Inflammation and Complex Regional Pain Syndrome: The role of alpha$_1$-adrenoceptors

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Declaration

I declare that:

a) The thesis is my own account of my research, except where other sources are acknowledged,

b) All co-authors, where stated and certified by my principal Supervisor or Executive Author, have agreed that the works presented in this thesis represent substantial contributions from myself, and

c) The thesis contains as its main content, work that has not been previously submitted for a degree at any other university.

Linda Kurnia Wijaya
Statement of Contributions

This thesis is comprised of four experimental chapters and is my original research. The first two chapters have been published in peer-reviewed journals and the last two chapters are written as manuscripts for publication. I am the first author of these publications, and substantially involved in conceptualization and project design, sample collection and laboratory work, data analysis and interpretation, and preparation and submission of the manuscripts.

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Abstract

A growing number of studies have investigated novel roles of the alpha1-adrenoceptor (α1-AR; a receptor for sympathetic neurotransmitter catecholamines) in inflammation. Stimulation of α1-AR in immune cells modulates cellular functions, including migration and inflammatory cytokine production. However, whether α1-ARs in the skin play a role in cutaneous inflammation is not well understood. Complex regional pain syndrome (CRPS) is a complex and debilitating type of neuropathic pain. In the acute stage of CRPS, excessive inflammation persists in the affected limb. Moreover, α1-AR expression is heightened in the skin of the affected limb. However, the causes of this upregulation of cutaneous α1-AR expression and whether this upregulation contributes to the persistent inflammation are unknown.

In this study, the role of α1-AR in cutaneous inflammation was explored, in both normal and pathological conditions, utilizing in vitro, in vivo and ex vivo approaches. It was hypothesized that inflammatory mediators produced by keratinocytes, as a response to injury, would up-regulate α1-AR expression in these cells, and that heightened expression of α1-AR would increase α1-AR sensitivity to stimulation, leading to further release of inflammatory mediators from keratinocytes. Moreover, in CRPS, it was hypothesized that this feedback loop would be amplified and the response stronger than in healthy controls.

A positive feedback interaction between α1-AR and inflammatory mediators in keratinocytes was demonstrated. In particular, α1-AR expression increased after exposure to the primary proinflammatory cytokine tumour necrosis factor α (TNFα). Furthermore, activation of α1-AR further induced a pro-inflammatory cytokine, interleukin 6 (IL-6), expression, thereby suggesting a positive feedback loop between α1-AR and IL-6 in keratinocytes. Interestingly, the interaction was stronger in keratinocytes obtained from CRPS patients, particularly those with high baseline levels of α1-AR expression, compared to healthy controls.

In conclusion, this study demonstrated a positive feedback interaction between α1-AR and IL-6 in the skin, which may play an important role in normal cutaneous inflammation. Maintaining homeostasis of this interaction could be crucial to prevent the development of persistent inflammation underlying pathophysiology in chronic diseases, such as CRPS.
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List of Abbreviations

-RT  No Reverse Transcriptase Control
ARs  Adrenoceptors
α-AR  Alpha-adrenoceptor
α1-AR  Alpha1-adrenoceptor
α1A-AR  Alpha1-adrenoceptor subtype A
α1B-AR  Alpha1-adrenoceptor subtype B
α1D-AR  Alpha1-adrenoceptor subtype D
α2-AR  Alpha2-adrenoceptor
β-AR  Beta-adrenoceptor
β2-AR  Beta2-adrenoceptor
bp  Base pair
Ca2+  Calcium
cAMP  Cyclic Adenosine Monophosphate
CREB  cAMP Response Element-Binding protein
CRPS  Complex Regional Pain Syndrome
Ct  Cycle threshold
CXCR4  Chemokine Receptor motif 4
CPT  Cold Pressor Test
DAG  Diacyl-glycerol
DC  Dendritic Cells
DMEM  Dulbecco’s Modified Eagle Medium
DNA  Deoxyribonucleic Acid
DRG  Dorsal Root Ganglia
ELISA  Enzyme-linked Immunosorbent Assay
ERK  Extracellular signal-Regulated Kinases
FBS  Fetal Bovine Serum
G  G Force (Relative Centrifugal Force)
GAPDH  Glyceraldehyde 3-phosphate Dehydrogenase
GPCRs  G Protein-coupled Receptors
h  Hour(s)
H & E  Hematoxylin and Eosin
HaCaT  Immortalized human keratinocyte cell line
HCC  Hepatocellular Carcinoma
HCl  Hydrochloric Acid
HIV  Human Immunodeficiency Viruses
HRP  Horseradish Peroxidase
IgE  Immunoglobulin E
IL-1β  Interleukin-1β
IL-1ra  interleukin-1 receptor antagonist
IL-2  Interleukin-2
IL-6  Interleukin-6
IL-8  Interleukin-8
IP₃  inositol (1,4,5) triphosphate
JAK-STAT  Janus Kinase/Signal Transducer and Activator of Transcription
JRA  Juvenile Rheumatoid Arthritis
KDa  Kilo Dalton
KM  Keratinocyte Media
LPS  Lipopolysaccharide
MAPK  Mitogen-Activated Protein Kinase
MCP-1  Monocyte Chemoattractant Protein-1
Mg²⁺  Magnesium
mRNA  messenger Ribonucleic Acid
NaCl  Sodium Chloride
NF-κB  Nuclear Factor kappa-light-chain-enhancer of activated B cells
NGF  Nerve Growth Factor
ns-KM  Non-supplemented Keratinocytes Media
OD  Optical Density
PBMCs  Peripheral Blood Mononuclear Cells
PE  Phenylephrine
PIP₂  Phosphatidylinositol 4,5-biphosphate
PKB/Akt  Protein Kinase B
PKC  Protein Kinase C
PLC  Phospholipase C
PZ  Prazosin
qRT-PCR  Quantitative Real-Time Polymerase Chain Reaction
RNA  Ribonucleic Acid
SD  Standard Deviation
SEM  Standard Error of the Mean
SNS    Sympathetic Nervous System
sTNF-R1 Soluble tumour necrosis factor (TNF) receptors
TBS    Tris-Buffered Saline
TBS-T  Tris-Buffered Saline supplemented with 0.05% Tween-20
TGF-β  Transforming Growth Factor beta
TLRs   Toll-like Receptors
TNFα   Tumour Necrosis Factor alpha
TNFR1  TNF receptor type 1
TNFR2  TNF receptor type 2
UVB    Ultraviolet B
WB     Western Blotting
Chapter 1
Chapter 1. Introduction

1.1. General Overview

A role of alpha₁-adrenoceptors (α₁-AR) in mediating cross-talk between the sympathetic nervous system and the immune system has been suggested. This highlights a possible role of α₁-AR in inflammation. In immune cells, key players in inflammation, a positive feedback interaction between the α₁-AR and inflammatory mediators has been demonstrated and may play an important role in normal inflammation. However, exaggeration and dysfunction of this interaction may contribute to the development of chronic inflammatory diseases.

The acute stage of complex regional pain syndrome (CRPS) is associated with excessive cutaneous inflammation, marked by increased inflammatory mediator levels and heightened α₁-AR expression in the skin (Huygen et al., 2002; Huygen et al., 2004a; Birklein et al., 2014; Drummond et al., 2014a; Drummond et al., 2014c; Finch et al., 2014). However, there are knowledge gaps in the role of α₁-AR in cutaneous inflammation; specifically, what causes an increase in cutaneous α₁-AR expression, what are the consequences of this upregulation, and whether α₁-AR interact with inflammatory mediators in the skin. In this study, I will investigate these research questions to provide better insight into the interaction between α₁-AR and inflammatory processes in the skin. This knowledge is important and may provide a potential therapeutic target to prevent the development of persistent inflammation in pathological conditions.

The first part of this chapter outlines fundamental literatures on the sympathetic nervous system, the α₁-AR and inflammation. The second part describes evidence on α₁-AR-mediated cross-talk between the sympathetic nervous system and the
immune system and how this cross-talk may contribute to the development of certain pathological conditions such as chronic inflammatory diseases and cancer. The last part identifies knowledge gaps in the role of $\alpha_1$-AR in cutaneous inflammation, and how this knowledge may be important in chronic pain conditions such as CRPS is discussed.

1.2. The sympathetic nervous system and adrenoceptors

The sympathetic nervous system (SNS) is part of the autonomic nervous system and plays a role in the fight-or-flight response, triggered by fear, threat and stress. The sympathetic chain is located next to the vertebral column with preganglionic sympathetic neurons arising from the thoracic (T1) to lumbar (L2) part of the spinal cord and each preganglionic neuron synapses with several postganglionic neurons (approximately 1:20 ratio) (McCorry, 2007). These postganglionic sympathetic neurons exit the chain and terminate on effector tissues such as heart (Coote & Chauhan, 2016), tracheobronchial blood vessels of the airways (Nadel & Barnes, 1984; van der Velden & Hulsmann, 1999), eyes (McDougal & Gamlin, 2015), kidney (Burnstock & Loesch, 2015), and cutaneous blood vessels (Ruocco et al., 2002). Upon activation, the SNS releases neurotransmitter catecholamines, such as noradrenaline, from post-ganglionic neurons. These catecholamines bind to adrenoceptors (ARs) to modulate the physiological function of target tissues, such as heart rate and blood pressure regulation (Carrier & Bishop, 1972; Herman & Sandoval, 1983; McCorry, 2007), bronchodilation (Nadel & Barnes, 1984), vasoconstriction (Alosachie, 1988), and pupil dilation (McDougal & Gamlin, 2015; Larsen & Waters, 2018). In the skin, the sympathetic nerves innervate several target tissues, such as hair follicles, arrector pili muscles, sweat glands and cutaneous blood vessels, which are involved in thermoregulation (Donadio et al., 2006; Glatte et al., 2019). The
catecholamine noradrenaline released from the SNS regulates skin cell function, such as keratinocyte proliferation to promote hair growth (Kong et al., 2015) and fibroblast proliferation and migration during wound healing (Wallert et al., 2011; Liao et al., 2014).

The ARs are transmembrane proteins and members of G protein-coupled receptors (GPCRs). These receptors are divided into two types, i.e. $\alpha$- and $\beta$-adrenoceptors, based on their response to catecholamine stimulation. The $\alpha$-ARs mediate excitatory functions of the tissues, while the $\beta$-ARs mediate inhibitory functions, with some exceptions depending on the tissues (Ahlquist, 1948). Each type of AR is further classified into the following subtypes: $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$, $\alpha_{2A}$, $\alpha_{2B}$, $\alpha_{2C}$, $\beta_{1}$, $\beta_{2}$, and $\beta_{3}$ (see review in (Civantos Calzada & Aleixandre de Artinano, 2001)). The summary of the AR classification is presented in Figure 1-1.

![Figure 1-1. A schematic figure of the adrenoceptor classifications (compiled by L. Wijaya).](image-url)
This study will focus on exploring the role of α₁-AR under normal and pathological conditions. The main aims of this study are to investigate the role of α₁-AR in regulating inflammation, and how alterations in this role may trigger the development of some inflammatory pathological conditions.

1.3. Alpha₁-adrenoceptors

The α₁-adrenoceptor (α₁-AR) is located post-synaptically and is mainly expressed in smooth muscles. As a member of GPCR subclass G<sub>q/11</sub>, catecholamine binding to α₁-AR activates the G<sub>q/11</sub> signalling pathway which results in intracellular Ca<sup>2+</sup> release from the endoplasmic reticulum. This Ca<sup>2+</sup> efflux works together with diacyl-glycerol (DAG) to activate protein kinase C (PKC), which mediates activation of other cellular responses (Figure 1-2; (Bruce et al., 2014)). Classically, a normal physiological role of α₁-AR is to modulate smooth muscle contraction, growth and proliferation, specifically in the cardiovascular system. The aorta, the biggest arterial vessel, has abundant smooth muscle cells in its tunica media layer. Incubation of rat aorta with the α₁-AR specific agonist phenylephrine stimulates its contraction (Doggrell, 1992). Moreover, α₁-AR stimulation in rat vascular smooth muscle cells induces DNA synthesis, cell growth and proliferation (Nakaki et al., 1990). As part of the cardiovascular system, the heart also expresses α₁-AR, specifically on the cardiac myocytes (review on (O’Connell et al., 2014)). Signalling through α₁-AR is cardioprotective. Normal physiological functions of α₁-AR in the heart include providing a protection against cardiac cell death, inducing cardio protection and positive inotropy, facilitating normal adaptive hypertrophy without affecting the contractile functions of the cardiomyocytes (O’Connell et al., 2014).
Chapter 1. Introduction

Figure 1-2. The G_q11 signalling pathway.

An activation of GPCR through the binding of its ligands results in a dissociation of the GPCR α-subunit from its complex (1). The α-subunit activates phospholipase C (PLC) (2) to hydrolyse phosphatidylinositol 4,5-biphosphate (PIP_2) into diacyl-glycerol (DAG) and inositol (1,4,5) triphosphate (IP_3) (3). A binding of IP_3 to calcium channels on the endoplasmic reticulum membrane (4) results in Ca^{2+} mobilization to cytoplasm (5). Furthermore, a phosphorylation of protein kinase C (PKC) by DAG (6) and Ca^{2+} efflux from the endoplasmic reticulum further activates other cellular responses ((Bruce et al., 2014) with some modifications compiled by L. Wijaya).

Based on growing molecular and pharmacological studies, the α_1-AR is further classified into several subtypes, i.e. α_1-AR subtype A (α_{1A}-AR), α_{1B}-AR and α_{1D}-AR (reviewed by (Piascik & Perez, 2001)). The expression levels and the function of each AR subtype vary depending on the cell or tissue in which they are expressed (Kikuchi-Utsumi et al., 1997; Jensen et al., 2011; O’Connell et al., 2014). In addition, α_1-AR stimulation results in differential regulation on each α_1-AR subtype expression. As an example, in adult rat cardiomyocytes, stimulation of α_1-AR leads to an increase in
α₁A-AR and a decrease in both α₁B-AR and α₁D-AR (Rokosh et al., 1996). Similar reduction in α₁B-AR expression following α₁-AR stimulation was also shown in neonatal rat cardiomyocytes (Luther et al., 2001).

Growing evidence shows that other tissues/cells, such as brain, liver, kidney, spleen (Price et al., 1994), spinal cord (Giroux et al., 1999) and dorsal root ganglia (DRG; Nicholson et al., 2005; Dawson et al., 2011), glial cells (Kulik et al., 1999), bone cells (Obata et al., 2007; Suga et al., 2010), immune cells (Heijnen et al., 1996; Ricci et al., 1999), and keratinocytes (Dawson et al., 2011; Li et al., 2013), also express α₁-AR. This has prompted investigation of novel physiological roles of α₁-AR. For example, in the brain, the expression of α₁-AR has been found widely in neurons and glial cells (Kulik et al., 1999). While the role of α₁-AR in the brain is not fully understood, some important roles of α₁-AR in facilitating normal brain function, such as neurogenesis, gliogenesis, and behavioural responses to an environmental change have been suggested (Stone et al., 1999; Gupta et al., 2009). Specifically, impaired α₁-AR signalling in the brain may be involved in depression, cognitive dysfunction and drug-induced locomotor activity (Nalepa et al., 2013). In osteoclasts, bone cells that have a role in bone remodelling, signalling through α₁-AR mediates nerve-bone cell interaction during osteoclastogenesis (development of osteoclasts; Suga et al., 2010)). Stimulation of α₁-AR leads to osteoclastic cell activation and production of interleukin (IL)-6, a cytokine that supports proliferation and differentiation of osteoclast progenitors. Since the discovery of α₁-AR expression in several types of immune cells, a growing number of studies have investigated the involvement of α₁-AR signalling in modulating immune cell functions (Ricci et al., 1999; Roupe van der Voort et al., 2000; Rouppe van der Voort et al., 2000; Kavelaars, 2002; Maestroni, 2006; Grisanti et al., 2011a), which includes their migration and inflammatory cytokine
production (Heijnen et al., 1996; Maestroni, 2000; Roupe van der Voort et al., 2000; Grisanti et al., 2011b). This highlights a possible involvement of $\alpha_1$-AR in inflammation.

1.4. Inflammation

Inflammation is a complex and important physiological process to combat infection and initiate repair after injury. Following an injury, acute inflammatory responses from local tissues are activated. Key pro-inflammatory cytokines, such as tumour necrosis factor $\alpha$ (TNF$\alpha$), interleukin (IL)-1$\beta$ and IL-6, and growth factors such as nerve growth factor (NGF), are released by immune cells and resident cells, such as keratinocytes in the skin, to promote an inflammatory response and initiate the healing process (Zhang & An, 2007; Bernard et al., 2012; Shi et al., 2013; Birklein et al., 2014).

In acute inflammation, some clinical presentations such as redness, swelling, heat, loss of function and pain, are observed at the site of injury. These physiological signs of inflammation also reflect changes at cellular levels, such as (1) activation of inflammatory pathways by harmful environmental stimuli, (2) inflammatory cytokine and growth factor release by both resident immune cells, such as macrophages, and resident structural skin cells, such as keratinocytes and fibroblasts, (3) recruitment of circulating immune cells to the site of injury, (4) an increased permeability of vascular endothelium to allow selective extravasation of plasma protein and neutrophils from blood vessels to the injured site, and (5) resolution of inflammation and initiation of tissue repair and remodelling (Libby, 2007; Medzhitov, 2008; Takeuchi & Akira, 2010; Chen et al., 2017a).

Inflammatory mediators activate nociceptors indirectly or directly by binding to their receptors on primary afferent nociceptors (Chao, 2003; Ji et al., 2014; Cook et al., 2018). Thus, inflammatory mediators induce pain. As examples, an injection of a pro-
inflammatory cytokine, such as TNFα or IL-1β, to uninjured animals induces neuropathic pain behaviours (hyperalgesia and allodynia), as intense as the pain that arises from peripheral nerve injury (Wagner & Myers, 1996a; Schafers et al., 2003; Zelenka et al., 2005). Moreover, this pain is aggravated when inflammatory mediators are administered locally to injured nerves (DeLeo et al., 1996; Schafers et al., 2003; Kim & Moalem-Taylor, 2011). On the contrary, administration of neutralizing agents to inflammatory mediators (Clatworthy et al., 1995; Schafers et al., 2001) or deletion of genes expressing these mediators, can alleviate the pain (Ramer et al., 1998; Kim & Moalem-Taylor, 2011). In normal inflammatory processes, this pain usually disappears once the healing process is completed.

TNFα is a pro-inflammatory cytokine and plays an important role in the innate immune system. In the skin, resident cells such as keratinocytes, fibroblasts and macrophages produce TNFα locally, following tissue injury (Fahey et al., 1995; Zelova & Hosek, 2013). TNFα signals through its receptors, mainly TNF receptor type 1 (TNFR1) and TNFR2, to activate different cellular responses (Bradley, 2008). Signalling through TNFR1 mediates activation of inflammatory signalling cascades and programmed cell death pathways (Bradley, 2008), while TNFR2 promotes cell migration and proliferation (Bradley, 2008). TNFα acts very rapidly. A study using cultured human keratinocytes showed that administration of TNFα induced expression of genes involved in innate immunity and inflammation as early as 1 hour after exposure (Banno et al., 2004).

Growing evidence has shown cross-talk between cytokines and growth factors, such as nerve growth factor (NGF), that contributes to the inflammatory process (Lindholm et al., 1987; Olgart & Frossard, 2001; Takei & Laskey, 2008b, 2008a). In vitro, the pro-inflammatory cytokines, IL-1 and TNFα, induce NGF secretion from non-neuronal cells isolated from sciatic nerves (Lindholm et al., 1987), human pulmonary fibroblasts
(Olgart & Frossard, 2001), and neuronal cells (Takei & Laskey, 2008b). Reciprocally, NGF induces TNFα expression in neuronal cells through the involvement of PKB/Akt (Protein Kinase B; an enzyme involved in the NGF signalling pathway) and NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells; a transcription factor involved in inflammatory signalling pathways) activation (Takei & Laskey, 2008b). This positive feedback interaction between TNFα and NGF is important for promoting neuronal cell survival; indeed, failure of this cross-talk may contribute to the pathophysiology of neurodegenerative diseases, such as Alzheimer’s disease (Takei & Laskey, 2008a).

Excessive inflammatory responses are thought to contribute to the development of persistent inflammation and complicate the pathophysiology of some diseases, such as chronic inflammatory diseases (Bernard et al., 2012; Nedoszytko et al., 2014; Kim & Moudgil, 2017), chronic wound healing (Eming et al., 2007; Zhao et al., 2016), and chronic pain (Kramer et al., 2011; Marinus et al., 2011; Sommer et al., 2018). For example, arthritis is a chronic inflammatory autoimmune disease in which pro-inflammatory cytokines, such as TNFα, IL-6, and IL-1, are elevated in biological tissues (synovial fluid, serum, cartilages) (Choy & Panayi, 2001; Tsuchida et al., 2014; Ren et al., 2018). These elevated pro-inflammatory cytokine levels are believed to contribute to the development of persistent inflammation and, eventually, the development of autoimmunity that leads to tissue damage (cartilage and bone) (Kim & Moudgil, 2017).

Administration of neutralizing agents to inhibit inflammatory mediator and NGF production has been shown to be beneficial for chronic pain conditions such as CRPS. In a case study, Huygen and colleagues (Huygen et al., 2004b) demonstrated the effectiveness of anti-TNFα (infliximab) in two CRPS patients. Following this treatment, the cutaneous levels of TNFα and IL-6 were significantly reduced in blister fluid
obtained from the affected limb. In addition, the clinical signs of CRPS and patient well-being were improved. In a double-blind, randomized, placebo-controlled trial, involving a larger number of CRPS patients, the benefit of anti-TNFα was further investigated (Dirckx et al., 2013). Although the trial was discontinued due to budgetary limitations, the preliminary report showed a trend towards similar benefits of anti-TNFα in CRPS patients (Dirckx et al., 2013). While anti-NGF has not been tested in clinical settings, in a tibia fracture rat model of CRPS, administration of anti-NGF antibody reduced NGF levels and neuropathic pain behaviour, such as hind paw mechanical allodynia and hindlimb unweighting (Sabsovich et al., 2008b; Wei et al., 2016).

1.5. Cross-talk between the sympathetic nervous system and immune system, mediated through α₁-adrenoceptors

There is growing evidence that α₁-AR mediate cross-talk between the SNS and the immune system. Stimulation of α₁-AR modulates immune cell (peripheral blood leukocytes, dendritic cells, monocytes, macrophages) functions, such as their migration and cytokine production (Heijnen et al., 1996; Maestroni, 2000; Roupe van der Voort et al., 2000; Grisanti et al., 2011b). As an example, noradrenaline administration to bone-marrow derived dendritic cells (DC) enhances cell migration and the effect is stronger in mature DCs compared to immature DCs (Maestroni, 2000). In vivo, topical administration of the α₁-AR antagonist prazosin on a mouse ear, but not the β-adrenergic antagonist propranolol, inhibits FITC-induced (fluorescein isothiocyanate, fluorescence molecules) Langerhans cell (skin DC) migration to lymph nodes (Maestroni, 2000). This suggests that sympathetic
catecholamine neurotransmitters modulate immune cell migration by acting on the \( \alpha_1 \)-AR.

Monocytes play an important role in the innate immune system. Utilizing an \textit{in vitro} approach, Grisanti and colleagues investigated the role of \( \alpha_1 \)-AR in modulating monocyte's responses to lipopolysaccharide (LPS; a gram-negative bacterial cell wall component that activates Toll-like receptor 4 (TLR4) in immune cells) in producing inflammatory cytokines (Grisanti et al., 2011b). They showed that administration of the \( \alpha_1 \)-AR agonist phenylephrine and LPS enhanced IL-1\( \beta \) production from a monocyte cell line, THP-1, and human primary monocytes, compared to the cells treated with phenylephrine or LPS only. However, the modulatory effect of phenylephrine on LPS-induced IL-1\( \beta \) production was blocked in the presence of the \( \alpha_1 \)-AR antagonist BE2254 (Grisanti et al., 2011b). Further investigation showed that the interaction between \( \alpha_1 \)-AR and TLR4 signalling pathways was mediated through activation of PKC/p38 MAPK (protein kinase C/p38 MAP kinase).

