A role for the $P2X_7$ receptor in the immune response to *Toxoplasma gondii*

by

Michael Lees
BSc (Hons) (UTS)

A thesis submitted for the degree of Doctor of Philosophy

Institute for the Biotechnology of Infectious Diseases
University of Technology, Sydney,
Australia

2009

Certificate

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Michael Lees

Acknowledgements

I started my PhD in 2005, under the early supervision of Dr Nicky Boulter and Prof Nick Smith. After Dr Boulter departed for a new job, my supervision fell to the hands of Prof Nick Smith and Dr Kate Miller. I whole heartedly thank my supervisors for all the help, support and input they have given me throughout the course of my PhD, with brain storming, lab work and reviewing my thesis. I have been fortunate to have such brilliant mentors.

My love of science began with my father, Mr Stephen Lees. His love of nature and a desire to understand his world, has rubbed off on me more than I am sure he realises. My Mother, Mrs Jennifer Lees, shaped me into the person I am. Mum, your strength and resolve with all aspects of your own life inspired me to go to university and undertake this PhD. Without the love and support I have received from you both, I would not have arrived where I am today. My moral values, scientific inspiration and loyalty to family and friends come from you, Dad and Mum. To Kylie, thank you for holding me together and loving me no matter what. Whatever comes next, I know we're ready for it.

I thank Prof James Wiley and Dr Steve Fuller for being supportive and productive collaborators and making the initial clinical observations with three toxoplasmosis patients which led to the beginning of this project.

During my PhD, it was a privilege spending three months at the University of Chicago, working in the laboratory of Prof Rima McLeod with samples from the NCCCTS. In collaboration with Prof Jennie Blackwell and Dr Sarra Jamieson, all subjects in the NCCCTS had previously been genotyped at eight $P2X_7$ receptor loci in order to conduct association testing for $P2X_7$ polymorphisms and congenital toxoplasmosis.

I sincerely thank Prof Rima McLeod, Dr Evan McLeod, Dr William Witola and Mr Ernest Mui for welcoming me to Chicago and helping me to realise my potential. I also thank Prof Jennie Blackwell and Dr Sarra Jamieson for making $P2X_7$ receptor genotyping and association testing data of the NCCCTS and EMSCOT available to me.

I also thank IBID and the Faculty of Science for my PhD scholarship and travel funding assistance, the ARC/NHMRC Research Network for Parasitology and the Australian Society for Parasitology for conference and travel funding assistance.

Very many of my friends deserve my gratitude. I thank Miss Amanda Hudson, Dr Catherine James, Mr Rowan Ikin, Dr Marilyn Katrib, Dr Jonathan Lowther, Dr Robert Walker and Miss Alana Zakrzewski for their friendship, input and regular help with my lab work. My friends are of course a very important part of my life, evidenced by the quality time we spent together, sensibly discussing the finer points of science and the arts over one (and never more than one) glass of wine or beer. Thank you for helping me in so many ways throughout my PhD, but particularly with maintaining my threadbare sanity.

Finally, I thank my long term friend, Sam, who was with me at the start but unfortunately left us for greener pastures before I could show him this thesis. ♥

To my wonderful parents.

Table of contents

CERTIFICATE I

ACKNOWLEDGEMENTS II

DEDICATION IV

TABLE OF CONTENTS V

LIST OF FIGURES AND TABLES VIII

ABBREVIATIONS X

ABSTRACT XIII

CHAPTER 1 – INTRODUCTION 1

1.1 – Toxoplasma gondii 1

1.2 – Infection and disease 4

1.3 – Treatment and vaccines 5

1.4 – Host cell invasion 7

1.5 – Immune response to T. gondii 9

1.6 – Immune evasion by T. gondii 14

1.7 – Clinical toxoplasmosis cases at Nepean Hospital 18

1.8 – Purinergic P2 receptors 21

1.9 – P2X$_7$ receptor 25

1.10 – P2X$_7$ receptor and intracellular bacteria 31

1.11 – P2X$_7$ receptor and intracellular parasites 35

1.12 – Project aim 37

1.13 – Objectives 38

CHAPTER 2 – GENERAL MATERIALS AND METHODS 39

2.1 - MATERIALS 39

2.2 – METHODS 43

2.2.1 – Ethics 43

2.2.2 – Statistical analysis 44

2.2.3 – In vitro tissue culture 44

2.2.4 – T. gondii tachyzoite culture – Vero cells 44

2.2.5 – T. gondii tachyzoite culture – HFF cells 45

2.2.6 – RAW 264.7 cell culture *45*
2.2.7 – Murine bone marrow macrophage culture *46*
2.2.8 – Human PBMC culture – Method 1 ..... *46*
2.2.9 – Human PBMC Culture – Method 2 ..... *47*
2.2.10 – P2X$_7$ receptor function assay *48*

CHAPTER 3 – *T. GONDII* VIABILITY AND REPLICATION ASSAYS – METHOD DEVELOPMENT **50**

3.1 - INTRODUCTION 50
3.2 – METHODS AND RESULTS 53
3.2.1 – Microplate cytometry replication assay *53*
3.2.2 – Flow cytometry viability assay *56*
3.2.3 – Comparative validation of flow cytometry viability assay *60*
3.3 – DISCUSSION 64

CHAPTER 4 – HUMAN P2X$_7$ RECEPTOR INVESTIGATION **68**

4.1 – INTRODUCTION 68
4.2 – METHODS AND RESULTS 70
4.2.1 – RH T. gondii viability following human P2X$_7$ receptor activation *70*
4.2.2 – RH T. gondii burden following polymorphic human P2X$_7$ receptor activation *74*
4.3 – DISCUSSION 80

CHAPTER 5 – *IN VITRO* MURINE P2X$_7$ RECEPTOR INVESTIGATION **83**

5.1 – INTRODUCTION 83
5.2 – METHODS AND RESULTS 84
5.2.1 – P2X$_7$ receptor investigation with RAW 264.7 cells *84*
5.2.2 – P2X$_7$ receptor investigation with murine BMM *91*
5.3 – DISCUSSION 94

CHAPTER 6 – *IN VIVO* MURINE P2X$_7$ RECEPTOR INVESTIGATION **100**

6.1 – INTRODUCTION 100
6.2 – METHODS AND RESULTS 100
6.2.1 – Murine infection with RH T. gondii – parasite burden *100*

6.2.2 – Murine infection with RH T. gondii – inflammatory cytokine analysis 102

6.3 – DISCUSSION 114

CHAPTER 7 – GENERAL DISCUSSION 118

7.1 – KEY FINDINGS 118

7.2 – FUTURE DIRECTIONS 124

7.3 – FINAL CONCLUSION 127

BIBLIOGRAPHY 129

List of figures and tables

CHAPTER 1 – INTRODUCTION 1

Figure 1.1 – Lifecycle of Toxoplasma gondii (Ferguson, 2002). 2

Figure 1.2 – Summary of the innate immune response to T. gondii. 10

Table 1.1 – Immunological investigation of toxoplasmosis subjects. 19

Figure 1.3 – $P2X_7$ receptor function of toxoplasmosis subjects. 20

Table 1.2 – $P2X_7$ receptor genotype of toxoplasmosis subjects. 21

Figure 1.4 – Membrane topology of P2X receptors. 24

CHAPTER 2 – GENERAL MATERIALS AND METHODS 39

CHAPTER 3 – *T. GONDII* VIABILITY AND REPLICATION ASSAYS – METHOD DEVELOPMENT 50

Figure 3.1 – YFP RH T. gondii fluorescence image. 55

Figure 3.2 – Validation of microplate cytometry T. gondii replication assay. 56

Figure 3.3 – Typical density plots obtained from flow cytometry T. gondii viability assay. 59

Figure 3.4 – Fluorescence microscopy assessment of T. gondii tachyzoite viability. 62

Figure 3.5 – Comparison of T. gondii tachyzoite viability assays. 63

CHAPTER 4 – HUMAN $P2X_7$ RECEPTOR INVESTIGATION 68

Figure 4.1 – $P2X_7$ receptor function of a normal/control donor. 71

Figure 4.2 – RH T. gondii viability and burden following human $P2X_7$ receptor activation. 73

Figure 4.3 – RH T. gondii burden in ATP-treated cells with functional $P2X_7$ receptors. 76

Figure 4.4 – RH T. gondii burden in ATP-treated cells with 1513C $P2X_7$ receptor polymorphism. 77

Figure 4.5 – Percent reduction of parasite burden by $P2X_7$ receptor genotypes 1 – 3. 79

CHAPTER 5 – *IN VITRO* MURINE P2X$_7$ RECEPTOR INVESTIGATION 83

Figure 5.1 – RAW 264.7 cell P2X$_7$ receptor expression. 86

Figure 5.2 – P2X$_7$ receptor function of RAW 264.7 cells. 87

Figure 5.3 – RH T. gondii viability following P2X$_7$ receptor activation of RAW 264.7 cells. 90

Figure 5.4 – P2X$_7$ receptor function of murine BMM. 92

Figure 5.5 – RH T. gondii viability following P2X$_7$ receptor activation of murine BMM. 94

CHAPTER 6 – *IN VIVO* MURINE P2X$_7$ RECEPTOR INVESTIGATION 100

Figure 6.1 – Parasite burden at site of infection in RH T. gondii infected mice. ... 102

Figure 6.2 – Serum IL-12p70 concentration in RH T. gondii infected mice. 105

Figure 6.3 – Serum IFN-γ concentration in RH T. gondii infected mice. 106

Figure 6.4 – Serum MCP-1 concentration in RH T. gondii infected mice. 107

Figure 6.5 – Serum IL-6 concentration in RH T. gondii infected mice. 108

Figure 6.6 – Serum TNF concentration in RH T. gondii infected mice. 109

Figure 6.7 – Serum IL-10 concentration in RH T. gondii infected mice. 110

Figure 6.8 – Serum IL-1β concentration in RH T. gondii infected mice. 112

Figure 6.9 – Serum NO concentration in RH T. gondii infected mice. 114

CHAPTER 7 – GENERAL DISCUSSION 118

Figure 7.1 – Downstream events following P2X$_7$ receptor activation. 120

Figure 7.2 – Apoptosis induced by P2X$_7$ receptor activation of RH T. gondii infected RAW 264.7 cells. 123

BIBLIOGRAPHY 129

Abbreviations

ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APLT	Aminophospholipid translocase
ATCC	American type culture collection
ATP	Adenosine triphosphate
BBG	Brilliant blue G
BCG	Bacillus Calmette-Guérin
BMM	Bone marrow macrophage
BN-PAGE	Blue native polyacrylamide gel electrophoresis
BSA	Bovine serum albumin
BzATP	Benzoyl-benzoyl adenosine triphosphate
CBA	Cytometric bead array
CD	Cluster of differentiation
CFU	Colony forming units
CNS	Central nervous system
CTL	Cytotoxic T-lymphocyte
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSCOT	European Multicentre Study on Congenital Toxoplasmosis
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FSW	FACS stain/wash solution
GM-CSF	Granulocyte macrophage-colony stimulating factor
HBSS	Hank’s balanced salt solution

HEPA	High efficiency particulate air
HFF	Human foreskin fibroblast
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
LPS	Lipopolysaccharide
MACS	Magnetically activated cell sorting
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MDM	Monocyte derived macrophage
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MyD88	Myeloid differentiation factor 88
NADPH	β-Nicotinamide adenine dinucleotide phosphate
NBS	Newborn bovine serum
NCCCTS	National Collaborative Chicago-based Congenital Toxoplasmosis Study
NEAA	Non-essential amino acids
NED	N-(1-naphthyl) ethylene diamine
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-κB
NIH	National Institutes of Health
NK	Natural killer
NO	Nitric oxide
NTP	Nucleotide triphosphate
oATP	Oxidised adenosine triphosphate
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PMA	Phorbol-12-myristate-13-acetate
PPADS	Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate
PS	Penicillin/streptomycin

PSF	Penicillin/streptomycin/fungicide
RNI	Reactive nitrogen intermediates
ROCK	Rho-effector kinase
ROI	Reactive oxygen intermediates
RPMI	Roswell Park Memorial Institute
SNP	Sodium nitroprusside
SSC	Side scatter
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor-β
TNF	Tumour necrosis factor
TLR	Toll-like receptor
USB	Universal serial bus
UV	Ultraviolet
YFP	Yellow fluorescent protein

Abstract

The $P2X_7$ receptor is a membrane bound cation channel expressed mainly on the surface of immune cells such as macrophages, lymphocytes and dendritic cells. $P2X_7$ receptor expression is up-regulated in response to the cytokine, IFN-γ, which also plays an integral role in the immune response to *Toxoplasma gondii*. Activation of the $P2X_7$ receptor is achieved through prolonged exposure to > 100μM ATP, which may be released from a variety of cellular sources, including activated platelets and dead/dying/damaged cells (including cellular damage caused by intracellular pathogens). Various studies have already demonstrated the ability of $P2X_7$ receptor activation to kill intracellular *Mycobacterium* spp., and have also linked a defective $P2X_7$ receptor with tuberculosis in humans. $P2X_7$ receptor activation is also known to kill intracellular *Chlamydia* spp., and has also been implicated in the immune response to *Leishmania* spp.

The hypothesis for this PhD project was that activation of the $P2X_7$ receptor results in the killing of intracellular *T. gondii*. Furthermore, that a defective $P2X_7$ receptor gene interferes with the normal immune response to *T. gondii*, rendering an individual more susceptible to severe disease following infection with *T. gondii*. Therefore the specific aims for this PhD project were to:

1. Develop fast, reliable methods to assess the viability and replication of intracellular *T. gondii* tachyzoites *in vitro*;
2. Assess the effect of ATP stimulation of human and murine immune cells on the viability and/or replication of Type I (RH) tachyzoites of *T. gondii*;
3. Assess the effect of deficiencies in $P2X_7$ receptor activity on the ability of ATP to affect the viability and/or replication of Type I (RH) tachyzoites of *T. gondii*;
4. Assess the effect of deficiencies in $P2X_7$ receptor activity on the production of inflammatory cytokines and mediators in response to infection with Type I (RH) tachyzoites of *T. gondii*.

Prior to investigating the role of the $P2X_7$ receptor in the immune response to *T. gondii*, two assays were developed that facilitated the accurate measurement of intracellular *T. gondii* tachyzoite viability or burden/replication. The viability assay used flow

cytometry to quickly and accurately quantify intracellular *T. gondii* tachyzoite viability, whereas the burden/replication assay used microplate cytometry to quantify intracellular *T. gondii* tachyzoite burden in host cells available in extremely limited quantities.

The human $P2X_7$ receptor was first investigated through *in vitro* experiments aimed at elucidating a role for $P2X_7$ receptor activation in the human immune response to *T. gondii*. Initially, RH *T. gondii* strain tachyzoites were infected into monocyte-derived macrophages cultured from a donor with full $P2X_7$ receptor function. ATP treatment of these cells to activate the receptor significantly reduced the viability of intracellular RH *T. gondii* (measured by the flow cytometry assay) and also reduced the number of intracellular YFP expressing RH *T. gondii* (measured by the microplate cytometry assay). Monocyte-derived macrophages from subjects with wild-type and polymorphic $P2X_7$ receptor genes were then infected with YFP expressing RH *T. gondii,* treated with ATP and parasite numbers monitored by microplate cytometry. Cells from donors with a polymorphism resulting in a loss of $P2X_7$ receptor function were unable to reduce the number of intracellular parasites whereas cells from donors with a wild type gene or a polymorphism that did not result in a loss of $P2X_7$ receptor function were able to reduce intracellular parasite numbers after ATP treatment

To complement the human investigation, experiments involving the murine $P2X_7$ receptor began with an *in vitro* investigation into the role of $P2X_7$ receptor activation in the murine immune response to *T. gondii*. These experiments definitively confirmed that ATP induced killing of RH *T. gondii* occurs via $P2X_7$ receptor activation, and not any other purinergic receptor/effect of ATP treatment. Blocking activation of the $P2X_7$ receptor in the immortalised RAW 264.7 mouse macrophage-like cell line by pre-treatment with the $P2X_7$ receptor antagonist, oATP, showed a reduction in ATP-induced RH *T. gondii* killing. Similarly, ATP treatment of bone marrow-derived macrophages cultured from $P2X_7$ receptor knockout mice did not result in a significant reduction in intracellular RH *T. gondii* viability.

The murine $P2X_7$ receptor investigation concluded with *in vivo* experiments aimed at understanding the phenotype of RH *T. gondii* infection in $P2X_7$ receptor knockout mice. These experiments showed that lack of $P2X_7$ receptor expression was associated with lower parasite burden at the site of infection and decreased serum concentration of

cytokines responsible for promotion and regulation of the inflammatory immune response.

The experiments conducted throughout this PhD have aided in the understanding of the immune response to *T. gondii*. It was previously known that $P2X_7$ receptor activation was important in the immune response to *Mycobacterium* spp., *Chlamydia* spp. and *Leishmania* spp. The role of the $P2X_7$ receptor may now also be extended to include *T. gondii*.

Chapter 1 – Introduction

1.1 – *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular protozoan parasite, belonging to the phylum Apicomplexa. The parasite is capable of causing a wide range of diseases in humans and other warm-blooded hosts. The organism was first described by Nicolle and Manceaux (1908) when it was observed in the blood, spleen, and liver of a North African rodent, *Ctenodactylus gondii*. There are three main phases of the *T. gondii* lifecycle, allowing for both sexual and asexual reproduction. Each phase consists of parasitic forms that ensure survival in the external environment, protection from the immune response and efficient spread and infection of new hosts (Figure 1.1).

The definitive host for *T. gondii* is the domestic cat or other Felidae, in which sexual replication of the parasite occurs inside intestinal epithelial cells. Very little is known about this stage of the lifecycle, and the degree to which the ratio of microgametes (male) and macrogametes (female) differs (Ferguson, 2002; West *et al.*, 2003). Nonetheless, sexual replication does occur in Apicomplexa, though, the development of unfertilised macrogametes into mature oocysts has also been proposed (Ferguson, 2002). Regardless of their method of production, oocysts are shed in the faeces of infected Felidae for 7 – 21 days, sporulating in the outside environment (Dubey *et al.*, 1970). The intermediate host for *T. gondii*, in which asexual replication occurs, may include humans or any other warm-blooded host such as cattle, sheep, pigs and birds.

Humans can become infected with *T. gondii* following ingestion of sporulated oocysts from the faeces of the definitive host. This may be due to poor hygiene when preparing food, improper hand-washing following the handling of cat litter, or being in close proximity to a cat that is shedding oocysts. An alternate route of infection is through the ingestion of tissue cysts containing bradyzoites in raw/undercooked meat (Figure 1.1). Tissue cysts are formed in the brain and musculature of infected intermediate hosts, after the mounting of a competent immune response forces the parasite to undergo stage conversion from the rapidly dividing tachyzoite stage to the slowly dividing bradyzoite stage, contained within tissue cysts.

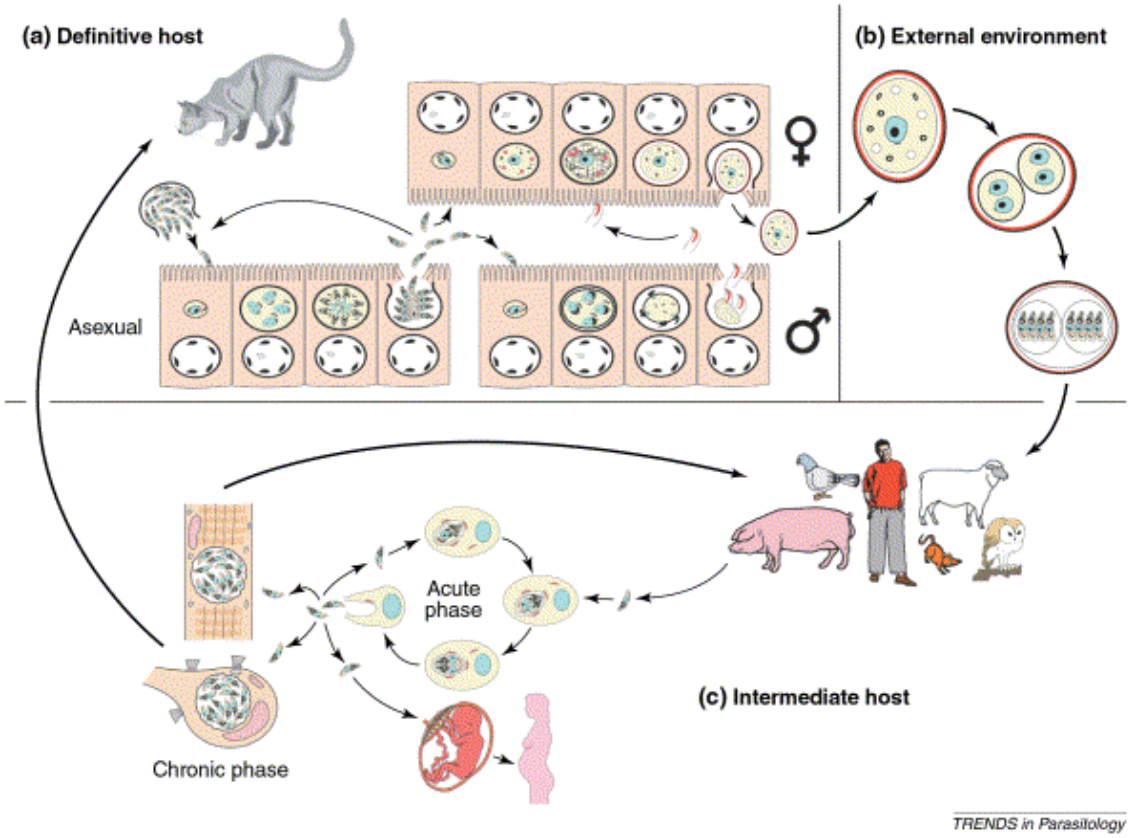

Figure 1.1 – Lifecycle of *Toxoplasma gondii* (Ferguson, 2002).
Sexual replication of *T. gondii* occurs in the feline definitive host, resulting in the production of oocysts by the fertilisation of female macrogametes by male microgametes. Oocysts sporulate in the external environment after their excretion in faeces. Humans and other intermediate hosts become infected following ingestion of sporulated oocysts, or by ingesting tissue cysts in raw or undercooked meat. After ingestion, oocysts/tissue cysts break open (excyst) and release sporozoites/bradyzoites respectively. Sporozoites/bradyzoites invade intestinal epithelial cells, replicating as tachyzoites primarily by endodyogeny, followed by dissemination through the blood and lymph to other tissues. The acute phase continues until a competent host immune response forces the organism into the chronic phase, during which it survives in the musculature and brain for the rest of the host's life, protected inside tissue cysts in the bradyzoite form. Cats, the definitive host, can also become infected with *T. gondii* by ingesting tissue cysts contained within the tissue of infected prey. Infection can also occur across the placenta from mother to foetus, through organ/blood transplants, or via needle stick injury in the laboratory.

Depending on location and other risk factors such as poor hygiene, seroprevalence of *T. gondii* typically ranges from 20 - 25% in the general population of the United States (Jones *et al.*, 2001) to 75 - 80% in El Salvador, France or Brazil (Ancelle *et al.*, 1996; Montoya and Liesenfeld, 2004). Despite high seroprevalence throughout the world,

infection has been steadily declining in recent years (Forsgren *et al.*, 1991; Smith *et al.*, 1996). Even so, *T. gondii* is still a parasite of medical and economical importance – over 1 billion people worldwide are infected.

There are three main clonal lineages of *T. gondii* that dominate in North America and Europe, termed types I, II and III, among which there is very little or no sexual recombination in the feline definitive host and differing only by a few percent at the DNA level for any given locus (Sibley and Boothroyd, 1992; Howe and Sibley, 1995). All three lineages can be isolated from humans; however type II isolates form the majority. Type II isolates have been mainly associated with symptomatic congenital toxoplasmosis and severe disease in AIDS patients (Ajzenberg *et al.*, 2002b; Howe *et al.*, 1997).

Notwithstanding the recent observations (Bowie *et al.*, 1997; Grigg *et al.*, 2001; Demar *et al.*, 2007) that epidemics of toxoplasmosis may be associated with atypical strains of *T. gondii*, various studies have confirmed the clonal lineage of *T. gondii*, which corresponds either to virulence or avirulence in the murine host. This notion has been supported by studies that have used techniques such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD)-PCR and microsatellites to identify markers that highlight the division between mouse-virulent and-avirulent isolates (Johnson, 1997; Ajzenberg *et al.*, 2002a).

While *T. gondii* strains share approximately 98% nucleotide homology, there are still striking phenotypic differences between isolates (Sibley and Boothroyd, 1992). Virulence of *T. gondii* is determined by the infectivity and subsequent mortality seen in the murine model of infection. Type I virulent strains of *T. gondii* cause a lethal infection in all strains of laboratory mice, even when infected at extremely low doses ($LD_{100} \approx 1$). In comparison, Type II and III avirulent strains of *T. gondii* have an $LD_{50} \geq 1000$ (Sibley and Boothroyd, 1992; Howe *et al.*, 1996). Therefore there are differences among these isolates that result in the ability of the parasite to cause devastating disease following infection.

1.2 – Infection and disease

Exposure to *T. gondii* and subsequent infection of an individual will result if the lifecycle stage of the parasite and route of infection are appropriate. The virulence of the parasite and infectious dose also play a role in the outcome of exposure to the parasite. In humans with a competent immune response, primary infection is mostly subclinical and asymptomatic (Georgiev, 1994; Fung and Kirschenbaum, 1996). The most common clinical manifestations of toxoplasmosis in immunocompetent individuals are non-specific symptoms, such as pyrexia, lymphadenopathy, malaise and myalgia. CNS involvement in immunocompetent individuals has been documented, but is very rare (Vastava *et al.*, 2002).

Contrary to the subclinical infection most evident with immunocompetent individuals, toxoplasmosis in immunocompromised individuals can result in life threatening disease. In patients infected with HIV, clinical manifestations of toxoplasmosis occur mostly due to the reactivation of a chronic infection following the depletion of the immune system associated with AIDS (Porter and Sande, 1992; Strittmatter *et al.*, 1992). In the USA 18% – 25% of patients infected with HIV and the subsequent onset of AIDS will suffer symptomatic toxoplasmosis, of which toxoplasmic encephalitis is the most frequent cause of morbidity and mortality (Luft and Remington, 1992; Kasper and Buzoni-Gatel, 1998). Toxoplasmic encephalitis causes the formation of multiple lesions within the brain, varying in size from microscopic to larger lesions affecting parts of the cerebral hemisphere (Navia *et al.*, 1986; Strittmatter *et al.*, 1992). Patients often present with clinical symptoms such as headache, confusion and fever, with disease progression possibly leading to disorientation, drowsiness, hemiparesis, reflex changes, convulsions, coma, and death (Porter and Sande, 1992).

Toxoplasma gondii has also evolved as an opportunistic pathogen in patients who receive organ transplants, or chemotherapy for malignancy. Primary infections in transplant recipients are mostly acquired from the donor organ, with survival of the parasite facilitated by immunosuppressive therapy that is used to prevent rejection of the transplant organ. While heart and heart – lung transplants (with donor seropositive for *T. gondii* and recipient seronegative for *T. gondii*) pose the greatest risk of

developing toxoplasmosis in the recipient (Couvreur *et al.*, 1992), transplant-associated toxoplasmosis has also been documented in kidney (Giordano *et al.*, 2002), liver (Mayes *et al.*, 1995) and bone marrow transplants (Weinrach and Oviedo, 2001), all of which can result in death of the patient. Toxoplasmosis in patients with malignancies is most frequently described in association with Hodgkin's disease (Whiteside and Begent, 1975; Hakes and Armstrong, 1983). It is not known if the mechanism of predisposal to toxoplasmosis in these patients is due to the immunosuppressive drugs used in treatment or a manifestation of Hodgkin's disease alone (Israelski and Remington, 1993).

Pregnant women and their unborn babies usually remain asymptomatic if the mother is chronically infected with *T. gondii* prior to pregnancy (Kravetz and Federman, 2005). However, if primary maternal infection occurs during pregnancy, the parasite can establish an infection in the placenta and spread to the foetus where it can cause severe disease. The risk of congenital infection with *T. gondii* following primary maternal infection increases dramatically with gestation time, from 6% in the first trimester to 70% in the third trimester (Dunn *et al.*, 1999). Fortunately, congenital disease is less severe with longer gestation time, resulting in the majority of congenitally infected neonates being asymptomatic at birth. However, most will still show retinal scarring in childhood or adolescence (Daffos *et al.*, 1988; Peyron *et al.*, 1996; Wallon *et al.*, 2004). The more rare and severe clinical presentation of congenital toxoplasmosis involves hydrocephaly, intracranial calcification and chorioretinitis, with the possibility of miscarriage or intrauterine death (Holliman, 1995).

1.3 – Treatment and vaccines

Immunocompetent adults and children with acute toxoplasmosis and accompanying lymphadenitis are not usually treated. However, if the condition persists or is unusually severe, a combination of pyrimethamine, sulfadiazine and folinic acid can be administered for 4 – 6 weeks (Georgiev, 1994; Fung and Kirschenbaum, 1996).

Upon the diagnosis of recently acquired maternal infection during pregnancy, oral spiramycin is administered to prevent transmission across the placenta to the foetus, whereas pyrimethamine/sulfadiazine combination is used for treatment of diagnosed

foetal infection (Wong and Remington, 1994). The rate of congenital toxoplasmosis has been demonstrated to be up to four times higher in infected neonates born to untreated mothers, compared with treated mothers (Ricci *et al.*, 2003). While pyrimethamine/sulfadiazine treatment is most effective in treating congenital toxoplasmosis, pyrimethamine is not recommended for treatment during the first trimester, due to its inhibition of dihydrofolate reductase and associated teratogenic effects (Harpey *et al.*, 1983; Wong and Remington, 1994).

Treatment of *T. gondii* infection in the immunocompromised patient can be divided into three situations: prevention of primary infection; treatment of the acute phase; and suppressive treatment. Orally administered trimethoprim-sulfamethoxazole is effective in preventing toxoplasmic encephalitis in patients with AIDS (Carr *et al.*, 1992; Gallant *et al.*, 1994) and transplant recipients on immunosuppressive drugs (Munoz *et al.*, 2003). Treatment of the acute phase of infection (most notably, toxoplasmic encephalitis) in immunocompromised patients usually involves a combination of pyrimethamine/sulfadiazine; however pyrimethamine/clindamycin treatment can be administered as an alternative in patients intolerant to sulfadiazine (Dannemann *et al.*, 1992). The same treatment used against the acute phase of infection is also administered as a suppressive maintenance treatment to prevent a relapse of toxoplasmic encephalitis in immunocompromised patients, due to the inability of any drugs to completely clear an infected individual of chronic infection (Pedrol *et al.*, 1990).

The development of a vaccine for human toxoplasmosis has so far been an elusive target. A vaccine for ovine toxoplasmosis, involving direct use of live tachyzoites of the attenuated S48 strain, is commercially available and successful (O'Connell *et al.*, 1988). However, this vaccine cannot be used in humans, due to the theoretically possible reversion to a pathogenic form of the parasite. Much attention has focused around developing a human vaccine based on surface antigens of tachyzoites, such as the membrane associated antigen SAG-1. Animal trials involving vaccination using recombinant SAG-1 or plasmid encoding the SAG-1 protein, have resulted in decreased morbidity and mortality compared with unvaccinated animals (Petersen *et al.*, 1998; Angus *et al.*, 2000), as well as decreased cyst production in the brain and reduced foetal transmission (Letscher-Bru *et al.*, 1998; Haumont *et al.*, 2000). Similar vaccines using either one or a combination of antigens from the apical complex have also been trialled

using animal models. These trials involved dense granule proteins (GRA-1, GRA-4 and GRA-7) (Desolme *et al.*, 2000; Vercammen *et al.*, 2000; Scorza *et al.*, 2003), rhoptry proteins (ROP-2) (Vercammen *et al.*, 2000) and microneme proteins (MIC-3) (Ismael *et al.*, 2003). These trials have also had some limited success in increasing survival time of vaccinated mice. However, it is unlikely that a human vaccine will be available in the near future, with emphasis more appropriately being directed towards prevention and surveillance schemes.

While current drug therapies are effective at treating toxoplasmosis in most patients, they have significant disadvantages. Drugs currently available are only effective against the stage of *T. gondii* involved in the acute phase of infection (tachyzoites – see Figure 1.1) and not against tissue cysts (containing bradyzoites – see Figure 1.1) (Fung and Kirschenbaum, 1996). This results in a high rate of relapse in immunocompromised patients following treatment for acute toxoplasmosis, as tissue cysts are not affected by the drugs (Leport *et al.*, 1988). In addition to this, adverse effects of the drugs used to treat toxoplasmosis are common, mainly involving dermatologic and haematologic side effects (Fung and Kirschenbaum, 1996). Of significant note is the dihydrofolate reductase inhibitor pyrimethamine, which causes bone marrow depletion (Boudes *et al.*, 1990). This side effect needs to be countered through the administration of folinic acid (Van Delden and Hirschel, 1996). Therefore, with the ineffectiveness of the drugs available, and their associated side effects, it is necessary for further research to be conducted in order to develop novel drug targets that are effective against all stages of the *T. gondii* life cycle and minimise side effects to the patient.

1.4 – Host cell invasion

Toxoplasma gondii tachyzoites exhibit a characteristic gliding motility dependent on parasite actin-myosin filaments, allowing the parasite to move through the extracellular environment and come into contact with a host cell (Dobrowolski and Sibley, 1996; Dobrowolski *et al.*, 1997). Host cell invasion is a rapid process taking significantly less time than phagocytosis (Morisaki *et al.*, 1995). Following invasion, the parasite resides within a parasitophorous vacuole composed of host cell membrane (Suss-Toby *et al.*, 1996). *Toxoplasma gondii* invasion is a passive process for the host cell, and no changes

are detected in host cell membrane ruffling, actin microfilament reorganization, or tyrosine phosphorylation of host proteins; events which occur during phagocytosis (Morisaki *et al.*, 1995). This is important because opsonisation and phagocytosis have been shown to result in fusion of lysosomes and acidification of the parasitophorous vacuole, causing death of the parasite (Sibley *et al.*, 1985; Mordue and Sibley, 1997).

Host cell invasion by *T. gondii* involves three distinct parasite organelles from the apical complex, and their sequential secretion at different times during the invasion process (Carruthers and Sibley, 1997). The release of intracellular parasite calcium stores initiates the exocytosis of a group of vesicles, known as micronemes (Lovett and Sibley, 2003). Micronemes contain proteins that bear homology to adhesive domains from higher eukaryotic proteins, such as thrombospondin-like domains, EGF-like domains and lectin-like domains, allowing attachment of the parasite to the surface of the host cell (Soldati *et al.*, 2001). Following attachment, the second phase of secretion involves club shaped organelles known as rhoptries, being secreted from the apical complex. They play a significant role in the formation of the parasitophorous vacuole (Beckers *et al.*, 1994). Final modifications are made when soluble dense granule proteins are secreted into the parasitophorous vacuole and incorporated into the parasitophorous vacuole membrane (Lecordier *et al.*, 1999; Carey *et al.*, 2000), or into an intravacuolar membranous network (Sibley *et al.*, 1995; Adjogble *et al.*, 2004). Their possible function may be as a surface extension to aid in exchange of nutrients between host cell and parasite.

Following host cell invasion, *T. gondii* tachyzoites divide asexually primarily by endodyogeny (a process in which two daughter cells grow inside a single mother cell) until the host cell cytoplasm has been consumed. The parasites then exit the host cell in search of another host cell to invade. A competent immune response is necessary to prevent this continual asexual replication.

1.5 – Immune response to *T. gondii*

As a direct result of the intracellular lifecycle of *T. gondii* and lack of any replicating extracellular stage, the development of a strongly biased Th-1 cell-mediated immune response is essential to halt the replication of the parasite (Denkers and Gazzinelli, 1998). In the absence of a competent immune response, *T. gondii* will replicate unabated and destroy infected tissues, leading to the severe pathology discussed earlier (Section 1.2).

Infection of immunocompetent individuals results in a cascade of immune response events that lead to the integrated development of an innate (Figure 1.2) and adaptive immune response. The innate response is responsible for initial control of parasite replication, forcing the parasite from the acute phase of infection into the chronic phase. In the long term, the parasite remains under the control of a T-cell based adaptive immune response.

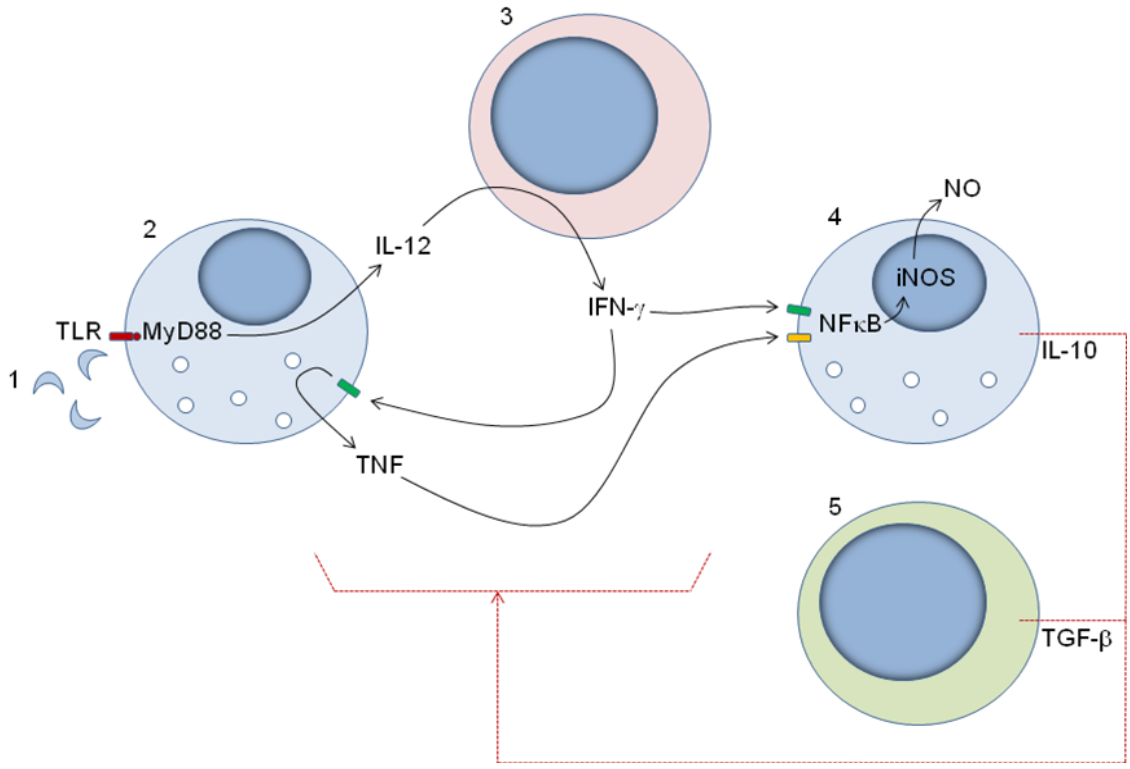

Figure 1.2 – Summary of the innate immune response to *T. gondii*.

T. gondii tachyzoites (1) come into contact with macrophages and dendritic cells (2), recognised through TLRs and signalling molecules such as MyD88 to promote the release of IL-12. IL-12 acts on NK cells and T-cells (3) resulting in the release of IFN-γ, which is essential for both the early control of, and continued resistance to reactivation, of *T. gondii* infection. IFN-γ then acts on macrophages (2), resulting in secretion of TNF, which acts synergistically with IFN-γ, binding to receptors on the surface of other macrophages (4), (or the same macrophage, not shown), resulting in the activation and nuclear translocation of NF-κB. The activation of NF-κB is essential in the production of NO, as it acts as a transcription factor for iNOS – an enzyme which catalyses the formation of NO. Upon formation of NO, there can be three parasite outcomes, depending on many host–parasite interactions involving host susceptibility/resistance and parasite virulence: intracellular parasites can be killed following NO production; proliferate for a short period of time in the acute phase; or avoid the immune response through the formation of bradyzoites within tissue cysts. The pro-inflammatory response is controlled through the secretion of IL-10 by macrophages (4) and TGF-β by regulatory T-cells (5), which act to regulate the transcription of pro-inflammatory cytokines such as IL-12, IFN-γ and TNF.

The initiation of the innate immune response to *T. gondii* is thought to begin when dendritic cells (Reis e Sousa *et al.*, 1997), neutrophils (Bliss *et al.*, 1999) and macrophages (Scharton-Kersten *et al.*, 1996) come into contact with *T. gondii* antigen, resulting in the production of IL-12. The importance of IL-12 in the innate immune response has been demonstrated by Khan *et al.*, (1994), who showed that exogenously administered IL-12 was able to increase the survival of mice infected with *T. gondii*,

with the effect being reversed following IL-12 depletion using neutralising antibody. The induction of IL-12 production in response to *T. gondii* antigen *in vivo* is dependent on the adaptor molecule myeloid differentiation factor 88 (MyD88). MyD88 acts as a signalling molecule for most toll-like receptors (TLR), which recognise and respond to pathogen-associated molecular patterns (PAMPs) such as lipids, carbohydrates and nucleic acids with the production of inflammatory cytokines (Takeda and Akira, 2003). The significance of MyD88 in IL-12 induction was shown by Scanga *et al*., (2002), with IL-12 production being dramatically reduced in *T. gondii*-infected MyD88 knockout mice, resulting in uncontrolled parasite replication. Interestingly, it has been noted that IL-12 production by macrophages is dependent on the strain of parasite with which the macrophages are infected. Robben *et al*., (2004) showed that macrophages infected with an avirulent Type II strain of *T. gondii* secreted IL-12, however, those infected with Type I virulent *T. gondii* did not, indicating that early IL-12 production is essential for parasite control.

Following its secretion, IL-12 acts on NK cells and T-cells resulting in the production of IFN-γ (Gazzinelli *et al.*, 1994). Both IL-12 and, particularly, IFN-γ are thought to be major cytokines involved in the immune response to *T. gondii*, as they are known to prime macrophages for enhanced microbial killing and promote a strongly biased Th-1 response (Seder *et al.*, 1993; Hsieh *et al.*, 1993). Suzuki *et al.*, (1988) first demonstrated the pivotal role of IFN-γ in murine toxoplasmosis, showing that administration of a monoclonal antibody against IFN-γ resulted in decreased macrophage activation and eventual death due to acute toxoplasmosis. IFN-γ treatment of *T. gondii*-infected murine macrophages drives parasite stage conversion from the rapidly dividing tachyzoite to the slowly dividing bradyzoite, indicated by the expression of bradyzoite specific antigens (Bohne *et al.*, 1993). IFN-γ was also shown to be important in minimising transmission to the unborn foetus, with IFN-γ knockout mice exhibiting higher parasite burdens in the placenta and uterus in comparison to the parental strain (Shiono *et al.*, 2007). Continued IFN-γ production is also essential in maintaining control of chronic *T. gondii* infection, with neutralising IFN-γ antibodies administered to chronically infected mice resulting in rapid parasite reactivation and subsequent host mortality.

While all the targets and roles of IFN-γ in the immune response to *T. gondii* are still in the process of being elucidated, it is known that one major function of IFN-γ is to promote the release of TNF from many cell populations, including macrophages, with both cytokines acting synergistically to promote the inflammatory response through NO production (Gazzinelli *et al.*, 1993; Janssen *et al.*, 2002). NO is a potent anti-microbial molecule capable of inhibiting proteins involved in the mitochondrial respiratory chain and is therefore toxic to *T. gondii* (Bohne *et al.*, 1994).

The synergistic action of IFN-γ and TNF in the production of NO is thought to act by promoting the nuclear translocation of NFκB. Sibley *et al.*, (1991) showed that recombinant TNF is able to trigger IFN-γ primed macrophages to kill intracellular *T. gondii*, however, IFN-γ primed macrophages were unable to kill *T. gondii* in the absence of another signal, confirming the important role of TNF in this process.

NFκB plays an important role in the series of events leading to NO production. NFκB dimers are ubiquitously expressed in the cytoplasm, usually composed of p50 and p65 (RelA) subunits, however, other combinations of subunits involving p52, c-Rel and RelB exist. The combination of subunits ultimately determines the gene for which the NFκB dimer acts as a transcription factor. In its inactive form in the cytoplasm, NFκB dimers are bound to IκB, an inhibitory molecule that masks the NFκB nuclear translocation signal (Baeuerle and Baltimore, 1988). Upon binding of TNF to the surface of the cell, IκB kinases are activated, resulting in the cleavage of NFκB from IκB, and its subsequent translocation to the nucleus (Karin and Ben-Neriah, 2000). The promoter of the murine gene encoding inducible nitric oxide synthase (iNOS) has two binding sites for NF-κB (Xie *et al.*, 1994). Following its translocation to the nucleus, NF-κB binds to the iNOS promoter where it enhances transcription of iNOS. iNOS is an enzyme that catalyses the formation of the microbicidal molecule NO from arginine (Xie *et al.*, 1994). NO produced by macrophages has been shown to be toxic to intracellular *T. gondii*, demonstrated by the reversal of macrophage induced *T. gondii* killing when treated with inhibitors that prevent the production of NO (Adams *et al.*, 1990).

Various other pro-inflammatory cytokines also contribute to the early control of *T. gondii*. It was shown that the administration of exogenous IL-18 to *T. gondii* infected mice was able to increase production of IFN-γ by NK cells, reducing parasite burden and time to death, indicating that IL-18 acts in synergism with IL-12 to promote IFN-γ release (Cai *et al.*, 2000). In addition, IL-1β has also been shown to be required for IL-12 induced production of IFN-γ by NK cells in *T. gondii* infected mice, with the ability of exogenous IL-12 to delay death in these mice being dependent on IL-1β (Hunter *et al.*, 1995).

It is important to also note that, while the mounting of an inflammatory response is important in host cell clearance of intracellular pathogens such as *T. gondii*, it is also extremely important that regulatory elements perform the function of limiting this inflammatory response in order to prevent immunopathology. Indeed, many of the symptoms of toxoplasmosis are due to the effects of immunopathology generated by the host immune response (Gazzinelli *et al.*, 1996). IL-10 and TGF-β are two cytokines central to regulation of the inflammatory response during *T. gondii* infection. IL-10 knockout mice are unable to control the inflammatory immune response to *T. gondii*, characterised by high levels of IL-12, IFN-γ and TNF, and succumb to immunopathology much faster than the parental strain of mice (Gazzinelli *et al.*, 1996). In response to pro-inflammatory cytokines, IL-10 secretion, predominantly by monocytes (de Waal Malefyt *et al.*, 1991), and subsequent binding to IL-10 receptors results in the intracellular activation of JAK1, which phosphorylates STAT3 (Moore *et al.*, 2001). STAT3 dimers then translocate to the nucleus, regulating (blocking) the transcription of various pro-inflammatory cytokines and molecules such as NFκB, IFN-γ and TNF (Lang *et al.*, 2002)., It has been known for some time that TGF-β, in synergy with IL-10, is capable of binding to macrophages, resulting in their deactivation (Tsunawaki *et al.*, 1988). While TGF-β is secreted mainly by $CD4^+/CD25^+$ regulatory T-cells, murine macrophages infected with *T. gondii* can also secrete TGF-β, acting to regulate IFN-γ and TNF-induced NO production (Bermudez *et al.*, 1993; Langermans *et al.*, 2001).

While the innate immune response is effective at coping with *T. gondii* infection during the acute phase, an adaptive immune response is necessary for long term control of the

parasite. Beaman *et al*., (1994) used SCID mice to show that infection with *T. gondii* elicits an innate immune response. However, mice succumbed to infection within 20 days unless reconstituted with T-cells. In the case of *T. gondii* infection, the early production of IL-12 by various cells results in the production of IFN-γ as earlier discussed. The importance of continued IFN-γ production is evident when mice chronically infected with *T. gondii* are administered neutralising antibodies directed against IFN-γ, resulting in reactivation of chronic infection and uncontrolled replication of *T. gondii* causing death within 12 days (Gazzinelli *et al.*, 1993). Therefore, not only does IFN-γ production help to control infection in the acute phase by priming macrophages for enhanced killing, but it also contributes to continued control of parasite replication in the chronic phase, forcing the parasite to stay within tissue cysts as bradyzoites (Gazzinelli *et al.*, 1994).

1.6 – Immune evasion by *T. gondii*

While a competent immune response is able to force *T. gondii* into the chronic phase of infection, the parasite exhibits mechanisms by which it is able to evade the immune response for both short and long periods of time. Initially, the fate of *T. gondii* inside macrophages depends upon the mechanism of entry. Opsonised parasites are phagocytosed, and contained within a phagosome which fuses with early endosomes, late endosomes and lysosomes whereas parasites that actively invade macrophages survive within a parasitophorous vacuole that avoids endosome and lysosome fusion (Mordue and Sibley, 1997; Mordue *et al.*, 1999). Therefore, with the ability to invade and replicate within the normally hostile macrophage, it has become evident that *T. gondii* exhibits mechanisms by which it can interfere with the normal pathways to macrophage-mediated pathogen killing.

One of the primary mechanisms of killing intracellular pathogens and restricting their replication, is via the induction of apoptosis of infected cells (Williams, 1994). Apoptosis is a highly ordered response that occurs following cellular damage or other apoptotic signals, originating from both intracellular and extracellular stimuli. The apoptotic process can be initiated intrinsically, via intracellular alterations to the mitochondrial membrane (Green and Reed, 1998), extrinsically, by ligation of various

surface receptors involved in the death receptor pathways (Tibbetts *et al.*, 2003) or by the release of granzymes and perforins by NK cells and CTL (Lieberman, 2003). Apoptotic pathways converge in the activation of various effector caspases, which cleave intracellular target proteins and result in DNA fragmentation, nuclear condensation, membrane blebbing, detachment of apoptotic bodies and phosphatidylserine exposure, tagging the apoptotic cell for removal by phagocytosis (Thornberry and Lazebnik, 1998; Messmer and Pfeilschifter, 2000).

