NOCICEPTIN (N/OFQ) AND IMMUNE MODULATION

Thesis submitted for the degree of

Master of Philosophy

at the University of Leicester

By

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2019

Abstract

Opioids are reported to modulate the functioning of the immune system through central and peripheral mechanisms, including direct interactions with opioid receptors on peripheral immune cells. Opioid receptors can be classified as classical (naloxone-sensitive); MOP (mu), KOP (kappa), DOP (delta) and non-classical (naloxone-insensitive); NOP (nociceptin/orphanin FQ as an endogenous ligand) receptors.

In this thesis, it is shown that classical (MOP, KOP and DOP) opioid receptor mRNA (and hence the receptor protein) is not expressed in peripheral whole human blood, whereas a consistent expression of (non-classical) NOP receptor mRNA is observed which also showed decreased NOP mRNA expression in response to *in vitro* sepsis.

The effects of NOP stimulation on cell migration (polymorphonuclear [PMN] and eosinophil-like cells [EOL-1]) and the release of inflammatory mediators *in vitro* were also investigated revealing concentration dependent inhibition of cell migration associated with NOP stimulation. The effect of NOP stimulation on cytokine balance was difficult to detect because of the small number of cells examined and a significant dilution effect.

Certain acute inflammatory conditions and disease processes change the balance between circulating neutrophils and lymphocytes. A high neutrophil to lymphocyte ratio (NLR) indicates a relatively higher number of circulating neutrophils accompanied by an imbalance of inflammatory mediators. The link between NLR and inflammatory cytokine *in vivo* was also investigated in this thesis but was proven to be inconclusive in view of the small sample size; however, it may warrant further investigation in the future.

These results indicate that the N/OFQ-NOP receptors—but not the classical opioid system—are involved in modulating peripheral immune cell function that may be a future therapeutic target.

Acknowledgements

I would like to dedicate this work to my wonderful wife Yusr and our children (Dania and Zain) for their love, support, and patience which kept me motivated throughout my journey.

My full appreciation and gratitude also go to my supervisors Professor David Lambert and Professor Jonathan Thompson, without whom none of this work would have been possible. I also appreciate all the support provided by Dr John McDonald, Dr Mark Bird, and all laboratory staff who played an instrumental part in supporting me throughout the numerous experiments that were part of this project. My full gratitude also goes to all the volunteers that participated in the studies that made this work possible.

I also would like to thank the Ernest Leach Research Fund (Royal College of Anaesthetists Research, Education and Travel grants) and the National Institute of Academic Anaesthesia for their financial support of this project. (https://www.niaa-hsrc.org.uk/2013)

Publications and presentations resulting from the work related to this thesis

Al-Hashimi M, McDonald J, Thompson JP, Lambert, DG. Evidence of nociceptin/orphanin FQ (NOP) but not MOP, DOP, or KOP opioid receptor mRNA in whole human blood (scientific paper). 2016, BJA;116(3): 423-9 (10 cites) (last accessed April 2019) Appendix 6

Al-Hashimi M, Scott SW, Thompson JP, Lambert DG. Opioids and immune modulation: more questions than answers (invited review). 2013, BJA; 111(1): 80-8. doi: 10.1093/bja/aet153 (101 cites) (last accessed April 2019) Appendix 6

Al-Hashimi M, McDonald J, Thompson JP, Lambert DG. Classical opioid receptor mRNA is not present in whole human blood (published abstract). 2013, BJA; 110 (5): 860P-885P.

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List of abbreviations in thesis

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
APC	Antigen presenting cells
B2M	Beta 2 microglobulin
BBB	Blood brain barrier
BMI	Body mass index
CBP	Cardiopulmonary bypass
cDNA	Copy deoxyribonucleic acid
CHO	Chinese hamster ovary
CI	Confidence interval
CRH	Corticotropin releasing hormone
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
DOP	Delta opioid receptor
∆Ct	Difference in cycle threshold
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EOL-1	Eosinophil-like cells – 1
EtBr	Ethidium bromide
FBS	Foetal bovine serum
FCS	Foetal calf serum

fMLP	N-formyl methionine-leucyl-phenylalanine
g	gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
g_{av}	Gravitational force
gDNA	Genomic deoxyribonucleic acid
HIV	Human immunodeficiency virus
HPA	Hypothalamic pituitary adrenal axis
HRP	Horseradish peroxidase
icv	Intracerebroventricular
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
IQ range	Interquartile range
IV	Intravenous
KOP	Kappa opioid receptor
LH	Luteinising hormone
LPS	Lipopolysaccharide
MC	Monocyte
Min	Minutes
ml	millilitre
MOP	Mu opioid receptor
mOsm	milliosmoles

mRNA	messenger ribonucleic acid
N/OFQ	Nociceptin/Orphanin FQ
NF	Nuclear factor
NK	Natural killer cells
NLR	Neutrophils to lymphocytes ratio
NOP	N/OFQ receptor
NTC	Non-template control
ORL-1	Opioid-receptor-like-one (now known as NOP)
PBS	Phosphate buffer saline
PEP-G	Peptidoglycan
PMNs	Polymorphonuclear cells
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cells
Rpm	Revolutions per minute
RPMI	Roswell park memorial institute
RT	Reverse transcriptase
rt-PCR	Real-time polymerase chain reaction
s	Seconds
SBG	Sybr green
SD	Standard deviation
TAE	Tri acetate buffer
TGF	Transforming growth factor

Tm	Annealing temperature
TMB	Tetramethylbenzidine
TNF-α	Tumour necrosis factor – alpha
UV	Ultraviolet
w/v	Percentage of weight to volume

1. Introduction

1.1. History of immune-modulatory effects of opioids

The link between opioids and alterations in the host's immune function has been referred to in numerous papers and reviews [1]. The increased incidence of various local and systemic infections in intravenous drug users has led to the conclusion that simply the injection process as the route of infection could not explain the causative link between intravenous (IV) drug abuse and infections. Instead, opioids themselves are acting as immune-modulators (tetanus and local abscess formation versus tuberculosis, pneumonia, HIV, endocarditis).

The increased incidence and severity of infections among opiate abusers was documented in the early 19th century, and similar observations appeared in the literature as early as the mid-1500s [2]. In 1898, Cantacuzene demonstrated the depression of the phagocytic and chemotactic functions of rodent phagocytes after their exposure to morphine *in vitro* and *in vivo* [3]. In 1950, Hussey and Katz published a landmark paper that included a list of infections associated with narcotic addiction [1].

An extensive amount of published research offers investigations of the immunomodulatory effects of opioids, their mechanisms, and the clinical significance of such effects. Opioids can also affect innate and adaptive immune functions. Their effects are summarised in Table 1.1 [4-7].

Table 1.1: Effects of opioids on immunity [8]

Adaptive immunity

↓ Splenic and thymic weight (rodents)

↓ T cell viability and proliferative response and T-helper cell function

↓ CD4/CD8 population *in vivo*

↓ IL1β, IL-2, TNF-α and IFN-γ (mouse splenocytes)

↓ Th1/Th2 ratio of T-helper cell population (PBMCs)

↓ Natural Killer (NK) cell activity and ↓ primary antibody response (B cells)

↓ B cells mitogenic response to bacterial LPS, ↓ macrophage activity

↑ TGF-β1 and IL-10 (anti-inflammatory cytokines)

↑ T cell apoptosis (NF-k β and AP-1/NFAT pathways)

Inhibition of CD3/CD28 mAb-induced IL-2 transcripts

Innate immunity

↓ Number of macrophages available to fight infections and Leukocyte migration

↓ Peritoneal macrophages phagocytosis

↓ Respiratory burst activity and chemotaxis

Inhibition of Fc γ receptor-mediated phagocytosis

↓ Superoxide production from neutrophils and macrophages

↓ Neutrophil cytokines involved in wound healing

↑ Apoptosis of macrophages impairing host defence barrier

↓ Leukocytes endothelial adhesion (↓ intracellular adhesion molecules expression)

Alteration of IL-8 induced neutrophil chemotaxis

Neuroendocrine system

↑ Growth hormone, prolactin, and thyroid stimulating hormone secretion in humans

↓ Sex hormones (LH and testosterone [hypogonadism]), oxytocin, and oestradiol

May affect the function of the HPA axis (ACTH, CRH) with risk of adrenal insufficiency

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Abbreviations are defined in a separate section (page 10-13)

1.2. Immune regulation

Immune function is tightly regulated to enable organisms to fight infections. Irregularities or modulations of immune function can lead to the increased incidence of infections or autoimmune diseases in which the immune system attacks the host tissues. The term “immune regulation” is usually used to refer to the interactions between different mediators and immune cells [9]. Immune function can be affected by many mechanisms through alterations in immune cell haematopoiesis, which affects the development of components of the immune system, antigen-antibody interactions, and positive/negative feedback control loops. Many triggers can affect immune function through direct peripheral receptor mechanisms; however, neuroendocrine and centrally mediated mechanisms are particularly important. Peripheral immune cells express a wide variety of receptors (e.g., corticosteroids, insulin, growth hormones, and adrenergic receptors).

However, the conflicting evidence of opioid receptor expression is addressed further in this thesis [10] [11]. A simplified illustration of immune cells’ haematopoiesis and regulation is shown in Figure 1.1, which highlights the potential sites of immune modulation [8].

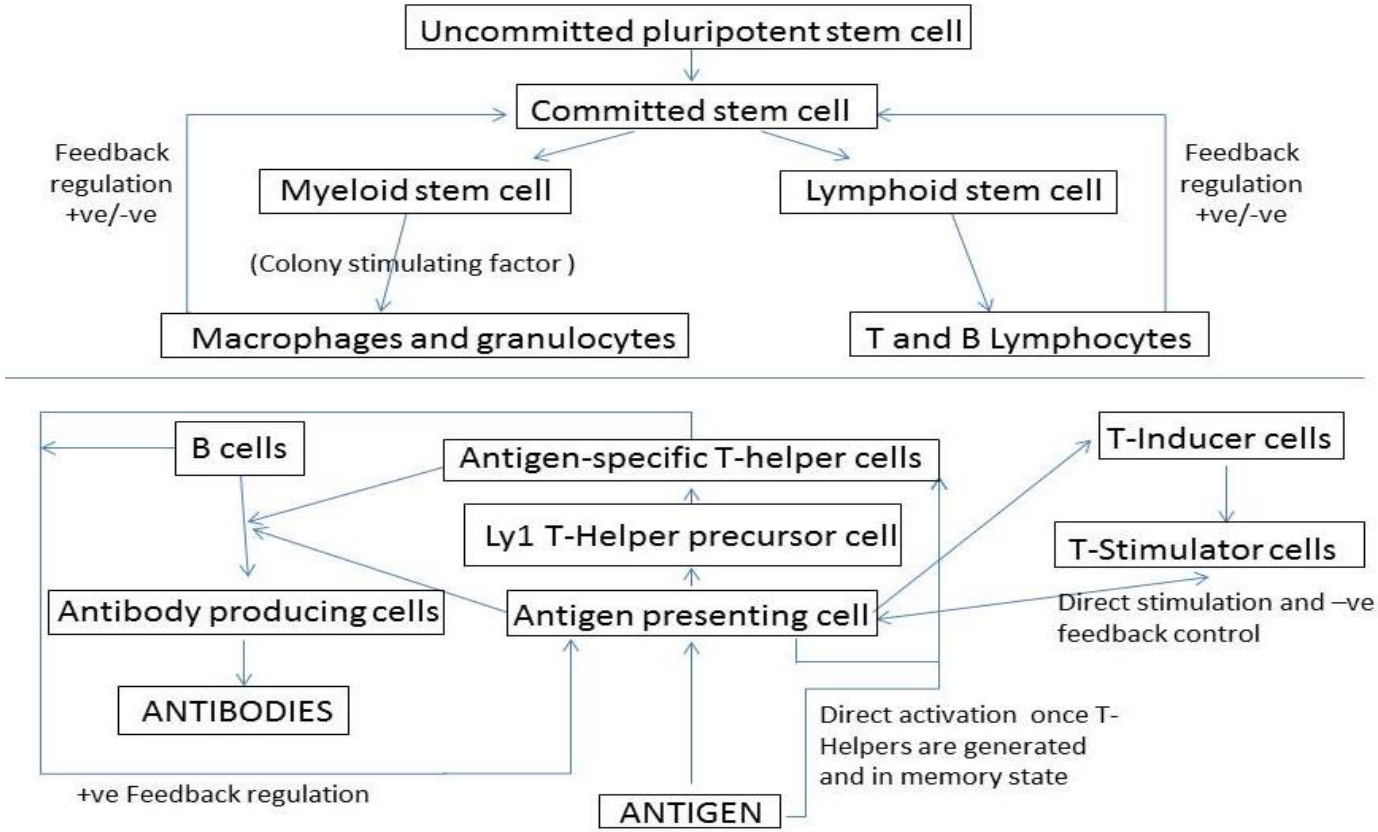

Figure 1.1: Immune regulation. The top half of the figure illustrates the process of immune cell generation from stem cells to individual cell populations, which then exert feedback control of stem cell maturation. The bottom half illustrates the antigen-antibody immune function. Antigens are bound by antigen presenting cells (APC-like macrophages), which then activate T-helper precursor cells and then form an antigen-specific T-helper cell, which is directly activated by the antigen and the APC [8].

1.3. Classification of opioid receptors

In 1973, Pert and Snyder were the first to demonstrate opioid receptors in neural tissue, which was followed by a demonstration of endogenous opioid ligands [12-14]. Endomorphins were not identified as endogenous ligands of the MOP receptor until 1997 [15]. Opioid receptors were formally identified by Kieffer *et al*. and Evans *et al*. in 1992 [16 17]. Research on the crystal structure of all opioid family members was published in 2012 [18-21]. The classification and nomenclature of opioid receptors have changed since their initial introduction. The most recent terminology, MOP (μ, mu), DOP (δ, delta), KOP (κ, kappa), and NOP, is listed in Table 1.2 [8].

The stimulation of classical opioid receptors causes analgesia to various degrees in addition to undesirable effects such as respiratory depression, nausea, vomiting, addiction, and dependence [22 23]. Naloxone-insensitive NOP receptors are widely expressed (their expression in immune cells is discussed in detail later). These receptors can cause analgesia spinally and hyperalgesia supraspinally, according to previous studies; however, new evidence suggests that supraspinal N/OFQ administration causes an anti-nociceptive response in primates when compared with the intrathecal instillation of substance P causing no increase in body scratching that is observed with classical opioid receptor agonist (morphine) administration [24 25].

The current literature sometimes describes numerous subtypes of individual opioid receptors based on evidence derived from variable modulations by pharmacological agents (MOP 1,2,3; DOP 1,2; and KOP 1a, 1b, 2a, 2b, 3) supporting the presence of putative opioid receptors [23]. Molecular and genetic knockout evidence supports the traditional thinking that contradicts the opioid receptor subtype theory because individual gene knockout results in a loss of function of all described types of that receptor. Throughout this thesis the current IUPHAR classifications of MOP, DOP, KOP, and NOP (table 1.2) are used [26].

Simultaneous applications of two pharmacological agents, such as the MOP agonist (morphine) and DOP antagonism (naltrindole), can stop dependence while achieving analgesia, which is also achievable utilizing a newer approach with bivalent molecules. Bivalent agonists/antagonists are two pharmacologically active molecules that target two receptors with specific space between them to facilitate optimum simultaneous receptor binding [27]. Opioid receptor classification is described in Table 1.2 along with the respective ligands and physiological effects. The role of NOP receptors in nociceptive transmission and their interplay with MOP receptors is illustrated in Figure 1.2 [22].

Table 1.2: Opioid receptor classification [8]

Current name	Other names	Main effects	Clinical ligand
(i) Classical members and naloxone sensitive			
MOP	μ, mu, MOR	Analgesia, respiratory depression, tolerance, Immunosuppression.	Almost all clinical opioids Morphine, Fentanyl [a]
DOP	δ, delta, DOR	Analgesia	NONE [b]
KOP	κ, kappa, KOR	Analgesia, diuresis	NONE [c]
(ii) Non-classical and naloxone insensitive			
NOP	ORL-1	Rodent - Analgesia-spinal Rodent - Hyperalgesia-icv Primate - Analgesia all routes	NONE [d]

icv – intracerebroventricular

Endogenous ligand [a], endomorphin, [b], enkephalin, [c] dynorphin and [d] Nociceptin /Orphanin FQ (N/OFQ). Buprenorphine also exhibits weak agonistic activity at NOP receptors.

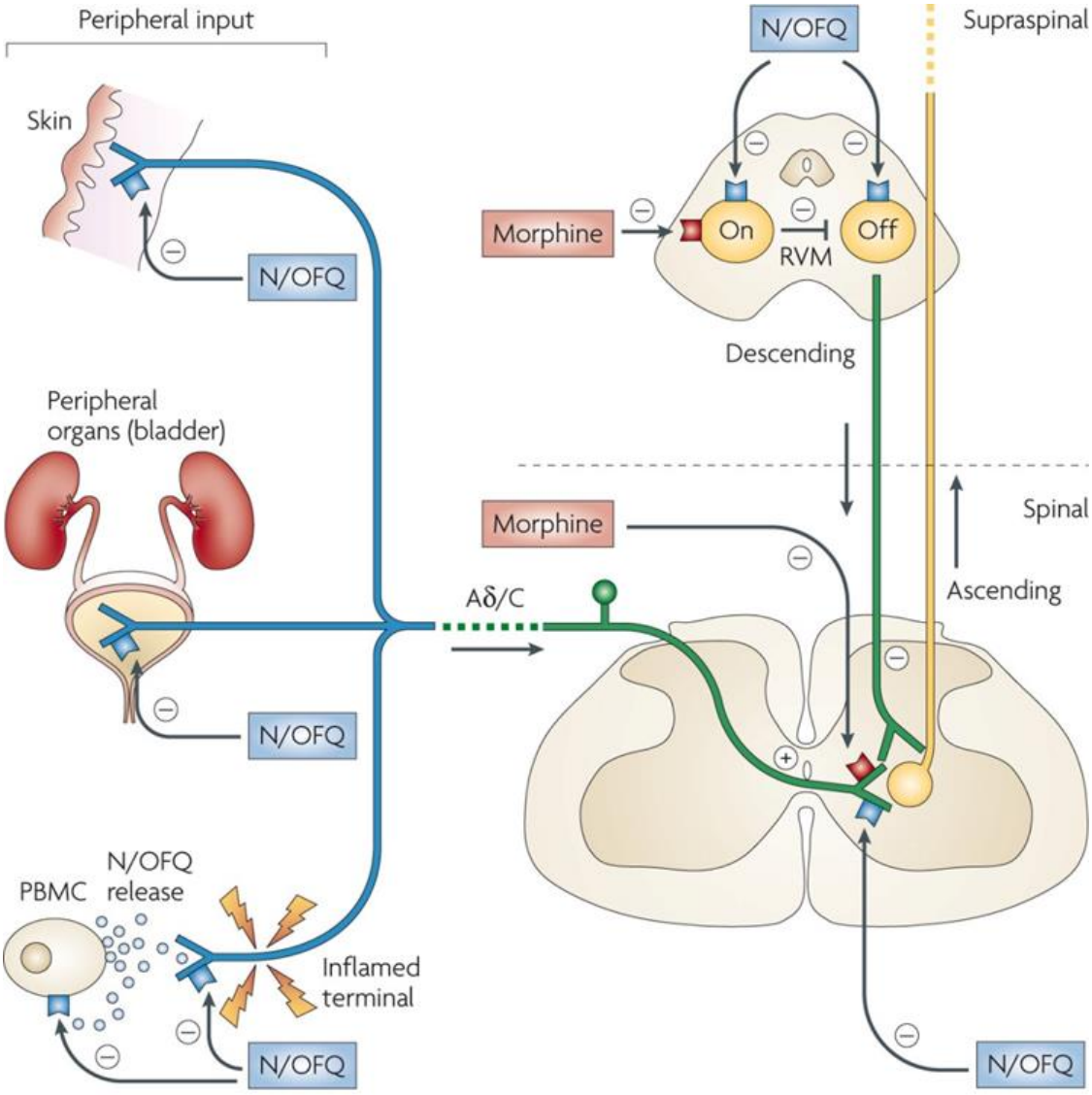

Figure 1.2: Role of NOP receptors in nociceptive transmission; central actions are relevant to rodents only after recent primate studies [22]

N/OFQ produces supraspinal antimorphine action by blocking the on and off cells in the rostral ventromedial medulla (RVM) which will stop the analgesic effect of morphine through MOP receptor stimulation that usually inhibits the "on" cells alone leaving the "off" cells to stop ascending nociceptive traffic. At a spinal level and peripherally, N/OFQ stops afferent nociceptive traffic causing analgesia [22].

1.4. Different opioids cause variable degree of immunomodulation

Several factors, such as the duration of exposure, pharmacokinetics, pharmacodynamics, and host factors can affect the immunomodulatory effects of different opioids [28]. Publications of the British Pain Society (Opioids for persistent pain: practice guide and Opioids for persistent pain: good practice January 2010) state, “patients must be aware of uncertainty regarding the long-term effects of opioids particularly in relation to endocrine and immune function.” The opioid practice guide also states that despite insufficient data it acknowledges the immunomodulatory effects of opioids and the fact that buprenorphine has no effect on immune function. Concerns about the use of opioids have led to the development of “Opioids Aware” a guide published by the Faculty of Pain Medicine and funded by Public Health England in 2015 to instruct healthcare professionals and better inform patients regarding opioids and their use in treating acute and chronic pain. Table 1.3 provides a summary of the immunomodulatory effects of opioids in animal studies, the results of which may not reflect the response in humans, where the duration of exposure to opioids changes the observed degree of immunomodulation [28-32]. Evidence published in the literature states that the immune-modulatory effects of different opioids depend on their molecular structure more than on the interaction with MOP receptors. The latter might suggest a non-opioid (receptor) site of action. However, no immune modulation has been observed in MOP knockout animals.

Clearly, interspecies variation is an important issue in studies of immune modulation by opioids.

Table 1.3: Degree of immunomodulation with different opioids in animal studies[8]

Strong immune modulation	Weak immune modulation
Codeine	Buprenorphine*
Methadone	Hydromorphone
Morphine	Oxycodone
Remifentanil	Tramadol
Fentanyl	Hydrocodone
	Oxycodone

Note: *Buprenorphine was shown to have the fewest (close to zero) immunomodulatory effects.

1.5. Potential sites of immunomodulation with opioids

The direct interactions of opioids on peripheral immune cells are widely suggested as a mechanism of immunomodulation; however, the presence of classical opioid receptors on these cells remains controversial, which is discussed later in this thesis [33]. Strong evidence links centrally mediated mechanisms of immunomodulation with opioids, which is supported by the fact that opioids that cross the blood brain barrier (BBB) cause more immunomodulation compared to those that do not. The degree of

immunomodulation has been shown to be even higher when opioids were delivered directly to the brain's ventricular system [34 35]. These centrally-mediated effects were linked to the central MOP receptors in studies of MOP in knockout mice, where centrally-administered opioids had no effect on immunity when no functioning MOP receptors were present [36-39]. Another mechanism could be through the stimulation of the sympathetic system, which would cause the suppression of immune cell function and proliferation with some effects on primary and secondary lymphoid tissue function [40 41].

The effects of opioids on the hypothalamic pituitary adrenal (HPA) system could explain both the central and peripheral immunomodulatory mechanisms. The HPA system can be affected by positive and negative feedback mechanisms that affect the production of glucocorticoids by the adrenal glands. The release of the hypothalamic corticotrophin hormone (CRH) stimulates the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland, which increases the secretion of cortisol by the adrenal gland [42]. The interaction of opioids with the HPA axis and its components (i.e. ACTH and cortisol production) is complex, species dependent, and time dependent (acute vs. chronic) [43]. In rodents, the acute administration of opioids increased ACTH and glucocorticoids, which may have immunosuppressive actions [44] However, chronic opioid administration has variable effects on ACTH and glucocorticoids (a decrease or no change). Because of handling and injection, it is difficult to separate stress-induced increases in cortisol from drug-dependent changes.

Although the data are limited, the evidence to date is that acute opioid administration results in either a reduction of or no change in ACTH or glucocorticoids. Chronic administration probably results in decreased HPA axis activity to the extent that adrenal insufficiency has sometimes been reported [8]. The HPA axis is affected by circadian rhythm; ACTH and cortisol levels are high in the morning and low in the evening. Some evidence suggests that opioids disrupt this rhythm, preventing low levels in the evening which leads to consistently elevated levels of ACTH and cortisol [45]. This effect might be enough to produce immunosuppression, although it does not seem plausible. A further point to consider is whether cortisol is a relevant marker of immunosuppression. Whether immunosuppressed (as compared to opioid treated patients/addicts) patients have elevated cortisol is unclear; however, the underlying status of the individual cannot be ignored. The presence of disease (e.g., cancer and organ failure) and nutritional status have the potential to affect the immune response via sites other than the HPA axis, including the release of immunosuppressive cytokines.

1.6. Opioid receptors on immune cells

The expression of opioid receptors on peripheral immune cells remains a widely controversial and debated subject. In the literature, it has been widely suggested that MOP receptors (mRNA and hence receptor proteins) are

expressed in peripheral immune cells [46-48]. However, the evidence is mainly historical, with many limitations existing in the methodology used to generate such evidence [49]. Furthermore, Williams *et al*. failed to identify classical opioid receptors on human peripheral blood PMN using competitive binding assays, florescent/immunofluorescent staining, real-time polymerase chain reaction (rt-PCR), and radio ligand binding. This casts doubt on the previous evidence, which our group believes should be interpreted with caution because no data (until this thesis) have been established using whole human blood RNA. The expression of non-classical opioid receptors (NOP) on immune cells is not as controversial, as the strong expression of mRNA using rt-PCR suggests the presence of receptor proteins in a wide variety of peripheral human blood immune cells, which may be linked to their immune function and role as a potential therapeutic target [8 11].

1.7. The Nociceptin/Orphanin FQ system (N/OFQ)

Nociceptin/Orphanin FQ is an endogenous 17-amino acid peptide. In 1995, it was identified as the endogenous ligand for the G-protein-coupled NOP receptor (formerly known as ORL-1) in the first successful case of reverse pharmacology (ORL-1 was identified before the peptide). In neurones, N/OFQ actions on the NOP receptor result in reduced neuronal excitability through reduced cyclic AMP formation, the closure of voltage gated calcium channels,

and the efflux of potassium ions through inwardly rectifying potassium channels [50-55].

Numerous studies have demonstrated the wide involvement of the N/OFQ-NOP system in many physiological functions including the immune system, cardiovascular system, and the central and peripheral nervous systems, which has been considered an obstacle to identifying a specific therapeutic target system [22]. However, the NOP-N/OFQ system might present a potential therapeutic target in certain conditions in which the simultaneous targeting of many systems involved in the disease process might be beneficial, including sepsis and modifying the host inflammatory process [22 56 57]. The wide physiological involvement of the N/OFQ-NOP system is illustrated in Figure 1.3, which was derived from a review article by Lambert [22].

Classical opioid receptors are upregulated in terminal nerve endings in response to local inflammation, which is also associated with an increase in opioid peptides that are released from circulating lymphocytes to cause a degree of peripheral analgesia [58 59]. It is therefore important to stress that, while there are no classical opioid receptors expressed on individual immune cells, they still play an important role in the neuroimmune axis modulation by releasing opioid peptides.

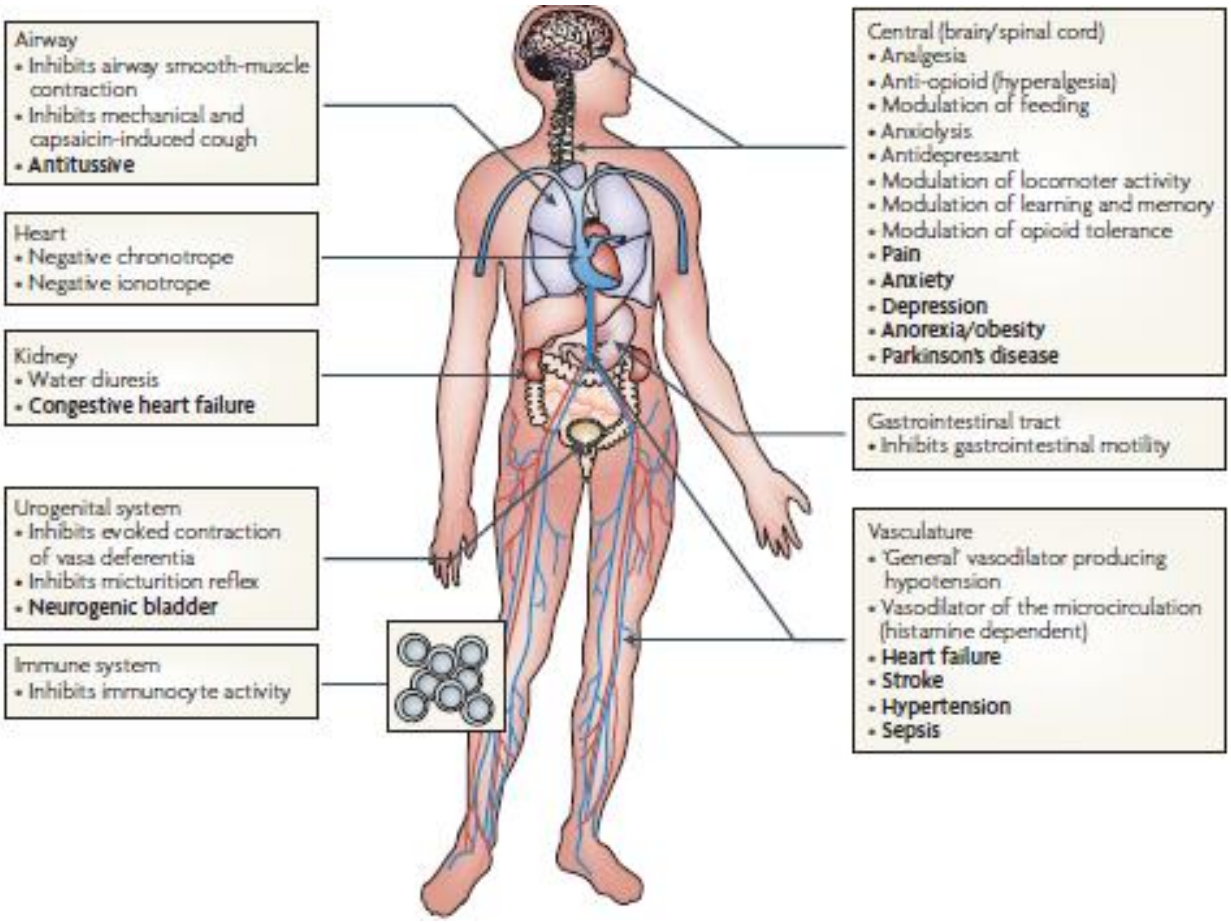

Figure 1.3: Pleiotropic effects of N/OFQ. Potential therapeutic targets are highlighted in bold [22].