Moreover, inflammatory cytokines, such as TNF\( \alpha \) and IL-1\( \beta \), modulate \( \alpha_1 \)-AR expression in immune cells. Resting THP-1 cells express \( \alpha_{1B} \)-AR and \( \alpha_{1D} \)-AR, without any detectable expression of \( \alpha_{1A} \)-AR (Rouppe van der Voort et al., 1999). Interestingly, administration of inflammatory cytokine TNF\( \alpha \) or IL-1\( \beta \) to THP-1 cells increases \( \alpha_{1A} \)-AR expression to detectable levels but decreases \( \alpha_{1B} \)-AR and \( \alpha_{1D} \)-AR expression levels (Heijnen et al., 2002). These studies indicate a positive feedback interaction between \( \alpha_1 \)-AR and inflammatory cytokines in immune cells, which may be important in normal inflammatory processes.

In healthy control peripheral blood mononuclear cells (PBMCs), the expression of \( \alpha_1 \)-AR is undetectable (Casale & Kaliner, 1984). However, in chronic inflammatory diseases, such as juvenile rheumatoid arthritis (JRA), PBMCs express functional \( \alpha_1 \)-
AR (Heijnen et al., 1996). Heijnen and colleagues demonstrated that stimulating JRA PBMCs with phenylephrine resulted in a significant increase in IL-6 production from the cells, while IL-6 production from healthy control PBMCs remained unchanged (Heijnen et al., 1996). On the contrary, application of the $\alpha_1$-AR antagonist doxazosin inhibited a stimulatory effect of phenylephrine on IL-6 production. This reveals that interaction between $\alpha_1$-AR and IL-6 in PBMCs is specifically mediated through $\alpha_1$-AR signalling. To investigate the significance of this interaction in vivo, Roupe van der Voort and colleagues administered a standardized cold pressor test (CPT; a stressor) to JRA patients and healthy controls to induce endogenous catecholamine production, which was confirmed by an immediate plasma noradrenaline spike measured within 1 min after the CPT (Roupe van der Voort et al., 2000). The CPT-induced noradrenaline augmented JRA PBMCs’ response to lipopolysaccharide challenge, resulting in elevated IL-6 production, while IL-6 production in healthy control PBMCs was unaltered (Roupe van der Voort et al., 2000). Investigation into the mechanism behind the $\alpha_1$-AR-induced IL-6 production in PBMCs showed that this interaction was mediated through activation of extracellular signal regulated kinases (ERKs) (Rouppe van der Voort et al., 2000).

In a canine model of chronic low-grade system of inflammation, administration of daily intraperitoneal injection of LPS in dogs for 2 weeks induced protein levels of TNF$\alpha$ and IL-6 in plasma and right atrial tissues (Chen et al., 2017b). Additional oral administration of the $\alpha_1$-AR antagonist doxazosin reduced TNF$\alpha$ and IL-6 protein levels compared to animals that were treated with LPS only. Moreover, doxazosin treatment also reduced NF-$\kappa$B activation, which may indicate the involvement of NF-$\kappa$B activation in mediating the interaction between $\alpha_1$-AR and inflammatory mediators in this in vivo model.
Chapter 1. Introduction

Pro-inflammatory cytokines, such as IL-1β, IL-6 and TNFα, have been suggested to mediate the development of depression symptoms. Utilizing an LPS-induced depression-like behaviour rat model, Kurosawa and colleagues demonstrated that intraperitoneal injection of LPS increased serum IL-1β, IL-6 and TNFα levels and the number of tyrosine-hydroxylase positive neurons (a marker for dopaminergic and noradrenergic neurons) around the locus coeruleus (Kurosawa et al., 2016). Further investigation revealed that intracerebroventricular injection of IL-1β and IL-6 activated tyrosine hydroxylase-positive neurons around the locus coeruleus and led to the development of depression-like behaviour. On the contrary, pre-treatment with prazosin prior to the intracerebroventricular cytokine injection abolished this behaviour. Interestingly, intracerebroventricular injection of phenylephrine alone also induced depression-like behaviour in the experimental rats. This suggests that α₁-AR plays an important role in mediating pro-inflammatory cytokine-induced depression symptoms.

Consumption of addictive stimulant drugs, such as cocaine, has been shown to alter the immune system. Specifically, septic complications are often reported among drug users. An in vivo experiment using rats demonstrated that intravenous injection of cocaine altered cardiovascular function and increased susceptibility to LPS-induced inflammation (Knuepfer et al., 2004). In addition, amongst rats with strong vascular responses to cocaine, LPS administration post-cocaine treatment resulted in heightened pupil dilation and inflammatory cytokine levels (IL-6 and IL-10), and ultimately death. However, pre-treatment of prazosin prior to cocaine-LPS administration reduced detrimental effects of cocaine on immune responses to infection, improved cardiovascular responses to cocaine and enhanced survival.

Studies have suggested that stressful events may induce inflammatory mediator levels and that this involves α₁-AR signalling. In an experiment involving human
participants, a hypoxic condition induced by high altitude (an environmental stressor) significantly increased resting plasma IL-6 levels compared to the levels measured at zero altitude (sea-level; (Mazzeo et al., 2001)). Performing high impact exercise (a physical stressor) resulted in elevated plasma IL-6 levels, which were higher at high altitude compared to sea-level. However, administration of prazosin inhibited the stress-induced increase in plasma IL-6. Chronic stress has been shown to enhance the progression of periodontitis (a chronic inflammatory disease affecting the gums) by inducing $\alpha_1$-AR-mediated inflammatory responses. In a rat model of periodontitis, $\alpha_1$-AR expression was increased in periodontal tissues and this expression was further elevated under chronic stress conditions (Lu et al., 2014). However, blocking this $\alpha_1$-AR signalling through perfusion of the non-selective $\alpha$-AR antagonist phentolamine reduced LPS-induced inflammatory responses in the periodontal tissues. Together, the aforementioned studies support an important role of $\alpha_1$-AR in mediating stress-induced inflammatory responses.

Cross-talk between $\alpha_1$-AR and inflammatory mediators may also play a role in cancer progression. In some type of cancers, an increased production of noradrenaline is positively correlated with the tumorigenesis and considered to be a risk factor in developing cancer (reviewed by (Fitzgerald, 2009)). In addition, chronic inflammation induced by excessive inflammatory responses contributes to tumour development and carcinogenesis (Karin & Greten, 2005). In patients suffering hepatocellular carcinoma (HCC), the density of sympathetic nerve innervations on the tumours correlates positively with tumour progression and negatively with disease-free survival (Huan et al., 2017). In animal models of chemical-induced HCC, tumour progression is also associated with an increased production of inflammatory cytokines from Kupffer cells (a resident macrophage in the liver) (Huan et al., 2017). In contrast, chemical sympathectomy or administration of the $\alpha_1$-AR antagonist, prazosin, prior to inducing HCC, supressed inflammatory cytokine production from
Kupffer cells and reduced the tumour size. Recent evidence also shows that $\alpha_1$-AR antagonists inhibit cancer cell proliferation and motility in other types of cancers (Lin et al., 2007; Assad Kahn et al., 2016; Colciago et al., 2016).

In other cell types, such as cardiomyocytes and $\alpha_1A$-AR-stably-expressed Rat-1 fibroblasts, Perez and colleagues demonstrated a positive feedback interaction between $\alpha_1$-AR and IL-6 (Perez et al., 2009). Cardiomyocytes obtained from mouse pups and Rat-1 fibroblasts were treated with epinephrine (adrenaline) in the presence of $\beta$-AR and $\alpha_2$-AR antagonists, propranolol and rauwolscine, respectively. Administration of epinephrine resulted in an increased cellular production of IL-6. Moreover, pre-treatment with the $\alpha_1$-AR antagonist prazosin blocked the effect of epinephrine on IL-6 levels (Perez et al., 2009). These results suggest that epinephrine induces IL-6 production from both cardiomyocytes and Rat-1 fibroblasts by acting through $\alpha_1$-AR. Investigations into the mechanisms behind the interaction between $\alpha_1$-AR and IL-6 showed that stimulation of $\alpha_1$-AR enhanced IL-6 transcription, mediated through activation of p38 MAPK and NF-κB (Perez et al., 2009).

Collectively, the studies mentioned above indicate that cross-talk between $\alpha_1$-AR and inflammatory cytokines may be important in normal inflammation. However, dysregulation of this cross-talk may alter normal inflammatory processes, which subsequently induces an unresolved and persistent inflammatory state.

An increasing number of studies also indicate that $\alpha_1$-AR-mediated cross-talk between the SNS and immune system may be involved in the pathophysiology of chronic pain. In particular, in neuropathic pain (a type of chronic pain that arises from an injury or disease affecting the nervous system (Treede et al., 2008)), excessive inflammation (Huygen et al., 2002; Huygen et al., 2004a; Li et al., 2013; Birklein et al., 2014) and heightened $\alpha_1$-AR expression are present in the skin of the affected limb.
of patients and in neuropathic pain animal models (Drummond et al., 2014a; Drummond et al., 2014c; Finch et al., 2014). In the present study, the role of $\alpha_1$-AR in mediating cross-talk between the SNS and immune system in complex regional pain syndrome (CRPS; a type of neuropathic pain) will be investigated, to determine whether this may contribute to the pathophysiological mechanism underlying this chronic pain condition. The following sections will review the involvement of the SNS and inflammation in the pathogenesis of CRPS, and the possible role of $\alpha_1$-AR in mediating cross-talk between the SNS and immune system.

1.6. Complex regional pain syndrome

Complex regional pain syndrome (CRPS) is a complex and debilitating type of neuropathic pain. This condition is debilitating as the pain causes disturbances in mobility, sleep and social activity (Smith & Torrance, 2012), frequently leading to the development of psychological problems, such as depression, anxiety, and stress (Haythornthwaite & Benrud-Larson, 2001). Like other types of neuropathic pain, CRPS is mainly characterized by hyperalgesia (an increased sensitivity to a stimulus that usually provokes pain – nociceptive stimulus) and allodynia (pain due to a stimulus that does not normally provoke pain – non-nociceptive stimulus) (Merskey & Bogduk, 1994; Li et al., 2000; Baron et al., 2010). Unfortunately, since the severity of pain experience varies between individuals with the same degree of lesion, diagnosing neuropathic pain is often difficult. In addition, mechanisms behind neuropathic pain are not fully understood, which makes it difficult to provide effective targeted treatment for these patients.

One of the triggers of CRPS is an injury to a limb (Brunner et al., 2017; Savas et al., 2017). Based on the evidence of major peripheral nerve damage, CRPS is further
classified into CRPS type I or type II, in which a lesion to peripheral nerves is absent or present, respectively. Although there is no evidence of nerve trunk damage in CRPS I, nerve fibre density in the skin is lower than normal (Albrecht et al., 2006; Oaklander et al., 2006; Morellini et al., 2018; Rasmussen et al., 2018; Drummond et al., 2019). Nevertheless, the burden and the clinical presentations of both CRPS types are similar. The first local population-based epidemiological study in 2003 revealed the incidence of CRPS to be around 6.28 per 100,000 person years (5.46 per 100,000 person years for CRPS type I and 0.82 per 100,000 person years for CRPS type II) (Sandroni et al., 2003). In 2007, a larger epidemiological study based on the Netherlands population updated the incidence rate of CRPS to 26.2 per 100,000 person years (de Mos et al., 2007). Nevertheless, these figures may be an underestimation. As this condition is very debilitating, it is important to clarify the pathophysiological mechanisms of CRPS.

The clinical presentations of CRPS include neuropathic pain behaviours (nociceptive hypersensitivity), autonomic dysfunction (swelling, oedema, excessive sweating), trophic changes (skin, hair and nail changes), motoric dysfunction (muscle weakness, atrophy, contractures). These clinical presentations are included in the Budapest criteria, a recent clinical diagnostic tool for CRPS (Harden & Bruehl, 2005; Harden et al., 2010b; Harden et al., 2010a). Skin temperature differences are observed between the affected and unaffected limb. Additionally, skin colour on the affected limb changes between the acute and chronic stages of CRPS. Both of these changes indicate vascular disturbances in CRPS. One proposed mechanism underlying vascular disturbances in CRPS is SNS dysfunction, mediated through $\alpha_1$-AR. Wasner and colleagues demonstrated the loss of sympathetic vasoconstrictor activity in the affected limb of CRPS patients at the acute stage (Wasner et al., 1999). This resulted in a warmer skin temperature (caused by vasodilation) on the affected limb compared to the contralateral site (“warm” CRPS). Moreover, the levels of blood norepinephrine
were reduced on the affected site (Wasner et al., 1999). It was hypothesized that the loss of cutaneous sympathetic vasoconstrictor activity and the reduction of norepinephrine levels at the affected site led to heightened $\alpha_1$-AR expression in the blood vessels and increased $\alpha_1$-AR sensitivity to catecholamines. This condition results in an increased vasoconstriction and decreased blood flow during sympathetic arousal that leads to the development of ‘cold’ CRPS seen in the chronic stage.

Persistent inflammation is present in the affected limb of CRPS patients, in particular in the acute stage of CRPS (Marinus et al., 2011). The pathophysiology of CRPS is not fully understood, but growing evidence shows that persistent inflammation and disturbances in SNS function in the acute stage of CRPS may contribute to the development of the condition into a chronic stage (Schlereth et al., 2014; Knudsen et al., 2019). Although inflammation is a normal physiological function designed to provide defence mechanisms and initiate healing processes after injury, there is evidence to show that inflammation becomes excessive in CRPS, resulting in a persistent inflammatory condition (Groeneweg et al., 2006; Heijmans-Antonissen et al., 2006; Kramer et al., 2011; Marinus et al., 2011; Birklein et al., 2014). Unfortunately, however, the mechanisms underlying this persistent inflammatory condition in CRPS are not fully understood. Moreover, whether the SNS dysfunction contributes to the development of this persistent inflammation requires further investigation.

1.6.1. Involvement of the sympathetic nervous system in CRPS

Sympathetic dysfunction contributes to the pathophysiology of CRPS. Sympathetic blocks, such as administration of local anaesthetic (Price et al., 1998; Stanton et al., 2013; O’Connell NE & Carr DB, 2016) or sympathectomy (Singh et al., 2003), have been used widely in clinical practice to reduce pain. As an example, stellate ganglion blocks have been commonly used as pain management for CRPS (Price et al., 1998;
Wei et al., 2014), although only short-term pain relief was achieved in some patients
(Price et al., 1998; Ackerman & Zhang, 2006; Cheng et al., 2019). This subset of
CRPS patients is considered to have sympathetically maintained pain. However, due
to the side effects and complications of sympathetic blocks (Doroshenko et al., 2020)
and the lack of good quality double blinded, placebo-controlled, and randomized
studies with sufficient samples size and follow-up period, the safety and efficacy of
sympathetic blockade as a treatment for CPRS needs to be further validated (Cepeda
et al., 2005; Stanton et al., 2013).

Under normal conditions, SNS activity does not usually affect the nociceptors.
However, cross-talk between the SNS and sensory neurons may contribute to the
development of persistent pain in this CRPS subset. Furthermore, studies show that
this cross-talk occurs through a direct interaction between the two nervous systems
or an adrenoceptor-mediated interaction (Davis et al., 1991; Chabal et al., 1992;
Torebjork et al., 1995; Choi & Rowbotham, 1997; Ali et al., 2000; Lee et al., 2000;
Nam et al., 2000; Mailis-Gagnon & Bennett, 2004).

A direct coupling between sympathetic and sensory neurons has been demonstrated
in animal models of neuropathic pain (Chung et al., 1993; McLachlan et al., 1993;
Janig et al., 1996; Ramer & Bisby, 1997; Xie et al., 2007). Several days following
nerve injury, abnormal innervations (sprouting) of postganglionic sympathetic fibres
have been observed surrounding surviving primary afferents in the dorsal root ganglia
(DRG) supplying the injured nerve. In addition, the extent of axon sprouting increases
with time (McLachlan et al., 1993). The sympathetic fibre sprouts are also present in
DRG of patients suffering from persistent neuropathic pain (Shinder et al., 1999).
Interestingly, the degree of this sprouting is correlated with the severity of neuropathic
pain behaviours (Chung et al., 1997). It can be postulated that the presence of
sympathetic nerve sprouts amongst the nociceptors in the DRG may facilitate direct
stimulation of nociceptors through neurotransmitters released by sympathetic nerves, thus establishing sympathetically maintained pain.

The adrenoceptor-mediated cross-talk between the SNS and nociceptors has been observed in several studies. Electrical stimulation of the SNS or a systemic adrenaline injection evoked primary afferent activity in a rat model of neuropathic pain (Devor et al., 1994). Similarly, in neuropathic pain patients with sympathetically maintained pain, an injection or topical administration of adrenergic ligands has been shown to evoke pain (Davis et al., 1991; Chabal et al., 1992; Torebjork et al., 1995; Choi & Rowbotham, 1997; Ali et al., 2000). Interestingly, in neuropathic pain patients who underwent successful sympathectomy, an intracutaneous injection or iontophoresis of noradrenaline into the skin of the symptomatic limb evoked pain similar to the original pain (Torebjork et al., 1995). However, administration of adrenergic ligands to an unaffected limb or in healthy controls did not induce pain (Choi & Rowbotham, 1997; Ali et al., 2000). An injection of the α-adrenoceptor antagonist phentolamine attenuated the excitatory effect of sympathetic stimulation on the DRG, while an injection of β-adrenoceptor antagonist propranolol, did not show any effects (Devor et al., 1994). This demonstrates that the α-adrenoceptors, rather than β-adrenoceptors, mediate the SNS-nociceptor coupling in neuropathic pain.

Studies utilizing a specific agonist and antagonist against α-AR types show that α₁-AR, instead of α₂-AR, is likely to be the primary mediator in the adrenoceptor-mediated coupling between the SNS and nociceptors in neuropathic pain (Davis et al., 1991; Choi & Rowbotham, 1997; Drummond et al., 2016; Drummond et al., 2018). An intradermal injection of the α₁-AR agonist phenylephrine to the affected/symptomatic limb of CRPS patients with sympathetically maintained pain, evoked persistent heightened pain, hyperalgesia and mechano-allodynia, while an injection of phenylephrine to a non-affected limb of CRPS patients or healthy people
evoked only momentary pain (Davis et al., 1991; Mailis-Gagnon & Bennett, 2004; Drummond et al., 2016; Drummond et al., 2018). In addition, pre-treatment with the \( \alpha_1 \)-AR antagonist prazosin reduced hyperalgesia and allodynia to brushing (Drummond et al., 2016). On the other hand, stimulation of \( \alpha_2 \)-AR through its agonist clonidine inhibited noradrenaline release and suppressed pain in patients with sympathetically maintained pain (Davis et al., 1991; Drummond et al., 2016) thus suggesting the analgesic property of \( \alpha_2 \)-AR.

Heightened expression of \( \alpha_1 \)-AR has been observed following nerve injury and may mediate adrenergic sensitivity in primary nociceptive afferents. In a cat model of spinal injury, an autoradiographic study using radioligand \([^3]H\)-prazosin revealed an increase in \( \alpha_1 \)-AR expression in the injured spinal cord, compared to uninjured cats (Giroux et al., 1999). In neuropathic pain rat models, gene expressions of \( \alpha_1 \)-AR were elevated in the DRG or spinal cord of rats with spinal nerve ligation (Xie et al., 2001), sciatic nerve injury (Maruo et al., 2006), or streptozotocin-induced diabetic neuropathy (Lee et al., 2000). This heightened \( \alpha_1 \)-AR expression is also observed in the skin, where primary afferent nociceptors are abundant. In rat models of neuropathic pain, peripheral nerve injury induced a significant increase in \( \alpha_1 \)-AR expression in the epidermis or on surviving dermal nerve bundles of the affected limb, compared to the contralateral site and sham controls (Drummond et al., 2014a; Drummond et al., 2014c). Cutaneous \( \alpha_1 \)-AR upregulation was also observed in the epidermis and dermal nerve fibres of the affected limb of patients suffering from CRPS (Drummond et al., 2014c; Finch et al., 2014; Drummond et al., 2018). This indicates that activation of cutaneous \( \alpha_1 \)-AR may contribute to pain processing in CRPS.
1.6.2. Evidence of excessive inflammation in CRPS

In the acute stage of CRPS, the signs of inflammation, such as pain, heat, redness, and swelling persist in the affected limb, even after the healing process is complete. This may indicate excessive and dysregulated inflammatory responses following injury in the acute stage of CRPS that may contribute to the development of this condition into a chronic stage (Birklein & Dimova, 2017).

Gene expression levels and protein production of inflammatory mediators, such as TNFα, IL-1β, IL-6, are significantly increased following experimental nerve injury in rats (DeLeo et al., 1996; Wagner & Myers, 1996b; Arruda et al., 1998; Okamoto et al., 2001; Pineau & Lacroix, 2007; Üçeyler et al., 2007). In rats that underwent sciatic nerve cryoneurolysis, heightened IL-6 expression was found in the ipsilateral (an affected site) dorsal horn of the spinal cord up to 35 days post-cryoneurolysis, and interestingly, the IL-6 levels were positively correlated with touch-evoked allodynia (DeLeo et al., 1996). Similar heightened IL-6 levels were also found at the injured nerves of other neuropathic pain rat models, such as spinal nerve cryoneurolysis and spinal nerve tight ligation, and these IL-6 levels were associated with touch-evoked alldynia (Arruda et al., 1998). A chronic constriction injury to rat sciatic nerves resulted in elevated TNFα and IL-1β mRNA levels at the injured nerves (Wagner & Myers, 1996b; Okamoto et al., 2001; Üçeyler et al., 2007), which were positively correlated with thermal hyperalgesia and mechanical allodynia (Okamoto et al., 2001). Utilizing immunohistochemistry, TNF-immunoreactivity was found to be increased in local resident cells of the injured sciatic nerves, such as endothelial cells, macrophages, fibroblasts and Schwann cells (Wagner & Myers, 1996b).

In rats with tibia fracture as a model for CRPS type I, levels of TNFα, IL-6, IL-1β, NGF were elevated in the hind paw skin of the fractured limb at 4-weeks post-fracture, compared to uninjured rats (Li et al., 2009a; Li et al., 2010). In addition, these rats
developed neuropathic pain behaviours, such as hind paw mechanical allodynia and unweighting. Interestingly, a sub-cutaneous injection of pro-inflammatory mediators, such as TNFα (Sabsovich et al., 2008a; Li et al., 2010), IL-1β (Li et al., 2009b), or IL-6 (Li et al., 2010) to the hind paw of normal rats induced similar neuropathic pain behaviour as seen in the fractured rats, while such injection aggravated neuropathic pain behaviours when administered locally to injured tissue (DeLeo et al., 1996; Schafers et al., 2003; Kim & Moalem-Taylor, 2011). On the contrary, administration of TNFα antagonist (sTNF-R1; (Sabsovich et al., 2008a)) or IL-1β antagonist (IL-1ra; (Li et al., 2009b)) alleviated neuropathic pain behaviours. Immunohistochemistry analysis of the ipsilateral hind paw skin revealed that inflammatory mediator mRNA and protein expression were significantly increased in keratinocytes.

Inflammatory mediators, such as TNFα and IL-6, were also elevated in the skin and skin blister fluid obtained from the symptomatic limb of CRPS patients (Huygen et al., 2002; Groeneweg et al., 2006; Heijmans-Antonissen et al., 2006; Kramer et al., 2011; Birklein et al., 2014), but not in the blood (Huygen et al., 2002; Schinkel et al., 2006; Schinkel et al., 2009). Collectively, these animal and human studies suggest that cutaneous immune responses may be important as an early response to nerve injury, and excessive accumulation of these inflammatory mediators may contribute to persistent pain in CRPS. However, the mechanisms behind this persistent cutaneous inflammation in CRPS patients are not totally understood.

1.7. Aims and hypotheses

In this study, the role of α1-AR in mediating the nerve-immune system interaction in normal inflammation and in CRPS was investigated, utilizing skin as an experimental model. The main cell types coordinating immune functions in the skin include resident
structural cells, such as keratinocytes and fibroblasts, that work with resident innate immune cells, such as macrophages and Langerhans cells, to orchestrate the local inflammatory process (Kuo et al., 2013; Matejuk, 2018). In addition, nociceptors and sympathetic nerve fibres innervate the skin layers (Ruocco et al., 2002; Donadio et al., 2019), which indicate a possible interaction between the nerves and resident skin cells.