It is well documented that *T. gondii* is capable of both interfering with and manipulating various aspects of the apoptotic cascade, allowing prolonged replication in the acute phase and minimising immune pressure. It has been known for some time that infection with *T. gondii* renders cells resistant to various pro-apoptotic signals (Nash *et al.*, 1998). The mechanisms by which *T. gondii* controls apoptotic pathways are still not fully understood, however, several studies have revealed that the parasite employs strategies that enable it to either inhibit or promote apoptosis in both infected and uninfected cells (Luder and Gross, 2005). The major mechanisms of *T. gondii*-mediated apoptosis interference/inhibition are discussed below.

Inhibition of apoptosis in *T. gondii* infected cells allows for continued parasite replication and minimises pressure from an inflammatory immune response. Inflammatory cells from mice infected with *T. gondii* were shown to exhibit up to a 300-fold increase in the expression of isoforms of the A1 anti-apoptotic protein (Orlofsky *et al.*, 2002). In addition, levels of poly(ADP-ribose) were found to be decreased in *T. gondii* infected cells, removing a mechanism that normally promotes cell death under conditions of excessive DNA damage, with this decrease being augmented by increased Mcl-1 expression, inhibition of caspase 3/9 activation and inhibition of cytochrome c release (Goebel *et al.*, 2001). Even in the presence of cytochrome c, *T. gondii* is capable of direct inhibition of apoptosis through blocking the activation of caspase 3/7 (Keller *et al.*, 2006).

A role for NFκB in the inhibition of apoptosis by *T. gondii* has also been identified. The ability of *T. gondii*-infected cells to resist apoptotic stimuli was lost in p65 knockout cells, indicating an integral role for the host cell transcription factor in apoptotic resistance (Payne *et al.*, 2003). The expression of anti-apoptotic genes of Bcl-1 and IAP

families was later shown to be dependent on NFκB, with the expression of these genes decreased in p65 knockout cells (Molestina *et al.*, 2003). There may also be other mechanisms by which *T. gondii* exploits host NFκB transcription of anti-apoptotic proteins. Kim and Denkers, (2006) recently found that protein kinase B activation via G_i-protein-coupled signalling in *T. gondii*-infected cells results in decreased levels of apoptosis. Protein kinase B is known to activate NFκB, promoting transcription of anti-apoptotic survival genes, deactivating caspase 9 and various other pro-apoptotic proteins (Kane *et al.*, 1999). From these studies, it is evident that *T. gondii* has more than one mechanism for promoting the survival of infected cells, with more research into this area likely to reveal additional anti-apoptotic strategies.

In contrast to the ability of *T. gondii* to inhibit various pathways to apoptotic host cell death, the parasite is also able to trigger apoptosis in other cell types. It has previously been shown that apoptosis of T lymphocytes induced by *T. gondii* infection may prolong parasite survival by restricting the immune response (Liesenfeld *et al.*, 1997; Wei *et al.*, 2002). Increased levels of apoptosis in splenocytes also accompanied unrestricted parasite replication and higher parasite burden in various other tissues of the host (Gavrilescu and Denkers, 2001). An important factor, however, is that the infecting parasite strain determined the levels of apoptotic host cell death, with more virulent strains inducing higher levels of apoptosis.

Heat shock proteins are a large, ubiquitous family of proteins that perform various functions in different organisms. It is likely that the function of *T. gondii* HSP70 is to interfere with the host immune response. It was noted by Lyons and Johnson (1995) that *T. gondii* HSP70 expression was detected only when virulent strains of the parasite were infected into immunocompetent mice, with little protein expressed when the parasites were infected into immunocompromised mice. This suggests that parasite HSP70 is expressed in response to stresses imposed by the host immune response. HSP70 expression has also been associated with stage conversion in avirulent strains of *T. gondii*, with Weiss *et al.*, (1998) demonstrating that decreasing HSP70 expression also prevented avirulent parasites from undergoing stage conversion to bradyzoites.

More recently, there have been important advances in the discovery of parasite genes that regulate resistance to macrophage mediated killing and prolonged survival during murine infection. Discovered through virulence mapping of the F_1 progeny from genetic crosses of Type II and Type III strains of *T. gondii*, the rhoptry protein ROP18 is a highly polymorphic serine-threonine kinase that is secreted by the parasite into the host cell during the invasion process (Saeij *et al.*, 2006). ROP18 has been shown to be a key factor in the virulence of Type I strains of *T. gondii*, with transfection of the Type I gene into an avirulent Type III strain resulting in a 4 to 5 fold increase in mortality upon murine infection (Taylor *et al.*, 2006). Also identified through genetic crosses of Type II and III strains of *T. gondii*, ROP16 is also a protein kinase that is secreted by the parasite during the host cell invasion process, altering downstream IL-12 secretion (Saeij *et al.*, 2007). Additionally, it has also been shown that Type I virulent strains of *T. gondii* are capable of evading rupture of the parasitophorous vacuole and subsequent parasite death facilitated by the host immunity related GTPases (Zhao *et al.*, 2009a). Avirulent strains however, are susceptible to host immunity related GTPase induced death, with a compromised parasitophorous vacuole preceding parasite death and eventual necrosis of the host cell (Zhao *et al.*, 2009b).

Perhaps the most obvious mechanism of long term immune evasion by *T. gondii* lies in its ability to form tissue cysts following a competent immune response. It has been shown that following infection of IFN-γ-treated murine macrophages with *T. gondii* tachyzoites, the subsequent decrease in parasite replication was accompanied by an increase in the expression of bradyzoite-specific antigens (Bohne *et al.*, 1993). Thus, long term survival of the parasite is facilitated by the formation of such tissue cysts containing bradyzoites. Another advantage in the formation of tissue cysts in the brain and musculature lies in the potential for transmission to other hosts through the ingestion of this tissue as food/prey. From this research, it can be seen that *T. gondii* possesses several mechanisms that allow both short term and long term survival, allowing continued replication and survival in the host and thus maintaining chronic infection.

1.7 – Clinical toxoplasmosis cases at Nepean Hospital

Recent clinical observations made by Prof James Wiley and Dr Stephen Fuller, University of Sydney, Nepean Hospital, NSW, Australia on three patients suffering unusually severe toxoplasmosis have provided clues that a previously unheralded factor in control of *T. gondii* may be the purinergic receptor, $P2X_7$.

Subjects were recruited from the Nepean Hospital after obtaining informed consent. Peripheral blood was collected from toxoplasmosis subjects and normal volunteers (the experimental protocol was approved by the Wentworth Area Health Service and University of Sydney Human Ethics Committees).

Subject 1, a 14-year-old male, presented with a 2-year history of tiredness, lethargy and generalised painless lymphadenopathy. An abdominal ultrasound showed splenomegaly. He had no response to multiple courses of antibiotic therapy. Serology for *T. gondii* was positive for acute infection. An excision biopsy of an enlarged left axillary lymph node showed lymphadenitis and the distinctive changes of reactive follicular hyperplasia with clusters of epithelioid histiocytes at the margins of germinal centers and sinuses distended with monocytoid cells, consistent with toxoplasmosis.

Subject 2, a 20-year-old female, presented with an enlarged submandibular lymph node (2 x 2 cm) 5 weeks following extraction of a right molar tooth. Excision biopsy and histology showed reactive lymphadenitis suggestive of toxoplasmosis. Serology for *T. gondii* was positive for both IgM and IgG. Because of persistent headaches and fatigue, a cerebral CT scan and ocular examination were performed, both of which were normal. Repeat serology after 2 years was positive for IgG and negative for IgM.

Subject 3, a 24-year-old female, (gravida 1, para 0) had an ultrasound scan at 18 weeks gestation, which showed borderline cerebral ventriculomegaly but no other abnormality. A repeat scan at 22 weeks showed gross cerebral ventriculomegaly. Amniocentesis showed normal karyotype and positive PCR diagnosis for *T. gondii*. The patient elected to terminate the pregnancy.

There was no evidence of an underlying immunodeficiency in any of the three subjects. HIV serology was negative in all subjects, and all had normal absolute lymphocyte numbers, normal T- lymphocyte subsets and normal values for serum immunoglobulins. There was no past history or family history of recurrent bacterial or viral infection. Table 1.1 summarises the investigation carried out to confirm immunocompetence of the three subjects.

Table 1.1 – Immunological investigation of toxoplasmosis subjects.
Hb = haemoglobin (g/L); WCC = white cell count (x 10^9 cells/L); Abs lymph = absolute lymphocytes (x 10^9 cells/L); CD4 = CD4+ lymphocytes (x 10^6 cells/L); CD8 = CD8+ lymphocytes (x 10^6 cells/L); Serum Ig = levels of serum immunoglobulins; HIV = serology for human immunodeficiency virus (data from Prof James Wiley and Dr Stephen Fuller, personal communication, used with permission).

Subject	Hb	WCC	Abs lymph	CD4	CD8	Serum Ig	HIV
Toxo 1	170	7.2	3.3	1122	1353	Normal	Neg
Toxo 2	120	7.6	2.2	ND	ND	Normal	Neg
Toxo 3	120	7.5	2.3	858	988	Normal	Neg

After confirming immunocompetence, $P2X_7$ receptor function of cultured macrophages taken from each subject was assessed by measuring the uptake of ethidium by flow cytometry after ATP addition. Activation of the $P2X_7$ receptor has been implicated in the immune response to other macrophage dwelling pathogens, which will be discussed in subsequent sections. This investigation was prompted by information in the literature which identified an important role for the $P2X_7$ receptor in the immune response to other intracellular prokaryotic and eukaryotic pathogens, sharing similar immune response and evasion characteristics with *T. gondii* (discussed in greater depth in Sections 1.10 and 1.11). In all toxoplasmosis subjects, $P2X_7$ receptor function was decreased or absent compared with normal controls (Figure 1.3).

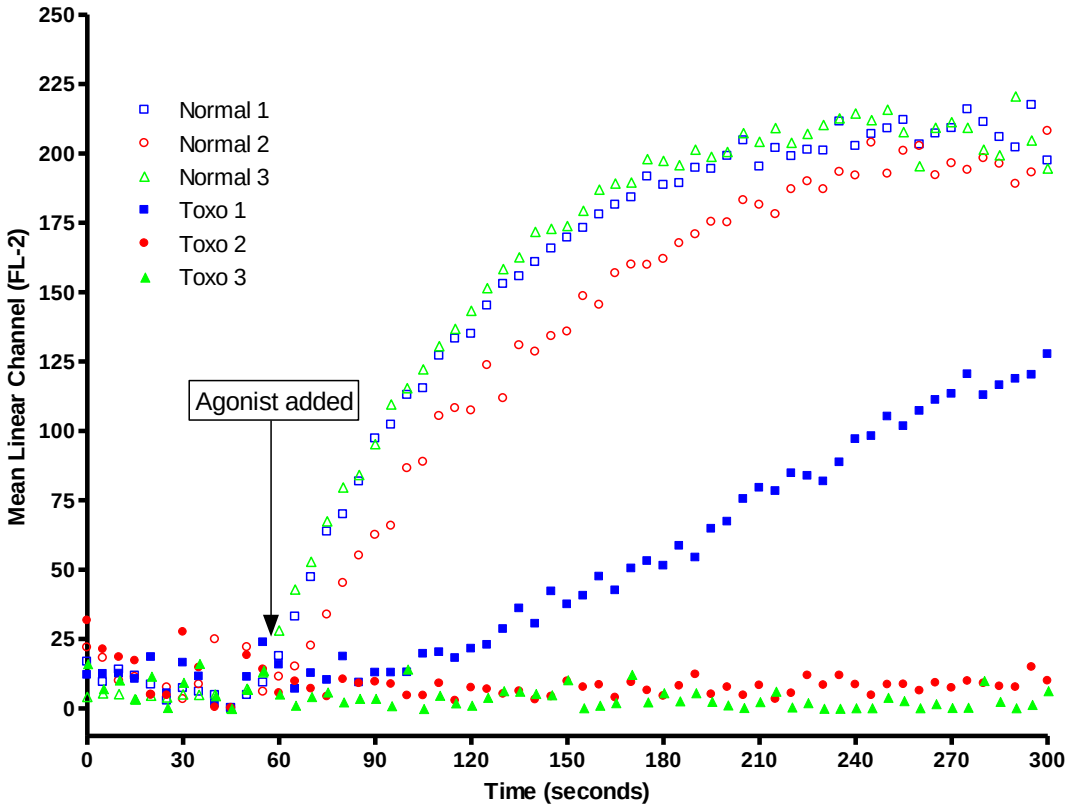

Figure 1.3 – $P2X_7$ receptor function of toxoplasmosis subjects.

Monocyte-derived macrophages from toxoplasmosis subjects were assessed for $P2X_7$ receptor function. Cells were treated with ATP, followed by quantification of ethidium flux through $P2X_7$ receptor generated pores by flow cytometry. $P2X_7$ receptor function of cultured monocyte derived macrophages from toxoplasmosis subjects 1 – 3 was decreased in comparison to three normal control subjects (data from Prof James Wiley and Dr Stephen Fuller, personal communication, used with permission).

Following the addition of ATP, cells from normal donors exhibited increased ethidium uptake via $P2X_7$ receptor dependent pore formation. In all three toxoplasmosis subjects, ethidium uptake was either reduced to approximately half that of normal subjects (Toxo 1), or completely absent (Toxo 2 and Toxo 3). This result demonstrates that all three toxoplasmosis subjects have decreased $P2X_7$ receptor protein function, prompting an investigation into the $P2X_7$ receptor sequence and genotype of all three individuals.

Following the confirmation that all three toxoplasmosis subjects exhibited low or absent $P2X_7$ receptor function, sequencing of the $P2X_7$ receptor gene showed the presence of one or more loss of function polymorphisms in the $P2X_7$ receptor gene of all three subjects (Table 1.2). Subject 1 was heterozygous for the Glu496 to Ala polymorphism, Subject 2 was heterozygous for both the Gln307 to Arg and the Thr357 to Ser

polymorphisms and Subject 3 was heterozygous for both the Gln307 to Arg and Ala496 to Glu polymorphisms.

Table 1.2 – $P2X_7$ receptor genotype of toxoplasmosis subjects.
Sequencing of the $P2X_7$ receptor gene after PCR amplification shows the presence of one or more loss of function SNPs in all three subjects. WT= wild-type allele (data from Prof James Wiley and Dr Stephen Fuller, personal communication, used with permission).

	$P2X_7$ receptor polymorphism			
Nucleotide	G946A	C1096G	A1513C	T1729A
Amino Acid	Gln-307-Arg	Thr-357-Ser	Glu-496-Ala	Ile-568-Asn
Toxo 1	WT	WT	A/C	WT
Toxo 2	G/A	C/G	WT	WT
Toxo 3	G/A	WT	A/C	WT

From these clinical data, it can be hypothesised that loss of $P2X_7$ receptor function may correlate with increased susceptibility to human infection with *T. gondii*, or render an individual more likely to suffer severe disease following infection. In order to further investigate this possibility, the role of $P2X_7$ receptor activation in the murine and human immune response to intracellular *T. gondii* was investigated in greater depth throughout the course of this PhD.

1.8 – Purinergic P2 receptors

The $P2X_7$ receptor is a purinergic receptor that is activated by extracellular ATP. In addition to its role in cellular metabolism, the purine nucleotide ATP acts as an important extracellular messenger in many pathways, producing its effects through the activation of a family of cell surface receptors, known as the purinergic P2 receptors. Extracellular ATP is present in a number of physiological situations and can be released from a variety of sources, including activated platelets (Malmgren, 1986; Beigi *et al.*, 1999), dead/dying/damaged cells (including cellular damage caused by pathogenic organisms) (Oshimi *et al.*, 1999) as well as viable cells through transport mechanisms such as the cystic fibrosis transmembrane conductase regulator (Crane *et al.*, 2005).

Burnstock and Kennedy (1985) originally suggested that P2 purinoceptors be divided into two subfamilies, based on the rank order of agonist potency of structural derivatives of ATP. Therefore, P2 receptors are divided into the P2X ligand-gated ion channels and the P2Y G-protein coupled receptors (Abbracchio and Burnstock, 1994). There are seven known molecularly unique members of the P2X group ($P2X_{1-7}$), and eight known molecularly unique members of the P2Y group ($P2Y_{1, 2, 4, 6,}$ and $P2Y_{11, 12, 13, 14}$) (Burnstock, 2004). P2X and P2Y receptors act on differing scales of time and distance within the cell. P2X receptors are the fast acting purinergic receptors, activating within milliseconds following ligand binding directly to the channel opening. As a result of this, most P2X receptors have a low affinity for ATP, usually within the μM range (North and Barnard, 1997). Conversely, P2Y receptors are the slow acting purinergic P2 receptors. This is due to the fact that their activation proceeds through second-messenger pathways, in the form of G-proteins. This amplifies and prolongs the state of activation from hundreds of milliseconds to seconds and ensures that P2Y receptors are responsive to much lower concentrations of ATP, in the nM range (North and Barnard, 1997).

P2Y receptors are expressed on a wide variety of cells. For example, in chickens and mammals, the $P2Y_1$ receptor is highly expressed in the brain, spinal cord, skeletal muscle, heart and pancreas (Simon *et al.*, 1995a; Tokuyama *et al.*, 1995; Ayyanathan *et al.*, 1996). The binding of ATP to P2Y receptors can lead to phospholipase C activation and inositol 1,4,5-triphosphate (IP3) production that eventually leads to the mobilisation of intracellular Ca^{2+} stores (Lustig *et al.*, 1993; Simon *et al.*, 1995b). P2Y receptors have been proposed as potential drug targets in the treatment of cystic fibrosis, due to their ability to promote normal solute transport across alveolar epithelial cells (Yerxa *et al.*, 2002). The activation of $P2Y_1$ and $P2Y_{12}$ receptors is also an important mechanism in platelet activation, through the stimulation of ADP production by agonists such as thrombin, thromboxane and collagen (Kunapuli *et al.*, 2003).

P2X receptors also have abundant tissue distribution and function. For example the $P2X_5$ receptor is expressed by stratified squamous epithelial cells (Groschel-Stewart *et al.*, 1999), while the $P2X_3$ receptor is expressed on dorsal root ganglia and other neural cells, aiding in nociception (Chen *et al.*, 1995). The seven molecularly unique P2X

receptor subunits share 40 – 50% nucleotide homology, and exhibit two transmembrane domains separated by a large extracellular loop (Figure 1.4). The transmembrane domains of P2X receptor subunits consist of hydrophobic regions that span the width of the plasma membrane (Figure 1.4). These are separated by the main portion of the polypeptide, the extracellular loop, which is approximately 280 amino acids in length (Valera *et al.*, 1994; North, 2002) (Figure 1.4). This extracellular polypeptide sequence contains ten scattered cysteine residues, which are conserved in all seven members of the P2X family and are thought to be involved in receptor trafficking to the cell surface and protein folding via disulphide bond formation (Ennion and Evans, 2002). Post-translational modifications to P2X subunits, such as N-linked glycosylation have also been shown to be essential for P2X receptor trafficking and functional expression (Torres *et al.*, 1998a). The intracellular carboxy terminus of P2X subunits can vary in length from 25 amino acids in the $P2X_6$ receptor, to 240 amino acids in the characteristically long carboxy terminus of the $P2X_7$ receptor (North, 1996).

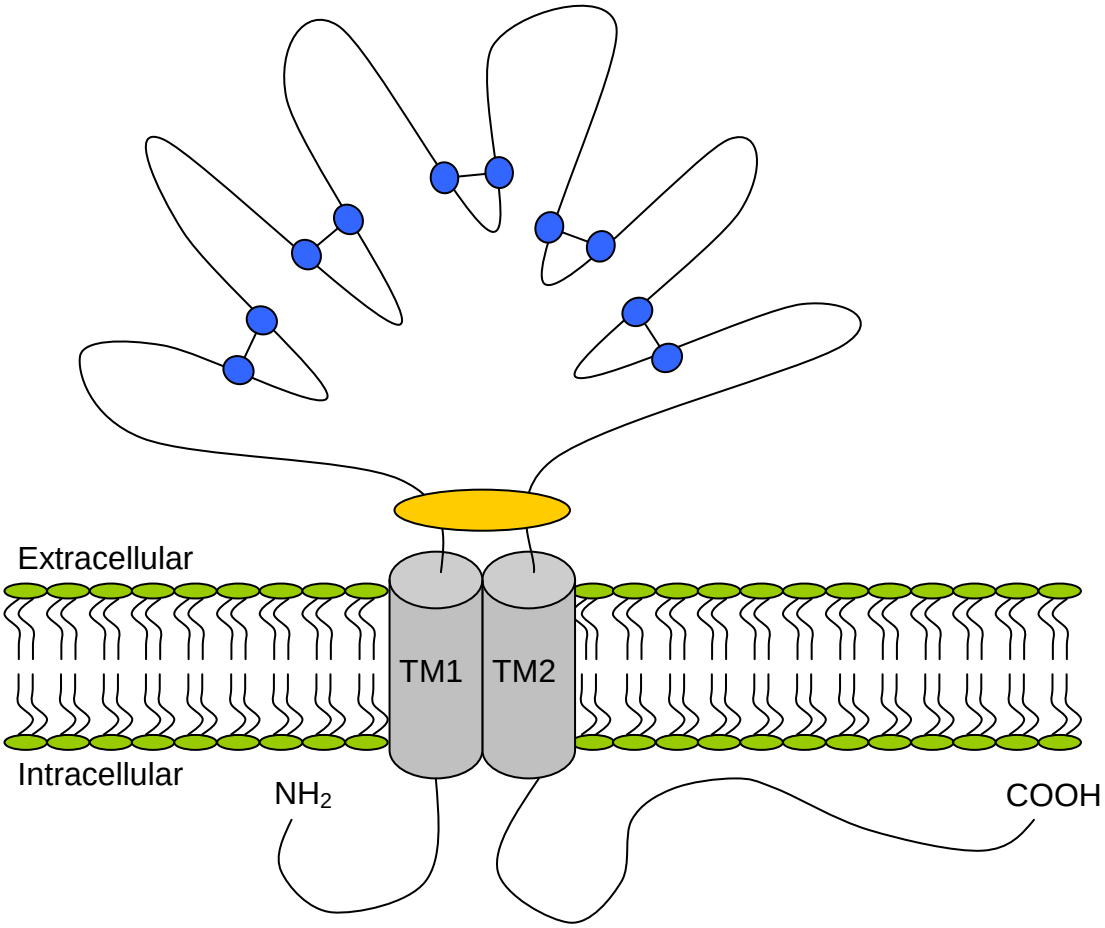

Figure 1.4 – Membrane topology of P2X receptors.

P2X receptors consist of two transmembrane domains (TM1 and TM2) separated by a large extracellular loop. Of significant note are the ten cysteine residues (blue) within the extracellular loop that are conserved among the seven members of the P2X family, and are thought to be cross-linked, thus contributing to the structure of the extracellular loop. Extracellular residues/regions important in nucleotide (ATP) binding are marked with an orange oval. The C-terminal domain is important in conferring many of the permeability properties on the receptor.

Ion channels form from the seven unique P2X receptor subunits, as oligomers of the same, or different subunits, resulting in the formation of homomeric or heteromeric P2X receptors with differing functional and pharmacological properties (North, 2002). For example, the defining phenotype for heteromeric $P2X_{1/5}$ receptors (expressed in HEK293 cells) is a sustained current when activated with αβmeATP, however, this phenotype is not seen for either of the $P2X_1$ or $P2X_5$ homomers when expressed separately (Torres *et al.*, 1998b). The quaternary structure of P2X receptors was investigated by Nicke *et al.*, (1998), showing, through the use of chemical cross linking

and BN-PAGE, that $P2X_1$ and $P2X_3$ receptors formed functional trimers of individual receptor subunits. This finding was later reinforced through the use of atomic force microscopy, showing that $P2X_2$ subunits also form a trimeric receptor (Barrera *et al.*, 2005).

Activation of all P2X receptors with ATP renders the cell permeable to small monovalent cations (Na^+, K^+ and Ca^{2+}). However, prolonged exposure of homomeric $P2X_2$, $P2X_4$ and $P2X_7$ receptors to ATP results in a second permeability state, which renders the cell permeable to larger molecules such as the fluorescent 314Da ethidium cation (Surprenant *et al.*, 1996; Wiley *et al.*, 1998; Khakh *et al.*, 1999; Virginio *et al.*, 1999). In addition, the homomeric $P2X_7$ receptor has several unique characteristics following activation that distinguish it from other homomeric and heteromeric P2X receptors. These downstream events identify the $P2X_7$ receptor as a potent tool that the immune system employs to kill and remove pathogenic organisms, a tool which may be important in the immune response to *T. gondii*.

1.9 – $P2X_7$ receptor

Located at chromosome position 12q24, the human $P2X_7$ receptor gene encodes a 595 amino acid polypeptide that exhibits the same membrane topology and structure as the other P2X subunits (Surprenant *et al.*, 1996; Buell *et al.*, 1998). The main structural feature of the $P2X_7$ receptor that distinguishes it from other P2X receptor subunits is its long intracellular C-terminal tail, which is 240 amino acids in length.

Based on previous co-immunoprecipitation experiments, it was originally thought that $P2X_7$ receptor subunits were the only P2X subunits that did not form heteromeric receptor complexes with other P2X receptor subunits, and instead only associated to form homomeric receptors (Torres *et al.*, 1999). However, Guo *et al.*, (2007) found that $P2X_7$ and $P2X_4$ receptor subunits were capable of forming a functional $P2X_7/P2X_4$ heteromeric receptor, demonstrated through co-immunoprecipitation from HEK 293 cells heterologously expressing both receptor subunits, and from murine bone marrow derived macrophages. Further investigation by Nicke (2008) confirmed that homo-trimeric $P2X_7$ receptors form the dominant assembly state of $P2X_7$ receptor subunits

and hypothesised that $P2X_7/P2X_4$ receptor heteromeric complexes were most likely unstable or underrepresented in the tissue types assessed.

$P2X_7$ receptor expression is localised primarily to epithelia and immune cells, most particularly antigen presenting cells such as macrophages (Collo *et al.*, 1997; Mutini *et al.*, 1999). Expression of the $P2X_7$ receptor by monocytes is up-regulated in response to both IFN-γ and TNF (cytokines which are known to be important in the immune response to *T. gondii*) (Humphreys and Dubyak, 1998). As a non-desensitising, non-selective cation channel, activation of the $P2X_7$ receptor requires concentrations of ATP approximately 10 – 100 times higher than those required for other P2X receptors in the presence of divalent cations, due to the low affinity for the agonist. Initial receptor activation causes the opening of a non-selective cation channel, resulting in rapid membrane depolarisation, Ca^{2+} influx and equilibration of Na^+ and K^+ membrane potentials (Khakh and North, 2006; North, 2002).

Prolonged activation of the $P2X_7$ receptor induces a potent and irreversible set of downstream events that ultimately lead to the death of the activated cell. This level of activation is characterised by the formation of a pore on the cell surface that is permeable to molecules up to 314Da in size (Wiley *et al.*, 1998; Surprenant *et al.*, 1996; Khakh *et al.*, 1999; Virginio *et al.*, 1999).

The specific mechanism of large pore formation following $P2X_7$ receptor activation has not been fully elucidated. Dual permeability states following $P2X_7$ receptor activation have been documented in various cell types, however, it was noted that dual permeability was absent from *Xenopus* oocytes transfected with $P2X_7$ receptor RNA (Petrou *et al.*, 1997). One possible explanation could be that the $P2X_7$ receptor forms a non-selective cation channel but there are other molecules that interact or signal with the $P2X_7$ receptor that allow the formation of the large pore or the transmission of an activation signal. In support of this hypothesis, it was originally shown that signalling through pannexin-1 was required for the activation of caspase-1 and subsequent release of IL-1β following $P2X_7$ receptor activation (Pelegrin and Surprenant, 2006; Pelegrin and Surprenant, 2007). In addition, co-expression of $P2X_7$ with pannexin-1 in *Xenopus* oocytes led to a complete $P2X_7$ response, including cytolytic cell death not previously seen in these cells when transfected with $P2X_7$ RNA only (Locovei *et al.*, 2007). More

recently, $P2X_7$ receptor-mediated current in J774 cells was found to be absent when pannexin-1 gene expression was knocked down using siRNA (Iglesias *et al.*, 2008). Therefore, it is likely that a $P2X_7$-pannexin-1 complex exists, with both elements required for the formation of a large pore on the cell surface and the subsequent downstream events following ligand binding and receptor activation.

While the exact mechanism of pore formation is still in the process of being elucidated, strong evidence exists showing that downstream events following $P2X_7$ receptor activation closely mirror, and in some cases act, synergistically with the innate immune response events that are already known to be important in infection with *T. gondii* and other macrophage dwelling pathogens. Depending on the cell type, activation of the $P2X_7$ receptor triggers a diverse range of cellular processes.

Apoptotic cell death is well known to be a potent mechanism by which the replication of intracellular pathogens is restricted, in many cases leading to death of the pathogen. ATP dependent apoptotic cell death was first demonstrated in a lymphocyte cell line by Zanovello *et al.*, (1990), through the measurement of ^{51}Cr release, membrane depolarisation, Ca^{2+} influx and DNA fragmentation after ATP treatment. This finding was later reinforced, with thymocytes and thioglycolate-elicited macrophages also shown to be capable of undergoing ATP-mediated apoptotic death (Hogquist *et al.*, 1991; Zheng *et al.*, 1991). While ATP was known to induce apoptosis, the $P2X_7$ receptor was not specifically implicated in this process until various other studies directly identified the $P2X_7$ receptor in the apoptotic cell death pathway, firstly by macrophages infected with *Mycobacterium tuberculosis* (Lammas *et al.*, 1997), then in mesangial cells, dendritic cells, and microglial cells (Schulze-Lohoff *et al.*, 1998; Coutinho-Silva *et al.*, 1999; Ferrari *et al.*, 1999a).

Like other inducers of apoptosis, $P2X_7$ receptor activation results in a defined set of cellular responses, resulting in various phenotypic changes leading to apoptosis. Phosphatidylserine (PS) is a phospholipid that is normally maintained on the inner leaflet of the plasma membrane by aminophospholipid translocase (APLT), and becomes externalised in the early stages of apoptotic cell death prior to nuclear condensation (Stuart *et al.*, 1998). APLT becomes deactivated during apoptosis, allowing for the translocation of PS to the cell surface where it is recognised by PS

receptors on macrophages, leading to phagocytosis of the apoptotic cell while minimising the inflammatory response (Tyurina *et al.*, 2007). Phosphatidylserine exposure has been demonstrated on the outer surface of thymocytes (Courageot *et al.*, 2004) and erythrocytes (Sluyter *et al.*, 2007) following $P2X_7$ receptor activation. These results were also in agreement with experiments showing that *T. gondii* infected RAW 264.7 murine macrophages undergo apoptotic death following $P2X_7$ receptor activation, indicated by phosphatidylserine exposure (Lees, 2004).

Many of the pathways leading to the promotion of the inflammatory response and the induction of apoptosis following $P2X_7$ receptor activation are still being elucidated. Ligation of the $P2X_7$ receptor with extracellular ATP is known to result in the activation of transcription factors NFAT (within minutes) and NFκB (within hours), with subsequent effects on various molecules capable of modulating the inflammatory response and apoptosis (Ferrari *et al.*, 1997; Ferrari *et al.*, 1999b). Stress-activated protein kinases (SAPK, also known as JNK or c-Jun N-terminal kinases) are members of the mitogen activated protein kinase (MAPK) family, which act to phosphorylate and activate various transcription factors in response to inflammatory cytokines and cellular stress, contributing to TNF and NFκB activation (Ip and Davis, 1998). Humphreys *et al.*, (2000) used $P2X_7$ receptor agonists and antagonists, to show that $P2X_7$ receptor activation on murine Bac-1 macrophages results in the phosphorylation of SAPK. Various other members of the MAPK family are now also known to be activated or accumulate following ligation of the $P2X_7$ receptor with ATP. Donnelly-Roberts *et al.*, (2004) identified that p38 MAPK inhibition prevented $P2X_7$ receptor-mediated pore formation in differentiated THP-1 cells, while hippocampal slices from $P2X_7$ receptor knockout mice demonstrated substantially lower p38 MAPK phosphorylation compared with the parental strain (Papp *et al.*, 2007). ERK1/2 (also known as p44/42 MAPK) was also found to be involved in $P2X_7$ receptor-mediated thymocyte death (Auger *et al.*, 2005), contributing to negative selection of thymocytes with high affinity for self antigen.

In addition to the involvement of various members of the MAPK family following $P2X_7$ receptor activation, $P2X_7$ receptor signalling also leads to activation of Rho-effector kinase (ROCK), leading to activation of the small GTPase Rho, which facilitates Rho dependent membrane blebbing and Phospholipase D activation (el-Moatassim and

Dubyak, 1992). Phospholipase D plays a particularly important role, hydrolysing phosphatidylcholine into phosphatidic acid and choline, thus aiding in the breakdown of the plasma membrane of $P2X_7$ receptor activated cells. In murine thymocytes, it was shown that $P2X_7$ receptor activation subsequently induced PLD activation, and was dependent on intracellular Ca^{2+} immediately after receptor activation rather than pore formation (Le Stunff *et al.*, 2004).

Metalloproteases are also activated following ligation of the $P2X_7$ receptor with extracellular ATP. These enzymes have been shown to proteolytically cleave cell surface proteins involved in lymphocyte migration and immunoglobulin binding, resulting in the shedding of L-selectin (CD62L) and CD23 from the cell surface (Jamieson *et al.*, 1996; Gu *et al.*, 1998; Sluyter and Wiley, 2002).

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that accumulates in the cytoplasm of monocytes and macrophages as an inactive 33kDa procytokine, expressed in response to inflammatory stimuli with transcription driven by NFκB (Kitaoka *et al.*, 2007). Inactive proIL-1β is cleaved by caspase-1, also known as IL-1β converting enzyme (ICE) to the active 17kDa IL-1β molecule (Cerretti *et al.*, 1992). Caspase-1 is activated by cleavage of the inactive zymogen procaspase-1 into p10 and p20 subunits, which assemble as a tetramer to form active caspase-1 (Wilson *et al.*, 1994). $P2X_7$ receptor activation has been shown to promote the activation of caspase-1, and subsequent IL-1β processing and release, through the efflux of K^+, causing cleavage of the inactive zymogen (Perregaux and Gabel, 1994; Solle *et al.*, 2001; Kahlenberg and Dubyak, 2004). $P2X_7$ receptor-deficient macrophages were subsequently shown to be incapable of the processing and release of active, mature IL-1β in response to extracellular ATP (Labasi *et al.*, 2002; Chessell *et al.*, 2005).

Sharing similar immunological characteristics with IL-1β, IL-18 (previously known as IFN-γ inducing factor) is a pro-inflammatory cytokine that is released by cells of the monocyte lineage, such as macrophages and dendritic cells (Stoll *et al.*, 1998; Kim *et al.*, 2000), and acts as a potent inducer of IFN-γ release from T-cells. As with IL-1β, IL-18 also requires processing by activated caspase-1 in order to cleave the 24kDa inactive proIL-18 to yield the biologically active 18kDa form of the cytokine (Ghayur *et al.*,

1997). Mature IL-18 is released following activation of the $P2X_7$ receptor, with this effect being first noted by Perregaux *et al.*, (2000) in ATP-treated monocytes. Subsequent experiments reinforced this observation, with anti-$P2X_7$ receptor monoclonal antibody and the $P2X_7$ receptor antagonist, oxidised ATP, blocking ATP-induced IL-18 release by human monocytes (Mehta *et al.*, 2001).

IL-18 and IL-1β are important pro-inflammatory cytokines that are released following activation of the $P2X_7$ receptor, with a loss of function polymorphism in the $P2X_7$ receptor gene shown to diminish both IL-1β and IL-18 release from human monocytes (Sluyter *et al.*, 2004a; Sluyter *et al.*, 2004b). Therefore, it is possible that $P2X_7$ receptor mediated release of IL-1β, IL-18 and other pro-inflammatory cytokines may contribute to the inflammatory response evident following human infection with *T. gondii*.

In addition to promoting the release of pro-inflammatory cytokines, it has been known for some time that exposure of haemopoietic cells such as neutrophils to extracellular ATP (or other sources of ATP), results in the production of reactive oxygen and nitrogen intermediates (ROI and RNI) such as the superoxide anion (O_2^-) (Kuhns *et al.*, 1988; Ward *et al.*, 1988). These data however, were not sufficient to identify which purinergic receptor was mediating the release of ROI and RNI species. More recently, the discovery of agonist/antagonist profiles for purinergic receptors, and the use of $P2X_7$ receptor knockout cells has demonstrated that the $P2X_7$ receptor is capable of mediating ROI and RNI release (Hewinson and Mackenzie, 2007). The $P2X_7$ receptor agonists, ATP and, more specifically, BzATP, were used to show superoxide anion and nitric oxide (NO) release by primary rat microglial cells (Parvathenani *et al.*, 2003), with this effect being blocked through the use of $P2X_7$ receptor antagonists, PPADS, oxidised ATP and BBG (Skaper *et al.*, 2006). Similar studies have also revealed $P2X_7$ receptor-dependent ROI and RNI release in various human cells, such as promyelocytes, eosinophils and neutrophils (Suh *et al.*, 2001; Ferrari *et al.*, 2000). In agreement with these data, the ability of the $P2X_7$ receptor to mediate ROI and RNI release was confirmed by Pfeiffer *et al.*, (2007), showing RAW 264.7 cells are capable of releasing ROI and RNI following $P2X_7$ receptor activation, with this effect being greatly reduced in $P2X_7$ receptor-deficient RAW 264.7 cells. Therefore, it is now known that an

important innate immune response mechanism, startlingly similar to that induced by *T. gondii*, is induced following activation of the $P2X_7$ receptor.

Specific aspects of $P2X_7$ receptor activation may also highlight important interactions with intracellular *T. gondii*. The early influx of Ca^{2+} into $P2X_7$ receptor activated cells (prior to the formation of the cytolytic pore) is of particular interest, as increased intracellular Ca^{2+} concentration is a well known stimulant for the egress of *T. gondii* from a host cell (Caldas *et al.*, 2007; Arrizabalaga and Boothroyd, 2004). One hypothesis could be that early signs of $P2X_7$ receptor activation in *T. gondii*-infected cells act as a signal that the cell is about to undergo apoptosis, alerting the parasite to eventual toxic downstream events after activation of the receptor and giving the parasite a chance to escape.

Considering the parallels between the cellular events that $P2X_7$ receptor activation initiates, and the immune response to *T. gondii*, it is logical to consider that activation of the $P2X_7$ receptor also plays a role in the immune response to *T. gondii*. There have already been various other studies that show the involvement and importance of $P2X_7$ receptor expression and functional activation in resistance to disease. Considering the similarities these organisms share with *T. gondii*, such as aspects of cellular preference and intracellular localisation, immune response events and pathogenicity make this research relevant to *T. gondii* and an important primer for the work conducted in this PhD project.

1.10 – $P2X_7$ receptor and intracellular bacteria

Mycobacterium tuberculosis is a pathogen that infects one third of the world's human population, with approximately 8 – 9 million new cases per year, of which 5 – 10% will develop clinical disease (WHO, 2007). Disease progression is characterised by slow loss of lung function eventually leading to death of the patient. Similarly to toxoplasmosis, the incidence of tuberculosis rises rapidly in areas where HIV is prevalent (De Cock and Chaisson, 1999).

Mycobacterium tuberculosis is a facultative intracellular pathogen, whose main reservoir within the host is the macrophage, the innate immune cell that also acts to regulate its growth and spread. Following phagocytosis, the pathogen is capable of surviving in the phagosome by arresting the process of phagosomal fusion with lysosomes, shown through the lack of vacuolar ATPase responsible for phagosomal acidification (Sturgill-Koszycki *et al.*, 1994). More specifically, the process of phagosome-lysosome maturation requires the gradual association of endosomal markers. Via *et al.*, (1997) were able to show that this process of phagosome-lysosome maturation was halted between the acquisition/recruitment of endosomal markers Rab5 and Rab7.

The ability of the $P2X_7$ receptor to mediate killing of intracellular mycobacteria was first demonstrated by Lammas *et al.*, (1997), in experiments that showed the ability of BCG-infected human macrophages to kill the intracellular pathogen post ATP treatment. Interestingly, this was shown to be via mechanisms that did not involve reactive nitrogen and oxygen intermediates. These experiments confirmed pathogen killing via $P2X_7$ receptor-dependent pathways through the use of $P2X_7$ receptor antagonists such as oxidised ATP, as well as the more potent agonist, benzoyl-benzoyl ATP. This study also suggested that there may be genetic variability within the $P2X_7$ receptor gene that impairs an individual's ability to kill the intracellular pathogen. The importance of $P2X_7$ receptors in controlling intracellular mycobacterial replication was also studied by Smith *et al.*, (2001), with the activation of $P2X_7$ receptors on bovine macrophages linked to reduced host cell and subsequently reduced *Mycobacterium bovis* viability.

Following on from experiments showing the ability of $P2X_7$ receptor activation to mediate killing of intracellular mycobacteria, Saunders *et al.*, (2003) confirmed that a single nucleotide polymorphism within the $P2X_7$ receptor gene (1513C) can result in full loss of receptor function resulting in inability of macrophages from patients homozygous for the polymorphism to kill the intracellular bacteria. These experiments involved the activation of $P2X_7$ receptors on BCG-infected macrophages from wild-type and homozygous 1513C donors. The results confirmed that $P2X_7$ receptor-activated wild-type macrophages undergo apoptosis and kill the intracellular bacteria, however, homozygous 1513C macrophages exposed to ATP fail to undergo apoptosis,

resulting in mycobacterial survival. The effect of multiple other $P2X_7$ receptor polymorphisms was further assessed by Fernando *et al.*, (2005) and Shemon *et al.*, (2006) in experiments that showed that several other $P2X_7$ receptor polymorphisms (946A, 1729A, 1747A, 155+1T and 699T) could also result in reduced macrophage apoptosis and mycobacterial killing. This effect was augmented in compound heterozygous donors (donors with a heterozygous loss of function polymorphism at more than one position on the $P2X_7$ receptor gene).

Although it has been shown that $P2X_7$ receptor activation induces the killing of intracellular mycobacteria *in vitro*, studies attempting to link some of the polymorphisms discussed earlier with clinical disease have been varied in their findings. Li *et al.*, (2002) studied the association between various $P2X_7$ receptor polymorphisms and clinical disease in a Gambian population. They found that a protective effect against tuberculosis was associated with a $P2X_7$ receptor promoter polymorphism at position -762 but the 1513C polymorphism discussed above did not show any significant association. However, Fernando *et al.*, (2007) more recently investigated the 1513C polymorphism in Southeast Asian cohorts, and found a strong association with extrapulmonary tuberculosis. The importance of the 1513C polymorphism as a possible risk factor for clinical tuberculosis has also been investigated by Nino-Moreno *et al.*, (2007) and Mokrousov *et al.*, (2008). These studies showed a significant association between clinical tuberculosis and the 1513C polymorphism in Mexican and Russian Slavic cohorts. Therefore, research conducted to date points to a role for the $P2X_7$ receptor in the immune response to tuberculosis both *in vitro* and *in vivo*, with disease progression and severity being influenced by multiple host defence and pathogen virulence genes.

In addition to the proposed role of the $P2X_7$ receptor in the immune response to *Mycobacterium* spp., the receptor is also implicated in the innate response to the intracellular bacteria, *Chlamydia* spp. *Chlamydia* spp., are obligate intracellular bacteria that enter epithelial mucosal cells by phagocytosis (Friis, 1972). Following phagocytosis, the non-metabolising elementary bodies inhibit phagosome-lysosome fusion and begin developing into metabolically active reticulate bodies (Eissenberg and Wyrick, 1981; Eissenberg *et al.*, 1983). After one day of reproduction inside a membrane bound inclusion, the reticulate bodies begin differentiation back into

elementary bodies that are released from infected cells two days post infection, allowing a new cycle of infection and reproduction to begin. *Chlamydia trachomatis* infects over 90 million people per year (WHO, 2001) and is capable of causing a sexually transmitted disease that can result in ectopic pregnancy, sterility and blindness (Ward, 1995). *Chlamydia psittaci* is capable of causing a zoonotic disease primarily affecting people in regular contact with birds, following the inhalation of aerosols from faeces and secretions from infected animals. The disease usually causes influenza-like symptoms, however, clinical presentation may also include mild respiratory infection to severe pneumonia and systemic illness leading to death (Petrovay and Balla, 2008).

As with the ability of infected macrophages to kill intracellular *M. tuberculosis*, it has also been shown that $P2X_7$ activation by ATP treatment is capable of killing intracellular *C. psittaci*. Coutinho-Silva *et al.*, (2001) showed that activation of $P2X_7$ receptors on J774 macrophages led to apoptosis and death of intracellular *C. psittaci*, however, host cells infected with the bacteria for one day exhibited decreased apoptosis in response to extracellular ATP, indicating that the intracellular bacteria may employ a mechanism to limit the effect of $P2X_7$ receptor activation. It was also shown that treatment of epithelial cells (the preferred target cell for chlamydiae) with $P2X_7$ receptor agonists reduced the infectiveness of the bacteria and was associated with activation of phospholipase D (Darville *et al.*, 2007). Darville *et al.*, (2007) also demonstrated that vaginally infected mice lacking $P2X_7$ receptors exhibited greater levels of acute inflammation and a small decrease in IL-1β release.

1.11 – $P2X_7$ receptor and intracellular parasites

Chagas disease (American trypanosomiasis) is caused by the intracellular protozoan parasite *Trypanosoma cruzi*, and is transmitted to humans through the bite of blood feeding arthropods, causing a chronic inflammatory disease characterised by cardiomyopathy and digestive disorders (Blum *et al.*, 2008; Ribeiro *et al.*, 2008). The disease is endemic in Latin America, with infection rates ranging from an estimated 6 – 8 million people infected in Brazil (Pinto Dias, 1995) to an estimated 17 million infected across all of Latin America, resulting in 45,000 deaths per year (Schmunis, 1991).

The immune response to *T. cruzi* requires a strongly polarised Th-1 response (similar to the response to *T. gondii*), with the production of IL-12, IFN-γ, TNF and NO, being associated with resistance and control of disease progression (Aliberti *et al.*, 1996; Martins *et al.*, 1999; Silva *et al.*, 1998). It has also been hypothesised that $P2X_7$ receptor modulation may also play a role in the progression of Chagas disease. Cascabulho *et al.*, (2008) showed that peritoneal macrophages from *T. cruzi* infected mice exhibited lower $P2X_7$ receptor expression compared with those obtained from uninfected mice. Decreased $P2X_7$ receptor expression on macrophages may reduce the immune pressure on parasites that would normally be created through the activation of $P2X_7$ receptors and the subsequent release of pro-inflammatory cytokines. Therefore, *T. cruzi* may be capable of manipulating the $P2X_7$ receptor by reducing expression of the receptor on cells that normally contribute to the inflammatory response.

Leishmaniasis is a complex of several diseases ranging in severity from small cutaneous nodules to the mass destruction of mucosal tissues, caused by infection with obligate intracellular parasites of the genus *Leishmania*. Globally, there are approximately 350 million people at risk of infection, with 1.5 – 2 million new cases and 70,000 deaths per year (WHO, 2004), with disease endemic throughout the Middle East, North Africa, parts of Europe, and central and South America (Alvar *et al.*, 1997; Herwaldt, 1999). Geographically, leishmaniasis is described as "Old World" (Middle East, North Africa and Europe) and "New World" (central and South America). The vector for leishmaniasis in the Old World is usually the female *Phlebotomus* sand fly, of which

there are approximately 30 different species capable of transmitting the disease to humans (Bates, 2007; Emami and Yazdi, 2008). New World leishmaniasis is usually transmitted to humans via the bite of the female *Lutzomyiz* sand fly (Oliveira *et al.*, 2006). The flagellate form of the parasite, known as the promastigote, is inoculated into the skin of the host through the bite of the sand fly, where it is rapidly phagocytosed by macrophages, neutrophils and dendritic cells. Upon phagocytosis, the parasite loses its flagellum and transforms into the replicative form, the amastigote, which replicates within the phagosome (Handman, 1999; Vannier-Santos *et al.*, 2002).

The species of parasite, as well as the host immune response, determines the type and severity of disease elicited following infection. Several species, including *Leishmania major*, *Leishmania tropica*, *Leishmania mexicana* and *Leishmania braziliensis* typically cause cutaneous leishmaniasis, a disease that may take the more benign form of localised cutaneous leishmaniasis (LCL), characterised by a self healing skin lesion, or may develop to diffuse cutaneous leishmaniasis (DCL), a non-healing form that can persist and spread throughout the skin (Bertho *et al.*, 2000; Reithinger *et al.*, 2007).

Also known as American tegumentary leishmaniasis, New World mucocutaneous leishmaniasis is endemic across Latin America and typically results in approximately 3 – 5% of patients following infection with *L. braziliensis* (Jones *et al.*, 1987), but may also result following infection with *L. amazonensis*, *Leishmania guyanensis* and *Leishmania panamensis* after a period of months to years following cutaneous leishmaniasis symptoms (Barral *et al.*, 1991; Osorio *et al.*, 1998). Disease progression is characterised by the non-healing destruction of nasal and pharyngeal mucosa, eventually resulting in severe facial disfiguration (Amato *et al.*, 1998).

Visceral leishmaniasis, also known as kala-azar, involves uncontrolled parasitism and progressive destruction of the liver, spleen and bone marrow, usually leading to death of the patient (Murray *et al.*, 2005). In Africa, visceral leishmaniasis usually results from infection with *Leishmania donovani* whereas the disease is mainly caused in the Indian sub-continent by *Leishmania infantum/chagasi*.

The innate immune response to *Leishmania* is very similar to that already described for *T. gondii* and *T. cruzi* (Liese *et al.*, 2008). Macrophages, dendritic cells and natural

killer cells recognise antigens on the surface of the parasite (via toll-like receptors) and contribute to a pro-inflammatory response involving early IL-12 production, followed by IFN-γ and TNF. As with *T. gondii*, production of IFN-γ during the innate response is crucial, the importance being highlighted by the lack of IFN-γ production by PBMC from patients suffering visceral leishmaniasis, caused by an over production of regulatory IL-10 (Ghalib *et al.*, 1995). In the murine model, a Th-1 response is critical for the resolution of infection, with the severity of disease in humans also associating a favourable outcome with a Th-1 response (Scott, 1991; Sypek *et al.*, 1993).