Animal studies have revealed a similar degree of NOP upregulation in response to lipopolysaccharide and a degree of immunomodulation in response to staphylococcal endotoxin-A administration, which may explain the reported high N/OFQ levels in septic human non-survivors [60-62]. In a study on inflammatory bowel disease, a link was shown between the NOP-N/OFQ system and inflammation when NOP knockout mice did not develop colitis after the administration of dextran sulphate sodium [22].

N/OFQ plays an important role in pro-inflammatory states and in sepsis [63] [64]. Stamer *et al*. demonstrated the modulated expression of NOP mRNA in human whole blood, especially in advanced cancer, major surgery, and severe sepsis, showing increased NOP mRNA expression in all patient groups using qPCR. Higher NOP expression was associated with higher mortality in patients admitted to the ICU [64]. Other associations included a reduction in N/OFQ precursor pp-N/OFQ and increased pro-inflammatory marker pro-calcitonin proportionate to increased NOP expression. This study had many limitations including its observational nature, low study candidate numbers, the use of unmatched controls, and the fact that plasma N/OFQ peptide concentrations were not measured; however, this study provides evidence that N/OFQ mRNA expression is modulated in pro-inflammatory states and extends the work of Williams *et al*. [60], which suggests an association between N/OFQ modulation and mortality. However, it should be remembered that mRNA measurements are not a measure of receptor functionality. Thompson *et al*. [65] described in a larger study that the N/OFQ system is modulated in ICU patients with sepsis and, to a lesser degree, after CPB where a significant increase of N/OFQ and a decrease in NOP mRNA and the precursor of N/OFQ (ppNOC) were observed. These changes in the NOP-N/OFQ system were also associated with significant changes in inflammatory mediators (IL-8, IL-10, and TNF-α), emphasizing the importance of this system in the inflammatory process and its potential therapeutic applications *in vivo* [65].

Animal studies continue to contribute to our understanding of the N/OFQ system in sepsis and inflammation. Anton *et al.* studied the effect of N/OFQ on spleen cells from mice, determining that antibody formation in exogenous sheep blood was suppressed [66]. This finding suggests that the nociceptin system has an important modulating effect on the adaptive immune response.

Furthermore, a recent paper confirmed the findings of an earlier study that examined the response to dextran sulphate-induced bowel colitis in knockout mice. By administering the N/OFQ antagonist SB612111 to dextran-sulphate induced colitic mice, Alt and colleagues, demonstrated an improvement in the mice's overall condition and a reduction in inflammatory cytokines and their mRNA expression [67]. The positive findings in genetically modified mice combined with the findings of this interventional study indicate the urgent need to investigate N/OFQ in inflammatory bowel disease in humans.

Carvalho *et al.* studied the effect of N/OFQ modulation on outcomes in a clinically-relevant mice sepsis model (Cecal ligation and perforation (CLP))[68]. In this study, Carvalho showed significant decrease in mortality, cell migration, and pro-inflammatory mediators release after the administration of high dose NOP antagonist (UFP-101) compared to increased mortality after NOP agonist (N/OFQ) administration in mice. This study highlighted the functional importance of the NOP-N/OFQ system in modulating the immune system and the host inflammatory response in sepsis [68].

Many studies to date have demonstrated associations of severe pro-inflammatory states with the N/OFQ system, yet little is known about the mechanisms that underlie these observations. *In vitro* work has demonstrated mRNA transcripts in immunocytes [49 60 69 70] and the release of N/OFQ peptide from stimulated polymorphonuclear cells (PMNs). Furthermore, N/OFQ has also been demonstrated to function as an immunocyte chemotactic agent [71 72].

Recent evidence has shown the effect of N/OFQ on increasing the activity of the critical sepsis-associated transcription factor NF-κB in human neuroblastoma cells (SH-SY5Y) [73]. However, our understanding of how the N/OFQ system modulates immunocyte and cytokine behaviour is still weak, and it remains an important area for further research.

1.8. Neutrophil to Lymphocyte ratio (NLR)

Peripheral immune cells are derived from myeloid and lymphoblastic precursors that can differentiate into PMN and lymphocytes, respectively, as shown in Figure 1.4 that highlights the expression of NOP receptors on all peripheral immune cell populations [49]. The NLR is the ratio of circulating neutrophils as a marker of the acute inflammatory component to the chronic inflammatory regulatory pathway, which is represented as the number of circulating lymphocytes. Neutrophilia and lymphopenia may indicate physiological stress,

and they affect NLR. In addition, other factors may affect NLR, such as smoking, age, gender, and obesity.

NLR has proven to have prognostic significance in inflammatory conditions, diseases of the cardiovascular system, infections, and certain cancers [74]. The exact normal range of NLR is still unknown, and it is a subject of intensive research. In a recent study using healthy volunteers, the mean NLR was 1.65 [±1.96 SD: 0.78–3.53] (95% CI [0.75–0.81] and [3.40–3.66]) [74]. PMN are also responsible for the inflammatory mediator imbalance (pro/anti-inflammatory cytokines) that can be detected in certain disease processes at variable degrees.

NLR could potentially be used as a surrogate for high PMN and facilitate individualised care of certain therapies that are more beneficial for patients suffering from severe neutrophilia and lymphopenia.

Our research group conducted two studies to establish the possible link between nociceptin modulation and acute inflammatory states in two groups of patients: those admitted to the Intensive Care Unit (ICU) with a clinical diagnosis of sepsis; and patients who had undergone cardiac surgery under cardiopulmonary bypass (CPB). CPB exposes human blood to artificial surfaces, with an ischaemia-reperfusion injury, tissue hypo-perfusion, and haemolysis. Together these factors can initiate and exacerbate an inflammatory

response, which can act as a perfect model to study sterile inflammatory processes that resemble the physiological changes during sepsis.

Inflammatory pathway activation during CPB induces both the humoral and cellular constituents of the immune system, leading to changes that can be initially manifested by an exaggerated inflammatory response; however, it can then lead to a temporary immunodeficiency state.

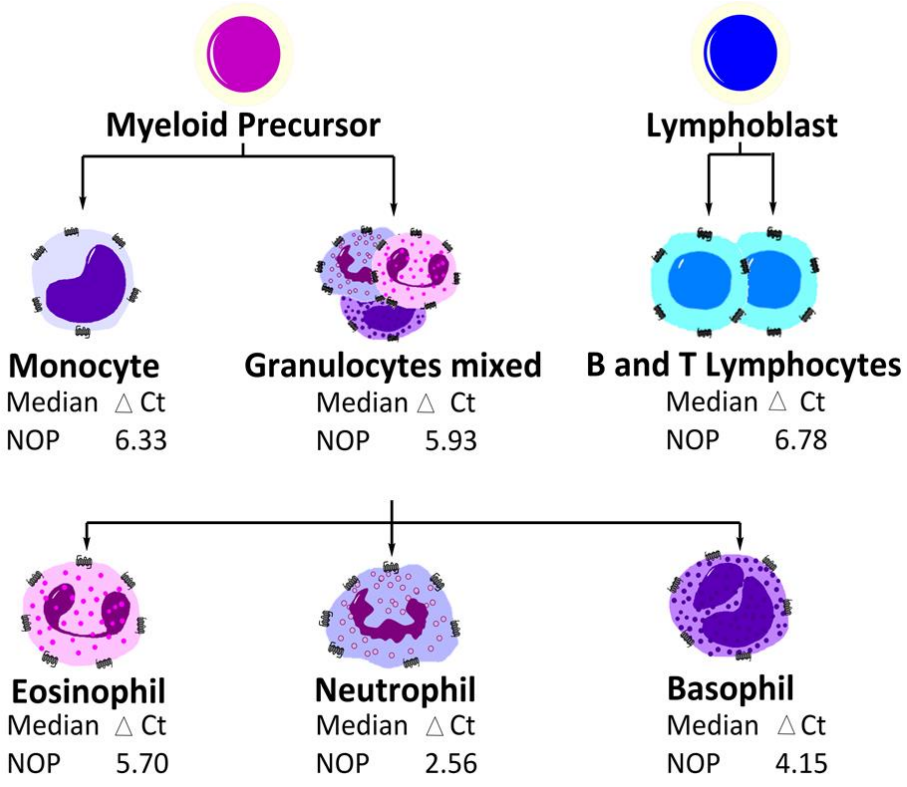

Figure 1.4: Peripheral immune cell population [75]

Peripheral human immune cell population differentiation cascade highlighting the expression on NOP receptors on all varieties of immune cells presented as the difference of expression (ΔCt) of NOP receptor mRNA compared to the endogenous gene GAPDH (further explained in qPCR methods section 2.3)

1.9. Hypothesis

This thesis' working hypothesis is that there are no functioning classical opioid receptors in peripheral human blood as opposed to widespread expression of NOP receptors that are affected in acute inflammatory response and sepsis.

In vitro sepsis and NOP stimulation is predicted to cause dynamic change in NOP mRNA expression.

NOP receptor agonists will also cause the inhibition of white blood cell migration and result in a measurable decrease in cytokine levels, which can be mirrored by a similar trend from a human study of the acute inflammatory response associated with sepsis syndrome and surgery under CPB procedures *in vivo.*

1.10. Aims

- To study NOP mRNA expression in peripheral whole venous human blood.
- To conclusively demonstrate the lack of expression of classical opioid receptor (MOP, KOP and DOP) mRNA in whole peripheral human blood.
- To study the effect of *in vitro* sepsis on the expression of classical and non-classical opioid receptor mRNA in whole human blood with or without the presence of variable opioid and N/OFQ agonists.
- To study the differences in transwell immune cell migration in response to the activation of NOP and classical opioid receptors.
- To detect any measurable change in inflammatory cytokines in line with cell migration.
- To analyse the Neutrophil to Lymphocyte (NLR) ratio plus the associated change in inflammatory mediators (*in vivo*) and attempt to link the acute inflammatory component with potential N/OFQ therapeutic applications.

2. Materials and Methods

2.1. Blood collection

Approval was obtained from the University of Leicester (volunteer) research ethics committee, and all volunteers gave written informed consent. A total of 30ml Venous blood was collected from 10 healthy volunteer members of the University of Leicester, Department of Cardiovascular Sciences. Fresh venous blood was collected in up to four EDTA-Monovette® tubes. The characteristics of the healthy volunteers are listed in Table 2.1.

Table 2.1: Weight, height, and age of healthy volunteers

Sample code	Age	Weight (kg)	Height (cm)
1	36	100	196
2	35	85	183
3	50	95	185
4	32	110	188
5	42	92	170
6	39	95	178
7	54	80	167
8	40	55	160
9	38	94	169
10	40	58	153

Mean age = 40.6 (32-54) years, mean weight = 86.4 (55-110) kg, mean = height 174.9 (153-196) cm, and six males: four females.

2.2. Blood handling and preparation

2.2.1. *In Vitro* sepsis and treatment conditions

Each whole venous blood sample was divided into 2-ml sterile tubes to which lipopolysaccharide from Escherichia coli 0111:B4 (LPS) 5 μg/ml; Staphylococcus aureus peptidoglycan (PepG), 20 μg/ml; fentanyl 10 μM; and morphine 10 μM were added in combination; the samples were incubated for 24 hrs at 37.0 °C in 5% CO_2 humidified air.

These concentrations represent supramaximal ranges and exceed the normal therapeutic levels used in clinical practice to increase the probability of detecting a difference if such a difference exists while cells are still functioning and viable outside host tissues. Similar reagent concentrations were used in similar studies to elicit a detectable response [76]. The different treatment conditions and reagents used are summarized in table 2.2.

Table 2.2: Incubation conditions and additives in the blood samples

Incubating condition	Drug / additive	Concentration
Plain	None	N/A
Media (PBS)	PBS and RPMI 1640	N/A
LPS	Gram-negative bacteria lipopolysaccharide	5μg/ml
Fentanyl	Fentanyl	10μM
Morphine	Morphine	10μM
PepG	Bacterial Peptidoglycans	20μg/ml

PBS is Phosphate buffered saline

2.2.2. PMN collection

The isolation of PMN leukocyte fractions from human blood was performed using the Polymorphprep™ density gradient method from Axis Shield (www.axis-shield-density-gradient-media.com). Polymorphprep™ is a ready-made sterile solution that contains 13.8% (w/v) sodium diatrizoate and 8.0% (w/v) polysaccharide at a density of 1.113 ± 0.001 g/ml and an osmolality of 445 ± 15 mOsm.

Polymorphprep™ has a shelf life of five years. The Polymorphprep solution is used regularly and aseptically in our laboratory, and it is stored away from direct sunlight at room temperature to ensure negligible contamination and iodine release. In utilising this method, the principle of leukocyte separation relies on the density gradient method in which erythrocytes aggregate after losing their water content as a result of high solution osmolality with the polysaccharide molecules, which causes them to sediment and sink to the bottom of the tube upon centrifugation.

Aided by the variable water content that is derived from the cells, distinct density layers form within the tube allowing different leucocytes to occupy predetermined due to the different water content in the surrounding media in the tube. The resulting sample, post-centrifugation, is illustrated in Figure 2.1 below, showing the plasma, monocyte, PMN and red blood cells (waste) layers.

Individual whole blood samples were layered on top of the Polymorphprep™ solution (room temperature) in 15-ml centrifuge tubes at a ratio of 1:1 (5mls each). The tubes were then centrifuged at 600 g_{av} and 20 °C for 45 min without deceleration to avoid disturbing the layers of the separated cells.

A pipette was then used to discard the plasma layer and collect the PMN cell layer in fresh 15-ml centrifuge tubes. Sterile phosphate buffered saline (PBS), prepared by adding a PBS tablet to 200 mls of sterile water, was then added to the collected PMN cell solution at a ratio of 1:1 to dilute any residual Polymorphprep™ which could prevent cell pelleting. The PBS PMN mixture was then centrifuged at 500 g_{av} and 20 °C for 10 min, and the supernatant and cells were finally re-suspended in RPMI 1640. The PMN cell numbers were quantified using a haemocytometer, which is described in section 2.6. PMN cells were then processed to extract the RNA that was used in qPCR experiments as described in section 2.3.

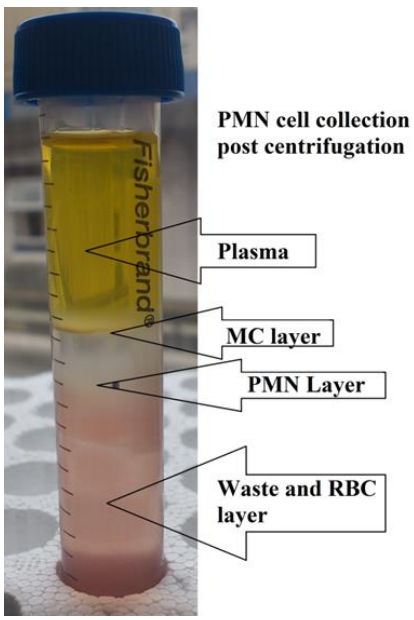

Figure 2.1: PMN preparation tube after centrifugation of whole blood

PMN= Polymorphonuclear cells

MC= Monocytes

RBC= Red Blood Cells

2.2.3. RNA Extraction

2.2.3.1. From PMN cells

RNA extraction from cultured PMN cells was performed using the Applied Biosystems/ThermoFisher Scientific mirVana™ miRNA Isolation Kit with Phenol (AM1560) (www.thermofisher.com). The PMN cells (10^4–10^6) were washed and mixed with 600 µl of a lysis binding solution to form homogenous lysate after gentle intermittent mixing (vortex). The organic extraction phase was then conducted by adding 60 µl of miRNA homogenous additive, vortexing the solution, and then resting it on ice for 10 min. Then, 600 µl of acid-phenol chloroform was added to the samples that were vortexed for 30–60s and then centrifuged at 10,000 g_{av} for 5 min. The fresh upper aqueous phase containing the genetic material was then recovered and transferred to new 2-ml tubes.

The RNA isolation step was performed by adding 1.25 volume of 100% (neat) ethanol to the recovered aqueous phase. Filter cartridge inserts were then placed in new tubes, and the resulting sample mixture was filtered through the inserts at 600 µl volume each time and then centrifuged at 10,000 g_{av} for 20 s before discarding the waste. Then 700 µl of wash solution was added (wash solution1 of the mirVana™ miRNA Isolation Kit). Subsequent wash steps were repeated by adding 500 µl of the second wash solution (wash solution 2/3 of the mirVana™ miRNA Isolation Kit) which was applied to the filter before centrifuging at 10,000 g_{av} for 20s each time. The waste fluid was discarded, and the tubes were centrifuged at 10,000 g_{av} for 1 min at the end to remove all

residual waste. The filters containing genetic material were then transferred to new tubes to which 100 µl of fresh preheated (95 °C) PCR-grade water was added before centrifuging at 10,000 g_{av} for 30s to recover the RNA from the filter that was collected and saved for the RNA quantification step as described in section 2.2.4. An Overview of the RNA extraction protocol is shown in figure 2.2 (Applied Biosystems/ThermoFisher Scientific mirVana™ miRNA Isolation Kit with Phenol manual (AM1560) (www.thermofisher.com)

2.2.3.2. From whole blood

Total extraction of RNA from the whole blood was performed utilising the Life Technologies™ RiboPure™ Blood Kit (AM 1928) (ThermoFisher Scientific) (www.thermofisher.com). The principles of RNA isolation are summarised in two steps. The first step involves cell lysis in a guanidinium-based solution followed by purification of the RNA using phenol/chloroform extraction. The second and final purification step was performed by solid-phase extraction utilising glass fibre filters. The blood collected from the healthy volunteers was divided into 0.5-ml samples in microcentrifuge tubes that were mixed gently by inverting the tube several times. Then, 800 µl of the lysis solution and 50 µl of sodium acetate were added to each 0.5ml blood sample, which was vortexed vigorously to lyse all cells, and 500 µl acid-phenol-chloroform from beneath the aqueous phase was added to the cell lysate and vortexed for 30s.

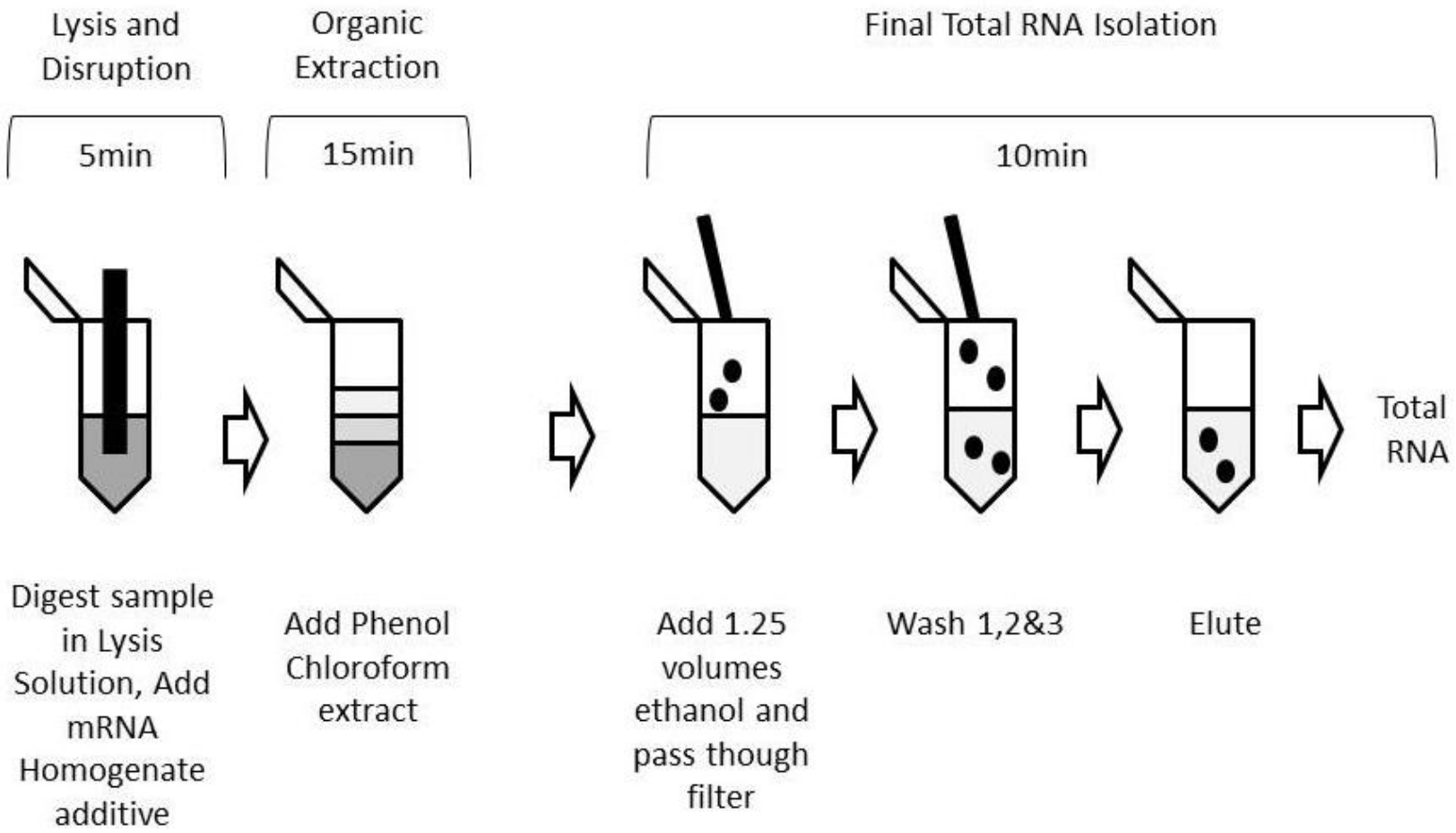

Figure 2.2: Outline of the RNA extraction protocol (adapted from Applied Biosystems/ThermoFisher Scientific mirVana™ miRNA Isolation Kit with Phenol manual (AM1560) (www.thermofisher.com)).

The mixture was stored for 5 min at room temperature and then centrifuged for 1 min to separate the aqueous phase (upper-RNA containing) from the organic (lower) phase. The aqueous phase that contained the RNA was then transferred to new 2-ml tubes at an average volume of 1-1.2 ml.

Then, 600 µl (half the volume of the aqueous phase) of 100% ethanol was then added to each tube. The tubes containing the aqueous phase and the ethanol were vortexed thoroughly and centrifuged for 1s.

Preparations for the final RNA purification step and collection of RNA were required where aliquots of the RNA elution solution were heated to around 75 °C in an RNase-free tube.

The assembling of fresh plastic tubes was also required; two tubes were labelled and used to wash and collect RNA from the glass fibre filters by adding the elution solution to the filter insert, which aids the collection of RNA from the filter inserts.

The aqueous phase and ethanol mix were applied to the glass fibre filter assembly (700 µl at a time) and then centrifuged for 5–10s at 10,000g_{av}, leaving the RNA captured in the filter.

The waste flow-through fluid was discarded followed by the loading of 700 µl of wash solution on the filter inserts (wash solution labelled 1 from the kit), which

were centrifuged for 5-10s at 10,000g_{av}. The same step was then repeated twice using the second wash solution (labelled wash solution 3/4 (from the extraction kit)) before discarding the flow-through with a final 1s quick spin of the filter tube assembly to eliminate any wash solution from the fibre glass filter before finally transferring the solution to a fresh RNA collection tube.

Then, 50 µl of the preheated elution solution was added to the fibre glass filter tube assembly and left to rest for 20s followed by centrifuging at maximum speed for 20–30s at 10,000g_{av} to recover the RNA from the filter.

Another 50 µl of the elution solution was loaded to the filter, which was centrifuged again for 1 min to ensure maximum RNA recovery.

The collection tube, containing a volume of almost 100 µl, was then stored in a freezer at -80 °C after the RNA quantification and quality control as described in section 2.2.4. A summary of RNA extraction from whole blood is shown in fig 2.3.

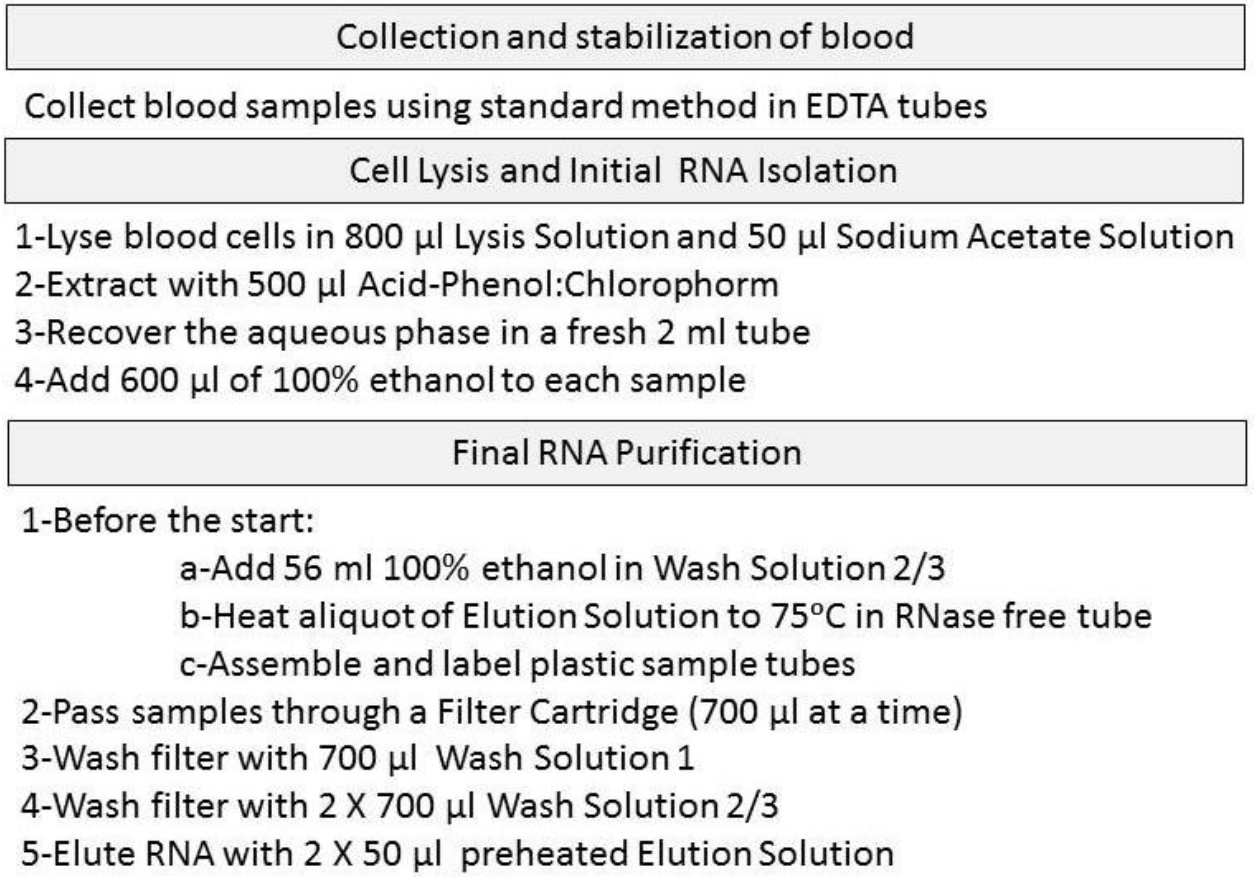

Figure 2.3: Summary of RNA extraction method from whole blood

Adapted from Life Technologies™ RiboPure™ Blood Kit (AM 1928) manual (ThermoFisher Scientific).

