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

by

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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

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To my wonderful parents.

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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*.