The $P2X_7$ receptor has also been implicated in the immune response to *Leishmania* spp, with Torres-Santos *et al.*, (2000) showing increased sensitivity of peritoneal macrophages and spleen cells to extracellular ATP when infected with *L. amazonensis*. Murine macrophages and cells cultured from cutaneous lesions were also shown to be more sensitive to $P2X_7$ receptor mediated pore formation upon infection with *L. amazonensis*, inhibiting growth of the intracellular parasite (Chaves *et al.*, 2009). Thus, the $P2X_7$ receptor may play an important role in the immune response to several important pathogens, and represents a possible aspect of the immune response to *T. gondii* that has not yet been considered.

1.12 – Project aim

My aims were to:

1. Develop fast, reliable methods to assess the viability and replication of intracellular *T. gondii* tachyzoites *in vitro*;
2. Assess the effect of ATP stimulation of human and murine immune cells on the viability and/or replication of Type I (RH) tachyzoites of *T. gondii*;
3. Assess the effect of deficiencies in $P2X_7$ receptor activity on the ability of ATP to affect the viability and/or replication of Type I (RH) tachyzoites of *T. gondii*; and
4. Assess the effect of deficiencies in P2X receptor activity on the production of inflammatory cytokines and mediators in response to infection with Type I (RH) tachyzoites of *T. gondii*.

1.13 – Objectives

The main objectives of this project are divided into four chapters:

1. Development and validation of assays allowing the quantification of intracellular *T. gondii* viability and burden/replication.
    a. Flow cytometry based *T. gondii* viability assay
    b. Microplate cytometry based *T. gondii* burden/replication assay
2. Investigation of the role of the human $P2X_7$ receptor during infection with *T. gondii*.
    a. Assessment of killing of *T. gondii* by primary human macrophages cultured from blood of wild-type donors.
    b. Selection and use of human samples with loss-of-function polymorphisms in the $P2X_7$ receptor gene to assess replicative ability of *T. gondii* following $P2X_7$ receptor activation.
3. Assessment of the role of the murine $P2X_7$ receptor during *T. gondii* infection *in vitro*.
    a. Assessment of killing of intracellular *T. gondii* by mouse macrophage-like RAW 264.7 cells following $P2X_7$ receptor activation.
    b. Assessment of killing of intracellular *T. gondii* by murine bone marrow-derived macrophages isolated from $P2X_7$ receptor knockout mice following $P2X_7$ receptor activation.
4. Assessment of the role of the murine $P2X_7$ receptor during infection of $P2X_7$ receptor knockout mice *in vivo*.
    a. Assessment of parasite burden at the site of infection.
    b. Assessment of serum samples for inflammatory cytokine levels.

Chapter 2 – General materials and methods

2.1 - Materials

Consumables:

Consumables are listed in alphabetical order with the manufacturer and relevant product/catalogue number for each consumable.

1640 RPMI with 2mM L-Glutamine – Gibco/Invitrogen, USA (22400)

2-mercaptoethanol – Gibco/Invitrogen, USA (21985-023)

DAPI – Sigma, USA (D9542)

Acridine orange – Sigma, USA (A8097)

AIM-V – Gibco/Invitrogen, USA (12055-091)

Alexa Fluor 647 protein labelling kit – Molecular Probes/Invitrogen, USA (A20173)

Anti-CD14-microbead conjugated monoclonal antibody (mouse IgG2a) – Miltenyi Biotec, Germany (130-050-201)

Anti-$P2X_7$ receptor polyclonal antibody (produced in rabbit) – Sigma, USA (P8232)

Anti-p30 *T. gondii* monoclonal antibody (mouse IgG2a) – HyTest, Finland (3Tx19)

Anti-rabbit-Alexa Fluor 488 conjugated antibody – Molecular Probes/Invitrogen, USA (A11008)

ATP – Sigma, USA (A7699)

BSA – Research Organics, USA (1328A)

Cell scrapers: 25cm – Sarstedt, Germany (83.1830)

CO_2 – BOC Gases, Australia

Cryotubes – Nunc, Denmark (377267)

CBA Mouse Inflammation Kit – Becton Dickinson, USA (552364)

DMSO – Sigma, USA (D5879)

EDTA – Sigma, USA (E9884)

Ethidium bromide – Sigma, USA (E1510)

Extravidin alkaline-phosphatase – Sigma, USA (E2636)

FACS tubes – Techno Plas, Australia (S7512)

Ficoll-Paque PLUS – GE Healthcare, UK (17-1440)

FBS – Cellgro, USA (35-010-CV) and Gibco/Invitrogen, USA (10099-141)

Gentamycin – Sigma, USA (G1397)

Glutamax – Gibco/Invitrogen, USA (35050-061)

Glycerol – Sigma, USA (G5516)

GM-CSF – Sigma, USA (G0282)

HBSS – Cellgro, USA (21-020-CV)

Heparin – Sigma, USA (H0878) and ThermoFisher Scientific, USA (10192980)

Histopaque 1077 – Sigma, USA (10771)

IMDM – Lonza, Switzerland (12-722F)

Isofluorane – Veterinary Companies of Australia (VCA), Australia

KCl – Sigma, USA (P9333)

KH_2PO_4 – Sigma, USA (P5655)

M-CSF – Sigma, USA (M6518)

Methanol (AR grade) – Fronine, Australia (JJ026)

MS column and magnet – Miltenyi Biotec, Germany (130-090-312)

Na_2CO_3 – Sigma, USA (S7795)

Na_2HPO_4 – Sigma, USA (S7907)

NaCl – Sigma, USA (S7653)

NADPH – Sigma, USA (N5130)

NaN_3 – Sigma, USA (S8032)

NEAA – Gibco/Invitrogen, USA (11140-050)

NED – Sigma, USA (70720)

Nitrate reductase – Sigma, USA (N7265)

Needles (21 gauge) – Terumo, USA (NN-2138R)

Needles (25 gauge) – Terumo, USA (NN-2538R)

Needles (26 gauge) – Terumo, USA (NN-2613R)

Needles (27 gauge) – Terumo, USA (NN-2713R)

NBS – Gibco/Invitrogen, USA (16010-159)

Nitrous oxide – BOC Gases - Australia

OptEIA Mouse IL-1β ELISA Set – Becton Dickinson, USA (559603)

oATP – Sigma, USA (A6779)

Oxygen – BOC Gases, Australia

PS solution – Gibco/Invitrogen, USA (15140-122)

PSF solution – Cellgro, USA (30-004-CI)

Polycarbonate filters: 3μm – Millipore, USA (TSTP04700)

Polypropylene filter unit – GE Osmonics, USA (1262579)

Sigma FAST (ρ-nitrophenyl phosphate) substrate tablet – Sigma, USA (N2770)

SNP – Sigma, USA (71778)

Sulphanilamide – Sigma, USA (S9251)

Syringes (1mL) – Terumo, USA (SS-01T)

Syringes (5mL) – Terumo, USA (SS-05L)

Syringes (20mL) – Terumo, USA (SS-20L)

Sytox Green – Molecular Probes/Invitrogen, USA (S7020)

Tissue culture chamber slides: 8-well – Lab-Tek – Nunc, Denmark (154534)

Tissue culture flasks ($75cm^2$) – Corning, USA (3275)

Tissue culture flasks ($80cm^2$) Nunclon Delta Surface – Nunc, Denmark (156499)

Tissue culture plates (384-well) flat/clear bottom, black sided – Corning, USA (3712)

Tissue culture plates (6-well) – Corning, USA (3516)

Tissue culture plates (6-well) Nunclon Delta Surface – Nunc, Denmark (140675)

Tissue culture plates (96-well) Nunclon Delta Surface – Nunc, Denmark (167008)

Trypsin (tissue culture grade) – Gibco/Invitrogen, USA (15090-046)

Tween-20 – Sigma, USA (P1379)

Vacutainers (lithium heparin coated) – Greiner Bio One, Germany (455084)

Media and buffers:

- BMM Wash Media – 1640 RPMI with 2mM L-Glutamine, 10% FBS and 100 units/mL penicillin/100 μg/mL streptomycin (PS) solution
- Coating buffer (ELISA) – 0.1M Na_2CO_3 pH 9.5
- Complete BMM Media – 1640 RPMI with 2mM L-Glutamine, 10% FBS (Gibco/Invitrogen, USA), 100 units/mL penicillin/100 μg/mL streptomycin (PS) solution and 10ng/mL GM-CSF
- Complete HFF Media –IMDM supplemented with 2mM Glutamax, 10% FBS (Cellgro, USA) and 100 units/mL penicillin/100 μg/mL streptomycin/0.25μg/mL amphotericin B (PSF) solution
- Complete Human Macrophage Media – 1640 RPMI with 2mM L-Glutamine, 10% FBS (Gibco/Invitrogen, USA), 0.1mM NEAA, 55μM 2-mercaptoethanol and 5 μg/mL gentamycin

- Complete RAW Media – 1640 RPMI with 2mM L-Glutamine, 5% FBS and 100 units/mL penicillin/100 μg/mL streptomycin (PS) solution
- Complete Vero Media – 1640 RPMI with 2mM L-Glutamine, 2% NBS and 100 units/mL penicillin/100 μg/mL streptomycin (PS) solution
- FSW – PBS, 1% BSA, 0.05% NaN_3
- Griess Reagent – Equal volumes of 1% sulphanilamide (diluted in H_2O) and 0.1% NED (diluted in 10% TCA)
- KCl buffer – 150mM KCl, 5mM NaCl, 10mM HEPES, 5mM D-glucose, 0.1% BSA
- Microscopy Blocking Solution – PBS, 3% BSA, 0.05% Tween-20
- MACS buffer – PBS, 0.5% BSA, 2mM EDTA
- NaCl buffer – 145mM NaCl, 5mM KCl, 10mM HEPES, 5mM D-glucose, 0.1% BSA
- PBMC cryopreservation media – AIM-V media with 20% FBS (Cellgro, USA) and 10% DMSO
- PBS – pH 7.4: 171mM NaCl, 3mM KCl, 13mM Na_2HPO_4, 2mM KH_2PO_4
- Wash buffer (ELISA) – PBS, 0.05% Tween-20

Cell lines, parasites and mice:

BALB/c mice – ARC, Australia

C57BL/6J mice – ARC, Australia

Human foreskin fibroblasts (HFF) – ATCC, USA

$P2X_7$ receptor knockout mice – Pfizer, USA

RAW 264.7 immortalised cell line – ATCC, USA and Cryosite, Australia

T. gondii tachyzoites (RH strain) – ATCC, USA and Cryosite, Australia

Vero immortalised cell line – ATCC, USA and Cryosite, Australia

YFP RH *T. gondii* – A kind gift from Dr Boris Striepen, University of Georgia, Athens, GA, USA

Instruments and equipment:

Acumen eX3 microplate cytometer – TTP Labtech, UK

Automated microplate reader (Powerwave HT) – BioTek Instruments, USA

Automated tissue culture incubator – ThermoFisher Scientific, USA

BD FACSCalibur flow cytometer – Becton Dickinson, USA

BD LSR II flow cytometer – Becton Dickinson, USA

F3 robot arm – ThermoFisher Scientific

Laminar flow hood (biohazard class II) – Gelman Sciences, Australia; BIOAIR/Euroclone, Italy

Olympus BX51 fluorescence microscope – Olympus, Japan

Olympus DP70 digital camera – Olympus, Japan

Tissue culture incubators – Sanyo Biomedical, Japan

Zeiss Axioplan 2 fluorescence microscope – Zeiss, Germany

Software:

Axiovision, version 4.5 – Zeiss, Germany

FCAP Array, version 1.0.1 – Soft Flow Ltd., Hungary

GraphPad Prism, version 5.00 – GraphPad, USA

Minitab, version 15 – Minitab, Inc., USA

WinMDI flow cytometry analysis software, version 2.9 – © Joseph Trotter

2.2 – Methods

2.2.1 – Ethics

All animal research (University of Technology, Sydney, Australia) was approved and conducted in accordance with the UTS Animal Care and Ethics Committee, with approval code: UTS ACEC 2008-30.

All human research (University of Technology, Sydney, Australia) was approved and conducted in accordance with the UTS Human Research Ethics Committee, with approval code: UTS HREC 2004-077A.

Ethical approval for the NCCCTS was obtained from the local Institutional Review Boards of the University of Chicago and Michael Reese Hospital and Medical Center, and oversight was provided by an Internal Data Safety Monitoring Committee, the Data Safety Monitoring Board, and NIH.

2.2.2 – Statistical analysis

Statistical analysis was performed using Minitab, version 15. Means were compared using a General Linear Model, 1 or 2-factor analysis of variance (ANOVA) with Tukey's post-hoc test.

2.2.3 – *In vitro* tissue culture

All tissue culture incubations were conducted in a humidified incubator at 37ºC with 5% CO_2. All tissue culture manipulations were carried out in a biohazard class II laminar flow hood which was sterilised for 15 minutes using a UV light and swabbed with 70% ethanol prior to each use.

2.2.4 – *T. gondii* tachyzoite culture – Vero cells

RH *Toxoplasma gondii* tachyzoites were maintained *in vitro* by continuous tissue culture with the immortalised Vero cell line, derived from African Green Monkey Kidney. Vero cells were grown in Complete Vero Media.

Confluent monolayers of Vero cells in 80cm^2 flasks were split twice weekly for expansion by removing the spent media, washing with 5mL PBS and incubating with 0.5mL tissue culture grade trypsin (diluted 1:10) for 5 minutes at 37ºC. Following incubation, the flask was gently tapped against a soft surface (such as the palm of the hand) to disrupt any remaining adherent cells. 8.5mL Complete Vero Media was added to the monolayer and the resulting 9mL cell suspension was divided evenly between three 80cm^2 flasks containing 22mL fresh Complete Vero Media and incubated until needed for infection with *T. gondii* tachyzoites.

RH *T. gondii* tachyzoites were infected into 80cm^2 flasks containing a confluent monolayer of Vero cells at varying multiplicities of infection (MOI). Upon the macroscopic and microscopic visualisation of host cell plaques and free tachyzoites in suspension, the monolayer was scraped with a 25cm cell scraper, forced twice through a 25 gauge needle and filtered using a 3μm polycarbonate filter to remove intact host cells

and debris. The filtered tachyzoite suspension was quantified using a haemocytometer and concentrated by centrifugation at 1500 x g for 10 minutes if necessary.

2.2.5 – *T. gondii* tachyzoite culture – HFF cells

YFP-expressing RH *T. gondii* tachyzoites were maintained *in vitro* by continuous tissue culture with primary human foreskin fibroblasts (HFF). HFF cells were cultured in 75cm^2 flasks in Complete HFF Media. HFF cells were split for expansion by removing spent media, washing with 10mL HBSS and incubating with 0.5mL tissue culture grade trypsin for 5 minutes at 37ºC. Following incubation, the flask was gently tapped against a soft surface (such as the palm of the hand) to disrupt any remaining adherent cells. 10mL Complete HFF Media was added to the monolayer and the cell suspension was removed and centrifuged at 200 x g for 10 minutes. Following centrifugation, the supernatant was removed and the pellet resuspended in 10mL Complete HFF Media. Aliquots of the cell suspension were added to 6-well plates and incubated until needed for infection with *T. gondii* tachyzoites.

T. gondii tachyzoites were infected into previously prepared 6-well plates containing a confluent monolayer of HFF cells at varying multiplicities of infection (MOI). Upon the macroscopic and microscopic visualisation of host cell plaques and free tachyzoites in suspension, the monolayer was scraped with a 25cm cell scraper, forced twice through a 25 gauge needle and filtered using a 3µm polycarbonate filter to remove intact host cells and debris. The filtered tachyzoite suspension was quantified using a haemocytometer and concentrated by centrifugation at 1500 x g for 10 minutes if necessary.

2.2.6 – RAW 264.7 cell culture

The immortalised mouse macrophage-like cell line RAW 264.7, was cultured in 80cm^2 tissue culture flasks containing 25mL Complete RAW Media and split twice weekly by removing spent media, replacing with 10mL fresh Complete RAW Media and scraping the adherent monolayer with a 25cm cell scraper.

For routine expansion, 1mL of the resulting cell suspension was added to a sterile 80cm^2 tissue culture flask containing 24mL fresh Complete RAW Media and incubated until needed. For other experiments, the undiluted RAW 264.7 cell suspension was quantified using a haemocytometer, and 1 x 10^6 cells incubated per well of a 6-well plate in a total volume of 3mL Complete RAW Media per well.

2.2.7 – Murine bone marrow macrophage culture

Murine bone marrow was isolated from BALB/c, C57BL/6J and $P2X_7$ receptor knockout mice. One mouse of each strain was euthanised by CO_2 inhalation and cervical dislocation. Femurs were removed and stored in a sterile tube containing 3mL BMM Wash Media.

Bone marrow was removed from femurs by flushing the marrow out with 3mL fresh BMM Wash Media into a sterile tube using a 5mL syringe equipped with a 25 gauge needle. A single cell suspension was achieved by drawing any visible cell clumps back through the 25 gauge needle and gently forcing the suspension back into the tube again. The cell suspension was quantified using a haemocytometer and 1 x 10^5 cells seeded per well of a 6-well plate, in a total volume of 3mL Complete BMM Media.

Bone marrow cells were incubated for 4 days to promote differentiation into macrophages prior to infection with *T. gondii* tachyzoites or use in $P2X_7$ receptor function assays.

2.2.8 – Human PBMC culture – Method 1

Peripheral blood (50mL) was collected using heparin-coated vacutainers. Blood was divided evenly between 3 sterile 50mL tubes and diluted to the 35mL graduation mark with PBS and mixed gently. Blood was underlayed with 15mL Ficoll-Paque PLUS and centrifuged at 400 x g for 30 minutes with no braking.

Following centrifugation, the mononuclear layer was carefully removed from each tube with a sterile transfer pipette and transferred to a sterile 50mL tube. Each 50mL tube

was diluted to 50mL with PBS and centrifuged at 400 x g for 15 minutes. Supernatants were discarded and pellets resuspended in 5mL PBS and pooled. The pooled cell suspensions were diluted to the 50mL graduation mark with PBS and centrifuged at 400 x g for 10 minutes.

The pellet was resuspended in 12mL Complete Human Macrophage Media and 3mL cultured per well of a 6-well plate, with approximately 5 x 10^5 cells per well for *T. gondii* viability assays. Monocytes were allowed to adhere to the plastic for 24 hours before non-adherent cells (such as lymphocytes) were removed by gently pipetting the media over the surface of the plastic (or gently swirling of the plate). The spent media containing non-adherent cells was aspirated and the adherent monolayer washed once more with 3mL PBS, followed by the removal of wash PBS and addition of 3mL fresh Complete Human Macrophage Media. Cells were incubated for 4 – 7 days until macrophage morphology was visible, with a media change after 3 days.

Alternatively for $P2X_7$ receptor function assays, the entire pellet was resuspended in 25mL Complete Human Macrophage media and cultured in an 80cm^2 tissue culture flask. Incubations and wash steps were performed as with 6-well plate culture, instead using 9mL PBS for wash steps. Cells were incubated for 4 – 7 days until macrophage morphology was visible, with a media change after 3 days.

2.2.9 – Human PBMC Culture – Method 2

Peripheral blood (50mL) was collected and transferred to an equal volume of heparinised PBS, consisting 1 IU heparin per mL PBS. 20 – 30mL blood/heparinised PBS mix was transferred to a sterile 50mL tube, underlayed with 10mL Histopaque 1077 and centrifuged at 400 x g for 30 minutes. Mononuclear cells were removed and transferred to a sterile tube and diluted 1:2 with cold HBSS prior to centrifuging at 250 x g for 10 minutes. The supernatant was discarded, resuspending the pellet in 10mL cold HBSS and centrifuging again at 250 x g for 10 minutes. The supernatant was again discarded and the pellet resuspended in PBMC cryopreservation media. Cells were quantified using a haemeocytometer and 4 x 10^6 cells pipetted into each cryotube for overnight freezing at -70°C followed by transfer to long term storage at -175°C.

One tube of cryopreserved PBL (4 x 10^6 cells per tube) was defrosted and transferred to a sterile 15mL tube, followed by gradual addition of cold HBSS (7mL). Cells were centrifuged at 300 x g for 10 minutes, the supernatant was discarded and the pellet resuspended in 80μL cold MACS buffer in a sterile 1.5mL tube. Anti-CD14-microbead conjugated monoclonal antibody (25μL) was added and mixed gently followed by incubation at 4°C for 15 minutes, with gentle mixing again after 7-8 minutes. Following incubation, 1mL cold MACS buffer was added and cells centrifuged at 300 x g for 10 minutes, the supernatant was removed and the pellet resuspended in 500μL MACS buffer. CD14-positive monocytes were then separated via a magnetic column assembly.

One magnetic separation MS column per sample was prepared by placing the column inside an MS magnet and adding 500μL MACS buffer, collecting the flow through in a 15mL tube. The antibody-labelled cell suspension was then added to the column, allowing the flow through to collect in the 15mL tube. When the flow stopped, the column was washed 3 x with 500μL of MACS buffer. The MS column was removed from the magnet and placed in a sterile 1.5mL tube. MACS buffer (1mL) was added to the MS column, and using the plunger supplied with the column, CD14-positive cells were flushed out. CD14-positive cells were centrifuged at 300 x g for 10 minutes, the supernatant discarded and the pellet resuspended in 200μL AIM-V supplemented with 50ng/mL M-CSF. Cells were counted using a haemocytometer and 5 x 10^4 cells added per well in flat/clear bottom, black sided 384 well plates and incubated for 4 days to promote differentiation to monocyte-derived macrophages.

2.2.10 – $P2X_7$ receptor function assay

Functioning of the $P2X_7$ receptor expressed by various immortalised (RAW 264.7) and primary (murine BMM and human PBMC) cells was assessed on a BD FACSCalibur flow cytometer, based on the ability of the receptor to form a large pore in response to the binding of extracellular ATP, allowing the ethidium cation to enter the cell and fluoresce from the nucleus.

3×10^6 cells were harvested, centrifuged at 400 x g for 10 minutes and resuspended in 3mL NaCl buffer. The cell suspension was centrifuged again, and the pellet resuspended in 1mL fresh KCl buffer. The cell suspension was divided evenly between two FACS tubes (500μL for basal and 500μL for ATP-treated ethidium flux measurements). 25μM ethidium bromide was added to the basal tube, and ethidium fluorescence was measured for 5 minutes on the FL-2 detector, with mean fluorescence intensities recorded in 5 second intervals. 25μM ethidium bromide was then added to the ATP-uptake tube and ethidium fluorescence measured for 55 seconds on the FL-2 detector, with mean fluorescence intensities recorded in 5 second intervals. At the 55 second time point, the tube was removed from the flow cytometer in order to add 1mM ATP then immediately replaced in the instrument for continued fluorescence measurement for a total of 5 minutes. Basal and ATP-treated samples were maintained at 37°C throughout the course of fluorescence measurement by immersing the tube in a beaker of water directly removed from a 37°C water bath.

Listmode data files were removed from the FACSCalibur Macintosh computer using a portable USB storage device and transferred to a PC for analysis with the free flow cytometry analysis software, WinMDI, version 2.9. Mean fluorescence intensities of the FL-2 detector were saved from WinMDI as “tabbed text” and imported for graphing using GraphPad Prism, version 5.00.

Chapter 3 – *T. gondii* viability and replication assays – method development

3.1 - Introduction

There are several gold standard methods for assessing *T. gondii* viability and replication that have been published and referenced for several years, such as plaque assays (Foley and Remington, 1969; Pfefferkorn and Pfefferkorn, 1976) and measurement of radioactive nucleotide incorporation (Pfefferkorn and Guyre, 1984). While validated, tested and referenced thoroughly, for a variety of reasons these methods were not applicable or amenable for the purposes of experiments described in this thesis. Part of this project constituted the development and validation of two separate assays that allowed for the accurate quantification of intracellular parasite viability, replication and burden.

The first assay was developed for use when samples were available in limited quantities, analysing parasite replication/burden in small quantities of host cells. The second assay developed utilised flow cytometry to directly assess *T. gondii* tachyzoite viability, with parasites obtained from an intracellular location allowing for fast and accurate assessment of parasite viability.

An opportunity arose to work with human PBMC samples from subjects in the National Collaborative Chicago-based Congenital Toxoplasmosis Study (NCCCTS), a trio study that tracks the health of children born following congenital infection with *T. gondii*. However, only a small number of cells (usually two hundred to four hundred thousand CD14 positive monocytes) were available per subject. With such a restriction on host cell numbers, a *T. gondii* replication assay was required that would allow for accurate and repeated quantification of intracellular *T. gondii*.

A previously published method by Gubbels *et al.*, (2003) used YFP-expressing *T. gondii* and a fluorescence plate reader to quantify intracellular parasite burden and replication in 96-well plates. This assay was used as a template to create a modified *T. gondii* replication assay. The newly modified assay employed 384-well plates for host cell culture, minimising the number of cells required per well and allowing for

replicates of experimental treatments to be conducted. The modified assay also employed the use of a microplate cytometer coupled with a robotics platform (including an automated cell culture incubator) contained within a sterile, HEPA filtered room. This sterile and automated setup allowed for repeated measurements of fluorescent *T. gondii* numbers in the same sample, without the need for a new sample at each time point thus further reducing the number of host cells required for each experiment.

Another goal of this project was to employ immortalised and primary cells to conduct a more detailed investigation into the effect of $P2X_7$ receptor activation on intracellular *T. gondii*. Traditional methods in the literature for directly assessing the viability of *T. gondii* were first published some years ago, using technology and methodology that may be considered outdated by the standards of today. One gold standard method for assessing *T. gondii* tachyzoite viability involves infection of parasites into pre-prepared 96-well plates containing confluent monolayers of host cells. Parasites were heavily diluted prior to infection in order to infect any well of the 96-well plate with not more than one parasite. This method ensured that any parasite plaques visible in the host cell monolayer after 5 – 6 days of incubation were due to one original viable parasite. This method, with slight variations, was published by Foley and Remington (1969) and Pfefferkorn and Pfefferkorn (1976), and is still used as a measure of parasite replicative ability/viability. This assay was originally planned for use with experiments in this project, however it was noted that the procedure was liable to experimental error and required long periods of incubation before results were obtained. The error was most notable with the dilution of parasites for infection into 96-well plates, and with repeated manipulation of tissue culture plates (such as repeated aspiration of spent media) over long incubation periods making contamination an increased risk. Therefore, with the method being prone to error and taking a long time to yield results, the plaque assay was deemed unsuitable for the experiments planned in this project.

Another method routinely used for quantification of *T. gondii* tachyzoite viability was described by Borel *et al.*, (1998), using the fluorescent dyes acridine orange and ethidium bromide to identify live and dead parasites, respectively, by fluorescence microscopy. This assay is advantageous in requiring relatively uncostly reagents, and a fluorescence microscope equipped with suitable filters that would be found in most research laboratories. The basis of the assay involves acridine orange uptake by all

tachyzoites (since this dye is permeable through the cell membrane), and ethidium bromide uptake only by non-viable cells. Viable *T. gondii* tachyzoites will fluoresce green with acridine orange fluorescence and exclusion of ethidium bromide. Non-viable tachyzoites will fluoresce red/orange following uptake of ethidium bromide through a compromised cell wall, causing fluorescence from the nucleus and quenching any green acridine orange fluorescence.

While inexpensive and fast, this assay was also not suitable for the purposes of this project. A major goal of this project was to assess the effect of $P2X_7$ receptor activation on intracellular *T. gondii*. Since an untreated, negative control is required for comparison of any experimental treatments; both acridine orange and ethidium bromide would need to first cross the plasma membrane of the host cell before gaining access to the intracellular parasite. The plasma membrane of an untreated cell remains intact, therefore it would be inappropriate to compare the results obtained from such a control with those obtained from a $P2X_7$ receptor activated condition (since $P2X_7$ receptor activation creates pores that render cells permeable to large, cationic molecules, including ethidium). Therefore, host cells would need to be mechanically lysed to release intracellular parasites prior to staining with viability stains.

Mechanical lysis of host cells introduces another problem, associated with identification of parasites among host cell debris by microscopy. Therefore a method for distinguishing parasites from host cell debris, without operator associated bias, was developed by conducting the assay using a flow cytometer, with a monoclonal antibody to identity *T. gondii* tachyzoites from host cell debris and a viability stain to quantify parasite viability.

Flow cytometry has been used previously to assess the viability of other parasites. In order to assess the effect of pentamidine on *Leishmania panamensis*, Delgado *et al.*, (2001) stained *L. panamensis* promastigotes with propidium iodide following drug treatment. Flow cytometry was then employed to measure levels of propidium iodide fluorescence, with live parasites excluding the dye and dead parasites allowing the dye to enter the cell via a compromised plasma membrane and stain DNA in the nucleus. In a similar method, *Cryptosporidium parvum* oocyst viability was also assessed by staining oocysts with propidium iodide, with the addition of a FITC-labelled

monoclonal antibody in order to confirm the identity of the population of oocysts isolated from soil samples (Kato and Bowman, 2002).

In addition to the already documented use for assessing parasite viability, flow cytometry has also been used to quantitatively measure bacterial viability. Roth *et al.*, (1997) used Sytox Green to measure antibiotic susceptibility of *Escherichia coli*. Sytox Green is available commercially and is excited by the argon 488nm laser of a bench top flow cytometer, and was found in this case to be more sensitive than previously used dyes such as propidium iodide.

Using this information, a flow cytometry based *T. gondii* tachyzoite viability assay was designed that allowed for the fast and accurate measurement of intracellular parasite viability. The main advantage of this assay was the ability to quantify the viability of several *T. gondii* tachyzoite samples in a short time, independent of operator bias, with a result being available in approximately one hour.

3.2 – Methods and results

3.2.1 – Microplate cytometry replication assay

A *T. gondii* burden/replication assay was developed based on a previously published method (Gubbels *et al.*, 2003). The modified assay utilised YFP expressing RH *T. gondii* and a microplate cytometer to monitor parasite replication based on fluorescent parasite numbers in a 384 well plate format, with all plate movements and manipulations carried out by a robotics platform including an automated tissue culture incubator, all contained within a clean, HEPA-filtered room.

For this assay, YFP RH *T. gondii* replication was monitored at the University of Chicago Cellular Screening Centre using an F3 robot arm coupled with an Acumen eX3 microplate cytometer. This assay was developed to assess the effect of $P2X_7$ receptor activation on the replication of RH *T. gondii* within small numbers of host cells.

For the quantification of intracellular YFP RH *T. gondii* burden, the Acumen eX3 microplate cytometer used a 488nm laser for excitation of YFP expressed by intracellular *T. gondii* tachyzoites with a 500 – 530nm filter for emission. Parasite fluorescence measurements consisted of the automated quantification of all YFP fluorescent events across the entire well for each sample.

Between fluorescence measurements, plates were incubated in an automated humidified incubator at 37°C with 5% CO_2, with all plate movements and manipulations carried out by the F3 robot arm. Fluorescent parasite numbers at each time point were plotted using GraphPad Prism, version 5.00.

In order to provide confirmation that the microplate cytometry assay was capable of quantifying varying numbers of intracellular parasites, HFF cells were cultured and trypsinised as previously described in section 2.2.5. 1 x 10^4 HFF cells were added per well in flat/clear bottom, black sided 384 well plates and incubated for 2 hours (total volume of 70μL per well). YFP RH *T. gondii* was cultured as previously described in section 2.2.5, harvested and infected into triplicate wells of previously prepared HFF cells in multiplicities of infection (MOI) ranging from 70 (70 parasites per HFF cell) to 0.1 (0.1 parasites per host cell) followed by overnight incubation. Following this, spent culture media was aspirated and extracellular *T. gondii* tachyzoites removed by washing all wells with complete HFF media. Fresh media was added to all wells and YFP RH *T. gondii* fluorescence was observed and photographed on a Zeiss Axiovert 200 inverted fluorescence microscope under the 100x/1.3 NA objective (with a YFP fluorescence filter cube) using a Hamamatsu Orca ER camera.

This confirmed that fluorescence was only visible in YFP RH *T. gondii* tachyzoites, with no fluorescence visible from HFF cells or other material in the culture vessel (Figure 3.1). Fluorescence in samples in the same plate was then quantified by the microplate cytometry method as described above, with results plotted using GraphPad Prism, version 5.00. The results comparing the number of parasites added per well with level of fluorescence are shown in Figure 3.2.

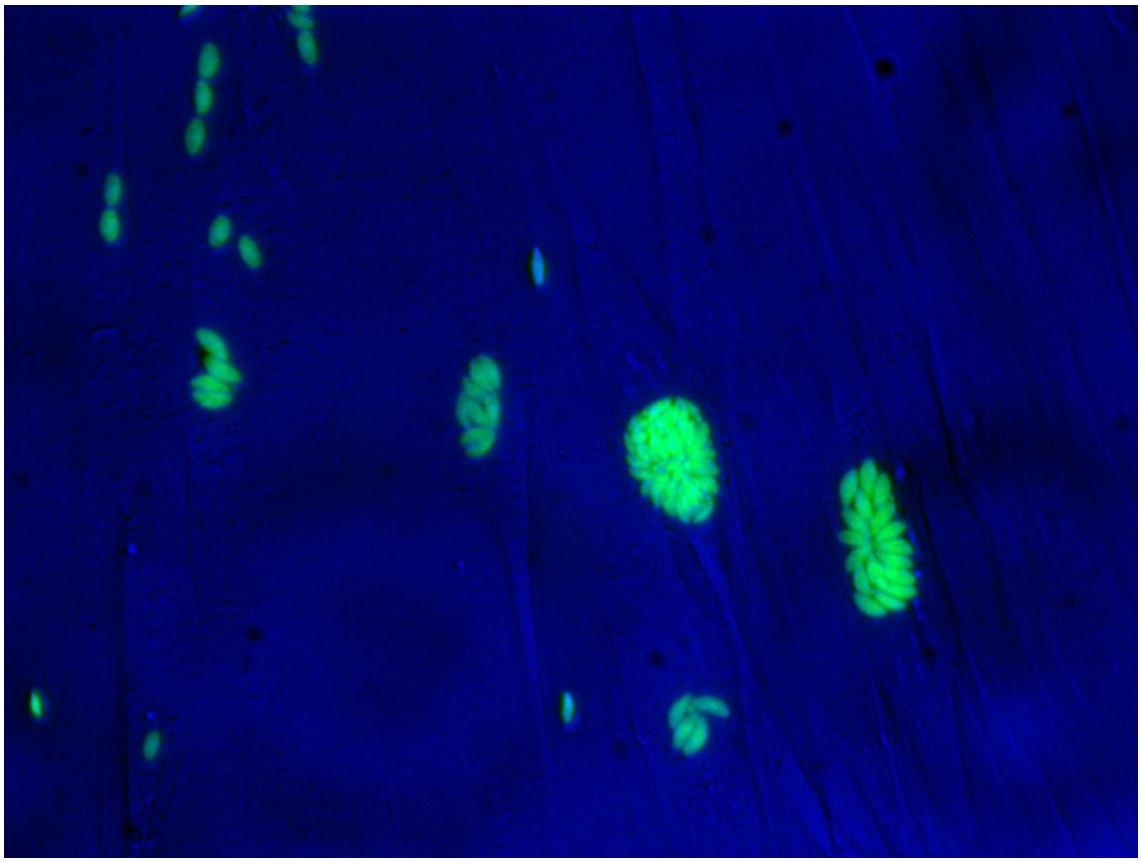

Figure 3.1 – YFP RH *T. gondii* fluorescence image.

Prior to parasite quantification by flow cytometry, YFP expressing RH *T.* gondii was visualised by fluorescence microscopy under the 100x objective. This figure shows the merged phase contrast/fluorescence microscopy image. Fluorescence was only visible in the YFP RH *T. gondii* tachyzoites (green), with no background fluorescence visible from HFF cells or other debris.

This experiment confirmed that the microplate cytometry method was capable of accurately quantifying increasing numbers of YFP-expressing RH *T. gondii* tachyzoites within small numbers of host cells. As the MOI increased, so too did the fluorescent event count, indicating that the fluorescence identified by the microplate cytometer correlated with the number of intracellular parasites. At an MOI of 2.2, the number of parasites quantified was thought to be sufficiently high enough as to not exceed the limits of detection for the assay. Therefore, to monitor increases or decreases in parasite numbers in subsequent experiments, an MOI of 2.5 was used.

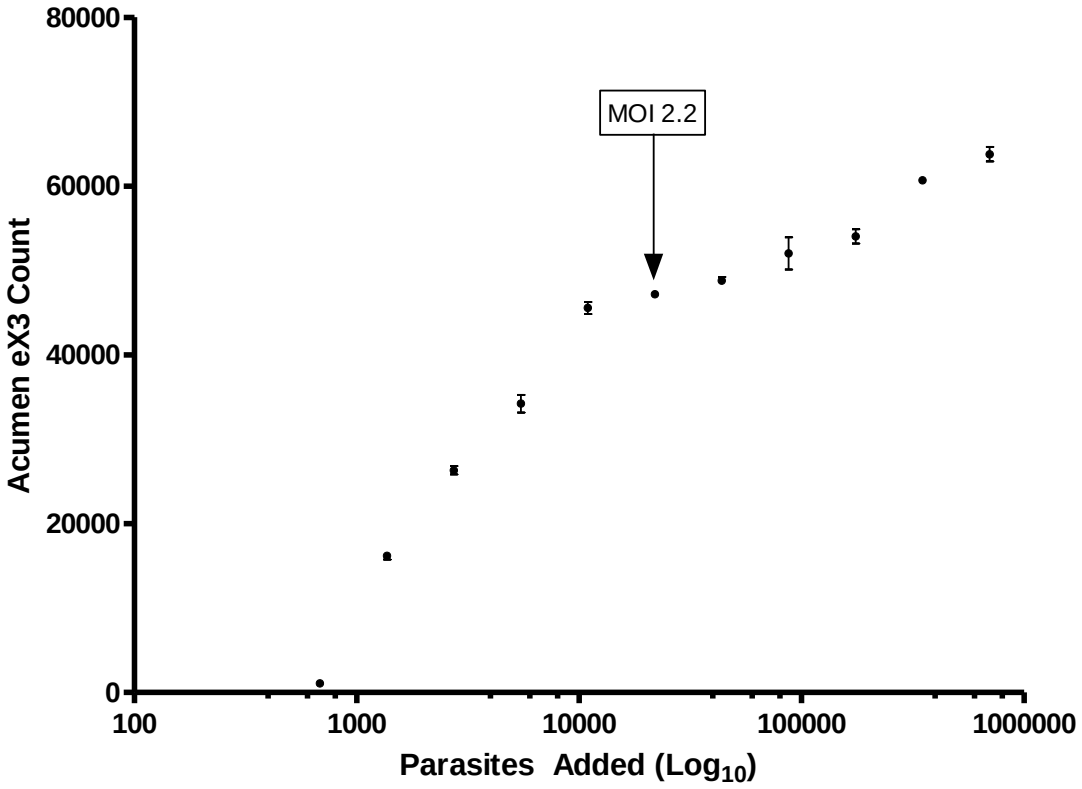

Figure 3.2 – Validation of microplate cytometry *T. gondii* replication assay.
YFP expressing RH *T. gondii* was infected into HFF host cells at varying multiplicities of infection. After overnight incubation, extracellular parasites were removed. The microplate cytometry method was used to quantify intracellular parasite burden by the fluorescent event count. This count was then plotted against the original number of parasites added to that sample. The data shows increased parasite count identified by the Acumen eX3 microplate cytometer in response to increasing numbers of parasites added to each sample.

3.2.2 – Flow cytometry viability assay

A flow cytometry-based viability assay for intracellular *T. gondii* tachyzoites was established that allowed the rapid assessment of the viability of thousands of intracellular *T. gondii* tachyzoites. RAW 264.7 cells were infected with RH *T. gondii* tachyzoites, incubated overnight and extracellular tachyzoites removed. Intracellular tachyzoites were released by mechanically lysing the host cells and parasite viability assessed by flow cytometry.

Prior to experiments, anti-p30 *T. gondii* monoclonal antibody was first conjugated to Alexa Fluor 647 using the Alexa Fluor 647 protein labelling kit according to the manufacturer's instructions. The antibody was combined with Alexa Fluor 647 dye, incubated at room temperature and separated from unbound dye by size exclusion chromatography.

T. gondii tachyzoite viability was assessed using flow cytometry by staining the cell suspension with anti-p30 *T. gondii*-Alexa Fluor 647 conjugated monoclonal antibody diluted 1:100 and Sytox Green diluted 1:10000. Previously prepared 97μL tachyzoite suspensions were combined with 1μL anti-p30 *T. gondii*-Alexa Fluor 647 conjugated monoclonal antibody and 2μL Sytox Green working solution (diluted 1:200 in FSW) and incubated at room temperature for 30 minutes protected from light, mixing the suspension gently after 15 minutes. Following incubation, 500μL FSW was added and the suspension transferred to a FACS tube for analysis on a BD FACSCalibur flow cytometer.

T. gondii tachyzoites were identified by log increases in anti-p30 *T. gondii*-Alexa Fluor 647 conjugated monoclonal antibody fluorescence, measured on the FL-4 detector. Tachyzoite viability was analysed based on Sytox Green uptake, measured on the FL-1 detector. Following the acquisition of 5000 gated tachyzoite events, listmode data files were removed from the FACSCalibur Macintosh computer using a portable USB storage device and transferred to a PC for analysis with the free flow cytometry analysis software, WinMDI, version 2.9. *Toxoplasma gondii* tachyzoites were gated for viability analysis, with the resulting bimodal populations corresponding to viable (Sytox Green non-fluorescent) and non-viable (Sytox Green fluorescent) tachyzoites. Percent viable *T. gondii* tachyzoites were plotted using GraphPad Prism, version 5.00.

Figure 3.3 shows the typical density plots generated from the flow cytometry viability assay. Panel A shows the forward scatter – side scatter density plot (indicators of the size and granularity of an event, respectively), showing that the cell suspension passing through the flow cytometer contained mixed populations. These populations corresponded to cell debris (1), *T. gondii* tachyzoites (2) and intact RAW 264.7 host cells (3). Panel B demonstrates that the introduction of an anti-*T. gondii* monoclonal antibody conjugated to Alexa Fluor 647 allowed the identification of two populations that exhibited log increases in their levels of fluorescence. These populations corresponded to smaller (with less forward scatter) *T. gondii* tachyzoites (1) and larger (with more forward scatter) intact host cells infected with *T. gondii* tachyzoites (2). Mechanical lysis of host cells was not 100% effective, and would always leave some cells intact. Panel C shows that the region R1 from panel B exhibited two distinct populations corresponding to viable *T. gondii* tachyzoites in the lower left quadrant (with no sytox green fluorescence) and non-viable *T. gondii* tachyzoites in the upper left quadrant (with log increases in sytox green fluorescence).In this sample, for example, 76.1% of tachyzoites were deemed viable and 23.9% non-viable. Similar scatter profiles and populations were seen in repeat experiments. This method of data analysis was used for all subsequent experiments involving the assessment of *T. gondii* tachyzoite viability by flow cytometry.

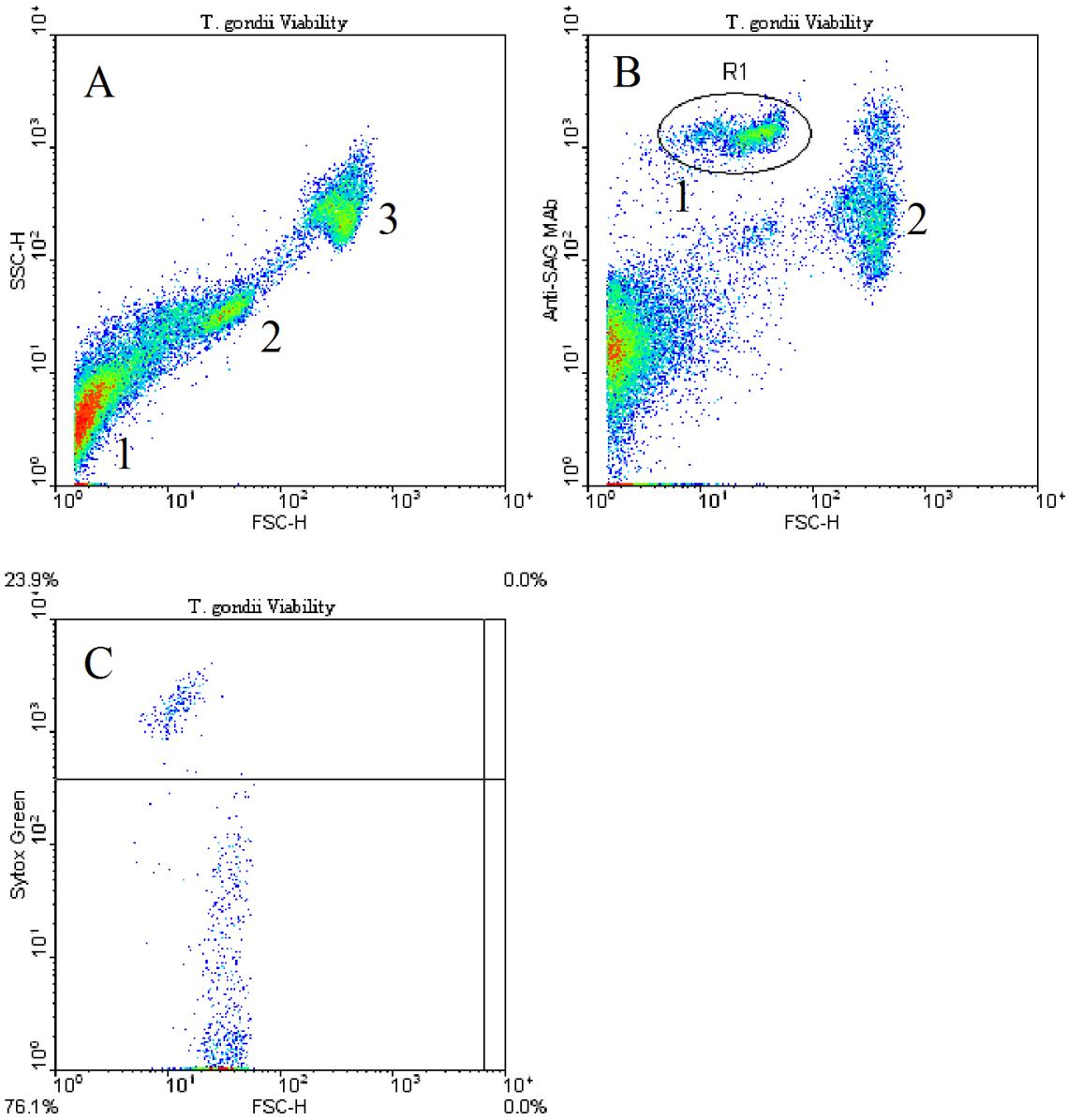

Figure 3.3 – Typical density plots obtained from flow cytometry *T. gondii* viability assay.

1 x 10^6 RAW 264.7 cells were infected with 3 x 10^6 RH *T. gondii* tachyzoites and incubated overnight (MOI = 3). Parasite viability was assessed by flow cytometry following mechanical lysis of host cells. Panel A shows the forward scatter – side scatter density plot, with debris (1), *T. gondii* tachyzoites (2) and intact RAW 264.7 cells (3). Panel B shows the density plot with populations exhibiting increased fluorescence with anti-*T. gondii*-Alexa Fluor 647 monoclonal antibody, corresponding to *T. gondii* tachyzoites (1) and *T. gondii* infected RAW 264.7 cells (2). Panel C shows the level of fluorescence of region R1 (gated from panel B) for the viability dye sytox green, confirming the *T. gondii* tachyzoites exhibit two populations corresponding to 76.1% viable and 23.9% non-viable.

3.2.3 – Comparative validation of flow cytometry viability assay

Results in Section 3.2.2 showed that the flow cytometry viability assay was capable of successfully quantifying the viability of intracellular *T. gondii* tachyzoites. It was still necessary however, to validate this method against a previously published method such as the acridine orange/ethidium bromide fluorescence microscopy method. For this experiment, a freshly prepared RH *T. gondii* tachyzoite monolayer was divided evenly between two tubes. One tube was incubated in a 60°C water bath for 5 minutes in order to prepare a suspension of dead tachyzoites, with the remaining tube left untreated to provide the untreated, viable tachyzoite control. The heat-treated dead tachyzoite suspension was combined with untreated tachyzoites in a total volume of 500μL in varying combinations (0%, 33%, 66% and 100% heat treated, prepared in duplicate) and centrifuged at 1500 x g for 10 minutes. The supernatant was discarded and the pellet resuspended in 97μL FSW for the flow cytometry method or 99μL FSW for the fluorescence microscopy method. Use of the fluorescence microscopy method was not problematic in this instance, since the parasites were not being removed from mechanically lysed host cells, and their viability was therefore easily quantifiable by fluorescence microscopy.

Toxoplasma gondii tachyzoite viability was assessed using fluorescence microscopy by staining the cell suspension with 3μg/mL acridine orange and 10μg/mL ethidium bromide (AO/EtBr). Previously prepared 99μL tachyzoite suspensions were combined with 1μL 100 x stock solution of AO/EtBr and incubated at room temperature for 30 minutes protected from light. 20μL of stained tachyzoites was placed on a microscope slide, a cover slip was applied and the slide viewed on an Olympus BX51 fluorescence microscope equipped with an Olympus DP70 digital camera using the 470 – 490nm band pass filter.

Five random fields of view were photographed using the 40 x objective for each sample and saved for further analysis. Analysis of the saved images was conducted blinded, with the percent viable tachyzoites calculated based on fluorescence of green, viable tachyzoites (only acridine orange fluorescence) and fluorescence of orange, non-

viable/dead (both acridine orange and ethidium bromide fluorescence) tachyzoites for each sample.

For example: Percent viable = Green ÷ (Green + Orange) x 100

After the viability of each sample was assessed by flow cytometry and fluorescence microscopy, the results were plotted for comparison using GraphPad Prism, version 5.00.

