Investigating the Role of Olfactory Receptors in Innate Immune Memory and Sepsis

Nigel Sammut

M.Sc. Applied Biomedical Science

Intake 2022

August 2024

Submitted to the Faculty of Health Sciences of the University of Malta in partial fulfilment of the requirements for the degree of Master of Science in Applied Biomedical Science.

Tutor: Dr Brendon P. Scicluna PhD

L-Università ta' Malta

FACULTY/~~INSTITUTE/CENTRE/SCHOOL~~ of Health Sciences

DECLARATIONS BY POSTGRADUATE STUDENTS

(a) Authenticity of Dissertation

I hereby declare that I am the legitimate author of this Dissertation and that it is my original work.

No portion of this work has been submitted in support of an application for another degree or qualification of this or any other university or institution of higher education.

I hold the University of Malta harmless against any third party claims with regard to copyright violation, breach of confidentiality, defamation and any other third party right infringement.

(b) Research Code of Practice and Ethics Review Procedures

I declare that I have abided by the University's Research Ethics Review Procedures. Research Ethics & Data Protection form code FHS-2022-00568.

As a Master's student, as per Regulation 77 of the General Regulations for University Postgraduate Awards 2021, I accept that should my dissertation be awarded a Grade A, it will be made publicly available on the University of Malta Institutional Repository.

Acknowledgements

I would like to express my heartfelt gratitude to my supervisor, Dr Brendon Scicluna, for his professional guidance, continuous assistance, mentorship and abundant patience throughout the course of this dissertation. Dr Scicluna's great knowledge and his drive for excellence have helped me elevate myself to the standard expected from him, both in the laboratory and beyond. He has been a continuous source of motivation that helped mature my outlook on the field of biomedical research in all its difficulties and triumphs alike and appreciate the importance of being more closely involved with the international biomedical research scene. I feel deeply honoured to have had him as my supervisor and look forward to working together with him once more in future research, whatever the challenge may be.

I would also like to thank the staff of the Faculty of Health Sciences and of the Centre for Molecular Medicine and Biobanking for allowing me to make frequent use of their facilities over the course of the dissertation. My gratitude further extends to all participants who agreed to partake in my study, and to Dr Nikolai Pace for his expertise, as my research could not have possibly carried on without them.

Furthermore, I would like to thank my family for their unending support, both in striving to the end, and in surmounting any obstacle encountered no matter how great. This also extends our dedicated lecturers at the Faculty of Health Sciences, who do their utmost to deliver quality lectures while maintaining an engaging learning environment. Finally, I would also like to thank Dr Scicluna's other students: Mikea Enriquez, Emilia Gellel, and Gillian Attard Montalto; for their enthusiasm and excellent laboratory conduct, which made the laboratory work associated with this dissertation a pleasant experience I will surely look back on in the future.

Abstract

Sepsis, defined as the abnormal host response to infection causing lethal organ dysfunction, remains one of the leading causes of patient morbidity and mortality worldwide. These mortality rates are thought to be contributed to heavily by the phenomenon of 'immune paralysis', a state of sustained immunosuppression. It is postulated that elucidating the molecular mechanisms that govern innate immune memory could in turn translate to effective treatments. The presence of ectopic olfactory receptors (ORs) on innate immune cells has been discovered in numerous studies in the past decade, linking their activity with specific pro- and anti-inflammatory functions. This study aims to test the hypothesis that ectopic ORs are associated with the host response to infection and the induction of innate immune memory. Using publicly available datasets, over 100 ectopic ORs were identified to be differentially upregulated in sepsis. Expression data from cell-sorted blood leukocytes revealed the differential expression of multiple ORs in sepsis, with *OR52B2* upregulation being singled out monocytes. Expression data for lipopolysaccharide (LPS)-treated healthy human monocyte-derived macrophages showed differential downregulation of *OR8G5* and upregulation of *OR52K3P*. OR kinetics observed through a human endotoxemia model dataset revealed continuous upregulation of *OR2B6*, a suspected monocyte-specific OR, over the span most significant to the development of innate immune memory, proving an attractive target for testing. A cohort of 8 healthy volunteers (4 females, 4 males; mean age 21 years) were recruited for this study. Heparinised blood was drawn with consent from each volunteer, from which monocytes were then harvested and enriched. Enriched monocytes were split into two categories, with one being subjected to a single controlled dose of LPS, while another was subjected to two doses with an overnight rest in between. ELISA testing revealed a stronger TNF-α response upon first exposure compared to restimulation, corroborated by quantitative real-time polymerase chain reaction, which showed much stronger *TNFA* expression in first exposure monocytes. The observed patterns closely mimic those demonstrated in several reports of monocytes in sepsis patients, characterised by sepsis-induced immune paralysis. *OR2B6* expression was not reliably detected during both first stimulation with LPS and in endotoxin tolerance (restimulation), suggesting that olfactory receptor gene expression may not be inducible by bacterial pathogen-associated molecular patterns. Although the impact of ectopic ORs on sepsis-induced immune paralysis remains largely unknown, this study provides multiple avenues through which further work may be carried out to elucidate the role of ORs in the host response during sepsis.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

5-parametric logistic	5-PL
Intra-abdominal infection	Abdo
Adenylate cyclase III	ACIII
Beta-actin Gene	*ACTB*
Androgen induced 1 Gene	*AIG1*
AU-rich binding element	ARE
Adenosine triphosphate	ATP
Adenosine triphosphatase phospholipid transporting 9A Gene	*ATP9A*
Fluorescence-activated cell sorting AriaIII machine	BD
Benjamini-Hochberg	BH
Bone marrow	BM
Cyclic adenosine monophosphate	cAMP
Community-acquired pneumonia	CAP
Cardiovascular infections	Cardio
Chemokine ligand 20 Gene	*CCL20*
Central Committee on Research Involving Human Subjects	CCMO
Chemokine receptor type 3 Gene	*CCR3*
CD24 Antigen Gene	*CD24*
CD177 Antigen Gene	*CD177*
Complementary DNA	cDNA
CCAAT/Enhancer-binding protein-Alpha	CEBPα
Cluster of differentiation	CD
Cyclic nucleotide gated	CNG
Chronic obstructive pulmonary disease	COPD
Coronavirus disease of 2019	COVID-19
Quantification Cycle	C_q
Clustered regularly interspaced short palindromic repeats	CRISPR
CRISPR-associated protein 9	CRISPR-Cas9
Coefficient of variation	CV

Detection above background	DABG
Damage-associated molecular pattern	DAMP
Dendritic cells	DCs
Death-inducing signalling complexes	DISC
Deoxyribonuclease	DNase
Deoxynucleoside trisphosphate	dNTP
Dulbecco's phosphate-buffered saline	DPBS
Escherichia coli	*E. coli*
Enzyme-linked immunosorbent assay	ELISA
Fluorescence-activated cell sorting	FACS
Foetal calf serum	FCS
Fibroblast growth factor binding protein 2 Gene	*FGFBP2*
Fluorescein isothiocynate	FITC
Forward scatter	FSC
GATA Binding Protein 3 Gene	*GATA3*
Gene expression omnibus	GEO
Granulocyte macrophage-colony stimulating factor	GM-CSF
G-protein coupled receptor	GPCR
G protein-coupled receptor 132	Gpr132
Genome Consortium human genome build 38	GRCh38
Guanosine triphosphatase	GTP-ase
Histone 3 lysine 9 trimethylation	H3K9me3
Histone 4 lysine 20 trimethylation	H4K20me3
Hospital-acquired pneumonia	HAP
Human bronchial epithelium / epithelial	HBE
Hierarchical Indexing for Spliced Alignment of Transcripts version 2	HISAT2
Hyper immunoglobulin D syndrome	HIDS
Hypoxia-inducible factor	HIF
Human Leukocyte Antigen	HLA
Major histocompatibility complex, Class II, DP Alpha 1 Gene	*HLA-DPA1*

Major histocompatibility complex, Class II, DM Alpha Gene	*HLA-DMA*
Human Leukocyte Antigen-Death Receptor	HLA-DR
High-mobility group box	HMGB
Haptoglobin Gene	*HP*
Hot start	HS
Haematopoietic stem cells	HSC
High-throughput sequencing	HTSeq
Intensive care unit	ICU
IDNK Glucokinase Gene	*IDNK*
Interleukin	IL
Lymphoid enhancer binding factor 1 Gene	*LEF1*
Linear Models for Microarray Data	Limma
Fold-change converted to a logarithmic scale	LogFC
Lipopolysaccharide	LPS
Lactotransferrin Gene	*LTF*
Lymphocyte antigen 96	LY96
Molecular Diagnosis and Risk Stratification of Sepsis	MARS
Monocyte chemotactic protein 1	MCP-1
Myeloid differentiation factor 2	MD-2
Monocyte-derived macrophage	MDM
Myeloid-derived suppressor cell	MDSC
Multiple EGF-like domains 6 Gene	*MEGF6*
Membrane metalloendopeptidase Gene	*MME*
Moloney Murine Leukaemia Virus	MMLV
Matrix metalloprotease 8 Gene	*MMP8*
Multipotent progenitors	MPP
Messenger ribonucleic acid	mRNA
Transcript abundance	N_0
Quantification threshold	N_p
National Center for Biotechnology Information	NCBI

Nucleotide-binding oligomerisation domain	NLRP
NLR family pyrin domain-containing protein 1	NLRP1
NLR family pyrin domain-containing protein 1 Gene	*NLRP1*
NLR family pyrin domain-containing protein 3	NLRP3
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κβ
Natural killer	NK
Nonsense-mediated decay	NMD
Optical density	OD
Olfactomedin 4 Gene	*OLFM4*
Mouse olfactory G protein-coupled receptor 2 Gene	*Olfr2*
Mouse olfactory G protein-coupled receptor 78 Gene	*Olfr78*
Olfactory receptor	OR
Olfactory receptor family 1 subfamily D member 5 Gene	*OR1D5*
Olfactory receptor family 2 subfamily A member 1 Gene	*OR2A1*
Olfactory receptor family 2 subfamily A member 7 Gene	*OR2A7*
Olfactory receptor family 2 subfamily A member 14 Gene	*OR2A14*
Olfactory receptor family 2 subfamily A member 42 Gene	*OR2A42*
Olfactory receptor family 2 subfamily AK member 2 Gene	*OR2AK2*
Olfactory receptor family 2 subfamily AT member 4 Gene	*OR2AT4*
Olfactory receptor family 2 subfamily B member 2 Gene	*OR2B2*
Olfactory receptor family 2 subfamily B member 6 Gene	*OR2B6*
Olfactory receptor family 2 subfamily B member 11 Gene	*OR2B11*
Olfactory receptor family 2 subfamily H member 2 Gene	*OR2H2*
Olfactory receptor family 2 subfamily G member 2 Gene	*OR2G2*
Olfactory receptor family 2 subfamily G member 6 Gene	*OR2G6*
Olfactory receptor family 2 subfamily L member 2 Gene	*OR2L2*
Olfactory receptor family 2 subfamily L member 5 Gene	*OR2L5*
Olfactory receptor family 2 subfamily L member 5 Gene	*OR2L8*
Olfactory receptor family 2 subfamily T member 1 Gene	*OR2T1*
Olfactory receptor family 2 subfamily T member 3 Gene	*OR2T3*

Olfactory receptor family 2 subfamily T member 10 Gene	*OR2T10*
Olfactory receptor family 2 subfamily T member 12 Gene	*OR2T12*
Olfactory receptor family 4 subfamily F member 15 Gene	*OR2F15*
Olfactory receptor family 5 subfamily A member 2 Gene	*OR5A2*
Olfactory receptor family 5 subfamily B member 21 Gene	*OR5B21*
Olfactory receptor family 6 subfamily A member 2 Gene	*OR6A2*
Olfactory receptor family 6 subfamily B member 2 Gene	*OR6B2*
Olfactory receptor family 6 subfamily P member 1 Gene	*OR6P1*
Olfactory receptor family 7 subfamily G member 3 Gene	*OR7G3*
Olfactory receptor family 8 subfamily A member 1 Gene	*OR8A1*
Olfactory receptor family 8 subfamily G member 5 Gene	*OR8G5*
Olfactory receptor family 10 subfamily AD member 1 Gene	*OR10AD1*
Olfactory receptor family 10 subfamily G member 3 Gene	*OR10G3*
Olfactory receptor family 10 subfamily H member 5 Gene	*OR10H5*
Olfactory receptor family 10 subfamily P member 1 Gene	*OR10P1*
Olfactory receptor family 10 subfamily T member 2 Gene	*OR10T2*
Olfactory receptor family 13 subfamily A member 1 Gene	*OR13A1*
Olfactory receptor family 51 subfamily A member 2 Gene	*OR51A2*
Olfactory receptor family 51 subfamily E member 2 Gene	*OR51E2*
Olfactory receptor family 51 subfamily G member 2 Gene	*OR51G2*
Olfactory receptor family 52 subfamily B member 2 Gene	*OR52B2*
Olfactory receptor family 52 subfamily E member 2 Gene	*OR52E2*
Olfactory receptor family 52 subfamily H member 1 Gene	*OR52H1*
Olfactory receptor family 52 subfamily K member 1 Gene	*OR52K1*
Olfactory receptor family 52 subfamily K member 2 Gene	*OR52K2*
Olfactory receptor family 52 subfamily K member 3 Pseudogene	*OR52K3P*
Olfactory receptor family 52 subfamily N member 1 Gene	*OR52N1*
Olfactory receptor family 52 subfamily N member 4 Gene	*OR52N4*
Olfactory receptor family 56 subfamily A member 1 Gene	*OR56A1*
Olfactory receptor family 56 subfamily B member 1 Gene	*OR56B1*

Mouse olfactory receptor 65 Gene	*Or65*
Mouse olfactory receptor 272 Gene	*Or272*
Mouse olfactory receptor 352 Gene	*Or352*
Mouse olfactory receptor 446 Gene	*Or446*
Mouse olfactory receptor 568 Gene	*Or568*
Mouse olfactory receptor 622 Gene	*Or622*
Mouse olfactory receptor 657 Gene	*Or657*
Mouse olfactory receptor 1014 Gene	*Or1014*
Pathogen-associated molecular patterns	PAMP
Peripheral blood mononuclear cells	PBMC
Phosphate-buffered saline	PBS
Polymerase chain reaction	PCR
Phycoerythrin	PE
Peridinin-Chlorophyll-Protein	PerCP
Paraformaldehyde	PFA
Pathogen-recognition receptors	PRR
Quantitative polymerase chain reaction	qPCR
Quantitative real-time polymerase chain reaction	qRT-PCR
Coefficient of determination	R^2
Retinol Binding Protein 7 Gene	*RPB7*
Restimulations	Restim
Ribonucleic acid	RNA
Ribonuclease	RNase
Reactive oxygen species	ROS
Carboxy-X-rhodamine	ROX
Roswell Park Memorial Institute	RPMI
Reverse-transcription quantitative polymerase chain reaction	RT-qPCR
S100 calcium-binding protein A8 Gene	*S100A8*
S100 calcium-binding protein A12 Gene	*S100A12*
Serum/glucocorticoid regulated kinase 1 Gene	*SGK1*

Single nucleotide polymorphism	SNP
Small proline rich protein 2A Gene	*SPRR2A*
Small proline rich protein 2E Gene	*SPRR2E*
Side scatter	SSC
Stomatin Gene	*STOM*
Streptavidin-Horseradish peroxidase	Streptavidin-HRP
Sulfatase 2 Gene	*SULF2*
Tumour-associated macrophage	TAM
Tricarboxylic acid	TCA
Transcription factor binding sites	TFBS
Toll-like receptor	TLR
Tumour necrosis factor	TNF
Tumour necrosis factor-Alpha	TNF-α
Regulatory T-Cells	Treg
Glutamine catabolism and uridine diphosphate N-acetylglucosamine	UDP-GlcNAc
University Research Ethics Committee Research Ethics and Data Protection	URECA-REDP
Urinary tract infections	Uros
Untranslated region	UTR
Window of Linearity	W-o-L
World Health Organisation	WHO

Chapter 1:

Literature Review

1 Introduction

Innate immune memory is a fundamental adaptation of innate immune cells that refers to the ability to develop a functional memory-like response following initial encounters with certain pathogens or stimuli. While the innate immune system is traditionally thought to lack specific memory, recent research has shown that innate immune cells can exhibit a form of memory-like behaviour, for example tolerance or trained immunity (Divangahi et al 2021). Innate immune memory is observed mainly in myeloid cells, including monocytes, macrophages, and natural killer cells (Netea et al 2011, Cerwenka and Lanier 2016). When these cells are exposed to a pathogen or stimulus, they are understood to undergo reprogramming involving epigenetic modifications, metabolic shifts, and altered transcriptional patterns that persist even after the initial stimulus has been cleared. Reprogramming of innate immune cells can have two opposing consequences, that is either enhanced or impaired responses upon subsequent encounters with the same or related pathogens. Enhanced responses, also known as priming or trained immunity, include increased production of pro-inflammatory cytokines, elevated phagocytic activity, heightened antimicrobial functions, and improved antigen presentation. On the other hand, impaired responses refer to regulatory mechanisms that prevent excessive or inappropriate responses to self-antigens or harmless environmental stimuli, also known as innate immune tolerance. Tolerance involves the dampening or suppression of the innate immune response, leading to a state of reduced inflammation and immune activation, whereas the mechanisms underlying priming or trained immunity potentiate the immune response (Divangahi et al 2021). In general, innate immune memory allows for a more rapid and robust response to subsequent infections, contributing to improved pathogen clearance and host defence. The mechanisms underlying innate immune memory involve various molecular pathways, including pattern

recognition receptors (PRRs), cytokines, and metabolic reprogramming. Specific molecules, such as histone modifications, microRNAs, and transcription factors, play important roles in mediating the long-term changes in innate immune cell function.

Innate immune memory should not be confused with immunological memory, which refers to the ability of the immune system to recognize and "remember" specific pathogens or antigens it has encountered in the past. Immunological memory is a fundamental feature of the adaptive immune response, which is the branch of the immune system responsible for mounting a targeted and specific defence against pathogens. When the immune system encounters a foreign substance, such as a bacterium or a virus, specialized cells called lymphocytes, particularly B cells and T cells, are activated to recognize and respond to the specific antigens associated with that pathogen (Janeway et al 2001). During this initial encounter, some of these lymphocytes undergo clonal expansion and differentiate into effector cells, which work to eliminate the pathogen. Other lymphocytes that have been activated transform into long-lasting memory cells (Parham 2014). If the same pathogen is encountered again in the future, the immune system can rapidly and efficiently mount a stronger and more targeted response. This secondary response is characterized by a faster production of specific antibodies by memory B cells and a rapid activation of memory T cells. The presence of memory cells allows for a quicker and more effective immune response upon re-exposure to the same antigen. This phenomenon is the basis for the effectiveness of vaccines, as they stimulate the immune system to generate memory cells without causing the disease (Parham 2014).

Recent studies have suggested that the mechanisms underlying innate immune memory can explain the aberrant immune reactions that characterize the pathophysiology of

sepsis (van der Poll et al 2017). Sepsis refers to the dysfunctional host response to an infection leading to lethal organ dysfunction (Singer et al 2016). Sepsis remains one of the oldest and most prominent clinical syndromes without a definitive therapeutic target in intensive care environments (Angus and van der Poll 2013). Our current understanding of sepsis pathophysiology involves a combination of both innate and adaptive immune responses, including the potential involvement of innate immune memory (van der Poll et al 2021). The role of innate immune memory in sepsis is still an area of active research, and its precise contributions and mechanisms in the context of sepsis pathophysiology are not yet fully understood. It is likely that the interplay between dysregulated immune responses, including both sustained inflammation and immunosuppression, and potential effects of innate immune memory mechanisms, contributes to the complex pathophysiology of sepsis.

Advances in genomics and computational biology have heralded an exponential increase in our understanding of previously unknown, but potentially crucial regulatory factors in sepsis immunopathology. A recent study on blood leukocytes obtained from sepsis patients of divergent infectious aetiologies, unmasked a transcriptional network of genes involved in olfactory receptor (OR) activity as a potential driver of the systemic response during sepsis (Scicluna et al 2020). ORs make up the largest family of G-Protein Coupled Receptors (GPCR), being themselves Class I GPCRs. For a long time, ORs have been thought to be exclusive to the cell surface of olfactory sensory neurons of the main olfactory epithelium. By binding exogenous chemical ligands referred to as odorants, ORs grant the sense of smell through a signalling pathway in which the receptor couples with the olfactory G-Protein (Pietraszewska-Bogiel et al 2019, Tong et al 2021). Although humans possess around 390 different OR genes (Tong et al 2021), so far only around 50 odorant-receptor relationships between odorants and their respective receptors have been identified (Pietraszewska-Bogiel

et al 2019). Despite initial assumptions on the localisation of ORs, recent studies have identified that ORs are not strictly localized to the olfactory epithelium but are also present in other non-olfactory tissues (Tong et al 2021). These ectopic ORs fulfil a variety of functions, including but not limited to cytokinesis (Zhang et al 2012), cell proliferation (Neuhaus et al 2009), sperm cell and monocyte chemotaxis (Spehr et al 2003), insulin and glucagon secretion (Munakata et al 2018, Kang et al 2015), regulation of breathing (Chang et al 2015), and myocardial activity (Kim et al 2015a). Although OR gene expression has been reported in macrophages of mice and humans during inflammation (Orecchioni et al 2022), the precise mechanisms and functional implications of OR gene expression in leukocytes during inflammation and inflammatory disorders is still evolving. These emerging reports certainly highlight the intriguing cross-talk between the olfactory system and the immune system. Whether ORs contribute to innate immune tolerance and sepsis pathophysiology is unexplored.

1.1 Regulation of the Inflammatory Response and Promoting Innate Immune Memory

Elucidating the molecular circuitry that regulates the inflammatory gene program, which in turn promotes innate immune memory to diminish disease pathology while maintaining antimicrobial immunity has been a major challenge in the field (Medzhitov and Horng 2009, Smale 2010, Ochando et al 2023). Although immune memory has been normally attributed to the adaptive immune system, the innate immune system can also “learn” from past exposures to pathogens or antigens. Innate immune cells can either gain the ability to mount a rapid and relatively more robust response to a secondary exposure, or in contrast, dampen their reactivity to repeated challenges. The latter functional adaptation is known as

immune tolerance, also referred to as endotoxin (e.g. lipopolysaccharide (LPS)) tolerance (Fan and Cook 2004, Biswas and Lopez-Collazo 2009), whereas the former has been termed trained immunity, which was first coined in 2011 (Netea et al 2011). Both processes are understood to be governed by multiple epigenetic changes that reprogram innate immune cells thereby modulating the immune response (Foster et al 2007, Netea et al 2016). Although cells go back to being inactive following the initial stimulus, epigenetic alterations that persist allow for a quicker and more potent response, or a dampened reaction when the antigen is encountered again (Divangahi et al 2021). The typical inducers of this 'training' are pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs); they can produce trained immunity and tolerance in the blood and tissues (peripheral trained immunity) as well as in hematopoietic stem cells in the bone marrow (central trained immunity) (Kaufmann et al 2018). Importantly, although dysfunctional innate immune responses may lead to chronic inflammatory illnesses and autoimmune disorders, trained immunity and tolerance may have evolved under continual evolutionary pressure to give greater protection against pathogens and/or injury (Bekkering et al 2021, Lajqi et al 2023).

Trained immunity and immune tolerance are currently regarded as opposing properties of innate immune cells (Ifrim et al 2014), however similarities are also evident (Divangahi et al 2021). It is important to understand that the plasticity of innate immune cells allows them to adapt to various situations in the highly diverse innate immune compartments. Insults such as traumatic injury, infections, environmental toxins and vaccination, promote adaptations to innate immune cells as they migrate out of the bone marrow tissue microenvironment and travel through the blood and other tissues. Notably, the magnitude and duration of exposure to insults can induce specific adaptations that either enhance or dampen the immune response to prevent excessive immunopathology. Prime examples of

these innate immune cell adaptive programs are differentiation, priming, tolerance and trained immunity (Divangahi et al 2021). Regulating innate immune memory can be a powerful therapeutic approach in various disease contexts. For instance, promoting immune tolerance in chronic inflammatory disorders or in organ transplantation are interesting therapeutic possibilities. It might be advantageous to establish trained immunity to support particular cancer treatments or to alleviate the sepsis-induced immunosuppression or immune paralysis.

1.1.1 Epigenetic Influences on Trained Immunity and Immune Tolerance

Trained immunity and immune tolerance have been reported in various innate immune cells, including monocytes, macrophages, dendritic cells (DCs) and Natural Killer (NK) cells (Foster et al 2007, Langenkamp et al 2000, Souza-Fonseca-Guimaraes et al 2012). The primary myeloid-derived cell-types that have been strongly tied to innate immune memory are the monocytes and macrophages, key orchestrators of the immune response. The plasticity of these myeloid cells enables them to adopt several functional states according to extracellular cues (Jakubzick et al 2017, Locati et al 2020). In a resting state, inflammatory gene loci of monocytes and macrophages have been associated with both open (euchromatin) and closed chromatin (heterochromatin) conformations, depending on the primacy of the protein product in the inflammatory response. For example, ribonucleic acid (RNA) polymerase II has been observed poised at promoters of prototypical pro-inflammatory cytokines that include Tumour Necrosis Factor (TNF) (Adelman et al 2009). In doing so, potent soluble mediators of the inflammatory response are readily produced and secreted upon PRR activation, such as the case of recognition of LPS by the Toll-like receptor (TLR) 4, myeloid differentiation factor (MD)-2 (also termed lymphocyte antigen 96 (LY96)) and cluster-of-differentiation (CD)14 receptor complex (Kawai et al 2010). TNF exerts profound effects on

systemic and tissue physiology and is understood to drive approximately half of the acute systemic inflammatory response *in vivo* (Scicluna et al 2013). Later stages of the inflammatory response in macrophages have been associated with chromatin remodelling events making specific regions of the genome accessible to numerous transcription factors and chromatin modifying factors (Medzhitov and Horng 2009). Such regions harbour genes involved in antimicrobial reactions, negative regulation of the inflammatory response and cell cycle control (Foster et al 2007).

1.1.2 Metabolic Influences on Trained Immunity and Immune Tolerance

Advances in genomics have permitted more comprehensive analyses of the inflammatory response to microbes and their ligands, including the identification of several novel cellular biological pathways in response to LPS exposure, particularly metabolic pathways that include oxidative phosphorylation, glycolysis, fatty-acid metabolism, cholesterol biosynthesis and pentose phosphate pathways (O'Neill and Pearce 2016). For example, the production of interleukin (IL-)1β after LPS stimulation of macrophages was strongly dependent on the activation of glycolysis, specifically mediated by the intermediate metabolite succinate (Tannahill et al 2013). Accumulation of succinate was responsible for the activation of the transcription factor hypoxia-inducible factor (HIF)1α, which potentiated the expression of pro-inflammatory genes. In addition, murine macrophage polarization to either a pro-inflammatory, antimicrobial state (termed M1), or to an M2-state characterized by anti-inflammatory and tissue repair activities were dependent on metabolic reprogramming (Jha et al 2015). Specifically, M2 polarization was attributed to glutamine catabolism and uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). Mouse M1 macrophages were characterized by a metabolic break at the enzyme that converts isocitrate to alpha-

ketoglutarate in the tricarboxcylic acid (TCA) cycle. Metabolic reprogramming and epigenetic mechanisms are understood to be integral to establishing immune tolerance or trained immunity in monocytes and macrophages (Dominguez-Andres and Netea 2019). For example, the metabolite mevalonate in the cholesterol biosynthesis pathway was identified as a key factor in promoting trained immunity in macrophages (Bekkering et al 2018). Moreover, in the same study monocytes of patients diagnosed with hyper immunoglobulin D syndrome (HIDS), characterized by deficiency in mevalonate kinase and abnormal accumulation of mevalonate, exhibited a constitutive trained immunity phenotype that may explain the sudden flares of sterile inflammation in these patients. In a separate study on immune tolerance, an epigenetic and transcriptional axis involving cholesterol biosynthesis genes was associated with the manifestation of an immune (endotoxin) tolerant phenotype in circulating monocytes of patients hospitalized due to community-acquired pneumonia relative to age and sex-matched control participants (Brands et al 2021). Of note, both trained immunity and tolerant phenotypes in monocytes lasted longer than the established 24 – 48-hour lifespan of circulating monocytes (Patel et al 2017). Trained monocytes persisted in the peripheral blood for at least three months (Kleinnijenhuis et al 2012), whereas immune tolerance exemplified by reduced production of IL-6 in monocytes persisted for at least one month after infection (Brands et al 2021). The persistence of these trained and tolerant monocytes is likely maintained by modifications of existing haematopoietic progenitor cells in the bone marrow, continuing to replenish the numbers as they turn over (Saeed et al 2014).

1.2 Sepsis, Immune Dysfunction and Innate Immune Memory

1.2.1 Epidemiology and Clinical Significance of Sepsis

Sepsis remains a global healthcare problem. It is defined as the dysregulated host response to infection that leads to lethal organ dysfunction (Singer et al 2016). In 2017, the global incidence of sepsis was estimated at 48.9 million cases, and 11 million attributable deaths representing 19.7% of all deaths worldwide (Rudd et al 2020). Respiratory infections, including pneumonia, intra-abdominal and urinary tract infections are causes of sepsis, particularly in older individuals (Vincent et al 2006) The sepsis incidence rate in Malta in 2017 was estimated at 1349 patients per year, and mortality rate at 20.2% (Rudd et al 2020). While age-standardized incidence and mortality have declined between 1990 and 2017 (Rudd et al 2020), mainly attributable to advances in antimicrobial therapy and supportive care (Prescott and Angus 2018), sepsis survivors continue to suffer long-term complications (Shankar-Hari and Rubenfeld 2016). These figures, however, may actually be much higher due to the difficulties in reporting cases of sepsis in countries of a lower socioeconomic standard (Dugani et al 2017). Globally, sepsis has been associated with an average of around 25 – 30% of hospital fatalities, a figure that rises to 40 – 50% in cases complicated by other co-morbidities and complications (Cohen et al 2015). Patients diagnosed with septic shock, a major complication of sepsis reflecting vascular endothelium failure, have mortality rates as high as 70% (Singer et al 2016, Angus and van der Poll 2013). It has been widely hoped that a revolution in treatment, that is immunotherapy, would provide much-needed aid to improve outcomes. However, numerous clinical trials targeting components of the host inflammatory response have so far resulted in no significant improvements (Marshall 2014, Prucha and et al 2017).

1.2.2 Global Healthcare Burden of Sepsis

It is evident that sepsis carries a high burden on patients, their relatives, health care systems and accompanied by significant socioeconomic costs. A study by Paoli et al (2018) on the healthcare costs of sepsis in the United States assessed that on average, patients aged around 65 years required $24,638, and $38,298 to treat sepsis and septic shock, respectively. These costs were found to fluctuate depending on whether a patient was hospitalised with sepsis upon admission, or whether the development of sepsis was the result of a nosocomial infection. In 2018, the treatment, management and rehospitalisation of patients due to sepsis in the United States is estimated to tally an annual cost of about $62 billion (Danna 2018). A more recent study concerning the evaluation of healthcare costs of sepsis was carried out by van den Berg et al (2022), who selected 26 studies dated between January 2010 and January 2022 for evaluation. This study found that the mean costs varied largely from country to country, with an average healthcare cost of €36,191 per person, an upper quartile of €53,349 and a lower quartile of €17,158. On average, countries were found to have spent 2.65% of their healthcare budgets on treating and managing sepsis, comprising 0.33% of their gross national product (GNP) (van den Berg et al 2022). Another recent study carried out by Schmidt et al (2022) in Germany focused on the associated healthcare costs of sepsis survivors after leaving intensive care. It was noted that patient re-hospitalisations within the first six months of discharge contributed the most towards increasing the healthcare burden, with an average of €13,787 per rehospitalisation. Without rehospitalisation, healthcare costs concerning management and recovery from sepsis were found to be noticeably lower (Schmidt et al 2022). Altogether, these alarming estimates and lack of an approved specific treatment prompted the World Health Organization (WHO) to pass a resolution in 2017 recognizing sepsis as a global health priority (Reinhart et al 2017).

1.2.3 Pathophysiology of Sepsis and Effects on Innate Immune Memory

One of the main pathophysiological features of sepsis is widespread simultaneous pro- and anti-inflammatory pathways (van der Poll et al 2021). Excessive pro-inflammatory reactions damage tissues during the acute phase, while sepsis-induced immunosuppression (or immune paralysis) increases the risk of nosocomial infections and maladapted tissue recovery (**Figure 1.1**). A consistent observation in patients with sepsis and mouse models of sepsis is that monocytes-macrophages are tolerant, or show a reduced ability to respond to LPS and other PAMPs and DAMPs. These observations have been credibly documented in studies of whole-blood organ culture, where LPS, high-mobility group box (HMGB) 1, or other stimulating chemicals are introduced directly into fresh whole blood taken from patients (Cavaillon and Adib-Conquy 2006, Monneret et al 2008). Monocytes in the blood of patients with sepsis release 80%–90% less TNF, IL-1, and other soluble mediators of the host inflammatory response compared to healthy individuals. Notably, this sensitivity deficit is not specific to LPS, but also to other TLR ligands (Monneret et al 2008, de Vos et al 2009). The mechanism underlying this tolerant condition is uncertain, however it may involve differentiation to the alternatively activated phenotype or M2 status, downregulation of *TLR4* expression, impaired NF-kB signalling (Iskander et al 2013), and more recently due to several widespread defects in immune cell metabolism (Cheng et al 2016). Analysis of post-mortem tissue revealed that cells taken from the spleen and lung exhibited suppressed cytokine responses, which was indicative of immunosuppression (Boomer et al 2011).

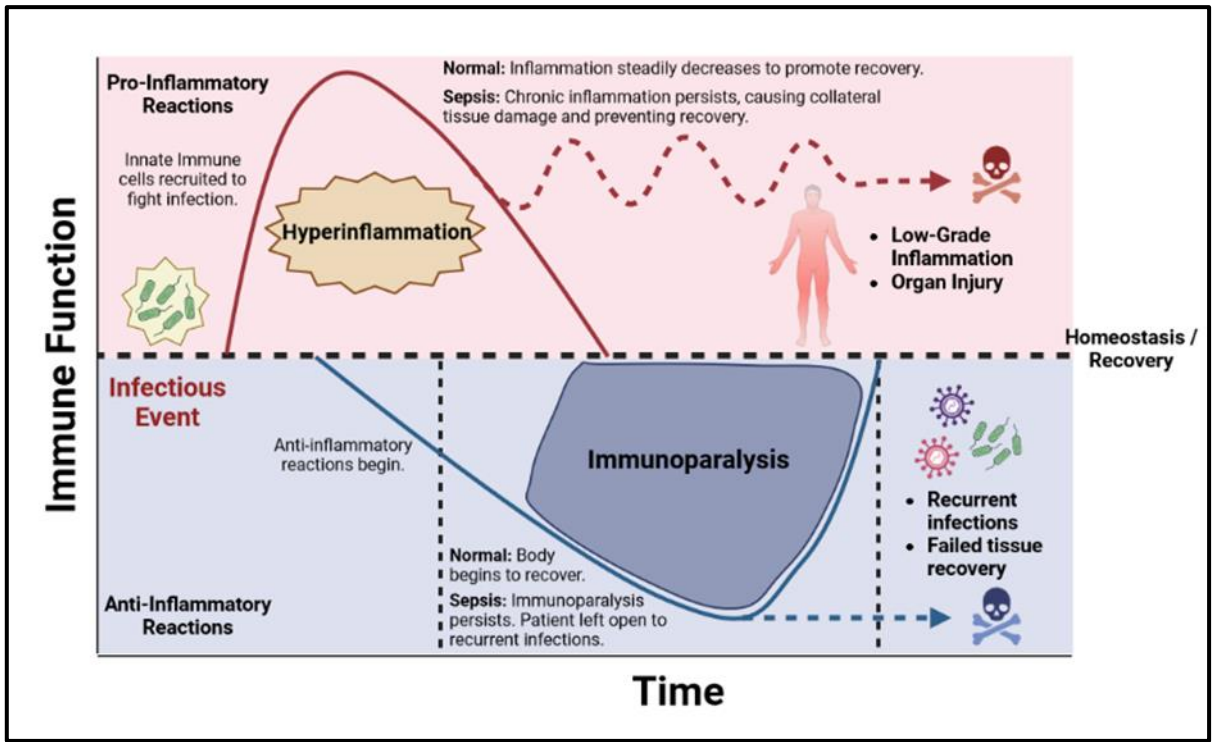


Figure 1.1: The paradigm of simultaneous pro- and anti-inflammatory reactions during the host response to infection. The host response to infection consists of a balance of pro-inflammatory and anti-inflammatory reactions. It begins with an acute hyperinflammatory phase in which innate immune cells are recruited en masse to the site of infection. To limit the damage caused by hyperinflammation, anti-inflammatory reactions commence simultaneously to gradually decrease the inflammatory response. Under normal circumstances, both pro- and anti-inflammatory reactions eventually balance out to maintain homeostasis. However, this homeostatic control is lost in sepsis, leading to cumulative organ damage, immunosuppression, and eventually death (Figure created using BioRender).

