The Role of the Purinergic P2X7 Receptor in Small Intestinal Inflammation

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

2015

Szu-Wei Huang
List of Contents

List of Contents ........................................................................................................................................... 2
List of Figures .................................................................................................................................................. 8
List of Tables .................................................................................................................................................. 11
Abstract .......................................................................................................................................................... 12
Declaration ...................................................................................................................................................... 14
Copyright Statement .................................................................................................................................. 15
Acknowledgements ...................................................................................................................................... 16
Abbreviations ............................................................................................................................................... 17
Chapter One General Introduction ........................................................................................................... 22
  1.1. Introduction ......................................................................................................................................... 23
  1.2. Gastrointestinal inflammation ......................................................................................................... 24
  1.3. Gastrointestinal protozoan infection ................................................................................................. 25
    1.3.1. Toxoplasma gondii .................................................................................................................... 26
    1.3.2. Life cycle of T. gondii .............................................................................................................. 26
    1.3.3. Immunity in response to T. gondii infection ............................................................................. 29
    1.3.4. Virulent factors and immune regulation ................................................................................... 31
    1.3.5. Mucosal responses .................................................................................................................. 33
    1.3.6. T. gondii-induced murine ileitis model ..................................................................................... 35
  1.4. Gastrointestinal helminthic infection ................................................................................................. 37
    1.4.1. Trichinella spiralis ...................................................................................................................... 37
    1.4.2. Life cycle of T. spiralis ............................................................................................................. 38
    1.4.3. Immunomodulation ................................................................................................................... 40
    1.4.4. Mucosal responses in T. spiralis infection ............................................................................... 41
    1.4.5. T. spiralis-induced murine ileitis model ................................................................................... 42
  1.5. Dendritic cell biology ......................................................................................................................... 42
1.5.1. Murine intestinal DCs .................................................................43
1.5.2. The origins of murine intestinal DCs ...........................................43
1.5.3. Murine intestinal DC subsets and their functions ......................44
1.5.4. Murine intestinal DC migration ..................................................47
1.5.5. DCs and *T. gondii* .....................................................................49
1.5.6. DCs and *T. spiralis* ...................................................................49
1.6. Pattern recognition receptors and damage-and pathogen-associated
    molecular patterns .............................................................................50
1.6.1. Infection and DAMPs/PAMPs .....................................................51
1.6.2. Pattern recognition receptors and *T. gondii* ..............................54
1.6.3. Pattern recognition and *T. spiralis* .............................................54
1.7. Intestinal epithelial cells and barrier function ..................................55
1.7.1. Intestinal epithelial cells and immunosurveillance ......................56
1.7.2. Intestinal epithelial cells and pattern recognition receptors ..........56
1.7.3. Intestinal epithelial cells and immune homeostasis ....................58
1.8. Purinergic P2X7 receptor .................................................................60
1.8.1. P2X7R signalling pathways .......................................................61
1.8.2. P2X7R in immune cells .............................................................64
1.8.3. P2X7R-mediated cell death .......................................................65
1.8.4. P2X7R-mediated killing of intracellular pathogens ....................66
1.8.5. P2X7R-mediated mucosal inflammatory responses ....................66
1.8.6. P2X7R−/− animal models .........................................................67
1.9. Hypothesis and aims .......................................................................69

References ..........................................................................................71