Figure 3.4 shows digital images taken by fluorescence microscopy of the *T. gondii* tachyzoite populations used to compare the acridine orange/ethidium bromide method with the flow cytometry method. These parasites have been stained with acridine orange/ethidium bromide and it can be seen that the number of orange/red tachyzoites increases as the proportion of heat treated tachyzoites in the cell suspension also increases. The number of green tachyzoites also correlates with the proportion of viable tachyzoites in each suspension. In order to provide a standard curve for comparison to the flow cytometry method, *T. gondii* tachyzoites were heat treated to reduce viability, combined with untreated tachyzoites and assessed for viability using both methods (Figure 3.5).

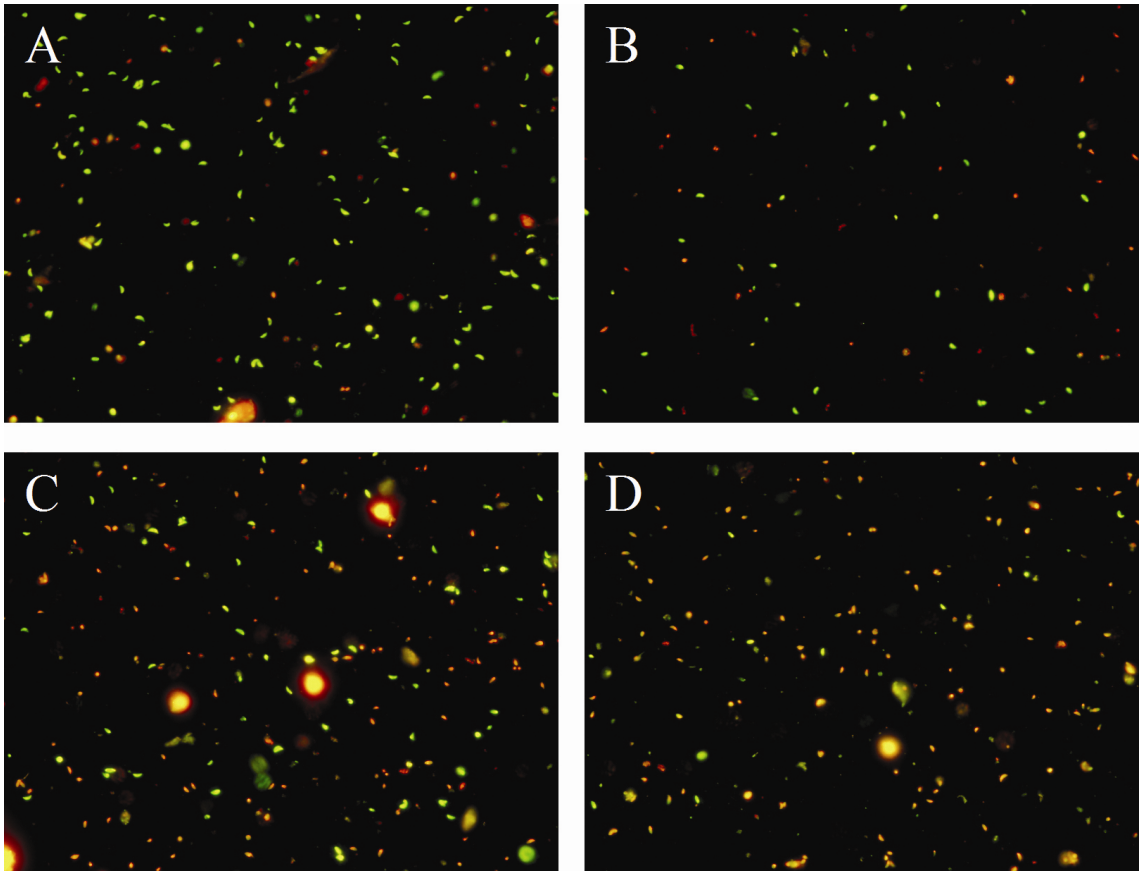

Figure 3.4 – Fluorescence microscopy assessment of *T. gondii* tachyzoite viability.
Cultured *T. gondii* tachyzoites were heat-treated in a hot water bath, combined with untreated tachyzoites with viability then assessed by fluorescence microscopy based on acridine orange (green, viable) and ethidium bromide (orange/red, non-viable) fluorescence. Panel A represents the sample containing 0% heat-treated (untreated) tachyzoites, with panels B, C, and D containing 33%, 66% and 100% heat treated tachyzoites respectively. Five random fields of view were photographed for each sample.

Concurrently, RH *T. gondii* viability was assessed by flow cytometry and fluorescence microscopy with parasite samples containing varying proportions of untreated (viable) and heat-treated (dead/non-viable) parasites. Both methods gave similar results when assessing the viability of the same sample, with no significant difference noted between methods with all samples ($P > 0.05$) when results were analysed using a General Linear Model, 2-factor ANOVA with Tukey's post-hoc test (Figure 3.5). In both methods, the untreated samples contained approximately 75 – 80% viable parasites. This result most likely represents the small proportion of tachyzoites that are non-viable in typical culture conditions. Additionally, in both methods the 100% heat-treated sample was identified as approximately 10 – 20% viable. This result could identify a limit of

detection for both assays, or simply show that not all *T. gondii* tachyzoites are rendered non-viable by heat-treatment at these conditions.

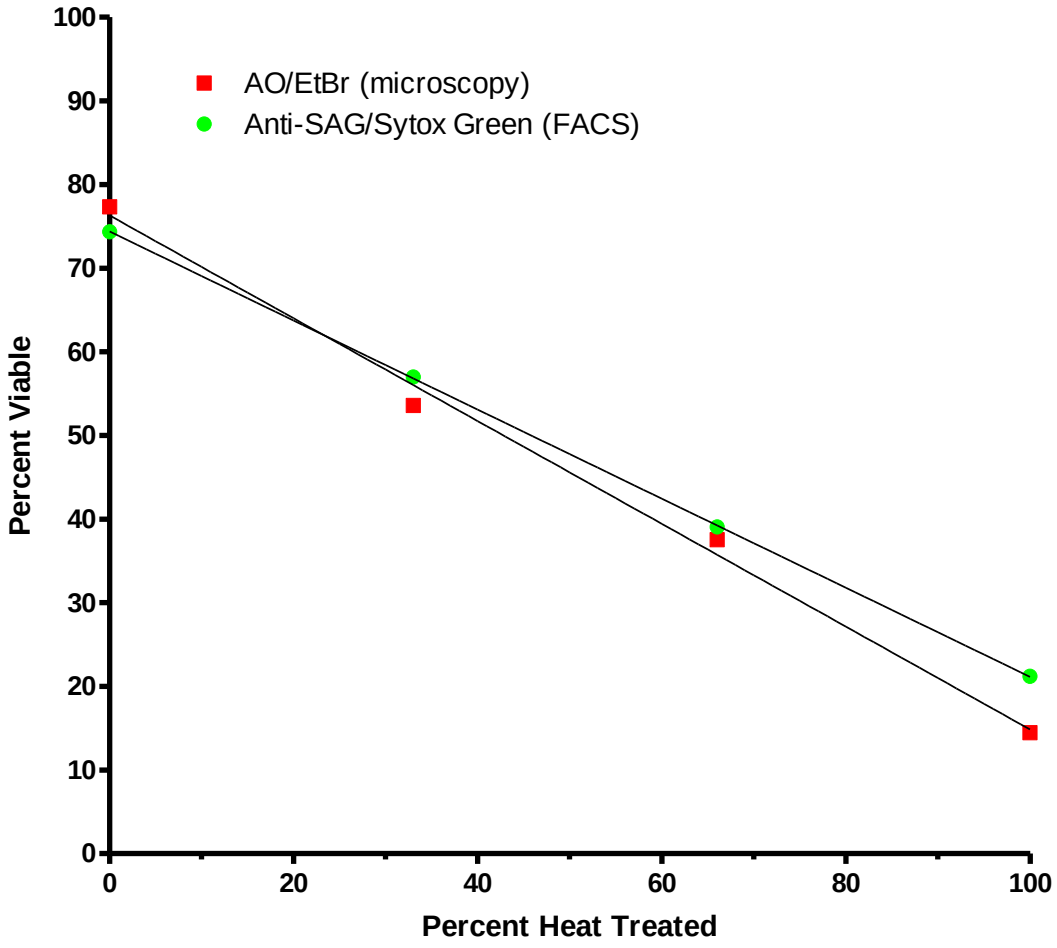

Figure 3.5 – Comparison of *T. gondii* tachyzoite viability assays.

Flow cytometry and fluorescence microscopy. *T. gondii* viability assays were used to assess heat-treated parasite viability. Points in this figure represent the parasite viability identified (y-axis) compared with percent heat treated (x-axis) for flow cytometry (green, circles) and acridine orange/ethidium bromide fluorescence microscopy (red, squares). 5000 *T. gondii* tachyzoite events were recorded for the flow cytometry assay and five random fields of view were photographed for the fluorescence microscopy assay. Data points = mean; n = 2. General Linear Model, 2-factor ANOVA with Tukey's post test revealed no significant difference ($P > 0.05$) between the mean viability identified by both assays for all samples.

3.3 – Discussion

Two novel assays for the quantification of intracellular *T. gondii* replication and viability were presented in this chapter. The first method was developed for use with host cell samples that were in limited supply. Experiments presented in Chapter 4 used cryopreserved PBMC samples from subjects in the NCCCTS, a study that tracks the health and development of children born with congenital toxoplasmosis. These samples were only available in extremely limited quantities, with approximately two hundred thousand to four hundred thousand cells per subject. Therefore, a *T. gondii* replication assay was needed that would allow for the monitoring of parasite replication within extremely small numbers of host cells with the ability to repeatedly measure parasite replication using the same samples over the course of the experiment.

With resources available at the University of Chicago Cellular Screening Center, a *T. gondii* replication assay was developed, based on a previously published method by Gubbels *et al.*, (2003). This method utilised *T. gondii* expressing yellow fluorescent protein (YFP) in order to monitor parasite replication based on fluorescence recorded on a fluorescence plate reader, using 96-well plates. This assay was modified, culturing cells in 384-well plates in order to decrease the number of cells required for each sample. Following infection of cells with YFP-expressing *T. gondii*, fluorescence measurements were initiated in a microplate cytometer capable of detecting and quantifying fluorescent events (such as YFP-expressing RH *T. gondii*) and outputting the data into a spreadsheet. Repeat fluorescence readings on the same sample, at later time points were made possible, since all plate manipulations and movements were conducted by a robotics platform in a HEPA filtered clean room. Since this assay quantifies intracellular parasite burden based on each fluorescing parasite, it may be possible to convert the number of fluorescent events to the real number of intracellular parasites. This possibility was not further explored, since arbitrary units were sufficient for the downstream uses of the assay.

To confirm that the modified YFP RH *T. gondii* replication assay was capable of accurately quantifying intracellular parasite burden/replication, YFP RH *T. gondii* was infected into 384-well plates containing HFF cells at varying multiplicities of infection.

Parasite fluorescence was then quantified, with the result showing that the fluorescence detected by the Acumen eX3 microplate cytometer increased as the number of parasites added to each well increased (Figure 3.2). Since this relationship was evident at the minimum and maximum MOI used, the limits of detection for the assay were not reached. This assay was then employed to assess the effect of $P2X_7$ receptor activation on intracellular *T. gondii*, with samples from subjects in the NCCCTS.

In addition to the purpose of assessing intracellular parasite replication, the microplate cytometry method may also be employed for other experimental purposes. It may be possible to use the assay to monitor parasite burden and dissemination in *T. gondii* infected mice, by monitoring YFP fluorescence in blood samples or homogenates from various tissues isolated post infection with YFP expressing *T. gondii*.

The second method was developed to provide a rapid and more accurate way of assessing parasite viability. Previously published *T. gondii* viability assays were not suitable for quickly assessing intracellular *T. gondii* viability. The major difficulty encountered with previously published methods was associated with delivering a viability stain to intracellular *T. gondii* tachyzoites through a host cell plasma membrane. If a viable host cell (with an intact plasma membrane) contained a non-viable parasite, the viability stain would not be able to pass the host cell plasma membrane, thus giving a false viable result for parasite viability. In addition, the quantification of parasite viability by previously published methods was often very time-consuming, requiring the counting of plaques on tissue culture monolayers or identification of fluorescent parasites by microscopy (both methods being open to human error and influence).

Studies in the literature showed that a flow cytometric assay for assessing viability of other organisms could also be applied to *T. gondii* (Delgado *et al.*, 2001; Kato and Bowman, 2002). This assay was developed around a 6-well plate format, allowing larger numbers of cells to be subjected to experimental treatments and easily recovered. Mechanical lysis of host cells allowed for the release intracellular parasites for viability analysis. Using flow cytometry, *T. gondii* tachyzoites could be identified and distinguished from host cell debris possessing a similar size and granularity by the use of a monoclonal antibody directed against a parasite surface antigen. The parasite

population could then be gated for viability analysis based on Sytox Green exclusion/uptake.

Typical scatter profiles for the *T. gondii* tachyzoite viability assay were shown with parasites removed from RW 264.7 cells exhibiting a viability of 76.1% based on the exclusion of the viability dye sytox green (Figure 3.3). This level of tachyzoite viability was expected, as the parasites had been growing in the RAW 264.7 mouse macrophage-like cell line and there is usually a small level of parasite death in typical cell culture conditions, especially when cells are taken from an intracellular location inside a macrophage host cell.

Initial experiments involving a flow cytometry based *T. gondii* viability assay focussed around comparing the new method with the previously accepted and published fluorescence microscopy method (Parks *et al.*, 1979; Handman and Remington, 1980; Borel *et al.*, 1998). Freshly cultured suspensions of extracellular *T. gondii* tachyzoites were heat treated to induce parasite death and combined with untreated tachyzoites in varying concentrations, with the viability of each suspension being assessed by both fluorescence microscopy and flow cytometry (Figure 3.5). Both methods gave similar results when assessing free, extracellular parasite viability. In addition, the use of flow cytometry removed the possibility of human error or influence on experimental samples, with all final parasite viability numbers generated by the flow cytometer and analysis software. The flow cytometry assay was also capable of assessing both extracellular and intracellular parasite viability, with intracellular parasites released by mechanical lysis of host cells prior to analysis. The use of a monoclonal antibody which recognises an epitope on the surface of *T. gondii* tachyzoites allowed the assay to distinguish between *T. gondii* tachyzoites and any other debris that interferes with parasite identification. After the validation procedure, the flow cytometry viability assay was used extensively throughout this project in experiments that showed the effect of human and murine $P2X_7$ receptor activation to on the viability of intracellular *T. gondii*.

There were however, limitations and disadvantages with the assay and there are improvements that could be made in order to increase efficiency and accuracy. The use of a monoclonal antibody to distinguish parasites from any other debris is an area where improvements could be made. The use of *T. gondii* expressing a fluorescent protein

such as YFP, would eliminate the need to use a monoclonal antibody and thus remove any possible errors associated with non-specific binding of the antibody to other proteins in the parasite cell suspension. This modification would also reduce the long term costs associated with continued purchase of antibodies and conjugation kits, as well as reducing the time required for sample preparation (with only one viability stain being required in the procedure).

The two assays that have been developed and validated in experiments presented in this chapter have facilitated investigations into the viability and replicative ability of *T. gondii* tachyzoites. The microplate cytometry replication assay made important progress in the ability to monitor the replication of *T. gondii* tachyzoites within host cells and samples of limited availability, tracking the number of parasites present in each sample. In addition, the development of the flow cytometry *T. gondii* viability assay allowed for the fast and accurate quantification of *T. gondii* viability, using reagents and equipment that most research laboratories would have local access to.

Chapter 4 – Human $P2X_7$ receptor investigation

4.1 – Introduction

$P2X_7$ receptor activation is already known to be important in the immune response to other pathogenic organisms. Lammas *et al.*, (1997) first showed ATP-mediated killing of intracellular BCG *Mycobacterium bovis*, with Smith *et al.*, (2001) and later Saunders *et al.*, (2003) conducting more detailed investigations, showing that ATP treatment exerted its effect through the $P2X_7$ receptor, resulting in decreased host cell and subsequently, decreased mycobacterial viability. $P2X_7$ receptor activation has also been implicated in the immune response to *Chlamydia* spp., with Coutinho-Silva *et al.*, (2001) demonstrating $P2X_7$ receptor-mediated killing of intracellular *C. psittaci* by human J774 macrophages. Darville *et al.*, (2007) reinforced this role, showing more efficient infection rates and greater levels of inflammation in *C. muridaru*- infected $P2X_7$ receptor knockout mice. In most of these studies, 3mM ATP was used to activate the $P2X_7$ receptor for *in vitro* assessment of the effect of receptor activation on intracellular pathogens. Therefore 3mM ATP was also used in experiments conducted in this thesis, aimed at assessing the effect of $P2X_7$ receptor activation on intracellular *T. gondii*. For assessing $P2X_7$ receptor function of primary and immortalised cells, 1mM ATP is used in order to facilitate a discernable difference between receptor function of various $P2X_7$ receptor genotypes.

The role for $P2X_7$ receptor activation in the immune response extends to *Leishmania* spp., with Torres-Santos *et al.*, (2000) showing increased sensitivity of peritoneal macrophages and spleen cells to ATP permeabilisation when infected with *L. amazonensis*. Increased sensitivity to ATP treatment resulted in $P2X_7$ receptor mediated pore formation and subsequent inhibition of parasite growth was also noted in murine macrophages and cells cultured from cutaneous lesions after infection of mice with *L. amazonensis* (Chaves *et al.*, (2009). Hence, the $P2X_7$ receptor not only plays a role in the immune response to intracellular bacteria, but also in the response to macrophage dwelling pathogens similar to *T. gondii*.

Clinical observations presented in Chapter 1 also implicated the $P2X_7$ receptor in the immune response to *T. gondii*. Three immunocompetent individuals presented with unusually severe toxoplasmosis and were subsequently found to have decreased $P2X_7$ receptor function due to the presence of loss-of-function polymorphisms. Considering the information from the literature and these clinical observations, the aim of experiments presented in this chapter was to assess the effect of ATP treatment and subsequent $P2X_7$ receptor activation, on the viability and replication of intracellular Type I (RH) *T. gondii* tachyzoites infected into normal/control human macrophages. The use of both flow cytometry- and microplate cytometry-based *T. gondii* viability and replication assays with human cells isolated from the same donor allowed for confirmation that both assays yielded the same/similar results upon $P2X_7$ receptor activation of *T. gondii*-infected human cells.

Additionally, the presence of loss-of-function polymorphisms within the human $P2X_7$ receptor gene was also investigated, assessing the effect that loss in $P2X_7$ receptor function would have on the fate of the intracellular parasite post-ATP treatment, using samples from NCCCTS subjects. The NCCCTS tracks the health and development of children born with congenital toxoplasmosis, aiming to understand the genetic basis for increased susceptibility to more severe disease seen with many congenitally infected children. Genomic DNA from one hundred and fifty one child/parent trios in the NCCCTS was genotyped for eight $P2X_7$ receptor single nucleotide polymorphisms (rs208293, rs28360457, rs1718119, rs2230911, rs2230912, rs3751143, rs1653624 and rs1621388) by Prof Jennie Blackwell and Dr Sarra Jamieson (University of Western Australia, WA, Australia). Experiments using NCCCTS samples were conducted using the $P2X_7$ receptor genotype information of individual subjects made available by Prof Blackwell and Dr Jamieson.

The main aim for the experiments presented in this chapter was to assess the effect of human $P2X_7$ receptor activation on intracellular RH *T. gondii* by:

1. Culturing monocyte-derived macrophages from a normal/control donor and:
    a. Assessing $P2X_7$ receptor function by flow cytometry;
    b. Assessing the effect of ATP treatment on the viability of intracellular RH *T. gondii* by flow cytometry;

c. Assessing the effect of ATP treatment on the intracellular burden/number of intracellular YFP RH *T. gondii* by microplate cytometry.

2. Culturing monocyte-derived macropahges from NCCCTS subjects with varying $P2X_7$ receptor function and assessing the effect of ATP treatment on the intracellular burden/number of intracellular YFP RH *T. gondii* by microplate cytometry.

4.2 – Methods and results

4.2.1 – RH *T. gondii* viability following human $P2X_7$ receptor activation

These experiments aimed to assess the effect of human $P2X_7$ receptor activation on the viability and/or replication of intracellular *T. gondii* tachyzoites. For this purpose, primary human monocytes freshly isolated from peripheral blood were found to be most amenable to prolonged culture and induction of macrophage morphology. Blood from a readily accessible donor was used for these initial experiments, showing first by flow cytometry that monocyte-derived macrophages (cultured as described in section 2.2.8) from this donor had a functional $P2X_7$ receptor. Activation of the $P2X_7$ receptor with extracellular ATP results in the formation of a large pore on the cell surface. The flow cytometry function assay takes advantage of this phenotype, by quantifying ethidium flux through this pore, and subsequent fluorescence following intercalation of the dye with DNA in the nucleus.

Macrophages from this donor exhibited a functional $P2X_7$ receptor, shown by the rapid increase in ethidium fluorescence post ATP treatment (Figure 4.1). Fresh cells were then isolated and cultured for use in flow cytometry- and microplate cytometry-based *T. gondii* viability/replication assays.

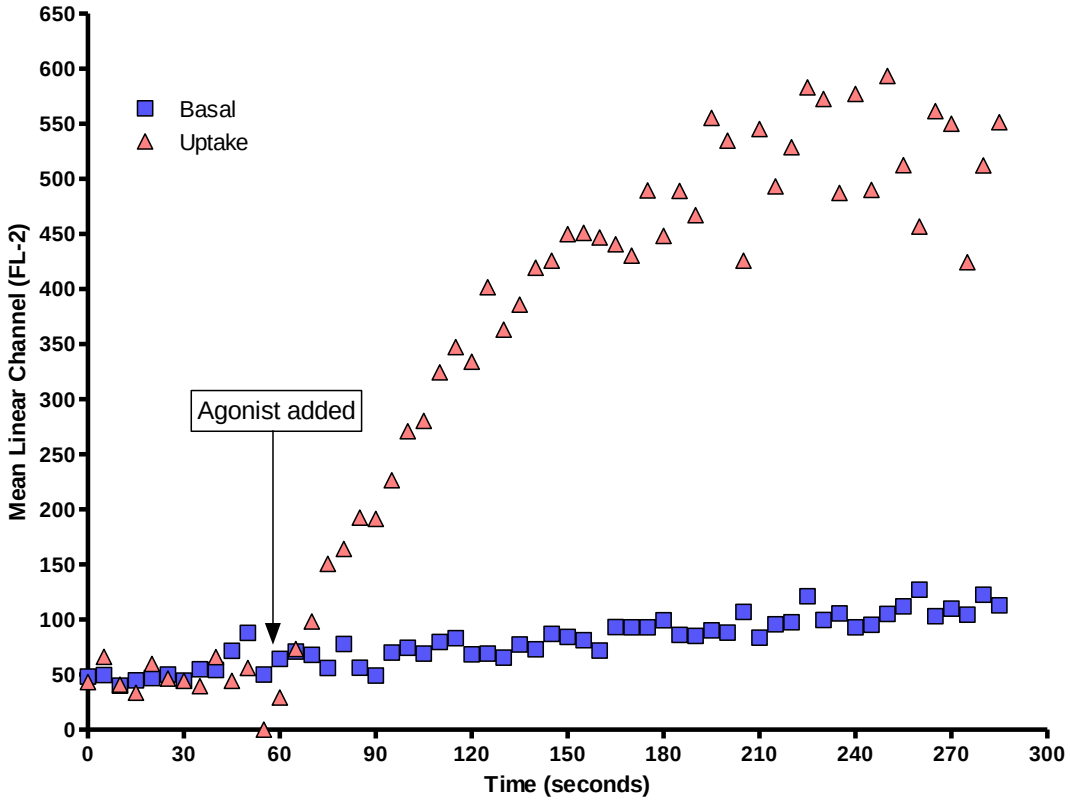

Figure 4.1 – $P2X_7$ receptor function of a normal/control donor.
Monocytes were purified from the blood of a normal/control donor and incubated for 7 days to induce macrophage differentiation. Cultured macrophages were assessed for $P2X_7$ receptor function by flow cytometry, by measuring ethidium flux through $P2X_7$ receptor mediated pores and fluorescence from the nucleus. The result shows ethidium uptake after ATP treatment, confirming that this donor has a functional $P2X_7$ receptor.

For the flow cytometry *T. gondii* viability assay, monocyte-derived macrophages were cultured in 6-well plates as described in section 2.2.8. Parasite viability was quantified by flow cytometry 24 hours following $P2X_7$ receptor activation. Each time point consisted of the following controls:

- Untreated (negative control – no parasite death); and
- ATP ($P2X_7$ receptor agonist).

A freshly cultured monolayer of RH *T. gondii* tachyzoites was prepared and quantified using a haemocytometer (as described in section 2.2.4). 3×10^6 tachyzoites were added to each well containing host cells and incubated overnight for 16 hours.

Following overnight incubation, extracellular tachyzoites were removed from all wells by aspirating the spent culture media and washing the wells with 3mL fresh Complete Human Macrophage Media. The wash media was removed and 3mL fresh Complete Human Macrophage Media added to each well, with 3mM ATP added to appropriate wells where appropriate.

Parasite viability was assessed by flow cytometry 24 hours after ATP addition (as described in Chapter 3). Tachyzoites were prepared for flow cytometry viability analysis by scraping each well with a 25cm cell scraper and forcing the cell suspension through a 27 gauge needle three times to lyse intact host cells. 500μL tachyzoite suspension was centrifuged at 1500 x g for 10 minutes, discarding the supernatant and resuspending the pellet in 97μL FSW.

For the microplate cytometry *T. gondii* burden assay, monocyte-derived macrophages were cultured in 384-well plates as described in section 2.2.9. A freshly cultured monolayer of YFP expressing RH *T. gondii* was prepared (as described in section 2.2.5) and quantified using a haemocytometer. Twenty thousand tachyzoites were added to each well containing MDM and incubated overnight for 16 hours.

Extracellular parasites were removed by aspirating spent media and adding 80μL fresh AIM-V. Wash media was removed and 80μL fresh AIM-V was added. YFP RH *T. gondii* replication in cultured MDM was then monitored at the University of Chicago Cellular Screening Centre using an F3 robot arm coupled with an Acumen eX3 microplate cytometer as described in Chapter 3. Hourly measurements of fluorescent parasites were conducted for a total period of 24 hours, with three initial measurements taken prior to the addition of 3mM ATP to half of the cell samples (to activate the $P2X_7$ receptor) immediately prior to the fourth YFP fluorescence measurement. Each time point consisted of the following controls:

- Untreated (negative control – no parasite death); and
- ATP ($P2X_7$ receptor agonist).

A significant decline in intracellular parasite viability was observed following ATP treatment using the flow cytometry assay (Figure 4.2, Panel A). Similarly, a significant

reduction in parasite burden was seen using the microplate cytometry assay following ATP treatment (Figure 4.2, Panel B). Statistical analysis was performed using a General Linear Model, 1/2-factor ANOVA with Tukey's post-hoc test

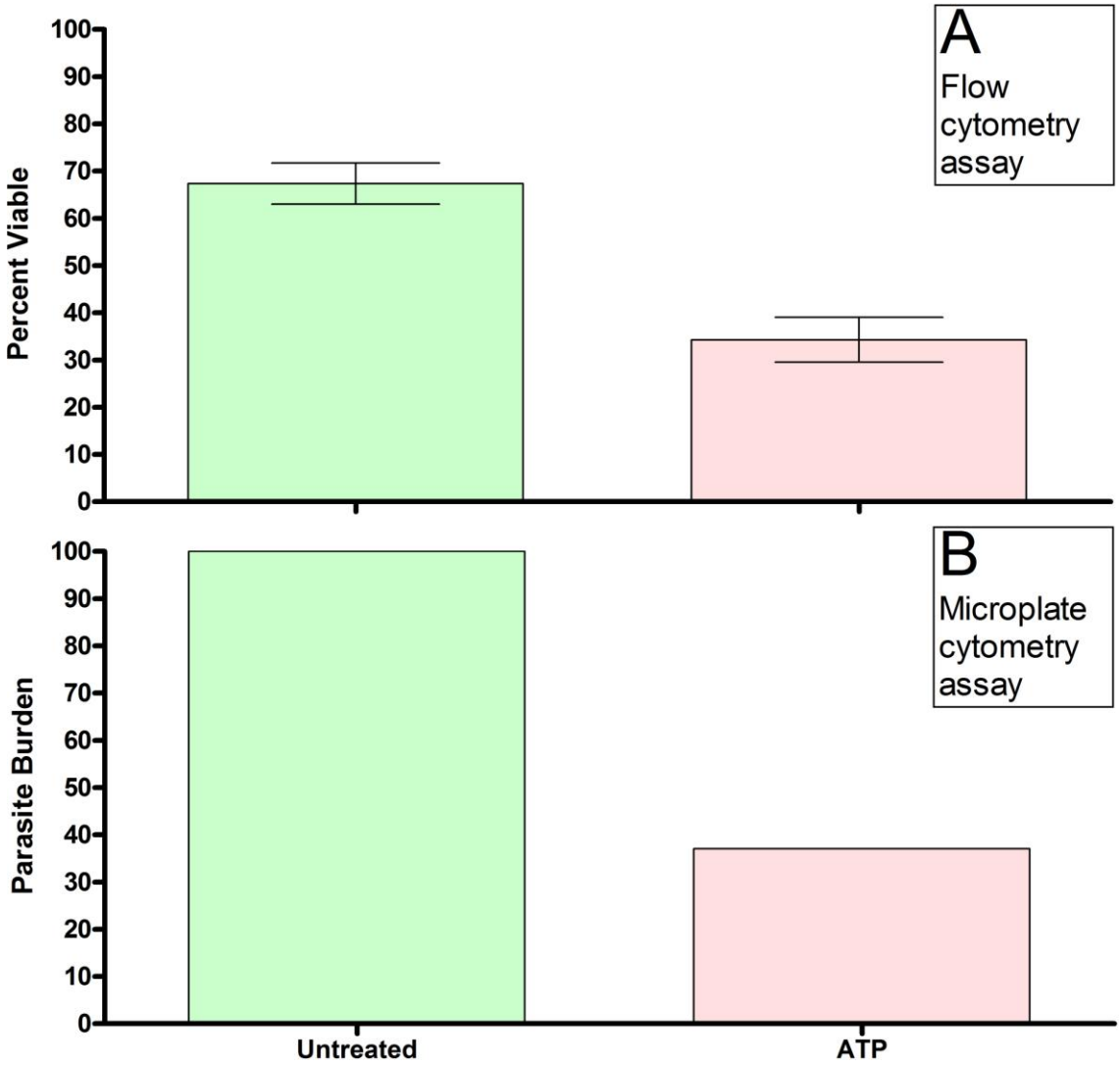

Figure 4.2 – RH *T. gondii* viability and burden following human $P2X_7$ receptor activation.

RH *T. gondii* or YFP RH *T. gondii* tachyzoites were infected into monocyte-derived macrophages cultured from a normal/control donor in 6-well (flow cytometry, n = 4; Results are the mean ± SEM. MOI = 10) or 384-well (microplate cytometry, n = 2. MOI = 2.5) plates. Following 3mM ATP treatment, viability (A) or parasite burden (B) was assessed 24 hours post ATP treatment. The result shows a significant decrease after ATP treatment in parasite viability measured by flow cytometry (A) ($P = 0.0021$) and parasite burden measured by microplate cytometry (B) ($P = 0.0002$) using a General Linear Model, 1-factor ANOVA with Tukey's post-hoc test. Additionally, there was no inter-assay difference with untreated ($P > 0.05$) or ATP-treated samples ($P > 0.05$) using a General Linear Model, 2-factor ANOVA with Tukey's post-hoc test, confirming that a reduction in parasite viability also corresponds to a reduction in parasite burden.

4.2.2 – RH *T. gondii* burden following polymorphic human $P2X_7$ receptor activation

The most common NCCCTS $P2X_7$ receptor genotypes had previously been determined using techniques and analysis methods which were also used for two other genes, COL2A1 and ABCA4 (Jamieson *et al.*, 2008). Patient samples exhibiting the three most common and experimentally relevant $P2X_7$ receptor genotypes in the NCCCTS were selected for further investigation. Experiments involving these samples aimed to investigate the phenotype following $P2X_7$ receptor activation of *T. gondii* infected monocyte-derived macrophages of varying $P2X_7$ receptor function. The following $P2X_7$ receptor genotypes were included:

1. Wild-type at the eight $P2X_7$ receptor positions genotyped (31% of cohort);
2. Homozygous polymorphism resulting in no changes at the amino acid level (non-loss of function SNPs) (14% of cohort); and
3. Homozygous 1513 A/C loss of function polymorphism (16% of cohort).

Section 4.2.1 showed that activation of a normal/control human $P2X_7$ receptor was capable of reducing the parasite viability and intracellular parasite burden of *T. gondii* infected monocyte-derived macrophages. Since both assays yielded similar results with the same treatment and cells isolated from the same donor, the microplate cytometry assay was employed in order to minimise the number of host cells required per assay with NCCCTS samples. This was of particular importance in experiments using samples from NCCCTS subjects since, as previously discussed, the number of CD14 positive monocytes available per NCCCTS subject was very limited.

Therefore, monocyte-derived macrophages were cultured from previously cryopreserved samples from NCCCTS subjects of $P2X_7$ receptor genotypes 1 – 3, infected with YFP expressing RH *T. gondii* and treated with ATP to activate $P2X_7$ receptors (as described for the normal/control donor in section 4.2.1). *Toxoplasma gondii* burden/replication was then quantified by the microplate cytometry assay as described in Chapter 3.

A reduction in parasite burden was observed following ATP treatment of monocyte-derived macrophages from NCCCTS $P2X_7$ receptor genotypes 1 (wild-type; Figure 4.3, Panels A – C) and 2 (non-loss of function polymorphism; Figure 4.3, Panels D – F). In comparison to the untreated control, ATP treatment caused a decrease in the number of YFP fluorescent RH *T. gondii* events recorded by the microplate cytometer. This was evident after three initial fluorescence readings prior to the addition of ATP (in order to establish a baseline comparison with the untreated control), with the number of YFP fluorescent events steadily decreasing thereafter.

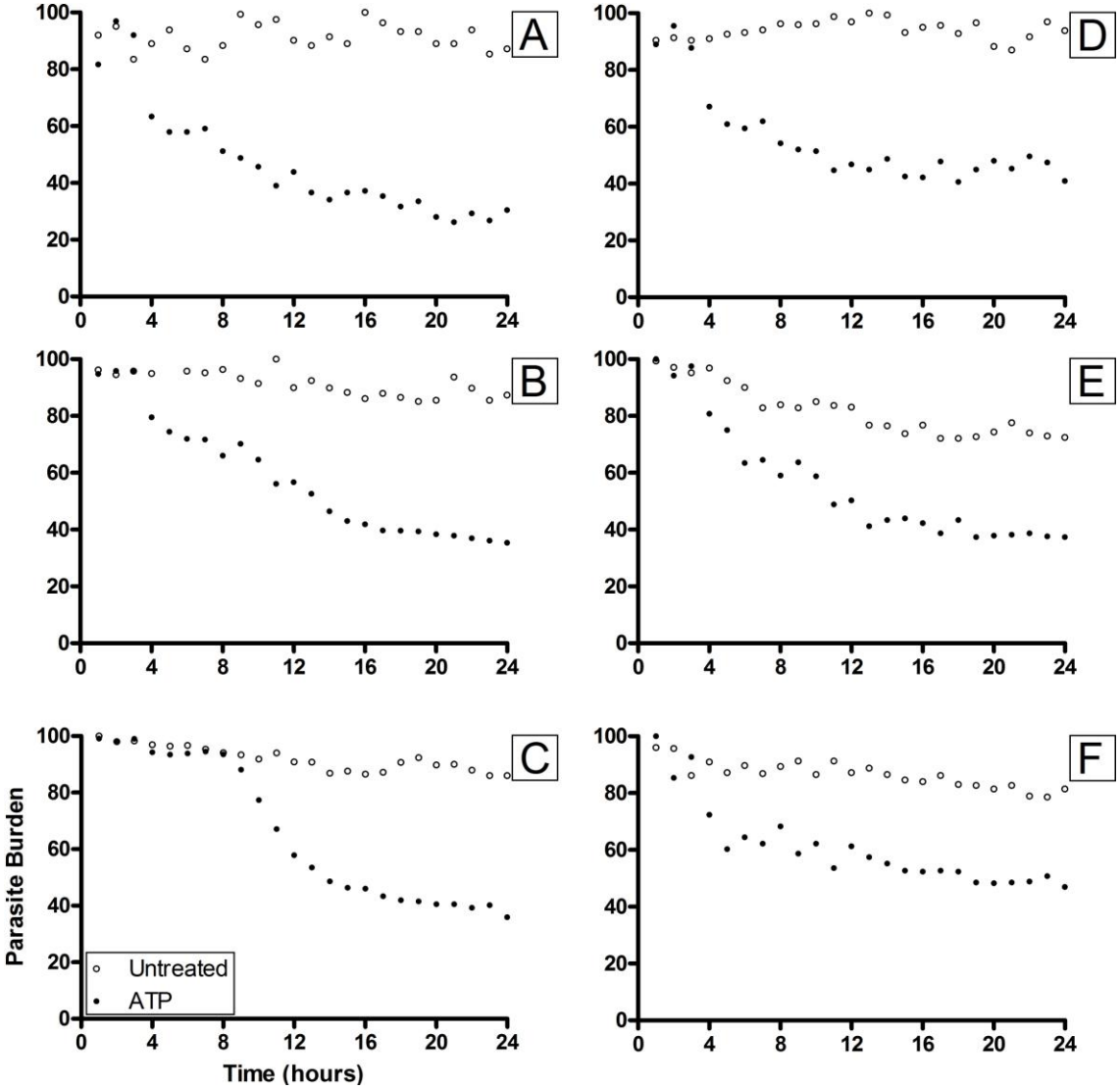

Figure 4.3 – RH *T. gondii* burden in ATP-treated cells with functional $P2X_7$ receptors.
Monocyte-derived macrophages from NCCCTS $P2X_7$ receptor genotypes 1 (wild-type; Panels A – C) and 2 (non-loss of function polymorphism; Panels D – F) were cultured for 4 days, infected with YFP expressing RH *T. gondii* and treated with 3mM ATP to activate $P2X_7$ receptors (MOI = 2.5). YFP RH *T. gondii* quantification was commenced prior to ATP treatment, with ATP addition immediately prior to the fourth measurement. The result shows a steady decrease in intracellular YFP RH *T. gondii* numbers after the addition of ATP.

Conversely, ATP treatment of monocyte-derived macrophages cultured from NCCCTS $P2X_7$ receptor genotype 3 (homozygous 1513C loss-of-function polymorphism) had minimal effect on the number of intracellular YFP RH *T. gondii* tachyzoites (Figure 4.4). Comparison of the untreated control with the ATP-treated condition showed no or minimal difference between these conditions, indicating that a fully functional $P2X_7$ receptor was required for ATP treatment to reduce intracellular parasite numbers.

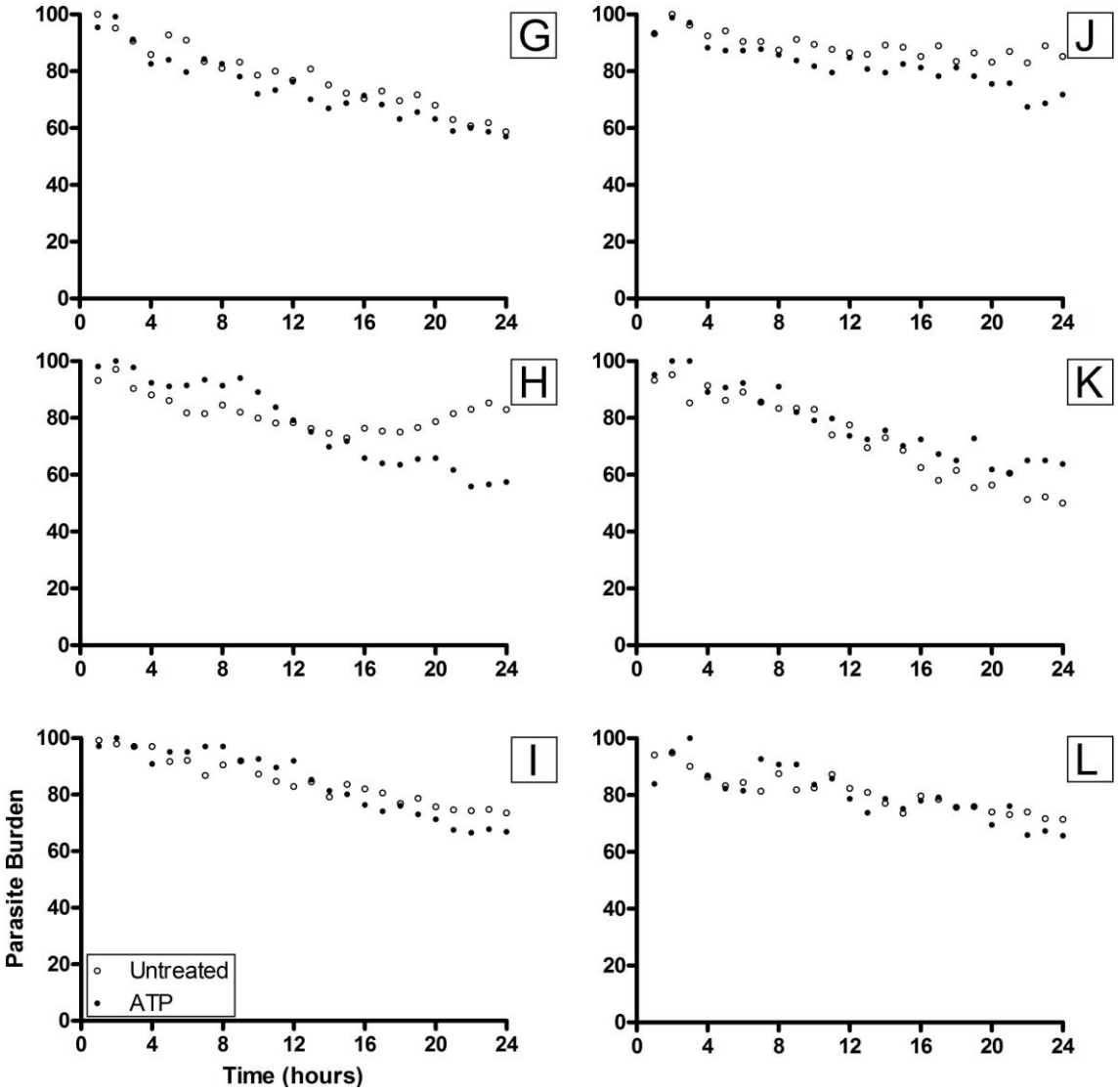

Figure 4.4 – RH *T. gondii* burden in ATP-treated cells with 1513C $P2X_7$ receptor polymorphism. Monocyte-derived macrophages from NCCCTS $P2X_7$ receptor genotype 3 (homozygous 1513C loss-of-function polymorphism; Panels G – L) were cultured for 4 days, infected with YFP expressing RH *T. gondii* and treated with 3mM ATP to activate $P2X_7$ receptors (MOI = 2.5). YFP RH *T. gondii* quantification was commenced prior to ATP treatment, with ATP addition immediately prior to the fourth measurement. The result shows no/minimal effect on intracellular YFP RH *T. gondii* numbers after the addition of ATP.

There was some variability in the effect of ATP treatment on some samples in Figure 4.4. For example, ATP treatment of monocyte derived macrophages from subject H seemed to result in a decrease in parasite numbers, however the sporadic trend seen in these results suggested that this result was not genuinely significant.

Results presented in this section have shown that a wild-type $P2X_7$ receptor is capable of reducing parasite burden following receptor activation, with the non-loss-of-function polymorphism also giving a similar result, as this polymorphism did not result in any amino acid change. In contrast, the presence of the 1513C loss of function polymorphism prevented parasite clearance following ATP treatment.

In order to compare these results, the percent reduction in parasite burden following ATP-treatment, was calculated for each subject for all three genotypes and plotted to allow for statistical analysis. This analysis confirmed that the 1513C polymorphism (3) resulted in significantly lower reduction in parasite burden at the 24 hour time point compared to wild-type (1) and non-loss-of-function polymorphism (2) following $P2X_7$ receptor agonist treatment, thus confirming that the result was statistically significant using a General Linear Mode, 1-factor ANOVA with Tukey's post-hoc test (Figure 4.5).

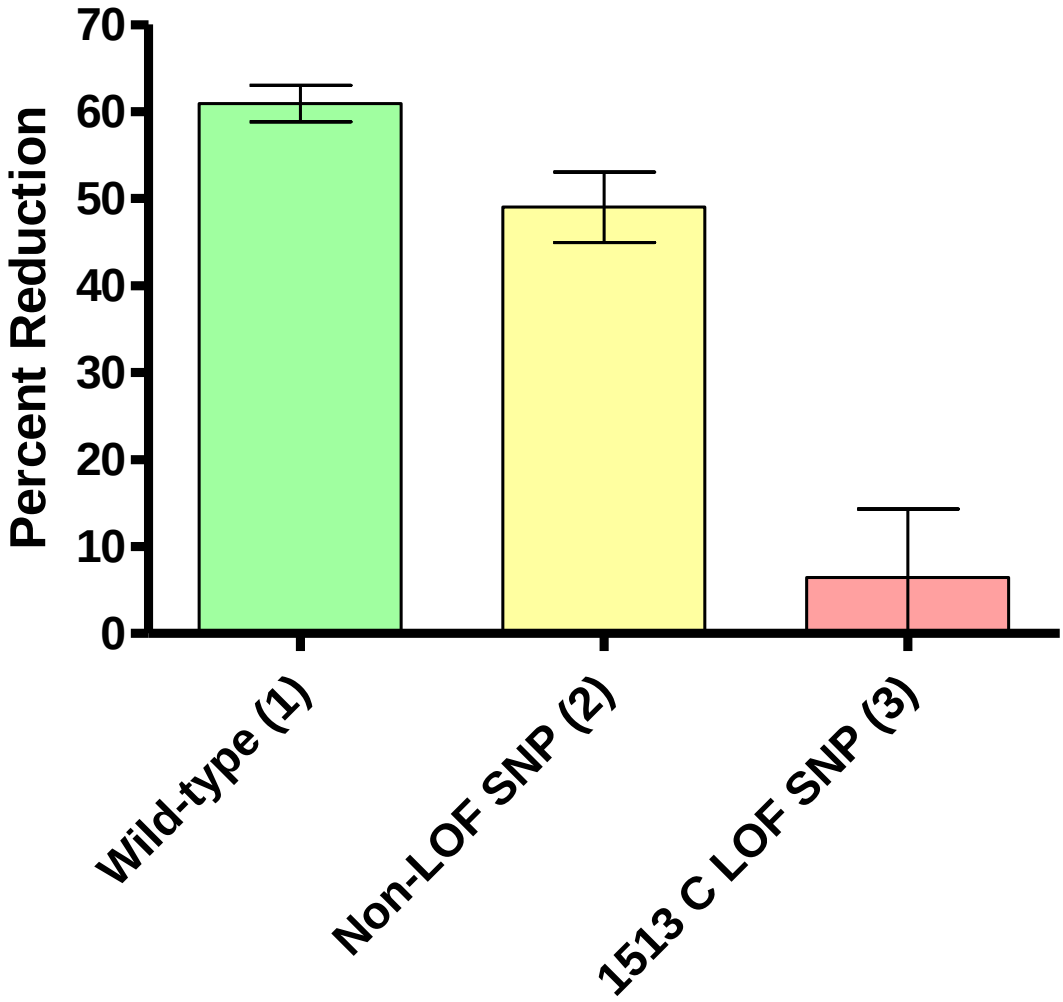

Figure 4.5 – Percent reduction of parasite burden by $P2X_7$ receptor genotypes 1 – 3.

This figure shows the percent reduction in parasite burden for NCCCTS $P2X_7$ receptor genotypes 1 – 3, 24 hours after ATP treatment (MOI = 2.5). This was performed by calculating reduction in ATP-treated fluorescence as a percentage of the untreated control for each subject. Subjects were grouped into respective genotypes. Genotypes 1 and 2, n = 3; Genotype 3, n = 6; Results are the mean ± SEM. Statistical analysis compared wild-type (genotype 1) with other genotypes (2 and 3) using a General Linear Model, 1-factor ANOVA with Tukey's post-hoc test. The results show a significant decrease in percent reduction of parasite burden (following ATP treatment) with cells possessing the 1513C loss-of function SNP (P = 0.0015).

4.3 – Discussion

The results presented in this chapter show, first, that human $P2X_7$ receptor activation is capable of killing intracellular RH *T. gondii* tachyzoites. Monocyte-derived macrophages were cultured from a normal/control donor. Following confirmation that this donor exhibited rapid ethidium uptake following ATP treatment (confirming a functional $P2X_7$ receptor), freshly cultured monocyte-derived macrophages were infected with RH *T. gondii* or YFP expressing RH *T. gondii*. The results from two separate assays showed that $P2X_7$ receptor activation of monocyte-macrophages from the normal/control donor had an effect on intracellular RH *T. gondii*. The flow cytometric assay demonstrated significant reduction in parasite viability, quantified through the direct measurement of parasite viability by sytox green exclusion/uptake by flow cytometry. In addition, monocyte-macrophages from the same donor were able to significantly reduce the number of intracellular parasites, quantified by the microplate cytometry assay, which directly quantified the number of intracellular parasites present in each sample. This result confirmed that human $P2X_7$ receptor activation significantly reduced the viability and number of intracellular RH *T. gondii* tachyzoites.

Furthermore, although the two assays operate on different principles, they both yielded similar results, thus strengthening the observation that ATP treatment is detrimental to the intracellular parasite. The similarity in results between assays also allowed for comparisons to be made between experiments in this chapter using human cells and, for experiments in following chapters, using murine cells.

