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Modulation of purinergic signalling in macrophages and T cells by *Trichinella spiralis* secreted proteins

Emily Eleftheriou

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Division of Cell and Molecular Biology

Imperial College London

I hereby declare that this thesis is my own work. Information derived from published or unpublished work of others has been acknowledged in the text and appropriately referenced.

Abstract

Extracellular nucleotides are signalling molecules whose purinergic receptor-mediated effects are known to be key regulators of inflammatory and other immune responses, and they are considered archetypal activators of the innate immune system. It has been shown by our group that infective larvae of *Trichinella spiralis* secrete enzymes which catalyze the degradation of extracellular nucleotides, and thus potentially interfere with host cell purinergic receptor signalling. Murine bone marrow-derived macrophages (BMMΦ) of various activation states and CD4+ T cells were used to examine the effects of *T. spiralis* secreted proteins on immune cell purinergic signalling. This study showed, by PCR and pharmacological analyses, that these cell types possess functional purinergic receptors and the sub-types of receptors responsible for the signalling events were identified. It was shown that the ecto-nucleotidases, CD39 and CD73, were functionally expressed on BMMΦ and CD4+ T cells. The signalling events which were triggered following purinergic receptor stimulation were inhibited by *T.spiralis* secreted enzymes due to metabolism of nucleotides to adenosine. It was shown that the action of *T.spiralis* secreted proteins enhanced anti-inflammatory processes, such as IL-10 release and arginase I activity in BMMΦ, while NO, TNF-α and IL-6 release were inhibited. In CD4+ T cells, proliferation and cytokine release were all inhibited by adenosine generation. Pharmacological characterisation of adenosine-induced effects on these cells showed that they were predominantly due to A_2R activation. In addition to the interference with purinergic signalling, *T.spiralis* secreted products were shown to have direct effects on BMMΦ and T cell effector functions and this was shown to be due to the glycan component of the secreted proteins. In BMMΦ, *T.spiralis* glycans appeared to activate C-type lectins, and in particular, the Macrophage Galactose Lectin (MGL). Thus the parasite, via its secreted proteins, appears to interfere with host purinergic signalling as well as having direct effects on immune cells by activation of C-type lectins.

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Abbreviations

β-ME	β-mercaptoethanol
5`NT	5`-nucleotidase
AAMΦ	Alternatively activated macrophages
Ab	Antibody
AC	Adenylyl cyclase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APC	Allophycocyanin
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
BCA	Bichinchonic assay
BLAST	Basic local alignment search tool
BMGY	Buffered glycerol complex medium
BMMY	Buffered methanol complex medium
BMMΦ	Bone marrow-derived macrophages
bp	Base pairs
BSA	Bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAMΦ	Classically activated macrophages
CPM	Counts per minute
cRPMI	complete (supplemented) RPMI 1640 medium
DAG	1,2-diacylglycerol
DC-SIGN	Dendritic cell specific intercellular adhesion molecule-3 grabbing nonintegrin

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	deoxy-nucleotide triphosphate
DP	Dipyridamole
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ES	Excretory/secretory proteins
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GalNAc	N-Acetylgalactosamine
GPCR	G-protein coupled receptor
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HRP	Horse radish peroxidise
IB-MECA	1-Deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol 1,4,5-triphosphate
kDa	Kilo Daltons

LPS	Lipopolysaccharide
MACS	Magnetic activated cell sorting
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor
MIP	Macrophage inhibitory protein
M-MLV	Moloney murine leukaemia virus
MR	Mannose receptor
mRNA	Messenger RNA
MW	Molecular weight
MyD88	Myeloid differentiation primary response gene 88
NDPK	Nucleotide diphosphate kinase
NK	Natural killer cell
NO	Nitric oxide
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.1% v/v Tween 20
PCR	Polymerase chain reaction
PE	Phycoerythrin
PIP_2	Phosphotidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
RAW 264.7	murine macrophage cell line

RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute culture medium
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TCA	Trichloroacetic acid
TEMED	Tetramethyl ethylene diamine
TGF-β	Transforming growth factor-β
Th	T-helper cell subset
TLR	Toll-like receptor
TMB	Tetramethyl benzene
TNF-α	Tumour necrosis factor-α
Tris	Tris (hydroxymethyl) aminomethane
Tween	Polyoxyethylene sorbitan monolaurate
UDP	Uridine diphosphate
YNB	Yeast nitrogen base

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Chapter 1

Introduction

1.1 Purinergic signalling

Extracellular nucleotides are perceived as danger signals and they can act as signalling molecules in host-pathogen interactions (Gallucci and Matzinger, 2001). Under conditions of cell damage, hypoxia, inflammation or mechanical stress, nucleotides can be released either as a result of cell lysis or via plasma membrane channels or transporters (Communi *et al*, 2000; Lazarowski *et al*, 2003). Adenosine 5`-triphosphate (ATP) and adenosine 5`-diphosphate (ADP) released by apoptotic cells also promote clearance of these cells by recruiting phagocytes (Elliot *et al*, 2009). Furthermore, these nucleotides, which can reach concentrations higher than 50μM locally, control inflammatory responses, release of tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) by macrophages and are involved in hemostasis and platelet aggregation (Trautmann, 2009). On the other hand, the breakdown product of ATP and ADP, adenosine, has opposite effects that suppress immune responses and is considered an endogenous regulator of innate immunity (Hasko and Cronstein, 2004). The effects triggered by nucleotides once in the extracellular milieu are mediated by selective plasma membrane receptors, namely, the purinergic receptors. Purinergic receptors are known to be present on all circulating hematopoietic cells, hence, nucleotides can potentially act as regulators of inflammation (Hasko *et al*, 2008). In addition to purinergic receptors, hematopoietic and epithelial cells also express surface proteins that can degrade extracellular nucleotides, called ecto-nucleotidases. These ecto-nucleotidases can alter the type and local concentration of nucleotides and act together with purinergic receptors in controlling responses triggered by nucleotides (Hasko *et al*, 2007). Specifically, adenosine is released in the extracellular milieu by neutrophils and endothelial cells at sites of inflammation and infection and is important in achieving a balance in purinergic receptor stimulation, as it triggers opposite responses to adenine nucleotides. The concentration of adenosine at its receptors is regulated by extracellular and intracellular breakdown of adenine

nucleotides catalyzed by nucleotidases as well as by release and re-uptake from cells via nucleoside transporters (summarised in Diagram 1). There are four equilibrative nucleoside transporters (ENT1–4) and three concentrative nucleoside transporters (CNT1–3) that are involved in recycling adenosine and the ENTs are inhibited by compounds such as dipyridamole, which is used in this study to prevent re-uptake of adenosine and prolong P1 receptor activation (Junger, 2011). In addition to adenosine removal by these transporters, adenosine-induced responses can be eliminated by catabolism of adenosine to inosine by adenosine deaminase (Bours *et al*, 2006).

Ecto-nucleotidases

Ecto-enzymes that are capable of catalyzing nucleotide hydrolysis are found on cell surfaces as well as in the interstitial medium following proteolytic cleavage. There are four known families of ecto-nucleotidases with similar expression patterns and substrate specificity: the ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) family, the ectonucleotide pyrophosphatase/phosphodiesterase (E-NPP) family, the alkaline phosphatases, and the ecto-5′-nucleotidase (CD73) (Bours *et al*, 2006). The E-NTPDases have apyrase activity catalysing the hydrolysis of nucleotide triphosphates and diphosphates such as ATP and ADP to ADP and AMP (adenosine 5`-monphosphate), respectively. CD39, which is a member of this family of enzymes, is expressed on vascular endothelial cells and is important in degrading ATP and ADP released from platelets. The E-NPP family includes enzymes with catalyze the hydrolysis of cAMP (cyclic AMP) to AMP and ATP to AMP and pyrophosphate. The GPI-anchored alkaline phosphatases release inorganic phosphate from nucleotide tri-, di- and monophosphates while the ecto-5′nucleotidase, CD73, specifically catalyzes the hydrolysis of AMP to adenosine (Communi *et al*, 2000). The accumulation of adenosine resulting from the above enzymes is regulated by the balance between activation of adenosine deaminase (ADA) and adenosine kinase. Ecto-ADA is

responsible for catalysing adenosine deamination to inosine which is irreversible due to further degradation to the stable end product, uric acid. Adenosine kinase is an intracellular enzyme which catalyzes the phosphorylation of adenosine to AMP, effectively removing adenosine and producing a concentration gradient which regulates entry of adenosine into cells (Kowaluk and Jarvis, 2000).

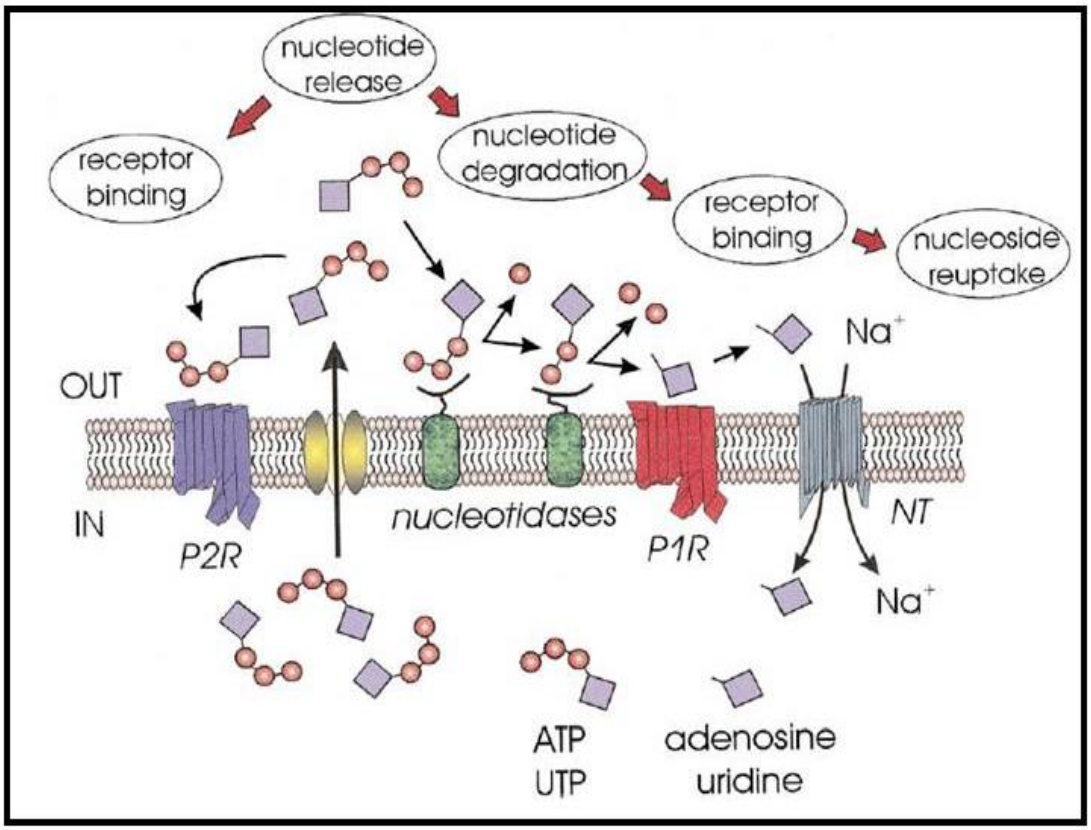


Diagram 1: The fate of extracellular nucleotides. Nucleotides can bind purinergic receptors to trigger intracellular signal transduction cascades. They can be metabolized by ecto-enzymes to adenosine which can be taken up by transporters on immune cell surfaces. (Diagram taken from Roman and Fitz, 1999).

Purinergic receptors

Purinergic signalling was first described in terms of the adenosine-mediated regulation of cell function following activation of specific surface receptors (Sattin and Rall, 1970). Since then purinergic receptors with different substrate specificities and intracellular signalling properties have been characterised (Di Virgilio *et al*, 2001). Purinergic receptor-mediated responses depend on receptor expression and density which differs according to cell type and activation state. For example, expression of certain purinergic receptor subtypes has been shown to be regulated by lipopolysaccharide (LPS), TNF-α and IFNγ (Xaus *et al*, 1999; Khoa *et al*, 2001; Nemeth *et al*, 2003).

Purinergic receptors belong to either the P1 or the P2 receptor families. The P1R family consists of four seven transmembrane-spanning G-protein coupled receptors, A_1, A_{2A}, A_{2B}, A_3, which respond to adenosine with differing affinity, and all except $A_{2B,}$ also respond to inosine. This means that, under conditions of metabolic stress when extracellular adenosine is abundant, the low affinity A_{2B} receptors are activated while the high affinity A_1, A_{2A}, and A_3 receptors are only activated when adenosine levels are low in the extracellular milieu, allowing cells to monitor extracellular adenosine levels (Hasko *et al*, 2007). These receptors differ in the type and efficacy of coupling to G-proteins thus the intracellular signalling pathways they trigger depend on activatory G-protein (Gq) or inhibitory G-protein (Gi) coupling (Fredholm *et al*, 2001). Gq coupling results in phospholipase C-β (PLC-β) activation with the concomitant generation of second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), that ultimately cause release of calcium ions from intracellular stores leading to activation of protein kinase C (PKC). Gi coupling results in inhibition of adenylyl cyclase and a subsequent decrease in cyclic AMP (cAMP). Conversely, G_S coupling involves activation of adenylyl cyclase leading to increase in cAMP levels (Bours *et al*, 2006; Communi *et al*, 2000). The A_1 receptors signal

through activation of PLC with G protein βγ-subunits, which also activate K+ channels, and negatively couple to adenylyl cyclase through their G α-subunits. The low affinity A_3 receptors couple to Gi and their activation results in signalling similar to that triggered by the A_1 receptors. Both A_{2A} and A_{2B} receptors are positively coupled to adenylyl cyclase via Gs which leads to cAMP formation, while A_{2B} receptor activation has been shown to couple to PLC via Gq resulting in mobilization of intracellular calcium (Linden, 2001; Sitkovsky *et al*, 2008). A summary of the above is presented in Table 1.1A. The mitogen activated protein kinases (MAPK) are activated following G-protein coupling of purinergic receptors and consist of the extracellular regulated kinases (ERK) and the stress activated protein kinases (SAPK), such as the p38 and the jun-N-terminal kinase (JNK). Coupling to Gq leads to activation of MAPK via MEK and Ras while A_{2A} receptor activation was also shown to increase MAPK activity. ERK1/2 was shown to be activated in a time- and dose-dependent manner by A_1 receptor coupling to Gi, whereas the A_{2B} receptor has also been shown to activate ERK1/2 in addition to p38 and JNK (Fredholm *et al*, 2000).

Cells usually express more than one adenosine receptor subtype, therefore their responsiveness to extracellular adenosine may not be pharmacologically typical of one specific receptor subtype. This is complicated further by the fact that, G protein-coupled receptors can dimerise to form oligomers, which, along with cross-talk of intracellular signalling pathways, can lead to diverse purinergic signalling outcomes (Linden, 2001). In order to control the type of P1 receptor activated as well as to prevent sustained activation, the presence of these receptors on the cell surface is down-modulated following ligation with their respective agonists. Specifically, it has been reported for A_{2A}, A_{2B} and A_3 receptors that they are desensitised by G protein-coupled receptor kinase (GRK) – mediated phosphorylation which targets them for internalisation via

lipid rafts or clathrin-coated pits to dampen GPCR signalling (Klaasse *et al*, 2008; Zezula and Freissmuth, 2008).

The P2 receptor family is subcategorised into the metabotropic P2Y and the ionotropic P2X, as a consequence of differences in molecular structure and signal transduction mechanisms (Di Virgilio *et al*, 2001; Burnstock *et al*, 2009). The P2Y family consists of eight receptors, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$, that have subtype-specific responsiveness to both purines (ATP, ADP) and pyrimidines (UTP, UDP) and these are also G-protein coupled. P2Y1, P2Y2, P2Y4 and P2Y6 receptors couple to Gq proteins signalling via increases in IP3 and cytosolic calcium while activation of the P2Y11 leads to elevation of both cAMP and IP3. The P2Y13 receptor, which routinely couples to Gi, can also couple to Gs at high ADP concentrations (Linden, 2001). P2Y14 is also a Gi-coupled receptor which is activated by UDP-glucose. (Summarised in Table 1.1B). The P2X family, comprised of seven members $P2X_{1-7}$, differs from the two families described so far in that it consists of ligand-gated ion channels that respond mainly to extracellular ATP and mediate fast permeability changes in monovalent and divalent cations such as Na^+, K^+ and Ca^{2+} (Ralevic and Burnstock, 1998). The P2X receptors will not be discussed further because this study focused on P1 and P2Y receptor activation due to their coupling to G-proteins that produce responses that could be measured *in vitro* by techniques used in our lab.

Receptor	Ligand	G-protein coupling
A_1	**Adenosine, Inosine**	**Gi**
A_{2A}	**Adenosine, Inosine**	**G_S**
A_{2B}	**Adenosine**	**G_S, Gq**
A_3	**Adenosine, Inosine**	**Gi, Gq**

Table 1.1A: P1 receptors, their natural agonists and their coupling to G-proteins.

Receptor	Ligand	G-protein coupling
$P2Y_1$	**ADP**	**Gq**
$P2Y_2$	**ATP, UTP**	**Gq**
$P2Y_4$	**UTP**	**Gq**
$P2Y_6$	**UDP**	**Gq**
$P2Y_{11}$	**ATP**	**Gq, G_S**
$P2Y_{12}$	**ADP**	**Gi**
$P2Y_{13}$	**ADP**	**Gi**
$P2Y_{14}$	**UDP-glucose**	**Gi**

Table 1.1B: P2Y receptors, their natural agonists and their coupling to G-proteins.

Purinergic signalling and immune cells

The purinergic receptor repertoire of immune cells varies according to their maturation and activation state and, as a result, the function of these cells in immunity is tightly regulated by purinergic signalling (Di Virgilio *et al*, 2001; Junger, 2011). For example, extracellular ATP is known to control chemotactic responses in dendritic cells, macrophages and neutrophils while macrophages exposed to ATP tend to exhibit a pro-inflammatory cytokine profile (Bours *et al*, 2006; Junger, 2011). The presence of ecto-nucleotidases (such as CD39 and CD73) on immune cells ensures that ATP-induced responses are not long-lived as they catalyze the hydrolysis of ATP to adenosine and exposure of these cells to adenosine leads to anti-inflammatory responses. Interestingly, it has been observed that the activity of CD39 and CD73 is up-regulated during macrophage differentiation thus these cells become more specialized in forming adenosine (Hasko *et al*, 2007). Activation of A_{2A} receptors by adenosine has been shown to inhibit the secretion of TNF-α and IL-12 and enhance IL-10 secretion by macrophages which also adopt an angiogenic phenotype (Hasko *et al*, 1996; Pinhal-Enfield *et al*, 2003). In neutrophils and leukocytes, activation of A_{2A} receptors leads to inhibition of chemokine and TNF-α release as well as degranulation, production of oxygen free radicals and transmigration across vascular endothelia (Hasko *et al*, 1996). An unusual role of P1 receptors has been shown whereby DC-expressed P1R act to anchor adenosine deaminase which binds CD26 on T cells providing an additional co-stimulatory signal, leading to Th1 expansion and pro-inflammatory cytokine production (Pacheco *et al*, 2005).

1.2 Macrophages

Immune responses require a tightly regulated balance of pro- and anti-inflammatory mediators released from immune cells. Macrophages are important immune effector cells which are involved in both innate and adaptive immune responses providing a link between the two and, in doing so, maintaining immune homeostasis. Macrophage populations arise from myeloid progenitors in the bone marrow or differentiate from monocytes in the bloodstream in response to immunoglobulins, cytokines and chemokines. Macrophages differentiated from murine bone marrow using macrophage colony-stimulating factor (M-CSF) are a good culture model as cells are generated in a state that has not been previously activated and can thus respond to any cytokine stimulus (Valledor *et al*, 1998). The effector functions of activated macrophages range from phagocytosis to release of pro-inflammatory mediators that promote killing, and anti-inflammatory mediators that enable repair. For example, interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) are important cytokines involved in dampening vigorous inflammatory responses triggered by the release of nitric oxide (NO) and TNF-α.

Macrophages and Pattern Recognition Receptors (PRRs)

Antigen presenting cells (APCs) perform their function in innate immunity by recognising antigens, and in particular pathogen associated molecular patterns (PAMPs), by means of their expression of pattern recognition receptors (PRRs). The most common PRRs are the Toll-like receptors (TLRs) which are important in handling microbial infections. TLRs are members of the Toll/IL-1 receptor (TIR) family, contain cytoplasmic TIR domains, and are capable of recognizing structurally diverse microbial ligands (Krishnan *et al*, 2007). TLR4 is the most studied because of its specificity for binding LPS, a major component of gram-negative bacterial

walls. Ligation of TLR4 by LPS is preceded by engagement to CD14 which enables LPS to be brought into direct contact with TLR4 and MD-2 (Beutler *et al*, 2003). CD14, which is expressed on myeloid cells, acts as an accessory molecule to TLR2 and TLR4 as is does not contain a transmembrane domain to link it with intracellular signal transduction (Taylor *et al*, 2005). Signalling via TLRs, which is dependent on other surface molecules such as CD14/MD-2 for ligand recognition, leads to cytokine production against the foreign antigen as well as inducing maturation of immune cells. Downstream signalling from TLR activation requires recruitment of adaptor proteins, the major one being MyD88 (Myeloid differentiation primary response gene 88), which is recruited by all TLRs except TLR3. Other adaptor proteins include MAL (MyD88 adapter like), TRIF (TIR domain-containing adapter protein inducing IFN-β) and TRAM (TRIF-related adapter molecule) which are recruited by different TLRs leading to variations in signalling specificity. Following LPS ligation and MyD88 recruitment, TRAM appears to act as an adaptor for TLR4 and TRIF. IRAKs (MyD88/interleukin-l receptor associated kinase) are next in the signalling cascade downstream of MyD88 which, when phosphorylated, dissociate from MyD88 resulting in activation of TRAF6 (TNF receptor associated factor), which is a member of the E3 ubiquitin ligase family (Chen, 2005). TRAF6 then activates the canonical NF-κB pathway which is where all TLR pathways converge. In the case of TLR3 and TLR4, downstream signalling can follow a MyD88-independent route that involves activation of IRF3. MyD88-independent signalling events downstream of TLR3 require TRIF while TLR4 recruits TRIF/TRAM in order to induce IRF3-dependent type I interferon production. In general, TLR engagement leads to signalling involving IκB kinase (IKK), MAPK (JNK/p38) and PI3K/Akt pathways, which ultimately lead to AP-1 and NF-κB activation and production of inflammatory cytokines and chemokines (Kawai and Akira, 2006).

TLRs are known to collaborate with other PRRs in PAMP recognition improving their affinity and effectively coupling innate and adaptive immune responses. Specifically, Dectin-1 which has a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM)-like sequence, cooperates with TLR2 in production of pro-inflammatory cytokines following recognition of *β*-glucan particles. Dectin-1 is a phagocytic receptor that also recognizes endogenous ligands on T cells and can act as a co-stimulatory molecule for T cell proliferation (Taylor *et al*, 2005). CLRs (C-type lectin receptors) are another group of PRRs that can collaborate with TLRs and they are known to act as antigen uptake receptors. CLRs bind a specific glycan repertoire via their carbohydrate recognition domains (CRDs) whose structural integrity is dependent on the presence of Ca^{2+} ions. Their binding properties are also influenced by positioning of carbohydrates on their ligands as well as by the proximity of CRDs often achieved by oligomerisation (Taylor and Drickamer, 2003). CLRs have roles in cell-cell or cell-matrix adhesion and glycoprotein turnover, while their function in immature DCs is antigen capture for presentation by MHC class I and II molecules to induce CD4+ and CD8+ T-cell responses. Therefore, they are important in pathogen recognition and promote cellular interactions that are required for pathogen internalisation and elimination. Examples of these CLRs capable of glycosylated antigen internalization are DC-SIGN, MGL, Dectin-1 and Mincle (Van Vliet, Garcia-Vallejo *et al*, 2008). The macrophage galactose lectin, MGL, (CD301) exists as a homo-oligomer and is found on myeloid APCs, especially on tolerogenic or alternatively activated subsets. Humans are known to express one MGL while mice express two closely related MGLs, MGL1 and MGL2, with unique carbohydrate specificities for Lewis X sugars as well as galactose and *N*-acetylgalactosamine (GalNAc). Human MGL specifically recognizes GalNAc sugars thus bearing more similarity to mMGL2, while mMGL1 binds Lewis X structures similar to rMGL (Sakakura *et al*, 2008; Singh *et al*, 2009). MGL shares a common internalisation motif to the

asialoglycoprotein receptor (ASGP-R) and DC-SIGN and can endocytose antigens which are directed to the endosomal-lysomal pathway for presentation via MHC II. No signalling properties have been attributed to MGL so far. MGL functions in controlling granulation of murine tissues as well as in distinguishing between healthy and tumour tissue (Van Vliet *et al*, 2008). In addition, MGL has been recently shown to bind terminal GalNAc moieties on T cell-expressed CD45 which affects T cell proliferation (Van Vliet *et al*, 2006). Finally, MGL1 and MGL2 were shown to be expressed in alveolar macrophages in an IL-4-dependent manner during asthma, suggesting that MGL can act as a marker of alternative activation (Raes *et al*, 2005).

The link between alternative activation of macrophages and lectins is also supported by the fact that, IL-4 stimulates a galectin-3 feedback loop leading to sustained PI3K activation via CD98 and is a requirement for alternative activation of macrophages. Galectin-3 is a carbohydrate-binding lectin which binds macrophage CD98 that triggers PI3K pathway activation. Its secretion is enhanced by IL-4 which provides a positive feedback loop resulting in sustained PI3K activation. Inhibition of galectin-3 suppresses the IL4-induced alternative activation in macrophages without affecting IFN-γ/LPS-induced classical activation or IL-10-induced deactivation (Mackinnon *et al*, 2008). Similarly, another PRR influenced by IL-4 stimulation is the macrophage mannose receptor. The MR (CD206) is an endocytic receptor involved in clearance of endogenous glycoproteins that recognizes mannose, fucose, and N-acetylglucosamine (Mukhopadhyay *et al*, 2009). Other PRRs such as the collectins and selectins are involved in both innate and adaptive immune responses. The collectins are soluble proteins, an example of which is MBP (mannose binding protein), are involved in the innate response by fixation of complement. The selectins are involved in adaptive immune responses by mediating

transient interactions between leukocytes and the endothelium for leukocyte trafficking (Weis *et al*, 1998).

Macrophages are known to express all cytokine receptors and PRRs in all their activation states allowing them to adjust to a changing local environment, as polarised macrophages can respond to both classical and alternative activation stimuli (Gratchev *et al*, 2006). For example, in parasite infections macrophages are able to respond to signals such as cytokines from other immune cells and PAMPs, which are recognized directly by macrophage PRRs. In fact, MGL as well as scavenger receptor and Dectin-1 are up-regulated in parasite infections.

Macrophage activation

The activation state of macrophages is important in determining the outcome of an inflammatory response. Distinct macrophage subsets with different expression profiles have been characterised in the presence of foreign antigens and T cells (Noel *et al*, 2004). Macrophages were originally considered as pro-inflammatory effector cells involved in cytotoxicity induced by T helper 1(Th1) cytokines [interferon-γ (IFN-γ) and TNF-a]. However, Stein and colleagues found that the T helper 2 (Th2) cytokine interleukin-4 (IL-4) activated murine macrophages in an alternative mode characterised by mannose receptor (MR) expression, suggesting the existence of an alternative activation state (Stein *et al*, 1992). Furthermore, Doyle and colleagues reported that the human macrophage MR was up-regulated by IL-4 which enhanced macrophage fusion, while interleukin-13 (IL-13), another Th2 cytokine, was found to produce a response that was between IFN-γ activation and IL-10 deactivation (Doyle *et al*, 1994). Deactivation of certain cell types, including macrophages, by IL-10 has been reported to involve inhibition of pro-inflammatory responses by reducing inflammatory cytokine production such as interleukin-6 (IL-6) and TNF-α

(Bogdan *et al*, 1991; Moore *et al*, 1993). In addition, enhancement of IL-10 production was shown to promote the anti-inflammatory phenotype of macrophages following expression and activation of AMP-activated kinases (Sag *et al*, 2008).

The most common macrophage activation phenotype, that arises in the presence of microbial stimuli, such as lipopolysaccharide (LPS), or IFN-γ, is known as the classically activated macrophage (CAMΦ) phenotype, and is involved in pro-inflammatory reactions by production of TNF-α, interleukin-1β and interleukin-12 and promotion of Th1 responses. CAMΦ are inhibited by Th2 cytokines and thus the macrophages generated in the presence of IL-4/IL-13 are termed alternatively activated macrophages (AAMΦ). Glucocorticoids have also been shown to induce an anti-inflammatory phenotype in macrophages that is very similar to IL-4 - stimulated AAMΦ (Edwards *et al*, 2006; Martinez *et al*, 2008). However, macrophage heterogeneity under different *in vivo* settings results in inaccurate classification of macrophage activation phenotypes (Naito *et al*, 1996). Despite the fact that certain macrophage features are shared by more than one phenotype, they are generally considered as either wound healing, regulatory or acting in host defence (Mosser and Edwards, 2008). In general, CAMΦ are aimed at dealing with threats by exhibiting aggressive action, which may lead to tissue destruction, whereas AAMΦ are responsible for tissue restoration. This AAMΦ characteristic is a result of preferential use of arginase I for processing L-arginine instead of inducible nitric oxide synthase (iNOS). This is the main distinguishing feature between the two modes of activation. These differences are summarised in Diagram 2.

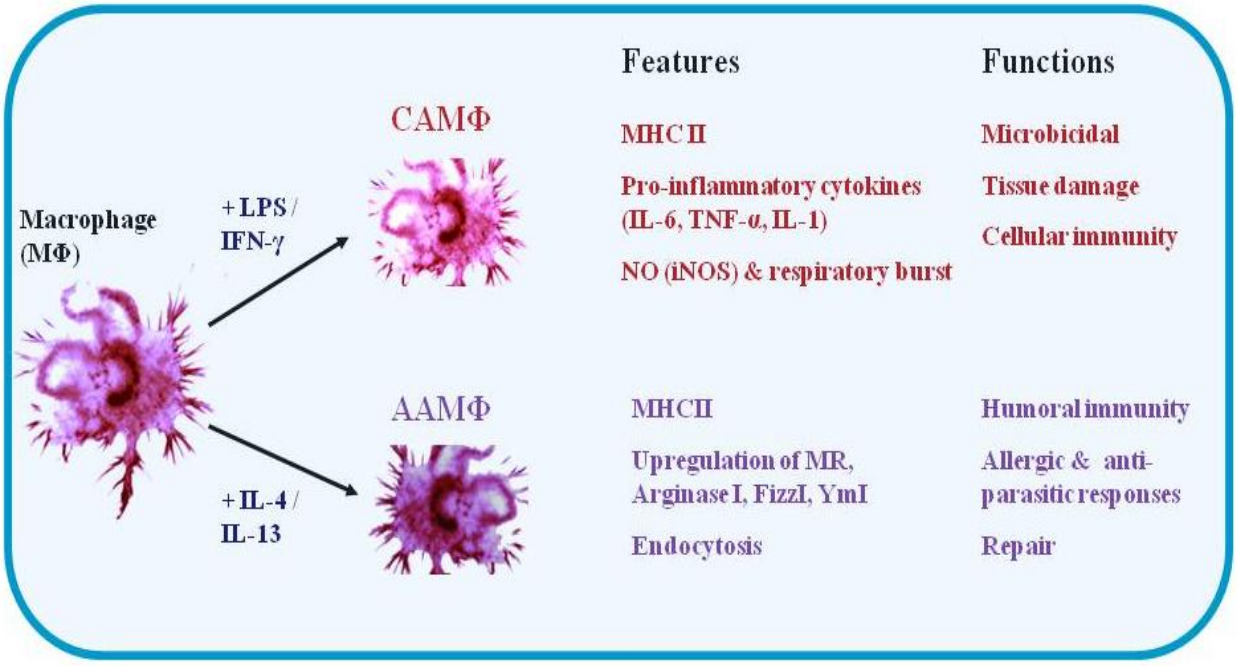


Diagram 2: Summary of macrophage activation. Microbial triggers (such as LPS) and Th1 cytokines (such as IFN-γ) lead to classical activation (CAMΦ) while Th2 cytokines (IL-4/IL-13) alternatively activate macrophages (AAMΦ). CAMΦ are considered pro-inflammatory while AAMΦ are anti-inflammatory. (iNOS = inducible nitric oxide synthase).

The balance between the activity of arginase I and iNOS is important in determining the state of macrophage activation and, specifically, arginase I induction is accompanied by suppression of iNOS (Corraliza *et al*, 1995). There are two known isoforms of arginase: arginase I is cytosolic and arginase II is mitochondrial. Both these enzymes convert L-arginine to L-ornithine which is essential for the production of polyamines for cell growth, and prolines which are major components of collagen (Hesse *et al*, 2001).

The process of L-arginine metabolism (summarised in Diagram 3) is regulated by its transporter, cationic amino acid transporter (CAT2), and macrophage activation results in a higher rate of L-arginine transport (Yeramian *et al*, 2006). In addition to controlling macrophage activation directly, arginase I, and the depletion of L-arginine, also regulates proliferation of T cells indirectly by interfering with formation of the T cell receptor (TCR) complex (Bronte and Zanovello, 2005).

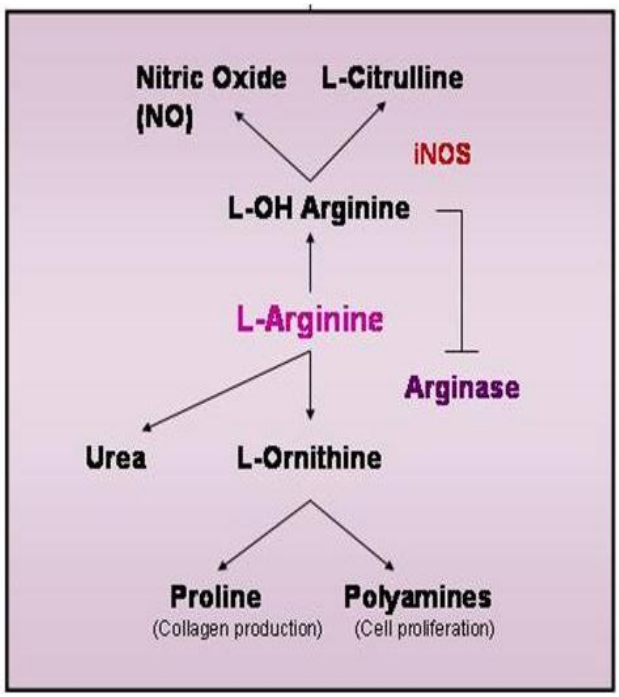


Diagram 3: Schematic representation of L-arginine metabolism. iNOS and arginase I are responsible for L-arginine metabolism. The products of the former include the pro-inflammatory nitric oxide (NO) while the latter provides products used in proliferation processes.

Classically activated macrophages (CAMΦ)

Classically activated macrophages have a major role in pro-inflammatory responses and are involved in the resolution of intracellular infections. This is achieved by the synthesis and release of cytotoxic reactive oxygen intermediates (ROI) and nitric oxide (NO) (Martinez *et al*, 2008). In addition, CAMΦ produce high levels of pro-inflammatory cytokines such as IL-6, TNF-α and IL-1 which also aid in eliminating pathogens by recruitment of additional effector cells. Furthermore, CAMΦ have increased antigen presenting capacity, as a result of the high levels of major histocompatibility complex II (MHCII) and CD86 they express, and this points to an important role in the establishment of adaptive immunity (Noel *et al*, 2004).

Classical activation is initiated during cell-mediated immune responses by IFN-γ produced by natural killer (NK) cells, responding to stress and infections, and then by Th1 cells. In addition, LPS-derived from the cell walls of Gram-negative bacteria also triggers classical activation following ligation of toll-like receptors (TLR) on macrophages (Mantovani *et al*, 2004). TLR ligation acts via MyD88 to induce TNF-α expression which provides sustained autocrine activation of CAMΦ as a result of cooperation with IFN-γ. In fact, lack of IFN-γ expression leads to increased susceptibility to various bacterial, protozoal and viral infections. Furthermore, certain pathogens can interfere with macrophage activation by preventing IFN-γ signalling (Mosser and Edwards, 2008). Activation of the IFN-γ receptor leads to expression of target genes via signal transducer and activator of transcription (STAT) molecules, while TLR and TNF receptor ligation activates nuclear factor-κB (NF-κB) and mitogen activated protein kinases (MAPKs). Gene expression downstream of these signal transducers involves, among others, the production of IL-1, IL-6 and IL-23 by CAMΦ which aids in the development of Th17 cells. In turn these cells produce IL-17, a cytokine that has been implicated in the development of

inflammatory autoimmune pathologies by recruitment of polymorphonuclear leukocytes (PMN) (O'Shea and Murray, 2008). Despite the beneficial role of CAMΦ in fighting infection, excessive responses are generally damaging to the host and it is at this stage that macrophages with remodelling and repair characteristics must be recruited to dampen CAMΦ effects.

Alternatively activated macrophages (AAMΦ)

Macrophages become alternatively activated following stimulation with IL-4 or IL-13 and are characterised by anti-inflammatory properties, although they are still able to respond to microbes by cytokine secretion (Varin *et al*, 2010). AAMΦ are distinguished from CAMΦ by the expression of certain markers. The MR, arginase I, Ym1 and Fizz1 are established markers of alternative activation although some discrepancies have been reported (Gordon, 2002). For example, arginase I expression has also been reported following activation of macrophages with TLR ligands (Huber *et al*, 2010). Human alternatively activated myeloid cells were not found to express arginase I and Ym1 (Raes *et al*, 2005). Finally, the MR is not always up-regulated in AAMΦ and the fact that this marker is also expressed by un-stimulated differentiating macrophages may suggest that the distinguishing feature of AAMΦ is an up-regulation of MR expression levels (Rouleux-Bonnin *et al*, 1998). The roles of these markers in AAMΦ are not clear but certain observations have been reported that implicate these markers in AAMΦ effector functions. Up-regulation of MR expression and activity by IL-4 has been shown to enhance macrophage ability to fuse (McNally *et al*, 1996). Ym1 is an inducible lectin synthesised and secreted by activated macrophages, which is homologous to mammalian chitinases. However, it has no known chitinase activity as its natural substrates are glucosamine (GlcN) oligomers and heparin (Chang *et al*, 2001). Fizz1 (Found in the inflammatory zone) is also known as RELM-α as it is a resistin-like secreted protein and it is up-regulated during allergic reactions and pulmonary inflammation in epithelial and alveolar cells (Raes *et al*, 2002). Fizz1 is an anti-

inflammatory molecule and its expression by AAMΦ in the lung has been shown to reduce pathogenesis resulting from type 2 inflammation (Nair *et al*, 2009).

Expression of markers of alternative activation is activated at the molecular level as a result of STAT6 and IRS-1 signalling recruited following IL-4Rα activation (Nelms *et al*, 1999). The IL-4Rα chain is shared by IL-4 and IL-13 mediated signalling as it is a major component of functional IL-4 and IL-13 receptor heterodimers (Zurawski *et al*, 1993). Association of the IL-4Rα chain with the common γ chain forms an IL-4R or type I receptor, while association with the IL-13Rα1 binding chain forms a type II, IL-13 receptor. Despite the fact that both IL-4Rs and IL-13Rs share a common chain, downstream signalling potencies are not the same suggesting that extracellular receptor interactions may vary (LaPorte *et al*, 2008). The IL-4R is a hematopoietic receptor that is associated with kinases, such as Src kinases, that allow signal transduction via STAT6 leading to activation of phosphatidylinositol 3-kinase (PI3K) and Ras-MAPK signalling (Varin and Gordon, 2009). IL-4Rs are also known to activate IRS2 for induction of target gene expression in macrophages (Heller *et al*, 2008).

IL-14 and IL-13 are known as Th2 cytokines and are produced by CD4+ T cells, basophils and mast cells. They activate and maintain Th2 responses by stimulating the production of IL-10 and IL-1R antagonist and inhibiting the expression of IL-1, TNF-a, IL-6, IL-12, macrophage inflammatory protein-1a (MIP-1a) thus providing protection from excessive inflammation (Fenton *et al*, 1992). In addition, they suppress NO production and increase scavenger receptor and MR for clearance by endocytosis while AAMΦ-derived TGF-β induces IL-2 production from T helper cells (Goerdt and Orfanos, 1999; Varin and Gordon, 2009). The specificity of these two Th2 cytokines to induce alternative activation is emphasised by the fact that, although IL-10 is also a type 2 cytokine, only IL-4 and IL-13 were found to induce arginase

I activity (Herbert *et al*, 2004). Furthermore, arginase I expression is down-regulated by IFN-γ and TNF-α. Recently, Huber and colleagues have reported that PD-L2 (Programmed Death-1 ligand 2) is up-regulated in AAMΦ and the authors suggest that it may be a more specific marker of alternative activation because of its STAT6-dependent expression (Huber *et al*, 2010). PPARγ (peroxisome proliferator activated receptor) was also shown to be induced in IL-4 stimulated macrophages, and is considered important in AAMΦ maturation and maintenance, by negative regulation of LPS and IFN-γ induced gene expression and enhancement of arginase I expression and activity (Odegaard *et al*, 2007).

Naturally occurring AAMΦ are found in the lung and the placenta where their role is to prevent unnecessary immune reactions, such as allergic reactions. In disease, AAMΦ are generally associated with the repair phase of acute inflammation or chronic inflammation such as rheumatoid arthritis (Martinez *et al*, 2009). Furthermore, AAMΦ down-regulate inflammation, cause angiogenesis and eliminate tissue debris which are important for restoration of damaged tissue (Goerdt and Orfanos, 1999). The recruitment of AAMΦ to wounds is critical for healing as they have enhanced expression of fibronectin, which is an extracellular matrix (ECM) glycoprotein, as well as collagen which is a product of their increased arginase I activity (Lucas, 2010). Excessive arginase-induced deposition of collagen has been suggested to promote fibrosis, however, Pesce and colleagues have shown that arginase I producing macrophages are beneficial for suppression of Th2-driven fibrosis (Pesce *et al*, 2009). However, the fact that wound macrophages also express TNF-α and IL-6 along with the fact that IL-4/IL-13 have not been found in wounds, has led to the suggestion that these macrophages are not purely AAMΦ (Daley *et al*, 2010). Alternatively, this may be due to the fact that the role of macrophages in wound healing is not continuous and that they are recruited at different stages to perform specialised

functions. AAMΦ have a protective role in Crohn's disease and inflammatory bowel disease (IBD), which are due to an excessive Th1 response, and Fizz1 and arginase I have been shown to be up-regulated when colon inflammation is reduced. In allergic asthma, the presence of AAMΦ, confirmed by detection of elevated levels of Fizz1 and Ym1, is not beneficial as IL-4 and IL-13 are associated with increased severity of this condition (Locksley, 2010). Tumour associated macrophages (TAMs) which promote tumour progression, have also been described as AAMΦ which inhibit MyD88-dependent TNF-α and NO production and express IL-10, Dectin-1, MGL1, TGF-β. However, they also express IFN-γ-inducible chemokines and there is no clear connection between IL-4/IL-13 and tumour progression, which may suggest that the phenotype of these macrophages is regulated by a variety of cytokines (Martinez *et al*, 2008).

Macrophages in nematode infections

The roles of macrophages in helminth infections are multifaceted owing to their involvement in many stages during the course of infection. Despite the fact that macrophages are typically associated with Th2-inducing infections, they can adopt different activation phenotypes in immune responses due to their versatile natureas. For example, nematode-elicited macrophages (NeMΦ) can be 're-programmed' to respond to Th1 stimuli (Mylonas *et al*, 2009; Varin *et al*, 2010). Functional modulation of macrophages as well as APCs is thought to be a fundamental event involved in establishing immune regulation by parasites. In general, helminths are known to induce Th2 responses characterised by increased numbers of CD4+ Th2 cells, eosinophils, mast cells and basophils and the release of Th2 cytokines such as IL-4, IL-5, IL-9, IL-10, IL-13 (Loke *et al*, 2007). Consequently, the phenotype of NeMΦ most closely resembles an alternative activation phenotype with characteristic elevation of Fizz1 and Ym1 expression and suppression of inflammatory cytokine production (Nair *et al*, 2003; Nair *et al*, 2005; Kreider *et al*, 2007).

F. hepatica secreted products were found to up-regulate alternative activation markers in macrophages both in *in vitro* and *in vivo* studies by inducing an IL-4-dependent Th2-type response (Donnelly *et al*, 2005; Flynn *et al*, 2007). In *N.brasiliensis* infection, all macrophages present after the first week of infection were AAMΦ aimed at reducing chronic pulmonary inflammation by Fizz1 and Ym1 expression, as they did not have a role in the expulsion of this parasite (Herbert *et al*, 2004; Marsland *et al*, 2008). AAMΦs, which are recruited to sites of infection in a variety of helminth infections (*Schistosoma mansoni, Nippostrongylus brasiliensis, Trichinella spiralis, Fasciola hepatica* and filarial parasites), orchestrate immune responses that lead to parasite expulsion, at the same time preventing any associated immune pathologies (Kreider *et al*, 2007). Pathology that may be associated with filarial infections is determined by the phenotype of macrophages as secreted products induce Th2 cytokine production and AAMΦ recruitment, while dying worms recruit CAMΦ as a result of endosymbiotic bacteria being released (Allen and Loke, 2001). Host survival is enhanced by AAMΦ as these cells protect the host by down-regulating the inflammation induced when parasite eggs are deposited, killing or promoting expulsion of adult worms, as well as repairing damaged tissue resulting from these processes. Additionally, AAMΦ may directly target chitin expressed by helminths as Fizz1 and Ym1 have been detected in sites of *T.cruzi* and *H.polygyrus* infection (Anthony *et al*, 2007). *T.spiralis* infection has also been shown to recruit macrophages with an alternative activation phenotype characterised by up-regulated Ym1 expression and secretion (Chang *et al*, 2001). Furthermore, IL-4/IL-13 was found to be necessary for expulsion of *T.spiralis* despite the fact that in the absence of IL-4R an effector response was still generated (Michels *et al*, 2009). This response is dependent on CD4+ T cells and IL-4 to generate AAMΦ which are involved in parasite clearance through arginase I activation.

The mode of action used by helminths to induce of alternative activation of macrophages is both direct modulation of macrophages as well as indirect through activation of other immune cells (Perrigoue *et al*, 2008). Specifically, the intestinal epithelium acts as a sensor of infection by gut parasites to which cells are recruited that can activate APCs indirectly (Artis and Grencis, 2008). This is achieved by induction of regulatory T cell subsets which are either recruited directly or activated by APCs, such as dendritic cells. Furthermore, helminths are known to directly interfere with immune cell recruitment and proliferation by production of molecules like MIF (macrophage inhibitory factor) which prevents macrophage accumulation (Maizels *et al*, 2004). Helminth secretory products contain glycan PAMPs that can be recognized by PRRs such as TLRs and C-type lectins (CTLs) on host APCs leading to innate immune responses. For example, LNFPIII (Lacto- *N*-fucopentaose III) which is a Lewis X sugar derived from *S.mansoni*, is recognized by TLR4 as is the ES-62 protein from *A.viteae* (Goodridge *et al*, 2005; Harn *et al*, 2009). Activation of TLR4 by these parasite products did not resemble LPS-induced signalling due to the release of a mixture of pro- and anti- inflammatory cytokines. The β-linked GalNAc in LDN (LacdiNAc) and Lewis X motifs of *S.mansoni* glycans were shown to bind the C-type lectin DC-SIGN which also recognizes *M.tuberculosis*, *L. mexicana* and *H. pylori* epitopes (Hokke and Yazdanbakhsh, 2005). Glycans from *T. canis* are also recognized by DC-SIGN, an event that is associated with granuloma formation *in vivo*. Helminths can also modulate immune cells by lipids such phosphatidylserine (PS) and lyso-PS, which induce IL-4 and IL-10 secreting T cells via DC activation through TLR2 (Maizels and Yazdanbakhsh, 2003). On the other hand, *T. canis* secreted products appear to mimic mammalian CTLs, such as the mannose receptor, suggesting an alternative mode of manipulation (Hewitson *et al*, 2009). In addition, helminths interfere with PAMP-induced TLR activation in DCs by conditioning them through PRR signalling to produce

cytokines, such as IL-10 and TGF-β, which also promotes induction of regulatory T cells (Everts *et al*, 2010).

The presence of AAMΦ during parasite infections is important in down-regulating effector cell pro-inflammatory functions by driving Th2 responses as well as repairing helminth-induced damage (Maizels *et al*, 2004). In turn, NeMΦ maintain the anti-inflammatory response by activating naïve T cells towards a Th2 phenotype (Flynn *et al*, 2010). This enhances AAMΦ recruitment as Th2 cells are important for their activation and maintenance even in the presence of innate cell-derived IL-4/IL-13. However, nematodes are also known to achieve their survival by suppressing immune responses through the promotion of regulatory T cell induction (Anthony *et al*, 2007). In fact, Treg produce IL-10 and TGF-β which suppress T helper cell subsets. It has been suggested that AAMΦ are actually a result of Th2 cell induction and are not important in the early stages of infection but rather act to stabilise Th2 cells. In fact, *B.malayi*-induced AAMΦ promote Th2 differentiation by inhibition of IFN-γ producing T cells instead of inducing IL-4 producing cells (Allen and Loke, 2001).

Helminth-induced Th2 responses are beneficial to both the host and the parasite as they protect themselves from clearance and prevent severe pathology to the host as a result of excessive tissue damage. As a result of this, helminth responses are considered beneficial for resolution of chronic inflammation characteristic of autoimmune diseases, allergy and Crohn's disease and the role of AAMΦ is recognized as a potential means of therapeutic intervention in these diseases (Namangala *et al*, 2001).

1.3 T cells

An important process in linking the innate and adaptive arms of the immune response is antigen presentation to T cells. APCs capture and process antigens which they present to T cells via MHCI and II. Therefore, T cell activation is dependent on the activation state of APCs such as macrophages, used in this study. T cells then mount a more specific response which cooperates with APCs to further eliminate pathogens (Hasko *et al*, 2007).

Naïve T cells originate in the thymus and develop into effector T cells that can either be T helper (CD4+), cytotoxic (CD8+), memory (CD4+/CD8+), regulatory (CD4+) or natural killer cells. T helper (Th) cells are subdivided into Th1 and Th2 subsets which are counter-regulatory. Th1 cells induced by intracellular pathogens, produce IFN-γ, GM-CSF and TNF-α and are involved in cell-mediated immunity, while Th2 cells induced by extracellular pathogens, are involved in humoral immunity as they produce IL-4, IL-5, IL-13 and IL-10. In addition, NO which is a known pro-inflammatory mediator, enhances Th1 cell development via a cGMP-induced mechanism of IL-12R up-regulation (Niedbala *et al*, 2006). Regulatory T cells (Treg) which are characterised by Foxp3 and CD25 expression (Foxp3+CD4+CD25+) and are constitutively expressed, develop in the thymus and regulate peripheral T cells, while 'inducible' Treg (Tr1 or Th3 cells) develop from peripheral CD4+ T cells in response to antigen or deactivating cytokines. Treg expressing markers such as Foxp3, CD25, CD62L, OX40, Glucocorticoid induced tumor necrosis factor receptor family related gene (GITR), Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) are known to produce IL-4, IL-10, TGF-β. Foxp3 is considered the most reliable marker as it is responsible for their development and function. In fact, low levels of Foxp3 lead to a Th2 phenotype that produces IL-4 even under Th1 conditions (Wan and Flavell, 2007). This is because Foxp3 expression represses cytokines controlled by

TCR activation such as IL-4, IFN-γ, TNF-α as well as altering surface expression of molecules that determine Treg responses to environmental cues (Gavin *et al*, 2007).

Naïve T cells are activated by APCs presenting antigen via MHC only in the presence of co-stimulatory molecules. T cells that encounter antigen without sufficient co-stimulation become anergic and their expansion is inhibited (Appleman and Boussiotis, 2003). T cell proliferation is also suppressed by Tregs and, although the mechanism is not entirely clear, different modes of suppression have been proposed. Antagonism either by competition with APCs for specific MHC:peptide complexes or by expression of inhibitory molecules is one possibility. Treg-expressed CTLA-4 is one such molecule while cytokines such as IL-10 and TGF-β are also considered as potential candidates of suppression. CTLA-4 binds the co-stimulatory molecules B7.1 and B7.2 and has also been shown to induce indoleamine dioxygenase (IDO), an enzyme whose metabolic products have immunosuppressive effects (Fehervari and Sakaguchi, 2004). This could also lead to modulation of APC activation providing an indirect mechanism of inhibition of T cell activation. Furthermore, suppression may also be mediated by 'metabolic disruption' which is achieved by two mechanisms. The presence of CD25 on Treg may result in uptake of IL-2 thus reducing the amount available for effector T cells in the vicinity. Ecto-enzymes such as CD39 and CD73 (discussed in section 1.1 of this chapter), which are now considered to be more specific markers for Tregs than CD25, participate in generation of extracellular adenosine which is also a potent suppressor of T cell effector functions (Vignali, 2008; Zarek *et al*, 2008).

T cell receptor (TCR) activation by MHC leads to recruitment and activation of Src kinases such as Lck and Fyn to the CD3 complex where they phosphorylate immune receptor-based activation motifs (ITAMs). Subsequently, the tandem Src homology 2 (SH2) domain-

containing tyrosine kinase Zap-70 is recruited to the phosphorylated CD3-ζ chain ITAMs resulting in an active TCR complex (Acuto *et al*, 2008). An important linker for signalling downstream of the TCR is the transmembrane adaptor protein linker for activation of T cells (LAT) which is phosphorylated by Zap-70/Syk producing a platform for the recruitment of PLCγ, and the p85 subunit of PI3K which initiate signalling events. These events ultimately result in binding of transcription factors such as NF-kB/NFAT, Ap-1 and CREB to initiate transcription of T cell activating genes (Torgersen *et al*, 2002). PKA (cAMP-dependent protein kinase) signalling is important for activation of several of these transcription factors as it can inhibit TCR-CD3 complex activation through activation of Csk (C-Src tyrosine kinase) which reduces the ability of Lck to phosphorylate the CD3-ζ chain (Vang *et al*, 2001). TCR signalling has also been shown to induce TLR2 and TLR4 expression which were proposed to act as co-receptors for T cell activation by antigens as well as enhancing Treg proliferation and immunosuppressive ability in response to LPS (Liu and Zhao, 2007). Furthermore, it has been reported that, NK cells produce IFN-γ in response to TLR activation, which they express constitutively, and that this leads to further recruitment of macrophages allowing for efficient resolution of microbial infections (Sawaki *et al*, 2007).

As a result of the potent suppressor ability of Tregs, they are generally considered to play a critical role in controlling inflammation and promoting self-tolerance and recruitment of these cells by parasites is beneficial for generating conditions favoring their survival and persistence (Belkaid *et al*, 2006). In the case of parasite infections, Tregs are beneficial to the host as they control immunopathology resulting from excessive responses aimed at eliminating the parasites. On the other hand, host death can result from uncontrolled parasite growth which is promoted by Treg-induced tolerance. Chronic parasite infections support survival and function of Tregs by triggering the release of TGF-β by cells in the site of infection (Belkaid *et al*, 2006). In

schistosome-infected mice, Tregs isolated from hepatic granulomas and lymphoid tissues were shown to produce IL-10, which promotes parasite persistence by targeting IL-12 production by dendritic cells (Hesse *et al*, 2004). Recruitment of Tregs was also observed in lung draining lymph nodes in *T.spiralis* infection which was suggested to improve lung function and reduce allergic airway inflammation (Park *et al*, 2011).

1.4 *Trichinella spiralis*

Trichinella spiralis is a parasitic nematode that infects mammalian species via the gastrointestinal tract, through which it enters the systemic circulation to penetrate muscle tissue. The uniqueness of *T.spiralis* infection is emphasised by the fact that it can exist both intracellularly and extracellularly in its host (Despommier *et al*, 1983). Furthermore, its life cycle is completed in one host and consists of an enteral and parenteral phase (Summarised in Diagram 4). Infective larvae are ingested in contaminated meat and enter the stomach where they are released during digestion. Larvae become activated in the stomach and by bile as they enter the small intestine where they penetrate mucosal epithelial cells and mature into adults. Adults are expelled once they have shed newborn larvae which then exit the intestine and migrate through the bloodstream to the striated muscle tissue (Patel *et al*, 2009). Following penetration of myofibres, larvae induce formation of a parasite-host cell complex. Infection by new-born larvae initiates muscle de-differentiation and is accompanied by increased angiogenesis as a result of subversion of host cell gene expression which also promotes expression of vascular endothelial growth factor (VEGF) (Shariati *et al*, 2009). In addition, parasite survival is enhanced by expression of IL-10 and TGF-β. Both these cytokines inhibit inflammation of infected muscle which protects larvae from the host immune response mounted against them (Beiting *et al*, 2007).

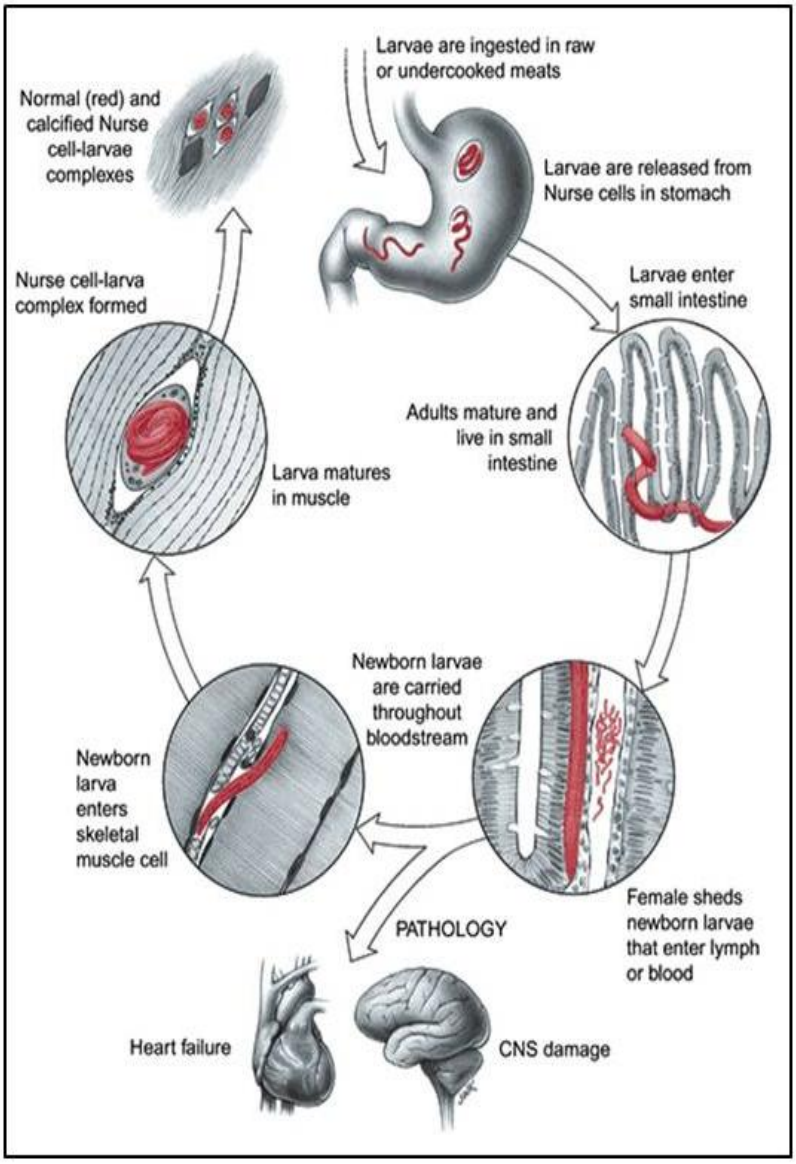


Diagram 4: The life cycle of *T.spiralis*. Parasites are released from contaminated meat into the stomach of the host. Larvae mature into adults in the small intestine and are expelled after newborn larvae are shed. Larvae migrate through the intestinal epithelium to the blood and lymphatics in search for striated muscle where they form a nurse cell complex (Taken from http://www.trichinella.org/bio_lifecycle.htm).

The gastrointestinal tract contains more immune cells than all the other immunological organs combined (Stokes and Bailey, 2000), therefore, products secreted by nematodes act in the parasite's advantage to ensure survival and prolong persistence until new larvae are released (Gibbs, 1982; Grencis, 1997). These secreted products consist of enzymes that are believed to down-regulate the host's immune system by generating mediators that serve to inhibit nucleotide induced activation of immune responses (Gounaris and Selkirk, 2005). A nucleotide metabolizing enzyme with dual apyrase and 5`-nucleotidase activity has been characterized that is capable of mimicking the endogenous ecto-enzymes, such as ecto-nucleotidases and ecto-NDPDases that are naturally expressed on cells of the immune system (Gounaris, 2002). This enzyme, known as 5`NT, catalyzes the hydrolysis of nucleoside 5`-diphosphates (ADP and UDP) and the 5`-monophosphate AMP at the same active site, thus limiting the availability of diphosphates and AMP (Gounaris *et al*, 2004). The importance of this potential evasion mechanism can be explained by the fact that, during parasite invasion the resulting tissue damage causes release of nucleotides such as ATP and ADP which act as warning signals to the immune system. Activation of purinergic signalling by these nucleotides leads to immediate inflammatory responses so the removal of these signals by *T.spiralis* enzymes, which is accompanied by generation of adenosine, could protect the parasite from host responses that would jeopardise its survival. The enzymatic cascade present in *T.spiralis* secreted proteins acting on extracellular nucleotides consists of nucleoside diphosphate kinases (NDPKs), adenosine deaminase and the 5`-nucleotidase mentioned above (Diagram 5). Extracellular nucleotides such as ATP act on P2 receptors expressed on immune cells leading to pro-inflammatory responses. The *T.spiralis* NDPK can convert these nucleotides to nucleoside diphosphates such as ADP which also activates P2 receptors. 5`NT is important in catalyzing the conversion of ADP to AMP and then to adenosine which acts on P1 receptors, also expressed on immune cells, to induce anti-

inflammatory responses that protect *T.spiralis* from inflammation-induced killing. Furthermore, inosine which is the breakdown product of adenosine from the action of adenosine deaminase, also acts on P1 receptors ensuring a sustained anti-inflammatory response. Interestingly, a similar mechanism of manipulation of purinergic signalling has also been proposed for ATP metabolising enzymes found on schistosome surfaces as well as in hematophagus insects (Gounaris and Selkirk, 2005; Bhardwaj and Skelly, 2009). Specifically, nucleoside triphosphate dephosphorylases (apyrases), which hydrolyze ATP and ADP to AMP, and 5`-nucleotidases, which hydrolyze AMP to adenosine, and adenosine deaminase are used by haematophagous insects to suppress immediate inflammatory responses (Gounaris and Selkirk, 2005). In addition, alkaline phosphatase, phosphodiesterase and ATP diphosphohydrolase 1 have been shown to be expressed on the tegumental surface of *S. mansoni* (Bhardwaj and Skelly, 2009).

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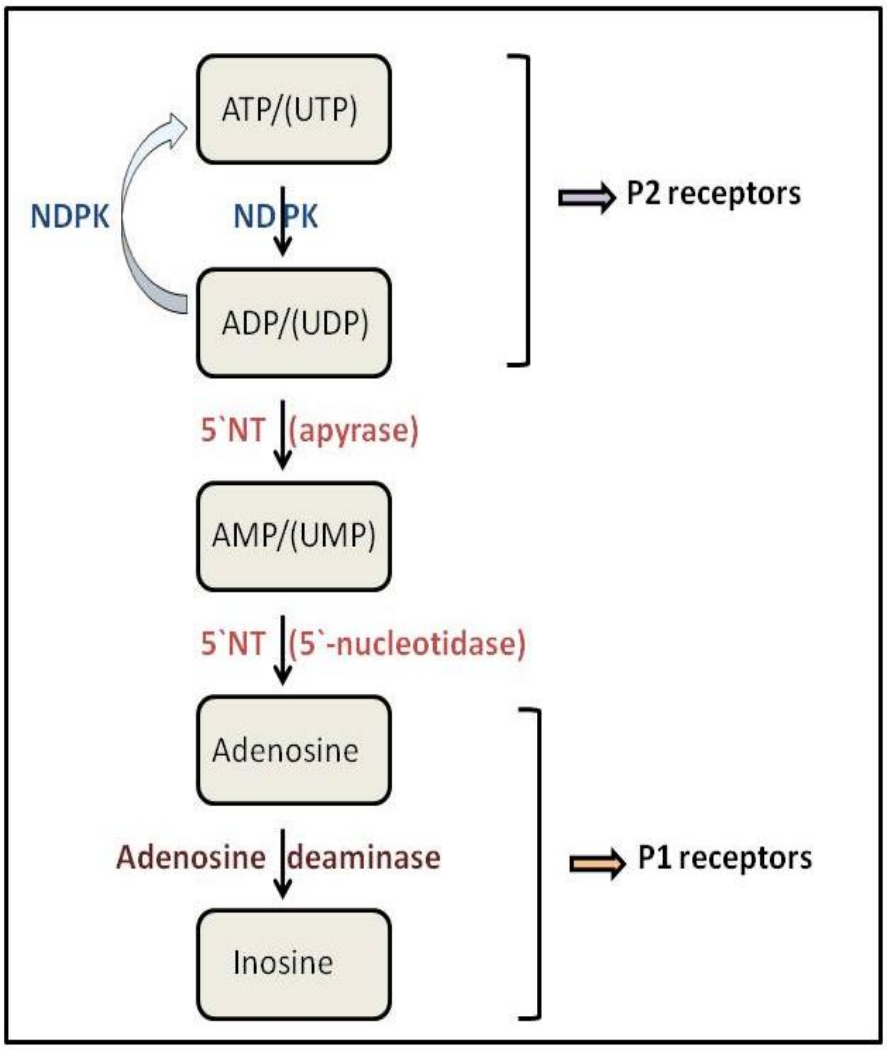


Diagram 5: Schematic representation of the enzymatic cascade catalyzed by *T. spiralis* secreted proteins. Extracellular nucleotides acting on P2 receptors are converted by nucleoside diphosphate kinase (NDPK) to nucleoside diphosphates like ADP/UDP, which are still P2 receptor agonists. The apyrase activity of 5`NT converts nucleoside diphosphates to monophosphates which have no known purinergic receptors. The 5`-nucleotidase activity of 5`NT then catalyzes the conversion of AMP to adenosine which is deaminated by adenosine deaminase to inosine and both these products act on P1 receptors.

1.5 Aims

Macrophages are involved in both innate and adaptive immune responses. Recently their role in nematode infection has also been established where they adopt a unique alternatively activated phenotype that promotes Th2 responses. The aims of this study were to investigate the effects of the parasitic nematode *Trichinella spiralis* on macrophage activation. In view of the parasite's potential to manipulate purinergic signalling of immune cells, the effects of *T.spiralis* secreted proteins on macrophage purinergic signalling were also investigated. Taking into account that macrophage activation can influence T cell responses either by antigen presentation or by release of soluble mediators, investigation of *T.spiralis* effects was also extended to T cells.

Chapter 2

Materials and Methods

2.1 Chemical reagents

Chemical reagents were purchased from Sigma-Aldrich (Dorset, UK), unless stated otherwise.

2.2 Animal strains

Wild type BALB/c mice were obtained at 6-8 weeks of age (Harlan, UK) for schedule 1 procedures to obtain bone marrow and spleens and Sprague-Dawley rats were obtained for maintenance of *Trichinella spiralis* and maintained at the Central Biological Services facility (CBS, Imperial College London).

2.3 Generation and maturation of mouse bone marrow-derived macrophages (BMMΦ)

Bone marrow was isolated from femurs and tibia of BALB/c mice sacrificed by cervical dislocation. Using forceps and scissors, fur was removed from the legs that were previously cut at the hip and placed in 15ml standard culture medium [RPMI-1640 (Lonza) supplemented with 2mM L-glutamine (Gibco), 100Uml^{-1} penicillin/ 100μg ml^{-1} streptomycin (Gibco), 50μM mercaptoethanol, 1mM sodium pyruvate (Lonza), 10mM HEPES (Lonza) and 10% v/v FCS (Biosera)]. After cleaning the bones with tissue paper, they were collected in 10ml of standard culture medium. They were then transferred to a sterile dish with 70% v/v ethanol and washed for 2 minutes, followed by a wash in culture medium. The ends were snipped with sterile scissors, and a hollow tube created with a fine needle (27GA1) attached to a 2ml syringe filled with culture medium to flush the bone marrow progenitor cells into a sterile dish containing standard

culture medium. After breaking up the cell clumps, the suspension was collected in a 50ml tube and centrifuged at 300 xg at 4 ^{o}C for 5 minutes. The red blood cells were lysed by resuspending the pellet in 0.83% ice cold ammonium chloride (1ml per leg) for 2 minutes at room temperature (RT). The cell suspension was made up to 50ml with standard culture medium and centrifuged at 300 xg at 4 ^{o}C for 5 minutes. The cell pellet was then resuspendend in 10ml standard culture medium for counting and then 3.5 x 10^{6}cells plated per Sterilin dish in 10ml of complete medium supplemented with 20% v/v M-CSF and incubated at 37^{o}C, 5% CO_2. L929 cell conditioned medium was used as a source of macrophage colony stimulating factor (M-CSF) obtained from NIMR, filtered and stored at -20 ^{o}C. On day 4, 10ml of fresh medium was added and after 7 days macrophages were obtained as a homogeneous population of adherent cells which were harvested by incubating with ice cold PBS at 4 ^{o}C for 15 minutes. Macrophage suspensions were centrifuged at 300 xg at 4 ^{o}C for 5 minutes and then resuspended in 10ml of standard culture medium for counting. Macrophages were then cultured in standard culture medium in 96-well or 6-well plates at 10^{6}cells/ml in 200µl and 2ml final volume per well, respectively.

2.4 Macrophage culture

Macrophages were seeded in plates as described above and allowed to adhere prior to exposure to the following treatments: LPS and interleukin-4 (IL-4) were used to activate macrophage populations at concentrations ranging from 0.1 to 50 ng/ml and 1 to 10 ng/ml, respectively (specified in the relevant text of Chapter 4). Ultrapure LPS and Pam3C were used at 10 ng/ml. Adenosine, NECA, AMP, ADP, β-ADP, ATP, ATP-γ and UDP were used at 100µM unless otherwise stated in the relevant figure legends. NECA (5′-N-Ethylcarboxamido adenosine) is an analogue of adenosine that has high affinity for the A_2 receptors and β-ADP and ATP-γ are slowly hydrolysable analogues of ADP and ATP, respectively. Purinergic receptor agonists CPA

(N^6-Cyclopentyladenosine) (A_1 receptor), CGS21680 (A_{2A} receptor), IB-MECA (A_3 receptor) were used at 100μM (purchased from Tocris Bioscience). P1R antagonists DPCPX (A_1 receptor), ZM241385 (A_{2A} receptor), MRS1754 (A_{2B} receptor), MRS1523 (A_3 receptor) were used at 10μM (purchased from Tocris Bioscience). Suramin (P2Y receptor) and PPADS (Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid) (P2Y receptor) were also used at 10μM. The following inhibitors of intracellular signalling pathways were used as indicated in the relevant text: cAMP inhibitor RP-cAMP (Adenosine- 3', 5'- cyclic monophosphorothioate, Rp- isomer), PLC inhibitor U73122 and its inactive analogue U73343, PI3 kinase inhibitor LY294002 (Calbiochem), NF-κB inhibitor Bay 11-7082, MEK1/2 inhibitor U0126. Cells were pre-incubated in 100μl medium with antagonists or inhibitors for 15 minutes at 37^{o}C before the addition of 100μl medium with LPS/IL-4 and/or agonists to yield the final concentrations specified. Rp-cAMP was added to cells and incubated at 37^{o}C for 1 hour prior to agonist addition. Elevators of cAMP (forskolin and rolipram) were used at concentrations indicated in the relevant text and were added to the cells 1 hour prior to addition of any other stimulant. For IL-10 neutralisation experiments, macrophages were incubated for 15 minutes at 37^{o}C with anti-IL-10 (R&D systems) to neutralise any IL-10 secreted by the cells into the medium, before addition of any other stimulant. Carbohydrates used to block macrophage surface lectins [D-mannose, mannan, laminarin, GalNAc, EGTA (ethylene glycol tetraacetic acid)] were added to macrophages incubated at 37^{o}C 45 minutes prior to *T.spiralis* ES addition in 100μl medium to yield final concentrations specified in the text. Cells were incubated in 200μl in triplicate for 20 hours at 37 ^{o}C, 5% CO_2 and 150μl supernatant per well were collected for analysis or stored at -20 ^{o}C.

2.5 Fluorescence Activated Cell Sorting (FACS) analysis

FACS analysis was carried out with 10^5-10^6 cells per FACS tube resuspended in FACS buffer (PBS + 1% w/v BSA + 0.1% v/v Sodium Azide). They were then washed twice with 0.5 or 1ml FACS buffer and centrifuged at 500 xg at 4^{o}C for 3 minutes. Pellets were resuspended by vortexing in 100µl FACS buffer per tube and 1µl of the relevant antibody (antibodies used are listed below) added to each tube, except for the negative controls which had FACS buffer only. Fc receptor block (anti-CD16/CD32, BD Biosiences) was added to the cell suspension 10 minutes prior to specific antibody incubations. Then cells were incubated with the relevant antibodies for 45 mins in the dark, on ice. Cells were washed again twice with 1ml of FACS buffer after which they were resuspended in 150µl of FACS buffer and analysed on a FACS Calibur. Antibodies used: Phycoerythrin/PE - conjugated anti - CD11b (Caltag), CD73 (BD Biosciences), anti-mouse CD124 (IL-4Rα). Unconjugated anti – CD39 (Santa Cruz), CD73 (BD Biosciences) and Fluorescein isothiocyanate/FITC - conjugated anti – mouse secondary (Sigma). Allophycocyanin/APC – conjugated anti - F4/80 (Caltag).

2.6 PCR analysis

2.6.1 RNA isolation. Bone marrow derived macrophages were homogenized directly in the culture dish or 6-well plate by adding 1ml of Trizol per 10cm^2. Cell lysates were passed several times through a pipette and then transferred to 1.5ml tubes at 1ml per tube. Samples were incubated for 5 mins at RT and then 0.2ml of chloroform was added per tube. The solutions were mixed vigorously by shaking for 15 seconds and incubated at RT for 2-3 mins. Samples were then centrifuged at 12000 xg for 15mins at 4 ^{o}C. The top aqueous phase of each sample was transferred to a fresh tube. 0.5ml of isopropyl alcohol was added per tube and mixed followed by

a 10 min incubation at RT. Samples were then centrifuged at 12000 xg for 10 min at 4^{o}C in order to precipitate the RNA. The supernatant was removed and pellets washed with 1ml of 75% ethanol per tube. Samples were mixed by vortexing and centrifuged at 7500 xg for 5 mins at 4 ^{o}C. Supernatants were removed and samples pulsed once more to remove any remaining supernatant before air-drying pellets for 10 mins at RT. Pellets were dissolved in RNase free water and incubated at 55^{o}C for 5 mins and cooled on ice before DNase digestion. To avoid any DNA contamination, samples were incubated with DNaseI (Qiagen) at RT for 45 mins. The RNeasy Kit (Qiagen) was then used to 'clean up' the RNA according to the manufacturer's protocol. Briefly, RNA was applied to the RNeasy Mini column and centrifuged with several buffers provided by the manufacturer. The concentration of isolated RNA for each sample was then determined by measuring absorbance at 260nm. Purity of the sample was confirmed by obtaining the OD_{260}/OD_{280} ratio.

2.6.2 cDNA synthesis. First strand synthesis of cDNA from RNA was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega). 2-3μg RNA of each sample were mixed with mRNA primer, Oligo (dt) (40μgml^{-1}; Biolabs) and an appropriate volume of RNase-free water to make a total volume of 13μl. Samples were incubated for 5minutes at 70^{o}C in a gradient cycler (MJ Peltier thermal cycler-200) followed by cooling on ice before the following were added to the annealed primer/template: M-MLV 5x reaction buffer (20% v/v; Promega) Ribonuclease Inhibitor (1μl of a 1.6Uμl^{-1} stock), dNTP (5μl of a 2.5mM stock; Fermentas) and M-MLV RT (1μl of a 8Uμl^{-1} stock). The samples at total volumes of 25μl were incubated in the gradient cycler for 1hour at 37^{o}C and subsequently for 5minutes at 94 ^{o}C.

2.6.3 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). RT-PCR was carried out to detect the presence of transcripts for markers of alternative activation, ecto-nucleotidases and purinergic receptors. The sequences of all the primers used in the RT-PCR are shown in Table 2.1 with their corresponding base pair product sizes. RT-PCR reactions contained 1μl of each cDNA sample, sense and anti-sense primers (1μl of each), 2.5mM dNTP, 2.5mM $MgCl_2$, 10x PCR buffer (10% v/v), water and Taq polymerase ($0.06Uml^{-1}$) in 25μl final volume and carried out in the GeneAmp PCR system 2700. The cycler conditions were as follows: 94^{o}C for 5minutes, followed by 35 cycles of 94 ^{o}C for 30sec, 54-57 ^{o}C for 30sec, 72 ^{o}C for 30sec and a final extension step of 72 ^{o}C for 10min. The annealing step varied according to the primer pair used; see Table 2.1 A, B, C and D.

Control β-actin primers were used (55 ^{o}C annealing temperature). PCR products were resolved by electrophoresis in 1.5% w/v ethidium bromide stained agarose gels and visualised under UV illumination.

2.6.4 Quantitative PCR analysis. RNA extracted as described above was serially diluted from 2μg/ml – 0.03125μg/ml reverse transcribed to cDNA and a standard curve was generated with β-actin in order to select an appropriate concentration of RNA to produce cDNA template in the linear range. 250ng of RNA was used in experiments to produce cDNA and primer-probes designed for each gene of interest used according to manufacturer's instructions (Applied Biosystems). Relative transcript levels were obtained by normalising against β-actin levels and then compared to controls as specified in the relative figure legends.

Primers	Sequence	Annealing Temperature (°C)	Product Size/bp
Actin Forward	5`- TGGAATCCTGTGGCATCCATGAAAC - 3`		
Reverse	5'- TAAAACGCAGCTCAGTAACAGTCCG - 3`	55	350
Arginase Forward	5`- CAGAAGAATGGAAGAGTCAG - 3`		
Reverse	5`- CAGATATGCAGGGAGTCACC - 3`	55	249
Mannose Rec. Forward	5`- CCATCGAGACTGCTGCTGAG - 3`		
Reverse	5`- AGCCCTTGGGTTGAGGATCC - 3`	55	355
Fizz Forward	5`- GGTCCCAGTGCATATGGATGAGACCATAGA - 3`		
Reverse	5`- CACCTCTTCACTCGAGGGACAGTTGGCAGC - 3`	55	296

Table 2.1A: Sequences, annealing temperatures and sizes for primers of markers of alternative activation

Primers	Sequence	Annealing Temperature (°C)	Product Size/bp
Actin Forward	5`- TGGAATCCTGTGGCATCCATGAAAC - 3`		
Reverse	5'- TAAAACGCAGCTCAGTAACAGTCCG - 3`	55	350
Arginase Forward	5`- CAGAAGAATGGAAGAGTCAG - 3`		
Reverse	5`- CAGATATGCAGGGAGTCACC - 3`	55	249
Mannose Rec. Forward	5`- CCATCGAGACTGCTGCTGAG - 3`		
Reverse	5`- AGCCCTTGGGTTGAGGATCC - 3`	55	355
Fizz Forward	5`- GGTCCCAGTGCATATGGATGAGACCATAGA - 3`		
Reverse	5`- CACCTCTTCACTCGAGGGACAGTTGGCAGC - 3`	55	296

Table 2.1B: Sequences, annealing temperatures and sizes for ecto-nucleotidase primers.

Primer	Sequence	Annealing Temperature (°C)	Product Size (bp)
P1 receptors			
A_1 Forward	5`-TTTGGCTGGAACAACCTGAGTGAG -3`	54	264
Reverse	5`-ATCTTGAGCTCCTTCCCGTAGTAC -3`		
A_{2A} Forward	5`- TTCAGCCTCTTGGCTATTGCCATC -3`	54	216
Reverse	5`- ATCCTTAGGTAGATGGCCAGCATG -3`		
A_{2B} Forward	5`- CCACCAACTACTTTCTGGTATCCC -3`	54	323
Reverse	5`- TCTTTACTGTTCCACCCCAGGAAC -3`		
A_3 Forward	5`- AGACCTGCATCCTCCAGGTTAATG -3`	54	260
Reverse	5`- GCTTCCATGCTGTCTCAGAAAAGC -3`		

Table 2.1C: Sequences, annealing temperatures and sizes for P1 receptor primers.

P2Y receptors			
Y_1		57	300
Forward	5`-CTGTGTCTTATATCCCTTTCC -3`		
Reverse	5`-CTCCATTCTGCTTGAACTC -3`		
Y_2		57	440
Forward	5`-ACCAGCGTGCGG GGAACC -3`		
Reverse	5`-GCATCTCGGGCAAAGCGGACAAGT -3`		
Y_4		57	499
Forward	5`-TGCCTCGTGCCCAACCTCTTCTTT –3`		
reverse	5`-CAGTTGTTCGGCGCTTAGGTGTGC -3`		
Y_6		57	452
Forward	5`-CCTGGCACTGGCGGACCTGAT- 3`		
Reverse	5`-GGCGGGCCATGCGACAATAAC -3`		
Y_{12}		55	300
Forward	5`-ATCGGTCTTTTGTCAGAACAAGG -3`		
Reverse	5`-TTCTCCCAGATGTTGATGTAGAG -3`		
Y_{13}		55	400
Forward	5`-TGGCATCAGGTGGTCAGTCACAC -3`		
Reverse	5`-CGCTTGATCCTGCTGTTCTTGCC -3`		
Y_{14}		55	264
Forward	5`-CGTCTTCTACACGGCCATCACG -3`		
Reverse	5`-TCCAGACACACATTGGCAGCCG -3`		

Table 2.1D: Sequences, annealing temperatures and sizes for P2Y receptor primers.

2.7 Cloning for sequencing

In order to sequence arginase I and A_3 receptor PCR products, they were cloned into TOPO vectors. PCR products were generated as described in section 2.3 and purified from agarose gels using a DNA purification kit (Qiagen) according to the manufacturer's instructions. Briefly, the gel was dissolved at 52^{o}C using the buffer provided and applied to a column to bind DNA which was washed by centrifuging several times with another buffer provided. The DNA was eluted with 30µl dH_2O and quantified (OD_{260}) before running 2µl of the sample on an agarose gel. Having confirmed that the correct bands for the PCR products were obtained on the gel, the DNA was concentrated to a volume of 8-10µl for use in the cloning reactions. These cloning reactions were carried out according to the TOPO-TA cloning kit (Invitrogen) protocol with 4µl of DNA, 1µl of salt solution and 1µl vector provided in the kit. This mixture was incubated on ice for 30 minutes prior to adding to competent DH5α-T1 and returning to ice for another 30 minutes. Cells were heat shocked for 30 seconds at 42^{o}C and transferred to ice immediately. Room temperature S.O.C. (Super Optimal Broth) medium was then added and the tubes incubated at 37°C for 1 hour, with shaking. Two different volumes from each transformation were spread onto pre-warmed selective LB agar plates (1% w/v Tryptone, 0.5% w/v Yeast extract, 1% w/v NaCl, 1.5% w/v agar plus 75µg/ml Kanamycin spread with X-gal 50mg/ml) and incubated overnight at 37°C. White colonies were selected to patch plate and colony PCR was carried out to confirm that the selected colonies contained PCR product inserts. The PCR reaction was carried out as described in Section 2.3 with a few changes outlined below. Colonies were diluted in 10µl of dH_2O and placed at 94°C for 10 minutes to lyse bacteria prior to setting up the usual PCR reaction. Products were resolved using agarose gel electrophoresis. Colonies positive for the insert were selected from the patch plate and grown in falcon tubes with

10ml LB (1% w/v Tryptone, 0.5% w/v Yeast extract, 1% w/v NaCl plus 75µg/ml Kanamycin) overnight at 37°C, shaking. DNA was isolated from the overnight cultures using a Qiaspin Mini prep kit (Qiagen) according to the manufacturer's instructions. Briefly, cells were lysed and neutralized prior to collecting supernatants which were applied to Qiaspin columns. These were washed a couple of times and DNA was eluted with 30µl of dH_2O. After DNA quantification, 100ng/µl of each cloning reaction were sent for sequencing (Cogenics).

2.8 Recombinant 5`-nucleotidase (5`NT) expression and purification

**2.8.1 Recombinant 5`NT expression.** *P.pastoris* transformed with pPICZα containing a 6 x His and c-Myc tagged *T.spiralis* recombinant 5`NT was streaked onto YPD agar [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar, 0.1 mg/ml zeocin] and incubated at 30^oC for 3 days. A single *P.pastoris* colony was used to inoculate 25ml of buffered glycerol complex medium [BMGY: 100mM potassium phosphate pH 6.0, 1% (w/v) yeast extract, 2% (w/v peptone 1.34% (w/v) yeast nitrogen base, YNB (with ammonium sulphate w/o amino acids) $4x10^{-5}$ % (w/v) Biotin, 1% (v/v) glycerol] and incubated at 30^oC with agitation until the culture reached an OD_{600} value between 2 and 6 units. The 25ml culture was then transferred into 400ml BMGY and incubated for a further 24 hours at 30^oC with shaking. Subsequently, cells were harvested by centrifugation at 3000 xg for 10 minutes at RT and resuspended in 400ml buffered methanol complex medium [BMMY: 100mM potassium phosphate pH 6.0, 1% (w/v) yeast extract, 2% (w/v peptone 1.34% (w/v) yeast nitrogen base, YNB (with ammonium sulphate w/o amino acids) $4x10^{-5}$ % (w/v) Biotin, 0.5% (v/v) methanol]. To induce protein expression, methanol was added at a final concentration of 0.5% (v/v) every 24 hours to cultures incubated at

30^{o}C with shaking for 48 hours. Cells were harvested again by centrifugation at 3000 xg for 10 minutes at 4 ^{o}C. The supernatant was then filtered through glass Whattman paper and 0.2 μm filters (Schleicher and Schuell) and concentrated to a final volume of approximately 10ml in an Amicon concentrator using a 30 kDa cut off membrane. The concentrated supernatant was then dialysed against 4L of dialysis buffer containing 20mM NaH_2PO_4, pH8, 300mM NaCl and 1mM Imidazole for 16 hours at 4 ^{o}C.

**2.8.2 5`NT purification.** Recombinant 5`NT was purified using nickel nitrilotriacetic acid (Ni-NTA) affinity chromatography. Ni-NTA-agarose beads were equilibrated with dialysis buffer (containing 20mM NaH_2PO_4, pH8, 300mM NaCl and 1mM Imidazole) and added to the concentrated 5′-NT yeast supernatant. Beads were incubated overnight with rotation at 4 ^{o}C. The slurry was added to a chromatography column (Bio-Rad) and beads allowed to sediment before the flow-through solution containing unbound proteins was decanted. The column was washed with 10ml of dialysis buffer to remove unbound proteins and bound proteins were eluted with 3 x 20ml washes of dialysis buffer with increasing Imidazole concentrations (10mM and 100mM). The eluted protein fractions were concentrated in a Centricon-YM10 micro-concentrator, according to the manufacturer's instructions to a final volume of ~1ml. Samples were then washed with 25mM HEPES pH7.5 and further concentrated to a volume of ~200μl. Aliquots were immediately stored at -20 ^{o}C. Proteins were analysed by SDS-PAGE and Western blotting using anti-c-myc antibody to confirm presence and purity. Protein concentration was determined using the BCA protein estimation assay (Bicinchoninic Acid protein assay, Pierce) according to the manufacturer's instructions.

2.8.3 Confirmation of 5`NT expression: Western analysis. Samples (25μl) containing SDS-PAGE loading buffer were loaded onto 12% polyacrylamide gels (recipes shown in appendix)

and electrophoresed at 15 mA/250V in running buffer (2.5mM Tris pH8.3, 19.2mM glycine, 0.01% w/v SDS) for an hour or until the tracking dye reached the bottom of the gel. The gels were either stained with Coomasie (40% v/v methanol, 10% v/v acetic acid, 01% w/v Coomasie Brilliant Blue) or transferred to nitrocellulose membranes (Appendix, Figure 1).

Staining of polyacrylamide gels with Coomasie was carried out overnight with shaking at RT and then gels were washed in destain solution (40% v/v methanol, 10% v/v acetic acid in dH_2O). Gels were set up for transfer onto nitrocellulose membranes, submerged in transfer buffer (2.5mM Tris pH8.3, 19.2mM glycine, 20% v/v methanol) and electrophoretic transfer was allowed for 2 hours at 10mA/500V at RT. Following transfer nitrocellulose membranes were placed in PBS and blocked with PBS/0.1% v/v Tween containing 1.25% w/v milk powder for an hour at RT on a shaker. Primary antibody (anti-His, mouse) diluted 1:1000 in PBS/0.1% v/v Tween 1.25% w/v milk powder was added and the membrane was incubated at RT on a shaker for 25 minutes. This is followed by 3 x 10 minute washes with PBS/0.1% v/v Tween at RT on a shaker before incubating with the secondary antibody (1:1000 goat anti-mouse IgG-HRP in PBS/0.1% v/v Tween) for 1 hour, shaking at RT. The membranes were washed as before followed by one wash with PBS for 10 minutes. Visualisation was achieved by a chemiluminescent detection system (ECL Western blotting analysis system, Amersham) following standard procedures.

2.9 Bicinchoninic acid assay (BCA)

The BCA assay was carried out in 96-well flat bottom plates according to the manufacturer's instructions (Pierce). Briefly, the protein concentration was estimated as follows: a standard curve was generated using BSA (2mg/ml) to produce samples of known

concentrations (dilutions shown in Table 2.2). 25µl of sample (standards/ blank-dH_20/ unknown sample) was added per well and then 200µl of BCA reagent added before incubating the plate at 37^oC for 30 minutes, in the dark. The absorbance at 550nm was then measured using a FLUOstar OPTIMA plate reader (BMG).

mg/ml BSA	BSA dilutions
1.5	115µl BSA stock + 35µl dH_20 (A)
1	100µl BSA stock + 100µl dH_20 (B)
0.75	100µl (A) + 100µl dH_20 (C)
0.5	100µl (B) + 100µl dH_20
0.25	100µl (C) + 100µl dH_20 (D)
0.125	100µl (D) + 100µl dH_20
0.025	10µl (D) + 90µl dH_20

Table 2.2: BSA dilutions for standard curve.

2.10 Preparation of *Trichinella spiralis* excretory/secretory proteins

2.10.1 *Trichinella spiralis* infection. In order to obtain muscle stage larvae of *Trichinella spiralis*, adult Sprague-Dawley rats were infected orally with 3500-4000 infective larvae. Infections were allowed to establish for 30 days before isolating muscle stage larvae.

2.10.2 Parasite recovery and culture. Parasites were recovered from infected rats which were culled by cervical dislocation. Rats were skinned and eviscerated and striated muscle tissue removed and chopped. The tissue was then digested by incubation, with agitation, in a solution of 1% (w/v) pepsin, 1% (v/v) HCl at 37^oC for 1hour. The digested tissue was then filtered through two layers of muslin cloth attached to a Baerman funnel connected to a collection tube and larvae were allowed to settle for 45minutes to 1hour. They were then washed in sterile PBS followed by sterile parasite culture medium [RPMI supplemented with 1% (w/v) glucose, 100U/ml penicillin,

100μg/ml streptomycin, 20μg/ml gentamycin, 20U/ml nystatin and 2mM L-glutamine], pre-warmed at 37 ^{o}C.

2.10.3 Collection of parasite secreted proteins. Muscle stage larvae were cultured for 3 days and the medium containing parasite secreted proteins was collected daily and replaced with fresh sterile culture medium pre-warmed at 37 ^{o}C. Medium containing parasite secreted proteins was concentrated and washed with 25mM HEPES, pH 7.5 in Vivaspin microconcentrators (Millipore). The concentrated protein solution was then aliquoted and stored at -20 ^{o}C.

2.11 Phosphate release Assay

**2.11.1 5`-Nucleotidase activity assay.** The 5`-nucleotidase activity of *T.spiralis* secreted proteins (ES) was measured by calculating the amount of free inorganic phosphate released from different nucleotide substrates. NaH_2PO_4 ranging from 1-10nmoles was used as a standard to generate a calibration curve. 1μl of nucleotide (ADP, AMP) was added to 20μl buffer (50mM Hepes, 300mM NaCl, 10mM $MgCl_2$, and 5mM DTT) to give a final concentration of 2.5mM to which 1μg of ES was added and the volume made up to 40μl with distilled water. Controls were included containing enzyme alone or nucleotides alone. Samples were incubated for 10 minutes at 37^{o}C following which 200μl of the chromogenic substrate malachite-green-phosphomolybdate (1.25mM Malachite green, 1.7N HCl, 8.6mM ammonium molybdate) were added to both the enzyme reactions and the standards simultaneously to ensure an accurate readout. Measurement of absorbance at 660nm using FLUOstar OPTIMA microplate reader (BMG Labtech) was then carried out. Background (control) absorbance values were subtracted from the actual readout of the enzyme sample.

2.11.2 Phosphate Release Assay; modified for phosphate release by ecto-nucleotidases.

BMMΦ were plated in 96 well plates at 10^5 cells per well and incubated overnight with 200µl of standard cell growth medium +/- stimuli. Cells were then washed twice with 200µl of assay buffer (10mM glucose, 20mM HEPES pH7.5, 5mM KCl, 120mM NaCl, 2mM $CaCl_2$, 5mM $MgCl_2$). Cells were allowed to adjust prior to nucleotide addition by incubating at 37^{o}C for 15 minutes with 100µl of assay buffer. Nucleotides were made up in assay buffer at 2x the final concentration and 100µl added per well already containing 100µl assay buffer. Cells were returned to 37^{o}C for 15-150 minutes. 40µl from each well was transferred to a new 96 well plate at the time points specified in the relevant figure legends and the standard curve was prepared as for the standard phosphate release assay. 200µl of Malachite green reagent (standard protocol) was added to each well and the absorbance measured at 660nm using a FLUOstar OPTIMA microplate reader (BMG Labtech). Absorbance values were converted to nmoles of phosphate and adjusted to represent total well volume corresponding to 10^5 cells.

2.12 Enzyme linked immunosorbent assay (ELISA)

Cytokine (IL-6, TNF-α and IL-10) levels in culture supernatants were detected by ELISA. ELISA plates were coated overnight at 4^{o}C with 0.8µg/ml of capture antibody (R&D systems) diluted in PBS. Plates were washed 3 times with PBS/ 0.05% v/v Tween 20 and then blocked with PBS/ 0.1% w/v BSA for 2hrs at RT. After 3 more washes, samples and standards were added in triplicates and incubated overnight at 4 ^{o}C.

