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The role of signal regulatory protein alpha in regulating macrophage subset activity in homeostasis and inflammation

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The role of signal regulatory protein alpha in regulating macrophage subset activity in homeostasis and inflammation

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Abstract

Macrophages (Mϕs) exist on a phenotypic spectrum characterised by the polar extremes of two subsets, pro-inflammatory M1s and regulatory M2s. Both subsets express signal regulatory protein alpha (SIRPα), receptor to CD47. Characterised as a "don't eat me" signal, SIRPα-CD47 ligation negatively regulates Mϕ phagocytic activity. This study developed a co-culture system between $CD47^+$ HL-60 and K562 cells, and $SIRP\alpha^+$ Mϕ subsets in order to investigate whether the regulatory effect of SIRPα-CD47 ligation extends to negative regulation of TLR-mediated TNFα production, and how this differs between Mϕ subsets and under various TNFα-inducing stimuli.

SIRPα and CD47 expression in THP-1-derived M1- and M2-like Mϕs, HL-60 pro-myelocytes and K562 lymphoblasts was measured using flow cytometry. THP-1 derived Mϕs were co-cultured with either HL-60 or K562 cells. Alone and in co-culture, Mϕs were stimulated with either *Staphylococcus aureus* LTA, *Escherichia coli* K12 LPS or *Porphyromonas gingivalis* LPS. TNFα production was measured by ELISA.

M2-like Mϕs were found to express higher levels of SIRPα than M1-like Mϕs, and K562 cells expressed greater CD47 than HL-60 cells. In Mϕ monoculture, all stimulation induced a TNFα response in both subsets, with M1s the higher TNFα producers in the case of K12 LPS and SA LTA stimulation, but no difference was present between subsets when stimulated with PG LPS. In all LPS co-culture conditions induced TNFα production was reduced compared to monoculture, with the higher SIRPα expressing M2 subset experiencing more suppression. The greatest suppressive effect was present when Mϕs were stimulated with K12 LPS, compared

to PG LPS. Co-culture did not inhibit SA LTA-induced TNFα production. When Mϕs were co-cultured with the higher CD47-expressing K562 cells, a greater suppression of LPS-induced TNFα response was present than when Mϕs were co-cultured with the lower CD47-expressing HL-60 cells.

These data indicate a role of SIRPα-CD47 ligation in the negative regulation of TLR4-mediated macrophage pro-inflammatory response, with TLR2-mediated TNFα production unaffected by co-culture. These findings identify SIRPα as a potential therapeutic target for TNFα-mediated pathologies of uncontrolled inflammation including Crohn's Disease and Rheumatoid Arthritis, presenting a novel mechanism for inducing functional shift from pro-inflammatory M1 towards regulatory M2 Mϕ phenotype.

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List of abbreviations

AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
BSA	Bovine serum albumen
CD	Cluster of differentiation
CML	Chronic myeloid leukaemia
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats - CRISPR-associated protein 9
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HRP	Horseradish peroxidase
IFNγ	Interferon gamma
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
IκK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric acid synthase
ITAM	Immunoreceptor tyrosine-based activatory motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus kinase
KD	Knockdown
KO	Knockout
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
Mϕ	Macrophage
M1	Pro-inflammatory macrophage
M2	Anti-inflammatory macrophage
MAPK	Mitogen-activate protein kinases
M-CSF	Macrophage colony-stimulating factor
MD-2	Lymphocyte antigen 96
MFI	Mean fluorescent intensity
mRNA	Messenger RNA
MyD88	Myeloid differentiation factor 88

NIBSC	National Institute for Biological Standards and Control
NF-κB	Nuclear factor kappa-B
NO	Nitric oxide
NS	Not significant
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol-12-myristate acetate
PRR	Pattern recognition receptor
R10	RPMI media supplemented with 10% foetal bovine serum
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SA	Staphylococcus aureus
SARM	Sterile α- and armadillo motif-containing protein
ShRNA	Short hairpin RNA
Shp2	Src homology region 2 domain-containing phosphatase-2
SIRPα	Signal regulatory protein alpha
SIRPγ	Signal regulatory protein gamma
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TACE	TNFα converting enzyme
TGFβ	Transforming growth factor beta
Th	T helper cell
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TRAM	Toll/interleukin-1 receptor domain-containing adaptor protein inducing IFNβ-related adaptor molecule
TRIF	Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon
VD_3	1,25-(OH)2-Vitamin D3
WT	Wild type

1 Chapter 1. General introduction

1.1 Macrophages, subsets and polarisation

Macrophages (Mϕs) are myeloid-derived patrolling sentinel cells of the innate immune system, often acting as first line of defence against pathogenic attack. Macrophages are present in nearly all somatic tissue, forming a heterogeneous population of antigen presentation phagocytes. Although a core component of the innate immune response, and as such non-specific and without memory, the initial response of Mϕs influences and guides the overall immune response to pathogenic assault. In this role as organisers and initiators of immune response, Mϕs are vital to ensuring an appropriate response is elicited. This requires a balance between optimal type, strength and duration of reaction for pathogen clearance, and mitigation of collateral damage to tissue, an unavoidable risk during active defence. Once the threat has been successfully eradicated, Mϕs are also responsible for ensuring that inflammatory signals cease and an anti-inflammatory state is induced to promote tissue healing and allow restoration of a homeostatic state. In some cases, aberrant signalling occurs and ongoing pro-inflammatory Mϕ activity continues, creating chronic inflammation, a causative feature of pathologies of uncontrolled inflammation. In this aberrant state, Mϕs are implicated in the pathogenesis of conditions including inflammatory bowel disease, rheumatoid arthritis and chronic periodontitis, all of which share a common feature of inappropriate Mϕ activation and a resultant continued pro-inflammatory state (Navegantes *et al.*, 2017).

Macrophages exist in a spectrum of phenotypes ranging from the activatory, pro-inflammatory M1s to the regulatory, anti-inflammatory M2s. Monocytes mature

towards the pro-inflammatory M1s *in vivo* via the classical activation pathway, T-helper (Th)-1-derived cytokine IFNγ in the presence of GM-CSF. These M1s are characterised as high in pro-inflammatory cytokines TNFα, IL-1β and IL-12, and also produce the nitic oxide inducer iNOS. Their polar counterparts, the M2s, are induced via the alternative activation pathway, Th-2-derived cytokines IL-4 and IL-13 in the presence of M-CSF (Gordon and Taylor, 2005; Martinez *et al.*, 2006). The cytokine repertoire of M2s is regulatory and anti-inflammatory: IL-10^{hi}, $TGF\beta^{+}$, $TNF\alpha^{lo}$. This extreme variation in potential Mϕ phenotypes allows for a variability of effector functions depending on the precise situation of activation. It must however be recognised that despite being a useful tool for modelling the vast range of Mϕ behaviours, the binary M1 and M2 paradigm is not an accurate reflection of the *in vivo* situation (Martinez and Gordon, 2014). The multitude of different factors that can influence the phenotypic profile of a Mϕ are such that a plethora of intermediate subsets exist. For the purposes of this investigation, for clarity and brevity, the terms M1-like and M2-like will be used to characterise the pro-inflammatory and anti-inflammatory Mϕ phenotypes respectively; however these are merely names which elicit a recognised understanding in the reader, and not an infallible description of a discrete cell type.

Within these phenotypes, the various mechanisms by which Mϕ behaviour is determined include direct stimulation by pathogens, soluble cytokine-mediated signalling and contact-dependent signalling.

1.2 Pattern recognition receptors and pathogen-associated molecular patterns

The presence of a pathogenic threat is sensed by pattern recognition receptors (PRRs) on the Mϕ cell surface. These receptors bind pathogen-associated molecular patterns (PAMPs), conserved motifs reliably expressed by pathogens of a certain group; downstream signalling then induces a response appropriate to the specific pathogenic threat. Amongst the various PRRs expressed by Mϕs are Toll-like receptors (TLRs), membrane spanning proteins which, for the most part, initiate an activatory signalling cascade in the Mϕ. These TLRs are expressed by a wide range of cells of the innate and adaptive immune systems, in addition to cells which are not traditionally regarded as part of the immune system, such as fibroblasts and epithelial cells (Takeda and Akira, 2018). Specific PAMPs bind specific TLRs, with many TLRs recognising bacterial PAMPs, as illustrated in **Table 1** below (McClure and Massari, 2014; Takeda and Akira, 2018; Yao *et al.*, 2015) .

Table 1. Human Toll-like receptors which recognise bacterial pathogen-associated molecular patterns

Pattern recognition receptor	**Pathogen-associated molecule pattern and source**
TLR1	Bacterial lipopeptides
TLR2	Bacterial lipoproteins and lipopeptides, bacterial peptidoglycans, zymosan from fungi, lipoteichoic acid from gram positive bacteria, lipopolysaccharides from gram-negative *Porphyromonas gingivalis* bacteria, tuberculin purified protein derivative
TLR4	Gram-negative bacterial lipopolysaccharide, heat shock proteins
TLR5	Bacterial flagellin, toxoplasma profilin
TLR6	Bacterial lipoproteins and lipopeptides, including lipoteichoic acid from gram-positive *Staphylococcus aureus*
TLR9	Unmethylated CpG Oligodeoxynucleotide DNA from viruses and bacteria

TLR4 and TLR2 are particularly implicated in Mϕ responses. Mϕ expressed TLR4 binds gram-negative bacterial PAMP lipopolysaccharide LPS lipid A, upon surface molecules CD14 and MD-2 forming a complex and aiding in the interaction between LPS and TLR4 (Shimazu *et al.*, 1999) . TLR2 binds both PAMPs including peptidoglycan and lipoteichoic acid (LTA), both associated with gram-positive bacteria (Schröder *et al.*, 2003). The exact role of TLR2 in LPS responsiveness has been a subject of controversy. Research indicates that TLR2 it is not implicated in LPS-induced immune response (Takeuchi *et al.*, 1999) and that it is the purification, or lack thereof, of the *Porphyromonas gingivalis*-derived LPS (PG-LPS) that accounted for the apparent TLR2-mediated responses found in early studies (Dziarski *et al.*, 2001; Manthey *et al.*, 1994). For this reason, when using PG-LPS level of purification is an important feature to consider, for ultra-pure should stimulate only TLR4, whereas less purified PG-LPS can be considered as a dual TLR2/TLR4 agonist. For pure stimulation of Mϕs via TLR2, gram-positive bacterial cell wall component LTA is a reliable agonist, utilising CD14 but not MD-2 or TLR4 to transduce signal (Schröder *et al.*, 2003).

Upon recognition of a PAMP, downstream signalling from TLRs induces a response from the Mϕ, via adaptor proteins, of which there are five: myeloid differentiation factor-88 (MyD88), TIR domain-containing adaptor protein (TIRAP, also known as MAL), toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon (IFN)β (TRIF), toll/interleukin-1 receptor domain-containing adaptor protein inducing IFNβ-related adaptor molecule (TRAM), and sterile α- and armadillo motif-containing protein (SARM) (Troutman *et al.*, 2012). All TLRs except TLR3 transduce signal via MyD88, with TLR4 using both MyD88-dependent and -independent pathways, the latter of which relies upon TRIF instead. TLR4 also

depends upon TIRAP (for its MyD88-depenent pathway) and TRAM (for TRIF-dependent signalling) as intermediate adaptors. An exception to the group, SARM is the one adaptor protein which instead of transducing signal inhibits the MyD88 and TRIF-dependent pathways. Following the action of these adaptor proteins, a series of mitogen-activate protein kinases (MAPKs) transduce signal until the final activation of dormant transcription factors is initiated. The final effect of TLR-mediated signalling can range from cell differentiation, expression of surface molecules (including co-stimulatory molecules which induce activation of other cells), and both inhibition and production of cytokines (Troutman *et al.*, 2012).

An overview of TLR2 and TLR4 signalling pathways is presented in **Figure 1** below.

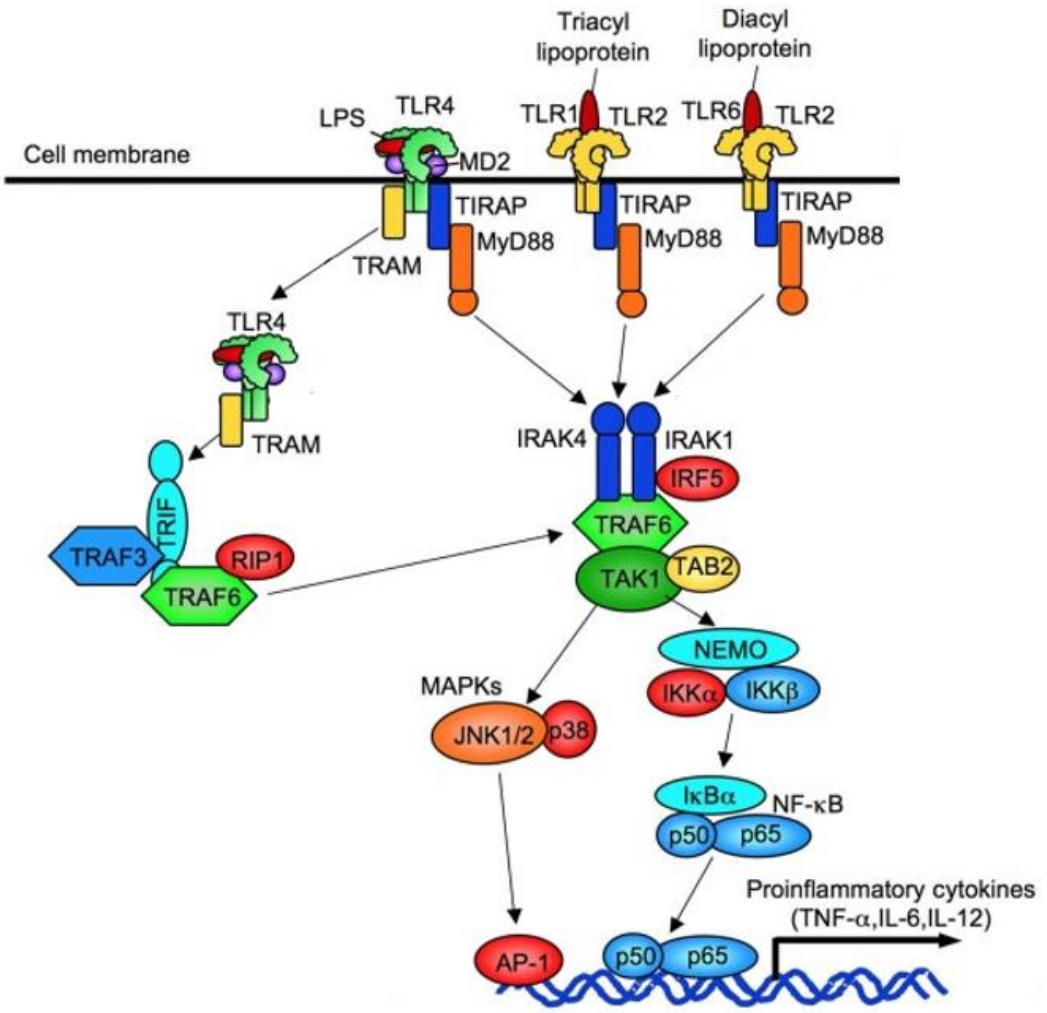

Figure 1. TLR2 and TLR4 signal transduction pathways (Adapted from Yang and Seki, 2012). Membrane-expressed TLR4 binds bacterial LPS. TLR2 heterodimerises with either TLR1, recognising triacylated lipoproteins, or TLR6, recognising diacetylated lipoproteins. Signal is transduced via either MyD88-dependent or independent pathways, activating NF-κB and inducing pro-inflammatory cytokine transcription.

1.3 Pro-inflammatory and anti-inflammatory cytokines

Cytokines form a communication network by which cells can inform each other of current events and co-ordinate an appropriate response. Cytokines signal between virtually all cells, however are most utilised by cells of the innate and adaptive immune systems. Cytokine production is determined by, amongst other factors, the type of pathogen being responded to. Upon release from the cell, cytokine molecules then bind to receptors on other cells, instructing those cells to perform

immunomodulatory, migratory and cell differentiation biological effects. The immunomodulatory effects of cytokines can be either pro-inflammatory or anti-inflammatory and cytokines can be broadly characterised as one or the other (Dinarello, 2007). The specific cocktail of cytokines present at any one time in a cell microenvironment can have a range of different effects of different cells; cytokines can synergise with each other increasing effects, inhibit specific effects, or act with redundancy, where more than one cytokine has the same downstream effect (Duque and Descoteaux, 2014). This contributes to a cellular microenvironment which influences the direction of polarisation of cells towards a pro- or anti-inflammatory phenotype.

Macrophage-produced pro-inflammatory cytokines include IFNγ, IL-1β, IL-6, IL-12 and TNFα (Duque and Descoteaux, 2014). TNFα is produced by macrophages in response to stimuli such as LPS in TLR4-mediated signalling, and LTA in TLR2-mediated signalling. The downstream effects of TNFα include inducing inflammatory cascades and cell death, essential for the clearance of pathogens but destructive to host tissue (Parameswaran and Patial, 2010). TNFα is also an endogenous pyrogen, inducing fever via the hypothalamus (Dinarello, C. A., 1999), induces secretion of acute phase proteins which alter plasma composition favouring pathogen clearance, at detriment to normal homeostasis (Gruys *et al.*, 2005) and inducer of chemokines, further recruits innate immune cells to site of inflammation, continuing the process. Pro-inflammatory cytokines IL-1β and IL-6 are also initiated by TLR2- and TLR4-dependent signalling and have overlapping downstream effects with TNFα, offering an element of redundancy to the pro-inflammatory cytokine repertoire (Tanaka *et al.*, 2014). Although effective in their roles as initiators of an immune response to

pathogenic attack, these pro-inflammatory cytokines are also involved in the pathogenesis of many pathologies of uncontrolled inflammation. These include rheumatoid arthritis (RA), in which TNFα, IL-1β and IL-6, amongst a host of other players, contribute to the ongoing inflammation, cellular infiltration into synovial fluid, auto-antibody development and Th1/ Th17 axis which result in the joint destruction typical of RA (Feldmann and Maini, 2008; McInnes and Schett, 2007) . TNFα additionally has roles in the pathogenesis of Crohn's disease and ulcerative colitis, encouraging the aggressive adaptive immune cell response to commensal gut bacteria and thereby promoting the gut damage (Adegbola *et al.*, 2018). A repertoire of pro-inflammatory cytokines have also been implicated in the tissue destruction and bone resorption characteristic of chronic periodontitis (Cardoso *et al.*, 2018).

Anti-inflammatory cytokines produced by Mϕs include TGFβ and IL-10. IL-10 is an essential component to the control and regulation of pro-inflammatory responses. The effects of IL-10 are wide ranging, covering a wide variety of cells of both the innate and adaptive immune responses, in this role as an immunoregulator it ameliorates unnecessary pro-inflammatory immune activation and mitigates against tissue damage. IL-10 is produced by many cells including monocytes, B cells and $CD4^+$ and $CD8^+$ T cells, when produced by Mϕs it is characteristic of the regulatory M2 subset. The effects of IL-10 are wide, exerted upon a variety of cell types, however its regulatory effects are largely reliant on its influence on Mϕ behaviour. IL-10 inhibits co-stimulator expression by Mϕs, preventing activation of the adaptive immune response, as well as inhibiting the production of pro-inflammatory cytokine including IL-1β, IL-6 and TNFα (Couper *et al.*, 2008). After binding to its receptors, IL-10 signal is transduced via JAK1 and STAT3, which upon translocation to the nucleus

suppresses NF-κB, production of suppressor of cytokine signalling SOCS3, and inhibits pro-inflammatory cytokine production (Qasimi *et al.*, 2006; Verma *et al.*, 2016).

1.4 Contact-dependent signalling

In additional to the soluble signalling of cytokines, cells also communicate via contact dependent signalling; surface-expressed protein receptors and ligands transduce signal when cells are in physical contact with one another. As with cytokine signalling, these signals can be activatory or inhibitory, either alone or acting as co-stimulation and co-inhibition signals for cell activation and maturation, as in the case of Mϕs control of the adaptive immune response via CD80/CD86 and CTLA4 (Sansom, 2000). Contact-dependent signalling is also present between cells of the same type, with anti-inflammatory contact-dependent signals act as negative regulators of immune response within Mϕs. The interaction between transmembrane protein CD47 and signal regulatory protein alpha (SIRPα) is an example of contact-dependent negative regulatory cell signalling between Mϕs.