Following the genotyping of all subjects in the NCCCTS at eight $P2X_7$ receptor loci, samples from subjects possessing the most common and experimentally relevant $P2X_7$ receptor genotypes were selected for further analysis. The $P2X_7$ receptor genotypes selected included: a wild-type gene, with all major alleles at the positions genotyped (1); non-loss-of-function polymorphism (2); and homozygous loss-of-function 1513C polymorphism, with complete lack of $P2X_7$ receptor function (3). Monocyte-macrophages cultured from cryopreserved samples obtained from subjects encompassing all three $P2X_7$ receptor genotypes were infected with YFP expressing RH *T. gondii* and parasite replication/burden monitored by the microplate cytometry method

at hourly intervals for 24 hours, with ATP addition immediately prior to the fourth measurement (in order to establish a baseline for comparison). The results show that macrophages cultured from subjects with a fully functional $P2X_7$ receptor (genotypes 1 and 2) were capable of reducing the number of intracellular parasites after the addition of ATP (Figure 4.3). Conversely, macrophages cultured from subjects with complete loss of $P2X_7$ receptor function due to the 1513C polymorphism (genotype 3), were unable to achieve this reduction (Figure 4.4). These samples showed significantly lower levels of parasite reduction post ATP treatment, in comparison to genotypes 1 and 2 at the 24 hour time point (Figure 4.5).

The microplate cytometry assay used in these experiments showed the ability of human monocyte-derived macrophages to reduce intracellular parasite burden following $P2X_7$ receptor activation. However, it must be emphasised that this assay only measures the number of fluorescent intracellular parasites, which is proportional to the level of fluorescence. It does not, however, give any indication as to whether the parasites become non-viable in these experiments. Therefore, this is one reason why experiments with NCCCTS samples were preceded with experiments that used monocyte-derived macrophages from a normal/control donor, with the flow cytometric *T. gondii* viability assay also confirming that a reduction in parasite burden by microplate cytometry directly correlates with reduced parasite viability.

It is already known that $P2X_7$ receptor activation plays an important role in the immune response to other intracellular pathogens. Experiments initially undertaken taken by Lammas *et al.*, (1997) and Smith *et al.*, (2001) to determine a role for the $P2X_7$ receptor in the immune response to *Mycobacterium* spp., showed $P2X_7$ receptor-mediated bacterial killing through decreased colony forming units (CFU) on agar growth plates with bacteria taken from $P2X_7$ receptor activated cells. Saunders *et al.*, (2003) extended this by showing a reduction in the number of CFU with bacteria taken from ATP-treated human cells exhibiting the loss-of-function 1513C $P2X_7$ receptor polymorphism, in comparison to cells from wild-type individuals. While Chaves *et al.*, (2009) showed decreased *L. amazonensis* infection in $P2X_7$ receptor activated cells *in vitro*, this study still only analysed the percent of infected cells at one time point through a single fluorescence measurement. There has therefore, still been no detailed investigation into the direct effect of $P2X_7$ receptor activation on intracellular *T. gondii*

burden/replication, or indeed, any other eukaryotic organism at all. Additionally, no study has assessed both the direct effect on the viability as well as a kinetic measurement of intracellular pathogen burden/replication in any organism.

The experiments presented in this chapter took a similar approach to those discussed for *Mycobacterium* spp., and *Leishmania* spp. However, this study utilised two novel assays to conduct a detailed investigation into the direct effect on parasite viability (through the flow cytometry viability assay) as well as a kinetic study of parasite burden in $P2X_7$ receptor activated human cells with fully functional and non-functional $P2X_7$ receptors (through the microplate cytometry burden assay).

While the experiments presented in this chapter provide a compelling argument that activation of the human $P2X_7$ receptor plays a role in the immune response to *T. gondii*, there are still limitations to the conclusions that may be drawn from this work. Since primary human cells isolated from whole blood were used in these experiments, there was a limit to the number of cells available per assay. This restricted the number of controls that were able to be incorporated into each experiment, with the inability to include controls such as $P2X_7$ receptor antagonists or a positive control for parasite death. In order to address this deficiency, similar experiments were conducted with primary and immortalised murine cells, incorporating these control treatments to strengthen the conclusions drawn from the experiments presented in this chapter.

Chapter 5 – *In vitro* murine $P2X_7$ receptor investigation

5.1 – Introduction

Chapter 4 investigated the role of human $P2X_7$ receptor activation in the immune response to *T. gondii*. While results from these experiments provided evidence that $P2X_7$ receptor activation was capable of killing and reducing the number of intracellular RH *T. gondii* tachyzoites, further investigation was required to definitively prove that ATP was acting through binding to the $P2X_7$ receptor and not some other purinergic receptor or alternative effect of extracellular ATP treatment. To address this requirement, an investigation was conducted using cells of murine origin to conduct a more in-depth investigation through the use of $P2X_7$ receptor agonist/antagonists and cells recovered from $P2X_7$ receptor knockout mice.

The $P2X_7$ receptor has a distinct agonist/antagonist profile that allows for reasonable differentiation from other purinergic receptors. Unlike the other P2X receptors, $P2X_7$ receptor activation requires ATP treatment at concentrations greater than 100μM, with BzATP being the most pure and effective form of the agonist available (North, 2002; Baraldi *et al.*, 2004). In addition, pre-treatment of cells for 1 – 2 hours with oxidised ATP (oATP) irreversibly prevents $P2X_7$ receptor activation (Surprenant *et al.*, 1996). Therefore, ATP and oATP were used with an immortalised mouse macrophage-like cell line to reinforce the findings in Chapter 4 and confirm the effect of $P2X_7$ receptor activation on intracellular RH *T. gondii* through the addition of the $P2X_7$ receptor antagonist, oATP.

To complement experiments with immortalised cells, bone marrow macrophages were cultured from mice with varying degrees of $P2X_7$ receptor function, to definitively confirm that ATP treatment acts through the $P2X_7$ receptor.

Therefore, the main aim for the experiments presented in this chapter was to confirm and extend the results obtained from human samples by:

1. Confirming $P2X_7$ receptor expression/function of RAW 264.7 cells by:
    a. Immunofluorescence microscopy;

b. Ethidium bromide uptake by flow cytometry.

2. Assessing the effect of the $P2X_7$ receptor agonist, ATP, and the $P2X_7$ receptor antagonist, oATP, on RH *T. gondii* tachyzoite viability in RAW 264.7 cells.
3. Confirming $P2X_7$ receptor function of murine bone marrow derived macrophages by:
    a. Ethidium bromide uptake by flow cytometry.
4. Assessing the effect of the $P2X_7$ receptor agonist, ATP, on RH *T. gondii* tachyzoite viability in bone marrow macrophages from mice with 100%, 50% and 0% $P2X_7$ receptor activity.

5.2 – Methods and results

5.2.1 – $P2X_7$ receptor investigation with RAW 264.7 cells

RAW 264.7 cells were assessed for $P2X_7$ receptor expression by immunostaining and fluorescence microscopy. RAW 264.7 cells were harvested (as described in Section 2.2.6), 5 x 10^4 cells were added per well of an 8-well chamber slide and incubated for 2 hours at 37°C/5% CO_2 to allow adherence to the surface of the slide.

Cells were fixed with pre-chilled methanol at -20°C for 15 minutes. Methanol was removed and the wells washed with PBS. Blocking to minimise non-specific binding of antibodies and permeabilisation of cells, was carried out using Microscopy Blocking Solution for 15 minutes.

Primary anti-$P2X_7$ receptor antibody was diluted 1:200 with PBS and applied to the appropriate wells for 2 hours, followed by ten washes with PBS to remove any unbound antibody. Secondary anti-rabbit-Alexa Fluor 488 conjugated antibody was diluted 1:10,000 in PBS, along with 1μg/mL DAPI and applied to the appropriate wells for 1 hour, followed by 10 washes with PBS to remove any unbound antibody and dye.

Following antibody incubations, the chamber was removed from the slide, 50μL 50% glycerol in PBS was added to each sample on the slide and a cover slip placed over the slide and sealed with nail polish. Slides were immediately examined on a Zeiss

Axioplan 2 fluorescence microscope. Phase contrast was used to locate RAW 264.7 cells, showing typical macrophage morphology. Fluorescence was then examined and photographed, with a z-stack of images taken through the plane of focus. Following acquisition, fluorescence deconvolution was conducted using Axiovision, version 4.5

Figure 5.1 shows a phase contrast and deconvolved fluorescence image of RAW 264.7 cells. Panel A shows RAW 264.7 cells, as visualised by phase contrast microscopy. After moving to fluorescence, a z-stack consisting of a consecutive series of fluorescence images was recorded through the plane of focus. This z-stack was deconvolved, with the middle fluorescence image of the deconvolved z-stack shown in panel B. Blue fluorescence from DAPI staining allowed localisation of nuclei, and green fluorescence showed that the anti-$P2X_7$ receptor antibody (followed by Alexa Fluor 488 secondary antibody) was binding to $P2X_7$ receptor protein expressed by RAW 264.7 cells. This shows therefore, that RAW 264.7 cells constitutively express $P2X_7$ receptors, with diffuse cytoplasmic staining and dense granular areas seen throughout the cytoplasm. In order to confirm specificity of the anti-$P2X_7$ receptor antibody, labelling was also conducted using only the Alexa Fluor 488 secondary antibody, resulting in no green fluorescence visible and a blank field of view under fluorescence (data not shown).

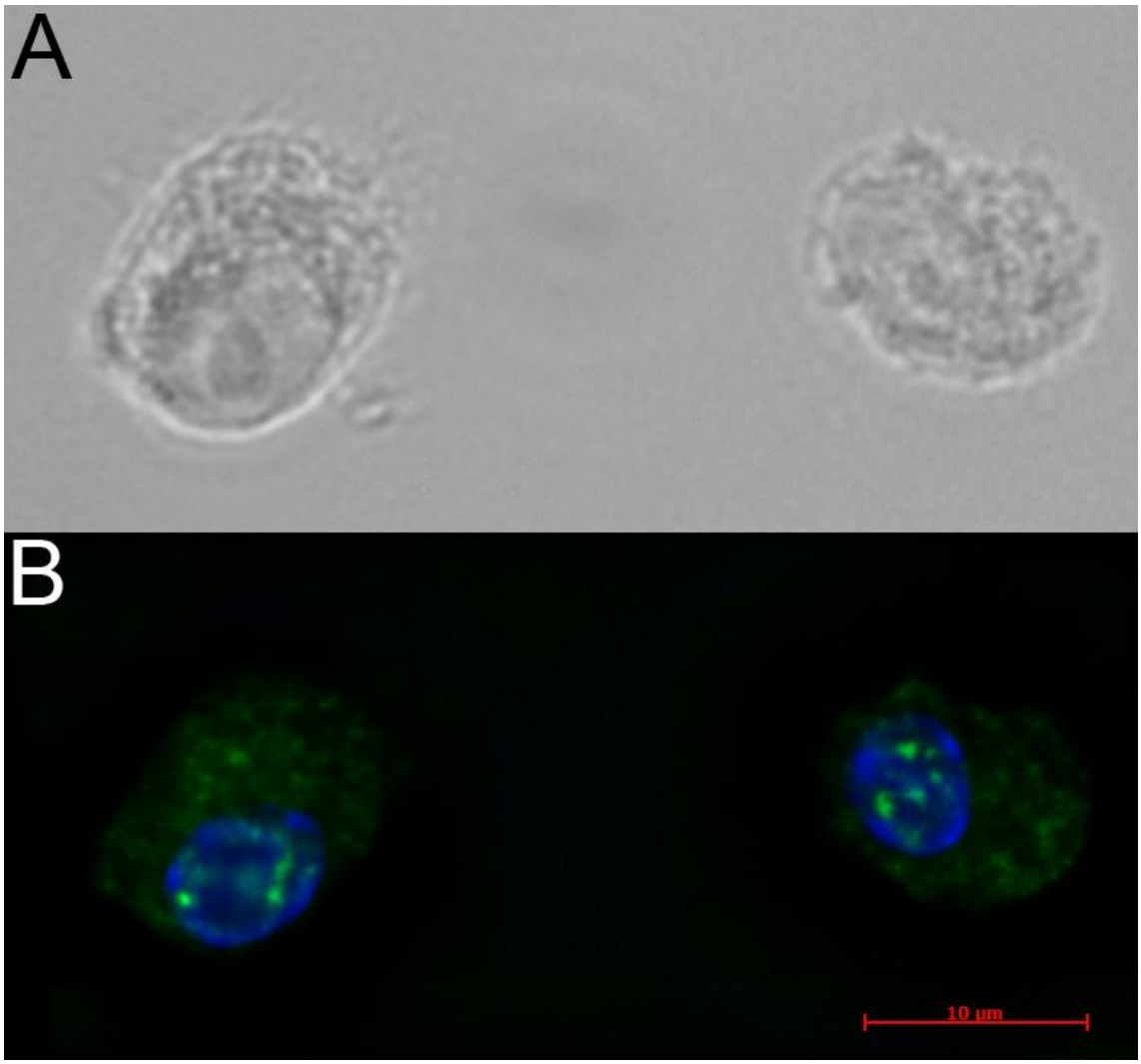

Figure 5.1 – RAW 264.7 cell $P2X_7$ receptor expression.

RAW 264.7 cells were cultured on chamber slides, stained with DAPI and anti-$P2X_7$ receptor antibody/Alexa Fluor 488 secondary antibody and examined under the 100x objective. Panel A shows the phase contrast image, with RAW 264.7 cells showing typical macrophage morphology. This region was chosen for fluorescence deconvolution with a z-stack of images taken through the plane of focus. Panel B shows the middle image of the deconvolved z-stack, allowing visualisation of nuclei (blue) and $P2X_7$ receptor expression (green).

Following confirmation of $P2X_7$ receptor expression by RAW 264.7 cells, the functional ability of the receptor was assessed by flow cytometry. This method assesses the ability of the $P2X_7$ receptor to form a large pore in response to prolonged application of extracellular ATP. The method involves time resolved measurement of ethidium bromide passage into $P2X_7$ receptor activated cells through the large pore that forms when the receptor is activated, as described in section 2.2.10. An untreated sample is used as a negative control.

Increased flux of ethidium across $P2X_7$ receptor generated pores in RAW 264.7 cells was observed following $P2X_7$ receptor activation with 1mM ATP (Figure 5.2). A similar effect was generated by treatment of cells with 300μM BzATP, a more selective $P2X_7$ receptor agonist (Figure 5.2) (North, 2002; Baraldi *et al.*, 2004). This confirms that RAW 264.7 cells express functional $P2X_7$ receptors.

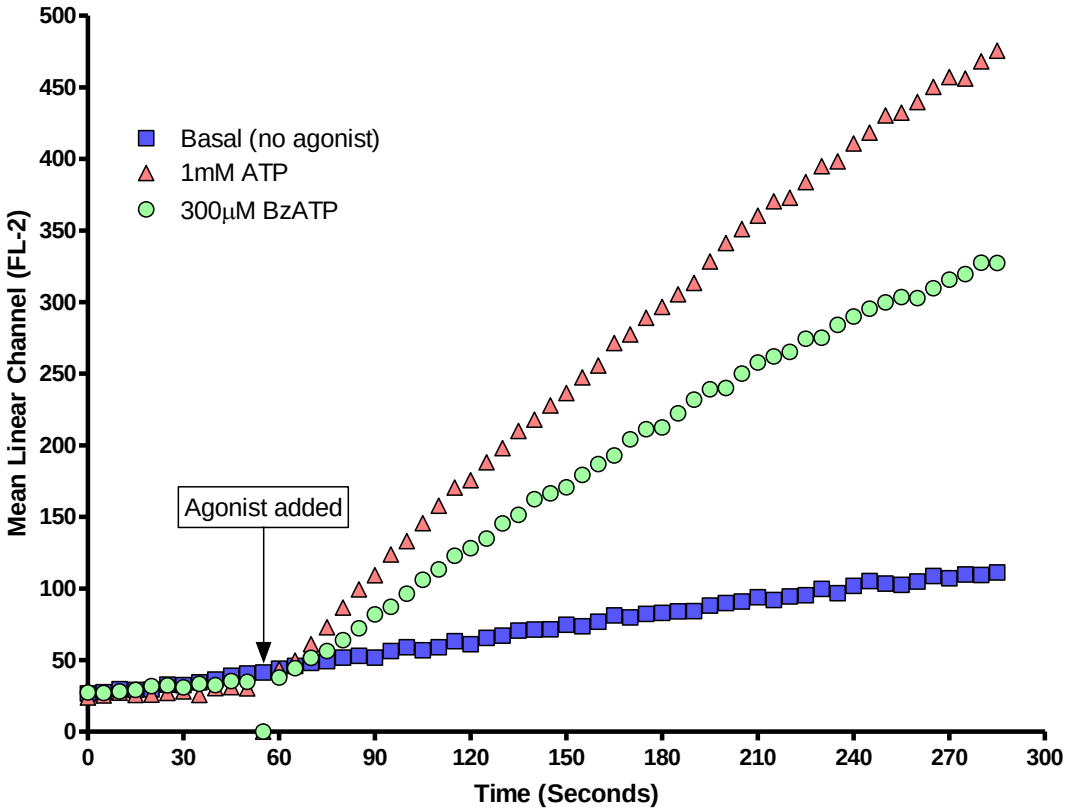

Figure 5.2 – $P2X_7$ receptor function of RAW 264.7 cells.

RAW 264.7 cell $P2X_7$ receptor function was assessed by quantifying ethidium flux through $P2X_7$ receptor generated pores following treatment with either 1mM ATP or 300μM BzATP at the 60 second time point. Ethidium fluorescence was quantified in 5 second intervals, with the result showing rapid uptake of the dye following agonist treatment. The y-axis shows ethidium fluorescence as mean linear channel on the FL-2 detector, with time course in seconds on the x-axis.

With confirmation that the mouse macrophage-like RAW 264.7 cell line expressed functional $P2X_7$ receptors, the effect of $P2X_7$ receptor activation on intracellular RH *T. gondii* viability was assessed. Parasite viability was quantified by flow cytometry 2, 8, 16 and 24 hours following $P2X_7$ receptor activation. Each time point consisted of the following controls:

- Untreated (negative control – no parasite death);
- ATP ($P2X_7$ receptor agonist);
- Oxidised ATP + ATP ($P2X_7$ receptor antagonist prevents activation by agonist); and
- SNP (positive control – nitric oxide donor induces parasite death).

RAW 264.7 cells were cultured and harvested (as described in section 2.2.6). 1×10^6 cells were added per well in 6-well plates and incubated for 2 hours to allow RAW 264.7 cells to adhere to the surface of the plastic prior to infection. A freshly cultured monolayer of RH *T. gondii* tachyzoites was prepared and quantified using a haemocytometer (as described in section 2.2.4). 3×10^6 tachyzoites were added to each well containing host cells and incubated overnight for 16 hours.

The following morning, oATP treatment was performed on appropriate samples. Spent culture media was removed and the monolayer washed with 3mL 1640 RPMI containing 2mM L-glutamine. Wash media was removed and 3mL fresh 1640 RPMI containing 2mM L-glutamine was added with 300μM oATP and incubated for 2 hours.

Following oATP incubation, extracellular tachyzoites were removed from all wells by aspirating the spent culture media and washing the wells with 3mL fresh Complete RAW Media. The wash media was removed and 3mL fresh Complete RAW Media added to each well, with 3mM ATP or 3mM SNP added to appropriate wells where appropriate.

Parasite viability was assessed by flow cytometry 2, 8, 16 and 24 hours after ATP or SNP addition (as described in Chapter 3). Tachyzoites were prepared for flow cytometry viability analysis by scraping each well with a 25cm cell scraper and forcing the cell suspension through a 27 gauge needle three times to lyse intact host cells.

500μL tachyzoite suspension was centrifuged at 1500 x g for 10 minutes, discarding the supernatant and resuspending the pellet in 97μL FSW.

No effect on parasite viability was seen at the earlier time points of 2 and 8 hours (Figure 5.3, Panels A and B). However, significant reduction in parasite viability was observed with ATP and SNP treatment 16 and 24 hours after their addition, with the effect of ATP being reversed by pre-treatment of cells with oATP (Figure 5.3, Panels C and D). Statistical analysis was performed using a General Linear Model 2-factor ANOVA with Tukey's post-hoc test.

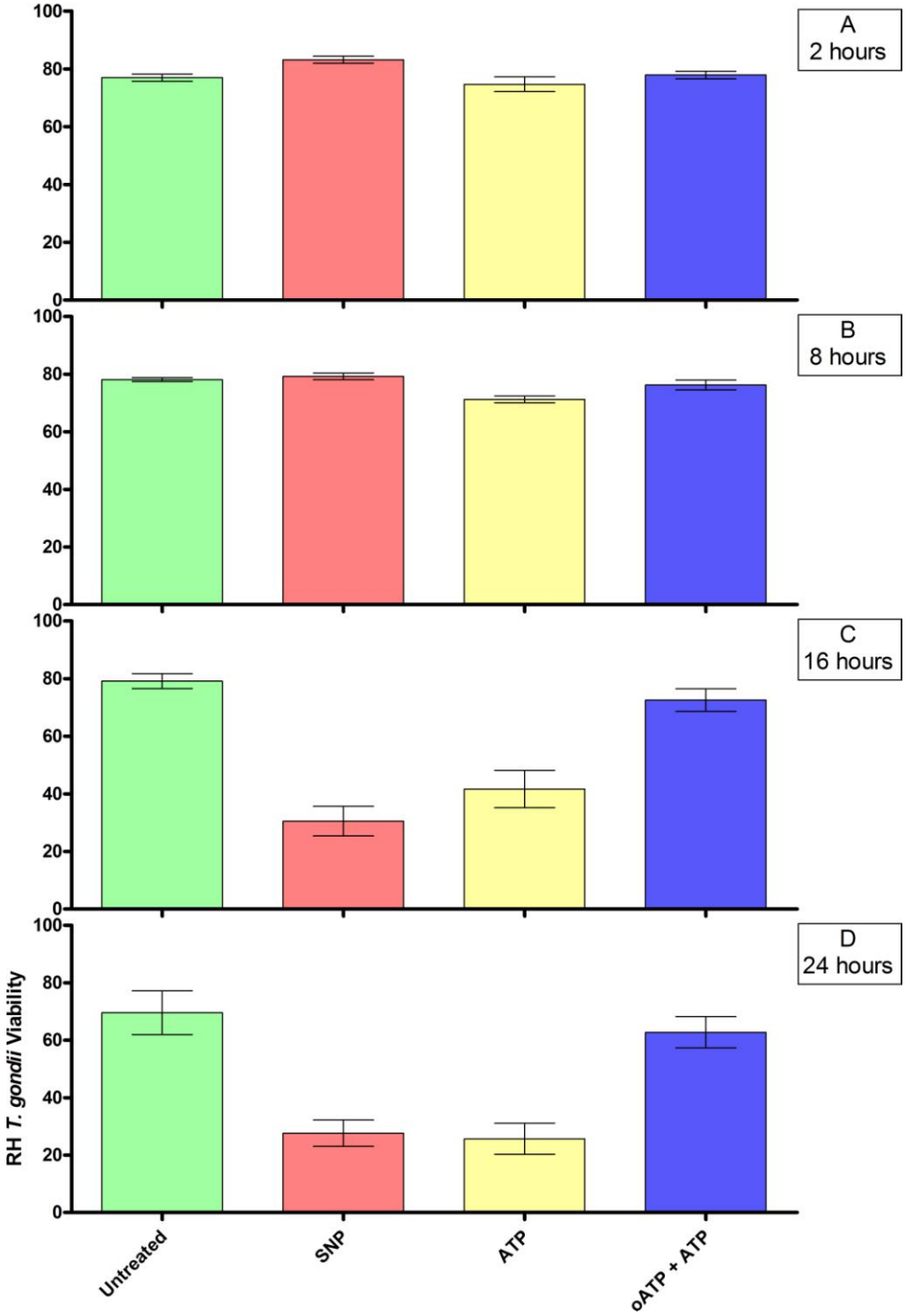

Figure 5.3 – RH *T. gondii* viability following $P2X_7$ receptor activation of RAW 264.7 cells.
1 x 10^6 RAW 264.7 cells in 6-well plates were infected with 3 x 10^6 RH *T. gondii* tachyzoites (MOI = 3). Parasite viability was assessed by flow cytometry at 2 (A), 8 (B), 16 (C) and 24 hours (D) after addition of either ATP or SNP. RH *T. gondii* viability was significantly reduced 16 hours after both ATP (P = 0.0000) and SNP (P = 0.0000) treatment and again 24 hours after ATP (P = 0.0000) and SNP (P = 0.0000) treatment, with the effect of ATP at 16 and 24 hours being reversed by pre-treatment of RAW 264.7 cells with oATP (P > 0.05). Results are the mean ± SEM; n = 4. Statistical analysis was performed using a General Linear Model 2-factor AVOVA with Tukey's post-hoc test, comparing the untreated control with experimental treatments at each time point.

5.2.2 – $P2X_7$ receptor investigation with murine BMM

RAW 264.7 cells demonstrated a striking ability to reduce the viability of intracellular RH *T. gondii* following ATP treatment. However, in order to reinforce this result and definitively confirm that ATP was acting through the $P2X_7$ receptor, RH *T. gondii* was infected into BMM cultured from three strains of mice with varying levels of $P2X_7$ receptor expression/function, in order to determine the effect of $P2X_7$ receptor activation on the viability of the intracellular parasite. The three strains of mice used in these experiments were:

1. BALB/c – possess a fully functional (wild-type) $P2X_7$ receptor;
2. C57BL/6J – possess a loss-of-function polymorphism in the $P2X_7$ receptor gene (Adriouch *et al.*, 2002) and thus exhibit approximately half $P2X_7$ receptor function compared to BALB/c mice; and
3. $P2X_7$ receptor knockout – lacking in $P2X_7$ receptor expression and function (on a C57BL/6J background).

$P2X_7$ receptor function of BMM derived from all three strains of mice was confirmed prior to assessing the effect of $P2X_7$ receptor activation on intracellular parasites. BMM were cultured in $80cm^2$ tissue culture flasks, as described in section 2.2.7. Cells were harvested using a 25cm cell scraper and assessed for $P2X_7$ receptor function as described in section 2.2.10.

Figure 5.4 shows the flux of ethidium across $P2X_7$ receptor mediated pores in murine BMM from the three different strains of mice with varying degrees of $P2X_7$ receptor function. In response to the addition of ATP, BMM from BALB/c mice showed the highest level of ethidium uptake. BMM derived from C57BL/6J mice showed approximately half that of BALB/c mice, while $P2X_7$ receptor knockout mouse BMM showed little or no ethidium uptake at all. ATP-stimulated ethidium flux measurements were made in comparison to a basal sample for each mouse strain, which was unstimulated.

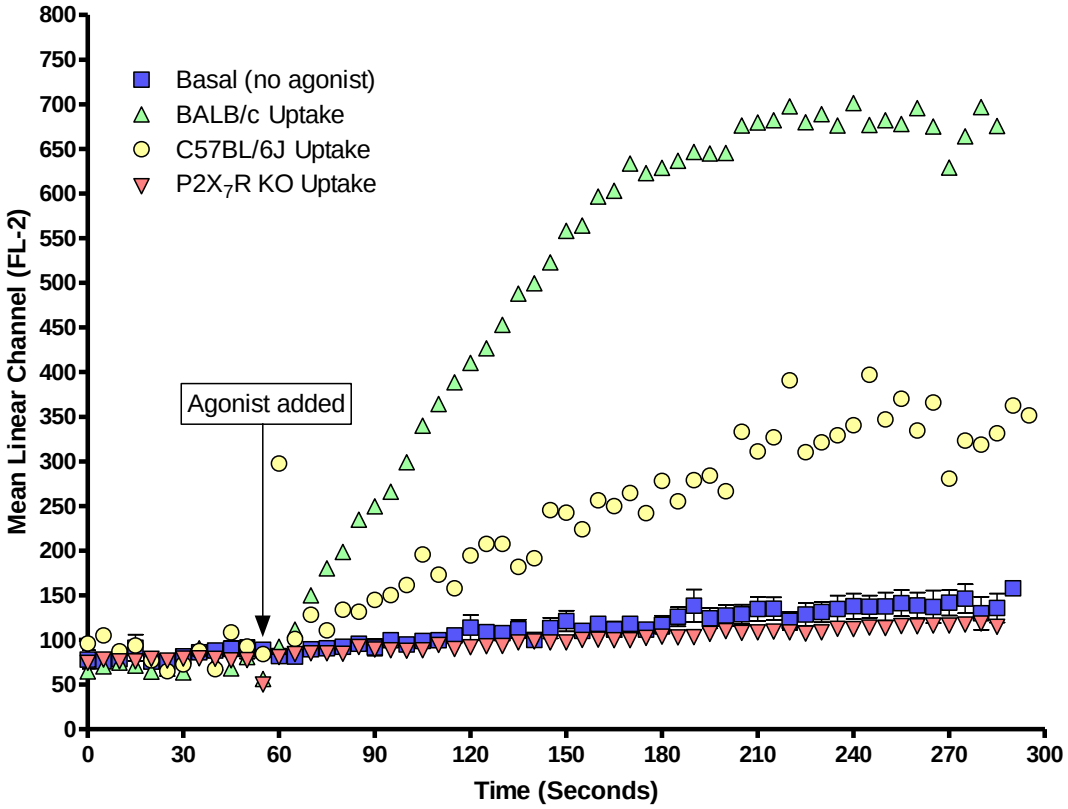

Figure 5.4 – $P2X_7$ receptor function of murine BMM.

Murine bone marrow cells were cultured for 4 days in the presence of GM-CSF to induce macrophage differentiation. BMM from BALB/c, C57BL/6J and $P2X_7$ receptor knockout mice were then assessed for $P2X_7$ receptor function by flow cytometry by quantifying ethidium flux through $P2X_7$ receptor generated pores following treatment with 1mM ATP at the 60 second time point. Ethidium fluorescence was quantified in 5 second intervals, with the result showing rapid uptake of the dye by BMM from BALB/c and C57BL/6J mice following agonist treatment. $P2X_7$ receptor knockout BMM exhibited very little ethidium flux after ATP treatment. Unstimulated (basal) ethidium uptake was recorded for each strain and pooled into one data set with error bars representing the mean ± SEM. The y-axis shows ethidium fluorescence as mean linear channel on the FL-2 detector, with time course in seconds on the x-axis. 1mM ATP was added at 55 seconds.

With confirmation that murine BMM from the three strains of mice exhibited the expected levels of $P2X_7$ receptor function, the effect of P2X receptor activation on intracellular RH *T. gondii* was assessed. Parasite viability was quantified by flow cytometry 24 hours following $P2X_7$ receptor activation. Each time point consisted of the following controls:

- Untreated (negative control – no parasite death); and
- ATP ($P2X_7$ receptor agonist).

BMM were cultured in 6-well plates as described in section 2.2.7. A freshly cultured monolayer of RH *T. gondii* tachyzoites was prepared and quantified using a haemocytometer (as described in section 2.2.4). 3 x 10^6 tachyzoites were added to each well containing host cells and incubated overnight for 16 hours.

Following overnight incubation, extracellular tachyzoites were removed from all wells by aspirating the spent culture media and washing the wells with 3mL fresh Complete BMM Media. The wash media was removed and 3mL fresh Complete BMM Media added to each well, with 3mM ATP added to appropriate wells where appropriate.

Parasite viability was assessed by flow cytometry 24 hours after ATP addition (as described in Chapter 3). Tachyzoites were prepared for flow cytometry viability analysis by scraping each well with a 25cm cell scraper and forcing the cell suspension through a 27 gauge needle three times to lyse intact host cells. 500μL tachyzoite suspension was centrifuged at 1500 x g for 10 minutes, discarding the supernatant and resuspending the pellet in 97μL FSW.

ATP treatment of BMM from BALB/c and C57BL/6J mice resulted in a significant reduction in parasite viability. This effect was not seen however, with $P2X_7$ receptor knockout mice (Figure 5.5). This result definitively confirms that ATP treatment and subsequent RH *T. gondii* killing acts through the $P2X_7$ receptor. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test.

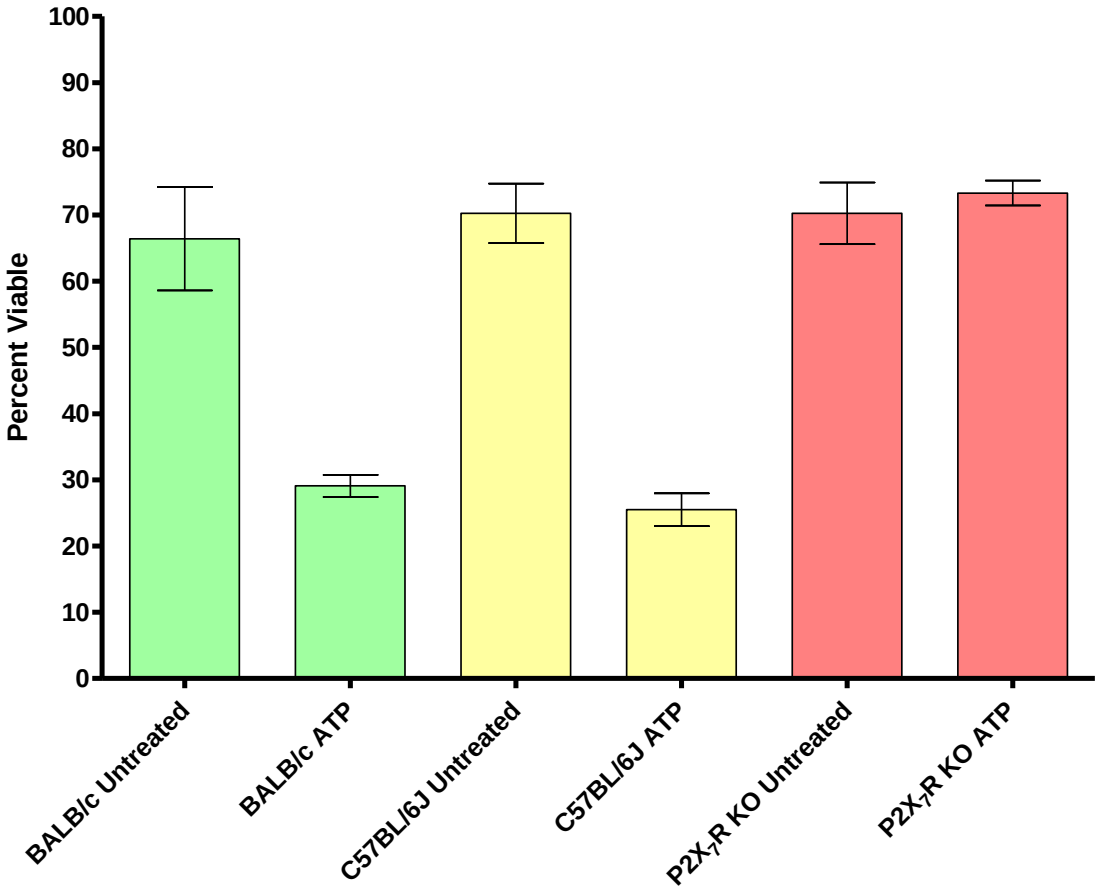

Figure 5.5 – RH *T. gondii* viability following $P2X_7$ receptor activation of murine BMM.
1 x 10^5 murine bone marrow cells were cultured in 6-well plates for 4 days in the presence of GM-CSF to induce macrophage differentiation. RH *T. gondii* tachyzoites (3 x 10^6) were added per well of BMM (MOI = 30). Parasite viability was assessed by flow cytometry 24 hours after addition of ATP. In comparison to the untreated control, RH *T. gondii* viability was significantly reduced in BMM isolated from BALB/c ($P = 0.0001$) and C57BL/6J ($P = 0.0000$) mice. BMM from $P2X_7$ receptor knockout mice were unable to reproduce this result ($P > 0.05$), confirming that ATP mediated killing of RH *T. gondii* was achieved through the $P2X_7$ receptor. Results are the mean ± SEM; n = 4. Statistical analysis was performed using a General Linear Model 1-factor AVOVA with Tukey's post-hoc test, comparing the untreated control with ATP treatment for each strain.

5.3 – Discussion

Activation of the $P2X_7$ receptor with extracellular ATP is known to induce a potent set of downstream events that may be toxic to intracellular pathogens such as *T. gondii*. Various studies have already revealed a role for $P2X_7$ receptor activation in the innate response to other macrophage dwelling pathogens, including *Mycobacterium* spp,

Chlamydia spp and *Leishmania* spp (discussed in sections 1.10 and 1.11). Experiments presented in Chapter 4 showed the ability of $P2X_7$ receptor activation to control *T. gondii*, through the use of cells isolated from human subjects with varying degrees of $P2X_7$ receptor function. The main aim for this chapter was to reinforce the findings from Chapter 4, using primary and immortalised murine cells to provide definitive proof that ATP treatment and the subsequent effect on intracellular RH *T. gondii* exerted its effect through the $P2X_7$ receptor.

Prior to *T. gondii* viability experiments being conducted, a suitable cell line was chosen based on $P2X_7$ receptor expression and function. The RAW 264.7 immortalised mouse macrophage-like cell line was created by infection of BALB/c mice with the Abelson murine leukaemia virus (Raschke *et al.*, 1978). It was thought that this would be an appropriate cell line for $P2X_7$ receptor studies, since the BALB/c mouse $P2X_7$ receptor is known to be fully functional, and does not exhibit any loss of function polymorphisms (Adriouch *et al.*, 2002). This hypothesis was confirmed through the use of immunofluorescence microscopy and flow cytometry to assess $P2X_7$ receptor expression and function of this cell line.

RAW 264.7 cells were shown to express $P2X_7$ receptor protein through the use of deconvolution immunofluorescence microscopy with a polyclonal antibody directed against a 20 amino acid epitope in the cytoplasmic C-terminal region. $P2X_7$ receptor expression in these cells was characterised by diffuse cytoplasmic staining with dense, granular areas throughout the cytoplasm (Figure 5.1). Immunofluorescence microscopy has been used previously to confirm $P2X_7$ receptor expression on various other cell types, including rat cortical astrocytes (Panenka *et al.*, 2001) and lymphocytes (Gu *et al.*, 2001). These investigations showed a similar staining pattern, with diffuse cytoplasmic and cell surface localisation as seen with RAW 264.7 cells in this chapter. With confirmation that RAW 264.7 cells expressed $P2X_7$ receptor protein, flow cytometry was used to confirm that the expressed protein was being trafficked to the cell surface and assembling as a functional receptor.

Flow cytometry can be employed to assess the functional ability of the $P2X_7$ receptor to form a large pore in response to agonist treatment. This assay takes advantage of the large pore formed by prolonged $P2X_7$ receptor activation, through quantification of

ethidium flux through this pore and subsequent increases in fluorescence caused by intercalation of the dye with DNA in the nucleus. This allows for a phenotype of $P2X_7$ receptor activation to be quantified in order to determine if the receptor is functional in any given cell type or sample. The results from such experiments conducted in this chapter confirmed $P2X_7$ receptor function of RAW 264.7 cells (isolated originally from a BALB/c mouse) and BMM cultured from BALB/c mice. These cells exhibited rapid ethidium flux following agonist treatment, reflecting the wild-type, fully functional $P2X_7$ receptor expressed by these mice. $P2X_7$ receptor function of BMM derived from BALB/c mice was compared with that of C57BL/6J BMM, which showed approximately half ethidium flux due to the P451L loss-of-function polymorphism (Adriouch *et al.*, 2002). As expected, $P2X_7$ receptor knockout mice showed negligible levels of ethidium uptake after ATP treatment, with very little increase in fluorescence compared with the basal (untreated) sample.

The flow cytometry $P2X_7$ receptor function assay has been used extensively in previous studies, with confirmation that various loss-of-function $P2X_7$ receptor polymorphisms result in decreased ethidium flux after agonist treatment in cell types including monocytes/monocyte-derived macrophages (Gu *et al.*, 2001; Wiley *et al.*, 2003), lymphocytes (Wiley *et al.*, 2002) and HEK-293 cells (Wiley *et al.*, 2003). The results seen in this chapter are similar to those seen in these studies, with cells exhibiting functional $P2X_7$ receptor genes responding to agonist treatment resulting in the rapid uptake of ethidium.

With confirmation that RAW 264.7 cells and BMM express functional $P2X_7$ receptors, the effect of ATP treatment on the viability of intracellular RH *T. gondii* was assessed by flow cytometry. In RAW 264.7 cell experiments, SNP was used as a positive control for NO induced *T. gondii* tachyzoite death and oATP as a $P2X_7$ receptor antagonist. SNP has previously been used as a direct inducer of *T. gondii* death in order to investigate parasite apoptotic death pathways induced by NO, with increasing SNP concentrations being associated with decreased parasite motility and survival (Peng *et al.*, 2003). Furthermore, Luder *et al.*, (2003) showed that the addition of SNP to *T. gondii* infected RAW 264.7 cells reduced parasite replication, in comparison to untreated *T. gondii* infected cells which showed decreased levels of NO upon infection

with the parasite. These findings were reflected in experiments in this chapter, with RH *T. gondii* viability being significantly reduced 16 and 24 hours after SNP addition.

Oxidised ATP inhibits $P2X_7$ receptor currents through irreversible binding to the agonist binding site. One or two hours pre-treatment before agonist application is sufficient to block the effect of ATP (Surprenant *et al.*, 1996). Various studies that have used oATP as an inhibitor of ATP-induced killing of pathogens such as *Mycobacterium* spp. and *Chlamydia* spp., have shown that the $P2X_7$ receptor was mediating this effect (Lammas *et al.*, 1997; Coutinho-Silva *et al.*, 2001).

With RH *T. gondii* infected RAW 264.7 cells, both SNP and ATP treatment induced a significant reduction in the viability of the intracellular parasite. The effect of ATP treatment was reversed through pre-treatment of RAW 264.7 cells with oATP prior to ATP addition (Figure 5.3). This result indicates that ATP acts through the $P2X_7$ receptor, inducing similar levels of parasite death to those seen with a known inducer of parasite death. The use of oATP further strengthens the conclusion that ATP-induced killing of RH *T. gondii* occurs through $P2X_7$ receptor activation. This is a strong indicator of the mechanism of parasite death, however oATP is also known to inhibit $P2X_1$ and $P2X_2$ receptor activation (Evans *et al.*, 1995). Even though $P2X_1$ and $P2X_2$ receptors are not thought to be involved in the immune response, antagonism of these receptors by oATP instils some uncertainty over the mechanism of ATP-induced parasite killing. Taking into account the non-specific nature of P2X receptor antagonism by oATP, further experiments aimed at definitively confirming the role of the $P2X_7$ receptor in ATP induced RH *T. gondii* killing were conducted.

Bone marrow-derived macrophages cultured from three different strains of mice (each with varying levels of $P2X_7$ receptor expression and function) were infected with RH *T. gondii*. BALB/c mice exhibit a fully functional, wild-type $P2X_7$ receptor gene; C57BL/6J mice exhibit the P451L loss-of-function polymorphism resulting in half function; and $P2X_7$ receptor knockout mice exhibit zero $P2X_7$ receptor expression/function (back-crossed to the C57BL/6J strain) (Adriouch *et al.*, 2002). Parasite viability was assessed by flow cytometry 24 hours after ATP addition to RH *T. gondii* infected BMM. Significant parasite killing was observed following ATP treatment of BALB/c and C57BL/6J BMM, but not with $P2X_7$ receptor knockout BMM

(Figure 5.5). This shows that RH *T. gondii* killing by ATP treatment can be attributed to the $P2X_7$ receptor. The MOI in this experiment was 30 (30 parasites per host cell), however this number would not be the MOI at the time of infection, as the original number of 1×10^5 cells added per well would have increased as the cells divided throughout the culturing process. One limitation of this work lies in the inability to discern the source of extracellular ATP for $P2X_7$ receptor activation *in vivo* and whether the typical sources of ATP would provide concentrations of ATP high enough for $P2X_7$ receptor activation.

One issue raised by this experiment however, is that the level of ATP induced parasite killing was the same with BALB/c and C57BL/6J macrophages, despite the C57BL/6J mice exhibiting approximately half $P2X_7$ receptor function (Figure 5.4). It might be expected that RH *T. gondii* killing via $P2X_7$ receptor mechanisms would be lower in C57BL/6J mice, than in BALB/c mice with full $P2X_7$ receptor function. This was not observed, however, with BALB/c and C57BL/6J mice exhibiting no difference in ATP induced RH *T. gondii* killing. It is possible that the parasite killing mechanism of $P2X_7$ receptor activation does not require a fully functional receptor, and that the partially functional C57BL/6J $P2X_7$ receptor is still capable of sufficiently inducing the downstream events responsible for parasite killing. This is a plausible explanation, since the P541L polymorphism in the C57BL/6J mouse $P2X_7$ receptor reduces sensitivity to ATP, but does not alter receptor expression or some downstream events after activation of the $P2X_7$ receptor, such as PLD activation (Adriouch *et al.*, 2002; Le Stunff *et al.*, 2004). Therefore, a threshold level of $P2X_7$ receptor activation in C57BL/6J BMM may be enough to affect *T. gondii in vitro*. Additionally, it may be evident that lower concentrations of ATP used in this killing assay would produce differences in the level of parasite killing seen between BALB/c and C57BL/6J mice. Such a dose response curve has not yet been conducted.

Therefore, the experiments presented in this chapter show that murine cells expressing a functional $P2X_7$ receptor are capable of significantly reducing the viability of intracellular RH *T. gondii* following ATP treatment. The mechanism of ATP induced parasite death was initially linked with the $P2X_7$ receptor, since oATP pre-treatment inhibited ATP induced parasite killing by RAW 264.7 cells. Further experiments definitively linked the $P2X_7$ receptor with ATP induced parasite killing, since BMM

isolated from $P2X_7$ receptor knockout mice were unable to reproduce the parasite killing seen in the parental C57BL/6J strain. With this confirmation, further experiments were conducted aimed at investigating the role of the $P2X_7$ receptor in RH *T. gondii* infected mice.

Chapter 6 – *In vivo* murine $P2X_7$ receptor investigation

6.1 – Introduction

Experimental results presented in Chapter 5 showed the *in vitro* effect of murine $P2X_7$ receptor activation on intracellular RH *T. gondii*. The use of $P2X_7$ receptor antagonists and cells from $P2X_7$ receptor knockout mice showed that activation of the $P2X_7$ receptor significantly reduces the viability of intracellular parasites. The next step was to determine what effect the lack of parasite killing via $P2X_7$ receptor dependent mechanisms would have on the progress of infection and the immune response *in vivo*.

Therefore, the aim for the experiments presented in this chapter was to elucidate any difference between mice with varying degrees of $P2X_7$ receptor function following infection with RH *T. gondii* by:

1. Assessing the effect of $P2X_7$ receptor deficiency on parasite burden *in vivo*; and
2. Assessing inflammatory cytokine and mediator responses in $P2X_7$ receptor deficient mice *in vivo*.

6.2 – Methods and results

6.2.1 – Murine infection with RH *T. gondii* – parasite burden

Male BALB/c, C57BL/6J and $P2X_7$ receptor knockout mice (6 – 8 weeks old) were infected with RH *T. gondii* for parasite burden and inflammatory cytokine analysis. RH *T. gondii* tachyzoites were cultured as described in Section 2.2.4. Culture flasks were scraped with a 25cm cell scraper, forced twice through a 25 gauge needle to lyse any intact host cells and filtered using a 3μm polycarbonate filter. The filtered tachyzoite suspension was centrifuged at 1500 x g for 10 minutes and resuspended in 0.9% NaCl in order to obtain a tachyzoite suspension of 5000 tachyzoites per mL, with the use of a haemocytometer to quantify the parasite suspension and dilutions carried out using 0.9% NaCl as needed.

Mice were restrained by hand and infected with 500 RH *T. gondii* tachyzoites by intra-peritoneal injection using a 1mL syringe and 26-gauge needle, injecting 100μL of the previously prepared tachyzoite suspension per mouse. Following infection, mice were monitored daily for signs of an adverse reaction, and for symptoms of acute toxoplasmosis (such as ruffled fur, hunched posture and failure to eat/drink) as specified by UTS ACEC protocol number 2008-30.

Two separate infection experiments were performed. For each infection experiment, ten mice of each strain (BALB/c, C57BL/6J and $P2X_7$ receptor knockout) were infected with RH *T. gondii* and six mice of each strain were injected with 100μL 0.9% NaCl only. Four and eight days post infection, five infected and three control mice were anaesthetised (2:1 nitrous oxide:oxygen, 2% isofluorane) and terminally bled using a 1mL syringe and 26-gauge needle, taking care not to rupture the peritoneal cavity. Blood was transferred to 1.5mL tubes and centrifuged at 13000 rpm for 10 minutes. Serum was transferred to sterile 1.5mL tubes and stored at -20°C for subsequent cytokine analysis by cytometric bead array (CBA) and ELISA.

Following cardiac puncture, mice were euthanised by cervical dislocation and peritoneal lavage performed by injecting 3mL 0.9% NaCl into the peritoneal cavity using a 5mL syringe and 21 gauge needle. The lavage fluid was then removed using the same needle/syringe and *T. gondii* tachyzoites quantified using a haemocytometer. The number of parasites recovered from the peritoneal cavity of BALB/c mice on day 8 was significantly higher than the number recovered from C57BL/6J and $P2X_7$ receptor knockout mice (Figure 6.1). There was no significant difference observed in parasite burden between C57BL/6J and $P2X_7$ receptor knockout mice, with statistical analysis performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test (Figure 6.1).