Recent large clinical genomics studies in whole blood leukocytes obtained from sepsis patients have shown that transcriptional signatures attuned to immunosuppressive patterns and endotoxin tolerance, suggestive of immune paralysis, were associated with poor prognosis (Davenport et al 2016, Scicluna et al 2017). A study by Boomer et al (2012) discovered an overall reduction of circulatory innate immune cells in cases of early acute sepsis, coupled with a decrease in pro-inflammatory and antimicrobial effector receptors on T-cells as the disease progressed (Boomer et al 2012). These changes were further compounded by the decrease of ligands for costimulatory receptors while those for inhibitory receptors increased. The shift in ligands is also accompanied by an increase of both regulatory

T-cell (Treg) and myeloid-derived suppressor cells (MDSC) in early sepsis, which further support the development of immune paralysis by inhibited action across the innate and adaptive immune responses (Boomer et al 2012, Bomans et al 2018, Nakamori et al 2021). Guisset et al (2007) discovered an overall reduction of circulatory dendritic cells during acute sepsis, while Hotchkiss et al (2002) noted a significant reduction of splenic follicular dendritic cells during sepsis. Both studies are suggestive of widespread apoptosis of effector cells during sepsis (Hotchkiss et al 2002, Guisset et al 2007), a process that is facilitated by increased caspase enzyme activity via the formation of death-inducing signalling complexes (DISC) (Nakamori et al 2021). Studies by De et al (2003) and Faivre et al (2007) also suggested that monocytes also demonstrated functional impairments in sepsis; being unable to affect a T-Cell proliferation response and differentiate into functional DCs *in vitro* (De et al 2003, Faivre et al 2007).

In addition, sepsis has been correlated with a severely reduced level of cell-surface human leukocyte antigen (HLA)-DR on circulating monocytes. HLA-DR is a cell surface molecule which is used as an indicator for monocyte and macrophage functional states, shown to be upregulated by IFN-γ and downregulated by IL-10 (Monneret et al 2008). The link between low HLA-DR expression and monocyte dysfunction in sepsis was investigated by Wolk et al (2000) using the immune tolerance model, wherein monocytes demonstrated decreased IFN-γ production and T-cell proliferation, consequently leading to a low expression of monocytic HLA-DR (Wolk et al 2000). Although no distinct cut-offs have been defined for HLA-DR, low levels of expression have frequently been linked with a poor patient outcome in the cases of sepsis (Monneret et al 2008, Nakamori et al 2021). Granulocyte macrophage-colony stimulating factor (GM-CSF) or interferon gamma administration may increase monocyte cytokine production, HLA-DR expression, and numbers of IL-17-expressing CD4+ T cells;

however, it is unknown whether this will improve organ function, physiological homeostasis, or survival.

During the host response to infection, haematopoietic stem cells (HSCs) in the bone marrow (BM) may be activated when exposed to LPS, proliferating and then differentiating from their long-term self-renewing phenotypes to form shorter-lived multipotent progenitors (MPPs) (de Laval et al 2020). These then differentiate into downstream progenitors based on transcriptional influences in the external environment, performing lineage-specific renewal effector myeloid or lymphoid cell populations (Mitroulis et al 2018, Schultze et al 2019). The resulting shift in epigenetic and transcriptional landscape following the acute proliferation stage is considered the foundation upon which innate immune memory at the progenitor level is established, (Netea et al 2016), facilitating rapid and efficient myelopoiesis upon secondary exposure (Mitroulis et al 2018, Schultze et al 2019). This is especially true in the case of granulocyte-monocyte progenitors, which are key to the proliferation of trained monocytes and the maintenance of longer-term innate immune memory (Bomans et al 2018). A study by Rodriguez et al (2009) provides evidence that the overall reduction of mature effector myeloid cells in sepsis may be linked to induced quiescence in HSCs, preventing them from differentiating into MPPs. HSCs exposed to septic conditions (i.e. repeated exposures to LPS) demonstrated expansive cell replication in response to immunogenic stimuli, but were noted to remain in a state of cell-cycle arrest. These ‘septic’ HSCs presented downregulated expression of transcription factors such as PU.1 and CCAAT/Enhancer-binding protein-Alpha (CEBPα) and marked increases in cell-cycle inhibitors such as p21 and the guanosine triphosphatase (GTP-ase) LRG47 (Rodriguez et al 2009).

1.3 Olfactory Receptors

1.3.1 The Structure and Signalling of Olfactory Receptors

ORs form part of the largest family of GPCRs, which are membrane-bound proteins. All GPCRs bear a similar segmented structure of seven alpha-helical hydrophobic transmembrane fragments, with the terminal ends consisting of an amino terminus extracellularly, and a carboxyl terminus intracellularly (Kobilka 2007, Latorraca et al 2017, Cong et al 2022). Both terminal structures demonstrate a high degree of variability, although the amino terminus has been shown to showcase a greater degree of recombination. Furthermore, a degree of variability has also been demonstrated in between the fifth and sixth transmembrane segments, which in combination with the variable termini, affect receptor function (Kobilka 2007, Latorraca et al 2017).

Native GPCR ligands may bind to their corresponding receptors relatively anywhere along the transmembrane segments in areas referred to as orthosteric sites (Kobilka 2007, Latorraca et al 2017). However, some ligands may also alter the structure, reactivity and sensitivity of GPCRs, modulating their affinity. These ligands are referred to as allosteric modulators and have proven pharmacologically popular for modulating the efficacy of various GPCR proteins. Molecular dynamics simulations have provided evidence that GPCRs can exist in many metastable intermediary conformational orientations, across which various transmembrane segments can exist in simultaneously active and inactive states (Latorraca et al 2017). Signal transduction across the GPCR is dependent on conformational changes across the seven transmembrane segments, which contain heterodimeric G proteins and intrinsic motifs such as ‘D(E)RY’, ‘CWLP’, and ‘NPxxY’ in the third, sixth and seventh transmembrane segments, respectively (Kobilka 2007, Latorraca et al 2017, Cong et al 2022).

In general, ORs contain an olfactory-specific G (G_{olf}) protein which activates adenylate cyclase 3 (AC3) in response to odorant binding (**Figure 1.2**). This enzyme produces cyclic adenosine monophosphate (cAMP), which binds to cyclic nucleotide gated ion channels (CNG), increasing their permeability to sodium and calcium. The resulting cationic influx results in depolarisation, triggering signal transduction. A simultaneous influx of chloride ions by calcium-activated anion channels further amplifies this signal. This signalling pathway is gated against activation in the absence of odorant ligands by high ambient concentrations of cations, generating a resting potential that determines the signal's duration, frequency and latency (Purves et al 2001, Antunes et al 2014).

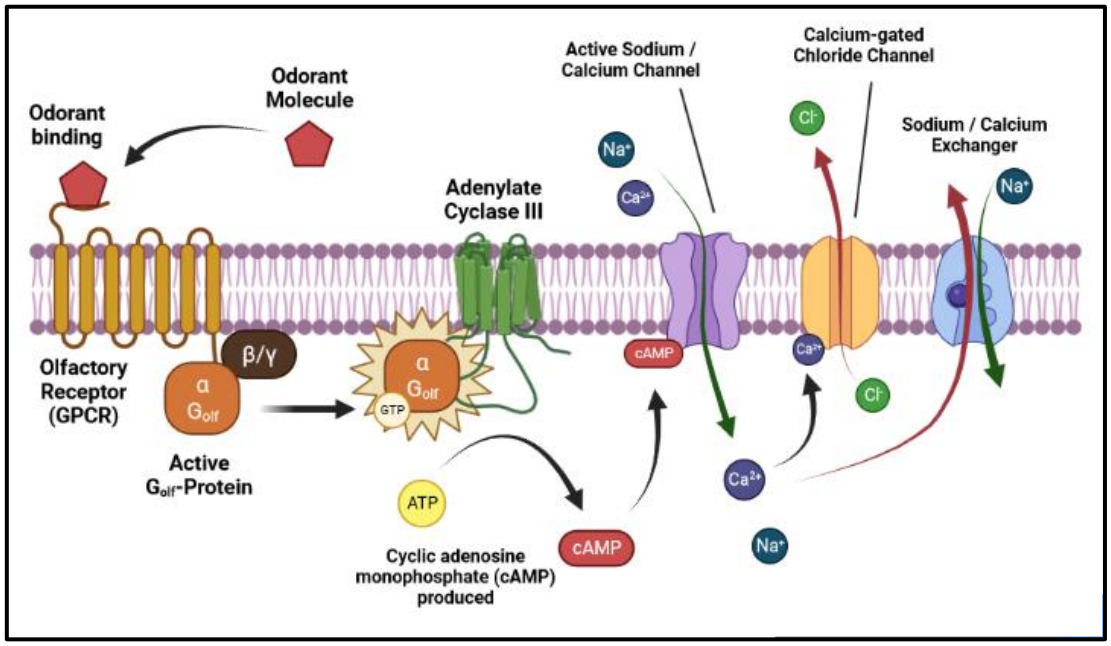


Figure 1.2: A general Olfactory Receptor signalling pathway. Olfactory receptors first bind to a corresponding odorant molecule which activates an olfactory-specific G_{olf} Protein. This protein then activates adenylate cyclase III (ACIII), which in turn utilises adenosine triphosphate (ATP) to produce cyclic adenosine monophosphate (cAMP). The cAMP then binds to sodium and ion channels, facilitating the influx of sodium and calcium ions into the cell. Calcium then binds to other calcium-gated ion channels and ion exchange proteins to amplify the signal. The duration, frequency and latency of this signal are determined by the potential generated by differing concentrations of cations within and outside of the cell (Figure created using BioRender, adapted using information from Kang and Koo 2012).

1.3.2 The Olfactory Receptor Genes

OR genes comprise the largest multi-gene family in mammalian genomes, themselves representing 2 – 5% of the total number of protein-coding genes in the human genome (Barnes et al 2020). OR genes are divided into two main classes: Class I; which are concentrated on a single cluster in chromosome 7, and Class II; which are more widespread across all chromosomes except chromosomes 18 and Y (Degl'Innocenti and D'Errico 2017). Although the exact number of protein-coding OR loci in the human genome is unknown, it is estimated that from around 857 OR genes, 391 were identified as intact with a further 466 being classified as pseudogenes (Olender et al 2016).

The vast majority of OR genes can be owed to repeated duplications and recombination, which became the genesis for new paralogue clusters required to match the expansive repertoire of corresponding odorants and their many isotypes. However, not all OR genes have been found to retain a close similarity to their origins, with some sharing as little overlap as 35% in their protein-coding sequence (Barnes et al 2020). Although previous models had suggested that most OR genes were single-exon genes lacking introns, more recent studies have found consistencies between protein-coding OR genes and multiple-exon gene models (Ibarra-Soria et al 2014, Barnes et al 2020). A study by Olender et al (2016) conducting RNA-sequencing on human intact OR genes narrowed down 120 transcripts which represented only around a tenth of the complete OR repertoire. The selected transcripts averaged little more than 4000 base pairs (bp) in length while spanning genomic lengths more than 8000 bp. Coding exons averaged an open reading frame of around 940 bp with a highly variable 3' untranslated region (UTR), and a 5' UTR typically little less than 400 bp in length. Splice sites per gene were found to vary between 1 – 5 sites with the most common variations being 2 – 4. The exon located close to the 3' end on most transcripts was discovered to contain

the full OR coding region, with a distinct lack of introns. On the 5' end, a higher level of recombination between OR transcripts were observed, both with exon count and intron sizes. OR transcripts also demonstrated anywhere from 1 – 4 non-coding exons in 5' UTR regions (Olender et al 2016). A study by Barnes et al (2020) later supported these findings with transcriptomic data obtained from human mucosa, accounting for more than double the amount (254 OR genes) of gene transcripts isolated in Olender et al's study. These transcripts also accounted for around half of the protein-coding OR loci discovered thus far, further increasing the coverage of the groundwork made by the previous study. Furthermore, RNA-sequencing data from ectopic ORs in this study implicated that their 5' UTR exons may be shared with adjacent genes, suggesting tissue specificity of ectopic ORs (Barnes et al 2020).

1.3.3 Regulation of Olfactory Receptor Gene Expression

One of the most important features that contributes to pre-transcriptional gene regulation is the nature of the promoter region for various OR genes (Ignatieva et al 2014). During cell development, all OR genes are initially suppressed across all genomic loci. These OR genes are then activated in small quantities by means of transcription factor-promoter binding, until a series of ORs become transcriptionally dominant and predominantly expressed across the cell (Degl'Innocenti and D'Errico 2017). Due to a high level of recombination in OR genes, many single nucleotide polymorphisms (SNPs) have been observed in a vast array of OR gene promoters. These variants may either lead to transcriptional impairments or enhancements and may also alter the biochemical properties of the resulting protein and its respective odorant ligand specificity (Ignatieva et al 2014). OR gene promoters are AT-rich, but typically lack a TATA-box, with a few exceptions. This AT-richness contributes to gene expression by sequential recombination to form transcription factor binding sites (TFBS) which

tend to be tissue-specific (Degl'Innocenti and D'Errico 2017). Some TFBS include ones specific for homeodomain, and early B transcription factors, among others. Various tissues may choose to express certain ORs out of a vast genomic repertoire through epigenetic exclusion of other unchosen promoter regions, being inactivated by histone marks such as H3K9me3, and H4K20me3 (Degl'Innocenti and D'Errico 2017, Sharma et al 2017).

OR genes can also be regulated by means of accessory proteins termed as 'elements.' However, rather than upregulate the expression of certain OR genes, elements are thought to influence the selection of one OR gene over another, being found close to their respective gene loci with a sequence consisting of similar TFBS to existing OR promoters, among some additional sites for an expanded repertoire of transcription factors. Despite their similarities, information as to their selection mechanism remains relatively scarce, postulated have both *in cis* and *in trans* influences across OR loci adjacent to their position along the human genome (Degl'Innocenti and D'Errico 2017, Sharma et al 2017).

Post-transcriptional regulation primarily focuses on interactions with the terminal 5' and 3' UTRs of the olfactory mRNA transcript, depending heavily on the terminal sequences and domains of such transcripts. The AU-rich nature of mRNA transcripts is one such feature that encourages the association of AU-rich binding elements (AREs); which are *cis* elements that encourage post-transcriptional deadenylation and consequent degradation of olfactory mRNAs. A study by Shum et al (2015) found OR mRNAs to be particularly enriched in AREs at the 3' UTR, correlating ARE-rich binding with upregulation in contrast to downregulation. These findings suggest that AREs may instead demonstrate stabilising effects in the case of OR transcripts, a function that some AREs have been known to perform. However, Shum et al

suggests that this pro-regulatory action may indeed be tissue-specific, as both up and down regulations were observed between different cell types (Shum et al 2015).

Another feature that may result in the premature degradation of mRNA transcripts is nonsense-mediated decay (NMD), which is another deadenylation pathway that affects mRNA transcripts with premature termination triggers. The most studied example of this trigger is an intron located just a short distance away from a terminal exonic stop codon, although other examples such as long 3' UTRs and upstream open reading frames, both of which can also house premature stop codons that lead to NMD (Shum et al 2015). Transcriptional OR mRNAs with a low GC-content confer greater stability and demonstrate more efficient translation. This is owed to less secondary structural folds in the translated protein, permitting a lower translational energy requirement. This low energy requirement allows for increased levels of translational expression compared to other classes of GPCRs with richer GC content, suggesting evolutionary pressures to achieve greater levels of expression among OR genes (Shum et al 2015).

1.3.4 Ectopic Olfactory Receptors and Innate Immune cells

Various studies have been performed over the last decade involving ectopic ORs on innate immune cells. Several different ectopic ORs have been identified thus far, with variances from one anatomical site to the next, and also being affected by the type of disease processes present. Their ability to bind an extensive arrangement of ligands including metabolic by-products and antigenic molecules makes them an indispensably utilitarian component in the innate immune response, although their defined roles in the immune response remain largely unknown (Li et al 2013).

A study by Li et al (2013) was among the first to discover ORs in macrophages. Li et al postulated that ectopic ORs could potentially have a role in the activation and differentiation of pulmonary macrophages. Ectopic ORs were found to be expressed alongside the vast array of receptors, initiating chemotactic processes which cause pulmonary macrophages to respond to local invasions by bacterial pathogens. Mouse airway tissue treated with LPS was found to significantly increase the expression of multiple ectopic ORs 12 hours after exposure, including: *Or65, Or272, Or352, Or446, Or568, Or622, Or657* and *Or1014*. Further stimulation with octanal; a known OR ligand formed from lipid peroxidation; resulted in a significant upregulation of monocyte chemotactic protein 1 (MCP-1), which selectively acts on and improves the recruitment of monocytes to a site of active infection. The findings of Li et al's study favour the implications of ORs in immune cell recruitment, suggesting that they may contribute towards increasing the efficacy of the innate immune response (Li et al 2013).

Another study into the function of ectopic ORs and their function in innate immune cells stems from Vadevoo et al (2021), which investigated the role of the ectopic *Olfr78* in tumour-associated macrophage (TAM) differentiation in mice. *Olfr78* (human ortholog; *OR51E2*) has been identified as a lactate-binding GPCR that is largely restricted to macrophages, interacting with Gpr132 to facilitate the conversion of naïve macrophages in the presence of IL-4 into anti-inflammatory M2 macrophages. These macrophages in turn decrease the activity of other immune cells at the tumour site, increasing the chances of tumour progression and penetration into deeper layers of organ tissue and favouring metastasis. This study suggested that not all ORs necessarily influence the differentiation of monocytes and macrophages into pro-inflammatory phenotypes but can also promote anti-inflammatory phenotypes (Vadevoo et al 2021). Additionally, another study by Lee et al (2019) documented the expression of OR51E2 in macrophages in the case of ulcerative colitis and its

use as an indicator for both innate immune cells and M2 macrophage polarisation. This study provides further evidence that aside from modulating antitumour immunity, OR51E2 may also possess a role in the suppression of inflammatory responses (Lee et al 2019).

Orecchioni et al (2022) investigated the pro-inflammatory effects of ectopic ORs by studying mouse *Olfr2*, an ortholog of human *OR6A2*; itself an olfactory receptor expressed in circulatory macrophages resident to atherosclerotic plaques. This receptor has been linked with the activation of reactive oxygen species (ROS) that consequently leads to inflammasome assembly with NLR family pyrin domain containing 3 (NLRP3). Another effect of the activation of the inflammasome by OR and LPS costimulatory signalling is the release of IL-1α and IL-1β. In the context of Orecchioni et al's study, however, the action of *OR6A2* in mediating the inflammatory response was found to be a contributor towards the aggravation of atherosclerosis. Nevertheless, the study also highlights that there are indeed various olfactory receptors that may recognise and bind circulating metabolites to initiate various signalling pathways that contribute towards the maintenance of the inflammatory response (Orecchioni et al 2022).

Ectopic ORs have been also detected directly influencing the phagocytic action of alveolar macrophages, as per Weidinger et al's (2020) study. Weidinger et al reported the effects of OR2AT4, an olfactory GPCR suspected to affect the performance of alveolar macrophages especially in chronic conditions such as chronic obstructive pulmonary disease (COPD). It was discovered that alveolar macrophages possess transmembrane OR2AT4 which exist in a dormant state in the absence of corresponding ligands. Upon activation, OR2AT4 interacts with transmembrane ion channels by direct signalling pathways to facilitate the intracellular uptake of calcium ions. The consequentially high concentration of intracellular

calcium ions in turn inhibits phagocytosis, effectively modulating the efficacy of the innate immune response (Weidinger et al 2020).

Altogether, these studies laid the foundation for further investigations into the expression patterns and functional roles of ORs outside of the *bonafide* olfactory system. Although the exact functions of the full repertoire of ectopic ORs remain largely unknown, there is sufficient evidence suggesting that these ORs may have significant links in regulating the innate immune response in humans. Evidence showing soluble metabolites as ligands of ORs raise the exciting possibility that ORs may influence the immunometabolic circuits, and in turn innate immune memory.

1.4 Hypothesis, Aims and Objectives of this Study

The study **hypothesis is that olfactory receptors are associated with the host response to infection and the induction of innate immune memory**. To test this hypothesis, the following aims will be addressed:

1. Describe the expression patterns of ORs in specific immune cell-types during infection and sepsis.
2. Test the inducibility of OR gene expression in innate immune cells after LPS stimulation *in vitro*.

To address these aims, this study will leverage on advances in integrative biology, by combining genomics, bioinformatics, immunology and cell biology. The combined bioinformatics and *in vitro* approach will be used to construct a model of OR gene expression patterns in sepsis patient leukocytes, as well as in the context of in vitro stimulations of innate immune cells that include monocytes, macrophages and epithelial cells. In doing so, prioritizing genes for subsequent *in vitro* functional tests in the context of either immune tolerance models or trained immunity. This study also seeks to complement existing knowledge regarding the functions of ectopic ORs in the human immune system, particularly their role in facilitating and modulating the innate immune response.

Chapter 2:

Materials & Method

2 Materials and Method

2.1 Critically Ill Patient Populations, Public Datasets and Ethics Declarations

Several publicly available sample populations containing genome-wide ribonucleic acid (RNA) expression data had been identified for the purposes of describing and mapping OR gene expression during infection and sepsis. The first dataset that was utilised in this study pertains to a collection of critically ill intensive care unit (ICU) patients diagnosed with sepsis (n=404) or non-infectious critical illness (n=93) on admission. Patients were included in the previously executed Molecular Diagnosis and Risk Stratification of sepsis (MARS) study between January 2011-June 2016, in the mixed ICUs of two tertiary teaching hospitals in the Netherlands, the University Medical Center Amsterdam, Amsterdam, and the University Medical Center Utrecht, Utrecht (ClinicalTrials.gov identifier NCT01905033) (Klein-Kouwenberg et al 2013, van Vught et al 2016, Scicluna et al 2017, Scicluna et al 2020). Sepsis patients were diagnosed with Community-Acquired Pneumonia (CAP) (n=101), Hospital-Acquired Pneumonia (HAP) (n=105), Intra-abdominal infections (n=116), Catheter-related bloodstream infections or primary bacteraemia (Cardiovascular infections; n=49), and genitourinary infections (n=33). Non-septic (non-infectious) controls (n=93) were critically ill patients who were selected based on primary site of suspected infection. Patients suspected of infection and reclassified retrospectively as non-infectious patients with for example respiratory distress due to exacerbated chronic obstructive pulmonary disease, or pulmonary embolisms, were termed no-CAP (n=33). No-CAP patients served as control population to CAP patients (Scicluna et al 2015) In addition, patients admitted to the ICU post-abdominal surgery not for infectious reasons, for example colorectal cancer surgery, were included as post-operative non-infectious controls (post-op_GI; n=42). Post-op GI patients served as controls

for patients diagnosed with intra-abdominal infections (Scicluna et al 2018). The study also involved a population of healthy volunteers (n=42) which were used to provide a baseline between a state of good health, sepsis and infection. Genome-wide RNA expression data of PaxGene™ whole blood specimens obtained from patients and healthy volunteers were obtained via the National Center for Biotechnology Information (NCBI) Gene Expression omnibus (GEO) accession GSE65682. RNA from each participant was processed and hybridized to Affymetrix U219 microarrays at the Cologne Centre for Genomics. A summary of the ICU patient population and healthy volunteers included in the MARS study is tabulated in **Table 2.1.**

Table 2.1: Summary and demographics of critically ill patients and healthy volunteers included in this thesis from the MARS Consortium Study.

Variable	**Sepsis**					**Non-Sepsis**		**Healthy**
Category	**CAP**	**HAP**	**Abdo**	**Cardio**	**Uros**	**CAP Ctrl**	**Abdo Ctrl**	**Volunteers**
No. of Subjects n (%)	101 (25.0)	105 (26.0)	116 (28.7)	49 (12.1)	33 (7.6)	33 (44.0)	42 (56.0)	42 (5.8)
Total No. of Subjects n (%)	*404 (77.5)*					*75 (14.4)*		*42 (8.1)*
Demographics								
Age (Years)	61 (52, 73)	61 (54, 72)	63 (56, 71)	61 (53, 70)	64 (56, 76)	55 (48, 67)	62 (54, 73)	46 (30, 63)
Gender								
Males, n	58	73	62	28	14	22	27	24
Females, n	43	32	54	21	19	11	15	18

Following up on OR expression patterns in whole blood, it was necessary to assess differential expression of ORs in sorted peripheral blood mononuclear cells (PBMCs) to identify which ORs may be involved in the functions of immune cells. To achieve this, a small dataset from the MARS project was obtained with RNA-sequencing data pertaining to sorted PBMCs (Khan et al 2020). In this study, heparinised whole blood (10ml) was collected from healthy subjects (n=4) and from a selection of critically ill patients diagnosed with sepsis secondary to CAP (n=6) within 24 hours of ICU admission. PBMCs were then purified by density gradient centrifugation (Ficoll-Paque) and specific cell-types purified using a fluorescence-activated cell sorting (FACS) AriaIII machine (BD) after staining with antibodies specific for cell-types, that is $CD4^+$ and $CD8^+$ T-cells, $CD14^+$ monocytes and $CD19^+$ B-cells. Total RNA was then isolated from these sorted cells and libraries sequenced accordingly. RNA-sequencing counts were obtained from NCBI GEO accession no. GSE136200. (Khan et al 2020).

All ICU patients included in the MARS study were older than 18 years admitted to the ICU with an expected length of stay longer than 24 hours via an opt-out method approved by the data protection and medical ethical committees of the participating hospitals (Institutional Review Board (IRB) No. 10-056). All healthy volunteers were included after providing informed consent via participant information brochures and consent cards approved by the University Medical Center Amsterdam, Amsterdam, data protection and medical research committee (IRB No. 10-056C).

2.2 Public Datasets of Human Endotoxemia and Innate Immune Cell Models

Public data from a study conducted by Malmström et al in 2022 was chosen for the purpose of investigating differential expression of ORs in monocyte-derived macrophages (MDMs) in response to bacterial stimuli (Malmström et al 2022). Monocyte-derived macrophages were chosen specifically for their importance as principal effector cells in the innate immune response (Jakubzick et al 2017, Locati et al 2020). Briefly, heparinized blood (2x9mL) was collected from healthy donors (n=4), diluted (1:1) with phosphate-buffered saline (PBS) and PBMCs were isolated by density-gradient centrifugation (Ficoll-Paque). CD14+ monocytes were purified using an anti-CD14–coated microbeads according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometry was used to ascertain the purity of CD14+ monocytes against CD15+ neutrophil or CD3+ lymphocyte or CD56+ natural killer cell contamination. Subsequently, CD14+ monocytes cells were seeded into six-well plates at a density of 1×10^6 cells per well in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, Amarillo, TX), which was supplemented with 10% sterile foetal calf serum (FCS), gentamicin (10 μg/ml; Lonza Bioscience, Durham, NC), 2 mM GlutaMAX (Thermo Fisher Scientific, Waltham, MA), 1 mM sodium pyruvate (Thermo Fisher Scientific, Waltham, MA), and recombinant human granulocyte macrophage-colony stimulating factor (5 ng/ml; Prospec, Ness-Ziona, Israel). These were cultured for 7 days, during which the RPMI medium was changed for fresh medium on the 3rd and 5th days. On the 7th day, cells were washed and harvested using Tryple-select (Thermo Fisher Scientific, Waltham, MA) and seeded to 500,000 cells per well of a 48-well tissue culture plate 24 h prior to stimulation. MDMs were then exposed to 100ng/mL LPS for 2, 6, or 24 hours. Total RNA was isolated from MDMs exposed to LPS or not (medium control) for 6 h. RNeasy mini kits (Qiagen, Hilden,

Germany) were used for total RNA isolation according to the manufacturer's instructions. RNA quality was assessed by bioanalysis (Agilent), with all samples having RNA integrity numbers > 7. Total RNA concentrations were determined by Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). RNA sequencing libraries were prepared from 300 ng of total RNA using KAPA RNA HyperPrep with RiboErase (Roche, Basel, Switzerland) library kits and sequenced using the Illumina HiSeq4000 instrument (Illumina) to generate single reads (50 bp). RNA expression data were obtained from NCBI GEO accession GSE201958 (Malmström et al 2022). Healthy volunteers for blood sampling were recruited in accordance with a study protocol that was reviewed by the University Medical Centre Amsterdam, Amsterdam, the Netherlands, data protection and medical ethical committee (IRB No. 2015_074). All donors signed informed consent cards prior to phlebotomy.

Expression patterns of OR genes were assessed in the context of human bronchial epithelial cells (HBE) cells. Publicly available RNA-sequencing data was obtained from a study by Ramirez-Moral et al in 2021. HBE cells were obtained anonymously with written consent from patient volunteers (n=3) who had been undergoing a lobectomy for lung cancer at the University Medical Centre Amsterdam, Amsterdam, the Netherlands (Ramirez-Moral et al 2021). Epithelial cells were isolated according to the methods described by Fulcher and Randell (Fulcher and Randell 2013). Briefly, HBE cells were obtained following isolation of healthy transbronchial tissue away from tumorous tissue cells and expanded in human type IV placental collagen coated porous support 24-well Transwell inserts (Corning, Corning, NY, USA) for differentiation in PneumaCult-Ex Plus media (StemCell Technologies, Vancouver, Canada). After observing confluency at the inserts, the medium was changed to PneumaCult-ALI medium (StemCell Technologies) supplemented with penicillin and streptomycin on the basolateral side. The medium on the apical side was removed to form an air–liquid interface.

After 30 days, well-differentiated HBE cells were then exposed to 1µg/ml bacterial flagellin originating from *Pseudomonas (P.) aeruginosa* for the duration of 24 hours. Total RNA was extracted using the High Pure RNA Isolation Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. (Ramirez-Moral et al 2021). RNA quality was assessed by bioanalysis (Agilent, Santa Clara, CA), with RINs >7. Next-generation sequencing libraries were prepared by means of the KAPA RNA HyperPrep with RiboErase (Roche, Basel, Switzerland) as per manufacturer's instructions. Resultant libraries were sequenced using the Illumina HiSeq4000 (Illumina, San Diego, CA, USA) to generate 50 bp single-end reads. RNA-sequencing data were obtained from NCBI GEO accession GSE164704. The University Medical Center Amsterdam, Amsterdam, the Netherlands data protection and medical ethics committee approved the study (IRB no. 2015-122#A2301550). All patients provided written informed permission prior to sampling.

To study the kinetics of OR gene expression in the context of *in vivo* acute systemic inflammation, publicly available data from a human endotoxemia study was selected (Scicluna et al 2020, Perlee et al 2018). Eight healthy volunteers were infused directly with a 2ng/kg dose of *Escherichia (E.) coli* LPS to elicit systemic inflammation in a controlled clinical setting (**Figure 2.1**). This method has been adopted to monitor functional changes in the cells of the human immune system over a given period, mirroring the innate immune responses during cases of sepsis (Andreasen et al 2008, van Lier et al 2019). In Scicluna et al's study (2020), whole blood was collected in PaxGene Blood RNA tubes before and 2, 4, 6, and 24 hours after LPS administration. Total RNA was then isolated from each timepoint using PaxGene Blood RNA isolation kits (Qiagen) and an automated QIAcube™ instrument (Qiagen) according to manufacturer's automated isolation instructions (Scicluna et al 2020). Subsequent bioanalysis (RINs > 6.0) and concentration determination by Qubit® 2.0 Fluorometry (Life Technologies,

Carlsbad, CA, USA), RNA was processed and hybridized to Affymetrix Human Transcriptome 2.0 microarrays at the Cologne Center for Genomics. Volunteers were part of a phase I, randomized, single-blind, parallel group, placebo-controlled study in 32 healthy male subjects (study acronym CELLULA; Trial Register number 2014-002537-63, clinicaltrials.gov identifier NCT02328612). The Kingdom of the Netherlands' Central Committee on Research Involving Human Subjects (CCMO), data protection and medical ethics committee of the University Medical Centre Amsterdam, Amsterdam, the Netherlands approved the study. Written informed consent was obtained from all subjects. For the purposes of the herein described project, only those samples from volunteers given placebo were used (Scicluna et al 2020). A pre-enrolment physical examination, haematological and biochemical screen, as well as electrocardiograms of all participants were done and ascertained as normal by attending clinicians. The HTA2.0 microarray data of human endotoxemia volunteers (placebo treated) were obtained from NCBI GEO accession GSE134356.

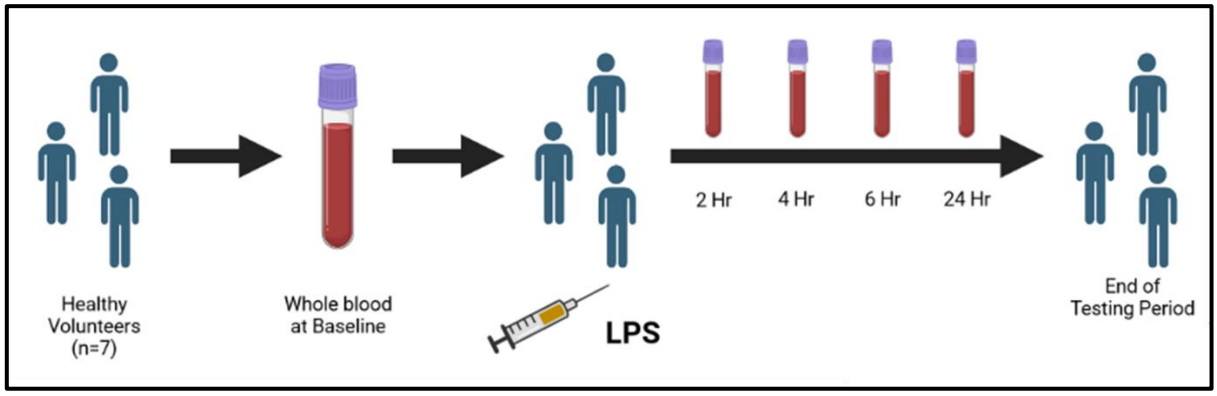


Figure 2.1: A visual summary of the experimental process used to investigate OR expression kinetics over the course of the *in vivo* innate immune response as carried out in Scicluna et al's study in 2020. Volunteers were first injected with a controlled dose of LPS to initiate an innate immune response. Whole blood was then drawn at the 2-, 4-, 6- and 24-hour time points after administration of LPS. OR expression kinetics over the course of the first 6 hours were investigated to better understand which ORs are involved in the development of trained immunity and endotoxin tolerance, respectively.