1.5 The interaction between CD47 and SIRPα, and their role in negative regulation of pro-inflammatory immune response: a mini-review

The transmembrane protein CD47 is expressed by all somatic cells and is often described as a marker of self or a 'don't eat me' signal. CD47 is comprised of five transmembrane regions and a single extracellular immunoglobulin superfamily (IgSF) domain. The inhibitory receptor to CD47, SIRPα is composed of three immunoglobulin superfamily (IgSF) domains, two C1-set domains and a single V-set domain, which is the binding site for ligation to the extracellular IgSF of CD47. Within the cytoplasmic region of SIRPα reside four tyrosine residues, forming

immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Barclay and van den Berg, 2014). Upon ligation, phosphorylation of these ITIMs mediates a downstream signalling pathway via Src homology (SH2) domain-containing tyrosine phosphatases Shp1 and Shp2. This induces a dephosphorylation cascade culminating in inhibition of phagocytosis; phagocytosis depends upon the ability of Mϕs to rearrange their cytoskeletons, and the downstream effects of CD47-SIRPα ligation prevents this cytoskeletal rearrangement (Gitik *et al.*, 2014). It is this inhibition of phagocytosis which was the first characterised role of CD47-SIRPα, with the interaction providing a mechanism for recognition and avoiding phagocytosis of healthy self-cells, and giving CD47 its 'don't eat me' name.

The observation that CD47 is upregulated in aberrant tumour cells has led to a great deal of research attention being paid to the CD47-SIRPα pairing for development of novel cancer immunotherapies (Jaiswal *et al.*, 2009; Majeti *et al.*, 2009; Zhang, M. *et al.*, 2016). Interrupting the 'don't eat me' message passed on by tumour cells will elicit a phagocytic response in Mϕs leading to clearance of cancer cells, with a wide variety of strategies including peptides, fusion proteins, blocking antibodies being utilised to interrupt the CD47-SIRPα axis (Hayat *et al.*, 2019). The $CD47^{hi}$ profile of cells derived from acute and chronic myeloid leukaemia patients has proved to be a useful tool in the study of CD47-SIRPα interactions.

As a result of research focused on cancer immunotherapy, much characterisation of the CD47-SIRPα interaction been undertaken. This has opened up the possibility of manipulating the axis for pathologies of uncontrolled inflammation, controlling not only phagocytic behaviour of Mϕs but also their output of pro-inflammatory cytokines. The involvement of CD47-SIRPα in Mϕ-mediated negative regulation of

pro-inflammatory immune response has attracted some brief early attention in the initial flurry of CD47-SIRPα research, however little current research exists exploring its potential as a therapeutic immune regulator in the context of Mϕ subsets and polarisation.

Early on in the history of CD47-SIRPα research, a tentative link was established between the CD47-SIRPα axis and regulation of Mϕ pro-inflammatory activity. Enticing hints were presented of SIRPα's involvement in Mϕ subset polarisation phenotype. **Table 2** below summarises the existing research examining the role of SIRPα in regulating pro-inflammatory activity of Mϕs. This collection of papers does not include the vast body of research focusing on CD47-SIRPα as a negative regulator of phagocytic behaviour.

Table 2. Summary of existing research examining the role of CD47-SIRPα in regulating and influencing macrophage pro-inflammatory activity

Study	Model	Findings
A novel MyD-1 (SIRP-1 α) signaling pathway that inhibits LPS-induced TNFα production by monocytes (Smith *et al.*, 2003)	PBMCs, U937	• SIRPα ligation reduces *E. coli* LPS-induced TNFα production by 50%, but had no effect on IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12 p70, IL-15 and IFNγ. • SIRPα ligation-induced reduction in TNFα is also present when induced by zymosan and tuberculin. • LPS-induced TNFα mRNA levels were unaffected by SIRPα ligation. • TNFα converting enzyme (TACE) expression is unaffected by SIRPα ligation. • SIRPα ligation induced IL-12 p70 in unstimulated cells, but did not affect LPS-induced IL-12 p70 production.
Signal Regulatory Protein α Ligation Induces Macrophage Nitric Oxide Production through JAK/STAT- and Phosphatidylinositol 3-Kinase/Rac1/NAPDH Oxidase/H2O2-Dependent Pathways (Alblas *et al.*, 2005)	Rat NR8383 alveolar macrophages	• Ligation of SIRPα induces NO production. • Ligation of SIRPα inhibits *E. coli* LPS-induced TNFα production.
LPS-induced down-regulation of signal regulatory protein α contributes to innate immune activation in macrophages (Kong *et al.*, 2007)	RAW264.7 Mϕs, SIRPα -/- mice	• LPS stimulation reduces SIRPα expression. • ShRNA knockdown of SIRPα enhances TLR signalling, prolonging MAPK activation.

		• SIRPα knockdown induced more LPS-induced TNFα, IL-6, and NO production. • SIRPα overexpression reduces LPS-induced TNFα IL-6 and NO. • SIRPα -/- mice are highly susceptible to LPS-induced endotoxic shock. • IL-4 and IL-10 expression is not altered by SIRPα knockdown. • Neither knockdown nor overexpression of SIRPα impacts TLR4 levels. • In TLR4 -/- mice there is no reduction in SIRPα expression after LPS treatment.
MicroRNA-17/20a/106a modulate macrophage inflammatory responses through targeting signal-regulatory protein α (Zhu *et al.*, 2013)	Murine alveolar Mϕs	• LPS treatment downregulates SIRPα expression. • In miR-17 (post-transcriptional regulator of SIRPα) overexpressing macrophages, TNFα, IL-6 and NO production is upregulated • Transfection with miRNA inhibitors reduces LPS-induced TNF-a, IL-6, and NO production.
Dual role of SIRPα in macrophage activation: inhibiting M1 while promoting M2 polarization via selectively activating SHP-1 and SHP-2 signal (Shi *et al.*, 2017)	WT mice and SIRPα -/- mice	• SIRPα -/- murine Mϕs treated with LPS/IFNγ (M1-like polarisation) have increased expression of M1 markers CD86, MHC-II, iNOS, IL-1β, IL-12 and TNFα. • SIRPα -/- murine Mϕs treated with IL-4 (M2-like polarisation) have decreased expression of M2 markers CD206, Arg1, IL-10 and TGFβ. • In WT Mϕs, CD47-SIRPα ligation suppresses M1 polarisation and enhanced M2 polarisation.
Notch signaling Modulates Macrophage Polarization and Phagocytosis Through	Murine BMDMs, RAW264.7	• SIRPα expression is downregulated Mϕs treated with LPS and IFNγ (M1-like).

Direct suppression of signal regulatory Protein α expression (Lin *et al.*, 2018)		• SIRPα expression is upregulated in the same cells treated with IL-4 (M2-like). • SIRPα overexpression downregulated LPS and IFNγ-induced expression of TNFα and IL-12, and upregulated expression of IL-10. • SIRPα knockdown upregulated LPS-induced TNFα and downregulated IL-4-induced IL-10.
Integrin CD11b negatively regulates Mincle-induced signaling via the Lyn–SIRPα–SHP1 complex (Zhang, Q. *et al.*, 2018)	Immortalised bone marrow macrophages	• *Mycobacterium tuberculosis* cord factor induced-TNF-α and IL-6 production is upregulated in SIRPα knockout Mϕs.
SIRPα deficiency accelerates the pathologic process in models of Parkinson disease (Wang *et al.*, 2019)	Primary murine microglia, SIRPα -/- mice	• SIRPα knockout upregulates LPS-induced TNFα and IL-6 in primary microglia • SIRPα knockout cells refractory to IL-4-induced production of M2 markers arginase1, CD206

The burgeoning collection of literature examining the role of CD47-SIRPα in regulating Mϕs pro-inflammatory response builds a clear and consistent picture: SIRPα exerts a negative regulatory effect on Mϕ pro-inflammation activity, as illustrated in **Figure 2**. Ligation of SIRPα to its ligand CD47, or a mimic of that ligand, reduces TNFα production when induced by a variety of PAMPs, including TLR4 agonist *E. coli* LPS, and the TLR2 agonists zymosan and tuberculin purified protein (Alblas *et al.*, 2005; Smith *et al.*, 2003).

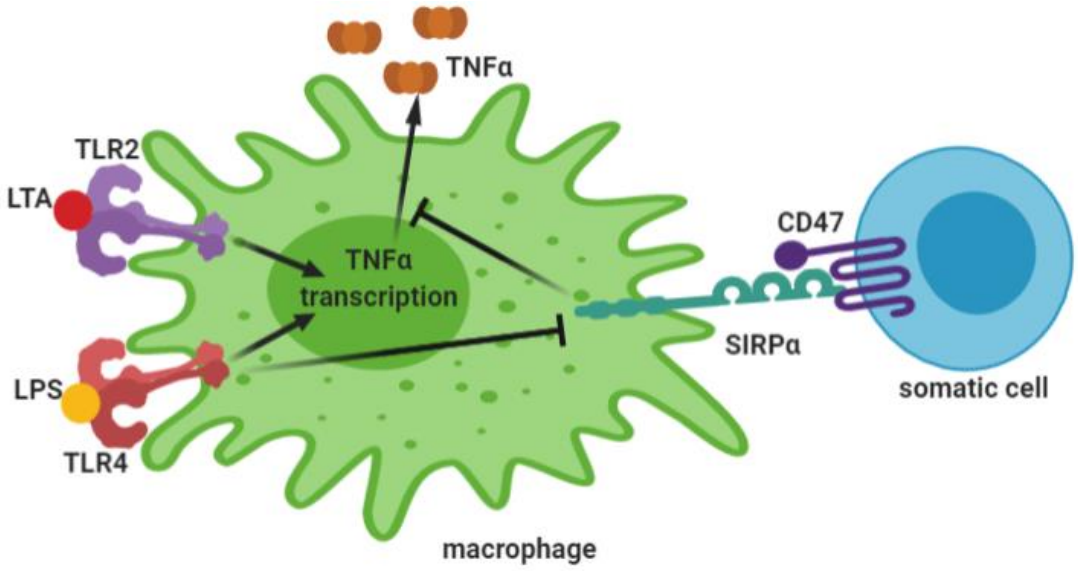

Figure 2. CD47 ligation to SIRPα inhibits TLR-mediated TNFα production and/or release, and TLR4 ligation inhibits SIRPα expression in macrophages

The suppression of TNFα is post-translational, as no effect on TNFα mRNA levels has been observed (Smith *et al.*, 2003). Further, the expression of TNFα-converting enzyme, TACE, is also unaffected by SIRPα ligation (Smith *et al.*, 2003). This indicates that when regulated by SIRPα, TNFα is being maintained intercellularly, with regulation occurring during TNFα migration to the membrane and not in its cleavage by TACE to a soluble form. Cytokines IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12 p70, IL-15 and IFNγ were unaffected by SIRPα ligation (Smith *et al.*, 2003), presenting a TNFα-

specific regulation. Curiously, SIRPα ligation was found to increase M1-associated pro-inflammatory IL-12 p70 and NO production in unstimulated cells (Alblas *et al.*, 2005; Smith *et al.*, 2003)

Knockdown and knockout studies using siRNA/ shRNA cell lines and SIRPα -/- mice have shown that SIRPα depletion enhances both LPS-induced production of TNFα (Kong *et al.*, 2007; Lin *et al.*, 2018; Shi *et al.*, 2017; Wang *et al.*, 2019; Zhu *et al.*, 2013) and that induced by *Mycobacterium tuberculosis* cord factor (Zhang, Q. *et al.*, 2018). This reduction in pro-inflammatory cytokine production within SIRPα depleted cells also extended to IL-6 (Kong *et al.*, 2007; Wang *et al.*, 2019; Zhang, Q. *et al.*, 2018; Zhu *et al.*, 2013), and NO (Kong *et al.*, 2007). The impact of knockdown on production of anti-inflammatory cytokines IL-4 and IL-10 has been shown to be unaffected by one study (Kong *et al.*, 2007), however another has found that their production was reduced (Lin *et al.*, 2018). SIRPα knockdown has also been shown to enhance TLR signalling, prolonging MAPK activation, yet no difference is made to the expression of TLR4 (Kong *et al.*, 2007). Fitting with the *in vitro* findings, SIRPα -/- mice are highly susceptible to LPS-induced endotoxic shock, suggesting a protective role of SIRPα against continued TLR activation (Kong *et al.*, 2007).

As expected, overexpression of SIRPα reduces the production of pro-inflammatory cytokines TNFα and IL-6, along with IL-12 (Kong *et al.*, 2007; Lin *et al.*, 2018), and curiously, considering its upregulation upon SIRPα ligation, NO (Kong *et al.*, 2007). SIRPα overexpression also increased expression of IL-10 (Lin *et al.*, 2018).

When stimulated with LPS, Mϕs reduce their expression of SIRPα (Kong *et al.*, 2007; Lin *et al.*, 2018; Zhu *et al.*, 2013), indicating that downregulation of SIRPα is necessary

for induction of an appropriate pro-inflammatory response. In TLR4 -/- mice, this reduction in LPS-induced SIRPα expression is absent, identifying TLR4 as a mediator for SIRPα downregulation, rather than SIRPα downregulation preceding the activation of TLR4 (Kong *et al.*, 2007). Downregulation of SIRPα is also present when Mϕs are treated with LPS and IFNγ to induce an M1-like state, whereas SIRPα is upregulated when Mϕs are treated with IL-4 to induce an M2-like state (Lin *et al.*, 2018). Murine SIRPα -/- Mϕs have an increased sensitivity to M1 induction, producing greater levels of M1 markers CD86, MHC-II, iNOS, IL-1β, IL-12 and TNFα, and are refractory to M2 induction, with decreased expression of CD206, arginase 1, IL-10 and TGFβ. (Shi *et al.*, 2017; Wang *et al.*, 2019). In WT murine Mϕs, CD47-SIRPα ligation suppressed M1 polarisation and enhanced M2 polarisation (Shi *et al.*, 2017).

A number of *in vivo* studies utilising experimental models of induced autoimmune conditions have found that interruption of CD47-SIRPα ameliorates the induced uncontrolled inflammation. CD47 -/- mice have been shown to have a reduced response to induced lupus nephritis (Shi *et al.*, 2015), experimental autoimmune encephalomyelitis (Gao *et al.*, 2016; Han *et al.*, 2012) and experimental colitis (Fortin *et al.*, 2009). These three pathologies all have a considerable TNFα involvement and so such studies question the concept of CD47-SIRPα as a potential tool for regulating TNFα pathologies. However, it should be noted that all the knockdowns focused on CD47 and not its receptor SIRPα. CD47 also binds other members of the SIRP family, including SIRPγ, a T-cell-expressed activatory molecule. Binding of CD47 to T-cell expressed SIRPγ is essential for T-cell transendothelial migration (Brooke *et al.*, 2004; Stefanidakis *et al.*, 2008) and as such inhibition of this process would prevent

induction of the T-cell mediated autoimmune pathologies in the above mentioned studies.

The overall picture painted by this body of research is one of SIRPα acting as a protective regulatory molecule, guarding against uncontrolled TNFα production. When PAMPs signal via TLRs 2 and 4, SIRPα expression is reduced to allow to production of pro-inflammatory cytokines. In SIRPα knockdown studies TLR signalling in prolonged, resulting in increased TNFα production and release. This feature of SIRPα -/- models implies that SIRPα negative regulation takes place by at least two separate mechanisms: firstly, as found by Smith *et al.* SIRPα ligation has no impact on TNFα mRNA and transcription. Regulation instead occurs due to a post-translational regulation of TNFα release, potentially by preventing migration to the membrane (Smith *et al.*, 2003). However, Kong *et al.* observed that upon SIRPα knockdown continued TLR activation is present, in the absence of increased TLR expression. This indicates a TLR-dependent influence on SIRPα-induced TNFα regulation upstream of TNFα transcription (Kong *et al.*, 2007). The stark contrast between the findings of these two studies leads to the inevitable question: is inhibition pre- or post-transcriptional? Delving deeper into the potential mechanistic pathways strengthens this position. Kong *et al.* suggested that in its recruitment of Shp2, SIRPα sequesters the phosphatase rendering it unavailable for utilisation by IκB kinases (Kong *et al.*, 2007). The interaction between Shp2 and IκK is essential for the disassociation of IκBα from NF-κB, and so unavailability of Shp2 would inhibit NF-κB translocation and subsequent TNFα transcription – a definite pre-transcriptional regulation.

An involvement of phosphoinositide 3-kinase (PI3K) has also been demonstrated in the SIRPα-induced regulation of TNFα production/ release. Smith *et al.* observed an increase in PI3K activity upon SIRPα ligation, and found that inhibition of PI3K with Wortmannin restores normal LPS-induced TNFα levels (Smith *et al.*, 2003). Further hints are provided by Toledano *et al.* who demonstrate an involvement of STAT3 in the SIRPα-mediated induction of anti-inflammatory Mϕ behaviour (Toledano *et al.*, 2013); STAT3 activation is heavily involved in the induction of tolerance in Mϕs, being intrinsic to the M2 phenotype, inhibition of pro-inflammatory cytokine production and production of anti-inflammatory cytokine IL-10 (Gur-Wahnon *et al.*, 2009). It is at present unclear whether these components all fit into one pathway or are evidence of a great deal of redundancy in the inhibitory actions and mechanisms of SIRPα, however it is clear that SIRPα plays a vital role in the negative regulation of Mϕ activity.

It has been indicated that SIRPα-mediated regulation exerts its effects on TNFα produced by both the TLR2 and TLR4 pathways, however differential involvement of these TLRs has not been examined in great detail. Also absent from the literature is an examination of the differential sensitivity to regulation between M1 and M2 subsets and their differential SIRPα expression profiles with and without LPS stimulation. Harnessing the SIRPα regulation pathway could become a tool for switching the phenotypes of Mϕs not only from M2 to M1, as is being examined in the context of cancer immunotherapies, but from M1 to M2, reducing TNFα production in pathologies of uncontrolled inflammation.

1.6 A note on the *in vitro* modelling of macrophage subsets

The study of Mϕs *in vitro* requires some creativity to model the *in vivo* situation as realistically as possible within the confines of a laboratory environment. Utilising Ficoll-centrifugation isolated peripheral blood mononuclear cells, selecting for monocytes via CD14+ positive selection and treatment with IFNγ and LPS or IL-4 and/or IL-13 (for M1- and M2-like Mϕs respectively) is one of the recognised methods for generation of primary polarised Mϕs *in vitro* (Gordon and Taylor, 2005). However, due to the difficulties in acquiring, isolating and differentiating sufficient PMBCs, cell line models are often used in the place of primary human cells. Further, PBMC isolation and differentiation results in Mϕs which are only viable in their polarised states for approximately one day (Martinez *et al.*, 2006). Even *in vivo*, classically activated M1 Mϕs circulate for only a day (Patel *et al.*, 2017), showing the limitations of even the best designed lab-based protocols. Originally, it was thought that polarised Mϕs were terminally differentiated and doomed to either a pro-inflammatory or anti-inflammatory fate; it has since emerged that there is more functional flexibility between phenotypes (Italiani *et al.*, 2014; Stout *et al.*, 2005) and that Mϕs maintain a proliferative capacity in the tissue (Hashimoto *et al.*, 2013). Unfortunately, however this doesn't include reversion to a naïve unstimulated M0 state, rendering laboratory induction of M1/M2 proliferation for convenience a questionable practice.

Instead, reliance on semi-immortalised cell lines has become a mainstay for fast, high-throughput preliminary experimental techniques requiring generation of polarised Mϕs. The pro-monocytic cell line, THP-1, has been established as a reliable tool for this purpose. Treatment of THP-1 cells with the phorbol ester phorbol-12-

myristate 13-acetate (PMA) for M1-like polarisation, and 1,25-$(OH)_2$-vitamin D_3 (VD_3) for M2-like induction produce two distinct subsets of Mϕs (**Figure 3**). These THP-1-derived Mϕ subsets express surface markers and respond to stimuli in a suitable approximation of pro- and anti-inflammatory Mϕs *in vivo*, with the PMA-treated M1-like cells having a pro-inflammatory $TNF\alpha^{hi}$ phenotype (Daigneault *et al.*, 2010; Schwende *et al.*, 1996), and the VD_3-treated M2-like cells displaying and $TNF\alpha^{lo}$ $IL\text{-}10^{hi}$ profile (Foey *et al.*, 2017; Foey and Crean, 2013; Liang *et al.*, 2019; Villaggio *et al.*, 2012).

