Potential peripheral biomarkers for chronic pain

Yuen H. Kwok BSc (Hons)

Discipline of Pharmacology, School of Medical Sciences

(Faculty of Health Sciences)

The University of Adelaide

June 2014

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Table of Contents

Abstract vii

Declaration ix

Acknowledgments xi

Abbreviations xiii

Chapter 1. Introduction 1

1.1. Current assessments for chronic pain 3

1.1.1. Pain scales 3

1.1.2. Pain assessments by observation of pain behaviours 6

1.1.3. Under treatment of chronic pain 6

1.1.4. Other issues related to the current methods for pain assessments 7

1.2. Biomarkers 9

1.2.1. What is a biomarker? 9

1.2.2. The need for biomarkers 9

1.2.3. Usefulness of biomarkers 10

1.2.4. Evaluation of biomarkers 11

1.2.5. Validation of biomarkers 12

1.2.6. Potential biomarker for chronic pain: neuroimaging 13

1.3. Neural mechanisms of chronic pain 15

1.3.1. Nociceptive pain 15

1.3.2. Inflammatory pain 16

1.3.3. Chronic pain 17

1.3.4. Structural changes after injury 18

1.3.5. Peripheral sensitization: ectopic activity 18

1.3.6. Central sensitization 19

1.4. Current treatments for chronic pain 21

1.4.1. Established treatments 22

1.4.2. Solutions for improving treatments for chronic pain 23

1.5. Neuroimmune contributions to chronic pain 25

1.5.1. How do peripheral immune cells contribute to chronic pain? 25

1.5.2. Immunocompotent cells in the CNS: glia 31

1.5.3. Microglia and astrocytes 32

1.5.4. Involvement of glia in chronic pain 33

1.5.5. Microglia can initiate chronic pain whilst astrocytes maintain it 34

1.5.6. Mediators released from glial cells indirectly contribute to chronic pain 35

1.5.7. Potential activators of glial cells 35

1.6. Toll-like receptors 36

1.6.1. TLR signalling pathways 36

1.6.2. Expressions and locations of TLRs 38

1.6.3. The involvement of TLR2 in chronic pain 39

1.6.4. The involvement of TLR4 in chronic pain 41

1.6.5. Influences other than TLR2 and 4 43

1.6.6. TLR7 activation contributes to itch and not pain 44

1.7. Role of inflammatory mediators in chronic pain 46

1.7.1. Cytokines 47

1.7.2. Role of interleukin-1 β in chronic pain 47

1.8. TLR responsiveness 51

1.9. Other techniques used in this project 55

1.9.1. Preclinical model: Graded chronic constriction injury model 55

1.9.2. Assessment of basal pain sensitivity: Cold pain test 55

1.10. Summary 57

1.11. Research Aims 58

Chapter 2. TLR responsiveness of the human peripheral blood mononuclear cells as a potential biomarker for chronic pain 61

Statement of Authorship 63

Chapter 3. Forward and backward translation of TLR responsiveness between a preclinical neuropathic pain model and a chronic pain patient cohort 72

Statement of Authorship 74

Chapter 4. Utility of TLR responsiveness for the assessment of a novel treatment 92

Statement of Authorship 94

Chapter 5. Conclusion 131

Chapter 6. References 137

Appendix A. Supplementary tables 162

Appendix B. Potential biomarkers for pain: Neopterin & reduced white cell count 174

B.1. Introduction 174

B.2. Materials and methods 176

B.2.1. Cell count 176

B.2.2. Neopterin assay 177

B.2.3. Statistical analysis 177

B.3. Results 177

B.4. Discussion 179

Appendix C. Development of data derived statistical models for study 2 and 3 181

C.1. R commands for the generation of models 182

C.1.1. For categorization of the pain presence (Model A) (Chapter 3 for rats and humans) & responders of ibudilast (Chapter 4) 182

C.1.2. For the prediction of pain severity in rats (Model B; Chapter 3) 184

C.1.3. For the prediction of f IL-1 β central output by models generated from peripheral outputs and prediction of pain presence in humans (Model D; Chapter 3) and responders of ibudilast (Chapter 4) 185

Appendix D. Appendix References 186

Table of Figures and Tables

Figure 1–1 Schematic representation of the three pain intensity scores. 4

Figure 1–2 Multiple sites that can be modified during neuropathic pain. 16

Figure 1–3 The interaction between neuron and glial cells in the central region. 32

Figure 1–4. A schematic representation of TLR4 signalling. 37

Figure B–1. The synthesis pathway of GTP (Tegeder et al., 2006) 175

Figure B–2. Number of cell count after isolation with the use of Optiprep™. 178

Figure B–3. No group differences were detected in the serum neopterin levels. 178

Table 1–1. Evidence of TLR responsiveness in humans with immune mediated diseases. 52

Table A–1. Individual information on chronic pain sufferers in Chapter 2 162

Table A–2. Best-fit logistic regression model results for the prediction of pain for rats post CCI. 165

Table A–3. Best-fit logistic regression model results for the prediction of the pain severity in rats post CCI. 168

Table A–4. Best-fit logistic regression model from rats (Peripheral only) and from humans to predict the presence of pain in chronic pain patients 173

Abstract

Chronic pain biomarkers can assist clinicians to diagnose patients, identify underlying mechanisms of disease, reduce the time and cost to reach a decision in early clinical trials and guide personalized pain treatments. Unfortunately, to date, there is no validated biomarker for chronic pain due to the difficulty in accessing the central nervous system. However, emerging literature has consistently provided evidence for the involvement of the immune system to play a substantial role in the modulation of chronic pain. Thus, this thesis examines components of the immune system such as Toll like receptor (TLR) signalling, peripheral immune cells and pro-inflammatory cytokine as an accessible source which may mirror similarities in brain immune cells and capture the changes in chronic pain state.

Therefore, the purpose of this thesis was to examine the use of peripheral immune cell reactivity as a potential biomarker for chronic pain. The first study was conducted in heterogeneous chronic pain and pain-free cohorts. Peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with various TLR agonists to generate a pro-inflammatory cytokine interleukin-1β (IL-1β) concentration response curve. Chronic pain patients displayed significantly enhanced expression of IL-1β compared with the pain-free cohort hence the TLR responsiveness demonstrated face validity as a chronic pain biomarker.

The second study demonstrated the translatability of the importance of TLR responsiveness in a preclinical neuropathic pain model. IL-1β levels were quantified from basal and TLR2/4 agonist stimulated isolated rat PBMCs and spinal cord tissues, and together with the behaviour responses were used to generate statistical models. The main findings of this study were the inclusion of basal and TLR agonist stimulated outputs were required to predict the presence of pain and severity of allodynia with high sensitivity and specificity and that peripherally collected outputs correlated with the outputs from the spinal cord, suggesting the ability of peripheral outputs to give insight into

central signalling. In addition, a mathematical model developed from rat studies using peripheral and central tissues was able to identify chronic pain patients by their peripheral blood response with high accuracy.

The final study assessed TLR responsiveness of isolated PBMCs collected from a cohort of medication overuse headache (MOH) patients to determine the efficacy of a novel treatment for headache (ibudilast). This study consisted of MOH patients on 8 weeks of either placebo or ibudilast treatments. After 8 weeks of treatment, both groups did not experience a change in their headache frequency or intensity. However, a significant reduction in the TLR responsiveness occurred in the ibudilast group. These data provide the first evidence of a biomarker for ibudilast treatment.

In sum, this thesis provides evidence that peripheral cells are a good source to be biomarker for chronic pain and the importance to assess TLR signalling as an approach to capture the dysregulated immune system as a result of chronic pain. The discovered biomarkers require further replication and validation before than can be routinely used. However, the present finding can assist with the development of future cellular biomarkers for chronic pain.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I acknowledge that copyright of published works contained within this thesis (as listed below) resides with the copyright holders of those works.

Kwok YH, Hutchinson MR, Gentgall MG & Rolan PE. (2012). Increased Responsiveness of Peripheral Blood Mononuclear Cells to In Vitro TLR 2, 4 and 7 Ligand Stimulation in Chronic Pain Patients. *PLoS ONE* 7(8), e44232.

Kwok YH, Tuke J, Nicotra LL, Grace PG, Rolan PE, Hutchinson MR (2013) TLR 2 and 4 Responsiveness from Isolated Peripheral Blood Mononuclear Cells from Rats and Humans as Potential Chronic Pain Biomarkers. *PLoS ONE* 8(10), e77799.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Yuen Hei Kwok Date

Acknowledgments

I would like to sincerely thank my supervisors Professor Paul Rolan and Professor Mark Hutchinson for giving me the opportunity to undertake such an exciting project. Their enthusiasm and immense knowledge really formed a concrete picture in my mind as to what "being passionate" is really about and I strive to be as passionate a researcher as them one day. Their ongoing encouragement, patience and guidance over the years are greatly appreciated and I could not have wished for better supervisors.

The research presented in this thesis would have been impossible without the financial support from the Faculty of Health Sciences Divisional PhD Scholarship as well as support from the Pain and Anaesthesia Research Clinic. The opportunities to present my work at numerous national and international conferences were generously supported by the Australasian Society for Clinical and Experimental Pharmacologists and Toxicologists Travel Grant, Australian Pain Society PhD Travel grant, Faculty of Health Sciences Postgraduate Travelling Fellowship, The School of Medical Sciences Postgraduate Travel Award and Professor Paul Rolan.

I would also like to acknowledge the work and express my gratitude to the following people: Melanie Gentgall and all the staffs at the Pain and Anaesthesia Research Clinic for teaching me how to run clinical studies as well as providing their clinical expertise; Dr Jonathan Tuke and Prof Mark Hutchinson for teaching me how to use and develop scripts for the statistical programming of R; Jacinta Johnson and Nicole Sumracki for their collaborations with the clinical studies; Lauren Nicotra for the collaboration with the preclinical study; Dr Janet Coller and Dr Dan Barratt for assistance with genotyping; I would like to thank Dr Dan Barratt again for his patience in training me on extracting RNA and providing insightful troubleshooting microarray advices; I'm also very grateful to Perona Ho who helped proofread my thesis; I would like to express my thanks to Gordon Crabbe and Karen Nunes-Vraz for their administrative support; I would like to thank all past and present members of the

Discipline of Pharmacology as they have provided me with valuable feedback throughout the years, which will be remembered; I would like to thank all the past and present students in the Discipline of Pharmacology and particularly to my colleagues in N529a and N511. They have been amazing and I will never forget the fun times we shared.

Furthermore, I am thankful to my friends for being so supportive and understanding over the years, whether it was chatting over lunch/dinner, hiking, watching the latest movies, singing karaoke or just hanging out -those moments have kept my life in balance!

Last but not least, I would like to thank my family, especially my parents. They have been my pillars of support both emotionally and financially and their constant encouragement really helped me get to where I am today and for that I am infinitely grateful.

Abbreviations

ATP	Adensoine triphosphate
cAMP	Cyclic adenosine monophosphate
CCI	Chronic constriction injury
CCL	Chemokine (C-C) motif ligand
CNS	Central nervous system
COX	Cyclooxygenase
DAMPs	Danger-associated molecular patterns
DRG	Dorsal root ganglia
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
FDA	Food and Drug Administration
fMRI	Functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
KO	Knockout
IL	Interleukin
IκB-α	NFκB inhibitor α
iNOS	Nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MOH	Medication overuse headache
MyD88	Myeloid differentiation primary response gene 88
MSU crystals	Monosodium urate crystals

NGF	Nerve growth factor
NF-κB	Nuclear factor- κB
NMDA	N-methyl-D-aspartate glutamate
NNT	Number needed to treat
NO	Nitric oxide
Nox2	NADPH oxidase 2
NRS	Numeric rating scale
NSAIDS	Nonsteroidal anti-inflammatory drugs
P2X4	Purinergic receptor
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
SSRIs	Selective serotonin reuptake inhibitors
StEP	Standardized Evaluation of Pain
SDSN	Supernatant of damaged sensory neurons
TCA	Tricyclics antidepressants
TIR	Toll/IL-1 receptor
TGF- β1	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TRAF6	Tumour necrosis factor receptor 6
TRIF	TIR-doman containing adapter-inducing interferon-β
VAS	Visual analogue scale
VRS	Verbal rating scale

Chapter 1. Introduction

Chronic pain impacts a large proportion of Australians and it affects more people than other chronic conditions such as diabetes, hypertension or asthma (Blyth et al., 2001). The prevalence of chronic pain is estimated to affect 20% of the population in Australia (Henderson et al., 2013) and other developed countries (Varrassi et al., 2010). Chronic pain sufferers have lower quality of life, as their daily activities are greatly impaired. Consequently, those affected with chronic pain incur indirect health and medical costs as well as lost productivity that imposes a significant social and economic burden to society (Gaskin and Richard, 2012).

Although the understanding of chronic pain has advanced, it remains to be a complex and multifactorial condition for which determination of the underlying pathology and appropriate treatments are difficult. Thus, there is an urgent need for the detection of the underlying mechanisms and development of disease modifying therapies, so pain can be better targeted and managed. The use of objective markers, known as biomarkers, have potential to improve the diagnosis and treatment of chronic pain by pinpointing the underlying pathophysiology and assisting with the development of personalized treatments. However, to date there is no validated biomarker for chronic pain due to the inaccessibility of the peripheral nervous system (PNS) and the central nervous system (CNS).

In the past 20 years there has been a paradigm shift in the understanding of pain with the recognition that the immune system also significantly contributes to the initiation and maintenance of chronic pain. Given the accessibility of peripheral immune cells from blood it is now plausible to search for potential biomarker candidates for chronic pain in regions that can be easily accessed and may provide a window to the inaccessible central region.

Based on this research gap, this thesis aims to find potential biomarkers for chronic pain in blood. Blood is a good starting point because it contains components of the immune system and can easily be sampled. Chapter 2 describes a potential biomarker for chronic pain by examining the reactivity of peripheral blood mononuclear cells (PBMCs) after stimulation with various Toll like receptor (TLR) agonists in a heterogeneous cohort of chronic pain patients. Chapter 3 continues to explore the concept of TLR responsiveness but in a preclinical model of graded neuropathic pain. The translational potential of the findings from humans was tested in a graded chronic constriction injury (CCI) rat model. Models constructed for the prediction of pain presence derived from the animal study was reapplied back to humans so the predictability of the animal findings could be confirmed. In addition, the question of whether the peripheral immune reactivity could mirror central immune reactivity was also examined. Chapter 4 further investigates the potential utility of the TLR responsiveness; whether it can serve as a biomarker to detect treatment efficacy of a novel drug (ibudilast) used to reduce headache symptoms in a cohort of medication overuse headache (MOH) patients. The process of the identification of potential peripheral biomarkers for chronic pain was demonstrated in both preclinical and clinical studies in this thesis.

The introduction will cover the background for the research topics listed above. Firstly, the need for chronic pain biomarkers will be highlighted with discussion about the limitations faced by the current assessments used for chronic pain. Secondly, the purpose of biomarkers will be introduced to demonstrate how they can address some of the issues faced in the current pain field. Thirdly, a brief background of the key understanding of chronic pain from the neuronal point of view and the limitations in the commonly used chronic pain interventions will be discussed. Fourthly, the involvement of the peripheral and central immune cells, TLR and pro-inflammatory cytokine, which are responsible for the initiation and maintenance of chronic pain will be reviewed. Finally, potential peripheral biomarkers of pain will be identified.

1.1. Current assessments for chronic pain

The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Hence, chronic pain is a complex subjective phenomenon that involves sensory and emotional aspects. An individual's perception of pain is dependent on many factors, such as past experience, age, gender, socioeconomic status and cultural background. As the CNS is difficult to access, clinicians try to unravel the cause and extent of pathology in patients by conventional documenting of pain history and performing physical examination to identify any potential pathophysiological and aetiological issues. Special tests to better understand the sensory responses such as pain tolerance (via cold pain test) (Turk and Rudy, 1987) are generally used in research. To reduce the bias associated with the subjective nature of symptom reporting, many rating scales have been developed in an attempt to quantify pain. These are not biomarkers but tools to assist with clinical assessment. Some of the frequently used pain assessments will be briefly discussed below.

1.1.1. Pain scales

In clinical practice, one-dimensional pain intensity scales such as the visual analogue scale (VAS) and numeric rating scale (NRS) are most commonly used to reflect patient response to pain and for the detection of changes after an intervention. Pain scales are useful as they are economical, easy to use, require minimal instructions and are believed to indicate the presence and extent of pain associated with tissue damage (Turk and Rudy, 1987, Breivik et al., 2008).

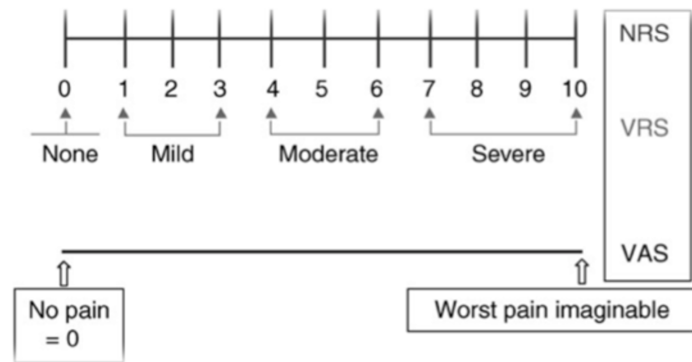


Figure 1–1 Schematic representation of the three pain intensity scores.

The 11-point numeric rating scale (NRS), the four-point categorical verbal rating scale (VRS) and the visual analogue scale (VAS) are currently used for pain assessment. Reprinted with permission from Oxford University Press, Assessment of pain, Vol. 101, p. 18, Breivlk et al., Figure 1, © 2008.

1.1.1.1. Visual analogue scale

A VAS consists of a line usually 100 mm in length with verbal descriptors at one end labelled as "no pain" whereas the other end is labelled with "worse pain ever experienced" (Turk and Rudy, 1987) (see Figure 1-1). VAS scores are found to be reliable over time, sensitive to pain change and psychomotor factor influences are minimal (Kremer et al., 1981, Williamson and Hoggart, 2005). However, when VAS were used in patients with cognitive impairment, they were found to be less likely to report pain and to give consistent results (Williamson and Hoggart, 2005). Moreover, 11% of chronic pain patients in a study population were found to be incapable of completing the VAS that were associated with older population, as their abstract thinking abilities were found to deteriorated with age (Kremer et al., 1981).

1.1.1.2. Numerical rating scale

A NRS is usually an 11-point scale with responses being represented as numbers. These scales are useful because they can be assessed verbally (Williamson and Hoggart, 2005) (see Figure 1-1). However, a major limitation with scales that display rank order or equal interval measures is the false

assumption that the difference between mild to moderate pain is the same as the difference between moderate to severe pain (Turk and Rudy, 1987). In addition, the use of a restricted range in a scale limits the sensitivity to detect small changes in treatment interventions (Turk and Rudy, 1987, Chapman et al., 1985).

1.1.1.3. Verbal rating scale

A verbal rating scale (VRS) uses a list of adjectives to correspond with the intensity of pain, but they are not as sensitive as the VAS or NRS for the detection of changes in pain, as such the VRS score requires a large change in the selected word choice before it can be detected (Williamson and Hoggart, 2005). A study conducted in neuropathic pain patients were assigned into groups on how likely they would have neuropathic pain based on their verbal description to a pain questionnaire. However, no group differences were detected between "definite neuropathic pain", "possible neuropathic pain" and "unlikely neuropathic pain" (Rasmussen et al., 2004). Another limitation with the VRS is the word choices available may be unfamiliar to the patient whereby the obtained responses may be incorrect due to the patient's limited vocabulary (Williamson and Hoggart, 2005). In addition, the choice of word and expression may have different implications in certain cultures so direct translation of words in VRS may provide inaccurate results (Chapman et al., 1985).

1.1.1.4. Multidimensional pain scales

VAS and NRS scales are limited with the assumption that pain is a uni-dimensional phenomenon that can only be described as "pain intensity". Accordingly, the obtained response is overly simplified and condenses a broad range of psychological experiences into a single response which may not be a true indicator of how a patient may feel (Turk and Rudy, 1987). Therefore, multidimensional pain questionnaires which consist of additional questions that assess other psychosocial factors can give an indication of how pain is impacting on patients' quality of life and the ability to perform daily activities (Turk and Rudy, 1987). A pain assessment tool known as StEP (Standardized Evaluation of Pain) which combines 6 interview questions and 10 physical tests was able to differentiate

neuropathic vs. nociceptive pain phenotypes with high sensitivity and specificity in a cohort of chronic pain patients (Scholz et al., 2009). The limitations of these multidimensional pain questionnaires are the questionnaires are too complex for use in very sick patients (Carlsson, 1983) and in patients other analgesic agents such as opioids may impair their judgement abilities (Chapman et al., 1985).

1.1.2. Pain assessments by observation of pain behaviours

In patients who cannot provide either accurate feedback or unlikely to perform the above scales reliably such as in patients with language difficulties, dementia, children, unconscious or are critically ill (Herr et al., 2011), pain is assessed through behaviours. The COMFORT scale is mostly used in paediatric intensive care units and the scale relies on the assessment of nine indicators: alertness, calmness or agitation, respiratory distress, crying, physical movement, muscle tone, facial tension, arterial pressure and heart rate. Clinicians will observe the patient against the nine indicators undisturbed for 2 mins and each indicator will be given a score from 1 to 5 and those nine scores will be added together to give an overall score. An overall score from 17 to 26 indicates adequate sedation and pain control (Breivik et al., 2008). However, behaviour scales are hindered with a weakness of the assessments being dependent on clinicians' expertise and experience therefore it cannot be validated. Also, the discrimination between unpleasantness and emotion such as fear can often be difficult to distinguish (Herr et al., 2011).

1.1.3. Under treatment of chronic pain

Pain is known to be pharmacologically undertreated and some of the contributing factors include: lack of knowledge about pain management, reluctance of clinicians in using opioids due to the potential regulatory scrutiny for the prescription of controlled substances and some patients related barriers that can cause patients' reluctance to report pain or follow their prescribed treatment regimen (such as psychological factors obstructing the assessment of pain, fear of addiction, tolerance and side effects) (Glajchen, 2001). Asian cancer patients were reported to experience a

greater prevalence of under treatment of pain when compared with Caucasians. The reason for the under treatment in Asian patients include fear of the use of analgesics, developing an addiction to opioids and the belief that a "good patient does not complain about pain" (Chen and Tang, 2012). Likewise, studies have found the presence of under treatment of chronic pain in the indigenous populations. The use of numerical pain scales has been reported in the Central Australian indigenous group (Fenwick, 2006). As some Aboriginal communities do not have the conceptual recognition of numbers above 5, so the commonly used NRS would provide inaccurate results because the scale utilises numbers from 1 to 10. Furthermore, the understanding of pain is culturally different, as Aboriginal people do not relate pain to oneself but to family members and the land. Hence, they are more likely to suppress or be reluctant to talk about their pain experiences as they view pain as a human weakness and breaking their law (Fenwick, 2006). Therefore, the current used of questionnaire to obtain an accurate level of their true pain level is insufficient resulting in under treatment of pain (Fenwick and Stevens, 2004). This illustrates the concept that people from different cultures and their perceptions can significantly impact the correct assessment of pain with the current used tools. So, the ability to assess pain objectively can address some of the shortcomings faced by the currently used subjective pain scales.

1.1.4. Other issues related to the current methods for pain assessments

Even though it has been said that pain is entirely subjective so that the only way to assess it "is to believe the patient and pain is what the patient says it is" (Williamson and Hoggart, 2005), a true reflection of the patient's response is dependent on the ability of the patient to understand the questions being asked as well as the ability of the clinicians to correctly interpret the responses (Williamson and Hoggart, 2005). However, there are instances when self-reports are limited by its subjective nature in that findings cannot be validated which allows dishonest patients to distort their pain responses for personal gain, or patients who does not have the ability to communicate (e.g. in young children and unconscious patients). Another limitation with questionnaires is the result of large

variability in the data due to heterogeneity in patient response. This can be problematic as within-patient variability may minimise or mask the accuracy of the true results obtain from the questionnaires.

Preclinical models are often utilised in the study of pain but as pain scales cannot be used in animals, the inability to translate research findings from animals to humans (Chizh et al., 2008) is problematic. Therefore, the use of an unbiased, objective marker can reduce the uncertainty of patient's response as well as variability caused by subjective perception and enable translation of research findings. It is noteworthy that the use of subjective reporting will not be replaced by biomarkers, but should be used in combination with existing pain assessment practices. The addition of biomarker usage can significantly reduce the incidence of incorrect chronic pain diagnoses and assist with the selection of adequate treatments. A more in-depth review on the uses and benefits of biomarkers will now followed.

1.2. Biomarkers

1.2.1. What is a biomarker?

The US National Institutes of Health has defined a biomarker as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Atkinson et al., 2001). Biomarkers are broad (Wagner, 2009) and can be any clinical measurements (Wagner, 2009) or models (Rolan, 1997) that can fulfil the above definitions (Mayeux, 2004).

1.2.2. The need for biomarkers

In comparison to 15 years ago, the cost of clinical drug development has continued to soar whereas the productivity has been slow (Woodcock, 2009, Gobburu, 2009). 90% of the drug failures have been attributed to the lack of efficacy or safety and 50% of late-phase clinical trials fail to differentiate the test drug from placebo (Arrowsmith, 2011). Consequently, the pharmaceutical industry is under immense pressure to discover new drugs that can demonstrate superiority over existing treatments or discover new drug targets.

Usage of biomarkers can improve the success rate, advance the medical value of new interventions as well as reduce developmental cost, and assist with the current issues faced by regulators and pharmaceutical companies (Woodcock, 2009). Biomarkers can improve the efficiency of drug development by shortening the time and process used to determine whether a novel drug would likely to demonstrate therapeutic and commercial success. It is interesting to note that the US Food and Drug Administration (FDA) has recognised the benefits that biomarkers exhibit, as it is now recommended that the development of a potential biomarker should occur before phase 1 clinical trials (LaVallie et al., 2008).

1.2.3. Usefulness of biomarkers

Biomarkers can provide valuable information about diseases which can assist clinicians in making medical decisions (Gobburu, 2009). For example, biomarkers can diagnosis the presence (or absence) of a disease, the extent (or stage) of the disease and identify patients with high likelihood of getting a disease (Atkinson et al., 2001).

Biomarkers may also be beneficial in drug development and in clinical trials as they may evaluate the effectiveness of novel interventions (Atkinson et al., 2001). In early drug development, biomarkers can assist with the lead compound and optimal dose selection, facilitate the design of clinical trials and aid with the evaluation of safety and efficacy for novel compounds (Atkinson et al., 2001). Biomarkers can give insight into disease pathways, mechanism of action for treatment interventions (Gobburu, 2009) and confirm pharmacological activity (Rolan, 1997). Biomarkers thereby provide a mechanistic approach for empirical results obtained in clinical trials so that specific molecular and cellular pathways can be identified (Atkinson et al., 2001). Furthermore, biomarkers can be used to understand the reason for clinical failure; whether ineffectiveness of the intervention is due to insufficient drug activity at the site or that the proposed mechanism is irrelevant to the disease (Rolan, 1997).

Clinical trials can be enriched with the use of biomarkers by selecting patients who are likely to benefit from a particular therapy or by excluding those who are unlikely to respond (Woodcock, 2009, Temple, 2008). This type of biomarker usage can therefore substantially reduce the risk in those who have been identified to be non-responders and enhance the benefit for patients who are responders of a treatment. Biomarker usage has been tremendously successful in oncology and cardiology research (Maisel et al., 2002, Desai et al., 2006) and in particular for the development of targeted therapies. For example, patients with mutated KRAS genes were excluded from the epidermal growth factor receptor targeting cancer drugs cetuximab and panitumumab, as they were

found to be unresponsive to therapy. This inclusion of likely-to-benefit groups and exclusion of unlikely-to-benefit groups enhanced the value of the clinical trials (Woodcock, 2009).

1.2.4. Evaluation of biomarkers

Potential biomarkers are evaluated against the characteristics of an ideal biomarker and the stringency of the correlation is dependent on the intended use. Listed below are the characteristics which an ideal biomarker will possess (Lesko and Atkinson, 2001).

1. **Clinical relevance.** The biomarker must have theoretically rational basis for its selection. It should fulfil the definition of a biomarker by reflecting measurement under normal process or disease process. To achieve this characteristic an understanding of the pathophysiology of the disease would be required. However, the ability for a biomarker to fulfil this characteristic would be difficult when the mechanisms for disease are unclear or when multiple mechanisms are involved.
2. **Sensitivity and specificity to treatment interventions.** The biomarker should only reflect changes specific to the intended therapeutic action of the drug and that it should not be affected by interferences from any unintended mechanisms of the drug. Likewise to the previous characteristic, the understanding of the mechanism of drug action would be necessary.
3. **Reliability.** The biomarker measurement should display acceptable accuracy, precision, robustness and reproducibility. This characteristic is closely associated with the rigor of laboratory techniques.
4. **Practicality.** In order for biomarkers to be accepted for widespread usage in clinical research, the biomarker would need to be obtained either non-invasively or be modestly invasive so that inconvenient and discomfort can be minimized for the patients. Body fluids such as blood and urine are usually well tolerated by patients but obtaining tissue biopsy

and cerebrospinal fluid should be avoided. The cost of obtaining the sample should also be reasonable (Mayeux, 2004).

5. **Simplicity.** Biomarkers should be obtained without the need for sophisticated equipment, neither skilled operator nor an extensive time commitment.

1.2.5. Validation of biomarkers

Validation for potential biomarkers is a rigorous and a continuous process that occurs in the later stage of biomarker discovery. The validation is particularly applied to biomarkers that can substitute for a clinical endpoint, also known as surrogate endpoints, as they are expected to predict clinical benefit and accelerate drug approval (Atkinson et al., 2001). Validation is the assessment of whether the selected biomarker displays characteristics that are "reliable for the intended application" (Lesko and Atkinson, 2001, Rolan, 1997, Lee et al., 2006). The performance characteristics include the following: sensitivity, specificity, reproducibility, accuracy and bias of measurement, probability of false positives / negatives, observer errors, variability due to assay techniques and biological variation (Atkinson et al., 2001, Rolan, 1997, Wagner, 2009).

There are three types of validities that can be applied to biomarkers and they are face validity, criterion validity and construct validity (Sim and Arnell, 1993). *Face* validity is the basic form of validation, where a test or measure is assessed at face value and determines whether it appears to measure what it is intended to measure (Sim and Arnell, 1993). *Criterion* validity is a measure of how well one variable or set of variables predicts the clinical variable based on previous clinical data. Lastly, *construct* validity determines whether the biomarker shares a causal mechanism with the clinical outcome (Rolan, 1997). This is often difficult to assess as the underlying mechanism for a disease or the mechanism of action for the novel interventions may be unknown. A good statistical correlation would be required between the biomarker and the true clinical outcome but it is not sufficient (Fleming and DeMets, 1996) as the two may share the same root but not share a common mechanism (Rolan, 1997).

It should be noted that biomarkers that are not well validated can be used to provide valuable information and should not be overlooked (Rolan, 1997). This is particularly the case in CNS disorders where there are no available objective markers that can help in the identification of disease assessments and novel interventions.

1.2.6. Potential biomarker for chronic pain: neuroimaging

The utility of biomarkers for more complex, multi-system neurological disorders such as chronic pain is of great interest. To date, there is no validated biomarker for chronic pain but neuroimaging has been considered the tool for use to identify the first pain biomarker. As this technique can identify "pain signatures", which is brain regions or structures (Borsook et al., 2011b) that reflect aberrant patterns of human brain activity which may be linked to the presence or the development of chronic pain conditions (Tracey, 2011). Use of neuroimaging is ideal for chronic pain as it can detect dynamic brain patterns (Farmer et al., 2012) and the identified "pain signatures" has been found to be sensitive to therapeutic interventions (Wise and Preston, 2010). Over the years, neuroimaging has contributed to the understanding of pain processing and studies have recognized that chronic pain patients process pain differently from healthy participants (Wartolowska and Tracey, 2009).

Functional magnetic resonance imaging (fMRI) was recently used to develop a pain signature that revealed discrimination between painful and non-painful stimulus, as well as between physical and social pain with high sensitivity and specificity (Wager et al., 2013). The identified signature was sensitive to treatment as the activation was substantially reduced after opioid (remifentanil) administration. Moreover, the regions activated were in agreement with previous reports of pain regions (primary and secondary somatosensory cortexes) (Wager et al., 2013).

Nevertheless, despite the surge of interest in the use of "pain signatures" identified from neuroimaging as a biomarker for chronic pain, there are several limitations that need to be addressed before it can be a valid biomarker. Firstly, brain activity can be misinterpreted because

there is no one "pain centre" in the brain (Wartolowska and Tracey, 2009). Regions that are activated in response to pain are widely distributed for our survival (Coghill et al., 1999) and are also associated with non-noxious sensory functions such as touch (Davis et al., 2012). Secondly, patients with cerebrovascular impairments (e.g. post stroke) have limited ability to show normal fMRI responses, which can cause erroneous results (Davis et al., 2012). Thirdly, a normal range for neuroimaging would need to be established from a large sample population which takes into the account any variability related to individual factors (such as sex, age and ethnicity) before use as a biomarker (Liu et al., 2008). Fourthly, chronic pain differs in location, type and clinical cause therefore, more than one pain signature would need to be developed for different imaging techniques and protocols used (Wager et al., 2013). Finally, neuroimaging is expensive, as it requires specialized equipment and trained technicians (Borsook et al., 2011a) therefore, with the lack of standardised procedures, it is not practical to be use routinely.

In summary, even though neuroimaging does have its merits, more extensive research is required to address the limitations and standardized procedures (Cruccu and Truini, 2009) to be utilised as a chronic pain biomarker. Consequently the identification of potential chronic pain biomarkers remains elusive and an urgent priority so that chronic pain can be better managed and treated.

Therefore, the aim of this thesis takes on a different approach for the hunt of potential biomarkers for chronic pain. Instead of using neuroimaging, this project focused on potential biomarkers at the peripheral level that are easy to access and may act as a window to the brain and the spinal cord. However, before potential candidates can be revealed, a brief background on the mechanisms and the mediators that involved in chronic pain will be required.

1.3. Neural mechanisms of chronic pain

1.3.1. Nociceptive pain

Pain is an essential mechanism for our protection and survival, as it can help detect and minimise contact with potentially damaging or noxious stimuli (Basbaum et al., 2009, Woolf and Ma, 2007). It is known to be adaptive and acts like an alarm system. Under normal conditions, intense or damaging noxious stimuli can produce pain via the activation of high intensity specialized peripheral terminals of primary sensory neurons also known as nociceptors (Woolf and Ma, 2007). Nociceptors are present on thinly myelinated Aδ afferents and unmyelinated C fibres (Basbaum et al., 2009) which can detect external incoming painful stimulus such as intense thermal, mechanical or chemical stimuli. Once the pain threshold reaches a noxious range (Woolf and Ma, 2007), the stimulus is transduced into action potentials and relayed the signal to central regions by synapsing onto pain-responsive interneurons and second order pain projection neurons (located in the dorsal horn of the spinal cord) (Scholz and Woolf, 2002, Basbaum et al., 2009). Neural plasticity allows the nervous system to modulate the pain threshold according to the incoming stimuli under normal and pathophysiological conditions (Scholz and Woolf, 2002).

At the spinal cord level, the second order neurons decussate and join ascending fibres of the anterolateral system to project to the supra-spinal pain processing centres (Milligan and Watkins, 2009). Information is also relayed from the brainstem back to the areas of origin of the ascending pathways (Hunt and Mantyh, 2001). The areas which are involved in the processing of pain are collectively known as the "pain matrix" and involves other multiple higher centres such as the brainstem, amygdala and midbrain periaqueductal gray (Scholz and Woolf, 2002).

When pain becomes chronic, such as in neuropathic pain conditions, abnormal pain modulation (facilitation and inhibition) can occur at multiple levels (see Figure 1-2), which further complicates the identification of the underlying mechanism as well as selecting the ideal treatment interventions.

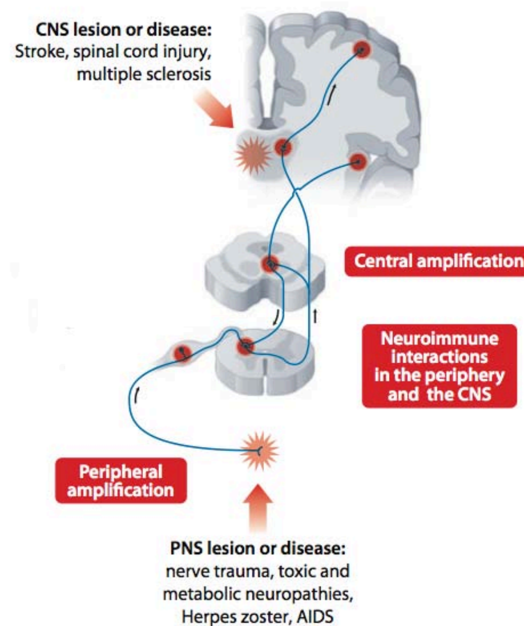


Figure 1–2 Multiple sites that can be modified during neuropathic pain.

Adapted with permission from Annual Review of Neuroscience, Neuropathic Pain: A Maladaptive Response of the Nervous System to Damage, Costigan, Scholz and Woolf, figure 1D, © 2009, 10.1146/annurev.neuro.051508.135531

1.3.2. Inflammatory pain

Inflammatory pain is similar to nociceptive pain, as it is both adaptive and protective (Woolf, 2010). In a preclinical inflammatory pain model induced by intraplantar administration of complete Freunds adjuvant in rats, the release of inflammatory mediators was detected as early as 4 hours post inflammatory pain and found at different sites such as from the inflamed paw, spinal cord and in various brain regions at later time points (Raghavendra et al., 2004). The detected mediators such as pro-inflammatory cytokines (such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor- α (TNF-α)), nerve growth factors, kinins (e.g. bradykinin), purines (e.g. ATP), amines

(e.g. histamines), prostanoids and ions (e.g. potassium and hydrogen) contribute to the increase responsiveness of the sensory nervous system (Raghavendra et al., 2004, Woolf et al., 1994, Woolf et al., 1997, Wang et al., 2005), so the injured site can be avoided and the healing and repairing processes can occur undisturbed (Woolf and Ma, 2007). Mediators can directly activate nociceptors and produce pain hypersensitivity (Vane et al., 1998), whereas others can act synergistically to sensitize the nervous system.

Allodynia and hyperalgesia are two characteristics that are common in both normal (adaptive) and chronic (maladaptive) pain (Costigan et al., 2009b). Allodynia occurs when normally innocuous stimuli such as light touch become painful. Pain can be elicited via the action of low threshold myelinated Aβ fibres or via a reduction in the threshold of nociceptor terminals in the periphery or a combination of both (Woolf and Mannion, 1999).

Hyperalgesia occurs when normally painful stimuli results in heightened pain, as the duration and amplitude of the response to abnormal noxious stimuli are amplified (Basbaum et al., 2009). Other characteristics of inflammatory pain includes increased neuronal excitability in the spinal cord (prompted by central sensitization), heightened sensitivity within and areas surrounding the inflamed region (Woolf and Salter, 2000).

As mentioned previously, inflammatory pain occurs after injury to accelerate the body's healing process thus, inflammatory pain induced hypersensitivity reverts back to normal once the injury has healed or the disease process has been controlled (Woolf and Salter, 2000). However, in chronic pain conditions the sensory system remains caught up in an ongoing excitation state and is similar to an ongoing false alarm which serves no useful purpose (Woolf, 2010).

1.3.3. Chronic pain

Chronic pain includes neuropathic and dysfunctional pain, with the former defined as "pain caused by a lesion or disease of the somatosensory system" (Jensen et al., 2011) and the latter occurs with

"no identifiable noxious stimuli nor any detectable inflammation or damage to the nervous system" (Costigan et al., 2009b). Neuropathic pain is stimulus independent and it is a pathology of the somatosensory system rather than caused by the original stimulus (Woolf and Salter, 2000). When pain persists for more than three months it becomes chronic and it is no longer protective nor does it support the healing and repair process.

1.3.4. Structural changes after injury

After partial peripheral nerve injury, a 20 % loss of superficial dorsal horn neurons have been reported which result in the injured axons, peripheral and central regions to undergo significant changes (Costigan et al., 2009b). New axonal sprouts are stimulated to re-form functional connections with peripheral targets (Kohama et al., 2000, Novakovic et al., 1998) trigger by genes responsible for regeneration and mediators released from the surrounding environment (Costigan et al., 2002). However, inappropriate synaptic formations can occur during the regeneration process as axons can sprout into peripheral nerve grafts, DRG (form baskets around the sensory neurons) and areas outside of normal projection fields (McLachlan et al., 1993). For example, allodynia can occur from misinterpretation of originally non-noxious signal as a result of Aβ fibres sprouting into lamina II (which normally receives painful stimuli from nociceptor C fibres (Woolf et al., 1992)).

1.3.5. Peripheral sensitization: ectopic activity

After a peripheral nerve injury or lesion, spontaneous pain can occur as a result of hyperexcitability in the primary sensory neurons and produces negative symptoms such as paraesthesia, dysthesia and pain (von Hehn et al., 2012). Spontaneous pain is driven by ectopic action potential discharge within the nociceptive pathways including site of injury, neuroma, cell body of injured dorsal root ganglia neurons and neighbouring axonal sites (Costigan et al., 2009b).

1.3.6. Central sensitization

Central sensitization is the term used for the augmentation (increased synaptic efficacy) that occurs in the spinal cord dorsal horn neurons following peripheral tissue injury or inflammation (Woolf, 1983, Ji et al., 2003). It is a major pathophysiological mechanism that contributes to tactile allodynia and secondary hypersensitivity and is common in inflammatory, neuropathic and dysfunctional pain (Campbell and Meyer, 2006).

Under normal conditions, the changes that lead to central sensitization cannot be maintained as the inhibitory mechanisms (GABAergic inhibitory mechanisms) are in place. Conversely, after nerve injury the nociceptive system, nervous system and other CNS regions undergo enormous changes (Woolf and Salter, 2000) leading to altered neuronal processing such as phenotypic shift of primary sensory neurons and increase expression of neuropeptides (Latremoliere and Woolf, 2009).

During inflammatory pain, central sensitization is generated by activated nociceptors that trigger homo- and heterosynaptic facilitation in the dorsal horn of the spinal cord. On the other hand, in neuropathic pain the ongoing excited state of damaged neurons becomes the trigger for central sensitization whilst, in dysfunctional pain the trigger is unknown (Costigan et al., 2009b, Bridges et al., 2001).

Once central sensitization is triggered, it can remain autonomous for some time and only required a low level of nociceptor input to be sustained. Central sensitization is predominantly driven by heterosynaptic potentiation (Woolf and Salter, 2000), where the initial input that activates nociceptor is different from the facilitated input (Campbell and Meyer, 2006).

Central sensitization can cause the following: increase in the membrane excitability, increase in pain responsiveness, prolonged aftereffects to noxious stimuli, expansion of the

receptive field to non-injured areas, as well as disinhibition (removal of inhibitory mechanisms) (Sivilotti and Woolf, 1994, Latremoliere and Woolf, 2009, Woolf and Mannion, 1999). As a result, the activation threshold is reduced and the neurons remain in an excited state hence the action potential can be easily activated and fired (Woolf, 2011). Evidences of central sensitization have been found in patients with osteoarthritis, fibromyalgia, headache, neuropathic pain and complex regional pain syndrome (Woolf, 2011).

Synaptic plasticity plays a key role in central sensitization which involves various pre- and postsynaptic changes (Bridges et al., 2001). However, it is now known that changes in glial cells, gap junctions, and gene transcription also contribute to the generation and maintenance of central sensitization (von Hehn et al., 2012, Gao et al., 2009). For more detailed explanation of central sensitization please refer to the following reviews (Ji et al., 2003, Latremoliere and Woolf, 2009). The next section will cover the treatments developed from the neuronal understanding of chronic pain and their limitations.

1.4. Current treatments for chronic pain

The understanding of the chronic pain mechanisms has developed considerably within the past 20 years, but research has not been matched by a similar growth in treatment efficacy (Finnerup et al., 2010). Chronic pain remains to be empirically treated and the established treatments rarely result in the complete resolution of the symptoms. As pain is heterogeneous and dynamic, only 30% of patients respond to approved treatments (Finnerup et al., 2010).

The number needed to treat (NNT) is commonly used to compare efficacy between different drug classes and it is an estimate of the number of patients that need to be treated for one patient to receive 50% pain relief (Sindrup and Jensen, 1999). The NNT for the currently used analgesics ranges from 1.7 to over 10. With the ideal NNT being 1, this shows that none of the commonly prescribed treatment regimens reliably produce adequate analgesic and that there is a gap in the current knowledge of the underlying pain mechanisms as well as the developed treatment interventions for chronic pain (Dray, 2008).

There are many pain management options including pharmacological treatments, functional neurosurgery, transcutaneous nerve stimulation, behavioural, and alternative treatments (Turk et al., 2011, Woolf and Mannion, 1999). Pharmacological treatments remain the most dominant therapeutic option for chronic pain (Finnerup et al., 2010). However, their use is limited either by the lack of efficacy or with the associated side effects (Sindrup and Jensen, 1999). As a result, a majority of the chronic pain patients still live with a certain degree of pain (Woolf and Mannion, 1999) so, there is an unmet clinical need for more effective and safe treatments to target the underlying mechanism rather than aimed at minimising the effect of symptoms (as one symptom may be generated by several pathophysiological mechanisms) (Scholz and Woolf, 2002, Finnerup et al., 2010, von Hehn et al., 2012). The most frequently used pharmacological treatments will be briefly mentioned follow by current limitations and potential solutions.

1.4.1. Established treatments

Nonsteroidal anti-inflammatory drugs (NSAIDs), tricyclic antidepressants and anticonvulsants were discovered to produce analgesic empirically and not through the understanding of their molecular target (Scholz and Woolf, 2002). NSAIDs are normally used for patients suffering from inflammatory based pain, such as osteoarthritis, rheumatoid arthritis and low back pain but are not used for neuropathic pain. NSAIDs usage is limited by the gastrointestinal side effects caused by blockade of cyclooxygenase (COX)-1 enzyme which normally protects stomach lining from gastric acid (Finnerup et al., 2010). COX-2 inhibitors (targets COX-2 enzymes which leads to inflammation and pain) were found to have fewer side effects than traditional NSAIDs but were associated with increased cardiovascular risks (Roelofs et al., 2008).

Opioids (such as morphine, codeine and its derivatives) are the most commonly prescribed drug class for the treatment of moderate to severe pain (Eriksen et al., 2006). Opioids produce analgesic by agonizing at the μ opioid receptor however, prolonged opioid usage can mediate unwanted side effects such as tolerance (requires higher dose to achieve the same effect), addiction, dependence, respiratory depression, sedation and euphoria (Rosenblum et al., 2008, Varrassi et al., 2010, Stein, 2013).

Recently, opioids have been demonstrated to be an agonist for TLR4 (Hutchinson et al., 2010) and blockade of glial activation in animal studies with the use of ibudilast or minocycline was found to reduce opioid reward, suppress the expression of opioid withdrawal, reduce opioid-induced respiratory depression, reduce conditioned place preference and enhance opioid analgesia (Hutchinson et al., 2009, Hutchinson et al., 2008b). Therefore, indicating prolonged opioid usage can potentially exacerbate glial activation (via TLR4 pathway) and cause the release of inflammatory mediators that shift the immune system to a pro-inflammatory state thus dysregulating the immune system. Opioid induced glial cells activation has been proposed to result in the development of opioid tolerance/withdrawal, oppose opioid analgesia, cause opioid-induced allodynia and

hyperalgesia (Johnston et al., 2004, Hutchinson et al., 2008a). Medication overuse headache (MOH) is thought to also be a form of opioid-induced allodynia as a result of continual glial activation from chronic headache and opioid usage.

Other established treatments for chronic pain include tricyclics antidepressants (TCAs, such as amitriptyline) or selective serotonin reuptake inhibitors (SSRIs, fluoxetine). TCAs work by directly blocking the reuptake of serotonin and noradrenalin (via monoamine transporters, cholinergic receptors, NMDA receptors or sodium channels (Sindrup et al., 2005)) but their usage is greatly limited by side effects, such as cardiovascular adverse events (arrhythmias), constipation and high doses can become toxic (Turk et al., 2011). On the other hand, SSRIs exhibit side effects that are related to the peripheral and central serotonin signalling actions such as nausea, anorexia and at high dose may be life threatening for the patient (Verdu et al., 2008).

Anticonvulsants (such as gabapentin and pregabalin) are also widely used to treat neuropathic pain. The mechanism of actions for anticonvulsants include: functional blockade of voltage-gated calcium or sodium channels, glutamate antagonism, enhancement of the inhibitory system (via GABA) or inhibition of the release of excitatory neurotransmitters which leads to the reduction of neuronal hyperexcitability (Czapinski et al., 2005, Soderpalm, 2002). Some of the common side effects associated with anticonvulsants include sedation, hepatotoxic effects (most anticonvulsants are metabolise by the liver), weight gain and skin rash (Swann, 2001).

1.4.2. Solutions for improving treatments for chronic pain

The field of chronic pain is in need of novel drugs, which can target the underlying mechanism as well as enhance the effects of currently used analgesia and reduce the side effects of pre-existing treatments. New interventions should not exclusively target the neurons, as there is strong evidence from animal studies that targeting only neurons cannot completely dampen pain. It is now known that

non-neuronal cells also play a pivotal role in pain processing (Marchand et al., 2005) and should be targeted so that better treatments can be generated.

Biomarkers can assist the identification of the underlying mechanisms and determine the potential utility of novel treatments. Personalised treatments for chronic pain may be possible with the use of biomarkers, which can recognize and reduce the heterogeneity in patient's response to treatment. One of the common weakness is the commonly use one-dimensional pain scale can introduce large variability in the data as it cannot reliably capture the complexity of patient's pain experience (Finnerup et al., 2010). In addition, other factors such as genes and genders also play a role and influence the underlying pain sensitivity and analgesic responsiveness. For example, differences in opioid receptor expression patterns and the polymorphism of the hepatic cytochrome P450 (responsible for drug metabolism) can explain why some patients are insensitive to certain treatments (Scholz and Woolf, 2002). As biomarkers are objective, so they can minimise patient's subjective variability and assist the identification of chronic pain patient subgroups based on their distinct sensory phenotypes, which allow the selection of mechanism-targeted treatments (Scholz and Woolf, 2002). The use of pain biomarkers can help transform diagnosis from empirical to evidence-based, work synergistically with the one-dimensional pain scales, help transform from treating symptoms to treating mechanism responsible, differentiate responders/ non-responders (Sindrup et al., 2012) that can support enriched trials and ultimately lower the NNT making analgesics more efficacious and safe.

As the search for centrally located biomarkers for chronic pain will be unpractical due to inaccessibility of the compartments, the following section will cover the hunt for potential peripheral biomarkers for chronic pain. Therefore, the role in which the immune system plays in the modulation of chronic pain will now be briefly reviewed.

1.5. Neuroimmune contributions to chronic pain

Traditionally, neurons were thought to be solely responsible for the generation of chronic pain hence treatments were developed to target neurons. However, the outcomes for current treatments are often poor as the treatments target only symptoms and are not disease modifying. This suggests neuronally focused approach is inadequate to prevent or reverse chronic pain. Consequently, there has been a shift in the focus of chronic pain initiation and maintenance from predominately neuronally based to include the involvement of non-neuronal cells of the peripheral and central immune system. Activation of the immune system has been demonstrated in preclinical models to have an important role in both peripheral and central abnormal sensory processing (Grace et al., 2011b, Grace et al., 2014). The contribution of the following peripheral immune cells during chronic pain will be briefly discussed below: mast cells, neutrophils, macrophages and lymphocytes.

1.5.1. How do peripheral immune cells contribute to chronic pain?

1.5.1.1. Mast cells

Mast cells are well known to play a key role in allergic reaction, acquired immune response and as effector cells for innate immunity (Galli et al., 2005). They are normally found in the peripheral nerves but after nerve injury, the number of mast cells was found to substantially increase (Moalem and Tracey, 2006). Mast cells can be activated through the endogenous peptide receptors or via the recognition of invading pathogens by the innate immune receptors such as Toll-like receptors (TLRs) (Galli et al., 2005). In a preclinical neuropathic pain model in which the sciatic nerve of rat was partially ligated, the activation of mast cells was found to initiate an inflammatory cascade that contributed to the development of hyperalgesia (Zuo et al., 2003).