2.3 Genome-wide Gene Expression Data Processing and Bioinformatics

Bioinformatics analysis was conducted using the RStudio statistical computing software (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/). Data from Affymetrix microarrays, specifically U219 and HTA2.0 arrays, were obtained in a normalized format as per seminal studies (Scicluna et al 2015, Scicluna et al 2017, Scicluna et al 2020). Briefly, probes were normalized using the RMA method and detection above background (DABG) probe level detection. Probes specific to *Homo sapiens* species with detection p-value < 0.05 in at least one sample were considered for further analyses. First, contrast tables were created using the 'Limma' (Linear Models for Microarray Data) method in R (version 3.36) (Smyth 2005). Limma is a powerful tool that utilises linear models to manage intricate experimental designs and leverages empirical Bayes approaches to leverage information from several genes, resulting in more stable variance estimates and improved statistical power. Limma is highly proficient at detecting genes that are expressed differently by comparing their expression levels across various circumstances, while also accounting for numerous comparisons to effectively manage false discovery rates. A Benjamini-Hochberg adjusted probability less than 0.05 (BH p < 0.05) demarcated significance (Benjamini and Hochberg 1995). The linear models included age and sex as additive covariates.

RNA-sequencing count data were obtained from NCBI GEO accessions of the respective seminal studies. Briefly, count data were generated by firstly assessing raw sequence data using FastQC (Andrews 2023) and, secondly after trimming Illumina adapters and poor-quality bases using Trimmomatic version 0.36 (Bolger et al. 2014) (trimmomatic parameters: leading = 3, trailing = 3, sliding window = 4:15, minimum length = 40). The

remaining high-quality reads were used to align against the Genome Consortium human genome build 38 (GRCh38) using the graph-based aligner HISAT2 version 2.1.0 (Kim et al. 2015b) with parameters as default. Count data were generated utilizing the HTSeq method (Anders et al., 2015), and downloaded as raw counts for the purposes of this project. After obtaining the data, counts were normalized using the DESeq2 method in R (Love et al 2014). DESeq2 is a tool that is specifically designed for analysing differential gene expression using RNA-sequencing data. The model is based on the negative binomial distribution of gene expression count data, which effectively incorporates the variability in read counts. Additionally, this method incorporates normalisation techniques to account for variations in sequencing depth and RNA composition among different samples. DESeq2 calculates gene-specific dispersion, using empirical Bayes shrinkage to improve the precision of fold changes, and applies Benjamini-Hochberg multiple testing adjustment to compensate for false discovery rates. A BH $p < 0.05$ demarcated significance.

Any significant differences in up- or down-regulated fold-changes for specific ORs were visualised by means of volcano plots against a background of overall gene expression. Dot plots were also used to compare between cases and controls to assess for any significant outliers in relative gene expression. Heatmaps were compiled to illustrate the relationships of specific ectopic OR genes, visualising the quantitative changes in OR gene expression. These statistical tools were employed in a step-by-step approach with the intent of narrowing down potential targets that were most likely to be involved in the innate immune response. The targets that were identified to be the best potential candidates were then chosen for further *in vitro* experimental laboratory testing.

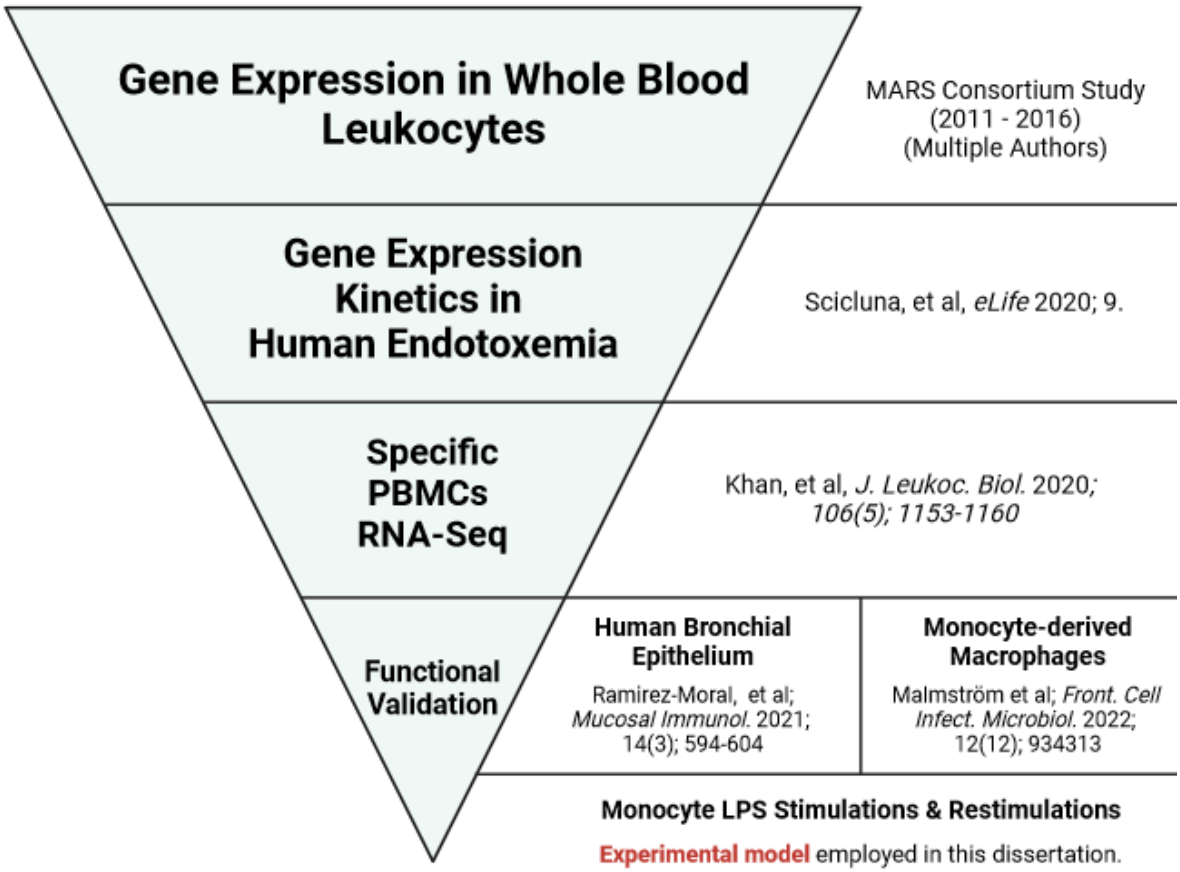


Figure 2.2: A visual summary of the overall approach carried out for the purposes of assessing olfactory receptor gene expression on innate immune cells in sepsis and infection. A top-down approach attuned to the concepts of integrative biology was employed. Olfactory receptor gene expression was first explored in blood leukocytes during sepsis, infection, and good health. Findings were further refined by searching for potential olfactory receptor targets over the course of the innate immune response through an *in vivo* human endotoxemia model. Further filtering for potential cell-specific olfactory receptor targets was performed using datasets for cell-sorted PBMCs, allowing for olfactory receptor gene expression to be assessed across specific innate immune cell types. Functional models on innate immune effector cells, including that used in this study, were pursued to validate the findings obtained by bioinformatics, and test the hypothesis about whether ectopic olfactory receptors are associated with the host response to infection and the induction of innate immune memory.

2.4 Validation Cohort and Ethical Declaration

For experimental validation, a cohort of 8 healthy volunteers (4 males; 4 females) older than 18 years were recruited between October 1st and November 30th, 2023, with mean age equating to 21 years (min. 19 years, max. 22 years). Exclusion criteria were (a) pregnancy, (b) drugs or alcohol use in the 48 hours prior to phlebotomy, (c) febrile illness over the two weeks prior to blood collection, (d) antimicrobial treatment in the two weeks prior to study enrolment, (e) clinically diagnosed cardiovascular disorders and/or primary immunodeficiency. Permission to recruit human volunteers and process human whole blood samples for this study was granted by the Faculty Research Ethics Committee of the Faculty of Health Sciences, and the University Research Ethics Committee Research Ethics and Data Protection (URECA-REDP) on the 28th of April 2023 (FREC No. FHS-2022-00568). A copy of this form can be found in **Appendix I** at the end of this document**.**

2.5 Whole blood Processing and Monocyte Enrichment

Heparinized blood (2x9mL) was collected from each volunteer. This volume was chosen to ensure that enough monocytes for testing were harvested, as monocytes make up the smallest population of circulating blood leukocytes (i.e. c. 0.26 x 10^9 monocytes per 6.07 x 10^9 white blood cells, as per Chen et al 2021). After collection, blood was diluted 1:1 in 1X Dulbecco's phosphate-buffered saline (DPBS; Gibco™ ThermoFisher Scientific, Waltham, United States) and layered carefully over Lymphoprep™ (Stem Cell Technologies, Vancouver, Canada) layer in a SepMate™-15 (15mL) (Stem Cell Technologies, Vancouver, Canada) tube. A polysucrose density gradient centrifugation step is a technique frequently employed for the purposes of PBMC isolation from whole blood. SepMate™ tubes provide a physical barrier

between the PBMC layer and the denser erythrocyte and granulocyte layer, allowing for easier direct retrieval of PBMCs without risking resuspension of the sediment. This makes SepMate™ tubes ideal for rapid and efficient density gradient centrifugations, especially when compared to more labour-intensive and longer retrieval techniques (Stabel and Wherry 2023). Before the addition of the diluted whole blood, the SepMate™-15 tubes were first filled with Lymphoprep™ at a density of 1.077 g/mL (Stem Cell Technologies 2023). The volume of Lymphoprep™ was made up to the barrier insert found inside the SepMate™ tube itself as per the manufacturer's instructions. Once layered, the tubes were centrifuged at 1200g for 10 minutes at room temperature (15 – 25°C) with acceleration set at 9 and break at 5. Notably, SepMate™ tubes are useful in negating the long waiting times required to allow the centrifuges to come to a halt as in a regular density gradient centrifugation setup.

After centrifugation was complete, the SepMate™ tubes consisting of separate blood cell layers and diluted plasma were retrieved and the PBMC cell-containing layer below the plasma was retrieved by pipetting. The PBMC layer were then transferred to a sterile 50mL conical Falcon tube (Fisher Scientific International, Hampton, United States), one for each volunteer sample, and washed three times before further processing. Washing steps were performed by adding 10mL of DPBS to the extracted PBMC cell layer, centrifuging at 400g for 10 minutes with the brakes fully engaged during each cycle (acceleration 9, brake 9). After washing, the enriched PBMCs were resuspended in 2ml DPBS. A volume of 20µL of resuspended PBMCs were taken and mixed with 20µL of Trypan Blue Stain (0.4%) (Gibco™ ThermoFisher Scientific, Waltham, United States), a diazo dye which selectively permeates compromised cell membranes of dead tissues and cells, while leaving live cells with intact membranes unstained. Therefore, stained cells which are counted during this step are considered non-viable, while those which remain unstained are considered live (Tran et al

2011). From this mixture, 10ul of the cells and trypan blue mix was then transferred to a Corning™ Counting Chamber (Corning Inc., Corning (NY), United States). Cell counts were then carried out using the Corning® Automated Cell Counter (Corning Inc. Corning (NY), United States). Cell counts were gated for cell size (larger than 7µm) to account for residual platelets, erythrocytes and cell debris that are typically smaller than 7µm.

Next, monocytes were purified from PBMCs by means of microbead technology according to the manufacturer's instructions for negative selection using the Miltenyi-Biotech Pan-monocyte enrichment kit (Miltenyi-Biotech). All volumes for magnetic labelling have been optimized for up to 10^7 total cells. PBMCs were transferred to a sterile 1.5mL eppendorf tube and centrifuged at 400g for 10 minutes while keeping the centrifuge cool at 10°C. After removing the supernatant, the cell pellet was resuspended in 30 µL of *buffer A* (composition: 0.5% BSA in PBS filter sterilized). To this, 10 µL of FcR Blocking Reagent was added, followed by 10 µL of Biotin-Antibody Cocktail. After mixing well, cells were incubated with antibody mix for 5 minutes in the refrigerator (2−8 °C). Subsequently, 30 µL of *buffer A* was added followed by 20 µL of Anti-Biotin MicroBead suspension. After mixing well, cells were left to incubate for 10 minutes in the refrigerator (2−8 °C). During this incubation step, a LS column per donor was assembled in the magnetic field of a Quadro MACS Separator and rinsed with 3mL *Buffer A* prior to use. 1mL *Buffer A* was added to the PBMC and microbead/antibody mix, and applied to the LS column, collecting flow-through containing un-labelled cells in 15mL falcon tube, representing the enriched monocytes. The column was washed 3X with 3mL (9mL total) *Buffer A* to ensure efficient wash through and collection of un-labelled cells (monocytes). Next, cells were centrifuged at 400g for 10 minutes (10°C), and resuspended in 1 mL full culture medium (composition: RPMI 1640 medium without L-Glutamine (Gibco™ ThermoFisher Scientific, Waltham, United States), 10% Foetal Bovine Serum (Gibco™ ThermoFisher Scientific), 500µM

GlutaMax (Gibco™ ThermoFisher Scientific, Waltham, United States), 500µM sodium pyruvate (Gibco™ ThermoFisher Scientific), and 250µM gentamicin (Gibco™ ThermoFisher Scientific)). Cells were then counted as per Trypan-blue exclusion test described above.

2.6 Primary Monocyte Stimulations

Following the cell counting protocol, the equivalent of 200,000 cells were seeded to each of 4 wells in a 48-well tissue culture plate in a maximum volume of 500µl full culture medium. The 4 wells were designated as (1) medium control, (2) medium control re-stimulation, (3) LPS stimulation, and (4) LPS re-stimulation. Cells were allowed to rest overnight, incubating at 37°C in 5% CO_2 and 90% humidity (Esco™ Scientific). After resting, two wells containing monocytes were stimulated for 24 hours with 10ng/mL lipopolysaccharide (LPS; Invivogen), and two wells left with full culture medium incubating at 37°C in 5% CO_2 and 90% humidity. The following day, supernatants were recovered for ELISAs from stimulation marked wells, and cells were harvested for RNA isolation by adding 200µl ice-cold PBS followed by 300µl RNAprotect cell reagent (Qiagen). Cells and supernatants were stored at -80°C until analysed. To the wells marked “re-stimulation”, cells were washed once with warm PBS (37°C) and allowed to rest overnight in full culture medium incubating at 37°C in 5% CO_2 and 90% humidity. The following day, cells were re-stimulated or not with LPS (10ng/mL) for 24 hours. Subsequent to re-stimulation, supernatants and cells were harvested as described previously for ELISA or RNA isolation.

2.7 Enzyme-Linked Immunosorbent Assay Protocol

A sandwich assay ELISA (RnD Systems, Minneapolis, United States) used to measure Human TNF-α produced by stimulated monocytes in response to LPS exposure. This was preferred over other types of ELISAs assays (e.g. direct, indirect, competitive), as a sandwich assay allows for specific biomolecules to be measured from impure substrates, such as supernatant rich in many different cytokines (Rao et al 1997, Sakamoto et al 2018). A working mixture (Concentration: 4µg/mL) of mouse anti-human TNF-α capture antibody (Stock solution: 480µg/mL) was prepared using 1X PBS. For this procedure, 96-well high-binding ELISA plates (Corning) were used. These were first coated with 100µL of capture antibody in each well, sealed and incubated overnight in the dark, at room temperature. The following day, all wells were then washed three times with wash buffer (0.05% Tween-20 in PBS) to remove any excess unbound capture antibody. The wells were then blocked with 300µL of reagent diluent (1% BSA in PBS, sterile filtered), sealed, and incubated at room temperature for 1 hour. Afterwards, the plates were washed vigorously three times with wash buffer, blotting on clean paper towels in between to ensure complete removal of the buffer.

TNF standards were prepared by serial dilution (1:1) of a 1ng/mL starting concentration as described by the manufacturer. To each well, 100µL of sample or TNF standards were added as required, and plates were then incubated for 2 hours at room temperature. At the end of incubation, all wells were aspirated and washed 3X with wash buffer. Subsequently, 100µL of biotinylated goat anti-human TNF-α detection antibody (stock solution: 3.0µg/mL), diluted to a working concentration of 50ng/mL with ELISA reagent diluent, were then added. The plates were sealed once more and incubated for a further 2 hours. A stock solution of streptavidin-horseradish peroxidase (Streptavidin-HRP) was diluted 40-fold with ELISA reagent diluent. Aliquots of streptavidin-HRP were then dispensed at a

volume of 100µL into each sample well, sealing the ELISA plate once more and incubating it in the dark for 20 minutes at room temperature. Next, the plates were washed 3X with wash buffer. Each well was then filled with 100µL aliquots of substrate solution, being covered and incubated in the dark for a further 20 minutes at room temperature. This was followed by 50µL of ELISA stop solution (1N sulfuric acid).

The plate was immediately read using a TECAN Spark® Multimode Microplate Reader (Tecan Life Sciences, Männedorf, Switzerland). OD readings were read at a wavelength of 450nm, with wavelength corrections of 540nm or 570nm being used in tandem. The manufacturer specifies a 5-parametric logistic (5-PL) regression curve to fit readings, however the spectrophotometer is not equipped with software to execute a 5-PL regression. For this reason, readings of the TNF standards were fit in a linear curve and concentrations derived from applying the resultant linear equation to samples.

2.8 Antibodies and Flow Cytometry

Around 100,000 directly harvested monocytes were added to each of two 1.5ml Eppendorf tubes. The tubes were centrifuged at 400g for 5 minutes. The cells were resuspended in 50 µl 1% BSA and 0.1% sodium azide, followed by an antibody mix composed of 5µl phycoerythrin (PE) anti-HLA-DR, 5µl Fluorescein isothiocyanate (FITC) anti-CD14, and 5µl Peridinin-Chlorophyll-Protein (PerCP) anti-human CD3 (all antibodies purchased from BioLegend, USA). A second tube was left unlabelled, serving as negative control. Both tubes were mixed properly and refrigerated at 2 – 8°C for 30 minutes. The cells were then centrifuged again at 400g for 5 minutes at 4°C and the supernatant was discarded. The cells were resuspended again in 100 µl 1% BSA, 0.1% sodium azide and 0.4% paraformaldehyde (PFA), and incubated

in the refrigerator for no more than 15 minutes to fix the cells and fluorophores. Cells were then centrifuged again at 400g for 5 minutes at 4°C and the supernatant was discarded, resuspending cells in 1% BSA and 0.1% sodium azide. The cells were stored in a refrigerator for no more than 48 hours before being analysed by using a BD FACS Calibur Flow Cytometer (BD biosciences). Data were analysed by means of FlowJo version 10.10.0 (BD Biosciences).

2.9 RNA Isolation

Total RNA was isolated using the RNeasy Micro Kit (50) (Qiagen, Hilden, Germany), as specified by the manufacturer. Cells were allowed to thaw over ice, followed by centrifugation at 5000g for 10 min. After removing supernatant, cell pellets were resuspended in 350µL RLT Buffer. The cells were thoroughly mixed in the RLT Buffer to ensure complete cell lysis. The lysate was homogenised by vortexing for 1 minute, after which 350µL absolute ethanol was added and mixed by aspiration. The mixtures were dispensed to spin columns and centrifuged at 8000g for 30 seconds, ensuring that no liquid had remained in the spin column. The collection tube with flow-through was discarded followed by the addition of 700µL of Buffer RW1. The spin column was then centrifuged at 10,000 RPM for 15 seconds. This flow-through was then discarded. Next, 350µL of Buffer RW1 were added and spin column was centrifuged a second time at 8000g for 15 seconds. This flow-through was then discarded. A volume of 10µL DNase I stock solution (1500 Kunitz units dissolved in 550µL of RNase-free water) was added to 70µL Buffer RDD, which were then mixed by gently inversion and centrifuged briefly to ensure complete collection of the mixture. The DNase I incubation mix was then added directly to the RNeasy spin column membrane, where the setup was left to stand at room temperature (25^{o}C) for 15 minutes. This was then followed by the addition of 350µL of Buffer

RW1, and a further centrifugation at 8000g for 15 seconds. The resulting flow-through was then discarded. A volume of 500µL of Buffer RPE was then added to the RNeasy spin column, after which the spin column was again sealed and centrifuged at 8000g for 15 seconds to wash the spin column thoroughly. A further 500µL of Buffer RPE were then added to the RNeasy spin column, which was later centrifuged at 8000g for 2 minutes to ensure complete removal of the RPE Buffer. The RNeasy spin column was then attached to a fresh 2mL collection tube to ensure no residual contamination of RPE Buffer, and a final centrifugation step at full speed for 1 minute was carried out to dry the RNeasy spin column. To collect the purified RNA, the dried RNeasy spin column was first transferred to a fresh 1.5mL collection tube. A volume of 15µL of RNase free water were then added directly to the spin column membrane and left to sit for 2 minutes. The RNeasy spin column was then centrifuged thoroughly at 8000g for 1 minute to ensure complete elution of the RNA, which was then collected as flow-through in the 1.5mL collection tube. The resulting concentrations of RNA were then quantified by using spectrophotometry, carried out using the ThermoFisher Scientific NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, United States).

2.10 Reverse-Transcription quantitative Polymerase Chain Reaction (RT-qPCR)

The cDNA Synthesis protocol used in this study was carried out using the TaKaRa PrimeScript™ RT Reagent Kit (TaKaRa Bio Inc., Kusatsu, Japan). The preparation of the cDNA synthesis template mRNA/primer mixture is outlined in **Table 2.2**. This mixture included a ready-to-use Oligo dT Primer mix, which is a primer that binds specifically to the poly-A tail of mRNA allowing for the synthesis of the first strand of cDNA in the presence of reverse transcriptase (ThermoFisher Scientific 2024a); deoxynucleoside triphosphate (dNTP) mixture,

which consists of multiple free nucleotides for cDNA synthesis and amplification; the template mRNA from which cDNA was to be produced; and RNase-free water. Once prepared and mixed with mRNA, the reaction mixture was then heated at 65^{o}C for 5 minutes, to allow for primer binding with the template mRNA. Due to the heat-sensitive nature of mRNA, the reaction mixtures were then cooled on ice to prevent heat degradation. This reaction mixture was then kept on ice while the reaction mixture was prepared.

Table 2.2: The volumes of components in the Template RNA/Primer mixture for each sample in cDNA Synthesis.

Component	Volumes for 1 Sample (µL)
Oligo dT Primer (50µM)	1
dNTP Mixture (10mM)	1
Template RNA (mRNA)	5
RNase-free dH_2O	3
Total	**10**

The preparation of the reaction mixture for cDNA synthesis is outlined in **Table 2.3**. This reaction mixture consisted of the template mRNA/primer mixture as prepared in **Table 2.2**; 5X PrimeScript Buffer, an RNase inhibitor, PrimeScript Reverse Transcriptase, a modified Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase enzyme, and RNase-free water. The PCR program was programmed and executed in a Biometra TProfessional Standard SL96 Real-Time PCR Thermocycler (Biometra).

Table 2.3: The volumes of components in the reaction mixture for each sample in cDNA Synthesis.

Component	Volumes for 1 Sample (µL)
Template RNA/Primer Mixture	10
5X PrimeScript Buffer	4
RNase Inhibitor	0.5
PrimeScript RT enzyme	1
RNase-free dH_2O	4.5
Total	**20**

The cDNA Synthesis PCR cycle was then performed as shown in **Figure 2.3**:

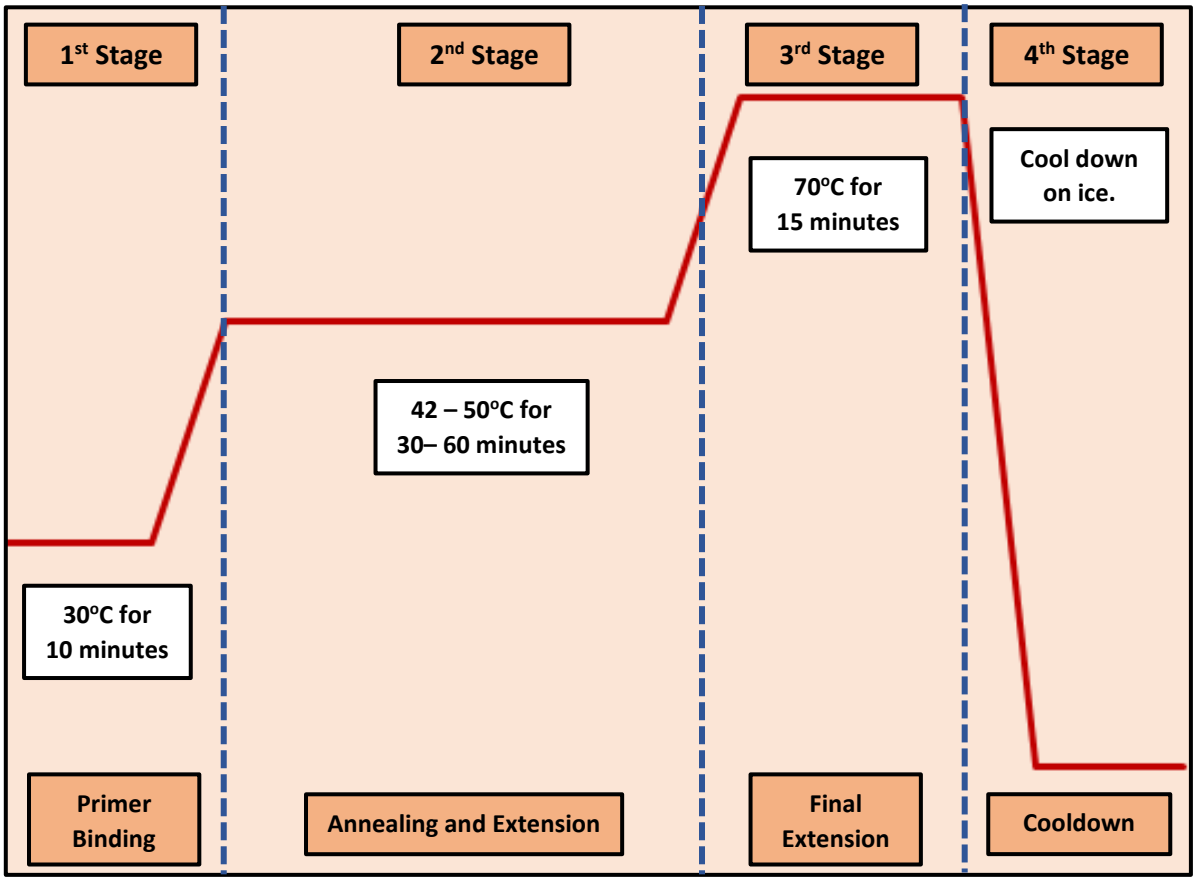


Figure 2.3: Thermal Profile for cDNA synthesis PCR program. The RT-PCR for cDNA synthesis consisted of four major stages, three of which were carried out on a thermal cycler, with a final cooling step on ice. The first stage allowed for binding of the primers to the mRNA template, being held at 30°C for 10 minutes. The second stage involved the annealing and extension steps of the cDNA strands from the mRNA template. A stage allowing for a final extension to minimise the potential for non-specific amplification produces followed, held at 70°C for 15 minutes. The amplified cDNA product was then transferred to a bed of ice for cooling.

RT-qPCR primers for *ACTB* (endogenous control), *TNFA* and *OR2B6* were designed using several online tools, including Ensembl (Howe et al 2020, available at: http://www.ensembl.org/index.html), which was used to research and select the target sequences for each gene, and Primer3Plus (Untergasser et al 2012, available at: https://www.primer3plus.com/index.html) for primer selection. The primers designed were then synthesized and purchased from Eurogentec (Brussels, Belgium). Aliquots from stock solutions were prepared based on the concentration for each primer mixture, being diluted down to a working concentration of 10µM for both forward and reverse primers, as per instructions provided as part of the protocol for the preparation of the TaKaRa Ex Taq Master Mix for RT-qPCR (TaKaRa Bio. Inc., Kusatsu, Japan). The forward and reverse primers used for RT-qPCRs were:

ACTB-forward: 5' TGCCCTGAGGCACTCTTC
ACTB-reverse: 5' GGATGTCCACGTCACACTTC
TNFA-forward: 5' GACAAGCCTGTAGCCCATGT
TNFA-reverse: 5' TCTCAGCTCCACGCCATT
OR2B6-forward: 5' GCCACTCTGTGACCCCTATG
OR2B6-reverse: 5' TGGAAGAGCTCACTGACAAGG

The preparation of the RT-qPCR Master Mix is outlined in **Table 2.4**, accordingly. The dominant component of the reaction mixture contained the TaKaRa TB Green Premix Eq Taq II (Stock Solution: X2) (TaKaRa Bio Inc., Kusatsu, Japan). This reagent contains several components, among which are TB Green, which is an intercalating dye that bears a strong resemblance to SYBR Green I; Tli RNase H, which is a heat-stable buffered RNase enzyme that degrades residual mRNA in the sample mixture (TaKaRa Bio Inc 2024a); TaKaRa Ex Taq HS, a hot-start

PCR enzyme associated with anti-Taq antibodies (TaKaRa Bio Inc 2024b); and ROX passive reference dye (TaKaRa Bio Inc 2024a).

Table 2.4: The volumes of components in the reaction mixture for each sample in qRT-PCR.

Component	Volumes for 1 Sample (µL)
TB Green premix Ex Taq II (2X) (TliRNase H plus, ROX Plus)	10
PC Forward Primer (10µM)	1
PC Reverse Primer (10µM)	1
Template DNA (<100 ng)	4
Sterile Purified Water	4
Total	**20**

The setup of the Shuttle RT-qPCR cycle employed in this study is outlined in **Figure 2.4.** Shuttle PCR is a rapid detection PCR method in which annealing, and amplification are performed simultaneously. Annealing temperature for all amplicons was 60^{o}C.

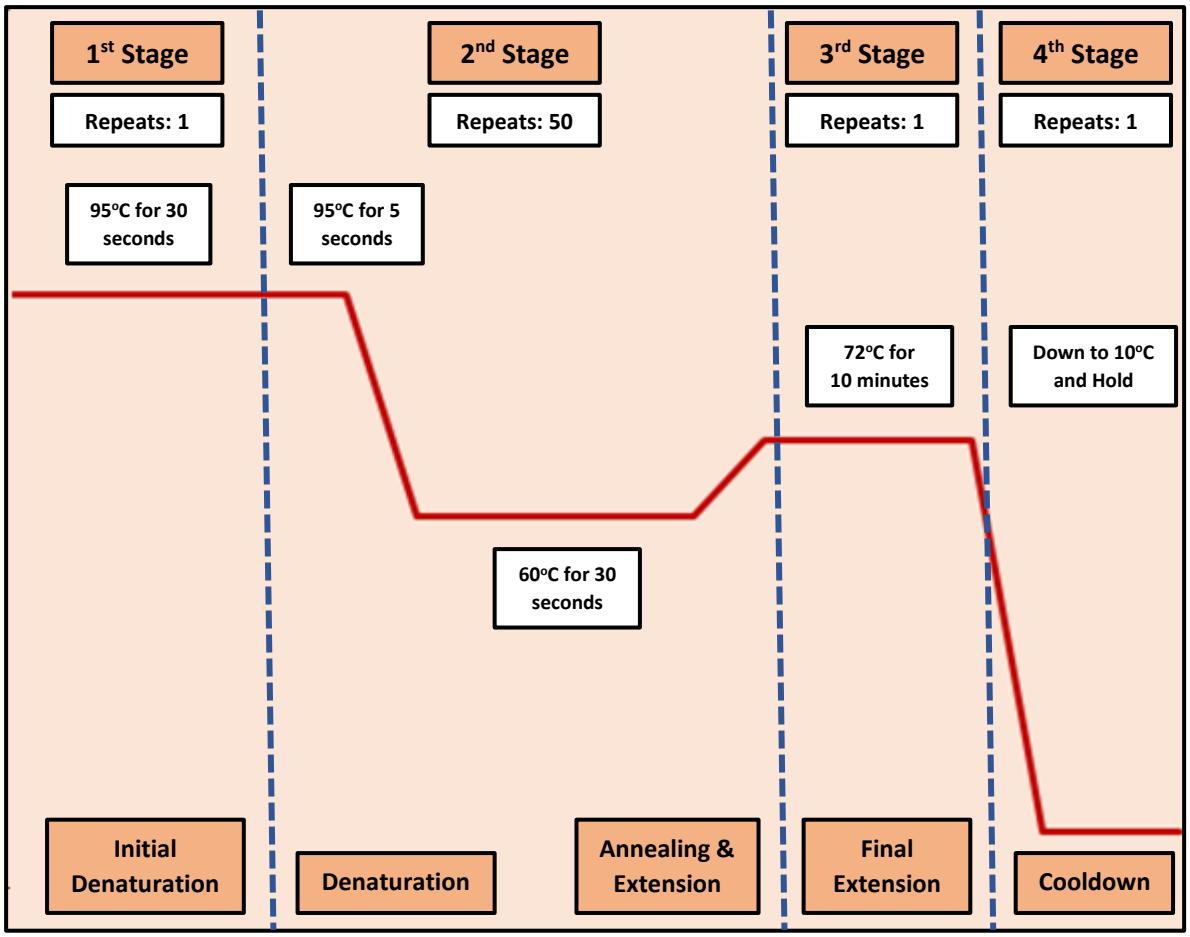


Figure 2.4: Thermal Profile for Shuttle qRT-PCR Run. The Shuttle qRT-PCR consisted of four distinct stages. The first involved the rapid denaturation of DNA at 95°C (Stage 1; 1 Repeat). The second stage was a continued denaturation at 95°C for a further 5 seconds, followed by a combined annealing and extension step at 60°C for 30 seconds (Stage 2; 50 Repeats). The third stage involved a final extension step at 72°C, for 10 minutes (Stage 3; 1 Repeat). The final stage entailed the conclusion of the shuttle qRT-PCR, lowering the temperature down to 10°C (Stage 4; 1 Repeat).

This PCR process was then performed on the Bio-Rad CFX 96 Real-Time PCR Detection System (Bio-Rad Laboratories, California, USA). Visual analysis of the standardised curves produced through RT-qPCR was then carried out using the Bio-Rad CFX Maestro Software (Bio-Rad Laboratories, California, USA). Raw data were subsequently analysed by using the LinRegPCR software (Untergasser et al 2021). First, a baseline fluorescence had to be established to account for incomplete fluorophore quenching and any non-specific primer annealing. In LinRegPCR, the estimation of the baseline fluorescence was automated, utilising a log(fluorescence) versus cycle number plot and applying baseline corrections until the upper

half of the amplification slope was steeper than the lower half of the slope. The corrections were then stepped up and down at increasingly minute increments until a <0.0001 difference was achieved. The exponential phase of amplification was then determined by identifying the period of cycles where a steadily continuous exponential increase of fluorescent signal could be detected, up to the point where a drop-off in intensity is observed (Untergasser at al 2021). The determination of PCR efficiency was essential to evaluating the true performance of the assay. The efficiency value of a PCR had a direct impact on the interpretation of gene expression levels, as it was applied when mathematically extrapolating expression values from the fluorescent signals generated during qRT-PCR (Ruijter et al 2021). While the determination of PCR efficiency is typically performed using a standard curve, LinRegPCR determined the PCR efficiency by selecting data points within a Window-of-Linearity (W-o-L) and calculating the Coefficient of Variation (CV) for each assay. For this process, a minimum of 3 data points over 3 separate cycles were required. The W-o-L was then gradually narrowed downward until the lowest possible CV was achieved. This process was then repeated for each assay, obtaining an PCR efficiency rating for each individual reaction. The overall PCR efficiency was then expressed as the average of the individual PCR efficiencies for all assays across the entire run. LinRegPCR also automatically excluded reactions where no amplification could be detected (Untergasser et al 2021). For interpretation and extrapolation of the estimated transcript abundance (N_0) to be made possible, a C_q value, also known as the Quantification Cycle, must be derived for each reaction. C_q refers to the fractional number of cycles required to reach the quantification threshold (N_q), the latter of which had to be established along the exponential phase. To account for variability between assays and PCR efficiency, LinRegPCR established a standardised common quantification threshold to be used ubiquitously for all PCR assays of the same run. The C_q value for each individual PCR assay was then mathematically derived

from the linear intersect of the exponential phase with the quantification threshold. By normalising the C_q values for all PCR assays, N_0 was derived for each reaction in the qRT-PCR. All N_0 values expressed were corrected for the PCR efficiency.

