
**Modulation of purinergic signalling
by *Trichinella spiralis* secreted
proteins**

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Abstract

Extracellular nucleotides are signalling molecules that can activate cell surface purinergic receptors, initiating or modulating cellular responses. The parasitic nematode *Trichinella spiralis* secretes nucleotide-metabolising enzymes potentially capable of interfering with this process. *In vitro* activation of bone marrow-derived mast cells (mBMMC) of a mucosal phenotype was used as a model to study the effects of *T. spiralis* excreted/secreted proteins (ES) on purinergic signalling.

RT-PCR, pharmacological and electrophysiological analyses demonstrated that mBMMC express functional A_{2B}, A₃, P2Y₁, P2Y₂, P2X₄ and putatively P2Y₆ and CysLT1 receptors, thus allowing the cells to respond to a range of nucleotides. *T. spiralis* ES modulated mBMMC purinergic receptor activation by metabolising extracellular nucleotides.

Adenine nucleotides induced mBMMC exocytosis, resulting in the release of mouse mast cell protease-1 (mMCP-1) and β -hexosaminidase. The *T. spiralis* secreted 5'-nucleotidase (5'-NT) mediated the inhibition of ADP-induced mMCP-1 release. Extracellular nucleotides were unable to mediate cytokine production or direct the migration of mBMMC. However, adenosine, ADP and ATP stimulated inhibitory pathways that led to the down-regulation of LPS-induced TNF- α generation. Furthermore, *T. spiralis* ES reduced the inhibitory effects of ADP. These results show that *T. spiralis* ES can modulate important mast cell effector functions induced by nucleotides.

Immunisation of mice with the *T. spiralis* recombinant 5'-NT elicited a T_H2-dominated systemic immune response generating high titres of antibodies that could neutralise the activity of the 5'-NT *in vitro*. However, immunisation did not generate a sufficiently potent intestinal mucosal immune response, and as such, did not confer protection against subsequent *T. spiralis* infection.

T. spiralis infection altered the qualitative expression of purinergic receptor subtypes in the intestinal epithelium. Strains of mice that expel parasites from the intestine at slow and fast rates expressed different purinergic receptor sub-types throughout the course of infection, suggesting that receptor expression may be linked to the rate of parasite expulsion.

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Abbreviations

(d)ATP	(deoxy) adenosine triphosphate
(d)CTP	(deoxy) cytosine triphosphate
(d)GTP	(deoxy) guanosine triphosphate
(d)TTP	(deoxy) thymidine triphosphate
(m)V	(milli)Volt
(n, m, μ)g	(nano, milli, micro) grams
(n, m, μ)l	(nano, milli, micro) litres
(n, m, μ)M	(nano, milli, micro) molar
5'-NT	5'-nucleotidase
A3P5PS	Adenosine 3'-phosphate 5'-phosphosulfate
ADA	Adenosine deaminase
ADK	adenosine kinase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BBG	Brilliant Blue G
BCA	Bichinchonic assay
BMGY	Buffered glycerol complex medium
BMMY	Buffered methanol complex medium
bp	Base pairs
BSA	Bovine serum albumin
C.P.M	Counts per minute
CysLTR	Cysteinyl leukotriene receptor
dH ₂ O	deionised water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNP	Dinitrophenyl
dNTPs	deoxynucleotide triphosphates
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
ELISA	Enzyme linked immunosorbant assay
ES	Excretory/secretory proteins
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FcεRI	High affinity IgE receptor
FITC	Fluorescein isothiocyanate
GI	Gastrointestinal
GPI	glycosylphosphatidylinositol
HSA	Human serum albumin
HCL	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HRP	Horse radish peroxidase
IB-MECA	1-Deoxy-1-[6-(((3-Iodophenyl)methyl)amino)-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide N ⁶ -(3-Iodobenzyl)adenosine-5'-N-methyluronamide
IFN-γ	Interferon- γ
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl thiogalactosidase
Kb	Kilo base pairs
kDa	Kilo Daltons
K _i	Inhibition constant
LPS	Lipopolysaccharide
LTB ₄	Leukotriene B ₄
LTD ₄	Leukotriene D ₄
mBMMC	mouse bone marrow-derived mast cells
MES-ADP	2-(Methylthio)adenosine 5'-diphosphate
MLN	Mesenteric lymph nodes
mMCP-1	mouse mast cell protease-1
MSL	Muscle stage larvae
MW	Molecular weight
NaOH	Sodium hydroxide

NBL	newborn larvae
NDPK	Nucleotide diphosphate kinase
NK	Natural killer cell
NO	Nitric oxide
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC12	rat phaeochromocytoma-12 cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PGT- β	mouse pineal gland tumour cells
pNP	p-nitrophenyl
PPADS	Pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid)
RAW 264.7	murine macrophage cell-line
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Rothwell Park Memorial Institute culture medium
RT-PCR	Reverse transcriptase-PCR
s.d.m	Standard deviation from the mean
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SDS	Sodium docecyl sulphate
SEB	Staphylococcal enterotoxin fragment B
sec	Seconds
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-tetramethyl ethylene diamine tetraacetic acid
TGF- β_1	Transforming growth factor- β_1
T _H	T-helper cell subset
TLR	Toll-like receptor
TMB	3, 3', 5, 5'-tetramethylbenzidine
TNF- α	Tumour necrosis factor- α

TNP-ATP	2',3'-O-(2,4,6-Trinitrophenyl) adenosine 5'-triphosphate
Tris	Tris (hydroxymethyl) aminomethane
Tween	Polyoxyethylenesorbitol monolaurate
U	Unit
UDP	Uridine diphosphate
UMP	Uridine monophosphate
UTP	Uridine triphosphate
v/v	volume to volume ratio
w/v	weight to volume ratio
WEHI-3B	Mouse myelomonocyte IL-3 producing cell-line
x g	Unit(s) of gravitational force
YNB	Yeast nitrogen base
β -Glc-NAc	N-acetyl- β -D-glucosamine
β -hex	β -hexosaminidase
μ Ci	micro Curie

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Chapter 1

General Introduction

1.1 Historical perspectives and classification of *Trichinella*

The genus *Trichinella* is composed of parasitic nematodes that belong to the order trichuroidea. These parasites constitute an unusual genus of organisms, in that they require only one host, and remain intracellular during the majority of their life cycle. *Trichinella* was initially described by Paget and Owen in 1835. They observed the coiled larvae in the diaphragm of a cadaver and named them *Trichina spiralis* from the Latin words for hair and coil. Later, in 1896, these parasites were renamed *Trichinella spiralis* the causative agent of the disease trichinellosis.

For more than a century after its discovery, *Trichinella* was considered to be a single species capable of infecting a wide variety of mammals. It was not until the 1970's, that distinct biological variants of *Trichinella* were established as separate species in their own right. Currently, the genus *Trichinella* is classified into two main clades; one includes species that encapsulate in host muscle tissue, and a second that does not encapsulate following host muscle cell dedifferentiation (Pozio *et al.*, 2001; La Rosa *et al.*, 2003; Gasser *et al.*, 2004; Zarlenga *et al.*, 2004). Except for the existence of a capsule and size differential in one of the non-encapsulated parasite groups, all species and genotypes of the genus *Trichinella* are morphologically indistinguishable at all developmental stages (Pozio *et al.*, 2005). More recently, to aid classification, molecular PCR methods have been developed to identify the genotype of individual parasite larvae (Zarlenga *et al.*, 1999; Pozio and La Rosa, 2003; Gasser *et al.*, 2004). To date, this has enabled the classification of eight species and three distinct genotypes of *Trichinella* (Pozio *et al.*, 2005) (summarised in Table 1.1).

The genus *Trichinella* has the widest geographical distribution and the largest range of host species of all parasitic nematodes (Wakelin and Goyal, 1996). Carnivorism and scavenging are essential components in parasite transmission as infection is acquired through the ingestion of raw or undercooked muscle tissue containing viable infective (L1) larvae (Despommier, 1983). In terms of epidemiology, the *Trichinella* life cycle has traditionally been considered as two distinct cycles: the domestic cycle, involving humans and domesticated animals; and the sylvatic cycle, involving wild animals (Campbell, 1983). As shown in Table 1.1, the species *T. spiralis* and *T. pseudospiralis* have the widest distribution of all the *Trichinella* species. The global prevalence of *T. spiralis* in both sylvatic and domestic cycles is thought to be due to its high reproductive capacity and the global increase in animal and meat trade, which

Clade	Species Name or genotype	Distinguishing characteristics	Distribution	Life cycle	Classification Reference
Encapsulated Clade 1	<i>T. spiralis</i>	Infects mammals, predominantly domestic and sylvatic swine	worldwide	Domestic and sylvatic	Owen, 1835
	<i>T. nativa</i>	Tolerant to freezing	Arctic and sub-arctic (cold climates)	Sylvatic	Britov and Boev, 1972
	<i>T. britovi</i>	Low pathogenicity in humans	Eurasia and Africa	Sylvatic	Pozio <i>et al.</i> , 1992
	<i>T. murelli</i>	Low infectivity in swine and rats	North America	Sylvatic	Pozio and La Rosa, 2000
	<i>T. nelsoni</i>	Low pathogenicity in humans	East Africa	Sylvatic	Pozio <i>et al.</i> , 1992
	Genotype T6	Related to <i>T. nativa</i>	Canada and U.S	Sylvatic	Pozio <i>et al.</i> , 1992
	Genotype T8	Related to <i>T. britovi</i>	South Africa Namibia	Sylvatic	Pozio <i>et al.</i> , 1992
	Genotype T9	Related to <i>T. britovi</i>	Japan	Sylvatic	Nagano <i>et al.</i> , 1999
Non-encapsulated Clade 2	<i>T. pseudospiralis</i>	Infects mammals and birds	Worldwide	Sylvatic	Garkavi, 1972
	<i>T. papuae</i>	Infects mammals and reptiles	Papua New Guinea	Sylvatic	Pozio <i>et al.</i> , 1999
	<i>T. zimbabwensis</i>	Infects mammals and reptiles	Zimbabwe, Ethiopia, Mozambique	Sylvatic	Pozio <i>et al.</i> , 2002

Table 1.1 *Trichinella* species and genotypes

A summary of the 8 classified species and 3 genotypes of the genus *Trichinella*. The genotypes T6, T8 and T9, are individually distinguishable by molecular analyses but have not gained species status, predominantly due to their ability to interbreed with related *Trichinella* species.

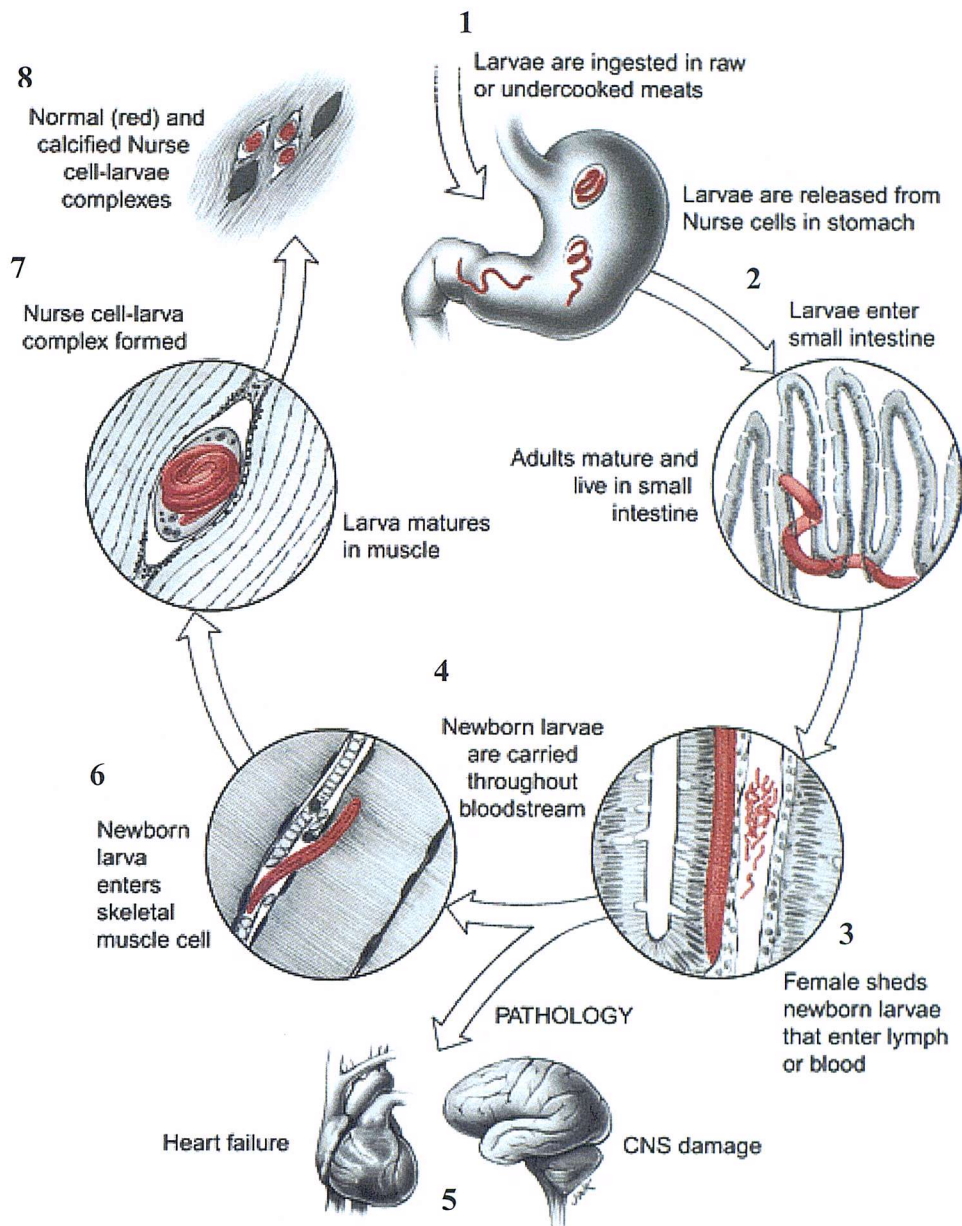
has transferred *T. spiralis* from endemic to non-endemic regions (Murrell and Pozio, 2000). *T. pseudospiralis* is thought to have been transmitted worldwide, due to its unique ability to infect avian hosts.

1.2 *Trichinella spiralis* life cycle

In contrast to many other types of parasitic nematodes that require multiple hosts or have free-living life-stages, the entire life cycle of *Trichinella* is completed within one host. As shown in Figure 1-1, the life cycle of *Trichinella* exhibits several unusual characteristics.

1.2.1 Enteral phase

The establishment of a successful infection requires the ingestion of infective L1 larvae existing in muscle tissue. The process of digestion releases the infective larvae from the muscle, and the parasites rapidly pass into the small intestine. Parasites are then directed to invade the columnar epithelial cells that line the small intestine, by as yet unknown environmental cues (Wright, 1979). It is thought that entry is unlikely to be *via* mechanical means as the infective L1 larvae do not have a stylet or other oral appendages (Bruce, 1970a). Experiments performed *in vitro* using epithelial cell lines have shown that *T. spiralis* infective larvae do not invade all cell types, a fact that argues that invasion is not merely mechanical, but requires a signal from host cells which permits or prohibits invasion (ManWarren *et al.*, 1997). Electron microscopic examination has shown that the parasites lie directly in the cytoplasm of epithelial cells, usually at the crypt-villus junction (Wright, 1979; Dunn and Wright, 1985). Migration of worms through enterocytes induces the fusion of adjacent lateral cell membranes resulting in a large pocket of shared cytoplasm, or ‘syncytium’ (Dunn and Wright, 1985). The infective larvae are approximately 1 mm long and can penetrate the cell membranes of up to 45 enterocytes at a time (Despommier, 1993). During the development of the parasites, female worms can grow to a length of 3 mm (Despommier, 1993). Thus, these parasites can damage significant numbers of cells present in the epithelium. It has been suggested that the worms continually move through the epithelium (Dunn and Wright, 1985; Wright *et al.*, 1987). It is thought that regions of enterocytes damaged by the parasites then die and are sloughed from the epithelium in the usual manner (Dunn and Wright, 1985; Wright *et al.*, 1987).



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Figure 1-1 The life cycle of *Trichinella*

Parasites enter a new host during the ingestion of raw/undercooked meat, and infective larvae (L1) are released from the nurse cell complex into the stomach [1]. Larvae then pass into the small intestine and invade the epithelium, where they moult four times, achieve adulthood and mate within 30 hours after being ingested [2]. On day 6 post-ingestion, females give birth to newborn larvae and the adult parasites begin to be expelled [3]. Newborn larvae then migrate around the body *via* the blood and/or lymphatics [4]. This phase is associated with occasionally fatal pathology [5]. Newborn larvae penetrate out of blood/lymph vessels into striated muscle myofibres [6]. Approximately twenty days after penetrating the muscle, the larva forms the nurse cell complex [7], and remains in this state unless calcification due to host immune defences occurs [8]. (Picture adapted from 'Parasitic Diseases' 4th Edition, Apple Trees Productions, New York).

Once established within the epithelial niche, the parasites rapidly moult four times over a period of 10-28 hours (Kozek, 1971). After the last moult, the mature adult worms immediately mate within a time-frame of 4-5 hours (Kozek, 1971). Individual males are known to mate more than once during an infection (Kozek, 1971). However, the mechanisms by which male and female worms communicate their position to each other in the intestinal milieu are unknown. The gravid females release approximately 500 to 1500 live juvenile newborn larvae (NBL) each, 3-5 days after mating (Khamboonruang, 1971). Once the adult parasites have successfully mated and released NBL, they are expelled from the intestine due to a combination of host immune responses (Wakelin and Denham, 1983).

Unlike the L1 infective larvae and adult parasites, the NBL have an anterior stylet. It is thought that they use this to mechanically penetrate into lymphatic vessels and capillaries innervating the lamina propria of the intestinal epithelium (Wang and Bell, 1986a). The NBL enter the general circulation *via* the hepatic portal vein and thoracic duct and circulate in the blood for up to 6 hours (Wang and Bell, 1986b). It is of note, that this migratory period is the only time in the life cycle when the parasites exist in the extracellular environment. The NBL then use their stylet to penetrate out of capillaries and migrate into the surrounding tissues (Despommier, 1993; Wang, 1997). This process leads to the destruction of cells and occasionally causes the death of the host (Wang, 1997). Only NBL that migrate into striated muscle will further develop into infective L1 larva.

1.2.2 Parenteral stage

Once in striated skeletal muscle, the larvae penetrates a muscle cell and enters a stage of development known as the parenteral phase. The parasite then directs the transformation of the muscle cell into a parasite-host cell complex termed the nurse cell (Figure 1-2), so called because it provides nutrients for the growth, development and maintenance of the larvae (Purkerson and Despommier, 1974). Transformation of the muscle cell takes 20 days to complete, during which time the parasite undergoes a period of rapid growth (Despommier, 1975). After this phase, the parasites are infective to the next host and enter a stage of arrest. The larvae remain within the nurse cell complex for the entire life of the host, until ingested by another host or, after a period of years, become calcified by host responses (Despommier, 1975).

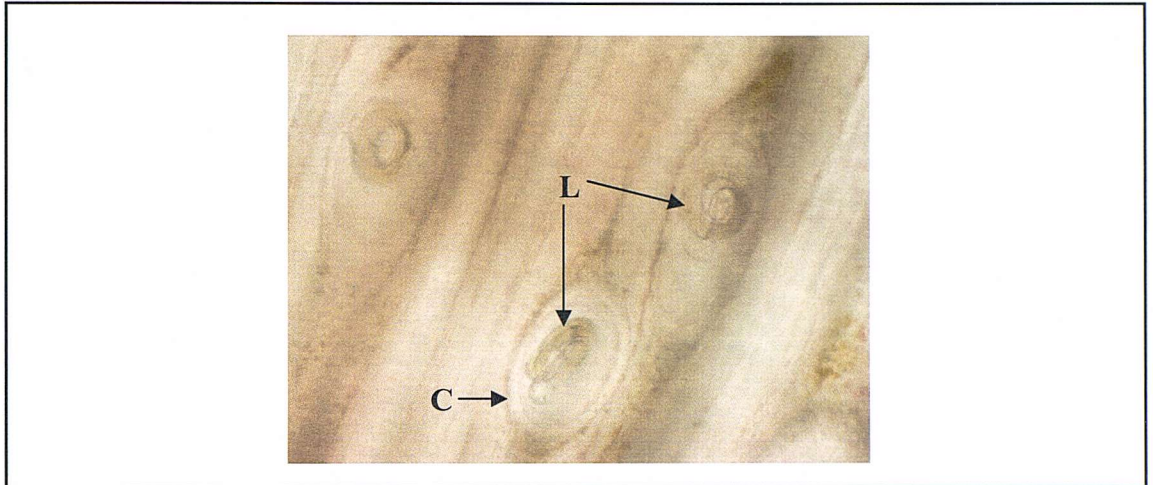


Figure 1-2 The *T. spiralis* nurse cell

T. spiralis larvae (L) form encapsulated nurse cell complexes within the striated muscle of vertebrate hosts. A dense collagenous capsule surrounds the parasite (C). Light microscopy showing the unstained diaphragm of an infected rat. Magnification x40

The formation of the nurse cell complex initially involves the loss of the classic characteristics of differentiated myofibres of muscle cells including the loss of myofibrillar proteins such as myosin heavy chain and α - and β -tropomyosin (Emerson, 1990; Jasmer, 1990). Furthermore, infected muscle cells are induced to re-enter the cell cycle from their terminally differentiated state at the G_0/G_1 stage. The host cell nuclei divide once and each nucleus then replicates its DNA, before the cell cycle is chronically suspended at the G_2/M boarder at approximately day 5 post infection (p. i.), resulting in a 4N complement of DNA in infected cell nuclei (Jasmer, 1993). The muscle cells also undergo re-differentiation to form the nurse cell, gaining characteristics such as the development of a collagen capsule, a circulatory network surrounding the infected cell and cytoplasmic changes including increased rough endoplasmic reticula, and increased Golgi-associated and lysosomal enzyme activity (Stewart, 1993).

The mechanisms driving the formation of the nurse cell complex have been the subject of a number of studies. The discovery that proteins secreted by the parasite were present within the cytoplasm and nucleus of the nurse cell, led to the hypothesis that the parasite was directing the development of the nurse cell (Despommier, 1990). However, it has also been postulated that re-entry of the myocyte into the cell cycle may account for many of the unusual characteristics of the nurse cell, and promote the growth and

survival of the parasite (Jasmer, 1995). Delineating the extent to which the parasite induces the development of the nurse cell complex is still far from being understood.

1.3 Trichinellosis

The most frequent source of human *Trichinella* infections is through the consumption of raw or under-cooked game animals, or pork products from pigs raised in farms where rodent populations are able to thrive (Pozio *et al.*, 1993). Herds of swine are usually infected by *T. spiralis* by the animals scavenging on infected rodents, eating offal or meat containing parasites in their feed, or as a result of direct transmission from other pigs (by cannibalism) (Pozio, 2000). Human infection can also result from the consumption of meat from herbivores such as horses (Boireau *et al.*, 2000). It is thought that these herbivorous animals become infected whilst grazing in areas contaminated with pork scraps or rat carcasses, or from feeds which may be made from contaminated meat (Pozio, 2000).

Trichinella infections have worldwide socioeconomic significance, and within Europe are of medical and veterinary concern in France, Germany, Italy, and Spain. Trichinellosis outbreaks are foremost in the east and central European countries, where infections are increasing (Pozio, 2001). Currently, the EU spends approximately €570 million per year on screening pigs, horses and boar for *Trichinella* infection to ensure the meat is safe for human consumption (Pozio, 1998; Kapel, 2005). However, these measures have not totally eradicated this parasite, and approximately 20,000 cases of trichinellosis were reported in Europe during 1991-2000 (Murrell and Pozio, 2000). A lack of adequate screening programmes, improper animal husbandry as well as cultural preferences for raw meat have led to *Trichinella* remaining endemic and even re-emerging in some areas of the world, particularly China and South and Central America (Bolpe and Bofi, 2000; Takahashi *et al.*, 2000).

1.4 *Trichinella spiralis* secreted proteins

T. spiralis larvae secrete a multitude of proteins, known as excretory–secretory (ES) proteins, during both the enteral and parenteral stages of infection (Despommier, 1983). The secreted proteins originate from a large secretory organelle termed the stichosome, which is characteristic of members of the Trichuroidea order of parasites (Chitwood, 1930), and occupies the entire anterior half of the *T. spiralis* infective larvae (Figure 1-3) (Despommier and Muller, 1976). The stichosome develops during

maturation of the infective larvae in the nurse cell, and is fully developed by day 16 p. i. The stichosome is retained by the infective L1 larvae and through to the adult stage parasites (Wu, 1955). Experiments using NBL *in vitro*, have reported that they secrete very little protein and have only a rudimentary, undeveloped stichosome (Zarlenga *et al.*, 2002; Parkhouse and Clark, 1983).

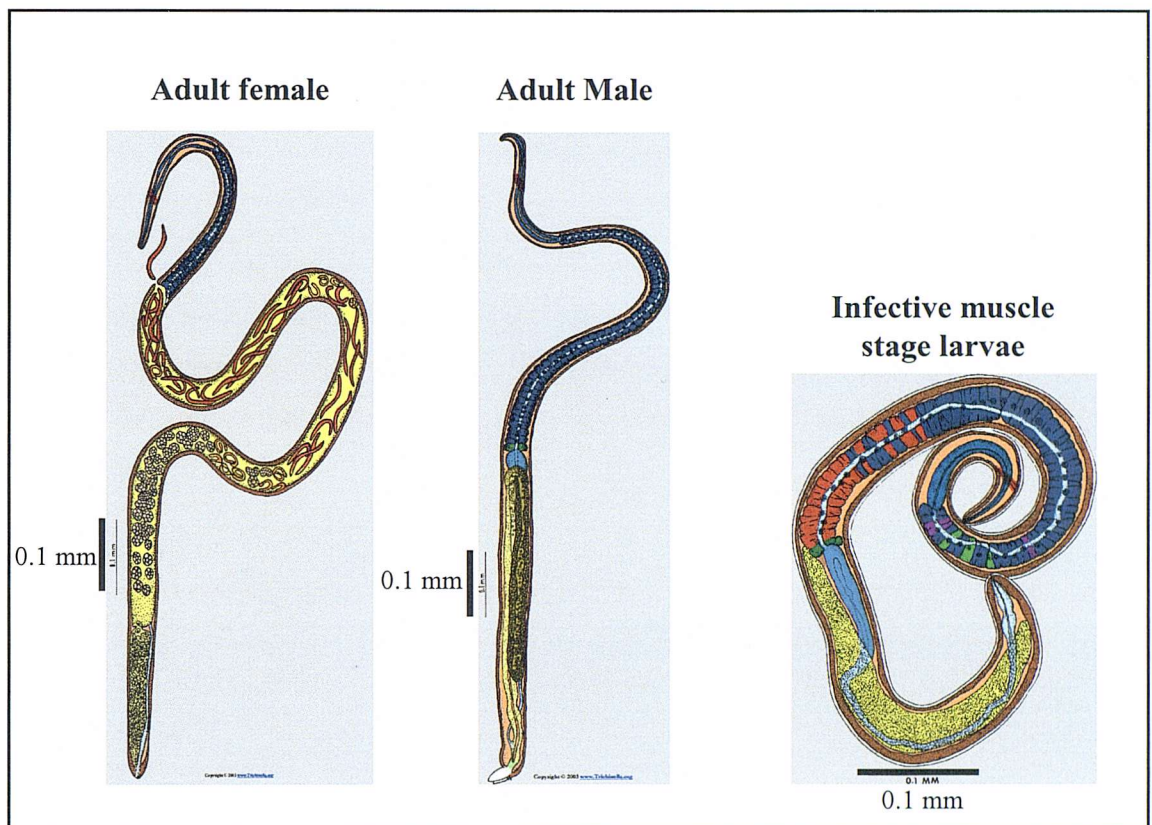


Figure 1-3 Morphology of *T. spiralis* adult and infective muscle stage larvae

The dark-blue region depicts the arrangement of stichocytes in the anterior portion of the adult male and female parasites. The dark-blue and red region depicts the arrangement of stichocytes in the anterior portion of infective larvae. Pictures reproduced from <http://www.Trichinella.org> (Villella, 1970).

The stichosome of *T. spiralis* infective larvae is composed of a row of 50 to 55 specialised discoid cells termed stichocytes, each of which has a diameter of approximately 25 μm (Villella, 1970; Despommier and Muller, 1976). Each stichocyte contains a single nucleus, mitochondria, Golgi-like complexes, a smooth and rough endoplasmic reticulum and multiple dense core secretory granules (Despommier and

Muller, 1976). The stichocytes are connected to the oesophageal lumen by a duct (Bruce, 1970b; Despommier and Muller, 1976). Upon degranulation, the contents of the secretory granules are released into the stichocyte duct, and are subsequently disgorged *via* the oesophagus (Despommier, 1983).

Proteins secreted by *T. spiralis* muscle stage larvae and adults can be collected from parasites cultured *in vitro*, thus allowing their content to be analysed. To date, substantial immunological, biochemical and molecular studies have focused on the muscle stage larvae due in part to the ease with which significant numbers of this stage can be recovered and the relatively large quantities of secreted proteins that can be collected for analysis compared to other life stages. *T. spiralis* secreted proteins have been detected in epithelial cells invaded by the parasite both *in vivo* and *in vitro*, suggesting that these proteins may play a role in invasion, migration, host immune response evasion, and parasite maintenance in this particular niche (Capo *et al.*, 1986; Manwarren *et al.*, 1997). Secreted proteins have also been detected in the cytoplasm and nuclei of developing nurse cells, where they are thought to be involved in the formation and maintenance of the nurse cell complex through regulating host cell gene expression (Despommier *et al.*, 1990; Lee *et al.*, 1991). Furthermore, this theory is supported by the observation that when proteins secreted by L1 larvae are injected into primary rat myocytes in culture, morphological and structural changes are elicited (Leung and Ko, 1997). Although the molecular mechanisms underlying the changes to the infected host cells are currently unknown, proteins that are released by the parasite into host cells are likely to be crucial for successful parasitism.

1.4.1 Biochemical characterisation of *T. spiralis* secreted proteins

The number and identity of different types of proteins secreted by *T. spiralis* is largely unknown. To date, only a handful of proteins secreted by *T. spiralis* have been biochemically characterised *in vitro*, and even fewer have been cloned. The majority of studies have focused on characterising enzymatic activities present within the total secreted proteins of muscle stage larvae. Advancement in this field has been hampered by the amount of secreted proteins recovered from cultured parasites, which in many cases precludes the purification of specific proteins. Recently, 2-D gel electrophoresis and proteomic analyses have shown that the *T. spiralis* L1 infective larvae secrete at least 52 proteins of different size and isoelectric point, and at least 13 different types of protein, some of which are completely novel (Robinson and Connolly, 2005). The

current knowledge regarding the biochemical activities present in *T. spiralis* ES is described below.

Nematode parasites including *T. spiralis* have been widely reported to secrete specific factors that modulate the immune responses of their host to facilitate their long-term survival. In terms of biochemical characterisation, homologues of human macrophage migration inhibitory factor (MIF), a protein that interferes with migration of cells from the monocytes lineage has been identified in the secreted proteins of a variety of parasites including *T. spiralis* (Pennock *et al.*, 1998; Tan *et al.*, 2001). The *T. spiralis* MIF was shown to inhibit monocyte migration and chemotaxis (Tan *et al.*, 2001). Human MIF can also profoundly influence T-cell proliferation. However, the *T. spiralis* MIF has no effect on T cells (Tan *et al.*, 2001). Tan *et al.*, (2001) suggested that the secreted MIF may serve to inhibit monocyte migration locally in infected tissue, thus reducing the ensuing inflammatory response and aiding parasite survival.

A number of enzymes that are normally associated with lysosomal function, such as proteases, endonucleases and glycosidases have also been reported to be secreted by *T. spiralis*. Proteases secreted by parasites are thought to be involved in a wide variety of adaptive functions including tissue penetration, degradation of the extracellular matrix, larval migration, immune evasion, digestion and moulting (McKerrow, 1989; Shapiro, 1998). Several authors have reported the presence of serine- and metallo- proteases in the secreted proteins of *T. spiralis* (Criado-Fornelio *et al.*, 1992; Takamoto and Sugane, 1993; De Armas-Serra *et al.*, 1995a and 1995b; Todorova *et al.*, 1995; Todorova, 2000; Lun *et al.*, 2003). It has also been proposed that proteolysis may facilitate enterocyte penetration by the larvae since under *in vitro* conditions the infective larvae secrete enzymes that exhibit gelatinolytic, collagenolytic and elastolytic activities and hence would be capable of degrading components of the extracellular matrix (Criado-Fornelio *et al.*, 1992; Lun *et al.*, 2003).

T. spiralis and other types of parasitic nematodes such as *Brugia malayi* (Murray *et al.*, 2005), *Nippostrongylus brasiliensis* (Dainichi *et al.*, 2001) and *Haemonchus contortus* (Newlands *et al.*, 2001) also secrete protease inhibitors. It is thought that nematode protease inhibitors may act as immuno-modulatory factors that contribute to the immune evasion strategies of the parasite, for example by inhibiting proteases involved in antigen processing and presentation (Nagano *et al.*, 2001; Robinson *et al.*, 2007). *T. spiralis* infective larvae secrete a serine protease inhibitor (Nagano *et al.*, 2001). A recent study has also reported the cloning of a novel cysteine protease-like

inhibitor secreted from both infective larvae and adults (Robinson *et al.*, 2007). This protein does not appear to exhibit any of the inhibitory effects that are usually characteristic of cystatins and has been classified as a divergent form of cystatin, the *in vivo* function of which is unknown (Robinson *et al.*, 2007).

Double-stranded DNA endonuclease activity was detected in the secreted proteins of *T. spiralis* infective larvae by Mak and Ko (1999). Subsequently, single-stranded DNA and RNA endonuclease activity (Mak *et al.*, 2000) and DNA-binding activity (Mak and Ko, 2001) have also been detected in the ES proteins. The functions of the endonucleases and the DNA binding proteins are still unknown. However, the endonuclease activity is postulated to play a role in degrading the host genome, dysregulating nucleic acid metabolism, creating chromosomal alterations, and inducing the arrest of the infected muscles at the G2/M phase of the cell cycle (Mak and Ko, 1999; Mak *et al.*, 2000; Jasmer, 1993). Furthermore, Mak and Ko (2001) have suggested that the DNA binding activity in ES may play a crucial role in the reorganisation of host muscle cells by modulating host gene expression.

Other lysosomal-type enzymes have also been detected in the secreted proteins of *T. spiralis*. The lysosomal degradation of glycosphingolipids with small carbohydrate chains requires the presence of non-enzymatic cofactors such as the ganglioside GM2-activator protein (GM2AP), and prosaposin, the precursor of sphingolipid activator proteins (saposins). Recently, a prosaposin homologue (Selkirk *et al.*, 2004) and a highly unusual GM2AP (Bruce *et al.*, 2006) were identified in the ES proteins of *T. spiralis*. The identification of classic lysosomal-type proteins in *T. spiralis* ES, and the localisation of the prosaposin homologue to secretory lysosome-type structures in the stichosome has led to speculation that secretion from lysosomes may constitute an additional pathway distinct from the stichocyte granules usually associated with secreted protein storage and release (Selkirk *et al.*, 2004). It has been postulated that the *T. spiralis* secreted prosaposin and GM2AP are involved in intestinal epithelial cell invasion either by disruption of the lipid membrane of the cell or through the activation of lipases, which in turn may play a role in epithelial cell invasion (Selkirk *et al.*, 2004; Bruce *et al.*, 2006).

Recently, Bruce and Gounaris (2006) also identified a novel *N*-acetyl- β -hexosaminidase activity in the secreted proteins of *T. spiralis*. The enzyme specifically hydrolyses glycans commonly occurring on mammalian glycoproteins and other glycoconjugates such as the major sulphated aminosugar in mucus glycoproteins (Bruce

and Gounaris, 2006). It has been proposed that by cleaving terminal carbohydrate residues from glycoconjugates during the intestinal phase of infection, the *N*-acetyl- β -hexosaminidase could act in concert with other hydrolytic enzymes to facilitate parasite invasion. Unexpectedly, although the *N*-acetyl- β -hexosaminidase and GM2AP would usually work together in the lysosome to degrade gangliosides, the secreted *T. spiralis* homologues do not (Bruce *et al.*, 2006); again highlighting how functionally divergent parasite proteins can be compared to their mammalian counterparts.

Phosphorylation of proteins by protein kinases can alter their enzymatic activities and other properties, and represents a major method of regulating metabolic pathways, gene expression, cell cycle progression and a plethora of other functions (Hunter, 1995). At least one and possibly two distinct serine/threonine protein kinases able to phosphorylate both endogenous and exogenous substrates have been detected in the secreted proteins of *T. spiralis* (Arden *et al.*, 1997). The major endogenous substrates phosphorylated among the proteins secreted by the parasite were polypeptides of 50 and 55 kDa (Arden *et al.*, 1997). These were identified as differentially glycosylated variants of a 35 kDa protein (Arden *et al.*, 1997). On the basis that secreted ES proteins were able to phosphorylate exogenous substrates such as myelin basic protein, MyoD and histone H1, Arden and co-workers (1997) suggested that these serine/threonine protein kinases may be involved in the invasion and migration of larvae through the intestinal epithelium, or the development of the nurse cell complex.

T. spiralis also secretes a range of enzymes capable of degrading and utilising extracellular nucleotides (Gounaris *et al.*, 2001; Gounaris, 2002; Gounaris *et al.*, 2004). Although the functions of these enzymes have been characterised biochemically, their exact role *in vivo* has yet to be determined. As nucleotides are extracellular signalling molecules capable of activating cell membrane-bound purinergic receptors, it has been postulated that the *T. spiralis*-secreted enzymes could regulate the availability of local nucleotides, resulting in alteration of purinergic receptor activation on immune cells and thus modulation of the ensuing inflammatory response (Gounaris, 2002; Gounaris and Selkirk, 2005). Ultimately, this may serve to prolong parasite survival in both the enteral and parenteral niche.

T. spiralis has been reported to secrete a 17 kDa nucleoside diphosphate kinase (NDPK) (Gounaris *et al.*, 2001). This kinase exhibits broad specificity in that it can readily accept γ -phosphate from either adenosine or guanine triphosphate (ATP and

GTP respectively). The NDPK uses the γ -phosphate to form a phosphohistidine intermediate prior to donating the phosphate to adenosine, guanine, uridine or cytidine diphosphates (ADP, GDP, UDP and CDP respectively) (Gounaris *et al.*, 2001). Additionally, this enzyme undergoes auto-phosphorylation using γ -phosphate derived from ATP and GTP substrates (Gounaris *et al.*, 2001). NDPKs are usually intracellular 'house-keeping' enzymes that maintain levels of intracellular nucleotide pools (de la Rosa *et al.*, 1995). Although the *in vivo* role of this secreted enzyme is unknown, it has been postulated that it might function to modulate levels of adenine and uridine nucleotides in order to influence cellular function *via* regulating cell membrane purinergic receptor activation (Gounaris *et al.*, 2001).

T. spiralis infective larvae also secrete a novel 5'-nucleotidase (5'-NT) of 67 kDa, which is homologous to enzymes of the 5'-nucleotidase family in terms of protein sequence, but unique in terms of its enzymatic properties (Gounaris *et al.*, 2004). This enzyme is unusual in that it exhibits both apyrase (nucleoside 5'-diphosphate hydrolysis, $\text{NDP} \rightarrow \text{NMP} + \text{Pi}$) and 5'-nucleotidase (nucleoside 5'-monophosphate hydrolysis, $\text{NMP} \rightarrow \text{N} + \text{Pi}$) activities (Gounaris *et al.*, 2004). The secreted 5'-NT catalyses the hydrolysis of nucleoside 5'-monophosphates with a preference for adenosine monophosphate (AMP), and 5'-diphosphates in the order of $\text{UDP} \gg \text{ADP}$. Furthermore, the secreted 5'-nucleotidase is unique amongst enzymes of this kind in that hydrolysis of diphosphate nucleosides is enhanced by Mg^{2+} ions and a reducing environment. Thus, it is possible that optimal conformation for catalysis is achieved upon secretion of the enzyme into the relatively reducing host environment. Unlike some other types of 5'-nucleotidases, this enzyme cannot catalyse the hydrolysis of nucleoside 5'-triphosphates (Gounaris *et al.*, 2004).

A secreted adenosine deaminase with an apparent molecular mass of 38 kDa has also been detected in the secreted proteins of *T. spiralis* (Gounaris, 2002). The secreted adenosine deaminase catalyses the irreversible hydrolytic deamination of adenosine to inosine and ammonia and appears to be similar in terms of activity to other enzymes of this kind (Cristalli *et al.*, 2001).

It has been proposed that the *T. spiralis* secreted 5'-nucleotidase and adenosine deaminase may form a nucleotide catabolising cascade, with the 5'-nucleotidase hydrolysing ADP/UDP to AMP/UMP and AMP to adenosine, which is further hydrolysed to inosine by the adenosine deaminase (Gounaris and Selkirk, 2005). During the enteral and migratory phase of infection, the *T. spiralis* parasites cause extensive

cellular damage as they migrate through the host, resulting in the release of nucleotides into the extracellular environment. These nucleotides could then bind to purinergic receptors present on surrounding cells, alerting the attention of immune cells to the presence of the parasite. Due to the far-reaching effects purinergic receptor activation can have on the functions of immune cells, the secretion of a cascade of nucleotide metabolising enzymes by the parasite may be a strategy to divert the host immune response, thus promoting parasite survival.

1.5 Purinergic signalling

As previously mentioned, purinergic signalling is a term used to describe the interaction of extracellular nucleotides with membrane-bound purinergic receptors found on many different cell types. The crucial factors that regulate purinergic signalling are the stimulation of nucleotide release from the intracellular environment, the metabolism of nucleotides by extracellular enzymes and the presence of specific purinergic receptor sub-types that selectively bind to the nucleotides and initiate signal transduction. This has imposed a layer of complexity to the understanding and quantification of the pharmacologically active nucleotides.

The first description of extracellular signalling by purine nucleotides was by Drury and Szent-Gyorgyi (1929) in a seminal paper reporting the activities of adenine compounds on the mammalian heart. However, the existence of plasma membrane receptors for extracellular nucleotides, were not formally recognised until 1976 (Burnstock, 1976). Spedding and Weetman (1976), subsequently suggested that separate receptors responded to adenosine and ATP. By 1978, receptors for purines were subdivided into P1 receptors responsive to adenosine, and P2 receptors responsive to ATP and ADP nucleotides (Burnstock, 1978). Several years later, subdivision of P2 receptors into P2X and P2Y sub-types was made on the basis of pharmacological data (Burnstock and Kennedy, 1985). The P2Y receptor family also includes receptors for extracellular pyrimidine nucleotides such as UDP and UTP (Seifert and Schultz, 1989). Hence, in this context, the term ‘purinergic signalling’ is a misnomer, as it is used to describe receptor activation induced by either purine or pyrimidine nucleotides. Over the past decade, purines and pyrimidines have been shown to be physiological regulators of wide range of cellular functions including cell growth, differentiation and death, and very importantly the development of pro- and anti-inflammatory immune responses. The following section describes the current families of known purinergic

receptor sub-types, and details some of the major effects nucleotides and nucleosides can have on different cell types.

1.5.1 P1 receptors

There are four known G-protein coupled P1 adenosine receptors, A₁, A_{2A}, A_{2B} and A₃, each with varying affinity for this agonist. The general characteristics of the different sub-types of P1 receptors are summarised in Table 1.2. A₁ and A_{2A} receptors are activated by nanomolar concentrations of adenosine, whereas A_{2B} and A₃ receptors require micromolar concentrations for activation (Jin *et al.*, 1997; Fredholm *et al.*, 2001a). Extracellular adenosine levels under physiological conditions are between 30 and 300 nM, which would be sufficient to induce 10 to 50% of the maximal response of A₁ and A_{2A} receptor activation (Winn *et al.*, 1981; Zetterström *et al.*, 1982). In inflammatory situations the extracellular levels of adenosine can rise to 10-100 µM, thus resulting in the additional activation of A_{2B} and A₃ receptors (Hasko *et al.*, 2004). Generally, adenosine produced in during inflammation reduces tissue injury and promotes repair by several receptor-mediated mechanisms, thus acting to dampen inflammatory responses (Fredholm, 2007).

The nucleoside inosine can bind to rodent A₃ receptors in the range of 10-50 µM (Jin *et al.*, 1997), and is also a weak agonist of recombinant, human A₁ and A₃ receptors (Fredholm *et al.*, 2001b). Normal interstitial concentrations of inosine are similar to those reported for adenosine. However, during tissue ischemia inosine concentrations can rise to greater than 1 mM, and activate A₃ receptors (Jones *et al.*, 1977; Ehlert, 1992). High extracellular concentrations of inosine have also been reported to have immunosuppressive properties when acting *via* A₃ receptors (Gomez and Sitkovsky, 2003).

P1 receptors usually transduce activation signals through heteromeric G proteins that can either stimulate (G_s) or inhibit (G_{i/o}) adenylyl cyclase, the enzyme that catalyses the formation of cAMP. The P1 receptors A_{2A} and A_{2B} signal primarily *via* G_s, resulting in activation of adenylyl cyclase, an increase in cyclic AMP production and activation of protein kinase A (PKA). A₁ and A₃ receptors have been shown to interact with multiple G proteins, with pathways varying between different cell systems that can result in the inhibition of adenylyl cyclase (Palmer *et al.*, 1995). Adenosine receptors are also coupled *via* distinct G-proteins to other effector systems, including potassium and calcium channels, phospholipase C, phosphodiesterases, cGMP and mitogen activated

protein kinases (Theil *et al.*, 2003). This enables P1 receptors to modulate many different cellular functions and explains how activation of these receptors has such diverse effects on different cell types.

Receptor	Agonist type and concentration required for activation	Affinity for adenosine	Primary G protein coupling/second messenger
A ₁	Adenosine (3-30 nM) Inosine (300 µM)§	High	G _{i/o} ; inhibition of adenylyate cyclase
A _{2A}	Adenosine (1-20 nM)	High	G _s ; activation of adenylyate cyclase
A _{2B}	Adenosine (5-20 µM)	Low	G _s ; activation of adenylyate cyclase
A ₃	Adenosine (>1 µM) Inosine (1 µM)§ Inosine* (10-50 µM)	Low	G _{i/o} ; inhibition of adenylyate cyclase

Table 1.2 Characteristics of P1 receptors

This table summarises the data on the potency of adenosine and inosine at human and rodent P1 receptors, and was adapted from Fredholm, 2007. § Inosine is a weak agonist at recombinant heterologously expressed human A₁ and A₃ receptors (Fredholm *et al.*, 2001b). * Inosine binds to rodent A₃ receptors (Jin *et al.*, 1997).

1.5.2 P2Y receptors

P2Y receptors are G-protein-coupled receptors (GPCR) composed of 7 hydrophobic transmembrane (7 TM) regions connected by 3 extracellular loops and 3 intracellular loops. On the basis of their structure, these receptors belong to the rhodopsin family of 7 TM GPCRs. Primitive P2Y-like receptors appear to have emerged early in evolution and are thought to have existed for more than 450 million years (Schultz and Schoneberg, 2003), consistent with the view that purinergic signalling may be among the oldest signalling mechanisms (Dranoff *et al.*, 2000). Structurally related groups of GPCRs within the rhodopsin family include cysteinyl leukotriene receptors, proteinase activated receptors and glycoprotein hormone receptors (Fredriksson *et al.*, 2003; Costanzi *et al.*, 2004). Currently, there are eight human, and seven murine P2Y receptors formally recognised by the ‘international union of pharmacology’: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃ and P2Y₁₄ (Abbracchio *et al.*, 2006). Rodents express orthologues of all the P2Y receptors except P2Y₁₁. Each receptor is responsive to a different range of nucleotide agonists as

summarised in Table 1.3. Species-specific differences in ligand specificity and affinity also occur within the group of mammalian P2Y receptors. Notably, although ATP and UTP activate the rodent P2Y₄ orthologues equipotently, ATP antagonises the human P2Y₄ receptor (Kennedy *et al.*, 2000).

Receptor	Agonist (Human)	Agonist (Murine)	Primary G protein coupling/second messenger	Selected references
P2Y ₁	ADP >>ATP	ADP>ATP	G _{q/11} ; PLCβ activation	Ayyanathan <i>et al.</i> , 1996; Janssens <i>et al.</i> , 1996; Tokuyama <i>et al.</i> , 1995
P2Y ₂	ATP=UTP	UTP=ATP	G _{q/11} ; PLCβ activation	Lustig <i>et al.</i> , 1993; Parr <i>et al.</i> , 1994; Lazarowski <i>et al.</i> , 1995
P2Y ₄	UTP	UTP=ATP	G _{q/11} ; PLCβ activation	Suarez-Huerta <i>et al.</i> , 2000; Communi <i>et al.</i> , 1996a
P2Y ₆	UDP>>UTP	UDP>UTP>ADP	G _{q/11} ; PLCβ activation	Lazarowski <i>et al.</i> , 2001; Communi <i>et al.</i> , 1996b
P2Y ₁₁	ATP	No receptor	G _{q/11} ; PLCβ activation	Communi <i>et al.</i> , 1999
P2Y ₁₂	ADP	ADP	G _{i/o} ; inhibition of adenylate cyclase	Von Kugelgen <i>et al.</i> , 2001; Hollopeter <i>et al.</i> , 2001
P2Y ₁₃	ADP	ADP>ATP	G _{i/o} ; inhibition of adenylate cyclase	Zhang <i>et al.</i> , 2002; Communi <i>et al.</i> , 2001; Marteau <i>et al.</i> , 2003
P2Y ₁₄	UDP-glucose>UDP-galactose	UDP-glucose	G _{i/o} ; inhibition of adenylate cyclase	Chambers <i>et al.</i> , 2000; Freeman <i>et al.</i> , 2001

Table 1.3 Cloned human and murine P2Y receptor sub-types

This table summarises information relating to cloned mammalian P2Y receptors that have been proven to mediate actions of extracellular nucleotides when functionally expressed in heterologous assay systems.

Recently, it has been reported that the cysteinyl leukotriene receptors 1 and 2 (CysLTR1 and 2) can be stimulated by nanomolar concentrations of UDP (Mellor *et al.*, 2001 and 2003). This showed for the first time, that other GPCRs of the rhodopsin family can respond to nucleotides.

There are several known orphan GPCRs with sequences related to those of the P2Y family, many of which have unknown functions (Fredricksson *et al.*, 2003). Very recently, the orphan GPCR, GPR17 originally discovered by Blasius *et al.*, 1998, has been reported to be activated by both CysLT receptor ligands and uridine nucleotides (Ciana *et al.*, 2006). This correlates with this GPCR being at an intermediate phylogenetic position between P2Y and CysLT receptors (Blasius *et al.*, 1998). As yet GPR17 has not been classified as a P2Y receptor. Potentially, a new classification system may be required to accommodate the growing number of dualistic receptor sub-types. The 'deorphanisation' of P2Y-like GPCRs and the identification of their roles is expected to provide new insight into the regulatory mechanisms of physiological processes (Howard *et al.*, 2001; Fredriksson *et al.*, 2003). Hence, it is likely that in the future many more orphan GPCR may be designated as P2Y receptors. Interestingly, purinergic receptors can also form heteromeric and homomeric associations with each other in the cell membrane, thus potentially altering receptor specificity. For example, the P1 receptor A₁ can associate with the P2Y receptors P2Y₁ and P2Y₂ *in vitro*, resulting in significant changes in ligand binding properties (Yoshioka *et al.*, 2001).

P2Y receptors can be subdivided into two sub-groups on the basis of their structure, phylogeny, amino acid sequence and coupling to G-proteins. P2Y₁, ₂, ₄, ₆ and P2Y₁₁ receptors all principally use G_q/G₁₁ to activate the PLCβ/IP₃ pathway and release intracellular calcium, whereas the receptors P2Y₁₂, ₁₃ and ₁₄ almost always couple to G_{i/o} family of G proteins (displayed in Table 1.3) (Abbrachio *et al.*, 2006). Current research is also focusing on evidence that suggests that P2Y receptors can couple directly to voltage-gated ion channels at the cell surface *via* activation of G proteins. Primarily, ion channel couplings of P2Y receptors are of major importance in brain neurons (Dolphin, 2003).

Most of the P2Y receptor sub-types are currently lacking selective, potent agonists and antagonists (Abbracchio *et al.*, 2001). Considering that most cell types express multiple types of purinergic receptors, this makes it very difficult to characterise the specific receptor subtypes(s) mediating a response. An extensive research effort to design and find selective agonists and antagonists is currently underway. Considerable

progress in exploring structure-activity relationships between receptor and ligand has been made for the P2Y₁ and P2Y₁₂ receptors and to a lesser extent for the P2Y₂ receptor (Abbracchio *et al.*, 2001). The majority of antagonists available are nucleotide-derivatives. For example, adenosine bisphosphate derivatives such as A3P5P act as partial antagonists for the P2Y₁ receptor (Boyer *et al.*, 1996). Non-nucleotide compounds are also used as purinergic receptor antagonists. These are usually highly negatively charged polycyclic compounds, for example the polysulfonate suramin, and the pyridoxal phosphate derivative PPADS, are general non-selective inhibitors of P2Y receptors. As yet, extensive structure-activity relationship manipulations of non-nucleotide compounds have not resulted in antagonists of nanomolar affinity.

1.5.3 P2X receptors

P2X receptors are ligand gated ion channels that gate extracellular cations in response to ATP only. There are seven different P2X receptor subtypes, P2X₁₋₇, which are 40-50% identical in amino acid sequence. Channels form as multimers of several subunits, where each subunit has two transmembrane domains. P2X receptor diversity is further expanded, as some sub-types can form heteromeric associations with other P2X receptor sub-types. To date, P2X₁/P2X₅ (Le *et al.*, 1999), P2X₂/P2X₆ (King *et al.*, 2000), and P2X₄/P2X₆ (Khakh *et al.*, 1999) receptor associations have been identified. Heterologous expression of cloned P2X receptor subtypes has shown that each receptor sub-type has a slightly different response profile in terms of agonist and antagonist affinity and selectivity, permeation properties and desensitisation/inactivation rate (North, 2002). Signal transduction occurs *via* fast Na⁺ and Ca²⁺ influx and K⁺ efflux, leading to depolarization of the plasma membrane and an increase in the concentration of cytosolic Ca²⁺. P2X receptors are found ubiquitously and also account for fast neurotransmission in excitatory tissues. The P2X₇ receptor is unusual amongst the other P2X sub-types in that it requires millimolar concentrations of ATP to achieve activation (Steinberg *et al.*, 1987; Greenberg *et al.*, 1988). P2X₇ receptor activation may also induce the opening of a nonselective pore permeable to large organic molecules up to 900 Da (North, 2002; North and Suprenant, 2000). Continuous activation of this receptor results in the formation of a large non-selective transmembrane pore that can cause perturbations in ion homeostasis and finally result in cell death (Di Virgilio, 1995; Suprenant *et al.*, 1996). The ability of P2X receptors to mediate the responses of many

types of immune cells has prompted a great deal of interest in this area over the recent years.

1.6 Nucleotide release and uptake

Nucleotides can be released from cells in both regulated and non-regulated manners. Tissue damage results in the non-regulated release of large amounts of nucleotides into the extracellular environment. However, nucleotides, particularly ATP are released in a regulated manner from many excitatory and secretory tissues. For example, neurons, chromaffin cells, platelets, mast cells and pancreatic acinar cells can all release ATP by regulated exocytosis (Evans *et al.*, 1992; Burnstock, 1997; Sorensen and Novak, 2001). Enhanced release of nucleotides also occurs after the mechanical stimulation of many different cell types, including endothelial, epithelial and glial cells (Vlaskovska *et al.*, 2001; Burnstock, 1999; Parr *et al.*, 1994). Nucleotides are also released from some non-secretory cell types in response to agonists. For example, studies have shown that treatment of coronary endothelial cells with ADP (Buxton *et al.*, 2001), and stimulation of COS-7 fibroblast-like cells and HEK-293 kidney cells with UTP results in the release of ATP (Ostrom *et al.*, 2000). An alternative mechanism for the release of nucleotides into the extracellular space *via* vesicular trafficking has also recently been proposed by Bodin and Burnstock (2001). They observed that ATP released from endothelial cells was dependent on vesicular formation from the Golgi apparatus, suggesting that ATP release could also be mediated by vesicular exocytosis.

Mechanisms also exist that allow cells to take-up extracellular adenosine. Movement of adenosine across the cell membrane is mediated by equilibrative nucleoside transporters (ENT) (Baldwin *et al.*, 2004). Adenosine formed intracellularly due to the metabolism of ATP will be transported out of cells until an equilibrium is reached with the external environment. In a similar manner, extracellular adenosine is transported into cells when intracellular adenosine levels are low. By regulating the concentration of adenosine available to cell surface receptors, these transporters are thought to influence many physiological and inflammatory processes (Musa *et al.*, 2002; Ackley *et al.*, 2003).

1.7 Extracellular nucleotide metabolism

The concentration and types of extracellular nucleotides available to interact with purinergic receptors is regulated by several families of ubiquitously distributed

membrane-bound and secreted nucleotide metabolising enzymes that rapidly catalyse the breakdown and interconversion of nucleotides (Zimmermann, 2000). Extracellular ecto-nucleotidases are expressed by most if not in all cell types. Several families of ectoenzymes participate in the degradation and interconversion of extracellular nucleotides.

Most prominent of these families are the members of the ecto-nucleotidase 5'-triphosphate diphosphohydrolase (E-NTPDase) family. Eight members of this family (E-NTPDase1-8) have been described so far. E-NTPDases 1, 2, 3 and 8 are cell surface-associated, whereas the remaining four types are intracellular enzymes. E-NTPDase 1 (otherwise known as ecto-ATPDase, CD39, apyrase), hydrolyses both nucleoside triphosphates and nucleoside diphosphates; E-NTPDase 2 (ecto-ATPase, CD39L1), hydrolyses nucleoside triphosphates; and E-NTPDase 3 (ectoATPDase, CD39L3), hydrolyses both nucleoside triphosphates and nucleoside diphosphates (Zimmermann, 2000; Lazarowski *et al.* 2003a). The secreted enzyme, NTPDase5 (CD39L4), may be responsible for soluble nucleotidase activities and preferentially catalyses the hydrolysis of nucleoside diphosphates and to a lesser extent nucleoside triphosphates (Mulero *et al.*, 1999). The more recently described E-NTPDase 8 hydrolyses adenine and uridine nucleotide di- and tri-phosphates (Bigonnesse *et al.*, 2004).

The ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family are cell surface trans-membrane metalloenzymes that can hydrolyse nucleoside 5'-triphosphate (NTP) to nucleoside monophosphate (NMP) and nucleoside 5'-diphosphate (NDP) to NMP. UDP-glucose, the natural agonist of the P2Y₁₄ receptor, and other nucleotide sugars are also substrates of E-NPP isoenzymes (Lazarowski, 2003b). The E-NPP family comprises five members. However, currently only the activities of E-NPP1-3 have been characterised (Goding *et al.*, 2003).

The abundant GPI-linked enzyme ecto-5'-nucleotidase (otherwise known as CD73) can convert NMP to nucleoside plus orthophosphate and is the major contributing factor to the accumulation of extracellular adenosine from AMP (Zimmermann, 1996).

Extracellular nucleotide interconverting activities such as nucleoside monophosphate- and diphosphate-kinases have also been described. For example, the activity of ecto-nucleoside diphosphate kinase (NDPK) catalyses transphosphorylation reactions such as the reversible phosphorylation of UDP or GDP by ATP (e.g., $\text{ATP} + \text{UDP} \rightleftharpoons \text{ADP} + \text{UTP}$) and adenylyl kinase can transphosphorylate 2ADP to ATP and

AMP ($2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP}$) (Yegutkin *et al.*, 2001 and 2002; Donaldson *et al.*, 2002; Picher and Boucher, 2003). In many instances both ATP-consuming and ATP-generating pathways co-exist on the cell surface, providing a mechanism for regulating the duration and magnitude of purinergic signalling (Yegutkin *et al.*, 2001).

Extracellular adenosine is mainly formed by the breakdown of intracellular or extracellular adenine nucleotides (Zimmermann, 2000). Extracellular adenosine can be catabolised to inosine by cell membrane-bound adenosine deaminase (ADA). Intracellular adenosine can either be deaminated to inosine by ADA, or phosphorylated to AMP by adenosine kinase (ADK). When ADA is blocked or genetically deleted as in SCID (severe combined immunodeficiency) mice, the enzymatic capacity of ADK is exceeded and adenosine levels rise rapidly. This leads to defective lymphocyte function, which is thought to be partly due to the accumulation of intracellular and extracellular adenosine (Apasov and Sitkovsky, 1999). This highlights the critical importance of adenosine metabolising enzymes in regulating immune cell function.

Local availability of extracellular nucleotides and nucleosides to cells of the immune system are implicated extensively in regulation of inflammation and are known to have immuno-modulatory effects (Cronstein, 1994; Linden, 2001). Notably, nucleotide metabolising enzymes such as CD39 and CD39L1 are expressed on a wide variety of immune cell types including dendritic cells, macrophages, and activated B lymphocytes (La Sala *et al.*, 2003). The presence of extracellular nucleotide metabolising enzymes play an important role in the modulation of immune responses. Recently, the coordinate expression of E-NTPDase 1 (CD39) and ecto-5'-nucleotidase (CD73) on T cells has been reported to be a marker of immuno-suppressive regulatory T cells (Deaglio *et al.*, 2007). Furthermore, T regulatory (Treg) cells from E-NTPDase 1-deficient mice (which have a lower capacity to metabolize extracellular ATP) show impaired suppressive properties *in vitro* and fail to block allograft rejection *in vivo* (Deaglio *et al.*, 2007). CD39 expression was found to be induced by the Treg cell-specific transcription factor Foxp3 and its catalytic activity was also enhanced by T-cell receptor (TCR) ligation. Activated Treg cells are therefore able to abrogate ATP-related effects such as P2 receptor-mediated cell toxicity and ATP-driven maturation of dendritic cells (Borsellino *et al.*, 2007). Other studies have also shown that nucleotide metabolising enzymes can have immunomodulatory effects. For example, monocyte/macrophages from E-NTPDase 1-null mice display an impaired chemotactic

response to ATP, associated with P2Y receptor-mediated signaling pathway desensitization (Goepfert et al., 2001).

1.8 Purinergic signalling in immune cells

Nucleotides released from cells are thought to act as ‘danger signals’ to alert the immune response of abnormal cell death or cell stress. Extracellular nucleotides and nucleosides play complex roles in activating and regulating the immune response. The balance between pro- and anti-inflammatory immune responses is critical for avoiding excess tissue damage. The modulation of inflammatory processes and immune responses by the P2Y and P2X receptor agonist ATP is complex and results from specific effects on a wide variety of immune cells. In many scenarios, ATP promotes inflammatory immune responses (La Sala *et al.*, 2003). The metabolism of ATP to adenosine by extracellular nucleotide metabolising enzymes results in the formation of adenosine which can act as a suppressive tissue protecting signal, in a negative feedback manner (Bours *et al.*, 2006). This paradigm holds true especially for neutrophils and macrophages/monocytes and to a lesser extent, lymphocytes and dendritic cells. The following section highlights some of the far-reaching effects nucleotides can have on the functions of many different immune cell types.

1.8.1 Dendritic cells

Dendritic cells (DCs) play a major role in the initiation and regulation of innate and adaptive immunity. Low concentrations of ATP released from damaged or stressed cells directs the migration of immature dendritic cells towards the source of ATP release. In this scenario the release of nucleotides acts as a ‘danger signal’, alerting the initiation of an appropriate immune response. In contrast, high concentrations of ATP inhibit the migratory capacity of DCs through P2Y₁₁ receptor activation (Idzko *et al.*, 2002). These differing responses highlight the concentration-dependent effects nucleotides can have on immune cell types, and may be a mechanism for limiting over-exuberant immune responses. Dendritic cells also express high levels of the receptor P2X₇, which explains why these cells are exceedingly sensitive to the cytotoxic activity of ATP and readily undergo apoptosis.

ATP released from stressed or damaged cells also induces an alternative pathway of DC maturation, characterized by the up-regulation of costimulatory membrane molecules but the absence of IL-12 and inflammatory cytokine and chemokine

production (la Sala *et al.*, 2001, 2002). This response appears to be mediated by activation of the P2Y₁₁ receptor (Wilkin *et al.*, 2001). DCs matured in the presence of ATP have an impaired capacity to promote type 1 polarization of naïve T lymphocytes, favouring instead the development of T_H2 lymphocytes (la Sala *et al.*, 2001, 2002). This is proposed to be beneficial to survival, by limiting inflammatory T_H1 responses (la Sala *et al.*, 2003).

1.8.2 Monocytes and Macrophages

An important component of the innate immune response, phagocytic circulating monocytes and tissue-dwelling macrophages principally contribute to chronic inflammatory responses. Monocytes and macrophages express multiple sub-types of P2Y and P2X receptors and all PI receptors (summarised in Bours *et al.*, 2006). It has been reported that during inflammatory and immune responses, extracellular ATP and ADP can induce upregulated expression of adhesion molecules on monocytes (Ventura and Thomopoulos, 1991), thus allowing them to firmly tether to endothelial cells and subsequently transmigrate through the endothelium and enter sites of inflammation (Goepfert *et al.*, 2001). UDP signalling *via* the P2Y₆ receptor stimulates monocytes to increase chemokine production, thus promoting the recruitment of other leukocytes to sites of inflammation (Warney *et al.*, 2001; Cox *et al.*, 2005). In contrast, adenosine has a more inhibitory effect on the adhesion of monocytes to the vascular endothelium and chemotactic responses (Pike and Snyderman, 1981; Okusa *et al.*, 2000), thus limiting the accumulation of macrophages at inflamed/damaged sites.

Extracellular nucleotides also have a considerable impact on cytokine production by monocytes and macrophages. Many studies have reported that high concentrations of ATP signalling *via* the P2X₇ receptor can stimulate the production of IL-1 α , IL-1 β , TNF- α , IL-6 and IL-18 (Bours *et al.*, 2006). In contrast, adenosine, acting through multiple PI receptor sub-types inhibits *de-novo* and LPS-induced proinflammatory cytokine production from monocytes and macrophages (Bours *et al.*, 2006; Hasko *et al.*, 1998). Hence, nucleotides and nucleosides can drastically promote or inhibit inflammatory immune responses.

An important function of macrophages is phagocytosis and removal of harmful pathogens. Activation of macrophages by LPS and ATP, ADP or UDP enhances nitric oxide (NO) synthesis (Tonetti *et al.*, 1994) and the production of reactive oxygen species (ROS) (Murphy *et al.*, 1993). Activation of the apoptotic P2X₇ receptor

expressed by monocytes/macrophages can also trigger cell death (Murgia *et al.*, 1992). These mechanisms act to enhance pathogen killing, even at the expense of the survival of the cell, and terminate potentially aberrant inflammatory responses by over-activated macrophages. Thus in general, nucleotides such as ATP dramatically activate pro-inflammatory responses of monocytes/macrophages, whereas adenosine acts to dampen down these responses.

1.8.3 Lymphocytes

Lymphocyte responsivity to nucleotides was discovered almost thirty years ago. In 1978, Gregory and Kern reported that extracellular ATP stimulated proliferation of thymocytes. Since then, the field of purinergic signalling in T lymphocytes has dramatically expanded, and the effects of nucleotides and adenosine on B lymphocyte activation have also been investigated, although the latter to a lesser extent.

T cells express several P2X receptor sub-types (Chused *et al.*, 1996; Gu *et al.*, 2000), and can upregulate the expression of P2Y receptors and release ATP during activation (Filippini *et al.*, 1990; Somers *et al.*, 1998). Extracellular ATP acting *via* P2X₇ receptors can modulate B and T lymphocyte migration by inducing the shedding of the adhesion molecule L-selectin (Jamieson *et al.*, 1996). Since this adhesion molecule is naturally shed upon lymphocyte activation, ATP may be involved in migration of activated lymphocytes to sites of inflammation. Extracellular ATP may also be involved in T lymphocyte proliferative responses. However, the data in this area is conflicting as ATP has been reported to inhibit (Duhant *et al.*, 2002), or induce the proliferation (Budagian *et al.*, 2003) of different types of T cells.

Adenosine can also affect lymphocyte function. T cells express a variety of A_{2A}, A_{2B} and A₃ receptor sub-types (summarised in Bours *et al.*, 2006), some of which are upregulated upon T cell receptor (TCR) activation (Mirabet *et al.*, 1999; Gessi *et al.*, 2004). Extracellular adenosine is thought to inhibit antigen-triggered TCR signalling in T cells, thus attenuating proliferation and generally playing a role in lymphocyte deactivation (Nishida *et al.*, 1984; Mirabet *et al.*, 1999). Cytokine production by activated T cells is also generally inhibited by extracellular adenosine (Erdmann *et al.*, 2005). Furthermore, activation of lymphocyte adenosine receptors *in vivo* has been shown to inhibit lymphocyte-driven inflammatory responses in mice, thus confirming the suppressive effects of adenosine (Odashima *et al.*, 2005). The effect of adenosine on B lymphocytes has not been as extensively studied as on T lymphocytes. However, B

cells are thought to express adenosine A_{2A} and A₁ receptors Conigrave *et al.*, 2001; Napieralski *et al.*, 2003). Currently, the effects of activation of these adenosine receptors on B cell function are still unknown (Conigrave *et al.*, 2001; Napieralski *et al.*, 2003).

1.8.4 Neutrophils

Neutrophils are the body's first line of defence against pathogens and are critical effectors in both innate and humoral immunity. A large body of evidence exists that suggests that nucleotides and nucleosides may contribute to the regulation of neutrophil function during inflammatory and immune responses. Neutrophils express multiple P2Y and P2X receptor sub-types and all four P1 receptors (summarised in Bours *et al.*, 2006). Generally, neutrophil effector functions appear to be enhanced by co-ordinated effects of high ATP levels, while increasing adenosine levels predominantly suppress effector functions of neutrophils.

ATP can stimulate neutrophil adhesion to endothelial cells (Freyer *et al.*, 1988), which is an important step in the recruitment of circulating neutrophils to extravascular inflammatory sites during the early stages of inflammation. Moreover, extracellular ATP may further facilitate neutrophil transmigration by increasing endothelial permeability *via* activation of P2Y receptors (Tanaka *et al.*, 2004). The effects of ATP on neutrophil migration are still controversial, as it has been reported that ATP can promote (Verghese *et al.*, 1996), inhibit (Elferink *et al.*, 1992), or not affect (Aziz and Zuzel, 2001) neutrophil migration. It has also been shown that ATP and UTP can prime neutrophil bactericidal mechanisms by inducing superoxide anion generation (Melloni *et al.*, 1986; Tuluc, 2005), and granule exocytosis (Aziz and Zuzel, 2001; Balazovich and Boxer, 1990). Other reports suggest extracellular ATP can delay neutrophil apoptosis in synergy with the neutrophil survival factor granulocyte macrophage colony-stimulating factor (GM-CSF), thus extending the functional lifespan of neutrophils (Gasmi *et al.*, 1996).

Extracellular adenosine plays a more complex role and acts predominantly to protect host tissues from the potentially damaging effects of neutrophils (Gasmi *et al.*, 1996). Adenosine at micromolar concentrations inhibits the oxidative burst (Cronstein *et al.*, 1983) and degranulation (Pasini *et al.*, 1985) of neutrophils through activation of A_{2A} and possibly A₃ receptors (Bouma *et al.*, 1997; Visser *et al.*, 2000). The effects of adenosine on neutrophil recruitment from the circulation appear to be bi-directional and

dependant upon its extracellular concentration. Sub-micromolar concentrations of adenosine can enhance neutrophil adhesion to the vascular endothelium (Cronstein *et al.*, 1992). In contrast, micromolar concentrations of adenosine inhibit adhesion of neutrophils to vascular endothelial cells (Kilian *et al.*, 2005). The inhibitory effects of adenosine probably dominate under inflammatory and hypoxic conditions during which extracellular adenosine levels rise markedly.

1.8.5 Eosinophils

Eosinophils are important effector cells in gastro-intestinal parasitic helminth infections (Dixon *et al.*, 2006; Coffman *et al.*, 1989) as well as different pathological conditions such as atopic diseases, rhinitis, eczema and asthma (Djukanovic *et al.*, 1990; Walsh, 1997). ATP, UTP and UDP nucleotides acting *via* P2Y and possibly P2X receptors can stimulate eosinophils in multiple ways. ATP is a chemoattractant for eosinophils and as such can direct them to sites of tissue damage (Saito *et al.*, 1991a; Burgers *et al.*, 1993). ATP, ADP and UTP are all able to induce the production of reactive oxygen metabolites in eosinophils (Idzko *et al.*, 2001) that further eosinophil-mediated toxicity by damaging parasite structures and surrounding tissues (Rothenberg, 1998; Frigas and Gleich, 1986). UDP and ATP also trigger the release of pro-inflammatory cytokines (Idzko *et al.*, 2003), and expression of cell surface adhesion molecules that facilitate migration of eosinophils into tissues (Dichmann *et al.*, 2000). Conversely, adenosine signalling *via* the A₃ receptor can downregulate C5a mediated degranulation and super oxide anion generation in eosinophils thus acting to limit inflammatory responses (Ezeamuzie and Phillips, 1999).

1.8.6 Mast cells

Mast cells are heavily granulated cells found throughout the body, particularly at mucosal sites such as the gastro-intestinal (GI) and respiratory tracts. Mast cells are important effector cells in acute IgE-antigen-associated allergic reactions and also mediate immunoregulatory functions, through their ability to produce cytokines. Upon stimulation, mast cells can be induced to secrete mediators that are either pre-formed and granule-associated (for example, histamine, proteoglycans and neutral proteases) or are synthesised *de-novo* (for example cytokines, chemokines, leukotriene C₄, prostaglandin D₂ and platelet activating factor).

Mast cells are known to express a range of P1, P2 and cysteinyl leukotriene receptors (Feng *et al.* 2004; Bulanova *et al.* 2005). Mast cells can respond in multiple ways when activated by different nucleotides/nucleosides. This can involve degranulation, chemotaxis and alteration of receptor expression. The effects of adenosine on mast cells has been the subject of intense research over the past 20 years, ever since it was discovered that inhaled adenosine, acting on lung mast cells, induced bronchoconstriction in asthmatic but not normal subjects (Cushley *et al.*, 1983; Rafferty *et al.*, 1987; Polosa *et al.*, 1995; Forsythe *et al.*, 1999). However, the effects of adenosine on mast cells *in vivo* are very different to the effects observed *in vitro*. Most types of mast cells do not degranulate in response to adenosine *in vitro* (Marquardt *et al.*, 1994; Schulman *et al.*, 1999), while in comparison the *in vivo* administration of adenosine results in the degranulation of mast cells *via* activation of A₃ receptors (Tilley *et al.*, 2000; Zhong *et al.*, 2003; Linden *et al.*, 1999), although the exact mechanisms controlling these events are however unknown.

Recently, experiments using bone marrow-derived mast cells (BMMC) from mice lacking the A_{2B} receptor have shown that loss of this receptor results in an excessive influx of extracellular calcium through store-operated calcium channels following antigen cross-linking of the high affinity IgE receptor FcεRI (Hua *et al.*, 2007). These findings imply that the A_{2B} receptor acts in concert with other signalling pathways to negatively regulate mast cell activation. In contrast, stimulation of mast cell A₃ receptors by adenosine results in amplification of FcεRI-mediated degranulation and cytokine production (Laffargue *et al.*, 2002).

Other experiments using BMMC have demonstrated that prolonged exposure to adenosine receptor antagonists mediates the up-regulation of adenosine receptor expression, resulting in a hyper-responsiveness to exogenous adenosine (Marquardt *et al.*, 1984). Conversely, chronic exposure to the adenosine analogue NECA, results in down-regulation of adenosine responsiveness and a decrease in the expression of adenosine receptors (Marquardt and Walker, 1987). The physiological relevance of these findings are still questionable, as extracellular adenosine is rapidly degraded or taken-up by cells, which would limit the long-term exposure of mast cells to this nucleoside. Again, these examples show the effects of adenosine on P1 receptor activation are complex, and may have unexpected impacts on mast cell activation.

The P2 receptor agonists ATP and ADP can directly induce the degranulation of murine mast cells *in vitro* (Nakamura *et al.*, 1989; Saito *et al.*, 1991b). This may suggest

that the presence of exogenous adenine nucleotides can promote inflammatory responses *via* the release of pre-formed mast cell mediators including histamine in mice (Nakamura *et al.*, 1989; Saito *et al.*, 1991b). However, these effects are species-dependent, as human mast cells can not be induced to degranulate in response to ATP (Schulman *et al.*, 1999).

The nucleotides ADP, ATP and UTP can also direct the migration of rat mast cells *in vitro* (McCloskey *et al.* 1999), suggesting that nucleotides released from sites of tissue damage may be able to recruit mast cells, thus promoting the development of an inflammatory response.

Nucleotides can also modulate mast cell effector functions activated by other signal transduction pathways. For example, antigen-dependent degranulation of mast cells and cytokine production is enhanced when purinergic receptors are co-stimulated with ATP, UTP, adenosine or inosine (Schulman *et al.*, 1999; Tilley *et al.*, 2000). Thus, the release of extracellular nucleotides can promote allergic and inflammatory mast cell responses.

Stimulation of mast cells with ADP and ATP suppresses pro-inflammatory cytokine production by mast cells stimulated with peptidoglycan (PGN), a ligand for the Toll-like receptor (TLR)-2/TLR-6 heterodimer that mediates innate immune responses to Gram-positive organisms (Feng *et al.*, 2004). In a similar manner, ADP can also suppress the generation of pro-inflammatory cytokine production by mast cells stimulated with the pro-inflammatory lipid mediator LTD₄ (Feng *et al.*, 2004). The activation of inhibitory pathways by adenine nucleotides may function to prevent the over-exuberant secretion of potentially harmful cytokines that would otherwise cause extensive tissue pathology.

1.9 Purinergic signalling in gastrointestinal epithelia

Epithelial cells maintain physiological homeostasis by regulating the amount of fluid in the GI lumen. This cell type achieves this by modifying nutrient and ion absorption, and ion secretion. Purinergic receptors play a significant role in regulating ion and fluid transport in many types of epithelial tissues including the epithelium of the gastro-intestinal (GI) tract. Purinergic receptors are found on both the basolateral and luminal membranes of nearly all epithelia that transports ions. The stimulation of ion transport and the flux of water into the lumen that accompanies it are thought to act as a

natural defence system that functions to flush away noxious stimuli in the setting of cellular damage or inflammation of the intestine.

All four P1 receptor subtypes are known to be involved in adenosine-mediated control of ion transport in the gastro-intestinal epithelium. Nucleoside transporters are also thought to play an important role in controlling the effect of adenosine at the epithelial surface by controlling the extracellular concentration (Szkotak *et al.*, 2003). The action of adenosine in the colon is a potent contributing factor in the development of secretory diarrhoea in the setting of inflammation. Neutrophils recruited to intestine during inflammatory responses are a major source of adenosine, as they release large amounts of adenine nucleotides that are rapidly metabolised to adenosine by ecto-5'-nucleotidase which is highly expressed on gut epithelium (Madara *et al.*, 1993). Adenosine induces a direct effect on Cl^- secretion in the colon *via* $\text{A}_{2\text{B}}$ -mediated G_s activation of adenylyl cyclase, leading to a rise in cAMP that directly activates the cystic fibrosis transmembrane conductance regulator protein (CFTR) Cl^- channel (Strohmeier *et al.*, 1995; Bucheimer and Linden, 2003).

Activation of P2 receptors can also activate K^+ secretion in the distal colon and Cl^- secretion in the small intestine. P2Y_4 receptor stimulation with ATP or UTP has been shown to increase Cl^- secretion leading to fluid accumulation in the lumen of the small intestine (Cressman *et al.*, 1999; Robaye *et al.*, 2003; Ghanem *et al.*, 2005). To a lesser extent, stimulation of basolateral P2Y_2 receptors can also stimulate chloride ion secretion in the small intestine (Ghanem *et al.*, 2005). Luminal ATP and UTP can induce K^+ secretion *via* the P2Y_2 and P2Y_4 receptors in the distal colon, resulting in an increase in the amount of fluid on the luminal surface of the epithelium (Kerstan *et al.*, 1998; Matos *et al.*, 2005).

P2X_7 receptors are functionally associated with apoptosis in many different cell types, and are also expressed in duodenal villus tip cells (Ralevic *et al.*, 1998; Schulze-Lohoff *et al.*, 1998). In the intestinal mucosa, epithelia are rapidly regenerated. Stem cells located in the crypt base move to the surface, apoptose and get exfoliated into the intestine. These dying cells eventually release ATP and may provide an extracellular 'death' signal. Thus, strategically located P2X_7 receptors present on the villus tips may ensure intestinal epithelial regeneration (Groschel-Stewart *et al.*, 1999).

1.10 Purinergic signalling in haemostatic responses

Vascular injury leads to the release of nucleotides that can directly activate platelets. Platelet activation is characterized by rapid shape change, release of granular contents, generation of lipid mediators, and aggregation (Kahner *et al.*, 2006). Activation of purinergic receptors results in signalling *via* unique signal transduction pathways that are important in the regulation of thrombosis and haemostasis. When exposed to ADP, P2Y₁ receptor activation triggers platelets to undergo shape change from smooth discs to spiculated spheres which facilitates a weak, transient aggregation response (Jin *et al.*, 1998; Hechler *et al.*, 1998a). Subsequent ADP-induced activation of platelet P2Y₁₂ receptors is required for complete platelet aggregation (Jin and Kunapuli, 1998). Activation of the P2Y₁₂ receptor plays a pivotal role in platelet function, as it amplifies aggregation responses induced by all known platelet agonists (Gachet, 2006).

Platelets also express P2X₁ receptors that are responsive to ATP (MacKenzie *et al.*, 1996). P2X₁ receptor activation can induce substantial and rapidly reversible shape change and centralisation of secretory granules without the release of granular contents outside of the cell (Rolf *et al.*, 2001; Toth-Zsomboki *et al.*, 2003). Although ATP is unable to directly trigger platelet aggregation, activation of the P2X₁ receptor is thought to play a priming role in the subsequent activation of the P2Y₁ receptor (Sage *et al.*, 2000; Vial *et al.*, 2002). The most significant function of P2X₁ receptor activation is through its amplifying of platelet responses mediated by lower concentrations of other platelet agonists. For example, low-dose collagen-mediated dense granule release and aggregation are potentiated in the presence of P2X₁ receptor activation (Oury *et al.*, 2001; 2002). More significantly, P2X₁ receptor activation has been reported to be essential for enhanced platelet adhesion and thrombus formation under high shear blood flow-rates (Hechler *et al.*, 2003).

Platelets also express the A_{2A} receptor (Haslam and Cusack, 1981). Activation of this receptor by adenosine inhibits platelet aggregation induced by other factors such as thrombin (Paul *et al.*, 1990). This is thought to act as a regulatory mechanism to prevent overexuberant aggregatory responses.

1.11 Modulation of purinergic signalling by pathogens

A variety of parasites and pathogens have evolved mechanisms to modulate purinergic signalling. Blood feeding insects and ticks (Ribeiro *et al.*, 1985; Ribeiro and Francischetti, 2003), parasitic nematodes such as *T. spiralis* (Gounaris *et al.*, 2001;

Gounaris, 2002; Gounaris and Selkirk, 2005), protozoans (Silverman *et al.*, 1998) and pathogenic bacteria (Zaborina *et al.*, 1999, 2000; Punj *et al.*, 2000; Melnikov *et al.*, 2000) have all been reported to secrete nucleotide-metabolising enzymes. In some pathogen/parasite types, the role of these secreted enzymes is very obvious. For example, blood-feeding ectoparasites cause tissue damage as they probe the host, resulting in the release of ADP and ATP which are proinflammatory and immediately stimulate platelet aggregation thus halting the flow of blood. To overcome host haemostatic responses, arthropods inject salivary apyrase to deplete ADP and ATP from the lesion produced by probing mouthparts (Champagne *et al.*, 1995; Arca *et al.*, 1999; Valenzuela *et al.*, 1998, 2001; Faudry *et al.*, 2004). Bacterial pathogens such as *Mycobacterium bovis* secrete ATPase and nucleoside diphosphate kinase (NDPK) enzymes that are able to prevent ATP-induced macrophage apoptosis. Therefore, it was suggested that depletion of extracellular ATP by these enzymes promotes the survival of mycobacterial-infected cells (Zaborina *et al.*, 1999).

In comparison, the role of secreted nucleotide-metabolising enzymes in the infectivity of parasitic nematodes is far more elusive due to the complex nature of the parasites lifecycle and the variety of host cell types it comes into contact with. However, interpretation of the physiological effects mediated by these enzymes can be predicted from their known substrate specificity. For example, *T. spiralis* secretes a 5'-NT able to hydrolyse ADP, UDP and AMP substrates, an adenosine deaminase and a NDPK with trans-phosphorylatory activities (Gounaris, 2002; Gounaris *et al.*, 2001, 2004). This suggests that the parasite may secrete these enzymes to modulate physiological and host immune responses directed by ADP, UDP and adenosine.

The nucleotide metabolising enzymes secreted by both ecto- and endo- parasites are similar to ubiquitous cellular enzymes involved in intracellular metabolic processes. This suggests that these parasites and pathogens have independently adapted genes involved in 'house-keeping' events to genes involved in pathogenicity, thus allowing them to survive in the otherwise inhospitable environment of their host. Due to the conserved nature of the genes encoding secreted nucleotide metabolising enzymes, it is likely that annotation of sequenced pathogen/parasite genomes generally categorises these enzymes as metabolic without investigating their potential to be secreted enzymes involved in pathogenicity. Thus, it is possible that many more species of parasites and pathogens may secrete these enzymes.

1.12 Host immune responses and effector mechanisms against the enteral stage of *T. spiralis* infection

Infection with intestinal nematodes induces strong immune responses in the host and profound pathological changes to the gut that are associated with eventual parasite expulsion. The pathology may have several causes, including direct damage from the migration/burrowing activities of the worm or secondary damage resulting from opportunistic bacterial infections, or the host's immune response to the parasite or bacteria.

Host genetic factors strongly influence the expulsion of *T. spiralis* from the murine small intestine. Inbred mice strains are classified into rapid- and slow-responders according to the speed at which they expel parasites from the intestine. Rapid-responder mice expel *T. spiralis* larvae from the intestine within 10 days, and can generate protective immune responses against *T. spiralis* infection when immunised with native parasite antigens (Wakelin, 1988; Robinson *et al.*, 1994, 1995a; Wakelin *et al.*, 1986). In comparison, slow-responder strains can take up to 17 days to expel parasites (Wakelin *et al.*, 1986), and these mice cannot be protected against infection by immunisation with native parasite antigens (Wakelin, 1988; Robinson *et al.*, 1994, 1995; Wakelin *et al.*, 1986).

Crucially, protection against *T. spiralis* infection is dependent upon the T cell-directed development of mucosal mastocytosis in the small intestine (Alizadeh and Murrell, 1984; Oku *et al.*, 1984). The following section highlights the specific immune responses and effector mechanisms that mediate expulsion of *T. spiralis* from the GI tract.