Standards were diluted at a range of 125-8000pg/ml in PBS/ 1% w/v BSA, including a blank of diluting buffer only. Plates were washed 4 times and incubated with 0.5µg/ml biotinylated detection antibody (R&D systems) diluted in PBS/ 1% w/v BSA for 2 hours at room

temperature. Plates were washed 6 times and streptavidin-HRP (R&D systems) diluted 1:1000 in PBS/ 1% w/v BSA added for 30 minutes at RT. The 6 washes were repeated and plates incubated for 20-30 minutes in the dark with substrate solution (0.11M acetate buffer pH5.5 with 0.1 mg/ml tetra-methylbenzidine in DMSO and 0.003% v/v H_2O_2). The reaction was stopped with 1.8M H_2SO_4 at 1:5 of the final volume per well. Levels of cytokines were determined by measuring the absorbance at 450nm using a FLUOstar OPTIMA microplate reader (BMG Labtech).

2.13 Arginase I activity assay

BMMΦ harvested on day 7 of culture were plated at 10^5/ well in a 96 well plate in standard RPMI medium and allowed to adjust and adhere for 1-2hrs. Either LPS or IL4 were then added to a final concentration of 10ng/ml or 5ng/ml, respectively. Cells were incubated at 37^{o}C for a specified length of time, as stated where appropriate, and then washed with 200µl PBS prior to the assay. 100µl of 0.1% v/v Triton + protease inhibitors (1 tablet per 10ml Triton of Complete Mini, Roche) were used to lyse the cells by incubating the plate at RT for 30 minutes, shaking. Then 100µl of 25mM Tris-HCl pH 7.5 were added per well and 100µl of lysate transferred to 1.5ml eppendorf tube. 10µl of 10mM $MnCl_2$ were added to the lysate and incubated at 56^{o}C to activate the enzyme. Then 100µl of 0.5M L-arginine pH 9.7 were added and the samples incubated at 37^{o}C for 65 minutes. Urea standards were prepared from a 10mg/ml urea stock and were ranging from 160 to 1.25 µg/ml in buffer containing 0.1% v/v Triton, 25mM Tris-HCl, 10mM $MnCl_2$ and acid mix (H_2SO_4: H_3PO_4: H_2O, 1:3:7) at the same final concentrations as the samples. The reaction was stopped by adding 800µl of acid mix. Finally, 40µl of ISPF (α-isonitrosopropiophenone) were added and incubated for 30 minutes at 95 ^{o}C. Samples and standards were kept in the dark for 10 minutes prior to transferring 200µl to a flat bottom 96-well plate and measuring absorbance at 540nm.

2.14 Nitric oxide (NO) assay

Activation status of BMMΦ was determined by monitoring NO release using Griess reagent. The Griess reagent was composed of two separate reagents (A and B) combined in a 1:1 ratio; Griess reagent A - 1% w/v sulphanilamide, 5% v/v phosphoric acid (H_3PO_4) and Griess reagent B – 0.1% w/v N-(1-Napthyl)ethylenediamine dihydrochloride (NEDD) kept at 4^{o}C in the dark. Sodium Nitrite ($NaNO_2$) (Sigma) was used to produce a standard curve. 100µl of Griess reagent was added to 100µl of sample supernatants in a 96-well plate. The absorbance at 550nm was measured using a [FLUOstar OPTIMA plate reader (BMG)] and nitrite concentration was calculated from the standard curve obtained from $NaNO_2$ in RPMI-1640.

2.15 Endotoxin detection

The presence of endotoxin was tested in protein solutions by using the QCL-1000 Chromogenic Limulus Amoebocyte Lysate Endpoint Assay (Lonza) according to the manufacturer's instructions. Briefly, an equal volume of sample and LAL reagent were incubated for 10 minutes at 37^{o}C prior to addition the chromogenic substrate. The reaction was stopped after 6 minutes with acetic acid. A standard curve of endotoxin was used to quantify levels reported as EU/ml. The absorbance was then measured at 410nm FLUOstar OPTIMA microplate reader (BMG Labtech).

2.16 Treatment of *T.spiralis* secreted proteins

2.16.1 Heat inactivation of *T.spiralis* secreted proteins

50µg/ml samples of *T.spiralis* secreted proteins in 1.5ml eppendorf tubes, sealed with parafilm to prevent evaporation, were placed in a heat block set at 100 °C. After a 10-minute incubation period, the tubes were removed and allowed to cool. Prior to using the samples in macrophage cultures, they were centrifuged at top speed for 1 minute to spin down any liquid collected on the lid or sides of the tube.

2.16.2 Chemical deglycosylation of *T.spiralis* secreted proteins

Chemical deglycosylation of *T.spiralis* secreted glycoproteins was carried out using a GlycoProfile IV, Chemical Deglycosylation Kit (Sigma) according to the manufacturer's instructions. Briefly, samples were lyophilized (1mg per reaction vial) using a vacuum pump (Dell group). A lyophilized RNase B Glycoprotein Standard, which was supplied in the kit, was also included as a control of the deglycosylation reaction. The Trifluoromethanesulfonic acid (TFMS) was cooled to 2–8 °C and added to each pre-cooled sample reaction vial. The mixtures were shaken for 2–5 minutes to dissolve the glycoproteins completely and the sample reaction vials were incubated on ice for an additional 25 minutes with occasional shaking. A drop of indicator dye (0.2% Bromophenol Blue Solution) was added to the pre-cooled sample reaction vial and mixed gently until a red colour was obtained. Pre-cooled 60% Pyridine Solution was added dropwise and mixed, keeping the sample on ice, until the colour of the solution changed to yellow and then to light purple. Samples were transferred to Vivaspin columns and washed with 10mM Hepes to obtain a clear solution before concentrating to 200µl and collecting for storage at -20 ^{o}C.

2.17 Macrophage Galactose Lectin (MGL) experiments

2.17.1 MGL-purification of *T.spiralis* ES. The human MGL CRD (HHL-416 – extracellular domain) was expressed and purified by affinity chromatography on a galactose–Sepharose column by A. Quintero-Martinez and K. Drickamer (as described in Lobst and Drickamer, 1996). The column was rinsed with 5 x 1ml loading buffer (1.25 M NaCl, 25mM Tris-Cl, pH 7.8, 25 mM $CaCl_2$) prior to incubating with ES for 15minutes. The flow-through was collected prior to 5 x 0.5ml washes with eluting buffer (1.25 M NaCl, 25 mM Tris-Cl, pH 7.8, 2.5 mM EDTA). Fractions were then analysed by SDS-PAGE as described above.

2.17.2 SDS-PAGE with radiolabelled MGL. All the fractions obtained from the MGL column were resolved by SDS-PAGE and then blotted for ^{125}I-labelled MGL by K. Drickamer as described in Coombs *et al*, 2005.

2.17.3 Competition Binding Assays. The binding affinity to an immobilised fragment of the MGL receptor was determined in the presence of increasing concentrations of the competing test ligand ^{125}I-Gal_{34}-BSA. Assays were performed in duplicate as described by Quesenberry and Drickamer, 1992 and Lobst and Drickamer, 1996. Briefly, polystyrene plates were coated with 10μg/ml MGL (HHL-416 – extracellular domain) and incubated overnight at 4 ^{o}C. Wells were blocked for 2 hours at 4^{o}C with 5% (w/v) BSA in low salt-loading buffer (150mM NaCl, 25mM Tris pH7.8, 25mM $CaCl_2$) prior to incubation of serial dilutions of ES or galactose with equal volumes of ^{125}I-Gal_{34}-BSA in binding buffer (0.3M NaCl, 50mM Tris pH7.8, 50mM $CaCl_2$, 0.2% (w/v) BSA), for 2 hours at 4 ^{o}C. Wells were washed in low salt-loading buffer and then counted for 2 minutes/well in a Packard PRIAS γ-counter. A nonlinear, least squares fitting program

(SigmaPlot, Jandel Scientific) was used to obtain *Ki* values, corresponding to the concentration of monosaccharide giving 50% inhibition of glycoprotein binding (Lobst and Drickamer, 1996).

2.18 CD4+ T cell Proliferation Assay

2.18.1 Generation of spleen cell suspensions. Spleens were removed under sterile conditions from BALB/c mice sacrificed by cervical dislocation. Spleens were placed in a Sterilin dish with 10ml medium [RPMI-1640 + 10% FCS, 2mM L-glutamine (Gibco), 100Uml^{-1} penicillin/ 100µg ml^{-1} streptomycin (Gibco)], and homogenised using pointed forceps to tear the tissue apart and clumps were broken up by passing through a 21G needle. The cell suspension was passed through a 100µm cell strainer to remove any remaining clumps and then centrifuged at 300 xg for 5mins at 4 ^{o}C. The cell pellet was then incubated at RT with 1ml ice-cold ammonium chloride (0.83%) for 2 minutes to lyse red blood cells and washed with 20ml medium to stop lysis (centrifuged at 300 xg, 5mins, 4^{o}C). Cells were resuspended in 10ml medium and counted.

2.18.2 CD4+ cell isolation and proliferation assay. CD4+ cells were isolated from the spleen cell suspension using anti-CD4 microbeads (Miltenyi Biotech) to purify on an LC column. Cells were centrifuged to remove all medium and the pellet was then resuspended in buffer (PBS/0.5% BSA+2mM EDTA) at 90µl per 10^7 cells and beads added at 10µl per 10^7 cells. The cells were incubated with the beads for 15mins on ice and then washed with 2ml of the above buffer per 10^7 cells, centrifuged at 300 xg for 10mins. The supernatant was then removed and 500µl buffer added and transferred to the LC column (previously equilibrated with 3ml of the above buffer). The column was washed with 3x3ml of the same buffer and the flow-through collected in a falcon tube. The column was then removed from the magnetic field and placed on a 15ml falcon

and CD4+ cells collected in 5ml buffer. CD4+ cell suspension and the flow-through, collectively referred to as APCs (antigen presenting cells), were centrifuged and counted. CD4+ cells were resuspended to $5x10^6$/ml and plated at 100µl per well in a 96-well round-bottom plate. They were placed at 37^{o}C while the APCs were irradiated for 7′21′′ (Irradiation facility, SAF). APCs were then centrifuged and resuspended at $2x10^6$/ml and added to the CD4+ cells at 100µl per well and transferred to a flat-bottom plate pre-coated overnight with 1µg/ml anti-CD3 (Serotec). Cells were cultured at 37^{o}C, 5% CO_2 for 3 days in a final volume of 200µl with treatments in quadruplicate as specified in Chapter 5. Proliferation was assessed by the addition of 1µCi of [^{3}H] – thymidine /well (10µl/well of 1:10 diluted [^{3}H] – thymidine) at the end of day 3 of culture. Prior to addition of [^{3}H] – thymidine, 100µl of supernatant was removed from each well for analysis or stored at -20 ^{o}C. Cells were harvested on day 4 of culture using a Tomtec harvester 96 Mach III. The level of [^{3}H] – thymidine incorporation was determined using a Trilux microbeta 1450 liquid scintillation counter.

3.19 Statistical Evaluation. Data values are expressed as the mean $\pm$ standard error of the mean (SEM). Student's t-test was used to assess the statistical significanc, except where stated otherwise. Paired t-test analysis was carried out for comparisons between groups of independent experiments. Results were taken to be statistically significant where $p<0.05$. Additionally, results were significant where $p<0.01$ and where $p<0.001$.

Chapter 3

Molecular analysis of bone marrow derived macrophage markers and purinergic receptors

3.1 Introduction

Macrophages have several biological functions ranging from phagocytosis and antigen presentation to pro- and/or anti-inflammatory responses resulting from signalling through a variety of cytokines and co-stimulatory molecules. Microbial antigens in conjunction with effector T cells (T helper (Th1), Th2 and Th3) and regulatory T cells influence the heterogeneity and state of activation of macrophages (Noel *et al,* 2004). Classically activated macrophages (CAMΦ) are the best characterised macrophage subset and this state is induced by pro-inflammatory microbial molecules such as LPS or a Th1 cytokine (IFN-γ) environment (Gordon 2003). CAMΦ characteristics include nitric oxide (NO) production and increased antigen presentation (Noel *et al,* 2004). However, a balance between pro- and anti-inflammatory responses is essential to limit tissue damage resulting from persistent inflammation that could jeopardise host survival.

In view of the fact that development of CAMΦ is inhibited by Th2 cytokines, macrophages arising in a Th2 environment were investigated and were found to perform a different activation program, resulting in a very different phenotype from CAMΦ. These alternatively activated macrophages (AAMΦ) develop in the presence of IL-4/IL-13 acting through the IL-4 receptor α chain to activate STAT6 and IRS-1 family members leading to macrophage survival and regulation of anti-inflammatory gene expression (Nelms *et al,* 1999). AAMΦ preferentially use arginase I instead of iNOS (inducible nitric oxide synthase) for metabolism of L-arginine thus skewing the pro-inflammatory phenotype of NO generation to a biosynthetic-repair phenotype.

In a murine model of nematode infection Nair and colleagues have found macrophages that display a phenotype that may be characteristic of chronic type 2 inflammation (Nair *et al,*

2003). These nematode-elicited macrophages (NeMΦ) are uniquely characterised by the ability to actively suppress proliferation of a broad range of cell types, and in addition exhibit a high level of expression of two novel macrophage genes, Ym1 and Fizz1. Fizz1, is a resistin-like molecule α (RELMα) that can promote deposition of extracellular matrix (Raes *et al*, 2002) while Ym1 is a member of the chitinase family with no known chitinase activity but possible interaction with extracellular matrix components in wound healing (Nair *et al*, 2003). It has been suggested recently that chitin, a structural polysaccharide expressed by many parasites, can induce eosinophil and basophil IL-4 production and also AAMΦ polarisation when administered *in vivo*, emphasising the ability of parasite products to influence immune environment (Kreider *et al*, 2007). NeMΦ show similar properties as AAMΦ derived *in vitro* such as the down-regulation of inflammatory cytokines (Nair *et al*, 2003; Loke *et al*, 2007) and the up-regulation of Fizz1 and Ym1 (Nair *et al*, 2005). Similar findings have been reported by Donnelly and colleagues in *in vivo* studies of *F.hepatica* infection or injection of *F.hepatica* derived molecules into mouse peritoneum which resulted in the presence of peritoneal macrophages with AAMΦ characteristics (Donnelly *et al*, 2005). Furthermore, *in vitro* cultures of *F.hepatica* secreted products with the RAW264.7 macrophage cell line showed induction of an AAMΦ phenotype determined by increased arginase I levels.

Overall, AAMΦ have been proposed to have several roles in parasite infection (Maizels *et al*, 2004). Firstly, they act to suppress inflammatory responses against the parasite as they are known to down-regulate pro-inflammatory functions of other effector cells such as T cells. In addition, their abundance in sites of infection makes them important effector cells which potentially target extracellular helminths and drive Th2 immune responses. Finally, their biosynthetic ability may be important in helminth-induced damage repair. With relevance to this

study, the activation state of macrophages is important in the case of *Trichinella spiralis* infection as a Th2 phenotype is important for parasite expulsion (Garside *et al*, 2000).

The parasitic nematode, *Trichinella spiralis*, which infects mammalian species including humans, is suggested to have evolved mechanisms for dampening immune cell responses by down-regulating the host's immune system during infection (Gounaris and Selkirk, 2005). In this scenario, it has been postulated that in order to evade host immune responses these parasites have adapted to utilise enzymes such as a secreted 5′-nucleotidase (5′NT) in the same way that the host immune system uses the endogenous ecto-enzymes, such as ecto-nucleotidases like CD39 and CD73 (Zimmerman, 2000). Extensive tissue damage to the host as a consequence of parasite invasion, results in nucleotide release which act as warning signals to the immune system. Hence, suppression of immediate inflammatory responses, leading to recruitment and degranulation of granulocytes, is achieved by enzyme action producing end-products such as adenosine that will abolish these warning signals by activating receptors linked to anti-inflammatory pathways.

Nucleotides mediate their effects by ligating purinergic receptors expressed on immune cells (as described in Chapter 1). Briefly, two distinct families of purinergic receptors have been characterised in terms of receptor coupling and the subtypes identified in terms of agonist type and affinity. The P1 receptor family consists of four G-protein coupled receptors that respond to adenosine and inosine with differential affinity. The P2 receptor family is subdivided into G-protein P2Y receptors with ligands including ATP, ADP, UTP and UDP and the ligand gated ion channel P2X receptors which are ATP receptors. Downstream signalling pathways activated following G-protein coupling of these receptors are diverse and can result in expression of a range of mediators involved in inflammatory responses, such as reactive oxygen species and cytokines.

Macrophages have major roles in host protection against excessive inflammation and pathogen invasion. Considering the emerging role of macrophages in nematode infection the aims of this study were to investigate the response of different macrophage subsets to *T.spiralis* secreted products. Firstly, this study aimed to characterise macrophages in terms of the known alternative activation markers expressed at the molecular level. In order to elucidate the molecular basis for any observed alteration in macrophage function in the context of *T.spiralis* infection, purinergic receptor profiles of the different subsets of murine macrophage activation were investigated. In addition, CD39/CD73 expression profiles were also investigated to determine their potential involvement in modulation of purinergic signalling.

3.2 Results

3.2.1 Flow cytometric analysis of macrophage purity

Bone marrow derived macrophages differentiated for 7 days with M-CSF were harvested and cultured for 20 hours with 10ng/ml LPS (CAMΦ), 5ng/ml IL-4 (AAMΦ) or medium (BMMΦ). Their purity was determined using FACS analysis to detect the macrophage markers F4/80 and CD11b (Figure 3.1). F4/80 is a 160kDa glycoprotein of the EGF TM7 family characterised as a macrophage marker by Hirsch and colleagues (Hirsch *et al,* 1981) and confirmed against the already characterised macrophage marker CD11b, also referred to as Mac-1, which is an alpha integrin (Springer *et al,* 1979). Approximately 10^6 cells were incubated with APC-conjugated F4/80 and PE-conjugated CD11b antibodies. Controls were included with unstained cells or PE/FITC conjugated IgG. The samples were analysed on the FL2 (PE) and FL4 (APC) channels of a FACS Calibur. Macrophage purity, as determined by double positive cells for both F4/80 and CD11b, was around 98% in all macrophage types as shown in Figure 3.1. Therefore, macrophage purity was not affected by the different activating stimuli used to generate the 2 activation phenotypes.

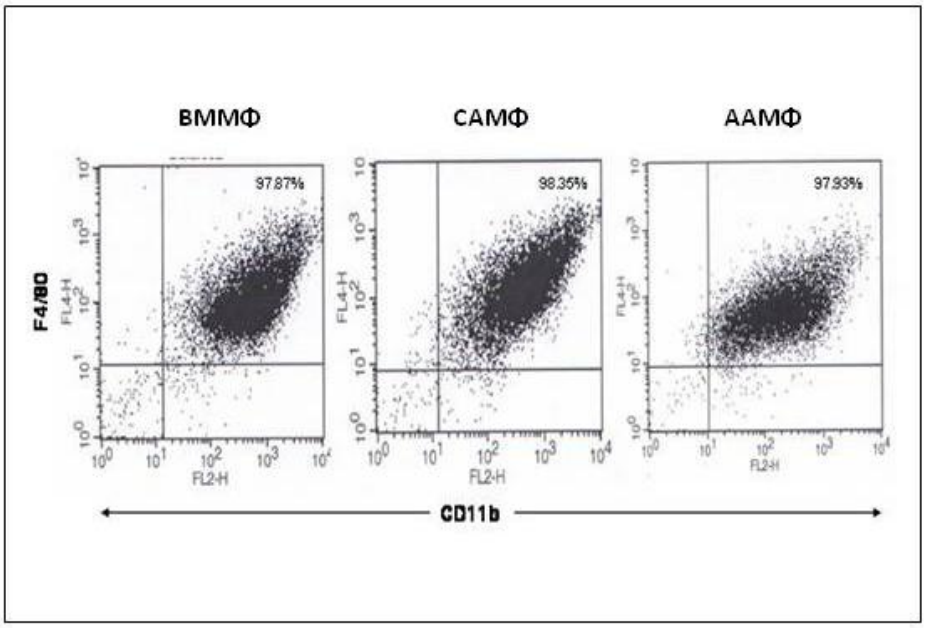


Figure 3.1: **Flow cytometric analyses of expression of F4/80 and CD11b on bone marrow-derived macrophages (BMMΦ), classically activated macrophages (CAMΦ) and alternatively activated macrophages (AAMΦ).** Percentage purity of macrophages, as determined by the presence of the markers F4/80 and CD11b, is shown in the top right quadrant.

3.2.2 Molecular analysis of macrophage marker expression

Alternative macrophage activation is characterised by the expression of certain markers, such as mannose receptor (MR), arginase I (Arg1), Fizz1 and Ym1. Therefore, the presence of the transcripts of these markers was used to distinguish between the macrophage types. cDNA was synthesised from RNA extracted from bone marrow derived macrophages (BMMΦ), classically activated macrophages (CAMΦ) and alternatively activated macrophages (AAMΦ) and was then amplified using specific primer pairs. Actin primer pairs were used as controls for the amplification reaction and as well as to determine if contaminating genomic DNA was present in the RNA used to make the cDNA (-RT control).

The MR transcript was found in all 3 macrophage types (Figure 3.2, 3.3 and 3.4). Arginase I, Fizz1 and Ym1 transcripts were present in macrophages cultured with IL-4 as early as 2 hours after IL-4 stimulation (Figure 3.4). At the 24-hour time point these transcripts appeared to be present at low levels in CAMΦ as well, while the Fizz1 transcript remained detectable even after 48 hours (Figure 3.3). Arginase I transcripts appeared in CAMΦ from the 16-hour time point, however, a non-specific product was also detected in RT-PCRs (Figure 3.3). This product, which was observed at 400-500bp, was sequenced and was found to be a result of non-specific primer binding to a cathepsin pre-protein. The sequence analysis is shown in the Appendix. Table 3.1 summarises the presence of marker transcripts detected in the 3 macrophage types by RT-PCR after 20 hours of culture, which was selected as an appropriate time point for macrophage stimulation due to consistent molecular expression of markers and purinergic receptors, shown in the following section (3.2.3).

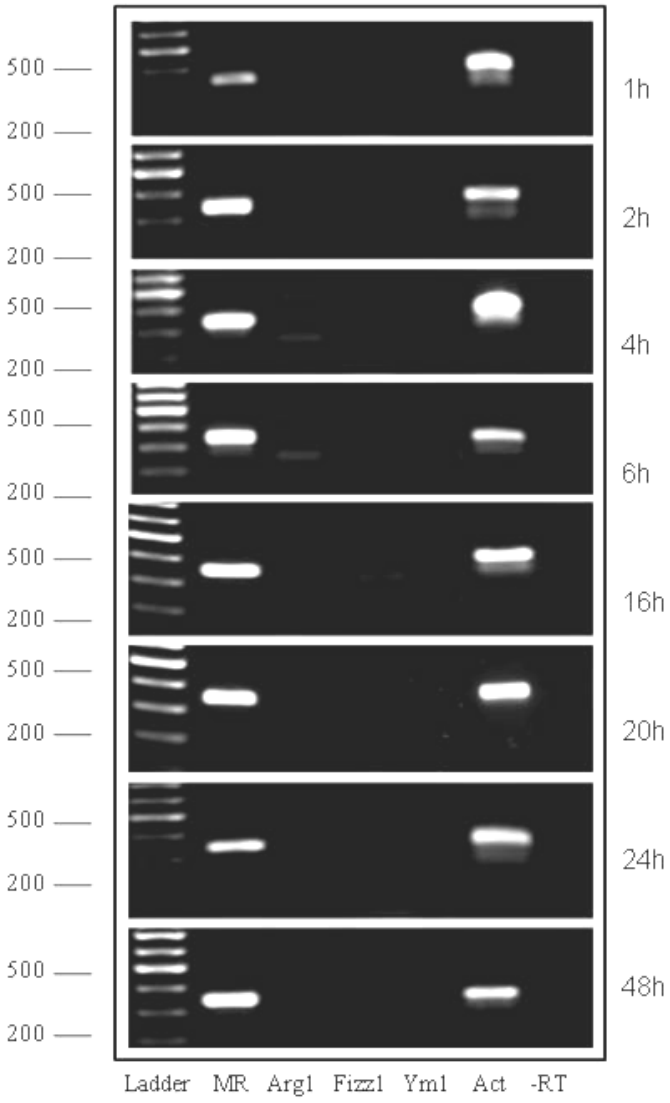


Figure 3.2: Determination of the presence of transcripts of alternative activation markers in BMMΦ following 48 hours of culture. PCR products obtained for mannose receptor (MR), arginase I (Arg 1), Fizz1 and Ym1 are shown and product sizes were determined by the ladder shown annotated on the left (in bp). Expected sizes were obtained for all transcripts detected (see Materials and Methods, Table 2.1A). Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

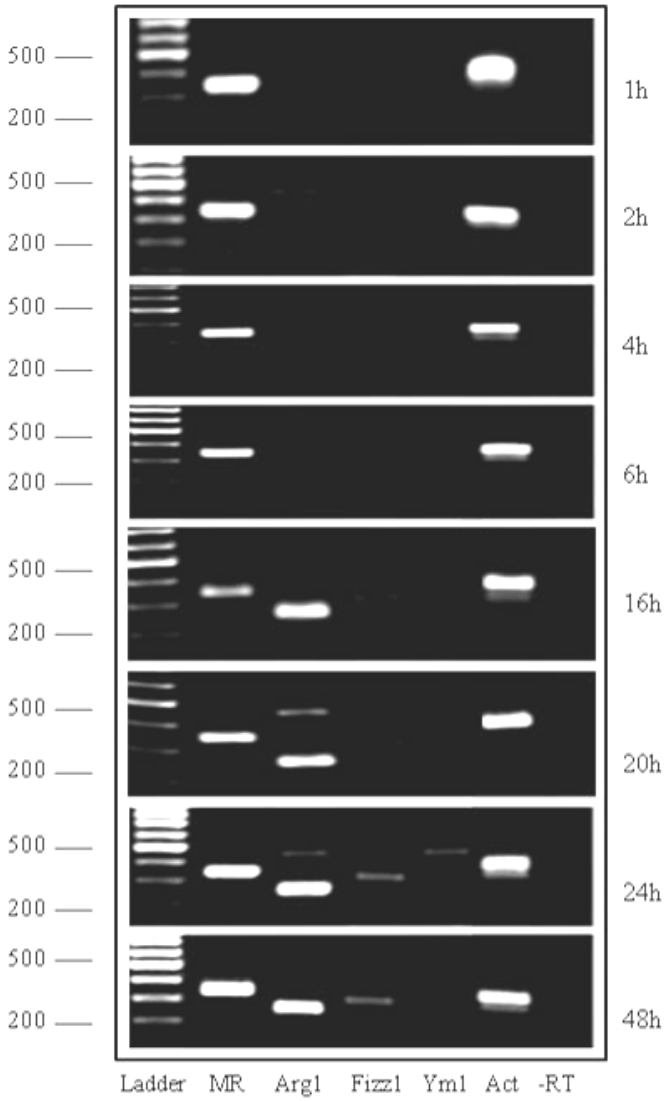


Figure 3.3: Determination of the presence of transcripts of alternative activation markers in CAMΦ following 48 hours of culture with 10ng/ml LPS. PCR products obtained for mannose receptor (MR), arginase I (Arg 1), Fizz1 and Ym1 are shown and product sizes were determined by the ladder shown annotated on the left (in bp). Expected sizes were obtained for all transcripts detected (see Materials and Methods, Table 2.1A) except the 500bp product in the arginase I lane which is non-specific, as explained in the text. Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

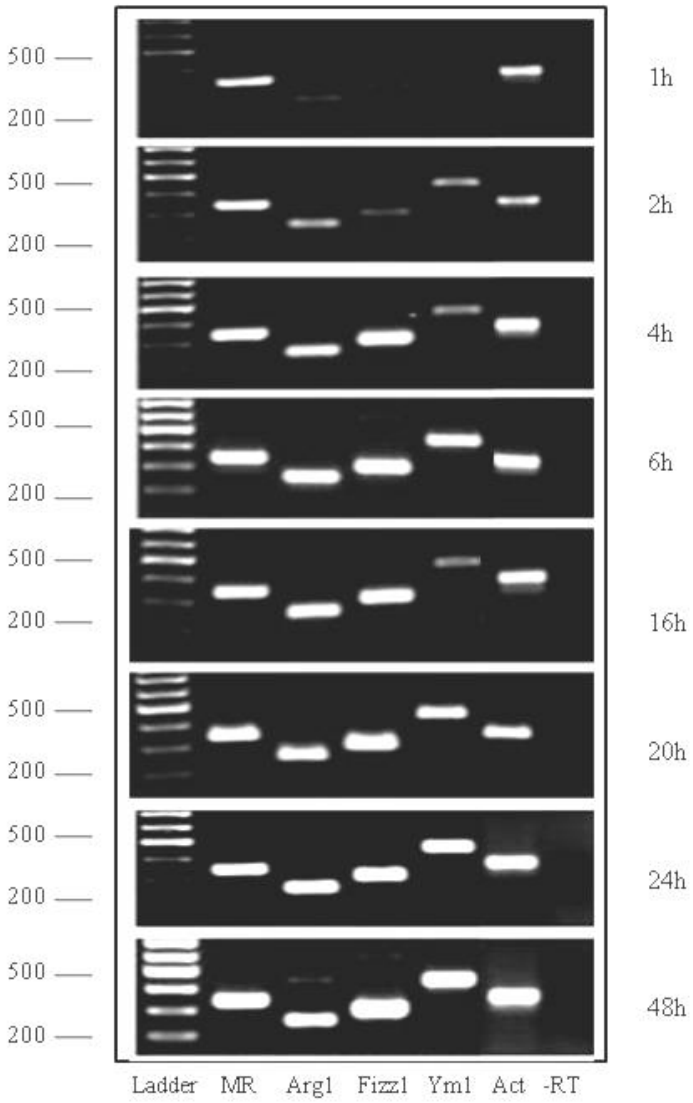


Figure 3.4: Determination of the presence of markers of alternative activation transcripts in AAMΦ following 48 hours of culture with 5ng/ml IL-4. PCR products obtained for mannose receptor (MR), arginase I (Arg 1), Fizz1 and Ym1 are shown and product sizes were determined by the ladder shown annotated on the left (in bp). Expected sizes were obtained for all transcripts detected (see Materials and Methods, Table 2.1A). Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

Marker / MΦ type	Mannose Receptor	Arginase 1	Fizz1	YM1
BMMΦ	+	-	-	-
CAMΦ	++	+	-	-
AAMΦ	++	++	++	++

Table 3.1: Summary of the presence of transcripts for markers of alternative activation in the different bone marrow derived macrophage types after 20 hours of culture. BMMΦ were cultured in standard medium only, CAMΦ were cultured in standard medium with 10ng/ml LPS and AAMΦ were cultured in standard medium with 5ng/ml IL-4. (-) = absent, (+) = present , (++) = present and abundant.

Quantitative PCR analysis was carried out to determine any differences in the relative levels of marker transcripts associated with alternative activation after 20 hours of culture. The 20 hour time point was selected for Q-PCR analysis as well as for functional assays as the molecular differences between the macrophage subsets were better defined at this time point. It is shown in Figure 3.5 that these transcripts increased only in AAMΦ while in CAMΦ only arginase I mRNA levels increased 35-fold relative to BMMΦ. As expected, this was not as high as the increase observed in AAMΦ arginase I mRNA levels (close to 900-fold). Fizz1 and Ym1 increased 10 and 150 fold, respectively, in AAMΦ when compared to BMMΦ as opposed to CAMΦ that appeared to have no difference in Fizz1 and Ym1 levels relative to BMMΦ. The mannose receptor transcript was expressed by all 3 subsets at similar levels and was only slightly up-regulated in AAMΦ by 2-fold.

In vitro generation of the alternative activation phenotype has been achieved by macrophage culture with IL-4 or IL-13 (Nair *et al,* 2003). In order to determine if marker levels are affected by a different combination of interleukins (IL-4 and IL-13) known to alternatively activate macrophages, as well as the anti-inflammatory cytokine, IL-10, Q-PCR analysis was carried out. Figure 3.6 shows the effect of combined cytokine treatment of macrophages compared to IL-4 alone. Macrophage culture in the presence of both IL-4 and IL-13 did not significantly affect marker up-regulation but appeared to slightly lower arginase I levels when compared to IL-4 alone. However, IL-10 enhanced IL-4-induced mannose receptor and arginase I up-regulation by 5-fold but had no effect on Fizz1 and Ym1. Taken together, these results show that macrophage activation states can clearly be defined in terms of expression or up-regulation of arginase I as well as Fizz1 and Ym1 transcript levels. In addition, anti-inflammatory cytokines such as IL-10 can further enhance alternative activation characteristics of AAMΦ.

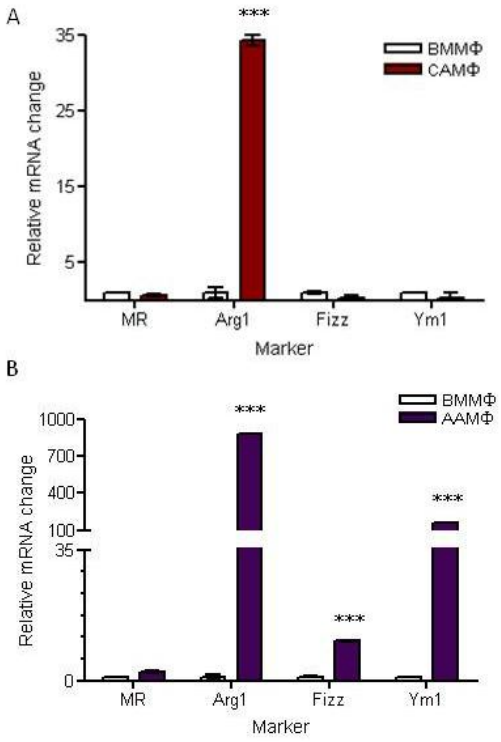


Figure 3.5: Quantitative PCR comparison of changes in alternative activation marker mRNA levels between different bone marrow derived macrophage activation types. RNA isolated from BMMΦ, CAMΦ and AAMΦ was used to produce cDNA used in quantitative PCR to determine changes of marker mRNA levels after 20 hours of cell culture with culture medium, in the presence of 10ng/ml LPS (A) or 5ng/ml IL-4 (B), respectively. The fold changes presented are relative to unstimulated BMMΦ after normalising to β-actin mRNA. Results show averages of experiments carried out in triplicate $\pm$ SEM (n = 3). Asterisks indicate statistical significance compared to absence of nucleotide stimulation. *** $p<0.001$

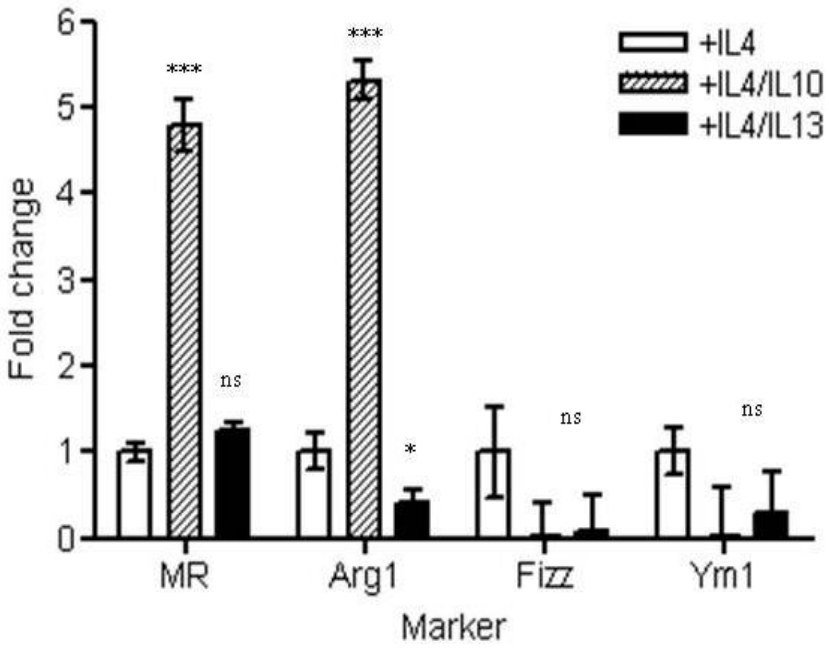


Figure 3.6: Quantitative PCR comparison of changes in alternative activation marker mRNA levels between different AAMΦ types. Bone marrow derived macrophages were activated with IL-4, IL-4/IL-10 or IL-4/IL-13 for 20 hours (5ng/ml of each cytokine) and cDNA synthesised from RNA extracted from these cells. Quantitative PCR was carried out to determine relative changes in mRNA levels of the mannose receptor (MR), arginase I, Fizz and Ym1. The fold changes presented are relative to macrophages treated with IL-4 only, after normalising to β-actin mRNA. Error bars represent standard error of the mean from one representative experiment. ns $p>0.05$, * $p<0.05$, *** $p<0.001$

3.2.3 Molecular analysis of macrophage purinergic receptor expression

Transcriptional profiling was used to determine purinergic receptor expression in macrophages. The presence of mRNA encoding these receptors was detected by RT-PCR. cDNA was synthesised from RNA extracted from unstimulated bone marrow derived macrophages (BMMΦ), classically activated macrophages (CAMΦ) and alternatively activated macrophages (AAMΦ) and was then amplified using specific primer pairs for P1 and P2 receptors. Actin primer pairs were used as positive controls for the amplification reaction and as negative controls to determine if contaminating genomic DNA was present in the RNA used to make the cDNA (-RT control).

PCR products of expected sizes were identified for the adenosine receptors. A_1, A_{2B} and A_3 mRNA was detected in all 3 macrophage subsets throughout the time course of culture (Figures 7, 8 and 9). A_{2A} transcripts were not found in BMMΦ and AAMΦ, while in CAMΦ a time-dependent expression was observed, as mRNA levels apparently increased between 2 and 6 hours of culture, remained detectable until the 20 hour time point and disappeared at 24 and 48 hours (Figure 3.8). The other P1 receptor transcripts did not appear to vary when analysed by RT-PCR. In the case of the A_3 receptor, two PCR products were obtained with the primers used. Sequencing of these products confirmed the higher molecular weight product being the correct A_3 receptor transcript while the other product was a result of non-specific binding to ferritin (light chain). Sequence analysis is shown in the Appendix. There was no contamination in the (-RT) of BMMΦ, CAMΦ or AAMΦ as shown in Figure 3.12, 3.13 and 3.14, respectively. Table 3.2 summarises the P1 receptor transcripts detected by RT-PCR in the 3 macrophage subsets after 20 hours of culture.

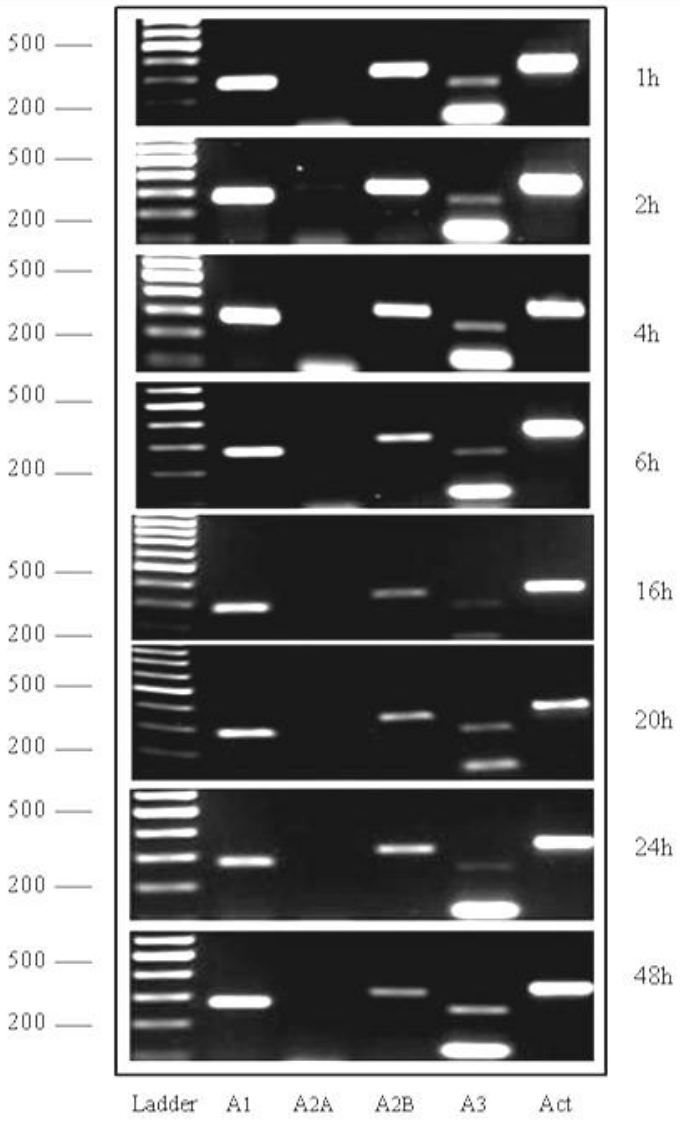


Figure 3.7: Purinergic receptor (P1R) transcripts in BMMΦ following 48 hours of culture, determined using RT-PCR. PCR products are shown and product sizes were determined by the ladder shown annotated on the left (in bp). Expected sizes were obtained for all transcripts detected (see Materials and Methods, Table 2.1C) except the 100bp product in the A3 lane which is non-specific as explained in the text. Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

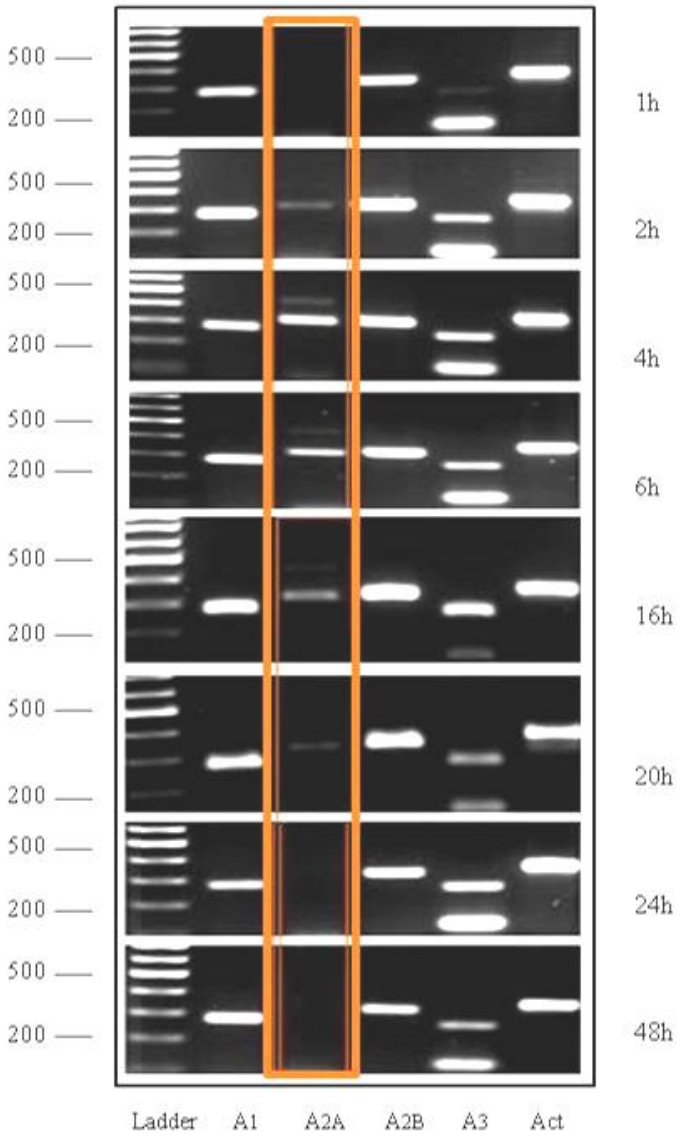


Figure 3.8: Purinergic receptor (P1R) transcripts in CAMΦ following 48 hours of culture with 10ng/ml LPS, determined using RT-PCR. PCR products are shown and product sizes were determined by the ladder shown annotated on the left (in bp). Expected sizes were obtained for all transcripts detected (see Materials and Methods, Table 2.1C) except the 100bp product in the A3 lane which is non-specific as explained in the text. The A_{2A} receptor products obtained are highlighted in the orange box as there appears to be a time-dependent regulation. Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

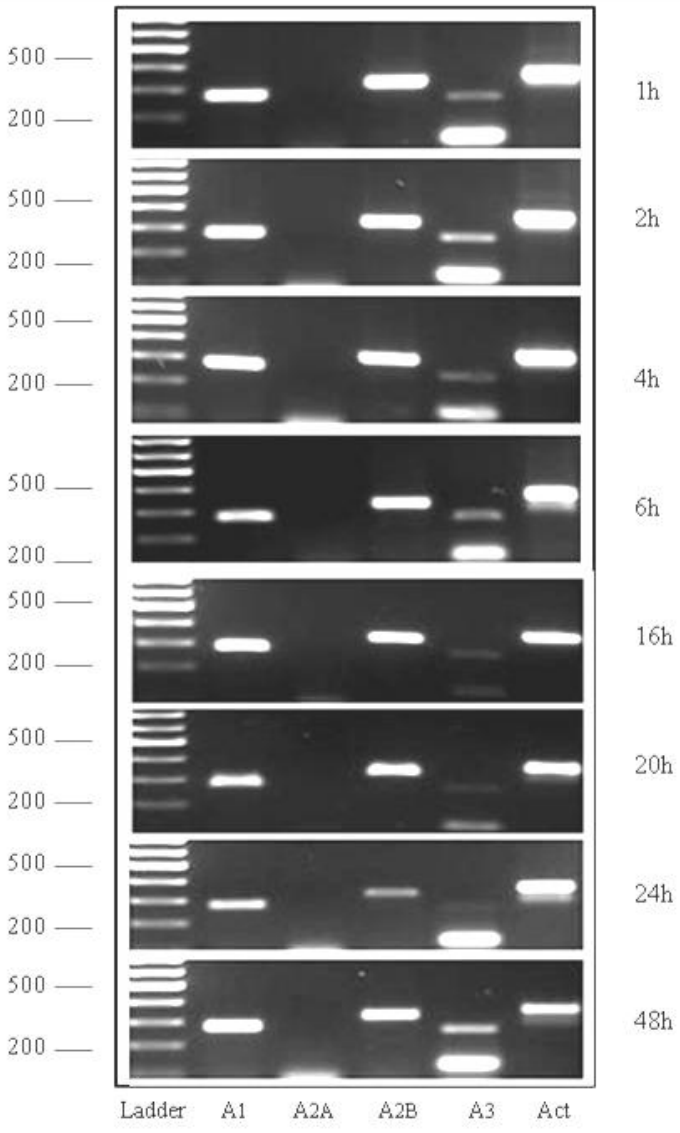


Figure 3.9: Purinergic receptor (P1R) transcripts in AAMΦ following 48 hours of culture with 5ng/ml IL-4, determined using RT-PCR. PCR products are shown and product sizes were determined by the ladder shown annotated on the left (in bp). Expected sizes were obtained for all transcripts detected (see Materials and Methods, Table 2.1C) except the 100bp product in the A3 lane which is non-specific as explained in the text. Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

P1 R / MΦ type	A1	A_{2A}	A_{2B}	A3
BMMΦ	++	-	++	+
CAMΦ	++	+ (*)	++	+
AAMΦ	++	-	++	+

Table 3.2: Summary of the presence of purinergic receptor (P1R) transcripts in the different bone marrow derived macrophage types after 20 hours of culture. BMMΦ were cultured in standard medium only, CAMΦ were cultured in standard medium with 10ng/ml LPS and AAMΦ were cultured in standard medium with 5ng/ml IL-4. (*) In CAMΦ the A_{2A} receptor showed a time-dependent expression up to 20hrs and was not detectable after 20hrs. (-) = absent, (+) = present , (++) = present and abundant.

Quantitative PCR analysis was carried out to detect any differences in P1 receptor levels between the BMMΦ, CAMΦ and AAMΦ. In Figure 3.10, the relative P1 receptor levels for the different macrophage subsets after 20 hours of culture are shown. A_1 and A_3 receptor levels appeared to be similar in all 3 subsets. A_{2A} receptor levels were 200-fold higher in CAMΦ when compared to BMMΦ while A_{2B} receptor levels were 25-fold higher in CAMΦ and 8-fold higher in AAMΦ.

The LPS time course of P1 receptor expression analysed by quantitative RT-PCR is shown in Figure 3.11. A_{2A}R transcript levels increased by 10-fold within 2 hours and reached a peak above 80-fold after 4 hours of culture in agreement with RT-PCR bands observed on the gel presented in Figure 3.8. A_{2A}R levels decreased after 6 hours of culture to 20-fold at 16 hours and remained at similar levels up to 20 hours. Similar expression patterns of the A_{2A}R have been detected in mouse peritoneal and human macrophages (Murphree *et al*, 2005). In addition, a 15-fold up-regulation of the A_3R transcript level was also observed at 4 hours which decreased at 6 hours and remained at a steady level (10-fold) until 24 hours.

These data suggest that, A_{2A} receptor expression was dependent on macrophage activation state and, in particular, LPS-induced classical activation. Furthermore, A_{2A} receptor expression displayed a time-dependent response to LPS stimulation as opposed to the other P1 receptor subtypes which were expressed steadily throughout the CAMΦ culture period.

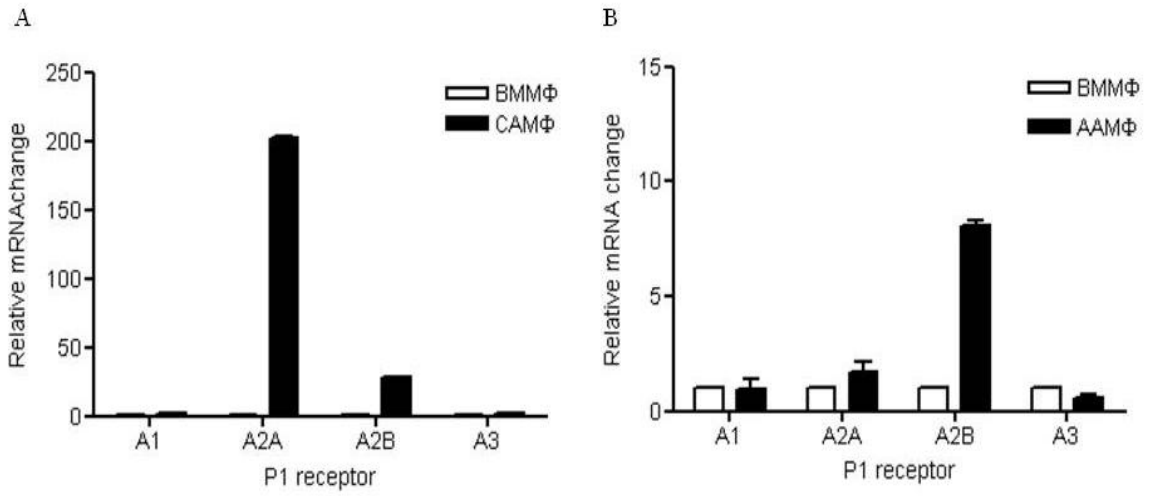


Figure 3.10: Quantitative PCR analyses of P1 receptor expression in the different activation phenotypes of bone marrow derived macrophages. Macrophages were treated with either (A) 10ng/ml LPS (CAMΦ) or (B) 5ng/ml IL-4 (AAMΦ) for 20 hours and mRNA levels of the P1 receptors determined and compared to levels detected in BMMΦ. The fold changes presented are relative to BMMΦ after normalising to β-actin mRNA and error bars show standard error of the mean from one representative experiment.

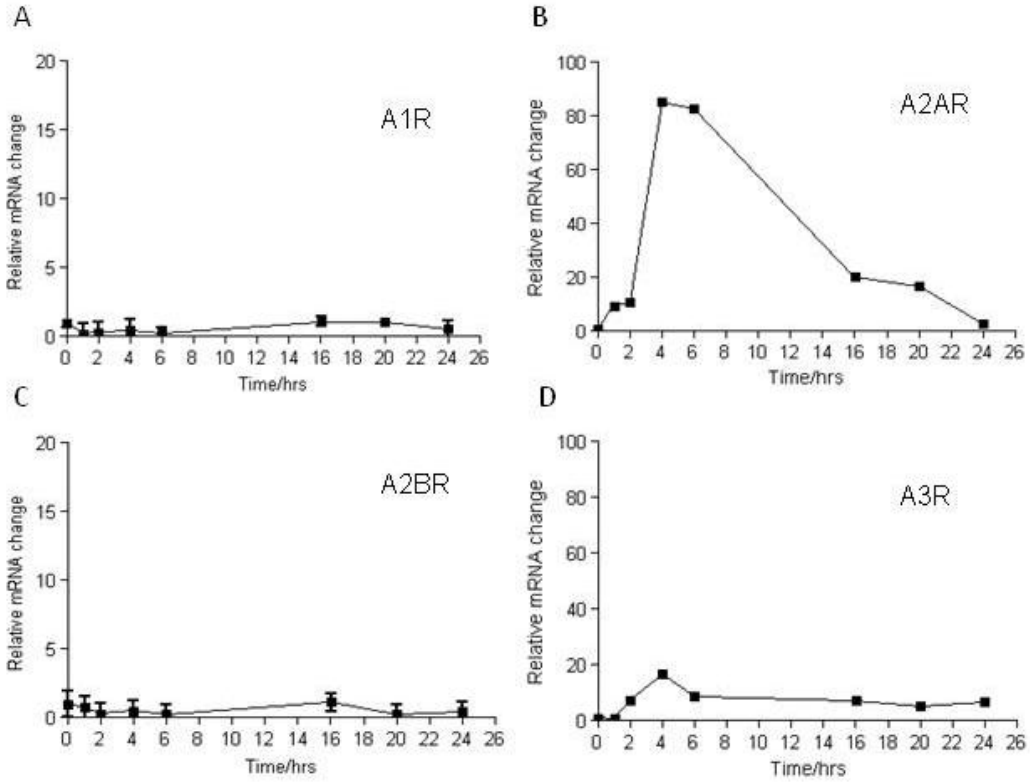


Figure 3.11: Quantitative PCR showing the changes in mRNA level of P1 receptor transcripts in CAMΦ during the 24 hour time course of culture with 10 ng/ml LPS. Fold mRNA change as well as standard errors are shown for (A) A_1 receptor (B) A_{2A} receptor (C) A_{2B} receptor (D) A_3 receptor relative to unstimulated BMMΦ, after normalising to β-actin mRNA.

The macrophage transcriptional profile of the other G-protein coupled purinergic receptors belonging to the P2Y subfamily was also characterised. P2Y receptor transcripts were detected at the expected sizes by RT-PCR (Figures 3.12, 3.13 and 3.14) and the profile detected at the 20 hour time point summarised in Table 3.3. The following receptor transcripts were present in all 3 types of macrophages: P2Y1, 2, 6, 12, 13, 14, although, P2Y13 and P2Y14 mRNA was not consistently detected exhibiting time-dependent up-regulation in all macrophage subsets. At 48 hours the P2Y14 receptor was detected at very low levels in BMMΦ and AAMΦ. The transcripts detected were not due to genomic DNA contamination as no β – actin products were obtained in the (-RT) controls (Figures 3.12, 3.13 and 3.14). The fact that transcripts of all P2Y receptors responding to ADP, ATP and UDP were present at the molecular level in all 3 macrophage subsets suggests that they can all potentially respond to extracellular nucleotide stimulation.

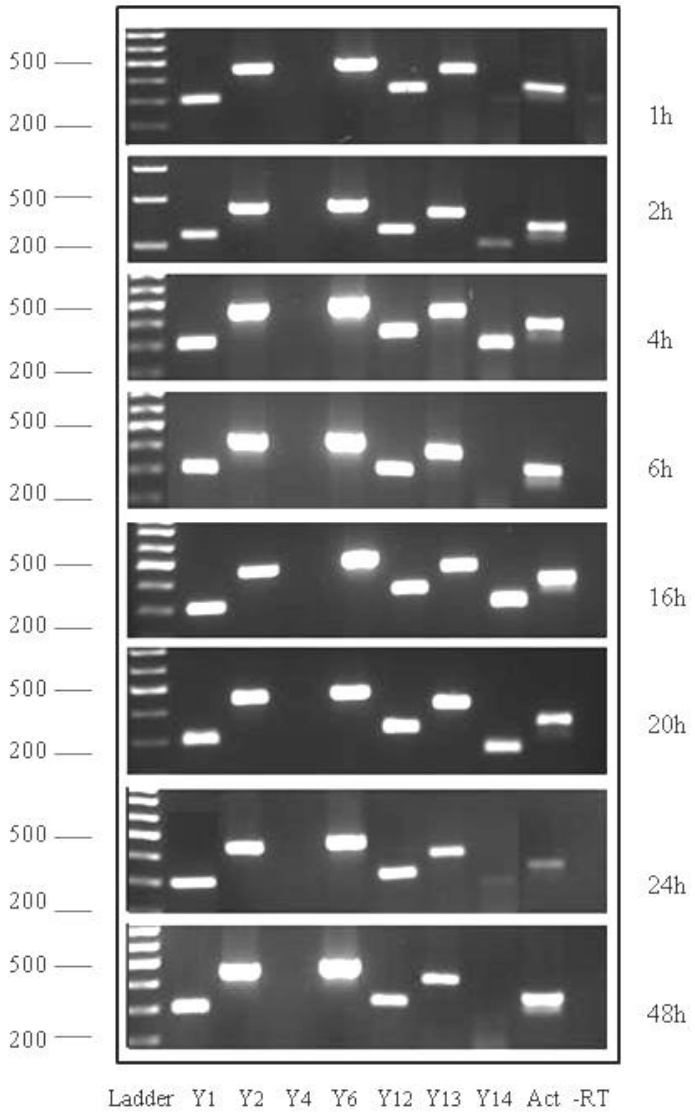


Figure 3.12: Purinergic receptor (P2YR) transcripts in BMMΦ following 48 hours of culture, determined using RT-PCR. PCR products are shown and product sizes were determined by the ladder shown annotated on the left (in bp). Expected sizes were obtained for all transcripts detected (see Materials and Methods, Table 2.1C). Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

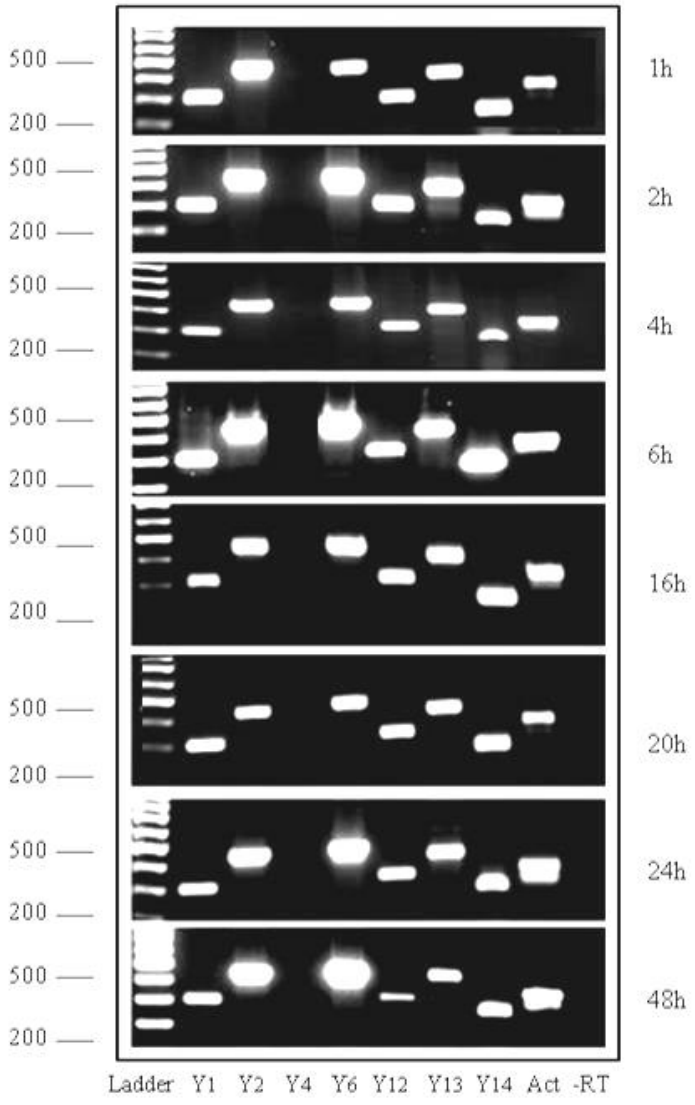


Figure 3.13: Purinergic receptor (P2YR) transcripts in CAMΦ following 48 hours of culture with 10ng/ml, determined using RT-PCR. PCR products are shown and product sizes were determined by the ladder shown annotated on the left (in bp). Expected sizes were obtained for all transcripts detected (see Materials and Methods, Table 2.1C). Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

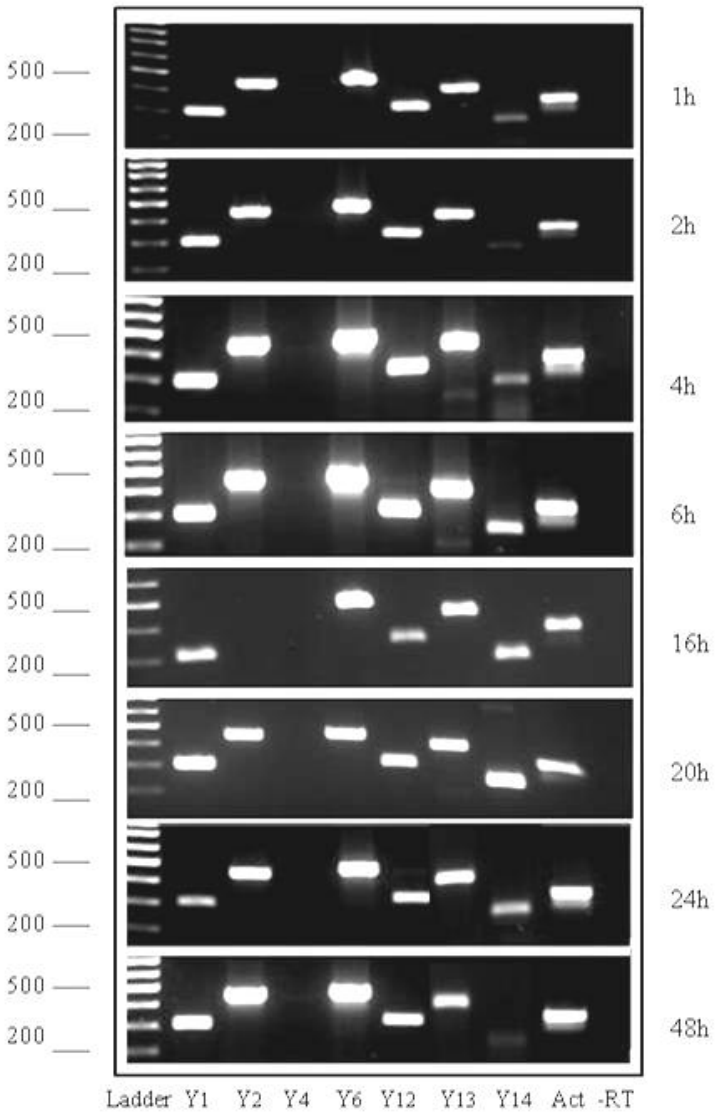


Figure 3.14: Purinergic receptor (P2YR) transcripts in AAMΦ following 48 hours of culture with 10ng/ml IL-4, determined using RT-PCR. PCR products are shown and product sizes were determined by the ladder shown annotated on the left (in bp). Expected sizes were obtained for all transcripts detected (see Materials and Methods, Table 2.1C). Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

P2R / MΦ type	P2Y1	P2Y2	P2Y4	P2Y6	P2Y12	P2Y13	P2Y14
BMMΦ	+	++	-	++	+	+	+
CAMΦ	+	++	-	++	++	++	++
AAMΦ	+	++	-	++	++	+	++

Table 3.3: Summary of the presence of purinergic receptor (P2YR) transcripts in the different bone marrow derived macrophage types after 20 hours of culture. BMMΦ were cultured in standard medium only, CAMΦ were cultured in standard medium with 10ng/ml LPS and AAMΦ were cultured in standard medium with 5ng/ml IL-4. (-) = absent, (+) = present, (++) = present and abundant.

3.2.4 Molecular analysis of macrophage ecto-nucleotidase expression

Purinergic signalling is dependent on extracellular nucleotide type and abundance. Therefore, ecto-nucleotidases present on immune cells that can affect both type and abundance of certain nucleotides are important in purinergic signalling. CD39 and CD73 are ecto-nucleotidases that have been detected on various immune cells, including T regulatory cells (Deaglio *et al*, 2007). In this study, CD39 and CD73 transcripts were detected in all the macrophage activation types as shown in Figure 3.15. No-RT controls were negative for DNA contamination (Figures 3.12, 3.13 and 3.14). Despite the fact that CD39 and CD73 transcripts were detected consistently throughout the time course tested, CD73 PCR products from BMMΦ and AAMΦ were not detected clearly at the 24 and 48 hour time point (Figure 3.15 A and C) while levels of CD73 mRNA in CAMΦ remained the same (Figure 3.15B). The decrease observed in CD73 transcripts correlates with the control β-actin levels which were also low at the 48 hour time point, which reflects poor mRNA/cDNA quality rather than an actual down-regulation of CD73 transcript levels. The rest of the time points had relatively similar levels of control β-actin so any decrease in CD39 and CD73 band intensities could be due to less abundant transcripts. Since this cannot be determined by RT-PCR as it is not a quantitative technique, quantitative PCR analysis was carried out (Figure 3.16 A, B and C). It appears that CD39 mRNA levels increased by 30-fold in BMMΦ between 16 and 24 hours of culture while a 45-fold increase was detected following 24 hours of IL-4 stimulation (AAMΦ). LPS stimulation of macrophages (CAMΦ) led to a 10-fold increase in CD39 transcript levels after 16 hours of culture and no further increase in the CD39 transcript levels was detected between 16 and 24 hours (Figure 3.16A).

CD73 mRNA levels increased 30-fold after 24 hours of BMMΦ culture and no difference was detected in the case of LPS stimulation (CAMΦ) during the same time period. In the case of IL-4 stimulation (AAMΦ), a 60-fold increase in CD73 transcript levels was detected after 24 hours of culture (Figure 3.16B). Absolute mRNA levels of CD39 and CD73 were compared in the 3 macrophage subsets after 20 hours of culture, as this was the time point selected for macrophage culture in subsequent functional assays (Figure 3.16C). It was observed that CAMΦ expressed similar levels of CD39 to BMMΦ while AAMΦ CD39 levels were 2-fold higher. CD73 transcripts were more abundant in BMMΦ when compared to CAMΦ and AAMΦ after 20 hours showing that LPS and IL-4 stimulation led to reduction in CD73 transcripts expression (Figure 3.16C).

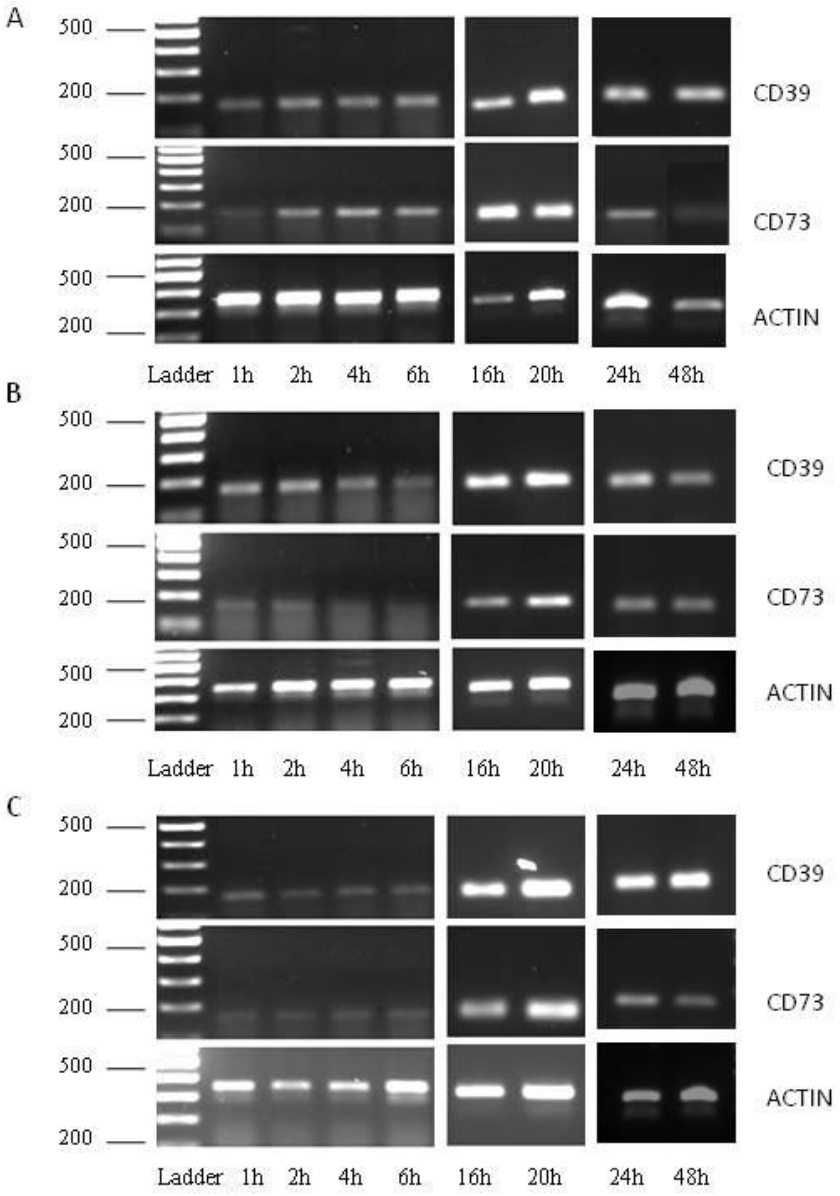
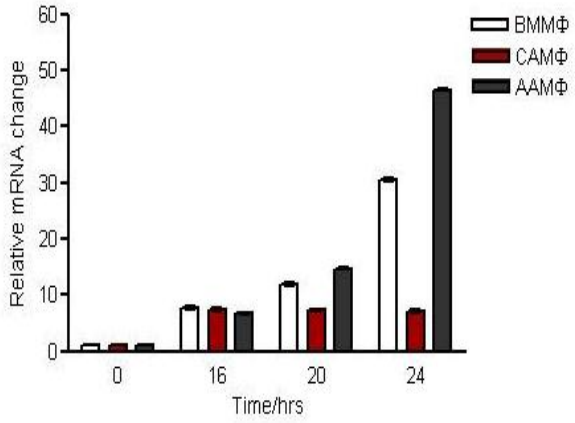
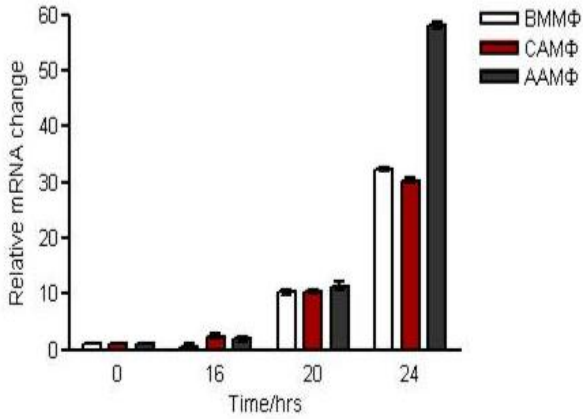


Figure 3.15: Determination of presence of CD39 and CD73 transcripts the different macrophage types. RNA isolated from BMMΦ (A), CAMΦ (B) and AAMΦ (C) markers was used to produce cDNA used in RT-PCR to detect the presence of CD39 and CD73 transcripts. PCR products of expected sizes for CD39 and CD73 were detected (see Materials and Methods) in BMMΦ, CAMΦ and AAMΦ over 48 hours of culture with standard medium, 10ng/ml LPS or 5ng/ml IL-4, respectively. Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

A



B



C

		CD39		CD73	
MΦ type	Time/hrs	Average normalised	SE	Average normalised	SE
BMMΦ	16	82.73	0.20	4.80	0.42
	20	209.94	0.5	6.30	0.25
	24	651.02	0.13	15.24	0.2
CAMΦ	16	197.60	0.14	3.83	0.42
	20	241.93	0.6	4.72	0.36
	24	245.69	0.36	14.24	0.46
AAMΦ	16	82.08	0.3	3.66	0.10
	20	432.40	0.9	5.43	0.62
	24	854.38	0.20	30.78	0.51

Figure 3.16: Quantitative PCR analyses comparing CD39 and CD73 levels in bone marrow derived macrophages. (A) and (B) Changes in CD39 and CD73 levels, respectively, at three different time points of culture for each macrophage type, relative to the respective levels detected in unstimulated macrophages. The fold changes are presented with standard error of the mean, after normalising to β-actin. (C) Table showing quantitative PCR data of CD39 and CD73 levels in BMMΦ, CAMΦ and AAMΦ compared at 16, 20 and 24 hours, after normalising to β-actin.

3.2.5 Macrophage ecto-nucleotidase surface expression and functional characterisation

Bone marrow derived macrophages were cultured for 20 hours with 10ng/ml LPS (CAMΦ), IL-4 (AAMΦ) or standard culture medium (BMMΦ) and harvested for flow cytometric analysis. The culture period selected for analysis of surface expression was selected to be between the 20 and 24 hour time point in order to see if the up-regulation detected at the molecular level affected surface expression. Cells were stained for CD39 and CD73 conjugated to FITC and PE, respectively, and analysed on the FL1 (FITC), FL2 (PE) of a FACS Calibur. Controls were included with unstained cells or PE/FITC conjugated IgG. The results obtained are shown in Figure 3.17. CD39 was detected in all 3 populations of macrophages as indicated by the shift in the trace peaks compared to the block trace representing IgG background staining (Figure 3.17). CD73 was also detected in all 3 macrophage subsets, however, the shift obtained relative to background IgG for BMMΦ and CAMΦ was not as pronounced as in AAMΦ, resulting in a slight overlap of IgG control and anti-CD73-stained cell populations. This might be due to lower CD73 expression levels in these macrophage populations when compared to AAMΦ.

In order to determine if the ecto-nucleotidases detected on macrophages were functional *in vitro*, macrophages were incubated with various nucleotides and nucleotidase activities determined by the presence of inorganic phosphate in culture supernatants. The results obtained are shown in Figure 3.18. Incubation of all macrophage activation types with either ADP or ATP resulted in detectable phosphate release after 35 minutes of culture while no phosphate release was detected in cultures with AMP (Figure 3.18A and B). These assays were carried out at a neutral pH (pH7.5-Figure 3.18A) as well as at a more alkaline pH (pH8.5-Figure 3.18B) in order to determine if the hydrolysis observed was due to alkaline phosphatase activity which is optimal at a higher pH. As shown in Figure 3.18A and B, increasing the pH did not significantly affect

nucleotide hydrolysis, therefore, alkaline phosphatases expressed on macrophages were not responsible for the phosphate release detected. This was confirmed by further experiments carried out in the presence of an alkaline phosphatase inhibitor, Tetramisole HCl which produced similar results (data not shown).

The different macrophage activation types were also incubated with nucleotides for 20 hours (Figure 3.18C) to determine their nucleotidase activity during the time period allowed for macrophage culture in experiments shown in subsequent chapters. After 20 hours, AMP was also metabolised by all macrophage types. In addition, it appears that some ATP-γ was degraded over time as the presence of phosphate in the culture medium was more pronounced after 20 hours reflecting the slowly hydrolysable nature of this ATP analogue. β-ADP was more stable than ATP-γ after 20 hours of culture as no phosphate was detected in culture supernatants. Taken together, these experiments confirmed that macrophages have functional ecto-enzymes capable of hydrolysing ATP and ADP as well as AMP, albeit with different kinetics.

The levels of phosphate present in the extracellular medium of AAMΦ stimulated by a different combination of interleukins (IL-4, IL-10 and IL-13) are shown in Figure 3.18D. There appears to be no difference between these differently stimulated AAMΦ, nor does this change the AMP metabolism.

Collectively, these data suggest that the ecto-nucleotidases CD39 and CD73 were expressed in all macrophage subsets both at the molecular and protein levels. Furthermore, these ecto-nucleotidases were expressed on the surface in a functional state and were capable of hydrolysing extracellular nucleotides *in vitro*. Their activity did not seem to be affected by macrophage activation states despite the higher expression levels detected in AAMΦ.

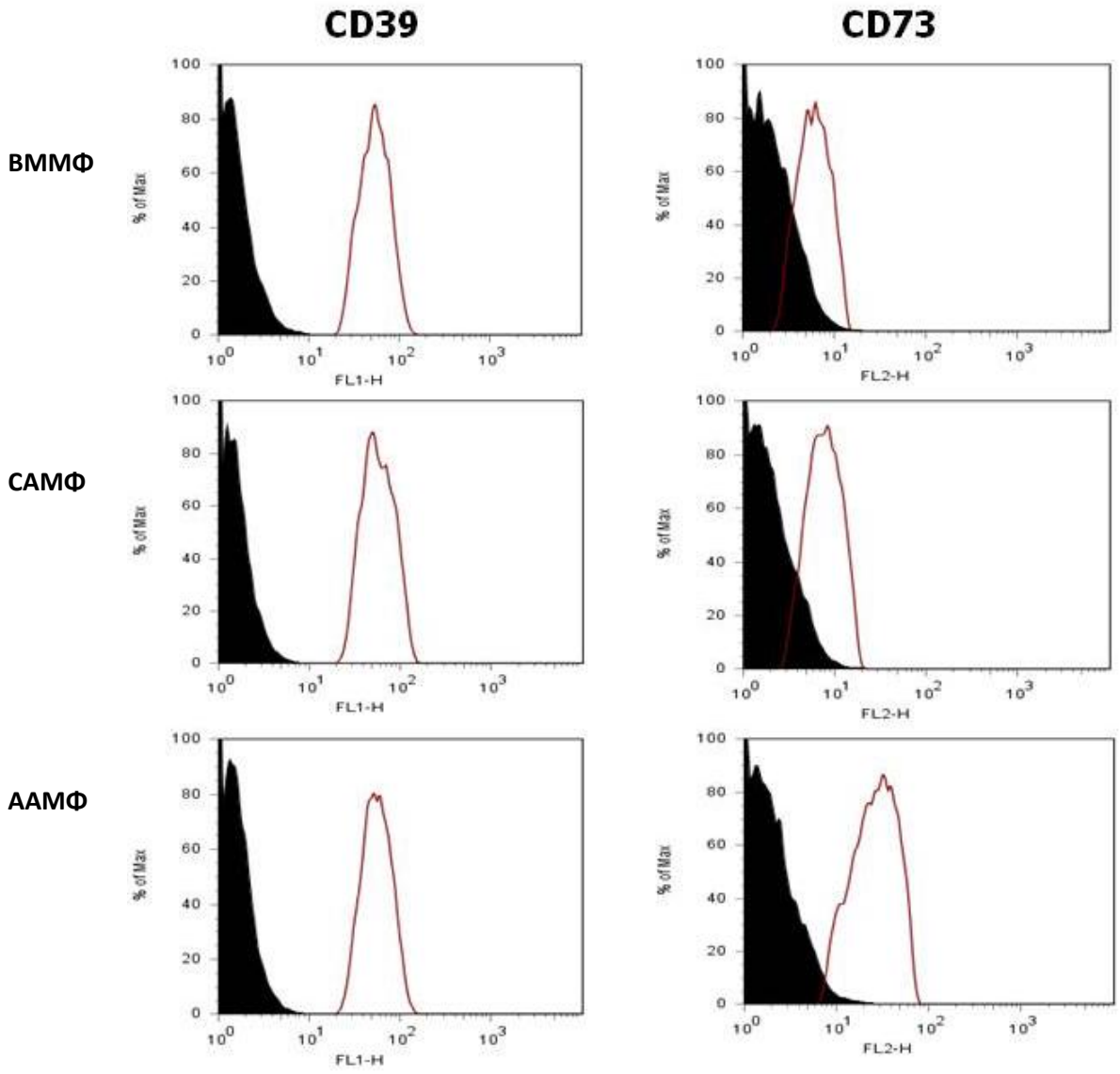


Figure 3.17: Flow cytometric analyses showing expression of CD39 and CD73 on macrophages. Bone marrow derived macrophages were harvested after 22 hours of culture with activating ligands (10ng/ml LPS or 5ng/ml IL-4). The block trace corresponds to IgG isotype control and the line trace corresponds to CD39/CD73-positive cells, presented as a % of the maximum number of cells.

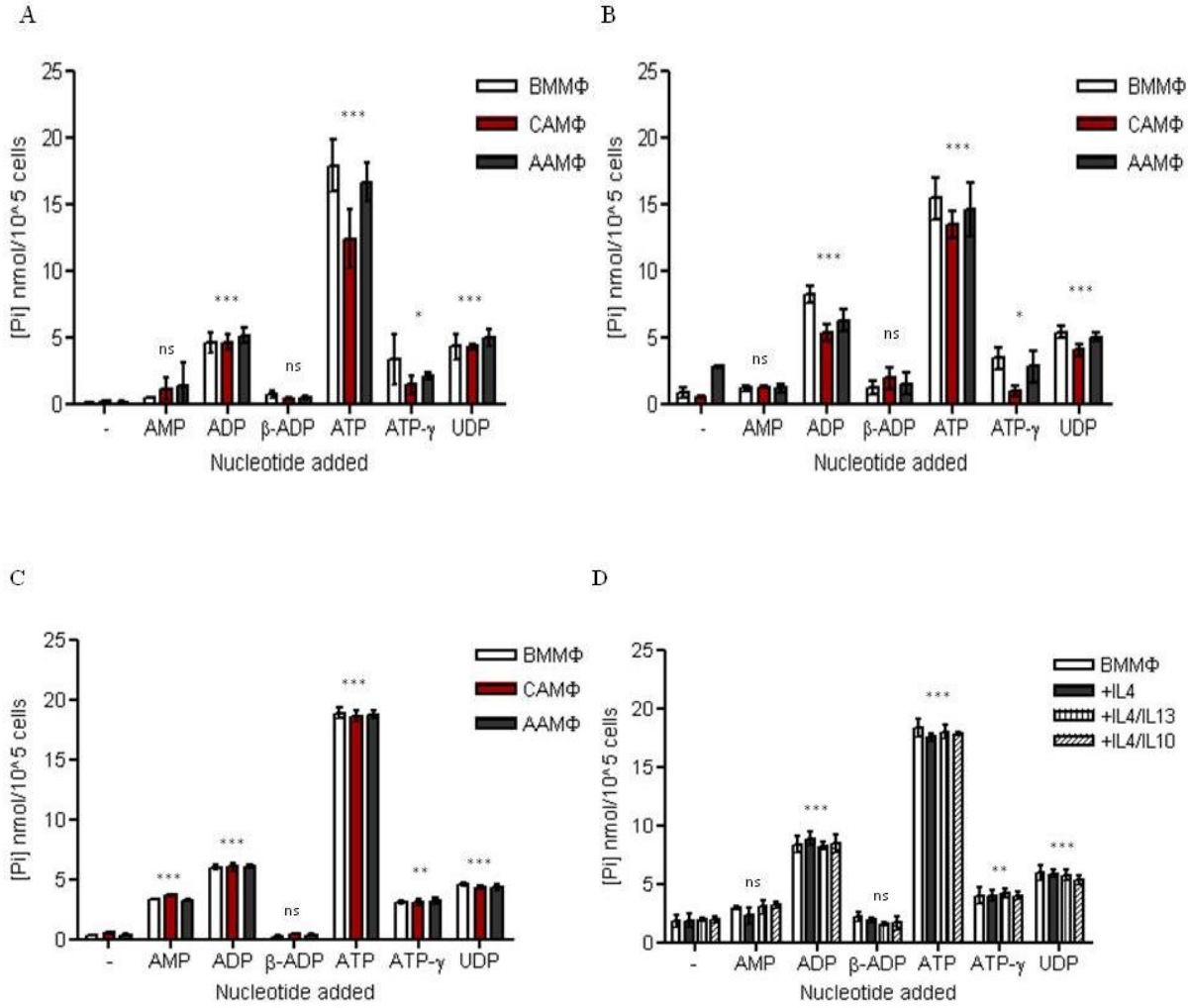


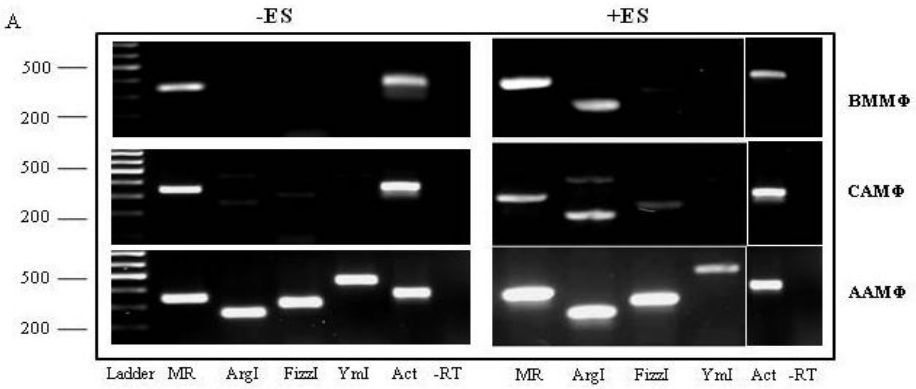
Figure 3.18: Phosphate release by macrophage ecto-nucleotidases. Nucleotides were incubated with bone marrow derived macrophage and the nmoles of phosphate released per 10^5 cells calculated. 100 μM of AMP, ADP, β-ADP, ATP, ATP-γ and UDP were added to BMMΦ, CAMΦ and AAMΦ for 35 minutes at pH7.5 (A) and pH8.5 (B), respectively. (C) The same experiment carried out at pH7.5 used to detect phosphate release from the 3 macrophage subsets after 20hrs of macrophage culture with indicated nucleotides (D) Pi release at pH7.5 from indicated nucleotides after 35 minutes from macrophages activated with different combinations of cytokines. Results show averages of experiments carried out in triplicate $\pm$ SEM (n = 3). Asterisks indicate statistical significance compared to absence of nucleotide stimulation. ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.6 Molecular analysis of the effects exerted by *T.spiralis* secreted proteins on macrophage transcript expression

Macrophages were treated with *T.spiralis* secreted proteins (ES) for 20 hours and RT-PCR was carried out in order to investigate whether they affect the expression of AAMΦ marker transcripts. As shown in Figure 3.19, arginase I levels appeared to be up-regulated in BMMΦ and CAMΦ. In addition, Fizz1 appeared clearly up-regulated in CAMΦ. There did not appear to be a difference in the AAMΦ as the levels were high in both cases. In order to determine if there were any differences in mRNA levels and to confirm the RT-PCR data, quantitative PCR was carried out. The Q-PCR data are presented in Figure 3.19B and confirm that arginase I levels indeed increased in all macrophage types. Specifically, in BMMΦ a more substantial up-regulation of arginase I levels was detected reaching 80-fold which is in agreement with the difference in band intensity observed in the Figure 3.19A. In addition, ES appeared to down-regulate Ym1 levels in AAMΦ by 6 fold. In order to characterise the nature of the ES component causing the changes observed, macrophages were cultured with ES treated to inactivate any enzyme activity (by heat inactivation) or ES treated to eliminate any carbohydrate components (by chemical deglycosylation). The effect of arginase I up-regulation observed in BMMΦ is reversed by deglycosylating ES while heat inactivating ES also partly reduces arginase I up-regulation (Figure 3.19 C).

Alternatively activated macrophages are the main macrophage phenotype detected in parasite infections (Noel *et al,* 2004). Therefore, the effect of ES on AAMΦ generated in different interleukin environments was also investigated. Figure 3.20 shows that when macrophages were activated by IL-4 in combination with IL-13 the effect of ES was similar to that observed when macrophages were activated with IL-4 alone. Interestingly, when macrophages were cultured with IL-4 and IL-10 Fizz1 and Ym1 expression was further up-regulated by ES reaching 8 and 6-fold, respectively.

A



B

MΦ type	Mannose Receptor	Arginase I	Fizz1	Ym1
BMMΦ	1.59±0.15	82.02±10.20	0.13±0.23	-0.11±0.1
CAMΦ	1.49±0.22	2.92±0.29	1.43±0.39	-0.7±0.6
AAMΦ	1.09±0.29	3.5±0.16	0.12±0.14	-6.07±0.47

C

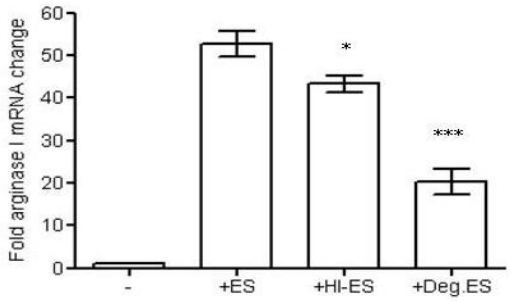


Figure 3.19: Molecular analysis of the effect of *T.spiralis* ES on bone marrow derived macrophage alternative activation markers. (A) PCR products obtained for mannose receptor (MR), arginase I (Arg 1), Fizz1 and Ym1 are shown and product sizes determined by the ladder shown annotated on the left (in bp). Expected sizes were obtained for all transcripts detected (see Materials and Methods) Actin (Act) was used as a positive control for cDNA quality as well as a negative control (-RT) for determination of potential RNA contamination. (B) Table of quantitative PCR data showing mRNA changes in response to ES when compared to the respective macrophage type cultured in the absence of ES. (C) The effect of heat inactivated (HI-) and deglycosylated (Deg-) ES on BMMΦ arginase I transcript levels, compared to unstimulated BMMΦ. Changes are calculated after normalising to β-actin mRNA. Standard errors of the mean shown are from one representative experiment of n=2 and asterisks indicate significance. * $p<0.05$, *** $p<0.001$

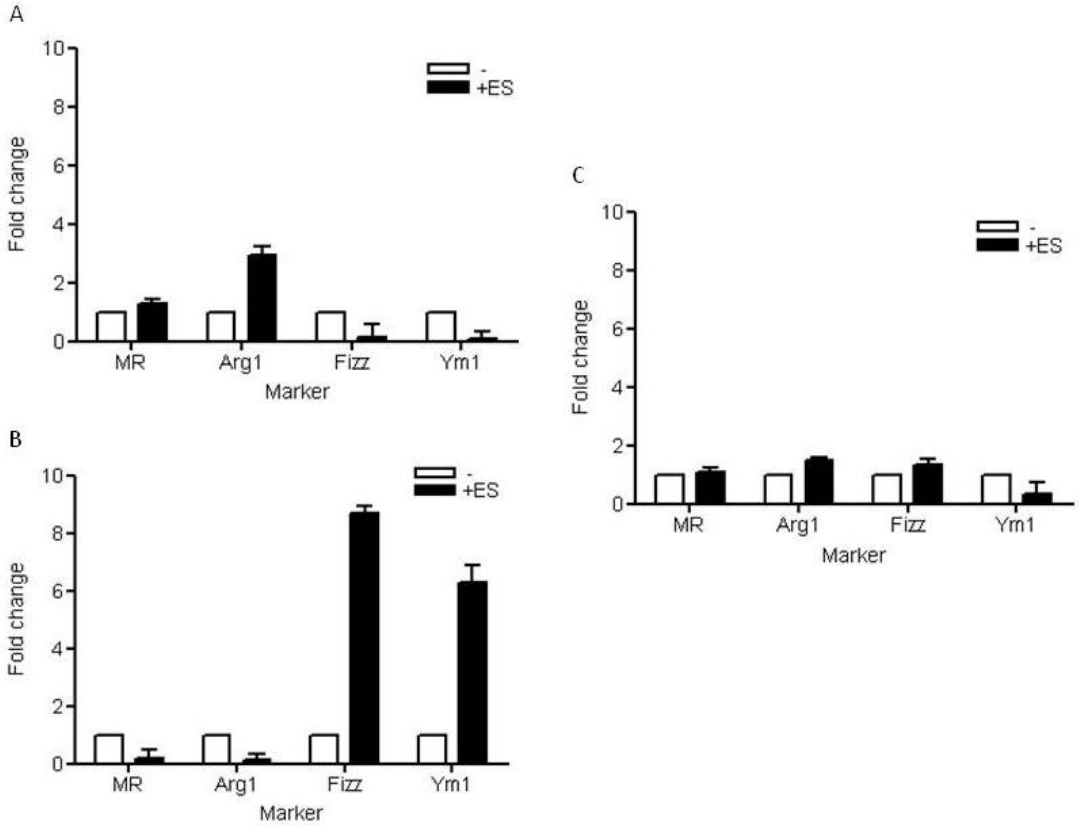


Figure 3.20: Quantitative PCR analyses of alternative activation marker transcript levels in AAMΦ treated with *T.spiralis* ES. Bone marrow derived macrophages were activated with (A) IL-4, (B) IL-4/IL-10 or (C) IL-4/IL-13 (5ng/ml of each cytokine) and co-cultured with *T.spiralis* ES for 20 hours. Mannose receptor (MR), arginase I, Fizz and Ym1 transcript levels were determined by q-PCR and the marker levels obtained by cells treated with ES were compared to activated cells cultured in the absence of ES (-). The fold changes are presented with standard errors after normalizing to β-actin.

Macrophage P1 receptor transcript levels were analysed by quantitative PCR to determine if treatment with ES altered P1 receptor expression at the molecular level. As shown in Figure 3.21A, ES induced A_{2A} receptor expression in BMMΦ as a 6-fold increase was detected, while A_3 receptor levels were increased 2-fold. This receptor up-regulation was also visible by RT-PCR shown in Figure 3.21B, especially for the A_{2A}R which was not detectable in the absence of ES. Deglycosylating ES reversed its effects while heat inactivation partly reduced the observed ES-induced effect. In CAMΦ, ES did not significantly change P1 receptor levels except for a slight inhibition of A_3 receptor transcript expression (Figure 3.21C), while in AAMΦ a small increase could be detected in A_{2A}R and A_3R levels (0.5- and 1-fold, respectively). As seen when BMMΦ were used, deglycosylating ES reduced any ES-induced effects.

Quantitative PCR analysis was also carried out to detect changes in CD39 and CD73 levels of macrophages cultured with ES. Figure 3.22 shows that CD39 transcript expression was down-regulated in BMMΦ and AAMΦ by ES, an effect reversed by deglycosylation and partly reduced by heat inactivation. CD73 was up-regulated by 3-fold in BMMΦ and 15-fold in AAMΦ which was brought down to 5-fold when ES was deglycosylated. In CAMΦ ES did not change CD73 transcript levels.