Activation of mast cells releases various inflammatory mediators such as histamine, pro-inflammatory cytokines, proteases and serotonin that are renowned to sensitize nociceptors, excite damaged sensory neurons (Sommer and Kress, 2004, Sommer, 2004) and cause the degranulation of mast cells (Gaboury et al., 1995, Malaviya and Abraham, 2000, Galli et al., 2005). Histamine is the

key mediator released by mast cells and neuronal histamine receptors were found to be upregulated in the DRG after nerve injury (Kashiba et al., 1999). When histamine was blocked with the use of histamine receptor antagonists, the development of both thermal and mechanical hyperalgesia was inhibited (Zuo et al., 2003). At the site of nerve lesion, degranulation of mast cells occurs which drives an influx of leukocytes (rolling and adhesion) and macrophages into the injured nerve for the clearance of the site of injury or infection (Zuo et al., 2003). Degranulation of mast cells was found to be driven by inflammatory mediators such as histamine, TNF-α, mast cell derived leukotrienes and P-selectins

The involvement of mast cells was further demonstrated with the use of a mast cell stabiliser (sodium cromoglycate). Despite being an incomplete stabiliser, sodium cromoglycate could prevent mast cell degranulation after nerve injury, reducing the infiltration of leukocytes and the density of macrophages to the injured site. Hyperalgesia which occurred as a result of nerve injury was also suppressed (Zuo et al., 2003).

1.5.1.2. Neutrophils

Neutrophils are one of the earliest inflammatory cell types to infiltrate the site of injury from the blood (Marchand et al., 2005) and are the most abundant polymorphonuclear leukocytes (Ren and Dubner, 2010). Leukocyte extravasation occurs after peripheral injury and contributes to mechanical allodynia by infiltration to the central regions and interacts with primary afferent cell bodies (Sweitzer et al., 2002, Morin et al., 2007). It should be noted that infiltrating neutrophils not only contribute to pain but can also display anti-nociceptive responses due to the presence of opioid peptides in neutrophils (Rittner et al., 2009) but this topic will not be covered in the thesis.

Neutrophils can be recruited and activated through various factors including leukotriene B4, selectins and nerve growth factor (NGF) which results in the production of inflammatory factors such as lipoxygenase products, nitric oxide (NO), superoxide, cytokines and chemokines (Zuo et al., 2003,

Marchand et al., 2005, Morin et al., 2007). Neutrophils can also be recruited by the vasoactive neuropeptides substance P and calcitonin gene-related peptide released from primary afferent neurons during neurogenic inflammation (inflammation without pathogens) (Ren and Dubner, 2010). Similar to mast cells, neutrophils contribute to peripheral nociceptive sensitization by the release of inflammatory factors, which can directly interact with nociceptors and release chemoattractants for monocytes and macrophages that further exacerbate the excite and sensitise surrounding sensory nerves (Pereira et al., 1990, Ren and Dubner, 2010).

After partially transected sciatic nerve injury in rats, substantial infiltration of endoneurial neutrophils was found in the damaged nerve (peaked at 24 hours post injury and maintained until seven days) (Perkins and Tracey, 2000). At the distal nerve and in the peripheral tissues innervated by the damaged nerve, fewer neutrophils were located (Perkins and Tracey, 2000). However, this finding contradicted another study where, after sciatic nerve injury, accumulation of neutrophils was detected at the rat paw (Levine et al., 1990). The differences in the findings were speculated to be due to the difference between the methods used to identify neutrophils and difference in the models used to generate nerve injury (Perkins and Tracey, 2000). Nevertheless, the studies have demonstrated the involvement of neutrophils in peripheral nerve injuries.

At the time of partial ligation of the sciatic nerve injury in rats, circulating neutrophils were depleted with the injection of a selective cytotoxic rabbit anti-rat neutrophil antibody and the induction of peripheral neuropathic pain (assessed with thermal hyperalgesia) was significantly reduced (Perkins and Tracey, 2000). The early depletion caused a delay in the onset of the inflammatory response following nerve injury (Perkins and Tracey, 2000) hence providing evidence of the vital role in which neutrophils display in the early induction of peripheral neuropathic pain. However, neutrophils may not play a role in the maintenance of chronic neuropathic pain because neutrophil depletion at eight days post injury did not reverse hyperalgesia (Perkins and Tracey, 2000). This suggests that neutrophils are only involved in the acute phase of neuronal injury.

1.5.1.3. Macrophage

Macrophages are part of the myeloid origin that also include monocytes and CNS microglia (Scholz and Woolf, 2007). Macrophages are highly plastic, heterogeneous (display various immunophenotypes) and exhibit different functions dependent on environment, location and time of injury (Lee and Zhang, 2012). Macrophages are classified as either resident macrophages, which are located within the peripheral nerve or blood-derived macrophages, which are located in the blood and are recruited to the nerve injury site by various triggers (Moalem and Tracey, 2006).

After peripheral nerve injury, massive macrophage infiltration was detected (resident macrophages are activated and blood-derived macrophages are recruited) at the DRG, injury sites and various areas proximal to the injured site (Hu and McLachlan, 2002). The number of macrophages was found to peak in the first 2 weeks (Shi et al., 2011) and remained in the injured site until 3 months after nerve injury and display phagocytic activity (remove degenerated axons, worn-out cells and debris) (Lee and Zhang, 2012). The recruitment of macrophages after injury can also be driven by endogenous ligands such as monocyte chemoattractant protein-1 (Hu and McLachlan, 2002), that are derivatives released from the damaged nerve (Shi et al., 2011).

Upon the activation and recruitment of macrophages, pro-nociceptive mediators such as NO, NGF, pro-inflammatory cytokines and prostaglandins are released (Brown et al., 1991, Sweitzer et al., 2002). Collectively, the recruitment and activation of macrophages lead to increased excitation/ degeneration of the axons, recruitment of other immune cells (such as Schwann cells, neutrophils and lymphocytes) to the injured site that ultimately leads to the development of persistent pain (Lee and Zhang, 2012, Heumann et al., 1987, Gomez-Nicola et al., 2008, Marchand et al., 2005). Interestingly, macrophages can also stimulate neuroprotective functions such as the secretion of anti-inflammatory cytokines (such as TGF-β1 and IL-10), tissue repair and regenerate injured peripheral nerves (Myers et al., 1996, Hohlfeld et al., 2000).

Several studies found a temporal correlation between the invasion of blood-derived macrophages and the development of neuropathic pain symptoms (allodynia or hyperalgesia) (Marchand et al., 2005). The development of thermal hyperalgesia was found to be impaired in mice with delayed recruitment of blood-derived macrophages to the site of nerve injury (Myers et al., 1996). Macrophages can also be activated by macrophage-attracting chemokines (CCL2/MCP-1 and CCL3/MIP-1), which are found to signal via TLR2 (Kim et al., 2011). The involvement of TLR2 was confirmed when a reduction in macrophage invasion and reduced neuropathic pain behaviour was observed with the use of TLR2 knockout (KO) mice after experimentally induced peripheral nerve injury (Kim et al., 2011). A study found the depletion of circulating monocytes/macrophages (via intravenous injection of liposome-encapsulated clodronate) in the injured nerve, to alleviate of thermal hyperalgesia and reduced degeneration of axons (Liu et al., 2000b). Conversely, another study found the depletion of circulating macrophages before the induction of peripheral nerve injury (L5 spinal nerve transection) as well as applying inactivated and activated macrophages to L4-L5 spinal peripheral nerves in sham-operated animals did not altered mechanical allodynia (Rutkowski et al., 2000). Despite the contradictory results, there is more evidence supporting the premise that macrophages do play a role in the generation of chronic pain (with their multiple roles) although how large a role it plays is yet to be determined.

1.5.1.4. Lymphocytes

Lymphocytes are classed as B- or T-lymphocytes. B-lymphocytes, which produce antibodies, are not detected in the dorsal horn after nerve injury so they will not be covered in this thesis (Cao and DeLeo, 2008). T-lymphocytes (T-cells) are mediators of the cellular immunity and are made from subpopulations of CD4+ (also known as T helper cells) and CD8+ (also known as cytotoxic T cells) (Moalem and Tracey, 2006). CD4+ cells can be classified as Th1, Th2 and Th17 subsets dependent on their cytokine secretion profile (Mosmann et al., 1986, Kleinschnitz et al., 2006).

After nerve injury, Th1 cells produce prolonged and enhanced released of pro-inflammatory cytokines (including IFN-γ, IL-2 and TNF-α) and activate/ recruit inflammatory cells such as resident macrophages and natural killer cells to be a part of the cellular immune response (London et al., 1998). On the other hand, Th2 cells produce anti-inflammatory cytokines (including IL-4 and IL-10), which can attenuate pain hypersensitivity by inhibition of pro-inflammatory cytokine production and suppress immune cell function. Th1 and Th2 cells are known to be regulators of each other (London et al., 1998, Moalem et al., 2004).

An increased in the number of T-cells and macrophages were detected within the DRGs in the rat for at least 3 months after sciatic or spinal nerve transection (Hu and McLachlan, 2002). Infiltration of T-cells was also detected in the injured sciatic nerve after CCI and the presence of T-cells also coincided with neuropathic behaviour (Moalem et al., 2004). After recruitment of T-cells into the dorsal horn, the release of cytokine interferon gamma by CD4+ T-cells was found to be upregulated after injury (Costigan et al., 2009a) and activate spinal microglia, which contribute to the pathogenesis of neuropathic pain (Tsuda et al., 2009).

After nerve injury, reduced neuropathic mechanical allodynia was detected in T-cells deficient rodents (Costigan et al., 2009a) and substantially less thermal hyperalgesia was identified compared to their heterozygous littermates (Moalem et al., 2004). Likewise, the injection of Th1 cell population via intraperitoneal (generated from splenocytes of sciatic nerve-injured heterozygous rats) into T-cell deficient rats found an increase in the pain behaviour and when Th2 cells were injected into heterozygous rats, the pain behaviour was slightly attenuated (Moalem et al., 2004). In agreement with previous study, the use of CD4+ T-cells KO mice found significant reduction in L5 nerve transection injury-induced mechanical hypersensitivity compared with sham post injury, which indicates the importance of T-cells contribution to neuropathic pain behaviour (Cao and DeLeo, 2008).

In summary, peripheral immune cells significantly contribute to the development and modulation of chronic pain by infiltrating injured site and releasing inflammatory mediators that either directly excites neurons or activate/recruit more neighbouring immune cells. The understanding of the involvement of peripheral immune cells has opened up new avenues for the discovery of potential cellular biomarkers that can be easily accessed. Therefore, this project examines whether the peripherally collected immune cells can be use as the medium and serve as a biomarker for chronic pain.

Other than the involvement of peripheral immune cells in chronic pain, in the past 20 years, immune cells located in the CNS, known as glial cells were also found to be involved in the establishment of chronic pain and will now be briefly discussed.

1.5.2. Immunocompotent cells in the CNS: glia

Glial cells constitute over 70% of the total cell population in the CNS and consist of oligodendrocytes, microglia and astrocytes (Moalem and Tracey, 2006) (see Figure 1-3). Glial cells were originally considered to act as passive bystanders in the CNS to provide housekeeping support for neurons (Allen and Barres, 2009), but accumulating evidence has indicated that glial cells exhibit dynamic key neuromodulatory, neurotrophic and neuroimmune functions in the CNS under both physiological and pathological conditions (Watkins and Maier, 2003, Moalem and Tracey, 2006, De Leo et al., 2006).

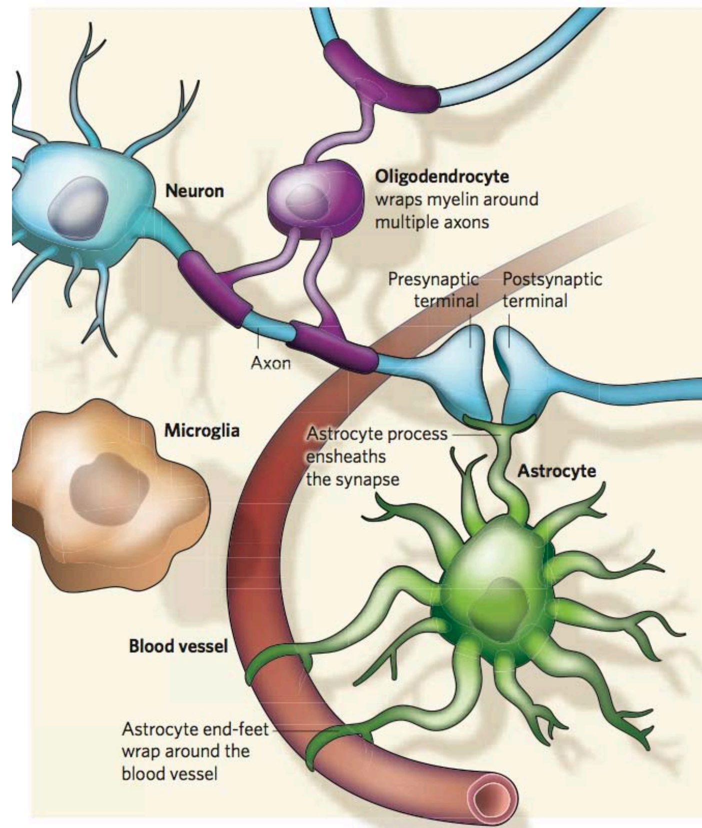


Figure 1–3 The interaction between neuron and glial cells in the central region.

Reprinted from Nature, Vol. 457, Allen and Barres, Neuroscience: Glia – more than just brain glue, 675-677, © 2009, with permission from Nature Publishing Group.

1.5.3. Microglia and astrocytes

Microglia are the resident host defence immune cells of the CNS (they express the same surface markers as macrophages and monocytes) and make up about 5 – 10% of the glial cells (Allen and Barres, 2009). Under basal conditions, microglia are in constant surveillance of the local environment to detect and respond to various stimuli such as pathogens, and damaged cells that threaten physiological homeostasis (Allen and Barres, 2009).

Microglia are usually the first cell type to respond to CNS injury and when activated, it is rapidly recruited to the site of injury followed by proliferation then undergo morphological and functional changes (become amoeboid in shape and act as a phagocyte), upregulate surface antigens and gene expression (Tanga et al., 2004, Beggs and Salter, 2010). Consequently, the

activation of microglia leads to the production of various inflammatory mediators such as cytokines (IL-1, IL-6 and TNF), chemokines, neurotrophic factors, NO, reactive nitrogen and oxygen intermediates (Hanisch, 2002), which can orchestrate the activation of nearby glial cells, neurons and recruit immune cells to the injured site (Moalem and Tracey, 2006, Milligan and Watkins, 2009). Recently, a new concept has come to light that activation of microglia from previous nerve injury (whether from peripheral or CNS tissues) dramatically alters the subsequent response from microglia when exposed to new challenges. This is known as "microglial priming" and has been speculated to contribute to the amplification of pain from microglial activation as a result of previous exposure to trauma or inflammation (Hains et al., 2010).

Astrocytes are the most abundant cells in the CNS and they regulate the synapses and assist with the maintenance of homeostasis in the brain via regulation of extracellular ion (in particular potassium), neurotransmitter concentrations and extracellular pH in the microenvironment (Moalem and Tracey, 2006, Allen and Barres, 2009). As glial cells are immunocompetent, they can express and release immune mediators under a variety of pathological conditions but for the purpose of this thesis only their contribution to chronic pain will be discussed. The use of the term 'glia' will refer to both microglia and astrocytes.

1.5.4. Involvement of glia in chronic pain

It was first discovered in 1991 that increase activation of spinal cord glia (astrocyte hypertrophy) was correlated to the degree of hyperalgesia following constriction injury of the sciatic nerve (Garrison et al., 1991). In a subsequent study, Garrison revealed rats treated with 7 days of systemic NMDA antagonist MK-801 following chronic constriction injury resulted in a reduction in glial activation (in the L4 spinal cord) at 7 and 14 days post injury which also corresponded with attenuation of injury-induced behavioural hyperalgesia (Garrison et al., 1994). Together, these two studies provided evidence that implicate the role of glial activation in chronic pain states.

Since the initial discovery, various studies have confirmed the involvement of glia in chronic pain (Watkins et al., 2007a, Cao and Zhang, 2008, Calvo and Bennett, 2012). Substantial microglial activation was found in the dorsal horn of the spinal cord, which was essential for tactile allodynia after peripheral nerve injury (Tsuda et al., 2003). Consistent with previous finding, the suppression of microglial activation after nerve injury was found to reduce inflammatory immune responses (at the lumbar spinal cord) that led to a reduction in allodynia and hyperalgesia (Raghavendra et al., 2003). Furthermore, spinal astrocytes are involved in chronic pain states via the activation and upregulation of MCP-1, as blockade of MCP-1 attenuated nerve injury-induced mechanical allodynia and heat hyperalgesia (Gao et al., 2009). Additionally, spinal astrocytes were found to play a role in the regulation of central sensitization (Gao et al., 2009). The relation between glial activation being causal to neuropathic pain is further supported by studies that attenuated pain with the use of a variety of glial inhibitors such as fluorocitrate, ibudilast and minocycline (Milligan et al., 2003, Raghavendra et al., 2003, Ledeboer et al., 2007).

1.5.5. Microglia can initiate chronic pain whilst astrocytes maintain it

When minocycline (an inhibitor of microglial activation) was used prophylactically, it reduced microglial activation and subsequent allodynia and hyperalgesia in rats post-L5 nerve transection. However, minocycline was ineffective for the attenuation of existing hyperalgesia and allodynia (Raghavendra et al., 2003). In agreement with the earlier finding, the activation of microglia was detected prior the activation of astrocytes and neurons in a neuropathic pain model (induced by ligation of the L5 spinal nerve) and in a peripheral inflammation model (induced by complete Freunds adjuvant) (Jin et al., 2003, Raghavendra et al., 2004). The activation of astrocytes was detected at a later stage of nerve injury (Kawasaki et al., 2008) thus giving insight of the role which astrocytes are likely to play in the maintenance of persistent pain (Zhuang et al., 2006).

1.5.6. Mediators released from glial cells indirectly contribute to chronic pain

When glial cells are activated after peripheral nerve injury, a variety of nociceptive mediators (such as pro-inflammatory cytokines, prostaglandins, COX-2, iNOS) are released which can act directly or indirectly on neurons and generate action potentials, upregulate neuronal receptors, modulate nociceptive neurons so that enhancement of spinal nociceptive transmission and increased pain behaviours occur (Watkins and Maier, 2003, Tsuda et al., 2003, Watkins et al., 2007b). Spinal cord glia and pro-inflammatory cytokines (IL-1, IL-6, TNF) are key mediators of pathological pain and allodynic behaviours (Raghavendra et al., 2004). As blockade of pro-inflammatory cytokines with the use of intrathecal pro-inflammatory cytokine antagonists for IL-1, TNF and IL-6 were found to reverse allodynia in a preclinical model of inflammatory neuropathy (Milligan et al., 2003). The involvement of pro-inflammatory cytokines released by activated glial cells and infiltrating peripheral immune cells together with pre- and post- synaptic terminals are now considered to be the new "pentapartite synapse" that contributes to chronic pain (reviewed by (Grace et al., 2011b)).

1.5.7. Potential activators of glial cells

Although it is clear that activation of glia is causal to chronic pain states, the signals that initiate the glial activation are currently inconclusive and controversial. There are several neuron-to-glia signals and receptors that are associated with nerve inflammation or trauma which have been implicated to trigger glial activation and induce behavioural hypersensitivity (such as fractalkine and P2X4 receptors) (Tsuda et al., 2003, Milligan et al., 2004). However, a particular family of transmembrane receptors, TLRs are of particular interest. The following section will briefly introduce TLRs and their role in chronic pain.

1.6. Toll-like receptors

In 2011, the discovery of Toll gene and the role of Toll-like receptors (TLRs) in innate immunity by Dr Hoffmann and Dr Beutler were rewarded with the Nobel Prize in Medicine (O'Neill et al., 2013), which confirms the important role in which TLRs play in living organisms. TLRs are a type of pattern-recognition receptors that are considered to be the first line of host defence and play a pivotal role as sensors for innate immunity (Lemaitre et al., 1996, Poltorak et al., 1998). TLRs can initiate innate immune response via the recognition of conserved molecular patterns known as pathogen-associated molecular patterns (PAMPs) (Akira and Sato, 2003) and endogenous ligands known as danger-associated molecular patterns (DAMPs) (Akira et al., 2006).

To date, 13 TLR subtypes have been identified (Bowie and O'Neill, 2000). Different TLR subtypes can recognise a variety of PAMPs derived from microorganisms such as viruses, bacteria, mycobacteria, fungi and parasites (Erridge, 2010) (Akira and Sato, 2003) as well as DAMPs released after tissue damage such as heat shock proteins (Erridge, 2010). DAMPs are also referred to as danger signals because most of the ligands are released during inflammatory responses or released after tissue damage (Miyake, 2007).

1.6.1. TLR signalling pathways

TLRs are evolutionarily conserved type I transmembrane receptors and comprise an ectodomain that assists with the recognition of PAMPs and DAMPs. The cytoplasmic portion of TLRs is similar to the interleukin-1 receptor and is referred to as the Toll/IL-1 receptor (TIR) domain. However, the extracellular portion is vastly different as TLRs are made up of leucine-rich repeats whilst the IL-1 receptor possesses an Ig-like domain (see Figure 1-4). The TLR domain is responsible for the activation of downstream TLR signalling pathways (Akira and Sato, 2003). The ultimate results of TLR activation are to upregulate, synthesise and secrete pro-inflammatory mediators so that invading pathogens can be eliminated from the body.

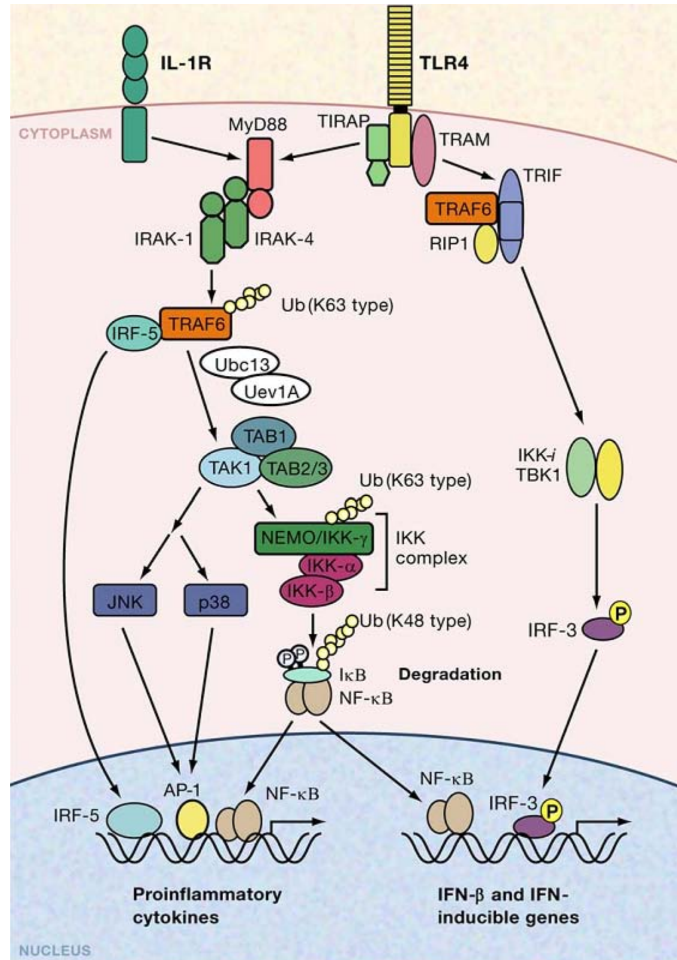


Figure 1–4. A schematic representation of TLR4 signalling.

Reprinted from Cell, Vol. 124, Akira, Uematsu and Takeuchi, Pathogen Recognition and Innate Immunity, page 783 - 801, © 2006, with permission from Elsevier.

A majority of TLRs (except for TLR3) share a common adaptor protein known as myeloid differentiation primary response protein 88 (MyD88) (Takeda and Akira, 2004) (see Figure 1-4). For the purpose of this thesis, TLR2 and TLR4 will be a particular focus as these receptors have been extensively characterised and demonstrated to modulate chronic pain. TLR7 will also be briefly discussed even though it does not contribute to chronic pain, but shares the same downstream signalling cascade as TLR2 and TLR4.

1.6.1.1. MyD88-dependent pathway used by TLR2, TLR4 and TLR7

When MyD88 becomes activated, it recruits IL-1 receptor-associated kinase (IRAK), then IRAK becomes activated via phosphorylation and associates with tumour necrosis factor receptor 6 (TRAF6). This then leads to the activation of two distinct signalling pathways and activates JNK and NF-κB (Takeda and Akira, 2004). TRAF6 is a part of the TRAF family that also simultaneously activates the mitogen-activated protein kinase (MAPK) signalling pathways and results in gene transcription and protein synthesis. The activation of MAPK pathways is a rapid process (within 15 minutes) and induces production of a variety of inflammatory mediators such as cytokines, chemokines and reactive NO (Liu et al., 2012).

1.6.1.2. TRIF-dependent signalling pathway used by TLR4

TLR4 can also signal via the TIR-domain containing adapter-inducing interferon-β (TRIF)-dependent pathway that leads to the activation of NF-κB and interferon regulatory factor 3 (Liu et al., 2012). Additionally, TLR3 also signals through this pathway and recruits the adaptor protein TRIF while TLR4 recruits the adaptor protein TRIF-related adaptor molecule (Akira and Sato, 2003).

1.6.2. Expressions and locations of TLRs

TLRs are located on the cell surface (TLRs 1, 2, 4, 5, 6 and 10), intracellularly in the endosomes or lysosomes (TLRs 3, 7/8 and 9) and some TLRs are expressed both in extracellular and in intracellular compartments (Takeuchi and Akira, 2010). TLRs form homo- or hetero-dimers resulting in complexes that can recognize a range of ligands, for example TLR4 dimerizes with another TLR4 whereas TLR2 can dimerize with either TLR1 or TLR6 (Liu et al., 2012, Takeuchi and Akira, 2010).

As TLRs protect the body via the recognition of invading pathogens and DAMPs, they are expressed throughout the CNS and PNS, including microglia, astrocytes, oligodendrocytes, macrophages, neutrophils, dendritic cells, B cells, specific types of T cells and Schwann cells. Non-immune cells such as neurons, fibroblasts and epithelial cells are also found to express functional

TLRs (Akira et al., 2006, Liu et al., 2012, Lehnardt et al., 2007). The expression of TLRs is dynamic and the expressions are dependent on the cell type and conditions of the cell. Under normal basal condition, the expressions of TLRs are usually low and difficult to detect (Akira et al., 2006). In a normal human brain, only a minority of cultured microglia cells expressed sufficient levels of TLR to be detectable by immunocytochemical staining which supports the notion that microglial TLR expression is tightly regulated and changes according to response to pathogens, cytokines or environmental stresses (Bsibsi et al., 2002).

1.6.3. The involvement of TLR2 in chronic pain

With the use of several preclinical models of neuropathy and TLR2 KO mice, studies have demonstrated TLR2 contributes to the activation of glial cells, enhances production of pro-inflammatory mediators and modulates pain behaviours. The key studies that provided the linkage of TLR2 with chronic pain modulation will be highlighted below.

1.6.3.1. TLR2 and glial activation

After peripheral nerve injury (induced by L5 nerve transection), an increased expression of TLR2 was detected in the glial cells isolated from the DRG (Kim et al., 2011). A recent study also found spinal microglial activation after spinal nerve injury was via the TLR2 induction of NADPH oxidase 2 (Nox2) mRNA expression. With the use of TLR2 KO mice, a reduction in the Nox2 expression was observed and appears to be specific for TLR2, as the Nox2 expression was unchanged in TLR3 or TLR4 KO mice (Lim et al., 2013). The relation between TLR2 and Nox2 expression was confirmed when intrathecal injection of a TLR2 agonist (lipoteichoic acid) induced Nox2 expression in the spinal cord microglia (Lim et al., 2013).

The detection of TLR2 in glial cells may be dependent on the location of tissue collection (DRG vs. spinal cord) as conflicting results have been reported. On the other hand, after partial nerve ligation, increased TLR2 mRNA was not detected in the activated microglia that were

infiltrating the lumbar spinal cord, despite the presence of profound activation of microglia. The study also demonstrated that the spinal microglial activation after nerve injury was not impaired in the TLR2 KO mice (Shi et al., 2011). The differences between the studies could be attributed to the time at which spinal cord microglia activation was measured, as one study measured nerve injury-induced spinal cord microglia activation at 14 day post injury (Shi et al., 2011), whereas the study performed by Kim et al observed a significant difference in the spinal cord microglial activation between TLR2 KO mice and wild type (WT) mice at 4 and 7 days post injury (Kim et al., 2011). Despite the inconsistencies, these studies demonstrate that TLR2 is likely to have a role in microglial activation, but it is dependent on time and location of tissue collection.

1.6.3.2. TLR2 induced pro-inflammatory mediators

The supernatant of damaged sensory neurons (SDSN) obtained from F11 cells (a hybrid cell line of mouse neuroblastoma and rat DRG sensory neurons) was found to activate glial cells (obtained from WT mice) and strongly induced the transcripts of various pain-mediating inflammatory genes (TNF-α, IL-1β, IL-6 and iNOS). The effects of the inflammatory gene induction caused by SDSN were completely abolished in the glial cells collected from the TLR2 KO mice, suggesting that the gene induction was mediated by TLR2 (Kim et al., 2007). The same study also confirmed that after spinal nerve injury, TLR2 induced the production of TNF- α, iNOS and p38 expression in spinal cord microglial cells because the expression was significantly diminished in TLR2 KO mice (Kim et al., 2007). This is in agreement with the significant increase of inflammatory genes (IκB-α and TNF- α) after partial sciatic nerve injury in WT mice but the expression of these genes was reduced in TLR2 KO mice (Shi et al., 2011). A markedly reduced induction of chemokines (CCL2/MCP-1 and CCL3/MIP-1α) and pro-inflammatory cytokines (IL-1β and TNF-α) were also detected in the DRG of TLR2 KO mice after experimentally induced neuropathic pain. The reduction in pro-inflammatory cytokines was accompanied by attenuation of nerve injury generated spontaneous pain hypersensitivity that was not detected in the WT mice (Kim et al., 2011).

1.6.3.3. TLR2 and peripheral immune cell activation

TLR2 plays an important role in the recruitment of macrophages in neuropathic pain. After experimentally induced partial sciatic nerve injury, increased expression of TLR2 was found on the peripheral macrophages that infiltrated the damaged nerve (Shi et al., 2011). TLR2 was found to orchestrate the recruitment of macrophage infiltration as the same process was impaired in the TLR2 KO mice (Shi et al., 2011). TLR2 induced macrophage infiltration to the DRG has also been reported in another study, which was also found to be attenuated in TLR2 KO mice (Kim et al., 2011).

1.6.3.4. TLR2 and behavioural changes

After peripheral nerve injury, it was demonstrated that thermal hyperalgesia was completely abolished whilst mechanical allodynia was partially reduced in TLR2 KO mice (Shi et al., 2011). Likewise, in another study, the use of TLR2 KO mice found the behavioural pain hypersensitivity as a result of spinal nerve damaged was partially inhibited but not completely reversed (Kim et al., 2007).

1.6.4. The involvement of TLR4 in chronic pain

TLR4 has also been demonstrated to be a modulator of chronic pain. In L5 spinal nerve transection and CCI models, TLR4 was identified to be a key molecular marker for glial activation and the activation of TLR4 was correlated with increased expression of pro-inflammatory cytokine genes as well as induction of allodynic behaviours (Tanga et al., 2004, Tanga et al., 2005, Hutchinson et al., 2008c).

1.6.4.1. TLR4 and glial activation

TLR4 was significantly up-regulated in the rat lumbar spinal cord after L5 nerve transection (Tanga et al., 2004). This was shown in TLR4 KO mice where both spinal microglia and astrocytes markers (expression of CD11b, CD14 and GFAP mRNA) were significantly reduced (Tanga et al., 2005). The TLR4 expression was linked to potential spinal cord damage as its expression was found to be

unchanged in sham operated animals. The up-regulation of microglia occurred 4 hours post-surgery until post-operative day 14 (Tanga et al., 2004), thus also confirming that microglia are involved in the initiation of neuropathic pain.

1.6.4.2. TLR4 induced pro-inflammatory cytokine release

In TLR4 KO mice post L5 spinal nerve transection, the expression of pro-inflammatory cytokine mRNA (INF-γ, IL-1β and TNF-α) in the mouse lumbar spinal cord was significantly attenuated compared with WT mice (Tanga et al., 2005). The study demonstrated that after peripheral nerve injury, functional knockout, or point mutation of the TLR4 gene in mice could significantly reduce the expression of spinal pro-inflammatory cytokine transcripts (Tanga et al., 2005). This further demonstrates the importance of TLR4 in the increase production of pro-inflammatory cytokines after neuropathic pain.

1.6.4.3. TLR4 and peripheral immune cells activation

The trafficking of peripheral leukocytes is speculated to be a contributor to the elevated TLR4 mRNA expression in the CNS (Tanga et al., 2004) however further investigation is required to confirm the finding.

1.6.4.4. TLR4 and behaviour changes

Mechanical allodynia was significantly attenuated after peripheral nerve injury in the TLR4 KO mice and in point mutant TLR4 mice when compared to the WT (Tanga et al., 2005). The involvement of TLR4 in mechanical allodynia was also demonstrated in the CCI model, with the use of two TLR4 antagonists (mutant LPS and LPS-RS) found to reverse mechanical allodynia over a period of time (Hutchinson et al., 2008c). The study also indicated that the μ-opioid antagonists, naloxone and naltrexone, could block TLR4 signalling as naloxone was previously used to inhibit LPS induced microglial production of NO and TNF-α (Liu et al., 2000a). Another study also showed robust

mechanical allodynia post LPS administration when injected into the spinal cord of male but not female mice (Sorge et al., 2011).

Hutchinson and colleagues found that intrathecally infusing naloxone in a rat 10 days after sciatic nerve injury and continued for 4 days caused a complete reversal of mechanical allodynia. L5 spinal cord revealed astrocytic marker (GFAP) was not suppressed by naloxone but markers of microglial activation (CD11b/c) were suppressed, which consequently links the behaviour of mechanical allodynia with TLR4 induced microglia activation (Hutchinson et al., 2008c). TLR4 was found to be vital in the maintenance of neuropathic pain as (+)-naloxone (inhibits TLR4 receptor signalling without antagonizing classical opioid receptors) was found to reverse allodynia in rats 2 months following spinal nerve ligation and 4 months following CCI (Lewis et al., 2012).

1.6.5. Influences other than TLR2 and 4

It should be noted that TLR2 and TLR4 induced glial activation could not be the sole receptors that are driving the change in the pain behaviour of the animals. TLR2 deficient mice do not display complete attenuation of mechanical allodynia after peripheral nerve injury and decreased microglial/macrophage expression of TLR4 mRNA was not accompanied by a decrease in the behaviour of the rats (tactile hypersensitivity) after L5 spinal nerve transection (Tanga et al., 2004). This implies other yet to be defined glial receptors or glial mediators may be responsible for the change in allodynia behaviour observed in animals as post nerve injury but TLR2 and TLR4 are modestly involved. (Tanga et al., 2004). The above studies collectively reflect that both TLR2 and TLR4 do contribute to the generation of chronic pain by orchestrating peripherally and centrally orientated actions that involves both glial and peripheral immune cells. It is also for this reason that the assessment of TLR2 and TLR4 signalling was included as potential biomarkers for the identification of chronic pain patients.

1.6.6. TLR7 activation contributes to itch and not pain

As mentioned previously, for a biomarker to be useful, it should demonstrate specificity. TLR7 shares the same downstream signalling as TLR2 and TLR4 but signalling via TLR7 does not result in pain but itch. Chronic itch and chronic pain are both debilitating conditions that produce distinct sensations; pain elicits a withdrawal response whilst itch evokes a desire to scratch (Ji, 2012). Both conditions arise as a result of the dysfunction of the immune and nervous system and hence share similar mechanisms, mediators and the primary sensory neurons which respond to both itch and pain stimulus (Liu and Ji, 2013). The establishment of chronic itch can be manifested by a combination of peripheral and central sensitization, loss of inhibitory control in the spinal cord, neuro-immune and neuro-glial interactions (Liu and Ji, 2013) which is similar to chronic pain. Therefore, TLR7 signalling was also examined in this project to determine whether the biomarker identified is specific to chronic pain.

In humans, TLR7 is found on dendritic cells (Ganguly et al., 2009) and DRG neurons (Qi et al., 2011). Several types of imidazoquinolines derivates such as imiquimod can activate TLR7 (Akira and Sato, 2003). Imiquimod has potent anti-viral and anti-tumour properties and it is commonly used for superficial and nodular basal cell-carcinoma. The use of topical imiquimod is frequently associated with pruritus in humans (Madan et al., 2010). Imiquimod was found to directly activate and excite itch-mediating fibres (Liu et al., 2010) and when applied on mice's skin induced psoriasis-like inflammation (van der Fits et al., 2009).

Despite similarities in the pain and itch pathways, TLR7 is not related to pain hypersensitivity as neuropathic pain induced by spinal nerve ligation and induced inflammatory pain was found to be unaltered in TLR7 KO mice (Liu et al., 2010). Furthermore, the TLR7 KO mice also demonstrated normal thermal and mechanical pain sensitivity and expectantly markedly reduced scratching behaviours compared with WT mice (Liu et al., 2010). TLR7 agonist in rats was found to increase the

activation of microglia in the spinal cord (Zhang et al., 2005) and the use of imiquimod elicited itch-like scratching but pain related behaviour was not observed.

1.7. Role of inflammatory mediators in chronic pain

There is a consensus that chronic pain patients have elevated pro-inflammatory cytokine levels (such as IL-2 mRNA, TNF mRNA, IL-2, and TNF) or chemokines (monocyte chemotactic, which can be correlated with worsening pain intensity (used pain scales). Elevated levels of pro-inflammatory cytokines and chemokines were found in patients with painful neuropathy (Uceyler et al., 2007a), female fibromyalgia patients (Ang et al., 2011, Garcia et al., 2013) and in a heterogeneous cohort of chronic pain patients (Koch et al., 2007). Some of the inflammatory mediators (such as IL-8, MCP-1 and NO) were found to significantly correlate with pain intensity (Ang et al., 2011, Brisby et al., 2002, Koch et al., 2007). Cytokine levels were found to predict treatment response as demonstrated in the level of interferon gamma immunoreactivity that was able to be used to predict treatment response in patients with lumbar degenerative and low back pain syndrome (Scuderi et al., 2009). Likewise, level of IL-6 collected in plasma was found to significantly inversely correlate with pain intensity (VAS score) in a chronic pain cohort who responded to treatments (Zin et al., 2010). However, studies that examine levels of basal cytokines and chemokines in chronic pain patients face the biggest limitation: reproducibility of the findings. This can be attributed to the lack of follow up after the initial discovery of the potential marker and the difficulty in assessing basal unstimulated cytokine expression. Therefore this project will not only measure the basal level of inflammatory mediators but after stimulation (evoked response).

However, before the evoked response can be introduced, the following section will go into more detail about the role that cytokines have in chronic pain so that the assessment of cytokines from TLR stimulated peripheral immune cells can be appreciated as a potential biomarker for chronic pain. Cytokines are often used as an inflammatory marker because they can give insight into changes that occur at gene level, transcription factor activity and protein level (Slade et al., 2011) that may correspond with behavioural changes.

1.7.1. Cytokines

Cytokines are small proteins that are synthesised by most cell types including both immune and non-immune cells (Uceyler and Sommer, 2008) and they can facilitate immune-nervous system communication (Watkins et al., 1999) by acting on themselves (autocrine), on nearby cells (paracrine) or on distance cells (endrocrine) (Zhang and An, 2007). They can act synergistically, antagonistically, can act on several different cell types (pleiotropic) and different cytokines can stimulate similar functions (redundant) (Dinarello, 2000, Zhang and An, 2007). Due to the many abilities of the cytokines, production is tightly regulated and is only produced when evoked by noxious stimuli such as during inflammation or after cell injury (Dinarello, 2000, Zhang and An, 2007).

Cytokines, which promote inflammation, are termed "pro-inflammatory" (such as IL-1β and TNF-α) whereas those that suppress the activity of pro-inflammatory cytokines are termed "anti-inflammatory" cytokines (such as IL-4 and IL-10) (Uceyler and Sommer, 2008). The maintenance of balance between pro- and anti- inflammatory cytokines is vital and can significantly impact on the outcome of the disease (Dinarello, 2000).

For the purpose of this thesis, the principal pro-inflammatory cytokine IL-1β will be examined as it has been demonstrated in many preclinical and clinical studies that the over production of IL-1β is a contributing factor for the development of chronic pain (Dinarello, 1996, Ren and Torres, 2009) and indicate dysregulation in the immune system by demonstrating the body to be in a pro-inflammatory shifted state with supressed anti-inflammatory cytokines.

1.7.2. Role of interleukin-1β in chronic pain

IL-1β signals through receptor IL-1 type 1 (IL-1RI) that is located on the cell surface and when bound to IL-1R1 (also found on neurons (Zhang and An, 2007)), a second receptor known as IL-1 receptor accessory protein is recruited at the cell membrane forming a high affinity binding receptor complex. This results in the initiation of several downstream signalling events (Moalem and Tracey, 2006, Ren and Torres, 2009). NF-κB is translocated into the nucleus and causes the up-regulation of several

gene transcriptions such as COX-2, inducible NO synthase, substance P, TNF, IL-1β and IL-6 (Tegeder et al., 2004). These inflammatory mediators are well-known to contribute to hyperalgesia (Ren and Torres, 2009).

Normally IL-1β exists in the inactive form (pro-IL-1β) and is converted into its mature form by the protease caspase-1 via the NALP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome (Martinon and Tschopp, 2007). Activation of the caspase-1 activating NALP3 inflammasome can occur with the use of monosodium urate (MSU) crystals with the addition of LPS and leads to the production of IL-1β. Gout was found to be a result of the aberrant activation of caspase-1 and NALP3 inflammasome due to deposition of MSU crystals (Martinon and Tschopp, 2007).

IL-1β is a potent pro-inflammatory cytokine and is involved in many important homeostatic functions such as regulation of feeding, core body temperature, sleep, learning and memory (Watkins et al., 1999). IL-1β can act either directly or indirectly on nociceptors, as demonstrated within 1 minute of IL-1β plantar injection into rat's hind-paw skin that substantially altered the effects of decreased nociceptor threshold and increased neural responsiveness to mechanical and thermal stimulation (Fukuoka et al., 1994).

As a result of nerve injury, the level of IL-1β in the periphery was found to indirectly induce the release of IL-1β in the brain by binding to the blood brain barrier cells that leads to the production of prostaglandin E2 (highly inflammatory) via COX-2 (Samad et al., 2001). As prostaglandin E2 is lipid soluble, it can pass through the blood brain barrier and activate neurons and glial cells (Ek et al., 2001) which lowers neuronal threshold, enhances hyperalgesic activity and contributes to central sensitization (Ren and Torres, 2009). The finding was reinforced when the intrathecal blockade of COX-2 could significantly reduced mechanical hyperalgesia (Samad et al., 2001).

1.7.2.1. IL-1β found to be elevated in chronic pain conditions

In intact nerves of rats, IL-1β is normally expressed at low levels to undetectable ranges (Rotshenker et al., 1992, Gillen et al., 1998). However, upregulation of IL-1 β mRNA in the intact nerves of rats was detected as early as 1 hour after CCI surgery (Uceyler et al., 2007b). Furthermore, expression of IL-1β mRNA in the spinal cord was found to increase 4-fold in rats that underwent traumatic spinal cord injury and remained elevated 72 hours post injury (Wang et al., 1997). Similarly, IL-1β mRNA expression in the sciatic nerve was found to be significantly greater than sham, at day 7 post CCI and accompanied with hyperalgesia (Okamoto et al., 2001). Likewise, in rats that have either undergone spared nerve injury or CCI, upregulation of IL-1β was detected in the hippocampus and the levels of IL-1β were found to closely correlate with mechanical allodynia in 2 different rat strains (del Rey et al., 2011).

Elevation of IL-1β levels has also been detected in various neuropathic models such as nerve crush injury (Wang et al., 1997), sciatic nerve transected injury (Sweitzer et al., 2001, Shamash et al., 2002) and 35 days post-sciatic cryoneuroloysis (DeLeo et al., 1997). The time of elevated IL-1β production positively correlated with the development of thermal hyperalgesia as well as mechanical allodynia thus further supporting IL-1β's role in the involvement of neuropathic pain behaviours after nerve injury. Consistent with preclinical models, IL-1β levels were found to be elevated in the semen of patients with chronic prostatitis/chronic pelvic pain syndrome (Alexander et al., 1998) and in the cerebrospinal fluid of chronic pain patients (Backonja et al., 2008). However, the detection of basal IL-1β in the blood proved to be difficult due to the low concentration which has led to undetectable levels in chronic pain patients (Koch et al., 2007).

1.7.2.2. Blocking the effects of IL-1β reduce neuropathic pain behaviours

The contributing role that IL-1β plays in chronic pain behaviour is further supported via the blockade of IL-1β which result in reduced allodynic behaviours. The combined effect of cytokine antagonists soluble TNF receptor and IL-1R antagonist (binds to IL-1R but prevents the interaction of IL-1 with its

receptors and behave as a natural inhibitor (Ren and Torres, 2009)) administrated in a rodent after L5 spinal nerve transection dose dependently attenuated mechanical allodynia (Sweitzer et al., 2001). The blockade of both TNF and IL-1R was found to exhibit additive effects to reduce pain-induced behaviours (Schafers et al., 2001) also demonstrating the synergistic actions in cytokine networks. The use of neutralizing antibody to IL1R was found to also dose-dependently reduce thermal threshold and mechanical allodynia (Sommer et al., 1999). The importance of IL-1 signalling was further shown in mutant mice with impaired IL-1 signalling as mechanical and thermal pain sensitivity after nerve injury was unchanged (Wolf et al., 2006). Similarly, the use of IL-1αβ gene-deficient mice after neuropathy displayed reduced mechanical allodynia compared with controls (Honore et al., 2006).

1.7.2.3. Administration of IL-1β produce pain behaviour

The relation between IL-1β and chronic pain behaviour is further demonstrated when IL-1β is injected into either peripheral regions (such as rat's paw (Ferreira et al., 1988), rat's sciatic nerve (Zelenka et al., 2005)) and central regions (such as into spinal cord (Sung et al., 2004)) all resulting in the production of hyperalgesia (Zhang and An, 2007). This therefore confirms IL-1β's role as a potent mechanical and thermal hyperalgesic agent (Ren and Torres, 2009).

In summary, IL-1β is upregulated after neuropathic pain and the level of IL-1β is correlated with pain behaviour. Collectively, the role in which IL-1β expression plays in chronic pain is vital for the generation and the development of pain behaviours in both preclinical and clinical models. However, as basal levels are variable and difficult to quantify, so in this thesis I have assessed the level of IL-1β in chronic pain patients after an evoked response (post TLR agonist stimulation). Hence, the next section will investigate the use of TLR agonist evoked responses to assess the status of the immune system.

1.8. TLR responsiveness

To date, the responsiveness of *in vitro* cytokine productions from stimulated PBMCs has mostly been studied within the context of immune system mediated diseases (see Table 1). TLR ligands are selected because they can induce innate cytokine responses via TLR pathways to and give insight into TLR signalling efficiency and downstream cellular immune responsiveness (Dunne et al., 2010). This approach is based on the premise that the measurement of evoked cellular responsiveness can identify certain "immunologic signatures" from the cytokines, thus discriminating patient groups from healthy controls (Davis et al., 2010).

Table 1–1. Evidence of TLR responsiveness in humans with immune mediated diseases.

	Blood	PBMCs	Measured outputs	Basal	Post TLR responsiveness	Ref
Irritable bowel syndrome		TLR4 ligand (1 ng/mL)	TNF-α, IL-6 & IL-1β	↑ TNF-α, IL-6 & IL-1β	↑ IL-6	1
Irritable bowel syndrome	TLR1/2, TLR2 (10µg/mL), TLR3 (1 µg/mL), TLR4, TLR5, TLR6/2, TLR7 and TLR8 ligands (The rest is 1 µg/mL)		Cortisol, IL-1β, IL-6, IL-8, TNF-α, IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-13 & IFN-γ	↑cortisol, IL-6 & IL-8	↑ IL-1β, IL-6 & TNF-α	2
Chronic fatigue syndrome		TLR4 ligand (1ng/mL) & phytohemagglutinin (4µg/mL)	IL-1β, IL-6, TNF-α, IL-2 & IL-4	↑TGF- β	↑ IL-1β, IL-6 & TNF- α ↓ TGF- β	3
Sepsis and post surgical stress		TLR4 ligand (1 µg/mL)	IL-1β, TNF-α & surface receptor expression	↑Basal TLR2 & 4 expression (both patient cohorts)	↑ IL-1β & TNF- α (Surgical patients) ↓ IL-1β (septic patients)	4
Rheumatoid Arthritis		TLR4 ligand (1, 10 and 100 ng/mL) & cytokine mix (IL-1, IL-6 and 100 ng.mL)	IL-6, TNF- & expression of TLR2 and 4 mRNA		↑ IL-6 & TNF-α	5
Septic patients	LPS (1 ng/mL and 1 µg/mL)		IL-1β, IL-6 & TNF-α		↓ IL-1β, IL-6 & TNF-α	6
Immunosuppressed		TLR1/2, TLR2/6, TLR4 & TLR5 ligands*	IL-8, TNF-α & surface expression of TLRs 1, 2 and 4	↑ Basal TLR 4 expression (both patient cohorts	↑ IL-8 & TNF-α	7
Sarcoidosis		TLR2, 4 and NOD2 ligands*	TNF-α, IL-1β, IL-6, IL-8, IL-10, IL012p70 & expression of TLR2 and 4	↑ TLR2 & 4 expression	↑ IL-1β & TNF- α	8

1: (Liebregts et al., 2007) 2: (McKernan et al., 2011) 3: (Chao et al., 1991) 4: (Tsujimoto et al., 2006) 5: (Kowalski et al., 2008) 6: (Ertel et al., 1995) 7: (Dunne et al., 2010) 8: (Wiken et al., 2009). * See references for specific concentrations used in the study

The over-responsiveness of TLR and cytokine activities found in PBMCs have provided evidence which suggests the presence of immune dysregulation (McKernan et al., 2011) and a primed immune system (as demonstrated with the exaggerated cytokine activities) in disease states that allowed the distinction between patients and healthy controls (Chao et al., 1991, Kowalski et al., 2008). On the contrary, reduction in TLR4 responsiveness was also found to be useful as it suggested that "endotoxin tolerance" occurs in septic patients and assisted in the understanding of sepsis (Ertel et al., 1995). Interestingly, some of the studies presented in Table 1-1 also assessed surface receptor expression, but there are inconclusive findings with whether change in levels of receptor expression is associated with change in cytokine levels (Tsujimoto et al., 2006, Dunne et al., 2010).

Despite the different approaches used to obtain assessed cytokine responsiveness after PBMCs stimulation (e.g. stimulating blood vs. stimulating PBMCs directly, differences in isolation techniques and measurement of different cytokines), there is a consensus in an enhancement of TLR responsiveness (except septic patients) when the immune system is dysregulated.

There are several advantages with the use of stimulated PBMCs. Firstly; circulating PBMCs come into contact with the microenvironment, which may influence the responsiveness of cytokine production (impacted by genetic, epigenetic and environmental factors). Therefore, the *in vitro* cytokine profiling may capture relevant but currently unknown factors that could affect disease phenotype (Davis et al., 2010). Secondly, the use of mixed cell populations limits the manipulation of the cells so it can preserve the natural state of the cells and allows cell-to-cell interactions, which may also affect cytokine responses (Hall et al., 2004). Thirdly, the measurement of an evoked response released from PBMCs may allow higher sensitivity for the detection of abnormal immune functions. The measurement of basal (unstimulated) cytokines is often shows high variability or poor sensitivity to differentiate between patients and healthy controls (Liebregts et al., 2007). Lastly, the use of PBMCs was previously demonstrated in our laboratory to be a good source of a potential pain

biomarker. The proliferative response from mitogen stimulated PBMCs were found to significantly correlate with cold pain tolerance scores in healthy volunteers (Hutchinson et al., 2004). Therefore, the assessment of PBMCs can be adapted so that it can be used as a biomarker to identified chronic pain patients.

1.9. Other techniques used in this project

1.9.1. Preclinical model: Graded chronic constriction injury model

Other than investigating the TLR responsiveness in isolated human PBMCs, a preclinical model was also utilised in this project to better understand the underlying mechanism. Even though the translation between human and animals is not perfect, preclinical models are frequently used in pain research as it can help understand the mechanisms at the cellular and subcellular levels. Preclinical models can provide a controlled environment in which physiological or pharmacological variables can be manipulated that would otherwise be difficult to achieve in humans (Chapman et al., 1985).

CCI has been commonly used as a neuropathic pain model since its developed in 1988 (Bennett and Xie, 1988) and has been mentioned countless times in the introduction. Four pieces of chromic gut sutures are tied around the exposed sciatic nerve and elicit characteristics of chronic pain such as hyperalgesia and allodynia, which can be tested using von Frey hair filaments. However, this model is limited by its binary response of either "a lot of pain" or "no pain" at all. As clinical pain is heterogeneous with regards to pain intensity; a modulation of the CCI model known as the graded model of CCI was selected. The graded model of CCI creates a graded pain response by varying the number of chromic gut ligated around the sciatic nerve and in the subcutaneous space (Grace et al., 2010), generating responses that better resembles the heterogeneity of the clinical population. Additionally, the graded model was found to demonstrate the role of peripheral immune cells in the development of nociceptive hypersensitivity (Grace et al., 2011a) which further supports its utility in pain biomarker discovery.