A large body of evidence presented in the above literature review indicates that the SNS, mediated through $\alpha_1$-AR, may play a role in cutaneous inflammation. This is based on the evidence that keratinocytes, the most abundant cells in the epidermis, express $\alpha_1$-AR and produce inflammatory mediators. Moreover, upregulation of $\alpha_1$-AR expression and heightened production of inflammatory mediators in the skin have been observed in several chronic inflammatory diseases and neuropathic pain. However, how $\alpha_1$-AR modulates cutaneous inflammation and what causes $\alpha_1$-AR upregulation and heightened inflammatory mediator production in pathological conditions are not fully understood.

The overall hypothesis investigated was that inflammatory mediator production by keratinocytes in response to an injury act in a feedback loop to up-regulate $\alpha_1$-AR expression in these cells. Consequently, this heightened expression of $\alpha_1$-AR could increase sensitivity to stimulation, leading to further release of inflammatory mediators from keratinocytes, thus establishing a positive feedback interaction. Furthermore, it was hypothesized that in keratinocytes derived from CRPS patients, this positive feedback loop may be stronger compared to keratinocytes isolated from the skin of healthy controls.

To address these hypotheses, in vitro, in vivo, and ex vivo approaches were used to investigate the role of $\alpha_1$-AR in cutaneous inflammation and a possible interaction between $\alpha_1$-AR and inflammatory mediators in the skin. An immortalized human
keratinocyte cell line (HaCaT) and the prototypical pro-inflammatory cytokine, TNFα, were used as an in vitro model and to induce an inflammatory state in cultured keratinocytes, respectively. In the in vivo approach, a skin microdialysis technique was used to determine the effects of α1-AR stimulation in acute skin inflammation, induced by UVB irradiation. In addition, primary keratinocytes were obtained from CRPS patients and healthy control volunteers to investigate the role of α1-AR in modulating inflammation in a neuro-pathological condition.

The specific aims of the study were:

1. To determine whether TNFα induced α1-AR expression in keratinocytes in vitro and to determine if this was restricted to a specific subtype of α1-AR. The results of this aim are presented in Chapter 2.

2. To assess if α1-AR stimulation in activated (TNFα-induced) HaCaT cells further induced inflammatory mediator production, thus establishing a positive feedback interaction between α1-AR and inflammatory mediators in keratinocytes in vitro. The results of this aim are presented in Chapter 3.

3. To investigate the effect of α1-AR stimulation in a UVB-irradiated skin, as an in vivo model for acute cutaneous inflammation. The results of this aim are presented in Chapter 4.

4. To investigate the interaction between α1-AR and inflammatory mediators in keratinocytes cultured ex vivo from the skin of CRPS patients and healthy control volunteers to determine whether the interaction was amplified in CRPS keratinocytes compared to healthy control keratinocytes. The results of this aim are presented in Chapter 5.
Chapter 2. Tumour necrosis factor α induces α₁B-adrenergic receptor expression in keratinocytes

2.1. Preface

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The role of α₁-AR in cutaneous inflammation is not fully understood. Heightened cutaneous α₁-AR expression and excessive inflammation have been identified in some inflammatory diseases and chronic pain, which suggests a potential interaction between α₁-AR and inflammatory processes in the skin. In this chapter, I present an *in vitro* study that identifies the effects of inflammatory signalling on α₁-AR expression in keratinocytes, as the first step to investigate this potential interaction.
Chapter 2. TNFα induces $\alpha_{1B}$-adrenoceptor expression in keratinocytes

2.2. Abstract

Keratinocytes produce cytokines and nerve growth factor (NGF) as part of a repair response to injury, disease or stress, and express alpha$_1$-adrenoceptors ($\alpha_1$-AR). The expression of these receptors is elevated in some inflammatory diseases and chronic pain conditions. In this study, we investigated whether inflammatory signalling affects $\alpha_1$-AR expression in keratinocytes in vitro. Tumour necrosis factor $\alpha$ (TNF$\alpha$) was administered to human keratinocytes, after which the levels of other key pro-inflammatory cytokines and NGF were measured. The production of these cytokines and NGF increased in cells treated with TNF$\alpha$ compared to untreated cells. Furthermore, exposure to TNF$\alpha$ increased gene expression of the $\alpha_1$-AR subtype B in keratinocytes. Our results suggest that inflammatory cytokines released during injury stimulate $\alpha_1$-AR expression in keratinocytes. The up-regulation of $\alpha_1$-AR may amplify the adrenergic sensitivity of these cells to catecholamines released during sympathetic nervous system activation after injury which, in turn, could heighten the inflammatory response.

2.3. Introduction

Cross-talk between the sympathetic nervous system (SNS) and immune system may play a role in inflammation. Inflammatory mediators released during inflammation modulate the expression of the alpha$_1$-adrenoceptor ($\alpha_1$-AR) (a target of the SNS neurotransmitter norepinephrine) in a range of cells, including immune cells, endothelial cells, and mesangial cells (Bucher et al., 2003). In healthy individuals, $\alpha_1$-AR expression in peripheral blood mononuclear cells (PBMCs) is undetectable (Rouppe van der Voort et al., 1999). However, in chronic inflammatory diseases, such as juvenile rheumatoid arthritis, PBMCs express functional $\alpha_1$-AR (Heijnen et al.,
1996). In addition, stress-induced inflammatory cytokines produced by the PBMCs induce $\alpha_1$-AR expression (Roupe van der Voort et al., 2000; Heijnen et al., 2002). As a result, the increase in $\alpha_1$-AR expression could further modulate immune cell activation, thereby influencing their migration and inflammatory cytokine production (Heijnen et al., 1996; Maestroni, 2000).

We recently found that $\alpha_1$-AR is expressed in epidermal keratinocytes (Dawson et al., 2011; Finch et al., 2014). These cells make up around 95% of the epidermis and provide a barrier to environmental hazards and produce inflammatory cytokines and nerve growth factor (NGF). In the present study, we aimed to elucidate further the interaction between inflammation and $\alpha_1$-AR in keratinocytes, and to investigate whether inflammatory cytokines can trigger $\alpha_1$-AR expression in these cells. We utilized tumour necrosis factor $\alpha$ (TNF$\alpha$), a pro-inflammatory cytokine that plays an important role in the innate immune response, to induce an inflammatory state in keratinocytes. It was hypothesized that TNF$\alpha$, or mediators released during the inflammatory cascade induced by TNF$\alpha$, would increase the expression of $\alpha_1$-AR in keratinocytes.

### 2.4. Materials and Methods

HaCaT cells (a spontaneously immortalized human keratinocytes cell line) (Boukamp et al., 1988) were cultured in DMEM media with 10% FBS. Human recombinant TNF$\alpha$ (PeproTech, USA) was administered to HaCaT cells under reduced-serum conditions (0.5% FBS) and incubated for 24 h. Each experimental condition was performed in six wells and repeated four times. For the naïve (control) condition, the same media without TNF$\alpha$ was applied to the cells.
A similar experiment was performed in primary human keratinocytes extracted from 3-mm punch skin biopsies obtained from 4 healthy volunteers (age range 33-55 years). Each participant provided their informed consent for the procedure, which was approved by the Murdoch University human research ethics committee. Primary keratinocytes were dissociated from the skin biopsy using the protocol described by Aasen and Belmonte (Aasen & Izpisua Belmonte, 2010) and maintained in keratinocyte complete media (EpiGro™, Merck, USA).

Prior to TNFα treatment, the media supplements (containing epinephrine, a ligand for α1-AR) were removed from the culture for 24 h and TNFα was applied. HaCaT cells and primary keratinocytes were collected and lysed to measure gene expression levels of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8 and NGF, and α1-AR-subtype B (α1B-AR), utilizing a SYBR® green-based qRT-PCR assay, followed by melting curve analysis. The mRNA levels of α1-AR-subtype A and D were undetectable or beyond the limit of quantification in this qRT-PCR assay (Ct values above 33), respectively (unpublished data). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. All primer sets used in this qRT-PCR assay were validated to amplify mRNA-derived transcripts but not genomic DNA (unpublished data) and are listed in the supplementary materials (Table 2-1; see page 38). A cycle threshold (Ct) value of each sample was collected at the end of the qRT-PCR run (40 cycles). The change in gene expression as a result of TNFα treatment compared to the naive group was represented by ΔΔCt (Yuan et al., 2006).

Protein levels of inflammatory cytokines and NGF secreted by HaCaT cells were measured in the media using a multiplex ELISA kit (Bio-Plex Pro™ Human Cytokine assay; Bio-Rad Laboratories, USA). These inflammatory cytokines were previously
Chapter 2. TNFα induces α1β-adrenoceptor expression in keratinocytes

shown to be elevated in chronic neuropathic pain conditions. The assay was performed according to the manufacturer’s protocol using undiluted media.

The mean ± SEM of the gene expression changes and the protein levels of four biological replicates are presented in the Results. A general linear model with repeated measures was applied to determine the effects of TNFα on inflammatory cytokine, NGF and α1-AR levels.

2.5. Results

In vitro exposure of HaCaT cells to TNFα significantly induced all cytokine mRNA levels, along with NGF, at doses of 1 ng/ml and greater, when compared to naïve cells (Figure 2-1 A-D). A TNFα dose-dependent increase in mRNA expression levels was observed for IL-1β, IL-8, and IL-6 (Figure 2-1 A-C), but not for NGF, which was maximally expressed after exposure to 1 ng/ml TNFα (Figure 2-1 D).

While protein production of IL-1β secreted by HaCaT cells was below the lowest detection limit of the kits (0.6 pg/ml – data not shown), TNFα increased protein production of IL-8, IL-6 and NGF in culture supernatants compared to naïve cells (Figure 2-1 E-F). Protein levels of IL-8 were highest amongst the inflammatory cytokines after exposure to 50ng/ml TNFα (Figure 2-1 E), consistent with mRNA expression levels, although a significant increase in IL-8 protein levels was only seen for cells that were treated with either 10 or 50 ng/ml TNFα (Figure 2-1 E). Increasing concentrations of TNFα applied to the cells induced significant production of IL-6, with IL-6 protein levels increasing in line with increasing levels of TNFα at 1 and 10 ng/ml, with no further increase at 50 ng/ml TNFα (Figure 2-1 F). Compared to IL-8 and IL-6, NGF levels showed a less dramatic increase when different concentrations of TNFα
were administered to the cells, with a significant increase in NGF only observed in cells treated with either 10 or 50 ng/ml TNFα (Figure 2-1 G).

To confirm that TNFα induced an inflammatory state in primary keratinocytes, 10 ng/ml TNFα was administered to the cells. This dose of TNFα increased mRNA levels of IL-1β, IL-8, and IL-6 (Figure 2-1 A, B, and C, respectively), compared to naïve cells, but less strongly than observed in HaCaT cells. However, in contrast to HaCaT cells, TNFα reduced NGF mRNA levels (Figure 2-1 D).

Protein concentrations of pro-inflammatory cytokines also increased, but these increases did not differ significantly from changes in naïve cells due to wide variation in response (Supplementary Materials, Table 2-2; see page 38). NGF protein did not change after exposing primary keratinocytes to TNFα (Supplementary Materials, Table 2-2; see page 38).
Chapter 2. TNFα induces α1B-adrenoceptor expression in keratinocytes
**Chapter 2. TNFα induces α1B-adrenoceptor expression in keratinocytes**

**Figure 2-1. TNFα effects on inflammatory cytokine and NGF gene expression and protein levels.**

The changes in inflammatory cytokine gene expression levels: (A) IL-1β, (B) IL-8, (C) IL-6 and (D) NGF, as a result of TNFα treatment compared to the naïve condition (untreated cells) were represented by ΔΔCt (ΔΔC_{ naïve}=0). The protein levels of (E) IL-8, (F) IL-6 and (G) NGF were measured using a multiplex ELISA assay. The bars represent mean ± SEM of the inflammatory cytokine gene expression and protein levels of four independent replicates (*represents a significant difference compared to naïve, p<0.05; **p<0.05, ***p<0.01, ****p<0.001 across different doses of TNFα).

Exposure to TNFα, or inflammatory mediators induced by TNFα, significantly increased α1B-AR levels in HaCaT cells at 10 or 50 ng/ml TNFα compared to the naïve condition, while there was no change in α1B-AR mRNA levels in the cells treated with the lowest dose of TNFα (Figure 2-2 A). Similarly, an exposure to 10 ng/ml TNFα increased α1B-AR mRNA levels in primary keratinocytes, compared to naïve cells, less strongly than seen in HaCaT cells. An agarose gel representing the correct size qRT-PCR products of α1B-AR as a single band at 112 bp is shown in Figure 2-2 B (grey arrow). Higher α1B-AR gene expression levels in HaCaT cells treated with 10 ng/ml TNFα were confirmed as a thicker band in lane 1 compared to the naïve condition in lane 2. As shown in lanes 3 and 4, genomic DNA was not amplified by the primer sets as only 55 bp primer-dimer bands remained (Figure 2-2 B, black arrow).
Chapter 2. TNFα induces α1B-adrenoceptor expression in keratinocytes

Figure 2-2. TNFα induced mRNA expression levels of α1B-AR in HaCaT cells and primary keratinocytes.

The changes in α1B-AR gene expression levels, as a result of TNFα exposure compared to naïve, are represented as mean ± SEM of the ∆∆Ct of four independent replicates (∆∆Ct_{naïve}=0) (A; *represents a significant difference compared to naïve, p<0.05). A gel view represents α1B-AR (GeneID: ADRA1B) products amplified by the primer sets as a single band at 112 bp (B; grey arrow). Lane 1 and 3 represent qRT-PCR products from HaCaT cells treated with 10ng/ml TNFα and its –RT control, respectively. Lane 2 and 4 represent qRT-PCR products from naïve HaCaT cells and its –RT control, respectively. Lane 5 represents non-template control. The black arrow indicates the remaining primer-dimers of qRT-PCR. L=DNA ladder; –RT control=no reverse transcriptase control; bp=base pair.

2.6. Discussion

In this study, we demonstrate that TNFα, a pro-inflammatory cytokine, is able to induce an inflammatory state in cultured keratinocytes, marked by increases in protein and/or mRNA expression for the key pro-inflammatory cytokines IL-1β, IL-6, IL-8 and NGF. In addition, TNFα, or a TNF-induced inflammatory state, was able to upregulate α1B-AR gene expression levels in keratinocytes. Previous studies have
shown differential effects of inflammatory cytokines on $\alpha_1$-AR subtype expression in several cell types (Heijnen et al., 2002; Bucher et al., 2003) but, to our knowledge, this is the first study to show that inflammatory cytokines modulate $\alpha_1$-AR expression in keratinocytes.

In a study by Heijnen and colleagues, administration of inflammatory cytokines to human monocytic cells resulted in a concentration-dependent increase in $\alpha_{1A}$-AR expression, while $\alpha_{1D}$-AR expression decreased and there was no change in $\alpha_{1B}$-AR expression (Heijnen et al., 2002). In primary human endothelial cells collected from umbilical veins, the expression of $\alpha_{1B}$-AR decreased significantly in the presence of inflammatory cytokines (Heijnen et al., 2002). A similar reduction in $\alpha_{1B}$-AR expression was seen in cultured mesangial cells (Bucher et al., 2003). This contrasts with our results showing that inflammatory cytokines increased $\alpha_{1B}$-AR expression in HaCaT cells and primary keratinocytes. This discrepancy indicates that changes in expression of $\alpha_1$-AR subtypes in response to inflammatory cytokine stimuli is cell-type specific (Heijnen et al., 2002; Bucher et al., 2003). Moreover, TNF$\alpha$ increased $\alpha_{1B}$-AR expression in primary keratinocytes less strongly than in HaCaT cells. The short lifespan of primary keratinocytes in culture may render these cells less responsive to exogenous TNF$\alpha$ than HaCaT cells. Moreover, the removal of media supplements from primary keratinocyte cultures may slow down cell proliferation. As a result, lesser inflammatory cytokines and NGF were produced by the cells, in particular NGF, as a normal proliferative stage is necessary for TNF$\alpha$-induced NGF secretion from keratinocytes. While there is still limited knowledge about how TNF$\alpha$ or inflammation regulates $\alpha_1$-AR expression, possible mechanisms may be mediation through involvement of NF-$\kappa$B, MAP kinases, and JAK/STAT signalling pathways, which are activated after TNF$\alpha$ binds to its receptor. Activation of the NF-$\kappa$B pathway down-regulates $\alpha_1$-AR expression during sepsis (Schmidt et al., 2009), but whether
activation of the other signalling pathways can stimulate $\alpha_1$-AR expression in keratinocytes is unknown.

Modulation of $\alpha_1$-AR by inflammatory cytokines and NGF plays an important role in wound healing, by influencing fibroblast proliferation and migration and secretion of the extracellular matrix needed for repair and remodelling (Liao et al., 2014). In addition, cytokines released by keratinocytes may stimulate $\alpha_1$-AR in immune cells to migrate and further release inflammatory cytokines to help with the healing process (Heijnen et al., 1996; Maestroni, 2000; Roupe van der Voort et al., 2000). While the interaction between $\alpha_1$-AR and inflammatory cytokines helps in healing and repair, unresolved inflammatory processes mediated through $\alpha_1$-AR may play a role in the maintenance of some chronic pain conditions (Schlereth et al., 2014) and the pathophysiology of some diseases (Heijnen et al., 1996; Pongratz & Straub, 2014).

In conclusion, our results show that TNF$\alpha$ can induce the release of pro-inflammatory cytokines from an immortalized human keratinocyte cell line, and can induce the expression of $\alpha_{1B}$-AR both in these cells and in primary human keratinocytes. Hence, inflammatory cytokines released by keratinocytes in response to injury may induce the expression of $\alpha_{1B}$-AR in keratinocytes in vivo. Such increase may heighten the response of keratinocytes to catecholamines released by the sympathetic nervous system after injury which, in turn, might amplify the inflammatory response. Such a cycle, if unresolved, could lead to persistent inflammation as observed in chronic disease conditions. Further studies utilizing primary keratinocytes, isolated from patients or animal disease models, may provide further insight into the effect of inflammatory cytokines on $\alpha_1$-AR expression in pain models and disease.
2.7. Supplementary Materials

Table 2-1. List of primer sets used in a SYBR® Green qRT-PCR assay

<table>
<thead>
<tr>
<th>Gene ID (Names)</th>
<th>GenBank Accession No</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Product Size (bp)</th>
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<tbody>
<tr>
<td>CXCL8 (IL-8)</td>
<td>NM_000584</td>
<td>5'-ATG ACT TCC AAG CTG GCC 3'</td>
<td>5'-TCC TTG GCA AAA CTG CAC CAT 3'</td>
<td>82</td>
</tr>
<tr>
<td>IL6 (IL-6)</td>
<td>NM_000600</td>
<td>5'-AGC CCT GAG AAA GGA GAC 3'</td>
<td>5'-ACC AGG CAA GTC TCC TCA TTG 3'</td>
<td>144</td>
</tr>
<tr>
<td>IL1B (IL-1β)</td>
<td>NM_000576</td>
<td>5'-CTG TCC TGC GTG TTG AAA GA 3'</td>
<td>5'-TTG GGT AAT TTT TGG GAT CTA CA 3'</td>
<td>70</td>
</tr>
<tr>
<td>NGF (NGF)</td>
<td>NM_002506</td>
<td>5'-TGT GAT CAG AGT GTA GAA CAA CA-3'</td>
<td>5'-CGG ACC CAA TAA CAG TTT TAC C-3'</td>
<td>97</td>
</tr>
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<td>GAPDH</td>
<td>NM_002046</td>
<td>5'-GGG TGT GAA CCA TGA GAA-3'</td>
<td>5'-GAC TGT GGT CAT GAG TCC T-3'</td>
<td>136</td>
</tr>
<tr>
<td>ADRA1B (α1B-AR)</td>
<td>NM_000679</td>
<td>PrimePCR™ SYBR® Green Assay (assay ID: qHsaCIP0030605).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2-2. Inflammatory cytokine and NGF protein levels in primary keratinocytes post-TNFα treatment at 10 ng/ml (Mean ± SEM).

<table>
<thead>
<tr>
<th>Analytes*</th>
<th>Naïve</th>
<th>10ng/mL TNFα</th>
<th>p value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>8.20 ± 6.17</td>
<td>13.34 ± 9.61</td>
<td>0.24</td>
</tr>
<tr>
<td>IL-8</td>
<td>467.44 ± 349.89</td>
<td>994.67 ± 557.55</td>
<td>0.17</td>
</tr>
<tr>
<td>IL-6</td>
<td>1829.34 ± 653.41</td>
<td>3942.50 ± 1410.89</td>
<td>0.08</td>
</tr>
<tr>
<td>NGF</td>
<td>0.96 ± 0.65</td>
<td>0.99 ± 0.52</td>
<td>0.89</td>
</tr>
</tbody>
</table>

* A different multiplex ELISA kit was used to measure cytokine and NGF protein levels in primary keratinocytes (Milliplex MAP Human Adipocytes kit, Merck, USA). This kit has higher sensitivity assay for IL-1β compared to the multiplex ELISA kit used in HaCaT cells.

† Statistical analysis was performed using a paired t-test.
Chapter 3
Chapter 3. A positive feedback loop between alpha$_1$-adrenoceptors and inflammatory cytokines in keratinocytes

3.1. Preface

This following is a published paper:


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Chapter 2 reveals that pro-inflammatory mediator TNF$_\alpha$ or a TNF$_\alpha$-induced Inflammatory state increased alpha$_1$-AR expression in cultured keratinocytes. In Chapter 3, the consequences of heightened alpha$_1$-AR expression are further investigated to determine whether a positive feedback mechanism between alpha$_1$-AR and inflammatory mediators, as seen in immune cells, may also be present in keratinocytes. Identifying this interaction will give better insight into the role of alpha$_1$-AR in normal cutaneous inflammation.
3.2. Abstract

A positive feedback loop between inflammatory cytokines and alpha\textsubscript{1}-adrenoceptors (\(\alpha\textsubscript{1}-\)AR) (a target of the sympathetic nervous system neurotransmitter norepinephrine) influences inflammatory responses in immune cells. This cross-talk between the sympathetic nervous system and immune system may play a role in promoting chronic inflammation. Emerging evidence shows that \(\alpha\textsubscript{1}-\)AR interact with inflammatory cytokines in keratinocytes, and this epidermal adrenergic signalling may contribute to skin inflammatory responses following injury, disease or stress. In this study, utilizing an \textit{in vitro} approach, we hypothesized that \(\alpha\textsubscript{1}-\)AR interact in a positive feedback loop with inflammatory mediators in keratinocytes. The pro-inflammatory cytokine tumour necrosis factor \(\alpha\) (TNF\(\alpha\)) was used to induce an inflammatory state in cultured keratinocytes. TNF\(\alpha\) increased interleukin (IL)-1\(\beta\), IL-6, IL-8 and nerve growth factor (NGF) production and gene expression levels of \(\alpha\textsubscript{1}-\)AR subtype B (\(\alpha\textsubscript{1B}-\)AR). Additional stimulation of \(\alpha\textsubscript{1}-\)AR further increased IL-6 levels, while maintaining high levels of IL-8 and decreasing levels of IL-1\(\beta\) and NGF. Our results suggest that reciprocal influences between \(\alpha\textsubscript{1}-\)ARs and inflammatory cytokines may play a role in normal inflammatory responses. However, if unchecked, this cycle could contribute to pathology (e.g. chronic inflammatory diseases, chronic pain conditions, and stress-induced cancer progression).

3.3. Introduction

Inflammation is a complex biological response to injury, stress or disease which promotes immune responses and assists repair and regeneration. Immune cells and other local tissue cells, such as keratinocytes in the skin, generate inflammatory responses through the release of inflammatory cytokines and growth factors (Bernard
et al., 2012; Shi et al., 2013; Birklein et al., 2014). The sympathetic nervous system (SNS) interacts with the immune system in regulating homeostasis of inflammatory processes (see reviews by (Janig, 2014; Pongratz & Straub, 2014)). Adrenoceptors (ARs) mediate SNS functions and are targets for catecholamines released by the SNS during injury or stress. The alpha1-adrenoceptor (α1-AR) is a member of the adrenoceptor family and is expressed mainly in smooth muscles. Thus, the classical role of α1-AR is to modulate smooth muscle contraction. Recent studies show, however, that α1-AR are not limited to smooth muscles but are also expressed in other cell types, including immune cells (Heijnen et al., 1996; Ricci et al., 1999), mesangial cells of kidney (Bucher et al., 2003), keratinocytes (Dawson et al., 2011; Li et al., 2013; Finch et al., 2014) and peripheral nociceptive neurons (Dawson et al., 2011). These findings have led to emergent studies investigating novel roles of α1-AR in different tissues.