1.12.1 The T cell response

The expulsion mechanism of adult *T. spiralis* worms from the small intestine is a complex immune-mediated process that is orchestrated by activated T helper type 2 (T_H2) cells. As *T. spiralis* burrows through the epithelium of the small intestine, villous atrophy, crypt hyperplasia, increased epithelial permeability, goblet cell and Paneth cell hyperplasia, eosinophilia, and infiltration of mucosal mast cell occurs (Garside *et al.*, 1992, 2000; Kamal *et al.*, 2001). Generally, these responses are controlled by cytokines produced by CD4⁺ T cells. Experiments performed in nude, thymectomised and cyclosporine A-treated mice that all lack fully functional T cells, have proven the T cell-dependency of both parasite expulsion and the accompanying intestinal pathology

(Garside *et al.*, 1992; Manson-Smith *et al.*, 1979). Furthermore, cell transfer studies have demonstrated that CD4⁺ T cells mediate parasite expulsion (Riedlinger *et al.*, 1986).

Protective immune responses against gastrointestinal (GI) nematode parasites are associated with the production of high levels of the T_H2 cytokines IL-3, IL-4, IL-5, IL-9 and IL-13 produced by CD4⁺ T cells, and high levels of IgG1 and IgE antibodies secreted by B cells (Grencis *et al.*, 1991; Urban *et al.*, 1993; Else *et al.*, 1994). Following infection, CD4⁺ T cells express IL-3, IL-4, IL-5, IL-9, and IL-10 in the mesenteric lymph nodes (MLN) (Grencis *et al.*, 1991; Behnke *et al.*, 1993), and are subsequently recruited to the intestinal mucosa (McDermott *et al.*, 2001), where they mediate immune responses in the intestinal epithelium.

Generally, due to the counter-regulatory mechanisms of T_H1 and T_H2 cytokines (Mosmann and Coffman, 1989), resistance to GI nematodes is impaired by T helper type 1 (T_H1) responses, which are characterised by elevated levels of IL-12, IFN- γ , IL-2 and the antibody isotype IgG2a. Worm expulsion is enhanced and larval establishment in the muscle is reduced in IFN- γ knock-out mice (Urban Jr. *et al.*, 2000). This effect appears to be caused by increased mastocytosis due to elevated levels of T_H2 cytokines, particularly IL-10, rather than an intrinsic effect of IFN- γ (Helmby and Grencis, 2003a). This was concluded on the basis that the *in vivo* neutralisation of IFN- γ in IL-10 knock-out mice does not alter the rate of expulsion of *T. spiralis* from the intestine (Helmby and Grencis, 2003a). Other cytokines such as IL-12 and IL-18 can also promote the development of a T_H1 response. IL-18 negatively regulates mastocytosis in an IFN- γ -independent manner *via* inhibition of T_H2 cytokine production particularly IL-10 and IL-13 (Helmby and Grencis, 2002). In a similar manner, the administration of IL-12 to *T. spiralis* infected animals also suppresses intestinal mast cell responses and transiently decreases early T_H2 cytokine production (Helmby and Grencis, 2003b). As mucosal mastocytosis is crucial for efficient parasite expulsion, it appears that these cytokines prolong parasite survival in the GI tract.

Different mechanisms of immunity operate against adult worms and NBL, and this immunity is in part regulated by IL-10 (Helmby and Grencis, 2003). IFN- γ is crucially involved in protection against NBL, but does not affect the expulsion of adults. The mechanism by which IFN- γ mediates protection against NBL may include enhanced cytotoxic killing by granulocytes, eosinophils and activated macrophages (Grove *et al.*, 1977; Gransmuller *et al.*, 1987). Recent work by Beiting *et al.*, (2007) has

suggested that CD4(+)CD25(-) effector T cells can suppress inflammation by an IL-10-dependent mechanism that limits IFN-gamma production and inducible NO synthase induction. They also reported that in the absence of IL-10, TGF- β participates in control of local inflammation in infected muscle and promotes parasite survival.

1.12.2 Innate responses of the intestinal epithelium

Infection with *T. spiralis* induces mucosal inflammation and changes in the general physiology of the small intestine. Alterations include goblet cell hyperplasia and increased mucous production, Paneth cell hyperplasia, hypercontractile smooth muscle and increased fluid secretion. Several lines of evidence now suggest that these physiological changes are directly controlled by activation of the immune system, rather than as a non-specific consequence of parasite antigen recognition. For example, efficient *T. spiralis* expulsion requires IL-4 receptor- α expression on non-bone marrow-derived cells, indicating the importance of non-immune gastrointestinal cells during infection (Urban *et al.*, 2001). Consequently, cells of the GI epithelium work as an extension of the immune system, directed by T_H2 cytokines produced by T cells.

The small intestinal epithelium is a rapidly renewing layer of cells composed of absorptive enterocytes on the villi, and their progenitors in the crypts, goblet cells and enteroendocrine cells, and also Paneth cells which occupy the crypts. The intestinal epithelium serves as a physical barrier between the external conditions of the gut lumen and the internal environment, and transports nutrients, ions and fluids.

Mouse models of infection using different GI nematodes, including *T. spiralis*, have shown a number of common effects on epithelial cell function. These alterations include increased permeability and decreased absorption of glucose, which leads to the accumulation of luminal fluid during infection (Madden *et al.*, 2004). Mucosal mast cells can also increase epithelial cell secretion through the release of soluble mediators such as PGE₂ and histamine (Castro *et al.*, 1987; Perdue *et al.*, 1991). Interestingly, in the absence of nerves (achieved by using neurotoxins), nematodes also have a direct antisecretory effect on the epithelial cells response to both PGE₂ and histamine (Madden *et al.*, 2004). These antisecretory effects are thought to reflect equilibrium between effects detrimental to the worm and effects detrimental to the host, thus allowing parasite expulsion with minimal disruption to the homeostasis of the GI tract.

During nematode infections, the level of fluid in the lumen increases and smooth muscle becomes hypercontractile (Vermillion and Collins, 1988). This is referred to as

the “weep and sweep” response which is thought to facilitate worm expulsion. Increased muscle contraction is dependent on CD4⁺ T cells and major histocompatibility complex II (MHC II) expression (Vallance *et al.*, 1999). Muscle hypercontraction markedly promotes parasite expulsion from the intestine, and cytokines, specifically IL-9, IL-4 and IL-13 secreted by T_H2 type cells, are thought to promote this effect (Khan and Collins, 2004; Khan *et al.*, 2003).

Goblet cell hyperplasia is also a feature of *T. spiralis* infection (Garside *et al.*, 1992). T_H2 cytokines are postulated to regulate the development of goblet cell hyperplasia in the gut during nematode infection *via* signal transducer and activator of transcription factor 6 (Stat6) activation, implicating IL-4 receptor α chain (IL-4R α) ligation by IL-4 or IL-13 (Khan *et al.*, 2001). Goblet cell mucins such as the secreted mucin Muc2, and the membrane-bound mucin Muc3, are up-regulated during *T. spiralis* infection (Shekels *et al.*, 2001). Transcripts corresponding to other goblet cell genes such as Siat4c involved in mucin sialylation, calcium chloride channel 5 (CLCA5) and goblet cell gene 4 (GOB4) involved in mucus secretion, are also upregulated during *T. spiralis* infection (Knight *et al.*, 2004). Expression of these genes promotes the altered and increased mucus secretion seen during infections (Miller, 1987), which acts to trap the worms and prevent attachment to the epithelial surface.

In addition, transcripts of a novel lectin termed intelectin-2 is rapidly upregulated in goblet cells and Paneth cells during *T. spiralis* infection (Pemberton *et al.*, 2004). Furthermore, upregulated expression of intelectin-2 is absent in strains of mice susceptible to *T. spiralis* and *T. muris* infections (Pemberton *et al.*, 2004; Datta *et al.*, 2005). The exact function of this novel intelectin variant is currently unknown, however the experiments reported by Pemberton *et al.*, (2004) and Datta *et al.*, (2005) imply that this molecule is closely associated with effective expulsion of GI parasites. Expression of the resistin-like molecule RELM β is also upregulated by goblet cells during *T. spiralis* infection (Artis *et al.*, 2004; Knight *et al.*, 2004). This expression is induced by IL-4 and IL-13 cytokines signalling *via* the IL-4R α and STAT-6-dependent pathway (Artis *et al.*, 2004). The role of this protein in *T. spiralis* infection is still unclear, however it has been suggested to mediate direct anti-parasite effects, tissue repair or immune regulation (Artis *et al.*, 2004).

1.12.3 The mucosal mast cell response

Mucosal mast cells play an essential role in host protection against some, but not all GI nematode parasites. Expulsion of *T. spiralis*, *Strongyloides ratti*, and *Strongyloides venezuelensis* is dependent on the recruitment and expansion of mast cells, whereas expulsion of *N. brasiliensis* is not (Abe *et al.*, 1992; Khan *et al.*, 1993; Ha *et al.*, 1983; Grecis *et al.*, 1993). *T. spiralis* infection is an ideal model to study the recruitment and expansion of mast cells, and the mast cell effector mechanisms deployed during intestinal inflammation. As such, this field of research has received much interest over the past 15 years, from mast cell biologists and parasitologists alike.

T. spiralis infection leads to hyperplasia, differentiation and activation of mucosal mast cells (MMC) in the gut and spleen (Gurish *et al.*, 2004; Woodbury *et al.*, 1984; Miller, 1996). Mast cells are derived from haematopoietic progenitor cells in the bone marrow. During the early stages of the infection, the bone marrow environment generates mast cell progenitors destined to home to the small intestine (Pennock and Grecis, 2004). These progenitor cells require the expression of the $\alpha_4\beta_7$ integrin to home to the small intestine (Artis *et al.*, 2000; Gurish *et al.*, 2001; Issekutz *et al.*, 2001; McDermott *et al.*, 2001). Furthermore, β_7 integrin-deficient mice do not possess a normal resting mast cell progenitor population in the small intestine, and during *T. spiralis* infection exhibit delayed mastocytosis and aberrantly distributed mast cells in the intra-epithelial compartment of the small intestine (Artis *et al.*, 2000).

During *T. spiralis* infection the main site of mast cell proliferation is the epithelium of the small intestine, rather than the bone marrow or blood (Dillon *et al.*, 1986). The mast cell hyperplasia seen in the gut is induced and regulated by factors acting in the local micro-environment of the intestinal epithelia. Mast cell precursors are induced to migrate into the epithelium under the direction of highly regulated integrin expression. In the small intestine, the integrin $\alpha_v\beta_6$, expressed by epithelial cells, activates epithelially secreted latent TGF- β_1 (Munger *et al.*, 1999). *In vitro* experiments have subsequently shown that TGF- β_1 induces mucosal mast cells to express the α_E chain of the integrin $\alpha_E\beta_7$ (Wright *et al.*, 2002; Smith *et al.*, 1994). The ligand for $\alpha_E\beta_7$ is cadherin (Cepek *et al.*, 1994) and this is exclusively expressed by epithelial cells (Kilshaw, 1999). Work by Brown *et al.*, (2004) showed that mice lacking the TGF- β_1 activating integrin $\alpha_v\beta_6$ exhibited reduced mucosal mast cell recruitment and expression of the integrin α_E . Hence, TGF- β_1 -mediated expression of the $\alpha_E\beta_7$ integrin enables the

mast cell progenitors to enter the epithelial compartment and allows the retention of mucosal mast cells as this cell population matures and expands.

Mucosal mastocytosis in the intraepithelial compartment is dependent on epithelially produced growth factors, as well as T_H2 type cytokines and therefore on the adaptive $CD4^+$ adaptive T cell response. Stem cell factor (SCF) is an essential growth factor for mast cells, and is particularly important for intestinal mucosal mastocytosis. The use of SCF receptor (c-kit)-deficient mice (W/W^v), and neutralising monoclonal antibodies to SCF or c-kit demonstrated that mastocytosis during *T. spiralis* infection was highly dependent on this growth factor (Grencis *et al.*, 1993; Alizadeh and Murrell, 1984). The growth factor TGF- β_1 also regulates important effector functions of mucosal mast cells. *In vitro* experiments using murine bone marrow-derived mast cells have reported that TGF- β_1 induces the upregulation of expression and constitutive secretion of the β -chymase, mouse mast cell protease-1 (mMCP-1), and the chemokine CCL2 (Miller *et al.*, 1999; Brown *et al.*, 2003). The release of mMCP-1 from mucosal mast cells plays a crucial role in *T. spiralis* expulsion from the gut of parasitized mice, as mice lacking the mMCP-1 gene (mMCP-1 ^{-/-}) have longer and more severe infections (Knight *et al.*, 2000). In addition, secretion of mMCP-1 also appears to be responsible for some of the enteropathy observed in the intestine during infection (Lawrence *et al.*, 2004). Mast cells releasing mast cell-specific proteases are directly responsible for degrading the tight junction protein ‘occludin’, thereby increasing epithelial paracellular permeability (McDermott *et al.*, 2003). Increased permeability of the epithelium has been postulated to be an important factor in parasite expulsion by allowing influx of solutes and water to ‘flush’ the parasites out (Murray *et al.*, 1971). It has been reported that other mast cell secretagogues such as histamine and prostaglandins mediate similar effects on fluid secretion in the presence of IL-4 (Madden *et al.*, 2002).

The specific association of T_H2 cytokines with mucosal mastocytosis has been widely shown. In IL-4-deficient mice, expulsion of *T. spiralis* from the small intestine is delayed (Lawrence *et al.*, 1998). Both IL-4 and IL-13 bind to the IL-4 receptor α chain; the ligation of this receptor results in the phosphorylation and activation of Stat6, which in turn leads to the activation of GATA3. *In vivo*, both IL-4R α and Stat6 are required to expel adult *T. spiralis*, and both IL-13 and IL-4 appear to contribute equally to protection (Urban *et al.*, 2000). The T_H2 cytokine IL-9 also promotes mucosal mastocytosis, which leads to the rapid expulsion of *T. spiralis* from the intestine (Faulkner *et al.*, 1997). In a similar manner, the injection of IL-3 prior to *T. spiralis*

infection in mice can promote accelerated parasite expulsion (Korenaga *et al.*, 1996). However, IL-3-deficient mice expel *T. spiralis* at a similar rate to wild-type controls, although mast cell number and consequently MCP-1 levels are reduced in the intestine (Pennock and Grencis, 2006). This has led to the suggestion that a threshold number of mast cells are required to achieve parasite expulsion (Pennock and Grencis, 2006).

Experimental *T. spiralis* infection using mast cell deficient mice (W/W^v) results in delayed worm expulsion, a reduced T_H2 response and a significantly increased T_H1 response (Lawrence *et al.*, 2004). However, the T_H2 response remains unaltered in mMCP-1 deficient mice (although expulsion is delayed), indicating a potential role for mast cells in promoting or amplifying the T_H2 responses required for protection against intestinal helminths (Lawrence *et al.*, 2004). In a similar manner, IL-3-deficient mice exhibit reduced mastocytosis, but also significantly reduced IL-5 and IL-10 production, again supporting a paracrine role for mast cells in the perpetuation of a T_H2 response (Pennock and Grencis, 2006).

1.12.4 The eosinophil response

IL-5-dependent eosinophilia is a marker of parasitic infection (Coffman *et al.*, 1989). However, although eosinophils are recruited to the gastrointestinal mucosa during *T. spiralis* infection, some reports have suggested that they are not necessary for parasite expulsion (Lammas *et al.*, 1992; Herndon and Kayes, 1992). This contrasts to the results reported by Grove and co-workers (1977), where treatment with a polyclonal anti-eosinophil anti-sera resulted in elevated levels of muscle stage larvae. Furthermore, other research has shown that eosinophils can kill *T. spiralis* larvae both *in vitro* (Wassom and Gleich, 1979; Kazura and Aikawa, 1980) and *in vivo* (Gurish *et al.*, 2002; Grove *et al.*, 1977). More recent research has shown that eosinophil recruitment is required to prevent the encystment of larvae in the muscles, rather than promote the expulsion of adult *T. spiralis* parasites from the intestine (Gurish *et al.*, 2002). This further supports the concept that eosinophils play a role in defending the host against the migratory phase of *T. spiralis* larvae. Interestingly, although IL-5 induced eosinophilia is not required for expulsion of *T. spiralis* adults from the intestine during a primary infection, eosinophilia does contribute to rapid parasite expulsion during a secondary infection, as delayed expulsion rates and an increase in encysted muscle larvae are observed in IL-5-deficient mice (Vallance *et al.*, 2000).

1.12.5 The humoral response

During helminth infections, elevated levels of IL-4, IL-5 and IL-13 induce the production of IgE and IgG1 antibody isotypes, characteristic of a T_H2 response. Consistent with this, the dominant antigen-specific immunoglobulin isotypes produced during *T. spiralis* infection are IgG1 and IgE, with other isotypes produced at lower levels (Crandall and Crandall, 1972; Goyal and Wakelin, 1993; Stewart *et al.*, 1999; Furze and Selkirk, 2005). Due to the time it takes to generate a humoral response against *T. spiralis* antigens, antibodies are generally regarded to be of more importance in protection against re-infection, rather than expulsion of a primary infection. In rats, during a secondary *T. spiralis* infection, ingested parasites are rapidly expelled from the intestine, and immunity is maintained for long periods (several weeks to several months) following a primary infection (Love *et al.*, 1976; Alizadeh and Wakelin, 1982). This response is mediated by antibodies, as the infusion of purified *T. spiralis* immune IgE into rats has been shown to confer rapid parasite expulsion (Ahmad *et al.*, 1991). However, rapid expulsion of adult *T. spiralis* parasites from the intestine of pre-infected animals does not occur in mice, where a less potent and short-lived, larva-specific immunity is demonstrated (Bell, 1992).

A range of studies have also investigated the role of antibodies, particularly IgE, during primary *T. spiralis* infections. A report by Perrudet-Badoux and co-workers (1978) showed that outbred mice selected for low antibody production had impaired immunity during infection. Other studies in rats also reported that the suppression of active IgE responses using anti-IgE antibodies markedly impaired parasite clearance, where the target of the IgE antibody for resistance seemed to be the muscle stage larvae (Bell *et al.*, 1992; Dessein *et al.*, 1981). In contrast, a separate investigation using relatively IgE-deficient SJA/9 inbred mice showed no defect, suggesting protection against adults and larvae was not IgE-dependent (Watanabe *et al.*, 1988). A more recent study by Gurish *et al.*, (2004) found IgE knock-out mice generated a normal mucosal mast cell response, but had lower serum mMCP-1 levels and expelled adult parasites more slowly, implying that IgE might promote mast cell activation. The exact role of parasite specific IgE responses in mast cell activation during *T. spiralis* infection is still not clear. However, evidence that supports a regulatory role for IgE is emerging, as decreased numbers of splenic mast cells were observed in IgE-null mice following *T. spiralis* infection suggesting that IgE may enhance mast cell survival *in vivo* (Gurish *et al.*, 2004).

1.13 Aims

One of the major aims of this study was to determine the modulatory effects *T. spiralis*-secreted proteins have on purinergic receptor-induced mucosal mast cell activation. This cell type is deemed important to study as intestinal mucosal mast cells play a major role in promoting the efficient expulsion of *T. spiralis* from the intestine. To achieve this aim, mast cells of a mucosal phenotype, derived from murine bone marrow, were used as an *in vitro* model of intestinal mucosal mast cells. The purinergic receptor sub-types expressed by this cell type were characterised functionally using a range of receptor agonists and antagonists. The effects of nucleotides on a variety of mast cell effector functions were also determined. The modulatory action of *T. spiralis*-secreted and recombinant proteins on mast cell purinergic receptor activation and purinergic receptor-mediated effector functions were also assessed *in vitro*.

The second major aim of this study was to determine whether immunisation of mice with the recombinant form of the *T. spiralis* 5'-nucleotidase (5'-NT) can provide protection against subsequent *T. spiralis* infection. These experiments were designed to examine whether the secreted 5'-NT is required for the promotion of parasite survival in the host. The effects of the immunisation on the progress of infection were also elucidated by monitoring the kinetics of *T. spiralis* expulsion from the small intestine, enumerating the establishment of muscle stage larvae, and recording the profile of the immune response generated.

The final aim of this thesis was to qualitatively characterise the expression of purinergic receptors in the intestinal epithelium during *T. spiralis* infection. As host genetic background can also influence the rate of expulsion of adult worms from the small intestine, these experiments were performed in strains of mice that expel parasites from the intestine at different rates. The objective of this was to ascertain whether alterations in host purinergic receptor expression correlate with the kinetics of parasite expulsion.

Chapter 2

Materials and Methods

2.1 Chemical reagents

All chemicals were purchased from Sigma-Aldrich, Fancy Road, Dorset, unless stated otherwise.

2.2 Animal procedures

All mice and rats were allowed to acclimatise for 7 days prior to use and were kept according to Home Office and institutional guidelines. All procedures were carried out at the Central Biomedical Services animal facilities at Imperial College London, South Kensington. All procedures were performed under project license number PPL 70/6073.

2.2.1 *Trichinella spiralis* infection

Trichinella spiralis were maintained in adult male Sprague-Dawley rats. To obtain muscle stage larvae, rats and mice were infected orally with 6000 and 500 infective larvae respectively. Infections were allowed to establish for at least 30 days before recovery of muscle stage larvae. To isolate adult and new born larvae from rats, animals were infected orally with 10,000 worms. Adults were collected from the intestine at either 3 or 6 days post infection.

2.2.2 Recovery of infective larvae

To isolate infective larvae, rats or mice were culled by cervical dislocation, skinned and eviscerated. Striated muscle tissue was removed from the carcass, chopped and blended in a solution of 1% (w/v) pepsin, 1% (w/v) HCl dispersed in tap-water, pre-warmed to 37°C. The homogenised tissue was digested by incubation, with agitation, for 1.5 hours at 37°C. The digested mix was filtered through two layers of cheese cloth attached to a Baerman funnel connected to a collection tube. Larvae were allowed to sediment for 1 hour, excess digestion fluid removed and larvae washed four times in sterile PBS pre-warmed to 37°C.

2.2.3 Recovery of adult parasites

The small intestine of infected animals was excised, opened longitudinally and laid onto a Baermann funnel covered with a single layer of muslin submerged in phosphate buffered saline (PBS) pre-warmed to 37°C. Worms were allowed to migrate

out over a 3 hour period. Adults were washed three times in sterile PBS and enumerated in a counting chamber (Weber Scientific International, Lancing).

2.2.4 Bone marrow extraction

Female BALB/c mice were sacrificed and their femurs removed under sterile conditions. Bone marrow was washed from the femurs using a 23-gauge needle and 20-mL syringe filled with Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were centrifuged at 230 x g for 7 minutes at room temperature. After resuspension in 1.5 ml DMEM containing 10% (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate (DMEM/FCS), cells were counted in an Improved Neubauer counting chamber (×40 magnification), using trypan blue (0.4% v/v) exclusion to measure viability (as described in section 2.6.1).

2.2.5 Immunisation of NIH mice with *T. spiralis* recombinant 5'-nucleotidase

To prepare the immunogen for test mice, the purified recombinant 5'-nucleotidase (5'-NT) was adjusted to a concentration of 500 µg/ml with sterile 25 mM HEPES; for control mice an equivalent volume of HEPES alone was used. Subsequently, an equal volume of 9% (w/v) potassium alum adjuvant (Sigma) dissolved in H₂O and one drop of phenol red pH indicator dye were added. Subsequently, 1 M NaOH was added drop-wise to the solution until the pH indicator dye turned pink (indicating a pH of 8.4) and the potassium alum precipitated out of solution. The 5'-NT/control-adjuvant solution was incubated at room temperature for 30 minutes. The samples were then centrifuged at 3000 x g for 10 minutes at room temperature, and the resulting supernatants removed and retained for use in a protein determination assay (as described in section 2.4.1) to elucidate the amount of protein that had bound to the adjuvant. The pellet of precipitated potassium alum-5'-NT/control were then washed twice in sterile PBS and the pellets resuspended in the appropriate volume of sterile PBS to result in a 500 µg/ml solution.

Prior to immunisation, a small volume of blood was collected from 5 female NIH mice *via* puncture of the tail vein. The blood was allowed to clot overnight at 4°C and subsequently centrifuged at 2500 x g for 10 minutes. The serum supernatant was removed and stored at -20°C. Groups of 15-test and 15-control female, NIH mice were injected subcutaneously with 25 µg 5'-NT-adjuvant (test), or an equivalent volume of

prepared adjuvant alone (control). The animals were subsequently boosted with 12.5 µg 5'-NT-adjuvant (test), or an equivalent volume of prepared adjuvant alone (control), 28 and 42 days after the initial immunisation.

Prior to challenge with *T. spiralis* infection, a small volume of blood was collected from every mouse in both the test and control groups *via* puncture of the tail vein, and the serum collected as described previously. Both test and control groups of mice were infected by oral gavage with 500 *T. spiralis* infective larvae suspended in 200 µl sterile PBS. At 4 days post-infection (p.i.), 5 mice from both test and control groups were sacrificed by intraperitoneal injection of 4 mg sodium pentobarbitone (supplied as Pentoject by Central Biomedical Services, Imperial College London). Mice were exsanguinated *via* the femoral artery and blood from individual mice was collected and the serum recovered as described previously. Mesenteric lymph nodes and spleen were removed aseptically and stored on ice in cold RPMI 1640 (Invitrogen Ltd., Paisley, UK). Intestines were removed and adult parasites extracted and counted as described in section 2.2.3. To determine the fecundity of adult female parasites, adults were subsequently cultured for 18 hours in NBL media (RPMI supplemented with 10 % (v/v) FCS, 1% (w/v) glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamycin, 20 U/ml mystatin and 2 mM L-glutamine), and the NBL collected and counted as described in section 2.3.2. At 7 days p.i., 5 mice from both test and control groups were sacrificed by cervical dislocation. The intestines were removed and adult and NBL parasites counted as for day 4 p.i. At 35 days p.i., 5 mice from both test and control groups were sacrificed by intraperitoneal injection of 4 mg sodium pentobarbitone. Blood from individual mice was collected and the serum recovered as described previously. Infective larvae were recovered from the muscle and enumerated as described in section 2.2.2.

2.2.6 Mucosal epithelial cell extraction from mouse intestine

6-8 week old Balb/c mice were sacrificed by cervical dislocation and the first 15 cm of small intestine from the pyloric sphincter removed. The intestine was flushed with PBS using a syringe and 21 gauge needle, then placed in fresh PBS. The lymphoid tissue was removed, and the jejunum bisected longitudinally and chopped into small, 0.5 cm segments. The tissue was then placed into a siliconised glass beaker and incubated in Hanks balanced salt solution (HBSS) (Invitrogen Ltd., Paisley, UK) containing 2 mM dithiothreitol (DTT), 5% foetal calf serum (FCS) (Invitrogen Ltd.,

Paisley, UK) for 15 minutes at 37°C with continuous stirring. The supernatant was subsequently removed and discarded. Tissue segments were then incubated in calcium and magnesium-free HBSS (HBSS-CMF) (Invitrogen Ltd., Paisley, UK) containing 2 mM DTT, 5% FCS, 1 mM EDTA at 37°C with continuous stirring. After 20 minutes the supernatant containing exfoliated epithelium was removed, filtered through a nylon 100 µm cell sieve and centrifuged at 300 x g for 5 minutes at room temperature. Fresh HBSS-CMF was added to the tissue and the procedure repeated three times. The cell pellets recovered containing epithelial cells were resuspended in PBS, transferred to 1.5 ml tubes and centrifuged at 500 x g for 5 minutes at room temperature. The supernatants were subsequently discarded and the cell pellets resuspended in 500 µl TRIzol (Invitrogen) and frozen at -80°C.

2.3 Preparation of parasite material

2.3.1 Parasite culture

Under sterile conditions isolated parasites were washed extensively in sterile parasite culture medium [RPMI (Gibco™, Paisley Scotland) supplemented with 1% (w/v) glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamycin, 20 U/ml mycostatin and 2 mM L-glutamine], pre-warmed to 37°C. Parasites were maintained in sterile parasite culture medium at 37°C, 5% CO₂. Infective larvae were cultured for up to 5 days and adults and NBL for up to 3 days. Medium containing parasite secreted proteins was removed daily and replaced with fresh sterile culture media pre-warmed to 37°C.

2.3.2 Recovery of newborn larvae

To collect newborn larvae (NBL), day 6 adults were washed extensively in sterile NBL media (RPMI supplemented with 10 % (v/v) FCS, 1% (w/v) glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamycin, 20 U/ml mycostatin and 2 mM L-glutamine) and cultured at 37°C, 5% CO₂. NBL were collected every 24 hours for a maximum of 3 days. Adult worms were allowed to sediment in the culture flask and the culture medium containing the NBL was removed. The NBL were either enumerated in a counting chamber (Weber Scientific International, Lancing), or sedimented by centrifugation at 1000 x g and washed three times in PBS, the supernatant removed and the pellet of NBL flash frozen in liquid nitrogen and stored at -80°C.

2.3.3 Collection of parasite secreted proteins

Medium containing parasite secreted proteins was pooled and filtered through a 0.2 µm filter (Schleicher and Schuell) and then concentrated in an Amicon concentrator (Amicon Inc., Beverly, MA) using a 10 kDa molecular weight cut off. The proteins were dialysed against 25 mM HEPES, pH 7.5 and further concentration was carried out in Centricon YM10 microconcentrators (Millipore, Bedford, MA). The concentrated protein solution was then aliquoted and stored at -20°C.

2.4 Protein techniques

2.4.1 Protein determination

The concentration of secreted proteins was determined using the Bichinchonic acid method (BCA assay) (Pierce, Rockford, IL) following the manufacturer's instructions. Bovine serum albumin ranging in concentration between 0.005-2.0 mg/ml was used as a standard. The absorbance at 560 nm was determined using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenbergl).

2.4.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

12% sodium-dodecyl sulphate (SDS) polyacrylamide resolving gels [12% (w/v) acrylamide mix (Protogel, National Diagnostics), 0.4 M Tris pH 8.8, 0.1% (w/v) ammonium persulphate, 0.1% (v/v) TEMED, 0.1% (w/v) SDS] and stacking gels [5% (w/v) acrylamide mix, 0.125 M Tris pH 6.8, 0.625% (w/v) ammonium persulphate, 0.15% (v/v) TEMED, 0.1% (w/v) SDS] were loaded with protein samples that had been boiled for 4 minutes in SDS-PAGE loading buffer [0.01% (v/v) β-mercaptoethanol, 0.01% (v/v) bromophenol blue, 0.1 M sucrose, 3% SDS (w/v), 62.5 mM Tris, 2 mM Na₂EDTA]. Samples were electrophoresed at a constant current of 20 mA in SDS-PAGE running buffer [25 mM Tris pH 8.3, 192 mM glycine and 1% (w/v) SDS] until the leading edge of the dye reached the bottom of the gel.

Proteins were visualised by staining gels for >1 hour at room temperature in a solution of 0.4% (w/v) Coomassie brilliant blue stain, 10% (v/v) acetic acid and 25% (v/v) propan-2-ol. Gels were then destained in a solution containing 10% acetic acid (v/v) and 25% (v/v) propan-2-ol.

2.4.3 Western blotting

Proteins were transferred to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia) using an electrophoretic transfer cell (Biorad) and Towbin buffer [25 mM Tris, 192 mM glycine and 20% (w/v) methanol], chilled to 4°C. Prior to transfer, the gel, membrane and blotting equipment were equilibrated for 10 minutes in chilled Towbin buffer. Electrophoresis was then carried out at 4°C for 2 hours at a constant current of 100 mA.

Following electrophoretic transfer of the protein, the membrane was incubated with agitation for 1 hour at room temperature in 50 ml blocking buffer composed of Tris buffered saline (TBS) containing: 100 mM Tris pH 7.5, 150 mM NaCl, 5% (w/v) skimmed milk powder and 0.3% (v/v) Tween 20. The blocking buffer was then removed and the membrane incubated overnight at 4°C with the appropriate primary antibody, diluted as required in blocking buffer (as above) containing 0.2% sodium azide. The membranes were then washed 3 times, for 15 minutes each, with TBS-0.3% (v/v) Tween 20 (TBS-Tween 20) at room temperature. The appropriate quantity and type of secondary horseradish peroxidase conjugated (HRP) antibody was diluted in TBS-Tween 20 and incubated with the membrane for 1 hour at room temperature. The membrane was washed 3 times with TBS-Tween 20 as before and finally with TBS for 15 minutes. Bound antibodies were then visualised using enhanced chemiluminescence (ECL), whereby membranes were submerged for 1 minute in a solution containing: 1.25 mM luminal, 0.1 M Tris pH 8.5, 0.009% (v/v) hydrogen peroxide and 6.8 μ M p-coumaric acid. Membranes were rinsed in distilled H₂O before exposure to X-ray film.

2.4.4 Expression of *T. spiralis* recombinant 5'-nucleotidase in *Pichia pastoris*

P. pastoris transformed with pPICZ α containing a 6xHis and c-Myc tagged *T. spiralis* recombinant 5'-NT (Gounaris *et al.*, 2004) 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°C for 3 days. Large scale expression was then carried out using a single *P. pastoris* colony to inoculate 25 ml of buffered glycerol complex medium (BMGY) (100 mM potassium phosphate pH 6.0, 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) yeast nitrogen base (with ammonium sulphate without amino acids, YNB), 4 x 10⁻⁵ % (w/v) biotin, 1% (v/v) glycerol) and incubated at 30°C with agitation until the culture was calculated to reach an OD₆₀₀ value of 2 to 6. The 25 ml of yeast culture was then transferred into 400 ml BMGY and incubated for a further

24 hours at 30°C with shaking. Cells were harvested by centrifugation at 3000 x g for 10 minutes at room temperature, the supernatant discarded and the cells resuspended in 800 ml buffered methanol complex medium (BMMY) [100mM potassium phosphate pH 6.0, 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% (w/v) YNB, 4×10^{-5} % (w/v) Biotin, 0.5% (v/v) methanol] to induce protein expression. Cultures were incubated at 30°C with shaking for 48 hours, with the addition of methanol at a final concentration of 0.5% (v/v) every 24 hours. Cells were then harvested by centrifugation at 3000 x g for 10 minutes at 4°C and the supernatant filtered through glass Whattman paper and 0.2 µm filters (Schleicher and Schuell). The supernatant was then concentrated to a final volume of approximately 10 ml in an Amicon concentrator (Amicon Inc., Beverly, MA) using a 30 KDa molecular weight cut off membrane. The concentrated supernatant was dialysed against 6 litres of dialysis buffer pH 8 containing 20 mM NaH₂PO₄, 300 mM NaCl and 1 mM Imidazole for 16 hours at 4°C.

2.4.5 Purification of the *T. spiralis* recombinant 5'-nucleotidase

The recombinant 5'-NT was purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. Ni-NTA-agarose beads (Quiagen) were equilibrated with dialysis buffer pH 8 (containing 20 mM NaH₂PO₄, 300 mM NaCl and 1 mM imidazole) and added to the concentrated 5'-NT yeast supernatant (0.5 ml beads per 400 ml pre-dialysis expression supernatant), then incubated overnight with rotation at 4°C. The slurry was added to a 0.8 x 4 cm, 10ml disposable chromatography column (Bio-Rad), the beads allowed to sediment, and the flow-through containing unbound proteins decanted. The column was then washed with 10 ml of dialysis buffer to remove unbound protein. Bound proteins were subsequently eluted with three 20 ml washes of dialysis buffer containing increasing concentrations of Imidazole (1 mM, 10 mM and 100 mM). The eluted fractions were then concentrated and washed with chilled dialysis buffer in a Centricon-YM10 micro-concentrator according to the manufacturer's instructions (Millipore, Bedford, MA), to a final volume of ~1 ml. Samples were then dialysed against 6 L of 25 mM HEPES pH 7.5 and further concentrated to a volume of ~200 µl. Samples were aliquoted immediately and stored at -20°C. Proteins were analysed by SDS-PAGE to confirm the purity of the protein, and Western blotting to confirm the presence of the protein using an antibody to the C-myc epitope.

2.5 Enzyme assays

2.5.1 5'-nucleotidase assay

5'-nucleotidase activity in *T. spiralis* secreted proteins was measured by calculating the amount of inorganic phosphate released from nucleotide diphosphate or monophosphate substrates. Samples of *T. spiralis* secreted proteins or recombinant 5'-nucleotidase were incubated for 10 minutes at 37°C in a reaction volume of 40 µl containing 25 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 2.5 mM dithiothreitol (DTT) and 2.5 mM of the appropriate nucleotide. Concentrations of NaH₂PO₄ ranging from 1-10 nmole were used as a source of inorganic phosphate to calculate a calibration curve. 200 µl of the chromogenic substrate malachite-green-phosphomolybdate (6 mM Malachite green, 2.1 N HCl, 17 mM ammonium molybdate) were then added to the reaction/phosphate standard and the absorbance measured at 600 nm using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenberg). In all experiments performed, background levels of phosphate detected in individual samples were subtracted from actual OD₆₀₀ values as appropriate.

2.5.2 β-hexosaminidase assay

Cell supernatants were collected from mast cell degranulation assays as described in Section 2.7.1. β-hexosaminidase activity was measured using a chromogenic p-nitrophenyl (pNP) linked glycoside as substrate (pNP-β-Glc-NAc) (Sigma). Reactions were carried out in 50 µl total volume, containing 15 µl of sample, citrate/phosphate buffer pH 4.4 and 2 mM pNP-β-Glc-NAc. Samples were incubated at 37°C for 30 minutes, and then the reaction was terminated by the addition of 50 µl 0.4 M Na₂CO₃. The amount of pNP released was determined spectrophotometrically by measuring the absorbance at 405 nm using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenberg). Results are expressed as the percentage β-hexosaminidase released into the supernatant compared to the amount present in the supernatant collected from resting cells.

2.6 Cell culture

2.6.1 Trypan Blue exclusion

Viable cells were counted by resuspending a sample of the cell preparation with an equal volume of 0.4% Trypan Blue solution. The number of viable cells that excluded dye uptake was determined by light microscopy using a haemocytometer.

2.6.2 Mouse bone marrow-derived mast cell culture

Bone marrow cell cultures were established in a humid 37°C, 5% CO₂ incubator using 162 cm² flat-bottomed flasks containing 5 × 10⁵ cells/ml in DMEM/FCS [DMEM (Invitrogen), 10% FCS (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, 1 mM sodium pyruvate] containing 15% (v/v) WEHI-3B-conditioned medium as a source of IL-3 (denoted as IL-3 throughout the text), 50 ng/ml recombinant rat stem cell factor (Amgen, Thousand Oaks, CA, and R&D Systems) and 5 ng/ml recombinant mouse IL-9 (R&D systems). Where stated, 1 ng/ml TGF-β₁ (R&D systems) was added to the culture media. Cultures were grown for up to 21 days and passaged at 7 day intervals by centrifuging the cells at 230 x g for 7 minutes and resuspending in fresh media and growth factors.

2.6.3 Culture of the WEHI-3B mouse myelomonocyte cell line

Cultures containing 1x10⁵ cells/ml were incubated in a humid 37°C, 5% CO₂ incubator. Cells were grown for 4-5 days in culture media (RPMI containing 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate) in a humid 37°C, 5% CO₂ incubator. Cells were centrifuged at 250 x g at 4°C for 5 minutes, the supernatant was then filtered through a 0.2 µm sterile filter and stored at -20°C. The cell pellet was then resuspended in RPMI, and 5x10⁵ cells used to seed 50 ml of fresh culture media.

2.6.4 Mast cell histology

5x10⁴ mBMMC were cytocentrifuged onto clean glass slides for 5 minutes at 40 x g. The cytosmears were then air-dried and fixed in Carnoys solution (60% v/v ethanol, 30% v/v chloroform, 10% v/v glacial acetic acid) for 1 hour. Slides were then stained with 0.5% (w/v) acidified toluidine blue for 15 minutes, rinsed in distilled water and counter stained for 30 seconds in eosin solution (VWR International Ltd., Poole, Dorset). Slides were then rinsed in distilled water prior to alcohol dehydration and mounting in DePeX (Fischer Scientific UK). Slides were photographed at x100 magnification using a Nikon Eclipse E400 microscope equipped with Lucia G software.

2.6.5 Culture of spleen and MLN cells for analysis of cytokine production

Spleen and MLN tissues were collected as described in section 2.2.5. Tissues were dissociated through a 100 µm sterile nylon cell strainer and centrifuged at 500 x g

for 5 minutes at 4°C. Cells were resuspended in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) FCS and counted as in section 2.6.1. Splenocytes or mesenteric lymph node cells were cultured at a density of 5×10^5 cells/well in round-bottom 96 well sterile plates at 37°C, 5% CO₂. Where indicated, cells were stimulated using either 8 µg/ml recombinant 5'-NT or 1 µg/ml staphylococcal enterotoxin B fragment (SEB). Cells from naïve mice cultured without stimulation were used as controls. Cells were incubated for 72 hours after which the supernatants were removed and stored at -20°C until use.

2.6.6 Cell proliferation assay

Cells were cultured as for cytokine production (section 2.6.6), using round bottom 96 well sterile plates. Cell proliferation was assessed by the addition of 1 µCi [³H]–thymidine per well for the final 16 hours of incubation. Cells were harvested using a Skatron semi-automatic cell harvester. The level of [³H]–thymidine incorporation was determined using a Packard tri-carb 1900TR liquid scintillation counter.

2.7 Mast cell assays

2.7.1 mBMMC degranulation and cytokine generation assays

Two week-old mBMMC were collected by centrifugation at 250 x g for 7 minutes at 4°C. The supernatant was discarded, and the cell pellet resuspended in fresh media and the cell concentration enumerated as described in section 2.6.1. mBMMC at a concentration of 2×10^6 /ml were then transferred into fresh media and growth factors (as detailed in section 2.6.2). 2×10^5 cells were then added to individual wells of a sterile 96-well microplate and the appropriate volume of test reagent/media added. In experiments involving *T. spiralis* ES or 5'-NT, the appropriate nucleotides were pre-incubated for 30 minutes with the amount of ES or 5'-NT as stated in a buffer containing a final concentration of 25 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl₂ and 2.5 mM dithiothreitol (DTT). As a positive control, mBMMC were passively sensitised with 10 µg/ml anti-DNP-albumin IgE (Sigma) for 2 hours, prior to washing and stimulation with 30 ng/ml DNP-albumin (Sigma) antigen. In some instances, 50 µg/ml of the mast cell secretagogue compound 48/80 (Sigma) was used as a positive control. For degranulation and cytokine generation experiments, mBMMC were incubated with test agonists for 2 hours and 24 hours respectively, at 37°C, in a

humidified incubator containing 5% CO₂. Samples were then centrifuged for 7 minutes at 250 x g, 4°C, and the supernatant carefully removed and stored at -20°C.

2.7.2 Freshly isolated bone marrow cell chemotaxis assay

Murine bone marrow was extracted from the femurs of female Balb/c mice as described in section 2.2.4. 20 µl of 1x10⁷ bone marrow cells/ml suspended in chemotaxis assay buffer were added to the upper wells of chemotaxis plates (ChemoTx, 5-µm pore size) and 30 µl of the indicated concentration of test chemoattractant added to the lower chamber. After 3 hours incubation at 37°C, migrated cells present in the lower wells were removed aseptically and cultured for 14 days with 1 ng/ml TGF-β₁ as described in section 2.6.3. Cells were lysed by freeze-thawing and the supernatants subsequently used in mMCP-1 specific ELISAs as detailed in section 2.9.1.

2.7.3 Immature mast cell chemotaxis assay

Murine bone marrow extracted from the femurs of female Balb/c mice (as described in section 2.2.4), was cultured in 15% WEHI-conditioned medium and DMEM/FCS (DMEM (Invitrogen), 10% FCS (Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, 1 mM sodium pyruvate). Growth media were replaced every 7 days and cells were cultured for a maximum of 14 days. Subsequently, 20 µl of 1x10⁷ immature mast cells/ml suspended in chemotaxis assay buffer were added to the upper wells of chemotaxis plates (ChemoTx, 5-µm pore size) and 30 µl of the indicated concentration of test chemoattractant added to the lower chamber. After 3 hours incubation at 37°C, migrated cells present in the lower wells were removed, and the wells washed with 25 µl of assay buffer. Cells were subsequently stained with rat-anti-mouse c-kit IgG2b-PE (Caltag Laboratories) diluted 1/100 (v/v), and counted using flow-cytometry as detailed in section 2.9.1. Assays were performed in duplicate, and results were expressed as the absolute number of cells migrating.

2.7.4 mBMMC chemotaxis assay

Chemotactic responses of mature mBMMCs were examined using 96-well chemotaxis plates (ChemoTx) with 5-µm pore size polycarbonate filters. 20 µl of BMMCs were added to the top wells at 2 x 10⁶ cells/ml in RPMI 1640 supplemented with 25 mM HEPES and 0.1% (w/v) BSA (chemotaxis assay buffer), and 30 µl of

agonist or buffer was added to the lower wells. After 3 hours incubation at 37°C, migrated cells present in the lower chamber were counted using a light microscope at x10 magnification. Assays were performed in triplicate, and results were expressed as the absolute number of cells migrating.

2.7.5 Calcium mobilisation assays

Changes in the cytosolic concentration of free Ca^{2+} were measured using the calcium indicator Fura-2-AM. The cells were washed and resuspended to a concentration of 0.5×10^7 cells/ml in Ca^{2+} flux buffer (Hank's balanced salt solution (HBSS) containing 1 mM CaCl_2 , 1 mM MgCl_2 , and 0.1% (w/v) BSA), and labelled with 5 μM Fura-2-AM for 30 min at 37°C in the dark. The labelled cells were washed by adding 10 ml of Ca^{2+} flux buffer and centrifuged at 500 x g for 7 minutes prior to resuspension in Ca^{2+} flux buffer to a concentration of 0.5×10^7 cells/ml. Subsequently 0.5×10^6 cells/well were added to a 96-well black fluorescence plate (Nunc), pre-warmed to 37°C. In some samples, the mBMMC were pre-treated with antagonist/vehicle as stated, for 10 minutes. In experiments involving *T. spiralis* ES or 5'-NT, the indicated nucleotide was pre-incubated for 30 minutes with the stated amount of ES or 5'-NT in a buffer containing a final concentration of 25 mM HEPES pH 7.5, 150 mM NaCl, 5 mM MgCl_2 and 2.5 mM dithiothreitol (DTT), prior to addition to cells. Changes in intracellular Ca^{2+} concentration induced by stimulation with different test agonists were recorded by monitoring the excitation at 340 nm and 380 nm and emission at 510 nm in a fluorescence microplate spectrophotometer (FLUOstar OPTIMA, BMG Labtech, Offenborg). The relative ratios of fluorescence emitted at 510 nm were recorded and displayed as a reflection of intracellular concentrations of Ca^{2+} .

2.7.6 Conditions for electrophysiology

Whole cell voltage-clamp recordings were made from mast cells that had been maintained in culture for 14 days. The composition of the control extracellular solution was (in mM) 145 NaCl, 2.5 KCl, 3 MgCl_2 , 1 CaCl_2 , 10 HEPES, (titrated to pH 7.4 with NaOH). Glass microelectrodes were pulled from thick-walled borosilicate glass capillaries. Fire-polished pipettes were back-filled with 0.2 μm -filtered intracellular solution (composition in mM: 150 KCl, 3 MgCl_2 , 5 EGTA, 10 HEPES; titrated to pH

7.4 with KOH). Cells were voltage-clamped using an Axopatch 1D amplifier (Axon instruments, USA) and low pass filtered at 5 kHz before sampling (2-10 kHz) and online capture. Data acquisition was carried out using pClamp software (clampex 7, Axon Instruments, USA). Mast cells were usually held at -60 mV then subjected to a step to -80 mV for 100 ms followed by a 500 ms step to -40 mV. A 100 ms step to -110 mV is followed by a 500 ms ramp change in voltage to $+20$ mV. Finally a 100 ms step to -80 mV precedes a step to the holding potential of -60 mV. This protocol was repeated once every 5 seconds and allowed simultaneous measurement of the steady state current at particular voltages (for example, the ATP-evoked current at -80 mV) and the currents evoked by ATP at different voltages following ramp changes in the voltage. All electrophysiological measurements were carried out at room temperature ($21 - 23^{\circ}\text{C}$). Data were analysed using Clampfit software (clampfit 8, Axon Instruments, USA), Excel (Microsoft Corporation, USA) and Origin (Microcal, USA).

2.8 Enzyme-linked immunosorbent assay (ELISA)

2.8.1 Mouse mast cell protease-1 (mMCP-1) ELISA

96-well plates (Nunc) were coated with $50\text{ }\mu\text{l}$ rat anti mMCP-1 monoclonal antibody ($2\text{ }\mu\text{g/ml}$) (Moredun), diluted in 0.1 M sodium carbonate buffer pH 9.6 and incubated overnight at 4°C . Plates were then washed 6 times with 154 mM NaCl, 0.05% (v/v) Tween 20 and incubated for one hour at room temperature with $50\text{ }\mu\text{l}$ of BMMC supernatant (collected as described in section 2.7.1), or $50\text{ }\mu\text{l}$ of mMCP-1 ranging from $0.5\text{-}20\text{ ng/ml}$ diluted in a solution of PBS, 4% BSA (w/v) and 0.05% (v/v) Tween 20. Plates were washed 6 times as before and $50\text{ }\mu\text{l}$ of rabbit anti-mMCP-1 HRP-conjugated antibody (Moredun) diluted 1:1500 in a solution of PBS containing 4% (w/v) BSA, 0.05% (v/v) Tween 20 was added. The plates were then incubated for 1 hour at room temperature. Plates were washed (as before) and $50\text{ }\mu\text{l}$ of tetramethylbenzidine (TMB) substrate (0.1 mg/ml TMB, 0.001% (v/v) hydrogen peroxide, 0.03 M acetic acid, 0.07 M sodium acetate) were added to each well and allowed to develop for 10-30 minutes. $50\text{ }\mu\text{l}$ of 0.1 N HCl acid was then added to each well to terminate the reaction. The absorbance at 450 nm was measured using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenberger) and the concentration of mMCP-1 in each test-sample determined using the mMCP-1 standard curve.

2.8.2 IL-4, IL-5, IL-13, TNF- α ELISAs

96-well plates (Nunc) were coated overnight at 4°C using 50 μ l of either anti-mouse IL-4 (R&D Systems) diluted to 2 μ g/ml in PBS, anti-mouse IL-5 (R&D Systems) diluted to 0.5 μ g/ml in PBS, anti-mouse IL-13 (R&D Systems) diluted to 4 μ g/ml in PBS, or anti-mouse TNF- α (R&D Systems) diluted to 0.8 μ g/ml in PBS. Subsequently, the coating antibody was aspirated and the plate washed 3 times with wash buffer (0.05% (v/v) Tween 20/PBS) prior to treating the plates with 200 μ l/well blocking buffer (1% (w/v) BSA/PBS) for 1 hour at room temperature. The plates were washed as before and 50 μ l of test supernatant or 50 μ l of the appropriate cytokine serially-diluted according to the manufacturers' instructions, added per well. All dilutions were made using dilution buffer (0.1% BSA (w/v)/PBS pH 7). Plates were sealed and incubated at room temperature for 2 hours. Subsequently, samples were aspirated and the plates flushed with wash buffer 6 times. 50 μ l/well of either 0.2 μ g/ml biotinylated anti-mouse IL-4 (R&D Systems), 0.1 μ g/ml biotinylated anti-mouse IL-5 (R&D Systems), 0.4 μ g/ml biotinylated anti-mouse IL-13 (R&D Systems) or 0.3 μ g/ml biotinylated anti-mouse TNF- α (R&D Systems) were added and the plates sealed and incubated at room temperature for 2 hours. Plates were then washed 3 times as before and 50 μ l of avidin-HRP conjugate (BD Biosciences) diluted 1:1000 in dilution buffer was added. Plates were sealed and incubated at room temperature for 30 minutes. Plates were then washed 6 times with wash buffer and 50 μ l of substrate solution (0.11 M acetate buffer pH 5.5, 100 μ g/ml (w/v) TMB diluted in DMSO, 0.1% (v/v) H₂O₂) added per well. Plates were incubated in the dark at room temperature for 30 minutes. The reaction was terminated by the addition of 40 μ l 1.8 M H₂SO₄ and the absorbance measured at 450 nm using a FLUOstar optima microplate reader (BMG Labtech, Offenberg).

2.8.3 cAMP ELISA

Assays for cAMP in cell lysates were carried out using a cAMP Biotrak Enzymeimmunoassay (EIA) system according to the manufacturer's instructions (Amersham Biosciences).

2.8.4 Antigen specific serum immunoglobulin ELISAs

Samples of *T. spiralis* recombinant 5'-NT were diluted to 5 μ g/ml in coating buffer (0.06 M sodium carbonate buffer pH 9.5) and 96-well flat bottom plates were coated

with 100 μ l/well. Plates were sealed and incubated overnight at 4°C. The 5'-NT coating-antigen was then aspirated and the plates washed 3 times with wash buffer (0.05% Tween 20 (v/v), PBS). Plates were subsequently treated with 300 μ l/well blocking buffer (5% (v/v) FCS, PBS pH 7.0) for 1 hour at room-temperature. Plates were washed as before and 100 μ l/well of sera diluted in blocking buffer added. Plates were sealed and incubated at room-temperature for 2 hours, prior to washing 3 times with wash buffer. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG1 (1:4000), IgG2a (1:2000), IgG2b (1:3000) and IgM (1:8000) antibodies (Zymed Laboratories, CA), and HRP-conjugated goat anti-mouse IgA (1:4000) antibody (Zymed Laboratories, CA) were diluted in blocking buffer and 100 μ l added per well. Plates were sealed and incubated for 2 hours at room-temperature. Plates were subsequently washed 6 times with wash buffer prior to addition of 100 μ l/well of substrate solution (0.11 M acetate buffer pH 5.5, 100 μ g/ml (w/v) TMB diluted in DMSO, 0.1% (v/v) H₂O₂). Plates were incubated in the dark at room-temperature for 30 minutes. The reaction was stopped by the addition of 50 μ l 1.8 M H₂SO₄ and the absorbance measured at 450 nm using a FLUOstar optima microplate reader (BMG Labtech, Offenberg).

2.9 Flow Cytometry

2.9.1 Flow cytometric analysis of mBMMC surface markers

BMMC were centrifuged at 250 x g for 7 minutes at 4°C and the supernatant discarded. Cells were resuspended in FACS buffer (PBS, 0.1% (w/v) BSA, 0.1% (w/v) azide) and counted (as described in section 2.6.1). Aliquots of 2×10^5 cells were then centrifuged at 250 x g for 7 minutes at 4°C, then resuspended in 100 μ l FACS buffer. Cells were stained by the addition of 1 μ g/ml of either rat anti-mouse c-kit phycoerythrin conjugated (R-PE) IgG2b (CALTAG laboratories), rat IgG2b-R-PE isotype control antibody (CALTAG laboratories), or the equivalent volume of FACS buffer added to them to act as a negative control. Samples were then incubated on ice in the dark for 30 minutes, washed twice with FACS buffer and then resuspended in 200 μ l PBS containing 3% (v/v) formaldehyde. To stain for the presence of the high affinity IgE receptor Fc ϵ RI, cells were prepared as before then incubated on ice with 10 μ g/ml anti-DNP-albumin IgE (clone SPE7), for 30 minutes. After washing with FACS buffer, cells were incubated on ice in the dark for a further 30 minutes with 1 μ g/ml fluorescein isothiocyanate (FITC)-conjugated rat anti mouse IgE (CALTAG laboratories). The cells

were washed twice with FACS buffer then resuspended in 200µl PBS containing 3% (v/v) formaldehyde. Cells were analysed using a FACSCalibur flow cytometer (Becton Dickinson) and Cell-Quest software with at least 30,000 events counted per sample. Forward and side light scatter were used to select cell populations for analysis using WinMDI software.

2.9.2 Cytometric bead analysis (CBA) assay

Assays for IL-2, IFN- γ , TNF- α , IL-4 and IL-5 were carried out using a mouse T_H1/T_H2 cytokine cytometric bead array kit according to the manufacturers instructions, using 25 µl of cell supernatant, capture beads, cytokine standards and detection reagent (BD Biosciences, Cowley, Oxford).

2.10 Molecular biology

2.10.1 RNA extraction from mBMMC

A Qiagen RNA extraction kit was used to purify RNA from 4x10⁶ mBMMC according to the manufacturer's instructions. The concentration and purity of the RNA was then quantified spectrophotometrically by measuring the absorbance at 260 nm and the absorbance ratio at 260 nm/280 nm respectively. To ensure there was no genomic DNA contaminating the sample the extracted RNA was digested with DNaseI using a micro-midi total RNA purification system (Invitrogen) according to manufacturer's instructions.

2.10.2 RNA extraction from epithelial cells and parasites

Parasites were frozen in liquid nitrogen and then disrupted using a percussive disruptor. The shattered pellet was transferred to a 1.5 ml tube and allowed to thaw. In a fume hood, 50µl of extracted epithelial cells or disrupted parasites were resuspended in 1 ml of TRIzol (Invitrogen). RNA was extracted according to the manufacture's instructions. Briefly, this involved incubating the suspension at room temperature for 5 minutes to allow dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added, the tube shaken vigorously for 15 seconds and then left to settle for 2-3 minutes at room temperature. Samples were then centrifuged at 12,000 x g for 15 minutes at 4°C. The clear upper aqueous phase containing the RNA was then carefully removed and transferred to a fresh eppendorf tube. The RNA was precipitated by adding 0.5 ml of isopropanol and the sample incubated for 10 minutes at room temperature prior to

centrifugation for 10 minutes at 12,000 x g, 4°C. The supernatant was removed and the gel-like pellet resuspended in 1 ml 75% (v/v) ethanol. The sample was then centrifuged at 7,500 x g for 5 minutes at 4°C, the ethanol removed and the RNA pellet left to air-dry for 5 minutes. The pellet was finally resuspended in 30 µl RNase free water and the RNA concentration and purity quantified by measuring the absorbance at 260 nm and the absorbance ratio at 260 nm/280 nm respectively, using a spectrophotometer. To ensure there was no genomic DNA contaminating the sample the extracted RNA was digested with DNaseI using a micro-midi total RNA purification system (Invitrogen) according to manufacturer's instructions.

2.10.3 cDNA synthesis

To a final volume of 13 µl dH₂O, 2 µg of RNA and 1 µg of oligo-dT (Promega) were added. The sample was then incubated at 70°C for 5 minutes (to denature the secondary structure of the RNA and allow oligo-dT primers to anneal). The sample was immediately placed on ice to prevent the secondary structure of the RNA reforming. 5 µl of 5X M-MLV (murine-Maloney leukaemia virus) buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂, 50 mM DTT), 40 U of RNase inhibitor, 200 U of M-MLV reverse transcriptase and 6.25 mM each of dATP, dTTP, dCTP and dGTP were then added to the reaction mix resulting in a final volume of 25 µl. The sample was incubated at 37°C for 1 hour and then either used for a PCR reaction directly afterwards or heated to 94°C for 5 minutes to denature the reverse transcriptase.

2.10.4 Polymerase chain reaction

PCRs to detect the expression of specific mRNA transcripts were carried out in a final volume of 25 µl containing 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.25 mM each of dATP, dGTP, dCTP, dTTP, 1 unit Taq DNA polymerase, 10 pmoles each of forward and reverse primer, and the indicated amount of template DNA. Amplification of template DNA was carried out using gene specific primers (detailed in Tables 2.1, 2.2 and 2.3) and the following thermal cycle:

- 1) 94°C for 10 minutes
- 2) 94°C for 30 seconds
- 3) Specified annealing temperature for 1 minute
- 4) 72°C for 2 minutes

Steps 2 to 4 were repeated for 34 cycles.

5) 72°C for 10 minutes

Reaction products greater than 250 bp were analysed on 1.5% (w/v) agarose gels, PCR products less than 250 bp were analysed on 2% (w/v) agarose gels.

2.10.5 Agarose gel electrophoresis

Agarose gels were made by mixing 1% (w/v) agarose with Tris-acetate electrophoresis buffer (0.04 M Tris-acetate, 1 mM EDTA) and heated until the agarose dissolved. Ethidium bromide (0.2 µg/ml) was then added and the gel allowed to set. DNA samples and 0.1 µg per mm of gel lane width of 1Kb DNA ladder (GeneRuler, Fermentas) were loaded onto gels and electrophoresed at 90V until the DNA fragments had separated to a sufficient resolution to be identified using UV illumination and photography.

2.10.6 DNA sequencing of PCR products

PCR products were separated from dNTPs and primers by agarose gel electrophoresis. The PCR product was subsequently excised from the gel using a clean scalpel blade. PCR products were then purified using a gel extraction kit (Qiagen) and eluted in 50 µl of dH₂O. The DNA concentration and purity was quantified by measuring the absorbance at 260 nm and the absorbance ratio at 260 nm/280 nm, using a spectrophotometer. PCR products were sequenced by the Advanced Biotechnology Centre (Imperial College, South Kensington) on a 3100 Genetic Analyzer (Applied Biosystems) using ABI BigDyes, where 120-300 ng of template DNA was amplified using 12.8 pmol of primer and the following thermal cycle:

1) 96°C for 10 seconds

2) 50°C for 5 seconds

3) 60°C for 4 minutes

Steps 1 to 3 were repeated for 35 cycles

Primer	Sequence	Annealing Temp (°C)	Fragment Size (bp)
CysLTR1 Forward	5'-GCACCAAGCAGACATTCCTGGAGA-3'	57	628 or 470
CysLTR1 Reverse	5'-AATCATGTATACTTGGAAGGCTGA-3'		
mMCP-1 Forward	5'-GGAAAACTGGAGAGAAAGAACCTAC-3'	57	460
mMCP-1 Reverse	5'-GACAGCTGGGGACAGAATGGGG-3'		
β -actin Forward	5'-CCCCAGCCATGTACGTAGCCATCC - 3'	55	425
β -actin Reverse	5'-AGAAGCCTCGGGGCATCGGAACGGC-3'		
<i>T. spiralis</i> Tubulin Forward	5'-GGTCACATATGCACCGGTGAT-3'	55	342
<i>T. spiralis</i> Tubulin Reverse	5'-GCTGTTGTATTAGATAACATGC-3'		
<i>T. spiralis</i> 5'-NT Forward	5'- GGTTGGGTGAAAACGGTG 3'	55	325
<i>T. spiralis</i> 5'-NT Reverse	5'- GGACAGTCTCCGCAACGTAG 3'		

Table 2.1 PCR primers

mMPC-1 primers were designed by Ghildyal *et al.*, 1992. CysLTR1 primers were designed by Maekawa *et al.*, 2001. 5'-NT and tubulin primers were designed by M. E. Selkirk (Imperial College, London). β -actin primers were designed by K. Kitchenen (Imperial College, London). Tubulin primers were designed by D. Guiliano (Imperial College, London).

Primer	Sequence	Annealing Temp (°C)	Fragment Size (bp)
A ₁ Forward	5'-TTTGGCTGGAACAACCTGAGTGAG - 3'	54	264
A ₁ Reverse	5'-ATCTTGAGCTCCTTCCCGTAGTAC - 3'		
A _{2A} Forward	5'- TTCAGCCTCTTGGCTATTGCCATC - 3'	54	216
A _{2A} Reverse	5'- ATCCTTAGGTAGATGGCCAGCATG - 3'		
A _{2B} Forward	5'- CCACCAACTACTTTCTGGTATCCC - 3'	54	323
A _{2B} Reverse	5'- TCTTTACTGTTCCACCCCAGGAAC - 3'		
A ₃ Forward	5'- AGACCTGCATCCTCCAGGTTAATG - 3'	54	260
A ₃ Reverse	5'- GCTTCCATGCTGTCTCAGAAAAGC - 3'		
P2Y ₁ Forward	5'- CTGTGICTTATATCCCTTTCC - 3'	57	410
P2Y ₁ Reverse	5'-CTCCATTCTGCTTGAAGTC - 3'		
P2Y ₂ Forward	5'- ACCAGCGTGCGGGGAACC - 3'	57	440
P2Y ₂ Reverse	5'- GCATCTCGGGCAAAGCGGACAAGT -3'		
P2Y ₄ Forward	5'- TGCCTCGTGCCCAACCTCTTCTTT -3'	57	499
P2Y ₄ Reverse	5'- CAGTTGTTTCGGCGCTTAGGTGTGC - 3'		
P2Y ₆ Forward	5'- CCTGGCACTGGCGGACCTGAT - 3'	57	452
P2Y ₆ Reverse	5'- GGCGGGCCATGCGACAATAAC - 3'		
P2Y ₁₂ Forward	5'- ATCGGTCTTTTGTGAGAACAAGG - 3'	55	300
P2Y ₁₂ Reverse	5'- TTCTCCCAGATGTTGATGTAGAG - 3'		
P2Y ₁₃ Forward	5'- TGGCATCAGGTGGTCAGTCACAC - 3'	55	400
P2Y ₁₃ Reverse	5'- CGCTTGATCCTGCTGTTCTTGCC - 3'		
P2Y ₁₄ Forward	5'- CGTCTTCTACACGGCCATCAC G - 3'	55	264
P2Y ₁₄ Reverse	5'- TCCAGACACACATTGGCAGCC G - 3'		

Table 2.2. P1 and P2Y receptor PCR primers

Primers were designed by S. Kock (Imperial College, London).

Primer	Sequence	Annealing Temp (°C)	Fragment Size (bp)
P2X ₁ Forward	5'- GAGAGTCGGGCCAGGACTTC - 3'	54	233
P2X ₁ Reverse	5'- GCGAATCCCAAACAACTTGA - 3'		
P2X ₂ Forward	5'- TCCCTCCCCCACCTAGTCAC - 3'	54	149
P2X ₂ Reverse	5'- CACCACCTGCTCAGTCAGAGC - 3'		
P2X ₃ Forward	5'- CTGCCTAACCTCACCGACAAG - 3'	54	150
P2X ₃ Reverse	5'- AATACCCAGAACGCCACCC - 3'		
P2X ₄ Forward	5'- CCCTTTGCCTGCCCAGATAT - 3'	54	145
P2X ₄ Reverse	5'- CCGTACGCCTTGGTGAGTGT - 3'		
P2X ₅ Forward	5'- GGATGCCAATGTTGAGGTTGA - 3'	54	81
P2X ₅ Reverse	5'- TCCTGACGAACCCTCTCCAGT- 3'		
P2X ₆ Forward	5'- CCCAGAGCATCCTTCTGTTCC - 3'	54	152
P2X ₆ Reverse	5'- GGCACCAGCTCCAGATCTCA - 3'		
P2X ₇ Forward	5'- GCACGAATTATGGCACCGTC - 3'	54	171
P2X ₇ Reverse	5'- CCCCACCTCTGTGACATTCT - 3'		

Table 2.3 P2X receptor PCR primers

P2X receptor primers were designed by S. Kock (Imperial College, London).

2.11 Statistics

Statistics were performed using the unpaired Student's *t*-test assuming unequal variances. Levels of significance are indicated as follows: * $P \leq 0.05$, ** $P \leq 0.01$ *** $P \leq 0.001$. In all graphs, error bars represent the standard deviation from the mean.

Chapter 3

Characterisation of the modulatory effects of *Trichinella spiralis* secreted proteins on mast cell purinergic receptor activation

3.1 Introduction

Extracellular nucleotides released from cells either due to damage or in a regulated manner are purinergic signalling molecules that induce a wide variety of cellular responses by binding to and activating cell surface purinergic receptors. There are two distinct families of purinergic receptors: the P1 and P2 classes. The P1 family are G-protein-coupled receptors of which there are 4 sub-types that differentially respond to adenosine and inosine (Fredholm *et al.*, 2001). The P2 family is further subdivided into G-protein-coupled P2Y receptors and ligand-gated ion channel P2X receptors. Currently, eight human (seven murine) P2Y receptors have been cloned and are differentially responsive to ADP, ATP, UDP, UTP and UDP-glucose, with ligand affinities depending on the receptor sub-type (von Kugelgen and Wetter, 2000). In addition, cysteinyl leukotriene receptors (CysLT1 and 2) (Mellor *et al.*, 2001; 2003) and the very recently described rat GPR17 receptor (Ciana *et al.*, 2006) have been shown to mediate the actions of extracellular UDP. There are seven sub-types of P2X receptors and they are all activated by ATP (North, 2002). Purinergic receptors couple to a wide variety of intracellular signalling pathways and thus can directly activate or modulate many different cellular responses.

Each purinergic receptor sub-type has a specific pharmacological profile in terms of agonist type and affinity, and most cells express multiple receptor sub-types (von Kugelgen, 2006). The combined use of sub-type preferential agonists and antagonists can allow the characterisation of most purinergic receptors. However, as purinergic receptor antagonists and agonists bind to multiple receptor sub-types with varying affinities, characterisation of specific receptor sub-types can be complex.

Recently *Trichinella spiralis* has been shown to secrete a range of nucleotide metabolising enzymes, specifically a 5'-nucleotidase (5'-NT) (Gounaris, 2002; Gounaris *et al.*, 2004), a nucleoside diphosphate kinase (NDPK) (Gounaris *et al.*, 2001) and an adenosine deaminase (Gounaris, 2002). The 5'-NT is unusual in that it can catalyse the hydrolysis of nucleoside 5'-diphosphates and 5'-monophosphates at the same active site (Gounaris *et al.*, 2004). The 5'-NT is mono-specific for AMP among the nucleoside 5'-monophosphates and catalyses the hydrolysis of nucleoside 5'-diphosphates in the order of UDP>>ADP. Thus, the action of the *T. spiralis* 5'-NT is hypothesised to limit the availability of extracellular nucleoside 5'-diphosphates to interact with P2Y receptors. The reaction product of the parasite 5'-nucleotidase acting on AMP would be adenosine,

which could act as a substrate for the secreted adenosine deaminase leading to the accumulation of inosine. The latter would activate A₃ receptors and result in limited activation of A₁, A_{2A} and A_{2B} adenosine receptors. The 5'-NT shows no activity against any nucleoside 5'-triphosphates (Gounaris *et al.*, 2004). However, the secreted NDPK can convert ATP to ADP or UTP to UDP in the presence of nucleoside phosphate acceptors, and could work in conjunction with other enzymes to reduce the concentration of extracellular nucleoside triphosphates. Secretion of these enzymes is expected to alter the amount of extracellular nucleotides available to bind host cell purinergic receptors. This is postulated to result in altered cell activation leading to an environment more favourable for parasite survival.

It has been known for many years that the resolution of *T. spiralis* infection is absolutely dependent on mast cells (Alizadeh and Murrell, 1984; Oku *et al.*, 1984). However, the exact role of mast cells in parasite clearance from the intestine has yet to be fully defined. During *T. spiralis* infection mast cell progenitors home to the small intestine where TGF- β_1 produced by epithelial cells induces them to secrete the β -chymase mouse mast cell protease-1 (mMCP-1) (Miller *et al.*, 1999; Wright *et al.*, 2002). Mice deficient in mMCP-1 are unable to expel *T. spiralis* although they develop mastocytosis (Knight *et al.*, 2000). One role of mMCP-1 is to degrade the tight-junction protein occludin, which increases the enteric 'leakiness' of the gut, disrupting epithelial barrier function leading to the influx of solutes and water into the lumen (McDermott *et al.*, 2003). This is thought to help flush the parasites out of the intestine (McDermott *et al.*, 2003; Murray *et al.*, 1971). Furthermore, TGF- β_1 also plays a role in the recruitment and distribution of mucosal mast cells in the intestinal epithelial compartment (Wright *et al.*, 2002; Smith *et al.*, 1994; Brown *et al.*, 2004). Thus, TGF- β_1 is a key regulator of mast cell effector function primarily by inducing the secretion of mMCP-1 and regulating mast cell distribution within the epithelium.

To establish a successful infection, *T. spiralis* infective larvae must burrow into the host intestinal epithelium, moult into adults and sexually reproduce (as discussed in Chapter 1). As the parasites migrate through the epithelium, considerable cell damage occurs. Large numbers of mast cells accumulate in the intestinal epithelium prior to the expulsion of *T. spiralis* from the intestine (Woodbury *et al.*, 1984). Thus, they are expected to come into close contact with areas of epithelium damaged by the invading parasites. The nucleotides released from damaged and dying cells would potentially be able to bind to and activate purinergic receptors on surrounding mucosal mast cells.

This may act to stimulate increased mast cell effector functions. Hypothetically, the secretion of nucleotide metabolising enzymes by *T. spiralis* may serve to inhibit the action of nucleotides in activating host immune responses. Assuming that the parasite secretes these enzymes to its advantage, it is possible that they act to specifically prolong parasite persistence in the intestinal tract. As mucosal mast cells are the key effector cells required to expel *T. spiralis* from the small intestine it was deemed pertinent to test the hypothesis that *T. spiralis* secreted proteins modulate mast cell purinergic receptor activation.

Mast cells of various types express P1, P2Y and P2X receptor sub-types that can be activated in both paracrine and autocrine ways (Feng *et al.*, 2004; Bulanova *et al.*, 2005; Osipchuk and Cahalan, 1992). Due to the difficulty in isolating significant numbers of mucosal mast cells free of contaminating cell types from the intestine (Fox *et al.*, 1985; Sellge and Bischoff, 2006), in this study *in vitro* bone marrow derived mast cells (mBMMC) displaying a mucosal phenotype were used. Studies by Miller *et al.*, (1999) and Wright *et al.*, (2002) have described cell culture conditions utilising IL-3, IL-9, SCF and TGF- β_1 that leads to the development of mature mast cells from murine bone marrow within 14 days. Importantly, these mast cells have a mucosal phenotype as assessed by mMCP-1 secretion and toluidine blue staining, thus providing a useful model to study the effects of *T. spiralis* secreted proteins on purinergic signalling.

This chapter focuses on the initial characterisation of mouse bone marrow-derived mast cell (mBMMC) cultures and the identification of functional purinergic receptors. A variety of selective purinergic receptor antagonists and agonists were used to define which mBMMC purinergic receptor sub-types were functionally expressed. Aspects of intracellular signal transduction pathways activated by nucleosides and nucleotides were also investigated to aid purinergic receptor characterisation. The regulatory effects of *T. spiralis* secreted nucleotide metabolising enzymes on the type and availability of extracellular nucleotides able to activate mBMMC purinergic receptors was investigated.

3.2 Results

3.2.1 Characterisation of mBMMC cultures

Previous work by Miller *et al.*, (1999) and Wright *et al.*, (2002) has described culture conditions conducive to developing mast cells from bone marrow in a relatively short, 14-day time frame. This *in vitro* cell type is similar to the mucosal mast cells found in the intestinal epithelium of *T. spiralis* infected mice as they can be induced to secrete the β -chymase mouse mast cell protease-1 (mMCP-1), a key effector molecule required for efficient parasite expulsion.

Culture conditions defined by Miller *et al.*, (1999) were used to generate mature mouse bone marrow-derived mast cells (mBMMC). Murine bone marrow cell suspensions were cultured with 15% WEHI-conditioned medium as a source of IL-3, 5 ng/ml IL-9 and 50 ng/ml stem cell factor (SCF). Throughout the text the 15% WEHI-conditioned medium is referred to as IL-3. Cells were cultured in the absence or presence of 1 ng/ml TGF- β_1 to ensure the known inhibitory effects of TGF- β_1 on Fc ϵ RI expression (Gomez *et al.*, 2005) did not adversely effect mast cell development. FACS analysis was utilised to monitor the development of the mBMMC cultures over a three-week period. Toluidine blue staining of day-14 mBMMC cultures was also used to confirm the sulphated acid mucopolysaccharide content of mast cell granules, indicative of mature mast cells.

Samples of cells were removed from the culture medium at, 0 (undifferentiated bone marrow), 4, 7, 14 and 21 days post-initiation of bone marrow cell culture. Cells were stained with antibodies corresponding to the mast cell markers c-kit and Fc ϵ RI, and analysed by FACS (isotype control staining is shown in Appendix 1-1). Mouse bone marrow cells were stained individually with anti-c-kit-PE and anti-IgE-FITC antibodies and dual-stained with both types of antibodies. Representative results showing log₁₀ fluorescence intensity dot plots of cells cultured in the absence or presence of TGF- β_1 are shown in Figures 3-1 and 3-2 respectively. Both bone marrow cell cultures, regardless of the presence of TGF- β_1 , showed increased staining for cell surface c-kit and Fc ϵ RI receptors as indicated by positive shifts in fluorescence intensity during 21 days of culture (Figures 3-1 and 3-2). As seen in the fluorescence intensity dot plots (Figures 3-1 and 3-2), from day-14 onwards, the culture conditions employed resulted in a relatively homogenous population of c-kit⁺ and Fc ϵ RI⁺ cells, unaffected by the addition of TGF- β_1 .

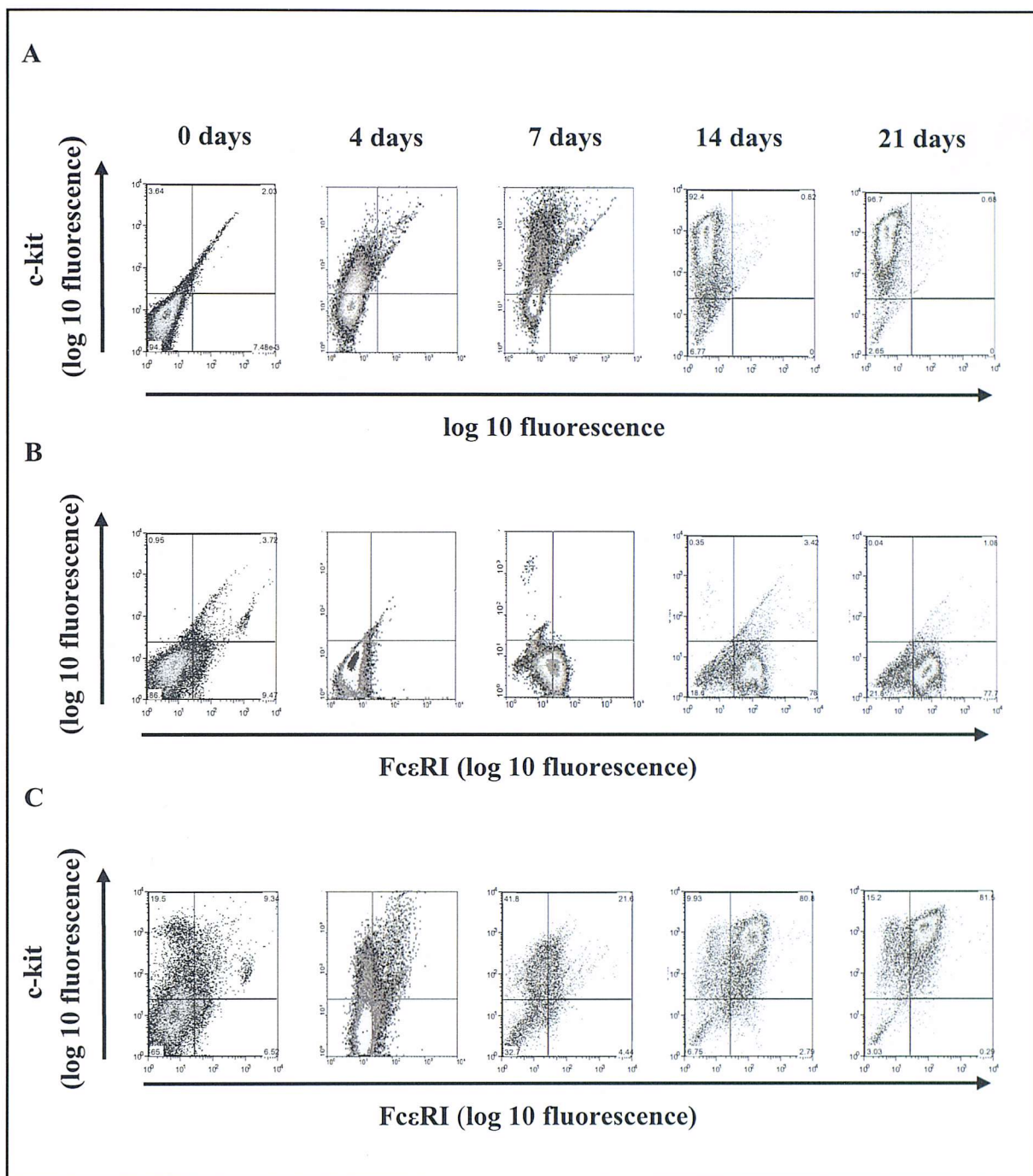


Figure 3-1 c-kit and FcεRI staining in mBMMC cultures grown in the absence of TGF-β₁

Representative density dot plots of total nucleated cells showing staining of mouse bone marrow cells with (A) anti-c-kit-PE antibody (c-kit), (B) IgE followed by anti-IgE-FITC antibody (FcεRI) and (C) both PE and FITC-labelled antibodies. Murine bone marrow was cultured with IL-3, IL-9 and SCF in the absence of TGF-β₁ and sampled at various time points up to 21 days. Samples were analysed by flow cytometry and data shown are representative of at least two separate experiments.

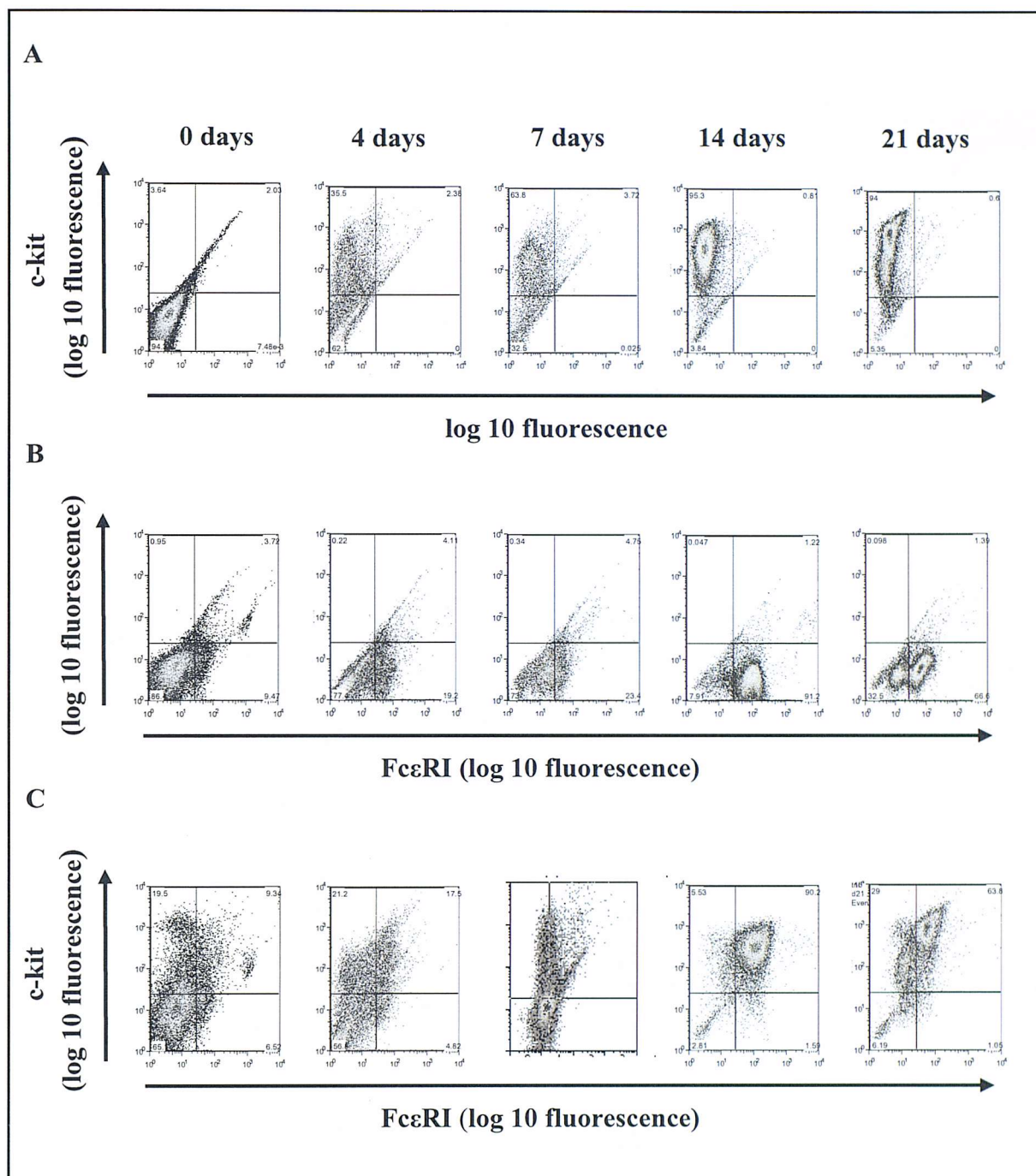


Figure 3-2 c-kit and FcεRI staining in mBMMC cultures grown in the presence of TGF-β₁

Representative density dot plots of total nucleated cells showing staining of mouse bone marrow cells with (A) anti-c-kit-PE antibody (c-kit), (B) IgE followed by anti-IgE-FITC antibody (FcεRI) and (C) both PE and FITC-labelled antibodies. Murine bone marrow was cultured with IL-3, IL-9 and SCF in the presence of TGF-β₁ and sampled at various time points up to 21 days. Samples were analysed by flow cytometry and data shown are representative of at least two separate experiments.

The percentage of cells stained positive for c-kit and FcεRI were calculated using the quadrant statistics obtained from the fluorescence dot plots (data from two separate FACS experiments) (Figure 3-3). Single c-kit and FcεRI staining are shown in Figure 3-3 (A) and (B) respectively and dual c-kit and FcεRI staining is shown in Figure 3-3 (C). The percentage of c-kit positive cells in the bone marrow cell population increased rapidly during the first 7 days of culture, and reached $99 \pm 0.5\%$ and $93 \pm 8\%$ by day-14, in the absence or presence of TGF- β_1 respectively [Figure 3-3 (A)]. During the first 7 days of culture, the development of FcεRI positively stained-cells increased at a slower rate than that observed for c-kit staining [compare Figure 3-3 (A) and (B)]. However, after an initial 7 day lag period, the percentage of cells expressing FcεRI rose very rapidly and by day-14 had reached $91 \pm 11\%$ and $94 \pm 5\%$ in the absence or presence of TGF- β_1 respectively [Figure 3-3 (B)]. Dual staining presented a similar 7 day lag phase [Figure 3-3 (C)]. However, from day-7 onwards c-kit positive cells upregulated FcεRI expression, peaking at day 14 where the percentage of cells positive for both mast cell markers was $90 \pm 6.7\%$ and $94.7 \pm 5\%$ in the absence or presence of TGF- β_1 respectively [Figure 3-3 (C)]. A trend of reduced c-kit and FcεRI cell surface expression was noted at day-21, however this was shown to be statistically non-significant (Figure 3-3).

FACS analysis measuring side scatter was also used to assess the size and granularity of the bone marrow cell population. Mature mast cells are very granular and as such have high side scatter properties when analysed by FACS. Uncultured bone marrow cells (day-0) had very low side scatter suggesting the absence of granules (Figure 3-4). From day-7 of culture onwards, the cell population showed an increase in side scatter consistent with the granularity associated with mature mast cells. No obvious differences in side scatter were associated with the presence or absence of TGF- β_1 [compare Figure 4-4 (A) and (B)]. This implies TGF- β_1 does not alter the overall granularity of the bone marrow cell population.