1.9.2. Assessment of basal pain sensitivity: Cold pain test

The other technique that was also used in this project is the cold pain test. Experimental pain tests are often used to understand pain sensitivity as they are found to be more accurate than measure of pain represented on VAS (Walsh et al., 1989). The cold pain test is an example of a non-invasive experimental pain model used for the induction of pain (painful cold sensation) so the basal pain

sensitivity (pain threshold and tolerance) can be assessed (Stening et al., 2007). Briefly, on immersion of the hand in cold water, an initial sensation of cold will be readily felt, followed by an aching or crushing pain. The sensation is reversible within a few seconds to minutes after removal of the hand from water (Walsh et al., 1989). The cold pain test has demonstrated good reliability (Mitchell et al., 2004), reproducibility and has good sensitivity to analgesics (demonstrated in morphine (Jones et al., 1988) or methadone-maintained (Doverty et al., 2001) patients. Reduction in pain tolerance has been reported in patients on opioids hence it was found to be the most sensitive test for the detection of opioid-related hyperalgesia (Younger et al., 2008, Krishnan et al., 2012).

The cold pain test is a tonic pain test mediated by both the A-δ fibres (responsible for the cold sensations) and C-fibres (responsible for the dull, aching cold pain) (Krishnan et al., 2012). With the use of fMRI the tonic stimulus induced by the cold pain test could strongly and reproducibly activate the periaqueductal gray (PAG) (La Cesa et al., 2014) and stimulate other descending pain pathways (Pickering et al., 2014). The cold pain test is a safe, inexpensive test used to assess the function in the descending pain modulatory system during tonic pain.

The generated cold pain tolerance score was found to be a useful biomarker for the identification of increased general pain sensitivity and central sensitization in chronic tension-type headache patients compared with control participants (Cathcart et al., 2009). Hence, the measurement of the basal pain sensitivity may behave as a chronic pain biomarker that can allow the identification of patients that respond abnormally to tonic pain so the presence of a neurological problem can be identified. The use of the cold pain test as a biomarker for pain will be further explored in later section, as a study has found a significant link between the immune system and basal nociceptive thresholds in healthy participants (Hutchinson et al., 2004) suggesting the utility of cold pain tolerance to demonstrate good utility as a biomarker.

1.10. Summary

To the author's knowledge, TLR responsiveness has yet to be investigated in chronic pain patients because the involvement of the immune system was only recently demonstrated. It is now clear from the literature that peripheral immune cells, TLR pathways and the release of pro-inflammatory cytokines all contribute to the shift in the immune system to a pro-inflammatory state during chronic pain conditions. Therefore, if the potential biomarker for chronic pain can include the mentioned candidates then it may capture the dysregulated state of the immune system from the periphery and serve as a useful biomarker. Therefore, the purpose of this thesis is to identify an immunologic signature from the periphery, which could differentiate chronic pain patients from pain free participants.

1.11. Research Aims

As outlined in the previous section, the currently used pain assessments do not inform clinicians about the underlying mechanisms responsible for the chronic pain, which then greatly impacts the quality of life and on the treatment selection. There is an urgent need for the discovery of chronic pain biomarkers that can assist in the identification of underlying mechanisms, assist clinicians in diagnosing patients as well as improving efficiency of decision making in early clinical phase and personalised treatments.

One of the current research gaps for chronic pain is the lack of a well-validated biomarker. This thesis examines the potential use of the peripheral immune cell reactivity as a potential biomarker for chronic pain. The hunt of a cellular chronic pain biomarker is hampered by the inaccessibility of central tissues (brain and spinal cord). However, as discussed in the introduction there has been a paradigm shift in the understanding of chronic pain and that the cells of immune system is now known to play an active role in the modulation of chronic pain.

This thesis consists of 2 clinical and 1 preclinical studies to examine the utility of peripheral immune cell responses post TLR ligand stimulation as potential chronic pain biomarkers.

Based on the existing literature, the major aims to be examined in this thesis are:

The first study was conducted in humans to identify potential biomarkers for chronic pain by examining TLR responsiveness in isolated PBMCs (chapter 2).

Aim 1: To characterise the IL-1β concentration curve in response to LPS, Pam3CSK4, imiquimod and MSU crystals from peripheral immune cells isolated from chronic pain patients and pain-free participants.

Aim 2: To determine whether chronic pain patients have dysregulated immune systems demonstrated from the cytokine production of PBMCs after TLR agonist stimulation.

Aim 3: To examine whether the TLR reactivity from isolated peripheral immune cells will be different between chronic pain patients and pain-free participants and determine whether peripheral immune cells can be a source to be a potential biomarker for chronic pain.

Aim 4: To investigate whether chronic pain patients on opioid treatments demonstrate an enhancement in cytokine production post TLR ligands stimulation when compared with chronic pain patients not on opioid and pain-free participants.

Chapter 3 consists of a preclinical study that will follow from findings obtained from chapter 2 to better understand the mechanisms of TLR responsiveness as a chronic pain biomarker. This chapter also examines the correlation between peripheral and central TLR signalling to determine similarity between the two regions. The translatability of the findings between rats and humans will also be investigated with the use of data-derived computer models.

Aim 5: To determine whether increased TLR reactivity can be found in rats with greater allodynia and to assess whether TLR stimulated responses from rat peripheral immune cells and spinal cord can predict the presence of pain and the severity of allodynia.

Aim 6: To examine whether a panel of IL-1β responses from TLR stimulated peripheral immune cells can be correlated with the IL-1β response released from lumbar spinal cord.

Aim 7: To determine if the biomarker panel that is based on the peripheral regions from the preclinical model can be used to distinguish pain from non-pain states in a cohort of chronic pain patients.

Chapter 4 will examine the utility of TLR responsiveness in isolated PBMCs to determine the efficacy of a novel glial targeted treatment (ibudilast) used to relieve migraine in a cohort of medication overuse headache patients.

Aim 8: To determine if ibudilast can lower the TLR reactivity in isolated peripheral immune collected from MOH patients.

Aim 9: To determine the efficacy of ibudilast can be correlated with the TLR reactivity from isolated peripheral immune cells collected in MOH patients.

Chapter 2. TLR responsiveness of the human peripheral blood mononuclear cells as a potential biomarker for chronic pain

Kwok YH, Hutchinson MR, Gentgall MG, Rolan PE (2012) Increased Responsiveness of Peripheral Blood Mononuclear Cells to In Vitro TLR 2, 4 and 7 Ligand Stimulation in Chronic Pain Patients. PLoS ONE 7(8): e44232. doi:10.1371/journal.pone.0044232. Reprinted with permission from PLoS ONE.

In the process of finding potential chronic pain biomarkers in the periphery, we believe that performing an exploratory clinical study with previously identified pain markers provides an optimal starting point. This study is an extension of the work previously conducted by Hutchinson, where the study found mitogen stimulated proliferative responses of peripheral blood mononuclear cells (PBMCs) were significantly correlated with cold pain sensitivity scores (Hutchinson et al., 2004). That study indicated that peripheral cells have the capacity to be a good cellular pain biomarker. The use of PBMCs is appealing because they express TLRs, which have been demonstrated to play a vital role in the activation of glial cells that is causal to the development of chronic pain (section 1.5.2 Immunocompotent cells in the CNS: glia). In addition, PBMCs may mirror central immune signalling and can be easily accessed which fulfils the characteristics of an ideal biomarker.

As this study is of exploratory in nature, heterogeneous cohort of chronic pain patients was selected. The study consisted of 2 chronic pain cohorts (on and not on opioid treatment) and a pain-free cohort for comparison. This study stimulated the isolated PBMCs with TLR2, TLR4, TLR7 and NLRP3 (MSU crystals) agonists and assessed TLR downstream signalling by measuring the pro-inflammatory cytokine, IL-1β. TLR agonists and IL-1β were intentionally selected because they have demonstrated substantial modulatory roles in various chronic pain conditions.

The investigation of TLR responsiveness in isolated PBMCs is not novel and has been extensively examined in different disease populations. However, to the author's knowledge *in vitro* TLR responsiveness has not been investigated in the chronic pain population compared with pain-free participants until this study. A gap in the present literature exists in the direction of the PBMCs' responses after stimulation because PBMCs are often stimulated with a single high concentration of TLR agonists. Therefore, we decided to use a range of TLR agonist concentrations to better understand and capture the stimulated cellular responses as this allows for better characterization of the PBMCs' reactivity.

The aim of this study was threefold: firstly, to determine whether isolated PBMCs could be a source of potential pain biomarker was investigated by measuring the pro-inflammatory output of the PBMCs after TLR agonist stimulations. Secondly, the study investigates the specificity of the PBMCs response with TLR and non-TLR agonists. Lastly, to determine whether chronic pain patients on opioids exhibit primed behaviour (exaggerated cytokine released) after TLR agonist stimulation was determined.

In this study we successfully found a potential pain biomarker that allowed significant differentiation between chronic pain patients maintained on opioids, opioid-free chronic pain patients and pain-free participants based on their TLR agonist stimulated cell reactivity. The expression of IL-1β was found to be significantly greater in chronic pain patients compared with pain-free participants. The finding supports that PBMCs in the periphery is a good source to be a chronic pain biomarker. Consequently, this study provides supporting evidence that the immune system of chronic pain patients is dysregulated and the profound cell reactivity allows the identification of patients objectively.

Statement of Authorship

Title of Paper Increased Responsiveness of Peripheral Blood Mononuclear Cells to In Vitro TLR 2, 4 and 7 Ligand Stimulation in Chronic Pain Patients.

Publication Status Published

Publication Details **Kwok YH**, Hutchinson MR, Gentgall MG & Rolan PE. (2012). Increased Responsiveness of Peripheral Blood Mononuclear Cells to In Vitro TLR 2, 4 and 7 Ligand Stimulation in Chronic Pain Patients. *PLoS One* 7, e44232.

Author Contributions

By signing the Statement of Authorship, each certifies that their states contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate) Yuen Hei Kwok

Contribution to the Paper: Had major input in the experimental design, recruited all study participants, performed all collection of blood samples, all cell culture experiments, ELISA measurements, statistical analysis, graphical presentation of the data collected, wrote the manuscript, prepared the manuscript for submission and acted as a corresponding author.

Signature Date 24/06/2014

Name of Co-Author Mark Hutchinson

Contribution to the Paper: Involved in the experimental design, contributed to the data interpretation and preparation of the manuscript.

Signature Date 24/06/2014

Name of Co-Author Melanie Gentgall

Contribution to the Paper: Responsible for the clinical trial logistics and provided editorial assistance.

Signature Date 19/06/2014

Name of Co-Author Paul Rolan

Contribution to the Paper: Involved in the experimental design, contributed to the data interpretation and preparation of the manuscript.

Signature Date 24/6/2014

OPEN ACCESS Freely available online

PLOS | ONE

Increased Responsiveness of Peripheral Blood Mononuclear Cells to In Vitro TLR 2, 4 and 7 Ligand Stimulation in Chronic Pain Patients

Yuen H. Kwok[1]*, Mark R. Hutchinson[2], Melanie G. Gentgall[3], Paul E. Rolan[1,3,4]

1 Discipline of Pharmacology, School of Medical Sciences, University of Adelaide, Adelaide, South Australia, Australia, 2 Discipline of Physiology, School of Medical Sciences, University of Adelaide, Adelaide, South Australia, Australia, 3 Pain and Anaesthesia Research Clinic, University of Adelaide, Adelaide, South Australia, Australia, 4 Pain Management Unit, Royal Adelaide Hospital, Adelaide, South Australia, Australia

Abstract

Glial activation via Toll-like receptor (TLR) signaling has been shown in animals to play an important role in the initiation and establishment of chronic pain. However, our ability to assess this central immune reactivity in clinical pain populations is currently lacking. Peripheral blood mononuclear cells (PBMCs) are an accessible source of TLR expressing cells that may mirror similarities in TLR responsiveness of the central nervous system. The aim of this study was to characterize the IL-1β response to various TLR agonists in isolated PBMCs from chronic pain sufferers (on and not on opioids) and pain-free controls. Venous blood was collected from 11 chronic pain sufferers on opioids (≥ 20 mg of morphine / day), 8 chronic pain sufferers not on opioids and 11 pain-free controls. PBMCs were isolated and stimulated *in vitro* with a TLR2 (Pam3CSK4), TLR4 (LPS) or TLR7 (imiquimod) agonist. IL-1β released into the supernatant was measured with ELISA. Significantly increased IL-1β expression was found in PBMCs from chronic pain sufferers (on and not on opioids) compared with pain-free controls for TLR2 ($F_{(6,\ 277)} = 15$, $P < 0.0001$), TLR4 ($F_{(8,\ 263)} = 3$, $P = 0.002$) and TLR7 ($F_{(2,201)} = 5$, $P = 0.005$) agonists. These data demonstrate that PBMCs from chronic pain sufferers were more responsive to TLR agonists compared with controls, suggesting peripheral cells may have the potential to become a source of biomarkers for chronic pain.

Citation: Kwok YH, Hutchinson MR, Gentgall MG, Rolan PE (2012) Increased Responsiveness of Peripheral Blood Mononuclear Cells to In Vitro TLR 2, 4 and 7 Ligand Stimulation in Chronic Pain Patients. PLoS ONE 7(8): e44232. doi:10.1371/journal.pone.0044232

Editor: Ari Waisman, Johannes Gutenberg University of Mainz, Germany

Received May 29, 2012; **Accepted** July 30, 2012; **Published** August 28, 2012

Copyright: © 2012 Kwok et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The study was funded by the Pain and Anaesthesia Research Clinic, University of Adelaide and was supported by Grant Number [DP110100297] from Australian Research Council (ARC). Its contents are solely the responsibility of the authors and the funders had no role in study design, date collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: yuen.kwok@adelaide.edu.au

Introduction

Traditionally, the mechanisms underlying chronic pain have thought to be solely neuronally based. However, there is now overwhelming evidence from animal studies [1,2,3,4,5,6,7,8] that pro-inflammatory activation of the immune-like cells (glia), found in the central nervous system (CNS), are central to the initiation and the progression of chronic pain such as neuropathic pain [9,10]. Despite this however, there remains a lack of human data demonstrating that these pathways are relevant to clinical pain.

Toll-like receptors (TLRs) are a key detection system via which glia become activated and release pro-inflammatory cytokines [8]. TLRs form a key part of the innate immune system, capable of the detection of "self" molecules known as danger-associated molecular patterns (DAMPs) or "non-self" molecules produced by microorganisms known as pathogen-associated molecular patterns (PAMPs). Examples of exogenous TLR agonists include: lipopeptides recognized by TLR2 homodimer or by TLR2-TLR1 or TLR2-TLR6 heterodimers, lipopolysaccharide by TLR4 and single-stranded viral RNA by TLR 7 [11]. Once TLRs bind PAMPs, a series of signal transduction cascades occur, leading to the activation of NF-κB and c-Jun N-terminal kinases (JNKs) [12] which induce the transcription of genes encoding cytokines and chemokines that are involved in the initiation of the inflammatory process [13].

TLRs have been implicated in preclinical models of chronic pain [8]. Blocking of TLRs, either genetically [14,15], or pharmacologically [16], has been found to reduce microglial activation, reduce pro-inflammatory cytokine level and produce a reduction in experimentally-induced neuropathic pain in animals [8]. Therefore, the propensity of an individual towards proinflammatory TLR signaling could be a critical contributor to chronic pain states.

Opioids are frequently prescribed for moderate to severe pain but their efficacy is limited by adverse effects such as tolerance [17]. Interestingly, it has recently been demonstrated that opioids such as morphine, are also TLR4 agonists [18,19,20,21] therefore it can induce central immune signaling events that reduces opioid analgesia and contribute to hyperalgesia [19,22]. The impact of opioid exposure on TLR signaling in clinical pain populations is highly relevant and needs to be examined in this study.

Despite the clear evidence from *in vitro* and *in vivo* preclinical models that glial activation and TLR signaling play a critical role in chronic pain and opioid responses, determining if the same occurs in human remains difficult due to the general inaccessibility of the CNS compartment. Excitingly, given the functional similarities between TLR signaling of immune cells in the

periphery and in the CNS, we hypothesize that indirect evidence of activity of the TLR pathways in the CNS may be obtained by examining the activity of the TLR pathways in the peripheral immune system. Therefore, the TLR responsivity of peripheral immune cells from different patient populations where TLR activity is hypothesized to contribute to the disease state should be different when compared to healthy controls. Such information could lead to improve mechanistic understandings of pain and to the discovery of objective pain biomarkers.

Thus, in this study we have examined *in vitro* responsiveness to 3 different specific TLR agonists in peripheral blood mononuclear cells from chronic pain patients maintained on opioids, opioid-free chronic pain patients and opioid-free healthy controls, to determine whether chronic pain patients have increased responsiveness compared to pain-free controls. Moreover, we sought to investigate the relationship between response scores obtained from the cold pain test with IL-1β expression from PBMCs after TLR agonist stimulation. We hypothesized that chronic pain patients would show increased responsiveness to TLR agonists and that patients on opioids would show a further increase in TLR-mediated IL-1β response.

Materials and Methods

Study Participants

This study was conducted at the Pain and Anaesthesia Research Clinic, Royal Adelaide Hospital, Adelaide, Australia. Ethical approval was obtained from the Human Research Ethics Committee of the Royal Adelaide Hospital, Adelaide, South Australia.

All participants gave written informed consent to participate after a detailed oral explanation of the study. All participants were paid $50 for their inconvenience upon completion of the study. Chronic pain patients were recruited from the general public through advertisements and from a pain management unit. Healthy participants were recruited from the Pain and Anaesthesia Research Clinic's healthy participant database. Thirty participants were recruited to the study and assigned into three groups according to their pain and opioid use status. Groups 1 and 2 consisted of chronic pain sufferers. Participants had to experience pain at least five days a week and for at least 3 months. There was no minimum pain score. For Group 1, patients had to be taking ongoing opioid therapy at a dose equivalent to at least 20 mg of oral morphine per day (calculated according to a comparative opioid chart [23]). Group 2 patients were not receiving chronic opioid therapy. Group 3 was the control group in which participants had no clinically significant chronic pain and were not taking opioids or other analgesics.

For all participants the key inclusion criteria were the following: aged between 18 and 65 years, be in good general health (other than chronic pain for Groups 1 and 2) without clinically significant renal, hepatic, cardiac or other diseases. Key exclusion criteria were: use of any immunosuppressant drugs (e.g. azathioprine); presence of an active inflammatory process; a clinically significant infection in the previous 4 weeks; Raynaud's phenomenon or disease or any other condition associated with abnormal sensitivity to cold pain; a positive urine screen for illicit drugs (except for prescribed opioids), pregnancy and/or lactation, and have a known history of hepatitis B, C or HIV.

Design

The study was of cross sectional design. The study physician was aware of the pain and medication status of the participants, but personnel performing the cold pain tests and laboratory evaluations of immune cell reactivity were blinded to the pain and opioid status of the participant.

Study schedule

Visit 1. On the first visit, information on pain history and medication use was recorded. Vital signs were recorded and participants underwent the cold pain test as previously described [24] in which cold pain threshold and endurance were assessed. The temperature of the room was set at 22.5°C at all times. Briefly, the cold pain test consisted of two temperature-controlled water baths of 34.5–35.5°C and 0.5–1.5°C. A water pump was placed in the cold-water container to prevent laminar warming around the immersed limb. Each participant's non-dominant forearm and hand (fingers wide apart) was placed vertically into the warm water for exactly 2 min so that all participants' starting temperature was the same. At 1 min 45 s, a blood pressure cuff was inflated to a pressure 20 mmHg below the diastolic blood pressure (to minimize the role of blood flow in determining the reaction to cold). At exactly 2 min, the forearm was placed into the cold water bath. The participant's eyes were covered for the entire procedure to minimize distraction. Once the arm was immersed in the cold water bath, participants were asked to indicate when they first experience pain (cold pain threshold), then asked to leave their arm submerged until they could no longer endure the pain (cold pain endurance) with a maximum cut-off time of 3 min. Upon removal of the arm from the cold water bath, participants were asked to indicate when the pain ceased. Endpoints were measured as time (s). Participants performed the cold pain test twice with a 20 min interval and the average was recorded.

Visit 2. On visit 2, information on health status, pain severity and pain medications were checked and recorded. Five ml of blood was collected into tubes containing EDTA and was sent for standard haematologic profile. Twenty-seven ml of blood were collected into tubes containing EDTA and left at room temperature for 1 h until isolation of peripheral blood mononuclear cells (PBMCs). PBMCs were isolated using Optiprep (Axis-Shield PoC AS, Oslo, Norway) as directed by the manufacturer using the mixer flotation method. Control wells minus the TLR agonist were also included. Isolated cells were diluted to 1×10^6 cells·ml^{-1} in enriched RPMI 1640 (10% foetal calf serum and 1% penicillin) and plated into 96 well plates (Nunc, Roskilde, Denmark) (100 ml per well). After allowing the PBMCs to sit for an hour, a range of concentrations of agonists were added into the wells in triplicate. TLR2 agonist: synthetic triacylated lipoprotein: Pam3CSK4 from 13 pg·ml^{-1} to 1 μg·ml^{-1} (Sigma); TLR4 agonist: lipopolysaccharide: LPS from 6 pg·ml^{-1} to 10 μg·ml^{-1} (Sigma); and TLR7 agonist: imiquimod from 50 pg·ml^{-1} to 100 μg·ml^{-1} (Sigma). Plates were incubated for 20 h at 37°C, 5% CO_2 in a humidified environment (Thermoline Scientific, Australia).

For non-TLR stimulation of inflammatory pathways, monosodium urate (MSU) crystals (suspended in phosphate buffered saline) (Sigma) primed with a low concentration of LPS (13 pg/mL) were used to stimulate the isolated PBMCs. The concentration of MSU crystals used ranged from 50 pg·ml^{-1} to 10 μg·ml^{-1}. MSU crystals are known activators of NALP3 or cryopyrin [25]. NALP3 consists of a protein complex that regulates the activity of caspase-1 which then cleaves pro IL-1β to IL-1β. However to respond to MSU crystals PBMCs require priming with pro-IL-1β hence we used a low concentration of LPS (TLR4 ligand) (13 pg·ml^{-1}). Our data show that the concentration selected for LPS alone cause minimal release of IL-1β.

IL-1β assay

IL-1β levels were determined by a commercially available ELISA (IL-1β ELISA; BD Bioscience, Australia) according to the manufacturer's instructions. The absorbance was quantified on a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany) at 450 nm with absorbance at 570 nm subtracted as per manufacturer's instructions. The manufacturer's limit of quantification of 0.8 $pg \cdot ml^{-1}$ was used.

Statistical analysis

Graphpad Prism 5.0 (GraphPad Software; San Diego, CA) was used for all statistical analysis and fitting of concentration-response curves. The Kruskal-Wallis test was used to assess the cold pain endurance scores. Student's t-test was used to calculate the difference between duration of chronic pain. The haematology data was normalized by log transformation and analyzed with one-way ANOVA. Age and the comparison between IL-1β expressions with the three groups at the unstimulated level were analyzed using one-way ANOVA. The concentration curves for TLR2, TLR7 agonists and MSU crystals were assessed using a sigmoidal concentration response equation. Assessments were first conducted to determine the type of model that best described the data using *F*-tests. In this case either 3 or 4 parameter sigmoidal concentration response curves were tested. In all cases except the biphasic TLR4 response described below a 3 parameter model was found to describe the data the best. The bottom responses were fixed at a value of 0 but other parameters were allowed to vary. For TLR4 a modified biphasic sigmoidal equation was used (Y = (YMin + (YMax-YMin)/(1+10^((LogEC50-X)*HillSlope)))+(YMin2 + (YMax2-YMin2)/(1+10^((LogEC502-X)*HillSlope2)))). The *F*-tests were used to ask the question of whether the best fit curves with the selected parameters (Emax, Emin and EC_{50}) differ so that group differences could be identify in the IL-1β expressed by PBMCs post TLR agonist/ MSU crystals stimulation. All significance was set at $P<0.05$.

Results

Demographic data

Basic demographics are listed in Table 1. Eleven chronic pain sufferers on opioids (referred to as CP+O group) (7 female, 4 male, (min-max) 33–65 years old; mean age 53), eight chronic pain sufferers not on opioids (referred to as CP group)(6 female, 2 male, 36–65 years old; mean age 52) and eleven pain-free participants (7 female, 4 male; 36–61 years old; mean age 51) took part in the study. Additional information of all chronic pain participants can be found in Table S1. The average duration of pain in the CP+O group was 10 years (min-max; 2–28) and for CP group was 4 years (1–10) ($P=0.08$). The mean daily dose (oral morphine equivalent) taken by CP+O was (mean ± SEM) 85±15 mg and 64% were also on other pain modifying medications such as pregabalin and amitriptyline. Thirty-eight percent of the CP group was on oral analgesics such as ibuprofen and gabapentin and 50% only took oral analgesics when required.

Cold pain endurance score

The cold pain endurance score obtained from the average of two testing sessions for CP+O group was: (median (interquartile range)) 28 (24–144) s, CP group was 32 (17–105) s and pain-free was 47 (28–106) s. There were no significant differences between either chronic pain group compared with pain-free participants (NS; $P=0.8$).

Haematology

There were no differences in absolute or relative white cell subtype counts (total white cell count, neutrophils, lymphocytes, monocytes, eosinophils and basophils) between the groups.

In vitro stimulation with TLR agonists

The basal level (unstimulated) of IL-1β expression was the same for all three groups: (Mean ± SEM) CP+O group: 1±0.2 $pg \cdot ml^{-1}$, CP group: 0.9±0.2 $pg \cdot ml^{-1}$ and for pain-free controls: 1±0.2 $pg \cdot ml^{-1}$ (NS, $P=0.9$).

(A) TLR2 agonist: Pam3CSK4. The observed concentration-response curves were mono-phasic, with the pain-free group appearing almost non-responsive to the TLR2 agonist Pam3CSK4. However, Pam3CSK4 induced significant concentration-dependent increases in IL-1β release in both pain groups with the CP+O group being the most responsive. The clear separation between the three groups resulted in an overall significant group effect in response to Pam3CSK4 ($F_{6,\ 277}=15$, $P<0.0001$; see Figure 1). These differences were also reflected in significantly different EC_{50} values: CP+O, 16 $ng \cdot ml^{-1}$ (95% confidence interval: 4 to 69 $ng \cdot ml^{-1}$); CP, 49 $ng \cdot ml^{-1}$ (95% CI: 12 to 210 $ng \cdot ml^{-1}$); and PF, 53 ng ml^{-1} (95% CI: 10 to 282 $ng \cdot ml^{-1}$). The E_{max} estimates were also significantly different with CP+O 827 $pg \cdot ml^{-1}$ (95% CI: 622 to 1031 $pg \cdot ml^{-1}$); CP, 584 $pg \cdot ml^{-1}$ (95% CI: 401 to 766 $pg \cdot ml^{-1}$); and PF 108 pg ml^{-1} (95% CI: 71 to 144 $pg \cdot ml^{-1}$).

(B) TLR4 agonist: LPS. The TLR4 agonist LPS induced significant concentration-dependent increases in IL-1β release in all groups. The observed concentration-response curve was best fitted to a biphasic curve, suggesting both low and high affinity systems, combining to produce the overall concentration curve. An increased response was observed for both chronic pain populations when compared with the controls with the CP+O group being the most responsive. This resulted in a significant group difference in

Table 1. Demographic summary.

	Chronic pain + Opioids	Chronic pain – Opioids	Control	*P*
Gender (M/F)	4/7	2/6	4/7	-
Age (Years)	53±3	52±4	51±3	0.89
Oral morphine equivalent dose (per day) (mg)	85±15	None	-	-
Duration of chronic pain (Years)	10±3	4±1	None	0.08

Data were collected from medical and family history. Data are expressed as mean ± S.E.M. One-way ANOVA and Student's t-test was used to determine significant differences (P-values shown).
doi:10.1371/journal.pone.0044232.t001

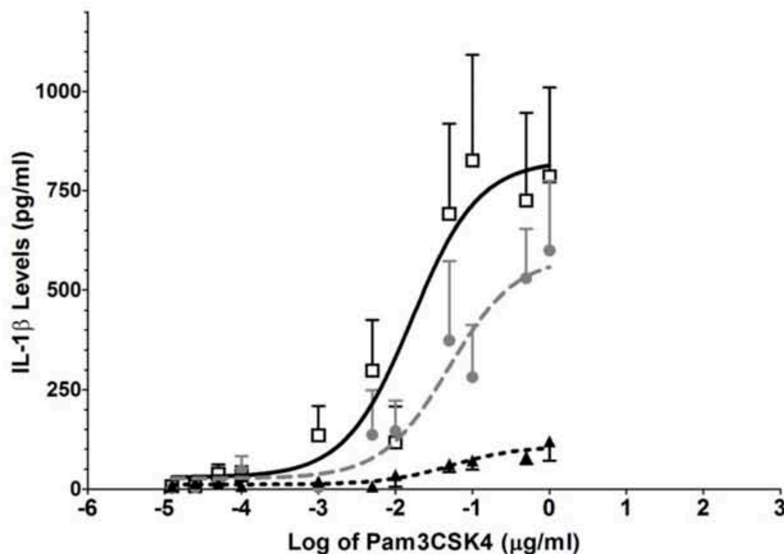


Figure 1. TLR 2 agonist stimulation caused significant enhanced release of IL-1β in chronic pain patients. Isolated white cells obtained from chronic pain sufferers on opioids (□), chronic pain sufferers not on opioids (●) and pain-free controls (▲) were stimulated with a range of Pam3CSK4 (TLR2) concentrations (13 pg·ml^{-1} to 1 μg·ml^{-1}) to generate the response curves and resulted in significant group differences (P<0.0001). Error bars on graphs represent standard error of the mean.
doi:10.1371/journal.pone.0044232.g001

the IL-1β release concentration-response ($F_{8,\ 263} = 3$, $P = 0.002$; see Figure 2). The EC_{50} of CP+O was 16 ng·ml^{-1} (95% CI: 4 to 69 ng·ml^{-1}); CP, 49 ng·ml^{-1} (95% CI: 12 to 210 ng·ml^{-1}); PF 53 ng·ml^{-1} (95% CI: 10 to 282 ng·ml^{-1}). The Emax were 827 pg·ml^{-1} (95% CI: 622 to 1031 pg·ml^{-1}) for CP+O, 584 pg·ml^{-1} (95% CI: 401 to 766 pg·ml^{-1}) for CP and 108 pg·ml^{-1} (95% CI: 71 to 144 pg·ml^{-1}) for PF.

(C) TLR7 agonist: Imiquimod. The TLR7 agonist imiquimod induced elevations in IL-1β release in only the CP+O group. The observed concentration-response curve was monophasic and due to the low amount of IL-1β expressed in response to TLR7 no response curve could represent the CP group and the controls. The differences were most prominent at the higher imiquimod concentrations causing a marked increase in the IL-1β ($F_{2,201} = 5$,

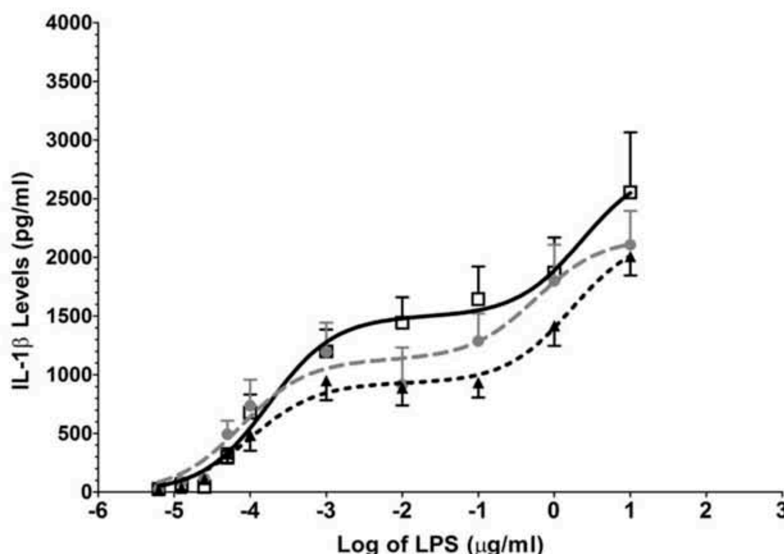


Figure 2. TLR 4 agonist stimulation caused significant enhanced release of IL-1β in chronic pain patients. Isolated white cells obtained from chronic pain sufferers on opioids (□), chronic pain sufferers not on opioids (●) and pain-free controls (▲) were stimulated with a range of LPS (TLR4) concentrations (6 pg·ml^{-1} to 10 μg·ml^{-1}) to generate the response curves and resulted in significant differences (P = 0.002). Error bars on graphs represent standard error of the mean.
doi:10.1371/journal.pone.0044232.g002

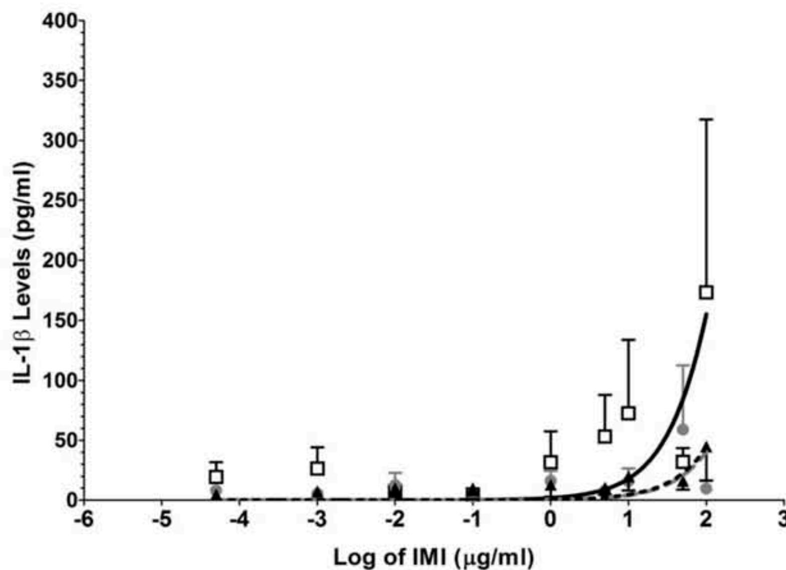


Figure 3. TLR 7 agonist stimulation allowed the differentiation between chronic pain patients and pain-free participants. Isolated white cells obtained from chronic pain sufferers on opioids (□), chronic pain sufferers not on opioids (●) and pain-free controls (▲) were stimulated with a range of imiquimod (TLR7) concentrations (50 pg·ml^{-1} to 100 μg·ml^{-1}) to generate the response curves and resulted in group differences ($P=0.0048$). Error bars on graphs represent standard error of the mean.
doi:10.1371/journal.pone.0044232.g003

$P=0.005$; see Figure 3). The EC_{50} for CP+O was 545 μg·ml^{-1} (95% CI: 283 to 1048 μg·ml^{-1}); CP, 2371 μg·ml^{-1} (95% CI: 1016 to 5535 μg·ml^{-1}); and PF, 2172 μg·ml^{-1} (95% CI: 1345 to 3506 μg·ml^{-1}) for PF.

(D) NALP3: TLR independent pathway: MSU crystals. The MSU crystals with one concentration of LPS (13 pg ml^{-1}) induced expression of IL-1β in all three groups (data not shown). All groups responded with a similar expression of IL-1β at each of the tested concentrations that resulted in no significant group or concentration differences (group effect: $P=0.2$ and concentration effect: $P=0.7$). The range of IL-1β detected was similar to the amount released only by LPS stimulation. For example, at 1 μg ml^{-1} of MSU crystals with 13 pg ml^{-1} of LPS, the amount of IL-1β expressed in CP+O group was (mean ± SEM) 75±27 pg·ml^{-1} (LPS alone 13 pg ml^{-1} was 73±18 pg·ml^{-1}), CP group was 112±34 pg·ml^{-1} (LPS alone 13 pg·ml^{-1} was 119±45 pg·ml^{-1}) and by PF was 82±15 pg·ml^{-1} (LPS alone 13 pg·ml^{-1} was 35±18 pg·ml^{-1}).

Discussion

The current study, to the best of the authors' knowledge, is the first to examine the IL-1β expression responsiveness of isolated PBMCs collected from chronic pain sufferers (on and not on opioid treatments) following TLR agonist stimulation. Although basal levels of IL-1β did not differ between groups, after TLR agonist stimulation significant group differences were detected in the IL-1β expression levels. The response appears to be selective for TLR pathways as there was no response found in all 3 populations after MSU crystals stimulation, (an inflammasome activator). These findings supported both our hypotheses of increased TLR responsiveness in pain patients and further increases in TLR responsiveness in pain patients on opioids.

The general technique *of in vitro* stimulation of human PBMCs with TLR agonists followed by quantification of cytokine expression is not novel. This approach can be used to identify a patient's immunologic "signature" [26] and to understand the TLR signaling pathway. Studies to evaluate TLR responsiveness in chronic pain patients have been limited. Our study is the first to show enhanced TLR responsiveness in PBMCs collected from chronic pain patients when compared with controls. Previous studies have demonstrated enhanced pro-inflammatory cytokine release from TLR agonist stimulation in PBMCs from patients with rheumatoid arthritis [27,28], primary biliary cirrhosis [29], inflammatory bowel diseases [30], chronic fatigue [26] and immunosuppressed patients with rheumatoid arthritis [31]. These conditions with the exception chronic fatigue, are defined by significant clinical inflammation and hence these findings are not surprising. We took care to exclude patients with clinical inflammation, suggesting that our findings are related to the pain state. The further enhanced sensitivity in patients taking opioids supports this. From the above studies, enhanced TLR-induced release of pro-inflammatory cytokines by PBMCs indicates a possible dysregulation in the innate immune system.

The increased responsiveness to TLR agonists could be attributable to the chronic pain patient's PBMC TLR system having been "primed" [32] following previous exposure of endogenous TLR ligands such as heat shock proteins [33] or HMGB-1 [34] which may be produced after a nerve injury. Such priming events are known to occur following painful insults. For example increased TLR2 mRNA [35], TLR4 mRNA and pro-inflammatory cytokine expression were found in murine models after L5 spinal nerve transection and sciatic nerve ligation [4,36]. Interestingly, these changes in immune responsiveness are not limited to the CNS with a recent report of increased peripheral immune cell activity in a neuropathic pain model in mice [37]. It is therefore possible that the increased PBMC responsiveness observed in the chronic pain groups may be a result of up regulation of the expression of the TLR pathways. In addition, the different responsiveness between the TLR2 and TLR4 agonists

could be attributed to the availability of the TLR4 accessory protein known as myeloid differentiation factor 2 (MD-2). LPS has to be captured by the LPS-binding protein then delivered to the TLR4/MD-2 complex that causes dimerization of the TLR4/MD-2 complex. In contrast, TLR2 does not need any accessory protein to activate downstream cascades [38]. Which pathways and the manner of alteration that lead to the changed responsiveness are a focus of future studies by our group.

Recently, a study unexpectedly found increased TLR4 surface expression on monocytes and enhanced pro-inflammatory cytokine (IL-8 and TNF-α) release after TLR stimulation (TLR1/2 heterodimer, TLR2/6 heterodimer, TLR4 and TLR5) in patients with autoimmune disease who were being treated with immunosuppressive medications, compared with patients not receiving immunosuppressive agents [31]. The authors hypothesized reduced TLR4 function as an explanation of increased infection associated with immunosuppressive therapy for autoimmune disease. The increased release of IL-8 and TNF-α after TLR stimulation was suggested to be as a result of altered TLR intracellular signaling because the surface expression of TLR1 and TLR2 did not differ from controls but the production of cytokines were enhanced. The innate immune system was concluded to be dysregulated and not simply suppressed. This finding parallels our study, despite differences in patient population, cell type and outcomes, where we observed enhanced immune response in patients receiving opioids despite opioid medications generally being regarded as being immunosuppressive [39] (see below).

Chronic morphine exposure is believed to cause immunosuppression as increased susceptibility to both bacterial and viral infections have been reported in drug users [39]. After morphine exposure, inhibition of the immune system, such as reduced monocyte function have been reported in humans [40] and decreased T-cell proliferation and NK cell function have been reported in rodents [41]. However the evidence is not all clearly in favor of immunosuppression, as hydrocodone and oxycodone were found to have no immunosuppressant properties with no effect on lymphocyte proliferation and natural killer activity found) [42]. Hence the concept of Dunne et al [31] of "dysregulation" may be more appropriate to the effects of opioids than simple concepts of enhanced or reduced immune activity. Such terms of enhanced or reduced immune activity fail to capture the complex interactions occurring within the immune system in which one part of the system may show increased activity while another part of action of the system is reduced.

A note of caution is needed with the assumption that the increased response in the CP+O group are causally related to the drugs using a cross sectional design. The CP+O group differed from the CP group by higher pain scores and duration of pain. It might seem paradoxical that patients on opioids have more pain, but since opioid use is recommended to be restricted to those with severe pain [43,44], and as the drugs have only modest efficacy [43,44] this finding is not surprising. Therefore, opioid use may be an indicator for more severe pain or pain of longer duration and these differences may be confounders in the interpretation. In future studies, controlling for pain intensity and duration between the groups might be interesting and would be important to examine in prospective longitudinal rather than cross-sectional study designs.

No group differences were found in the basal level of IL-1β release by PBMCs. This result is not surprising as basal level of cytokines have yielded inconsistent findings in other patient populations such as in rheumatoid arthritis patients. One study reported the basal level to be the same as controls [28] whilst another study found lower cytokine levels in rheumatoid arthritis patients compared to controls [27]. This could be due to the very low circulating cytokine levels but difference in sensitivity of the system may only be revealed after stimulation.

There is clear heterogeneity in inter-laboratory reporting of the sensitivity of PBMCs to LPS. At 1 ng/mL of LPS, the IL-1β released from PBMCs in the control group in our study (950 pg/mL) was higher than previous reports (600 pg/(5×10^5/ml) [26]. Higher concentrations of LPS (1 μg/mL) produced lower amounts of IL-1β (2008 pg/mL) compared to other studies (5000 pg/mL) [45]. The differences could be attributed to the lack of a standard operating procedure and specific experimental conditions in the investigation of cytokine release from PBMCs. Other differences in the following will also influence the outcome: the serotype of TLR agonists used (e.g LPS), cell population used (mixed populations of cells versus monocytes only), method of PBMC isolation, cell concentration used and apparatus used for cytokine measurements (bead assay, multiplex ELISA and standard ELISA kits). No studies have investigated the TLR responsiveness of PBMCs from chronic pain patients. One key difference between our study and the bulk of the literature in the area is that we determined extensive concentration-response curves whereas in most other studies only a single concentration of agonist was used. Such single point determinations may miss the complexity of response, like the biphasic response we observed to LPS which has not been reported previously.

Biphasic cytokine responses (IL-8) have been reported following LPS stimulation in whole blood [46]. However, in this study the secondary wave observed was due to the effect of time (first wave was from 6–12 hours and the secondary wave was from 12–24 hours) instead of concentration of LPS (as was observed in the current study). The secondary wave was attributed to the LPS-induced release of TNF and IL-1 as demonstrated with the disappearance of the wave with the use of anti-TNF and anti-IL-1 neutralizing antibodies. In the current study the biphasic nature of the IL-1 response may have arisen from the activation of two systems (possibly in two cell types) that are characterized as high affinity but low capacity, verses lower affinity but higher capacity. This response characteristic may not have been examined before as most studies usually only employ one high concentration of agonist.

The specificity of the TLR agonists response was tested with the use of the non-TLR signaling stimulus, monosodium urate monohydrate (MSU) crystals [47]. MSU crystals are reported to be an inflammasome activator and cause the conversions of procaspase-1 to active caspase 1 allowing pro-IL-1β can be cleaved to active IL-1β via the NALP3 inflammasome [48]. No detectable IL-1β from collected PBMCs were found with pure MSU crystals. Therefore, a low concentration of LPS (second stimulus) was added with MSU crystal so the synergistic activation could be measured. However, no group differences were detected between the 3 groups. Hence it confirms our finding of the increased responsiveness in pain patients may be TLR-specific and not related to other pathway such as the NALP3 inflammasome.

Previously, our group found a strong correlation (Pearson r = 0.9) between morphine-enhanced concanavalin A proliferation (in isolated PBMCs) with cold pain endurance score in a healthy voluntary population[49]. Therefore we wished to investigate whether cold pain endurance could be correlated with TLR agonist induced IL-1β levels in the PBMCs collected in the current experiment. However no group differences were found with the cold pain endurance scores and no correlation was found with the TLR agonists.

Some limitations of this study are: (1) the small sample size and heterogeneity of pain diagnosis and severity. The heterogeneity

was deliberate as we hypothesized that it was the chronic pain process *per se* and not the underlying diagnosis that was associated with increased immune responsiveness. In future studies of larger sample size, selecting a range of clearly defined pathologies and controlling for pain severity would be useful. (2) Potential confounding effects of concomitant depression and changes in the in the hypothalamic-pituitary-adrenal axis status were not studied, but in future work this would be appropriate as depression may be associated with changes in glial activity [50]. (3) Only one pro-inflammatory cytokine was measured and IL-1β was chosen as it plays a pivotal role in chronic pain. Future studies should examine a broader range of cytokines and chemokines.

In summary, TLR agonists (TLR2, TLR4, and TLR7) were found to cause elevation in IL-1β release that could significantly differentiate from chronic pain sufferers on opioids, chronic pain sufferers not on opioids and pain-free participants. This study is the first in providing evidence in human cells that TLRs are more responsive in chronic pain sufferers. As we were able to significantly differentiate three groups on the basis of their IL-1β output, it appears this response of *in vitro* stimulation of isolated immune cells with TLR agonists may serve to be a potential test for identifying biomarkers for chronic pain from readily accessible peripheral blood samples.

Supporting Information

Table S1 Individual information on chronic pain sufferers in this study.
(DOCX)

Acknowledgments

We would like to thank the staff at the Pain and Anaesthesia Research Clinic for assistance with the conduct of this study.

Author Contributions

Conceived and designed the experiments: YHK MRH MGG PER. Performed the experiments: YHK. Analyzed the data: YHK MRH. Contributed reagents/materials/analysis tools: MRH PER. Wrote the paper: YHK MRH PER. Revision of manuscript: PER MRH. Interpretation of data: YHK MRH PER.

References

1. Watkins LR, Maier SF (2003) Glia: a novel drug discovery target for clinical pain. Nat Rev Drug Discov 2: 973–985.
2. McMahon SB, Cafferty WB, Marchand F (2005) Immune and glial cell factors as pain mediators and modulators. Exp Neurol 192: 444–462.
3. Tsuda M, Mizokoshi A, Shigemoto-Mogami Y, Koizumi S, Inoue K (2004) Activation of p38 mitogen-activated protein kinase in spinal hyperactive microglia contributes to pain hypersensitivity following peripheral nerve injury. Glia 45: 89–95.
4. Raghavendra V, Rutkowski MD, DeLeo JA (2002) The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats. J Neurosci 22: 9980–9989.
5. Ledeboer A, Sloane EM, Milligan ED, Frank MG, Mahony JH, et al. (2005) Minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. Pain 115: 71–83.
6. Raghavendra V, Tanga F, DeLeo JA (2003) Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. J Pharmacol Exp Ther 306: 624–630.
7. Grace PM, Rolan PE, Hutchinson MR (2011) Peripheral immune contributions to the maintenance of central glial activation underlying neuropathic pain. Brain Behav Immun: 1322–1332.
8. Nicotra L, Loram LC, Watkins LR, Hutchinson MR (2011) Toll-like receptors in chronic pain. Exp Neurol: 316–329.
9. Milligan ED, Watkins LR (2009) Pathological and protective roles of glia in chronic pain. Nat Rev Neurosci 10: 23–36.
10. De Leo JA, Tawfik VL, LaCroix-Fralish ML (2006) The tetrapartite synapse: path to CNS sensitization and chronic pain. Pain 122: 17–21.
11. Guo LH, Schluesener HJ (2007) The innate immunity of the central nervous system in chronic pain: the role of Toll-like receptors. Cell Mol Life Sci 64: 1128–1136.
12. Muzio M, Natoli G, Saccani S, Levrero M, Mantovani A (1998) The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). J Exp Med 187: 2097–2101.
13. Trinchieri G, Sher A (2007) Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol 7: 179–190.
14. Tanga FY, Nutile-McMenemy N, DeLeo JA (2005) The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. Proc Natl Acad Sci U S A 102: 5856–5861.
15. Kim D, Kim MA, Cho IH, Kim MS, Lee S, et al. (2007) A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. J Biol Chem 282: 14975–14983.
16. Hutchinson MR, Zhang Y, Brown K, Coats BD, Shridhar M, et al. (2008) Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). Eur J Neurosci 28: 20–29.
17. Chu LF, Clark DJ, Angst MS (2006) Opioid tolerance and hyperalgesia in chronic pain patients after one month of oral morphine therapy: a preliminary prospective study. J Pain 7: 43–48.
18. Hutchinson MR, Northcutt AL, Chao LW, Kearney JJ, Zhang Y, et al. (2008) Minocycline suppresses morphine-induced respiratory depression, suppresses morphine-induced reward, and enhances systemic morphine-induced analgesia. Brain Behav Immun.
19. Hutchinson MR, Bland ST, Johnson KW, Rice KC, Maier SF, et al. (2007) Opioid-induced glial activation: mechanisms of activation and implications for opioid analgesia, dependence, and reward. ScientificWorldJournal 7: 98–111.
20. Wang X, Loram LC, Ramos K, de Jesus AJ, Thomas J, et al. (2012) Morphine activates neuroinflammation in a manner parallel to endotoxin. Proc Natl Acad Sci U S A 109: 6325–6330.
21. Hutchinson MR, Zhang Y, Shridhar M, Evans JH, Buchanan MM, et al. (2010) Evidence that opioids may have toll-like receptor 4 and MD-2 effects. Brain Behav Immun 24: 83–95.
22. Hutchinson MR, Zhang Y, Shridhar M, Evans JH, Buchanan MM, et al. (2009) Evidence that opioids may have toll-like receptor 4 and MD-2 effects. Brain Behav Immun.
23. Rossi S (2011) Australian Medicines Handbook 2011. Available: http://www.amh.net.au. Accessed 2 February 2012.
24. Doverty M, White JM, Somogyi AA, Bochner F, Ali R, et al. (2001) Hyperalgesic responses in methadone maintenance patients. Pain 90: 91–96.
25. Sutterwala FS, Ogura Y, Zamboni DS, Roy CR, Flavell RA (2006) NALP3: a key player in caspase-1 activation. J Endotoxin Res 12: 251–256.
26. Chao CC, Janoff EN, Hu SX, Thomas K, Gallagher M, et al. (1991) Altered cytokine release in peripheral blood mononuclear cell cultures from patients with the chronic fatigue syndrome. Cytokine 3: 292–298.
27. Kowalski ML, Wolska A, Grzegorczyk J, Hilt J, Jarzebska M, et al. (2008) Increased responsiveness to toll-like receptor 4 stimulation in peripheral blood mononuclear cells from patients with recent onset rheumatoid arthritis. Mediators Inflamm 2008: 132732.
28. Davis JM, 3rd, Knutson KL, Strausbauch MA, Crowson CS, Therneau TM, et al. (2010) Analysis of complex biomarkers for human immune-mediated disorders based on cytokine responsiveness of peripheral blood cells. J Immunol 184: 7297–7304.
29. Mao TK, Lian ZX, Selmi C, Ichiki Y, Ashwood P, et al. (2005) Altered monocyte responses to defined TLR ligands in patients with primary biliary cirrhosis. Hepatology 42: 802–808.
30. Kovarik JJ, Tillinger W, Hofer J, Holzl MA, Heinzl H, et al. (2010) Impaired anti-inflammatory efficacy of n-butyrate in patients with IBD. Eur J Clin Invest 41: 291–298.
31. Dunne DW, Shaw A, Bockenstedt LK, Allore HG, Chen S, et al. (2010) Increased TLR4 expression and downstream cytokine production in immunosuppressed adults compared to non-immunosuppressed adults. PLoS One 5: e11343.
32. Perry VH, Hume DA, Gordon S (1985) Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. Neuroscience 15: 313–326.
33. Beg AA (2002) Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. Trends Immunol 23: 509–512.
34. Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, et al. (2006) High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol 290: C917–924.
35. Shi XQ, Zekki H, Zhang J (2011) The role of TLR2 in nerve injury-induced neuropathic pain is essentially mediated through macrophages in peripheral inflammatory response. Glia 59: 231–241.
36. Tanga FY, Raghavendra V, DeLeo JA (2004) Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. Neurochem Int 45: 397–407.