2.2.4. RNA quantification using UV spectrophotometry

RNA quantification was performed utilising the Eppendorf BioPhotometer® (https://www.eppendorf.com/OC-en/). RNA samples diluted at a ratio of 1:10 (5 µl sample plus 45 µl PCR-grade RNA free water) were loaded into microcuvette tubes for quantification. The principal of RNA quantification depends on the amount of 260 nm and 280 nm light absorption through a 1-cm path in the spectrophotometer chamber. A value of A_{260} =1 is equivalent to 40 µg RNA/ml in a 1-cm chamber. The RNA concentration can be calculated by multiplying the A_{260} absorbance value by the dilution factor used in the experiment that was 10 to derive the actual sample RNA concentration (µg/ml). The higher the A_{260} value, the higher the RNA yield at average target values of 0.02, which corresponds to the dilution ratio of 1:20. The A_{260}/A_{280} ratio is a measure of RNA purity. The ideal target ratio is between 1.8 and 2.2. Lower ratios indicate protein or phenol contamination. The device used displays RNA concentration on the screen without the need for any calculations because the dilution factor is entered in the initial step. The RNA concentration (µg/µl) in each sample was recorded, and 5 µg of total RNA was then processed using a DNase enzyme to remove DNA contamination.

2.2.5. DNA contamination removal

The presence of DNA will cause false positive results upon analysis of qPCR because genomic DNA will be amplified rather than cDNA (synthesised from RNA). Despite every effort in avoiding DNA contaminants, some DNA will still be present in small amount that should be eradicated. DNA contamination was reduced by up to 5 million fold by utilising the TURBO DNA-free™ Kit containing TURBO™ DNAse Treatment and Removal Reagents from Ambion® by Life Technologies® (www.thermofisher.com) (Catalogue number AM1907, publication number 1907M).

To ensure the highest RNA yield, up to 5 µg of RNA was processed; where samples had low RNA yields, the maximum volume suggested by the manufacturer was used up to a maximum volume of 50 µl. Then, 0.1 volumes of the RNA containing sample (i.e. 5 µl) of TURBO DNase buffer and 1 µl TURBO DNase were added to the RNA samples with gentle mixing. The samples were then incubated for 30 min at 37 °C, after which a similar volume of the TURBO DNase (i.e. 1 µl) of DNAse inactivation reagent was added with thorough mixing. After 5 min of incubation, intermittent mixing was needed before centrifuging the tubes at 10,000g_{av} for 1.5 min. RNA samples that are soluble in the solution were then collected in fresh tubes, excluding the cloudy DNA pellet in the bottom of each tube.

2.2.6. cDNA synthesis

Copy DNA (cDNA) was synthesised from the extracted RNA using a High Capacity cDNA Reverse Transcription Kit from Applied Biosystems® (Part Number 4375575 Rev. E 06/2010). The first step was to prepare the reverse transcription master mix by allowing the master mix components to thaw on ice. The required master mix volume was calculated according to the number of reactions described in table 2.3.

Each RNA sample was diluted in RNA free water up to 10 µl volume mixed with 10 µl RT positive (including the reverse transcriptase enzyme) and RT negative (excluding reverse transcriptase) master mix to generate single stranded cDNA and the respective negative control (see table 2.3). 20 µl volume, 10 µl master mix, and 10 µl sample were placed in a thermocycler plate that was sealed, labelled, and then loaded in the thermocycler. The thermocycler was run for 2 h and 15 min, heating the samples to different temperatures according to the programme detailed in Table 2.4. The RT positive and negative samples were then stored at -20°C before processing using a real-time polymerase chain reaction (qPCR).

Table 2.3: Reverse transcription reaction reagents

Component	Volume per Reaction (µl)	
	Kit with RNase inhibitor	Kit without RNase inhibitor
10X RT Buffer	2.0	2.0
25X dNTP Mix (100mM)	0.8	0.8
10X RT Random Primers	2.0	2.0
MultiScribe™ Reverse Transcriptase	1.0	1.0
RNase inhibitor	1.0	-
Nuclease-free H_2O	3.2	4.2
Total volume per reaction	10.0	10.0

Table 2.4: Thermocycler reverse transcription reaction programme

	Step1	Step 2	Step 3	Step 4
Temperature °C	25	37	85	4
Time	10min	120min	5min	Hold

2.3. Quantitative polymerase chain reaction (qPCR)

Gene expression was studied utilising Real Time – Quantitative - Polymerase Chain Reaction after reverse transcription, which aims to detect the presence or absence of specific genetic sequence in a target sample that is specific for target receptor protein. Each human cell contains up to a 30,000 DNA gene sequence that is divided into exons and introns where exons are responsible for

coding specific target proteins. The exon regions of the DNA sequence are used to code mRNA in the cell nucleus that is used in protein synthesis in the cytoplasm [77].

PCR methodology detects the presence of a gene of interest by the amplification and detection of specific cDNA sequences. The core steps of PCR rely on the presence of forward and reverse primers (5' and 3' respectively) that will build the nucleic acid sequence resembling the gene of interest using bacterial DNA polymerases which are resistant to heat, unlike human DNA polymerase. The process of heating the PCR materials to 90 degrees Celsius will result in the uncoupling of the DNA double strands that will be followed by the synthesis of two copies of DNA aided by the presence of ribonucleotides and a DNA polymerase enzyme, a process that is repeated many times to cause an exponential increase in the original genetic material (cDNA) sample content. The quantitative reaction utilised Taqman® methodology relies on the principle of florescence emitted by a fluorophore attached to the forward 5' primer when it is separated from the quencher molecule attached to the backward 3' primer when the PCR reaction amplifies the original genetic material and separates the two molecules. This enables the quantification of fluorescence, which is proportional to gene expression, as illustrated by figure 2.4 (from the ThermoFisher Scientific Guide). The expression of the gene of interest can be quantified through comparison with the expression of an endogenous housekeeper control target, which represents a gene consistently

expressed. The qPCR results are presented as cycle threshold that indicates the number of amplification cycles needed for the threshold florescence to be detectable above a passive reference dye. Comparative PCR experiments compare the cycle threshold between the genes of interest against the endogenous gene, and the results are expressed as ΔCt, which is calculated by subtracting the mean Ct of the endogenous housekeeper gene from the mean Ct of the genes of interest. PCR experiments traditionally include negative control samples that should show no RNA sample amplification.

Figure 2.5 presents a snapshot from a comparative PCR experiment taken from the desktop software of PCR Applied Biosystems StepOne® V2.2.2 illustrating the comparative gene expression of MOP to the endogenous gene (GAPDH) shown as plate sample layout on the right and amplification plot on the left. Curves that are higher than baseline signify positive expression of GAPDH with the number of cycles on the bottom of the grid signifying the cycle threshold. The coloured lines that are not showing any change from baseline are the negative non-template control samples and the MOP samples that showed no expression. Figure 2.6 shows the different variables that can be seen in a typical PCR amplification plot as described in the text above.

The selection of the housekeeper gene (positive endogenous control) is important in qPCR experiments because of the possibility of variable expression in different tissues and disease sensitivity [78].

The geometric mean of two housekeeper genes (GAPDH and B2M) was used to make results more robust and to make ΔCt values less dependent on volunteer covariates. The results were usually expressed as the cycle threshold (Ct) and the difference in cycle threshold (ΔCt).

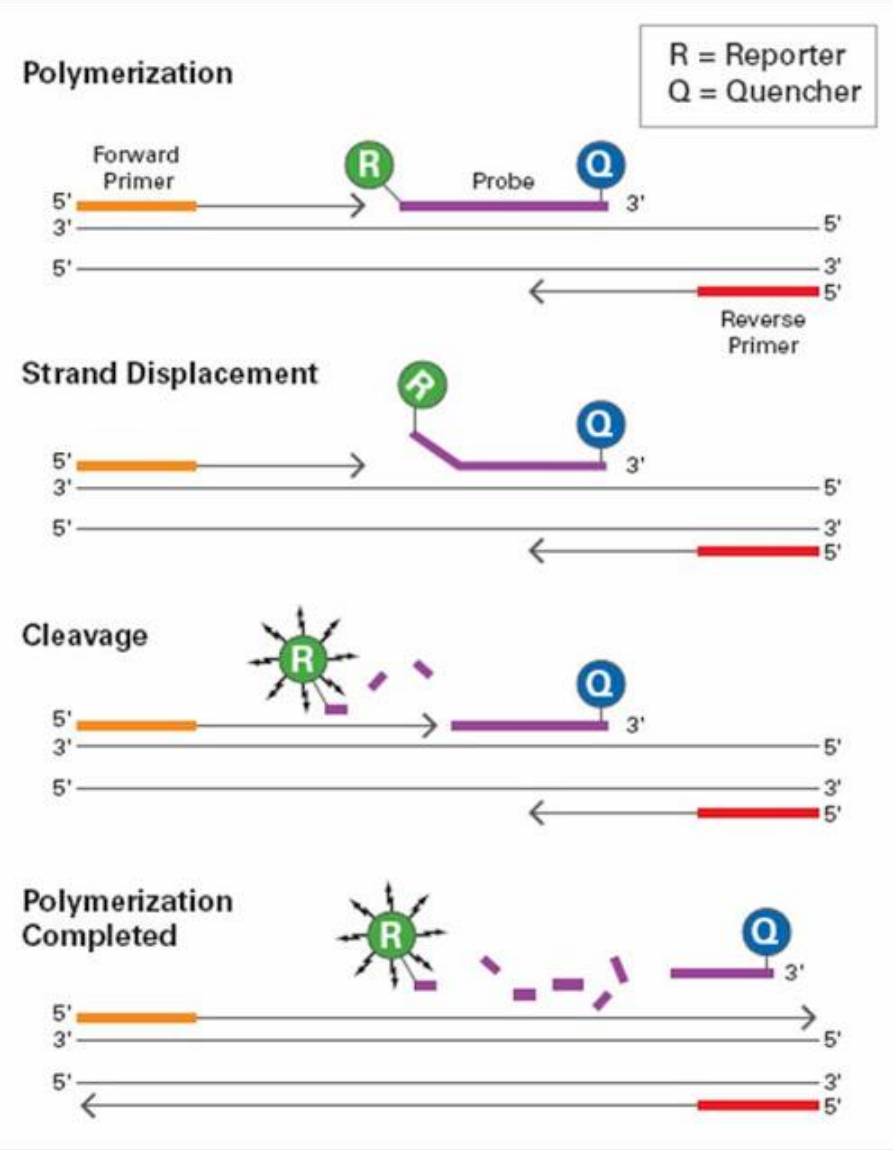

Figure 2.4: Taqman® assay principle

Rt-PCR assay principle as described by ThermoFisher Scientific https://www.thermofisher.com/in/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics/how-taqman-assays-work.html

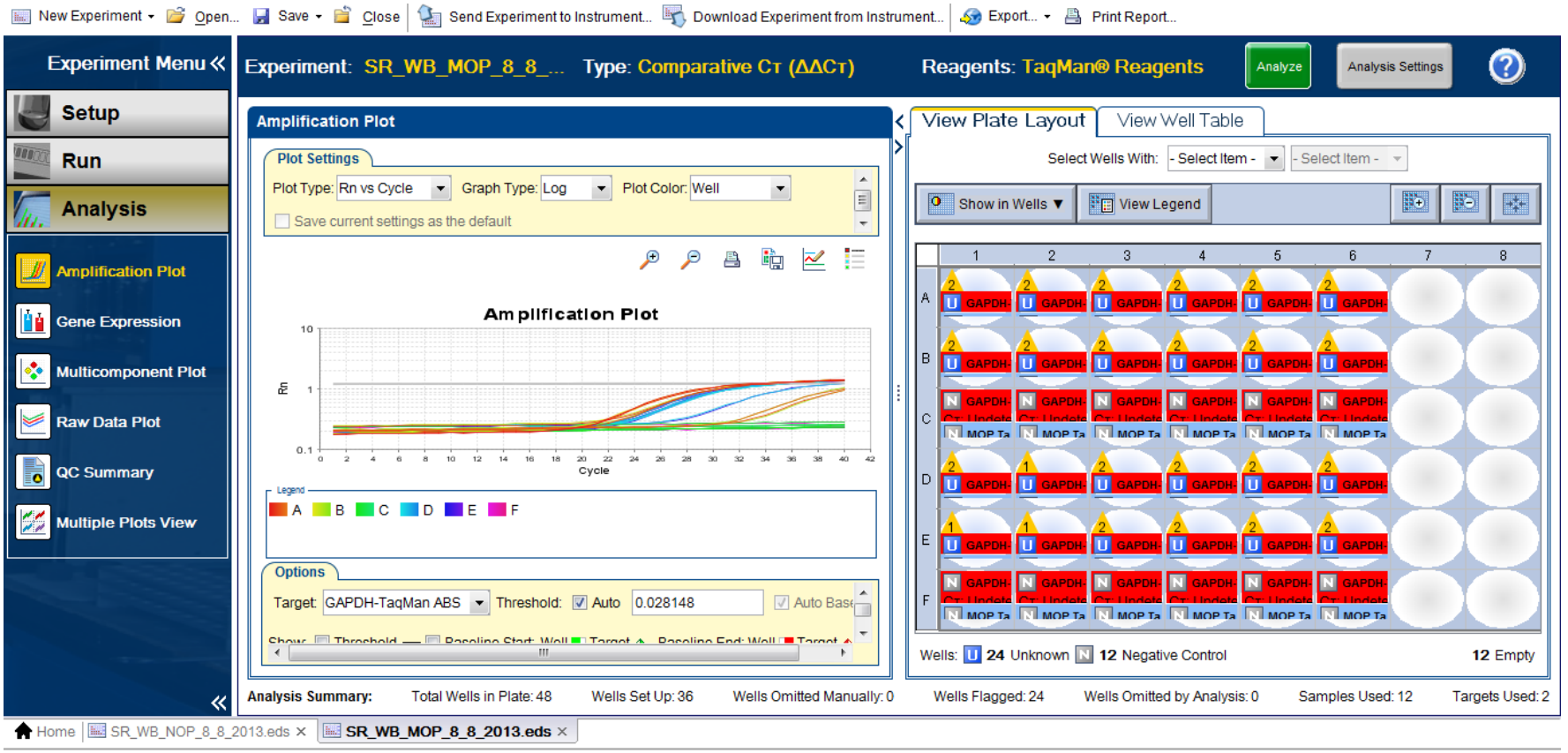

Figure 2.5: Desktop snapshot of Applied Biosystems StepOne® V2.2.2 qPCR

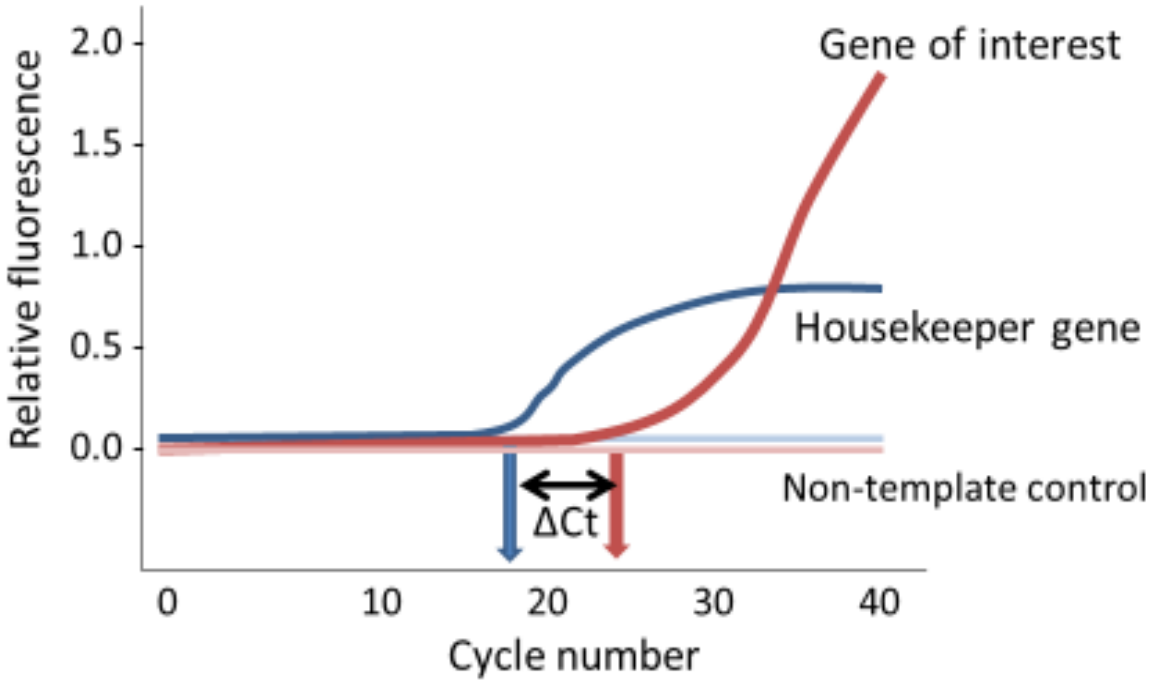

Figure 2.6: example amplification plot of qPCR experiment

Dark red and blue lines represent the amplification plot of the gene of interest and the housekeeping gene respectively. Coloured arrows represent respective cycle threshold value (Ct) and the black arrow represents the difference in cycle threshold (ΔCt). Lighter shades coloured flat lines represent respective negative non-template controls .

All qPCR work was undertaken in a dedicated area to minimise contamination. All surfaces and instruments were cleaned using hypochlorite spray. The PCR reaction reagents mix was prepared as shown in Table 2.5 according to the number of reactions planned and their respective negative controls by adding 18 µl of master mix and 2 µl of the samples in the positive wells.

Table 2.5: qPCR reagents per reaction

Component	Volume for one reaction (µl)
TaqMan gene expression master mix	10
Gene of interest probe	1
Control endogenous gene probe	1
PCR-grade water	6
Sample	2

The qPCR programme was then prepared by running 48 wells quantitatively for the standard 2 h. The data are presented as Ct (the lower the number, the higher the expression), and ∆Ct signifies the degree of gene expression relative to the endogenous control (the lower the number the closer the expression of the gene of interest relative to the housekeeper gene, which is typically expressed in abundance).

TaqMan® probes, which are commercially available, predesigned gene expression assays, were used to study the expression of the MOP, KOP, and NOP mRNA. The assay IDs are included in Table 2.6.

Table 2.6: Sample TaqMan® probes used in the experiments

Target	Type	Sequence/ Assay ID (supplier)	Abbreviation
KOP (OPRK1)	TaqMan assay	Hs00175127_m1 (Life Technologies)	TM-KOP1
MOP (OPRM1)	TaqMan assay	Hs01053957_m1 (Life Technologies)	TM-MOP1
NOP (OPRL1)	TaqMan assay	Hs00173471_m1 (Life Technologies)	TM-NOP1

Full list of probes used in PCR experiments is listed in Appendix 2

The limitations surrounding commercially available DOP TaqMan probes are discussed further in section 2.4. These limitations led us to resort to gel electrophoresis methodology to probe non-template control positive reactions.

2.4. Agarose gel electrophoresis

Agarose gel electrophoresis was utilised to further probe DOP receptor expression; this was done to address the issue of the ability of commercially available DOP qPCR probes to amplify both genomic DNA and cDNA in my experiments. Agarose gel electrophoresis relies on the fact that negatively charged DNA fragments travel toward the positive electrodes through the gel at

a distance that is proportional to their size. Hence, long fragments travel shorter distances compared to shorter fragments. The distance travelled is compared to a predetermined DNA ladder that consists of differently sized fragments to indicate the target cDNA fragment size when the gel is processed to detect the position of the cDNA band.

First, 3% agarose gel was prepared by mixing 1.5 g agarose powder with 50 ml of buffer solution containing a mixture of Tris base, acetic acid and EDTA (1x-TAE buffer) in a flask, which was then heated in a microwave for 1-3 min until all the agarose was melted. Then, 2.5 µl of ethidium bromide (EtBr) was added to the flask to facilitate the future visualisation of DNA under ultraviolet (UV) light after binding to DNA. The agarose gel was then left to cool, after which it was poured into a gel tray with grid combs in place while ensuring that it was on a flat surface and that no bubbles were present before it solidified.

The solidified gel was then placed in the gel box (i.e., the electrophoresis unit) after the grid comb was gently removed, leaving equal rectangular pits in the gel in which to load the samples. The gel box was then filled with Tri acetate buffer (1xTAE) mixed with 0.05 M EDTA until the gel was covered.

Then a 1000base-pair ladder (mixed with 1µl loading buffer and 4µl water) was loaded in the first lane of the gel to act as an indicator of genetic material sample size in adjacent wells. Electrodes were then connected to the gel cell at 80v. The run time was 1–2 h, and it was stopped when the dye had travelled

80% of the gel. The gel was then carefully removed, and the cDNA bands were visualised under UV light as depicted in figure 2.7.

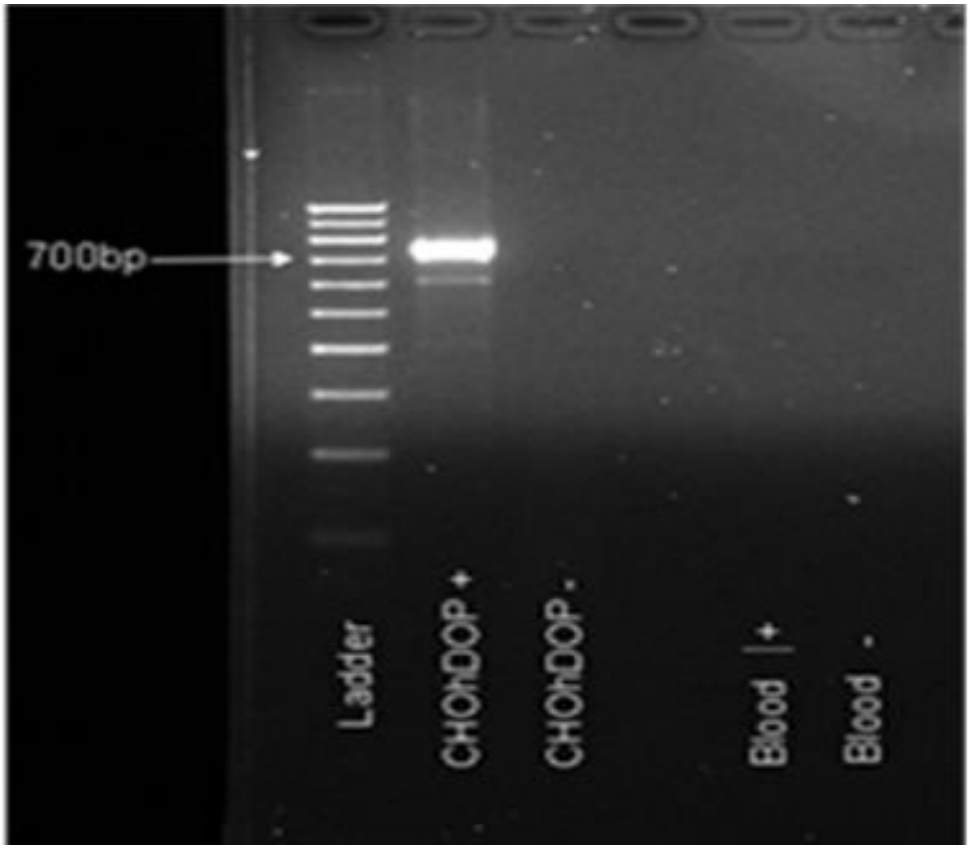

Figure 2.7: Gel electrophoresis UV visualisation and printout example.

The figure shows a UV picture printout of the Gel illustrating the ladder on the left portion of the picture pointing to a gene of interest size of 700bp (100bp each ladder step). The white band in the position adjacent to the ladder represents a DOP positive CHO cells with its negative control sample in the position immediately to its right. Blood samples did not amplify the same size genetic material and are therefore considered negative.

2.5. Transwell migration assay (Boyden chamber assay)

Transwell migration assay was performed to study the effect of receptor-agonist interactions on cell activities and to link receptor expression results with function to ascertain the *in vivo* role of such receptors.

The transwell migration assay is used to study the migration of activated cells from two media through a porous membrane in which the pore sizes are smaller than the resting size of the cells to avoid the non-specific cell gravitational migration effect. The upper chamber usually contains cells that are suspended in culture media mixed with different reagents to study their effects on cell migration. The lower chamber usually contains culture media and a chemo-attractant agent that facilitates the activated migration of cells that will be collected and counted at a later stage. Before the cells and reagents were loaded, the plates, filters, and plain culture media were incubated for 1 h to ensure that the culture media had reached 37 °C.

2.5.1. Reagents

- N/OFQ: natural peptide agonist of NOP receptors.
- [des-phe^{1}]N/OFQ: structurally inactive N/OFQ derivative that lacks affinity to NOP receptors.

- PWT2-N/OFQ: peptide welding technology compound consists of four N/OFQ molecules welded onto a cyclam core molecule that acts as a super agonist of the NOP receptors with long duration of action.
- Morphine: potent classical opiate receptors agonist
- fMLP: N-Formylmethionyl-leucyl-phenylalanine is a potent PMN chemotactic agent and a macrophage activator.
- Eotaxin: protein molecule produced in abundance in lung tissue of asthmatic patients that can be used as a chemoattractant for eosinophils.

2.5.2. PMNs

PMN cells were prepared from peripheral whole venous human blood as per the protocol described in section 2.2.2 and then suspended in an RPMI 1640 tissue culture media mixed with 10% foetal calf serum (FCS). Then, 3-µm transwell inserts were placed in 96 well plates and labelled according to different incubation conditions. $1*10^4$ PMNs were loaded in the top chamber and mixed with different reagents at varying concentrations (plain control, N/OFQ (300, 30, 3, 0.3, and 0.03 nM), [des-phe^1] N/OFQ, PWT2-N/OFQ and Morphine at 300 nM concentrations) to a volume of 300 µl. The lower chamber was filled with tissue culture media and fMLP (100 µM) to a volume of 450 µl apart from the plain negative control well, which had no fMLP.

The tissue culture well plates were incubated for 2 h at 37 °C in a 5% CO_2 humidified air incubator. Then the bottom chamber media containing the migrating cells were collected in fresh 2-ml tubes that were centrifuged to collect cell pellets that were re-suspended in a 200-µl volume. The migrated PMNs were counted using the haemocytometer method (section 2.6). The media from the bottom chamber were also saved for further analysis and inflammatory mediator quantification utilising ELISA, which is described in section 2.7.

2.5.3. Eosinophil-like cells (EOL-1)

EOL-1 cell migration assays were undertaken using EOL-1 cell culture from Sigma-Aldrich (www.sigmaaldrich.com), the biological origin of which is peripheral human blood in human eosinophilic leukaemia. The cells were cultured in 1640 RPMI media supplemented by 2 mM glutamine and 10% FBS. The cell cultures were incubated at 37 °C in 5% CO_2 until they become confluent, but not adherent to each other in clusters after which point cells were retrieved, quantified, and re-suspended in fresh culture media. Then, $1*10^6$ cells were loaded in 8-µm pore size filter inserts (bigger pores were selected because of the difference in cell size of EOL-1 compared to PMN) in a 96 well plate mixed with different reagents as described in the PMN migration assay (section 2.5.1). Eotaxin (200 ng/ml) was used in the lower chamber to promote EOL-1 cell migration through chemotaxis. The well plates were then incubated

for 2 h after which the cells in the lower chamber were collected in fresh 2-ml tubes and re-suspended in 200 µl of fresh culture media before the cells were counted using a haemocytometer. The cell media from the bottom chambers were saved for use in the ELISA analysis of inflammatory mediators (section 2.7).

2.6. Cell counting using the haemocytometer

The haemocytometer is usually used to quantify cells in any given volume by counting the number of cells in a fixed area under the microscope, which allows for calculating the number of cells in the original volume. The haemocytometer chamber is a glass slide with a known area of depression in the middle. It is made up of nine large equally sized squares each of which is divided into 16 equal small squares, as shown in figure 2.8. The large corner squares are used to count the cells in low-density samples, whereas the middle squares are further divided to facilitate counting the cells in high cell density samples. In cell migration assays, the four large corner squares and the middle squares were used for cell counting and were then averaged to quantify the number of cells.

The haemocytometer slide was first thoroughly cleaned and then covered by a coverslip. Moisture was applied to form a tight seal between the hemocytometer and the coverslip. Then, 10µl samples were loaded by pipetting at the angle of

the coverslip to ensure that the sample was equally distributed in a thin layer all over the hemocytometer grid by the effect of capillarity.

