receptor expression in PBMC fractions (Fig. 5.2)[156]. As expected CD14, was

detected only in the larger, more-granular population of cells, as assessed by

forward and side scatter; a finding consistent with monocyte-specific expression

of CD14 (Fig. 5.2 D&E). Staining with the pan-α1-AR antibody showed a similar

pattern of expression as CD14, mostly restricted to large granular cells, but with

some positive staining also being observed in the population of cells usually

categorized as lymphocytes (Fig. 5.2 B&C). Dual staining-confirmed that all

CD14+ cells were also pan-α1-AR+, with a second population of cells staining as

CD14-/pan-α1-AR+, probably representing lymphocytes or other small non-

granular cells (Fig. 5.2A). Staining protocols in which the primary antibody was

excluded or replaced by an isotype control antibody were negative. Staining with

a β2-AR showed positive staining in all PBMCs, consistent with previous findings

of widespread β2-AR expression[151] (Appendix Fig. 4).
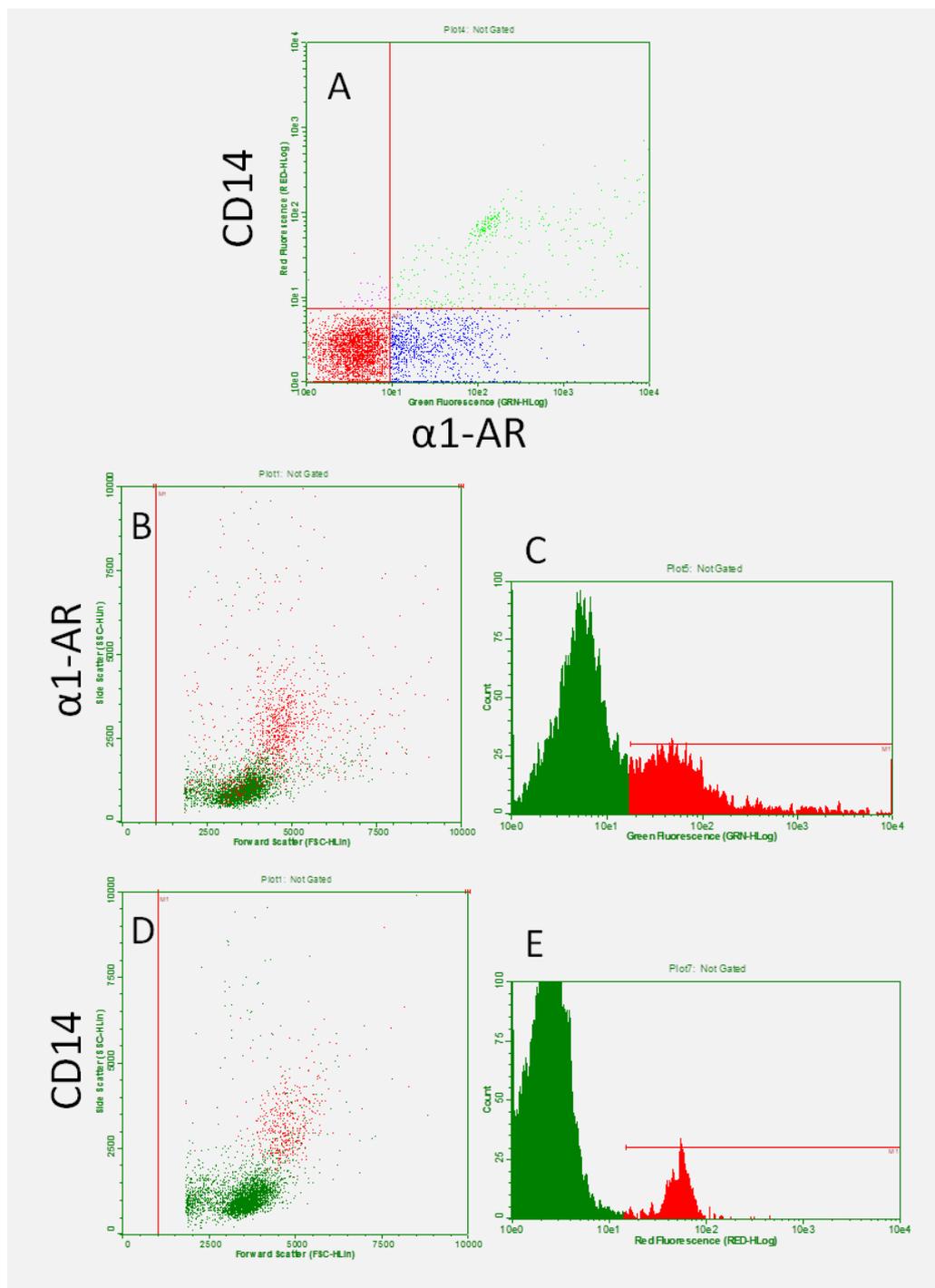
Figure 5.2 – Identification of alpha 1 adrenergic receptor-expressing peripheral blood cells

Peripheral blood cells isolated by density dependent centrifugation were stained directly using a PerCP conjugated anti-human CD14 antibody or indirectly using a pan-α1adrenergic receptor antibody with an Alex Fluor 488 secondary antibody. **A**) Dual staining of PBMCs shows a majority of CD14/pan-α1 double positive cells. **B&C**) Backgating of pan-α1+ cells shows expression is predominantly restricted to large granular cells, as assessed by forward and side scatter. **D&E**) Backgating of CD14+ cells shows staining is restricted to the large granular cell population attributed to peripheral blood monocytes.

Abbreviations: α1-AR = α1-adrenergic receptors; PBMC = Peripheral blood mononuclear cells

ii. *Alpha 1 Adrenergic Receptor Expression in CRPS patient Monocytes*

To investigate any differences in α1-AR expression in CRPS patients compared to healthy controls, PBMCs were isolated from the peripheral blood of CRPS patients by density -dependent centrifugation, followed by the purification of monocytes by negative magnetic isolation. Monocytes were then stained (as above) and the expression of α1-AR measured as a percentage of dual-positive cells (Fig. 5.3). The mean percentage of cells showing dual-positive expression was 86% ±4.38 and 85% ± 5.05, for healthy control and CRPS monocytes, respectively. There was no significant difference between the means of the two groups.