In immune cells, signalling through α1-AR regulates physiological functions including migration and differentiation, and the production of inflammatory cytokines (Heijnen et al., 1996; Maestroni, 2000; Roupe van der Voort et al., 2000; Grisanti et al., 2011b). While the expression of α1-AR is undetectable in peripheral blood mononuclear cells (PBMCs) of healthy control volunteers, PBMCs in patients with juvenile rheumatoid arthritis (JRA – a chronic inflammatory and autoimmune disease) express functional α1-AR. Stimulation of α1-AR by norepinephrine released during cold-pressor stress induces elevated production of inflammatory cytokines IL-6 and IL-8 in JRA PBMCs compared to PMBCs from healthy people (Roupe van der Voort et al., 2000). In addition, inflammatory cytokines released during inflammation induce the expression of α1-AR in immune cells (Heijnen et al., 2002). Thus, reciprocal interactions between inflammatory cytokines and α1-AR in immune cells may modulate inflammatory responses in JRA.
Chapter 3. A positive feedback loop between $\alpha_1$-AR and inflammatory cytokines

Growing evidence suggests that $\alpha_1$-AR signalling in the skin may regulate normal inflammatory processes and wound healing. Elevated levels of $\alpha_1$-AR are expressed in rat keratinocytes after injuries such as a burn (Drummond et al., 2015), nerve injury (Drummond et al., 2014a; Drummond et al., 2014c) or fracture (Drummond et al., 2014b), possibly due to inflammatory cytokines released by local tissue after injury. We recently reported that the pro-inflammatory cytokine tumour necrosis factor $\alpha$ (TNF$\alpha$) induced inflammatory cytokine and NGF production in a human keratinocyte cell line, HaCaT cells, and in cultured primary human keratinocytes (Wijaya et al., 2020b). Moreover, TNF$\alpha$, or a TNF-induced inflammatory state, evoked $\alpha_1$-AR subtype B ($\alpha_{1B}$-AR) expression in these cells. However, whether further stimulation of $\alpha_1$-AR in these activated (TNF$\alpha$-treated) keratinocytes promotes further pro-inflammatory cytokine release is unknown.

In the present study, we aimed to investigate the effect of $\alpha_1$-AR stimulation on inflammatory cytokine and nerve growth factor (NGF) production in normal and TNF-activated keratinocytes. It was hypothesized that TNF$\alpha$ would upregulate $\alpha_1$-AR expression on keratinocytes, and that subsequent stimulation of $\alpha_1$-AR would further increase the production of a range of inflammatory cytokines and also NGF in these cells.

3.4. Material and Methods

3.4.1. Cell maintenance

The materials used for maintenance of the cells were purchased from Gibco™ (Thermo Fisher Scientific, MA, USA), unless otherwise stated. The immortalized human keratinocyte cell line (HaCaT cells) was obtained from the German Cancer
Chapter 3. A positive feedback loop between $\alpha_1$-AR and inflammatory cytokines

Research Centre (DKFZ, Heidelberg, Germany; Cat No. 300493) (Boukamp et al., 1988). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Serana, Bunbury, Australia) and maintained in a controlled incubator at 37°C with 5% CO$_2$. For the experiment, HaCaT cells were plated in a 24-well cell culture plate at 50,000 cells/well overnight prior to TNF$\alpha$ administration. The cells reached approximately 50-60% confluence on the treatment day.

3.4.2. TNF$\alpha$ treatments and $\alpha_1$-AR stimulation

All treatments were performed in DMEM under reduced-serum conditions (0.5% FBS) to minimise the effect of cell proliferation and growth on $\alpha_1$-AR expression (Gonzalez-Cabrera et al., 2004). Human recombinant TNF$\alpha$ (Peprotech, New Jersey, USA) was administered to HaCaT cells at 10 ng/ml and incubated for 24 h. We have previously shown that 10 ng/ml TNF$\alpha$ administered to HaCaT cells for 24 h significantly increases inflammatory cytokine and NGF production (Wijaya et al., 2020b). A selective agonist of $\alpha_1$-AR, phenylephrine (Sigma-Aldrich, NSW, Australia), was applied to the cells at a final concentration of 1 or 10 $\mu$M for 6 h, following TNF$\alpha$ treatments. The naïve cells were treated with media only. Cells that were treated with either TNF$\alpha$ or phenylephrine only, along with the naïve condition, were used as experimental controls. Each experimental condition was performed in six wells (six technical replicates) and each set of experiments was repeated four times. At the end of the experiment, media was collected to measure protein levels of inflammatory agents and the cells were rinsed with Hank’s balanced salt solution and lysed with lysis buffer supplemented with 0.05% $\beta$-mercaptoethanol (Aurum™ total RNA mini kit, Bio-Rad Laboratories, CA, USA). The cell lysates from the technical replicates were pooled prior to RNA isolation.
Chapter 3. A positive feedback loop between $\alpha_1$-AR and inflammatory cytokines

3.4.3. $\alpha_1$-AR blockade

To determine whether the effects of $\alpha_1$-AR stimulation on inflammatory cytokine levels were mediated specifically through the $\alpha_1$-AR receptor, a selective $\alpha_1$-AR inverse agonist, prazosin (Sigma-Aldrich, NSW, Australia), was administered to HaCaT cells prior to $\alpha_1$-AR stimulation with phenylephrine. Prazosin was administered to the cells at 1 $\mu$M for 1 h, followed by phenylephrine treatment at 10 $\mu$M for 6 h. Cells treated with media only (naïve), prazosin or phenylephrine only were used as experimental controls. Each experimental condition was performed in six wells (six technical replicates) and each set of experiments was repeated four times. The effects of $\alpha_1$-AR blockade on inflammatory cytokine and $\alpha_1$-AR levels were determined through gene expression analysis.

3.4.4. Quantitative real-time PCR

Total RNA was extracted from the cell lysates according to the manufacturer’s protocol (Aurum™ total RNA mini kit, Bio-Rad Laboratories, CA, USA). The amount of total RNA was determined using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA, USA). One $\mu$g total RNA was converted to cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, CA, USA), with the following cycle: 5 min priming step at 25°C, followed by a reverse transcription step at 42°C for 30 min and reverse transcriptase inactivation at 95°C for 5 min. A SYBR® Green-based quantitative real-time PCR (qRT-PCR) assay was used to measure gene expression levels of $\alpha_1$-AR-subtype B ($\alpha_{1B}$-AR) and of the inflammatory cytokines interleukin (IL)-1$\beta$, IL-6, IL-8 and NGF. We previously showed that HaCaT cells do not express $\alpha_1$-AR-subtype A ($\alpha_{1A}$-AR) and have a very low expression of $\alpha_1$-AR-subtype D ($\alpha_{1D}$-AR), beyond the limit of quantification in qRT-PCR (Ct value above 33) (Wijaya et al., 2020b). The primer sets used in qRT-PCR are listed in Table...
Chapter 3. A positive feedback loop between $\alpha_1$-AR and inflammatory cytokines

3-1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. All of the primers were validated to amplify mRNA-derived transcripts but not genomic DNA (unpublished data). The qRT-PCR assay was performed using a Viia™ 7 Real-Time PCR System (Applied Biosystems™; Thermo Fisher Scientific, MA, USA). A cycle threshold (Ct) value of each sample was collected at the end of the qRT-PCR run (40 cycles). Changes in gene expression as a result of the treatments compared to the naïve group were represented in $\Delta\Delta$Ct, where $\Delta\Delta$Ct=$(\Delta$Ct$_{\text{naïve}}-\Delta$Ct$_{\text{treatment}})$, $\Delta$Ct$_{\text{naïve}}=$(Ct$_{\text{naïve}}$–Ct$_{\text{GAPDH}}$), and $\Delta$Ct$_{\text{treatment}}=$(Ct$_{\text{treatment}}$–Ct$_{\text{GAPDH}}$) (Yuan et al., 2006; Schmittgen & Livak, 2008). The mean ± SEM of the gene expression changes between biological replicates is presented in the Results section.

### Table 3-1. List of primer sets used in SYBR® Green qRT-PCR assay

<table>
<thead>
<tr>
<th>Gene ID (Names)</th>
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<th>Reverse Sequence ’5 – 3’</th>
<th>Product Size (bp)</th>
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<td>NM_000584</td>
<td>ATG ACT TCC AAG CTG GCC GT</td>
<td>TCC TTG GCA AAA CTG CAC CT</td>
<td>82</td>
</tr>
<tr>
<td>IL6 (IL-6)</td>
<td>NM_000600</td>
<td>AGC CCT GAG AAA GGA GAC ATG</td>
<td>ACC AGG CAA GTC TCC TCA TTG</td>
<td>144</td>
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<tr>
<td>IL1B (IL-1β)</td>
<td>NM_000576</td>
<td>CTG TCC TGC GTG TTG AAA GA</td>
<td>TTG GGT AAT TTT TGG GAT CTA CA</td>
<td>70</td>
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<td>NGF (NGF)</td>
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</table>
3.4.5. Multiplex enzyme-linked immunosorbent assay

Conditioned media collected from technical replicates were pooled prior to the measurement of inflammatory cytokine and NGF protein levels. A magnetic-bead based multiplex enzyme-linked immunosorbent assay (ELISA) technology that allows simultaneous measurement of different proteins (analytes) in one assay was used to quantify the protein levels. Several important inflammatory cytokines that were previously shown to be elevated in chronic neuropathic pain conditions were chosen from the Bio-Plex Pro™ Human Cytokine assay (Bio-Rad Laboratories, CA, USA) (Heijmans-Antonissen et al., 2006; Uceyler et al., 2010; Kramer et al., 2011; Birklein et al., 2014). These cytokines were IL-1β, IL-6, IL-8 (Bio-Plex Pro™ Human Cytokine Group I) and NGF (Bio-Plex Pro™ Human Cytokine Group II). The validation data provided by the company has shown no cross-reactivity between the analytes. The assay was performed according to the manufacturer’s protocol using undiluted media, and the assay signal was measured with a Bio-Plex® 200 System (Bio-Rad Laboratories, CA, USA).

3.4.6. Statistical analysis

A general linear model with repeated measures statistical analysis was applied to determine the effects of TNFα and phenylephrine on inflammatory cytokine, NGF and α1-AR gene expression levels. The ΔΔCt value was used as the dependent variable in the gene expression statistical analysis (Yuan et al., 2006). The values generated from the multiplex ELISA assay were analysed to determine the effects of TNFα and phenylephrine on inflammatory cytokine and NGF protein levels. All statistical analysis was performed using SPSS® statistical analysis software version 24 (IBM, New York, USA).
3.5. Results

3.5.1. Differential effects of $\alpha_1$-AR stimulation on inflammatory cytokine and NGF mRNA levels in HaCaT cells

In line with our previous findings, 10 ng/ml TNF$\alpha$ induced a significant increase in pro-inflammatory cytokine (Figure 3-1 A-C) and NGF (Figure 3-1 D) mRNA levels in HaCaT cells compared to the naïve condition (Wijaya et al., 2020b). Following TNF$\alpha$ treatment, stimulation of $\alpha_1$-AR through its agonist phenylephrine (PE) at 1 or 10 $\mu$M, significantly reduced IL-1$\beta$ mRNA levels, while there was no change in IL-1$\beta$ levels in cells that were only treated with PE (Figure 3-1 A). There was no effect of $\alpha_1$-AR stimulation on IL-8 mRNA levels (Figure 3-1 B). Importantly, PE induced IL-6 gene expression in the cells that were treated with PE alone when compared to the naïve condition and this expression was further increased in the cells administered with both TNF$\alpha$ and PE, compared to the cells treated with TNF$\alpha$ only (Figure 3-1 C). In contrast, PE significantly reduced NGF mRNA levels in the cells pre-treated with or without TNF$\alpha$, compared to TNF$\alpha$-treated cells only or cells in the naïve condition, respectively (Figure 3-1 D). The effects of 1 $\mu$M PE on inflammatory cytokine and NGF gene expression levels were similar to those of 10 $\mu$M PE (Figure 3-1).
Chapter 3. A positive feedback loop between $\alpha_1$-AR and inflammatory cytokines

Figure 3-1. The effects of TNF$\alpha$ and phenylephrine treatments on inflammatory cytokine and NGF mRNA levels in HaCaT cells.

TNF$\alpha$ was administered to HaCaT cells at 10 ng/ml in reduced serum media for 24 h. Following TNF$\alpha$ administration, the $\alpha_1$-AR agonist phenylephrine (PE) was added to the cells at a final concentration of 1 or 10 $\mu$M and incubated for another 6 h. PE alone at 1 or 10 $\mu$M was administered to untreated cells as an experimental control. Naïve cells treated with media only were used to estimate baseline expression level. Gene expression levels of inflammatory cytokines: (A) IL-1$\beta$, (B) IL-8, (C) IL-6 and (D) NGF were measured. Changes in gene expression as a result of the treatments compared to the naïve condition are represented as $\Delta\Delta$Ct ($\Delta\Delta$Ct$_{Naive}$=0). Data are represented as mean ± SEM of four independent replicates (*represents a significant difference compared to naïve, p<0.05; $\phi$represents a significant difference compared to PE-treated cells only at the same PE concentration, p<0.05; $#$represents a significant difference compared to TNF$\alpha$-treated cells, p<0.05).
3.5.2. Phenylephrine reduces $\alpha_{1B}$-AR mRNA levels in HaCaT cells

Exposure of HaCaT cells to 10 ng/ml TNF$\alpha$ for 24 h increased $\alpha_{1B}$-AR mRNA levels significantly compared to the naïve condition (Figure 3-2). Specific stimulation of $\alpha_{1}$-AR with PE resulted in a significant reduction in $\alpha_{1B}$-AR mRNA levels, both in the presence and absence of TNF$\alpha$ (Figure 3-2). The effect of PE on the reduction $\alpha_{1B}$-AR mRNA levels in HaCaT cells exposed to TNF$\alpha$ was not dose-dependent (Figure 3-2).

![Figure 3-2. The effects of TNF$\alpha$ and phenylephrine treatments on $\alpha_{1B}$-AR mRNA levels in HaCaT cells.](image)

Gene expression levels of $\alpha_{1B}$-AR in HaCaT cells were determined using qRT-PCR. Different treatments of either 10 ng/ml TNF$\alpha$, 1 $\mu$M phenylephrine (PE), 10 $\mu$M PE, or combinations of TNF$\alpha$ and PE were applied to HaCaT cells. Naïve cells that were treated with media only were used to estimate baseline expression level. Changes in gene expression as a result of the treatments compared to the naïve group are represented as $\Delta\Delta$Ct ($\Delta\Delta$Ct$_{naïve}$=0). Data are represented as mean $\pm$ SEM of the $\Delta\Delta$Ct of four independent replicates (*represents a significant difference compared to naïve,
Chapter 3. A positive feedback loop between $\alpha_1$-AR and inflammatory cytokines

p<0.05; *represents a significant difference compared to PE-treated cells only at the same PE concentration, p<0.05; #represents a significant difference compared to TNF$\alpha$-treated cells, p<0.05).

3.5.3. Stimulation of $\alpha_1$-AR, following TNF$\alpha$ treatment, further increases IL-6 protein levels in HaCaT cells

Inflammatory cytokine and NGF protein levels secreted from HaCaT cells were measured from the conditioned media using multiplex ELISA kits. The levels of IL-1$\beta$ secreted by HaCaT cells were below the lowest detection limit of the kits (0.6 pg/ml - data not shown). The results for IL-8, IL-6 and NGF are presented in Figure 3-3. TNF$\alpha$ treatment to HaCaT cells significantly increased IL-8 (Figure 3-3 A), IL-6 (Figure 3-3B) and NGF (Figure 3-3 C) protein levels. There were no effects of PE stimulation on IL-8 protein levels (Figure 3-3 A), consistent with the IL-8 mRNA levels (Figure 3-1 B). PE treatment to naïve cells resulted in an upward trend in IL-6 protein levels compared to the naïve condition. Of particular note, following TNF$\alpha$ treatment, stimulation of $\alpha_1$-AR with 10 $\mu$M PE resulted in a further significant increase in IL-6 protein levels, compared to cells treated with TNF$\alpha$ only (Figure 3-3 B). While NGF mRNA levels were significantly reduced in cells that were treated with PE (Figure 3-1 D), phenylephrine did not influence NGF protein levels (Figure 3-3 C).
Chapter 3. A positive feedback loop between $\alpha_1$-AR and inflammatory cytokines

Figure 3-3. The effects of TNF$\alpha$ and phenylephrine treatments on inflammatory cytokine and NGF protein levels in HaCaT cells.

Inflammatory cytokine and NGF protein produced by HaCaT cells post-TNF$\alpha$ and phenylephrine (PE) stimulation were measured in the conditioned media using multiplex ELISA. TNF$\alpha$ was administered to the cells at 10 ng/ml for 24 h, while the naïve cells were treated with media only. PE was administered to the cells with or without pre-treatment of TNF$\alpha$, at a final concentration of 1 or 10 $\mu$M PE for another 6 h. Data are represented as mean $\pm$ SEM of the four independent replicates (*represents a significant difference compared to naïve, p<0.05; $\Phi$represents a significant difference compared to PE-treated cells only at the same PE concentration, p<0.05; #represents a significant difference compared to TNF$\alpha$-treated cells, p<0.05).
3.5.4. $\alpha_1$-AR signalling specifically modulates IL-6 production in HaCaT cells

To confirm that $\alpha_1$-AR signalling specifically modulated IL-6 levels in HaCaT cells, the $\alpha_1$-AR selective inverse agonist prazosin (PZ) was used to competitively block $\alpha_1$-AR signalling prior to $\alpha_1$-AR stimulation. The changes in both IL-6 mRNA and protein levels were investigated. There was a consistent increase in IL-6 mRNA (Figure 3-4 A) and protein levels (Figure 3-4 B) in HaCaT cells treated with PE, compared to naïve cells. Pre-treatment with 1 $\mu$M PZ prior to $\alpha_1$-AR stimulation with 10 $\mu$M PE resulted in a trend towards decreasing levels of IL-6 mRNA (a reduction in IL-6 mRNA levels with pre-treatment of PZ was observed in three of four biological replicates) (Figure 3-4 A). At the protein level, there was a significant inhibitory effect of PZ on IL-6 production, compared to cells that were treated with PE only (Figure 3-4 B). This confirmed that signalling through $\alpha_1$-AR can modulate IL-6 production in keratinocytes. PE treatment significantly reduced $\alpha_{1B}$-AR mRNA levels compared to the naïve condition, but pre-treatment with PZ prior to PE did not alter this effect (Figure 3-4 C).
Figure 3-4. The effect of $\alpha_{1B}$-AR blockade on PE-induced IL-6 levels and $\alpha_{1B}$-AR expression in HaCaT cells.

To investigate whether $\alpha_{1B}$-AR mediates the effect of phenylephrine (PE) stimulation on IL-6 levels, the $\alpha_{1}$-AR selective inverse agonist prazosin (PZ) was pre-administered to HaCaT cells prior to PE stimulation, at 1 $\mu$M for 1 h. PE was then added at a final concentration of 10 $\mu$M for a further 6 h. Naïve cells treated with media only were used to estimate baseline expression level. Cells treated either with PZ or PE only were used as a comparison. The gene expression levels of IL-6 (A) and $\alpha_{1B}$-AR (C) were determined by qRT-PCR analysis, while IL-6 protein levels (B) were measured by ELISA. Changes in gene expression as a result of any treatment compared to the naïve condition were represented as $\Delta\Delta Ct$ ($\Delta\Delta Ct_{Naive}=0$). Data are represented as mean ± SEM of the $\Delta\Delta Ct$ (n=4) and of the IL-6 protein levels (n=3) (*represents a significant difference compared to naïve, #represents a significant difference compared to PE-treated cells, $\phi$represents a significant difference compared to PZ-treated cells, p<0.05).
3.6. Discussion

In this study, the effects of $\alpha_1$-AR stimulation in both normal and activated (TNF$\alpha$ treated) keratinocytes were examined \textit{in vitro}, and a positive feedback interaction between $\alpha_1$-AR and levels of the inflammatory cytokine IL-6 was found. Consistent with our previous findings, the pro-inflammatory cytokine TNF$\alpha$ induced an inflammatory state in cultured HaCaT keratinocytes, characterised by a significant increase in production of key pro-inflammatory cytokines and NGF (Wijaya et al., 2020b). In addition, this inflammatory state upregulated $\alpha_{1B}$-AR mRNA levels in these cells. Stimulation of $\alpha_1$-AR, following TNF$\alpha$ treatment, resulted in differential effects on inflammatory cytokine and NGF levels in HaCaT cells. Specifically, we showed that stimulation of $\alpha_1$-AR in activated keratinocytes resulted in a further increase in IL-6 levels, thus potentially establishing a positive feedback loop.

Levels of IL-1$\beta$ mRNA decreased in response to the combined TNF$\alpha$ and phenylephrine treatment, compared to cells treated with TNF$\alpha$ only. In contrast, Li and colleagues showed that $\alpha_1$-AR stimulation through its agonist cirazoline increased the production of IL-1$\beta$ mRNA in rat keratinocytes compared to untreated cells (Li et al., 2013). One possible explanation for this discrepancy is that, in our system, TNF$\alpha$ stimulated IL-1$\beta$ expression levels to reach a ceiling and further stimulation of $\alpha_1$-AR in these activated cells downregulated IL-1$\beta$ mRNA levels, in order to maintain cell homeostasis (Zhu & Kanneganti, 2017). However, as this does not account for the downward trend in IL-1$\beta$ mRNA levels after treatment with phenylephrine alone, this requires further investigation. In addition to its $\alpha_1$-AR agonist properties, cirazoline is a nonselective antagonist to $\alpha_2$-AR (Ruffolo & Waddell, 1982). Further work is required to determine whether effects at the $\alpha_2$-AR
explain the positive effect of cirazoline on IL-1β production in rat keratinocytes (Li et al., 2013).

Phenylephrine did not change mRNA or protein levels of IL-8 either in normal or activated keratinocytes. This indicates that IL-8 levels in these cells are regulated through inflammatory signalling pathways independently of cross-talk with α₁-AR. In a human monocyte cell line, THP-1, complex cross-talk between α₁-AR and inflammatory signalling pathways was shown (Grisanti et al., 2011a). α₁-AR stimulation increased IL-8 protein levels slightly in THP-1 cells, but when inflammatory responses were induced by lipopolysaccharide (LPS), stimulation of α₁-AR reduced LPS-induced IL-8 protein production compared to cells treated with LPS only.

NGF is an important neurotrophin that supports neuronal growth and prevents neuronal death. After injury, NGF produced by peripheral tissue promotes peripheral nerve regeneration and axonal outgrowth (Gaudet et al., 2011). Prolonged stimulation of ARs in myocardial tissue triggers a reduction in NGF levels and results in sympathetic nerve loss and heart failure (Kaye et al., 2000; Kimura et al., 2010). The reduction in NGF mRNA is reversed through pre-administration of prazosin (Kaye et al., 2000). In this study, α₁-AR stimulation reduced NGF mRNA levels both in normal and activated keratinocytes. However, NGF protein levels remained unchanged, possibly due to the stability of NGF protein in culture. Correlation between transcription and translation processes depends on factors such as mRNA and protein stability and the degree of post-translational modification of proteins. In contrast to NGF, transcription and translation processes appeared to operate in tandem for other cytokines, as expressed and secreted interleukin levels changed in parallel after the TNFα and phenylephrine treatments. In vivo, a reduction in skin NGF levels may hinder peripheral nerve regeneration and axonal growth after injury. A
Chapter 3. A positive feedback loop between \( \alpha_1 \)-AR and inflammatory cytokines

Reduction in cutaneous nerve fibre density has been identified in skin biopsies collected from patients with painful conditions including diabetic neuropathy (Quattrini et al., 2008), small nerve fibre neuropathy (Uceyler et al., 2010), leprosy (Facer et al., 2000), chronic postsurgical sciatica (Drummond et al., 2019) and complex regional pain syndrome (Oaklander et al., 2006; Morellini et al., 2018; Rasmussen et al., 2018). It may be relevant that some of these conditions are associated with an upregulation of \( \alpha_1 \)-AR (Drummond et al., 2014c; Finch et al., 2014; Drummond et al., 2019).