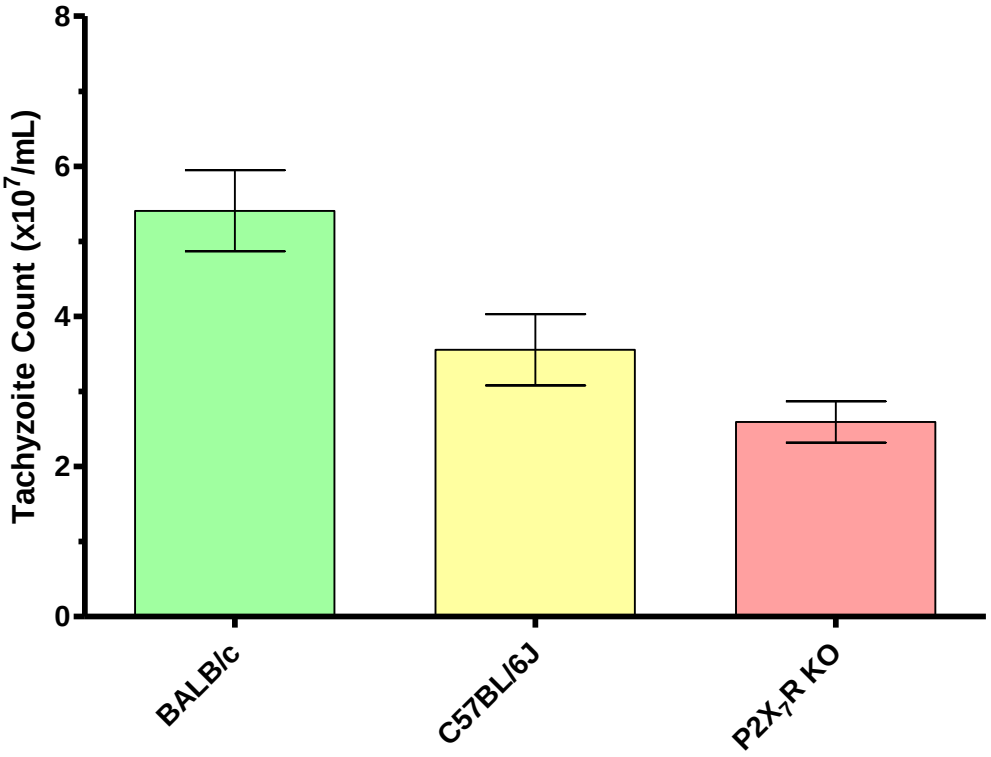

Figure 6.1 – Parasite burden at site of infection in RH *T. gondii* infected mice.
Mice were infected by intra-peritoneal injection with 500 RH *T. gondii* tachyzoites and euthanised 8 days post infection. Peritoneal lavage was performed, with parasites quantified in peritoneal lavage fluid using a haemocytometer. Results are the mean ± SEM; BALB/c, n = 16; C57BL/6J, n = 18; $P2X_7$ receptor knockout, n = 10. Compared to BALB/c mice, the result shows a significant reduction in parasite burden of C57BL/6J (P = 0.0179) and $P2X_7$ receptor knockout mice (P = 0.0018). Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test.

6.2.2 – Murine infection with RH *T. gondii* – inflammatory cytokine analysis

Inflammatory cytokines were quantified in mouse serum samples obtained from RH *T. gondii* infections using the Cytometric Bead Array (CBA) Mouse Inflammation Kit according to the manufacturer's instructions (Becton Dickinson, USA). This method allowed for the simultaneous detection and quantification of IL-6, IL-10, MCP-1, IFN-γ, TNF and IL12p70 in a single 50µL serum sample. In this method, antibodies directed against individual cytokines are immobilised on capture beads with distinct fluorescence intensities for each cytokine. When combined with the sample, the capture bead/antibody binds to the cytokine, which is then detected/quantified with an additional antibody conjugated to a PE-detection reagent, with the fluorescence then recorded by flow cytometry.

Prior to beginning, FACS tubes were prepared by labelling with experimental sample identifier or standard dilution. Forty-eight tubes per infection were required for experimental samples and ten tubes for standards.

Standards for all cytokines were prepared in a single 15mL tube, by resuspending the lyophilised spheres (each sphere containing a standardised amount of recombinant mouse protein) in 2mL assay diluent and incubating for 15 minutes at room temperature. Eight serial 1:2 dilutions were then performed using assay diluent, by transferring 300μL resuspended standards to a new tube containing 300μL assay diluent. This process was repeated until the top standard had been diluted 1:256.

Ten microlitres of each individually bottled capture bead (each directed against an individual antibody) was required per sample/standard, therefore sufficient quantities were mixed together in one 15mL tube. With ten standards plus 48 experimental samples (per infection) making a total of 58 samples, 600μL of each capture bead was added to a 15mL tube (in order to have a small amount of extra pooled capture beads to account for pipetting error and inconsistency). Mixed capture beads (50μL) were then added to all previously labelled FACS tubes.

Undiluted experimental serum samples (50μL) and previously prepared standards were then added to each appropriate FACS tubes, followed by 50μL PE detection reagent and incubated, protected from light, at room temperature for 2 hours. Following incubation, 1mL wash buffer was added to all tubes followed by centrifugation at 200 x g for 5 minutes. Supernatants were carefully removed and discarded, resuspending the bead pellet in 300μL wash buffer prior to data acquisition on a BD LSR II flow cytometer.

During the 2 hour incubation, cytometer setup beads were prepared and voltage/compensation settings adjusted on the flow cytometer. Cytometer setup beads (50μL) were added to three FACS tubes labelled A, B and C. FITC beads (50μL) were added to tube B and 50μL PE beads added to tube C. All tubes were then incubated, protected from light at room temperature for 30 minutes. Following incubation, 450μL wash buffer was added to tube A and 400μL added to tubes B and C. Voltage and

compensation settings were then adjusted on the LSR II flow cytometer according to the instructions supplied with the kit, using setup beads A, B and C. FSC, SSC and fluorescence detectors were set to log mode with a SSC threshold of 650. Fluorescence was recorded on the APC (capture beads) and PE (detection antibody) detectors, with compensation carried out on the FITC, and PerCP detectors.

Following data acquisition, standard curves and cytokine quantification were carried out using FCAP Array, version 1.0.1. Inflammatory cytokine analysis was carried out using mouse serum samples from the same experiments determining parasite burden in RH *T. gondii* infected mice. Inflammatory cytokine analysis by CBA included quantification of IL-12p70, IFN-γ, MCP-1, IL-6, TNF and IL-10. IL-1β was quantified later by ELISA. There was no detectable cytokine response in any mice at the day 4 time point, so only day 8 time point data are presented.

Figure 6.2 shows the serum concentration of IL-12p70 in control and RH *T. gondii* infected mice 8 days post infection, quantified using CBA. Serum concentration of IL-12p70 in control and infected mice of each strain was either non-variant/zero or negligible. Statistical analysis was therefore not performed as it was concluded that the levels of IL-12p70 were attributable to background levels of detection.

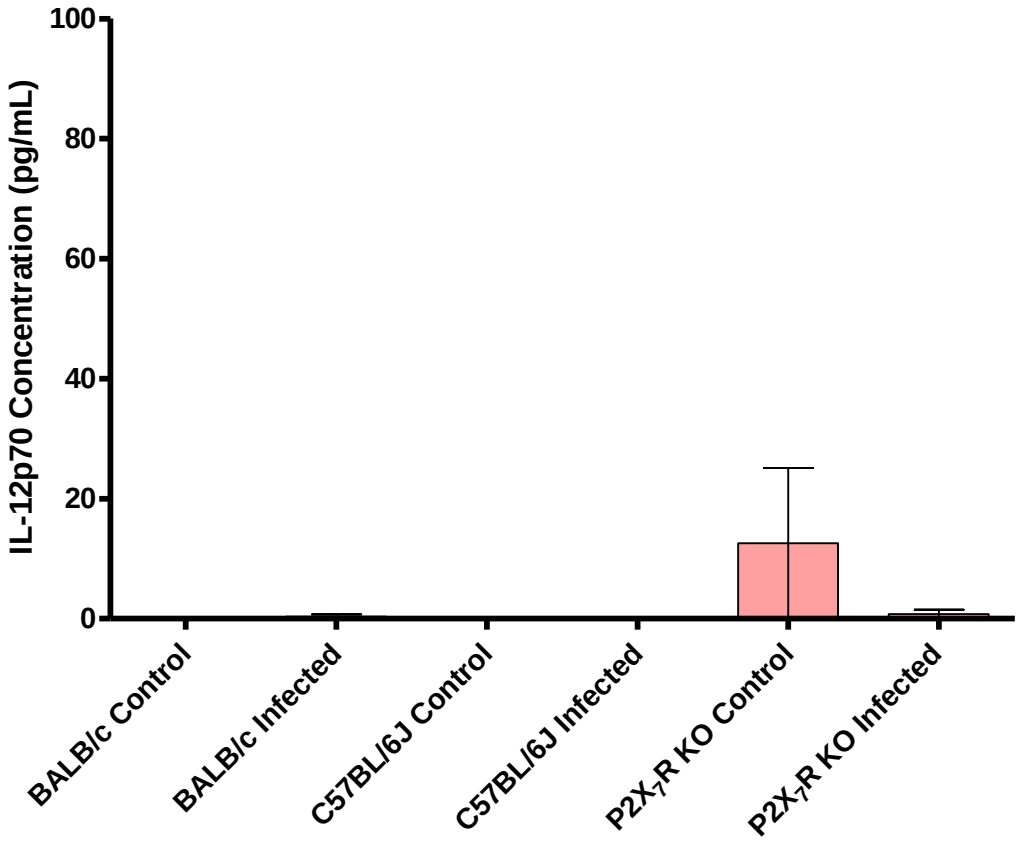

Figure 6.2 – Serum IL-12p70 concentration in RH *T. gondii* infected mice.

Mice were infected by intra-peritoneal injection with 500 RH T. gondii tachyzoites and euthanised 8 days post infection. Serum was collected for IL-12p70 quantification by CBA. Serum from control mice was also quantified, with the result showing no difference in IL-12p70 concentration between control and infected mice of each strain. Results are the mean ± SEM; BALB/c control/infected, n = 8/12; C57BL/6J control/infected, n = 8/12; $P2X_7$ receptor knockout control/infected, n = 8/9. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

Figure 6.3 shows the serum concentration of IFN-γ in control and RH *T. gondii* infected mice 8 days post infection, quantified using CBA. Levels of IFN-γ in serum of control mice were either non-variant/zero or negligible. When comparing infected mice of each strain, $P2X_7$ receptor knockout mice showed significantly lower levels of serum IFN-γ in comparison to the parental C57BL/6J strain and BALB/c strain. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

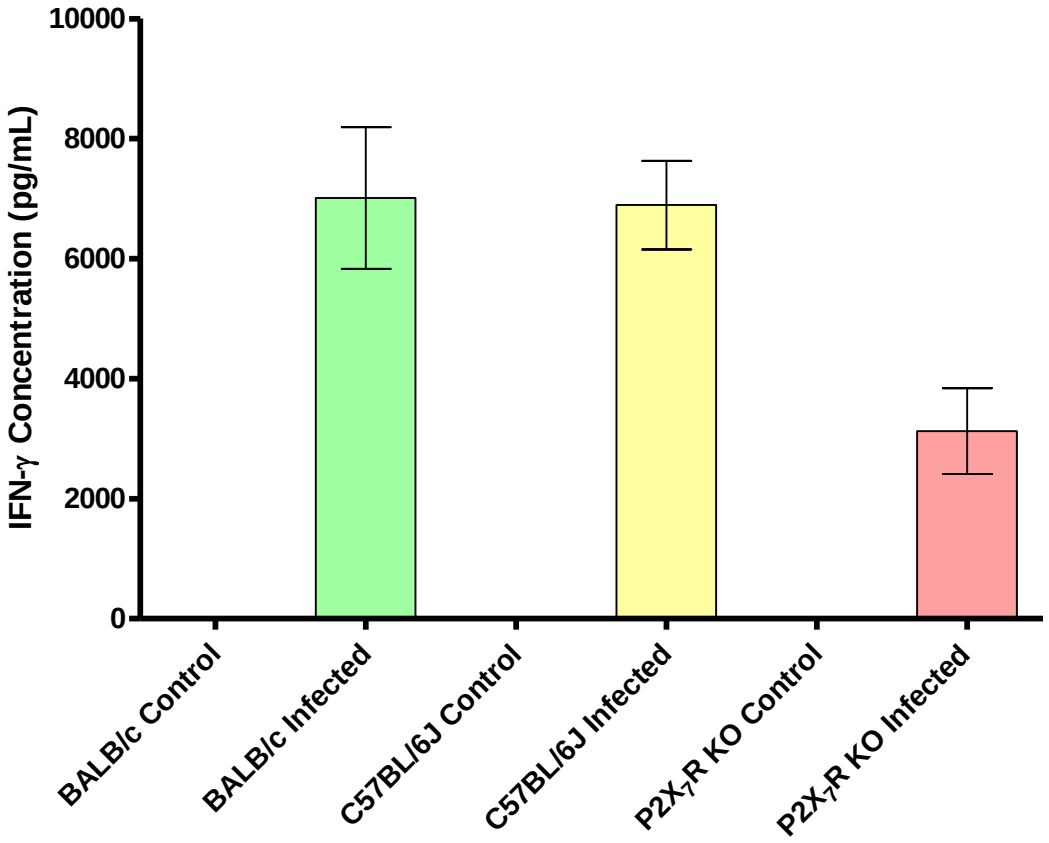

Figure 6.3 – Serum IFN-γ concentration in RH *T. gondii* infected mice.

Mice were infected by intra-peritoneal injection with 500 RH *T. gondii* tachyzoites and euthanised 8 days post infection. Serum was collected for IFN-γ quantification by CBA. Serum from control mice was also quantified, showing little/no IFN-γ in comparison to infected mice of each strain. The result shows RH *T. gondii* infected $P2X_7$ receptor knockout mice exhibit significantly decreased levels of serum IFN-γ in comparison to the parental C57BL/6J strain (P = 0.0274) and BALB/c mice (P = 0.0224). Results are the mean ± SEM; BALB/c control/infected, n = 8/12; C57BL/6J control/infected, n = 8/12; $P2X_7$ receptor knockout control/infected, n = 8/9. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

Figure 6.4 shows the serum concentration of MCP-1 in control and RH *T. gondii* infected mice 8 days post infection, quantified using CBA. Levels of MCP-1 in serum of control mice were either non-variant/zero or negligible. When comparing infected mice of each strain, $P2X_7$ receptor knockout mice showed significantly lower levels of serum MCP-1 in comparison to the parental C57BL/6J strain. BALB/c mice also showed a significant reduction in serum MCP-1 in comparison to C57BL/6J mice. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

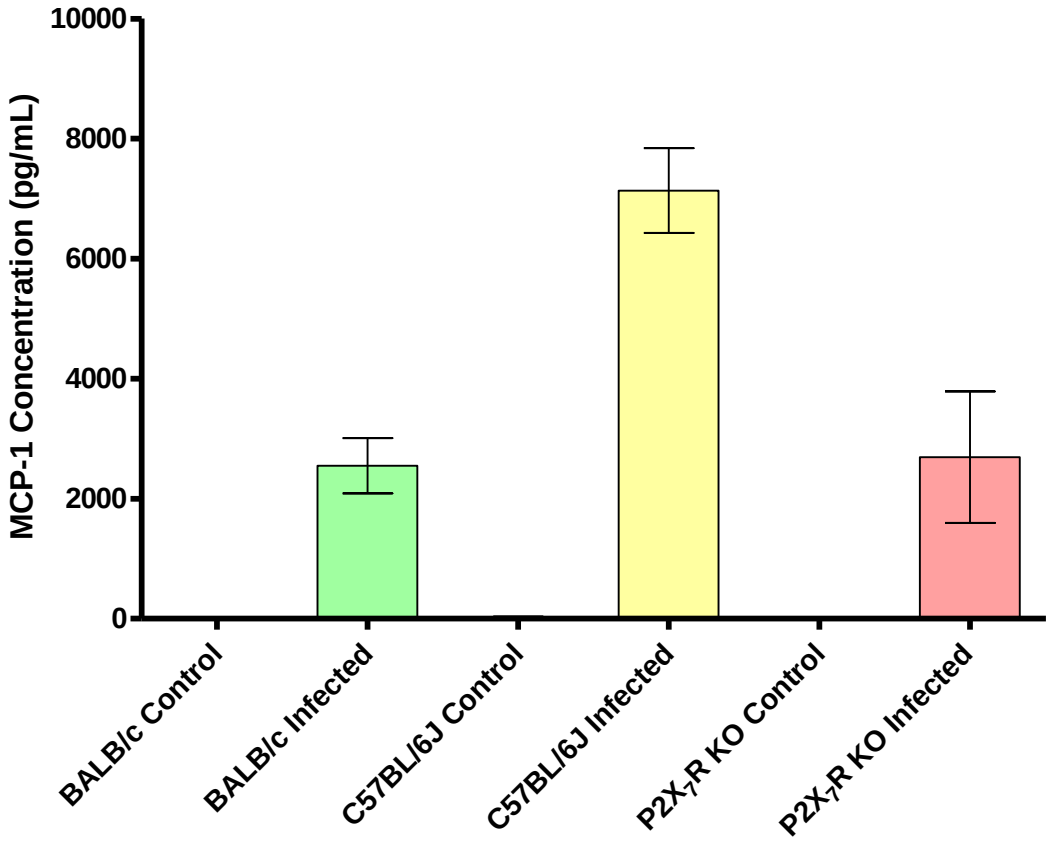

Figure 6.4 – Serum MCP-1 concentration in RH *T. gondii* infected mice.

Mice were infected by intra-peritoneal injection with 500 RH T. gondii tachyzoites and euthanised 8 days post infection. Serum was collected for MCP-1 quantification by CBA. Serum from control mice was also quantified, showing little/no MCP-1 in comparison to infected mice of each strain. The result shows significantly higher MCP-1 in RH *T. gondii* infected C57BL/6J mice in comparison to BALB/c ($P = 0.0002$) and $P2X_7$ receptor knockout mice ($P = 0.0008$). Results are the mean ± SEM; BALB/c control/infected, n = 8/12; C57BL/6J control/infected, n = 8/12; $P2X_7$ receptor knockout control/infected, n = 8/9. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

Figure 6.5 shows the serum concentration of IL-6 in control and RH *T. gondii* infected mice 8 days post infection, quantified using CBA. Levels of IL-6 in serum of control mice were either non-variant/zero or negligible. When comparing infected mice of each strain, $P2X_7$ receptor knockout mice showed what appeared to be lower levels of serum IL-6 in comparison to the parental C57BL/6J strain and BALB/c strain. This difference, however, was not statistically significant. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

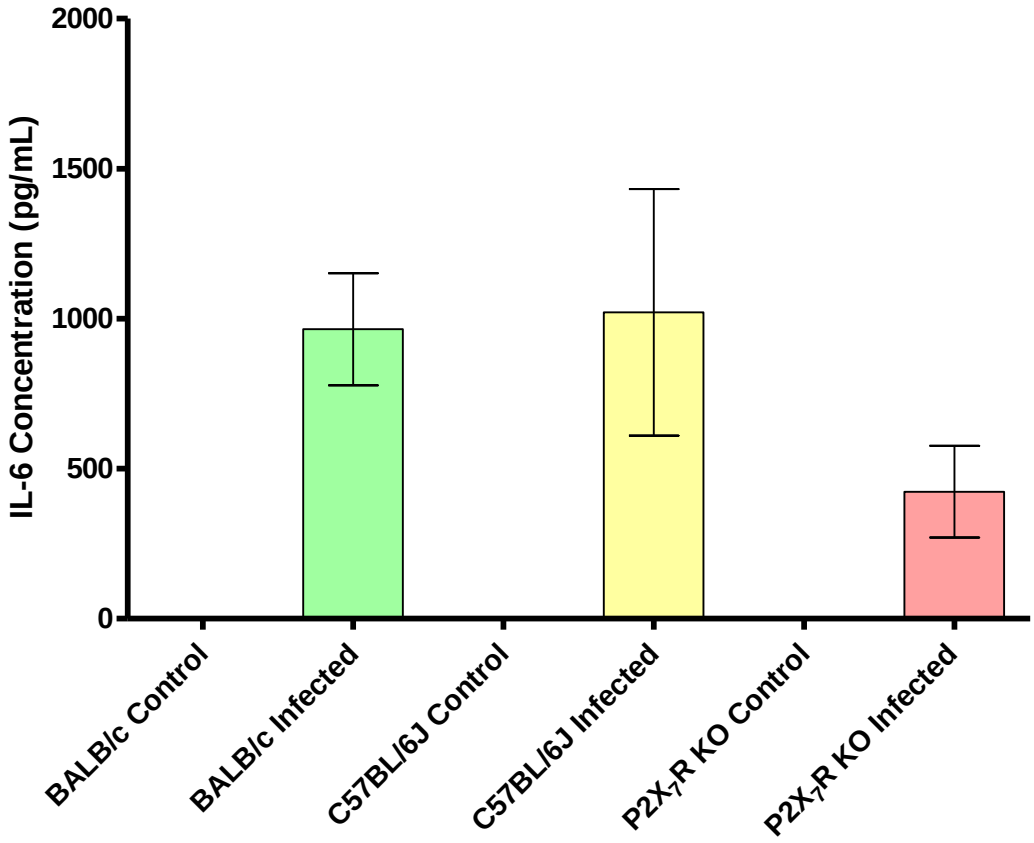

Figure 6.5 – Serum IL-6 concentration in RH *T. gondii* infected mice.

Mice were infected by intra-peritoneal injection with 500 RH *T. gondii* tachyzoites and euthanised 8 days post infection. Serum was collected for IL-6 quantification by CBA. Serum from control mice was also quantified, showing little/no IL-6 in comparison to infected mice of each strain. The result shows RH *T. gondii* infected $P2X_7$ receptor knockout mice exhibit decreased levels of serum IL-6 in comparison to the parental C57BL/6J strain and BALB/c mice. This result however, was not statistically significant ($P > 0.05$). Results are the mean ± SEM; BALB/c control/infected, n = 8/12; C57BL/6J control/infected, n = 8/12; $P2X_7$ receptor knockout control/infected, n = 8/9. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

Figure 6.6 shows the serum concentration of TNF in control and RH *T. gondii* infected mice 8 days post infection, quantified using CBA. Levels of TNF in serum of control mice were either non-variant/zero or negligible. When comparing infected mice of each strain, $P2X_7$ receptor knockout mice showed significantly lower levels of serum TNF in comparison to the parental C57BL/6J strain. BALB/c mice also showed a significant reduction in serum TNF in comparison to C57BL/6J mice similar to the $P2X_7$ receptor knockout strain. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

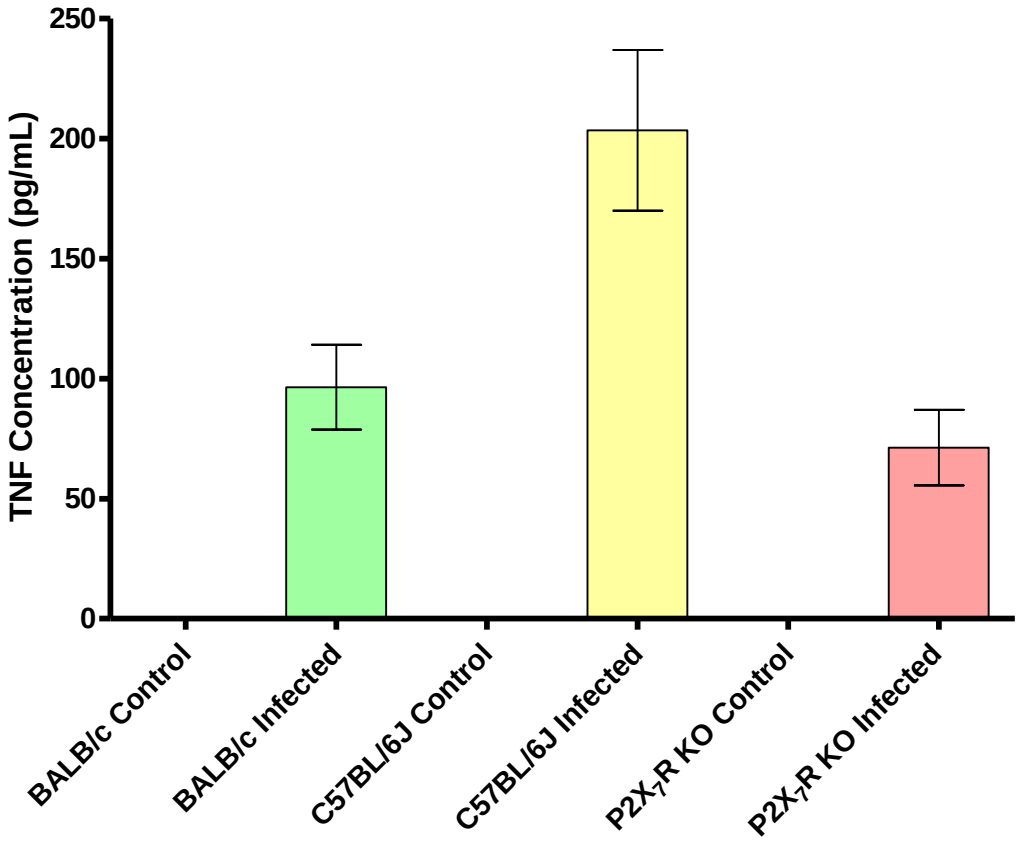

Figure 6.6 – Serum TNF concentration in RH *T. gondii* infected mice.

Mice were infected by intra-peritoneal injection with 500 RH T. gondii tachyzoites and euthanised 8 days post infection. Serum was collected for TNF quantification by CBA. Serum from control mice was also quantified, showing little/no TNF in comparison to infected mice of each strain. The result shows significantly higher TNF in RH T. gondii infected C57BL/6J mice in comparison to BALB/c (P = 0.0100) and $P2X_7$ receptor knockout mice (P = 0.0031). Results are the mean ± SEM; BALB/c control/infected, n = 8/12; C57BL/6J control/infected, n = 8/12; $P2X_7$ receptor knockout control/infected, n = 8/9. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

Figure 6.7 shows the serum concentration of IL-10 in control and RH *T. gondii* infected mice 8 days post infection, quantified using CBA. Levels of IL-10 in serum of control mice were either non-variant/zero or negligible. When comparing infected mice of each strain, $P2X_7$ receptor knockout mice showed what appeared to be lower levels of serum IL-10 in comparison to the parental C57BL/6J strain and BALB/c strain. This difference, however, was not statistically significant. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

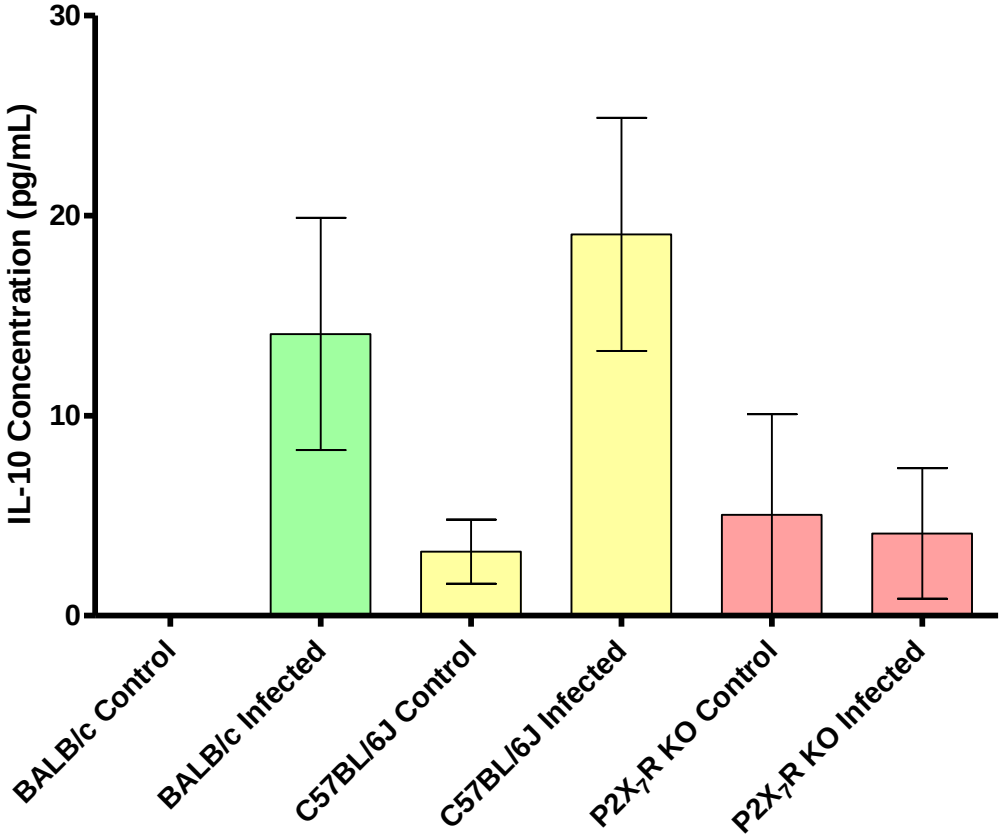

Figure 6.7 – Serum IL-10 concentration in RH *T. gondii* infected mice.

Mice were infected by intra-peritoneal injection with 500 RH *T. gondii* tachyzoites and euthanised 8 days post infection. Serum was collected for IL-10 quantification by CBA. Serum from control mice was also quantified, showing little/no IL-10 in comparison to infected mice of each strain. The result shows RH *T. gondii* infected $P2X_7$ receptor knockout mice exhibit decreased levels of serum IL-10 in comparison to the parental C57BL/6J strain and BALB/c mice. This result however, was not statistically significant ($P > 0.05$). Results are the mean ± SEM; BALB/c control/infected, n = 8/12; C57BL/6J control/infected, n = 8/12; $P2X_7$ receptor knockout control/infected, n = 8/9. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

IL-1β was quantified in the remaining mouse serum samples obtained from one RH *T. gondii* infection using the OptEIA Mouse IL-1β ELISA Set. Ninety-six-well plates were coated with 100µL per well of capture antibody diluted 1:250 in coating buffer, and incubated overnight at 4°C. Following incubation, coating antibody was aspirated and all wells washed three times with ≥ 300µL wash buffer. All wells were then blocked by incubating with 200µL assay diluent consisting of PBS with 10% FBS, for 1 hour at room temperature. Following blocking, assay diluent was removed and all wells washed three times with wash buffer.

Experimental serum samples were prepared by diluting 50μL serum 1:2 with 50μL assay diluent. Lyophilised standard was prepared by resuspending with 1mL ddH_2O and equilibrating at room temperature for 15 minutes. The reconstituted standard was diluted by combining 358μL standard with 242mL assay diluent to obtain a top standard of 2000 pg/mL. Six 1:2 serial dilutions were then performed by transferring 300μL to a sterile tube containing 300μL assay diluent to eventually obtain a low standard of 31.3 pg/mL.

Each standard or diluted experimental serum sample (50μL) was added to duplicate wells on the 96-well plate and incubated at room temperature for 2 hours. Following incubation, standards/experimental samples were aspirated and all wells washed five times with wash buffer. Detection antibody (100μL) diluted 1:500 in assay diluent, was then added to all wells and incubated for 1 hour at room temperature. Detection antibody was then aspirated and all wells washed five times with wash buffer.

Enzyme reagent, Extravidin alkaline-phosphatase, was diluted 1:3000 and 100μL added to each well, followed by incubation at 37°C for 1 hour. Following incubation, enzyme reagent was aspirated and all wells washed seven times with wash buffer.

One Sigma FAST (ρ-nitrophenyl phosphate) substrate tablet was reconstituted in 20mL ddH_2O and 100μL added to each well. Absorbance was read in an automated microplate reader at 405nm at regular intervals, with data saved for analysis when the top standard absorbance reading had increased to between 2.0 and 3.0. Following data acquisition, a standard curve, line of best fit and line equation was generated using GraphPad Prism, version 5.00 with experimental serum sample IL-1β concentrations determined using the A405nm and equation generated by GraphPad Prism.

Figure 6.8 shows the serum concentration of IL-1β in control and RH *T. gondii* infected mice 8 days post infection, quantified using CBA. Levels of IL-1β in serum of control mice were negligible. When comparing infected mice of each strain, $P2X_7$ receptor knockout mice showed lower levels of serum IL-1β in comparison to the parental C57BL/6J strain and BALB/c strain. This difference, however, was not statistically

significant. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

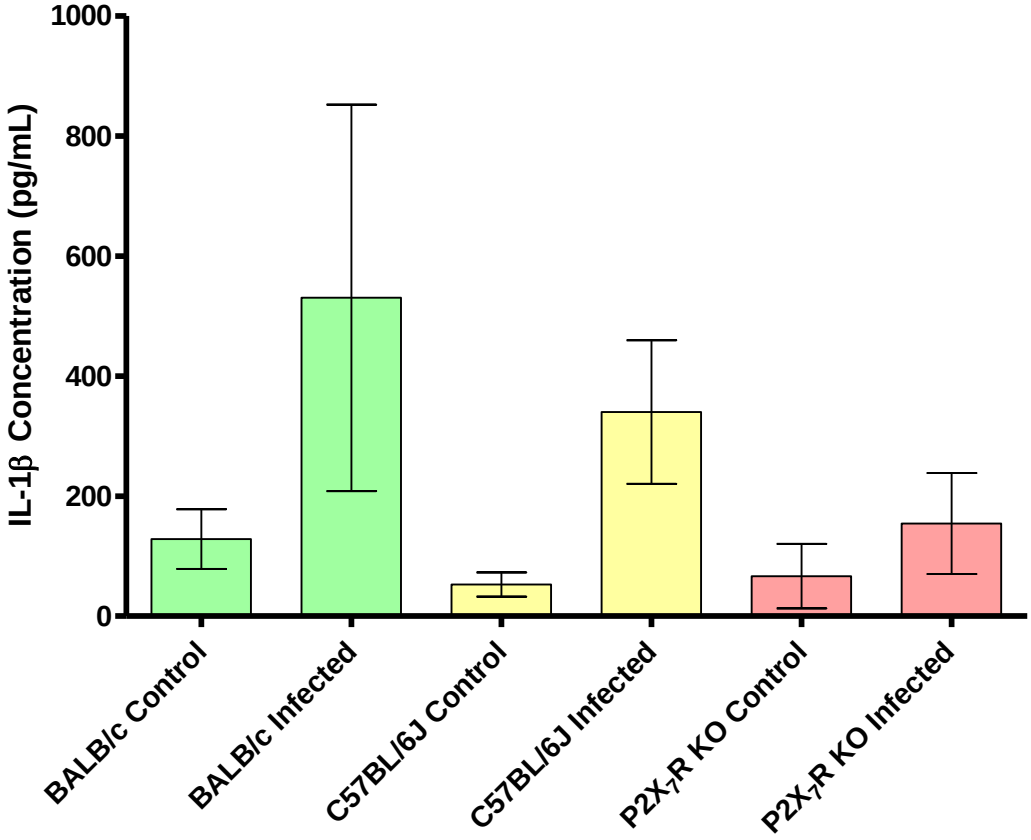

Figure 6.8 – Serum IL-1β concentration in RH *T. gondii* infected mice.
Mice were infected by intra-peritoneal injection with 500 RH *T. gondii* tachyzoites and euthanised 8 days post infection. Serum was collected for IL-1β quantification by ELISA. Serum from control mice was also quantified, showing lower IL-1β concentration in comparison to infected mice of each strain. The result shows RH *T. gondii* infected $P2X_7$ receptor knockout mice exhibit decreased levels of serum IL-1β in comparison to the parental C57BL/6J strain and BALB/c mice. This result however, was not statistically significant ($P > 0.05$). Results are the mean ± SEM; BALB/c control/infected, n = 3/5; C57BL/6J control/infected, n = 3/5; $P2X_7$ receptor knockout control/infected, n = 3/5. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

Reactive nitrogen intermediates (including NO) was quantified in the remaining mouse serum samples obtained from one RH *T. gondii* infection by quantifying the levels of nitrite (NO_2^-) and nitrate (NO_3^-), as described previously by Rockett *et al.*, (1994). Nitrite and nitrate standard solutions were prepared in a concentration range of 0 – 500μM in pooled, uninfected normal mouse serum.

In sterile 1.5mL tubes, 15μL of each experimental serum sample or 15mL nitrite standard was combined with 10μL distilled water and incubated at room temperature for 20 minutes.

In separate, sterile 1.5mL tubes, 15μL of each experimental serum sample or nitrate standard was combined with 7.5μL of 1.25 mg/mL NADPH and 2.5μL of 5U/mL nitrate reductase and incubated at room temperature for 20 minutes.

Following incubation, 50μL Griess Reagent, followed by 10μL 10% TCA was added to all tubes (experimental sera and standards for both nitrite and nitrate). All samples were mixed by vortexing and centrifuged at 10,000 x g for 5 minutes. 100μL of each supernatant was transferred to separate wells of a 96-well plate and A_{540} quantified in an automated microplate reader.

Serum nitrite and nitrate for each experimental serum sample was calculated by reading directly from a standard curve constructed for each analyte using the standards. Reactive nitrogen intermediates (NO) were then expressed as a sum of the concentrations of nitrite plus nitrate.

Figure 6.9 shows the serum concentration of reactive nitrogen intermediates (including NO) in control and RH *T. gondii* infected mice 8 days post infection, quantified by the Griess Assay. Levels of NO in serum of control mice were either non-variant/zero or negligible. When comparing infected mice of each strain, $P2X_7$ receptor knockout mice showed the highest levels of serum NO in comparison to the parental C57BL/6J strain and BALB/c strain. This difference, however, was not statistically significant. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

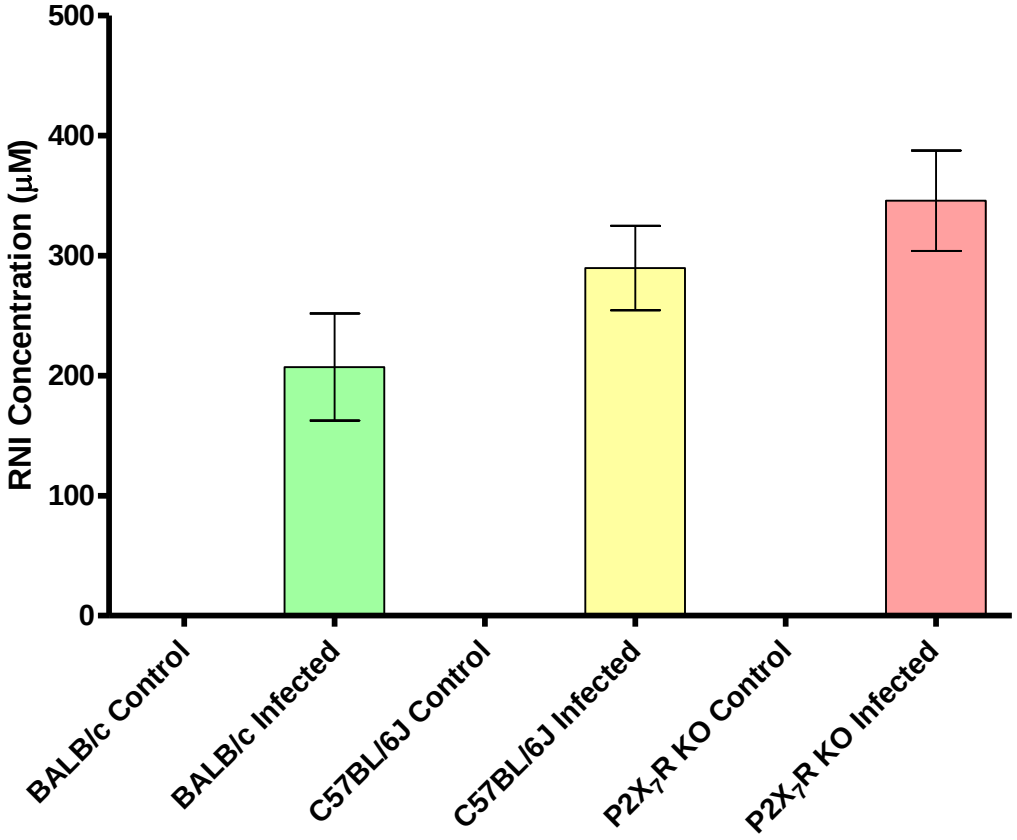

Figure 6.9 – Serum NO concentration in RH *T. gondii* infected mice.

Mice were infected by intra-peritoneal injection with 500 RH *T. gondii* tachyzoites and euthanised 8 days post infection. Serum was collected for NO quantification by the Griess Assay. Serum from control mice was also quantified, showing little/no NO in comparison to infected mice of each strain. The result shows RH *T. gondii* infected $P2X_7$ receptor knockout mice exhibit increased levels of serum NO in comparison to the parental C57BL/6J strain and BALB/c mice. This result however, was not statistically significant ($P > 0.05$). Results are the mean ± SEM; BALB/c control/infected, n = 5/8; C57BL/6J control/infected, n = 5/9; $P2X_7$ receptor knockout control/infected, n = 5/5. Statistical analysis was performed using a General Linear Model 1-factor ANOVA with Tukey's post-hoc test, comparing infected mice of each strain.

6.3 – Discussion

Results presented in Chapters 4 and 5 definitively confirmed a role for the $P2X_7$ receptor in the human and mouse immune response to RH *T. gondii*. Having confirmed this role, experiments conducted in this chapter investigated the phenotype of *in vivo* infection in mice with varying levels of $P2X_7$ receptor expression/function. As in Chapter 5, this study included BALB/c mice (with wild-type, fully functional $P2X_7$ receptors); C57BL/6J (with the loss-of-function P451L polymorphism and half $P2X_7$

receptor function) (Adriouch *et al.*, 2002) and the $P2X_7$ receptor knockout mouse with no $P2X_7$ receptor expression or function.

The $P2X_7$ receptor knockout mouse is commercially available from Pfizer, and was back-crossed for several generations from the C57BL/6J x DBA/2 F1 mouse strain into the C57BL/6J mouse strain (Solle *et al.*, 2001). After generation of the $P2X_7$ receptor knockout mouse, Adriouch *et al.*, (2002) discovered that the C57BL/6J mouse $P2X_7$ receptor gene exhibits a naturally occurring polymorphism at amino acid position 451, where proline is substituted for leucine. This reduces receptor function to approximately half that of the wild-type gene (such as that present in BALB/c mice). This result was confirmed by functional experiments presented in Chapter 5, with C57BL/6J mouse bone marrow derived macrophages (BMM) exhibiting approximately half ethidium flux through $P2X_7$ receptor mediated pores compared with BALB/c BMM (Figure 5.4). As expected, $P2X_7$ receptor knockout BMM showed minimal ethidium uptake in response to ATP confirming the successful knockout of the $P2X_7$ receptor in these mice. Taking this discovery into account, it was decided that experiments conducted in this chapter should include cells derived from all three strains of mice.

In murine infection with *T. gondii*, severity of disease has been known for some time to depend on several factors, including the strain of parasite (Masihi and Werner, 1977) and the strain of mouse (Araujo *et al.*, 1976). The route of infection also determines the severity of disease, with genetically susceptible C57BL/6J mice being more susceptible to oral infection with *T. gondii* compared with the resistant BALB/c mouse, with little difference between the strains following intra-peritoneal infection (Johnson, 1984).

In the intra-peritoneal infections performed in this chapter, BALB/c mice exhibited significantly higher parasite burden in the peritoneal cavity in comparison to those seen with C57BL/6J and $P2X_7$ receptor knockout mice. Furthermore, $P2X_7$ receptor knockout mice exhibited a non-significant reduction in parasite burden in comparison to the parental C57BL/6J strain (Figure 6.1). This result may seem to contradict earlier results from viability assays showing that $P2X_7$ receptor activation kills intracellular parasites, as one would expect the presence of a functional/semi-functional $P2X_7$ receptor to reduce parasite viability and therefore lower parasite burden in BALB/c and C57BL/6J mice. The results seen here, however, suggest lack of $P2X_7$ receptor

expression is associated with a lower parasite burden at the site of infection. This may reflect the ability of parasites infected into $P2X_7$ receptor knockout mice, to more freely disseminate to other organs and tissues around the body due to a lack of immune pressure. While speculative, this hypothesis could be investigated by a more detailed infection study in $P2X_7$ receptor knockout mice, looking at parasite burden in various tissues/organs after *T. gondii* infection.

As well as assessing parasite burden in the peritoneal cavity, the inflammatory cytokine response was assessed in the serum of infected mice by cytometric bead array (CBA) and ELISA. CBA allows for the simultaneous quantification of several cytokines in a single serum sample, through the use of fluorescent micro beads (of varying fluorescence intensity) conjugated to monoclonal antibodies directed against the cytokine of interest. The experimental process operates in a similar principle to a conventional ELISA, but only requires a single incubation and wash step, with quantification performed by flow cytometry and analysis software. The CBA used in this analysis incorporated IL-6, IL-10, MCP-1, IFN-γ, TNF and IL-12p70. In addition, IL-1β was quantified in remaining serum samples by ELISA. The aim of this analysis was to quantify various pro-inflammatory cytokines that are already known to be important in both $P2X_7$ receptor activation and the immune response to *T. gondii*.

There was a trend in the inflammatory cytokine analysis suggesting a decreased inflammatory response in $P2X_7$ receptor knockout mice in comparison to the parental C57BL/6J strain. IFN-γ, MCP-1 and TNF were significantly reduced in the serum of $P2X_7$ receptor knockout mice, with IL-6 and IL-1β showing similar (non-significant) reduction compared with the parental C57BL/6J strain. Both TNF and IFN-γ are central cytokines already known to be important in the immune response to *T. gondii* (Sibley *et al.*, 1991; Gazzinelli *et al.*, 1994; Hunter *et al.*, 1995; Scharton-Kersten *et al.*, 1996; Lieberman and Hunter, 2002), with MCP-1 also important for recruitment of macrophages to the site of inflammation and tissue damage (Brenier-Pinchart *et al.*, 2000). IL-6 and IL-1β also promote the inflammatory response after secretion from T-cells and monocytes/macrophages.

As we know from the literature (and discussed in Chapter 1.5), a robust cell mediated immune response is required for innate immunity and control of *T. gondii* replication. Additionally, intra-peritoneal infection of BALB/c mice with RH *T. gondii* has previously been associated with the early overproduction of IL-12, IFN-γ and TNF, eventually leading to death by immunopathology (Nguyen *et al.*, 2003). Therefore a balanced and controlled inflammatory response is crucial for resistance to *T. gondii* infection while minimising immunopathology. Any genetic defect that upsets this balance is likely to sway the outcome of infection to either an uncontrolled immunopathology, or an insufficient inflammatory response allowing continued parasite replication. Further evidence of this is seen with lower (albeit non-significant) IL-10 levels in $P2X_7$ receptor knockout mice following infection, further suggesting that the lack of $P2X_7$ receptor expression interferes with signalling pathways involved not only in the promotion of, but also the regulation of, the immune response to *T. gondii.*

The results of the inflammatory cytokine analysis suggest that a lack of $P2X_7$ receptor expression *in vivo* interferes with the normal inflammatory response to *T. gondii*. There is not sufficient information in the literature to confirm this result with *T. gondii* or other organisms. However, these findings are in agreement with previously published experiments making use of $P2X_7$ receptor knockout mice, showing decreased IL-6 release in response to ATP injection *in vivo*, as well as decreased IL-1β processing and release by macrophages *in vitro* (Solle *et al.*, 2001).

When interpreted in a broad context, the results presented in this chapter both confirm a role for the $P2X_7$ receptor in the murine immune response to RH *T. gondii in vivo*, and also highlight a defect in the development of a competent, controlled inflammatory response. Lack of $P2X_7$ receptor expression may also be associated with the inability to restrict dissemination of the parasite to other organs/tissues. Observations made in this chapter lead to the requirement of a more detailed *in vivo* investigation into the role of the $P2X_7$ receptor in the murine immune response to RH and other strains of *T. gondii*.

Chapter 7 – General discussion

7.1 – Key findings

The physiological effect of extracellular purine nucleotides was first documented by Drury and Szent-Györgyi, (1929), noting a transient bradycardia in guinea pigs following the injection of a heart muscle preparation. Following this, the concept of purinergic receptor signalling via ATP was introduced by Burnstock, (1972), with initial scepticism surrounding the possibility that such an important and ubiquitous molecule in cellular respiration could also act as an extracellular messenger. Subsequent cloning of purinergic receptors confirmed the existence of specific cell surface receptors for purine nucleotides, with the cloning of the rat $P2X_7$ receptor by Surprenant *et al.*, (1996), followed by the human $P2X_7$ receptor by Rassendren *et al.*, (1997). Following cloning and preliminary characterisation, it has been shown that activation of the $P2X_7$ receptor by extracellular ATP is capable of inducing a potent set of downstream cellular events that promote the inflammatory response and modulate apoptosis (North, 2002). With this knowledge, $P2X_7$ receptor activation was implicated, and subsequently confirmed, to play a role in the immune response to various intracellular pathogens that share similar host immune responses and mechanisms of immune evasion to *T. gondii*. Armed with this knowledge, the main goal of the research conducted in this PhD was to extend the role of $P2X_7$ receptor activation to incorporate the immune response to *T. gondii*.

The experiments in this thesis demonstrate that activation of the human and murine $P2X_7$ receptor with extracellular ATP leads to a significant reduction in both the viability and replication of virulent RH *T. gondii* tachyzoites *in vitro*. There are three main findings that support this. First, human macrophages lacking $P2X_7$ receptor function (due to the 1513C loss-of-function polymorphism) were unable to reduce intracellular RH *T. gondii* burden. Second, the use of the $P2X_7$ receptor antagonist, oATP on the murine macrophage-like cell line, RAW 264.7 showed that ATP induced killing of RH *T. gondii* was through a P2X receptor, most likely the $P2X_7$ receptor. Third the use of BMM derived from $P2X_7$ receptor knockout mice confirmed that the killing of intracellular tachyzoites observed *in vitro* was through the $P2X_7$ receptor.

Investigation into the role of the $P2X_7$ receptor in the murine immune response *in vivo* employed the use of $P2X_7$ receptor knockout mice. Infection of mice with RH *T. gondii* resulted in a significant decrease in parasite burden in $P2X_7$ receptor knockout mice in comparison to the BALB/c strain (with a similar, non-significant decrease compared to the parental C57BL/6J strain). It is possible that the decrease in parasite burden may reflect an increased ability of parasites to more freely disseminate to other parts of the body in $P2X_7$ receptor knockout mice. This hypothesis was reinforced by experiments that assessed the serum levels of inflammatory cytokines that are important in both the promotion and regulation of the immune response to *T. gondii*.