iii. *Alpha Adrenergic receptor subtype expression in CRPS monocytes*

As discussed previously, it has shown that changes in adrenergic receptor subtype expression can affect cellular response, and so a pre-optimized TaqMan™ qPCR assay was used to assay for differences in α1- and α2-AR subtype expression in peripheral blood monocytes. Human heart cDNA was used as a positive control for the primers and β-actin used as an internal reference gene for normalisation. No transcripts were detected for any of the α1-ARs in cDNA generated from either CRPS or control monocytes (Fig. 5.3). Of the three α2-AR subtypes analysed, both α2b and α2c showed a trend towards increased levels of expression in healthy cells when compared to CRPS, but the difference was not statistically significant.
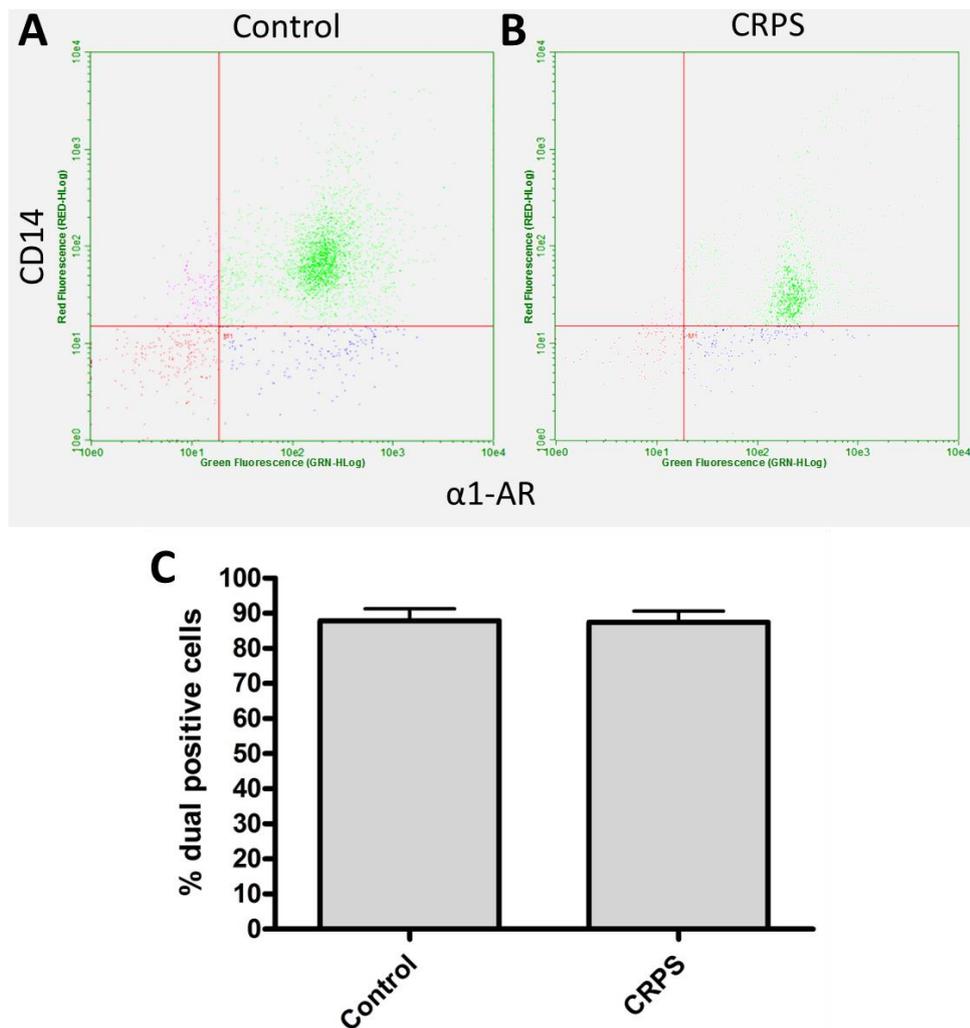
Figure 5.3 – Expression of α1-adrenergic receptors on CD14+ monocytes in CRPS

After peripheral blood monocytes were purified by negative magnetic isolation, cells were stained directly using a PerCP conjugated anti-human CD14 antibody or indirectly using a pan-α1 adrenergic receptor antibody with an Alex Fluor 488 labelled secondary antibody. **A&B**) representative scatter plots of dual-stained cells showing α1-adrenergic receptor expression is predominantly restricted to CD14+ monocytes. **C**) There was no significant difference in the percentage of α1-adrenergic receptor/CD14 dual positive monocytes isolated from CRPS donors compared to healthy controls. Data shown is the mean of 3 experiments. Error bars show the mean ±SEM.

Abbreviations: α1-AR = α1-adrenergic receptors

(p = 0.266 & p = 0.283 respectively). The α2a subtype was not detected in either CRPS or control, monocytes. β2-AR transcripts were detected in all samples, with no observable difference in levels following normalisation. As expected from previous work, mRNA representing all of the adrenergic receptor types were detected in cDNA from human heart tissue[284]. The apparently decreased levels of expression in heart tissue compared to monocyte cDNA may be a product of both a reduced level starting material compared to the monocyte assays and also differential expression of the reference gene in heart tissue i.e. increased levels of β-actin relative to adrenergic receptors in heart tissue compared to the same relative difference in monocytes. The conflicting results of adrenergic receptor expression are discussed in more detail later.

iv. Adrenergic receptor mediated monocyte activation in CRPS

Following the detection of α1-AR protein by antibody staining and the contrasting absence of specific transcripts at the mRNA level, the effects of adrenergic receptor stimulation on monocytes were examined. It has previously been demonstrated that monocyte activation, measured by IL-1β production, in response to LPS stimulation is synergistically increased in the presence of phenylephrine (PE) in a α1-AR specific manner[260]. It has also been hypothesised that auto-antibodies may play a role in maintenance of CRPS disease by binding to and activating adrenergic receptors[109]. To investigate the possible role of auto-
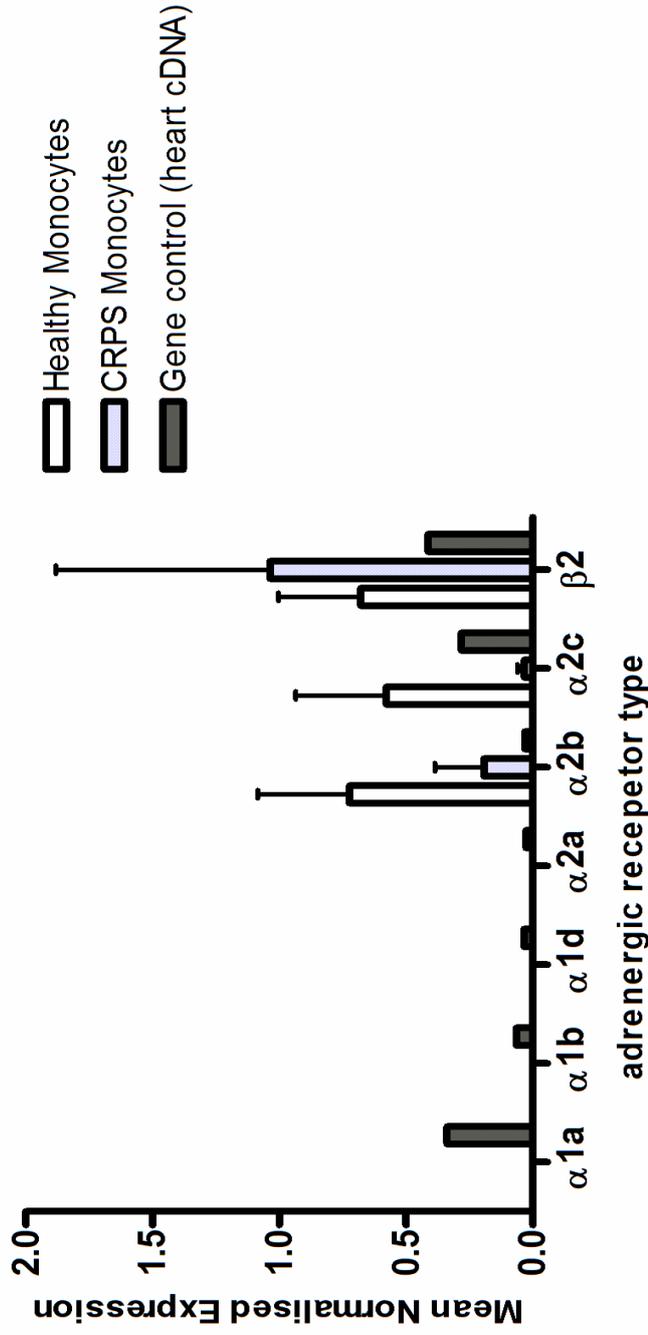
Figure 5.4– Expression of α1, α2- and β2- adrenergic receptor RNA transcripts in monocytes in CRPS

RNA was extracted from negatively-selected peripheral blood monocytes isolated from CRPS patients and healthy controls. Following reverse transcription PCR adrenergic receptor gene expression was assessed by qPCR using pre-optimised TaqMan™ primer assays to detect all α1- and α2-adrenergic receptor subtypes and the β2-adrenergic receptor. β-actin was also analysed as an internal reference gene and human heart cDNA was included as a positive control. Cycle threshold values were normalised to the internal reference gene and transcript levels expressed as mean normalised expression. No transcripts for α1-adrenergic receptor subtypes were detected in monocytes and there was no significant difference in the expression of any of the α2-adrenergic receptor subtypes or the β2-adrenergic receptor. Data shown is the mean of 3 repeats with the exception of human heart cDNA (n = 1). Error bars show the mean ±SEM.

antibody-mediated monocyte activation, previous experiments, showing

synergistic IL-1β production following TLR and α1-AR stimulation[260], were first

replicated using negatively-selected healthy monocytes stimulated with 25ng/ml

LPS and 10μM PE for 3 h (Fig 5.5). Protein lysates from stimulated cells were

subject to SDS-PAGE and western blotting for IL-1β and actin. Densitometry was

used to assess protein level relative to unstimulated cells and data presented as

fold change normalised to actin (Fig 5.5 A&C). In line with previous observations

by others[260], LPS treatment significantly increased IL-1β production, compared to

control (p = 0.0161) and the addition of PE caused a further increase in IL-1β

production which was significantly greater than the increase observed with LPS

alone (p = 0.0391).