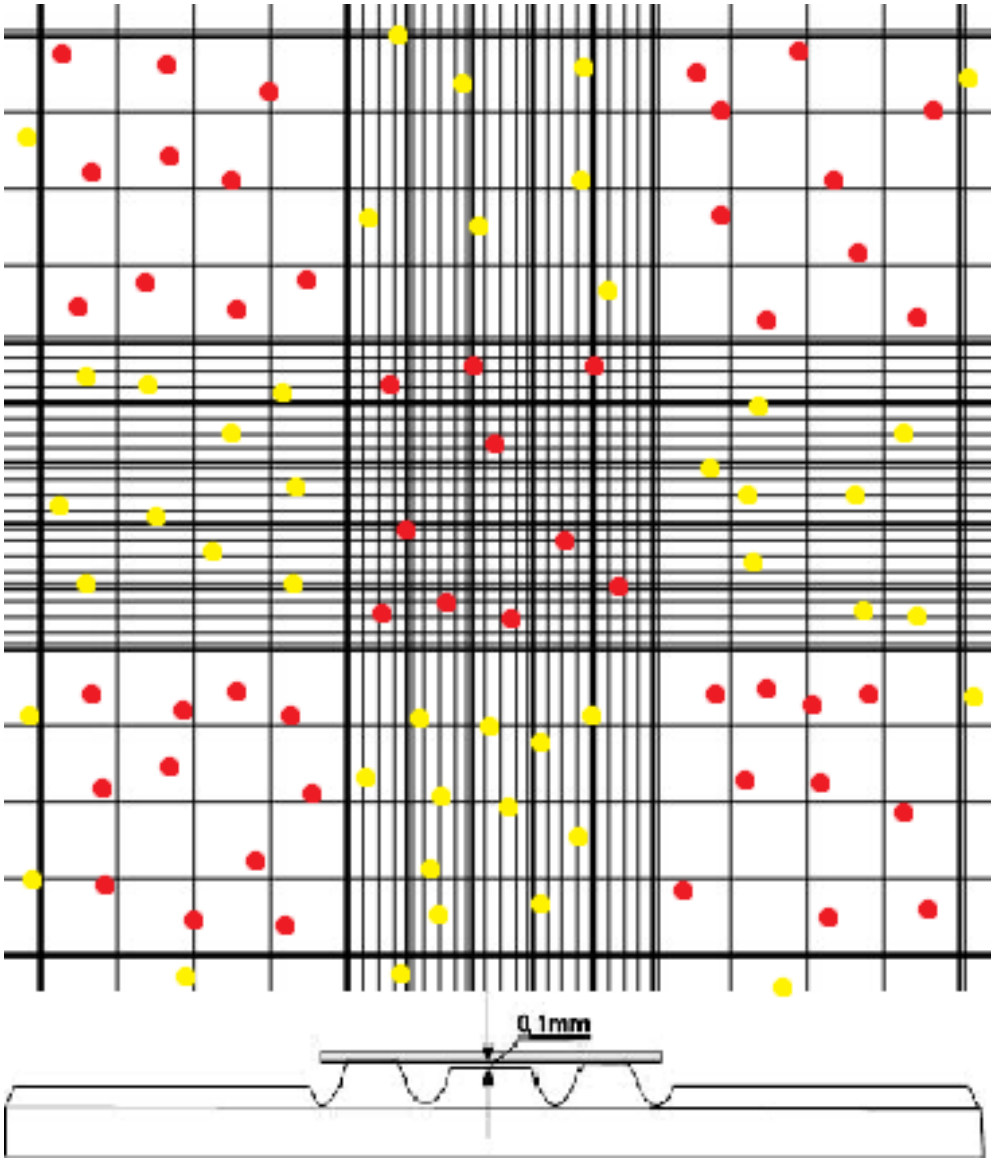

Figure 2.8: Haemocytometer chamber grid and profile section example

Haemocytometer grid example illustrating marked cell counting area at the top and cut section area at the bottom. The red dots represent included cells and the yellow dots represent excluded cells.

The haemocytometer slide was then placed under the microscope and the cells were counted. The counting was done by ignoring the left and bottom limb of each square to avoid double counting. The cells were included in the count if they were more than half way across the lines dividing the small squares. The number of cells per 1 ml was derived by multiplying the number of cells in one square by 16 and then multiplying it by 10^4, giving the number of cells in 1 ml.

2.7. Enzyme linked immunosorbent assay (ELISA)

ELISA was developed by Engvall and Perlmann in 1971 to detect and quantify specific proteins in a specimen mixture [79]. The basic principles of ELISA can be summarised in four steps: 1) coating and capture, in which specific antigens are adhered to the polystyrene microplate wells. 2) plate blocking, in which additional molecules are added to the microplate wells to cover all residual surface binding sites. 3) probing and detection involves incubating the plates after the introduction of antigen-specific antibodies with high binding affinity to the target antigen. 4) signal measurement, in which the signal generated by the tag is measured on the specific antigen.

Acute phase cell-signalling cytokine proteins, including the tumour necrosis factor (TNFα) and interleukins (IL-8, IL-10), were tested in the cell migration samples to detect any pro- or anti-inflammatory responses that were associated with the migration of the immune cells. TNF-α is a strong proinflammatory

pleiotropic cytokine [80]. IL-10 is also known as human cytokine synthesis inhibitory factor and is a strong anti-inflammatory mediator [81]. IL-8 is a chemoattractant cytokine that plays an important role in PMN activation hence is considered as a pro-inflammatory cytokine [82].

Plasma concentrations of tumour necrosis factor (TNFα), interleukins IL-8, and IL-10 were determined using a DuoSet ELISA Development kit from R&D Systems (Biotechne). The wells of Microlite-2 plates were coated by incubating overnight with 100uL of PBS containing 4ug/ml of anti-human TNFα, IL-8 and 2µg/ml of anti-human IL-10. The wells were washed with PBS and subsequently blocked for 2 h with PBS containing 10% (v/v) foetal calf serum, after which they were washed three times with wash buffer consisting of 1.5mmol/l NaH_2PO_4, 8mmol/l Na_2HPO_4, 340mmol/l NaCl, 0.5g/l Tween and 0.1g/l sodium azide. Following this 100µl of assay buffer, consisting of 1.5mmol/l NaH_2PO_4, 8mmol/l Na_2HPO_4, 140mmol/l NaCl, 1mmol/l EDTA, 1g/l BSA, 0.1% (v:v) Triton-X 100 and 0.1g/l sodium azide, was added to all wells. This was followed by 100µl of sample or standard (recombinant human TNFα, IL-8, or IL-10 diluted in either assay buffer or media). The plates were incubated overnight and were then washed, as before, followed by the addition of 100µl biotinylated goat anti-human IL-8, IL-10, or TNF-α at working concentrations of 20ng/ml, 75ng/ml and 400ng/ml respectively, for 2 h. After this, the plates were washed and methylacridinium ester labelled streptavidin [2×10^6 relative light units (RLU)/100µl of assay buffer] was added and incubated at room temperature for

2 h. The plates were washed a final time with wash buffer, and chemiluminescence was measured in a Dynex MLX luminometer. Chemiluminescence was measured after the wells were initiated with sequential injections of 100µl of 100mmol/l HNO_3 containing 0.05% hydrogen peroxide and, 4s later, 100µl of 250mmol/l NaOH containing 0.25% cetyl triethylammonium bromide. The measurements were expressed in RLU. The concentrations of TNF-α, IL-8, and IL-10 in the media samples were determined by extrapolating them against standard curves of the known concentrations of human TNFα, IL-8, and IL-10 (see Figure 2.9).

2.8. Neutrophil to lymphocyte ratio (NLR)

The NLR is the ratio of circulating neutrophils (which is a marker of an acute inflammatory component) to the number of circulating lymphocytes (representing the inflammatory regulatory pathway). Neutrophilia and lymphopenia may indicate physiological stress, and they affect NLR. In addition, other factors affect NLR, such as smoking, age, gender, and obesity. NLR has proven to have prognostic significance in inflammatory conditions, diseases of the cardiovascular system, infections, and certain cancers [74].

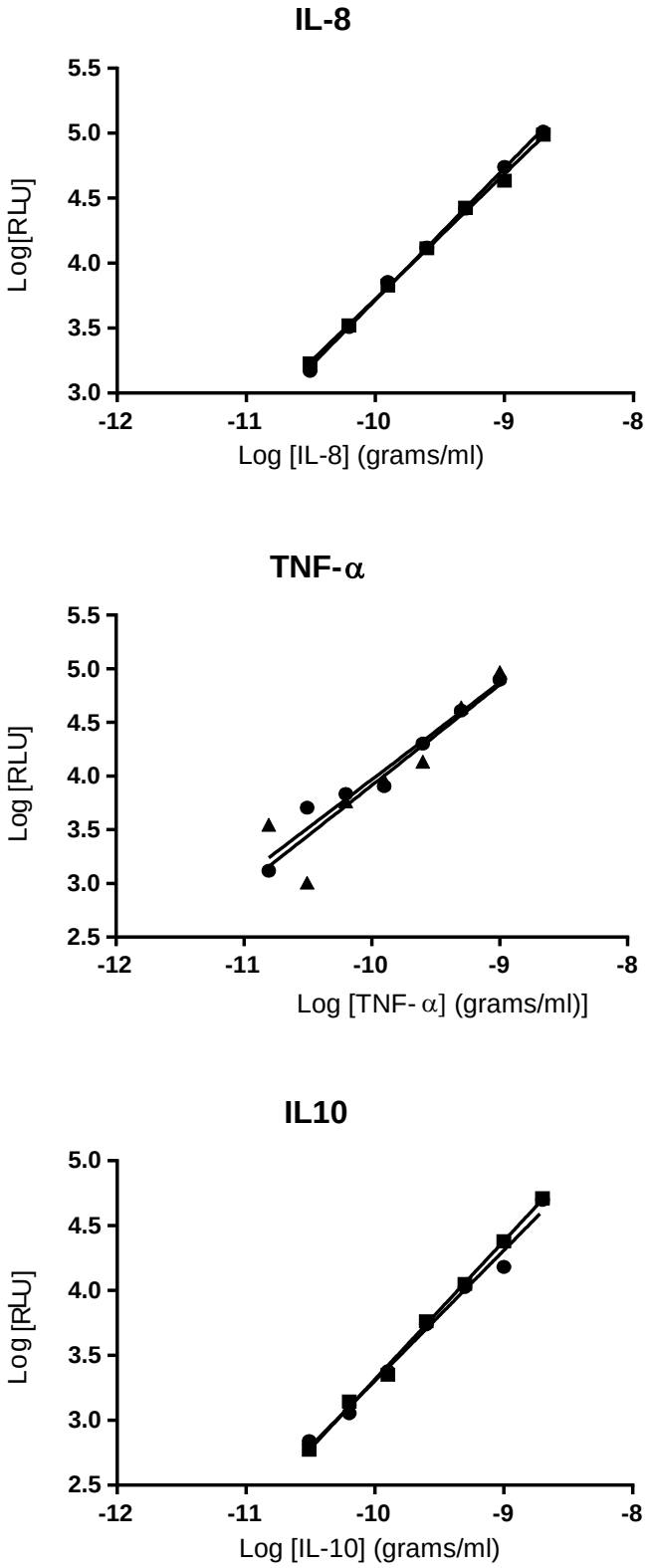

Figure 2.9: ELISA standard curves for IL-8, IL-10 and TNF-α expressed as Log Relative Light Unit (RLU) and Log cytokine concentration (gram/ml)

The rationale of looking into the NLR data is since NLR can be easily calculated at different time points and could be mapped against pro and anti-inflammatory cytokines levels to determine which patient population would benefit from therapeutic interventions that would decrease PMN migration and inflammatory cytokine release. A future step would mean that validated NLR levels could be used as a surrogate to discern therapeutic interventions and predict outcomes without measuring circulatory cytokines.

Our group conducted a prospective observational cohort study in 82 patients admitted to ICU with a clinical diagnosis of sepsis and 40 patients who were undergoing cardiac surgery requiring CPB [63 65]. The control group included 63 age- and sex-matched healthy volunteers. Neutrophil and lymphocyte concentrations were recorded in peripheral whole blood, and the differential white cell counts were processed in central hospital laboratories at the University Hospitals of Leicester NHS Trust using commercially standardised assay kits. Circulating cytokine (TNF-α, IL10, IL8) levels of the patients in the ICU were recorded using the ELISA protocol (described in section 2.7) on days 1 and 2, and upon clinical recovery from sepsis; and in patients undergoing cardiac surgery at 3 and 24 hours after CPB. The results of this study are fully discussed in section 3.5 to further explore the relationship between NLR, inflammatory response, and NOP expression.

2.9. Data analysis

A basic statistical analysis that includes measuring the Mean, Median, Mode and range was calculated using Microsoft Office Excel software. GraphPad Prism 7 software was used to analyse migration, ELISA and NLR results including testing for statistical significance, variance and spread of samples (standard deviation and standard error of the mean). Graphic representation of the results was produced utilising GraphPad Prism 7 software. All software was obtained from the University of Leicester (home licence).

3. Results

3.1. Human whole blood RNA extraction and quantification

The blood samples were incubated under various conditions in two sets of experiments based on an *in vitro* sepsis model with LPS and PepG. The RNA extracted from peripheral whole human venous blood was quantified using UV spectrophotometry (section 2.2.4) to determine quantity and purity under various incubation conditions as indicated in appendix 1. Median and mean RNA content and the A260/A280 ratio of samples in all incubation condition was 0.11, 0.112 µg/µl and 1.74, 1.76 respectively. Individual sample RNA content and the A260/A280 ratio are listed in appendix 1.

3.2. qPCR

3.2.1. MOP, KOP, and NOP receptors

The results of the qPCR experiment using TaqMan probes to target the MOP, KOP, and NOP receptors are summarised in Table 3.1. The results are presented as cycle threshold (Ct) and the difference in cycle threshold (ΔCt) (as explained in section 2.3) between the target gene and the geometric mean of the two endogenous housekeeper genes (GAPDH and B2M).

The samples were treated with lipopolysaccharide (LPS) 5 µg/ml, Staphylococcus aureus peptidoglycan (PepG) 20 µg/ml, fentanyl (F) 10µM, and morphine (M) 10µM in the combinations shown in appendix 1.

Consistent with previous results, the MOP and KOP transcripts could not be detected in contrast with NOP transcripts that were expressed in all samples. The geometric mean of the Ct values derived from B2M and GAPDH was used to determine the ∆Ct values for the expression of the NOP receptor. The results are presented as mean (interquartile range). LPS was used in all 10 samples compared to PepG, which was used in five out of ten experiments to study any differences in mixed sepsis model, hence the difference in the number of samples (5-10) is indicated in table 3.1.

The dynamic change in NOP mRNA expression in variable incubation conditions is further emphasised in figure 3.1, which shows a significant difference in NOP mRNA expression in all samples incubated with LPS and in samples incubated with PepG when combined with other reagents. The data are presented as fold difference in Ct values from the baseline and analysed using Kruskal-Wallis test with Dunn's multiple comparisons test.

Table 3.1: Expression of classical and non-classical opioid receptor transcripts in mRNA extracted from whole blood samples

	MOP and KOP receptors				NOP receptor		
Sample	Ct GAPDH (IQ range)	Ct B2M (IQ range)	Ct MOP	Ct KOP	Geometric Mean Ct (GAPDH / B2M)	NOP Ct (IQ range)	ΔCt (IQ range)
Plain (n = 10)	24.16 (22.93-24.93)	21.89 (20.58-22.18)	ND	ND	23.17 (21.49-23.58)	32.75 (31.75-34.17)	10.40 (8.56-11.24)
Media (n = 10)	23.88 (22.54-25.09)	21.07 (20.00-22.01)	ND	ND	22.88 (21.15-23.47)	32.82 (31.89-34.29)	10.71 (8.41-11.64)
Morphine (n = 10)	24.23 (22.68-24.89)	21.29 (20.67-21.87)	ND	ND	22.93 (22.29-23.48)	34.07 (32.19-34.47)	10.68 (9.21-11.70)
Fentanyl (n = 10)	24.11 (23.24-24.90)	21.52 (20.98-21.73)	ND	ND	23.17 (22.34-23.30)	33.45 (31.92-34.61)	10.67 (8.21-11.48)
LPS (n = 10)	25.75 (25.02-27.27)	21.19 (19.77-22.09)	ND	ND	23.37 (22.54-24.37)	35.19 (34.11-35.66)	11.40 (11.21-12.98)
LPS + M (n = 10)	25.35 (24.79-26.63)	20.05 (19.44-21.46)	ND	ND	22.82 (21.93-23.58)	35.08 (34.58-36.04)	11.93 (10.74-13.12)

LPS + F (n = 10)	25.46 (24.94-26.83)	20.72 (19.82-22.57)	ND	ND	23.20 (22.57-24.70)	35.66 (34.34-36.27)	11.75 (10.79-13.18)
PepG (n = 5)	24.71 (22.82-28.18)	20.74 (19.49-23.26)	ND	ND	22.84 (21.57-25.40)	33.91 (31.21-36.35)	10.75 (8.54-12.19)
PepG + M (n = 5)	26.00 (24.32-26.92)	20.76 (20.11-22.14)	ND	ND	23.22 (22.24-24.75)	34.71 (32.31-35.70)	11.52 (8.41-13.08)
PepG + F (n = 5)	24.43 (22.43-26.81)	20.54 (19.43-22.51)	ND	ND	22.41 (21.43-24.48)	34.10 (30.51-36.24)	11.02 (8.26-12.90)
PepG + LPS (n = 5)	25.01 (23.12-26.34)	20.33 (19.76-21.17)	ND	ND	22.70 (21.94-23.57)	35.61 (31.33-36.00)	12.36 (8.99-13.54)
PepG+LPS+M+F (n=5)	25.59 (22.99-27.66)	20.23 (19.15-22.89)	ND	ND	22.38 (21.66-25.15)	35.29 (32.94-36.47)	12.28 (9.33-13.58)

IQR=Inter quartile range, Ct= Cycle threshold, ∆Ct= Difference in cycle threshold, ND= Not detected, LPS= Lipopolysaccharide, PepG=Peptidoglycan, SD= Standard Deviation, and SE= Standard error of mean.

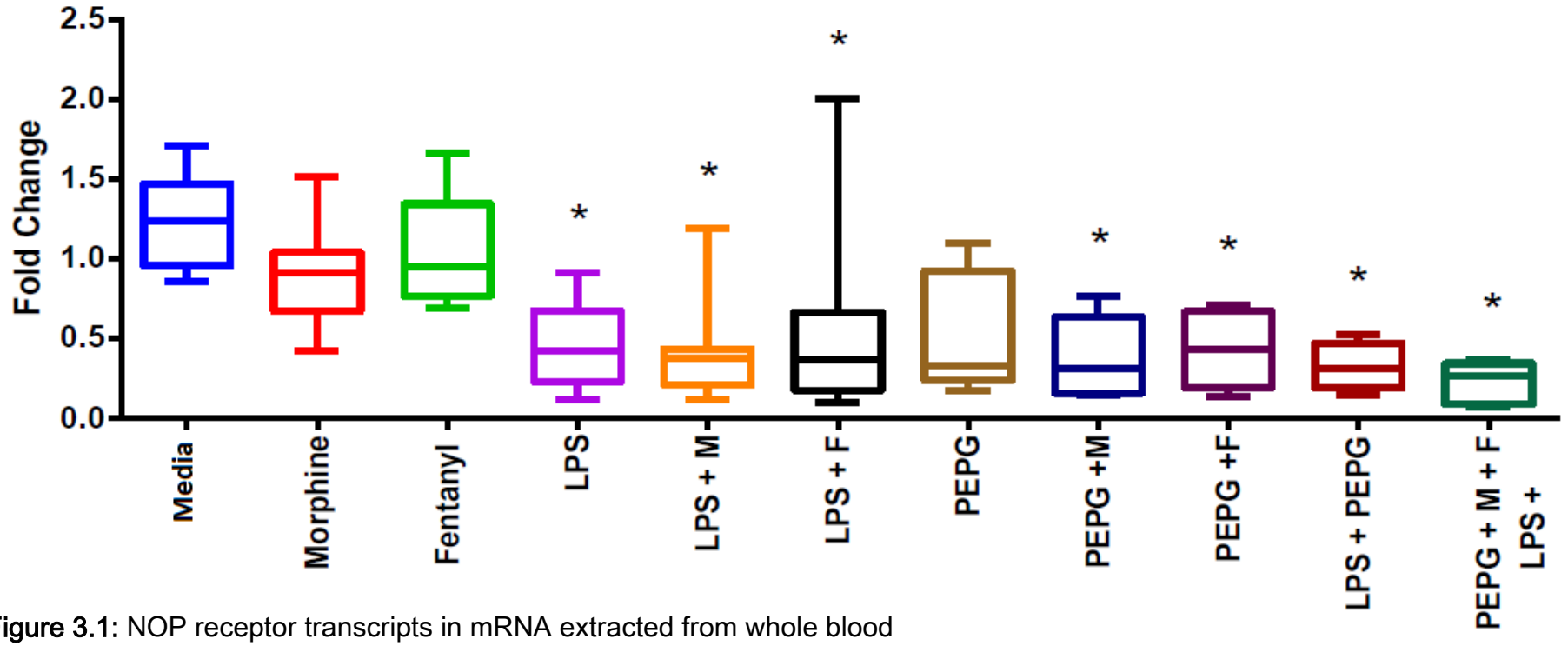

Figure 3.1: NOP receptor transcripts in mRNA extracted from whole blood

Expression of NOP receptor transcripts in mRNA extracted from whole blood samples treated with lipopolysaccharide (LPS) 5µg/ml, Staphylococcus Aureus Peptidoglycan (PepG) 20 µg/ml, fentanyl (F) 10µM, and morphine(M) 10µM in the combinations shown. Data presented as fold change in Ct value relative to “plain” sample treated with a vehicle control “media” where 0.5-fold difference means 50% change in expression. *samples showed statistically significant difference to plain samples using Kruskal-Wallis test with Dunn’s multiple comparisons test.

3.2.2. DOP PCR and Gel electrophoresis

The qPCR experiments that utilised the TaqMan® probes were designed and validated for the human DOP receptor, TM-DOP 1-3 (Appendix 2). The results showed amplification in both positive controls, cDNA from CHO cells and in cDNA from the whole blood samples. Moreover, the amplification of the DOP receptor transcript was also detected in the non-template controls (NTC) from the whole blood samples. The NTC was a negative control for each sample in which the reverse transcriptase enzyme, which converts mRNA to cDNA, was removed. Therefore, amplification in the NTC samples most likely represents genomic DNA contamination, not mRNA expression, which is the precursor of protein (receptor) expression. The high Ct values found with TM-DOP1-3 primers in both the cDNA samples and the related NTC again suggest gDNA rather than mRNA/cDNA amplification.

Alternative commercially-available primer pairs were sourced and used in SYBR green experiments using a Quantifast SYBR Green PCR Master Mix (Qiagen). The thermal profile of reactions with SBG-DOP1&2 primer pairs was 5 min at 95 ˚C followed by 40 cycles of 10 s at 95 ˚C and 30 s at 60 ˚C. Again, the amplification of DOP receptor transcripts was detected in cDNA samples and NTCs from the whole blood samples and in the CHO_{hDOP} controls using both primer pairs SBG-DOP1&2.

To determine any differences in the PCR products amplified from sample wells and NTCs wells, a melt curve analysis was performed. A melt curve analysis was carried out by a final melting step at the end of the PCR reaction; this consisted of 15 s at 95 ˚C followed by the construction of the dissociation curve comprising increasing temperatures in increments from 0.3 ˚C to 60˚C. In the preliminary experiment (n = 1) using whole blood RNA, expected differences were determined in the measured melting temperature (Tm) values of the PCR products. Tm is the temperature at which one half of the DNA duplex will dissociate to become single stranded which will be amplified by different primer pairs: 77.67 (TM-DOP1), 81.29 (TM-DOP3), 85 (SBG-1), and 87.25 (SBG-2), based on differences of the amplicon size and sequences amplified by the different primer pairs.

However, when a single TaqMan probe or primer pair was used, no differences in the measured Tm values were determined between the cDNA samples and the NTCs: 77.67 and 77.67 for TM-DOP1, 81.29 and 81.44 for TM-DOP3, 85 and 85.3 for SBG-1, and 87.25 and 87.25 for SBG-2. This result suggests that the PCR product in the cDNA samples and NTCs was the same, which was confirmed by imaging the PCR products using gel electrophoresis for TM-DOP1 (~100 bp) as shown Figure 3.2. This result suggests a false positive, and the amplified DNA must be genomic in origin.

To remove gDNA contamination beyond that removed by DNAse treatment alone, a test was performed using Raji cells known to express DOP receptors.

The mRNA from Raji cells was reverse transcribed using the Quantitect Reverse Transcription Kit, which incorporates an additional genomic DNA elimination reaction.

Using the mRNA extracted from Raji cells, the reverse transcription reaction with genomic DNA elimination reaction showed no effect on the results. The Ct values determined, using TM-DOP1 probes, relative to the standard reverse transcription reaction, were 36.17 and 36.13 in the cDNA samples and 36.59 and 36.38 in NTC. Overall, the extra genomic DNA elimination reaction was deemed ineffective, and it was not used to process the blood samples drawn from the volunteers.

To distinguish cDNA from gDNA, primers were designed to span an exon/exon boundary using “primer-BLAST” against the NCBI Reference Sequence NM_000911.3 for the Homo sapiens opioid receptor, delta 1 (OPRD1) mRNA. These primers, designated as STD-DOP-1, were applied in an end-point PCR experiment using an AmpliTaq Gold® 360 Master Mix. In an Eppendorf Mastercycler, the thermal profile consisted of an initial denaturing step at 95 °C for 10 min, 40 cycles of 30 s at 95 °C, annealing for 30 s intervals at temperatures from 51 °C–71.5 °C, and extension at 72 °C for 60 s. A final extension was carried out for 10 min at 72 °C. The PCR products were run on a 3% agarose gel stained with ethidium bromide and imaged under UV illumination. DOP gel electrophoresis and end point PCR results are illustrated in Figure 3.2.

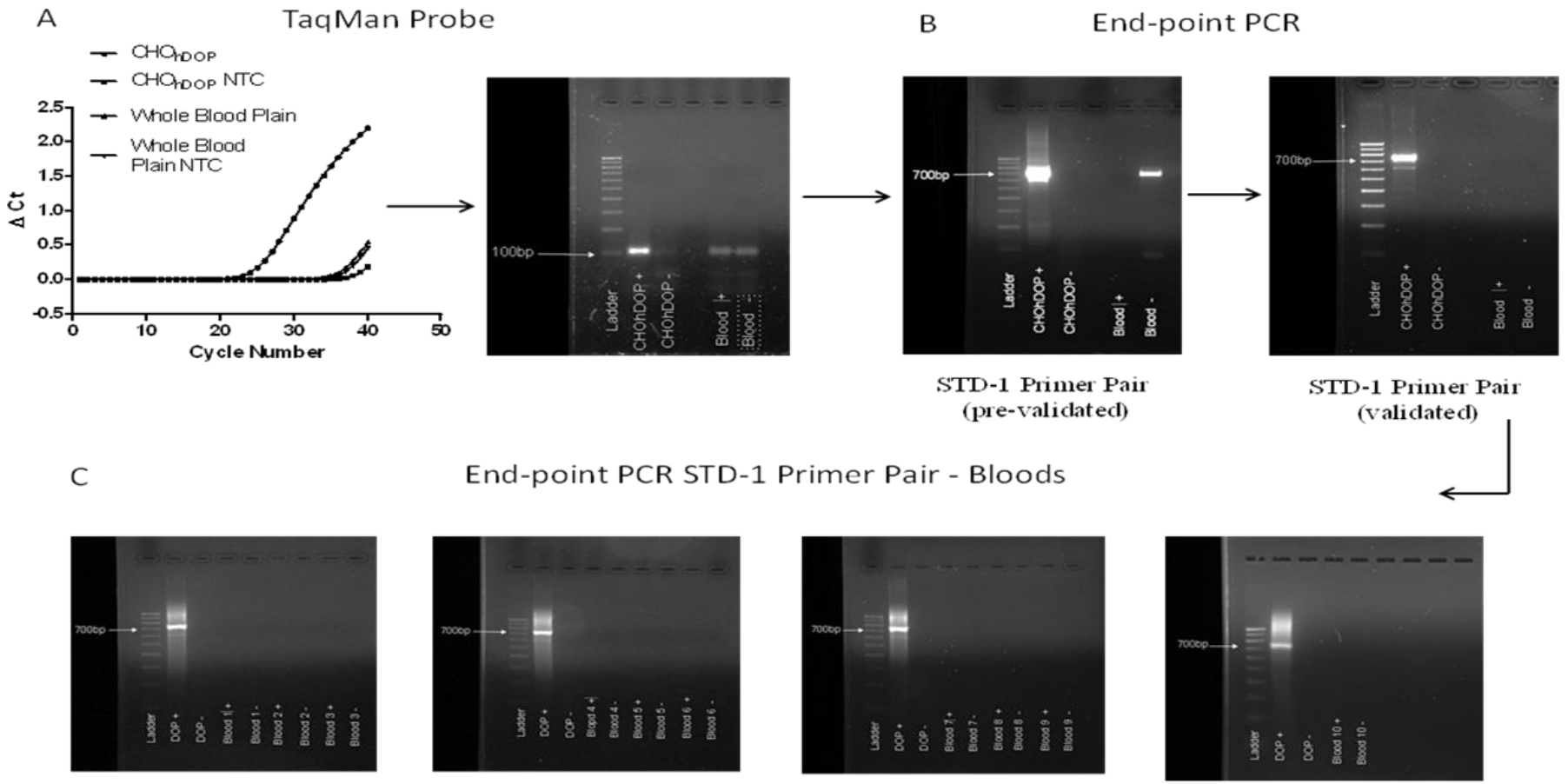

Figure 3.2: Panel A displays the qPCR amplification plot in whole blood RNA using TaqMan probes targeting the DOP receptor. Amplification in both reverse transcribed (RT) and non-RT samples is shown, and the equivalent gel confirms the presence and size of both products. Panel B displays gel electrophoresis for the PCR products from end-point PCR using STD-1 primers-pairs pre- and post-validation, in which the annealing temperatures and primer concentrations were optimised. Panel C displays the gel electrophoresis of PCR reactions using STD-1 primer pairs under authenticated conditions in the RNA from the whole blood samples.