Chapter Two A Novel Role for P2X7 Receptor in Promoting Recruitment of CD103+
Dendritic Cells and Regulating Immunity to *Toxoplasma gondii*
infection ...............................................................................................96
ABSTRACT ........................................................................................................................................ 98
INTRODUCTION ................................................................................................................................ 99
METHODS ......................................................................................................................................... 100
RESULTS .......................................................................................................................................... 106
Early *T. gondii* induced-cell death is P2X7R dependent................................................................. 106
Delayed recruitment of CD103+ DCs to the small intestinal epithelium in P2X7R−/− mice. 107
P2X7R−/− animals have reduced development of Th1 immunity. ................................................. 110
P2X7R−/− mice have a higher parasite burden post-infection...................................................... 111
P2X7R deficiency has no effect on small intestinal pathology after infection. ....................... 112
P2X7R−/− DCs can migrate and present antigen normally *in vitro*. ........................................... 113
Impaired chemotactic function in P2X7R−/− intestinal epithelial cells................................. 114
Epithelial Responses to Infection..................................................................................................... 116
Discussion ....................................................................................................................................... 118
ACKNOWLEDGEMENTS .................................................................................................................. 120
REFERENCES ................................................................................................................................... 121
SUPPLEMENTARY DATA ................................................................................................................ 125
Chapter Three Epithelial Expression of P2X7 Receptor Promotes DC Recruitment in
*Trichinella spiralis*-induced Intestinal Inflammation .............................................................. 127
ABSTRACT ....................................................................................................................................... 129
INTRODUCTION ................................................................................................................................ 130
METHODS ....................................................................................................................................... 132
RESULTS ......................................................................................................................................... 136
P2X7R mice have reduced inflammation in response to infection is critical to protect
animals from *T. spiralis* infection.................................................................................................. 136
Delayed recruitment of CD103+CD11b+ DCs to the small intestinal epithelium in P2X7R−/−
mice. .............................................................................................................................................. 138
P2X7R−/− intestinal epithelial cells have impaired chemokine responses. .............................. 141
Reduced Epithelial Responses to Infection.................................................................142
P2X7R deficiency impairs the development of Th2 immunity.................................143
DISCUSSION ..................................................................................................................147
ACKNOWLEDGEMENTS ...............................................................................................150
REFERENCES ................................................................................................................150
SUPPLEMENTARY DATA .................................................................................................154
Chapter Four In vitro P2X7 Receptor-mediated Promotion of Chemokine and Pro-
inflammatory Cytokine Responses in Intestinal Epithelial Cells .......................156
ABSTRACT .......................................................................................................................158
INTRODUCTION .............................................................................................................159
METHODS ......................................................................................................................162
RESULTS .........................................................................................................................164
P2X7R signalling promotes CCL5 and proinflammatory cytokine production in response to
T. gondii infection .................................................................................................164
Apoptosis is not required for P2X7R-dependent regulation of CCL5.....................166
P2X7R regulates TLR signalling-associated CCL5 response .....................................168
NF-κB signalling is involved in the CCL5 production in response to infection ........170
The Kinetics of T. gondii infection are altered by P2X7R inhibition.........................172
DISCUSSION ..................................................................................................................174
ACKNOWLEDGEMENTS ...............................................................................................178
REFERENCES ................................................................................................................178
Chapter Five Overall Discussion ...............................................................................182
5.1 Key findings ............................................................................................................183
5.2 P2X7 receptor as a potential initiator of intestinal inflammation? .....................183
5.3 How important are DCs in the initiation of immunity to intestinal infections? ......185
5.4 How does P2X7R affect the chemoattraction of immune cells? .........................190
5.5 How does CCL5 expression up-regulate in IECs in response to infection? ........192
5.6 How does P2X7R modulate CCL5 induction in response to *T. gondii* infection? .......... 193
5.7 How does P2X7R antagonism cause the reduction in *T. gondii* infectivity? ............ 194
5.8 Does P2X7R signalling play a protective role in intestinal infection? .................. 196
5.9 Conclusions .................................................................................................................. 199
5.10 Future work and significance ..................................................................................... 200
References ....................................................................................................................... 203