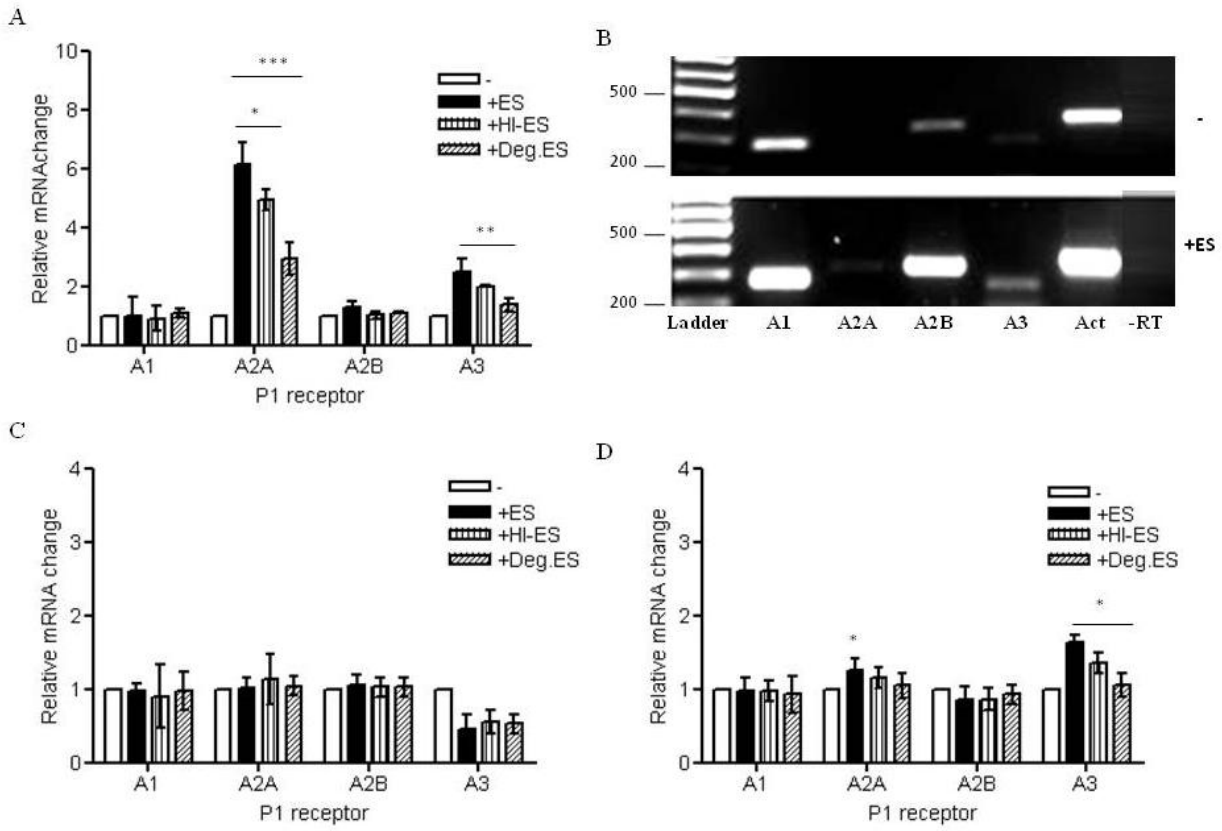


Figure 3.21: Analysis of the effect of *T.spiralis* ES on macrophage P1 receptor transcripts. Bone marrow derived macrophages cultured with medium (A and B), 10ng/ml LPS (C) or 5ng/ml IL-4 (D) were treated with *T.spiralis* ES, heat inactivated (HI-) or deglycosylated (Deg-) ES for 20 hours. RNA extracted was analysed by RT-PCR and quantitative PCR. (A) The effect of ES on BMMΦ P1 receptor transcript levels and reversal of the effect by deglycosylated ES. (B) Results from RT-PCR of P1 receptors from BMMΦ treated with ES. Quantitative PCR was carried out to determine the effect of ES, HI-ES and Deg-ES on CAMΦ (C) and AAMΦ (D) P1 receptor transcript levels. The relative mRNA changes when compared to the respective macrophage type cultured in the absence of ES (-) were calculated after normalizing to β-actin. Standard errors of the mean shown are from one representative experiment of n=2 and asterisks indicate significance. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

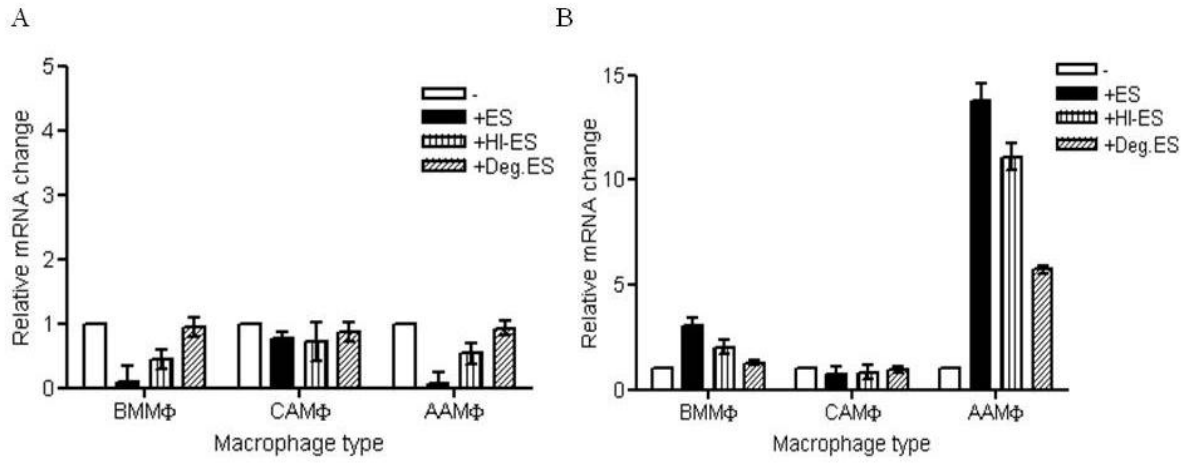


Figure 3.22: Quantitative PCR analyses of *T.spiralis* ES effect on macrophage CD39 and CD73 levels. Bone marrow derived macrophages cultured with medium (BMMΦ), 10ng/ml LPS (CAMΦ) or 5ng/ml IL-4 (AAMΦ) were cocultured with ES, heat inactivated (HI-) or deglycosylated (Deg-) ES for 20 hours. CD39 (A) and CD73 (B) levels were determined by q-PCR and compared to levels obtained by the respective macrophage type in the absence of ES (-). Relative mRNA changes presented with standard errors were calculated after normalizing to β-actin from one representative experiment of n=2.

The effect of ES on macrophage CD39 and CD73 surface expression was also investigated. Flow cytometric analyses of CD39 and CD73 expression from macrophages cultured for 20 hours with ES are shown in Figure 3.23 presented as scatter plots of 4 independent experiments. CD39 surface expression did not change in either of the macrophage subsets (Figure 3.23A and 3.23C) as opposed to CD73 expression which increased by 10% in both BMMΦ and AAMΦ cultured with ES (Figure 3.23B and 23D). In CAMΦ ES did not change ecto-nucleotidase expression (Figure 3.23E). Having established the up-regulation of CD73 surface expression by ES, further experiments were carried out with heat inactivated (HI) and deglycosylated (Deg) ES. Figure 3.24 shows that when BMMΦ (Figure 3.24A and 24B) and AAMΦ (Figure 3.24C and D) were cultured with either HI- or Deg-ES CD73 expression was restored to initial levels.

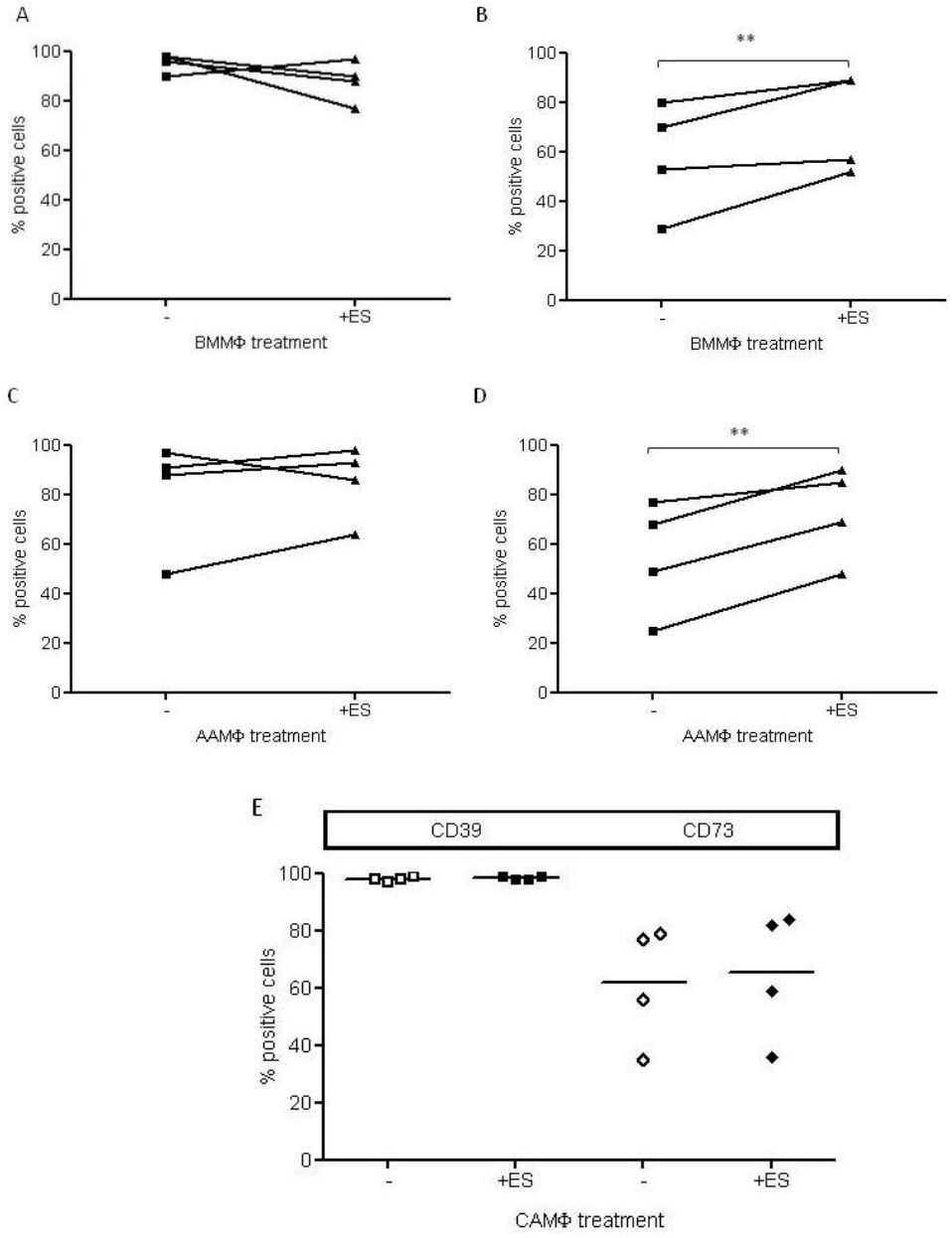


Figure 3.23: Flow cytometric analyses of the effect of *T.spiralis* ES on macrophage CD39 and CD73 surface expression. Bone marrow derived macrophages cultured with medium (BMMΦ), 10ng/ml LPS (CAMΦ) or 5ng/ml IL-4 (AAMΦ) were cocultured with *T.spiralis* ES for 22 hours and then stained with anti-CD39 or anti-CD73 antibodies for FACS analysis. Data obtained from 4 different experiments as % positive cells are presented in paired and unpaired scatter plots. (A) and (B) Plots of % positive BMMΦ for CD39 and CD73, respectively. (C) and (D) Plots of % positive AAMΦ for CD39 and CD73, respectively. (E) A combined plot of % positive CAMΦ for CD39 and CD73. Statistical significance was determined by paired analysis between data from each individual experiment and the overall significance between (-) and (+ES) is shown on the plots (** $p<0.01$).

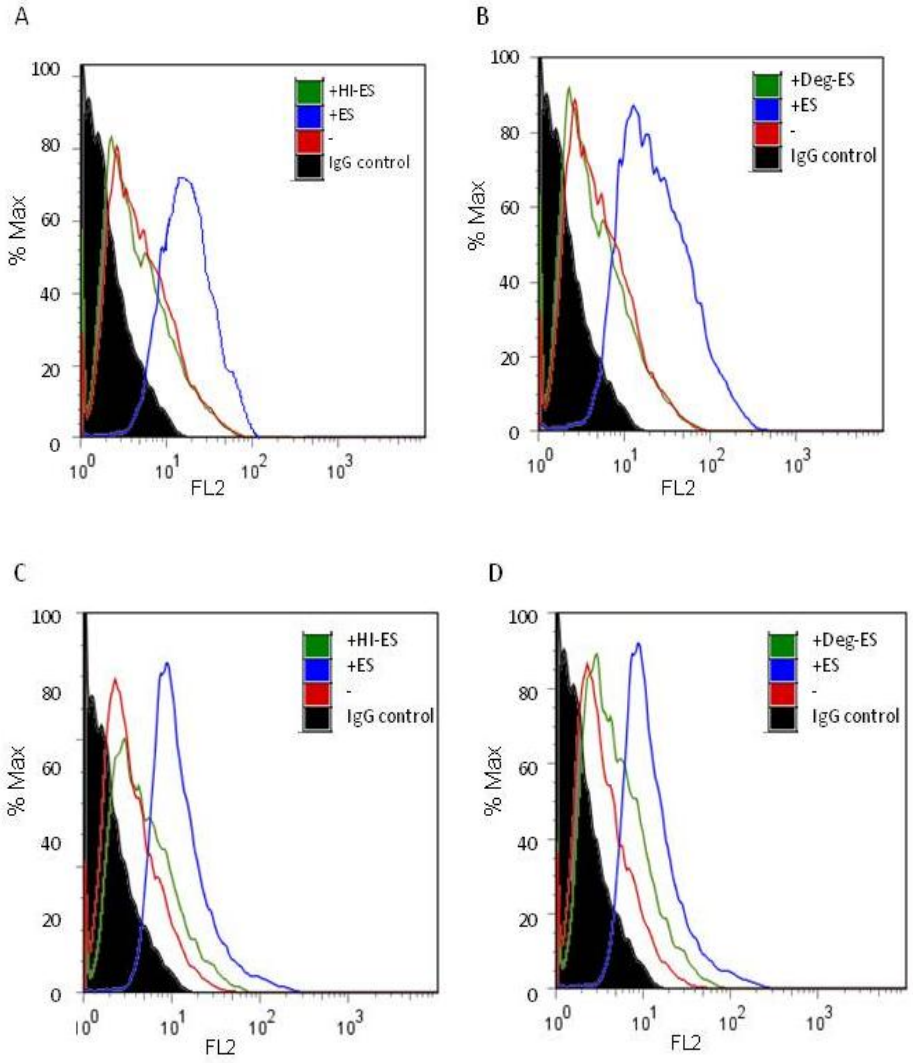


Figure 3.24: Flow cytometric analysis of *T.spiralis* ES effects on bone marrow derived macrophage CD73 surface expression. BMMΦ (A and B) and AAMΦ (C and D) were cultured with ES, heat inactivates (HI-) or deglycosylated (Deg-) ES for 22 hours and then stained for CD73. (A) and (C) Traces of BMMΦ and AAMΦ, respectively, cultured with heat inactivated ES. (B) and (D) Traces of BMMΦ and AAMΦ, respectively, cultured with deglycosylated ES. The block trace represents the IgG control, red represents cells in the absence of ES, blue represents cells with ES and green represents cells with either heat inactivated or deglycosylated ES.

The IL-4R is essential for IL-4-dependent up-regulation of alternative activation markers. Therefore, the effect of ES on macrophage IL-4R was investigated. All 3 macrophage subsets were cultured for 20 hours with ES and stained with PE conjugated anti-IL-4R antibody. Flow cytometric analysis was carried out on the FL2 channel of a FACS Calibur. Figure 3.25 shows scatter plots of the results obtained from 4 independent experiments. In BMMΦ, ES up-regulated IL-4R expression by approximately 20% (Figure 3.25A) while the increase observed in AAMΦ was less than 10% (Figure 3.25C). CAMΦ IL-4R expression did not seem to be affected by ES (Figure 3.25B). Having established that the increase in BMMΦ IL-4R expression was significant experiments were then carried out with heat inactivated (HI) and deglycosylated (Deg) ES. As shown in Figure 3.26 IL-4R expression was restored to initial levels when macrophages were cultured with either HI- or Deg-ES.

Together these results demonstrate that *T.spiralis* secreted products are capable of shifting macrophage activation to an anti-inflammatory mode by up-regulating arginase I transcript levels as well as IL-4R expression which makes cells more responsive to IL-4. In terms of purinergic signalling, the A_{2A} receptor was up-regulated at the molecular level while CD73 was up-regulated at the molecular and protein levels. Inactivation of *T.spiralis* secreted protein activity partly reduced these effects which were generally reversed by removing the glycan component by protein deglycosylation.

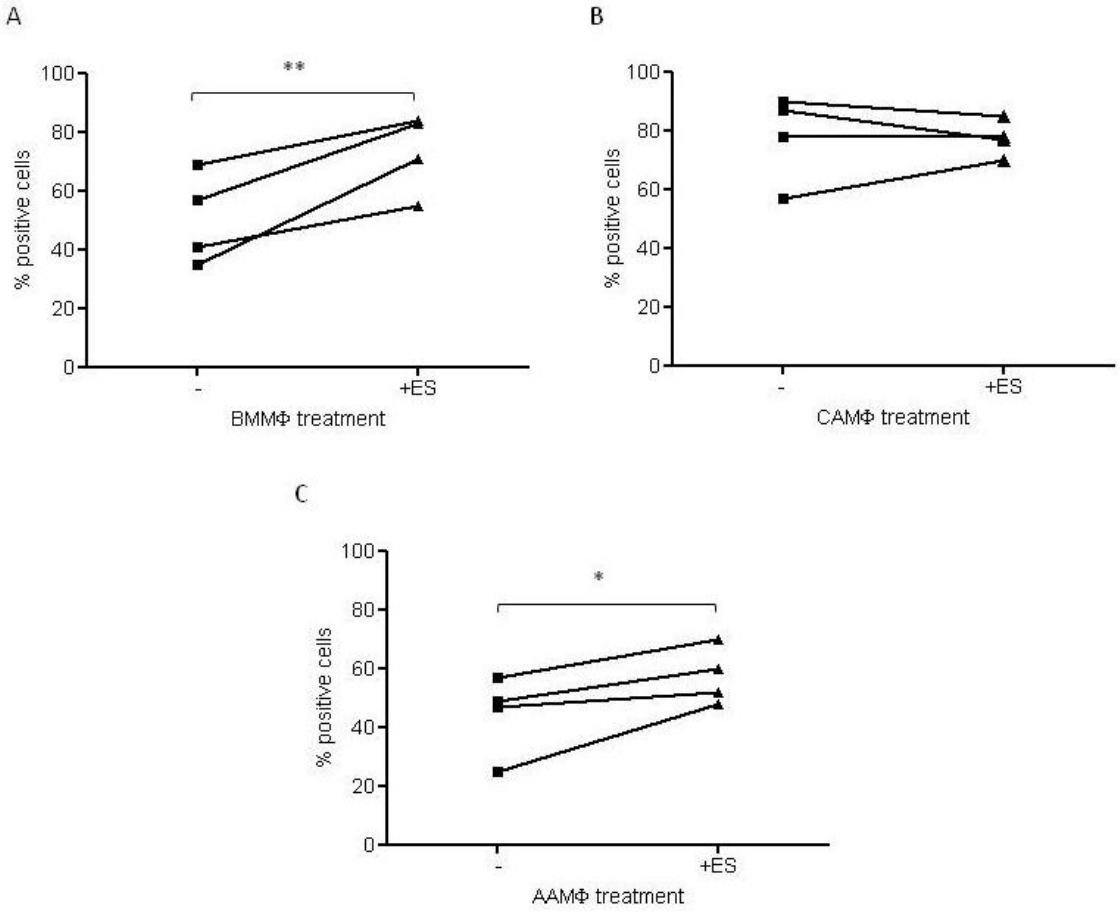


Figure 3.25: Flow cytometric analyses of the effect of *T.spiralis* ES on macrophage IL-4R surface expression. BMMΦ (A), CAMΦ (B) and AAMΦ(C) were cocultured with *T.spiralis* ES for 22 hours and then stained with anti- IL-4Rα antibodies for FACS analysis. Data obtained from 4 different experiments as % positive cells are presented in paired scatter plots. Statistical significance was determined by paired analysis between data from each individual experiment and the overall significance between (-) and (+ES) is shown on the plots (*$p<0.05$, ** $p<0.01$).

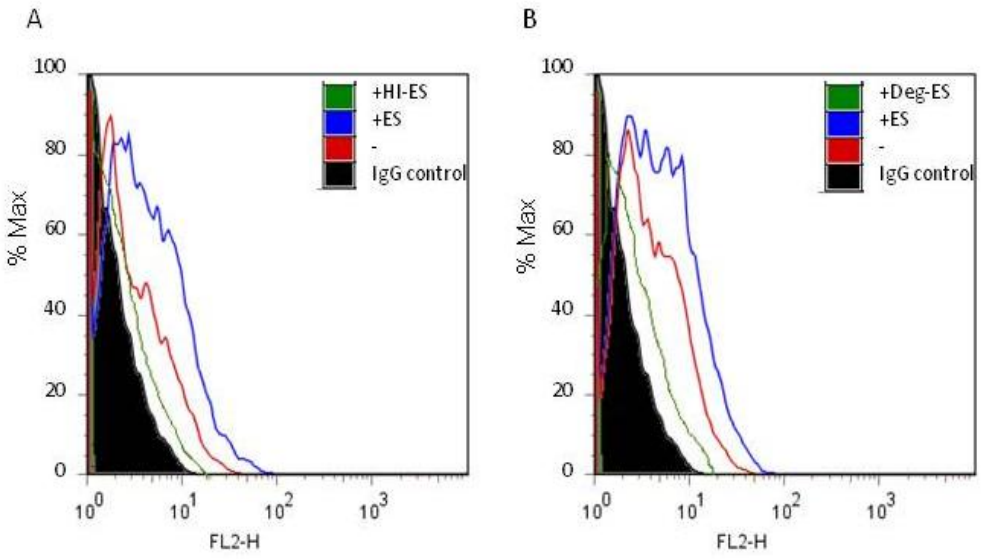


Figure 3.26: Flow cytometric analysis of ES effect on bone marrow derived macrophage IL-4R surface expression. BMMΦ were cultured with ES, heat inactivates (HI-) or deglycosylated (Deg-) ES for 22 hours and then stained for IL-4Rα. (A) BMMΦ treated with ES or HI-ES. (B) BMMΦ treated with ES or Deg-ES. The block trace represents the IgG control, red represents cells in the absence of ES, blue represents cells with ES and green represents cells with either heat inactivated or deglycosylated ES.

3.6 Discussion

Macrophages participate in the innate and adaptive immune response to pathogens as well as in different stages of inflammatory processes exhibiting phenotypic plasticity in response to local changes in their environment. Classically activated macrophages (CAMΦ) generated in a Th1 (IFN-γ) environment or in the presence of LPS are pro-inflammatory while anti-inflammatory macrophages are alternatively activated (AAMΦ) by Th2 cytokines (IL-4 and IL-13) and are involved in parasite infections (Gordon, 2003; Kreider *et al*, 2007). In this way a balance is achieved to overcome an exuberant immune response to pathogen invasion that could be deleterious to the host. In addition to the cytokine environment macrophages are capable of responding to extracellular nucleotides by means of purinergic receptors expressed on their surface. Nucleotide effects are controlled by enzymes capable of metabolising them to their respective nucleoside by hydrolysis of terminal phosphates. Consequently, different purinergic receptors are engaged resulting in switches between pro- and anti-inflammatory responses. Enzymes secreted by parasites such as *T.spiralis* have similar properties to these endogenous enzymes suggesting a way to mimic and thus evade the immune response (Gounaris and Selkirk, 2005). In this study, the aims were to characterise bone marrow derived macrophages in terms of alternative activation marker expression and, subsequently, in terms of their purinergic receptor profile. In addition, the effect of *T.spiralis* secreted products on their phenotype and activation was investigated.

Macrophages were differentiated from murine bone marrow with M-CSF and a 98% purity of the final population was determined using FACS analysis to identify surface macrophage markers, F4/80 and Cd11b. After confirming that differentiation with M-CSF

produced a pure macrophage population, macrophages were then activated with either LPS or IL-4 to produce CAMΦ and AAMΦ, respectively. In an attempt to confirm that we can distinguish between the two activation phenotypes investigated, macrophages were first characterised in terms of the known markers of alternative activation (Gordon, 2003).

The mannose receptor was first reported to be up-regulated by murine peritoneal macrophages in an IL-4 environment by Stein and colleagues in 1992 and has since been used as a marker of alternative activation. In this study mannose receptor up-regulation was not as pronounced as expected at the molecular level as in AAMΦ mannose receptor levels were only two fold higher than the other subsets. The presence of IL-13 did not alter IL-4-induced mannose receptor levels. This may be explained by the fact that mannose receptor up-regulation may be a result of increased protein synthesis and activity rather than increased levels of mRNA (Stein *et al*, 1992). Furthermore, being a receptor involved in phagocytosis it is even possible that regulation is achieved by increased recycling to the surface. Interestingly, the presence of IL-10 in macrophage culture with IL-4 induced mannose receptor up-regulation suggesting synergism with an alternative pathway for increasing its transcription. In fact, Dewals *et al* have reported an IL-4Rα-independent up-regulation of mannose receptor that appears to be achieved by IL-10R signalling (Dewals *et al*, 2010).

Arginase I is an inducible enzyme whose expression is up-regulated by Th2 cytokines and down-regulated by IFN-γ and TNF-α as confirmed in a murine model of granuloma (Hesse *et al*, 2001). A ubiquitous feature of alternative activation is the up-regulation of arginase I, which is always accompanied by an inhibition of inducible nitric oxide synthase (iNOS) (Munder *et al*, 1998). This shift in enzyme expression enhances the AAMΦ protective effect through increased proline and collagen production essential in tissue repair. In this study, arginase I was the most

robustly up-regulated marker of alternative activation expressed at high levels in AAMΦ. Arginase I transcripts were also detected in CAMΦ but at comparatively lower levels. The observation that LPS induces up-regulation of arginase I levels was first made in mouse peritoneal macrophages by Ryan and colleagues (Ryan *et al,* 1980) and further characterised by later studies as a means employed by pathogens to control iNOS activity thus diverting macrophage responses away from killing (El Kasmi *et al,* 2008). Furthermore, this LPS-induced up-regulation was found to be via a STAT6-independent pathway used for induction of C/EBPβ that involved MyD88 (Sonoki *et al,* 1997; El Kasmi *et al,* 2008). Another possible by-product of LPS-induced signalling was detected in this study. A non-specific product was observed in CAMΦ arginase I RT-PCR reactions only, which on subsequent cloning, was found to encode a cathepsin protein. As arginase I levels increased with time this product remained detectable which, taken together with its absence from BMMΦ, rules out a possible preferential primer binding that may occur in the absence of the specific arginase I transcript. A possible explanation for the presence of cathepsin in CAMΦ is the TLR-induced activation of Interferon Regulatory Factor (IRF8) which is a hematopoietic transcription factor with target genes involved in myeloid cell differentiation (Savitsky *et al,* 2010). Interestingly, Tamura and colleagues have identified cathepsin as one of the targets of IRF8 that are rapidly up-regulated upon IRF8 induction (Tamura *et al,* 2005).

AAMΦ arginase I levels were shown in this study to be further up-regulated by IL-10, which was also previously observed in a study investigating the effect of a sequentially changing cytokine environment on macrophage phenotype (Stout *et al,* 2005). Indeed, Lang and colleagues have reported that IL-10 increases IL-4R levels leading to synergistic induction of arginase I (Lang *et al,* 2002). In addition, gene profiling revealed that IL-10 induced up-regulation of

arginase I. These effects were induced via a STAT3 dependent pathway which taken with the up-regulation of mannose receptor by IL-10 observed in this study might point to a STAT3-STAT6 pathway cross-talk. In contrast to IL-10-induced effects, IL-13 appeared to slightly reduce IL-4-induced arginase I up-regulation which may be explained by the fact that IL-13Rα, which is also engaged on IL-13 stimulation, has been characterised as a decoy receptor that can dampen IL-13 responses (Wood *et al*, 2003).

Fizz1 and Ym1 were first characterised as markers of alternative activation of macrophages recruited in nematode infections (NeMΦ) (Loke *et al*, 2002). Fizz1, also referred to as RELM-α, is a resistin-like molecule which is up-regulated in pulmonary inflammation and can inhibit the effects of nerve growth factor, while Ym1 is a secretory lectin of the chitinase family with the ability to form crystals in alveolar macrophages (Loke *et al*, 2002; Nair *et al*, 2003). In this study, both Fizz1 and Ym1 were shown to be significantly up-regulated in AAMΦ and neither IL-13 nor IL-10 made a difference in their expression levels. The presence of Fizz1 and Ym1 in CAMΦ was only detected at low levels which was also seen in RT-PCR analysis presented by Raes and colleagues (Raes *et al*, 2002). These workers showed that, both Fizz1 and Ym1 were detected faintly in LPS-treated thioglycollate-elicited and resident peritoneal macrophages suggesting that an up-regulation of these markers is indicative of alternative activation.

In addition to manipulation of macrophage phenotype by the parasite, it is suggested that *Trichinella spiralis* secreted products can interfere with immune cell purinergic signalling by metabolising extracellular nucleotides (Gounaris and Selkirk, 2005). The presence of purinergic receptors on macrophages is an important determinant of nucleotide-induced effects (Bours *et al*, 2006). Macrophages were characterised in terms of their purinergic receptor profile in order to

determine if macrophage phenotype can alter responsiveness to nucleotides. During a 48 hour course of culture the P1 receptor transcripts were shown to be present consistently in BMMΦ, CAMΦ and AAMΦ except for the A_{2A} receptor which was absent in BMMΦ and AAMΦ but showed time-dependent levels in CAMΦ. Murphree and colleagues have also reported a time-dependent up-regulation of the A_{2A} receptor in response to LPS in human and mouse macrophages and have suggested this to be a protective pathway to limit inflammatory responses to bacterial infection (Murphree *et al*, 2005). Even after A_{2A} receptor levels decreased in CAMΦ, after 20 hours of culture, they were still 200-fold higher than those observed in BMMΦ. This difference in P1 receptor repertoire may influence macrophage responses to nucleotides depending on activation status as a result of downstream intracellular pathways. The P2Y receptor profiles were the same for all 3 subsets showing that they can all potentially respond to ADP, ATP and UDP. The only P2Y receptor not detected in this study was the P2Y4 receptor which responds to UTP. P2Y4 was found to be expressed in spleen macrophages and J774 by Coutinho-Silva and colleagues indicating that macrophage localisation may play a role in the expression of this receptor. Indeed, a control experiment carried out with mouse splenocytes showed that the primers used in this study can detect P2Y4 transcripts (Chapter 5).

The fact that *T.spiralis* secreted proteins can mimic the function of ecto-nucleotidases expressed on immune cells may indicate that this is one of the major mechanisms of endogenous control of purinergic signalling in the host. Recent work by Borsellino *et al*, 2007 and Deaglio *et al*, 2007 has shown that T cell subsets express the extracellular nucleotidases, nucleoside triphosphate diphosphohydrolase/apyrase (CD39) and ecto5′-nucleotidase (CD73). This finding is relevant to this study in the context of *T.spiralis* secreted products which have been shown to contain a 5′-nucleotidase which functions as both of these mammalian counterparts (Gounaris *et*

al, 2004). Conventional RT-PCR analysis did not reveal major differences in CD39 and CD73 expression between the macrophage subsets, although PCR products appeared to be less abundant than PCR products obtained for β-actin controls. Therefore, quantitative PCR analysis was carried out which revealed that the apparent increase observed in CD73 product bands in RT-PCR with time was true as CD73 levels increased significantly between 20 and 24 hours of culture in all subsets and especially in AAMΦ, suggesting a potential anti-inflammatory mechanism. In contrast to CD73, CD39 transcript levels only increased significantly in BMMΦ and AAMΦ. These observations could be important when looking at nucleotide-induced effects as, depending on the time course of macrophage culture, the presence of these enzymes could significantly alter nucleotide levels.

Nevertheless, transcriptional profiling can only give an indication of receptor expression as it can only detect the first step of the expression process. In order to determine if CD39 and CD73 are actually expressed on the surface of macrophages, FACS analysis was carried out on unstimulated and activated macrophages. It was shown that, both CD39 and CD73 were also present at the protein level, with CD39 being robustly expressed in all macrophage subsets. CD73 surface expression was actually higher in AAMΦ, a result that correlates with the increased transcript levels detected in AAMΦ. Aiming to dissect functionality of these ecto-enzymes, nucleotidase assays were carried out concurrently, and it was found to support the surface expression analysis. In these assays, ATP and ADP metabolism was evidenced by phosphate released into the extracellular medium, showing that the surface CD39 detected is indeed functional. AMP was only metabolised successfully after prolonged culture with the ecto-nucleotidases a result that can be attributed to delayed expression of CD73 or slower degradation kinetics of this enzyme. In view of this, it would appear that CD73 is the rate-limiting enzyme in

this pathway thus a possible point for regulation. In addition, the presence of these functional ecto-nucleotidases introduces a factor to be taken into account when investigating the effects of nucleotides on macrophage purinergic signalling as they would be responsible for conversion of ATP/ADP to AMP to adenosine.

Having established the basal expression profiles of markers, purinergic receptors and ecto-nucleotidases in macrophages, the effect of *T.spiralis* secreted proteins (ES) was then investigated. It is now well established that helminth infections induce an anti-inflammatory macrophage phenotype driven by Th2 cytokines (Noel *et al*, 2004). *T.spiralis* infection was also reported to give rise to macrophage responses that were not characterised by the classical activation pathway (Dzik *et al*, 2004). In addition to Th2 cytokine-induced alternative activation of macrophages, Donnelly and colleagues have reported induction of alternative activation by *F.hepatica* secreted products (Donnelly *et al*, 2005). In particular, Flynn and Mulcahy have suggested that *F.hepatica*-induced effects are due to glycans binding to TLRs (Flynn and Mulcahy, 2008). Furthermore, carbohydrates present on *S.mansoni* egg antigen (SEA) have been reported to cause a Th1 to Th2 switch via LNFPIII (lacto-N-fucopentaose) binding to surface TLRs (Okano *et al*, 2001). In view of these findings, the effect of *T.spiralis* ES on transcript and surface expression levels was investigated. Arginase I levels were increased in all macrophage subsets and more significantly in BMMΦ, probably because AAMΦ already have high basal levels of arginase I. However, Ym1 levels of AAMΦ were down-regulated by ES and were only up-regulated in the presence of IL-10 suggesting that a synergism of cytokine effects possibly operates *in vivo* to achieve the observed nematode-elicited macrophage characteristics. Indeed, Atochina and colleagues have reported that the up-regulation of Ym1 together with arginase I is initiated by LNFPIII *in vivo* (Atochina *et al*, 2008). It is worth noting that when taken together,

the effect of ES on different combinations of cytokine treatment of macrophages is additive in that different markers are up-regulated for every combination supporting the view of potential synergism *in vivo*. In an attempt to gain some insight into the mechanism of action of *T.spiralis* secreted proteins, chemical deglycosylation was carried out to remove glycan residues, or the enzyme activity eliminated by boiling. ES-induced effects were generally reversed by deglycosylation of proteins and only reduced by heat inactivation. This may be explained by the fact that boiling could have the same effect as removal of glycans because protein denaturation induces conformational changes that would reposition conjugated glycans to become dynamically unfavourable for binding macrophage surface receptors. Hence, the reason for retaining some effect after boiling is that the glycans are still present and can still bind albeit with lower efficiency.

T.spiralis ES induced a pronounced increase in BMMΦ A_{2A} receptor levels but had no effect on the existing CAMΦ receptor expression. AAMΦ also had up-regulated levels of this receptor but they could only be detected by the more sensitive quantitative PCR approach. Ecto-nucleotidase expression was also affected by ES and was detectable at the molecular and protein expression levels. In particular, CD73 transcript levels were up-regulated in AAMΦ and less so in BMMΦ while CD39 levels were reduced, an effect only observed at the transcript level as surface expression was not altered significantly. Taken together with the A_{2A} receptor up-regulation the increase in CD73 levels suggests a higher responsiveness to adenosine. The importance of the glycan component in ES-induced effects was observed again as transcript levels and surface expression of CD73, and A_{2A} receptor were restored to initial levels following deglycosylation of ES. *T.spiralis* ES had no effect on CAMΦ levels of ecto-nucleotidases and P1 receptors which may be due to some TLR interference of ES glycan binding or signalling.

Taking into account that alternative activation is at least partly dependent on the expression of the IL-4R, the capacity of ES to increase levels of this receptor was investigated. Indeed, ES but not deglycosylated ES, significantly increased the IL-4Rα surface expression on BMMΦ indicating a skewing of macrophage responsiveness to Th2 cytokines, and in particular IL-4 and IL-13. This effect was again more significant in BMMΦ suggesting that in the time frame allowed in this study changes in expression of proteins investigated occurred more readily in the naïve macrophage subset. It is possible that increasing culture periods could ultimately lead to macrophage switching as it has been reported in monocyte derived macrophages cultured for 4 days with a variety of cytokines (Porcheray *et al*, 2005). Similarly in bone marrow derived macrophages cultured for sequential 24 hour periods in different cytokine environments 'reversible adaptation' of the macrophage phenotype was achieved (Stout *et al,* 2005).

In conclusion, macrophage activation phenotypes were found to vary in more aspects than the markers investigated giving each the potential to respond appropriately in the different stages of infection. Specifically, A_{2A} receptor expression was found to be a distinguishing feature of classical activation and was only up-regulated on treatment of unstimulated macrophages with *T.spiralis* secreted proteins. CD73 which is responsible for the extracellular generation of adenosine was also up-regulated by *T.spiralis*, pointing to a possible mechanism of increasing the macrophage anti-inflammatory response as a result of adenosine-induced purinergic signalling. Furthermore, *T.spiralis* proteins can potentially skew macrophage activation by causing up-regulation of the IL-4 receptor thus making them more responsive to Th2 cytokines. Finally, the component responsible for *T.spiralis*-induced effects is most likely the carbohydrate portion of its secreted glycoproteins. Therefore, this parasitic nematode does not differ from other organisms of

its family in this direct mode of host cell manipulation as many helminths have been reported to interfere with immune cell signalling via similar structures present on their secreted products.

Chapter 4

Effect of purinergic signalling on macrophage effector functions *in vitro*

4.1 Introduction

Purinergic receptors control immune cell responses due to their ubiquitous expression on cells of the hematopoietic lineage, especially on antigen presenting cells (APC). APCs, including macrophages, B cells and dendritic cells, are the first line of defence against infection and present foreign antigen to naïve T lymphocytes using the Major Histocompatibility Complex (MHC) thereby initiating specific lymphocyte-effector pathways (Banchereau *et al*, 2000).

Distinct macrophage subsets have been found, the best characterised being the classically activated macrophages induced by pro-inflammatory microbial molecules, such as LPS, and in a Th1 cytokine environment by IFN-γ (Gordon, 2003). Classically activated macrophages (CAMΦ) have the ability to produce nitric oxide (NO), have a high capacity of antigen presentation and show increased expression of MHCII and CD86 (Noel *et al*, 2004). Their essential role in protection against intracellular pathogens is also a consequence of increased oxidative burst and NO release. In addition, their ability to secrete NO and pro-inflammatory cytokines (TNF-α, IL-1, IL-6) enhances their anti-proliferative and cytotoxic activities. However, if these pro-inflammatory effector functions are not controlled by the host the balance might shift to a more toxic environment that could be deleterious to the host. In an attempt to control excessive inflammation, macrophages can have an anti-inflammatory function when they develop in the presence of IL-4/IL-13 and are known as alternatively activated macrophages (AAMΦ) (Nelms *et al*, 1999). AAMΦ differ from CAMΦ in that they use an alternative pathway for metabolism of L-arginine thus preventing nitric oxide generation from iNOS. In addition, they do not secrete pro-inflammatory cytokines but produce IL-10 *in vivo* which is an anti-inflammatory cytokine (Gordon, 2003).

Studies have shown that nematode elicited macrophages (NeMΦ) have characteristics of alternative activation as they down-regulate inflammatory cytokines (Donnelly *et al*, 2005, Loke *et al*, 2007; Nair *et al*, 2003) and they up-regulate alternative activation markers such as arginase I, Fizz1 and Ym1 (Flynn *et al*, 2007; Nair *et al*, 2005). Inoculation of mice with the enzyme peroxiredoxin (Prx), which is an excretory/secretory product of *F.hepatica*, created a population of macrophages with suppressed iNOS mNRA levels and elevated TGF-β levels (Flynn *et al*, 2007). With relevance to *T.spiralis*, which is an intestinal nematode, parasite invasion can cause damage to the intestinal epithelium of the host which leads to initiation of inflammatory reactions. Nucleotides released by host immune cells at sites of inflammation are danger signals that trigger purinergic signalling, which is an essential means of immune cell communication. The importance of host purinergic signalling in dealing with *T.spiralis*-induced damage is emphasised by the fact that *T.spiralis* secreted proteins have nucleotide metabolising ability and can thus potentially interfere with this process. In this study, purinergic signalling was investigated in different macrophage types in order to determine if macrophages activated in different stages of inflammation and infection altered their responses to nucleotides.

The purinergic receptors investigated in this study are G-protein coupled receptors (GPCRs) and depending on the G-protein subunit associated with each receptor the downstream events that ensue following receptor ligation have different outcomes on macrophage effector functions. Specifically, stimulatory G-protein signalling results in cAMP generation or Phospholipase C (PLC) activation, while inhibitory G-protein signalling prevents cAMP accumulation. In addition, Toll-like receptor (TLR) ligation on classically activated macrophages ultimately leads to activation of NF-κB, the major transcription factor involved in pro-inflammatory gene transcription. NF-κB is found in the cytosol of unstimulated cells and upon

stimulation it translocates to the nucleus following phosphorylation and degradation of its inhibitory binding protein, IκB (Rothwarf and Karin, 1999). Pathways leading to NF-κB activation are overlapping and redundant and may be subject to interference by other pathways originating from G-protein activation at the cell surface. For example, PI3 kinase, which is known to be involved in pro-inflammatory responses, mainly due to the fact that NF-κB is one of its downstream targets, can be activated both by TLR signalling and GPCR signalling (Dos Santos *et al*, 2007). Therefore, cross-talk between these pathways can lead to unique activation profiles of macrophages that encounter extracellular nucleotides.

Macrophages are known to express purinergic receptors, as shown in Chapter 3, which are important, among other surface proteins, in host protection against excessive inflammation and pathogen invasion. Firstly, this study aimed to functionally characterise bone marrow derived macrophages in terms of known effector functions like nitric oxide release, arginase I activity and cytokine release. Then, the effect of purinergic receptor activation on these functions was investigated by treating the different macrophage types with nucleotides such as AMP, ADP and ATP as well as adenosine, the product resulting from adenine nucleotide hydrolysis. Finally, functional purinergic receptors involved in macrophage responses to nucleotides were identified by using a variety of selective purinergic receptor antagonists and agonists. Furthermore, an attempt was made at characterising intracellular signal transduction pathways triggered by purinergic receptor activation.

4.2 Results

4.2.1 Effect of extracellular nucleotides on murine macrophage effector functions

Nitric oxide release vs arginase I activity

Nitric oxide (NO) is a key effector molecule of CAMΦ only as it is released in response to stimulation by IFN-γ or microbial products like LPS. NO release was assayed to determine the effect of nucleotides on bone marrow derived macrophages (BMMΦ), CAMΦ and AAMΦ. In order to determine the effect of nucleotides on NO release and arginase I activity, macrophages were stimulated with a range of LPS or interleukin-4 (IL-4) concentrations to obtain an optimal concentration for further experiments. Firstly, the optimal concentration of LPS required to trigger robust NO release by macrophages was investigated (Figure 4.1A). The first concentration that produced significant levels of NO release was 0.5ng/ml LPS while 10ng/ml produced a robust response, which was selected for classically activating macrophages in all further experiments. In order to determine an optimal concentration of IL-4 for alternatively activating macrophages, a range of IL-4 concentrations was used to activate macrophages and the resulting phenotype assayed by arginase I activity (Figure 4.1B). The arginase I activity assay was carried out as described by Corraliza and colleagues (Corraliza *et al*, 1994). Arginase I activity appeared to be robustly and consistently up-regulated at 5ng/ml of IL-4 so this concentration was selected for further AAMΦ experiments. Characteristic of alternative macrophage stimulation is an up-regulation of arginase I activity leading to a shift of L-arginine metabolism from iNOS to arginase. The arginase I activity of macrophages was assayed to determine differences in L-arginine metabolism and a possible shift of phenotype. In Figure 4.1D a comparison of arginase I activity between macrophage activation subsets is shown. CAMΦ have significantly higher levels of arginase I activity than BMMΦ but not as high as AAMΦ which have close to 10-fold higher.

L-arginine was administered exogenously to see if the low arginase I activity measured in CAMΦ was due to substrate depletion by iNOS. As can be seen in Figure 4.1D, arginase I activity was not enhanced by administering L-arginine to CAMΦ. In addition, the levels of NO released by CAMΦ were actually enhanced by the lower concentration of exogenous L-arginine administered, while five-fold more L-arginine (10mM) enhanced NO release levels to a lesser extent (Figure 4.1C). Exogenous L-arginine administered to AAMΦ did not enhance arginase I activity levels and became inhibitory at the higher concentration used (Figure 4.1D). In addition, a slight increase in NO release by AAMΦ was detected at the higher L-arginine concentration which, although not visible in the graph when compared to NO release by CAMΦ, was significant when compared to background levels of NO released by AAMΦ.

Having selected optimal concentrations of LPS and IL-4, BMMΦ, CAMΦ and AAMΦ were incubated with nucleotides in order to determine their effect on NO release and arginase I activity. Stimulation by nucleotides did not cause NO release by BMMΦ and AAMΦ (Figure 4.2A). It is shown here that, all nucleotides tested inhibited NO release from CAMΦ, except for the slowly hydrolysable analogue of ADP (β-ADP) and UDP (a P2Y6 ligand) (Figure 4.2B). This shows that ADP-induced inhibition of NO release by CAMΦ is not due to P2Y receptor activation so it could be a result of ADP hydrolysis to adenosine in culture which acts on P1 receptors. In addition, P2Y6 receptor activation does not cause inhibition of NO release either. ATP-γ, which is a slowly-hydrolysable analogue of ATP, also inhibited NO release to some extent (30% decrease) but this inhibition was less pronounced than the 50% reduction in NO release induced by adenosine and NECA (an analogue of adenosine that is not taken up by nucleoside transporters). This shows that the inhibition of NO release caused by ATP may either be due to P2Y receptor activation or P1 receptor activation by adenosine generated in culture.

Only one concentration of nucleotides is shown here that was selected from a titration carried out for all nucleotides (shown in Appendix, Figures A.5 and A.6).

In order to investigate if inhibition induced by purinergic receptor activation required the involvement of a specific TLR, different TLR ligands were used to activate macrophages. The effect of nucleotides on CAMΦ resulting from TLR4 (Ultrapure LPS) and TLR2 (Pam3C) activation, respectively, is shown in Figure 4.2C and D. It appears that TLR activation of any kind resulted in the same inhibition of NO release following purinergic receptor activation.

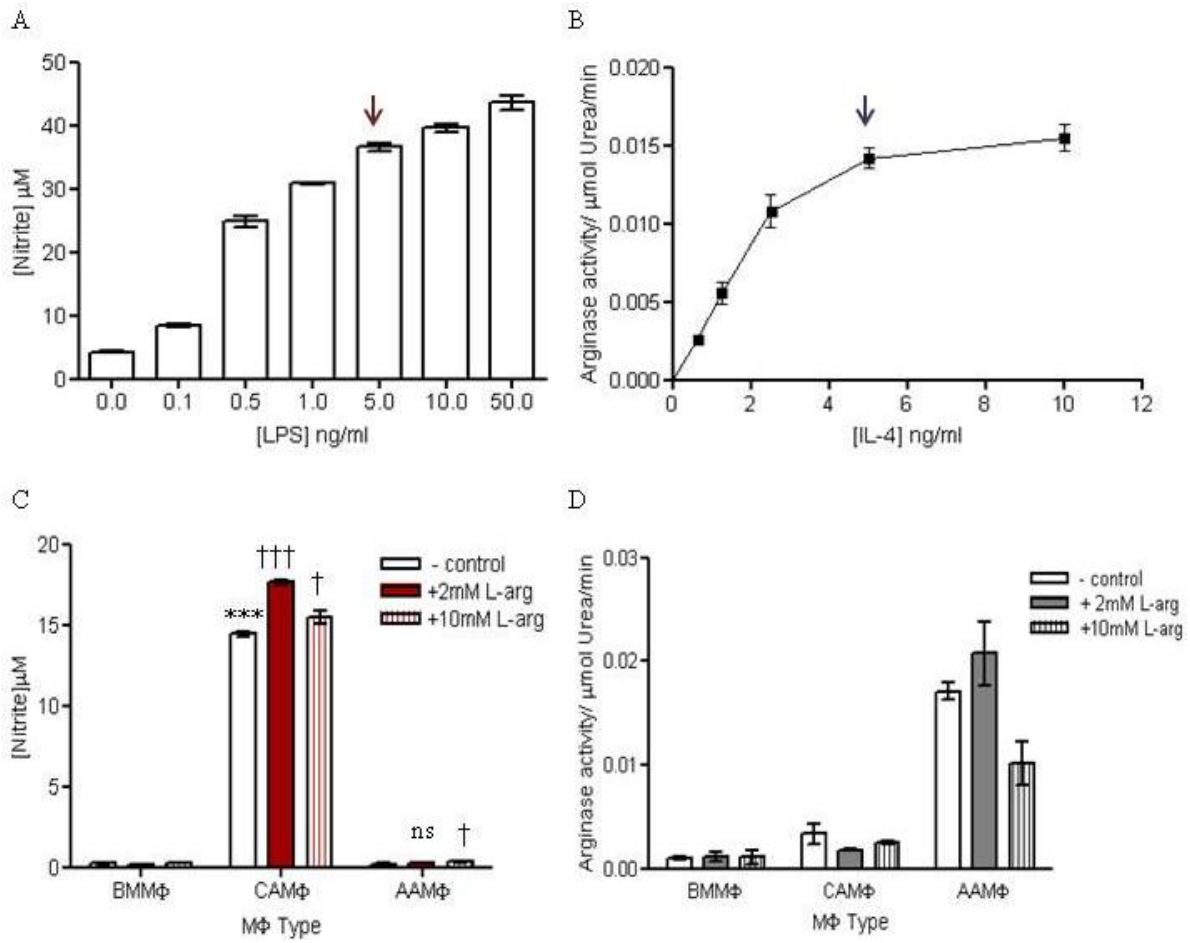


Figure 4.1: **Bone marrow-derived macrophage characterisation in terms of nitric oxide (NO) release and arginase I activity. (**A) The effect of a range of LPS concentrations on nitric oxide (NO) release by bone marrow-derived macrophage (BMMΦ). Levels of NO are expressed in µM nitrite measured in the supernatants of BMMΦ stimulated with different concentrations of LPS for 20 hours. (B) The effect of a range of IL-4 concentrations on arginase I activity of bone marrow-derived macrophage (BMMΦ). Macrophages were stimulated with increasing IL-4 concentrations for 20 hours to determine the optimal concentration required to obtain a robust response. Arginase I activity is expressed as µmol Urea/min per 5 x 10^4 cells. Arrows indicate the concentration of LPS and IL-4 selected for subsequent experiments. (C) A comparison of the levels of NO released by different BMMΦ activation subsets. Macrophages were classically or alternatively activated macrophages with 10ng/ml LPS or 5ng/ml IL-4, respectively and co-stimulated with exogenous L-arginine at 2mM and 10mM for 20 hours. Levels of NO release are expressed in µM nitrite. (D) A comparison of the levels of arginase I activity in the BMMΦ activation subsets. Data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3. (*) is compared to untreated BMMΦ. ** $p < 0.01$, *** $p < 0.001$. (†) is compared to the untreated control of the respective macrophage type. † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$, ns - not significant ($p > 0.05$).

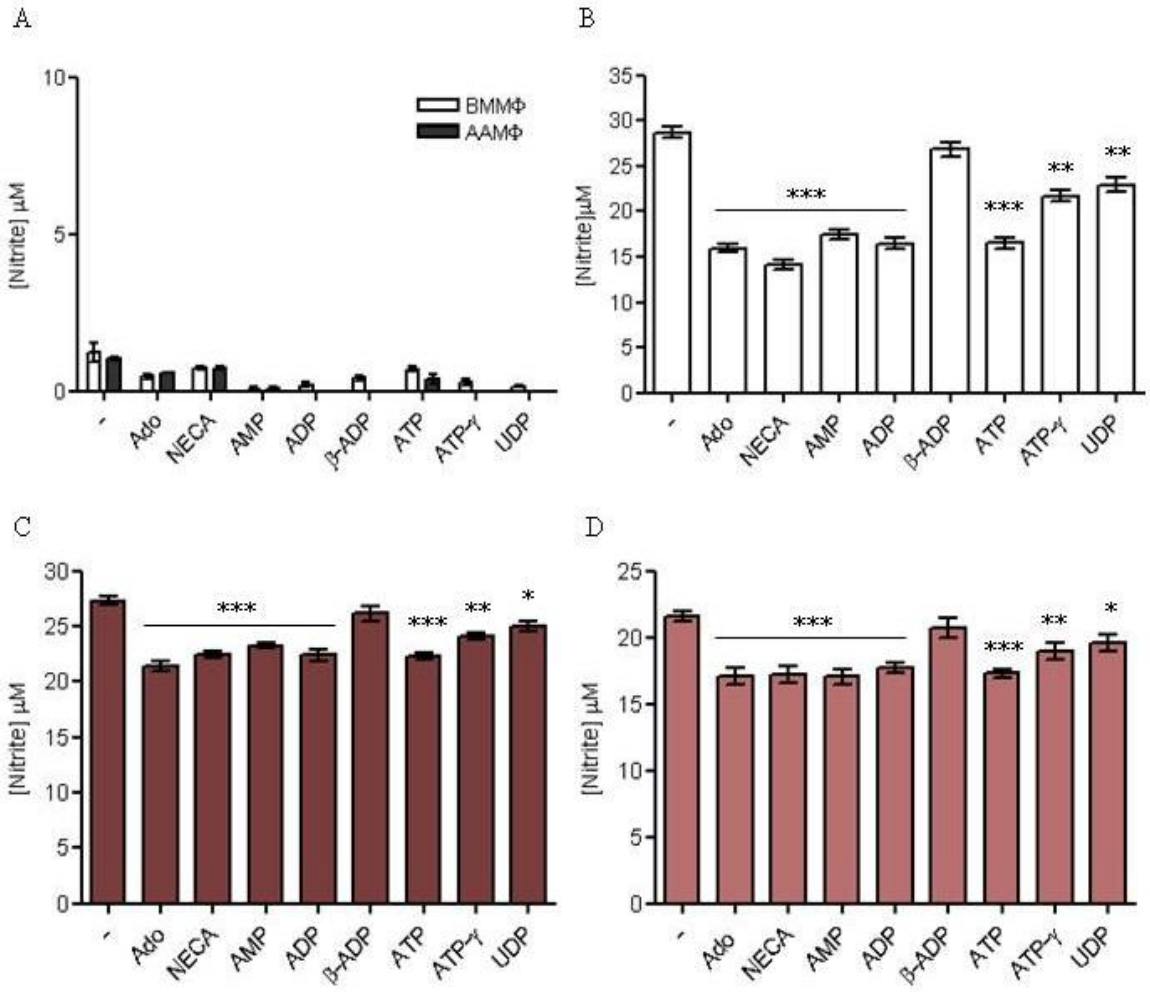


Figure 4.2: The effect of nucleotides on NO release by bone marrow-derived macrophages. Bone marrow-derived macrophages (BMMΦ), classically activated macrophages (CAMΦ) and alternatively activated macrophages (AAMΦ) were treated with 100µM adenosine (Ado), NECA, nucleotides or the slowly-hydrolysable analogues of ADP and ATP (β-ADP and ATP-γ, respectively) for 20 hours. 10µM of the adenosine transporter inhibitor, Dipyridamole, was co-administered with adenosine, AMP, ADP and ATP. Levels of NO release, expressed in µM nitrite, were measured in supernatants from (A) untreated BMMΦ and AAMΦ, (B) CAMΦ activated with 10ng/ml LPS, (C) CAMΦ activated through TLR4 with 10ng/ml ultrapure LPS and (D) CAMΦ activated through TLR2 with 10ng/ml Pam3C. Data presented are an average of triplicates $\pm$ SEM from one representative xperiment of $n > 3$ and asterisks indicate statistical significance compared to the untreated control.* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

The effect of purinergic receptor activation by nucleotides on macrophage arginase I activity was investigated to determine if the inhibition of NO release was accompanied by an enhancement of arginase I activity, considering that it is the alternative pathway of L-arginine metabolism. The effects of purinergic signalling on CAMΦ arginase I activity are shown in Figure 4.3A. Adenosine increased arginase I activity while its analogue, NECA, caused an even higher increase as it cannot be taken up by cells or be metabolised. It should be noted that Dipyridamole, a nucleoside transporter inhibitor used in other assays to prevent adenosine uptake, was not used in arginase I activity assays as it was found to interfere with this assay. β-ADP stimulation did not increase arginase I activity while stimulation with ATP-γ caused a slight increase, possibly due to slow hydrolysis to adenosine over time (Figure 4.3A).

The up-regulatory effect of adenosine stimulation on CAMΦ arginase I activity was restricted to LPS-activated macrophages, as IFN-γ-activated macrophages did not respond to adenosine stimulation in the same way, despite the fact that stimulation of macrophages with IFN-γ also results in classical activation (Figure 4.3B). Therefore, it appears that TLR activation is necessary for up-regulation of CAMΦ arginase I activity by P1R activation. CAMΦ were stimulated with adenosine, NECA and ADP at three different concentrations and the optimal concentration for the increase observed was 150μM, while NECA produced a similar increase at 100μM (Figure 4.3C). In CAMΦ, NECA always induced higher levels of arginase I activity than adenosine and ADP, which induced similar levels at 100μM and 150μM.

AAMΦ arginase I activity was also affected by nucleotides although to a lesser extent due to the higher basal levels (Figure 4.4A). Adenosine/NECA induced the highest increase while AMP, ADP, ATP, UDP induced a slight but significant increase. β-ADP stimulation did not lead to an increase in arginase I activity while stimulation with ATP-γ produced a slight increase.

AAMΦ were stimulated with adenosine, NECA and ADP at three different concentrations to determine if there was any concentration dependence (Figure 4.4B). NECA produced a higher increase at 100μM, while the increase was comparatively lower for all the concentrations of ADP tested. Adenosine induced a more gradual increase in arginase I activity than NECA in AAMΦ.

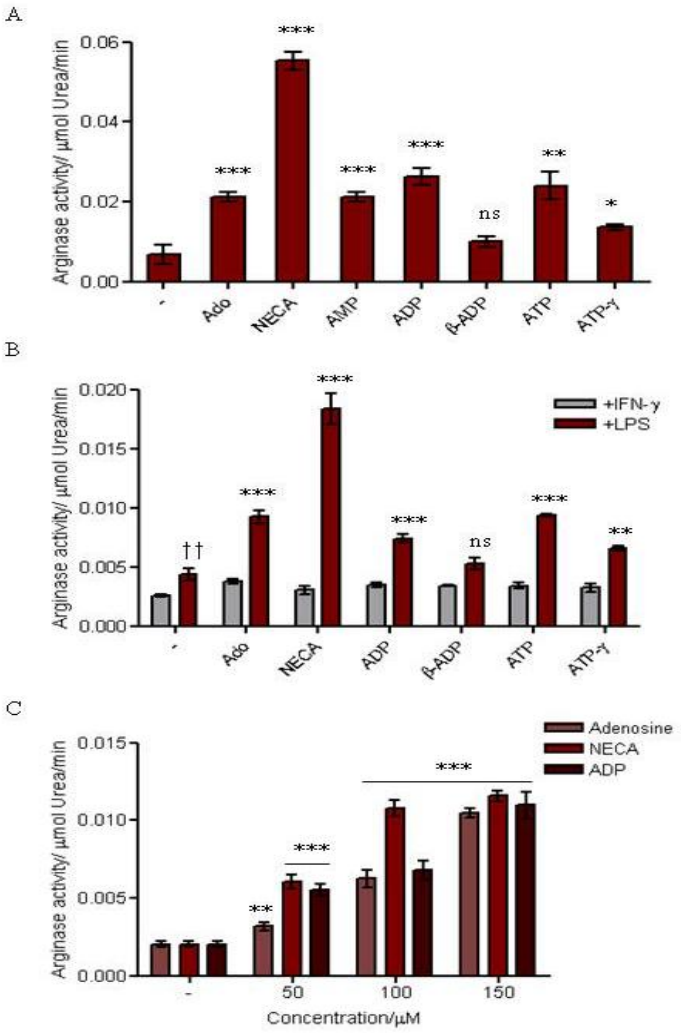


Figure 4.3: The effect of nucleotides on classically activated macrophage (CAMΦ) arginase I activity. Macrophages were classically activated either with 10ng/ml LPS or IFN-γ and incubated with nucleotides for 20 hours before measuring arginase I activity. (A) The levels of arginase I activity measured after incubation of LPS-activated CAMΦ with 100µM adenosine, NECA, AMP, ADP, ATP or the slowly hydrolysable analogues of ADP and ATP (β-ADP and ATP-γ, respectively). (B) A comparison of arginase I activity in IFN-γ and LPS - activated macrophages. (C) The effect of 3 different concentrations of adenosine, NECA and ADP on CAMΦ arginase I activity is shown after 20 hours of culture. 10µM of the adenosine transporter inhibitor, Dipyridamole, was co-administered with adenosine, AMP, ADP and ATP. Arginase I activity is expressed as µmol Urea/ min per 5 x 10^4 cells. Data presented are an average of triplicates ± SEM from one representative experiment of n > 3 and asterisks indicate statistical significance. (*) is compared to untreated BMMΦ.* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, ns - not significant ($p> 0.05$). (†) compares to basal arginase I activity in CAMΦ activated with LPS. †† $p< 0.01$, ns - not significant ($p> 0.05$).

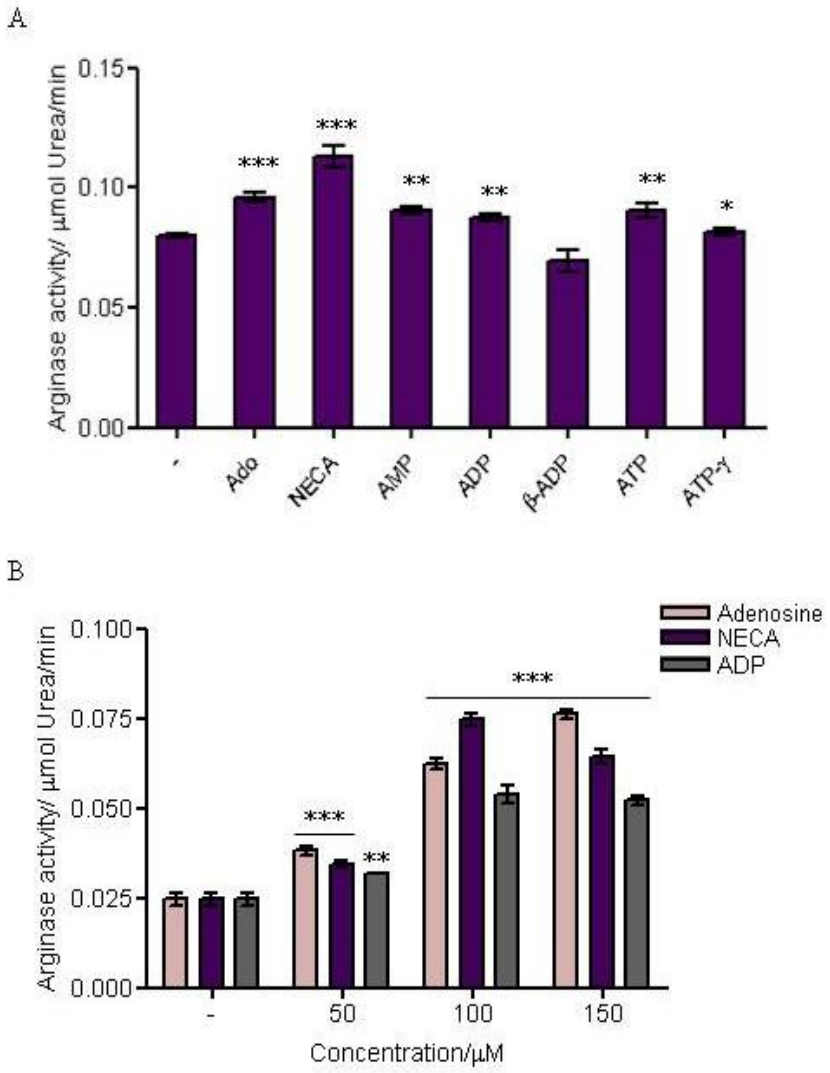


Figure 4.4: The effect of nucleotides on alternatively activated macrophage (AAMΦ) arginase I activity. Macrophages were alternatively activated with 5ng/ml IL-4 and incubated with nucleotides for 20 hours before measuring arginase I activity. (A) The levels of arginase I activity measured after incubation of AAMΦ with 100µM adenosine, NECA, AMP, ADP, ATP or the slowly hydrolysable analogues of ADP and ATP (β-ADP and ATP-γ, respectively). (B) The effect of 3 concentrations of adenosine, NECA and ADP on AAMΦ arginase I activity after 20 hours of culture. Arginase I activity is expressed as µmol Urea/ min per 5 x 10^4 cells. Data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate statistical significance compared to the untreated control. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

Cytokine release

Cytokine release is another major effector function of macrophages which differs depending on macrophage activation state. Pro-inflammatory cytokines such as IL-6 and TNF-α are produced by CAMΦ in a Th1 environment or in response to LPS. LPS also induces IL-10 production by CAMΦ, while BMMΦ and AAMΦ do not produce any cytokines *in vitro*. In the presence of adenosine or nucleotides, IL-6 and TNF-α release from CAMΦ were reduced while IL-10 release was up-regulated (Figure 4.7).

Firstly, the optimal concentration of LPS that induced cytokine release was investigated by titrating the LPS concentration (Figure 4.5). It appears that 0.5-1ng/ml LPS produced submaximal cytokine release, while stimulation with concentrations above 1ng/ml resulted in a plateau for IL-6 and IL-10. Hence, 10ng/ml LPS was used a suitable concentration for triggering optimal cytokine release for further experiments. The effects adenosine and nucleotides exerted on the different macrophage subsets were then assayed after having determined an optimal concentration (Appendix, Figures 7-10). Stimulation with exogenous nucleotides had no effect on cytokine release from BMMΦ and AAMΦ and the low background levels of IL-6 that were observed were not significant (Figure 4.6).

Pro-inflammatory cytokine release from CAMΦ in response to purinergic receptor stimulation by nucleotides was similar to the inhibitory pattern observed for NO release (Figure 4.7A and B). On the other hand, purinergic receptor stimulation led to an enhancement in the levels of IL-10 released by CAMΦ (Figure 4.7C). Stimulation with the slowly hydrolysable analogue of ADP, β-ADP had no effect on cytokine release levels, therefore, the fact that ADP stimulation had an effect on cytokine levels was probably a result of hydrolysis to adenosine.

ATP and ATP-γ stimulation had similar inhibitory effect on cytokine release, although ATP-γ-induced effects were always less pronounced. The response to adenosine and nucleotides was further investigated pharmacologically to dissect which purinergic receptors are involved.

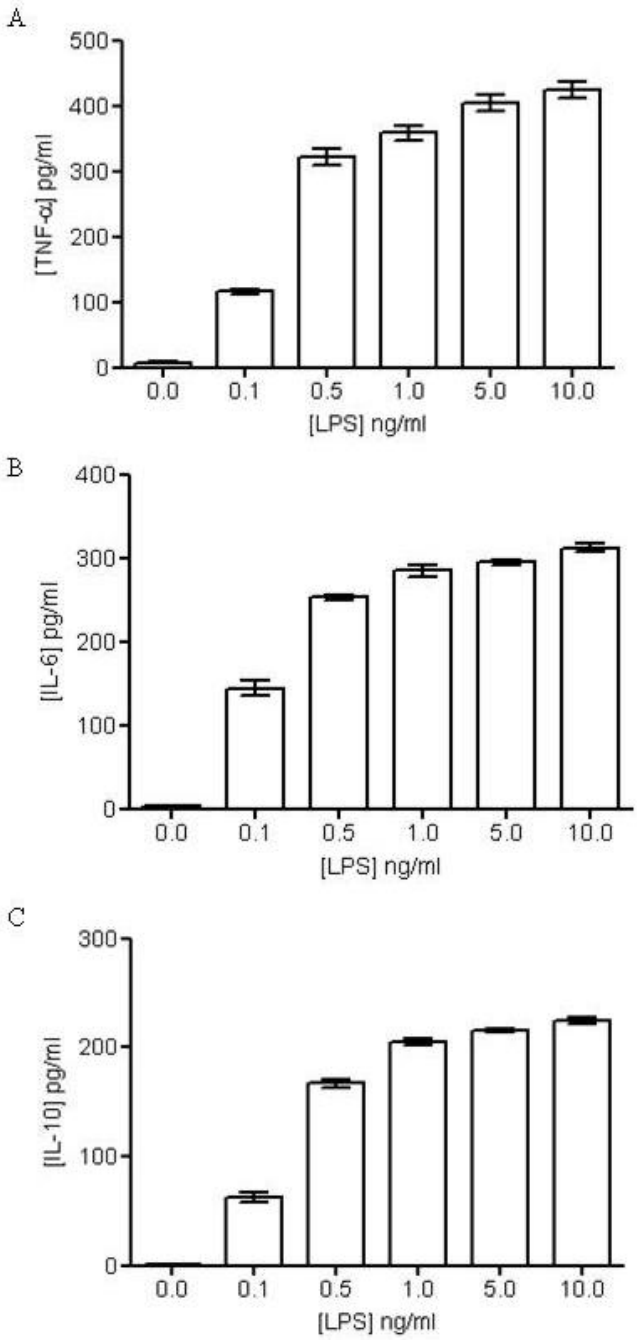


Figure 4.5: The effect of LPS concentration on cytokine release from bone marrow-derived macrophage (BMMΦ). Levels of cytokine release measured in the supernatants of BMMΦ stimulated with different concentrations of LPS for 20 hours are expressed in pg/ml. (A) The effect of LPS on TNF-α release, (B) The effect of LPS on IL-6 release and (C) The effect of LPS on IL-10 release. Data presented are an average of triplicates ± SEM from one representative experiment of n > 3.

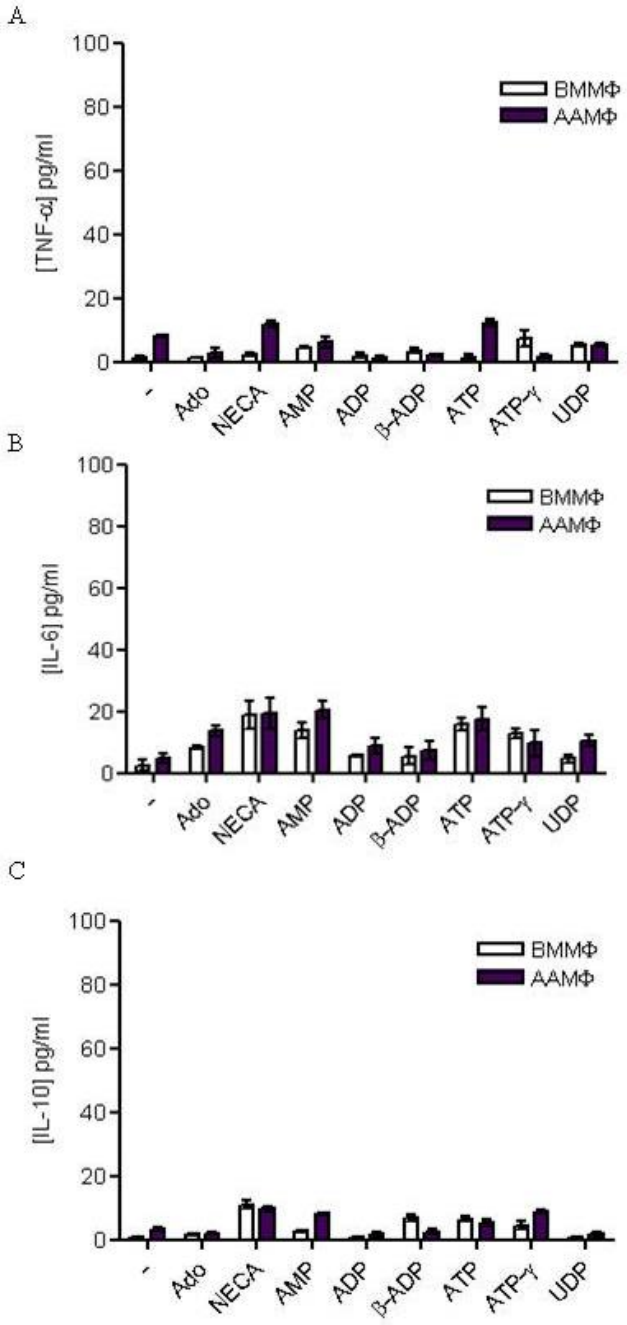


Figure 4.6: The effect of nucleotides on cytokine release from BMMΦ and AAMΦ. Macrophages were alternatively activated with 5ng/ml IL-4 or cultured with standard medium and incubated with nucleotides for 20 hours. The levels of cytokine release after stimulation of BMMΦ and AAMΦ with 100μM adenosine, NECA, AMP, ADP, ATP or the slowly hydrolysable analogues of ADP and ATP (β-ADP and ATP-γ, respectively) were measured after 20 hours of culture. 10μM of the adenosine transporter inhibitor, Dipyridamole, was co-administered with adenosine, AMP, ADP and ATP. (A) The effect on TNF-α release, (B) The effect on IL-6 release and (C) The effect on IL-10 release. Data presented are an average of triplicates $\pm$ SEM from one representative experiment of $n > 3$.

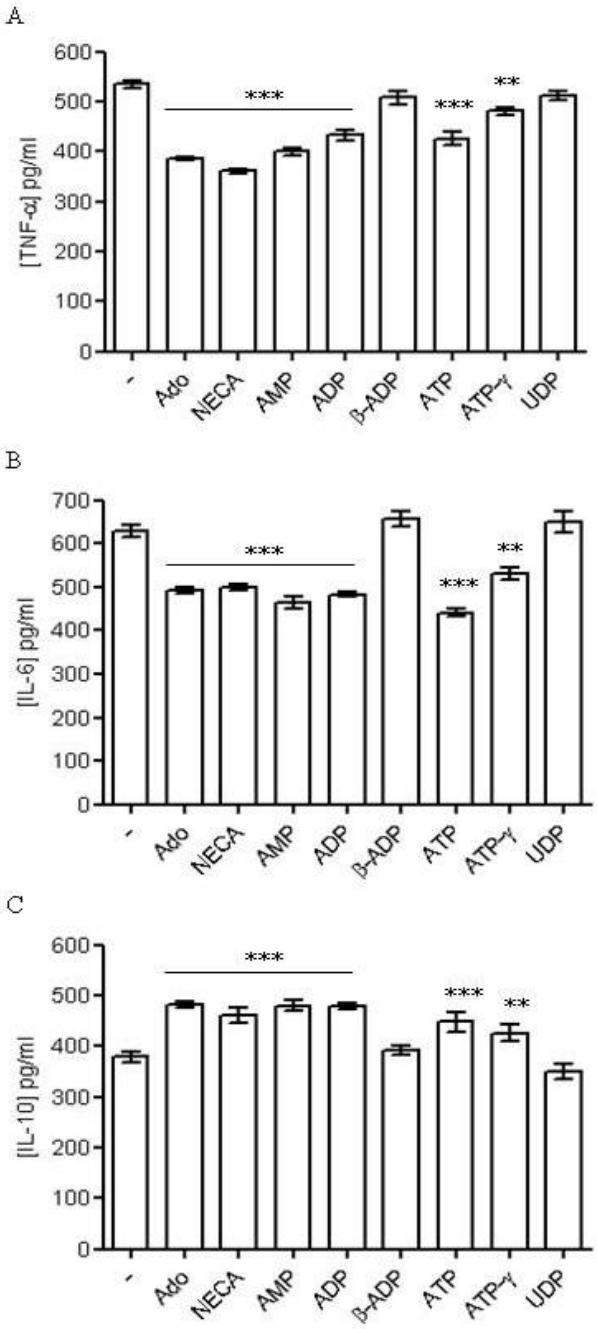


Figure 4.7: The effect of nucleotides on cytokine release from CAMΦ. Macrophages were classically activated with 10ng/ml LPS and incubated with nucleotides for 20 hours. The levels of cytokine release measured after incubation of CAMΦ with 100μM adenosine, NECA, AMP, ADP, ATP or the slowly hydrolysable analogues of ADP and ATP (β-ADP, ATP-γ, respectively). 10μM of the adenosine transporter inhibitor, Dipyridamole, was co-administered with adenosine, AMP, ADP and ATP. (A) The effect on TNF-α release, (B) The effect on IL-6 release and (C) The effect on IL-10 release. Data presented are an average of triplicates $\pm$ SEM from one representative experiment of $n > 3$. ** $p < 0.01$, *** $p < 0.001$.

4.2.2 Characterisation of the receptors involved in murine macrophage purinergic signalling

Nitric oxide release vs arginase I activity

In order to confirm and further characterise the effects induced by adenosine administered directly or resulting from hydrolysis of adenine nucleotides on nitric oxide release and arginase I activity, P1 receptor (P1R) agonists and antagonists were used. The concentration of agonists and antagonists used (Materials and Methods, 2.4) was selected from a range of concentrations tested (data not shown). Considering that adenosine acts on all P1 receptors with varying affinities, agonists that act on specific P1 receptors were used in order to see if adenosine-induced effects could be mimicked by stimulation of one particular receptor. As can be seen in Figure 4.8A, A_{2A} receptor ($A_{2A}R$) activation is also capable of inhibiting NO release from CAMΦ which is also supported by the fact that NECA, which has a higher affinity for the A_2 receptors, is also a potent inhibitor of NO release. An A_{2B} receptor ($A_{2B}R$) agonist has been used by Eckle *et al*, 2007 but is restricted to use by Bayer Healthcare and was not available for use in this study.

In addition to using P1R antagonists to characterise the receptor via which adenosine inhibited NO release and enhanced arginase I activity, they were also used to determine if AMP, ADP and ATP also inhibited NO release via P1R stimulation as a result of adenosine generation in culture. The results obtained and presented in Figures 8 and 9 show that antagonising A_{2B} receptor activation reverses the effects induced by adenosine, and its stable analogue NECA, on NO release and arginase I activity. Specifically, it is shown in Figure 4.8B and C that, the $A_{2B}R$ antagonist MRS1754 significantly inhibited both adenosine and NECA-induced effects restoring the levels of NO release to levels detected when CAMΦ were cultured alone (-). Therefore,

adenosine mediated its effects via $A_{2B}R$ activation and it appears that purinergic receptor activation by nucleotides also involved the A_{2B} receptor. Specifically, it can be seen in Figure 4.8D, E and F that AMP, ADP and ATP-induced inhibition of NO release was also reversed by $A_{2B}R$ antagonism. Furthermore, the fact that no AMP purinergic receptor has been reported suggests that ADP and ATP-induced effects are not due to AMP generation and that AMP-induced effects are due to its hydrolysis to adenosine.

In terms of arginase I activity, the use of P1R agonists showed that the up-regulation induced by adenosine was mimicked by A_2R activation by NECA as well as by $A_{2A}R$-activation specifically by CGS21680 (Figure 4.9A). Similarly to NO release, P1R antagonism showed that up-regulation of arginase I activity induced by adenosine was also inhibited by targeting the $A_{2B}R$. This was observed in both CAMΦ and AAMΦ as can be seen in Figure 4.9B.

Further confirmation that nucleotide-induced effects were mediated via P1 receptor activation was obtained from experiments with P2Y receptor (P2YR) antagonists. As is shown in Figure 4.10, the inhibition in NO release observed when CAMΦ were stimulated with ADP and ATP was not reversed by the P2YR antagonists, Suramin (A) or PPADS (B). Furthermore, the slowly hydrolysable nucleotides β-ADP and ATP-γ, which were also used as controls to ensure P2YR activation, induced the same effects on NO release both in the presence and absence of P2YR antagonists. In fact, the slight decrease in NO release observed with ATP-γ was not reversed by P2YR antagonists, so it was not due to P2YR ligation by ATP, or ADP that could be generated from ATP degradation.

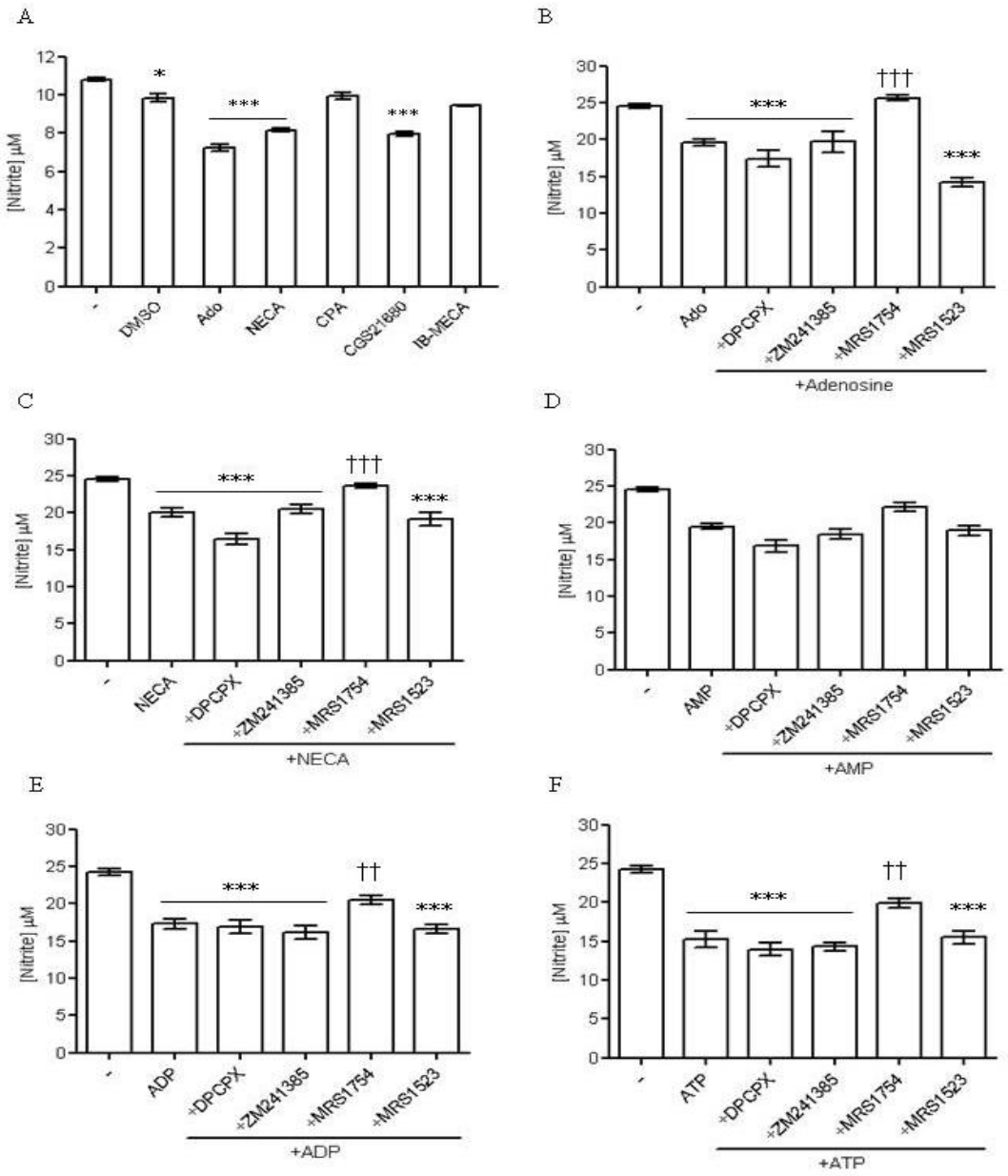


Figure 4.8: Pharmacological characterisation of P1R-mediated effects on NO release from CAMΦ. Macrophages were cultured with 10ng/ml LPS in the presence or absence of P1 receptor agonists (A) or antagonists (B-F). (A) The effect of the following agonists: 100 µM of adenosine + 10µM Dipyridamole (DP), NECA (A_2R), CPA (A_1R), CGS21680 ($A_{2A}R$) or IB-MECA (A_3R). Antagonists were used to reverse effects elicited by (B) adenosine, (C) NECA, (D) AMP, (E) ADP, (F) ATP. Antagonists: DPCPX (A_1R), ZM241385 ($A_{2A}R$), MRS1753 ($A_{2B}R$) or MRS1523 (A_3R). Levels of NO release are expressed as µM nitrite and the data presented are an average of triplicates ± SEM from one representative experiment of n > 3 and asterisks indicate statistical significance compared to the untreated control. * $p< 0.05$, *** $p< 0.001$. (†) is compared to the effect induced by the respective nucleoside/nucleotide. †† $p< 0.01$, ††† $p< 0.001$.

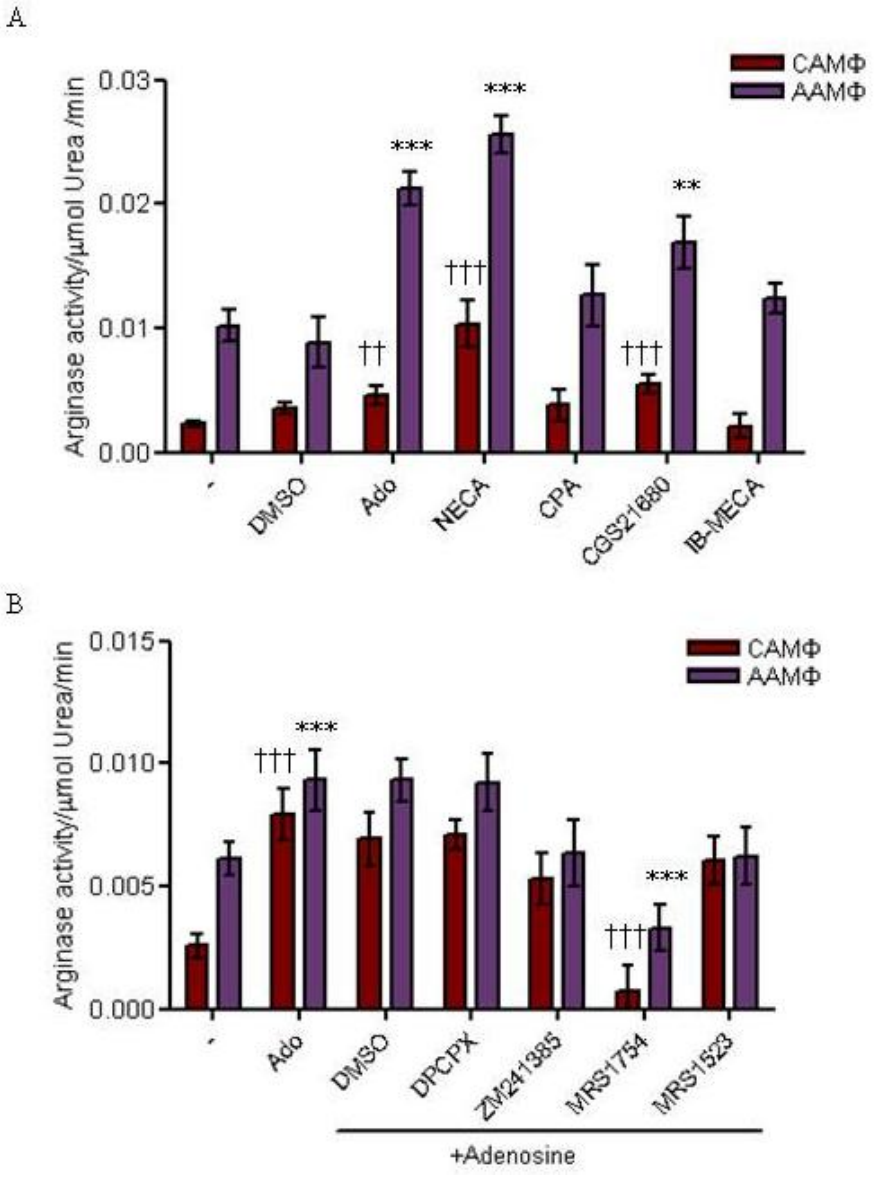


Figure 4.9: Pharmacological characterisation of P1R-mediated effects on CAMΦ and AAMΦ arginase I activity. Macrophages were cultured with 5ng/ml IL-4 in the presence or absence of P1 receptor agonists (A) or antagonists (B). Agonists: 100µM of adenosine + 10µM Dipyridamole (DP), NECA, CPA (A_1R agonist), CGS21680 (A_{2A}R agonist) or IB-MECA (A_3R agonist). Antagonists: DPCPX (A_1R antagonist), ZM241385 (A_{2A}R antagonist), MRS1753 (A_{2B}R antagonist) or MRS1523 (A_3R antagonist). Arginase I activity is expressed as µmol Urea/ min per 5 x 10^4 cells. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks/daggers indicate statistical significance compared to the untreated control. ** $p< 0.01$, *** $p< 0.001$. (†) is compared to the respective untreated control. †† $p< 0.01$, ††† $p< 0.001$.

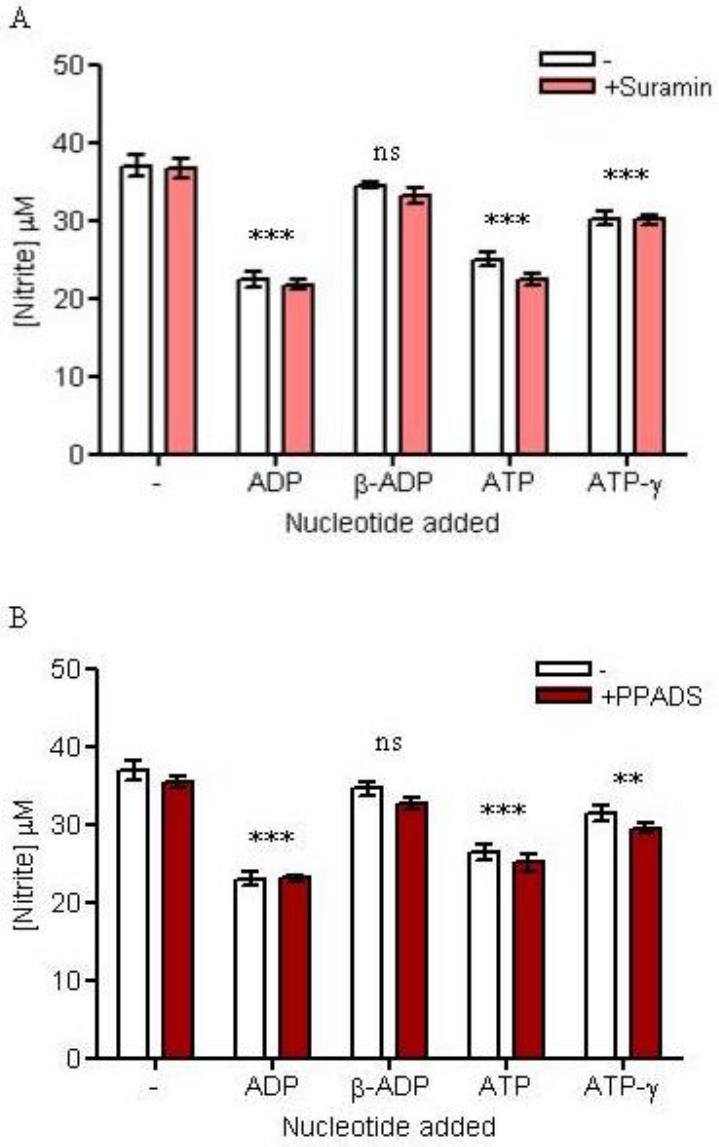


Figure 4.10: Pharmacological characterisation of P2YR-mediated effects on NO release from CAMΦ. Macrophages were activated with 10ng/ml LPS and incubated with 100µM nucleotides in the presence or absence of 2 different non-selective P2Y receptor antagonists for 20 hours. (A) The effect of Suramin on ADP- and ATP-induced inhibition of NO release by CAMΦ (B) The effect of PPADS (Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid) on ADP- and ATP-induced inhibition of NO release by CAMΦ. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate statistical significance compared to the untreated control. ** $p < 0.01$, *** $p < 0.001$, ns - not significant ($p > 0.05$).

Cytokine release

Cytokine responses following activation of purinergic signalling were also characterised pharmacologically. P1 receptor antagonists were used to antagonise adenosine/NECA-induced effects as well as the effects induced by nucleotides that could potentially be converted to adenosine in culture. The results presented in Figure 4.11 show that TNF-α release was inhibited by adenosine via both A_{2A} and A_{2B} receptors, as antagonism of both receptors by ZM241385 and MRS1754, respectively, led to partial reversal of adenosine inhibition. Levels of IL-6 release appeared to be affected in the same way as TNF-α release by adenosine stimulation as a similar pattern of antagonism was observed (Figure 4.11A and B). In addition to characterising the P1R involved in adenosine-induced inhibition of TNF-α and IL-6 release from CAMΦ, AMP, ADP and ATP were also used to stimulate cells in the presence of P1R antagonists. As can be seen in Figure 4.11 C, D and E, the inhibition of TNF-α release by AMP, ADP and ATP was partially reversed both by A_{2A} and A_{2B} receptor antagonists. Similarly, the levels of IL-6 release were partially restored to CAMΦ background levels when AMP, ADP and ATP were used to stimulate cells in the presence of A_{2A} and A_{2B} receptor antagonists (Figure 4.12 C, D and E). As was observed with both TNF-α and IL-6 release, antagonism of adenosine/NECA and AMP resulted in more efficient reversal of inhibition when compared to the reversal achieved with ADP and ATP. In order to confirm that both A_{2A} and A_{2B} receptors have an additive effect in adenosine-induced inhibition of IL-6 and TNF-α release which cannot be properly reversed by inhibiting one of the two receptors, experiments were carried out using a combination of both A_{2A} and A_{2B} receptor antagonists (Figure 4.13). Indeed, when a cocktail of A_2 receptor antagonists was used the adenosine and NECA-induced inhibition of IL-6 and TNF-α release was completely reversed and levels were restored to basal levels detected when CAMΦ were cultured in medium (-).