To test the hypothesis that adrenergic receptor-activating auto-antibodies

present in CRPS may produce similar synergistic effects to PE when added in

conjunction with LPS serum, samples from CRPS patients and matched (age &

sex) healthy controls were subject to protein G affinity chromatography to

isolate IgG fractions. Following elution, IgG fractions were adjusted to

approximate physiological concentrations (7-16mg/ml)[285] and incubated with

monocytes prior to protein isolation and western blotting as above (Fig 5.5 B&C).

A total of 3 matched IgG samples from CRPS patients and controls were tested

on healthy donor monocytes in each experiment. LPS stimulated cells showed

significantly greater levels of IL-1β compared to unstimulated (ctrl) cells after the

addition of both CRPS IgG (p = 0.0229) and healthy control IgG (p = 0.0197)
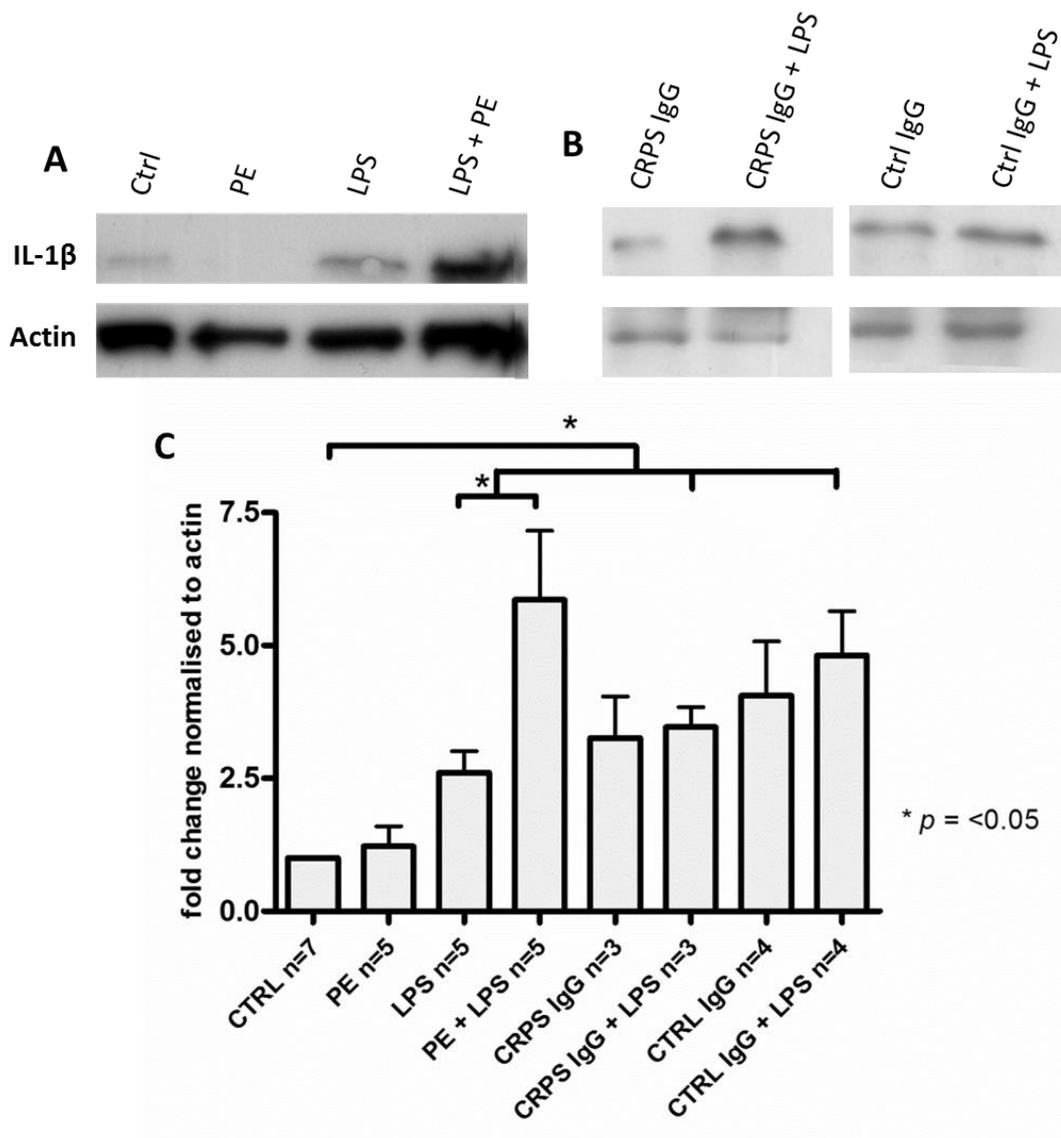
Figure 5.5– IL-1β production in monocytes stimulated with LPS and either phenylephrine, or IgG fractions from CRPS and healthy donors.

Negatively-selected monocytes isolated from healthy donors were stimulated with LPS in the presence of phenylephrine (10μM) or human IgG fractions from CRPS and healthy donors (7-16mg/ml) for 3 h. Cell protein was then extracted and subject to SDS-PAGE before western blotting with an anti-human IL-1β antibody. Protein levels were assessed by densitometry and data expressed as fold change normalised to actin **A**) A representative western blot showing that LPS stimulation increases expression of IL-1β in monocytes and the addition of phenylephrine causes a further significant increase which is not observed in cells treated with phenylephrine alone **B**) A representative western blot showing that stimulation with IgG fractions from both healthy and CRPS serum alone, induce increased IL-1β expression in monocytes and that addition of LPS further increases IL-1β production. **C**) Analysis of mean normalised expression showed monocytes stimulated with LPS (with any additions) significantly increased IL-1β expression ($p < 0.05$) and that the synergistic increase in production following the addition of phenylephrine was also statistically significant ($p < 0.05$). Error bars show the mean ±SEM. Data analysed using paired t-tests or Wilcoxon signed ranked test for non-normal data (bars 4-8).

Abbreviations: LPS = lipopolysaccharide; PE = phenylephrine; IL- = interleukin

analysed by paired t-test (or Wilcoxon signed ranked test for non-normal data sets). Treatment with both CRPS and healthy control IgG in the absence of LPS resulted in a marked increase in IL-1β production, but this difference did not reach statistical significance in either case (p = 0.1001 and p = 0.0569, respectively). There was no significant difference between LPS stimulated cells and those stimulated with LPS in the presence of CRPS IgG; and despite a trend towards increased IL-1β production in monocytes stimulated with both LPS and healthy control IgG, compared with LPS alone, the difference was not significant (p = 0.1015). These data suggest that although there may be low levels of IgG-mediated activation of monocytes, it is independent of LPS stimulation and thus does not mimic the synergistic increase in IL-1β production apparent in the presence of PE. These data suggest that any auto-antibodies present in CRPS IgG are not functionally binding to ARs in a manner which could influence cell behaviour, similar to NA or PE but that non-specific activation may be occurring through other mechanisms. Findings which have direct bearing on the regulation of monocytes in CRPS patients with longstanding disease.