With *in vitro* experiments in this thesis, a relatively high concentration of ATP (3mM) was used to activate the $P2X_7$ receptor. This is a standard concentration of ATP used in experiments aimed at assessing the effect of $P2X_7$ receptor activation on other intracellular pathogens (Lammas *et al.*, 1997; Coutinho-Silva *et al.*, 2001; Fairbairn *et al.*, 2001; Smith *et al.*, 2001; Saunders *et al.*, 2003; Fernando *et al.*, 2005; Shemon *et al.*, 2006). However, with $P2X_7$ receptor activation requiring an extracellular ATP concentration of greater than 100μM (North, 2002), this raises the issue of whether the ATP generated at the site of a toxoplasmosis lesion is sufficient to activate the $P2X_7$ receptor. There are several potential sources for the generation of ATP that may result in $P2X_7$ receptor activation *in vivo*. These include ATP released from activated platelets (Malmgren, 1986; Beigi *et al.*, 1999) or dead/dying/damaged cells (Oshimi *et al.*, 1999) as well as ATP released from viable cells through transport mechanisms such as the cystic fibrosis transmembrane conductase regulator (Crane *et al.*, 2005).

Intracellular *T. gondii* replication eventually results in lysis of the host cell as parasites egress in search of a new host cell to invade, therefore potentially providing a source of extracellular ATP for localised $P2X_7$ receptor activation. The concentration of ATP inside cells can be between 5 – 10mM, meaning lysis of *T. gondii* infected cells could liberate sufficient quantities of ATP to provide a localised concentration of approximately 3mM (Di Virgilio, 1995; Lammas *et al.*, 1997). While this partially justifies the concentration of ATP used in this thesis and other studies, one possible avenue for further research would be to conduct *in vivo* experiments where ATP is quantified at the site of active *T. gondii* lesions in various tissues (such as eye, brain,

musculature and lymph nodes), to determine if the amount of ATP released in this manner would be sufficient to activate the $P2X_7$ receptor *in vivo*.

Although $P2X_7$ receptor activation has been shown to result in the death of intracellular RH *T. gondii*, the intracellular events downstream of $P2X_7$ receptor activation that lead to parasite death still remain relatively unexplored. As discussed in Chapter 1.9, $P2X_7$ receptor activation leads to a variety of downstream events. The majority of $P2X_7$ receptor-mediated events contribute to promotion of the inflammatory response, activation of various transcription factors and induction apoptotic cell death (Figure 7.1).

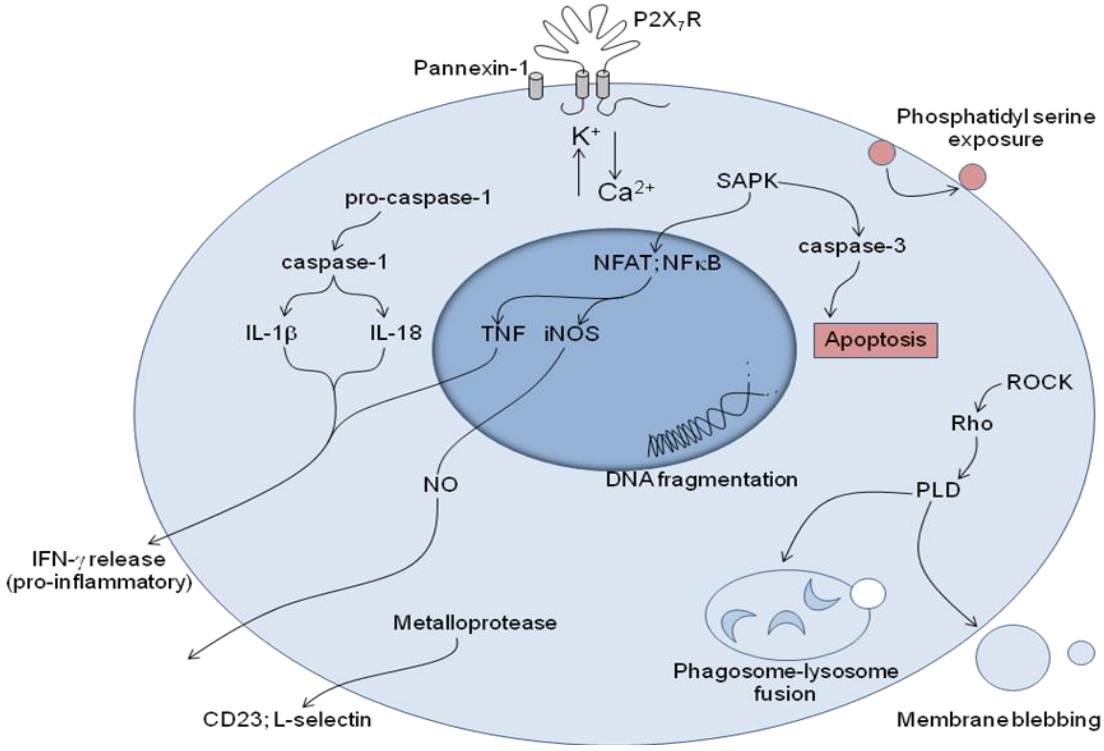

Figure 7.1 – Downstream events following $P2X_7$ receptor activation.

Activation of the $P2X_7$ receptor (possibly in a signalling complex with Pannexin-1) leads to a variety of downstream events, starting with influx of Ca^{2+} and efflux of K^+ and ultimately culminating in promotion of the inflammatory immune response and the induction of apoptotic cell death. SAPK activation leads to activation of caspase-3, resulting in DNA fragmentation and apoptosis with externalisation of phosphatidylserine (flagging the apoptotic cell for phagocytosis). SAPK activation also results in nuclear translocation of transcription factors NFκB and NFAT, promoting the transcription of iNOS (leading to NO formation) and TNF. Caspase-1 activation is known to promote the release of active IL-1β and IL-18, synergising with TNF to promote IFN-γ release from T-cells and drive the inflammatory response. Metalloprotease activation induces the shedding of CD23 and L-selectin, influencing lymphocyte migration. PLD activation, preceded by Rho and ROCK activation, leads to phagosome-lysosome fusion and membrane blebbing.

From the findings presented in this thesis, it is likely that many downstream events following $P2X_7$ receptor activation act synergistically to both kill the intracellular parasite and aid in the mounting of a competent, controlled inflammatory response. It is well known that various pro-inflammatory cytokines are important in host resistance to *T. gondii* (Sibley *et al.*, 1991; Gazzinelli *et al.*, 1994; Hunter *et al.*, 1995; Scharton-Kersten *et al.*, 1996; Janssen *et al.*, 2002). Experiments in this thesis showed the absence of the $P2X_7$ receptor significantly impairs the release of such cytokines, with significant reduction in the concentration of IFN-γ and TNF. Decreased levels of MCP-1 were also associated with a lack of $P2X_7$ receptor expression. MCP-1 is important in recruiting monocytes to the site of a *T. gondii* lesion, aiding also in the activation of T-cells and NK cells (Brenier-Pinchart *et al.*, 2000). $P2X_7$ receptor activation is also known to promote the shedding of CD23 and L-selectin, promoting lymphocyte migration (Gu *et al.*, 1998; Jamieson *et al.*, 1996). Therefore, it seems that the release of pro-inflammatory and chemotactic cytokines is one arm of the $P2X_7$ receptor-mediated immune response to *T. gondii.*

NO is a molecule that is already known to be capable of killing the parasite (and was even used successfully as a positive control for parasite death in this thesis) (Bohne *et al.*, 1994). The fact that elevated levels of NO were seen in infected $P2X_7$ receptor knockout mice in association with reduced peritoneal cavity parasite burden could be taken as further evidence for a role for NO in controlling *T. gondii.* However, this conclusion would need to be taken with extreme caution and, it could be argued, that it is flawed on several counts. First, as discussed already, the reduced number of parasites in the peritoneal cavity may reflect enhanced dissemination of the parasite in the absence of immune pressure and/or the lack of suitable host cells in the peritoneal cavity of $P2X_7$ receptor knockout mice. Second, cells with deficiencies in $P2X_7$ receptor activity were not able to control *T. gondii in vitro.* And, third, the levels of NO seen in the sera of BALB/c mice were almost certainly sufficient to affect *T. gondii.* More interesting, in this context, is the possible link between elevated NO levels and reduced IL-10 levels seen in the $P2X_7$ receptor knockout mice (albeit that the later observations were not statistically significant); decreased IL-10 and associated elevation of NO levels is a known cause of immunopathology in *T. gondii*-infected mice (Suzuki *et al.*, 2000). There is very little data relating to a potential anti-inflammatory, regulatory role for the

$P2X_7$ receptor but there are some indications that prolonged exposure to ATP can result in inhibition of production of pro-inflammatory cytokines such as IL-1β, 1L-12, TNF and IFN-γ and promotion of IL-10 production (la Sala *et al.*, 2003; Kaufmann *et al.*, 2005). Thus, a role for the $P2X_7$ receptor in regulation of IL-10 in response to *T. gondii* is a genuinely novel notion that deserves further investigation. In any case, on balance, it appears that $P2X_7$ receptor mediated killing of RH *T. gondii* is not dependent on NO, an observation that is in agreement with previous studies, showing that $P2X_7$ receptor mediated killing of *Mycobacterium* spp. was also independent of NO (Lammas *et al.*, 1997; Fairbairn *et al.*, 2001).

Via the activation of effector kinases, $P2X_7$ receptor activation also results in the downstream activation of PLD, which is known to promote phagosome-lysosome fusion. $P2X_7$ receptor-mediated phagosome-lysosome fusion via PLD activation was shown to kill intracellular mycobacteria (Fairbairn *et al.*, 2001). This, however, is not likely to be an important mechanism of $P2X_7$ receptor-mediated *T. gondii* killing, since *T. gondii* tachyzoites actively invade host cells and reside within an immunologically inert parasitophorous vacuole, not a phagosome (Morisaki *et al.*, 1995; Mordue and Sibley, 1997; Sacks and Sher, 2002).

It has previously been shown that $P2X_7$ receptor activation of RH *T. gondii* infected RAW 264.7 cells rapidly induces apoptotic host cell death (similar results are also seen in uninfected RAW 264.7 cells) (Lees, 2004) (Figure 7.2). Experiments conducted in this thesis confirmed the death and reduced numbers of the intracellular parasite following $P2X_7$ receptor activation.

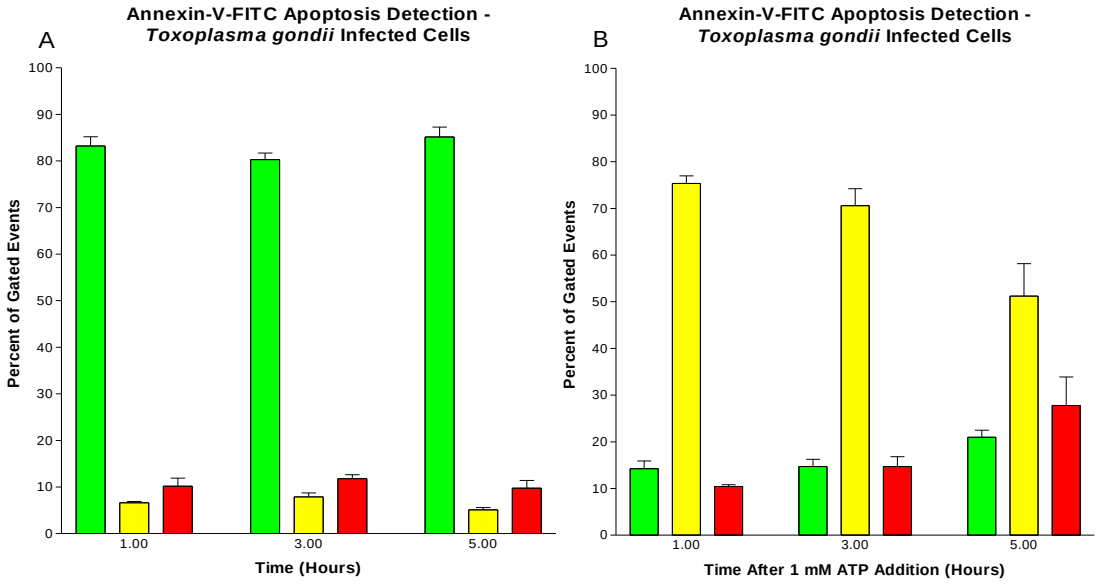

Figure 7.2 – Apoptosis induced by $P2X_7$ receptor activation of RH *T. gondii* infected RAW 264.7 cells. RAW 264.7 cells (1 x 10^6) were infected with RH *T. gondii* tachyzoites (3 x 10^6). After overnight incubation, RAW 264.7 cells were untreated (A) or $P2X_7$ receptors activated with 1mM ATP (B), with apoptosis/necrosis quantified by Annexin-V-FITC/propidium iodide staining and flow cytometry 1, 3 and 5 hours after ATP addition. Green, yellow and red bars represent percent viable, apoptotic and necrotic, respectively. Error bars = mean ± SEM; n = 3. Results show rapid induction of apoptosis in $P2X_7$ receptor activated cells. Data reproduced from Lees (2004).

Therefore, it seems likely that apoptosis of *T. gondii* infected cells is another arm of the immune response to which $P2X_7$ receptor activation contributes. While it may be argued that inducing apoptotic death of these cells simply deprives the parasite of cells in which to replicate, this is still a likely mechanism of parasite killing *in vivo*. *Toxoplasma gondii* is well known to interfere with the normal pathways to apoptosis (Nash *et al.*, 1998; Goebel *et al.*, 2001; Payne *et al.*, 2003; Luder and Gross, 2005; Keller *et al.*, 2006; Kim and Denkers, 2006). $P2X_7$ receptor activation, therefore, may provide an additional pathway to apoptosis of *T. gondii* infected cells, resulting both in the death of the intracellular parasite (even if simply depriving it of a safe intracellular reservoir), and also promoting the inflammatory response, attracting immune cells to the site of *T. gondii* infection.

7.2 – Future directions

The findings presented in this thesis progress our understanding of the immune response to *T. gondii*. There are however, avenues for continued research to explore both host and parasite-derived factors that may contribute to susceptibility or resistance to toxoplasmosis. In terms of host susceptibility to *T. gondii* infection at the genetic level, the $P2X_7$ receptor gene may be in linkage with other nearby genes that also affect outcome after infection with *T. gondii*. An interesting approach could be to try and understand if there are any other genes located close to the $P2X_7$ receptor at 12q24, which are likely to influence susceptibility to more severe toxoplasmosis. We now know that the $P2X_7$ receptor plays a role in the immune response to *T. gondii*, however if there are other polymorphic genes that are in linkage with those that also effect $P2X_7$ receptor function, by identifying these genes we may gain further understanding of the role that other host factors play in toxoplasmosis.

Studies attempting to associate $P2X_7$ receptor polymorphisms with other infectious diseases in human cohorts have been varied in their findings. For example, the 1513C $P2X_7$ receptor polymorphism was not associated with tuberculosis in a Gambian cohort (Li *et al.*, 2002), however, the same polymorphism was associated with disease in various other cohorts from Southeast Asia (Fernando *et al.*, 2007), Mexico (Nino-Moreno *et al.*, 2007) and Russia (Mokrousov *et al.*, 2008). Considering the variability in these findings, it would be interesting to conduct a thorough analysis of $P2X_7$ receptor genotypes in both the EMSCOT (European Multicentre Study on Congenital Toxoplasmosis) and NCCCTS (National Collaborative Chicago-based Congenital Toxoplasmosis Study), in order to determine if $P2X_7$ receptor polymorphisms are associated not only with the incidence of congenital toxoplasmosis, but also the type and severity of disease and any long-term symptoms following congenital infection and a lack of $P2X_7$ receptor function. Such analysis may prove difficult however, since polymorphic $P2X_7$ receptor variants form the majority in the general population with the wild-type gene only contributing to approximately 30% of the general population. With this problem, association studies would need to constitute much larger numbers than those available in EMSCOT and NCCCTS to gain enough power to definitively link polymorphic $P2X_7$ receptor variants with disease.

As well as host factors that may contribute to susceptibility to toxoplasmosis, there may also be clinical consequences that arise from parasite-derived factors. In addition to the work conducted during this PhD using samples from the NCCCTS, it would be very interesting to determine the clonal type of parasite infecting mothers and children in this study. Performing this analysis would yield information that may identify if there is any particular clonal type of parasite that is associated with congenital toxoplasmosis, or if there is an overrepresentation of atypical *T. gondii* isolates, similar to those seen in recent outbreaks of toxoplasmosis (Bowie *et al.*, 1997; Grigg *et al.*, 2001; Demar *et al.*, 2007). Such analyses may also identify if the infecting type of parasite also determines the clinical symptoms in congenitally infected children (for example, incidence and/or severity of eye lesions).

In determining the effect of $P2X_7$ receptor activation on intracellular *T. gondii*, the Type I virulent RH strain was used in experiments throughout this thesis. Further experiments assessing the *in vitro* effect of $P2X_7$ receptor activation on other parasite clonal types should also be conducted, for example, in determining whether Type II or III strains of *T. gondii* are also susceptible to $P2X_7$ receptor-mediated killing. Such experiments may identify differences in susceptibility to $P2X_7$ receptor-mediated killing among isolates.

Additionally, a more detailed *in vivo* investigation monitoring the course of infection of $P2X_7$ receptor knockout mice with different strains of *T. gondii* would also complement the proposed *in vitro* analysis. These experiments would allow further investigation into the rate of dissemination of *T. gondii* tachyzoites, following the observation of lower parasite burden in the peritoneal cavity of RH *T. gondii* infected P2X receptor knockout mice. It was hypothesised that absence of the $P2X_7$ receptor allowed faster parasite dissemination through the body, thus resulting in lower parasite numbers at the site of infection. If this is the case, one would also expect to see higher parasite counts in other tissues/organs throughout the body.

Another opportunity for further investigation lies with determining possible direct mechanisms of parasite interference with the $P2X_7$ receptor. It is well known that Type I virulent strains of *T. gondii* such as RH are capable of delaying the immune response for a short period of time, thus allowing the parasite to replicate in the acute phase and

cause pathology prior to immune regulation (Sacks and Sher, 2002). One interesting mechanism of *T. gondii* virulence is the excreted nucleotide triphosphate hydrolase (NTPase), an immunodominant antigen in mice (Asai *et al.*, 1987) and humans (Asai *et al.*, 1992), which has been proposed as a determinant/indicator of virulence (Johnson *et al.*, 2003). It is known that all *T. gondii* strains exhibit the less active NTPaseII isoform, whereas only virulent *T. gondii* strains exhibit two NTPase isoforms, the highly active NTPaseI and less active NTPaseII, with virulent parasite attenuation accompanied by lower NTPaseI expression (Asai *et al.*, 1995).

The ability of all *T. gondii* isolates to degrade extracellular ATP through an excreted enzyme could act to decrease the available concentration of ATP that would normally be available to bind to $P2X_7$ receptors in the extracellular milieu. It is possible that, *in vivo, T. gondii* tachyzoites have the ability to degrade sufficient quantities of ATP to prevent or delay $P2X_7$ receptor activation via the excreted NTPases. It would be of interest to conduct *T. gondii* infections with $P2X_7$ receptor knockout mice, using either wild-type parasites, or NTPase knockout variants. This would allow us to determine/assess the role of parasite driven $P2X_7$ receptor agonist degradation in the pathogenicity of toxoplasmosis.

In addition to elucidating the role of the excreted NTPase in the pathogenicity of toxoplasmosis, there may also be other parasite-derived molecules or strategies that interact with the $P2X_7$ receptor and/or avoid downstream events following receptor activation. If different *T. gondii* strains are indeed truly variable in their susceptibility to $P2X_7$ receptor mediated killing, it would be very interesting to elucidate the underlying reasons for this phenotype. The process of creating genetic crosses using two separate isolates of *T. gondii* has been previously published, with tissue cysts from two separate parasite strains infected into kittens where sexual replication occurs between the two strains, resulting in oocyst progeny that are a genetic cross of the two parental strains. These oocysts are sporulated and reintroduced into *in vitro* culture and cloned for phenotype testing. Genetic crosses of various *T. gondii* clonal types have been created, with groundbreaking work conducted by Pfefferkorn and colleagues (Pfefferkorn and Pfefferkorn, 1976; Pfefferkorn and Pfefferkorn, 1980; Pfefferkorn and Kasper, 1983). Genetic cross *T. gondii* strains may be used in similar experiments to those presented in this thesis, similar to those already conducted for parasite virulence genes ROP18 and

ROP16 (Saeij *et al.*, 2006; Saeij *et al.*, 2007). Such experiments may allow for the identification of a chromosomal region and, eventually, a candidate parasite gene that is important in parasite resistance or susceptibility to $P2X_7$ receptor-mediated killing. With this knowledge, we gain more information about mechanisms of *T. gondii* virulence and immune evasion/interaction, and will thus be better prepared for future drug and vaccine development.

Finally, some effort to resolving the relative roles of apoptosis and NO production in $P2X_7$ receptor-mediated control of *T. gondii* is required. This could be achieved by (a) assessing the effects of $P2X_7$ receptor deficiency on various steps in the signalling pathways that lead to apoptosis and/or NO production in *T. gondii*-infected cells and (b) by use of inhibitors of apoptosis and NO *in vitro* and *in vivo* (where possible, for example, through the use of NO deficient cell lines).

7.3 – Final conclusion

Toxoplasma gondii is a parasite of worldwide distribution, with significant medical and economic importance. With high rates of *T. gondii* infection in the human population and the continued spread of HIV, toxoplasmosis will continue to emerge as an increasingly important pathogen of immunocompromised individuals. In addition, the effects of congenital toxoplasmosis on the unborn foetus can also be devastating, and with current debate over whether screening for congenital toxoplasmosis should continue (Gilbert and Peckham, 2002), we may be approaching a rather alarming era of increasing numbers of neonates being born with debilitating symptoms of congenital infection.

It is imperative therefore, that we make efforts to understand the host immune response to *T. gondii* and elucidate interactions between the parasite and the host immune response. By understanding these areas of interaction, approaches to developing novel drug and vaccine targets will be aided. The experiments conducted throughout this PhD have aided in the understanding of the immune response to *T. gondii*. It was previously known that $P2X_7$ receptor activation was important in the immune response to *Mycobacterium* spp., *Chlamydia* spp. and *Leishmania* spp. The role of the $P2X_7$

receptor may now also be extended to include *T. gondii*. The shared preference for the infection of macrophages by these pathogens seems to be a common factor in the need to achieve $P2X_7$ receptor activation, resulting in pathogen killing and promoting the inflammatory response. This may be reinforced by the observation that there is no information thus evident in the literature pertaining to a need for $P2X_7$ receptor activation in the clearance of non-macrophage dwelling pathogens. Considering this information, it is evident that activation of the $P2X_7$ receptor is important in the ability to clear infection with pathogens that safely reside within cells that should normally be capable of killing them.

Bibliography

Abbracchio, M.P. and Burnstock, G. (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacology & Therapeutics* **64**: 445-475.

Adams, L.B., Hibbs, J.B., Jr., Taintor, R.R. and Krahenbuhl, J.L. (1990) Microbiostatic effect of murine-activated macrophages for Toxoplasma gondii. Role for synthesis of inorganic nitrogen oxides from L-arginine. *Journal of Immunology* **144**: 2725-2729.

Adjogble, K.D., Mercier, C., Dubremetz, J.F., Hucke, C., Mackenzie, C.R., Cesbron-Delauw, M.F. and Daubener, W. (2004) GRA9, a new Toxoplasma gondii dense granule protein associated with the intravacuolar network of tubular membranes. *International Journal for Parasitology* **34**: 1255-1264.

Adriouch, S., Dox, C., Welge, V., Seman, M., Koch-Nolte, F. and Haag, F. (2002) Cutting edge: a natural P451L mutation in the cytoplasmic domain impairs the function of the mouse $P2X_7$ receptor. *Journal of Immunology* **169**: 4108-4112.

Ajzenberg, D., Banuls, A.L., Tibayrenc, M. and Darde, M.L. (2002a) Microsatellite analysis of Toxoplasma gondii shows considerable polymorphism structured into two main clonal groups. *International Journal for Parasitology* **32**: 27-38.

Ajzenberg, D., Cogne, N., Paris, L., Bessieres, M.-H., Thulliez, P., Filisetti, D., Pelloux, H., Marty, P. and Darde, M.-L. (2002b) Genotype of 86 Toxoplasma gondii isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. *Journal of Infectious Diseases* **186**: 684-689.

Aliberti, J.C., Cardoso, M.A., Martins, G.A., Gazzinelli, R.T., Vieira, L.Q. and Silva, J.S. (1996) Interleukin-12 mediates resistance to Trypanosoma cruzi in mice and is produced by murine macrophages in response to live trypomastigotes. *Infection & Immunity* **64**: 1961-1967.

Alvar, J., Canavate, C., Gutierrez-Solar, B., Jimenez, M., Laguna, F., Lopez-Velez, R., Molina, R. and Moreno, J. (1997) Leishmania and human immunodeficiency virus coinfection: the first 10 years. *Clinical Microbiology Reviews* **10**: 298-319.

Amato, V., Amato, J., Nicodemo, A., Uip, D., Amato-Neto, V. and Duarte, M. (1998) Treatment of mucocutaneous leishmaniasis with pentamidine isothionate. *Annales de Dermatologie et de Venereologie* **125**: 492-495.

Ancelle, T., Goulet, V., Tirard-Fleury, V., Baril, L., Du Mazaubrin, C. and Thulliez, P. (1996) La toxoplasmose chez la femme enceinte en France en 1995. *Bulletin Épidémiologique Hebdomadaire* **51**: 227-228.

Angus, C.W., Klivington-Evans, D., Dubey, J.P. and Kovacs, J.A. (2000) Immunization with a DNA plasmid encoding the SAG1 (P30) protein of Toxoplasma gondii is immunogenic and protective in rodents. *Journal of Infectious Diseases* **181**: 317-324.

Araujo, F.G., Williams, D.M., Grumet, F.C. and Remington, J.S. (1976) Strain-dependent differences in murine susceptibility to toxoplasma. *Infection & Immunity* **13**: 1528-1530.

Arrizabalaga, G. and Boothroyd, J.C. (2004) Role of calcium during Toxoplasma gondii invasion and egress. *International Journal for Parasitology* **34**: 361-368.

Asai, T., Kim, T.J., Kobayashi, M. and Kojima, S. (1987) Detection of nucleoside triphosphate hydrolase as a circulating antigen in sera of mice infected with Toxoplasma gondii. *Infection & Immunity* **55**: 1332-1335.

Asai, T., Mizuno, F., Kojima, S., Takeuchi, T., Kobayashi, A. and Suzuki, Y. (1992) High correlation in antibody titers between the Sabin-Feldman dye test and an enzyme-linked immunosorbent assay detecting immunoglobulin G antibodies to the nucleoside triphosphate hydrolase of Toxoplasma gondii. *Journal of Clinical Microbiology* **30**: 1291-1293.

Asai, T., Miura, S., Sibley, L.D., Okabayashi, H. and Takeuchi, T. (1995) Biochemical and molecular characterization of nucleoside triphosphate hydrolase isozymes from the parasitic protozoan Toxoplasma gondii. *Journal of Biological Chemistry* **270**: 11391-11397.

Auger, R., Motta, I., Benihoud, K., Ojcius, D.M. and Kanellopoulos, J.M. (2005) A role for mitogen-activated protein kinase(Erk1/2) activation and non-selective pore formation in $P2X_7$ receptor-mediated thymocyte death. *Journal of Biological Chemistry* **280**: 28142-28151.

Ayyanathan, K., Webbs, T.E., Sandhu, A.K., Athwal, R.S., Barnard, E.A. and Kunapuli, S.P. (1996) Cloning and chromosomal localization of the human $P2Y_1$ purinoceptor. *Biochemical & Biophysical Research Communications* **218**: 783-788.

Baeuerle, P.A. and Baltimore, D. (1988) IκB: a specific inhibitor of the NF-κB transcription factor. *Science* **242**: 540-546.

Baraldi, P.G., Di Virgilio, F. and Romagnoli, R. (2004) Agonists and antagonists acting at $P2X_7$ receptor. *Current Topics in Medicinal Chemistry* **4**: 1707-1717.

Barral, A., Pedral-Sampaio, D., Grimaldi Junior, G., Momen, H., McMahon-Pratt, D., Ribeiro de Jesus, A., Almeida, R., Badaro, R., Barral-Netto, M., Carvalho, E.M. and et al. (1991) Leishmaniasis in Bahia, Brazil: evidence that Leishmania amazonensis produces a wide spectrum of clinical disease. *American Journal of Tropical Medicine & Hygiene* **44**: 536-546.

Barrera, N.P., Ormond, S.J., Henderson, R.M., Murrell-Lagnado, R.D. and Edwardson, J.M. (2005) Atomic force microscopy imaging demonstrates that $P2X_2$ receptors are trimers but that $P2X_6$ receptor subunits do not oligomerize. *Journal of Biological Chemistry* **280**: 10759-10765.

Bates, P.A. (2007) Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. *International Journal for Parasitology* **37**: 1097-1106.

Beaman, M.H., Araujo, F.G. and Remington, J.S. (1994) Protective reconstitution of the SCID mouse against reactivation of toxoplasmic encephalitis. *Journal of Infectious Diseases* **169**: 375-383.

Beckers, C.J., Dubremetz, J.F., Mercereau-Puijalon, O. and Joiner, K.A. (1994) The Toxoplasma gondii rhoptry protein ROP 2 is inserted into the parasitophorous vacuole membrane, surrounding the intracellular parasite, and is exposed to the host cell cytoplasm. *Journal of Cell Biology* **127**: 947-961.

Beigi, R., Kobatake, E., Aizawa, M. and Dubyak, G.R. (1999) Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. *American Journal of Physiology* **276**: C267-278.

Bermudez, L.E., Covaro, G. and Remington, J. (1993) Infection of murine macrophages with *Toxoplasma gondii* is associated with release of transforming growth factor beta and downregulation of expression of tumor necrosis factor receptors. *Infection & Immunity* **61**: 4126-4130.

Bertho, A.L., Santiago, M.A., Da-Cruz, A.M. and Coutinho, S.G. (2000) Detection of early apoptosis and cell death in T $CD4^+$ and $CD8^+$ cells from lesions of patients with localized cutaneous leishmaniasis. *Brazilian Journal of Medical & Biological Research* **33**: 317-325.

Bliss, S.K., Marshall, A.J., Zhang, Y. and Denkers, E.Y. (1999) Human polymorphonuclear leukocytes produce IL-12, TNF-α, and the chemokines

macrophage-inflammatory protein-1 α and -1 β in response to Toxoplasma gondii antigens. *Journal of Immunology* **162**: 7369-7375.

Blum, J.A., Zellweger, M.J., Burri, C. and Hatz, C. (2008) Cardiac involvement in African and American trypanosomiasis. *The Lancet Infectious Diseases* **8**: 631-641.

Bohne, W., Heesemann, J. and Gross, U. (1993) Induction of bradyzoite-specific *Toxoplasma gondii* antigens in gamma interferon-treated mouse macrophages. *Infection & Immunity* **61**: 1141-1145.

Bohne, W., Heesemann, J. and Gross, U. (1994) Reduced replication of Toxoplasma gondii is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion. *Infection & Immunity* **62**: 1761-1767.

Borel, E., Mayencon, M., Kaiser, K., Picot, S. and Peyron, F. (1998) Fluorogenic detection of viable Toxoplasma gondii. *Parasite* **5**: 371-373.

Boudes, P., Zittoun, J. and Sobel, A. (1990) Acute pancytopenia induced by pyrimethamine during treatment of cerebral toxoplasmosis associated with AIDS. Role of dihydrofolate reductase inhibitors. *Annales de Medecine Interne* **141**: 183-186.

Bowie, W.R., King, A.S., Werker, D.H., Isaac-Renton, J.L., Bell, A., Eng, S.B. and Marion, S.A. (1997) Outbreak of toxoplasmosis associated with municipal drinking water. The BC Toxoplasma Investigation Team. *Lancet* **350**: 173-177.

Brenier-Pinchart, M.P., Pelloux, H., Simon, J., Ricard, J., Bosson, J.L. and Ambroise-Thomas, P. (2000) *Toxoplasma gondii* induces the secretion of monocyte chemotactic protein-1 in human fibroblasts, in vitro. *Molecular & Cellular Biochemistry* **209**: 79-87.

Buell, G.N., Talabot, F., Gos, A., Lorenz, J., Lai, E., Morris, M.A. and Antonarakis, S.E. (1998) Gene structure and chromosomal localization of the human $P2X_7$ receptor. *Receptors & Channels* **5**: 347-354.

Burnstock, G. (1972) Purinergic nerves. *Pharmacological Reviews* **24**: 509-581.

Burnstock, G. and Kennedy, C. (1985) Is there a basis for distinguishing two types of P2-purinoceptor? *General Pharmacology* **16**: 433-440.

Burnstock, G. (2004) Introduction: P2 receptors. *Current Topics in Medicinal Chemistry* **4**: 793-803.

Cai, G., Kastelein, R. and Hunter, C.A. (2000) Interleukin-18 (IL-18) enhances innate IL-12-mediated resistance to *Toxoplasma gondii*. *Infection & Immunity* **68**: 6932-6938.

Caldas, L.A., de Souza, W. and Attias, M. (2007) Calcium ionophore-induced egress of Toxoplasma gondii shortly after host cell invasion. *Veterinary Parasitology* **147**: 210-220.

Carey, K.L., Donahue, C.G. and Ward, G.E. (2000) Identification and molecular characterization of GRA8, a novel, proline-rich, dense granule protein of Toxoplasma gondii. *Molecular & Biochemical Parasitology* **105**: 25-37.

Carr, A., Tindall, B., Brew, B.J., Marriott, D.J., Harkness, J.L., Penny, R. and Cooper, D.A. (1992) Low-dose trimethoprim-sulfamethoxazole prophylaxis for toxoplasmic encephalitis in patients with AIDS. *Annals of Internal Medicine* **117**: 106-111.

Carruthers, V.B. and Sibley, L.D. (1997) Sequential protein secretion from three distinct organelles of Toxoplasma gondii accompanies invasion of human fibroblasts. *European Journal of Cell Biology* **73**: 114-123.

Cascabulho, C.M., Menna-Barreto, R.F.S., Coutinho-Silva, R., Persechini, P.M. and Henriques-Pons, A. (2008) $P2X_7$ modulatory web in Trypanosoma cruzi infection. *Parasitology Research* **103**: 829-838.

Cerretti, D.P., Kozlosky, C.J., Mosley, B., Nelson, N., Van Ness, K., Greenstreet, T.A., March, C.J., Kronheim, S.R., Druck, T., Cannizzaro, L.A. and et al. (1992) Molecular cloning of the interleukin-1β converting enzyme. *Science* **256**: 97-100.

Chaves, S.P., Torres-Santos, E.C., Marques, C., Figliuolo, V.R., Persechini, P.M., Coutinho-Silva, R. and Rossi-Bergmann, B. (2009) In *Microbes & Infection*.

Chen, C.C., Akopian, A.N., Sivilotti, L., Colquhoun, D., Burnstock, G. and Wood, J.N. (1995) A P2X purinoceptor expressed by a subset of sensory neurons. *Nature* **377**: 428-431.

Chessell, I.P., Hatcher, J.P., Bountra, C., Michel, A.D., Hughes, J.P., Green, P., Egerton, J., Murfin, M., Richardson, J., Peck, W.L., Grahames, C.B.A., Casula, M.A., Yiangou, Y., Birch, R., Anand, P. and Buell, G.N. (2005) Disruption of the $P2X_7$ purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain* **114**: 386-396.

Collo, G., Neidhart, S., Kawashima, E., Kosco-Vilbois, M., North, R.A. and Buell, G. (1997) Tissue distribution of the $P2X_7$ receptor. *Neuropharmacology* **36**: 1277-1283.

Courageot, M.-P., Lepine, S., Hours, M., Giraud, F. and Sulpice, J.-C. (2004) Involvement of sodium in early phosphatidylserine exposure and phospholipid scrambling induced by $P2X_7$ purinoceptor activation in thymocytes. *Journal of Biological Chemistry* **279**: 21815-21823.

Coutinho-Silva, R., Persechini, P.M., Bisaggio, R.D., Perfettini, J.L., Neto, A.C., Kanellopoulos, J.M., Motta-Ly, I., Dautry-Varsat, A. and Ojcius, D.M. (1999) P2Z/$P2X_7$ receptor-dependent apoptosis of dendritic cells. *American Journal of Physiology* **276**: C1139-1147.

Coutinho-Silva, R., Perfettini, J.L., Persechini, P.M., Dautry-Varsat, A. and Ojcius, D.M. (2001) Modulation of P2Z/$P2X_7$ receptor activity in macrophages infected with Chlamydia psittaci. *American Journal of Physiology - Cell Physiology* **280**: C81-89.

Couvreur, J., Tournier, G., Sardet-Frismand, A. and Fauroux, B. (1992) Heart or heart-lung transplantation and toxoplasmosis. *Presse Medicale* **21**: 1569-1574.

Crane, J.K., Naeher, T.M., Choudhari, S.S. and Giroux, E.M. (2005) Two pathways for ATP release from host cells in enteropathogenic Escherichia coli infection. *American Journal of Physiology - Gastrointestinal & Liver Physiology* **289**: 407-417.

Daffos, F., Forestier, F., Capella-Pavlovsky, M., Thulliez, P., Aufrant, C., Valenti, D. and Cox, W.L. (1988) Prenatal management of 746 pregnancies at risk for congenital toxoplasmosis. *New England Journal of Medicine* **318**: 271-275.

Dannemann, B., McCutchan, J.A., Israelski, D., Antoniskis, D., Leport, C., Luft, B., Nussbaum, J., Clumeck, N., Morlat, P., Chiu, J., Vilde, J., Orcllana, M., Fcigal, D., Bartok, A., Heseltine, P., Leedom, J. and Remington, J. (1992) Treatment of toxoplasmic encephalitis in patients with AIDS. A randomized trial comparing pyrimethamine plus clindamycin to pyrimethamine plus sulfadiazine. The California Collaborative Treatment Group. *Annals of Internal Medicine* **116**: 33-43.

Darville, T., Welter-Stahl, L., Cruz, C., Sater, A.A., Andrews, C.W., Jr. and Ojcius, D.M. (2007) Effect of the purinergic receptor $P2X_7$ on Chlamydia infection in

cervical epithelial cells and vaginally infected mice. *Journal of Immunology* **179**: 3707-3714.

De Cock, K.M. and Chaisson, R.E. (1999) Will DOTS do it? A reappraisal of tuberculosis control in countries with high rates of HIV infection. *International Journal of Tuberculosis & Lung Disease* **3**: 457-465.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G. and de Vries, J.E. (1991) Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *Journal of Experimental Medicine* **174**: 1209-1220.

Delgado, G., Puentes, F., Moreno, A. and Patarroyo, M.E. (2001) Flow cytometry, a useful tool for detecting the lethal effect of pentamidine on Leishmania viannia complex promastigote forms. *Pharmacological Research* **44**: 281-286.

Demar, M., Ajzenberg, D., Maubon, D., Djossou, F., Panchoe, D., Punwasi, W., Valery, N., Peneau, C., Daigre, J.-L., Aznar, C., Cottrelle, B., Terzan, L., Darde, M.-L. and Carme, B. (2007) Fatal outbreak of human toxoplasmosis along the Maroni River: epidemiological, clinical, and parasitological aspects. *Clinical Infectious Diseases* **45**: e88-95.

Denkers, E.Y. and Gazzinelli, R.T. (1998) Regulation and function of T-cell-mediated immunity during Toxoplasma gondii infection. *Clinical Microbiology Reviews* **11**: 569-588.

Desolme, B., Mevelec, M.N., Buzoni-Gatel, D. and Bout, D. (2000) Induction of protective immunity against toxoplasmosis in mice by DNA immunization with a plasmid encoding Toxoplasma gondii GRA4 gene. *Vaccine* **18**: 2512-2521.

Di Virgilio, F. (1995) The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death. *Immunology Today* **16**: 524-528.

Dobrowolski, J.M. and Sibley, L.D. (1996) Toxoplasma invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. *Cell* **84**: 933-939.

Dobrowolski, J.M., Carruthers, V.B. and Sibley, L.D. (1997) Participation of myosin in gliding motility and host cell invasion by Toxoplasma gondii. *Molecular Microbiology* **26**: 163-173.

Donnelly-Roberts, D.L., Namovic, M.T., Faltynek, C.R. and Jarvis, M.F. (2004) Mitogen-activated protein kinase and caspase signaling pathways are required for $P2X_7$ receptor ($P2X_7R$)-induced pore formation in human THP-1 cells. *Journal of Pharmacology & Experimental Therapeutics* **308**: 1053-1061.

Drury, A.N. and Szent-Györgyi, A. (1929) The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *The Journal of Physiology* **68**: 213-237.

Dubey, J.P., Miller, N.L. and Frenkel, J.K. (1970) The Toxoplasma gondii oocyst from cat feces. *Journal of Experimental Medicine* **132**: 636-662.

Dunn, D., Wallon, M., Peyron, F., Petersen, E., Peckham, C. and Gilbert, R. (1999) Mother-to-child transmission of toxoplasmosis: risk estimates for clinical counselling. *Lancet* **353**: 1829-1833.

Eissenberg, L.G. and Wyrick, P.B. (1981) Inhibition of phagolysosome fusion is localized to Chlamydia psittaci-laden vacuoles. *Infection & Immunity* **32**: 889-896.

Eissenberg, L.G., Wyrick, P.B., Davis, C.H. and Rumpp, J.W. (1983) Chlamydia psittaci elementary body envelopes: ingestion and inhibition of phagolysosome fusion. *Infection & Immunity* **40**: 741-751.

el-Moatassim, C. and Dubyak, G.R. (1992) A novel pathway for the activation of phospholipase D by P2z purinergic receptors in BAC1.2F5 macrophages. *Journal of Biological Chemistry* **267**: 23664-23673.

Emami, M.M. and Yazdi, M. (2008) Entomological survey of phlebotomine sand flies (Diptera: Psychodidae) in a focus of visceral leishmaniasis in central Iran. *Journal of Vector Borne Diseases* **45**: 38-43.

Ennion, S.J. and Evans, R.J. (2002) Conserved cysteine residues in the extracellular loop of the human $P2X_1$ receptor form disulfide bonds and are involved in receptor trafficking to the cell surface. *Molecular Pharmacology* **61**: 303-311.

Evans, R.J., Lewis, C., Buell, G., Valera, S., North, R.A. and Surprenant, A. (1995) Pharmacological characterization of heterologously expressed ATP-gated cation channels (P2x purinoceptors). *Molecular Pharmacology* **48**: 178-183.

Fairbairn, I.P., Stober, C.B., Kumararatne, D.S. and Lammas, D.A. (2001) ATP-mediated killing of intracellular mycobacteria by macrophages is a $P2X_7$-dependent process inducing bacterial death by phagosome-lysosome fusion. *Journal of Immunology* **167**: 3300-3307.

Ferguson, D.J.P. (2002) Toxoplasma gondii and sex: essential or optional extra? *Trends in Parasitology* **18**: 355-359.

Fernando, S.L., Saunders, B.M., Sluyter, R., Skarratt, K.K., Wiley, J.S. and Britton, W.J. (2005) Gene dosage determines the negative effects of polymorphic alleles

of the $P2X_7$ receptor on adenosine triphosphate-mediated killing of mycobacteria by human macrophages. *Journal of Infectious Diseases* **192**: 149-155.

Fernando, S.L., Saunders, B.M., Sluyter, R., Skarratt, K.K., Goldberg, H., Marks, G.B., Wiley, J.S. and Britton, W.J. (2007) A polymorphism in the $P2X_7$ gene increases susceptibility to extrapulmonary tuberculosis. *American Journal of Respiratory & Critical Care Medicine* **175**: 360-366.

Ferrari, D., Wesselborg, S., Bauer, M.K. and Schulze-Osthoff, K. (1997) Extracellular ATP activates transcription factor NF-κB through the P2Z purinoreceptor by selectively targeting NF-κB p65. *Journal of Cell Biology* **139**: 1635-1643.

Ferrari, D., Los, M., Bauer, M.K., Vandenabeele, P., Wesselborg, S. and Schulze-Osthoff, K. (1999a) P2Z purinoreceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death. *FEBS Letters* **447**: 71-75.

Ferrari, D., Stroh, C. and Schulze-Osthoff, K. (1999b) $P2X_7$/P2Z purinoreceptor-mediated activation of transcription factor NFAT in microglial cells. *Journal of Biological Chemistry* **274**: 13205-13210.

Ferrari, D., Idzko, M., Dichmann, S., Purlis, D., Virchow, C., Norgauer, J., Chiozzi, P., Di Virgilio, F. and Luttmann, W. (2000) P2 purinergic receptors of human eosinophils: characterization and coupling to oxygen radical production. *FEBS Letters* **486**: 217-224.

Foley, V.L. and Remington, J.S. (1969) Plaquing of *Toxoplasma gondii* in secondary cultures of chick embryo fibroblasts. *Journal of Bacteriology* **98**: 1-3.

Forsgren, M., Gille, E., Ljungstrom, I. and Nokes, D.J. (1991) Toxoplasma gondii antibodies in pregnant women in Stockholm in 1969, 1979, and 1987. *Lancet* **337**: 1413-1414.

Friis, R.R. (1972) Interaction of L cells and Chlamydia psittaci: entry of the parasite and host responses to its development. *Journal of Bacteriology* **110**: 706-721.

Fung, H.B. and Kirschenbaum, H.L. (1996) Treatment regimens for patients with toxoplasmic encephalitis. *Clinical Therapeutics* **18**: 1037-1056.

Gallant, J.E., Moore, R.D. and Chaisson, R.E. (1994) Prophylaxis for opportunistic infections in patients with HIV infection. *Annals of Internal Medicine* **120**: 932-944.

Gavrilescu, L.C. and Denkers, E.Y. (2001) IFN-γ Overproduction and High Level Apoptosis Are Associated with High but Not Low Virulence Toxoplasma gondii Infection. *Journal of Immunology* **167**: 902-909.

Gazzinelli, R.T., Eltoum, I., Wynn, T.A. and Sher, A. (1993) Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-α and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. *Journal of Immunology* **151**: 3672-3681.

Gazzinelli, R.T., Wysocka, M., Hayashi, S., Denkers, E.Y., Hieny, S., Caspar, P., Trinchieri, G. and Sher, A. (1994) Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with Toxoplasma gondii. *Journal of Immunology* **153**: 2533-2543.

Gazzinelli, R.T., Wysocka, M., Hieny, S., Scharton-Kersten, T., Cheever, A., Kuhn, R., Muller, W., Trinchieri, G. and Sher, A. (1996) In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-γ and TNF-α. *Journal of Immunology* **157**: 798-805.

Georgiev, V.S. (1994) Management of toxoplasmosis. *Drugs* **48**: 179-188.

Ghalib, H.W., Whittle, J.A., Kubin, M., Hashim, F.A., el-Hassan, A.M., Grabstein, K.H., Trinchieri, G. and Reed, S.G. (1995) IL-12 enhances Th1-type responses in human Leishmania donovani infections. *Journal of Immunology* **154**: 4623-4629.

Ghayur, T., Banerjee, S., Hugunin, M., Butler, D., Herzog, L., Carter, A., Quintal, L., Sekut, L., Talanian, R., Paskind, M., Wong, W., Kamen, R., Tracey, D. and Allen, H. (1997) Caspase-1 processes IFN-γ-inducing factor and regulates LPS-induced IFN-γ production. *Nature* **386**: 619-623.

Gilbert, R.E. and Peckham, C.S. (2002) Congenital toxoplasmosis in the United Kingdom: to screen or not to screen? *Journal of Medical Screening* **9**: 135-141.

Giordano, L.F.C., Lasmar, E.P., Tavora, E.R.F. and Lasmar, M.F. (2002) Toxoplasmosis transmitted via kidney allograft: case report and review. *Transplantation Proceedings* **34**: 498-499.

Goebel, S., Gross, U. and Luder, C.G. (2001) Inhibition of host cell apoptosis by Toxoplasma gondii is accompanied by reduced activation of the caspase cascade

and alterations of poly(ADP-ribose) polymerase expression. *Journal of Cell Science* **114**: 3495-3505.

Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis. *Science* **281**: 1309-1312.

Grigg, M.E., Ganatra, J., Boothroyd, J.C. and Margolis, T.P. (2001) Unusual abundance of atypical strains associated with human ocular toxoplasmosis. *Journal of Infectious Diseases* **184**: 633-639.

Groschel-Stewart, U., Bardini, M., Robson, T. and Burnstock, G. (1999) Localisation of $P2X_5$ and $P2X_7$ receptors by immunohistochemistry in rat stratified squamous epithelia. *Cell & Tissue Research* **296**: 599-605.

Gu, B., Bendall, L.J. and Wiley, J.S. (1998) Adenosine triphosphate-induced shedding of CD23 and L-selectin (CD62L) from lymphocytes is mediated by the same receptor but different metalloproteases. *Blood* **92**: 946-951.

Gu, B.J., Zhang, W., Worthington, R.A., Sluyter, R., Dao-Ung, P., Petrou, S., Barden, J.A. and Wiley, J.S. (2001) A Glu-496 to Ala polymorphism leads to loss of function of the human $P2X_7$ receptor. *Journal of Biological Chemistry* **276**: 11135-11142.

Gubbels, M.-J., Li, C. and Striepen, B. (2003) High-throughput growth assay for *Toxoplasma gondii* using yellow fluorescent protein. *Antimicrobial Agents & Chemotherapy* **47**: 309-316.

Guo, C., Masin, M., Qureshi, O.S. and Murrell-Lagnado, R.D. (2007) Evidence for functional $P2X_4/P2X_7$ heteromeric receptors. *Molecular Pharmacology* **72**: 1447-1456.

Hakes, T.B. and Armstrong, D. (1983) Toxoplasmosis. Problems in diagnosis and treatment. *Cancer* **52**: 1535-1540.

Handman, E. and Remington, J.S. (1980) Antibody responses to toxoplasma antigens in mice infected with strains of different virulence. *Infection & Immunity* **29**: 215-220.

Handman, E. (1999) Cell biology of Leishmania. *Advances in Parasitology* **44**: 1-39.