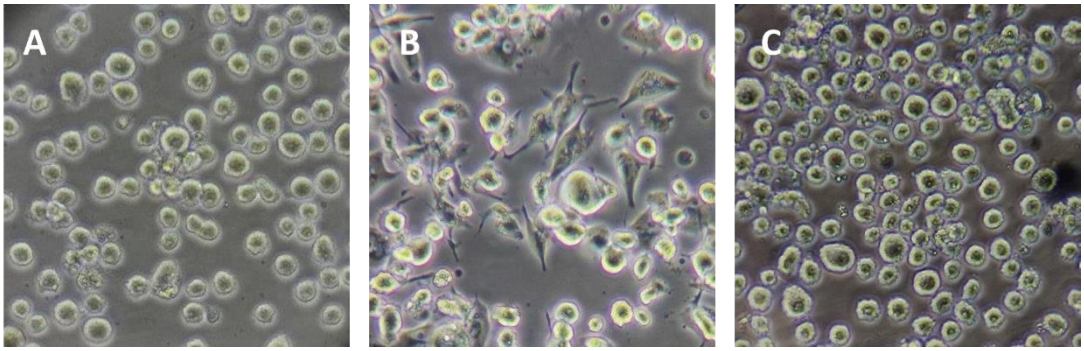

Figure 3. THP-1 cells and THP-1 derived M1-like and M2-like Mϕs. THP-1 promonocytes (A) were differentiated into M1-like (B) and M2-like (C) Mϕ subsets in the presence of either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 8 days respectively. M1-like cells become adherent with extended pseudopodia. M2-like cells are distinguishable from THP-1 cells by their mild adherence and increased granularity.

1.7 Aim and objectives

The aim of this study was to investigate the differential effects of CD47-SIRPα ligation upon pro-inflammatory cytokine production in Mϕ subsets in both homeostatic and inflamed states, utilising THP-1-derived M1-like and M2-like Mϕs. Induction of CD47-SIRPα ligation was investigated by using a co-culture model with Mϕs alongside $CD47^+$ cell lines HL-60 and K562. Differences in sensitivity to regulation between TLR2- and TLR4-mediated TNFα production were also explored, to further understand the mechanisms behind CD47-SIRPα-mediated negative regulation.

Objectives were as follows:

1 Characterisation of the TNFα and IL-6 production profiles of M1- and M2-like Mϕs in stimulated and unstimulated states, utilising TLR2, TLR4 and dual TLR2 and 4 binding PAMPs.

2 Characterisation of the SIRPα expression profiles of the two Mϕ subsets in stimulated and unstimulated states, in addition to the CD47 expression of HL-60 and K562 cells.

3 Induction of TNFα inhibition by introducing a co-culture of Mϕ and $CD47^{+/hi}$ cells, representing a slightly altered homeostatic environment.

4 Elucidation of the mechanisms behind the CD47-SIRPα negative regulation, employing various molecular tactics to blockade the pathway.

1.8 Experimental plan

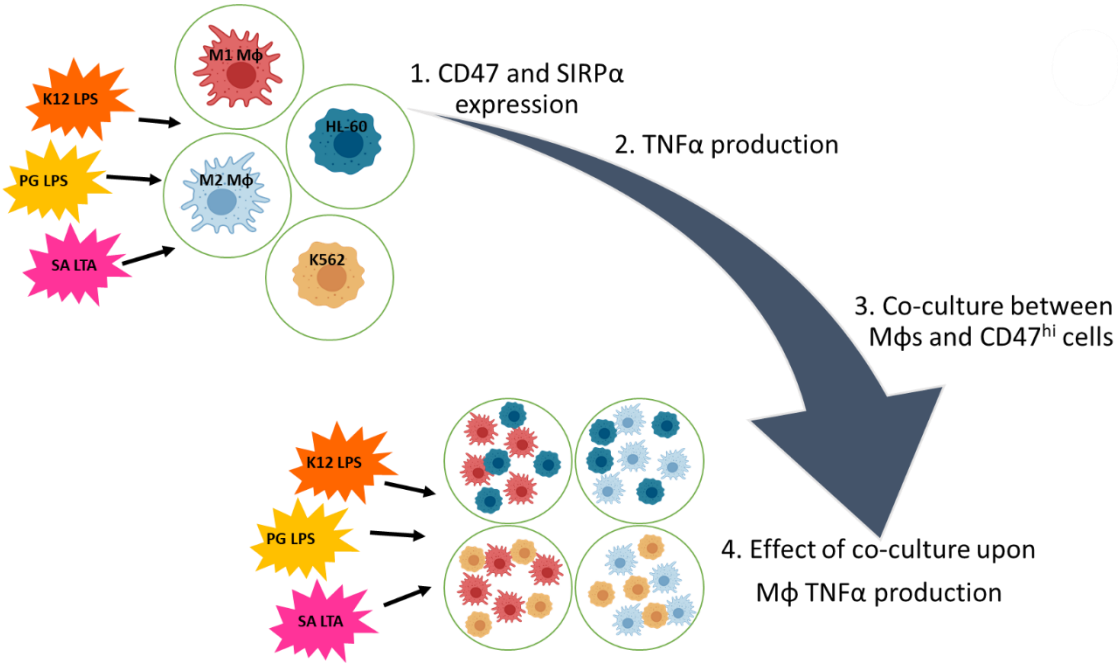

Figure 4. Schematic diagram of experimental plan. 1. Analysis of stimulated and unstimulated CD47 and SIRPα expression in M1-like Mϕs, M2-like Mϕs, HL-60 and K562 cells. 2. Analysis of TNFα production in individual cell types. 3. Development of a Mϕ and $CD47^{+}$ co-culture system. 4. Analysis of co-culture effect upon PAMP-induced Mϕ TNFα response.

2 Chapter 2. Materials and methods

2.1 Materials

Sources and product codes for all materials and reagents are included in appendix 1.

2.2 General methodology

2.2.1 THP-1 cell culture

The promonocytic THP-1 cell line was obtained from ECACC and utilised between passages 9 to 20. Frozen THP-1 cell stocks were stored in 50% FBS, 42.5% RPMI and 7.5% DMSO, within liquid nitrogen. Upon defrosting, cells were seeded at 5×10^5 cells/mL in R10 medium, comprised of RPMI 1640 medium supplemented with 10% FBS and 2 mM L-glutamine. Cells were cultured in perforated flasks, incubated in 5% CO_2 at 37°C, and passaged at a 1 in 4 passage every three to four days, in order to maintain cell densities below 1×10^6 cells/mL.

Differentiation of THP-1 cells into M1-like Mϕs was performed in the presence of 25 ng/mL phorbol 12-myristate 13-acetate (PMA) when cultures were growing in log phase. Cells were incubated for 4 days, with a wash and refresh of R10 media performed on the 3rd and 4th days.

Differentiation of THP-1 cells into M2-like Mϕs was induced with 10 nM 1,25-(OH)2-vitamin D3 during log phase. Cells were incubated for 8 days, with a 1 in 2 passage and 10 nM VD3 refresh performed on the 4th day of incubation.

2.2.2 HL-60 cell culture

Promyelocytic HL-60 cells were used between passages 35 and 45. Cells were stored and maintained as previously described for THP-1 cells.

2.2.3 K562 cell culture

Lymphoblastic K562 cells were used between passages 38 and 48 and were stored and maintained as previously described for THP-1 cells.

2.2.4 Co-culture

Macrophages were co-cultured at a effector to target ratio ranging from 0.01:1 through to 1:1, with either HL-60 cells or K562 cells. Co-culture was performed in either 24 well plates (total 1×10^6 cells per well) or 48 well plates (total 5×10^5 cells per well). Mϕs were differentiated and plated before the co-culture cell types was seeded. Cells were then allowed to rest for one hour prior to further experimentation.

2.2.5 Conditioned supernatants

Cell-free supernatants derived from HL-60 and K562 suspensions were collected from the culture flasks containing the effector cells utilised for co-culture experiments after centrifugation, and used immediately at a concentration matched to the cell densities for co-culture.

2.2.6 Cell stimulation

Cells were initially stimulated with titrations of PAMPs ranging up to 1 µg/mL K12 LPS, 1 µg/mL PG LPS and 5 µg/mL SA LTA in order to select a suitable concentration for further study. An incubation time of 18 hours was chosen for all experiments due to its alignment with a high TNFα production window, but also its ability to cover the production of most cytokines of potential future interest within this study (Agarwal *et al.*, 1995; Chanteux *et al.*, 2007; Segura *et al.*, 2002) . PAMPs were vortexed for five minutes prior to dilution in R10 medium. Cell-free supernatants were collected either without disturbing cells for adherent/ semi-adherent cells, or for suspension

cells after well-plate centrifugation at 200 × g. Supernatants were either analysed immediately or stored at -20°C.

2.2.7 Blocking peptide treatment of cells

SIRPα blocking peptide (Sigma Aldrich) was used at a range of concentrations from 200 ng/mL to 4 µg/mL. Cells were washed twice in DPBS, then incubated with the peptide for 20 minutes at 37°C.

2.2.8 ELISA cytokine analysis

Analysis of cytokine presence in cell supernatants was performed using sandwich ELISA. 96-well high binding microplates were coated with capture antibody diluted in PBS at 50µL per well. Plates were incubated overnight at 4°C. Plates were then blocked with 2% w/v BSA in PBS and incubated for 3 hours at room temperature. Three washes with 0.05% Tween-20 in PBS were performed, before samples were added in triplicate at 50 µL per well. A standard curve ranging from was prepared and plated in duplicate at 50 µL per well. Samples and standards were incubated overnight (approx. 16 hours) at 4°C. Plates were washed three times with 0.05% Tween-20/ PBS; 50 µL per well biotinylated detection antibody was added, diluted in 2% BSA/ PBS, and incubated for three hours at room temperature. After another three washes as previously described, streptavidin-HRP at 1/250 dilution in 2% BSA/ PBS was added at 50 µL per well and incubated for one hour at room temperature. Three more washes were performed, then 100 µL per well of TMB substrate added. Colour development was allowed to progress until distinction between appropriate standards and samples was apparent; the reaction was then stopped with 1.8M H_2SO_4. Plates were read colourimetrically at 450 nm on a Versa Max Tunable microplate reader and analysed with Softmax Pro version 5.4 software.

Antibodies and standards were used in the concentrations listed in **Table 3** below.

Table 3. ELISA antibodies and standard ranges in use throughout this research

Target	Capture Ab (µg/mL)	Detection Ab (µg/mL)	Standards (pg/mL)
TNFα	4	0.5	5 - 7000
IL-6	1	0.5	10 - 13000
IL-10	1	1	5 - 7000
IL-1β	1	0.1	5 - 7000

2.2.9 Fluorescent flow cytometry

Cells were centrifuged at 200 ×g and washed twice in Ca^{2+} and Mg^{2+} free DPBS. Cells were resuspended at 5×10^6 cells/mL. Individual samples each containing at least 1×10^6 cells (up to a maximum of 2.5×10^6 cells) were stained with 5 uL of either FITC-labelled anti-target antibody or appropriate matched isotype control, then incubated on ice in the dark for 20 minutes.

Cells were then washed three times in DPBS as above, before being resuspended in DPBS and run through the FACSCalibur flow cytometry (excitation: 488 nm; emission: 520 nm; Laser: blue), acquiring 10,000 events per sample. Analyses were run in triplicate where cell numbers allowed it, however in certain samples this was not achievable and duplicates were used. A live gating strategy was utilised. Post-acquisition analysis was performed using FCS Express 7 (De Novo Software).

2.2.10 Statistical analysis

Normality was assessed using Shapiro-Wilk test. Normally distributed data were analysed with unpaired T Test (two data sets) or one-way ANOVA (more than two data sets) with Holm-Sidak corrected multiple comparisons analysis. For data which were not normally distributed, Mann-Whitney (two data sets) or Kruskal-Wallis with Dunn's multiple comparisons (more than two data sets) analyses were employed.

3 Chapter 3. Characterisation of M1-like and M2-like Mϕs, and CD47^{+} cell lines, and examination of the effect co-culture has upon PAMP-induced TNFα production by Mϕs

In order to characterise the pro-inflammatory activity of THP-1-derived M1-like and M2-like Mϕs, HL-60 and K562 cells, a series of initial experiments were conducted to establish the baseline and stimulated responses of these Mϕs to K12 LPS, PG LPS and SA LTA. Macrophages produce TNFα when stimulated with PAMPs, however some baseline production of TNFα may be present even in unstimulated cells. Media component foetal bovine serum (FBS) is known to stimulate TNFα production in Mϕ cell culture, due to contamination with trace, or not so trace, amounts of endotoxin (Kirikae *et al.*, 1997). This immunogenicity varies considerably between various batches of FBS, and in order to maintain a low TNFα producing resting state an appropriate batch must be chosen. The immunogenicity of FBS batches in terms of baseline TNFα production of M1-like and M2-like Mϕs were analysed, in addition to the ability of Mϕs to respond to PAMPs in a manner significantly different from the baseline of untreated controls. This preliminary data is shown in appendix 2, and a suitable, low immunogenic FBS selected for future experiments. The next series of experiments focused on selecting a suitable PAMP concentration which elicits a TNFα response that falls within the linear portion of the ELISA standard curve, in order that TNFα analyte concentration can be accurately determined, with opportunity for either reduction or increase of the TNFα output whilst remaining within the reliable detectable ELISA range of 7 - 5000 pg/mL.

Prior to utilising HL-60 or K562 cells in co-culture with Mϕs, it was necessary to determine if either of these cell lines respond to the chosen stimuli in a pro-

inflammatory or anti-inflammatory manner, and so titrations of K12 LPS, PG LPS and SA LTA were also performed on these cells. Although undifferentiated HL-60 and K562 cells have been characterised as unresponsive to PAMPs in a TLR2 or TLR4-dependent cytokine producing manner due to their lack of these TLRs or TLR4 co-receptor CD14 (Li *et al.*, 2002; Okamoto *et al.*, 2009; Sullivan *et al.*, 2007; Yu *et al.*, 1998), characterisation of the particular cells in use was performed.

Next, the characterisation of CD47 and SIRPα expression profiles of M1-like and M2-like Mϕs, HL-60 and K562 cells was undertaken. As a mainly myeloid-expressed receptor, it is to be expected that THP-1-derived M1-like and M2-like Mϕs, HL-60 and K562 cells express SIRPα. Additionally, due to its ubiquitous nature, all somatic cell types are expected to express CD47 (Brown and Frazier, 2001). The relative levels of these surface molecules on the cell lines in use for this research require elucidating in order that differential outputs could potentially be linked to the relative CD47/ SIRPα expression.

It has been established that the pro-inflammatory activity of Mϕ-like cells can be inhibited by *in vitro* induction of SIRPα ligation with a synthetic soluble CD47 molecule or mimic (Alblas *et al.*, 2005; Smith *et al.*, 2003). Unfortunately, this neatly orchestrated *in vitro* modelling falls short of representing a true *in vivo* microenvironment. Although it may serve well to prove discrete concepts in the lab, isolation of an individual molecular interaction overlooks the cross-talk occurring between many groups of cell-expressed receptor-ligand pairings. The influence these peripheral interactions have upon Mϕ cell behaviour cannot be disregarded if attempting to apply laboratory findings to anything other than a laboratory context. Within the dynamic *in vivo* milieu, the amount of available CD47 expressed by

surrounding cells may influence the pro-inflammatory behaviour of Mϕs, yet mitigation via alternative contact-dependent and soluble signalling could likely abrogate any net effect upon TNFα production and release.

In an attempt to provide a more holistic picture, representative of *in vivo* cellular interactions, a co-culture model between Mϕs and $CD47^+$ cells was developed. This was designed to investigate whether TNFα production by SIRPα expressing M1-like and M2-like cells is inhibited while they are in the company of $CD47^+$ cells. Co-culture cell lines were selected based upon their $CD47^+$ profile and lack of inherent immunogenicity when co-cultured alongside Mϕ-like cells.

In addition to looking at TNFα production, two other pro-inflammatory Mϕ-derived cytokines, IL-6 and IL-1β were also examined within this co-culture system. Further attention was paid to the contact-dependent nature of the $CD47^+$ cell-Mϕ interaction, with the effect of $CD47^+$ cell supernatants and production of anti-inflammatory cytokine IL-10 also examined.

3.1 Experimental aims

The aims of these experiments were:-

1. Characterisation of unstimulated and stimulated cytokine production profiles of THP-1-derived M1-like and M2-like Mϕs, HL-60 and K562 cells;
2. To establish the immunogenicity of HL-60 and K562 cells in co-culture with M1-like and M2-like Mϕs in terms of Mϕ TNFα response;

3. Characterisation of the CD47 and SIRPα expression profile of THP-1, M1-like, M2-like, HL-60 and K562 cells in relation to their effects upon the co-culture system;

4. To determine if co-culture with $CD47^{+}$ cell lines elicits an inhibitory effect upon PAMP-induced TNFα production in M1-like and M2-like Mϕs;

5. To establish if this interaction is contact-dependent or soluble cytokine mediated.

3.2 Results

3.2.1 Undifferentiated THP-1 cells express both CD47 and SIRPα

Prior to differentiating THP-1 cells to M1-like or M2-like subsets, the CD47 and SIRPα expression was examined using fluorescent flow cytometry. As expected for a constitutive marker of self and for cells derived from an AML patient, these THP-1 cells expressed CD47 (net MFI 8.55) (**Figure 5 A)**. SIRPα expression was also present in THP-1 cells (net MFI 33.84) (**Figure 5 B)**.

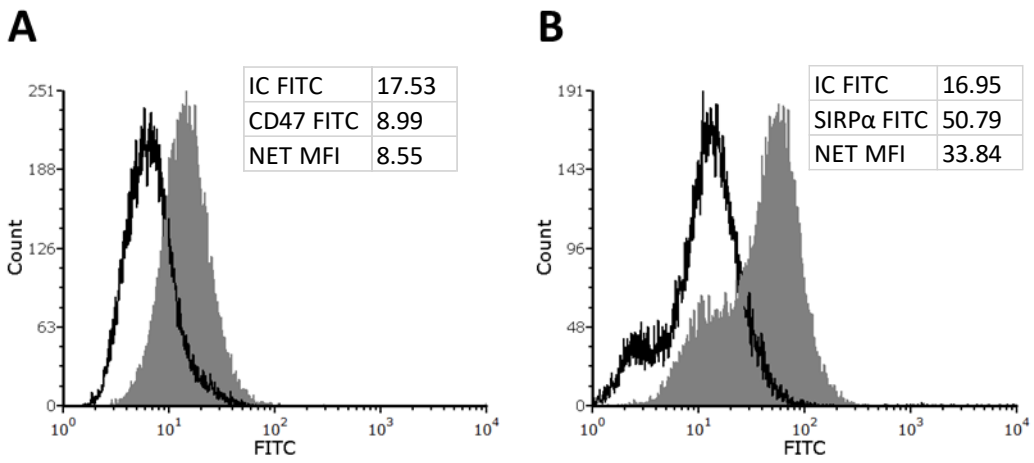

Figure 5. CD47 (A) and SIRPα (B) expression in undifferentiated THP-1 cells. Cells were labelled with anti-CD47 (A) or anti-SIRPα (B) FITC antibody (grey) and analysed by flow

cytometry, compared to FITC labelled isotype control (black). Data displays one representative experiment from n = 2 experiments, with triplicate samples.

3.2.2 K562 cells express higher levels of CD47 that HL-60 cells

HL-60 and K562 cells are known to overexpress CD47, due to their AML and CML origins. The differential expression between the cell lines was investigated. HL-60 cells had a net MFI of 198.8 (**Figure 6 A)** whereas K562 cells expressed four-fold greater, with a net MFI of 821.65. (**Figure 6 B**).