In the case of IL-10 release, which was enhanced in response to adenosine, pharmacological characterisation showed that only one P1R was involved. As it is shown in Figure 4.14A, only the A_{2B} receptor antagonist (MRS1754) reversed the adenosine-induced enhancement of IL-10 release by CAMΦ. This was also the case for NECA, AMP, ADP and ATP as can be seen in Figure 4.14 B, C D and E, suggesting that the active ligand is adenosine resulting from nucleotide hydrolysis in culture. Furthermore, the A_{2B}R antagonist caused complete restoration of IL-10 release levels to basal levels when incubated with all the nucleotides tested. This observation may be due to the fact that only one receptor was involved, therefore, adenosine stimulation of this particular receptor could be blocked by a specific P1R antagonist.

Taken together these data show that, similar to the observations made when investigating NO release and arginase I activity in this study, the cytokine responses triggered in the presence of AMP, ADP and ATP were reversed in a way that points to adenosine as the active ligand in activation of purinergic signalling.

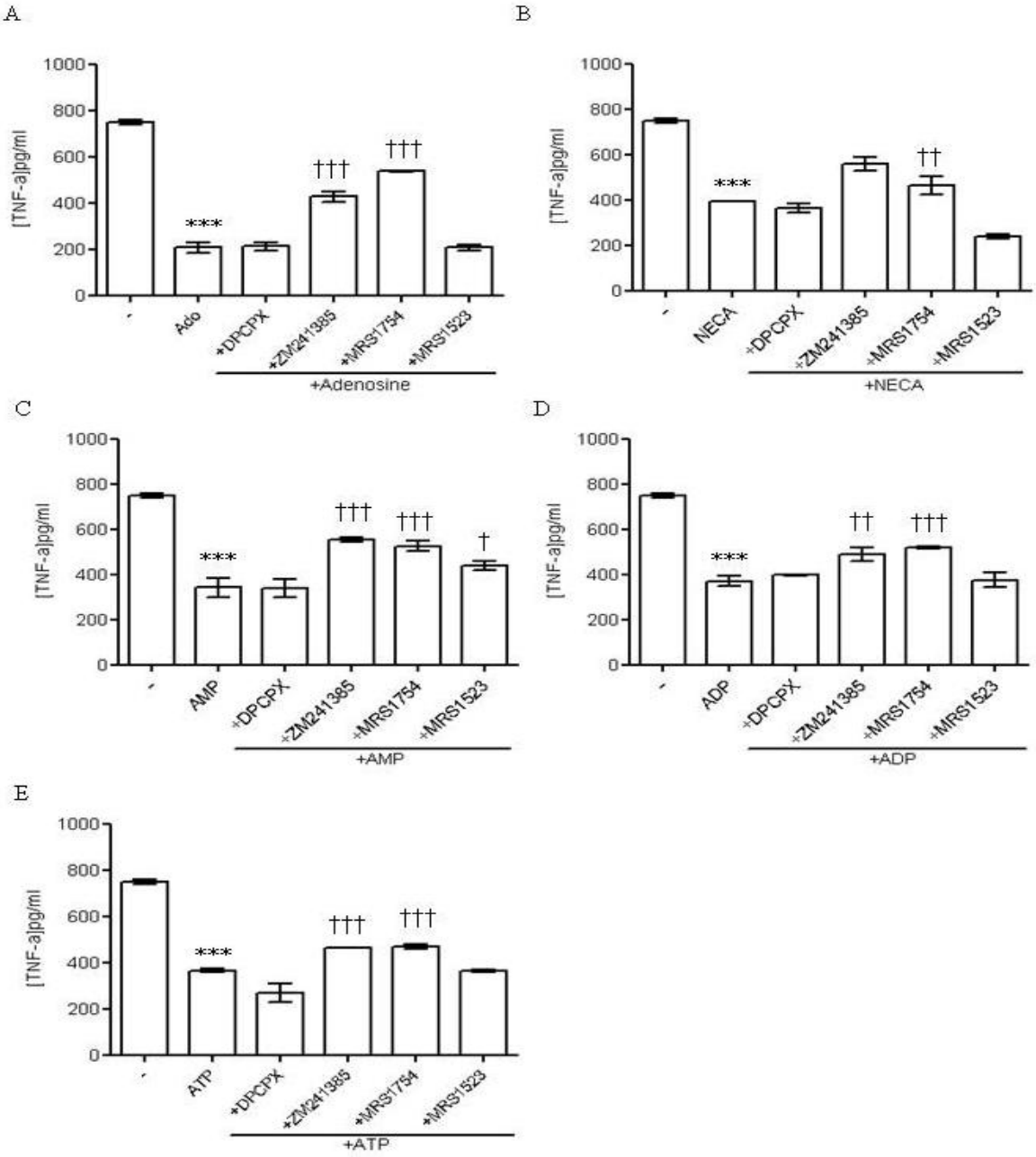


Figure 4.11: Pharmacological characterisation of P1R-mediated effects on TNF-α release from CAMΦ. Macrophages were cultured with 10ng/ml LPS in the presence or absence of P1 receptor antagonists (A-E). Antagonists were used to reverse effects elicited by (A) adenosine, (B) NECA, (C) AMP, (D) ADP, (E) ATP. DPCPX (A_1R antagonist), ZM241385 (A_{2A}R antagonist), MRS1753 (A_{2B}R antagonist) or MRS1523 (A_3R antagonist). Levels of TNF-α release are expressed as pg/ml and the data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate statistical significance compared to the untreated control. *** $p < 0.001$. (†) is compared to the effect induced by the respective nucleoside/nucleotide. † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$.

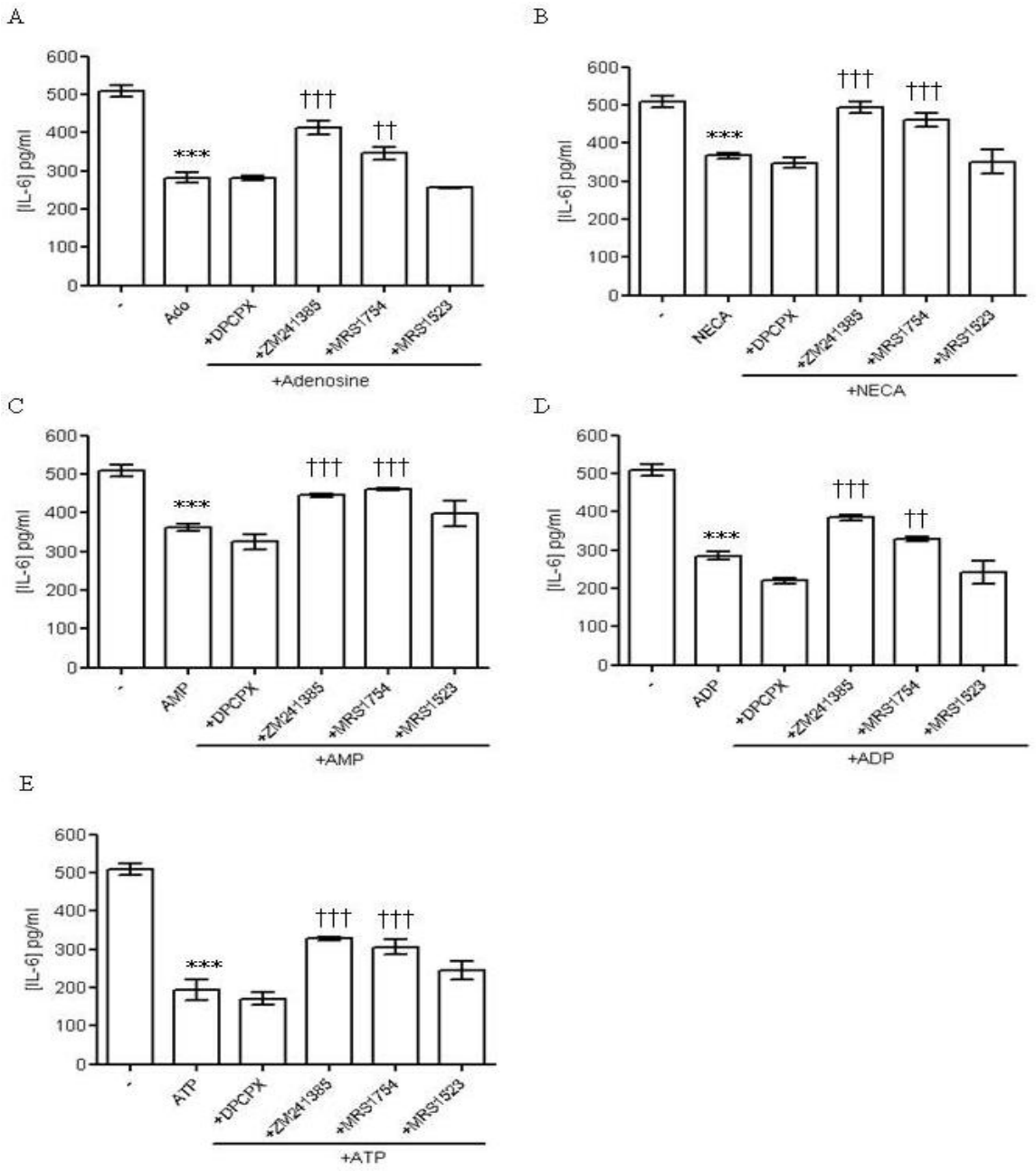


Figure 4.12: Pharmacological characterisation of P1R-mediated effects on IL-6 release from CAMΦ. Macrophages were cultured with 10ng/ml LPS in the presence or absence of P1 receptor antagonists (A-E). Antagonists were used to reverse effects elicited by (A) adenosine, (B) NECA, (C) AMP, (D) ADP, (E) ATP. DPCPX (A_1R antagonist), ZM241385 (A_{2A}R antagonist), MRS1753 (A_{2B}R antagonist) or MRS1523 (A_3R antagonist). Levels of IL-6 release are expressed as pg/ml and the data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate statistical significance compared to the untreated control. *** $p < 0.001$. (†) is compared to the effect induced by the respective nucleoside/nucleotide. †† $p < 0.01$, ††† $p < 0.001$.

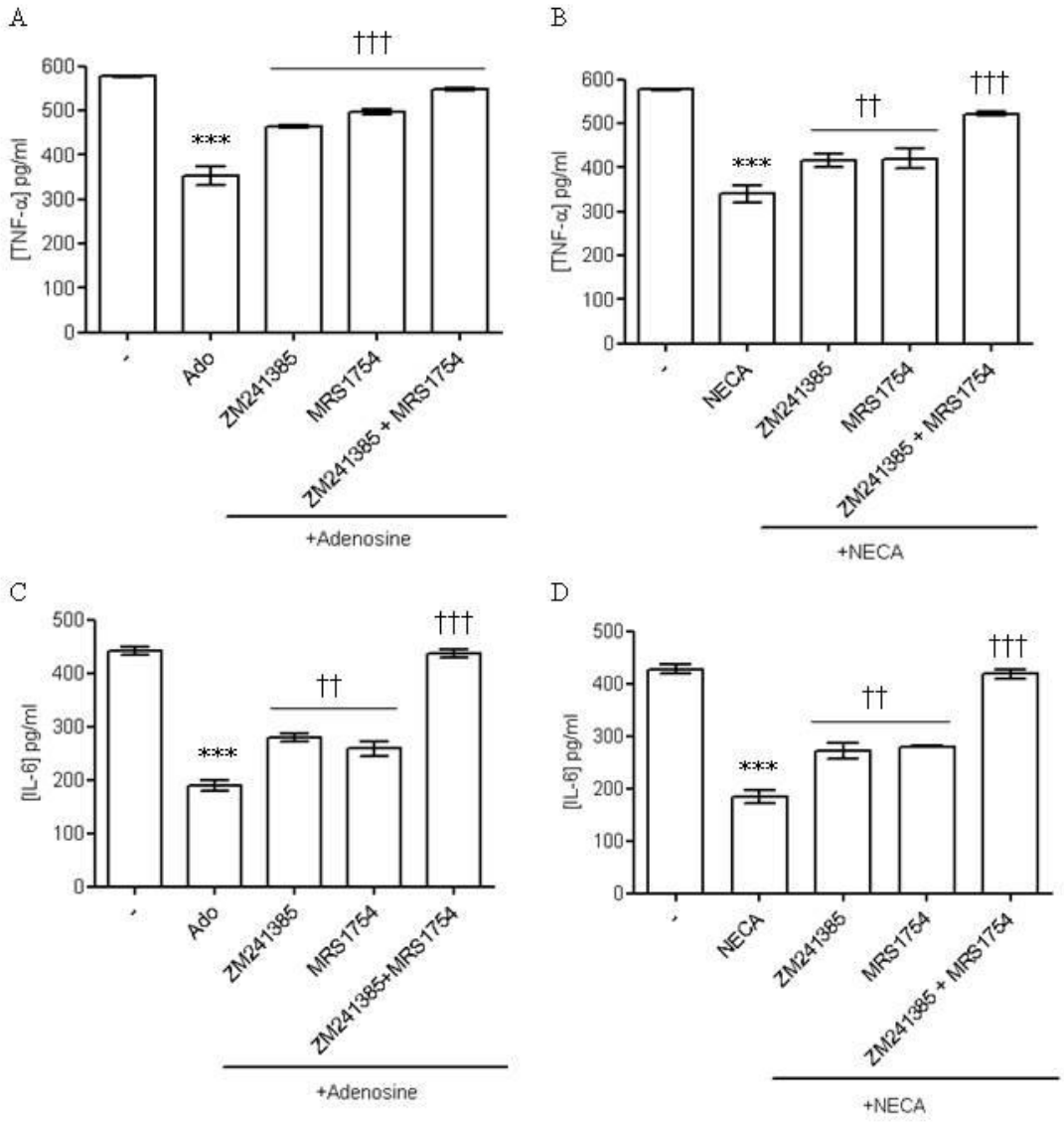


Figure 4.13: Pharmacological characterisation of P1R-mediated effects on TNF-α and IL-6 release from CAMΦ. Macrophages were cultured with 10ng/ml LPS in the presence or absence of P1 receptor antagonists (A-D). Antagonists, ZM241385 (A_{2A}R antagonist) and MRS1753 (A_{2B}R antagonist), were used to reverse effects elicited by (A, C) adenosine, (B, D) NECA. The levels of TNF-α (A, B) and IL-6 (C, D) release are expressed as pg/ml and the data presented are an average of triplicates ± SEM from one representative experiment and asterisks indicate statistical significance compared to the untreated control. *** $p < 0.001$. (†) is compared to the effect induced by adenosine or NECA alone. †† $p < 0.01$, ††† $p < 0.001$.

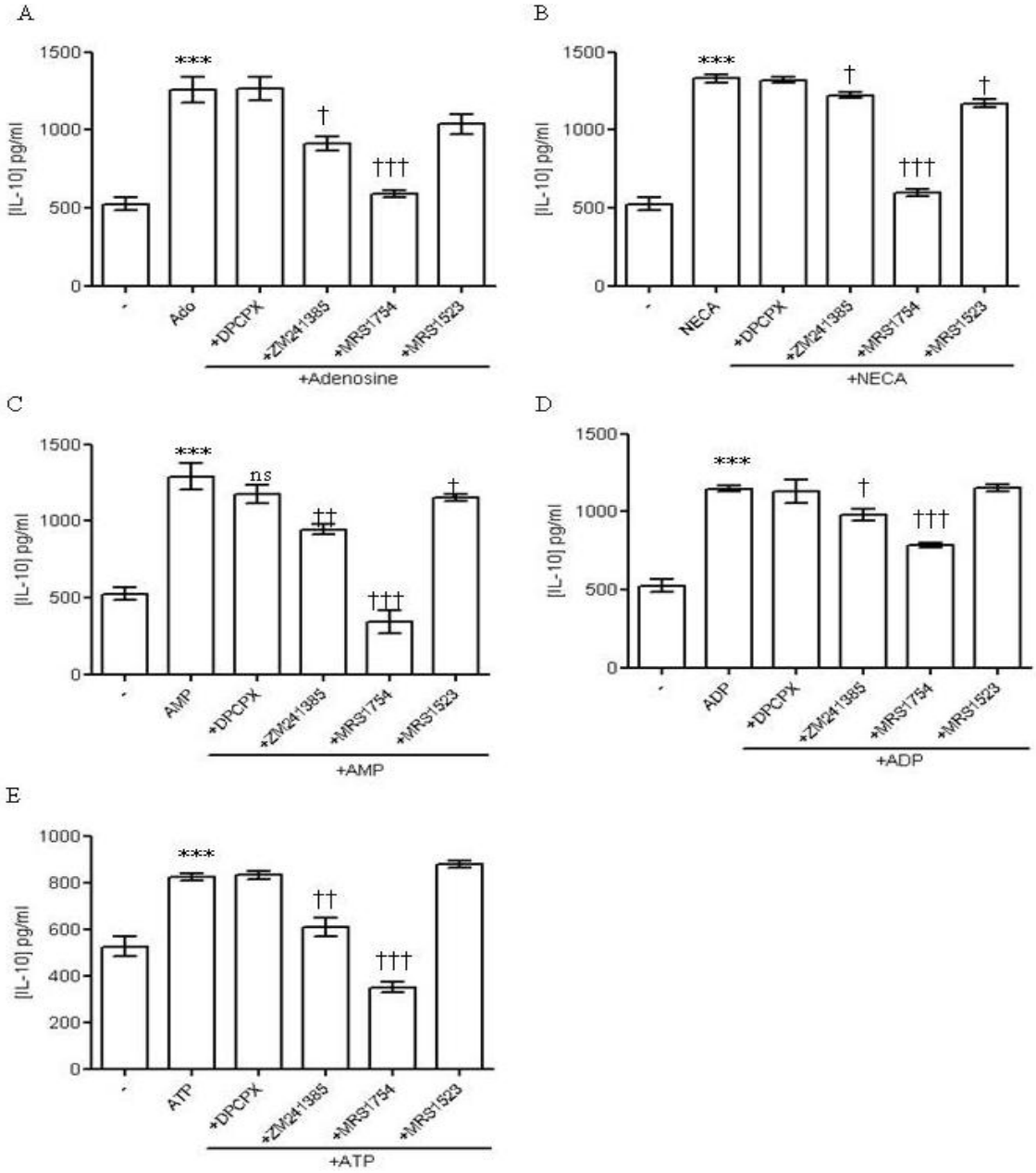


Figure 4.14: Pharmacological characterisation of P1R-mediated effects on bone marrow derived macrophage IL-10 release. Macrophages were cultured with 10ng/ml LPS in the presence or absence of P1 receptor antagonists (A-E). Antagonists were used to reverse effects elicited by (A) adenosine, (B) NECA, (C) AMP, (D) ADP, (E) ATP. DPCPX (A_1R antagonist), ZM241385 (A_{2A}R antagonist), MRS1753 (A_{2B}R antagonist) or MRS1523 (A_3R antagonist). The levels of IL-10 release are expressed as pg/ml and the data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate statistical significance compared to the untreated control. *** $p< 0.001$. (†) is compared to the effect induced by the respective nucleoside/ nucleotide. † $p< 0.05$, †† $p< 0.01$, ††† $p< 0.001$, ns - not significant ($p> 0.05$).

Despite the fact that purinergic receptor-induced cytokine responses were blocked by P1R antagonists, P2Y receptor involvement could not be ruled out. Therefore, P2YR antagonists were used to determine if ADP and ATP-induced the cytokine responses observed by direct action on their respective P2YR, before being degraded to adenosine. As it is shown in Figure 4.15A and B, the ADP and ATP-induced inhibition of TNF-α and IL-6 release from CAMΦ was not reversed by the P2YR antagonist, Suramin. The same result was obtained with a second P2YR antagonist, PPADS which is shown in Figure 4.16A and B. These P2YR antagonists were ineffective at reversing the ADP and ATP-induced enhancement of IL-10 release (Figure 4.15C and 16C). The slowly hydrolysable analogue of ADP (β-ADP) did not affect cytokine release from CAMΦ, which shows that ATP-induced cytokine responses were not due to generation of ADP. Furthermore, the fact that the slowly hydrolysable analogue of ATP (ATP-γ) induced similar responses to ATP that were not reversed by the P2YR antagonists shows that, ATP-induced responses were not due to P2YR activation either.

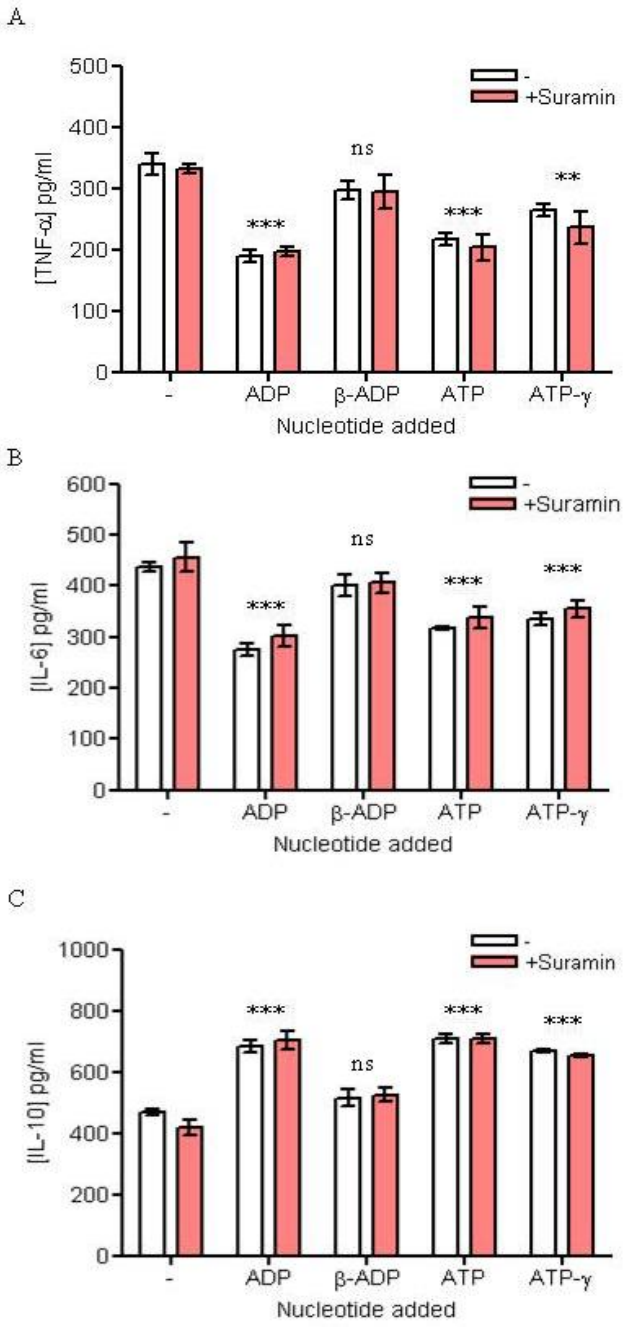


Figure 4.15: Pharmacological characterisation of P2YR-mediated effects on cytokine release from CAMΦ. Macrophages were cultured with 10ng/ml LPS with 100μM nucleotides in the presence or absence of a non-selective P2Y receptor antagonist for 20 hours. The effect of Suramin on ADP- and ATP-induced effects on cytokine release by CAMΦ was investigated for (A) TNF-α, (B) IL-6 and (C) IL-10 release. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate statistical significance compared to the respective untreated control.** $p < 0.01$, *** $p < 0.001$, ns - not significant ($p > 0.05$).

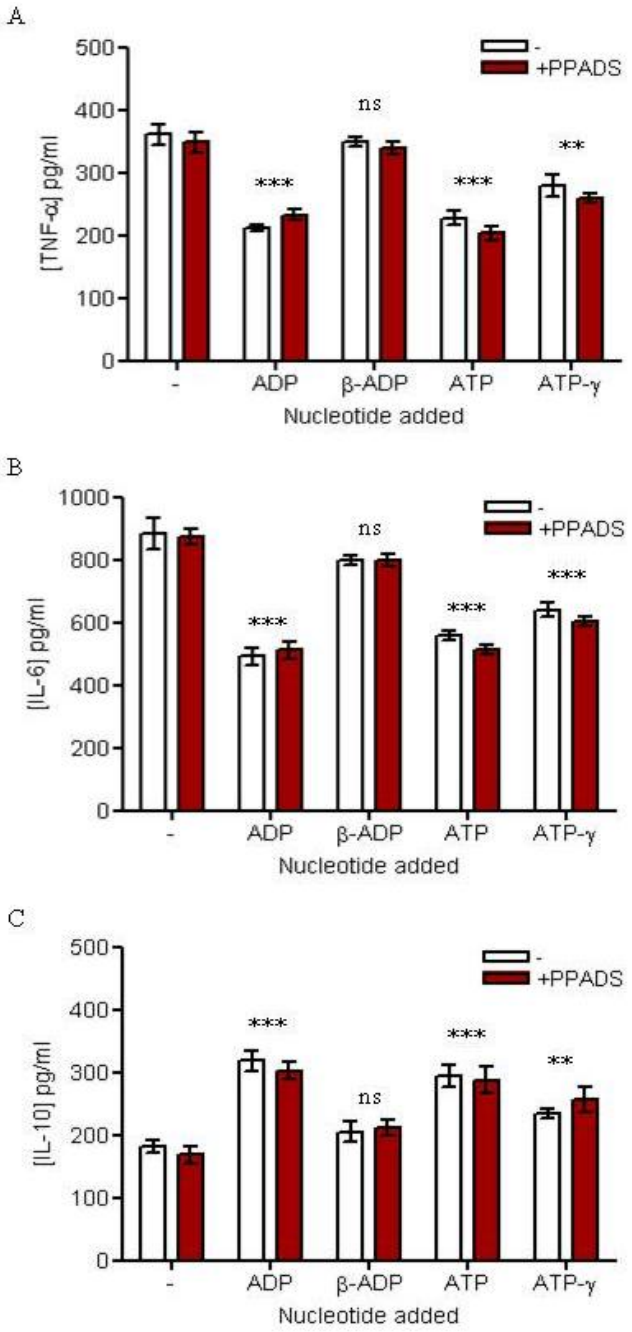


Figure 4.16: Pharmacological characterisation of P2YR-mediated effects on cytokine release from CAMΦ. Macrophages were cultured with 10ng/ml LPS with 100μM nucleotides in the presence or absence of a non-selective P2Y receptor antagonist for 20 hours. The effect of PPADS (Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid) on ADP- and ATP-induced effects on (A) TNF-α, (B) IL-6 and (C) IL-10 release by CAMΦ was investigated. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate statistical significance compared to the respective untreated control. ** $p < 0.01$, *** $p < 0.001$, ns - not significant ($p > 0.05$).

4.2.3 Downstream effectors of purinergic signalling in murine macrophages

Purinergic receptors are G-protein coupled so they mediate their effects by engaging different G subunits, namely Gs, Gi or Gq. Gs and Gi subunits act via adenylyl cyclase activation or inhibition, respectively, and thus control levels of cAMP. Gq subunits act via phospholipase C (PLC) and/or PI3 kinase activation. Ultimately these pathways converge to signal transcriptional activation in the nucleus either via NF-κB or p38/MAPK pathways. Inhibitors acting at certain points in the pathways mentioned were used in order to dissect how nucleotides mediate their effects.

Involvement of cAMP in purinergic signalling

In order to determine if the effects downstream of adenosine stimulation were Gs/i – mediated, macrophages were first incubated with an analogue of cAMP, Rp-cAMP. Unlike cAMP, Rp-cAMP is resistant to metabolism by phosphodiesterase (PDE) and can bind protein kinase A to prevent its activation by cAMP. The effect exerted by Rp-cAMP on P1R-induced inhibition of NO release from CAMΦ is shown in Figure 4.17A, B, C. Inhibition of NO release induced by adenosine and NECA was partly restored at the higher concentration of Rp-cAMP used (50μM). Similarly, when ultrapure LPS was used to classically activate macrophages via TLR4, adenosine-induced inhibition of NO release was partly restored by 25μM Rp-cAMP while at 50μM Rp-cAMP, NO release levels were almost completely restored. Rp-cAMP had no effect on BMMΦ and AAMΦ NO release.

Arginase I activity has been shown so far to change reciprocally to NO release, therefore, arginase I activity following inhibition of cAMP accumulation was also investigated. As it is shown in Figure 4.18A, the increase in CAMΦ arginase I activity induced by adenosine/NECA

was inhibited by 50μM Rp-cAMP by approximately 75%, while 25μM Rp-cAMP only caused a 20% inhibition. In addition, Rp-cAMP also inhibited basal arginase I activity in CAMΦ. TLR4-activated CAMΦ, stimulated with ultrapure LPS, whose arginase I activity was enhanced by adenosine/NECA, also required cAMP elevation for this response, as Rp-cAMP reversed adenosine and NECA induced up-regulation of arginase I activity by 50% at the higher concentration used (Figure 4.18B).

The effect of inhibiting cAMP elevation on arginase I activity was also investigated in AAMΦ. The results shown in Figure 4.18C, confirm that up-regulation of arginase I activity requires cAMP accumulation as Rp-cAMP administration to AAMΦ resulted in a 10 to 20% reduction in basal arginase I activity levels. Furthermore, the increase in arginase I activity induced by adenosine and NECA was restored to background levels in AAMΦ by both concentrations of Rp-cAMP tested (Figure 4.18C).

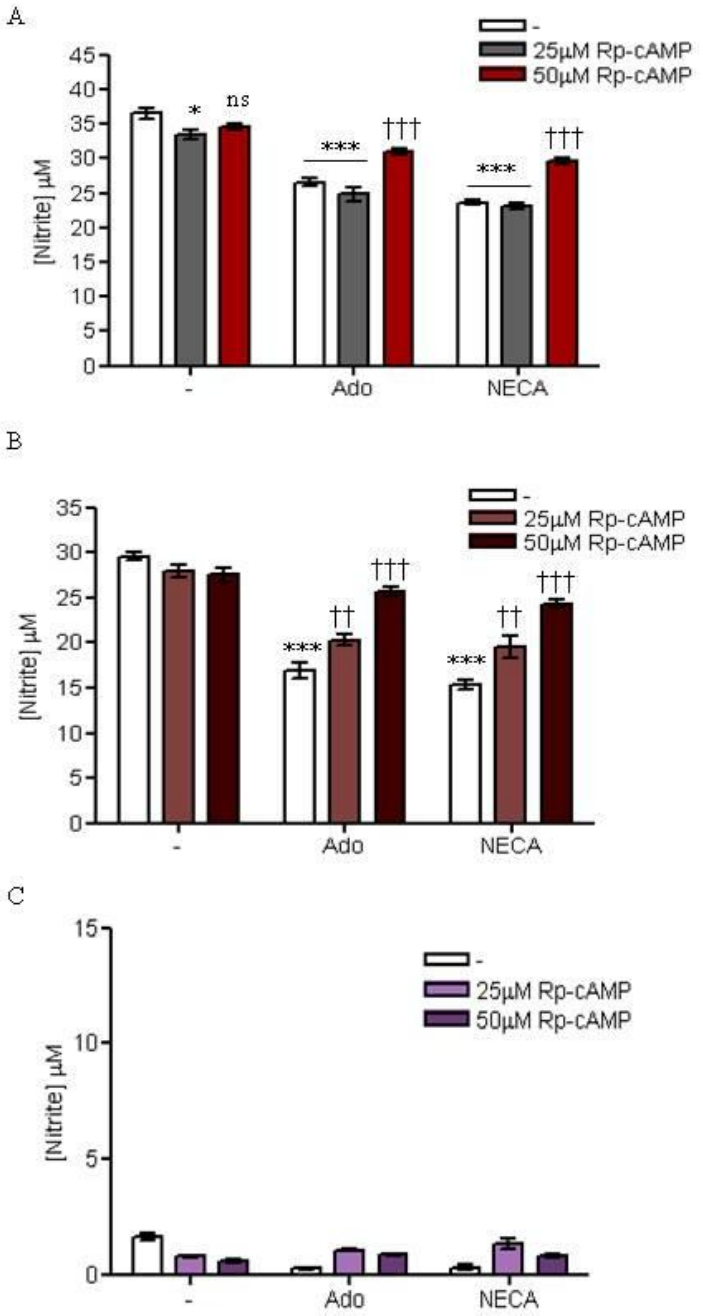


Figure 4.17: The effect of cAMP inhibition on adenosine-induced inhibition of NO release from macrophages. Macrophages were classically activated with 10ng/ml LPS (A) or 10ng/ml ultrapure LPS (B) or alternatively activated with 5ng/ml IL-4 (C). The inhibiting cAMP elevation with 2different concentrations of Rp-cAMP on the adenosine-induced inhibition of NO release after 20 hours of culture was investigated. Levels of NO release are expressed as µM nitrite and the data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate significance compared to untreated control.* $p< 0.05$, *** $p< 0.001$. (†) is compared to the effect induced by adenosine or NECA alone. †† $p< 0.01$, ††† $p< 0.001$, ns - not significant ($p> 0.05$).

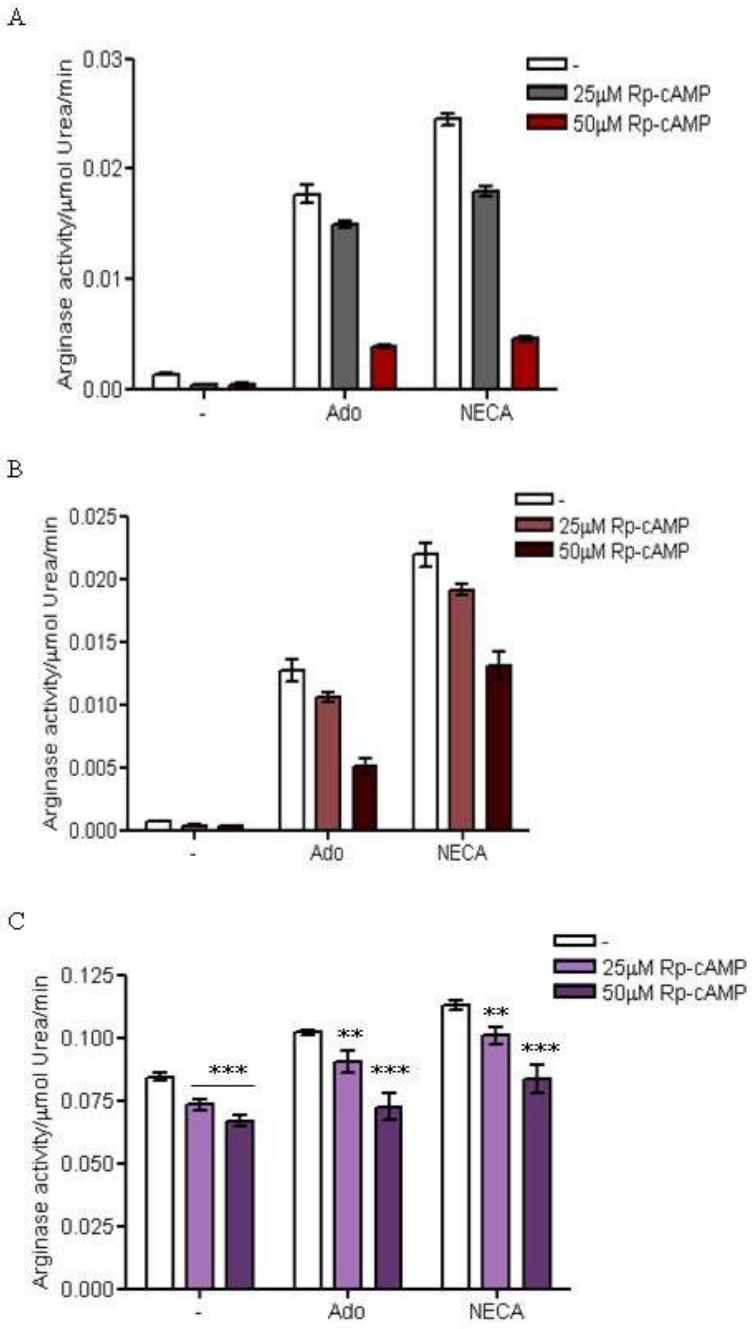


Figure 4.18: The effect of cAMP inhibition on adenosine-induced enhancement of macrophage arginase I activity. Macrophages were classically activated with 10ng/ml LPS (A) or 10ng/ml ultrapure LPS (B) or alternatively activated with 5ng/ml IL-4 (C). The effect of inhibiting cAMP elevation with 2 different concentrations of Rp-cAMP on the adenosine-induced enhancement of arginase I activity after 20 hours of culture was investigated. Arginase I activity is expressed as µmol Urea/ min per 5 x 10^4 cells and the data presented are an average of triplicates $\pm$ SEM from one representative experiment of $n > 3$ and asterisks indicate significance com pared to the respective untreated control. ** $p < 0.01$, *** $p < 0.001$.

Compounds which elevate cAMP levels were then used to see if elevating cAMP levels exogenously was enough to mimic the adenosine-induced responses. Forskolin is an adenylate cyclase activator (Schneyer *et al*, 1983) which causes increased cAMP production while rolipram is a selective Phosphodiesterase (PDE) IV inhibitor which prevents cAMP turnover (Greten *et al*, 1995; Griswold *et al*, 1993). In CAMΦ, arginase I activity was increased by treatment with forskolin or rolipram (Figure 4.19A) which was accompanied by a decrease in NO release at 10μM (Figure 4.19B). The concentration of DMSO used to solubilise these compounds was tested on cells to determine their DMSO tolerance. The final concentration of DMSO in 50μM forskolin was toxic to the cells and caused inhibition of NO release so forskolin was used at 10μM in further experiments. The final concentration of DMSO in rolipram was much lower than in forskolin and since rolipram was effective at both concentrations tested, 5μM was selected for subsequent experiments. A comparison of NO release in different CAMΦ (LPS, Pam3C for TLR2 and ultrapure LPS for TLR4) was carried out (Figure 4.19C). It appears that, 10μM forskolin and 5μM rolipram had the same inhibitory effect in all types of CAMΦ. LPS-activated CAMΦ generally produced higher levels of NO which might be due to the presence of more than one TLR ligand, leading to activation of more TLRs resulting in an additive response.

In order to further investigate cAMP involvement, cAMP elevators were also combined with adenosine and NECA. The levels of NO released from CAMΦ stimulated with adenosine or NECA combined with forskolin or rolipram were measured and the results are shown in Figure 4.20. Forskolin and rolipram caused a 10% decrease in NO release from CAMΦ, while the inhibition induced by adenosine and NECA was enhanced by elevating cAMP levels. Specifically, as shown in Figure 4.20A, forskolin increased adenosine and NECA-induced inhibition of NO release by 10%, while rolipram caused a further 50% decrease (Figure 4.20B).

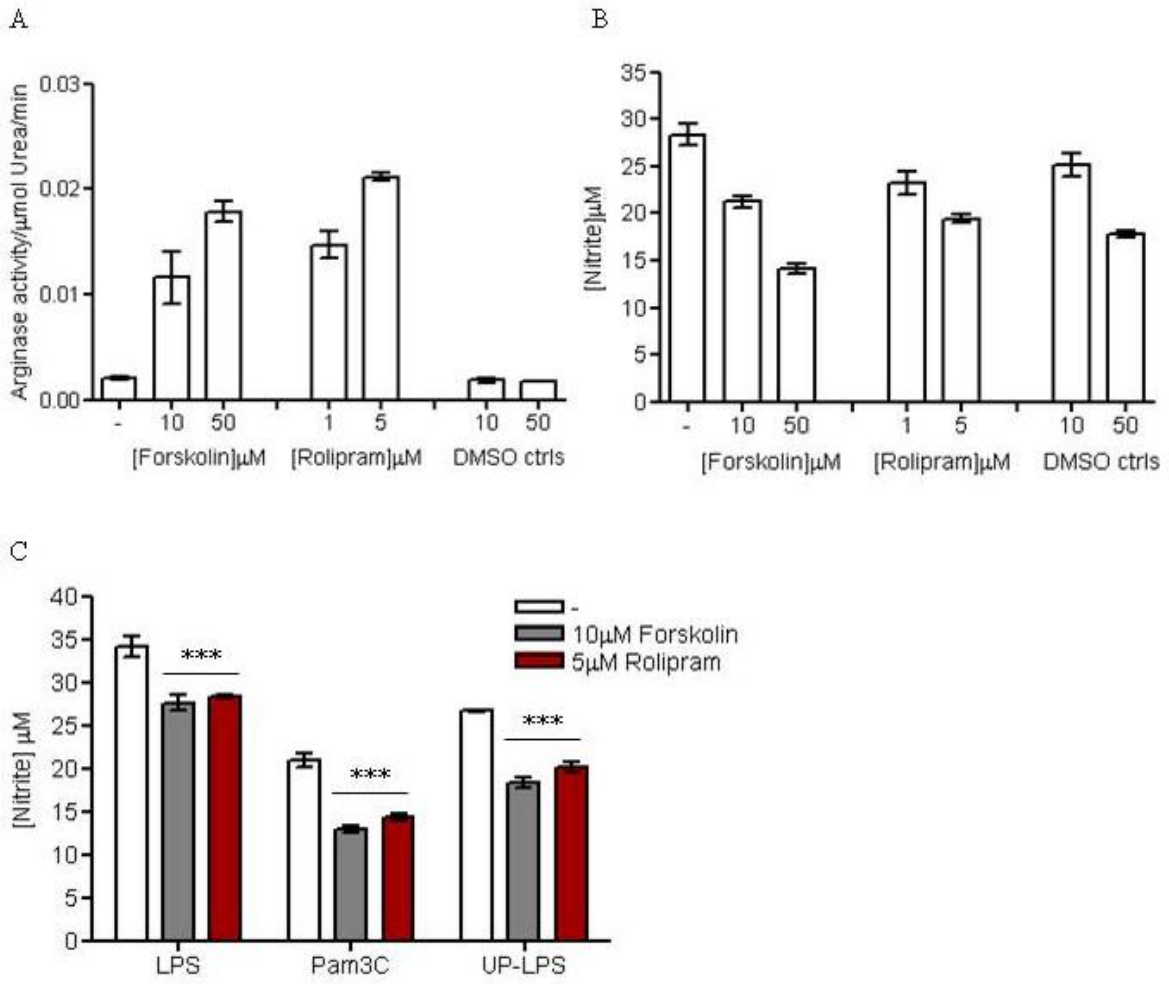


Figure 4.19: The effect of cAMP elevators on NO release by macrophages and arginase I activity. Macrophages were cultured in standard medium or classically activated with 10ng/ml LPS, ultrapure LPS (UP-LPS) or Pam3C for 20 hours. (A) The effect of 2 concentrations of forskolin and rolipram on BMMΦ arginase I activity, expressed as µmol Urea/ min per 5 x 10^4 cells. (B) The effect of forskolin and rolipram on CAMΦ (LPS) NO release, expressed as µM nitrite. DMSO controls corresponding to the final concentration of DMSO used to solubilise compounds were also included. The final concentration of DMSO in forskolin was the same as the forskolin concentration while 5µM rolipram corresponds had a final concentration of 10µM DMSO. (C) The effect of 10µM forskolin and 5µM rolipram on the levels of NO released by CAMΦ (LPS, Pam3C and ultrapure LPS – activated). The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. *** $p < 0.001$.

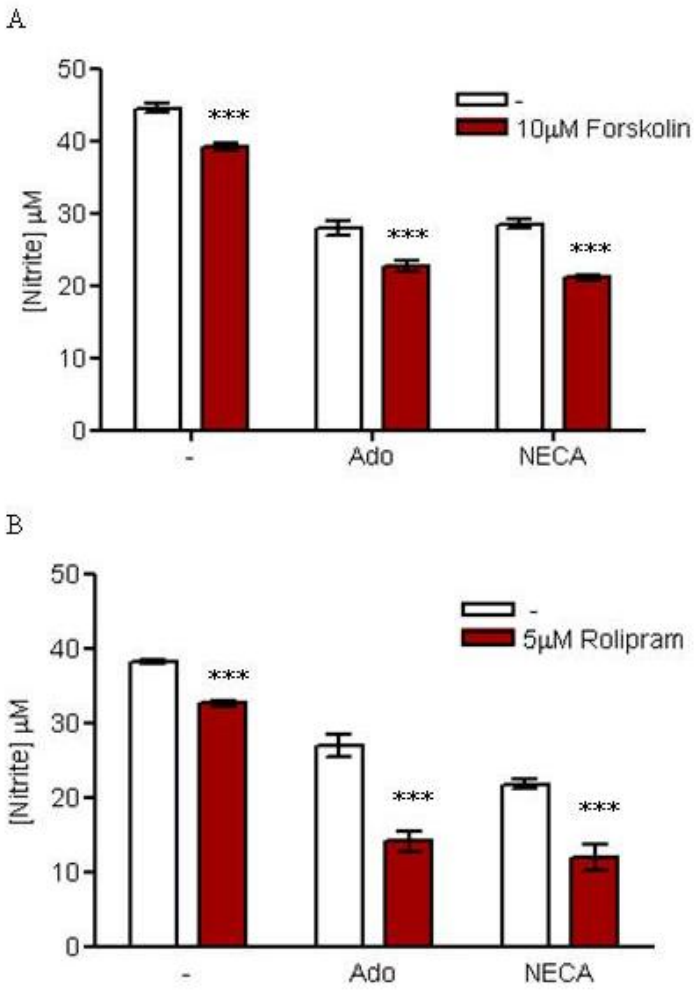


Figure 4.20: **The effect of cAMP elevators on adenosine/NECA-induced inhibition of NO release by CAMΦ.** Macrophages were classically activated with 10ng/ml LPS and the effect of 2 cAMP elevators on adenosine – induced inhibition of NO release investigated after 20 hours of culture. (A) The effect of 10µM forskolin on the inhibition of NO release by CAMΦ induced by 100µM adenosine or 100µM NECA, expressed as µM nitrite. (B) The effect of 5µM rolipram on the inhibition of NO release by CAMΦ induced by 100µM adenosine or 100µM NECA, expressed as µM nitrite. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance. *** $p < 0.001$.

Finally, the dependence of cytokine release on cAMP elevation was also investigated in CAMΦ and the results obtained are shown in Figure 4.21. cAMP elevation by forskolin and rolipram caused a reduction in the levels of IL-6 (Figure 4.21A) and TNF-α (Figure 4.21B), while the opposite was true for the levels of IL-10 (Figure 4.21C). The concentration of DMSO used to solubilise these compounds was tested on cells to determine their DMSO tolerance. The final concentration of DMSO in 50μM forskolin was again toxic to the cells as IL-6 and TNF-α release levels were reduced by DMSO alone. The levels of IL-10 release were not affected by the DMSO levels in these compounds. Having selected 10μM forskolin and 5μM rolipram as optimal concentrations for inducing a measurable difference in cytokine levels, CAMΦ were stimulated with adenosine or NECA in the presence of forskolin or rolipram. Both these compounds caused a 50% reduction in the levels of TNF-α (Figure 4.22A) and IL-6 (Figure 4.22B) released by CAMΦ that were stimulated with either adenosine or NECA. The decrease in the levels of these cytokines induced in CAMΦ stimulated with adenosine/NECA was more pronounced than the reduction detected in basal CAMΦ levels showing that, exogenous elevation of cAMP levels led to an additive response. As shown in Figure 4.22C, IL-10 release was enhanced following cAMP elevation as stimulation with both forskolin and rolipram resulted in the levels of IL-10 released by CAMΦ being 10% higher.

In general, it was observed that when cAMP elevators were used to stimulate macrophages, effector functions such as NO and cytokine release were affected in the same way as when adenosine was present in culture. The effect of adenosine on cAMP levels was further analysed at the molecular level to see if the shift caused between iNOS and arginase I metabolism of L-arginine was due to transcriptional up-regulation of arginase I or due to a shift in metabolism of L-arginine by iNOS.

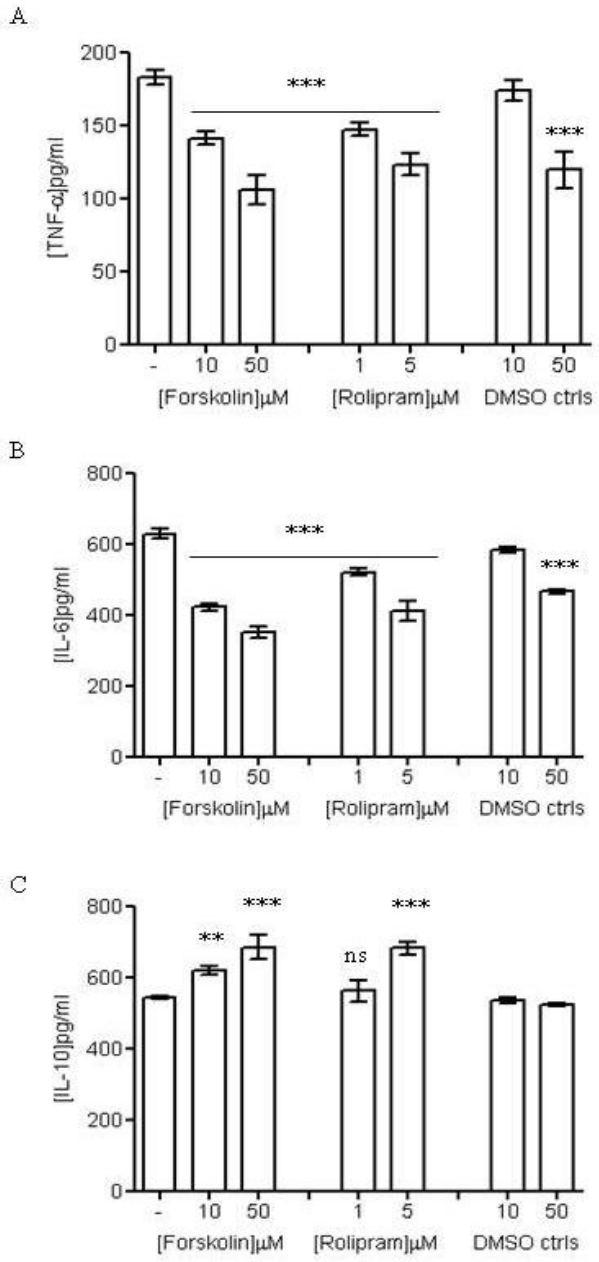


Figure 4.21: The effect of cAMP elevators on cytokine release from CAMΦ. Macrophages were classically activated with 10ng/ml LPS and the effect of 2 cAMP elevators on cytokine release investigated after 20 hours of culture. The effect of 2 concentrations of forskolin and rolipram on TNF-α (A) IL-6 (B) and IL-10 (C) release from CAMΦ is shown expressed in pg/ml. DMSO controls corresponding to the final concentration of DMSO used to solubilise compounds were also included. The final concentration of DMSO in forskolin was the same as the forskolin concentration while 5μM rolipram corresponds had a final concentration of 10μM DMSO. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance. ** $p < 0.01$, *** $p < 0.001$, ns - not significant ($p > 0.05$).

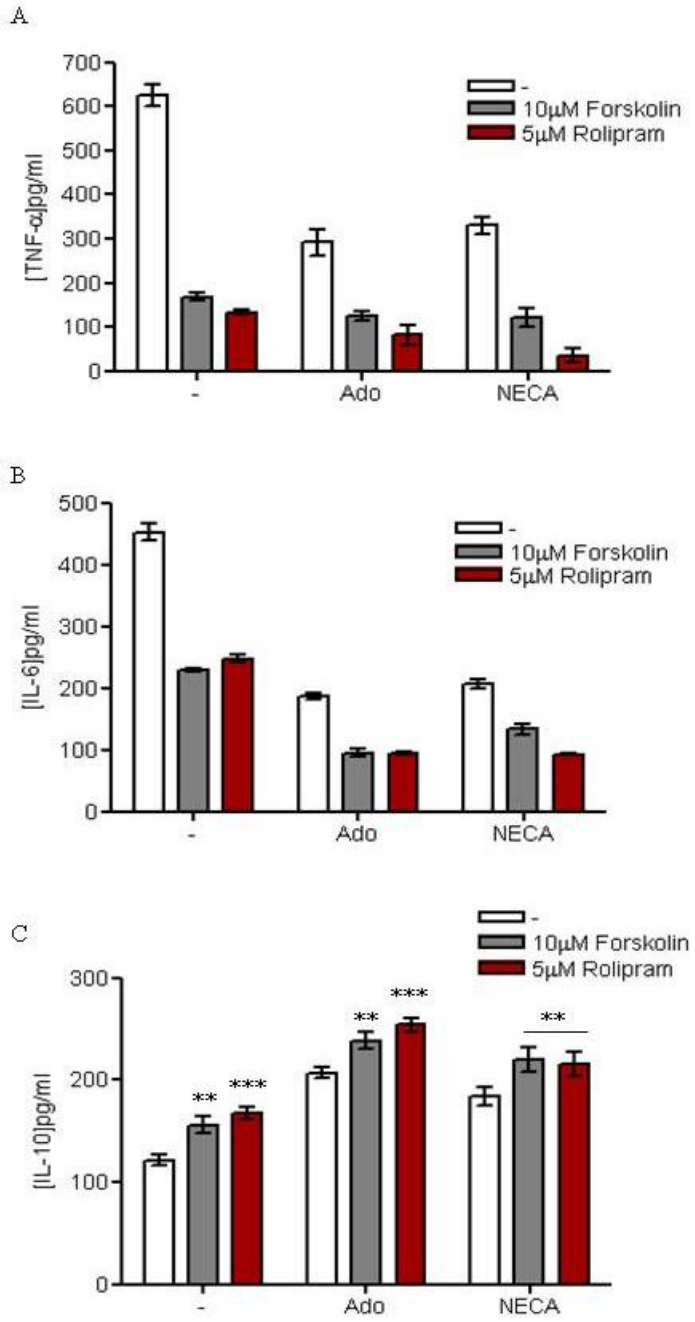


Figure 4.22: The effect of cAMP elevators on cytokine release from CAMΦ following stimulation with adenosine/NECA. Macrophages were classically activated with 10ng/ml LPS and the effect of 2 cAMP elevators on adenosine-induced inhibition of cytokine release investigated. The effect of 10μM forskolin and 5μM rolipram on TNF-α (A), IL-6 (B) and IL-10 (C) release by CAMΦ stimulated with 100μM adenosine or 100μM NECA is shown expressed in pg/ml. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate significance. ** $p < 0.01$, *** $p < 0.001$.

Quantitative PCR (Q-PCR) was carried out to determine arginase I mRNA levels following macrophage stimulation with the two cAMP elevators used previously in functional assays. Arginase I transcript levels were up-regulated in all 3 macrophage types following stimulation with forskolin or rolipram (Figure 4.23). In particular, it is shown in Figure 4.23A that, forskolin led to a 5-fold increase in arginase I mRNA levels in BMMΦ and CAMΦ, while in AAMΦ only a 3-fold increase was detected. Rolipram stimulation of BMMΦ and AAMΦ led to a 2-fold and 3-fold increase in arginase I mRNA levels, respectively, while CAMΦ arginase I mRNA levels were up-regulated by 15-fold (Figure 4.23B).

In order to see if adenosine stimulation of macrophages caused a similar up-regulation in arginase I transcripts, RT-PCR analysis was carried out on mRNA extracted from cells stimulated with the adenosine analogue, NECA, to ensure sustained P1R stimulation. In addition to arginase I mRNA, the presence of transcripts of markers of alternative activation in response to NECA was also investigated. The RT-PCR analysis is shown in Figure 4.24. Arginase I transcripts were detected in CAMΦ stimulated with NECA. The presence of the non-specific arginase I PCR product in the un-stimulated CAMΦ reaction, which is absent from the NECA-stimulated CAMΦ, suggests that, the levels of arginase I mRNA were probably increased by NECA-stimulation because binding of arginase I primers to non-specific cDNA was prevented by the higher abundance of specific arginase I cDNA. A comparison was carried out between P1R stimulation with NECA and P2YR stimulation with β-ADP to determine if the latter also leads to up-regulation of marker transcripts. Interestingly, β-ADP stimulation of CAMΦ led to inhibition of arginase I transcript expression. Neither NECA nor β-ADP stimulation of BMMΦ induced any detectable increase in the transcript levels of the markers of alternative activation tested. In AAMΦ, the high basal levels of all markers of alternative activation made it hard to distinguish

between the different stimulants used. For this reason as well as to clarify if arginase I transcripts were indeed up-regulated in CAMΦ, Q-PCR was also carried out on the same mRNA samples used for RT-PCR.

It is evident from the Q-PCR analysis shown in Figure 4.25 that, stimulation of BMMΦ, CAMΦ and to a lesser extent, AAMΦ, led to up-regulation of arginase I transcript levels. In addition, it can be seen that, although not detected by RT-PCR, arginase I transcript levels were also up-regulated significantly in BMMΦ (Figure 4.25A). However, the increase in arginase I transcript levels induced by NECA in CAMΦ (Figure 4.25B) was almost 2-fold more than the increase induced in BMMΦ, which may explain why this increase was only detected by the more sensitive Q-PCR technique. In AAMΦ, arginase I transcript levels only increased by 5-fold following NECA stimulation possibly due to the fact that, arginase I transcripts are already abundantly expressed in this macrophage type (Figure 4.25C).

In the case of β-ADP, the results shown in Figure 4.26 did not reveal any significant changes in the levels of markers of alternative activation expressed by BMMΦ (Figure 4.26A) and CAMΦ (Figure 4.26B). An increase in Fizz1 and Ym1 levels was apparent in AAMΦ stimulated with β-ADP corresponding to 4 and 10-fold, respectively (Figure 4.26C). In all the macrophage types, arginase I transcript levels were not affected by P2YR stimulation with β-ADP.

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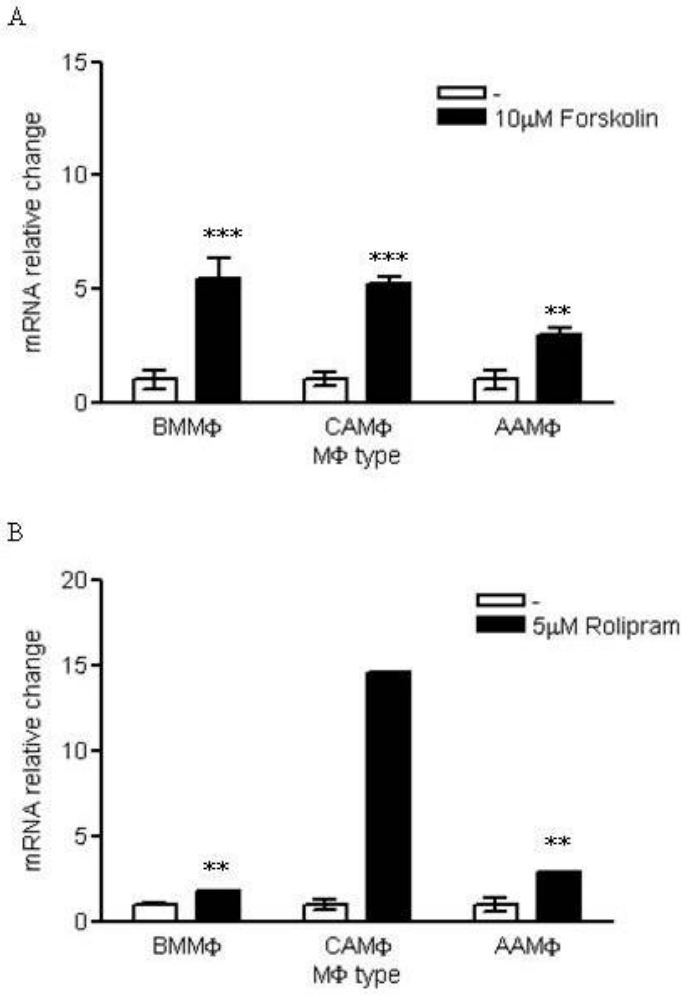


Figure 4.23: The effect of cAMP elevators on BMMΦ arginase I transcript levels. Macrophages were cultured in standard medium and treated with either 10μM forskolin (A) or 5μM rolipram (B) for 20 hours. The fold changes presented are relative to untreated BMMΦ after normalising to β-actin mRNA and error bars show standard error of the mean from one experiment carried out in triplicate. Asterisks indicate significance. ** $p < 0.01$, *** $p < 0.001$.

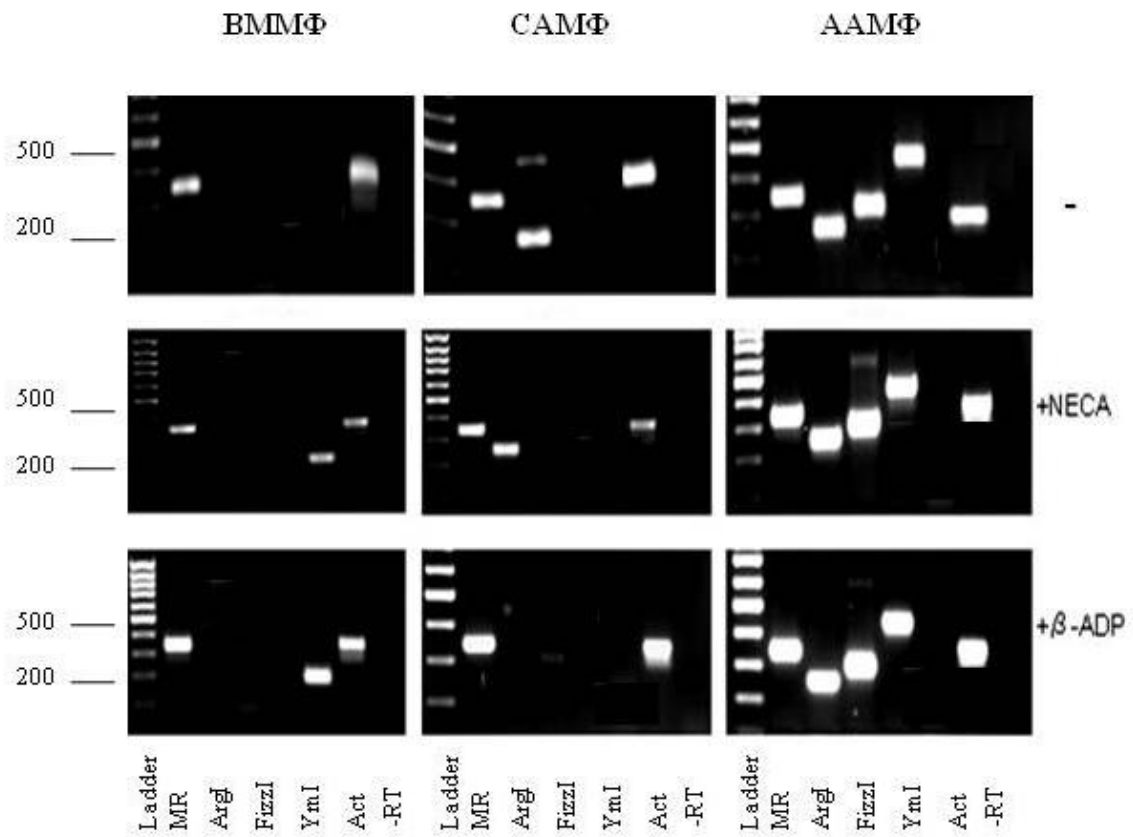


Figure 4.24: Detection of transcripts of markers of alternative activation following NECA or β-ADP stimulation. Macrophages were stimulated with 100μM NECA or 100μM β-ADP for 20 hours and mRNA levels of mannose receptor (MR), arginase I (Arg1), Fizz1 and Ym1 were detected using RT-PCR. PCR products of expected sizes, determined by the ladder shown annotated on the left in bp, were obtained (see Materials and Methods, Table 2.1A). Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

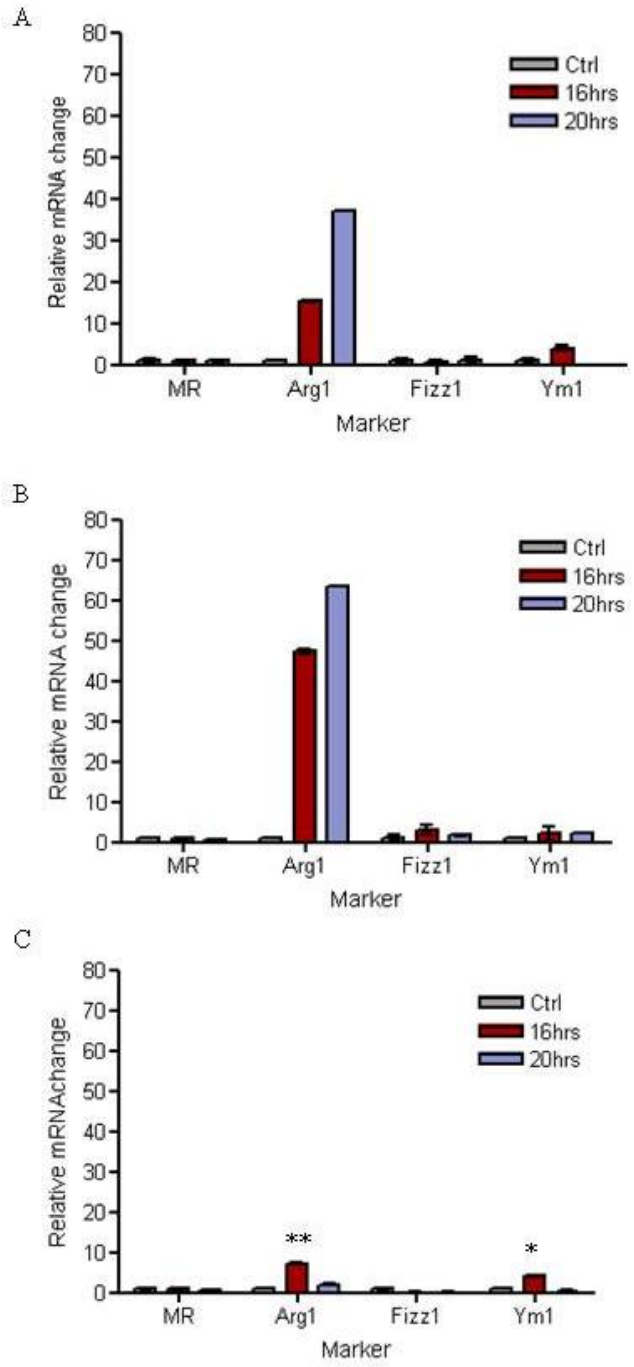


Figure 4.25: Changes in mRNA levels of AAMΦ markers in response to NECA. RNA isolated from BMMΦ (A), macrophages activated with 10ng/ml LPS-CAMΦ (B) and macrophages activated with 5ng/ml IL-4-AAMΦ (C) stimulated with 100μM NECA for 16 or 20 hours was used to produce cDNA for quantitative PCR to determine changes in mRNA levels of mannose receptor, arginase, Fizz1 and Ym1. The fold changes presented are relative to the respective macrophage type cultured without NECA, after normalising to β-actin mRNA. * $p < 0.05$, ** $p < 0.01$.

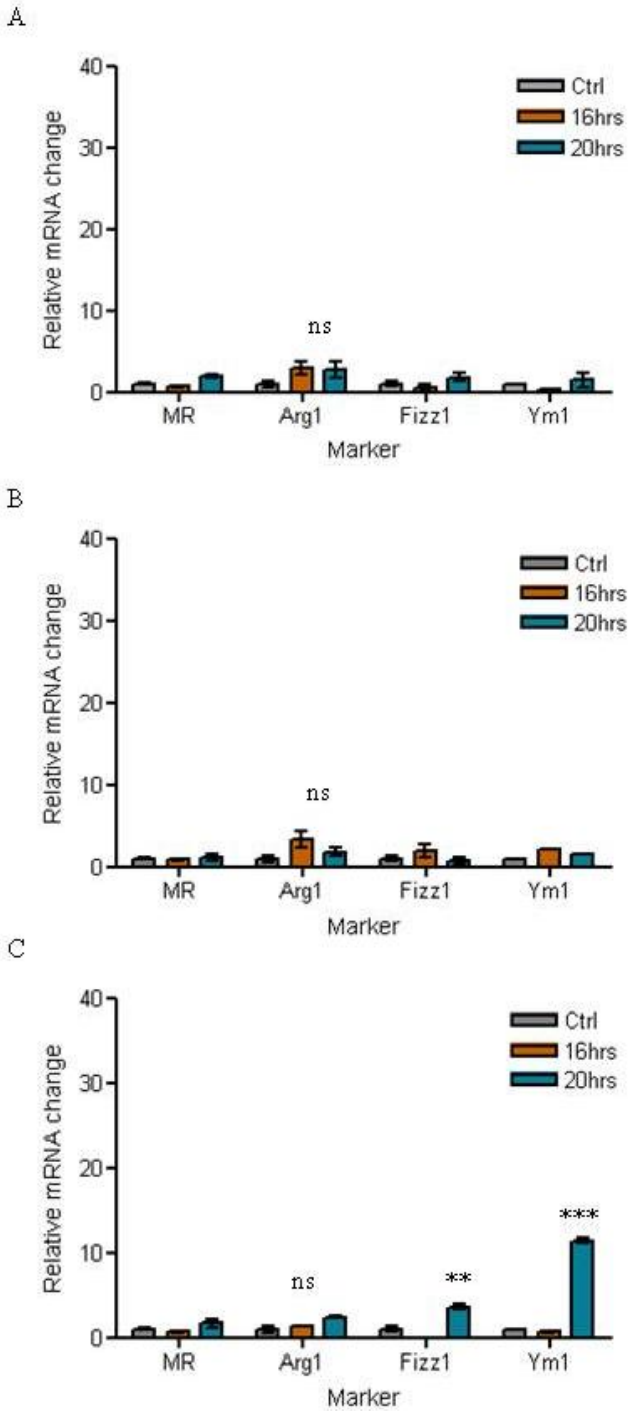


Figure 4.26: Changes in mRNA levels of AAMΦ markers in response to β-ADP. RNA RNA isolated from BMMΦ (A), macrophages activated with 10ng/ml LPS-CAMΦ (B) and macrophages activated with 5ng/ml IL-4-AAMΦ (C) stimulated with 100μM β-ADP for 16 or 20 hours was used to produce cDNA for quantitative PCR to determine changes in mRNA levels of mannose receptor, arginase, Fizz1 and Ym1. The fold changes presented are relative to the respective macrophage type cultured without β-ADP, after normalising to β-actin mRNA. ** $p < 0.01$, *** $p < 0.001$, ns - not significant ($p > 0.05$).

Involvement of PLC in purinergic signalling

Gq involvement was investigated by inhibiting phospholipase C (PLC) in macrophages treated with adenosine and NECA. This was achieved by using U73122 which is a PLC, and PLA_2 inhibitor, that prevents G-protein coupling to PLC and generation of the second messengers IP3 and DAG (Grinberg *et al*, 2009). In Figure 4.27 a comparison is shown of the effect of inhibiting cAMP (Rp-cAMP) or PLC (U73122) signalling on inhibition of NO release by adenosine/NECA and nucleotides. An inactive analogue of U73122 (U73433) was also available and was used to confirm that any responses inhibited by the PLC inhibitor were only due to blocking PLC activity and not due to intrinsic compound-induced effects. Inhibition of PLC activity (Figure 4.27A) did not reverse the nucleotide-induced inhibition of NO release which was only achieved when Rp-cAMP was used in macrophage cultures (Figure 4.27B). Taken together with the fact that PLC inhibition appeared to enhance the AMP-induced inhibition of NO release (Figure 4.27A), it is evident that elevation of cAMP was necessary for inhibition of NO release by CAMΦ following P1R stimulation.

A comparison between cAMP and PLC activation was also carried out to determine which pathway was involved in nucleotide-induced cytokine responses by CAMΦ. As it is shown in Figure 4.28A and B, the levels of TNF-α and IL-6 inhibited by stimulation of CAMΦ with adenosine, NECA, AMP, ADP or ATP were restored to basal levels only by blocking cAMP accumulation. In addition, IL-10 levels that were enhanced by stimulation of CAMΦ with nucleotides were reduced to initial levels when Rp-cAMP was used to inhibit cAMP signalling. CAMΦ stimulated with adenosine, NECA, AMP or ADP were pre-incubated with the PLC inhibitor U73122 to determine if PLC activation was involved in the effects induced by these compounds on cytokine release. It should be noted that the slowly hydrolysable analogue of ADP

(β-ADP) was used in these experiments to determine if PLC activation via the P2Y receptors was responsible for ADP and ATP-induced effects. Inhibition of PLC activity did not reverse the nucleotide-induced reduction of TNF-α and IL-6 release from CAMΦ as shown in Figure 4.29A and B. Similarly, the levels of IL-10 release, which were up-regulated in response to nucleotide stimulation, remained higher than the basal levels even when PLC activity was blocked (Figure 4.29C).

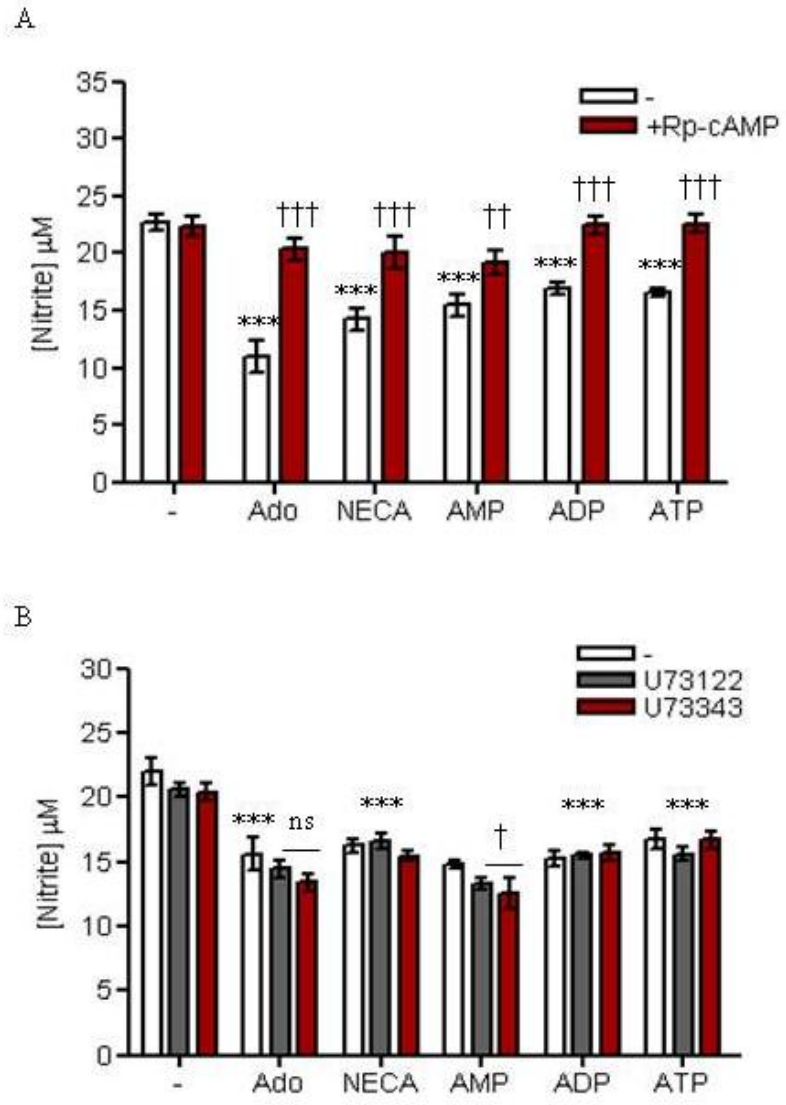


Figure 4.27: The effect of cAMP and PLC inhibition on adenosine-induced inhibition of NO release from CAMΦ. Macrophages were classically activated with 10ng/ml LPS and treated with 100µM adenosine/NECA or nucleotides for 20 hours. Cells were pre-incubated either with the cAMP inhibitor, Rp-cAMP, or with the PLC inhibitor U73122 or the mock inhibitor U73343 and then stimulated with adenosine, NECA or the nucleotides shown. (A) The effect of inhibiting cAMP elevation with 50µM Rp-cAMP on the adenosine and nucleotide – induced inhibition of NO release by CAMΦ is expressed in µM nitrite. (B) The effect of inhibiting PLC with 10µM P U73122 on the adenosine and nucleotide – induced inhibition of NO release by CAMΦ is expressed in µM nitrite. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance compared to untreated control. *** $p< 0.001$. (†) is compared to the effect induced by the respective nucleoside/nucleotide. † $p< 0.05$, †† $p< 0.01$, ††† $p< 0.001$, ns - not significant ($p> 0.05$).

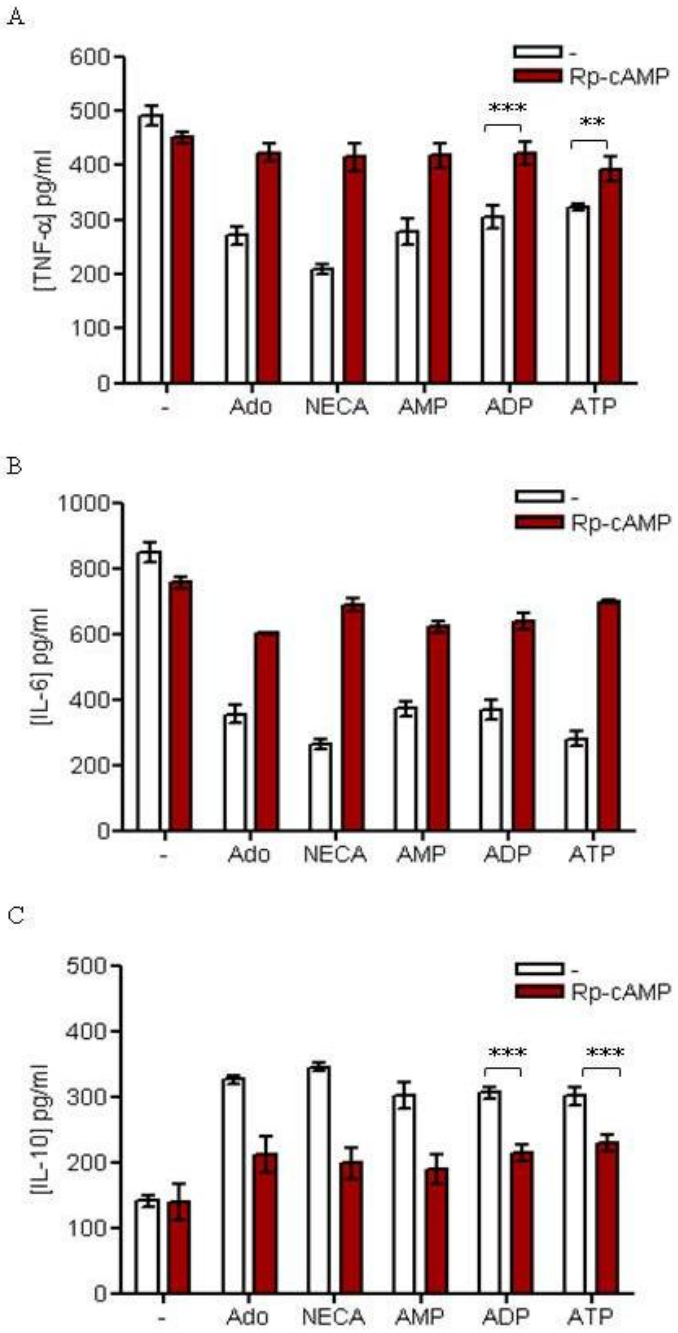


Figure 4.28: The effect of cAMP inhibition on adenosine-induced inhibition of cytokine release by CAMΦ. Macrophages were classically activated with 10ng/ml LPS and treated with 100μM adenosine/NECA or nucleotides for 20 hours. Cells were pre-incubated with the cAMP inhibitor, Rp-cAMP and then stimulated with adenosine, NECA or the nucleotides shown. The effect of inhibiting cAMP elevation with 50μM Rp-cAMP on the adenosine and nucleotide – induced changes in TNF-α (A), IL-6 (B) and IL-10 (C) release by CAMΦ is shown expressed in pg/ml. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance compared to untreated control. ** $p < 0.01$, *** $p < 0.001$.

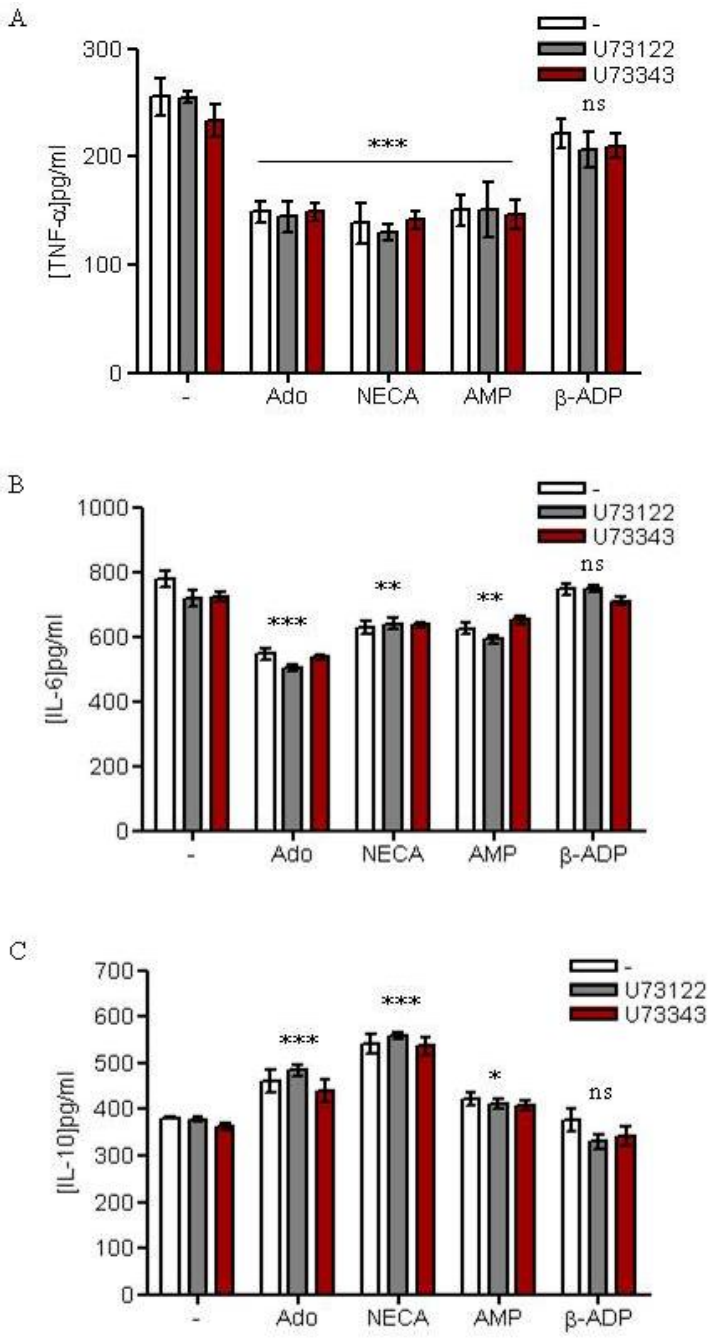


Figure 4.29: The effect of PLC inhibition on adenosine-induced inhibition of cytokine release by CAMΦ. Macrophages were classically activated with 10ng/ml LPS and treated with 100μM adenosine, NECA or 100μM of the nucleotides shown for 20 hours. The effect of inhibiting PLC with 10μM U73122 on the adenosine and nucleotide – induced changes in TNF-α (A), IL-6 (B) and IL-10 (C) release by CAMΦ is shown expressed in pg/ml. U73343 is an inactive analogue of U73122 used at the same concentration. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns - not significant ($p > 0.05$).

Involvement of PI3 kinase in purinergic signalling

Macrophage PI3 kinase (PI3K) activity was inhibited by a known PI3K inhibitor, LY294002 (Koga *et al*, 2009), and the resulting NO and cytokine release by CAMΦ were assayed. Before testing the effect of this inhibitor on adenosine/NECA-induced inhibition it was observed that basal NO release by CAMΦ was inhibited even at the lowest concentration of LY29002 used (10μM) (Figure 4.30). Furthermore, when LY294002 was combined with adenosine or NECA, a further inhibition was observed proportional to the inhibition seen in the control (-).

Similar to the inhibitory effect of LY294002 on NO release by CAMΦ, the basal levels of TNF-α, IL-6 and IL-10 release were all inhibited by 20% to 50% when PI3K activity was blocked (Figure 4.31). In fact, even in the presence of adenosine or NECA, IL-10 release levels were reduced by the same amount by both concentrations of LY29004 used to block PI3K activity (Figure 4.31C). As can be seen in Figure 4.31A and B, the adenosine/NECA-induced inhibition of TNF-α and IL-6 release was enhanced by both concentrations of LY29004 used.

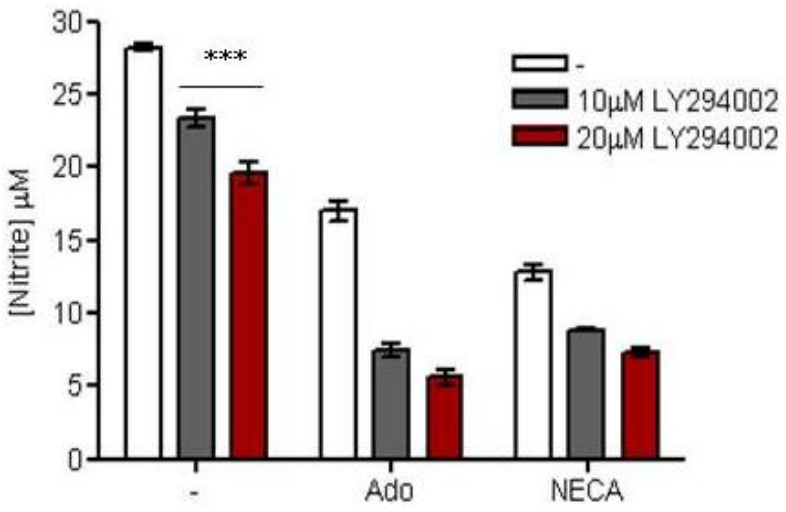


Figure 4.30: The effect of PI3 kinase inhibition on adenosine-induced inhibition of NO release by CAMΦ. Macrophages were classically activated with 10ng/ml LPS and treated with 100µM adenosine or NECA for 20 hours. The effect of inhibiting PI3 kinase activity with 2 different concentrations of LY294002 was investigated by measuring the nitrite levels in cell supernatants. Levels of NO release are expressed as µM nitrite and the data presented are an average of triplicates $\pm$ SEM from one representative experiment of $n > 3$ and asterisks indicate significance compared to the respective untreated control. *** $p < 0.001$.

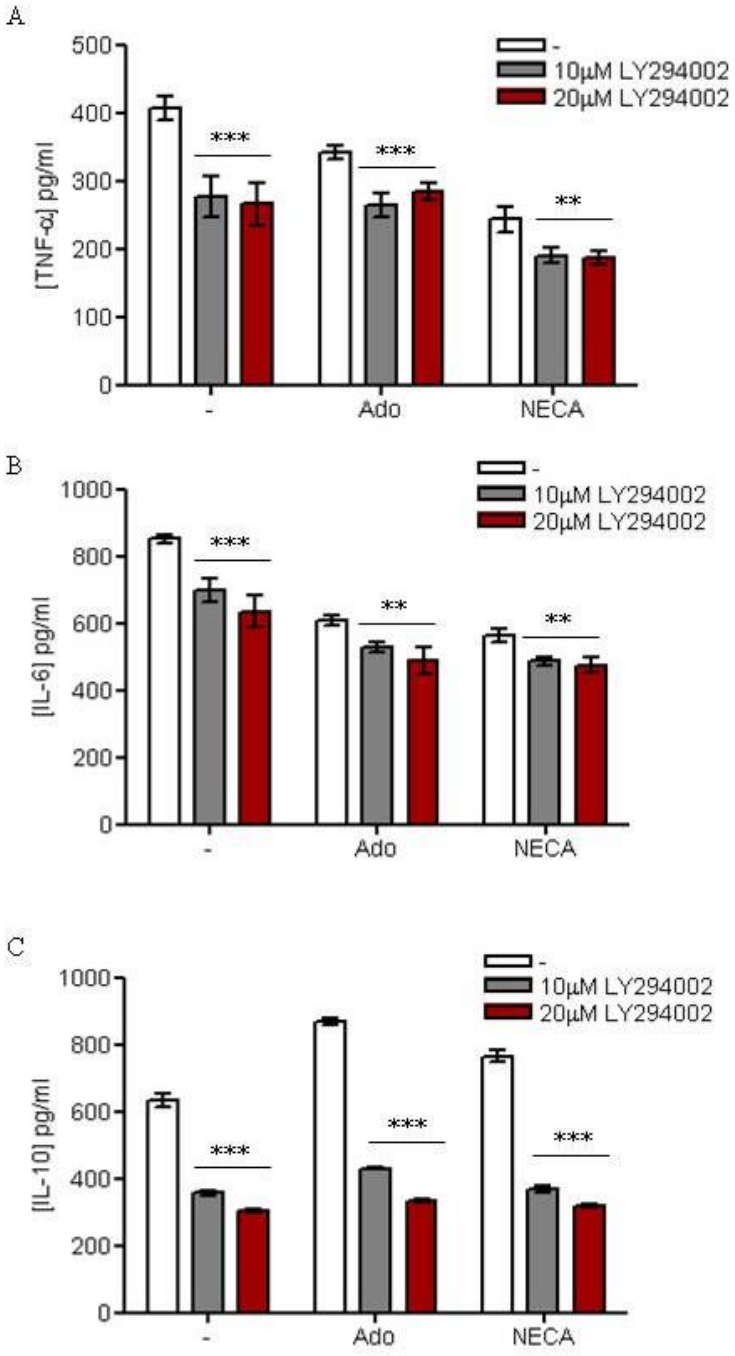


Figure 4.31: The effect of PI3 kinase inhibition on adenosine-induced inhibition of cytokine release by CAMΦ. Macrophages were classically activated with 10ng/ml LPS and treated with 100μM adenosine or NECA for 20 hours. The effect of inhibiting PI3 kinase activity with 2 different concentrations of LY294002 was investigated by measuring the cytokine levels in cell supernatants. Levels of cytokine release are expressed in pg/ml and the data presented are an average of triplicates $\pm$ SEM from one representative experiment of $n > 3$ and asterisks indicate significance compared to the respective untreated control.** $p < 0.01$, *** $p < 0.001$.

The effects of IL-10R signalling

Having established that P1 receptor stimulation led to an increase in the levels of IL-10 released by CAMΦ (Figure 4.7C), the effect of IL-10R signalling was investigated. CAMΦ were pre-incubated with an anti-IL-10 antibody prior to adenosine/NECA or AMP stimulation in order to block the action of IL-10 released in response to P1R activation. Firstly, a range of anti-IL-10 concentrations was used to determine if there were any concentration-dependent effects on the basal levels of TNF-α and IL-6 release by CAMΦ. As it is shown in Figure 4.32A and B, administering IL-10 exogenously inhibited both TNF-α and IL-6 release from CAMΦ by 10% and 90%, respectively. As the concentration of anti-IL-10 was increased to 2.5μg/ml, the levels of TNF-α and IL-6 released from CAMΦ increased (Figure 4.32A and B). The positive control testing the effect of anti-IL-10 on IL-10 release showed that, after 1.5μg/ml IL-10 release was completely neutralised (Figure 4.32C). In fact, this corresponds to the concentration after which both TNF-α and IL-6 release reached maximal levels.

Having selected 2.5μg/ml as the optimal concentration of anti-IL-10 required to neutralise extracellular IL-10, anti-IL-10 was added to cultures of CAMΦ stimulated with adenosine, NECA or AMP to determine if eliminating IL-10 released by these cells relieved inhibition of TNF-α and IL-6 release. The inhibition induced by adenosine on IL-6 release by CAMΦ was almost completely reversed by neutralising IL-10, but only a partial restoration of TNF-α levels was achieved (Figure 4.32 D and E). In the case of NECA-induced inhibition of TNF-α and IL-6 release, almost 100% reversal was detected when extracellular IL-10 was neutralised. Finally, the inhibition of TNF-α and IL-6 release from CAMΦ that was detected by stimulation with AMP was 50% less when IL-10 accumulation was prevented.

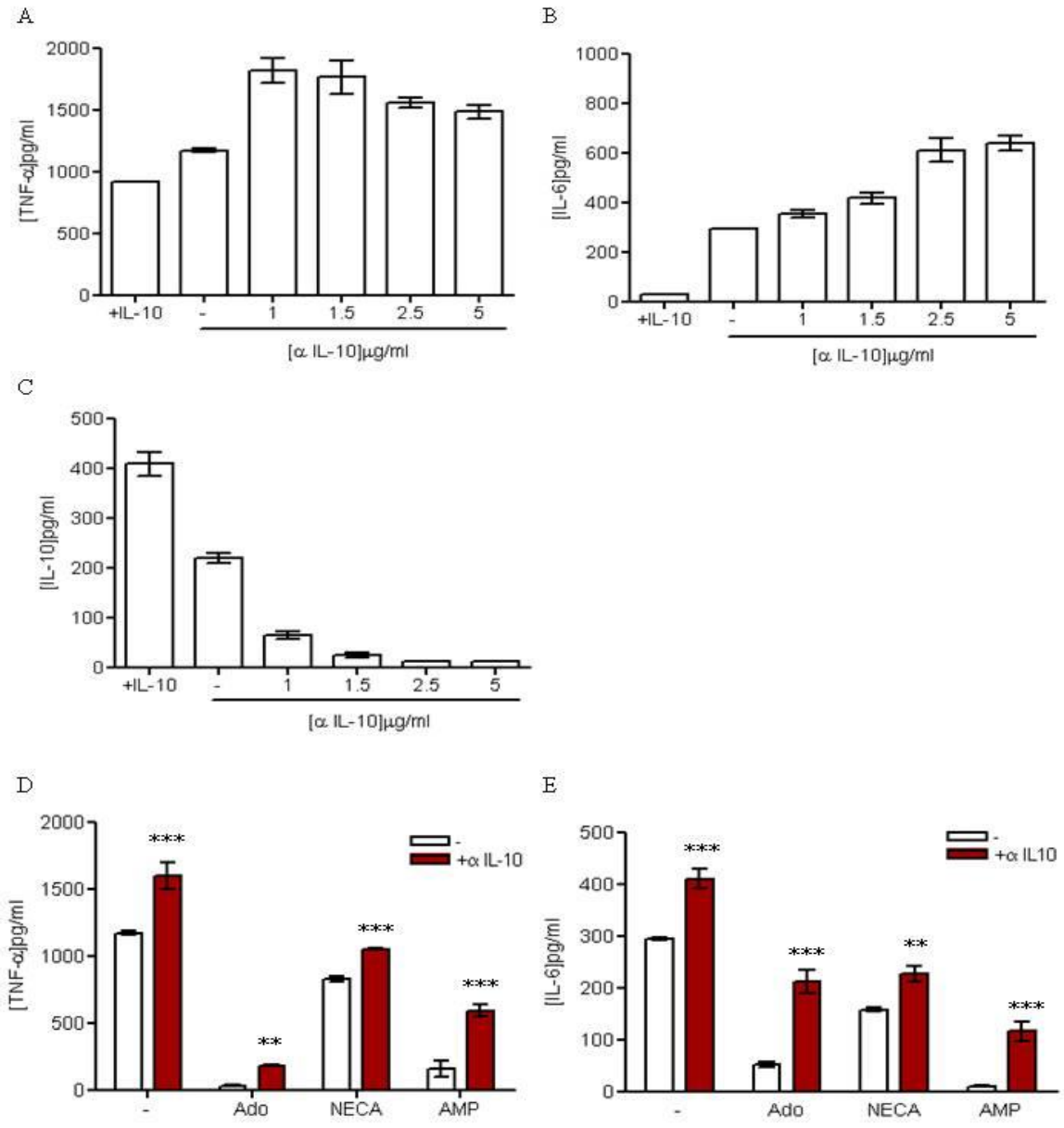


Figure 4.32: The effect of extracellular IL-10 neutralisation on cytokine release from CAMΦ. Macrophages were classically activated with 10ng/ml LPS and cultured for 20 hours with 100µM adenosine, NECA or AMP. Supernatants were then assayed for TNF-α (A and D), IL-6 (B and E) and IL-10 (C) and levels are expressed in pg/ml. The effect of a range of α-IL-10 concentrations on (A) TNF-α, (B) IL-6 and (C) IL-10 release from CAMΦ. (D) The effect of neutralising IL-10 with 2.5µg/ml α-IL-10 on the inhibition of TNF-α release from CAMΦ induced by 100µM adenosine. (E) The effect of neutralising IL-10 with 2.5µg/ml α-IL-10 on the inhibition of IL-6 release from CAMΦ induced by 100µM adenosine. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control (-). ** $p < 0.01$, *** $p < 0.001$.