Chapter Six Supplementary Materials and Methods....................................................... 211
6.1 Animals and cell culture............................................................................................... 212
6.1.1 Animals .................................................................................................................. 212
6.1.2 Cell culture ........................................................................................................... 212
6.2 Parasite specific techniques......................................................................................... 212
6.2.1 Culture of *Toxoplasma gondii* in human foreskin fibroblasts.............................. 212
6.2.2 Maintenance and recovery of *Trichinella spiralis* ............................................... 213
6.2.3 Preparation of *T. spiralis* antigen ........................................................................ 213
6.2.4 *In vivo* and *in vitro* Infections ........................................................................... 214
6.2.5 Reagents used in *in vitro* cell line model ............................................................ 214
6.3 *Ex vivo* analysis ....................................................................................................... 215
6.3.1 Assessment of *T. spiralis* burden in the gut ....................................................... 215
6.3.2 Mouse bone marrow-derived dendritic cells ...................................................... 215
6.3.3 Isolation of the small intestinal cells ................................................................... 216
6.3.4 Isolation and culture of the primary small intestinal crypt cells ......................... 216
6.3.5 Cytokine and chemokine analysis ....................................................................... 217
6.4 Histology .................................................................................................................... 219
6.4.1 Tissue processing ................................................................................................. 219
6.4.2 Haematoxylin and eosin staining ........................................................................ 219
6.5 Immunohistochemistry ............................................................................................... 220
6.5.1 Tissue processing ................................................................................................. 220
6.5.2 TUNEL staining .............................................................................................................. 220
6.5.3 Staining of CD11c+CD103+ dendritic cells ..................................................................... 221
6.6 Flow cytometry .................................................................................................................. 222
6.6.1 FACS analysis for small intestinal cells ........................................................................ 222
6.6.2 FACS analysis for intracellular cytokine expression..................................................... 222
6.7 Molecular Biology ........................................................................................................... 223
6.7.1 Isolation of small intestinal epithelial cells for RNA extraction ........................................ 223
6.7.2 RNA extraction and Preparation .................................................................................. 224
6.7.3 cDNA conversion ....................................................................................................... 225
6.7.4 Quantitative PCR ......................................................................................................... 225
6.7.5 Genotyping of wildtype and P2X7R-/- mice ................................................................. 226
6.8. Generation of bone-marrow chimeras ............................................................................ 227
References ........................................................................................................................... 229
Appendix ............................................................................................................................... 230
List of Figures

Figure 1.1. The complex composition and environment of the GI tract. ........................................... 24
Figure 1.2. The life cycle of *Toxoplasma gondii*. ........................................................................ 28
Figure 1.3. The Mucosal Response against *T. gondii* infection. ..................................................... 35
Figure 1.4. The Life Cycle of *Trichinella spiralis*. .................................................................... 39
Figure 1.5. The basic structure of P2X7R. .................................................................................... 61
Figure 1.6. A summary of P2X7R activation-mediated intracellular signalling and control of transcriptional regulators. ................................................................. 64
Figure 1.7. The constructs of P2X7R−/− Pfizer and P2X7R−/− Glaxo mice..................................... 69
Figure 2.1. P2X7R promotes infection induced-cell death. ............................................................. 107
Figure 2.2. Impaired early recruitment of CD11c+CD103+CD11b− dendritic cells (DCs) in
P2X7R−/− mice after infection. ....................................................................................................... 109
Figure 2.3. P2X7R deficiency impaired development of Th1 immunity against infection. 111
Figure 2.4. Acute *T. gondii* induced pathology is not dependent on P2X7R.......................... 113
Figure 2.5. Epithelial P2X7R is necessary for the intraepithelial recruitment of dendritic cells (DCs). ........................................................................................................ 115
Figure 2.6. P2X7R promotes CCL5 production in intestinal epithelial cells in response to
infection ........................................................................................................................................ 117
Supplementary Figure S2.1. P2X7R deficiency does not affect the recruitment of small
intestinal CD103+ DCs and F4/80+CD11c+ macrophages in response to
infection ......................................................................................................................................... 125
Supplementary Figure S2.2. P2X7R−/− mice had significantly fewer inflammatory foci in the
liver at day 8 post infection. ........................................................................................................... 126
Figure 3.1. P2X7R−/− mice have delayed gut inflammation in response to infection. ................. 137
Figure 3.2. Impaired recruitment of CD11c+CD103+CD11b+ dendritic cells in P2X7R−/− mice
in response to infection .............................................................................................................. 140
Figure 3.3. Epithelial P2X7R is required for CD103\(^+\)CD11b\(^+\) dendritic cell recruitment.....142