To further confirm the granularity of mature day 14 mBMMC, histological analysis was used. Cells were immobilised on glass slides, fixed with Carnoy's solution, stained with toluidine blue and photographed at X100 magnification (Figure 3-5). Cells cultured in the absence or presence of TGF- β_1 stained positive with toluidine blue and only a few contaminating cell types were observed [Figure 3-5 (A)]. Cells cultured with TGF- β_1 appeared to have more densely stained granules than cells cultured without TGF- β_1 . The optimal time point to use the mBMMC was chosen to be 14 days as

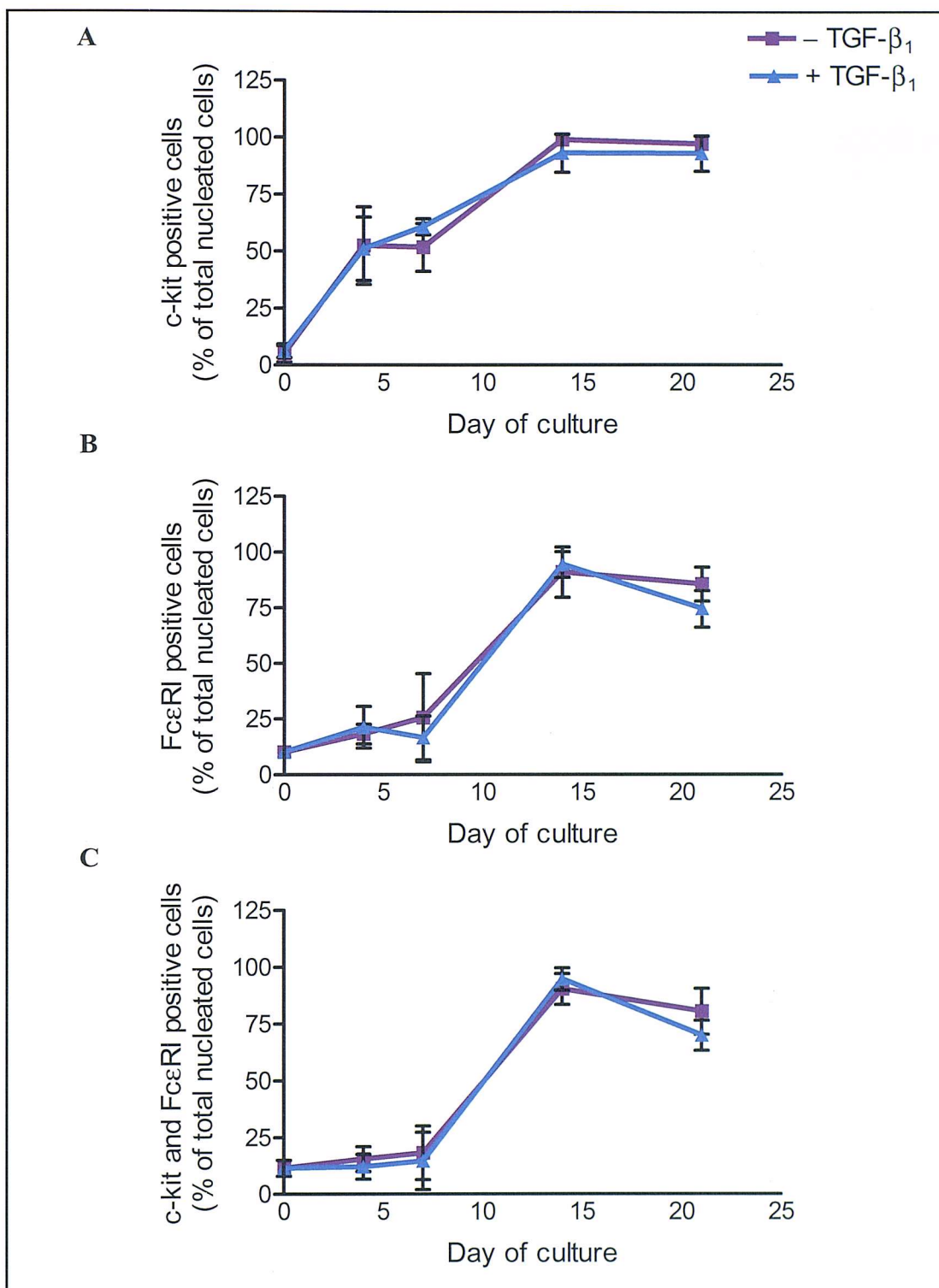


Figure 3-3 The percentage of c-kit⁺ and FcεRI⁺ cells in mBMMC cultures grown in the presence and absence of TGF-β₁

Murine bone marrow cells cultured with IL-3, IL-9 and SCF in the presence or absence of TGF-β₁ were sampled at various time points up to 21 days. Flow cytometry was used to calculate the percentage of total nucleated cells positive for c-kit and FcεRI. Data shown are $\pm$ s.d.m. of at least two separate experiments.

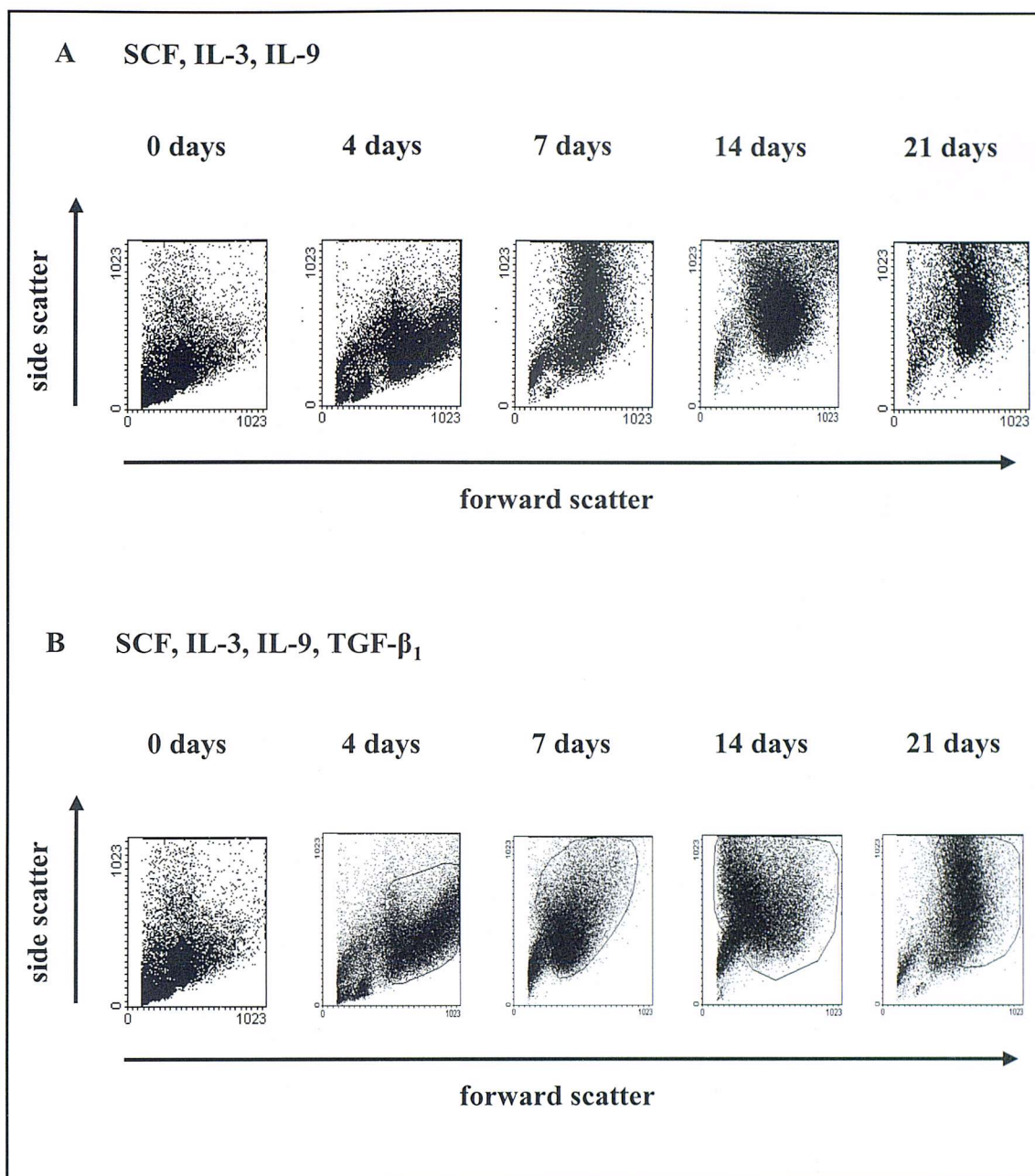


Figure 3-4 The granularity of mBMMC cultures grown in the presence or absence of TGF- β_1

Representative dot plots showing forward and side scatter properties of murine bone marrow during 3 weeks of culture in media containing (A) SCF, IL-3, IL-9 and (B) SCF, IL-3, IL-9 and TGF- β_1 . Samples were analysed by flow cytometry and data shown are representative of at least two separate experiments.

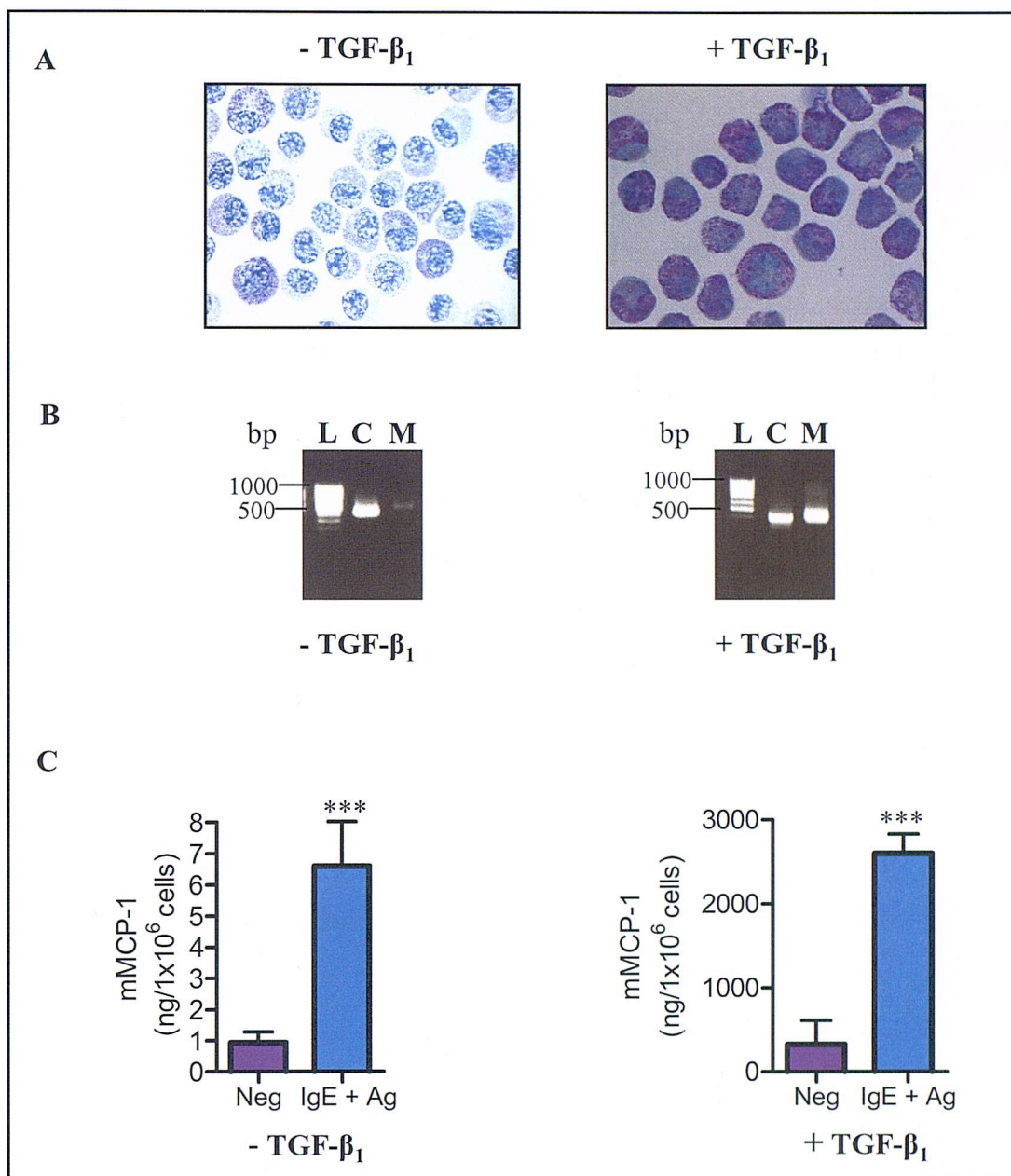


Figure 3-5 The expression of mMCP-1 and the morphology of mBMMC grown in the absence or presence of TGF- β_1

A) Toluidine blue stained cytopins of mBMMC cultured in the absence or presence of TGF- β_1 . Representative photographs at x100 magnification are shown. B) Representative reverse transcriptase (RT)-PCR analysis of mMCP-1 expression (lane M) in mBMMC cultured in the absence or presence of TGF- β_1 . PCR amplification of β -actin was used as a control (lane C). Samples were analysed on a 1.5 % (w/v) agarose gel and PCR fragment size was evaluated using a 1000 base pair (bp) DNA ladder (lane L). C) mBMMC cultured in the absence or presence of TGF- β_1 were stimulated for 2 hrs by crosslinking the Fc ϵ RI receptor using IgE and antigen (Ig+Ag), or using media alone (Neg). Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone, *** *p* < 0.001.

maximal cell surface expression of c-kit and FcεRI was observed and the cells appeared granular by FACS and toluidine blue staining.

The expression and secretion of mMCP-1 was also assessed to confirm the mBMMC were of a mucosal phenotype. Non-quantitative reverse transcriptase-PCR (RT-PCR) using mMCP-1-specific primers highlighted expression of mRNA transcripts corresponding to mMCP-1 in mBMMC regardless of the culture conditions used (Figure 3-5 (B)). As shown in Figure 3-5 (C), mBMMC cultured with TGF-β₁ spontaneously secreted approximately 1000-fold more mMCP-1 than mBMMC cultured without TGF-β₁. These results confirm that TGF-β₁ induces the mBMMC to spontaneously secrete more mMCP-1 which is consistent with the cells being of a mucosal phenotype. Cross-linking of FcεRI using IgE and antigen induced the mBMMC to degranulate releasing significant amounts of mMCP-1 [Figure 3-5(C)]. This further confirms the cells are mature and express active cell surface FcεRI.

3.2.2 Characterisation of mBMMC purinergic receptor expression

Upon the establishment of an *in vitro* culture system for the generation of mBMMC, a non-quantitative reverse transcriptase-PCR (RT-PCR) approach was utilised to determine the expression of mRNA transcripts in mBMMC that correspond to P1, P2Y and P2X receptors. The presence of mRNA transcripts does not directly correspond to levels of cell surface protein expression and conversely absence of mRNA transcripts does not necessarily correspond to absence of the expressed protein. However, this method was used to give a general insight into the purinergic receptors expressed by mBMMC.

To ensure all the purinergic receptor primer pairs amplified PCR products of the expected size, control template cDNA isolated from murine spleen (or other organs where indicated) was used and the results displayed in Appendix 1-2. Unless stated otherwise, all primer pairs amplified PCR products of the expected size from template mBMMC cDNA (expected PCR product sizes are displayed in the Materials and Methods section and are also depicted in Appendix 1.2). In order to standardise cDNA levels between batches of RNA, β-actin primers were used as a positive control. To exclude the possibility of genomic DNA contaminating mBMMC cDNA preparations, all samples of RNA were digested with DNase prior to the RT reaction. To control for genomic DNA contamination in RNA preparations, PCRs were carried out using

DNase-treated RNA preparations and β -actin primers. No PCR products were generated from all batches of mBMMC RNA used (data shown in Appendix 1-3). Representative RT-PCR analysis of expression of all purinergic P1, P2Y and P2X receptor mRNA transcripts extracted from day 14 mBMMC cultured in the absence or presence of TGF- β_1 are shown in Figures 3-6, 3-7 and 3-8 respectively.

Of the P1 receptors, only the A_{2B} and A₃ transcripts were found to be expressed at high levels, and this pattern did not seem to be altered by the presence of TGF- β_1 (Figure 3-6). Low levels of transcripts corresponding to the A₁ and A_{2A} receptors were weakly detected by RT-PCR in mBMMC cultured in the absence of TGF- β_1 , and this was less apparent in mBMMC cultured in the presence of TGF- β_1 (Figure 3-6). Regardless of the culture conditions used, mBMMC strongly expressed transcripts corresponding to the P2Y₁ receptor and to a lesser extent, transcripts for the P2Y₂ and P2Y₁₄ receptors (Figure 3-7). As the cysteinyl leukotriene receptor 1 (CysLT1) has recently been found to bind, and be activated by UDP (Mellor *et al.*, 2001), primers were used to detect expression of this receptor transcript (Figure 3-7). Because murine CysLT1 receptor cDNA is known to have two isoforms that results from the alternate splicing of a gene with 4 exons (Maekawa *et al.*, 2001), primers were used that detected both isoforms resulting in PCR products of two different sizes (628 bp and 470 bp) depending on the isoforms present. PCR performed using mBMMC cDNA as a template produced PCR products of approximately 900 bp and 200 bp, which differed from the expected sizes (Figure 3-7). To determine whether the PCR fragments produced were an aberrant result due to non-specific primer binding, the PCR fragments were purified from the agarose gel and the 3'-end of the PCR product partially sequenced using the reverse primer. The sequencing results confirmed the small (~200 bp) PCR fragment corresponded to murine CysLT1 cDNA, the results of which are shown in Appendix 1-5. However, the large PCR fragment corresponded to prosaposin mRNA, indicating the primers had non-specifically amplified template cDNA (Appendix 1-6). These results indicate that mBMMC express transcripts corresponding to the CysLTR1, but do not highlight the type of splice variant expressed.

RT-PCR using primers designed for the detection of P2X receptors, amplified transcripts corresponding to the sub-types P2X₁ and P2X₄ (Figure 3-8). The PCR fragments produced were at very low levels and no differences between culture conditions were observed (Figure 3-8).

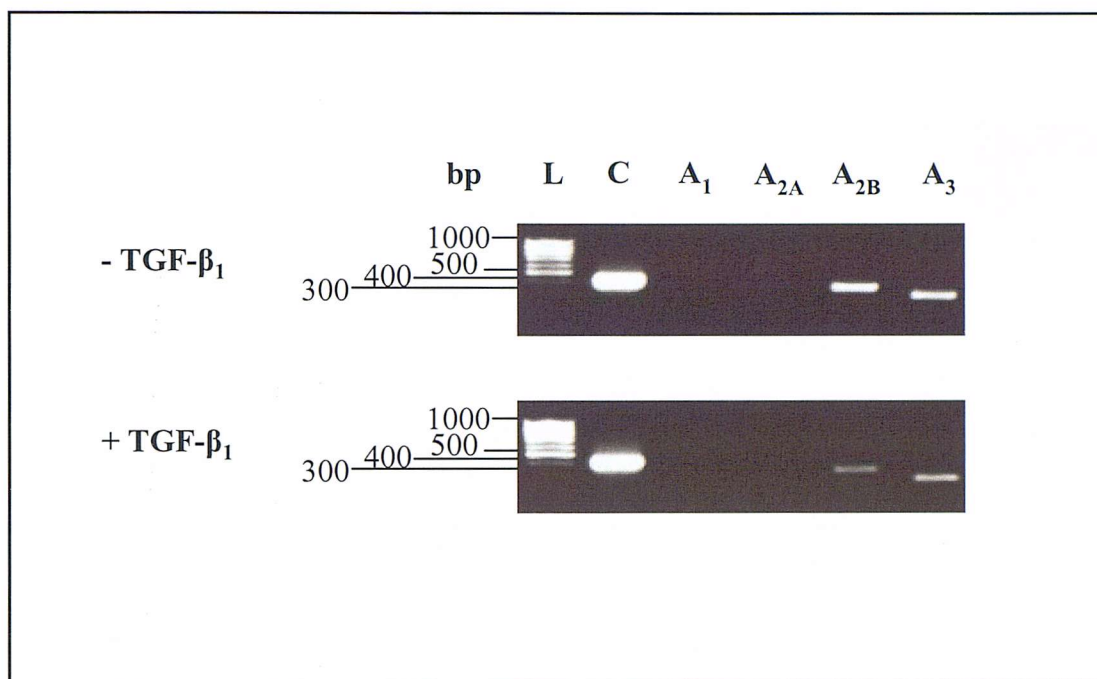


Figure 3-6 Expression of purinergic P1 receptor subtypes in mBMMC

Representative reverse transcriptase (RT)-PCR analysis of P1 receptor expression in mBMMC cultured in the absence or presence of TGF-β₁. PCR amplification of β-actin was used as a control (lane C). Samples were analysed on a 1.5 % (w/v) agarose gel and PCR fragment size was evaluated using a 1000 base pair (bp) DNA ladder (lane L).

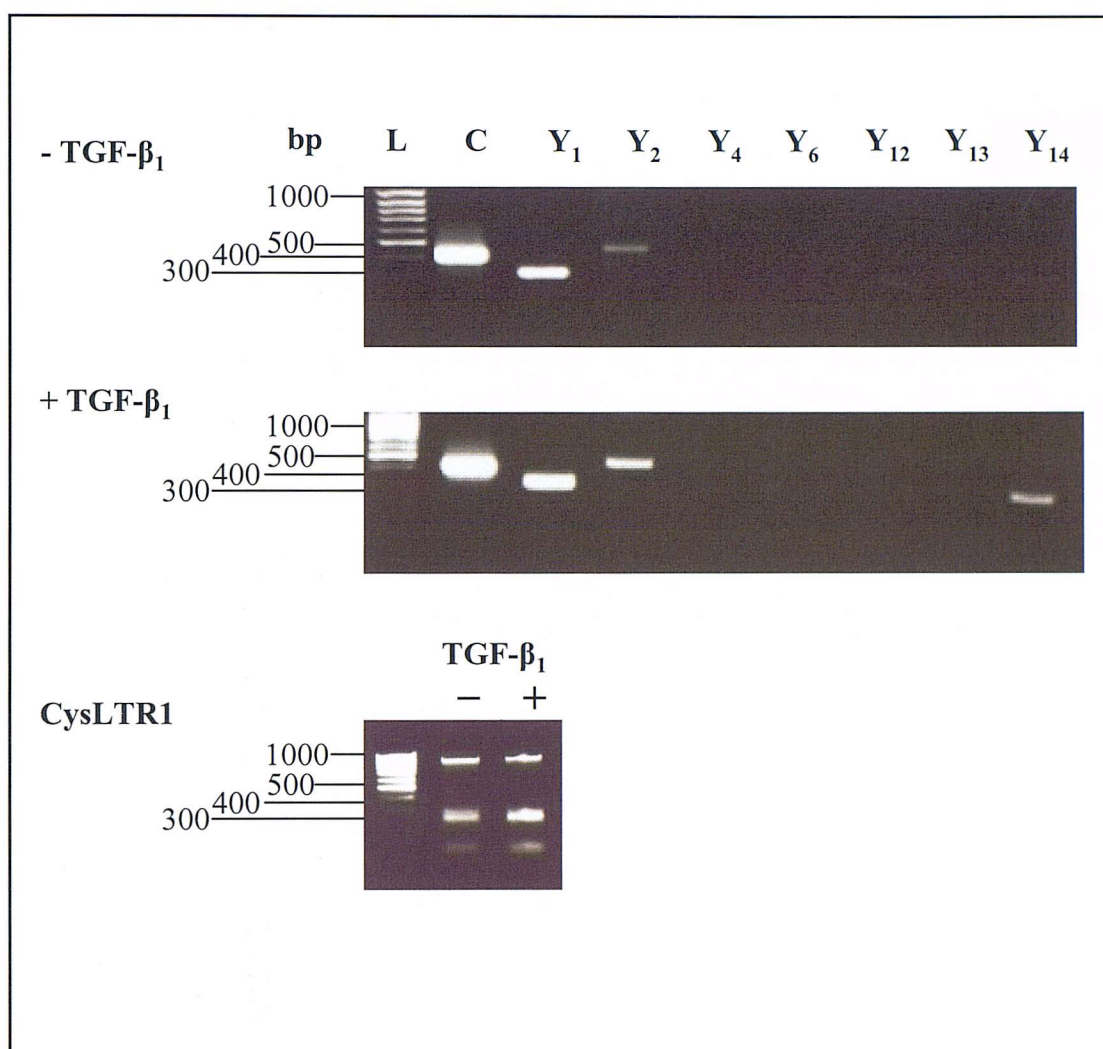


Figure 3-7 Expression of purinergic P2Y receptor subtypes and CysLTR1 in mBMMC

Representative reverse transcriptase (RT)-PCR analysis of P2Y receptor expression in mBMMC cultured in the absence or presence of TGF- β_1 . PCR amplification of β -actin was used as a control (lane C). Samples were analysed on a 1.5 % (w/v) agarose gel and PCR fragment size was evaluated using a 1000 base pair (bp) DNA ladder (lane L).

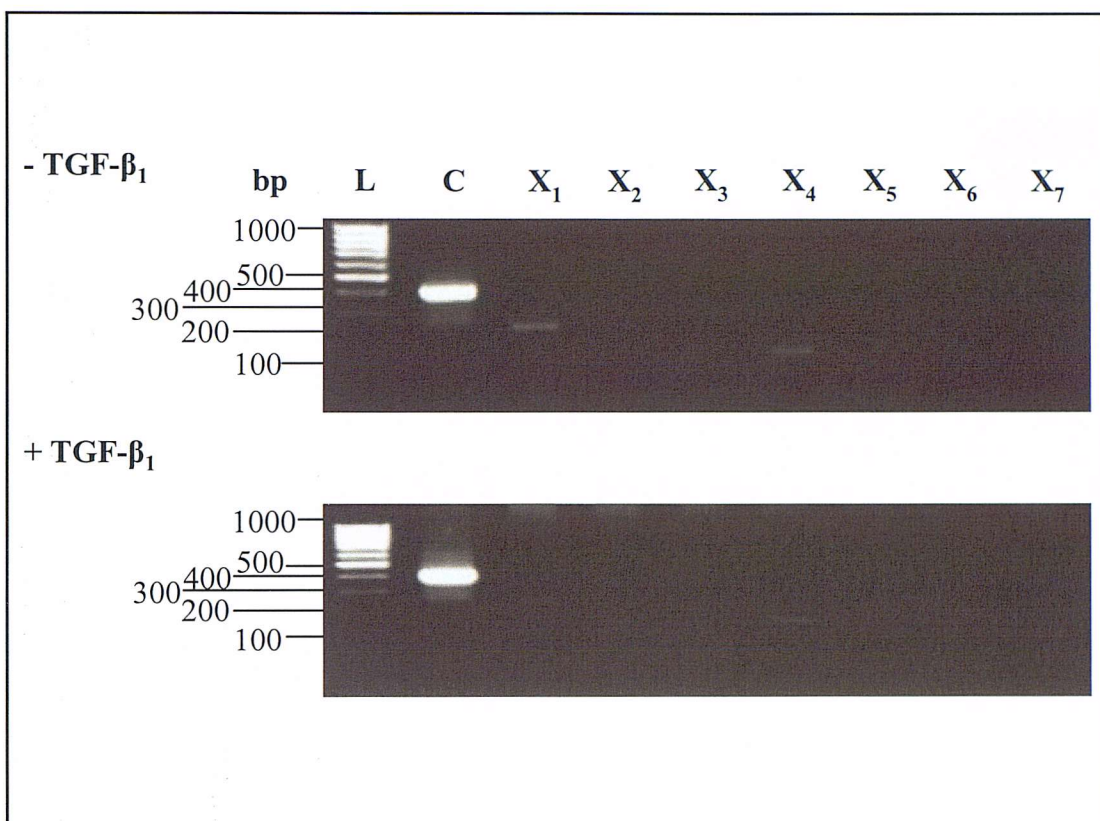


Figure 3-8 Expression of purinergic P2X receptor subtypes in mBMMC

Representative reverse transcriptase (RT)-PCR analysis of P2X receptor expression in mBMMC cultured in the absence or presence of TGF-β₁. PCR amplification of β-actin was used as a control (lane C). Samples were analysed on a 2 % (w/v) agarose gel and PCR fragment size was evaluated using a 1000 base pair (bp) DNA ladder (lane L).

3.2.3 Pharmacological characterisation of mBMMC purinergic receptors

The initial characterisation of the development of mBMMC cultures described in sections 3.2.1 and 3.2.2 shows the presence of TGF- β_1 does not adversely affect the development of mBMMC during the first 14 days of culture and does not noticeably alter purinergic receptor expression at the mRNA level. Furthermore, as TGF- β_1 induced the secretion of large quantities of mMCP-1 from mBMMC *in vitro*, it was deemed important to include this growth factor in all mBMMC cultures used. In all further experiments unless stated otherwise, the term mBMMC refers to cells cultured with IL-3, IL-9, SCF and TGF- β_1 for 14 days.

To determine whether mBMMC expressed functional receptors for nucleotides and nucleosides, qualitative changes in intracellular Ca^{2+} levels were monitored as an indicator of receptor activation. mBMMC at a concentration of $0.5 \times 10^7/\text{ml}$ were incubated with the inactive, esterated (AM) form of the fluorescent probe Fura-2, which can passively diffuse across cell membranes. Once inside the cell, Fura-2-AM is cleaved by esterases resulting in the active cell impermeable indicator Fura-2. The excitation peak for Fura-2 shifts from 380 nm in the absence of Ca^{2+} to 340 nm in the presence of Ca^{2+} . Changes in the 340/380 fluorescence ratio emitted from labelled mBMMC were monitored for a maximum of 150 seconds post receptor stimulation and are displayed as an indicator of changes in cytosolic Ca^{2+} levels.

To investigate the presence of functional P1 receptors, of which there are four different sub-types (A_1 , A_{2A} , A_{2B} and A_3), Fura-2-AM-loaded mBMMC were stimulated with the P1 receptor ligands adenosine and inosine. Stimulation of mBMMC with the A_3 receptor ligand, inosine, at concentrations ranging from 1 mM to 100 nM did not induce any changes in levels of cytosolic Ca^{2+} (Figure 3-9). This suggests inosine does not activate an intracellular signalling pathway that leads to an increase in intracellular Ca^{2+} levels. In contrast, adenosine, the P1 receptor agonist capable of signalling *via* all 4 receptor sub-types (A_1 , A_{2A} , A_{2B} and A_3) elicited rapid, sustained elevations in intracellular Ca^{2+} levels in a dose dependent manner when used at concentrations ranging from 1 mM to 1 μM (Figure 3-9). Only a very small increase in cytosolic Ca^{2+} levels were observed when 1 μM concentrations of adenosine were used, and no changes in Ca^{2+} levels were seen when 100 nM concentrations of adenosine was applied to the mBMMC (Figure 3-9).

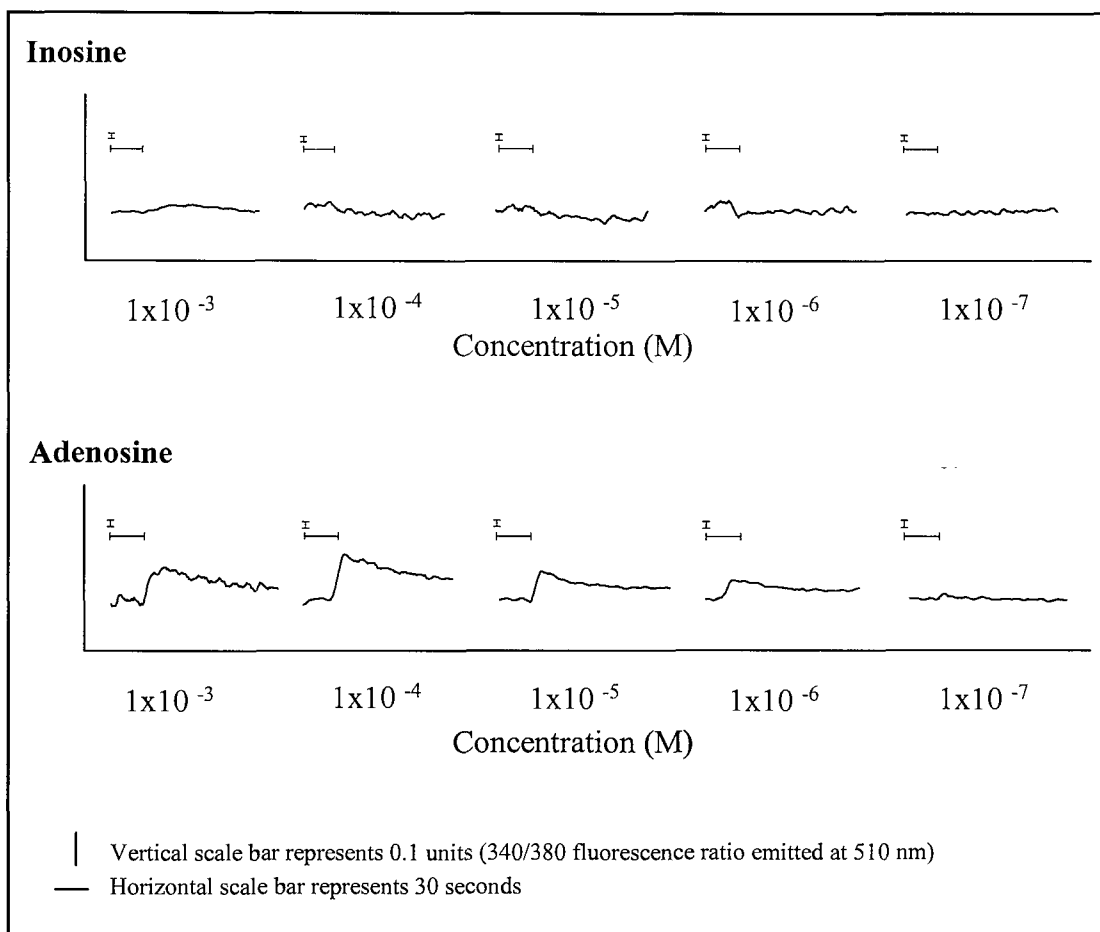


Figure 3-9 Calcium mobilisation in mBMMC stimulated with nucleosides

Calcium mobilisation induced by stimulation of 0.5×10^6 fura-2-AM loaded mBMMC with the indicated concentration of inosine and adenosine. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

3.2.3.1 Pharmacological characterisation of P1 receptor sub-types

To further investigate which P1 receptor sub-type adenosine was signalling *via*, a range of P1 receptor antagonists and agonists were used. A standard method of pre-incubating the antagonist with fura-2-AM-loaded mBMMC for at least 10 minutes at 37°C was used for all experiments described. Many of the antagonists used were only soluble in non-physiological vehicles such as DMSO and ethanol (as detailed in the Materials and Methods section) which can interfere with normal cell function. To ensure the effect of all the antagonists tested were due to intrinsic antagonistic properties and not the vehicle they were dissolved in, mBMMC were pre-incubated with the relevant concentration of vehicle alone and stimulated with the nucleotide as a positive control.

To determine whether adenosine was signalling *via* the A₁ receptor, the competitive A₁ receptor antagonist DPCPX was used. A small reduction in adenosine (100 µM) induced Ca²⁺ mobilisation was observed when 1 mM concentrations of DPCPX were used (Figure 3-10). However, DPCPX was unable to completely antagonise adenosine (100 µM) induced Ca²⁺ mobilisation in mBMMC at concentrations ranging from 1 mM to 100 µM (Figure 3-10). Considering DPCPX has previously been reported to antagonise A₁ receptors in a variety of rat cell types with K_i values in the low nM range (Lohse *et al.*, 1987), it appears unlikely that the A₁ receptor is mediating the adenosine induced Ca²⁺ flux. This is consistent with the RT-PCR data, where expression of the A₁ receptor was not detected.

To investigate the involvement of the A_{2A} receptor in adenosine mediated Ca²⁺ mobilisation, the A_{2A} receptor antagonist ZM241385 was utilised. ZM241385 was pre-incubated with mBMMC at concentrations ranging from 1 mM to 1 µM. When used at 1 µM, ZM241385 was capable of partially reducing adenosine (100 µM)-induced Ca²⁺ mobilisation (Figure 3-10). Concentrations of ZM241385 at and above 10 µM completely inhibited adenosine induced Ca²⁺ mobilisation (Figure 3-10). Concentrations of ZM241385 below 1 µM are required to inhibit the rodent A_{2A} receptor (Poucher *et al.*, 1995; Koshiba *et al.*, 1997). Hence the results observed here do not conclusively confirm the involvement of the A_{2A} receptor.

To characterise the involvement of the A_{2B} receptor in adenosine mediated Ca²⁺ mobilisation, the relatively widely used antagonist of murine A_{2B} receptors alloxazine, and a recently described A_{2B} receptor antagonist MRS1754, were tested in the Ca²⁺ mobilisation system. Alloxazine inhibited Ca²⁺ mobilisation in a dose-dependent

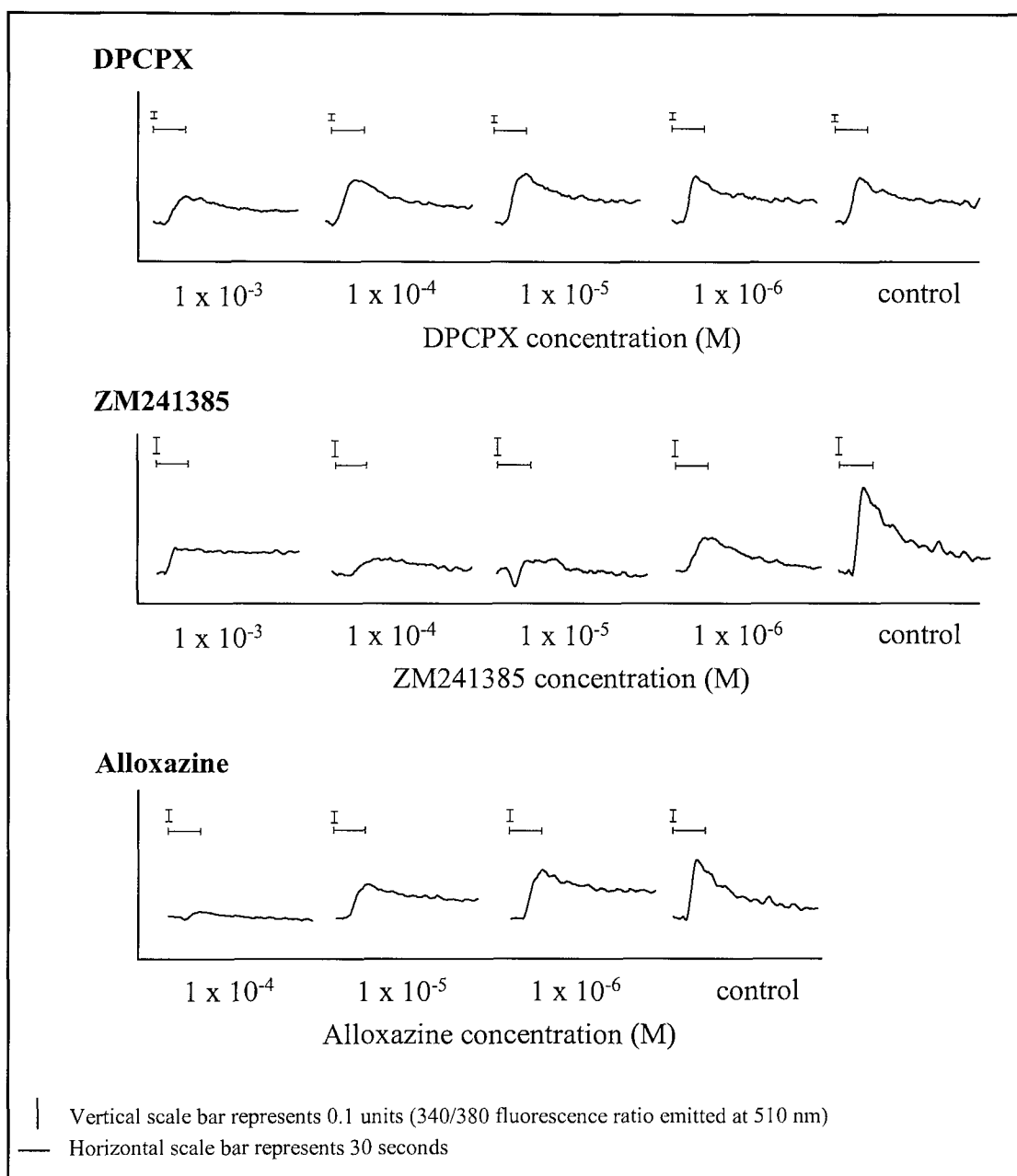


Figure 3-10 Calcium mobilisation in mBMMC pre-incubated with P1 receptor antagonists and stimulated with adenosine

Calcium flux induced by pre-incubating 0.5×10^6 fura-2-AM loaded mBMMC for 10 minutes with the indicated concentration of the A₁ receptor antagonist DPCPX, the A_{2A} receptor antagonist ZM241385 and the A_{2B} receptor antagonist Alloxazine. Calcium flux was subsequently induced by stimulation with 100 μ M adenosine in the presence and absence (control) of antagonist. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

manner and completely blocked 100 μM adenosine elicited Ca^{2+} mobilisation when used at 100 μM concentrations (Figure 3-10). The specificity of alloxazine for murine $\text{A}_{2\text{B}}$ receptors is relatively low. Previous reports have concluded that it is only 9-fold more selective for the $\text{A}_{2\text{B}}$ receptor than for the $\text{A}_{2\text{A}}$ receptor (Brackett *et al.*, 1994). Hence the antagonistic effect may be attributed to interactions with $\text{A}_{2\text{B}}$ or other P1 receptors.

The $\text{A}_{2\text{B}}$ receptor antagonist, MRS1754, at concentrations ranging from 10 to 100 μM was unable to block either 10 μM or 100 μM concentrations of adenosine-induced Ca^{2+} mobilisation in mBMMC (Figure 3-11). MRS1754 has been shown to have excellent selectivity at human $\text{A}_{2\text{B}}$ receptors but is only 8.5 fold more selective for rat $\text{A}_{2\text{B}}$ receptors than for A_1 receptors (Kim *et al.*, 2000). However, no antagonism was observed, potentially indicating that neither the $\text{A}_{2\text{B}}$ nor A_1 receptors are mediating the adenosine-induced increase in cytosolic Ca^{2+} .

To assess the contribution of the A_3 receptor to adenosine-mediated calcium mobilisation, the A_3 receptor-selective agonist IB-MECA was used. Notably, IB-MECA was able to induce Ca^{2+} mobilisation in mBMMC at concentrations ranging from 100 μM to 100 nM (Figure 3-12). As IB-MECA is a selective A_3 receptor agonist at concentrations below 1 μM (Fredholm *et al.*, 2001), this result strongly supports the involvement of an active cell surface A_3 receptor. To further investigate the involvement of the A_3 receptor in adenosine mediated Ca^{2+} mobilisation, the moderately selective A_3 receptor antagonist MRS1523 was used. This antagonist inhibited adenosine induced Ca^{2+} mobilisation in a dose dependent manner at concentrations ranging from 100 μM to 1 μM (Figure 3-12). As this antagonist has been reported to inhibit the A_3 receptor at 5 μM concentrations (Gao *et al.*, 2000), this potentially indicates adenosine is signalling *via* the A_3 receptor in mBMMC.

3.2.3.2 Pharmacological characterisation of P2Y receptor sub-types

To investigate the presence of functional P2Y receptors, of which there are seven different sub-types (P2Y_1 , P2Y_2 , P2Y_4 , P2Y_6 , P2Y_{12} , P2Y_{13} and P2Y_{14}) Fura-2-AM-loaded mBMMC were stimulated with a variety of P2Y receptor ligands. Each P2Y receptor has a different profile of selectivity and specificity for nucleotide 5'-diphosphates and 5'-triphosphates. To initially determine whether mBMMC express any P2Y receptors that couple to pathways that induce increased cytosolic Ca^{2+} levels, cells were stimulated with a variety of different nucleotides at a range of concentrations.

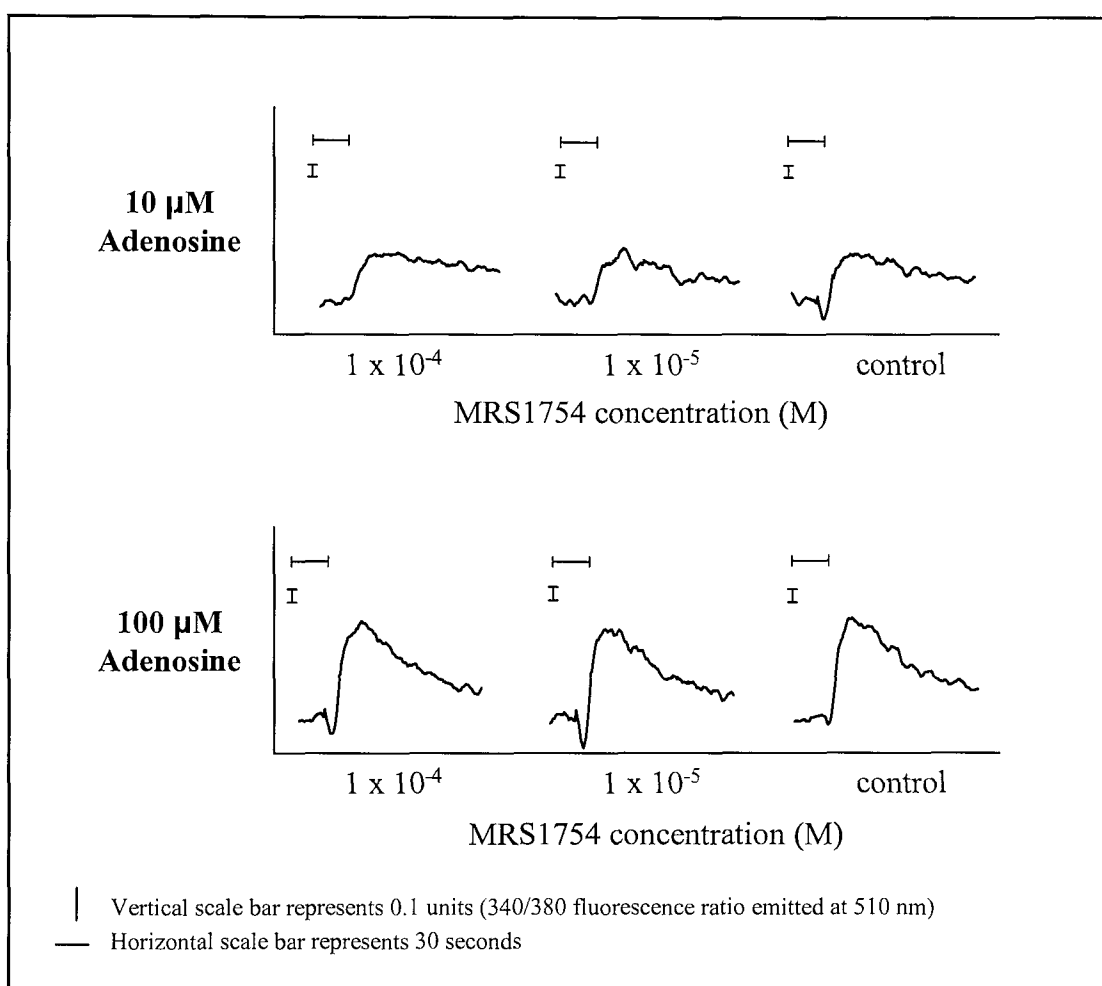


Figure 3-11 Calcium mobilisation in mBMMC pre-incubated with the A_{2B} receptor antagonist MRS1754 and stimulated with adenosine

Calcium flux induced by pre-incubating 0.5×10^6 fura-2-AM loaded mBMMCs for 10 minutes with the indicated dose of the A_{2B} receptor antagonist MRS1754 and subsequently stimulated with the indicated concentration of adenosine. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

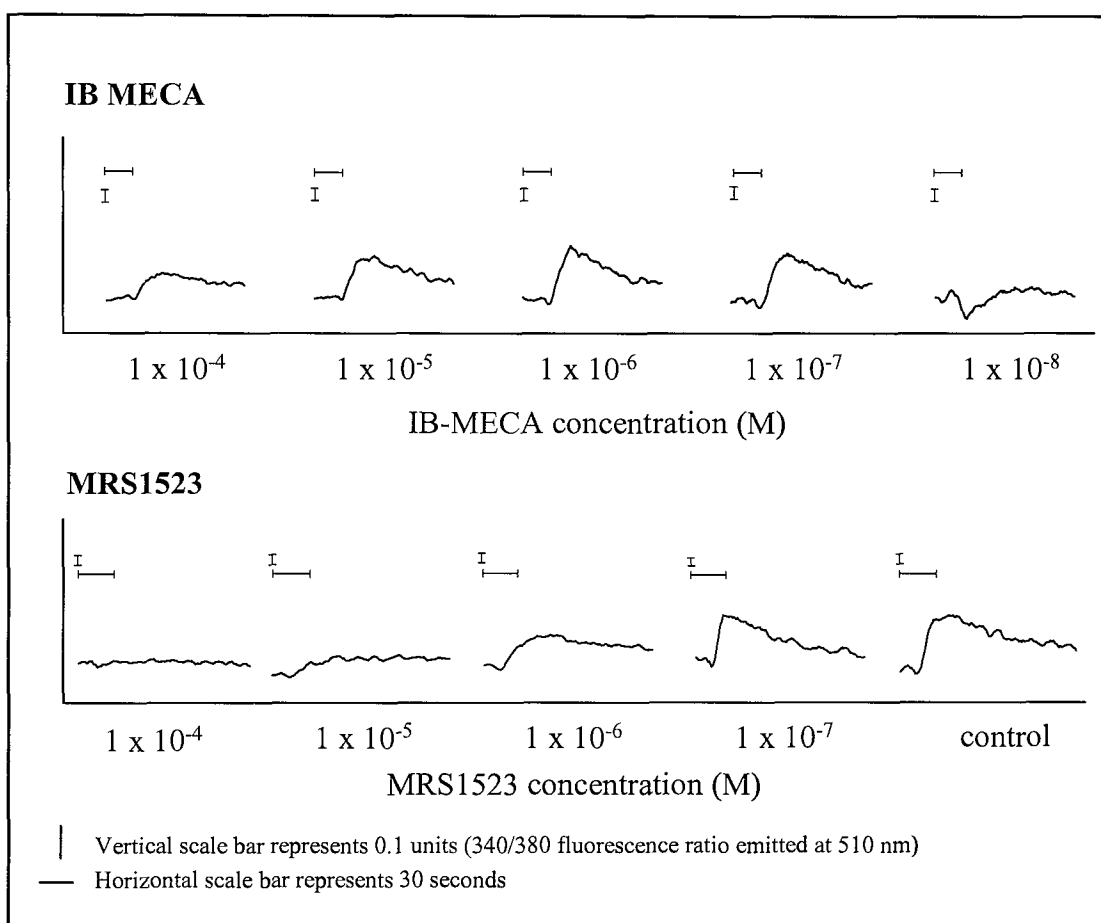


Figure 3-12 Calcium responses of mBMMC stimulated with the A₃ receptor agonist IB-MECA and the A₃ receptor antagonist MRS1523

Calcium mobilisation induced by stimulation of 0.5×10^6 fura-2-AM loaded mBMMCs with the indicated concentration of the A₃ receptor specific agonist IB-MECA. 0.5×10^6 fura-2-AM loaded mBMMC were pre-incubated for 10 minutes with indicated concentration of MRS1523 and subsequently stimulated with 10^{-6} M adenosine. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

Stimulation of mBMMC with AMP at concentrations ranging from 1 mM to 100 nM did not lead to any alterations of intracellular Ca^{2+} levels (Figure 3-13). This result is consistent with there being no known cell surface P2Y receptor for AMP (Abbracchio *et al.*, 2005). Stimulation of mBMMC with ADP at concentrations ranging from 100 μM to 10 nM stimulated a rapid, sustained increase in cytosolic Ca^{2+} levels and this effect was concentration-dependent (Figure 3-13). In theory, the ADP-induced signal could be mediated *via* multiple P2Y receptor sub-types that bind ADP. The P2Y₁, P2Y₁₂ and P2Y₁₃ receptors can all be activated by ADP (von Kugelgen, 2006). As shown in Figure 3-13, ATP at concentrations ranging from 100 μM to 100 nM also induced rapid elevations in intracellular Ca^{2+} levels in mBMMC. The magnitude of the Ca^{2+} response induced was dependent on the concentration of ATP (Figure 3-13). ATP could be signalling *via* a variety of murine P2Y receptor sub-types, as it preferentially binds to the P2Y₂ and P2Y₄ receptors and to a lesser extent the P2Y₁ and P2Y₁₃ receptors. In addition, ATP can also signal *via* ligand-gated ion channel P2X receptors.

To determine whether mBMMC have functional cell surface P2Y receptors responsive to uridine nucleotides, cells were stimulated with UMP, UDP and UTP. UMP at concentrations ranging from 1 mM to 100 nM did not induce any alterations in cytosolic Ca^{2+} levels (Figure 3-14). This is consistent with there being no known purinergic receptor that binds to UMP. UDP at concentrations at and above 100 μM elicited rapid sustained increases in intracellular Ca^{2+} levels (Figure 3-14). UDP signals *via* the P2Y₆ receptor and can also signal *via* the CysLT1 receptor, so it is likely that mBMMC express either one or both of these receptors at the cell surface. UTP at concentrations at and above 10 μM was also capable of inducing Ca^{2+} mobilisation in mBMMC in a concentration-dependent manner (Figure 3-14). The P2Y₂, P2Y₄ and P2Y₆ receptors can all bind, and be activated by, UTP. Hence it is possible that any one, or a combination, of these receptors could be mediating the UTP-induced Ca^{2+} mobilisation in mBMMC.

The data suggested that a wide variety of active P2Y receptors are present on the mBMMC surface as indicated by their responsiveness to ADP, ATP, UDP and UTP. To determine which receptor sub-type(s) each nucleotide was signalling *via*, a range of antagonists and agonists were utilised. To confirm ADP was signalling *via* P2Y receptors, the broad specificity P2Y receptor antagonists PPADS and Suramin were employed. Both antagonists, when used at concentrations at and above 100 μM completely blocked 1 μM ADP-induced Ca^{2+} mobilisation (Figure 3-15). Lower

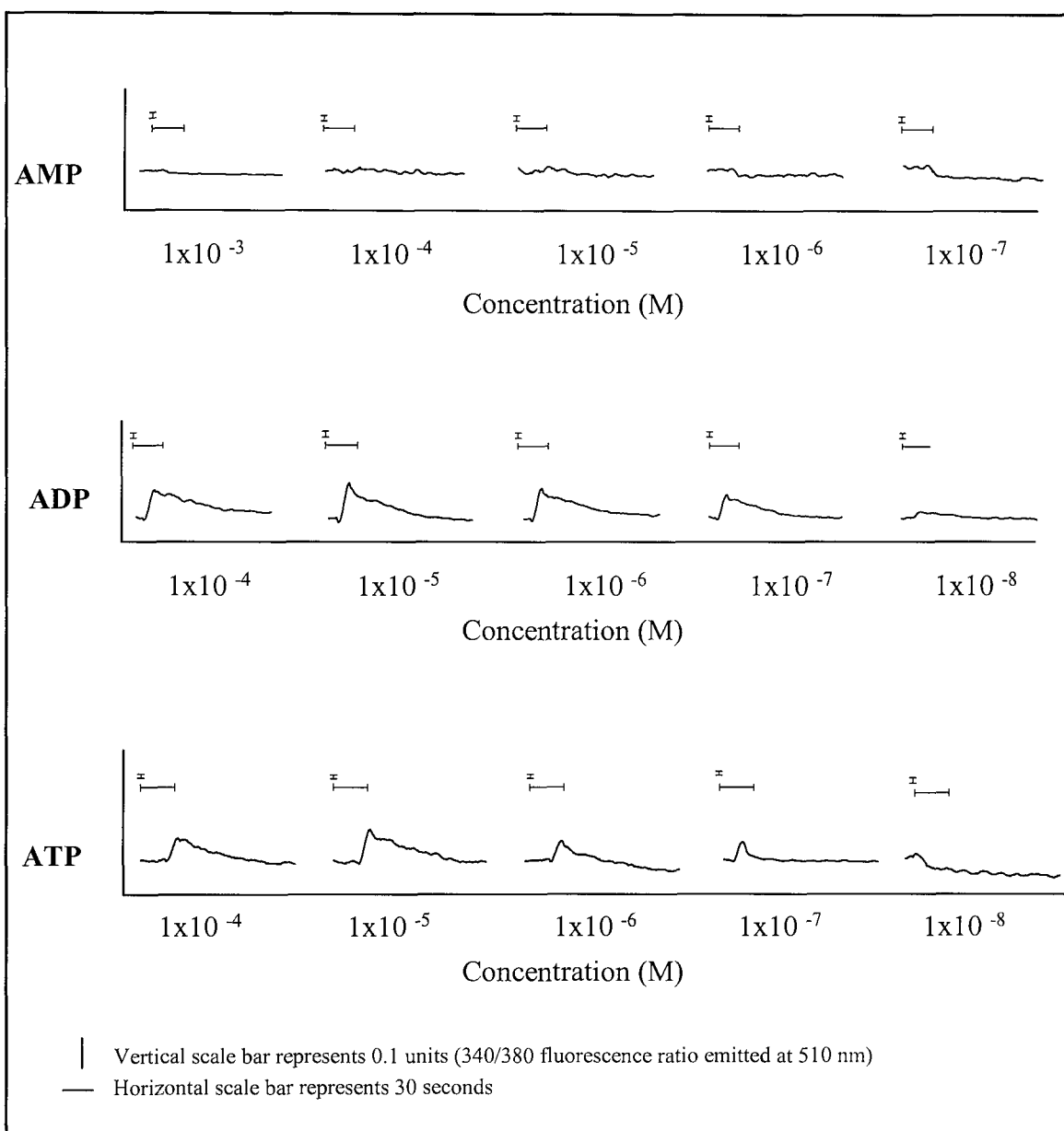


Figure 3-13 Calcium mobilisation in mBMMC stimulated with adenine nucleotides

Calcium mobilisation induced by stimulation of 0.5×10^6 fura-2-AM loaded mBMMC with the indicated dose of AMP, ADP and ATP. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

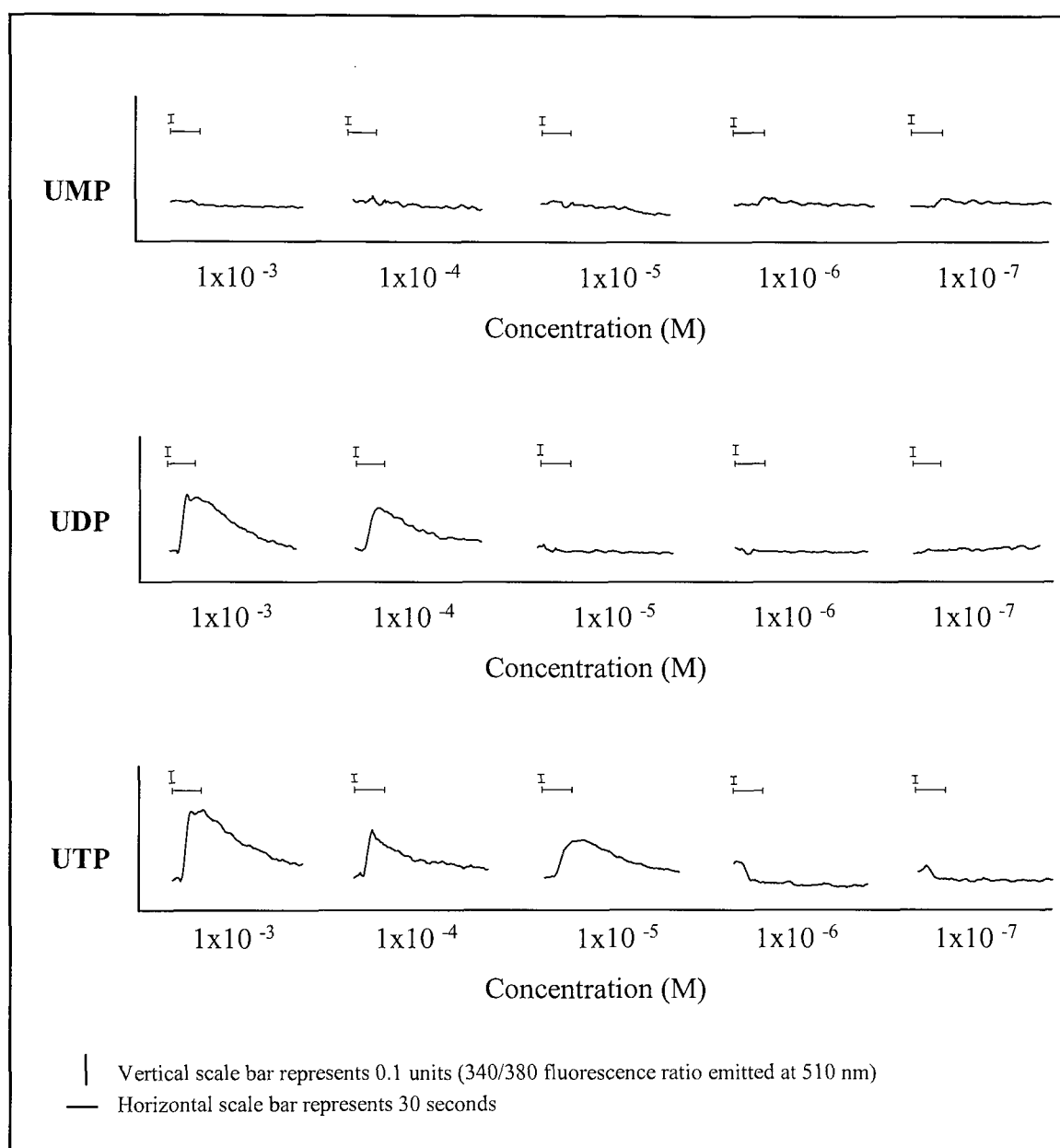


Figure 3-14 Calcium mobilisation in mBMMC stimulated with uridine nucleotides

Calcium flux induced by stimulation of 0.5×10^6 fura-2-AM loaded mBMMC with the indicated dose of UMP, UDP and UTP. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

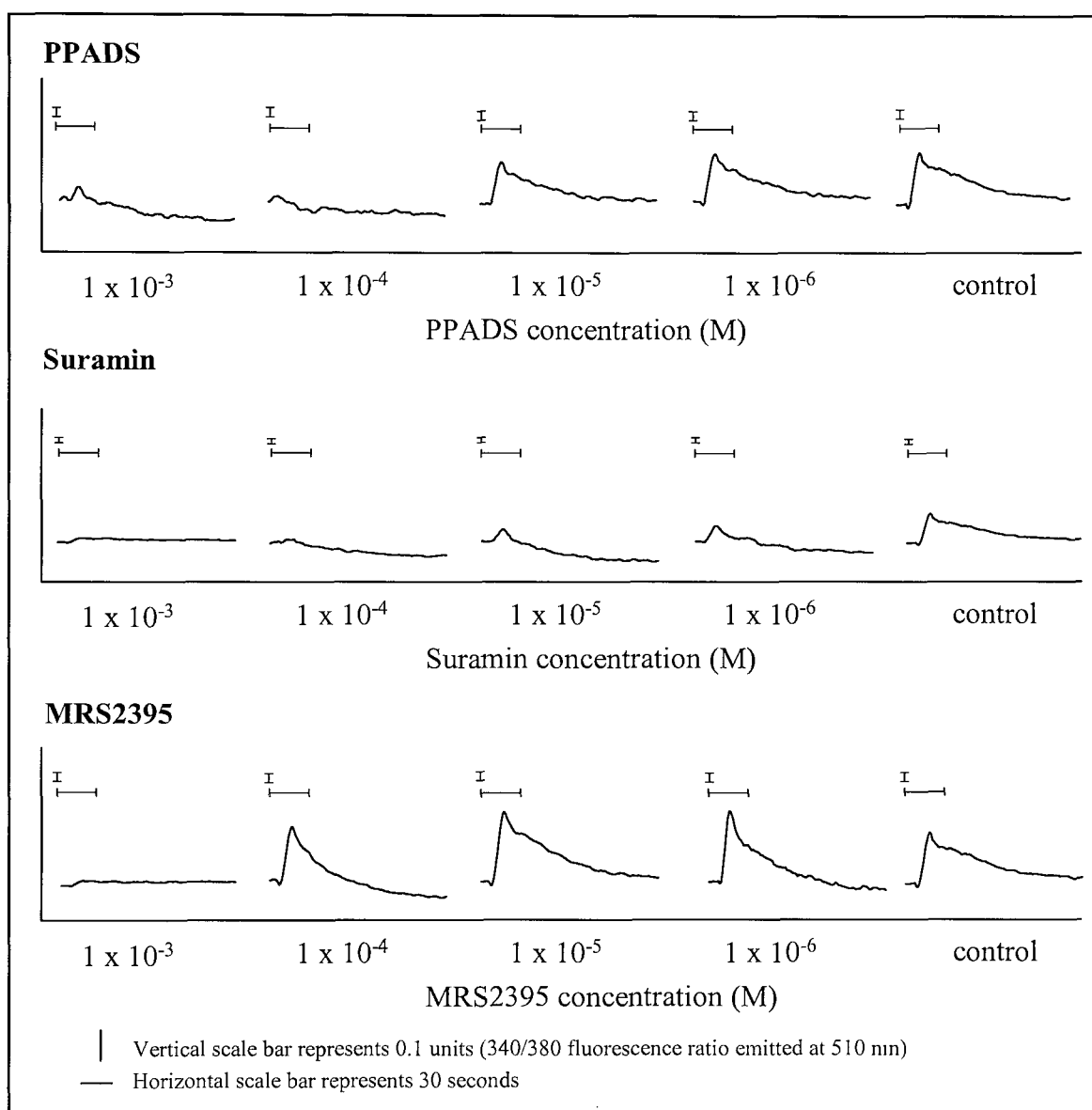


Figure 3-15 Calcium mobilisation in mBMMC pre-incubated with P2Y receptor antagonists and stimulated with ADP

0.5 x 10⁶ fura-2-AM loaded mBMMC were pre-incubated for 10 minutes with the indicated dose of the non-sub-type specific purinergic receptor antagonists PPADS and suramin, and the P2Y₁₂ receptor antagonist MRS2395. Calcium flux was subsequently induced by stimulation with 1 μM ADP in the presence and absence (control) of antagonist. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

concentrations of suramin and PPADS (less than 100 μM) inhibited ADP induced calcium mobilisation in a dose-dependant manner (Figure 3-15). This indicates that ADP was signalling *via* P2Y receptors and also implies that ADP was not being metabolised to adenosine (which would activate A₃ receptors) by cell surface exonucleotidases. To identify the specific P2Y receptor mediating the ADP-induced Ca²⁺ mobilisation, the P2Y₁₂-specific receptor antagonist MRS2395 was used. As shown in Figure 3-15, MRS2395 could only inhibit ADP (1 μM) induced Ca²⁺ mobilisation when used at 1 mM concentrations. Previous studies have found MRS2395 to antagonise P2Y₁₂ receptors at low- μM concentrations (Xu *et al.*, 2002). Hence, it is likely that the antagonistic effects observed using 1 mM concentrations of MRS2395 was due to non-selective P2Y receptor blockade and that P2Y₁₂ receptor activation is not involved in ADP-induced Ca²⁺ flux.

ADP can also signal *via* the P2Y₁ receptor, so the P2Y₁ receptor antagonist A3P5PS was utilised in the Ca²⁺ mobilisation assays. A3P5PS completely inhibited ADP (1 μM)-induced Ca²⁺ mobilisation when used at a concentration of 1 mM (Figure 3-16). 100 μM concentrations of A3P5PS partially reduced ADP-induced increases in cytosolic Ca²⁺, but had no effect when used at lower concentrations (Figure 3-16). Reports in the literature have also documented A3P5PS antagonising P2Y₁ receptors at concentrations in the 100 μM range (Jin *et al.*, 1998). Therefore, it is possible that the P2Y₁ receptor is mediating the ADP-induced Ca²⁺ mobilisation. To investigate this further, the relatively newly described competitive P2Y₁ receptor antagonist MRS2179 was utilised. This antagonist when used at concentrations at and above 10 μM completely inhibited ADP (1 μM)-induced Ca²⁺ flux (Figure 3-16). To determine whether this antagonist was capable of inhibiting higher concentrations of ADP, the experiment was repeated using 10 μM and 100 μM concentrations of ADP as the stimulus. When used at concentrations at and above 10 μM , MRS2179 was capable of inhibiting 10 μM ADP elicited Ca²⁺ flux in a dose dependant manner (Figure 3-16). However, MRS2179 was unable to block 100 μM ADP induced Ca²⁺ mobilisation (Figure 3-16). Similar levels of antagonism using MRS2179 have been reported in the literature (Feng *et al.*, 2004) indicating that this response is mediated, at least in part, by the P2Y₁ receptor. This is consistent with the RT-PCR data, where the P2Y₁ receptor was readily detectable.

In order to ascertain whether UDP was signalling *via* a P2Y receptor, the broad specificity purinergic receptor antagonist PPADS and the P2Y₆ receptor-specific

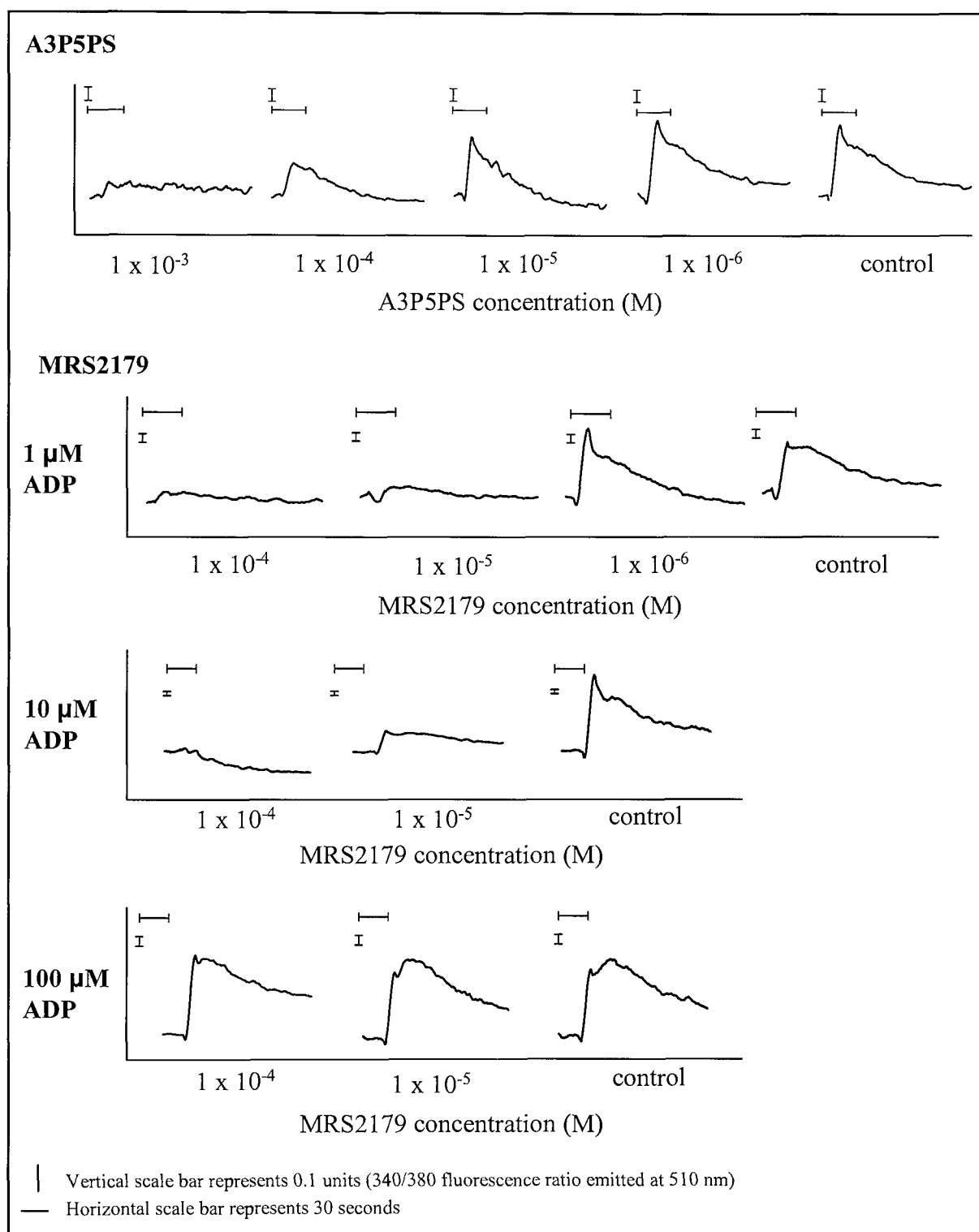


Figure 3-16 Calcium responses of mBMMC pre-incubated with the P2Y₁ receptor antagonists and stimulated with ADP

0.5 $\times 10^6$ fura-2-AM loaded mBMMC were pre-incubated for 10 minutes with the indicated concentration of the P2Y₁ receptor antagonists MRS2179 and A3P5PS. Cells were subsequently stimulated with 1 μ M or the indicated concentration of ADP. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

antagonist MRS2578 were used. When mBMMC were stimulated with 100 μ M UDP, PPADS at concentrations at and above 10 μ M completely inhibited intracellular calcium mobilisation (Figure 3-17) suggesting that UDP was signalling *via* a P2Y receptor. As shown in Figure 3-17, the more specific P2Y₆ receptor antagonist MRS2578 inhibited UDP-induced fluxing when used at 100 μ M concentrations. Doses of MRS2578 lower than 100 μ M did not antagonise UDP-induced Ca²⁺ flux in mBMMC (Figure 3-17). To confirm whether the CysLT1 receptor was also mediating the UDP-induced increases in cytosolic Ca²⁺ in mBMMC, the competitive CysLT1 receptor antagonist MK-571 was used. Concentrations of MK-571 ranging from 1 μ M to 100 μ M were unable to completely inhibit UDP (100 μ M)-induced increases in cytosolic Ca²⁺ (Figure 3-17). Potentially, the relatively high concentration (100 μ M) of UDP required to activate the mBMMC may compete with the antagonist preventing it blocking receptor activation.

Both Ca²⁺ mobilisation assays and electrophysiological studies were used to determine the P2Y/P2X receptors involved in mediating ATP-induced dose dependent elevations in cytosolic Ca²⁺ levels in mBMMC. To initially confirm the involvement of P2Y/P2X receptors, the broad specificity purinergic receptor antagonist suramin was utilised. As shown in Figure 3-18, 1 mM concentrations of suramin completely inhibited ATP (1 μ M) induced Ca²⁺ mobilisation in mBMMC. When used at 100 μ M concentrations, suramin partially inhibited Ca²⁺ mobilisation in mBMMC and had no effect when used at lower doses (Figure 3-18). Previous reports have shown that rat P2X receptors are not inhibited by 100 μ M concentrations of suramin (Buell *et al.*, 1996), but P2Y receptors are (von Kugelgen, 2006). This potentially indicates the involvement of P2X receptors. To investigate this further, specific P2X receptor antagonists were used.

To examine whether the P2X₁ receptor was participating in ATP-induced Ca²⁺ mobilisation, the partially selective P2X₁ receptor antagonist TNP-ATP was used. This antagonist completely inhibited 1 μ M ATP-induced Ca²⁺ mobilisation when used at concentrations ranging from 100 μ M to 1 mM (Figure 3-18). 10 μ M concentrations of TNP-ATP partially inhibited the stimulatory effects of ATP and lower concentrations had no antagonistic effects (Figure 3-18). TNP-ATP has been reported to antagonise P2X₁ and P2X₃ receptors at low nM concentrations (Virginio *et al.*, 1998). Considering that 100 μ M concentrations of TNP-ATP were required to inhibit ATP-induced Ca²⁺ mobilisation in mBMMC, it is unlikely the P2X₁ or P2X₃ receptors mediate ATP signalling. As the P2X₇ receptor has been associated with ATP evoked signalling in

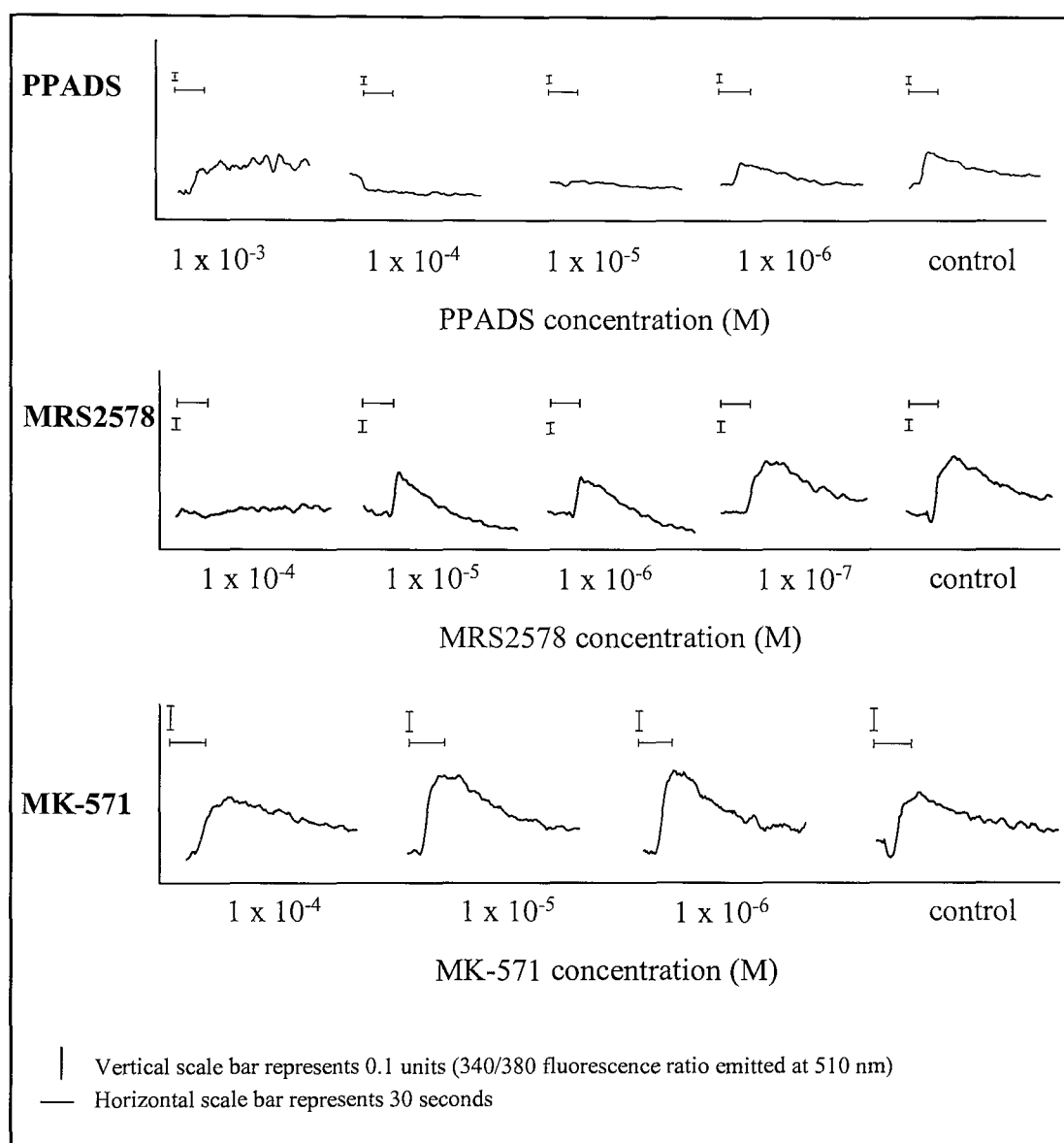


Figure 3-17 Calcium mobilisation in mBMMC pre-incubated with P2Y₆ receptor antagonists and CysLT1 receptor antagonist stimulated with UDP

0.5 x 10⁶ fura-2-AM loaded mBMMC were pre-incubated for 10 minutes with the indicated concentration of the non-sub-type specific purinergic receptor antagonist PPADS and the P2Y₆ receptor specific antagonist MRS2578. Cells were subsequently stimulated with 100 μM UDP. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

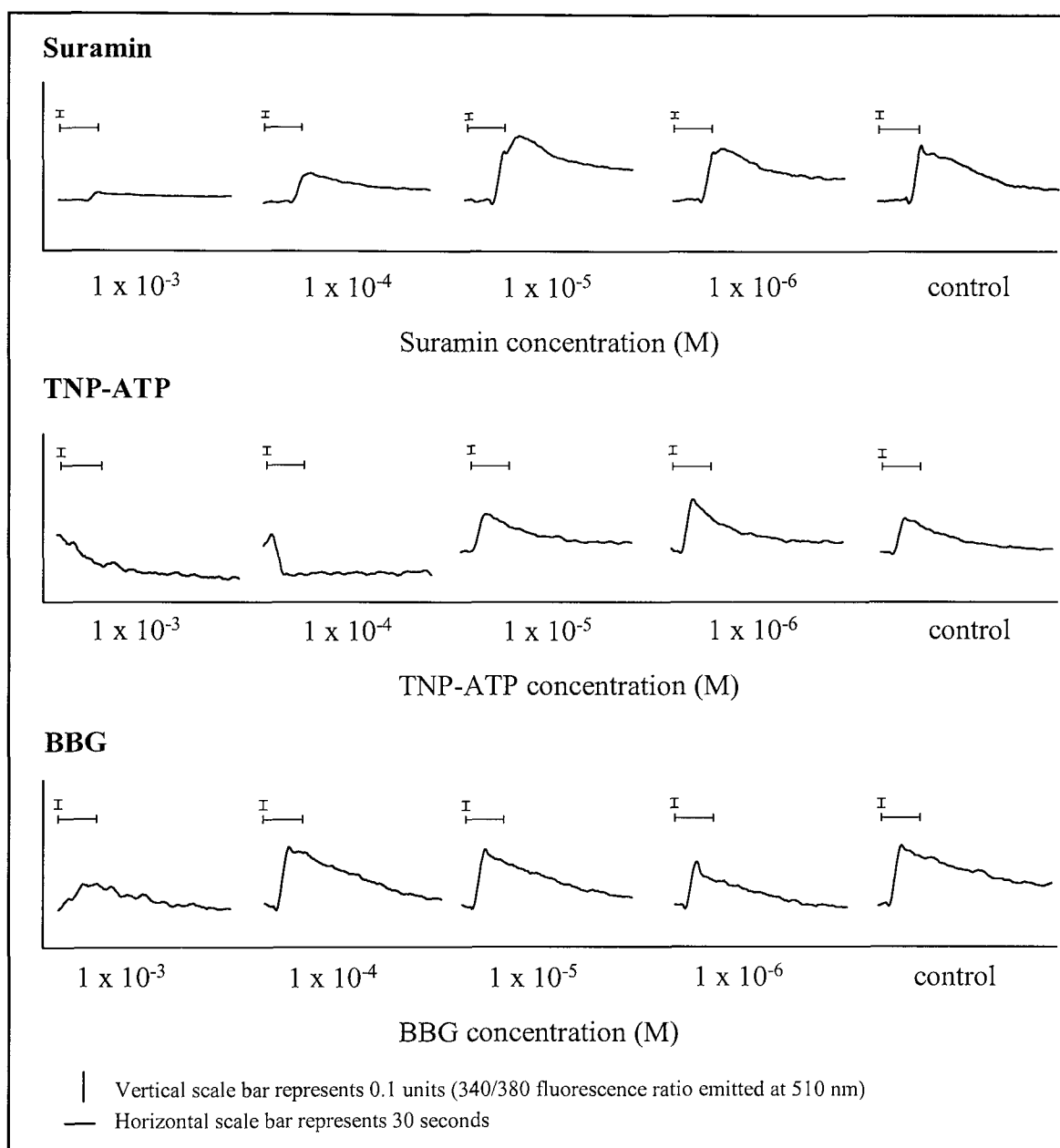


Figure 3-18 Calcium mobilisation in mBMMC pre-incubated with P2X receptor antagonists and stimulated with ATP

0.5 x 10⁶ fura-2-AM loaded mBMMC were pre-incubated for 10 minutes with the indicated concentration of the non-sub-type specific purinergic receptor antagonist suramin, the P2X₁ receptor antagonist TNP-ATP and the P2X₇ receptor antagonist BBG. Cells were subsequently stimulated with 1 μM ATP. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

murine mast cells previously (Bulanova *et al.*, 2005), the P2X₇ receptor antagonist BBG was also tested in the Ca²⁺ mobilisation experimental system. 1 mM concentrations of BBG partially inhibited ATP (1 μM)-induced Ca²⁺ mobilisation but at lower concentrations had no effect (Figure 3-18). BBG has been reported to inhibit rat P2X₇ receptors at concentrations in the nM range (Jiang *et al.*, 2000) which is not consistent with the results seen in this study. This indicates the P2X₇ receptor is unlikely to be mediating ATP induced Ca²⁺ mobilisation in mBMMC, which also correlates with the RT-PCR data where transcripts corresponding to the P2X₇ receptor were not detected.

3.2.3.3 Pharmacological characterisation of P2X receptor sub-types

P2X receptors are membrane-bound ion channels that open in the presence of ATP and have been extensively characterised using electrophysiology (North, 2002). To confirm the involvement of P2X receptors in ATP-mediated mBMMC activation, electrophysiology studies were performed to determine whether ATP could induce current in mBMMC. As shown in Figure 3-19 (A), ATP (10 μM) evoked current in patch clamped mBMMC, indicating the definite involvement of P2X receptors. The decline in the current elicited by ATP during the continued presence of ATP (desensitisation) was slow, which is indicative of P2X₂ or P2X₄ receptor activation (Garcia-Guzman *et al.*, 1997; Séguéla *et al.*, 1996; Wang *et al.*, 1996) [Figure 3-19 (A)]. The most useful distinguishing feature of ATP-evoked currents at P2X₄ receptors is their potentiation by ivermectin (Khakh *et al.*, 1999). To investigate the involvement of the P2X₄ receptor further, ATP was applied to the mBMMC in the presence of ivermectin. As shown in Figure 3-19 (B), the application of ivermectin (3 μM) greatly increased the magnitude of current elicited by ATP and reduced the rate of desensitisation [compare the time scales in Figure 3-19 (A) and (B)]. This definitively indicates the involvement of the P2X₄ receptor. The integrated values of current elicited in mBMMC when stimulated with ATP and ATP + ivermectin are summarised in Figure 3-19 (C). To further confirm the involvement of the P2X₄ receptor, the effect of ivermectin on ATP-induced Ca²⁺ mobilisation was also investigated. mBMMC were pre-incubated for 10 minutes with 10 μM ivermectin and subsequently stimulated with 1 μM and 10 μM concentrations of ATP. As shown in Figure 3-20, stimulation of mBMMC with ATP in the presence of ivermectin induced a more prolonged and sustained elevation in levels of intracellular Ca²⁺ than cells treated with ATP only (Figure 3-20). This was most notable when 10 μM concentrations of ATP were used.