37. Jang Y, Song HK, Yeom MY, Jeong DC (2012) The Immunomodulatory Effect of Pregabalin on Spleen Cells in Neuropathic Mice. Anesth Analg.
38. Akira S, Sato S (2003) Toll-like receptors and their signaling mechanisms. Scand J Infect Dis 35: 555–562.
39. Risdahl JM, Khanna KV, Peterson PK, Molitor TW (1998) Opiates and infection. J Neuroimmunol 83: 4–18.
40. Menzebach A, Hirsch J, Nost R, Mogk M, Hempelmann G, et al. (2004) [Morphine inhibits complement receptor expression, phagocytosis and oxidative burst by a nitric oxide dependent mechanism]. Anasthesiol Intensivmed Notfallmed Schmerzther 39: 204–211.
41. Budd K (2006) Pain management: is opioid immunosuppression a clinical problem? Biomed Pharmacother 60: 310–317.
42. Sacerdote P, Manfredi B, Mantegazza P, Panerai AE (1997) Antinociceptive and immunosuppressive effects of opiate drugs: a structure-related activity study. Br J Pharmacol 121: 834–840.
43. Chou R, Fanciullo GJ, Fine PG, Adler JA, Ballantyne JC, et al. (2009) Clinical Guidelines for the Use of Chronic Opioid Therapy in Chronic Noncancer Pain. The Journal of Pain 10: 113–130.
44. Anon. (2010) Canadian Guideline for Safe and Effective Use of Opioids for Chronic Non-Cancer Pain. Canada: National Opioid Use Guideline Group (NOUGG) Available from: http://nationalpaincentre.mcmaster.ca/opioid/. Accessed 30 January 2012.
45. Tsujimoto H, Ono S, Majima T, Efron PA, Kinoshita M, et al. (2006) Differential toll-like receptor expression after ex vivo lipopolysaccharide exposure in patients with sepsis and following surgical stress. Clin Immunol 119: 180–187.
46. DeForge LE, Remick DG (1991) Kinetics of TNF, IL-6, and IL-8 gene expression in LPS-stimulated human whole blood. Biochem Biophys Res Commun 174: 18–24.
47. Chen CJ, Shi Y, Hearn A, Fitzgerald K, Golenbock D, et al. (2006) MyD88-dependent IL-1 receptor signaling is essential for gouty inflammation stimulated by monosodium urate crystals. J Clin Invest 116: 2262–2271.
48. Ogura Y, Sutterwala FS, Flavell RA (2006) The inflammasome: first line of the immune response to cell stress. Cell 126: 659–662.
49. Hutchinson MR, La Vincente SF, Somogyi AA (2004) In vitro opioid induced proliferation of peripheral blood immune cells correlates with in vivo cold pressor pain tolerance in humans: a biological marker of pain tolerance. Pain 110: 751–755.
50. Rajkowska G, Miguel-Hidalgo JJ (2007) Gliogenesis and glial pathology in depression. CNS Neurol Disord Drug Targets 6: 219–233.

Chapter 3. Forward and backward translation of TLR responsiveness between a preclinical neuropathic pain model and a chronic pain patient cohort

Kwok YH, Tuke J, Nicotra LL, Grace PG, Rolan PE, Hutchinson MR (2013) TLR 2 and 4 Responsiveness from Isolated Peripheral Blood Mononuclear Cells from Rats and Humans as Potential Chronic Pain Biomarkers. PLoS ONE 8(10): e77799. doi:10.1371/journal.pone.0077799. Reprinted with permission from PLoS ONE.

This chapter is a logical progression from Chapter 2, which demonstrated that PBMCs isolated from chronic pain patients displayed increase TLR responsiveness when compared with pain-free participants. However, due to the cross-sectional design of the study, only correlations could be drawn between the chronic pain and enhanced TLR responsiveness.

This chapter takes a "backward translation approach" and explores the potential causal relationship between chronic pain and TLR responsiveness by using a prospective study design in a well-control preclinical model of neuropathic pain. The "graded chronic constriction injury model" was selected, as it grades the severity of allodynia that better mimics the clinical pain population making the findings more relevant. Basal and TLR stimulated outputs collected from both the peripheral and central regions allow the generation of statistical models that could predict the presence of pain and the severity of allodynia. The use of a preclinical model also allowed access to the central region and so a panel of peripheral outputs was used to give insight as to whether central region's responses could be mirrored. As many researches are lost in translation from rats to humans, a model generated from the rat's peripheral outputs was used to determine the pain state in a new cohort of chronic pain patients to determine the potential translational value.

This study found that the basal outputs could be used to accurately predict pain presence as well as the severity of allodynia in rats post-graded CCI but even higher sensitivity and specificity scores were found with the addition of TLR stimulated outputs. This confirms the importance of TLR responsiveness as an important factor for the identification of a chronic pain biomarker. The response from the central region (lumbar spinal cord) was significantly correlated with the panel of peripheral outputs. Excitingly, the peripherally derived model developed from the preclinical study could also be applied to humans for accurately predicting pain presence in a cohort of chronic pain patients (consisting of sciatica and medication overuse headache patients) thus indicating potential translational value.

Statement of Authorship

Title of Paper	TLR 2 and 4 Responsiveness from Isolated Peripheral Blood Mononuclear Cells from Rats and Humans as Potential Chronic Pain Biomarkers.
Publication Status	Published
Publication Details	Kwok YH, Tuke J, Nicotra LL, Grace PG, Rolan PE, Hutchinson MR (2013) TLR 2 and 4 Responsiveness from Isolated Peripheral Blood Mononuclear Cells from Rats and Humans as Potential Chronic Pain Biomarkers. PLoS ONE 8(10): e77799. doi:10.1371/journal.pone.0077799.

Author Contributions

By signing the Statement of Authorship, each certifies that their states contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Yuen Hei Kwok	
Contribution to the Paper	Had a major input in the experimental design, performed most surgeries, some behavioural testing, tissue collection, all cell culture experiments, all ELISA assessments, statistical analysis and all graphical presentation of the data collected, wrote the manuscript, prepared the manuscript for submission and acted as a corresponding author.	
Signature		Date 24/06/2014

Name of Co-Author	Jonoathan Tuke	
Contribution to the Paper	Assisted with the statistical modelling and contributed to the preparation of the manuscript particularly the statistics section of the Methods.	
Signature		Date 20/06/2014

Name of Co-Author	Lauren Nicotra	
Contribution to the Paper	Performed rat surgeries, performed some behavioural testing, assisted with tissue extraction and provided editorial assistance.	
Signature		Date 20/06/2014

Name of Co-Author	Peter Grace
Contribution to the Paper	Helped in the experimental design and provided editorial assistance.
Signature	Date 19/06/2014

Name of Co-Author	Paul Rolan
Contribution to the Paper	Invovled in the experimental design, contributed to the data interpretation and the preparation of the manuscript.
Signature	Date 24/06/2014

Name of Co-Author	Mark Hutchinson
Contribution to the Paper	Involved in the experimental design, assisted with statistical analysis, contributed to the data interpretation and the preparation of the manuscript.
Signature	Date 24/06/2014

OPEN ACCESS Freely available online

PLOS | ONE

TLR 2 and 4 Responsiveness from Isolated Peripheral Blood Mononuclear Cells from Rats and Humans as Potential Chronic Pain Biomarkers

Yuen H. Kwok[1]*, Jonathan Tuke[2], Lauren L. Nicotra[1], Peter M. Grace[1,3], Paul E. Rolan[1,4,5], Mark R. Hutchinson[3,6]

1 Discipline of Pharmacology, School of Medical Sciences, University of Adelaide, Adelaide, South Australia, Australia, **2** School of Mathematical Sciences, University of Adelaide, Adelaide, South Australia, Australia, **3** Department of Psychology and The Center for Neuroscience, University of Colorado at Boulder, Boulder, Colorado, United States of America, **4** Pain and Anaesthesia Research Clinic, University of Adelaide, Adelaide, South Australia, Australia, **5** Pain Management Unit, Royal Adelaide Hospital, Adelaide, South Australia, Australia, **6** Discipline of Physiology, School of Medical Sciences, University of Adelaide, Adelaide, South Australia, Australia

Abstract

Background: Chronic pain patients have increased peripheral blood mononuclear cell Interkeukin-1β production following TLR2 and TLR4 simulation. Here we have used a human-to-rat and rat-to-human approach to further investigate whether peripheral blood immune responses to TLR agonists might be suitable for development as possible systems biomarkers of chronic pain in humans.

Methods and Results: Study 1: using a graded model of chronic constriction injury in rats, behavioral allodynia was assessed followed by *in vitro* quantification of TLR2 and TLR4 agonist-induced stimulation of IL-1β release by PBMCs and spinal cord tissues (n = 42; 6 rats per group). Statistical models were subsequently developed using the IL-1β responses, which distinguished the pain/no pain states and predicted the degree of allodynia. Study 2: the rat-derived statistical models were tested to assess their predictive utility in determining the pain status of a published human cohort that consists of a heterogeneous clinical pain population (n = 19) and a pain-free population (n = 11). The predictive ability of one of the rat models was able to distinguish pain patients from controls with a ROC AUC of 0.94. The rat model was used to predict the presence of pain in a new chronic pain cohort and was able to accurately predict the presence of pain in 28 out of the 34 chronic pain participants.

Conclusions: These clinical findings confirm our previous discoveries of the involvement of the peripheral immune system in chronic pain. Given that these findings are reflected in the prospective graded rat data, it suggests that the TLR response from peripheral blood and spinal cord were related to pain and these clinical findings do indeed act as system biomarkers for the chronic pain state. Hence, they provide additional impetus to the neuroimmune interaction to be a drug target for chronic pain.

Citation: Kwok YH, Tuke J, Nicotra LL, Grace PM, Rolan PE, et al. (2013) TLR 2 and 4 Responsiveness from Isolated Peripheral Blood Mononuclear Cells from Rats and Humans as Potential Chronic Pain Biomarkers. PLoS ONE 8(10): e77799. doi:10.1371/journal.pone.0077799

Editor: Theodore John Price, University of Arizona, United States of America

Received March 21, 2013; **Accepted** September 4, 2013; **Published** October 30, 2013

Copyright: © 2013 Kwok et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The study was funded by the Pain and Anaesthesia Research Clinic, University of Adelaide and was supported by Grant Number [DP110100297] from Australian Research Council (ARC). Its contents are solely the responsibility of the authors and the funders had no role in study design, date collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: yuen.kwok@adelaide.edu.au

Introduction

Pain as defined by the International Association for the Study of Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. Pain is a subjective experience, and hence it is conventionally assessed by patient reports, sometimes with added rating scales. Preclinical animal pain research cannot use such measures, but instead relies on behavioral responses to infer the pain experience. There is a large failure rate in clinical translation of therapies that are efficacious in standard preclinical animal studies, possibly in part because of these different assessments [1]. Biomarkers that reflect pain biology and which could be used in both preclinical animal and clinical human studies have the potential for improving translational success in pain research. Additionally, practical human pain biomarkers have potential uses in enriching clinical trial populations, assisting in the selection of patient treatment and monitoring treatment efficacy [2].

Development of pain biomarkers is problematic because of difficulty in accessing the central nervous system (CNS) where the chronic pain pathology likely resides. Although neuroimaging has emerged as a potential biomarker for chronic pain, by providing "pain signatures" of the brain [3–6], there are several limitations to its usage [7]. Instead, we have sought evidence that peripheral tissues reflect functional changes of the CNS and hence have the potential to be accessible human pain biomarkers.

Over the past 20 years substantial evidence has accumulated indicating the involvement of non-neuronal cells playing a pivotal role in chronic pain. In particular, the immunocompetent cells of the CNS, glia, respond to pain signals releasing additional pro-nociceptive proinflammatory mediators that in turn sensitize neighboring neurons and glia facilitating the heightened pain state [8–12]. Interestingly, this research points to attenuating proinflammatory glial activation is a promising new target for the treatment of neuropathic pain, as drugs that attenuate pro-inflammatory glial activation results in a reduction in allodynia [13–16].

A key mediator in the initiation of proinflammatory glial reactivity associated with chronic pain is Toll Like Receptors (TLRs). TLRs are an innate immune receptor family that recognize danger-associated molecular patterns (DAMP) and pathogen-associated molecular patterns [17]. Activation of TLRs causes the production of pro-inflammatory mediators such as pro-inflammatory cytokines (such as IL-1β) [9]. It is clear from preclinical models that glia assume a proinflammatory reactive state following activation by TLRs and that blockade of glial TLRs significantly reduces experimentally induced neuropathic pain [18–20]. Interestingly, we have recently demonstrated that in chronic pain patients, peripheral blood mononuclear cells (PBMCs) also have increased TLR2 and TLR4 responsiveness compared with pain-free participants [21], suggesting that this could be a potential pain biomarker.

However, given the cross-sectional nature of the human data which hinders cause-and-effect analysis, we have sought whether similar findings occur in a prospective graded animal model to support that interpretation that this biomarkers lie on the causal pathway rather than being bystander effects. Additionally we have sought whether a biomarker panel based on TLR-induced IL-1β production by PBMCs can distinguish pain from non-pain states in two separate clinical pain populations (medication overuse headache and sciatica) to explore potential clinical utility.

Materials and Methods

Study 1: Graded Chronic Constriction Injury Surgery and Sample Preparation

Animals. Pathogen-free adult male Sprague–Dawley rats (300–350 g; University of Adelaide, Laboratory Animal Services, Waite Campus, Urrbrae, Australia) were used in all experiments. Rats were housed in a temperature-controlled (18–21°C) and light-controlled (12 h light/dark cycle; lights on at 07:00 h) rooms with standard rodent chow and water available *ad libitum*. Animals were habituated to the holding facility for 1 week prior to experimentation. All procedures were approved by the Animal Ethics Committee of the University of Adelaide and were conducted in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Surgery. A graded neuropathic pain model, the "Grace model" was used [22]. Surgery was conducted under isofluorane (3% oxygen) anaesthesia. Briefly, the sciatic nerve was exposed at the mid-thigh level of the left leg as previously described [23]. Between zero and 4 sterile chromic gut sutures (cuticular 4–0 chromic gut, FS-2; Ethicon, Somerville, NJ, USA) were loosely tied around the gently isolated sciatic nerve to produce varying degrees of allodynia. Once the superficial muscle overlying the nerve was sutured, the animals had varying numbers of chromic gut suture (equivalent length) placed in the subcutaneous space. For sham treatment, the sciatic nerve was identically exposed and isolated but not tied. Animals were monitored postoperative (PO) until fully ambulatory prior to the return of their cage and checked daily for signs of infection. No such cases occurred in this study.

Experimental groups and design. Experimental groups used in the Grace model were also selected in this study. The sciatic nerve was loosely ligated with chromic gut sutures, with the number of perineural sutures indicated by the designation N0, N1, N2 or N4. Additional pieces of chromic gut designated S4, S3, S2 or S0 respectively were also placed in the subcutaneous space, to keep the total number of ligatures to 4, in order to keep the non-specific immunological stimulus constant between the groups. This model has been shown to produce graded neuropathic pain in relation to the number of ligatures around the nerve. Two additional groups (N1S0 and N2S0) with only ligatures to the sciatic nerve were also introduced to examine only neuronal insults. For the sham control the nerve was isolated but there was no exposure to chromic gut. N0S4 was a control group for the presence of chromic gut. The experimental groups (6 rats/group) were N0S0 (sham control), N0S4, N1S0, N1S3, N2S0, N2S2 and N4S0.

Behavioral testing: von frey test. Rats were habituated for at least three sessions (60 min) to the test environment prior to von Frey testing. Testing was performed blinded with respect to the experimental group. The von Frey test was performed within the sciatic innervation area of the hind paw. Assessments were at baseline, PO day 3, 7, 10 and day of cull and the development of allodynia was assessed. Animals were followed to at least PO day 18 to ensure the neuropathic pain was well established. A logarithmic series of ten calibrated Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA) were applied randomly to the left hind paw to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. Log_{10} (milligrams×10) hair stiffness ranged from 3.61 (0.407 g) to 5.18 (15.136 g). The behavioral responses were used to calculate the 50% paw withdrawal threshold (absolute threshold), by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method using the program PsychoFit [24]. This fitting method allows parametric analyses that otherwise would not be appropriate.

Peripheral blood and spinal cord collection. On the day of cull (at least PO 18 day), rats were anesthetized with sodium pentobarbital and blood (approximately 7 mL) was collected via cardiac puncture into tubes containing EDTA. The rat was then transcardially perfused with 15 ml of chilled 0.9% isotonic saline and the lumbar spinal cord was quickly removed and dissected into 3 equal lengths. The isolated spinal cord was incubated for 20 h at 37°C, 5% CO_2 in a humidified environment (Thermoline Scientific, Australia). Added to the incubation medium were either: 10 μg/mL of TLR2 agonist synthetic triacylated lipoprotein (Pam3CSK4) or 10 μg/mL of TLR4 agonist lipopolysaccharide (LPS) from Sigma-Aldrich (Castle Hill, NSW, Australia) or RPMI medium only (control).

Stimulation of rat peripheral blood mononuclear cells (PBMCs) and plasma collection. PBMCs were isolated using Optiprep Sigma-Aldrich (Castle Hill, NSW, Australia) as directed by the manufacturer using the mixer flotation method. Plasma was also collected and stored at −70°C until the ELISA. Isolated cells were diluted to 1×10^6 cells·ml^{-1} in enriched RPMI 1640 (10% fetal calf serum and 1% penicillin) and plated into 96 well plates (Nunc, Roskilde, Denmark) (100 μl per well). When insufficient cells were obtained (5 rats), data were adjusted to 1×10^6 cells (by multiplication of the factor to obtain a response for IL-1β 1×10^6 cells). A range of concentrations was added into the wells, TLR2 agonist (Pam_3CSK_4) from 10 ng·ml^{-1} to 1 μg·ml^{-1} and TLR4 agonist (LPS) from 10 ng·ml^{-1} to 10 μg·ml^{-1}. Control wells

minus the TLR agonist were also included. Plates were incubated for 20 h at 37°C, 5% CO_2 in a humidified environment (Thermoline Scientific, Australia).

Spinal cord sample preparation. Briefly, after 20 h of incubation the supernatant of the spinal cord was stored at −80°C until assay. The spinal cord sections were removed and sonicated using a Labsonic 1510 probe sonicator (B. BRAUN, Melsungen, Germany) in ice-cold extraction buffer containing Iscove's medium with 5% FCS and a cocktail enzyme inhibitor (including: 100 mM amino-n-caproic acid, 10 mM EDTA, 5 mM benzamidine-HCL, and 0.2 mM phenylmethylsulfonyl fluoride) all obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Sonicated samples were centrifuged with the supernatant and stored at −70°C until assay.

Study 2: Chronic Pain and Pain-free Participants

Study participants. The data presented here was obtained from 1 published study [21] and 2 unpublished clinical studies. Ethical approval was obtained from the Human Research Ethics Committee of the Royal Adelaide Hospital, Adelaide, South Australia. All studies were conducted at the Pain and Anaesthesia Research Clinic (PARC), Royal Adelaide Hospital, Adelaide, Australia.

All participants gave written informed consent to participate after a detailed oral explanation of the study. All participants were paid for their inconvenience upon completion of the study. Chronic pain patients were recruited from the PARC volunteer database, by public advertisements and from a pain management unit. Healthy participants were recruited from the PARC's healthy participant database. Sixty-four participants were recruited and participants were divided into 2 cohorts: published cohort (consisted of participants from a previous study [21]) and an expanded cohort.

The published cohort consisted of chronic pain participants and pain-free participants. Chronic pain participants had to experience pain at least five days a week and for at least 3 months. The pain-free participants had no clinically significant chronic pain and were not taking opioids or other analgesics. The expanded cohort consisted of mainly unilateral sciatica and medication-overuse headache participants. Unilateral sciatica participants had to experience pain at least five days a week and for at least 3 months. For the medication-overuse headache participants, the inclusion criteria included regular use for at least 3 months of opioid-containing analgesics (10≥ days per month) headache present on at least 15 days/month (for at least 2 months), headache developed or markedly worsened during medication-overuse and primary indication for analgesics is a headache disorder.

There was no minimum pain score for eligibility. Chronic pain patients from both cohorts could be taking ongoing opioid therapy or not on any chronic opioid therapy. For all participants the key inclusion criteria were the following: aged between 18 and 65 years, be in good general health (other than chronic pain patients) without clinically significant renal, hepatic, cardiac or other diseases. Key exclusion criteria were: use of any immunosuppressant drugs (e.g. azathioprine); presence of an active inflammatory process; a clinically significant infection in the previous 4 weeks; a positive urine screen for illicit drugs (except for prescribed opioids), pregnancy and/or lactation, and have a known history of hepatitis B, C or HIV.

Human blood collection and PBMCs isolation. On the study day, information on pain history and medication use was recorded. Twenty-seven ml of blood were collected into tubes containing EDTA and the same procedure mentioned previously

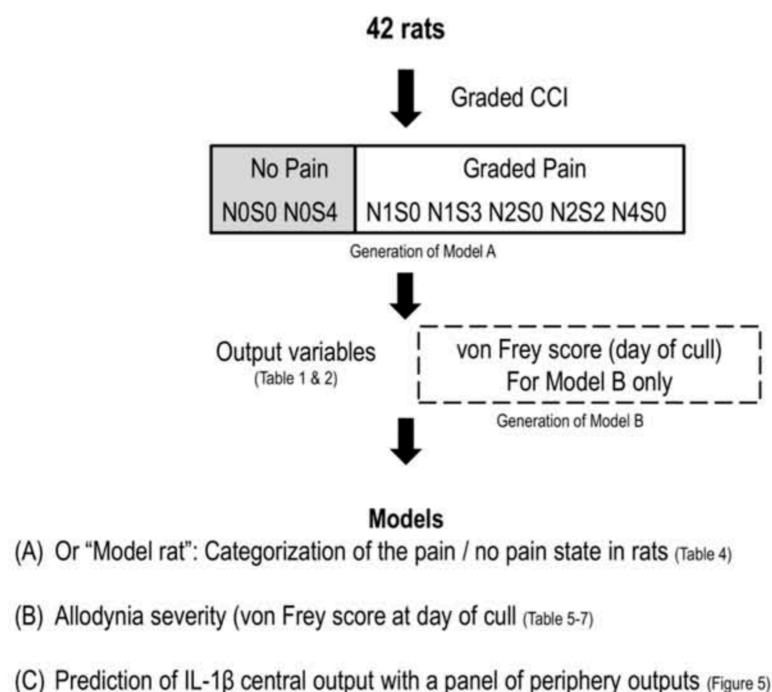


Figure 1. Overview of the generation of models in post graded CCI rats.
doi:10.1371/journal.pone.0077799.g001

in "Stimulation of rat PBMCs and plasma collection" was performed. Sufficient cells were obtained from all participants and plasma was not collected in humans. A range of concentrations of TLR agonists were added into the wells in triplicate, Pam3CSK4 from 13 $pg \cdot ml^{-1}$ to 1 $\mu g \cdot ml^{-1}$ (Sigma) and LPS from 6 $pg \cdot ml^{-1}$ to 10 $\mu g \cdot ml^{-1}$ (Sigma). Control wells minus the TLR agonist were also included.

Rat and Human IL-1β Assay

IL-1β level was determined by a commercially available ELISA (rat IL-1β ELISA; eBioscience, San Diego, CA and for human IL-1β ELISA; BD Bioscience, Australia). For the rat's ELISA kit, the manufacturer's instructions were modified by extending the standard curve from 39 pg/mL to 5 pg/mL so that lower concentration of IL-1β could be detected. The extended standard curve was accepted for each ELISA when the R-square (goodness of fit) was above 0.99. For the human's ELISA kit the IL-1β levels were determined according to the manufacturer's instructions. UV absorbance was quantified on a BMG PolarStar microplate reader (BMG Labtechnologies, Offenburg, Germany) at 450 nm with absorbance at 570 nm subtracted. The modified limit of quantification of 5 $pg \cdot ml^{-1}$ was used for the rat's ELISA kit and for the human's ELISA kit the manufacturer's limit of quantification of 0.8 $pg \cdot ml^{-1}$ was used.

Study 1 Development of Models from Peripheral and Central Obtained IL-1β Released from Post Graded CCI Rats

Overview of modeling. The overview of the modeling is summarize in Figure 1. All the collected outputs from the rat (presented in Table 1 and 2.) were imported into the statistical computing environment R (R Development Core Team, 2007). In order to determine whether models constructed with the collected output variables allow: (A) categorization of the pain/no pain states in rats (B) the detection of the allodynia severity in rats (C) whether in rats, central outputs can be predicted with peripheral outputs. The generalized linear model (glm) and the R function stepAIC were used to generate models. StepAIC function [25] performs stepwise model selection (backward and forward selection) using the Akaike information criterion (AIC) as a variable selection criterion. The functions glm and stepAIC are both found in the Modern Applied Statistics with S (MASS) package (From the statistical software R; www.r-project.org).

The glm function assesses how much each output variable contributes to a response; the responses in question were (A) pain, (B) von Frey score and (C) spinal cord IL-1β output (Figure 1). The stepAIC function was used to refine the model by identifying specific output variables that contributed the most to the model and removed others that added no value to the model.

Grouping of output variables. To further dissect which output variables were needed to create the best model to predict the presence and severity of allodynia. The output variables were divided according to anatomical locations and by stimulations as outlined in Figure 2. "Dataset" contained all output variables collected from all anatomical locations and from all stimulations. Whereas subsets contained specific output variables group from either specific location (e.g. Central subset only consist output variables obtained from central region) or from specific stimulation (e.g. TLR2 subset consist output variables stimulated only with TLR2 agonists). The 5 subsets were Peripheral, Central, Basal, TLR2 and TLR4.

To explore the interaction between the effects of nerve alone and combined suture placement, experimental groups were also divided into "**Neuronal and subcutaneous**" (N0S0, N0S4, N1S3, N2S2 and N4S0) and "**Neuronal**" (N0S0, N1S0, N2S0 and N4S0) groups. Within the group, the output variables were further divided into the 5 different subsets as mentioned above.

Best model A selection: To predict the presence of pain. N0S0 and N0S4 were experimental groups which considered to have no pain (assigned as 0) because on the day of cull the behavior score indicated there were no group differences between N0S0 (sham) and N0S4. The 5 experimental groups considered to have pain were assigned as 1 and consists of N1S0, N1S3, N2S0, N2S2 and N4S0 (see Figure 1). The glm function was used to predict the presence of pain for the following datasets and subsets from **all** experimental groups:

- **All** dataset and the following 5 subsets: **Peripheral**, **Central**, **Basal**, **TLR2** and **TLR4**

The stepAIC function was performed to select output variables that contributed significantly to the refined model. Receiver Operating Characteristic (ROC) curves were generated from the refined model and the area under the curve was calculated. One-way ANOVA was used to compare the model generated from the **All** dataset with the 5 subsets (see Figure 1).

The same process was repeated with the **Neuronal and subcutaneous** and **Neuronal** only experimental groups and their corresponding 5 subsets (e.g. Peripheral).

Best model B selection: To predict the severity of allodynia. The glm function was used to predict the severity of allodynia (von Frey score at day of cull) and the stepAIC function was used to identify the refined model for **All** experimental groups:

- **All** dataset and the following 5 subsets: **Peripheral**, **Central**, **Basal**, **TLR2** and **TLR4**

A Pearson correlation was chosen to determine the relationship between the actual von Frey score and the data predicted by the refined model. The adjusted R-square was used as it takes into account the number of variables introduced to the refined model. One-way ANOVA was used to compare the model generated from the **All** dataset with the 5 subsets (see Figure 2) to determine which models is a better predictor of the severity of allodynia.

The same process was repeated with the **Neuronal and subcutaneous** and **Neuronal** only experimental groups and their corresponding 5 subsets (e.g. Peripheral).

Best model C selection: Prediction of IL-1β central output by models generated from peripheral outputs. The glm function and the stepAIC function were used to generate the refined model to predict the basal spinal cord supernatant IL-1β with output variables obtained from the **Peripheral** subset (from **All** and for **Neuronal and subcutaneous** experimental groups). A Pearson correlation was used to determine the relationship between the predicted values (from the refined model) with the actual IL-1β released from the basal spinal cord supernatant. The adjusted R-square was used and the same procedure was used to generate the model to predict the IL-1β released from the lumbar spinal cord supernatant (post TLR2 and TLR4) and the lumbar spinal cord (basal, post TLR2 and TLR4) response.

Table 1. Summary of variables collected from rats post CCI in the periphery region.

Variables									**P- value**
Peripheral (PBMCs)		**N0S0**	**N0S4**	**N1S3**	**N2S2**	**N4S0**	**N1S0**	**N2S0**	
Non-stimulated	**Plasma IL-1β (pg/mL)**	1.7±0.4	2.7±0.7	1.4±0.4	3.3±0.3	10±5.3	4±0.3	5.5±1.7	0.03
	Cell count (^6 cells/mL)	3.4±1.8*	7.4±1.5	9.2±2.8*#	3.5±0.8	5.9±1	1.8±0. 7#	5.6±2.1	0.004
	Cells IL-1β (pg/mL)	6.3±1.7	4.9±0.6	5.7±1	7.5±2.8	4.6±0.4	4.8±1.1	4.7±0.4	1
Pam3CSK4stimulated (TLR2) IL-1β (pg/mL)	**Minimum**	2.3±0.7	3.1±0.9	2.5±0.4	3.6±0.5	2.2±0.7	3.7±0.7	3.8±0.7	0.5
	Maximum	8.4±1.8	13.9±4	14.5±3.2	12.7±3.5	9.5±1.4	8.6±2.4	10.5±2.3	0.7
	Slope	−0.6±0.5	−2.1±1.7	1.1±1.7	0.3±0.9	−0.1±0.8	−0.09±0.6	−1.9±1.4	0.02
	Intercept	5.4±1.1	8.7±2.6	5.8±0.6	7±1	5.6±0.5	5.7±0.9	6.4±1.04	0.4
LPS stimulated (TLR4) IL-1β (pg/mL)	**Minimum**	12±3.3	14.5±6.1	26.4±10.2	23.6±7.2	16±3.1	12.8±4.7	16±7.3	0.2
	Maximum	24.5±6.8	31.4±10.6	70.5±39.7	65.4±19.1	27.7±5.8	41.5±19	45.3±25.3	0.7
	Slope	3.7±1.8	2.8±2	−17.4±17.5	11.8±7.5	3.2±1.9	9.2±4.8	8.9±9	0.5
	Intercept	13.7±3	20.1±6.8	60.7±35.8	28.6±7.7	18.2±3.1	16.3±6.1	18.9±5.6	0.8
		Control	**Neuronal and Subcutaneous**			**Neuronal**			
		Experimental Groups							

Data are presented as mean ± SEM. Peripheral blood mononuclear cells (PBMCs) were isolated from 41 rats and stimulated with LPS (10 ng·ml^{-1} to 10 µg·ml^{-1}) and with Pam3CSK4 (from 10 ng·ml^{-1} to 1 µg·ml^{-1}) for 20 h. No PBMCs could be obtained from 1 rat in N2S0. Plasma were collected from 42 rats. Cell counts were normalized by log transformation and analyzed using one-way ANOVA followed by Bonferroni. It should be noted the following rats did not have enough PBMCs therefore the rat's PBMCs reactivity were normalize to 10^6 cells in the following rats: 2 rats from N0S0, 3 rats from N1S0 and 1 rat from N2S0. Higher cell counts were detected in experimental group N1S3 vs. N0S0 (indicated with *, $P = 0.049$) and N1S0 vs. N1S3 (Indicated with #, $P = 0.035$). Data (except cell count) were analyzed using Kruskal-Wallis one-way ANOVA.

doi:10.1371/journal.pone.0077799.t001

Study 2 Development of Model D to Predict the Presence of Pain in Humans (Chronic Pain and Pain-free Participants) and Compare Models Generated from Study 1

The overview of the modeling is summarized in Figure 3. The chronic pain patients in the published cohort [21] were the group considered to have pain therefore assigned as 1. The pain-free participants were considered to have no pain hence assigned as 0. The "Model human" was the refined model constructed from the collected outputs (Table 3) from the "published cohort" [21] with the use of the glm and stepAIC function.

Comparison of different models developed from rats and humans. "Model rat to human" used the output variables selected by the **Peripheral** subset obtained from rats (from **all** experimental groups) and applied to the clinical data obtained from the "published cohort" [21] (listed in Table 3). A ROC curve was generated from both models and the area under the curve was calculated. One-way ANOVA was used to compare the models

Table 2. Summary of variables collected from rats post CCI in the central region.

Variables									**P- value**
Central (lumbar spinal cord)		**N0S0**	**N0S4**	**N1S3**	**N2S2**	**N4S0**	**N1S0**	**N2S0**	
Non-stimulated IL-1β (pg/mL)	**Spinal cord supernatant**	4.4±0.4	34.4±24.9	5.1±0.8	9.3±2.9	10.4±5.1	16.5±7.5	7.3±1.9	0.7
	Spinal cord	66.6±11.4	60±10.6	55.4±15.2	50.4±10.4	50.5±10.9	50±15.6	67.4±14.2	0.8
Pam3CSK4stimulated (TLR2) IL-1β (pg/mL)	**Spinal cord supernatant**	5.2±0.6	7.1±1.5	6.1±1.4	7±2	6±1.3	9.2±2.7	6.7±1.5	0.9
	Spinal cord	62±12	42.6±2.	50.5±12.2	64.4±12.8	79±7.2	64.8±10.6	83.5±13.1	0.2
LPS stimulated (TLR4) IL-1β (pg/mL)	**Spinal cord supernatant**	6.8±0.7	7.7±2.5	11.1±4.6	9±1.8	7±1.3	11.8±5.5	6.2±0.8	0.9
	Spinal cord	48.5±7.1	50.3±7.1	61.6±10.3	81.8±16.9	51.7±8.2	67.2±11.8	66.3±8.9	0.6
		Control	**Neuronal and Subcutaneous**			**Neuronal**			
		Experimental Groups							

Data are presented as mean ± SEM. Spinal cord sections were collected from 42 rats and stimulated with LPS at 100ug/mL and Pam3CSK4 at 100 ug/mL. Experimental groups were analyzed using Kruskal-Wallis one-way ANOVA.

doi:10.1371/journal.pone.0077799.t002

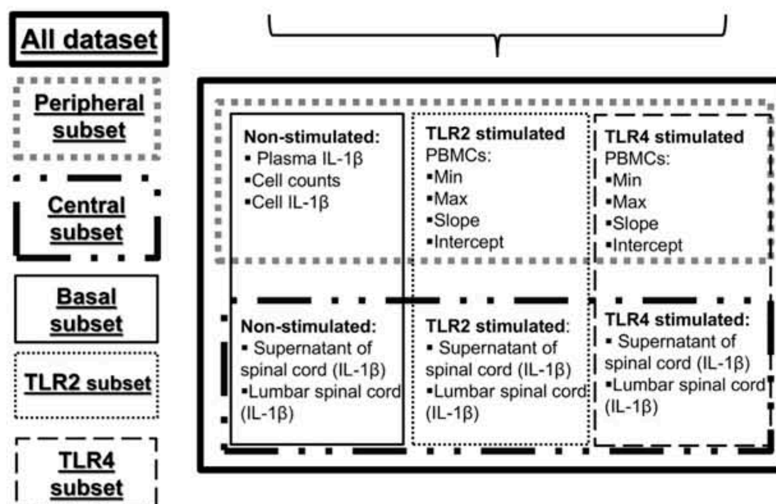


Figure 2. The schematic representation of the breakdown of data according to anatomical location and stimulations. Experimental groups N0S0 and N0S4 are present in both "neuronal and subcutaneous" (italics) and in "neuronal" (underline).
doi:10.1371/journal.pone.0077799.g002

generated from rats and from humans to predict the presence of pain.

Validation of Models to Predict the Presence of Pain in a New Chronic Pain Cohort

The predict function in R was used to determine which of the constructed models (see Figure 3 and also listed below) is the best predictor of pain presence in the "expanded cohort" (all chronic pain participants). Participant with the predicted score between 0 to 0.5 was considered to have no pain and score above 0.5 was considered to have pain.

I. Model rat: The output variables were from the **Peripheral** subset refined model obtained from rats and the data used in this model was also from rats.

II. Model rat to human: "Model rat" was used however the rat data was replaced with human data ("published cohort").

III. Model human: Model generated from the published cohort.

Statistical Analyses

Graphpad Prism version 6.0 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) was used for basic statistical analysis and correlation graphs unless otherwise stated. Data were tested for normality with the D'Agostino-Pearson omnibus normality test and when the data did not fit normal distribution a non-parametric test was chosen instead.

For study 1, data from the von Frey test were analyzed as the interpolated 50% threshold (absolute threshold) in log base 10 of stimulus intensity (monofilament stiffness in milligrams *10). The

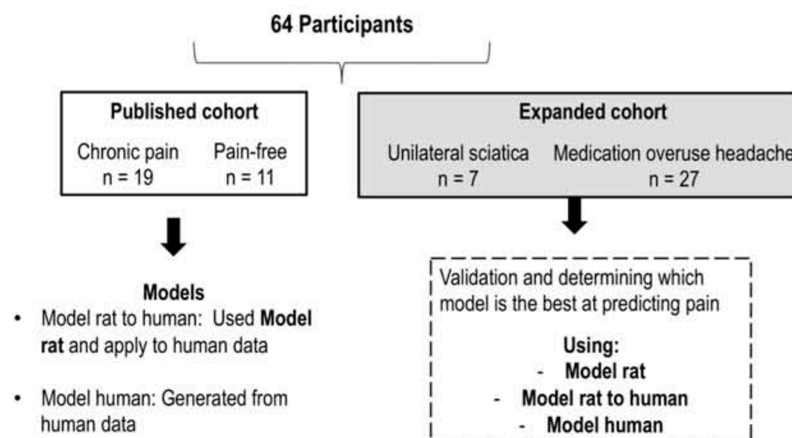


Figure 3. Overview of the generation and comparison of models generated from post graded CCI rats and humans (chronic pain and pain-free participants).
doi:10.1371/journal.pone.0077799.g003

Table 3. Summary of variables collected from humans in the periphery region.

Variables		Published Cohort		Expanded Cohort
		Chronic Pain	Pain-Free	Chronic Pain
Non-stimulated	**Cell count (^7)**	1.1±0.09	1±0.12	1.2±0.07
	Cells (pg/mL)	0.9±0.2	1.1±0.2	12±3
Pam3CSK4 stimulated (TLR2) IL-1β (pg/mL)	**Minimum**	3.5±2.5	2.1±1.1	30±8.2
	Maximum	929.8±164.4	162.6±40.5	524.5±93.9
	Slope	155.4±33.1	31.60±14.2	65.75±15.4
	Intercept	677.5±148.3	93.4±19.3	361.7±69.6
LPS stimulated (TLR4) IL-1β (pg/mL)	**Minimum**	107.6±82	15.2±7.3	112.3±37.3
	Maximum	2231±202.2	2008±162	1975±145.3
	Slope	358.8±40.1	289.2±24.6	322.5±51.1
	Intercept	2065±207.6	1530±150.1	1930±239.2

Data are presented as mean ± SEM. Peripheral blood mononuclear cells (PBMC) were isolated from 64 participants and were stimulated with LPS (6 pg·ml^{-1} to 10 µg·ml^{-1}) and with Pam3CSK4 (from 13 pg·ml^{-1} to 1 µg·ml^{-1}) for 20 h.
doi:10.1371/journal.pone.0077799.t003

cell count data was normalized by log transformation. Differences between experimental groups in von Frey score and *in vitro* IL-1β post TLR agonist were analyzed using repeated measures two-way ANOVAs followed by Bonferroni *post hoc* test. The experimental groups differences on the day of cull and the cell count was analyzed with one-way ANOVA followed by Bonferroni *post hoc* test. For the other variables: plasma, basal IL-1β level, TLR agonist stimulated IL-1β curves (min, max, slope and intercept), TLR agonist stimulated IL-1β from spinal cord the experimental groups differences were calculated with Kruskal-Wallis one-way ANOVA.

For study 2, the age difference between the chronic pain participants (published and expanded cohort) and the pain-free participants was analyzed using one-way ANOVA. The daily morphine used and the duration of pain between the 2 chronic pain cohorts was analyzed using Mann-Whitney test. To determine the group differences between the new cohort of chronic pain patients and pain-free participants, previously published clinical data was used [21]. The concentration-response curve for the TLR2 agonist was assessed using a sigmoidal concentration response equation. For the TLR4 agonist concentration-response, curve a modified biphasic curve as described previously was used [21]. The *F-tests* were used to determine if the best fit curves with the selected parameters (E_{max}, E_{min} and EC_{50}) differed, thus reflecting group differences in the IL-1β expressed by PBMCs post TLR agonist stimulation.

For both studies, the concentration-response curves for the TLR2 and TLR4 agonists obtained from rats and humans were fitted by linear regression. The minimum, maximum, slope and intercept were calculated from the curves obtained from each rat group, chronic pain patients and pain-free participants. All significance was set at $P<0.05$.

Results

Study 1: Post Graded CCI Rat Model

Rats developed allodynia after CCI surgery. At baseline all rats had similar behavior scores revealed by one-way ANOVA (NS, P = 0.8) (Figure 4). After CCI surgery, two-way ANOVA revealed a significant effects of group (P<0.0001) and time (P<0.0001) (data not shown). On the day of cull (at least PO day 18 to PO day 27), two-way ANOVA revealed a significant group effect was observed (P<0.0001) and Bonferroni *post hoc* test showed N1S3 (P = 0.03), N2S0 (P = 0.0002), N2S2 (P = 0.0002) and N4S0 (P = 0.0002) having significantly greater allodynia when compared to sham N0S0. The following experimental groups: N2S0 (P = 0.02), N2S2 (P = 0.02) and N4S0 (P = 0.02) were also found to have significantly greater allodynia scores than N0S4 (no nerve ligatures) confirming the important component of nerve involvement (Figure 4).

IL-1β outputs (basal and stimulated) collected from rats in central and peripheral regions did not differ between

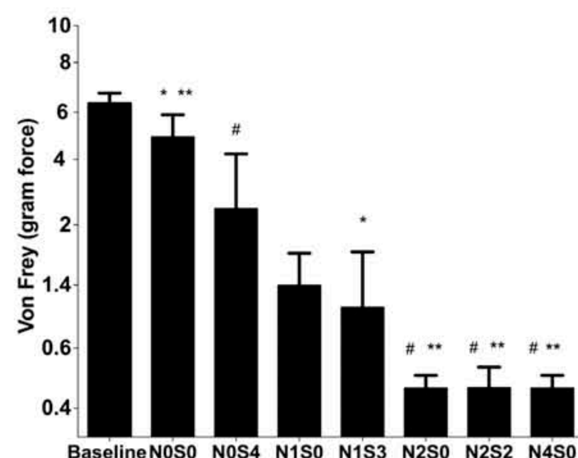


Figure 4. Allodynia quantification at day of cull (At least postoperative day 18). Graded neuropathy was induced by varying the number of chromic gut pieces ligating the nerve (N) and/or distributed in the subcutaneous (S) compartments. The treatment groups were N0S0, N0S4, N1S0, N1S3, N2S0, N2S2 and N4S0 (n = 6/group). At baseline all rats responded very similarly and was not included in the statistical analysis. A significant group effect was observed at day of cull (*P*<0.0001) and with some of the experimental groups (**P* = 0.03, N0S0 vs. N1S3; ***P* = 0.0002, N0S0 vs. N2S0, N0S0 vs. N2S2, N0S0 vs. N4S0; #*P* = 0.02, N0S4 vs. N2S0, N0S4 vs. N2S2, N0S4 vs. N4S4). Error bars on graphs represent standard error of the mean and significance is set at *P*<0.05.
doi:10.1371/journal.pone.0077799.g004

experimental groups. The basal cell level (un-stimulated) of IL-1β expression was revealed by one-way ANOVA to be the same for all 7 experimental groups (p = 1) (see Table 1). In contrast to the previous human study, two-way ANOVA revealed there were no significant group effect between all 7 experimental groups post TLR2 (p = 0.9) or TLR4 (p = 0.1) agonist stimulation in the isolated PBMCs but a significant concentration effects was found for both TLR2 (p = 0.0002) and TLR4 (p = 0.001). No significant group effect was found by the Kruskal-Wallis one-way ANOVA for the lumbar spinal cord (Basal p = 0.8, Post TLR2 stimulated p = 0.2 and Post TLR4 stimulated p = 0.6) or from the supernatant (Basal p = 0.7, Post TLR2 stimulated p = 0.9 and Post TLR4 stimulated p = 0.9) showed in Table 2.

In all experimental groups Model A: Central outputs best predicted the presence of pain in rats. The panel of output variables that best predicted the presence of pain was from **Central** region (Table 4). The area under the ROC curve (ROC AUC) was 0.9 indicating a very good ability to determine the presence of pain. The IL-1β released by the basal spinal cord played a significant contribution in the model (P = 0.04). The order of best to worse models for the predictor of pain collected from the dataset and subsets are as follows: **All>Basal>TLR2** and **Peripheral>TLR4** (ROC AUC: 0.87>0.8>0.76>0. 61) (Additional information can be found in Table S1 in File S1). ANOVA analysis revealed the model generated from the **Complete** dataset did not differ with the model collected from the **Peripheral** (p = 0.09) but it was significantly different from the models generated from output variables collected from **Central** (p = 0.04), **TLR2** (p = 0.02), **TLR4** (p = 0.003) and **Basal** (p = 0.01).

In all experimental groups Model B: A combination of all outputs best predicted the severity of allodynia in rats. The combined output variables (include all regions and stimulations) that best predicted the severity of allodynia was from **All** outputs (see Table 5). The actual von Frey score was significantly correlated with the predicted von Frey score generated from a panel of output variables (adjusted R-square = 0.44, P = 0.0044). The following output variables played a significant contribution to the model: TLR2 stimulated PBMC responses (max (P = 0.01), min (P = 0.01) and intercept (P = 0.008), basal spinal cord (P = 0.007) and TLR2 stimulated spinal cord (P = 0.003) responses. The order of the other models generated from the different subsets are as follows: (from best to worse predictor of allodynia severity) **Central>TLR2>Peripheral>" Basal>TLR4** (adjusted R-square: 0.32 (P = 0.0035) >0.17 (P = 0.05) >0.17 (P = 0.11) >0.1 (NS P = 0.07) >0.0081, (NS P = 0.37) (Additional information can be found in Table S2 in File S1). ANOVA analysis revealed the model generated from all outputs was significantly different from the **Peripheral** (p = 0.004), **TLR2** (p = 0.01), **TLR4** (p = 0.003) and the **Basal** (p = 0.01) subsets but it did not differ with **Central** (p = 0.09) collected outputs.

In neuronal and subcutaneous experimental groups Model A: A combination of all outputs best predicted the presence of pain in rats. The panel of output variables that best predicted the presence of pain was from **All** output variables (Table 4). The ROC AUC was 0.9 and the order of best to worse predictor of pain of the other models collected from the other subsets are as follows: **Peripheral** and **Central>TLR2> TLR4> Basal** (ROC AUC: 0.88>0.77>0.64>0.58) (Table S1 in File S1). ANOVA analysis revealed the model generated from the **All** outputs variables did not differ with model collected from **Peripheral** (p = 0.2), **Central** (p = 0.2), **TLR2** (p = 0.2), **TLR4** (p = 0.05) or **Basal** (p = 0.1).

In neuronal and subcutaneous experimental groups Model B: TLR2 outputs best predicted the severity of allodynia in rats. The panel of output variables that best predicted the severity of allodynia was from **TLR2** IL-1β outputs

Table 4. Best-fit logistic regression model results for the prediction of pain for rats post CCI.

Experimental Groups:	Dataset	Variables	Estimate	SE	*P*	Null deviance	df	Residual deviance	df	AUC	ANOVA
All	Central	TLR4 stimulated supernatant of spinal cord	0.21	0.12	0.07.	39.90	34	23.57	29	0.9	0.004
		Non-stimulated supernatant of spinal cord	−0.052	0.052	0.31						
		TLR4 stimulated spinal cord	0.099	0.052	0.058						
		TLR2 stimulated spinal cord	0.067	0.038	0.078						
		Non-stimulated spinal cord	−0.062	0.029	0.036						
Neuronal and subcutaneous	Complete	Peripheral non-stimulated plasma	0.78	0.60	0.19	31.76	23	16.76	18	0.9	–
		Peripheral non-stimulated cells	−0.31	0.23	0.17						
		Peripheral TLR4 stimulated min	0.10	0.059	0.085						
		Central TLR4 stimulated spinal cord	0.070	0.056	0.21						
		Central non-stimulated spinal cord	−0.073	0.049	0.14						
Neuronal	Complete	Peripheral non-stimulated cells	−1.23	0.77	0.11	23.05	20	14.51	18	0.86	–
		Peripheral TLR4 stimulated intercept	0.43	0.31	0.17						

Notes: Significant variables are shown in bold. The discrimination probabilities (D, area under ROC curve) are presented in the table. One-way ANOVA was used to compare which subsets (Central/Peripheral/TLR2/TLR4 or Basal) when compare with all outputs is a better model. The residual deviance for the model includes predictor variables, whereas the null deviance for the model does not. SE, standard error.

doi:10.1371/journal.pone.0077799.t004

Table 5. Best-fit logistic regression model results for the prediction of the pain severity in rats post CCI in all experimental groups.

Dataset	Variables	Estimate	SE	P	Null deviance	df	Residual deviance	df	Adjusted R-square:	P-value
Complete	Peripheral non-stimulated cell count	-1.99×10^{-8}	1.30×10^{-8}	0.14	5.42	34	2.15	24	0.44	0.0044
	Peripheral non-stimulated plasma	-1.22×10^{-2}	8.84×10^{-3}	0.18						
	Peripheral TLR4 stimulated min	1.56×10^{-2}	8.69×10^{-3}	0.086						
	Peripheral TLR4 stimulated intercept	-8.13×10^{-3}	4.89×10^{-3}	0.11						
	Peripheral TLR4 stimulated slope	-1.37×10^{-2}	6.80×10^{-3}	0.055						
	Peripheral TLR2 stimulated max	-5.29×10^{-2}	1.91×10^{-2}	0.011						
	Peripheral TLR2 stimulated min	-1.25×10^{-1}	4.46×10^{-2}	0.0099						
	Peripheral TLR2 stimulated intercept	1.19×10^{-1}	4.11×10^{-2}	0.0078						
	Central TLR2 stimulated spinal cord	-7.74×10^{-3}	2.32×10^{-3}	0.0028						
	Central non- stimulated spinal cord	6.36×10^{-3}	2.15×10^{-3}	0.0069						
	Spinal cord	0.0050	0.0024	0.043						

Notes: Significant variables are shown in bold. The residual deviance for the model includes predictor variables, whereas the null deviance for the model does not. SE, standard error.

doi:10.1371/journal.pone.0077799.t005

(see Table 6). The von Frey score was significantly correlated with the variables from the **TLR2** IL-1β outputs (adjusted R-square: 0.37, P = 0.02). Within the output variables selected, the following outputs have significant contribution: TLR2 stimulated PBMC (max (P = 0.03), intercept (P = 0.04)) and TLR2 stimulated spinal cord (P = 0.01). The order of the other models collected from different dataset and subsets are as follows: (from best to worse predictor of allodynia severity) **Central >Peripheral>Basal>All>TLR4** (adjusted R-square: 0.34 (P = 0.02) >0.29 (P = 0.04) >0.23 (P = 0.04) >0.56 (NS P = 0.06)>−0.019 (NS P = 0.5) (Table S2 in File S1). ANOVA analysis revealed the model generated from **All** output variables was not significantly different from the **Peripheral** (p = 0.15), **Central** (p = 0.19), **TLR2** (p = 0.2), **TLR4** (p = 0.05) and **Basal** (p = 0.12) specific output variables.

In neuronal experimental groups Model A: A combination of all outputs best predicted the presence of pain in rats. The panel of output variables that best predicted the presence of pain was from the **All** outputs variables (Table 4). The ROC AUC was 0.86 indicating a very good ability to determine the presence of pain. The order of the best to worse predictor of allodynia of the other models collected from the other subsets are as follows: **Central** and **Basal>TLR2>Peripheral>TLR4** (ROC AUC: 0.76>0.66>0.63>0.6) (Table S1 in File S1). ANOVA analysis revealed the outputs generated from **Peripheral** (p = 0.009) and **TLR2** (p = 0.006) were significantly different from **All** output variables but not with **Central** (p = 0.16). The ANOVA could not be calculated for the model generated from **TLR4** output variables owing to incompatibility of the models.

In neuronal experimental groups Model B: A combination of all outputs best predicted the severity of allodynia in rats. The output variables that best predicted the severity of allodynia were from **All** output variables (see Table 7). The von Frey score was significantly correlated with the combined output variables (adjusted R-square: 0.67, P = 0.0048). Within the output variables selected the following outputs have significant contribution: non-stimulated cells (P = 0.001), TLR4 stimulated PBMC (max (P = 0.02) and slope (P = 0.03)) and TLR2 stimulated PBMC max (P = 0.02). The orders of the other models collected from the other subsets are as follows: (from best to worse predictor of allodynia severity) **Peripheral>Basal>Central>TLR2** (adjusted R-square: 0.62 (P = 0.004)>0.35 (P = 0.03)>0.16 (NS P = 0.08)>0.08 (NS P = 0.1) (Table S2 in File S1). Correlation between von Frey score and TLR4 only outputs was not obtained due to the refined model not being

Table 6. Best-fit logistic regression model results for the prediction of the pain severity in rats post CCI in neuronal and subcutaneous experimental groups.