v. IgG-mediated monocyte activation in CRPS

To examine the direct effects of IgG stimulation on monocytes independently of LPS stimulation, negatively-selected monocytes were stimulated as above but
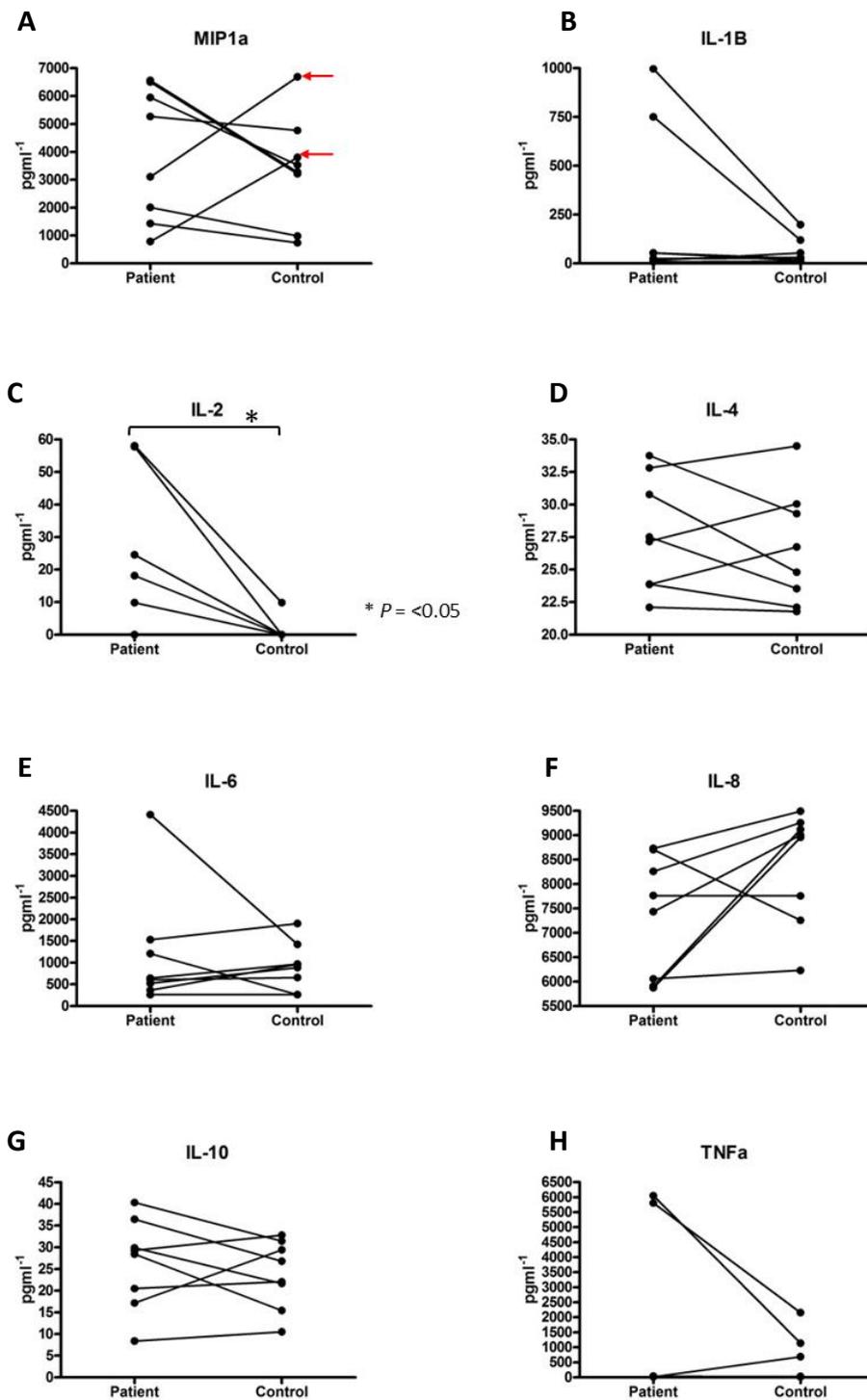
Figure 5.6 – Cytokine secretion by monocytes stimulated with CRPS or healthy IgG fractions.

Negatively-selected monocytes from healthy donors were stimulated with IgG fractions from CRPS serum or matched healthy controls for 6 h. Cell culture supernatants were then harvested and assayed by multiplex ELISA for the above cytokines. Each data point represents a single experiment from the same donor monocytes stimulated with matched IgG samples. **A-H**) IL-2 secretion was significantly increased in cells stimulated using CRPS IgG compared to matched controls (p = <0.05), while the increase in MIP-1α also reached significance following exclusion of outliers (shown by red arrows). Data analysed by paired t-test or Wilcoxon signed ranked test for non-normal data sets (graphs B,C,F & H)

Abbreviations: MIP1a = macrophage inflammatory protein 1α; IL- = interleukin; TNFa = tumor necrosis factor α

was specific for 12 different analytes (IL's -1β, -2, -4, -5, -6, -8, -10, -17, GM-CSF, IFNγ, TNFα, and MIP1α). Cytokine concentrations were then interpolated from standard curves of known concentration standards and expressed pgml^{-1} of cell culture supernatant. Of the 12 analytes assayed IL-5, IFNγ, GM-CSF and IL-17 were all below the reliable minimum threshold of the assay, while the results of the remaining 8 cytokines are shown below in Figure 5.6. Each pair of data points is a single experiment using healthy monocytes from a single donor, stimulated with either CRPS IgG or an age- and sex-matched healthy control IgG fraction.

Paired t-tests (or Wilcoxon signed ranked test for non-normal data sets) for each analyte were used to assess statistically significant differences. Only the increase in IL-2 secretion in CRPS IgG-stimulated monocytes was shown to be significant, when compared to matched controls (p = 0.0415) (Fig 5.6 C). CRPS IgG-treated monocytes also showed a general trend towards increased levels of MIP1α secretion, but this did not reach statistical significance, as 2 experimental repeats, both using IgG from the same CRPS patient, displayed an opposing pattern (red arrows in Fig 5.6 A). Statistical analysis, excluding experiments using this patient's IgG did show significantly increased levels of MIP1α secretion in CRPS IgG-treated monocytes when compared to matched controls (p = 0.0164) suggesting differential effects in different patient samples. Both IL-1β and TNFα displayed outliers within the CRPS patient group, but these were IgG fractions from different patients and previous repeats on different donor cells did not produce the same response (Fig 5.5 B&H). IL-8

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showed a weak trend towards decreased secretion in monocytes stimulated with CRPS IgG when compared to matched controls, but the trend did not reach statistical significance (p = 0.1026). Statistical significance was achieved by excluding a single data point, but in this case there were no justification for exclusion as there was no indication that the IgG fractions or the donor cells for that experiment behaved unusually. Analysis of IL-4, -6 and -10 levels were not significantly different following incubation with IgG from CRPS patients or healthy controls (Fig. 5.5 D,E&G).

Although based on small data sets these results present a potentially crucial difference in monocyte response to IgG when comparing longstanding CRPS patients to matched healthy controls. Despite the above data showing that functional auto-antibody binding was not occurring via α1-ARs it does appear that healthy monocytes produce a more inflammatory response to CRPS IgG than healthy IgG. Although further work is needed to establish the mechanism of this regulation, discussed below, it could indicate a crucial mechanism for immune dysregulation in longstanding patients and thus improve treatment and diagnosis of this patient cohort.

5D) Discussion

In summary, the key finding of this chapter are:

- α-Adrenergic receptor protein is expressed on peripheral blood monocytes from both healthy individuals and CRPS patients.

- qPCR analysis of adrenergic receptor gene transcripts showed no expression of any α1-AR subtypes and only limited expression of α2-AR subtypes in CRPS or control monocytes.

- There was no difference between levels of adrenergic receptor expression detected at either the protein or RNA level, in CRPS monocytes compared to healthy controls.

- Stimulation of healthy monocytes with IgG fractions from CRPS serum, in conjunction with LPS, did not induce a synergistic increase in IL-1β expression, in contrast to the effects of PE and LPS.