Growing evidence shows cross-talk between \( \alpha_1 \)-AR signalling and IL-6 levels in different cell types and tissues (Li et al., 2013; Huan et al., 2017; Rossi et al., 2018). In a study by Perez and colleagues, \( \alpha_1 \)-AR stimulation increased IL-6 levels in mouse cardiomyocytes and in \( \alpha_{1A} \)-AR-over-expressed rat fibroblasts. This response was mediated through a stabilization of IL-6 mRNA and a maintenance of IL-6 transcription factor, involving the p38 MAPK and NF-\( \kappa \)B inflammatory signalling pathways (Perez et al., 2009). Activation of these pathways triggers inflammatory signalling cascades and leads to production of pro-inflammatory cytokines (Zarubin & Han, 2005; Liu et al., 2017). In this study, cross-talk between \( \alpha_1 \)-AR signalling and IL-6 levels in keratinocytes was demonstrated. In keratinocytes not exposed to TNF\( \alpha \) treatment, \( \alpha_1 \)-AR stimulation induced IL-6 production and this increase was reduced by pre-treatment with the \( \alpha_1 \)-AR antagonist prazosin. Interestingly, stimulation of \( \alpha_1 \)-AR following TNF\( \alpha \) treatment further increased both mRNA and protein levels of IL-6. We postulate that TNF\( \alpha \) activated inflammatory signalling pathways, such as NF-\( \kappa \)B, JAK-STAT, and MAPK, to induce IL-6 production (Tanabe et al., 2010). Subsequently, stimulation of \( \alpha_1 \)-AR stabilized the production of IL-6 mRNA induced through TNF signalling pathways and maintained transcription factors for IL-6 activated through TNF-induced NF-\( \kappa \)B and MAPK pathways (Perez et al., 2009). This
cross-talk between $\alpha_1$-AR and inflammatory signalling pathways may trigger overproduction of IL-6, and dysregulation of this interaction could maintain inflammatory conditions in keratinocytes. Although this seems plausible, further studies are required to confirm that the NF-κB and MAPK pathways mediate the interaction between $\alpha_1$-AR and IL-6 in keratinocytes. Persistently high levels of IL-6 have been detected in autoimmune and chronic inflammatory diseases (Tanaka & Kishimoto, 2012) and chronic pain conditions (Sommer et al., 2018), and targeting IL-6 has been proposed as a therapeutic treatment for these conditions (Tanaka & Kishimoto, 2012; Kishimoto et al., 2015). Whether $\alpha_1$-AR blockade suppresses IL-6 production in these conditions requires further study.

Stimulation of $\alpha_1$-AR reduced $\alpha_{1B}$-AR gene expression levels, both in naïve and TNF$\alpha$-treated keratinocytes. In cardiomyocytes, $\alpha_1$-AR stimulation results in differential regulation of $\alpha_1$-AR subtype expression, i.e. an increase in $\alpha_{1A}$-AR mRNA levels, and a down-regulation of $\alpha_{1B}$-AR and $\alpha_{1D}$-AR mRNA expression (Li et al., 1995; Rokosh et al., 1996; Autelitano & Woodcock, 1998). In addition, in rabbit aortic smooth muscles cells, stimulation of $\alpha_1$-AR transiently reduced $\alpha_1$-AR mRNA levels at 4 h and the expression level gradually returned to basal levels over 24 h (Izzo et al., 1990). This downregulation of $\alpha_{1B}$-AR expression following $\alpha_1$-AR stimulation was mediated through an inhibition on $\alpha_1$-AR gene transcription and a reduction in $\alpha_1$-AR mRNA stability (Izzo et al., 1990; Rokosh et al., 1996). For unknown reasons, pre-treatment of $\alpha_1$-AR antagonist prazosin did not block the effects of $\alpha_1$-AR stimulation on $\alpha_1$-AR downregulation in keratinocytes in the present study. Thus, further investigation is warranted.

ARs have been suggested to play an important role in regulation of normal inflammatory processes (Scanzano & Cosentino, 2015). However, dysregulation of ARs during inflammation may amplify inflammatory responses and transform the
acute pathological process of the disease or condition into a chronic state (Scanzano & Cosentino, 2015). For example, stimulation of $\alpha_1$-AR during chronic stress in human testicular peritubular cells elevates production of inflammatory cytokines such as IL-6 and monocyte chemoattractant protein-1 (MCP-1); this inflammatory state is associated with male infertility (Rossi et al., 2018). In a chemically-induced hepatocellular carcinoma rat model, sympathetic nerve fibre innervation increased in the liver, accompanied by high levels of inflammatory cytokines IL-6, TGF-β, and MCP-1 (Huan et al., 2017). Activation of $\alpha_1$-AR in Kupffer cells (macrophage-like inflammatory cells in the liver) induced the production of inflammatory cytokines, thereby contributing to hepatocarcinogenesis (Huan et al., 2017). In chronic pain conditions, such as complex regional pain syndrome (CRPS), increased levels of inflammatory cytokines and $\alpha_1$-AR were detected in skin isolated from the symptomatic (affected) limb of CRPS patients and in animal models of CRPS (Birklein et al., 2014; Drummond et al., 2014b; Drummond et al., 2014a; Drummond et al., 2014c; Finch et al., 2014; Drummond et al., 2018). Moreover, stimulation of $\alpha_1$-AR provoked pain and hyperalgesia in a subgroup of patients with CRPS (Drummond et al., 2018), whereas administration of the $\alpha_1$-AR inverse agonist prazosin alleviated mechanical allodynia in patients (Drummond et al., 2016) and unweighting of the affected limb in a fracture model of CRPS (Drummond et al., 2014b). Our current study suggests that $\alpha_1$-AR may influence inflammatory cytokine production in the skin in a positive feedback loop. Inflammatory cytokines produced by local tissue in the skin directly bind to their receptors on peripheral nociceptive neurons and increase nociceptive neuron sensitization (Ji et al., 2014; Obreja et al., 2018). This may be an important source of pain in conditions such as CRPS.

In the tightly-controlled in vitro cell culture model used in this study, a reciprocal interaction between $\alpha_1$-AR and inflammatory cytokines in keratinocytes resulted in a synergistic increase in IL-6 levels. In addition, stimulation of $\alpha_1$-AR reduced NGF
production. These findings are intriguing; nonetheless, it is important to establish whether the interaction between inflammatory cytokines and $\alpha_1$-AR plays a role in amplifying normal inflammatory processes, and whether chronic exposure (weeks/months/years) to TNF$\alpha$, as in some disease states, results in chronic upregulation of $\alpha_1$-AR and IL-6 production. In particular, if left unchecked, the interaction could augment chronic inflammatory diseases, chronic pain, and stress-induced cancer progression (Heijnen et al., 1996; Perez et al., 2009; Li et al., 2013; Birklein et al., 2014; Huan et al., 2017; Rossi et al., 2018). If so, targeting this interaction may help to prevent progression of diseases into a chronic stage. Conversely, as stimulation of $\alpha_1$-AR reduced NGF production in our cell culture model, $\alpha_1$-AR blockade might prevent diminution of NGF levels in keratinocytes after injury and support normal regeneration of peripheral nerves. An in vivo approach will be required to investigate these hypotheses.
Chapter 4
Chapter 4. Alpha₁-adrenoceptor stimulation induces interleukin-6 production in UVB-induced skin inflammation

4.1. Preface

This following chapter is written as a manuscript for submission to a peer-reviewed journal.

Chapter 2 and Chapter 3 identify a positive feedback interaction between α₁-AR and inflammatory mediators in keratinocytes, in vitro, which may play an important role in cutaneous inflammation. In Chapter 4, using UVB-induced skin inflammation as an experimental model, the positive feedback interaction between α₁-AR and inflammatory mediator is further assessed to determine whether the interaction seen in vitro is also present in vivo.
Chapter 4. The effects of $\alpha_1$-adrenoceptor stimulation in vivo

4.2. Abstract

The alpha$_1$-adrenoceptor ($\alpha_1$-AR) may play a role in cutaneous inflammation. $\alpha_1$-AR interact in a vicious cycle with inflammatory mediators in cultured keratinocytes. In this study, the role of $\alpha_1$-AR in cutaneous inflammation was further elucidated in vivo, utilizing a skin microdialysis technique. UVB irradiation to the skin was used to induce skin inflammation in 11 healthy participants. Phenylephrine, an $\alpha_1$-AR agonist, was perfused through microdialysis fibres that were inserted both in UVB-exposed skin and the contralateral normal skin. Saline was perfused through separate fibres, in parallel with the phenylephrine fibres, as an experimental control. UVB irradiation induced skin inflammation, marked by histological changes in the skin. This was enhanced by $\alpha_1$-AR stimulation post-UVB exposure, as evidenced by cutaneous IL-6 production. These results suggest that $\alpha_1$-AR signalling enhances cutaneous inflammatory responses post-injury, and that maintaining a homeostatic interaction between $\alpha_1$-AR and inflammatory mediators may be important in preventing the development of a persistent inflammatory state in the skin.

4.3. Introduction

The alpha$_1$-adrenoceptor ($\alpha_1$-AR) is a transmembrane receptor and a target of sympathetic catecholamine neurotransmitters, such as adrenaline and noradrenaline. The $\alpha_1$-AR is expressed primarily in smooth muscle cells where it regulates smooth muscle contraction. However, recent studies have shown that $\alpha_1$-AR is also expressed in other types of cells, such as immune cells (Heijnen et al., 1996; Ricci et al., 1999), and keratinocytes (Dawson et al., 2011; Li et al., 2013; Wijaya et al., 2020b). This has prompted investigations into novel physiological roles of $\alpha_1$-AR. Particularly, in the past few decades, growing evidence indicates important roles of $\alpha_1$-AR in
modulating inflammatory responses in immune cells and resident cells, such as keratinocytes in the skin.

Resident cells at the site of injury release inflammatory mediators (cytokines and growth factors) to provide protection, fight infection and assist with repair. We recently reported that tumour necrosis factor \( \alpha \) (TNF\( \alpha \)), a pro-inflammatory cytokine, induced \( \alpha_1 \)-AR expression in a human immortalized keratinocyte cell line (Wijaya et al., 2020b), and that further stimulation of \( \alpha_1 \)-AR heightened the production of interleukin (IL)-6 in these cells (Wijaya et al., 2020a). These findings imply a vicious cycle between \( \alpha_1 \)-AR and IL-6 in keratinocytes.

In this study, the role of \( \alpha_1 \)-AR in modulating the inflammatory response in inflamed skin of healthy people was further investigated, in vivo, utilizing local UVB irradiation to induce inflammation in the skin. Stimulation of \( \alpha_1 \)-AR in non-inflamed and inflamed skin was performed by perfusing the \( \alpha_1 \)-AR agonist phenylephrine through microdialysis fibres. Saline solution was perfused simultaneously through separate microdialysis fibres, in parallel with the phenylephrine, as an experimental control. It was hypothesized that \( \alpha_1 \)-AR stimulation in inflamed skin would trigger more IL-6 production, compared to saline in inflamed skin or phenylephrine and saline in non-inflamed skin of healthy people.

4.4. Material and methods

This study was an exploratory study to follow-up the previous in vitro studies investigating the role of \( \alpha_1 \)-AR in cutaneous inflammation (Wijaya et al., 2020b, 2020a) and to confirm whether previous in vitro observation was also present in vivo. This study was not registered as a clinical trial. The primary outcome of the study was to
investigate whether $\alpha_1$-AR stimulation can increase IL-6 production in inflamed skin.

As the study was exploratory, no study power analysis was calculated.

**4.4.1. Participants**

The study group consisted of 11 men aged between 18 and 50 years (mean age $\pm$ SD 24.7 $\pm$ 8.7 years) in good health. Due to the time limitation of the study, only a few participants were available for recruitment. None of the participants had a medical condition that affected their heart, blood vessels, skin, liver or kidneys that required regular treatment with medication, had a known sensitivity to adrenergic drugs or had severe hypertension, arrhythmias, hyperthyroidism or hyperglycaemia. Each participant provided written informed consent for the procedures, which were approved by the human research ethics committee at Murdoch University.

**4.4.2. Microdialysis and UVB exposures**

**Microdialysis:** After cooling the skin with ice, two microdialysis fibres (internal diameter 200 µm) were inserted intradermally for 15 mm into the volar aspect of the forearm about 1 cm apart, and another two microdialysis fibres were inserted several cm distally in the same forearm. The fibres consisted of a semi-permeable membrane with an upper cut-off of 3000 kDa. Saline (0.9%) was perfused at 4 µL/min through the fibres for 60 minutes, and then one pair of fibres was flushed with $5 \times 10^{-6}$ M phenylephrine (Leis et al., 2004). Over the next 60 minutes, $5 \times 10^{-6}$ M phenylephrine was perfused through this pair of fibres and saline was perfused through the second pair of fibres. The dialysate was collected on ice and then stored at -80°C.

**UVB exposure:** To determine optimal settings for UV exposure, ten 1-cm diameter spots on the volar aspect of the left or right forearm near the wrist were exposed to
graded intensities of UVB radiation (Durham Erythema Tester Device; Hybec, UK). A mask was placed over the UVB lamp such that filtering at the ten spots ranged from zero to 84%. To minimize unnecessary exposure to UVB, the duration of exposure was adjusted for skin pigmentation in line with calibration charts provided with the lamp (longer exposure for more darkly pigmented skin). Twenty-four hours later, the skin was examined to identify the minimum dose of UVB that had evoked slight reddening (erythema). Subsequently, two 2 x 3 cm sites on the volar aspect of the contralateral forearm, separated by 1 cm, were exposed simultaneously to UVB radiation at twice the minimum erythema dose.

**Microdialysis after UVB exposure**: Microdialysis was repeated 48 h after sites on the volar forearm had been exposed to UVB radiation. One of the exposed sites was perfused with phenylephrine and the other with saline for 60 minutes. The dialysate was collected, as described above.

### 4.4.3. Skin biopsies

Two 3-mm diameter punch biopsies were collected from each patient under local anaesthesia: one from a site that had been exposed to UVB radiation but 1-2 cm away from the microdialysis fibres, and the other from a mirror image site on the contralateral forearm. Biopsies were fixed in Zamboni’s solution (5% formalin, 1% picric acid, 0.9% saline), embedded in paraffin and cut at 10 µm for histochemical staining.

### 4.4.4. Magnetic based enzyme-linked immunosorbent assay

The dialysates collected from microdialysis fibres were used to measure IL-6 protein levels using a magnetic-bead based enzyme-linked immunosorbent assay (ELISA)
technology. The IL-6 assay kit was part of the MILLIPLEX MAP Human Adipocyte panel (Merck, Darmstadt, Germany). The assay was performed according to the manufacturer’s protocol using undiluted dialysates. The assay signal was measured with a Bio-Plex® 200 System and analysed using Bio-Plex Manager software (Bio-Rad Laboratories, CA, USA).

4.4.5. Histochemical staining and UVB damage score

Prior to staining, tissue sections were de-paraffinized in xylene and rehydrated in a descending series of ethanol. A standard laboratory hematoxylin and eosin staining (H & E) protocol was applied to each section and used to determine the extent of UVB damage for each individual. For control and UVB irradiated skin, one image per section was taken with an Olympus BX51 light microscope at x20 magnification. The histological changes in the skin after UVB exposure were observed and four different parameters were used to determine the extent of UVB damage, with the operator blinded to the experimental group (Bissett et al., 1987; Soter, 1990). These parameters included: 1) an increase in epidermal thickness, 2) presence of necrotic cells in the epidermis, 3) dysplasia of epidermis; and 4) an increase in number of cellular infiltrates in the dermis. To measure epidermal thickness, namely the distance between the basal keratinocytes and the outer most epidermal layer, five different locations across the section were measured using Image J software (version 1.50d, National Institutes of Health, USA). These measures were averaged and the percent change in epidermal thickness from UVB irradiation compared to the control was calculated. An increase in epidermal thickness of > 10% was given a score of 1. Each of the other parameters were scored 0 or 1, to give a total score of 4 for UVB damage.
4.4.6. Statistical approach

Since the data for IL-6 protein levels showed a highly skewed distribution, a natural logarithm transformation was performed prior to statistical analysis. The changes in IL-6 protein levels after $\alpha_1$-AR stimulation in normal or UVB treated skin were investigated in a Site (UVB, control) x Drug (phenylephrine, saline) repeated measures analysis of variance. The statistical analysis was performed using SPSS® statistical analysis software version 24 (IBM, New York, USA).

4.5. Results

4.5.1. Histological changes in the skin post-UVB exposure

An acute exposure of the skin to UVB changed the histological structure compared to the unexposed skin (control). These changes included a thickened epidermal layer, the presence of necrotic cells in the epidermis, dysplasia of the epidermis, and an increase in cellular infiltrates in the dermis (Figure 4-1). These parameters were used to determine the extent of UVB damage (UVB damage score) in the skin of each participant (Table 4-1). In all but one case, epidermal thickness was at least 10% greater in UVB-treated than control skin.
Figure 4-1. Skin histological changes post-UVB exposure

Histological changes in the skin were compared between control and post-UVB exposure. The tissues were cut at 10 µm thickness and stained using haematoxylin & eosin staining. The representative pictures were taken with a light microscope at x40 magnification. Ep=epidermis, Sc=stratum corneum, D=dermis, TEp=thickened epidermis, N=necrotic cells. The red box represents an increased number of cellular infiltrates in the dermis.
Chapter 4. The effects of $\alpha_1$-adrenoceptor stimulation in vivo

Table 4-1. UVB damage score for each participant

<table>
<thead>
<tr>
<th>Subject No</th>
<th>Increased epidermal thickness*</th>
<th>Necrotic cells in epidermis*</th>
<th>Dysplasia of epidermis*</th>
<th>Cellular infiltrates in dermis (qualitative measurement)*</th>
<th>Total score (out of 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<td>2</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
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* 0 = absent; 1 = present

4.5.2. IL-6 protein levels

The protein levels of IL-6 measured in dialysates varied highly among individuals. Thus, to reduce the skewness of this data, IL-6 protein levels were transformed to a natural logarithm for statistical analysis and presented in Figure 4-2. UVB exposure did not change IL-6 protein levels, compared to the untreated skin (control). Moreover, perfusion of the $\alpha_1$-AR agonist phenylephrine in the control skin did not change IL-6 protein levels measured in the dialysates (Figure 4-2 A). However, changes in IL-6 production at phenylephrine-perfused sites after UVB treatment were greater than changes in IL-6 production at saline-perfused sites after the UVB treatment [Site x
Drug interaction $F(1,10) = 5.27, p = 0.045$. This interaction remained statistically significant after excluding the participant with no evidence of skin damage from the analysis ($p = 0.047$). Investigation of this interaction indicated a nett increase in IL-6 levels after UVB exposure during phenylephrine perfusion relative to saline perfusion, represented as the IL-6 difference score (Figure 4-2 B).

**Figure 4-2. IL-6 protein levels in dialysates**

(A) IL-6 protein levels were measured by ELISA from skin microdialysis dialysates. 
(B) Changes in IL-6 production at phenylephrine-perfused sites after UVB treatment in comparison to changes in IL-6 production at saline-perfused sites after the UVB treatment [Site x Drug interaction $F(1,10) = 5.27, p = 0.045$]. The IL-6 difference score $= [\text{phenylephrine}_{(UVB)} - \text{phenylephrine}_{(control)}] - [\text{saline}_{(UVB)} - \text{saline}_{(control)}]$. The graphs represent the mean ± SEM of IL-6 protein levels that were transformed to natural logarithmic (ln) values in pg/ml.
4.6. Discussion

This study demonstrated that acute UVB irradiation caused an injury to the skin, characterized clinically by erythema (redness) and histologically by structural changes in the skin (Soter, 1990). The skin histological changes included an increase in epidermal thickness, necrotic keratinocytes, a dysplasia of the epidermis, and an increased number of cellular infiltrates in the dermis, such as mast cells, neutrophils and monocytes (Soter, 1990). However, the extent of UVB damage differed among participants, possibly due to differing skin types (D’Orazio et al., 2013). While signs of skin inflammation due to UVB exposure were observed clinically and histologically, we did not observe any consistent change in inflammatory cytokine IL-6 levels after the UVB treatment.

A microdialysis fibre consists of semipermeable membrane that allows a passive diffusion between solutions inside and outside the membrane. The skin microdialysis technique was utilized to investigate the effect $\alpha_1$-AR stimulation in cutaneous inflammation, in vivo. Perfusion of the $\alpha_1$-AR agonist phenylephrine in UVB-induced skin inflammation enhanced cutaneous IL-6 protein production relative to changes after saline perfusion. Keratinocytes are the primary IL-6 producing cells in the skin and express $\alpha_1$-AR (Dawson et al., 2011; Li et al., 2013; Wijaya et al., 2020b). We have previously shown that $\alpha_1$-AR stimulation induced IL-6 production in cultured keratinocytes (Wijaya et al., 2020a). Other resident cells in the skin, such as resident immune cells, Langerhans cells and dermal fibroblasts also secrete IL-6 in smaller amounts (Castells-Rodellas et al., 1992; Feghali et al., 1992; Wang et al., 2004) and express $\alpha_1$-AR (Maestroni, 2000; Sterin-Borda et al., 2007; Grisanti et al., 2011a; Grisanti et al., 2011b; Liao et al., 2014). Grisanti and colleagues showed that stimulation of $\alpha_1$-AR in peripheral immune cells heightened lipopolysaccharide-induced cytokine production (Grisanti et al., 2011b). However, whether other resident
skin cells contributed to this α₁-AR-induced IL-6 production in dialysates in the current study requires further investigation.

IL-6 is a pleiotropic cytokine which plays an important role in normal skin inflammatory responses. However, persistently high levels of cutaneous IL-6 have been found in several chronic pain conditions (Huygen et al., 2002; Birklein et al., 2014) and chronic inflammatory skin diseases (Goodman et al., 2009; Tanaka et al., 2014). UVB irradiation is a common type of skin injury and prolonged UVB exposure is associated with heightened risk of developing melanoma. Melanocytes and melanomas produce IL-6 (Krüger-Krasagakes et al., 1995), and persistent high levels of IL-6 may play an important role in cancer progression (reviewed by (Hoejberg et al., 2012)). Thus, a vicious cycle between α₁-AR and IL-6 in the skin, following prolonged UVB exposure, if unchecked, might contribute to melanoma malignancy progression.

We acknowledge that a larger or more homogenous sample than that used in this study might be needed to detect significant change in IL-6 levels after UVB exposure. In addition, an alternative technique to induce cutaneous inflammation that is independent from skin type, such as tape stripping (Holzmann et al., 2004), might induce inflammation more consistently than UVB exposure. Further studies involving additional participants and a stronger model of cutaneous inflammation could provide a clearer insight into the role of α₁-AR in cutaneous inflammation and its interaction with inflammatory mediators, in vivo.

Nevertheless, the present findings suggest that activation of α₁-AR following in an injury to the skin, enhances cutaneous inflammation, reflected by increased levels of IL-6 in dialysates. While a positive feedback interaction between α₁-AR and IL-6 in the skin may be important for normal inflammatory responses, dysregulation of this interaction could play a key role in the development of chronic cutaneous inflammation and associated skin disease.
Chapter 5
Chapter 5. Inflammation and complex regional pain syndrome: The role of alpha\textsubscript{1}-adrenoceptors

5.1. Preface

This following chapter is written as a manuscript for submission to a peer-reviewed journal.