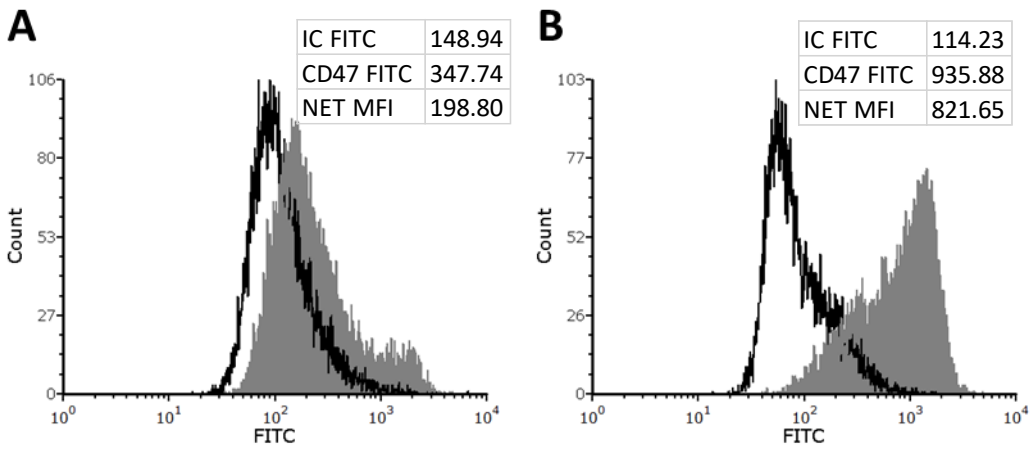

Figure 6. CD47 expression of HL-60 and K562 cells. HL-60 cells (A) and K562 cells (B) were labelled with anti-CD47 FITC antibody (grey) and analysed by fluorescent flow cytometry, compared to FITC-labelled isotype control (black). Data displays one representative experiment from n = 3 experiments, with triplicate samples.

3.2.3 M1-like and M2-like cells differentially express CD47, with M2s being the higher expressors

Both M1-like and M2-like Mϕs constitutively express the ubiquitous marker of self, CD47, however M1-like cells (**Figure 7 A)** are shown to express less CD47 than M2-like cells (**Figure 7 B)**; the net MFI of M1-like cells was 1214, compared to 1782 for M2-like cells.

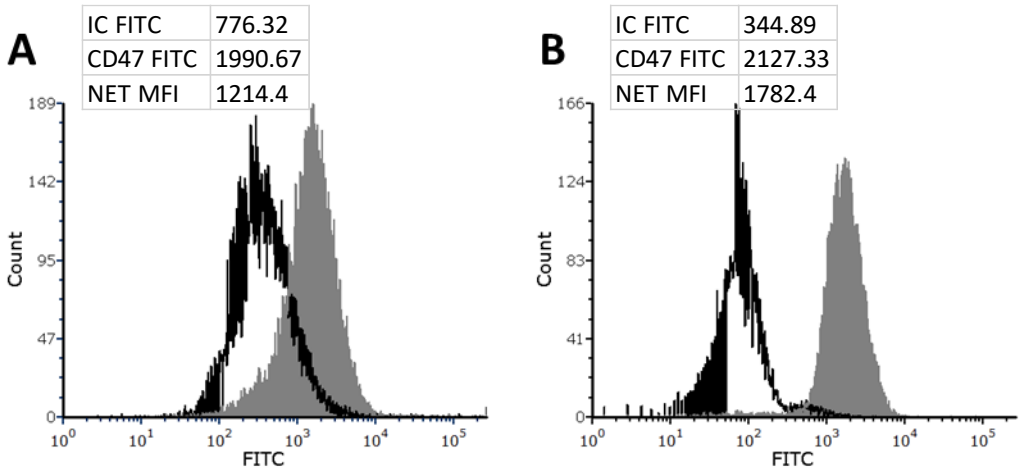

Figure 7. CD47 expression in unstimulated M1-like and M2-like cells. THP-1 derived M1-like Mϕs (A) and M2-like Mϕs (B) were labelled with anti-CD47 FITC antibody (grey) and analysed by fluorescent flow cytometry, compared to FITC-labelled isotype control (black). Data displays one representative experiment from n = 2 experiments, with triplicate samples.

3.2.4 M1-like and M2-like cells differentially express SIRPα, with M2s being the higher expressors

Analysis of SIRPα expression in M1-like and M2-like cells demonstrates that M2s express over three-fold greater levels of SIRPα compared to M1s, with a net MFI of 1668 for M2s, compared to 513 for M1s.

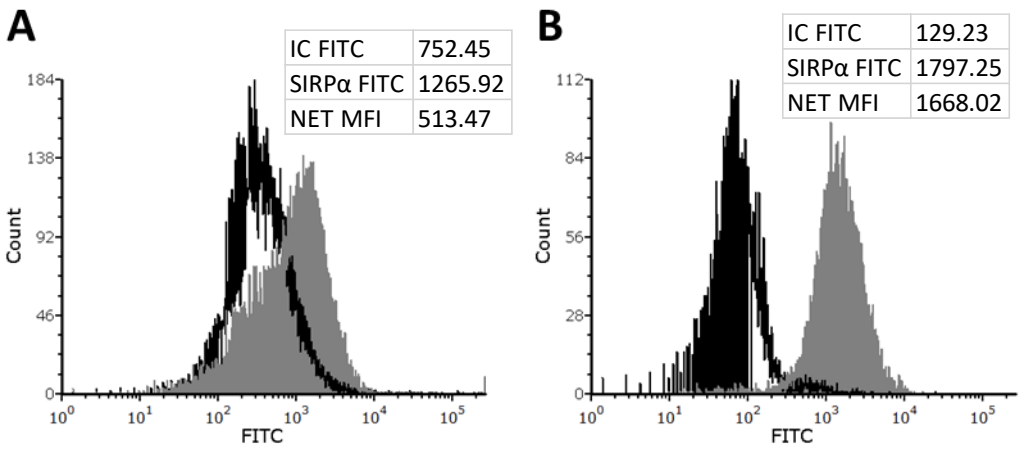

Figure 8. SIRPα expression in unstimulated M1-like and M2-like cells. THP-1 derived M1-like Mϕs (A) and M2-like Mϕs (B) were labelled with anti-SIRPα FITC antibody (grey) and

analysed by fluorescent flow cytometry, compared to FITC-labelled isotype control (black). Data displays one representative experiment from n = 2 experiments, with triplicate samples.

3.2.5 SIRPα expression in THP-1-derived M1-like Mϕs is downregulated upon stimulation with K12 LPS, PG LPS and SA LTA, yet is unchanged in M2-like Mϕs

In order to examine if stimulation with TLR2 and TLR4 agonists have varying effects upon SIRPα expression in M1-like and M2-like Mϕs, these cells were stimulated with K12 LPS, PG LPS and SA LTA, before the addition of anti-SIRPα FITC conjugated antibody and flow cytometric analysis. In M1-like cells, all stimuli marginally reduced SIRPα expression compared to unstimulated cells; K12 LPS had the least reduction of 3.76 relative MFI, SA LTA reduced the MFI by 6.08, and PG LPS reduced by 11.03. For M2-like cells an even more marginal increase in SIRPα expression was induced by stimuli, with PG LPS increasing MFI by 0.39, SA LTA by 0.91, and K12 LPS by 3.43. None of these changes were statistically significant, however a tentative trend could be observed: decrease of SIRPα expression in stimulated M1s and increase of SIRPα expression in stimulated M2s.

Table 4. SIRPα expression in unstimulated M1-like and M2-like Mϕs. THP-1 derived M1-like Mϕs (A) and M2-like Mϕs (B) were labelled with anti-SIRPα FITC antibody and analysed by flow cytometry, compared to FITC labelled isotype control. Data representative experiment from n = 2 experiments, with triplicate samples.

M1-like cells		**M2-like cells**	
Stimuli	Δ SIRPα expression compared to unstimulated baseline (net MFI)	Stimuli	Δ SIRPα expression compared to unstimulated baseline (net MFI)
K12 LPS	-3.76	K12 LPS	+3.43
PG LPS	-11.03	PG LPS	+0.39
SA LTA	-6.08	SA LTA	+0.91

3.2.6 M1-like and M2-like Mϕs produce differential TNFα production profiles in response to K12 LPS and SA LTA, however do not differ in their response to PG LPS

The TNFα production profile of M1-like and M2-like Mϕs stimulated with titrations of K12 LPS, PG LPS or SA LTA were investigated to determine the differential effects of TLR4 and TLR2 agonists on M1-like and M2-like Mϕs (**Figure 9**). For K12 LPS, significant differences in TNFα production between M1-like and M2-like cells were present for all K12 LPS concentrations ($p < 0.0001$) with M1s the higher TNFα producer, however no difference occurred in unstimulated states. Stimulation with PG LPS did not consistently produce a significant difference in TNFα production between subsets; only 10 ng/mL PG LPS produced a significant difference between M1-like and M2-like cells, with M1s producing 63 pg/mL compared to the M2-produced 17.4 pg/mL. Despite the lack of statistical significance, in all PG LPS concentrations M1-like Mϕs produced more TNFα than M2-like Mϕs. In the SA LTA treatment all concentrations produced significant difference in TNFα production between M1-like and M2-like cells ($p < 0.0001$), with M1s producing greater amounts of TNFα than M2s. Interestingly, the TNFα production between untreated M1 and M2 controls in this group also displayed a significant difference ($p < 0.0001$).

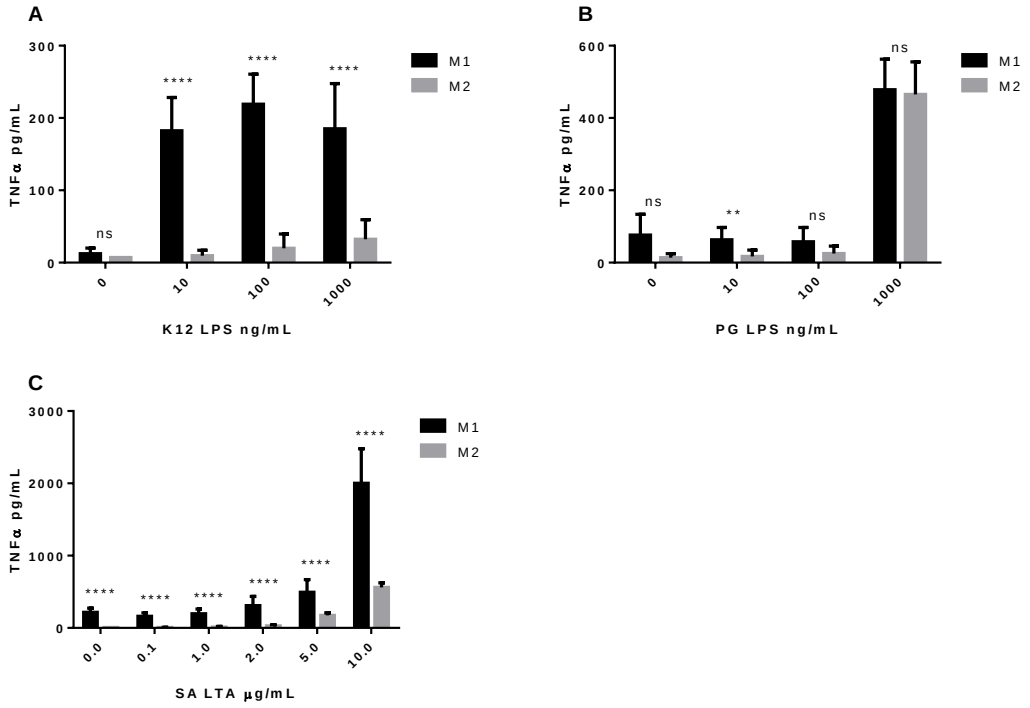

Figure 9. Differential effect of K12 LPS, PG LPS and SA LTA upon TNFα production in M1-like and M2-like Mϕs. M1-like and M2-like Mϕ subsets were generated from THP-1 promonocytes in the presence of either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 8 days respectively. Mϕs were stimulated with K12 LPS (A), PG LPS (B) or SA LTA (C) for 18 hours at varying concentrations, in addition to an unstimulated control. TNFα production is displayed as the mean ± SD in pg/ml. Data represents n = 2 experiments, with triplicate samples. Differences in TNFα production between M1-like and M2-like Mϕs are indicated with ****p < 0.0001, ** p < 0.01 and ns, not significant.

3.2.7 Neither HL-60 nor K562 cells produce TNFα or IL-10 in response to K12 LPS, PG LPS nor SA LTA.

Prior to utilising HL-60 or K562 cells in co-culture with Mϕs, it was necessary to determine if either of these cell lines respond to the chosen stimuli in a pro-inflammatory or anti-inflammatory manner. Upon stimulation with K12 LPS, PG LPS or SA LTA in the titrated concentrations used above, all cytokine production data for these experiments were at or below the lower level of detection, namely ≤ 7 pg/mL.

3.2.8 Co-culture with HL-60 or K562 cells does not induce a TNFα response in M1-like or M2-like Mϕs

Prior to utilising any $CD47^+$ cell line in a co-culture system with Mϕs, it was necessary to establish if any inherent immunogenicity existed between the cell lines. In the absence of any stimulation, M1-like and M2-like Mϕs co-cultured alongside $CD47^+$ HL-60 cells do not differ in their TNFα production compared to monoculture. Observable trends and significance were absent for all cell densities of HL-60. For M1-like cells (**Figure 10 A**), *p* values were as follows for varying HL-60 cell densities: 1×10^4, 1×10^5 and 1×10^6 cells/mL, $p > 0.9999$, > 0.999 and 0.3659 respectively. For TNFα production by M2-like cells co-cultured with HL-60 (**Figure 10 B**), TNFα production was also not significantly different from monoculture, with $p > 0.9999$ for all HL-60 cell densities. When co-cultured with K562 cells, TNFα production of M1-like Mϕs (**Figure 10 C**) observed a downward trend with increased K562 cell concentration, however this only became significant at the greatest K562 cell density of 1×10^6 cells/mL. *p* values for K562 cells densities 1×10^4, 1×10^5 and 1×10^6 cells/mL were > 0.9999, 0.3075 and 0.0005 respectively. For M2-like cells (**Figure 10 D**), TNFα production did not differ from monoculture at any cell density co-culture with K562 cells, $p > 0.9999$ for all comparisons.

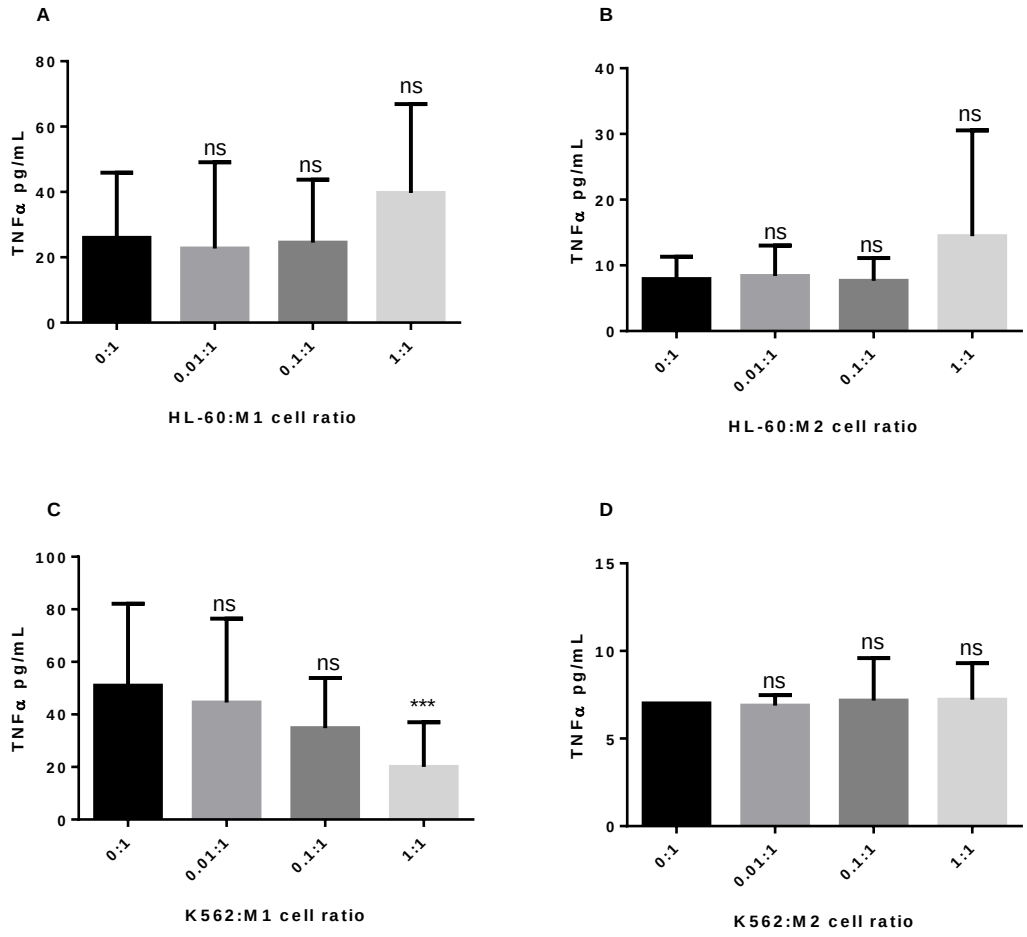

Figure 10. TNFα production by M1-like and M2-like Mϕs in the presence of HL-60 and K562 cells at varying cell densities. M1-like and M2-like Mϕ subsets were generated from THP-1 promonocytes in the presence of either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 8 days respectively. TNFα production (pg/mL) in the absence and presence of co-culture with increasing densities of HL-60 cells up to 1:1 is shown for M1-like cells (A) and M2-like cells (B). TNFα production by Mϕs alone and in co-culture with K562 cells is shown for M1-like cells (C) and M2-like cells (D). Data represents n = 3 experiments, with triplicate samples. Differences in TNFα production compared to monoculture are indicated with *** $p < 0.001$ and ns, not significant.

3.2.9 Co-culture between Mϕs and CD47$^+$ cell lines differentially inhibits TNFα release induced by K12 LPS, PG LPS and SA LTA

M1-like and M2-like Mϕs were co-cultured alongside equal cell density of HL-60 and K562 cells before being stimulated with 1 µg/mL K12 LPS, 1 µg/mL PG LPS or 5 µg/mL SA LTA. In all K12 LPS and PG LPS induced TNFα production, co-culture with either

HL-60 or K562 cells reduced TNFα production in both subsets. In all cases, M2-like cells displayed a greater sensitivity to co-culture-induced reduction of TNFα than M1-like cells. A trend was present where co-culture between the higher CD47 expressing K562 cells induced a greater reduction than that induced by HL-60 during K12 LPS induced TNFα production, yet this was not statistically significant. The sensitivity to reduction was greatest in K12 LPS-induced response, and least (or absent) in SA LTA-induced response.

Co-culture with HL-60 cells reduced K12 LPS-induced TNFα production in M1 cells by 35.8% (p = 0.0489), and for M2 cells K12 LPS-induced TNFα production was reduced by 48.71% (p 0.0440), (**Figure 11** A). When stimulated with PG LPS, HL-60 co-culture reduced TNFα production in M1s by 35.01% (p = 0.0267), and in M2s by 40.41% (p = 0.0098) (**Figure 11** B). For Mϕs stimulated with SA LTA, HL-60 co-culture did not produce a significant reduction in either M1-like or M2-like TNFα production (p > 0.9999 and 0.0730 respectively) (**Figure 11** C).