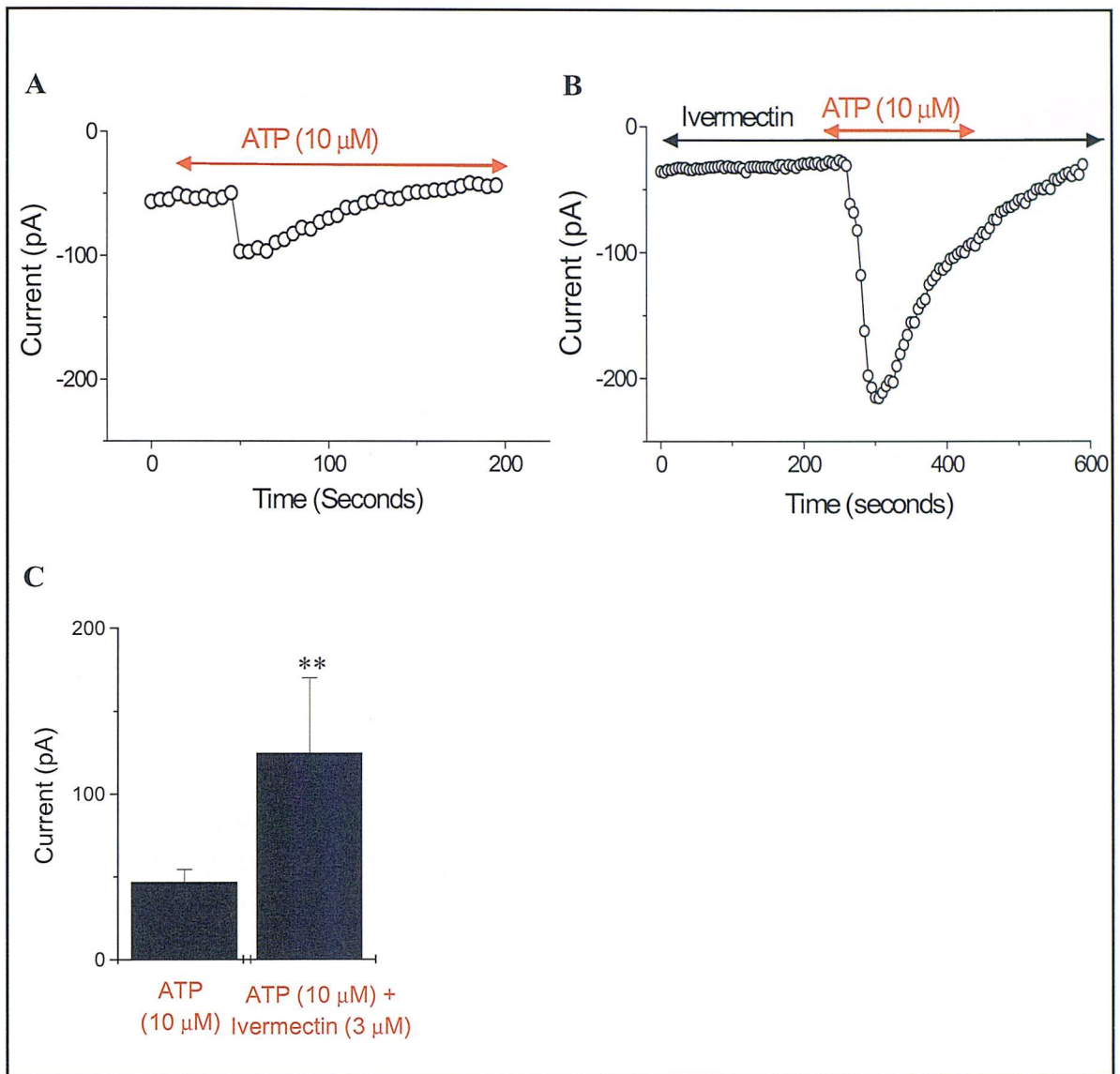


Figure 3-19 The ability of ATP to elicit currents in mBMMC

Representative whole cell patch clamping recordings displaying 10 μ M ATP evoked inwardly rectifying current in (A) the absence and (B) the presence of 3 μ M ivermectin. In (B), cells were incubated with ivermectin (black line) prior to and throughout the application of ATP (red line). (C) shows a bar chart display of the relative ability of the indicated concentration of ATP and ivermectin plus ATP, to elicit currents in mBMMC. Data shown are + s.d.m. of 4 separate experiments. Significant values are relative to cells treated without ivermectin ** $p \leq 0.01$.

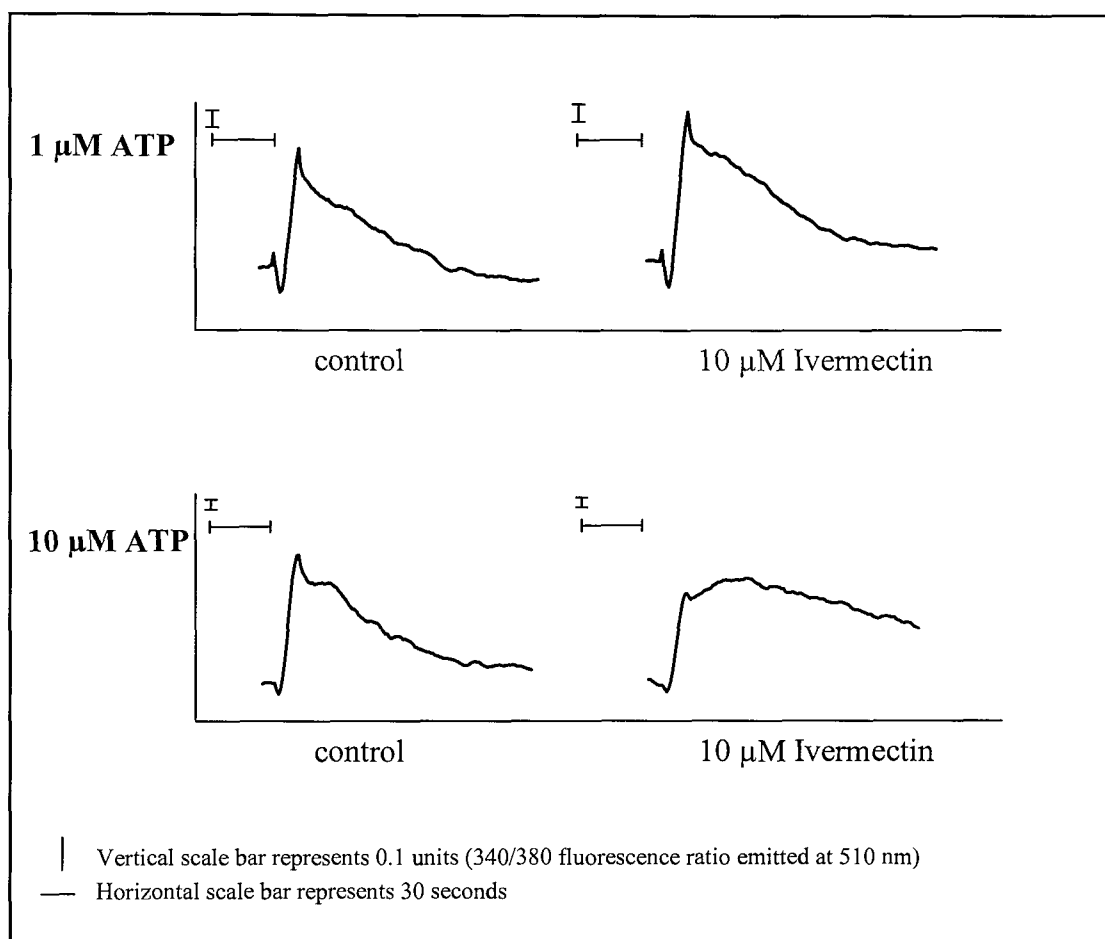


Figure 3-20 Calcium mobilisation in mBMMC pre-incubated with ivermectin and stimulated with ATP

Calcium mobilisation induced by stimulation of 0.5×10^6 fura-2-AM loaded mBMMCs pre-incubated with 10 μM ivermectin for 10 minutes and stimulated with 1 μM and 10 μM ATP. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

This result correlates with the data recorded from the patch-clamping study and implies that ATP-induced mBMMC activation is mediated by the P2X₄ receptor.

3.2.4 The effects of *T. spiralis* ES and recombinant 5'-NT on mBMMC purinergic receptor activation

It has been reported that *T. spiralis* secretes a range of nucleotide-metabolising enzymes which act on ADP, UDP, AMP and adenosine (Gounaris, 2002). As demonstrated in 'Section 3.2.3', mBMMC express functional purinergic receptors that can be activated by ADP, ATP, UDP, UTP and adenosine. *T. spiralis* secreted proteins (ES) could potentially modulate mBMMC purinergic receptor activation by degrading extracellular nucleotides/nucleosides thus limiting the concentration and type of nucleotides/nucleosides available to interact with mBMMC purinergic receptors. To investigate this hypothesis, adenosine, ADP, ATP and UDP were applied to mBMMC in the presence of *T. spiralis* ES or recombinant 5'-nucleotidase (5'-NT), and intracellular Ca²⁺ levels monitored.

To establish that *T. spiralis* ES itself does not induce signalling events that lead to a rise in intracellular Ca²⁺ within the mBMMC, cells were stimulated in the presence or absence of ES. No alterations in cytosolic Ca²⁺ levels were observed [Figure 3-21 (control)] indicating that ES can not directly activate the cells in a manner that would affect the Ca²⁺ mobilisation assay. To investigate the effects of *T. spiralis* ES on mBMMC P1 receptor activation, a range of concentrations of adenosine were utilised in the presence of ES. As shown in Figure 3-21, *T. spiralis* ES prevented adenosine at either 1 µM or 10 µM concentrations inducing an increase in the level of cytosolic Ca²⁺ in mBMMC. This suggests that the *T. spiralis* ES can abolish mBMMC P1 receptor activation. This effect could be mediated by the secreted adenosine deaminase, which can metabolise adenosine to inosine. The presence of *T. spiralis* ES did not alter Ca²⁺ mobilisation induced by 100 µM concentrations of adenosine (Figure 3-21).

The effect of *T. spiralis* ES and the recombinant 5'-NT on UDP-induced Ca²⁺ mobilisation was also investigated. As shown in Figure 3-22, ES completely ablated UDP (100 µM)-induced Ca²⁺ mobilisation in mBMMC. This effect could be attributed to the 5'-NT activity present in *T. spiralis* ES which can degrade extracellular UDP to UMP, thus preventing P2Y/CysLT receptor activation. To verify that this was due to the action of the *T. spiralis* 5'-nucleotidase, UDP was applied to the mBMMC in the

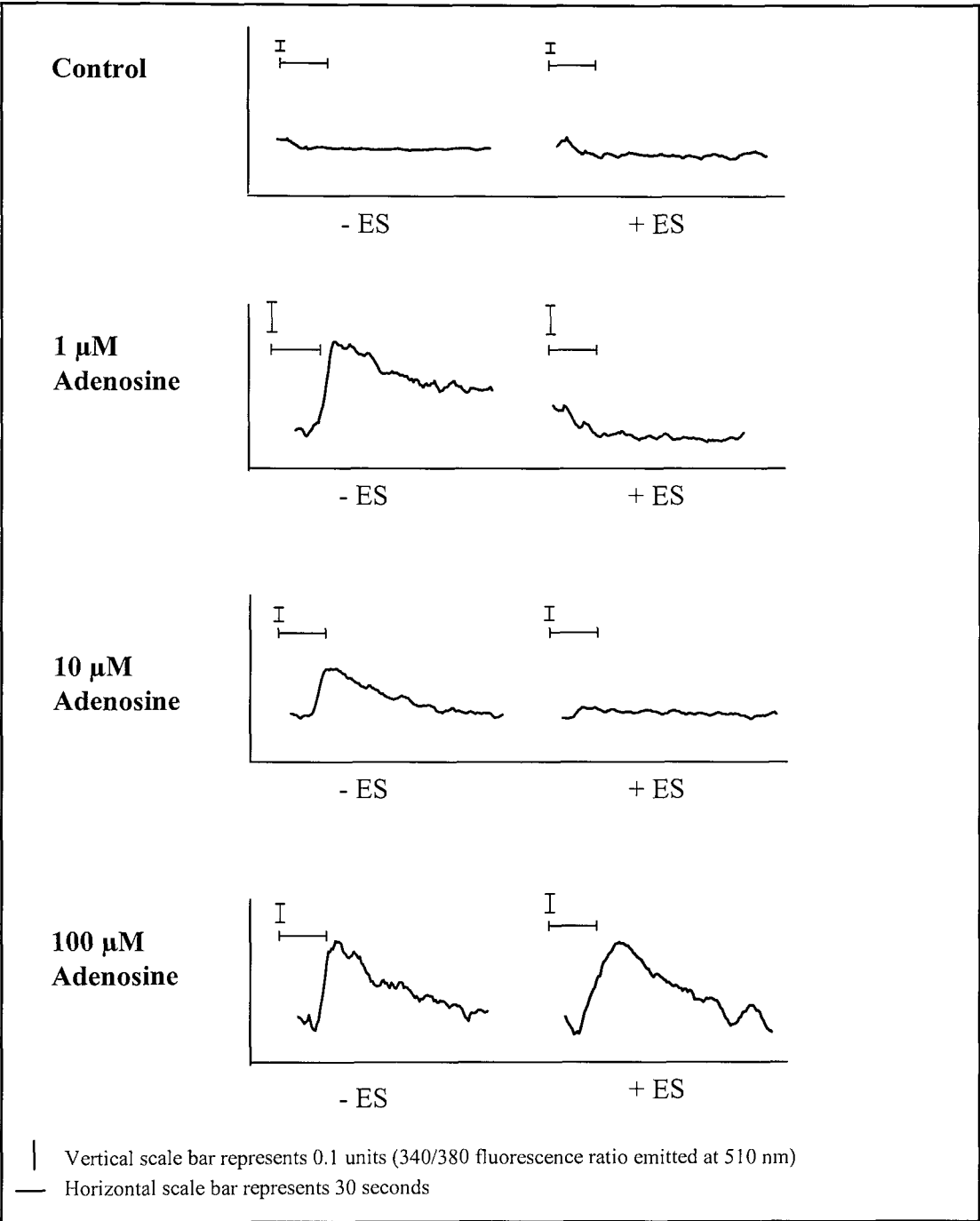


Figure 3-21 Calcium mobilisation in mBMMC stimulated with adenosine in the presence of *T. spiralis* ES

Calcium mobilisation induced by stimulation of 0.5×10^6 fura-2-AM loaded mBMMCs with the indicated concentration of adenosine in the presence or absence of 40 µg/ml *T. spiralis* secreted products (ES) as described in the Materials and Methods section. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

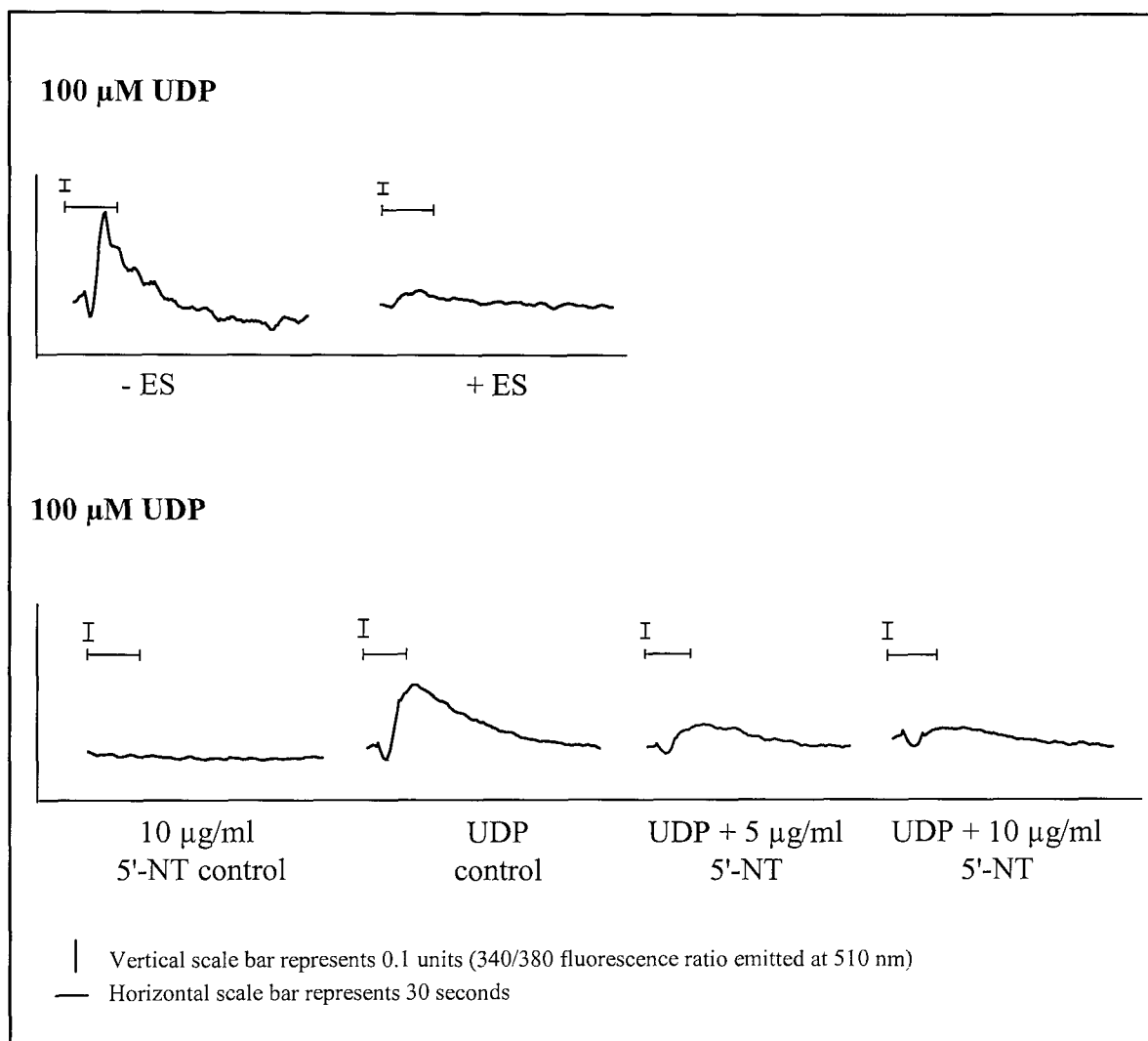


Figure 3-22 Calcium mobilisation in mBMMC stimulated with UDP pre-incubated with *T. spiralis* ES and recombinant 5'-nucleotidase (5'-NT)

Calcium mobilisation induced by stimulation of 0.5×10^6 fura-2-AM loaded mBMMC with 100 μ M UDP in the presence or absence of 40 μ g/ml *T. spiralis* secreted products (ES) and 10 μ g/ml 5'-NT and 100 μ M UDP as described in the materials and methods section. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

presence of 5 or 10 µg/ml recombinant 5'-NT. The presence of the recombinant *T. spiralis* 5'-NT alone, did not induce any changes in cytosolic Ca²⁺ levels (Figure 3-22), indicating that it was not directly activating the mBMMC in this assay. Application of either 5 µg/ml or 10 µg/ml concentrations of 5'-NT reduced the magnitude of intracellular Ca²⁺ mobilised by UDP to virtually basal levels (Figure 3-22). This correlates with the results seen when UDP was applied to mBMMC in the presence of *T. spiralis* ES. This confirms that the ablation of UDP-induced Ca²⁺ mobilisation was due to the action of the *T. spiralis* 5'-NT, which catalyses the hydrolysis of UDP to UMP, thus preventing mBMMC P2Y₆/CysLT receptor activation.

In order to determine the effect of *T. spiralis* ES on ADP-induced Ca²⁺ mobilisation in mBMMC, 1, 10 and 100 µM concentrations of ADP were applied in the presence or absence of ES. The presence of ES resulted in complete ablation of Ca²⁺ mobilisation in mBMMC stimulated with 1 µM ADP (Figure 3-23). This suggests that ADP was metabolised, preventing activation of mBMMC ADP and adenosine receptors. The presence of ES did not alter the increase in cytosolic Ca²⁺ induced by 10 µM and 100 µM concentrations of ADP (Figure 3-23). This suggests that the mBMMC were still being stimulated either by P2Y or P1 receptors. This was investigated further by use of the A₃ and P2Y₁ receptor antagonists MRS1523 and MRS2179 respectively, at concentrations previously shown to antagonise mBMMC activation. As shown in Figure 3-23, the P2Y₁ receptor antagonist MRS2179 did not alter the level of cytosolic Ca²⁺ induced by 10 µM ADP applied in the presence of ES. In contrast, preincubation of the mBMMC with the A₃ receptor antagonist MRS1523 completely inhibited Ca²⁺ mobilisation induced by 10 µM ADP applied in the presence of ES (Figure 3-23). This result shows that the continued elevation in cytosolic Ca²⁺ levels observed when 10 µM ADP is used in the presence of ES, is due to activation of the A₃ receptor by adenosine. When the same experiment was performed using 100 µM ADP, the preincubation of mBMMC with MRS2179 slightly reduced the level of cytosolic Ca²⁺ induced by ADP applied in the presence of ES (Figure 3-23). The A₃ receptor antagonist partially inhibited Ca²⁺ mobilisation induced by 100 µM ADP applied in the presence of ES, indicating that the Ca²⁺ mobilisation was mediated by both the A₃ and P2Y₁ receptors at these concentrations (Figure 3-26).

To further identify the role of *T. spiralis* 5'-NT in mediating the effects observed with ES on ADP (Figure 3-24), concentrations of ADP ranging from 1 µM to 100 µM were applied to mBMMC in the presence of 5 µg/ml or 10 µg/ml 5'-NT (Figure 3-24).

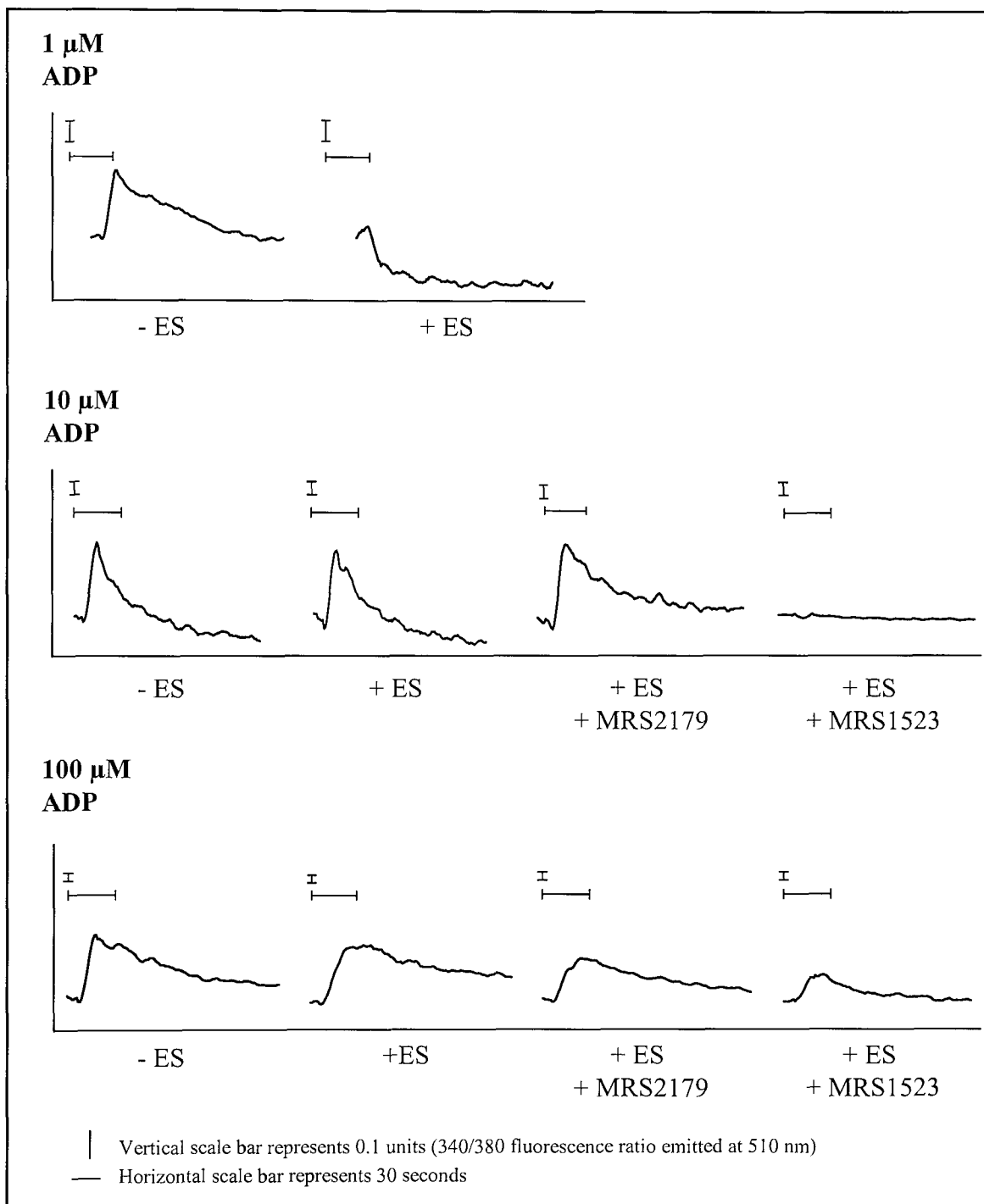


Figure 3-23 Calcium mobilisation in mBMMC pre-treated with the P2Y₁ and A₃ receptor antagonists and subsequently stimulated with ADP in the presence of *T. spiralis* ES

0.5 x 10⁶ fura-2-AM loaded mBMMC were pre-incubated for 10 minutes with 100 µM of the P2Y₁ receptor antagonist MRS2179 or 10 µM of the A₃ receptor antagonist MRS1523. Cells were subsequently stimulated with the indicated concentration of ADP in the absence or presence of 40 µg/ml *T. spiralis* secreted products (ES) as described in the Materials and Methods section. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

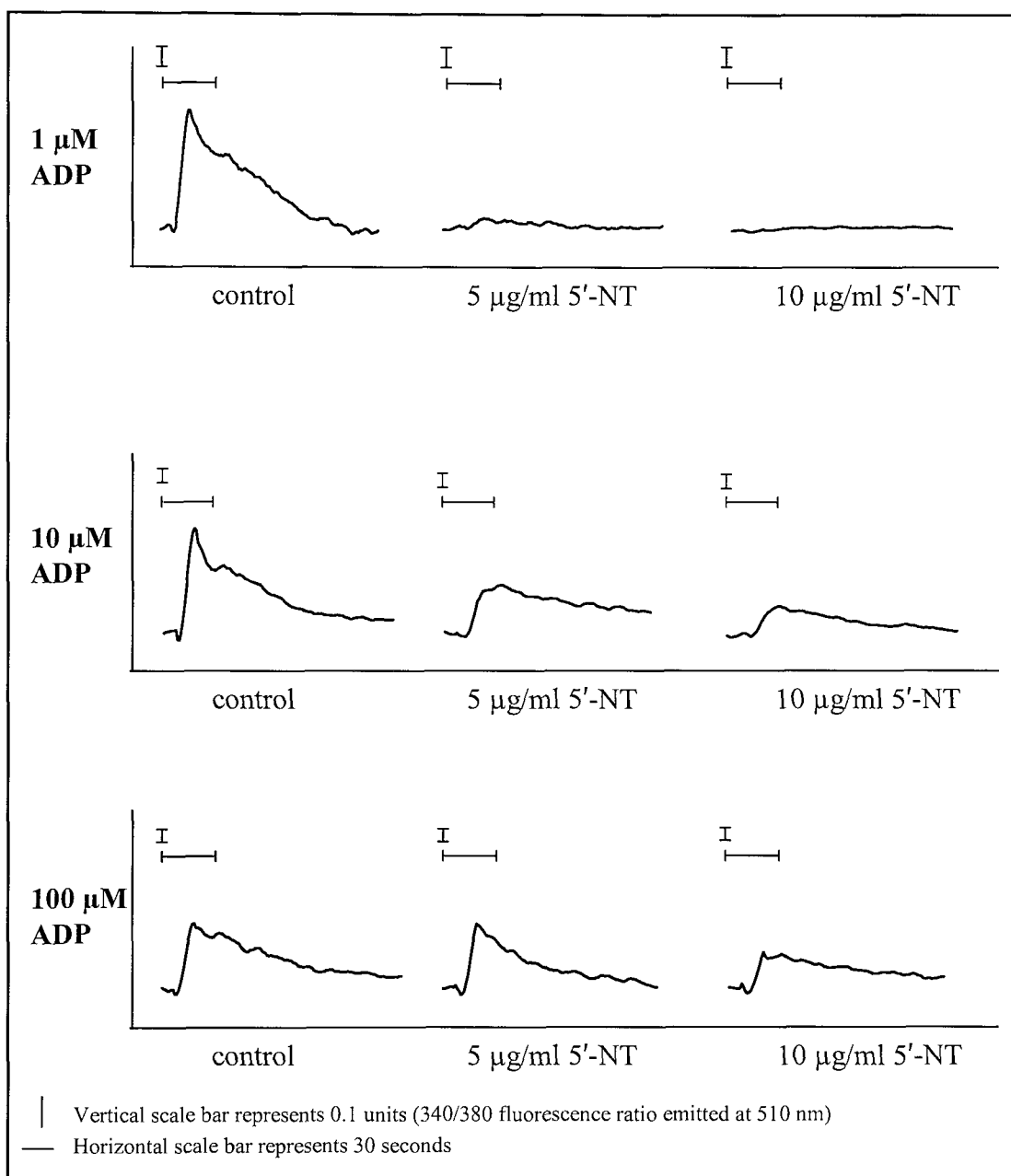


Figure 3-24 Calcium mobilisation in mBMMC stimulated with ADP in the presence of *T. spiralis* recombinant 5'-nucleotidase (5'-NT)

Calcium flux induced by stimulation of 0.5×10^6 fura-2-AM loaded mBMMCs stimulated with the indicated concentration of ADP, in the absence or presence of 5 and 10 μ g/ml 5'-NT as described in the Materials and Methods section. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

The presence of either concentration of 5'-NT was sufficient to completely inhibit 1 μ M ADP-induced calcium flux (Figure 3-24), indicating the prevention of P2Y and P1 receptor activation. The magnitude of Ca^{2+} mobilisation induced by 10 μ M and 100 μ M concentrations of ADP was reduced in a dose dependent manner by the presence of increasing concentrations of 5'-NT (Figure 3-24). The continued fluxing observed when 10 μ M and 100 μ M concentrations of ADP were applied to mBMMC in the presence of either concentration of 5'-NT implies cell surface P2Y and/or P1 receptors were still activated.

To determine whether activation of P1 receptors by adenosine or activation of P2Y receptors by ADP was responsible for the continued fluxing observed in mBMMC stimulated with ADP in the presence of 5'-NT, the P2Y₁ receptor antagonist MRS2179 and the A₃ receptor antagonist MRS1523 were utilised at concentrations previously shown to antagonise mBMMC activation. As shown in Figure 3-25, the P2Y₁ receptor antagonist MRS2179 did not alter the level of cytosolic Ca^{2+} induced by 10 μ M ADP applied in the presence of 5'-NT. In contrast, preincubation of the mBMMC with the A₃ receptor antagonist MRS1523 completely inhibited Ca^{2+} mobilisation induced by 10 μ M ADP applied in the presence of 5'-NT (Figure 3-25). This result shows that the continued elevation in cytosolic Ca^{2+} levels observed when 10 μ M ADP was applied in the presence of 5'-NT, is due to activation of the A₃ receptor by adenosine. When the same experiment was performed using 100 μ M ADP, the preincubation of mBMMC with MRS2179 did not noticeably alter the level of cytosolic Ca^{2+} induced by ADP applied in the presence of 5'-NT (Figure 3-25). The A₃ receptor antagonist nearly completely inhibited Ca^{2+} mobilisation induced by 100 μ M ADP applied in the presence of 5'-NT, indicating the increase in cytosolic Ca^{2+} levels was mediated by the A₃ receptor (Figure 3-25).

It has been reported that *T. spiralis* ES does not contain an enzyme capable of ATP hydrolysis (Gounaris. 2002). Hence, ATP is not expected to be hydrolysed by *T. spiralis* ES. To confirm this, two different concentrations of ATP were applied to mBMMC in the presence of ES. As shown in Figure 3-26 *T. spiralis* ES had no effect on ATP induced Ca^{2+} mobilisation.

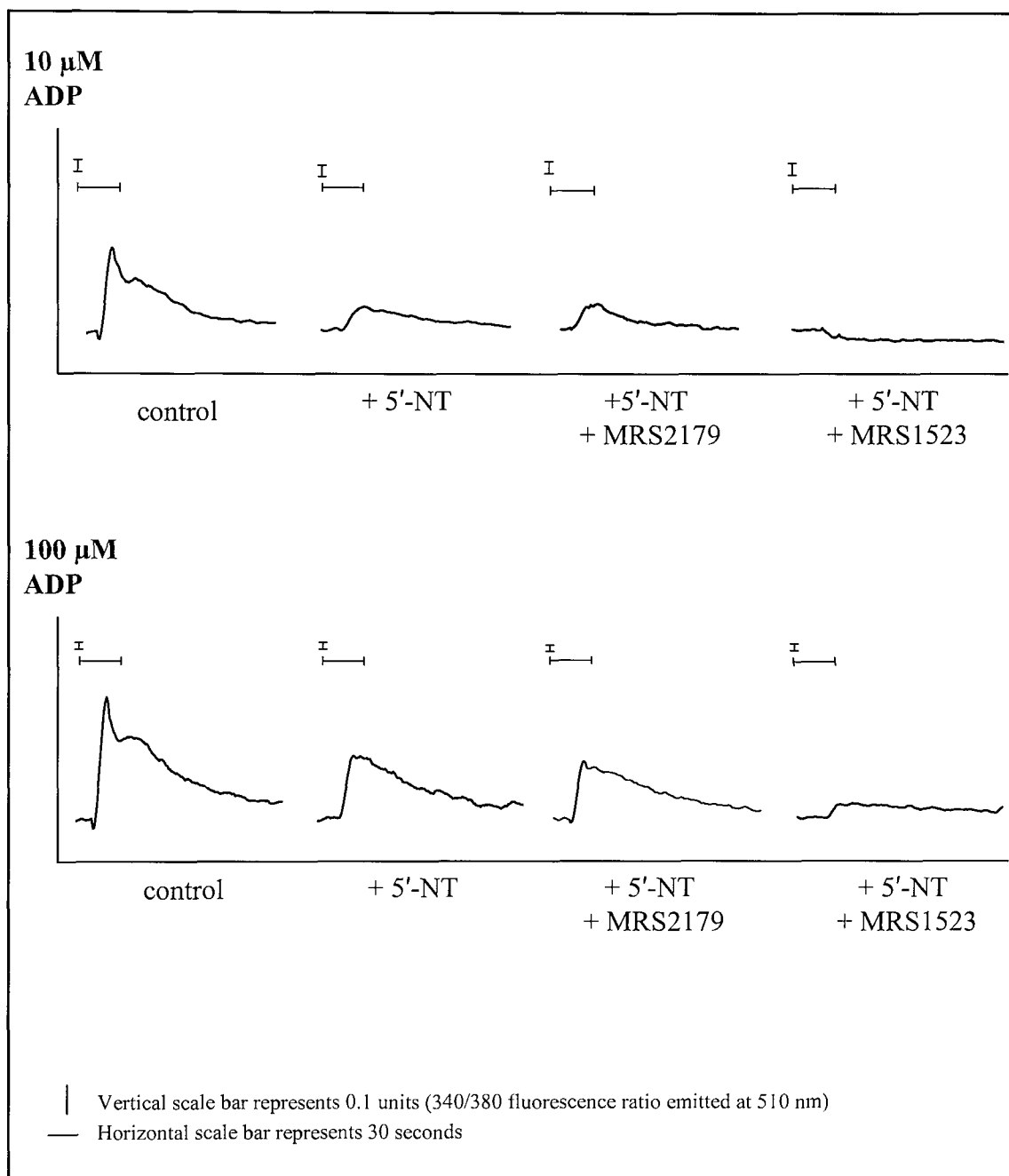


Figure 3-25 Calcium mobilisation in mBMMC pre-treated with the P2Y₁ and A₃ receptor antagonists and subsequently stimulated with ADP in the presence of *T. spiralis* recombinant 5'-nucleotidase

0.5 x 10⁶ fura-2-AM loaded mBMMC were pre-incubated for 10 minutes with 100 μ M of the P2Y₁ receptor antagonist MRS2179 or 10 μ M of the A₃ receptor antagonist MRS1523. Cells were subsequently stimulated with the indicated concentration of ADP in the absence or presence of 10 μ g/ml 5' -NT as described in the Materials and Methods section. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

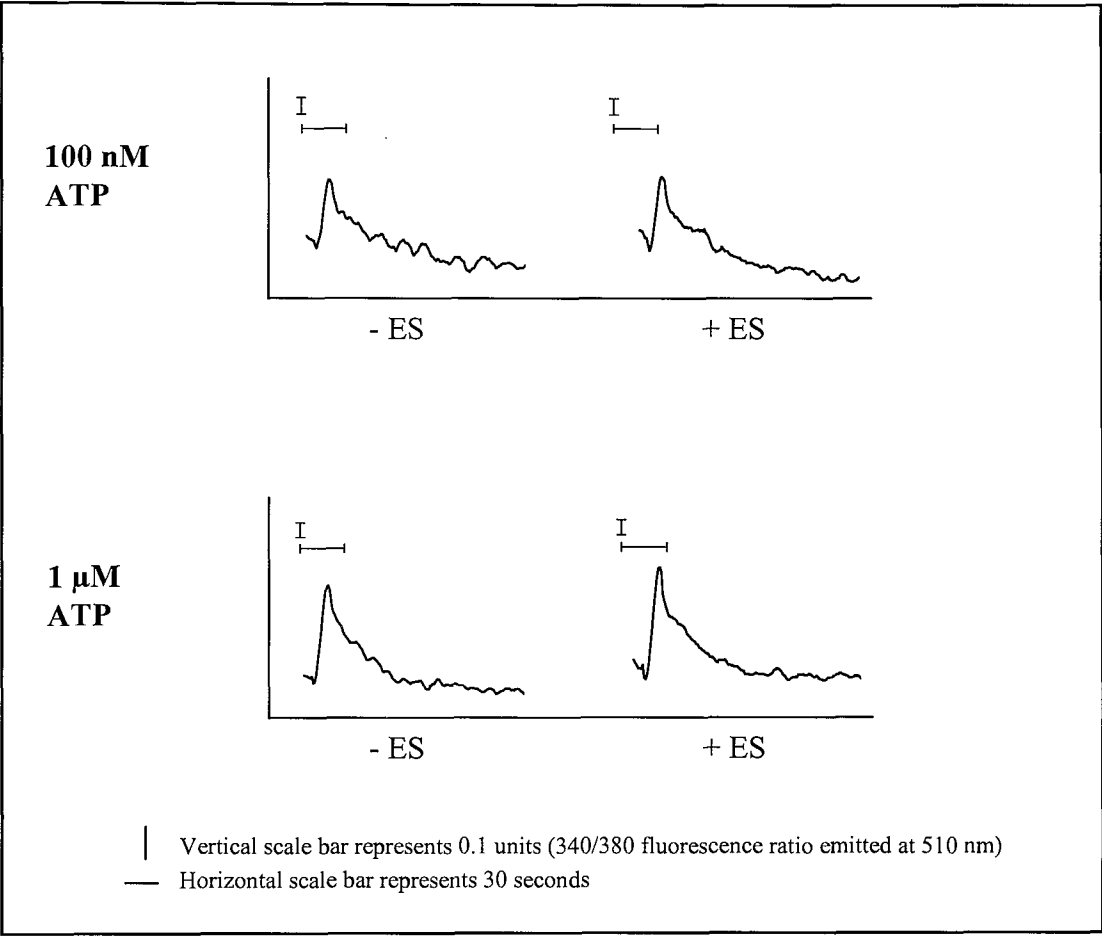


Figure 3-26 Calcium mobilisation in mBMMC stimulated with ATP in the presence of *T. spiralis* ES

Calcium flux induced by stimulation of 0.5×10^6 fura-2-AM loaded mBMMCs with the indicated concentration of ATP in the absence or presence of 40 µg/ml *T. spiralis* secreted products (ES) as described in the Materials and Methods section. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

3.2.5 The effect of nucleotides on intracellular signalling pathways

All cloned and functionally defined P1 and P2Y receptors are able to couple *via* a range of G-proteins that activate phospholipase C (PLC)-dependent pathways and stimulate or inhibit adenylyl cyclase activity. The specific downstream signalling transduction pathway is thought to depend not only on the purinergic receptor sub-type activated, but also on the cell type expressing the receptor (Ralevic and Burnstock, 1998). However, some purinergic receptors, especially those of the P1 receptors primarily signal *via* the same intracellular transduction pathways regardless of the cell type they are expressed in (Fredholm *et al.*, 2001). Hence, characterisation of intracellular signalling pathways can aid determination of some mBMMC purinergic receptor sub-types. As shown thus far, adenosine, ADP, ATP, UDP and UTP can all induce increased levels of cytosolic Ca^{2+} in mBMMC. This suggests the involvement of the signalling enzyme phospholipase C (PLC), which induces an increase in inositol phosphates and leads to the mobilisation of Ca^{2+} from intracellular stores (Mak *et al.*, 2001). The increase in intracellular Ca^{2+} levels can then directly control cell function by activating other pathways.

To investigate whether PLC was required for the Ca^{2+} mobilisation observed when nucleosides/nucleotides were applied to mBMMC, assays were performed using the PLC antagonist U73122. As a control for any potential non-specific action of U73122, the non-reactive analogue U73433 was used as a negative control. U73122 (10 μM) completely inhibited cytosolic Ca^{2+} mobilisation induced by 100 nM concentrations of ADP and ATP (Figure 3-27). When 1 μM concentrations of ADP were used as stimuli, U73122 (10 μM) inhibited the majority of the Ca^{2+} mobilisation response (Figure 3-27). In contrast U73122 (10 μM) only partially inhibited 1 μM ATP-induced Ca^{2+} mobilisation (Figure 3-27). Preincubation of mBMMC with U73122 (10 μM) inhibited adenosine (10 μM) and UDP (100 μM) induced Ca^{2+} fluxing (Figure 3-28). The negative control, U73433, had no negative effect on nucleotide induced calcium mobilisation in mBMMC (see Figures 3-27 and 3-28). These results suggest that mBMMC purinergic receptor activation by ADP, adenosine and UDP requires PLC activity for mobilisation of cytosolic Ca^{2+} . Higher concentrations (1 μM) of ATP were not totally dependent on PLC activation for cytosolic Ca^{2+} mobilisation. This potentially indicates the involvement of P2X receptors that can directly allow the influx of extracellular Ca^{2+} without PLC activation.

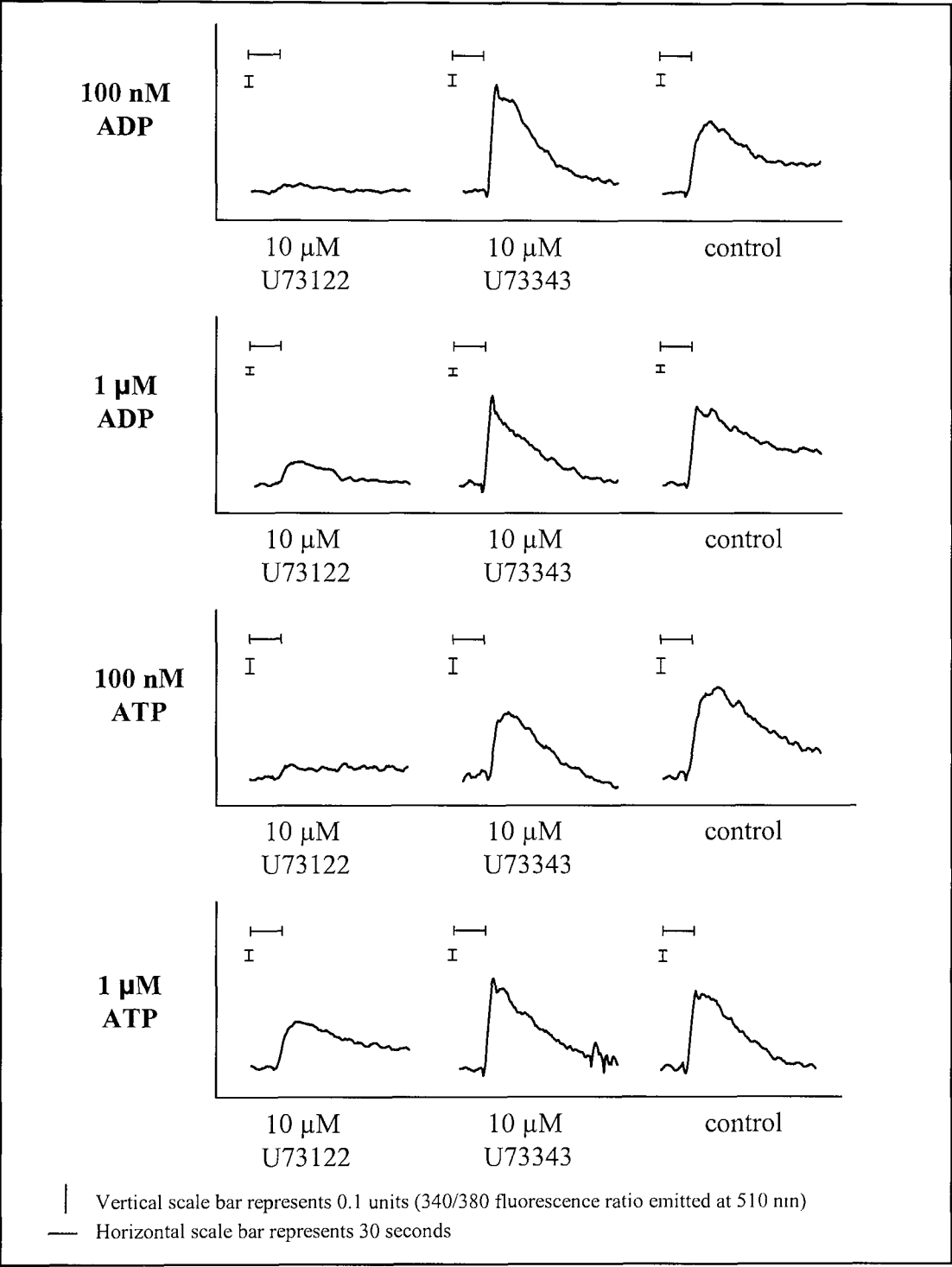


Figure 3-27 Adenine nucleotide induced PLC related calcium mobilisation in mBMMC

Calcium mobilisation was induced by application of the indicated concentration of nucleotide to 0.5×10^6 fura-2-AM loaded mBMMC pre-incubated for 10 minutes with the indicated dose of the PLC antagonist U73122, the inactive negative control analogue U73433 and media alone (control). Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

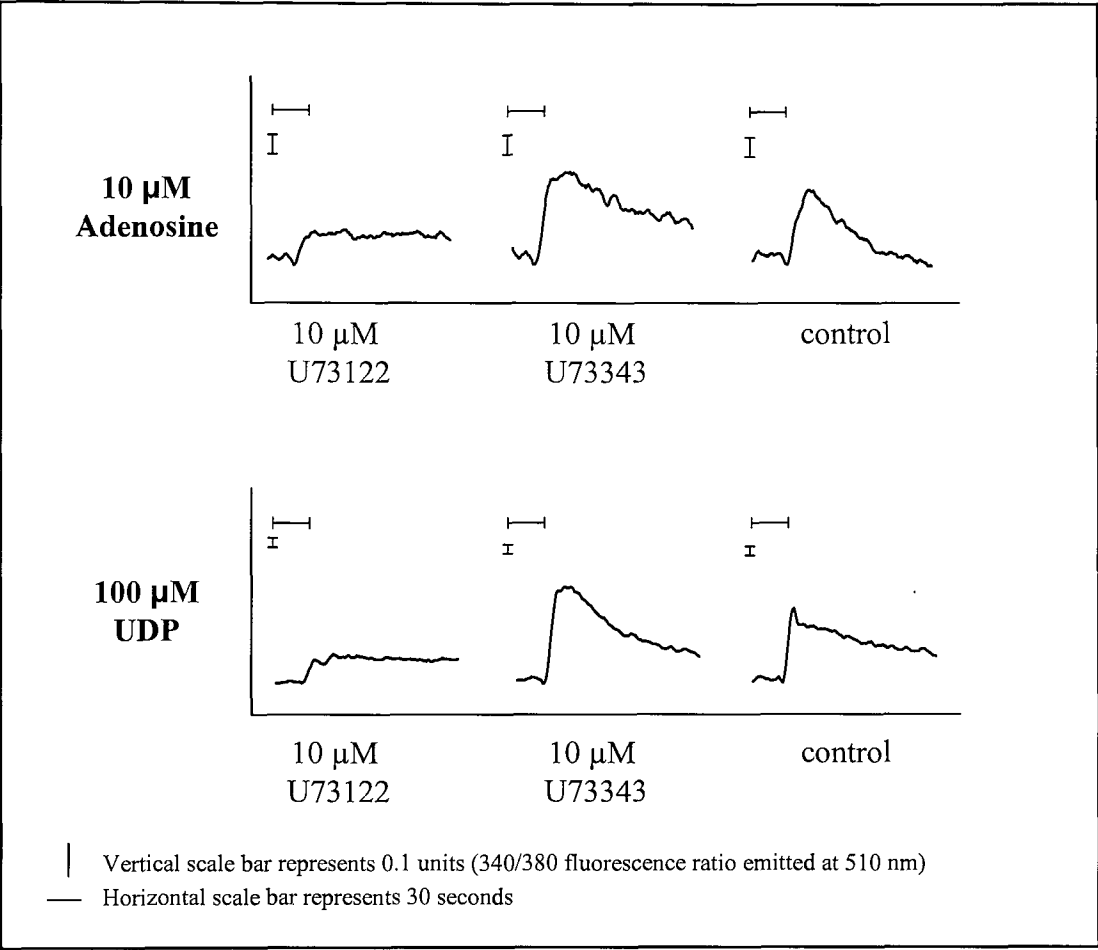


Figure 3-28 UDP and adenosine induced PLC related calcium mobilisation in mBMMC

Calcium mobilisation induced by stimulation of 0.5×10^6 fura-2-AM loaded mBMMC pre-incubated for 10 minutes with the indicated dose of the PLC antagonist U73122 and the inactive negative control analogue U73433 and subsequently stimulated with the indicated concentration of nucleotide/nucleoside. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

To determine whether these nucleosides and nucleotides could induce mobilisation of intracellular stores of Ca^{2+} , assays were carried out in the absence of extracellular Ca^{2+} . As shown in Figure 3-29, the absence of extracellular Ca^{2+} greatly reduced the level of cytosolic Ca^{2+} mobilised when mBMMC were treated with 10 μM ADP and 10 μM ATP. This suggests only a small proportion of cytosolic Ca^{2+} mobilised by purinergic receptors activated by ADP and ATP is from intracellular stores. The majority of the response is due to extracellular Ca^{2+} entering the cell. When mBMMC were stimulated with adenosine, UDP or UTP in the absence of extracellular Ca^{2+} , no mobilisation of cytosolic Ca^{2+} was observed (Figure 3-29). Considering that the Ca^{2+} mobilisation induced by adenosine and UDP was dependent on PLC activation (Figure 3-28), it is likely that stores of intracellular Ca^{2+} were mobilised, but were beyond the limit of detection by the instrument used to monitor the assay. Similar to the results observed using ADP and ATP (Figure 3-27), the majority of the response was due to extracellular Ca^{2+} entering the cell.

To determine whether adenosine, inosine, ADP and UDP exhibited their effects through receptors that also coupled to adenylyl cyclase stimulation, cAMP was measured in mBMMC incubated for 10 minutes with various concentrations of these nucleotides. As displayed in Figure 3-30 (A), adenosine and ADP at concentrations ranging from 1 μM to 100 μM were equipotent at increasing cAMP levels in a dose dependent manner. This suggests the purinergic receptors activated by adenosine and ADP couple to intracellular signalling pathways *via* G-proteins that stimulate adenylyl cyclase. 100 μM concentrations of UDP were found not to stimulate cAMP accumulation (the adenylyl cyclase activator forskolin (10 μM) was used as a positive control) [Figure 3-30 (B)]. This suggests G-proteins that stimulate adenylyl cyclase were not coupled to the receptor *via* which UDP was signalling.

As presented earlier (Figure 3-21) application of adenosine (10 μM) in the presence of *T. spiralis* ES ablated Ca^{2+} mobilisation in mBMMC. To confirm this also prevented adenosine induced adenylyl cyclase accumulation, the experiment was repeated and cAMP measured. As shown in Figure 3-31 the addition of 10 μM adenosine in the presence of *T. spiralis* ES prevented cAMP accumulation in mBMMC, indicating adenylyl cyclase was not activated. Inosine (10 μM) did not induce adenylyl cyclase activation as no increases in cAMP were observed [Figure 3-31 (A)]. These results suggest that *T. spiralis* ES prevents mBMMC PI receptor activation by degradation of adenosine to inosine. To determine whether *T. spiralis* ES could also

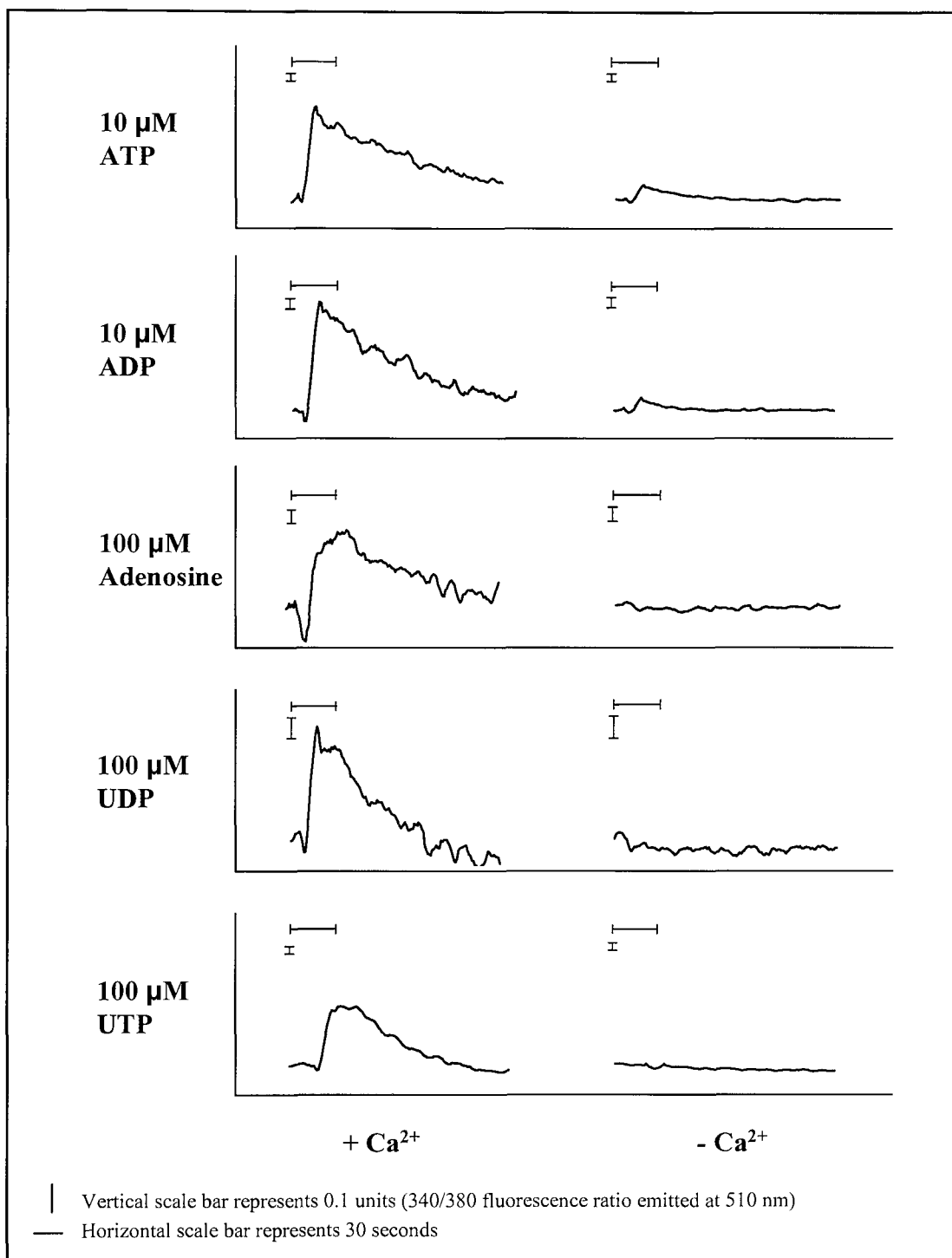


Figure 3-29 The effect of extracellular calcium on purinergic receptor mediated calcium mobilisation in mBMMC

Calcium mobilisation induced by stimulation of 0.5×10^6 fura-2-AM loaded mBMMC with the indicated type and concentration of nucleoside/nucleotide in the presence or absence of 1 mM extracellular calcium. Traces display the 340/380 fluorescence ratio emitted at 510 nm over a time period of 150 seconds. The depicted results are from single experiments representative of at least three performed.

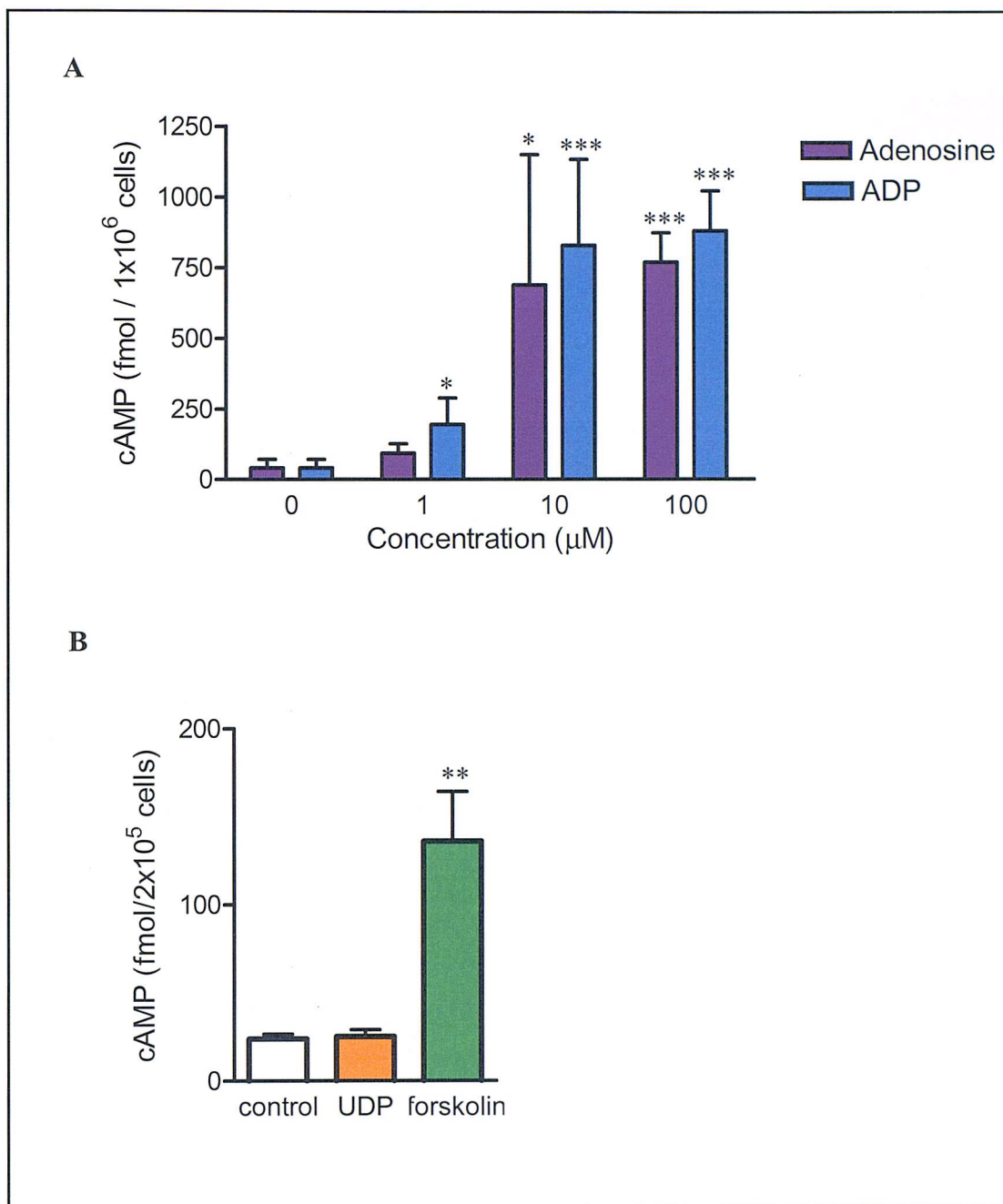


Figure 3-30 Nucleotide induced adenylyl cyclase related signalling in mBMMC

Accumulation of cAMP in mBMMC stimulated for 10 minutes with the indicated concentration of (A) adenosine and ADP and (B) 100 μ M UDP and 10 μ M forskolin. Data were analysed using the unpaired two-tail Student's *t*-test and represent the mean $\pm$ s.d.m of at least triplicate cultures. Significance values are shown relative to cells treated with media alone *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

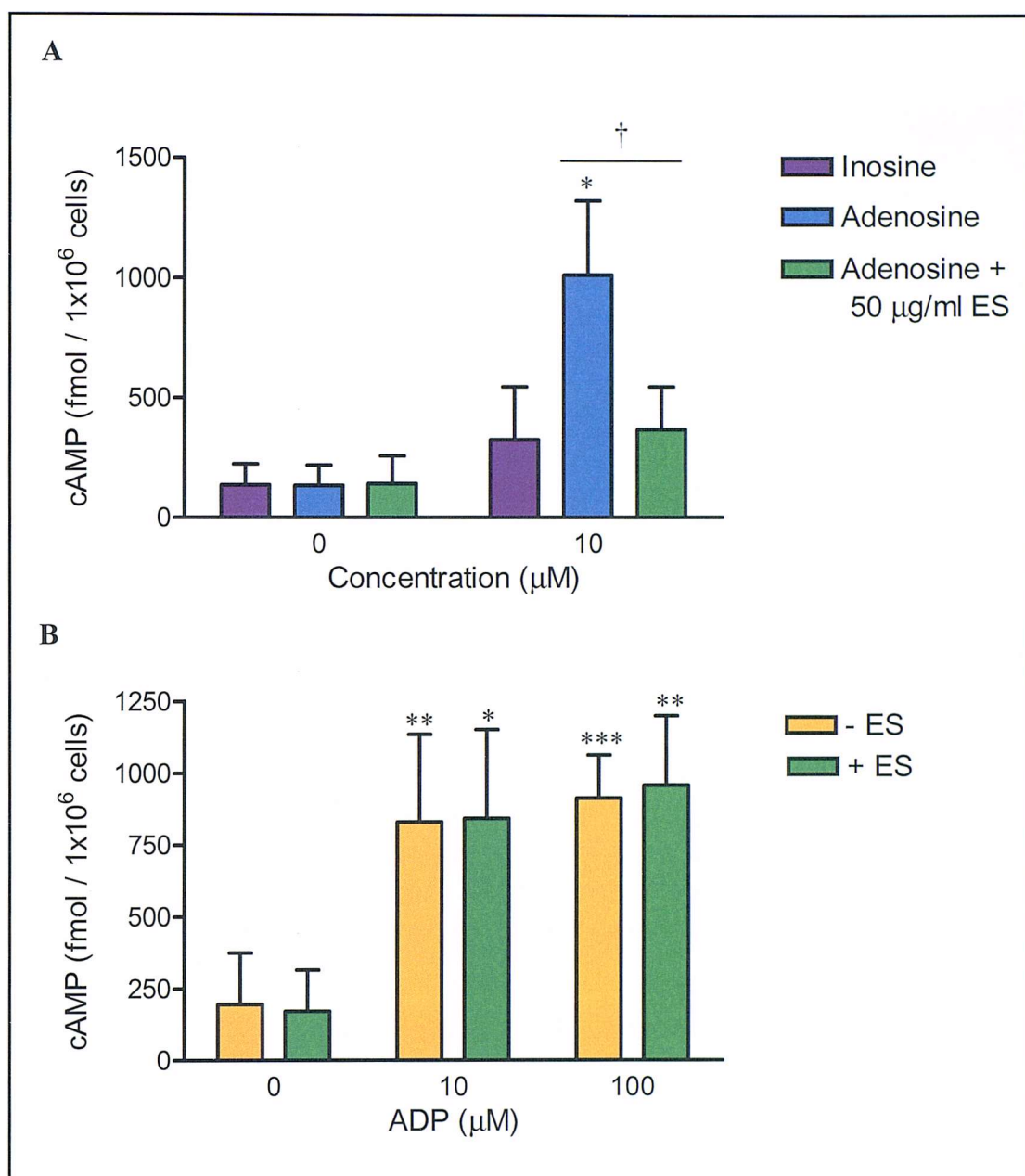


Figure 3-31 The effect of *T. spiralis* ES on adenosine and ADP induced adenylyl cyclase related signalling in mBMMC

Accumulation of cAMP in mBMMC stimulated for 10 minutes (in the presence of 10 μ M rolipram) with the indicated concentration of adenosine (A) and ADP (B) pre-incubated at 37 °C for 1 hour, in the presence/absence of 50 μ g/ml *T. spiralis* secreted proteins (ES). Data were analysed using the unpaired two-tail Student's *t*-test and represent the mean $\pm$ s.d.m of at least triplicate cultures. Significance values are shown relative to untreated cells *** $p \leq 0.001$, ** $p \leq 0.01$ * $p \leq 0.05$ and cells treated with the indicated concentration of nucleotide in the presence/absence of *T. spiralis* ES, † $p \leq 0.05$.

inhibit ADP-induced adenylyl cyclase activation, ADP and ES were applied to mBMMC. As shown in Figure 3-31 (B), application of 10 and 100 μM concentrations of ADP in the presence of ES did not abolish cAMP accumulation. This correlates with the Ca^{2+} flux data shown in Figure 3-23, where calcium was mobilised even in the presence of ES due to stimulation of P1 receptors by adenosine. This suggests that continuous purinergic receptor stimulation was occurring in the mBMMC *via* both P2Y and P1 receptors.

3.3 Discussion

Mucosal mast cells are an important component of the immune response associated with the enteric stage of *Trichinella spiralis* infection. Recent work suggests that mucosal mast cells expressing mast cell protease-1 (mMCP-1) directly mediate the expulsion of *T. spiralis* adults from the intestine and contribute to the development of the local inflammatory response and the consequent enteropathy seen at this stage of the infection (Lawrence *et al.*, 2004; Knight *et al.*, 2000). The level of cellular damage caused by adult parasites migrating through the intestinal epithelium is likely to be substantial, and result in the release of an abundance of nucleotides and nucleosides into the extracellular environment. Considering that *T. spiralis* secretes nucleotide-metabolising enzymes, and also the effects nucleotides and nucleosides have on mast cell function, it was of interest to determine whether *T. spiralis* ES could modulate mast cell purinergic receptor activation.

This study was initially designed to characterise the type and nature of purinergic receptors present on mucosal mast cells. Mouse bone marrow derived mast cells (mBMMC) grown in the presence of transforming growth factor- β_1 (TGF- β_1) are highly analogous to intestinal mucosal mast cells in that they express the mucosal mast cell-specific protease, mMCP-1 (Miller *et al.*, 1999; Wright *et al.*, 2002). An *in vitro* culture method utilising IL-3, IL-9 and SCF was used to generate mouse bone marrow-derived mast cells (mBMMC) (Miller *et al.*, 1999). Cultures were grown in the absence or presence of TGF- β_1 and analysed in terms of purity and maturation state for three weeks of culture.

Similar to the findings of Wright *et al.*, (2002), the data presented here show that culture of bone marrow cells in the presence of SCF, IL-3 and IL-9, independent of TGF- β_1 addition, resulted in ~50% of cells differentiating into a c-kit-positive phenotype within four days. Approximately 25% of the total cell population expressed the Fc ϵ RI four days post-initiation of culture, which was similar to that recorded by Wright *et al.*, (2002). Previous work by Gomez *et al.*, (2005) reported that bone marrow-derived mast cells cultured with IL-3 and SCF, then supplemented with TGF- β_1 for 3 days only, resulted in a ~50 % decrease in surface Fc ϵ RI expression. In contrast to this report, no reduction in cell surface Fc ϵ RI expression was observed in day-14 mBMMC cultured with TGF- β_1 . This could potentially be explained by the presence of IL-9, which is thought to synergise with SCF in promoting mucosal mast cell

maturation (Miller *et al.*, 1999), or be a reflection of the different culture conditions used. Consistent with other studies (Miller *et al.*, 1999), after 2 weeks in culture the population of bone marrow cells consisted of granular, toluidine blue-positive cells. Transcripts corresponding to the mMCP-1 gene were detected by non-quantitative RT-PCR in day-14 mBMMC regardless of the culture conditions used. This agrees with other studies that have suggested that the presence of IL-9 is associated with upregulated expression of mMCP-1, albeit to low levels (Miller *et al.*, 1999; Eklund *et al.*, 1993). Consistent with other studies, spontaneous secretion of mMCP-1 from mBMMC was massively upregulated in the presence of TGF- β_1 (Miller *et al.*, 1999; Wright *et al.*, 2002). Together these results show day-14 mBMMC cultured in the presence of TGF- β_1 are mature, granular and of a mucosal phenotype.

It has previously been reported that human and rodent mast cells express a variety of functional purinergic P1, P2Y and P2X receptor sub-types (Feng *et al.*, 2004; Jaffar and Pearce, 1990; Schulman *et al.*, 1999; Tilley *et al.*, 2000; Jin *et al.*, 1997; Mellor *et al.*, 2001 and 2003). In the current study, the profile of purinergic receptor sub-types functionally expressed by mucosal mBMMC was investigated. Non-quantitative reverse transcriptase-PCR (RT-PCR) analyses showed that the mBMMC expressed a variety of purinergic receptor sub-types. Notably, the presence of TGF- β_1 did not alter the type of purinergic receptors expressed by the mBMMC. This suggests that TGF- β_1 cannot induce alteration of purinergic receptor transcription. Subsequently, all functional purinergic receptor characterisation studies were performed on mBMMC cultured in the presence of TGF- β_1 .

Changes in cytosolic Ca^{2+} levels were monitored in mBMMC as an initial indicator of active purinergic receptors at the cell surface. After binding to their cognate ligands, P2Y and P1 receptors initiate signalling through multiple types of G-proteins (Communi *et al.*, 2000; von Kugelgen, 2006; Fredholm *et al.*, 2001b). This usually couples to stimulation of phospholipase C (PLC) followed by increases in inositol phosphates and mobilisation of Ca^{2+} from intracellular stores. Furthermore, Ca^{2+} released from intracellular stores can induce the entry of Ca^{2+} from the extracellular environment by activating cell-surface store-operated Ca^{2+} channels. Activation of these channels is linked to the depletion of IP_3 sensitive Ca^{2+} stores and represents the principal Ca^{2+} entry pathway into the cell (Putney *et al.*, 2001; Parekh *et al.*, 1997). The exact activation pathway of these Ca^{2+} channels is still being unravelled (Lewis, 2007). ATP-induced opening of P2X receptors can also allow influx of external Ca^{2+} (North,

2002). The collective increase in cytosolic Ca^{2+} is critical to the induction of many different mast cell effector functions such as release of granular contents and chemotaxis (Hua *et al.*, 2007; McCloskey *et al.*, 1999; Gilfillan and Tkaczyk, 2006).

Adenosine, ADP, ATP, UDP and UTP induced Ca^{2+} mobilisation in the mBMMC used in this study. A variety of agonists and antagonists were then used to elucidate which purinergic receptor sub-type(s) were activated by the nucleosides/nucleotides tested. The reported effectiveness of a chosen agonist/antagonist critically depends on its affinity and selectivity for a species-specific receptor sub-type. Ideally, agonists and antagonists should differ in potency by at least two orders of magnitude at different receptors to be really useful in receptor classification. However, most purinergic receptor antagonists and agonists can bind to multiple receptor sub-types, and therefore cannot be used to definitively identify a receptor (Fredholm *et al.*, 2001b; von Kugelgen, 2006). Still, the combined use of antagonists, agonists and characterisation of the intracellular signalling pathways activated can aid functional receptor sub-type identification.

The known pharmacological profile of P1 receptors is a valuable tool when deciphering the specific receptor sub-types activated. The A_1 and $\text{A}_{2\text{A}}$ receptors have high affinities for adenosine and bind sub-micromolar concentrations; in contrast the $\text{A}_{2\text{B}}$ and A_3 receptors have low affinity for adenosine and bind micromolar amounts (Fredholm *et al.*, 2001a). These differences in ligand affinity have important implications, as tissue levels of adenosine are believed to increase into the micromolar range during periods of inflammation (Schrader and Gerlach, 1976; Mentzer *et al.*, 1975). In this study, concentrations of adenosine above $1\mu\text{M}$ activated cell surface adenosine receptors, implying the involvement of the $\text{A}_{2\text{B}}$ and/or A_3 receptor. This correlates with the data obtained by RT-PCR, where of the P1 receptors, only the $\text{A}_{2\text{B}}$ and A_3 receptors were detected. This is consistent with other studies that have also observed the expression of the $\text{A}_{2\text{B}}$ and A_3 receptors in other types of murine mast cells (Salvatore *et al.*, 2000; Marquardt *et al.*, 1994).

To further confirm that the A_1 receptor was not mediating the adenosine induced cytosolic Ca^{2+} mobilisation, the competitive A_1 receptor antagonist DPCPX was used. DPCPX is widely used to identify the involvement of A_1 receptors in rat models, where it has been reported to antagonise A_1 receptors with K_i values in the low nM range (Lohse *et al.*, 1987). DPCPX only antagonised adenosine-induced Ca^{2+} mobilisation

when used at 1 mM concentrations. This again suggests the lack of involvement of the A₁ receptor.

As many types of murine and human mast cells express functional A_{2A} receptors (Marquardt *et al.*, 1994; Salvatore *et al.*, 2000; Feoktistov and Biaggioni, 1998; Suzuki *et al.*, 1998) it was deemed important to investigate whether this receptor was involved in adenosine related signalling. ZM241385 is a widely used A_{2A} receptor antagonist and was originally shown to competitively antagonise rat A_{2A} receptors present in rat PC12 (adrenal pheochromocytoma cell line) cell membrane preparations. It exhibits 80-fold selectivity for A_{2A} versus A_{2B} receptors, as well as 500-1000-fold and 500,000-fold selectivities for A_{2A} versus A₁ and A₃ receptors, respectively (Poucher *et al.*, 1995). Concentrations of ZM241385 at or above 10 μ M were required to inhibit adenosine-induced Ca²⁺ mobilisation in mBMMC. This is in contrast to the effects of this antagonist on murine T cells (Koshiba *et al.*, 1997) and human cord blood derived mast cells (Suzuki *et al.*, 1998), where lower concentrations of ZM241385 antagonise A_{2A} receptor activation. However, considering A_{2A} receptors are classically activated by sub-micromolar concentrations of adenosine (Fredholm *et al.*, 2001a) and this receptor was not detected by RT-PCR analysis using mBMMC RNA, it appears unlikely that this receptor is mediating adenosine-induced mBMMC activation.

Characterisation of A_{2B} receptors has in the recent past been notoriously difficult. For this reason, a recently described A_{2B} receptor antagonist MRS1754 and a relatively widely used antagonist of murine A_{2B} receptors, alloxazine, were tested in the Ca²⁺ mobilisation system. Originally described by Kim *et al.*, (2000) MRS1754 was found to have approximately 200-fold selectivity at human A_{2B} versus A₁ and A₃ receptors. On the basis of the reported human data, all of the concentrations of MRS1754 used would have been expected to block mBMMC A_{2B} receptors. However, no inhibition of adenosine-induced Ca²⁺ mobilisation in mBMMC was seen. Currently, there have been no reports in the literature characterising the effects of MRS1754 on murine or rat A_{2B} receptors, so the species selectivity of this antagonist is unknown.

To further investigate the involvement of the A_{2B} receptor in adenosine-mediated Ca²⁺ mobilisation, alloxazine was utilised. The experiments reported here demonstrated that 100 μ M concentrations of alloxazine completely ablated adenosine-elicited Ca²⁺ mobilisation in mBMMC. This result is in keeping with other studies that reportedly required relatively high concentrations of alloxazine to inhibit the effects of adenosine (Németh *et al.*, 2005). However, the antagonistic effects observed with

alloxazine needs to be interpreted with caution as a previous pharmacology study has shown it can also antagonise the murine A_{2A} receptor and is only ~9-fold more selective for the A_{2B} receptor (Brackett and Daly, 1994). These results potentially implicate the involvement of the A_{2B} receptor in adenosine-mediated signalling in the mBMMC used here. This correlates with the pharmacological profile of A_{2B} receptor activation, and the RT-PCR data where the A_{2B} receptor was readily detectable.

To assess the contribution of the A₃ receptor to adenosine-mediated Ca²⁺ mobilisation, A₃ receptor-selective agonists and antagonists were used. IB-MECA is a potent selective agonist of A₃ receptors at concentrations below 1 µM (Fredholm *et al.*, 2001a) and is 50-fold selective for rat A₃ versus either A₁ or A_{2A} receptors (Gallo-Rodriguez *et al.*, 1994; Jacobson *et al.*, 1995). IB-MECA-induced Ca²⁺ mobilisation in mBMMC at concentrations as low as 100 nM, which is consistent with concentrations of IB-MECA required to activate A₃ receptor-mediated Ca²⁺ mobilisation in murine primary lung mast cells (Zhong *et al.*, 2003). This result strongly supports the involvement of the A₃ receptor in adenosine-mediated signalling and also correlates with the RT-PCR and ligand affinity data discussed earlier. To reinforce this conclusion, MRS1523, a compound reported to be a moderately selective antagonist for the rat (but not human) A₃ receptor, with a K_i of 519 nM was used (Li *et al.*, 1998). This antagonist can also bind to A₁ and A_{2A} receptor sub-types (binding to A_{2B} receptors has not been studied) and is only selective for the A₃ receptor at concentrations at or below 1 µM (Li *et al.*, 1998). In this study, MRS1523 inhibited adenosine-induced Ca²⁺ mobilisation in mBMMC at concentrations at or above 1 µM, which is consistent with the inhibitory action of this antagonist in other cell types (Gao *et al.*, 2001). The stimulation of mBMMC with IB-MECA and the antagonism observed using MRS1523, strongly indicate that the A₃ receptor is the dominant P1 receptor sub-type mediating adenosine-induced Ca²⁺ mobilisation. This also correlates with the RT-PCR data and adenosine affinity profile of mBMMC. Furthermore, this is also concordant with other studies using murine mast cells where A₃ receptors are responsible for the effects seen with adenosine stimulation (Reeves *et al.*, 1997; Tilley *et al.*, 2000; Tilley *et al.*, 2003; Zhong *et al.*, 2003).

The signal transduction mechanisms of adenosine-activated mBMMC were also investigated to help further determine the sub-types of P1 receptors activated. It is generally regarded that A₁ and A₃ receptors couple to G proteins that inhibit adenylyl cyclase, and A_{2A} and A_{2B} receptors couple to G proteins that stimulate adenylyl cyclase

(Fredholm *et al.*, 2001b; Zhou *et al.*, 1992). In this study, adenosine stimulated adenylyl cyclase and coupled to a PLC-dependent increase in cytosolic Ca^{2+} levels. Hence, it is likely that activation of the $\text{A}_{2\text{B}}$ receptor is occurring in parallel with A_3 receptor activation. Potentially, activation of $\text{A}_{2\text{B}}$ receptor-subtypes that stimulate adenylyl cyclase can mask the inhibitory effects of A_3 receptor activation on adenylyl cyclase accumulation. This result re-enforces the concept that adenosine is signalling *via* more than one P1 receptor, most likely the $\text{A}_{2\text{B}}$ and A_3 receptors.

The use of the PLC antagonist U73122 demonstrated that PLC activation was required for the adenosine-induced increase in cytosolic Ca^{2+} levels in mBMMC. However, in the absence of extracellular Ca^{2+} , no increase in cytosolic Ca^{2+} levels was observed. This result was surprising, as PLC activation is required for the production of IP_3 which functions to mobilise Ca^{2+} from intracellular stores such as the endoplasmic reticulum. This results in the release of Ca^{2+} into the cytosol that would be detected by Fura-2. It is likely that the amount of Ca^{2+} mobilised was too small to detect, but sufficient to induce the entry of Ca^{2+} ions from the extracellular environment. This probably occurs *via* stimulation of cell surface store-operated Ca^{2+} channels, which are activated to open when intracellular stores of Ca^{2+} are depleted (Lewis, 2007).

Inosine is known to bind to heterologously expressed recombinant rat A_3 receptors in the range of 10-50 μM , and couple to Ca^{2+} mobilisation and inhibition of adenylyl cyclase (Jin *et al.*, 1997). However, in the experiments performed here, inosine could not induce Ca^{2+} mobilisation or alter the basal level of cAMP in mBMMC. This was unexpected, as the use of IB-MECA (the A_3 receptor agonist) definitively confirmed active A_3 receptors were present on the mBMMC surface, and RT-PCR showed the receptor was expressed. Potentially, the A_3 receptor expressed by mBMMC may not respond to inosine. Alternatively, inosine may bind to A_3 receptors on mBMMC, but not activate a PLC-dependent signalling pathway that leads to an increase in intracellular Ca^{2+} . The differential responsiveness of the A_3 receptor to adenosine and inosine potentially suggests that the receptor can couple to alternate signal transduction pathways depending on the ligand bound.

To determine whether *T. spiralis* secreted proteins (ES) could modulate mBMMC P1 receptor activation, adenosine and ES were applied to mBMMC and cytosolic Ca^{2+} levels monitored. Previous biochemical analysis of *T. spiralis* ES has confirmed the presence of a secreted adenosine deaminase (ADA) capable of deaminating adenosine to inosine and ammonia (Gounaris, 2002). *T. spiralis* ES

inhibited $\leq 10 \mu\text{M}$ concentrations of adenosine mediating mBMMC activation in terms of adenylyl cyclase stimulation and cytosolic Ca^{2+} mobilisation. This effect could be attributed to the action of the *T. spiralis* ADA which would deaminate adenosine to inosine. The accumulation of inosine would not induce increased levels of intracellular Ca^{2+} or stimulate adenylyl cyclase in mBMMC. Stimulation of mBMMC with higher ($100 \mu\text{M}$) concentrations of adenosine was not altered in the presence of ES, indicating adenosine levels were still sufficiently high enough ($\geq 1 \mu\text{M}$) to activate $\text{A}_3/\text{A}_{2\text{B}}$ receptors. Considering that the specific activity of the adenosine deaminase in *T. spiralis* ES is relatively low (51 mU/mg of ES, where 1 unit is equivalent to the deamination of $1 \mu\text{mol}$ adenosine/minute) (Gounaris, 2002), this could account for the continued mobilisation of Ca^{2+} observed when $100 \mu\text{M}$ concentrations of adenosine was applied in the presence of ES. The results displayed here have definitively shown that *T. spiralis* secreted proteins can modulate mBMMC activation by altering the amount of adenosine available to interact with P1 receptors. Secretion of adenosine deaminase by *T. spiralis* during the enteral stage of infection may function to interfere with adenosine-mediated signalling in host mucosal mast cells. This could interfere with mast cell effector functions mediated by adenosine, and potentially lead to the enhanced survival of the parasite.

To characterise the specific P2Y receptors mediating the effects of adenine and uridine nucleotides, a range of general and selective P2Y receptor antagonists were used. The mBMMC used in this study responded to low (100 nM) concentrations of ADP. Non-selective P2Y receptor antagonists PPADS and suramin blocked ADP-mediated signalling, confirming the involvement of P2Y receptors. This also indicated that ADP was not hydrolysed by cell surface ecto-nucleotidases to adenosine which could in turn activate P1 receptors. ADP is known to signal via murine P2Y_1 , P2Y_{12} and P2Y_{13} receptors (Tokuyama *et al.*, 1995; von Kugelgen *et al.*, 2001; Zhang *et al.*, 2002), so receptor selective antagonists were used to further define the involvement of these receptor sub-types. The recently described P2Y_{12} receptor antagonist MRS2395 (Xu *et al.*, 2002) failed to inhibit ADP-induced signalling when used at concentrations below 1 mM . Considering that MRS2395 has previously been shown to inhibit ADP-induced aggregation in rat platelets with a K_i of $3.1 \mu\text{M}$ (Xu *et al.*, 2002), it is likely that the antagonistic results obtained using 1 mM concentrations of MRS2395 was due to non-selective P2Y receptor inhibition. This indicates the P2Y_{12} receptor does not mediate the

effects of ADP, which correlates with the RT-PCR data where P2Y₁₂ receptor mRNA was not detected.

The P2Y₁ receptor antagonist, A3P5PS, was subsequently used to identify a role for the P2Y₁ receptor in ADP-mediated signalling. Previously, A3P5PS has been found to block turkey erythrocyte P2Y₁ receptor-mediated responses (Boyer *et al.*, 1996) and inhibit ADP (10 μ M)-induced shape change of human platelets when used at concentrations of 300 μ M (Jin *et al.*, 1998). Concordant with these studies, A3P5PS was found to partially inhibit ADP-mediated responses in mBMMC when used at 100 μ M concentrations and completely blocked Ca²⁺ mobilisation when used at 1 mM concentrations, indicating the involvement of the P2Y₁ receptor.

To further confirm ADP was signalling via the P2Y₁ receptor, the more selective, competitive, P2Y₁ receptor antagonist MRS2179 was utilised. Pharmacologically relevant (10 μ M) concentrations of MRS2179 (Boyer *et al.* 1998; Waldo *et al.* 2002) completely blocked 1 μ M and 10 μ M concentrations of ADP-mediated Ca²⁺ mobilisation. Considering that MRS2179 is known to be unresponsive at the P2Y₂, 4, 5, 12 and 13 receptors (von Kugelgen, 2006) it can be suggested that the response to ADP is mediated by the P2Y₁ receptor. Overall, these data confirm the involvement of the P2Y₁ receptor and also correlates with the RT-PCR results where P2Y₁ receptor mRNA was readily detectable.

Upon activation by ADP, P2Y₁ receptors couple *via* G_q proteins, leading to stimulation of PLC followed by increases in inositol phosphates and mobilisation of intracellular Ca²⁺ (von Kugelgen and Wetter, 2000). In concordance with the usual intracellular signalling pathway activated by P2Y₁ receptors, ADP at concentrations at or above 100 nM mediated PLC-dependent Ca²⁺ mobilisation in mBMMC. Slightly higher, 1 μ M concentrations of ADP were not totally dependent on PLC activation, potentially due to the incomplete inhibition of PLC by the antagonist U73122. Concentrations of ADP above 1 μ M also stimulated adenylyl cyclase. This result correlates with a similar study using human cord blood-derived mast cells that reported that comparable concentrations of ADP initiated Ca²⁺ fluxing and adenylyl cyclase activation *via* the P2Y₁ receptor (Feng *et al.*, 2004). The majority of intracellular Ca²⁺ mobilised by ADP in mBMMC was due to the influx of extracellular Ca²⁺. This suggests ADP activation of the P2Y₁ receptor induced rapid release of Ca²⁺ from the endoplasmic reticulum store, followed by a slow and sustained entry of extracellular Ca²⁺ through cell surface store-operated Ca²⁺ channels. This is consistent with other

studies that have reported store operated calcium channels in the plasma membrane of mast cells (Chang *et al.*, 2006; Prakriya and Lewis, 2003). As expected, AMP did not induce calcium mobilisation in mBMMC as there is no known receptor for AMP (Abbracchio *et al.*, 2005).

The nucleotide metabolising properties of *T. spiralis* ES are postulated to also alter the amount of ADP available to activate purinergic receptors. Consistent with this hypothesis, ES and the recombinant 5'-nucleotidase (5'-NT) completely inhibited ADP (1 μ M)-induced intracellular Ca^{2+} mobilisation in mBMMC. This result was replicated using the recombinant 5'-NT strongly suggesting that this was due to the specific-action of the 5'-NT which hydrolysed ADP to adenosine thus preventing activation of mBMMC P2Y₁ receptors. This also resulted in the accumulation of sub-micromolar concentrations of adenosine which were insufficient to activate A₃/A_{2B} receptors. This effect was concentration dependent, as ES could not inhibit higher concentrations of ADP (>1 μ M) activating purinergic receptors that couple to Ca^{2+} mobilisation or adenylyl cyclase stimulation.