Involvement of MEK1/2 and NF-kB in purinergic signalling

The effect of purinergic receptor stimulation on downstream signalling was further investigated by looking at the involvement of two pathways, namely, the MAP Kinase Kinase (MEK1/2) and NF-κB pathways. CAMΦ were pre-incubated with known inhibitors of MEK1/2 (Bay11-7082) and NF-κB pathways (U0126) (Gringhuis *et al*, 2007). In CAMΦ, basal NO release was inhibited by Bay 11-7082 as opposed to U0126 which slightly enhanced NO release upon TLR stimulation with Pam3C and Ultrapure LPS (Figure 4.33A). Inhibition of these two pathways was also tested in terms of P1R stimulation by the analogue of adenosine, NECA. As it is shown in Figure 4.33B, the NECA-induced inhibition of NO release by CAMΦ was almost completely reversed by U0126.

In terms of cytokine release, it is shown in Figure 4.34 that Bay 11-7082 and U0126 inhibited all cytokines released by LPS, Pam3C and ultrapure LPS-activated CAMΦ. TLR2 (Pam3C)-activated CAMΦ produced higher levels of TNF-α and lower IL-10 levels than LPS and ultrapure LPS-activated CAMΦ, whereas TLR4 (ultrapure LPS)-activated CAMΦ released comparable cytokine levels to LPS-activated CAMΦ (Figure 4.34A, B and C). Despite the fact that activation via different TLRs led to different basal levels of cytokine release, inhibition of both the NF-κB and MEK1/2 pathways resulted in equal inhibition of cytokine release by all the CAMΦ types.

Since the NF-κB inhibitor was toxic to macrophages and inhibited basal levels of CAMΦ NO and cytokine release, NF-κB involvement could not be confirmed. The fact that the MEK1/2 inhibitor reversed some effects could point to MEK1/2 involvement but it cannot be concluded that the effects observed are solely dependent on this pathway.

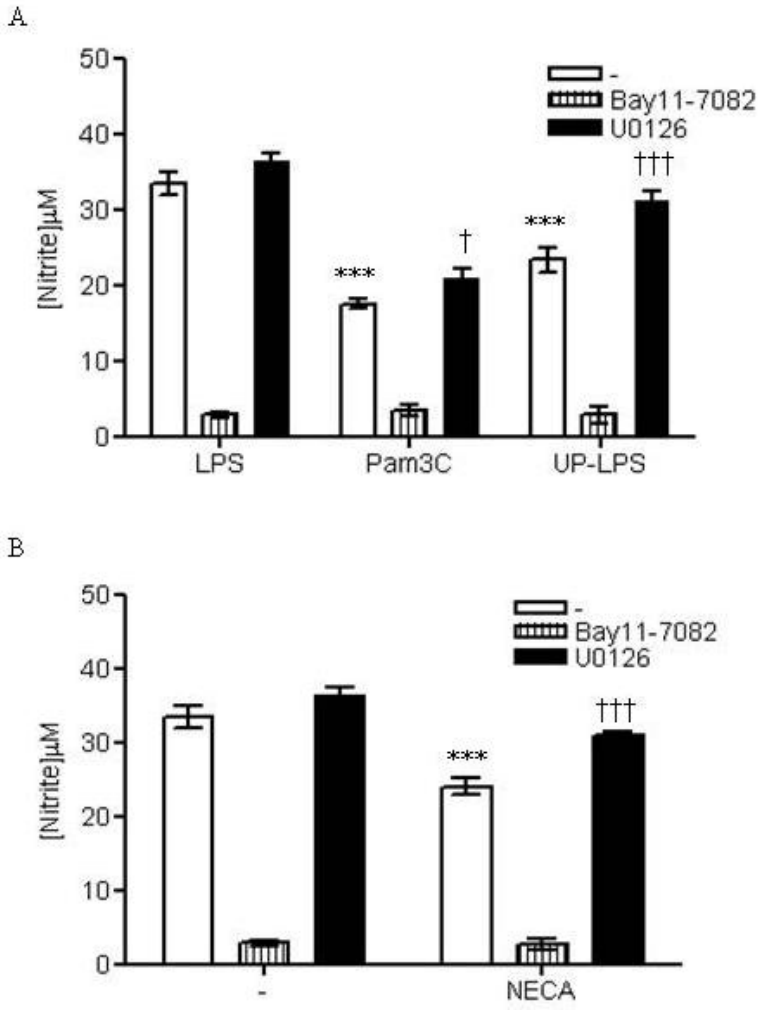


Figure 4.33: The effect of NF-κB and MEK1/2 inhibition on NO release by CAMΦ and inhibition of NO release by CAMΦ stimulated with NECA. (A) Macrophages were classically activated with 10ng/ml LPS, Pam3C or ultrapure LPS (UP-LPS) for 20 hours and the effect of 5µM Bay 11-7082 (NF-κB selective inhibitor**)** and 5µM U0126 (MEK1/2 inhibitor) on NO release was investigated. (B) The effect of inhibiting NF-κB and MEK1/2 on NO release by LPS-activated macrophages stimulated with 100µM NECA for 20 hours. Levels of NO release are expressed as µM nitrite. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. *** $p< 0.001$. (†) is compared to the respective control cultured in the absence of inhibitors. † $p< 0.05$, ††† $p< 0.001$.

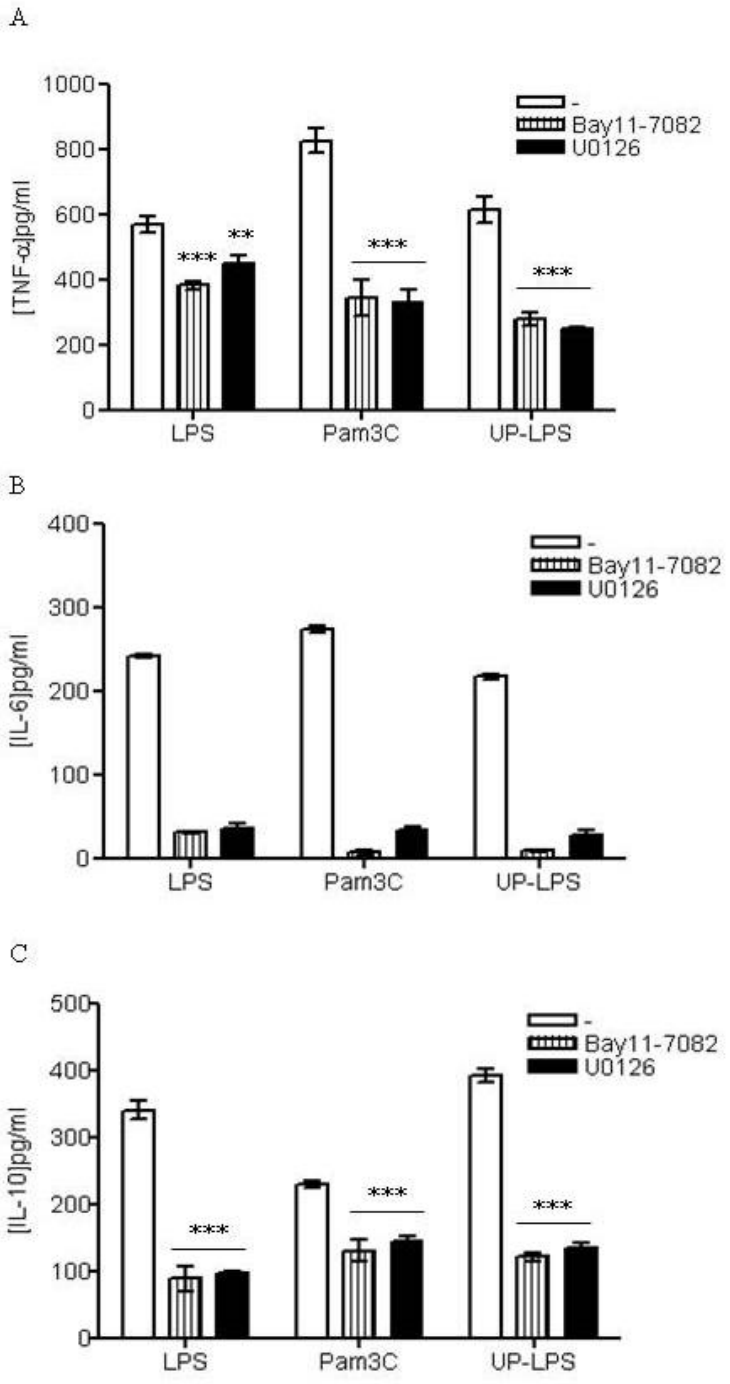


Figure 4.34: The effect of NF-κB and MEK1/2 inhibition on cytokine release from CAMΦ. Macrophages were classically activated with 10ng/ml LPS, Pam3C or ultrapure LPS (UP-LPS) for 20 hours and the effect of 5μM Bay 11-7082 (NF-κB selective inhibitor**)** and 5μM U0126 (MEK1/2 inhibitor) on cytokine release investigated. Supernatants were assayed for TNF-α (A), IL-6 (B) and IL-10 (C) release, expressed in pg/ml. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. ** $p < 0.01$, *** $p < 0.001$.

4.3 Discussion

The phenotype of activated macrophages is characterised by a number of effector functions as well as expression of specific proteins. These effector functions are generally either pro- or anti- inflammatory and the balance between these two states is achieved by the ability of macrophages to switch from the pro-inflammatory classical activation mode to the anti-inflammatory alternative activation mode. The state of macrophage activation can dictate how they respond to danger signals they may encounter in their local environment. Nucleotides, which are found at high concentrations intracellularly, are released from damaged or lysed cells at sites of inflammation and are therefore considered to be ubiquitous danger signals for cells that possess purinergic receptors. Having established that murine bone marrow derived macrophages (BMMΦ) express purinergic receptors at the molecular level (Chapter 3), purinergic signalling in BMMΦ was investigated. Furthermore, the different macrophage activation states, known as classical and alternative, were characterised in terms of effector functions and the impact of purinergic signalling on these functions studied for each macrophage activation phenotype.

Characterisation of macrophage activation phenotype was carried out for the two activation states used in this study in terms of their effector functions. In order to generate optimal conditions for classical and alternative activation, macrophages were activated with TLR ligands or IL-4, respectively, and nitric oxide (NO) release was assayed and compared to arginase I activity. The reason for selecting NO release and arginase I activity to distinguish between the two macrophage activation phenotypes is that, in the absence of microbial stimulation or in the presence of Th2 cytokines, L-arginine metabolism proceeds via the arginase I pathway which generates polyamines used for biosynthesis and repair as opposed to the cytotoxic NO produced by the inducible NO synthase (iNOS) pathway (Munder *et al*, 1998; Hesse *et al*, 2001). In an

attempt to investigate the possible switch of L-arginine metabolism from iNOS to arginase I that could cause a phenotype shift, both macrophage subsets were assayed for NO release and arginase I activity to see if there was a pattern of reciprocal activation of the two enzymes. LPS was used to induce classical activation of macrophages producing a robust response between 0.5ng/ml and 10ng/ml with the latter concentration significantly eliciting NO release. Alternative activation was achieved using 5ng/ml IL-4, as determined by arginase I activity levels which were optimal at this concentration. IL-13, the other Th2 cytokine capable of inducing alternative activation, was also found to optimally induce arginase I activity in the macrophage cell line, J774A.1, when used at this concentration (Chang *et al,* 2000), while IL-4 used at 8ng/ml produced submaximal arginase I activity levels in peritoneal macrophages (Waddington *et al,* 1998).

Increasing LPS concentration increased NO release by BMMΦ while arginase I activity followed a similar pattern of induction with increasing IL-4 concentrations, confirming previously published work showing reciprocal activation of iNOS and arginase I depending on the macrophage local cytokine environment (Modolell *et al,* 1995). In this study, CAMΦ were found to have arginase I activity that was significantly higher than that observed in BMMΦ. However, AAMΦ arginase I activity was 10-fold higher than the arginase I activity detected in CAMΦ. Interestingly, TLR activation by LPS has been shown to be responsible for arginase I up-regulation in peritoneal macrophages and has been proposed to be a means of controlling NO levels in chronic bacterial infections (Ryan *et al,* 1980; Sonoki *et al,* 1997; Sosroseno *et al,* 2006). In addition, no increase in arginase I activity was observed with IFN-γ in this study showing that, it is an LPS-dependent effect rather than a Th1-dependent response and also

confirming published observations showing that this effect is due to a MyD88-dependent pathway, as IFN-γ acts via a different pathway (El Kasmi *et al*, 2008).

The balance between iNOS and arginase I can be achieved by altering enzyme availability (by means of protein expression), enzyme activity or by indirect control resulting from substrate availability. In order to account for limiting L-arginine being the cause of lower arginase I activity in CAMΦ in this study, L-arginine was administered exogenously. This did not appear to alter CAMΦ arginase I activity levels significantly while in the case of AAMΦ exogenous L-arginine led to decreased arginase I activity levels. In fact, exogenous L-arginine appeared to enhance NO release levels in both CAMΦ and AAMΦ, the increase in CAMΦ NO release being more significant, suggesting that L-arginine supplementation resulted in a shift to its use by iNOS, so it can be considered both as a substrate and regulator of this pathway. It has been shown in a study using IL-13 to induce arginase I activity in peritoneal macrophages that extracellular arginine depletion had the same effect on macrophage iNOS activity as IL-13 itself and that administration of exogenous L-arginine could reverse the IL-13-induced inhibition of iNOS expression (El-Gayar *et al*, 2003). These observations point to iNOS regulation by Th2 cytokines being a result of lack of substrate, depleted as a result of arginase I up-regulation, as well as inhibition of iNOS synthesis. Therefore, interaction between these two pathways is important in pathophysiologic conditions as a counteracting mechanism in case of NO over-production.

Cytokine release is another important macrophage function that can determine the outcome of immune responses. In this study, cytokine release from bone marrow derived macrophages was only detected when cells were cultured in the presence of TLR ligands such as LPS and Pam3C. Specifically, titrating LPS revealed a concentration-dependent increase in the release of all cytokines tested, namely TNF-α, IL-6 and IL-10. Similar to NO release, 1-10ng/ml

LPS were sufficient to produce a robust response. Having selected an LPS concentration for optimally inducing classical activation of macrophages as determined by their effector functions, the effect of purinergic receptor activation on these effector functions was investigated. Taking into account that BMMΦ and AAMΦ do not release cytokines *in vitro*, the only way of assaying the effect of purinergic signalling on AAMΦ was by means of arginase I activity.

Purinergic receptor activation was investigated in both murine macrophages activation subsets by exogenously administering various nucleotides and testing the resulting macrophage responses in terms of effector functions such as NO release, arginase I activity and cytokine release. Firstly, the effect of purinergic receptor activation on the switch between classical and alternative macrophage activation was investigated by assaying CAMΦ NO release versus arginase I activity. NO release was only affected in CAMΦ as the other macrophage subsets do not produce NO in the presence or absence of nucleotides**.** In general, adenosine inhibited NO release from CAMΦ as did all adenine nucleotides and UDP which is known to be a ligand for P2Y6 expressed by monocytes (Cox *et al*, 2005). pharmacological characterisation of purinergic receptor stimulation suggested that the active ligand was adenosine. Indeed, taking into account data obtained in this study and presented in Chapter 3, macrophages have functional, surface-expressed enzymes capable of degrading nucleotides. Therefore, it can be expected that nucleotides encountered by macrophages in culture for more than a few hours are degraded by CD39 and CD73 to adenosine. This can be confirmed by the fact that AMP also had an inhibitory effect on NO release, despite the fact that there is no known receptor for this ligand, suggesting that CD73 was able to metabolise AMP to adenosine. Inhibition of NO release by a slowly hydrolysable analogue was only observed when ATP-γ was used as the slowly hydrolysable β-ADP did not have an inhibitory effect. This could be due to the fact that these two compounds are

not the same structurally so that, ATP-γ is made in a way that is more susceptible to hydrolysis in culture either by cell ecto-nucleotidases or by degradation due to low stability in the culture conditions used.

The adenosine-induced inhibition of NO release was further investigated to see if it was accompanied by an enhancement of arginase I activity, taking into account that it is the alternative pathway of L-arginine metabolism. Indeed, stimulation by adenosine or nucleotides led to an increase of arginase I activity in both CAMΦ and AAMΦ while NECA caused an even higher increase probably due to the fact that it is an adenosine analogue that cannot be taken up by cells or metabolised. The slowly hydrolysable ADP analogue did not increase arginase I activity while ATP-γ caused a slight increase probably due to slow hydrolysis to adenosine over time. When comparing adenosine, NECA and ADP-induced up-regulation of arginase I activity, NECA appeared to induce a more pronounced increase in arginase I activity levels, while ADP-induced arginase I activity followed a similar pattern to adenosine suggesting that purinergic activation by ADP was via adenosine. In AAMΦ, arginase I basal levels were always too high to allow significant up-regulation by adenosine and nucleotides, however, the slight increase detected in every case was significant. β-ADP did not increase arginase I activity as opposed to the slight increase observed with the stable ATP analogue. When investigating arginase I activity levels in response to stimulation with increasing concentrations of adenosine, NECA and ADP, adenosine appeared to be more efficient in up-regulating arginase I activity in AAMΦ as it produced a more steady increase than NECA. This might be due to the fact that, as presented in Chapter 3, AAMΦ do not express A_{2A} receptors ($A_{2A}R$) so the use of an A_2 receptor agonist like NECA does not greatly differ to P1R stimulation by adenosine. In addition, CAMΦ responses to P1R activation that led to arginase I up-regulation may involve other downstream pathways

whose effects are amplified by an A_2 receptor-specific ligand like NECA since CAMΦ have been shown to express both A_2 receptors during the incubation period used in this study (Chapter 3). In fact, the effect observed in CAMΦ with adenosine was restricted to macrophages activated with LPS as IFN-γ, which is an endogenous trigger of classical activation, had no such effect. This apparent TLR synergy was also investigated for NO release by stimulating macrophages with different TLR ligands and, indeed, TLR activation of any kind resulted in adenosine-induced inhibition of NO release. In addition, the adenosine receptor – TLR synergy does not appear to be specific to a particular TLR as adenosine receptor activation had the same effect with both TLR2 and TLR4 ligands suggesting that it is a MyD88-dependent effect since both these TLRs act through this downstream pathway.

In view of the fact that only CAMΦ produce detectable cytokine levels *in vitro*, the effects of purinergic receptor activation on cytokine release were only investigated in this macrophage subset. Similar to NO release, TNF-α and IL-6 release were inhibited by adenosine and nucleotides while slowly hydrolysable nucleotides did not affect cytokine levels. The anti-inflammatory cytokine IL-10 was positively regulated by direct P1 receptor activation showing that in the presence of adenosine pro-inflammatory macrophage functions are inhibited. The slowly hydrolysable β-ADP had no effect on cytokine levels suggesting that the effects observed with ADP were a result of adenosine generation. The other slowly hydrolysable nucleotide used, ATP-γ, had a similar effect to ATP which, as explained earlier, was probably a result of its intrinsic instability in culture. Furthermore, this effect was not due to degradation to ADP since experiments with β-ADP did not mirror ATP-γ-induced effects.

Since the immunomodulatory effects of nucleotides on macrophage effector functions are mediated by ligation of specific purinergic receptors these effects were further investigated

pharmacologically, in order to identify the specific receptor involved. P1 receptors were targeted on macrophages with specific antagonists and the effects induced by adenosine on NO release and arginase I activity were only reversed by the A_{2B} receptor ($A_{2B}R$) antagonist MRS1754. In addition, the inhibitory effect elicited on CAMΦ NO release by nucleotides was also reversed by antagonising the $A_{2B}R$. The effect of adenosine on arginase I activity in both CAMΦ and AAMΦ was shown to be the opposite to that observed for NO release and, as expected, targeting the $A_{2B}R$ in both CAMΦ and AAMΦ led to inhibition of adenosine-induced up-regulation of arginase I activity. When looking at the effect of P1R agonists, $A_{2A}R$ activation using CGS21680 also resulted in up-regulation of arginase I activity in CAMΦ and AAMΦ. Unfortunately, the $A_{2B}R$ agonist used in published work by Eckle and colleagues (Eckle *et al*, 2007) was not available for use in this study so the involvement of the $A_{2B}R$ could only be shown using the P1R antagonists. In addition, despite the fact that the $A_{2A}R$ agonist had an up-regulatory effect on arginase I activity, the $A_{2A}R$ antagonist was not found to significantly inhibit adenosine-induced effects, suggesting that the $A_{2A}R$ is either dispensable and may only be activated in conjunction to the $A_{2B}R$, or that the $A_{2A}R$ agonist acts non-specifically on the $A_{2B}R$. Similarly in dendritic cells it has been reported that NECA-induced inhibition of NO release is accompanied by an increase in arginase I expression and this effect is $A_{2B}R$ mediated (Ben Addi *et al*, 2008). In the same study, it is suggested that NECA-induced effects on dendritic cell effector functions occur in the presence of LPS stimulation due to TLR synergism with the $A_{2A}R$. This LPS/NECA synergy has also been reported to cause VEGF up-regulation in murine macrophages, an important component of angiogenesis and wound healing both of which are major functions attributed to AAMΦ (Ramanathan *et al*, 2009). In fact, Pinhal-Enfield and colleagues showed that this up-regulation of VEGF can result from synergy between the $A_{2A}R$ and the TLRs 2,4,7,9 (Pinhal-Enfield *et al*, 2003).

P1R antagonists also confirmed the fact that the inhibitory effect of nucleotides on CAMΦ NO release was due to their degradation to adenosine as AMP, ADP and ATP-induced effects were only reversed by the A_{2B}R antagonist, MRS1754. Furthermore, confirmation of P1R involvement in effects observed after stimulation by ADP and ATP was obtained by the fact that, the P2Y inhibitors suramin and PPADS had no effect on the changes observed with ADP and ATP, as well as their slowly hydrolysable forms. In addition, this showed that ATP-γ-induced inhibition of NO release was not due to the intermediate breakdown product, ADP. Since AMP has no known receptor, CAMΦ responses to nucleotides are mainly due hydrolysis to adenosine and this is also suggested by the observation that reversal of AMP-induced effects is achieved in a similar mode to adenosine. The existence of AMP receptors is debatable as their presence has been reported in neuronal systems but this has not been confirmed for cells of the immune system (Ross *et al*, 1998), while a recently de-orphanised GPCR was mistakenly characterised as a purinergic receptor (P2Y15) responding to AMP and adenosine (Abbracchio *et al*, 2005). Based on the results obtained in this study and discussed above, responses to AMP stimulation were most likely due to adenosine generation.

The mode of regulation of cytokine release by purinergic signalling was found to be due to CAMΦ P1 receptor activation by adenosine similar to regulation of NO release and arginase I activity. However, the release of the pro-inflammatory cytokines, TNF-α and IL-6, was not affected in the same way as IL-10 in that, both the A_{2A} and A_{2B} receptors were involved in inhibition of TNF-α and IL-6 as opposed to IL-10 whose levels were up-regulated by adenosine ligation of the A_{2B}R alone. Indeed, this has also been reported by Nemeth and colleagues who also showed that the adenosine-induced increase of IL-10 expression levels in murine macrophages was a result of post transcriptional control following A_{2B}R activation (Nemeth *et al*,

2005). All nucleotides appeared to act via their degradation product, adenosine, as antagonism at the A_2 receptors was successful at reversing their effects on cytokine release. Reversal was generally better for IL-10 and this was confirmed to be due to the fact that only one receptor is responsible for adenosine-induced up-regulation. Indeed, when a cocktail of A_2R antagonists was used, TNF-α and IL-6 release was fully restored, suggesting that inhibition of these cytokines is a result of an additive effect of A_2R receptor activation and it can be achieved by activating either one of these two receptors. These data are in agreement with published work by Hasko and colleagues who reported that, inhibition of TNF-α release by adenosine from murine peritoneal macrophages was not only dependent on the $A_{2A}R$ (Hasko, Kuhel *et al*, 2000). This was confirmed by another study carried out on murine peritoneal macrophages which further showed that the other receptor responsible for adenosine-induced suppression of TNF-α was the $A_{2B}R$ (Kreckler *et al*, 2006).

All macrophage effector functions tested in this study were influenced by purinergic signalling via P1 receptor activation due to generation of adenosine in culture. As was the case for NO release, antagonism at P2 receptors with suramin and PPADS showed that ADP/ATP-induced effects on cytokine release were not P2YR-mediated for the culture period in this study. It is possible that initial encounters to nucleotides trigger responses via the P2Y receptors but these responses are short-lived due to CD39 expression on murine macrophages that effectively reduces ADP and ATP concentrations in the local macrophage environment. Furthermore, CD73 which is also expressed on murine macrophages, completes conversion of AMP to adenosine leading to increased P1R stimulation that masks any initial effects induced by P2YR activation.

Having established that adenosine was the active ligand in macrophage responses to adenine nucleotides and following pharmacological confirmation of A_2R involvement,

downstream signalling events were analysed taking into account that the A_2 receptors act via coupling to Gs, Gi and Gq subunits (Fredholm *et al*, 2000). Hence, regulation of cAMP levels was investigated to determine if Gs/Gi are involved while PLC and PI3 kinase activation were investigated to determine Gq involvement (Ralevic and Burnstock, 1998). Signalling cascades activated downstream of these G proteins result in activation of known pathways of transcriptional control within the nucleus two of which were investigated in this study, namely the NF-κB or MEK pathways.

Ligation of receptors coupled to Gs results in adenylyl cyclase activation and subsequent cAMP accumulation leading to Protein Kinase A (PKA) activation (Ralevic and Burnstock, 1998). Rp-cAMP is a PKA inhibitor used to inhibit cAMP-induced effects *in vtiro* (Zang and Hintze, 2001; Zhao *et al*, 2006). In this study administration of Rp-cAMP to CAMΦ partly restored the adenosine-induced suppression of NO release, especially when CAMΦ were activated via TLR4. Inhibition of cAMP did not affect basal NO release levels significantly nor was it enough to induce NO release by BMMΦ and AAMΦ. Conversely, basal CAMΦ arginase I activity was inhibited by Rp-cAMP while in AAMΦ the increased levels caused by adenosine were restored to basal activity typical of AAMΦ. These data suggested that adenosine-induced effects via the A_{2B}R were due to cAMP elevation as a result of Gs coupling. In an attempt to determine if cAMP elevation alone was responsible for these effects and in order to confirm Gs coupling, cAMP elevation was achieved superficially by administration of forskolin and rolipram which are known to increase cAMP levels by different mechanisms. Both forskolin and rolipram led to inhibition of NO release by CAMΦ activated by the different TLR ligands used, while adenosine-induced inhibition of NO release was additive when macrophages were co-cultured with adenosine and cAMP elevators. Control of NO release by cAMP has been shown to be time

and concentration-dependent. Specifically, Greten and colleagues have reported that NO release by RAW 264.7 macrophages increased in response to rolipram for up to one hour and then was inhibited for the rest of the culture period (Greten *et al*, 1995). Mullet and colleagues showed that NO release by a murine macrophage cell line was enhanced by cAMP elevation but that at high concentrations cAMP became inhibitory leading them to suggest that, NO release was biphasic in response to cAMP levels and that regulation could be autocrine as a result of cAMP-induced cytokine release (Mullet *et al*, 1997).

In this study, cAMP elevation was found to be sufficient to induce arginase I activity even in BMMΦ suggesting that the increase caused by adenosine was mainly due to Gs coupling and subsequent cAMP accumulation. It should be noted though that for this to happen *in vitro* some kind of co-stimulation is necessary, such as TLR (CAMΦ) or IL-4R (AAMΦ) activation, since P1R ligation alone had no effect on BMMΦ arginase I activity. This increase in arginase I activity could be a result of transcriptional or translational control. From molecular analysis using both RT-PCR and quantitative PCR (Q-PCR) approaches, it was apparent that the increase in arginase I activity achieved by cAMP elevation, either by adenosine or by chemical means, was accompanied by an increase in arginase I transcript levels. This is in agreement with work published by Nebes and Morris who showed that liver arginase I was up-regulated by cAMP-induced increase of arginase I mRNA levels (Nebes and Morris, 1998). Furthermore, Haffner and colleagues have shown that in RAW 264.7 macrophages arginase I up-regulation was PKA-HDAC dependent (Haffner *et al*, 2007). The fact that BMMΦ arginase I transcript levels were found to be increased in this study by the adenosine analogue, NECA, despite an apparent lack of enhancement in activity, may suggest that there is some form of post-transcriptional control as well, possibly affecting mRNA stability. The Q-PCR analysis also confirmed the fact that ADP

does not cause an increase in arginase I expression and that any up-regulation detected when macrophages were cultured in the presence of ADP or ATP was due to adenosine generation. In addition, the data obtained from the molecular analysis showed that the shift between iNOS and arginase I metabolism of L-arginine was due to increasing arginase I levels and not just an alteration in enzyme preference. Interestingly, it has been reported that the inhibition in NO release caused by IL-13 is via up-regulation of arginase I through cAMP/PKA-mediated *de novo* synthesis of arginase I mRNA and protein (Chang *et al*, 2000).

Similar to NO release, TNF-α and IL-6 release by CAMΦ was found to be suppressed by cAMP elevation in agreement with published work by Greten and colleagues who showed that, similar concentrations of rolipram to the ones used in this study were enough to inhibit TNF-α release (Greten *et al*, 1995). In addition, adenosine-induced effects were reversed by cAMP inhibition with Rp-cAMP and enhanced with cAMP elevators. IL-10 release was enhanced by cAMP elevation showing that the same pathway downstream of P1R activation led to differential regulation of pro-inflammatory and anti-inflammatory cytokine expression. Similarly, cAMP elevating agents were found to inhibit TNF-α and IL-12 from RAW 264.7 cells and bone marrow derived dendritic cells, while IL-10 release was increased by up-regulation of c-fos mRNA that led to reduced transcription of TNF-α genes (Koga *et al*, 2009). The fact that Rp-cAMP reversed adenosine-induced effects shows that cAMP-induced effects are mediated via activation of PKA instead of Epac, another kinase downstream of cAMP, which is confirmed by published work by Kreckler and colleagues who showed that, NECA inhibited TNF-α release by murine LPS-stimulated macrophages through cAMP activation of PKA and not Epac (Kreckler *et al*, 2009). The requirement for TLR-P1R crosstalk is highlighted by another study which showed that, the reciprocal regulation of IL-10/TNF-α in RAW 264.7 macrophages was PKA-dependent and that

LPS-induced phosphorylation of CREB downstream of PKA only becomes inhibitory in terms of TNF-α expression in the presence of a G-protein coupled receptor (GPCR) ligand (Avni *et al*, 2010). Furthermore, simultaneous stimulation with LPS and a GPCR ligand capable of activating the PKA-CREB pathway was shown in the same study to be essential for up-regulation of IL-10 expression.

Another pathway coupled to G proteins and particularly to Gq subunits is the PLC-mediated generation of second messengers such as IP3 and DAG that ultimately leads to Ca^{2+} mobilization. Inhibition of PLC activation did not reverse nucleotide-induced effects on any of the effector functions tested in this study. Specifically, adenosine and nucleotide-induced inhibition of NO release was only reversed effectively by cAMP inhibition by Rp-cAMP as no such reversal was achieved by PLC inhibition, showing that even nucleotide-induced effects were due to adenosine-induced elevation of cAMP. Furthermore, the fact that PLC inhibition enhanced the AMP-induced inhibition of NO release may suggest that blocking Gq coupling of the $A_{2B}R$ shifts coupling to Gs. Likewise with cytokine release PLC inhibition was not sufficient to reverse adenosine-induced changes in cytokine levels detected in CAMΦ supernatants. These data further confirm that TNF-α and IL-6 release, shown in this study to be controlled by both A_{2A} and A_{2B} receptor activation by adenosine, is via cAMP and not by $A_{2B}R$ coupling to the PLC pathway. Furthermore, IL-10 up-regulation by adenosine activation of A_{2B} receptors is also cAMP-dependent and is not a result of PLC activation. Despite the fact that A_{2A} receptors are not known to couple to PLC, Grinberg and colleagues have reported that the VEGF up-regulation in murine peritoneal macrophages and RAW 246.7 cells is a PLC-induced effect resulting from $A_{2A}R$ activation (Grinberg *et al*, 2009).

Gq subunits may also couple to PI3 kinase. In this study, blocking PI3 kinase activation with LY294002 led to inhibition of basal CAMΦ NO, TNF-α and IL-6 release. Similar observations were made by Zang and Hintze who showed that LY294002 inhibited NO release by endothelial cells (Zang and Hintze, 2001). The adenosine-induced inhibition was not reversed by LY294002 but was further enhanced. Basal IL-10 release levels were also affected by LY294002 which appeared to cause the same reduction even in the presence of adenosine. Therefore, no conclusions can be drawn regarding PI3 kinase involvement in purinergic signalling.

An indirect mode of control for the adenosine-induced effects observed in this study was also investigated based on the observation that adenosine caused a significant increase in IL-10 release by CAMΦ. IL-10 has been reported to deactivate macrophages in the sense that, IL-10R signalling prevents activation of other pathways and in doing so makes macrophages refractory to further activation (Porcheray *et al*, 2005). In this study, when extracellular IL-10 levels were completely neutralised TNF-α and IL-6 release by CAMΦ was evidently increased. Fiorentino and colleagues also showed that IL-6 release from peritoneal macrophages is inhibited by IL-10, by neutralising extracellular endogenously-produced IL-10 (Fiorentino, Zlotnik *et al*, 1991). Interestingly, adenosine, NECA and AMP-induced inhibition of TNF-α and IL-6 release was not completely reversed suggesting that, this is also a direct effect of cAMP elevation as well as an indirect response to the cAMP-induced increase in IL-10 release. Indeed, this autocrine mechanism of IL-10 control has been suggested in a study using human monocytes showing that IL-10 produced by LPS-activated monocytes occurs later than the other cytokines thus allowing control of pro-inflammatory cytokine production (de Waal Malefyt *et al*, 1991). This further explained their observation that exogenously administered IL-10 produced a more effective

inhibition on TNF-α and IL-6 production, an effect also observed in this study. IL-10 has been reported by many studies to be a suppressor of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1 in murine peritoneal macrophages and macrophage cell lines (Bogdan *et al*, 1991; Fiorentino, Zlotnik *et al*, 1991). The mechanism via which IL-10 mediates reduction in alveolar macrophage cytokine levels has been shown to be dependent on inhibition of NF-κB nuclear localisation (Lentsch *et al*, 1997). According to the authors, this is achieved by increasing IκB mRNA levels through stabilisation thus leading to its preservation which maintains NF-κB in its inactive state in the cytosol.

In order to further characterise signalling pathways downstream of G-protein activation, two pathways leading to activation of transcription in the nucleus were selected. NF-κB activation was blocked by using an inhibitor of cytokine-induced Iκ-B phosphorylation (Bay 11-7082) while MEK1/2 activation was blocked using a specific inhibitor, U0126 (Zhao *et al*, 2006; Gringhuis *et al*, 2007). NF-κB inhibition led to suppression of basal CAMΦ NO release as opposed to MEK1/2 inhibition which appeared to improve NO release by TLR2 and TLR4-activated CAMΦ. In addition, MEK1/2 inhibition partly relieved NECA-induced suppression of NO release. As for cytokine release, both inhibitors had similar effects on all cytokines tested suggesting that, pathways downstream of cytokine receptors converge to these two pathways or that there is some means of cross-talk operating somewhere between the cell surface and the nucleus. The fact that the p38/MSK1 pathway (which is downstream of the MEK1/2 activation) is important for LPS-induced TNF-α expression has been recently reported by Avni and colleagues (Avni *et al*, 2010). Since NF-κB inhibition suppressed LPS activation of macrophages even prior to nucleotide addition and inhibited all macrophage effector functions, it could not be determined if purinergic signalling has an inhibitory effect on NF-κB. However, it could be

suggested that purinergic receptor activation does not have an activatory effect on NF-κB either, as P1R activation was not capable of outcompeting the inhibitor to allow NF-κB translocation to the nucleus. However, it should be noted that in order to confirm this a titration of P1R activation would have to be carried out. Nevertheless, Majumdar and Agarwal have shown that TNF-α inhibition by adenosine is in fact due to A_{2A}R-induced NF-κB inhibition (Majumdar and Agarwal, 2003). MEK1/2 inhibition led to some reversal of adenosine/NECA-induced effects and this could lead to the assumption that, MEK1/2 activation is part of the downstream signalling events, however, it may not be the only pathway responsible for purinergic signalling-induced effects.

In conclusion, this study has shown that both murine macrophage activation states can be influenced by purinergic signalling at least in terms of the effector functions tested here. In particular, adenosine activation of A_{2B} receptors is responsible for shifting macrophage L-arginine metabolism from iNOS to arginase I effectively suppressing NO release. In addition to pro-inflammatory NO release adenosine also inhibits pro-inflammatory cytokine release, such as TNF-α and IL-6, by activation of both A_{2A} and A_{2B} receptors. Furthermore, production of the anti-inflammatory cytokine IL-10 is enhanced by adenosine activation of the A_{2B}R. It can be concluded that adenosine, present in culture either by direct addition or through generation by nucleotide hydrolysis, mediates these effects via cAMP elevation through the A_{2B}R. These data point to a versatility of macrophage activation in response to exogenous danger signals such as nucleotides that allows resolution of inflammation without having to recruit naïve macrophages.

Chapter 5

Effects of *Trichinella Spiralis* secreted proteins on macrophage effector functions *in vitro*

5.1 Introduction

Helminth parasites are known to colonise a variety of organs and tissues in the host, such as the gastrointestinal tract and the lymphatics, and they employ a range of immunomodulatory mechanisms in order to evade host detection and achieve successful colonisation. In the case of the parasitic nematode, *Trichinella spiralis*, invasion during the gastrointestinal phase of infection results in tissue damage generating molecular signals capable of activating the immune system. Extracellular nucleotides are major molecular signals encountered by cells of the hematopoietic lineage which are capable of controlling immune responses as a result of ubiquitous purinergic receptor expression. So far, this study has shown that bone marrow derived macrophages (BMMΦ) possess functional purinergic receptors and are thus subject to regulation by nucleotides (Chapter 3 and 4). *T.spiralis* appears to take advantage of this feature of immune cells as nucleotide metabolising enzymes have been characterised in its secretory products (ES) which effectively convert pro-inflammatory signals (ATP and ADP) to anti-inflammatory adenosine, thus allowing for parasite survival (Gounaris, 2002). In view of this, the effect of *T.spiralis* ES on BMMΦ purinergic signalling was investigated and is presented in this chapter.

In addition to their role in immune regulation by means of their purinergic receptor expression, macrophages are also an important component of the innate immune response due to their ability, as antigen presenting cells (APCs), to recognize a broad spectrum of molecular patterns known as pathogen-associated molecular patterns (PAMPs). APCs are known to express pattern recognition receptors (PRRs) that, upon ligation by PAMPs, induce inflammatory cytokine production. PAMPs have been widely characterised for viruses, bacteria and lower eukaryotes and shown to include lipopolysaccharides and peptidoglycans. Toll-like receptors (TLRs) are type I transmembrane PRRs that are sensors of these molecular patterns and function

in linking innate and adaptive immune responses (Venugopal *et al*, 2008). The responses elicited by different pathogens vary depending on TLR engagement and may even involve interaction of multiple TLRs. These responses are predominantly pro-inflammatory in nature resulting from the generation of classically activated macrophages (CAMΦ). Recently, components of higher eukaryotes such as helminths have been identified as potential PAMPs that are recognized by host PRRs including TLRs and C-type lectins. In particular, glycoproteins and glycophospholipids have been shown to induce a Th2 phenotype in APCs via TLR ligation. Indeed, this is a unique mode of host manipulation made even more significant when considering that different stages of parasite development within the host are accompanied by changes in the glycan combinations expressed (Van der kleij *et al*, 2002).

Immune cell responses triggered by PAMPs may be exploited by parasites to act in their favour. Specifically, helminth parasites are capable of triggering highly polarised immune responses due to increased numbers of CD4+ Th2 cells, eosinophils, mast cells and basophils (Loke *et al,* 2007). These cells coordinate an effective response ultimately leading to parasite expulsion in addition to their role in the regulation of associated pathologic inflammation. Functional modulation of macrophages, as well as other APCs, is a fundamental event involved in establishing immune regulation by parasites supported by the fact that, alternatively activated macrophages (AAMΦ) were consistently shown to be recruited in Th2 responses to nematode infections (Kreider *et al,* 2007). In particular, arginase I which is a major marker of alternative activation that can be up-regulated by IL4, IL-10 or TGF-β, has also been shown to be up-regulated either by parasites directly or by their secreted products (Stempin *et al,* 2009). Alternative activation of macrophages is important in limiting collateral tissue damage that generally results from an excessive Th1 response and, additionally, it is advantageous for parasite survival and proliferation. With relevance to this study, *T.spiralis* ES glycans have been reported

to bind the macrophage mannose receptor (MR), an event partly responsible for triggering NO release by these cells (Gruden-Movsesijan and Milosavljevic Lj, 2006). The same authors showed that, *T.spiralis* ES carbohydrates can potentially activate another arm of the innate immune response via mannose-binding lectin (MBL) ligation leading to complement component deposition on larvae which is suggested to act in a protective manner (Gruden-Movsesijan *et al*, 2003).

Macrophages are emerging as an important immune cell type involved in nematode infection so this study aimed to delineate the effector responses of different macrophage subsets to *T.spiralis* secreted products. Having established that macrophage effector functions such as arginase I activity, nitric oxide (NO) and cytokine release are affected by administration of extracellular nucleotides and considering that *T.spiralis* secretory proteins contain nucleotide metabolising activity, the effect of combining the two was investigated. Finally, *T.spiralis* ES-induced effects were characterised in terms of downstream pathway activation and an attempt was made at determining which component of ES was responsible for macrophage responses elicited.

5.2 Results

The objectives of experiments presented in this chapter were to determine how the parasitic nematode, *T.spiralis*, interferes with host macrophage effector functions. Macrophage responses to *T.spiralis* secreted products were investigated as well as the effect of these products on extracellular nucleotide-induced responses, bearing in mind that *T.spiralis* proteins are known to metabolise nucleotides (Gounaris, 2002). This was confirmed in this study prior to using *T.spiralis* ES in stimulation of macrophages by carrying out phosphate release assays of ES with AMP, ADP and ATP (results shown in the appendix, Figure A.3).

5.2.1 Effect of *T.spiralis* secreted proteins on murine macrophage effector functions

The effect of *T.spiralis* secreted proteins (ES) on macrophage functions was first investigated. ES triggered cytokine release from BMMΦ and AAMΦ but did not seem to affect the existing levels produced by CAMΦ (Figure 5.3 and 5.4). This effect was concentration-dependent. In order to dissect the origin of this stimulation, ES was subjected to heating to inactivate any enzyme activity as well as to deglycosylation to remove carbohydrates. In order to test if the effect observed was due to mannose-type carbohydrates acting on the mannose receptor of macrophages, D-mannose was used to stimulate cells. In addition, the effect of other carbohydrates on macrophage C-type lectin activation was also investigated to determine the source of ES-induced effects.

Effects of ES on nitric oxide release

Bone marrow derived macrophages (BMMΦ) were classically (CAMΦ) or alternatively (AAMΦ) activated and cultured for 20 hours with a range of ES concentrations. The effect of titrating the concentration of ES used to stimulate macrophages on NO release is shown in Figure

5.1. A concentration dependent NO release was detected in BMMΦ and AAMΦ that gradually increased up to 75μg/ml for both BMMΦ and AAMΦ but at 100μg/ml BMMΦ released higher levels of NO. However, when compared to CAMΦ the increase in NO released from BMMΦ elicited by ES was only 20% of the NO released following LPS stimulation. Activation of macrophages to produce CAMΦ and AAMΦ was tested at two different concentrations of LPS and IL-4, respectively, to examine if the concentration of the activating stimulus affected the macrophage response to ES. There appeared to be no difference in the response elicited by ES.

However, the fact that NO release was triggered by ES could also suggest the presence of endotoxin/LPS in ES, as NO release is typical of classically (LPS) activated macrophages. Therefore, ES was tested for endotoxin using the Limulus Amoebocyte Lysate assay and a positive result was obtained for the presence of endotoxin (data not shown). The data were inconclusive due to the fact that the levels detected were too high for the sterile preparation of ES material used. In addition, when examined in the context of the final concentration of this detected 'endotoxin' that would end up in the cell culture, the response observed was a lot higher than would be expected by the corresponding concentration of LPS cultured with cells (data of LPS titration on BMMΦ shown in Chapter 4, Figure 4.1A). In addition, previous work carried out in this lab involving ES treatment of other immune cells like mucosal mast cells (Holly Afferson PhD thesis) and dendritic cells did not suggest ES contamination by LPS. It should be noted that the basis of the endotoxin detection assay involves activation of a pro-enzyme in the presence of endotoxin, which could explain why ES may be capable of activating this enzyme, since it has been found to contain certain proteases, such as serine proteases (Nagano *et al*, 2003).

Effects of ES on arginase I activity

Macrophages whose supernatants were used to measure NO release were also assayed for arginase I activity levels post ES treatment. As shown in Figure 5.2, arginase I activity was increased in BMMΦ and CAMΦ (A and B, respectively) when in response to stimulation with ES with the effect being more significant at the concentration of 100μg/ml. The increase observed never reached basal AAMΦ levels which were approximately 10-fold higher. Furthermore, the slight increase observed in AAMΦ arginase I activity by ES was not as high when compared to untreated AAMΦ (Figure 5.2C). Therefore, ES only affected arginase I activity levels in BMMΦ and CAMΦ.

Effects of ES on cytokine production

Supernatants from ES-treated macrophages assayed for NO release were also used to measure cytokine levels. In BMMΦ and AAMΦ, cytokine release was significant after stimulation with 25μg/ml ES while 50μg/ml produced a submaximal response and was selected for further experiments (Figure 5.3). In BMMΦ, 100μg/ml ES stimulated production of higher levels of TNF-α than in AAMΦ, while AAMΦ activated with 20ng/ml IL-4 appeared be limited in their production of IL-6 following stimulation with ES. ES induced higher levels of TNF-α release from BMMΦ at 75 and 100μg/ml when compared to AAMΦ, while the levels of IL-10 produced by both BMMΦ and AAMΦ were comparable for all the concentrations of ES tested. In fact, the levels of IL-10 released by BMMΦ cultured with 100μg/ml ES were only 70% of the levels induced by LPS in CAMΦ (Figure 5.4). The levels of TNF-α and IL-6 released by BMMΦ stimulated with 100μg/ml ES were the same as the levels released following stimulation of

CAMΦ with ES. It should be noted that, cytokine release by CAMΦ activated LPS was not affected by any of the ES concentrations tested. At the concentration of ES selected for further experiments (50μg/ml), cytokine release levels elicited in BMMΦ were only 50% of the levels released following macrophage stimulation with LPS (CAMΦ).

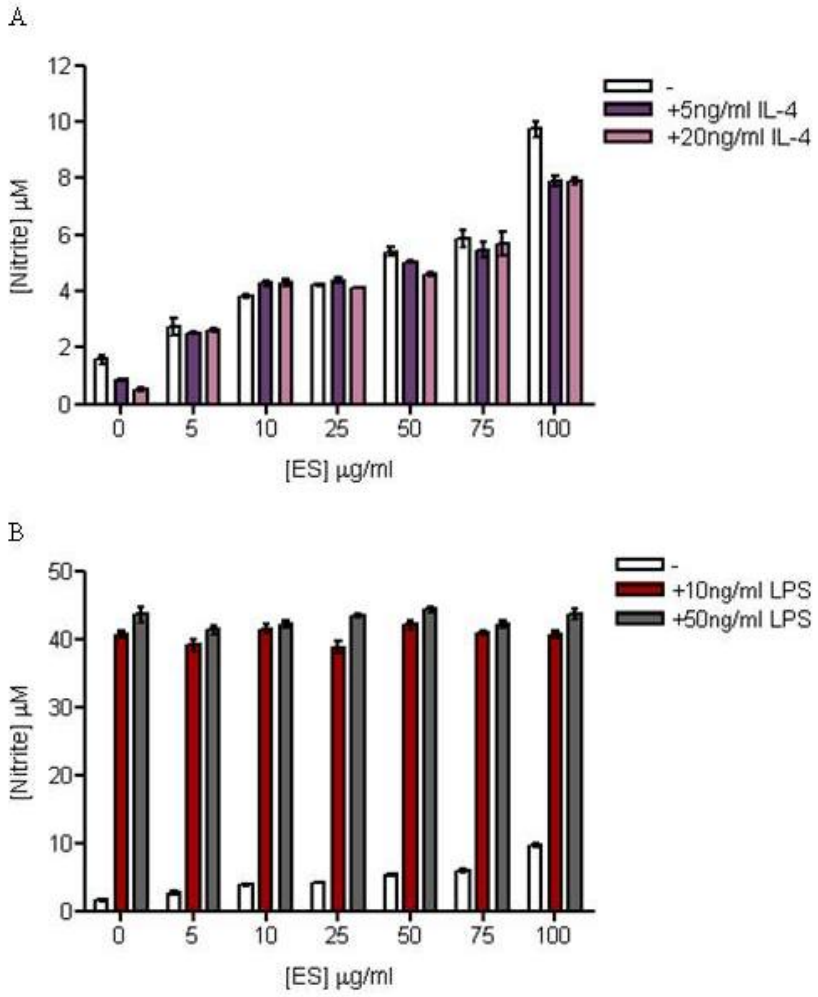


Figure 5.1: **The effect of *T.spiralis* ES on nitric oxide (NO) release by bone marrow derived macrophages.** Macrophages cultured with standard medium (BMMΦ), 10 or 50ng/ml LPS (CAMΦ), or 5 or 20ng/ml IL4 (AAMΦ) were treated with *T.spiralis* ES at a range of concentrations (5-100µg/ml) for 20hrs. Nitrite levels were measured in supernatants and are presented in µM. (A) The effect of ES on BMMΦ and AAMΦ NO release and (B) The effect of ES on BMMΦ and CAMΦ NO release. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3.

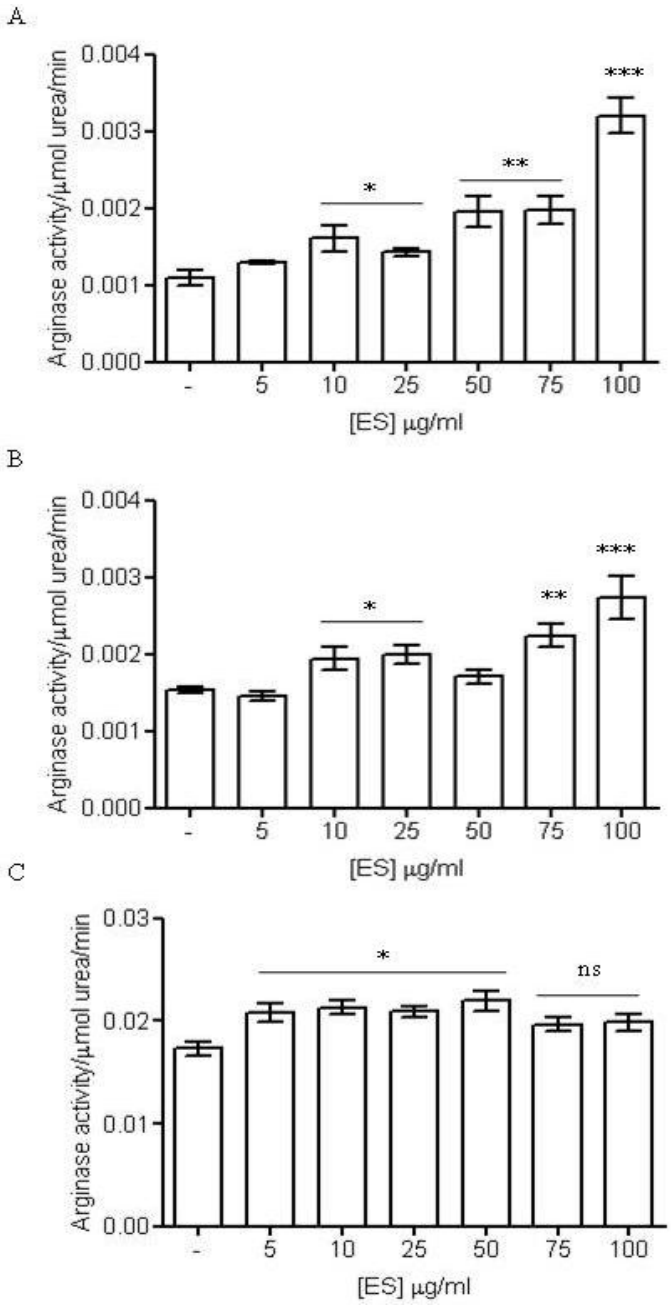


Figure 5.2: **The effect of *T.spiralis* ES on bone marrow derived macrophage arginase I activity.** Macrophages cultured with standard medium (BMMΦ), 10 ng/ml LPS (CAMΦ), or 5 ng/ml IL4 (AAMΦ) were treated with *T.spiralis* ES at a range of concentrations (5-100µg/ml) for 20hrs. Arginase I activity levels were measured and are presented in µmol urea per minute per 5 x 10^4 cells. The effect of ES on BMMΦ (A) on CAMΦ (B) and on AAMΦ (C) arginase I activity. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 2 and asterisks indicate significance compared to the respective untreated control. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, ns - not significant ($p> 0.05$).

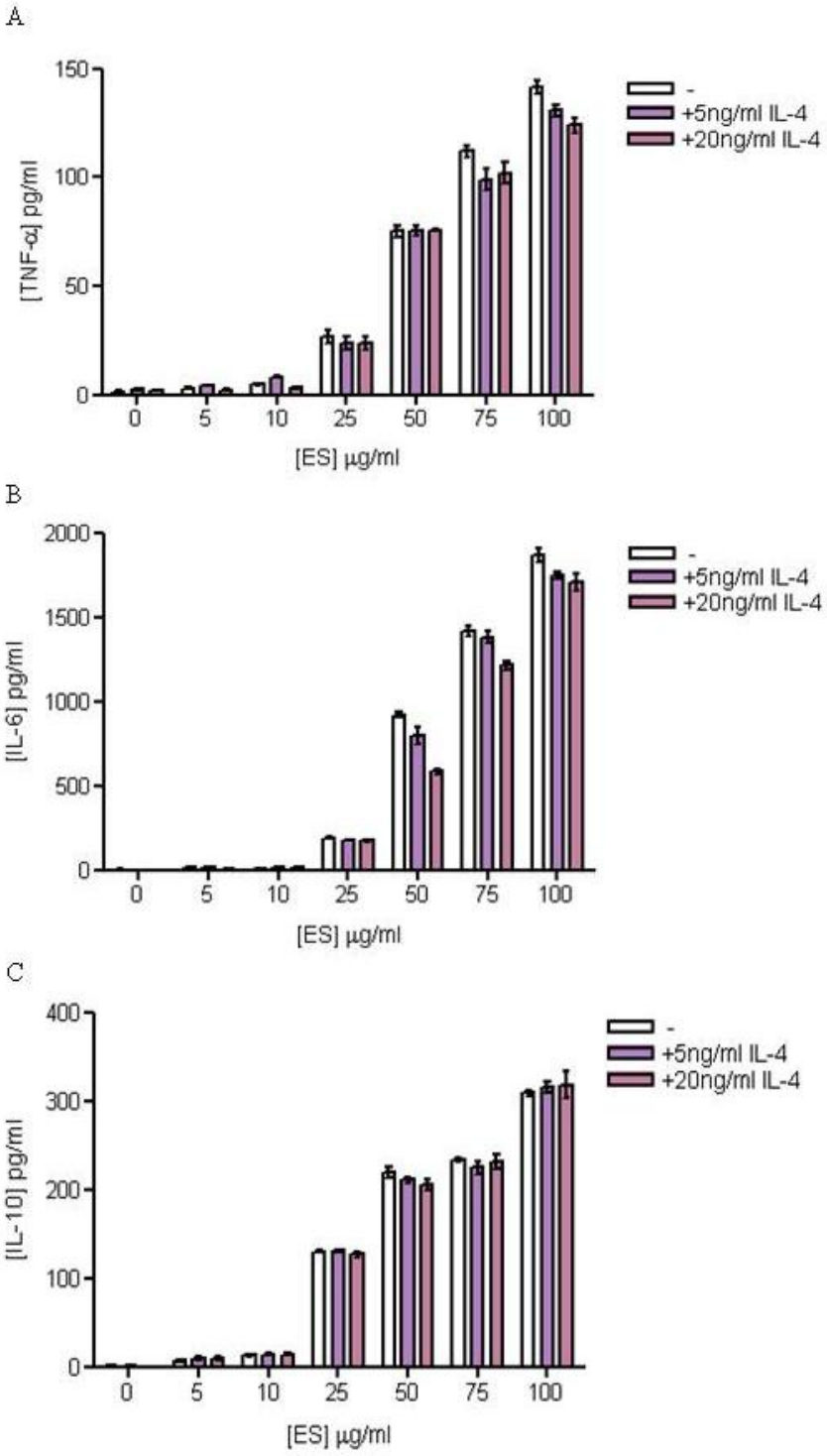


Figure 5.3: The effect of *T.spiralis* ES on cytokine release by bone marrow derived macrophages. Macrophages cultured with standard medium (BMMΦ), 5 or 20ng/ml IL4 (AAMΦ) were treated with *T.spiralis* ES at a range of concentrations (5-100µg/ml) for 20hrs. The levels of TNF-α, IL-6 and IL-10 released from BMMΦ and AAMΦ are shown in (A), (B) and (C), respectively. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3.

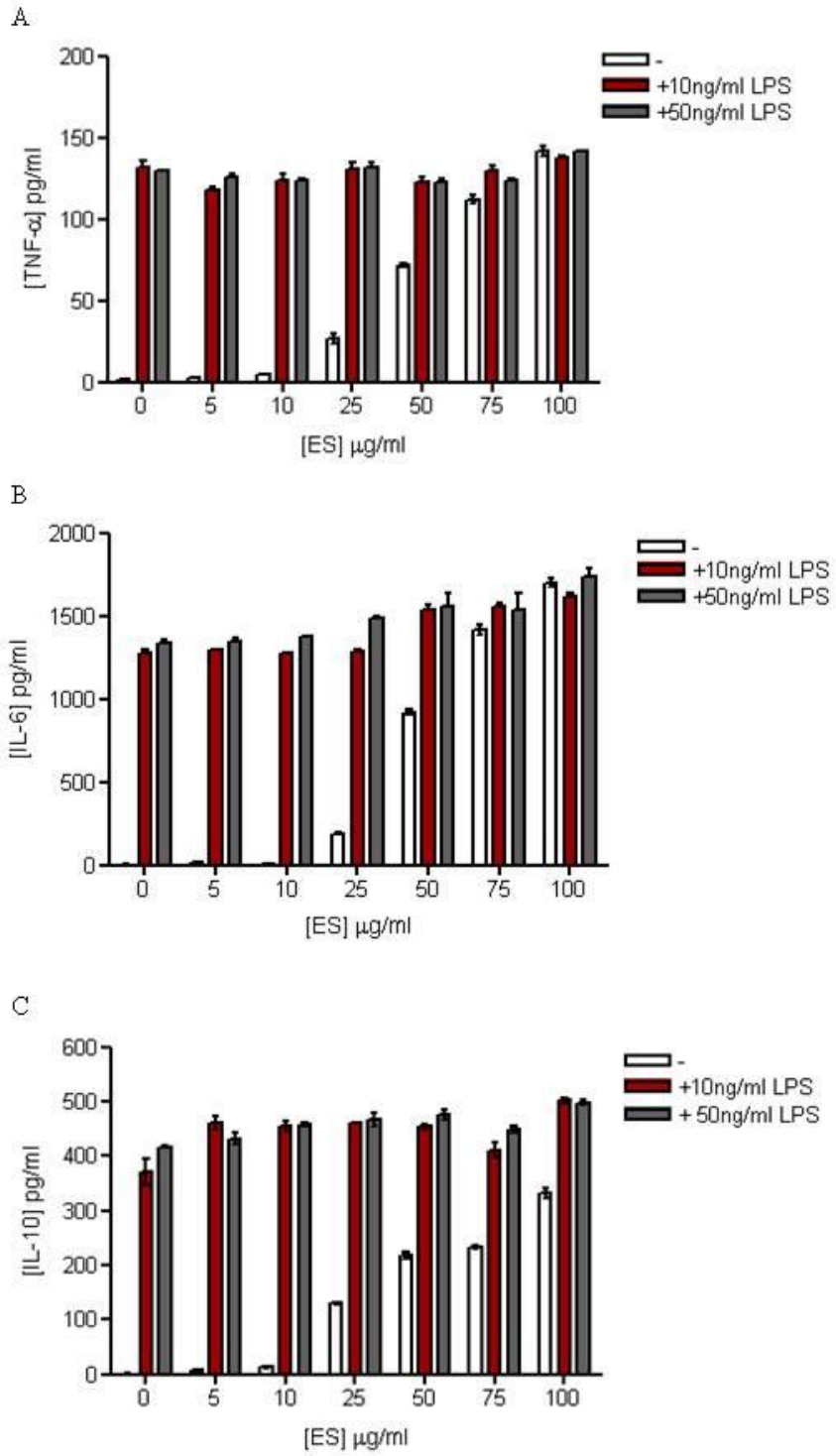


Figure 5.4: The effect of *T.spiralis* ES on cytokine release by classically activated macrophages (CAMΦ). Macrophages cultured with standard medium (BMMΦ), 10 or 50ng/ml LPS (CAMΦ) were treated with *T.spiralis* ES at a range of concentrations (5-100µg/ml) for 20hrs. The levels of TNF-α, IL-6 and IL-10 released by BMMΦ and CAMΦ are shown in (A), (B) and (C) respectively. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3.

5.2.2 Effect of *T. spiralis* secreted proteins on murine macrophage purinergic signalling

Taking into account that *T.spiralis* secreted proteins (ES) have nucleotide metabolising activity (Gounaris, 2002), the effect of ES on nucleotide-induced macrophage effector functions was investigated. Macrophages were treated with nucleotides in the presence of *T.spiralis* ES and were assayed for NO and cytokine release and arginase I activity. ES was used at a concentration of 50μg/ml.

Effect of ES on nucleotide-induced NO release

After verifying the effect of nucleotides on NO release from CAMΦ, *T.spiralis* ES was included in macrophage treatment with nucleotides. In addition, having established that ES treatment of macrophages resulted in detectable NO release, BMMΦ and AAMΦ were also treated with ES combined with nucleotides. As shown in Figure 5.5, the ES-induced NO release detected in BMMΦ and AAMΦ (A and C, respectively) was abolished by adenosine or adenine nucleotides. This suggests that ES enzymes converted nucleotides to adenosine which, in turn, reversed the increase in NO release induced by ES components. In CAMΦ (Figure 5.5B), adenine nucleotides inhibited NO release further and NO release reached levels similar to those induced by adenosine/NECA when incubated with ES. This was probably due to the additive effect of hydrolysis by ES enzymes and macrophage CD39 and CD73 resulting in generation of adenosine. The effect of incubating UDP in the presence of ES was also investigated since ES enzymes can also hydrolyse UDP. UDP did not affect NO release by any of the macrophage types which was also the case when UDP was co-administered with ES.

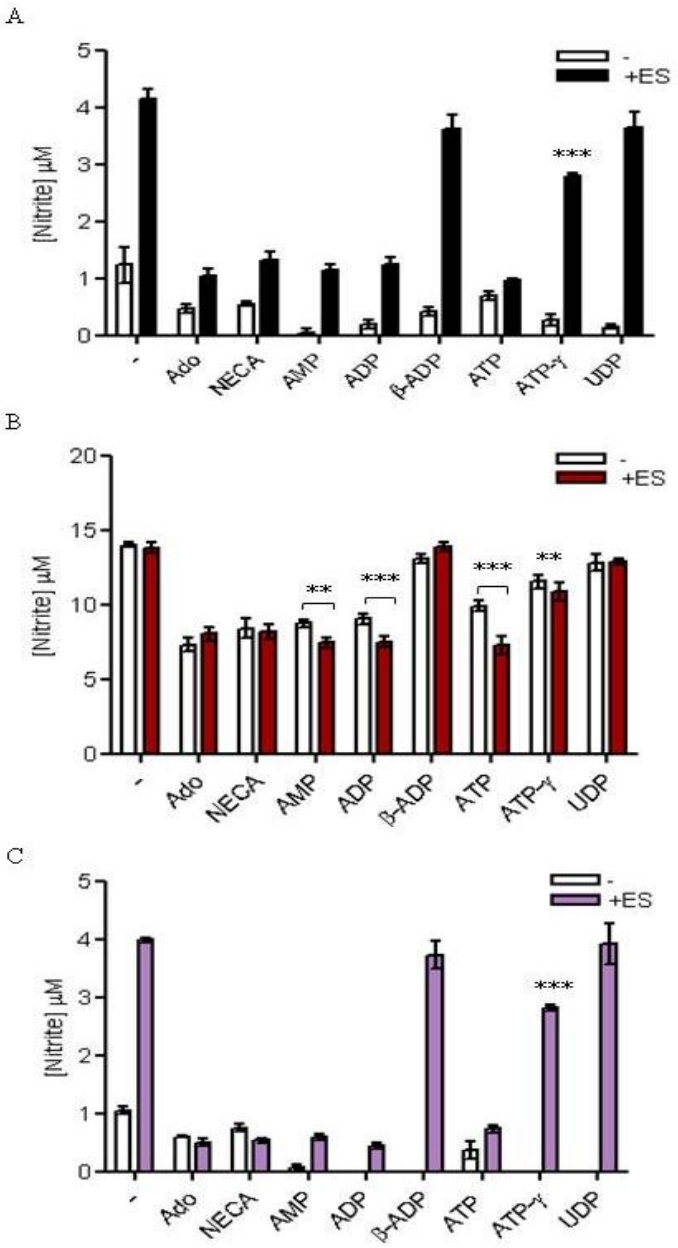


Figure 5.5: The effect of *T.spiralis* ES on NO released by bone marrow derived macrophages following purinergic receptor activation. Macrophages were cultured with standard medium (BMMΦ), classically or alternatively activated with 10ng/ml LPS or 5ng/ml IL-4, respectively, and treated for 20 hours with 100µM adenosine, NECA, AMP, ADP, ATP or the slowly hydrolysable analogues of adenosine, ADP and ATP (β-ADP and ATP-γ, respectively). The effect of nucleotides, in the presence of 50µg/ml ES, on NO released from BMMΦ (A), CAMΦ (B) and AAMΦ (C).The levels of NO release are expressed in µM. The data presented are an average of triplicates ± SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control (-ES). ** $p < 0.01$, *** $p < 0.001$.

Effect of ES on nucleotide-induced arginase I activity

Macrophage arginase I activity in response to nucleotides has also been investigated in this study (Chapter 4), therefore, the effect of ES on nucleotide-induced arginase I activity was assayed. Arginase I activity was only assayed in CAMΦ and AAMΦ since BMMΦ were not found to have significant arginase I activity levels (Chapter 4, Figure 4.1). The effect of ES on nucleotide-induced arginase I activity is shown in Figure 5.6. In CAMΦ, the adenosine/NECA-induced increase in arginase I activity was not affected by ES, while ES only caused a slight increase on its own, similar to the increase observed in Figure 5.2. This increase in arginase I activity in response to ES alone can be attributed to an increase in arginase I transcripts, as determined by Q-PCR presented in Chapter 3. When CAMΦ were stimulated with nucleotides in the presence of ES, a 10-fold increase was detected in arginase I activity which is of similar magnitude to the increase induced by adenosine/NECA. Therefore, ES enzymes catalyzed the hydrolysis of adenine nucleotides to adenosine in culture. Similarly in AAMΦ, when adenine nucleotides were co-administered with ES, the increase in arginase I activity produced was comparable to the adenosine/NECA-induced increase. It should be noted that, because of the high basal levels of arginase I activity in AAMΦ, the fold increase was not as high as in CAMΦ. The effect of UDP on arginase I activity levels was also tested as it is a P2YR agonist that can be hydrolysed by ES enzymes. As it can be seen in Figure 5.6, UDP caused an increase in CAMΦ arginase I activity and a decrease in AAMΦ arginase I activity, which was not affected when UDP was added in the presence of ES. The observation that UDP had different effects on arginase I activity in the two macrophage types could be due to different levels of expression of the P2Y6R (which responds to UDP) or different coupling to intracellular signalling pathways by CAMΦ and AAMΦ.

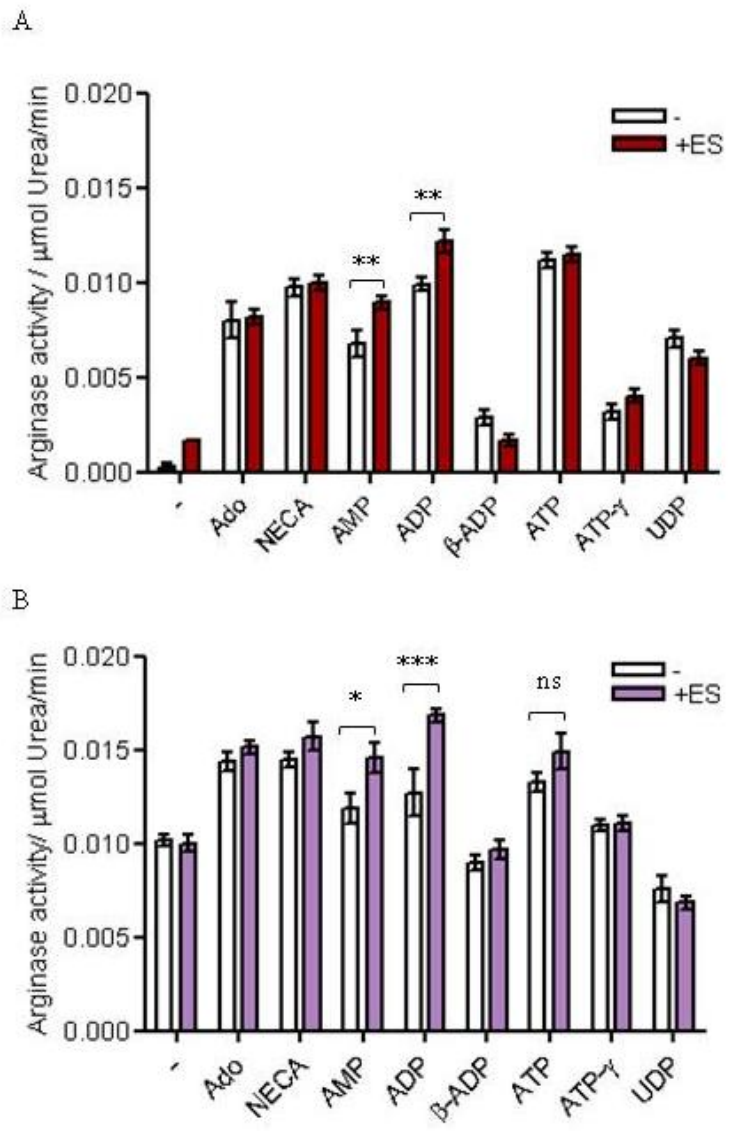


Figure 5.6: The effect of *T.spiralis* ES on arginase I activity of purinergic receptor activated bone marrow derived macrophage. Macrophages were classically or alternatively activated with 10ng/ml LPS (A) or 5ng/ml IL-4 (B), respectively, and treated for 20 hours with 100µM adenosine, NECA, AMP, ADP, ATP or the slowly hydrolysable analogues of adenosine, ADP and ATP (β-ADP and ATP-γ, respectively). The effect of nucleotides in the presence of 50µg/ml ES on CAMΦ (A) and AAMΦ (B) arginase I activity is expressed as µmol Urea/ min for every 5 x 10^4 cells. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, ns - not significant ($p> 0.05$).

Effect of ES on nucleotide-induced cytokine production

Having determined macrophage cytokine release levels in response to nucleotides and *T.spiralis* ES separately, the effect of combining nucleotides with ES was then investigated. As shown in Figures 5.7, 5.8, 5.9 the effect of ES on nucleotide-induced cytokine release in BMMΦ and AAMΦ was similar to the pattern observed with NO release. Specifically, the ES-induced release of TNF-α and IL-6 from BMMΦ and AAMΦ was inhibited by adenosine/NECA and the same was true for adenine nucleotides, probably due to the fact they were hydrolysed to adenosine by ES enzymes. The ES-induced release of IL-10 was further enhanced by adenosine/NECA as well as by adenine nucleotides converted to adenosine in culture. In BMMΦ and AAMΦ, β-ADP did not affect the ES-induced cytokine release while ATP-γ-induced effects were not consistent. Specifically, TNF-α and IL-6 released from BMMΦ stimulated with ES was not affected by ATP-γ but the release of these cytokines from AAMΦ was inhibited by ATP-γ (Figure 5.7 and 5.8). The ES-induced IL-10 release from both BMMΦ and AAMΦ was slightly enhanced by ATP-γ (Figure 5.9).

In CAMΦ, the effects of ES were only tested with respect to nucleotide hydrolysis as ES on its own did not affect background cytokine levels. Indeed, nucleotides such as AMP, ADP and ATP that could be hydrolysed by ES enzymes affected cytokine release by CAMΦ in a similar manner to adenosine/NECA. In cases where nucleotides (such as AMP, ADP and ATP) produced an effect on cytokine release even in the absence of ES, this effect was enhanced by co-stimulation with ES due to the additive effect of hydrolysis by enzymes present in ES as well as macrophage CD39 and CD73 (discussed in Chapter 3). The slowly hydrolysable analogues of ADP and ATP (β-ADP and ATP-γ) had similar effects when used to stimulate CAMΦ both in the presence and absence of ES, therefore, ES enzymes were not capable of hydrolysing these

nucleotides in this form. β-ADP did not affect release of any of the cytokines tested while ATP-γ inhibited TNF-α and IL-6 release and slightly increased IL-10 release.

In addition to testing the effect of ES with adenine nucleotides, UDP was also included as it is a P2YR agonist that is also potentially hydrolysed by ES enzymes. The ES-induced cytokine release from BMMΦ and AAMΦ was not affected by UDP, while hydrolysis of UDP by ES enzymes did not change CAMΦ responses to this nucleotide.

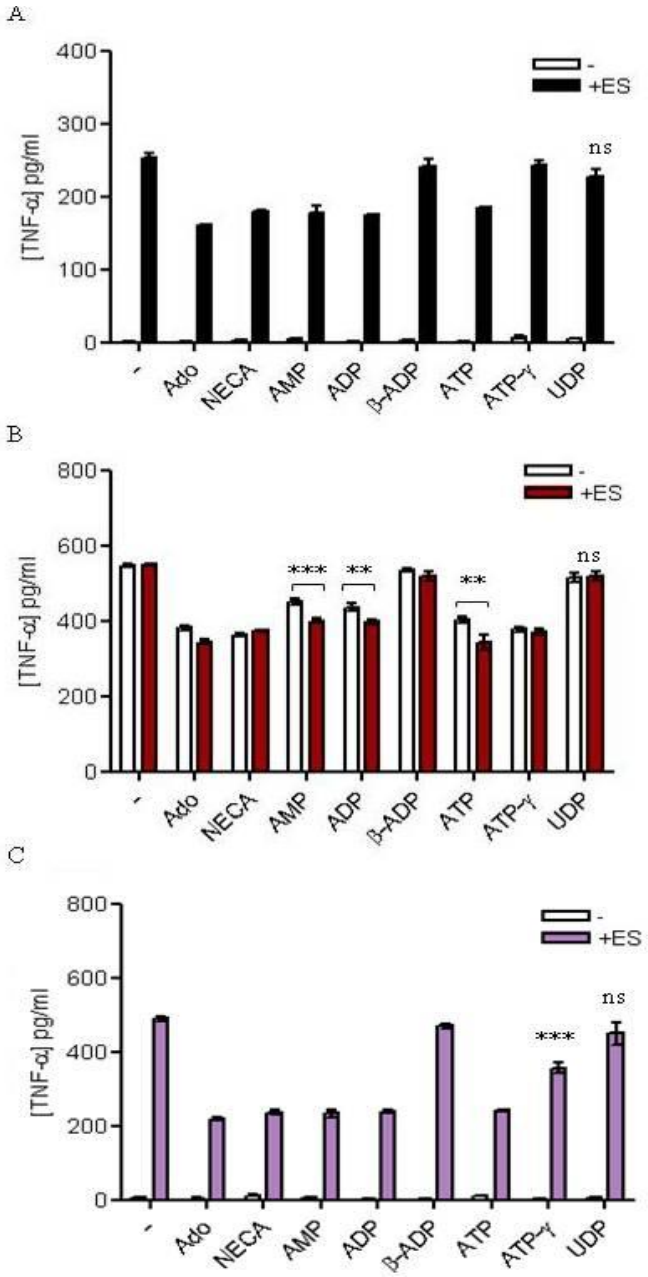


Figure 5.7: The effect of *T.spiralis* ES on TNF-α released from purinergic receptor activated bone marrow derived macrophages. Macrophages were cultured with standard medium (BMMΦ), classically or alternatively activated with 10ng/ml LPS or 5ng/ml IL-4, respectively, and treated for 20 hours with adenosine, NECA, nucleotides or their slowly hydrolysable analogues. The effect of nucleotides in the presence of 50μg/ml ES on TNF-α released from BMMΦ (A), CAMΦ (B) and AAMΦ (C).The levels of TNF-α release are expressed in pg/ml. The data presented are an average of triplicates ± SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. ** $p < 0.01$, *** $p < 0.001$, ns - not significant ($p > 0.05$).

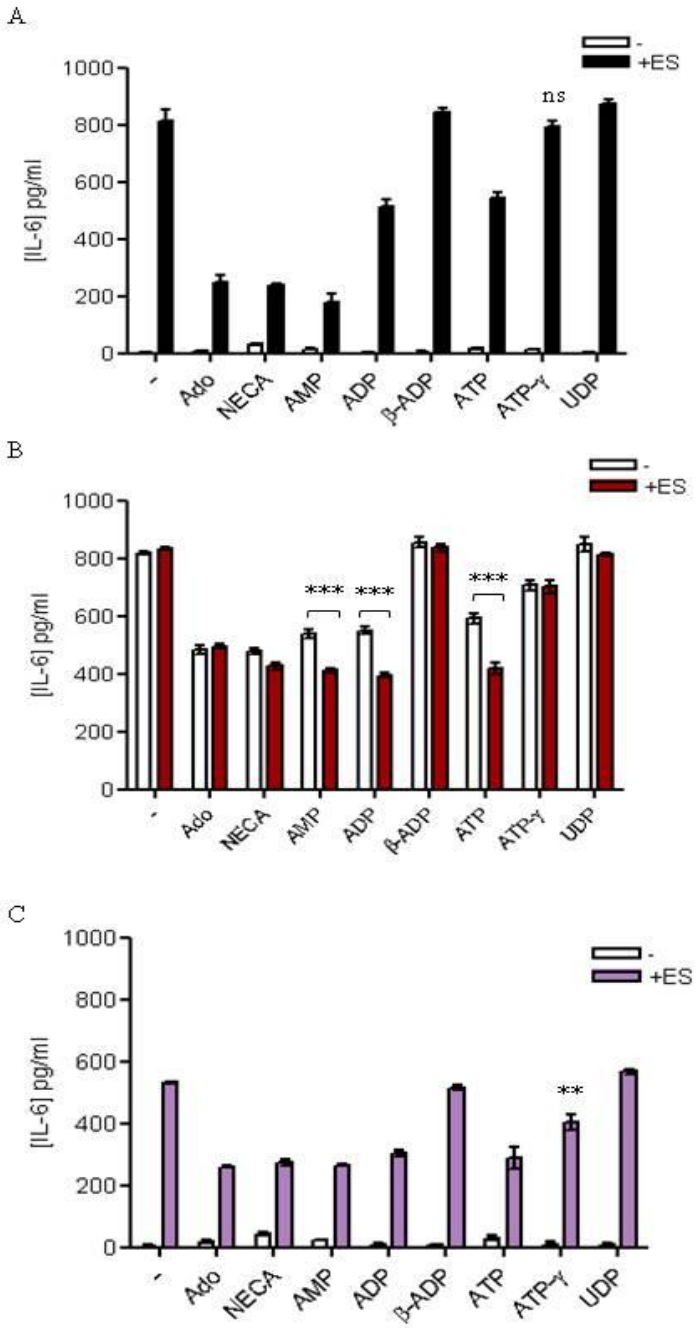


Figure 5.8: The effect of *T.spiralis* ES on IL-6 released from purinergic receptor activated bone marrow derived macrophages. Macrophages were cultured with standard medium (BMMΦ), classically or alternatively activated with 10ng/ml LPS or 5ng/ml IL-4, respectively, and treated for 20 hours with adenosine, NECA, nucleotides or their slowly hydrolysable analogues. The effect of nucleotides in the presence of 50µg/ml ES on IL-6 released from BMMΦ (A), CAMΦ (B) and AAMΦ (C). The levels of IL-6 release are expressed in pg/ml. The data presented are an average of triplicates ± SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. ** $p < 0.01$, *** $p < 0.001$, ns - not significant ($p > 0.05$).

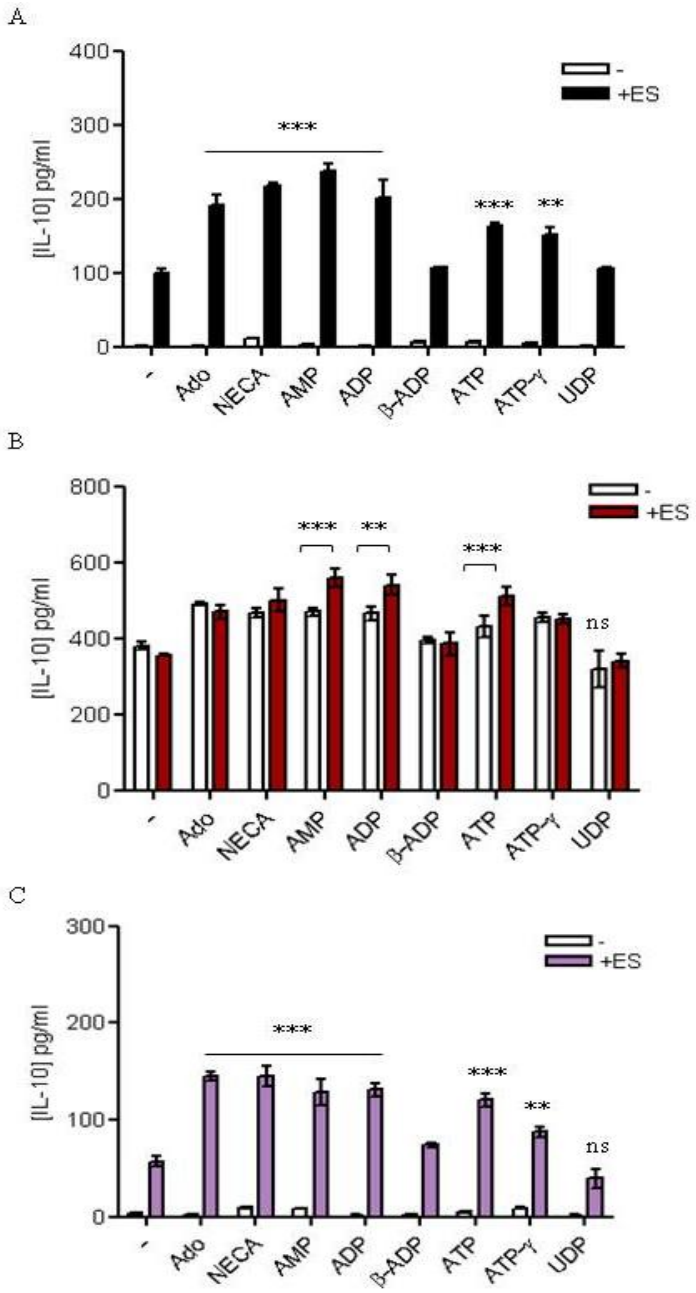


Figure 5.9: The effect of *T.spiralis* ES on IL-10 released from purinergic receptor activated bone marrow derived macrophages. Macrophages were cultured with standard medium (BMMΦ), classically or alternatively activated with 10ng/ml LPS or 5ng/ml IL-4, respectively, and treated for 20 hours with adenosine, nucleotides or their slowly hydrolysable analogues. The effect of nucleotides in the presence of 50µg/ml ES on IL-10 released from BMMΦ (A), CAMΦ (B) and AAMΦ (C). The levels of IL-10 release are expressed in pg/ml. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. ** $p < 0.01$, *** $p < 0.001$, ns - not significant ($p > 0.05$).

Effect of ES on nucleotides mediated by 5`NT

It is known that one of the enzymes present in *T.spiralis* ES has unique catalytic properties in that it can convert ADP to AMP and AMP to adenosine (Gounaris *et al,* 2004). This enzyme was recombinantly expressed and was available for *in vitro* use in order to examine whether this enzyme was responsible for the ES-induced effects on macrophage responses to nucleotides. Expression and purification of 5`NT was carried out and its activity confirmed *in vitro* prior to treatment of macrophages (Appendix, Figures A.1 and A.2). As shown in Figure 5.10, the changes in nucleotide-induced effects observed when ES was present in culture of CAMΦ were due to the action of 5`NT. Experiments were only carried out with CAMΦ because the other macrophage subsets do not produce NO in the absence of ES. NO release and arginase I activity were assayed when nucleotides were incubated with 5`NT instead of ES (Figure 5.10). The results obtained followed the same pattern as that detected with ES, except for the ATP-induced effects which were not as pronounced with 5`NT due to the fact that this enzyme is not responsible for catalysing hydrolysis of ATP by ES. The rest of the nucleotides incubated with 5`NT had similar effects to adenosine, which was an inhibition of NO release from CAMΦ accompanied by an increase in arginase I activity. 5`NT itself did not affect basal NO release or arginase I activity levels.

When ES was replaced by recombinant 5`NT in cultures of CAMΦ with nucleotides, the levels of TNF-α and IL-6 release were inhibited further when AMP and ADP were present in culture while the levels of IL-10 were enhanced. This was also observed when ES was co-administered with these nucleotides showing that the 5`NT activity of ES was responsible for the nucleotide hydrolysis to adenosine. The only difference was observed with ATP which was degraded by ES (Figure 5.7-9) but it is not a substrate for 5`NT-mediated hydrolysis.

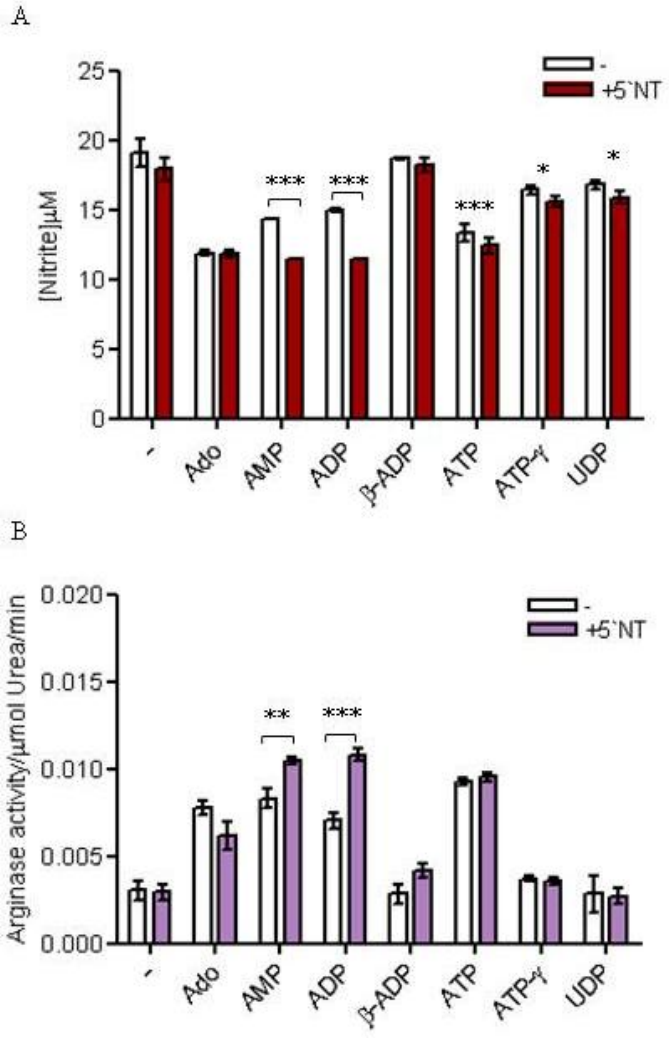


Figure 5.10: The effect of recombinant 5`NT on the levels of NO release and arginase I activity in purinegic receptor activated CAMΦ. Macrophages were classically activated with 10ng/ml LPS (A) and treated for 20 hours with adenosine, nucleotides or their slowly hydrolysable analogues. The effect of nucleotides in the presence of 15µg/ml 5`NT on NO release by CAMΦ (A), expressed in µM nitrite, and arginase I activity (B), expressed as µmol Urea/ min for every 5 x 10^4 cells, is shown. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

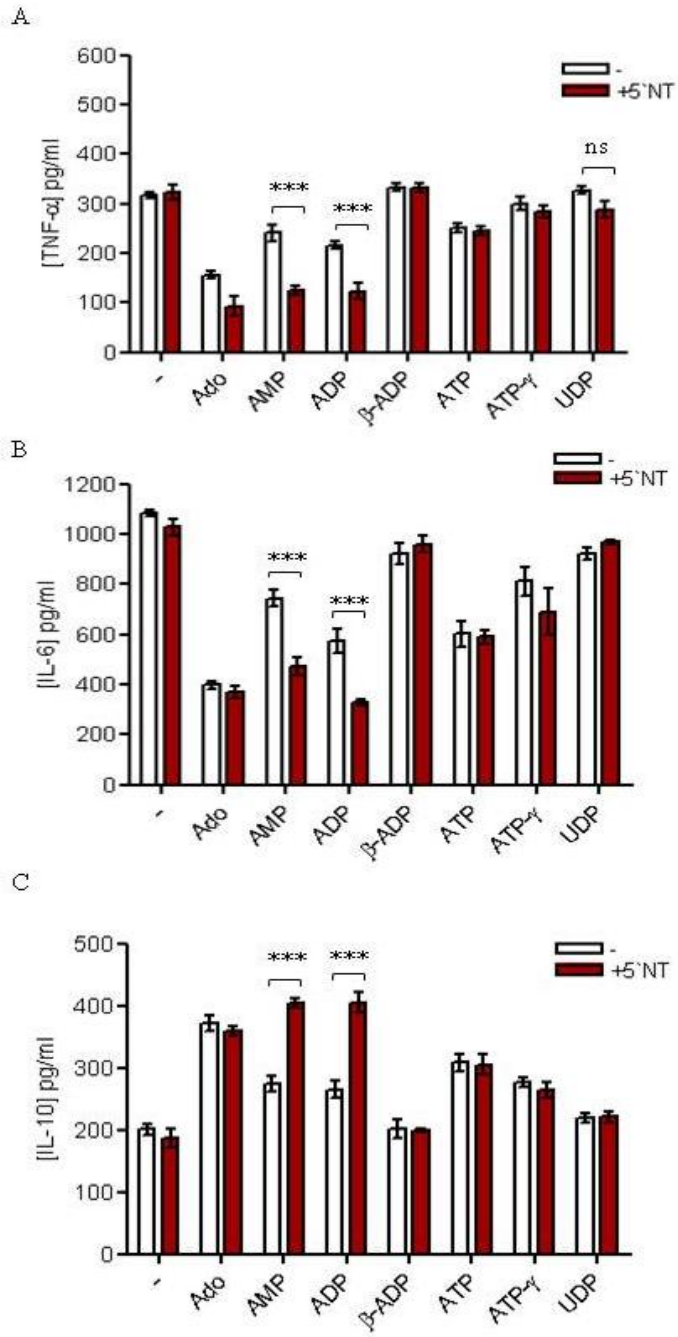


**Figure 5.11: The effect of recombinant 5`NT on cytokine released from purinergic receptor activated CAMΦ.** Macrophages were classically activated with 10ng/ml LPS and treated for 20 hours with adenosine, nucleotides or their slowly hydrolysable analogues. The effect of nucleotides in the presence of 15µg/ml 5`NT on the levels of TNF-α (A), IL-6 (B) and IL-10 (C) released by CAMΦ. Cytokine levels are expressed in pg/ml. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. *** $p < 0.001$, ns - not significant ($p > 0.05$).

5.2.3 Characterisation of *T.spiralis* ES-induced effects

Involvement of downstream signalling pathways

In order to characterise the pathways involved in mediating *T.spiralis* ES-induced effects, macrophages cultured with ES were pre-incubated with compounds known to interfere with or activate some of the known intracellular signalling pathways. Taking into account that adenosine inhibited ES-induced effects and knowing that adenosine mediated its effects via cAMP elevation (as discussed in Chapter 4), ES-treated macrophages were pre-incubated with forskolin to determine if the adenosine-induced effects could be mimicked just by raising cAMP levels. The effect of forskolin on ES-induced NO release is shown in Figure 5.12. In BMMΦ, cAMP elevation with two different concentrations of forskolin inhibited the ES-induced NO release. The effect of forskolin could not be tested at a higher concentration due to the fact that DMSO used to solubilise this compound was toxic to the cells.

TNF-α and IL-6 release from BMMΦ triggered by ES was also partly reversed by cAMP elevation (Figure 5.13A, B). On the other hand, cAMP elevation enhanced the ES-induced IL-10 release by BMMΦ. In the case of cytokine release, DMSO-induced toxicity was observed at the lower concentration of forskolin used. However, it was evident that the forskolin-induced inhibition of TNF-α and IL-6 release were due to cAMP elevation and not to DMSO-induced toxicity as the inhibition observed in the presence of forskolin was much more pronounced. In addition, no DMSO-induced effects were observed in the case of IL-10 release.

Taken together, these data indicate that cAMP elevation can interfere with the ES-induced release of NO and cytokines from BMMΦ. This could either be a direct cAMP effect or might be due to interference with PLC signalling.