- Stimulation of healthy monocytes with CRPS IgG, induced a significant increase in IL-2 secretion compared to control, and also a significant increase in MIPα1 secretion in two of the three CRPS patients tested.

i. Alpha adrenergic receptor expression in monocytes

Initial work aimed at identifying α-adrenergic receptor subtypes expressed on peripheral blood cells, was hampered by difficulties in characterising proteins of similar structure and function (Fig. 5.1). The structural similarities of the α1-AR subtypes make them difficult to distinguish by techniques such as western blotting. Indeed, a study using selective α1-AR family knockout mice showed that many commercially available antibodies, claiming to be subtype specific, were in fact binding to multiple subtypes[286]. Given the appearance of three bands on the western blot using an antibody that is marketed to be specific for only the α1a-AR subtype (Fig. 5.1), indicated that the antibody used in this chapter was also non-specific. The relative predicted molecular weights of the detected bands suggests that the three subtypes were detected i.e. the 80kDa band is equivalent to the α1d-AR, with each receptor running with apparent molecular masses at approximately 20kDa greater than the molecular weight predicted from the amino acid sequence. However, this theory is based on identical post translational modification for each receptor type.

In order to address the issue of subtype-specific adrenergic receptor expression, a multi-format approach was developed based on live cell staining with a pan-α1-AR and flow cytometry, and RNA analysis for specific adrenergic receptor subtypes. Initial characterisation of α1-ARs, indicated that expression was mostly restricted to CD14+ monocytes (Fig 5.2). This finding conflicts with previous work suggesting that peripheral blood monocytes do not express α1-ARs, based on

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functional response to PE stimulation and mRNA expression [155,159,160]. Another study, using a combination of antibody staining, mRNA analysis and radio ligand binding, reported expression of all three α1-AR subtypes in PBMCs and the same group also used *in situ* hybridization and immunocytochemistry to show α1-AR expression (predominantly the α1a- and α1b- subtypes) in PBMCs[287,288]. Similarly evidence for functional α1-AR expression in purified monocytes has also been described[289]. However, doubts remain over the specificity of subtype specific ligands and, as previously described, subtype specific antibodies[286,290]. The reliance on mRNA analyses is also subject to criticism, as studies in mononuclear cells from murine bone marrow and the human monocytic cell line THP-1, indicate that both cell types express mRNA for α1-AR suggesting, translation and protein expression may occur early in cellular development [155,291]. The conflicting opinions on adrenergic receptor expression in immune cells are summarised by Grisanti, Perez & Porter[292].

Analysis of negatively-selected monocytes from CRPS patients and healthy controls showed no difference in the expression of α1-ARs, when analysed using a pan-specific antibody (Fig. 5.3). It has previously been shown that monocyte α1-AR subtype expression is differentially modulated by different pro- and anti-inflammatory stimuli, including PHA and LPS, and glucocorticoid and dexamethasone [157,160]. If different adrenergic receptor profiles were expressed on CRPS monocytes compared to healthy cells then these may not be distinguished using the pan-specific antibody. However, on examination of

monocyte mRNA, no α1-AR transcripts were detected in monocytes from either healthy controls or CRPS donors (Fig. 5.4). These data are consistent with the current literature, as discussed above[156,292]. There are several possible explanations for these findings. The first possibility is that α1-AR expression at the protein level occurs at an early point in cell development, and further gene expression only occurs following direct cell stimulation, as previously described [157,160]. Assuming this hypothesis is correct, the similarity in CRPS and healthy monocyte transcription profiles suggest that there is no altered expression profile in CRPS monocytes, as has been shown in other inflammatory conditions[159,160]. Alternatively, the pan-α1-AR antibody used may be cross-reacting with α2-ARs expressed on monocytes, transcripts of which were detected by qPCR: thus, α1-AR may not be expressed by peripheral blood monocytes. One final possibility is that the qPCR assay was not sensitive enough to detect low copy numbers of transcripts. However, the β2-AR receptor has also been shown to have relatively low transcript copy numbers in various tissues[156]. As β2-AR transcripts were detected in all samples and subtype specific α2-ARs were also detected, this explanation seems unlikely. Likewise, cDNA from human heart tissue, at a minimum concentration for the qPCR assay, was positive for all the adrenergic receptors tested (Fig. 5.4).

ii. Effects of CRPS immunoglobulin G on monocyte function

Previous work has established that a subset of CRPS patients possess auto-antibodies directed against nervous system structures, including adrenergic

receptors[109,113,114]. A synergistic increase in monocyte IL-1β production has also been described following treatment with LPS, in the presence of the α1-AR specific agonist PE[260]. The above data replicates previous work by Grisanti *et al*[260] and shows a synergistic increase in LPS-induced IL-1β expression when stimulated in the presence of PE (Fig 5.5 A). This also supports earlier data that peripheral blood monocytes express functional α1-AR, despite the lack of transcripts detected at the RNA level.

To explore the possible role of α1-AR activating auto-antibodies in CRPS disease, IgG fractions from CRPS patients and healthy controls were used in conjunction with LPS stimulation and IL-1β western blotting, to assess the synergistic effects with α1-ARs (Fig. 5.5 B). Despite increased levels of IL-1β production in IgG stimulated cells, there was no synergy when co-incubated with LPS (Fig 5.5 C). Only those cells treated with LPS showed significantly increased levels of IL-1β production, compared to untreated cells and only the LPS-PE combination showed significantly more IL-1β than LPS alone. Because monocytes are known to express IgG specific Fc-γ receptors, which induce cell activation once cross-linked, it is possible that the trend toward activation in IgG-treated cells is Fc receptor mediated[293].

One interesting aspect of CRPS pathology is that intravenous immunoglobulin (IVIG) therapy has proven to be effective in some patients, and it has been suggested that IVIG efficacy in a variety of inflammatory and auto-immune conditions, is due to the up regulation of an inhibitory Fc receptor[26,118,294].

To examine the direct effects of CRPS IgG on healthy monocytes, independently of the LPS-IL-1β synergy model, cell culture supernatants were analysed by multiplex ELSIA following cell stimulation (Fig 5.6). Healthy monocytes stimulated with CRPS IgG, secreted significantly greater amounts of IL-2, compared to healthy controls ($p< 0.05$) and two of the three CRPS patient IgG fractions also stimulated significantly increased MIP-1α secretion ($p< 0.05$) (Fig 5.6 A&C). This could indicate different methods of activation through which CRPS IgG, but not healthy IgG, produces a more inflammatory monocyte profile.

Auto-antibody activation of α1-AR receptors is unlikely to be responsible for these differences, given the absence of synergy in the LPS-IL-1β model. Furthermore, functional binding via other auto-antibody epitopes, such as the β2-AR, would be expected to produce an anti-inflammatory response[114,295]. Cross-linking of Fc-γ receptors would cause cell activation but this is commonly-associated with TNFα and IL-6 secretion, which was not apparent in the above data[296,297] (Fig 5.6 E&H). Increased MIP-1α may have particular relevance to CRPS disease, as it has been demonstrated to induce pain through nociceptors sensitisation[298]. IL-2 has also been reported as elevated in the blood of chronic CRPS patients in a recent meta-analysis, but the same analysis showed increased levels of IL-4 and TNFα, which was not seen in the above experiments.

iii. Conclusion

In conclusion the data presented in this chapter show that, despite conflicting evidence on adrenergic receptor expression in peripheral blood monocytes, CRPS

patient IgG does not functionally bind to ARs on these cells in a manner similar to NA or PE. Thus it would appear that activating auto-antibodies do not modulate monocyte cell function via this pathway. However, further data shows that CRPS patient IgG does produce a more inflammatory cytokine profile in healthy monocytes when compared to healthy control. This suggests that, although the hypothesised anti-α1-AR antibodies may not play a role in modulating immune cell function, there may be an alternative mechanism which is specific to CRPS patients.