The effect of ES and 5'-NT on ADP-induced mBMMC receptor activation was investigated further using P2Y₁ and A₃ receptor antagonists. Inhibition of A₃ receptors using MRS1523 completely ablated Ca^{2+} mobilisation in mBMMC stimulated with 10 μ M ADP in the presence of ES. The prevention of P2Y₁ receptor activation indicates that the *T. spiralis* ES degraded all available ADP, resulting in the formation of adenosine which then activated A₃ receptors on mBMMC. Although Ca^{2+} flux induced by 100 μ M concentrations of ADP was not altered by the presence of *T. spiralis* ES, the use of A₃ and P2Y₁ receptor antagonists each partially reduced the level of cytosolic Ca^{2+} mobilised. This suggests that the *T. spiralis* ES did not degrade all of the available ADP, which would account for the small Ca^{2+} flux observed when the A₃ receptor was blocked. Furthermore, adenosine accumulated to sufficient levels to also activate the mBMMC A₃ receptor. To characterise the specific involvement of the *T. spiralis* 5'-NT in mediating the degradation of ADP, the experiment was repeated using the recombinant protein. Application of ADP in the presence of the 5'-NT and purinergic receptor antagonists showed the continued Ca^{2+} mobilisation was due to stimulation of A₃ receptors by adenosine. This confirms that the *T. spiralis* 5'-NT is the enzyme in *T. spiralis* ES responsible for ADP hydrolysis.

As discussed earlier, *T. spiralis* ES can also inhibit adenosine-mediated mBMMC stimulation, putatively *via* the action of the adenosine deaminase (ADA). The

specific involvement of the ADA in the pathway of ADP hydrolysis in mBMMC activation is not fully clear. However, the results suggest that the rate of adenosine accumulation due to the metabolism of ADP by the 5'-NT occurs more rapidly than the rate of deamination of adenosine to inosine by the ADA. This is consistent with the ADA having a lower specific activity and rate of substrate hydrolysis than that of the 5'-NT in *T. spiralis* ES (Gounaris, 2002). In summary, *T. spiralis* ES can modulate mBMMC purinergic receptor activation by preventing ADP activating P2Y₁ receptors and inducing stimulation of A₃ adenosine receptors. Potentially, this could significantly perturb ADP-induced mBMMC functions.

Considering mBMMC expressed functional P2Y receptors, it was of interest to determine whether they could also respond to uridine-nucleotides. As expected, UMP did not induce Ca²⁺ mobilisation, which is consistent with there being no known receptors for this nucleotide. UDP can signal *via* the murine P2Y₆ receptor (Lazarowski *et al.*, 2001), the human CysLT1 receptor (Mellor *et al.*, 2001) and the very recently described rat GPR17 receptor (Ciana *et al.*, 2006). Nanomolar concentrations of UDP are required to stimulate cytosolic Ca²⁺ mobilisation in murine P2Y₆ receptors heterologously expressed in human astrocytoma cells (Lazarowski *et al.*, 2001). In contrast, another study has reported that the peak response due to P2Y₆ receptor activation in neurons and glial cells was induced by much higher, 100 µM concentrations of UDP (Calvert *et al.*, 2004). Relatively high (100 µM) concentrations of UDP induced PLC-dependent Ca²⁺ mobilisation in mBMMC, suggesting the mBMMC express functional low-affinity receptors for UDP.

To determine whether UDP was signalling *via* a P2Y receptor the broad-specificity antagonist PPADS was used. PPADS inhibited UDP-induced calcium mobilisation, which is consistent with other studies where similar concentrations of PPADS reduced UDP-induced activation of recombinant human P2Y₆ receptors by 70% (Robaye *et al.*, 1997). Of the P2Y receptors, UDP has been reported to only signal *via* the P2Y₆ receptor (Lazarowski *et al.*, 2001), hence the more selective, P2Y₆ receptor antagonist MRS2578 was used. MRS2578 has an IC₅₀ value of 98 nM at recombinant rat P2Y₆ receptors expressed in human astrocytes (Mamedova *et al.*, 2004). Currently, the competitive nature of this antagonist is unknown as there are no radioligands for the P2Y₆ receptor (Mamedova *et al.*, 2004). MRS2578 completely inhibited Ca²⁺ mobilisation in mBMMC when used at 100 µM concentrations, implying the

involvement of the P2Y₆ receptor. However, P2Y₆ receptor mRNA was not detected by RT-PCR, indicating this receptor is not actively expressed in resting mBMMC.

Nanomolar concentrations of UDP have recently been reported to be able to additionally signal *via* the CysLT1 receptor in human mast cells (Mellor *et al.*, 2001). The competitive CysLT1 receptor antagonist MK-571 can totally inhibit equimolar concentrations of UDP-induced calcium mobilisation in human mast cells, and is reported to have no effect at P2Y₆ receptors (Mellor *et al.*, 2001). MK-571 partially reduced UDP-induced Ca²⁺ mobilisation in mBMMC when used at 100 μM concentrations, suggesting the CysLT1 receptor may be mediating the UDP-induced response. This correlates with the RT-PCR data where CysLT1 receptor mRNA was readily detectable. The CysLT1 receptor PCR products generated using mBMMC cDNA as a template differed from the expected sizes. Partial sequence analysis of the two PCR products produced showed that only one of them corresponded to the CysLT1 receptor. This indicates that mBMMC do express CysLT1 receptor mRNA transcripts, but due to the primers binding non-specifically to the template cDNA, does not show which splice variant they express.

In terms of intracellular signalling pathways activated in mBMMC, UDP signalled *via* a receptor coupled to a PLC dependent mobilisation of intracellular Ca²⁺, and did not couple to stimulation of adenylyl cyclase. This is consistent with either P2Y₆ or CysLT1 receptor activation which are both able to signal through the IP₃ pathway and couple to G-proteins that do not stimulate adenylyl cyclase (Lazarowski *et al.*, 2001; Brink *et al.*, 2003; Capra *et al.*, 2006). All of the intracellular Ca²⁺ mobilised by UDP in mBMMC was due to the influx of extracellular Ca²⁺. This again suggests that receptors that were activated by UDP induced entry of extracellular Ca²⁺ through cell surface store-operated Ca²⁺ channels.

On the basis of the results discussed thus far, it is apparent that UDP is signalling *via* a P2Y receptor, most likely the P2Y₆ receptor and perhaps also the CysLT1 receptor. The dose-response profile of UDP is atypical, indicating that a low affinity receptor is expressed by the mBMMC. GPR17, a Gi-coupled orphan receptor at an intermediate phylogenetic position between P2Y and CysLT receptors, is specifically activated by micromolar concentrations of UDP and induces both adenylyl cyclase inhibition and intracellular Ca²⁺ increases (Ciana *et al.*, 2006). Due to the very recent publication of the GPR17 receptor (Ciana *et al.*, 2006), the expression of this receptor in

mBMMC was not investigated. However, involvement of this receptor in UDP-mediated mBMMC activation cannot be ruled out.

The *T. spiralis* 5'-NT preferentially hydrolyses UDP>>ADP (Gounaris *et al.*, 2004). To investigate whether *T. spiralis* ES and recombinant 5'-NT could prevent UDP-induced receptor activation in mBMMC, UDP was applied to cells in the presence of ES. Interestingly, Ca²⁺ mobilisation in mBMMC was completely abolished. It was postulated that the 5'-NT present in the native ES was responsible for this effect by converting UDP to UMP and P_i, which would not stimulate the cells. This was shown to be highly likely as application of UDP in the presence of recombinant 5'-NT also inhibited UDP-induced Ca²⁺ flux. Notably, the *T. spiralis* ES and recombinant 5'-NT were able to inhibit UDP-induced signalling in mBMMC even though relatively high (100 µM) concentrations of UDP were applied. This can be explained by the fact that mBMMC were insensitive to ten-fold lower concentrations of UDP. This suggests only 10% of the UDP present had to be degraded to prevent receptor stimulation. In the parasitized intestine where extracellular nucleotides released from damaged cells would accumulate to high concentrations, the release of secreted 5'-NT by the parasite could decrease UDP levels to sub-100 µM concentrations. This could be sufficient to prevent activation of intestinal mucosal mast cells. Potentially, this may affect mast cell effector function in a way that would aid parasite survival.

The effect of UTP on Ca²⁺ mobilisation in mBMMC was also characterised as the cells expressed mRNA transcripts corresponding to the P2Y₂ receptor, of which UTP is a principle agonist. UTP at concentrations at and above 10 µM induced Ca²⁺ mobilisation in mBMMC, potentially signalling *via* P2Y₂, or P2Y₄ receptors (Lustig *et al.*, 1993; Suarez-Huerta *et al.*, 2001; Lazarowski *et al.*, 2001 respectively). The profile of Ca²⁺ mobilisation in mBMMC in response to UTP was similar to that observed in human mast cells, where ≥5 µM concentrations of UTP stimulated receptor activation (Feng *et al.*, 2004). As there are currently no P2Y₂ receptor selective antagonists/agonists, previous studies on human mast cells have concluded that UTP signals *via* the P2Y₂ receptor mainly on the basis of RT-PCR data (Schulman *et al.*, 1999; Feng *et al.*, 2004). In light of these studies and the RT-PCR data observed using mBMMC, it is likely that UTP also signals *via* the P2Y₂ receptor in the mBMMC used here. The effects of P2Y₁₄ receptor ligands (such as UDP-glucose, UDP-Galactose, UDP-glucuronic acid and N-acetylglucosamine) on mBMMC activation were not investigated due to time limitations. Further work characterising the effects of these

ligands on P2Y₁₄ receptor activation in mBMMC could prove interesting as mBMMC expressed this receptor.

ATP signals *via* a range of P2Y and P2X receptors. In the experiments reported here, the application of ATP at concentrations at or above 100 nM stimulated a rapid increase in cytosolic Ca²⁺ levels that required PLC activation. This indicates the presence of functional receptors on mBMMC that respond to ATP. This is consistent with other studies that have reported that murine, rat and human mast cells express functional P2Y and/or P2X receptors that respond to ATP (Bulanova *et al.*, 2005; Jaffar and Pearce. 1990; Schulman *et al.*, 1999; Feng *et al.*, 2004).

To aid characterisation of the P2X/P2Y receptor(s) involved in ATP-mediated mBMMC activation, the non-sub-type-selective antagonist suramin was used. ATP-induced Ca²⁺ mobilisation in mBMMC was only completely inhibited by very high-1 mM concentrations of suramin. This suggests the possible involvement of P2X₄ receptors, as they are not inhibited by concentrations of suramin up to 100 µM (Buell *et al.*, 1996). To further determine whether mBMMC express functional P2X receptors, the mBMMC were patch-clamped and stimulated with ATP. Electrophysiological studies (carried out by A. Mathie, G. Sutton and E. Veale) confirmed that ATP elicited current in mBMMC, indicating activation of P2X receptors. Sustained application of ATP to mBMMC resulted in slow desensitisation, indicative of P2X₂, P2X₄ or P2X₇ receptor activation (North, 2002). Ivermectin is known to potentiate ATP elicited responses at the P2X₄ receptor (Khakh *et al.*, 1999) and when used in our experiments greatly enhanced the magnitude and length of ATP-evoked currents in mBMMC. Ivermectin also induced more sustained elevations in ATP-mediated Ca²⁺ mobilisation in mBMMC, further indicating the involvement of P2X₄ receptors. These results are consistent with the RT-PCR data, where P2X₄ receptor mRNA was the most abundant P2X receptor sub-type expressed by the mBMMC.

As P2X₇ receptors have recently been reported to be responsible for a range of effects on mBMMC cultured with IL-3 only (Bulanova *et al.*, 2005), the P2X₇ receptor antagonist BBG was used. BBG did not antagonise ATP-induced cytosolic Ca²⁺ mobilisation at pharmacologically relevant levels (Jiang *et al.*, 2000). This suggests that the P2X₇ receptor is not mediating the response observed using ATP, which also correlates with the RT-PCR data that suggested the P2X₇ receptor was not expressed in mBMMC.

As the expression of P2X₁ receptor mRNA was detected by RT-PCR, it was possible that this receptor was also functional at the mBMMC surface. The data discussed thus far indicates that this is unlikely as P2X₁ receptors are known to be rapidly desensitised by ATP (North, 2002) and can be blocked by suramin (Evans *et al.*, 1995). However, to further investigate any potential involvement of the P2X₁ receptor the semi-selective antagonist TNP-ATP was used. TNP-ATP can antagonise P2X₁ and P2X₃ receptors at nanomolar concentrations and is at least 1000-fold less effective in blocking currents in cells expressing P2X₂, P2X₄, or P2X₇ receptors (Virginio *et al.*, 1998). Concentrations of TNP-ATP at or above 100 μ M were required to inhibit ATP-induced calcium mobilisation in mBMMC, indicating that it is unlikely the P2X₁ receptor mediates ATP signalling.

Application of ATP in the absence of extracellular Ca²⁺ induced a small mobilisation of Ca²⁺ from intracellular stores. However, the ATP-induced increase in cytosolic Ca²⁺ levels in mBMMC occurred mainly due to the influx of external Ca²⁺. In addition to being a stimulus for P2X receptors, ATP is also a ligand for G-protein coupled P2Y receptors, notably P2Y₁, P2Y₂ and P2Y₄. These P2Y receptors can all couple through intracellular transduction pathways to activate PLC (Harden *et al.*, 1995; Schachter *et al.*, 1996, 1997). The increase in cytosolic Ca²⁺ induced by 100 nM concentrations of ATP was dependent on PLC activation. However, Ca²⁺ mobilisation induced by stimulation of mBMMC with higher concentrations of ATP (1 μ M) was only partially dependent on PLC activation. Considering P2X₄ receptors operate as cation-selective channels permeable to Ca²⁺ (Soto *et al.*, 1996), activation by ATP directly allows Ca²⁺ to enter the cell, acting parallel to the PLC response. Potentially, lower concentrations of ATP may be acting *via* P2Y receptors and higher concentrations *via* the P2X₄ receptor. This could explain why PLC activation was not required at all concentrations of ATP tested. Alternatively, the PLC antagonist (U73122) may not have been able to fully inhibit such a strong stimulus. Further characterisation of the intracellular signalling pathways activated by ATP in mBMMC would prove interesting but was beyond the scope of this study.

The secreted proteins of *T. spiralis* exhibit 5'-nucleotidase and apyrase activities, and as such can degrade nucleoside 5'-diphosphates and nucleoside 5'-monophosphates. In contrast, no ATPase activity against any nucleoside 5'-triphosphates has been detected in *T. spiralis* ES (Gounaris, 2004). However, *T. spiralis* larvae also secrete serine/threonine kinases and a nucleoside diphosphate kinase (NDPK) (Arden *et al.*,

1997; Gounaris *et al.*, 2001). These enzymes would utilise ATP, and the NDPK would convert ATP to ADP or UTP to UDP in the presence of nucleoside phosphate acceptors. To investigate the potential modulatory effects of *T. spiralis* ES on ATP-induced mBMMC activation, ATP was applied to mBMMC in the presence of ES. ATP-induced mobilisation of cytosolic Ca^{2+} in mBMMC was not affected by *T. spiralis* ES, which correlates with the lack of known ATPase activity present in ES.

In summary, mast cells of a mucosal phenotype were successfully derived from murine bone marrow. These mBMMC expressed functional $\text{A}_{2\text{B}}$, A_3 , P2Y_1 , P2Y_2 and P2X_4 receptors. The P2Y_6 and CysLT1 receptors were also putatively active at the mBMMC surface. Expression of these purinergic receptor sub-types enabled the mBMMC to respond to nanomolar concentrations of ADP and ATP, micromolar concentrations of adenosine and UTP, and 100 μM concentrations of UDP. All of the nucleosides/nucleotides discussed induced influx of extracellular Ca^{2+} into the mBMMC. In terms of mast cell-function, the influx of Ca^{2+} is critical for mast cell degranulation (Hua *et al.*, 2007; McCloskey *et al.*, 1999; Gilfillan and Tkaczyk, 2006), indicating adenosine, ADP, ATP, UDP and UTP may directly induce mast cell mediator release. Importantly, *T. spiralis* ES was able to modulate mBMMC purinergic receptor activation by degrading extracellular ADP, UDP and adenosine. Overall, this appeared to generally result in reduced P2Y receptor activation, and increased P1 receptor activation. This result shows for the first time that *T. spiralis* ES can directly modulate mast cell activation.

Chapter 4

Modulation of purinergic receptor-induced mast cell effector functions by *Trichinella spiralis* secreted proteins

4.1 Introduction

It has been known for many years that resolution of the intestinal phase of *Trichinella spiralis* infection is absolutely dependent on mucosal mast cells (Alizadeh and Murrell, 1984; Oku *et al.*, 1984). Although the exact role of mast cells in parasite clearance from the intestine is yet to be fully defined, the effector functions of mucosal mast cells are central to the hosts' mucosal immune response (Pennock and Grencis, 2006). Recent work suggests that mucosal mast cells contribute to intestinal pathology and amplify the protective T_H2 immune response (Lawrence *et al.*, 2004). Thus, the regulation of mast cell effector functions is crucial for efficient parasite expulsion.

Mast cells contain cytoplasmic granules that store several preformed inflammatory mediators, such as TNF- α , histamine, heparin, serotonin, and proteases and other enzymes which are released immediately upon activation of mast cells after cross-linkage of Fc ϵ RI (Benoist and Mathis, 2002; Piliponsky and Levi-Schaffer, 2000). Activation of mast cells also induces *de novo*-synthesis of inflammatory mediators, including a plethora of pro-inflammatory cytokines, T_H2 cytokines, immunoregulatory cytokines, chemokines, prostaglandins and leukotrienes (Benoist and Mathis, 2002; Piliponsky and Levi-Schaffer, 2000; Robbie-Ryan and Brown, 2002; Wedemeyer *et al.*, 2000). During *T. spiralis* infection, the release of these products from mucosal mast cells is thought to have drastic effects on the gut and can result in increased gastrointestinal motility, leukocyte recruitment and activation, and increased epithelial permeability (Khan and Collins, 2004; Broaddus and Castro, 1994; Moqbel *et al.*, 1987; McDermott *et al.*, 2003). It has recently become apparent that mucosal mast cells may also play an important role in amplifying the mucosal T_H2 immune response during *T. spiralis* infection by releasing pre-formed and *de novo* synthesized mediators (Lawrence *et al.*, 2004). The release of mediators such as TNF- α , amplify and perpetuate inflammatory responses through the recruitment of other cells (Wedemeyer *et al.*, 2000; Yu and Perdue, 2001; Aoki *et al.*, 1999; Stenton *et al.*, 1998). TNF- α and mMCP-1 production by mucosal mast cells are also thought to contribute heavily to the intestinal pathology observed during *T. spiralis* infection (Lawrence *et al.*, 2004)

Mast cells from a variety of sources are known to express a range of P1, P2Y and P2X receptors (Feng *et al.* 2004; Bulanova *et al.* 2005). Extracellular nucleosides and nucleotides such as adenosine, ADP, ATP, UTP and UDP can directly activate an array of mast cell effector functions. For example, inosine and adenosine acting *via* the

A₃ receptor can directly induce degranulation (Jin *et al.*, 1997; Zhong *et al.*, 2003) and prevent apoptosis of mast cells (Gao *et al.*, 2001). ATP can induce the release of β -hexosaminidase and leukotrienes from mast cells (Sudo *et al.*, 1996; Nakamura *et al.*, 1989; Saito *et al.*, 1991b). Furthermore, high (1 mM) concentrations of ATP signalling *via* the P2X₇ receptor can directly stimulate pro-inflammatory cytokine production and induce apoptosis in mast cells (Bulanova *et al.*, 2005). UDP acting *via* the CysLT1 receptor can induce pro-inflammatory cytokine production in mast cells primed with IL-4 (Mellor *et al.*, 2002). The P2Y receptor ligands, ADP, ATP and UTP are also effective chemoattractants for rat mast cells (McCloskey *et al.*, 1999).

In many scenarios, nucleosides and nucleotides acting *via* purinergic receptors do not directly stimulate immune cells, but ‘fine-tune’ immune responses already activated by other stimuli (La Sala *et al.*, 2003). Activation of purinergic receptors on mast cells can modulate many different effector functions induced in response to other stimuli. For example, stimulation of the A₃ receptor by adenosine results in amplification of Fc ϵ RI-mediated degranulation and cytokine production (Laffargue *et al.*, 2002). ATP can also enhance anti-IgE-mediated histamine release from mast cells (Schulman *et al.*, 1999). In other situations, nucleotides can inhibit mast cell effector functions. For example, ADP and ATP acting *via* P2Y receptors can inhibit cytokine generation in response to stimulation with peptidoglycan and leukotriene D₄ (Feng *et al.*, 2004). Although these studies are not all representative of mucosal mast cells, taken together they support the concept that mast cells in general express multiple purinergic receptor sub-types that can activate and modulate a range of mast cell effector functions. This is of significance when considering *T. spiralis* ES contains nucleotide metabolising enzymes which can alter mBMMC purinergic receptor activation (as discussed in Chapter 3).

During the intestinal stage of *T. spiralis* infection, infective larvae invade the epithelium, moult, copulate and release new born larvae, prior to expulsion from the intestine. The invasion process damages cells of the epithelium and is expected to liberate high concentrations of nucleotides and nucleosides into the extracellular environment. The faster and more efficient the host immune response is during this stage of the infection, the quicker the parasites are expelled, and the lower the chance of infective larvae successfully carrying out their life-cycle (Wassom *et al.*, 1983; Goyal and Wakelin, 1993; Brown *et al.*, 2003). Mast cells are a crucial component of the host immune response at this stage of the infection, and are required for efficient parasite

expulsion (Ha *et al.*, 1983; Donaldson *et al.*, 1996; Knight *et al.*, 2000). Potentially, secretion of nucleotide metabolising enzymes by the parasite may serve to limit the activation of purinergic receptors on mucosal mast cells by hydrolysis of extracellular nucleosides and nucleotides. This could benefit parasite survival by restricting the activation of mast cell effector functions, which would slow-down the ensuing inflammatory response, potentially prolonging parasite persistence in the intestinal tract.

As shown in chapter 3, mBMMC displaying a mucosal phenotype express functional P1, P2Y and P2X receptors that can be activated by a range of nucleosides and nucleotides. The aim of this chapter, therefore, was to investigate whether nucleosides and nucleotides could induce or modulate mast cell effector functions *in vitro* and determine whether *T. spiralis* secreted proteins could perturb these effects. The effects of nucleosides and nucleotides on major mucosal mast cell effector functions, such as degranulation, protease secretion, *de novo* cytokine generation and chemotaxis were characterised *in vitro*. The regulatory effects of *T. spiralis* secreted nucleotide metabolising enzymes on the type and availability of extracellular nucleotides able to activate mBMMC effector functions were also investigated.

4.2 Results

4.2.1 Characterisation of mast cell secretory responses to stimulation with nucleosides and nucleotides

Mast cells are densely packed with cytoplasmic granules that store a variety of preformed mediators that have diverse functions. Upon antigen-dependent stimulation, the enzyme β -hexosaminidase (β -hex) is co-released with other pre-formed mast cell effector molecules such as histamine, heparin proteoglycan, chondroitin sulphate E proteoglycan and leukotriene C₄ (Razin *et al.*, 1983; Schwartz *et al.*, 1979, 1981 and 1982). As β -hex is associated with mast cell granules, the activity of this enzyme was measured as a general marker of mast cell degranulation. Murine mast cell protease-1 (mMCP-1) is a crucial effector molecule required for efficient expulsion of *T. spiralis* from the intestine of parasitized animals (Knight *et al.*, 2000). A substantial body of evidence exists to support the hypothesis that TGF- β ₁ plays a central role in mMCP-1 secretion from mast cells by a mechanism that is distinct from antigen-mediated degranulation (Wastling *et al.*, 1997; Miller *et al.*, 1999; Wright *et al.*, 2002). Although a significant proportion of mMCP-1 is secreted by a process that by-passes the secretory granules (Brown *et al.*, 2003) mBMMC can also release mMCP-1 when stimulated with general mast cell secretagogues such as compound 48/80 (De Jonge *et al.*, 2004). Thus, it was deemed pertinent to investigate whether activation of purinergic receptors by nucleosides and nucleotides could induce mMCP-1 release from mBMMC. In order to assess the ability of nucleosides and nucleotides to induce the release of β -hex and mMCP-1, mBMMC were stimulated with a range of concentrations of nucleosides and nucleotides for a short 2-hour period.

4.2.1.1 β -hexosaminidase release from mBMMC

mBMMC that had been washed in DMEM to remove background levels of β -hex activity were resuspended in fresh media and growth factors (IL-3, IL-9, SCF and TGF- β ₁) to maintain cell viability. 2×10^5 mBMMC were subsequently stimulated with a variety of different types of nucleosides and nucleotides at a range of concentrations. The level of β -hexosaminidase activity in mBMMC supernatants and lysed mBMMC were measured to calculate the overall percentage release, which was calculated according to the formula $[S/L] \times 100$, where S and L refer to the absorbance detected in

equal volumes of supernatant and lysed cells respectively, after subtraction of the background absorbance of media alone.

The background level of spontaneous β -hex release from mBMMC was very low, as shown in Figures 4-1, 4-2 and 4-3. This indicates the cells were viable and had not degranulated prior to stimulation with the test agonist. To confirm the mBMMC were responsive to extracellular stimuli, the mBMMC were passively sensitised with anti-DNP IgE and subsequently stimulated with DNP-antigen to induce degranulation. mBMMC were also passively sensitised with anti-DNP IgE alone, to control for any potential effects the addition of IgE could have on the cells. As shown in Figures 4-1, 4-2 and 4-3, the addition of anti-DNP IgE alone did not significantly alter the background level of spontaneous β -hex released from mBMMC. Cross-linkage of mBMMC Fc ϵ RI using anti-DNP IgE and DNP antigen induced a 29.6 ± 5.9 % increase in β -hex release (Figures 4-1, 4-2 and 4-3), indicating that the mBMMC have functional Fc ϵ RI. To confirm the responsiveness of mBMMC to non-antigen-dependent stimulation, the non-specific, polyamine mast cell secretagogue compound 48/80 (c48/80) was also used as an additional positive control. c48/80 is thought to activate mast cells by directly stimulating G $_i$ proteins (Mousli *et al.*, 1989, 1990) and can induce degranulation resulting in β -hex release. As shown in Figures 4-1, 4-2 and 4-3, c48/80 induced high levels of β -hex release (59.7 ± 10.7 %), further confirming the responsiveness of mBMMC to external stimuli.

The nucleosides inosine and adenosine did not significantly alter β -hex release at any of the concentrations tested (Figures 4-1, (A) and (B) respectively). This indicates inosine and adenosine cannot activate intracellular signalling pathways that induce mBMMC degranulation. As displayed in Figure 4-2, the adenine nucleotides ADP and ATP could not induce β -hex release when used at concentrations ranging from 1 to 10 μ M. However, at concentrations of 50 μ M and 100 μ M, both ADP and ATP induced small, but statistically significant increases in β -hex release (Figure 4-2 (A) and (B) respectively). This suggests that adenine nucleotides at high micromolar concentrations can weakly induce exocytosis in mBMMC. Both ADP and ATP induced concentration dependent β -hex release from mBMMC, and this effect was maximal when 1 mM concentrations were used ($37.6 \pm 12.9\%$ and $32.4 \pm 7.2\%$ for ADP and ATP respectively). These results suggest binding of ADP and ATP to purinergic receptors expressed on mBMMC, can couple to intracellular signal transduction pathways that directly stimulate mBMMC degranulation. In contrast, the uridine

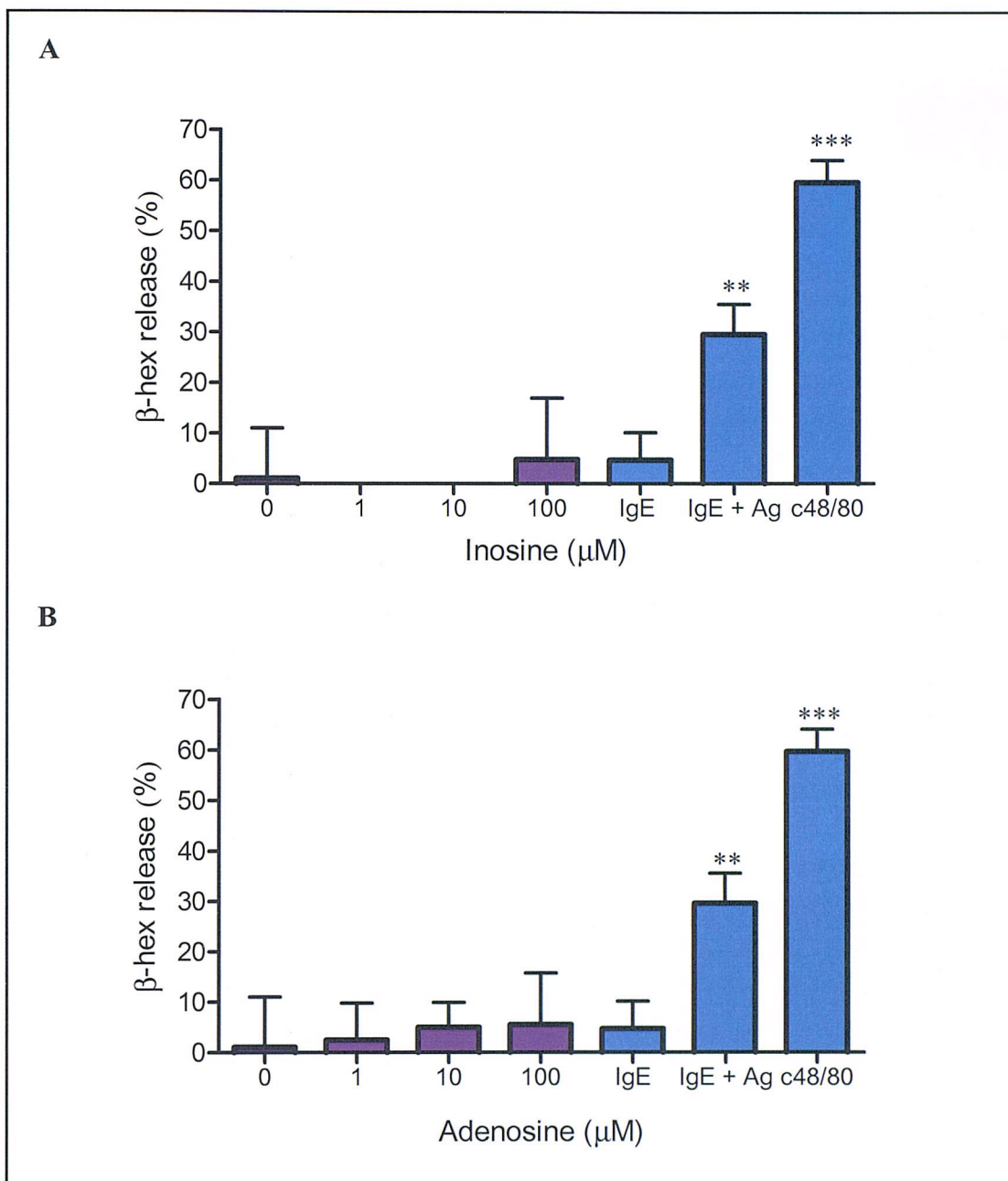


Figure 4-1 β-hexosaminidase release from mBMMC in response to nucleosides

mBMMC were incubated for two hours with the indicated concentrations of (A) inosine and (B) adenosine. Released β-hexosaminidase (β-hex) was measured as described in the 'Materials and Methods' section and is displayed as a percentage relative to total cell content. 50 μg/ml of the non-specific secretagogue c48/80 and stimulation of the FcεRI receptor by crosslinking IgE and antigen (IgE + Ag) were used as positive controls. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone *** $p \leq 0.001$, ** $p \leq 0.01$.

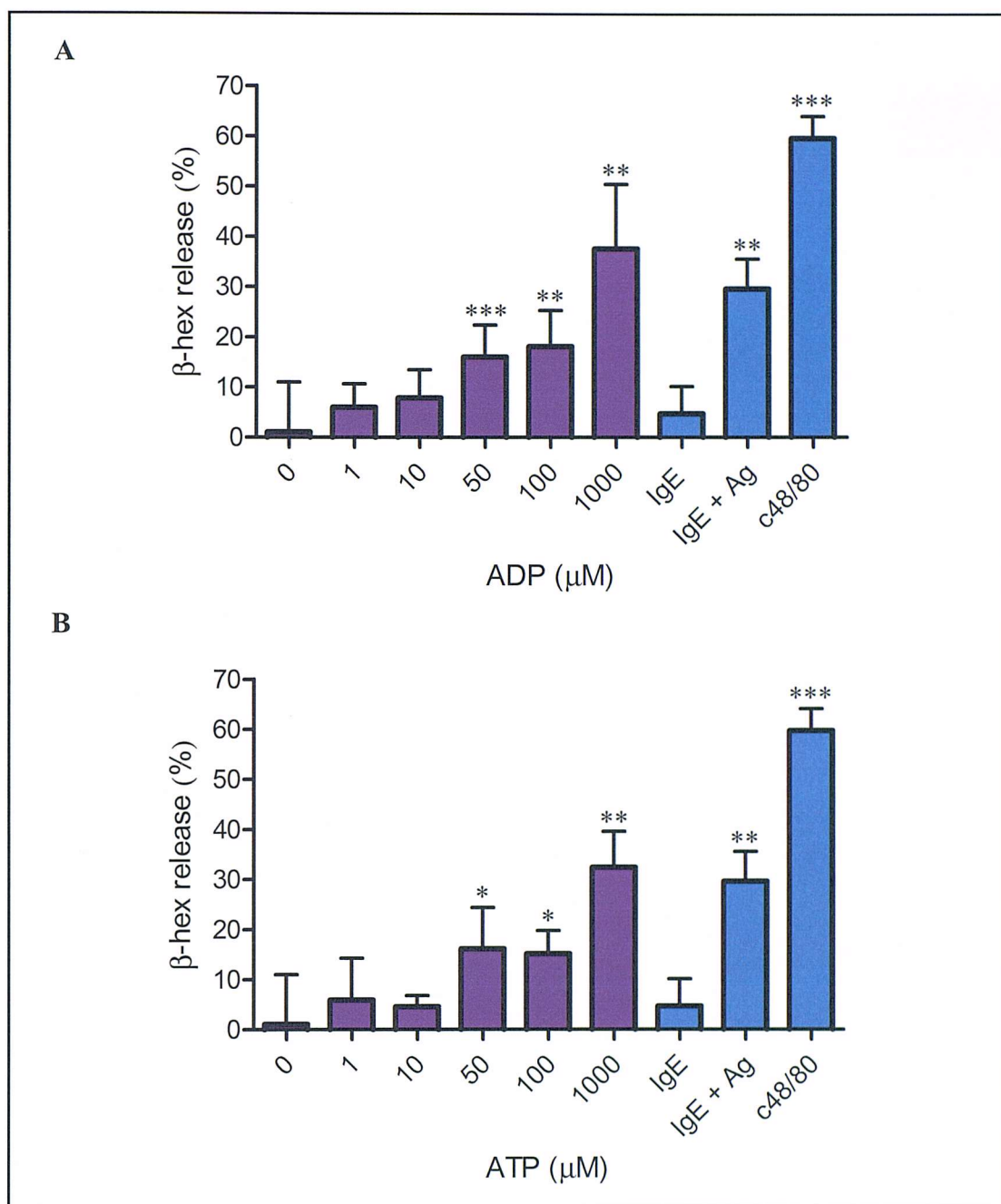


Figure 4-2 β-hexosaminidase release from mBMMC in response to adenine nucleotides

mBMMC were incubated for two hours with the indicated concentrations of (A) ADP and (B) ATP. Released β-hexosaminidase (β-hex) was measured as described in the 'Materials and Methods' section and is displayed as a percentage relative to total cell content. 50 μg/ml of the non-specific secretagogue c48/80 and stimulation of the FcεRI receptor by crosslinking IgE and antigen (IgE + Ag) were used as positive controls. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone *** $p \leq 0.001$, ** $p \leq 0.01$.

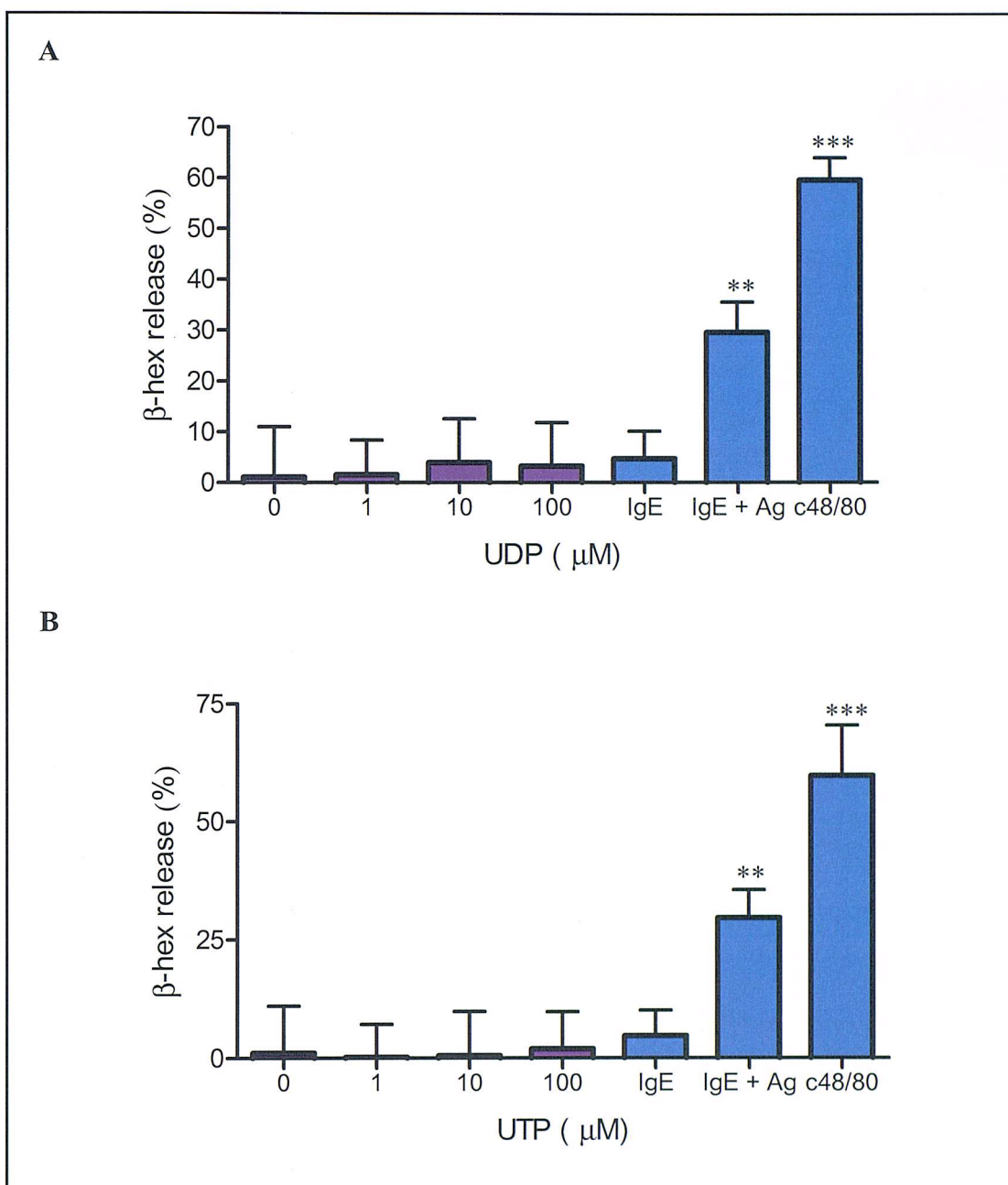


Figure 4-3 β-hexosaminidase release from mBMMC in response to uridine nucleotides

mBMMC were incubated for two hours with the indicated concentrations of (A) UDP and (B) UTP. Released β-hexosaminidase (β-hex) was measured as described in the 'Materials and Methods' section and is displayed as a percentage relative to total cell content. 50 μg/ml of the non-specific secretagogue c48/80 and stimulation of the FcεRI receptor by crosslinking IgE and antigen (IgE + Ag) were used as positive controls. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone *** $p \leq 0.001$, ** $p \leq 0.01$.

nucleotides, UDP and UTP, at concentrations ranging from 1 μ M to 100 μ M did not effect β -hex release (Figure 4-3), indicating that the mBMMC purinergic receptors activated by these nucleotides do not couple to intracellular signal transduction pathways that induce granule exocytosis.

4.2.1.2 mMCP-1 release from mBMMC

To determine whether nucleosides and nucleotides could induce the release of mMCP-1 from mast cell granules, mBMMC cultured in the absence or presence of TGF- β_1 were stimulated with a range of concentrations of inosine, adenosine, ADP, ATP, UDP and UTP. The experiment was initially executed using mBMMC that had been cultured without TGF- β_1 . This was performed to ensure the up-regulatory effect TGF- β_1 has on mMCP-1 expression and secretion did not mask any potential modulatory effects of nucleosides and nucleotides on mMCP-1 release. To remove background levels of mMCP-1 and to maintain cell viability, mBMMC were washed in DMEM and re-suspended in fresh media and growth factors (IL-3, IL-9, SCF +/- TGF- β_1). 2×10^5 mBMMC were subsequently stimulated with a variety of different types of nucleosides and nucleotides at a range of concentrations, and mMCP-1 levels measured by ELISA.

The background level of mMCP-1 release from mBMMC cultured without TGF- β_1 was low (0.9 ± 0.3 ng/ml) (Figures 4-4, 4-5 and 4-6), indicating that the cells were viable and had not degranulated prior to stimulation with the test agonist. Cross-linkage of Fc ϵ RI using anti-DNP IgE and DNP-antigen was used as a positive control. This induced the secretion of 6.6 ± 1.4 ng/ml mMCP-1, which approximately equates to a 7.3 fold increase in mMCP-1 release (Figures 4-4, 4-5 and 4-6). This suggests that mBMMC can release mMCP-1 in response to antigenic stimuli. mBMMC were also passively sensitised with anti-DNP IgE alone, to control for any potential effects the addition of IgE could have on the cells. As shown in Figures 4-4, 4-5 and 4-6, the addition of anti-DNP IgE alone did not significantly alter the background level of spontaneous mMCP-1 released from mBMMC, suggesting IgE alone cannot induce mBMMC degranulation.

As in the case of β -hex release (Figure 4-1), the nucleosides inosine and adenosine did not affect mMCP-1 release (Figure 4-4 (A) and (B) respectively). This implies inosine and adenosine cannot bind purinergic receptors that stimulate the release of mMCP-1-containing granules from mBMMC. In contrast, ADP and ATP induced a

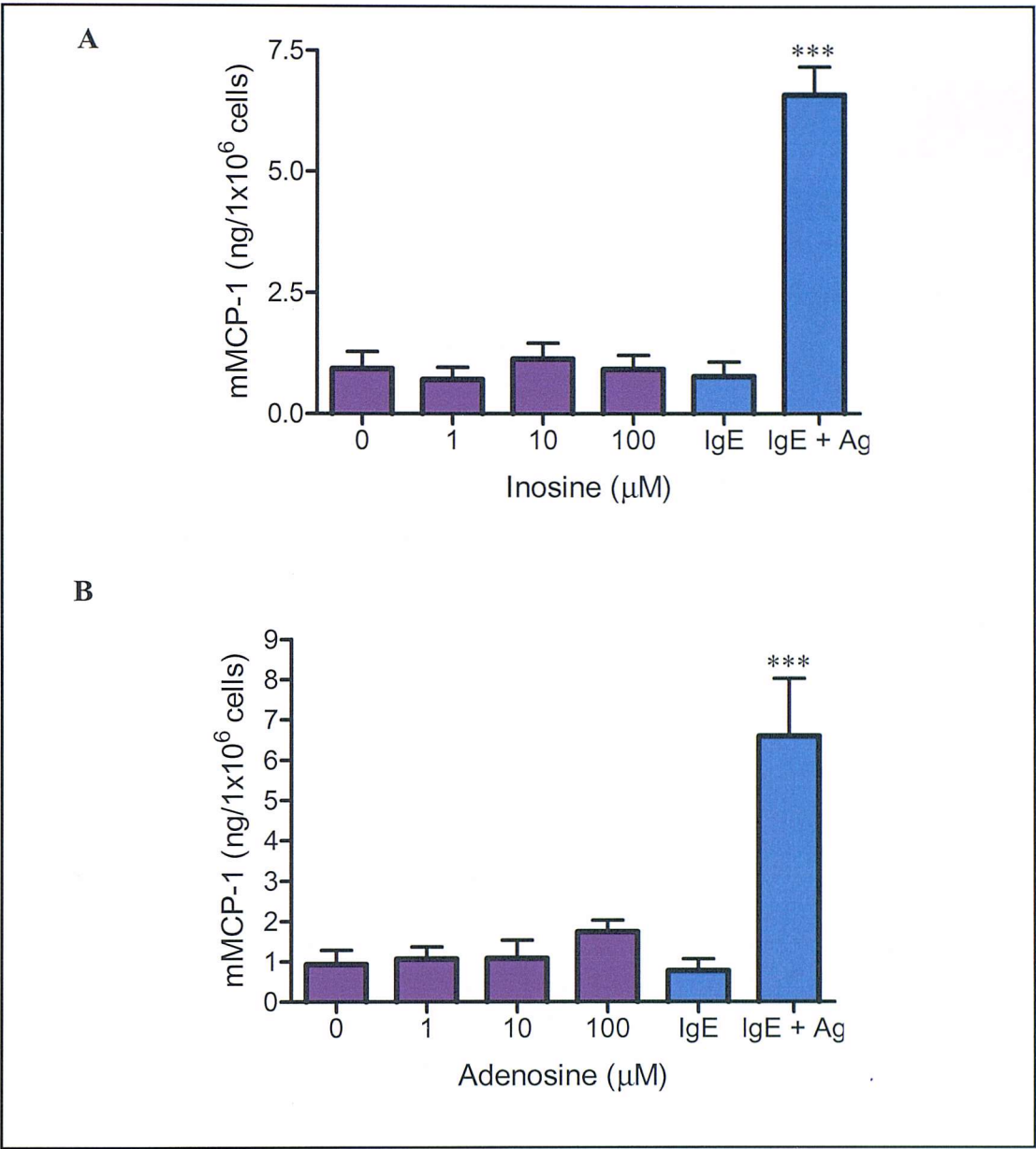


Figure 4-4 mMCP-1 secretion by mBMMC (-TGF-β₁) in response to nucleosides

mBMMC cultured without TGF-β₁ were stimulated with the indicated concentration of (A) inosine and (B) adenosine. Stimulation of the FcεRI receptor by crosslinking IgE and antigen (IgE + Ag) was used as a positive control. Culture supernatants were tested for mMCP-1 by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone, *** *p* ≤ 0.001.

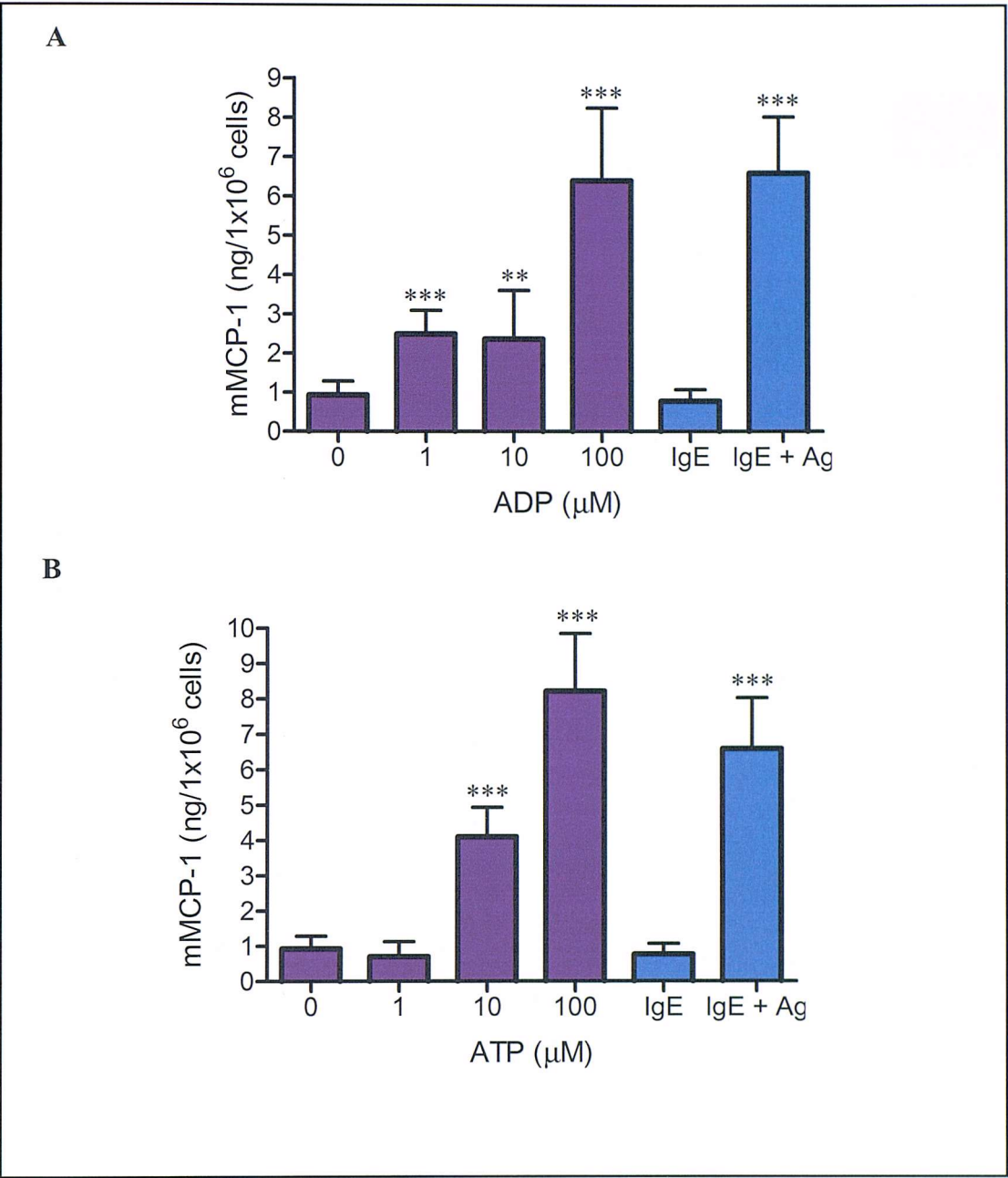


Figure 4-5 mMCP-1 secretion by mBMMC (-TGF-β₁) in response to adenine nucleotides

mBMMC cultured without TGF-β₁ were stimulated with the indicated concentration of (A) ADP and (B) ATP. Stimulation of the FcεRI receptor by crosslinking IgE and antigen (IgE + Ag) was used as a positive control. Culture supernatants were tested for mMCP-1 by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone, *** $p \leq 0.001$, ** $p \leq 0.01$.

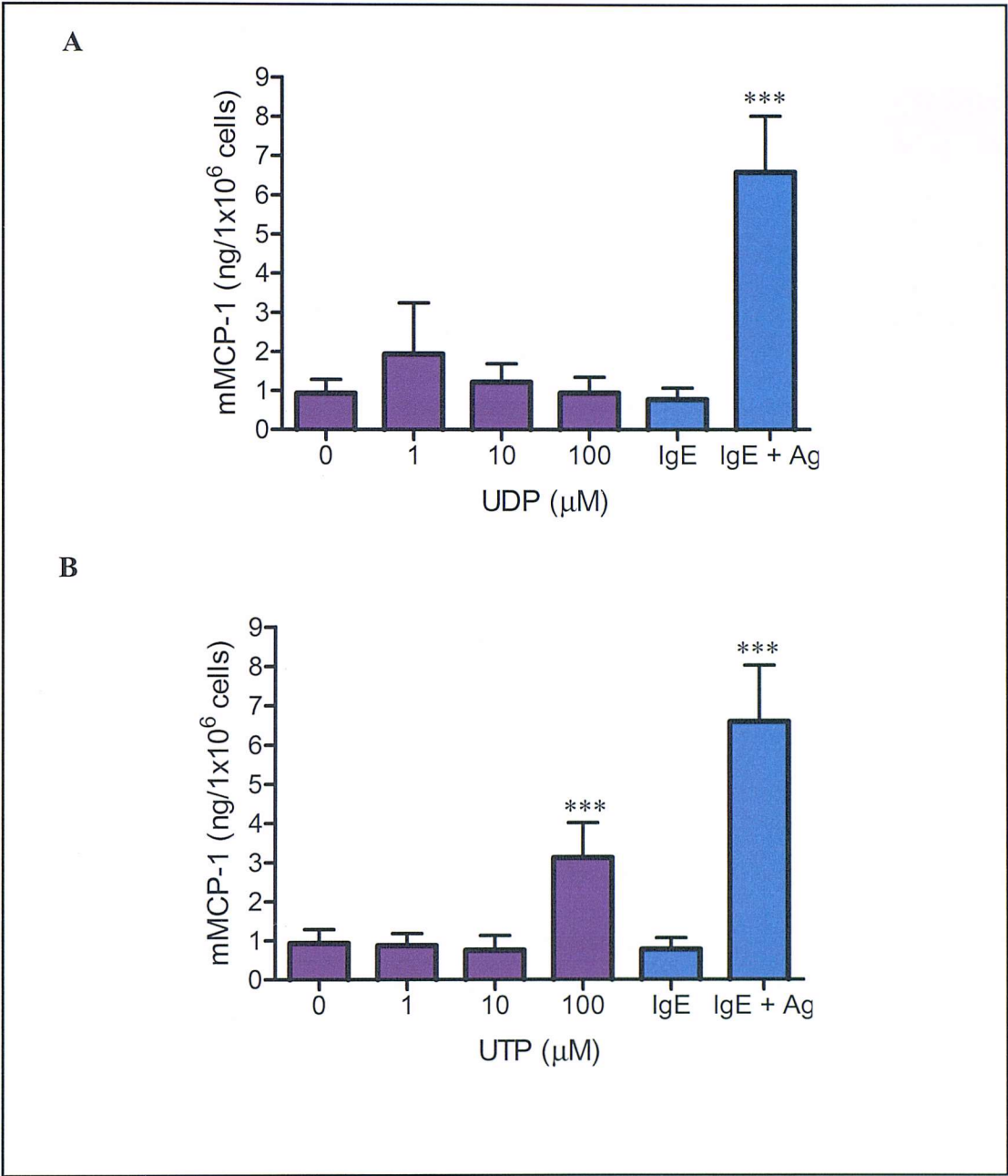


Figure 4-6 mMCP-1 secretion by mBMMC ($-TGF-\beta_1$) in response to uridine nucleotides

mBMMC cultured without $TGF-\beta_1$ were stimulated with the indicated concentration of (A) UDP and (B) UTP. Stimulation of the $Fc\epsilon RI$ receptor by crosslinking IgE and antigen (IgE + Ag) was used as a positive control. Culture supernatants were tested for mMCP-1 by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone, *** $p \leq 0.001$.

dose-dependent increase in mMCP-1 release from mBMMC (Figure 4-5). ADP at concentrations ranging from 1 μ M to 100 μ M induced a statistically significant increase in mMCP-1 secretion [Figure 4-5 (A)]. ATP at concentrations ranging from 10 μ M to 100 μ M also induced a statistically significant increase in mMCP-1 release [Figure 4-5 (B)]. This result suggests ADP and ATP activate purinergic receptors that induce mMCP-1 release. Furthermore, ADP and ATP appear to be more potent stimulators of mMCP-1 secretion than β -hex release (compare Figure 4-2 with Figure 4-5). As shown in Figure 4-6 (A), stimulation of mBMMC with UDP at concentrations ranging from 1 μ M to 100 μ M did not alter mMCP-1 release. In a similar manner, UTP at concentrations ranging from 1 μ M to 10 μ M could not induce increased mMCP-1 release [Figure 4-6 (B)]. However, UTP at 100 μ M concentrations induced a statistically significant increase in mMCP-1 release from mBMMC [Figure 4-6 (B)].

To investigate whether the presence of TGF- β_1 altered the effects of nucleosides and nucleotides on mMCP-1 secretion, the experiments were repeated using mBMMC cultured with TGF- β_1 . Cultures maintained with TGF- β_1 spontaneously secreted significantly higher basal levels of mMCP-1 than mBMMC cultured in the absence of TGF- β_1 (0.27 ± 0.28 μ g/ml and 0.9 ± 0.3 ng/ml respectively) (compare Figure 4-1 with Figure 4-7). This was expected, as TGF- β_1 is known to upregulate mMCP-1 secretion from mBMMC by 100-1000 fold (Miller *et al.*, 1999). Antigen-dependent stimulation of mBMMC induced by cross-linkage of Fc ϵ RI using anti-DNP IgE and DNP-antigen, stimulated an approximately 9.6-fold increase in mMCP-1 release (Figures 4-7, 4-8, 4-9). This shows that antigenic stimulation of mBMMC can induce the release of statistically significant amounts of mMCP-1. The presence of IgE alone did not significantly alter background levels of mMCP-1 secretion (Figure 4-7, 4-8, 4-9), which is consistent with the results obtained using mBMMC cultured without TGF- β_1 .

The addition of inosine and adenosine did not induce increased mMCP-1 secretion from mBMMC [Figure 4-7 (A) and (B) respectively]. This is consistent with the results observed using mBMMC cultured without TGF- β_1 (Figure 4-4) and the β -hex activity assays (Figure 4-1).

As shown in Figure 4-8 (A) and (C), ADP and ATP respectively, at concentrations ranging from 10 μ M to 100 μ M induced a dose dependent increase in mMCP-1 secretion from mBMMC. Again, this correlates with the results observed when mBMMC cultured without TGF- β_1 were used [compare Figure 4-5 (A) and (B) with Figure 4-8 (A) and (C)]. To confirm ADP and ATP were not metabolised by cell-

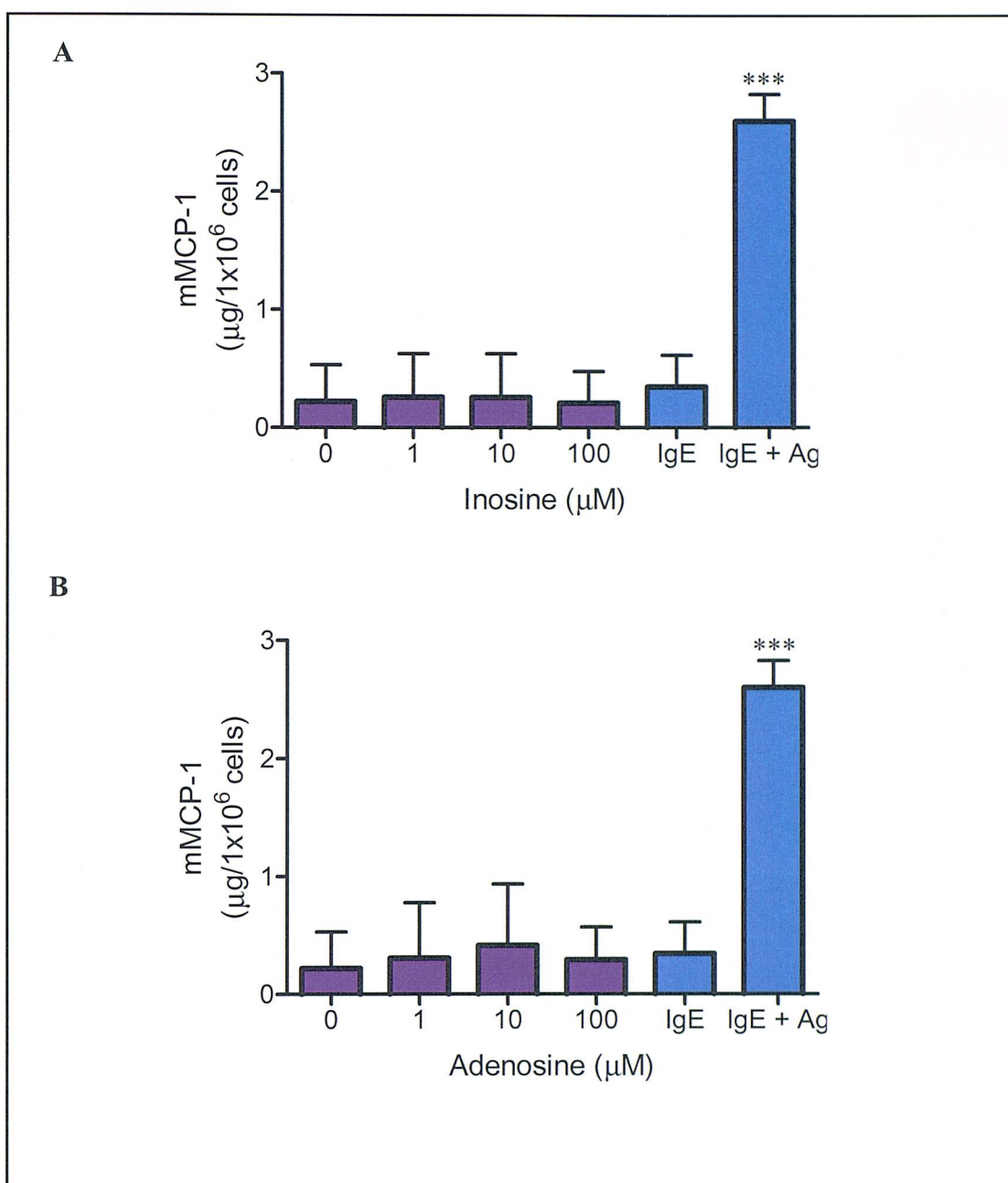


Figure 4-7 mMCP-1 secretion by mBMMC in response to nucleosides

mBMMC cultured with TGF- β_1 were stimulated with the indicated concentration of (A) inosine and (B) adenosine. Stimulation of the Fc ϵ RI receptor by crosslinking IgE and antigen (IgE + Ag) was used as a positive control. Culture supernatants were tested for mMCP-1 by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone, *** $p \leq 0.001$

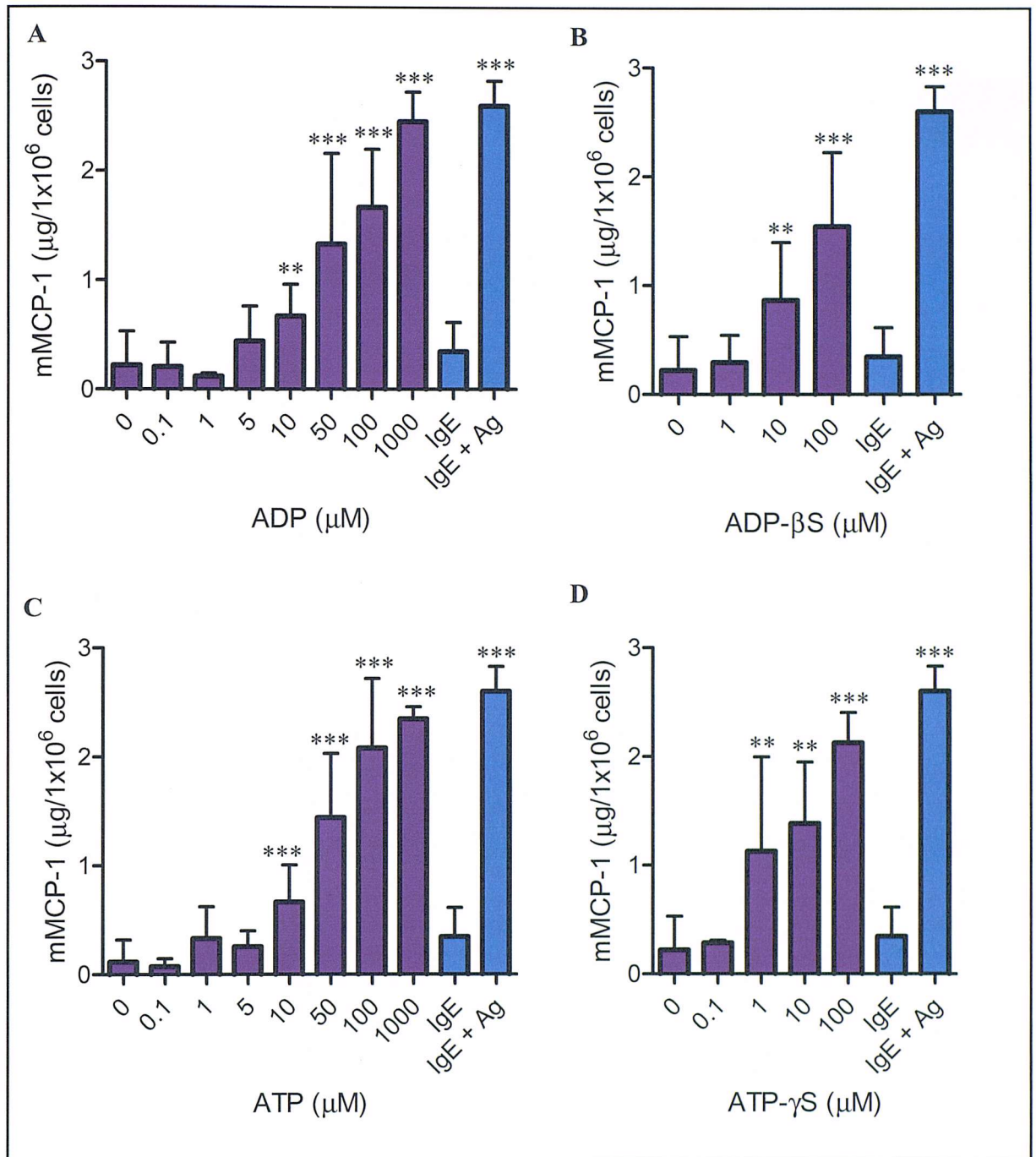


Figure 4-8 mMCP-1 secretion by mBMMC in response to adenine nucleotides and their analogues

mBMMC were stimulated with the indicated concentration of (A) ADP, (B) ADP-βS, (C) ATP and (D) ATP-γS. Stimulation of the FcεRI receptor by crosslinking IgE and antigen (IgE + Ag) was used as a positive control. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

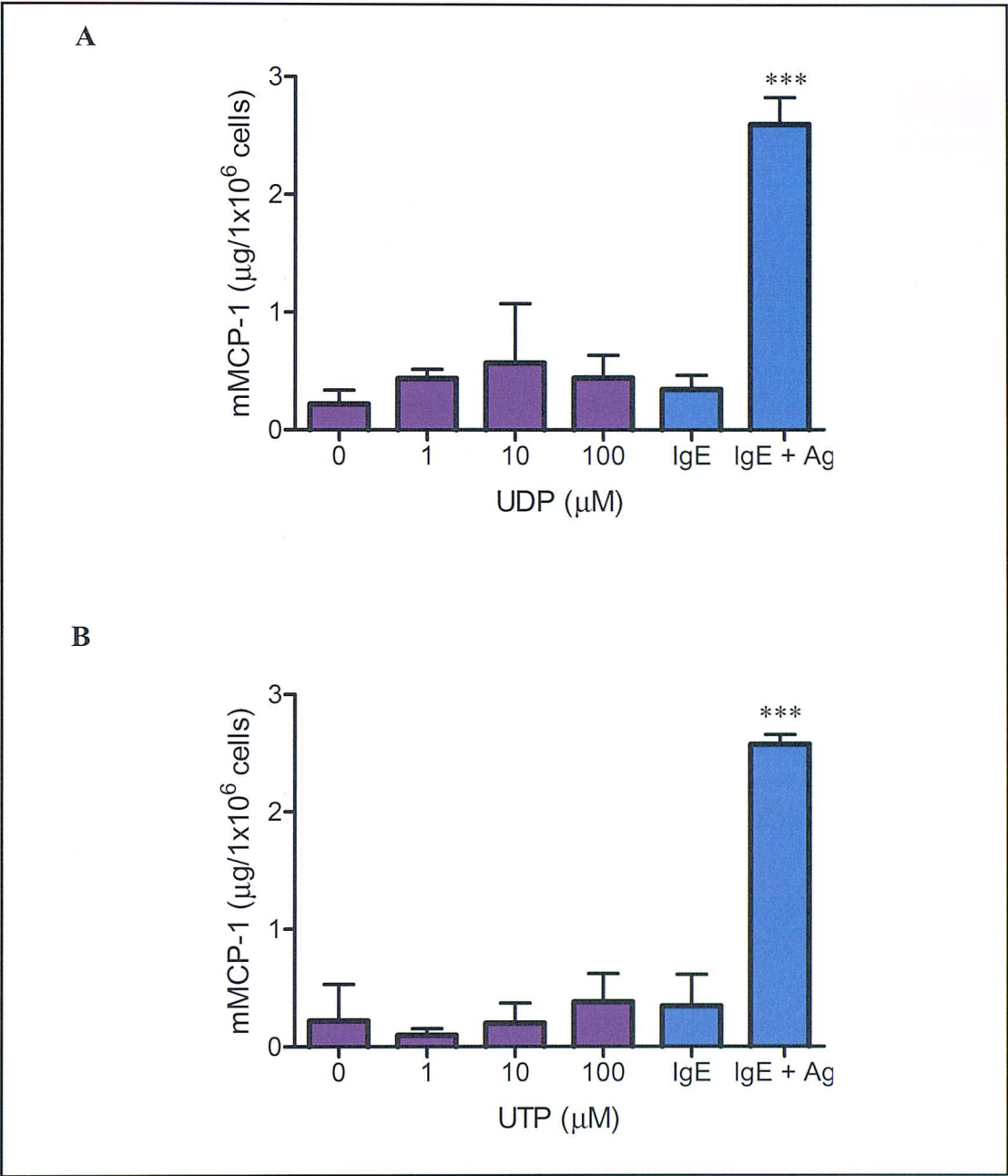


Figure 4-9 mMCP-1 secretion by mBMMC in response to uridine nucleotides

mBMMC were stimulated for 2 hrs with the indicated concentration of (A) UDP and (B) UTP. Stimulation of the FcεRI receptor by crosslinking IgE and antigen (Ig+Ag) was used as a positive control. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone, *** $p \leq 0.001$.

surface ecto-nucleotidases to a product that was responsible for the observed effects, the highly stable, non-hydrolysable nucleotide analogues ADP- β S and ATP γ S were used. Both analogues at concentrations ranging from 10 μ M to 100 μ M induced a dose-dependent increase in mMCP-1 release [Figure 4-8 (B) and (D)], confirming that it was indeed ADP and ATP that induced the mMCP-1 release.

UDP at concentrations ranging from 1 μ M to 100 μ M could not induce increased mMCP-1 secretion from mBMMC [Figure 4-9 (A)]. This correlates with the results observed when UDP was incubated with mBMMC cultured without TGF- β_1 [compare Figures 4-6 (A) with 4-9 (A)]. As shown in Figure 4-9 (B), UTP at concentrations ranging from 1 μ M to 100 μ M could not induce increased mMCP-1 secretion from mBMMC. The effects of UTP on mMCP-1 secretion from mBMMC appears to be inconsistent, as mBMMC cultured without TGF- β_1 appeared to release mMCP-1 in response to high (100 μ M) concentrations of UTP [compare Figure 4-6 (B) with Figure 4-9(B)].

4.2.1.3 Characterisation of P2Y receptor-induced mMCP-1 secretion

As shown in Chapter 3, mBMMC express functional P2Y receptors that are responsive to a wide range of ADP concentrations (100 nM-1 mM). Furthermore, the use of antagonists pin-pointed the P2Y $_1$ receptor as the major P2Y receptor sub-type *via* which ADP was signalling. Considering ADP can induce mMCP-1 release from mBMMC, it was of interest to determine whether this also occurred *via* P2Y $_1$ receptor activation. To investigate this, the non-subtype-selective P2Y receptor antagonists suramin and PPADS, and the P2Y $_1$ -selective receptor antagonist MRS2179 were used. mBMMC were preincubated with the indicated concentrations of antagonist for 10 minutes prior to stimulation with concentrations of ADP that induced statistically significant increases in mMCP-1 release. Unexpectedly, application of the antagonists alone (suramin, PPADS and MRS2179) induced a small but significant increase in the background level of mMCP-1 secretion (Figure 4-10). This could be an artefact of the experimental procedure, or indicate that the antagonists generally do slightly increase the basal level of mMCP-1 secretion. However, the small increase in background mMCP-1 levels did not interfere with the overall purpose of the experiment.

Pre-incubation of mBMMC with suramin or PPADS significantly reduced the amount of mMCP-1 released in response to 10 μ M concentrations of ADP [Figure 4-10 (A) and (B)]. These effects occurred in an ADP-concentration-dependent manner due to

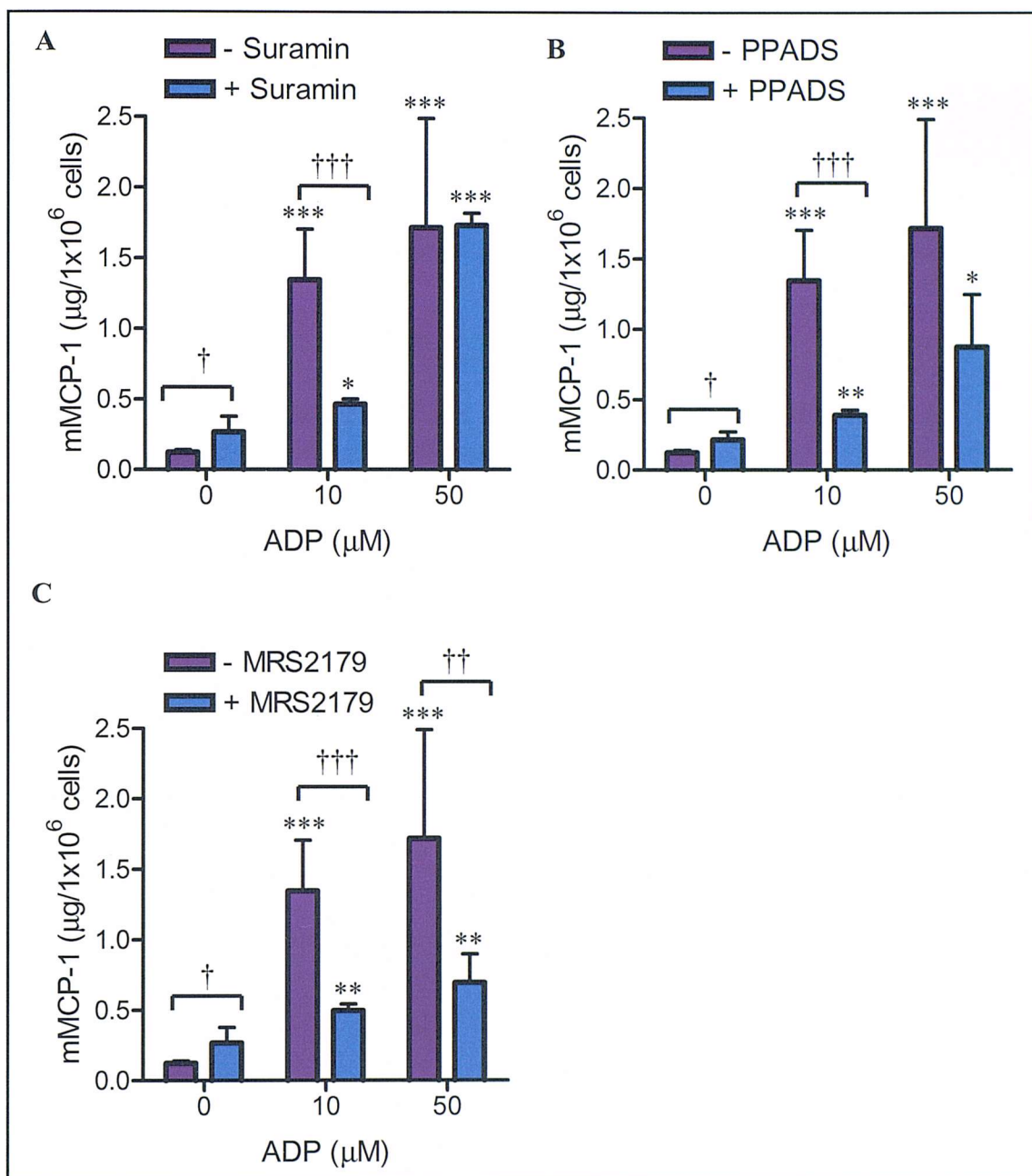


Figure 4-10 ADP induced mMCP-1 secretion by mBMMC treated with P2Y receptor antagonists

mBMMC were pre-incubated for 10 minutes with 100 µM of the general P2Y receptor antagonists (A) suramin and (B) PPADS, and with (C) 100 µM of the P2Y₁ specific antagonist MRS2179. Cultures were subsequently stimulated with the indicated concentration of ADP and cell supernatants were tested for mMCP-1 by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media/antagonist alone *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ and the indicated concentration of ADP in the absence/presence of antagonist, † † † $p \leq 0.001$, † † $p \leq 0.01$, † $p \leq 0.05$.

suramin and PPADS being surmountable antagonists of P2Y receptors. To further characterise *via* which receptor sub-type(s) ADP was signalling, the more specific P2Y₁ receptor antagonist, MRS2179 was utilised [Figure 4-10 (C)]. MRS2179 significantly reduced the level of mMCP-1 released in response to 10 and 50 μ M concentrations of ADP. This indicates ADP is signalling *via* the P2Y₁ receptor, which correlates with the observations reported in Chapter 3.

4.2.1.4 Modulation of adenine nucleotide-induced mMCP-1 secretion by *T. spiralis* secreted proteins and recombinant 5'-NT.

T. spiralis secretes a range of nucleotide-metabolising enzymes which can directly metabolise extracellular ADP, AMP, and adenosine (Gounaris, 2002). As demonstrated in Chapter 3, mBMMC P2Y₁ receptor activation can be modulated by *T. spiralis* excretory/secretory proteins (ES). This effect was attributed to the action of the *T. spiralis* 5'-nucleotidase (5'-NT), which hydrolysed ADP to AMP and AMP to adenosine, thus limiting P2Y₁ receptor activation. *T. spiralis* ES does not contain ATPase activity against any nucleoside 5'-triphosphates (Gounaris, 2002), and thus cannot directly hydrolyse ATP. However, *T. spiralis* larvae also secrete serine/threonine kinases and a nucleoside diphosphate kinase (NDPK) (Arden *et al.*, 1997; Gounaris *et al.*, 2001). The serine/threonine kinases would utilise ATP, and the NDPK would convert ATP to ADP in the presence of nucleoside diphosphate acceptors.

Considering that ADP and ATP both stimulate mBMMC to release mMCP-1, it was deemed important to investigate the potential modulatory effects that *T. spiralis* ES and recombinant 5'-nucleotidase (5'-NT) have on adenine nucleotide-induced mMCP-1 secretion. In order to investigate this, the indicated concentration of nucleotide was applied to mBMMC in the presence of ES or 5'-NT (as described in the Materials and Methods section). Initially, to establish that *T. spiralis* ES and the recombinant 5'-NT do not induce signalling events that lead to mMCP-1 release from mBMMC, they were applied to the mBMMC in the absence of nucleotides. As shown in Figure 4-11, ES and the 5'-NT did not alter the background level of mMCP-1 secretion. The presence of ES significantly reduced the amount of mMCP-1 secreted from mBMMC when the latter were stimulated with 10 μ M concentrations of ADP [Figure 4-11 (A)]. This indicates that *T. spiralis* ES can modulate mBMMC effector functions *in vitro*. Potentially, this effect could be mediated by the secreted 5'-NT, which would hydrolyse ADP to AMP, and AMP to adenosine, thus preventing P2Y₁ receptor activation. The inhibitory effect

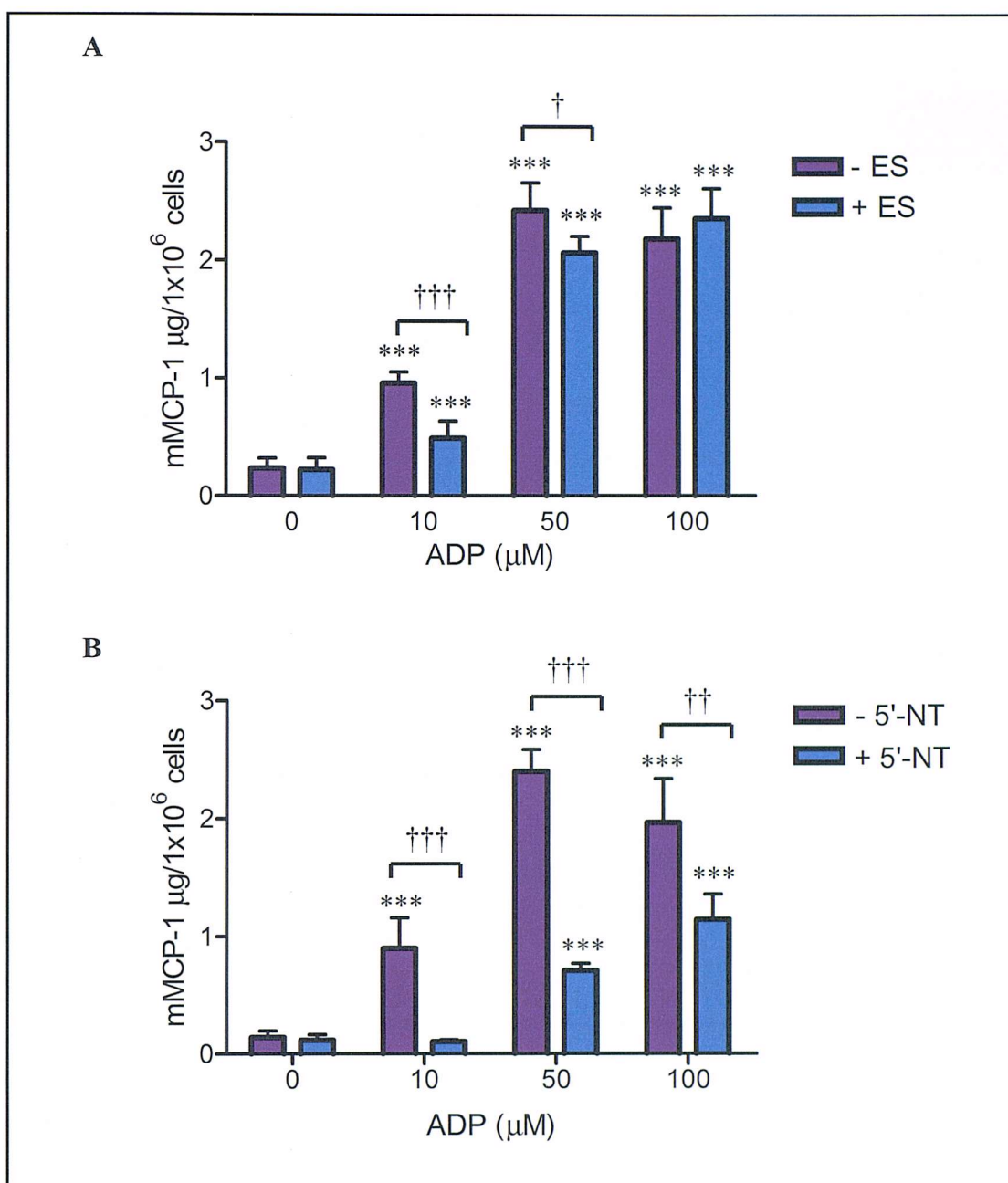


Figure 4-11 mMCP-1 secretion by mBMMC in response to ADP in the presence of *T. spiralis* ES and 5'-NT

mBMMC were stimulated with the indicated concentration of ADP in the presence of (A) 100 $\mu\text{g}/\text{ml}$ ES and (B) 15 $\mu\text{g}/\text{ml}$ 5'-NT, as described in the Materials and Methods section. Culture supernatants were removed and tested for mMCP-1 by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated without ADP, *** $p \leq 0.001$ and cells treated with ADP in the presence/absence of ES/5'-NT, † † † $p \leq 0.001$, † † $p \leq 0.01$.

of ES on ADP-induced mMCP-1 secretion was limited by the rate of ADP hydrolysis, as ES could not inhibit mMCP-1 release from mBMMC stimulated with 50 μM or 100 μM concentrations of ADP [Figure 4-11 (A)]. To confirm the ADP metabolising properties observed in ES were due to the action of the 5'-NT, the recombinant 5'-NT was used in the mMCP-1 secretion assay. The presence of the recombinant 5'-NT induced a statistically significant reduction in mMCP-1 release in response to 10 μM , 50 μM and 100 μM concentrations of ADP [Figure 4-11 (B)]. The degree of inhibition was dependent on the concentration of ADP used, where lower ADP concentrations were antagonised by 5'-NT more readily than higher ADP concentrations. This result confirms the modulatory effect *T. spiralis* ES has on ADP-induced mMCP-1 release is due to the action of the 5'-NT.

The potential modulatory effects of *T. spiralis* ES were also analysed using ATP as a substrate. The presence of ES significantly reduced ATP (10 μM)-induced mMCP-1 secretion from mBMMC [Figure 4-12 (A)], which was unexpected as ES did not alter ATP-induced Ca^{2+} mobilisation (as discussed in Chapter 3). This effect was dependent on the rate of ATP hydrolysis as ES did not alter the effect of 50 μM or 100 μM concentrations of ATP on mMCP-1 release from mBMMC [Figure 4-12 (A)]. This putatively suggests *T. spiralis* ES contains enzymes that can metabolise low but not high concentrations of ATP. To determine whether the modulatory effects of ES on low concentrations ATP were due to the action of the 5'-nucleotidase, the experiment was repeated using the *T. spiralis* recombinant 5'-NT. As shown in Figure 4-12 (B), the recombinant 5'-NT did not alter the amount of mMCP-1 released from mBMMC in response to 10 μM , 50 μM and 100 μM concentrations of ATP. This suggests that the 5'-NT is not the enzyme in *T. spiralis* ES responsible for ATP metabolism.