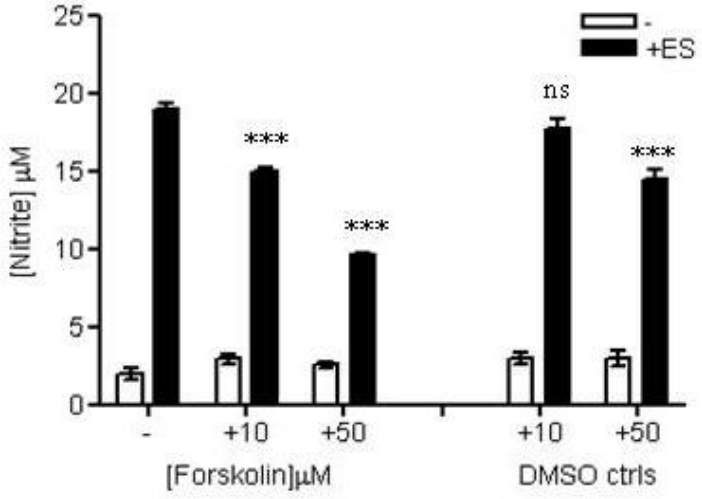


Figure 5.12: The effect of cAMP elevation on *T.spiralis* ES-induced NO release from BMMΦ. Macrophages were cultured with standard medium for 20 hours and treated with 50µg/ml *T.spiralis* ES. Prior to ES addition cells were incubated with the cAMP elevator, forskolin. DMSO controls corresponding to the final concentration of DMSO for forskolin were also included. DMSO 10 and 50 correspond to the 10µM and 50µM forskolin used. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the untreated control (-). *** $p < 0.001$, ns - not significant ($p > 0.05$).

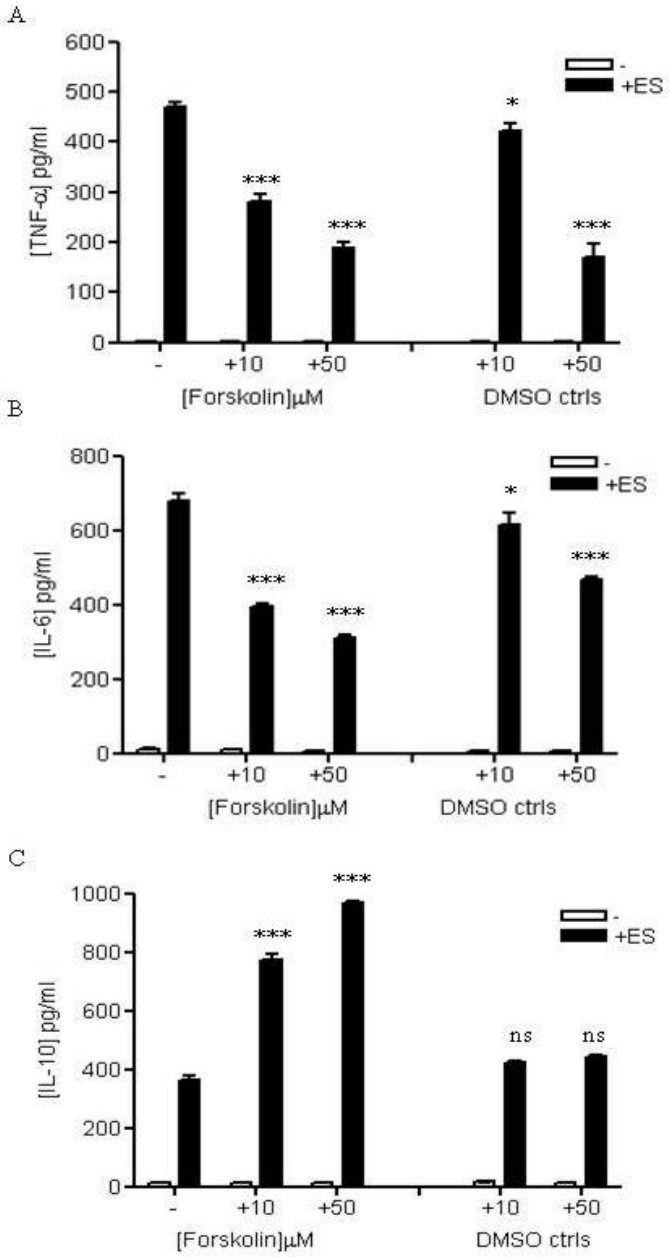


Figure 5.13: The effect of cAMP elevation on *T.spiralis* ES – induced cytokine release from BMMΦ. Macrophages were cultured with standard medium for 20 hours and treated with 50μg/ml *T.spiralis* ES. Prior to ES addition cells were incubated with the cAMP elevator, forskolin. DMSO controls corresponding to the final concentration of DMSO for forskolin were also included. DMSO 10 and 50 correspond to the 10μM and 50μM forskolin used. Supernatants were assayed for TNF-α (A), IL-6 (B) and IL-10 (C) release, and the levels of cytokines are expressed in pg/ml. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the untreated control (-). * $p< 0.05$, *** $p< 0.001$, ns - not significant ($p> 0.05$).

The involvement of PLC in macrophage effector functions induced by ES was also investigated. As shown in Figure 5.14A, the ES-induced increase in NO release by BMMΦ was completely reversed by the PLC inhibitor U73122 while the mock inhibitor had no effect, as expected. Downstream pathways were also blocked at different points using Bay 11-7082, U0126 and LY294002 to inhibit NF-κB, MEK1/2 and PI3 kinase, respectively (Figure 5.14B). Inhibiting NF-κB and MEK1/2 also inhibited the ES-induced effect but not as dramatically as PLC inhibition. However, blocking PI3 kinase resulted in a 50% inhibition of ES-induced NO release.

The effect of blocking downstream pathways on ES-induced cytokine release was also investigated. As shown in Figure 5.15, the PLC inhibitor did not have the same effect on all the cytokines tested. Specifically, U73122 reversed the ES-induced TNF-α and IL-10 release but so did the mock PLC inhibitor showing that the effect was non-specific. However, it should be noted that, the inhibition of TNF-α release observed with U73122 was more pronounced than that caused by the inactive analogue. In the case of IL-6 release, only the PLC inhibitor blocked the ES-induced increase. These data show that only the IL-6 release induced by ES was affected by inhibiting activation of the PLC pathway since the data obtained for TNF-α and IL-10 release were inconclusive.

The NF-κB, MEK1/2 and PI3 kinase pathways were also blocked to determine if ES-induced cytokine release was dependent on their activation (Figure 5.16). As shown in Figure 5.16A, Bay 11-7082, U0126 and LY294002 increased basal TNF-α release. When the inhibitors were co-administered with ES, the ES-induced increase was reduced by 20% when NF-κB and MEK1/2 were inhibited and by 50% when PI3 kinase was blocked. In the case of IL-6 release only LY294002 increased BMMΦ basal levels while all inhibitors reversed the ES-induced IL-6 release by approximately 70% (Figure 5.16B). Finally, basal IL-10 release levels were increased

by all 3 inhibitors, however, when BMMΦ were co-cultured with ES and the respective inhibitor, Bay 11-7082, U0126 and LY294002, ES-induced effects were reduced by 50% in all cases.

In general, the NO and cytokine release triggered by ES varied according to the ES batch used so only one representative experiment is shown. In addition, ES was used at a concentration that triggered a submaximal response, especially when inhibitors were tested to avoid saturation effects that would lead to inefficient antagonism.

Taken together these data show that ES-induced effects were mediated by an increase in PLC levels and/or a decrease in cAMP levels. In addition, ES-induced NO and cytokine release required NF-κB and MEK1/2 activation at some point in the signalling cascade, however, the lack of dependence on one particular pathway points to overlap as these pathways converge before activating nuclear transcription factors. PI3 kinase involvement is also a possibility, however, it should be taken into account that the inhibitor used also blocked background NO and cytokine release by CAMΦ, which may suggest toxicity or that PI3 kinase is a ubiquitous enzyme in macrophage signalling cascades.

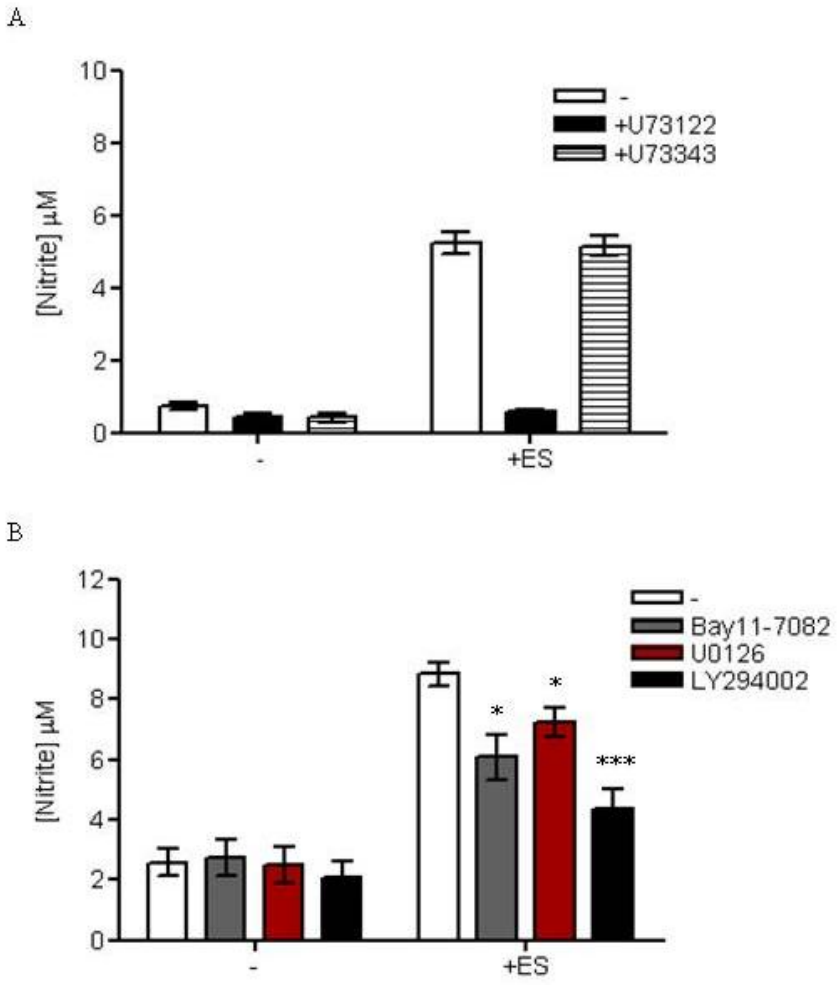


Figure 5.14: The effect of PLC, NF-κB, MEK and PI3 kinase inhibition on *T.spiralis* ES-induced NO release from BMMΦ. Macrophages were cultured in standard medium for 20 hours and treated with 50µg/ml *T.spiralis* ES. Prior to ES addition cells were incubated with different inhibitors for 15 minutes at 37˚C. (A) The effect of PLC inhibition with 10µM U73122 on ES-induced effects. U73343 is a mock PLC inhibitor. (B) The effect of 5µM Bay11-7082 (NF-κB), 5µM U0126 (MEK1/2) and 10µM LY294002 (PI3 kinase) on ES – induced NO release from BMMΦ. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control.* $p < 0.05$, *** $p < 0.001$.

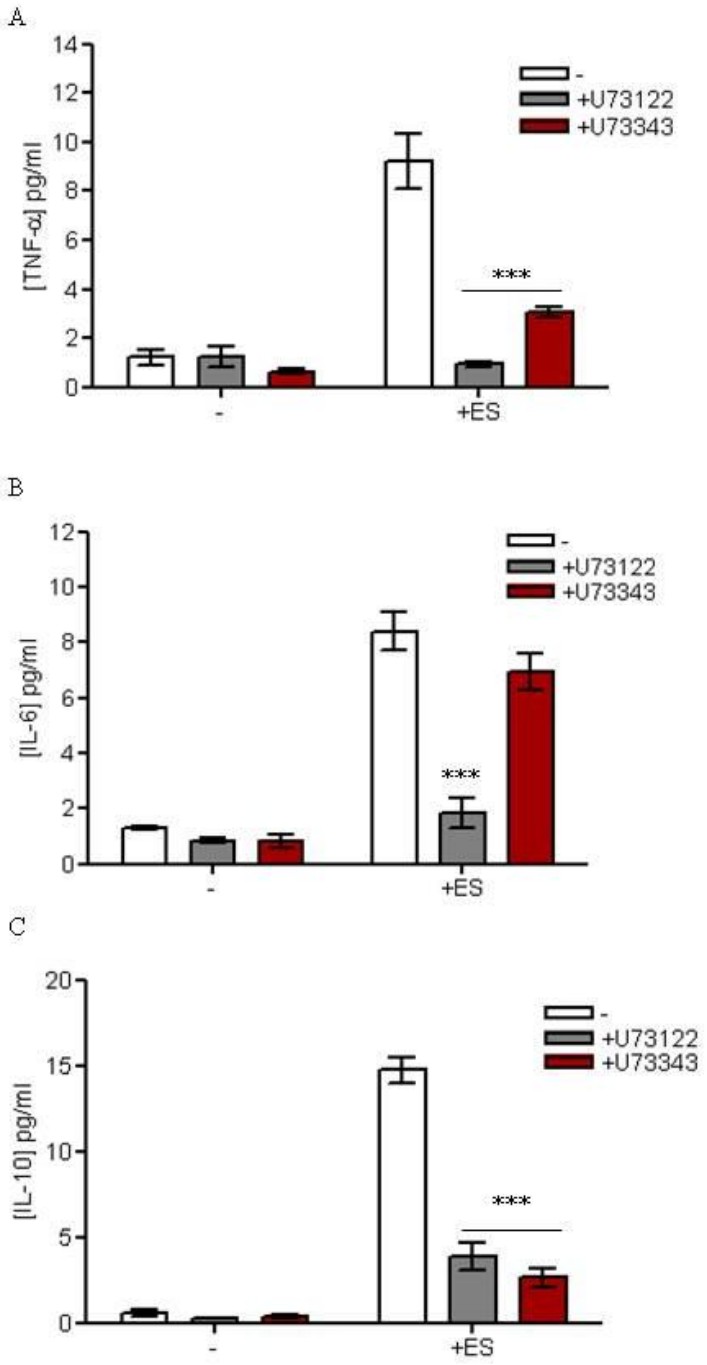


Figure 5.15: The effect of PLC inhibition on *T.spiralis* ES-induced cytokine release from BMMΦ. Macrophages were pre-incubated with 10μM of the PLC inhibitor U73122 or the mock inhibitor U73343 and then cultured in standard medium for 20 hours with 50μg/ml *T.spiralis* ES. Cell supernatants were then assayed for TNF-α (A), IL-6 (B) and IL-10 (C) release, expressed in pg/ml. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control.*** $p < 0.001$.

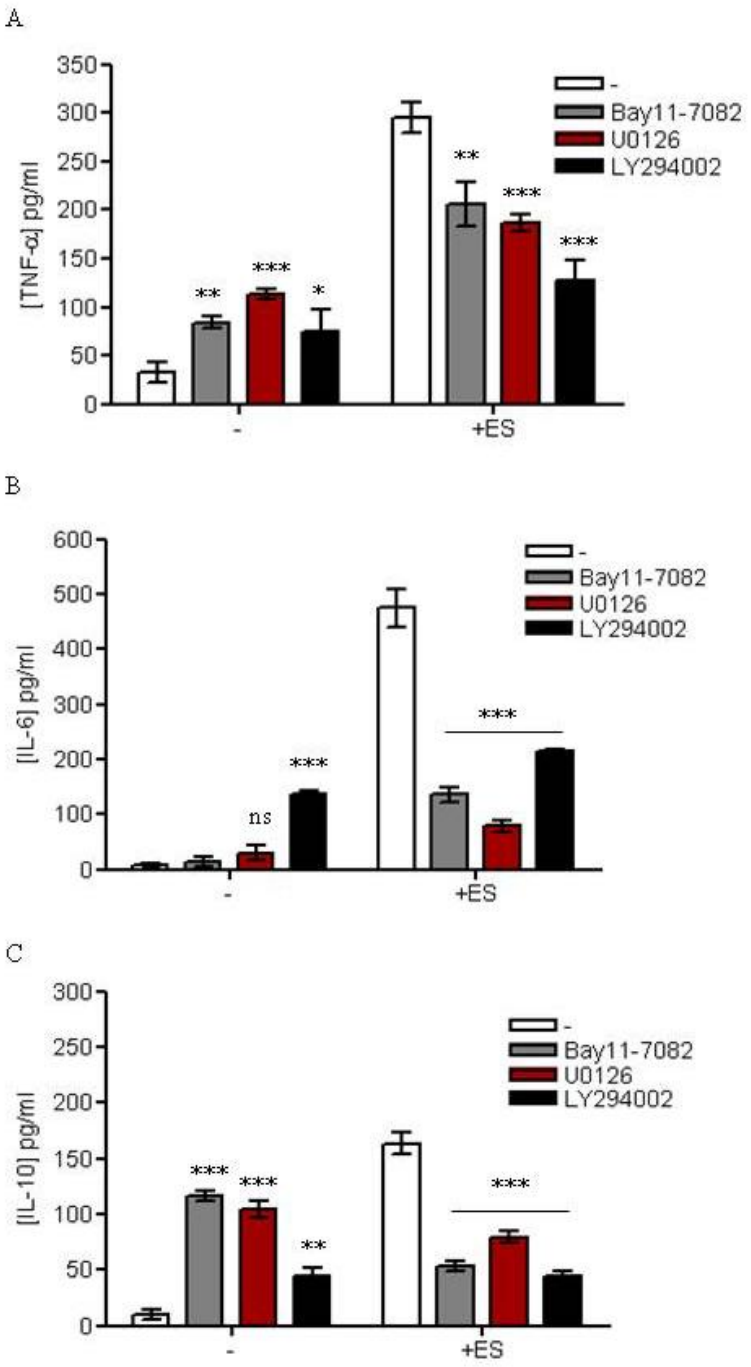


Figure 5.16: The effect of NF-κB, MEK and PI3 kinase inhibition on *T.spiralis* ES-induced cytokine release from BMMΦ. Macrophages were pre-incubated with 5μM Bay 11-7082 (NF-κB), 5μM U0126 (MEK1/2) or 10μM LY294002 (PI3 kinase) and then cultured in standard medium for 20 hours with 50μg/ml *T.spiralis* ES. Cell supernatants were then assayed for the presence of TNF-α (A), IL-6 (B) and IL-10 (C). Cytokine levels are expressed in pg/ml. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control.* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, ns - not significant ($p> 0.05$).

Effect of heat inactivation of ES

In an attempt to characterise which component of ES was responsible for the induction of macrophage effector functions, ES was subjected to different treatments to alter or eliminate certain structural characteristics. Firstly, ES was heat-inactivated by incubating at 100˚C for 10 minutes in order to inhibit any enzymatic activity prior to macrophage stimulation. The effect of heat inactivating ES on arginase I activity and NO release is shown in Figure 5.17. In BMMΦ the increase in NO release and arginase I activity was reduced by heat inactivation. No effect was observed in CAMΦ and AAMΦ NO release or arginase I activity.

Cytokine release was also assayed and as it is shown in Figure 5.18, heat inactivation partly but significantly reduced TNF-α release from BMMΦ and AAMΦ by 20-30%. Likewise, IL-6 release was also reduced in BMMΦ and AAMΦ by 50% and 20%, respectively. There was no significant change in CAMΦ TNF-α and IL-6 release levels. Interestingly, IL-10 release by BMMΦ and AAMΦ was almost completely reversed by heat inactivating ES. In this particular set of experiments the batch of ES used appeared to increase CAMΦ IL-10 release as well, however, this increase was also reversed by heat inactivation.

Glycan involvement in ES-induced effects

To further characterise the ES-induced effects, the involvement of glycans conjugated onto ES proteins was investigated. ES was chemically deglycosylated by the TFMS method which eliminates all O- and N-linked glycans whilst preserving the core proteins. These proteins were recovered and used to stimulate BMMΦ. As shown in Figure 5.19, deglycosylating ES completely inhibited the NO release triggered in BMMΦ while in AAMΦ, where stimulation with ES caused a smaller increase, the reduction was less pronounced but still significant.

Similarly, the increase in cytokine release elicited by ES in BMMΦ and AAMΦ was also reversed by deglycosylation, as can be seen in Figure 5.20. As with NO release, the ES-induced effects on cytokine release were more pronounced in BMMΦ which made the reversal more apparent than in AAMΦ. Nevertheless, AAMΦ cytokine release levels up-regulated by ES were also significantly reduced when the deglycosylated protein was administered instead.

Taken together these results suggest that the glycan components of *T.spiralis* ES are responsible for macrophage activation. The fact that heat inactivation also partly reversed ES-induced effects may point to the importance of correct positioning of glycans, which is directly related to protein structural integrity.

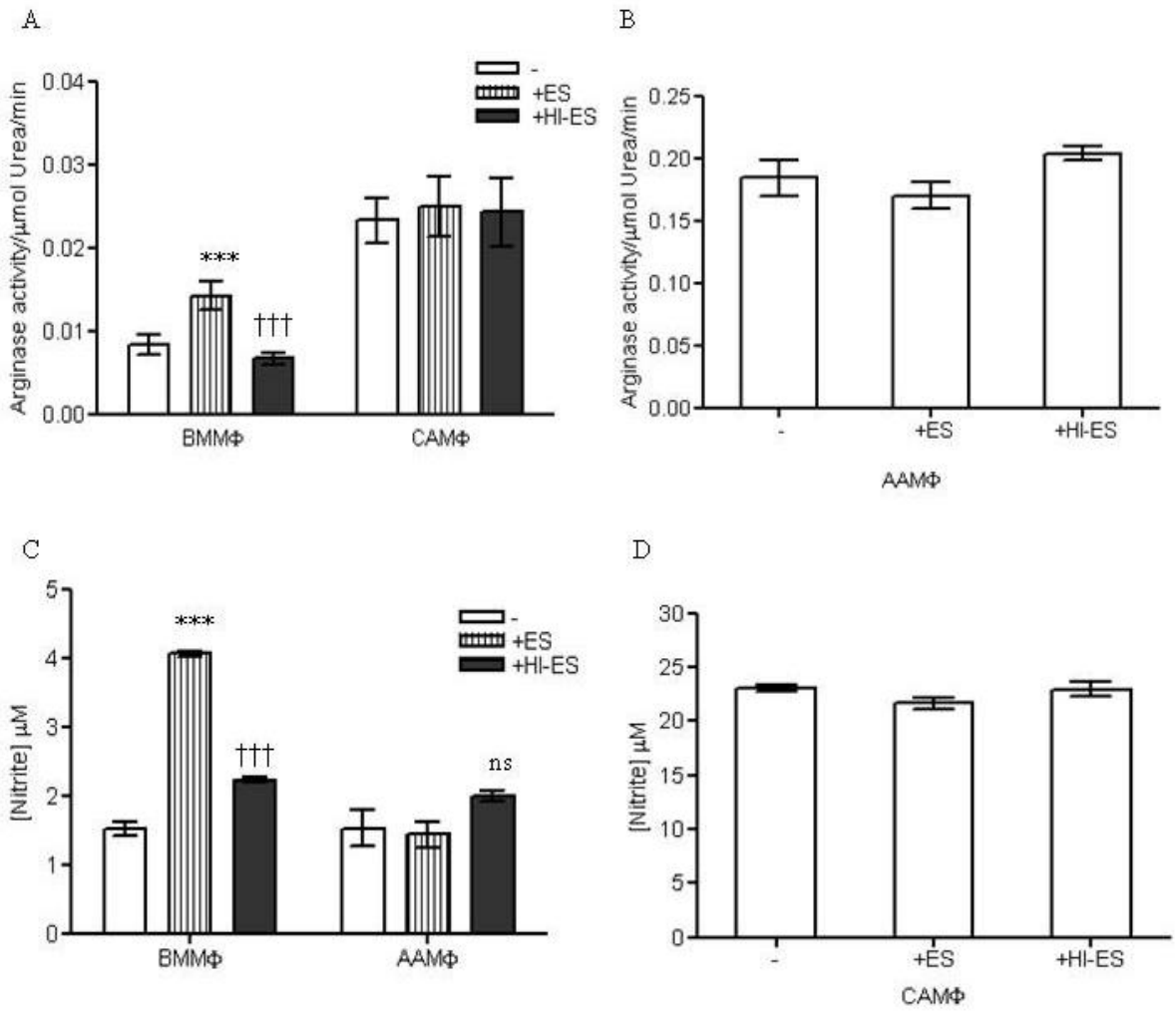


Figure 5.17: The effect of heat inactivating *T.spiralis* ES on arginase I activity and NO released from bone marrow derived macrophages. Macrophages cultured with standard medium (BMMΦ), 10ng/ml LPS (CAMΦ) or 5ng/ml IL4 (AAMΦ) were treated with 50 µ g/ml *T.spiralis* ES that was boiled at 100˚C for 10 minutes (HI-ES). (A) and (B) The effect on the levels of arginase I activity in the 3 macrophage types expressed as µmol Urea/ min for every 5 x 10^4 cells. (C) and (D) The effect on nitric oxide released by the 3 macrophage types expressed in µM nitrite. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. *** $p < 0.001$. (†) is compared to the respective macrophage type treated with ES only. ††† $p < 0.001$, ns - not significant ($p > 0.05$).

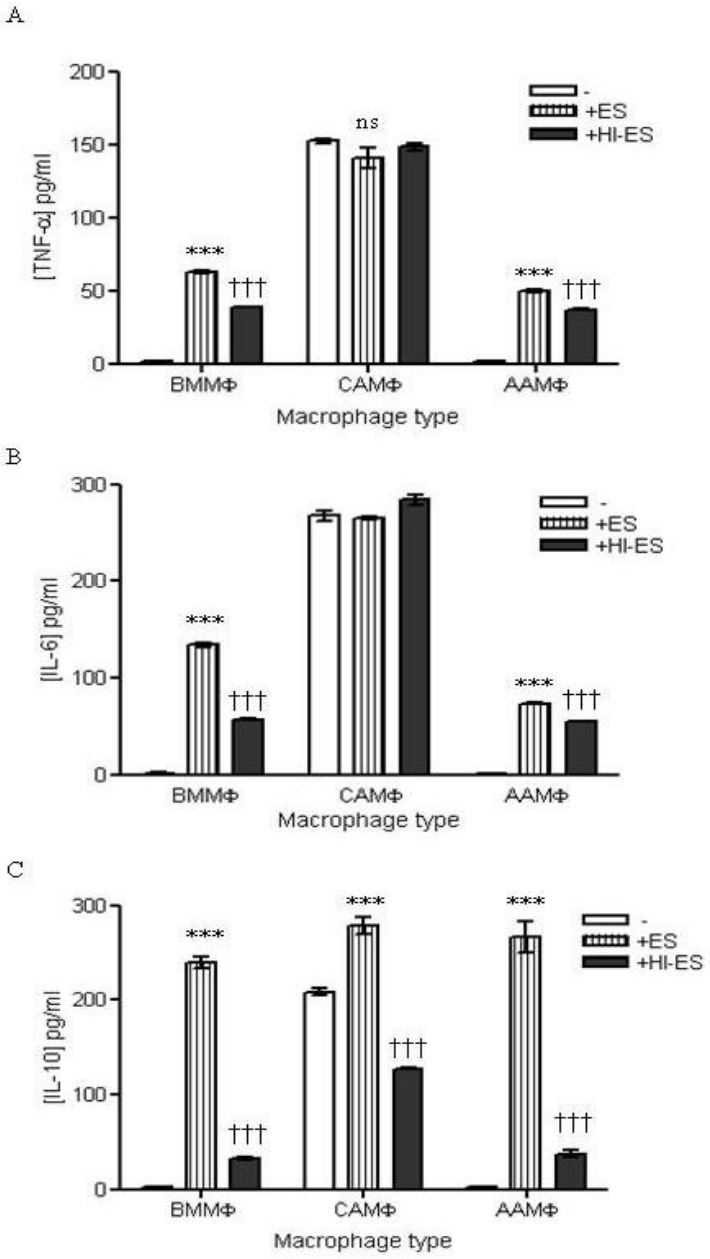


Figure 5.18: The effect of heat inactivating *T.spiralis* ES on cytokine release by bone marrow derived macrophages. Macrophages cultured with standard medium (BMMΦ), 10ng/ml LPS (CAMΦ) or 5ng/ml IL4 (AAMΦ) were treated with 50 µ g/ml *T.spiralis* ES that was boiled at 100˚C for 10 minutes (HI-ES). Cell supernatants were assayed for the presence of TNF-α (A), IL-6 (B) and IL-10 (C). Cytokine levels are expressed in pg/ml. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. *** $p < 0.001$, ns - not significant ($p > 0.05$). (†) is compared to the respective macrophage type treated with ES only. ††† $p < 0.001$.

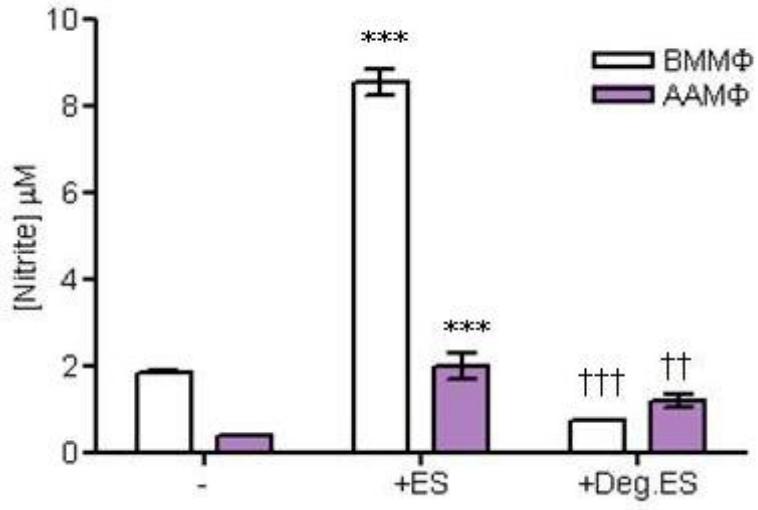


Figure 5.19: The effect of deglycosylating *T.spiralis* ES on induction of NO release from BMMΦ and AAMΦ. Macrophages were cultured in standard medium (BMMΦ) or 5ng/ml IL-4 (AAMΦ) for 20 hours and treated with 50µg/ml *T.spiralis* ES or deglycosylated ES (Deg.ES). Cell supernatants were assayed for the presence of NO. NO levels are expressed as µM nitrite. The data presented are an average of triplicates ± SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. *** p< 0.001. (†) is compared to the respective macrophage type treated with ES only. †† p< 0.01, ††† p< 0.001.

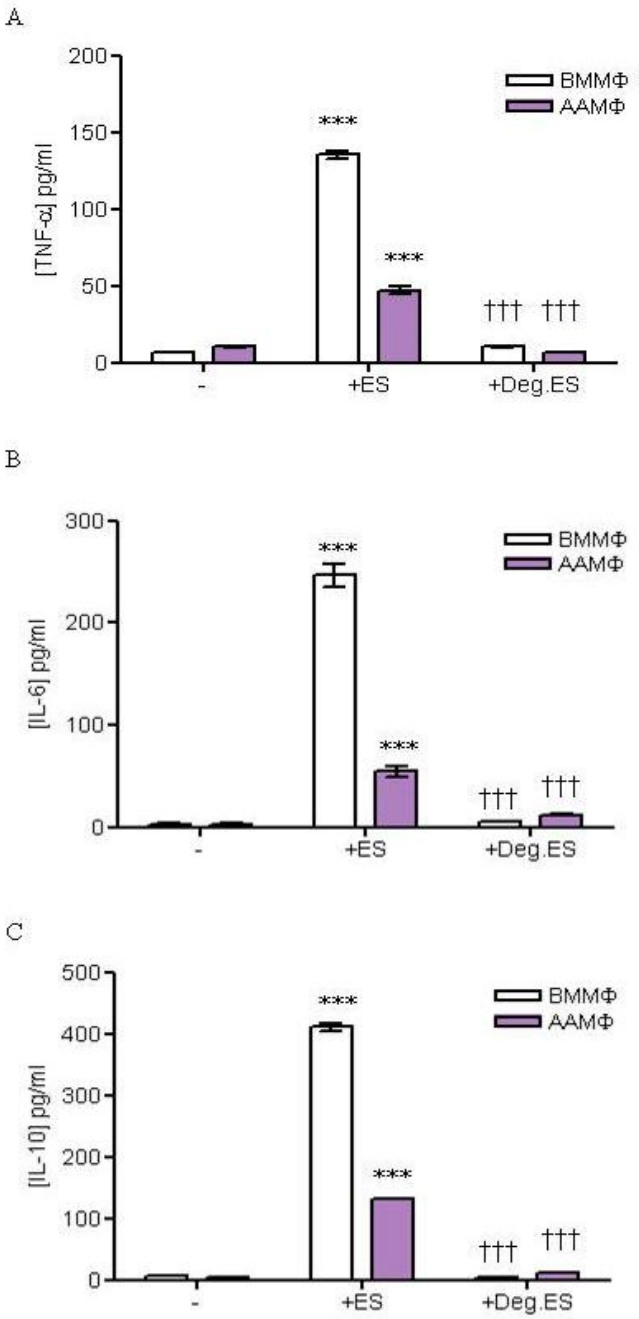


Figure 5.20: The effect of deglycosylating *T.spiralis* ES on induction of cytokine release from BMMΦ and AAMΦ. Macrophages were cultured in standard medium (BMMΦ) or 5ng/ml IL-4 (AAMΦ) for 20 hours and treated with 50μg/ml *T.spiralis* ES or deglycosylated ES (Deg.ES). Cell supernatants were assayed for the presence of TNF-α (A), IL-6 (B) and IL-10 (C). Cytokine levels are expressed in pg/ml. The data presented are an average of triplicates ± SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. *** $p < 0.001$. (†) is compared to the respective macrophage type treated with ES only. ††† $p < 0.001$.

Investigation of macrophage glycan receptor activation

Having established that the glycan component of *T.spiralis* ES was responsible for the activation of BMMΦ by ES, the involvement of specific macrophage receptors of the C-type lectin class was investigated. This was achieved by pre-incubating macrophages with carbohydrates known to bind C-type lectins prior to treatment with *T.spiralis* ES.

Firstly, BMMΦ, CAMΦ and AAMΦ were incubated with D-mannose to see if ES-induced effects could be mimicked by mannose receptor activation. As shown in Figure 5.21, D-mannose had no effect on macrophage arginase I activity and NO release. Cytokine release in response to D-mannose was also assayed but no increase was detected throughout the D-mannose concentration range tested (Figure 5.22).

Macrophage cytokine and NO release were then measured in response to ES after blocking surface lectins for one hour with specific carbohydrates, such as, D-mannose (mannose receptor), mannan (DC-SIGN and mannose receptor), laminarin (Dectin-1), EGTA (all c-type lectins) and GalNAc (Macrophage Galactose binding Lectin-MGL). The effect of blocking lectins was only investigated in BMMΦ and AAMΦ which generally showed a greater responsiveness to ES. EGTA completely abolished NO release triggered by ES while GalNAc also significantly inhibited NO release (Figure 5.23). D-mannose and laminarin had no effect on NO release but mannan seemed to enhance the ES-induced NO production. Cytokine release was similarly affected by blocking macrophage surface lectins. As shown in Figure 5.24, blocking the effects induced by ES with GalNAc and EGTA inhibited release of all the cytokines measured. The effects induced by mannan and laminarin on NO release were not as significant in the case of IL-6 and IL-10 release and were not detected in the case on TNF-α release. Taken together, these data point to the involvement of MGL in the ES-induced NO and cytokine release by BMMΦ.

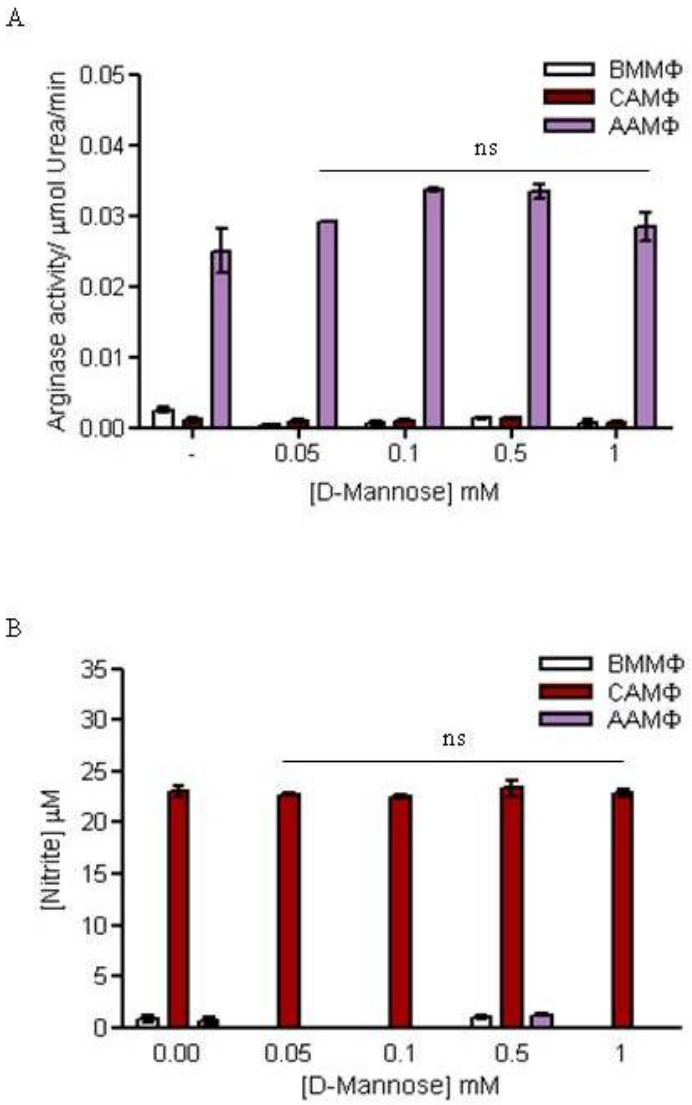


Figure 5.21: The effect of D-Mannose on arginase I activity and NO released by bone marrow derived macrophages. Macrophages cultured with standard medium (BMMΦ), 10ng/ml LPS (CAMΦ), or 5ng/ml IL4 (AAMΦ) were treated with D-Mannose at a range of concentrations (0.05-1mM) for 20hrs. Arginase I activity (A) and nitric oxide release (B) levels were determined. Arginase I activity is expressed as µmol Urea/ min for every 5 x 10^4 cells and nitrite levels detected are presented in µM. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. ns - not significant ($p > 0.05$).

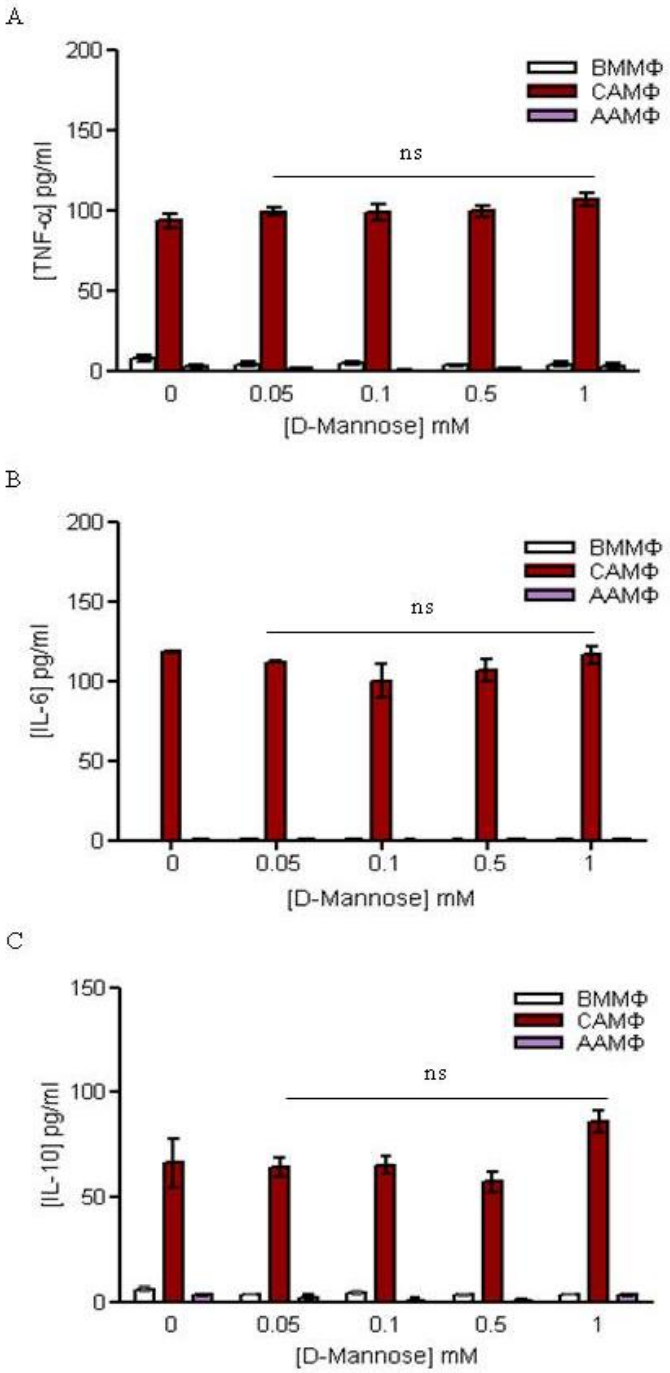


Figure 5.22: The effect of D-Mannose on cytokine release from bone marrow derived macrophages. Macrophages cultured with standard medium (BMMΦ), 10 ng/ml LPS (CAMΦ), or 5 ng/ml IL4 (AAMΦ) were treated with D-Mannose at a range of concentrations (0.05-1mM) for 20hrs. Supernatants were collected and ELISA carried out to determine TNF-α (A), IL-6 (B) and IL-10 (C) levels. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. ns - not significant ($p > 0.05$).

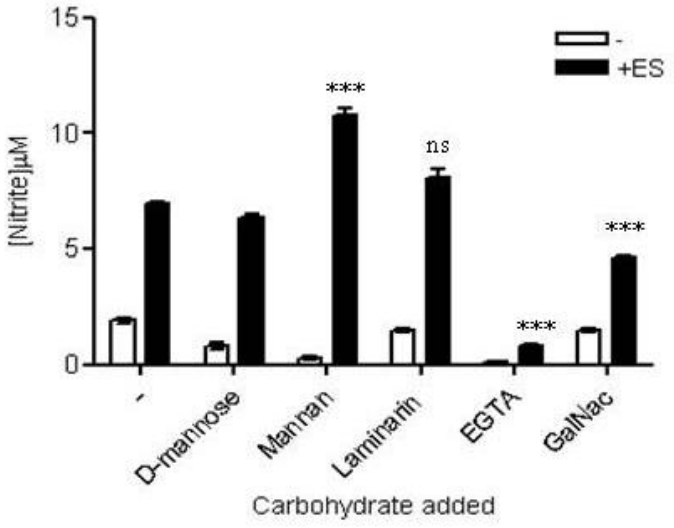


Figure 5.23: The effect of blocking BMMΦ surface lectins on *T.spiralis* ES – induced NO release. Macrophages were cultured with standard medium for 20 hours and treated with 50µg/ml *T.spiralis* ES. Prior to ES addition cells were incubated with the following carbohydrates: 100µg/ml mannan (blocking DC-SIGN and MR), 200µg/ml laminarin (blocking Dectin-1), 10mM EGTA (blocking all c-type lectins) and 100mM GalNAc (blocking MGL). Cell supernatants were assayed for NO release, expressed as µM nitrite. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. *** $p < 0.001$, ns - not significant ($p > 0.05$).

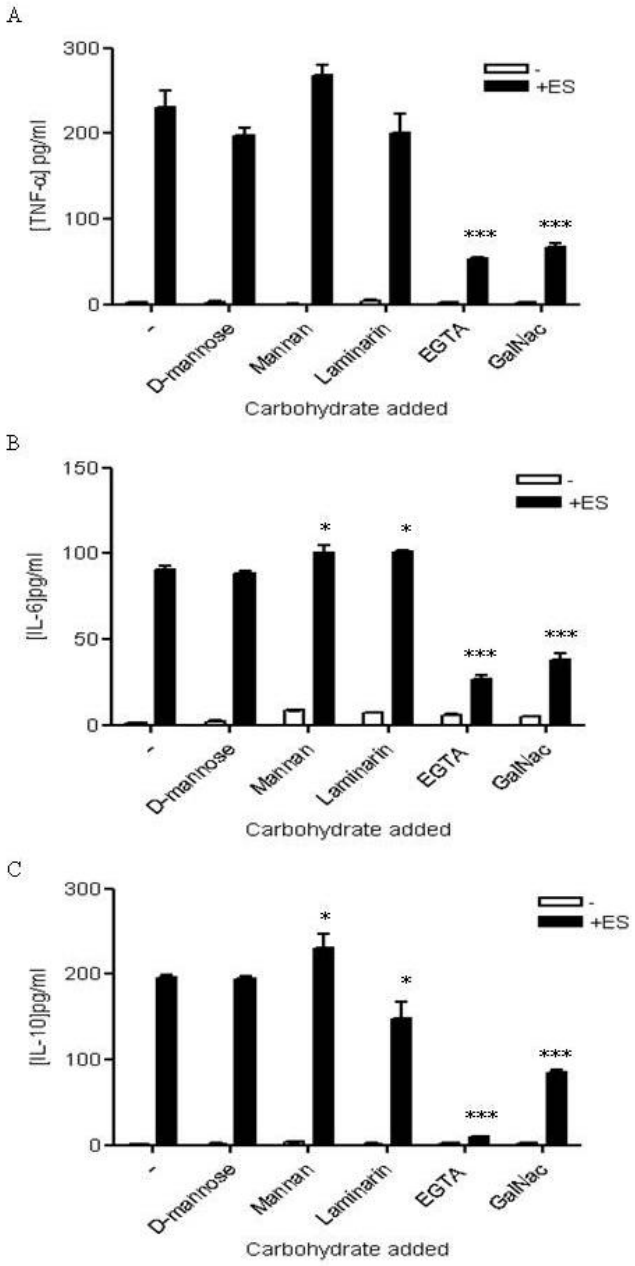


Figure 5.24: The effect of blocking BMMΦ surface lectins on *T.spiralis* ES – induced cytokine release. Macrophages were cultured with standard medium for 20 hours and treated with 50μg/ml *T.spiralis* ES. Prior to ES addition cells were incubated with the following carbohydrates: 100μg/ml mannan (blocking DC-SIGN and MR), 200μg/ml laminarin (blocking Dectin-1), 10mM EGTA (blocking all c-type lectins) and 100mM GalNAc (blocking MGL). Cell supernatants were assayed for the presence of TNF-α (A), IL-6 (B) and IL-10 (C). Cytokine levels are expressed in pg/ml. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of $n > 3$ and asterisks indicate significance compared to the respective untreated control. * $p< 0.05$, *** $p< 0.001$.

Macrophage Galactose Lectin (MGL) involvement

Having concluded from macrophage functional assays that MGL is responsible for *T.spiralis* ES-induced effects, MGL-binding fractions in ES were purified. The human macrophage galactose lectin CRD was expressed and purified by affinity chromatography on a galactose–Sepharose column (by A. Quintero-Martinez, K. Drickamer as described in Lobst and Drickamer, 1996) and incubated with ES in loading buffer containing Ca^{2+} ions required for MGL stability. Unbound or weakly-bound fractions were collected by washing with loading buffer and bound ES proteins eluted by removal of Ca^{2+} ions (elution buffer – Ca^{2+}). BMMΦ were then cultured with the fractions obtained and NO and cytokine release assayed. The effect of MGL-purified fractions of ES on NO release by BMMΦ is shown in Figure 5.25. As shown in Figure 5.25A, the flow-through (F), the first fraction collected after application of ES, retained some activity that was comparable to the levels measured with ES and the MGL-bound ES fraction. In order to determine if the residual activity was due to low affinity fractions of GalNAc-conjugated proteins present in the flow-through, macrophages were blocked with GalNAc prior to their treatment with the different fractions (Figure 5.25B). As expected, the activity of all fractions tested was significantly inhibited by blocking macrophage MGL with GalNAc. This also confirmed that the bound fraction was specific for MGL on macrophages since GalNAc completely inhibited the Bound-ES-induced NO release.

Cytokine release in response to MGL-purified fractions was also measured in macrophage supernatants. A similar pattern to NO release was observed for all cytokines tested (Figure 5.26). Specifically, the activity detected in the first two fractions that were collected from the column gradually decreased with each wash so that no activity was detected in the last wash. The cytokine release triggered by the flow-through (F) and Bound fractions was inhibited by pre-

treatment of macrophages with GalNAc and in the case of IL-10 the effect was completely reversed (Figure 5.27).

In an attempt to identify ES proteins that were eluted from the MGL column, SDS-PAGE analysis was carried out. SDS-PAGE gels used to resolve the different ES fractions that resulted from MGL-purification are shown in Figure 5.28. Protein bands that were detected in the bound-ES fraction were very faint and corresponded to 120, 50, 45, 20 kDa. A band at 37kDa was unique for the bound-ES fraction and could be due to leaching of MGL from the column during the elution step. The rest of the bands detected in the bound-ES fraction were also observed in the flow-through (F) and wash (W1 and W2) fractions as well. This could indicate the presence of active ES components in F and W which would also explain the persisting activity in these fractions.

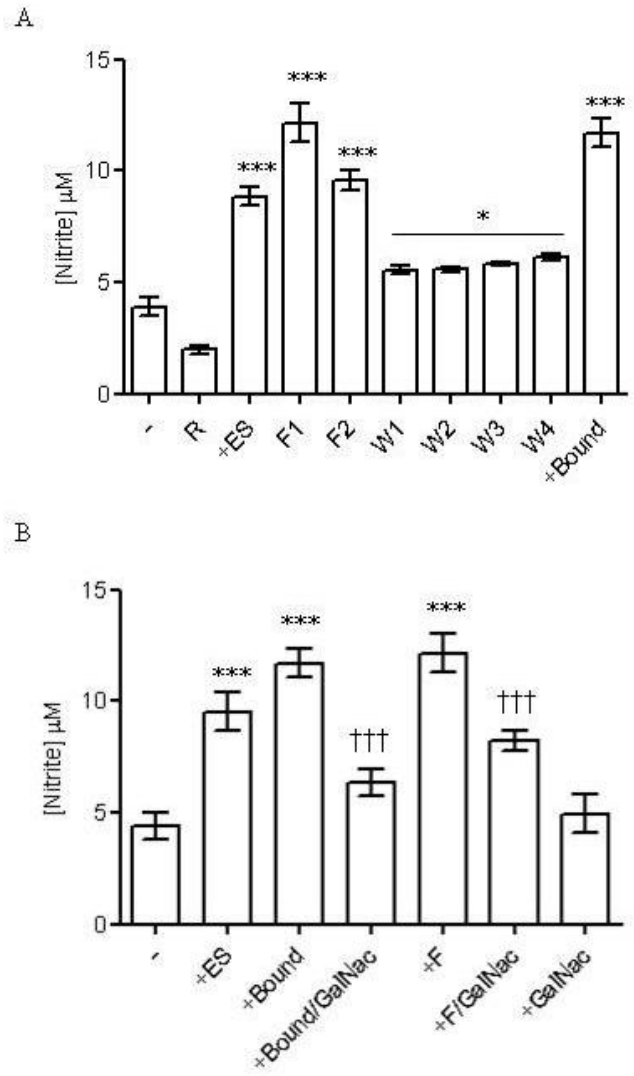


Figure 5.25: The effect of MGL-purified *T.spiralis* ES fractions on NO release from BMMΦ. Macrophages were cultured in standard medium and treated with 50µg/ml *T.spiralis* ES. Cells were also incubated with separated ES fractions obtained from applying ES to an MGL-conjugated column. (A) The effect of incubating BMMΦ with the following fractions: R (column rinse), F1 and F2 (flow through 1 and 2), W 1, 2, 3 and 4 (Column washes with loading buffer) and Bound ES (eluted products). (B) The effect of incubating purified fractions with 100mM GalNAc. NO release was assayed in cell supernatants and expressed as µM nitrite. The data presented are an average of triplicates ± SEM from one experiment of n > 3 and asterisks indicate significance compared to the respective untreated control.* $p < 0.05$, *** $p < 0.001$. (†) is compared to the respective fraction not treated with GalNAc. ††† $p < 0.001$.

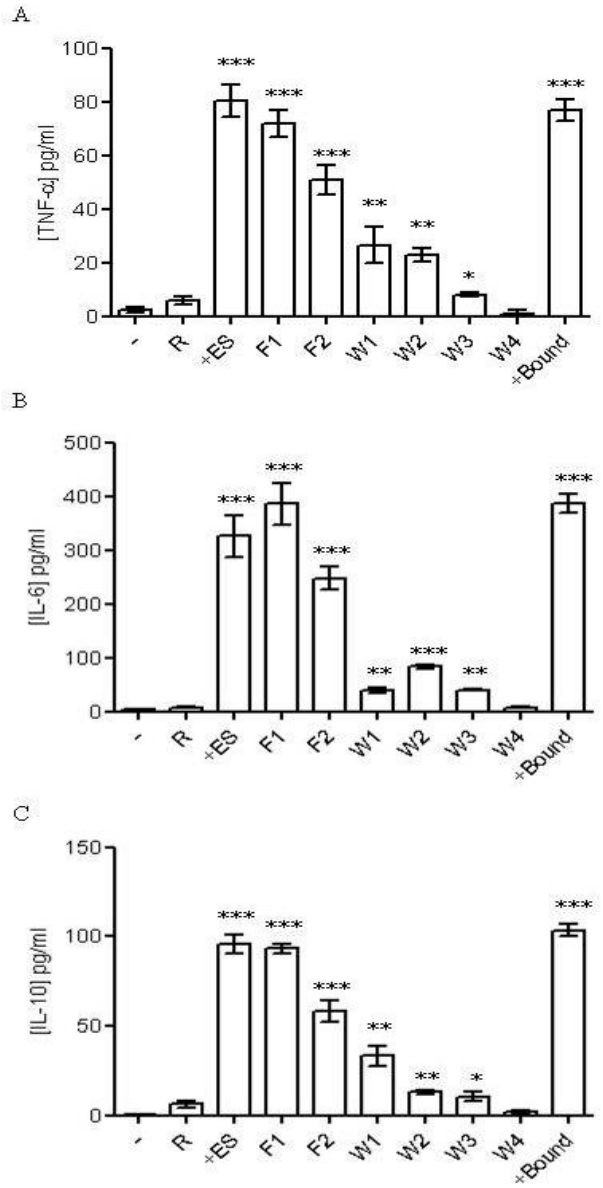


Figure 5.26: The effect of MGL-purified *T.spiralis* ES fractions on cytokine release from BMMΦ. Macrophages were cultured in standard medium and treated with 50µg/ml *T.spiralis* ES. Cells were also incubated with separated ES fractions obtained from applying ES to an MGL-conjugated column. The effect of incubating BMMΦ with the following fractions: R (column rinse), F1 and F2 (flow through 1 and 2), W 1, 2, 3 and 4 (Column washes with loading buffer) and Bound ES (eluted products) was investigated and cell supernatants were assayed for the presence of TNF-α (A), IL-6 (B) and IL-10 (C). Cytokine levels are expressed in pg/ml. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control.* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

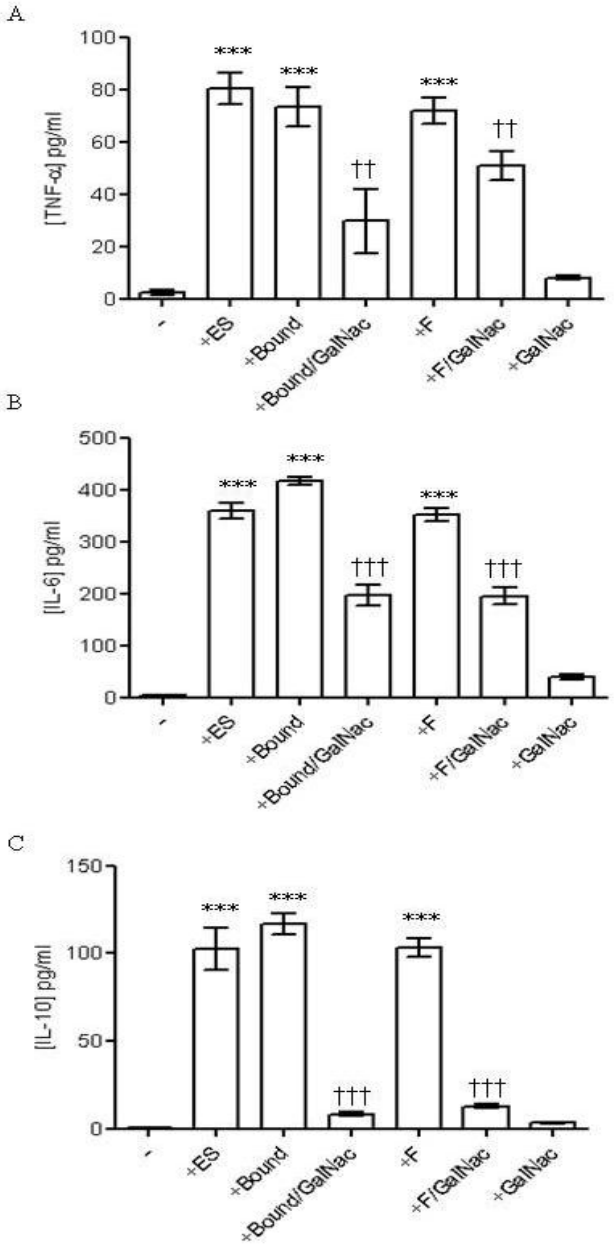


Figure 5.27: The effect of MGL-purified *T.spiralis* ES fractions on NO release from BMMΦ. Macrophages were cultured in standard medium and treated with 50μg/ml *T.spiralis* ES. Cells were also incubated with separated ES fractions obtained from applying ES to an MGL-conjugated column. The effect of incubating purified fractions with 100mM GalNAc was investigated and cell supernatants were assayed for the presence of TNF-α (A), IL-6 (B) and IL-10 (C). Cytokine levels are expressed in pg/ml. The data presented are an average of triplicates ± SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control.*** $p < 0.001$. (†) is compared to the respective fraction not treated with GalNAc. †† $p < 0.01$, ††† $p < 0.001$.

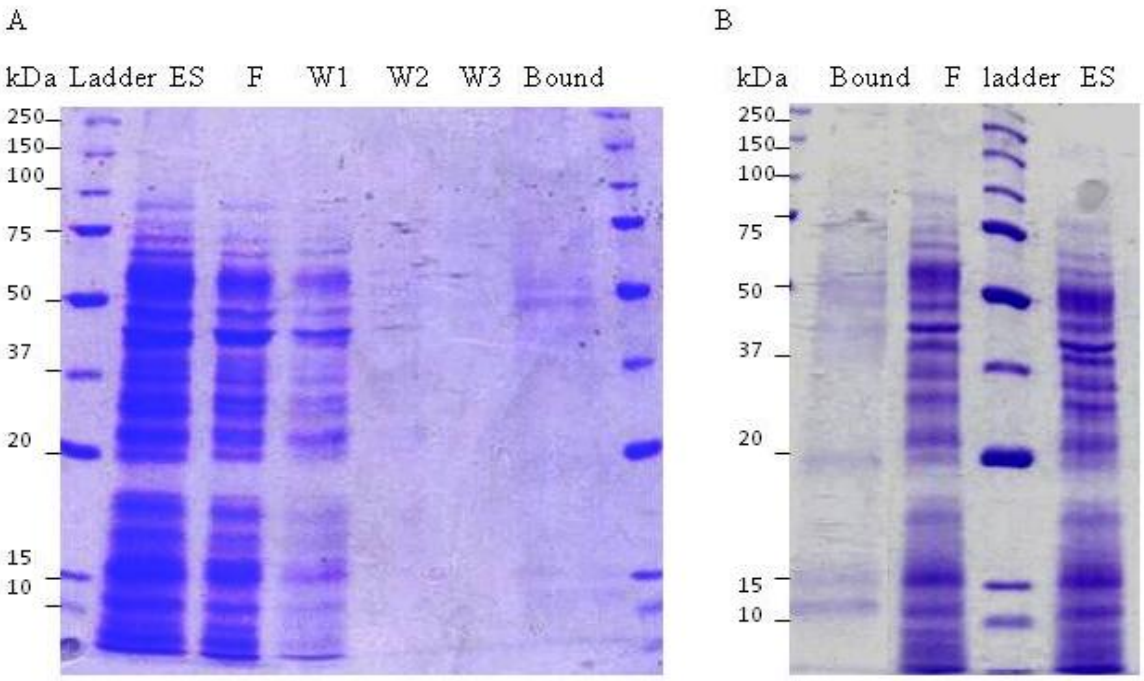


Figure 5.28: SDS-PAGE analysis of MGL-purified *T.spiralis* ES proteins. *T.spiralis* secretory proteins were applied to an MGL-conjugated column and the fractions obtained after elution were analysed by SDS-PAGE. (A) SDS-PAGE of all the fractions obtained: F (Flow through), W1,2 and 3 (column washes with loading buffer) and Bound (fraction eluted from the column). (B) SDS-PAGE of Bound and Flow through fractions as well as ES for band comparison. Product sizes were determined by the protein ladder annotated with sizes in kDa.

In conjunction to MGL-specific purification of ES proteins, competition assays were carried out to determine the specificity and affinity of ES components to immobilised MGL (as described by Quesenberry and Drickamer, 1992 and Lobst and Drickamer, 1996). The binding affinity of ES components to an immobilised fragment of the MGL receptor was determined by competition of increasing concentrations of ES with radioiodinated galactose-BSA (Figure 5.29). The results obtained revealed a high affinity of ES proteins for MGL that was comparable to the galactose control.

In order to confirm that the activity detected in MGL-purified ES fractions was due to GalNAc residues, all the fractions obtained from the MGL column were resolved by SDS-PAGE and then blotted for ^{125}I-labelled MGL (Figure 5.30). This was carried out by K. Drickamer as described in Coombs *et al*, 2005. Indeed, ^{125}I-MGL was found to bind to all the fractions with varying affinity. Flow-through and Wash fractions appeared to be more enriched in GalNAc residues that bound ^{125}I-MGL as band intensity was higher in lanes loaded with these fractions. ^{125}I-MGL binding was also detected in the Eluted (or Bound-ES) fractions confirming the presence of GalNAc residues on glycoproteins retained by the MGL-column.

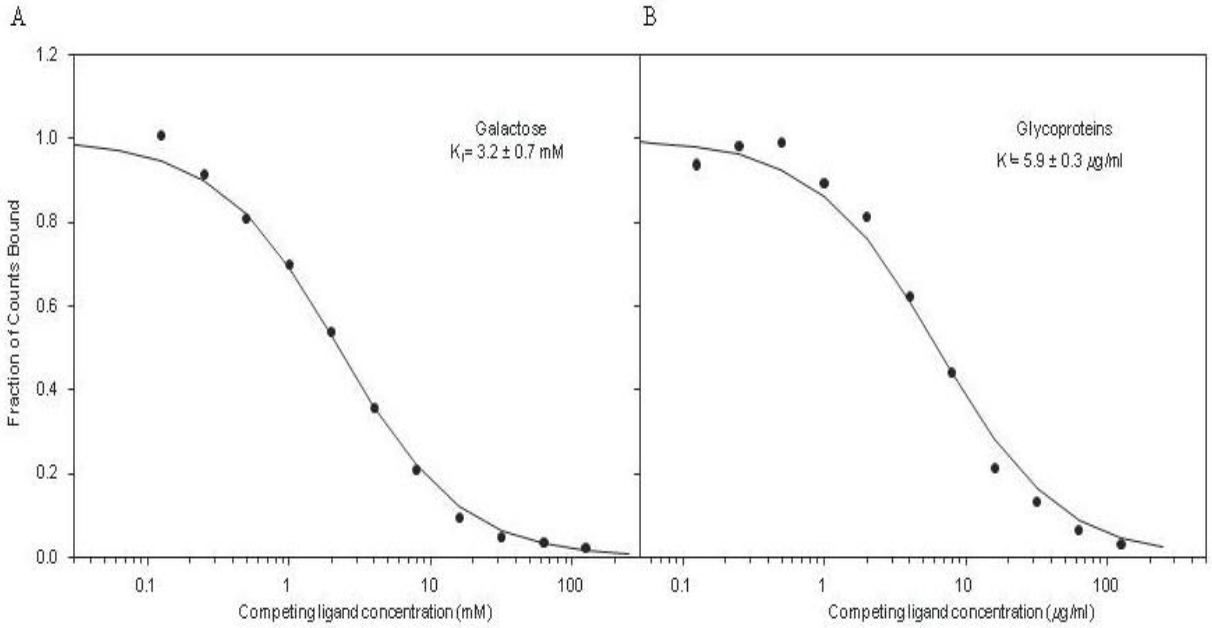


Figure 5.29: Binding competition assays to characterise *T.spiralis* glycoprotein binding to recombinant MGL. MGL extracellular domain was immobilised on wells and probed with ^{125}I-Galactose-BSA in the presence of varying concentrations of ligands. (A) Competition with varying concentrations of the MGL ligand Galactose. (B) Competition with varying concentrations of *T.spiralis* secretory proteins. (Kurt Drickamer)

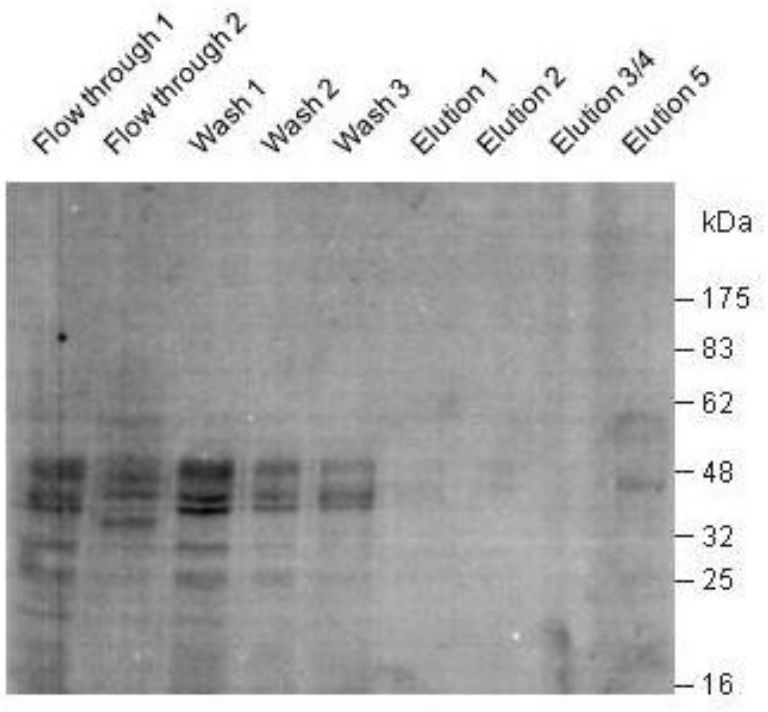


Figure 5.30: SDS-PAGE and blot analysis of *T.spiralis* ES fractionated on human MGL. *T.spiralis* secretory products were applied to an MGL-conjugated column and the fractions obtained after elution were analysed by SDS-PAGE and blotted with ^{125}I-MGL. Fractions resolved are annotated on the blot. Elutions 1,2,3/4 and 5correspond to the 'Bound' fractions. Product sizes were determined by the protein ladder annotated with sizes in kDa (right). (Kurt Drickamer, Adrián Quintero-Martínez)

5.3 Discussion

Macrophages are important innate immune cells known to express a wide range of surface receptors capable of interacting with natural and altered-self host components as well as a range of microorganisms. Following recognition the signalling that ensues contributes to macrophage activation which can directly or indirectly affect host defence effector mechanisms to pathogens by linking innate and adaptive immunity. In general, the sequence of events triggered by pathogen invasion is considered an inflammatory response aiming to kill or expel the invading organism. However, helminth parasites have evolved to survive in hostile host environments by evading these responses and this has been shown to involve recruitment of AAMΦs which are stimulated optimally in the context of a Th2 cytokine environment (Kreider *et al*, 2007). This chapter focused on the effects induced by the parasitic nematode *Trichinella spiralis* on macrophage effector functions. In particular, the parasite secreted proteins (ES) were used to stimulate macrophages and the effects on nitric oxide release, arginase I activity and cytokine release investigated. In addition, bearing in mind that *T.spiralis* ES contains nucleotide metabolising activity, the impact on macrophage purinergic signalling was also investigated.

T.spiralis ES induced a concentration-dependent NO release from BMMΦ and AAMΦ that was lower than the release observed from CAMΦ. This is consistent with studies carried out with *F.hepatica* secretory products which similarly showed that the levels of NO released from monocyte-derived macrophages were not as high as the levels released from LPS-stimulated macrophages (Flynn and Mulcahy, 2008). In order to investigate if the concentration of the activating stimulus affected the macrophage response to ES, ES-induced effects were measured at two different concentrations of LPS or IL4 in CAMΦ and AAMΦ, respectively. No difference in the response elicited by ES was observed. In fact, the minimal effect of ES on CAMΦ NO release

was found to be insignificant. Interestingly, in studies carried out with the macrophage cell line RAW264.7, *T.spiralis* ES was found to inhibit LPS-induced NOS2 via mRNA down-regulation, however, the authors have not shown a correlation with NO release levels (Bian *et al*, 2005).

Arginase I activity was also increased in BMMΦ and CAMΦ by high ES concentrations but this effect was a lot more significant at the concentration of 100μg/ml which is higher than ES concentrations encountered *in vivo*. Nevertheless, arginase I activity levels were never as high as basal AAMΦ levels. Furthermore, basal AAMΦ arginase I activity was not significantly affected by ES which might be due to the fact that arginase I activity levels are maximal and further enhancement in activity is either not possible or not detectable by the *in vitro* assays used. A number of studies on the effects of parasite products have shown that arginase I up-regulation is a common characteristic of macrophage manipulation by parasites. In guinea pig alveolar macrophages arginase I levels were found to be increased by *T.spiralis* as well as *T.cruzi* infection but no effect on NO release was detected (Dzik *et al*, 2004). Cuervo and colleagues also showed that *T.cruzi* infection caused arginase I up-regulation in heart-infiltrating macrophages (Cuervo *et al*, 2008). Increases in both arginase I and YmI have been reported in mouse peritoneal macrophages treated with LNFPIII from schistosome egg antigen (SEA) which did not seem to be dependent on the presence of IL-4 or IL-13 (Atochina *et al*, 2008). In addition, Omega-1 from SEA was also found to be a potent Th2 inducer in a study with human monocyte-derived dendritic cells (Everts *et al*, 2009).

Both NO release levels and arginase I activity levels were found to be increased by *T.spiralis* ES in this study which can be attributed to the fact that, the immune response switches from a Th1 pro-inflammatory response to a Th2 protective response, as NO can act as an effector against parasites. The increase in arginase I ultimately leads to depletion of L-arginine which,

being an iNOS substrate, may eventually shift macrophage phenotype away from classical activation by disabling iNOS activity. It is known that a Th2 response is required to expel gastrointestinal nematodes (Jenkins and Allen, 2009). Specifically, in the case of *T.spiralis* infection it has been reported that the Th2 response and, in particular IL-4, is necessary for parasite expulsion (Garside *et al*, 2000). In fact, mice deficient in IL-4R or lacking IL-13 showed a reduction in parasite expulsion (Scales *et al*, 2007). Therefore, it is conceivable that Th1 responses synergise with Th2 responses to kill parasites.

The involvement of the Th2 arm of the immune response has also been reported in *N.brasiliensis* infection where AAMΦ recruitment was detected in mouse lungs as early as 4 days post-infection and, although initially dependent on Th2 cytokines IL-4 and IL-13, appeared to be influenced by additional factors developing in the pulmonary environment by parasite infection (Reece *et al*, 2006; Siracusa *et al*, 2008). AAMΦs have also been detected in *Brugia malayi* infection and this was found to result from the synergy of *B.malayi*-generated MIF and IL-4 (Prieto-Lafuente *et al*, 2009). The macrophage inhibitory factor (MIF) has been shown to synergise with LPS to upregulate IL-6 and TNF-α production as well, which is another example of parasite products inducing both pro- and anti-inflammatory responses in the host (Vermeire *et al*, 2008). Similarly in this study, *T.spiralis* ES was found to elicit a significant cytokine release from BMMΦ and AAMΦ at the concentration selected for *in vitro* experiments. The TNF-α levels induced in BMMΦ were not detected in AAMΦ activated with high levels of IL4 suggesting that the presence of IL-4 limited pro-inflammatory cytokine induction by ES. This induction of Th1 cytokines by *T.spiralis* ES even in the AAMΦ subset is in agreement with published work by Ishikawa and colleagues that reports a role for the Th1 lymphocyte subset in early phases of *T.spiralis* infection (Ishikawa *et al*, 1998). In addition, *T.spiralis* ES has been

shown to induce IL-6 release in peritoneal macrophages *in vitro* (Gruden-Movsesijan and Milosavljevic, 2006). In the current study, IL-10 release was also induced by *T.spiralis* ES in BMMΦ and AAMΦ and were similar to CAMΦ background levels, an effect that was not observed with TNF-α and IL-6 levels which never reached CAMΦ levels. Consistent with this, Van der Kleij and colleagues have reported that LDN-DF (difucosylated LacDiNac) from schistosome eggs induced IL-6, TNF-α and IL-10 production by PBMCs (Van der Kleij *et al*, 2002). Similar to NO release, basal CAMΦ cytokine levels were not affected by ES at either concentration of LPS used. The observation that parasite products can induce both Th1 and Th2 responses was also made by Dissanayake and colleagues in studies using *T.crassiceps* glycans (Dissanayake *et al*, 2004). The authors suggested a two step mechanism for induction of the Th2 response by *T.crassiceps* whereby glycans are recognized by macrophage TLR4 resulting in IL6 release which subsequently aids in producing a Th2 cytokine environment, possibly by blocking Th1 cells and promoting differentiation of IL-4 producing T cells.

In addition to the ability of *T.spiralis* secreted products to generate both Th1 and Th2 responses, its secreted proteins can potentially manipulate host danger signals to evade any response aimed to eliminate the parasite. Specifically, it is known that the enzymatic activity of *T.spiralis* secreted proteins can interfere with purinergic signalling by altering extracellular nucleotide type and concentration. In Chapter 4 the effects of nucleotides on CAMΦ effector functions were established showing a general trend where the presence of adenosine, either by administration or by degradation of adenine nucleotides by macrophage ecto-nucleotidases, resulted in enhancement of anti-inflammatory functions such as arginase I activity and IL-10 release and inhibition of pro-inflammatory cytokine and NO release. In the presence of ES the effects of nucleotides on CAMΦ NO and cytokine release were more pronounced as the

combination of ES enzymes with macrophage ecto-nucleotidases resulted in more efficient conversion to adenosine. Since ES had no effect on CAMΦ basal cytokine levels, the effects of nucleotide metabolising enzymes were clear as the only differences in purinergic responses were solely due to manipulation of the extracellular nucleotide pool. In this chapter ES was found to induce NO and cytokine release in BMMΦ and AAMΦ so the effect of nucleotides on this induction was also investigated. Interestingly, the presence of adenosine reversed ES-induced NO, IL-6 and TNF-α release detected in BMMΦ and AAMΦ while enhancing IL-10 release, which further supports its anti-inflammatory role in the context of parasite infection. Furthermore, arginase I activity was also enhanced by hydrolysis of nucleotides to adenosine by ES enzymes producing an additive increase in arginase I activity in BMMΦ and CAMΦ. Therefore, ES-induced effects leading to a pro-inflammatory phenotype were inhibited by generation of adenosine in the presence of ES while anti-inflammatory responses were enhanced.

The generation of adenosine by *T.spiralis* ES enzymes has been attributed to a nucleotidase that has unique catalytic properties in that it can convert ADP to AMP and AMP to adenosine (Gounaris *et al,* 2004). This enzyme, 5`-nucleotidase (5`NT), has been recombinantly expressed in our lab so it was used to determine if nucleotide manipulation by *T.spiralis* was indeed due to 5`NT. Since BMMΦ and AAMΦ displayed effector functions that were only induced in the presence of ES, 5`NT-induced effects were only investigated in CAMΦ and indeed the manipulation of nucleotide-induced effects observed with ES was mirrored by the action of the recombinant 5`NT. The only difference was observed when ATP was present due to the fact that this nucleotide is not metabolised by 5`NT in ES, but it is possible that other enzymes in ES are capable of metabolising ATP directly. Arginase I activity in CAMΦ and AAMΦ was enhanced by incubating nucleotides with 5`NT in a similar manner to ES, once again

confirming that when nucleotides are in culture with *T.spiralis* ES, macrophage effector functions are dictated by generation of adenosine.

Adenosine mediates its anti-inflammatory effects predominantly by downstream signalling involving cAMP elevation as shown in the previous chapter (Chapter 4). Combining this result with the observation that adenosine inhibited ES-induced effects, studies were carried out to determine if cAMP was involved in ES-induced effects and this was then extended to determine involvement of other intracellular signalling pathways. Interestingly, cAMP elevation by exogenous agents like forskolin had similar effects to adenosine in that NO, IL-6 and TNF-α release elicited by ES were inhibited while IL-10 release was further enhanced. Therefore, cAMP elevation induced by adenosine is probably the major event responsible for dampening the ES-induced responses. Taking into account that this could also be a result of indirect effects on PLC signalling, this was the downstream pathway investigated next. Inhibition of PLC also inhibited ES-induced NO, IL-6 and TNF-α release, although the reversal obtained for TNF-α release was not as clear as that obtained for NO and IL-6 release. On the other hand, IL-10 release was not affected by PLC inhibition indicating that cAMP is a major signalling component upstream of IL-10 release. As opposed to IL-10, TNF-α and IL6 production was found to be more reliant on the PLC pathway in bacterial ligand-induced APC activation via TLR2 and 4 (Aki *et al*, 2008).

The involvement of other signalling components such as PI3 kinase and the MEK1/2 pathway were also determined as well as the effect of inhibiting NF-κB. ES-induced NO release was more dramatically inhibited by blocking PI3 kinase activity when compared to blocking MEK1/2 and NF-κB. This could be attributed to the fact that PI3 kinase is higher up in the signalling cascade so blocking the pathway at an initial stage abolishes effects more efficiently than when blocking PLC activation and cAMP generation. Conversely, inhibition of MEK1/2

may not have profound effects as other MAP kinases may be used as alternative pathways for signals to reach the nucleus due to the redundancy of intracellular signalling pathways.

A study with *T.crassiceps* glycans has shown that LewisX galactose residues increased IFN-γ via NF-κB induction following TLR activation of mouse peritoneal macrophages (Dissanayake and Shahin, 2007). Despite the similarity in APC activation observed by parasite glycans to LPS activation, it is generally accepted that the pathways downstream are altered by glycans so that they no longer follow the canonical TLR signalling, resulting in the generation of APCs with an immature phenotype that produce decreased levels of pro-inflammatory cytokines (Harn *et al*, 2009). Specifically, a phosphorylcholine-containing glycoprotein ES-62 from the nematode *A.viteae* that induces a Th2 polarised immune response has been shown to act via TLR4 and in doing so it interferes with APC activation by a variety of TLR ligands (Goodridge *et al*, 2005). LNFPIII was shown to activate bone marrow derived dendritic cells to a Th2 inducing phenotype via a TLR4-dependent mechanism (Thomas *et al*, 2003). When comparing LPS with LNFPIII (SEA) signalling, the LPS-induced activation of NF-κB via MAPK/JNK/p38 is replaced by ERK activation and NF-κB activation is not accompanied by IκB degradation (Harn *et al*, 2009). DC-SIGN activation, a major event in SEA manipulation of DCs, was shown to act via Raf-1 kinase to activate NF-κB (Gringhuis *et al*, 2007). Binding of *H.pylori* LewisX, another DC-SIGN ligand, on gastric DCs has been shown to block Th1 development (Bergman *et al*, 2004). Recently, van Stijn and colleagues reported that DC-SIGN acts as co-receptor for TLR4 in SEA-induced effects which may explain the mode of manipulation of TLR signalling (Van Stijn *et al*, 2010). However, SEA-induced effects have also been shown to be independent of TLR activation (Kane *et al*, 2008). This may indicate that the TLR pathway is not necessary for SEA-induced effects but, if active, SEA interactions act to inactivate it in order to promote a

Th2 phenotype. Finally, *S.mansoni* larvae were shown to inhibit cytokine production by macrophages via a TLR4 and MyD88-dependent mechanism and this was suggested to be due to the presence of mannose receptor ligands found in SEA (Jenkins *et al*, 2005). With relevance to *T.spiralis* secreted products in this study, the fact that macrophage phenotype was shifted to an alternative mode of activation by ES suggested the possibility that macrophages could respond to ES via mannose receptor ligation since this is a marker of alternative activation. However, the effects of *T.spiralis* secreted proteins on macrophage effector functions were not found to be due to mannose structures in ES, as treatment of macrophages with D-mannose did not appear to activate them in terms of any of the effector functions tested. In fact, another study has shown that NO release induced by *T.spiralis* in peritoneal macrophages was only partly due to the MR (Gruden-Movsesijan and Milosavljevic, 2006). Similarly, deSchoolmeester and colleagues have reported that cytokine released from BMMΦ in response to *T.muris* ES was also partly due to MR binding (deSchoolmeester *et al*, 2009).

T.spiralis ES is known to contain heavily glycosylated proteins and analysis of these glycoproteins has revealed N- and O-linked structures of oligosaccharides, including mannose (Reason *et al*, 1994; Gruden-Movsesijan *et al*, 2002). Having eliminated mannose as a possible trigger of ES-induced effects, the hypothesis that other glycans may be involved in macrophage activation was investigated. In addition, in order to test the possibility that ES glycoproteins mediated their effects via the protein component, any enzyme activity was deactivated prior to macrophage stimulation by heat denaturation. In general, heat-inactivation partly restored some of the effector functions induced by ES, however, this was inconsistent for NO, TNF-α and IL-6 release while arginase I activity did not seem to be significantly affected. On the other hand, IL-10 release seemed to be more consistently reversed by heat inactivation of ES suggesting once

again that the mechanism of IL-10 regulation is not the same as for NO, TNF-α and IL-6. The fact that heat-inactivation had a partial effect on ES-induced responses suggests that the protein component is important but not essential. This points to a structural role for the protein part as denaturation probably led to protein unfolding that interfered with glycan positioning, an explanation also supported by the fact that some ES activity was retained after heating probably as a result of less efficient glycan binding. In order to further confirm that the glycan component of ES was responsible for activating macrophages, secreted products were chemically deglycosylated to remove N- and O-linked carbohydrates. As expected, ES-induced effects were completely reversed when the glycans were no longer present. Interestingly, in a study published by Flynn and Mulcahy using *F.hepatica* ES, the increase in arginase I induced in human PBMC-derived macrophages was also only abolished by removal of glycans by oxidation (Flynn and Mulcahy, 2008).

Glycans mediate their effects following recognition by APCs via C-type lectin receptors (van Vliet *et al*, 2008, Geijtenbeek and Gringhuis, 2009). In *S.mansoni*-treated immature human monocyte derived DCs, SEA was found to be localized in compartments for MHC II presentation following internalization through the C-type lectins DC-SIGN, MGL (macrophage galactose lectin) and the mannose receptor and was suggested to be important in regulation of TLR-induced DC activation (van Liempt *et al*, 2007). This was determined by van Liempt and colleagues by blocking DC surface lectins with carbohydrates known to bind and activate specific lectins. A variation of this experiment was carried out in this study in an attempt to block *T.spiralis* ES-induced effects and to determine the receptor via which these effects were elicited. Confirmation of C-type lectin involvement was first obtained by the fact that EGTA abolished all responses triggered by ES, since EGTA is a Ca^{2+} chelator that effectively removes calcium ions from the

extracellular milieu thus destabilising lectins which require this ion for structural integrity (Drickamer, 1999). Mannan appeared to enhance ES-induced effects instead of blocking them which may suggest synergy of DC-SIGN or MR with the lectin actually involved in ES glycan binding. This may be explained by published work referred to earlier in this section reporting partial involvement of the MR in *T.spiralis*-induced NO and cytokine release (Gruden-Movsesijan and Milosavljevic, 2006). Only GalNAc significantly inhibited all ES-induced effects on macrophage effector functions pointing to the involvement of the macrophage galactose lectin (MGL) in recognition of ES glycoproteins. The fact that ES responses were not completely abolished by blocking MGL may be a result of involvement of more than one C-type lectin in glycan recognition or may be due to lectin redundancy allowing for recognition to occur even if MGL is not available for binding. Indeed, Saunders and colleagues have reported that SIGNR1 (a mouse paralogue of DC-SIGN) binding to SEA was redundant since in the knockout was still capable of SEA recognition (Saunders *et al*, 2009). Interestingly, MGL has been characterised by Raes and colleagues as a novel marker of alternative macrophage activation by parasites which becomes relevant in the context of *T.spiralis*-induced macrophage alternative activation in this study (Raes *et al*, 2005). Therefore, AAMΦ would be expected to respond better to ES glycans due to the fact that they already have higher MGL levels as a result of IL4-induced up-regulation. However, this was not so clear and may be due to insufficient levels of IL-4 to induce MGL up-regulation *in vitro*. In addition, the lack of Th2-independent signals in systems of *in vitro* studies that activate macrophages with IL-4 and IL-13 produces characteristics that are less pronounced than those elicited during a natural infection.

Characterisation of MGL as the lectin responsible for ES glycan activation of macrophages was extended by purifying ES fractions on a MGL-conjugated column provided by

Prof. K. Drickamer and using these fractions to detect those positive for NO and cytokine release from macrophages. Indeed, bound fractions were capable of activating macrophage responses, however, some activity was still present in unbound fractions. This could be due to low affinity binding of GalNAc to the column-conjugated MGL or a result of recognition of other ES glycans by other lectins. In fact, when trying to block this activity by pre-incubation of active fractions with GalNAc, activity was not always completely inhibited suggesting that other glycans may be involved. However, this could also be attributed to the fact that MGL immobilised on a column is not as efficient at binding all GalNAc-positive glycoproteins due to steric hindrance which is avoided *in vivo* due to mobility of lectins on the macrophage surface. In addition, MGL activation on macrophages may be additive so that GalNAc from neighbouring glycoproteins can come together to cluster MGL molecules to produce robust responses. Indeed, studies have shown that binding of multiple glycan branches to multiple recognition domains of receptor oligomers enhances interactions as a result of membrane receptor geometry (Coombs *et al*, 2009). The importance of multivalency of glycan recognition can be seen by the fact that incubation of macrophages with carbohydrates was not sufficient to activate them, an observation also made by van der Kleij and colleagues who reported that free synthetic oligosaccharides were unable to stimulate monocytes to produce cytokines (Van der Kleij *et al*, 2005). Another factor that needs to be taken into account is that, the MGL available for *in vitro* studies carried out was of human origin that may explain differences in specificities leading to inefficient isolation of GalNAc-containing residues that the mouse MGL would respond to (Coombs *et al*, 2006). It is known that there are differences between structure and specificity of mouse and human C-type lectins. In fact, murine cells are known to express MGL-1 and MGL-2 whose glycan specificities have been characterised and are different (Singh *et al*, 2009). Furthermore, carbohydrate profiling of human MGL binding specificity showed that it more closely resembled MGL-2 (Van Vliet *et al*, 2005).

In addition, Singh and colleagues suggest that cellular MGL has better affinity for its ligands than soluble MGL, suggesting that the efficiency of purification of MGL-specific glycoproteins may not have been good enough in the current study (Singh *et al*, 2009). When MGL was immobilised on plates and used in competition assays to determine the specificity and affinity of ES, the affinity was found to be comparable to galactose, which is the natural ligand of MGL used in *in vitro* competition assays (K.Drickamer personal communication) (Powelsland *et al*, 2010).

The glycoproteins obtained from MGL purification were analysed by SDS-PAGE but there were no specific bands that only came up in the MGL-eluted fractions except for the 37kDa, which was probably a result of some conjugated MGL being pulled down with the bound glycoprotein on elution. The SDS-PAGE analysis was further used to confirm the presence of GalNAc-positive fractions by blotting for ^{125}I-labelled MGL (K. Drickamer) and ^{125}I-MGL did indeed bind to all the fractions with flow-through and wash fractions binding ^{125}I-MGL with higher affinity. The fact that GalNAc residues appear to be recognized by MGL suggests that *T.spiralis* glycoproteins have exposed GalNAc perhaps from tetra-antennary complex chains that lack terminal sugars. These sugars have been characterised to decorate all *T.spiralis* glycoproteins, namely tyvelose (Reason *et al*, 1994). The fact that MGL-column purification did not produce clear GalNAc eluted fractions may also be explained by the existence of this unique *T.spiralis* capping sugar. It is conceivable that it is in fact tyvelose that is recognized by macrophage MGL and that no GalNAc residues are actually exposed. Therefore, effects elicited by ES tyvelose are blocked by GalNAc because of competition for receptor binding but when the receptor is incubated with ES on a column the interactions resulting under these conditions are not strong enough to purify all active glycoproteins.

Parasites have evolved to modulate the host immune response to ensure long-term survival and they do so in many ways. Macrophages are an important link between the innate and acquired immune response as they are capable of detecting the presence of invading pathogens on the basis of the composition of carbohydrate moieties expressed. *T.spiralis* secreted proteins used in this study were found to induce pro-and anti-inflammatory cytokine responses in macrophages which indicates a role for these structures in stimulating innate immunity, besides the effects resulting from manipulation of purinergic signalling. Therefore, *T.spiralis* has developed multiple mechanisms of evading host defences to invasion protecting itself long enough to allow establishment of a long-term infection.

Chapter 6

Purinergic signalling and the effect of *T.spiralis* secreted proteins on T cell effector functions *in vitro*

6.1 Introduction

T cell effector functions ranging from pro- to anti- inflammatory to regulatory are dependent on the mode of activation following T cell receptor (TCR) ligation as a result of costimulation, source of antigen and local cytokine environment. Naïve CD4+ T cell activation leads to proliferation and differentiation into T helper (Th) cells which are subcategorized into Th1 and Th2 based on their cytokine profile and immunomodulatory effects (O'Garra, 1998). Th1 cells, which arise in the presence of IL-12, IL-18 and IFN-γ, are generally involved in pro-inflammatory cell-mediated reactions as they produce IFN-γ and TNF-β. On the other hand, Th2 cells develop in the presence of IL-4 and produce IL-4, IL-5, IL-10 and IL-13. It is evident that differentiation into Th1 or Th2 will determine the outcome of an immune response as the resulting cytokine environment gives rise to pro-inflammatory or anti-inflammatory immune cell activation, respectively. In addition to the cytokine influence on T cell differentiation another important factor is costimulation achieved through antigen presentation by APCs. TCR activation in response to antigen recognition requires specific costimulatory molecules such as CD28 expressed on APCs, in the absence of which TCR activation leads to T cell anergy (Schwartz, 2003).

Regulatory T (Treg) cells are emerging as an important regulator of T cell differentiation and proliferation as well as activation of other immune cells such as APCs. Regulation is either contact-dependent or is due to modulation of the local cytokine environment. Another means of control used by Treg cells is an indirect mechanism involving metabolism of extracellular nucleotides (Bynoe and Viret, 2008). Recent characterisation of T cell activation markers revealed that, the surface expression of CD73, which is responsible for catabolism of AMP to adenosine, is a novel marker of Treg. activation as it is up-regulated in CD4+CD25+Foxp3+

Treg. cells (Deaglio *et al*, 2007). In addition, it has been reported that Foxp3 in human spleen and lymph CD4+ T cells drives expression of CD39, an ecto-nucleotidase responsible for conversion of ATP to ADP and ADP to AMP (Borsellino *et al*, 2007; Eckle *et al*, 2007). The up-regulated expression of these ecto-nucleotidases on Treg. cells means that these cells have the capacity to hydrolyse extracellular levels of ATP thus dampening its pro-inflammatory effects. In addition to the action of CD39 which leads to removal of ATP, CD73 activity leads to generation of adenosine which is known to have anti-inflammatory effects (Lappas *et al*, 2005; Kobie *et al*, 2006).

Helminth infections are known to induce "modified" Th2 responses as a means of improving their survival (Hewitson *et al*, 2009). In the case of the intestinal parasite *Trichuris muris*, increased recruitment of Foxp3+ T cells was detected in the gut of infected mice and was found to be important for protection of the host from parasite driven pathology as well as for providing more efficient parasite expulsion (D'Elia *et al*, 2009). *Trichinella spiralis* infection also involves an intestinal phase and persistence of the parasite during this phase is essential for successful establishment in the host. In addition, *T.spiralis* secreted products (ES) are known to possess nucleotide metabolising activity and thus have the capacity to manipulate host purinergic signalling by generation of extracellular adenosine, much like the mode of action of Treg. ecto-nucleotidases (Gounaris, 2002).

The aims of this study were to investigate purinergic signalling in CD4+ T cells in terms of purinergic receptor expression as well as the effects of extracellular nucleotides on T cell proliferation and cytokine release. The expression of ecto-nucleotidases CD39/CD73 was investigated as the presence of these enzymes on T cells would also influence the outcome of purinergic receptor stimulation. Finally, the direct effects of *T.spiralis* ES on T cell effector functions were investigated prior to determining ES-induced effects on T cell purinergic signalling.

6.2 Results

CD4+ T cells were isolated from Balb/c mouse spleens and the remaining cells, mostly antigen presenting cells (APCs), were irradiated to prevent effector functions like proliferation and cytokine release. T cell proliferation was measured following culture of the isolated CD4+ cell population with the irradiated APCs in the presence of anti-CD3 to trigger TCR activation. The effects induced by extracellular nucleotides on proliferation and cytokine release by T cells were investigated. Molecular characterisation of CD4+ T cells was carried out in terms of purinergic receptor expression and ecto-nucleotidase expression as they both influence purinergic signalling. The effects of *T.spiralis* secreted products (ES) on expression of these two classes of surface expressed proteins was investigated as well as the resulting T cell proliferation and cytokine release in the presence of these products. Finally, an attempt was made at characterising downstream pathways responsible for the effects observed with nucleotides and *T.spiralis* ES.

6.2.1 T cell purinergic signalling

Molecular characterisation of purinergic receptor expression

In order to investigate the effects of purinergic signalling on T cell responses, the purinergic receptor profile of resting splenic CD4+ T cells used in this study was determined by RT-PCR analysis (Figure 6.1). In general, CD4+ T cells express all purinergic receptor transcripts at the molecular level except for the P2Y4R. All P1 receptor transcripts were detected. In order to confirm that the primers used for the RT-PCR analysis of purinergic receptors were capable of detecting transcripts correctly, mRNA extracted from the original splenocyte population was used as a positive control. Indeed, purinergic receptor transcripts of expected sizes were detected with all primers used and PCR products were of similar intensity.