Dataset	Variables	Estimate	SE	P	Null deviance	df	Residual deviance	df	Adjusted R-square:	P-value	ANOVA
TLR2 agonist stimulation only	**Peripheral stimulated max**	−0.041	0.017	0.031	4.52	23	2.24	18	0.37	0.02	0.2
	Peripheral stimulated min	−0.073	0.058	0.23							
	Peripheral stimulated intercept	0.084	0.038	0.041							
	Central stimulated spinal cord	0.0091	0.0033	0.013							
	Central spinal cord supernatant	0.0046	0.0026	0.096.							
	Central spinal cord	0.010	0.0036	0.010							

Notes: Significant variables are shown in bold. One-way ANOVA was used to compare which subsets (Central/Peripheral/TLR2/TLR4 or Basal) when compare with all outputs is a better model. The residual deviance for the model includes predictor variables, whereas the null deviance for the model does not. SE, standard error.

doi:10.1371/journal.pone.0077799.t006

Table 7. Best-fit logistic regression model results for the prediction of the pain severity in rats post CCI in neuronal experimental groups.

Dataset	Variables	Estimate	SE	P	Null deviance	Residual df deviance	df	Adjusted R-square:	P-value
Complete	Peripheral non-stimulated plasma	−0.013	0.007	0.088.	3.12	20 0.56	11	0.67	0.0048
	Peripheral non-stimulated cells	0.12	0.027	0.00094					
	Peripheral TLR4 stimulated max	−0.024	0.0089	0.022					
	Peripheral TLR4 stimulated slope	0.068	0.027	0.03					
	Peripheral TLR2 stimulated max	−0.037	0.014	0.022					
	Central TLR4 stimulated spinal cord supernatant	−0.023	0.015	0.15					
	Central non-stimulated spinal cord supernatant	0.013	0.0096	0.21					
	Central TLR2 stimulated spinal cord	−0.0045	0.003	0.16					
	Central non-stimulated spinal cord	0.0042	0.0021	0.065.					
	Peripheral plasma	-1.29×10^{-2}	9.89×10^{-3}	0.22					
	Peripheral cell	7.66×10^{-2}	2.79×10^{-2}	0.014					
	Central spinal cord supernatant	-1.267×10^{-2}	6.041×10^{-3}	0.052.					

Notes: Significant variables are shown in bold. The residual deviance for the model includes predictor variables, whereas the null deviance for the model does not. SE, standard error.

doi:10.1371/journal.pone.0077799.t007

solved. ANOVA analysis revealed the model generated from **All** output variables was significantly different from the following outputs collected in **Central** (p = 0.0089), **TLR2** (p = 0.0063), **Basal** (p = 0.022) but it was not different from the outputs collected from **Peripheral** (p = 0.16).

Model C: Selected Peripheral Outputs can be Significantly Correlated with the IL-1β from the Lumbar Spinal Cord

All experimental groups. The refined model from the **Peripheral** subset correlated the best with the IL-1β released from the basal spinal cord culture supernatant (adjusted R-square: 0.26, P = 0.003) followed by TLR4 stimulated spinal cord culture supernatant (adjusted R-square: 0.22; P = 0.008) and lastly by TLR2 stimulated spinal cord culture supernatant (adjusted R-square: 0.08, P = 0.05). The same refined model was only correlated with the TLR2 stimulated spinal cord however it did not reach significance (adjusted R-square: 0.06, P = 0.24). Correlations could not be obtained from the TLR4 stimulated spinal cord or from the non-stimulated spinal cord.

Neuronal and subcutaneous experimental groups. The refined model from the **Peripheral** subset best correlated with the basal spinal cord culture supernatant (adjusted R-square: 0.56, P = 0.0005) (Figure 5A) followed by TLR2 stimulated spinal cord culture supernatant (adjusted R-square: 0.52; P = 0.01) (Figure 5C) and lastly by TLR4 stimulated spinal cord culture supernatant (adjusted R–square: 0.48, P = 0.02) (Figure 5E). The refined model generated from output variables collected from **Peripheral** location best correlated with the non-stimulated spinal cord culture supernatant (adjusted R-value: 0.56, P = 0.0005) (Figure 5B) followed by TLR2 stimulated spinal cord culture supernatant (adjusted R-square: 0.52; P = 0.01) (Figure 5D) and lastly TLR4 stimulated spinal cord culture supernatant (adjusted R–square: 0.48, P = 0.02) (Figure 5F).

Study 2: Chronic Pain and Pain-free Participants

Human participant demographic data. Basic demographics are listed in Table 8. In the published cohort [21] there are nineteen chronic pain patients (13 female, 6 male, (min-max) 33–65 years old; mean age 52), and eleven pain-free participants (7 female, 4 male; 36–61 years old; mean age 51). In the expanded cohort there are thirty-four chronic pain patients (25 female, 9 male, 23–64 years old, mean age 46). Additional information on the pain diagnosis of chronic pain patients can be found in Table 9. The average duration of pain in the published cohort was 7 years (min-max; 1–28) and for the expanded cohort was 21 years (min – max, 3–55). The mean daily dose (oral morphine equivalent) taken by the published cohort was (mean ± SEM) 49±13 mg and for the expanded cohort was 13±3 mg.

Increased TLR responsiveness was observed in the isolated PBMCs collected from the expanded cohort compared with pain-free participants.. The TLR2 agonist Pam3CSK4 induced significant concentration-dependent increases in IL-1β release in the isolated PBMCs collected from the chronic pain patients (expanded cohort) when compared with pain-free participants from the previously published cohort [21]. The clear separation between the two groups resulted in an overall significant group effect in response to Pam3CSK4 ($F_{3,\ 452} = 13$, $P<0.0001$; see Figure 6A).

The TLR4 agonist LPS induced elevations in IL-1β in the isolated PBMCs collected from the chronic pain patients in the expanded cohort and in pain-free participants from the published cohort [21]. There was a significant group difference ($F_{1,\ 385} = 5$, $P<0.03$; see Figure 6B).

Which Model is Best at Predicting Pain Presence

Models generated from peripheral derived models from both rats and humans have a good ability to predict presence of pain in human. The ROC AUC generated from the "Model rat to human" was 0.94 indicating a very good ability to determine the presence of pain in humans (published cohort; Figure 7A). Likewise, the "Model human" had an ROC AUC of 0.92 (Figure 7B) (Additional information of the model can be seen in Table S3 in File S1) also indicating a very good ability to detect the presence of pain. ANOVA analysis revealed the two models were found to be not significantly different (Table S3 in File S1).

Model rat was found to predict presence of pain accurately in a cohort of chronic pain participants. All participants in the expanded cohort should be 1 (pain) however

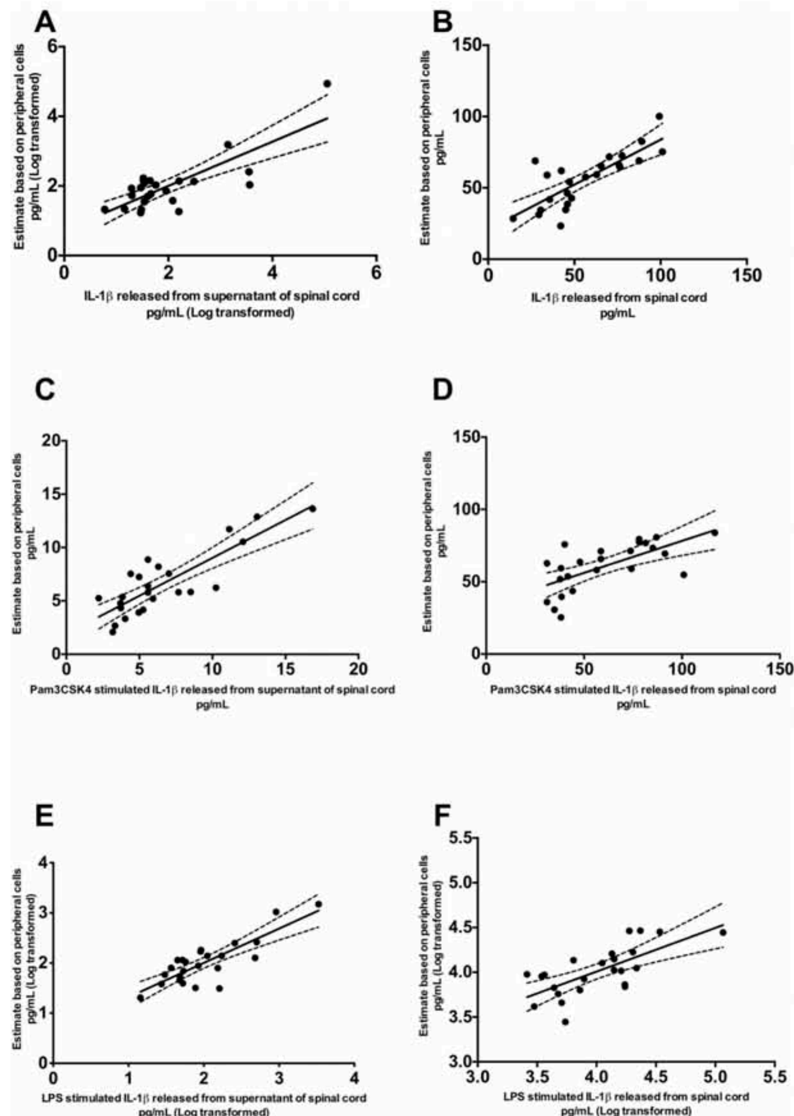


Figure 5. Rat spinal cord (basal, post TLR2 and TLR4 agonist stimulation) was positively correlated with periphery outputs. IL-1β level released from (A) basal spinal cord supernatant ($P = 0.00048$, adjusted R-square = 0.56) (B) basal spinal cord ($P = 0.011$, adjusted R-square = 0.47) (C) spinal cord supernatant ($P = 0.01$, adjusted R-square = 0.52) (D) spinal cord post Pam3CSK4 ($P = 0.04$, adjusted R-square = 0.29) (TLR2) stimulation at 100 μg/mL (E) spinal cord supernatant ($P = 0.02$, adjusted R-square = 0.48) (F) spinal cord ($P = 0.09$, adjusted R-square = 0.26) post LPS (TLR4) stimulation at 100 μg/mL was found to be significantly correlated with the estimated values predicted from peripheral tissue outputs in rats from Neuronal and subcutaneous. Pearson correlation was used and data shown in panel (A, E and F) have been log transformed and linear regression with 95% confidence interval curves are shown on the graph.
doi:10.1371/journal.pone.0077799.g005

according to all models some participants were predicted to have no pain (Figure 8). The number of participants predicted to have no pain/pain (from best to worst predictors) by "Model rat" was 6/28, for "Model rat to human" was 21/13 and for Model human" was 14/20.

Discussion

Despite the wealth of pre-clinical evidence implicating TLR-mediated neuroinflammation and chronic pain [9–11,26], the relevance of neuroinflammation and TLR signaling in human pain conditions is lacking largely due to the inaccessibility of the CNS [3]. However, we have recently published functional evidence of this relationship by demonstrating that low dose intravenous endotoxin (LPS; TLR4 activator) markedly enhanced the flare, hyperalgesia and allodynia responses to intradermal capsaicin in healthy volunteers [27]. Despite this important finding, this model is not practical as a pain biomarker in large patient populations. Hence more practical biomarkers of the neuroimmune activation status in chronic pain are needed. In this study we have two major findings relevant to this aim. The first is

Table 8. Demographic summary.

	Published Cohort		Expanded Cohort	
	Chronic pain (n = 19)	Pain-free (n = 11)	Chronic Pain (n = 34)	P
Gender (M/F)	6/13	4/7	9/25	–
Age (Years)	52 (33–65)	51 (36–61)	46 (23–64)	0.17
Oral morphine equivalent dose (per day) (mg)	49±13	–	13±3	0.44
Duration of chronic pain (Years)	7±2	–	21±2	<0.0001

Data were collected from medical and family history. Data are expressed as mean ± S.E.M except age is expressed as mean ± min–max. One-way ANOVA was used to determine the age difference and the non-parametric Mann-Whitney test was used to daily morphine dose and duration of chronic pain between the chronic pain groups (P-values shown).

doi:10.1371/journal.pone.0077799.t008

that we have replicated our earlier conclusion that *in vitro* PMBC stimulation to be a biomarker of the pain state in two distinct patient populations. Sciatica was selected as it has high face validity to the CCI model used in our animal experiments. Medication overuse headache was selected in contrast as there is no evidence of peripheral pathology in this condition, yet we have hypothesized that opioid-induced glial activation is a major contributing pathology [28]. However cross-sectional studies demonstrating correlation cannot demonstrate a causal relationship. Hence our second major finding is that in a prospective graded rat model we have shown that immune activation in peripheral blood and in spinal cord were related to the pain state in a "dose"-related manner supporting a causal relationship. There are two main implications of these findings. Firstly, this confirms the likely role of TLR signaling in human chronic pain, providing support to the search for inhibitors of these systems as potential new treatment for pain. Secondly, excitingly, as the sensitivity is measured in the readily accessible tissue of peripheral blood, these assessments have the potential to act as biomarkers.

Biomarkers of pain have several potential clinical utilities. One important potential role would be to support patient stratification for

Table 9. Primary diagnoses and medications of chronic pain patients (n = 53).

Diagnosis	Number (%)	
	Published Cohort	Expanded Cohort
Chronic back or shoulder or leg pain	26.3	–
Fibromyalgia	10.6	–
Sciatica	10.6	20.5
Osteoarthritis	31.5	–
Medication-overuse headache	–	79.5
Others	21*	–
Medications		
Opioids	58	79.4
On medications other than opioids:	32	8.8
Not on medications	10	11.8

*Other pain diagnosis include: complex regional pain syndrome (n = 1), atypical trigeminal neuralgia (n = 1), neuropathic Pain syndrome (n = 1) and non cardiac chest pain (n = 1).

doi:10.1371/journal.pone.0077799.t009

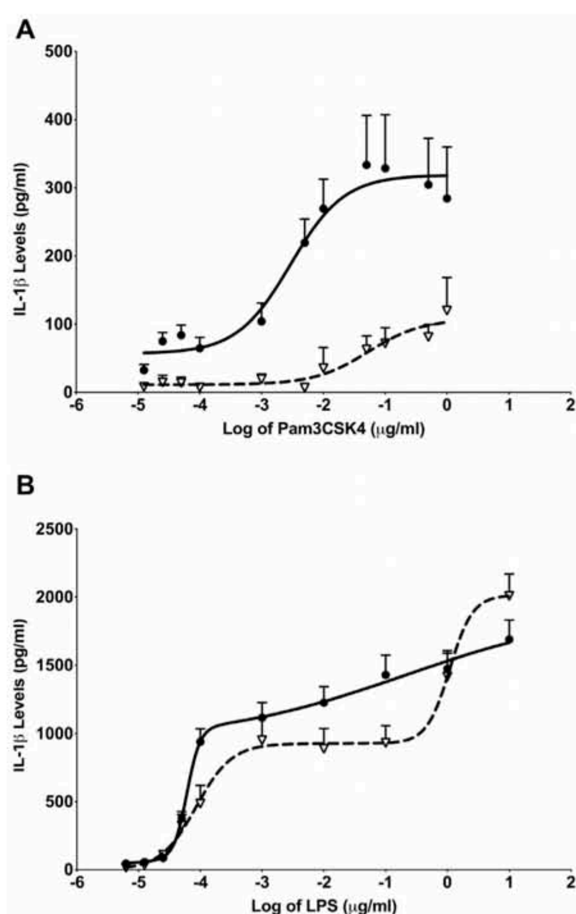


Figure 6. TLR agonist stimulation caused significant group differences in the release of IL-1β in chronic pain patients and pain-free participants. Isolated white cells obtained from new chronic pain patients (closed circle) and pain-free controls from previous study (open triangle) were stimulated with a range of (A) Pam3CSK4 (TLR2) concentrations (13 $pg \cdot ml^{-1}$ to 1 $\mu g \cdot ml^{-1}$) and (B) LPS (TLR4) concentrations (6 $pg \cdot ml^{-1}$ to 10 $\mu g \cdot ml^{-1}$) to generate the response curves and resulted in significant group differences (Pam3CSK4; $P<0.0001$ and LPS, $P=0.004$). Error bars on graphs represent standard error of the mean.

doi:10.1371/journal.pone.0077799.g006

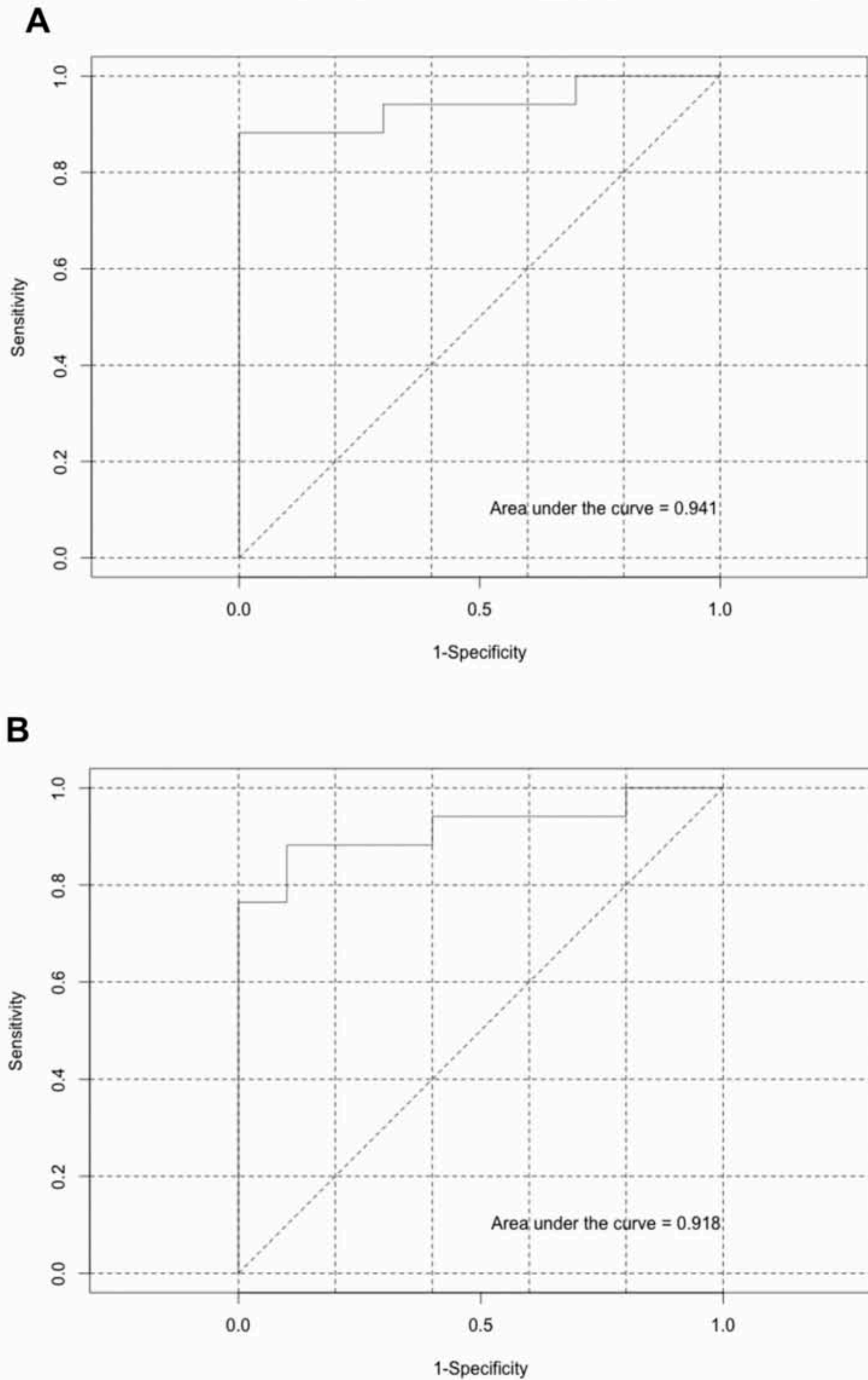
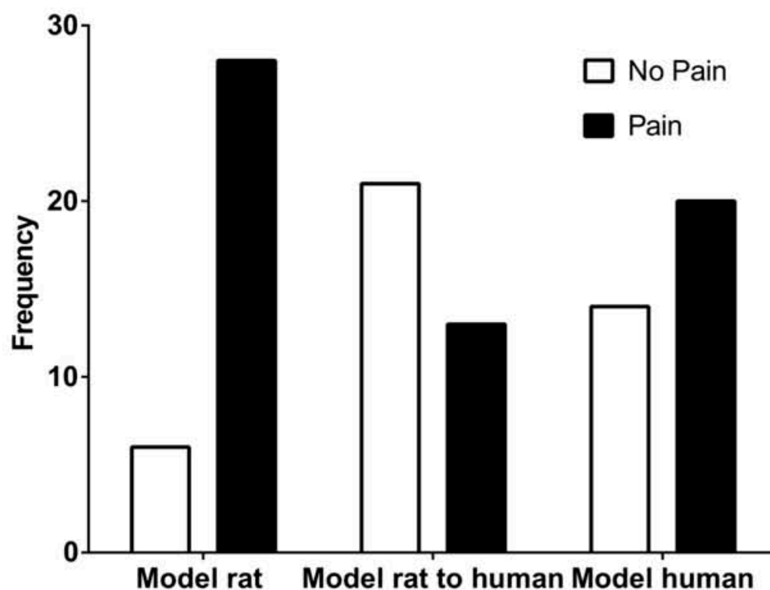


Figure 7. Representation of ROC curves for the detection of pain presence. Models generated from (A) rat data and (B) human data obtained from peripheral collected output variables.
doi:10.1371/journal.pone.0077799.g007



Bin Center	Model rat	Model rat to human	Model human
0	5	9	0
0.1	0	4	4
0.2	1	3	3
0.3	0	1	2
0.4	0	3	4
0.5	0	1	1
0.6	0	1	6
0.7	0	0	0
0.8	0	3	2
0.9	0	4	4
1	28	5	8

Figure 8. Representation of the constructed models Model rat, Model rat to human and Model human that predicted the presence of pain in the expanded cohort. Bin center 0 represents no pain and 1 represents pain.
doi:10.1371/journal.pone.0077799.g008

enriched clinical trials or for monitoring the response to intervention [29,30], making such trials more sensitive to interventions and more meaningful. Another potential role is in patients who cannot communicate well, e.g. children, patients with cognitive impairment, or where cultural and language barriers prevent meaningful evaluation or comparison between populations [31].

The Superior Discrimination of Stimulated vs. Basal Immune Responses

Our data have indicated that the innate immune responses following TLR2 and TLR4 stimulation are both linked to the presence of chronic pain. However, in humans, no group difference was detected from the basal (i.e. unstimulated) cell activity. In contrast, under stimulation conditions, discrimination between the pain/no pain groups was possible. In rats, a combination of basal output responses could identify the pain presence and the severity of allodynia. However, more sensitive and specific findings were obtained with the addition of the stimulated response. Therefore, it is important to not only examine the basal but also dynamic stimulation, as it allows for integrated ligand/receptor interaction, receptor to intracellular signaling, transcriptional to translational modification including genetic variability and epigenetic contributions. By only examining the basal response the above integration is lost and the elicit response has proven to be important for the discovery of potential pain biomarkers.

How has the Rodent Work Added to Our Previous Findings?

Firstly, although cross-sectional studies in patients are easy to perform, they suffer from the inherent weakness of potential selection bias, and are hence at best hypothesis generating. Prospective longitudinal studies (from pre to post injury) in humans with neuropathic pain are difficult, as studying the patients before the onset of injury is probably only possible in post-surgical neuropathic pain, which is only one facet of the condition. Since only a minority of patients experience such complications, such studies are difficult because of very large sample sizes required potentially and complicated by the pathology for which

the surgery is indicated (eg the altered immune milieu in cancer). Hence there are several advantages of studying the neuroimmune processes involved in pain in animals. Firstly, a study published recently found putative pain biomarkers from blood-based RNA transcriptome using the same preclinical graded model of pain [32]. Many of the genes identified encode for proteins that have a recognized role in nociceptive and immune signaling thus providing validation for the use of this model. Secondly, animals can be studied in a prospective manner in disease-free groups of little heterogeneity. More importantly, CNS tissue can be accessed directly. In this study we have shown that peripheral blood TLR signaling sensitivity performed in a similarly predictive manner to that from CNS-tissue sensitivity, providing construct validation to our findings in humans. However, the discrimination of pain states and degree of allodynia in rats was not possible on single derived parameters from either TLR 2 or 4 stimulation, unlike in our result in humans.

IL-1β Expression from Lumbar Spinal Cord Predicted by Peripheral Combination Outputs

The output variables collected from peripheral sites proved to be informative in predicting central responses. Here we demonstrated that IL-1β expression from the rat's lumbar spinal cord was related to peripheral immune cell responsivity. Even though the adjusted R-square was low, the significance of the correlation should not be dismissed. The findings imply that samples collected from the readily accessible peripheral circulation may provide information as to how the CNS is responding. Thus a peripheral marker for proinflammatory glial reactivity may be achieved without the need to access central tissues. It is speculated that the peripheral variables collected from chronic pain sufferers could also predict the IL-1β expression in the central region. Further studies are required to validate this exciting hypothesis.

The Usefulness of Neuronal and Subcutaneous Experimental Groups

The graded CCI model allows for a better pain prediction when chromic gut is placed both around the sciatic nerve and in the subcutaneous space. This is supported by ROC AUC being 0.9 indicating the high accuracy to predict pain presence. The peripheral outputs collected from the neuronal and subcutaneous experimental groups could also be significantly correlated with central tissue IL-1β outputs. Peripheral immune cells are known to play a pivotal role in the establishment of chronic pain by infiltration into central sites [33] and interact with glia causing the release of pro-inflammatory mediators [34]. The neuronal and subcutaneous experimental group is recommended for the understanding of chronic pain as it better mimics the clinical heterogeneous phenotype rather than the standard binomial model of CCI [23].

TLR Responsiveness in the New Cohort of Chronic Pain Patients

The level of IL-1β released from the new cohort of chronic pain patients was not as high as previously published chronic pain patients [21] and could be attributed the fact that the underlying pain is very different in medication-overuse headache and sciatica patients compared with the heterogeneous chronic pain population employed previously. The involvement of TLR signaling with medication-overuse headache patients is currently unknown and has not yet been reported. It should be noted the mean daily morphine equivalent dose of opioids in the new cohort was significantly less even though pain was experienced longer compared with the published cohort.

From the previous study and confirmed in the current study, chronic pain patients have greater TLR-induced IL-1β release from PBMCs than pain-free participants. The mechanistic cause of the PMBC phenotype that resulted in elevated TLR-induced IL-1β release is currently unknown. It is speculated that in chronic pain patients PBMCs are primed by previous exposure with DAMPs and hence following subsequent exposure to a TLR stimulus will produce an exaggerated response (increased in IL-1β release).

The technique of stimulating acutely isolated human PBMCs with TLR agonists and measuring cytokines has been previously used to examine innate immune function in patients. With the use of this cell culture technique, differences in cell reactivity have been detected between healthy controls and patients with the following conditions: surgery [35], rheumatoid arthritis [36], immunosuppression [37] and chronic fatigue syndrome [38]. This supports the usefulness of this technique to reflect the dysregulation of the immune function via the assessment of TLR signaling efficiency. Further research is required to identify which populations of cells in chronic pain patients are responsible for this increased IL-1β production. Using this acute isolation and culturing approach, little time is provided to allow the cells to differentiate away from their in vivo phenotype, thus providing results as close to the in vivo setting as experimentally possible. We speculate the underlying mechanisms lie in the intracellular TLR signaling as surface TLR expression does not always correspond to the PBMCs' output as reported previously [37].

Models Generated from Rat Data can Predict the Presence of Pain in Humans

The model generated from the rat was found to have very good prediction ability for the pain presence in the new cohort of chronic pain patients. This indicates the findings from rats could be translated to humans. The ability of the rat model to be able to predict the presence of pain could be attributed to more output variables being selected. Despite the high accuracy in the prediction of pain in chronic pain participant we do not believe that the current biomarker is as yet a diagnostic for pain. However, it does provide further evidence in humans of the importance of peripheral and central reactivity and that this biomarker approach might be useful in assessing the response to selecting intervention for evaluation and reflecting the response for novel treatments that target the TLR pathways. For it to be a clinically usable biomarker it will need to fulfill additional criteria such as discrimination between other disease states and sensitivity to treatment responses.

Limitations of Current Study

There are several limitations in this study. Only 1 pro-inflammatory cytokine was examined in the study as we were testing a simplified system. As we were validating a previous finding here we did not wish to introduce new pain mediators. Secondly, this study only undertook collection of rat biological samples on the day of cull. It would be informative to conduct a longitudinal study to examine the time sequence and evolution in the sensitivity of the output variables to predict pain. Lastly, the same pain-free participants were used as a comparison to investigate whether increased TLR responsiveness was also observed with the expanded subject cohort and a larger control group would be useful.

Conclusions

In summary, our study is novel, in that the data-driven approach was able to accurately predict pain presence and degree of allodynia in rats after graded CCI surgery. The peripherally derived model identified from rats could also be applied to humans and allowed the prediction of pain presence with accuracy. In addition, IL-1β levels in the central tissue could be predicted by the peripheral outputs obtained from the rats. Collectively, these results provide further evidence of the potential of peripheral cells in being a source of potential pain biomarkers that can be easily accessed and that supporting the role of TLR pathways in playing a vital role in the understanding of chronic pain.

Supporting Information

File S1 Contains: Table S1. Best-fit logistic regression model results for the prediction of pain for rats post CCI. **Table S2.** Best-fit logistic regression model results for the prediction of the pain severity in rats post CCI. **Table S3.** Best-fit logistic regression model from rats (Peripheral only) and from humans to predict the presence of pain in chronic pain patients.
(DOCX)

Acknowledgments

We would like to thank Jacinta Johnson and Nicole Sumracki for their help with the recruitment of participants for the expanded cohort.

Author Contributions

Conceived and designed the experiments: YHK PMG PER MRH. Performed the experiments: YHK LLN. Analyzed the data: YHK JT MRH. Wrote the paper: YHK PER MRH.

References

1. Yassen A, Passier P, Furuichi Y, Dahan A (2012) Translational PK-PD modeling in pain. Journal of pharmacokinetics and pharmacodynamics.
2. J. Atkinson J, Colburn WA, DeGruttola VG, DeMets DL, Downing GJ, et al. (2001) Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 69: 89–95.
3. Borsook D, Becerra L, Hargreaves R (2011) Biomarkers for chronic pain and analgesia. Part 1: the need, reality, challenges, and solutions. Discov Med 11: 197–207.
4. Borsook D, Becerra L, Hargreaves R (2011) Biomarkers for chronic pain and analgesia. Part 2: how, where, and what to look for using functional imaging. Discov Med 11: 209–219.
5. Wartolowska K, Tracey I (2009) Neuroimaging as a tool for pain diagnosis and analgesic development. Neurotherapeutics 6: 755–760.
6. Wager TD, Atlas LY, Lindquist MA, Roy M, Woo CW, et al. (2013) An fMRI-based neurologic signature of physical pain. N Engl J Med 368: 1388–1397.
7. Davis KD, Racine E, Collett B (2012) Neuroethical issues related to the use of brain imaging: can we and should we use brain imaging as a biomarker to diagnose chronic pain? Pain 153: 1555–1559.
8. Watkins LR, Maier SF (2003) Glia: a novel drug discovery target for clinical pain. Nat Rev Drug Discov 2: 973–985.
9. Nicotra L, Loram LC, Watkins LR, Hutchinson MR (2011) Toll-like receptors in chronic pain. Exp Neurol: 316–329.
10. De Leo JA, Tawfik VL, LaCroix-Fralish ML (2006) The tetrapartite synapse: path to CNS sensitization and chronic pain. Pain 122: 17–21.
11. DeLeo JA, Tanga FY, Tawfik VL (2004) Neuroimmune activation and neuroinflammation in chronic pain and opioid tolerance/hyperalgesia. Neuroscientist 10: 40–52.
12. Hutchinson MR, Shavit Y, Grace PM, Rice KC, Maier SF, et al. (2011) Exploring the neuroimmunopharmacology of opioids: an integrative review of mechanisms of central immune signaling and their implications for opioid analgesia. Pharmacol Rev 63: 772–810.
13. Ledeboer A, Liu T, Shumilla JA, Mahoney JH, Vijay S, et al. (2006) The glial modulatory drug AV411 attenuates mechanical allodynia in rat models of neuropathic pain. Neuron Glia Biol 2: 279–291.
14. Hutchinson MR, Northcutt AL, Chao LW, Kearney JJ, Zhang Y, et al. (2008) Minocycline suppresses morphine-induced respiratory depression, suppresses morphine-induced reward, and enhances systemic morphine-induced analgesia. Brain Behav Immun 22: 1248–1256.
15. Hutchinson MR, Lewis SS, Coats BD, Skyba DA, Crysdale NY, et al. (2009) Reduction of opioid withdrawal and potentiation of acute opioid analgesia by systemic AV411 (ibudilast). Brain Behav Immun 23: 240–250.
16. Raghavendra V, Tanga F, DeLeo JA (2003) Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. J Pharmacol Exp Ther 306: 624–630.
17. Miyake K (2007) Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. Semin Immunol 19: 3–10.
18. Tanga FY, Nutile-McMenemy N, DeLeo JA (2005) The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. Proc Natl Acad Sci U S A 102: 5856–5861.
19. Hutchinson MR, Zhang Y, Brown K, Coats BD, Shridhar M, et al. (2008) Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). Eur J Neurosci 28: 20–29.
20. Kim D, Kim MA, Cho IH, Kim MS, Lee S, et al. (2007) A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. J Biol Chem 282: 14975–14983.
21. Kwok YH, Hutchinson MR, Gentgall MG, Rolan PE (2012) Increased Responsiveness of Peripheral Blood Mononuclear Cells to In Vitro TLR 2, 4 and 7 Ligand Stimulation in Chronic Pain Patients. PLoS One 7: e44232.
22. Grace PM, Hutchinson MR, Manavis J, Somogyi AA, Rolan PE (2010) A novel animal model of graded neuropathic pain: Utility to investigate mechanisms of population heterogeneity. J Neurosci Methods 193: 47–53.
23. Bennett GJ, Xie YK (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33: 87–107.
24. Milligan ED, Mehmert KK, Hinde JL, Harvey LO, Martin D, et al. (2000) Thermal hyperalgesia and mechanical allodynia produced by intrathecal administration of the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein, gp120. Brain research 861: 105–116.
25. Venables WNR, Ripley BD (2002) Modern Applied Statistics with S. New York: Springer.
26. Milligan ED, Watkins LR (2009) Pathological and protective roles of glia in chronic pain. Nat Rev Neurosci 10: 23–36.
27. Tawfik VL, LaCroix-Fralish ML, Nutile-McMenemy N, DeLeo JA (2005) Transcriptional and translational regulation of glial activation by morphine in a rodent model of neuropathic pain. J Pharmacol Exp Ther 313: 1239–1247.
28. Johnson JL, Hutchinson MR, Williams DB, Rolan P (2012) Medication-overuse headache and opioid-induced hyperalgesia: A review of mechanisms, a neuroimmune hypothesis and a novel approach to treatment. Cephalalgia : an international journal of headache 33: 52–64.
29. Lesko LJ, Atkinson AJ Jr (2001) Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation, strategies. Annu Rev Pharmacol Toxicol 41: 347–366.
30. Chizh BA, Greenspan JD, Casey KL, Nemenov MI, Treede RD (2008) Identifying biological markers of activity in human nociceptive pathways to facilitate analgesic drug development. Pain 140: 249–253.
31. Herr K, Coyne PJ, McCaffery M, Manworren R, Merkel S (2011) Pain assessment in the patient unable to self-report: position statement with clinical practice recommendations. Pain management nursing : official journal of the American Society of Pain Management Nurses 12: 230–250.
32. Yoshikawa M, Suzumura A, Tamaru T, Takayanagi T, Sawada M (1999) Effects of phosphodiesterase inhibitors on cytokine production by microglia. Mult Scler 5: 126–133.
33. Austin PJ, Moalem-Taylor G (2010) The neuro-immune balance in neuropathic pain: involvement of inflammatory immune cells, immune-like glial cells and cytokines. Journal of neuroimmunology 229: 26–50.
34. Grace PM, Rolan PE, Hutchinson MR (2011) Peripheral immune contributions to the maintenance of central glial activation underlying neuropathic pain. Brain Behav Immun: 1322–1332.
35. Tsujimoto H, Ono S, Majima T, Efron PA, Kinoshita M, et al. (2006) Differential toll-like receptor expression after ex vivo lipopolysaccharide exposure in patients with sepsis and following surgical stress. Clin Immunol 119: 180–187.
36. Kowalski ML, Wolska A, Grzegorczyk J, Hilt J, Jarzebska M, et al. (2008) Increased responsiveness to toll-like receptor 4 stimulation in peripheral blood mononuclear cells from patients with recent onset rheumatoid arthritis. Mediators Inflamm 2008: 132732.
37. Dunne DW, Shaw A, Bockenstedt LK, Allore HG, Chen S, et al. (2010) Increased TLR4 expression and downstream cytokine production in immunosuppressed adults compared to non-immunosuppressed adults. PLoS One 5: e11343.
38. Chao CC, Janoff EN, Hu SX, Thomas K, Gallagher M, et al. (1991) Altered cytokine release in peripheral blood mononuclear cell cultures from patients with the chronic fatigue syndrome. Cytokine 3: 292–298.

Chapter 4. Utility of TLR responsiveness for the assessment of a novel treatment

Yuen H. Kwok, Jacinta L. Johnson, Mark R. Hutchinson and Paul E. Rolan (2014) Ibudilast caused reduction in TLR responsiveness in isolated peripheral blood mononuclear cells collected from medication overuse headache patients. *Text in manuscript format.*

The following study continues to explore the utility of chronic pain patient's TLR responsiveness from peripheral cells as a source of a chronic pain biomarker. Chapter 2 made the initial discovery of increased TLR responsiveness in chronic pain patients and demonstrated face validity as a chronic pain biomarker to differentiate patients from pain-free participants. Chapter 3 demonstrated the translatability of the TLR responsiveness from humans to rats as both basal and TLR stimulated outputs collected from a preclinical model of neuropathic pain were required to construct models with high accuracy for the prediction of pain presence and severity of allodynia. In addition, peripheral outputs could accurately predict central IL-1β response, which confirms the usefulness of peripheral cells. Furthermore, a model generated to predict the presence of pain from the preclinical model was transferable back to humans, which suggest its relevance. Collectively the 2 studies provided good evidence that the assessment of TLR responsiveness in peripheral cells is a good marker for chronic pain.

Medication overuse headache (MOH) is the third most frequent form of headache and is caused by the overuse of headache analgesics (such as opioids, triptans and ergots). In particular, the overuse of opioid analgesics has been associated with worsening headache. Recently, the involvement of glial activation has been speculated in opioid-induced headache, therefore the blockade of glial activation may unravel the current understanding of the underlying mechanisms for MOH and be a mechanism-specific treatment (Johnson et al., 2012).

Ibudilast is a non-selective phosphdiesterase inhibitor and preclinical models have revealed ibudilast can alternate glial activation, which leads to a reduction in pro-inflammatory cytokines, and results in reduced experimental-induced pain in animals. Hence, ibudilast is considered to be an excellent potential treatment for pain as it can cross the blood brain barrier and reduce the side effects of opioids (Ledeboer et al., 2007).

This study explores whether the discovered chronic pain biomarker can assess the utility of ibudilast as a novel treatment for chronic pain. A homogeneous cohort of MOH patients was treated with ibudilast and the efficacy was quantified with the use of TLR responsiveness. The primary aim of this study was to investigate whether TLR responsiveness could reflect the efficacy of ibudilast in MOH patients reflected in the patient's headache frequency and intensity. The secondary aim was to characterize TLR2 and TLR4 IL-1β concentration response curve in MOH patients.

This study found that ibudilast may not be an effective treatment for opioid-induced MOH patients. Eight weeks of ibudilast treatment did not alter the frequency or intensity of headache experienced by the MOH patients. Another PhD student, Jacinta Johnson, was responsible for the design and execution of the clinical phase of the study. However, ibudilast did produce changes in the MOH patient's TLR responsiveness as a significant reduction in the TLR-stimulated IL-1β reactivity was found after 8 weeks. Changes in the TLR responsiveness were not present in the placebo treated group. This finding indicates ibudilast did modulate peripheral cells' phenotype but the targeted pathway was not causal to the generation of headache experience by MOH patients. This study reinforces the important utility of biomarkers as vital information can be obtained quickly and assist in the decision as to whether a novel treatment has the potential to be further investigated.

Statement of Authorship

Title of Paper	Ibudilast caused reduction in TLR responsiveness in isolated peripheral blood mononuclear cells collected from medication overuse headache patients.
Publication Status	Publication Style
Publication Details	N/A

Author Contributions

By signing the Statement of Authorship, each certifies that their states contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Yuen Hei Kwok		
Contribution to the Paper	Conducted all cell culture experiments, all ELISA assessments, statistical modelling and analysis, all graphical presentation of the data collected and wrote the manuscript.		
Signature		Date	24/06/2014

Name of Co-Author	Jacinta Johnson		
Contribution to the Paper	Responsible for the design of the study, recruited all study participants and provided editorial assistance.		
Signature		Date	21/06/2014

Name of Co-Author	Mark Hutchinson		
Contribution to the Paper	Contributed to the data interpretation and the preparation of the manuscript.		
Signature		Date	24/06/2014

Name of Co-Author	Paul Rolan		
Contribution to the Paper	Involved in the experimental design, contributed to the data interpretation and the preparation of the manuscript.		
Signature		Date	24/06/2014

Title: Ibudilast caused reduction in TLR reactivity in isolated peripheral blood mononuclear cells collected from medication overuse headache patients.

Authors: Yuen H. Kwok[1], Jacinta L. Johnson[1], Mark R. Hutchinson[2] and Paul E. Rolan [1, 3, 4]

Affiliations:

1. Discipline of Pharmacology, School of Medical Sciences, University of Adelaide, Adelaide, South Australia, Australia

2. Discipline of Physiology, School of Medical Sciences, University of Adelaide, Adelaide, South Australia, Australia.

3. Pain and Anaesthesia Research Clinic, University of Adelaide, Adelaide, South Australia, Australia.

4. Pain Management Unit, Royal Adelaide Hospital, Adelaide, SA, Australia

Competing interest:

P.E.R is a co-holder of a provisional patent on the use of ibudilast in medication overuse headache and is the principal investigator in the clinical trial. P.E.R was not compensated as a consultant.

Corresponding author: Yuen H.Kwok

Discipline of Pharmacology, School of Medical Sciences, University of Adelaide, Adelaide, South Australia, Australia

e: yuen.kwok@adelaide.edu.au

t: 61 8 8303 5188

f: 61 8 8224 0685

Keywords: biomarkers, ibudilast, medication overuse headache, TLR responsiveness, chronic pain, clinical, IL-1β

Abstract

Chronic pain patients have an increase in interkeukin-1β (IL-1β) production in isolated peripheral blood mononuclear cells (PBMCs) after TLR2 and TLR4 stimulation. Here we investigated whether IL-1β production after TLR agonist stimulation in PBMCs collected from medication overuse headache (MOH) patients will (1.) decrease after 8 weeks of ibudilast treatment and (2.) determine whether ibudilast will correspond to changes in headache frequency or intensity. The study is a parallel, double blind, randomized, placebo-controlled design in MOH patients. Twenty-three patients diagnosed with opioid-induced MOH were enrolled in the study and patients were randomised to receive either ibudilast (80 mg daily) ($n = 9$) or placebo ($n = 17$) for 8 weeks. PBMCs were collected at baseline and 8 weeks post treatment for the assessment of IL-1β production after stimulation with TLR2 (Pam3CSK4) and TLR4 (LPS) agonists. IL-1β released into the supernatant was measured with ELISA. Headache index and intensity scores were collected throughout the study using a headache diary for all patients. Ibudilast did not improve the headache intensity ($P = 0.8$) or headache index ($P = 0.9$) after 8 weeks of treatment compared with placebo. However, PBMCs obtained from the ibudilast group demonstrated a significant reduction in the IL-1β production when compared with placebo in both TLR2 ($P < 0.0001$) and TLR4 agonist stimulations ($P = 0.01$). Ibudilast did not improve the headache index or intensity but it did exhibit a peripheral anti-inflammatory effect. This study found ibudilast may not be targeting the underlying mechanism that is responsible for MOH as indicated in the unchanged headache frequency or intensity. However, the assessment of TLR reactivity in isolated PBMCs showed potential as a biomarker to assess the efficacy of ibudilast in the periphery.

Introduction

Medication overuse headache (MOH) represents a clinically challenging problem that can occur from the overuse of acute analgesics such as opioids, triptans and ergots (Silberstein et al., 2005). In particular opioid use has been associated with higher risk of MOH (Bigal et al., 2008) and recognized by the International Classification of Headache Disorders that opioid-induced MOH is linked with highest relapse rates after medication withdrawal. MOH is a leading risk factor for the development of chronic daily headache (Meng and Cao, 2007) and often occurs in people who already have headache disorders such as migraine or tension-type headache (Evers and Marziniak, 2010).

The current treatment for MOH is drug withdrawal (Evers and Marziniak, 2010). However, severe withdrawal headache, nausea, anxiety, vomiting, tachycardia and sleep disturbance can develop (Diener and Limmroth, 2004). Therefore, better treatments are needed to treat MOH and to cease the use of medications with ease.

The underlying mechanism responsible for MOH is currently unknown (Perrotta et al., 2010). However, it has been indicated that genetic, up-regulation of pro-nociceptive systems (via changes in expression and sensitization of nociceptors), dysfunction of pain processing (brain stem or diencephalic nuclei), central sensitization and psychological factors (such as positive conditioning) all play a role in the development of headache chronification (Diener and Limmroth, 2004, Srikiatkhachorn, 2002).

Recent evidence supports the involvement of glial activation as a potential mechanism for opioid induced MOH. A link between MOH and abnormal excitation in the spinal nociceptive system was

found, which may be an indicator of glial activation (Perrotta et al., 2010). Glial cells are located in the central nervous system (CNS) and under basal condition perform housekeeping functions and maintain neural homeostasis (Watkins, Milligan et al. 2001). However, when glial cells become activated, they become immunoresponsive and modulate pain by the release of neuroexcitatory signals that can increase neuronal excitability, activate neighbouring glial cells and cause the production of nociceptive mediators (such as nitric oxide, excitatory amino acids and proinflammatory cytokines) (Milligan and Watkins, 2009). In preclinical models, blockade of glial activation and proinflammatory mediators released by glia caused reduction in hypersensitivity and allodynia respectively (Raghavendra et al., 2003, Sweitzer et al., 1999).

Opioids such as morphine can act on glia and the classical opioid receptors (to provide pain relief). However, repeated exposure or increased dose of opioid (such as in the case of MOH patients) can cause increased expression of glial activation markers (astrocyte marker GFAP expression and microglial activation CD11b) (Song and Zhao, 2001) and enhance proinflammatory mediator expression (Hutchinson et al., 2009). Opioids have been demonstrated to induce the development of opioid tolerance, hyperalgesia and dependence via TLR4 signalling pathway (Hutchinson et al., 2010).

It is hypothesized that MOH is a form of opioid-induced hyperalgesia (OIH) (Johnson et al., 2012). OIH is a paradoxical phenomenon where continuous use of opioids leads to an increased sensitivity to pain. It has been convincingly demonstrated in clinical trials that OIH can occur however whether it actually occurs in humans remains debatable (Tompkins and Campbell, 2011). Therefore, by targeting opioid induced glial activation or neutralizing or antagonizing the action of pro-inflammatory

cytokines may enhance efficacy of morphine. Ibudilast has been demonstrated to attenuate glial reactivity and hence may be a good treatment for opioid induced MOH.

Ibudilast is a non-selective phosphodiesterase (PDE) inhibitor which has been used in Japan for 20 years to treat bronchial asthma (Kawasaki et al., 1992), post stroke dizziness (Fukuyama et al., 1993), and ocular allergies (Rolan et al., 2009). As ibudilast has the properties of being bioavailable orally, having no serious adverse effects and having good blood brain barrier permeability (Ledeboer et al., 2006, Rolan et al., 2008), it is an ideal candidate for the treatment of neuropathic pain. In preclinical models, ibudilast attenuated microglial activation (reduced withdrawal behaviours), reduced production of pro-inflammatory cytokines, enhanced production of anti-inflammatory cytokines, improved the safety profile of opioids (such as reduced tolerance to opioids and attenuated reward and dependence) (Rolan et al., 2009, Mizuno et al., 2004, Suzumura et al., 1999, Hutchinson et al., 2009). Ibudilast exhibited immunomodulatory actions in multiple sclerosis patients and also displayed inhibition of pro-inflammatory cytokines from peripheral leukocytes (Feng et al., 2004). In a case report, open-label Ibudilast (20 mg daily) was able resolve one MOH patient's daily headache intensity and frequency within one week of treatment and she was able to discontinue the overuse of analgesic medications after treatment (Shimomura et al., 1991). Given the preclinical findings, there is sufficient evidence to undertake a parallel, placebo-controlled, randomised trial of ibudilast in MOH patients.

The levels of cytokines in headache sufferers are found to fluctuate significantly (Perini et al., 2005) leading to disagreement in the literature about the trend of pro-inflammatory cytokine activities during headache attacks. Some studies have found increase pro-inflammatory cytokine expression (such as TNF-α, IL-1β and IL-10) (Perini et al., 2005) (Munno et al., 2001) while others found no change

(Mueller et al., 2001, van Hilten et al., 1991). These studies provide evidence that headache patients have dysregulated immune system. The discrepancies in the cytokine levels reflect the difficulty in the detection of cytokine concentration at basal state. Therefore the quantification of stimulated responses may prove to be advantageous, as the functional changes would be amplified hence it would be easier to assess.

This study hypothesized that ibudilast may provide headache relief in opioid induced MOH patients and consequently decrease TLR reactivity in PBMCs compared with placebo treated MOH patients. Therefore, the first aim of the study was to assess whether ibudilast demonstrated 'on target' activity in MOH patients by measurement of TLR reactivity in isolated PBMCs. The second aim was to determine whether the efficacy of ibudilast would be correlated with the corresponding clinical endpoints (change in headache experienced by MOH patients).

Methods

Study approval and design

Ethical approval was obtained from the Human Research Ethics Committee of the Royal Adelaide Hospital, Adelaide, South Australia. All patients gave their written informed consent before commencement of the study. The study was conducted at the Pain and Anaesthesia Research Clinic (PARC), Royal Adelaide Hospital, Adelaide, Australia and in accordance with the Principles of International Conference on Harmonization Good Clinical Practice as well as the Declaration of Helsinki and the Australian National Statement on Ethical Conduct in Research Involving Humans.

The study design was double-blind, randomized, placebo-controlled with the use of parallel groups. Patients were randomized to receive ibudilast or placebo in a 1 : 1 ratio by the Royal Adelaide Hospital pharmacy department clinical trials staff. Upon completion of the study the treatment status (ibudilast/placebo) of each patient was disclosed.

Patient selection

The study is currently on going therefore only patients recruited from December 2011 to October 2012 were included in this study. The study population included patients with MOH defined by the revised second edition of the International Classification of Headache Disorders who overuse analgesic preparations containing codeine or other opioids.

Twenty-eight patients were recruited from the general population through advertisements and from the PARC volunteer database. Patients were eligible for the study enrolment when all of the following criteria applied:

1. Regular user of opioid containing analgesics on ≥ 10-days/ month for at least 3 months.
2. Experienced headache at least 15 days/ month for at least 2 months.
3. Headache developed or markedly worsened during medication overuse.
4. Primary indication for analgesics was headache disorder.

Key exclusion criteria were the following: unable to provide written informed consent; age < 18 years old at the time of screening; taking codeine for reasons other than headache; severe psychiatric disorders; diabetic neuropathy; immunosuppressant drugs user (e.g. azathioprine); have active inflammatory diseases; recent or current active infection; recent history of drug or alcohol abuse; spinal cord injury; known hypersensitivity to ibudilast or excipients in Pinatos® formulation; renal or hepatic impairment; recent history of significant trauma; current malignancy; a positive urine screen for illicit drugs (except for prescribed opioids); and for women of child bearing potential: pregnancy, lack of adequate protection and breastfeeding.