The role of \alpha\textsubscript{1}-AR in cutaneous inflammation in normal conditions, \textit{in vitro} and \textit{in vivo}, has been described in the previous three chapters. In Chapter 5, the role of \alpha\textsubscript{1}-AR in skin inflammation is further elucidated in primary keratinocytes obtained from healthy controls and CRPS patients, as \textit{ex vivo} pathological models, to determine the role of \alpha\textsubscript{1}-AR in inflammation under pathological conditions.
5.2. Abstract

Previous studies have shown that alpha$_1$-adrenoceptors (alpha$_1$-AR) and inflammatory mediators (inflammatory cytokines and growth factors) may play important roles in the development and maintenance of persistent inflammation and pain in a subset of patients with complex regional pain syndrome (CRPS). In particular, we have shown that alpha$_1$-AR and inflammatory mediators interacted in a vicious cycle to promote inflammatory mediator production in an immortalized keratinocyte cell line. In this study, this vicious cycle was further investigated in primary keratinocytes obtained from healthy control volunteers and from CRPS patients. It was hypothesized that the vicious cycle between alpha$_1$-AR and inflammatory mediators would be stronger in CRPS compared to control primary keratinocytes. A pro-inflammatory cytokine, tumour necrosis factor alpha (TNF$\alpha$) was administered to cultured primary keratinocytes for 24 hours to provide an environment resembling skin inflammation. Following this treatment, the alpha$_1$-AR agonist phenylephrine (PE) was added and incubated for 6 hours. Cells treated with media, TNF$\alpha$, or PE without TNF$\alpha$ were used as experimental controls. Exposure to TNF$\alpha$ induced inflammatory cytokine mRNA and protein levels and reduced NGF mRNA levels in primary keratinocytes. Additional stimulation of alpha$_1$-AR using PE resulted in a heightened production of IL-6 mRNA and protein, and a further reduction in NGF mRNA levels. In CRPS keratinocytes, baseline alpha$_1$-AR protein expression was strongly associated with IL-6 mRNA production after alpha$_1$-AR stimulation with PE. These findings suggest that a positive feedback interaction between alpha$_1$-AR and inflammatory mediators in CRPS keratinocytes contributes to the development of persistent inflammation in this syndrome. Hence, targeting this interaction at an early stage of CRPS may be a useful therapeutic target to prevent the progression of the condition into the chronic stage.
5.3. Introduction

Sympathetic nervous system (SNS) dysfunction may be involved in the development and maintenance of pain in a subgroup of patients with complex regional pain syndrome (CRPS), a type of neuropathic pain (Drummond PD, 2004; Knudsen et al., 2019). A cutaneous injection of adrenergic ligands, noradrenaline or adrenaline, into the symptomatic (affected) limbs evokes greater and prolonged pain compared with injection into unaffected limbs (Davis et al., 1991; Ali et al., 2000), whereas sympathetic blockade reduces pain (Yoo et al., 2011; van Eijs et al., 2012; Harden et al., 2013). Our findings and those of others indicate that adrenoceptors (ARs) may facilitate this coupling between the SNS and nociceptors (Li et al., 2013; Drummond et al., 2014a; Drummond et al., 2014c; Finch et al., 2014; Drummond et al., 2016; Drummond et al., 2018). Our particular interest is in the alpha\textsubscript{1}-adrenoceptors (\(\alpha_1\)-AR), as their expression is heightened in the epidermis and dermal nerves bundles in the symptomatic limb of CRPS patients (Finch et al., 2014; Drummond et al., 2018) and in animal models of neuropathic pain (Drummond et al., 2014a; Drummond et al., 2014c). Moreover, stimulation of this receptor through its agonist phenylephrine (PE) induces prolonged mechanical hyperalgesia (Davis et al., 1991; Drummond et al., 2018), while \(\alpha_1\)-AR antagonist administration alleviates hyperalgesia (Davis et al., 1991; Ali et al., 2000), mechanical allodynia (Drummond et al., 2016) and unweighting (Drummond et al., 2014b).

One of the triggers of CRPS is injury to a limb, involving fracture or soft tissue injury (CRPS type I) or major peripheral nerve damage (CRPS type II). Inflammatory cytokines (e.g. tumour necrosis factor \(\alpha\) (TNF\(\alpha\)), interleukin (IL)-1\(\beta\), IL-6) and growth factors (e.g. nerve growth factor (NGF)), are released by resident tissue, such as skin, to assist healing, repair and regeneration after injury. In normal conditions, inflammation resolves once the injury is healed. However, in CRPS patients, the inflammatory state persists. This exaggerated inflammatory response is associated
with elevated levels of inflammatory cytokines, such as TNFα, IL-1β, and IL-6, in the skin and blister fluid obtained from the affected limb (Huygen et al., 2002; Groeneweg et al., 2006; Heijmans-Antonissen et al., 2006; Kramer et al., 2011; Birklein et al., 2014) and is believed to complicate the pathophysiology of CRPS. Moreover, the presence of inflammatory mediators and NGF induces neuropathic pain behaviours when injected into uninjured (Wagner & Myers, 1996a; Schafers et al., 2003; Zelenka et al., 2005) or injured tissue (DeLeo et al., 1996; Schafers et al., 2003; Kim & Moalem-Taylor, 2011). However, the mechanisms that underlie this persistent inflammatory condition in CRPS are not fully understood.

Growing evidence suggests that α1-AR signalling modulates inflammatory responses in several types of cells, such as immune cells (Rouppe van der Voort et al., 2000; Grisanti et al., 2011b), Kupffer cells (macrophage-like inflammatory cells in liver) (Huan et al., 2017), and human testicular peritubular cells (Rossi et al., 2018). We recently reported that an inflammatory cytokine, TNFα, induced α1-AR subtype B (α1B-AR) expression in an immortalized human keratinocyte cell line (HaCaT cells; Wijaya et al., 2020b)) and that further stimulation of this activated α1-AR enhanced the production of the inflammatory cytokine IL-6 from these cells (Wijaya et al., 2020a), suggesting a positive feedback loop between α1-AR and inflammatory cytokines in keratinocytes.

In the current study, we further investigated the positive feedback interaction between α1-AR and inflammatory cytokines in keratinocytes obtained from the skin of CRPS patients and healthy control volunteers (primary keratinocytes). Following activation of these cells with the pro-inflammatory cytokine TNFα, the α1-AR agonist PE was administered to determine whether stimulation of this receptor would generate heightened production of pro-inflammatory cytokines. It was hypothesized that α1-AR would interact in a vicious cycle with inflammatory cytokines in cultured keratinocytes.
ex vivo and that this interaction would be stronger in keratinocytes that originated from CRPS patients than healthy controls.

5.4. Material and methods

This study was an exploratory study to investigate the positive feedback interaction between $\alpha_1$-AR and inflammatory cytokines in keratinocytes obtained from the skin of CRPS patients and healthy control volunteers. Due to time limitations and patient availability, no study power analysis was calculated.

5.4.1. Participants

This study was approved by the human ethics committee of Murdoch University. Nine CRPS patients, who met the Budapest diagnostic criteria for CRPS (Harden & Bruehl, 2005; Harden et al., 2010b), were recruited from a local pain clinic. Both patients and healthy volunteers gave their informed consent at the beginning of the study.

On the morning of the visit, patients were requested not to take analgesic medication, but took other scheduled medications as usual. Patients underwent a short clinical interview and sensory tests. The sensory tests were performed to both affected (ipsilateral) and unaffected (contralateral) limbs in a temperature-controlled room between 21 and 24°C. The tests included assessment to pressure-pain threshold, pinprick sharpness, von Frey filament touch sensitivity to a single or 5 applications, and mechanical allodynia to skin brushing (light touch response). Pressure-pain threshold was measured using an algometer (FDX; Wagner Instruments, Greenwich, CT, USA) with a modified 8-mm diameter hemispheric rubber tip that was applied once at each site at 100 g/second until the participant reported pain. A spring-loaded metal pin at a force of 40 g (NeuroPen; Owen Mumford, Woodstock, Oxfordshire,
Chapter 5. Inflammation and CRPS: The role of $\alpha_1$-adrenoceptors

United Kingdom) was applied for 2 seconds and a rating of sharpness was recorded. A von Frey nylon monofilament with a bending force of 10 g (NeuroPen; Owen Mumford, Woodstock, Oxfordshire, United Kingdom) was applied once and then five times at 1-second intervals. The sharpness of these applications was rated at the end of the application series. In addition, skin temperature was measured on the dorsal surface of the base of each digit on the affected and unaffected limbs using an infrared thermometer. The temperature difference between affected and unaffected limbs was determined by taking the average of temperature of digits on each limb. For the control group, eight age-matched healthy volunteers, without any acute or chronic pain or inflammation, were also recruited.

5.4.2. Isolation and culture of primary keratinocytes from skin biopsies

A 3-mm diameter punch skin biopsy was collected from the affected limb of CRPS patients or at a similar site in healthy control volunteers, under local anaesthesia. The skin biopsies were washed in cold PBS (no $\text{Mg}^{2+}/\text{Ca}^{2+}$) containing 100 U/mL penicillin and 100 $\mu$g/mL streptomycin. All the materials used in the isolation and maintenance of primary keratinocyte culture were obtained from Gibco™ (Thermo Fisher Scientific, MA, USA) unless otherwise stated. The isolation and dissociation of primary keratinocytes from the skin biopsy were performed using protocols published previously with some modifications (Aasen & Izpisua Belmonte, 2010). Briefly, a skin biopsy was incubated in a digestive enzyme solution, containing 1.3 U/mL dispase, 1 $\mu$g/mL fungizone (Amphotericin B) and 200 $\mu$g/mL kanamycin in keratinocyte media (KM; EpiGRO™ Human Epidermal Keratinocyte Complete Culture Media Kit, Merck, Germany), for 12-16 h, at 4°C to separate the epidermal layer. After several washes in KM to remove dispase, the epidermis was slowly separated from the dermis using tweezers and incubated in trypsin solution (1X TrypLE™ Express Enzyme) in a sterile culture dish, with the basal layer of epidermis facing down, for 20-30 min at room
temperature. Primary keratinocytes were gently dissociated from the epidermis by gently rubbing the epidermis against the base of the dish. The cells were collected and centrifuged for 5 min at 200 G at room temperature. The cell pellet was resuspended gently in KM media and seeded in a 10-mm cell culture dish. The media was changed after 4 days, and every 2 days thereafter. The cultivation and maintenance of the keratinocytes were performed in a controlled incubator at 37°C and 5% CO₂.

5.4.3. Experimental procedure

The second or third passage of CRPS and healthy keratinocytes was plated in 24-well plates. To abolish the effect of epinephrine (neurotransmitter ligand for α₁-AR) in the media supplement, primary keratinocytes were incubated in non-supplemented KM (nsKM) for 24 h. Human recombinant TNFα (Peprotech, New Jersey, USA) was diluted in nsKM at a final concentration of 10 ng/mL and was administered to the cells for 24 h. This concentration has been shown previously to induce inflammatory cytokines, nerve growth factor (NGF), and α₁-AR subtype B (α₁B-AR) expression in a human keratinocyte cell line, HaCaT cells (Wijaya et al., 2020b). The α₁-AR selective agonist, phenylephrine (PE), was then added to the cells at a final concentration of 1 μM or 10 μM and the cells were incubated for another 6 h. Cells treated with nsKM, PE, or TNFα only were used as experimental controls. Each treatment was performed in 4 wells (4 technical replicates). At the end of the incubation period, the conditioned media was collected to measure inflammatory cytokine and NGF protein levels. In addition, the cells were lysed with lysis buffer and technical replicates were pooled prior to isolation of total RNA.
5.4.4. Quantitative real-time PCR

Total RNA was isolated and purified from the cell lysates using an Isolate II RNA mini kit (Bioline, NSW, Australia). The amount of RNA extracted from the cells was quantified using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, DE, USA). One μg total RNA was converted to cDNA using a Sensifast™ cDNA synthesis kit (Bioline, NSW, Australia), according to the manufacturer’s protocol. A SYBR® Green-based quantitative real-time PCR (qRT-PCR) assay, followed by melting curve analysis, was used to measure gene expression levels of α1-AR subtype B (α1B-AR) and inflammatory cytokines IL-1β, IL-6, IL-8, and NGF, while a hydrolysis probe-based qRT-PCR assay was used to measure levels of α1-AR subtype A (α1A-AR) and D (α1D-AR). The primer sets used in qRT-PCR are listed in Table 5-1 and Table 5-2, for SYBR® Green-based and hydrolysis probe-based assays, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control in both assays. All of the primers were validated to amplify only mRNA and not genomic DNA (unpublished data). The qRT-PCR assay was performed using a ViiA™ 7 Real-Time PCR System (Applied Biosystems™, MA, USA). A cycle threshold (Ct) value of each sample was collected at the end of the qRT-PCR run (40 cycles). The change in gene expression as a result of treatments compared to the untreated cells (naïve) was represented by ΔΔCt as described previously (Yuan et al., 2006). The mean ± SEM of the gene expression change in each treatment are presented in the Results.
Table 5-1. Primer sets used in a SYBR® Green qRT-PCR assay

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<tr>
<th>Gene ID (Names)</th>
<th>GenBank Accession</th>
<th>Forward Sequence ‘5 – 3’</th>
<th>Reverse Sequence ‘5 – 3’</th>
<th>Product Size (bp)</th>
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<tr>
<td>CXCL8 (IL-8)</td>
<td>NM_000584</td>
<td>ATG ACT TCC AAG CTG GCC GT</td>
<td>TCC TTG GCA AAA CTG CAC CT</td>
<td>82</td>
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<tr>
<td>IL6 (IL-6)</td>
<td>NM_000600</td>
<td>AGC CCT GAG AAA GGA GAC ATG</td>
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<td>144</td>
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<tr>
<td>IL1B (IL-1β)</td>
<td>NM_000576</td>
<td>CTG TCC TGC GTG TTG AAA GA</td>
<td>TTG GGT AAT TTT TGG GAT CTA CA</td>
<td>70</td>
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<tr>
<td>NGF (NGF)</td>
<td>NM_002506</td>
<td>TGT GAT CAG AGT GTA GAA CCA CA</td>
<td>CGG ACC CAA TAA CAG TTT TAC C</td>
<td>97</td>
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<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>GGG TGT GAA CCA TGA GAA</td>
<td>GAC TGT GGT CAT GAG TCC T</td>
<td>136</td>
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<tr>
<td>ADRA1B (α1B-AR)</td>
<td>NM_000679</td>
<td>PrimePCR™ SYBR® Green Assay (assay ID: qHsACIP0030605)</td>
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Table 5-2. TaqMan® primers used in a hydrolysis probe-based qRT-PCR assay

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<th>Assay ID</th>
<th>Amplicon Size (bp)</th>
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<tr>
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<td>NM_000678</td>
<td>Hs00169865_m1</td>
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<td>NM_001256799</td>
<td>Hs02758991_g1</td>
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5.4.5. Multiplex enzyme-linked immunosorbent assay

Conditioned media collected from each treatment was used to measure inflammatory cytokine and NGF protein levels from primary keratinocytes. The protein levels were measured using a magnetic-bead based multiplex enzyme-linked immunosorbent assay (ELISA) technology that allows simultaneous measurement of different proteins (analytes) in one assay. Several important inflammatory cytokines that were
previously shown to be elevated in chronic neuropathic pain conditions and CRPS (Heijmans-Antonissen et al., 2006; Uceyler et al., 2010; Kramer et al., 2011; Birklein et al., 2014) were chosen from the MILLIPLEX MAP Human Adipocyte panel (Merck, Darmstadt, Germany). The assay was performed according to the manufacturer’s protocol using undiluted media. The assay signal was measured with a Bio-Plex® 200 System and analysed using Bio-Plex Manager software (Bio-Rad Laboratories, CA, USA).

5.4.6. Western blotting

The materials used for the western blotting (WB) method were acquired from Thermo Fisher Scientific (MA, USA), unless stated separately. The WB technique was used to determine baseline $\alpha_1$-AR protein expression levels in CRPS and healthy control keratinocytes. Keratinocytes from each participant were lysed using NP40 lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet-P40 or Triton X-100) supplemented with cOmplete™ protease inhibitor cocktail (Roche, via Merck, Darmstadt, Germany; 1 tablet / 10 ml NP40 lysis buffer). The lysates were incubated on ice for 30 min with application of vigorous vortexing every 10 min. At the end of the incubation period, the lysates were centrifuged at 12,000 G for 15 min at 4°C. The supernatants were collected and total protein concentration was determined using a Pierce™ BCA protein assay kit. Twenty-five $\mu$g total protein was prepared in NuPAGE™ LDS Sample Buffer with reducing agent, boiled at 95°C for 10 min, and loaded into NuPage™ 4-12% Bis-Tris gels. Gel electrophoresis was performed in 1X NuPAGE™ MES SDS running buffer at 90 volts (constant), for 2.5 h. The protein was transferred onto a 0.45 $\mu$m nitrocellulose membrane using a wet-transferred system (Mini Trans-Blot® Cell, Bio-Rad Laboratories, CA, USA) in 1X transfer buffer (192 mM glycine, 25 mM Tris) with 20% (v/v) methanol, at 100 volts for 1.5 h. Immunoblotting steps were performed at room temperature (unless stated separately) and with gentle rocking.
Following several washes in tris-buffered saline pH 7.4 (TBS; 10 mM Tris, 150 mM NaCl) to remove excess methanol, membranes were incubated in 5% skimmed milk in TBS buffer for 1 h. Primary antibodies against \( \alpha_1 \)-AR or GAPDH (as a loading control) were diluted in TBS, supplemented with 0.05% Tween-20 (TBS-T; Sigma-Aldrich, St. Louis, MO) and 0.5% skimmed milk. The incubation of primary antibody was performed overnight at 4°C. The next day, membranes were washed with TBS-T (3 x 15 min) prior to secondary antibody incubation in TBS-T with 0.5% skimmed milk for 1 h. Primary and secondary antibodies are listed in Table 5-3. After three subsequent washes with TBS-T (15 min each), enhanced chemiluminescence substrate solution (Clarity™, Bio-Rad Laboratories, CA, USA) was added and the chemiluminescence signals were captured using ChemiDoc MP (Bio-Rad Laboratories, CA, USA). The protein expression levels of \( \alpha_1 \)-AR and GAPDH were determined through measurement of band signal (in optical density – OD) at 55 kDa and 37 kDa, respectively, using Image™ Lab analysis software (Bio-Rad Laboratories, CA, USA). As the protein size of \( \alpha_1 \)-AR and GAPDH are similar, the detection of GAPDH was performed following WB for \( \alpha_1 \)-AR, using WB membrane stripping protocol: membranes were washed in TBS-T (3 x 15 min), incubated with Restore™ WB stripping buffer for 15 min, washed in TBS-T (3 x 15 min) and rinsed in TBS. WB steps were repeated for GAPDH detection following this stripping protocol.
Table 5-3. Primary and secondary antibodies

<table>
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<th>Dilution</th>
<th>Product Code and Source</th>
<th>Protein Size (kDa)</th>
</tr>
</thead>
<tbody>
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<td>Anti-α₁-AR, rabbit monoclonal</td>
<td>1:1000</td>
<td>ab192614; Abcam, Victoria, Australia</td>
<td>55</td>
</tr>
<tr>
<td>Anti-GAPDH, mouse monoclonal</td>
<td>1:5000</td>
<td>#97166; Cell Signalling Technology, MA, USA</td>
<td>37</td>
</tr>
<tr>
<td>Anti-rabbit-HRP</td>
<td>1:5000</td>
<td>NA934; GE Healthcare, Freiburg, Germany</td>
<td></td>
</tr>
<tr>
<td>Anti-mouse-HRP</td>
<td>1:5000</td>
<td>NA931; GE Healthcare, Freiburg, Germany</td>
<td></td>
</tr>
</tbody>
</table>

5.4.7. Statistical approach

Multiple comparison analysis, utilizing a general linear model with repeated measures, was performed to determine mean differences between: (1) treatment conditions and; (2) CRPS and healthy control groups. The change in gene expression levels caused by treatments (in ΔΔCt) and the values generated from multiplex ELISA assays were investigated in the statistical analyses. The mean values of baseline α₁-AR protein expression (in OD) of CRPS and healthy control keratinocytes were analysed using an independent-samples Student’s t-test to determine the mean difference between groups. A Pearson’s correlation analysis was performed to investigate the association between baseline α₁-AR protein expression and inflammatory cytokine and NGF levels. All statistical analyses were performed using SPSS® statistical analysis software version 24 (IBM, New York, USA).
5.5. Results

5.5.1. Demographic characteristics of participants

Nine CRPS patients and eight healthy control volunteers participated in this study. There was no age difference between CRPS patients (51.1 ± 11.9 years; Mean ± SD) and healthy control volunteers (49.9 ± 10.7 years). The percentage of female participants was 44% and 50% in the CRPS and healthy control groups respectively. Eight of the patients had chronic CRPS (duration more than 36 months) when the skin biopsies were obtained, and the other patient had CRPS for 23 months. Other demographic characteristics are summarised in Table 5-4 and Table 5-5.

Table 5-4. Summary of demographic characteristics of CRPS patients and healthy controls

<table>
<thead>
<tr>
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<th>CRPS</th>
<th>Control</th>
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<tr>
<td>Age ± SD (years)</td>
<td>51.1 ± 11.9</td>
<td>49.9 ± 110.7</td>
</tr>
<tr>
<td>Female</td>
<td>44%</td>
<td>50%</td>
</tr>
<tr>
<td>Duration ± SD (months)</td>
<td>114 ± 83.5</td>
<td></td>
</tr>
<tr>
<td>Extremity affected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left upper extremity</td>
<td>22%</td>
<td></td>
</tr>
<tr>
<td>Right upper extremity</td>
<td>22%</td>
<td></td>
</tr>
<tr>
<td>Left lower extremity</td>
<td>11%</td>
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<tr>
<td>Right lower extremity</td>
<td>44%</td>
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</table>
Table 5-5. Demographic data of healthy control volunteers

<table>
<thead>
<tr>
<th>Control No</th>
<th>Age</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>F</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
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<td>3</td>
<td>41</td>
<td>M</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>F</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>F</td>
</tr>
<tr>
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<td>M</td>
</tr>
<tr>
<td>8</td>
<td>65</td>
<td>F</td>
</tr>
</tbody>
</table>

The results of individual quantitative sensory tests from CRPS patients are presented in Table 5-6. Significant differences in pressure pain threshold, pinprick hyperalgesia and touch sensitivity measured by a single or multiple application of von Frey hair were shown between affected and unaffected limbs. Different responses to light touch were observed between patients, while there was no consistent difference in temperature between affected and unaffected limbs. In Patients 1 and 4 pain had spread globally and to the contralateral limb, respectively. The skin biopsy was taken from the most painful limb of Patient 4.
### Table 5-6. Quantitative sensory tests of CRPS patients

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Age</th>
<th>Sex</th>
<th>Pressure Pain Threshold (kg) (t=-2.599; p=0.0320*)</th>
<th>Pain rating to Pinprick (t=0.873; p=0.000*)</th>
<th>Pain rating to von Frey filaments (1X) (t=3.545; p=0.000*)</th>
<th>Pain rating to von Frey filaments (5X) (t=0.117; p=0.901*)</th>
<th>Brush (light touch responses)</th>
<th>Temperature (°C) (t=0.969; p=0.361)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Affected Contralateral</td>
<td>Affected Contralateral</td>
<td>Affected Contralateral</td>
<td>Affected Contralateral</td>
<td>Affected Contralateral</td>
<td>Affected Contralateral</td>
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<tr>
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<td>2</td>
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<td>0.965</td>
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<td>7</td>
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<tr>
<td>9</td>
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<td>F</td>
<td>0.460</td>
<td>0.740</td>
<td>9</td>
<td>7</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td><strong>Mean ± S.D.</strong></td>
<td></td>
<td></td>
<td>0.553 ± 0.247</td>
<td>1.696 ± 1.313</td>
<td>5.4 ± 2.1</td>
<td>2.8 ± 2.6</td>
<td>4.9 ± 2.8</td>
<td>2.1 ± 2.5</td>
</tr>
</tbody>
</table>

*Pain rating scale is from 0 (not at all painful) to 10 (the most unbearable pain ever experienced)

*The pain had intensified in the contralateral limb. The skin biopsy was obtained from contralateral site that was more problematic (inflamed and painful) on the visit day.
5.5.2. Baseline $\alpha_1$-AR protein levels in healthy control and CRPS patient primary keratinocytes

Baseline $\alpha_1$-AR protein levels in healthy control and CRPS keratinocytes were determined using western blotting (WB). The individual $\alpha_1$-AR protein levels, along with its GAPDH loading control, are presented in Figure 5-1. Individual variation in $\alpha_1$-AR protein levels was greater in the CRPS group than in the control group.