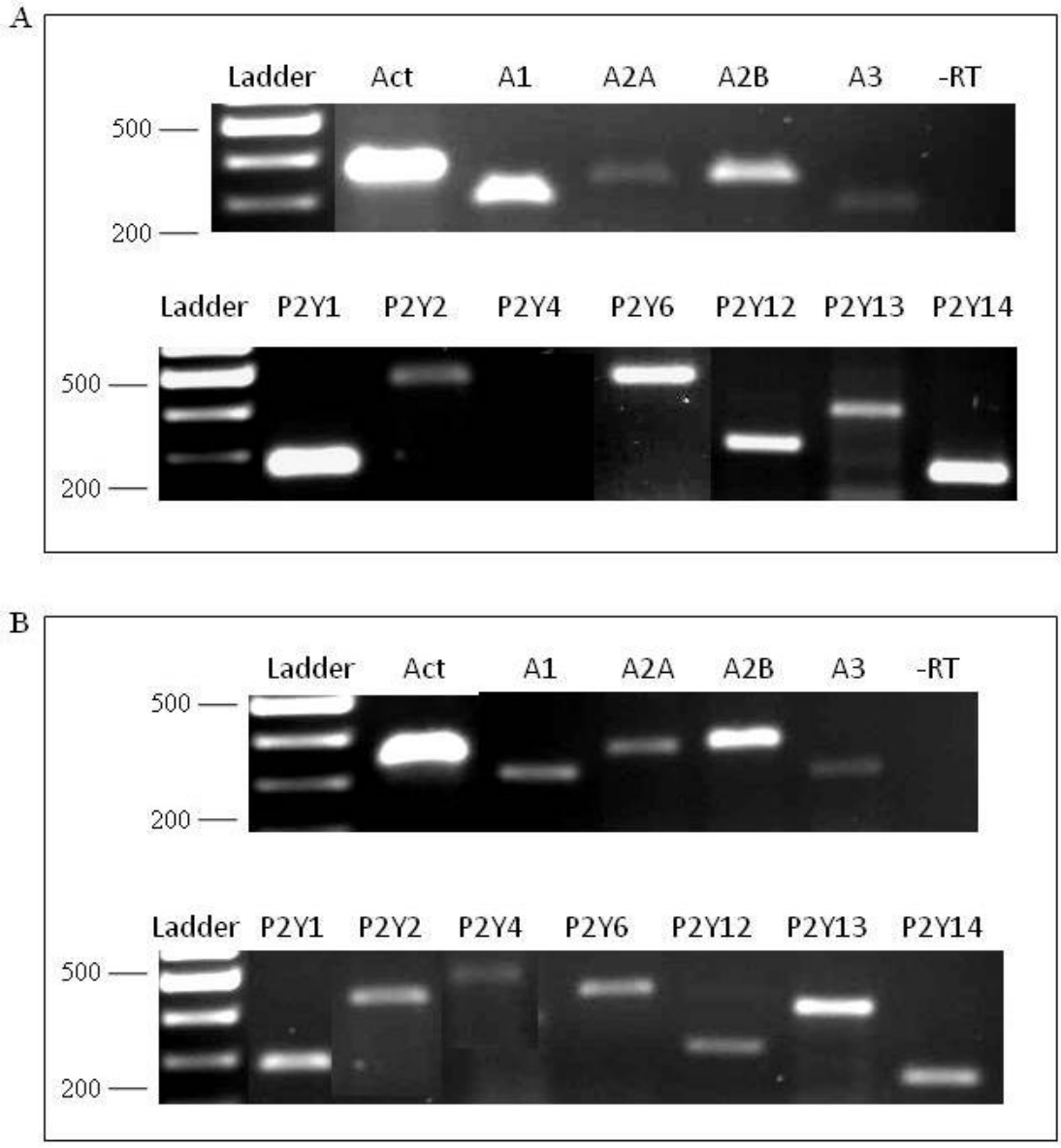


Figure 6.1: Purinergic receptor (PR) transcripts in murine CD4+ T cells and spleen determined using RT-PCR. A) The PR transcripts detected in resting CD4+ T cells purified from mouse spleen and B) PR transcripts from mouse whole spleen. PCR products are shown and product sizes were determined by the ladder shown annotated on the left (in bp). Expected sizes were obtained for all transcripts detected (see Materials and Methods, Table 2.1C). Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

Effect of purinergic signalling on T cell proliferation in vitro

Purinergic signalling-induced effects were first investigated on T cell proliferation *in vitro*. CD4+ T cells isolated from mouse spleen were stimulated with 50μM nucleotides (as determined from trial experiments not shown) and then transferred to anti-CD3 coated plates and co-cultured with irradiated APCs from the same spleen for 4 days. Proliferation was measured by ^{3}H-thymidine incorporation during the final 16 hours of culture and the results obtained presented in Figure 6.2. T cell proliferation was inhibited by all nucleotides. Considering the similarity in nucleotide responses to adenosine it is possible that nucleotide-induced effects were due to their breakdown to adenosine. The fact that, in some cases, nucleotide stimulation resulted in lower levels of proliferation than adenosine was probably due to adenosine uptake by nucleoside transporters, since the nucleoside transporter inhibitor, dipyridamole, was not used in T cell experiments due to interference with this assay (data not shown). For this reason, NECA which is an analogue of adenosine that is not taken up by cells, was used to confirm the involvement of P1R activation.

In order to confirm if the inhibitory effect on proliferation was indeed due to generation of adenosine and P1 receptor activation, P1R agonists were used to examine if these effects were indeed due to P1R activation. P1R agonists also inhibited proliferation except for IB-MECA which acts on A_3 receptors (A_3R) (Figure 6.3A). Inhibition obtained with the A_1 receptor agonist CPA was not as significant as that obtained with adenosine, NECA and the A_{2B} receptor (A_{2B}R) agonist (CGS21680), which points to A_{2A} and A_{2B} receptor involvement (Figure 6.3A). In fact, P1 receptor antagonism showed that the A_{2A}R and A_{2B}R antagonists, ZM241385 and MRS1754 respectively, reversed adenosine-induced effects almost completely (Figure 6.3B). Similarly, the 50% inhibition in proliferation induced by AMP was completely reversed by the A_{2A} and A_{2B}

receptor antagonists (Figure 6.4A) as was the 20% inhibition induced by ADP (Figure 6.4B). These data confirm that nucleotide-induced effects on T cell proliferation were in fact due to P1 receptor activation by adenosine generated from their degradation.

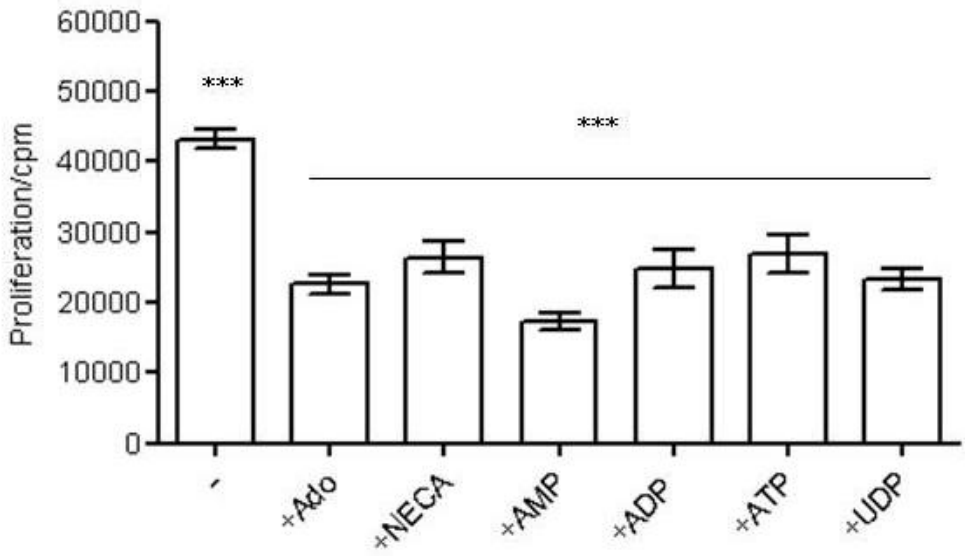


Figure 6.2: The effect of nucleotides on T cell proliferation. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 to induce cell proliferation, measured by means of ^{3}H-thymidine incorporation. The effect of 50μM adenosine, AMP, ADP, ATP, their slowly hydrolysable analogues, inosine and UDP on T cell proliferation was measured after 4 days of culture. Data presented are an average of triplicates $\pm$ SEM from one representative experiment of $n > 3$. * $p < 0.05$, *** $p < 0.001$, ns - not significant ($p > 0.05$) compared to the untreated control.

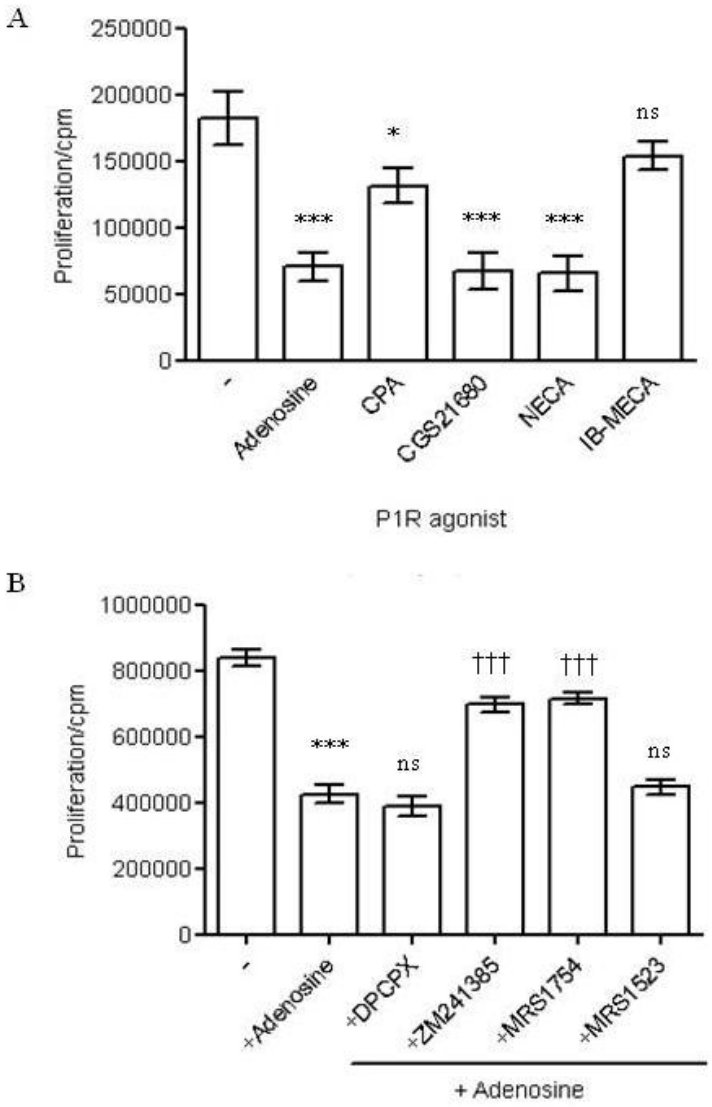


Figure 6.3: Pharmacological characterisation of P1R-mediated effects on T cell proliferation. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 to induce cell proliferation, measured by means of ^{3}H-thymidine incorporation cultured in the presence or absence of P1 receptor agonists (A) or antagonists (B) for 4 days. The following agonists and antagonists (10μM) were added 15 minutes prior to adenosine addition: 50 μM of adenosine, CPA (A_1R agonist), CGS21680 (A_{2A}R agonist), NECA or IB-MECA (A_3R agonist), DPCPX (A_1R antagonist), ZM241385 (A_{2A}R antagonist), MRS1753 (A_{2B}R antagonist) or MRS1523 (A_3R antagonist). The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks/daggers indicate statistical significance compared to the untreated control.* $p< 0.05$, *** $p< 0.001$. (†) is compared to treatment with adenosine only. ††† $p< 0.001$, ns - not significant ($p> 0.05$).

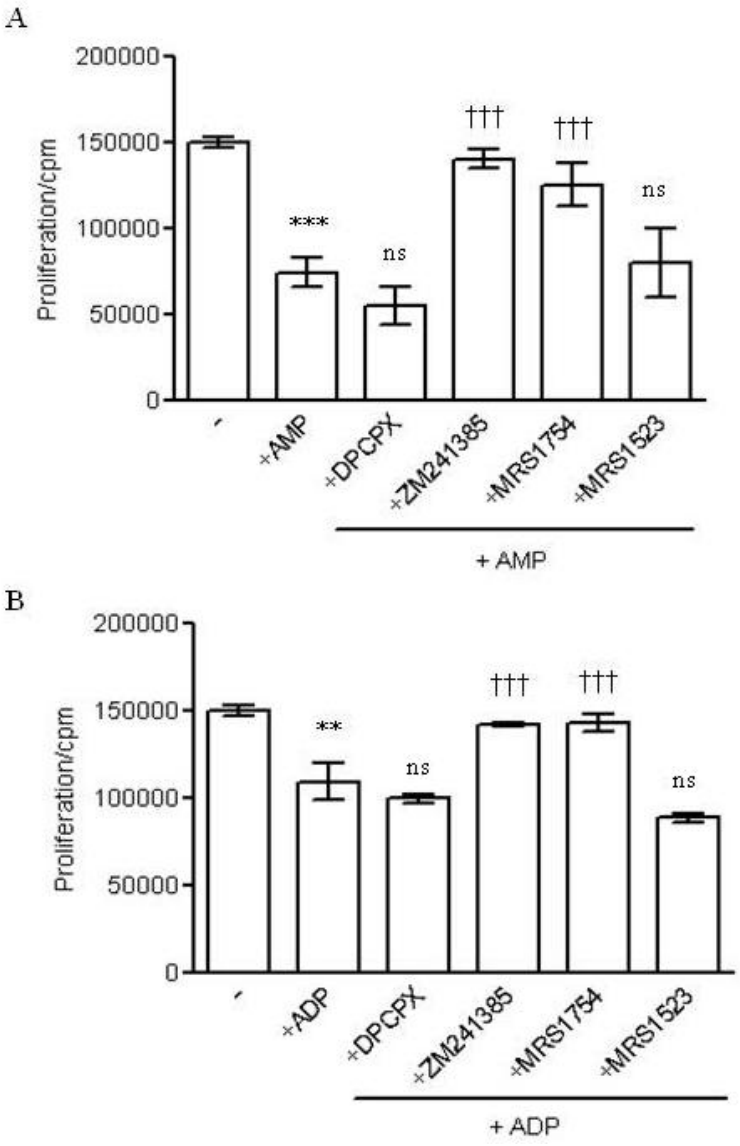


Figure 6.4: Pharmacological characterisation of P1R-mediated effects on T cell proliferation. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 to induce cell proliferation, measured by means of ^{3}H-thymidine incorporation in the presence of P1R antagonists, after 4 days of culture. The following antagonists (10μM) were added 15 minutes prior to addition of 50μM (A) AMP or (B) ADP: DPCPX (A_1R antagonist), ZM241385 (A_{2A}R antagonist), MRS1753 (A_{2B}R antagonist) or MRS1523 (A_3R antagonist). The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks/daggers indicate statistical significance compared to the untreated control. ** $p< 0.01$, *** $p< 0.001$. (†) is compared to treatment with AMP or ADP only. ††† $p< 0.001$, ns - not significant ($p> 0.05$).

In an attempt to determine if adenosine-induced effects were a result of coupling to Gs protein subunits and activation of adenylate cyclase leading to cAMP elevation, known cAMP elevators were administered exogenously to see if they mimicked adenosine-induced effects. Indeed, cAMP elevation also led to inhibition of proliferation and this effect was additive when NECA was combined with rolipram (Figure 6.5A). Investigation of signalling downstream of adenosine-induced activation of P1 receptors showed that only the MEK1/2 inhibitor (U0126) reversed adenosine-induced effects. The PLC and NF-κB inhibitors (U73122 and Bay 11-7082) blocked proliferation completely as did the MEK1/2 inhibitor while the PLC mock inhibitor (U73343) had no effect showing that, the inhibition in proliferation observed with the PLC inhibitor was in fact due to inactivation of PLC and not a compound interference effect (Figure 6.5B).

Collectively, these data show that CD4+ T cells express most of the purinergic receptors and all the P1 receptors which respond to adenosine, either administered or generated in culture by degradation of adenine nucleotides. Furthermore, these data confirm that the effects of AMP and ADP are due to P1R stimulation as P1R antagonists, and specifically of the $A_{2A}R$ and $A_{2B}R$, reversed these effects. Finally, exogenous cAMP elevation was sufficient to mimic adenosine-induced effects showing that adenosine mediates its effects mainly by cAMP elevation. In addition, inhibition of MEK1/2 was also effective in reversing adenosine-induced effects, however, it cannot be concluded that other pathways are not involved since the inhibitors used to test involvement of other pathways produced background effects on proliferation.

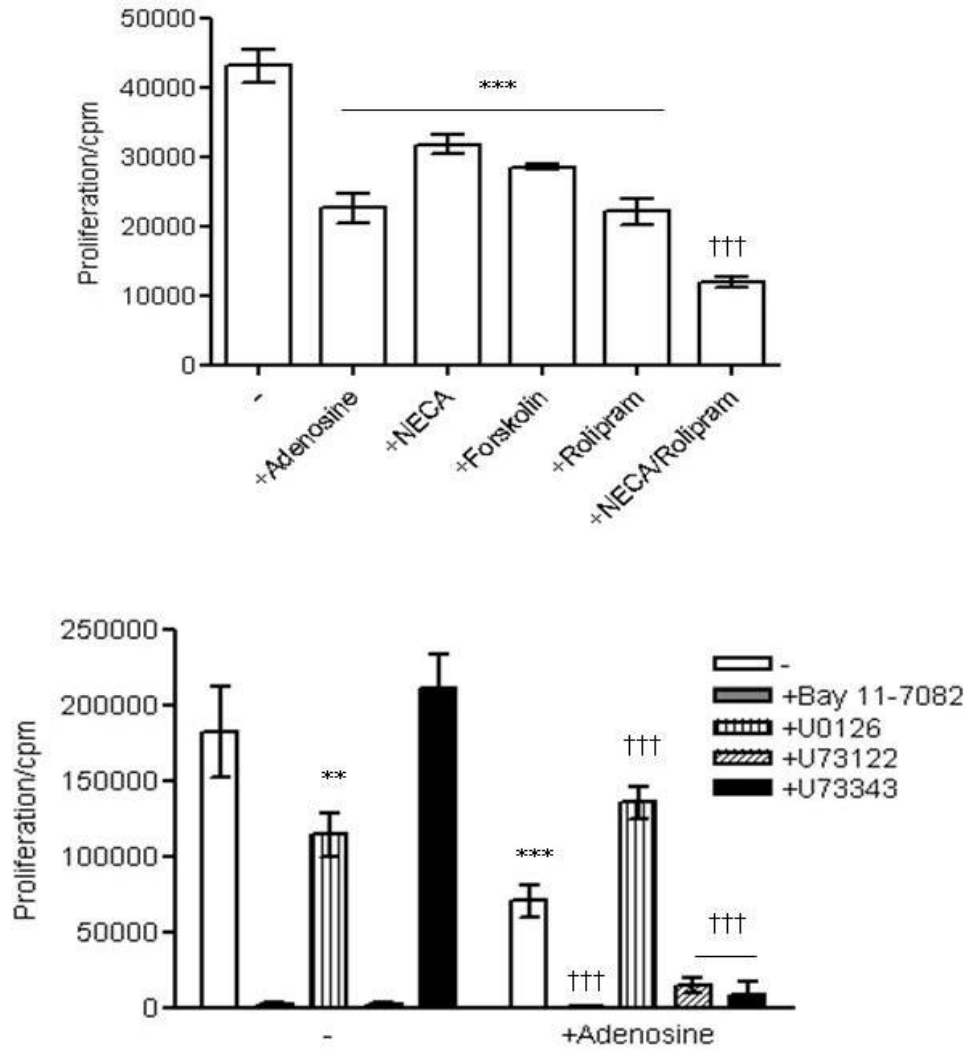


Figure 6.5: The effect of downstream signalling pathways on P1R-mediated effects on T cell proliferation. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 to induce cell proliferation, measured by means of ^{3}H-thymidine incorporation. (A) The effect of the cAMP elevators forskolin (10μM) and rolipram (5μM) on T cell proliferation compared to and combined with P1R agonists adenosine and NECA. (B) The effect of PLC, NF-κB and MEK inhibition on P1R-mediated inhibition of T cell proliferation. The effect of 10μM U73122 (PLC inhibitor), 5μM Bay 11-7082 (NF-κB selective inhibitor**)** and 5μM U0126 (MEK1/2 inhibitor) on adenosine-induced inhibition of T cell proliferation was measured after 4 days of culture. Cells were pre-incubated with the all inhibitors for 15 minutes prior to adenosine addition. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. ** $p< 0.01$, *** $p< 0.001$. (†) is compared to treatment with 50μM (A) NECA or (B) adenosine, only. ††† $p< 0.001$.

Effect of purinergic signalling on cytokine release from T cells

Cytokine release is another important effector function that can determine the outcome of an immune response as it can influence activation of APCs amongst other immune cells. Cytokine release from T cells was similarly affected by nucleotides as in this case all nucleotides inhibited IL-4, IL-10 and IFN-γ release (Figure 6.6). In general, adenosine and NECA inhibited IL-4 and IFN-γ by 50% while in the case of IL-10 inhibition was more pronounced. The similarity of nucleotide-induced effects on cytokine release to adenosine again suggests that nucleotides were hydrolysed and generation of adenosine led to P1R stimulation. As explained previously, the fact that adenosine-induced inhibition of cytokine release was higher with nucleotides than with adenosine was due to adenosine uptake into cells. UDP appeared to inhibit IL-4 and IFN-γ release only (Figure 6.6).

Confirmation of P1R effects on cytokine release was obtained by using P1R agonists which also inhibited cytokine release in general (Figure 6.7). Specifically, IL-4 release was inhibited by all agonists used except for the A_1R and A_3R agonists (CPA and IB-MECA, respectively). IL-10 release was inhibited by all agonists but more significantly by the $A_{2B}R$ agonist (CGS21680), NECA and adenosine. IFN-γ release was inhibited by adenosine, NECA and CGS21680 but not by IB-MECA (Figure 6.7). Therefore, activation of the $A_{2A}R$ and $A_{2B}R$ resulted in inhibition of all cytokines tested. This was further confirmed by the fact that P1R antagonists acting on $A_{2A}R$ (ZM241385) and $A_{2B}R$ (MRS1754) reversed adenosine-induced inhibition of cytokine release (Figure 6.8). In order to confirm the hypothesis that AMP and ADP inhibit cytokine release from T cells by P1R activation as a result of their conversion to adenosine, AMP and ADP were co-administered with P1R antagonists. As it is shown in Figure 6.9, the AMP-induced inhibition of cytokine release from T cells is restored by $A_{2A}R$ and $A_{2B}R$

antagonists. A similar restoration of cytokine release was observed when ADP was co-administered with either the $A_{2A}R$ or the $A_{2B}R$ antagonist (Figure 6.10).

Collectively these data show that, $A_{2A}R$ and $A_{2B}R$ activation is responsible for adenosine-induced inhibition of cytokine release from T cells. Adenine nucleotides inhibit cytokine release from T cells and this is also due to $A_{2A}R$ and $A_{2B}R$ activation by adenosine generated in culture from hydrolysis of these nucleotides. In addition to P1R activation, P2Y6R activation by UDP also results in inhibition of IL-4 and IFN-γ release from T cells.

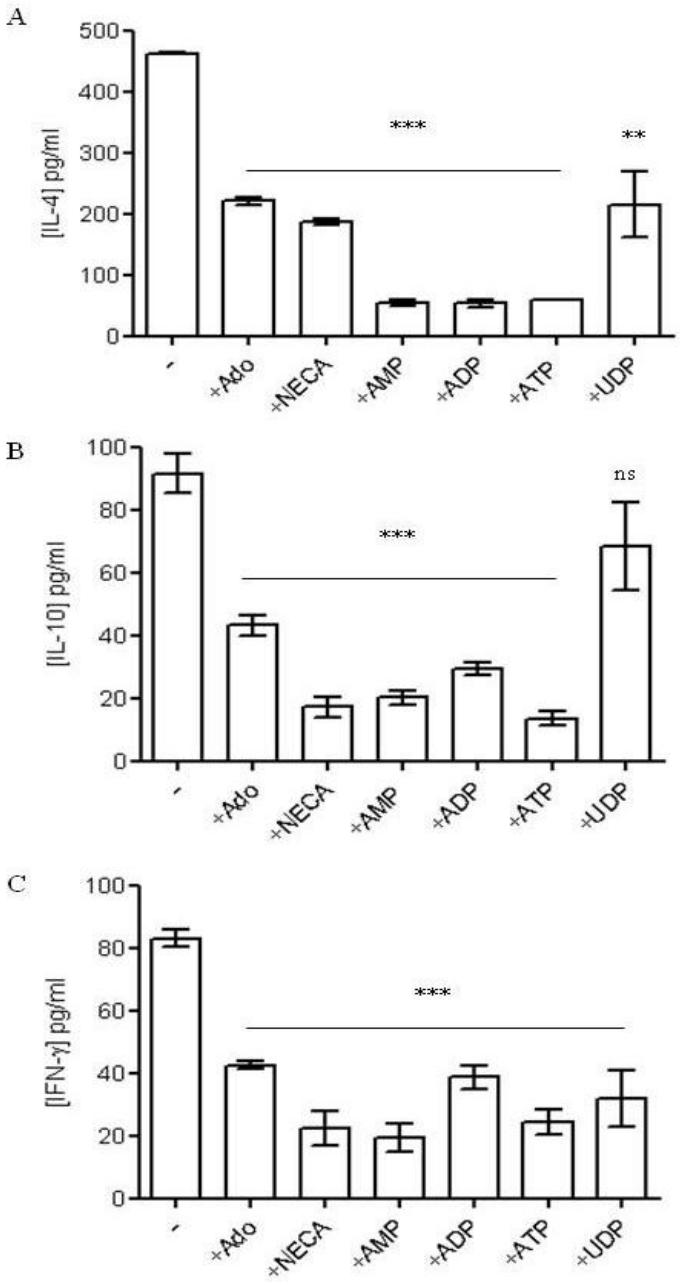


Figure 6.6: The effect of nucleotides on cytokine release from T cells. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 and cytokine release measured after 4 days of culture. The effect of 50μM adenosine, AMP, ADP, inosine and UDP or the slowly-hydrolysable analogues of adenosine, ADP and ATP (NECA, β-ADP, ATP-γ, respectively, on (A) IL-4 release, (B) IL-10 release and (C) IFN-γ release. Cytokine levels are expressed in pg/ml. Data presented are an average of triplicates $\pm$ SEM from one representative experiment of $n > 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns - not significant ($p > 0.05$) compared to the untreated control.

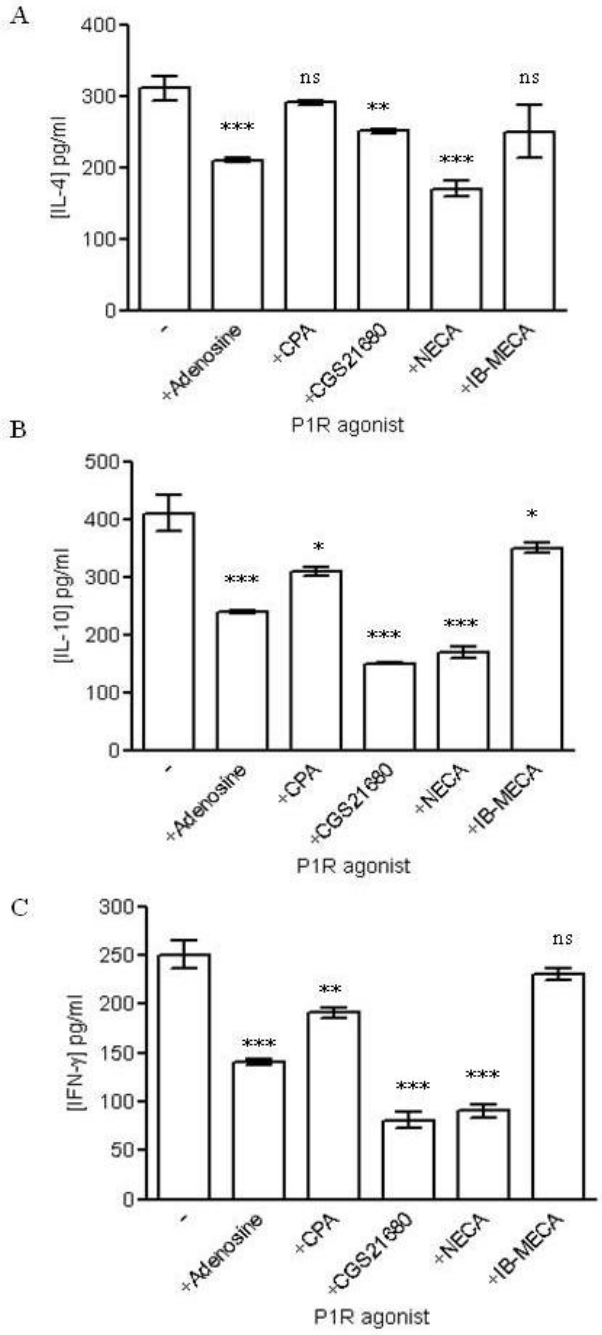


Figure 6.7: Pharmacological characterisation of P1R-mediated effects on cytokine release from T cells. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 and cytokine release measured after 4 days of culture. The effect of 50μM of the following P1R agonists on (A) IL-4 release, (B) IL-10 release and (C) IFN-γ release: 50 μM of adenosine, CPA (A_1R agonist), CGS21680 (A_{2A}R agonist), NECA or IB-MECA (A_3R agonist). Cytokine levels are expressed in pg/ml. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate statistical significance compared to the untreated control.* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$, ns - not significant ($p> 0.05$).

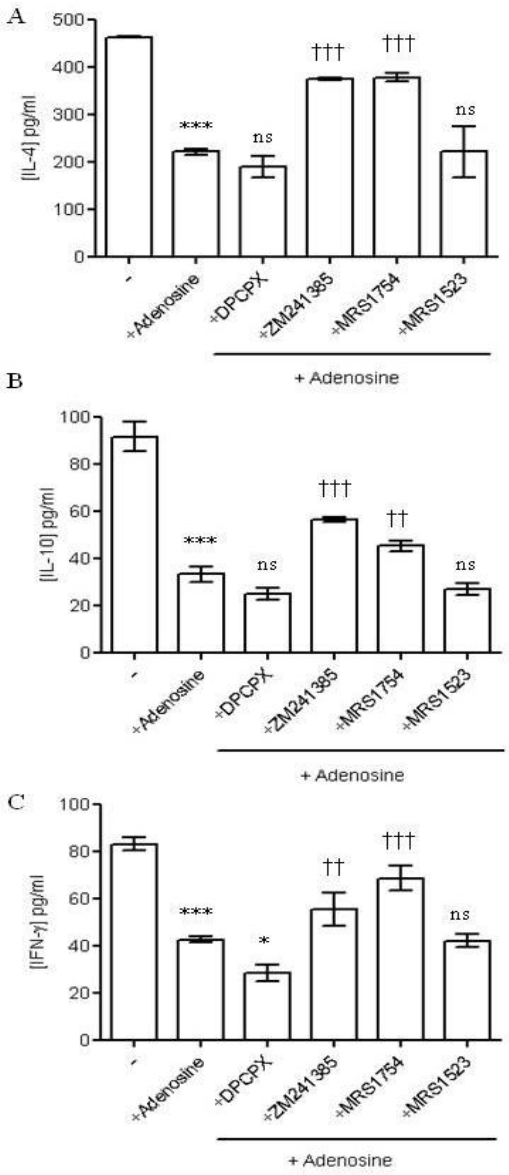


Figure 6.8: Pharmacological characterisation of P1R-mediated effects on cytokine release from T cells. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 and cytokine release measured after 4 days of culture. The effect of 50μM adenosine in the presence of P1R antagonists (10μM) on (A) IL-4 release, (B) IL-10 release and (C) IFN-γ release. Cytokine levels are expressed in pg/ml. The following antagonists were added 15 minutes prior to adenosine addition: DPCPX (A_1R antagonist), ZM241385 (A_{2A}R antagonist), MRS1753 (A_{2B}R antagonist) or MRS1523 (A_3R antagonist). The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks/daggers indicate statistical significance compared to the untreated control.* $p< 0.05$, *** $p< 0.001$. (†) is compared to treatment with adenosine only. †† $p< 0.01$, ††† $p< 0.001$, ns - not significant ($p> 0.05$).

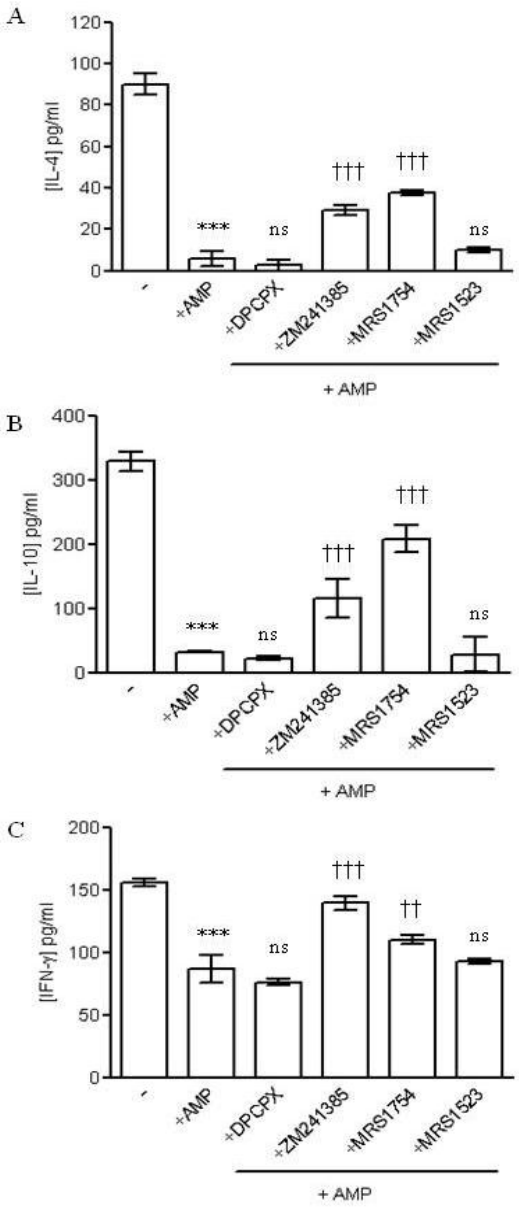


Figure 6.9: Pharmacological characterisation of P1R-mediated effects on cytokine release from T cells. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 and cytokine release measured after 4 days of culture. The effect of 50μM AMP in the presence of P1R antagonists on (A) IL-4 release, (B) IL-10 release and (C) IFN-γ release. Cytokine levels are expressed in pg/ml. The following antagonists (10μM) were added 15 minutes prior to adenosine addition: DPCPX (A_1R antagonist), ZM241385 (A_{2A}R antagonist), MRS1753 (A_{2B}R antagonist) or MRS1523 (A_3R antagonist). The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks/daggers indicate statistical significance compared to the untreated control. *** $p < 0.001$. (†) is compared to treatment with AMP only. †† $p < 0.01$, ††† $p < 0.001$, ns - not significant ($p > 0.05$).

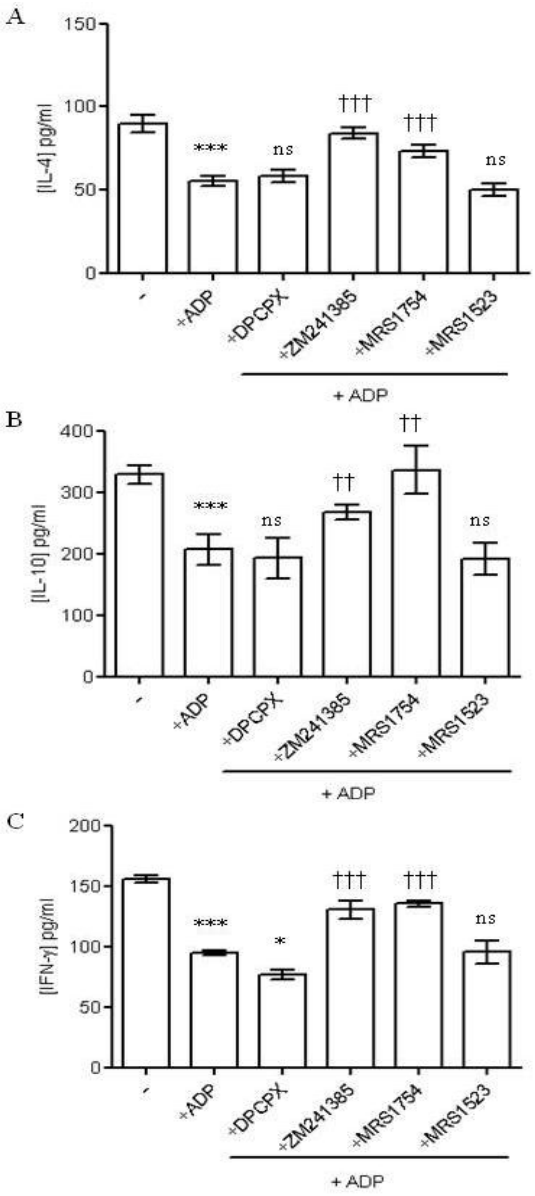


Figure 6.10: Pharmacological characterisation of P1R-mediated effects on cytokine release from T cells. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 and cytokine release measured after 4 days of culture. The effect of 50μM ADP in the presence of P1R antagonists was measured on (A) IL-4 release, (B) IL-10 release and (C) IFN-γ release. Cytokine levels are expressed in pg/ml. The following antagonists (10μM) were added 15 minutes prior to adenosine addition: DPCPX (A_1R antagonist), ZM241385 (A_{2A}R antagonist), MRS1753 (A_{2B}R antagonist) or MRS1523 (A_3R antagonist). The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks/daggers indicate statistical significance compared to the untreated control.* $p< 0.05$, *** $p< 0.001$. (†) is compared to treatment with ADP only. †† $p< 0.01$, ††† $p< 0.001$, ns - not significant ($p> 0.05$).

The involvement of downstream pathways in adenosine-induced inhibition of cytokine release was also investigated. Similar to T cell proliferation, cAMP elevation also mimicked the pattern of cytokine release obtained with adenosine as it inhibited all cytokine release. The combination of NECA and rolipram had an additive effect of inhibition for IL-4 and IFN-γ release while IL-10 levels were similar for all cAMP elevators (Figure 6.11). When components of other downstream pathways were inhibited it was evident that most of these components were essential for basal levels of cytokine release. Specifically, all inhibitors used inhibited IL-4 release except for the PLC mock (U73343) while the NF-κB and PLC inhibitors (Bay 11-7082 and U73122) blocked IL-10 and IFN-γ release. Adenosine-induced inhibition of IL-4 release was only slightly reversed by inhibition of PLC while in the case of IL-10 there was no significant reversal by any of the inhibitors tested. IFN-γ release was restored more significantly by the PLC inhibitor while the NF-κB and MEK1/2 inhibitors partly reversed adenosine inhibition.

Taken together, these results show that P1 receptor activation and, in particular the $A_{2A}R$ and $A_{2B}R$, was responsible for adenosine-induced inhibition of cytokine release by CD4+ T cells. This inhibition was mainly due to receptor coupling to Gs leading to accumulation of cAMP. In addition, blocking MEK1/2 and PLC activation also reversed adenosine-induced inhibition of IL-4 and IFN-γ release although these effects were not very clear due to inhibitor interference with basal cytokine levels.

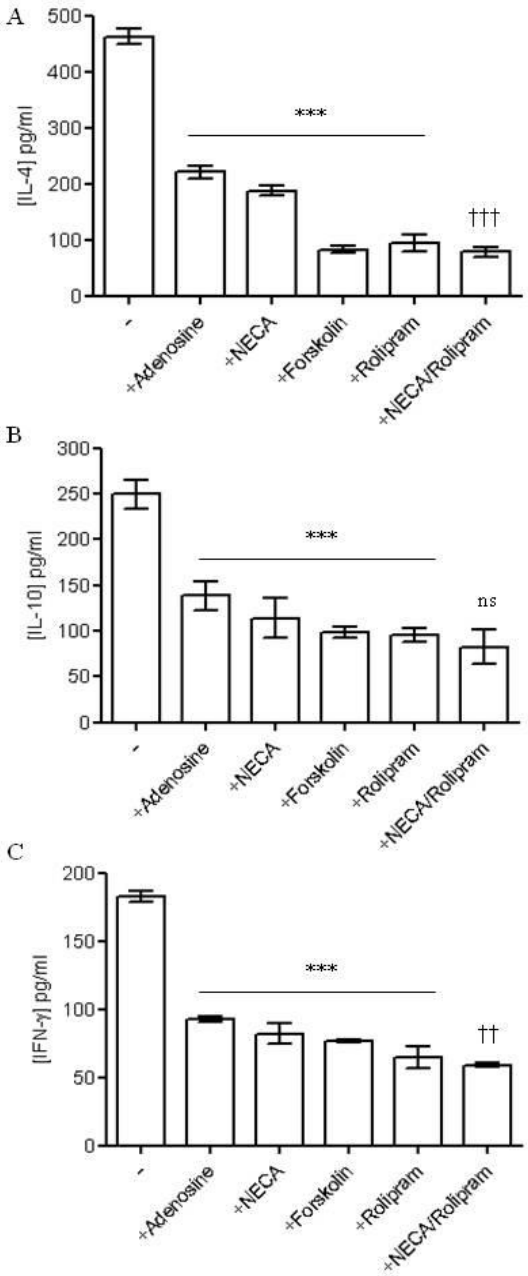


Figure 6.11: The effect of cAMP elevators on P1R-mediated effects on cytokine release from T cells. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 and cytokine release measured after 4 days of culture. The effect of the cAMP elevators forskolin (10μM) and rolipram (5μM) on cytokine release from T cells compared to and combined with P1R agonists 50μM adenosine and 50μM NECA. A) IL-4 release, (B) IL-10 release and (C) IFN-γ release are shown expressed in pg/ml. The cAMP elevators were added 15 minutes prior to adenosine/NECA addition. The data presented are an average of triplicates ± SEM from one experiment of n = 3 and asterisks indicate significance. *** $p < 0.001$. (†) is compared to treatment with NECA only. †† $p < 0.01$, ††† $p < 0.001$, ns - not significant ($p > 0.05$).

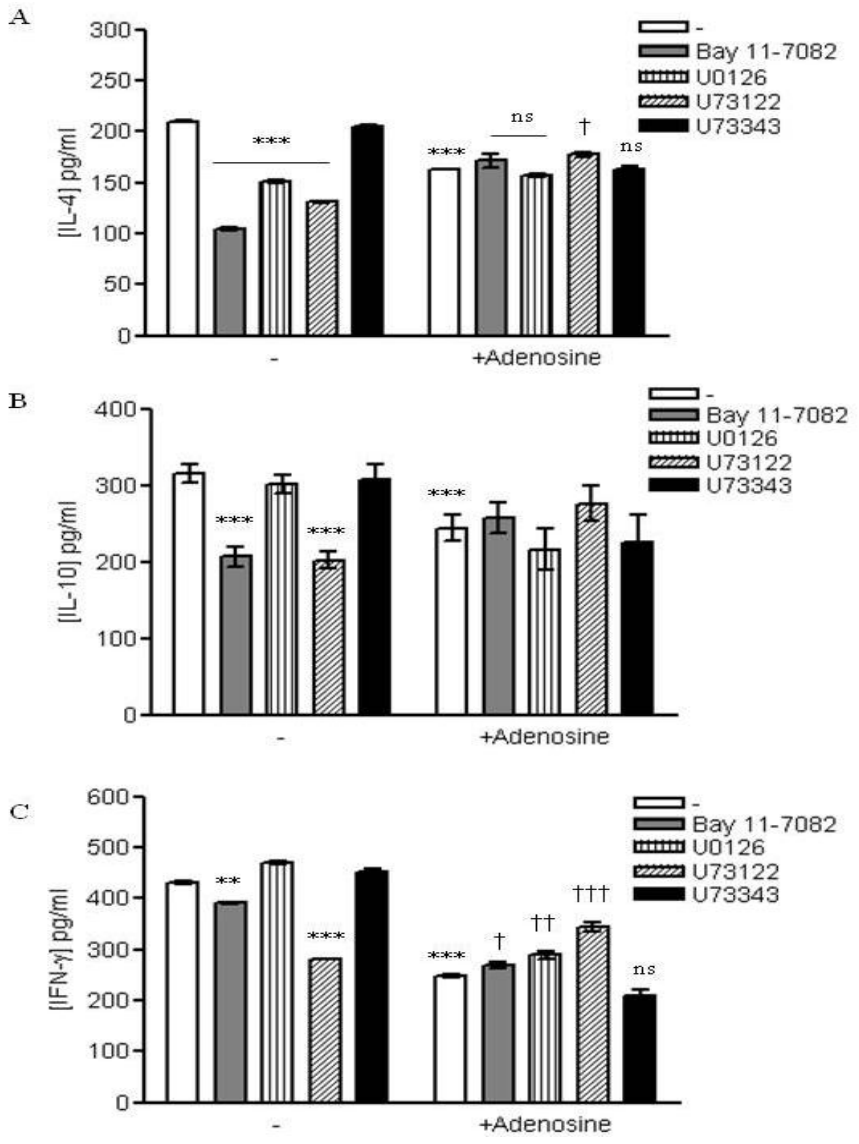


Figure 6.12: The effect of downstream signalling pathways on P1R-mediated effects on cytokine release from T cells. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 and cytokine release measured after 4 days of culture. The effect of 10μM U73122 (PLC inhibitor), 5μM Bay 11-7082 (NF-κB selective inhibitor**)** and 5μM U0126 (MEK1/2 inhibitor) on adenosine-induced inhibition of cytokine release from T cells was measured after 4 days of culture. Cells were pre-incubated with the all inhibitors for 15 minutes prior to adenosine addition. The effect on the release of the following cytokines was measured: (A) IL-4, (B) IL-10, and (C) IFN-γ and expressed in pg/ml. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. ** $p< 0.01$, *** $p< 0.001$. (†) is compared to treatment with 50μM adenosine only. † $p< 0.05$, †† $p< 0.01$, ††† $p< 0.001$, ns - not significant ($p> 0.05$).

6.2.2 T cell ecto-nucleotidases

Characterisation of ecto-nucleotidase expression and function

Most immune cells are known to express ecto-nucleotidases on their surface and, specifically in T cells, these proteins have been shown to act as markers of their activation. Hence, in this study, CD39/CD73 expression was analysed by RT-PCR and both ecto-nucleotidases were found to be present on CD4+ T cells while both CD39 and CD73 PCR products were detected (Figure 6.13A). In order to determine if these ecto-nucleotidases were in fact functionally expressed, CD4+ T cells were cultured for 24 hours in the presence of nucleotides and levels of inorganic phosphate measured in culture supernatants. Phosphate was released from all nucleotides tested and the levels of phosphate detected were in the order ATP > ADP > AMP (Figure 6.13B).

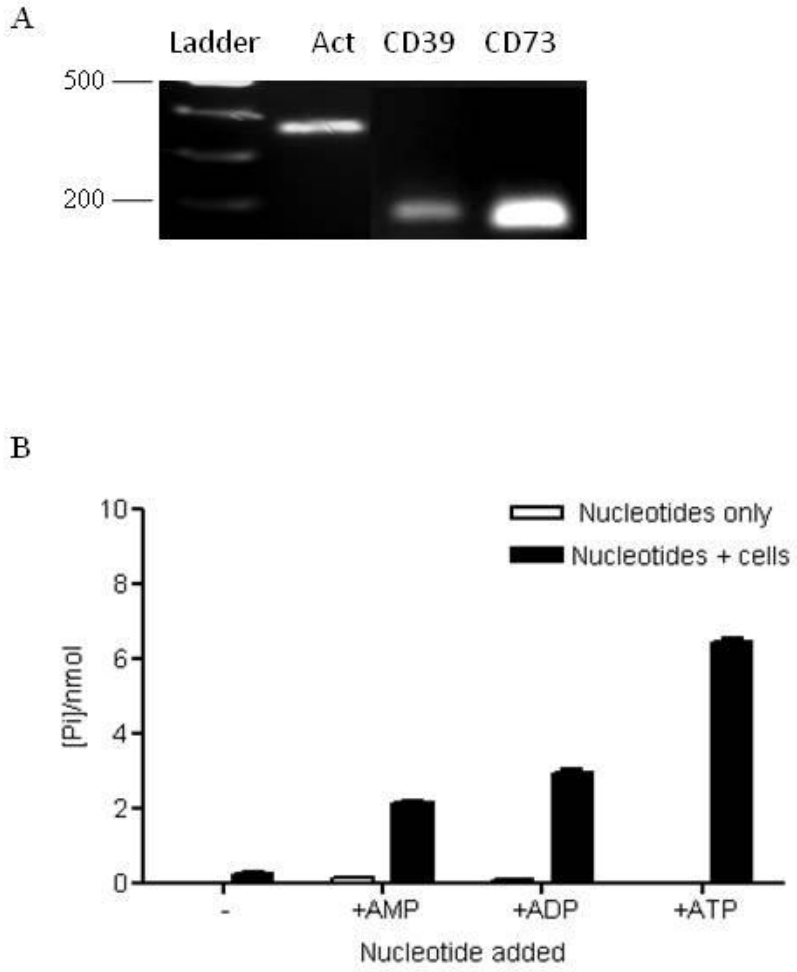


Figure 6.13: Determination of the presence and function of CD39 and CD73 in CD4+ T cells. (A) RNA isolated from resting CD4+ T cells purified from mouse spleen was used to produce cDNA used in RT-PCR to detect the presence of CD39 and CD73 transcripts. PCR products of expected sizes for CD39 and CD73 were detected (see Materials and Methods, Table 2.1B) in untreated T cells. Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT). (B) Nucleotides were incubated with spleen derived CD4+ T cells and the nmoles of phosphate released per 10^5 cells calculated. 50 μM of AMP, ADP, β-ADP, ATP, ATP-γ and UDP were added to T cells for 24 hours. Results show averages of experiments carried out in triplicate $\pm$ SEM (n = 2).

Effect of T.spiralis ES on ecto-nucleotidase expression

The effect of *T.spiralis* secreted proteins (ES) on ecto-nucleotidase expression on T cells was investigated in order to examine if they interfere with T cell activation. The RT-PCR analysis presented in Figure 6.14 showed that ES appeared to reduce CD73 transcripts while heat inactivated ES (HI-ES) and deglycosylated ES (Deg-ES) did not. CD39 transcripts appeared to be the same for all T cell treatments (Figure 6.14). Since RT-PCR is qualitative a quantitative approach was used in order to determine if this apparent difference in transcript levels was real.

The reduction in CD73 levels was confirmed by Q-PCR, however, a similar reduction was also detected in CD39 levels. The ES-induced inhibition of CD39 and CD73 transcript levels was reversed partly by heat inactivation of ES (HI-ES) while complete reversal was obtained by deglycosylation of ES (Deg-ES) (Figure 6.15A). In order to investigate if this increase in transcript levels resulted in up-regulation of CD39 and CD73 protein expression, FACS analysis was carried out. CD39 protein levels were not affected by ES as all conditions tested produced overlapping traces (Figure 6.15B). In contrast, ES inhibited CD73 expression to levels that were no longer detectable by FACS as the trace obtained was similar to the isotype control. CD73 expression was restored to basal CD4+ T cell levels by HI-ES and Deg-ES (Figure 6.15C).

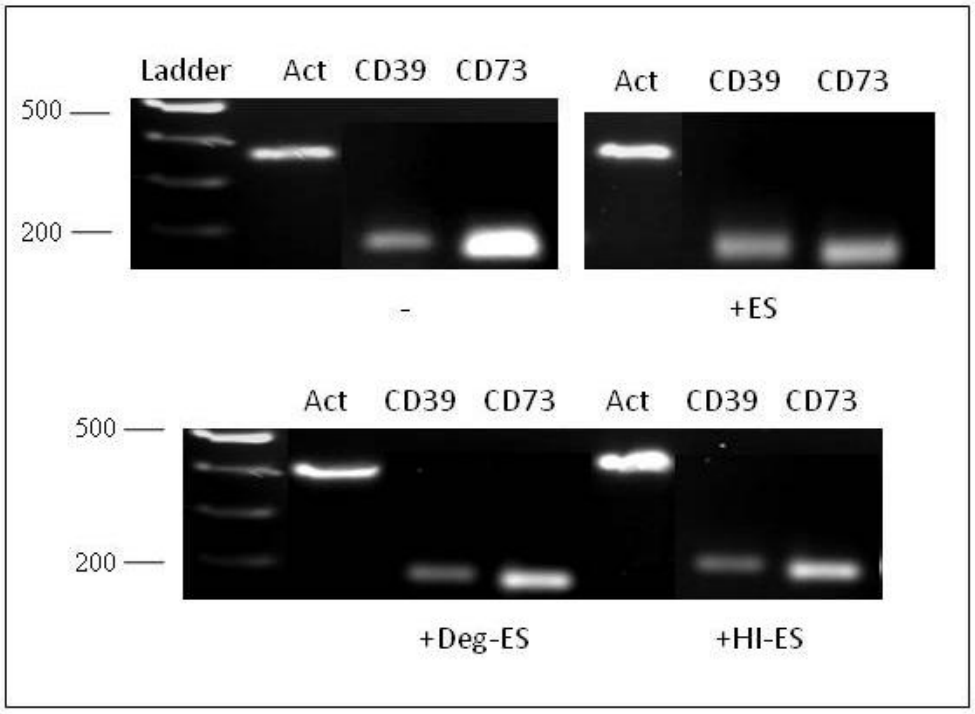


Figure 6.14: Determination of the presence of CD39 and CD73 transcripts in CD4+ T cells and effect of *T.spiralis* ES. RNA isolated from CD4+ T cells purified from mouse spleen was used to produce cDNA used in RT-PCR to detect the presence of CD39 and CD73 transcripts. PCR products of expected sizes for CD39 and CD73 were detected (see Materials and Methods, Table 2.1B) in untreated T cells or T cells treated with ES or heat-inactivated (HI-) ES. Actin primers were used in reactions to confirm cDNA quality (Act) and in reactions to determine if RNA was contaminated with DNA (-RT).

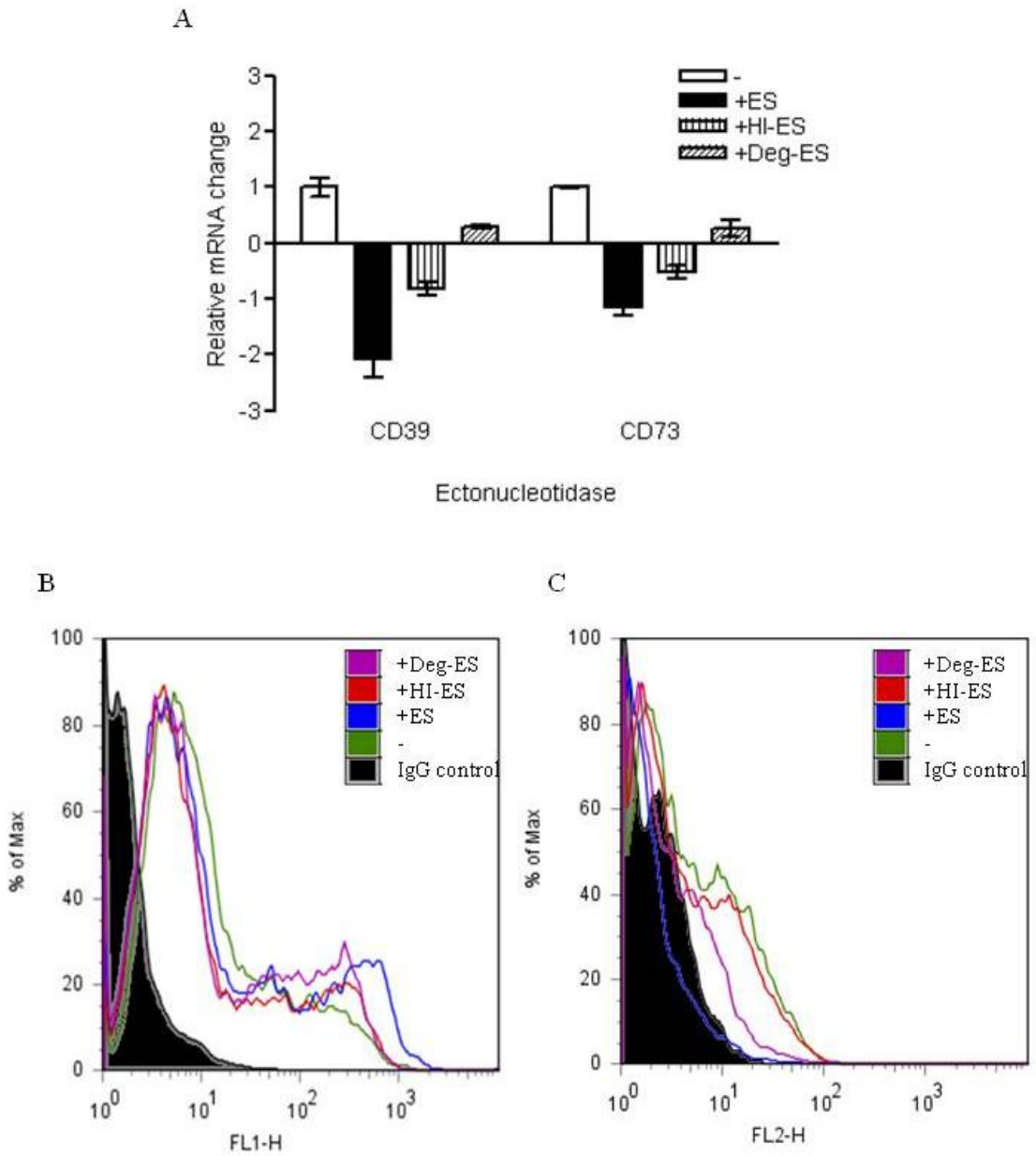


Figure 6.15: Flow cytometric analyses showing expression of CD39 and CD73 on CD4+ T cells. CD4+ T cells were isolated from mouse spleen and cultured for 24 hours in culture medium only or in the presence of 50µg/ml *T.spiralis* ES, HI-ES or deglycosylated (Deg-) ES. (A) The effect on CD39 and CD73 transcript levels. (B) The effect on CD39 expression. (C) The effect on CD73 expression. In the flow cytometric analysis of ES-induced effects, the block trace corresponds to IgG isotype control and the line trace corresponds to CD39/CD73-positive cells, presented as a % of the maximum number of cells. In the quantitative PCR analyses of ES-induced effects on T cell CD39 and CD73 expression, relative mRNA changes presented with standard errors were calculated after normalizing to β-actin from one representative experiment of n=2.

6.2.3 Effects of *T.spiralis* ES on T cell effector functions

Effect of ES on T cell proliferation in vitro

The effect of *T.spiralis* ES on anti-CD3-activated CD4+ T cell proliferation in the presence of irradiated APCs was then investigated. ES induced an inhibition of T cell proliferation which reached 40% after 4 days of culture (Figure 6.16A). This inhibition was enhanced when ES was co-administered with ATP due to generation of adenosine in culture by ecto-nucleotidases on T cells, as ATP alone caused the same inhibition of proliferation as when it was combined with ES. The activity of T cell ectonucleotidases in culture was confirmed by carrying out phosphate release assays, the results of which are shown in Figure 6.13B. In order to investigate if inhibition of T cell proliferation is a common characteristic of parasite infection, *Brugia malayi* microfilarial and adult ES which was available in our lab, was used. As opposed to the *T.spiralis* ES-induced inhibition of proliferation, *Brugia malayi* ES from both microfilaria and adults seemed to increase proliferation by 10-20% (Figure 6.16B and C). ATP inhbited the increase in proliferation induced by *B.malayi* ES which was probably due to generation of adenosine by T cell CD39 and CD73, as well as by *B.malayi* ES enzymes which are also capable of catalysing ATP hydrolysis, as shown in the Appendix, Figure A.3 B and C.

Taking into account that *T.spiralis* ES contains nucleotide metabolising enzymes and that it was found previously in this section that adenosine inhibits proliferation, P1R antagonists were used to see if ES-induced effects were due to generation of adenosine from nucleotides potentially released during culture. However, ES-induced effects on proliferation were not due to P1R activation as none of the P1R antagonists were capable of reversing the ES-induced inhibition (Figure 6.17).

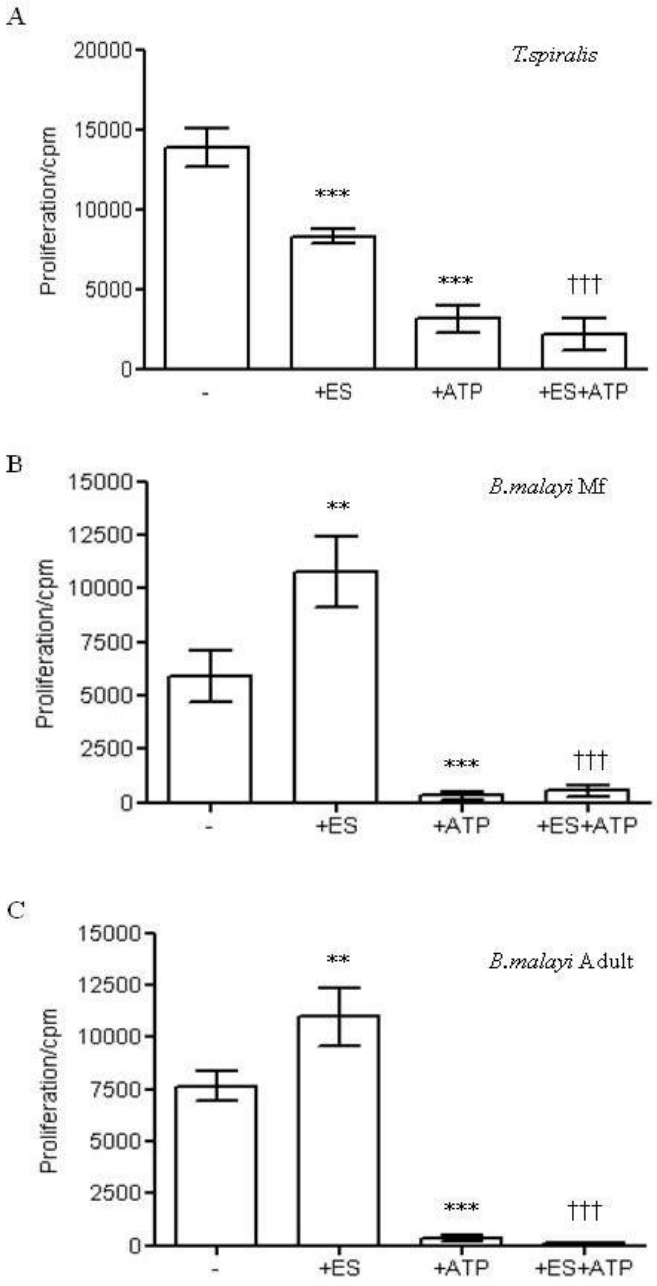


Figure 6.16: The effect of *T.spiralis* ES on T cell proliferation; comparison with *B.malayi* ES. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 to induce cell proliferation, measured by means of ^{3}H-thymidine incorporation, after 4 days of culture. CD4+ T cells were pre-incubated with 50µg/ml *T.spiralis* ES and 10µg/ml of *B.malayi* ES for 1 hour prior to co-culture with iAPCs for 3 days before ^{3}H-thymidine addition. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n $\geq$ 2 and asterisks indicate statistical significance compared to the untreated control. ** $p < 0.01$, *** $p < 0.001$. . (†) is compared to treatment with ES only. ††† $p < 0.001$.

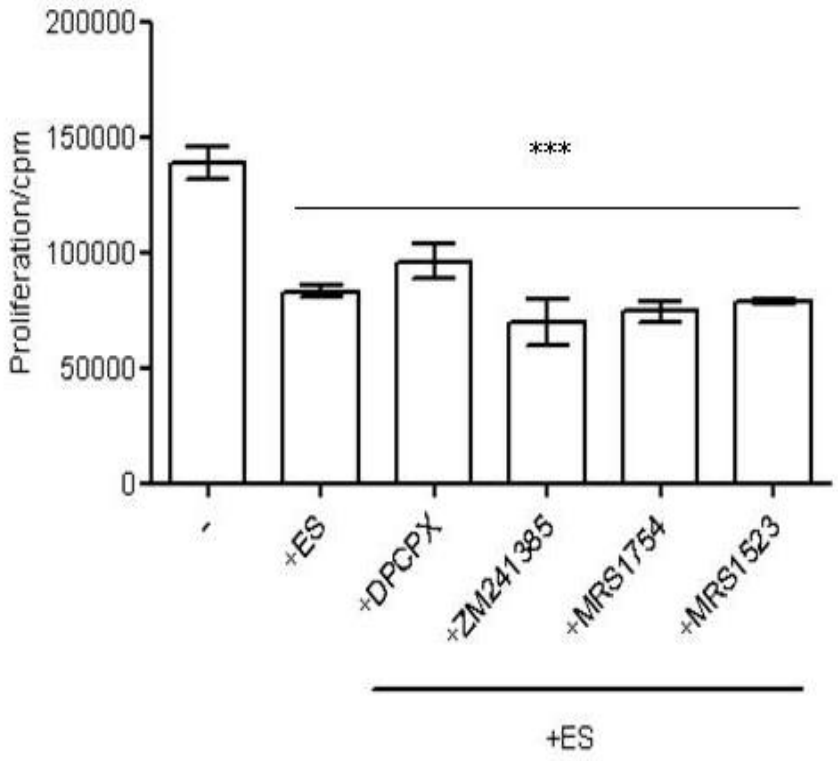


Figure 6.17: Pharmacological characterisation of *T.spiralis* ES-mediated effects on T cell proliferation. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 to induce cell proliferation, measured by means of ^{3}H-thymidine incorporation, after 4 days of culture. Antagonists (10μM) DPCPX (A_1R antagonist), ZM241385 (A_{2A}R antagonist), MRS1753 (A_{2B}R antagonist) or MRS1523 (A_3R antagonist) were added 15 minutes prior to addition of 50μg/ml ES. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate statistical significance compared to the untreated control. *** $p< 0.001$.

In order to further analyse ES-induced inhibition of T cell proliferation, downstream pathways were blocked with specific inhibitors. All inhibitors blocked basal levels of proliferation, except for the PLC mock inhibitor (U73343) suggesting that the inhibition induced by the PLC inhibitor was not due to compound effects. The MEK1/2 inhibitor (U0126) also reduced basal levels of proliferation but it induced complete reversal of the ES-induced inhibition suggesting that activation MEK1/2 is necessary for inhibiting T cell proliferation (Figure 6.18).

Having established in the previous chapter (Chapter 5) that ES-induced effects on macrophages were mediated via activation MGL, the effect of blocking MGL using GalNAc was also investigated on T cell proliferation. In fact, GalNAc partly reversed inhibition of proliferation by ES despite the fact that GalNAc administered in the absence of ES inhibited proliferation. Glycan involvement was further supported by the fact that both HI-ES and Deg-ES reversed the inhibition completely. EGTA which blocks all C-type lectins also inhibited proliferation due to interference with the T cell receptor (Figure 6.19).

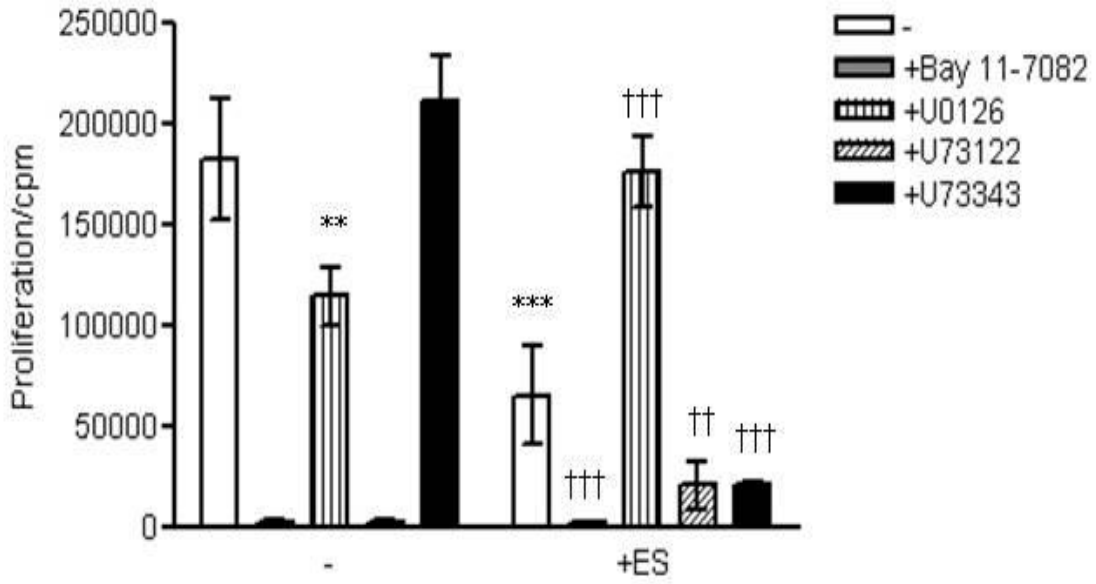


Figure 6.18: The effect of downstream signalling pathways on *T.spiralis* ES-mediated effects on T cell proliferation. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 to induce cell proliferation, measured by means of ^{3}H-thymidine incorporation, after 4 days of culture. The effect of 10μM U73122 (PLC inhibitor), 5μM Bay 11-7082 (NF-κB selective inhibitor**)** and 5μM U0126 (MEK1/2 inhibitor) on ES-induced inhibition of T cell proliferation was measured after 4 days of culture. Cells were pre-incubated with the all inhibitors for 15 minutes prior to addition of 50μg/ml ES. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. ** $p< 0.01$, *** $p< 0.001$. (†) is compared to treatment with ES only. †† $p< 0.01$, ††† $p< 0.001$.

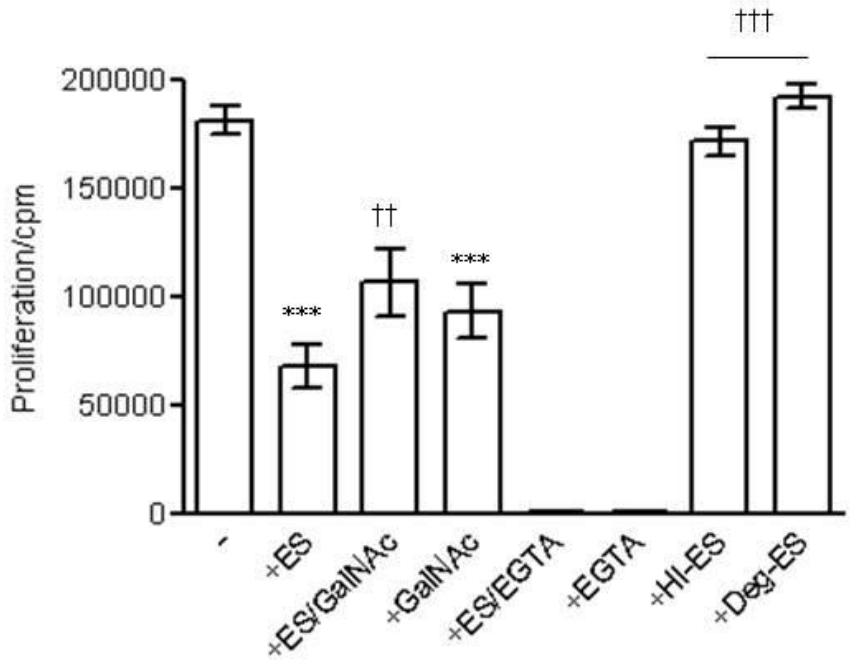


Figure 6.19: The effect of blocking T cell surface lectins on *T.spiralis* ES-mediated inhibition of proliferation. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 to induce cell proliferation, measured by means of ^{3}H-thymidine incorporation, after 4 days of culture. Prior to addition of 50µg/ml ES, cells were incubated with 100mM GalNAc (blocking MGL). Cells were also treated with either heat-inactivated ES or deglycosylated ES. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks/daggers indicate significance compared to the respective untreated control. *** $p< 0.001$. (†) is compared to treatment with ES only. †† $p< 0.01$, ††† $p< 0.001$.

Effect of ES on cytokine release from T cells

Cytokine release from CD4+ T cell was also affected by *T.spiralis* ES. Similar to its effects on proliferation, ES inhibited IL-10 and IFN-γ release by 30-40%. However, IL-4 release was increased in the presence of ES by about 20% (Figure 6.20). Inhibitors were used to investigate involvement of downstream pathways. IL-4 release was inhibited by all the inhibitors used (Figure 6.20A). The ES-induced increase in IL-4 release was inhibited by all inhibitors, however, since these compounds also inhibited basal IL-4 release it may not be a specific inhibition of the ES-induced increase. IL-10 release was inhibited slightly by the NF-κB and PLC inhibitors (Bay 11-7082 and U73122). ES inhibited IL-10 release and this inhibition was reversed by both the NF-κB and MEK1/2 (U0126) inhibitors (Figure 6.20B). Since the MEK1/2 inhibitor did not affect basal IL-10 release from T cells, the fact that the ES-induced inhibition of IL-10 release was reversed by blocking MEK1/2 activation implies that this pathway is activated by ES proteins. IFN-γ release was inhibited by the NF-κB and PLC inhibitors (Figure 6.20). ES inhibited IFN-γ release and the NF-κB inhibitor (Bay 11-7082) reversed this inhibition. Blocking MEK1/2 with U0126 led to a similar reversal of ES-induced inhibition which suggests that activation of the MEK1/2 pathway is also required for inhibition of IFN-γ release by ES as U0126 did not affect basal levels (Figure 6.20C). As was the case for all cytokines tested, the PLC mock did not affect basal release levels showing that the inhibition induced by the PLC inhibitor was actually due to interference with PLC activation and not a compound effect.

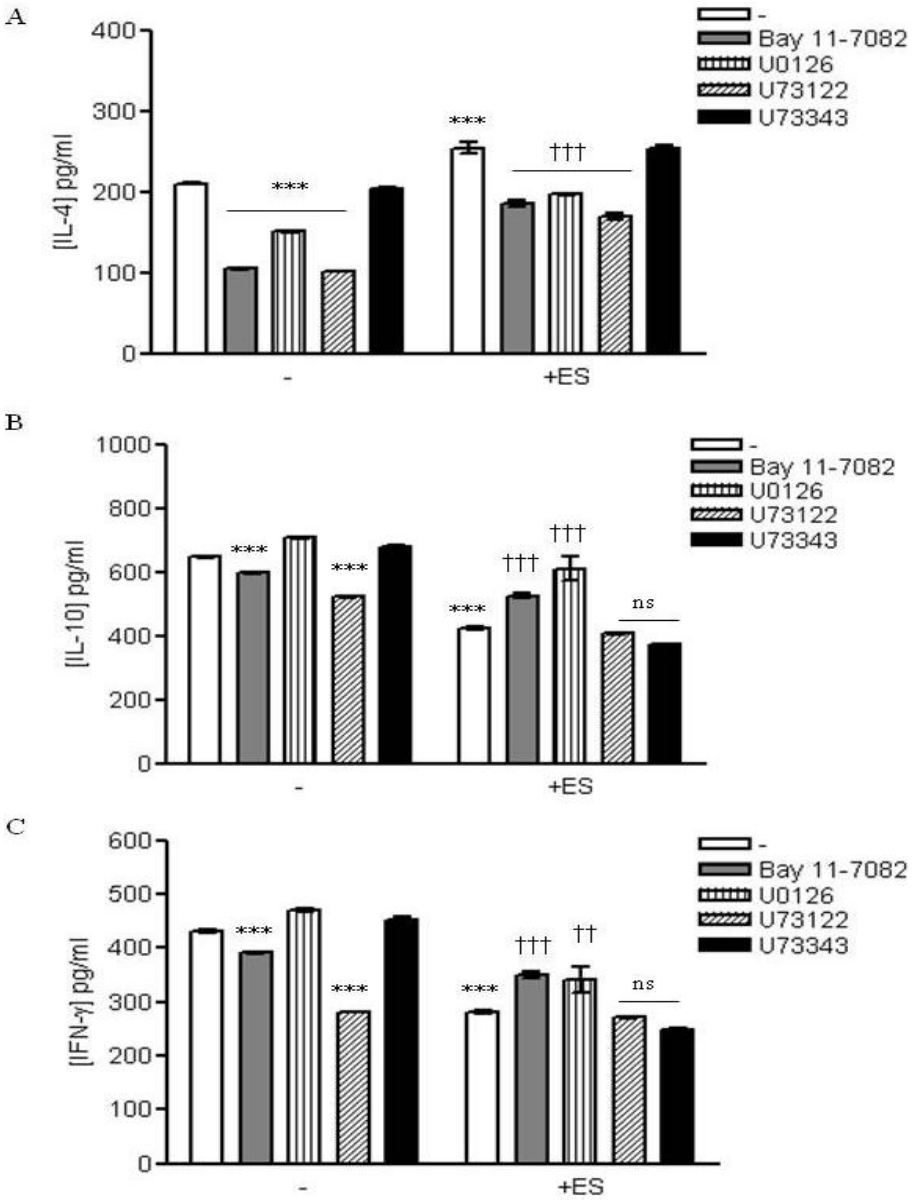


Figure 6.20: The effect of downstream signalling pathways on *T.spiralis* ES-mediated effects on cytokine release from T cells. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 and cytokine release measured after 4 days of culture. The effect of 10μM U73122 (PLC inhibitor), 5μM Bay 11-7082 (NF-κB selective inhibitor**)** and 5μM U0126 (MEK1/2 inhibitor) on ES-induced inhibition of T cell (A) IL-4 release, (B) IL-10 release and (C) IFN-γ release. Cytokine levels are expressed in pg/ml. Cells were pre-incubated with the all inhibitors for 15 minutes prior to addition of 50μg/ml ES. The data presented are an average of triplicates ± SEM from one representative experiment of n = 3 and asterisks indicate significance compared to the respective untreated control. *** $p < 0.001$. (†) is compared to treatment with ES only. †† $p < 0.01$, ††† $p < 0.001$, ns - not significant ($p > 0.05$).

Since heat inactivation and deglycosylation of ES reversed the ES-induced effects on cytokine release from CD4+ T cells, shown in Figure 6.21 A, B and C, the involvement of ES glycans was also investigated. The increase in IL-4 release induced by ES was inhibited by GalNAc, while GalNAc administered in the absence of ES had no effect on IL-4 release from T cells (Figure 6.21A). The ES-induced inhibition of IL-10 was reversed by GalNAc and this was specific to ES-induced effects as basal levels were not affected (Figure 6.21B). IFN-γ release was inhibited both by ES and GalNAc which, when administered together, produced an additive inhibitory effect (Figure 6.21C). Similar to T cell proliferation, EGTA blocked all cytokine release suggesting that interference with the TCR affects all T cell effector functions (Figure 6.21).

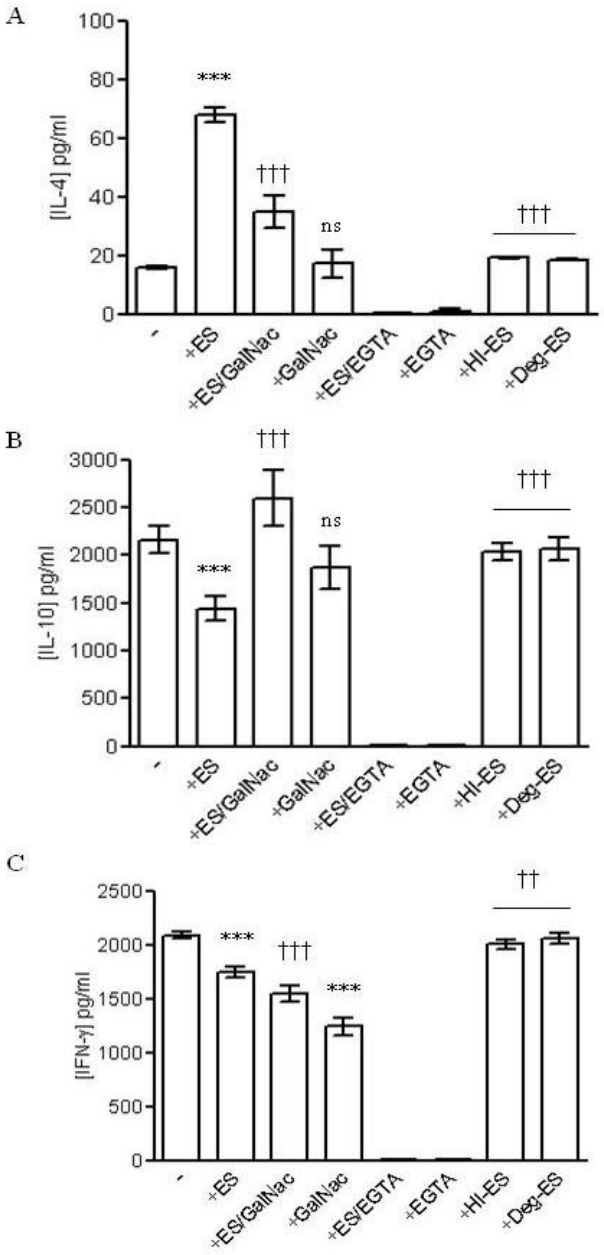


Figure 6.21: The effect of blocking T cell surface lectins on *T.spiralis* ES-mediated inhibition of cytokine release. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 and cytokine release measured after 4 days of culture. Prior to addition of 50µg/ml ES, cells were incubated with 100mM GalNAc (blocking MGL). Cells were also treated with either heat-inactivated ES or deglycosylated ES. (A) IL-4 release, (B) IL-10 release and (C) IFN-γ release are shown expressed in pg/ml. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. *** $p < 0.001$, ns - not significant ($p > 0.05$). (†) is compared to treatment with ES only. †† $p < 0.01$, ††† $p < 0.001$.

ES-induced effects on purinergic signalling

In order to determine if *T.spiralis* ES interfered with purinergic signalling at the purinergic receptor expression level in addition to ecto-nucleotidase expression, P1R analysis by RT-PCR was carried out. ES seemed to reduce $A_{2A}R$ and A_3R transcript levels and this was reversed by heat inactivating or deglycosylating ES (Figure 6.22). Q-PCR analysis confirmed the apparent inhibitory effect of ES on $A_{2A}R$ mRNA levels but no effect was observed on A_1R and A_3R mRNA levels. Furthermore, $A_{2B}R$ levels were up-regulated by ES by approximately 2-fold. All ES effects were reversed by heat inactivation and deglycosylation (Figure 6.22).

Taking into account that ES contains nucleotide metabolising enzymes, and in particular 5`NT, the effect of ES on nucleotide-induced effects was also investigated. However, since ES induces an increase in IL-4 release and inhibition of IL-10 and IFN-γ release as well as inhibition of T cell proliferation when administered to cells, it would not be possible to distinguish between these effects and any effects induced by ES administered with nucleotides. Since the major enzyme responsible for nucleotide hydrolysis by *T.spiralis* ES is 5`NT, a recombinantly expressed 5`NT was used. Recombinant 5`NT on its own had no effect on basal T cell proliferation, however, when combined with nucleotides it enhanced the AMP and ADP-induced inhibition of proliferation (Figure 6.23). Furthermore, 5`NT had no effect on adenosine or ATP-induced inhibition of proliferation which was expected as only AMP, ADP and UDP are 5`NT substrates (phosphate release from nucleotides by 5`NT is shown in the Appendix, Figure A.2). As a result of UDP hydrolysis by 5`NT, the UDP-induced inhibition of proliferation was no longer observed. 5`NT had no effect on basal IL-4, IL-10 and IFN-γ release by T cells but led to increased inhibition of cytokine release by nucleotides like AMP and ADP. ATP-induced effects

were not affected while the UDP-induced inhibition of IL-4 and IFN-γ release was reversed as a result of 5`NT-mediated hydrolysis of UDP (Figure 6.24).

Collectively, these data show that *T.spiralis* ES inhibits CD4+ T cell expression of CD39 at the molecular level and CD73 expression at both the molecular and protein levels. In addition, ES inhibits $A_{2A}R$ transcript expression and enhances $A_{2B}R$ expression. Furthermore, ES inhibits T cell proliferation as well as IL-10 and IFN-γ release while it enhances IL-4 release. These effects are generally counteracted by blocking or removing ES glycans and by interference with MGL binding and MEK1/2 activation. In addition to modulating CD4+ T cell effector functions directly, ES can influence T cell responses to nucleotides via 5`NT-mediated hydrolysis.

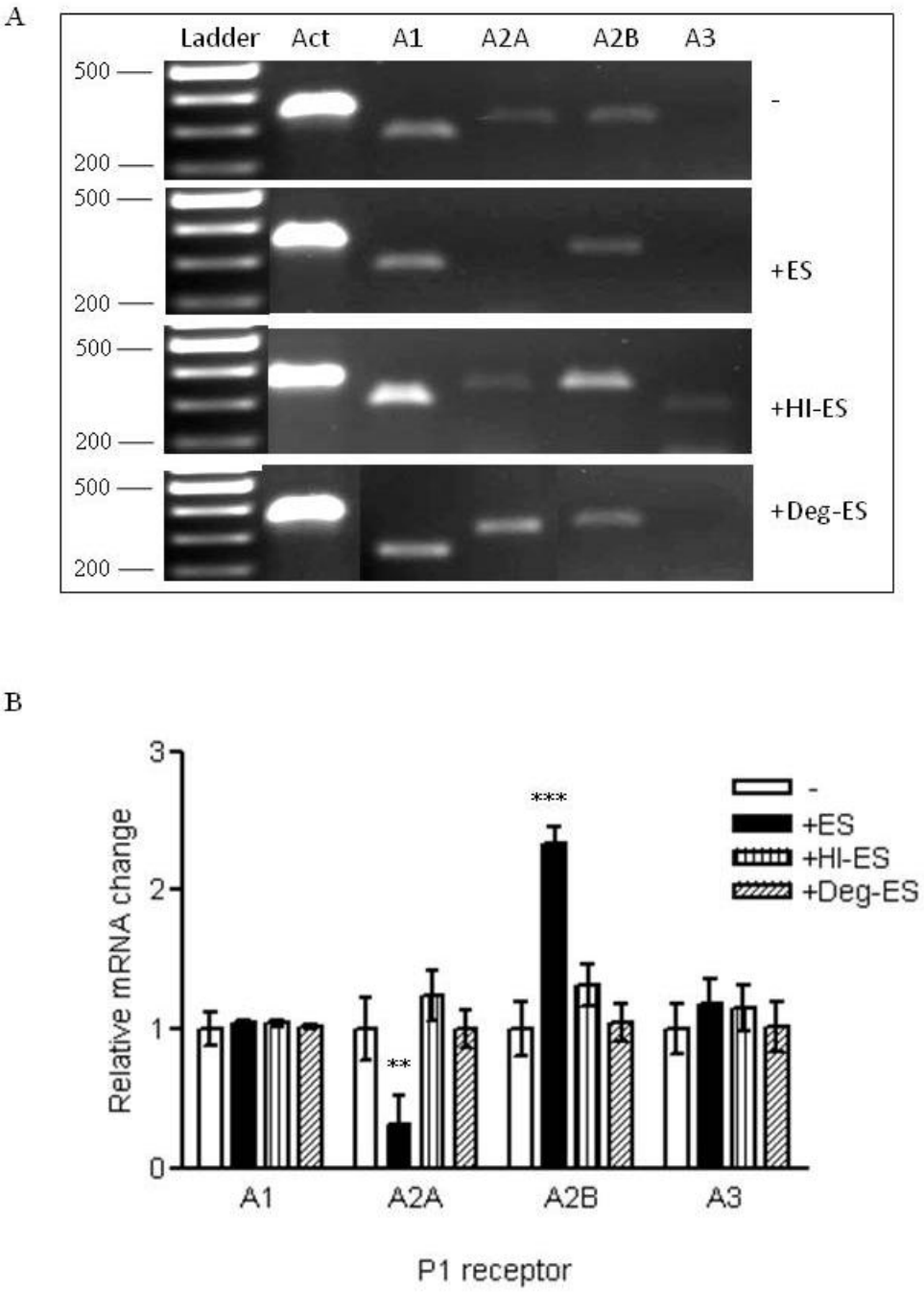


Figure 6.22: Analysis of the effect of *T.spiralis* ES on CD4+T cell P1 receptor transcripts. RNA isolated from CD4+ T cells purified from mouse spleen was used to produce cDNA used in RT-PCR to detect the presence of P1R transcripts in the presence of 50μg/ml *T.spiralis* ES or HI-ES. (A) PR transcripts detected in CD4+ T cells purified from mouse spleen by RT-PCR. (B) Quantitative PCR analyses of P1R mRNA levels and the fold changes presented are relative to untreated T cells after normalising to β-actin mRNA. Standard errors of the mean shown are from one representative experiment of n=2. ** $p < 0.01$, *** $p < 0.001$.

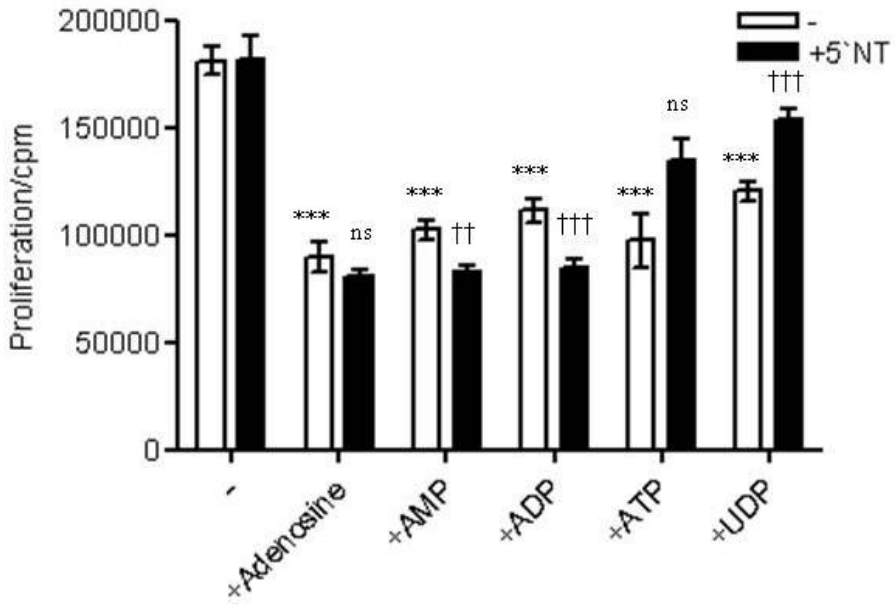


Figure 6.23: The effect of recombinant 5`NT on nucleotide-mediated effects on CD4+T cell proliferation. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 to induce cell proliferation, measured by means of ^{3}H-thymidine incorporation. The effect of 50μM adenosine/nucleotides in the presence of 15μg/ml 5`NT on T cell proliferation was measured after 4 days of culture. The data presented are an average of triplicates $\pm$ SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. *** $p < 0.001$. (†) is compared to treatment with the respective nucleotide. †† $p < 0.01$, ††† $p < 0.001$, ns - not significant ($p > 0.05$).

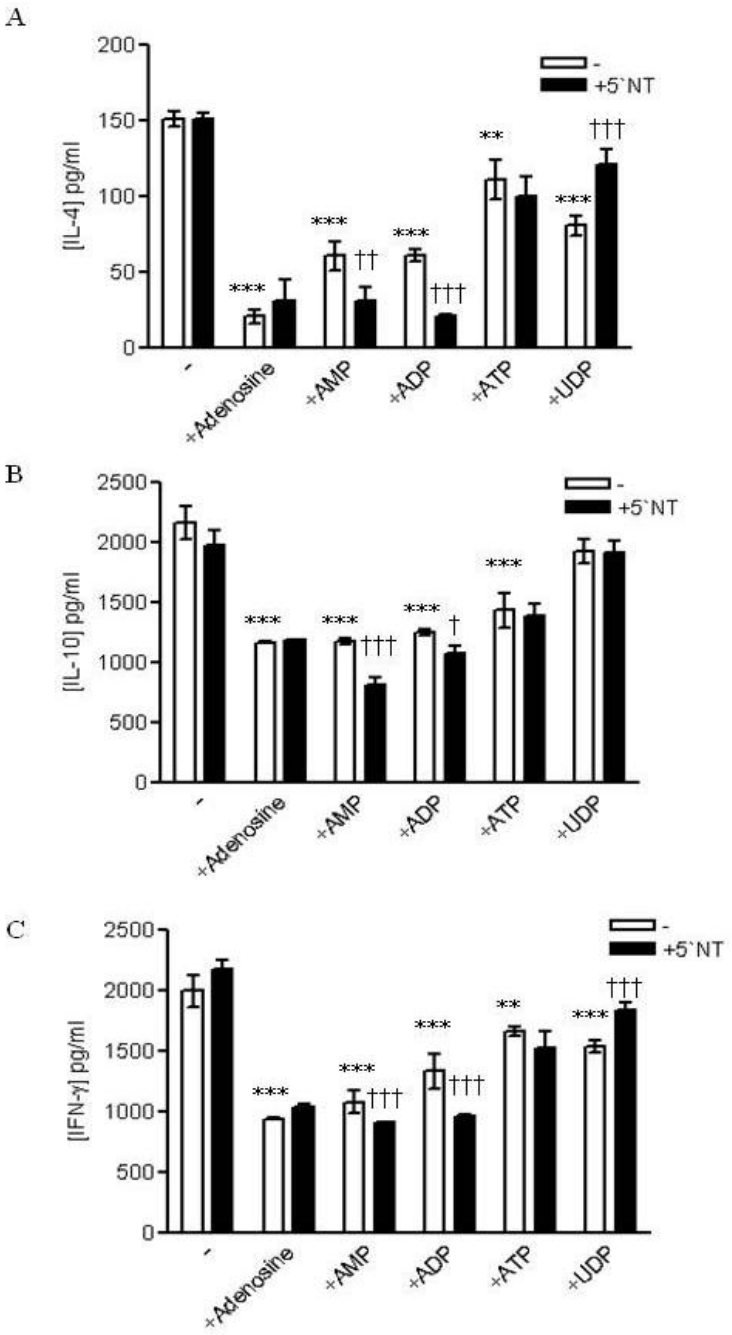


Figure 6.24: The effect of recombinant 5`NT on purinergic signalling-induced cytokine release from T cells. CD4+ cells isolated from mouse spleen were cultured with irradiated APCs from the same original splenocyte population in the presence of anti-CD3 and cytokine release measured after 4 days of culture. The effect of (50μM) adenosine/nucleotides in the presence of 15μg/ml 5`NT on (A) IL-4 release, (B) IL-10 release and (C) IFN-γ release are shown expressed in pg/ml. The data presented are an average of triplicates ± SEM from one representative experiment of n > 3 and asterisks indicate significance compared to the respective untreated control. ** p< 0.01, *** p< 0.001. (†) is compared to treatment with the respective nucleotide. † p< 0.05, †† p< 0.01, ††† p< 0.001.