STD-DOP-1 primers were initially used at a concentration of 0.4 μM with an annealing temperature of 59 °C. They were determined to amplify a product of predicted size: ~700bp in cDNA from positive control CHO_{hDOP} cells. In the NTC wells of the whole blood samples, the STD-DOP 1 primer pair was observed to amplify a product of ~700 bp and another of ~200 bp; however, this result was not consistent in all the NTC samples tested. To improve the stringency of the STD-DOP 1 primer pair, temperature and concentration gradients were performed which led to the determination of the optimal annealing temperature at 56.5 °C and a primer pair concentration of 40 nM. Under these conditions, in cDNA from CHO_{hDOP} cells, the amplification was of the predicted size; ~700 bp and no product was detected in the NTCs. The STD-DOP 1 primers were subsequently used under the same experimental conditions to probe the samples for the expression of DOP in whole blood samples. No expression was detected. Figure 3.2, panel C.

3.3. Cell migration assay

3.3.1. PMNs migration assay

PMNs were counted in duplicate using the haemocytometer. The baseline (plain) number of cells migrating without any chemoattractant or any other agent was set as 1.0 against which the rest of the results were presented as the number of fold differences in migration.

The PMN migration assay was repeated to compare different active and inactive N/OFQ agonists (PWT2-N/OFQ and [desPhe[1]] N/OFQ) and the MOP agonist (morphine). PWT N/OFQ is a tetra-branched synthetic peptide that is a derivative of N/OFQ in which four N/OFQ molecules are linked by a central moiety (as shown in figure 3.3), resulting in a compound that is 40-fold more potent and 10-fold longer acting than N/OFQ both *in vitro* and *in vivo*. Moreover, it maintains the selective NOP agonist activity that is reversed by the NOP selective antagonist SB-612111 [83] [84].

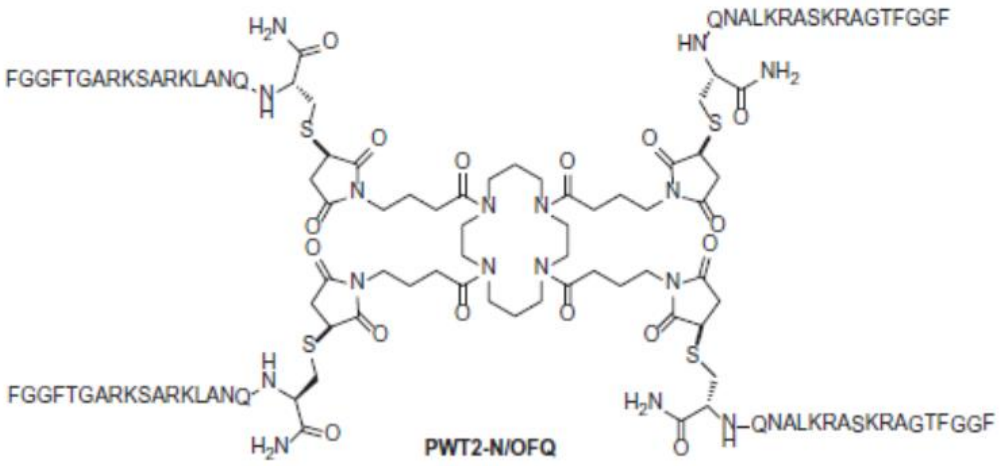

Figure 3.3: PWT2-N/OFQ, molecular structure [85].

[desPhe[1]]N/OFQ is produced as an inactive metabolite of N/OFQ by aminopeptidases that lack affinity to NOP receptors. It is usually considered an inert compound that has a structural resemblance to N/OFQ [86].

[desPhe^1]N/OFQ is usually used as a negative control in pharmacological studies because of its lack of NOP receptor activation.

PMN cell migration was studied using 100 μM fMLP as a chemoattractant. The inhibition of PMNs chemoattraction/migration with N/OFQ stimulation was concentration dependent. The N/OFQ concentration that caused 50% maximum inhibition of cell migration (IC_{50}) was 7.7pM as calculated from the nonlinear curve plot (Figure 3.4).

PMN migration assay was repeated with 300 nM each of PWT2-N/OFQ, [desPhe^1] N/OFQ, and the MOP agonist morphine as shown in figure 3.4. There was no significant difference in mean cell migration compared to fMLP in both [desPhe^1] N/OFQ and morphine (unpaired t-test P values 0.056 and 0.537, respectively). However, there was a significant difference in mean cell migration compared to fMLP with PWT2-N/OFQ ($p<0.05$).

3.3.2. EOL-1 cell migration assay

Our research group highlighted the expression of NOP and the prepronociceptin (ppN/OFQ) which is the precursor of endogenous N/OFQ peptide mRNA on eosinophils in asthmatic patients previously [87]. Eosinophils account for 1–3% of circulating white blood cells in humans. Because they have a lifespan of only a few hours in outside human circulation, it is challenging to study them *in vitro*. Hence, experimental models are based on an eosinophil-like cell line (EOL-1)

where cells differentiate into eosinophil cells by histone acetylation. Commercially available Eosinophil-like cell lines were used in the experiments that were previously shown to express NOP receptors as mentioned above. EOL-1 transwell cell migration was inhibited by N/OFQ in a concentration-dependent manner with an IC_{50} of 41.7pM. EOL-1 cell migration studies were conducted using 200ng/ml eotaxin, which is a small protein that is produced in the lungs of asthmatic patients and acts as a strong chemoattractant for eosinophils.

The EOL-1 cell migration in response to 300 nM PWT2-N/OFQ, [desPhe^1]N/OFQ, and the MOP agonist morphine using eotaxin as chemoattractant showed no significant difference in cell migration with morphine and [desPhe1] N/OFQ (P value > 0.20). However, it showed a significant inhibition of cell migration with PWT2-N/OFQ (P value <0.05) as shown in figure 3.4.

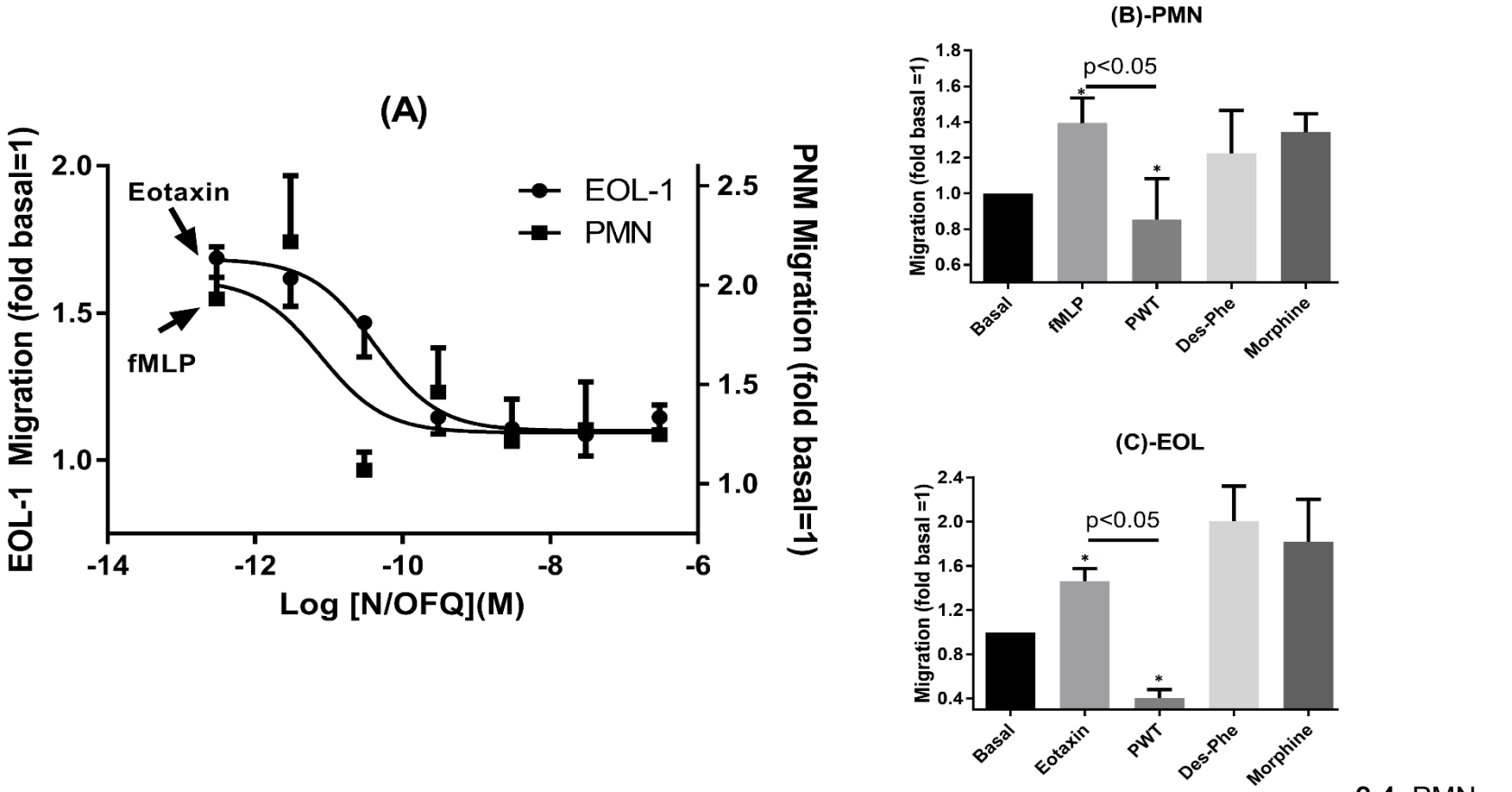

Figure 3.4: PMNs and EOL-1 cell migration assay presented as log N/OFQ concentrations in mole; -12.5 in Panel A represent fMLP and eotaxin migration responses and all data are number of folds of cell migration from the baseline (i.e.1). Panel A illustrates the concentration response curve for both cell types (PMN IC_{50} 7.7pM and EOL-1 IC_{50} 41.7pM). B and C show a range of NOP ligands and morphine for comparison, * represent significant PMN and EOL cell migration inhibition with 300nM PWT expressed as folds difference from baseline (SEM 0.11, 0.04) (Data are n=5).

3.4. ELISA

To establish any possible link between the pattern of cell migration and inflammatory responses, the media from migration experiments for both human PMN cells and EOL-1 cells were assayed for the presence of several inflammatory mediators. The media from migration experiments using human PMN cells that were treated with a variety of opioid ligands; N/OFQ, PWT2-N/OFQ, [desPhe1]N/OFQ, morphine and the chemoattractant fMLP and eotaxin. They were assayed using ELISAs for the cytokines IL-8, IL-10, and TNF-α.

The concentrations of IL-10 and TNF-α were very low or below the level of detection in all the experiments (see Appendix 4). It was therefore not possible to carry out a full analysis of the data with these cytokines.

The yields for IL8 were low but detectable through interpolation from the IL8 standard curve; however, sample yields were below the lowest IL-8 standard of 31.3pg/ml, (see Figure 3.5).

In a similar series of experiments, the media from EOL-1 migration experiments were tested for IL-8, IL-10, and TNF-α. Similarly, the levels of IL10 and TNF-α were such that it was not possible to analyse the data. For IL-8, the levels were measurable, although the presence of the eosinophil chemoattractant Eotaxin and the opioid ligands PWT2-N/OFQ, [desPhe1]N/OFQ, and morphine had no statistically significant effect on the levels of IL8 as seen in figure 3.6.

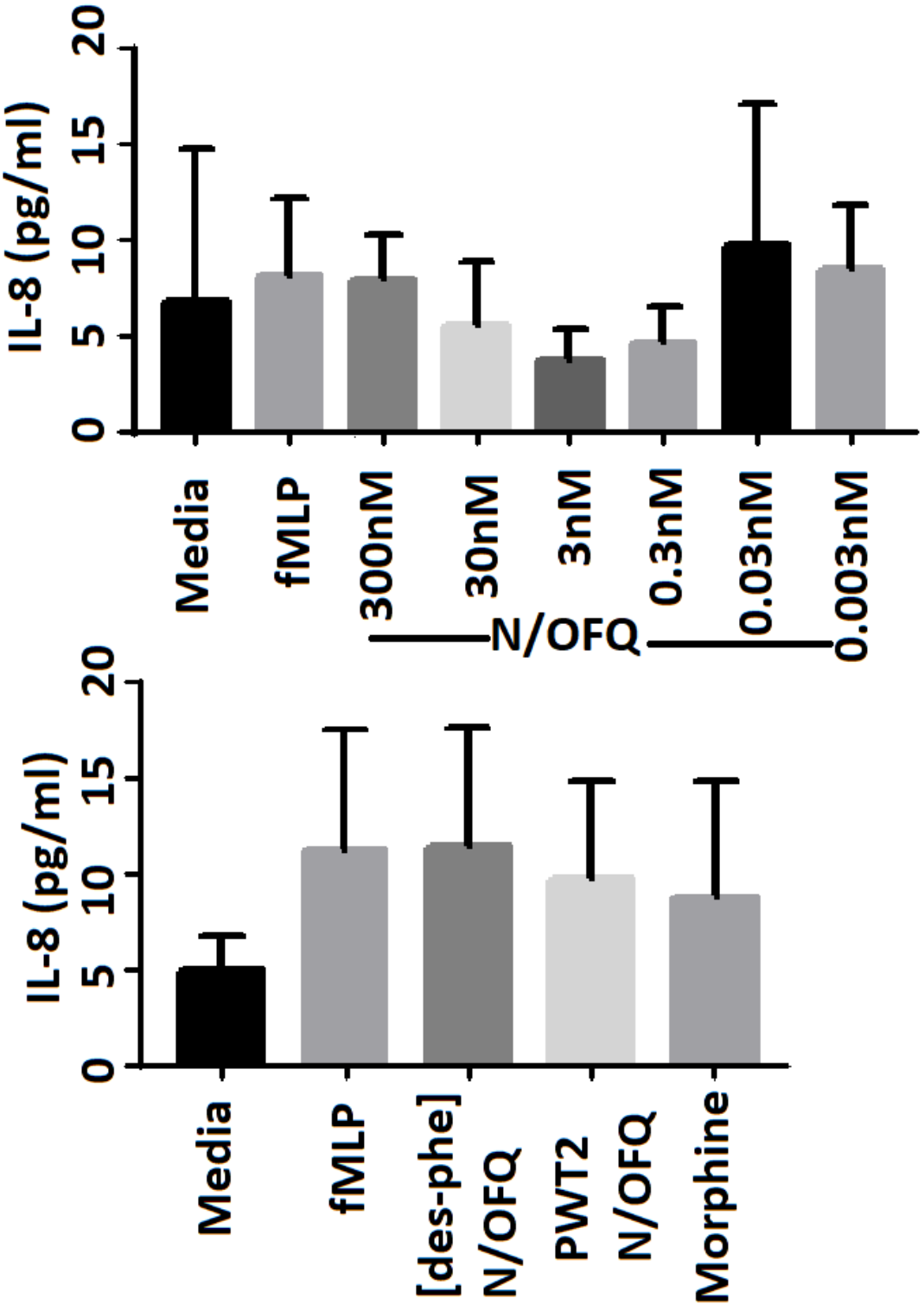

Figure 3.5: IL-8 data using ELISA, Raw data and statistics is detailed in Appendix 4

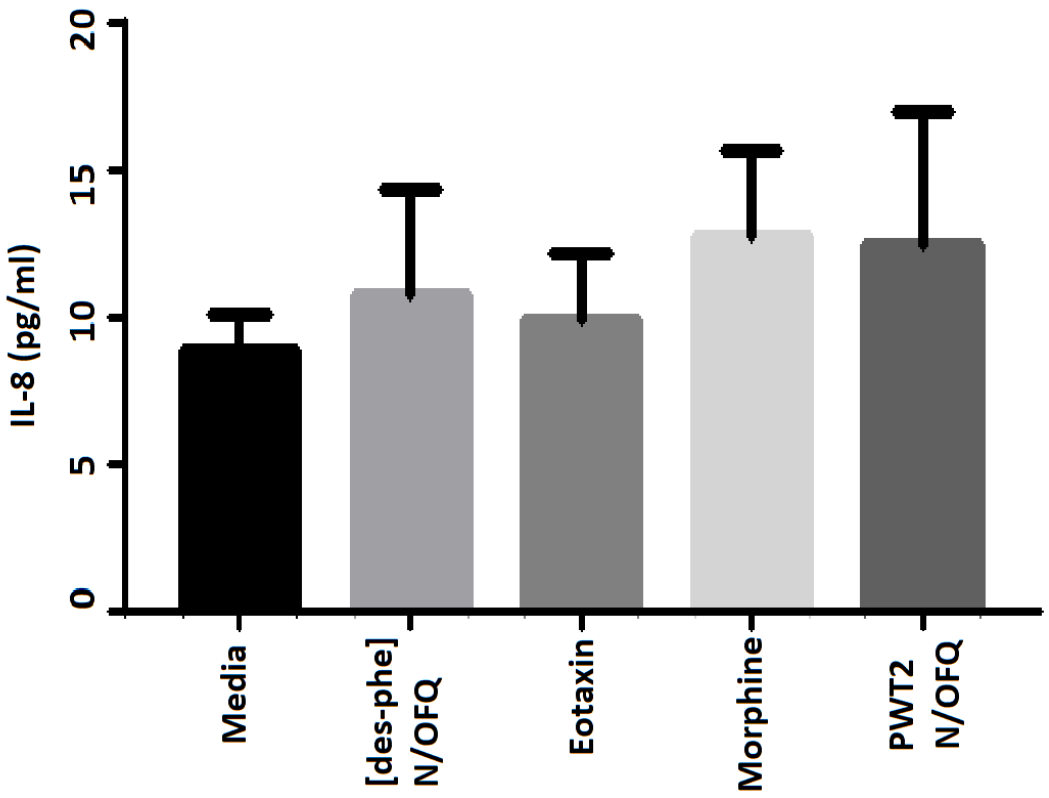

Figure 3.6: IL-8 levels in variable incubation conditions, Raw data and statistics is detailed in appendix 4.

This analysis of the cytokine levels was from media which has been recovered from migration assays. It was considered that the undetectable and low levels of cytokine were a result of the number of cells and the assay volume used in the original migration experiments. To determine the effect of opioid ligands on the release of cytokines in the EOL-1 cells, a series of experiments with increasing cell numbers was carried out. Four different quantities of cells were utilised, $1*10^6$, $2*10^6$, $3*10^6$, and $4*10^6$ and exposed to a range of opioid receptor ligands and eotaxin in a

reduced assay volume. Subsequently, the levels of IL-8 were tested utilising the same ELISA protocol from previous experiments. IL-8 was chosen for the standardization experiments because it is the only cytokine that showed detectable levels in the previous experiment.

The IL-8 yields were higher than in the media extracted from migration assays, 76.6pg/ml ($1*10^6$ cells) relative to 4.99pg/ml. Increasing the number of cells from $1*10^6$ to $2*10^6$ and $3*10^6$ leads to an increase in the concentration of IL-8 from 76.6 to 81 and 97. However, there was an apparent decrease with $4*10^6$ cells (see figure 3.7).

The maximum capacity of the transwell migration assay exceeds the desired cell counts to produce a detectable cytokine rise, which limited the ability to repeat these experiments.

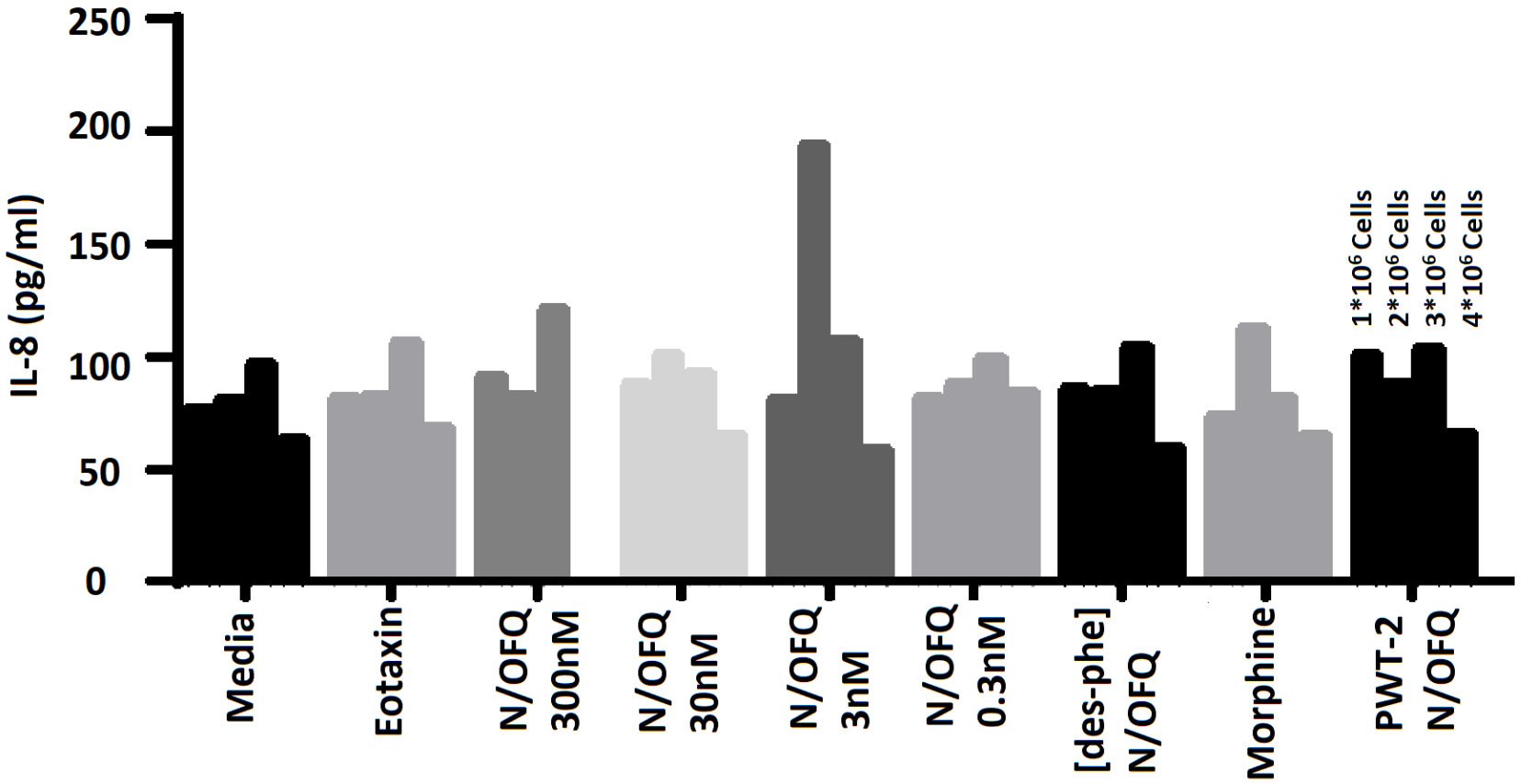

Figure 3.7: Effect of increasing cell count on IL-8 levels (pg/ml) obtained from ELISA in variable conditions using four cell count levels as labelled on the last cluster of columns, highest IL-8 levels were observed in $3*10^6$ cells.

3.5. Neutrophil to Lymphocyte ratio (NLR) *in vivo*

This thesis illustrated that targeting NOP receptors causes concentration dependent inhibition of cell migration *in vitro*; however, such migration inhibition was not linked previously to patient related outcomes *in vivo*. NLR can be used as a surrogate marker of acute/chronic inflammation imbalance where a higher ratio indicates higher neutrophils. If linked to a negative outcome, NLR could pave the way for clinical trials to test the theory that targeting NOP receptors could be a therapeutic target in acute inflammatory conditions.

The NLR data presented here have not been previously presented nor published by our research group in the context of cell migration and the inflammation link. The results of the study that was conducted to analyse the NLR included 61 healthy volunteers, 69 patients with clinical sepsis, and 37 patients undergoing cardiac surgery requiring cardio pulmonary bypass (CPB). The healthy volunteers were matched according to sex and age to the patients diagnosed with sepsis to minimise the effects of covariates. The ages and BMI in the database are illustrated in Figure 3.8.

The NLR value is affected by age, and it is significantly higher in advancing age [88]. Hence, the NLR data on the CPB patients were excluded from the analysis because of the significant difference in mean age in that group. However, the neutrophil numbers were compared to illustrate the acute inflammatory component and the link to circulating cytokines. Figure 3.9 shows the mean

NLR in the healthy volunteers, patients suffering from clinical sepsis, and the same patients upon recovery from clinical sepsis.

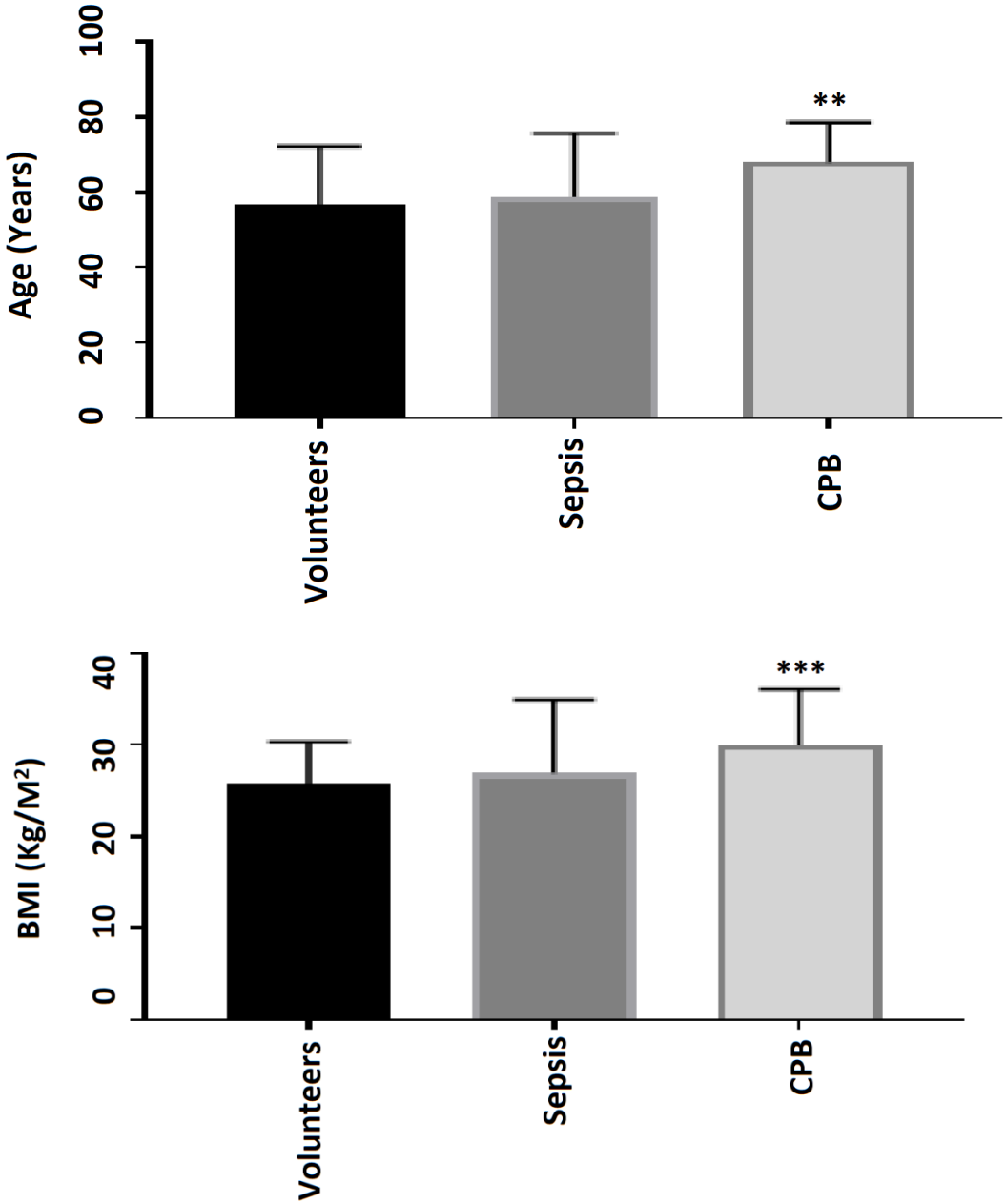

Figure 3.8: Age (mean in years) and BMI (mean in Kg/m^2) in healthy volunteers (56.80) (25.76), patients suffering from clinical sepsis (58.68) (26.94), and patients undergoing CPB surgery (68.0) (29.89) are illustrated in a mean and standard deviation bar which shows no differences between the volunteers and the septic patients. However, CPB patients had significantly higher mean age** and mean BMIs*** (p=0.01 and 0.001 respectively), which could alter the NLR.