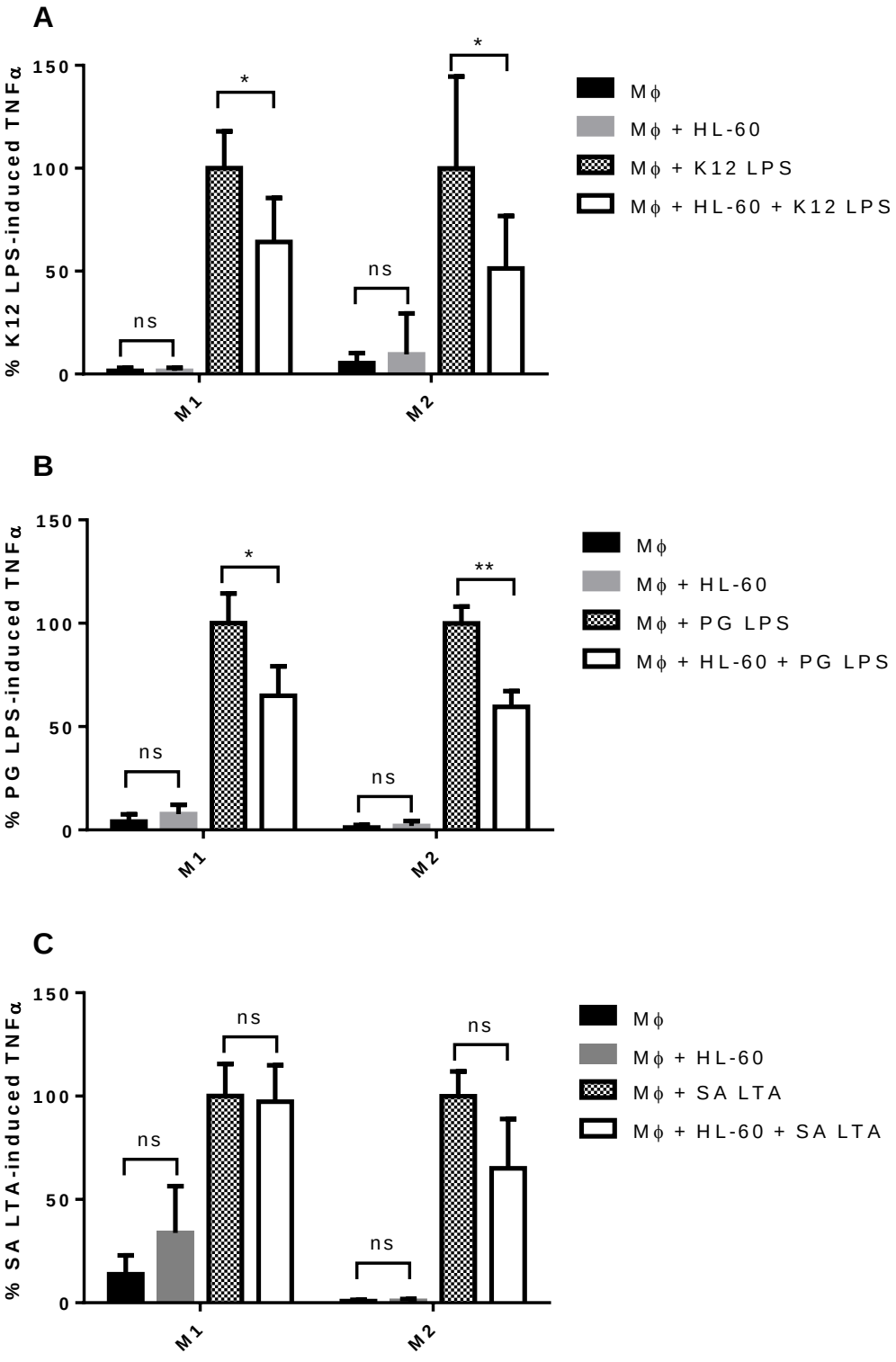

Figure 11 TNFα production by K12 LPS, PG LPS and SA LTA stimulated M1-like and M2-like Mϕs in 1:1 co-culture with HL-60 cells. M1-like and M2-like Mϕ subsets were generated from THP-1 promonocytes in the presence of either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 8 days respectively. 1 µg/mL K12 LPS- (A), 1 µg/mL PG LPS- (B) and 5 µg/mL SA LTA-induced (C) TNFα production is shown in the absence and presence of co-culture with HL-60, and displayed as percentage of each subset's K12 LPS-induced TNFα production. Data represents n = 3+ experiments, with

triplicate samples. Differences in TNFα production compared to monoculture are indicated with ** $p < 0.01$, * $p < 0.1$ and ns, not significant.

K562 co-culture reduced K12 LPS-induced TNFα production in M1 cells by 65.25 % ($p < 0.0001$) and in M2 cells by 72.1% ($p < 0.0001$) (**Figure 12 A**). When stimulated with PG LPS, K562 co-culture induced reduction in TNFα in M1s was 34.62% ($p = 0.0261$), and in M2s 50.15% ($p < 0.0001$) (**Figure 12 B**). For SA LTA stimulation, K562 co-culture did not significantly impact TNFα production in M1s ($p = 0.1150$), but reduced M2-derived TNFα by 34.63% ($p = 0.0015$) (**Figure 12 C**).

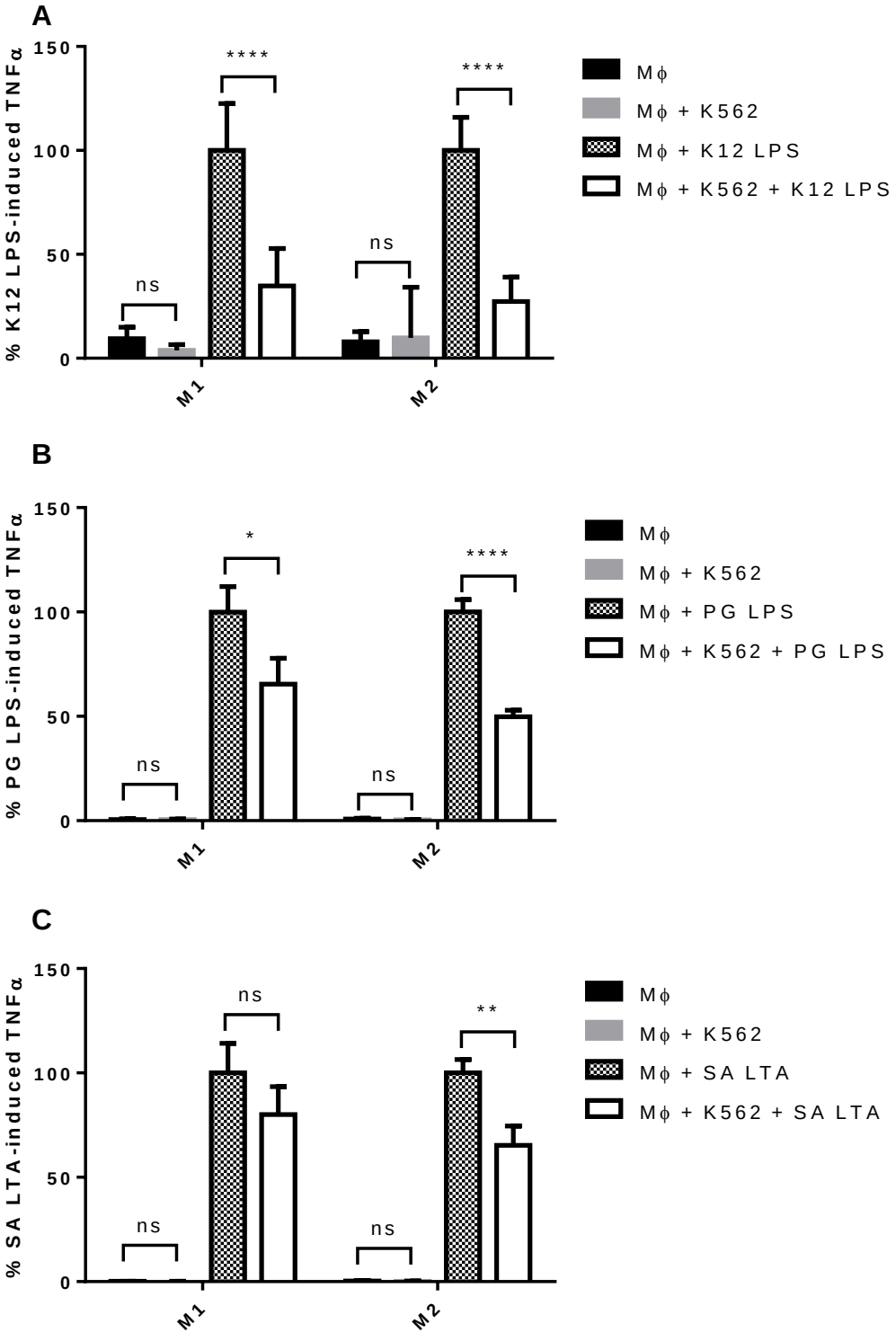

Figure 12. TNFα production by K12 LPS, PG LPS and SA LTA stimulated M1-like and M2-like Mϕs in 1:1 co-culture with K562 cells. M1-like and M2-like Mϕ subsets were generated from THP-1 promonocytes in the presence of either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 8 days respectively. 1 µg/mL K12 LPS- (A), 1 µg/mL PG LPS- (B) and 5 µg/mL SA LTA-induced (C) TNFα production is shown in the absence and presence of co-culture with K562, and displayed as percentage of K12 LPS-induced TNFα production. Data represents n = 3+ experiments, with triplicate samples.

Differences in TNFα production compared to monoculture are indicated with ** $p < 0.01$, * $p < 0.1$ and ns, not significant.

3.2.10 Co-culture inhibition of pro-inflammatory cytokine production in Mϕ subsets does not extend to IL-1β or IL-6, nor is IL-10 induced.

Pro-inflammatory TNFα acts in concert with other cytokines including IL-1β and IL-6, and a great deal of redundancy is often displayed amongst these cytokines. Additionally, the potential of co-culture inducing IL-10 production in Mϕs required exploration. The activity of K12 LPS-induced IL-1β, IL-6 and IL-10 in the K562-Mϕ co-culture system was examined. In unstimulated M1 cells (**Figure 13 A**) K562 co-culture induced an upregulation in IL-6 production from 6.412 % of K12 LPS-induced IL-6 to 78.56% (p = 0.0035). The effect of K562 co-culture upon K12 LPS-induced IL-6 production was also notable, with an increase of 217.9% (p = 0.0100). In M2 cells (**Figure 13 B**) co-culture with K562 did not induce upregulation of IL-6 production, however upon stimulation with K12 LPS over five-fold upregulation of IL-6 production was induced ($p < 0.0001$). Co-culture with K562 did not induce production of IL-1β either stimulated or unstimulated M1-like or M2-like cells, $p > 0.9999$ for all comparations except stimulated M1 vs stimulated M1 with K562 cells, where p = 0.7162 (**Figure 13 C and D**). Production of IL-10 in unstimulated and stimulated M1-like and M2-like cells alone or in co-culture with K562 was undetectable above the ELISA lower level of detection (7 pg/mL) (data not displayed).

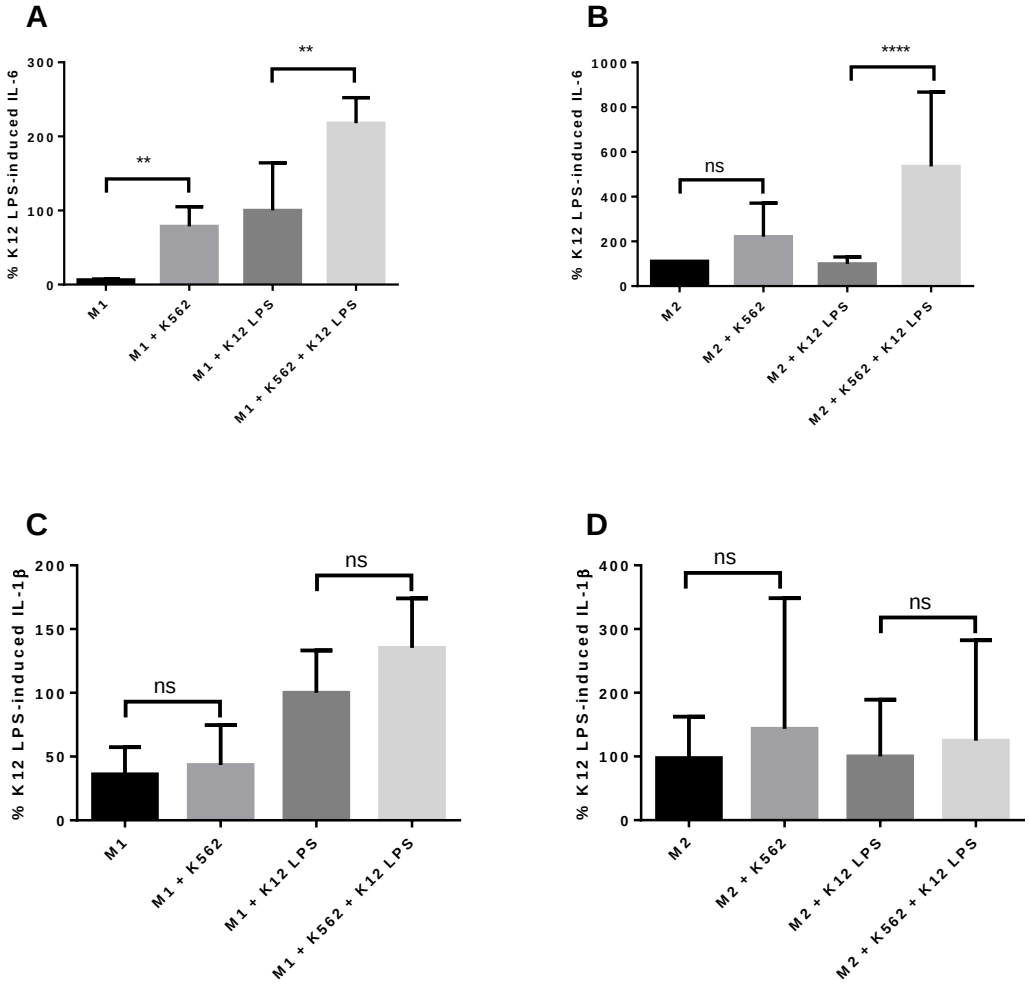

Figure 13. K562 co-culture effect on K12 LPS-induced IL-6 and IL-1β production in M1-like and M2-like Mϕs. M1-like and M2-like Mϕ subsets were generated from THP-1 promonocytes in the presence of either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 8 days respectively. IL-6 production is shown in the absence and presence of co-culture with K562 for M1-like cells (A) and M2-like cells (B). IL-1β production is shown for M1-like cells (C) and M2-like cells (D). Data are displayed as percentage of K12 LPS-induced TNFα production. Data represents n = 2 experiments, with triplicate samples. Differences in TNFα production compared to monoculture are indicated with ** $p < 0.01$, * $p < 0.1$ and ns, not significant.

3.2.11 The inhibitory effects of K562 co-culture upon K12 LPS-induced Mϕ TNFα production are mainly contact-dependent

The co-culture system and stimulus that produced the greatest inhibitory effect on Mϕ TNFα response, namely K562 cells in co-culture with K12 LPS stimulated Mϕs, was then selected for further examination as to the contact-dependent nature of the

interaction. **Figure 14** below shows a comparison between the TNFα production of K12 LPS-stimulated Mϕs, K12 LPS-stimulated Mϕs in co-culture with K562, and K12 LPS-stimulated Mϕs treated with K562 supernatants. For M1-like cells (**Figure 14 A**) a small reduction in the monoculture K12 LPS induced-TNFα production was present when M1s were treated with K562 supernatants, a reduction of 12.26% compared to the 39.34% reduction induced by co-culture. This reduction however was no statistically significant, p =0.1829. For M2-like cells (**Figure 14 B**) the K562 cell-free supernatant reduced the TNFα production by 25.34% (p = 0.0224), however this was much reduced from the 73.65% reduction induced by the co-culture system.

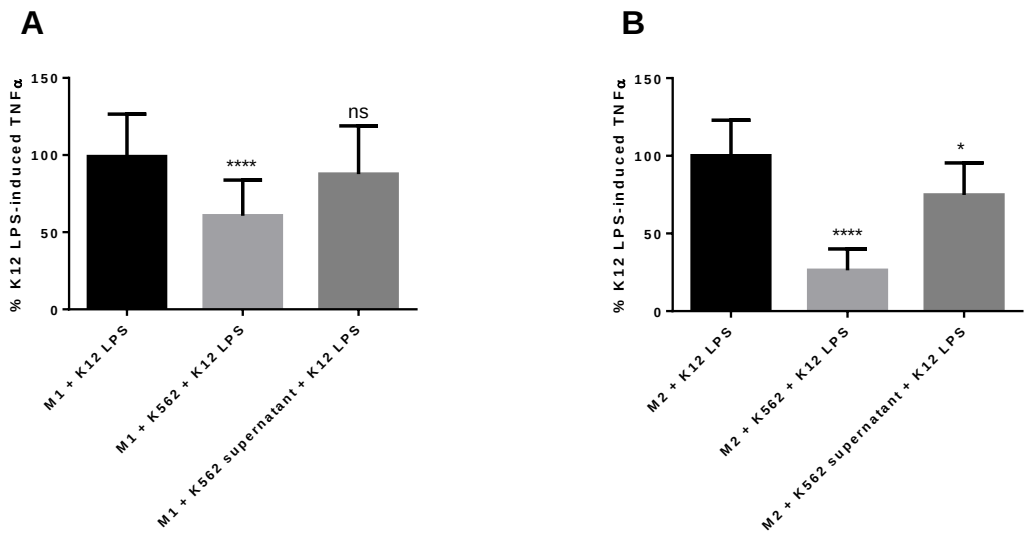

Figure 14. TNFα production by K12 LPS stimulated M1-like and M2-like Mϕs either alone, in co-culture with K562 cells, or when treated with K562 cell-free supernatant. M1-like and M2-like Mϕ subsets were generated from THP-1 promonocytes in the presence of either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 8 days respectively. K12 LPS-induced TNFα production is displayed in the absence and presence of co-culture with K562 cells and K562 cell-free supernatant. Data represents n = 3 experiments, with triplicate samples. Differences in TNFα production compared to monoculture are indicated with **** p < 0.0001, * p < 0.1 and ns, not significant.

3.2.12 Blockade of CD47-SIRPα with SIRPα blocking peptide does not abrogate co-culture-induced downregulation of K12 LPS-induced TNFα response

The introduction of a blocking peptide into the co-culture system was examined for its effect on the co-culture induced downregulation of K12 LPS-induced TNFα production. Curiously, for M1-like cells, these experiments did not show the HL-60 co-culture induced TNFα reduction which had been reliably present in n = 3 earlier experiments (3.2.9). Addition of SIRPα blocking peptide at 2 μg/mL and 4 μg/mL had no effect upon the K12 LPS-induced TNFα response in M1 and HL-60 co-culture (**Figure 15 A**). For M2-like cells (**Figure 15 B**), TNFα reduction in co-culture with HL-60 cells corresponded to previous experiments. Addition of the peptide caused a small upward trend in TNFα production upon increasing concentration when compared to the non-peptide treated HL-60 co-culture condition; untreated TNFα production in the co-culture system was 20.33 pg/mL, addition of 2 μg/mL peptide raised this to 22.33 pg/mL, and at 4 μg/mL peptide concentration this further raised to 27.44 μg/mL. Although observable, this trend was not statistically significant when compared to the untreated co-culture TNFα production ($p > 0.9999$ for 2 μg/mL peptide, $p = 0.1059$ for 4 μg/mL peptide).

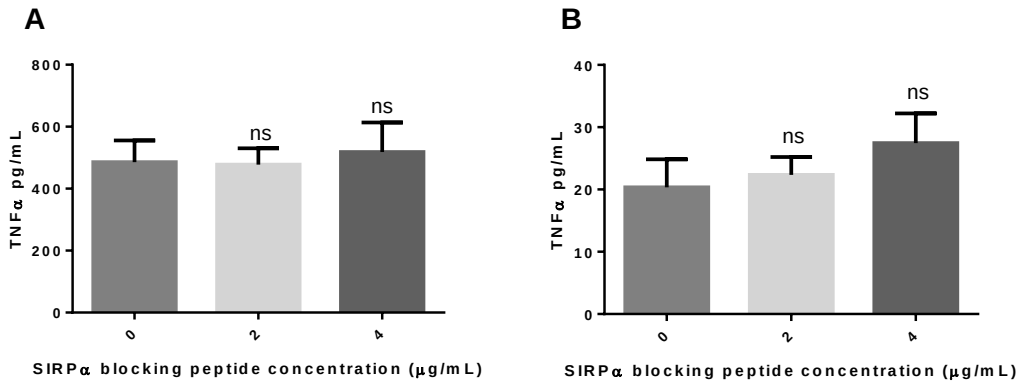

Figure 15. TNFα production by M1-like and M2-like Mϕs in co-culture with HL-60s and in the presence or absence of SIRPα blocking peptide treatment. M1-like and M2-like Mϕ subsets were generated from THP-1 promonocytes in the presence of either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 8 days respectively. K12 LPS-induced TNFα production is displayed in the absence and presence of treatment with SIRPα blocking peptide at 2 μg/mL and 4 μg/mL. Data represents n = 2 experiments, with triplicate samples.