4.2.1.5 The secretory pathway of adenine nucleotide induced mMCP-1 secretion

The constitutive release of mMCP-1 from mBMMC has previously been reported to be partially dependent on *de novo* protein synthesis and Golgi directed transport (Brown *et al.*, 2003), which explains how mMCP-1 is constitutively released from mBMMC in the absence of antigen mediated IgE/Fc ϵ RI activation. mMCP-1 is packaged in the Golgi complex and then stored in the granules of mBMMC (Brown *et al.*, 2003; Miller *et al.*, 1999; Wright *et al.*, 2002), thus it was of interest to determine whether adenine nucleotides induced mMCP-1 release from mast cell granules, or increased the constitutive secretion of mMCP-1. To investigate this, Brefeldin A (BFA)

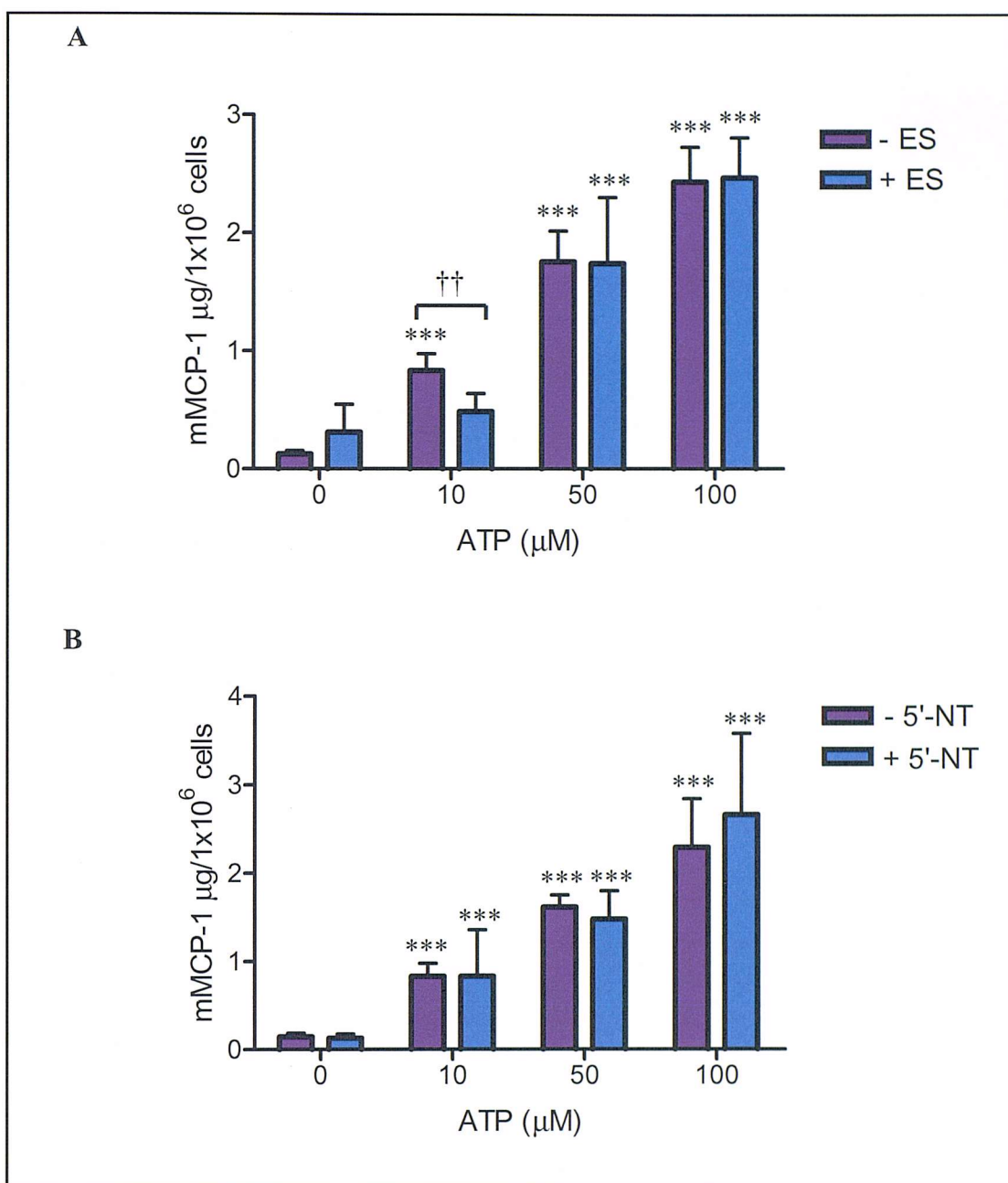


Figure 4-12 mMCP-1 secretion by mBMMC in response to ATP pre-incubated with *T. spiralis* ES and 5'-NT

mBMMC were stimulated with the indicated concentration of ATP in the presence of (A) 100 µg/ml ES and (B) 15 µg/ml 5'-NT as described in the Materials and Methods section. Culture supernatants were removed and tested for mMCP-1 by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated without ATP, *** $p \leq 0.001$ and cells treated with ATP in the presence/absence of ES/5'-NT, †† $p \leq 0.01$.

was used to disrupt the Golgi complex, and cyclohexamide (CHX) was used to inhibit protein synthesis. Considering mBMMC are highly susceptible to BFA- and CHX-induced cell death, these inhibitors were used at concentrations known not to affect mBMMC viability (Brown *et al.*, 2003).

mBMMC were cultured for 24 hours with media supplemented with either CHX, BFA or vehicle alone [Figure 4-13 (A) and (B)]. Cells were subsequently stimulated with 10 μ M and 100 μ M concentrations of ADP and ATP (Figure 4-13 (A) and (B) respectively). In the absence of nucleotide stimulation, CHX and BFA significantly reduced the background level of mMCP-1 secretion by $44 \pm 9.8\%$ and $46 \pm 13.1\%$ respectively (Figure 4-13). This is consistent with the inhibitory effects of CHX and BFA on mMCP-1 secretion observed by Brown *et al.*, (2003) and suggests CHX had inhibited protein synthesis and BFA had disrupted the Golgi complex.

The application of 10 μ M ADP in the presence of CHX or BFA induced a statistically significant increase in mMCP-1 release, however this was lower than the amount of mMCP-1 released from mBMMC stimulated with 10 μ M ADP only [Figure 4-13 (A)]. The presence of CHX or BFA did not alter 100 μ M concentrations of ADP inducing mMCP-1 release from mBMMC [Figure 4-13 (A)]. These results suggest that the Golgi complex and *de novo* protein synthesis are not required for ADP-induced mMCP-1 release from mBMMC.

The application of 10 μ M and 100 μ M concentrations of ATP induced a statistically significant increase in mMCP-1 release, and this was independent of the presence of CHX or BFA [Figure 4-13 (B)]. This suggests that the Golgi complex and *de novo* protein synthesis are not required for ATP-induced mMCP-1 release from mBMMC. Furthermore, these results may suggest that ADP and ATP induce the release of pre-formed mMCP-1 from mBMMC granules.

4.2.2 Investigating the role of nucleosides and nucleotides as mast cell chemoattractants

As nucleotides have previously been reported to induce migration of mature rat mast cells (McCloskey *et al.*, 1999), it was thought important to investigate whether nucleosides and nucleotides could induce migration of mBMMC. The frequency of mast cell precursors in murine bone marrow, and the ability of mast cells to proliferate determine the extent of mast cell hyperplasia observed in the intestine during *T. spiralis* infection (Reed *et al.*, 1988; Brown *et al.*, 2003; Pennock and Grencis, 2004). Thus, it

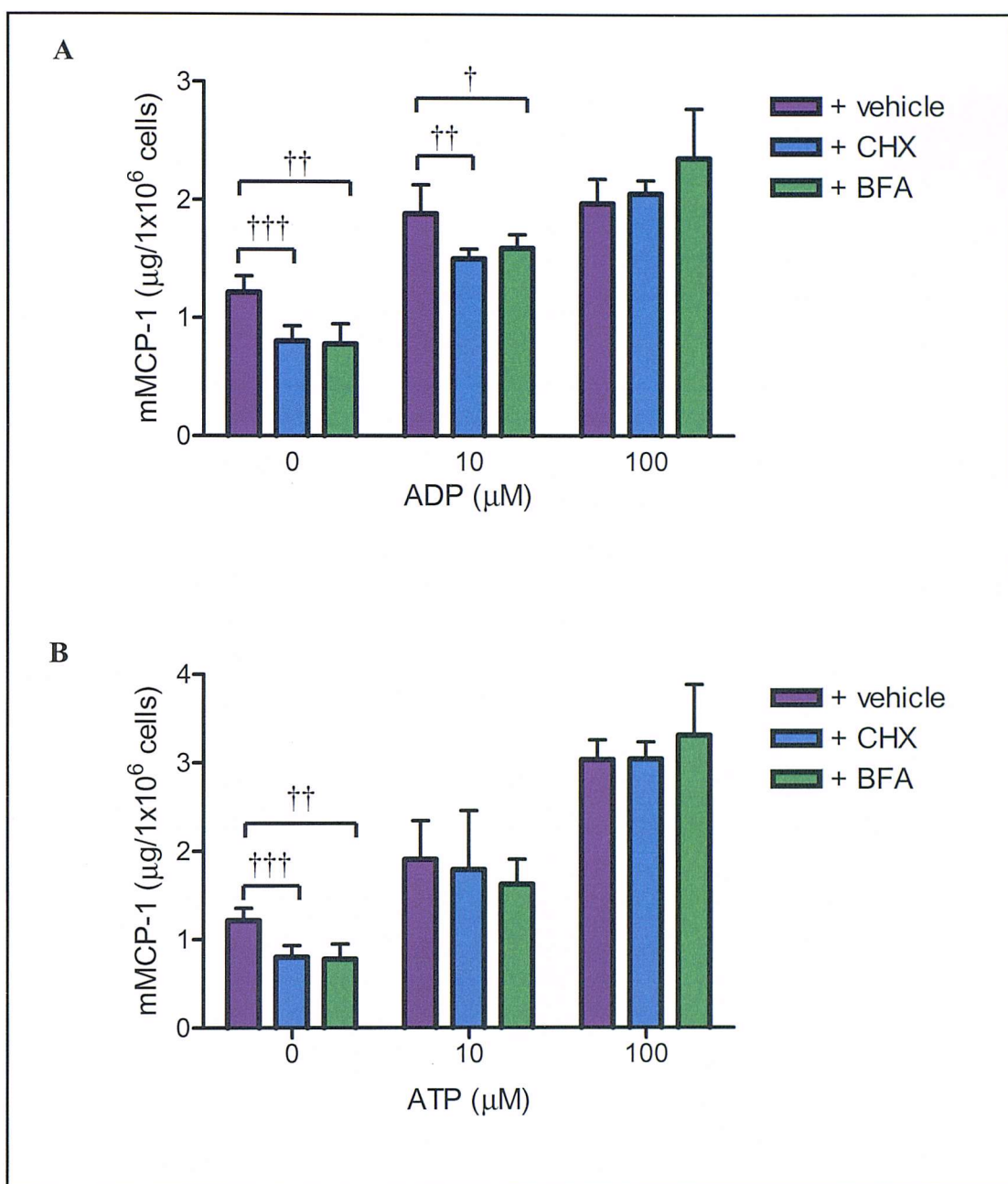


Figure 4-13 mMCP-1 secretion by mBMMC pre-incubated for 24 hours with cyclohexamide and brefeldin A and subsequently stimulated with adenine nucleotides

mBMMC were pre-incubated for 24 hours with methanol vehicle, 120 ng/ml cyclohexamide (CHX) and 120 ng/ml brefeldin A (BFA). Cultures were subsequently stimulated with the indicated concentration of (A) ADP and (B) ATP, and culture supernatants subsequently tested for mMCP-1 by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with vehicle alone and the indicated concentration of nucleotide † † † $p \leq 0.001$, † † $p \leq 0.01$, † $p \leq 0.05$.

was of interest to determine if nucleotide and nucleotides could direct the migration of mast cell precursors. Furthermore, mature mast cells also release factors during activation that are chemotactic for immature mast cells (Weller *et al.*, 2005). Considering ADP and ATP induce mBMMC to release hexosaminidase and mMCP-1 from their granules, it was thought interesting to investigate whether stimulation of mBMMC with nucleosides and nucleotides could also induce the release of chemoattractants for mast cell progenitors.

4.2.2.1 Mature mBMMC chemotaxis

Chemotactic responses of mature mBMMC were measured using 96-well microchemotaxis plates. After 3 hours incubation at 37°C, cells that had migrated to the lower chambers were counted using a light microscope. The mast cell growth factor SCF is a chemoattractant for murine mast cells (Meininger *et al.*, 1992), and was used as a positive control in the mBMMC migration assay. As shown in Figure 4-14, SCF induced a dose-dependent increase in mBMMC migration, which peaked when 1 nM concentrations of SCF were used. This result confirms that the mBMMC were able to undergo migration in the assay system and were responsive to chemotactic factors. Inosine, adenosine, ADP, ATP, UDP and UTP at concentrations ranging from 1 µM to 100 µM were subsequently utilised in the chemotaxis assay. None of the nucleosides and nucleotides tested induced a statistically significant increase in mBMMC migration (Figure 4-14). Thus, it appears that these compounds are not chemotactic agents for mBMMC.

4.2.2.2 The chemotactic effect of nucleosides and nucleotides on 2 week-old immature mast cells.

To determine if nucleosides and nucleotides could direct the migration of immature mast cells, mouse bone marrow cells that had been cultured for 2 weeks with 15% WEHI conditioned medium as a source of IL-3 were used in the chemotaxis assay. These cells are deemed 'immature' by their ability to populate tissues *in vivo* after intravenous injection in mice (Nakano *et al.*, 1985; Weller *et al.*, 2005). These cells were approximately 42 % c-kit⁺ as determined by FACS analysis after two weeks of culture [Figure 4-15 (A)], thus providing a significant number of c-kit⁺ cells to use in this assay system. The population of immature mast cells, and the indicated concentration of test agonist, were applied to the upper and lower wells of the

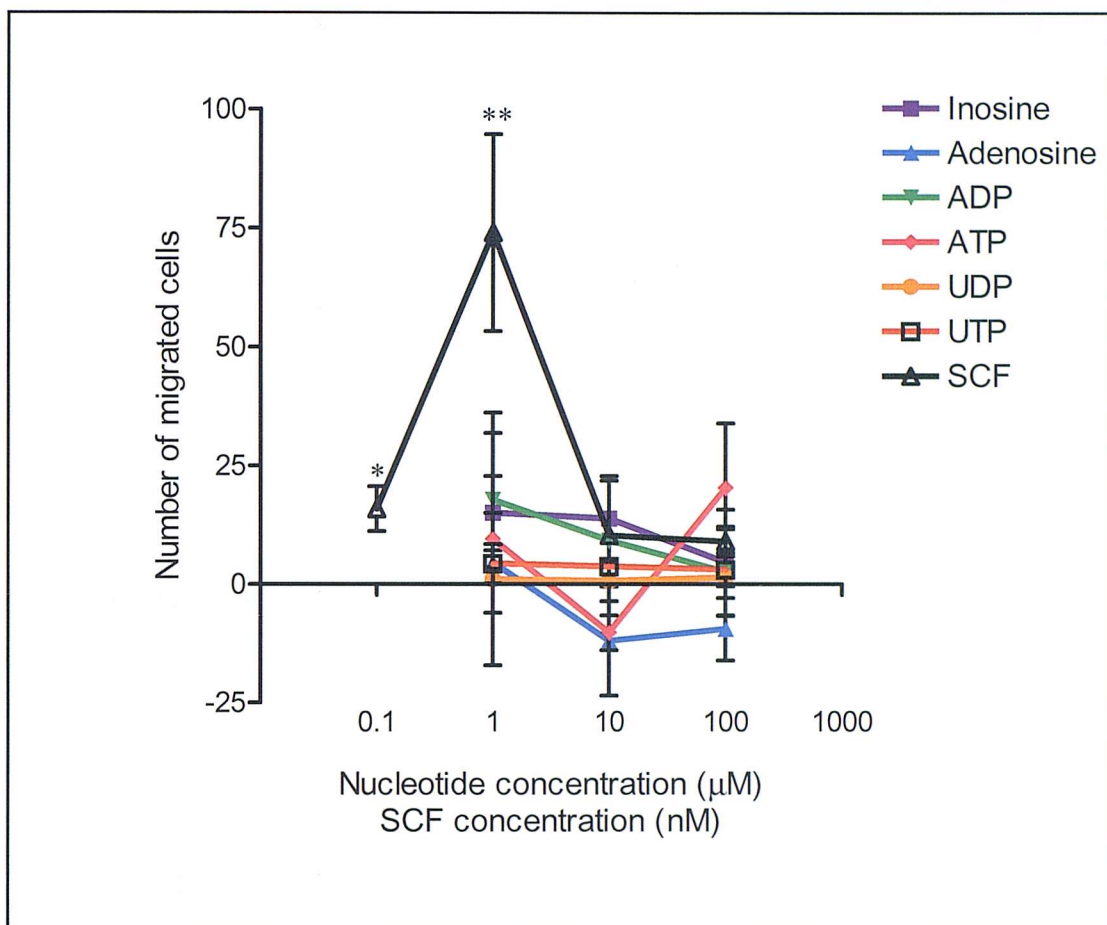


Figure 4-14 The effects of nucleosides and nucleotides on mature mBMMC migration

mBMMC were added to the top wells of a microchemotaxis plate. The indicated concentrations of test chemotactic factors or nucleotides were added to the lower wells. After 3 hours incubation, migrated cells were removed from the lower wells and counted using a light microscope at X10 magnification. The average number of cells migrated to media alone was subtracted from absolute cells counts. Data were analysed using the unpaired, two-tail Student's *t*-test and represent the mean $\pm$ s.d.m of triplicate cell cultures. Significance values are shown relative to the migration of cells to media alone. * $p \leq 0.05$, ** $p \leq 0.01$.

microchemotaxis plate respectively. After 3 hours incubation at 37°C, migrated cells were removed from the lower chambers and stained with anti-c-kit-PE. FACS analysis was subsequently used to determine the number of c-kit⁺ cells that had migrated. As shown in Figure 4-15 (B), the immature mast cell chemoattractant leukotriene (LT) B₄ (Weller *et al.*, 2005), at concentrations of 100 nM, induced a statistically significant increase in the migration c-kit⁺ cells. Inosine, adenosine, ADP, ATP, UDP and UTP at 100 µM concentrations did not act as chemotactic agents for immature mast cells as no alterations in migration were observed [Figure 4-15 (B)].

4.2.2.3 The chemotactic effect of nucleosides and nucleotides on freshly isolated mouse bone marrow preparations

Chemotactic responses of mast cell progenitors were also measured using 96-well microchemotaxis plates. Freshly isolated mouse bone marrow cell suspensions, and the indicated concentration of test agonist, were applied to the upper and lower wells of the microchemotaxis plate respectively. After 3 hours incubation at 37°C, migrated cells were removed from the lower chambers and cultured for 14 days with IL-3, IL-9, SCF and TGF-β₁ to induce the growth of mature mBMMC high in mMCP-1 levels. Cells were then lysed by freeze-thawing and the released mMCP-1 quantified by ELISA. The amount of mMCP-1 measured was used as an indicator of mast cell precursor migration.

The lipid mediator LTB₄ is a chemoattractant for mast cell progenitors (Weller *et al.*, 2005), and was used as a positive control in the migration assay. As shown in Figure 4-16 (A), LTB₄ induced a concentration-dependent increase in mMCP-1 levels that peaked when 1 nM was used. This indicates that the mast cell precursors were responsive to external stimuli and were able to migrate in the assay system used. Inosine, adenosine, ADP, ATP, UDP and UTP at 100 µM concentrations failed to induce a statistically significant increase in mMCP-1 levels, indicating mast cell progenitors had not been induced to migrate [Figure 4-16 (B)].

To identify whether nucleosides and nucleotides could induce mature mast cells to release chemotactic factors that would lead to the recruitment of immature mast cell progenitors, cell supernatants were taken from mBMMC stimulated for 2 hours with 100 µM inosine, adenosine, ADP, ATP, UDP and UTP. As a positive control, supernatants were taken from mBMMC sensitised with anti-DNP-IgE and activated by the specific antigen DNP-HSA. As shown in Figure 4-16 (C), supernatants from antigen

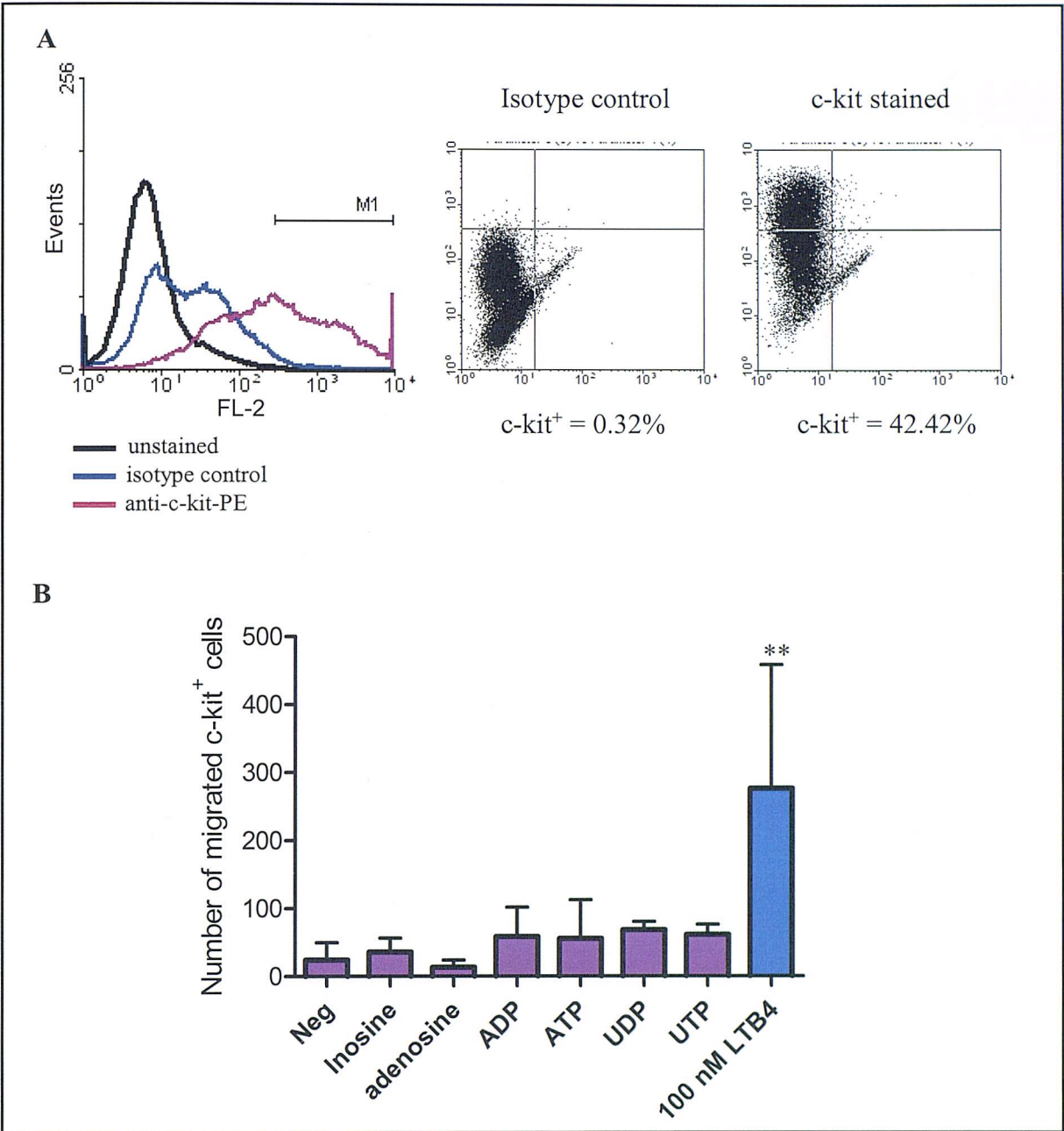


Figure 4-15 The effects of nucleosides and nucleotides on immature mast cell migration

The percentage of c-kit⁺ cells in the total BMBC population was measured by flow cytometry. (A) Representative histogram and dot plots showing c-kit staining of mBMBC cultured for 2 weeks with 15% WEHI-conditioned medium as a source of IL-3. (B) 2 week old mBMBC were added to the top wells of a microchemotaxis plate. Leukotriene B₄ or 100 μ M concentrations of the indicated nucleoside/nucleotides were added to the lower wells. After 3 hours incubation, migrated cells were removed from the lower wells and stained with anti-c-kit-PE. The number of migrated c-kit⁺ mBMBC were counted by FACS. Data were analysed using the unpaired, two-tail Student's *t*-test and represent the mean + s.d.m of triplicate cell cultures. Significance values are shown relative to the migration of cells to media alone. ** $p \leq 0.01$.

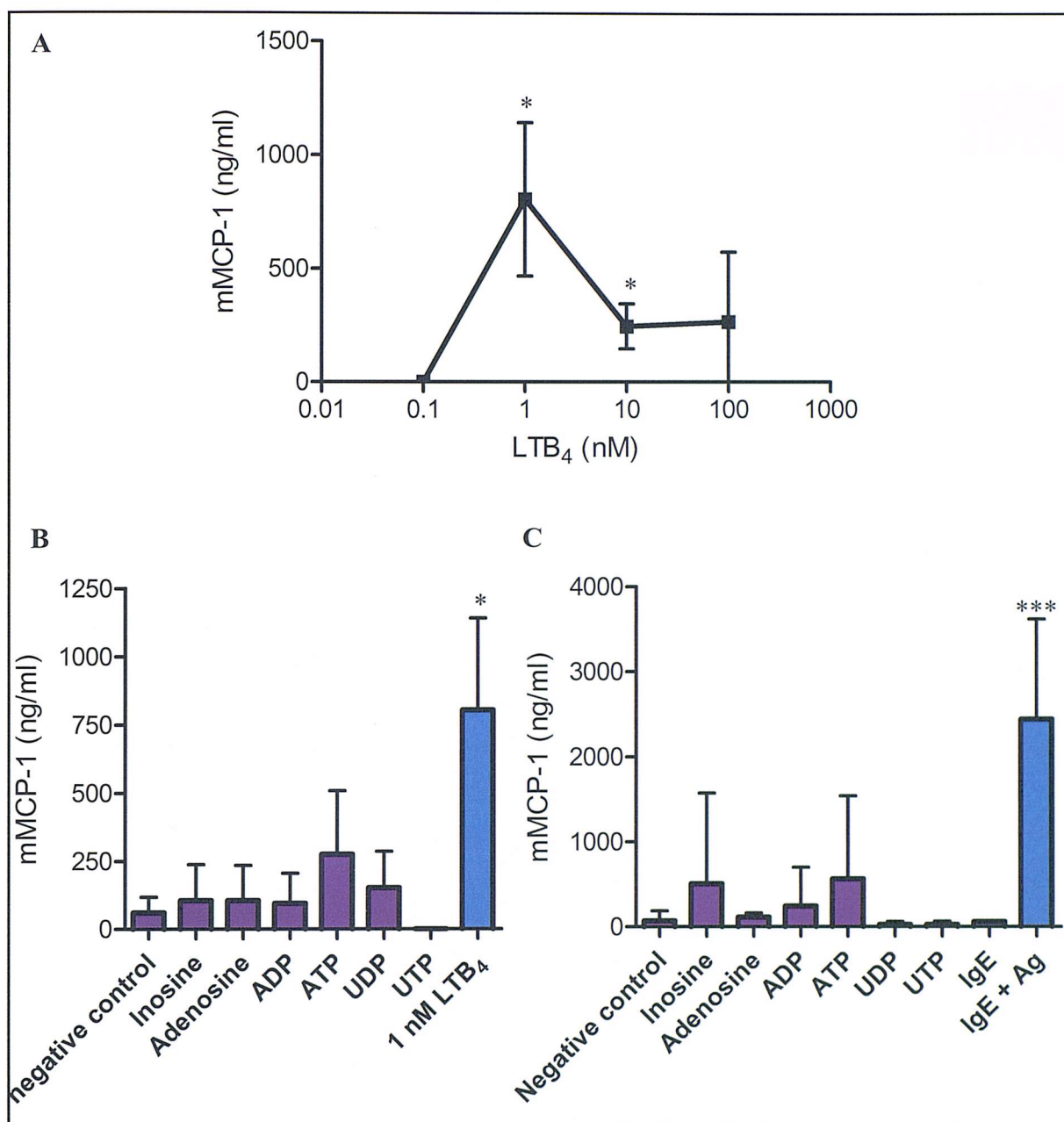


Figure 4-16 The effects of nucleosides and nucleotides on the migration of freshly isolated bone marrow cells

Freshly isolated bone marrow cells were added to the top wells of a microchemotaxis plate and the indicated concentration of test chemoattractant was added to the bottom well. After a 3 hour incubation, migrated cells were cultured for 14 days in the presence of IL-3, IL-9, SCF and TGF- β_1 . On day 14 the migrated cells were frozen, lysed, and assayed for levels of mMCP-1 by ELISA. (A) Dose-dependent migration of bone marrow cells in response to LTB₄. (B) Migration of bone marrow cells in response to 100 μ M nucleotides and 1 nM LTB₄. (C) Migration of bone marrow cells in response to supernatant collected from day-14 mBMMC stimulated with 100 μ M of the indicated nucleotides and IgE + Antigen. Data were analysed using the unpaired two-tail Student's *t*-test and represent the mean + s.d.m of triplicate bone marrow preparations. Significance values are shown relative to cells treated with media alone. * $p \leq 0.05$, *** $p \leq 0.001$.

dependent FcεRI stimulated mBMMC induced a statistically significant increase in mMCP-1 levels. This indicates mast cell progenitors were induced to migrate and again suggests the mast cell precursors were responsive to external stimuli and able to migrate in the assay system used. Supernatants from mature mBMMC stimulated with nucleosides and nucleotides did not induce a statistically significant increase in mMCP-1 levels [Figure 4-16 (C)]. This suggests stimulation of mBMMC with nucleosides and nucleotides does not induce the release of factors such as LTB₄ that are chemotactic for mast cell progenitors.

4.2.3 The effect of nucleotides and *T. spiralis* ES on cytokine generation in mBMMC

Activation of mast cells by a variety of stimuli can induce *de novo*-synthesis of cytokines. More specifically, adenosine, ATP and UDP have been reported to directly induce cytokine production in some mast cell types (Feoktistov and Biaggioni, 1995; Ryzhov *et al.*, 2006; Bulanova *et al.*, 2005; Mellor *et al.*, 2002). Furthermore, ADP and ATP have been reported to modulate mast cell cytokine production initiated by another stimulus (Feng *et al.*, 2004). Thus, it was deemed pertinent to determine whether nucleosides and nucleotides could induce *de novo* generation of cytokine production, or modulate cytokine production in mBMMC.

4.2.3.1 The effect of nucleotides on cytokine generation

To determine whether nucleotides could induce the *de novo* generation of cytokines, mBMMC were stimulated with 100 μM of inosine, adenosine, ADP, ATP, UDP and UTP. To ensure the mBMMC could generate cytokines, the cells were passively sensitised with IgE and subsequently stimulated with antigen (IgE and Ag). Supernatants were harvested 24 hours after stimulation and cytokine levels subsequently measured by ELISA. FcεRI cross-linkage of mBMMC induced statistically significant increased levels of TNF-α, IL-4, IL-5 and IL-13 cytokine production (Figure 4-17 (A)-(D) respectively). This suggests the mBMMC were able to generate cytokines in response to external stimuli, and correlates with other studies that have shown FcεRI cross-linkage of mast cells induces *de novo* cytokine generation (Bradding *et al.*, 1992; Boyce, 2003; Zhao *et al.*, 2005). When used at concentrations of 100 μM, inosine, adenosine, ADP, ATP, UDP and UTP did not elicit statistically significant increased TNF-α, IL-4, IL-5 or IL-13 cytokine generation [Figure 4-17 (A)-

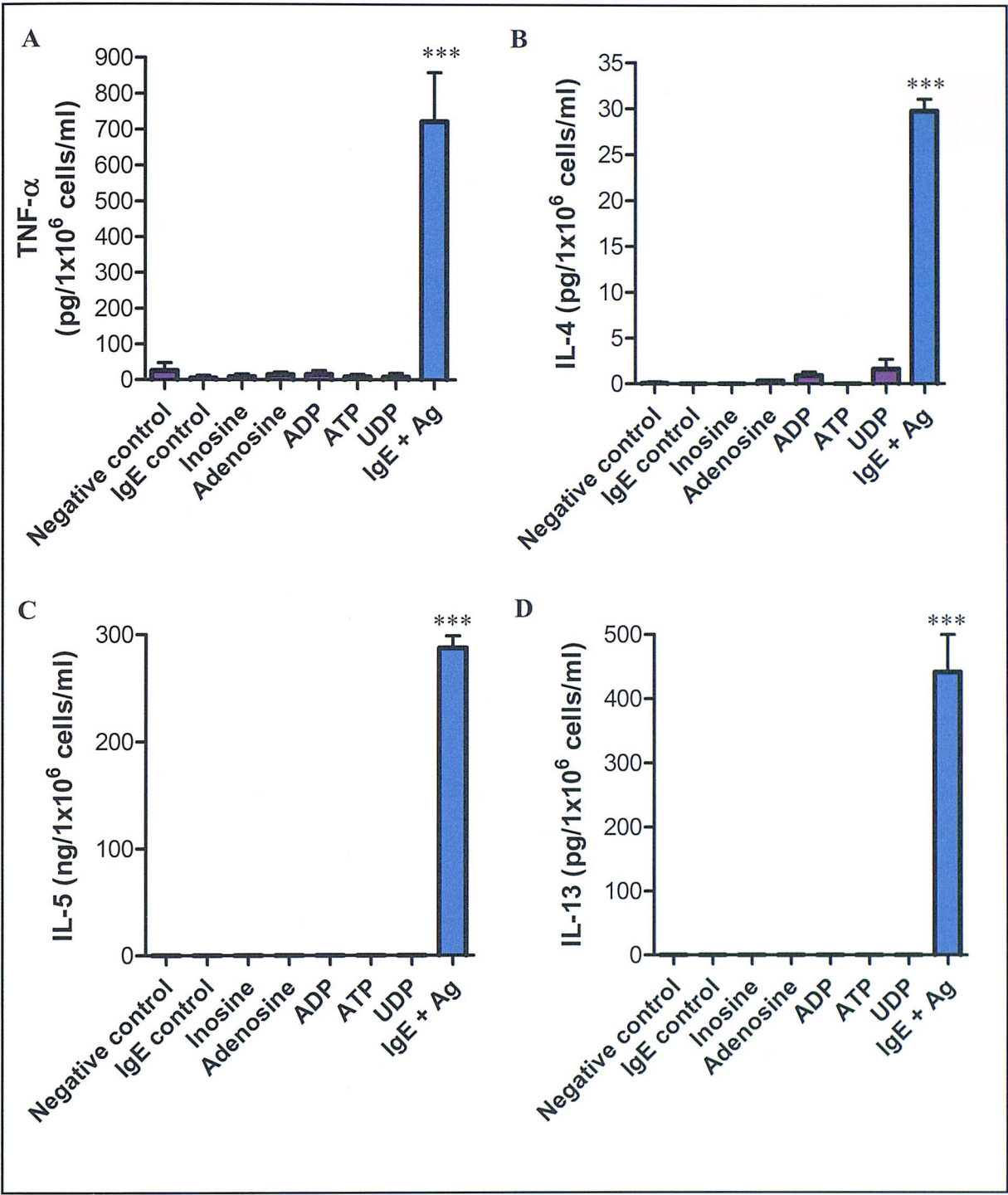


Figure 4-17 Cytokine production by mBMMCs in response to nucleotides

mBMMC were stimulated for 24 hrs with 100 μ M nucleotides and IgE + Antigen (IgE + Ag). Culture supernatants were tested by ELISA for the presence of the cytokines (A) TNF- α , (B) IL-4, (C) IL-5 and (D) IL-13. Data were analysed using the unpaired two-tail Student's *t*-test and represent the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone. *** $p \leq 0.001$.

(D)]. This implies that nucleosides and nucleotides cannot directly induce mBMMC to generate pro-inflammatory or T_H2-type cytokines, even when used at relatively high concentrations.

4.2.3.2 The modulatory effects of nucleosides and nucleotides on cytokine generation

As nucleosides and nucleotides could not directly induce cytokine generation in mBMMC, the possibility that nucleosides and nucleotides could modulate the effects of other stimuli was investigated. It has recently been shown that mast cells express toll-like receptors (TLRs) (McCurdy *et al.*, 2001; Supajatura *et al.*, 2001; Varadaradjalou *et al.*, 2003). Given that during *T. spiralis* infection large numbers of mast cells reside in the epithelium of the intestine, TLR activation could be an important, but as yet unappreciated aspect of their role in intestinal immunology. The TLR-2 ligand peptidoglycan (PGN) (a component of *Staphylococcus aureus* cell walls), and the TLR-4 ligand lipopolysaccharide (LPS) (from *Escherichia coli*), activate human mast cells to generate TNF- α production (Varadaradjalou *et al.*, 2003). Furthermore, the nucleotides ADP and ATP acting as a secondary stimulus have been reported to inhibit PGN-induced TNF- α generation in human mast cells (Feng *et al.*, 2004).

To confirm that LPS could induce TNF- α generation in mBMMC *via* activation of TLR-4, cells were stimulated for 24 hours with concentrations of LPS ranging from 1 ng/ml to 10 μ g/ml. As shown in Figure 4-18, LPS induced a concentration dependent increase in TNF- α production, suggesting that the mBMMC express functional TLR-4. To determine if nucleosides and nucleotides could modulate LPS-induced TNF- α production, mBMMC were incubated for 10 minutes with inosine, adenosine, ADP, ATP, UDP and UTP at concentrations ranging from 1-100 μ M and subsequently stimulated with 100 ng/ml LPS. As shown in Figure 4-19 (A), inosine did not alter LPS induced TNF- α generation in mBMMC. In contrast, when added as a concomitant stimulus, adenosine, ADP and ATP induced statistically significant reductions in LPS-induced TNF- α production (Figure 4-19 (B), (C) and (D) respectively). The inhibitory effect occurred in a dose-dependent manner and was maximal when 100 μ M concentrations of adenosine, ADP and ATP were used. The uridine nucleotides, UDP and UTP did not affect LPS-induced TNF- α generation (Figure 4-19 (E) and (F) respectively). These results show adenosine, ADP and ATP are able to attenuate TLR-4-induced TNF- α generation in mBMMC.

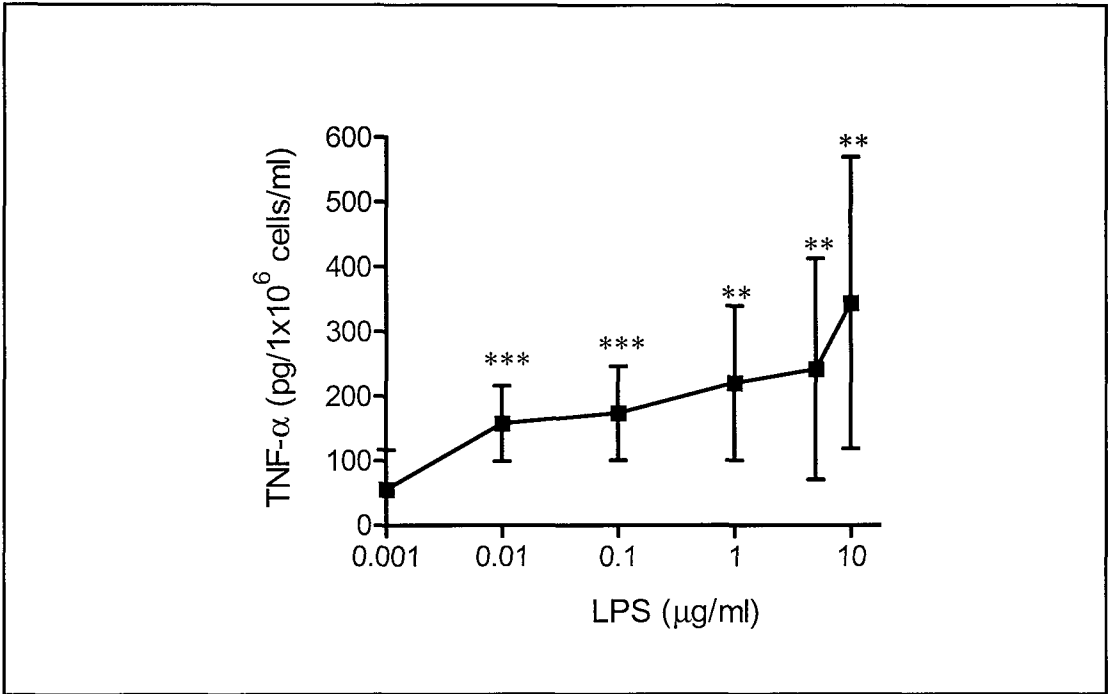


Figure 4-18 TNF-α production by mBMMCs in response to LPS

mBMMC were stimulated for 24 hrs with a range of LPS concentrations. Culture supernatants were then tested for TNF-α production by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent the mean $\pm$ s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone. ** $p \leq 0.01$, *** $p \leq 0.001$.

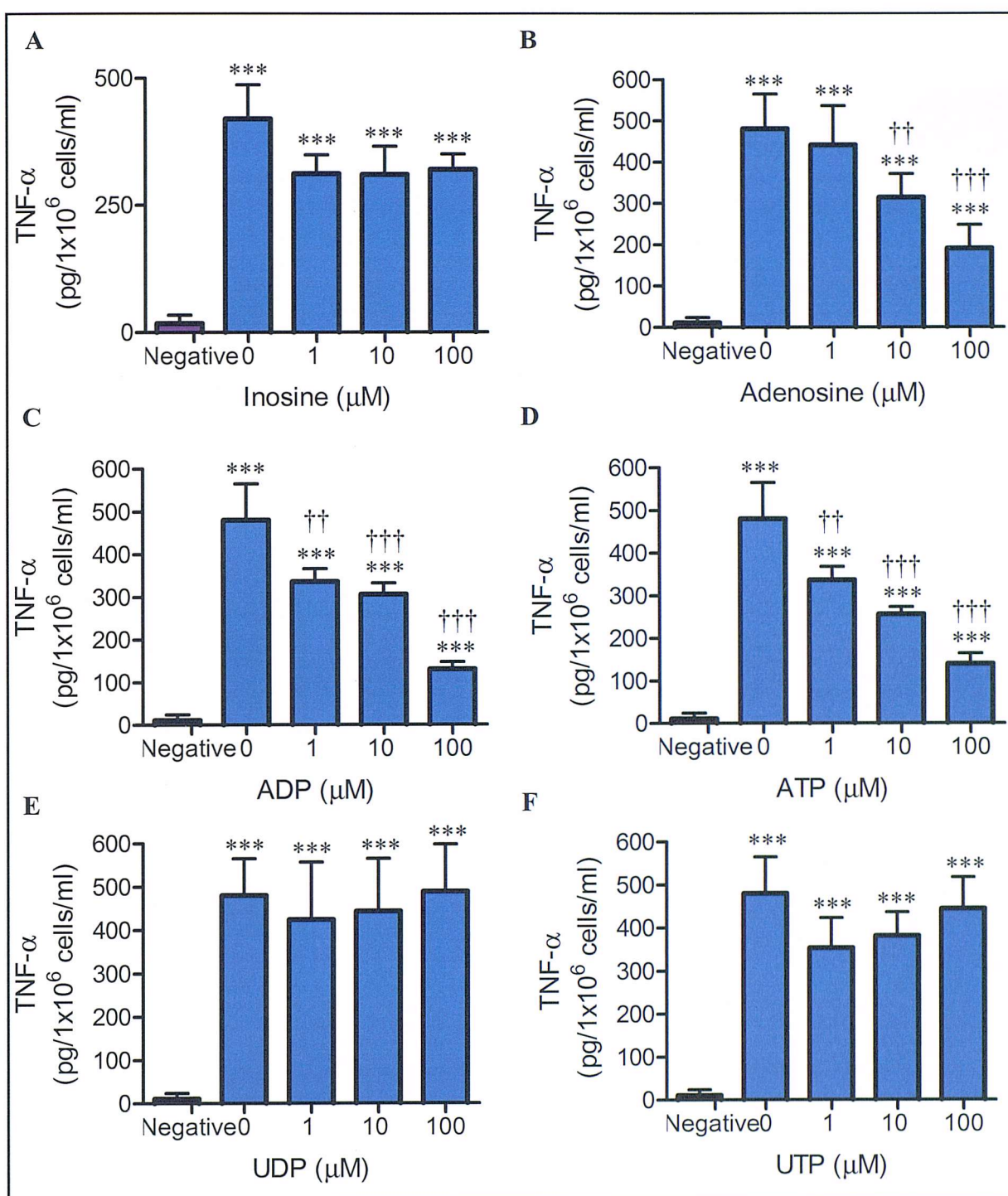


Figure 4-19 TNF- α production by mBMMCs in response to 100 ng/ml LPS and nucleotides

mBMMC were stimulated for 24 hrs with 100 ng/ml LPS and the indicated concentration of (A) Inosine, (B) Adenosine, (C) ADP, (D) ATP, (E) UDP and (F) UTP. Culture supernatants were then tested for TNF- α production by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent at least one experiment, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with media alone, *** $p \leq 0.001$ and cells treated with 100 ng/ml LPS, †† $p \leq 0.01$, ††† $p \leq 0.001$.

4.2.3.3 The effect of *T. spiralis* ES and recombinant 5'-NT on nucleotide-modulated cytokine generation

T. spiralis ES can modulate adenosine- and ADP-induced mBMMC stimulation in terms of purinergic receptor activation and mMCP-1 release (section 3.2.4 and section 4.2.1.4 respectively). Thus, it was of interest to determine if *T. spiralis* ES could also modulate the inhibitory effects of adenosine and ADP on TLR-4-induced TNF- α generation in mBMMC. Adenosine ranging in concentration from 1-100 μ M was applied to mBMMC in the absence or presence of *T. spiralis* ES for 10 minutes prior to stimulation with 100 ng/ml LPS. As shown in Figure 4-20, concentrations of adenosine at 10 μ M and 100 μ M induced a statistically significant reduction in LPS induced TNF- α production. The presence of *T. spiralis* ES did not significantly alter the inhibitory effect of 10 μ M and 100 μ M concentrations of adenosine (Figure 4-20). These results suggest that in this scenario, *T. spiralis* ES cannot modulate the inhibitory effects of adenosine on TLR-4-induced TNF- α generation in mBMMC.

As shown in Figure 4-21 (A), the inhibitory effect of 1 μ M concentrations of ADP on LPS-induced TNF- α generation in mBMMC was ablated in the presence of *T. spiralis* ES. This effect could be mediated by the secreted 5'-NT, and adenosine deaminase, thus limiting P2Y and P1 receptor activation. This effect was limited by the rate of ADP and/or adenosine metabolism, as the ablation of ADP mediated inhibition was not observed when 10 μ M and 100 μ M concentrations of ADP were applied to mBMMC in the presence of ES [Figure 4-21 (A)]

To further investigate the role of the 5'-NT in this bio-assay, LPS and ADP were applied to the mBMMC in the absence or presence of the recombinant 5'-NT. As shown in Figure 4-21 (B), the inhibitory effect of 1 μ M concentrations of ADP was ameliorated in the presence of the 5'-NT. These results suggest that the 5'-NT activity present in *T. spiralis* ES is responsible for the observed modulation of the inhibitory effects of low (1 μ M) ADP concentrations on TNF- α generation in mBMMC. However, the addition of the 5'-NT did not significantly alter the inhibitory effects of 10 μ M and 100 μ M concentrations of ADP on LPS-induced TNF- α generation in mBMMC [Figure 4-21 (B)]. The hydrolysis of ADP by the 5'-NT will result in the accumulation of adenosine which also inhibits LPS-induced TNF- α production. Hence, the lack of inhibition observed, is likely to be a result of adenosine stimulating P1 receptors which will continue to suppress TNF- α generation in mBMMC.

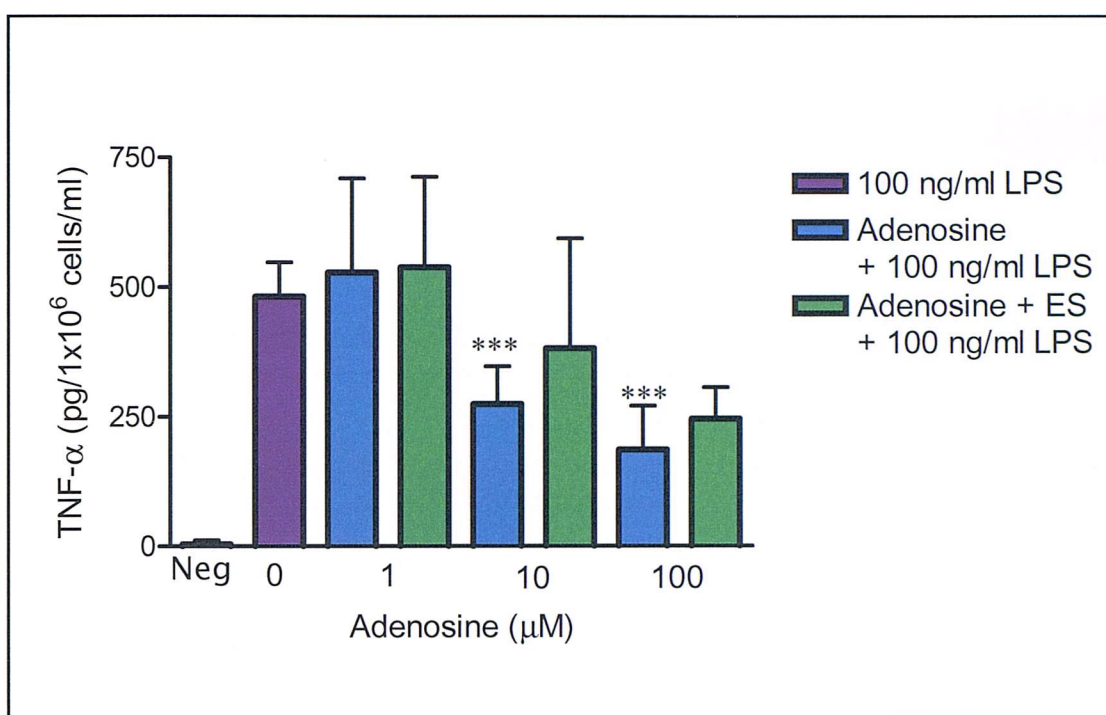


Figure 4-20 TNF- α production by mBMMCs in response to 100 ng/ml LPS and adenosine in the presence of *T. spiralis* ES

mBMMC were stimulated for 24 hrs with 100 ng/ml LPS and the indicated concentration of adenosine in the absence or presence of *T. spiralis* ES (as described in the Materials and Methods section). Culture supernatants were then tested for TNF- α production by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with 100 ng/ml LPS, *** $p \leq 0.001$ and cells treated with 100 ng/ml LPS and the indicated concentration of adenosine † $p \leq 0.05$, † † $p \leq 0.01$.

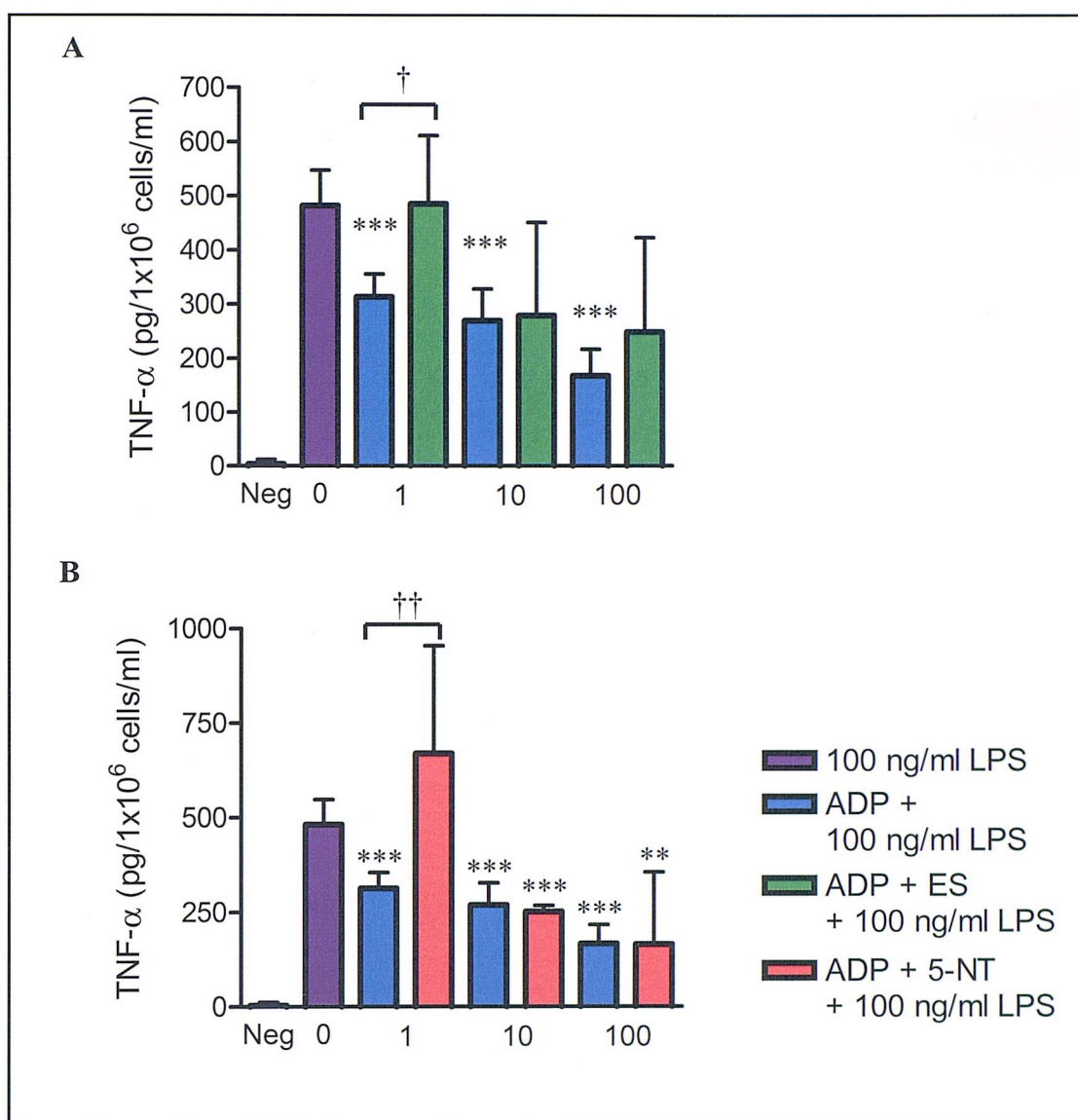


Figure 4-21 TNF- α production by mBMMCs in response to 100 ng/ml LPS and adenine nucleotides in the presence of *T. spiralis* ES and 5'-NT

mBMMC were stimulated for 24 hrs with 100 ng/ml LPS and the indicated concentration of ADP in the absence or presence of *T. spiralis* ES and 5'-NT (as described in the Materials and Methods section). Culture supernatants were then tested for TNF- α production by ELISA. Data were analysed using the unpaired two-tail Student's *t*-test and represent two experiments, showing the mean + s.d.m of triplicate cultures. Significance values are shown relative to cells treated with 100 ng/ml LPS, *** $p \leq 0.001$, ** $p \leq 0.01$ and cells treated with 100 ng/ml LPS and the indicated concentration of ADP † $p \leq 0.05$, †† $p \leq 0.01$.

4.3 Discussion

As demonstrated and discussed in Chapter 3, mBMMC of the mucosal phenotype express multiple functional purinergic receptor sub-types responsive to a range of nucleosides and nucleotides. This prompted the study presented in this chapter, in which the complementary or opposing roles of purinergic receptor activation on mast cell effector functions were explored. As *T. spiralis* secretes nucleotide-metabolising enzymes (Gounaris, 2002; Gounaris *et al.*, 2004; Arden *et al.*, 1997; Gounaris *et al.*, 2001), that can modulate mBMMC purinergic receptor activation, it was important to determine whether mast cell effector functions were also affected. This is discussed in the context of the intestinal phase of *T. spiralis* infection, where mucosal mast cell effector functions play a pivotal role in efficient parasite expulsion.

A wealth of conflicting data surrounds the effects of nucleosides and nucleotides on mast cell degranulation. To unequivocally define if inosine, adenosine, ADP, ATP, UDP and UTP are secretagogues for mBMMC of the mucosal phenotype, the activity of β -hexosaminidase (β -hex) secreted into cell supernatants was monitored. Inosine has been reported to directly induce degranulation of the rat mast cell-derived basophilic leukemia cell line RBL-2H3 *in vitro*, and induce degranulation of mouse mast cells *in vivo* (Shepherd and Duling, 1996; Jin *et al.*, 1997; Tilley *et al.*, 2000). However, the results observed in this study showed that neither inosine nor adenosine could induce degranulation of mBMMC. This is consistent with other studies that have reported that these nucleosides do not directly induce degranulation of a similar type of mBMMC (cultured with IL-3 only) (Marquardt *et al.*, 1994), and human lung mast cells (Schulman *et al.*, 1999).

ADP and ATP at concentrations at or above 50 μ M induced a dose-dependent increase in β -hex release. This correlates with another study that suggested that ADP could induce modest exocytosis in human mast cells (Feng *et al.*, 2004). ATP has also been shown to directly induce exocytosis in the mouse mast cell-like cell line MC-9 (Sudo *et al.*, 1996) and murine BMMC (cultured with IL-3 only) (Nakamura *et al.*, 1989). In contrast, a study using human lung mast cells reported that ATP could not directly induce degranulation, but only modulate anti-IgE-induced degranulation (Schulman *et al.*, 1999), thus emphasizing the functional heterogeneity of mast cells from different sources. The uridine nucleotides UDP and UTP did not induce degranulation of mBMMC. These results correlate with similar studies that have

reported that UDP can not induce exocytosis in human cord blood-derived mast cells (Mellor *et al.*, 2002), and that UTP can only enhance antigen-dependent degranulation of human lung mast cells (Schulman *et al.*, 1999).

Mast cells secreting MCP-1 are central to the efficient expulsion of *T. spiralis* infective larvae from the gut (Knight *et al.*, 2000). During *T. spiralis* infection TGF- β_1 expressed by the epithelium induces mucosal mast cells to constitutively secrete MCP-1 (Wastling *et al.*, 1997; Miller *et al.*, 1999; Wright *et al.*, 2002). This occurs in the absence of Fc ϵ RI cross-linkage, suggesting that antigen-dependent degranulation is not required. Considering that extracellular nucleosides and nucleotides play significant roles in activating mast cells, it was deemed pertinent to investigate whether they could also influence mMCP-1 release from mBMMC.

The adenine nucleotides ADP and ATP induced a dose-dependent increase in mMCP-1 release from mBMMC independent of the presence of TGF- β_1 . Use of the stable nucleotide analogues ADP- β S and ATP- γ S also induced a dose-dependent increase in mMCP-1 release from mBMMC, confirming that this effect was due to the adenine nucleotides rather than their degradation products. These results show for the first time that extracellular nucleotides can directly activate mast cells to secrete the β -chymase mMCP-1. Of the nucleosides and nucleotides tested, only adenine nucleotides consistently induced both β -hex and mMCP-1 release, potentially implying that these two mediators may be released *via* stimulation of signalling pathways that lead to secretion. Unexpectedly, 100 μ M concentrations of UTP induced a modest increase in mMCP-1 release from mBMMC cultured in the absence of TGF- β_1 . Potentially, the small increase in mMCP-1 release may not have been detected in cells cultured with TGF- β_1 due to the higher background level of mMCP-1 secretion. As UTP did not induce mMCP-1 release from mBMMC cultured with TGF- β_1 , this phenomenon was not investigated further.

As discussed in Chapter 3, ADP and ATP were shown to signal *via* the P2Y₁ and P2X₄ receptors respectively, in mBMMC. However, due to the lack of P2X₄ receptor antagonists (North, 2002), the purinergic receptor *via* which ATP was inducing mMCP-1 secretion could not be confirmed. The use of the broad-specificity purinergic receptor antagonists suramin and PPADS reduced the amount of mMCP-1 released from ADP-stimulated mBMMC, suggesting that this process was definitely mediated by purinergic receptors. Use of the antagonist MRS2179 confirmed that ADP was

signalling *via* the P2Y₁ receptor, which correlates with the calcium mobilisation data shown in Chapter 3.

mMCP-1 is packaged in the Golgi complex and subsequently stored in granules (Brown *et al.*, 2003). However, the mechanism of constitutive secretion of mMCP-1 from mBMMC is still not fully understood. Currently, constitutive mMCP-1 secretion has been shown to be partially dependent on *de novo* protein synthesis and subsequent Golgi-directed transport (Brown *et al.*, 2003). Some stimuli, such as c48/80 can also invoke the release of mMCP-1 directly from mast cell granules (De Jonge *et al.*, 2004). To investigate the mechanism of adenine nucleotide-induced mMCP-1 release from mBMMC, the inhibitor cyclohexamide (CHX) was used to prevent *de novo* protein synthesis, and Brefeldin A (BFA) was used to disrupt the Golgi complex. In keeping with the findings of Brown *et al.* (2003), 24 hour incubation with CHX or BFA reduced the constitutive secretion of mMCP-1 from mBMMC. The subsequent application of adenine nucleotides induced a substantial dose-dependent increase in mMCP-1 release. This suggests *de novo* protein synthesis coupled with Golgi-directed transport is not the major mechanism by which adenine nucleotides induce mMCP-1 release from mBMMC. Therefore, this implies that adenine-nucleotide induced degranulation is the likely mechanism by which pre-formed mMCP-1 is released from mBMMC.

T. spiralis ES significantly reduced mMCP-1 secretion from mBMMC stimulated with concentrations of ADP up to 50 μ M. The modulatory effect of *T. spiralis* ES was due to 5'-nucleotidase activity, as the recombinant 5'-NT also significantly reduced the amount of mMCP-1 released from mBMMC stimulated with concentrations of ADP up to 100 μ M. These results suggest that the *T. spiralis* secreted 5'-nucleotidase (5'-NT) metabolised the extracellular ADP to adenosine, thus preventing mBMMC P2Y₁ receptor activation that mediates mMCP-1 release. This effect was dependent on the rate of ADP hydrolysis by the secreted 5'-NT.

T. spiralis ES also mediated a significant reduction in ATP-induced mMCP-1 release from mBMMC. This was unexpected, as *T. spiralis* ES does not contain an enzyme capable of ATP hydrolysis (Gounaris, 2002). However, *T. spiralis* larvae secrete serine/threonine kinases (Arden *et al.*, 1997) and a nucleoside diphosphate kinase (NDPK) (Gounaris *et al.*, 2001), which are capable of utilising ATP. Potentially, the secreted serine/threonine kinases and NDPK could utilise and sequester the extracellular ATP thus reducing the overall concentration of ATP available to stimulate mBMMC. Due to time limitations this parameter was not investigated further. *T.*

spiralis ES did not inhibit mMCP-1 release from mBMMC when stimulated with concentrations of ATP above 50 μ M, indicating that the action of ATP-utilising enzymes present in ES were reaction rate-limited. Use of the recombinant 5'-NT did not modulate ATP-induced mMCP-1 release from mBMMC, further reinforcing the concept that ATP-utilising/metabolising enzymes were responsible for the observed modulatory effects of ES.

Murray *et al.* (1971), originally postulated that an influx of solutes and water into the gut lumen during enteric nematode infection may be a method by which the host attempts to expel the parasite. More recently, the mechanism that induces increased permeability of the intestinal epithelium has been reported to be mediated at least in part, by mMCP-1 (McDermott *et al.*, 2003). Degradation of the tight-junction protein occludin, by mMCP-1, aids parasite expulsion by increasing the paracellular permeability of the jejunum (McDermott *et al.*, 2003). Thus, activation of mast cells with extracellular adenine nucleotides will potentiate the release of mMCP-1, further contributing to the generation of an inhospitable environment for parasites and aiding parasite expulsion from the intestine. The secretion of nucleotide metabolising enzymes by *T. spiralis* ES may serve to reduce the amount of extracellular adenine nucleotides available to bind to and activate P2Y and P2X purinergic receptors on mucosal mast cells, thus limiting mMCP-1 release. This could favour parasite survival by reducing the permeability of the intestine, thus restraining the influx of solutes and water that would usually flush the parasite away.

T. spiralis infected mice undergo a transient, but pronounced T cell-dependent expansion in the number of their jejunal mast cells (Miller and Jarrett, 1971; Ruitenberg and Elgersma, 1976). Jejunal mast cells change their mMCP-1 expression profile in response to the stage of infection and their positioning within the jejunum (Friend *et al.*, 1996; 1998). Thus, the location of mast cells within the intestinal epithelium has functional consequences during *T. spiralis* infection. During this phase of infection, large amounts of nucleotides are postulated to be released from damaged intestinal cells as the larvae migrate through the epithelium. The nucleotides ADP, ATP and UTP have previously been reported to direct the migration of rat bone marrow-derived cells *in vitro* (McCloskey *et al.*, 1999). Thus, it was hypothesised that extracellular nucleosides and nucleotides may play a role in regulating the migration of the mature intestinal mucosal mast cell population. When this was investigated *in vitro*, inosine, adenosine, ADP, ATP, UDP and UTP were not chemotactic for mBMMC, even though the cells

were able to migrate towards the known mast cell chemoattractant SCF (Meininger *et al.*, 1992). It is of note, that the rat BMMC used by McCloskey *et al.* (1999) were an adherent cell type and did not detach from the polycarbonate filter used in the chemotaxis assay. In comparison, the mBMMC used in this study were non-adherent in culture, and did not remain attached to the underside of the filters during the migration assay. Potentially, the inherent differences between these mast cell types may account for the discrepancies observed between these studies.

Mast cell precursors are derived from pluripotent haematopoietic stem cells in the bone marrow (Kitamura *et al.*, 1979; 1978). During *T. spiralis* infection mast cell progenitors expressing $\alpha 4/\beta 7$ integrins are produced in the bone marrow directly in response to infection (Pennock and Grecis, 2004). The immature precursor cells circulate in the periphery prior to homing to the small intestine where they take 3 days to mature into toluidine blue-positive mucosal mast cells (Pennock and Grecis, 2004). Immature mast cells acquire a mucosal phenotype as they move from the submucosa to the intraepithelial area of the lamina propria of the small intestine (Friend *et al.*, 1996). Immigration of mast cell precursors through the small intestine occurs at the same time as *T. spiralis* parasites are causing massive cellular damage (day 5-8 post infection) (Pennock and Grecis, 2004). It was hypothesised that the expected release of nucleosides and nucleotides into the intestinal epithelium could potentially act as migratory cues to the mast cell precursors, thus aiding maturation of the cells. The immature mast cell chemoattractant Leukotriene B₄ (LTB₄) induced the migration of c-kit⁺ immature mast cell progenitors, but inosine, adenosine, ADP, ATP, UDP and UTP used at relatively high (100 μ M) concentrations could not induce migration. This result suggests that purinergic receptor activation of immature mast cell progenitors does not stimulate migration.

The mechanism by which *T. spiralis* infection induces qualitative and quantitative changes in bone marrow haematopoiesis is still unknown (Pennock and Grecis, 2004). Hypothetically, nucleotides and nucleosides released at the intestinal site of infection may also serve to recruit mast cell progenitor cells at distal sites. Upon access to the general circulation, nucleotides and nucleosides could potentially induce the release of increased number of mast cell progenitors from the bone marrow by establishing a chemotactic gradient across the sinus endothelium. To investigate this idea, freshly isolated bone marrow containing mast cell progenitors were used in chemotaxis assays. Although the immature mast cell chemoattractant LTB₄ induced the

migration of mast cell progenitors, 100 μ M concentrations of inosine, adenosine, ADP, ATP, UDP and UTP could not induce migration.

Activation of mature mast cells by cross-linkage of their Fc ϵ RI results in the production of factors such as LTB₄ that are potent chemoattractants for mast cell progenitors (Weller *et al.*, 2005). Considering that nucleotides could induce the degranulation of mature mBMMC, it was of interest to determine whether this also resulted in the release of chemoattractants for mast cell progenitors. However, supernatants taken from mature mBMMC incubated with inosine, adenosine, ADP, ATP, UDP and UTP did not direct the migration of any mast cell progenitors. These results suggest that stimulation of mBMMC with nucleosides and nucleotides cannot induce LTB₄ release. Taken together these results indicate that nucleosides and nucleotides are not chemoattractants for murine bone marrow derived mast cells at any stage of their life-history. Further experiments to determine whether nucleotides can induce chemokinesis and thus enhance chemotaxis in the presence of other chemotactic stimuli could prove interesting.

Rodent and human mast cells activated *in vitro* by cross linkage of their Fc ϵ RI with IgE and antigen can transcribe, translate and secrete several types of proinflammatory and regulatory cytokines (Walsh *et al.*, 1991; Toru *et al.*, 1998; Kobayashi *et al.*, 1998; Okayama *et al.*, 1995). Recent reports suggest that mucosal mast cells contribute to the induction of protective T_H2 responses during *T. spiralis* infection (Lawrence *et al.*, 2004). The mechanisms by which mast cells amplify the inflammatory response during *T. spiralis* infection is unknown at present, but is thought to be mediated, in part, by cytokines (Lawrence *et al.*, 2004). It has been reported that a variety of mast cell types, and mast cell-like cell lines secrete *de novo* generated cytokines when stimulated with nucleosides and nucleotides. For example, adenosine signalling *via* the A_{2B} receptor in the human mast cell line HMC-1 can induce IL-4, IL-13 and IL-8 secretion (Ryzhov *et al.*, 2004, 2006; Feoktistov *et al.*, 1995). It has been shown that activation of the apoptotic P2X₇ receptor by very high concentrations of ATP (3 mM) can induce small increases in IL-6 and IL-13 secretion from mBMMC (cultured with IL-3 only) (Bulanova *et al.*, 2005). Currently, there are no reports in the literature that implicate the involvement of P2Y receptors in *de novo* generation of cytokines in mast cells. However, a recent study reported that stimulation of the CysLT₁ receptor with UDP, induces IL-4-primed human cord-blood derived mast cells to secrete IL-5, TNF- α and MIP- β (Mellor *et al.*, 2002).

To determine whether nucleosides and nucleotides could directly stimulate the production of cytokines in mBMMC, a panel of T_H2 cytokines (IL-4, IL-5, IL-13) that have previously been shown to be important in protective responses to helminth infections (Mosmann and Coffman, 1989) were examined. These cytokines are typically produced by CD4⁺ T cells present in the local mesenteric lymph nodes during the intestinal phase of infection (Grencis *et al.*, 1991). However, mast cells of various types can also generate and release IL-4, IL-5 and IL-13 upon cross-linkage of FcεRI (Masuda *et al.*, 2002). Each of these cytokines mediate one or more of the central features of intestinal inflammation in mouse models. IL-4 is required for efficient expulsion of *T. spiralis* from the intestine (Lawrence *et al.*, 1998) and both IL-4 and IL-13 can induce mucus secretion from goblet cells (Urban, Jr *et al.*, 2001). IL-13 also promotes goblet cell hyperplasia (McKenzie *et al.*, 1998). IL-5 plays a pivotal role in regulating eosinophilia (Coffman *et al.*, 1989) and is required to reduce the number of larvae encysting in the muscle (Gurish *et al.*, 2002). As mast cells are also a potent source of TNF-α, which can act directly and indirectly to promote intestinal inflammation (Bischoff *et al.*, 1999; Lawrence *et al.*, 2004), this cytokine was also measured.

Following passive IgE sensitisation, stimulation of mBMMC with antigen induced the release of significant amounts of IL-4, IL-5, IL-13 and TNF-α, which is consistent with reports from other groups that have used murine bone marrow-derived mast cells (Masuda *et al.*, 2002; Nakamura *et al.*, 1995; Burd *et al.*, 1995; Gordon and Galli, 1990). However, relatively high (100 μM) concentrations of inosine, adenosine, ADP, ATP, UDP and UTP in the absence of IgE could not induce the *de novo* generation of any of the cytokines measured. A variety of studies have also reported that these nucleosides and nucleotides can not induce cytokine production in other types of mast cells. For example, Feng *et al.* (2004) found that ADP and ATP could not directly stimulate cytokine release from human cord blood-derived mast cells. Conversely, it has been reported that adenosine can induce cytokine generation in the mast cell line HMC-1 (Rhyzov *et al.*, 2004, 2006; Feokistov *et al.*, 2003). Interestingly, UDP can also induce cytokine generation in human mast cells that have been primed with IL-4 (Mellor *et al.*, 2002). Further experiments using IL-4 primed mBMMC could prove useful in determining whether exposure to this cytokine can alter the responsiveness of mBMMC to nucleotides.

Within the field of purinergic signalling, there is an increasing understanding that stimulation of purinergic receptors by nucleosides and nucleotides may act to modulate, rather than directly stimulate mast cell activation. For example, it has been widely reported that adenosine and inosine potentiate antigen dependent mast cell activation (Tilley *et al.*, 2000; Reeves *et al.*, 1997; Marquardt *et al.*, 1978). Furthermore, ADP and ATP have been shown to attenuate peptidoglycan (PGN)-induced TNF- α production in human mast cells (Feng *et al.*, 2004).

The intact intestinal mucosal barrier is relatively impervious to bacteria and macromolecules that reside within the lumen of the gut. However, during infection with *T. spiralis*, infective larvae invade and migrate through the intestinal epithelium, leaving in their wake a trail of damaged cells (Wright, 1979). Furthermore, the mMCP-1-dependent increased paracellular permeability disrupts the intestinal epithelial barrier function (McDermott *et al.*, 2003; Madden *et al.*, 2002). The altered intestinal environment during infection may allow bacterial LPS and other pathogen-associated molecular patterns (PAMPS) usually present in the lumen of the gut to come into contact with mucosal mast cells and other cell types found in the epithelium. Toll-like receptors (TLR) serve as sensors against pathogens that invade the host by recognising (PAMPS) (Akira and Hemmi, 2003). Although intestinal mast cells have not been shown to express TLRs *in vivo*, it has been demonstrated that TLR-2, TLR-4 and TLR-6 are expressed by murine bone marrow-derived mast cells *in vitro* (McCurdy *et al.*, 2003; Supajatura *et al.*, 2002; Matsushima *et al.*, 2004). Given the prime location of mucosal mast cells in the intestinal epithelium, recognising PAMPS could be an important but as yet uncharacterised aspect of their role in gut immunology.

TLR-4 is activated by LPS derived from Gram-negative bacteria such as *Escherichia coli* (Hoshino *et al.*, 1999). Activation of mBMMC TLR-4 induces the release of TNF- α and other inflammatory cytokines (Supajatura *et al.*, 2001). TNF- α can directly and indirectly promote inflammation during the intestinal phase of *T. spiralis* infection (Scales *et al.*, 2004; Lawrence *et al.*, 2004). Furthermore, this cytokine is predicted to be released from intestinal mucosal mast cells during *T. spiralis* infection (Lawrence *et al.*, 2004). Although the *in vivo* effects of TLR activation on the kinetics and pathology of *T. spiralis* infection still remain to be determined, it has been postulated that opportunistic bacterial infections may affect the intestinal pathology and host immune response (Garside *et al.*, 2000).

The TLR-4 ligand LPS induced a dose-dependent increase in TNF- α secretion from mBMMC, at levels consistent with that reported by Supajatura *et al.* (2001). Pre-treatment of mBMMC with adenosine at concentrations at and above 10 μ M, prior to activation with LPS, inhibited TNF- α secretion in a dose-dependent manner. This is consistent with a body of evidence that suggests adenosine acts as an anti-inflammatory molecule (Linden, 2001; la Sala *et al.*, 2003; Thiel *et al.*, 2003). For example, adenosine acting *via* P1 receptors can attenuate TLR-induced TNF- α generation in macrophages and monocytes (Hasko *et al.*, 1996, 2000; Pinhal-Enfield *et al.*, 2003). Furthermore, *in vivo* experiments have shown that adenosine can mediate inhibition of LPS-stimulated TNF- α production (Salvatore *et al.*, 2000). However, the results presented here show for the first time that adenosine can modulate TLR-4 activation in mast cells. Further experiments to elucidate the P1 receptor sub-type mediating the inhibitory effect of adenosine could prove interesting.

The adenine nucleotides ADP and ATP at concentrations at or above 1 μ M also strikingly suppressed TNF- α production by mBMMC stimulated with LPS. This is consistent with a similar study that reported that adenine nucleotides inhibit cytokine generation in human mast cells stimulated with the TLR-2/TLR-6 ligand peptidoglycan (PGN) (Feng *et al.*, 2004). In the study reported by Feng and co-workers (2004), the specific purinergic receptor sub-types mediating this effect could not be elucidated, despite a range of receptor antagonists being used. Other nucleosides and nucleotides such as inosine, UDP and UTP did not affect LPS-induced TNF- α generation. This implies a potential lack of involvement of the A₃, P2Y₆/CysLTR1 and P2Y₂ purinergic receptors *via* which these ligands signal. Further experiments are required to fully characterise the antagonistic action of adenosine, ADP and ATP at other TLR-types, and to define the purinergic receptors and the intracellular signalling mechanisms involved in this phenomenon. Potentially, adenosine and adenine nucleotides signalling *via* purinergic receptors may stimulate a negative feedback mechanism to control inflammation in the intestine.

During *T. spiralis* infection the concurrent stimulation of TLR-4 and inhibitory pathways mediated by adenosine, ADP or ATP, released from damaged or activated cells, could potentially prevent over-exuberant secretion of pro-inflammatory cytokines such as TNF- α , thus limiting the enteropathy observed during the infection. This may benefit the host by reducing the debilitating effects inflammation causes, but also benefit the parasite by maintaining an environment in which it can survive.

Paradoxically, however, *T. spiralis* secretes enzymes able to metabolise nucleotides, and as a result may be able to inhibit the down-regulatory action of extracellular nucleotides. This may actually promote inflammatory cytokine production from mast cells, thus potentially enhancing intestinal pathology. When this was investigated *in vitro*, the presence of *T. spiralis* ES did not alter the inhibitory effect of adenosine on LPS-induced TNF- α production. The relatively low specific activity of the adenosine deaminase in *T. spiralis* ES (51 mU/mg of ES, where 1 unit is equivalent to the deamination of 1 μ mol adenosine/minute) (Gounaris, 2002), could account for the lack of modulation of adenosine-induced inhibition observed when concentrations of adenosine greater than 10 μ M were used.

The presence of *T. spiralis* ES reversed the inhibitory effects of low (1 μ M) concentrations of ADP on TLR-4-mediated TNF- α production. Use of the recombinant 5'-nucleotidase (5'-NT) showed that this was due to the action of the secreted 5'-NT which would metabolise the available ADP to adenosine, thus limiting P2Y receptor activation. The amount of adenosine resulting from ADP hydrolysis (≤ 1 μ M) would be insufficient to inhibit TLR-4 induced TNF- α secretion. The presence of either *T. spiralis* ES or recombinant 5'-NT was unable to modulate the inhibitory effects of concentrations of ADP at or above 10 μ M. Considering that the product of the enzymatic action of the 5'-NT acting on ADP would be adenosine, the inhibition of TLR-4 mediated TNF- α secretion could be elicited by a combination of both non-hydrolysed ADP and high concentrations of accumulated adenosine. As mentioned previously, the slower rate of adenosine metabolism by the adenosine deaminase may account for the continued inhibition observed when concentrations of ADP above 10 μ M were used in the assay.

Thus, it appears that *T. spiralis* ES can modulate the inhibitory effects of relatively low (1 μ M) concentrations of adenosine and ADP on TLR-4 activation. This suggests that *T. spiralis* ES may promote inflammatory cytokine release from mast cells. The relevance of this observation *in vivo* remains to be determined, as one would have expected this would be deleterious to the survival of parasites in the host intestinal tract. Further investigation of the effects of *T. spiralis* secreted proteins and nucleotides on TLR activation in mast cells is required, especially considering other parasitic helminth antigens can regulate TLR responses in diverse immune cells types (Goodridge *et al.*, 2005; Kane *et al.*, 2004).

In summary, this section reveals that ADP and ATP can induce mBMMC exocytosis. Importantly, these nucleotides elicit the release of mMCP-1 *via* a mechanism that is not dependent on *de novo* protein synthesis or the Golgi complex, indirectly suggesting adenine nucleotides induce exocytosis of granules containing pre-formed mMCP-1. ADP appeared to mediate this effect *via* stimulation of the P2Y₁ receptor. The purinergic receptor *via* which ATP was acting was not confirmed, but was likely to be the P2X₄ receptor on the basis of the calcium flux data presented in chapter 3. Interestingly, *T. spiralis* ES inhibited ADP-induced mMCP-1 release, and this was mediated by the action of the secreted 5'-NT. *T. spiralis* ES also inhibited ATP-induced mMCP-1 release, possibly *via* the action of the secreted serine/threonine kinases and NDPK. Putatively, this could act to perturb mMCP-1 release during the intestinal stage of *T. spiralis* infection, thus reducing the rate of parasite expulsion. The range of nucleosides and nucleotides tested could not direct the migration of progenitor, immature or mature mBMMC, suggesting these compounds are not chemoattractants for this type of mast cell. Furthermore, nucleosides and nucleotides alone could not induce the *de novo* generation of inflammatory or T_H2 cytokines in mBMMC. However, the simultaneous activation of TLR-4 and purinergic receptors responsive to adenosine, ADP or ATP stimulated inhibitory pathways that led to the down-regulation of pro-inflammatory TNF- α generation. In the presence of *T. spiralis* ES, the inhibitory effects of low (1 μ M) concentrations of ADP were reduced, suggesting that parasite secreted proteins may also act to enhance the amount of pro-inflammatory cytokines generated by TLR activation.

Chapter 5

The effect of immunisation with the recombinant *T. spiralis* 5'-nucleotidase on infection outcome in the mouse

5.1 Introduction

During *Trichinella spiralis* infection, ingested infective larvae invade the small intestine. These parasites then rapidly develop into adults and release new born larvae that migrate through the lymphatic and circulatory systems into the host muscle where they form nurse cells. Although the intestinal phase of infection is eventually resolved, adult parasites are not expelled from the intestine until they have released new born larvae, successfully completing their life cycle. Primary *T. spiralis* infection stimulates strong immune responses in the mouse which are CD4⁺ T cell-dependent (Grencis *et al.*, 1985) and polarized towards the T_H2 phenotype (Grencis *et al.*, 1991; Finkelman *et al.*, 1997). The expulsion of *T. spiralis* from the intestinal compartment is thought to involve the complex interplay of both innate and parasite-specific (adaptive) effector mechanisms (Knight *et al.*, 2004; Miller, 1987). Primary *T. spiralis* infection in mice leads to a strong long lasting immunity to reinfection, and this immunity can be adoptively transferred using T cells present in the mesenteric lymph nodes that are rapidly restimulated upon challenge (Wakelin and Wilson, 1979; Grecnis and Wakelin, 1982). Transfer of immunity to a primary *T. spiralis* infection in the mouse can also be achieved using immune sera from animals immunised with parasite antigens (Jarvis and Pritchard, 1992; Robinson *et al.*, 1995b).

High levels of protection against *T. spiralis* infection can be achieved in mice by systemic immunisation with a variety of different types of parasite antigens (summarised in Table 5-1). When immunised animals are challenged with *T. spiralis*

Antigen preparation	Author
γ -irradiated infective larvae	Larsh <i>et al.</i> , 1959
crude parasite homogenate preparations	Despommier <i>et al.</i> , 1977; Despommier and Laccetti, 1981
secreted parasite antigens (ES)	Wakelin <i>et al.</i> , 1986; Robinson <i>et al.</i> , 1994
purified secreted antigens	Siberstein and Despommier, 1984
parasite-surface antigens	Grencis <i>et al.</i> , 1986

Table 5.1 Native parasite antigens that provide protection against *T. spiralis* infection

infection, worms are expelled more rapidly from the intestine and their growth and fecundity are reduced. Thus, it appears that immunisation interferes with the development of *T. spiralis* during the early stages of the enteral phase of its life cycle.

The majority of immunisation-challenge studies have used native parasite antigens and only one example of a non-native protein conferring resistance to *T. spiralis* infection exists (Robinson *et al.* 1995c). Robinson and co-workers (1995c) demonstrated that a synthetic 40-mer peptide of the immunodominant 43 kDa glycoprotein secreted by the infective larval L1 stage, was able to reduce intestinal parasite burden in mice by ~50%. Currently, there are no examples in the literature of recombinant proteins being used to immunise animals against subsequent *T. spiralis* infection. However, recombinant proteins have successfully been used to immunise animals against other types of intestinal helminth infections (Loukas *et al.*, 2004; Ball *et al.*, 2007; Tsuji *et al.*, 2001).

Considering that the secreted *T. spiralis* 5'-nucleotidase (5'-NT) has already been cloned and biochemically characterised (Gounaris *et al.*, 2004), it seemed important to establish whether immunisation with this catalytically active recombinant protein could protect mice from *T. spiralis* infection. This enzyme has been postulated to affect the function of immune cells (Gounaris *et al.*, 2004) and, as shown in Chapters 3 and 4 of this work, can modulate mast cell effector functions *in vitro*. It may therefore contribute to the survival of the parasite during the enteric phase of infection. The aim of the work described in this chapter was to examine whether immunisation with the recombinant 5'-NT could protect mice from *T. spiralis* infection. Ultimately, the purpose of this experiment was to provide insight into the *in vivo* function of the secreted 5'-NT. Moreover, the outcome of this experiment could have implications for other intestinal parasites, as it has been suggested that secretion of nucleotide metabolising enzymes may be common to other nematode species (Gounaris and Selkirk, 2005). Specifically, 5'-nucleotidase activity has been detected in the secreted proteins of *Nippostrongylus brasiliensis* and close homologues of the 5'-nucleotidase are present in the *Trichuris muris* expressed sequence tag dataset (K. Gounaris and M.E. Selkirk, unpublished).

To determine whether immunisation with the recombinant 5'-NT could induce a protective effect, the kinetics of *T. spiralis* expulsion from the small intestine and the establishment of muscle stage larvae were characterised. As previous immunisation studies using native parasite antigens had shown that the main effect of vaccination was

to increase and accelerate antibody and cellular responses to subsequent *T. spiralis* infection (Robinson *et al.*, 1995a), the profile of the immune response generated by immunisation with the 5'-NT was also investigated both before and after infection.

Immunisation of mice with the 5'-NT was expected to generate the production and maintenance of high affinity neutralising antibodies, and the induction of a rapid secondary immune response involving a number of immune cell types. To confirm this had occurred, the serum-titres of 5'-NT-specific antibody isotypes were monitored as the course of immunisation was expected to generate plasma cells that secrete isotype-switched high affinity 5'-NT-specific antibodies. The presence of high titres of neutralising antibodies prior to *T. spiralis* infection would potentially be capable of systemically blocking the nucleotide metabolising activity of the 5'-NT present in *T. spiralis* secreted proteins. To examine whether B cell responses stimulated by the immunisation had generated neutralising antibodies that would inhibit the nucleotide metabolising activity of the 5'-NT, sera from immunised animals were used in 5'-NT enzyme activity assays.

Immunisation of mice with the 5'-NT was also expected to generate 5'-NT-specific T cells in the mesenteric lymph nodes and spleen. These would be capable of rapidly responding to processed parasite-derived 5'-NT-antigen displayed on antigen presenting cells during the infection *in vivo*. To confirm that the immunisation process had primed T cells, 5'-NT-specific proliferative responses of mesenteric lymph node (MLN) and spleen cells were measured. The profile of 5'-NT-specific cytokine production in the MLN was also investigated as a marker of T-helper cell subset activity.

5.2 Results

5.2.1 Expression of active recombinant *T. spiralis* 5'-nucleotidase.

The previously cloned *T. spiralis* 5'-nucleotidase (5'-NT) (Gounaris *et al.*, 2004) was expressed in the yeast *Pichia pastoris* as a secreted protein. Nickel-chelating chromatography was used to purify the protein *via* a C-terminal polyhistidine tag. As shown in Figure 5-1 (A), the purified protein resolved by SDS-PAGE with an apparent molecular mass of ~66-68 kDa, corresponding very closely to that of the native protein (67 kDa) which lacks the histidine tag (Gounaris, 2002). To confirm that the Coomassie blue-stained protein eluted by nickel-chelating chromatography was the recombinant 5'-nucleotidase, Western blotting using an antibody to the C-myc epitope, which had been incorporated into the recombinant protein during cloning, was carried out. A single band of approximately 66-68 kDa was observed [Figure 5-1(B)], confirming successful purification of the recombinant 5'-NT.