2.11 Statistics

ELISA and RT-qPCR data were analysed by means of a Kruskal-Wallis and Dunn's post-hoc test. Data were presented as boxplots, dot plots, or bar graphs using the RStudio software. A p-value < 0.05 demarcated significance.

Chapter 3:

Results & Statistics

3 Results

3.1 Olfactory receptor gene expression in whole blood of critically ill patients with sepsis or non-infectious aetiologies

To investigate the expression patterns of ectopic ORs in whole blood between sepsis, infection and health, whole blood leukocyte gene expression data of the MARS study were analysed, and contrast tables constructed for the following categories: all-cause sepsis (n=404), non-infectious controls (n=93), and healthy volunteers (n=42). In line with seminal studies (Scicluna et al 2015, Scicluna et al 2017, Scicluna et al 2019), substantial changes in gene expression indices (adjusted $p \leq 0.05$) were uncovered for all-cause sepsis relative to healthy control subjects depicted in a volcano plot (integrating -log transformed adjusted p-values and log2 transformed fold expression) (**Figure 3.1**). Overall, 7341 genes (2708 elevated; 4633 reduced) were differentially expressed. Highly expressed pro-inflammatory and anti-microbial markers (e.g. S100 family genes, and *MMP8*) were highlighted for reference to very high degrees of fold-changes with high levels of significance. Low expression genes in sepsis versus health include *GATA3*, *CCR3*, and *LEF1*, which have linked with immunosuppression when downregulated in sepsis (Venet et al 2004, Huang et al 2016, Chen et al 2023). OR gene expression was found to be elevated in the case of all-cause sepsis when compared to healthy volunteers (**Figure 3.2**). Over 100 ectopic OR genes were found to be differentially expressed in all-cause sepsis, with two OR genes exceeding a log2-transformed fold-change of 0.25, namely *OR10G3* and *OR2A14*. In comparison to highly expressed pro-inflammatory and anti-microbial genes, ectopic OR gene expression was found to be relatively low. Nevertheless, the statistically significant (adjusted p-value ≤ 0.05) overexpression of over 100 OR genes in the case of all-cause sepsis relative to health suggests that ectopic ORs may be a novel feature of the systemic host response during sepsis.

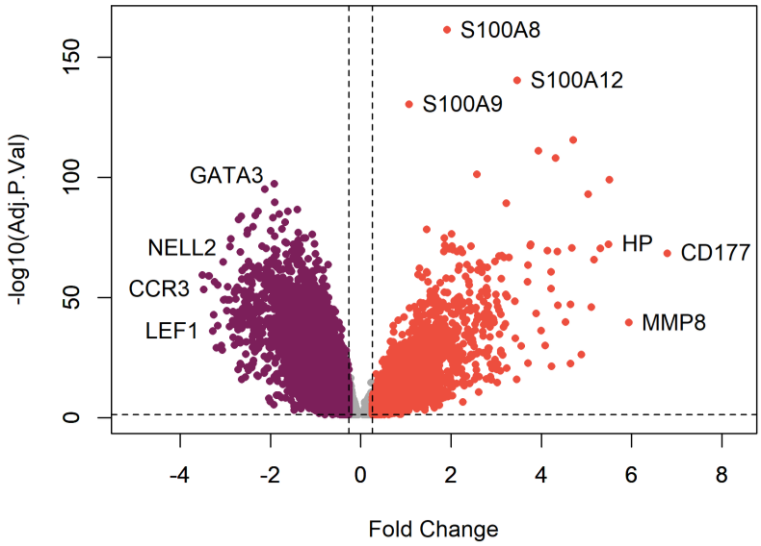


Figure 3.1: Overall patterns of gene expression in whole blood of all-cause sepsis patients compared to healthy volunteers. Volcano plot depicting overall changes in gene expression patterns of leukocytes highlighting a considerably elevated activity in pro-inflammatory and antibacterial effector genes (e.g. *S100A8, S100A9, MMP8, CD177,* and *HP*). Orange dots denote high expression, purple dots denote low expression. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

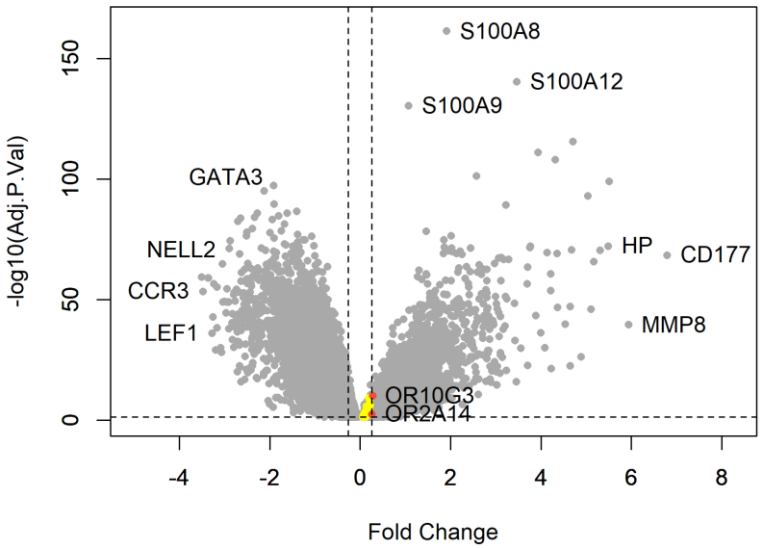


Figure 3.2: Patterns of gene expression for olfactory receptors (highlighted in yellow and orange) in whole blood of all-cause sepsis patients compared to healthy volunteers. Volcano plot depicting changes in olfactory receptor gene expression patterns against a background of overall gene expression (grey dots). All olfactory receptors (highlighted in yellow and orange dots) presented some increase in expression, with *OR10G3* and *OR2A14* demonstrating particularly high expression. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

Following the assessment of differential OR expression between sepsis and health, the differences between all-cause sepsis and all-cause non-infectious critical illness were also investigated. Some changes in pro-inflammatory and anti-microbial markers were notably increased in sepsis compared to non-infectious aetiologies, as illustrated in **Figure 3.3**. Altogether, 1859 genes (1063 elevated; 796 reduced) were differentially expressed. High expression genes include *CD177, MMP8, AIG1* and *OLFM4*. Low expression genes in sepsis versus non-infectious aetiologies include *NLRP1, MEGF6, RBP7, MME* and *SULF2*. One OR gene, that is *OR6P1*, retained statistical significance (adjusted $p \leq 0.05$) while demonstrating a marginal upregulation in sepsis when compared to non-infectious control patients, while several OR genes (*OR6B2, OR5A2, OR2T1, OR56A1, OR1D5* and *OR52E2*) showed marginal downregulation as shown in **Figure 3.4.** Considering fold changes relative to health as a baseline, ectopic OR expression in both all-cause septic patients and non-infectious control patients were tightly correlated with a Spearman's coefficient equating to 0.99 (p-value = 2.2×10^{-16}) (**Figure 3.5**). A line-of-best-fit was also established, with a coefficient of determination (R^2) equating to 0.98. This result indicates that 98% of the variation in leukocyte gene expression in all-cause sepsis patients on ICU admission can be explained by gene expression changes in non-infectious critical illness, related to health as a baseline. Therefore, these findings suggest that changes in OR gene expression may not be specific to sepsis, but a general feature of the host response in critical illness.

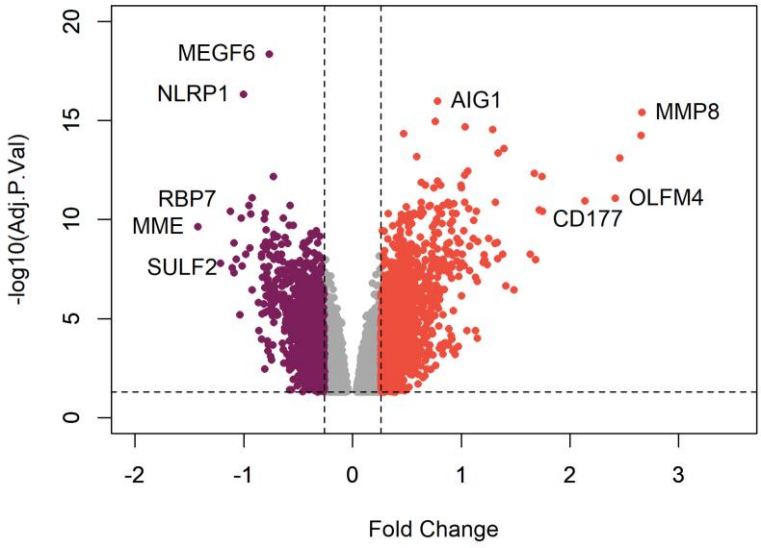


Figure 3.3: Patterns of overall gene expression in whole blood of all-cause sepsis patients compared to non-infectious ICU controls. Volcano plot depicting overall changes in gene expression patterns of leukocytes, showing considerable differences in gene expression in cases of both upregulation (e.g. *CD177, MMP8*, *OLFM4*) and downregulation (e.g. *NLRP1*, *SULF2*, *MME*). Orange dots denote high expression, purple dots denote low expression. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

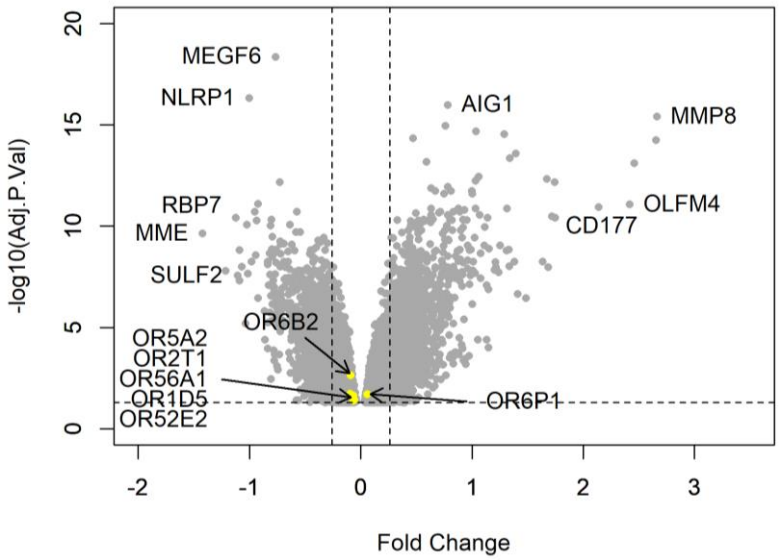


Figure 3.4: Patterns of olfactory receptor gene expression in whole blood of all-cause sepsis patients compared to non-infectious ICU controls. Volcano plot depicting changes in olfactory receptor gene expression patterns against a background of overall gene expression (grey dots). Olfactory receptors (highlighted in yellow) showed both increases (*OR6P1*) and decreases (*OR6B2, OR5A2, OR2T1, OR56A1, OR1D5, OR52E2*) in gene expression. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

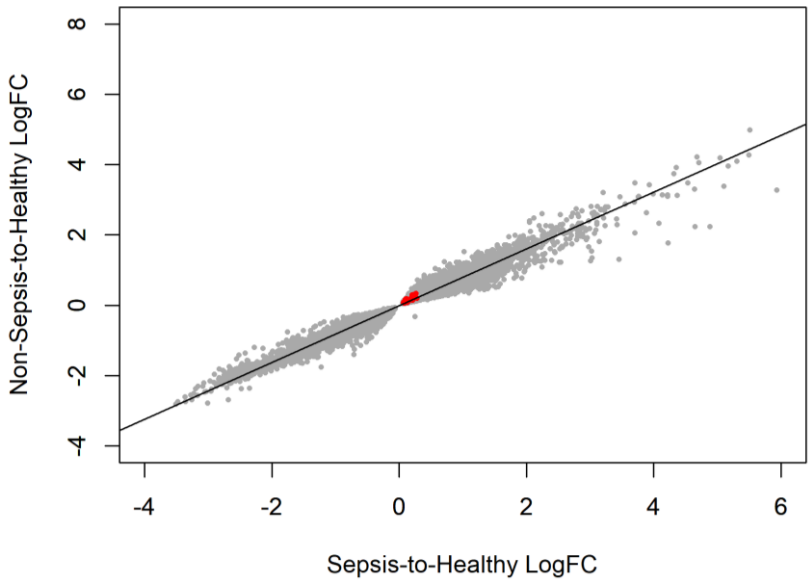


Figure 3.5: Dot Plot representing the trends of olfactory receptor gene expression (highlighted in red) within sepsis patients and non-infectious controls, compared to healthy volunteers. Linear dot plot depicting changes in olfactory receptor gene expression patterns against a background of overall gene expression (grey dots). Olfactory receptors (highlighted in red) showed a general increase in gene expression in both sepsis patients and grouped non-infectious controls when compared to healthy volunteers. A line of best fit was also established, demonstrating a coefficient of determination of 0.98, explaining that 98% of gene expression changes in all-cause sepsis can be explained by changes in grouped non-infectious controls, relative to health. Transcripts shown had achieved statistical significance (adjusted p-value < 0.05).

3.2 Source-specific olfactory receptor gene expression in sepsis

Here, an anatomical approach was adopted, comparing whole blood gene expression in sepsis caused by infections at different anatomical sites, that is pneumonia (also classified as community or hospital-acquired), intra-abdominal, genitourinary and cardiovascular. For the purposes of this investigation, contrast tables were constructed for CAP, HAP, intra-abdominal infections, cardiovascular, and genitourinary infections, all relative to health as a common baseline. The relationships in overall gene expression changes, and those pertaining to OR genes between site-specific causes of sepsis are illustrated in a series of dot plots, which were compiled into **Figure 3.6.** In line with previous reports (van Vught et al 2016, Peters-Sengers et al 2022), leukocyte gene expression patterns in sepsis patients grouped as primary

anatomical sites of infection or setting in the context of pneumonia (community versus hospital-acquired), were largely similar with highest Spearman's coefficient of 0.99 (p-value = $2.2x10^{-16}$) for CAP versus HAP, and a lowest Spearman's coefficient of 0.98 (p-value = $2.2x10^{-16}$) for Abdo versus Uros. Ectopic OR gene expression (marked in red in **Figure 3.6**) remained largely similar across all infection sites. These findings suggest changes in OR gene expression in sepsis may not be dependent on primary anatomical site of infection nor setting.

Figure 3.6: Dot plots representing comparisons of olfactory gene expression (highlighted in red) across different anatomical sites in sepsis patients. Collection of linear dot plots depicting changes in olfactory receptor gene expression patterns against a background of overall gene expression (grey dots) between categories of site-specific sepsis. Olfactory receptors (highlighted in red) showed no noteworthy deviations regardless of anatomical site. A line of best fit was established for each comparison, with the highest coefficient of determination being found between CAP-to-HAP (0.99), and the lowest coefficient of determination being found between Abdo-to-Uros (0.97). Therefore, olfactory receptor gene expression can be said to remain largely similar in sepsis between different anatomical sites. Transcripts shown had achieved statistical significance (adjusted p-value < 0.05).

The dynamics of expression between sepsis in specific anatomical sites and their respective controls were also tested for any significant differences. The purpose of this step was to highlight any ORs which may possess a more significant impact in site-specific sepsis when compared to non-infectious controls. To achieve this, the OR gene expression patterns in CAP and intra-abdominal sepsis cases were contrasted directly against the corresponding expression in their respective controls, that is no-CAP, and post-op GI patients, respectively. These comparisons are illustrated using volcano plots as in **Figures 3.7 – 3.9**. No statistically significant differences (adjusted p-value ≤ 0.05) in OR gene expression was found for CAP versus no-CAP. However, statistically significant differences (adjusted p-value ≤ 0.05) in OR gene expression were noted in the case of intra-abdominal sepsis versus post-op GI controls. Four ectopic OR genes, namely *OR5A2*, *OR6B2*, *OR1D5*, and *OR52E2* were expressed to a lower degree in abdominal sepsis patients, whereas two ectopic OR genes *(OR10P1* and *OR10H5*) were elevated in abdominal sepsis (**Figure 3.9**). These findings may allude to the possibility of variable OR gene expression between the states of sepsis and infection in the case of intraabdominal infections, potentially changing the landscape of potential targets of OR genes in sepsis attributed to this anatomical site.

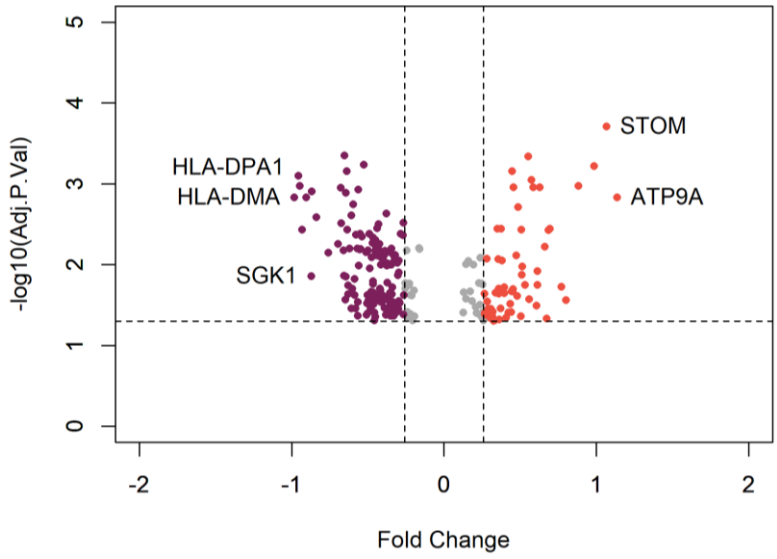


Figure 3.7: Patterns of overall gene expression in whole blood of septic CAP patients and non-infectious CAP controls. Volcano plot depicting overall changes in gene expression patterns of leukocytes, showing considerable differences in gene expression in cases of both upregulation (e.g. *CD24, STOM, ATP9A*) and downregulation (e.g. *HLA-DPA1, HLA-DMA, SGK1*). Orange dots denote high expression, purple dots denote low expression. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

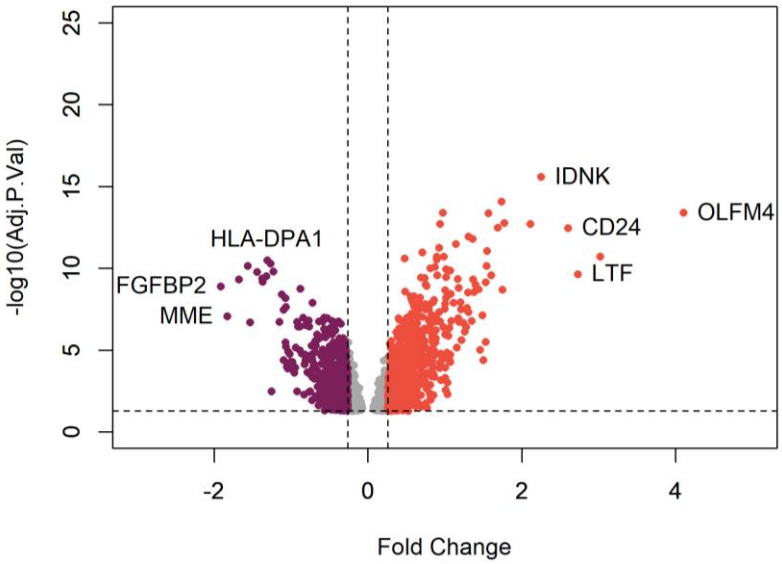


Figure 3.8: Patterns of overall gene expression in whole blood of septic patients with intraabdominal infections, and their respective ICU controls. Volcano plot depicting overall changes in gene expression patterns of leukocytes, showing considerable differences in gene expression in cases of both upregulation (e.g. *CD24, OLFM4, IDNK, LTF*) and downregulation (e.g. *HLA-DPA1, FGFBP2, MME*). Orange dots denote high expression, purple dots denote low expression. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

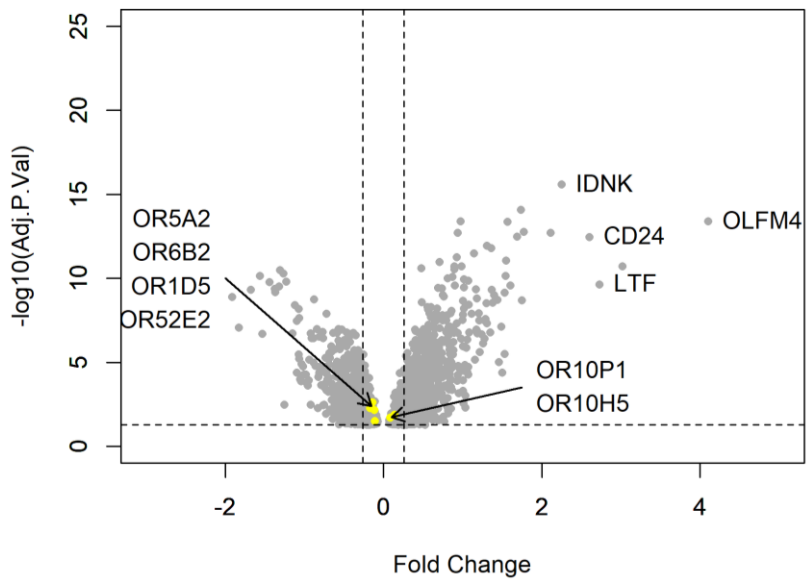


Figure 3.9: Patterns of olfactory receptor gene expression in the whole blood of septic patients with intraabdominal infections, and their respective ICU controls. Volcano plot depicting changes in olfactory receptor gene expression patterns against a background of overall gene expression (grey dots). Olfactory receptors (highlighted in yellow) showed both increases (*OR10P1, OR10H5*) and decreases (*OR5A2, OR6B2, OR1D5, OR52E2*) in gene expression in abdominal sepsis compared to non-infectious controls. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

3.3 Olfactory receptor gene expression in specific peripheral blood mononuclear cells

To further investigate the differential expression and potential functions of ectopic ORs in innate immune cells, the study by Khan et al (2020) was chosen as it provided datasets of RNA gene expression for pure populations of PBMCs. This conveys the advantage of investigating differential OR gene expression which can be solely attributed to a particular cell type, an effect that cannot be directly measured in whole blood. Cells in this study were separated by FACS based on their cluster of differentiation (CD) markers: $CD14^+$ Monocytes, $CD4^+$ T-Cells, $CD8^+$ T-Cells, and $CD19^+$ B-Cells (Khan et al 2020). Specific PBMCs were sorted from heparinized whole blood obtained from patients with sepsis secondary to community-acquired pneumonia (n=6) or healthy age- and sex-matched healthy control subjects (n=4).

Blood samples from patients were obtained on ICU admission. Using DESeq2 method (see Methods), transcriptomes of each specific cell-type were compared between sepsis and health, revealed the most alterations in gene expression in CD4 T-Cells (3 upregulated, 10 down-regulated), with a lowest number of changes observed for CD8 T-Cells (1 upregulated, 2 down-regulated) (**Figures 3.10 – Figure 3.13**). Considering adjusted p-values ≤ 0.05, *OR52B2* gene expression was found significantly upregulated in monocytes of sepsis patients relative to health (**Figure 3.10**). Considering statistical significance, OR gene expression remained relatively unchanged across both B-Cells and T-Cells. However, several ORs were nevertheless expressed in all specific cell-types, although with differing levels of expression. In addition, some ORs were also found to be commonly expressed by multiple cell-types. A list of these common ORs was compiled into **Table 3.1**.

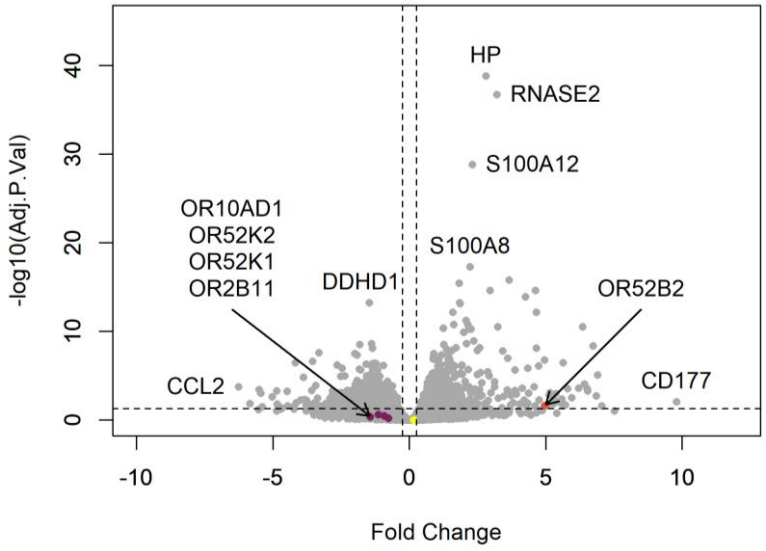


Figure 3.10: Olfactory receptor gene expression patterns in CD14-positive monocytes, sorted PBMCs study (Khan et al 2020). Volcano plot depicting changes in olfactory receptor gene expression patterns against a background of overall gene expression (grey dots). Highly expressed pro-inflammatory and antibacterial genes (e.g. *CD177, S100A12, S100A8, HP*) are visible. Olfactory receptors which demonstrated upregulation (*OR52B2*) are highlighted as orange dots, while those which were downregulated (*OR10AD1, OR52K2, OR52K1, OR2B11*) are highlighted as purple dots. *OR52B2* was also found to have achieved statistical significance (p-value < 0.05). Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

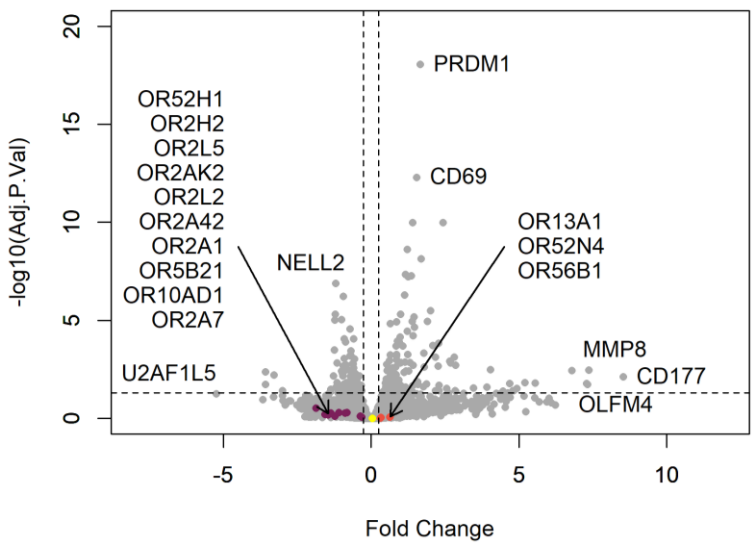


Figure 3.11: Olfactory receptor gene expression patterns in CD4-positive T-cells. Volcano plot depicting changes in olfactory receptor gene expression patterns against a background of overall gene expression (grey dots). Highly expressed cell activation and antibacterial genes (e.g. *CD177, MMP8*) are visible. Olfactory receptors which demonstrated upregulation (*OR13A1, OR52N4, OR56B1*) are highlighted as orange dots, while those which were downregulated (*OR52H1, OR2H2, OR2L5, OR2AK2, OR2L2, OR2A42, OR2A1, OR5B21, OR10AD1, OR2A7*) are highlighted as purple dots. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

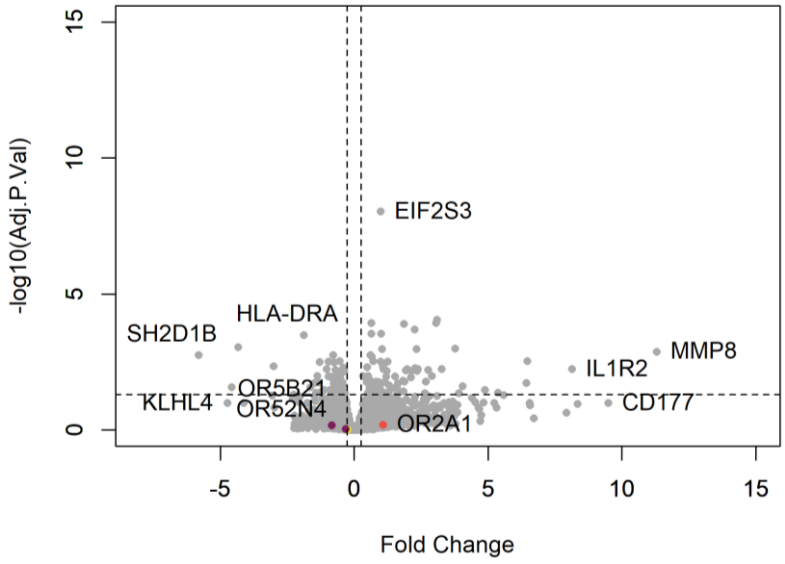


Figure 3.12: Olfactory receptor gene expression patterns in CD8-positive T-cells. Volcano plot depicting changes in olfactory receptor gene expression patterns against a background of overall gene expression (grey dots). Highly expressed pro-inflammatory and antibacterial genes (e.g. *CD177, MMP8, IL1R2*) are visible. Olfactory receptors which demonstrated upregulation (*OR2A1*) are highlighted as orange dots, while those which were downregulated (*OR5B21, OR52N4*) are highlighted as purple dots. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

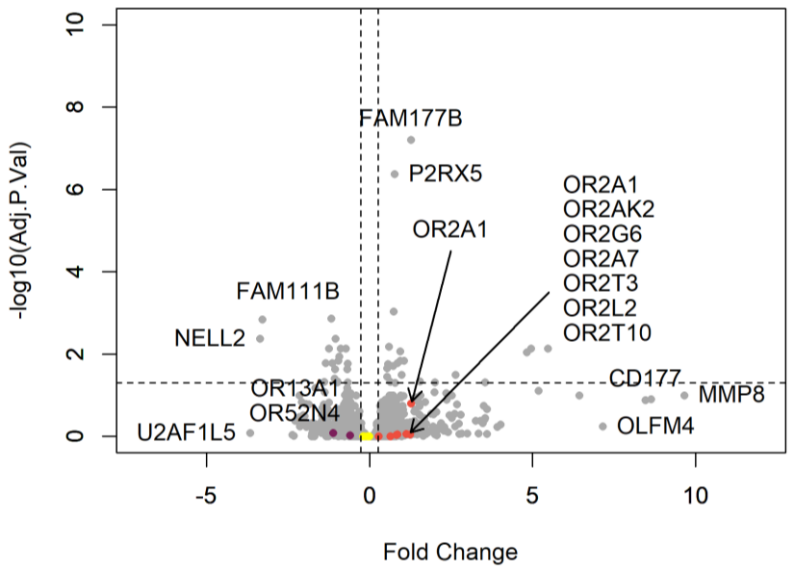


Figure 3.13: Olfactory receptor gene expression patterns in CD19-positive B-cells. Volcano plot depicting changes in olfactory receptor gene expression patterns against a background of overall gene expression (grey dots). Highly expressed cell activation and antibacterial genes (e.g. *CD177, MMP8*) are visible. Olfactory receptors which demonstrated upregulation (*OR2A1, OR2AK2, OR2G6, OR2A7, OR2T3, OR2L2, OR2T10*) are highlighted as orange dots, while those which were downregulated (*OR13A1, OR52N4*) are highlighted as purple dots. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

Table 3.1: Olfactory Receptor genes that were found to be commonly expressed between two or more types of PBMCs in Khan et al (2020)'s study. Although many olfactory receptor genes were found to be commonly expressed between several types of PBMCs, not all shared similar levels of differential expression between cell types. While some olfactory receptor genes were upregulated or downregulated similarly across multiple cell types, some cell types showed distinct upregulations of some olfactory receptor genes while all other populations expressed downregulations, hinting that some olfactory receptor genes found to be commonly expressed among cells may indeed also have cell-specific functions. **Red** denotes statistical difference (adjusted p-value < 0.05) between sepsis and health.

Monocytes (CD14)		T-Cells (CD4)		T-Cells (CD8)		B-Cells (CD19)	
Downregulated	**Upregulated**	**Downregulated**	**Upregulated**	**Downregulated**	**Upregulated**	**Downregulated**	**Upregulated**
OR10AD1	*OR52B2*	*OR52H1*	***OR13A1***	***OR5B21***	***OR2A1***	***OR13A1***	*OR2A1*
OR52K2		*OR2H2*	***OR52N4***	***OR52N4***		***OR52N4***	***OR2AK2***
OR52K1		*OR2L5*	*OR56B1*				*OR2G6*
OR2B11		***OR2AK2***					***OR2A7***
		OR2L2					*OR2T3*
		OR2A42					***OR2L2***
		OR2A1					*OR2T10*
		OR5B21					
		OR10AD1					
		OR2A7					

Genes that have been highlighted in bold were found to be commonly expressed across multiple PBMC types (CD14s, CD4s, CD8s, CD19s).

3.4 Time course of olfactory receptor gene expression in human endotoxemia

To better understand the temporal patterns of OR gene expression response, a human endotoxemia model with genome-wide transcriptomic data at different time points was utilized (Scicluna BP et al 2020). The human endotoxemia model was established by intravenous administration of a 2ng/kg blous of *E. coli* LPS to 8 healthy male volunteers who were followed in a clinically controlled setting for the duration of 24 hours having blood drawn in PaxGene blood tubes before, and after 2, 4, 6, and 24 hours LPS injection. During the first 2 hours of human endotoxemia, 12 ORs were found to be significantly (adjusted p-value ≤ 0.05) elevated, while no ORs showed noteworthy downregulation as shown in **Figure 3.14.** The most significantly altered OR gene after 2 hours of human endotoxemia was *OR52K1* overexpression. After 4 hours of human endotoxemia, 30 and 4 ORs were found to be significantly (adjusted p-value ≤ 0.05) elevated and reduced, respectively, some of which are shown in **Figure 3.15.** The most significantly altered OR after 4 hours of human endotoxemia was *OR52N4* downregulation. After 6 hours of human endotoxemia, 7 and 2 ORs were found to be significantly (adjusted p-value ≤ 0.05) elevated and reduced, respectively, as shown in **Figure 3.16.** The most significantly altered OR after 6 hours of human endotoxemia was *OR52K2* overexpression. No transcriptome changes were observed after 24 hours of human endotoxemia. Next, to explore the potential cell-type specific ramifications of these human endotoxemia findings, time-specific OR genes were matched to those found in specific PBMCs of sepsis patients (**Section 3.3** above) and tabulated as **Table 3.2 – 3.4**. Altogether, these data suggest that 4 hours after human endotoxemia initiation is associated with a greater number of OR genes, and *OR2B6* expression may be a monocyte-derived gene exhibiting expression patterns consistent across all human endotoxemia time points.