Since IgG fractions were isolated from patients with longstanding disease this finding may indicate a mechanism by which peripheral immune cells are dysregulated in CRPS patients with longstanding disease. This is an important observation as it highlights on-going changes in the immune system in the absence of active inflammation and suggests future therapy and research could target this disease mechanism. Furthermore, if CRPS patient monocytes are differentially regulated via auto-antibody interactions it could have downstream effects on terminal differentiation, such as monocytes recruited to skin tissue in order to develop LCs. Further research is needed to investigate these possible links and elucidate potential avenues for therapy or diagnosis using this knowledge.

CHAPTER 6: Discussion

CRPS is a chronic pain condition which causes severe debilitation in many affected individuals and significantly reduces quality of life[17]. The wide variety of symptoms and the progression from acute to chronic disease displays the complex underlying disease etiology[18]. Despite clear evidence that the immune system plays a key role in CRPS disease significant gaps remain within our understanding of how the immune system might contribute directly to disease development and maintenance[109,299,300]. Furthermore this lack of understanding contributes to the difficulty in establishing definitive and reliable diagnoses of CRPS due to the absence of reliable biomarkers for disease. It is likely that different underlying disease etiologies exist in different patients and that these diverse mechanisms of disease combine to produce the wider syndrome. This concept is summarised in the integrative conceptual model of CRPS (Fig. 1.2)[18].

The aim of this research was to better understand how the immune cell population in CRPS affected tissue may vary from that in healthy tissue and also how immune cell status may be modulated by aspects of the nervous system, a relationship of particular relevance to CRPS disease[301].

In chapter 3, I examined skin tissue from patients with longstanding CRPS. The data from the histological sections showed there was no difference in immune cell infiltration or mast cell density in CRPS affected tissue compared to non-affected or healthy control tissue (Fig 3.2). In early stage CRPS there is visible

swelling and inflammation in the affected limb and recently it has been reported that mast cell numbers are increased in early stage patients even after the resolution of obvious inflammation[86,302]. The role of mast cells in facilitating neurogenic inflammation is well described and mast cells have been identified as potential targets for treatment in CRPS[73,86,271].

As discussed previously it is possible that mast cell involvement is a factor in early stage disease and thus normal mast cell densities are restored in patients with longstanding disease. Interestingly mast cell activation is classically associated with allergic responses (through IgE specific Fc-Ɛ receptors) and T-helper 2 immune activation[303]. Activation of mast cells in the early stages of disease could lead to secretion of class switching cytokines such as IL-4 which in turn would bias T-cell responses in affected tissue towards a Th-2 phenotype[304]. The finding that tissue resident T-cells are biased towards a Th-2 phenotype in CRPS affected tissue compared to non-affected tissue supports this hypothesis (Fig 3.6). It has also previously been suggested that peripheral blood cells in CRPS patients may exhibit a Th-2 phenotype[92]. However the Th-2 inducing potential of mast cells has mostly been described in relation to allergic sensitization or pathogens such as helminths and it is not clearly understood if neurogenic stimulation in the absence of other sources of stimulation can produce the same Th-2 polarisation[305,306].

The difference in LC densities observed in CRPS affected limbs compared to non-affected tissue could also have implications for wider immunity (Fig 3.4).

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As discussed earlier, the difference in LC density is more difficult to interpret as it could represent either a relative increase (in non-affected tissues) or relative decrease (in CRPS affected tissue) or combination of the two. One interesting link between the T-cell data and LCs is that the neuropeptide CGRP which has been shown to induce Th-2 responses in LCs including enhanced antigen presentation to Th-2 T-cell clones, increased secretion of IL-4 in activated LCs and a skew towards Th-2 chemokine secretion[216,218]. If cortical reorganisation occurs in long standing cases of CRPS then it is possible that efferent nervous signalling is altered. Given the interaction between LCs and neurogenic signalling molecules like CGRP it is entirely possible that this re-organisation is responsible for the changes observed in LC density. Indeed if there are underlying changes to peripheral nerve fibres, as a result of acute inflammation during early stage CRPS, the altered efferent nervous signalling may have more effect in CRPS non-affected limbs i.e. increasing LC density in non-affected limbs and supressing Th-2 differentiation. Alternatively the altered nerve signalling to LCs could be restricted to CRPS affected tissues i.e. increasing LC density and increasing Th-2 polarisation. Furthermore the recently established role of densely innervated hair follicles in the recruitment and replenishment of LCs in the epidermis supports the hypothesis that altered efferent nerve signalling could have significant impact on LC populations[275].

In chapter 4, I showed how monocyte derived Langerhans cells (MoLCs) express the ubiquitin processing protein UCH-L1 (Fig. 4.1 & 4.2). UCH-L1

expression in LCs is particularly relevant to pain research as various animal models of chronic pain have shown UCH-L1 expression develops concomitantly with the onset of pain[237–239]. By inhibiting the activity of UCH-L1 in MoLCs I have also shown that there is no effect on activation marker expression or cytokine secretion (Fig. 4.5 & 4.6) suggesting that the expression of the deubiquitinating enzyme is not related to cell activation. This observation is important in establishing that UCH-L1 expression is not directly involved in cell activation, a mechanism which could lead to the induction of auto-immunity. However, inhibition of UCH-L1 function did have significant effects on MoLC chemotaxis (Fig 4.7).

Although no study has examined this issue directly in CRPS, UCH-L1 positive LCs have not been observed in studies using PGP9.5 staining to study epidermal nerve fibre density in CRPS patients[130]. However the observation of UCH-L1 positive LCs in animal models follows closely after epidermal denervation whereas there are no biopsy studies in CRPS patients during the acute phase of inflammation where nerve damage may be most apparent. Given the constitutive expression of UCH-L1 in MoLCs it is possible that UCH-L1 expressing LCs in animal models of pain are in fact monocyte derived cells recruited from circulation following nerve injury, mirroring the type monocyte recruitment seen under inflammatory condition[194]. Another possibility given the apparent role of UCH-L1 in MoLC chemotaxis is that expression is switched on in skin resident LCs following epidermal denervation in order to facilitate LC

migration away from the tissue. This finding is indirectly supported by previous work describing an interaction between UCH-L1 and α2-AR expression as signalling through adrenergic receptors has been shown to alter LC migration[226,227,307]. Further work examining the relationship between epidermal nerve fibres and LCs is required to understand this relationship in the context of chronic pain.

In the final chapter I explored how the presence of auto-antibodies in CRPS disease may have systemic effects on immune regulation. Previous work has established that auto-antibodies are present in a subset of CRPS patients and that these antibodies are directed towards nervous system epitopes, including adrenergic receptors[109,113,114]. The therapeutic application of intravenous immunoglobulin (IVIG) in CRPS has also been shown to reduce pain in some patients suggesting a direct link between the action auto-antibodies and maintenance of pain[26]. To establish if auto-antibodies to adrenergic receptor epitopes could modulate immune cell function in CRPS, I first explored the expression of adrenergic receptors on peripheral blood monocytes. Despite the obvious expression of α1-ARs on CD14+ peripheral blood monocytes (Fig 5.2) no transcripts for any of the α1-ARs were detected at the in cell RNA (Fig. 5.4). Furthermore there were no differences in expression when comparing healthy and CRPS monocytes at either the protein or RNA level. As discussed earlier the historical problems with specificity in commercially available adrenergic receptor antibodies casts doubt on the accuracy of this method, although this

risk was minimized by using a novel pan-specific antibody[286]. Furthermore RNA transcripts for α1-AR are reportedly of very low abundance in human blood cells making detection difficult[155]. Due to the contrasting results in measuring adrenergic receptor expression, a function assay based on LPS stimulation in monocytes was used to show that IgG from CRPS patients does not modulate monocyte activation in a similar way to the α1-AR agonist phenylepherine[260] (Fig 5.5). However, stimulation with CRPS IgG alone did produce differential effects in healthy monocytes when compared to healthy IgG stimulations, including increases in IL-2 and MIP1α (Fig. 5.6).