3.3 Discussion

The overall findings of this study demonstrate a contact-dependent regulation of Mϕ pro-inflammatory activity upon co-culture with $CD47^+$ cells lines. The greatest regulation was present in co-culture between the highest CD47 expressing effector cells, K562, and M2-like cells, the highest SIRPα expressors of the two Mϕ subsets.

Undifferentiated THP-1 cells are shown to express both CD47 and SIRPα, aligning with the characterisation of CD47 as ubiquitously expressed (Brown and Frazier, 2001), and SIRPα as a myeloid-expressed receptor (Barclay and Brown, 2006). M2-like cells were the higher expressors of both CD47 and SIRPα, showing a higher propensity for both regulation and regulatability, fitting with this characterisation as a regulatory subset.

The SIRPα expression of M1s was marginally reduced upon stimulation with TLR4 agonist K12 LPS, yet a greater reduction was present in TLR2-binding SA LTA, and the

greatest reduction present when stimulated with PG LPS. It should be noted at this point that the PG LPS utilised in this study was standard level purity, it was not ultra-purified and so can be regarded as dual stimulator of TLR2 and TLR4 due to the presence of TLR2-stimulating endotoxin protein contaminants (Dziarski *et al.*, 2001). These data therefore indicate that both TLR2 and TLR4 stimulation have a minor downregulatory effect upon SIRPα expression in M1-like cells, which is compounded upon dual stimulation of the TLRs. TLR4-mediated downregulation of SIRPα expression is supported by the findings of Kong *et al.* (2007) and Zhu *et al.*(2013), who used RAW264.7 Mϕs and primary murine Mϕs respectively. Interestingly, Kong *et al.* also found that TLR4 knockdown removed LPS-induced SIRPα downregulation, however it is not stated what source of LPS was used, nor was any other TLR agonist utilised. To further explore the effect of TLR2 ligation upon SIRPα expression, treatment with another TLR2 agonist such as Pam2CSK4 could be utilised. Additionally, to rule out the potential of TLR4-ligating contaminants in supposed TLR2-specific agonists, the effect upon SIRPα expression of TLR4 knockdown prior to TLR2 stimulation should be examined. In M2-like cells a small increase in SIRPα expression was present upon stimulations with all PAMPs. This observation does not align with the literature for non-polarised Mϕs, however could be indicative of the stimulation furthering the Mϕs down the high SIRPα expressing M2-like route, in effect increasing their polarisation and strengthening the SIRPα^{hi} phenotype. A full time course experiment would provide further insight into these observations, in addition to examination of the SIRPα mRNA expression response to stimulation.

K562 cells were demonstrated to express higher levels of CD47 than HL-60 cells, identifying K562s as a co-culture component which could induce more CD47-SIRPα

binding to Mϕs than HL-60. It has been observed in the unpublished findings of colleagues that CD47 expression in both HL-60 and K562 cells increases with passage number, and so to maintain consistency these cells for all experiments within only 10 passages of defrosting.

In keeping with the existing literature, the initial PAMP stimulation experiments have characterised M1-like Mϕs as TNFα^{hi}, and M2-like cells as TNFα^{lo} when stimulated with K12 LPS and SA LTA. Interestingly, PG LPS stimulation did not produce a differential response between subsets, with M2-like cells eliciting a TNFα response of the same level as M1-like cells. This hyper-responsivity is uncharacteristic of M2-like cells and a departure from the findings of previous research studies (Foey and Crean, 2013). The choice of K12 LPS, PG LPS and SA LTA concentrations were based upon a TNFα response which would be within the reliable range of ELISA detection, whilst maintaining the potential for either decrease or increase in response to future experimental treatments. For this reason, 1 µg/mL K12 LPS, 1 µg/mL PG LPS and 5 µg/mL SA LTA were selected as appropriate concentrations for all further experiments.

The unresponsiveness of HL-60 and K562 to K12 LPS, PG LPS and SA LTA in terms of TNFα or IL-10 production identifies these cell lines are suitable co-culture choices for a Mϕ co-culture system for examination of contact-dependent effects between the two cell types. The lack of attention paid to further HL-60 and K562 cytokines is somewhat mitigated by later experiments aimed at establishing a contact-dependent focus on the co-culture system; however this characterisation step could be much improved by analysing a full panel of pro- and anti-inflammatory cytokines produced by HL-60 and K562 cells, with further attention given to the potential production of

other anti-inflammatory cytokines, especially IL-4 and IL-13, due to their influence on Mϕ polarisations towards a regulatory, M2-like phenotype.

Despite some inconsistencies with previously published findings, these experiments provide a justification for continuing with the use of THP-1 derived M1-like and M2-like cells for studies of induced TNFα responses. The Mϕ subset specific responses in terms of TNFα production were reliable for K12 LPS and SA LTA stimulation, demonstrating M1s as TNFα^{hi} and M2s as TNFα^{lo}. M2-like cells were shown to have higher SIRPα expression and CD47 expression than M1s, again aligning with their regulatory characterisation. HL-60 and K562 cells do not produce a TNFα or IL-10 response to K12 LPS, PG LPS or SA LTA, and they both express high, but differentially so, levels of CD47. These findings supported the continued development of a co-culture model between each Mϕ subset and CD47^{+} HL-60 or K562 cells, to examine the impact that such co-culture has on PAMP-induced TNFα production by Mϕs, and the level to which CD47-SIRPα interaction is implicated.

The Mϕ and HL-60/ K562 co-culture data builds a picture of a co-culture system which induces a clear and substantial inhibitory effect upon TLR4-mediated TNFα production in M1-like and M2-like Mϕs. The two CD47^{+} cell lines chosen for this co-culture model, HL-60 and K562 were not inherently immunogenic towards M1-like or M2-like Mϕs, nor did they elicit an IL-10 response in either subset. Co-culture with the CD47^{+} HL-60 cells and CD47^{hi} K562 cells reduced K12 LPS and PG LPS-induced TNFα production in both subsets, with the greatest reduction consistently present in M2-like cells. Interestingly, co-culture had no impact upon SA LTA-induced TNFα production. This aligns with and further strengthens the existing research findings that SIRPα ligation inhibits TLR4-mediated TNFα production (Alblas *et al.*, 2005; Smith

et al., 2003), with the current study's results indicating that this does not extend to TLR2-mediated TNFα production. In unstimulated M1-like cells, co-culture with an equal number of K562 cells downregulated baseline TNFα production, indicating that the inhibitory effect of K562 cells upon Mϕ pro-inflammatory response is present even without external PAMP stimulation, an observation which merits further study into the potential mechanisms. The cause of baseline TNFα production in 'unstimulated' M1-like Mϕs is likely to be low level endotoxin contamination within the FBS (Kirikae *et al.*, 1997), although FBS batches were screened for the lowest level baseline TNFα response in M1-like and M2-like cells, this could not be entirely mitigated against.

Although attempts were made to confirm the contact-dependent nature of this K562/ Mϕ induced downregulation of TNFα production, secreted soluble mediators produced by, and acting upon, Mϕs may represent an intermediate component in the observed TNFα downregulation. A novel finding of these series of experiments exists in the upregulation of IL-6 production in M1-like and M2-like cells by co-culture with K562 cells, occurring alongside the downregulation of TNFα. Although this may be viewed as an indication of the redundancy which exists between TNFα and IL-6 in their pro-inflammatory activities, it must also be noted that IL-6 has roles as an anti-inflammatory cytokine, directly inhibiting the production of TNFα (Aderka *et al.*, 1989; Scheller *et al.*, 2011). It is within this context that the present findings become particularly interesting, with the suggestion that K562 co-culture induces IL-6 upregulation in Mϕs which in turn inhibits an LPS-induced TNFα response. A potential further avenue for exploring this would be the inclusion of an IL-6 inhibitor such as tocilizumab within the co-culture. This monoclonal antibody competitively binds IL-6

receptors preventing signalling, and so would identify if the observed co-culture induced TNFα reduction is in part, or whole, mediated by IL-6 signalling between Mϕs.

Along these same lines, the lack of observable IL-10 induced by K562 cells in co-culture with M1-like and M2-like cells does not necessarily indicate that IL-10 is unimplicated within this interaction. The lack of detectable LPS-induced IL-10 production in the M2-like subset within this study hints at the possibility of membrane-bound IL-10. This has been observed in multiple studies and its endogenous anti-inflammatory effect upon Mϕs recognised despite the lack of secreted soluble IL-10 (Fleming, S D and Campbell, 1996; Fleming, Sherry D. *et al.*, 1999). The use of an anti-IL-10 neutralising antibody would assist in determining the extent to which membrane-bound IL-10 is inhibiting TNFα release in the co-culture systems examined within this study (Foey *et al.*, 2017).

Further examination of the exact role of CD47-SIRPα interaction in the co-culture induced downregulation of LPS-induced TNFα mainly focused on the use of a commercially available blocking peptide. This experiment was performed only for the HL-60 co-culture system, and so further work would examine its effect upon the K562 co-culture system; however these early results indicated that the peptide was ineffective at blockading CD47-SIRPα binding and biological activity in the concentrations examined, and therefore not a practical, economically viable tool to continue utilising for such purpose. Some elucidation of the lack of efficacy was provided by a follow-up experiment (data not shown) examining the effect of the SIRPα blocking peptide upon FITC-labelled anti-SIRPα binding; these data demonstrated that the SIRPα blocking peptide had no blockade effect on anti-SIRPα antibody binding in to Mϕs. This hints that the blocking peptide binds to a domain of

SIRPα which doesn't prevent any future binding. This revelation encouraged further examination of the blocking peptide's sequence, and the knowledge that it binds 17 amino acids at the C-terminus of SIRPα, whereas the Ig-like V-set domain of SIRPα which binds extracellular IgSF of CD47 is located at the N-terminus (Hatherley *et al.*, 2007). This introduces the potential that the peptide was not in fact binding or blocking the same domain through which signal is transduced upon CD47 ligation. Further work should consider the use of a SIRPα neutralising antibody instead of a blocking peptide.

During this study some early proof-of-concept studies into the efficacy of SIRPα and CD47 knockdown and knockout in THP1-derived Mϕs and K562 cells were also completed. This work represented a methods development exploration, which identified a number of issues with the use of siRNA or CRISPR in THP-1-derived Mϕs; the use of such knockdown and knockout techniques upon a terminally differentiated population of non-dividing cells with a limited useable lifespan is problematic due to the amount of time required for transformation to take place. Additionally, the lipid-based transfection protocols utilised were identified as largely ineffective for use on suspension cell lines such as those utilised within this study. Should transfection occur prior to differentiation of THP-1 cells into M1-like and M2-like Mϕs, knockout of SIRPα would likely inhibit attempts to push cells towards an M2-like phenotype, as shown in previous studies demonstrating an upregulation of M1-like phenotypic traits and a downregulation of M2-like traits (Lin *et al.*, 2018; Wang *et al.*, 2019). Knockdown/ knockout of CD47 is equally problematic, especially if such cells are introduced into a co-culture system with Mϕs, as many studies have shown that $CD47^{-/-}$ cells are readily phagocytosed by Mϕs, rendering such a system inappropriate

for study of pro-inflammatory cytokine production analysis and more towards a phagocytosis study.

In summary, this study has developed a co-culture system between THP-1-derived Mϕs and $CD47^+$ cell lines which downregulates TLR4-dependent TNFα production in both Mϕ subsets:

1. K12 LPS-induced TNFα had the largest reduction upon co-culture.
2. The interaction appears to be contact-dependent, induced when $CD47^+$ cells are in direct contact with SIRPα-expressing Mϕs.
3. Higher CD47-expressing effector cells induce greater reduction upon co-culture with Mϕs.
4. Higher SIRPα-expressing target cells are more sensitive to co-culture-induced TNFα inhibition.

The precise role that CD47-SIRPα ligation plays in this interaction cannot be stated with confidence, as the degree of redundancy and effect of additional receptor-ligand pairings hasn't been elucidated; however with the future work suggested above, further examination of this co-culture system could lead to a greater understanding of the role of cell-to-cell contact mediated suppression of Mϕ pro-inflammatory response. These early findings have the potential to contribute to therapeutic alteration of the inflammatory *in vivo* microenvironment, shifting Mϕ behaviour towards a regulatory phenotype and downregulating TNFα production.

Bibliography

Adegbola, S. O., Sahnan, K., Warusavitarne, J., Hart, A., and Tozer, P. (2018). Molecular Sciences Anti-TNF Therapy in Crohn's Disease. *International Journal of Molecular Sciences* 19(2244).

Aderka, D., Le, J. M., and Vilcek, J. (1989). IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes , U937 cells , and in mice . Information about subscribing to The Journal of Immunology is online at : *The Journal of Immunology* 143(11): 3517–3523.

Agarwal, S., Piesco, N. P., Johns, L. P., and Riccelli, A. E. (1995). Differential Expression of IL-1β, TNF-α, IL-6, and IL-8 in Human Monocytes in Response to Lipopolysaccharides from Different Microbes. *Journal of Dental Research* 74(4): 1057–1065.

Alblas, J., Honing, H., Renardel de Lavalette, C., Brown, M. H., Dijkstra, C. D., and van den Berg, T. K. (2005). Signal Regulatory Protein Ligation Induces Macrophage Nitric Oxide Production through JAK/STAT- and Phosphatidylinositol 3-Kinase/Rac1/NAPDH Oxidase/H2O2-Dependent Pathways. *Molecular and Cellular Biology* 25(16): 7181–7192.

Barclay, A. N., and Brown, M. H. (2006). The SIRP family of receptors and immune regulation. *Nature Reviews Immunology* 6(6): 457–464.

Barclay, A. N., and van den Berg, T. K. (2014). The Interaction Between Signal Regulatory Protein Alpha (SIRP α) and CD47: Structure, Function, and Therapeutic Target . *Annual Review of Immunology* 32(1): 25–50.

Brooke, G., Holbrook, J. D., Brown, M. H., and Barclay, A. N. (2004). Human Lymphocytes Interact Directly with CD47 through a Novel Member of the Signal Regulatory Protein (SIRP) Family. *The Journal of Immunology* 173(4): 2562–2570.

Brown, E. J., and Frazier, W. A. (2001). Integrin-associated protein (CD47) and its ligands. *Trends in Cell Biology* 11(3): 130–135.

Cardoso, E. M., Reis, C., and Manzanares-Céspedes, M. C. (2018). Chronic periodontitis, inflammatory cytokines, and interrelationship with other chronic diseases. *Postgraduate Medicine*.

Chanteux, H., Guisset, A. C., Pilette, C., and Sibille, Y. (2007). LPS induces IL-10 production by human alveolar macrophages via MAPKinases- and Sp1-dependent mechanisms. *Respiratory Research* 8(1): 71.

Couper, K. N., Blount, D. G., and Riley, E. M. (2008). IL-10: The Master Regulator of Immunity to Infection. *The Journal of Immunology* 180(9): 5771–5777.

Daigneault, M., Preston, J. A., Marriott, H. M., Whyte, M. K. B., and Dockrell, D. H. (2010). The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PLoS ONE* 5(1).

Dinarello, C. A. (1999). Cytokines as Endogenous Pyrogens. *The Journal of Infectious Diseases* 179(s2): S294–S304.

Dinarello, C. a (2007). Historical Review of Cytokines. *European Journal of Immunology* 37(SUPPL. 1): S34.

Duque, G. A., and Descoteaux, A. (2014). Macrophage cytokines: Involvement in

immunity and infectious diseases. *Frontiers in Immunology*. Frontiers Media S.A.

Dziarski, R., Wang, Q., Miyake, K., Kirschning, C. J., and Gupta, D. (2001). MD-2 Enables Toll-Like Receptor 2 (TLR2)-Mediated Responses to Lipopolysaccharide and Enhances TLR2-Mediated Responses to Gram-Positive and Gram-Negative Bacteria and Their Cell Wall Components. *The Journal of Immunology* 166(3): 1938–1944.

Feldmann, M., and Maini, S. R. N. (2008/June). Role of cytokines in rheumatoid arthritis: An education in pathophysiology and therapeutics. *Immunological Reviews*.

Fleming, S D, and Campbell, P. A. (1996). Macrophages have cell surface IL-10 that regulates macrophage bactericidal activity. *Journal of immunology (Baltimore, Md. : 1950)* 156(3): 1143–50.

Fleming, Sherry D., Leenen, P. J. M., Freed, J. H., and Campbell, P. A. (1999). Surface interleukin-10 inhibits listericidal activity by primary macrophages. *Journal of Leukocyte Biology* 66(6): 961–967.

Foey, A. D., and Crean, S. J. (2013). Macrophage Subset Sensitivity to Endotoxin Tolerisation by Porphyromonas gingivalis. *PLoS ONE* 8(7).

Foey, A. D., Habil, N., Al-Shaghdali, K., and Crean, S. J. (2017). Porphyromonas gingivalis-stimulated macrophage subsets exhibit differential induction and responsiveness to interleukin-10. *Archives of Oral Biology* 73: 282–288.

Fortin, G., Raymond, M., Van, V. Q., Rubio, M., Gautier, P., Sarfati, M., and Franchimont, D. (2009). A role for CD47 in the development of experimental colitis mediated by SIRPα $^{+}$ CD103 $^{-}$ dendritic cells. *The Journal of Experimental Medicine* 206(9): 1995–2011.

Gao, Q., Zhang, Y., Han, C., Hu, X., Zhang, H., Xu, X., Tian, J., Liu, Y., Ding, Y., Liu, J., Wang, C., Guo, Z., Yang, Y., and Cao, X. (2016). Blockade of CD47 ameliorates autoimmune in fl ammation in CNS by suppressing IL-1-triggered in fi ltration of pathogenic Th17 cells. *Journal of Autoimmunity* : 1–12. doi:10.1016/j.jaut.2016.03.002.

Gitik, M., Kleinhaus, R., Hadas, S., Reichert, F., and Rotshenker, S. (2014). Phagocytic receptors activate and immune inhibitory receptor SIRPα inhibits phagocytosis through paxillin and cofilin. *Frontiers in Cellular Neuroscience* 8(1).

Gordon, S., and Taylor, P. R. (2005/December). Monocyte and macrophage heterogeneity. *Nature Reviews Immunology*.

Gruys, E., Toussaint, M. J. M., Niewold, T. A., and Koopmans, S. J. (2005). Acute phase reaction and acute phase proteins. *Journal of Zhejiang University: Science* 6 B(11): 1045–1056.

Gur-Wahnon, D., Borovsky, Z., Liebergall, M., and Rachmilewitz, J. (2009). The induction of APC with a distinct tolerogenic phenotype via contact-dependent STAT3 activation. *PLoS ONE* 4(8).

Han, M. H., Lundgren, D. H., Jaiswal, S., Chao, M., Graham, K. L., Garris, C. S., et al. (2012). Janus-like opposing roles of CD47 in autoimmune brain inflammation in humans and mice. *The Journal of Experimental Medicine* 209(7): 1325–1334.

Hashimoto, D., Chow, A., Noizat, C., Teo, P., Beasley, M. B., Leboeuf, M., et al. (2013). Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* 38(4): 792–804.

Hatherley, D., Harlos, K., Dunlop, D. C., Stuart, D. I., and Barclay, A. N. (2007). The structure of the macrophage signal regulatory protein α (SIRPα) inhibitory receptor reveals a binding face reminiscent of that used by T cell receptors. *Journal of Biological Chemistry* 282(19): 14567–14575.

Hayat, S. M. G., Bianconi, V., Pirro, M., Jaafari, M. R., Hatamipour, M., and Sahebkar, A. (2019). CD47: role in the immune system and application to cancer therapy. *Cellular Oncology*. doi:10.1007/s13402-019-00469-5.

Italiani, P., Mazza, E. M. C., Lucchesi, D., Cifola, I., Gemelli, C., Grande, A., Battaglia, C., Bicciato, S., and Boraschi, D. (2014). Transcriptomic profiling of the development of the inflammatory response in human monocytes in vitro e87680. *PLoS ONE* 9(2).

Jaiswal, S., Jamieson, C. H. M., Pang, W. W., Park, C. Y., Chao, M. P., Majeti, R., Traver, D., van Rooijen, N., and Weissman, I. L. (2009). CD47 Is Upregulated on Circulating Hematopoietic Stem Cells and Leukemia Cells to Avoid Phagocytosis. *Cell* 138(2): 271–285.