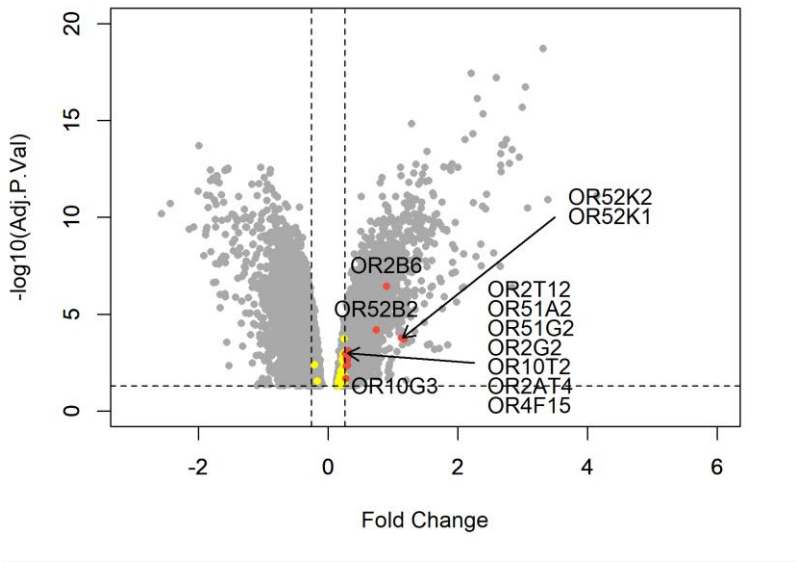


Figure 3.14: Overall olfactory receptor gene expression patterns for whole blood, during the 2nd hour of the course of the Human Endotoxemia Model (Scicluna et al 2020). Volcano plot depicting changes in olfactory receptor gene expression patterns in whole blood leukocytes against a background of overall gene expression (grey dots). Olfactory receptors which demonstrated upregulation (*OR52K2, OR52K1, OR2B6, OR52B2, OR2T12, OR51A2, OR51G2, OR2G2, OR10T2, OR2AT4, OR4F15, OR10G3*) are highlighted as orange dots. No olfactory receptors showed significant downregulation during this time point. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

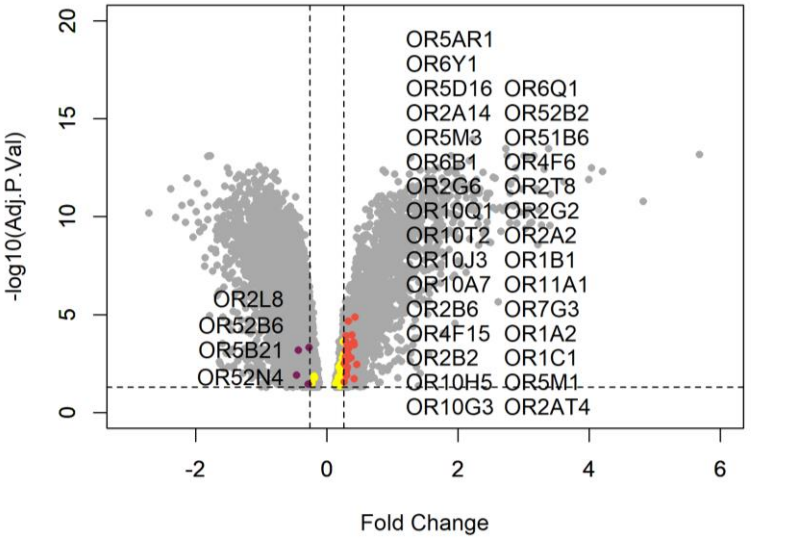


Figure 3.15: Overall olfactory receptor gene expression patterns for whole blood, during the 4th hour of the course of the Human Endotoxemia Model. Volcano plot depicting changes in olfactory receptor gene expression patterns in whole blood leukocytes against a background of overall gene expression (grey dots). Olfactory receptors which demonstrated upregulation (*OR2B6, OR52B2, OR7G3, OR2B2, OR2A14*, etc, as above) are highlighted as orange dots. Olfactory receptors which were found to be downregulated (*OR2L8, OR52B6, OR5B21, OR52N4*) are highlighted as purple dots. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

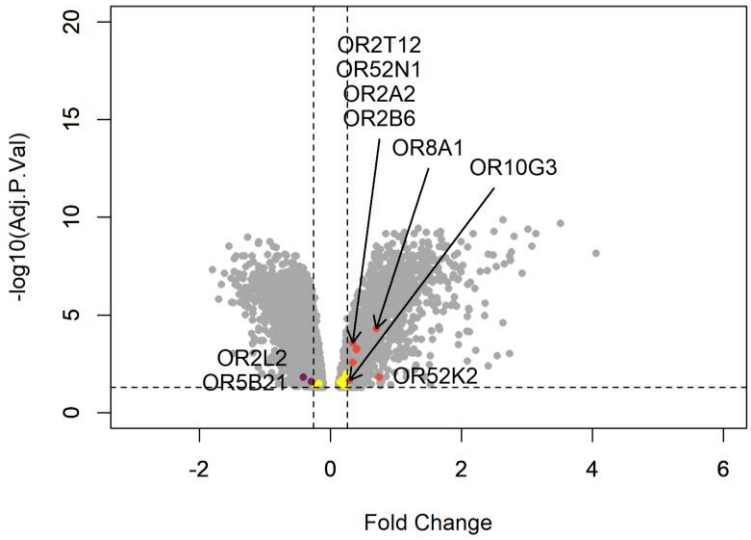


Figure 3.16: Overall olfactory receptor gene expression patterns for whole blood, during the 6th hour of the course of the Human Endotoxemia Model. Volcano plot depicting changes in olfactory receptor gene expression patterns in whole blood leukocytes against a background of overall gene expression (grey dots). Olfactory receptors which demonstrated upregulation (*OR52K2, OR8A1, OR2T12, OR52N1, OR2A2, OR2B6, OR10G3*) are highlighted as orange dots. Olfactory receptors which were found to be downregulated (*OR2L2, OR5B21*) are highlighted as purple dots. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

Table 3.2: Table of olfactory receptor genes expressed by multiple PBMC types at the 2-hour time point of the Human Endotoxemia Model. A list of olfactory receptors known to be expressed on each specific cell type was compiled and matched with results obtained from the 2-hour time point of the human endotoxemia model. Some olfactory receptors were found to be unique to specific cell-types. Olfactory receptors such as *OR52K2* and *OR2B6* were found to be exclusive to monocytes. *OR52K1,* which was found to be upregulated, was found to be shared between monocytes and CD4 T-Cells.

HUMAN ENDOTOXEMIA MODEL – 2 HOUR TIME POINT			
Monocytes (CD14)	**T-Cells (CD4)**	**T-Cells (CD8)**	**B-Cells (CD19)**
Upregulated	**Upregulated**	**Upregulated**	**Upregulated**
OR52K1	*OR52K1*	***OR52B2***	***OR52B2***
OR52K2	***OR52B2***	*OR4F15*	
OR2B6			
OR52B2			

Genes that have been highlighted in bold were found to be commonly expressed across all four PBMC types (CD14s, CD4s, CD8s, CD19s). Furthermore, all genes as shown above are sorted in descending order from top to bottom, based on their recorded fold changes.

OR52B2 was commonly expressed across all cell types. Olfactory receptors which were found to have been commonly shared between all cell types were highlighted accordingly.

Table 3.3: Table of olfactory receptor genes expressed by multiple PBMC types at the 4-hour time point of the Human Endotoxemia Model. A list of olfactory receptors known to be expressed on each specific cell type was compiled and matched with results obtained from the 4-hour time point of the human endotoxemia model. Some olfactory receptors were found to be unique to specific cell-types. *OR2B6* was once again found to be upregulated while remaining unique to monocytes, with the upregulated *OR52B2* remaining commonly expressed across all cell types. *OR2B2*, which was found to be upregulated was also found to be shared by both monocytes and B-Cells. The downregulated olfactory receptors *OR52N4, OR52B6* and *OR5B21*, were commonly expressed across all cell types.

HUMAN ENDOTOXEMIA MODEL – 4 HOUR TIME POINT			
Monocytes (CD14)	**T-Cells (CD4)**	**T-Cells (CD8)**	**B-Cells (CD19)**
Upregulated	**Upregulated**	**Upregulated**	**Upregulated**
OR2B6	***OR52B2***	***OR52B2***	***OR52B2***
OR2B2	*OR2G6*	*OR4F15*	*OR2G6*
OR52B2			*OR2B2*
Downregulated	**Downregulated**	**Downregulated**	**Downregulated**
OR52N4	***OR52N4***	***OR52N4***	***OR52N4***
OR52B6	***OR5B21***	***OR5B21***	***OR5B21***
OR5B21	***OR52B6***	***OR52B6***	***OR52B6***

Genes that have been highlighted in bold were found to be commonly expressed across all four PBMC types (CD14s, CD4s, CD8s, CD19s). Furthermore, all genes as shown above are sorted in descending order from top to bottom, based on their recorded fold changes.

Olfactory receptors which were found to have been commonly shared between all cell types were highlighted accordingly.

Table 3.4: Table of olfactory receptor genes expressed by multiple PBMC types at the 6-hour time point of the Human Endotoxemia Model. A list of olfactory receptors known to be expressed on each specific cell type was compiled and matched with results obtained from the 6-hour time point of the human endotoxemia model. Some olfactory receptors were found to be unique to specific cell-types. *OR2B6* remained consistently upregulated across all time points of the model, while *OR52K2*, which was upregulated once again at the 6-hour time point, had been previously upregulated during the 2-hour time point. Both *OR2B6* and *OR52K2* remained specific to monocytes. The olfactory receptor gene *OR5B21* was once again found to be downregulated across all cell types, having been identified during the 4-hour time point. Olfactory receptors which were found to have been commonly shared between all cell types were highlighted accordingly.

HUMAN ENDOTOXEMIA MODEL – 6 HOUR TIME POINT			
Monocytes (CD14)	**T-Cells (CD4)**	**T-Cells (CD8)**	**B-Cells (CD19)**
Upregulated	**Upregulated**	**Upregulated**	**Upregulated**
OR52K2		*OR52N1*	
OR2B6			
Downregulated	**Downregulated**	**Downregulated**	**Downregulated**
OR5B21	*OR2L2*	*OR2L2*	*OR2L2*
	OR5B21	***OR5B21***	***OR5B21***

Genes that have been highlighted in bold were found to be commonly expressed across all four PBMC types (CD14s, CD4s, CD8s, CD19s). Furthermore, all genes as shown above are sorted in descending order from top to bottom, based on their recorded fold changes.

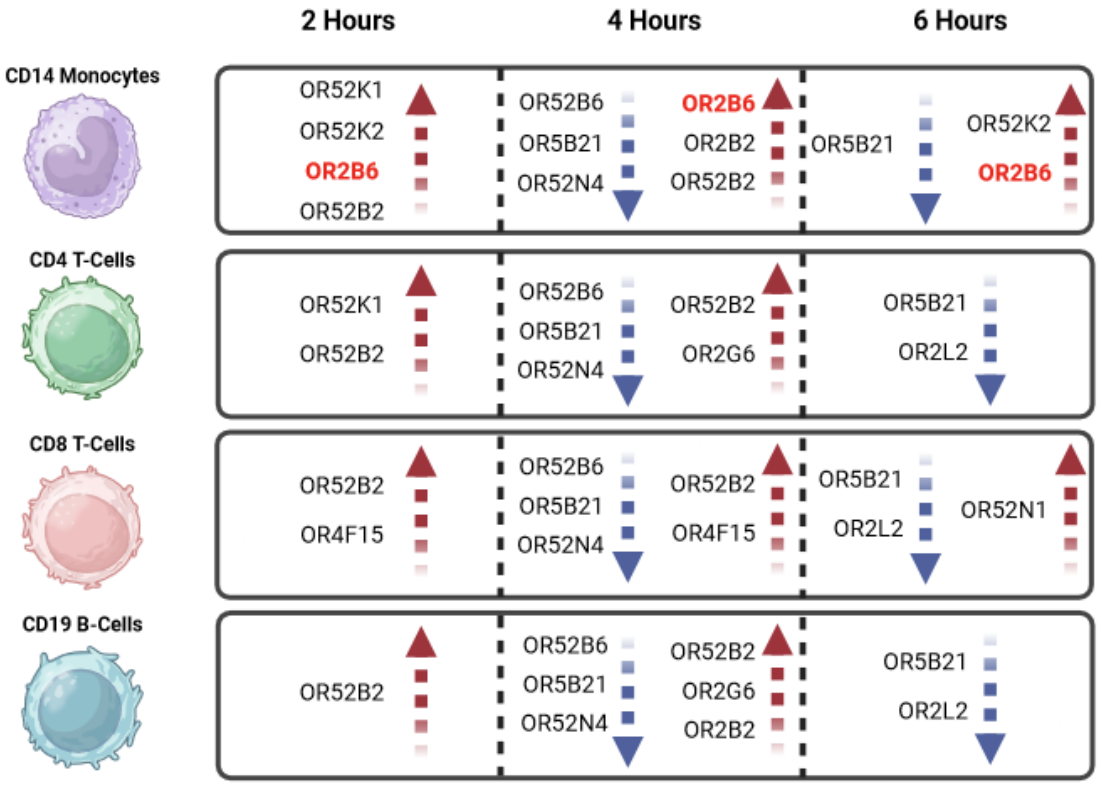


Figure 3.17: Visual summary of OR expression kinetics in peripheral whole blood over the course of the first 6 hours of the human endotoxemia model. ORs for each cell type were identified using a shortlist of cell-specific olfactory receptors. Cell types shown include monocytes, T-cells and B-cells. *OR2B6* (highlighted in **red**) on CD14+ monocytes was found to be continually upregulated over the course of the 2-, 4- and 6-hour time points of the human endotoxemia model. *OR52K1* and *OR52K2,* which were found to be uniquely expressed by monocytes, were also overexpressed during the 2-hour time point, while *OR52B2* remained a commonly upregulated olfactory receptor between all cell types during the 2- and 4-hour time points. Furthermore, all olfactory receptors which were found to be downregulated over the 4- and 6-hour time points were shared by all or most cell types tested. The expression patterns demonstrated by *OR2B6, OR52K1, OR52K2, OR52B2* provide intriguing targets to be considered for further sequencing to determine whether olfactory receptor gene expression may truly be inducible by PAMPs (e.g. LPS). All OR genes shown had reached statistical significance (adjusted p-value < 0.05).

3.5 Olfactory gene expression in human monocyte-derived macrophages and bronchial epithelial cells

To investigate the inducibility of OR gene expression, two different *in vitro* models with genome-wide transcriptomes were analysed, that is a primary human monocyte-derived macrophage model stimulated with *E. coli* LPS (Malmström et al 2020), and a bronchial epithelial cell model stimulated with *Pseudomonas aeruginosa* flagellin (Ramirez-Moral et al's 2021). Analysis of human MDMs stimulated with LPS for 6 hours revealed several statistically significant (adjusted p-value ≤ 0.05) differences in gene expression that corroborate the seminal study (Malmström et al 2020), with *OR8G5* (log2 fold change = -0.63) and *OR52K3P* (log2 fold change = 1.54) significantly down-regulated and up-regulated, respectively **(Figure 3.18)**. In contrast, OR gene expression was not altered in human bronchial epithelial cells stimulated with flagellin (**Figure 3.19**). Overall, these results suggest that OR gene expression may be induced by PAMPs.

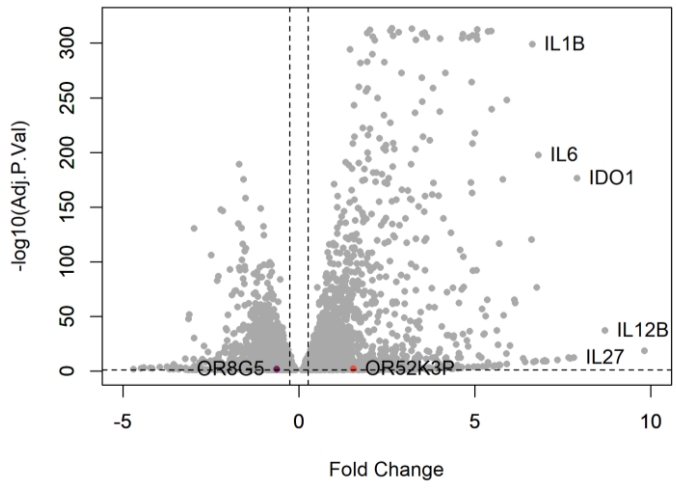


Figure 3.18: Olfactory receptor gene expression patterns in human monocyte-derived macrophages in response to LPS stimulus (Malmström et al 2020). Volcano plot depicting changes in olfactory receptor gene expression patterns in human monocyte-derived macrophages against a background of overall gene expression (grey dots). Pro-inflammatory cytokine-producing genes (e.g. *IL27, IL1B, IL12B, IL27*) showed visible overexpression, as shown above. Olfactory genes which retained statistical significance (p-value < 0.05) were *OR52K3P* (orange dot) and *OR8G5* (purple dot), which were upregulated and downregulated, respectively. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

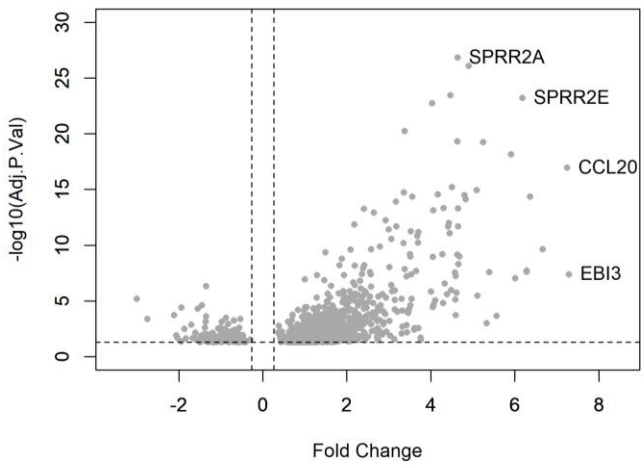


Figure 3.19: Olfactory receptor gene expression patterns in human bronchial epithelium following stimulation by bacterial flagellin, after filtering out for statistical significance (Ramirez-Moral et al 2021). Volcano plot depicting changes in gene expression (grey dots) in human bronchial epithelium. Pro-inflammatory and bactericidal genes (e.g. *SPRR2A, SPRR2E, CCL20*) showed visible overexpression. Although olfactory receptors were detected, these failed to maintain statistical significance (p-value < 0.05) and were filtered out. No olfactory receptors of statistical significance could thus be identified in human bronchial epithelium. Vertical dashed lines depict the log2-fold change threshold of 0.25, which equates to an absolute fold change of 1.2. Horizonal dashed lines depict statistical significance (adjusted p-value < 0.05).

3.6 Inducibility of *OR2B6* in stimulated primary human monocytes and endotoxin tolerance

Thus far, analyses of publicly available datasets revealed that OR gene expression is relatively low in the context of whole blood leukocytes, specific PBMCs and immune functional cell models. In addition, some OR genes may be induced as a function of sepsis or stimulated by PAMPs. To provide more robustness, a human volunteer study was created to evaluate the inducibility of OR gene expression in primary monocytes. After enriching for monocytes using negative selection microbeads, flow cytometry was used to evaluate the purity and functional state of the monocytes. Unstained control cells were used to define gates (**Appendix II, Figure 1**). Enriched monocytes encompassed more than 80% of the fraction obtained after microbead treatment, with an average of 92% CD14+ (min. 85.6%; max. 97.8%) across all

donor samples (**Figure 3.20**). Moreover, average CD14+HLA-DR+ double positive monocytes equated to 98% (min. 93.9%; max. 99.1%).

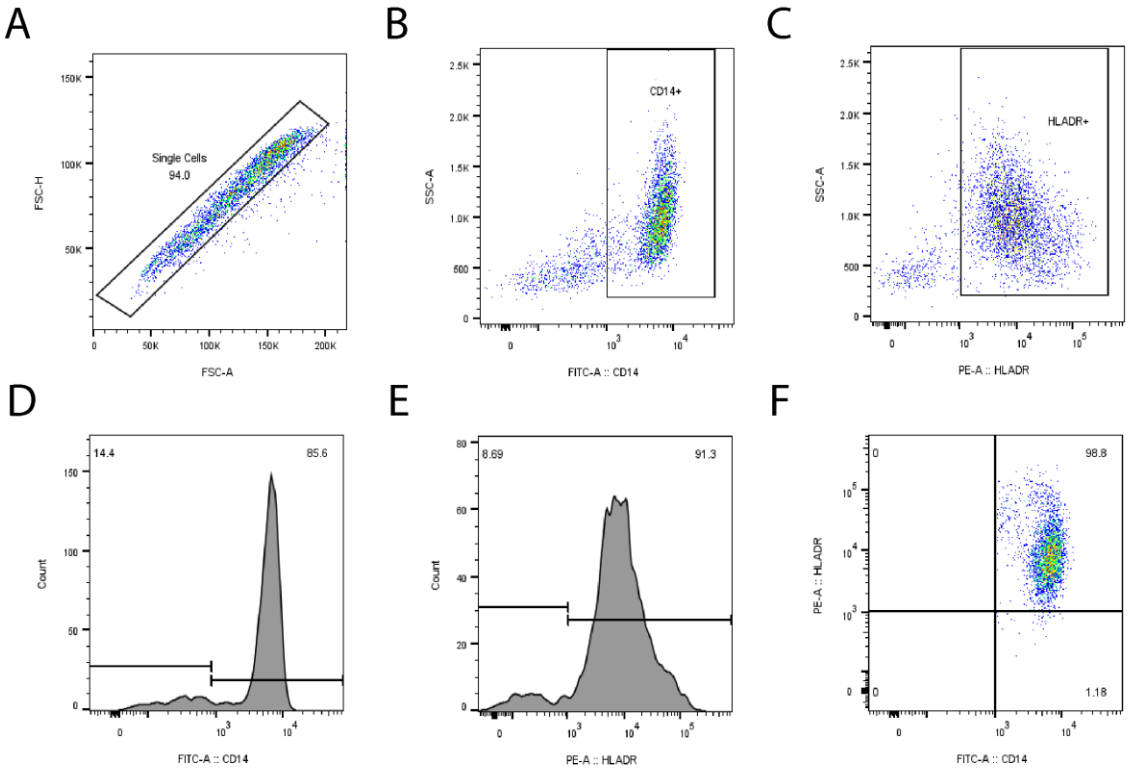


Figure 3.20: Representative flow cytometry data of enriched monocytes. (A) Scatter plot of single cell gating; **(B)** CD14+ single cells; **(C)** HLA-DR+ single cells; **(D)** and **(E)** histograms depicting CD14+ and HLA-DR+ percentages of all single cells, respectively. **(F)** Scatter plot of CD14+,HLA-DR+ double positive percentage of monocytes in the CD14+ gate. Analysis performed in FlowJo version 10.10.0. FSC, forward scatter; SSC, side scatter; FITC, Fluorescein isothiocyanate; PE, Phycoerythrin.

Stimulation of monocytes with LPS resulted in a robust TNF-α response (**Figure 3.21**). This is in line with expectations that monocytes would respond strongly upon first exposure to LPS, generating large quantities of pro-inflammatory cytokines such as TNF-α (Foster et al 2007, Brands et al 2021). Re-stimulation with LPS resulted in a dampened TNF-α response, indicative of endotoxin tolerance (Foster et al 2007, Biswas and Lopez-Collazo 2009, Divangahi et al 2021). The standardised linear graph upon which sample data was further extrapolated and derived can be found in **Appendix I, Figure 2**.

TNFA gene expression (relative to *ACTB*) followed the same patterns as for the secreted protein product, with strong induction in *TNFA* mRNA after stimulation with LPS, and a dampened response after re-stimulation (**Figure 3.22**). Gene expression analysis of a selected OR candidate, that is, *OR2B6*, resulted in no noticeable induction of mRNA after LPS stimulation (**Figure 3.23)**. PCR amplification profiles are illustrated in **Appendix II Figures 3 – 5**.

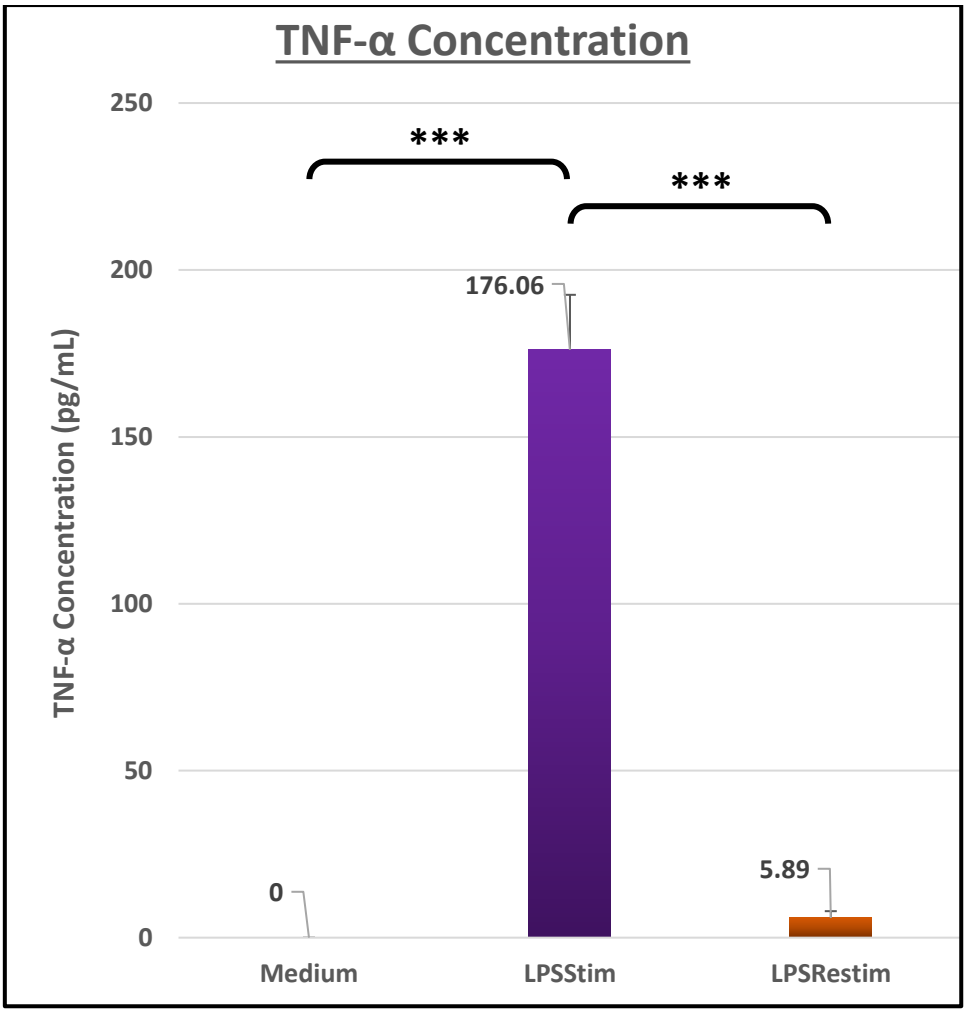


Figure 3.21: Bar Graph showcasing increases in TNF-α concentration following stimulations and restimulations of PBMCs by TLR-4-mediated signalling with LPS. TNF-α concentrations obtained by ELISA, derived from a standard curve. Values shown above were obtained from the average TNF-α concentration for each experimental category: Medium, LPS and Restim. Wilcoxon Rank Sum Tests were carried out to determine statistically significant differences between each category. The degree of statistical significance between each category shown above can be interpreted as follows *** = p-value < 0.005 (LPSStim vs. Medium, LPSStim vs. LPSRestim)

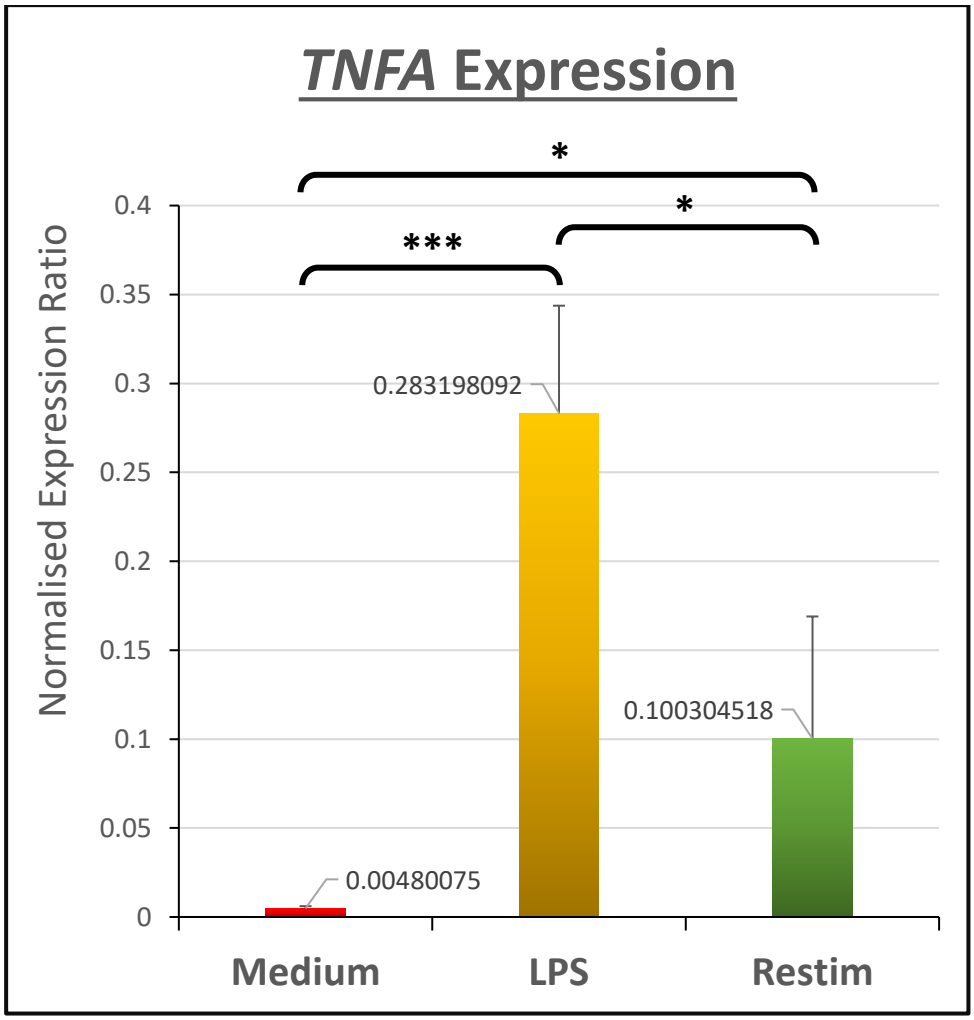


Figure 3.22: Bar Chart visualising differences in expression ratios for *TNFA* in PBMCs exposed to culture medium, primary LPS stimulation, and secondary LPS restimulation, respectively. Normalised expression ratios of *TNFA* expression obtained by real-time quantitative PCR against reference gene *ACTB.* Ratios shown above were obtained from the average of normalised ratios for each experimental category: Medium, LPS and Restim. Wilcoxon Rank Sum Tests were carried out to determine statistically significant differences between each category. The degree of statistical significance between each category shown above can be interpreted as follows: *** = p-value < 0.005 (LPS vs. Medium), * = p-value < 0.05 (LPS vs. Restim, Restim vs. Medium).

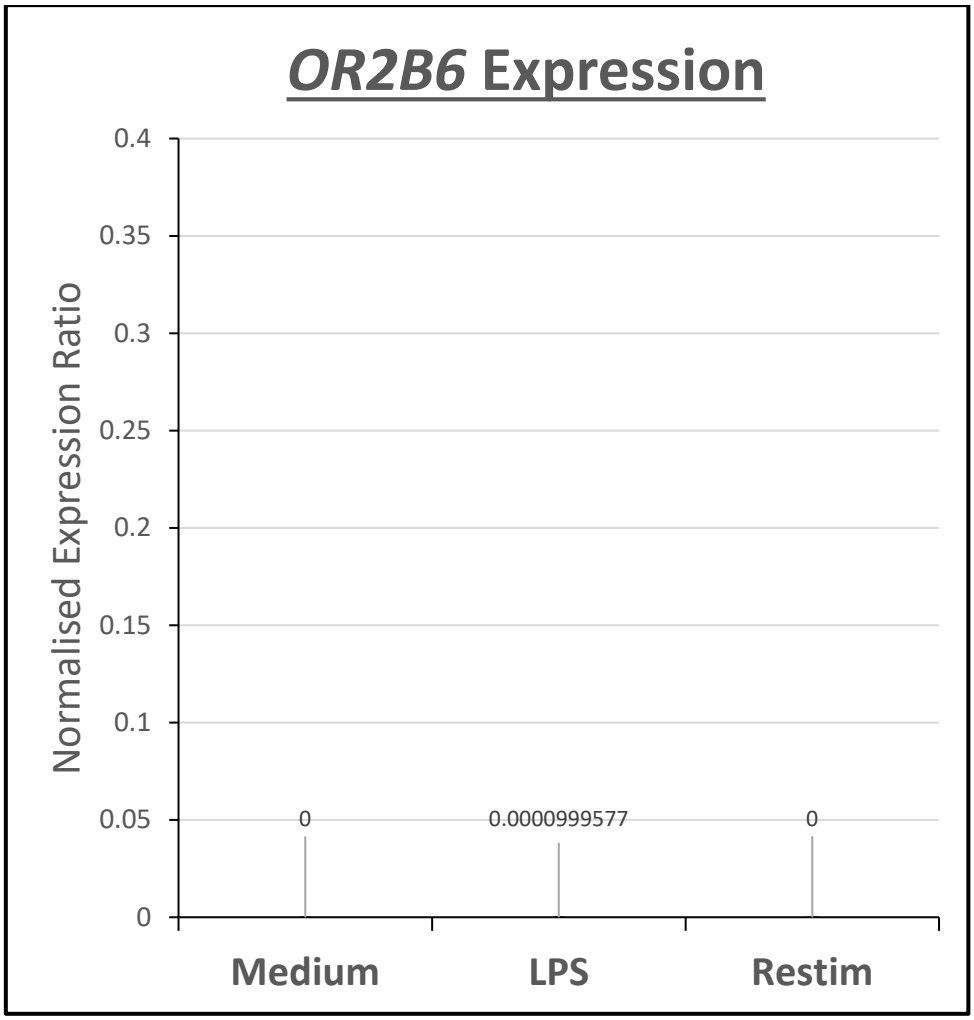


Figure 3.23: Bar Chart visualising differences in expression ratios for *OR2B6* in PBMCs exposed to culture medium, primary LPS stimulation, and secondary LPS restimulation, respectively. Normalised expression ratios of *TNFA* expression obtained by real-time quantitative PCR against reference gene *ACTB.* Ratios shown above were obtained from the average of normalised ratios for each experimental category: Medium, LPS and Restim. No noticeable expression of *OR2B6* was detected across all three experimental conditions.

Chapter 4:

Discussion

4 Discussion

In this project, OR gene expression patterns and inducibility were evaluated in the context of immune cells, particularly monocytes and macrophages. Bioinformatics analysis of independent publicly available leukocyte transcriptomes from sepsis patients, non-infectious ICU patients, human endotoxemia, and *in vitro* LPS stimulations of macrophages revealed members of the OR gene family were expressed. This corroborates a seminal study that found ectopic OR gene expression in leukocytes of critically ill patients with all-cause sepsis (Scicluna et al 2020). Moreover, OR gene expression was significantly altered in sepsis relative health, as well as throughout a human endotoxemia time course relative to baseline (before LPS infusion). No differences in OR gene expression was uncovered in source-specific sepsis compared to non-infectious ICU patients, suggesting that induction of OR gene expression may not be specific to infection, but may represent a general feature of critical illness. Furthermore, *OR2B6* expression in primary human monocytes was not induced by LPS nor detectable in endotoxin tolerance. Altogether, OR gene expression was altered in the context of critical illness and an acute systemic inflammatory model, albeit expression levels of ORs were relatively low and may not be inducible by PAMPs (**Figure 4.1**).