Study Drugs

The Royal Adelaide Hospital pharmacy department was responsible for the randomization of patients to be either on ibudilast or placebo treatment. The study used a generic delayed-release ibudilast product, Pinatos®, which was obtained from Taisho Pharmaceuticals Industries, Ltd (Koka, Japan). The placebo capsule for this study was also manufactured by Taisho Pharmaceuticals under Japanese Current Good Manufacturing Practices and was supplied by MediciNova. The placebo capsules were identical in appearance to Pinatos® and the manufacturing process was identical to that used for the active drug formulation. An equivalent amount of d-mannitol was used in place of the active ingredient, ibudilast. Each capsule of drug product contained 10 mg of ibudilast. 40mg of ibudilast or placebo was self administered by patients orally, twice daily for 8 weeks.

Study schedule

4 weeks prior to baseline visit

Patients were provided with a headache diary to complete for at least 4 weeks prior to the baseline visit to confirm study eligibility. Standardized education and instructions on how to complete the diary was provided by the experimenter and the patient was required to fill in the headache diary daily. The headache diary recorded a comprehensive assessment of the headache experienced by patients and consisted of head pain characteristics, headache frequency, average headache intensity (11 point numerical rating scale), duration of headache (number of hours) and intake of symptomatic headache treatments (timing, type and amount of medication consumed). The headache index was calculated by the summation of headache duration (hours) with headache intensity (11-point numerical rating scale). The dose of opioid-containing analgesics used by each patient was converted to oral morphine equivalent dose by a comparative opioid chart obtained from the Pain Management Unit at Royal Adelaide Hospital and another comparative opioid chart was used for the calculation for pethidine (Foley, 1985).

Baseline visit

During the baseline visit, the headache diary was collected and patients who were eligible to continue in the study were randomized to either ibudilast or placebo treatment. Patients were asked to rate their current headache intensity on a 100 mm visual analogue scale (VAS) with the endpoint marked with "no pain" (0 mm) or "worst pain ever experienced" (100 mm). Twenty-seven ml of blood was collected from the venous blood to assess the reactivity of the PBMCs after TLR agonist stimulations.

Patients were advised to take only minimum amount of medications required to control their pain however they were not specifically informed to reduce their analgesic intake. Patients were asked to complete another headache diary for the 8-week treatment period. The study medication was dispensed to the patients prior to discharge.

Observations visits

Throughout the treatment period, patients were asked to report for any adverse effects potentially related to the study drug. Blood biochemistry (including the assessment of renal function), liver function tests and haematology were monitored as safety measures however the results will not be reported in this study.

Patients visited PARC at week 2 and 4 to obtain additional diary pages and study drugs. At the completion of the ibudilast or placebo treatment (at week 8), patients were asked to bring their diary and study drug for review and accountability purposes. Similar to baseline visit, patients were asked to rate their current headache intensity on the VAS and blood was collected for the determination of PBMC reactivity to TLR2 and TLR4 agonists after the 8 weeks of treatment.

PBMC isolation and stimulation

PBMC isolation and stimulation techniques reported previously were used (Kwok et al., 2012). Briefly, twenty-seven mL of blood was collected into tubes containing EDTA and PBMCs were isolated using Optiprep (Axis-Shield PoC AS, Oslo, Norway) (Kwok et al., 2012). Isolated cells were diluted to 1 X 10^6 cells·ml^{-1} in enriched RPMI 1640 and plated into 96 well plates (Nunc, Roskilde, Denmark) (100

μl per well). A range of agonist concentration was added into the wells in triplicate. TLR2 agonist: synthetic triacylated lipoprotein: Pam3CSK4 from 13 pg·ml^{-1} to 1 μg·ml^{-1} (Sigma) and TLR4 agonist: lipopolysaccharide: LPS from 6 pg·ml^{-1} to 10 μg·ml^{-1} (Sigma). Control wells minus the TLR agonist were included. Plates were incubated for 20 h at 37 °C, 5 % CO_2 in a humidified environment (Thermoline Scientific, Australia). Plasma were also collected and stored at -70°C until analysis.

IL-1β assay

IL-1β levels were quantified according to the manufacturer's instructions by a commercially available ELISA kit (IL-1β ELISA; BD Bioscience, Australia). The absorbance was quantified on a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany) at 450 nm with absorbance at 570 nm subtracted as per manufacturer's instructions. The manufacturer's limit of quantification of 0.8 pg·ml^{-1} was used.

Statistical analysis

Organisation of data and model development

The TLR2 and TLR4 agonist concentration-response curves were fitted to a linear regression. The minimum, maximum, slope and intercept were calculated from the curve. The output differences between the baseline visit to post 8 weeks visit were presented in Table 2. The baseline headache index score was the average scores obtained from 4 weeks prior to the baseline visit whereas post 8-week headache index score was the average score obtained from day 63 to day 84.

All the collected outputs from the MOH patients (presented in Table 1) were imported into the statistical package R (R Core Team, 2012). A model was generated from the collected output

variables to determine whether patients on ibudilast could be identified. Patients on ibudilast were assigned as 1 and patients on placebo were assigned as 0. A generalized linear model (glm) was initially constructed using the glm() function from MASS package in R. Then the glm model was refined with the stepAIC () function from the MASS package, by the removal of output variables that did not contributed to the identification of ibudilast treated patients determined by the Akaike's Information Criterion (AIC) (Venables and Ripley, 2002). A receiver operating characteristic curve (ROC) curve was generated from the refined model and the area under the curve was calculated.

To identify biomarker non-responders to ibudilast, the predict function in R was used to generate predicted values from the refined model. Then a Spearman correlation was used to determine the relationship between the actual treatment assignment and the predicted assignment. Patients with the predicted score from 0 to 0.5 were determined to be biomarker non-responders for ibudilast and patients with score above 0.5 were determined to be biomarker responders. For more information on the exact R commands used to generate above models please see Appendix C. Biomarker responders for treatment were then selected and their VAS and headache indices were analysed with the two-way ANOVA with repeated measures to identify any potential treatment differences.

Graphpad Prism 6.0 for windows (GraphPad Software; San Diego, CA) was used for all basic statistical analysis and fitting of concentration-response curves. The concentration-response curves for TLR2 and TLR4 agonist were assessed with the use of a sigmoidal and a modified biphasic sigmoidal equation previously reported (Kwok et al., 2012). The F-test was used to detect group differences between the IL-1β concentration curves post TLR agonist stimulation. The two-way ANOVA with repeated measures was used to detect any treatment or visit differences in plasma (IL-1β), VAS and headache index scores. The treatment difference between age, headache history and

morphine dose used was analysed using Mann-Whitney test whereas, treatment difference between the output variables was analysed using the unpaired t-test. When significance was detected it was followed by the Bonferroni post-hoc. All significance was set at $P < 0.05$.

Results

Demographics of MOH patients

Twenty-eight MOH patients were randomly assigned to receive either ibudilast (n = 13) or placebo (n = 15). Five patients (four patients were treated with ibudilast) did not complete the study to week 8 therefore they were excluded from the analysis. Of these five patients, two were withdrawn by an investigator because of worsening headaches (one of the two patients also experienced nausea), one patient was excluded because that patient's codeine usage did not meet with the inclusion criteria and two patients were withdrawn for non study-related reasons.

Patients' basic demographics are listed in Table 2. The mean age for the ibudilast treated group was 44 years old ((min-max) 23 – 59) and the placebo treated group was 46 years old (35 – 62). The ibudilast group consisted of 2 men and 7 women while placebo group consisted of 3 men and 11 women. The average duration of MOH in the ibudilast group was 26 years (min – max; 3 - 52) and for placebo group was 20 years (min - max; 4 - 55). The mean daily dose (oral morphine equivalent) used by ibudilast treated group was (mean ± SEM) 9 ± 2 mg and for placebo group was 17 ± 5 mg. The age ($P = 0.9$), duration of MOH ($P = 0.4$) and mean daily dose ($P = 0.5$) were not different between the ibudilast or placebo groups.

Ibudilast did not change the IL-1β level in plasma or in the supernatant of un-stimulated-PBMCs.

Unstimulated IL-1β level in plasma was found to be the same between treatments ($P = 0.9$) and visits ($P = 0.2$). The plasma IL-1β concentration at: baseline was (mean ± SEM) 0.5 ± 0.2 pg/mL (placebo) vs. 0.6 ± 0.3 pg/mL (ibudilast) and post 8 weeks of treatment was 1 ± 0.3 pg/mL (placebo)

vs. 0.9 ± 0.3 pg/mL (ibudilast). Likewise unstimulated supernatant from PBMCs did not differ between treatments (P = 0.5) or visits (P = 0.06). The IL-1β concentration in un-stimulated supernatant from PBMCs was (mean ± SEM) 8 ± 3 pg/mL (placebo) vs. 5 ± 2 pg/mL (ibudilast) and post 8 weeks of treatment was 2 ± 0.6 pg/mL (placebo) vs. 2 ± 0.4 pg/mL (ibudilast).

Ibudilast did not cause a reduction in the headache intensity but overall headache index was reduced after 8 weeks.

The headache intensity (obtained from the VAS) was not different between treatment groups at baseline (p = 0.9, mean (range) placebo vs. ibudilast: 35 (0 – 76) vs. 34 (0 – 82)) or after 8 weeks of treatment (mean (range) placebo vs. ibudilast: 29 (0 – 85) vs. 26 (0 – 94)) (see Figure 1).

A significantly lower headache index score was obtained 8 weeks after treatment compared to baseline (P = 0.01) irrespective of treatment groups. However no treatment differences were detected at baseline (P = 0.9, mean (range) placebo vs. ibudilast: 1200 (0 – 3000) vs. 1182 (0 – 4116)) or after 8 weeks of treatment (mean (range) placebo vs. ibudilast: 899 (0 – 2664) vs. 788 (0 – 3192)) (see Figure 2).

Ibudilast caused a reduction in the IL-1β level released from isolated PBMCs after TLR2 and 4 agonist stimulation.

(A) TLR2 agonist stimulation

At baseline there were no treatment differences between the placebo and the ibudilast group (P = 0.3, $F_{(3,221)}$=1.2) (see Figure 3A). However, after 8 weeks of treatment, there was a significant reduction in the release of IL-1β in the ibudilast group when compared with placebo (P < 0.0001, F

$_{(3,221)}$ =11.5) (see Figure 3B). A significant concentration-dependent increase was found at both baseline (P = 0.009) and 8 weeks after treatment (P = 0.0051).

(B) TLR4 agonist stimulation

At baseline, no treatment differences were detected between the placebo and the ibudilast group (P = 0.1, $F_{(1, 159)}$ = 2.09) (see Figure 4A). Eight weeks post-treatment, the ibudilast group showed a significant reduction in the release of IL-1β (P = 0.01, $F_{(4, 210)}$ = 3.3) (see Figure 4B) after TLR4 agonist stimulation compared with the placebo group. A significant concentration-dependent increase was also found at both baseline (P < 0.0001) and 8 weeks after treatment (P < 0.0001).

Statistical model derived from IL-1β output from stimulated PBMCs could accurately predict who was on ibudilast.

The panel of selected output variables that best predicted who was on ibudilast treatment with great accuracy consisted only of IL-1β released TLR2 and TLR4 agonist stimulation (change in: TLR4 stimulated min (P = 0.1), TLR4 stimulated max (P = 0.1), TLR4 stimulated slope (P = 0.1) and TLR2 stimulated min (P = 0.05); see Table 3). The area under the ROC curve was 0.9 indicating a very good ability to determine responders of ibudilast (see Figure 5).

Removal of treatment non-responders assessed with the biomarker did not lead to any treatment difference in headache index or headache intensity.

By using the refined model (Table 3), 2 biomarker non-responders of placebo (out of the 14) and 3 biomarker non-responders of ibudilast (out of 9) were removed and the headache intensity and the

index scores were recalculated. No differences were detected between the treatment groups for the headache intensity or the headache index (data not shown).

Discussion

In this study, for the first time in the authors' knowledge, ibudilast caused a significant reduction in the IL-1β levels quantified from MOH patient's isolated PBMCs after TLR2 and TLR4 agonist stimulations. Although the reduction in the IL-1β levels in MOH patients did not correspond with the clinical endpoint, the data provided by the biomarker used in this study suggests ibudilast displays "on target" and anti-inflammatory activities. These data suggest good adherence to study treatment, therefore a lack of efficacy due to poor adherence is unlikely. The current finding cannot conclude whether glial cells are involved in MOH or if ibudilast can be used to treat headaches experienced by MOH patients. However, the assessment of TLR responsiveness was found to be a good marker for assessing the effect of ibudilast reactivity in peripheral cells.

After 8 weeks of treatment, ibudilast treated patients did not experience a reduction in headache intensity or frequency compared with placebo treated patients. With the use of the statistical model derived from TLR stimulated PBMC outputs, biomarker non-responders of either treatments were removed, but the headaches remained the same between both groups. Interestingly, after 8 weeks, the headache indices for both treatment groups were significantly reduced compared to baseline, which could be an indicator of patients having a positive expectancy towards the study drug (Bingel et al., 2011). After investigation of this effect in the ongoing study with more participants included in the study, the potential positive expectancy effect was absent and could be attributed to the inclusion of more patients so the effect was diminished.

Even though the clinical endpoints were not improved after 8 weeks of intervention, ibudilast was found to significantly reduce the levels of IL-1β in PBMCs (isolated from MOH patients) after LPS and Pam3CSK4 stimulation. Ibudilast exhibited an important shift from a pro-inflammatory to an anti-

inflammatory effect that is in agreement with another reported study conducted in multiple sclerosis patients. In the reported study, 60 mg of ibudilast was given over 4 weeks and caused a significant immunological change in the reduction of pro-inflammatory mRNA (IFN-γ and TNF-α) obtained from isolated PBMCs (Feng et al., 2004). Additional studies have also found ibudilast can markedly suppress the release of a chemokine (monocyte chemoattractant protein-1) dose-dependently in LPS treated primary rat microglial cell cultures (Ledeboer et al., 2006) and reduce infiltration of inflammatory cells to the lumbar spinal cord of preclinical models (Fujimoto et al., 1999). In combination with our finding, ibudilast exhibits effects at immune cells, mRNA and cytokine/chemokine secretion levels that should be targeted in future studies. In this study, ibudilast was found to demonstrate specificity as plasma IL-1β level and in the un-stimulated supernatant of PBMCs were unaltered. This is in agreement with previous report of ibudilast having no effect on the basal response thresholds (Ledeboer et al., 2007), which suggests ibudilast does not interfere with normal processes and only target-activated pathways or cells. Collectively, ibudilast display specificity and the ability to reduce pro-inflammatory activities in both the peripheral and the central region.

The biomarker (assessment of peripheral cell reactivity to TLR agonists in isolated PBMCs) used in this study provided valuable information about the efficacy of ibudilast. Significant reduction in the IL-1β levels after 8 weeks of ibudilast intervention was observed with both TLR2 and TLR4 agonist stimulations. Even though the chronic pain biomarker used in this study was imperfect, it provided vital information that would be absent if self-reports were used (Rolan, 1997). The reduction in IL-1β in the PBMCs acted as evident to confirm patients have been compliant with the study drug, there was sufficient drug activity at the site (adequate dose selected) and that ibudilast was hitting the target as cells were responding differently. Even though the biomarker used in the study was not linked to the clinical endpoints (changes in the headache frequency and intensity), it provided

insights that the anti-inflammatory effects demonstrated by the ibudilast may not be relevant to MOH (due to the clinical endpoint being unchanged).

There are several likely reasons that the clinical endpoints were not captured by the biomarker used in the study. Firstly, a single biomarker is unlikely to capture the underlying complex mechanisms responsible for MOH (Atkinson et al., 2001). In week 8, placebo treated MOH patients were found to display enhanced IL-1β levels after TLR2 agonist stimulation that did not correlate with increased headache intensity or frequency. Hence, suggesting TLR pathway may not be involved in the modulation of headache in MOH patients. Secondly, the study population presented in this study is heterogeneous and due to the low number of participants there was large variability in the response obtained from the clinical endpoints, consequently any potential differences could not be deciphered. However, there was not even a trend towards benefit in the ibudilast group, indicating a clinically meaningful effect unlikely.

The reduction in pro-inflammatory cytokine from PBMCs could be due to the properties of ibudilast being a non-selective PDE inhibitor. PDE inhibitor can increase cytoplasmic cAMP that causes down-regulation of the nuclear factor NF-kappa B (NF-κB) and the up-regulation of cAMP responsive element binding protein. As a result, they can suppress the activation and translocation of NF-κB and lead to the depression of pro-inflammatory cytokine production (Mizuno et al., 2004). PDE inhibitors have found to suppress the activation and production of pro-inflammatory cytokines released by peritoneal macrophages and microglia (Yoshikawa et al., 1999) and LPS stimulated cultured microglial cells (Zhang et al., 2002). In addition, PDE inhibitors can suppress pro-inflammatory processes by a feedback mechanism involving the enhance production of anti-inflammatory IL-10 (Yoshikawa et al., 1999, Mizuno et al., 2004) and IL-4 cytokine production (Feng et al., 2004). The

effects of ibudilast is likely to have additional effects other than PDE actions, as a PDE3 inhibitor (propentofylline) was found to have poor efficacy in a preclinical model of neuropathic pain (Ledeboer et al., 2006). More research is required to elucidate the mechanism of action for ibudilast.

It is unclear from this study whether glial cells are involved in MOH and that opioid induced MOH is a form of OIH. Ibudilast has demonstrated good CNS partitioning (Ledeboer et al., 2006) but as it was not found to have any effect in the clinical endpoints suggests the mechanisms responsible for MOH may be signalling through another pathway or neurons may play a stronger role. So more research would be required to address whether glial activation is a likely contributor for MOH. Even though there have been reports of pro- and anti-inflammatory cytokines being a predictor of headaches, the examination of peripheral targets may not be adequate. In a chronic migraine study, elevation of TNF-α was found in the cerebrospinal fluid but not in the plasma (Rozen and Swidan, 2007) indicating migraines may not involve systemic inflammation. Consequently, the use of a peripherally derived biomarker may not be suitable assessment tools for migraine but this biomarker is suitable for assessment of ibudilast efficacy.

There are several limitations identified in this study. Firstly, the sample size for this sub- study is small due to practical constraints and as a result only a subset of patients was included in the biomarker assessment. In addition, some patients on ibudilast treatment were withdrawn from the study before week 8 which resulted in uneven number in the groups. However, this study serves as a pilot study to provide important information on the characterization of PBMC reactivity to TLR before and after 8 weeks of ibudilast treatment. Secondly, the biomarker of assessing TLR responsiveness in PBMCs may be of interest to investigate in other cytokines, preferably anti-inflammatory cytokine such as IL-10, to obtain a clearer understanding about the balances of cytokine effects.

In summary, this study suggests that ibudilast was not effective in the reduction of headache in patients with MOH, but demonstrated anti-inflammatory effects by significantly reducing the level of IL-1β in the isolated peripheral immune cells after TLR agonist stimulation. The assessment of TLR responsiveness in the peripheral cells showed that ibudilast had "on target activity" in the MOH patients. Moreover, the use of a biomarker, even though it is not well validated, can provide vital information that can assist in the understanding of whether a novel drug is having an effect in the patients.

References

Atkinson, J., Colburn, W. A., Degruttola, V. G., Demets, D. L., Downing, G. J. & Hoth, D. F. (2001). Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther*, 69, 89-95.

Bigal, M. E., Serrano, D., Buse, D., Scher, A., Stewart, W. F. & Lipton, R. B. (2008). Acute migraine medications and evolution from episodic to chronic migraine: a longitudinal population-based study. *Headache*, 48, 1157-68.

Bingel, U., Wanigasekera, V., Wiech, K., Ni Mhuircheartaigh, R., Lee, M. C., Ploner, M. & Tracey, I. (2011). The effect of treatment expectation on drug efficacy: imaging the analgesic benefit of the opioid remifentanil. *Sci Transl Med*, 3, 70ra14.

Diener, H. C. & Limmroth, V. (2004). Medication-overuse headache: a worldwide problem. *Lancet Neurol*, 3, 475-83.

Evers, S. & Marziniak, M. (2010). Clinical features, pathophysiology, and treatment of medication-overuse headache. *Lancet Neurol*, 9, 391-401.

Feng, J., Misu, T., Fujihara, K., Sakoda, S., Nakatsuji, Y., Fukaura, H., Kikuchi, S., Tashiro, K., Suzumura, A., Ishii, N., Sugamura, K., Nakashima, I. & Itoyama, Y. (2004). Ibudilast, a nonselective phosphodiesterase inhibitor, regulates Th1/Th2 balance and NKT cell subset in multiple sclerosis. *Mult Scler*, 10, 494-8.

Foley, K. M. (1985). The treatment of cancer pain. *N Engl J Med*, 313, 84-95.

Fujimoto, T., Sakoda, S., Fujimura, H. & Yanagihara, T. (1999). Ibudilast, a phosphodiesterase inhibitor, ameliorates experimental autoimmune encephalomyelitis in Dark August rats. *J Neuroimmunol*, 95, 35-42.

Fukuyama, H., Kimura, J., Yamaguchi, S., Yamauchi, H., Ogawa, M., Doi, T., Yonekura, Y. & Konishi, J. (1993). Pharmacological effects of ibudilast on cerebral circulation: a PET study. *Neurological research*, 15, 169-73.

Hutchinson, M. R., Lewis, S. S., Coats, B. D., Skyba, D. A., Crysdale, N. Y., Berkelhammer, D. L., Brzeski, A., Northcutt, A., Vietz, C. M., Judd, C. M., Maier, S. F., Watkins, L. R. & Johnson, K. W. (2009). Reduction of opioid withdrawal and potentiation of acute opioid analgesia by systemic AV411 (ibudilast). *Brain Behav Immun*, 23, 240-50.

Hutchinson, M. R., Zhang, Y., Shridhar, M., Evans, J. H., Buchanan, M. M., Zhao, T. X., Slivka, P. F., Coats, B. D., Rezvani, N., Wieseler, J., Hughes, T. S., Landgraf, K. E., Chan, S., Fong, S., Phipps, S., Falke, J. J., Leinwand, L. A., Maier, S. F., Yin, H., Rice, K. C. & Watkins, L. R. (2010). Evidence that opioids may have toll-like receptor 4 and MD-2 effects. *Brain Behav Immun*, 24, 83-95.

Johnson, J. L., Hutchinson, M. R., Williams, D. B. & Rolan, P. (2012). Medication-overuse headache and opioid-induced hyperalgesia: A review of mechanisms, a neuroimmune hypothesis and a novel approach to treatment. *Cephalalgia : an international journal of headache*, 33, 52-64.

Kawasaki, A., Hoshino, K., Osaki, R., Mizushima, Y. & Yano, S. (1992). Effect of ibudilast: a novel antiasthmatic agent, on airway hypersensitivity in bronchial asthma. *J Asthma*, 29, 245-52.

Kwok, Y. H., Hutchinson, M. R., Gentgall, M. G. & Rolan, P. E. (2012). Increased Responsiveness of Peripheral Blood Mononuclear Cells to In Vitro TLR 2, 4 and 7 Ligand Stimulation in Chronic Pain Patients. *PLoS One*, 7, e44232.

Ledeboer, A., Hutchinson, M. R., Watkins, L. R. & Johnson, K. W. (2007). Ibudilast (AV-411). A new class therapeutic candidate for neuropathic pain and opioid withdrawal syndromes. *Expert Opin Investig Drugs*, 16, 935-50.

Ledeboer, A., Liu, T., Shumilla, J. A., Mahoney, J. H., Vijay, S., Gross, M. I., Vargas, J. A., Sultzbaugh, L., Claypool, M. D., Sanftner, L. M., Watkins, L. R. & Johnson, K. W. (2006). The glial modulatory drug AV411 attenuates mechanical allodynia in rat models of neuropathic pain. *Neuron Glia Biol*, 2, 279-91.

Meng, I. D. & Cao, L. (2007). From migraine to chronic daily headache: the biological basis of headache transformation. *Headache*, 47, 1251-8.

Milligan, E. D. & Watkins, L. R. (2009). Pathological and protective roles of glia in chronic pain. *Nat Rev Neurosci*, 10, 23-36.

Mizuno, T., Kurotani, T., Komatsu, Y., Kawanokuchi, J., Kato, H., Mitsuma, N. & Suzumura, A. (2004). Neuroprotective role of phosphodiesterase inhibitor ibudilast on neuronal cell death induced by activated microglia. *Neuropharmacology*, 46, 404-11.

Mueller, L., Gupta, A. K. & Stein, T. P. (2001). Deficiency of tumor necrosis factor alpha in a subclass of menstrual migraineurs. *Headache*, 41, 129-37.

Munno, I., Marinaro, M., Bassi, A., Cassiano, M. A., Causarano, V. & Centonze, V. (2001). Immunological aspects in migraine: increase of IL-10 plasma levels during attack. *Headache*, 41, 764-7.

Perini, F., D'andrea, G., Galloni, E., Pignatelli, F., Billo, G., Alba, S., Bussone, G. & Toso, V. (2005). Plasma cytokine levels in migraineurs and controls. *Headache*, 45, 926-31.

Perrotta, A., Serrao, M., Sandrini, G., Burstein, R., Sances, G., Rossi, P., Bartolo, M., Pierelli, F. & Nappi, G. (2010). Sensitisation of spinal cord pain processing in medication overuse headache involves supraspinal pain control. *Cephalalgia*, 30, 272-84.

R Core Team 2012. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Raghavendra, V., Tanga, F. & Deleo, J. A. (2003). Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *J Pharmacol Exp Ther*, 306, 624-30.

Rolan, P. (1997). The contribution of clinical pharmacology surrogates and models to drug development--a critical appraisal. *Br J Clin Pharmacol*, 44, 219-25.

Rolan, P., Gibbons, J. A., He, L., Chang, E., Jones, D., Gross, M. I., Davidson, J. B., Sanftner, L. M. & Johnson, K. W. (2008). Ibudilast in healthy volunteers: safety, tolerability and pharmacokinetics with single and multiple doses. *Br J Clin Pharmacol*, 66, 792-801.

Rolan, P., Hutchinson, M. & Johnson, K. (2009). Ibudilast: a review of its pharmacology, efficacy and safety in respiratory and neurological disease. *Expert Opin Pharmacother*, 10, 2897-904.

Rozen, T. & Swidan, S. Z. (2007). Elevation of CSF tumor necrosis factor alpha levels in new daily persistent headache and treatment refractory chronic migraine. *Headache*, 47, 1050-5.

Shimomura, T., Kowa, H. & Takahashi, K. (1991). Analgesic-induced headaches: successful treatment with ibudilast. *Headache*, 31, 483.

Silberstein, S. D., Olesen, J., Bousser, M. G., Diener, H. C., Dodick, D., First, M., Goadsby, P. J., Gobel, H., Lainez, M. J., Lance, J. W., Lipton, R. B., Nappi, G., Sakai, F., Schoenen, J. & Steiner, T. J. (2005). The International Classification of Headache Disorders, 2nd Edition (ICHD-II)--revision of criteria for 8.2 Medication-overuse headache. *Cephalalgia*, 25, 460-5.

Song, P. & Zhao, Z. Q. (2001). The involvement of glial cells in the development of morphine tolerance. *Neurosci Res*, 39, 281-6.

Srikiatkhachorn, A. (2002). Chronic daily headache: a scientist's perspective. *Headache*, 42, 532-7.

Suzumura, A., Ito, A., Yoshikawa, M. & Sawada, M. (1999). Ibudilast suppresses TNFalpha production by glial cells functioning mainly as type III phosphodiesterase inhibitor in the CNS. *Brain Res*, 837, 203-12.

Sweitzer, S. M., Colburn, R. W., Rutkowski, M. & Deleo, J. A. (1999). Acute peripheral inflammation induces moderate glial activation and spinal IL-1beta expression that correlates with pain behavior in the rat. *Brain Res*, 829, 209-21.

Tompkins, D. A. & Campbell, C. M. (2011). Opioid-induced hyperalgesia: clinically relevant or extraneous research phenomenon? *Current pain and headache reports*, 15, 129-36.

Van Hilten, J. J., Ferrari, M. D., Van Der Meer, J. W., Gijsman, H. J. & Looij, B. J., Jr. (1991). Plasma interleukin-1, tumour necrosis factor and hypothalamic-pituitary-adrenal axis responses during migraine attacks. *Cephalalgia*, 11, 65-7.

Venables, W. N. & Ripley, B. D. 2002 *Modern Applied Statistics with S*, New York, Springer.

Yoshikawa, M., Suzumura, A., Tamaru, T., Takayanagi, T. & Sawada, M. (1999). Effects of phosphodiesterase inhibitors on cytokine production by microglia. *Mult Scler*, 5, 126-33.

Zhang, B., Yang, L., Konishi, Y., Maeda, N., Sakanaka, M. & Tanaka, J. (2002). Suppressive effects of phosphodiesterase type IV inhibitors on rat cultured microglial cells: comparison with other types of cAMP-elevating agents. *Neuropharmacology*, 42, 262-9.

Figures, Tables and Legends

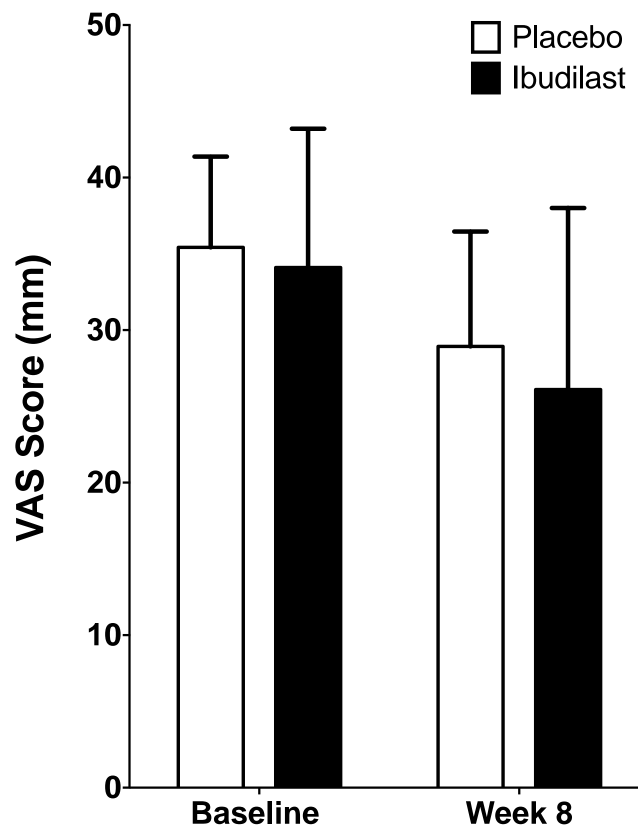


Figure 1. Ibudilast did not reduce headache intensity. MOH patients rated their headache experience on a visual analogue scale at baseline and 8 weeks after treatment (placebo: white bar and ibudilast: black bar). No significant differences were found between treatment (P = 0.9) and visit (P = 0.2). The values are presented as mean ± SEM. Scores on the headache pain VAS range from 0 (no pain) to 100 (worst pain possible).

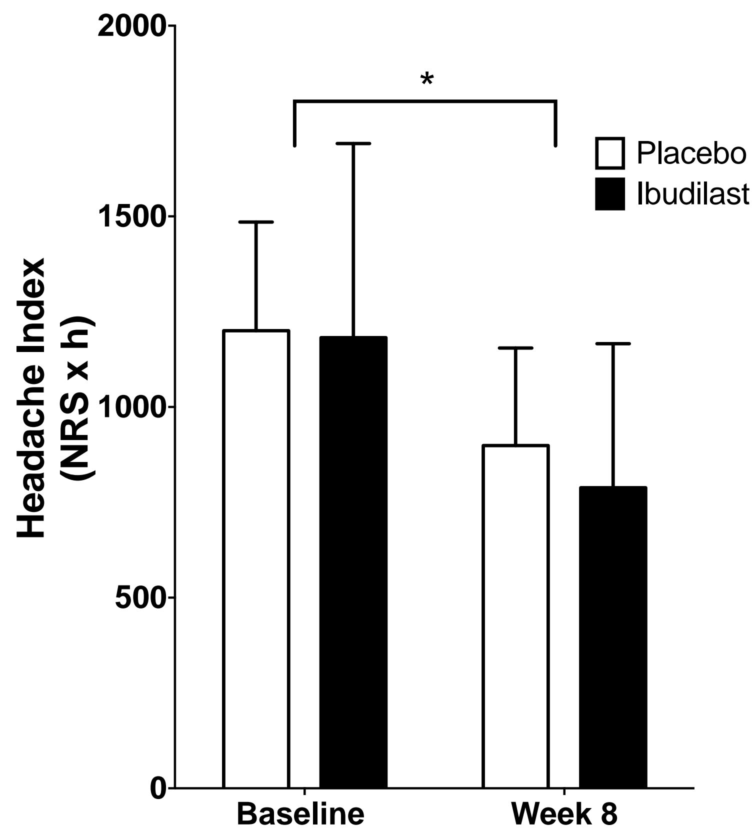


Figure 2. Ibudilast did not reduce the headache index. There were no differences between the treatments (P = 0.9) however there was a significant reduction in the headache indices at 8 weeks post treatment compared with the baseline visit (*, P = 0.01). Baseline score was taken from the average of 28 days prior to the baseline visit and the 8 weeks post treatment score was the average from day 56 to day 84 (placebo: white bar and ibudilast: black bar). The headache index was calculated by the summation of headache duration multiple by the headache intensity (11 point numerical rating scale; NRS). The values are presented as mean ± SEM.

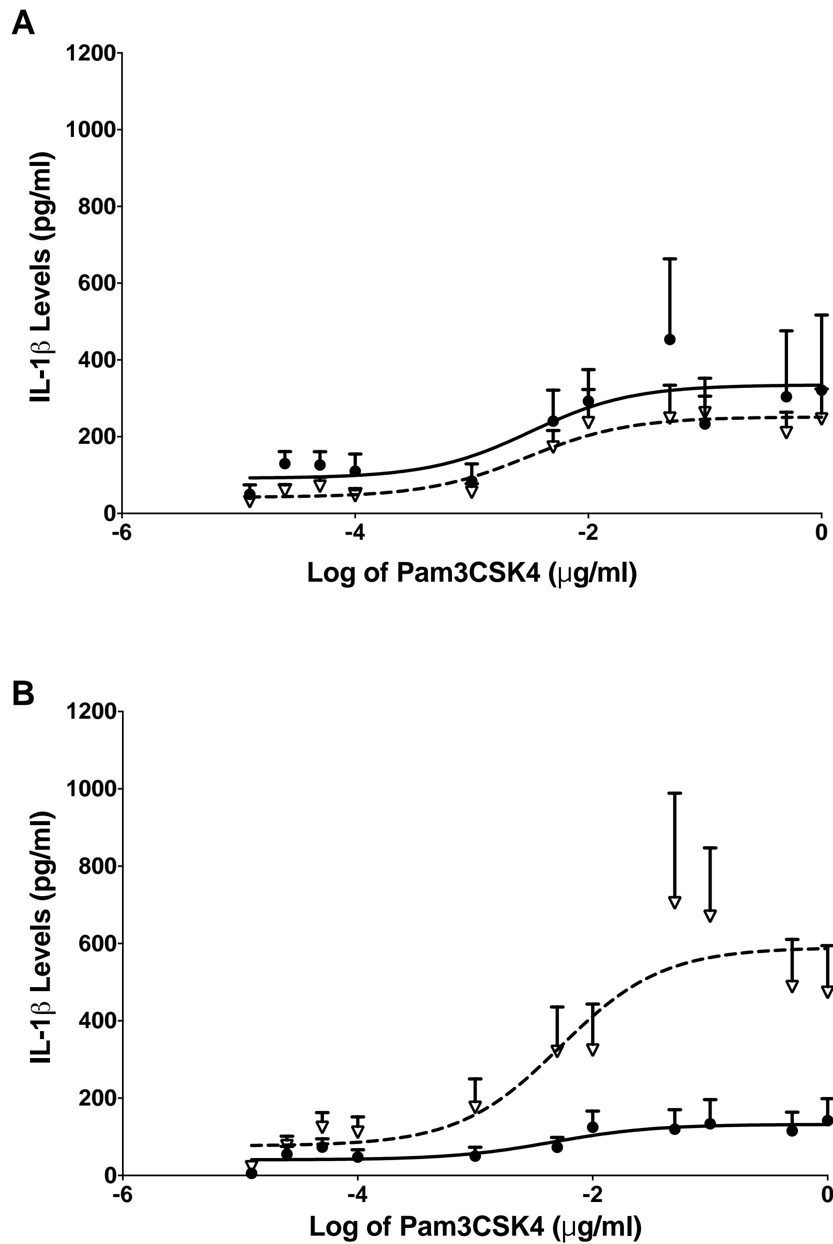


Figure 3. Ibudilast treatment caused a significant reduction in IL-1β level in the isolated PBMCs post TLR2 agonist stimulation in MOH patients. Isolated PBMCs obtained from MOH patients on ibudilast treatment (closed circle) and on placebo (open triangle) were stimulated with a range of Pam3CSK4 (TLR2) concentrations (13 $pg{\cdot}ml^{-1}$ to 1 $\mu g{\cdot}ml^{-1}$) to generate the response curves. At baseline (3A), there were no group differences ($P = 0.3$). After 8 weeks post treatment (3B), ibudilast caused a reduction in IL-1β that resulted in a significant treatment difference ($P < 0.0001$). Error bars on graphs represent standard error of the mean.

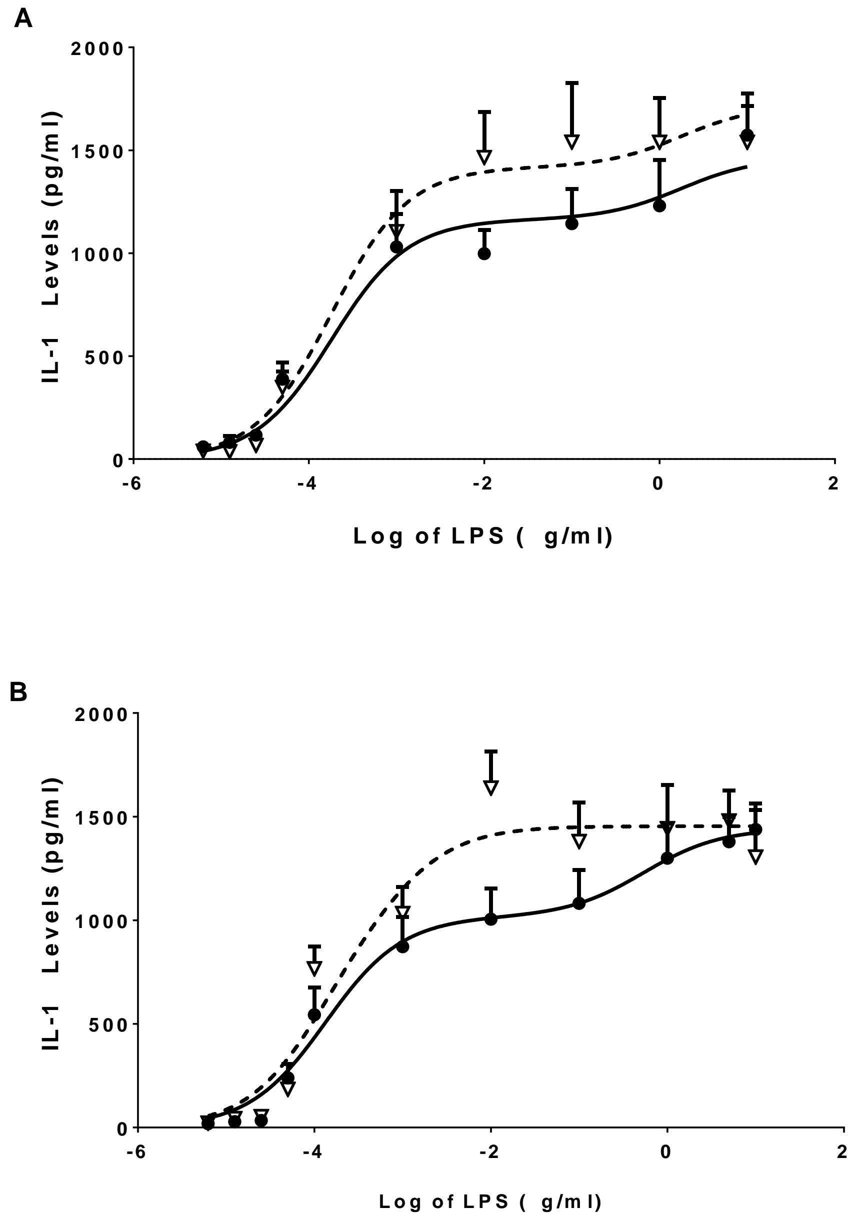


Figure 4. Ibudilast treatment caused a significant reduction in the IL-1β level in the isolated PBMCs post TLR4 agonist stimulation in MOH patients. Isolated PBMCs obtained from MOH patients on ibudilast treatment (closed circle) and on placebo (open triangle) were stimulated with a range of LPS (TLR4) concentrations (6 pg·ml^{-1} to 10 µg·ml^{-1}) to generate the response curves. At baseline (4A) both groups were not difference (P = 0.1) however after 8 weeks post treatment (4B), ibudilast caused a reduction in IL-1β that resulted in significant treatment difference (P = 0.01). Error bars on graphs represent standard error of the mean.

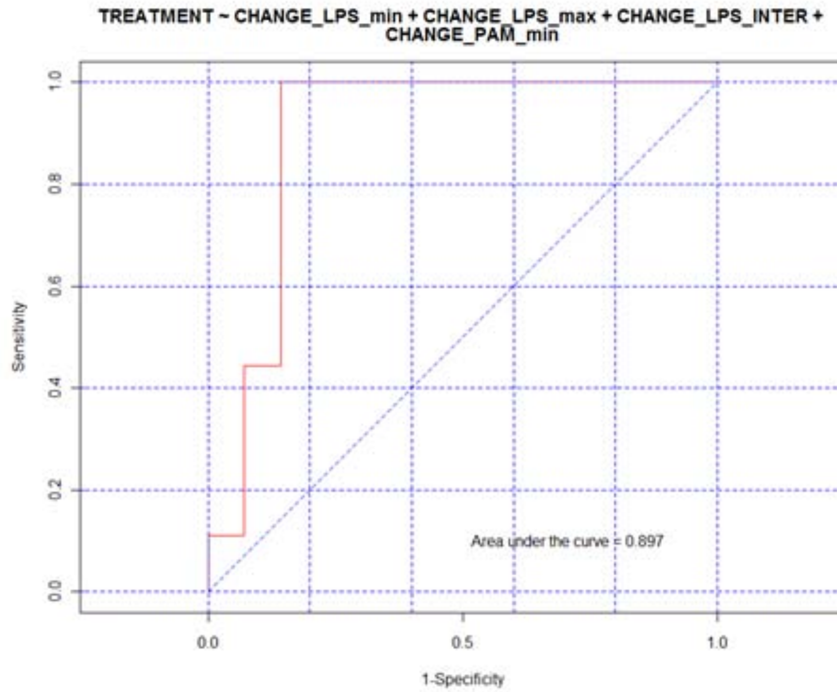


Figure 5. Representation of ROC curve for the prediction of ibudilast responders. Model was generated from peripheral collected output variables (from TLR2 and 4 agonists stimulated responses).

Table 1. Demographic summary. Data were collected from medical and family history. Data are expressed as mean ± S.E.M except age is expressed as mean (min – max). Mann Whitney test was used to determine the treatment differences (P-values shown).

	Placebo (n = 14)	Ibudilast (n = 9)	P - value
Gender (M/F)	3/11	2/7	-
Age (Years)	46 (35 – 62)	44 (23 – 59)	NS, 0.9
Age of headache onset (Years)	24 ± 4	19 ± 4	NS, 0.6
Duration of headache (Years)	20 ± 4	26 ± 5	NS, 0.4
Oral daily morphine equivalent dose (mg)	9 ± 2	17 ± 5	NS, 0.5
Primary headache diagnosis			
Migraine	86 %	56 %	
Tension type headache	7 %	22 %	
Migraine and tension type headache	7 %	22 %	

Table 2. Summary of isolated PBMC variables collected from codeine induced MOH patients. Data are presented as mean ± SEM. Peripheral blood mononuclear cells (PBMC) were isolated from 23 patients and were stimulated with LPS (6 pg·ml^{-1} to 10 µg·ml^{-1}) and with Pam3CSK4 (from 13 pg·ml^{-1} to 1 µg·ml^{-1}) for 20 h. All data are analysed with the unpaired t-test.

		Medication overuse headache patients			
			Placebo	Ibudilast	P - value
		Variables			
Morphine	4 weeks prior baseline (mg)	Daily	9.1 ± 1.9	17 ± 5.3	0.11
		Cumulative	254 ± 54	478 ± 149	0.11
		Standard deviation or change in morphine	5.4 ± 0.83	4.9 ± 1.7	0.7
	4 weeks post treatment (mg)	Daily	9.3 ± 2.2	16 ± 5.5	0.18
		Cumulative	261 ± 61	459 ± 154	0.18
		Standard deviation or change in morphine	6.2 ± 1.2	6.5 ± 2.3	0.91
Change in	Non-stimulated	Plasma (pg/mL)	0.52 ± 0.36	0.46 ± 0.31	0.91
		Cell count (^6 cells/mL)	0.99 ± 2	1.4 ± 1.6	0.89
		Cells IL-1β (pg/mL)	-5.5 ± 3.4	-3.5 ± 1.7	0.67
	TLR2 stimulated (Pam3CSK4) IL-1β (pg/mL)	Minimum	-0.0045 ± 14	-54 ± 23	*, 0.04
		Maximum	494 ± 183	-440 ± 247	**, 0.0055
		Slope	93 ± 39	-37 ± 35	*, 0.03
		Intercept	424 ± 169	-224± 154	*, 0.015
	TLR4 stimulated (LPS) IL-1β (pg/mL)	Minimum	-80 ± 51	-124 ± 81	0.63
		Maximum	-30 ± 246	-242 ± 184	0.54
		Slope	2.4 ± 55	69 ± 47	0.40
		Intercept	-108 ± 231	15 ± 155	0.70

Table 3. Best-fit logistic regression model results for the prediction of treatment (ibudilast or placebo) for medication overuse headache patients.

Outputs	Estimate	SE	P	Null deviance	Df	Residual deviance	Df	AUC
Change in TLR4 stimulated min	0.008	0.005	0.11	30.79	22	20.15	18	0.90
Change in TLR4 stimulated max	-0.0022	0.0014	0.10					
Change in TLR4 stimulated slope	0.013	0.0079	0.10					
Change in TLR2 stimulated min	-0.03	0.015	0.053					

Notes: The discrimination probabilities (D, area under ROC curve) are presented in the table. The residual deviance for the model includes predictor variables, whereas the null deviance for the model does not. SE, standard error.

Chapter 5. Conclusion

This project has documented a process in which a potential biomarker for chronic pain was discovered in the periphery. The first study initially found the level of IL-1β released from pain patients' isolated PBMCs post TLR2, TLR4 and TLR7 agonist stimulation was able to differentiate pain patients from pain-free participants. This study replicated a previous finding discovered in our laboratory that confirmed peripheral cells could be a source of a pain biomarker as the PBMC reactivity was found to significantly correlate with cold pain sensitivity scores (Hutchinson et al., 2004). Enhanced levels of pro-inflammatory cytokine were released by chronic pain patients' isolated PBMCs, which suggest the body had been previously primed (from previous exposure of similar TLR agonists) that caused a change in the PBMCs' reactivity. Therefore, when expose to the TLR agonists, chronic pain patient's PBMCs would cause an exaggerated response and this change in the PBMC's phenotype could be captured in the *in vitro* model that was used throughout this project. The technique of PBMCs isolation and stimulation with TLR agonists is not novel and has been used in other known immune mediated diseases to reflect innate immune dysregulation. However, this is the first study to indicate increased TLR responsiveness in pain patient without overt inflammation (Kwok et al., 2012).

There is extensive evidence that PBMCs, TLR and pro-inflammatory cytokines all play a vital role in experimental pain studies in animals but the translation to human pain is unclear. The quantification of isolated PBMCs after TLR stimulation has revealed a change in the chronic pain patient's innate immune response to shift towards pro-inflammatory state. The reactivity of the PBMCs was able to capture this distinction between the chronic pain patients and pain-free participants indicating the peripheral cells to be a potential biomarker. This study has important implications as research of pain biomarkers was previously hindered by an inability to access the brain and spinal cord region, which resulted in researchers turning to very few non-invasive methods

for accessing CNS function such as neuroimaging to identify pain biomarkers. Therefore, if peripheral markers are able to define chronic pain patients, this is another lead that should be investigated as it has the advantage of being simpler to access.

The second study explored the causal relationship between chronic pain and TLR responsiveness in a prospective graded neuropathic pain rat model. Unlike the clinical study, the discrimination of pain states and severity of allodynia were not possible on a single derived parameter in rat's PBMCs or from lumbar spinal cord from either TLR2 or TLR4 stimulation. Therefore, a different approach was used with the use of statistical models generated with all the collected outputs from the rats. The models created could predict the presence of pain and severity of allodynia with high accuracy. The outputs selected to best predict pain states were found to include both basal and TLR stimulated responses, which indicated the important contribution of evoked TLR responsiveness. It is noteworthy that the preclinical model of neuropathic pain selected in this study assisted with the contribution of TLR responsiveness with pain severity, as we did not examine pain levels in the clinical population. The use of the graded CCI model which generated graded nociceptive hypersensitivity in the rat in an objective manner (by varying the number of chromic gut sutures in the subcutaneous space and sciatic nerve) is believed to be a better model for the testing of pain biomarker research when compared with the traditional CCI model.

The study also found IL-1β expression from the rat's lumbar spinal cord was significantly correlated to peripheral immune cell responsivity. This indicates that the peripheral cells could give insight into how the CNS is responding; thus further supporting the use of peripheral cells in its ability to provide a window to the central regions in particular the lumbar spinal cord. To assessed the translational ability of the preclinical finding, a statistical model developed from the rats was applied to a cohort of chronic pain patients (consisted of sciatica and medication overuse headache patients) and displayed high predictive utility as the model could correctly classified a majority of patients with

pain. Collectively, results generated from study 2 confirm that the immune system, at present, is the most appropriate avenue for biomarker discovery.

The third study examined the utility of TLR responsiveness to assess a novel intervention, ibudilast, in a cohort of patients with medication overuse headache (MOH). MOH patients were randomised into either ibudilast or placebo treatment and blood was collected and stimulated with TLR2 and TLR4 agonists at baseline and 8 weeks post treatment. From the findings obtained in study 1, agonists selected to stimulate isolated PBMCs were refined so MSU crystals and TLR7 agonist were excluded, as they did not demonstrate utility as a pain biomarker.

After 8 weeks, ibudilast was found to have no effect on the frequency or intensity of headaches experienced by patients. However, intriguingly, ibudilast caused a significant reduction in the expression of IL-1β after TLR2 and TLR4 stimulation that was not observed in the placebo treated patients. Ibudilast was found to alter the phenotype of the peripheral cells so the ability to produce IL-1β via TLR pathway (particularly TLR2) was greatly impacted. A statistical model was derived from the peripherally collected outputs to identified biomarker responders of ibudilast. However even after the removal of biomarker non-responders, the frequency and intensity of headache remained the same between the two patient cohorts.

In week 8, the study also found the placebo treated MOH patients revealed a substantially increase expression of IL-1β after TLR2 agonist stimulation that was not correlated with more headaches. This suggests the TLR pathway may not be involved in the modulation of headaches, which supports the lack of efficacy found with the use of ibudilast to treat headache. Or the headaches experienced by MOH patients could be caused by central mechanisms, which at this stage could not be captured by the peripheral-based biomarker. Although, the biomarker used did not correlate with the unchanged headaches experienced by the patients, it was found to be useful in demonstrating ibudilast did exhibit an effect on the peripheral cell reactivity. The finding in the third

study also reflects the need of multiple biomarkers for different chronic pain conditions originating from different regions.

There are certain limitations associated with the investigation of TLR responsiveness as a biomarker for chronic pain in both clinical and preclinical studies. The main limitation for the two clinical studies is the limited number of participants that were recruited. As study 1 was the first clinical study to demonstrate increased responsiveness in a heterogeneous cohort of chronic pain patients, this study should be replicated in a larger patient cohort. On the other hand, study 3 was part of an ongoing study, so only participants who had completed the 8 weeks treatment intervention during the specified time period could be included. Despite the small sample sizes both studies provided important findings as: study 1 allowed the observation of the utility of TLR responsiveness as a biomarker for chronic pain, whereas study 3 used TLR responsiveness to demonstrate the effects of ibudilast. Another potential limitation identified is the lack of female rats used in study 2, as female patients dominate in chronic pain conditions. Hence, for future research, female rats should also be included to reflect the clinical populations.

We believe despite the promising findings identified in this project, TLR responsiveness cannot be used as a biomarker for pain in its current state and may only be useful for some chronic pain conditions. The findings presented in this thesis has to be rigorously tested and validated in a well-powered study before routine usage because of the important role biomarkers have in decision making. The current findings raise further questions that should be address in the future such as to unravel and understand the underlying mechanisms responsible for the increase of TLR responsiveness in chronic pain patients. The examination of the transcriptome of basal and post TLR2 stimulated human PBMCs would greatly assist in the understanding and identifying of genes and gene activities which undergo changes that are responsible for the enhanced IL-1β expression after TLR2 stimulation. Another should be investigated is the identification of the cell types within PBMCs that are responsible for:

- The enhanced release of IL-1β in study 1
- The cell types best correlated with central tissue IL-1β levels in study 2
- The cell types that are responsible for the reduction of IL-1β after ibudilast usage in study 3

As the identification and extraction of specific cell types can improve the specificity of future findings with TLR responsiveness, non-responder cells can be excluded so downstream signals may be even more enhanced.