**Figure 5-1. Baseline $\alpha_1$-AR protein levels in control and CRPS keratinocytes**

Baseline protein levels of $\alpha_1$-AR were measured in cultured keratinocytes using western blotting. Detection of $\alpha_1$-AR and GAPDH (a loading control) proteins was performed by western blot using antibodies listed in Table 5-3. The $\alpha_1$-AR and GAPDH protein levels are depicted as bands at 55 kDa and 37 kDa, respectively (A). The individual band intensity for each participant was measured, normalized to its GAPDH expression, and presented in the graph (B). The bars represent the Mean ± SEM of each group. M=Marker of protein weight in kilo Dalton (kDa); OD=optical density; n$_{CRPS}$=9 and n$_{Control}$=8; numbers 1-8 and 1-9 match healthy control and CRPS patient numbers as listed in Table 5-5 and Table 5-6, respectively.
5.5.3. The effects of TNFα and/or phenylephrine treatments on inflammatory cytokine and NGF levels

There were no differences in inflammatory cytokine or NGF mRNA and protein levels between CRPS and healthy control groups; thus, the data from both groups were combined and analysed together to determine the effects of TNFα and phenylephrine treatments on inflammatory cytokine and NGF levels. TNFα treatment significantly increased both inflammatory cytokine mRNA expression (Figure 5-2 A-C) and protein levels (Figure 5-2 E-G) compared to the untreated cells (naïve). In contrast, TNFα significantly reduced NGF mRNA levels (Figure 5-2 D), while there was no change in protein levels (Figure 5-2 H).

Phenylephrine (PE) administration, at either 1 or 10 μM, altered inflammatory cytokine and NGF levels. Administration of PE alone reduced IL-1β mRNA levels at 10 μM, compared to naïve cells (Figure 5-2 A); however, IL-1β gene expression levels did not change when PE was administered to activated (TNF-treated) keratinocytes. Changes in IL-1β mRNA levels were not reflected at the protein level. Here, administration of 1 μM PE reduced IL-1β levels in normal keratinocytes but did not alter IL-1β levels in activated keratinocytes (Figure 5-2 E). Ten μM PE did not alter IL-1β protein levels when administered to normal or activated keratinocytes, compared to naïve and TNF-treated cells, respectively (Figure 5-2 E).

Phenylephrine-mediated stimulation of α1-AR significantly reduced IL-8 gene expression levels in normal keratinocytes and this reduction was dose dependent (Figure 5-2 B). However, PE administration to TNFα-activated keratinocytes did not alter IL-8 mRNA (Figure 5-2 B) or protein (Figure 5-2 F) levels.

Exposure to PE significantly augmented IL-6 mRNA levels (Figure 5-2 C), consistent with the dose-dependent increase in IL-6 protein levels (Figure 5-2 G), when PE was applied alone to resting keratinocytes. Stimulation of α1-AR following TNFα treatment
resulted in an increase in IL-6 mRNA (Figure 5-2 C) and protein levels (Figure 5-2 G), with a dose-dependent increase at the mRNA level.

In contrast to IL-6, administration of PE alone reduced NGF mRNA levels compared to naïve cells (Figure 5-2 D). Interestingly, there were further reductions in NGF mRNA levels when PE was administered to TNF-treated cells. However, these changes were not reflected at the protein level (Figure 5-2 H).
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Gene Expression

A. IL-1$\beta$
B. IL-8
C. IL-6
D. NGF

Protein

E. IL-1$\beta$
F. IL-8
G. IL-6
H. NGF
Chapter 5. Inflammation and CRPS: The role of $\alpha_1$-adrenoceptors

Figure 5-2. Inflammatory cytokine and NGF mRNA and protein levels in both healthy control and CRPS keratinocytes

Healthy control and CRPS keratinocytes were administered 10 ng/ml TNF$\alpha$ (TNF) or media only (naïve) for 24 h. One or 10 $\mu$M phenylephrine (PE) was added to the TNF-treated cells (TNF + PE condition) or naïve cells (PE condition) for an additional 6 h. At the end of the treatment, gene expression levels of inflammatory cytokines IL-1$\beta$ (A), IL-8 (B), IL-6 (C) and NGF (D) were measured using a qRT-PCR. Changes in gene expression as a result of treatment are represented as $\Delta\Delta$Ct ($\Delta$Ct$_{\text{naïve}}$=0). Protein levels of inflammatory cytokine IL-1$\beta$ (E), IL-8 (F), IL-6 (G) and NGF (H) secreted by cells into the media were measured using multiplex ELISA and are represented as mean ± SEM. White bars represent control keratinocytes (n=8) and grey bars represent CRPS keratinocytes (n=9). There were no significant differences either in gene expression or protein levels between control and CRPS groups. ★p<0.05 compared to naïve cells; *p<0.05 compared to all groups; †p<0.05.

5.5.4. The effects of TNF$\alpha$ and/or phenylephrine treatments on $\alpha_{1B}$-AR gene expression and NGF levels

A preliminary experiment was performed to characterize gene expression levels of $\alpha_1$-AR subtype A ($\alpha_{1A}$-AR), B ($\alpha_{1B}$-AR) and D ($\alpha_{1D}$-AR) in primary keratinocytes. Consistent with $\alpha_1$-AR subtype expression seen in an immortalized human keratinocyte cell line (HaCaT cells; (Wijaya et al., 2020b)), primary keratinocytes did not express $\alpha_{1A}$-AR. In addition, $\alpha_{1B}$-AR mRNA levels were too low to be accurately measured and quantified (Ct value > 33), while $\alpha_{1B}$-AR was readily expressed (unpublished data). TNF$\alpha$ exposure increased $\alpha_{1B}$-AR gene expression levels in both control and CRPS keratinocytes (Figure 5-3). Administration of PE to normal or
activated (TNF-treated) keratinocytes reduced $\alpha_{1B}$-AR mRNA levels in a dose-dependent manner. However, there were no differences between control and CRPS keratinocytes in response to either treatment.

Figure 5-3. $\alpha_{1B}$-AR gene expression levels in healthy control and CRPS keratinocytes

TNF$\alpha$ was administered to keratinocytes at 10 ng/ml for 24 h. Following TNF$\alpha$ treatment, 1 or 10 $\mu$M phenylephrine (PE) was added to the cells and incubated for another 6 h. Naïve cells treated with media only were used to estimate baseline $\alpha_{1}$-AR expression and cells treated with PE alone were used as an experimental control. Changes in gene expression as a result of the treatments are represented as $\Delta \Delta Ct$ ($\Delta \Delta Ct_{\text{Naïve}}=0$). White bars represent control keratinocytes (n=8) and grey bars represent CRPS keratinocytes (n=9). Data are represented as mean ± SEM. There were no differences between control and CRPS keratinocyte responses to either of the treatments. ★p<0.05 compared to naïve cells (at zero); ◊p<0.05 compared to all groups; *p<0.05.
5.5.5. Correlation analysis

Several CRPS patients with high levels of baseline $\alpha_1$-AR protein produced high levels of IL-6 in response to $\alpha_1$-AR stimulation. Therefore, we were interested to investigate the association between the baseline $\alpha_1$-AR protein expression and IL-6 production in healthy control and CRPS keratinocytes. No correlation between $\alpha_1$-AR baseline protein levels and IL-6 mRNA levels (Figure 5-4 A-C, E) was seen in the healthy control group apart from a positive correlation for cells treated with TNF and 1 µM PE (Figure 5-4 D). In contrast, strong positive correlations between baseline $\alpha_1$-AR protein expression and IL-6 mRNA levels were observed both in normal (Figure 5-4 F-G) and activated (TNF-treated; Figure 5-4 I-J) CRPS keratinocytes that were treated with phenylephrine. No correlation was observed in CRPS keratinocytes treated with TNF$\alpha$ only (Figure 5-4 H).
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Figure 5-4. Correlation analysis between baseline $\alpha_1$-AR protein expression and IL-6 mRNA levels

Scatterplot graphs with linear regression represent the correlation between baseline $\alpha_1$-AR protein levels and IL-6 gene expression changes ($\Delta\Delta$Ct) in response to treatments of: 1 $\mu$M PE (A, F), 10 $\mu$M PE (B, G), 10 ng/ml TNF$\alpha$ (C, H), TNF$\alpha$ and 1 $\mu$M PE (D, I), or TNF$\alpha$ and 10 $\mu$M PE (E, J), in healthy controls (A–E; n=8) and CRPS patients (F–J; n=9). R= Pearson’s correlation coefficient; OD= optical density. *p<0.05.

5.6. Discussion

In this study, interactions between $\alpha_1$-AR and inflammatory mediators (cytokines and NGF) were investigated in primary keratinocytes obtained from CRPS patients and healthy control volunteers. TNF$\alpha$ induced an inflammatory state marked by a significant increase in inflammatory cytokine production in primary keratinocytes and upregulated $\alpha_{1B}$-AR gene expression levels, while reducing NGF mRNA levels. Stimulation of activated (TNF-induced) $\alpha_1$-AR further augmented both gene expression and protein levels of IL-6, which suggests a positive feedback interaction between $\alpha_1$-AR and IL-6 in primary keratinocytes. In contrast to IL-6, NGF mRNA levels were further reduced following stimulation of $\alpha_1$-AR in activated keratinocytes, although these changes were not reflected at the protein level. While there were no significant differences between control and CRPS keratinocytes in responses to TNF$\alpha$ and $\alpha_1$-AR stimulation, interestingly, $\alpha_1$-AR baseline protein levels correlated positively with the CRPS cells’ response to $\alpha_1$-AR stimulation in producing IL-6. These results suggest that whilst both CRPS and control keratinocytes behaved similarly in culture, inflammatory and sympathetic signalling may drive inflammatory mediator
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production differently \textit{in vivo}, as differences in local skin inflammatory mediator levels were observed between CRPS patients and healthy controls (Huygen et al., 2002; Groeneweg et al., 2006; Heijmans-Antonissen et al., 2006; Kramer et al., 2011; Birklein et al., 2014).

In the acute stage of CRPS, an excessive inflammatory response in local tissues at the injury site plays an important role in the development of a persistent inflammatory condition. Inflammatory cytokines, such as TNF$\alpha$ and IL-6, are elevated in the epidermis (Birklein et al., 2014) and skin blister fluid (Huygen et al., 2002; Groeneweg et al., 2006; Heijmans-Antonissen et al., 2006; Kramer et al., 2011) in the affected limb of CRPS patients, but not in the blood (Huygen et al., 2002; Schinkel et al., 2006; Schinkel et al., 2009). This heightened local inflammatory response appears to be specific to CRPS (Kramer et al., 2011; Lenz et al., 2013). While clinical signs of inflammation are often reduced at the affected site in the chronic stage of CRPS, levels of IL-6 in skin blister fluid obtained from the affected site are higher than the contralateral site (Munnikes et al., 2005; Dirckx et al., 2015). In the present study, keratinocytes obtained from chronic CRPS patients were sensitive to $\alpha_1$-AR stimulation, and this triggered an increase in the production of IL-6 in proportion to basal $\alpha_1$-AR protein levels. While the responsiveness of CRPS keratinocytes obtained at the acute stage (the inflammatory phase) to $\alpha_1$-AR stimulation in producing IL-6 might be different, these results provide evidence that an abnormal local inflammatory response exists in some CRPS patients in the chronic stage which may be activated by $\alpha_1$-AR stimulation (e.g., during sympathetic nervous system arousal caused by stress).

Signs of persistent inflammation are often detected in the affected limb of CRPS patients. While the mechanisms that mediate chronic inflammation are not fully understood, the current study suggests the contribution of a positive feedback interaction between $\alpha_1$-AR and inflammatory mediators, in particular IL-6 and NGF,
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in the development of persistent inflammation and pain. Perez and colleagues showed that such cross-talk may be facilitated through the involvement of the p38 MAPK and NF-κB inflammatory signalling pathways (Perez et al., 2009). Utilizing mouse cardiomyocytes and $\alpha_{1A}$-AR-over-expressed rat fibroblasts, stimulation of $\alpha_1$-AR resulted in a stabilization of IL-6 mRNA and a maintenance of IL-6 transcription factor, involving the aforementioned pathways (Perez et al., 2009). In the present study, stimulation of TNF-induced $\alpha_1$-AR further augmented IL-6 mRNA and protein levels, compared to cells that were treated only with phenylephrine or TNFα. It is postulated that TNFα induced IL-6 production through classical inflammatory signalling pathways, such as NF-κB, JAK-STAT, and MAPK (Tanabe et al., 2010). Subsequent $\alpha_1$-AR stimulation might then have stabilized TNF-induced IL-6 mRNA and maintained transcription factors for IL-6 (Perez et al., 2009).

Elevated IL-6 levels at the site of injury have been reported in neuropathic pain animal models and CRPS patients in association with neuropathic pain behaviour (DeLeo et al., 1996; De Jongh et al., 2003; Sabsovich et al., 2008b; Li et al., 2010; Li et al., 2013; Birklein et al., 2014; Guo et al., 2014; Guo et al., 2018). IL-6 evokes nociceptive hypersensitivity and pain when injected into normal skin (DeLeo et al., 1996; De Jongh et al., 2003). Additionally, systemic administration of an IL-6 receptor antagonist, TB-2-081, reduced neuropathic pain behaviour in a rat tibia fracture model of CRPS (Li et al., 2013). Studies utilizing IL-6 knockout mice showed that elimination of IL-6 expression reduced mechanical allodynia in a neuropathic pain mouse model (Ramer et al., 1998) and in a chemotherapy-induced neuropathy mouse model (Kiguchi et al., 2008). Together, this evidence supports the important role of IL-6 in nociception. Therefore, persistently high levels of IL-6 may contribute to persistent pain seen in CRPS patients. Importantly, IL-6 also stimulates antibody (immunoglobulin) production from B lymphocytes (Tanaka et al., 2014). Persistent and heightened production of IL-6 may trigger the development of autoimmunity and
autoantibody production (Tanaka et al., 2014; Kishimoto et al., 2015), and may thus contribute to the development of the autoimmune component in CRPS pathophysiology (Goebel & Blaes, 2013; David Clark et al., 2018).

NGF is a neurotrophic factor that plays an important role in peripheral nerve survival and regeneration after injury. In keratinocytes, NGF acts as a chemotactic factor for axonal outgrowth and peripheral nerve innervation during wound healing. While TNFα has been previously shown to induce NGF expression in an immortalized human keratinocyte cell line (HaCaT cells) (Takaoka et al., 2009; Wijaya et al., 2020b), we demonstrated that TNFα reduced NGF mRNA levels in primary keratinocytes. This may be due to the short life span of primary keratinocytes and attenuation of cell growth caused by the removal of media supplement (Di Marco et al., 1991). This might have reduced the sensitivity of keratinocytes to TNFα induction and caused aberrant cross-talk between TNFα and NGF that decreased NGF production (Takei & Laskey, 2008a).

Stimulation of α1-AR in cardiomyocytes attenuates NGF mRNA levels (Kaye et al., 2000; Ieda et al., 2004) and this effect is reversed through pre-administration of the α1-AR antagonist prazosin (Kaye et al., 2000). Chronic stimulation of adrenoceptors reduces NGF levels and results in the loss of sympathetic nerves in myocardial tissue (Kaye et al., 2000; Kimura et al., 2010). Consequently, this condition leads to heart failure. In this study, α1-AR stimulation reduced NGF mRNA levels in primary keratinocytes, with the greatest decreases seen in primary keratinocytes treated with a combination of TNFα and phenylephrine. This was not reflected at the protein level, possibly due to the stability of NGF protein in culture (Nguyen et al., 2000) and the complexity of transcription-translation mechanisms (Greenbaum et al., 2003). Nonetheless, in vivo, attenuated skin NGF levels may hinder peripheral nerve regeneration and axonal outgrowth after injury and result in a reduction in cutaneous nerve fibre density. In patients with painful neuropathy, such as diabetic neuropathy
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(Quattrini et al., 2008), HIV neuropathy (Obermann et al., 2008), small nerve fibre neuropathy (Uceyler et al., 2010), leprosy (Facer et al., 2000) and CRPS (Oaklander et al., 2006; Morellini et al., 2018; Rasmussen et al., 2018), a reduction in cutaneous nerve fibre density has been identified in skin biopsies collected from the affected limb, and is associated with the pain experience.

Similar to other G-protein coupled receptors, $\alpha_1$-AR undergo rapid internalization to maintain homeostasis when the receptors are stimulated (Fonseca et al., 1995; Stanasila et al., 2008; Castillo-Badillo et al., 2015); this internalization leads to desensitization and down regulation of the receptors. Eventually, the receptors are recycled back to the plasma membrane or degraded by the lysosome (Castillo-Badillo et al., 2015). These mechanisms may explain phenylephrine-induced $\alpha_1$-AR mRNA downregulation in primary keratinocytes. Unfortunately, due to the limited supply of primary keratinocytes, these changes could not be confirmed at the protein level. Nevertheless, the mRNA reduction did not hinder the effect of $\alpha_1$-AR signalling on inflammatory mediator levels. These results may indicate that the induction of IL-6 levels through $\alpha_1$-AR signalling was rapid, possibly preceding $\alpha_1$-AR downregulation.

In summary, our findings indicate that keratinocytes may play an important role in local inflammatory responses and the development of persistent inflammation in CRPS. This may be mediated through a positive feedback interaction between $\alpha_1$-AR and inflammatory mediators, in particular IL-6 and NGF. We acknowledge that the small number of participants (CRPS patients and healthy volunteers) was a limitation of this study. Nevertheless, a vicious cycle between $\alpha_1$-AR and IL-6 in CRPS keratinocytes, particularly those with high baseline levels of $\alpha_1$-AR expression, was identified. Thus, targeting the interaction between $\alpha_1$-AR and inflammatory mediators may be a useful therapeutic strategy to hinder the development of persistent inflammation and pain in CRPS patients and, ultimately, to prevent the progression of CRPS into a chronic stage.
Chapter 6
Chapter 6. General Discussion

6.1. Results summary

In this study, the role of alpha-1-adrenoceptors (α1-AR) in cutaneous inflammation was investigated utilizing in vitro, in vivo and ex vivo approaches. In Chapter 2, it was shown that in vitro administration of the pro-inflammatory mediator tumour necrosis factor α (TNFα) induced an inflammatory state in an immortalized human keratinocyte cell line (HaCaT cells), characterised by increased levels of the pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8, and nerve growth factor (NGF). Moreover, TNFα or the inflammatory state induced by TNFα, heightened the expression of α1-AR subtype B (α1B-AR) in these cells. In Chapter 3, the consequences of this heightened α1-AR expression were further investigated. The α1-AR agonist phenylephrine was administered to activated HaCaT cells following TNFα treatment, with the results showing that stimulation of α1-AR on activated HaCaT cells further increased production of IL-6 from these cells compared to HaCaT cells that were treated with TNFα only. These results suggested a positive feedback interaction between α1-AR and TNFα in promoting pro-inflammatory IL-6 production in keratinocytes.

In Chapter 4, an in vivo model of cutaneous inflammation was used to further investigate the role of α1-AR in skin inflammation and to confirm the observations seen in vitro. UVB irradiation was applied to the skin of healthy people to induce inflammation, as evidenced by histological changes in the skin, including a thickened epidermal layer, the presence of necrotic cells in the epidermis, dysplasia of the epidermis, and an increase in cellular infiltrates in the dermis although, unexpectedly, there were no changes in inflammatory cytokine production. However, a perfusion of
phenylephrine through microdialysis fibres induced greater IL-6 production in UVB-exposed skin compared to saline perfusion in UVB-exposed skin, again suggesting a synergy between keratinocyte activation and α1-AR signalling in promoting pro-inflammatory IL-6 production.

In the final experiments (Chapter 5), the interaction between inflammatory mediators and α1-AR was investigated in primary keratinocytes obtained from patients with complex regional pain syndrome (CRPS), as an ex vivo pathological model. One of the clinical presentations seen in CRPS is persistent cutaneous inflammation. Similar to the results seen in HaCaT cells, administration of TNFα to primary keratinocytes, obtained both from healthy control volunteers and CRPS patients, resulted in an increase in inflammatory cytokines IL-1β, IL-6, IL-8, whereas NGF levels were decreased. Following TNFα treatment, phenylephrine administration to these cells further increased IL-6 and further decreased NGF levels. Due to a small number of participants, no differences were observed in inflammatory mediator levels produced by control and CRPS keratinocytes as a response to both TNFα and phenylephrine treatments. Interestingly, α1-AR baseline protein levels in CRPS keratinocytes were positively correlated with IL-6 production from these cells in response to α1-AR stimulation. This suggests that CRPS keratinocytes with high baseline α1-AR protein expression responded strongly to α1-AR stimulation and resulted in a higher production of IL-6.

In summary, throughout all the chapters, a positive feedback interaction between α1-AR and IL-6 was demonstrated in vitro, in vivo and ex vivo, suggesting an important role of this interaction in cutaneous inflammation.
6.2. Mechanisms behind cross-talk between $\alpha_1$-AR and inflammatory mediators

The mechanisms behind cross-talk between $\alpha_1$-AR and inflammatory mediators in keratinocytes are not totally understood. However, utilizing mouse cardiomyocytes and $\alpha_1\alpha$-AR-over-expressed rat fibroblasts as models, Perez and colleagues investigated the mechanisms behind cross-talk between $\alpha_1$-AR and IL-6 in these cells. They showed that $\alpha_1$-AR stimulation stabilized IL-6 mRNA and maintained IL-6 transcription factor, involving the p38 MAPK and NF-$\kappa$B inflammatory signalling pathways, which resulted in a higher production of IL-6 (Perez et al., 2009). In this study, a positive feedback interaction between $\alpha_1$-AR and IL-6 was demonstrated in keratinocytes. It is postulated that TNF$\alpha$ induced IL-6 production from keratinocytes through activation of canonical inflammatory signalling pathways, such as NF-$\kappa$B, JAK-STAT, and MAPK (Tanabe et al., 2010). Subsequently, stimulation of $\alpha_1$-AR following TNF$\alpha$ treatment stabilized the production of IL-6 mRNA previously induced through TNF signalling pathways and maintained transcription factors for IL-6 activated through TNF-induced NF-$\kappa$B and MAPK pathways (Perez et al., 2009).

In murine splenocytes (leukocytes harvested from spleen), stimulation of $\alpha_1$-AR resulted in an increased production of IL-2 from these cells, accompanied by elevated phosphorylation of ERKs (extracellular signal-regulated kinases) and CREB (cAMP response element-binding protein), a protein kinase involved in the MAPK/ERK pathway and a cellular transcription factor, respectively (Priyanka & ThyagaRajan, 2013). A recent study by Tripathi and colleagues suggested that cross-talk between $\alpha_1$-AR and inflammatory mediators was mediated through a direct interaction between $\alpha_1$-AR and inflammatory mediator receptors (Tripathi et al., 2015). They found a heteromerization of $\alpha_1$-AR and chemokine receptor motif 4 (CXCR4) in
vascular smooth muscle cells. Thus, the binding of chemokine ligands to CXCR4 also modulates $\alpha_1$-AR physiological function in these cells, and *vice versa*.

The mechanisms behind cross-talk between $\alpha_1$-AR and inflammatory mediators in keratinocytes requires further investigation. Measurement of proteins involved in (1) downstream $\alpha_1$-AR signalling pathway activation, such as phospholipase-C (PLC) (Xiao et al., 2010), (2) transcription and translation regulation pathways, such as in MAPK/ERK and JAK/STAT pathways, and (3) inflammatory signalling pathways, such as in the NF-κB pathway, will give insights into the mechanisms involved in cross-talk between $\alpha_1$-AR and inflammatory mediator production in keratinocytes.

### 6.3. Possible implications behind $\alpha_1$-AR and inflammatory mediator interactions

Modulation of inflammatory mediator production from cells or tissues via $\alpha_1$-AR activation has been demonstrated in several studies (Grisanti et al., 2011b; Huan et al., 2017; Rossi et al., 2018). While this may be a normal physiological process, dysregulation of this interaction may compromise normal inflammatory processes and normal physiological functions of the cells and tissues.

Activation of immune cells, such as peripheral blood mononuclear cells (PBMCs) and monocytes, increases $\alpha_1$-AR expression to detectable levels compared to the expression in resting cells (Heijnen et al., 1996; Rouppe van der Voort et al., 1999, 2000). Stimulation of $\alpha_1$-AR in these activated cells further induces inflammatory cytokine production (Grisanti et al., 2011b). Subsequently, these inflammatory cytokines enhance $\alpha_1$-AR expression in these cells (Heijnen et al., 2002), thus establishing a positive feedback loop between $\alpha_1$-AR and inflammatory cytokines. Maintaining homeostasis of this vicious cycle is important because, if unchecked, this interaction may enhance inflammatory responses.
and result in the development of a chronic inflammatory condition such as juvenile rheumatoid arthritis (Heijnen et al., 1996; Roupe van der Voort et al., 2000).