The hypothesis that altered adrenergic receptor expression profiles in immune cells can modify the immune cell response to neurogenic signalling, including stress induced NA, has been described previously[155,160,292]. This theory is appealing in CRPS as it provides another link between aberrant nervous signalling and immune cell activation. Furthermore, as established earlier, peripheral blood monocytes are able to act as precursor cells for epidermal LCs by migrating to the epidermis under inflammatory conditions[277]. Thus altered adrenergic receptor expression in circulating monocytes in CRPS patients could also contribute to the observed differential LC densities in these patients. However, the above data indicate that receptor expression is not altered in CRPS monocytes when compared to healthy controls. The data also suggest that CRPS IgG differentially activates healthy monocytes independently of α1-AR as, despite increases in IL-2 and MIP1α secretion, no change in IL-1β was

observed in the LPS synergy assay. One possible explanation for IgG mediated activation of monocytes is Fc-receptor activation as described earlier[293]. This could have interesting implications of IVIG treatment in CRPS as one postulated mechanism for the anti-inflammatory effects of this treatment is the up-regulation of so called "inhibitory Fc-Receptors"[294]. Thus there may be a role for auto-antibodies in CRPS, possibly through the cross-linking of Fc-receptors in peripheral blood monocytes, however it would appear to be independent of direct activation and/or modulation of adrenergic receptors on immune cells.

Final Summary -

In summary my work over the course of this project has shown that there are significant differences between immune cells in CRPS affected and non-affected tissues in late stage patients including altered LC densities and T-cell polarisation. By exploring the functional relevance of UCH-L1 expression in MoLCs I was able to show that UCH-L1 plays a role in cell chemotaxis and describe how this may be relevant to chronic pain by linking neurogenic and immune signalling in the epidermis. Finally by examining the expression of adrenergic receptors in CRPS monocytes and the effects of CRPS IgG on healthy monocytes I was able to show differential activation of these cells in a manner which was independent of α1-AR binding. These findings contribute to our understanding of CRPS and taken together indicate that the immune system involvement continues into late stage disease. By further understanding the

mechanisms of this immune dysregulation we may identify key pathways responsible for the maintenance of CRPS disease in chronic sufferers.

Further Work -

Various avenues for further research have been elucidated during the course of this project. Given the differences in LC density between affected and non-affected limbs in CRPS patients exploring UCH-L1 expression in these tissues would provide answers regarding intra-epidermal nerve fibre density, and its relationship to LC density, and also provide further detail on UCH-L1 expression in CRPS LCs, this work is underway currently by a collaborator with experience in intra-epidermal nerve fibre quantification.

The effects of CRPS IgG on healthy monocytes would be improved by expanding sample numbers and by exploring any correlation between monocyte activation and the presence of known auto-antibodies. Furthermore inhibition of Fc-receptor binding may provide answers on the exact mechanism of activation in these cells.

Finally, by expanding on the methods used in this study to examine LCs in greater detail (markers of activation or cell migration, adrenergic receptor expression etc.) a better understanding of LCs *in situ* in CRPS tissue would be developed and thus provide direct clues as to the nature of neuro-immune signalling in the late stages of this disease.

Taking a broader view the data presented in this thesis suggest that there is on-going immune involvement in late stage CRPS but that it is distinct from the acute phase of disease. Finding such as these may provide a more reliable diagnostic indicator of the stages or CRPS disease, i.e. when a patient is progressing to late stage disease, and also help improve diagnosis of patients presenting with late stage symptoms.

Furthermore the indication that previously undiscovered mechanisms may be at work during late stage disease could have implications for treatment. This finding further reinforces the hypothesis that classic anti-inflammatory medication may only have a limited window of effectiveness within the acute phase of the disease. Following disease progression a more complex neuro-immune interaction begins to predominate which is poorly understood but which does still involve immune elements. Thus a combined approach including immuno-modulating therapies, such as IVIG, may be the most effective way forward when treating late stage disease and is deserving of further research.

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APPENDIX:

Appendix Table 1. Histological assessment table

| Histological Assessment	Score
Healthy tissue	0
Slightly increased cell infiltration or tissue disruption	1
Cell infiltration or tissue disruption indicative of inflammation	2
Obvious cellular infiltrate and active inflammation	3
Severe inflammation with obvious cellular infiltrate	4

Appendix Table 2. Serum Cytokine Concentrations Measured by Multiplex ELISA (duplicate wells)

|	IL-1b	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	GMCSF	IFN-g	TNF-a	IL-17a	MIP1-a	IFN-a
p1	*0.25	*1.49	*5.36	OOR <	*3.22	*19.39	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p1	OOR <	*1.37	*4.68	OOR <	*2.30	*20.13	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p10	OOR <	*2.66	*5.36	OOR <	OOR <	73.84	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p10	OOR <	*2.22	*5.36	OOR <	OOR <	66.21	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p2	OOR <	*0.76	*4.68	OOR <	OOR <	*7.77	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p2	OOR <	*1.03	*4.68	OOR <	OOR <	*12.79	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p3	OOR <	*0.71	*4.68	OOR <	OOR <	*5.76	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p3	OOR <	*0.82	*4.68	OOR <	OOR <	*8.94	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p4	OOR <	*0.92	*4.68	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p4	OOR <	*0.82	*5.36	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p5	OOR <	*0.71	*4.68	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p5	OOR <	*0.71	*4.01	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p6	OOR <	*0.82	*4.68	OOR <	OOR <	*11.34	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p6	OOR <	*0.82	*4.68	OOR <	*0.20	*9.79	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p7	OOR <	*0.61	*4.68	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p7	OOR <	*0.66	*4.68	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p8	*0.25	*1.03	*4.68	OOR <	OOR <	53.97	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p8	OOR <	*1.03	*4.68	OOR <	OOR <	57.29	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p9	*16.39	*4.48	*6.74	OOR <	OOR <	*3.02	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
p9	*6.48	*3.12	*5.36	OOR <	OOR <	*2.82	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
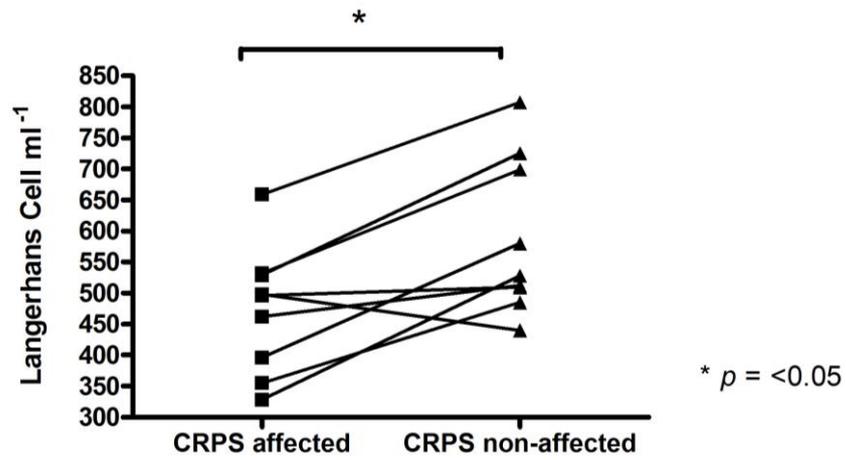
Appendix Figure 1 - Langerhans Cell Densities in CRPS Affected tissues.