In addition, the best way to move forward with all biomarker discoveries would be to develop a consensual, framework model which allows multi-centres to recruit well-powered and well-controlled studies (Wagner, 2009, Lesko and Atkinson, 2001). As researchers from different laboratories tend to use different techniques and procedures in the interpretation of data, hence the differences in the findings may be attributed to variation in experimental approaches and not from the underlying disease. Therefore, the development of standardized operating procedures (Woosley, 2012) for PBMC isolation and measurement of TLR responsiveness as well as the sharing of data will assist in the advancement of biomarker discoveries.

This project only investigated IL-1β expression and it was found in all three studies to provide vital information that contributed to the differentiation of chronic pain patients, generation of pain models and demonstrate the efficacy of ibudilast. Therefore, the investigation of other pro-inflammatory cytokines (such as IL-6 and TNF-α) would be advised after the above future studies are performed, as the introduction of other variables at this stage would complicate the findings in this thesis that were not originally tested.

This project was very exciting yet daunting as it takes the ambitious step towards identifying and testing several unknown potential pain biomarkers. It started with several potential leads that were associated with the immune system as a target for the determination of chronic pain states. Throughout the search for potential pain biomarkers several potential candidates were eliminated

(see Appendix B). However, the TLR responsiveness from PBMCs was found to demonstrate face validity as a pain biomarker. Not only was the importance of TLR responsiveness in a preclinical model confirmed, the findings were translated back to a clinical pain population and demonstrated good translational value. The utility of TLR responsiveness was also found to give insight into the effects of ibudilast.

I believe the results of the studies presented in this thesis will contribute to the discovery of a cellular pain biomarker, which can be expanded and further investigated. Even though the process of pain biomarker identification remains to be a challenge as pain itself is complex and multi-dimensional, I believe the cellular pain biomarker identified in this project, along with existing behaviour tests, clinical history, neuroimaging and genetic makeup, will assist in capturing the complexity of pain in a more accurate manner. The integrative approach can work synergistically to provide a multidimensional view for the identification of pain and the mechanisms that underlies it so that in the near future, clinicians can utilise cellular pain biomarkers as one of the tools to improve the lives of chronic pain patients around the world.

Chapter 6. References

Akira, S. & Sato, S. (2003). Toll-like receptors and their signaling mechanisms. *Scand J Infect Dis*, 35, 555-62.

Akira, S., Uematsu, S. & Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell*, 124, 783-801.

Alexander, R. B., Ponniah, S., Hasday, J. & Hebel, J. R. (1998). Elevated levels of proinflammatory cytokines in the semen of patients with chronic prostatitis/chronic pelvic pain syndrome. *Urology*, 52, 744-9.

Allen, N. J. & Barres, B. A. (2009). Neuroscience: Glia - more than just brain glue. *Nature*, 457, 675-7.

Ang, D. C., Moore, M. N., Hilligoss, J. & Tabbey, R. (2011). MCP-1 and IL-8 as Pain Biomarkers in Fibromyalgia: A Pilot Study. *Pain Med*, 12, 1154-61.

Arrowsmith, J. (2011). Trial watch: phase III and submission failures: 2007-2010. *Nat Rev Drug Discov*, 10, 87.

Atkinson, J., Colburn, W. A., Degruttola, V. G., Demets, D. L., Downing, G. J. & Hoth, D. F. (2001). Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther*, 69, 89-95.

Avanzas, P., Arroyo-Espliguero, R. & Kaski, J. C. (2009). Neopterin and cardiovascular disease: growing evidence for a role in patient risk stratification. *Clin Chem*, 55, 1056-7.

Avanzas, P., Arroyo-Espliguero, R., Quiles, J., Roy, D. & Kaski, J. C. (2005). Elevated serum neopterin predicts future adverse cardiac events in patients with chronic stable angina pectoris. *Eur Heart J*, 26, 457-63.

Backonja, M. M., Coe, C. L., Muller, D. A. & Schell, K. (2008). Altered cytokine levels in the blood and cerebrospinal fluid of chronic pain patients. *J Neuroimmunol*, 195, 157-63.

Basbaum, A. I., Bautista, D. M., Scherrer, G. & Julius, D. (2009). Cellular and molecular mechanisms of pain. *Cell*, 139, 267-84.

Beggs, S. & Salter, M. W. (2010). Microglia-neuronal signalling in neuropathic pain hypersensitivity 2.0. *Current opinion in neurobiology*, 20, 474-80.

Bennett, G. J. & Xie, Y. K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain*, 33, 87-107.

Bigal, M. E., Serrano, D., Buse, D., Scher, A., Stewart, W. F. & Lipton, R. B. (2008). Acute migraine medications and evolution from episodic to chronic migraine: a longitudinal population-based study. *Headache*, 48, 1157-68.

Bingel, U., Wanigasekera, V., Wiech, K., Ni Mhuircheartaigh, R., Lee, M. C., Ploner, M. & Tracey, I. (2011). The effect of treatment expectation on drug efficacy: imaging the analgesic benefit of the opioid remifentanil. *Sci Transl Med*, 3, 70ra14.

Blyth, F. M., March, L. M., Brnabic, A. J., Jorm, L. R., Williamson, M. & Cousins, M. J. (2001). Chronic pain in Australia: a prevalence study. *Pain*, 89, 127-34.

Borsook, D., Becerra, L. & Hargreaves, R. (2011). Biomarkers for chronic pain and analgesia. Part 2: how, where, and what to look for using functional imaging. *Discov Med*, 11, 209-19.

Bowie, A. & O'neill, L. A. (2000). The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *Journal of leukocyte biology*, 67, 508-14.

Breivik, H., Borchgrevink, P. C., Allen, S. M., Rosseland, L. A., Romundstad, L., Hals, E. K., Kvarstein, G. & Stubhaug, A. (2008). Assessment of pain. *Br J Anaesth*, 101, 17-24.

Bridges, D., Thompson, S. W. & Rice, A. S. (2001). Mechanisms of neuropathic pain. *Br J Anaesth*, 87, 12-26.

Brisby, H., Olmarker, K., Larsson, K., Nutu, M. & Rydevik, B. (2002). Proinflammatory cytokines in cerebrospinal fluid and serum in patients with disc herniation and sciatica. *Eur Spine J*, 11, 62-6.

Brown, M. C., Perry, V. H., Lunn, E. R., Gordon, S. & Heumann, R. (1991). Macrophage dependence of peripheral sensory nerve regeneration: possible involvement of nerve growth factor. *Neuron*, 6, 359-70.

Bsibsi, M., Ravid, R., Gveric, D. & Van Noort, J. M. (2002). Broad expression of Toll-like receptors in the human central nervous system. *Journal of neuropathology and experimental neurology*, 61, 1013-21.

Calvo, M. & Bennett, D. L. (2012). The mechanisms of microgliosis and pain following peripheral nerve injury. *Exp Neurol*, 234, 271-82.

Campbell, J. N. & Meyer, R. A. (2006). Mechanisms of neuropathic pain. *Neuron*, 52, 77-92.

Cao, H. & Zhang, Y. Q. (2008). Spinal glial activation contributes to pathological pain states. *Neuroscience and biobehavioral reviews*, 32, 972-83.

Cao, L. & Deleo, J. A. (2008). CNS-infiltrating CD4+ T lymphocytes contribute to murine spinal nerve transection-induced neuropathic pain. *Eur J Immunol*, 38, 448-58.

Carlsson, A. M. (1983). Assessment of chronic pain. I. Aspects of the reliability and validity of the visual analogue scale. *Pain*, 16, 87-101.

Carru, C., Zinellu, A., Sotgia, S., Serra, R., Usai, M. F., Pintus, G. F., Pes, G. M. & Deiana, L. (2004). A new HPLC method for serum neopterin measurement and relationships with plasma thiols levels in healthy subjects. *Biomed Chromatogr*, 18, 360-6.

Cathcart, S., Winefield, A. H., Lushington, K. & Rolan, P. (2009). Effect of mental stress on cold pain in chronic tension-type headache sufferers. *J Headache Pain*, 10, 367-73.

Celik, C., Erdem, M., Cayci, T., Ozdemir, B., Ozgur Akgul, E., Kurt, Y. G., Yaman, H., Isintas, M., Ozgen, F. & Ozsahin, A. (2010). The association between serum levels of neopterin and number of depressive episodes of major depression. *Prog Neuropsychopharmacol Biol Psychiatry*, 34, 372-5.

Chao, C. C., Janoff, E. N., Hu, S. X., Thomas, K., Gallagher, M., Tsang, M. & Peterson, P. K. (1991). Altered cytokine release in peripheral blood mononuclear cell cultures from patients with the chronic fatigue syndrome. *Cytokine*, 3, 292-8.

Chapman, C. R., Casey, K. L., Dubner, R., Foley, K. M., Gracely, R. H. & Reading, A. E. (1985). Pain measurement: an overview. *Pain*, 22, 1-31.

Chen, C. H. & Tang, S. T. (2012). Meta-analysis of cultural differences in Western and Asian patient-perceived barriers to managing cancer pain. *Palliative medicine*, 26, 206-21.

Chizh, B. A., Greenspan, J. D., Casey, K. L., Nemenov, M. I. & Treede, R. D. (2008). Identifying biological markers of activity in human nociceptive pathways to facilitate analgesic drug development. *Pain*, 140, 249-53.

Coghill, R. C., Sang, C. N., Maisog, J. M. & Iadarola, M. J. (1999). Pain intensity processing within the human brain: a bilateral, distributed mechanism. *J Neurophysiol*, 82, 1934-43.

Costigan, M., Befort, K., Karchewski, L., Griffin, R. S., D'urso, D., Allchorne, A., Sitarski, J., Mannion, J. W., Pratt, R. E. & Woolf, C. J. (2002). Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. *BMC Neurosci*, 3, 16.

Costigan, M., Moss, A., Latremoliere, A., Johnston, C., Verma-Gandhu, M., Herbert, T. A., Barrett, L., Brenner, G. J., Vardeh, D., Woolf, C. J. & Fitzgerald, M. (2009a). T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity. *J Neurosci*, 29, 14415-22.

Costigan, M., Scholz, J. & Woolf, C. J. (2009b). Neuropathic Pain: A Maladaptive Response of the Nervous System to Damage. *Annu Rev Neurosci*, 32, 1-32.

Cruccu, G. & Truini, A. (2009). Tools for assessing neuropathic pain. *PLoS Med*, 6, e1000045.

Czapinski, P., Blaszczyk, B. & Czuczwar, S. J. (2005). Mechanisms of action of antiepileptic drugs. *Curr Top Med Chem*, 5, 3-14.

Dale, R. C., Brilot, F., Fagan, E. & Earl, J. (2009). Cerebrospinal fluid neopterin in paediatric neurology: a marker of active central nervous system inflammation. *Dev Med Child Neurol*, 51, 317-23.

Davis, J. M., 3rd, Knutson, K. L., Strausbauch, M. A., Crowson, C. S., Therneau, T. M., Wettstein, P. J., Matteson, E. L. & Gabriel, S. E. (2010). Analysis of complex biomarkers for human immune-mediated disorders based on cytokine responsiveness of peripheral blood cells. *J Immunol*, 184, 7297-304.

Davis, K. D., Racine, E. & Collett, B. (2012). Neuroethical issues related to the use of brain imaging: can we and should we use brain imaging as a biomarker to diagnose chronic pain? *Pain*, 153, 1555-9.

De Leo, J. A., Tawfik, V. L. & Lacroix-Fralish, M. L. (2006). The tetrapartite synapse: path to CNS sensitization and chronic pain. *Pain*, 122, 17-21.

Del Rey, A., Yau, H. J., Randolf, A., Centeno, M. V., Wildmann, J., Martina, M., Besedovsky, H. O. & Apkarian, A. V. (2011). Chronic neuropathic pain-like behavior correlates with IL-1beta expression and disrupts cytokine interactions in the hippocampus. *Pain*, 152, 2827-35.

Deleo, J. A., Colburn, R. W. & Rickman, A. J. (1997). Cytokine and growth factor immunohistochemical spinal profiles in two animal models of mononeuropathy. *Brain Res*, 759, 50-7.

Desai, M., Stockbridge, N. & Temple, R. (2006). Blood pressure as an example of a biomarker that functions as a surrogate. *AAPS J*, 8, E146-52.

Diener, H. C. & Limmroth, V. (2004). Medication-overuse headache: a worldwide problem. *Lancet Neurol*, 3, 475-83.

Dinarello, C. A. (1996). Biologic basis for interleukin-1 in disease. *Blood*, 87, 2095-147.

Dinarello, C. A. (2000). Proinflammatory cytokines. *Chest*, 118, 503-8.

Doverty, M., White, J. M., Somogyi, A. A., Bochner, F., Ali, R. & Ling, W. (2001). Hyperalgesic responses in methadone maintenance patients. *Pain*, 90, 91-6.

Dray, A. (2008). Neuropathic pain: emerging treatments. *Br J Anaesth*, 101, 48-58.

Dunne, D. W., Shaw, A., Bockenstedt, L. K., Allore, H. G., Chen, S., Malawista, S. E., Leng, L., Mizue, Y., Piecychna, M., Zhang, L., Towle, V., Bucala, R., Montgomery, R. R. & Fikrig, E. (2010). Increased TLR4 expression and downstream cytokine production in immunosuppressed adults compared to non-immunosuppressed adults. *PLoS One*, 5, e11343.

Ek, M., Engblom, D., Saha, S., Blomqvist, A., Jakobsson, P. J. & Ericsson-Dahlstrand, A. (2001). Inflammatory response: pathway across the blood-brain barrier. *Nature*, 410, 430-1.

Eriksen, J., Sjogren, P., Bruera, E., Ekholm, O. & Rasmussen, N. K. (2006). Critical issues on opioids in chronic non-cancer pain: an epidemiological study. *Pain*, 125, 172-9.

Erridge, C. (2010). Endogenous ligands of TLR2 and TLR4: agonists or assistants? *J Leukoc Biol*, 87, 989-99.

Ertel, W., Kremer, J. P., Kenney, J., Steckholzer, U., Jarrar, D., Trentz, O. & Schildberg, F. W. (1995). Downregulation of proinflammatory cytokine release in whole blood from septic patients. *Blood*, 85, 1341-7.

Evers, S. & Marziniak, M. (2010). Clinical features, pathophysiology, and treatment of medication-overuse headache. *Lancet Neurol*, 9, 391-401.

Farmer, M. A., Baliki, M. N. & Apkarian, A. V. (2012). A dynamic network perspective of chronic pain. *Neurosci Lett*, 520, 197-203.

Feng, J., Misu, T., Fujihara, K., Sakoda, S., Nakatsuji, Y., Fukaura, H., Kikuchi, S., Tashiro, K., Suzumura, A., Ishii, N., Sugamura, K., Nakashima, I. & Itoyama, Y. (2004). Ibudilast, a nonselective phosphodiesterase inhibitor, regulates Th1/Th2 balance and NKT cell subset in multiple sclerosis. *Mult Scler*, 10, 494-8.

Fenwick, C. (2006). Assessing pain across the cultural gap: Central Australian Indigenous peoples' pain assessment. *Contemp Nurse*, 22, 218-27.

Fenwick, C. & Stevens, J. (2004). Post operative pain experiences of central Australian aboriginal women. What do we understand? *The Australian journal of rural health*, 12, 22-7.

Ferreira, S. H., Lorenzetti, B. B., Bristow, A. F. & Poole, S. (1988). Interleukin-1 beta as a potent hyperalgesic agent antagonized by a tripeptide analogue. *Nature*, 334, 698-700.

Finnerup, N. B., Sindrup, S. H. & Jensen, T. S. (2010). The evidence for pharmacological treatment of neuropathic pain. *Pain*, 150, 573-81.

Fleming, T. R. & Demets, D. L. (1996). Surrogate end points in clinical trials: are we being misled? *Ann Intern Med*, 125, 605-13.

Foley, K. M. (1985). The treatment of cancer pain. *N Engl J Med*, 313, 84-95.

Fujimoto, T., Sakoda, S., Fujimura, H. & Yanagihara, T. (1999). Ibudilast, a phosphodiesterase inhibitor, ameliorates experimental autoimmune encephalomyelitis in Dark August rats. *J Neuroimmunol*, 95, 35-42.

Fukuoka, H., Kawatani, M., Hisamitsu, T. & Takeshige, C. (1994). Cutaneous hyperalgesia induced by peripheral injection of interleukin-1 beta in the rat. *Brain Res*, 657, 133-40.

Fukuyama, H., Kimura, J., Yamaguchi, S., Yamauchi, H., Ogawa, M., Doi, T., Yonekura, Y. & Konishi, J. (1993). Pharmacological effects of ibudilast on cerebral circulation: a PET study. *Neurological research*, 15, 169-73.

Gaboury, J. P., Johnston, B., Niu, X. F. & Kubes, P. (1995). Mechanisms underlying acute mast cell-induced leukocyte rolling and adhesion in vivo. *J Immunol*, 154, 804-13.

Galli, S. J., Nakae, S. & Tsai, M. (2005). Mast cells in the development of adaptive immune responses. *Nat Immunol*, 6, 135-42.

Ganguly, D., Chamilos, G., Lande, R., Gregorio, J., Meller, S., Facchinetti, V., Homey, B., Barrat, F. J., Zal, T. & Gilliet, M. (2009). Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. *J Exp Med*, 206, 1983-94.

Gao, Y. J., Zhang, L., Samad, O. A., Suter, M. R., Yasuhiko, K., Xu, Z. Z., Park, J. Y., Lind, A. L., Ma, Q. & Ji, R. R. (2009). JNK-induced MCP-1 production in spinal cord astrocytes contributes to central sensitization and neuropathic pain. *J Neurosci*, 29, 4096-108.

Garcia, J. J., Cidoncha, A., Bote, M. E., Hinchado, M. D. & Ortega, E. (2013). Altered profile of chemokines in fibromyalgia patients. *Annals of clinical biochemistry*.

Garcia-Gonzalez, M. J., Dominguez-Rodriguez, A. & Abreu-Gonzalez, P. (2006). Diurnal variations in serum neopterin levels are associated with the pineal hormone melatonin circadian rhythm in healthy human subjects. *J Pineal Res*, 40, 288-9.

Garrison, C. J., Dougherty, P. M. & Carlton, S. M. (1994). GFAP expression in lumbar spinal cord of naive and neuropathic rats treated with MK-801. *Exp Neurol*, 129, 237-43.

Garrison, C. J., Dougherty, P. M., Kajander, K. C. & Carlton, S. M. (1991). Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. *Brain Res*, 565, 1-7.

Gaskin, D. J. & Richard, P. (2012). The economic costs of pain in the United States. *The journal of pain : official journal of the American Pain Society*, 13, 715-24.

Gillen, C., Jander, S. & Stoll, G. (1998). Sequential expression of mRNA for proinflammatory cytokines and interleukin-10 in the rat peripheral nervous system: comparison between immune-mediated demyelination and Wallerian degeneration. *J Neurosci Res*, 51, 489-96.

Glajchen, M. (2001). Chronic pain: treatment barriers and strategies for clinical practice. *J Am Board Fam Pract*, 14, 211-8.

Gobburu, J. V. (2009). Biomarkers in clinical drug development. *Clin Pharmacol Ther*, 86, 26-7.

Gomez-Nicola, D., Valle-Argos, B., Suardiaz, M., Taylor, J. S. & Nieto-Sampedro, M. (2008). Role of IL-15 in spinal cord and sciatic nerve after chronic constriction injury: regulation of macrophage and T-cell infiltration. *J Neurochem*, 107, 1741-52.

Grace, P. M., Hutchinson, M. R., Bishop, A., Somogyi, A. A., Mayrhofer, G. & Rolan, P. E. Adoptive transfer of peripheral immune cells potentiates allodynia in a graded chronic constriction injury model of neuropathic pain. *Brain Behav Immun*, 25, 503-13.

Grace, P. M., Hutchinson, M. R., Maier, S. F. & Watkins, L. R. (2014). Pathological pain and the neuroimmune interface. *Nat Rev Immunol*, 14, 217-31.

Grace, P. M., Hutchinson, M. R., Manavis, J., Somogyi, A. A. & Rolan, P. E. (2010). A novel animal model of graded neuropathic pain: Utility to investigate mechanisms of population heterogeneity. *J Neurosci Methods*, 193, 47-53.

Grace, P. M., Rolan, P. E. & Hutchinson, M. R. (2011). Peripheral immune contributions to the maintenance of central glial activation underlying neuropathic pain. *Brain Behav Immun*, 25, 1322-32.

Hains, L. E., Loram, L. C., Weiseler, J. L., Frank, M. G., Bloss, E. B., Sholar, P., Taylor, F. R., Harrison, J. A., Martin, T. J., Eisenach, J. C., Maier, S. F. & Watkins, L. R. (2010). Pain intensity and duration can be enhanced by prior challenge: initial evidence suggestive of a role of microglial priming. *The journal of pain : official journal of the American Pain Society*, 11, 1004-14.

Hall, S. K., Perregaux, D. G., Gabel, C. A., Woodworth, T., Durham, L. K., Huizinga, T. W., Breedveld, F. C. & Seymour, A. B. (2004). Correlation of polymorphic variation in the promoter region of the interleukin-1 beta gene with secretion of interleukin-1 beta protein. *Arthritis and rheumatism*, 50, 1976-83.

Hanisch, U. K. (2002). Microglia as a source and target of cytokines. *Glia*, 40, 140-55.

Henderson, J. V., Harrison, C. M., Britt, H. C., Bayram, C. F. & Miller, G. C. (2013). Prevalence, causes, severity, impact, and management of chronic pain in Australian general practice patients. *Pain Med*, 14, 1346-61.

Herr, K., Coyne, P. J., Mccaffery, M., Manworren, R. & Merkel, S. (2011). Pain assessment in the patient unable to self-report: position statement with clinical practice recommendations. *Pain management nursing : official journal of the American Society of Pain Management Nurses*, 12, 230-50.

Heumann, R., Lindholm, D., Bandtlow, C., Meyer, M., Radeke, M. J., Misko, T. P., Shooter, E. & Thoenen, H. (1987). Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: role of macrophages. *Proc Natl Acad Sci U S A*, 84, 8735-9.

Hoffmann, G., Wirleitner, B. & Fuchs, D. (2003). Potential role of immune system activation-associated production of neopterin derivatives in humans. *Inflamm Res*, 52, 313-21.

Hohlfeld, R., Kerschensteiner, M., Stadelmann, C., Lassmann, H. & Wekerle, H. (2000). The neuroprotective effect of inflammation: implications for the therapy of multiple sclerosis. *J Neuroimmunol*, 107, 161-6.

Honore, P., Wade, C. L., Zhong, C., Harris, R. R., Wu, C., Ghayur, T., Iwakura, Y., Decker, M. W., Faltynek, C., Sullivan, J. & Jarvis, M. F. (2006). Interleukin-1alphabeta gene-deficient mice show reduced nociceptive sensitivity in models of inflammatory and neuropathic pain but not post-operative pain. *Behav Brain Res*, 167, 355-64.

Hu, P. & Mclachlan, E. M. (2002). Macrophage and lymphocyte invasion of dorsal root ganglia after peripheral nerve lesions in the rat. *Neuroscience*, 112, 23-38.

Huber, C., Batchelor, J. R., Fuchs, D., Hausen, A., Lang, A., Niederwieser, D., Reibnegger, G., Swetly, P., Troppmair, J. & Wachter, H. (1984). Immune response-associated production of neopterin. Release from macrophages primarily under control of interferon-gamma. *J Exp Med*, 160, 310-6.

Hunt, S. P. & Mantyh, P. W. (2001). The molecular dynamics of pain control. *Nat Rev Neurosci*, 2, 83-91.

Hutchinson, M. R., Buijs, M., Tuke, J., Kwok, Y. H., Gentgall, M., Williams, D. & Rolan, P. (2013). Low-dose endotoxin potentiates capsaicin-induced pain in man: evidence for a pain neuroimmune connection. *Brain Behav Immun*, 30, 3-11.

Hutchinson, M. R., Coats, B. D., Lewis, S. S., Zhang, Y., Sprunger, D. B., Rezvani, N., Baker, E. M., Jekich, B. M., Wieseler, J. L., Somogyi, A. A., Martin, D., Poole, S., Judd, C. M., Maier, S. F. & Watkins, L. R. (2008a). Proinflammatory cytokines oppose opioid-induced acute and chronic analgesia. *Brain Behav Immun*, 22, 1178-89.

Hutchinson, M. R., La Vincente, S. F. & Somogyi, A. A. (2004). In vitro opioid induced proliferation of peripheral blood immune cells correlates with in vivo cold pressor pain tolerance in humans: a biological marker of pain tolerance. *Pain*, 110, 751-5.

Hutchinson, M. R., Lewis, S. S., Coats, B. D., Skyba, D. A., Crysdale, N. Y., Berkelhammer, D. L., Brzeski, A., Northcutt, A., Vietz, C. M., Judd, C. M., Maier, S. F., Watkins, L. R. & Johnson, K. W. (2009). Reduction of opioid withdrawal and potentiation of acute opioid analgesia by systemic AV411 (ibudilast). *Brain Behav Immun*, 23, 240-50.

Hutchinson, M. R., Northcutt, A. L., Chao, L. W., Kearney, J. J., Zhang, Y., Berkelhammer, D. L., Loram, L. C., Rozeske, R. R., Bland, S. T., Maier, S. F., Gleeson, T. T. & Watkins, L. R. (2008b). Minocycline suppresses morphine-induced respiratory depression, suppresses morphine-induced reward, and enhances systemic morphine-induced analgesia. *Brain Behav Immun*, 22, 1248-56.

Hutchinson, M. R., Zhang, Y., Brown, K., Coats, B. D., Shridhar, M., Sholar, P. W., Patel, S. J., Crysdale, N. Y., Harrison, J. A., Maier, S. F., Rice, K. C. & Watkins, L. R. (2008c). Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). *Eur J Neurosci*, 28, 20-9.

Hutchinson, M. R., Zhang, Y., Shridhar, M., Evans, J. H., Buchanan, M. M., Zhao, T. X., Slivka, P. F., Coats, B. D., Rezvani, N., Wieseler, J., Hughes, T. S., Landgraf, K. E., Chan, S., Fong, S., Phipps, S., Falke, J. J., Leinwand, L. A., Maier, S. F., Yin, H., Rice, K. C. & Watkins, L. R. (2010). Evidence that opioids may have toll-like receptor 4 and MD-2 effects. *Brain Behav Immun*, 24, 83-95.

Jensen, T. S., Baron, R., Haanpaa, M., Kalso, E., Loeser, J. D., Rice, A. S. & Treede, R. D. (2011). A new definition of neuropathic pain. *Pain*, 152, 2204-5.

Ji, R. R. (2012). Recent progress in understanding the mechanisms of pain and itch. *Neurosci Bull*, 28, 89-90.

Ji, R. R., Kohno, T., Moore, K. A. & Woolf, C. J. (2003). Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci*, 26, 696-705.

Jin, S. X., Zhuang, Z. Y., Woolf, C. J. & Ji, R. R. (2003). p38 mitogen-activated protein kinase is activated after a spinal nerve ligation in spinal cord microglia and dorsal root ganglion neurons and contributes to the generation of neuropathic pain. *J Neurosci*, 23, 4017-22.

Johnson, J. L., Hutchinson, M. R., Williams, D. B. & Rolan, P. (2012). Medication-overuse headache and opioid-induced hyperalgesia: A review of mechanisms, a neuroimmune hypothesis and a novel approach to treatment. *Cephalalgia : an international journal of headache*, 33, 52-64.

Johnston, I. N., Milligan, E. D., Wieseler-Frank, J., Frank, M. G., Zapata, V., Campisi, J., Langer, S., Martin, D., Green, P., Fleshner, M., Leinwand, L., Maier, S. F. & Watkins, L. R. (2004). A role for proinflammatory cytokines and fractalkine in analgesia, tolerance, and subsequent pain facilitation induced by chronic intrathecal morphine. *J Neurosci*, 24, 7353-65.

Jones, S. F., Mcquay, H. J., Moore, R. A. & Hand, C. W. (1988). Morphine and ibuprofen compared using the cold pressor test. *Pain*, 34, 117-22.

Kashiba, H., Fukui, H., Morikawa, Y. & Senba, E. (1999). Gene expression of histamine H1 receptor in guinea pig primary sensory neurons: a relationship between H1 receptor mRNA-expressing neurons and peptidergic neurons. *Brain Res Mol Brain Res*, 66, 24-34.

Katoh, S., Sueoka, T., Matsuura, S. & Sugimoto, T. (1989). Biopterin and neopterin in human saliva. *Life Sci*, 45, 2561-8.

Kawasaki, A., Hoshino, K., Osaki, R., Mizushima, Y. & Yano, S. (1992). Effect of ibudilast: a novel antiasthmatic agent, on airway hypersensitivity in bronchial asthma. *J Asthma*, 29, 245-52.

Kawasaki, Y., Xu, Z. Z., Wang, X., Park, J. Y., Zhuang, Z. Y., Tan, P. H., Gao, Y. J., Roy, K., Corfas, G., Lo, E. H. & Ji, R. R. (2008). Distinct roles of matrix metalloproteases in the early- and late-phase development of neuropathic pain. *Nat Med*, 14, 331-6.

Kim, D., Kim, M. A., Cho, I. H., Kim, M. S., Lee, S., Jo, E. K., Choi, S. Y., Park, K., Kim, J. S., Akira, S., Na, H. S., Oh, S. B. & Lee, S. J. (2007). A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. *J Biol Chem*, 282, 14975-83.

Kim, D., You, B., Lim, H. & Lee, S. J. (2011). Toll-like receptor 2 contributes to chemokine gene expression and macrophage infiltration in the dorsal root ganglia after peripheral nerve injury. *Mol Pain*, 7, 74.

Koch, A., Zacharowski, K., Boehm, O., Stevens, M., Lipfert, P., Von Giesen, H. J., Wolf, A. & Freynhagen, R. (2007). Nitric oxide and pro-inflammatory cytokines correlate with pain intensity in chronic pain patients *Inflamm Res*, 56, 32-7.

Kohama, I., Ishikawa, K. & Kocsis, J. D. (2000). Synaptic reorganization in the substantia gelatinosa after peripheral nerve neuroma formation: aberrant innervation of lamina II neurons by Abeta afferents. *J Neurosci*, 20, 1538-49.

Kowalski, M. L., Wolska, A., Grzegorczyk, J., Hilt, J., Jarzebska, M., Drobniewski, M., Synder, M. & Kurowski, M. (2008). Increased responsiveness to toll-like receptor 4 stimulation in peripheral blood mononuclear cells from patients with recent onset rheumatoid arthritis. *Mediators Inflamm*, 2008, 132732.

Kremer, E., Atkinson, J. H. & Ignelzi, R. J. (1981). Measurement of pain: patient preference does not confound pain measurement. *Pain*, 10, 241-8.

Krishnan, S., Salter, A., Sullivan, T., Gentgall, M., White, J. & Rolan, P. (2012). Comparison of pain models to detect opioid-induced hyperalgesia. *J Pain Res*, 5, 99-106.

Kwok, Y. H., Hutchinson, M. R., Gentgall, M. G. & Rolan, P. E. (2012). Increased Responsiveness of Peripheral Blood Mononuclear Cells to In Vitro TLR 2, 4 and 7 Ligand Stimulation in Chronic Pain Patients. *PLoS One*, 7, e44232.

La Cesa, S., Tinelli, E., Toschi, N., Di Stefano, G., Collorone, S., Aceti, A., Francia, A., Cruccu, G., Truini, A. & Caramia, F. (2014). fMRI pain activation in the periaqueductal gray in healthy volunteers during the cold pressor test. *Magn Reson Imaging*, 32, 236-40.

Laich, A., Neurauter, G., Wirleitner, B. & Fuchs, D. (2002). Degradation of serum neopterin during daylight exposure. *Clin Chim Acta*, 322, 175-8.

Latremoliere, A. & Woolf, C. J. (2009). Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain*, 10, 895-926.

Lavallie, E. R., Dorner, A. J. & Burczynski, M. E. (2008). Use of ex vivo systems for biomarker discovery. *Current Opinion in Pharmacology*, 8, 647-653.

Ledeboer, A., Hutchinson, M. R., Watkins, L. R. & Johnson, K. W. (2007). Ibudilast (AV-411). A new class therapeutic candidate for neuropathic pain and opioid withdrawal syndromes. *Expert Opin Investig Drugs*, 16, 935-50.

Ledeboer, A., Liu, T., Shumilla, J. A., Mahoney, J. H., Vijay, S., Gross, M. I., Vargas, J. A., Sultzbaugh, L., Claypool, M. D., Sanftner, L. M., Watkins, L. R. & Johnson, K. W. (2006). The glial modulatory drug AV411 attenuates mechanical allodynia in rat models of neuropathic pain. *Neuron Glia Biol*, 2, 279-91.

Lee, J. W., Devanarayan, V., Barrett, Y. C., Weiner, R., Allinson, J., Fountain, S., Keller, S., Weinryb, I., Green, M., Duan, L., Rogers, J. A., Millham, R., O'brien, P. J., Sailstad, J., Khan, M., Ray, C. & Wagner, J. A. (2006). Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm Res*, 23, 312-28.

Lee, S. & Zhang, J. (2012). Heterogeneity of macrophages in injured trigeminal nerves: cytokine/chemokine expressing vs. phagocytic macrophages. *Brain Behav Immun*, 26, 891-903.

Lehnardt, S., Lehmann, S., Kaul, D., Tschimmel, K., Hoffmann, O., Cho, S., Krueger, C., Nitsch, R., Meisel, A. & Weber, J. R. (2007). Toll-like receptor 2 mediates CNS injury in focal cerebral ischemia. *J Neuroimmunol*, 190, 28-33.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. & Hoffmann, J. A. (1996). The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell*, 86, 973-83.

Lesko, L. J. & Atkinson, A. J., Jr. (2001). Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation, strategies. *Annu Rev Pharmacol Toxicol*, 41, 347-66.

Levine, J. D., Coderre, T. J., White, D. M., Finkbeiner, W. E. & Basbaum, A. I. (1990). Denervation-induced inflammation in the rat. *Neurosci Lett*, 119, 37-40.

Lewis, S. S., Loram, L. C., Hutchinson, M. R., Li, C. M., Zhang, Y., Maier, S. F., Huang, Y., Rice, K. C. & Watkins, L. R. (2012). (+)-naloxone, an opioid-inactive toll-like receptor 4 signaling inhibitor, reverses multiple models of chronic neuropathic pain in rats. *The journal of pain : official journal of the American Pain Society*, 13, 498-506.

Liebregts, T., Adam, B., Bredack, C., Roth, A., Heinzel, S., Lester, S., Downie-Doyle, S., Smith, E., Drew, P., Talley, N. J. & Holtmann, G. (2007). Immune activation in patients with irritable bowel syndrome. *Gastroenterology*, 132, 913-20.

Lim, H., Kim, D. & Lee, S. J. (2013). Toll-like receptor 2 mediates peripheral nerve injury-induced NADPH oxidase 2 expression in spinal cord microglia. *J Biol Chem*, 288, 7572-9.

Liu, B., Du, L., Kong, L. Y., Hudson, P. M., Wilson, B. C., Chang, R. C., Abel, H. H. & Hong, J. S. (2000a). Reduction by naloxone of lipopolysaccharide-induced neurotoxicity in mouse cortical neuron-glia co-cultures. *Neuroscience*, 97, 749-56.

Liu, T., Gao, Y. J. & Ji, R. R. (2012). Emerging role of Toll-like receptors in the control of pain and itch. *Neurosci Bull*, 28, 131-44.

Liu, T. & Ji, R. R. (2013). New insights into the mechanisms of itch: are pain and itch controlled by distinct mechanisms? *Pflugers Arch*, 465, 1671-85.

Liu, T., Van Rooijen, N. & Tracey, D. J. (2000b). Depletion of macrophages reduces axonal degeneration and hyperalgesia following nerve injury. *Pain*, 86, 25-32.

Liu, T., Xu, Z. Z., Park, C. K., Berta, T. & Ji, R. R. (2010). Toll-like receptor 7 mediates pruritus. *Nat Neurosci*, 13, 1460-2.

Liu, Y. M., Zhu, S. M., Wang, K. R., Feng, Z. Y. & Chen, Q. L. (2008). Effect of tramadol on immune responses and nociceptive thresholds in a rat model of incisional pain. *J Zhejiang Univ Sci B*, 9, 895-902.

London, C. A., Abbas, A. K. & Kelso, A. (1998). Helper T cell subsets: heterogeneity, functions and development. *Vet Immunol Immunopathol*, 63, 37-44.

Madan, V., Lear, J. T. & Szeimies, R. M. (2010). Non-melanoma skin cancer. *Lancet*, 375, 673-85.

Maisel, A. S., Krishnaswamy, P., Nowak, R. M., Mccord, J., Hollander, J. E., Duc, P., Omland, T., Storrow, A. B., Abraham, W. T., Wu, A. H., Clopton, P., Steg, P. G., Westheim, A., Knudsen, C. W., Perez, A., Kazanegra, R., Herrmann, H. C. & Mccullough, P. A. (2002). Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure. *The New England journal of medicine*, 347, 161-7.

Malaviya, R. & Abraham, S. N. (2000). Role of mast cell leukotrienes in neutrophil recruitment and bacterial clearance in infectious peritonitis. *J Leukoc Biol*, 67, 841-6.

Marchand, F., Perretti, M. & Mcmahon, S. B. (2005). Role of the immune system in chronic pain. *Nat Rev Neurosci*, 6, 521-32.

Martinon, F. & Tschopp, J. (2007). Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ*, 14, 10-22.

Mayeux, R. (2004). Biomarkers: potential uses and limitations. *NeuroRx*, 1, 182-8.

Mckernan, D. P., Gaszner, G., Quigley, E. M., Cryan, J. F. & Dinan, T. G. (2011). Altered peripheral toll-like receptor responses in the irritable bowel syndrome. *Aliment Pharmacol Ther*, 33, 1045-52.

Mclachlan, E. M., Janig, W., Devor, M. & Michaelis, M. (1993). Peripheral nerve injury triggers noradrenergic sprouting within dorsal root ganglia. *Nature*, 363, 543-6.

Meng, I. D. & Cao, L. (2007). From migraine to chronic daily headache: the biological basis of headache transformation. *Headache*, 47, 1251-8.

Milligan, E. D., Twining, C., Chacur, M., Biedenkapp, J., O'connor, K., Poole, S., Tracey, K., Martin, D., Maier, S. F. & Watkins, L. R. (2003). Spinal glia and proinflammatory cytokines mediate mirror-image neuropathic pain in rats. *J Neurosci*, 23, 1026-40.

Milligan, E. D. & Watkins, L. R. (2009). Pathological and protective roles of glia in chronic pain. *Nat Rev Neurosci*, 10, 23-36.

Milligan, E. D., Zapata, V., Chacur, M., Schoeniger, D., Biedenkapp, J., O'connor, K. A., Verge, G. M., Chapman, G., Green, P., Foster, A. C., Naeve, G. S., Maier, S. F. & Watkins, L. R. (2004). Evidence that exogenous and endogenous fractalkine can induce spinal nociceptive facilitation in rats. *Eur J Neurosci*, 20, 2294-302.

Mitchell, L. A., Macdonald, R. A. & Brodie, E. E. (2004). Temperature and the cold pressor test. *J Pain*, 5, 233-7.

Miyake, K. (2007). Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin Immunol*, 19, 3-10.

Mizuno, T., Kurotani, T., Komatsu, Y., Kawanokuchi, J., Kato, H., Mitsuma, N. & Suzumura, A. (2004). Neuroprotective role of phosphodiesterase inhibitor ibudilast on neuronal cell death induced by activated microglia. *Neuropharmacology*, 46, 404-11.

Moalem, G. & Tracey, D. J. (2006). Immune and inflammatory mechanisms in neuropathic pain. *Brain Res Rev*, 51, 240-64.

Moalem, G., Xu, K. & Yu, L. (2004). T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats. *Neuroscience*, 129, 767-77.

Morin, N., Owolabi, S. A., Harty, M. W., Papa, E. F., Tracy, T. F., Jr., Shaw, S. K., Kim, M. & Saab, C. Y. (2007). Neutrophils invade lumbar dorsal root ganglia after chronic constriction injury of the sciatic nerve. *J Neuroimmunol*, 184, 164-71.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*, 136, 2348-57.

Mueller, L., Gupta, A. K. & Stein, T. P. (2001). Deficiency of tumor necrosis factor alpha in a subclass of menstrual migraineurs. *Headache*, 41, 129-37.

Munno, I., Marinaro, M., Bassi, A., Cassiano, M. A., Causarano, V. & Centonze, V. (2001). Immunological aspects in migraine: increase of IL-10 plasma levels during attack. *Headache*, 41, 764-7.

Murr, C., Widner, B., Wirleitner, B. & Fuchs, D. (2002). Neopterin as a marker for immune system activation. *Curr Drug Metab*, 3, 175-87.

Myers, R. R., Heckman, H. M. & Rodriguez, M. (1996). Reduced hyperalgesia in nerve-injured WLD mice: relationship to nerve fiber phagocytosis, axonal degeneration, and regeneration in normal mice. *Exp Neurol*, 141, 94-101.

Novakovic, S. D., Tzoumaka, E., Mcgivern, J. G., Haraguchi, M., Sangameswaran, L., Gogas, K. R., Eglen, R. M. & Hunter, J. C. (1998). Distribution of the tetrodotoxin-resistant sodium channel PN3 in rat sensory neurons in normal and neuropathic conditions. *J Neurosci*, 18, 2174-87.

O'neill, L. A., Golenbock, D. & Bowie, A. G. (2013). The history of Toll-like receptors - redefining innate immunity. *Nat Rev Immunol*, 13, 453-60.

Okamoto, K., Martin, D. P., Schmelzer, J. D., Mitsui, Y. & Low, P. A. (2001). Pro- and anti-inflammatory cytokine gene expression in rat sciatic nerve chronic constriction injury model of neuropathic pain. *Exp Neurol*, 169, 386-91.

Pereira, H. A., Shafer, W. M., Pohl, J., Martin, L. E. & Spitznagel, J. K. (1990). CAP37, a human neutrophil-derived chemotactic factor with monocyte specific activity. *J Clin Invest*, 85, 1468-76.

Perini, F., D'andrea, G., Galloni, E., Pignatelli, F., Billo, G., Alba, S., Bussone, G. & Toso, V. (2005). Plasma cytokine levels in migraineurs and controls. *Headache*, 45, 926-31.

Perkins, N. M. & Tracey, D. J. (2000). Hyperalgesia due to nerve injury: role of neutrophils. *Neuroscience*, 101, 745-57.

Perrotta, A., Serrao, M., Sandrini, G., Burstein, R., Sances, G., Rossi, P., Bartolo, M., Pierelli, F. & Nappi, G. (2010). Sensitisation of spinal cord pain processing in medication overuse headache involves supraspinal pain control. *Cephalalgia*, 30, 272-84.

Pickering, G., Pereira, B., Dufour, E., Soule, S. & Dubray, C. (2014). Impaired modulation of pain in patients with postherpetic neuralgia. *Pain Res Manag*, 19, e19-23.

Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. & Beutler, B. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*, 282, 2085-8.

Qi, J., Buzas, K., Fan, H., Cohen, J. I., Wang, K., Mont, E., Klinman, D., Oppenheim, J. J. & Howard, O. M. (2011). Painful pathways induced by TLR stimulation of dorsal root ganglion neurons. *J Immunol*, 186, 6417-26.

R Core Team 2012. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Raghavendra, V., Tanga, F. & Deleo, J. A. (2003). Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *J Pharmacol Exp Ther*, 306, 624-30.

Raghavendra, V., Tanga, F. Y. & Deleo, J. A. (2004). Complete Freunds adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. *Eur J Neurosci*, 20, 467-73.

Rasmussen, P. V., Sindrup, S. H., Jensen, T. S. & Bach, F. W. (2004). Symptoms and signs in patients with suspected neuropathic pain. *Pain*, 110, 461-9.

Ren, K. & Dubner, R. (2010). Interactions between the immune and nervous systems in pain. *Nat Med*, 16, 1267-76.

Ren, K. & Torres, R. (2009). Role of interleukin-1beta during pain and inflammation. *Brain Res Rev*, 60, 57-64.

Rittner, H. L., Hackel, D., Yamdeu, R. S., Mousa, S. A., Stein, C., Schafer, M. & Brack, A. (2009). Antinociception by neutrophil-derived opioid peptides in noninflamed tissue--role of hypertonicity and the perineurium. *Brain Behav Immun*, 23, 548-57.

Roelofs, P. D., Deyo, R. A., Koes, B. W., Scholten, R. J. & Van Tulder, M. W. (2008). Nonsteroidal anti-inflammatory drugs for low back pain: an updated Cochrane review. *Spine*, 33, 1766-74.

Rolan, P. (1997). The contribution of clinical pharmacology surrogates and models to drug development--a critical appraisal. *Br J Clin Pharmacol*, 44, 219-25.

Rolan, P., Gibbons, J. A., He, L., Chang, E., Jones, D., Gross, M. I., Davidson, J. B., Sanftner, L. M. & Johnson, K. W. (2008). Ibudilast in healthy volunteers: safety, tolerability and pharmacokinetics with single and multiple doses. *Br J Clin Pharmacol*, 66, 792-801.

Rolan, P., Hutchinson, M. & Johnson, K. (2009). Ibudilast: a review of its pharmacology, efficacy and safety in respiratory and neurological disease. *Expert Opin Pharmacother*, 10, 2897-904.

Rosenblum, A., Marsch, L. A., Joseph, H. & Portenoy, R. K. (2008). Opioids and the treatment of chronic pain: controversies, current status, and future directions. *Experimental and clinical psychopharmacology*, 16, 405-16.

Rotshenker, S., Aamar, S. & Barak, V. (1992). Interleukin-1 activity in lesioned peripheral nerve. *J Neuroimmunol*, 39, 75-80.

Rozen, T. & Swidan, S. Z. (2007). Elevation of CSF tumor necrosis factor alpha levels in new daily persistent headache and treatment refractory chronic migraine. *Headache*, 47, 1050-5.

Rutkowski, M. D., Pahl, J. L., Sweitzer, S., Van Rooijen, N. & Deleo, J. A. (2000). Limited role of macrophages in generation of nerve injury-induced mechanical allodynia. *Physiology & behavior*, 71, 225-35.

Samad, T. A., Moore, K. A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J. V. & Woolf, C. J. (2001). Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature*, 410, 471-5.

Sauerland, S., Hensler, T., Bouillon, B., Rixen, D., Raum, M. R., Andermahr, J. & Neugebauer, E. A. (2003). Plasma levels of procalcitonin and neopterin in multiple trauma patients with or without brain injury. *J Neurotrauma*, 20, 953-60.

Schafers, M., Brinkhoff, J., Neukirchen, S., Marziniak, M. & Sommer, C. (2001). Combined epineurial therapy with neutralizing antibodies to tumor necrosis factor-alpha and interleukin-1 receptor has an additive effect in reducing neuropathic pain in mice. *Neurosci Lett*, 310, 113-6.

Scholz, J., Mannion, R. J., Hord, D. E., Griffin, R. S., Rawal, B., Zheng, H., Scoffings, D., Phillips, A., Guo, J., Laing, R. J., Abdi, S., Decosterd, I. & Woolf, C. J. (2009). A novel tool for the assessment of pain: validation in low back pain. *PLoS Med*, 6, e1000047.

Scholz, J. & Woolf, C. J. (2002). Can we conquer pain? *Nat Neurosci*, 5 Suppl, 1062-7.

Scholz, J. & Woolf, C. J. (2007). The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci*, 10, 1361-8.

Schumacher, M., Halwachs, G., Tatzber, F., Fruhwald, F. M., Zweiker, R., Watzinger, N., Eber, B., Wilders-Truschnig, M., Esterbauer, H. & Klein, W. (1997). Increased neopterin in patients with chronic and acute coronary syndromes. *J Am Coll Cardiol*, 30, 703-7.

Scuderi, G. J., Cuellar, J. M., Cuellar, V. G., Yeomans, D. C., Carragee, E. J. & Angst, M. S. (2009). Epidural interferon gamma-immunoreactivity: a biomarker for lumbar nerve root irritation. *Spine (Phila Pa 1976)*, 34, 2311-7.

Shamash, S., Reichert, F. & Rotshenker, S. (2002). The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. *J Neurosci*, 22, 3052-60.

Shi, X. Q., Zekki, H. & Zhang, J. (2011). The role of TLR2 in nerve injury-induced neuropathic pain is essentially mediated through macrophages in peripheral inflammatory response. *Glia*, 59, 231-41.

Shimomura, T., Kowa, H. & Takahashi, K. (1991). Analgesic-induced headaches: successful treatment with ibudilast. *Headache*, 31, 483.

Silberstein, S. D., Olesen, J., Bousser, M. G., Diener, H. C., Dodick, D., First, M., Goadsby, P. J., Gobel, H., Lainez, M. J., Lance, J. W., Lipton, R. B., Nappi, G., Sakai, F., Schoenen, J. & Steiner, T. J. (2005). The International Classification of Headache Disorders, 2nd Edition (ICHD-II)--revision of criteria for 8.2 Medication-overuse headache. *Cephalalgia*, 25, 460-5.

Sim, J. & Arnell, P. (1993). Measurement validity in physical therapy research. *Phys Ther*, 73, 102-10; discussion 110-5.

Sindrup, S. H., Finnerup, N. B. & Jensen, T. S. (2012). Tailored treatment of peripheral neuropathic pain. *Pain*, 153, 1781-2.

Sindrup, S. H. & Jensen, T. S. (1999). Efficacy of pharmacological treatments of neuropathic pain: an update and effect related to mechanism of drug action. *Pain*, 83, 389-400.

Sindrup, S. H., Otto, M., Finnerup, N. B. & Jensen, T. S. (2005). Antidepressants in the treatment of neuropathic pain. *Basic Clin Pharmacol Toxicol*, 96, 399-409.

Sivilotti, L. & Woolf, C. J. (1994). The contribution of GABAA and glycine receptors to central sensitization: disinhibition and touch-evoked allodynia in the spinal cord. *J Neurophysiol*, 72, 169-79.

Soderpalm, B. (2002). Anticonvulsants: aspects of their mechanisms of action. *Eur J Pain*, 6 Suppl A, 3-9.

Sommer, C. (2004). Serotonin in pain and analgesia: actions in the periphery. *Mol Neurobiol*, 30, 117-25.

Sommer, C. & Kress, M. (2004). Recent findings on how proinflammatory cytokines cause pain: peripheral mechanisms in inflammatory and neuropathic hyperalgesia. *Neurosci Lett*, 361, 184-7.

Sommer, C., Petrausch, S., Lindenlaub, T. & Toyka, K. V. (1999). Neutralizing antibodies to interleukin 1-receptor reduce pain associated behavior in mice with experimental neuropathy. *Neurosci Lett*, 270, 25-8.

Song, P. & Zhao, Z. Q. (2001). The involvement of glial cells in the development of morphine tolerance. *Neurosci Res*, 39, 281-6.

Srikiatkhachorn, A. (2002). Chronic daily headache: a scientist's perspective. *Headache*, 42, 532-7.

Stein, C. (2013). Opioids, sensory systems and chronic pain. *Eur J Pharmacol*, 716, 179-87.

Stening, K., Eriksson, O., Wahren, L., Berg, G., Hammar, M. & Blomqvist, A. (2007). Pain sensations to the cold pressor test in normally menstruating women: comparison with men and relation to menstrual phase and serum sex steroid levels. *Am J Physiol Regul Integr Comp Physiol*, 293, R1711-6.

Sung, C. S., Wen, Z. H., Chang, W. K., Ho, S. T., Tsai, S. K., Chang, Y. C. & Wong, C. S. (2004). Intrathecal interleukin-1beta administration induces thermal hyperalgesia by activating inducible nitric oxide synthase expression in the rat spinal cord. *Brain Res*, 1015, 145-53.

Suzumura, A., Ito, A., Yoshikawa, M. & Sawada, M. (1999). Ibudilast suppresses TNFalpha production by glial cells functioning mainly as type III phosphodiesterase inhibitor in the CNS. *Brain Res*, 837, 203-12.

Svoboda, P., Ko, S. H., Cho, B., Yoo, S. H., Choi, S. W., Ye, S. K., Kasai, H. & Chung, M. H. (2008). Neopterin, a marker of immune response, and 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress, correlate at high age as determined by automated simultaneous high-performance liquid chromatography analysis of human urine. *Anal Biochem*, 383, 236-42.