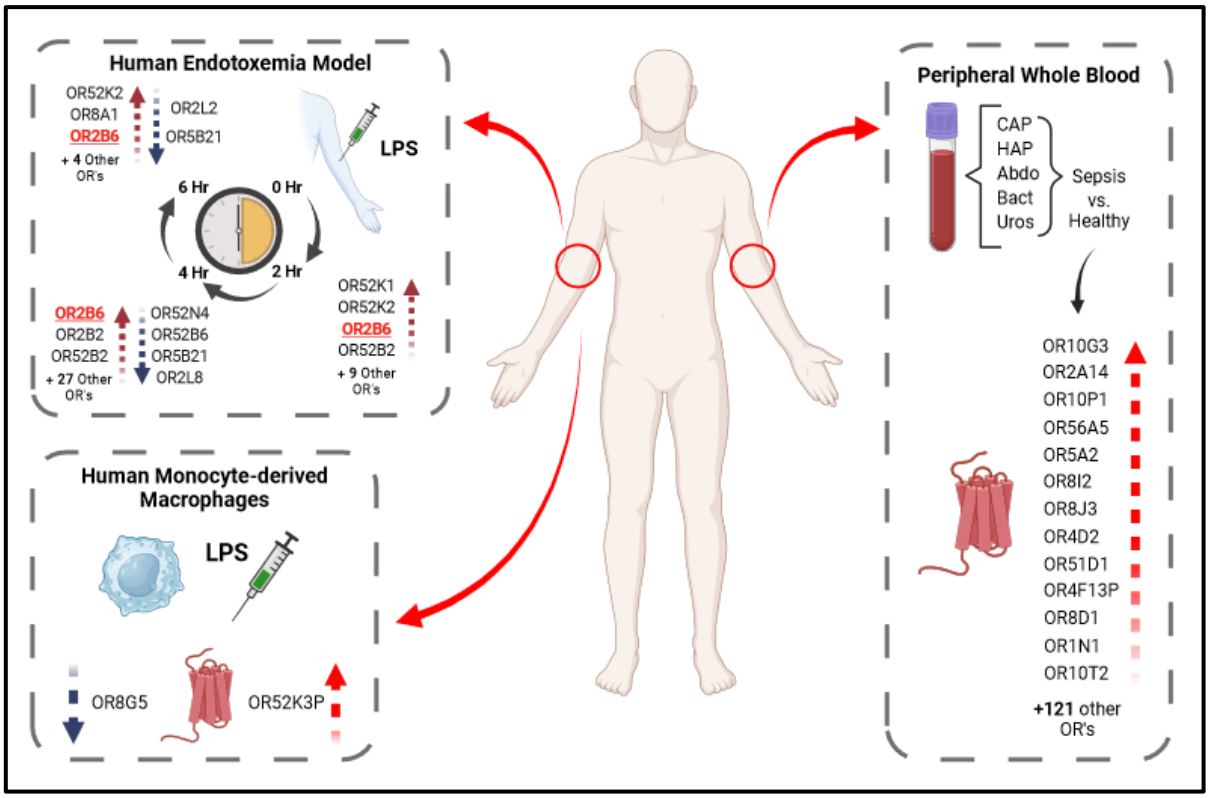


Figure 4.1: Overview of Olfactory Receptor (OR) gene expression findings. Critical illness was associated with elevated expression of OR genes, as well as in the context of an acute systemic inflammatory model (human endotoxemia), with *OR2B6* substantially altered. PAMPs, for example *E. coli* LPS, may not induce OR gene expression.

While previously thought not to possess any degree of specific immune memory, the innate immune system has since demonstrated an interesting capability to develop a short-term functional memory which enables it to incite rapid immune responses towards certain pathogens or stimuli (Divangahi et al 2021). This behaviour reveals itself in the form of trained immunity and immune tolerance, two opposing processes which can enhance or modulate the immune response, respectively (Ifrim et al 2014). These features are primarily observed in highly plastic myeloid-derived monocytes, macrophages and natural killer cells (Langenkamp et al 2000, Foster et al 2007, Netea et al 2011, Souza-Fonseca-Guimaraes et al 2012, Cerwenka and Lanier 2016), with monocytes and macrophages being the greatest contributors (Jakubzick et al 2017, Locati et al 2020). Trained immunity refers to the primed state in which

innate immune cells may elicit more robust immune responses in future encounters, so long as these cells continue to persist (Divangahi et al 2021). Immunological "training" of innate immune cells typically begins with the exposure of cellular PRRs (e.g. TLR-4) to PAMPs (e.g. LPS) present on foreign pathogens, or DAMPs (e.g. ATP) released by damaged tissue. However, the excessive amounts of primed cells may be detrimental due to the increasing severity of subsequent inflammatory responses which have a high propensity for collateral damage in healthy tissues. In the face of repeated exposures to immunogenic stimuli, an opposing state of immune tolerance is instead favoured (Kaufmann et al 2018), with the aim of deliberately impairing the immune response to prevent such extensive damage (Divangahi et al 2021). The most widely investigated form of tolerance has been that established by repeated exposures of innate immune cells, primarily monocytes or macrophages, to LPS termed endotoxin tolerance (Foster et al 2007, Biswas and Lopez-Collazo 2009). The mechanisms underlying the establishment of endotoxin tolerance have been advocated as potentially key features of sepsis-induced immune paralysis (Cheng et al 2016). Sepsis causes both extensive tissue damage during its acute inflammatory phase and then suppresses the innate and adaptive immune system in the longer-term, increasing vulnerability to secondary infections and impairing tissue recovery (Cavaillon and Adib-Conquy 2006, Monneret et al 2008, van der Poll et al 2017). The transcriptional profiles of leukocytes in sepsis patients on ICU admission have been shown to exhibit signs of sustained endotoxin tolerance (Davenport et al 2016, Cheng et al 2017). Whether OR activity is a feature of sepsis pathophysiology and/or endotoxin tolerance is unknown. Hence, the findings of this project lend weight to the concept of ectopic OR gene expression in the context of critical illness due to sepsis and acute systemic inflammation. When comparing source-specific sepsis cases with their respective ICU controls, some differences in OR gene expression were found, particularly intra-abdominal infections

relative to post-operative ICU patients. These findings suggest a more variable OR landscape in the case of sepsis caused by intra-abdominal infections than other types of sources, for example lungs. Therefore, OR gene expression in leukocytes may not be specific to infection. Despite this observation, leukocyte transcriptomes during a human endotoxemia time course exhibited significant shifts in OR gene expression. The 2-hour time point during human endotoxemia is considered the most significant time point in the innate immune response during which the inflammatory response reaches its peak and visible symptoms of fever begin to manifest (Lowry 2005). It is at this time point where monocytes are most likely to be experiencing significant epigenetic and metabolic reorientations to best adapt towards the role of effector pro-inflammatory immune cells (Divangahi et al 2021). A total of 3 monocyte-specific ORs (*OR52K1, OR52K2, OR2B6*) were identified to be statistically significant and notably upregulated during the 2-hour time point of the innate immune response as shown in Scicluna et al's (2020) study, with a fourth upregulated OR being shared amongst multiple other innate immune cell types (*OR52B2*). The 4 hours' time point in human endotoxemia has been reported as highly relevant to endotoxin tolerance (Scicluna et al 2020, Davenport et al 2016, Cheng et al 2017, de Vos et al 2009). Therefore, OR gene expression found to be altered during this phase may be potentially involved in the development of immune tolerance. *OR2B6* was strongly expressed after 4 and 6 hours of human endotoxemia. This observation was key to selecting *OR2B6* for experimental inducibility evaluation. Notably, the human endotoxemia model and critical illness due to sepsis or non-infectious aetiologies are characterized by substantial shifts in cell composition. In the context of human endotoxemia, approximately 40% of the variance in whole blood leukocyte gene expression can be explained by shifts in cell counts and differentials (Khan et al 2019). Therefore, it is completely plausible that differences in OR gene expression may reflect changes in cell composition rather than

bonafide differential gene expression. To better understand the cell-specific implications of OR gene expression and whether they respond to extracellular cues, for example infection, RNA-sequencing data generated from specific PBMCs purified from sepsis patients or healthy control subjects was evaluated. When comparing the data for CD14+ monocytes between sepsis patients and healthy controls, both upregulated and downregulated ORs were identified, with *OR52B2* both found to be statistically significant and strongly upregulated. Thus, these results suggest that ORs may be differentially expressed in specific PBMCs in response to sepsis. Exploring the expression patterns in a larger cohort of patients with cell-type specific transcriptomes, including neutrophils would undoubtedly assist in better understanding their mechanistic implications.

Olfactory receptors are the largest family of membrane-bound GPCRs (Kobilka 2007, Latorraca et al 2017, Cong et al 2022), constituting the largest multi-gene family in mammalian genomes (Barnes et al 2020). While ORs had been initially thought to be exclusively localised to the olfactory tissue, studies over the last two decades have discovered ectopic ORs located in non-olfactory tissues and cells (Tong et al 2021), fulfilling various other functions (Spehr et al 2003, Neuhaus et al 2009, Zhang et al 2012, Chang et al 2015, Kang et al 2015, Munakata et al 2018). The discovery of ectopic ORs in pulmonary mouse macrophages and their chemotactic functions (Li et al 2013) has since suggested that these ORs may also possess roles in the innate immune response to infection and cell activation. Other ORs have since been implicated in anti-inflammatory macrophage differentiation (Lee et al 2019, Vadevoo et al 2021), inflammasome formation (Orecchioni et al 2022), and phagocytosis in alveolar macrophages (Weidinger et al 2020). Attempts at investigating parallels between peripheral whole blood and other tissues involved in innate immune were made by studying the action and expression patterns of ectopic ORs in human bronchial epithelium (Ramirez-Moral et al

2021). A dataset containing gene expression data for HBE prior to and after exposure against bacterial flagellin was analysed, attempting to isolate any potential ORs of interest which could be more profoundly expressed in response to an immunogenic stimulus. Although several different ORs were initially found to be differentially up- and downregulated in response to bacterial flagellin, these were all attenuated after filtering out for statistical significance and thus were not reliable enough to draw any targets from, most likely due to the low sample size of the study (n=3). Following up on evidence that suggested ORs to be increasingly expressed during infection, and the inconclusive evidence towards differential expression in immune tissues, the expression of ORs in dedicated innate immune effector cells was thus explored. The cells chosen for this study were human monocyte-derived macrophages, highly plastic immune effector cells differentiated from naïve human monocytes. These MDMs were later exposed to a bacterial stimulus (i.e. LPS) with gene expression profiles before and after the stimulatory event being available (Malmström et al 2020). Analysis of the OR gene expression patterns showed evidence of upregulation and suppression of two specific ORs: *OR52K3P* and *OR8G5*, respectively. These findings suggested that these ORs were differentially expressed in response to a well-known PAMP, thus strengthening the idea that ORs may indeed be inducible in innate effector cells such as human monocytes and macrophages in response to pathogen recognition. In the experimental procedure of this study, monocytes from healthy donors were exposed to LPS for 24 hours prior to harvesting, with another select population of cells being allowed to rest for a full day thereafter. After this first resting period, the monocytes were re-stimulated with LPS to establish endotoxin tolerance as previously described (Cheng, Scicluna et al 2017, de Vos et al 2009, Bekkering et al 2018, Saeed et al 2014). As expected, testing by ELISA showed a significantly marked increase in TNF-α production after LPS stimulation, with a dampened

response to re-stimulation. Moreover, *TNFA* gene expression reflected protein secretion profiles, further reinforcing the establishment of tolerance. In this context, *OR2B6* was not reliably expressed. Only 1 of 8 cell samples returned a detectable signal which alluded to expression of *OR2B6*. Whether ectopic OR gene expression can be induced in innate immune cells after stimulation remains an open question.

The study has limitations. First, changes in OR gene expression in sepsis patients relative to ICU control patients or health, as well as in the human endotoxemia model may relate to shifts in cell counts and differentials. The variance in OR gene expression explained by shifts in cell composition was not evaluated. Albeit, OR gene expression was altered in specific PBMCs purified from sepsis patients relative to health, which lends weight to the potential for *bonafide* OR gene expression responses to infection, as well as potential cell-specific functions across different committed blood leukocyte lineages. Second, the RNA-sequencing data assessed in this study lacked neutrophil-specific data. Third, bronchial epithelial cell and MDM RNA-sequencing data were low in sample size, precluding robust conclusions on OR gene expression inducibility. Fourth, the experimental validation was centred on one PAMP as a trigger, that is LPS. It is certainly plausible that in the context of sepsis several other PAMPs (e.g. peptidoglycan) or DAMPs (e.g. ATP or *HMGB1*) may elicit changes in OR gene expression. Furthermore, OR gene expression inducibility may be triggered by cytokine signalling.

In the future, more comprehensive analyses of OR gene expression dynamics in broader patient cohorts encompassing other infectious diseases, for example: tuberculosis, dengue fever, and COVID-19; that is, not only sepsis patients, as well as evaluating a larger range of PAMPs, DAMPs and cytokines (e.g. IL-1β, IL-10, IL-6) in the context of immune

functional assays is certainly warranted. Moreover, designing antibodies against specific ORs and testing their surface expression by means of flow cytometry or western blots may allow for targeted investigations of cell-specific ORs, following up on results for cell-specific ORs obtained via bioinformatics. Genome editing in specific cells (e.g. CRISPR-Cas9 editing of ORs) can be utilised to evaluate cell-specific functions of potential OR targets, especially after further investigation on cell-specific OR surface expression has been performed, expediting the transition to a potential mechanism versus the aforementioned correlative approaches. These molecular mechanisms can then be further investigated in relation to the development of innate immune memory and endotoxin tolerance. Furthermore, the OR landscape and associated mechanisms can be further studied in MDMs through targeted cell culturing and testing, allowing for a deeper understanding of their effects in terminally differentiated innate immune effector cells.

In conclusion, ORs represent the largest family of receptor proteins that have been traditionally confined to the olfactory system. Recent reports and results generated in this study lend weight to the concept of ectopic OR activity in innate immune cells during sepsis. The expression patterns of ORs may not be influenced by PAMP recognition in innate immune cells. Altogether, the study herein described may facilitate further functional exploration of ectopic ORs in the context of infection. Whether innate immune cells can “smell” danger by means of ORs remains an outstanding and interesting question to pursue in future studies.

References

ABELES, R.D., MCPHAIL, M.J., SOWTER, D., ANTONIADES, C.G., VERGIS, N., VIJAY, G.K., XYSTRAKIS, E., KHAMRI, W., SHAWCROSS, D.L., MA, Y., WENDON, J.A., VERGANI, D., 2012, CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14(hi) /CD16(neg) monocytes: Expansion of CD14(hi) /CD16(pos) and contraction of CD14(lo) /CD16(pos) monocytes in acute liver failure, *Cytometry A.*, vol. 81, no. 10, pp. 823 – 834, doi: 10.1002/cyto.a.22104

ADELMAN, K., KENNEDY, M.A., NECHAEV, S., GILCHRIST, D.A., MUSE, G.W., CHINENOV, Y., ROGATSKY, I., 2009, Immediate mediators of the inflammatory response are poised for gene activation through RNA polymerase II stalling, *Proc. Natl. Acad. Sci. USA,* vol. 106, no. 43, pp. 18207 – 18212, doi: 10.1073/pnas.0910177106

ADEOLA, F., 2018, Normalization of Gene Expression by Quantitative RT-PCR in Human Cell Line: comparison of 12 Endogenous Reference Genes, *Ethiop. J. Health Sci.*, vol. 28, no. 6, pp. 741 – 748, doi: 10.4314/ejhs.v28i6.9

AKAVIPAT, P., THINKHAMROP, J., THINKHAMROP, B., SRIRAJ, W., 2019, Acute Physiology And Chronic Health Evaluation (Apache) II Score – The Clinical Predictor In Neurosurgical Intensive Care Unit, *Acta Clinica Croatica*, vol. 58, pp. 50 – 56, doi: 10.20471/acc.2019.58.01.07

ANDERS, S., PYL, P.T., HUBER, W., 2015, HTSeq—a Python framework to work with high-throughput sequencing data, *Bioinformatics*, vol. 31, no. 2, pp. 166 – 169, doi: 10.1093/bioinformatics/btu638

ANDREASEN, A.S., KRABBE, K.S., KROGH-MADSEN, R., TAUDORF, S., PEDERSEN, B.K., MØLLER, K., 2008, Human endotoxemia as a model of systemic inflammation, *Curr. Med. Chem.*, vol. 15, no. 17, pp. 1697 – 1705, doi: 10.2174/092986708784872393

ANDREWS, S., 2023-last update, FastQC [Babraham Bioinformatics], [Online]. Available at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (Jul 23, 2024)

ANGUS, D.C., VAN DER POLL, T., PHD, 2013, Severe Sepsis and Septic Shock, *New England Journal of Medicine,* vol. 369, pp. 840 – 851, doi: 10.1056/NEJMra1208623

ANTUNES, G., SEBASTIÃO, A.M., DE SOUZA, F.M., 2014, Mechanisms of Regulation of Olfactory Transduction and Adaptation in the Olfactory Cilium, *PLoS One*, vol. 9, no. 8, doi: 10.1371/journal.pone.0105531

ARTIKA, I.M., DEWI, Y.P., NAINGGOLAN, I.M., SIREGAR, J.E., ANTONJAYA, U., 2022, Real-Time Polymerase Chain Reaction: Current Techniques, Applications, and Role in COVID-19 Diagnosis, *Genes (Basel)*, vol. 13, no. 12, p. 2387, doi: 10.3390/genes13122387

ARUTA, M.G., LARI, E., DE SIMONE, D., SEMPLICI, B., SEMPLICI, C., DALE, H., CHIRWA, E., KADWALA, I., MBEWE, M., BANDA, H., ITURRIZA-GOMARA, M., GORDON, M., NYIRENDA, T., PIU, P., PIZZA, M., SCORZA, F.B., GRAPPI, S., CANALS, R., ROSSI, O., 2023, Characterization of

Enzyme-Linked Immunosorbent Assay (ELISA) for Quantification of Antibodies against Salmonella Typhimurium and Salmonella Enteritidis O-Antigens in Human Sera, *BioTech (Basel)*, vol. 12, no. 3, p. 54, doi: 10.3390/biotech12030054

BALS, R., HEIMSTRA, P.S., 2004, Innate immunity in the lung: how epithelial cells fight against respiratory pathogens, *European Respiratory Journal*, vol. 23, pp. 327 – 333, doi: 10.1183/09031936.03.00098803

BARNES, I.H., IBARRA-SORIA, X., FITZGERALD, S., GONZALEZ, J.M., DAVIDSON, C., HARDY, M.P., MANTHRAVADI, D., VAN GERVEN, L., JORISSEN, M., ZHENG, Z., KHAN, M., MOMBAERTS, P., HARROW, J., LOGAN, D.W., FRANKISH, A., 2020, Expert curation of the human and mouse olfactory receptor gene repertoires identifies conserved coding regions split across two exons, *BMC Genomics*, vol. 21, no. 196, doi: 10.1186/s12864-020-6583-3

BEKKERING, S., ARTS, R.J., NOVAKOVIC, B., KOURTZELIS, I., VAN DER HEIJDEN, C.D., LI, Y., POPA, C.D., TER HORST, R., VAN TUIJL, J., NETEA-MAIER, R.T., VAN DE VEERDONK, F.L., CHAVAKIS, T., JOOSTEN, L.A., VAN DER MEER, J.W., STUNNENBERG, H., RIKSEN, N.P., NETEA, M.G., 2018, Metabolic Induction of Trained Immunity through the Mevalonate Pathway., *Cell*, vol. 172, no. 1 – 2, pp. 135 – 146, doi: 10.1016/j.cell.2017.11.02

BEKKERING, S., DOMÍNGUEZ-ANDRÉS, J., JOOSTEN, LA.., RIKSEN, N.P., NETEA, M.G., 2021, Trained Immunity: Reprogramming Innate Immunity in Health and Disease, *Annu. Rev. Immunol.*, vol. 39, pp. 667 – 693, doi: 10.1146/annurev-immunol-102119-073855

BENJAMINI, Y., HOCHBERG, Y., 1995, "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing", *Journal of the Royal Statistical Society Series B*, Oxford University Press, United Kingdom, pp. 289 – 300

BISWAL, J.K., RANJAN, R., DAHIYA, S.S., MALLICK, S., MOHAPATRA, J.K., 2021, Regenerated silica-based RNA purification columns to address the short supply of RNA purification kits for COVID-19 diagnosis, *Mol. Biol. Rep.*, vol. 48, no. 10, pp. 6871 – 6877, doi: 10.1007/s11033-021-06688-0

BISWAS, S.K., LOPEZ-COLLAZO, E., 2009, Endotoxin tolerance: new mechanisms, molecules and clinical significance, *Trends. Immunol.*, vol. 30, no. 10, pp. 475 – 487, doi: 10.1016/j.it.2009.07.009.

BOLGER, A.M., LOHSE, M., USADEL, B., 2014, Trimmomatic: a flexible trimmer for Illumina sequence data, *Bioinformatics*, vol. 30, no. 15, pp. 2114 – 2120, doi: 10.1093/bioinformatics/btu170

BOMANS, K., SCHENZ, J., SZTWIERTNIA, I., SCHAACK, D., WEIGAND, M.A., UHLE, F., 2018, Sepsis Induces a Long-Lasting State of Trained Immunity in Bone Marrow Monocytes, *Front. Immunol.*, vol. 9, no. 2685, doi: 10.3389/fimmu.2018.02685

BOOMER, J.S., SHUHERK-SHAFFER, J., HOTCHKISS, R.S., GREEN, J.M., 2012, A prospective analysis of lymphocyte phenotype and function over the course of acute sepsis, *Critical Care*, vol. 16, no. R112, doi: 10.1186/cc11404

BOOMER, J.S., TO, K., CHANG, K.C., TAKASU, O., OSBORNE, M.D., WALTON, A.H., BRICKER, T.L., JARMAN, S.D., KREISEL, D., KRUPNICK, A.S., SRIVASTAVA, A., SWANSON, P.E., GREEN, J.M., HOTCHKISS, R.S., 2011, Immunosuppression in Patients Who Die of Sepsis and Multiple Organ Failure, *JAMA,* vol. 306, no. 23, pp. 2594 – 2605, doi: 10.1001/jama.2011.1829

BRANDS, X., HAAK, B.W., KLARENBEEK, A.M., BUTLER, J., UHEL, F., QIN, W., OTTO, N.A., JAKOBS, M.E., FABER, D.R., LUTTER, R., WIERSINGA, W.J., VAN DER POLL, T., SCICLUNA, B.P., 2021, An epigenetic and transcriptomic signature of immune tolerance in human monocytes through multi-omics integration, *Genome Medicine*, vol. 13, no. 131, doi: 10.1186/s13073-021-00948-1

BUSTIN, S., HUGGETT, J., 2017, qPCR primer design revisited, *Biomolecular Detection and Quantification*, vol. 14, pp. 19 – 28, doi: 10.1016/j.bdq.2017.11.001

BUSTIN, S., NOLAN, T., 2004, "Analysis of mRNA Expression by Real-time PCR", SAUNDERS, N.A., LEE, M.A. (eds), *Real-Time PCR: Advanced Technologies and Applications*, Caister Academic Press, Poole (UK).

BUTCHER, S.K., O'CARROLL, C.E., WELLS, C.A., CARMODY, R.J., 2018, Toll-Like Receptors Drive Specific Patterns of Tolerance and Training on Restimulation of Macrophages, *Front. Immunol.*, vol. 9, p. 933, doi: 10.3389/fimmu.2018.00933

CAVAILLON, J.M., ADIB-CONQUY, M., 2006, Bench-to-bedside review: Endotoxin tolerance as a model of leukocyte reprogramming in sepsis, vol. 10, no. 5, p. 233, doi: 10.1186/cc5055

CERWENKA, A., LANIER, L. L., 2016, Natural killer cell memory in infection, inflammation and cancer., *Nature Reviews Immunology*, vol. 16, pp. 112 – 123, doi: 10.1038/nri.2015.9

CHAND, M., ARMSTRONG, T., BRITTON, G., NASH, G., 2007, How and why do we measure surgical risk?, *Journal of the Royal Society of Medicine*, vol. 100, no. 11, pp. 508 – 512, doi: 10.1258/jrsm.100.11.508

CHANG, A.J., ORTEGA, F.E., RIEGLER, J., MADISON, D.V., KRASNOW, M.A., 2015, Oxygen control of breathing by an olfactory receptor activated by lactate, *Nature,* vol. 527, no. 7577, pp. 240 – 244, doi: 10.1038/nature15721

CHEN, D., LI, K., PAN, L., WU, Y., CHEN, M., ZHANG, X., XU, J., LOU, T., 2023, TCF7 and LEF-1 downregulation in sepsis promotes immune suppression by inhibiting CD4+ T cell proliferation, *Microb. Pathog*., vol. 184, doi: 10.1016/j.micpath.2023.106362.

CHEN, W., WANG, J., YE, B., ZHOU, J., WANG, W., 2021, The population characteristics of the main leukocyte subsets and their association with chronic diseases in a community-dwelling population: a cross-sectional study, *Prim. Health Care Res. Dev.*, vol. 22, p. e18, doi: 10.1017/S1463423621000153

CHENG, S.C., SCICLUNA, B.P., ARTS, R.J., GRESNIGT, M.S., LACHMANDAS, E., GIAMARELLOS-BOURBOULIS, E.J., KOX, M., MAJERI, G.R., WAGENAARS, J.A., CREMER, O.L., LEENTJENS, J., VAN DER MEER, A.J., VAN DE VEERDONK, F.L., BONTEN, M.J., SCHULTZ, M.J., WILLEMS, P.H., PICKKERS, P., JOOSTEN, L.A., VAN DER POLL, T., NETEA, M.G., 2016, Broad defects in the energy metabolism of leukocytes underlie immunoparalysis in sepsis, *Nat. Immunol.*, vol. 17, no. 4, pp. 406 – 413, doi: 10.1038/ni.3398

CHIEN, A., EDGAR, D.B., TRELA, J.M., 1976, Deoxyribonucleic acid polymerase from the extreme thermophile Thermus aquaticus., *J. Bacteriol.*, vol. 127, no. 3, pp. 1550 – 1557, doi: 10.1128/jb.127.3.1550-1557.1976

COHEN, J., VINCENT, J., ADHIKARI, N.,K., MACHADO, F.R., ANGUS, D.C., CALANDRA, T., JATON, K., GIULIERI, S., DELALOYE, J., OPAL, S., TRACEY, K., VAN DER POLL, T., PELFRENE, E., 2015, Sepsis: a roadmap for future research, *Lancet. Infect. Dis.*, vol. 15, no. 5, pp. 581 – 614, doi: 10.1016/S1473-3099(15)70112-X.

CONG, X., REN, W., PACALON, J., XU, R., XU, L., LI. X., DE MARCH, C.A., MATSUNAMI, H., YU, H., YU, Y., GOLEBIOWSKI, J., 2022, Large-Scale G Protein-Coupled Olfactory Receptor–Ligand Pairing, *ACS Cent. Sci.*, vol. 8, no. 3, pp. 379 – 387, doi: 10.1021/acscentsci.1c01495

DANNA, D.M., 2018, Hospital Costs Associated with Sepsis Compared with Other Medical Conditions, *Crit. Care. Nurs. Clin. North Am.*, vol. 30, no. 3, pp. 389 – 398, doi: 10.1016/j.cnc.2018.05.007

DAVENPORT, E.E., BURNHAM, K.L., RADHAKRISHNAN, J., HUMBURG, P., HUTTON, P., MILLS, T.C., RAUTANEN, A., GORDON, A.C., GARRARD, C., HILL, A.V., HINDS, C.J., KNIGHT, J.C., 2016, Genomic landscape of the individual host response and outcomes in sepsis: a prospective cohort study, *Lancet Resp. Medicine*, vol. 4, no. 4, pp. 259 – 271, doi: 10.1016/S2213-2600(16)00046-1

DE, A.K., LAUDANSKI, K., MILLER-GRAZIANO, C.L., 2003, Failure of Monocytes of Trauma Patients to Convert to Immature Dendritic Cells is Related to Preferential Macrophage-Colony-Stimulating Factor-Driven Macrophage Differentiation, *J. Immunol*., vol. 170, no. 12, pp. 6355 – 6362, doi: 10.4049/jimmunol.170.12.6355

DE LAVAL, B., MAURIZIO, J., KANDALIA, P.K., BRISOU, G., SIMONNET, L., HUBER, C., GIMENEZ, G., MATCOVITCH-NATAN, O., REINHARDT, S., DAVID, E., MILDNER, A., LEUTZ, A., NADEL, B., BORDI, C., AMIT, I., SARRAZIN, S., SIEWEKE, M.H., 2020, C/EBPβ-Dependent Epigenetic Memory Induces Trained Immunity in Hematopoietic Stem Cells, *Stem Cell*, vol. 26, no. 5, pp. 657 – 674, doi: 10.1016/j.stem.2020.01.017

DEGL'INNOCENTI, A., D'ERRICO, A., 2017, Regulatory Features for Odorant Receptor Genes in the Mouse Genome, *Front. Genet.*, vol. 8, no. 19, doi: 10.3389/fgene.2017.00019

DE VOS, A.F., PATER, J.M., VAN DEN PANGAART, P.S., DE KRUIF, M.D., VAN'T VEER, C., VAN DER POLL, T., 2009, In vivo lipopolysaccharide exposure of human blood leukocytes induces cross-tolerance to multiple TLR ligands, *J. Immunol.*, vol. 183, no. 1, pp. 533 – 542, doi: 10.4049/jimmunol.0802189

DIVANGAHI, M., AABY, P., ABDUL, K., BARREIRO, L.B., BEKKERING, S., CHAVAKIS, T., VAN CREVEL, R., CURTIS, N., DINARDO, A.R., DOMINGUEZ-ANDRES, J., DUIVENVOORDEN, R., FANUCCHI, S., FAYAD, Z., FUCHS, E., HAMON, M., JEFFREY, K.L., KHAN, N., JOOSTEN, L.A., KAUFMANN, E., LATZ, E., MATARESE, G., VAN DER MEER, J.W., MHLANGA, M., MOORLAG, S.J., MULDER, W.J., NAIK, S., NOVAKOVIC, B., O'NEILL, L., OCHANDO, J., OZATO, K., RIKSEN, N.P., SAUERWEIN, R., SHERWOOD, E.R., SCHLITZER, A., SCHULTZE, J.L., SIEWEKE, M.H., STABELL BENN, C., STUNENNBERG, H., SUN, J., VAN DE VEERDONK, F.L., WEIS, S., WILLIAMS, D.L., XAVIER, R., NETEA, M.G., 2021, Trained immunity, tolerance, priming and differentiation: distinct immunological processes, *Nat. Immunol.*, vol. 22, no. 1, pp. 2 – 6, doi: 10.1038/s41590-020-00845-6.

DOMINGUEZ-ANDRES, J., NETEA, M.G., 2019, Long-term reprogramming of the innate immune system, *J, Leukoc. Biol.,* vol. 105, no. 2, pp. 329 – 338, doi: 10.1002/JLB.MR0318-104R

DUGANI, S., PHD, VEILLARD, J., PHD, KISSOON, N., 2017, Reducing the global burden of sepsis, *CMAJ.*, vol. 189, no. 1, pp. E2 – E3, doi: 10.1503/cmaj.160798

ESCOBAR, M.D., HUNT, J.L., 2017, A cost-effective RNA extraction technique from animal cells and tissue using silica columns, *J. Biol. Methods*, vol. 4, no. 2, p. e72, doi: 10.14440/jbm.2017.184

2024, Ex Taq DNA Polymerase [TaKaRa Bio. Inc.], [Online]. Available: https://www.takarabio.com/products/pcr/high-yield-pcr/ex-taq-products/ex-taq-polymerase-hot-start (Jan 22, 2024)

FAIVRE, V., LUKASZEWICZ, A., ALVES, A., CHARRON, D., PAYEN, D., HAZIOT, A., 2007, Accelerated in vitro differentiation of blood monocytes into dendritic cells in human sepsis, *Clinical and Experimental Immunology*, vol. 147, no. 3, pp. 426 – 439, doi: 10.1111/j.1365-2249.2006.03287.x

FAN, H., COOK, J.A., 2004, Review: Molecular mechanisms of endotoxin tolerance, *Journal of Endotoxin Research*, vol. 10, no. 2, pp. 71 – 84, doi: 10.1177/09680519040100020301

FOSTER, S.L., HARGREAVES, D.C., MEDZHITOV, R., 2007, Gene-specific control of inflammation by TLR-induced chromatin modifications, *Nature,* vol. 447, no. 7147, pp. 972 – 978, doi: 10.1038/nature05836

FULCHER, M.L., RANDELL, S.H., 2013, Human nasal and tracheo-bronchial respiratory epithelial cell culture, *Methods. Mol. Biol.*, vol. 945, pp. 109 – 121, doi: 10.1007/978-1-62703-125-7_8.

2023, Gentamicin (10 mg/mL), [ThermoFisher Scientific], [Online]. Available at: https://www.thermofisher.com/order/catalog/product/15710064 (Dec 20, 2023)

GHANNAM, M.G., VARACALLO, M., 2023-last update, Biochemistry, Polymerase Chain Reaction [StatPearls], [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK535453/ (Jan 20, 2024)

2023, GlutaMAX™ Supplement, [ThermoFisher Scientific], [Online]. Available at: https://www.thermofisher.com/order/catalog/product/35050061 (Dec 20, 2023)

GUISSET, O., DILHUYDY, M., THIÉBAUT, R., LEFÈVRE, J., CAMOU, F., SARRAT, A., GABINSKI, C., MOREAU, J., BLANCO, P., 2006, Decrease in circulating dendritic cells predicts fatal outcome in septic shock, *Intensive Care Medicine*, vol. 33, pp. 148 – 152, doi: 10.1007/s00134-006-0436-7

HEUBECK, A., SAVAGE, A., HENDERSON, K., ROLL, C., HERNANDEZ, V., TORGERSON, T., BUMOL, T., READING, J., 2022, Cross-platform immunophenotyping of human peripheral blood mononuclear cells with four high-dimensional flow cytometry panels, *Cytometry Part A*, vol. 103, no. 6, pp. 500 – 517, doi: 10.1002/cyto.a.24715

HOTCHKISS, R.S., TINSLEY, K.W., SWANSON, P.E., GRAYSON, M.H., OSBORNE, D.F., WAGNER, T.H., COBB, J.P., COOPERSMITH, C., KARL, I.E., 2002, Depletion of Dendritic Cells, But Not Macrophages, in Patients with Sepsis, *J. Immunol.*, vol. 168, no. 5, pp. 2493 – 2500, doi: 10.4049/jimmunol.168.5.2493

HUANG, P., ZHOU, Y., LIU, Z., ZHANG, P., 2016, Interaction between ANXA1 and GATA-3 in Immunosuppression of CD4+ T Cells, *Mediators Inflamm.,* doi: 10.1155/2016/1701059

IBARRA-SORIA, X., LEVITIN, M.O., SARAIVA, L.R., LOGAN, D.W., 2014, The Olfactory Transcriptomes of Mice, *PLoS Genetics*, vol. 10, no. 9, doi: 10.1371/journal.pgen.1004593

IFRIM, D.C., QUINTIN, J., JOOSTEN, L.A., JACOBS, C., JANSEN, T., JACOBS, L., GOW, N.A., WILLIAMS, D.L., VAN DER MEER, J.W., NETEA, M.G., 2014, Trained Immunity or Tolerance: Opposing Functional Programs Induced in Human Monocytes after Engagement of Various Pattern Recognition Receptors, *Clin. Vaccine Immunol.*, vol. 21, no. 4, pp. 534 – 545, doi: 10.1128/CVI.00688-13

IGNATIEVA, E.V., LEVITSKY, V.G., YUDIN, N.S., MOSHKIN, M.P., KOLCHANOV, N.A., 2014, Genetic basis of olfactory cognition: extremely high level of DNA sequence polymorphism in promoter regions of the human olfactory receptor genes revealed using the 1000 Genomes Project dataset, *Front. Psychol.*, vol. 5, no. 247, doi: 10.3389/fpsyg.2014.00247

ISHINO, S., ISHINO, Y., 2014, DNA polymerases as useful reagents for biotechnology – the history of developmental research in the field, *Front. Microbiol.*, vol. 5, p. 465, doi: 10.3389/fmicb.2014.00465

ISKANDER, K.N., OSUCHOWSKI, M.F., STEARNS-KUROSAWA, D.J., KUROSAWA, S., STEPIEN, D., VALENTINE, C., REMICK, D.G., 2013, Sepsis: Multiple Abnormalities, Heterogeneous Responses, and Evolving Understanding, *Physiol. Rev.*, vol. 93, no. 3, pp. 1247 – 1288, doi: 10.1152/physrev.00037.2012

ITALIANI, P., BORASCHI, D., 2014, From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation, *Front. Immunol.*, vol. 5, p. 514, doi: 10.3389/fimmu.2014.00514

JAATINEN, T., LAINE, J., 2007, Isolation of mononuclear cells from human cord blood by Ficoll-Paque density gradient, *Curr. Protoc. Stem Cell Biol.*, doi: 10.1002/9780470151808.sc02a01s1

JANEWAY, C.A., TRAVERS, P., WALPORT, M., SHLOMCHIK, M.J., 2001, Chapter 9 – The Humoral Immune Response, *Immunobiology: The Immune System in Health and Disease 5th Edition*, United States, New York: Garland Science

JAKUBZICK, C.V., RANDOLPH, G.J., HENSON, P.M., 2017, Monocyte differentiation and antigen-presenting functions, *Nat. Rev. Immunol.*, vol. 17, no. 6, pp. 349 – 362, doi: 10.1038/nri.2017.28

JHA, A.K., HAUNG, S.C., SERGUSHICHEV, A., LAMPROPOULOU, V., IVANOVA, Y., LOGINICHEVA, E., CHMIELEWSKI, STEWART, K.M., ASHALL, J., EVERTS, B., PEARCE, E.J., DRIGGERS, E.M., ARTYOMOV, M.N., 2015, Network Integration of Parallel Metabolic and Transcriptional Data Reveals Metabolic Modules that Regulate Macrophage Polarization, *Immunity*, vol. 42, no. 3, pp. 419 – 430, doi: 10.1016/j.immuni.2015.02.005

JOSHI, C.J., KE, W., DRANGOWSKA-WAY, A., O'ROURKE, E.J., LEWIS, N.E., 2022, What are housekeeping genes?, *PLoS Comput. Biol.*, vol. 18, no. 7, pp. e1010295, doi: 10.1371/journal.pcbi.1010295

JULIAN, M.W., STRANGE, H.R., BALLINGER, M.N., HOTCHKISS, R.S., PAPENFUSS, T.L., CROUSER, E.D., 2015, Tolerance and Cross-Tolerance following Toll-Like Receptor (TLR)-4 and -9 Activation Are Mediated by IRAK-M and Modulated by IL-7 in Murine Splenocytes, *PLoS One*, vol. 10, no. 7, p. e0132921, *doi: 10.1371/journal.pone.0132921*

KANG, N., BAHK, Y.Y., LEE, N., JAE, Y., CHO, Y.H., KU, C.R., BYUN, Y., LEE, E.J., KIM, M., KOO, J., 2015, Olfactory receptor Olfr544 responding to azelaic acid regulates glucagon secretion in α-cells of mouse pancreatic islets, *Biochem. Biophys. Res. Commun.*, vol. 460, no. 3, pp. 616 – 621, doi: 10.1016/j.bbrc.2015.03.078

KANG, N., KOO, J.H., 2012, Olfactory receptors in non-chemosensory tissues, *BMB Reports*, vol. 45, no. 11, pp. 612 – 622, doi: 10.5483/BMBRep.2012.45.11.232

KAUFMANN, E., SANZ, J., DUNN, J.L., KHAN, N., MENDONÇA, L.E., PACIS, A., TZELEPIS, F., PERNET, E., DUMAINE, A., GRENIER, J., MAILHOT-LÉONARD, F., AHMED, E., BELLE, J., BESLA, R., MAZER, B., KING, I.L., NIJNIK, A., ROBBINS, C.S., BARREIRO, L.B., DIVANGAHI, M., 2018, BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis, *Cell,* vol. 172, no. 1 – 2, pp. 176 – 190, doi: 10.1016/j.cell.2017.12.031

KAWAI, T., AKRIA, S., 2010, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, *Nat. Immunol.*, vol. 11, no. 5, pp. 373 – 384, doi: 10.1038/ni.1863

KHAN H.N., BRANDS X., AUFIERO S., HOOGENDIJK A.J., KLARENBEEK A.M., VAN ENGELEN T.S., HAAK, B.W., VAN VUGHT L.A., HORN J., SCHULTZ M.J., ZWINDERMAN A.H., VAN DER POLL T., SCICLUNA B.P., 2020, The circular RNA landscape in specific peripheral blood mononuclear cells of critically ill patients with sepsis, *Crit Care*, vol. 24, no. 1, p. 423.