CD1a stained epidermal sheets, including those from patients with control biopsies from non-contralateral sites, were imaged by confocal microscopy and a 3 dimensional picture constructed of the epidermal sheet following which LC were quantified and cell density recorded as cells per mm^2 of epidermis. Paired analysis between LC densities in CRPS affected and non-CRPS affected tissue from the same individual showed a significant difference (p <0.05)
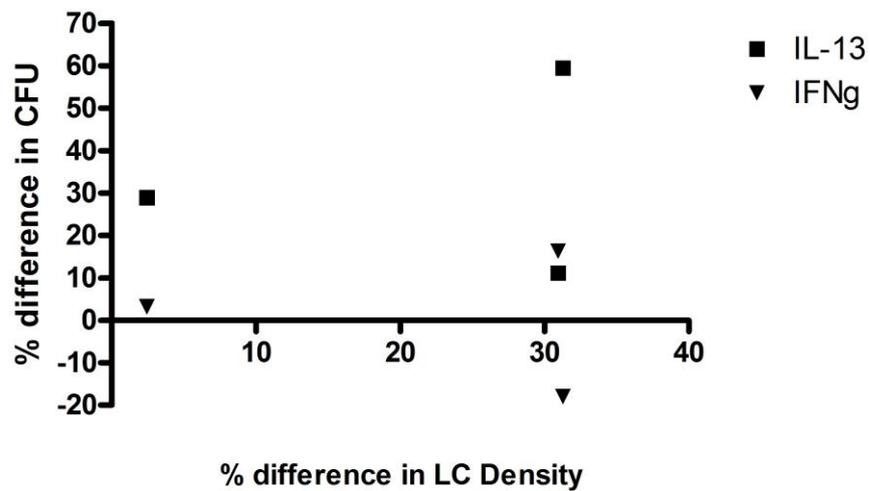
Appendix Figure 2 – The Relationship between altered Langerhans Cell Density and Tissue Resident T-cell phenotypes in CRPS affected skin.

The percentage difference in LC density in 3 patients was plotted against the percentage difference in SFU produced by tissue resident T-cells from the same tissue samples. There was no correlation between these data and thus no established relationship between altered LC densities and skewing of tissue resident T-cell phenotypes.
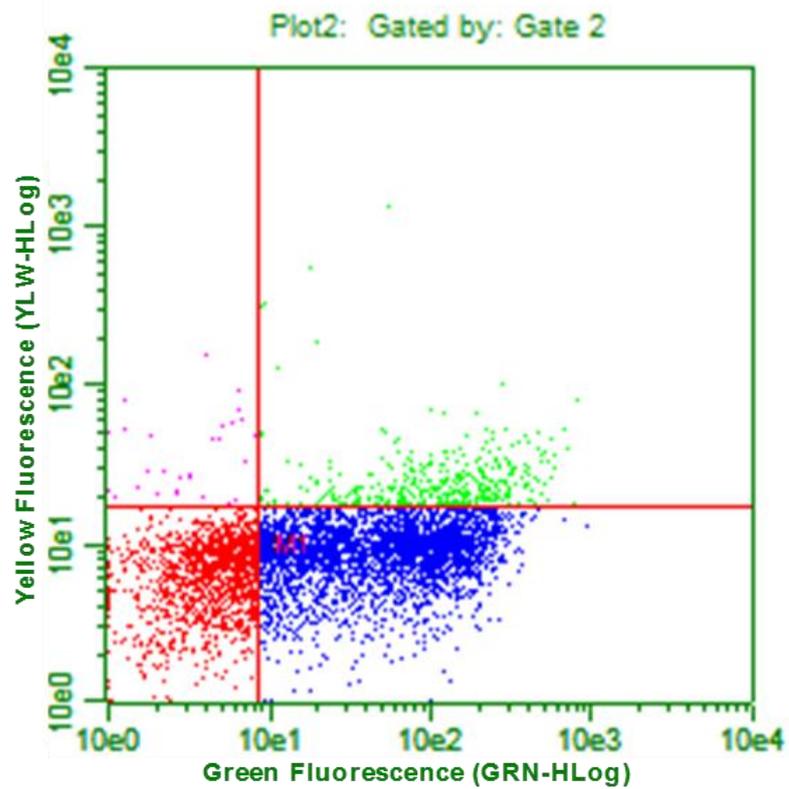
Appendix Figure 3 – PGP 9.5 and CD1a staining in non-permeabilized MoLCs

Following negative magnetic isolation negatively selected peripheral blood monocytes were differentiated into MoLCs over 7 d and fixed in PFA. Cells were dual stained for CD1a and PGP 9.5 and analysed by flow cytometry. Minimal levels of PGP 9.5 were detected using this method whilst CD1a staining remained prominent.

Abbreviations: MoLC = Monocyte derived Langerhans Cell; PFA = paraformaldehyde

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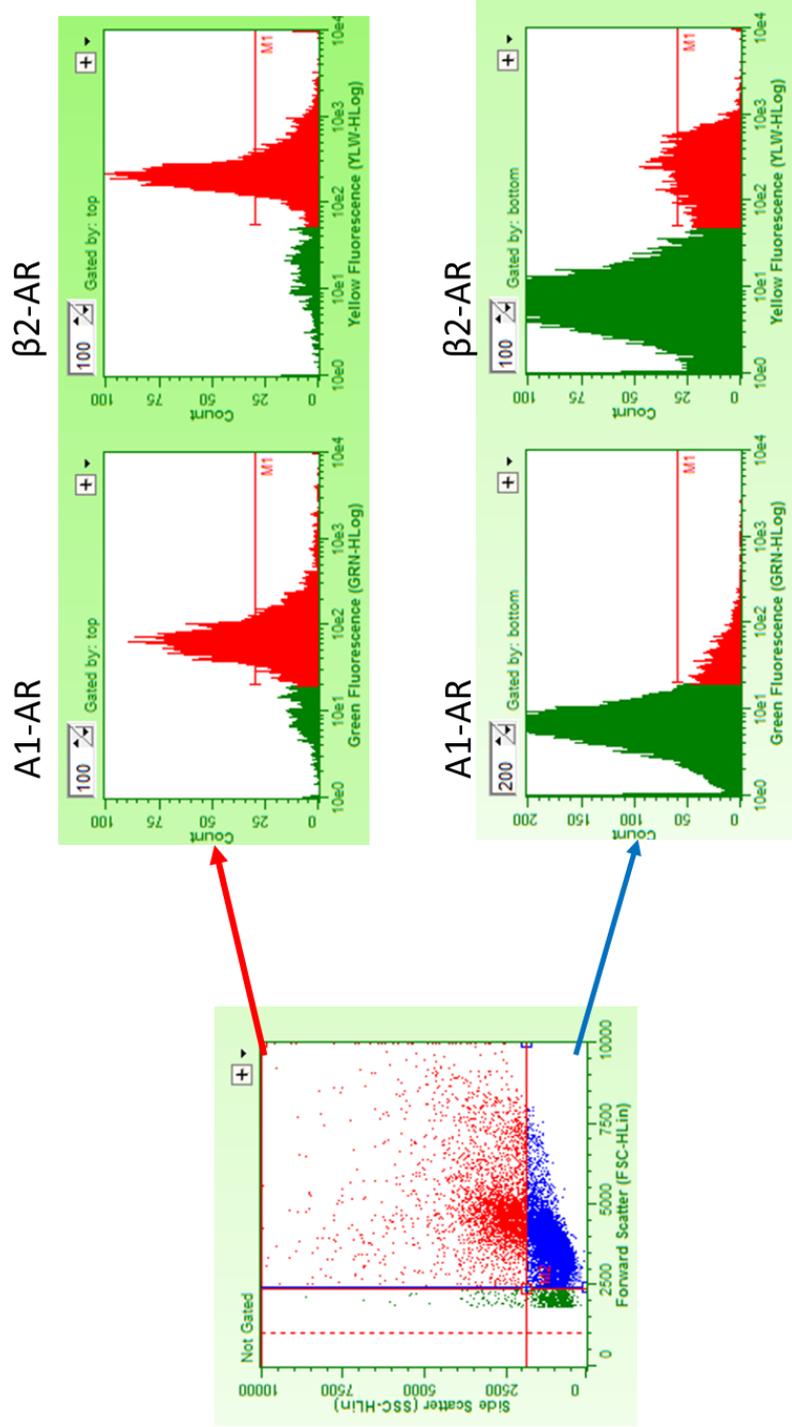
Appendix Figure 4 – β2- and α1-Adrenergic receptor Expression on PBMCs

Peripheral blood cells isolated by density dependent centrifugation were stained indirectly using anti-human β2-AR and anti-human pan-α1-AR antibodies with Alex Fluor 488 and R-PE conjugated secondary antibodies. Whilst α1-AR expression is restricted to larger granular cells β2-AR expression is uniformly expressed by the majority of PBMCs.