The purified recombinant 5'-nucleotidase and *T. spiralis* ES were analysed for 5'-nucleotidase activity by measuring the amount of inorganic phosphate released from nucleotide substrates (as detailed in the Materials and Methods section). The nucleotides AMP and UDP, already known to be the preferential substrates for the *T. spiralis* 5'-NT (Gounaris *et al.*, 2004), were used as substrates in the activity assays. As shown in Figure 5-2 (A), using excess substrate and optimal buffer conditions (as described by Gounaris *et al.*, 2004), the specific activity of the 5'-nucleotidase present in *T. spiralis* ES was established to be 3.77 ± 0.4 $\mu\text{mol/min/mg}$ and 0.4 ± 0.1 $\mu\text{mol/min/mg}$ for AMP and UDP respectively. This is consistent with previous reports (Gounaris, 2002). In further experiments, 1 μg and 40 ng were chosen as the optimal amounts of ES to use in 5'-nucleotidase activity assays utilising UDP and AMP as substrates respectively. The specific activity of the recombinant 5'-nucleotidase utilising AMP and UDP as substrates were 36.4 ± 3.5 $\mu\text{mol/min/mg}$ and 3.9 ± 1.3 $\mu\text{mol/min/mg}$ respectively [Figure 5-2 (B)]. The purified recombinant 5'-NT exhibited high specific activity and was approximately 5 times more active than the enzyme purified previously (Gounaris *et al.*, 2004). In further experiments, 100 ng and 15 ng of recombinant 5'-NT were used in activity assays utilising UDP and AMP as substrates respectively.

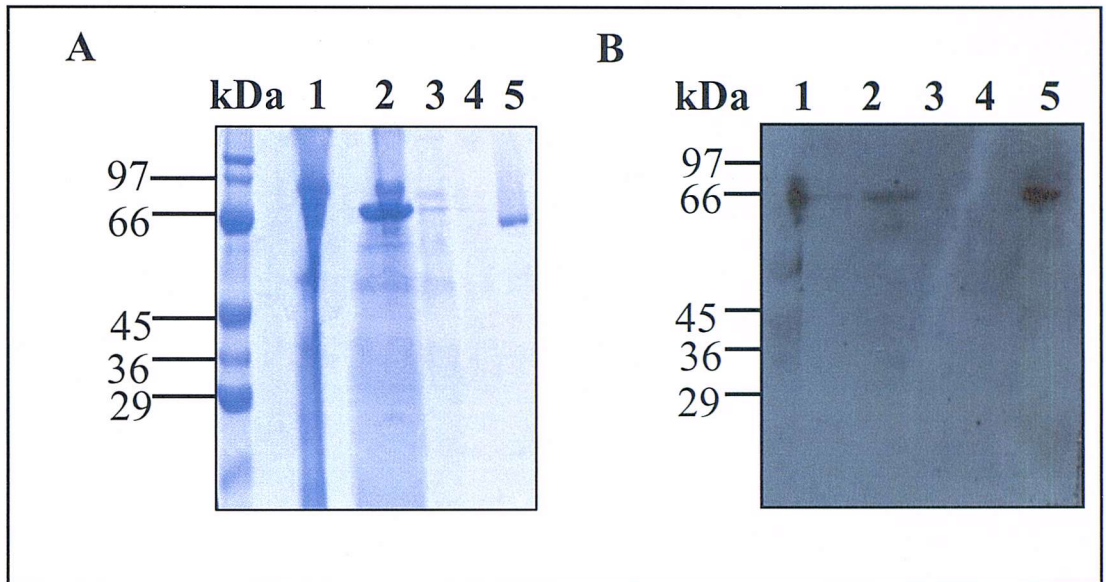


Figure 5-1 Ni-NTA purification of the recombinant *T. spiralis* 5'-NT

Proteins were resolved by 12% (w/v) SDS-PAGE and visualised by (A) Coomassie staining and (B) Western blotting using an antibody to the C-myc epitope. Yeast culture supernatant was concentrated, dialysed and applied to a Ni-NTA matrix (lane 1). Unbound proteins were removed (lane 2) and the matrix washed with buffer containing 1 mM imidazole (lane 3) and 5 mM imidazole (lane 4). Strongly bound proteins were eluted using 100 mM imidazole (lane 5). The migration of molecular mass markers is indicated in kDa.

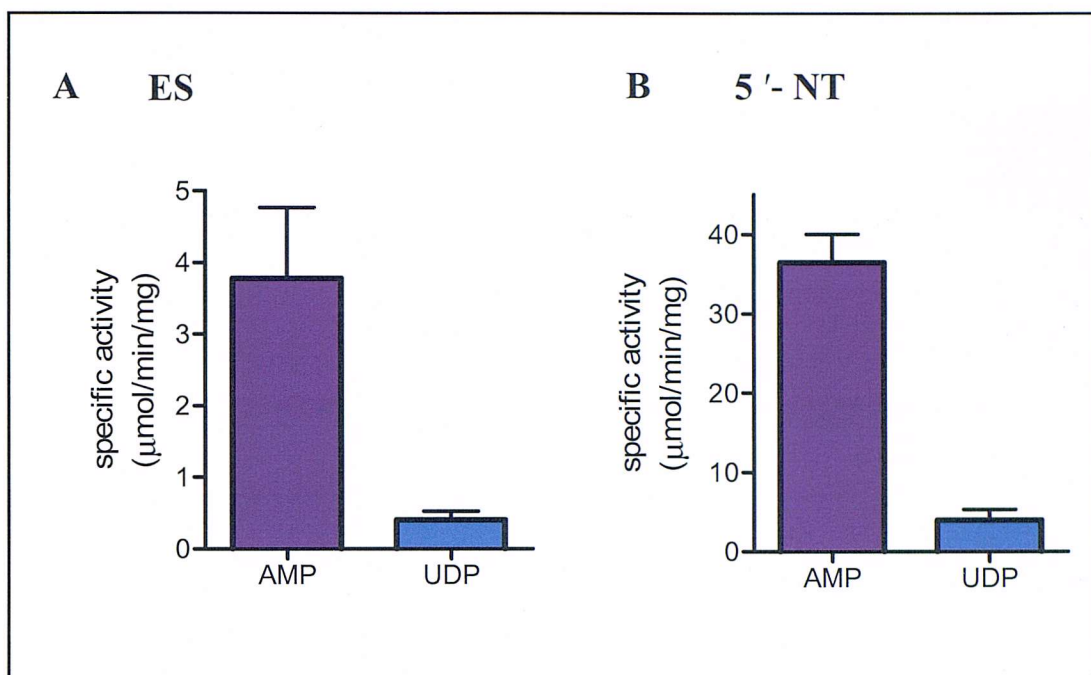


Figure 5-2 Nucleotide substrate specificity of *T. spiralis* ES and 5'-NT

T. spiralis ES and 5'-NT were used in phosphate-release assays as detailed in Materials and Methods to determine the activity of the native secreted and recombinant 5'-nucleotidase. Specific 5'-nucleotidase activities of *T. spiralis* ES (A) and 5'-NT (B) were determined using the substrates AMP and UDP. Data represent the mean + s.d.m of 3 batches of ES and one batch of expressed 5'-nucleotidase. Experiments were performed in triplicate.

5.2.2 Analysis of 5'-nucleotidase expression and secretion by different *T. spiralis* life-stages.

To determine the stage specificity of expression of the 5'-NT, reverse transcriptase (RT)-PCRs were carried out using DNase-digested RNA extracted from muscle stage, adult and new born larvae. To exclude the possibility of genomic DNA contaminating cDNA preparations, control PCRs were carried out using DNase-treated RNA and tubulin primers. No PCR products were generated from all batches of RNA used, indicating genomic DNA was not contaminating (data shown in Appendix 1-4). In order to standardise cDNA levels between batches of RNA, tubulin primers were used as a positive control. Representative RT-PCR analyses of *T. spiralis* 5'-NT expression are displayed in Figure 5-3 (A). Adult, newborn larvae and muscle stage larvae strongly expressed transcripts corresponding to the 5'-NT [Figure 5-3 (A)], indicating that this gene is expressed throughout the parasite life cycle.

T. spiralis muscle stage larvae secrete the 5'-NT (Gounaris, 2002), thus it was of interest to determine whether this enzyme is secreted by other parasite life stages. To examine the stage specificity of 5'-NT secretion, ES proteins were collected from adult and muscle stage larvae. Western blot analysis using rabbit anti-*T. spiralis* 5'-NT polyclonal antibody sera detected single bands of the correct size (67 kDa) in both adult and muscle stage larvae secreted proteins [Figure 5-3 (B)], suggesting this enzyme is secreted throughout the enteral and parenteral stages of the life-cycle.

5.2.3 The effects of immunisation with the recombinant 5'-NT on *T. spiralis* infection

The extent of protective immunity to *T. spiralis* infection in the mouse has been shown to be greatly influenced by host genetic factors. For example NIH mice naturally expel *T. spiralis* infections more rapidly than C57BL/10 mice (Wakelin and Donachie, 1983; Wakelin, 1988). Furthermore, immunisation of rapid-responder NIH mice with native parasite antigens confers a significant degree of protection against subsequent *T. spiralis* infection. In contrast, immunisation does not affect the expulsion kinetics of slow-responder C57BL/10 mice (Robinson *et al.*, 1994, 1995a; Wakelin *et al.*, 1986). For this reason, high-responder NIH mice were used in the immunisation trial described here.

In most model antigen systems, the generation of significant and long-lasting immune responses is dependent upon enhancing the immunogenicity of the antigen by

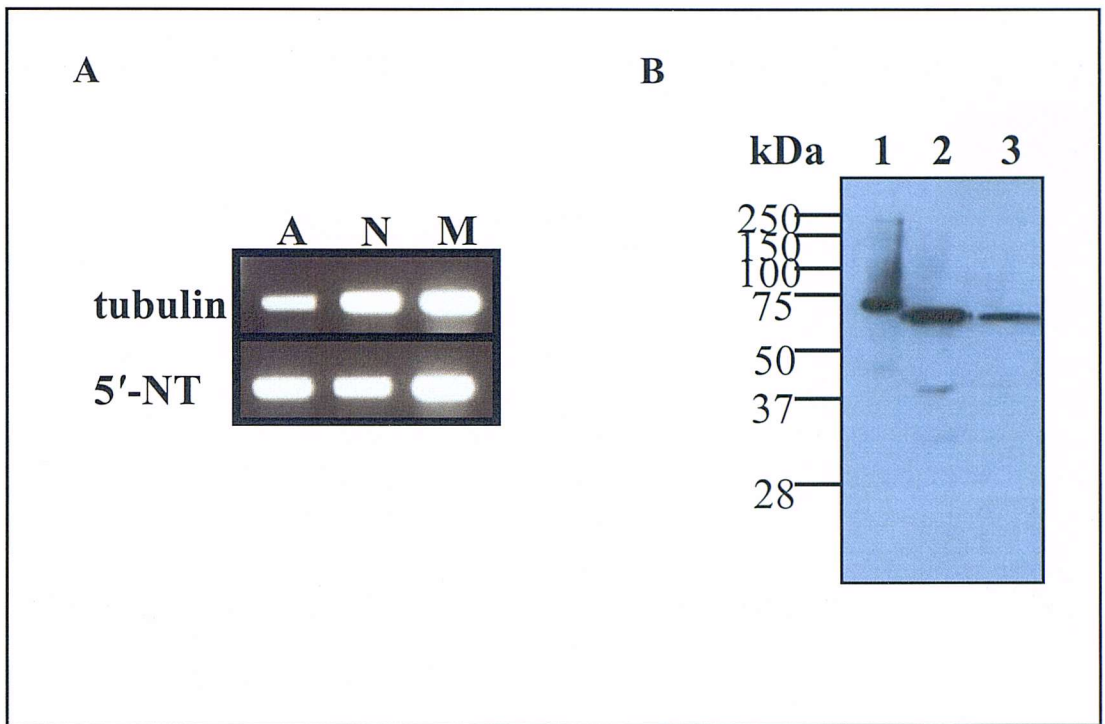


Figure 5-3 Stage specificity of 5'-NT expression and secretion

(A) Reverse transcriptase-PCR analyses of 5'-nucleotidase (5'-NT) and tubulin expression using RNA extracted from *T. spiralis* adult (lane A), newborn larvae (lane N) and muscle stage larvae (lane M). (B) Parasite proteins were resolved by SDS-PAGE and transferred to nitrocellulose by Western blotting. The lanes represent: (1) 2 µg recombinant 5'-NT, (2) 20 µg muscle stage larvae ES, (3) 20 µg adult ES. The migration of molecular mass markers is indicated in kDa. The blot was probed using rabbit anti-*T. spiralis* 5'-NT polyclonal antibody sera diluted 1/500 (vol/vol).

delivery with an adjuvant. The mineral gel adjuvant potassium alum has been reported to induce T_H2-type responses (Grun and Maurer, 1989), and has successfully protected mice from *T. spiralis* infection when used as an adjuvant in immunisation trials using *T. spiralis* antigen preparations (Robinson *et al.*, 1994). Considering that the generation of a T_H2-type immune response is associated with the expulsion of helminth parasites, this adjuvant was used to promote the development of a T_H2-type immune response directed toward the 5'-NT.

Mice were initially immunised with a relatively high dose of 5'-NT (25 µg) and boosted with lower doses (12.5 µg) 28 and 42 days later. Separate groups of control mice were sham-immunised with adjuvant only, in parallel to the 5'-NT-immunised animals. The timing of the immunisation schedule was used to allow the development of high affinity B cell clones, which require low levels of antigen to undergo affinity maturation (Goidl *et al.*, 1968). 49 days after the original immunisation, immunised and control mice were subsequently challenged with 500 *T. spiralis* infective larvae. The kinetics of parasite expulsion from the small intestine were monitored at days 4 and 7 post infection. The fecundity of female parasites was also assessed in terms of new born larvae production per adult parasite. The effect of immunisation on muscle stage larvae establishment was also evaluated 35 days post infection.

The immune response elicited in immunised-infected and sham-immunised-infected mice were compared to naive mice in terms of 5'-NT-specific antibody isotype profiles, cytokine responses and cellular proliferation in antigen-recall experiments. The titres of 5'-NT-specific neutralising antibodies present in the sera of immunised-infected, sham-immunised-infected and naïve animals were also determined by using the 5'-NT activity assay.

5.2.3.1 Anti-recombinant 5'-NT antibody isotype profile

To determine whether immunisation with the recombinant 5'-NT had induced antibody production, relative amounts of 5'-NT-specific IgG1, IgG2a, IgG2b, IgM and IgA in the serum were determined by ELISA. Serum was titrated and the levels of 5'-NT-specific antibody present expressed as optical density at 450 nm. As expected, infected sham-immunised animals had low levels of IgG1, IgG2a, IgG2b, IgA and IgM antibody isotypes present in their sera, which were not significantly different to uninfected sham-immunised animals (Figure 5-4). This is typical of a primary response to *T. spiralis* infection, where elevated levels of these antibody isotypes are not

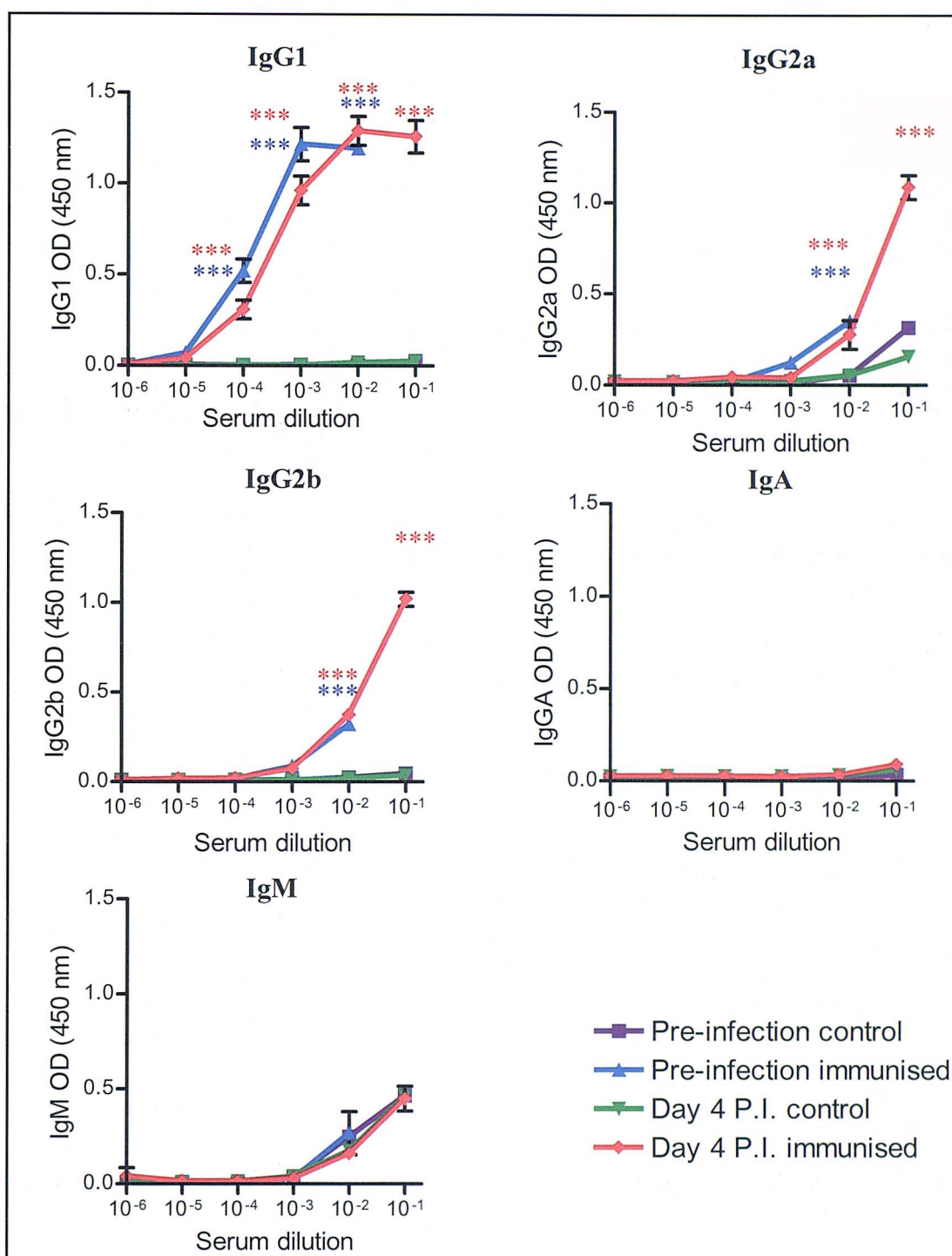


Figure 5-4 Levels of antigen specific antibody isotypes in sera from 5'-NT-immunised mice pre and post *T. spiralis* infection.

Sera were collected from control and immunised mice prior to and 4 days post *T. spiralis* infection (D4 PI). Sera were pooled and analysed for 5'-NT-specific antibodies by sandwich ELISA using plates coated with 5 µg/ml recombinant 5'-NT. Data were analysed using the unpaired students *t*-test and represent the mean $\pm$ s.d.m (n=3). Significant differences between immunised and control animals are indicated *** $p \leq 0.001$, ** $p \leq 0.01$.

observed until much later in infection (Goyal and Wakelin, 1993; Furze and Selkirk, 2005).

From the data shown in Figure 5-4, it is clear that the principal isotype induced by immunisation with the recombinant protein was IgG1, suggesting immunisation had stimulated a T_H2 type immune response. Levels of 5'-NT-specific IgG1 antibodies were elevated in 5'-NT-immunised animals prior to challenge *T. spiralis* infection and continued to bind antigen at dilutions of 1:10000. Levels of the IgG subclasses IgG2a and IgG2b were also elevated in animals immunised with the recombinant 5'-NT, and sera-titrations of these antibody isotypes continued to bind antigen at dilutions of 1:100 (Figure 5-4). Infection with *T. spiralis* did not appear to alter IgG1, IgG2a or IgG2b antibody isotype levels in 5'-NT-immunised animals.

Immunisation of mice with the recombinant 5'-NT did not elevate serum levels of IgM or IgA antibody isotypes (Figure 5-4). Furthermore, serum levels of these isotypes were not affected by *T. spiralis* infection (Figure 5-4). In general, these results suggest immunisation of mice with the recombinant 5'-NT prepared in alum adjuvant, successfully generated 5'-NT-specific antibody responses dominated by an IgG1 antibody isotype.

5.2.3.2 Cellular proliferation

To determine whether immunisation with the 5'-NT had induced antigen-specific T cells in the mesenteric lymph nodes (MLN) and spleen, cellular responsiveness to 5'-NT antigen was assessed (antigen recall). Considering that peak cellularity and lymphoblast activity in the MLN occurs on day 4 post-infection during secondary *T. spiralis* infection (Grencis and Wakelin, 1982), proliferative and cytokine responses in the MLN were monitored at day 4 post-infection.

Cells from the MLN [Figure 5-5 (A)], and spleen [Figure 5-5 (B)] of naïve (uninfected control), infected-5'-NT-immunised and infected-sham-immunised mice were stimulated using 8 µg/ml 5'-NT, and proliferation assessed by [3 H] thymidine incorporation. The super-antigen, staphylococcal enterotoxin B fragment (SEB), was used as a positive control to directly stimulate T cells. In order to determine if 5'-NT-specific responses were in part due to intrinsic mitogenic properties of the recombinant protein, cells from naïve animals were stimulated with the recombinant 5'-NT. As shown in Figure 5-5, the recombinant 5'-NT did not induce cellular proliferation of

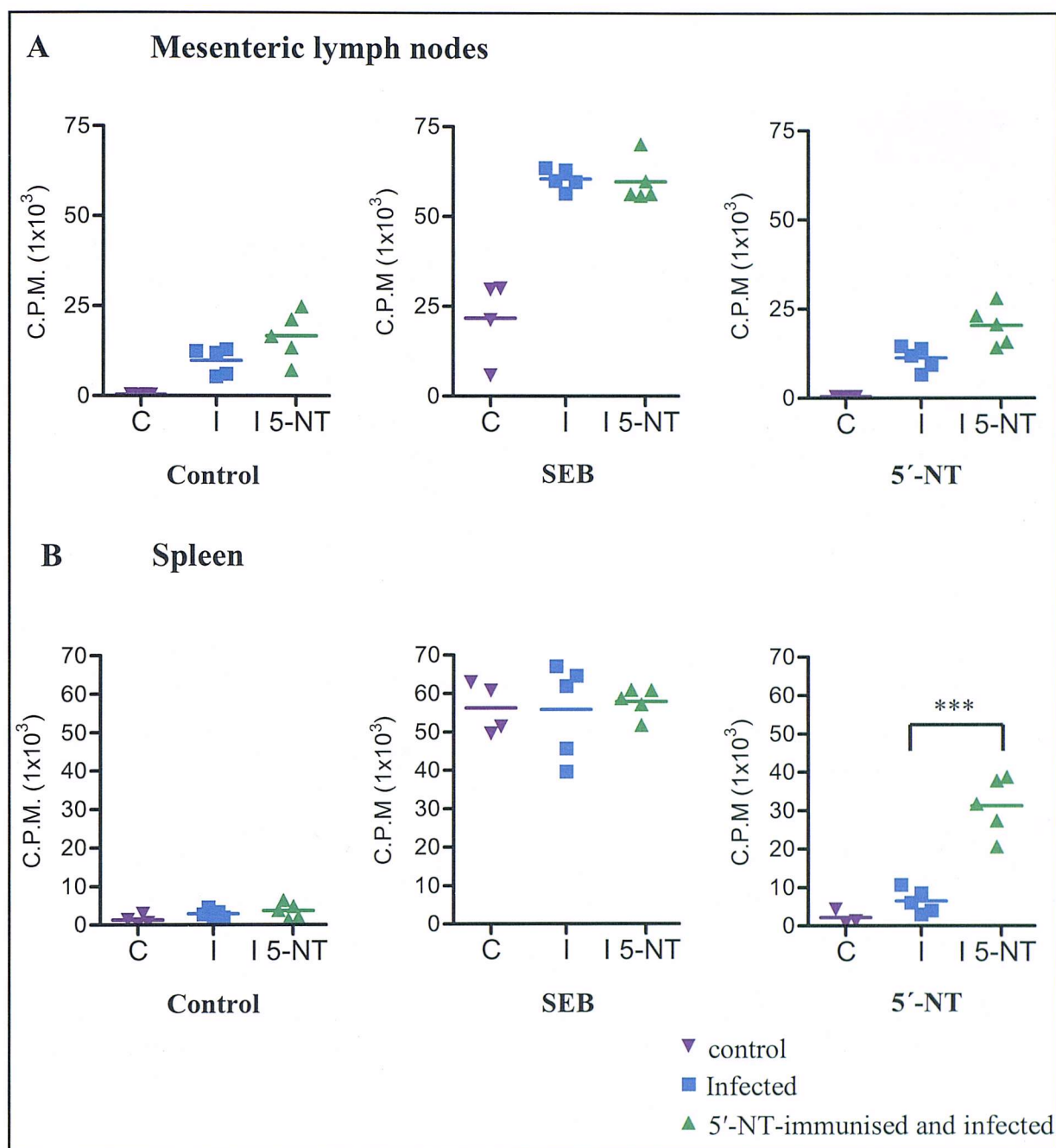


Figure 5-5 5'-NT-antigen specific induction of cellular proliferation in the spleen and mesenteric lymph nodes during the enteric phase of *T. spiralis* infection

Cells were isolated from naïve, *T. spiralis* infected and *T. spiralis* infected-5'-NT immunised mice, 4 days post infection. Mesenteric lymph node cells (A) or spleen cells (B) were cultured *ex vivo* for 72 hours and treated with media (control), 1 µg/ml Staphylococcal Enterotoxin B fragment (SEB) and 8 µg/ml 5'-NT, with the addition of 1 µCi [³H] thymidine for the final 24 hours of culture. Data are shown with each symbol representing individual mice. Data were analysed using the unpaired Student's *t*-test. Significance values are shown relative to infected sham-immunised animals and infected 5'-NT-immunised animals only *** $p \leq 0.001$.

MLN or spleen cells extracted from naïve animals, suggesting that it was not acting as a non-specific stimulus.

Cellular proliferative responses were measured at 4 days post-*T. spiralis* infection, as at this time point both MLN and spleen cells can respond to antigen (Grencis and Wakelin, 1982; Robinson *et al.*, 1995a; Wakelin *et al.*, 1985). As shown in Figure 5-5, MLN and spleen cells removed from *T. spiralis* infected animals exhibited higher basal rates of proliferation than naïve animals, indicative of generalised activation by infection. When stimulated with SEB, MLN cells removed from infected-sham-immunised, and infected-5'-NT-immunised mice, exhibited elevated proliferation in comparison to naïve animals [Figure 5-5 (A)], further implying that the infection had stimulated an immune response local to the intestine. Stimulation with the recombinant 5'-NT did not induce increased proliferation of MLN cells isolated from infected-5'-NT-immunised animals, suggesting immunisation with the recombinant 5'-NT had not induced the expansion of 5'-NT-specific T cells in the MLN [Figure 5-5 (A)]. Stimulation with the recombinant 5'-NT did not induce elevated proliferation in naïve or *T. spiralis* infected animals either [Figure 5-5 (A)].

When stimulated with SEB, spleen cells removed from naïve, infected-5'-NT-immunised, and infected-sham-immunised mice exhibited similar levels of elevated proliferation [Figure 5-5 (B)]. This suggests that at this time point the infection had not yet induced a systemic immune response. Stimulation with the recombinant 5'-NT induced increased proliferation of spleen cells taken from infected-5'-NT-immunised mice only [Figure 5-5 (B)], indicating that the immunisation process had expanded the population of 5'-NT-specific T cells in the spleen.

5.2.3.3 Cytokine response

In order to determine if immunisation with the 5'-NT induced differing cytokine profiles during *T. spiralis* infection, MLN cells were isolated and cultured *ex vivo* for 72 hours with 8 µg/ml 5'-NT. Antigen-specific IL-2, IL-4, IL-5, IFN-γ and TNF-α levels were assessed by using the BD™ cytometric bead array assay. Animals immunised with the 5'-NT prior to *T. spiralis* infection generated significantly higher levels of IL-4 and IL-5 (Figure 5-6), indicative of a T_H2 type cytokine environment typical of nematode infections. Notably, this response was dominated by IL-5, with levels reaching 2573 ± 1296 pg/ml (Figure 5-6).

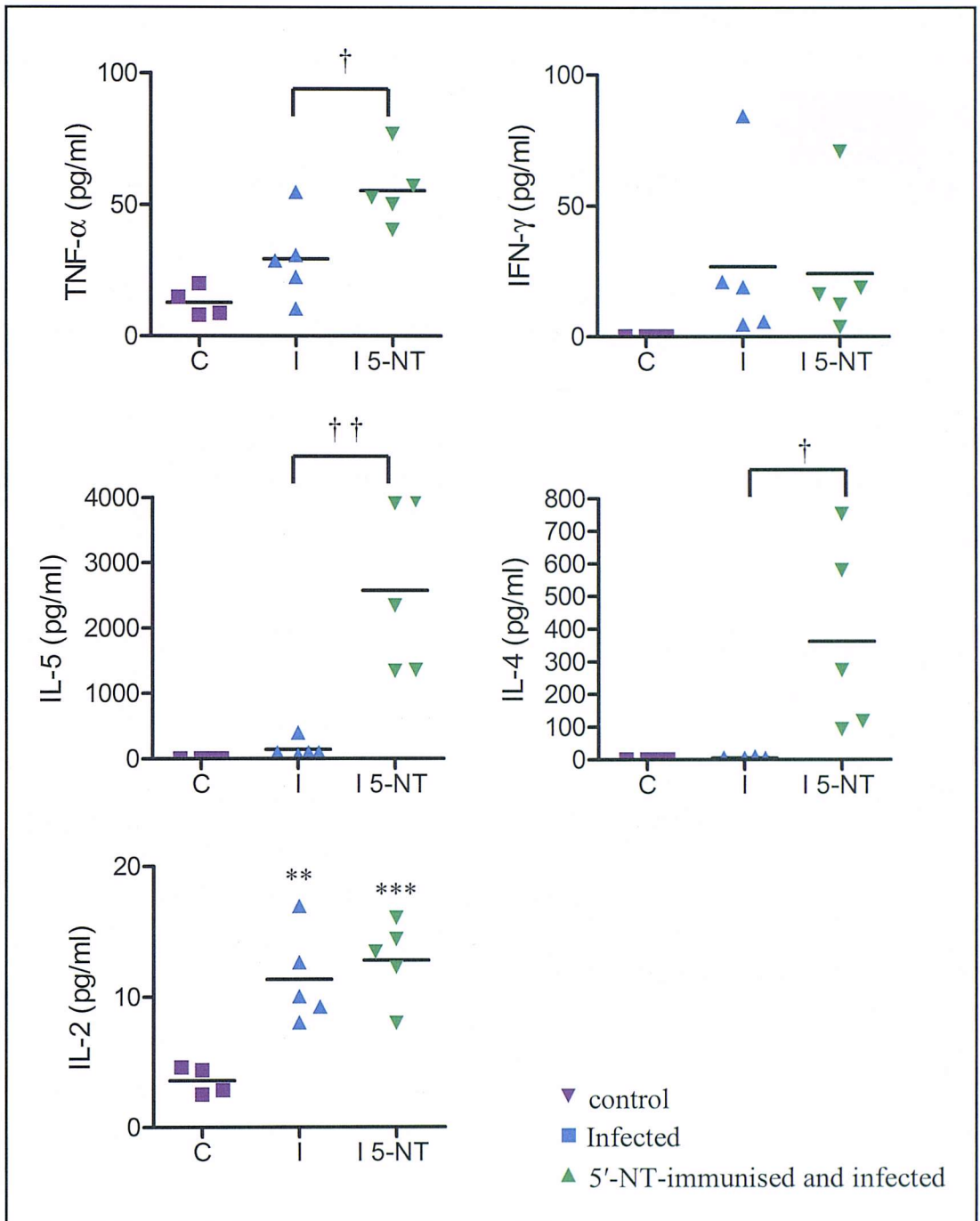


Figure 5-6 5-NT antigen specific cytokine production in mesenteric lymph node cultures 4 days post *T. spiralis* infection

Cells were isolated from naïve (C), *T. spiralis* infected (I), and *T. spiralis* infected-5'-NT immunised (I 5'-NT) mice, 4 days post infection. Cells were cultured *ex vivo* for 72 hours with 8 µg/ml 5'-NT. The concentration of cytokines in supernatants was determined by cytometric bead analysis. Data are shown with each symbol representing individual mice. Data were analysed using the unpaired Student's *t*-test. Significance values are shown relative to uninfected controls (C) *** $p \leq 0.001$, ** $p \leq 0.01$ and infected controls (I) † $p \leq 0.05$, †† $p \leq 0.01$.

Levels of the pro-inflammatory type-1 cytokine TNF- α was also elevated in infected-5'-NT-immunised mice compared to infected-sham-immunised animals (Figure 5-6). Production of cytokines associated with a T_H1 type environment such as IL-2 and IFN- γ was slightly increased in *T. spiralis* infected animals (Figure 5-6). This appeared to be independent of immunisation with the 5'-NT as stimulation with the recombinant protein did not alter levels of IL-2 or IFN- γ .

5.2.3.4 *T. spiralis* parasite burden in mice immunised with the recombinant 5'-NT.

NIH mice expel *T. spiralis* adult worms from the intestine within 10 days of infection (Robinson *et al.*, 1994). Hence, worm burdens following immunisation with *T. spiralis* recombinant 5'-NT were monitored 4 and 7 days post-infection. No significant differences between the numbers of adult worms recovered from sham or 5'-NT vaccinated mice at 4 or 7 days post-infection were observed [Figure 5-7 (A)]. The fecundity of female worms was also tested by incubating adult worms (both male and female) for 18 hours in media containing FCS. The numbers of NBL released were counted and are expressed relative to total number of adults cultured. No statistically significant differences between 5'-NT-immunised or sham-immunised mice were observed [Figure 5-7 (B)], suggesting that immunisation does not appear to affect the fecundity of female worms. To determine whether immunisation with the 5'-NT induced protective immune responses against establishment of parasites in the muscle, either by interfering with new born larvae survival and migration, or development of the nurse cell complex, muscle-stage larvae (MSL) were extracted from mice 35 days post-infection. As shown in Figure 5-7 (C), slightly reduced numbers of MSL were recovered from mice immunised with the 5'-NT, but this was not statistically significant.

5.2.4 The effect of sera on 5'-NT activity

In order to determine whether the immunisation induced the production of neutralising antibodies capable of blocking the activity of *T. spiralis* recombinant 5'-NT, sera at a final dilution of 1/100 were used in the phosphate-release assays. Unexpectedly, sera from uninfected control mice increased the activity of the recombinant 5'-NT by approximately 3-fold, in terms of both AMP and UDP hydrolysis [Figure 5-8 (A) and (B)]. However, the activity of the recombinant protein was not enhanced when sera from 5'-NT-immunised animals was used [Figure 5-8 (A) and (B)].

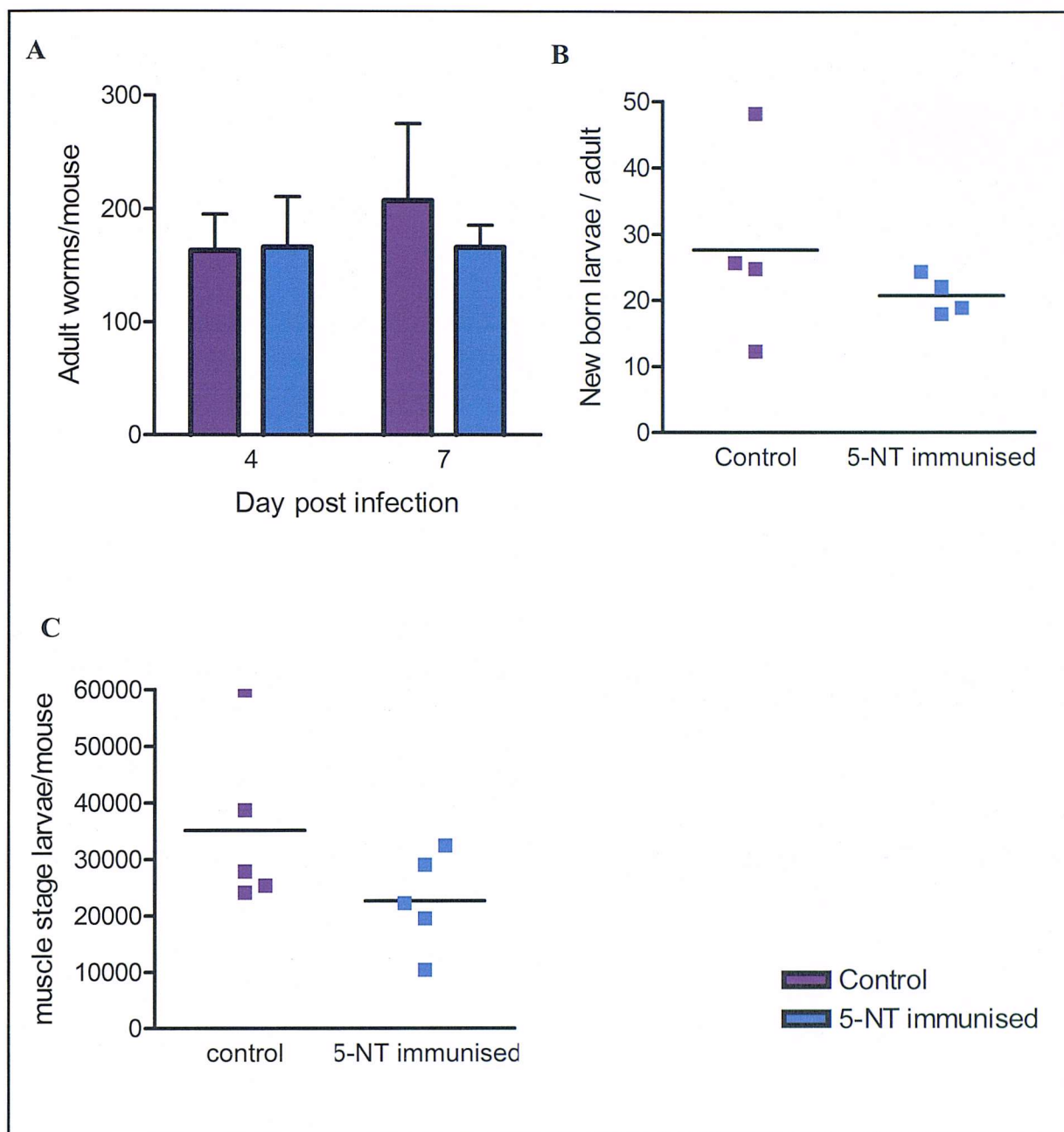


Figure 5-7 The effect of immunisation with the recombinant *T. spiralis* 5'-NT on *T. spiralis* parasite burden in mice

Mice were immunised with adjuvant (control) or adjuvant + 5'-NT (5'-NT immunised) and subsequently infected with 500 *T. spiralis* infective larvae. Groups of mice were sacrificed at days 4 and 7 days post infection and the total number of adult parasites were enumerated (A). Adults extracted at day 7 were cultured overnight in worm media containing FCS and the following day new born larvae were collected and counted (B). To determine the muscle stage larvae burden, mice were sacrificed 35 days post infection and the level of parasites enumerated (C). Data are shown with each symbol representing individual mice. Data were analysed using the unpaired Student's t-test and represent the mean + s.d.m.

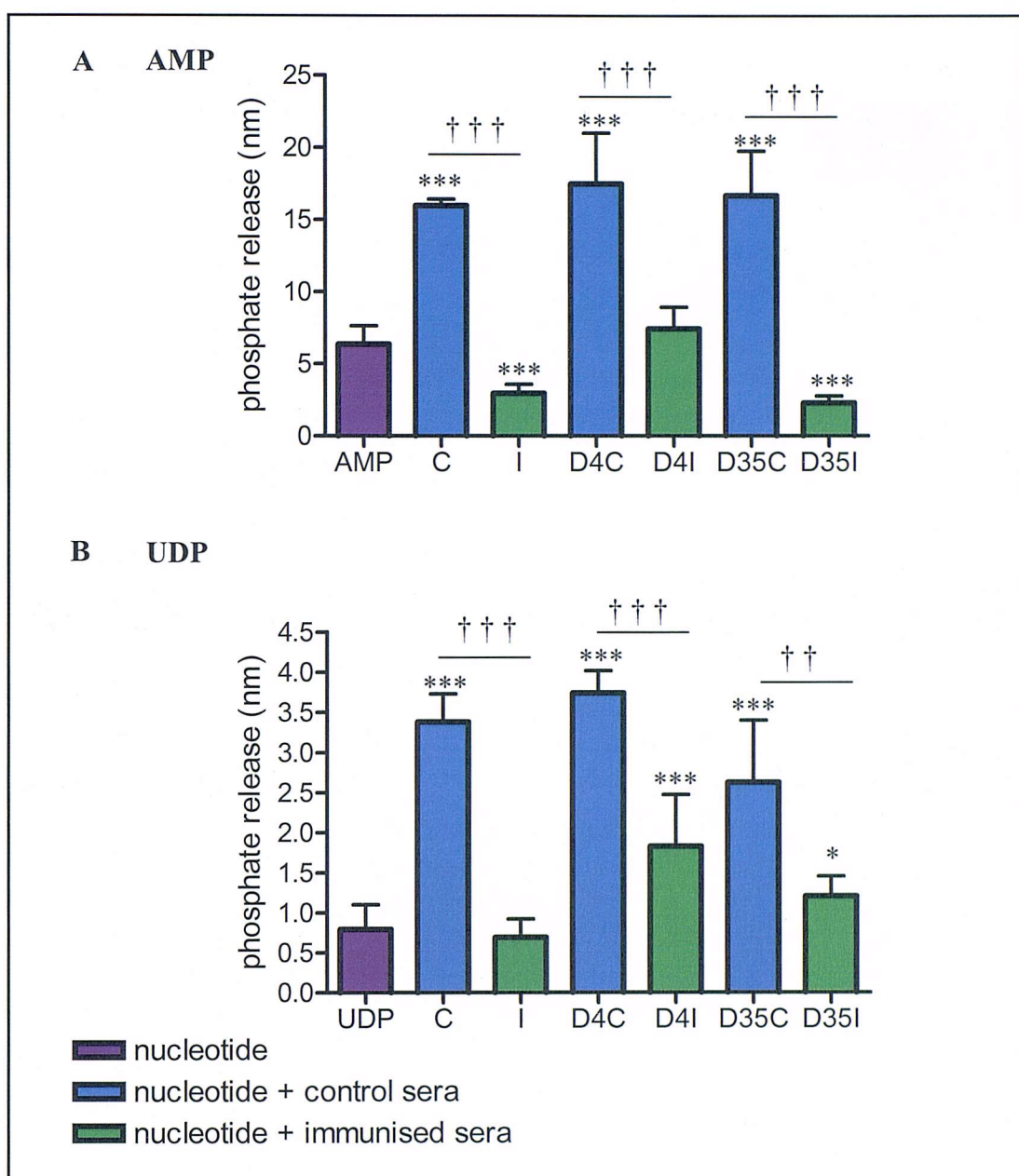


Figure 5-8 The effect of sera from control and 5'-NT-immunised mice on 5'-NT activity

Prior to infection sera were collected from control (C) and 5'-NT immunised (I) mice. Sera were also collected from the same animals 4 days (D4C, D4I) and 35 days (D35C, D35I) post *T. spiralis* infection. Sera were diluted 1/100 and used in a phosphate release assay utilising (A) 2.5 mM AMP and 15 ng 5'-NT, and (B) 2.5 mM UDP and 40 ng 5'-NT. Data were analysed using the unpaired two-tail Student's *t*-test and represent the mean + s.d.m of assays performed in duplicate using sera from groups of at least 4 mice mice. Significance values are shown relative to nucleotide + 5'-NT, ** $p \leq 0.01$, *** $p \leq 0.001$, and nucleotide + 5'-NT + sera from immunised and control animals at the equivalent time point ††† $p \leq 0.01$, †† $p \leq 0.001$.

This suggests the presence of a factor, most likely antibody, in the immune serum that inhibited the enhanced activity of the enzyme. It is important to note that control reactions (as detailed in the Materials and Methods section) were performed to exclude the possibility that enhanced 5'-NT activity or elevated levels of nucleotides were present in the sera. Sera extracted from 5'-NT-immunised mice at day 4 and 35 post *T. spiralis* infection consistently inhibited the activity of the recombinant protein [Figure 5-8 (A) and (B)], indicating that neutralising antibodies were present throughout the *T. spiralis* infection.

To investigate whether this phenomenon was restricted to the recombinant 5'-NT, the experiment was repeated using *T. spiralis* ES containing native 5'-NT activity. As shown in Figure 5-9, sera from naïve and sham-immunised animals also potentiated the hydrolysis of AMP and UDP by the native 5'-NT. In correlation with the data observed using the recombinant 5'-NT (Figure 5-8), sera from 5'-NT-immunised animals prior to and during *T. spiralis* infection consistently inhibited the hydrolysis of both AMP and UDP [Figure 5-9 (A) and (B)]. This suggests that the neutralising antibodies present in the sera of immunised mice recognised both native and recombinant forms of the 5'-NT.

To determine if the inhibitory factor present in sera from 5'-NT-immunised mice was an antibody or other protein, sera were heated to 90°C for 10 minutes to denature all heat-labile proteins. As shown in Figure 5-10, the inhibitory effect of sera from 5'-NT-immunised mice on recombinant 5'-NT activity was lost upon heat treatment indicating that this effect was due to a heat-labile protein. Heat treatment of sera from immunised animals resulted in the potentiation of enzyme activity (Figure 5-10), in a similar manner to sera from naïve mice (compare Figure 5-9 and 5-10). Therefore, this suggests that the factor that resulted in the enhancement of 5'-NT activity was most likely not a protein.

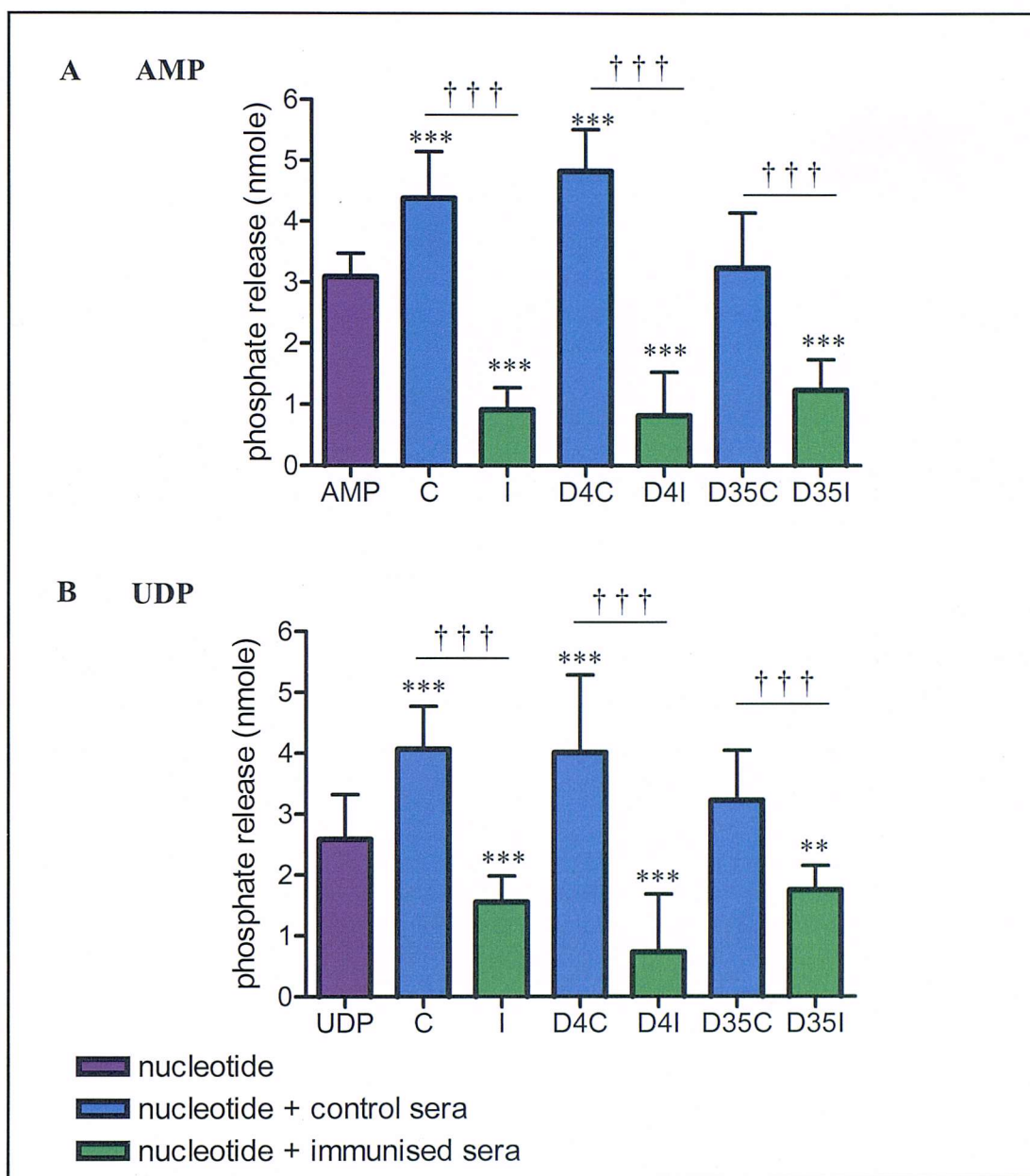


Figure 5-9 The effect of sera from control and 5'-NT immunised mice on 5'-NT activity in *T. spiralis* ES

Prior to infection sera were collected from control (C) and 5'-NT immunised (I) mice. Sera were also collected from the same animals 4 days (D4C, D4I) and 35 days (D35C, D35I) post *T. spiralis* infection. Sera were diluted 1/100 and used in a phosphate release assay utilising (A) 2.5 mM AMP and 100 ng ES, and (B) 2.5 mM UDP and 1 μ g ES. Data were analysed using the unpaired two-tail Student's *t*-test and represent the mean + s.d.m of assays performed in duplicate using sera from groups of at least 4 mice. Significance values are shown relative to nucleotide + ES, ** $p \leq 0.01$, *** $p \leq 0.001$ and sera from immunised and control animals at the equivalent time point † † † $p \leq 0.001$.

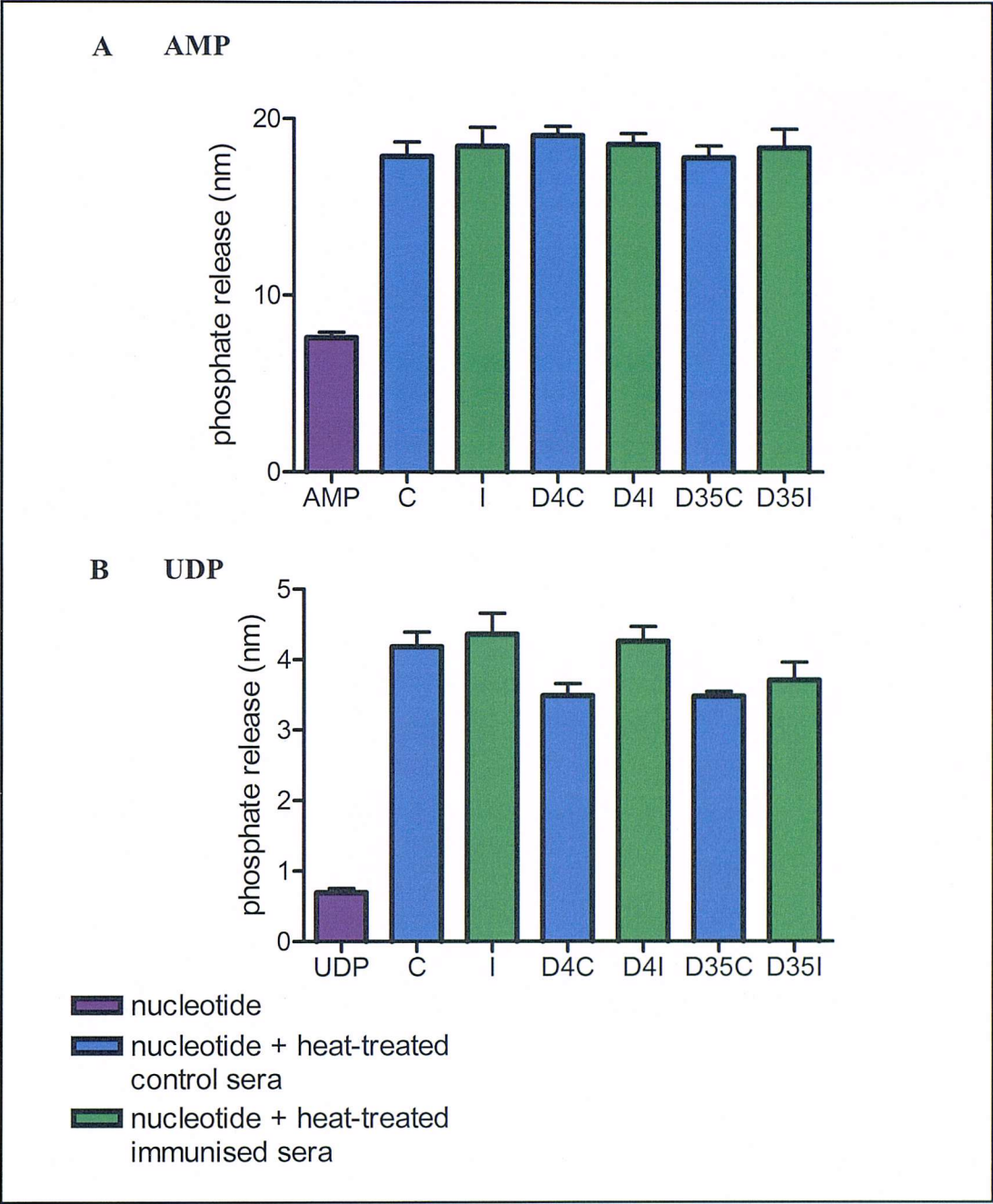


Figure 5-10 The effect of heat treated sera from control and 5'-NT immunised mice on 5'-NT activity

Prior to infection sera were collected from control (C) and 5'-NT immunised (I) mice. Sera were also collected from the same animals 4 days (D4C, D4I) and 35 days (D35C, D35I) post *T. spiralis* infection. Sera were diluted 1/100 and used in a phosphate release assay utilising (A) 2.5 mM AMP and 20 ng 5'-NT, and (B) 2.5 mM UDP and 50 ng 5'-NT. Data were analysed using the unpaired two-tail Student's *t*-test and represent the mean + s.d.m of assays performed in duplicate using sera from groups of at least 2 mice. All assays containing heat-treated sera were significantly different to nucleotide + 5'-NT alone, $p \leq 0.001$.

5.3 Discussion

The aim of this study was to determine whether immunisation of mice with the *T. spiralis* recombinant 5'-nucleotidase could confer protection against subsequent *T. spiralis* challenge infection. This was performed to provide new insight into the *in vivo* role of the secreted 5'-nucleotidase. Enzymes secreted into the host environment by parasites such as *T. spiralis* are likely to be involved in the invasion process and/or the survival and development of the parasite (Despommier, 1983). As shown here, the 5'-NT is expressed and secreted by all the major life-stages of the parasite. This potentially suggests that secretion of the 5'-NT is not specifically involved in one particular aspect of the parasites life cycle, and may play a role at multiple stages of the infection. This implies that all the parasite life-stages have the potential to modulate purinergic signalling by altering the amount and type of nucleotides available to bind to host purinergic receptors.

To determine whether immunisation with the recombinant 5'-NT successfully primed the immune response, various immunological parameters at both the local and systemic level were characterised. CD4⁺ T cells play a central role in protective immune responses against *T. spiralis* infection (Grencis *et al.*, 1985; Ramaswamy *et al.*, 1994). During the early phase of *T. spiralis* infection, CD4⁺ T cells residing within the MLN respond to parasite antigen, proliferate (Krco *et al.*, 1982) and migrate (Riedlinger *et al.*, 1986; Wang *et al.*, 1990) to the intestinal tissue, where they orchestrate the inflammatory changes that are ultimately responsible for parasite expulsion from the intestine. Consistent with other studies, infection with *T. spiralis* stimulated the basal level of MLN cell proliferation and potentiated T cell proliferation in response to the super-antigen SEB (Furze and Selkirk, 2005). These effects were not affected by immunisation with the 5'-NT. Other studies have reported that after immunisation with native parasite antigens, mice remain immune to challenge infections reflecting the persistence of a primed T cell population that can be rapidly restimulated upon challenge (Robinson *et al.*, 1994). However, antigen recall experiments using the recombinant 5'-NT did not induce MLN cell proliferation, suggesting the immunisation regime used did not stimulate the clonal expansion of 5'-NT-specific T cells in the MLN. Potentially, this could have been due to the fact that the 5'-NT was administered sub-cutaneously during immunisation, thus generating a systemic rather than mucosal immune response.

In contrast to MLN cells, splenocytes extracted from 5'-NT-immunised mice proliferated when stimulated with the recombinant 5'-NT. Furthermore, *T. spiralis* infection had no effect on the level of basal or SEB-induced splenocyte proliferation, which is indirectly consistent with other reports that suggest lymphocytes do not infiltrate the spleen until 7-15 days post *T. spiralis* infection (Furze and Selkirk, 2005). This result clearly indicates that immunisation with the 5'-NT generated a systemic rather than a mucosal immune response.

Protective immune responses and expulsion of parasitic nematodes in mice are dependent on the generation of a type 2 immune response (Grencis *et al.*, 1991; Else *et al.*, 1992; Lawrence *et al.*, 1998; Finkelman *et al.*, 1997). MLN cells from *T. spiralis*-infected mice transiently secrete high levels of the T_H2 cytokines, IL-3, IL-4, IL-5, IL-9, IL-10 and IL-13 that peak during the first 4-13 days of infection (Grencis *et al.*, 1991; Robinson *et al.*, 1995a; Helmby and Grencis, 2003; Furze and Selkirk, 2005). In correlation with the observation that MLN cells extracted from 5'-NT-immunised mice 4 days post *T. spiralis* infection did not proliferate in response to the recombinant 5'-NT when stimulated *in vitro*, levels of IL-2 secretion were also not elevated in response to the 5'-NT. Considering that IL-2 produced by activated T cells is required to drive T cell proliferation and differentiation, these data explain why elevated proliferation was not observed in MLN cells extracted from 5'-NT-immunised mice. In addition, this suggests that a 5'-NT-specific T cell response had not been primed in the MLN. Notably, the basal level of IL-2 secretion was elevated in *T. spiralis*-infected animals, which is a characteristic of this phase of the infection (Zhu and Bell, 1989).

During *T. spiralis* infection, T_H2 cytokine production in response to recall with parasite antigen is dominated by IL-5, and to a lesser extent IL-4, in MLN draining the intestine (Grencis *et al.*, 1991; Furze and Selkirk, 2005). This response peaks 7 days post infection. In a similar manner, MLN cells extracted from 5'-NT-immunised mice 4 days post-*T. spiralis* infection and stimulated with the recombinant 5'-NT secreted significantly more of the T_H2 cytokines IL-4 and IL-5, and the pro-inflammatory cytokine TNF- α . It is likely that these cytokines were produced by 5'-NT-specific T cells in the MLN. Considering that IL-4 plays an important role in driving the development of T_H2 cells and the generation of protective immunity to primary *T. spiralis* infection (Finkelman *et al.*, 1997; Lawrence *et al.*, 1998), the elevated levels of IL-4 in the immunised animals could act to help in protecting the host. In a similar manner, elevated levels of IL-5 could promote the generation and recruitment of

eosinophils (Coffman *et al.*, 1989), which may also enhance parasite expulsion (Gurish *et al.*, 2002). The elevated levels of TNF- α in 5'-NT-immunised mice could also activate macrophages and further promote inflammation. The 5'-NT-specific cytokine responses of splenocytes were not investigated in this study, as the focus was on determining whether an immune response was generated in the vicinity of the intestine i.e. the MLN.

Transfer of immunity to a primary *T. spiralis* infection in the mouse can be achieved using immune sera from vaccinated animals (Jarvis and Pritchard, 1992; Robinson *et al.*, 1995). Thus, it was deemed important to determine whether immunisation had generated an antigen-specific antibody response. The immunisation strategy used here successfully gave rise to high titres of 5'-NT-specific antibodies. During the initiation of a humoral immune response, IgM is the initial isotype produced and subsequent class switching is influenced by the type of cytokines present in the external milieu. Class switching to the IgG1 and IgG2b isotype is driven by IL-4 and IL-5 produced by T_H2 cells, whereas switching to IgG2a is more indicative of a T_H1 environment (Gracie and Bradley, 1996). The antibody response generated during the 5'-NT-immunisation process was dominated by IgG1 and to a lesser extent IgG2b and IgG2a antibody isotypes. This suggests that immunisation using alum adjuvant generated a mixed immune response dominated by T_H2 cells. This also correlates with the observation that elevated levels of the T_H2-type cytokines, IL-4 and IL-5 were present in the supernatants of MLN cells extracted from 5'-NT immunised mice during *T. spiralis* infection.

IgM levels were not elevated in 5'-NT-immunised mice, which is inconsistent with another similar immunisation study that reported that vaccination of NIH mice using alum-adjuvant and *T. spiralis* ES induced predominantly mixed IgG1 and IgM antibody isotypes prior to and at 7 days post infection (Robinson *et al.*, 1994). Considering that the early stages of antibody responses are dominated by IgM produced by B cells that have not yet undergone isotype switching or affinity maturation, it can be suggested that the immunisation strategy used by Robinson *et al.*, (1994) was still stimulating naïve B cells. In contrast, the complete lack of 5'-NT-specific IgM observed in the immunisation strategy used here implies that naive B cells were no longer being stimulated by the 5'-NT antigen, and the humoral response had already undergone isotype switching. This discrepancy could be due to the differences in the time course of

immunisation, as the regime used in this study was twice as long as that used by Robinson and co-workers.

During the normal course of *T. spiralis* infection in mice, IgA levels in the sera are generally low and peak at day 15 post-infection (Goyal and Wakelin, 1993; Furze and Selkirk, 2005). Immunisation did not generate 5'-NT-specific IgA antibody isotypes in the sera, again suggesting a mucosal immune response had not been activated. A previous immunisation study using native *T. spiralis* antigens has also reported low levels of IgA in the sera of immunised animals, but elevated levels of IgA in intestinal lavage fluid (Robinson *et al.*, 1995a). Potentially, elevated levels of IgA may also have been present in the intestines of 5'-NT-immunised animals, however this parameter was not measured.

Immunisation using native parasite ES greatly increases the magnitude of the antibody response induced during the early phase of infection (Robinson *et al.* 1994, 1995a). In contrast, infection of 5'-NT-immunised animals with *T. spiralis* did not stimulate a rapid secondary humoral immune response during the early phase of infection (day 4 p.i). This result may suggest that the amount of 5'-NT secreted into the host by *T. spiralis* during the intestinal phase of infection was insufficient to further boost antibody levels. This idea is consistent with the fact that the native secreted 5'-NT constitutes only a very small percentage of the total proteins secreted by *T. spiralis* (Gounaris, 2002). This discrepancy probably reflects the greater complexity of native ES which contains multiple immunogenic proteins that are secreted in great quantities by the parasite, and can thus be detected more readily by the host.

Immune responses towards antigens usually involve antibodies directed at multiple epitopes, only a proportion of which will neutralise the function of the antigen. When this property was investigated, sera from 5'-NT-immunised animals inhibited recombinant 5'-NT activity, suggesting neutralising antibodies were present. Unexpectedly, sera from naive and sham-immunised, infected animals enhanced the enzymatic activity of the recombinant 5'-NT by approximately 3-fold. Sera from naive or infected animals also potentiated the activity of the native 5'-NT present in ES. Furthermore, sera from 5'-NT-immunised mice extracted prior to, or throughout the course of infection, significantly reduced the activity of the recombinant and native 5'-NT towards either 5'-monophosphate or -diphosphate substrates. This suggests that immunisation using the 5'-NT would generate antibodies that could neutralise the native *T. spiralis* 5'-NT secreted by the parasite during the course of the infection.

When sera from 5'-NT-immunised and sham-immunised mice were heat-treated and subsequently used in 5'-NT activity assays, sera from both groups of mice enhanced the activity of the 5'-NT. This suggests that a heat-stable molecule present in the sera of both immunised and non-immunised animals was responsible for enhancing the activity of the 5'-NT. Furthermore, heat-treatment of sera taken from immunised animals also showed that the neutralisation of 5'-NT activity was attributed to a heat labile protein, most likely IgG1 considering that this was the most abundant isotype produced during the immunisation. Further experiments using a protein-A column to purify IgG antibodies from the sera would have been useful to determine whether this was the case.

A possible scenario that explains the enhancing effect of sera on 5'-NT activity could involve the presence of a small molecule/ion/metabolite in the serum binding to the recombinant 5'-NT at a site other than the active site. This could induce a conformational change in the 5'-NT which results in increased activity of the enzyme. Potentially, the activating factor may already be present in *T. spiralis* ES, as sera did not enhance the activity of the native 5'-NT as potently as the recombinant 5'-NT. Further experiments to investigate whether serum alters the kinetic profile of the recombinant 5'-NT and native 5'-NT could prove interesting to determine whether allostery is occurring.

Despite immunisation with the 5'-NT generating a systemic immune response resulting in high titres of 5'-NT-specific antibodies in the sera and increased numbers of 5'-NT-primed T cells in the spleen, no protection was conferred to *T. spiralis* infection. There was no difference in the kinetics of parasite expulsion or fecundity of adults between immunised and non-immunised animals. Potentially, the absence of protection against the enteric phase of infection in 5'-NT-immunised animals may have been due to the immunisation strategy not priming a 5'-NT-specific T cell response in the local draining lymph nodes of the intestine.

As the immunisation failed to evoke a localised immune response, the results of this experiment are inconclusive, and the role of the 5'-NT in the enteric phase of infection still remains unknown. Further experiments that pursue immunisation as a tool to determine the role of the 5'-NT in *T. spiralis* infection should be aimed at generating a mucosal immune response. Potentially, this could be aided by use of different adjuvants or routes of administering the recombinant protein. For example other studies have reported the successful generation of an enhanced mucosal immune response by

orally or nasally immunising mice with native or synthetic parasite antigens and cholera toxin (Robinson *et al.*, 1995c; McGuire *et al.*, 2002)

Immunisation of mice with the 5'-NT also failed to significantly affect the numbers of parasites encysting in the muscle, despite the presence of neutralising, 5'-NT-specific antibodies in the sera. This may suggest that 5'-NT activity is not required for the migration of newborn larvae or nurse cell formation in the muscle. Alternatively, the neutralising antibodies generated by immunisation may not have been sufficient to completely inhibit all of the 5'-NT secreted by the parasites. This may have allowed the parasite to successfully complete its life-cycle.

In summary, these experiments have shown that the 5'-NT is expressed by adult, newborn larvae and muscle stage larval parasites. Immunisation of mice with this non stage-specific recombinant protein generated a T_H2-dominated systemic immune response, but did not induce protective adaptive immune responses in the intestinal environment. The immunisation strategy used generated high titres of 5'-NT-specific antibodies that could neutralise the activity of the 5'-NT *in vitro*. Unexpectedly, sera from naive mice actually increased the enzymatic activity of the 5'-NT *in vitro*, potentially indicating this enzyme may have a higher specific activity than previously thought. Challenge of immunised animals with *T. spiralis* infective larvae did not confer protection against any stage of the parasites life cycle. The lack of protection against *T. spiralis* infection inferred by immunisation with the 5'-NT may be due to the inadequate T cell response generated in the MLN. In conclusion, sub-cutaneous immunisation of mice with the recombinant 5'-NT did not generate a sufficiently potent mucosal immune response and hence the importance of this secreted enzyme in *T. spiralis* infection still remains elusive.

Chapter 6

Purinergic receptor expression in the murine intestinal epithelium during *T. spiralis* infection

6.1 Introduction

The intestinal epithelium serves as a physical barrier between the external environment of the gut lumen and the internal milieu, and transports nutrients, ions and fluids. Epithelial cells maintain physiological homeostasis by regulating the amount of fluid in the lumen. This is achieved by modifying nutrient and ion absorption and ion secretion, and can also be further modulated by the enteric nervous system. Purinergic receptors play a significant role in regulating ion transport in epithelial tissues such as the gastrointestinal tract (Bucheimer and Linden, 2004). Extracellular nucleotide signalling in intestinal epithelia is a complex process and distinct receptors are involved in mediating responses at the apical (luminal) and basolateral cell membranes (Dubyak, 2003). Moreover, different combinations of purinergic receptors are expressed by the multiple cell types that comprise the complex epithelial tissue. Elucidation of how extracellular nucleotides modulate purinergic receptor-induced epithelial cell function is further complicated by the presence of cell surface ecto-nucleotidases which readily metabolise nucleotides to products that can then interact with other purinergic receptor sub-types.

During nematode infections the level of fluid in the lumen increases and smooth muscle becomes hypercontractile. This is referred to as the “weep and sweep” response which is thought to facilitate worm expulsion (Murray *et al.*, 1971). The response is mediated by a variety of mechanisms involving epithelial cells, immune effector cells and the enteric nervous system. Nematode infections generally induce increased epithelial cell permeability, which may allow protective proteins from lymph and serum to gain access to the gut lumen, where they might be able to damage the parasite (McDermott *et al.*, 2003; Madden *et al.*, 2004). The absorptive properties of the intestinal epithelium are also decreased, which further contributes to the increased fluid accumulation in the lumen (Madden *et al.*, 2002; 2004). The effect of nematode infection on epithelial cell ion secretion is more complex. For instance, ion secretion by epithelial cells is enhanced by enteric nerves through a reflex pathway that involves serotonin (5-hydroxytryptamine (5-HT)) and cholinergic stimulation. Mucosal mast cells release a number of soluble mediators, such as prostaglandin (PG) E₂ and histamine, which also increase epithelial cell secretion, putatively by enhancing neuroimmune interactions (Perdue *et al.*, 1991; Madden *et al.*, 2004). In contrast, when enteric nerves are inhibited by antagonists, it has been reported that nematode infection

directly inhibits the secretory responses of epithelial cells when activated by mast cell mediators (Madden *et al.*, 2004). A parasite mediated inhibition of secretory responses would potentially limit the accumulation of fluid in the lumen that would usually facilitate worm expulsion, thus benefiting the parasites.

Purinergic receptor stimulation of chloride ion secretion could also contribute to the secretory-flush response observed during intestinal nematode infections, as a variety of purinergic receptors are known to mediate chloride ion secretion from epithelial cells. For example, P2Y₄ receptor stimulation with ATP or UTP has been shown to increase Cl⁻ secretion leading to fluid accumulation in the lumen (Cressman *et al.*, 1999; Robaye *et al.*, 2003; Ghanem *et al.*, 2005). To a lesser extent, stimulation of basolateral P2Y₂ receptors can also stimulate chloride ion secretion in the small intestine (Ghanem *et al.*, 2005). Adenosine signalling *via* A₁ receptors present on luminal cell membranes and A_{2B} receptors found on basolateral cell membranes, can stimulate chloride ion secretory responses from epithelial cells (Strohmeier *et al.*, 1995; Ghanem *et al.*, 2005). The A_{2B} adenosine receptor (Barrett *et al.*, 1989; Strohmeier *et al.*, 1995) and the P2Y₁ and P2Y₆ receptors (Leipziger *et al.*, 1997; Kottgen, 2003) have also been implicated in the regulation of ion transport in the colon.

Infective *T. spiralis* larvae actively invade jejunal epithelial cells and live within the intestinal epithelium (Wright, 1979; ManWarren *et al.*, 1997). Infection is associated with acute pathological changes within the epithelium such as villus atrophy and crypt hyperplasia, increased epithelial cell permeability, infiltration by mucosal mast cells and paneth and goblet cell hyperplasia (Kamal *et al.*, 2001; Garside *et al.*, 2000). *T. spiralis* invasion of the epithelium results in the release of nucleotides from damaged cells. This response may play a role in host defence by activating purinergic receptors on epithelial cells which stimulate ion secretion, leading to water efflux into the lumen. Potentially, the release of nucleotide metabolising enzymes by the parasite may serve to limit the accumulation of fluid in the lumen that would ordinarily facilitate worm expulsion. This may also act to dampen the potential debilitating effects of excess fluid loss on the host through diarrhoea.

Host genetic factors strongly influence the expulsion of *T. spiralis* from the murine small intestine. Inbred mice strains are classified into rapid- and slow-responders according to the speed at which they expel parasites from the intestine. Rapid-responder NIH mice expel parasites from the intestine within 10 days of infection and have lower muscle stage larvae burdens in comparison to slow-responder

C57BL/10 mice which can take up to 17 days to expel parasites (Wakelin *et al.*, 1986). This is partially determined by the mouse major histocompatibility complex (MHC) H-2 (Wakelin and Donachie, 1983), as well as non-MHC linked responses (Wassom *et al.*, 1983). For example, the mast cell response in C57BL/10 mice is slower and of a lower magnitude than NIH mice (Tuohy *et al.*, 1990). Innate immune responses in the epithelium also differ between strains, as exemplified by the expression of the lectin, intelectin 2. This molecule is suggested to serve a protective role as it is rapidly upregulated in the jejunum of NIH mice during *T. spiralis* infection, but is absent in slow-responder C57BL/10 mice (Pemberton *et al.*, 2004).

The aim of this investigation was to characterise the different sub-types of P1, P2Y and P2X receptors expressed in the murine jejunal epithelium during *T. spiralis* infection, using qualitative RT-PCR analyses. This would determine which types of extracellular nucleotides would potentially be able to interact with purinergic receptors expressed by the epithelium during *T. spiralis* infection. As host genetic factors determine the rate of expulsion of adult worms from the small intestine, fast (NIH) and slow (C57BL/10) responder strains of mice were used. This was performed to ascertain whether alterations in host purinergic receptor expression correlated with the kinetics of parasite expulsion.

6.2 Results

Cells of the intestinal epithelium were extracted from the jejunum of mice using a method described by Russell *et al.*, (2000) which was a modification of the EDTA perfusion technique originally pioneered by Bjerknes and Cheng (1981). This method is based on the principle that EDTA exfoliates both villus and crypt epithelium from the underlying basal lamina and muscularis mucosae. The extracted epithelial cells were subsequently used to generate RNA preparations.

To exclude the possibility of genomic DNA contaminating epithelial RNA preparations, all samples of RNA were digested with DNase and used as templates in PCR reactions using β -actin primers. No PCR products were generated from all batches of epithelial RNA used (data shown in Appendix 1-7), indicating the absence of contaminating genomic DNA. cDNA was subsequently prepared from the RNA, and the amounts used standardised between different RNA batches via β -actin-specific PCR. Two mice of each strain (NIH and C57BL/10) were used per time point and mouse mast cell protease-1 transcripts were monitored by RT-PCR to confirm that the infection had been successful.

6.2.1 P1 purinergic receptor expression in the intestinal epithelium of rapid- and slow-responder mice during *T. spiralis* infection

As shown in Figure 6-1, naïve, slow-responder C57BL/10 mice expressed relatively high levels of A₁ receptor transcripts (Figure 6-1). Naïve rapid-responder NIH mice expressed extremely low levels of A₁ receptor transcripts and also low levels of A_{2B} receptor transcripts (Figure 6-2). These results suggest that the two strains differ in their profile of basal P1 receptor sub-type expression.

RT-PCR analyses of P1 receptor transcripts in both strains of mouse showed that expression in the small intestine varied during the course of *T. spiralis* infection (summarised qualitatively in Table 6-1). Notably, between days 7 and 18 post infection (p.i.), basal level of A₁ receptor expression was decreased in the epithelium extracted from C57BL/10 mice (Figure 6-1). This implies that during the peak of intestinal pathology and parasite expulsion, the epithelium would not be responsive to adenosine. Levels of A₁ receptor expression did not return to pre-infection levels until day 21 p.i. (Figure 6-1), which correlates with the initiation of the resolution of intestinal pathology.

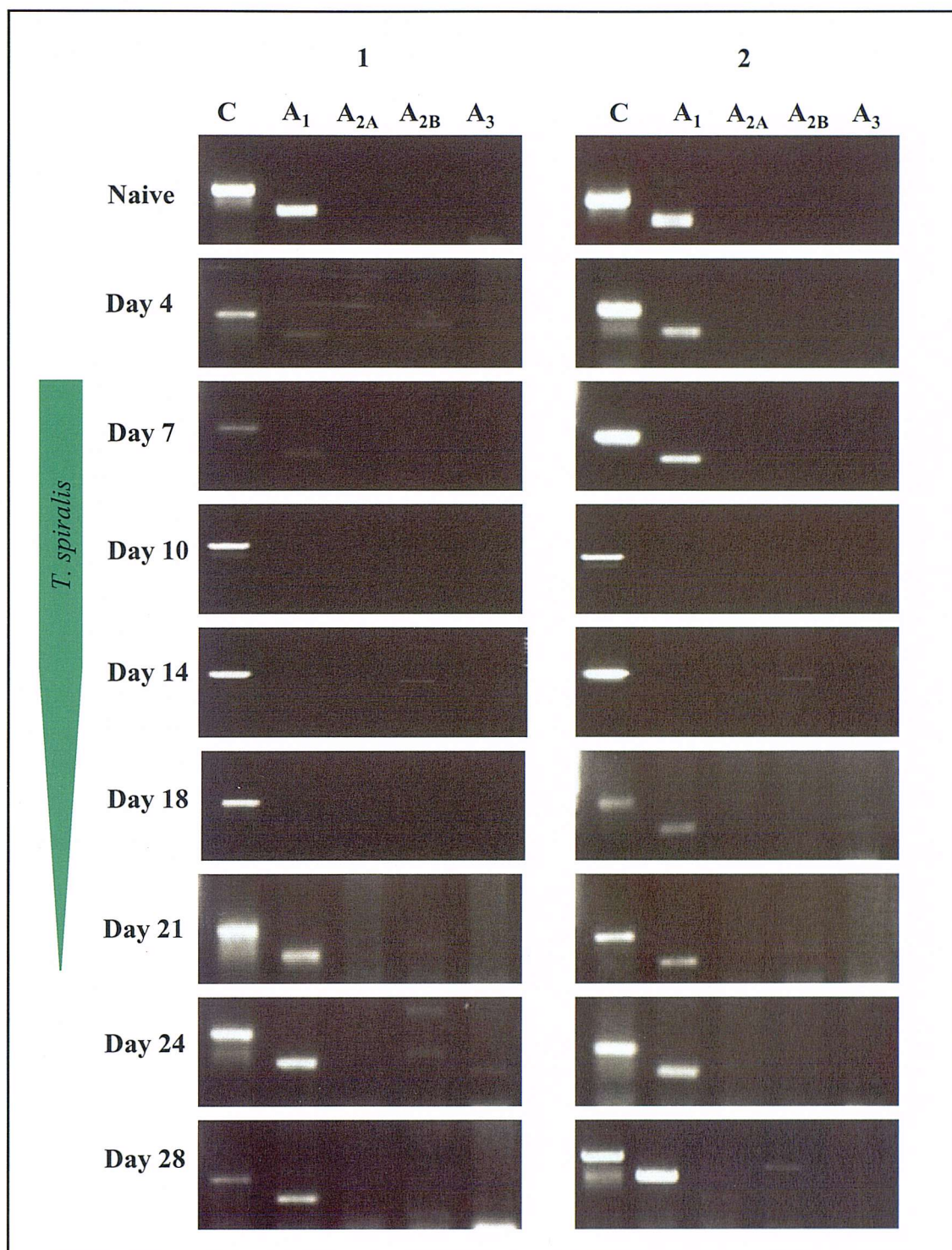


Figure 6-1 Purinergic P1 receptor expression in C57BL/10 intestinal epithelium during *T. spiralis* infection

C57BL/10 mice were infected with 500 *T. spiralis* larvae and intestinal epithelium extracted at the indicated time points post infection. RNA was extracted, Dnase digested and 2 µg used in a reverse transcriptase reaction to produce cDNA. 1 µl of cDNA was used as a template in PCRs utilising receptor specific primers. Data show the results obtained from 2 mice (1 and 2). The green triangle represents the presence of parasites in the intestine.

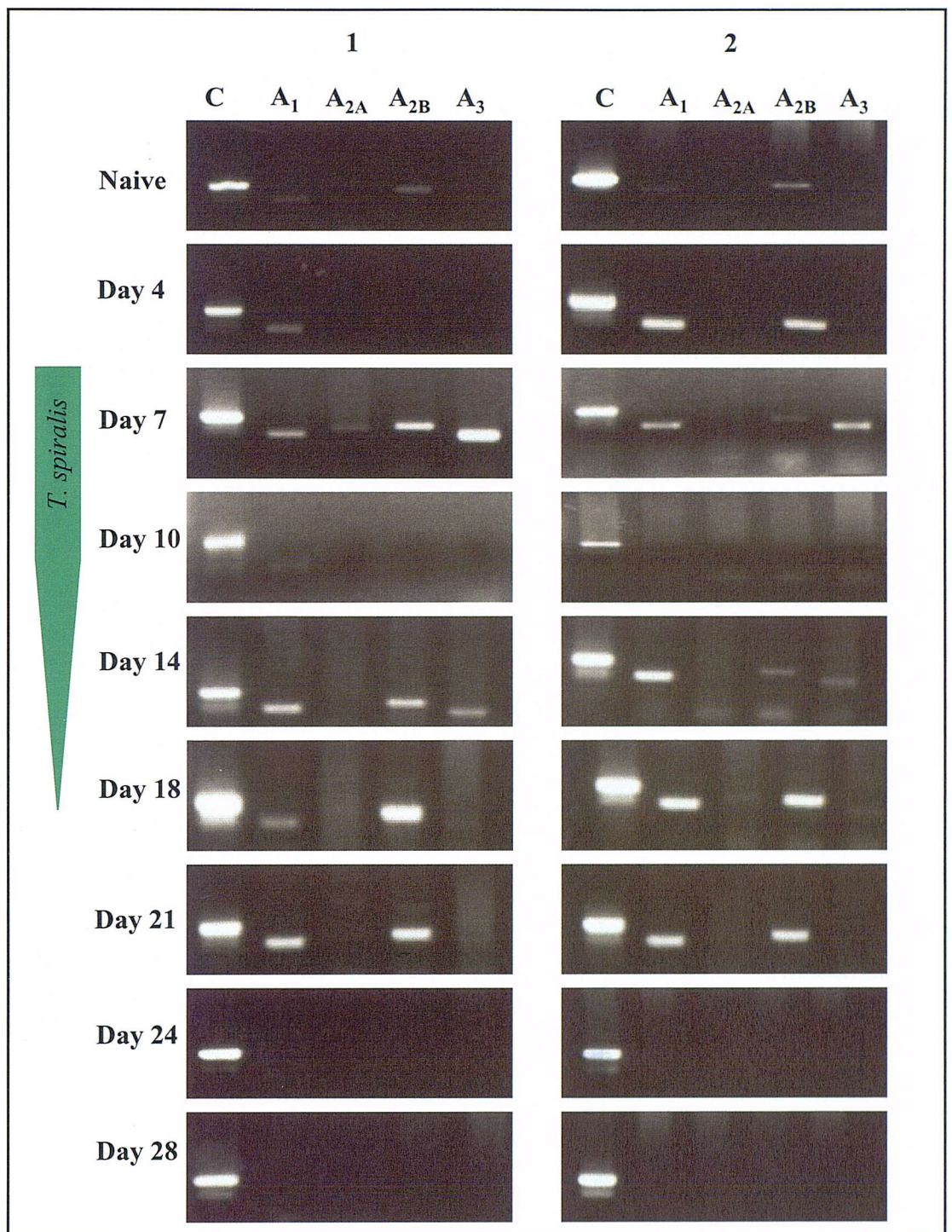


Figure 6-2 Purinergic P1 receptor expression in NIH intestinal epithelium during *T. spiralis* infection

NIH mice were infected with 500 *T. spiralis* larvae and intestinal epithelium extracted at the indicated time points post infection. RNA was extracted, Dnase digested and 2 µg used in a reverse transcriptase reaction to produce cDNA. 1 µl of cDNA was used as a template in PCRs utilising receptor specific primers. Data show the results obtained from 2 mice (1 and 2). The green triangle represents the presence of parasites in the intestine.

C57BL/10

Time Point	Actin		A ₁		A _{2A}		A _{2B}		A ₃	
	1	2	1	2	1	2	1	2	1	2
Naïve	+	+	+	+						
	+	+	+	+						
	+	+	+	+						
D4	+	+	+	+			+			
	+	+		+						
		+								
D7	+	+	+	+						
	+	+		+						
		+								
D10	+	+								
	+	+								
D14	+	+					+	+		
	+	+								
	+	+								
D18	+	+	+	+		+	+			
	+	+	+	+						
	+		+							
D21	+	+	+	+			+	+		
	+	+	+	+						
	+	+								
D24	+	+	+	+			+		+	
	+	+	+	+						
	+	+								
D28	+	+	+							
	+	+	+							
		+								

NIH

Time Point	Actin		A ₁		A _{2A}		A _{2B}		A ₃	
	1	2	1	2	1	2	1	2	1	2
Naïve	+	+	+	+			+	+		
	+	+								
	+	+								
D4	+	+	+	+				+		
	+	+	+	+				+		
		+								
D7	+	+	+	+	+	+	+	+	+	+
	+	+							+	+
	+	+								+
D10	+	+	+	+						
	+	+								
	+									
D14	+	+	+	+			+	+	+	+
	+	+	+	+			+			
	+	+								
D18	+	+	+	+		+	+	+	+	+
	+	+		+			+	+		
	+	+								
D21	+	+	+	+			+	+		
	+	+	+	+			+	+		
	+	+								
D24	+	+	+							
	+	+								
	+	+								
D28	+	+								
	+	+								
	+	+								

+ = m1

++ = m2

+ = low expression level

++ = medium expression level

+++ = high expression level

Table 6.1 Qualitative summary of purinergic P1 receptor expression in C57BL/10 and NIH intestinal epithelium during *T. spiralis* infection

Agarose gel pictures of PCR products amplified from cDNA derived from intestinal epithelial extracts of C57BL/10 and NIHmice infected with 500 *T. spiralis* larvae were analysed qualitatively and summarised according to the relative intensity of the PCR product formed. Data are categorised according to days post infection and murine epithelial extracts 1 and 2.

T. spiralis infection of NIH mice appeared to promote A₁, A_{2B} and to a lesser extent A₃ receptor expression (summarised qualitatively in Table 6-1). RT-PCR analyses showed that A₁ and A_{2B} receptors were upregulated as early as day 4 p.i. (Figure 6-2), which correlates with the initiation of pathological changes that occur in the epithelium. Notably, at day 10 p.i., a temporal down regulation of A₁, A_{2B} and A₃ receptor expression occurred (Figure 6-2), suggesting that at the peak of parasite expulsion the epithelium of NIH mice may not be responsive to adenosine. This effect was short-lived as relatively high levels of A₁, A_{2B} and to a lesser extent A₃ receptor transcripts were detected again between days 14-21 post infection. Expression of P1 receptor transcripts were not reduced to preinfection levels until day 24 post infection (Figure 6-2), which correlates with the resolution phase of intestinal pathology.

6.2.2 P2Y purinergic receptor expression in the intestinal epithelium of rapid- and slow-responder mice during *T. spiralis* infection

To investigate whether infection with *T. spiralis* alters the ability of the host intestinal epithelium to respond to nucleotides such as UDP, UTP, ADP and ATP, P2Y purinergic receptor expression was monitored. Naïve C57BL/10 mice expressed high levels of transcripts corresponding to the P2Y₁ receptor and low levels of transcripts corresponding to the P2Y₁₃ receptor (Figures 6-3 and 6-4), which could indicate the epithelium was responsive to ADP. Naïve NIH mice strongly expressed P2Y₁ receptor transcripts and low levels of transcripts corresponding to the P2Y₂ receptor. These results suggest that the intestinal epithelium of fast-responder mice may be responsive to ADP, ATP and UTP nucleotides. Thus, it appears that the two strains of mice express different profiles of P2Y receptors in the naïve state.