KHAN, H.N., PERLEE, D., SCHOENMAKER, L., VAN DER MEER, A.-J., FRANITZA, M., TOLIAT, M.R., NÜRNBERG, ZWINDERMANN, A.H., VAN DER POLL, T., SCICLUNA, B.P., 2019, Leukocyte transcriptional signatures dependent on LPS dosage in human endotoxemia, *J. Leukoc. Biol.*, vol. 106, no. 5, pp. 1153 – 1160, doi: 10.1002/JLB.4A0219-050R

KHEHRA, N., PADDA, I.S., SWIFT, C.J., 2023-last update, Polymerase Chain Reaction (PCR) [StatPearls], [Online]. Available: https://www.ncbi.nlm.nih.gov/books/NBK589663/ (Jan 19, 2024)

KIM, D., LANGMEAD, B., SALZBEG, S.L., 2015, HISAT: a fast spliced aligner with low memory requirements, *Nature Methods*, vol. 12, pp. 357 – 360, doi: 10.1038/nmeth.3317

KIM, S., YOON, Y.C., LEE, A.S., KANG, N., KOO, J., RHYU, M., PARK, J., 2015, Expression of human olfactory receptor 10J5 in heart aorta, coronary artery, and endothelial cells and its functional role in angiogenesis, *Biochem. Biophys. Res. Commun.*, vol. 460, no. 2, pp. 404 – 408, doi: 10.1016/j.bbrc.2015.03.046

KLEIN KLOUWENBERG, P.M., ONG, D.S., BOS, L.D., DE BEER, F.M., VAN HOOIJDONK, R.T., HUSON, M.A., STRAAT, M., VAN VUGHT, L.A., WIESKE, L., HORN, J., SCHULTZ, M.J., VAN DER POLL, T., BONTEN, M.J., CREMER, O.L., 2013, Interobserver agreement of Centers for Disease Control and Prevention criteria for classifying infections in critically ill patients, *Crit. Care Med.*, vol. 41, no. 10, pp. 2373 – 2378, doi: 10.1097/CCM.0b013e3182923712.

KLEINNIJENHUIS, J., QUINTIN, J., PREIJERS, F., JOOSTEN, L.A., IFRIM, D.C., SAEED, S., JACOBS, C., VAN LOENHOUT, J., DE JONG, D., STUNNENBERG, H.G., XAVIER, R.J., VAN DER MEER, J.W., VAN CREVEL, R., NETEA, M.G., 2012, Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes, *Proc. Natl. Acad. Sci. USA,* vol. 109, no. 43, pp. 17537 – 17542, doi: 10.1073/pnas.1202870109

KOBILKA, B.K., 2007, G Protein Coupled Receptor Structure and Activation, *Biochim. Biophys. Acta.*, vol. 1768, no. 4, pp. 794 – 807, doi:

LAJQI, T., KÖSTLIN-GILLE, N., BAUER, R., ZAROGIANNIS, S.G., LAJQI, E., AJETI, V., DIETZ, S., KRANIG, S.A., RÜHLE, J., DEMAJ, A., HEBEL, J., BARTOSOVA, M., FROMMHOLD, D., HUDALLA, H., GILLE, C., 2023, Training vs. Tolerance: The Yin/Yang of the Innate Immune System, *Biomedicines*, vol. 11, no. 3, p. 766, doi: 10.3390/biomedicines11030766

LANGENKAMP, A., MESSI, M., LANZAVECCHIA, A., SALLUSTO, F., 2000, Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells, *Nat. Immunol.*, vol. 1, no. 4, pp. 311 – 316, doi: 10.1038/79758

LATORRACA, N.R., VENKATAKRISHNAN, A.J., DROR, R.O., 2017, GPCR Dynamics: Structures in Motion, *Chem. Rev.*, vol. 117, no. 1, pp. 139 – 155, doi: 10.1021/acs.chemrev.6b00177

LEE, B.J., KIM, N.J., PARK, S.B., KOO, J.S., JEEN, Y.T., PARK, J.J., JOO, M.K., 2019, P065 Olfactory receptor, OR51E2 is a marker for innate immune cells in ulcerative colitis, *Journal of Crohn's and Colitis*, doi: 10.1093/ecco-jcc/jjy222.189

LI, Z., JU, X., SILVEIRA, P.A., ABADIR, E., HSU, W.-H., HART, D.N., CLARK, G.J., 2019, CD83: Activation Marker for Antigen Presenting Cells and Its Therapeutic Potential, *Front. Immunol.*, vol. 10, doi: 10.3389/fimmu.2019.01312

LI, J.J., TAY, H.L., PLANK, M., ESSILFIE, A., HANSBRO, P.M., FOSTER, P.S., YANG, M., 2013, Activation of Olfactory Receptors on Mouse Pulmonary Macrophages Promotes Monocyte Chemotactic Protein-1 Production, *PLoS One*, vol. 8, no. 11, doi: 10.1371/journal.pone.0080148

LI, Z., YANG, L., WANG, J., SHI, W., PAWAR, R.A., LIU, Y., XU, C., CONG, W., HU, Q., LU, T., XIA, F., GUO, W., ZHAO, M., ZHANG, Y., 2010, beta-Actin is a useful internal control for tissue-specific gene expression studies using quantitative real-time PCR in the half-smooth tongue sole Cynoglossus semilaevis challenged with LPS or Vibrio anguillarum, *Fish Shellfish Immunol.*, vol. 29, no. 1, pp. 89 – 93, doi: 10.1016/j.fsi.2010.02.021

LOCATI, M., CURTALE, G., MANTOVANI, A., 2020, Diversity, Mechanisms and Significance of Macrophage Plasticity, *Annu. Rev. Pathol.*, vol. 15, pp. 123 – 147, doi: 10.1146/annurev-pathmechdis-012418-012718

LOPEZ-COLLAZO, E., DEL FRESNO, C., 2013, Pathophysiology of endotoxin tolerance: mechanisms and clinical consequences, *Crit. Care*, vol. 17, no. 6, p. 242, doi: 10.1186/cc13110

LOVE, M.I., HUBER, W., ANDERS, S., 2014, Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2, *Genome Biology,* vol. 15, no. 550, doi: 10.1186/s13059-014-0550-8

LOWRY, S.F., 2009, HUMAN ENDOTOXEMIA: A MODEL FOR MECHANISTIC INSIGHT AND THERAPEUTIC TARGETING, *Shock*, vol. 24, pp. 94 – 100, doi: 10.1097/01.shk.0000191340.23907.a1

2023, Lymphoprep™, [Stem Cell Technologies], [Online]. Available at: https://www.stemcell.com/products/lymphoprep.html (Dec 18, 2023)

MALMSTRÖM E., KHAN H.N., VEER C.V., STUNNENBERG M., MEIJER M.T., MATSUMOTO H., OTTO N.A., GEIJTENBEEK T.B., DE VOS A.F., VAN DER POLL T., SCICLUNA B.P., 2022, The Long Non-Coding Antisense RNA JHDM1D-AS1 Regulates Inflammatory Responses in Human Monocytes, *Front Cell Infect Microbiol.*, vol. 12, no. 12, p. 934313

MARINOWIC, D.R., ZANIRATI, G., RODRIGUES, F.V., GRAHL, M.V., ALCARÁ, A.M., MACAHDO, D.C., DA COSTA, J.C., 2021, A new SYBR Green real-time PCR to detect SARS-CoV-2, *Scientific Reports*, vol. 11, no. 2224, doi: doi.org/10.1038/s41598-021-81245-0

MARIUZZA, R.A., AGNIHOTRI, P., ORBAN, J., 2020, The structural basis of T-cell receptor (TCR) activation: An enduring enigma, *J. Biol. Chem.*, vol. 295, no. 4, pp. 914 – 925, doi: 10.1074/jbc.REV119.009411

MARMUR, J., DOTY, P., 1962, Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature, *Journal of Molecular Biology*, vol. 5, no. 1., pp. 109 – 118, doi: 10.1016/S0022-2836(62)80066-7

MARSHALL, J.C., 2014, Why have clinical trials in sepsis failed?, *Trends in Molecular Medicine*, vol. 20, no. 4, pp. 195 – 203, doi: 10.1016/j.molmed.2014.01.007

MCKINNON, K.M., 2019, Flow Cytometry: An Overview, *Curr. Protoc. Immunol*. vol. 120, pp. 5.1.11 – 5.1.11, doi: 10.1002/cpim.40

MEDZHITOV, R., HORNG, T., 2009, Transcriptional control of the inflammatory response, *Nat. Rev. Immunol.*, vol. 9, no. 10, pp. 692 – 703, doi: 10.1038/nri2634

MEIS, J.E., KHANNA, A., 2009, RNA amplification and cDNA synthesis for qRT-PCR directly from a single cell, *Nature Methods*, vol. 6, pp. 12 – 13, doi: 10.1038/nmeth.f.280

MENGOS, A.E., GASTINEAU, D.A., GUSTAFSON, M.P., 2019, The $CD14^{+}HLA\text{-}DR^{lo}/^{neg}$ Monocyte: An Immunosuppressive Phenotype That Restrains Responses to Cancer Immunotherapy, *Front. Immunol.*, vol. 10, doi: 10.3389/fimmu.2019.01147

MITROULIS, I., KALAFATI, L., HAJISHENGALLIS, G., CHAVAKIS, T., 2018, Myelopoiesis in the Context of Innate Immunity, *J. Innate Immun.*, vol. 10, no. 5 – 6, pp. 365 – 372, doi: 10.1159/000489406

MONNERET, G., VENET, F., PACHOT, A., LEPAPE, A., 2008, Monitoring Immune Dysfunctions in the Septic Patient: A New Skin for the Old Ceremony, *Molecular Medicine*, vol. 14, pp 64 – 78, doi: 10.2119/2007-00102.Monneret

MUNAKATA, Y., YAMADA, T., IMAI, J., TAKAHASHI, K., TSUKITA, S., SHIRAI, Y., KODAMA, S., ASAI, Y., SUGISAWA, T., CHIBA, Y., KANEKO, K., UNO, K., SAWADA, S., HATAKEYAMA, H., KANZAKI, M., MIYAZAKI, J., OKA, Y., KATAGIRI, H., 2018, Olfactory receptors are expressed in pancreatic β-cells and promote glucose-stimulated insulin secretion, *Scientific Reports*, vol. 8, no, 1499, doi: 10.1038/s41598-018-19765-5

NAKAMORI, Y., PARK, E.J., SHIMAOKA, M., 2021, Immune Deregulation in Sepsis and Septic Shock: Reversing Immune Paralysis by Targeting PD-1/PD-L1 Pathway, *Front. Immunol.*, vol. 11, doi: 10.3389/fimmu.2020.624279

NATAJARAN, S., REMICK, D.G., 2008, The ELISA Standard Save: Calculation of sample concentrations in assays with a failed standard curve, *J. Immunol. Methods*, vol. 336, no. 2, pp. 242 – 245, doi: 10.1016/j.jim.2008.04.001

NETEA, M.G., JOOSTEN, L.A., LATZ, E., MILLS, K.H., NATOLI, G., STUNENNBERG, H.G., O'NEILL, L.A., XAVIER, R.J., 2016, Trained immunity: a program of innate immune memory in health and disease, *Science*, vol. 352, no. 6284, doi: 10.1126/science.aaf1098

NETEA, M.G., QUINTIN, J., VAN DER MEER, J.W., 2011, Trained Immunity: A Memory for Innate Host Defense, *Cell Host Microbe,* vol. 9, no. 5, pp. 355 – 361, doi: 10.1016/j.chom.2011.04.006

NEUHAUS, E.M., ZHANG, W., GELIS, L., DENG, Y., NOLDUS, J., HATT, H., 2009, Activation of an Olfactory Receptor Inhibits Proliferation of Prostate Cancer Cells, *J. Biol. Chem.*, vol. 284, no. 24, pp. 16218 – 16225, doi: 10.1074/jbc.M109.012096

NIELSEN, M.C., ANDERSEN, M.N., MØLLER, H.J., 2020, Monocyte isolation techniques significantly impact the phenotype of both isolated monocytes and derived macrophages in vitro, *Immunology*, vol. 159, no. 1, pp. 63 – 74, doi: 10.1111/imm.13125

NOWAK-TERPILOWSKA, A., SLEDZINSKI, P., ZEYLAND, J., 2021, Impact of cell harvesting methods on detection of cell surface proteins and apoptotic markers, *Braz. J. Med. Biol. Res.*, vol. 54, no. 2, p. e10197, doi: 10.1590/1414-431X202010197

OCHANDO, J., MULDER, W.J., MADSEN, J.C., NETEA, M.G., DUIVENVOORDEN, R., 2022, Trained immunity — basic concepts and contributions to immunopathology, *Nature Reviews Nephrology*, vol. 19, pp. 23 – 37, doi: 10.1038/s41581-022-00633-5

OLENDER, T., KEYDAR, I., PINTO, J.M., TATARSKYY, P., ALKELAI, A., CHIEN, M., FISHILEVICH, S., RESTREPO, D., MATSUNAMI, H., GILAD, Y., LANCET, D., 2016, The human olfactory transcriptome, *BMC Genomics*, vol. 17, no. 619, doi: 10.1186/s12864-016-2960-3

2024, Olgi(dT)$_{12\text{-}18}$ Primer [ThermoFisher Scientific], [Online]. Available at: https://www.thermofisher.com/order/catalog/product/18418012 (Jan 18, 2024)

O'NEILL, L.A., PEARCE, E.J., 2016, Immunometabolism governs dendritic cell and macrophage function, *J. Exp. Med.*, vol. 213, no. 1, pp. 15 – 23, doi: 10.1084/jem.20151570

ORECCHIONI, M., KOBIYAMA, K., WINKELS, H., GHOSHEH, Y., MCARDLE, S., MIKULSKI, Z., KIOSSES, W.B., FAN, Z., WEN, L., JUNG, Y., ROY, P., ALI, A.J., MIYAMOTO, Y., MANGAN, M., MAKINGS, J., WANG, Z., DENN, A., VALLEJO, J., OWENS, M., DURANT, C.P., BRAUMANN, S., MADER, N., LI, L., MATSUNAMI, H., ECKMANN, L., LATZ, E., WANG, Z., HAZEN, S.L., LEY, K., 2022, Olfactory receptor 2 in vascular macrophages drives atherosclerosis by NLRP3-dependent IL-1 production, *Science*, vol. 375, no. 6577, pp. 214 – 221, doi: 10.1126/science.abg3067

OSMEKHINA, E., NEUBAUER, A., KLINZING, K., MYLLHARJU, J., NEUBAUER, P., 2010, Sandwich ELISA for quantitative detection of human collagen prolyl 4-hydroxylase, *Microb. Cell Fact.*, vol. 9, p. 48, doi: 10.1186/1475-2859-9-48

PAOLI, C.J., REYNOLDS, M.A., PHD, SINHA, M., GITLIN, M., CROUSER, E., 2018, Epidemiology and Costs of Sepsis in the United States—An Analysis Based on Timing of Diagnosis and Severity Level, *Crit. Care Med.*, vol. 46, no. 12, pp. 1889 – 1897, doi: 10.1097/CCM.0000000000003342

PARHAM, P., 2014, *The Immune System Fourth Edition*, New York, United States: Garland Science

PATEL, A.A., ZHANG, Y., FULLERTON, J.N., BOELEN, L., RONGVAUX, A., MAINI, A.A., BIGLEY, V., FLAVELL, R.A., GILROY, D.W., ASQUITH, B., MACALLAN, D., YONA, S., 2017, The fate and lifespan of human monocyte subsets in steady state and systemic inflammation, *J. Exp. Med.*, vol. 214, no. 7, pp. 1913 – 1923, doi: 10.1084/jem.20170355

PERLEE, D., VAN VUGHT, L.A., SCICLUNA, B.P., MAAG, A., LUTTER, R., KEMPER, E.M., VAN'T VEER, C., PUNCHARD, M.A., GONZÁLEZ, J., RICHARD, M.P., DALEMANS, W., LOMBARDO, E., DE VOS, A.F., VAN DER POLL, T., 2018, Intravenous Infusion of Human Adipose Mesenchymal Stem Cells Modifies the Host Response to Lipopolysaccharide in Humans: A Randomized, Single-Blind, Parallel Group, Placebo Controlled Trial, *Stem Cells*, vol. 36, no. 11, pp. 1778 – 1788, doi: 10.1002/stem.2891.

PETERS-SENGERS, H., BUTLER, J.M., UHEL, F., SCHULTZ, M.J., BONTEN, M.J., CREMER, O.L., SCICLUNA, B.P., VAN VUGHT, L.A., VAN DER POLL, T., MARS CONSORTIUM, 2022, Source-specific host response and outcomes in critically ill patients with sepsis: a prospective cohort study, *Intensive Care Med*, vol. 48, no. 1, pp 92 – 102, doi: 10.1007/s00134-021-06574-0.

PRAUSMÜLLER, S., SPINKA, G., STASEK, S., ARFSTEN, H., BARTKO, P.E., GOLIASCH, G., HÜLSMANN, M., PAVO, N., 2022, Neutrophil Activation/Maturation Markers in Chronic Heart Failure with Reduced Ejection Fraction, *Diagnostics (Basel)*., vol. 12, no. 2, p. 444, doi: 10.3390/diagnostics12020444

PRESCOTT, H.C., ANGUS, D.C., 2018, Enhancing Recovery From Sepsis, *JAMA*, vol. 319, no. 1, pp. 62 – 75, doi: 10.1001/jama.2017.17687

2024, PrimeScript Reverse Transcriptase [Scientifix], [Online]. Available at: https://scientifix.com.au/primescript-reverse-transcriptase.html (Jan 18, 2024)

PRUCHA, M., ZAZULA, R., RUSSWURM, S., 2017, Immunotherapy of Sepsis: Blind Alley or Call for Personalized Assessment?, *Arch. Immunol. Ther. Exp. (Warsz).*, vol. 65, no. 1, pp. 37 – 49, doi: 10.1007/s00005-016-0415-9

PULEO, A., CARROLL, C., MAECKER, H.T., GUPTA, R., 2017, Isolation of Peripheral Blood Mononuclear Cells Using Vacutainer® Cellular Preparation Tubes (CPTTM), *Bio Protoc.*, vol. 7, no. 2, p. e2103, doi: 10.21769/BioProtoc.2103

PURVES, D., AUGUSTINE, G.J., FITZPATRICK, D., KATZ, L.C., LAMANTIA, A., MCNAMARA, J.O., WILLIAMS, S.M., 2001, The Transduction of Olfactory Signals, In: PURVES, D., AUGUSTINE, G.J., FITZPATRICK, D., KATZ, L.C., LAMANTIA, A., MCNAMARA, J.O., WILLIAMS, S.M. (eds.), *Neuroscience 2nd Edition*, Sunderland, Massachusetts, United States: Sinauer Associates

RAMIREZ-MORAL, I., YU, X., VAN WEEGHEL, M., OTTO, N.A., LIMA FERREIRA, B., VAN MAELE, L., SIRARD, J.C., DE VOS, A.F., DE JONG, M.D., HOUTKOOPER, R.H., VAN DER POLL, T., 2021, mTOR-driven glycolysis governs induction of innate immune responses by bronchial epithelial cells exposed to the bacterial component flagellin, *Mucosal Immunol.*, vol. 14, no. 3, pp. 594 – 604, doi: 10.1038/s41385-021-00377-8

RAP, A., SHYAM, D., 2014, Scoring systems in the intensive care unit: A compendium, *Indian Journal of Critical Care Medicine*, vol. 18, no. 4, pp. 220 – 228, doi: 10.4103/0972-5229.130573

RAO, M.K., KAPILA, K., GUPTA, R.M., 1997, ENZYME LINKED IMMUNOSORBENT ASSAYS REVISITED, *Med. J. Armed Forces India,* vol. 53, no. 1, pp. 45 – 51, doi: 10.1016/S0377-1237(17)30644-5

REINHART, K., DANIELS, R., KISSOON, N., MACHADO, F.R., SCHACHTER, R.D., FINFER, S., 2017, Recognizing Sepsis as a Global Health Priority — A WHO Resolution, *N. Eng. J. Med.*, vol. 377, pp. 414 – 417, doi: 10.1056/NEJMp1707170

RODRIGUEZ, S., CHORA, A., GOUMNEROV, B., MUMAW, C., GOEBEL, W.S., FERNANDEZ, L., BAYDOUN, H., HOGENESCH, H., DOMBKOWSKI, D.M., KARLEWICZ, C.A., RICE, S., RAHME, L.G., CARLESSO, N., 2009, Dysfunctional expansion of hematopoietic stem cells and block of myeloid differentiation in lethal sepsis, *Blood*, vol. 114, no. 19, pp. 4064 – 4076, doi: 10.1182/blood-2009-04-214916

2024, RNase Inhibitor [ThermoFisher Scientific], [Online]. Available at: https://www.thermofisher.com/order/catalog/product/N8080119 (Jan 18, 2024)

2023, RPMI 1640 Medium, [ThermoFisher Scientific], [Online]. Available at: https://www.thermofisher.com/order/catalog/product/11875093 (Dec 20, 2023)

RUDD, K.E., JOHNSON, S.C., AGESA, K.M., SHACKELFORD, K.A., TSOI, D., KIEVLAN, D.R., COLOMBARA, D.V., IKUTA, K.S., KISSOON, N., FINFER, S., FLEISCHMANN-STRUZEK, C., MACHADO, F.R., REINHART, K.K., ROWAN, K., SEYMOUR, C.W., WATSON, R.S., WEST, T.E., MARIHNHO, F., HAY, S.I., LOZANO, R., LOPEZ, A.D., ANGUS, D.C., MURRAY, C.J., NAGHAVI, M., 2020, Global, regional, and national sepsis incidence and mortality, 1990-2017: analysis for the Global Burden of Disease Study., *Lancet (London, England),* vol. 395, no. 10219, pp. 200 – 211, doi: 10.1016/s0140-6736(19)32989-7

RUIJTER, J.M., BARNEWALL, R.J., MARSH, I.B., SZENTIRMAY, A.N., QUINN, J.C., VAN HOUDT, R., GUNST, Q.D., VAN DEN HOFF, M.J., 2021, Efficiency Correction Is Required for Accurate Quantitative PCR Analysis and Reporting, *Clinical Chemistry*, vol. 67, no. 6, pp. 829 – 842, doi: 10.1093/clinchem/hvab052

SAEED, S., QUINTIN, J., KERSTENS, H.H., RAO, N.A., AGHAJANIREFAH, A., MATARESE, F., CHENG, S., RATTER, J., BERENTSEN, K., VAN DER ENT, M.A., SHARIFI, N., JANSSEN-MEGENS, E.M., TER HUURNE, M., MANDOLI, A., VAN SCHAIK, T., NG, A., BURDEN, F., DOWNES, K., FRONTINI, M., KUMAR, V., GIAMARELLOS-BOURBOULIS, E.J., OUWEHAND, W.H., VAN DER MEER, J.W., JOOSTEN, L.A., WIJMENGA, C., MARTENS, J.H., XAVIER, R.J., LOGIE, C., NETEA, M.G., STUNNEBERG, H.G., 2014, Epigenetic programming during monocyte to macrophage differentiation and trained innate immunity, *Science*, vol. 345, no. 6204, doi: 10.1126/science.1251086

SAKAMOTO, S., PUTALUN, W., VIMOLMANGKANG, S., PHOOLCHAOREN, W., SHOYAMA, Y., TANAKA, H., MORIMOTO, S., 2018, Enzyme-linked immunosorbent assay for the

quantitative/qualitative analysis of plant secondary metabolites, *J. Nat. Med.*, vol. 72. No. 1, pp. 32 – 42, doi: 10.1007/s11418-017-1144-z

SCHMIDT, K.F., HUELLE, K., REINHOLD, T., PRESCOTT, H.C., GEHRINGER, R., HARTMANN, M., LEHMANN, T., MUELLER, F., REINHART, K., SCHNEIDER, N., SCHROEVERS, M.J., KOSILEK, R.P., VOLLMAR, H.C., HEINTZE, C., GENSICHEN, J.S., SMOOTH STUDY GROUP, 2022, Healthcare Utilization and Costs in Sepsis Survivors in Germany–Secondary Analysis of a Prospective Cohort Study, *J. Clin. Med.*, vol. 11, no. 4, p. 1142, doi: 10.3390/jcm11041142

SCICLUNA B.P., KLEIN KLOUWENBERG P.M., VAN VUGHT L.A., WIEWEL M.A., ONG D.S., ZWINDERMAN A.H., FRANITZA M., TOLIAT M.R., NÜRNBERG P., HOOGENDIJK A.J., HORN J., CREMER O.L., SCHULTZ, M.J., BONTEN M.J., VAN DER POLL T., 2015, A molecular biomarker to diagnose community-acquired pneumonia on intensive care unit admission, *Am J Respir Crit Care Med.*, vol. 192, no. 7, pp. 826 – 835.

SCICLUNA, B.P., UHEL, F., VAN VUGHT, L.A., WIEWEL, M.A., HOOGENDIJK, A.J., BAESSMAN, I., FRANITZA, M., NÜRNBERG, P., HORN, J., CREMER, O.L., BONTEN, M.J., SCHULTZ, M.J., VAN DER POLL, T., MOLECULAR DIAGNOSIS AND RISK STRATIFICATION IN SPESIS (MARS) CONSORTIUM, 2020, The leukocyte non-coding RNA landscape in critically ill patients with sepsis, *eLife*, vol. 9, no. e58597, doi: 10.7554/eLife.58597

SCICLUNA B.P., VAN VUGHT L.A., ZWINDERMAN A.H., WIEWEL M.A., DAVENPORT E.E., BURNHAM K.L., NÜRNBERG P., SCHULTZ M.J., HORN J., CREMER O.L., BONTEN M.J., HINDS C.J., WONG H.R., KNIGHT, J.C., VAN DER POLL T., MARS CONSORTIUM, 2017, Classification of patients with sepsis according to blood genomic endotype: a prospective cohort study, *Lancet Respir Med*, vol. 5, no. 10, pp. 816 – 826.

SCICLUNA B.P., WIEWEL M.A., VAN VUGHT L.A., HOOGENDIJK A.J., KLARENBEEK A.M., FRANITZA M., TOLIAT M.R., NÜRNBERG P., HORN J., BONTEN M.J., SCHULTZ M.J., CREMER O.L., VAN DER POLL T., 2018, Molecular Biomarker to Assist in Diagnosing Abdominal Sepsis upon ICU Admission, *Am J Respir Crit Care Med*, vol. 197, no. 8, pp. 1070 – 1073.

SCHULTZE, J.L., MASS, E., SCHLITZER, A., 2019, Emerging Principles in Myelopoiesis at Homeostasis and during Infection and Inflammation, *Immunity*, vol. 50, no. 2, pp. 288 – 301, doi: 10.1016/j.immuni.2019.01.019

SCICLUNA, B.P., UHEL, F., VAN VUGHT, L.A., WIEWEL, M.A., HOOGENDIJK, A.J., BAESSMAN, I., FRANITZA, M., NÜRNBERG, P., HORN, J., CREMER, O.L., BONTEN, M.J., SCHULTZ, M.J., VAN DER POLL, T., MOLECULAR DIAGNOSIS AND RISK STRATFICATION IN SEPSIS (MARS) CONSORTIUM, 2020, The leukocyte non-coding RNA landscape in critically ill patients with sepsis, *eLife*, vol. 9, doi: 10.7554/eLife.58597

SCICLUNA, B.P., VAN'T VEER, C., NIEUWDORP, M., FELSMANN, K., WLOTZKA, B., STROES, E.S., VAN DER POLL, T., 2013, Role of Tumor Necrosis Factor-α in the Human Systemic Endotoxin-Induced Transcriptome, *PLoS One*, vol. 8, no. 11, p. e79051, doi: 10.1371/journal.pone.0079051

SCICLUNA, B.P., VAN VUGHT, L.A., ZWINDERMAN, A.H., WIEWEL, M.A., DAVENPORT, E.E., BURNHAM, K.L., NÜRNBERG, P., SCHULTZ, M.J., HORN, J., CREMER, O.L., BONTEN, M.J., HINDS, C.J., WONG, H.R., KNIGHT, J.C., VAN DER POLL, T., 2017, Classification of patients with sepsis according to blood genomic endotype: a prospective cohort study, *The Lancet Resp. Medicine*, vol. 5, no. 10, pp. 816 – 826, doi: 10.1016/S2213-2600(17)30294-1

2023, SepMate™-15 (RUO), [Stem Cell Technologies], [Online]. Available at: https://www.stemcell.com/products/sepmate-15-ruo.html (Dec 16, 2023)

SHANKAR-HARI, M., RUBENFELD, G.D., 2016, Understanding Long-Term Outcomes Following Sepsis: Implications and Challenges, *Curr. Infect. Dis. Rep.*, vol. 18, no. 11, p. 37, doi: 10.1007/s11908-016-0544-7

SHARMA, M., 2021, BASIC CONCEPTS OF PRIMER DESIGNING: A MINIREVIEW, *International Journal of Latest Trends in Engineering and Technology*¸ vol. 17, no. 4, pp. 10 – 12, doi: 10.21172/1.174.03e-ISSN:2278-621X

SHARMA, R., ISHIMARU, Y., DAVISON, I., IKEGAMI, K., CHIEN, M., YOU, H., CHI, Q., KUBOTA, M., YOHDA, M., EHLERS, M., MATSUNAMI, H., 2017, Olfactory receptor accessory proteins play crucial roles in receptor function and gene choice, *eLife*, vol. 6, doi: 10.7554/eLife.21895

SHUM, E.Y., ESPINOZA, J.L., RAMAIAH, M., WILKINSON, M.F., 2015, Identification of novel post-transcriptional features in olfactory receptor family mRNAs, *Nucleic Acids Research*, vol. 43, no. 19, pp. 9314 – 9326, doi: 10.1093/nar/gkv324

SINGER, M., DEUTSCHMANN, C.S., SEYMOUR, C.W., SHANKAR-HARI, M., ANNANE, D., BAUER, M., BELLOMO, R., BERNARD, G.R., CHICHE, J., COOPERSMITH, C.M., HOTCHKISS, R.S., LEVY, M.M., MARSHALL, J.C., MARTIN, G.S., OPAL, S.M., RUBENFELD, G.D., VAN DER POLL, T., VINCENT, J., ANGUS, D.C., 2016, The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3), *JAMA,* vol. 315, no. 8, pp. 801 – 810, doi: 10.1001/jama.2016.0287

SMALE, S.T., 2010, Selective Transcription in Response to an Inflammatory Stimulus, *Cell*, vol. 140, no. 6, pp. 833 – 844, doi: 10.1016/j.cell.2010.01.037

SMYTH, G.K., 2005, "LIMMA: Linear models for microarray data", *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*, Springer, New York, pp. 397 – 420

2023, Sodium Pyruvate (100 mM), [ThermoFisher Scientific], [Online]. Available at: https://www.thermofisher.com/order/catalog/product/11360070 (Dec 20, 2023)

SOUZA-FONSECA-GUIMARAES, F., PARLATO, M., PHILIPPART, F., MISSET, B., CAVAILLON, J., ADIB-CONQUY, M., CAPTAIN STUDY GROUP, 2012, Toll-like receptors expression and interferon-γ production by NK cells in human sepsis, *Critical Care*, vol. 16, no. R206, doi: 10.1186/cc11838

SPEHR, M., GISSELMANN, G., POPLAWSKI, A., RIFFELL, J.A., WETZEL, C.H., ZIMMER, R.K., HATT, H., 2003, Identification of a testicular odorant receptor mediating human sperm chemotaxis, *Science*, vol. 299, no. 5615, pp. 2054 – 2058, doi: 10.1126/science.1080376

STABEL, J.R., WHERRY, T.L., 2023, Comparison of methods to isolate peripheral blood mononuclear cells from cattle blood, *Journal of Immunological Methods*, vol. 512, p. 113407, doi: 10.1016/j.jim.2022.113407

TANNAHILL, G.M., CURTIS, A.M., ADAMIK, J., PALSSON-MCDERMOTT, E.M., MCGETTRICK, A.F., GOEL, G., FREZZA, C., BERNARD, N.J., KELLY, B., FOLEY, N.H., ZHENG, L., GARDET, A., TONG, Z., JANY, S.S., CORR, S.C., HANEKLAUS, M., CAFFERY, B.E., PIERCE, K., WALMSLEY, S., BEASLEY, F.C., CUMMINS, E., NIZET, V., WHYTE, M., TAYLOR, C.T., LIN, H., MASTERS, S.L., GOTTLIEB, E., KELLY, V.P., CLISH, C., AURON, P.E., XAIVER, R.J., O'NEILL, L.A., 2013, Succinate is a danger signal that induces IL-1β via HIF-1α, *Nature*, vol. 496, no. 7444, pp. 238 – 242, doi: 10.1038/nature11986

2024, TB Green Premix Ex Taq II (Tli RNase H Plus), [TaKaRa Bio. Inc], [Online]. Available: https://www.takarabio.com/products/real-time-pcr/real-time-pcr-kits/dye-based-qpcr-mixes/tb-green-premix-ex-taq-ii-(tli-rnase-h-plus) (Jan 22, 2024)

2023, The Basics of Fetal Bovine Serum Use in Cell Culture, [ThermoFisher Scientific], [Online]. Available at: https://www.thermofisher.com/it/en/home/references/gibco-cell-culture-basics/cell-culture-environment/culture-media/fbs-basics.html (Dec 20, 2023)

2023, THE RNA Storage Solution, [ThermoFisher Scientific], [Online]. Available at: https://www.thermofisher.com/order/catalog/product/AM7000 (Dec 22, 2023)

TONG, T., WANG., Y., KANG, S., HUANG, K., 2021, Ectopic Odorant Receptor Responding to Flavor Compounds: Versatile Roles in Health and Disease, *Pharmaceutics*, vol. 13, no. 8, p. 1314, doi: 10.3390/pharmaceutics13081314

TRAN, S., PUHAR, A., NGO-CAMUS, M., RAMARAO, N., 2011, Trypan Blue Dye Enters Viable Cells Incubated with the Pore-Forming Toxin HlyII of Bacillus cereus, *PLoS One*, vol. 6, no. 9, p. e22876, doi: 10.1371/journal.pone.0022876

UDVARDI, M.K., CZECHOWSKI, T., SCHEIBLE, W., 2008, Eleven Golden Rules of Quantitative RT-PCR, *Plant Cell.*, vol. 20, no. 7, pp. 1736 – 1737, doi: 10.1105/tpc.108.061143

UNTERGASSER, A., RUIJTER, J.M., BENES, V., VAN DEN HOFF, M.J., 2021, Web-based LinRegPCR: application for the visualization and analysis of (RT)-qPCR amplification and melting data, *BMC Bioinformatics*, vol. 22, no. 398, doi: 10.1186/s12859-021-04306-1

USHACH, I., ZLOTNIK, A., 2016, Biological role of granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) on cells of the myeloid lineage, *J. Leukoc. Biol.*, vol. 100, no. 3, pp. 481 – 489, doi: 10.1189/jlb.3RU0316-144R

VADEVOO, S.M., GUNASSEKARAN, G.R., LEE, C., LEE, N., LEE, J., CHAE, S., PARK, J., KOO, J., LEE, B., 2021, The macrophage odorant receptor Olfr78 mediates the lactate-induced M2 phenotype of tumor-associated macrophages, *PNAS*, vol. 118, no. 37, doi: 10.1073/pnas.2102434118

VAN DEN BERG, M., VAN BEUNINGEN, F.E., TER MAATEN, J.C., BOUMA, H.R., 2022, Hospital-related costs of sepsis around the world: A systematic review exploring the economic burden of sepsis, *Journal of Critical Care*, vol. 71, doi: 10.1016/j.jcrc.2022.154096

VAN DER POLL, T., SHANKAR-HARI, M., WIERSINGA, W.J., 2021, The immunology of sepsis, *Immunity*, vol. 54, no. 11, pp. 2450 – 2464, doi: 10.1016/j.immuni.2021.10.012

VAN DER POLL, T., VAN DE VEERDONK, F.L., SCICLUNA B.P., NETEA, M.G., 2017, The immunopathology of sepsis and potential therapeutic targets, *Nat. Rev. Immunol.*, vol. 17, no. 7, pp. 407 – 420, doi: 10.1038/nri.2017.36

VAN LIER, D., GEVEN, C., LEIJTE, G.P., PICCKERS, P., 2019, Experimental human endotoxemia as a model of systemic inflammation, *Biochimie*, vol. 159, pp. 99 – 106, doi: 10.1016/j.biochi.2018.06.014

VAN VUGHT, L.A., KLEIN KLOUWENBERG, P.M., SPITONI, C., SCICLUNA, B.P., WIEWEL, M.A., HORN, J., SCHULTZ, M.J., NURNBERG, P., BONTEN, M.J., CREMER, O.L., VAN DER POLL, T., MARS CONSORTIUM, 2016, Incidence, Risk Factors, and Attributable Mortality of Secondary Infections in the Intensive Care Unit After Admission for Sepsis, *JAMA*, vol. 315, no. 14, pp. 1469 – 1479, doi: 10.1001/jama.2016.2691.