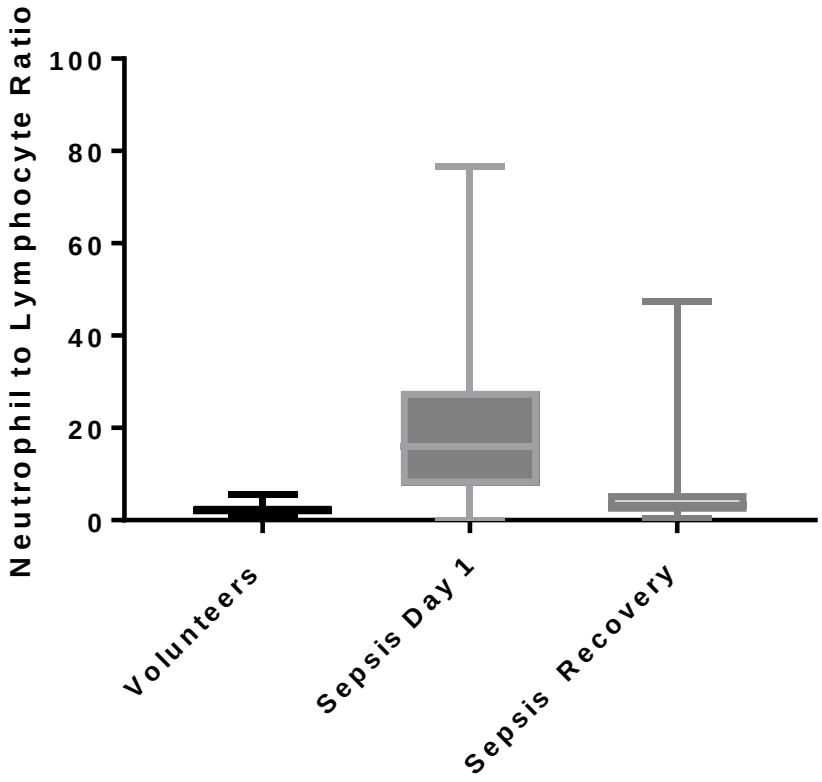

Figure 3.9: NLR results presented as mean, IQR, and minimum-maximum bars in healthy volunteers (n=61) (mean 2.251[SD 0.927]), patients suffering from clinical sepsis (n=69) (mean 19.43[SD 19.43]), and the same patients upon recovery from sepsis (mean 5.45[SD 8.41]). ($p<0.05$)

The number of circulating neutrophils was also noted in different patient groups. The time points illustrated in figure 3.10 show a significant rise in the neutrophil count in patients suffering from clinical sepsis, which decreased toward normal in recovery and after 3 h after CPB.

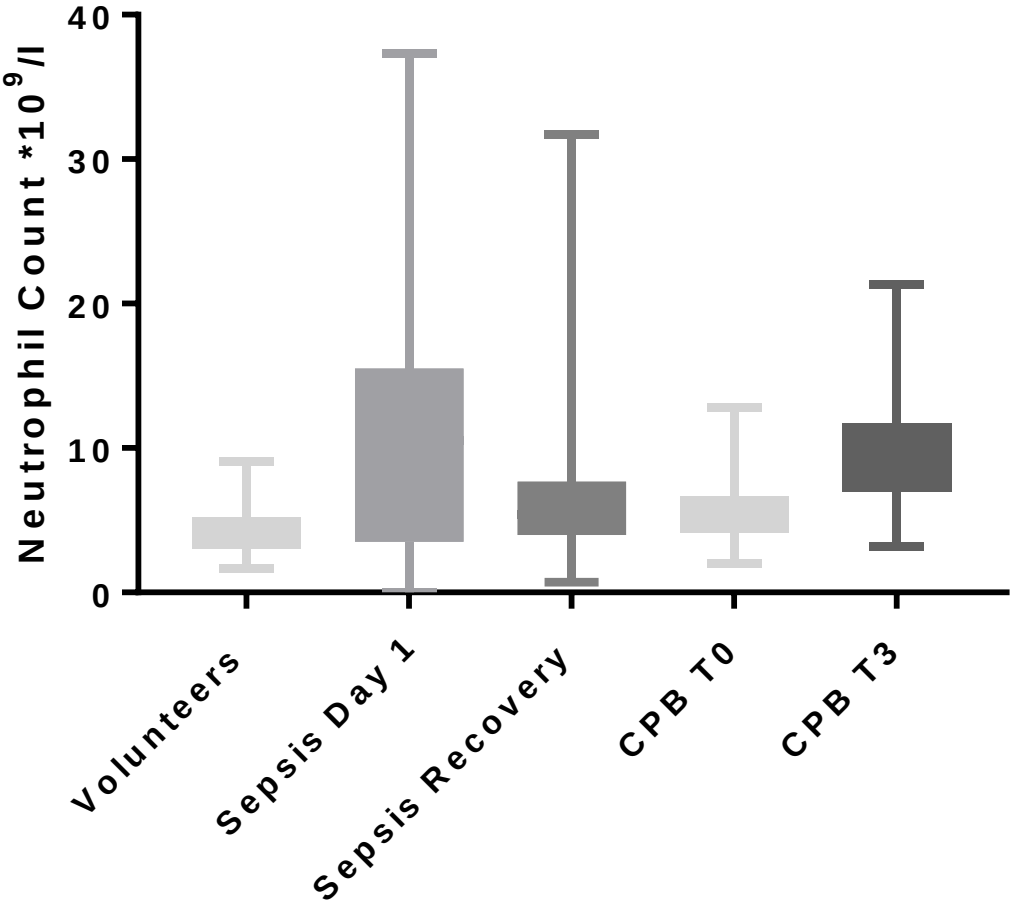

Figure 3.10: Neutrophil count (10^9/l) in healthy volunteers (mean 4.18, SD1.58), patients suffering from clinical sepsis on day one (mean 11.04, SD 8.30) and after clinical recovery (mean 6.44, SD 4.61), and patients undergoing CBS at time zero (mean 5.84, SD 2.56) and 3 h after the initiation of CPB (mean 9.60, SD 3.82). ($P<0.05$).

Circulating pro-inflammatory (IL-8 and TNF-α) and anti-inflammatory (IL-10) cytokine concentrations were measured in peripheral blood. The results revealed that an increase in peripheral neutrophils was linked to an increase in peripheral cytokine concentrations with a significant drop in cytokine levels in the recovery samples where the peripheral neutrophil count was notably lower, which is shown in figure 3.11.

No statistically significant differences were noted between the mean TNF-α log concentrations upon the diagnosis of clinical sepsis and after clinical recovery (mean ± SD 1.919 ± 0.084 and 1.686 ± 0.1587 respectively (P = 0.1594). However, IL-8 and IL-10 log concentrations at the same time points were significantly different (mean ± SD 2.277 ± 0.123 and 1.819 ± 0.154 [P = 0.027]) for IL-8, 2.24 ± 0.098 and 1.786 ± 0.195 (P = 0.024) for IL-10, respectively.

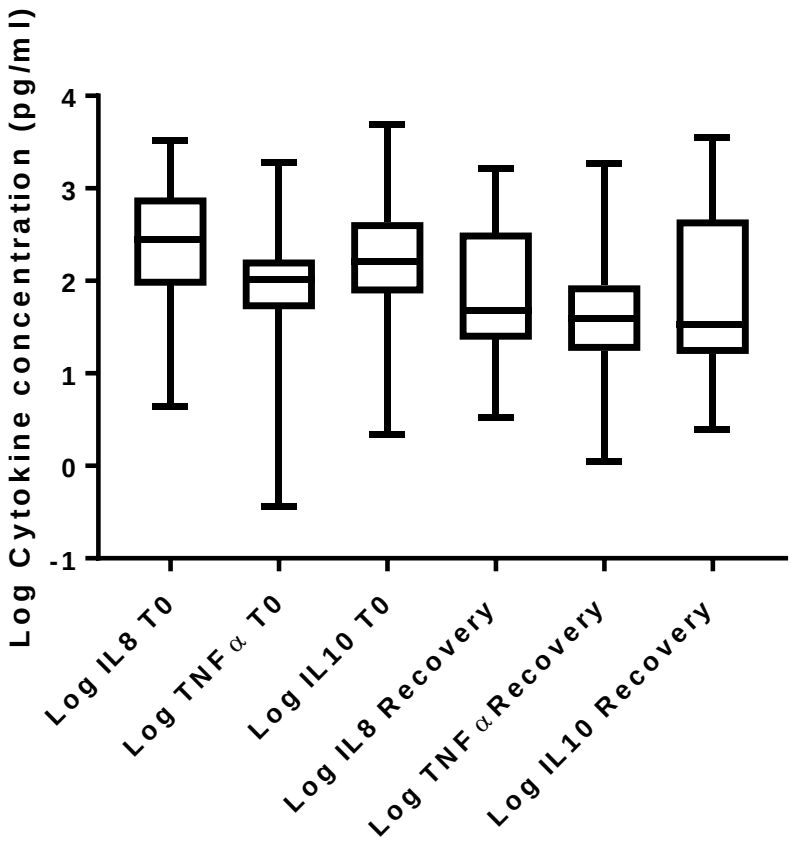

Figure 3.11: Log peripheral cytokines concentration (pg/ml) upon diagnosis of clinical sepsis and after recovery, which illustrate the difference between the mean of each cytokine at both time points. Mean log IL-8 2.27 and 1.81, Mean log TNFα 1.91 and 1.68, mean log IL-10 2.24 and 1.76 for diagnosis and recovery samples respectively that is significantly different ($p<0.05$).

4. Discussion

Epidemiological studies have demonstrated the increased incidence of opportunistic viral and bacterial infections among intravenous drug abusers compared to non-users, which could be linked to the immunomodulation resulting from opiate abuse [89]. It is important to understand the mechanisms of such immunomodulation to devise potential management strategies against those infections and to address the issue of immunomodulation associated with opioid use whether recreational or medicinal. Opioids are a diverse group of drugs that cause analgesia and many other host responses depending on the opiate receptor subtype and its endogenous ligand that is stimulated. Classical naloxone sensitive opiate receptors are classified as three types of G-protein coupled receptors that are usually referred to as MOP (mu, μ), KOP (k, kappa), and DOP (delta, δ). The activation of these classical opiate receptors causes centrally-mediated analgesia and adverse side effects depending on the degree of activation of the subtypes of the various opiate receptors. Regarding modulating immune function, the exact mechanism of opiate immunomodulation is still not fully understood. It has been a subject of intensive clinical research that has posited that it could be attributed to central and peripheral mechanisms [90]. The expression of classical opiate receptors on peripheral immune cells is widely theorised in the literature, and it is usually deemed to cause the immune-modulatory effect of opioids. However, the evidence is controversial. Moreover, it was found to be non-reproducible when individual populations of peripheral

immune cells were studied by our research group, which makes this theory unlikely [8 11 49]. However, because the non-classical naloxone-insensitive opiate receptor NOP (opiate receptor like – 1, ORL-1) is widely expressed in peripheral human blood cells, including immunocytes, these receptors are of special interest regarding inflammation and peripherally mediated immune modulation [49 63 65].

In understanding the immunomodulatory effects of classical and non-classical opiate receptor systems, it is important to address the controversial issue of opiate receptors expression on peripheral immune cells. The whole human blood RNA extracted from healthy volunteers contains the entire genetic fingerprint of all receptors that are expressed in peripheral human blood. The absence of the opiate receptor gene expression in peripheral whole blood would refute the theory that opiate immune modulation could be the result of direct opiate interaction with peripheral immune cells.

Quantitative polymerase chain reaction experiments (qPCR) were conducted to determine the differences in the mRNA gene expression of MOP, KOP, DOP, and NOP. The quantification of gene expression was made by a comparison of the endogenous control genes GAPDH and B2M.

The proximity of the cycle threshold of the gene of interest to the endogenous control gene reflects the degree of gene expression in the sample. Commercially available TaqMan® probes are mononucleotides that are

specifically designed to bind to a specific target sequence, which results in the release of the florescence and quencher upon annealing to the target sequence. Quantitative PCR reactions are run in 40 cycles at varying temperatures, which are optimal for the various phases of the enzymatic reaction, and which allow extension and amplification that result in the annealing process that leads to the detection of the Ct value.

In this study, the methodology employed consisted of two-step, real-time PCR reactions. The reverse transcription reaction was performed first to synthesise the cDNA before running the qPCR step. This methodology has the advantage of producing stable cDNA that can be used in repeated experiments with samples of the gene of interest, and the reference genes are amplified together. However, the trade-off is that multiple pipetting steps are required, which results in the increased potential for DNA contamination and the need for better optimisation. The reverse transcription methodology involved using random primers that were six to nine bases long and annealed at multiple spots on the RNA transcript not only on the mRNA portion. The results showed good amplification in the samples with low starting genetic material. However, this methodology may result in the synthesis of truncated DNA or excessive cDNA diluting mRNA signals.

PCR probe primers should ideally span the mRNA exon-exon boundary part of the genetic material—not the exon-intron part— and therefore avoid the amplification of genomic DNA contaminants and ensure that the PCR

amplification is only caused by the cDNA sequence. It is sometimes difficult for commercially available PCR primer probes to be designed in this way because the target gene sequence is neither unique nor possesses an identifiable exon-exon junction, which causes false positive results. As previously discussed, in the literature some false positive results have inferred DOP receptor expression in peripheral human blood. Although RNA samples should be treated with RNase-free DNAse to eliminate genomic DNA contamination, this enzymatic reaction has a limited capacity and depends on the initial degree of genomic DNA contamination. Genomic DNA contamination can be verified by running parallel negative reverse transcriptase samples that contain all reagents and samples aside from the RT enzyme; any amplification is then attributed to the genomic DNA that was observed in the DOP qPCR samples.

Our qPCR data revealed that mRNA for the MOP and KOP classical opioid receptors were not detectable in peripheral human blood. The results showed some false positive expression of DOP receptors, which was studied further using gel electrophoresis. Non-classical NOP receptor mRNA was expressed in peripheral human blood with dynamic changes in this expression in response to the *in vitro* sepsis model (LPS and PepG) after 2 h of incubation.

The false positive signals were due to the exon-exon limitation discussed above, and we therefore conducted a series of experiments using gel electrophoresis using specifically designed probes at various annealing temperatures in the thermocycler to achieve the optimal programme enabling

the amplification of mRNA signals but not the genomic DNA signals. The results of the gel electrophoresis showed that the DOP receptors were not expressed in peripheral human blood whole RNA, and no amplification occurred in the negative control samples. A positive control assay was run in parallel utilising Chinese hamster ovary cells (CHO) that were transfected with and hence positive for the DOP gene; there was no DOP expression in the respective negative control samples using the same methodology.

The *in vitro* sepsis model was applied by incubating the cells with lipopolysaccharide from Escherichia coli and peptidoglycan from Staphylococcus aureus to mimic mixed pathogen sepsis and the acute inflammatory process. The samples were incubated in physiological conditions (temperature 37 °C and 5.0 kPa CO_2 enriched air) for 24 h, after which the genetic material was processed using whole blood RNA extraction. The results revealed statistically significant differences in NOP mRNA gene expression in the *in vitro* sepsis model. Morphine and fentanyl in the absence of 'sepsis' caused no change in the expression of NOP mRNA.

It was then important to determine whether the activation of NOP receptors on immune cells would result in any functional alteration in white blood cells. A series of transmembrane/transwell migration assays were conducted to mimic tissue migration and the response of inflammatory markers *in vivo*. The transwell migration assay mimics the trans-epithelial migration of activated white blood cells in response to local and systemic inflammation as the host

tries to deliver high concentrations of cytokines to the affected organs. PMNs from healthy volunteers were incubated for 2 h in physiological conditions after loading them on filter inserts that were mixed with varying concentrations of NOP and MOP agonists (N/OFQ, morphine, PWT2 N/OFQ, and [desphe^{1}] N/OFQ). The bottom chamber of the migration assay tray was mixed with a chemoattractant agent that is utilised to promote cell migration. The results showed concentration-dependent inhibition of PMNs cell migration in response to N/OFQ. No statistically significant differences were observed in PMN cell migration with a single concentration (300 nM) of morphine (MOP agonist) or the structurally inactive [desphe^{1}] N/OFQ. However, PWT2 N/OFQ caused statistically significant differences in cell migration. PWT2 N/OFQ caused the most significant inhibition to PMNs migration, which was expected because of its superior potency and longer duration of action (as quoted in the literature) compared to other NOP agonists.

Eosinophils also play a major part in the host immune function, namely fighting parasitic infections, allergic reactions, and inflammatory responses. Human eosinophils express the N/OFQ precursor (preproN/OFQ) mRNA as described by our research group previously [87]. It is exceedingly difficult for human eosinophils to be studied *in vitro* because of their small numbers and their inability to survive outside the circulatory system. Hence, we studied eosinophil like -1 cell line [EOL-1] and their transwell migration responses in similar conditions to the reagents used in the PMN migration assays. The results also

showed significant, concentration-dependent EOL-1 cell migration inhibition in which N/OFQ. PWT2-N/OFQ showed similarly higher inhibitions of EOL-1 cell migration. In contrast, no inhibition of EOL-1 migration with morphine or the structurally inactive [desphe1] N/OFQ was noted.

The supernatant obtained from the cell migration experiments was collected to determine changes in the cytokine concentrations (IL-10, IL-8, and TNF-α) associated with the cell migration and to compare them with the results obtained from patients diagnosed with sepsis or undergoing surgical interventions requiring CPB, which constituted infective and sterile models of inflammation, respectively. The results of the interleukin assay did not demonstrate any trend to match the changes in cell migration because the interleukin concentrations were low and often below detection limits. The three interleukins were selected because they represent the pro- and anti-inflammatory mediators that are usually found in response to local and systemic inflammatory processes. Another experiment was conducted to study the effects of cell numbers on the concentration of inflammatory mediators. As expected, in different numbers of cells, a linear relationship was shown between the number of cells and the interleukin concentration. The number of cells causing the most IL-8 release was $3*10^6$ after which, an increase in cell numbers caused a drop in IL-8 concentration.

Because the results showed a significant inhibition of PMN cell migration after brief incubation with the N/OFQ agonist, an analysis was conducted on a data

set obtained *in vivo* from patients suffering from clinical sepsis and patients undergoing CPB. It was observed that the absolute number of circulating neutrophils was significantly higher after 3 h of CPB compared to the baseline. The number was also higher in patients at the diagnosis of sepsis compared to that in blood samples collected upon their recovery. It was also observed that the change in the interleukin concentrations in peripheral blood drawn from those patients mirrored the change in the number of neutrophils, which indicates that NOP agonists could be a therapeutic option in cases of systemic inflammation.

Previous studies have suggested that the NLR is a valuable marker of inflammatory balance and that a high ratio may indicate the severity of acute inflammation, signifying a worsened prognosis [74 91]. Our results showed that NLR was significantly higher in patients diagnosed with sepsis compared to the healthy volunteers. This result could be utilised to determine which patients would benefit from NOP agonists administration, thus preventing an increased number of circulating neutrophils from migrating to tissues and causing changes in inflammation. NLR mirrored the change in circulating PMN and cytokines, which means that higher NLR can be used as a surrogate for higher circulating cytokine levels; however, special attention should be paid to covariates that can affect NLR levels irrespective of acute disease processes.

5. Summary

In this thesis, the peripheral direct immunomodulatory effects of opioids were examined. The data presented refutes the theory of direct classical opioid interactions with immune cells. In contrast, the NOP receptor mRNA was present, and the activation of NOP caused a concentration dependent inhibition of immune cell migration which could be linked to survival benefits in patients suffering from conditions causing increasing numbers of circulating PMN, higher NLR, and elevated pro-inflammatory cytokines.

NOP and N/OFQ remains an active area of research, a Google Scholar search for Nociceptin as keyword reported 1,180 publications since 2018 (May 2019). Future direction of research could be to study further the effect of NOP agonists/antagonists on the inflammatory/immune system *in vivo* to achieve survival benefits. The NLR is another active area of research with 17,900 publications since 2018 (Google Scholar search May 2019). Combining NLR and Cytokine imbalance results could be further studied to pave the way for NOP receptor targeting (agonist/antagonist) tailored for specific NLR in varied disease processes where multisystem targeting is deemed appropriate.

6. Appendices

Appendix 1:

Whole blood RNA concentrations and purity markers in all samples

Sample	Incubating condition	RNA content (µg/µl)	A260/A280
1	Plain (Control)	0.1001	1.64
	Culture media	0.1726	1.55
	Media + LPS	0.0903	1.58
	Media + Fentanyl	0.0821	1.68
	Media + Morphine	0.1121	1.64
	Media + LPS + Fentanyl	0.056	1.66
	Media + LPS + Morphine	0.0355	1.81
2	Plain (Control)	0.129	1.74
	Culture media	0.109	1.84
	Media + LPS	0.119	1.71
	Media + Fentanyl	0.04	1.93
	Media + Morphine	0.131	1.76
	Media + LPS + Fentanyl	0.156	1.70
	Media + LPS + Morphine	0.182	1.93
3	Plain (Control)	0.172	1.7
	Culture media	0.19	1.58
	Media + LPS	0.145	1.61
	Media + Fentanyl	0.097	1.82
	Media + Morphine	0.1229	1.67
	Media + LPS + Fentanyl	0.06	1.65
	Media + LPS + Morphine	0.11	1.62
4	Plain (Control)	0.127	1.70
	Culture media	0.15	1.68
	Media + LPS	0.06	1.84
	Media + Fentanyl	0.1001	1.79

	Media + Morphine	0.105	1.8
	Media + LPS + Fentanyl	0.072	1.78
	Media + LPS + Morphine	0.066	1.82
5	Plain (Control)	0.1255	1.90
	Culture media	0.14	1.84
	Media + LPS	0.084	1.80
	Media + Fentanyl	0.176	1.83
	Media + Morphine	0.145	1.89
	Media + LPS + Fentanyl	0.096	1.82
	Media + LPS + Morphine	0.079	1.84
6	Plain (Control)	0.1001	1.64
	Culture media	0.1726	1.55
	Media + LPS	0.0903	1.58
	Media + Fentanyl	0.0821	1.68
	Media + Morphine	0.1121	1.64
	Media + LPS + Fentanyl	0.056	1.66
	Media + LPS + Morphine	0.0355	1.81
7	Plain (Control)	0.129	1.74
	Culture media	0.109	1.84
	Media + LPS	0.119	1.71
	Media + Fentanyl	0.04	1.93
	Media + Morphine	0.131	1.76
	Media + LPS + Fentanyl	0.156	1.70
	Media + LPS + Morphine	0.182	1.93
8	Plain (Control)	0.172	1.7
	Culture media	0.19	1.58
	Media + LPS	0.145	1.61
	Media + Fentanyl	0.097	1.82
	Media + Morphine	0.1229	1.67
	Media + LPS + Fentanyl	0.06	1.65
	Media + LPS + Morphine	0.11	1.62
9	Plain (Control)	0.127	1.70

	Culture media	0.15	1.68
	Media + LPS	0.06	1.84
	Media + Fentanyl	0.1001	1.79
	Media + Morphine	0.105	1.8
	Media + LPS + Fentanyl	0.072	1.78
	Media + LPS + Morphine	0.066	1.82
10	Plain (Control)	0.1255	1.90
	Culture media	0.14	1.84
	Media + LPS	0.084	1.80
	Media + Fentanyl	0.176	1.83
	Media + Morphine	0.145	1.89
	Media + LPS + Fentanyl	0.096	1.82
	Media + LPS + Morphine	0.079	1.84

Appendix 2

Assay IDs and sequences for the different TaqMan probes and primer pairs used throughout the study

Target	Type	Sequence/ Assay ID (supplier)	Abbreviation
KOP (OPRK1)	TaqMan assay	Hs00175127_m1 (Life Technologies)	TM-KOP1
MOP (OPRM1)	TaqMan assay	Hs01053957_m1 (Life Technologies)	TM-MOP1
NOP (OPRL1)	TaqMan assay	Hs00173471_m1 (Life Technologies)	TM-NOP1
DOP (OPRD1)	TaqMan assay	Hs00538331_m1 (Life Technologies)	TM-DOP1
DOP (OPRD1)	TaqMan assay	Hs00357182_mh (Life Technologies)	TM-DOP2
DOP (OPRD1)	QuantiFast Probe Assay	Hs_OPRD1_QF_1 (Qiagen)	TM-DOP3
DOP (OPRD1)	QuantiTect Primer Assay (SYBR Green)	Hs_OPRD1_1_SG (Qiagen)	SBG-DOP1
DOP (OPRD1)	KiCqStart™ Primers H_OPRD1_1	FH1(Sigma) RH1(Sigma)	SBG-DOP2
DOP (OPRD1) set4	Oligonucleotide primer pairs	Forward GGCATCGTCCGGTACACTA Reverse GTCGAGGAAAGCGTAGAGCA	STD-DOP-1

Appendix 3

ELISA plate 1 data

Plate 1	IL10 (pg/ml)	IL8 (pg/ml)	TNF (pg/ml)
a Plain	24.92642526	0.742796759	Not Determined
b Plain	Not Determined	1.67694956	Not Determined
c Plain	0.138937666	18.05759881	6.206832
d Plain	Not Determined	Not Determined	19.39188
e Plain	Not Determined	6.700234458	13.41838
EOL-1 17 Plain	3.507841809	7.70708236	1.997469
EOL-1 18 Plain	Not Determined	5.782158602	20.80224
EOL1 Plain	Not Determined	5.387781814	13.01248
a FMLP	26.72144872	12.90149386	0.003176
b FMLP	Not Determined	2.95753573	Not Determined
c FMLP	Not Determined	8.355645382	Not Determined
d FMLP		Not Determined	Not Determined
e FMLP	6.895567276	8.21977633	Not Determined
EOL-1 17 Eotaxin	10.91566	6.868153613	9.340497
EOL-1 18 Eotaxin	4.262849499	9.34006718	Not Determined
EOLeut	Not Determined	12.03178326	Not Determined
a 300nM	6.880500013	5.966230116	3.975301
b 300nM	Not Determined	7.357834246	15.52888
c 300nM	Not Determined	7.263233397	Not Determined
d 300nM	0.108200682	Not Determined	30.75459
e 300nM	Not Determined	11.36920245	Not Determined
EOL-1 17 30nM	Not Determined	10.07929137	Not Determined
EOL-1 18 30nM	Not Determined	10.4308572	Not Determined
Eolnofq 300	10.40974282	4.599811133	39.25365
a 30nM	83.59301703	10.02028329	15.52673
b 30nM	4.589760246	2.06747373	Not Determined
c 30nM	Not Determined	4.583845804	Not Determined
d 30nM	Not Determined	Not Determined	Not Determined

e 30nM	Not Determined	5.522554367	Not Determined
EOL-1 17 0.3nM	Not Determined	7.865023055	Not Determined
EOL-1 18 0.3nM	Not Determined	17.81804289	Not Determined
Eolnofq 30	Not Determined	6.318587442	Not Determined
a 3nM	Not Determined	4.44877	Not Determined
b 3nM	Not Determined	5.159647	0.908071
c 3nM	Not Determined	4.094586	8.510009
d 3nM	Not Determined	Not Determined	Not Determined
e 3nM	12.92736	1.487271	Not Determined
EOL-1 17 0.003nM	Not Determined	5.722687	Not Determined
EOL-1 18 0.003nM	Not Determined	7.239524	Not Determined
Eolnofq 3	Not Determined	6.049368	5.13074
a 0.3nM	Not Determined	4.389045	40.02211
b 0.3nM	Not Determined	3.572646	24.32596
c 0.3nM	Not Determined	7.367837	15.36243
d 0.3nM	7.47257	Not Determined	22.86652
e 0.3nM	Not Determined	3.482892	Not Determined
EOL-1 17 0.003nM	Not Determined	4.585852	Not Determined
EOL-1 18 0.003nM	Not Determined	6.011876	Not Determined
Eolnofq 3	Not Determined	4.49842	Not Determined
a 0.03nM	23.70992	8.667024	35.96168
b 0.03nM	Not Determined	1.920658	7.859049
c 0.03nM	0.358468	19.71469	3.044739
d 0.03nM	Not Determined	Not Determined	10.23458
e 0.03nM	5.605186	8.578276	18.49056
Eolnofq 0.03	Not Determined	Not Determined	Not Determined
	Not Determined	Not Determined	Not Determined
	Not Determined	4.605746	3.100202

a 0.003nM	Not Determined	13.19715	Not Determined
b 0.003nM	0.230962	5.92475	5.918069
c 0.003nM	Not Determined	8.621642	21.44371
d 0.003nM	Not Determined	Not Determined	13.79653
e 0.003nM	3.593437	6.370008	13.03707
	Not Determined	Not Determined	Not Determined
	Not Determined	Not Determined	Not Determined
Eolnofq 0.003	Not Determined	4.871918	Not Determined

Samples in bold had 50ul of sample (not 100ul). Interpolated value doubled.