6.3 Discussion

T cell differentiation from naïve cells, unlike other immune cells, is known to give rise to at least 3 major subsets, T helper 1 (Th1), T helper 2 (Th2) and regulatory T cells (Treg). The phenotype of T cell differentiation is important as the resulting cytokine environment influences the development and recruitment of other immune cells like APCs. The presence of ecto-nucleotidases on T cells has been documented as a marker of their activation, and provides a basis of immunosuppression as a result of generation of adenosine from nucleotides the latter being considered as inducers of pro-inflammatory responses (Bynoe and Viret, 2008). Purinergic signalling is important in APC effector functions and adenosine in particular has been shown in this study to induce an anti-inflammatory phenotype in murine bone marrow derived macrophages (Chapter 4). Therefore, this study further aimed to determine if purinergic signalling had any direct effects on T cell effector functions as well. In addition, considering that *T.spiralis* ES is known to interfere with macrophage purinergic signalling-induced effector functions as was shown in this study (Chapter 5), the effect of ES on T cells was also investigated. The results obtained and presented in the previous section are discussed below.

Purinergic signalling is dependent on purinergic receptor expression and on the availability of extracellular nucleotides. Immune cells can control both these parameters by regulating receptor expression, as well as the surface expression of ecto-nucleotidases which can alter nucleotide type and concentration. Analysis of CD4+ T cell purinergic receptor expression at the molecular level in this study showed that T cells express all P1 receptors and all of the known P2Y receptors except P2Y4. P1 receptor expression by murine splenic T cells has also been reported by Huang and colleagues (Huang *et al*, 1997). Having established that CD4+ T cells express purinergic receptors their functionality was then investigated. T cell proliferation

was inhibited by adenosine as well as by nucleotides due to their degradation to adenosine. P1 receptor agonists were used to confirm that inhibition of proliferation is achieved by P1 receptor stimulation, a fact also confirmed by the lack of inhibition in the case of the slowly-hydrolysable analogue of ADP (β-ADP). This is in agreement with published work by Kobie and colleagues which reported that adenosine inhibits proliferation of murine splenic CD4+ T cells while Ohta and colleagues have shown that increasing concentration of the adenosine analogue NECA inhibited cytotoxic T lymphocyte proliferation *in vitro* (Kobie *et al*, 2006; Ohta *et al*, 2009). In addition, Duhant and colleagues reported that extracellular adenine nucleotides inhibit CD4+ T cell proliferation in response to CD3 and CD28 ligation (Duhant *et al*, 2002). Furthermore, P1 receptor antagonism confirmed that inhibition of T cell proliferation obtained with adenosine, AMP and ADP was in fact due to $A_{2A}R$ and $A_{2B}R$ activation (Figures 6.3 and 6.4). This inhibition was most likely due to cAMP elevation resulting from A_2R coupling to Gs as cAMP elevation by administration of cAMP elevators mimicked the adenosine-induced effects. Similarly, in a study with mouse splenocytes, it was found that inhibition of TCR-activated effector functions by extracellular adenosine was mimicked by an increase in cAMP when using dbcAMP and a PDE inhibitor (Huang *et al*, 1997). When investigating downstream pathway involvement, the only pathway that seemed to interfere with adenosine-induced inhibition of T cell proliferation was the MEK1/2 pathway, as blocking MEK1/2 led to restoration of basal levels of proliferation (Figure 6.5). It is probable that other pathways are also involved, however, it was not possible to draw any conclusions in this study. The reason for this is that inhibition of the PLC and NF-κB pathways interfered with basal T cell proliferation which shows that these pathways are necessary and non-redundant.

Cytokine release from CD4+ T cells was similarly affected by nucleotides and in particular by generation of extracellular adenosine. P1 receptor stimulation with specific agonists as well as use of specific P1 receptor antagonists to reverse adenosine-induced effects showed that, inhibition of cytokine release was again due to $A_{2A}R$ and $A_{2B}R$ stimulation. Therefore, nucleotides probably act via their breakdown product adenosine. Many studies have reported similar effects of adenosine on T cells, specifically, studies using murine splenic T cells which generally attribute these effects to $A_{2A}R$ activation (Alam *et al*, 2009; Erdmann *et al*, 2005; Kobie *et al*, 2006; Ohta *et al*, 2009). In this study it was observed that, in some experiments, nucleotides had more pronounced effects than adenosine. This could be due to adenosine uptake by cells which would mean that after the first 24 hours in culture adenosine may no longer be available, while nucleotides provide a more sustained source of extracellular adenosine. Another explanation may be that adenosine is degraded by the action of adenosine deaminase (ADA) which is known to be expressed on T cells (Martin *et al*, 1995).

Th1 and Th2 cytokines were inhibited by adenine nucleotides an observation also made by Duhant and colleagues who reported the inhibition of both Th1 and Th2 cytokines in response to CD3 and CD28 ligation (Duhant *et al*, 2002). Furthermore, Csoka and colleagues have reported that $A_{2A}R$ activation down-regulates both IFN-γ and IL-4 mRNA (Csoka *et al*, 2008). Recently, Yang and colleagues have shown that adenosine inhibits T cell activation and release of IL-2 and IFN-γ and that these effects were due to cAMP elevation via Gs coupling of P1 receptors (Yang *et al*, 2010). This is in agreement with results presented in this study showing that cAMP elevation, even when produced by administration of cAMP elevators like rolipram and forskolin, mimicked adenosine-induced inhibition of cytokine release (Figure 6.11). Lappas and colleagues have also shown that the adenosine-induced inhibition of TCR-activated T cell

IFN-γ release was due to cAMP elevation as they were able to mimic and enhance adenosine-induced effects when rolipram was co-administered (Lappas *et al*, 2005). Furthermore, Raskovalova and colleagues have reported that adenosine-induced inhibition of T lymphocyte cytokine production via A2AR stimulation involves cAMP-PKA (Protein kinase A) signalling (Raskovalova *et al*, 2007).

Interestingly, blocking PLC which is activated by P1 receptor coupling to Gq also appeared to reverse adenosine-induced inhibition of IL-4 and IFN-γ release, despite background effects produced by the inhibitor used. As was observed with T cell proliferation, MEK1/2 inhibition also reversed adenosine-induced effects on cytokine release. Duhant and colleagues have reported that inhibition of TCR signalling by adenine nucleotides was not a result of Ca^{2+} mobilization which rules out this downstream pathway originating from PLC activation (Duhant *et al*, 2005). In addition, they showed that ERK2 and p38 MAPK were involved which may explain the inhibition of cytokine release shown in this study.

In addition to regulation of purinergic receptor expression and stimulation, purinergic signalling is also regulated by the availability of extracellular nucleotides. So far, the results presented in this study have shown that extracellular nucleotide-induced effects are mediated by generation of adenosine by degradation of adenine nucleotides. This is achieved by ecto-nucleotidases, CD39 and CD73, which are expressed on immune cells and are known to be expressed on lymphocytes, and are considered as the main system of termination of ADP/ATP purinergic effects (Yegutkin *et al*, 2002). Kobie and colleagues have suggested that CD4+ T cells suppress effector functions due to the presence of CD73 which converts AMP to adenosine (Kobie *et al*, 2006). Interestingly, this system of inactivation of purinergic signalling is also used by *T.spiralis* which has been shown to secrete an enzyme with activity similar to CD39 and

CD73 called 5`-nucleotidase (5`NT) (Gounaris, 2002). Therefore, it was of interest to determine if *T.spiralis* secreted products (ES) potentially interfere with T cell effector functions and expression of ecto-nucleotidases. In fact, it was found that, ES affects expression of both CD39 and CD73 with more pronounced effects observed in CD73 molecular and surface expression (Figure 6.13). CD73 has been reported to be a marker of T regulatory cell differentiation so this decrease in CD73 may reflect a reduction in Treg cells, at least within the first 24 hours of T cells encountering *T.spiralis*, since PCR experiments were carried out using mRNA obtained from 24 hour cultures of CD4+ T cells with ES. In conjunction with the inhibitory effect of ES on IFN-γ release and upregulatory effect on IL-4 release, this may suggest that the initial response triggered by *T.spiralis* is a Th2 response, which would be beneficial for parasite persistence in the gut prior to expulsion. In agreement with this, it has been reported that depletion of Treg cells in *T.spiralis* infection results in exaggerated Th2 responses (Beiting *et al*, 2007). This subversion of the T cell response to a less damaging phenotype has also been suggested as a means of facilitation of *Trichuris muris* survival in the gut (D'Elia *et al*, 2009). Recently, Dvoroznakova and colleagues showed that *T.spiralis* induced a Th2 response during the intestinal phase and T cell proliferation at both the intestinal and muscle phases (Dvoroznakova *et al*, 2010). However, in this study, *T.spiralis* ES inhibited CD4+ T cell proliferation *in vitro* and this was not due to exogenously generated adenosine, since P1 receptor antagonists were not able to reverse ES-induced effects (Figure 6.16 and 6.17). On the other hand, *B.malayi* ES had the opposite effects on CD4+ T cell proliferation as both microfilarial and adult ES induced an increase in CD3-activated T cell proliferation. It has been reported that *B.malayi* infection of mice induces a Treg response, while injection of both microfilaria and adults have been shown to induce up-regulation of Treg markers thus skewing CD4+ T cell phenotype to a regulatory mode (McSorley *et al*,

2008). Interestingly, this study has shown that ATP had an inhibitory effect on *B.malayi*-induced T cell proliferation (Figure 6.16).

The only inhibitor that was capable of reversing the inhibitory effects of ES on proliferation, IFN-γ and IL-10 release was the MEK1/2 inhibitor. Having established from Chapter 5 that *T.spiralis* ES mediates its direct effects on macrophages via MGL activation by ES glycans, the effects on CD4+ T cells were also investigated following glycan removal. Indeed, all ES-induced effects were reversed by removing glycans by deglycosylation or by structural destabilisation by heating. In addition, blocking MGL using GalNAc also reversed ES-induced effects. Despite the fact that GalNAc was preincubated with T cells and then added to the irradiated APC population, it may be possible that some effects could be a result of blocking MGL on APCs. In fact, van Vliet and colleagues have reported that T cell regulation by APCs can occur via interaction of MGL with T cell CD45 (van Vliet *et al*, 2006).

T.spiralis ES-induced effects were also analysed in terms of purinergic receptor expression and it was found that CD4+ T cell $A_{2A}R$ transcript expression was inhibited while $A_{2B}R$ expression was enhanced. In order to further investigate ES-induced effects on purinergic signalling, the recombinant 5`NT from *T.spiralis* expressed in our lab was used since ES had direct effects on T cell effector functions and the results obtained when combining ES with nucleotides would not be entirely due to purinergic signalling. As expected, when 5`NT was co-administered with nucleotides the resulting effector functions were typical of adenosine-induced responses. T cell proliferation and cytokine release inhibited by nucleotides were inhibited further as a result of combined action of ecto-nucleotidases on cells and *T.spiralis* 5`NT, while 5`NT on its own had no effect on T cell functions.

In conclusion, this study has shown that purinergic signalling induced in CD4+ T cells by adenosine ligation of A_2 receptors inhibits proliferation as well as Th1 and Th2 responses. This points to the hypothesis that extracellular adenosine acts as an agent of induction of peripheral tolerance by skewing T cell responses to a regulatory phenotype. Furthermore, Treg cells are known to protect the host from intestinal pathology resulting from parasites like *T.spiralis* indicating another beneficial consequence of *T.spiralis*-induced adenosine generation.

Chapter 7

General Discussion

7. Discussion

Cell-mediated immunity is critical in host defence against extracellular pathogens. In particular, macrophages are known to participate in a variety of biological processes aimed at fighting infection as well as remodelling and repair. This functional diversity suggests that changes in their local environment can have an impact on their differentiation and activation. For example, classically activated macrophages (CAMΦ), linked with Th1 responses, are generally involved in combat against bacterial infections while Th2 responses to parasitic infections are associated with alternative activation of macrophages (AAMΦ). Pathogen invasion also results in tissue damage that introduces additional danger signals in their extracellular environment. Hence, immune cells are sensitive to any changes in extracellular nucleotide levels due to purinergic receptor surface expression and activation. Nucleotide-induced effects are controlled by enzymes capable of metabolising them to their respective nucleoside by hydrolysis of terminal phosphates. Consequently, different purinergic receptors are engaged resulting in switches between pro- and anti-inflammatory responses. Enzymes secreted by parasites such as *T.spiralis* have similar properties to these endogenous enzymes suggesting a way to mimic host regulation of purinergic signalling in order to evade the immune response (Gounaris and Selkirk, 2005). In this study, the aims were to characterise murine bone marrow derived macrophages (BMMΦ) and murine spleen derived T cells, in terms of their purinergic receptor profile and functionality. In addition, this study aimed to investigate nucleotide-induced responses in macrophages and T cells as well as the effect of *T.spiralis* secreted products on their phenotype and activation.

In this study, the purinergic receptor profile of BMMΦ that were differentiated *in vitro* was shown to include G-protein coupled purinergic receptors except for the $A_{2A}R$ and P2Y4R, although the $A_{2A}R$ mRNA expression was up-regulated in response to LPS (CAMΦ), an

observation that has also been made by Murphree and colleagues in human and mouse macrophages (Murphree *et al*, 2005). Interestingly, the A_{2A}R has been shown to be involved in the macrophage switch from an inflammatory to an angiogenic, anti-inflammatory phenotype in the presence of LPS stimulation (Grinberg *et al*, 2009). Furthermore, the switch to a wound healing phenotype has been shown to result from synergy of the A_{2A}R with MyD88 recruited following LPS stimulation (Macedo *et al*, 2007). Stimulation of BMMΦ with IL-4 (AAMΦ) did not change the purinergic receptor profile detected in the absence of IL-4, except for a slightly up-regulated A_{2B}R expression which was also achieved by LPS stimulation, showing that only A_{2A}R induction in macrophages is LPS-specific (Chapter 3). This is in agreement with quantitative studies in RAW 264.7 macrophages showing that LPS stimulation up-regulated both the A_{2A}R and A_{2B}R (Streitova *et al*, 2010). Furthermore, it has been shown that, TLR4 stimulation of human lung macrophages induces up-regulated expression of the A_{2A}R as a means of controlling inflammation, due to its down-regulatory properties on pro-inflammatory cytokine production and inhibition of inflammatory cell recruitment (Buenestado *et al*, 2010). Similarly, it has been shown that glucocorticoids can up-regulate the A_3R, stimulation of which leads to activation of ERK which is responsible for protection of monocytes, suggesting a mechanism for down-regulation of inflammation (Barczyk *et al*, 2010). In addition, Xaus and colleagues have shown that IFN-γ up-regulates the A_{2B}R in bone marrow derived macrophages by *de novo* synthesis, while Khoa and colleagues have shown that the inflammatory cytokines IL-1 and TNF-α regulate A_{2A}R function by inducing its expression at both the mRNA and protein levels (Xaus *et al*, 1999; Khoa *et al*, 2001). Therefore, these data show that Th1 cytokines like IFN-γ and IL-1 that promote classical activation of macrophages, as does TNF-α, have similar effects as LPS stimulation of TLR4.

The modulation of P1R expression becomes more important when considering that extracellular nucleotides can act on these receptors, following metabolism by ecto-nucleotidases to adenosine, which is a known regulator of innate immune responses (Kumar and Sharma, 2009). In the current study, all nucleotides that could be degraded to adenosine had an inhibitory effect on macrophage pro-inflammatory functions such as nitric oxide (NO), TNF-α and IL-6 release, while they enhanced IL-10 release and arginase I activity. This is in agreement with published work on RAW 264.7 macrophages showing that, adenosine-induced effects are generally anti-inflammatory due to P1R activation as binding of P1R agonists such as adenosine or its metabolite inosine was found to negatively regulate pro-inflammatory NO production (Hasko *et al*, 1996; Min *et al*, 2000). These adenosine-induced effects were pharmacologically characterised as A_2R-specific and cAMP-mediated (Chapter 4). In fact, activation of the A_{2B}R enhanced IL-10 release while pro-inflammatory TNF-α and IL-6 release was inhibited by activation of both A_{2A} and A_{2B} receptors. In support of this finding, it has been reported that the A_{2A}R is responsible for adenosine-induced inhibition of TNF-α production in murine peritoneal macrophages (Ezeamuzie and Khan, 2007). In addition, adenosine receptor agonists have been shown to differentially regulate IL-10, TNF-α and NO production in RAW 264.7 macrophages (Hasko *et al*, 1996; Hu *et al*, 1998). Adenosine was also shown to inhibit TNF-α release by a human macrophage cell line via the A_3R (McWhinney *et al*, 1996; Sajjadi *et al*, 1996). In fact, A_3R activation in RAW 264.7 macrophages inhibits LPS-induced TNF-α by Ca^{2+}-dependent activation of NF-κB and ERK (Martin *et al*, 2006). The P1 receptors have also been shown to be involved in inhibition of the LPS-induced respiratory burst in human monocytes (Thiele *et al*, 2004).

Taking into account that ecto-nucleotidases were found to be present and functional on all macrophage activation subsets, nucleotide hydrolysis was probably combined with P1R stimulation in this study. Specifically, it has been reported that ecto-nucleotidase generation of adenosine and activation of the $A_{2B}R$ are coordinated in hypoxic conditions (Eltzschig *et al*, 2003). Therefore, ATP and ADP-induced effects can depend on their conversion to AMP and adenosine, as was the case in the current study, and has also been reported previously by Moody and colleagues (Moody *et al*, 1984). However, ATP can also affect macrophage activation by stimulation of P2 receptors. It is known that ATP causes stimulation of the respiratory burst by rat alveolar macrophages as a result of P2R activation leading to Ca^{2+} mobilization (Murphy *et al*, 1993). In addition, extracellular ATP has been shown to enhance mRNA levels of iNOS and TNF-α in LPS-treated RAW 264.7 macrophages (Tonetti *et al*, 1995). This was later found to be due to the P2XR (Sperlagh *et al*, 1998). Nevertheless, P2YR agonists were also shown to inhibit iNOS expression by macrophages via Ca^{2+} signalling (Denlinger *et al*, 1996). However, as shown in Chapter 4, ATP effects on macrophage effector functions were found to be predominantly due to its conversion to adenosine, although it is possible that P2YR stimulation was masked by P1R signalling.

Macrophage activation phenotypes, characterised by the presence of known markers of alternative activation, can potentially respond differently to purinergic stimulation as a result of the variations in their P1R profile detected in this study. On the other hand, this study has shown that A_2R activation causes up-regulation of one of these markers of alternative activation. Specifically, arginase I was up-regulated by A_2R-dependent mechanisms and its activity enhanced by $A_{2B}R$ activation, thus shifting macrophage L-arginine metabolism from iNOS to arginase, which effectively suppresses NO release. As presented in Chapter 3, BMMΦ and

AAMΦ were not found to express the $A_{2A}R$ at the molecular level. Therefore, the fact that the $A_{2A}R$ agonist had an effect on AAMΦ arginase I activity points to agonist activation of other P1 receptors, most likely the $A_{2B}R$, possibly due to non-specific binding. In fact, cross-talk between Gs and Gq coupled pathways following $A_{2B}R$ activation in human mast cells has been shown to regulate IL-4 release (Ryzhov *et al*, 2006). This may be an indirect means of controlling the macrophage activation phenotype by producing a cytokine environment that promotes alternative activation.

Arginase I expression is known to be controlled by the presence of Th2 polarising cytokines and, in particular, by the Th1/Th2 balance (Munder *et al*, 1999). Interestingly, CAMΦ were also found to express arginase I although the levels detected were not comparable to levels obtained following IL-4R stimulation in AAMΦ (Chapter 3). In support of this finding, arginase I mRNA and protein have been shown to be up-regulated in rodent peritoneal macrophages stimulated by LPS (Louis *et al*, 1998). The ability of macrophages to express both enzymes of the L-arginine metabolic cascade allows them to respond to changes in their local environment that can control their activation phenotype. For example, Rutschman and colleagues have reported that NO production is regulated by substrate depletion of iNOS as a result of IL-4R activation which up-regulates arginase I activity (Rutschman *et al*, 2001). In addition to IL-4-dependent regulation of arginase I activity, the current study showed that IL-10 induced up-regulation of arginase I in synergy with IL-4/IL-13 (Chapter 3). Considering that the arginase I mRNA appears at a later time point of CAMΦ culture, its up-regulation may also be due to autocrine stimulation effects of the IL-10 produced by CAMΦ. In fact, Hesse and colleagues have reported arginase I activity in macrophages stimulated with IFN-γ/TNF-α which is attributed to autocrine production of IL-10 (Hesse *et al*, 2001). This observation led the authors to suggest that this may be a

mechanism used to counterbalance NOS activity during Th1 responses. Furthermore, autocrine IL-10 has been shown to suppress production of pro-inflammatory mediators (Yan *et al*, 2001). This observation was also made in the current study (Chapter 4) whereby neutralisation of IL-10 released by macrophages led to enhancement of TNF-α and IL-6 secretion.

In the current study, cAMP was found to be the major downstream effector of P1R signalling implying coupling to Gs subunits (Sitkovsky *et al*, 2008). This is in agreement with the pharmacological evidence showing that adenosine and adenine nucleotide-induced effects were mediated by A_2R stimulation (Chapter 4). In support of these findings, studies have shown that cAMP involvement downstream of adenosine stimulation of the A_2R is responsible for manipulating macrophage effector functions. For example, the $A_{2A}R$ has been shown to inhibit matrix-induced inflammation by signalling involving cAMP generation (Scheibner *et al*, 2009). Furthermore, elevated levels of cAMP were found to reduce NO production in a macrophage cell line while in a study by Beshay and colleagues using the cAMP elevators rolipram and forskolin, macrophage suppression of NO production both *in vitro* and *in vivo* was shown to be a result of regulation of iNOS mRNA levels (Bulut *et al*, 1993; Beshay *et al*, 2001). Interestingly, cAMP is generated by adenylate cyclase from the intracellular pathogen *M.tuberculosis* to manipulate macrophage signalling and, in particular, TNF-α production (Agarwal *et al*, 2009). Studies in RAW264.7 macrophages have shown that the presence of cAMP affects activation of NF-κB by LPS, as stimulation with LPS in combination with a cAMP agonist led to enhancement of p50-p50 dimers, which are considered to be transcriptional repressors at NF-κB-dependent promoters (Wall *et al*, 2009). This was proposed by the authors to be partly responsible for the cAMP-mediated suppression of pro-inflammatory cytokines induced by LPS. Chen and colleagues have shown that LPS-induced effects involve activation of kinases such as PKC, ERK, and p38 MAPK

and activation of the latter is necessary for induction of iNOS in RAW264.7 by LPS-induced activation of NF-κB (Chen and Wang, 1999; Chen *et al*, 2001). The same authors have reported that MEK inhibition had no effect on LPS-induced signalling, which is relevant to the current study showing that adenosine-induced signalling can be inhibited by interference with MEK as opposed to LPS-induced signalling. In support of this, it has been reported that Gs coupled to the $A_{2A}R$ signals via ARNO to MEK1/2 before ERK activation of Fos-induced transcription (Zezula and Freissmuth, 2008).

The ability of macrophages to switch between activation states has been reported in a number of studies (Porcheray *et al*, 2005; Mylonas *et al*, 2009). This allows macrophages to act more effectively as the first line of defence since inflammation can thus be resolved without the requirement to recruit new macrophages with the appropriate phenotype. Porcheray and colleagues reported that monocyte-derived macrophages of a specific phenotype can switch to an alternate phenotype quickly upon stimulation (Porcheray *et al*, 2005). Furthermore, Mylonas and colleagues showed that peritoneal macrophages obtained from *B.malayi* infection in an alternatively activated state could be switched to a classically activated state upon LPS/IFN-γ stimulation (Mylonas *et al*, 2009). Macrophage phenotype switching may play a key role in their involvement in infections. In fact, as the current study has shown, *T.spiralis* secreted proteins (ES) up-regulated arginase I in BMMΦ and FizzI and YmI in CAMΦ skewing their phenotype to an alternative activation mode. Furthermore, ES up-regulated $A_{2A}R$ expression in BMMΦ as well as CD73 expression in AAMΦ indicating a possible mechanism of macrophage switching to the anti-inflammatory phenotype. This is further supported by the fact that ES up-regulated the IL-4R making macrophages more responsive to stimulation that would result in alternative activation. The fact that *T.spiralis* ES interferes with P1R expression points to host cell manipulation of

purinergic signalling by making cells more responsive to adenosine-induced anti-inflammatory effects. In addition, any pro-inflammatory responses elicited by ES, such as NO and TNF-α release, were inhibited by adenosine-induced cAMP elevation (Chapter 5). As it was established in Chapter 3, BMMΦ and AAMΦ do not express the $A_{2A}R$, therefore, adenosine-induced effects could be mediated via the $A_{2B}R$, which confirms studies that show involvement of both receptors in adenosine-induced effects (Hasko *et al*, 2000; Csoka *et al*, 2010). However, it is also possible that adenosine acts via the $A_{2A}R$ which was found to be up-regulated in macrophages treated with *T.spiralis* ES (Chapter 3).

The *T.spiralis*-induced skewing of macrophage activation to the AAMΦ phenotype observed in the current study is not surprising as AAMΦ have been proposed to have several roles in parasite infection (Maizels *et al*, 2004). Firstly, they act to suppress inflammatory responses to the parasite as they are known to down-regulate other effector cell pro-inflammatory functions. In addition, their abundance in sites of infection makes them important effector cells which potentially target extracellular helminths and drive Th2 immune responses. Finally, their biosynthetic ability may be important in helminth-induced damage repair. *T.spiralis* ES enhanced both pro-inflammatory and anti-inflammatory cytokine release as well as NO release and arginase I activity (Chapter 5). This is in agreement with published work showing that *T.spiralis* infection increased IL-10 and TGF-β production in mouse airway tract (Park *et al*, 2011). The ES-induced effects elicited in macrophages in the current study resulted from the direct action of glycans conjugated on ES proteins, and this was confirmed by the fact that removal of these carbohydrates abrogated ES-induced effects. Furthermore, the receptor responsible for recognition of glycans on macrophages was not the mannose receptor, as might be expected due to its ubiquitous expression on these immune cells, but the macrophage galactose lectin (MGL)

which was determined by blocking C-type lectins on macrophages. Evidently, *T.spiralis* employs a similar mechanism of direct host cell manipulation to other helminths which skew immune cell responses by interaction of carbohydrates with pattern recognition receptors (PRRs). In fact, it is known that N-linked glycans are present in *T.spiralis* ES (Reason *et al*, 1994; Morelle *et al*, 2000). Furthermore, it has been reported that *T.spiralis* TSL-1 antigens are highly immunogenic and contain glycoproteins with GalNAc residues (Yepez-Mulia *et al*, 2007). In this study, *T.spiralis* ES was deglycosylated by chemical means which are known to remove 0- and N-linked glycans and, in this way, inhibition of ES-induced effects was achieved (Reason *et al*, 1994; Edge, 2003).

Helminth parasites, like all pathogens, are first encountered by APCs which are the first line of defence, however, not all APCs respond in the same way. Although macrophages and dendritic cells (DCs) have almost identical basal gene expression patterns, macrophages exposed to microfilaria are known to produce both IL-12 and IL-10 while DCs do not (Semnani and Nutman, 2004). The data obtained in the current study similarly showed that macrophages respond to parasite products from *T.spiralis* by producing cytokines such as IL-10, IL-6 and TNF-α. Many studies have shown that helminth parasites mediate their effects on APCs by direct interactions with PRRs, like C-type lectins and TLRs, or by regulation of their expression. For example, live *B.malayi* microfilaria can activate TLR2 directly, but not TLR4 or TLR3 (Venugopal *et al*, 2009). In addition, it has been shown that filarial nematode ES-62 can activate TLR4 via phosphorylcholine (PC), although signalling was not found to be similar to that induced by TLR4 when activated by LPS (Harnett *et al*, 2010; Harnett and Harnett, 2010). *S.mansoni* secreted products activate DC TLR2 and TLR4 due to the presence of phosphatidylserine (PS) but the resulting DC activation profiles are distinct from those obtained following bacterial activation (Van Riet *et al*, 2009). In fact, it has been reported that parasites

use TLR4 as a co-receptor for PRRs to induce type II responses important in their expulsion as well as in host tissue repair (Diaz and Allen, 2007). Interestingly, it was recently reported that, Korean mistletoe lectin, which has been shown to bind GalNAc and galactose, activates mouse peritoneal macrophages via TLR4 (Park *et al*, 2010). Another C-type lectin expressed on macrophages, called mincle, was shown to be responsible for the *C.albicans*-induced TNF-α release from murine macrophages (Wells *et al*, 2008). Furthermore, Dissanayake and colleagues have reported that *T.crassiceps* carbohydrates induce IL-6 and IL-10 release by macrophages (Dissanayake *et al*, 2002).

The importance of AAMΦ in parasitic infections is further emphasised by the fact that *M.corti* infections of IL-4-deficient mice were lethal due to reduction of Th2 cytokines and enhancement of Th1 cytokines leading to reduced generation of AAMΦ (O'Connell *et al*, 2009). In addition, alternative activation of macrophages was shown to enhance survival in *S.mansoni* infection due to the fact that they down-regulate Th1 responses and iNOS toxic effects (Herbert *et al*, 2004). On the other hand, *T.spiralis* has been shown to down-regulate iNOS during the gut inflammatory phase, which again points to alternative activation of APCs, as they are the main producers of NO during inflammation (Bian *et al*, 2001). However, in the current study *T.spiralis* ES was found to increase NO release by macrophages. This may be an initial event when macrophages encounter ES that is replaced by activation of arginase I later on. In fact, arginase I was also up-regulated in this study although it was not consistent in all macrophage subsets, which may imply that it is indeed a response that develops later in the stimulation process. Furthermore, ES was found to increase both pro- and anti-inflammatory cytokine release. Interestingly, TNF-α has been reported to be involved in the development of the Th2 response required for expulsion of *T.spiralis* (Ierna *et al*, 2009). Nevertheless, *T.spiralis* effects on macrophages appear to depend on the presence of AAMΦ characteristics like MGL expression,

which has recently been characterised as a marker of AAMΦ recruited in parasite infections (Raes *et al*, 2005).

Signalling events downstream of *T.spiralis*-induced effects were found to involve activation of all signalling pathways tested to some extent, although the cAMP/PLC and MEK1/2 pathways appeared to be involved consistently. Specifically, the increase in NO and IL-6 release was inhibited by targeting PLC activation, while cAMP elevation was found to reverse ES-induced effects in general. This could point to reciprocal control of the signalling pathways involved since cAMP elevation inhibited PLC activation, thus acting like an indirect PLC inhibitor. The involvement of MEK1/2 could be explained by the fact that MGL activation induces Ras-Raf signalling which is upstream of MEK1/2. It has been shown that signalling pathways originating from the cytoplasmic domain of proteins containing the YXXL motif, which is also found a C-type lectin called Dectin-1, result in transcription and release of IL-10 (Brown, 2006; Saba *et al*, 2009). In the same study, the authors reported that murine macrophages lacking MGL-1 produced less IL-10 suggesting that intracellular signalling triggered by MGL activation may also require this motif. Therefore, it is possible that activation of MGL by *T.spiralis* ES triggers YXXL-mediated recruitment of factors that initiate intracellular signalling via the MEK-ERK pathway, which then activates transcription and release of IL-10. Similarly, another carbohydrate derived from *S.mansoni* called LNFPIII activates DC-SIGN which has been shown to signal via ERK (Harn *et al*, 2009). Another mode of activation of macrophages by *T.spiralis* ES was found in guinea pig alveolar macrophages involving PKC phosphorylation and this was shown to be due to PAFR activation by PC present in *T.spiralis* homogenates (Dzik *et al*, 2010). Therefore, the fact that *T.spiralis* ES can potentially activate different pathways may explain the up-regulation of both pro- and anti-inflammatory mediators detected in this study (Chapters 3 and 4).

T cell responses are either due to direct activation of T cells or to APC-mediated stimulation either by soluble mediators or by contact-dependent mechanisms, or by a combination of both. It has been reported that IL-6 induces the expression of IL-4 by T cells leading to Th2 differentiation while at the same time induction of IFN-γ inhibits Th1 differentiation (Diehl and Rincon, 2002). Fiorentino and colleagues have reported that IL-10 (commonly derived from APCs) inhibits IL-12 and IFN-γ production by Th1 cells impairing APC function by contact dependent mechanisms (Fiorentino *et al*, 1991). Regulatory T cells were reported to induce high levels of IL-10 production by APCs as well as stimulating co-stimulatory molecule expression, rendering them immunosuppressive (Kryczek *et al*, 2006). Furthermore, studies have shown that IL-10 can inhibit Th1 cells in the presence of macrophages, and this may be an indirect effect resulting from macrophage expression of T cell stimulatory or inhibitory products or by MHCII regulation (de Waal Malefyt *et al*, 1991; Bogdan *et al*, 1991). On the other hand, Schebesch and colleagues showed that human AAMΦ inhibited CD4+ T cell proliferation and that this was not dependent on IL-10, NO or costimulatory molecules (Schebesch *et al*, 1997). An important mechanism by which macrophages are known to modulate T cell function is L-arginine consumption, which has been shown to affect the CD3ζ chain in T lymphocytes, and therefore their ability to be activated via the T cell receptor (TCR) (Rodriguez *et al*, 2003). Since AAMΦ are known to have up-regulated arginase I levels and activity, an observation also made in the current study, this suggests that generation of a macrophage anti-inflammatory phenotype will affect T cell activation. Furthermore, macrophage-expressed Fizz1 (RELM-α), which is another established marker of alternative activation, has also been reported to suppress T cell proliferation (Nair *et al*, 2009).

As this study has shown, activation of purinergic signalling via the P1 receptors by adenosine can influence macrophage activation, skewing it to an AAMΦ phenotype with

characteristic up-regulation of arginase I activity. Therefore, the mechanism of T cell inhibition via depletion of L-arginine outlined above could be partly responsible for adenosine-induced effects on T cells, at least *in vivo*. Indeed, adenine nucleotides inhibited T cell proliferation and cytokine release and, although this was found to be a result of P1R activation on T cells, the indirect effect of adenosine on arginase I levels cannot be ruled out. However, since APCs were irradiated in this study, transcription and translation were minimal, therefore, the effects were probably due to the direct action on T cells. However, it should be taken into account that, in an *in vivo* setting, soluble mediators produced by macrophages, such as the ones tested in this study, will also influence T cell proliferation and cytokine production.

It has been established in this study that, purinergic signalling effects on T cell effector functions were due to adenosine generated in culture by ecto-nucleotidases CD39 and CD73 which were functionally expressed on T cells. It is now widely accepted that these ecto-nucleotidases are better markers of Treg cell activation than CD25, which was the original marker of Treg activation together with Foxp3 (Zarek *et al*, 2008). In fact, CD73 has also been reported to play a co-stimulatory role in T cell activation (Resta *et al*, 1998). However, the major role of CD73 is the generation of extracellular adenosine from adenine nucleotides, like ATP, which are released by T cells and first metabolised by CD39 to AMP (Di Virgilio *et al*, 2009). In the current study, activation of A_2 receptors by adenosine inhibited T cell proliferation as well as Th1 and Th2 responses. Adenosine has been shown to shift T cell differentiation to a regulatory phenotype by increasing TGF-β production and decreasing IL-6 production following $A_{2A}R$ activation (Zarek *et al*, 2008). This has been described as a means of inducing peripheral tolerance (Zarek *et al*, 2008; Sitkovsky *et al*, 2009). Activation of the $A_{2A}R$ was shown to be involved in suppression of pro-inflammatory cytokine production in T cell-mediated colitis while IL-10 and TGF-β production remained unaffected (Naganuma *et al*, 2006). Although $A_{2A}R$

activation inhibited cytokine secretion by CD4+ T cells in this study, it has been shown that activation of the $A_{2A}R$ in natural killer T cells enhanced IL-4 and IL-10 secretion, suggesting that not all T cells respond to adenosine in the same way (Nowak *et al*, 2009).

With reference to intracellular signalling events involved in T cell suppression, AAMΦ have been shown to inhibit T cell proliferation by STAT6-dependent mechanisms by up-regulating PD-L2, which is a B7 family member that binds Th2 cells (Huber *et al*, 2010). This confirms previous studies showing that the inhibition of T cell proliferation by nematode-elicited AAMΦ is achieved by cell contact (Loke *et al*, 2000). With relevance to the current study, *T.spiralis*-induced effects on T cell effector functions could either be due to direct activation of CD4+ T cell PRRs by ES or by cytokines produced by APCs in response to ES. In addition, *T.spiralis* ES was shown to up-regulate expression of the low affinity $A_{2B}R$ by T cells while expression of the high affinity $A_{2A}R$ was inhibited, making cells more responsive to low levels of extracellular adenosine. The inhibitory effects of extracellular adenosine on T cell effector functions have been attributed to increases in intracellular cAMP which antagonises TCR-signalling (Koshiba *et al*, 1997). A study has shown that a stable analogue of ATP also inhibits CD4+ T cell cytokine secretion by inhibiting TCR signalling and this was found to involve inhibition of ERK and p38 MAPK downstream of cAMP activation (Duhant *et al*, 2005). In the current study, MEK1/2 and cAMP were found to be responsible for both ES and adenosine-induced proliferation effects, in agreement with the studies discussed above.

Taking into account that APCs are the first to encounter invading organisms, the type of compounds recognized by these cells will determine to a large extent the nature of T cell polarisation promoted by APCs. For example, AAMΦ isolated from *T. crassiceps*-infected mice were shown to inhibit naïve T cell proliferation (Reyes and Terrazas, 2007). In addition,

macrophages isolated from mice treated with *N.brasiliensis* ES were also found to suppress T cell proliferation *in vitro* (Allen and McDonald, 1998). Th1 cell recruitment in response to *M.tuberculosis* as well as the IFN-γ-dependent functions were not affected by macrophage IL-10, which caused macrophage alternative activation in an autocrine manner (Schreiber *et al*, 2009). However, filarial infection induced IL-10 expression by CD4+ cells that affected IFN-γ and IL-12 levels in a malaria infection model (Metenou *et al*, 2009). In the case of microfilarial infection it was reported that inhibition of cellular proliferation was not due to macrophage derived factors like NO, prostaglandins and catalase (Allen *et al*, 1996). On the other hand, NO has been shown to inhibit Th1 cell expansion while low levels of NO promoted differentiation of Th1 cells by IL-12R induction (Niedbala *et al*, 2006). In the current study, NO released by macrophages in response to *T.spiralis* ES could, therefore, influence T cell differentiation promoting a Th2 or even a Treg phenotype. In fact, Belkaid and colleagues have found that regulatory T cells are the main producers of IL-10 in *S.mansoni* infected mice and high levels of this cytokine were also secreted by T cells in the current study (Belkaid *et al*, 2006). However, Hesse and colleagues report that although IL-10 is important for polarizing the schistosome egg-specific Th2 response, it is not essential to generate alternatively activated, arginase I expressing cells, although synergistic effects between IL-10 and IL-4/-13 remain possible (Hesse *et al*, 2001). In support of this, Zhao and colleagues have reported that macrophages are not required for initiation of the cytokine response to *N.brasiliensis* infection and are activated as a result of increased Th2 cytokine production (Zhao *et al*, 2009). Furthermore, Treg cell expansion in filarial infection was found to occur even in the absence of AAMΦ and a Th2 response showing that immune response is shifted toward Treg differentiation (McSorley *et al*, 2008). In fact the authors suggest that Tregs are the first cells to be recruited in filarial infection and AAMΦ development is a secondary event. This hypothesis is further supported by another study reporting that human

AAMΦ are activated in the presence of Tregs (Tiemessen *et al*, 2007). They showed that Tregs can produce IL-10 and IL-4/13 which, in co-cultures with macrophages enhance alternative activation markers, while they inhibit TNF-α and IL-6 and enhance IL-10 production. With relevance to the current study which showed that *T.spiralis* ES increased expression of the Treg cell marker CD73, Tregs have been found to be recruited to airways of *T.spiralis* infected animals (Park *et al*, 2011). Regulation of effector T cells by APCs has also been shown to be due to MGL from APCs binding to T cell CD45, which is a protein tyrosine phosphatase that controls TCR activation via Lck dephosphorylation (Van Vliet *et al*, 2006). Interestingly, the *T.spiralis*-induced inhibition of T cell proliferation and IL-10 and IFN-γ release showed some MGL dependence (Chapter 6). Similarly, a surface lectin of *E.histolytica* was reported to suppress T-cell proliferation while promoting Th2 cytokine production (Loukas and Maizels, 2000). Another mechanism of T cell responsiveness by parasites has been reported whereby filarial infection leads to reduced TLR expression by T cells leading to reduced cytokine production, such as IFN-γ and IL-2 (Babu *et al*, 2006). As opposed to its effects on other T cell effector functions, *T.spiralis* ES induced an increase in IL-4 release (Chapter 6). This finding is supported by the fact that, although in some cases IL-4/IL-13 produced by innate cells may be sufficient to trigger their differentiation, Th2 cells are generally required for their optimal activation, recruitment and expansion. It is likely that the lack of Th2-dependent signals in other systems of study, like *in vitro*, is the reason why the IL-4 and IL-13 activated AAMΦ phenotype is considerably less intense in nature than the AAMΦ generated during a natural infection (NeMΦ).

In summary, this study has shown that *T.spiralis*, like other parasites, has evolved to modulate the host immune response on different levels. Manipulation of host purinergic signalling by *T.spiralis* secreted enzymes has been hypothesised (Gounaris and Selkirk, 2005) and this study has confirmed that macrophages and T cells are also a part of this mode of immune

suppression. In addition to this indirect modulation of macrophage signalling, this study has shown that *T.spiralis* secreted proteins can also influence macrophage functions by binding to MGL. It is known that macrophages provide a link between innate and adaptive immune responses by contact-dependent and cytokine-dependent activation of T cells, therefore, the macrophage phenotype influences the T cell subsets that develop. *T.spiralis* secreted proteins used in this study were found to induce both Th1 and Th2 responses either by direct effects on T cells or by switching the macrophage activation phenotype. It is evident from this study that, *T.spiralis* can evade host immune responses by regulating the activation of immune cells in a way that is beneficial for its survival throughout the different stages of infection allowing it to establish itself successfully in the host.

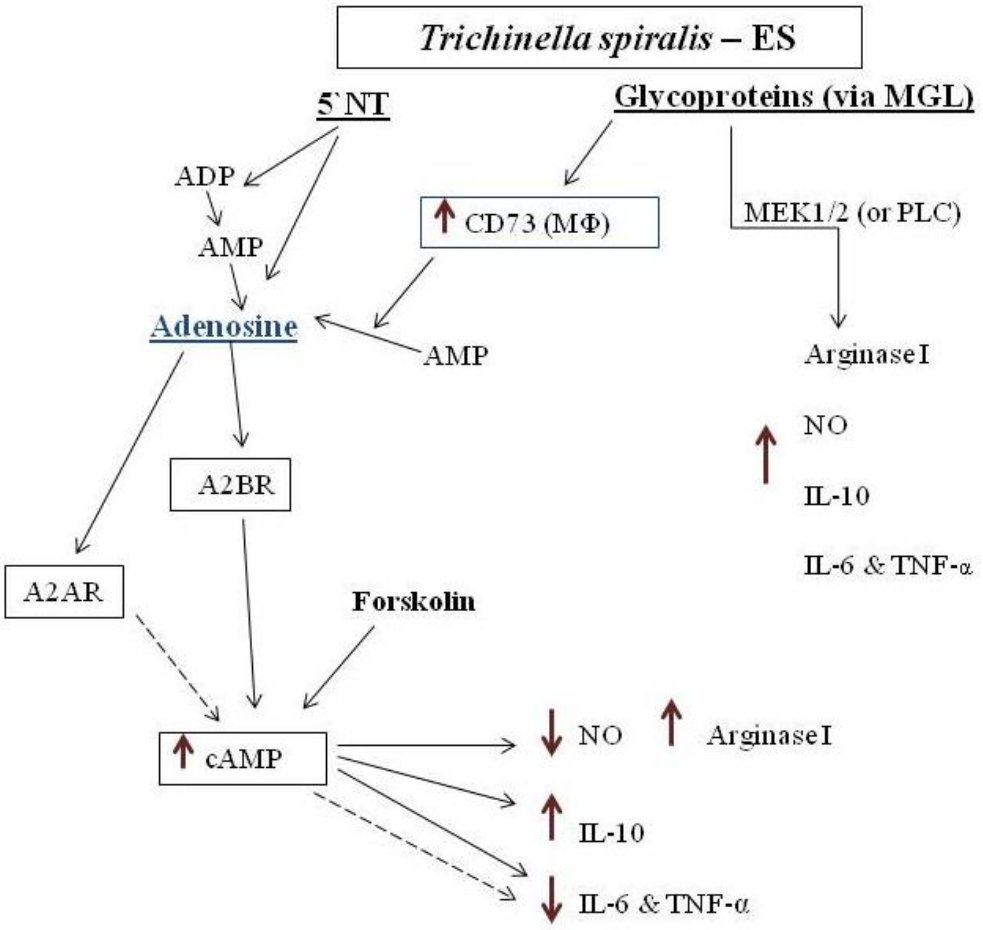


Figure 7.1: Summary of *T.spiralis* ES-induced effects on BMMΦ. *T.spiralis* ES affects BMMΦ effector functions in 2 ways. Firstly, 5`-nucleotidase (5`NT) converts ADP to AMP and AMP to adenosine which acts on A2 receptors ($A_{2A}R$ or $A_{2B}R$). This results in the elevation of cAMP levels in macrophages causing an increase in arginase activity and IL-10 release and inhibition of nitric oxide (NO) production, IL-6 and TNF-α release. Forskolin, an exogenous elevator of cAMP, was used in this study to confirm the cAMP involvement in macrophage effector functions. Secondly, ES glycoproteins also affect macrophage effector functions via activation of the MGL (macrophage galactose lectin) receptor which results in elevation of arginase activity, NO production as well as cytokine release. This was shown to proceed via the MAP kinase kinase (MEK1/2) pathway. In addition, ES induced an elevation in CD73 expression levels which further enhances extracellular adenosine generation.

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Appendix

A.1 Polyacrylamide gels

Stacking gel

Final concentration	Volume(ml)/5ml
30% v/v Acrylamide mix	0.83
1M Tris (pH 6.8)	0.63
10% v/v SDS	0.05
10% v/v Ammonium persulphate (APS)	0.05
TEMED	0.0005
dH_2O	3.4

Resolving gel (12%)

Final concentration	Volume(ml)/5ml
30% v/v Acrylamide mix	2
1M Tris (pH 8.8)	1.3
10% v/v SDS	0.05
10% v/v Ammonium persulphate (APS)	0.05
TEMED	0.002
dH_2O	1.6

A.2 5′NT Expression and Purification

Recombinant 5′-NT was expressed and purified as described in Materials and Methods. The purified proteins were resolved on an SDS-PAGE gel to confirm presence of pure recombinant 5′-NT. Western analysis carried out is shown in Figure 1 below. Following purification, r5′-NT was assayed for nucleotidase activity using a phosphate release assay as described previously. The specific activity when 5′-NT was incubated with AMP was calculated to be 10 μmol Pi/min/mg.

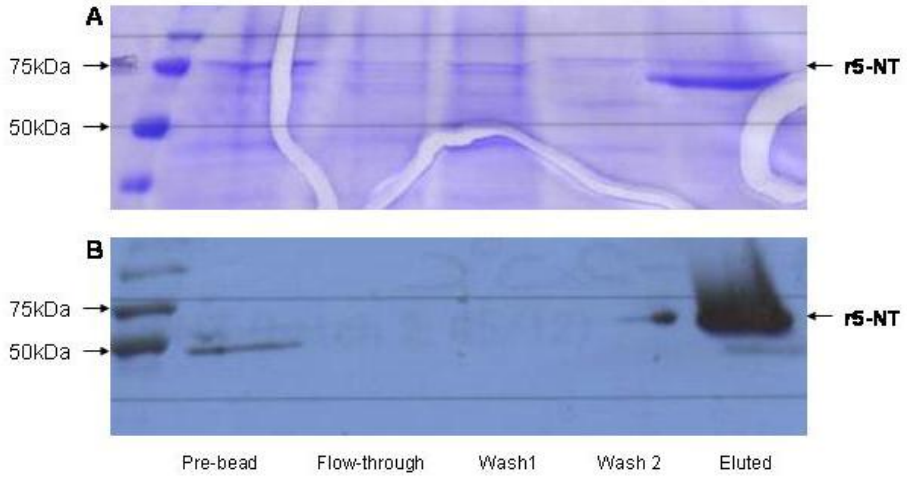


Figure A.1: Recombinant 5-nucleotidase (r5′NT) expression and purification. The c-myc/6His – tagged *T.spiralis* recombinant 5′NT was expressed from transformed *P.pastoris* and purified by Ni-NTA affinity chromatography. Purified protein was visualised by Coomasie staining (A) and Western analysis using the ECL detection system (B). The expected size of 5′NT is 67kDa.

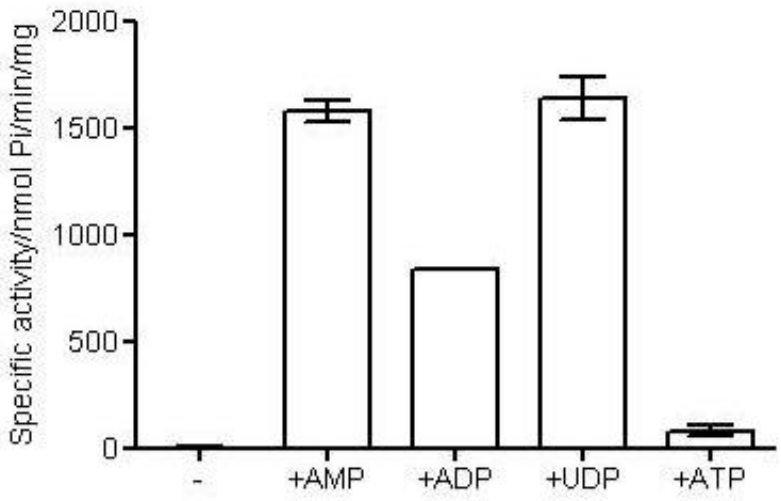


**Figure A.2: 5`NT substrate specificity.** AMP, ADP, UDP and ATP were incubated with 5`NT for 10 minutes and the nmoles of phosphate released per mg of protein calculated. This was done by detecting changes in absorbance of a chromogenic detector of free phosphate. The specific activity of 5`NT for every nucleotide is plotted here.

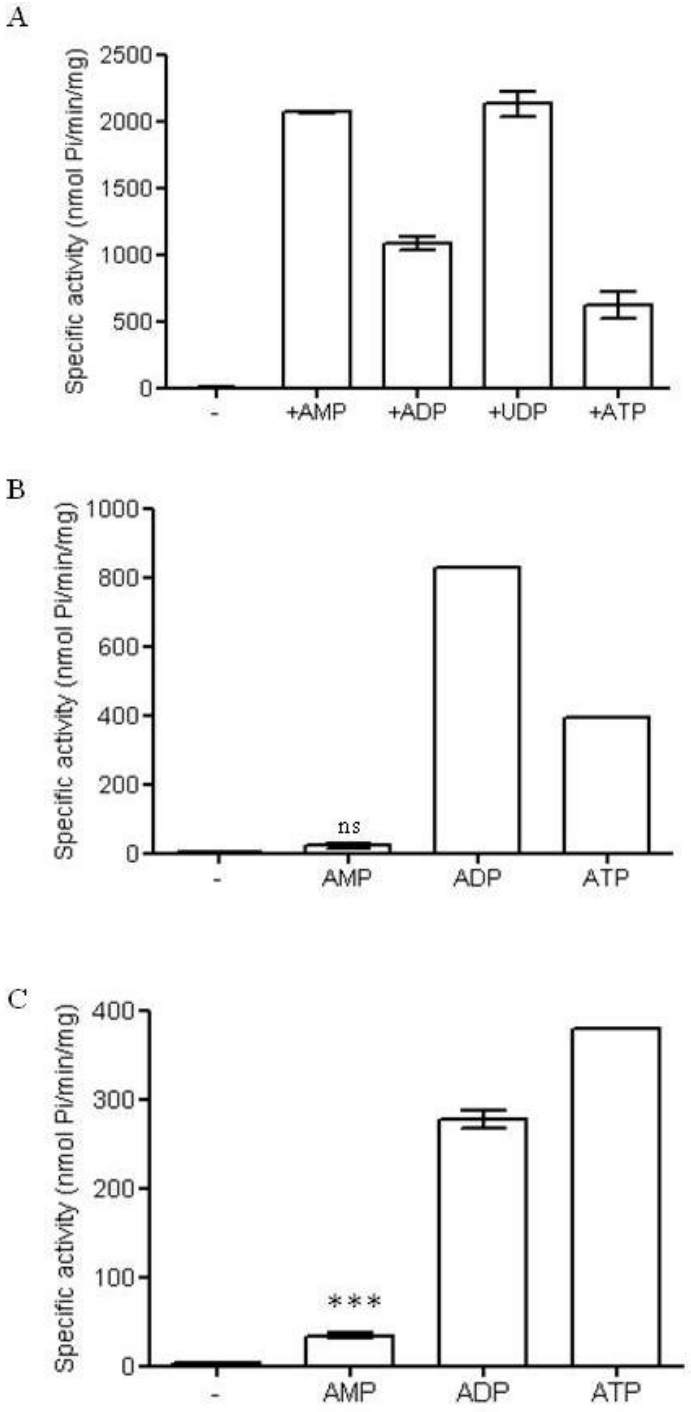


Figure A.3: Parasite ES substrate specificity. AMP, ADP and ATP were incubated with ES from (A) *T.spiralis*, (B) *B.malayi* microfilaria and (C) *B.malayi* adult ES for 10 minutes and the nmoles of phosphate released per mg of protein calculated. This was done by measuring changes in absorbance of a chromogenic detector of free phosphate. The specific activity of ES proteins for every nucleotide is plotted here. *** $p < 0.001$, ns - not significant ($p > 0.05$) compared to the untreated control.

Sequencing non-specific bands

A.3 Sequencing

PCR products were cloned into pCR2.1-TOPO (Figure 2) for sequencing (Cogenics). The sequences obtained were then aligned to known transcripts using BLAST and then ClustalW was used to align to the primers used for the PCR reactions to determine sequence homology.

A3 receptor clones

Sequences producing significant alignments to Sequence 1 (260bp):

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NM_009631.3	Mus musculus adenosine A3 receptor (Adora3), transcript variant 1, mRNA	472	472	100%	1e-130	99%

```
Sequence 1  AGACCTGCATCCTCCAGGTTAATGGGAAGGCAGGGATAAGCGGCTGAGGTTGATGGTCCT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NM_009631.3  AGACCTGCATCCTCCAGGTTAATGGGAAGGCAGGGATAAGCGGCTGAGGTTGATGGTCCT  187

Sequence 1  GTAACAGGCCCTTTCTGAGAAGAGTCTCTAAGAGAGAGTCATACCGGAAGGAATGAGCAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NM_009631.3  GTAACAGGCCCTTTCTGAGAAGAGTCTCTAAGAGAGAGTCATACCGGAAGGAATGAGCAA  247

Sequence 1 GTTGTGAGTTCTCTGGACTGTTGTGACATTCTAACTTCTGGGCAGAAGTCTGACAAGAGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NM_009631.3  GTTGTGAGTTCTCTGGACTGTTGTGACATTCTAACTTCTGGGCAGAAGTCTGACAAGAGC  307

Sequence 1  TGGGTCCACTGGCCCATACACAACCTGCTGAAGAGCCACGGAAGCTTCCGCCTGAAGCTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NM_009631.3  TGGGTCCACTGGCCCATACACAACCTGCTGAAGAGCCACGGAAGCTTCCGCCTGAAGCTT  367

Sequence 1  TTCTGCGACAGCATGGAA  258
            ||||| ||||||||||||
NM_009631.3  TTCTGAGACAGCATGGAA  385
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A3              CAGCAGAAGCCCTGACTCTGTTTGCCTGGGGAAGTAAGAACGGTGGCCCTCTCGGATTTG 120

F               ------------------------------------------------------------

A3              GCTGCAGAGACCTGCATCCTCCAGGTTAATGGGAAGGCAGGGATAAGCGGCTGAGGTTGA 180

F               -------AGACCTGCATCCTCCAGGTTAATG---------------------------- 24

                       ************************

A3              TGGTCCTGTAACAGGCCCTTTCTGAGAAGAGTCTCTAAGAGAGAGTCATACCGGAAGGAA 240

F               ------------------------------------------------------------

A3              TGAGCAAGTTGTGAGTTCTCTGGACTGTTGTGACATTCTAACTTCTGGGCAGAAGTCTGA 300

R               ------------------------------------------------------------

A3              CAAGAGCTGGGTCCACTGGCCCATACACAACCTGCTGAAGAGCCACGGAAGCTTCCGCCT 360

R               ------------------------------------------------------------

A3              GAAGCTTTTCTGAGACAGCATGGAAGCCGACAACACCACGGAGACGGACTGGCTGAACAT 420

R               ---GCTTTTCTGAGACAGCATGGAAGC-------------------------------- 24

                   ************************
```

Sequences producing significant alignments to Sequence 2 (100-200bp):

Accession	**Description**	E value	Max ident
XM_001478411.1	PREDICTED: Mus musculus similar to ferritin light chain 1 (LOC634386), mRNA	2e-40	96%
XR_031806.1	PREDICTED: Mus musculus similar to ferritin light chain 2 (LOC633082), misc RNA	2e-40	96%
NM_010240.2	Mus musculus ferritin light chain 1 (Ftl1), mRNA	2e-40	96%

```
Sequence 2      CCTCCAGGTTAATGAACCTCTCAAGCCACTAGGCAGCTTTGTAACCGCCCTGGAGCCTCT  70
                ||||||  | ||||||||||||||||||||||||||||||||||||||||||||||||
XM_001478411.1  CCTCCACCTGAATGAACCTCTCAAGCCACTAGGCAGCTTTGTAACCGCCCTGGAGCCTCT  938

Sequence 2      GTCAAGTCTTGGACCAAGTAAAAATAAAGCTTTTCTGAGACAGCA  115
                |||||||||||||||||||||||||||||| ||||||||||
XM_001478411.1  GTCAAGTCTTGGACCAAGTAAAAATAAAGCTTTT-TGAGACAGCA  982
```

```
A3 ns         GGGCTCCCCCCTCTGCTCTGCACCAGCCCGCCCTGGGACCTCCACCTGAATGAACCTCTC 900

F             ----------------------------AGACCTGCATCCTCCAGGTTAATG-------- 24

                                          * ****   ******  * ****

A3 ns         AAGCCACTAGGCAGCTTTGTAACCGCCCTGGAGCCTCTGTCAAGTCTTGGACCAAGTAAA 960

F             ------------------------------------------------------------

A3 ns         AATAAAGCTTTTTGAGACAGCAAAAAAAACAAAAAAACAAAAAAAAAAAAAAAAAAAAA 1020

R             -----GCTTTTCTGAGACAGCATGGAAGC------------------------------ 24

                     *** **********   **

A3 ns         AAAGAGATAACTTTAT 1036

R             ----------------
```

Arginase I clones

Sequences producing significant alignments to Sequence 1 (249bp):

Accession	Description	E value	Max ident
NM_007482.2	Mus musculus Arginase I, liver (Arg1), mRNA	1e-124	99%

```
Sequence 1    CAGATATGCAGGGAGTCACCCAGGAGAATCCTGGTACATCTGGGAACTTTCCTTTCAGTT  68
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NM_007482.2   CAGATATGCAGGGAGTCACCCAGGAGAATCCTGGTACATCTGGGAACTTTCCTTTCAGTT  531

Sequence 1    CCTTCAGGAGAAAGGACACAGGTTGCCCATGCAGATTCCCAGAGCTGGTCGTCAGGGGAG  128
              |||||||||||||||||||||||||||||||||||||||||||| ||||||||||
NM_007482.2   CCTTCAGGAGAAAGGACACAGGTTGCCCATGCAGATTCCCAGAGCTGGTTGTCAGGGGAG  471

Sequence 1    TGTTGATGTCAGCGTGAGCATCCACCCAAATGACACATAGGTCAGGGTGGACCCTGGCGT  188
                ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
NM_007482.2   TGTTGATGTCAGTGTGAGCATCCACCCAAATGACACATAGGTCAGGGTGGACCCTGGCGT  411

Sequence 1    GGCCAGAGATGCTTCCAACTGCCAGACTGTGGTCTCCACCCAGCACCACACTGACTCTTC  248
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NM_007482.2   GGCCAGAGATGCTTCCAACTGCCAGACTGTGGTCTCCACCCAGCACCACACTGACTCTTC  351

Sequence 1    CATTCTTCTG  258
                ||||||||||
NM_007482.2   CATTCTTCTG  341
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```
Arg1            CTTTGTTGATGTCCCTAATGACAGCTCCTTTCAAATTGTGAAGAACCCACGGTCTGTGGG 300

F               ------------------------------------------------------------

Arg1            GAAAGCCAATGAAGAGCTGGCTGGTGTGGTGGCAGAGGTCCAGAAGAATGGAAGAGTCAG 360

F               ----------------------------------------CAGAAGAATGGAAGAGTCAG 20

                                                         ********************

Arg1            TGTGGTGCTGGGTGGAGACCACAGTCTGGCAGTTGGAAGCATCTCTGGCCACGCCAGGGT 420

F               ------------------------------------------------------------

Arg1            CCACCCTGACCTATGTGTCATTTGGGTGGATGCTCACACTGACATCAACACTCCCCTGAC 480

F               ------------------------------------------------------------

Arg1            AACCAGCTCTGGGAATCTGCATGGGCAACCTGTGTCCTTTCTCCTGAAGGAACTGAAAGG 540

R               ------------------------------------------------------------

Arg1            AAAGTTCCCAGATGTACCAGGATTCTCCTGGGTGACTCCCTGCATATCTGCCAAAGACAT 600

R               ------------------------------GGTGACTCCCTGCATATCTG---------- 20

                                               ********************        Arg1

CGTGTACATTGGCTTGCGAGACGTAGACCCTGGGGAACACTATATAATAAAAACTCTGGG 660

R               ------------------------------------------------------------
```

Sequences producing significant alignments to Sequence 2 (400-500bp):

Accession	Description	E value	Max ident
NW_001030728.1	Mus musculus chromosome 3 genomic contig, alternate assembly (based on Celera assembly)	0.0	98%
NT_039240.7	Mus musculus chromosome 3 genomic contig, strain C57BL/6J	0.0	98%

```
Sequence 2      TATGCAGGGAGTCACCATTCTGTGTTGCTGGTCCGTTTTAATGCTGACGCCATGCTGTTT  64
                  ||| |||||||||||||||||||||||| ||||||||||||||||||||||||||||||
NW_001030728.1   TATACAGGGAGTCACCATTCTGTGTTGCTGATCCGTTTTAATGCTGACGCCATGCTGTTT  1370157

Sequence 2      GGCTGCTGTAGCTCAGGAATGCACTCTCAGGTTAGATATTGTGATGCCTCTTTTCCTCAT  124
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NW_001030728.1   GGCTGCTGTAGCTCAGGAATGCACTCTCAGGTTAGATATTGTGATGCCTCTTTTCCTCAT  1370097

Sequence 2      TTTGTTCAAAGGGCTTTGGCTGTGGAGGGTCTCCATGGTTTCGTGCACTCTTTAGGATTA  184
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NW_001030728.1   TTTGTTCAAAGGGCTTTGGCTGTGGAGGGTCTCCATGGTTTCGTGCACTCTTTAGGATTA  1370037

Sequence 2      GTCTGCCGAGTTTTGTGAAGAATCTTACTGACATTTGACTCTTCACTCTAAACAGAGGAT  244
                  |||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
NW_001030728.1   GTCTGCCGAGTTTTGTGAAGAATCTTACTGACATTTGACTCTTCTCTCTAAACAGAGGAT  1369977

Sequence 2      AGAGCTAAAGCTTTCACCTCTGTGCACGCATCACTTCGTGTCTCTCTAAGCACTTTTCTG  304
                  | ||||||||||||||||||||||||||||||||  ||||||||||||||||||||||
NW_001030728.1   AAAGCTAAAGCTTTCACCTCTGTGCACGCATCACTTTATGTCTCTCTAAGCACTTTTCTG  1369917

Sequence 2      CTCTCTGGATAAATTTATTATTAGCTCTCTCCATAGTTGGAAGTGGGATTGCTTTCTTGG  364
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NW_001030728.1   CTCTCTGGATAAATTTATTATTAGCTCTCTCCATAGTTGGAAGTGGGATTGCTTTCTTGA  1369857

Sequence 2      TTTTTAGTTTGTATAATGCACTCTTCATGGATTAAAAAACAAACAAACA  413
                 ||||||||||||||||||||||||||||||||||||||||||||||||
NW_001030728.1   TTTTTAGTTTGTATAATGCACTCTTCATGGATTAAAAAACAAACAAACA  1369808
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Arg ns          ----TATACAGGGAGTCACCATTCTGTGTTGCTGATCCGTTTTAATGCTGACGCCATGCT 56

R               CAGATATGCAGGGAGTCACC---------------------------------------- 20

                    *** ************

Arg1 ns         GTTTGGCTGCTGTAGCTCAGGAATGCACTCTCAGGTTAGATATTGTGATGCCTCTTTTCC 116

R               ------------------------------------------------------------

Arg1 ns         TCATTTTGTTCAAAGGGCTTTGGCTGTGGAGGGTCTCCATGGTTTCGTGCACTCTTTAGG 176

R               ------------------------------------------------------------

Arg1 ns         ATTAGTCTGCCGAGTTTTGTGAAGAATCTTACTGACATTTGACTCTTCTCTCTAAACAGA 236

R               ------------------------------------------------------------

Arg1 ns         GGATAAAGCTAAAGCTTTCACCTCTGTGCACGCATCACTTTATGTCTCTCTAAGCACTTT 296

F               ------------------------------------------------------------

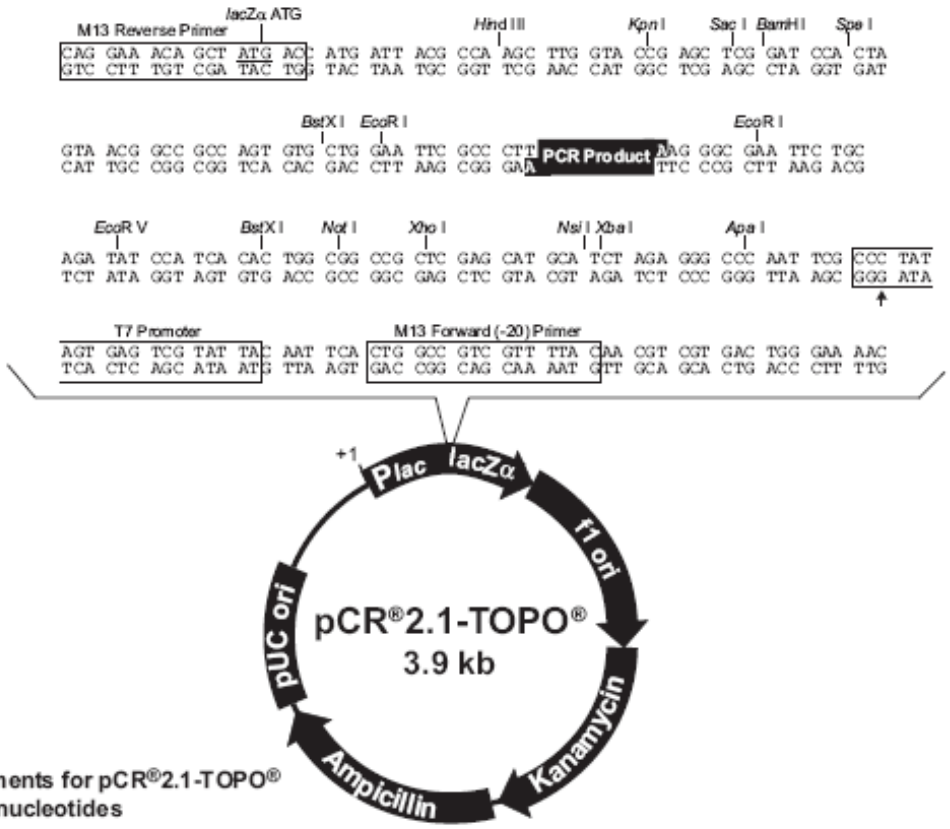
Arg1 ns         TCTGCTCTCTGGATAAATTTATTATTAGCTCTCTCCATAGTTGGAAGTGGGATTGCTTTC 356

F               ------------------------------------------------------------

Arg1 ns         TTGATTTTTAGTTTGTATAATGCACTCTTCATGGATTAAAAAACAAACAAACA 409

F               --------CTGACTCTTCCATTCTTC-TG-------------------- 20

                         **  *  * ** ***** **
```



Comments for pCR®2.1-TOPO®
3931 nucleotides

*Lac*Zα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Figure A.4: Map of pCR2.1-TOPO showing the cloning site used for PCR product insertion as well as Kanamycin gene used for selection and growth of vector-containing bacteria.

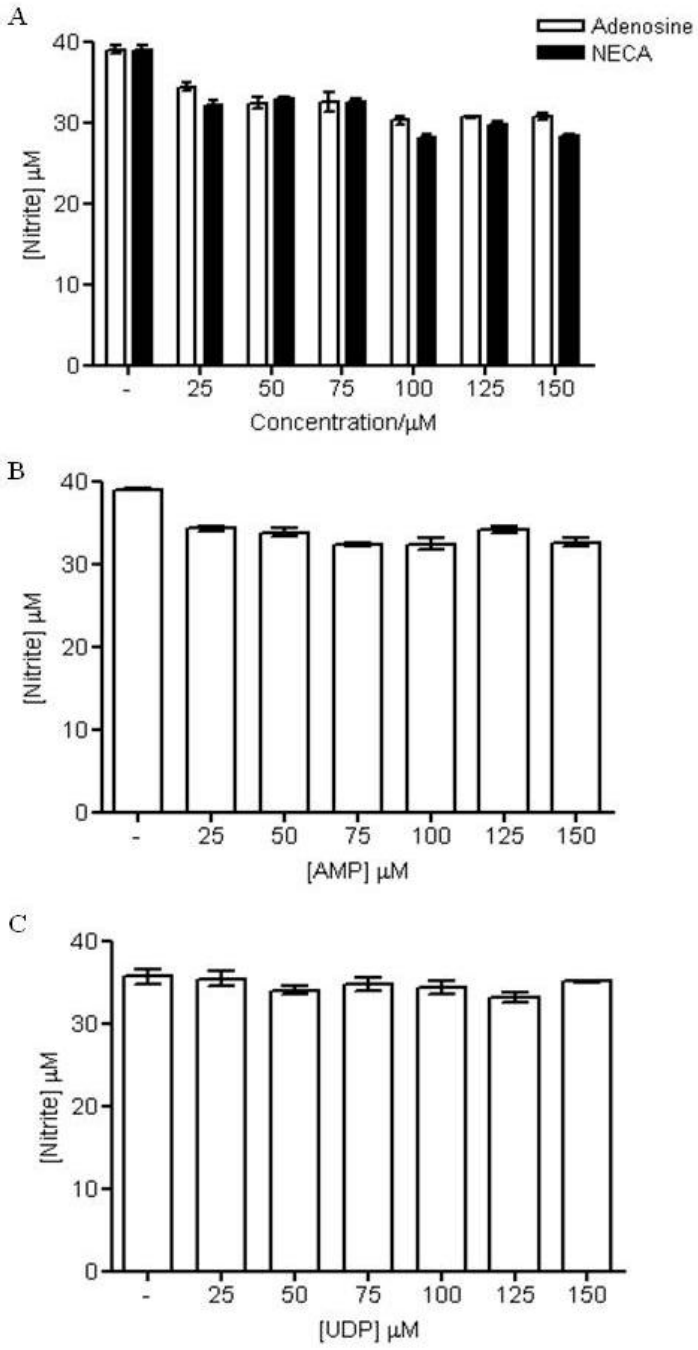


Figure A.5: The effect of adenosine, AMP and UDP titration on NO release from CAMΦ. Macrophages were classically activated with 10ng/ml LPS and the effect of titrating the concentration of adenosine (+10µM Dipyridamole) and its slowly hydrolysable analogue NECA, AMP and UDP on NO release by CAMΦ was measured after 20 hours of culture. (A)The effect of adenosine/NECA on NO release, (B) The effect of AMP on NO release and (C) The effect of UDP on NO release, expressed as nitrite in µM. Data presented are an average of triplicates ± SEM from one representative experiment of n = 2.

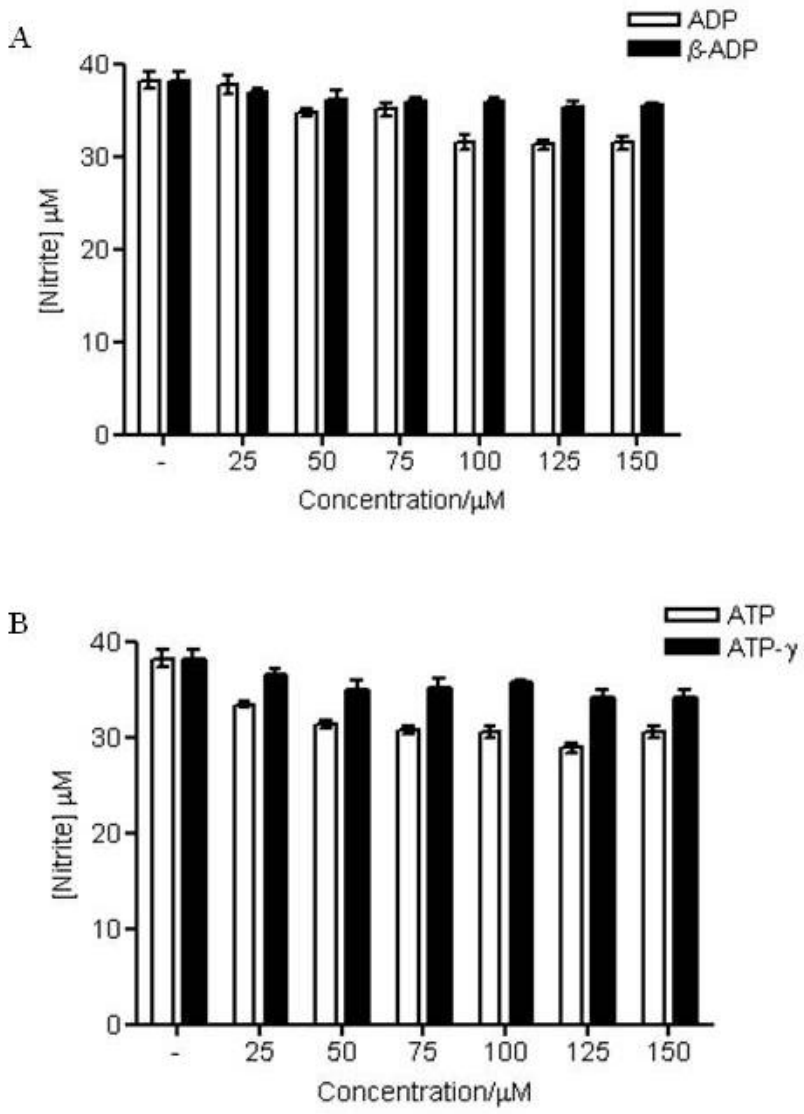


Figure A.6: The effect of ADP and ATP titration on NO release from CAMΦ. Macrophages were classically activated with 10ng/ml LPS and the effect of titrating the concentration of ADP or ATP (+10µM Dipyridamole) and their slowly hydrolysable analogues, β-ADP and ATP-γ, on NO release by CAMΦ was measured after 20 hours of culture. (A) The effect of ADP/β-ADP on NO release. (B) The effect of ATP/ATP-γ on NO release, expressed as nitrite in µM. Data presented are an average of triplicates ± SEM from one representative experiment of n = 2.

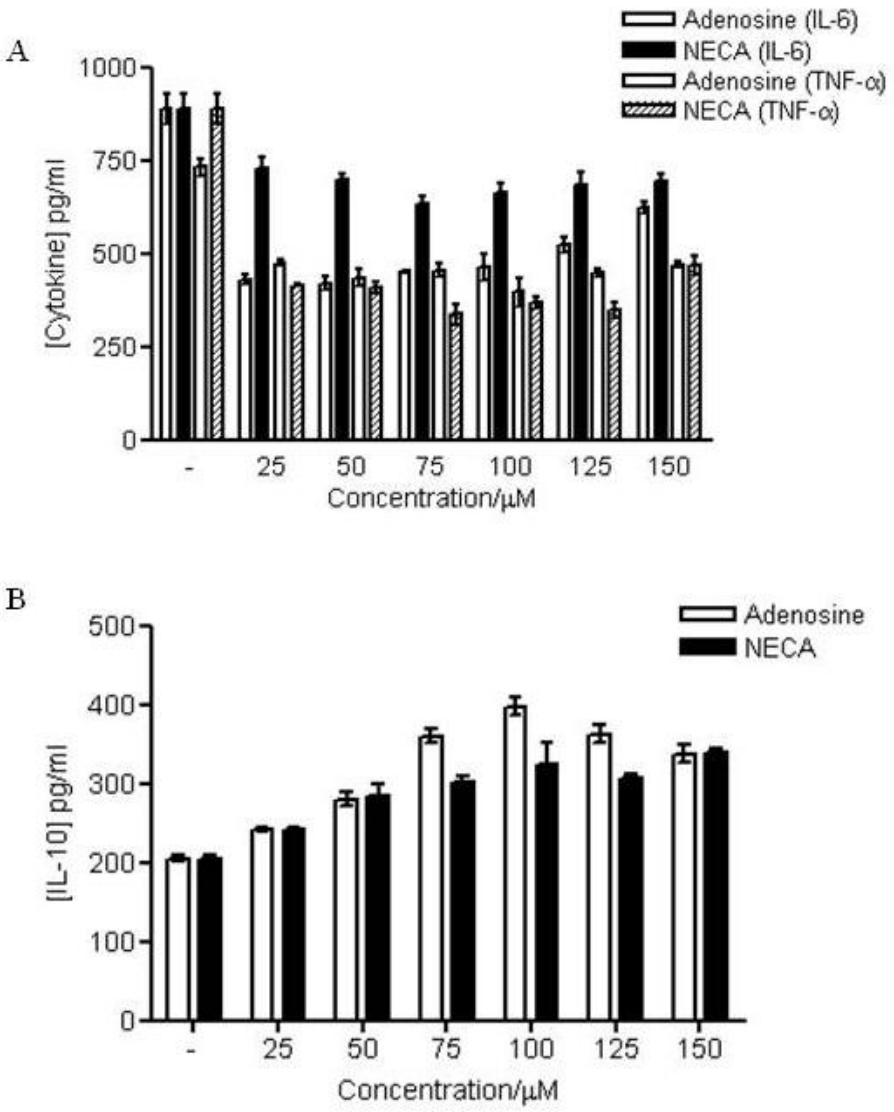


Figure A.7: The effect of adenosine/NECA titration on cytokine release from CAMΦ. Macrophages were classically activated with 10ng/ml LPS and the effect of titrating the concentration of adenosine (+10μM Dipyridamole) and its slowly hydrolysable analogue NECA on cytokine release by CAMΦ cytokine was meausered after 20 hours of culture. (A) The effect on IL-6 and TNF-α release. (B) The effect on IL-10 release. Cytokine release is expressed in pg/ml. Data presented are an average of triplicates ± SEM from one representative experiment of n = 3.

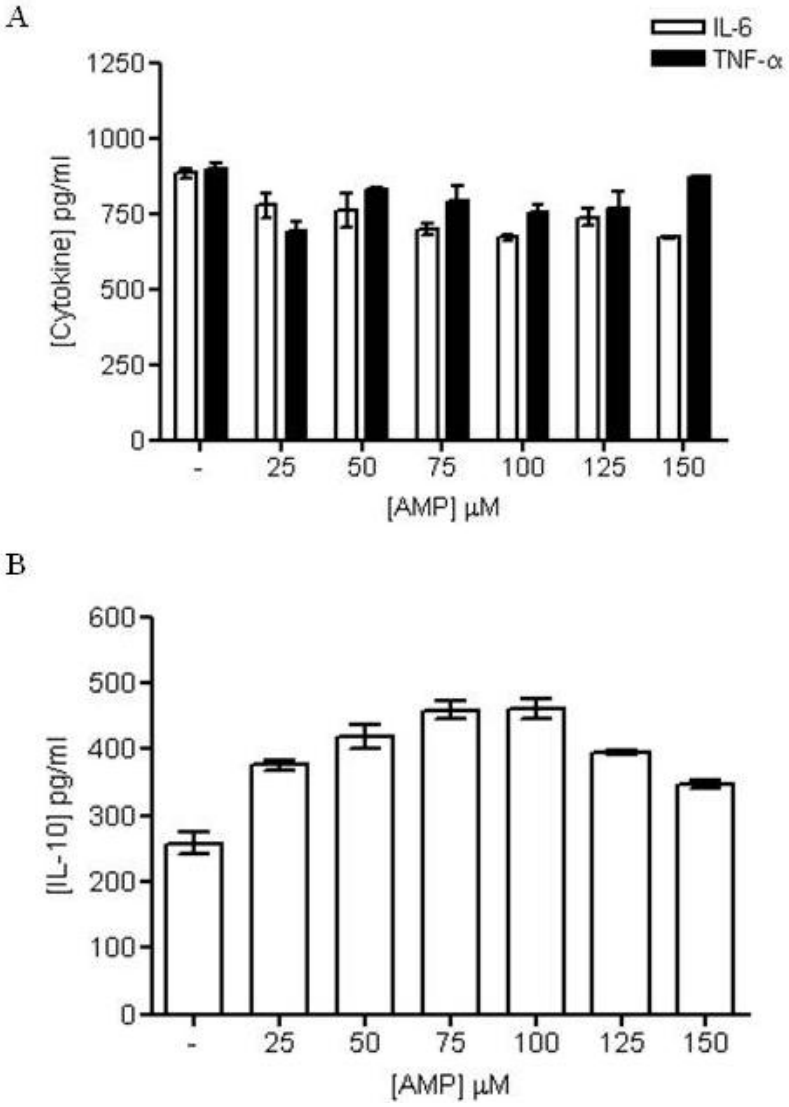


Figure A.8: The effect of AMP titration on cytokine release from CAMΦ. Macrophages were classically activated with 10ng/ml LPS and the effect of titrating the concentration of AMP on cytokine release by CAMΦ was measured after 20 hours of culture. (A) The effect of AMP on IL-6 and TNF-α release. (B) The effect on IL-10 release. Cytokine release is expressed in pg/ml. Data presented are an average of triplicates $\pm$ SEM from one representative experiment of n = 3.

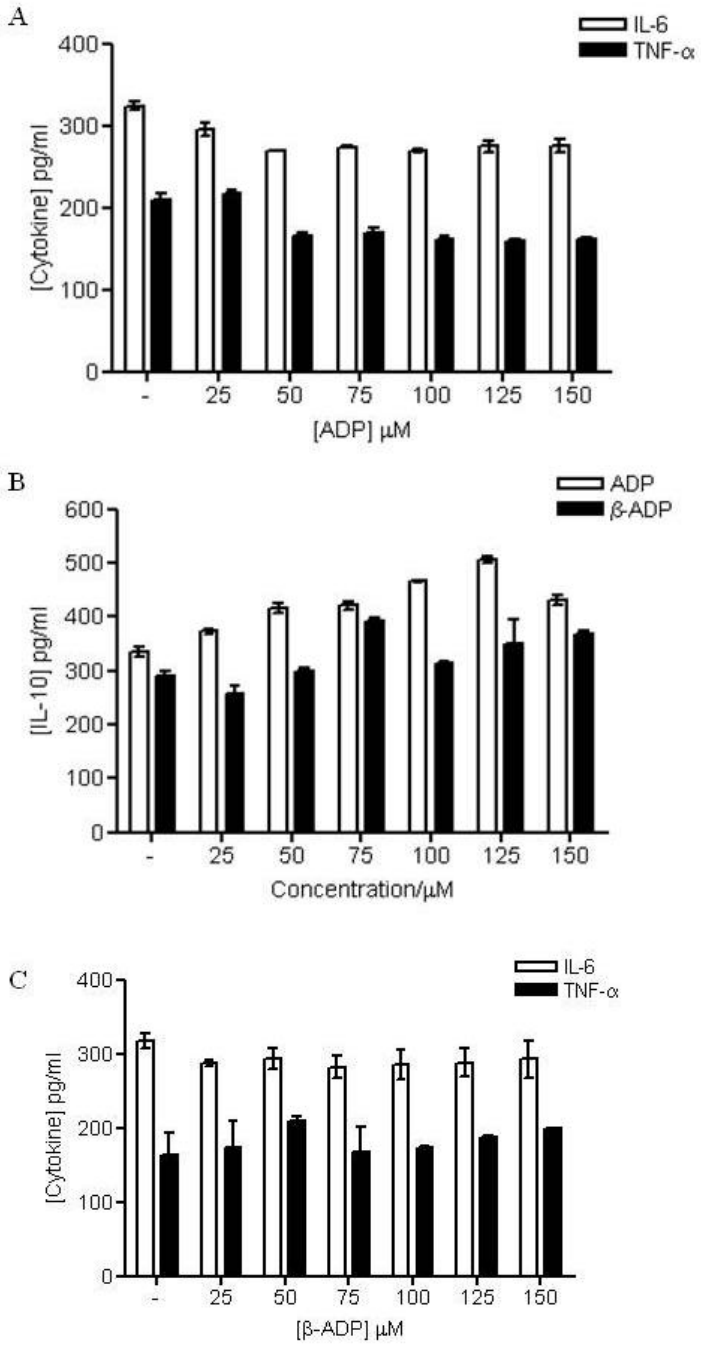


Figure A.9: The effect of ADP titration on cytokine release from CAMΦ. Macrophages were classically activated with 10ng/ml LPS and the effect of titrating the concentration of ADP (+10μM Dipyridamole) and its slowly hydrolysable analogue β-ADP on cytokine release by CAMΦ was measured after 20 hours of culture. (A) The effect of ADP on IL-6 and TNF-α release. (B) The effect of ADP or β-ADP on IL-10 release. (C) The effect of β-ADP on IL-6 and TNF-α release. Cytokine release is expressed in pg/ml. Data presented are an average of triplicates ± SEM from one representative experiment of n = 2.

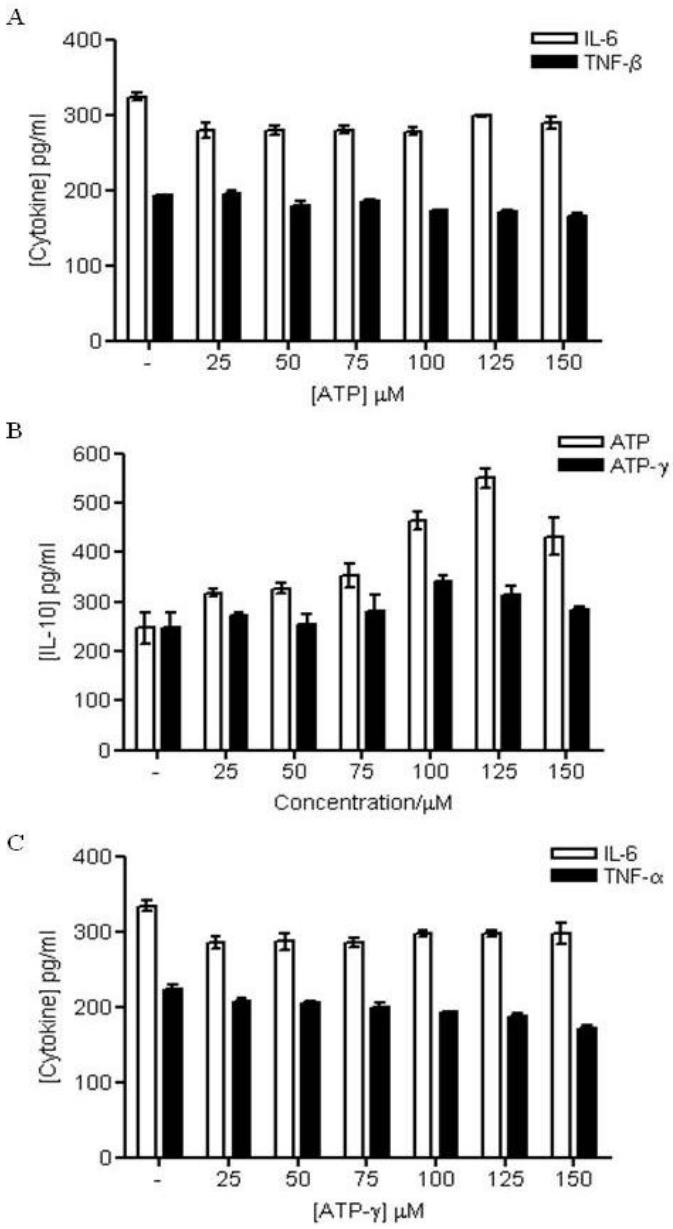
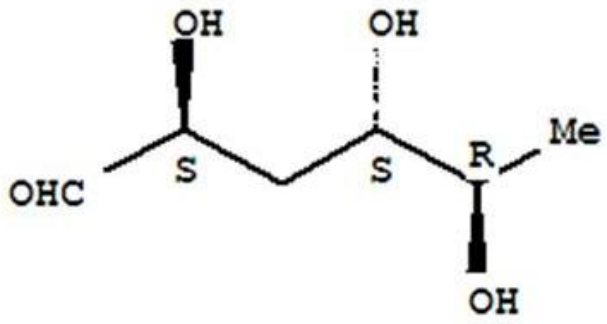


Figure A.10: The effect of ATP titration on cytokine release from CAMΦ. Macrophages were classically activated with 10ng/ml LPS and the effect of titrating the concentration of ATP (+10μM Dipyridamole) and its slowly hydrolysable analogue ADP-γ on cytokine release by CAMΦ was measured after 20 hours of culture. (A) The effect of ATP on IL-6 and TNF-α release. (B) The effect of ATP or ATP-γ on IL-10 release. (C) The effect of ATP-γ on IL-6 and TNF-α release. Cytokine release is expressed in pg/ml. Data presented are an average of triplicates ± SEM from one representative experiment of n = 2.

A 3-6 dideoxy arabinohexose (tyvelose)



B GalNAc

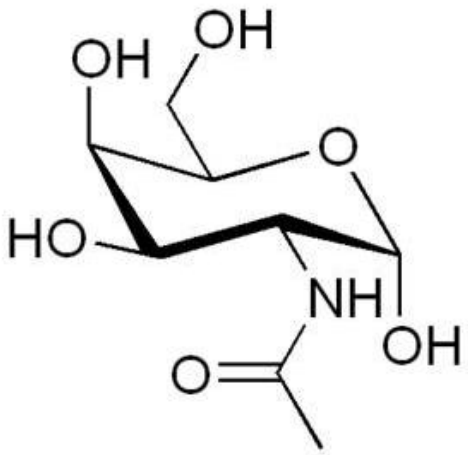


Figure A.11: Schematic representation of the structure of (A) Tyvelose and (B) GalNAc (N-acetylgalactosamine).

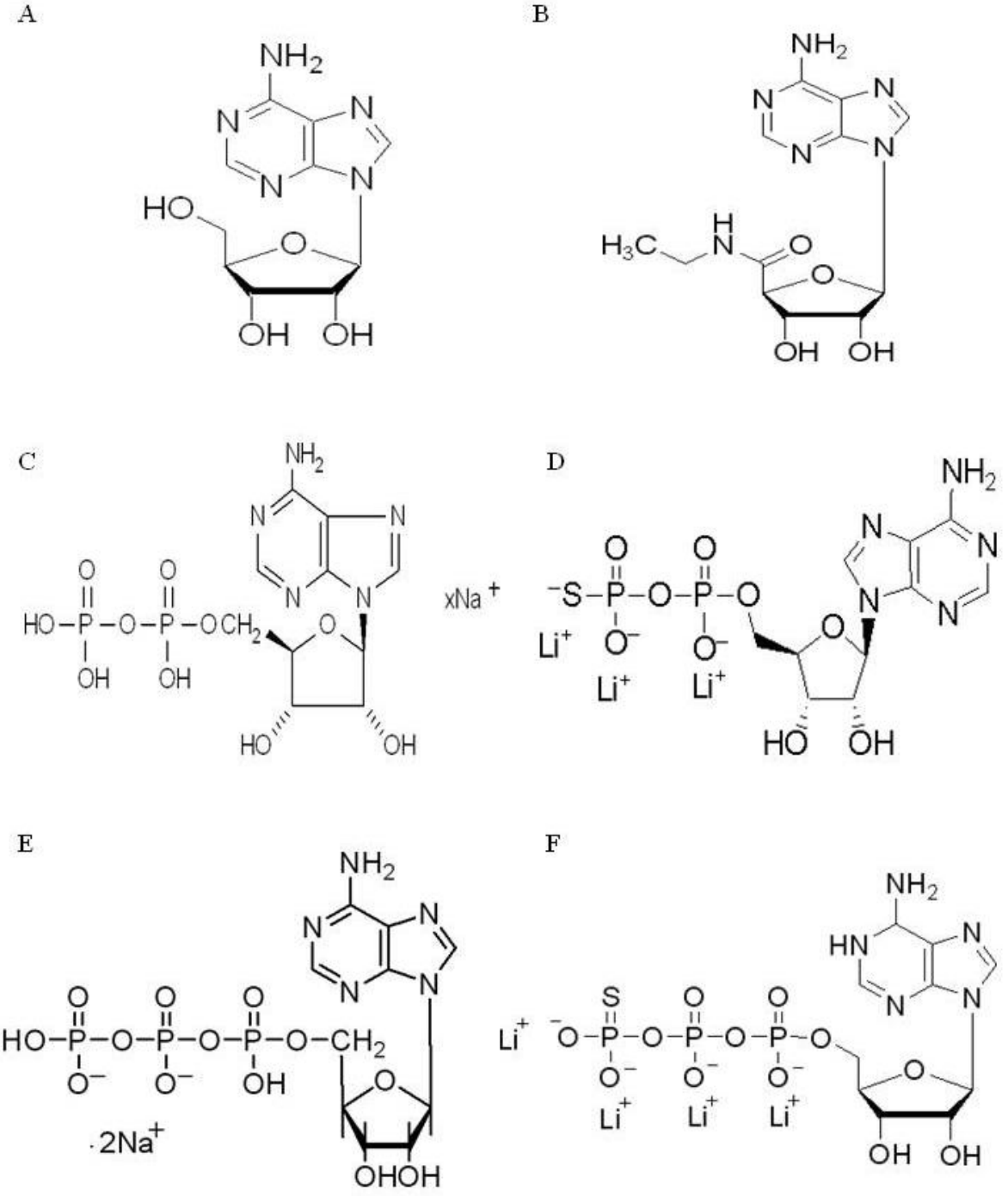


Figure A.12: Schematic representation of the structure of adenosine (A) and its slowly hydrolysable analogue NECA (B), ADP (C) and β-ADP (D), ATP (E) and ATP-γ (F).