VAN VUGHT, L.A., SCICLUNA, B.P., WIEWEL, M.A., HOOGENDIJK, A.J., KLEIN KLOUWENBERG, P.M., FRANITZA, M., TOLIAT, M.R., NÜRNBERG, P., CREMER, O.L., HORN, J., SCHULTZ, M.J., BONTEN, M.M., VAN DER POLL, T., 2016, Comparative Analysis of the Host Response to Community-acquired and Hospital-acquired Pneumonia in Critically Ill Patients, *Am J Respir Crit Care Med*, vol. 194, no. 11, pp. 1366 – 1374, doi: 10.1164/rccm.201602-0368OC.

VENET, F., LEPAPE, A., DEBARD, A.-L., BIENVENU, J., BOHÉ, J., MONNERET, G., The Th2 response as monitored by CRTH2 or CCR3 expression is severely decreased during septic shock, *Clin. Immunol.*, vol. 113, no. 3, pp. 278 – 284, doi: 10.1016/j.clim.2004.07.005.

VERGARDI, E., VAPORIDI, K., TSATSANIS, C., 2018, Regulation of Endotoxin Tolerance and Compensatory Anti-inflammatory Response Syndrome by Non-coding RNAs, *Front. Immunol.*, vol. 9, no. 20, p. 2705, doi: 10.3389/fimmu.2018.02705

VINCENT, J.L., PHD, SAKR, Y., PHD, SPRUNG, C.L., RANIERI, V.M., REINHART, K., GERLACH, H., MORENO, R., CARLET, J., LE GALL, J.R., PAYEN, D., SEPSIS OCCURRENCE IN ACUTELY ILL

PATIENTS INVESTIGATORS, 2006, Sepsis in European intensive care units: results of the SOAP study, *Crit. Care Med.*, vol. 34, no. 2, pp. 344- 353, doi: 10.1097/01.ccm.0000194725.48928.3a

WAGNER, D.P., DRAPER, E.A., 1984, Acute physiology and chronic health evaluation (APACHE II) and Medicare reimbursement, *Health Care Financ. Rev.*, vol. 1984(Suppl), pp. 91 – 105.

WEIDINGER, D., ALISCH, D., JAMEEL, K.J., RUHE, M., YUSUF, F., ROHDE, S., SCHALK, J., KAUFMANN, P., KRONSBEIN, J., PETERS, M., GIANNAKIS, N., HATT, H., KNOBLOCH, J., 2020, Function of the olfactory receptor OR2AT4 in alveolar macrophages, *Pneumologie,*, vol. 74, no. 1, p. 19, doi: 10.1055/s-0039-3403101

WHITSETT, J.A., ALENGHAT, T., 2015, Respiratory epithelial cells orchestrate pulmonary innate immunity, *Nature Immunology*, vol. 16, pp. 27 – 35, doi: 10.1038/ni.3045

WOLK, K., DÖCKE, W., VON BAEHR, V., VOLK, H., SABAT, R., 2000, Impaired antigen presentation by human monocytes during endotoxin tolerance, *Blood*, vol. 96, no. 1, pp. 218 – 223, doi: 10.1182/blood.V96.1.218

WONG, M.L., MEDRANO, J.F., 2005, Real-time PCR for mRNA quantitation, *Biotechniques*, vol. 39, no. 1, doi: 10.2144/05391RV01

YE, J., COULOURIS, G., ZARETSKAYA, I., CUTCUTACHE, I., ROZEN, S., MADDEN, T.L., 2012, Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction, *BMC Bioinformatics*, vol. 13, no. 134, doi: 10.1186/1471-2105-13-134

ZHANG, X., BEDIGIAN, A.V., WANG, W., EGGERT, U.S., 2012, G-protein-coupled receptors participate in cytokinesis, *Cytoskeleton (Hoboken)*, vol. 69, no. 10, pp. 810 – 818, doi: 10.1002/cm.21055

Appendix I

Copy of URECA REDP Form – FREC No. FHS-2022-00568

Researcher Actions

Submit REDP form

Draft/submitted forms

Account

Logout

REDP Form

University of Malta staff, students, or anyone else planning to carry out research under the auspices of the University, must complete this form. The UM may also consider requests for ethics and data protection review by External Applicants.

Ahead of completing this online form, please read carefully the University of Malta Research Code of Practice and the University of Malta Research Ethics Review Procedures. Any breach of the Research Code of Practice or untruthful replies in this form will be considered a serious disciplinary matter. It is advisable to download a full digital version of the form to familiarise yourself with its contents (https://www.um.edu.mt/research/ethics/resources/umdocuments/). You are also advised to refer to the FAQs (https://www.um.edu.mt/research/ethics/faqs).

Part 1: Applicant and Project Details

Applicant Details

Name:
Nigel

Surname:
Sammut

Email:
nigel.sammut.18@um.edu.mt

Applicant Status:
Student

Please indicate if you form part of a Faculty, Institute, School or Centre: *
Faculty of Health Sciences

Department: *
Department of Applied Biomedical Science

Principal Supervisor's Name: *
Dr. Brendon Scicluna

Principal Supervisor's Email: *
brendon.scicluna@um.edu.mt

Co-Supervisor's Name:

Study Unit Code: *
PMSCABSFTR2

Course Title: *

Student Number: *
12101L

Researcher Actions

Submit REDP form

Draft/submitted forms

Account

Logout

Project Details

Title of Research Project: *

Investigating the Role of Olfactory Receptors in Establishing Immune Cell Tolerance and Tra

Project description, including research question/statement and method, in brief: *

Sepsis is defined as an abnormal host response to infection that in turn leads to fatal organ dysfunction. In fact, sepsis presently remains one of the leading causes of death worldwide, a proportion which exceeds that of all combined cancer-related fatalities. Despite this significance, no specific therapy for sepsis has yet been clinically approved, as although

Will project involve collection of primary data from human participants?

Yes / Unsure

Explain primary data collection from human participants:

Please explain the following aspects with regard to data collection from human participants.

a. Salient participant characteristics (e.g. min-max participants, age, sex, other): *

The study will aim to recruit 10 volunteers as participants. The criteria for participants include an age of 18 years or greater, with no history of cardiovascular disease; non-smoker; no chronic medication; free from any febrile illness in the month preceding sample collection; no antibiotic treatment in the month preceding sample collection;

b. How will they be recruited (e.g. sampled, selected, contacted, etc.): *

By means of electronic media such as the Faculty of Health Sciences Social Media platform or Newspoint and advertisement on campus digital boards.

c. What they will be required to do and for how long: *

All participants will be presented with a consent form to provide all necessary information about the study and the nature of the biological specimen to be collected. If consent has been provided by the participant, they will be subject to a venepuncture procedure, which will be carried out by Dr. Nikolai Pace (Medical Doctor and Senior

d. If inducements/rewards/compensation are offered: *

None.

e. How participants/society may benefit: *

Identification of pathways that can modulate and affect immune tolerance could in turn be a stepping stone in developing potential therapeutic strategies reverse the deleterious effects of immune paralysis caused by sepsis.

f. Is the participant's identity recorded at any stage of the research (e.g. in consent forms, records, publications): *

No, as all samples should be anonymised.

g. The manner in which you will manage and store the data: *

All data that is obtained will be accumulated and stored into a centralised database utilising the University of Malta's secure Google folder structure. All calculations and handling of data will be performed within the R Statistical Computing and Graphics Environment.

Will project involve collection of primary data from animals?

No

Researcher Actions

Submit REDP form

Draft/submitted forms

Account

Logout

Part 2: Self Assessment and Relevant Details

In what follows, all questions have been answered as "No or Not Applicable" by default. Please mark "Yes or Unsure" to any of the issues that may apply to your research. In such cases, your research proposal presents potential issues in the domain of research ethics and/or data protection. Therefore, you are kindly asked to elaborate upon the specific issue/s you indicate, and you will need to seek F/REC permission before data collection.

Human Participants

Skip questions 1-10 if your project does NOT involve primary data collection from human participants (or their tissue/ samples).

1. Risk of harm to participants:

Are your participants at risk of harm? (Physical, psychological, legal, economic, social, etc.)

No / N.A.

2. Physical intervention:

Does your research involve non-harmful physical intervention on participants which may raise ethical concerns in your discipline?

No / N.A.

3. Vulnerable participants:

Do you include participants who, in your study or discipline, would be considered vulnerable or may be more at risk of being in vulnerability than others (e.g. children, persons who are older, with dementia, patients, subject to discrimination, with disabilities, with mental health conditions, unable to give consent, in prostitution, incarcerated, substance abusers, economically or educationally disadvantaged or minorities)?

No / N.A.

4. Identifiable participants:

Are there participants in your research whose identity may be revealed in your research data, even though they have not given explicit consent to be so identified/attributed?

No / N.A.

5. Special Categories of Personal Data (SCPD):

Do you plan to collect SCPD, which, for identifiable participants (in records, data and/or publication), reveals race or ethnic origin, political opinions, religious or philosophical beliefs, membership in a trade union, genetic or biometric data that may uniquely identify a natural person, health, sex life and/or sexual orientation?

Yes / Unsure

Which of the following data categories are collected, if any? i. race and ethnic origin; ii. political opinions; iii. religious and philosophical beliefs; iv. trade union memberships; v. health status; vi. sex life or sexual orientation; vii. genetic information; viii. biometric data that may uniquely identify a natural person. Please describe. Please submit a Data Management Plan. *

For the purposes of this study, the participant details to be recorded are age and sex. No unique identifying credentials will be required from any of the participants of this study, as all biological samples will be assigned custom numerical identifiers to anonymize them.

6. Human tissue/samples:

Will your research involve the collection of human tissue/samples?

Yes / Unsure

Please elaborate on: i. the nature of materials and/or biological tissue/samples, their storage, security, traceability, identifiability, and who has access to them; ii. plans for retention and destruction. *

Blood will be collected into PaxGene, heparin and serum tubes. PaxGene tubes will be stored in the CMMB facility after collection, whereas heparin and serum tubes will be processed immediately to obtain monocytes for in vitro stimulation. Following stimulation, supernatants will be used to measure cytokine levels, whereas RNA

7. Withheld info assent/consent:

Do you plan to withhold information from potential participants regarding the nature of the research when you seek to obtain assent/consent?

Researcher Actions

Submit REDP form

Draft/submitted forms

Account

Logout

No / N.A.

8. 'opt-out' recruitment:

Will you use the 'opt-out' instead of the 'opt-in' method of obtaining consent when recruiting participants (i.e. will any participants be included in your study without providing explicit consent)?

No / N.A.

9. Deception in data generation:

Do you plan to actively provide false/misleading information or passively withhold information during the process of data generation (e.g. experiments, use of placebos, scenarios, games)?

No / N.A.

10. Incidental findings:

Could your research generate incidental findings that may need to be communicated to participants?

No / N.A.

Unpublished secondary data

Answer questions 11-13 if your research involves the use of secondary data. Otherwise skip to question 14.

11. Human:

Was the data collected from human participants?

No / N.A.

12. Animal:

Was the data collected from animals?

No / N.A.

13. No written permission:

Is written permission from the data controller of the original data still to be obtained?

No / N.A.

Animals

Answer questions 14 - 16 if your project involves primary data collection from animals (non-human vertebrates and cephalopods) or their tissue/samples. Otherwise skip to question 17.

14. Live animals, lasting harm:

Does your research involve taking live animals out of their natural habitat for use in procedures or where such removal may cause the animals lasting harm?

No / N.A.

15. Live animals, harm:

Is there a risk that your research causes harm to live animals?

No / N.A.

16. Source of dead animals, illegal:

Does your research involve the use of dead animals (or their tissue/samples) that have not been acquired legally or from a legal source?

No / N.A.

General Considerations

These questions are to be considered for all projects.

17. Cooperating institution:

If you need permission from a cooperating institution, do you require F/REC approval prior to approaching the institution?

Yes / Unsure

Researcher Actions

Submit REDP form

Draft/submitted forms

Account

Logout

Please explain: I. how the cooperating institution will be contacted; II. whether the approval of another Research Ethics Committee or Data Protection Office is required. *

The Department of Applied Biomedical Science, under the Faculty of Healthy Sciences, is aware of this research project as part of the Postgraduate Special Projects as part of the M.Sc. (by Research) for Applied Biomedical Science. No other committee other than FREC and UREC are required for approval.

18. Risk to researcher/s:

Does this research expose any members of the research team to any significant foreseeable risk that would require precautionary measures over and above those typically required in their line of work?

No / N.A.

19. Risk to environment:

Is there significant foreseeable risk that your research can cause harm to the environment?

No / N.A.

20. Commercial sensitivity:

Does your research make use of data that may be commercially sensitive?

No / N.A.

Other Potential Risks

Other potential risks for research ethics and data projection may arise from conflict of interest; harvesting social media data; the involvement of low income and/or lower middle income countries; the import and/or export of records, data, materials and specimens; the need for special permits/licenses to employ specific constructs/tests; the researcher/s having a dual role; and/or the dual use/misuse of research, among other. Applicants are advised to refer to the FAQs in case of doubt (https://www.um.edu.mt/research/ethics/faqs).

21. Other potential risks:

Does your research run other potential ethical or data protection risks?

No / N.A.

22. Official statement: Do you require an official statement from the F/REC that this submission has abided by the UM's REDP procedures?

Please note that answering "Yes / Unsure" here means that you may not start data collection until you receive F/REC approval. *You may also request a formal letter at a later date if you need it for publication/funding purposes.*

Yes / Unsure

Part 3: Submission

Which F/REC are you submitting to? *

Faculty applicants will submit automatically to the Faculty FREC. Non-Faculty applicants may refer to the UREC webpages and seek guidance from their Institute, Centre or School to identify which F/REC they should apply to.

Faculty of Health Sciences

Attachments:

Please indicate which of the following materials you are attaching. Failure to provide these, where relevant, risks delaying approval to proceed with the research. Materials should be attached below. Students may be required to produce their DRAFT letters of request ahead of sending to cooperating institutions and to obtain their supervisor's signature on consent and assent forms.

- University_of_Malta_Mail_-_Communications_Office_July_2022.pdf (size: 141.8 KB, uploaded on: 01/12/2022 18:32:52)
- Information_Letter__Consent_Form_-_Nigel_Sammut_M.Sc._Thesis_V5.pdf (size: 191.6 KB, uploaded on: 25/04/2023 13:29:11)
- Nigel_Sammut_M.Sc._Social_Media_Post_for_FREC_Review.pdf (size: 111.6 KB, uploaded on: 28/04/2023 21:11:15)

*Please produce these materials in English and/or Maltese and/or any other relevant language (or equivalent text that may be communicated orally for those who do not read).

Researcher Actions

Submit REDP form

Draft/submitted forms

Account

Logout

- [x] Information and/or recruitment letter*
- [x] Consent forms (adult participants)*
- [ ] Consent forms for legally responsible parents/guardians, in case of minors and/or adults unable to give consent*
- [ ] Assent forms in case of minors and/or adults unable to give consent*
- [ ] Data collection tools (interview questions, questionnaire etc.)
- [ ] Data Management Plan
- [ ] Data controller permission in case of use of unpublished secondary data
- [ ] Licence/permission to use research tools (e.g. constructs/tests)
- [ ] Any permits required for import or export of materials or data
- [ ] Letter granting institutional approval for access to participants
- [ ] Institutional approval for access to data
- [ ] Letter granting institutional approval from person directly responsible for participants
- [x] Other

Please feel free to add a cover note or any remarks to F/REC

If this is a re-submission of a Form to F/REC, please include previous form reference number. Please indicate if you require written approval for institutional/funding purposes. Please include reference to project grant and/or any previous ethics/data protection approval of related parent project. Please elaborate on any additional attachments you are providing.

Declarations: *

- [x] I hereby confirm having read the University of Malta Research Code of Practice and the University of Malta Research Ethics Review Procedures.
- [x] I hereby confirm that the answers to the questions above reflect the contents of the research proposal and that the information provided above is truthful.
- [x] I hereby give consent to the University Research Ethics Committee to process my personal data for the purpose of evaluating my request, audit and other matters related to this application. I understand that I have a right of access to my personal data and to obtain the rectification, erasure or restriction of processing in accordance with data protection law and in particular the General Data Protection Regulation (EU 2016/679, repealing Directive 95/46/EC) and national legislation that implements and further specifies the relevant provisions of said Regulation.

Applicant Signature: *

Write your full name here. By doing so and submitting this form you are effectively signing the declaration.

Nigel Sammut

Date of Submission: *

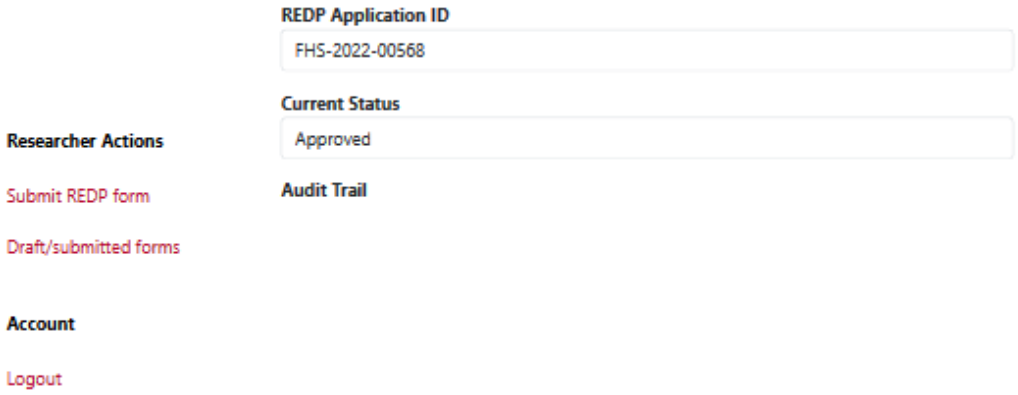
28 / 04 / 2023

If applicable: Date collection start date

Please insert envisaged date of data collection.

dd / mm / yyyy

Administration


REDP Application ID
FHS-2022-00568
Current Status
Approved
Researcher Actions
Audit Trail
Submit REDP form
Draft/submitted forms
Account
Logout

Appendix II

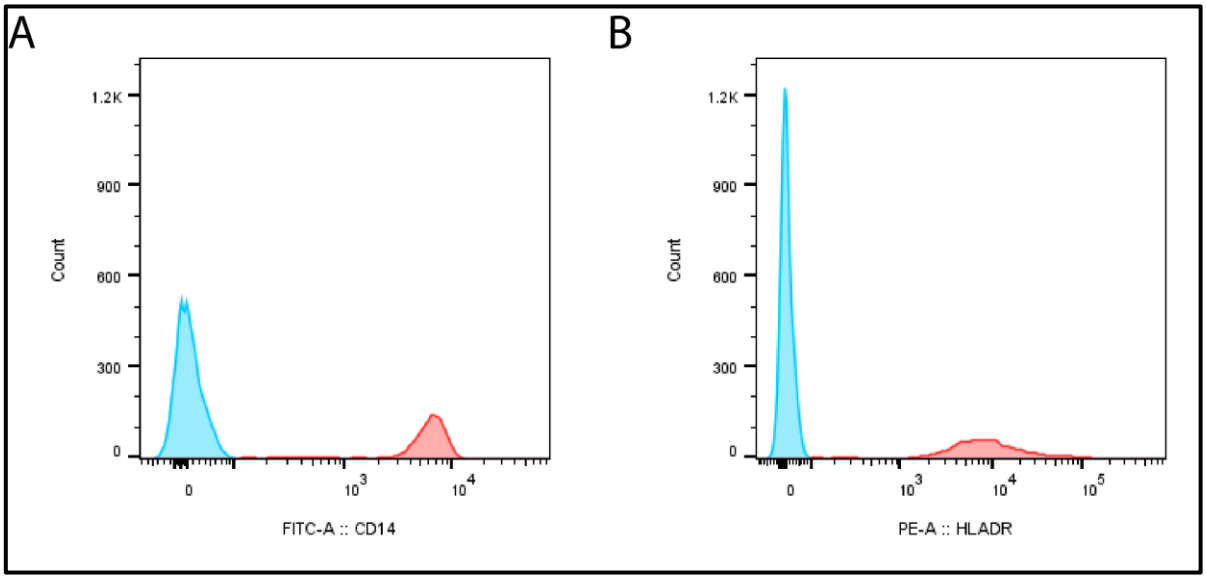


Figure 1: Flow cytometry of unstained and stained monocyte controls to set gating strategy. **(A)** Unstained (blue) and FITC-CD14 stained (red) histogram overlay, **(B)** unstained (blue) and PE-HLA-DR stained (red) histogram overlay.

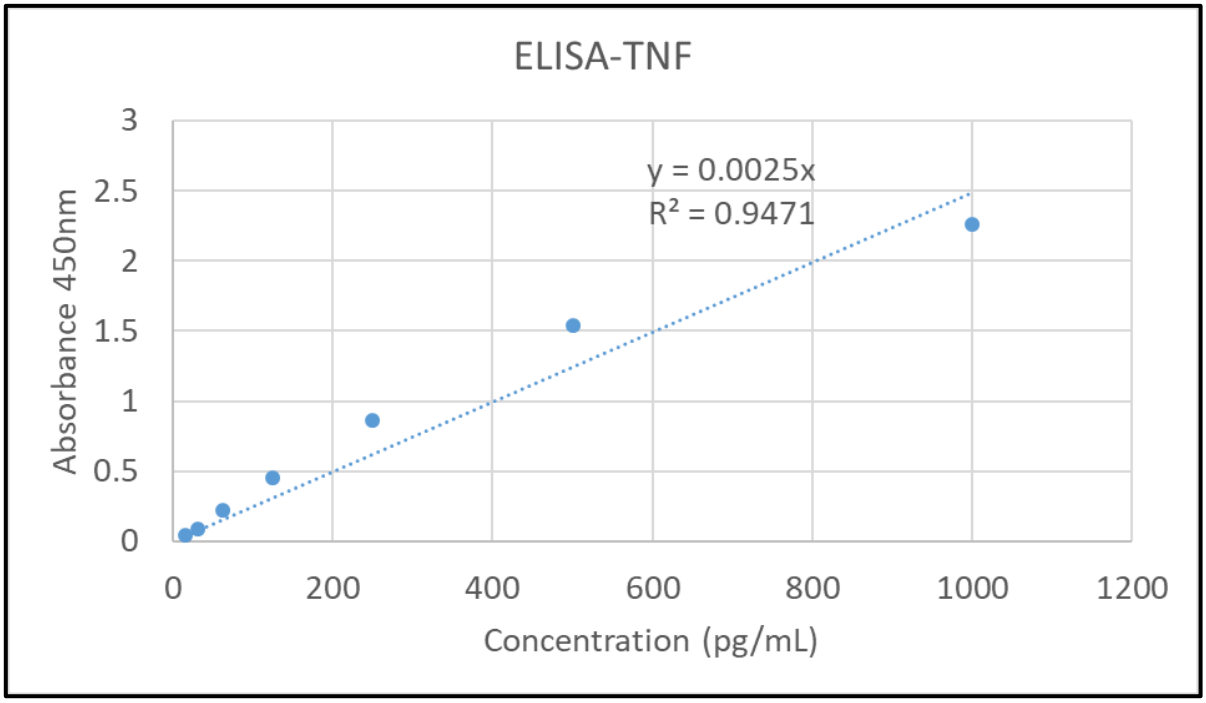


Figure 2: TNF-α standard curve for R&D Duo set ELISA.

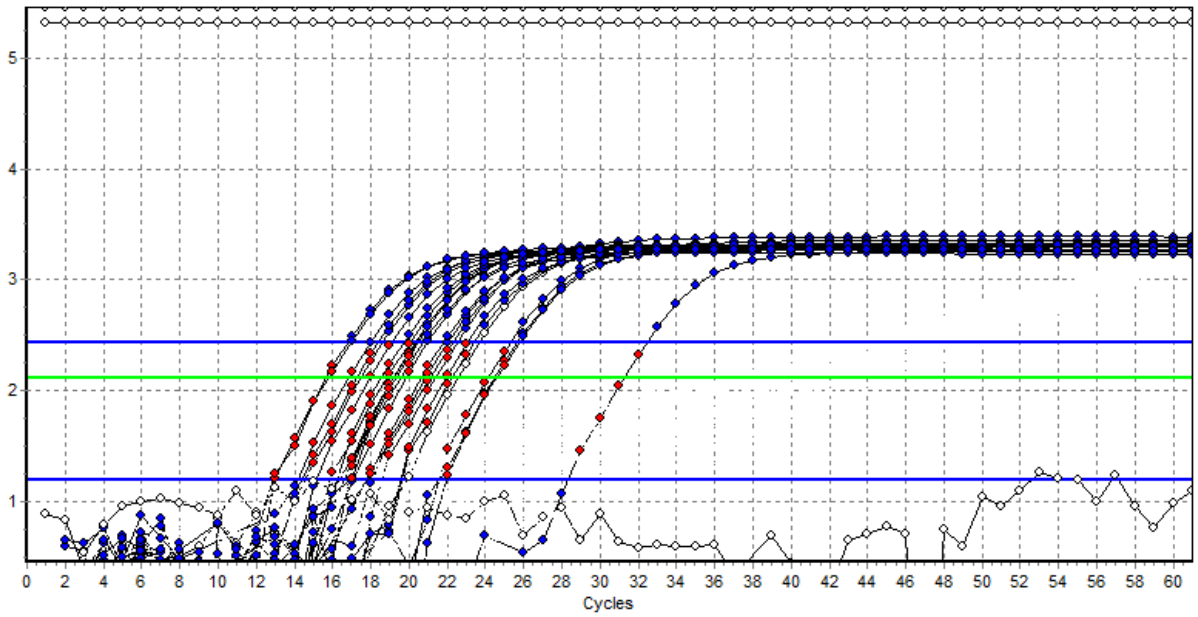


Figure 3: PCR amplification curves of *ACTB* (beta-actin) in isolated monocytes. Curves were generated in linregPCR. No amplification observed for negative control.

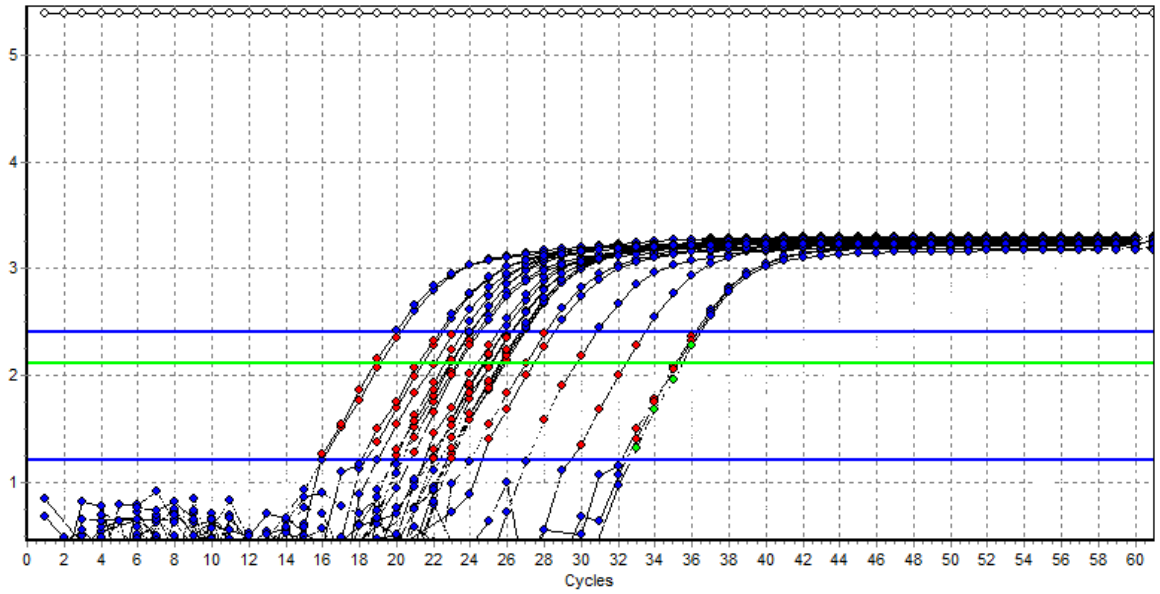


Figure 4: PCR amplification curves of *TNFA* (tissue necrosis factor-alpha) in isolated monocytes. Curves were generated in linregPCR. No amplification observed for negative control.

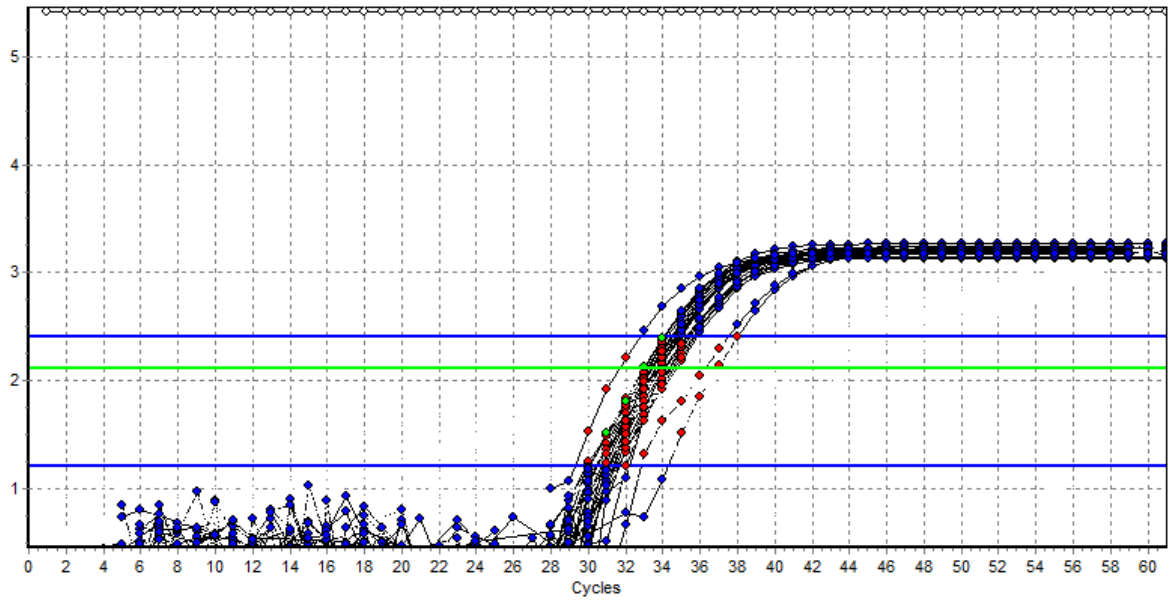


Figure 5: PCR amplification curves of *OR2B6* (olfactory receptor family 2 subfamily B member 6) in isolated monocytes. Curves were generated in linregPCR. No amplification observed for negative control. Expression of *OR2B6* was not reliably detected, as most signals for *OR2B6* amplification originated after the cut-off point derived from the negative control.