Appendix 4

ELISA IL-10, IL-8, and TNF-α level

Series 1 (5 volunteers assayed)								
Condition	Plain	FMLP	N/OFQ (300nM)	N/OFQ (30nM)	N/OFQ (3nM)	N/OFQ (0.3nM)	N/OFQ (0.03nM)	N/OFQ (0.003nM)
IL10 (pg/ml) (Samples detected)	24.93, 0.14 (2)	26.72,6.90 (2)	6.88, 0.11 (2)	83.59,4.59 (2)	12.93 (1)	7.473 (1)	23.71, 0.36, 5.61 (3)	0.23, 3.59 (2)
IL8 (pg/ml) (Samples detected)	6.79±3.98 (4)	8.10±2.03 (4)	7.99±1.17 (4)	5.55±1.66 (4)	3.80±0.80 (4)	4.70±0.91 (4)	9.72±3.69 (4)	8.53±1.66 (4)
TNF-α	6.21, 19.39, 3.42 (3)	0.003 (1)	3.98, 15.53, 30.75 (3)	15.53 (1)	0.91, 8.51 (2)	25.64±5.18 (4)	15.12±5.78 (5)	13.55±3.17 (4)

Series 2 (7 volunteers assayed)								
Condition	Plain	FMLP	PWT	DESPHE	Morph			
IL10 (pg/ml) (Samples detected)	10.79±2.36 (4)	9.91 (1)	10.24 (1)	6.11, 17.99 (2)	11.24, 7.82 (2)			
IL8 (pg/ml) (Samples detected)	4.99±0.81 (5)	11.22±2.37 (7)	11.4±2.34 (7)	9.77±1.93 (7)	8.73±2.28 (7)			
TNF-α	No detection	12.02, 10.42 (2)	No detection	29.27 (1)	8.82, 9.81 (2)			

IL-10, IL-8, and TNF-α concentration detected utilizing ELISA listed as mean values and standard deviation as illustrated in Figure 3.5.

Appendix 5

Blood collection volunteer consent form

Participant name & DOB

CONSENT FORM

Study Title: Collection of blood sample for Anaesthesia, ICU, Pain and Cardiovascular related research (volunteer).

Investigators: Prof DG Lambert, Dr S Scott, Dr M Al-Hashimi, Dr J Thompson, Dr J McDonald, Mrs S Bowrey, Miss N Rich, Miss R Orr.

Participant to initialise boxes

1. I confirm that I have read and understood the participant information sheet dated 05 Oct 2011, v.2, and have had the opportunity to ask questions. ☐
2. I understand that any blood samples are a gift and that I will not benefit from any intellectual property that results from their use. ☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time. ☐
4. I hereby consent to donate blood samples (up to 30 ml), and to have these samples stored in the laboratory only until processing of the samples for stress hormone levels, after which they will be destroyed. ☐
5. I understand that relevant sections of my research notes may be looked at by responsible individuals from the University of Leicester to make sure that the research is being conducted properly. I give permission for these people to access my research records ☐
6. I agree to take part in the above study. ☐

Name of Participant _____

Signature of Participant _____ Date _____

Name of Researcher _____

Signature of Researcher _____ Date _____

Original form to be held in University Department, copy to Participant

Appendix 6

Papers published in connection to this thesis

British Journal of Anaesthesia **111** (1): 80–8 (2013)
doi:10.1093/bja/aet153

BJA

Opioids and immune modulation: more questions than answers

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Editor's key points

- There is increasing evidence for immunemodulatory effects of opioids.
- The precise sites of immune modulation are controversial and unclear.
- Urgent translational research is needed, as increasing numbers of people are on long-term opioids.

Summary. Opioid addicts are more likely to present with infections suggesting opioids are immune modulators. The potential sites/mechanism(s) for this modulation are controversial and on close inspection not well supported by the current literature. It has long been assumed that opioid-induced immune modulation occurs via a combination of direct actions on the immune cell itself, via the hypothalamic-pituitary-adrenal (HPA) axis, or both. Opioid receptors are classified as MOP (μ, mu), DOP (δ, delta), and KOP (κ, kappa)—classical naloxone sensitive receptors—or NOP (the receptor for nociceptin/orphanin FQ), which is naloxone insensitive. Opioids currently used in clinical practice predominantly target the MOP receptor. There do not appear to be classical opioid receptors present on immune cells. The evidence for HPA activation is also poor and shows some species dependence. Most opioids used clinically or as drugs of abuse do not target the NOP receptor. Other possible target sites for immune modulation include the sympathetic nervous system and central sites. We are currently unable to accurately define the cellular target for immune modulation and suggest further investigation is required. Based on the differences observed when comparing studies in laboratory animals and those performed in humans we suggest that further studies in the clinical setting are needed.

Keywords: HPA axis; immunomodulation; opioid receptors

The link between opioids and an alteration in host immune function is often referred to in the literature. The increased incidence of various local and systemic infections in intravenous drug users led to the conclusion that the causative link between i.v. drug abuse and infections could not be simply explained by the injection process being the route of infection, but that the opiates themselves were acting to modulate immune function. The increased incidence and severity of infections among opiate abusers was documented in the early 19th century with similar observations appearing in the literature as early as the mid 1500s when Professor Fallopious of Pisa recorded the death of a prisoner probably from malaria after experimental opium administration.[1] In 1950, Hussey and Katz published a landmark paper describing the growing list of infections associated with narcotic addiction.[2] As then, extensive research has been directed at investigating the immunomodulatory effects of opioids and mechanisms and the clinical significance of such effects.

Opioids can affect innate and adaptive immune function and their effects are summarized in Table 1.[3–28] In this review, we explore the possible mechanisms of immune modulation caused by opioids in the current literature. The evidence discussed in this article should be taken in the context of the diversity and complexity of the subject, immune function and the group of patients/cells/species in which those studies were conducted.

Immune regulation

The immune system is intricate and diverse; its main function is fighting infections in the host environment. Regulation of the immune system is very important and, when imbalanced, may result in either increased susceptibility to infections or autoimmune disease status. Immune regulation refers to the interactions between immune cells and mediators.[29] Immune regulation can be affected by any stimuli that can affect immune cell haemopoiesis, immune system component development, and antigen-antibody feedback mechanisms. Other factors that can affect immune regulation are neuroendocrine control, mental and physical stress, and genetic predisposition. There are numerous receptors on different populations of immune cells that interact with corticosteroids, insulin, growth hormones, β-adrenergic agonists, acetylcholine, and many others that may potentially affect immune function.[30]

A simplified representation of the immune cell generation process and the antigen-antibody immunity pathway is shown in Figure 1. The term 'immunemodulatory' refers to any endogenous or exogenous stimulus that can potentially

Table 1 Opioid effects on immunity. IL, interleukin; TNF-α, Tumour Necrosis Factor-Alpha; IFN-γ, Interferon-gamma; PBMC, Peripheral Blood Mononuclear Cell; NK, Natural Killer; LPS, Lipopolysaccharides; TFG-β, Transforming Growth Factor Beta; NF-κb, Nuclear Factor kappa Beta; AP-1, Activator Protein 1; NFAT, Nuclear factor of activated T-cells; Fc, fragment crystallisable region; HPA, Hypothalamic Pituitary Adrenal axis; ACTH, Adrenocorticotropic hormone; CRH, Corticotropin releasing hormone; LH, Luteinizing hormone

Adaptive immunity
↓ Splenic and thymic weight (rodents)[4–6]
↓ T cell viability and proliferative response
↓ T-helper cell function
↓ CD4/CD8 population *in vivo*
↓ IL1β, IL-2, TNF-α and IFN-γ (mouse splenocytes)
↓ Th1/Th2 ratio of T-helper cell population (PBMCs)[7]
↓ NK cell activity[8]
↓ Primary antibody response (B cells)[9]
↓ B cells mitogenic response to bacterial LPS[4 10 11]
↓ Macrophage activity[9]
↑ TFG-β1 and IL-10 (anti-inflammatory cytokines)[12]
↑ T cell apoptosis (NF-k β and AP-1/NFAT pathways)[13]
Inhibition of CD3/28 mAb induced IL-2 transcripts[14]
Innate immunity
↓ Number of macrophages available to fight infections[15]
↓ Leucocyte migration[16]
↓ Peritoneal macrophages phagocytosis
↓ Respiratory burst activity and chemotaxis[17]
Inhibition of Fc γ receptor mediated phagocytosis[18]
↓ Superoxide production from neutrophils and macrophages[19–21]
Alteration of IL-8 induced neutrophil chemotaxis[22 23]
↓ Neutrophil cytokines involved in wound healing[24]
↑ Apoptosis of macrophages impairing host defence barrier[25]
↓ Leucocytes endothelial adhesion (↓ intracellular adhesion molecules expression)[26 27]
Neuroendocrine system
↑ Growth hormone, prolactin, and thyroid stimulating hormone secretion in humans[28]
May affect the function of the HPA axis (ACTH and CRH) with risk of adrenal insufficiency[28]
↓ Sex hormones [LH and testosterone (hypogonadism)], oxytocin, and estradiol[28]

alter immune function by effects on the generation, function, and maturation of immune cells.

Classification of opioid receptors

Opioid receptors were first demonstrated in neural tissue in 1973 by Pert and Snyder[31] followed by the characterization of a range of endogenous opioid ligands.[32 33] Interestingly, the endomorphins were only identified as endogenous ligands for the MOP receptor in 1997.[34] Despite the long history describing the existence of endogenous opioid-binding sites, opioid receptors were only formally identified in 1992 by the pioneering work of Kieffer and colleagues[35] and Evans and colleagues[36], the crystal structures of all members of the opioid family were published in 2012.[37–40] There have been several attempts to classify the opioid receptors. Table 2 lists the current classification system for opioid receptors: MOP (μ, mu), DOP (δ, delta), and KOP (κ, kappa) for classical naloxone-sensitive receptors, and NOP for the non-classical nociceptin/orphanin FQ (N/OFQ) receptor, which is naloxone-insensitive. All of the classical opioid receptors are capable of producing analgesia along with variable side-effects including respiratory depression, tolerance, dependence, and immunosuppression.[41] Activation of the NOP receptor with its endogenous peptide ligand N/OFQ produces analgesia spinally and hyperalgesia/anti-opioid actions supraspinally. Unlike classical opioids, there is no respiratory depression and little evidence for tolerance/dependence with NOP receptor activation. NOP receptors are known to be located on immune cells.[42] In the literature, there are numerous reports of subtypes of the classical receptors (MOP, KOP, and DOP) but data from knockout experiments, where single receptor gene knockouts result in a complete loss of function, indicate that these proposed subtypes are unlikely to represent structurally distinct receptor proteins.[41] In addition, there is good evidence to support the simultaneous targeting of multiple opioid receptors to improve analgesia and adverse effect profiles.[43] Throughout the remainder of this article, we will use the MOP, DOP, KOP, and NOP terminology but in describing some of the older studies, other terminology (as in Table 2) will be used and qualified.

Some opioids cause more immunomodulation than others

Different opioids affect immune function differently depending on drug factors, host factors, and the duration of exposure.[44] A recent publication from the British Pain Society[45] states that 'patients must be aware of uncertainty regarding the long-term effects of opioids, particularly in relation to endocrine and immune function'. The practice guide also acknowledges the immune modulatory effects of opioids but states that buprenorphine has no impact on immune function.[45] Table 3[44 46–49] summarizes the immune modulatory effects of opioids in animal studies; these may not reflect responses in humans where the duration of exposure to opioids changes the observed degree of immune modulation. Some data suggest that the immune modulatory effects of different opioids depend more on their molecular structure than their interaction with the MOP receptor,[49] which might imply a non-opioid receptor site of action. However, in MOP knockout animals, no immune modulation is seen.[8 50 51] Clearly, inter-species variability is an important issue in studies of immune modulation (see below).

Potential sites of immunomodulation

The immune modulatory effects of opioids (Fig. 2) are often linked to central neuro-endocrine/neuro-paracrine and peripheral mechanisms; it has often been suggested that peripheral actions are mediated by MOP receptors on immunocytes,

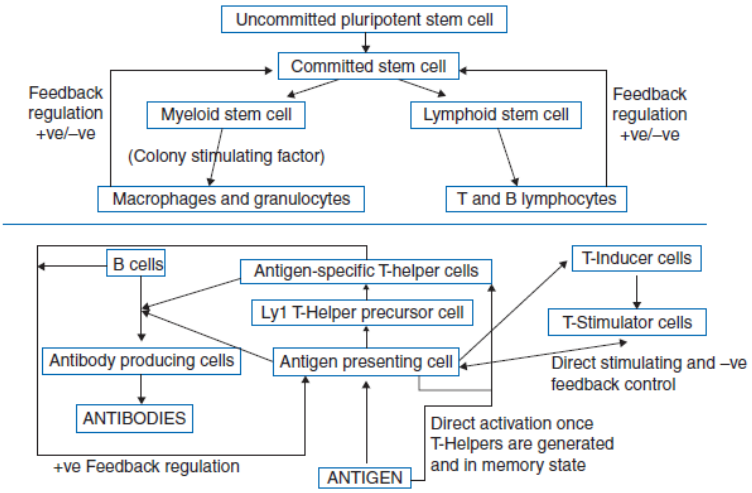

Fig 1 Immune-regulation. The top half illustrates the process of immune cell generation from stem cells into individual cell populations which then exert feedback control onto stem cell maturation. The bottom half illustrates antigen-antibody immune function. Antigens will be bound by antigen presenting cells (APC like macrophages) which in turn will activate T-helper precursor cells and end up forming an antigen specific T-helper cell that once formed can be directly activated by the antigen and the APC. These antigen specific T cells help B cells (with some help from the APC) to form antibodies by antibody producing cells, a process that exerts a feedback control mechanism on the APC. Another function of the APC is to activate the T inducer and T stimulator cells, a process that has feedback loop control as well affecting the function of the APC.

Table 2 Opioid receptor classification. i.c.v., intracerebroventricular. Buprenorphine has activity at NOP. Endogenous ligand: *endomorphin; †enkephalin; ‡dynorphin; ¶Nociceptin/orphanin FQ (N/OFQ)

Current name	Other names	Main effects	Clinical ligand
Classical members and naloxone sensitive			
MOP	μ, mu, MOR	Analgesia, respiratory depression, tolerance, immunosuppression	Almost all clinical opioids; morphine fentanyl*
DOP	δ, delta, DOR	Analgesia	None†
KOP	κ, kappa, KOR	Analgesia, diuresis	None‡
Non-classical and naloxone insensitive			
NOP	ORL-1	Analgesia spinal Hyperalgesia-i.c.v., anti opioid	None¶

though their presence on immune cells is controversial. MOP knockout mice showed no immune modulation effects after central administration of opioids suggesting that the central immune modulatory effect of opioids is mediated by MOP receptors.[8 50 51] The importance of centrally mediated mechanisms is supported further by the observation that opioids that cross the blood–brain barrier exert more immune modulatory effects than opioids that do not cross the blood–brain barrier; the latter only cause immune modulation when injected directly into the ventricular system of the brain.[52 53]

Although the cardiovascular effects of opioids are largely attributed to decreasing central sympathetic nervous system outflow, opioids can also cause sympathetic nervous activation that may suppress the proliferation and function of some immune cell populations and primary and secondary lymphoid tissues.[54 55] The central immune modulatory effect of opioids has also been linked to the HPA axis (see section below). Many authors repeat the assertion that the peripherally-mediated immune modulatory effects of opioids are mediated by interactions with opioid receptors on immunocytes,[56] but the evidence for this is not convincing.

Table 3 Degree of immune modulation with different opioids in animal studies.[44 46–49] *Buprenorphine was shown to have the least (almost no) immune modulatory effect

Strong immune modulation	Weaker immune modulation
Codeine	Buprenorphine*
Methadone	Hydromorphone
Morphine	Oxycodone
Remifentanil	Tramadol
Fentanyl	Hydrocodone
	Oxycodone

The immunocytes controversy

Several studies have suggested that MOP receptors are expressed on peripheral blood mononuclear cells (PBMCs).[57–62] The presence of opioid receptors in peripheral blood and immune cells was evaluated by our group[63 64] in a series of experiments using competitive-binding assays, fluorescent/immunofluorescent staining, real-time polymerase chain reaction (RT-PCR) and radioligand binding. Despite using multiple validated methodologies, we could not detect MOP, KOP, or DOP receptors or mRNA transcripts on PBMCs from venous blood in contrast to previous reports. In a very recent study, we used RT-PCR to analyse whole blood (containing the full immunocyte component) for MOP, DOP, KOP, and NOP messenger RNA (mRNA) transcripts. Consistent with our previous work, we failed to detect mRNA for classical (MOP, DOP, and KOP) receptors; however, mRNA transcripts for NOP were detected.[65] The absence of mRNA for the receptor suggests a lack of a functional receptor. Limitations in previous RT-PCR studies that detected the presence of opioid receptors on immunocytes could be attributed to the designs of the assay used and its inability to differentiate genomic DNA from cDNA.[64] Moreover, poor specificity of antibodies used in the detection of opioid receptors is also an important issue.[64] We therefore suggest that data indicating the presence of opioid receptors on immunocytes should be interpreted with caution. This apparent lack of receptor on immunocytes indicates that ascribing immune modulation to immune cells is unsafe and alternative sites/mechanisms need to be considered.

Opioids and hypothalamic-pituitary-adrenal axis

Another potential site for immune suppression is the hypothalamic-pituitary-adrenal (HPA) axis (Fig. 3). Simply put, corticotrophin releasing hormone (CRH) activates the anterior pituitary to produce adrenocorticotrophic hormone (ACTH) which then activates the adrenal cortex to produce glucocorticoids.[66] Glucocorticoids can affect the function of various components of the innate and adaptive immune system through gene modulation.[67] Exogenous glucocorticoids can suppress cellular immunity, promote humoral immunity, and induce tolerance against certain antigens through the alteration of T and B cell function.[67] Agents that activate the HPA axis have the potential to depress the immune response by the resulting increased plasma concentrations of glucocorticoids. The interaction of opioids with the HPA axis and its components (ACTH and cortisol production) is complex, species dependent and time dependent, with different effects after acute and chronic administration.[28] In rodents, the acute administration of opioids increases ACTH and glucocorticoids; this may have immunosuppressive actions. However, chronic opioid administration has variable effects on ACTH and glucocorticoids (decrease or no change). It is difficult to separate stress-induced increases in cortisol (because of animal handling/injection) from drug dependent changes. In man, there are little data; however, the evidence to date is that acute administration of opioids results in either a reduction or no change in ACTH or glucocorticoids.[28] Chronic administration probably results in a decrease in HPA axis activity to an extent that adrenal insufficiency results.[28] The HPA axis is subject to circadian rhythm with high levels of ACTH and cortisol in the morning and low levels in the evening.[66] There is evidence that opioids attenuate this rhythm leading to consistently increased circulating ACTH and cortisol.[68] This might be sufficient to produce immune suppression but, taken at face value, does not seem plausible.

A further point to consider is whether plasma cortisol concentrations are a relevant marker of immune suppression. It is not clear as to whether plasma cortisol concentrations are increased in patients with depressed immune function (compared with opioid-treated patients/addicts) but what cannot be ignored is the underlying 'status' of the individual. The presence of disease (e.g. cancer and organ failure) and nutritional status potentially affects the immune response via sites other than the HPA axis, including release of immunosuppressive cytokines (i.e. altered immune cell function). Glucocorticoid feedback inhibits both the hypothalamus and anterior pituitary. The question is, therefore, could low-circulating concentrations of glucocorticoids indicate *increased* HPA axis activity to an extent that feedback inhibition has occurred? Would this lead to immune suppression? What has happened to the effect site concentration (i.e. that surrounding the immune cell)? These are all questions that lend themselves to study and a further detailed evaluation of multiple markers of HPA activity for opioids of different classes in carefully grouped patients would seem sensible. In conclusion, while it would seem that the HPA is an attractive (indirect) site for immune modulation the supportive evidence is lacking.

Non-classical opioid receptors (NOP) involvement in inflammation, sepsis, and immunity

The role of the nociceptin system in inflammation and sepsis was recently reviewed in this journal.[69] Further studies have subsequently confirmed an important role for N/OFQ in pro-inflammatory states. Of particular clinical relevance is a study by Stamer and colleagues[70] who demonstrated modulated expression of whole blood mRNA for nociceptin components associated with advanced cancer, major surgery, and

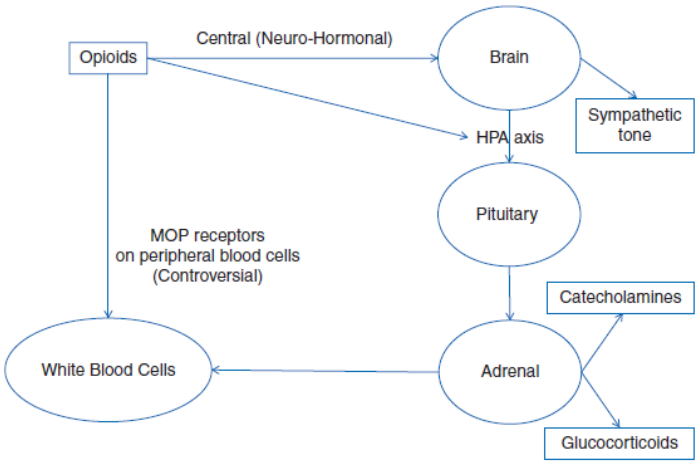

Fig 2 Potential sites of immunomodulatory effects of opioids that can be categorized into central and peripheral effects.

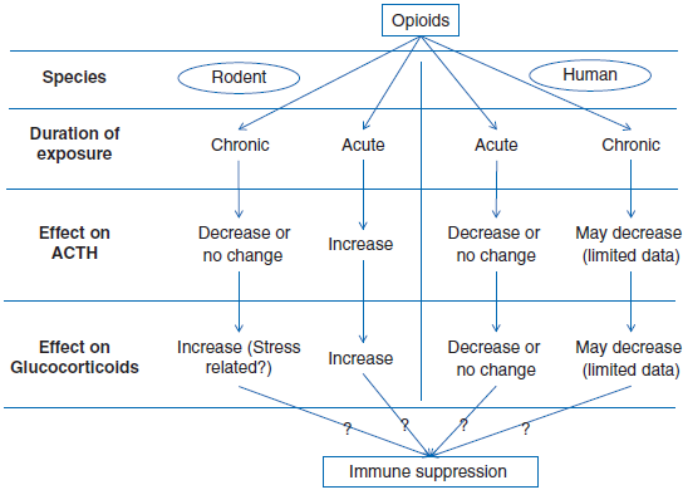

Fig 3 Effects of opioids on markers of HPA axis activity. ACTH, adrenocorticotrophic hormone.

severe sepsis. Whole blood was sampled from patients with cancer (n=113), patients admitted to the intensive care unit (ICU) with severe sepsis (n=18), postoperative patients after major surgery (n=20), and analysed against a control group of unmatched healthy volunteers (n=20). mRNA from whole blood samples was extracted and subjected to PCR analysis for NOP and pre-pro-N/OFQ transcripts. When compared with the healthy volunteers, NOP expression was significantly increased in all sample groups. Higher NOP expression in ICU patients was associated with mortality. pp-N/OFQ expression was also seen to be significantly reduced in the septic and cancer groups and associated with increases in the inflammatory marker pro-calcitonin. While limited by its observational nature, low numbers and the use of unmatched controls, this study represents more evidence that N/OFQ mRNA expression is modulated in pro-inflammatory states. A major limitation is

that N/OFQ peptide was not measured, only mRNA. Despite this, Stamer's work adds to the work of Williams and colleagues[71] suggesting an association between nociceptin modulation and mortality. However, it should be remembered that mRNA measurements are not an accurate reflection of functionality. Further studies measuring N/OFQ peptide and receptor activity are needed to confirm and extend these findings.

Animal studies continue to contribute to our understanding of nociceptin system in sepsis and inflammation. Anton and colleagues[72] have studied the effect of N/OFQ on spleen cells from mice, determining that antibody formation to exogenous sheep blood was suppressed. This suggests that the nociceptin system has an important modulating effect on the adaptive immune response. Furthermore, a recent paper has confirmed the findings of an earlier study that examined the response to dextran-sulphate induced bowel colitis in NOP knockout mice. By administering the N/OFQ antagonist SB612111 to these mice, Alt and colleagues[73] demonstrated an improvement in clinical condition and a reduction in inflammatory cytokines and their mRNA expression. The combination of positive findings in genetically modified mice with this interventional study suggests that studies investigating the effects of N/OFQ in human inflammatory bowel disease are required.

Many of the studies published to date have demonstrated associations of severe pro-inflammatory states with the Nociceptin system, yet little is known about the mechanisms behind these observations. *In vitro* work has demonstrated mRNA transcripts in immunocytes,[74–76] and N/OFQ peptide measured as being released from stimulated polymorphonuclear cells.[76] Furthermore, N/OFQ has also been demonstrated as functioning as an immunocyte chemotaxis agent.[77 78] More recently, there has been some evidence of an effect of N/OFQ on increasing activity of the critical sepsis-associated transcription factor NF-κB in human neuroblastoma cells (SH-SY5Y).[79] However, our understanding of how the Nociceptin system modulates immunocyte and cytokine behaviour is still poor and remains an important area for further research.

Opioid effects on sympathetic tone

Opioids can affect the cardiovascular system; for example, high-dose opioids can cause a vagally mediated bradycardia without depressing contractility in non-diseased myocardium.[80] Opioids showed some cardio-protective properties in ischaemic preconditioning animal models which was thought to be due to the direct cellular effect of opioids on mitochondrial potassium channels preventing calcium influx and cell apoptosis.[81 82] These effects were identical in intact and 'sympathectomized' myocardial cells; however, it was noted that opioid receptors can crosstalk physiologically and functionally with β-adrenergic receptors which may further emphasize the sympathetic-opioid link.[83] Felten and colleagues[84] described catecholamine involvement in the alteration of immune function mediated by opioids that is related to the heavy sympathetic innervation of bone marrow, spleen, and lymph nodes.[54] Adrenoceptors (α and β) were identified on rodent lymphocytes and macrophages[85–87] and these receptors were linked to the immunomodulatory effects of opioids. Acute administration of opioids can alter sympathetic system outflow altering immune function in a similar way to the effect of ganglion stimulants.[87] Sympathetically mediated opioid immune modulation includes depression of natural killer (NK) cell activity and suppression of peripheral blood lymphocyte proliferation.[54] Whether modulation of sympathetic tone alone can explain opioid immunomodulation is debatable but, in our opinion, unlikely.

Conclusions

We know that: (i) opioids produce immune modulation in both humans and experimental animals; (ii) there are differences amongst opioids in immune modulatory effects; (iii) MOP receptors are probably the main target for classical opioid immune modulation (with possible N/OFQ receptor involvement); and (iv) there are differences between species. There are several proposed mechanisms and sites of action including: (i) a direct action on the immunocyte; (ii) modulation of the HPA axis; (iii) modulation of sympathetic activity; and (iv) other sites, including central immune modulation. In essence, this illustrates a highly controversial area of enquiry.

Contrary to recent published opinion, this review indicates that direct action at the immunocyte by classical opioids is unlikely and modulation of the activity of the HPA axis at best partly explains immune modulation and, at worst, does not help at all. The presence of co-existing disease in previous experimental models is likely to have confounded conclusions and caution should be exercised in comparing data from animals and the highly heterogeneous situation in human clinical studies. Based on MOP knockout animal data, the MOP receptor seems to be the molecular target but the cellular/tissue/organ targets remain elusive. There is a need for a systematic study of the immune modulatory effects of classical opioids to provide firm evidence for site(s) of action and aid evidence-based opioid use in patients where immune suppression is to be avoided. Moreover, the specific targeting of the NOP receptor should be a focus for future work as this is a receptor that is expressed on immune cells. We conclude that with respect to opiods and immune modulation there are certainly currently more questions than answers.

Declaration of interest

The authors have collaborative links with University of Ferrara Peptides (UFPeptides) that is involved in the development of opioid ligands. D.G.L. holds a consultancy with Grunenthal GmbH. D.G.L. is an editorial board member and director of the *BJA*. J.P.T. is an editorial board member and editor of the *BJA*.

Funding

British Journal of Anaesthesia/Royal College of Anaesthetists funds current opioid work.

BJA

British Journal of Anaesthesia, 116 (3): 423–9 (2016)

doi: 10.1093/bja/aev540
Translational Research

TRANSLATIONAL RESEARCH

Evidence for nociceptin/orphanin FQ (NOP) but *not* μ (MOP), δ (DOP) or κ (KOP) opioid receptor mRNA in whole human blood

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Abstract

Background: While it is well known that opioids depress the immune system, the site(s) of action for this depression is highly controversial. Immune modulation could occur directly at the immune cell or centrally via the hypothalamic-pituitary-adrenal axis. In a number of studies using individual enriched immune cell populations we have failed to detect classical μ (MOP), δ (DOP) and κ (KOP) receptors. The non-classical nociceptin/orphanin FQ (N/OFQ) receptor (NOP) is expressed on all cells examined thus far. Our hypothesis was that immune cells do not express classical opioid receptors and that using whole blood would definitively answer this question.

Methods: Whole blood (containing all immune cell types) was incubated with opioids (morphine and fentanyl) commonly encountered in anaesthesia and with agents mimicking sepsis [lipopolysaccharide (LPS) and peptidoglycan G (PepG)]. Opioid receptor mRNA expression was assessed by endpoint polymerase chain reaction (PCR) with gel visualisation and quantitative PCR.