Kirikae, T., Tamura, H., Hashizume, M., Kirikae, F., Uemura, Y., Tanaka, S., Yokochi, T., and Nakano, M. (1997). Endotoxin contamination in fetal bovine serum and its influence on tumor necrosis factor production by macrophage-like cells J774.1 cultured in the presence of the serum. *International Journal of Immunopharmacology* 19(5): 255–262.

Kong, X. N., Yan, H. X., Chen, L., Dong, L. W., Yang, W., Liu, Q., Yu, L. X., Huang, D. D., Liu, S. Q., Liu, H., Wu, M. C., and Wang, H. Y. (2007). LPS-induced down-regulation of signal regulatory protein α contributes to innate immune activation in macrophages. *Journal of Experimental Medicine* 204(11): 2719–2731.

Li, C., Wang, Y., Gao, L., Zhang, J., Shao, J., Wang, S., Feng, W., Wang, X., Li, M., and Chang, Z. (2002). Expression of toll-like receptors 2 and 4 and CD14 during differentiation of HL-60 cells induced by phorbol 12-myristate 13-acetate and 1 alpha, 25-dihydroxy-vitamin D(3). *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* 13(1): 27–38.

Liang, S., Cai, J., Li, Y., and Yang, R. (2019). 1,25-Dihydroxy-Vitamin D3 induces macrophage polarization to M2 by upregulating T-cell Ig-mucin-3 expression. *Molecular Medicine Reports* 49(5): 3707–3713.

Lin, Y., Zhao, J.-L., Zheng, Q.-J., Jiang, X., Tian, J., Liang, S.-Q., Guo, H. W., Qin, H.-Y., Liang, Y.-M., and Han, H. (2018). Notch Signaling Modulates Macrophage Polarization and Phagocytosis Through Direct Suppression of Signal Regulatory Protein α Expression. *Frontiers in Immunology* 9: 1744.

Majeti, R., Chao, M. P., Alizadeh, A. A., Pang, W. W., Jaiswal, S., Gibbs, K. D., van Rooijen, N., and Weissman, I. L. (2009). CD47 Is an Adverse Prognostic Factor and Therapeutic Antibody Target on Human Acute Myeloid Leukemia Stem Cells. *Cell* 138(2): 286–299.

Manthey, C. L., Perera, P. Y., Henricson, B. E., Hamilton, T. A., Qureshi, N., and Vogel, S. N. (1994). Endotoxin-induced early gene expression in C3H/HeJ (Lpsd) macrophages. *Journal of immunology (Baltimore, Md. : 1950)* 153(6): 2653–63.

Martinez, F. O., and Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000Prime Reports* 6(13).

Martinez, F. O., Gordon, S., Locati, M., and Mantovani, A. (2006). Transcriptional

Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and Patterns of Gene Expression. *The Journal of Immunology* 177(10): 7303–7311.

McClure, R., and Massari, P. (2014). TLR-dependent human mucosal epithelial cell responses to microbial pathogens. *Frontiers in Immunology*.

McInnes, I. B., and Schett, G. (2007/June). Cytokines in the pathogenesis of rheumatoid arthritis. *Nature Reviews Immunology*.

Navegantes, K. C., Souza Gomes, R., Pereira, P. A. T., Czaikoski, P. G., Azevedo, C. H. M., and Monteiro, M. C. (2017/15/February). Immune modulation of some autoimmune diseases: The critical role of macrophages and neutrophils in the innate and adaptive immunity. *Journal of Translational Medicine*. BioMed Central Ltd.

Okamoto, M., Hirai, H., Taniguchi, K., Shimura, K., Inaba, T., Shimazaki, C., Taniwaki, M., and Imanishi, J. (2009/November). Toll-like Receptors (TLRs) are expressed by myeloid leukaemia cell lines, but fail to trigger differentiation in response to the respective TLR ligands: Correspondence. *British Journal of Haematology*.

Parameswaran, N., and Patial, S. (2010). Tumor Necrosis Factor-α Signaling in Macrophages. *Advances in Global Change Research* 39: 415–419.

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., and Yona, S. (2017). The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *Journal of Experimental Medicine* 214(7): 1913–1923.

Qasimi, P., Ming-Lum, A., Ghanipour, A., Ong, C. J., Cox, M. E., Ihle, J., Cacalano, N.,

Yoshimura, A., and Mui, A. L. F. (2006). Divergent mechanisms utilized by SOCS3 to mediate interleukin-10 inhibition of tumor necrosis factor α and nitric oxide production by macrophages. *Journal of Biological Chemistry* 281(10): 6316–6324.

Sansom, D. M. (2000). CD28, CTLA-4 and their ligands: Who does what and to whom? *Immunology*. Wiley-Blackwell.

Scheller, J., Chalaris, A., Schmidt-Arras, D., and Rose-John, S. (2011/May). The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta - Molecular Cell Research*.

Schröder, N. W. J., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zähringer, U., Göbel, U. B., Weber, J. R., and Schumann, R. R. (2003). Lipoteichoic acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *Journal of Biological Chemistry* 278(18): 15587–15594.

Schwende, H., Fitzke, E., Ambs, P., and Dieter, P. (1996). Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *Journal of Leukocyte Biology* 59(4): 555–561.

Segura, M., Vadeboncoeur, N., and Gottschalk, M. (2002). CD14-dependent and -independent cytokine and chemokine production by human THP-1 monocytes stimulated by Streptococcus suis capsular type 2. *Clinical and Experimental Immunology* 127(2): 243–254.

Shi, L., Bian, Z., Chen, C. X. J., Guo, Y., Lv, Z., Zeng, C., Liu, Z., and Zen, K. (2015). CD47

deficiency ameliorates autoimmune nephritis in Fas lpr mice by suppressing IgG autoantibody production. *JJournal of Pathology* 237(July): 285–295.

Shi, L., Bian, Z., and Liu, Y. (2017). Dual role of SIRP α in macrophage activation : inhibiting M1 while promoting M2 polarization via selectively activating SHP-1 and SHP-2 signal. *The Journal of Immunology* 198(1 Supplement): 2017.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999). MD-2, a Molecule that Confers Lipopolysaccharide Responsiveness on Toll-like Receptor 4. *Journal of Experimental Medicine* 189(11): 1777–1782.

Smith, R. E., Patel, V., Seatter, S. D., Deehan, M. R., Brown, M. H., Brooke, G. P., Goodridge, H. S., Howard, C. J., Rigley, K. P., Harnett, W., and Harnett, M. M. (2003). A novel MyD-1 (SIRP-1α) signaling pathway that inhibits LPS-induced TNFα production by monocytes. *Blood* 102(7): 2532–2540.

Stefanidakis, M., Newton, G., Lee, W. Y., Parkos, C. A., and Luscinskas, F. W. (2008). Endothelial CD47 interaction with SIRP{gamma} is required for human T-cell transendothelial migration under shear flow conditions in vitro. *Blood* 112(4): 1280–1289.

Stout, R. D., Jiang, C., Matta, B., Tietzel, I., Watkins, S. K., and Suttles, J. (2005). Macrophages Sequentially Change Their Functional Phenotype in Response to Changes in Microenvironmental Influences. *The Journal of Immunology* 175(1): 342–349.

Sullivan, K. E., Reddy, A. B. M., Dietzmann, K., Suriano, A. R., Kocieda, V. P., Stewart, M., and Bhatia, M. (2007). Epigenetic Regulation of Tumor Necrosis Factor Alpha.

Molecular and Cellular Biology 27(14): 5147–5160.

Takeda, K., and Akira, S. (2018). Toll-like receptors in innate immunity. *International Immunology* 17(1): 1–14.

Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 11(4): 443–451.

Tanaka, T., Narazaki, M., and Kishimoto, T. (2014). Il-6 in inflammation, Immunity, And disease. *Cold Spring Harbor Perspectives in Biology* 6(10).

Toledano, N., Gur-Wahnon, D., Ben-Yehuda, A., and Rachmilewitz, J. (2013). Novel CD47: SIRPα Dependent Mechanism for the Activation of STAT3 in Antigen-Presenting Cell. *PLoS ONE* 8(9).

Troutman, T. D., Bazan, J. F., and Pasare, C. (2012/1/October). Toll-like receptors, signaling adapters and regulation of the pro-inflammatory response by PI3K. *Cell Cycle*. Taylor and Francis Inc.

Verma, R., Balakrishnan, L., Sharma, K., Khan, A. A., Advani, J., Gowda, H., Tripathy, S. P., Suar, M., Pandey, A., Gandotra, S., Prasad, T. S. K., and Shankar, S. (2016). A network map of Interleukin-10 signaling pathway. *Journal of Cell Communication and Signaling* 10(1): 61–67.

Villaggio, B., Soldano, S., and Cutolo, M. (2012). 1,25-dihydroxyvi1. Villaggio B, Soldano S, Cutolo M. 1,25-dihydroxyvitamin D3 downregulates aromatase expression and inflammatory cytokines in human macrophages. Clin Exp Rheumatol [Internet]. [cited 2019 Sep 28];30(6):934–8. Available from: http://www.nc. *Clinical*

and experimental rheumatology 30(6): 934–8.

Wang, J., Ding, X., Wu, X., Liu, J., Zhou, R., Wei, P., Zhang, Q., Zhang, C., Zen, K., and Li, L. (2019). SIRPα deficiency accelerates the pathologic process in models of Parkinson disease. *Glia* (July): 1–17. doi:10.1002/glia.23689.

Yang, L., and Seki, E. (2012). Toll-like receptors in liver fibrosis: Cellular crosstalk and mechanisms. *Frontiers in Physiology* 3(138).

Yao, C., Oh, J. H., Lee, D. H., Bae, J. S., Jin, C. L., Park, C. H., and Chung, J. H. (2015). Toll-like receptor family members in skin fibroblasts are functional and have a higher expression compared to skin keratinocytes. *International Journal of Molecular Medicine* 35(5): 1443–1450.

Yu, W., Soprana, E., Cosentino, G., Volta, M., Viale, G., Vercelli, D., and Lichenstein, H. S. (1998). Soluble CD141-152confers responsiveness to both lipoarabinomannan and lipopolysaccharide in a novel HL-60 cell bioassay. *Journal of Immunology* 161(8): 4244–4251.

Zhang, M., Hutter, G., Kahn, S. A., Azad, T. D., Gholamin, S., Xu, C. Y., Liu, J., Achrol, A. S., Richard, C., Sommerkamp, P., Schoen, M. K., McCracken, M. N., Majeti, R., Weissman, I., Mitra, S. S., and Cheshier, S. H. (2016). Anti-CD47 treatment stimulates phagocytosis of glioblastoma by M1 and M2 polarized macrophages and promotes M1 polarized macrophages in vivo. *PLoS ONE* 11(4).

Zhang, Q., Lee, W. Bin, Kang, J. S., Kim, L. K., and Kim, Y. J. (2018). Integrin CD11b negatively regulates mincle-induced signaling via the lyn–SIRPα–SHP1 complex. *Experimental and Molecular Medicine* 50(2): e439-17.

Zhu, D., Pan, C., Li, L., Bian, Z., Lv, Z., Shi, L., Zhang, J., Li, D., Gu, H., Zhang, C. Y., Liu, Y., and Zen, K. (2013). MicroRNA-17/20a/106a modulate macrophage inflammatory responses through targeting signal-regulatory protein α. *Journal of Allergy and Clinical Immunology* 132(2): 426-436.e8.

Appendices

Appendix 1. Reagents and materials

Tissue culture reagents and plasticwares

Product	Catalogue no.	Supplier
1, 25-$(OH)_2$ Vitamin D_3 (VD_3)	D1530	Sigma-Aldrich, Poole, UK
Cell culture plasticwares	Flasks, plates, pipette tips, serological pipettes, falcon tubes	Greiner Bio-One, Stonehouse, UK
Dimethyl sulfoxide (DMSO)	BP231-100	Fisher Scientific, Loughborough, UK
Dulbecco's phosphate buffered saline (DPBS)	17-512F	Lonza, Slough, UK
Escherichia coli K12 LPS	tlrl-peklps	Invivogen, Toulouse, France
Foetal bovine serum	F9665, Batch BCBW3204	Sigma-Aldrich, Poole, UK
HL-60 cell line		ECACC, Salisbury, UK
K562 cell line		ECACC, Salisbury, UK
L-Glutamine 200nM	17-605E	Lonza, Slough, UK
Staphylococcus aureus lipoteichoic acid (LTA)	LTA-SA TLR2 Agonist	Invivogen, Toulouse, France
Phorbol-12-myristate acetate (PMA)	P1585	Sigma-Aldrich, Poole, UK
Porphyromonas gingivalis LPS	tlrl-pglps	Invivogen, Toulouse, France
RPMI 1640 (without L-glutamine)	15-040-CVR	SLS, Nottingham, UK
SIRP alpha Blocking Peptide	SBP3500202	Sigma-Aldrich, Poole, UK
THP-1 cell line		ECACC, Salisbury, UK

ELISA reagents

Product	Catalogue no.	Supplier
Bovine serum albumin (BSA)	A30075	Melford, Ipswich, UK
IL-10 biotin anti-human detection antibody	554499	BD Biosciences, Wokingham, UK
IL-10 cytokine standard	93/722	NIBSC, Hertfordshire, UK
IL-10 purified mouse anti-human capture antibody	554497	BD Biosciences, Wokingham, UK
IL-6 biotin anti-human detection antibody	554546	BD Biosciences, Wokingham, UK
IL-6 cytokine standard	89/548	NIBSC, Hertfordshire, UK
IL-6 purified rat anti-human capture antibody	554543	BD Biosciences, Wokingham, UK
Phosphate buffered saline tablets	P4417	Sigma-Aldrich, Poole, UK
Streptavidin HRP	DY998	R&D Systems, Abingdon, UK
TMB microwell peroxidase/ substrate system	5120-0047	Insight Bio (KPL), Wembley, UK
TNFα biotin mouse anti-human detection antibody	554511	BD Biosciences, Wokingham, UK
TNFα cytokine standard	12/154	NIBSC, Hertfordshire, UK
TNFα mouse anti-human capture antibody	551220	BD Biosciences, Wokingham, UK
Tween 20	P1379	Sigma-Aldrich, Poole, UK

Flow cytometry reagents

Product	Catalogue no.	Supplier
CD172a (SIRP alpha) Mouse anti-Human, FITC,	15556506	Fisher Scientific, Loughborough, UK

Clone: 15-414		
CD282 (TLR2), FITC, clone: TL2.1	15536646	Fisher Scientific, Loughborough, UK
CD47 Mouse anti-Human, FITC, Clone: B6H12,	15516416	Fisher Scientific, Loughborough, UK
IgG1 kappa, FITC, clone: P3.6.2.8.1, Isotype Control	15104218	Fisher Scientific, Loughborough, UK
IgG2a, K Mouse, FITC, Clone: eBM2a, Isotype Control,	12700780	Fisher Scientific, Loughborough, UK

siRNA reagents

Product	**Catalogue no.**	**Supplier**
CD47 antibody (B6H12)	sc-12730	Santa Cruz Biotechnology, Heidelberg, Germany
CD47 siRNA (h)	sc-35006	Santa Cruz Biotechnology, Heidelberg, Germany
Control siRNA-B	sc-44230	Santa Cruz Biotechnology, Heidelberg, Germany
siRNA Transfection Medium	sc-36868	Santa Cruz Biotechnology, Heidelberg, Germany
siRNA Transfection Reagent	sc-29528	Santa Cruz Biotechnology, Heidelberg, Germany
SIRP-α Antibody (C-7)	sc-376884	Santa Cruz Biotechnology, Heidelberg, Germany
SIRP-α siRNA (h)	sc-44106	Santa Cruz Biotechnology, Heidelberg, Germany

CRISPR-Cas9 reagents

Product	**Catalogue no.**	**Supplier**
CD47 CRISPR/Cas9 KO Plasmid (h2)	sc-400508-KO-2	Santa Cruz Biotechnology, Heidelberg, Germany

Control CRISPR/Cas9 Plasmid	sc-418922	Santa Cruz Biotechnology, Heidelberg, Germany
Plasmid transfection medium	sc-108062	Santa Cruz Biotechnology, Heidelberg, Germany
UltraCruz® Transfection Reagent	sc-395739	Santa Cruz Biotechnology, Heidelberg, Germany

Appendix 2. FBS batch testing

The TNFα production profile of unstimulated and stimulated M1-like and M2-like Mϕs are affected by the batch of FBS used in culture medium

The varying immunogenicity between batches of FBS in terms of TNFα production in unstimulated Mϕs is shown in **Figure A1**. Current FBS batch A produced the lowest baseline TNFα response in unstimulated M1-like cells at 15.28 pg/mL. FBS batch B was the next lowest TNFα-inducer in unstimulated M1-like cells, inducing 44.22 pg/mL TNFα in M1-like Mϕs. Batch D induced the lowest TNFα response M2-like cells, 7.45 pg/mL compared to the 8.56 pg/mL of batch A. Batch B was the only batch that had no statistically significant difference with batch A for both M1-like and M2-like TNFα production.

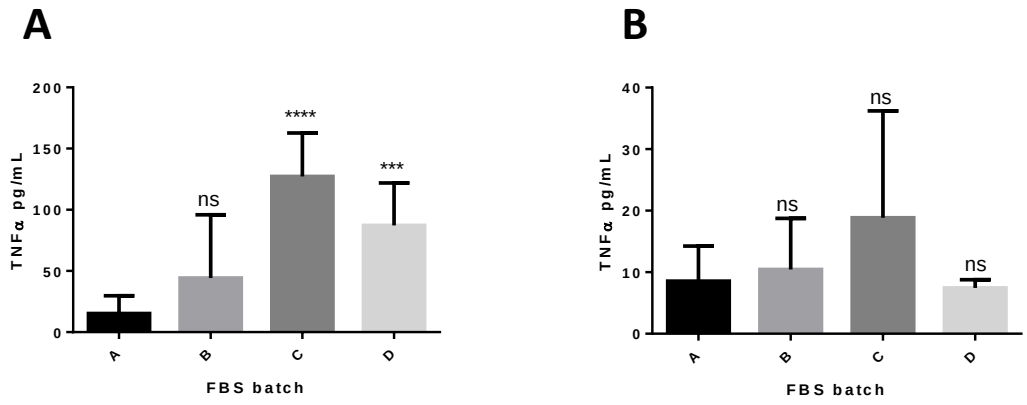

Figure A1. FBS batch effect on TNFα production by unstimulated THP-1-derived M1-like and M2-like Mϕs. M1-like (A) and M2-like (B) Mϕ subsets were generated from THP-1 promonocytes in the presence of either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 8 days respectively. THP-1 cell culture and differentiation of M1-like and M2-like cells took place in one of four FBS batches, A, B, C or D. TNFα production is displayed as the mean ± SD in pg/ml.

Data represents n = 4 experiments. Differences in TNFα production compared to batch A are indicated with ****$p < 0.0001$, *** $p < 0.001$ and ns, not significant.

In their separate FBS batches, M1-like and M2-like Mϕs were stimulated with *E. coli* K12 LPS at concentrations ranging from 10 ng/mL to 1 µg/mL (**Figure A2**). In M1-like cells TNFα production induced by all K12 LPS concentrations were significantly different to the unstimulated control for all batches of FBS. In the M2 subset all K12 LPS concentrations in batches A and C were of significant difference to the unstimulated control, whereas for batch B only the 1 µg/mL K12 LPS concentration was distinct. In FBS batch D, no K12 LPS-induced TNFα differed from the baseline TNFα production in unstimulated controls. At 1 µg/mL the TNFα production levels for M1-like cells in FBS batch A was 394.8 pg/mL, batch B 185.1 pg/mL, batch C 396.7 pg/mL and in batch D 172.6 pg/mL. For M2-like cells stimulated with 1 µg/mL K12 LPS, TNFα production for batch A was 46.22 pg/mL, batch B 32.44 pg/mL, batch C 39.22 pg/mL and batch D 17.11 pg/mL.