Swann, A. C. (2001). Major system toxicities and side effects of anticonvulsants. *J Clin Psychiatry*, 62 Suppl 14, 16-21.

Sweitzer, S., Martin, D. & Deleo, J. A. (2001). Intrathecal interleukin-1 receptor antagonist in combination with soluble tumor necrosis factor receptor exhibits an anti-allodynic action in a rat model of neuropathic pain. *Neuroscience*, 103, 529-39.

Sweitzer, S. M., Colburn, R. W., Rutkowski, M. & Deleo, J. A. (1999). Acute peripheral inflammation induces moderate glial activation and spinal IL-1beta expression that correlates with pain behavior in the rat. *Brain Res*, 829, 209-21.

Sweitzer, S. M., Hickey, W. F., Rutkowski, M. D., Pahl, J. L. & Deleo, J. A. (2002). Focal peripheral nerve injury induces leukocyte trafficking into the central nervous system: potential relationship to neuropathic pain. *Pain*, 100, 163-70.

Takeda, K. & Akira, S. (2004). TLR signaling pathways. *Semin Immunol*, 16, 3-9.

Takeuchi, O. & Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell*, 140, 805-20.

Tanga, F. Y., Nutile-Mcmenemy, N. & Deleo, J. A. (2005). The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A*, 102, 5856-61.

Tanga, F. Y., Raghavendra, V. & Deleo, J. A. (2004). Quantitative real-time RT-PCR assessment of spinal microglial and astrocytic activation markers in a rat model of neuropathic pain. *Neurochem Int*, 45, 397-407.

Tegeder, I., Costigan, M., Griffin, R. S., Abele, A., Belfer, I., Schmidt, H., Ehnert, C., Nejim, J., Marian, C., Scholz, J., Wu, T., Allchorne, A., Diatchenko, L., Binshtok, A. M., Goldman, D., Adolph, J., Sama, S., Atlas, S. J., Carlezon, W. A., Parsegian, A., Lotsch, J., Fillingim, R. B., Maixner, W., Geisslinger, G., Max, M. B. & Woolf, C. J. (2006). GTP cyclohydrolase and tetrahydrobiopterin regulate pain sensitivity and persistence. *Nat Med*, 12, 1269-77.

Tegeder, I., Niederberger, E., Schmidt, R., Kunz, S., Guhring, H., Ritzeler, O., Michaelis, M. & Geisslinger, G. (2004). Specific Inhibition of IkappaB kinase reduces hyperalgesia in inflammatory and neuropathic pain models in rats. *J Neurosci*, 24, 1637-45.

Temple, R. (2008). Complexities in drug trials: enrichment, biomarkers and surrogates. Interview with Robert Temple. *Biomarkers in medicine*, 2, 109-12.

Tompkins, D. A. & Campbell, C. M. (2011). Opioid-induced hyperalgesia: clinically relevant or extraneous research phenomenon? *Current pain and headache reports*, 15, 129-36.

Tracey, I. (2011). Can neuroimaging studies identify pain endophenotypes in humans? *Nature reviews. Neurology*, 7, 173-81.

Tsuda, M., Masuda, T., Kitano, J., Shimoyama, H., Tozaki-Saitoh, H. & Inoue, K. (2009). IFN-gamma receptor signaling mediates spinal microglia activation driving neuropathic pain. *Proc Natl Acad Sci U S A*, 106, 8032-7.

Tsuda, M., Shigemoto-Mogami, Y., Koizumi, S., Mizokoshi, A., Kohsaka, S., Salter, M. W. & Inoue, K. (2003). P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature*, 424, 778-83.

Tsujimoto, H., Ono, S., Majima, T., Efron, P. A., Kinoshita, M., Hiraide, H., Moldawer, L. L. & Mochizuki, H. (2006). Differential toll-like receptor expression after ex vivo lipopolysaccharide exposure in patients with sepsis and following surgical stress. *Clin Immunol*, 119, 180-7.

Turgan, N., Habif, S., Parildar, Z., Ozmen, D., Mutaf, I., Erdener, D. & Bayindir, O. (2001). Association between homocysteine and neopterin in healthy subjects measured by a simple HPLC-fluorometric method. *Clin Biochem*, 34, 271-5.

Turk, D. C. & Rudy, T. E. (1987). Towards a comprehensive assessment of chronic pain patients. *Behav Res Ther*, 25, 237-49.

Turk, D. C., Wilson, H. D. & Cahana, A. (2011). Treatment of chronic non-cancer pain. *Lancet*, 377, 2226-35.

Uceyler, N., Rogausch, J. P., Toyka, K. V. & Sommer, C. (2007a). Differential expression of cytokines in painful and painless neuropathies. *Neurology*, 69, 42-9.

Uceyler, N. & Sommer, C. (2008). Cytokine regulation in animal models of neuropathic pain and in human diseases. *Neurosci Lett*, 437, 194-8.

Uceyler, N., Tscharke, A. & Sommer, C. (2007b). Early cytokine expression in mouse sciatic nerve after chronic constriction nerve injury depends on calpain. *Brain Behav Immun*, 21, 553-60.

Van Der Fits, L., Mourits, S., Voerman, J. S., Kant, M., Boon, L., Laman, J. D., Cornelissen, F., Mus, A. M., Florencia, E., Prens, E. P. & Lubberts, E. (2009). Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol*, 182, 5836-45.

Van Hilten, J. J., Ferrari, M. D., Van Der Meer, J. W., Gijsman, H. J. & Looij, B. J., Jr. (1991). Plasma interleukin-1, tumour necrosis factor and hypothalamic-pituitary-adrenal axis responses during migraine attacks. *Cephalalgia*, 11, 65-7.

Vane, J. R., Bakhle, Y. S. & Botting, R. M. (1998). Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol*, 38, 97-120.

Varrassi, G., Muller-Schwefe, G., Pergolizzi, J., Oronska, A., Morlion, B., Mavrocordatos, P., Margarit, C., Mangas, C., Jaksch, W., Huygen, F., Collett, B., Berti, M., Aldington, D. & Ahlbeck, K. (2010). Pharmacological treatment of chronic pain - the need for CHANGE. *Curr Med Res Opin*, 26, 1231-45.

Venables, W. N. & Ripley, B. D. 2002 *Modern Applied Statistics with S*, New York, Springer.

Verdu, B., Decosterd, I., Buclin, T., Stiefel, F. & Berney, A. (2008). Antidepressants for the treatment of chronic pain. *Drugs*, 68, 2611-32.

Von Hehn, C. A., Baron, R. & Woolf, C. J. (2012). Deconstructing the neuropathic pain phenotype to reveal neural mechanisms. *Neuron*, 73, 638-52.

Wager, T. D., Atlas, L. Y., Lindquist, M. A., Roy, M., Woo, C. W. & Kross, E. (2013). An fMRI-based neurologic signature of physical pain. *N Engl J Med*, 368, 1388-97.

Wagner, J. A. (2009). Biomarkers: principles, policies, and practice. *Clin Pharmacol Ther*, 86, 3-7.

Walsh, N. E., Schoenfeld, L., Ramamurthy, S. & Hoffman, J. (1989). Normative model for cold pressor test. *Am J Phys Med Rehabil*, 68, 6-11.

Wang, C. X., Olschowka, J. A. & Wrathall, J. R. (1997). Increase of interleukin-1beta mRNA and protein in the spinal cord following experimental traumatic injury in the rat. *Brain Res*, 759, 190-6.

Wang, H., Kohno, T., Amaya, F., Brenner, G. J., Ito, N., Allchorne, A., Ji, R. R. & Woolf, C. J. (2005). Bradykinin produces pain hypersensitivity by potentiating spinal cord glutamatergic synaptic transmission. *J Neurosci*, 25, 7986-92.

Wartolowska, K. & Tracey, I. (2009). Neuroimaging as a tool for pain diagnosis and analgesic development. *Neurotherapeutics*, 6, 755-60.

Watkins, L. R., Hansen, M. K., Nguyen, K. T., Lee, J. E. & Maier, S. F. (1999). Dynamic regulation of the proinflammatory cytokine, interleukin-1beta: molecular biology for non-molecular biologists. *Life Sci*, 65, 449-81.

Watkins, L. R., Hutchinson, M. R., Ledeboer, A., Wieseler-Frank, J., Milligan, E. D. & Maier, S. F. (2007a). Norman Cousins Lecture. Glia as the "bad guys": implications for improving clinical pain control and the clinical utility of opioids. *Brain Behav Immun*, 21, 131-46.

Watkins, L. R., Hutchinson, M. R., Milligan, E. D. & Maier, S. F. (2007b). "Listening" and "talking" to neurons: implications of immune activation for pain control and increasing the efficacy of opioids. *Brain Res Rev*, 56, 148-69.

Watkins, L. R. & Maier, S. F. (2003). Glia: a novel drug discovery target for clinical pain. *Nat Rev Drug Discov*, 2, 973-85.

Wiken, M., Grunewald, J., Eklund, A. & Wahlstrom, J. (2009). Higher monocyte expression of TLR2 and TLR4, and enhanced pro-inflammatory synergy of TLR2 with NOD2 stimulation in sarcoidosis. *J Clin Immunol*, 29, 78-89.

Williamson, A. & Hoggart, B. (2005). Pain: a review of three commonly used pain rating scales. *J Clin Nurs*, 14, 798-804.

Wise, R. G. & Preston, C. (2010). What is the value of human FMRI in CNS drug development? *Drug Discov Today*, 15, 973-80.

Wolf, G., Gabay, E., Tal, M., Yirmiya, R. & Shavit, Y. (2006). Genetic impairment of interleukin-1 signaling attenuates neuropathic pain, autotomy, and spontaneous ectopic neuronal activity, following nerve injury in mice. *Pain*, 120, 315-24.

Woodcock, J. (2009). Chutes and ladders on the critical path:comparative effectiveness, product value, and the use of biomarkers in drug development. *Clin Pharmacol Ther*, 86, 12-4.

Woolf, C. J. (1983). Evidence for a central component of post-injury pain hypersensitivity. *Nature*, 306, 686-8.

Woolf, C. J. (2010). What is this thing called pain? *The Journal of clinical investigation*, 120, 3742-4.

Woolf, C. J. (2011). Central sensitization: Implications for the diagnosis and treatment of pain. *Pain*, 152, S2-15.

Woolf, C. J., Allchorne, A., Safieh-Garabedian, B. & Poole, S. (1997). Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. *Br J Pharmacol*, 121, 417-24.

Woolf, C. J. & Ma, Q. (2007). Nociceptors--noxious stimulus detectors. *Neuron*, 55, 353-64.

Woolf, C. J. & Mannion, R. J. (1999). Neuropathic pain: aetiology, symptoms, mechanisms, and management. *Lancet*, 353, 1959-64.

Woolf, C. J., Safieh-Garabedian, B., Ma, Q. P., Crilly, P. & Winter, J. (1994). Nerve growth factor contributes to the generation of inflammatory sensory hypersensitivity. *Neuroscience*, 62, 327-31.

Woolf, C. J. & Salter, M. W. (2000). Neuronal plasticity: increasing the gain in pain. *Science*, 288, 1765-9.

Woolf, C. J., Shortland, P. & Coggeshall, R. E. (1992). Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature*, 355, 75-8.

Woosley, R. L. (2012). Is it possible for FDA regulatory scientists and industry scientists to work together? *Clin Pharmacol Ther*, 91, 390-2.

Yoshikawa, M., Suzumura, A., Tamaru, T., Takayanagi, T. & Sawada, M. (1999). Effects of phosphodiesterase inhibitors on cytokine production by microglia. *Mult Scler*, 5, 126-33.

Younger, J., Barelka, P., Carroll, I., Kaplan, K., Chu, L., Prasad, R., Gaeta, R. & Mackey, S. (2008). Reduced cold pain tolerance in chronic pain patients following opioid detoxification. *Pain Med*, 9, 1158-63.

Zelenka, M., Schafers, M. & Sommer, C. (2005). Intraneural injection of interleukin-1beta and tumor necrosis factor-alpha into rat sciatic nerve at physiological doses induces signs of neuropathic pain. *Pain*, 116, 257-63.

Zhang, B., Yang, L., Konishi, Y., Maeda, N., Sakanaka, M. & Tanaka, J. (2002). Suppressive effects of phosphodiesterase type IV inhibitors on rat cultured microglial cells: comparison with other types of cAMP-elevating agents. *Neuropharmacology*, 42, 262-9.

Zhang, J. M. & An, J. (2007). Cytokines, inflammation, and pain. *Int Anesthesiol Clin*, 45, 27-37.

Zhang, Z., Trautmann, K. & Schluesener, H. J. (2005). Microglia activation in rat spinal cord by systemic injection of TLR3 and TLR7/8 agonists. *J Neuroimmunol*, 164, 154-60.

Zhuang, Z. Y., Wen, Y. R., Zhang, D. R., Borsello, T., Bonny, C., Strichartz, G. R., Decosterd, I. & Ji, R. R. (2006). A peptide c-Jun N-terminal kinase (JNK) inhibitor blocks mechanical allodynia after spinal nerve ligation: respective roles of JNK activation in primary sensory neurons and spinal astrocytes for neuropathic pain development and maintenance. *J Neurosci*, 26, 3551-60.

Zin, C. S., Nissen, L. M., O'callaghan, J. P., Moore, B. J. & Smith, M. T. (2010). Preliminary study of the plasma and cerebrospinal fluid concentrations of IL-6 and IL-10 in patients with chronic pain receiving intrathecal opioid infusions by chronically implanted pump for pain management. *Pain Med*, 11, 550-61.

Zuo, Y., Perkins, N. M., Tracey, D. J. & Geczy, C. L. (2003). Inflammation and hyperalgesia induced by nerve injury in the rat: a key role of mast cells. *Pain*, 105, 467-79.

Appendix A. Supplementary tables

Table A–1. Individual information on chronic pain sufferers in Chapter 2

Participant	Age/ Gender	Primary pain diagnosis	Opioid	Other analgesics	Oral morphine equivalent dose (mg)	Duration of pain (years)
Group 1: Chronic Pain sufferers on opioid						
1	63/F	Sciatica	Morphine	Amitriptyline, pregabalin & paracetamol	200	10
2	43/M	Chronic back and leg pain	Oxycodone		80	28
3	65/F	Osteoarthritis	Oxycodone		120	3
4	49/F	Chronic low back pain	Oxycodone	Diclofenac	36	16
5	54/M	Osteoarthritis of hip	Oxycodone		80	2
6	59/F	Back and shoulder pain	Methadone		30	20
7	45/M	Osteoarthritis	Morphine	Venlafaxine, quetiapine & diazepam	70	3
8	42/M	Fibromyalgia	Paracetamol/ codeine	Duloxetine	30	3
9	64/F	Osteoarthritis	Tramadol	Celecoxib & paracetamol/ codeine	107	14
10	62/F	Back Pain & migraine	Morphine	Oxycodone hydrochloride & paracetamol/ codeine	40	7
11	33/F	Complex regional pain syndrome	Oxycodone	Pregabalin & paracetamol/ codeine	120	7

Group 2: Chronic pain sufferers not on opioids						
12	36/F	Osteoarthritis of knee	None	Ibuprofen	N/A	2
13	65/M	Osteoarthritis of knee	None	Ibuprofen#	N/A	1
14	60/M	Sciatica	None	Citalopram & paracetamol#	N/A	4
15	40/F	Atypical trigeminal neuralgia	None	Amitriptyline#, carbamazepine# & pregabalin#	N/A	2
16	64/F	Chronic low back pain	None		N/A	11
17	45/F	Neuropathic pain syndrome	None	Gabapentin & amitriptyline	N/A	4
18	57/F	Non cardiac chest pain	None	Paracetamol#	N/A	4
19	46/F	Fibromyalgia and back pain	None		N/A	5

# denotes when required

THIS PAGE WAS INTENTIONALLY LEFT BLANK

Table A–2. Best-fit logistic regression model results for the prediction of pain for rats post CCI.

	Dataset	Variables	Estimate	SE	*P*	Null deviance	Df	Residual deviance	Df	AUC	ANOVA
All data	**Complete**	**Peripheral non-stimulated plasma**	0.91	0.41	0.027	39.90	34	26.27	32	0.87	-
		Central TLR4 stimulated spinal cord	0.063	0.032	0.050						
	Peripheral	Non-stimulated cell count	9.17e-08	1.02e-07	0.37	49.57	40	40.65	33	0.76	0.09
		Non-stimulated plasma	5.11e-01	2.97e-01	0.086 .						
		TLR4 stimulated max	5.88e-02	9.18e-02	0.52						
		TLR4 stimulated intercept	- 2.21e-02	1.11e-01	0.84						
		TLR4 stimulated slope	-9.43e-02	1.91e-01	0.62						
		TLR2 stimulated max	-7.38e-02	6.7e-02	0.29						
		TLR2 stimulated min	-1.58e-03	2.79e-01	0.1						
	Central	TLR4 stimulated supernatant of spinal cord	0.21	0.12	0.07.	39.90	34	23.57	29	0.9	0.004
		Non-stimulated supernatant of spinal cord	-0.052	0.052	0.31						
		TLR4 stimulated spinal cord	0.099	0.052	0.058						
		TLR2 stimulated spinal cord	0.067	0.038	0.078						
		Non-stimulated spinal cord	-0.062	0.029	0.036						
	TLR2 stimulated only (peripheral & central)	Peripheral stimulated max	0.25	0.14	0.085	39.90	34	31.76	30	0.76	0.02
		Peripheral stimulated min	0.55	0.36	0.13						
		Peripheral stimulated intercept	-0.53	0.34	0.12						
		Central stimulated spinal cord	0.039	0.022	0.074 .						
	TLR4 stimulated only (peripheral & central)	Central stimulated spinal cord	0.042	0.024	0.087 .	39.9	34	35.88	33	0.61	0.003

	Non-stimulated (peripheral & central)	**Plasma**	0.83	0.39	0.034	39.9	34	31.74	33	0.8	0.01
Neuronal & subcutaneous :	**Complete**	Peripheral non-stimulated plasma	0.78	0.60	0.19	31.76	23	16.76	18	0.9	-
		Peripheral non-stimulated cells	-0.31	0.23	0.17						
		Peripheral TLR4 stimulated min	0.10	0.059	0.085						
		Central TLR4 stimulated spinal cord	0.070	0.056	0.21						
		Central non-stimulated spinal cord	-0.073	0.049	0.14						
	Periphery	Non-stimulated plasma	0.53	0.45	0.24	40.38	29	22.89	19	0.88	0.2
		Non-stimulated cells	-0.8	0.49	0.10						
		TLR4 stimulated max	0.77	0.36	0.034						
		TLR4 stimulated min	1.4	0.70	0.045						
		TLR4 stimulated intercept	-1.94	0.93	0.037						
		TLR4 stimulated slope	-1.86	0.90	0.038						
		TLR2 stimulated max	0.76	0.62	0.22						
		TLR2 stimulated min	1.3	1.01	0.20						
		TLR2 stimulated intercept	-1.76	1.47	0.23						
		TLR2 stimulated slope	-1.34	1.037	0.20						
	Central	TLR2 stimulated supernatant of spinal cord	0.54	0.34	0.11	31.76	23	18.03	18	0.88	0.2
		Non-stimulated supernatant of spinal cord	-0.16	0.10	0.11						
		TLR4 stimulated spinal cord	0.12	0.069	0.093						
		TLR2 stimulated spinal cord	0.053	0.036	0.14						
		Non-stimulated spinal cord	-0.075	0.036	0.039						
	TLR2 stimulated only (peripheral & central)	Peripheral stimulated slope	0.4	0.27	0.13	31.76	23	26.59	21	0.77	0.2
		Central stimulated spinal cord	0.032	0.022	0.16						
	TLR4 stimulated only (peripheral & central)	Central stimulated spinal cord	0.039	0.026	0.14	31.76	23	28.70	22	0.64	0.05
	Basal	Peripheral plasma	0.55	0.39	0.16	31.76	23	27.40	22	0.58	0.1
Neuronal Only:	**Complete**	Peripheral non-stimulated cells	-1.23	0.77	0.11	23.05	20	14.51	18	0.86	-
		Peripheral TLR4 stimulated intercept	0.43	0.31	0.17						
	Periphery	Non-stimulated cell count	8.11e-08	1.48e-07	0.58	26.40	22	26.08	21	0.63	0.009
	Central	TLR2 stimulated supernatant of spinal cord	0.22	0.22	0.32	23.05	20	21.56	19	0.76	0.16
	TLR2 agonist stimulation only	Peripheral stimulated max	0.19	0.19	0.33	23.05	20	21.77	19	0.66	0.0062
	TLR4 agonist stimulation only	Central stimulated spinal cord	0.038	0.032	0.24	23.05	20	21.27	19	0.60	N/A
	Basal	Peripheral cell count	3.05e-07	2.6e-07	0.24	23.05	20	21.13	19	0.76	0.022

Appendix A. Supplementary tables

Notes: Significant variables are shown in bold. The residual deviance for the model includes predictor variables, whereas the null deviance for the model does not. The discrimination probabilities (D, area under ROC curve) are presented in the table. One-way ANOVA was used to compare which sub groups (central /peripheral/TLR2 agonist stimulation/ TLR4 agonist stimulation or Basal) when compare with all data set is a better model. The residual deviance for the model includes predictor variables, whereas the null deviance for the model does not. SE, standard error.

Table A–3. Best-fit logistic regression model results for the prediction of the pain severity in rats post CCI.

	Dataset	Variables	Estimate	SE	*P*	Null deviance	Df	Residual deviance	Df	Adjusted R square:	P-value	ANOVA
All data	**Complete**	Peripheral non-stimulated cell count	-1.99e-08	1.30e-08	0.14	5.42	34	2.15	24	0.44	0.0044	
		Peripheral non-stimulated plasma	-1.22e-02	8.84e-03	0.18							
		Peripheral TLR4 stimulated min	1.56e-02.	8.69e-03	0.086							
		Peripheral TLR4 stimulated intercept	-8.13e-03	4.89e-03	0.11							
		Peripheral TLR4 stimulated slope	-1.37e-02	6.80e-03	0.055							
		Peripheral TLR2 stimulated max	-5.29e-02	1.91e-02	0.011							
		Peripheral TLR2 stimulated min	-1.25e-01	4.46e-02	0.0099							
		Peripheral TLR2 stimulated intercept	1.19e-01	4.11e-02	0.0078							
		Central TLR2 stimulated spinal cord	-7.74e-03	2.32e-03	0.0028							
		Central non- stimulated spinal cord	6.36e-03	2.15e-03	0.0069							
	Periphery	**Non-stimulated cell count**	-3.19e-08	1.49e-08	0.040	6.47	40	4.30	32	0.17	0.11	0.0036
		Non-stimulated plasma	-1.36e-02	1.05e-02	0.20							
		TLR4 stimulated max	-5.58e-03	3.24e-03	0.095 .							
		TLR4 stimulated min	1.04e-02	6.91e-03	0.14							
		TLR4 stimulated intercept	5.87e-03	3.65e-03	0.12							
		TLR2 stimulated max	-5.32e-02	2.33e-02	0.029							
		TLR2 stimulated min	-1.36e-01	4.94e-02	0.0095							
		TLR2 stimulated intercept	1.24e-01	4.93e-02	0.017							
	Central	**TLR4 stimulated spinal cord supernatant**	-0.018	0.0083	0.037	5.42	34	3.26	30	0.32	0.0035	0.093
		Non-stimulated spinal cord supernatant	0.0049	0.0023	0.043							
		TLR2 stimulated spinal cord	-0.0076	0.0024	0.0041							
		Non-stimulated spinal cord	0.0079	0.0023	0.0016							

	TLR2 agonist stimulation only	Peripheral stimulated max	-0.030	0.016	0.077	5.42	34	3.96	30	0.17	0.05	0.015
		Peripheral stimulated min	-0.12	0.045	0.010							
		Peripheral stimulated intercept	0.062	0.038	0.11							
		Central stimulated spinal cord	-0.0055	0.0025	0.037							
	TLR4 agonist stimulation only	Peripheral stimulated min	0.015	0.010	0.15	5.42	34	4.9	31	0.0081	0.37	0.0029
		Peripheral stimulated intercept	-0.010	0.0058	0.089 .							
		Peripheral stimulated slope	-0.014	0.0079	0.094							
	Basal	Spinal cord supernatant	0.0034	0.0024	0.172	5.42	34	4.57	32	0.10	0.07	0.0096
		Spinal cord	0.0050	0.0024	0.043							
Neuronal and subcutaneous	**Complete**	Peripheral non-stimulated cell count .	-3.47e-08	1.7e-08	0.075	4.51777	23	0.68	8	0.56	0.06	
		Peripheral TLR4 stimulated max	1.15e-01	3.54e-02	0.012							
		Peripheral TLR4 stimulated min	1.82e-01	5.077e-02	0.0071							
		Peripheral TLR4 stimulated intercept	-2.89e-01	8.49e-02	0.0093							
		Peripheral TLR4 stimulated slope	-2.82e-01	8.053e-02	0.0080							
		Peripheral TLR2 stimulated max	-1.53e-01	6.43e-02	0.044							
		Peripheral TLR2 stimulated min	8.98e-02	1.04e-01	0.41							
		Peripheral TLR2 stimulated intercept	1.11e-01	1.2e-01	0.38							
		Peripheral TLR2 stimulated slope	2.16e-01	1.39e-01	0.16							
		Central TLR4 stimulated spinal cord supernatant	1.016e-01	4.12e-02	0.039							
		Central TLR2 stimulated spinal cord supernatant	-1.64e-01	5.62e-02	0.02							
		Central non-stimulated spinal cord supernatant	3.5e-02	1.38e-02	0.035							
		Central TLR4 stimulated spinal cord	-6.59e-03	4.93e-03	0.22							
		Central TLR2 stimulated spinal cord	-1.023e-02	4.16e-03	0.039							
		Central non-stimulated spinal cord	1.53e-02	5.35e-03	0.021							

	Periphery	**Non-stimulated cell count**	-3.44e-08	1.67e-08	0.05	5.52	29	3.38	24	0.29	0.04	0.15
		Non-stimulated cells	7.89e-02	3.24e-02	0.022							
		TLR4 stimulated max	-1.81e-02	6e-03	0.0059							
		TLR4 stimulated intercept	1.65e-02	6.12e-03	0.013							
		TLR4 stimulated min	-7.053e-02	4.71e-02	0.15							
	Central	TLR4 stimulated spinal cord supernatant	-0.016	0.012	0.19	4.52	23	2.46	19	0.34	0.02	0.19
		Non-stimulated spinal cord supernatant	0.0049	0.0025	0.070 .							
		TLR2 stimulated spinal cord	-0.0077	0.0032	0.029							
		Non-stimulated spinal cord	0.011	0.0033	0.0039							
	TLR2 agonist stimulation only	**Peripheral stimulated max**	-0.041	0.017	0.031	4.52	23	2.24	18	0.37	0.02	0.2
		Peripheral stimulated min	-0.073	0.058	0.23							
		Peripheral stimulated intercept	0.084	0.038	0.041							
		Central stimulated spinal cord	0.0091	0.0033	0.013							
	TLR4 agonist stimulation only	Peripheral stimulated min	0.018	0.0136	0.2	4.52	23	4.0018	20	-0.019	0.48	0.052 .
		Peripheral stimulated intercept	-0.014	0.0087	0.13							
		Peripheral stimulated slope	-0.019	0.013	0.15							
	Basal	Peripheral cells	0.029	0.021	0.18	4.52	23	3.03	20	0.23	0.04	0.12
		Central spinal cord supernatant	0.0046	0.0026	0.096 .							
		Central spinal cord	0.010	0.0036	0.010							
Neuronal Only:	**Complete**	Peripheral non-stimulated plasma	-0.013	0.007	0.088.	3.12	20	0.56	11	0.67	0.0048	
		Peripheral non-stimulated cells	0.12	0.027	0.00094							
		Peripheral TLR4 stimulated max	-0.024	0.0089	0.022							
		Peripheral TLR4 stimulated slope	0.068	0.027	0.03							
		Peripheral TLR2 stimulated max	-0.037	0.014	0.022							
		Central TLR4 stimulated spinal cord supernatant	-0.023	0.015	0.15							
		Central non-stimulated spinal cord supernatant	0.013	0.0096	0.21							
		Central TLR2 stimulated spinal cord	-0.0045	0.003	0.16							
		Central non-stimulated spinal cord	0.0042	0.0021	0.065 .							

	Periphery	Non-stimulated plasma	-0.017	0.0081	0.051 .	3.4	22	1.068	15	0.62	0.0038	0.16
		Non-stimulated cells	0.12	0.025	0.00028							
		TLR4 stimulated max	-0.02	0.0074	0.019							
		TLR4 stimulated slope	0.064	0.023	0.012							
		TLR2 stimulated max	-0.034	0.016	0.05							
		TLR2 stimulated min	-0.087	0.049	0.096 .							
		TLR2 stimulated slope	-0.061	0.040	0.15							
	Central	**TLR2 stimulated spinal cord**	-0.0078	0.0035	0.038	3.11	20	2.35	18	0.16	0.078	0.0089
		Non-stimulated spinal cord	0.005	0.0029	0.1							
	TLR2 agonist stimulation only	Central spinal cord -	0.0056	0.0034	0.12	3.11	20	2.73	19	0.076	0.12	0.0063
	TLR4 agonist stimulation only	N/A										
	Basal	Peripheral cell count	-2.86e-08	2.26e-08	0.22	3.11	20	1.63	16	0.35	0.027	0.022
		Peripheral plasma	-1.29e-02	9.89e-03	0.22							
		Peripheral cell	7.66e-02	2.79e-02	0.014							
		Central spinal cord supernatant	-1.267e-02	6.041e-03	0.052 .							

Notes: Significant variables are shown in bold. One-way ANOVA was used to compare which subsets (Central/ Peripheral/ TLR2/ TLR4 or Basal) when compare with all outputs is a better model. The residual deviance for the model includes predictor variables, whereas the null deviance for the model does not. SE, standard error.

THIS PAGE WAS INTENTIONALLY LEFT BLANK

Table A–4. Best-fit logistic regression model from rats (Peripheral only) and from humans to predict the presence of pain in chronic pain patients

	Variables	Estimate	SE	p-value	Null deviance	Df	Residual deviance	Df	AUC	ANOVA
Model rat to human	Non-stimulated cell count	-4.04e-1	2.67	0.88	35.59	26	16.68	21	0.94	
	TLR4 stimulated max	4.52e-8	2.11e-7	0.83						
	TLR4 stimulated intercept	5.55e-3	3.93	0.16						
	TLR2 stimulated max	5.32e-3	2.66e-3	0.045*						
	TLR2 stimulated min	-1.63e-1	1.26e-1	0.195						
Model human	TLR4 stimulated max	-0.0038	0.0031	0.22	35.59	26	18.16	23	0.92	NS
	TLR4 stimulated intercept	0.0042	0.003	0.16						
	TLR2 stimulated max	0.0045	0.0023	0.054						

Notes: Significant variable is shown in bold. The residual deviance for the model includes predictor variables, whereas the null deviance for the model does not. The discrimination probabilities (D, area under ROC curve) are presented in the table. One-way ANOVA was used to compare Model human with Model rat to human. The residual deviance for the model includes predictor variables, whereas the null deviance for the model does not. SE, standard error.

Appendix B. Potential biomarkers for pain: Neopterin & reduced white cell count

B.1. Introduction

Neopterin is an immune modulator that belongs to a class of pteridines derived from guanosine triphosphate (GTP) (Murr et al., 2002, Carru et al., 2004). Neopterin is secreted from activated monocytes and macrophages after the release of interferon gamma by T-cells (Huber et al., 1984). As the concentration of neopterin correlates with disease progression, it is considered to be a circulating inflammatory marker that can reflect the intensity of the cell mediated immune response (Avanzas et al., 2009).

Neopterin can be easily quantified in various locations (such as serum (Turgan et al., 2001), urine (Svoboda et al., 2008), saliva (Katoh et al., 1989) and cerebrospinal fluid (Dale et al., 2009)) and displays good stability in biological fluids. Since 1994 in Austria, concentration of neopterin has been screened in blood donations and when elevated neopterin levels (> 11 nmol/L) are detected, the blood donation is excluded for transfusion (Laich et al., 2002). Aside from its use in identifying viral infection, numerous studies have established the usefulness of neopterin in various situations including its usefulness in aiding the prediction of cardiovascular diseases (such as chronic stable angina) (Avanzas et al., 2005), autoimmune disorders and complications in transplant recipients (Hoffmann et al., 2003).

To date, there is a paucity of data regarding the role of neopterin as a biomarker for chronic pain but an indirect association between neopterin and pain has been found. A genetic polymorphism of GTP cyclohydrolase was found to be associated with reduced pain sensitivity and chronicity in human as the production of tetahydrobiopterin (BH4) was reduced. As neopterin is an intermediate product in the synthesis of BH4 from GTP cyclohydrolase (see Figure B1),

changes in GTP cyclohyrolase protein would correspond with neopterin levels (Tegeder et al., 2006) thus linking neopterin to BH4 and pain. The level of BH4 was found to increase in rat doral root ganglia after axonal injury (Tegeder et al., 2006).

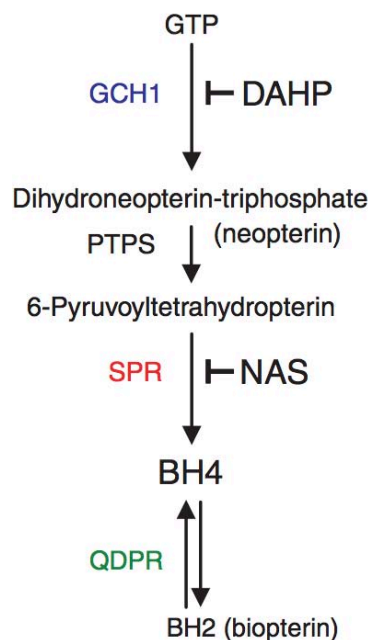


Figure B–1. The synthesis pathway of GTP (Tegeder et al., 2006)

Neopterin can also stimulates NF-κB translocation to the nucleus leading to the expression of pro-inflammatory cytokines (Avanzas et al., 2009), which is known to contribute to alloydnia and hypersensitivity (Marchand et al., 2005). The neopterin appears to be a valuable tool to reflect immune status, thus his study hopes to give insight into the immune status of chronic pain patients and to investigate the potential use of neopterin as a pain biomarker.

Another potential biomarker for pain arises from two independent researchers in our laboratory who observed a reduced harvest of white cells in both healthy participants and a chronic pain patient after systemic administration of opioids. The reduced harvest of white cells was first observed after the separation of whole blood with the use of the density gradient kit,

OptiPrep and it was found that the population of white cells was reduced by approximately 50%. The reduced harvest was not due to abnormally low circulating numbers of white cells hence was an artefact of the preparation. It was found when the white cells were incubated with morphine *in vitro*, the white cells harvest was not reduced. To our knowledge this has not been reported previously and might reflect a change in white blood cell behaviour as a marker of *in vivo* opioid exposure and hence could be a potential biomarker.

Therefore, the aims of this study are:

- To investigate whether reduced white cell harvest occurs when isolated with OptiPrep following *in vitro* TLR ligands and opioid exposure.
    a. If verified, to investigate the mechanisms involved with reduced white cells harvest.
- To compare serum levels of neopterin between chronic pain patients and pain-free participants.

B.2. Materials and methods

B.2.1. Cell count

All participants recruited in Chapter 2 (Kwok et al., 2012) were included in this study. After the isolation of PBMCs with the use of Optiprep™ (Axis-Shield PoC AS, Oslo, Norway), the cell suspension was collected into an eppendorf tube. Then 10 µL of the cell suspension was transferred to a clean eppendorf tube and mixed gently with 90 µL of enriched RPMI (RPMI 1640 with 10% foetal calf serum and 1% penicillin) and 100 µL of trypan blue. 10 µL of the cell suspension containing trypan blue was used to fill each of the chambers on the haemocytometer. The number of live cells (unstained by trypan blue) was counted on the haemocytometer and recorded.

B.2.2. Neopterin assay

Nine ml of blood was collected from venous blood and transferred into a collected in a serum separator tube and allowed to clot for 30 min. Then it was centrifuged at 1200g for 10 min and the serum was collected and kept frozen at -70°C until analysis. Neopterin levels were determined by a commercially available ELISA kit (Neopterin ELISA; ARP American Research Products, USA) and performed according to the manufacturer's instructions. The absorbance was quantified on a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany) at 450 nm per manufacturer's instructions. The manufacturer's limit of quantification of 0.7 nmol/L was used.

B.2.3. Statistical analysis

Graphpad Prism 6.0 (GraphPad Software; San Diego, CA) was used for all statistical analysis. The D'Agostino and Peason omnibus was performed all on dataset to determine normality. The Kruskal-Wallis test was used to assess the PBMCs count between the three groups, whereas one-way ANOVA was used to analyse the level of neopterin. All significance was set at $P < 0.05$.

B.3. Results

There were no group differences ($P = 0.7$) between the number of PBMCs isolated from all three groups (10^9 mean ± SEM): CP+O group: 11 ± 1.5, CP group: 9.7 ± 1.1 and PF: 9.9 ± 1.2 (see Figure B-2). Likewise, no group differences ($P = 0.5$) were detected in the level of neopterin collected from the serum: CP+O group: 2 ± 0.09, CP group: 2.2 ± 0.1 and PF group: 2.2 ± 0.1 (see Figure B-3).

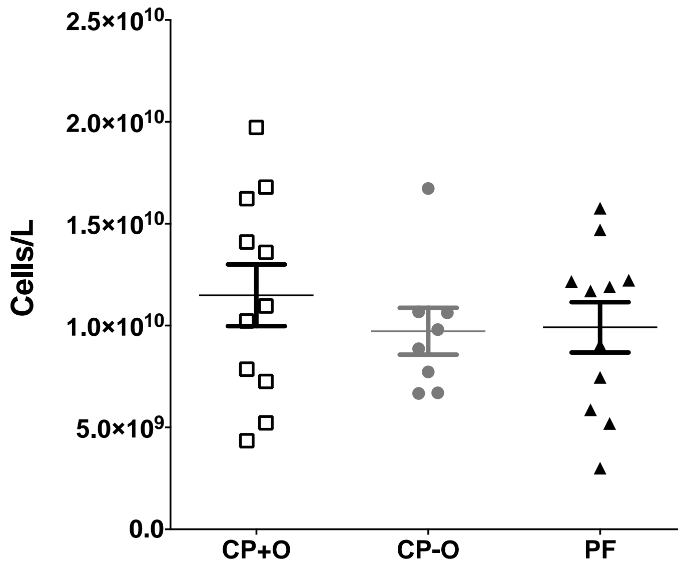


Figure B–2. Number of cell count after isolation with the use of Optiprep™.

Isolated white cells obtained from chronic pain patients on opioid, chronic pain patients not on opioids and pain-free participants were found have no group differences (P = 0.7). Each point represents a participant and the error bars on graph represent standard error of the mean.

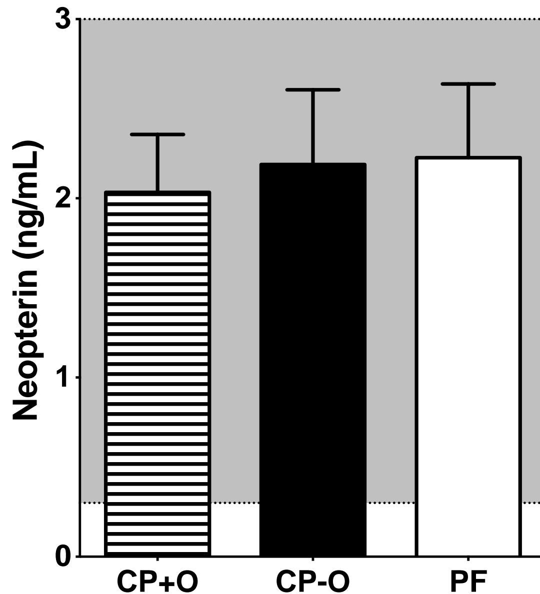


Figure B–3. No group differences were detected in the serum neopterin levels.

Neopterin levels were found to be the same between chronic pain patients on opioid, chornic pain patients not on opioids and pain-free participants (P = 0.5). Neopterin was quantified with

the use of ELISA and the section between the dotted lines represents the normal physiological ranges. Error bars on graph represent standard error of the mean.

B.4. Discussion

This is the first study to investigate the level of neopterin as a biomarker for chronic pain but no group differences could be detected between the chronic pain and pain-free cohorts. The result indicates that the immune system of all participants in this study was not overtly activated. The other potential pain biomarker of reduced white cell count in participants after systemic administration of opioids was not observed in the chronic pain patients currently on opioid treatment. As the finding of reduced white cells was not found, the previous reports could be coincidental.

The level of neopterin found in the study was quite low compared to other studies (Schumacher et al., 1997, Sauerland et al., 2003, Avanzas et al., 2005). However, it is noteworthy that the detected range of neopterin in this study lies within the normal range. Neopterin is known to be daylight sensitive (Laich et al., 2002) so from the time of blood collection to the quantification of neopterin with ELISA, all tubes containing test samples were wrapped with aluminium foil to avoid light exposure. Hence we do not believe the lower concentration found to be resulted from degradation. Also potential circadian effects of melatonin have been reported on neopterin levels (Garcia-Gonzalez et al., 2006) and all participants had their blood taken during day time which also could not have influence the levels of neopterin.

The level of neopterin reported in the study is in agreement with the findings reported in Chapter 2 as the use of basal cytokine levels in PBMCs were not able to differentiate between patient cohorts. It was only post TLR agonist stimulations that the differences were observed. This suggests that in chronic pain patients, it is only after stimulation that the cells would produce an exaggerated response hence indicating the possibility of primed cells in chronic pain patients.

Therefore, it would be of interest to test the neopterin level of chronic pain patients after a stimulus such as capsaicin with endotoxin as was previously reported (Hutchinson et al., 2013).

For future study, the use of at least 30 participants would be required to determine the utility of neopterin as a potential marker for chronic pain or other diseases. As a reported study found a correlation between neopterin and recurrent depression (the p-value obtained was < 0.001) with 30 participants (Celik et al., 2010). In another study, which investigated the use of neopterin in cardiovascular diseases, included an upward of 300 participants and p-value was detected to be 0.02 (Avanzas et al., 2005). Collectively, the number of participants needed to detect potential differences in neopterin would be dependent on the underlying disease and the involvement of the immune system. As a result, the current testing of neopterin as a potential biomarker for pain is inconclusive and the potential contribution of neopterin to chronic pain should be determine in a larger sample size study.

Appendix C. Development of data derived statistical models for study 2 and 3

A detailed explanation of the commands used for the generation of models for Chapter 3 and 4 is presented here. The explanation of the following models will be best understood if viewed together with the method section in Chapter 3.

A generalized linear model was fitted to all models with commands that look like this:

The following command generated a model to determine how much of the output variables plasma and cell count predict the pain response.

```
modelP <- glm (painresponse ~ plasma + cell count, family = "binomial", data = all)
```

1. modelP is the name assign to this fitted model to determine pain response and the results of this model can accessed by entering "modelP".
2. glm = Generalized linear model function that fits data linearly which requires to specify the variance and link functions such as, bionomial or gaussian variance.
3. ~ = Is modelled as
4. + = The plus sign means "to combine" the output variables and in the above command, it combined plasma and cell count
5. Family = Specifying which variance and as the pain response is either 0 (no pain) or 1 (pain), it is binomial.
6. Data = The file in which the data are stored that contains data for painresponse, plasma and cell count.

C.1. R commands for the generation of models

C.1.1. For categorization of the pain presence (Model A) (Chapter 3 for rats and humans) & responders of ibudilast (Chapter 4)

		Response	
		0	**1**
Chapter 3	**Rat model**	N0S0 N0S4	N1S0 N1S3 N2S0 N2S2 N4S0
	Human model	Pain-free participants	Chronic pain patients
Chapter 4	**Ibudilast model**	Placebo treated MOH patients	Ibudilast treated MOH patients

All of the commands below also apply to Chapter 4, so the response would be "treatment" along with the output variables collected in from MOH patients.

First, the column for the "presence of pain" or "treatments of ibudilast" has to be changed into a factor. In this example, "all data" is the name of the file where all data are stored. "Pain" is the name of the column where the status of pain presence is stored.

```
alldata$pain <- factor(alldata$pain)
```

Then the generalized linear model is fitted to all output variables from the dataset "alldata" to determine which best predict the presence of pain. The "summary" command provides a table with information about each output variable's estimates and standard errors. The null and residual deviance with degrees of freedom is also provided. Output variable that contribute significantly to the response (such as "presence of pain") will be flagged with stars dependent on the p-values.

```
modelA.glm <- glm(pain~., family = "binomial", data = alldata)

summary(modelA.glm)
```

Then a stepwise logistic regression is performed on the glm model by taking a series of step (based on Akaike's information criterion (AIC) which is defined as "-2 maximized log-likelihood +

2 number of parameters") to assist in the selection for deleting or adding different output variables that best contribute to the response of "presence of pain".

```
modelA.aic <- stepAIC(modelA.glm)

summary(modelA.aic)
```

For this particular model, after the stepAIC function, the following output variables were selected to best contribute to the presence of pain. So a new glm model was created.

```
remodelA.glm <- glm(pain~ SUPER_SC_LPS+ SUPER_SC_CON + SPINALCORD_LPS
+SPINALCORD_PAM + SPINALCORD_CON, family = "bionomial", data=alldata)

summary(remodelA.glm)
```

A ROC curve was generated with the lroc command to determine how specific and sensitive the model is to predict the presence of pain.

```
Lroc(remodelA.glm, title=TRUE, auc.coords=c(.5.1))
```

The above commands were used in study 2 to generate Model A for all experimental groups in all dataset and the following subsets: Peripheral, Central, Basal, TLR2 and TLR4 (presented in Table 4 in Chapter 2). The command to calculate the ANOVA between the models generated from each subset was then compared with all dataset.

```
realldata.glm <- all

recentraldata.glm <- central

compareallcentral <- anova(all, central)

summary(compareallcentral)
```

C.1.2. *For the prediction of pain severity in rats (Model B; Chapter 3)*

The response for the following model would be the von Frey score of the rat on the day of cull. The glm and stepwise logistic regression functions were used to generate the models. As the response is no longer binary the variance of the data is not restricted and the parameter for "family" can be omitted.

```
modelB.glm <- glm(vf_left~., data = behave)

summary(modelB.glm)

modelB.aic <- stepAIC(modelB.glm)

remodelB.glm <- glm(vf_left~cell_count + plasma + LPS_min + LPS_intercept + LPS_slope + PAM_max + PAM_min + PAM_intercept + SPINALCORD_PAM + SPINALCORD_CON, data = behave)
```

The use of the predict function allows "predicted values" to be generated from the refined model.

```
rat.sev.pre <- predict(remodelB.glm, new data = behave, se.fit = TRUE, interval = "confidence", type = "response")
```

Then the predicted value was correlated with the actual con Frey score obtained from the rats to determine the adjusted R-square (performed in Graphpad Prism version 6.0 for Windows).

C.1.3. For the prediction of f IL-1β central output by models generated from peripheral outputs and prediction of pain presence in humans (Model D; Chapter 3) and responders of ibudilast (Chapter 4)

All the functions outlined in D2 were used for the generation of models with replacements in the response, output variables and data for the following:

- Predict IL-1β central output by models from peripheral outputs (Model C)
- Predicting the presence of pain:
    - In the expanded cohort with rat model (Model D; Model rat)
    - In the expanded cohort with rat model and human data (Model D; Model rat to human)
    - In the expanded cohort with the model generated from the published cohort (Model D: Model human)
- Predicting responders of ibudilast

All of the above information related to R can be found in (Venables and Ripley, 2002).

Appendix D. Appendix References

Avanzas, P., Arroyo-Espliguero, R. & Kaski, J. C. (2009). Neopterin and cardiovascular disease: growing evidence for a role in patient risk stratification. *Clin Chem*, 55, 1056-7.

Avanzas, P., Arroyo-Espliguero, R., Quiles, J., Roy, D. & Kaski, J. C. (2005). Elevated serum neopterin predicts future adverse cardiac events in patients with chronic stable angina pectoris. *Eur Heart J*, 26, 457-63.

Carru, C., Zinellu, A., Sotgia, S., Serra, R., Usai, M. F., Pintus, G. F., Pes, G. M. & Deiana, L. (2004). A new HPLC method for serum neopterin measurement and relationships with plasma thiols levels in healthy subjects. *Biomed Chromatogr*, 18, 360-6.

Celik, C., Erdem, M., Cayci, T., Ozdemir, B., Ozgur Akgul, E., Kurt, Y. G., Yaman, H., Isintas, M., Ozgen, F. & Ozsahin, A. (2010). The association between serum levels of neopterin and number of depressive episodes of major depression. *Prog Neuropsychopharmacol Biol Psychiatry*, 34, 372-5.

Dale, R. C., Brilot, F., Fagan, E. & Earl, J. (2009). Cerebrospinal fluid neopterin in paediatric neurology: a marker of active central nervous system inflammation. *Dev Med Child Neurol*, 51, 317-23.

Garcia-Gonzalez, M. J., Dominguez-Rodriguez, A. & Abreu-Gonzalez, P. (2006). Diurnal variations in serum neopterin levels are associated with the pineal hormone melatonin circadian rhythm in healthy human subjects. *J Pineal Res*, 40, 288-9.

Hoffmann, G., Wirleitner, B. & Fuchs, D. (2003). Potential role of immune system activation-associated production of neopterin derivatives in humans. *Inflamm Res*, 52, 313-21.

Huber, C., Batchelor, J. R., Fuchs, D., Hausen, A., Lang, A., Niederwieser, D., Reibnegger, G., Swetly, P., Troppmair, J. & Wachter, H. (1984). Immune response-associated production of neopterin. Release from macrophages primarily under control of interferon-gamma. *J Exp Med*, 160, 310-6.

Hutchinson, M. R., Buijs, M., Tuke, J., Kwok, Y. H., Gentgall, M., Williams, D. & Rolan, P. (2013). Low-dose endotoxin potentiates capsaicin-induced pain in man: evidence for a pain neuroimmune connection. *Brain Behav Immun*, 30, 3-11.

Katoh, S., Sueoka, T., Matsuura, S. & Sugimoto, T. (1989). Biopterin and neopterin in human saliva. *Life Sci*, 45, 2561-8.

Kwok, Y. H., Hutchinson, M. R., Gentgall, M. G. & Rolan, P. E. (2012). Increased Responsiveness of Peripheral Blood Mononuclear Cells to In Vitro TLR 2, 4 and 7 Ligand Stimulation in Chronic Pain Patients. *PLoS One*, 7, e44232.

Laich, A., Neurauter, G., Wirleitner, B. & Fuchs, D. (2002). Degradation of serum neopterin during daylight exposure. *Clin Chim Acta*, 322, 175-8.

Marchand, F., Perretti, M. & Mcmahon, S. B. (2005). Role of the immune system in chronic pain. *Nat Rev Neurosci*, 6, 521-32.

Murr, C., Widner, B., Wirleitner, B. & Fuchs, D. (2002). Neopterin as a marker for immune system activation. *Curr Drug Metab*, 3, 175-87.

Sauerland, S., Hensler, T., Bouillon, B., Rixen, D., Raum, M. R., Andermahr, J. & Neugebauer, E. A. (2003). Plasma levels of procalcitonin and neopterin in multiple trauma patients with or without brain injury. *J Neurotrauma*, 20, 953-60.

Schumacher, M., Halwachs, G., Tatzber, F., Fruhwald, F. M., Zweiker, R., Watzinger, N., Eber, B., Wilders-Truschnig, M., Esterbauer, H. & Klein, W. (1997). Increased neopterin in patients with chronic and acute coronary syndromes. *J Am Coll Cardiol*, 30, 703-7.

Svoboda, P., Ko, S. H., Cho, B., Yoo, S. H., Choi, S. W., Ye, S. K., Kasai, H. & Chung, M. H. (2008). Neopterin, a marker of immune response, and 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress, correlate at high age as determined by automated simultaneous high-performance liquid chromatography analysis of human urine. *Anal Biochem*, 383, 236-42.

Tegeder, I., Costigan, M., Griffin, R. S., Abele, A., Belfer, I., Schmidt, H., Ehnert, C., Nejim, J., Marian, C., Scholz, J., Wu, T., Allchorne, A., Diatchenko, L., Binshtok, A. M., Goldman, D., Adolph, J., Sama, S., Atlas, S. J., Carlezon, W. A., Parsegian, A., Lotsch, J., Fillingim, R. B., Maixner, W., Geisslinger, G., Max, M. B. & Woolf, C. J. (2006). GTP cyclohydrolase and tetrahydrobiopterin regulate pain sensitivity and persistence. *Nat Med*, 12, 1269-77.

Turgan, N., Habif, S., Parildar, Z., Ozmen, D., Mutaf, I., Erdener, D. & Bayindir, O. (2001). Association between homocysteine and neopterin in healthy subjects measured by a simple HPLC-fluorometric method. *Clin Biochem*, 34, 271-5.

Venables, W. N. & Ripley, B. D. 2002 *Modern Applied Statistics with S*, New York, Springer.