Harpey, J.P., Darbois, Y. and Lefebvre, G. (1983) Teratogenicity of pyrimethamine. *Lancet* **2**: 399.

Haumont, M., Delhaye, L., Garcia, L., Jurado, M., Mazzu, P., Daminet, V., Verlant, V., Bollen, A., Biemans, R. and Jacquet, A. (2000) Protective immunity against

congenital toxoplasmosis with recombinant SAG1 protein in a guinea pig model. *Infection & Immunity* **68**: 4948-4953.

Herwaldt, B.L. (1999) Leishmaniasis. *Lancet* **354**: 1191-1199.

Hewinson, J. and Mackenzie, A.B. (2007) $P2X_7$ receptor-mediated reactive oxygen and nitrogen species formation: from receptor to generators. *Biochemical Society Transactions* **35**: 1168-1170.

Hogquist, K.A., Nett, M.A., Unanue, E.R. and Chaplin, D.D. (1991) Interleukin 1 is processed and released during apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **88**: 8485-8489.

Holliman, R.E. (1995) Congenital toxoplasmosis: prevention, screening and treatment. *Journal of Hospital Infection* **30 (Supplement)**: 179-190.

Howe, D.K. and Sibley, L.D. (1995) Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease. *Journal of Infectious Diseases* **172**: 1561-1566.

Howe, D.K., Summers, B.C. and Sibley, L.D. (1996) Acute virulence in mice is associated with markers on chromosome VIII in *Toxoplasma gondii*. *Infection & Immunity* **64**: 5193-5198.

Howe, D.K., Honore, S., Derouin, F. and Sibley, L.D. (1997) Determination of genotypes of Toxoplasma gondii strains isolated from patients with toxoplasmosis. *Journal of Clinical Microbiology* **35**: 1411-1414.

Hsieh, C.S., Macatonia, S.E., Tripp, C.S., Wolf, S.F., O'Garra, A. and Murphy, K.M. (1993) Development of TH1 $CD4^+$ T cells through IL-12 produced by Listeria-induced macrophages. *Science* **260**: 547-549.

Humphreys, B.D. and Dubyak, G.R. (1998) Modulation of $P2X_7$ nucleotide receptor expression by pro- and anti-inflammatory stimuli in THP-1 monocytes. *Journal of Leukocyte Biology* **64**: 265-273.

Humphreys, B.D., Rice, J., Kertesy, S.B. and Dubyak, G.R. (2000) Stress-activated protein kinase/JNK activation and apoptotic induction by the macrophage $P2X_7$ nucleotide receptor. *Journal of Biological Chemistry* **275**: 26792-26798.

Hunter, C.A., Chizzonite, R. and Remington, J.S. (1995) IL-1β is required for IL-12 to induce production of IFN-γ by NK cells. A role for IL-1β in the T cell-independent mechanism of resistance against intracellular pathogens. *Journal of Immunology* **155**: 4347-4354.

Iglesias, R., Locovei, S., Roque, A., Alberto, A.P., Dahl, G., Spray, D.C. and Scemes, E. (2008) $P2X_7$ receptor-Pannexin1 complex: pharmacology and signaling. *American Journal of Physiology - Cell Physiology* **295**: C752-760.

Ip, Y.T. and Davis, R.J. (1998) Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. *Current Opinion in Cell Biology* **10**: 205-219.

Ismael, A.B., Sekkai, D., Collin, C., Bout, D. and Mevelec, M.-N. (2003) The MIC3 gene of Toxoplasma gondii is a novel potent vaccine candidate against toxoplasmosis. *Infection & Immunity* **71**: 6222-6228.

Israelski, D.M. and Remington, J.S. (1993) Toxoplasmosis in patients with cancer. *Clinical Infectious Diseases* **17 (Supplement 2)**: S423-435.

Jamieson, G.P., Snook, M.B., Thurlow, P.J. and Wiley, J.S. (1996) Extracellular ATP causes of loss of L-selectin from human lymphocytes via occupancy of P2Z purinocepters. *Journal of Cellular Physiology* **166**: 637-642.

Jamieson, S.E., de Roubaix, L.-A., Cortina-Borja, M., Tan, H.K., Mui, E.J., Cordell, H.J., Kirisits, M.J., Miller, E.N., Peacock, C.S., Hargrave, A.C., Coyne, J.J., Boyer, K., Bessieres, M.-H., Buffolano, W., Ferret, N., Franck, J., Kieffer, F., Meier, P., Nowakowska, D.E., Paul, M., Peyron, F., Stray-Pedersen, B., Prusa, A.-R., Thulliez, P., Wallon, M., Petersen, E., McLeod, R., Gilbert, R.E. and Blackwell, J.M. (2008) Genetic and epigenetic factors at COL2A1 and ABCA4 influence clinical outcome in congenital toxoplasmosis. *PLoS ONE [Electronic Resource]* **3**: e2285.

Janssen, R., Van Wengen, A., Verhard, E., De Boer, T., Zomerdijk, T., Ottenhoff, T.H.M. and Van Dissel, J.T. (2002) Divergent role for TNF-α in IFN-γ-induced killing of *Toxoplasma gondii* and *Salmonella typhimurium* contributes to selective susceptibility of patients with partial IFN-γ receptor 1 deficiency. *Journal of Immunology* **169**: 3900-3907.

Johnson, A.M. (1984) Strain-dependent, route of challenge-dependent, murine susceptibility to toxoplasmosis. *Zeitschrift fur Parasitenkunde* **70**: 303-309.

Johnson, A.M. (1997) Speculation on possible life cycles for the clonal lineages in the genus *Toxoplasma*. *Parasitology Today* **13**: 393-397.

Johnson, M., Broady, K., Angelici, M.C. and Johnson, A. (2003) The relationship between nucleoside triphosphate hydrolase (NTPase) isoform and Toxoplasma

strain virulence in rat and human toxoplasmosis. *Microbes & Infection* **5**: 797-806.

Jones, J.L., Kruszon-Moran, D., Wilson, M., McQuillan, G., Navin, T. and McAuley, J.B. (2001) Toxoplasma gondii infection in the United States: seroprevalence and risk factors. *American Journal of Epidemiology* **154**: 357-365.

Jones, T.C., Johnson, W.D., Jr., Barretto, A.C., Lago, E., Badaro, R., Cerf, B., Reed, S.G., Netto, E.M., Tada, M.S., Franca, T.F. and et al. (1987) Epidemiology of American cutaneous leishmaniasis due to Leishmania braziliensis braziliensis. *Journal of Infectious Diseases* **156**: 73-83.

Kahlenberg, J.M. and Dubyak, G.R. (2004) Mechanisms of caspase-1 activation by $P2X_7$ receptor-mediated K^+ release. *American Journal of Physiology - Cell Physiology* **286**: C1100-1108.

Kane, L.P., Shapiro, V.S., Stokoe, D. and Weiss, A. (1999) Induction of NF-κB by the Akt/PKB kinase. *Current Biology* **9**: 601-604.

Karin, M. and Ben-Neriah, Y. (2000) Phosphorylation meets ubiquitination: the control of NF-κB activity. *Annual Review of Immunology* **18**: 621-663.

Kasper, L.H. and Buzoni-Gatel, D. (1998) Some Opportunistic Parasitic Infections in AIDS: Candidiasis, Pneumocystosis, Cryptosporidiosis, Toxoplasmosis. *Parasitology Today* **14**: 150-156.

Kato, S. and Bowman, D.D. (2002) Using flow cytometry to determine the viability of Cryptosporidium parvum oocysts extracted from spiked environmental samples in chambers. *Parasitology Research* **88**: 326-331.

Kaufmann, A., Musset, B., Limberg, S.H., Renigunta, V., Sus, R., Dalpke, A.H., Heeg, K.M., Robaye, B. and Hanley, P.J. (2005) "Host tissue damage" signal ATP promotes non-directional migration and negatively regulates toll-like receptor signaling in human monocytes. *Journal of Biological Chemistry* **280**: 32459-32467.

Keller, P., Schaumburg, F., Fischer, S.F., Hacker, G., Gross, U. and Luder, C.G.K. (2006) Direct inhibition of cytochrome c-induced caspase activation in vitro by Toxoplasma gondii reveals novel mechanisms of interference with host cell apoptosis. *FEMS Microbiology Letters* **258**: 312-319.

Khakh, B.S., Bao, X.R., Labarca, C. and Lester, H.A. (1999) Neuronal P2X transmitter-gated cation channels change their ion selectivity in seconds. *Nature Neuroscience* **2**: 322-330.

Khakh, B.S. and North, R.A. (2006) P2X receptors as cell-surface ATP sensors in health and disease. *Nature* **442**: 527-532.

Khan, I.A., Matsuura, T. and Kasper, L.H. (1994) Interleukin-12 enhances murine survival against acute toxoplasmosis. *Infection & Immunity* **62**: 1639-1642.

Kim, L. and Denkers, E.Y. (2006) Toxoplasma gondii triggers Gi-dependent PI 3-kinase signaling required for inhibition of host cell apoptosis. *Journal of Cell Science* **119**: 2119-2126.

Kim, Y.M., Im, J.Y., Han, S.H., Kang, H.S. and Choi, I. (2000) IFN-γ up-regulates IL-18 gene expression via IFN consensus sequence-binding protein and activator protein-1 elements in macrophages. *Journal of Immunology* **165**: 3198-3205.

Kitaoka, Y., Munemasa, Y., Nakazawa, T. and Ueno, S. (2007) NMDA-induced interleukin-1β expression is mediated by nuclear factor-κ B p65 in the retina. *Brain Research* **1142**: 247-255.

Kravetz, J.D. and Federman, D.G. (2005) Toxoplasmosis in pregnancy. *American Journal of Medicine* **118**: 212-216.

Kuhns, D.B., Wright, D.G., Nath, J., Kaplan, S.S. and Basford, R.E. (1988) ATP induces transient elevations of $[Ca^{2+}]i$ in human neutrophils and primes these cells for enhanced O_2^- generation. *Laboratory Investigation* **58**: 448-453.

Kunapuli, S.P., Dorsam, R.T., Kim, S. and Quinton, T.M. (2003) Platelet purinergic receptors. *Current Opinion in Pharmacology* **3**: 175-180.

la Sala, A., Ferrari, D., Di Virgilio, F., Idzko, M., Norgauer, J. and Girolomoni, G. (2003) Alerting and tuning the immune response by extracellular nucleotides. *Journal of Leukocyte Biology* **73**: 339-343.

Labasi, J.M., Petrushova, N., Donovan, C., McCurdy, S., Lira, P., Payette, M.M., Brissette, W., Wicks, J.R., Audoly, L. and Gabel, C.A. (2002) Absence of the $P2X_7$ receptor alters leukocyte function and attenuates an inflammatory response. *Journal of Immunology* **168**: 6436-6445.

Lammas, D.A., Stober, C., Harvey, C.J., Kendrick, N., Panchalingam, S. and Kumararatne, D.S. (1997) ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z($P2X_7$) receptors. *Immunity* **7**: 433-444.

Lang, R., Patel, D., Morris, J.J., Rutschman, R.L. and Murray, P.J. (2002) Shaping gene expression in activated and resting primary macrophages by IL-10. *Journal of Immunology* **169**: 2253-2263.

Langermans, J.A., Nibbering, P.H., Van Vuren-Van Der Hulst, M.E. and Van Furth, R. (2001) Transforming growth factor-beta suppresses interferon-gamma-induced toxoplasmastatic activity in murine macrophages by inhibition of tumour necrosis factor-alpha production. *Parasite Immunology* **23**: 169-175.

Le Stunff, H., Auger, R., Kanellopoulos, J. and Raymond, M.-N. (2004) The Pro-451 to Leu polymorphism within the C-terminal tail of $P2X_7$ receptor impairs cell death but not phospholipase D activation in murine thymocytes. *Journal of Biological Chemistry* **279**: 16918-16926.

Lecordier, L., Mercier, C., Sibley, L.D. and Cesbron-Delauw, M.F. (1999) Transmembrane insertion of the Toxoplasma gondii GRA5 protein occurs after soluble secretion into the host cell. *Molecular Biology of the Cell* **10**: 1277-1287.

Lees, M.P. (2004) A role for the $P2X_7$ receptor in host cell clearance of *Toxoplasma gondii*. **Honours Thesis**. *University of Technology, Sydney, Faculty of Science*.

Leport, C., Raffi, F., Matheron, S., Katlama, C., Regnier, B., Saimot, A.G., Marche, C., Vedrenne, C. and Vilde, J.L. (1988) Treatment of central nervous system toxoplasmosis with pyrimethamine/sulfadiazine combination in 35 patients with the acquired immunodeficiency syndrome. Efficacy of long-term continuous therapy. *American Journal of Medicine* **84**: 94-100.

Letscher-Bru, V., Villard, O., Risse, B., Zauke, M., Klein, J.P. and Kien, T.T. (1998) Protective effect of vaccination with a combination of recombinant surface antigen 1 and interleukin-12 against toxoplasmosis in mice. *Infection & Immunity* **66**: 4503-4506.

Li, C.M., Campbell, S.J., Kumararatne, D.S., Bellamy, R., Ruwende, C., McAdam, K.P.W.J., Hill, A.V.S. and Lammas, D.A. (2002) Association of a polymorphism in the $P2X_7$ gene with tuberculosis in a Gambian population. *Journal of Infectious Diseases* **186**: 1458-1462.

Lieberman, J. (2003) The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nature Reviews* **Immunology. 3**: 361-370.

Lieberman, L.A. and Hunter, C.A. (2002) The role of cytokines and their signaling pathways in the regulation of immunity to Toxoplasma gondii. *International Reviews of Immunology* **21**: 373-403.

Liese, J., Schleicher, U. and Bogdan, C. (2008) The innate immune response against Leishmania parasites. *Immunobiology* **213**: 377-387.

Liesenfeld, O., Kosek, J.C. and Suzuki, Y. (1997) Gamma interferon induces Fas-dependent apoptosis of Peyer's patch T cells in mice following peroral infection with Toxoplasma gondii. *Infection & Immunity* **65**: 4682-4689.

Locovei, S., Scemes, E., Qiu, F., Spray, D.C. and Dahl, G. (2007) Pannexin1 is part of the pore forming unit of the $P2X_7$ receptor death complex. *FEBS Letters* **581**: 483-488.

Lovett, J.L. and Sibley, L.D. (2003) Intracellular calcium stores in Toxoplasma gondii govern invasion of host cells. *Journal of Cell Science* **116**: 3009-3016.

Luder, C.G.K., Algner, M., Lang, C., Bleicher, N. and Gross, U. (2003) Reduced expression of the inducible nitric oxide synthase after infection with *Toxoplasma gondii* facilitates parasite replication in activated murine macrophages. *International Journal for Parasitology* **33**: 833-844.

Luder, C.G.K. and Gross, U. (2005) Apoptosis and its modulation during infection with Toxoplasma gondii: molecular mechanisms and role in pathogenesis. *Current Topics in Microbiology & Immunology* **289**: 219-237.

Luft, B.J. and Remington, J.S. (1992) Toxoplasmic encephalitis in AIDS. *Clinical Infectious Diseases* **15**: 211-222.

Lustig, K.D., Shiau, A.K., Brake, A.J. and Julius, D. (1993) Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proceedings of the National Academy of Sciences of the United States of America* **90**: 5113-5117.

Lyons, R.E. and Johnson, A.M. (1995) Heat shock proteins of Toxoplasma gondii. *Parasite Immunology* **17**: 353-359.

Malmgren, R. (1986) ATP-secretion occurs as an initial response in collagen induced platelet activation. *Thrombosis Research* **43**: 445-453.

Martins, G.A., Vieira, L.Q., Cunha, F.Q. and Silva, J.S. (1999) Gamma interferon modulates CD95 (Fas) and CD95 ligand (Fas-L) expression and nitric oxide-induced apoptosis during the acute phase of Trypanosoma cruzi infection: a possible role in immune response control. *Infection & Immunity* **67**: 3864-3871.

Masihi, K.N. and Werner, H. (1977) Immunization of NMRI mice against virulent Toxoplasma gondii. Differing efficacy of eleven cyst-forming Toxoplasma strains. *Zeitschrift fur Parasitenkunde* **54**: 209-216.

Mayes, J.T., O'Connor, B.J., Avery, R., Castellani, W. and Carey, W. (1995) Transmission of Toxoplasma gondii infection by liver transplantation. *Clinical Infectious Diseases* **21**: 511-515.

Mehta, V.B., Hart, J. and Wewers, M.D. (2001) ATP-stimulated release of interleukin (IL)-1β and IL-18 requires priming by lipopolysaccharide and is independent of caspase-1 cleavage. *Journal of Biological Chemistry* **276**: 3820-3826.

Messmer, U.K. and Pfeilschifter, J. (2000) New insights into the mechanism for clearance of apoptotic cells. *Bioessays* **22**: 878-881.

Mokrousov, I., Sapozhnikova, N. and Narvskaya, O. (2008) *Mycobacterium tuberculosis* co-existence with humans: making an imprint on the macrophage $P2X_7$ receptor gene? *Journal of Medical Microbiology* **57**: 581-584.

Molestina, R.E., Payne, T.M., Coppens, I. and Sinai, A.P. (2003) Activation of NF-κB by Toxoplasma gondii correlates with increased expression of antiapoptotic genes and localization of phosphorylated IκB to the parasitophorous vacuole membrane. *Journal of Cell Science* **116**: 4359-4371.

Montoya, J.G. and Liesenfeld, O. (2004) Toxoplasmosis. *Lancet* **363**: 1965-1976.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L. and O'Garra, A. (2001) Interleukin-10 and the interleukin-10 receptor. *Annual Review of Immunology* **19**: 683-765.

Mordue, D.G. and Sibley, L.D. (1997) Intracellular fate of vacuoles containing Toxoplasma gondii is determined at the time of formation and depends on the mechanism of entry. *Journal of Immunology* **159**: 4452-4459.

Mordue, D.G., Hakansson, S., Niesman, I. and Sibley, L.D. (1999) Toxoplasma gondii resides in a vacuole that avoids fusion with host cell endocytic and exocytic vesicular trafficking pathways. *Experimental Parasitology* **92**: 87-99.

Morisaki, J.H., Heuser, J.E. and Sibley, L.D. (1995) Invasion of Toxoplasma gondii occurs by active penetration of the host cell. *Journal of Cell Science* **108**: 2457-2464.

Munoz, P., Arencibia, J., Rodriguez, C., Rivera, M., Palomo, J., Yanez, J. and Bouza, E. (2003) Trimethoprim-sulfamethoxazole as toxoplasmosis prophylaxis for heart transplant recipients. *Clinical Infectious Diseases* **36**: 932-933; author reply 933.

Murray, H.W., Berman, J.D., Davies, C.R. and Saravia, N.G. (2005) Advances in leishmaniasis. *Lancet* **366**: 1561-1577.

Mutini, C., Falzoni, S., Ferrari, D., Chiozzi, P., Morelli, A., Baricordi, O.R., Collo, G., Ricciardi-Castagnoli, P. and Di Virgilio, F. (1999) Mouse dendritic cells express the $P2X_7$ purinergic receptor: characterization and possible participation in antigen presentation. *Journal of Immunology* **163**: 1958-1965.

Nash, P.B., Purner, M.B., Leon, R.P., Clarke, P., Duke, R.C. and Curiel, T.J. (1998) Toxoplasma gondii-infected cells are resistant to multiple inducers of apoptosis. *Journal of Immunology* **160**: 1824-1830.

Navia, B.A., Petito, C.K., Gold, J.W., Cho, E.S., Jordan, B.D. and Price, R.W. (1986) Cerebral toxoplasmosis complicating the acquired immune deficiency syndrome: clinical and neuropathological findings in 27 patients. *Annals of Neurology* **19**: 224-238.

Nguyen, T.D., Bigaignon, G., Markine-Goriaynoff, D., Heremans, H., Nguyen, T.N., Warnier, G., Delmee, M., Warny, M., Wolf, S.F., Uyttenhove, C., Van Snick, J. and Coutelier, J.P. (2003) Virulent *Toxoplasma gondii* strain RH promotes T-cell-independent overproduction of proinflammatory cytokines IL12 and gamma-interferon. *Journal of Medical Microbiology* **52**: 869-876.

Nicke, A., Baumert, H.G., Rettinger, J., Eichele, A., Lambrecht, G., Mutschler, E. and Schmalzing, G. (1998) $P2X_1$ and $P2X_3$ receptors form stable trimers: a novel structural motif of ligand-gated ion channels. *EMBO Journal* **17**: 3016-3028.

Nicke, A. (2008) Homotrimeric complexes are the dominant assembly state of native $P2X_7$ subunits. *Biochemical & Biophysical Research Communications* **377**: 803-808.

Nicolle, C. and Manceaux, L. (1908) Sur une infection à corps de Leishman (ou organismes voisins) du gondi. *Cahiers Recherche de la Herbdomaire Séances Academie Science* **147**: 763-766.

Nino-Moreno, P., Portales-Perez, D., Hernandez-Castro, B., Portales-Cervantes, L., Flores-Meraz, V., Baranda, L., Gomez-Gomez, A., Acuna-Alonzo, V., Granados, J. and Gonzalez-Amaro, R. (2007) $P2X_7$ and NRAMP1/SLC11 A1 gene polymorphisms in Mexican mestizo patients with pulmonary tuberculosis. *Clinical & Experimental Immunology* **148**: 469-477.

North, R.A. (1996) Families of ion channels with two hydrophobic segments. *Current Opinion in Cell Biology* **8**: 474-483.

North, R.A. and Barnard, E.A. (1997) Nucleotide receptors. *Current Opinion in Neurobiology* **7**: 346-357.

North, R.A. (2002) Molecular physiology of P2X receptors. *Physiological Reviews* **82**: 1013-1067.

O'Connell, E., Wilkins, M.F. and Te Punga, W.A. (1988) Toxoplasmosis in sheep. II. The ability of a live vaccine to prevent lamb losses after an intravenous challenge with *Toxoplasma gondii*. *New Zealand Veterinary Journal* **36**: 1-4.

Oliveira, A.G.d., Galati, E.A.B., de Oliveira, O., de Oliveira, G.R., Espindola, I.A.C., Dorval, M.E.C. and Brazil, R.P. (2006) Abundance of Lutzomyia longipalpis (Diptera: Psychodidae: Phlebotominae) and urban transmission of visceral leishmaniasis in Campo Grande, state of Mato Grosso do Sul, Brazil. *Memorias do Instituto Oswaldo Cruz* **101**: 869-874.

Orlofsky, A., Weiss, L.M., Kawachi, N. and Prystowsky, M.B. (2002) Deficiency in the anti-apoptotic protein A1-a results in a diminished acute inflammatory response. *Journal of Immunology* **168**: 1840-1846.

Oshimi, Y., Miyazaki, S. and Oda, S. (1999) ATP-induced Ca^{2+} response mediated by P2U and P2Y purinoceptors in human macrophages: signalling from dying cells to macrophages. *Immunology* **98**: 220-227.

Osorio, L.E., Castillo, C.M. and Ochoa, M.T. (1998) Mucosal leishmaniasis due to Leishmania (Viannia) panamensis in Colombia: clinical characteristics. *American Journal of Tropical Medicine & Hygiene* **59**: 49-52.

Panenka, W., Jijon, H., Herx, L.M., Armstrong, J.N., Feighan, D., Wei, T., Yong, V.W., Ransohoff, R.M. and MacVicar, B.A. (2001) $P2X_7$-like receptor activation in astrocytes increases chemokine monocyte chemoattractant protein-1 expression via mitogen-activated protein kinase. *Journal of Neuroscience* **21**: 7135-7142.

Papp, L., Vizi, E.S. and Sperlagh, B. (2007) $P2X_7$ receptor mediated phosphorylation of p38MAP kinase in the hippocampus. *Biochemical & Biophysical Research Communications* **355**: 568-574.

Parks, D.R., Bryan, V.M., Oi, V.T. and Herzenberg, L.A. (1979) Antigen-specific identification and cloning of hybridomas with a fluorescence-activated cell sorter. *Proceedings of the National Academy of Sciences of the United States of America* **76**: 1962-1966.

Parvathenani, L.K., Tertyshnikova, S., Greco, C.R., Roberts, S.B., Robertson, B. and Posmantur, R. (2003) $P2X_7$ mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease. *Journal of Biological Chemistry* **278**: 13309-13317.

Payne, T.M., Molestina, R.E. and Sinai, A.P. (2003) Inhibition of caspase activation and a requirement for NF-κB function in the Toxoplasma gondii-mediated blockade of host apoptosis. *Journal of Cell Science* **116**: 4345-4358.

Pedrol, E., Gonzalez-Clemente, J.M., Gatell, J.M., Mallolas, J., Miro, J.M., Graus, F., Alvarez, R., Mercader, J.M., Berenguer, J., Jimenez de Anta, M.T. and et al. (1990) Central nervous system toxoplasmosis in AIDS patients: efficacy of an intermittent maintenance therapy. *AIDS* **4**: 511-517.

Pelegrin, P. and Surprenant, A. (2006) Pannexin-1 mediates large pore formation and interleukin-1β release by the ATP-gated $P2X_7$ receptor. *EMBO Journal* **25**: 5071-5082.

Pelegrin, P. and Surprenant, A. (2007) Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1β release through a dye uptake-independent pathway. *Journal of Biological Chemistry* **282**: 2386-2394.

Peng, B.W., Lin, J., Lin, J.Y., Jiang, M.S. and Zhang, T. (2003) Exogenous nitric oxide induces apoptosis in *Toxoplasma gondii* tachyzoites via a calcium signal transduction pathway. *Parasitology* **126**: 541-550.

Perregaux, D. and Gabel, C.A. (1994) Interleukin-1β maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *Journal of Biological Chemistry* **269**: 15195-15203.

Perregaux, D.G., McNiff, P., Laliberte, R., Conklyn, M. and Gabel, C.A. (2000) ATP acts as an agonist to promote stimulus-induced secretion of IL-1β and IL-18 in human blood. *Journal of Immunology* **165**: 4615-4623.

Petersen, E., Nielsen, H.V., Christiansen, L. and Spenter, J. (1998) Immunization with E. coli produced recombinant T. gondii SAG1 with alum as adjuvant protect mice against lethal infection with Toxoplasma gondii. *Vaccine* **16**: 1283-1289.

Petrou, S., Ugur, M., Drummond, R.M., Singer, J.J. and Walsh, J.V., Jr. (1997) $P2X_7$ purinoceptor expression in Xenopus oocytes is not sufficient to produce a pore-forming P2Z-like phenotype. *FEBS Letters* **411**: 339-345.

Petrovay, F. and Balla, E. (2008) Two fatal cases of psittacosis caused by Chlamydophila psittaci. *Journal of Medical Microbiology* **57**: 1296-1298.

Peyron, F., Wallon, M. and Bernardoux, C. (1996) Long-term follow-up of patients with congenital ocular toxoplasmosis. *New England Journal of Medicine* **334**: 993-994.

Pfefferkorn, E.R. and Pfefferkorn, L.C. (1976) *Toxoplasma gondii*: isolation and preliminary characterization of temperature-sensitive mutants. *Experimental Parasitology* **39**: 365-376.

Pfefferkorn, E.R. and Kasper, L.H. (1983) *Toxoplasma gondii*: genetic crosses reveal phenotypic suppression of hydroxyurea resistance by fluorodeoxyuridine resistance. *Experimental Parasitology* **55**: 207-218.

Pfefferkorn, E.R. and Guyre, P.M. (1984) Inhibition of growth of *Toxoplasma gondii* in cultured fibroblasts by human recombinant gamma interferon. *Infection & Immunity* **44**: 211-216.

Pfefferkorn, L.C. and Pfefferkorn, E.R. (1980) Toxoplasma gondii: genetic recombination between drug resistant mutants. *Experimental Parasitology* **50**: 305-316.

Pfeiffer, Z.A., Guerra, A.N., Hill, L.M., Gavala, M.L., Prabhu, U., Aga, M., Hall, D.J. and Bertics, P.J. (2007) Nucleotide receptor signaling in murine macrophages is linked to reactive oxygen species generation. *Free Radical Biology & Medicine* **42**: 1506-1516.

Pinto Dias, J.C. (1995) Natural history of Chagas disease. *Arquivos Brasileiros de Cardiologia* **65**: 359-366.

Porter, S.B. and Sande, M.A. (1992) Toxoplasmosis of the central nervous system in the acquired immunodeficiency syndrome. *New England Journal of Medicine* **327**: 1643-1648.

Raschke, W.C., Baird, S., Ralph, P. and Nakoinz, I. (1978) Functional macrophage cell lines transformed by Abelson leukemia virus. *Cell* **15**: 261-267.

Rassendren, F., Buell, G.N., Virginio, C., Collo, G., North, R.A. and Surprenant, A. (1997) The permeabilizing ATP receptor, $P2X_7$. Cloning and expression of a human cDNA. *Journal of Biological Chemistry* **272**: 5482-5486.

Reis e Sousa, C., Hieny, S., Scharton-Kersten, T., Jankovic, D., Charest, H., Germain, R.N. and Sher, A. (1997) In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas. *Journal of Experimental Medicine* **186**: 1819-1829.

Reithinger, R., Dujardin, J.-C., Louzir, H., Pirmez, C., Alexander, B. and Brooker, S. (2007) Cutaneous leishmaniasis. *The Lancet Infectious Diseases* **7**: 581-596.

Ribeiro, B.M., Crema, E. and Rodrigues, V., Jr. (2008) Analysis of the cellular immune response in patients with the digestive and indeterminate forms of Chagas' disease. *Human Immunology* **69**: 484-489.

Ricci, M., Pentimalli, H., Thaller, R., Rava, L. and Di Ciommo, V. (2003) Screening and prevention of congenital toxoplasmosis: an effectiveness study in a population with a high infection rate. *Journal of Maternal-Fetal & Neonatal Medicine* **14**: 398-403.

Robben, P.M., Mordue, D.G., Truscott, S.M., Takeda, K., Akira, S. and Sibley, L.D. (2004) Production of IL-12 by macrophages infected with *Toxoplasma gondii* depends on the parasite genotype. *Journal of Immunology* **172**: 3686-3694.

Rockett, K.A., Awburn, M.M., Rockett, E.J., Cowden, W.B. and Clark, I.A. (1994) Possible role of nitric oxide in malarial immunosuppression. *Parasite Immunology* **16**: 243-249.

Roth, B.L., Poot, M., Yue, S.T. and Millard, P.J. (1997) Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain. *Applied & Environmental Microbiology* **63**: 2421-2431.

Sacks, D. and Sher, A. (2002) Evasion of innate immunity by parasitic protozoa. *Nature Immunology* **3**: 1041-1047.

Saeij, J.P., Boyle, J.P., Coller, S., Taylor, S., Sibley, L.D., Brooke-Powell, E.T., Ajioka, J.W. and Boothroyd, J.C. (2006) Polymorphic secreted kinases are key virulence factors in toxoplasmosis. *Science* **314**: 1780-1783.

Saeij, J.P., Coller, S., Boyle, J.P., Jerome, M.E., White, M.W. and Boothroyd, J.C. (2007) Toxoplasma co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature* **445**: 324-327.

Saunders, B.M., Fernando, S.L., Sluyter, R., Britton, W.J. and Wiley, J.S. (2003) A loss-of-function polymorphism in the human $P2X_7$ receptor abolishes ATP-mediated killing of mycobacteria. *Journal of Immunology* **171**: 5442-5446.

Scanga, C.A., Aliberti, J., Jankovic, D., Tilloy, F., Bennouna, S., Denkers, E.Y., Medzhitov, R. and Sher, A. (2002) Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. *Journal of Immunology* **168**: 5997-6001.

Scharton-Kersten, T.M., Wynn, T.A., Denkers, E.Y., Bala, S., Grunvald, E., Hieny, S., Gazzinelli, R.T. and Sher, A. (1996) In the absence of endogenous IFN-γ, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to control acute infection. *Journal of Immunology* **157**: 4045-4054.

Schmunis, G.A. (1991) Trypanosoma cruzi, the etiologic agent of Chagas' disease: status in the blood supply in endemic and nonendemic countries. *Transfusion* **31**: 547-557.

Schulze-Lohoff, E., Hugo, C., Rost, S., Arnold, S., Gruber, A., Brune, B. and Sterzel, R.B. (1998) Extracellular ATP causes apoptosis and necrosis of cultured mesangial cells via P2Z/P2X_7 receptors. *American Journal of Physiology* **275**: F962-971.

Scorza, T., D'Souza, S., Laloup, M., Dewit, J., De Braekeleer, J., Verschueren, H., Vercammen, M., Huygen, K. and Jongert, E. (2003) A GRA1 DNA vaccine primes cytolytic $CD8^+$ T cells to control acute Toxoplasma gondii infection. *Infection & Immunity* **71**: 309-316.

Scott, P. (1991) IFN-γ modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *Journal of Immunology* **147**: 3149-3155.

Seder, R.A., Gazzinelli, R., Sher, A. and Paul, W.E. (1993) Interleukin 12 acts directly on $CD4^+$ T cells to enhance priming for interferon γ production and diminishes interleukin 4 inhibition of such priming. *Proceedings of the National Academy of Sciences of the United States of America* **90**: 10188-10192.

Shemon, A.N., Sluyter, R., Fernando, S.L., Clarke, A.L., Dao-Ung, L.-P., Skarratt, K.K., Saunders, B.M., Tan, K.S., Gu, B.J., Fuller, S.J., Britton, W.J., Petrou, S. and Wiley, J.S. (2006) A Thr357 to Ser polymorphism in homozygous and compound heterozygous subjects causes absent or reduced P2X_7 function and impairs ATP-induced mycobacterial killing by macrophages. *Journal of Biological Chemistry* **281**: 2079-2086.

Shiono, Y., Mun, H.-S., He, N., Nakazaki, Y., Fang, H., Furuya, M., Aosai, F. and Yano, A. (2007) Maternal-fetal transmission of *Toxoplasma gondii* in interferon-γ deficient pregnant mice. *Parasitology International* **56**: 141-148.

Sibley, L.D., Weidner, E. and Krahenbuhl, J.L. (1985) Phagosome acidification blocked by intracellular Toxoplasma gondii. *Nature* **315**: 416-419.

Sibley, L.D., Adams, L.B., Fukutomi, Y. and Krahenbuhl, J.L. (1991) Tumor necrosis factor-α triggers antitoxoplasmal activity of IFN-γ primed macrophages. *Journal of Immunology* **147**: 2340-2345.

Sibley, L.D. and Boothroyd, J.C. (1992) Virulent strains of Toxoplasma gondii comprise a single clonal lineage. *Nature* **359**: 82-85.

Sibley, L.D., Niesman, I.R., Parmley, S.F. and Cesbron-Delauw, M.F. (1995) Regulated secretion of multi-lamellar vesicles leads to formation of a tubulo-vesicular network in host-cell vacuoles occupied by Toxoplasma gondii. *Journal of Cell Science* **108**: 1669-1677.

Silva, J.S., Aliberti, J.C., Martins, G.A., Souza, M.A., Souto, J.T. and Padua, M.A. (1998) The role of IL-12 in experimental Trypanosoma cruzi infection. *Brazilian Journal of Medical & Biological Research* **31**: 111-115.

Simon, J., Webb, T.E. and Barnard, E.A. (1995a) Characterization of a P2Y purinoceptor in the brain. *Pharmacology & Toxicology* **76**: 302-307.

Simon, J., Webb, T.E., King, B.F., Burnstock, G. and Barnard, E.A. (1995b) Characterisation of a recombinant P2Y purinoceptor. *European Journal of Pharmacology* **291**: 281-289.

Skaper, S.D., Facci, L., Culbert, A.A., Evans, N.A., Chessell, I., Davis, J.B. and Richardson, J.C. (2006) $P2X_7$ receptors on microglial cells mediate injury to cortical neurons in vitro. *GLIA* **54**: 234-242.

Sluyter, R. and Wiley, J.S. (2002) Extracellular adenosine 5'-triphosphate induces a loss of CD23 from human dendritic cells via activation of $P2X_7$ receptors. *International Immunology* **14**: 1415-1421.

Sluyter, R., Dalitz, J.G. and Wiley, J.S. (2004a) $P2X_7$ receptor polymorphism impairs extracellular adenosine 5'-triphosphate-induced interleukin-18 release from human monocytes. *Genes & Immunity* **5**: 588-591.

Sluyter, R., Shemon, A.N. and Wiley, J.S. (2004b) Glu496 to Ala polymorphism in the $P2X_7$ receptor impairs ATP-induced IL-1β release from human monocytes. *Journal of Immunology* **172**: 3399-3405.

Sluyter, R., Shemon, A.N. and Wiley, J.S. (2007) $P2X_7$ receptor activation causes phosphatidylserine exposure in human erythrocytes. *Biochemical & Biophysical Research Communications* **355**: 169-173.

Smith, K.L., Wilson, M., Hightower, A.W., Kelley, P.W., Struewing, J.P., Juranek, D.D. and McAuley, J.B. (1996) Prevalence of Toxoplasma gondii antibodies in

US military recruits in 1989: comparison with data published in 1965. *Clinical Infectious Diseases* **23**: 1182-1183.

Smith, R.A., Alvarez, A.J. and Estes, D.M. (2001) The $P2X_7$ purinergic receptor on bovine macrophages mediates mycobacterial death. *Veterinary Immunology & Immunopathology* **78**: 249-262.

Soldati, D., Dubremetz, J.F. and Lebrun, M. (2001) Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite Toxoplasma gondii. *International Journal for Parasitology* **31**: 1293-1302.

Solle, M., Labasi, J., Perregaux, D.G., Stam, E., Petrushova, N., Koller, B.H., Griffiths, R.J. and Gabel, C.A. (2001) Altered cytokine production in mice lacking $P2X_7$ receptors. *Journal of Biological Chemistry* **276**: 125-132.

Stoll, S., Jonuleit, H., Schmitt, E., Muller, G., Yamauchi, H., Kurimoto, M., Knop, J. and Enk, A.H. (1998) Production of functional IL-18 by different subtypes of murine and human dendritic cells (DC): DC-derived IL-18 enhances IL-12-dependent Th1 development. *European Journal of Immunology* **28**: 3231-3239.

Strittmatter, C., Lang, W., Wiestler, O.D. and Kleihues, P. (1992) The changing pattern of human immunodeficiency virus-associated cerebral toxoplasmosis: a study of 46 postmortem cases. *Acta Neuropathologica* **83**: 475-481.

Stuart, M.C., Damoiseaux, J.G., Frederik, P.M., Arends, J.W. and Reutelingsperger, C.P. (1998) Surface exposure of phosphatidylserine during apoptosis of rat thymocytes precedes nuclear changes. *European Journal of Cell Biology* **76**: 77-83.

Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P., Haddix, P.L., Collins, H.L., Fok, A.K., Allen, R.D., Gluck, S.L., Heuser, J. and Russell, D.G. (1994) Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase.[see comment][erratum appears in Science 1994 Mar 11;263(5152):1359]. *Science* **263**: 678-681.

Suh, B.C., Kim, J.S., Namgung, U., Ha, H. and Kim, K.T. (2001) $P2X_7$ nucleotide receptor mediation of membrane pore formation and superoxide generation in human promyelocytes and neutrophils. *Journal of Immunology* **166**: 6754-6763.

Surprenant, A., Rassendren, F., Kawashima, E., North, R.A. and Buell, G. (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). *Science* **272**: 735-738.

Suss-Toby, E., Zimmerberg, J. and Ward, G.E. (1996) Toxoplasma invasion: the parasitophorous vacuole is formed from host cell plasma membrane and pinches off via a fission pore. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 8413-8418.

Suzuki, Y., Orellana, M.A., Schreiber, R.D. and Remington, J.S. (1988) Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science* **240**: 516-518.

Suzuki, Y., Sher, A., Yap, G., Park, D., Neyer, L.E., Liesenfeld, O., Fort, M., Kang, H. and Gufwoli, E. (2000) IL-10 is required for prevention of necrosis in the small intestine and mortality in both genetically resistant BALB/c and susceptible C57BL/6 mice following peroral infection with *Toxoplasma gondii*. *Journal of Immunology* **164**: 5375-5382.

Sypek, J.P., Chung, C.L., Mayor, S.E., Subramanyam, J.M., Goldman, S.J., Sieburth, D.S., Wolf, S.F. and Schaub, R.G. (1993) Resolution of cutaneous leishmaniasis: interleukin 12 initiates a protective T helper type 1 immune response. *Journal of Experimental Medicine* **177**: 1797-1802.

Takeda, K. and Akira, S. (2003) Toll receptors and pathogen resistance. *Cellular Microbiology* **5**: 143-153.

Taylor, S., Barragan, A., Su, C., Fux, B., Fentress, S.J., Tang, K., Beatty, W.L., Hajj, H.E., Jerome, M., Behnke, M.S., White, M., Wootton, J.C. and Sibley, L.D. (2006) A secreted serine-threonine kinase determines virulence in the eukaryotic pathogen *Toxoplasma gondii*. *Science* **314**: 1776-1780.

Thornberry, N.A. and Lazebnik, Y. (1998) Caspases: enemies within. *Science* **281**: 1312-1316.

Tibbetts, M.D., Zheng, L. and Lenardo, M.J. (2003) The death effector domain protein family: regulators of cellular homeostasis. *Nature Immunology* **4**: 404-409.

Tokuyama, Y., Hara, M., Jones, E.M., Fan, Z. and Bell, G.I. (1995) Cloning of rat and mouse P2Y purinoceptors. *Biochemical & Biophysical Research Communications* **211**: 211-218.

Torres, G.E., Egan, T.M. and Voigt, M.M. (1998a) N-Linked glycosylation is essential for the functional expression of the recombinant $P2X_2$ receptor. *Biochemistry* **37**: 14845-14851.

Torres, G.E., Haines, W.R., Egan, T.M. and Voigt, M.M. (1998b) Co-expression of $P2X_1$ and $P2X_5$ receptor subunits reveals a novel ATP-gated ion channel. *Molecular Pharmacology* **54**: 989-993.

Torres, G.E., Egan, T.M. and Voigt, M.M. (1999) Hetero-oligomeric assembly of P2X receptor subunits. Specificities exist with regard to possible partners. *Journal of Biological Chemistry* **274**: 6653-6659.

Torres-Santos, E.C., Mantuano, M.B., Persechini, P.M., Coutinho-Silva, R. and Rossi-Bergmann, B. (2000) Enhanced macrophage permeabilization by ATP upon leishmania infection. *Drug Development Research* **50**: 61.

Tsunawaki, S., Sporn, M., Ding, A. and Nathan, C. (1988) Deactivation of macrophages by transforming growth factor-beta. *Nature* **334**: 260-262.

Tyurina, Y.Y., Basova, L.V., Konduru, N.V., Tyurin, V.A., Potapovich, A.I., Cai, P., Bayir, H., Stoyanovsky, D., Pitt, B.R., Shvedova, A.A., Fadeel, B. and Kagan, V.E. (2007) Nitrosative stress inhibits the aminophospholipid translocase resulting in phosphatidylserine externalization and macrophage engulfment: implications for the resolution of inflammation. *Journal of Biological Chemistry* **282**: 8498-8509.

Valera, S., Hussy, N., Evans, R.J., Adami, N., North, R.A., Surprenant, A. and Buell, G. (1994) A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. *Nature* **371**: 516-519.

Van Delden, C. and Hirschel, B. (1996) Folinic acid supplements to pyrimethamine-sulfadiazine for Toxoplasma encephalitis are associated with better outcome. *Journal of Infectious Diseases* **173**: 1294-1295.

Vannier-Santos, M.A., Martiny, A. and de Souza, W. (2002) Cell biology of Leishmania spp.: invading and evading. *Current Pharmaceutical Design* **8**: 297-318.

Vastava, P.B., Pradhan, S., Jha, S., Prasad, K.N., Kumar, S. and Gupta, R.K. (2002) MRI features of toxoplasma encephalitis in the immunocompetent host: a report of two cases. *Neuroradiology* **44**: 834-838.

Vercammen, M., Scorza, T., Huygen, K., De Braekeleer, J., Diet, R., Jacobs, D., Saman, E. and Verschueren, H. (2000) DNA vaccination with genes encoding Toxoplasma gondii antigens GRA1, GRA7, and ROP2 induces partially protective immunity against lethal challenge in mice. *Infection & Immunity* **68**: 38-45.

Via, L.E., Deretic, D., Ulmer, R.J., Hibler, N.S., Huber, L.A. and Deretic, V. (1997) Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. *Journal of Biological Chemistry* **272**: 13326-13331.

Virginio, C., MacKenzie, A., Rassendren, F.A., North, R.A. and Surprenant, A. (1999) Pore dilation of neuronal P2X receptor channels. *Nature Neuroscience* **2**: 315-321.

Wallon, M., Kodjikian, L., Binquet, C., Garweg, J., Fleury, J., Quantin, C. and Peyron, F. (2004) Long-term ocular prognosis in 327 children with congenital toxoplasmosis. *Pediatrics* **113**: 1567-1572.

Ward, M.E. (1995) The immunobiology and immunopathology of chlamydial infections. *APMIS* **103**: 769-796.

Ward, P.A., Cunningham, T.W., McCulloch, K.K., Phan, S.H., Powell, J. and Johnson, K.J. (1988) Platelet enhancement of O2-. responses in stimulated human neutrophils. Identification of platelet factor as adenine nucleotide. *Laboratory Investigation* **58**: 37-47.

Wei, S., Marches, F., Borvak, J., Zou, W., Channon, J., White, M., Radke, J., Cesbron-Delauw, M.-F. and Curiel, T.J. (2002) Toxoplasma gondii-infected human myeloid dendritic cells induce T-lymphocyte dysfunction and contact-dependent apoptosis. *Infection & Immunity* **70**: 1750-1760.

Weinrach, D.M. and Oviedo, A. (2001) Toxoplasmosis in a bone marrow transplant patient. *Archives of Pathology & Laboratory Medicine* **125**: 707.

Weiss, L.M., Ma, Y.F., Takvorian, P.M., Tanowitz, H.B. and Wittner, M. (1998) Bradyzoite development in Toxoplasma gondii and the hsp70 stress response. *Infection & Immunity* **66**: 3295-3302.

West, S.A., Reece, S.E. and Read, A.F. (2003) Toxoplasma gondii, sex and premature rejection. *Trends in Parasitology* **19**: 155-157; discussion 157-158.

Whiteside, J.D. and Begent, R.H. (1975) Toxoplasma encephalitis complicating Hodgkin's disease. *Journal of Clinical Pathology* **28**: 443-445.

WHO (2001) Global Prevalence and Incidence of Selected Curable Sexually Transmitted Infections: Overview and Estimates. Geneva.

WHO (2004) The world health report 2004. Changing history. Geneva.

WHO (2007) Global tuberculosis control - surveillance, planning, financing. Geneva/New York/Paris.

Wiley, J.S., Gargett, C.E., Zhang, W., Snook, M.B. and Jamieson, G.P. (1998) Partial agonists and antagonists reveal a second permeability state of human lymphocyte P2Z/P2X$_7$ channel. *American Journal of Physiology* **275**: C1224-1231.

Wiley, J.S., Dao-Ung, L.P., Gu, B.J., Sluyter, R., Shemon, A.N., Li, C., Taper, J., Gallo, J. and Manoharan, A. (2002) A loss-of-function polymorphic mutation in the cytolytic P2X$_7$ receptor gene and chronic lymphocytic leukaemia: a molecular study. *Lancet* **359**: 1114-1119.

Wiley, J.S., Dao-Ung, L.-P., Li, C., Shemon, A.N., Gu, B.J., Smart, M.L., Fuller, S.J., Barden, J.A., Petrou, S. and Sluyter, R. (2003) An Ile-568 to Asn polymorphism prevents normal trafficking and function of the human P2X$_7$ receptor. *Journal of Biological Chemistry* **278**: 17108-17113.

Williams, G.T. (1994) Programmed cell death: a fundamental protective response to pathogens. *Trends in Microbiology* **2**: 463-464.

Wilson, K.P., Black, J.A., Thomson, J.A., Kim, E.E., Griffith, J.P., Navia, M.A., Murcko, M.A., Chambers, S.P., Aldape, R.A., Raybuck, S.A. and et al. (1994) Structure and mechanism of interleukin-1β converting enzyme. *Nature* **370**: 270-275.

Wong, S.Y. and Remington, J.S. (1994) Toxoplasmosis in pregnancy. *Clinical Infectious Diseases* **18**: 853-861.

Xie, Q.W., Kashiwabara, Y. and Nathan, C. (1994) Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *Journal of Biological Chemistry* **269**: 4705-4708.

Yerxa, B.R., Sabater, J.R., Davis, C.W., Stutts, M.J., Lang-Furr, M., Picher, M., Jones, A.C., Cowlen, M., Dougherty, R., Boyer, J., Abraham, W.M. and Boucher, R.C. (2002) Pharmacology of INS37217 [P(1)-(uridine 5')-P(4)- (2'-deoxycytidine 5')tetraphosphate, tetrasodium salt], a next-generation P2Y$_2$ receptor agonist for the treatment of cystic fibrosis. *Journal of Pharmacology & Experimental Therapeutics* **302**: 871-880.

Zanovello, P., Bronte, V., Rosato, A., Pizzo, P. and Di Virgilio, F. (1990) Responses of mouse lymphocytes to extracellular ATP. II. Extracellular ATP causes cell type-dependent lysis and DNA fragmentation. *Journal of Immunology* **145**: 1545-1550.

Zhao, Y., Ferguson, D.J., Wilson, D.C., Howard, J.C., Sibley, L.D. and Yap, G.S. (2009a) Virulent *Toxoplasma gondii* evade immunity-related GTPase-mediated parasite vacuole disruption within primed macrophages. *Journal of Immunology* **182**: 3775-3781.

Zhao, Y.O., Khaminets, A., Hunn, J.P. and Howard, J.C. (2009b) Disruption of the *Toxoplasma gondii* parasitophorous vacuole by IFNgamma-inducible immunity-related GTPases (IRG proteins) triggers necrotic cell death. *PLoS Pathogens* **5**:

Zheng, L.M., Zychlinsky, A., Liu, C.C., Ojcius, D.M. and Young, J.D. (1991) Extracellular ATP as a trigger for apoptosis or programmed cell death. *Journal of Cell Biology* **112**: 279-288.