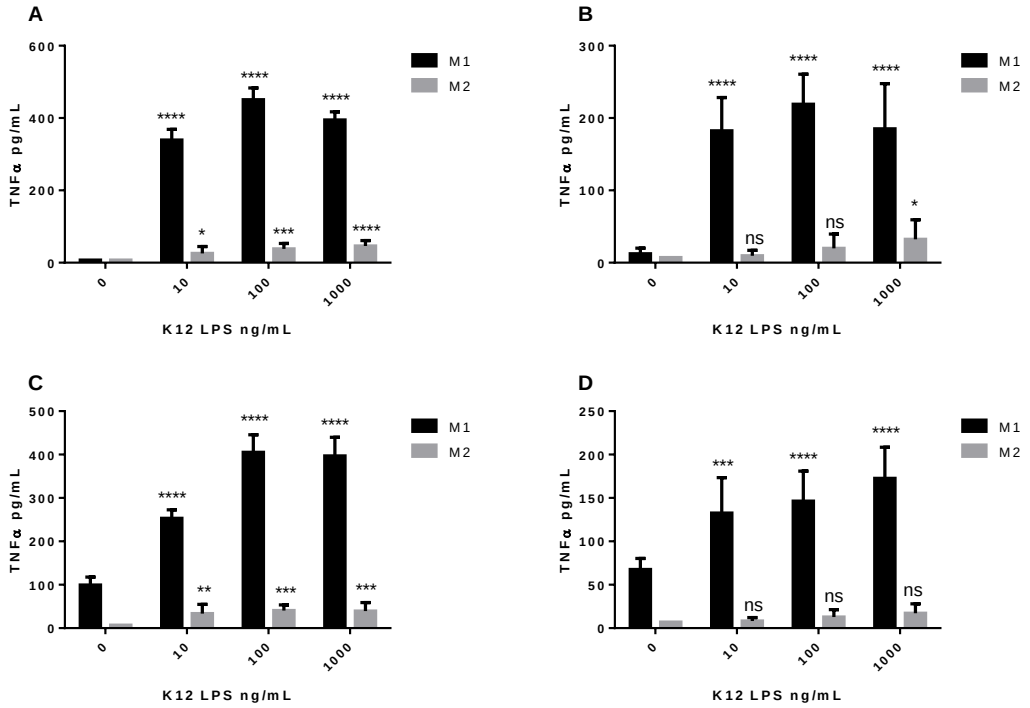

Figure A2. FBS batch effect on K12 LPS-induced TNFα production by THP-1-derived M1-like and M2-like Mϕs. M1-like and M2-like Mϕ subsets were generated from THP-1 promonocytes in the presence of either 25 ng/ml phorbol 12-myristate 13-acetate (PMA) for 4 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 8 days respectively. THP-1 cell culture and differentiation of M1-like and M2-like cells took place in one of four FBS batches, A, B, C or D. Mϕs were then stimulated with K12 LPS for 18 hours at varying concentrations, in addition to an unstimulated control. TNFα production is displayed as the mean ± SD in pg/ml. Data represents n = 2 experiments. Differences in TNFα production compared to unstimulated control are indicated with ****p < 0.0001, ***p < 0.001, ** p < 0.01 and ns, not significant.

M1-like and M2-like Mϕs growing in separate batches of FBS were then stimulated with TLR2/ TLR4 agonist PG LPS at concentrations ranging from 10 ng/mL to 1 µg/mL,

and TNFα production was analysed as before (**Figure A3**). Only the highest concentration, 1 µg/mL of PG LPS produced a statistically significant difference in TNFα production from the unstimulated control. For M1-like cells this occurred in FBS batches A, B and C; in batch D no concentration of PG LPS produced a significant difference from unstimulated. M2-like cells in all batches of FBS responded significantly to 1 µg/mL PG LPS, however none of the lower concentrations produced a significant response. At 1 µg/mL PG LPS stimulation, the TNFα production of M1-like Mϕs in batch A was 513 pg/mL, batch B 478.4, batch C 1031 pg/mL and batch D 119.1 pg/mL. In M2-like Mϕs TNFα production in batch A was 324.8 pg/mL, batch B 465.2 pg/mL, batch C 287.4 pg/mL and batch D 140.7 pg/mL.

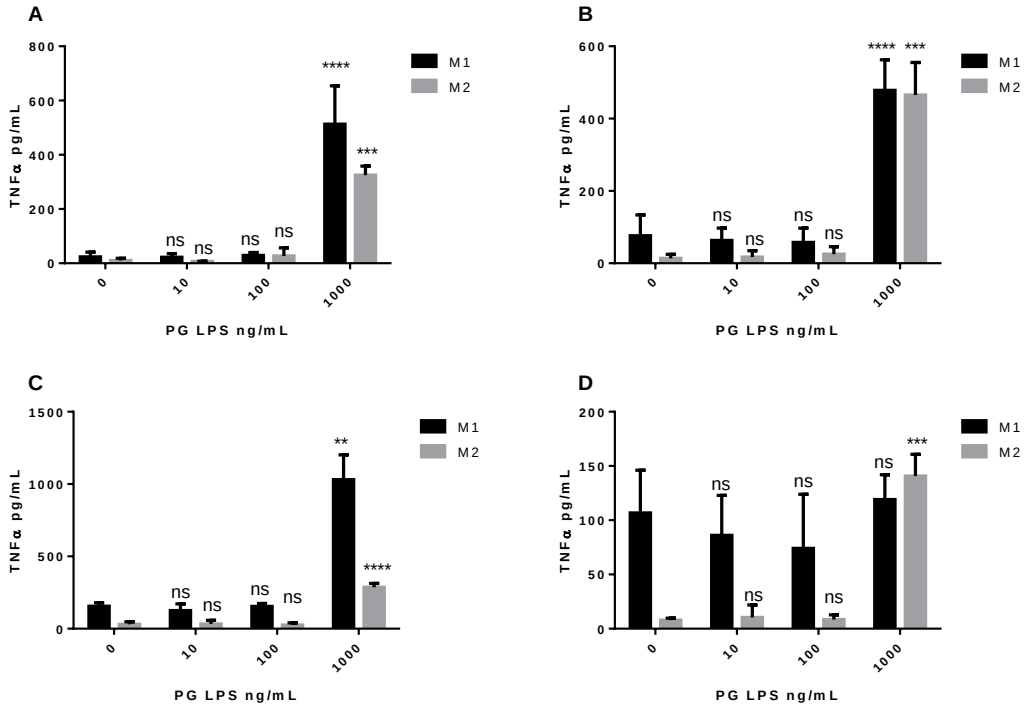

Figure A3. FBS batch effect on PG LPS-induced TNFα production by THP-1-derived M1-like and M2-like Mϕs. M1-like and M2-like Mϕ subsets were generated from THP-1 promonocytes in the presence of either 25 ng/ml phorbol 12-myristate 13-

acetate (PMA) for 4 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 8 days respectively. THP-1 cell culture and differentiation of M1-like and M2-like cells took place in one of four FBS batches, A, B, C or D. Mϕs were then stimulated with PG LPS for 18 hours at varying concentrations, in addition to an unstimulated control. TNFα production is displayed as the mean ± SD in pg/ml. Data represents n = 2 experiments. Differences in TNFα production compared to unstimulated control are indicated with ****p < 0.0001, ***p < 0.001, ** p < 0.01 and ns, not significant.

Due to its low immunogenicity on unstimulated cells, FBS batch B was chosen to use for all further cell culture maintenance and experiments. This batch also allowed for a consistent response to 1 μg/mL K12 and PG LPS, the chosen concentration for all future experiments (unless otherwise stated).

Appendix 3. Co-culture raw data

Table A1. Raw data, TNFα production by M1 macrophages alone and in co-culture with HL-60 cells, unstimulated and stimulated with 1 μg/mL K12 LPS

n = 3 experiments; n.d. = no data

	TNFα production (pg/mL)			
	M1	**M1 + HL-60**	**M1 + K12**	**M1 + K12 + HL-60**
	7	7	208	86
	7	7	184	162
	7	7	199	119
	7	7	106	163
	7	6	149	101
	7	7	129	130
	7	7	276	110
	7	7	240	119
	7	7	242	144
	41	49	7977	6608
	38	51	7710	6925
	42	71	7729	6439
	29	96	7940	6616
	35	82	7531	6421
	30	69	7699	6882
	32	41	6482	5650
	33	44	7180	6853
	82	38	n.d.	6497
	3	7	2607	1157
	61	7	2967	1263
	4	7	2953	1225
	7	7	2515	897
	7	7	2472	866
	7	7	2409	934
	17	7	2472	933
	7	7	2218	838
	7	7	2288	1105
Mean	**20.19**	**24.67**	**3264.69**	**2564.56**

Table A2. Raw data, TNFα production by M2 macrophages alone and in co-culture with HL-60 cells, unstimulated and stimulated with 1 μg/mL K12 LPS

n = 3 experiments; n.d. = no data

	TNFα production (pg/mL)			
	M2	**M2 + HL-60**	**M2 + K12**	**M2 + K12 + HL-60**
	7	7	50	12
	7	27	35	60
	7	2	125	30
	7	7	62	6.8
	7	7	13	6.8
	7	4	37	36
	7	57	53	32
	7	7	6.8	6.8
	7	7	141	12
	7	64	1263	975
	24	7	1429	997
	12	7	1366	986
	7	7	1251	1062
	7	37	1168	1029
	10	36	1187	1006
	7	24	1351	874
	7	12	1246	900
	7	8	1151	915
	7	7	225	86
	7	7	212	95
	7	7	227	94
	7	7	224	86
	7	7	259	86
	7	7	222	86
	7	7	263	100
	7	7	252	81
	7	7	218	96
Mean	**7.93**	**14.44**	**519.88**	**361.35**

Table A3. Raw data, TNFα production by M1 macrophages alone and in co-culture with HL-60 cells, unstimulated and stimulated with 1 μg/mL PG LPS

n = 3 experiments; n.d. = no data

	TNFα production (pg/mL)			
	M1	**M1 + HL-60**	**M1 + PG**	**M1 + PG + HL-60**
	21	187	6059	3883
	20	190	5376	4753
	6	188	5183	3446
	14	172	4265	3165
	21	183	4835	3978
	76	167	5669	4013
	21	159	5078	4418
	55	213	5504	5447
	69	237	5644	4975
	142	237	2255	1582
	137	210	2528	1619
	133	235	2521	1702
	97	176	2956	1980
	88	173	3252	1899
	99	213	3329	2064
	43	142	3546	1844
	67	159	3568	1355
	97	184	4474	1940
	165	207	1547	1116
	119	204	1584	1136
	176	231	1750	1066
	156	239	1712	1034
	127	243	1852	890
	160	240	1808	980
	169	266	1999	1151
	154	271	2035	1103
	170	318	2092	1151
Mean	**96.37**	**209.04**	**3423.00**	**2358.89**

Table A4. Raw data, TNFα production by M2 macrophages alone and in co-culture with HL-60 cells, unstimulated and stimulated with 1 μg/mL PG LPS

n = 3 experiments; n.d. = no data

	TNFα production (pg/mL)			
	M2	**M2 + HL-60**	**M2 + PG**	**M2 + PG + HL-60**
	103	192	4138	2512
	132	180	4170	2728
	103	190	3807	2666
	133	186	3951	2075
	54	231	3759	2190
	146	226	3905	2455
	105	273	4290	1883
	129	259	3286	1691
	137	n.d.	4786	1908
	7	7	1438	942
	7	7	1642	1095
	7	7	1467	1057
	7	7	1570	792
	7	7	1755	765
	7	7	1637	795
	7	7	1387	921
	7	7	1445	1006
	7	7	1344	1001
	7	7	2443	1393
	7	7	2469	1505
	7	7	2748	1560
	7	7	2483	1550
	7	7	2525	1675
	7	7	2554	1718
	7	7	2597	1555
	7	7	2483	1583
	7	7	n.d.	1591
Mean	**43.26**	**71.65**	**2695.35**	**1578.22**

Table A5. Raw data, TNFα production by M1 macrophages alone and in co-culture with HL-60 cells, unstimulated and stimulated with 5 μg/mL SA LTA

n = 3 experiments; n.d. = no data

	TNFα production (pg/mL)			
	M1	**M1 + HL-60**	**M1 + LTA**	**M1 + LTA + HL-60**
	56	111	307	279
	64	137	348	290
	71	150	352	256
	79	142	299	205
	70	156	230	209
	62	161	311	207
	59	127	219	238
	65	138	243	273
	64	165	264	265
	20	53	121	117
	19	58	126	124
	26	58	127	126
	21	65	115	124
	27	63	123	111
	17	67	133	122
	20	61	124	140
	19	63	130	149
	18	60	132	151
	276	310	9885	17160
	292	361	10184	8747
	260	440	16378	13290
	245	312	9161	10269
	220	318	13363	9257
	248	297	9264	10633
	176	303	13377	11415
	192	255	13085	14805
	227	341	9998	11778
Mean	**107.89**	**176.74**	**4014.78**	**4101.48**

Table A6. Raw data, TNFα production by M2 macrophages alone and in co-culture with HL-60 cells, unstimulated and stimulated with 5 μg/mL SA LTA

n = 3 experiments; n.d. = no data

	TNFα production (pg/mL)			
	M2	**M2 + HL-60**	**M2 + LTA**	**M2 + LTA + HL-60**
	7	7	601	200
	7	7	632	188
	7	7	605	214
	7	7	532	212
	7	7	583	219
	7	7	566	210
	7	7	526	244
	7	7	651	252
	7	7	693	276
	1	1	208	111
	1	1	205	104
	1	1	213	108
	1	1	163	123
	6	1	151	122
	1	2	161	145
	1	1	155	122
	1	2	157	161
	1	9	163	106
	7	7	2177	1384
	7	7	1852	1410
	7	7	2144	1610
	7	7	1687	1731
	7	7	2124	1948
	7	7	2539	1786
	7	7	1817	2186
	7	7	2307	2226
	7	7	1951	2069
Mean	**5.19**	**5.37**	**946.78**	**721.00**

Table A7. Raw data, TNFα production by M1 macrophages alone and in co-culture with K562 cells, unstimulated and stimulated with 1 μg/mL K12 LPS

n = 3 experiments; n.d. = no data

	TNFα production (pg/mL)			
	M1	**M1 + K562**	**M1 + K12**	**M1 + K12 + K562**
	4	7	383	89
	7	7	381	72
	7	7	390	73
	7	7	221	57
	2	7	216	66
	2	7	241	73
	86	39	438	251
	66	42	347	329
	58	35	549	343
	67	54	616	252
	62	48	675	298
	60	46	619	290
	56	36	672	347
	56	34	612	383
	42	30	601	392
	108	10	450	84
	98	16	507	102
	94	8	440	86
	60	7	441	109
	71	7	445	116
	58	7	421	97
	57	1	725	117
	47	15	619	141
	42	7	742	143
Mean	**50.71**	**20.17**	**489.63**	**179.58**

Table A8. Raw data, TNFα production by M2 macrophages alone and in co-culture with K562 cells, unstimulated and stimulated with 1 μg/mL K12 LPS

n = 4 experiments; n.d. = no data

TNFα production (pg/mL)			
M2	M2 + K562	M2 + K12	M2 + K12 + K562
7	7	130	6
7	7	140	7
7	7	147	12
7	3	262	23
7	7	126	7
7	7	123	18
7	70	62	15
7	7	47	7
7	7	47	21
7	7	49	12
7	7	35	7
7	16	39	7
7	7	49	7
7	7	38	7
7	7	34	7
3	7	232	99
20	7	228	102
5	7	225	113
6	7	236	113
13	7	251	92
27	1	234	99
10	7	271	88
18	7	245	100
17	7	266	102
19	7	189	63
12	7	186	62
14	7	203	51
16	3	166	64
8	2	196	51
23	2	182	43
6	8	207	53
11	7	209	62
6	8	216	58
4	7	178	52
4	7	193	61
10	7	182	65
19	7	176	49
21	8	202	67
19	8	198	58
6	13	204	48
15	13	205	57

	12	0	212	51
Mean	**10.69**	**8.36**	**167.14**	**49.67**

Table A9. Raw data, TNFα production by M1 macrophages alone and in co-culture with K562 cells, unstimulated and stimulated with 1 μg/mL PG LPS

n = 3 experiments; n.d. = no data

	TNFα production (pg/mL)			
	M1	**M1 + K562**	**M1 + PG**	**M1 + PG + K562**
	7	7	1177	716
	7	7	1261	772
	7	7	1146	757
	7	7	1124	n.d.
	7	7	1268	n.d.
	7	7	1379	n.d.
	7	7	1540	790
	1	6	1546	840
	8	13	1487	811
	7	7	572	754
	9	7	970	720
	7	7	976	721
	7	7	1026	343
	7	7	998	364
	5	7	990	647
	3	7	937	696
	14	7	1014	702
	12	7	882	683
	7	7	1007	918
	2	7	972	795
	1	7	958	849
	7	1	976	644
	7	7	1048	640
	4	7	967	659
	3	7	1205	617
	10	1	1192	656
	10	3	1144	639
Mean	**6.67**	**6.59**	**1102.30**	**697.21**

Table A10. Raw data, TNFα production by M2 macrophages alone and in co-culture with K562 cells, unstimulated and stimulated with 1 μg/mL PG LPS

n = 3 experiments; n.d. = no data

	TNFα production (pg/mL)			
	M2	**M2 + K562**	**M2 + PG**	**M2 + PG + K562**
	3	7	1668	944
	20	7	1738	911
	5	7	1935	933
	6	7	1520	895
	13	7	1800	864
	27	1	1816	923
	10	7	1720	758
	18	7	1824	833
	17	7	1825	925
	19	7	1585	656
	12	7	1405	742
	14	7	1587	742
	16	3	1502	723
	8	2	1392	691
	23	2	1465	778
	6	8	1385	777
	11	7	1442	777
	6	8	1482	763
	4	7	1620	781
	4	7	1660	751
	10	7	1585	767
	19	7	1632	826
	21	8	1561	767
	19	8	1585	737
	6	13	1401	783
	15	13	1432	724
	12	7	1412	663
Mean	**12.74**	**6.85**	**1591.81**	**793.85**

Table A11. Raw data, TNFα production by M1 macrophages alone and in co-culture with K562 cells, unstimulated and stimulated with 5 μg/mL SA LTA

n = 3 experiments; n.d. = no data

	TNFα production (pg/mL)			
	M1	**M1 + K562**	**M1 + LTA**	**M1 + LTA + K562**
	7	7	6831	5434
	7	7	6214	5198
	7	7	6526	5490
	7	7	6678	5393
	7	7	6331	5312
	7	7	6148	5552
	7	7	6649	6061
	1	6	6854	5080
	8	13	6427	6283
	7	7	3965	3714
	9	7	3399	4149
	7	7	3727	3590
	7	7	2861	n.d.
	7	7	3220	2339
	5	7	3506	3045
	3	7	4611	1419
	14	7	4933	3028
	12	7	5320	3296
	7	7	6853	6018
	2	7	6334	5402
	1	7	6024	5788
	7	1	5339	4302
	7	7	6048	4752
	4	7	6567	4662
	3	7	7475	4973
	10	1	8520	5232
	10	3	6567	4896
Mean	**6.67**	**6.59**	**5701**	**4631.08**

Table A12. Raw data, TNFα production by M2 macrophages alone and in co-culture with K562 cells, unstimulated and stimulated with 5 µg/mL SA LTA

n = 4 experiments; n.d. = no data

	TNFα production (pg/mL)			
	M2	**M2 + K562**	**M2 + LTA**	**M2 + LTA + K562**
	3	7	3343	2608
	20	7	3371	2373
	5	7	3339	2518
	6	7	3099	2306
	13	7	3141	2379
	27	1	2825	2333
	10	7	3342	2232
	18	7	3744	2400
	17	7	3578	2322
	19	7	2754	1931
	12	7	2852	1939
	14	7	2837	2049
	16	3	2778	1730
	8	2	2713	1575
	23	2	2354	1742
	6	8	2512	1900
	11	7	2690	1896
	6	8	2817	1983
	4	7	3415	2238
	4	7	3231	2289
	10	7	3055	2365
	19	7	3312	2187
	21	8	3135	2182
	19	8	2968	2135
	6	13	3169	2288
	15	13	3212	2169
	12	7	3524	2158
	7	7	2787	1307
	7	7	2744	1320
	7	7	2693	1350
	7	7	2689	1430
	7	7	2903	1450
	7	7	2863	1501
	7	7	2912	1609
	7	7	2939	1684
	7	7	3373	1701
Mean	**11.31**	**6.89**	**3028.14**	**1988.31**