In a rat model of endotoxemia (acute sepsis), an increase in cytokines released as a response to an intravenous injection of bacterial lipopolysaccharide (LPS), was associated with a decrease in $\alpha_1$-AR expression in peripheral tissues, such as heart, lung, kidney, and liver (Bucher et al., 2003). This $\alpha_1$-AR downregulation explains the unresponsiveness of these patients to noradrenaline treatment to treat sepsis-induced hypotension, which leads to the development of heart failure. However, targeting an $\alpha_1$-AR and inflammatory cytokines interaction via inhibition of the NF-$\kappa$B inflammatory signalling pathway attenuated cytokine production and prevented $\alpha_1$-AR downregulation in this model (Schmidt et al., 2009). In contrast, in the current study TNF-$\alpha$ (or an inflammatory state induced by TNF-$\alpha$) caused an upregulation of $\alpha_1$-AR in keratinocytes. This discrepancy may be explained by the complexity of innate inflammatory signalling pathways activated by LPS through its receptors, toll-like receptors (TLRs) on multiple cell types (Biron, 2016). Activation of TLRs by LPS in sepsis results in over production of a range of inflammatory cytokines to create a cytokine storm that may over-stimulate $\alpha_1$-AR and subsequently lead to a compensatory downregulation of $\alpha_1$-AR.

6.4. The interaction between the sympathetic nervous system neurotransmitters, adrenoceptors and inflammatory mediators: implications in chronic pain conditions and chronic skin inflammatory diseases

Inflammation is an important physiological process in our body. However, in some pathological conditions, such as chronic pain or chronic inflammatory skin diseases, excessive inflammatory responses, influenced by sympathetic dysfunction, may contribute to the progression of these conditions into a chronic stage.
Neuropathic pain is a type of chronic pain that arises from an injury or disease affecting the somatosensory system. Growing evidence from our laboratory and others has shown that $\alpha_1$-AR expression is increased in the skin of the symptomatic limb of CRPS patients (Finch et al., 2014; Drummond et al., 2018) and in skin of neuropathic pain animal models (Drummond et al., 2014a; Drummond et al., 2014c). In addition, elevated pro-inflammatory cytokine levels are also observed in the skin or blister fluid obtained from the affected limb of patients suffering from CRPS (Huygen et al., 2002; Groeneweg et al., 2006; Heijmans-Antonissen et al., 2006; Kramer et al., 2011; Birklein et al., 2014). The results of the current project suggest that inflammatory mediators released from keratinocytes as a response to an injury induce $\alpha_1$-AR expression (Chapter 2; Wijaya et al., 2020b)). Subsequently, catecholamines released through activation of the sympathetic nervous system may further stimulate this heightened $\alpha_1$-AR expression and result in an increased production of inflammatory mediators (Chapter 3; Wijaya et al., 2020a). This vicious cycle between $\alpha_1$-AR and inflammatory mediators appears to be dysfunctional in neuropathic pain and may promote a persistent inflammatory state in the affected limb. In addition, inflammatory mediators can induce pain by acting on their receptors on the primary afferent sensory neurons that innervate the epidermis (Chao, 2003; Ji et al., 2014; Cook et al., 2018). Thus, excessive production of inflammatory mediators, as a result of dysregulation of $\alpha_1$-AR, may contribute to the development of persistent pain. While persistent inflammation is present in the acute stage of CRPS and attenuates as the condition progresses to the chronic stage, this study has shown that keratinocytes isolated from patients with chronic CRPS were sensitive to $\alpha_1$-AR stimulation, and this triggered an increase in production of IL-6 in proportion to basal $\alpha_1$-AR protein levels. Hence, catecholamines released during stressful events may re-activate the vicious cycle, particularly in a subset of CRPS patients with evidence of sympathetically maintained pain.
In chronic inflammatory skin diseases, such as psoriasis and atopic dermatitis, levels of noradrenaline are increased in the affected skin (Hall et al., 2012). The aetiology and pathophysiology of these skin diseases are not totally understood; however, psychological stress is known to aggravate these conditions. Basal catecholamine (Buske-Kirschbaum et al., 2002a) and immunoglobulin E (IgE) levels (Buske-Kirschbaum et al., 2002b) were elevated in atopic dermatitis patients compared to that in healthy controls and interestingly, both catecholamines and IgE levels were significantly elevated after administration of a standardized psychosocial stressor. In addition, an increased number of mast cells is observed in affected skin compared to unaffected skin (Lonne-Rahm et al., 2008). Moroni and colleagues showed that $\alpha_1$-AR mediated noradrenaline-induced histamine release from murine neoplastic skin mast cells (Moroni et al., 1977) and this could further induce inflammatory responses from other resident skin cells (Navi et al., 2007). Together, this may imply that in atopic dermatitis, enhanced catecholamine release during stressful events may enhance cutaneous inflammatory responses, mediated by $\alpha_1$-AR.

6.5. The role of keratinocytes in mediating cross-talk between the sympathetic nervous system and immune system

Keratinocytes, the most abundant cells in the epidermis, provide a barrier against environmental hazards and produce inflammatory mediators. An injury triggers pro-inflammatory cytokine production, such as TNF$\alpha$, IL-1$\beta$, and IL-6, from keratinocytes and these cytokines act as signals to recruit other immune cells from the circulation. Moreover, activated Langerhans cells (a type of dendritic cell, which resides in the basal layer of the epidermis) and resident macrophages also release inflammatory cytokines to enhance the attractant signals to recruit peripheral immune cells to the injury site. Both Langerhans cells and macrophages are considered messengers
between the innate and adaptive immune system. Inflammatory cytokines also induce the expression of α₁-AR in keratinocytes (Chapter 2; (Wijaya et al., 2020b)) and immune cells (Heijnen et al., 2002), which increases the responsiveness of these cells to adrenergic stimulation.

Sympathetic nerve endings innervate the epidermal layer of the skin and are in close contact with keratinocytes (Albers et al., 1994). Following injury, catecholamines released from sympathetic nerve endings stimulate adrenoceptors expressed in skin cells and resident immune cells to further release inflammatory cytokines and initiate wound healing processes (Heijnen et al., 1996; Sang et al., 2007; Grisanti et al., 2011b; Wallert et al., 2011; Lei et al., 2013; Drummond et al., 2014b; Drummond et al., 2014a; Drummond et al., 2014c; Drummond et al., 2015; Wijaya et al., 2020a). Physiological levels of noradrenaline play an important role in wound healing. In normal rats, noradrenaline released after injury enhances the recruitment of neutrophils and macrophages to the injury site, reduces wound angiogenesis and induces keratinocyte migration to augment wound re-epithelization (Gosain et al., 2006). However, excessive stimulation of adrenoceptors may lead to impaired wound healing (Lei et al., 2013; Sivamani et al., 2014). Together, this evidence highlights the importance of keratinocytes as a mediator for cross-talk between the SNS and immune cells in the skin.

### 6.6. Limitations of this study

This study has demonstrated a positive feedback interaction between α₁-AR and the inflammatory cytokine IL-6 *in vitro, ex vivo and in vivo*. However, it is acknowledged that there were some experimental limitations. First, an immortalized keratinocyte cell line (HaCaT) was used as an *in vitro* model to simplify the investigation into the role
of $\alpha_1$-AR in cutaneous inflammation. However, since skin is a complex organ, the interaction between $\alpha_1$-AR and inflammatory mediators \textit{in vivo} might be slightly different and involve interaction with other resident cells, such as fibroblasts, macrophages, and sensory neurons. This includes the interaction with $\alpha_1$-AR expressed on those cells and inflammatory mediators produced by these cells. Thus, further studies investigating this interaction in other resident skin cells and utilizing a co-culture system to investigate the interaction between the cell types will be beneficial to provide better insights into the interaction between $\alpha_1$-AR and inflammatory mediators in the skin.

Second, while human primary keratinocytes obtained from skin biopsies most likely model \textit{in vivo} processes more closely than HaCaT cells, their short lifespan in culture was one of the limitations in this study. Indeed, primary keratinocytes were less proliferative and less responsive to exogenous TNF$\alpha$ than HaCaT cells. This might have resulted in a reduction of inflammatory cytokines and NGF production from primary keratinocytes, compared to HaCaT cells.

Third, in the microdialysis experiment it was found that cutaneous inflammation induced by UVB exposure to the skin varied among individuals, possibly due to differing skin types. In addition, we found that the skin response to $\alpha_1$-AR stimulation post-UVB irradiation resulted in inconsistent IL-6 production. Therefore, further studies involving additional participants and a stronger model of cutaneous inflammation (Holzmann et al., 2004) could provide a clearer insight into the role of $\alpha_1$-AR in cutaneous inflammation and its interaction with inflammatory mediators, \textit{in vivo}.

Lastly, the recruitment of CRPS patients was difficult due to the low incidence of CRPS (Sandroni et al., 2003; de Mos et al., 2007) and because of time constraints in this study, only a small number of participants (both CRPS patients and healthy
volunteers) could be recruited for the primary keratinocyte experiments. Moreover, we found that there were great differences in individual baseline $\alpha_1$-AR expression levels, which may result in high variability of primary keratinocyte responses to $\alpha_1$-AR stimulation. In addition, the complex diversity of CRPS phenotypes, which may involve different pathophysiological mechanisms, may also contribute to these individual differences. Together, this may explain why there were no significant differences in inflammatory cytokine and NGF production between CRPS and healthy control keratinocytes post-$\alpha_1$-AR stimulation. Therefore, including more participants would enrich our current results. In addition, a higher number of CRPS patients would allow us to categorize them into different CRPS subtypes (type I, type II, acute stage, chronic stage, warm and cold CRPS, etc) and investigate whether the interaction between $\alpha_1$-AR and inflammatory mediators differed between the subtypes.

### 6.7. Future directions

At the beginning of the study, an *in vitro* scratch assay was utilized to apply a mechanical injury to keratinocytes and subsequently induce inflammatory mediator production from these cells. Following thorough assessment, the scratch assay was found not to be a suitable technique to induce an inflammatory state in these cells (see Appendix A). Thus, administration of TNF$\alpha$ to keratinocytes was used as an alternative approach to induce an inflammatory state. As demonstrated in this study, TNF$\alpha$ increased inflammatory mediator production and $\alpha_1$-AR expression in keratinocytes. In turn, stimulation of this heightened $\alpha_1$-AR expression further induced IL-6 production from keratinocytes. Although the scratch was not successful in inducing inflammation, it is important to explore other approaches (e.g., mechanical trauma; exposure to ultra-violet radiation; or low pH), as this might more closely mimic inflammation *in vivo*. 
Future studies can be proposed to provide more insights into the role of $\alpha_1$-AR in cutaneous inflammation and its positive feedback interaction with inflammatory mediators in the skin. An important next step would be to clarify the involvement of several signalling pathways, such as MAPK/ERK, NF-κB, JAK-STAT, underlying this positive feedback interaction in keratinocytes.

Since this study showed that IL-6 production was heightened by synergism between $\alpha_1$-AR and inflammatory mediators in the skin, future studies investigating the consequences of this heightened IL-6 expression in the skin will be needed. Specifically, over-production of IL-6 has been associated with autoimmune and chronic inflammatory disease, such as rheumatoid arthritis and systemic lupus erythematosus (Hirano, 2010). This might also be important in CRPS, as an autoimmune component may contribute to CRPS (Goebel & Blaes, 2013). Moreover, further studies involving co-culturing techniques using keratinocytes and other types of skin cells, such as fibroblasts, sensory neurons and macrophages, could be utilized to further investigate the role of $\alpha_1$-AR in cutaneous inflammation and how this cell-to-cell interaction affects synergism between $\alpha_1$-AR and inflammatory mediators.

6.8. Conclusions

The results from these studies confirm that inflammatory mediators such as TNF$\alpha$ can induce $\alpha_1$-AR expression on keratinocytes in the skin that, together with catecholamines released by sympathetic nerves following skin injury, could act synergistically to activate keratinocytes via elevated $\alpha_1$-AR expression levels. As a result, heightened signalling through $\alpha_1$-AR may synergistically induce over-production of IL-6 from resident keratinocytes, thus acting to promote an inflammatory cascade. Although this interaction may be a normal cutaneous inflammatory process,
restoring the homeostasis levels of $\alpha_1$-AR following injury could be crucial to prevent the development of persistent inflammation. Furthermore, targeting this interaction may be a useful therapeutic target to hinder the development of persistent inflammation and pain, such as in CRPS, and, ultimately, to prevent the progression of this condition into a chronic stage.
Appendix
Appendix A. Assessment of *in vitro* scratch assay to induce inflammation in keratinocytes

A.1. Introduction

Epidermal keratinocytes and other resident skin cells produce inflammatory mediators, such as tumour necrosis factor alpha (TNF$\alpha$), interleukin (IL)-1$\beta$, IL-8 and IL-6, to promote acute inflammatory responses and initiate the healing process after injury (Zhang & An, 2007). Moreover, keratinocytes express $\alpha_1$-AR (Dawson et al., 2011; Li et al., 2013; Finch et al., 2014) and the expression is heightened in the skin isolated from the affected limb of patients with chronic pain conditions, such as complex regional pain syndrome (CRPS; (Birklein et al., 2014; Drummond et al., 2014b; Drummond et al., 2014a; Drummond et al., 2014c; Finch et al., 2014; Drummond et al., 2018). However, the role of $\alpha_1$-AR in cutaneous inflammation and a potential interaction between $\alpha_1$-AR and inflammatory processes in the skin are not well understood.

The aim of this study was to investigate whether inflammatory mediators released from keratinocytes can trigger $\alpha_1$-AR expression in these cells. This is the first step to elucidate if the interaction between inflammatory mediators and $\alpha_1$-AR is present in keratinocytes. The *in vitro* scratch assay (Liang et al., 2007) was utilized to apply a mechanical injury to an immortalized human keratinocyte cell line (HaCaT cells; (Boukamp et al., 1988)). It was hypothesized that the scratch assay would induce inflammatory cytokine production from HaCaT cells and that this scratch-induced inflammatory state would then heighten $\alpha_1$-AR expression in HaCaT cells.
Appendix A. Assessment of in vitro scratch assay to induce inflammation

A.2. Material and Methods

A.2.1. Cell maintenance

The materials used for maintenance of the cells were purchased from Gibco™ (Thermo Fisher Scientific, MA, USA), unless otherwise stated. The immortalized human keratinocyte cell line (HaCaT cells) was obtained from the German Cancer Research Centre (DKFZ, Heidelberg, Germany; Cat No. 300493) (Boukamp et al., 1988). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Serana, Bunbury, Australia) and maintained in a controlled incubator at 37°C with 5% CO₂.

A.2.2. In vitro scratch assay

The in vitro scratch assay was performed according to Liang et al. (Liang et al., 2007) with some slight modifications. Briefly, three straight lines were drawn underneath each well of a 6-well cell culture plate using an ultrafine tip marker and used as a guide for placing the scratches. The first line was drawn in the middle of the well and the other two lines were 5 mm away to the left and to the right from the middle line. For the experiment, HaCaT cells were plated in marked cell culture plates at 150,000 cells/well for 2 days prior to applying the scratches. Three scratches per well were applied on the guide lines using 200 μl pipette tips. A glass slide was used as a straight edge while making the scratches. Three-vigorous PBS washes were applied to remove the remaining cell debris around the scratch/wound area. To determine the optimal time to induce inflammatory cytokine production from HaCaT cells, the cells were further incubated in reduced serum media (0.2% FBS) for 6, 12, 24, 33 or 48 h. At the end of each time point, an image of HaCaT cells was taken at the middle scratch under a phase-contrast light microscope (Olympus CK40 inverted microscope).
Appendix A. Assessment of in vitro scratch assay to induce inflammation

at 4x magnification. The cells were then washed once with PBS and lysed with lysis buffer for total RNA isolation. Each set of experiments was repeated three times.

A.2.3. Quantitative real-time PCR

Total RNA was isolated and purified from the cell lysates using an Aurum™ total RNA mini kit (Bio-Rad Laboratories, CA, USA). One µg total RNA was converted to cDNA using an iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, CA, USA), with the following cycle: 5 min priming step at 25°C, followed by a reverse transcription step at 42°C for 30 min and reverse transcriptase inactivation at 95°C for 5 min. A SYBR® Green-based quantitative real-time PCR (qRT-PCR) assay, followed by melting curve analysis, was used to measure gene expression levels of α1-AR subtype B (α1B-AR) and inflammatory cytokines IL-1β, IL-6 and IL-8. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. The primer sets used in qRT-PCR are listed in Table A-1. All of the primers were validated to amplify mRNA-derived transcripts but not genomic DNA (unpublished data). The qRT-PCR assay was performed using a ViiA™ 7 Real-Time PCR System (Applied Biosystems™; Thermo Fisher Scientific, MA, USA). A cycle threshold (Ct) value of each sample was collected at the end of the qRT-PCR run (40 cycles). Changes in gene expression as a result of the treatments compared to the naïve (non-scratch) group at each time point were represented in ∆∆Ct, where ∆∆Ct= (∆Ct naïve−∆Ct scratch), ∆Ct naïve=(Ct naïve−Ct GAPDH), and ∆Ct scratch=(Ct scratch−Ct GAPDH) (Yuan et al., 2006; Schmittgen & Livak, 2008). The mean ± SEM of the gene expression changes between biological replicates is presented in the Results section.
Appendix A. Assessment of in vitro scratch assay to induce inflammation

Table A-1. Primer sets used in a SYBR® Green qRT-PCR assay

<table>
<thead>
<tr>
<th>Gene ID (Names)</th>
<th>GenBank Accession</th>
<th>Forward Sequence ‘5 – 3’</th>
<th>Reverse Sequence ‘5 – 3’</th>
<th>Product Size (bp)</th>
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</thead>
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<tr>
<td>CXCL8 (IL-8)</td>
<td>NM_000584</td>
<td>ATG ACT TCC AAG CTG GCC GT</td>
<td>TCC TTG GCA AAA CTG CAC CT</td>
<td>82</td>
</tr>
<tr>
<td>IL6 (IL-6)</td>
<td>NM_000600</td>
<td>AGC CCT GAG AAA GGA GAC ATG</td>
<td>ACC AGG CAA GTC TCC TCA TTG</td>
<td>144</td>
</tr>
<tr>
<td>IL1B (IL-1β)</td>
<td>NM_000576</td>
<td>CTG TCC TGC GTG TTG AAA GA</td>
<td>TTG GGT AAT TTT TGG GAT CTA CA</td>
<td>70</td>
</tr>
<tr>
<td>NGF (NGF)</td>
<td>NM_002506</td>
<td>TGT GAT CAG AGT GTA GAA CAA CA</td>
<td>CGG ACC CAA TAA CAG TTT TAC C</td>
<td>97</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>GGG TGT GAA CCA TGA GAA</td>
<td>GAC TGT GGT CAT GAG TCC T</td>
<td>136</td>
</tr>
<tr>
<td>ADRA1B (α1B-AR)</td>
<td>NM_000679</td>
<td>PrimePCR® SYBR® Green Assay (assay ID: qHsaCIP0030605).</td>
<td></td>
<td>112</td>
</tr>
</tbody>
</table>

A.2.4. Statistical analysis

At each time point, the ΔCt mean values of naïve \[\Delta C_{t\text{naive}}= (C_{t\text{naive}} - C_{t\text{GAPDH}})\] and scratch \[\Delta C_{t\text{scratch}}= (C_{t\text{scratch}} - C_{t\text{GAPDH}})\] cells were analysed using a paired sample t-test to determine the mean difference between treatments. All statistical analysis was performed using SPSS® statistical analysis software version 24 (IBM, New York, USA).
Appendix A. Assessment of in vitro scratch assay to induce inflammation

A.3. Results

Following the scratch assay, images of HaCaT cells were acquired under a phase-contrast microscopy at the end of each time point to observe the wound closure. As seen in Figure A-1, HaCaT cell migration to the wound area was observed over time and the wound was nearly closed at 48 h post-scratch. However, the scratch application did not alter inflammatory cytokine (Figure A-2 A-C) or α1B-AR (Figure A-2 D) gene expression levels in HaCaT cells at any time point, except a reduction in IL-6 gene expression levels in the scratch condition compared to naïve cells at 24 h post-scratch. Moreover, both inflammatory cytokine and α1B-AR gene expression levels varied highly among the biological replicates.

![Image: Figure A-1. HaCaT cells migration following in vitro scratch assay](image)

The scratch assay was applied to HaCaT cells using 200 μl pipette tips. The representative images were acquired at 0 (non-scratch/naïve), 6, 12, 24, 33 and 48 h (hours) post-scratch with a phase-contrast light microscope at 4x magnification.
Appendix A. Assessment of in vitro scratch assay to induce inflammation

Figure A-2. Inflammatory mediator and $\alpha_{1B}$-AR gene expression levels post-scratch in HaCaT cells

The scratch assay was applied to HaCaT cells using 200 $\mu$l pipette tips. Following the scratch assay, the cells were further incubated in reduced serum media for 6, 12, 24, 33, and 48 h (hours). Naïve cells (non-scratch cells) treated with reduced-serum media only were used to estimate baseline expression level at each time point. Gene expression levels of inflammatory cytokines: (A) IL-1$\beta$, (B) IL-8, (C) IL-6, and (D) $\alpha_{1B}$-AR were measured at each time point. Changes in gene expression as a result of the scratch assay compared to the naïve condition are represented as $\Delta\Delta$Ct ($\Delta\Delta$Ct$_{naive}=0$). Data are represented as mean ± SEM of three independent replicates; *p<0.05 compared to naïve at the same time point.
A.4. Discussion

The effects of the scratch assay on inflammatory mediator production in HaCaT cells were investigated in vitro. The scratch assay was utilized to mimic a mechanical injury to cultured HaCaT cells. Keratinocyte migration and wound closure were observed over time and this wound closure was almost completed at 48 h post-scratch. An inflammatory state was not induced by the scratches, marked by no changes in inflammatory cytokine gene expression levels in HaCaT cells. Furthermore, the scratch assay did not alter $\alpha_{1B}$-AR gene expression levels.

The in vitro scratch assay has been widely utilized in wound healing and cell migration studies (Walter et al., 2010; Sivamani et al., 2014). However, utilization of this technique to induce inflammation in vitro has not been reported. The results from this study demonstrated that applying the scratch assay to cultured keratinocytes did not induce inflammatory cytokine production in these cells, although it induced keratinocyte proliferation and migration that supports the wound closure. This may be because the scratch only scraped the cells from the growth surface without inducing any inflammatory processes in the keratinocytes. In addition, in vivo, the inflammatory cytokines released from keratinocytes following an injury may be induced by pro-inflammatory mediators released by local inflammatory cells that are involved in acute inflammatory responses, such as neutrophils and macrophages (Yeh et al., 2017).

The reduction of IL-6 gene expression levels observed in the scratch condition at 24 h post-scratch was unexpected and the mechanism behind this scratch-reduced IL-6 levels needs to be explored further. However, as the decrease in IL6 was significant at only one time point, 24 h after the scratch, it may have been a chance effect.

In conclusion, while the in vitro scratch assay is useful in wound healing and cell migration studies, this technique was not suitable as a technique to induce an inflammatory state in cultured keratinocytes. Administration of pro-inflammatory
cytokines that are present at the inflammatory phase following an injury, such as TNF\(\alpha\) (Robson et al., 2001), may be a better approach to induce inflammatory conditions in cultured keratinocytes and to investigate the effects of inflammatory cytokines on \(\alpha_1\)-AR expression in keratinocytes.
Appendix B. Peer-review process and copyright statement from Cytokine (Elsevier).

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Appendix C. Peer-review process and copyright statement from Experimental Cell Research (Elsevier).

EXPERIMENTAL CELL RESEARCH
Emphasizing Molecular Approaches to Cell Biology

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DESCRIPTION

*Experimental Cell Research* welcomes articles that further our understanding of cell biology in its widest sense by providing significant insights into the organisation and activity of cells in both tissue culture assays as well as in animal models, not the least in models that recapitulate key aspects of human diseases. We welcome both mammalian and non-mammalian model organisms. In addition to regular articles we also publish review articles in regular as well as in special issues. Our scope includes but is not limited to areas such as:

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- The cytoskeleton
- Intracellular trafficking
- Cell-cell and cell-matrix interactions
- Cell motility and migration
- Cell proliferation
- Cellular differentiation
- Signal transduction
- Programmed cell death

AUDIENCE

Molecular and cell biologists, cancer researchers.

IMPACT FACTOR

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