Results: Classical MOP, DOP, and KOP receptors were not detected in any of the samples tested either at rest or when challenged with opioids, LPS or PepG. Commercial primers for DOP did not perform well in quantitative PCR, so the absence of expression was confirmed using a traditional gel-based approach. NOP receptors were detected in all samples; expression was unaffected by opioids and reduced by LPS/PepG combinations.

Conclusions: Classical opioid receptors are not expressed on circulating immune cells.

Key words: immune cells; morphine; nociceptin; opioid receptors; PCR

Opioid receptors are classified as both classical [μ (MOP), δ (DOP), and κ (KOP)] and non-classical [nociceptin/orphanin FQ peptide (NOP)] receptors. A causal link between opioids and immune function has been historically presumed, based on observations of opioid addicts having an increased incidence and severity of infections.[1 2] More recent evidence highlights how opioids can have an effect on endocrine and immune function.[3] However, the immunomodulatory effects of opioids have not been determined precisely, with various opioid drugs having different effects on immune function, despite targeting a single receptor subtype, the MOP receptor.[4–6] Moreover, elucidating the mechanism(s) by which opioids modulate immune function is confounded by interspecies differences in their immunomodulatory effects. Hence opioid immunological interactions are both drug and species dependent. In MOP receptor knockout animal studies, no opioid immune modulation is

† J.P.T. and D.G.L. are joint senior authors.

Accepted: November 24, 2015

Editor's key points

- The site of action of opioids in depressing immune function remains controversial.
- Authors incubated whole blood with opioids or the mediators of sepsis.
- Classical MOP, DOP, and KOP receptors were not detected in any of the samples tested either at rest or when challenged with opioids or the mediators of sepsis.
- The study provides strong evidence that classical opioid receptors are not expressed on circulating immune cells.

seen, providing robust evidence that this receptor is the biological target.[7 8]

One of the proposed mechanisms for the immunomodulatory action of opioids is a direct action on immunocytes; however, there is huge controversy as to whether classical opioid receptors are expressed on these cells. While there is still a strong belief that peripherally mediated immunomodulatory effects of opioids are facilitated through receptors expressed on immunocytes, and several studies have determined the presence of opioid receptors on peripheral blood mononuclear cells (PBMCs), our group has been unable to detect classical opioid receptor expression on immunocytes in several settings and using a series of detection methodologies, including fluorescent/immunofluorescent labelling, quantitative polymerase chain reaction (PCR) and western blotting.[9–13] Previous studies have focused on individual populations of immune cell types. In one study, NOP receptor mRNA and mRNA for the precursor protein of N/OFQ (ppNOC) was differentially detected in granulocytes, monocytes, and lymphocytes; MOP receptor mRNA was not detected in any cell type.[11–15] We therefore aimed to take a more systematic approach using several PCR methodologies to investigate opioid receptor expression in unstimulated blood from healthy volunteers. It is possible that opioid treatment or the presence of sepsis might upregulate expression, so we additionally treated whole blood with supraclinical concentrations of morphine and fentanyl and lipopolysaccharide (LPS) and peptidoglycan G (PepG), agents commonly used to mimic sepsis.

Our hypothesis was that immune cells do not express classical opioid receptors and that using whole blood would definitively answer this question.

General methodology

Blood collection

Venous blood was collected from 10 healthy volunteers from the Department of Cardiovascular Sciences, Division of Anaesthesia, Critical Care and Pain Management, with approval from the University of Leicester (volunteer) Research Ethics Committee and with informed consent. Up to 30 ml of venous blood was collected using EDTA-monovette® tubes and divided into sterile 2 ml tubes to which LPS from *Escherichia coli* 0111:B4 (Sigma, Dorset, UK) 5 μg ml^{-1}, *Staphylococcus aureus* PepG (Sigma, Dorset, UK) 20 μg ml^{-1}, fentanyl 10 μM (Tocris Bioscience, Bristol, UK), and morphine 10 μM (Sigma, Dorset, UK) were added separately and in combinations as indicated in the results. Samples were incubated for 24 h at 37.0°C in 5% CO_2 humidified air.

Tissue culture

Positive control samples were prepared using Chinese hamster ovary cells (CHO) expressing human KOP, DOP, and NOP receptors and human embryonic kidney (HEK) cells expressing human MOP. $CHO_{hKOP/DOP}$ cells were cultured in Hams F12, CHO_{hNOP} in DMEM/Hams F12 (1:1), and HEK_{hMOP} cells in MEM, and all media were supplemented with 10% FCS, penicillin (100 IU ml^{-1}), Fungizone (2.5 μg ml^{-1}), and streptomycin (100 μg ml^{-1}). For maintaining expression of inserted genes, stock cultures of cells were additionally supplemented with 200 μg ml^{-1} of G418 for classical opioid receptors MOP/KOP/DOP, and 200 μg ml^{-1} of G418 and Hygromycin B for CHO_{hNOP}. Raji cells (lymphoblast-like cell) were used as an additional cell expressing DOP and cultured in RMPI 1640 supplemented with 10% FCS, Penicillin (100 IU ml^{-1}), Fungizone (2.5 μg ml^{-1}), streptomycin (100 μg ml^{-1}), and glutamine (2 mM). All cells were cultured at 37.0°C in 5% CO_2 humidified air. All tissue culture media were from Sigma (UK) and supplements were from Thermo Fisher, Paisley (UK).

RNA extraction, copy DNA (cDNA) synthesis, and quantitative PCR (qPCR)

Total RNA was purified from whole blood samples using a RiboPure™ RNA Purification Kit (Thermo Fisher Scientific, UK) as per the manufacturer's instructions and from CHO cells and Raji cells using a preparatory RNA isolation kit (*mir*Vana, Thermo Fisher Scientific) in which 1 ml of *mir*Vana lysis buffer was used for extraction of RNA from confluent 25 cm^2 flasks for adherent cells or 1×10^7 for non-adherent cells. In all cases, final RNA samples were resuspended in PCR-grade water, the mass of RNA determined using a NanoDrop 2000 (LabTech) and purity assessed from the 260/280 nm ratio, which was >1.8. Isolated RNA was processed using a Turbo DNA-*free*® kit for the removal of possible genomic DNA (gDNA) contamination before reverse transcription and production of cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, UK). For some samples, additional production of cDNA using the Quantitect Reverse Transcription Kit (Qiagen, UK), which included an additional genomic DNA elimination reaction, took place. For all samples, a non-template control (in which the reverse transcriptase enzyme was omitted from reverse transcription reactions), was included during PCRs/qPCRs.

Statistical analysis

qPCR experimental data are expressed as cycle threshold (C_t; one cycle representing a doubling of starting material) relative to the geometric mean of two housekeeper gene C_t values; human β_2-microglobulin (β_2M) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) representing the ΔC_t. The effect of treatments on mRNA expression was determined by normalizing the target gene expression level using the equation $2^{(-\Delta\Delta Ct)}$, where $\Delta\Delta C_t = \Delta C_t$ (treated)$-\Delta C_t$ (control). Data were analysed using Kruskal–Wallis analysis of variance (ANOVA) with Dunn's multiple comparison test. *P*-values <0.05 were considered statistically significant.

Methods and results

Volunteers included four females and six males, with a mean age of 40.6 years (range 32–54), mean weight 86.4 kg (range 55–110) and mean height 174.9 cm (range 153–196).

MOP/KOP

Whole blood was collected and incubated overnight with the inflammatory mediators LPS and PepG in the absence and presence

of clinical MOP receptor agonists morphine and fentanyl (supra-clinical concentrations) as noted earlier. Additional untreated (plain) and media (vehicle)-treated controls were included. cDNA from whole blood samples and cell lines were probed for gene transcripts using qPCR with commercially available TaqMan probes (Thermo Fisher Scientific, UK), SYBR Green, and pre-designed, target-validated primer pairs (Qiagen and Sigma, UK) (Table 1). Relative gene expression quantification was determined using TaqMan endogenous control probes, human β_2M and human GAPDH, and assays run in a duplex format. qPCRs were run on a StepOne instrument (Applied Biosystems, UK). The thermal profile for reactions with TM-MOP1, TM-KOP1, TM-NOP1, TM-DOP1&2 and endogenous control probes was 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C and for TM-DOP3 probes 3 min at 95°C followed by 40 cycles of 3 s at 95°C and 30 s at 60°C.

In the qPCR experiments with mRNA extracted from whole blood samples, probing for KOP and MOP expression, no amplification of these receptor transcripts could be detected. In the same samples there was robust amplification of endogenous controls β_2M and GAPDH, which was consistent across the various blood treatments, with C_t values not varying significantly from one another (Kruskal–Wallis and Dunn's multiple comparison $P>0.05$) (Table 2). Positive-control cDNA from CHO_{hKOP} and HEK_{hMOP} cells verified the probes with C_t values of 21.95 and 24.06, respectively. This absence of KOP and MOP receptor expression is similar to our previous studies in more discrete populations of white blood cells, PBMCs, and polymorphonuclear cells.[10 12 14 15]

NOP

Relative gene expression quantification for NOP receptor transcripts was determined using two TaqMan endogenous control probes, human β_2M and human GAPDH in a duplex assay format with NOP TaqMan probes. NOP expression was detected in untreated whole blood samples [median ΔC_t of 10.40 (range 8.56–11.24)] and in all treated samples (Table 2).

The effect of LPS, PepG, or a combination of the two on NOP mRNA was determined by normalized target gene expression level using the equation $2^{(-\Delta\Delta C_t)}$, where $\Delta\Delta C_t=\Delta C_t$ (treated)$-\Delta C_t$ (control; media). The calculated fold change $[2^{(-\Delta\Delta C_t)}]$ of NOP

Table 1 Assay IDs and sequences for the different TaqMan probes and primer pairs used throughout the study

Target	Type	Sequence/assay ID (supplier)	Abbreviation
KOP (OPRK1)	TaqMan assay	Hs00175127_m1 (Thermo Fisher)	TM-KOP1
MOP (OPRM1)	TaqMan assay	Hs01053957_m1 (Thermo Fisher)	TM-MOP1
NOP (OPRL1)	TaqMan assay	Hs00173471_m1 (Thermo Fisher)	TM-NOP1
DOP (OPRD1)	TaqMan assay	Hs00538331_m1 (Thermo Fisher)	TM-DOP1
DOP (OPRD1)	TaqMan assay	Hs00357182_mh (Thermo Fisher)	TM-DOP2
DOP (OPRD1)	QuantiFast Probe Assay	Hs_OPRD1_QF_1 (Qiagen)	TM-DOP3
DOP (OPRD1)	QuantiTect Primer Assay (SYBR Green)	Hs_OPRD1_1_SG (Qiagen)	SBG-DOP1
DOP (OPRD1)	KiCqStart™ Primers H_OPRD1_1	FH1(Sigma) RH1(Sigma)	SBG-DOP2
DOP (OPRD1) set4	Oligonucleotide primer pairs	Forward: GGCATCGTCCGGTACACTA Reverse: GTCGAGGAAAGCGTAGAGCA	STD-DOP-1

Table 2 Expression of classical and non-classical opioid receptor transcripts in mRNA extracted from whole blood samples treated with lipopolysaccharide (LPS) 5 μg ml^{-1}, *Staphylococcus aureus* peptidoglycan (PepG) 20 μg ml^{-1}, fentanyl (F) 10 μM, and morphine (M) 10 μM in the combinations shown. Data presented are expressed as median (interquartile range) cycle threshold values (C_t) for endogenous controls (β_2M and GAPDH) and genes of interest (MOP, KOP, NOP). Consistent with previous data, MOP and KOP transcripts could not be detected. Positive control cDNA from CHO_{hNOP}, CHO_{hKOP}, CHO_{hDOP}, and HEK_{hMOP} cells verified the probes with respective C_t values of 17.95, 21.95, 24.72, and 24.06. The geometric mean of C_t values derived from β_2M and GAPDH were used to determine ΔC_t values for expression of NOP receptor transcripts. Data are for n=5–10 volunteers

Whole blood treatment	Classical opioid receptors				Nociceptin/orphanin FQ receptor (NOP)		
	C_t GAPDH	C_t β_2M	C_t MOP	C_t KOP	Geometric mean C_t (GAPDH/β_2M)	NOP C_t	ΔC_t
Plain (n=10)	24.16 (22.93–24.93)	21.89 (20.58–22.18)	ND	ND	23.17 (21.49–23.58)	32.75 (31.75–34.17)	10.40 (8.56–11.24)
Media (n=10)	23.88 (22.54–25.09)	21.07 (20.00–22.01)	ND	ND	22.88 (21.15–23.47)	32.82 (31.89–34.29)	10.71 (8.41–11.64)
Morphine (n=10)	24.23 (22.68–24.89)	21.29 (20.67–21.87)	ND	ND	22.93 (22.29–23.48)	34.07 (32.19–34.47)	10.68 (9.21–11.70)
Fentanyl (n=10)	24.11 (23.24–24.90)	21.52 (20.98–21.73)	ND	ND	23.17 (22.34–23.30)	33.45 (31.92–34.61)	10.67 (8.21–11.48)
LPS (n=10)	25.75 (25.02–27.27)	21.19 (19.77–22.09)	ND	ND	23.37 (22.54–24.37)	35.19 (34.11–35.66)	11.40 (11.21–12.98)
LPS+M (n=10)	25.35 (24.79–26.63)	20.05 (19.44–21.46)	ND	ND	22.82 (21.93–23.58)	35.08 (34.58–36.04)	11.93 (10.74–13.12)
LPS+F (n=10)	25.46 (24.94–26.83)	20.72 (19.82–22.57)	ND	ND	23.20 (22.57–24.70)	35.66 (34.34–36.27)	11.75 (10.79–13.18)
PepG (n=5)	24.71 (22.82–28.18)	20.74 (19.49–23.26)	ND	ND	22.84 (21.57–25.40)	33.91 (31.21–36.35)	10.75 (8.54–12.19)
PepG+M (n=5)	26.00 (24.32–26.92)	20.76 (20.11–22.14)	ND	ND	23.22 (22.24–24.75)	34.71 (32.31–35.70)	11.52 (8.41–13.08)
PepG+F (n=5)	24.43 (22.43–26.81)	20.54 (19.43–22.51)	ND	ND	22.41 (21.43–24.48)	34.10 (30.51–36.24)	11.02 (8.26–12.90)
PepG+LPS (n=5)	25.01 (23.12–26.34)	20.33 (19.76–21.17)	ND	ND	22.70 (21.94–23.57)	35.61 (31.33–36.00)	12.36 (8.99–13.54)
PepG+LPS+M+F (n=5)	25.59 (22.99–27.66)	20.23 (19.15–22.89)	ND	ND	22.38 (21.66–25.15)	35.29 (32.94–36.47)	12.28 (9.33–13.58)

mRNA levels decreased significantly relative to vehicle-treated control samples after treatment with LPS, PepG, or the two combined (Fig. 1).

DOP

In the qPCR experiments with TaqMan probes designed and validated for the human DOP receptor TM-DOP1–3, amplification in both positive control cDNA from CHO cells transfected with the human DOP receptor and in cDNA from whole blood samples was detected. Moreover, amplification of DOP receptor transcripts was also detected in non-template controls (NTCs) from whole blood samples for all TaqMan probes tested (Table 1). NTCs represent a negative control run in parallel for each sample for which the reverse transcriptase enzyme, which converts mRNA to cDNA, is removed and therefore amplification in these samples represents genomic DNA contamination and not mRNA expression, which is the precursor for protein (receptor) expression. Amplification in NTCs suggests the amplification measured in sample cDNA could represent a false positive. The high C_t values seen with TM-DOP1–3 and those similarly measured C_t values between sample cDNA and the related NTC data also suggest this (Fig. 2).

Alternative commercially available primer pairs were sourced and used in SYBR green experiments, using a Quantifast SYBR green PCR master mix (Qiagen, UK). The thermal profile of reactions with SBG-DOP1&2 primer pairs was 5 min at 95°C followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. Melt curve analysis was carried out by a final melting of 15 s at 95°C followed by dissociation curve construction comprising increasing temperature by 0.3°C increments from 60°C. Amplification of DOP receptor transcripts was detected in cDNA and NTCs from whole blood samples and CHO_{hDOP} controls using both primer pairs (Fig. 2).

To determine whether there was a difference in the PCR products amplified from sample wells and NTC wells, melt curve analysis was performed to determine the dissociation temperature (T_m) of the PCR product. In a preliminary experiment (n=1) using whole blood RNA, expected differences were determined in the measured T_m values of PCR products amplified by different primer pairs; 77.67°C (TM-DOP1), 81.29°C (TM-DOP3), 85°C (SBG-1), and 87.25°C (SBG-2). However, when using a single TaqMan probe or primer pair, no differences in measured T_m values was determined between cDNA samples and NTCs: 77.67°C and 77.67°C for TM-DOP1, 81.29°C and 81.44°C for TM-DOP3, 85°C and 85.3°C for SBG-1 and 87.25°C and 87.25°C for SBG-2. This suggests the PCR product in the cDNA samples and NTCs is the same, and imaging of PCR products using gel electrophoresis for TM-DOP1 confirms these findings with all products (~100 bp; Fig. 2).

In an effort to reduce gDNA contamination beyond that removed by DNase treatment alone, a test was performed using Raji cells that were reverse transcribed using the Quantitect reverse transcription kit, which incorporates an additional genomic DNA elimination reaction (Qiagen, UK). Using mRNA extracted from Raji cells, a reverse transcription reaction that incorporated an extra genomic DNA elimination reaction had no effect. The C_t values determined using TM-DOP1 probes, relative to the standard reverse transcription reaction, were 36.17 and 36.13 in cDNA samples and 36.59 and 36.38 for NTCs, respectively. Overall, the extra genomic DNA elimination reaction was deemed ineffective and was not used in the processing of volunteer sample bloods.

In an effort to distinguish cDNA from gDNA, primers were designed that span an exon/exon boundary using primer-BLAST against the NCBI reference sequence NM_000911.3 for the *Homo sapiens* opioid receptor δ1 (OPRD1) mRNA. These primers, designated STD-DOP-1, were used in endpoint PCR using AmpliTaq Gold® 360 Master Mix (Thermo Fisher Scientific, UK) on an Eppendorf Mastercycler with an initial 95°C 10 min denaturing step followed by 40 cycles of 30 s at 95°C, annealing for 30 s at temperatures of 51°C –71.5°C and extension at 72°C for 60 s with a final extension for 10 min at 72°C. PCR products were run on a 3% agarose gel stained with ethidium bromide and imaged under ultraviolet illumination.

STD-DOP-1 primers were initially used at a concentration of 0.4 µM and with an annealing temperature of 59°C and were determined to amplify a product of predicted size (~700 bp in cDNA from CHO_{hDOP}). In NTC wells from whole blood samples the STD-DOP-1 primer pair was seen to amplify a product of ~700 bp and another ~200 bp, although this was not consistent for all NTC samples tested. In order to improve the stringency of the STD-DOP-1 primer pair, temperature and concentration gradients were performed, leading to the determination of an optimum annealing temperature at 56.5°C and primer pair concentration

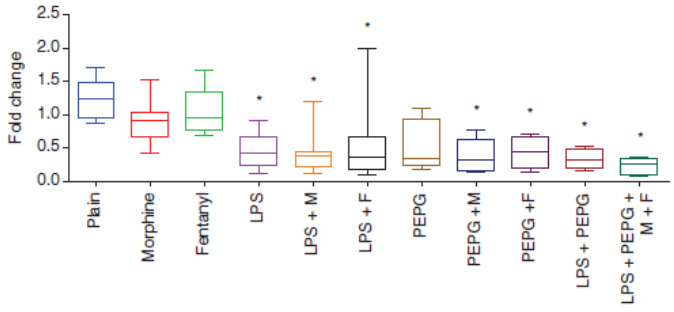

Fig 1 Expression of NOP receptor transcripts in mRNA extracted from whole blood samples treated with lipopolysaccharide (LPS) 5 µg ml^{-1}, *Staphylococcus aureus* peptidoglycan (PepG) 20 µg ml^{-1}, fentanyl (F) 10 µM, and morphine (M) 10 µM in the combinations shown. Data presented as the fold change relative to vehicle control (media). *Samples are statistically different from the plain sample using a Kruskal–Wallis test with Dunn's multiple comparisons test.

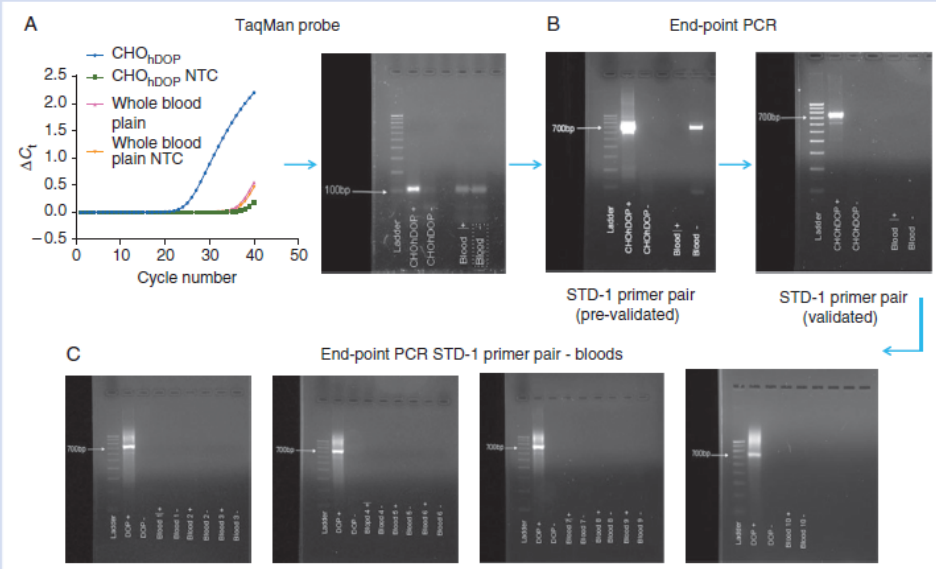

Fig 2 (A) The QPCR amplification plot in whole blood RNA using TaqMan probes targeting the DOP receptor, amplification in both reverse transcribed (RT) and non-RT samples can be seen, and the equivalent gel confirms both product presence and size. (B) Gel electrophoresis for the PCR products from endpoint PCR using STD-1 primer pairs pre- and post-validation, in which annealing temperatures and primer concentrations were optimised. (C) Gel electrophoresis of PCRs using STD-1 primer pairs under authenticated conditions in RNA from whole blood samples.

of 40 nM. Under these conditions, in cDNA from CHO_{hDOP} cells, amplification was of a product of predicted size (~700 bp), and no product could be detected in NTCs. STD-DOP-1 primers were subsequently used under the same experimental conditions to probe samples for expression of DOP in whole blood samples and no expression could be detected (Fig. 2).

Discussion

Our main finding using qPCR is that mRNA encoding for either the MOP or KOP receptor could not be detected in peripheral whole human blood under resting conditions or after incubation with a variety of inflammatory mediators and opioids. A false-positive DOP receptor mRNA signal was initially detected, since a variety of the commercially available TaqMan probes for this receptor transcript did not differentiate genomic from copy DNA. The presence of DOP receptor transcript was subsequently disproved through the development of a primer pair utilised in endpoint PCR experiments. These data further underscore the need for carefully controlled PCR protocols. Transcripts encoding for the NOP receptor were consistently detected in whole blood samples, and these decreased in the presence inflammatory stimuli (LPS and PepG).

In this study, the full complement of cell populations in whole blood, comprising red blood cells, white blood cells, and platelets, were utilised with a range of experimental conditions, to include the effects of inflammatory stimuli (alone and in combination) and a number of clinically used MOP receptor agonists. This approach was used in conjunction with a comprehensive series of opioid receptor primers to fully establish expression, and to allow for measuring changes in expression of both classical and non-classical opioid receptor subtypes, in the mRNA from whole blood. Expression of classical opioid receptors on immune cells is hugely controversial and has profound consequences for studies into and clinical modulation of immune function by opioid drugs.[3 10 15 16–18] A comprehensive and carefully controlled (methodologically) study such as this is needed where the entire circulating immune cell population is present.

The physiological and cellular mechanisms that result in the immunosuppressant effect of opioid drugs seen in humans and whole animal studies is hypothesised to be mediated either through a direct action on immunocytes, altering of sympathetic activity, or modulation of the hypothalamic-pituitary-axis.[17] In addition, there is evidence for further central effects at glia. Findings from our laboratory have shown that PBMCs do not express classical opiate receptors and therefore the functional action of opioid drugs on immune functioning is unlikely to be through opioid receptors expressed on these cells.[10 14–16] This finding is disputed by some studies that have identified the presence of MOP receptors.[5 11 15 17 18] In order to explore the hypothesis that opioid drugs can have a direct action on immunocytes, the expression of opioid receptor subtypes was determined using total RNA extracted from whole blood. It was believed that this approach could allow for detection of opioid receptor transcripts that may have been missed in discrete cell types. Consistent with the data of Williams and colleagues,[10] both MOP and KOP receptor transcripts could not be detected in RNA from whole blood.

MOP knockout species implicate the receptor as central to the immunomodulatory effects of opioids, and while MOP receptor expression could not be detected under 'basal' conditions, it has been considered that expression on immunocytes occurs only after their exposure to pro-inflammatory mediators and cytokines. To determine if the immunomodulatory role of opioids could be influenced, or even established, a number of regulators of the immune system where tested to see if they had an impact on opioid receptor expression in whole blood. Incubation of whole blood samples with the pro-inflammatory mediators LPS and PepG for 24 h had no effect on the expression of either MOP, DOP, or KOP receptor transcripts. Other authors have shown that administration of TNF-α to immune cells up-regulates the MOP receptor, which may suggest that the type, concentration, and exposure time of the pro-inflammatory mediators used in this study were not optimal.[14 19] It is worthy of mention that the Kraus study did use multiple rounds of PCR to detect MOP receptor transcripts, which suggests an extremely low quantity of transcripts.[14]

cDNA from whole blood RNA was initially probed for the presence of human DOP receptor transcripts using TaqMan probe Hs00538331_m1 (Thermo Fisher Scientific, UK), which revealed amplification of product, i.e. the presence of the human DOP receptor. However, this was a false positive, with amplification measured in parallel non-template control samples in which the reverse transcriptase enzyme had been removed in the conversion of whole blood RNA to cDNA, suggesting the amplification in both samples to be genomic DNA. The same false-positive amplification was seen with further commercially available TaqMan probes for the DOP receptor [Hs00357182_mh (Thermo Fisher Scientific, UK) and Hs_OPRD1_QF_1 (Qiagen, UK)]. Such gDNA amplification was despite RNA samples being processed with a DNase enzyme designed to remove DNA contaminates, providing a cautionary tale and pointing to the need for use of the correct negative controls. To distinguish cDNA from gDNA, primers were designed that span an exon/exon boundary on the OPRD1 mRNA sequence. While these primers initially yielded amplification in some NTC samples, through a series of validating experiments the stringency with which they amplified specifically cDNA for the DOP receptor was increased. Using the STD-DOP-1 primer pair, under the correct experimental conditions, no amplification of the DOP receptor transcript could be detected, while robust and consistent amplification was determined in positive control samples (cDNA from CHO_{hDOP} cells). Again this highlights the importance and possibility of detecting a false positive if the correct experimental conditions are not defined.

Recent studies have indicated that the nociceptin system has a role in inflammation and sepsis.[20 21] Clinical evidence from intensive care unit (ICU) patients diagnosed with sepsis has shown how there is a decrease in mRNA encoding for the NOP receptor in septic patients when compared with a matched control volunteer. Previously we demonstrated that while PBMCs lack expression of classical opioid receptors (MOP, DOP, and KOP), they do express NOP receptor transcripts, and further studies have revealed differential expression on different white cell types: ΔC_t=6.43 in monocytes, 8.05 in lymphocytes, and 7.73 in granulocytes.[15] In mRNA extracted from whole untreated blood, expression of the NOP receptor was further detected, with a ΔC_t of 10.40. In the presence of LPS and PepG the calculated fold change [$2^{(-\Delta\Delta C_t)}$] for NOP mRNA levels decreased (Fig. 2). These findings are similar to the decrease in NOP mRNA measured in ICU patients diagnosed with sepsis.[20]

Limitations

One obvious limitation of this study is the fact that we did not show the presence or absence of opioid receptor protein. There are two points of note in this regard. First, without the presence of a transcribed gene, i.e. no receptor mRNA, there can be no translated protein. Second, and despite considerable effort (see Niwa *et al.*[22]), there are no antibodies with proven selectivity for a particular member of the opioid receptor family.

In summary, using whole blood from healthy volunteers that should contain the full range of circulating immune cells, we failed to detect transcripts for MOP, DOP, or KOP receptors, but NOP mRNA was present. We therefore conclude that immune modulation produced by MOP agonists in clinical practice (morphine and fentanyl) cannot occur at the level of the circulating immune cell, as there is no target for interaction.

Authors' contributions

J.P.T., D.G.L.: study design/planning.
M.Al-H., J.McD.: study conduct.
M.Al-H., J.McD., J.P.T., D.G.L.: data analysis.
M.Al-H., J.McD., J.P.T., D.G.L.: writing paper.
All authors: manuscript revision

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