Levels of P2Y receptor transcripts expressed in the epithelial compartment appeared to fluctuate throughout the time-course of infection in both strains of mice (summarised in Tables 6-2 and 6-3). Transcripts corresponding to the P2Y₁ receptor were detected throughout the infection time-course in both strains of mouse (Figures 6-3 and 6-4). This suggests that the infection did not alter P2Y₁ receptor expression and implies the intestinal epithelium was continually responsive to ADP.

A transient but low-level up-regulation of P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptor expression was observed between days 14-18 p.i. in epithelium extracted from C57BL/10 mice (Figure 6-3). This correlates with parasite expulsion and the resolution of *T. spiralis* infection. Elevated levels of the P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors would

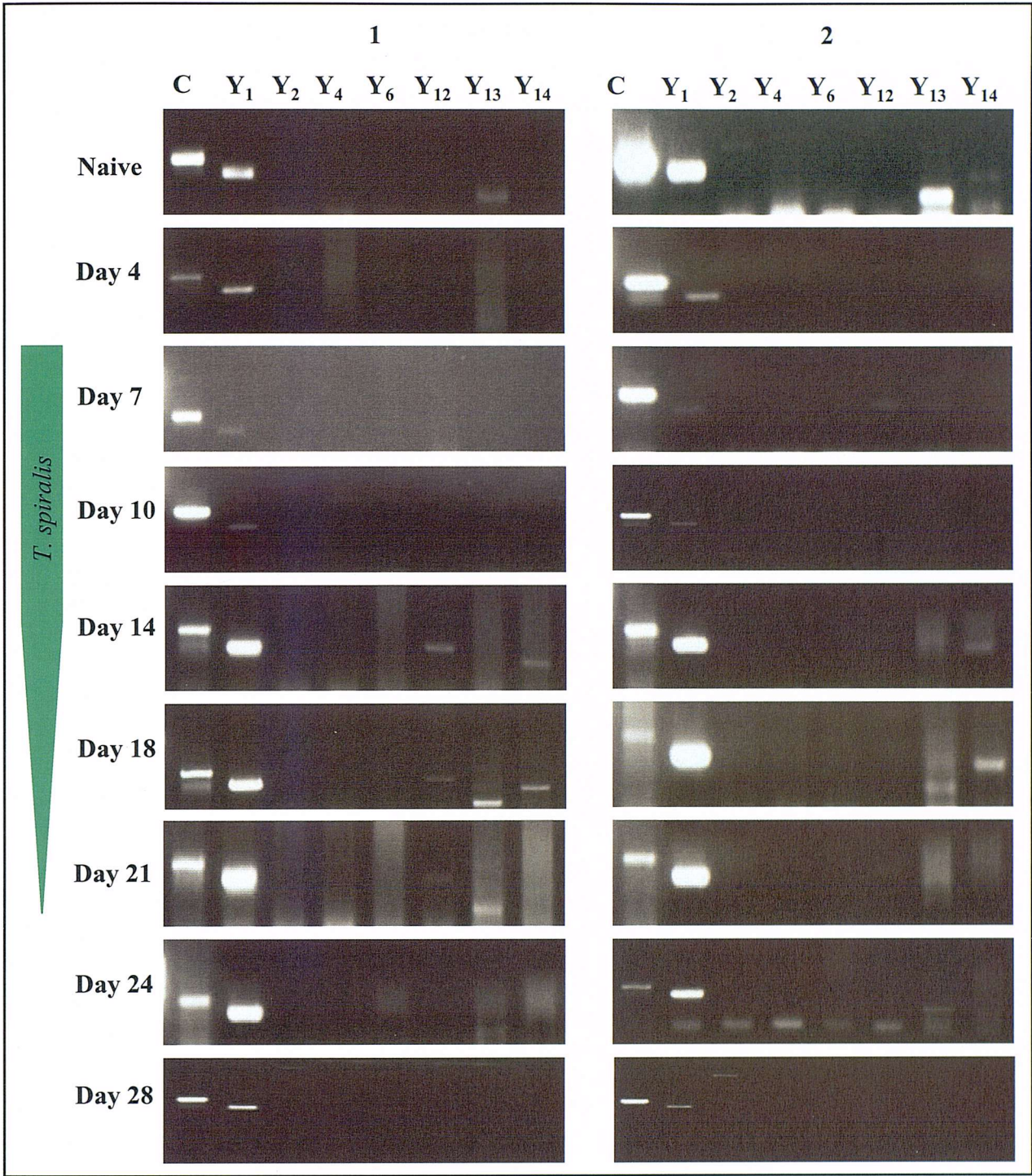


Figure 6-3 Purinergic P2Y receptor expression in C57BL/10 intestinal epithelium during *T. spiralis* infection

C57BL/10 mice were infected with 500 *T. spiralis* larvae and intestinal epithelium extracted at the indicated time points post infection. RNA was extracted, Dnase digested and 2 µg used in a reverse transcriptase reaction to produce cDNA. 1 µl of cDNA was used as a template in PCRs utilising receptor specific primers. Data show the results obtained from 2 mice (1 and 2). The green triangle represents the presence of parasites in the intestine.

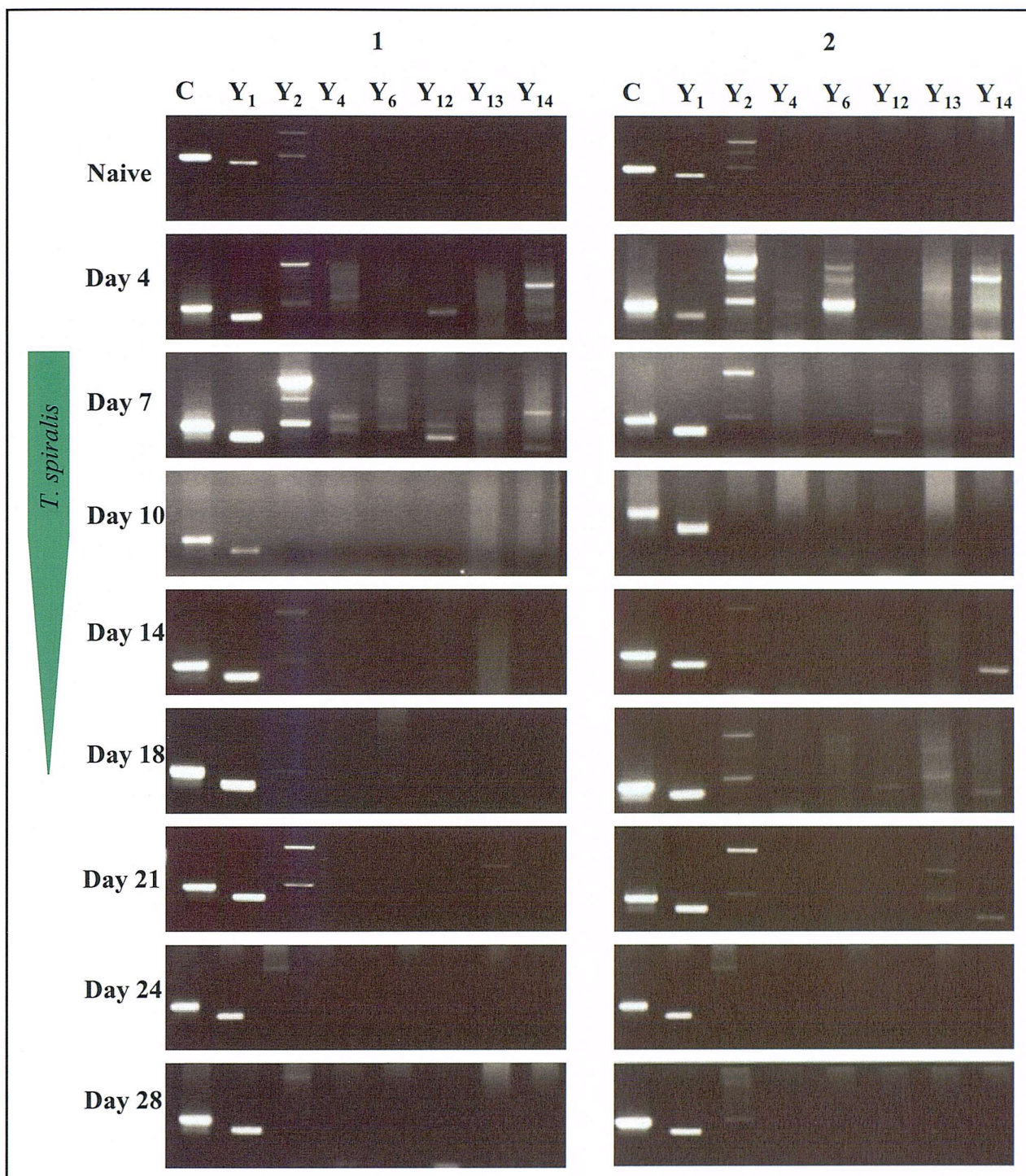


Figure 6-4 Purinergic P2Y receptor expression in NIH intestinal epithelium during *T. spiralis* infection

NIH mice were infected with 500 *T. spiralis* larvae and intestinal epithelium extracted at the indicated time points post infection. RNA was extracted, Dnase digested and 2 µg used in a reverse transcriptase reaction to produce cDNA. 1 µl of cDNA was used as a template in PCRs utilising receptor specific primers. Data show the results obtained from 2 mice (1 and 2). The green triangle represents the presence of parasites in the intestine.

Time Point	Actin		P2Y ₁		P2Y ₂		P2Y ₄		P2Y ₆		P2Y ₁₂		P2Y ₁₃		P2Y ₁₄	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Naïve	+	+	+	+									+	+		
	+	+	+	+										+		
	+	+		+												
D4	+	+	+	+												
	+	+	+	+												
		+														
D7	+	+	+	+								+		+		
	+	+														
	+	+														
D10	+	+	+	+												
	+	+														
	+	+														
D14	+	+	+	+							+	+		+	+	+
	+	+	+	+										+		
	+	+	+	+												
D18	+	+	+	+							+	+	+	+	+	+
	+	+	+	+												
	+	+	+	+												
D21	+	+	+	+							+	+				
	+	+	+	+												
	+	+	+	+												
D24	+	+	+	+												
	+	+	+	+												
	+	+														
D28	+	+	+	+		+										
	+	+	+	+												
	+	+														

+= m1

+= m2

+= low expression level

++ = medium expression level

+++ = high expression level

Table 6.2 Qualitative summary of purinergic P2Y receptor expression in C57BL/10 intestinal epithelium during *T. spiralis* infection

Agarose gel pictures of PCR products amplified from cDNA derived from intestinal epithelial extracts of C57BL/10 mice infected with 500 *T. spiralis* larvae were analysed qualitatively and summarised according to the relative intensity of the PCR product formed. Data are categorised according to days post infection and murine epithelial extracts 1 and 2.

Time Point	Actin		P2Y ₁		P2Y ₂		P2Y ₄		P2Y ₆		P2Y ₁₂		P2Y ₁₃		P2Y ₁₄	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Naïve	+	+	+	+	+	+										
	+	+	+	+												
	+	+														
D4	+	+	+	+	+	+	+	+		+	+				+	+
	+	+	+	+	+	+				+						
	+	+		+		+				+						
D7	+	+	+	+	+	+	+		+		+	+			+	
	+	+	+	+	+	+										
	+	+	+	+	+											
D10	+	+	+	+												
	+	+	+	+												
	+	+		+												
D14	+	+	+	+	+	+										+
	+	+	+	+												
	+	+	+	+												
D18	+	+	+	+	+	+						+		+		+
	+	+	+	+		+										
	+	+	+	+												
D21	+	+	+	+	+	+							+	+		+
	+	+	+	+	+	+										
	+		+	+												
D24	+	+	+	+	+	+										
	+	+	+	+												
	+	+														
D28	+	+	+	+	+	+										
	+	+	+	+												
	+	+	+	+												

+= mouse 1

+ = mouse 2

+= low expression level

++ = medium expression level

+++ = high expression level

Table 6.3 Qualitative summary of purinergic P2Y receptor expression in NIH intestinal epithelium during *T. spiralis* infection

Agarose gel pictures of PCR products amplified from cDNA derived from intestinal epithelial extracts of NIH mice infected with 500 *T. spiralis* larvae were analysed qualitatively and summarised according to the relative intensity of the PCR product formed. Data are categorised according to days post infection and murine epithelial extracts 1 and 2.

potentially make the epithelial compartment responsive to ADP and UDP-glucose at this time-point in the infection.

In contrast to the C57BL/10 mice, transcripts corresponding to P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors were transiently upregulated much earlier during the course of infection (day 4-7 p.i.) in the NIH strain (Figure 6-4, Table 6-3). Expression of these receptors appeared to be downregulated at days 10-14 post infection as transcripts were not detected (Figure 6-4). However, transcripts corresponding to these receptors also appeared to be transiently expressed in the epithelium of NIH mice quite inconsistently and at very low levels, later in the infection time-course (day 14-21 p.i.). At day 10 p.i., P2Y receptor expression appeared to be generally down-regulated in NIH mice, with only transcripts corresponding to the P2Y₁ detected (Figure 6-4). This would suggest that at the peak of parasite expulsion (day 10 p.i.) the epithelial compartment would be responsive to ADP. From day 14 p.i. onwards, P2Y₂ receptor expression was gradually upregulated reaching pre-infection levels by day 18 p.i. (Figure 6-4). This correlates with the initiation of the resolution of intestinal pathology.

In some cases, the P2Y receptor primers amplified PCR products of multiple sizes (Figure 6-3 and 6-4). This may be due to non-specific primer binding, or a splice variant form of the P2Y receptor. Notably, the P2Y₂ receptor primer pair consistently amplified a PCR product of the expected size (440 bp), but also a larger PCR fragment of approximately 1 Kb (Figure 6-4). In some instances an intermediate sized band of ~800 bp was also detected (Figure 6-4). To investigate whether this was due to non-specific primer binding, the P2Y₂ PCR product of the expected size (440 bp), and the largest PCR product (~1 Kb) were purified from agarose gels, and the 3' end of the gene sequenced using the reverse primer. As shown in Appendix 1-8 and 1-9, the sequence derived from both PCR products corresponded to the published P2Y₂ receptor sequence, indicating the primers were detecting the P2Y₂ receptor only.

6.2.3 P2X purinergic receptor expression in the intestinal epithelium of rapid- and slow-responder mice during *T. spiralis* infection

The expression of ligand gated ion channel P2X receptors during *T. spiralis* infection was also monitored to determine whether cells in the epithelial compartment could respond to ATP. Generally, very low levels of P2X receptors were expressed in the uninfected epithelium of either mouse strain. However, some variation in P2X

receptor expression was observed throughout the course of infection (summarised in Tables 6-4 and 6-5).

Transcripts corresponding to P2X receptors were not detected in the epithelium extracted from naïve C57BL/10 mice (Figure 6-5). Apart from a temporal, inconsistent expression of P2X₂, P2X₃, and P2X₄ receptors at day 7 p.i., P2X receptors were not expressed in the epithelium of C57BL/10 mice during the first 14 days of infection (Figure 6-5). However, a transient upregulation of P2X₂, P2X₃, and P2X₄ receptor expression was observed between days 18 and 21 p.i. (Figure 6-5). This occurred after parasite expulsion, during the resolution of intestinal pathology. From day 24 p.i. onwards, P2X receptor expression appeared to be downregulated as transcripts were not consistently detected (Figure 6-5), suggesting restoration of the basal state.

Very low levels of P2X₄ and P2X₇ receptor transcripts were detected in naïve NIH mice (Figure 6-6). Notably, the P2X₇ receptor primers amplified multiple PCR products that were larger than the expected size (171 bp) (Figure 6-6). Potentially, this was due to non-specific primer binding, or a splice variant form of the P2X₇ receptor. At day 10 p.i., P2X receptor transcripts in NIH intestinal epithelium were not detected by RT-PCR analyses, potentially indicating that the very low level of P2X₄ and P2X₇ receptor expression observed in naïve NIH mice had been downregulated, or the cells expressing these receptors were no longer present (Figure 6-6). Interestingly, at day 14 p.i., P2X₄ receptor transcripts were detectable again, as well as very low levels of P2X₁, P2X₂ and P2X₇ receptor transcripts (Figure 6-6). Low levels of P2X₄ receptor transcripts could be detected at days 18 and 21 p.i., but expression of this receptor was not consistently observed from day 24 p.i. onwards (Figure 6-6). As shown in Figure 6-6, levels of transcripts corresponding to P2X₄ and P2X₇ receptors were not detected at 24 or 28 days post infection, indicating that expression of these receptor sub-types had not returned to basal levels. This potentially suggests the infection had induced a long-term down-regulation of P2X receptor expression in NIH mice.

6.2.4 mMCP-1 expression in the intestinal epithelium of rapid- and slow-responder mice during *T. spiralis* infection

Mast cells are usually uncommon in the intestinal mucosae of normal mice, but during *T. spiralis* infection extensive intraepithelial mast cell recruitment occurs, where mast cells migrate into the epithelium and express mast cell protease-1 (mMCP-1). In order to confirm the infection had been successful, mastocytosis was monitored

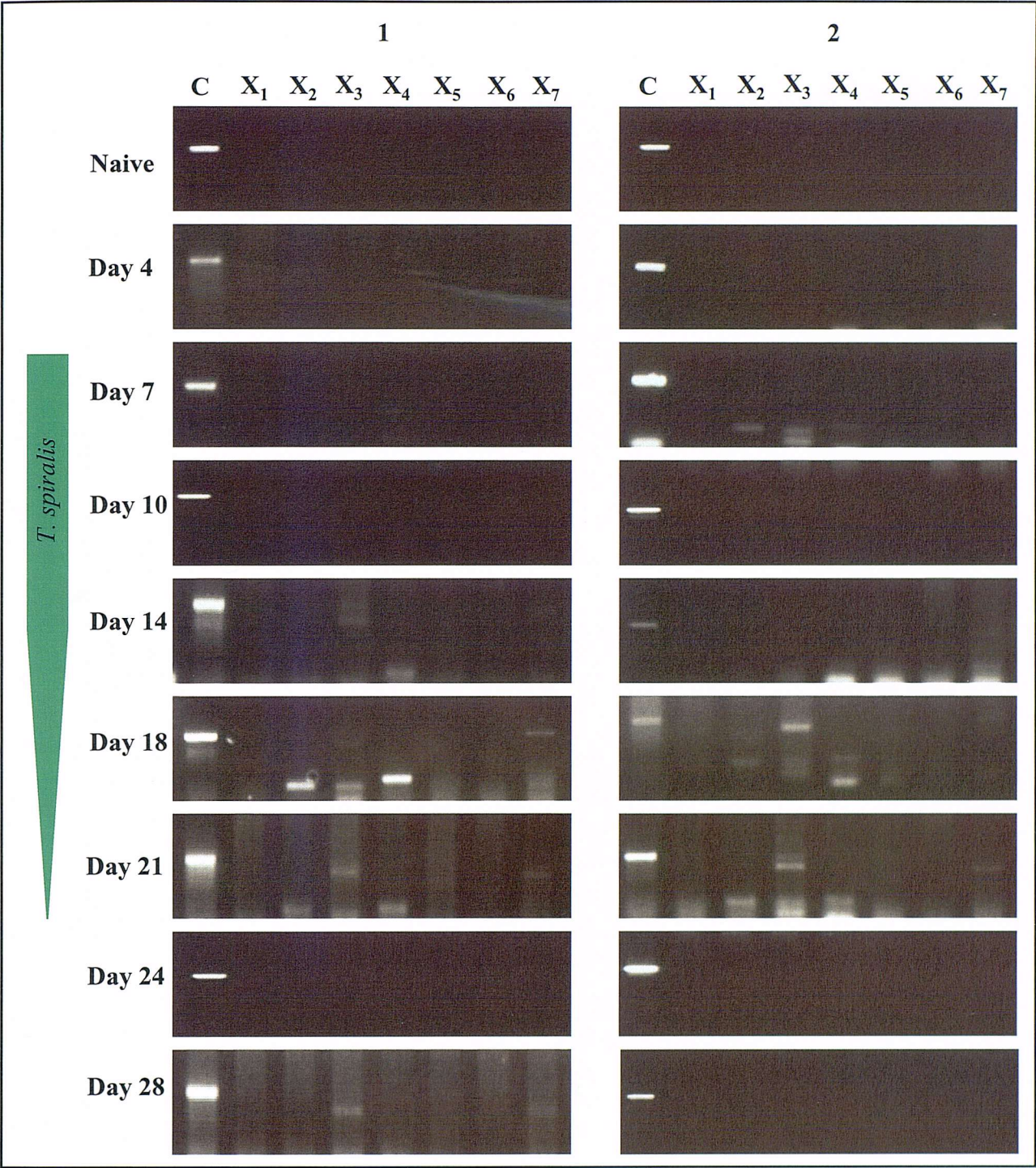


Figure 6-5 Purinergic P2X receptor expression in C57BL/10 intestinal epithelium during *T. spiralis* infection

C57BL/10 mice were infected with 500 *T. spiralis* larvae and intestinal epithelium extracted at the indicated time points post infection. RNA was extracted, Dnase digested and 2 µg used in a reverse transcriptase reaction to produce cDNA. 1 µl of cDNA was used as a template in PCRs utilising receptor specific primers. Data show the results obtained from 2 mice (1 and 2). The green triangle represents the presence of parasites in the intestine.

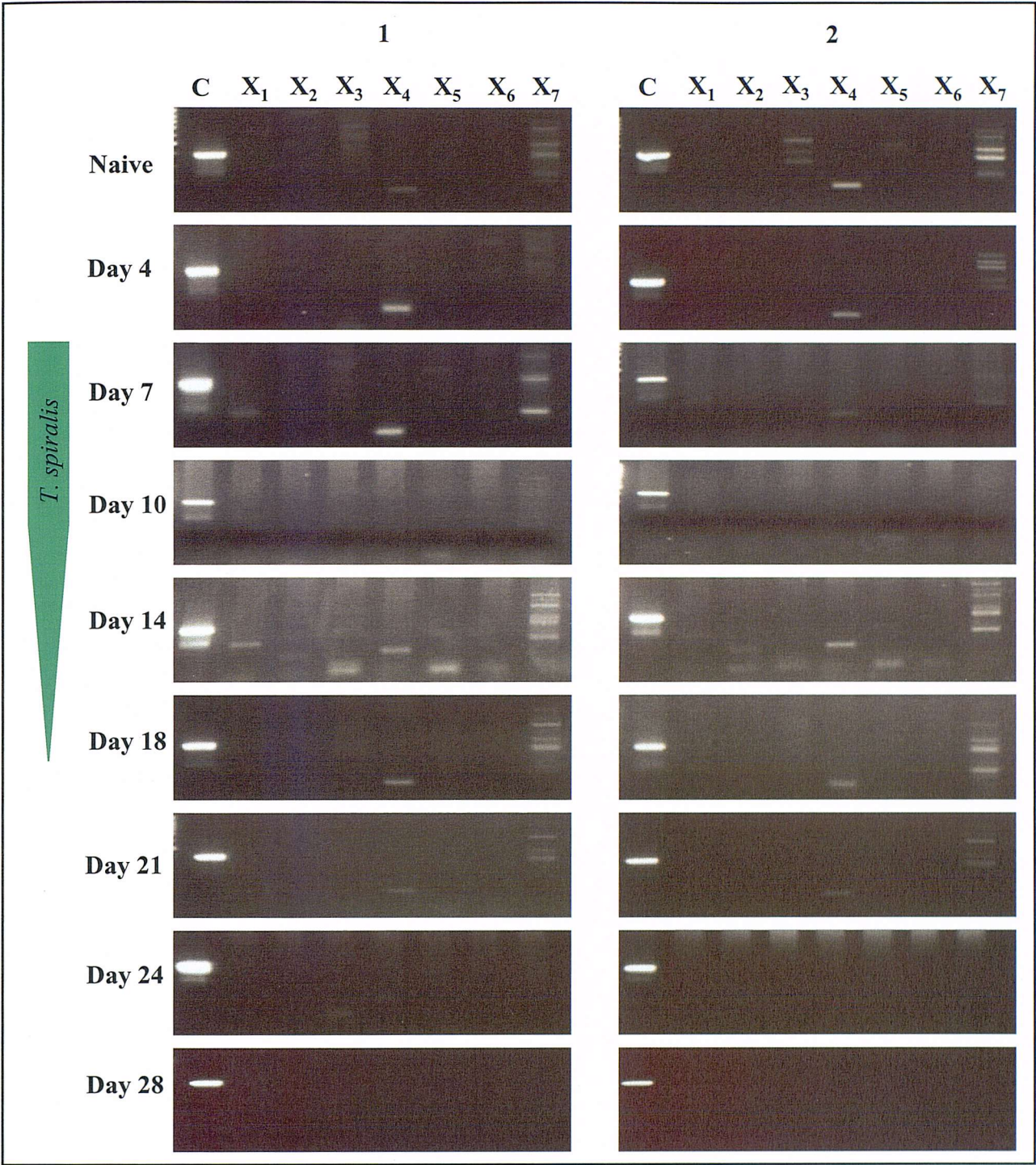


Figure 6-6 Purinergic P2X receptor expression in NIH intestinal epithelium during *T. spiralis* infection

NIH mice were infected with 500 *T. spiralis* larvae and intestinal epithelium extracted at the indicated time points post infection. RNA was extracted, Dnase digested and 2 µg used in a reverse transcriptase reaction to produce cDNA. 1 µl of cDNA was used as a template in PCRs utilising receptor specific primers. Data show the results obtained from 2 mice (1 and 2). The green triangle represents the presence of parasites in the intestine.

Time Point	Actin		P2X ₁		P2X ₂		P2X ₃		P2X ₄		P2X ₅		P2X ₆		P2X ₇	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Naïve	+	+														
	+	+														
	+	+														
D4	+	+														
	+	+														
		+														
D7	+	+				+	+	+		+						
	+	+														
		+														
D10	+	+														
	+	+														
	+	+														
D14	+	+							+	+						
	+	+														
	+															
D18	+	+			+	+	+	+	+	+						
	+	+														
	+															
D21	+	+			+	+	+	+	+	+						
	+	+														
	+	+														
D24	+	+														
	+	+														
	+	+														
D28	+	+														
	+	+														
		+														

+= mouse 1

+ = mouse 2

+= low expression level

++ = medium expression level

+++ = high expression level

Table 6.4 Qualitative summary of purinergic P2X receptor expression in C57BL/10 intestinal epithelium during *T. spiralis* infection

Agarose gel pictures of PCR products amplified from cDNA derived from intestinal epithelial extracts of C57BL/10 mice infected with 500 *T. spiralis* larvae were analysed qualitatively and summarised according to the relative intensity of the PCR product formed. Data are categorised according to days post infection and murine epithelial extracts 1 and 2.

Time Point	Actin		P2X ₁		P2X ₂		P2X ₃		P2X ₄		P2X ₅		P2X ₆		P2X ₇	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Naïve	+	+							+	+					+	+
	+	+														
	+	+														
D4	+	+							+	+					+	+
	+	+							+	+						
	+	+														
D7	+	+	+	+	+	+			+	+					+	+
	+	+							+							
		+														
D10	+	+														
	+	+														
D14	+	+	+	+	+	+			+	+					+	+
	+	+														
	+	+														
D18	+	+							+	+					+	+
	+	+														
	+	+														
D21	+	+							+	+					+	+
	+	+														
	+	+														
D24	+	+							+							
	+	+														
	+	+														
D28	+	+														
	+	+														

+

= mouse 1

+

= mouse 2

+

= low expression level

++

= medium expression level

+++

= high expression level

Table 6.5

Qualitative summary of purinergic P2X receptor expression in NIH intestinal epithelium during *T. spiralis* infection

Agarose gel pictures of PCR products amplified from cDNA derived from intestinal epithelial extracts of NIH mice infected with 500 *T. spiralis* larvae were analysed qualitatively and summarised according to the relative intensity of the PCR product formed. Data are categorised according to days post infection and murine epithelial extracts 1 and 2.

indirectly by measuring murine mast cell protease-1 (mMCP-1) expression in epithelial samples. Numbers of mucosal mast cells in the epithelium begin to increase within one week after infection with *T. spiralis* and peaks between 10 and 14 days p.i. depending on the genetic strain of mouse (Tuohy *et al.*, 1990). As shown in Figure 6-7, in both NIH and C57BL/10 strains of mice, mMCP-1 transcript levels were barely detectable in uninfected animals. During the course of infection levels of mMCP-1 transcripts increased (Figure 6-7). This is consistent with similar studies that have used RNA extracted from total jejunum (Ghildyal *et al.*, 1992; Knight *et al.*, 2000) or epithelial isolates (Knight *et al.*, 2004). Furthermore, levels of mMCP-1 transcripts appeared to peak earlier in NIH mice (day 7-10 p.i) than C57BL/10 mice (day 10-14), which correlates with the observation that NIH develop mastocytosis more rapidly than C57BL/10 mice (Tuohy *et al.*, 1990). Overall, these analyses suggest the infections had proceeded normally, and the epithelial cell isolating technique used had been successful.

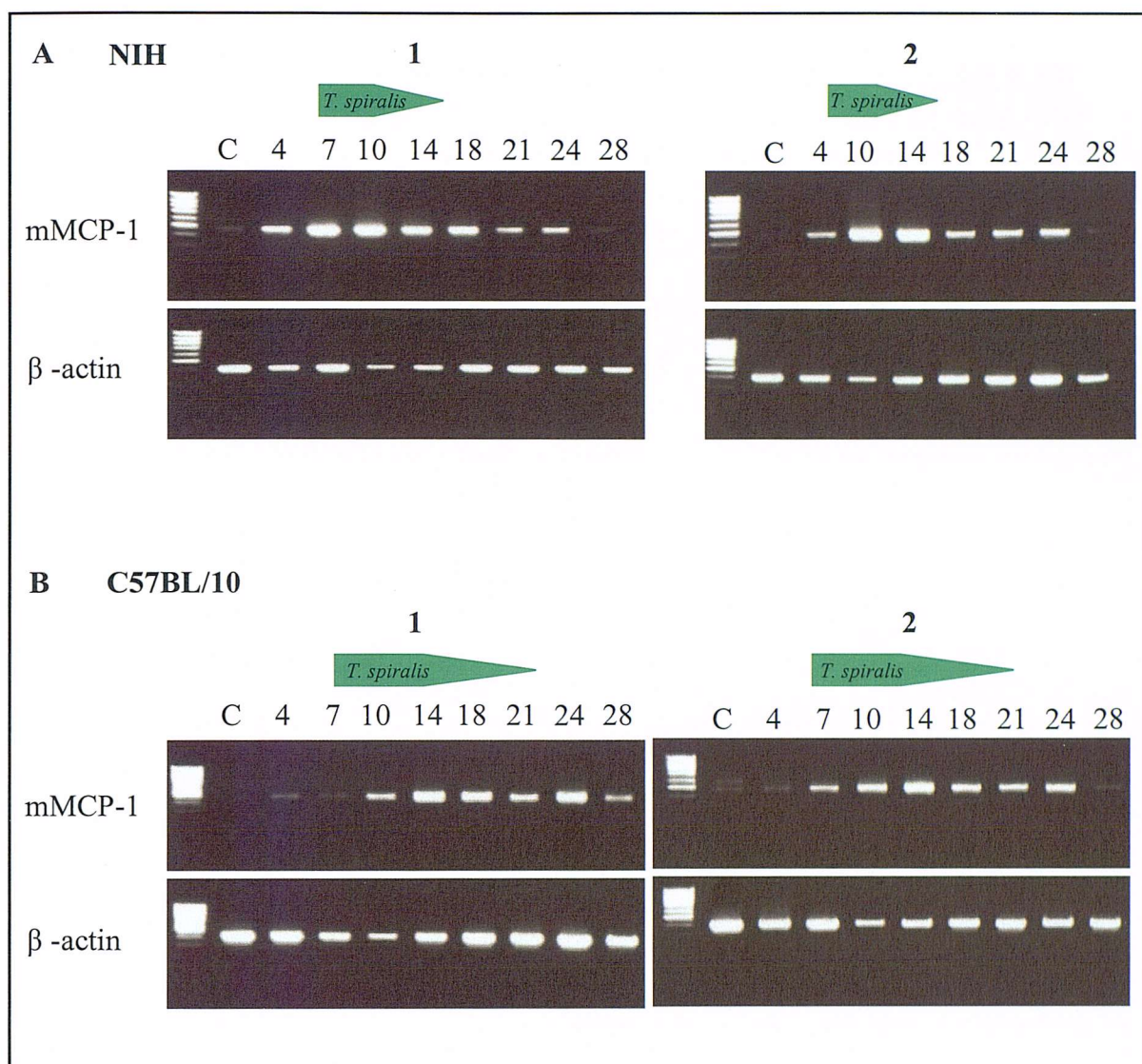


Figure 6-7 mMCP-1 expression in intestinal epithelium during *T. spiralis* infection

NIH (A) and C57BL/10 (B) mice were infected with 500 *T. spiralis* larvae and intestinal epithelium extracted on the indicated days post infection. RNA was extracted, Dnase digested and 2 μ g used in a reverse transcriptase reaction to produce cDNA. 1 μ l of cDNA was used as a template in PCRs utilising mMCP-1 specific primers. β -actin was used as a positive control to confirm the integrity of the cDNA. Data show the results obtained from 2 mice (1 and 2). The green triangle represents the presence of parasites in the intestine.

6.3 Discussion

The aim of this study was to compare purinergic receptor expression in the small intestine of rapid- and slow-responder strains of mice during *T. spiralis* infection. This would help to elucidate whether purinergic receptor expression correlated with parasite expulsion and pathological changes within the intestinal epithelium.

Generally, rapid- and slow-responder strains of mice expressed distinct profiles of purinergic receptor expression prior to infection and exhibited altered purinergic receptor expression throughout the course of infection. There are two mechanisms by which altered expression could occur: i) up/down-regulation of purinergic receptor expression, ii) increase/decrease in populations of specific cell types within the epithelium expressing purinergic receptor sub-types. It is possible that both mechanisms may contribute to the alterations in expression observed here. Indeed, there are several studies reporting that both mechanisms may cause changes in transcript expression levels in the epithelial compartment during helminth infections. For instance, expression of intelectin 2 is upregulated by goblet cells during *T. spiralis* infection (Pemberton *et al.*, 2004), and acetylcholinesterase expression is upregulated in epithelial cells during *Nippostrongylus braziliensis* infection (Russell *et al.*, 2000). In contrast, upregulated mMCP-1 expression during *T. spiralis* infection is due to the migration of mucosal mast cells into the intestinal epithelium (Tuohy *et al.*, 1990; Knight *et al.*, 2000). It is important to note that altered transcription of a gene may not alter levels of the corresponding protein in a predictable manner. However, the functional significance of these observations will be discussed in terms of altered protein expression.

A₁ and A_{2B} receptors were detected in the epithelium of naïve mice. However, mouse jejunum has previously been shown to express all P1 receptor sub-types (Ghanem *et al.*, 2005). These discrepancies in P1 receptor expression are likely to be due to differences in the type of method used to extract epithelium from the jejunum. The EDTA-stripping technique used in this study is likely to be far more selective than the method used by Ghanem and co-workers (2005), which involved scraping cells off the epithelium.

Adenosine stimulates chloride ion secretory responses from epithelial cells at both the luminal and basolateral membrane. The action of luminal adenosine is mediated by the A₁ receptor (Ghanem *et al.*, 2005), whereas A_{2B} receptor activation has been postulated to stimulate chloride ion secretion from the basolateral membrane

(Strohmeier *et al.*, 1995; Ghanem *et al.*, 2005). Neutrophils migrate into the intestinal epithelium during *T. spiralis* infection (Stadnyk *et al.*, 2000). Upon transmigration into the intestinal epithelium, neutrophils are known to release AMP (Madara *et al.*, 1993), which is rapidly hydrolysed to adenosine by 5'-ectonucleotidase (CD73) expressed on the apical surface of enterocytes (Strohmeier *et al.*, 1997). Accumulation of adenosine in the intestinal lumen would stimulate A₁ receptors resulting in chloride ion secretion from epithelial cells and the resulting influx of fluid into the lumen. During *T. spiralis* infection, a transient decrease in A₁ receptor transcripts were detected in the epithelium of C57BL/10 mice during the peak of parasite expulsion (day 10-14 p.i.). Potentially, this may be a protective response by the host to limit A₁ receptor activation by the luminal adenosine, which may serve to decrease the amount of fluid accumulating in the intestine and prevent the potential debilitating effects of fluid loss on the host through diarrhoea. *T. spiralis* also secretes an adenosine deaminase (Gounaris, 2002) which may serve to restrict A₁ receptor-mediated fluid secretion from enterocytes. By converting extracellular adenosine to inosine, luminal A₁ receptor activation will not occur and the parasite will prevent the fluid accumulation that would usually flush it away. These observations may reflect an equilibrium between effects detrimental to the host versus effects detrimental to the parasite.

In comparison, naïve NIH mice expressed relatively low levels of the A₁ receptor, levels of which appeared to be slightly elevated during the infection (day 4 to day 21). Upregulation of A₁ receptor expression might suggest that the epithelial cells were more responsive to adenosine, which could induce increased chloride ion secretion and therefore the secretory-flush response of the intestine. Potentially, this difference between the two strains may contribute to the more rapid parasite expulsion observed in NIH mice.

A transient reduction in P1 receptor expression in the epithelium of NIH mice was noted at day 10 post-infection, which correlates with the peak of parasite expulsion. This would suggest that the epithelial compartment was unresponsive to adenosine at this crucial time-point, and again could indicate a damage-limitation response by the host, by controlling excess fluid loss. Further investigation into this observation could provide new insight into the role of P1 receptors during parasite expulsion. A transient up-regulation of A₃ and A_{2B} receptor expression was also observed at days 7-14 p.i. and days 7-21 p.i. respectively (excluding the day 10 time point, where expression levels appeared to be decreased), which roughly correlates with parasite expulsion. As shown

in chapter 3, mucosal mast cells express the A_{2B} and A₃ receptors. Hence, up-regulation of these receptor sub-types may reflect the influx of mucosal mast cells that occurs around this time-point during the infection (Alizadeh and Wakelin, 1982). The lack of A_{2B} and A₃ receptor expression in the epithelium extracted from C57BL/10 could be due to the less intense mast cell response generated in this strain (Tuohy *et al.*, 1990).

Both rapid- and slow-responder strains of mice expressed transcripts corresponding to the P2Y₁ receptor. This is consistent with previous work that has shown that this receptor is expressed in the crypts and luminal epithelial surface of the small intestine (Giaroni *et al.*, 2002). P2Y₁ receptors are also expressed in the myenteric neurones and smooth muscle of the murine small intestine (Giaroni *et al.*, 2002). The majority of work surrounding the role of the P2Y₁ receptor in the intestine has focused on the ability of nucleotides to act as gut neurotransmitters and neuromodulators. Activation of P2Y₁ receptors by ATP or ADP has been reported to mediate relaxation of the longitudinal muscle in the mouse small intestine (Giaroni *et al.*, 2002). Furthermore, sub-mucosal, and to a lesser extent, mucosal ATP can induce increased ion secretion from epithelial cells of the small intestine by stimulating P2Y₁ receptors expressed on secretomotor neurones (Fang *et al.*, 2006). This may also contribute to parasite expulsion through the secretory-flush response. However, the role of non-neuronal P2Y₁ receptors in the intestinal epithelium is currently not known.

Interestingly, P2Y₁ receptor expression appeared to be slightly down-regulated in C57BL/10 mice prior to parasite expulsion (day 4-10 p.i.). In contrast, no alteration in P2Y₁ receptor expression was observed in NIH mice throughout the course of infection. Due to the extensive expression of the P2Y₁ receptor by epithelial cells (Giaroni *et al.*, 2002), it is likely that these differences may represent down-regulation of receptor expression, rather than altered cell populations. Potentially, down-regulation of P2Y₁ receptor expression in C57BL/10 mice may dampen-down as yet unknown extracellular nucleotide-mediated epithelial cell responses.

Extracellular ATP can stimulate mucin secretion from the rat airway goblet cell line SPOC1 (Abdullah *et al.*, 2003) and conjunctival goblet cells (Jumblatt and Jumblatt, 1998) via the P2Y₂ receptor. Furthermore, ATP and UTP can stimulate basolaterally expressed P2Y₂ receptors to mediate chloride ion secretion in the small intestine (Ghanem *et al.*, 2005). P2Y₂ receptor expression, albeit at low levels, were detected in the epithelium of NIH mice only, and expression levels were not altered throughout the course of infection. It is possible that ATP and UTP released during the

invasion of the epithelium by infective larvae would be able to stimulate P2Y₂ receptor mediated chloride ion secretion from epithelial cells, and mucin secretion from goblet cells. This may promote the secretory-flush response and contribute to the rapid expulsion of parasites observed in the NIH strain. The primers used to detect P2Y₂ receptor transcripts amplified multiple PCR products, of which only one was the expected size. Following sequencing of the first 200-300 bp of the 3' end, both PCR products corresponded to the published murine P2Y₂ receptor sequence, implying that the primers were not non-specifically amplifying cDNA. It is unlikely that a splice variant of this gene was expressed as this receptor does not contain introns and there have been no reports in the literature detailing alternate expression of the P2Y₂ receptor. The multiple PCR products formed are probably an artefact of the PCR reaction.

C57BL/10 mice expressed low levels of the P2Y₁₃ receptor prior to infection. The P2Y₁₃ receptor was not expressed by naïve NIH mice, again highlighting differences between the two strains of mice. To date, there have been no studies in the literature characterising P2Y₁₃ receptor expression in the gastrointestinal tract, so the possible function of this receptor remains unknown. Considering that this receptor is known to be highly expressed in nervous tissue (Fumagalli *et al.*, 2004), it may play a role in neuronal ADP-mediated secretory responses as discussed previously.

During *T. spiralis* infection, P2Y₁₂, P2Y₁₃ and P2Y₁₄ receptors were generally upregulated in both strains of mice. This appeared to occur earlier during the infection in NIH mice (day 4-7 p.i.) and later (day 14-21) in C57BL/10 mice, potentially correlating with the fast and slow rates of parasite expulsion in the NIH and C57BL/10 strains respectively. The P2Y₁₂ receptor has previously been reported to be expressed in the rat distal colon submucosa, however a role for this receptor has not yet been assigned (Christofi *et al.*, 2004). There have been no reports to date that have characterised P2Y₁₄ receptor expression in the intestinal tract. However, other studies have reported that P2Y₁₄ receptors, activated by UDP-glucose, can stimulate IL-8 secretion from airway epithelial cells (Muller *et al.*, 2005). Further experiments are required to determine whether P2Y₁₄ receptor activation stimulates intestinal epithelial cells to secrete proinflammatory cytokines. Potentially, the low level of P2Y₁₄ receptor expression observed during the infection may be due to other cell types that migrate to the epithelium during the infection. For example, neutrophils (Scrivens and Dickenson, 2006), T cells (Scrivens and Dickenson, 2005) and immature dendritic cells (Skelton *et*

al., 2003) all express the P2Y₁₄ receptor and are also present during the enteric stage of *T. spiralis* infection.

In the intestinal mucosa, epithelia are rapidly regenerated. Stem cells located in the crypt base move to the surface, apoptose and get exfoliated into the intestine. P2X₇ receptors are strategically located on the villus tips, and are thought to ensure intestinal epithelial regeneration (Groschel-Stewart *et al.*, 1999). Prior to *T. spiralis* infection, P2X₇ receptor expression was only detected in the epithelium of NIH mice (albeit at low levels) and not in C57BL/10 mice. Expression levels of the apoptotic P2X₇ receptor appeared to remain constant throughout the infection in NIH mice, putatively suggesting the number of apoptotic cells expressing this receptor in the villus tips remained the same. PCR reactions utilising the P2X₇ primers generated multiple PCR products of differing sizes. This is most likely due to differential splicing of the P2X₇ mRNA, as this receptor has previously been reported to produce up to seven different splice-variants (Cheewatrakoolpong *et al.*, 2005).

P2X₂, P2X₃, P2X₄ and P2X₆ receptors are not expressed by epithelial cells of the duodenal epithelium (Groschel-Stewart *et al.*, 1999). However, P2X receptors have been shown to play an important role in synaptic transmission within the neural pathway mediating motor behaviour in the intestine (Katayama and Morita, 1989; Heinemann *et al.*, 1999). Moreover, sub-mucosal neurones have been suggested to express functional P2X₁, P2X₂, P2X₃ and P2X₄ receptors in the mouse jejunum (De Man *et al.*, 2003; Xiang and Burnstock, 2004). Hence, it is possible that the slightly upregulated expression of P2X₂, P2X₃ and P2X₄ receptors in the epithelium of C57BL/10 mice is due to the presence of these receptors in the sub-mucosal nerve fibres that innervate the epithelial crypts.

In other studies, antibodies have been successfully used to define the profile of purinergic receptor expression in the intestinal epithelium (Groschel-Stewart *et al.*, 1999; Giaroni *et al.*, 2002), and so a similar approach could be used to follow-up the work presented here. This would determine whether alterations in receptor transcript expression actually translate into changes in protein levels. Furthermore, this would identify which cell types were expressing the receptors. The use of purinergic receptor knock-out animals would also be an extremely useful tool in determining the role of purinergic signalling in parasite expulsion. However, this would be a more general approach as all cell types would lack the purinergic receptor involved. Further work is also required to determine whether *T. spiralis* infection alters the effects of adenosine

and nucleotides on intestinal epithelial cell resistance, Na^{2+} -linked glucose absorption and ion secretion. This would not only provide insight into purinergic signalling in the small intestine during the infection, but could also determine the effects of parasite secreted proteins on intestinal function.

In summary, this chapter has shown that purinergic receptor expression in the intestinal epithelium is altered throughout the course of infection, and that this appears to correlate with pathological changes in the epithelium. Furthermore, this seems to be dependent on the genetic background of the host, as rapid- and slow-responder strains of mice not only express slightly different sub-types of purinergic receptors, but display different patterns of receptor expression throughout the course of *T. spiralis* infection. Purinergic receptors implicated in the secretory-flush response appear to be expressed earlier in the fast responder strain, potentially indicating a role for these receptors in aiding parasite expulsion.

Conclusions

Extracellular nucleotides are autocrine and paracrine signalling molecules whose receptor mediated effects regulate a wide range of physiological responses in mammalian tissues. Numerous studies have demonstrated that extracellular nucleotides can modulate inflammatory and other immune responses. Secretion of nucleotide-metabolising enzymes by both ecto- and endo-parasites is thought to be an adaptation that has evolved to modulate the availability and local concentrations of extracellular nucleotides (Gounaris and Selkirk, 2005). This would be advantageous to all species of parasite that, through tissue damage or residence in the vascular system, induce the release of proinflammatory nucleotides. Presumably, these secreted enzymes serve to regulate purinergic receptor activation, thus potentially limiting a range of inflammatory responses.

The hypothesis that *T. spiralis* secretes nucleotide-metabolising enzymes to actively down-regulate nucleotide-induced immune responses during the invasive stages of its life-cycle was investigated. The actions of these enzymes are postulated to promote parasite survival within the host. The results presented here have shown for the first time that the *T. spiralis* secreted nucleotide-metabolising enzymes can interfere with the activation of key immune-cell types *in vitro*.

Mucosal mast cells expressing mast cell protease-1 (mMCP-1) directly mediate the expulsion of adult *T. spiralis* parasites from the small intestine, and contribute to the development of the local inflammatory response and the consequent enteropathy seen at this stage of the infection (Lawrence *et al.*, 2004; Knight *et al.*, 2000). *In vitro* activation of bone marrow-derived mast cells that express mMCP-1 (and hence were of a mucosal phenotype), was used as a model to characterise the effects of purinergic signalling on mast cells. Concordant with other mast cell types, it was shown that these cells expressed multiple sub-types of purinergic receptors, including functional A_{2B}, A₃, P2Y₁, P2Y₂, P2X₄ and putatively P2Y₆ and CysLT1 receptors. Expression of these purinergic receptor sub-types enabled the mBMMC to respond to nanomolar concentrations of ADP and ATP, and micromolar concentrations of adenosine, UDP, and UTP. The 5'-NT activity present in *T. spiralis* ES was able to modulate mBMMC purinergic receptor activation by degrading extracellular nucleotides. Overall, this appeared to result in reduced P2Y receptor activation, and where relevant, increased P1 receptor activation. *T. spiralis* secreted proteins also modulated mBMMC activation by

altering the amount of adenosine available to interact with P1 receptors. This effect could be attributed to the action of the *T. spiralis* adenosine deaminase which would deaminate adenosine to inosine, and prevent receptor activation. These results demonstrated for the first time that *T. spiralis* ES can directly modulate mast cell purinergic receptor activation.

Subsequently, the complementary or opposing roles of purinergic receptor activation on mast cell effector functions were explored. Further experiments showed that ADP and ATP could induce mBMMC exocytosis resulting in the release of β -hexosaminidase and mMCP-1. Importantly, these nucleotides elicited the release of mMCP-1 *via* a mechanism that was not dependent on *de-novo* protein synthesis or the Golgi complex, indirectly suggesting adenine nucleotides induced the exocytosis of granules containing mMCP-1. Further characterisation of the purinergic receptor subtypes involved in mediating this effect showed that ADP appeared to signal *via* stimulation of the P2Y₁ receptor. The purinergic receptor that mediated the effects of ATP was not fully characterised due to the lack of available antagonists. However, ATP was postulated to mediate its effects *via* stimulation of the P2X₄ receptor on the basis of electrophysiological analysis. Degradation of the tight-junction protein occludin, by mMCP-1, aids parasite expulsion by increasing the paracellular permeability of the jejunum (McDermott *et al.*, 2003). Thus, activation of mast cells with extracellular adenine nucleotides will potentiate the release of mMCP-1, further contributing to the generation of an inhospitable environment for parasites and aid parasite expulsion from the intestine.

T. spiralis ES inhibited ADP-induced mMCP-1 release. This was mediated by the action of the secreted 5'-NT, which metabolised ADP to adenosine. *T. spiralis* ES also inhibited low concentrations of ATP inducing mMCP-1 release, possibly *via* the action of the secreted serine/threonine kinases and NDPK. The secretion of nucleotide metabolising enzymes by *T. spiralis* ES may serve to limit P2Y and P2X purinergic receptor activation on mucosal mast cells, thus limiting mMCP-1 release. This could favour parasite survival by reducing the permeability of the intestine, thus reducing the influx of solutes and water that would usually flush the parasite away.

The effects that nucleosides and nucleotides have on a range of other mast cell functions were also investigated to determine the extent of the effects these signalling molecules could have on mucosal mast cells. Nucleosides and nucleotides could not direct the migration of mature mast cells, immature mast cells, or mast cell progenitors,

suggesting that these molecules are not directly involved in the migration of mast cells to areas of damaged tissue. Furthermore, treatment of mature mast cells with nucleosides or nucleotides could not induce the release of factors that were chemoattractive to mast cell progenitors, implying that these signalling molecules could not promote the recruitment of mast cells either. Nucleotides and nucleosides were also unable to mediate *de-novo* generation of inflammatory and T_H2 cytokines in mast cells. This was consistent with a variety of other studies that have shown that nucleotides can not directly stimulate cytokine production in other types of mast cells (Feng *et al.* 2004).

Stimulation of purinergic receptors by nucleosides and nucleotides have been reported to modulate, rather than directly stimulate mast cell activation (Tilley *et al.*, 2000; Reeves *et al.*, 1997; Marquardt *et al.*, 1978; Feng *et al.*, 2004). During the intestinal phase of *T. spiralis* infection the increase in epithelial permeability has been postulated to allow the invasion of luminal bacteria (Lawrence *et al.*, 2004), thus potentially exposing mucosal mast cells to pathogen associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS). Due to the potential importance of TLR activation and purinergic-signalling in mucosal mast cells during *T. spiralis* infection, the effect of nucleosides and nucleotides on lipopolysaccharide (LPS)-induced TLR-4 stimulation of mBMMC was investigated.

Interestingly, the simultaneous activation of TLR-4 and purinergic receptors responsive to adenosine, ADP or ATP stimulated inhibitory pathways that led to the down-regulation of pro-inflammatory cytokine generation. In the presence of *T. spiralis* ES, the inhibitory effects of low concentrations of ADP were reduced. This suggests that parasite secreted proteins may act to enhance the amount of pro-inflammatory cytokines generated by TLR activation. The benefit of this to the parasite is not obvious, as this would promote pathology in the intestinal epithelium, possibly aiding parasite expulsion. Potentially, further work examining the characteristics of *T. spiralis* infection in TLR-4-deficient or MyD88-deficient mice may determine the importance of TLR-activation during the intestinal phase of the infection.

As *in vitro* experiments had shown the *T. spiralis* secreted 5'-NT could directly modulate purinergic signalling in immune cell types, it seemed important therefore to establish whether immunisation with this recombinant protein could protect mice from subsequent *T. spiralis* infection. Ultimately, the purpose of this experiment was to provide insight into the *in vivo* function of the secreted 5'-NT.

Challenge of 5'-NT-immunised animals with *T. spiralis* infective larvae resulted in a slightly reduced parasite burden in the muscle, however this did not reach statistical significance. This was despite the generation of high titres of 5'-NT-specific antibodies that could neutralise the activity of the 5'-NT *in vitro*. Notably, although the immunisation generated a T_H2-dominated systemic immune response, it did not induce protective adaptive immune responses in the MLN local to the intestine. Thus, further work is still required to develop an immunisation strategy that will generate a potent enough immune response in the intestinal environment. Considering that interest in mucosal adjuvants and vaccine delivery is currently an intense area of research, the next few years are likely to witness an increase in the rate of progress in this field. Potentially, if the immune response can be directed towards the gut mucosa, new opportunities for discovering the *in vivo* role of this enzyme will become available.

An unexpected outcome of the 5'-NT immunisation experiment was discovering that sera from naive mice actually increased the enzymatic activity of the 5'-NT *in vitro*. This potentially indicates that this enzyme may have a higher specific activity than previously thought. Elucidating what kind of small molecule was causing this effect was beyond the scope of this project, but any kind of trace element, co-factor or metabolite present in sera could be responsible.

This thesis has also addressed the broader aspects of purinergic receptor expression in the intestinal epithelial compartment during *T. spiralis* infection. This was performed to determine whether infection modulates the expression of purinergic receptors. Interestingly, purinergic receptor expression was altered throughout the course of *T. spiralis* infection, and this appeared to correlate with pathological changes in the epithelium. Furthermore, this seemed to be dependent on the genetic background of the host, as fast- and slow-responder strains of mice not only expressed slightly different sub-types of purinergic receptors, but displayed different patterns of receptor expression throughout the course of infection. Purinergic receptors implicated in the secretory-flush response appear to be expressed earlier in the fast responder strain, potentially indicating a role for these receptors in aiding parasite expulsion.

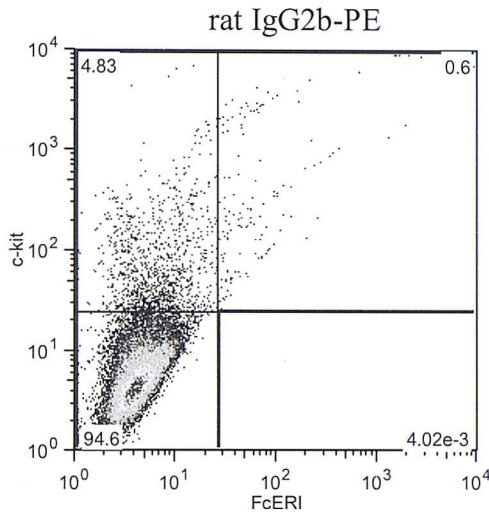
Altered receptor expression could occur due to the up/down-regulation of purinergic receptors expressed by individual cell types, or an increase/decrease in specific cell populations within the epithelium expressing purinergic receptor sub-types. Further experiments using antibodies to define the profile of purinergic receptor expression on specific cell types in the epithelium would be a useful extension to the

work presented in this thesis. Furthermore, this would determine whether alterations in receptor transcript expression actually translate into changes in protein levels.

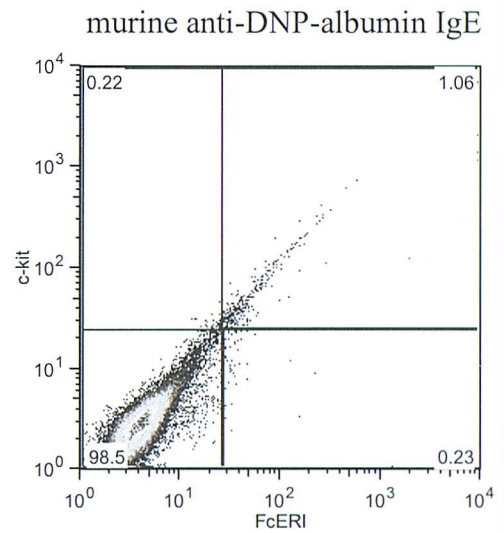
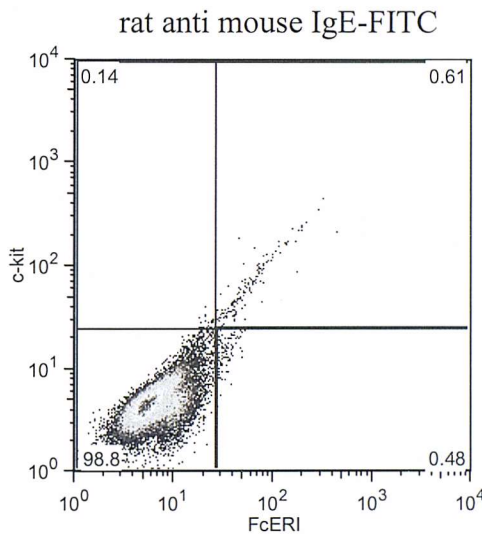
Experimental models of parasitic infection have been key tools to further understand how the immune system functions to combat infection and prevent disease. Currently, the mechanisms that initially alert the immune response to parasite invasion are still unknown. It is a tantalising prospect to consider that purinergic signalling, one of the most evolutionarily ancient signalling pathways known, may alert the immune response to the presence of invading parasites. The work described in this thesis has demonstrated that the nucleotide metabolising enzymes secreted by *T. spiralis* can modulate purinergic receptor activation and ultimately cellular functions. These experiments contribute towards dissecting how *T. spiralis* manages to out-run the host immune response, especially in the epithelial compartment. Further work aiming to define the complex roles purinergic signalling events play in helminth infections may potentially aid understanding of how intestinal inflammatory responses are induced and maintained, and go some way towards explaining why these parasites are so successful.

Appendix

A Isotype control for c-kit staining

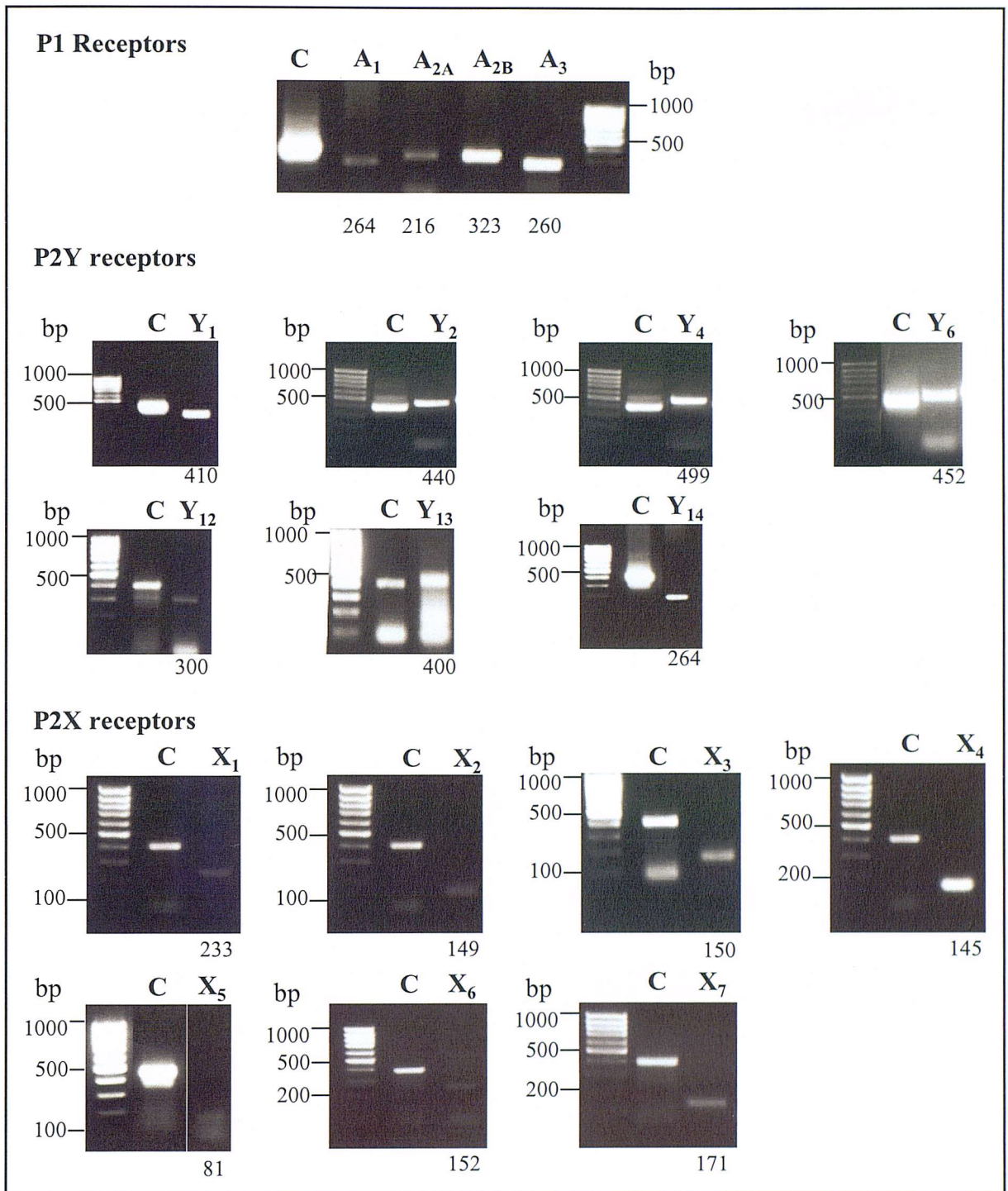


B Control for FcεRI staining



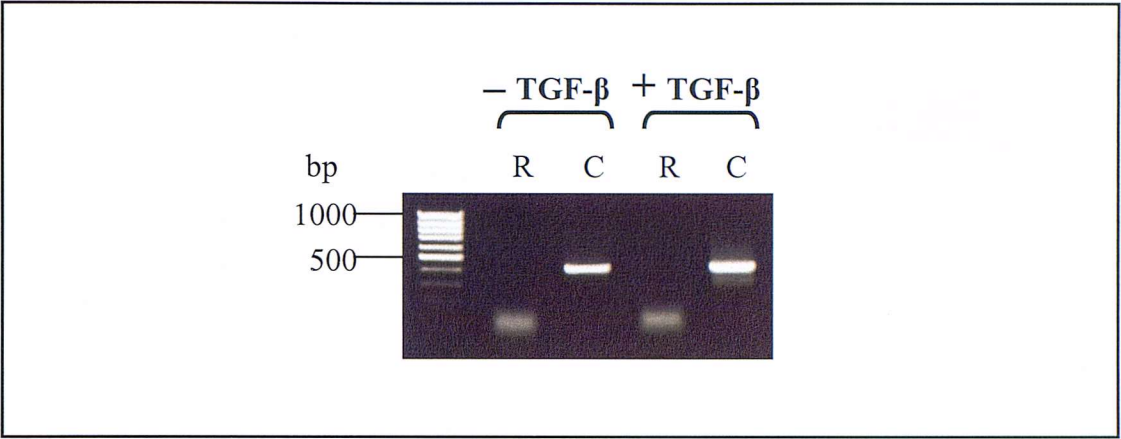
Appendix 1-1 Isotype control staining for FACS data

mBMMC were stained for c-kit and FcεRI at a range of time points during culture. Isotype controls were analysed alongside corresponding antibodies at each time point and representative data are shown. Cells were stained with isotype control antibodies in order to set quadrants for subsequent analysis and to exclude non-specific staining. All isotype controls were used at the same concentration and under the same conditions as the corresponding fluorescently labelled antibody. Samples were analysed by flow cytometry and data are presented as fluorescence intensity dot plots, as determined by forward and side scatter properties. For c-kit control staining, cells were incubated with (A) 1 µg/ml rat IgG2b-PE. For FcεRI control staining, cells were incubated with (B) 1 µg/ml rat anti-mouse IgE-FITC or (C) 1 µg/ml murine anti-DNP-albumin IgE.



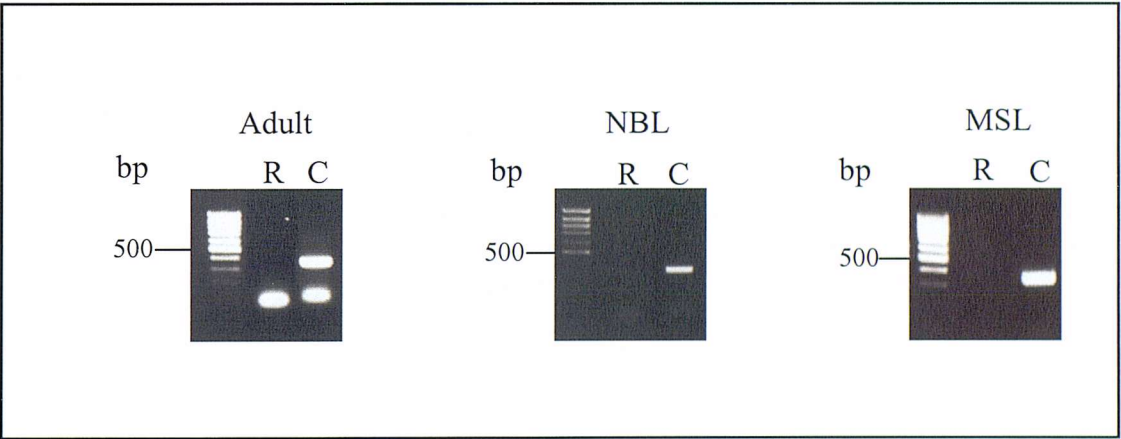
Appendix 1-2 Purinergic receptor control RT-PCRs

Positive control Reverse transcriptase (RT)-PCR data displaying PCR products of the expected sizes. Spleen cDNA was used as a template for the P1, P2Y₂, P2Y₄, P2Y₆, P2Y₁₂ and P2Y₁₃ receptor primer pairs. Mouse brain cDNA was used as a template for the P2X₆ receptor. mBMMC cDNA was used as a templates for the P2X₁, P2X₄, P2Y₁ and P2Y₁₄ receptor primer pairs. Mouse intestinal epithelial cell cDNA was used as a template for P2X₂ and P2X₃ receptor primer pairs. CMT-93 epithelial cell cDNA was used as a template for P2X₅ and P2X₇ receptor primer pairs. PCR amplification of β -actin was used as a control (lane C). Samples were analysed on a 1.5 % (P1 and P2Y receptors) or 2 % (P2X receptors) (w/v) agarose gel and PCR fragment size was evaluated using a 1000 base pair (bp) DNA ladder. The actual size (in bp) of the PCR products generated are indicated below each gel photo



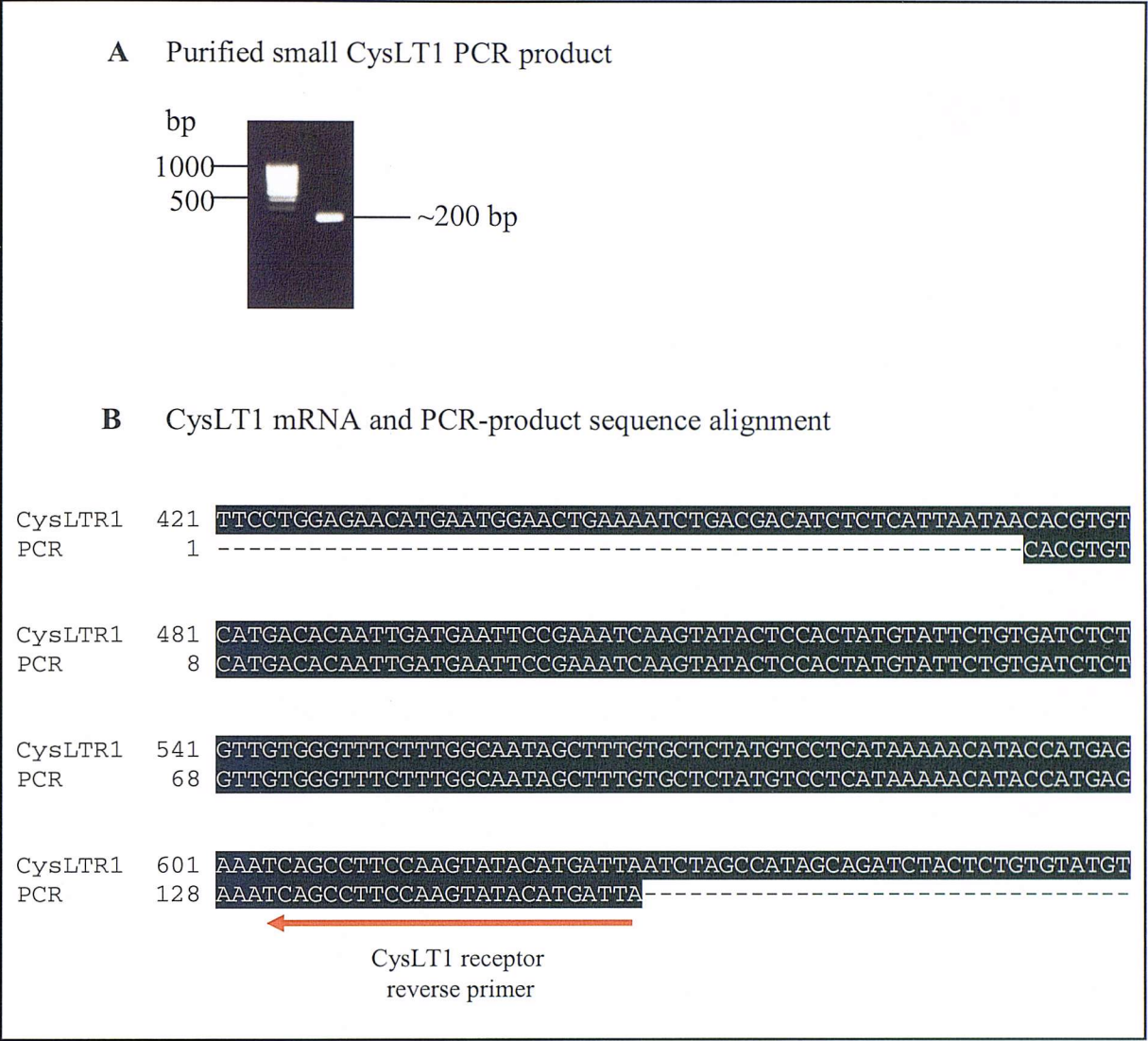
Appendix 1-3 PCRs to control for genomic DNA contamination in RNA preparations extracted from mBMMC cultured in the absence or presence of TGF-β

Control PCRs were carried out using DNase-treated RNA preparations and β-actin primers (lane R). PCR amplification of β-actin using mBMMC cDNA as a template was used as a positive control (lane C). Samples were analysed on a 1.5 % (w/v) agarose gel and PCR fragment size was evaluated using a 1000 base pair (bp) DNA ladder.



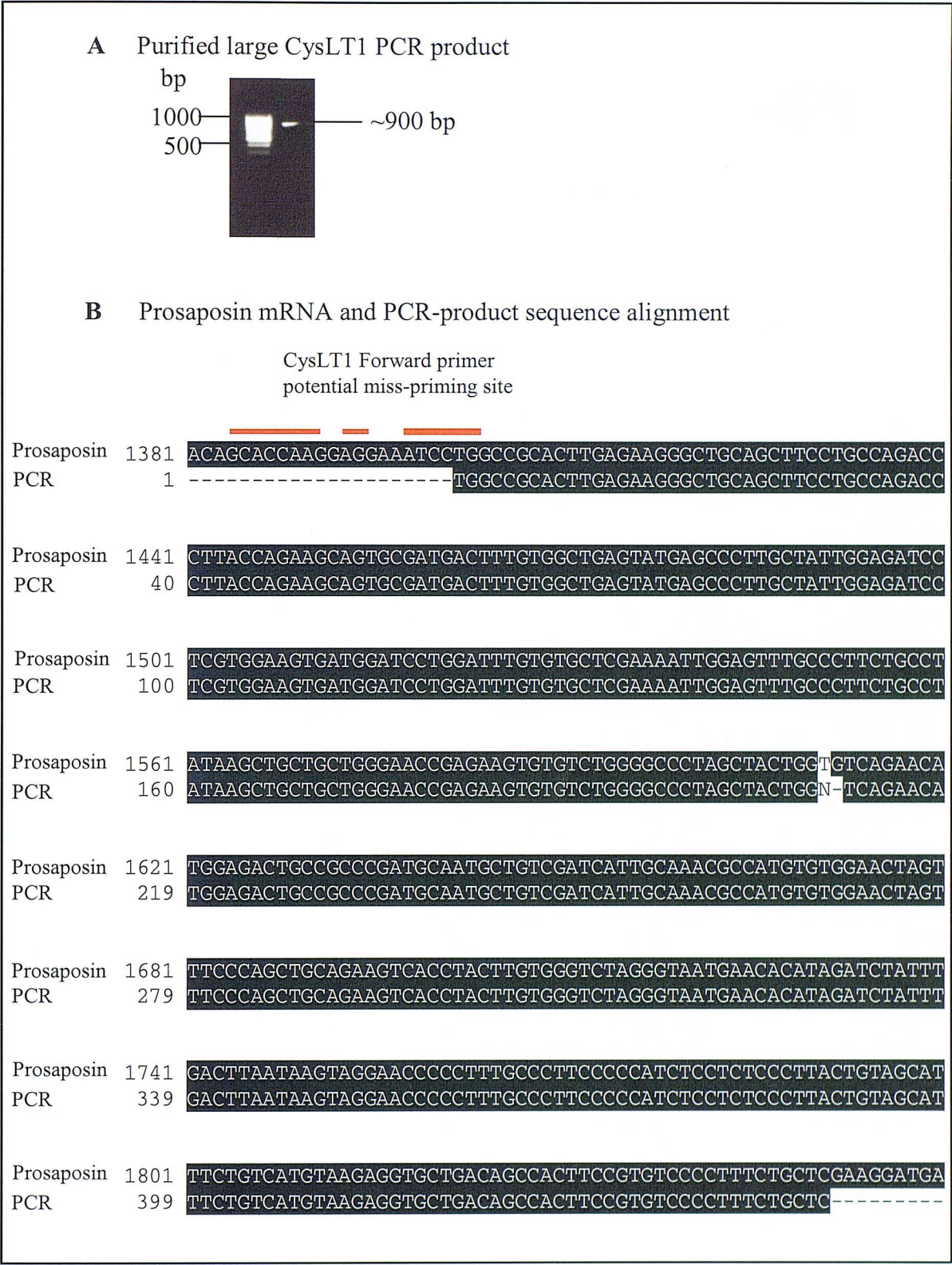
Appendix 1-4 PCRs to control for genomic DNA contamination in RNA preparations extracted from adult, NBL, MSL *T. spiralis* parasites

Control PCRs were carried out using DNase-treated RNA preparations and tubulin primers (lane R). PCR amplification of tubulin using adult, NBL or MSL cDNA as a template was used as a positive control (lane C). Samples were analysed on a 1.5 % (w/v) agarose gel and PCR fragment size was evaluated using a 1000 base pair (bp) DNA ladder.



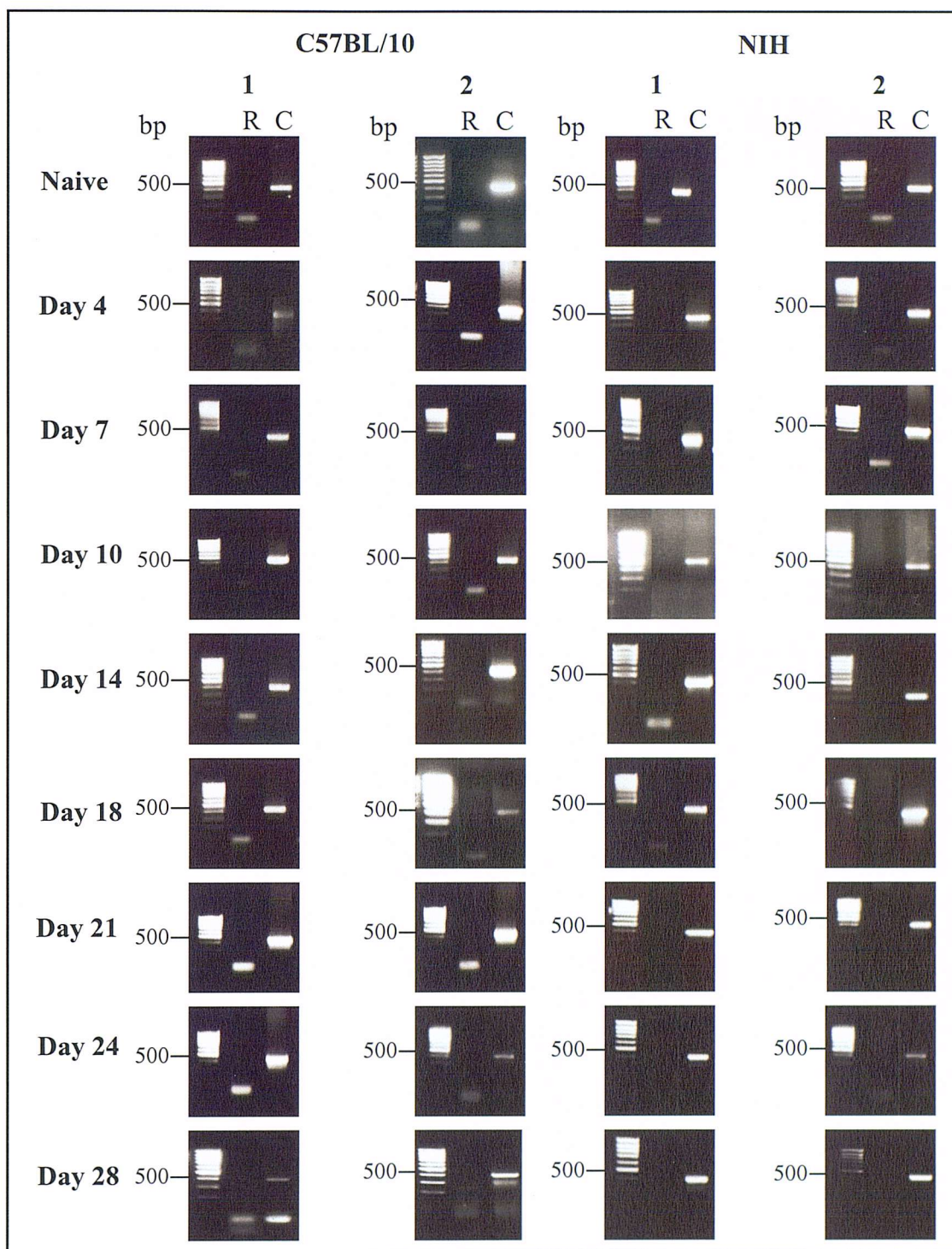
Appendix 1-5 Cysteinyl Leukotriene Receptor1 (CysLT1) ~200 bp PCR-product sequence alignment

The purified ~200 bp CysLTR1 PCR fragment (A) was sequenced and queried using the WU-Blast2 tool to search the EMBL nucleotide database. The PCR product (PCR) was subsequently aligned with the sequence of highest homology (murine CysLT1 mRNA NCBI accession number NM 021476) using the ClustalW (EMBI) programme (B). Alignments are displayed using BoxShade 3.21 software.



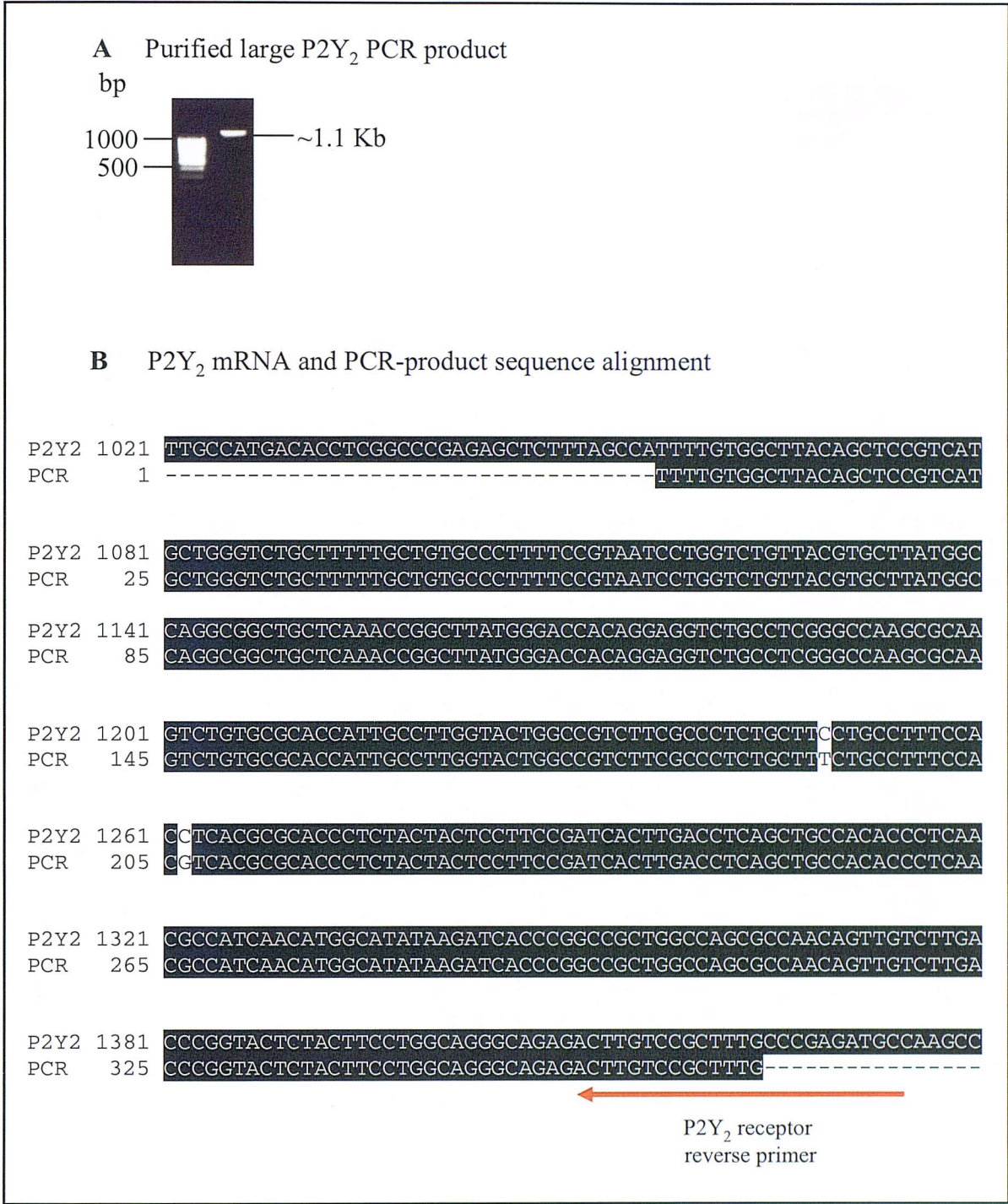
Appendix 1-6 Cysteinyl Leukotriene Receptor1 (CysLT1) ~900 bp PCR-product sequence alignment

The purified ~900 bp CysLT1 PCR fragment (A) was sequenced and queried using the WU-Blast2 tool to search the EMBL nucleotide database. The PCR product (PCR) was subsequently aligned with the sequence of highest homology (murine prosaposin mRNA NCBI accession number NM 011179) using the ClustalW (EMBI) programme (B). Alignments are displayed using BoxShade 3.21 software.



Appendix 1-7 PCRs to control for genomic DNA contamination in mouse epithelial RNA preparations

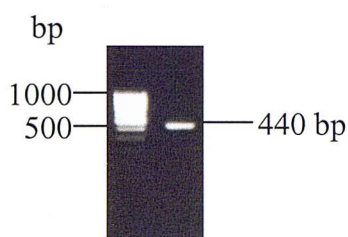
Control PCRs were carried out using DNase-treated epithelial cell RNA preparations and β -actin primers (lane R). PCR amplification of β -actin using epithelial cell cDNA as a template was used as a positive control (lane C). Data show the results obtained from 2 mice (1 and 2) of C57BL/10 and NIH strain. Samples were analysed on a 1.5 % (w/v) agarose gel and PCR fragment size was evaluated using a 1000 base pair (bp) DNA ladder.



Appendix 1-8 P2Y₂ receptor ~1.1 kb PCR-product sequence alignment

The purified ~1.1 kb P2Y₂ receptor PCR fragment (A) was sequenced and queried using the WU-Blast2 tool to search the EMBL nucleotide database. The PCR product (PCR) was subsequently aligned with the sequence of highest homology (murine P2Y₂ mRNA NCBI accession number NM_008773) using the ClustalW (EMBI) programme (B). Alignments are displayed using BoxShade 3.21 software.

A Purified small P2Y₂ PCR product



B P2Y₂ mRNA and PCR-product sequence alignment

P2Y ₂	1081	GCTGGGTCTGCTTTTTGCTGTGCCCTTTTCCGTAATCCTGGTCTGTTACGTGCTTATGGC
PCR	1	-----
P2Y ₂	1141	CAGGCGGCTGCTCAAACCGGCTTATGGGACCACAGGAGGCTGTCCTCGGGCCAAGCGCAA
PCR	1	--GGCGGCTGCTCAAACCGGCTTATGGGACCACAGGAGGCTGTCCTCGGGCCAAGCGCAA
P2Y ₂	1201	GTCTGTGCGCACCATTGCCTTGGTACTGGCCGTCTTCGCCCTCTGCTTCTGCCTTTCCA
PCR	59	GTCTGTGCGCACCATTGCCTTGGTACTGGCCGTCTTCGCCCTCTGCTTCTGCCTTTCCA
P2Y ₂	1261	CTCACGCGCACCCCTCTACTACTCCTTCCGATCACTTGACCTCAGCTGCCACACCCTCAA
PCR	119	CNTCACGCGCACCCCTCTACTACTCCTTCCGATCACTTGACCTCAGCTGCCACACCCTCAA
P2Y ₂	1321	CGCCATCAACATGGCATATAAGATCACCGGCCGCTGGCCAGCGCCAACAGTTGTCTTGA
PCR	179	CGCCATCAACATGGCATATAAGATCACCGGCCGCTGGCCAGCGCCAACAGTTGNTTGA
P2Y ₂	1381	CCCGGTACTCTACTTCCTGGCAGGGCAGAGACTTGTCGGCTTTGCCCGAGATGCCAAGCC
PCR	239	CCCGGTACTCTACTTCCTGGCAGGGCAGAGACTTGTCGGCTTTGCC-----

←
P2Y₂ receptor
reverse primer

Appendix 1-9 P2Y₂ receptor 440 bp PCR-product sequence alignment

The purified 440 bp P2Y₂ receptor PCR fragment (A) was sequenced and queried using the WU-Blast2 tool to search the EMBL nucleotide database. The PCR product (PCR) was subsequently aligned with the sequence of highest homology (murine P2Y₂ mRNA NCBI accession number NM_008773) using the ClustalW (EMBI) programme (B). Alignments are displayed using BoxShade 3.21 software.

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