Figure 3.4. Reduced epithelial CCL5 production in P2X7R\(^{-/-}\) mice. .................................143

Figure 3.5. Cytokine Responses and worm burden in P2X7R\(^{-/-}\) mice......................146

Supplementary Figure S3.1. The recruitment of CD103 CD11b\(^+\) DCs, CD103 CD11b\(^-\) DCs
and F4/80 CD11c\(^+\) macrophages were unaffected in P2X7R\(^{-/-}\) mice. ....154

Supplementary Figure S3.2. P2X7R\(^{-/-}\) BMDCs are functional. ...............................155

Figure 4.1. Inhibition of P2X7R down-regulates \textit{T. gondii}-induced chemokine and pro-
inflammatory cytokine responses..........................................................166

Figure 4.2. Inhibition of P2X7R has no effect on infection-induced cell death..........168

Figure 4.3. Inhibition of P2X7R down-regulates LPS-induced CCL5 response. ............170

Figure 4.4. Infection of type I toxoplasma RH strain does not induce CCL5 production in
IECs. ........................................................................................................ 171

Figure 4.5. The infection-induced CCL5 production is partially via activation of NF-\(\kappa\)B
signalling. .................................................................................................172

Figure 4.6. Inhibition of P2X7R delays \textit{T. gondii} infection in CMT-93 cells. .........174

Figure 5.1. The mechanism of \textit{P2X7R}-dependent initiation of inflammation in response to
intestinal infection. ................................................................................ 185

Figure 6.1. Genotyping for WT and P2X7R\(^{-/-}\) mice. ..................................................227

Figure A.1. Gate setting of FACS analysis for small intestinal cells. .........................233

Figure A.2. Representative plots of small intestinal DC and macrophages population in IEL
in response to \textit{T. gondii} infection..............................................................234

Figure A.3. Representative plots of small intestinal DC and macrophages population in LPL
in response to \textit{T. gondii} infection..............................................................235

Figure A.4. Representative FACS plots showing P2X7R deficiency impairs Th1 response to
\textit{T. gondii} infection..............................................................................236

Figure A.5. Representative plots of small intestinal DC and macrophages population in IEL
in response to \textit{T. spiralis} infection............................................................237
Figure A.6. Representative plots of small intestinal DC and macrophages population in LPL in response to *T. spiralis* infection........................................................ 238

Figure A.7. Representative FACS plots showing P2X7R deficiency impairs Th2 response to *T. spiralis* infection................................................................. 239
List of Tables

Table 3.1 Inflammatory Scoring for Assessment of Ileal Damage ..............................................134

Table 6.1: Primer sequences for qPCR analysis of small intestinal epithelial cells (shown from 5’ to 3’). ........................................................................................................226

Table 6.2: Primer sequences for genotyping (shown from 5’ to 3’). ........................................227

Table A.1: Chemokines and their roles in the gut and enteric infection .......................231
Abstract

The purinergic P2X7 receptor (P2X7R), an adenosine triphosphate (ATP)-gated receptor, is widely distributed in a variety of cell types such as neuron cells, immune cells and epithelial cells. P2X7R on cells senses extracellular ATP released from dying cells which then acts as a danger signal and initiates inflammation. Activation of P2X7R results in various downstream events, including Ca$^{2+}$ influx, nonselective membrane pore formation, cell death, assembly of the inflammasome, and killing of intracellular pathogens. Epithelial cells in the gut also express P2X7R and act as a sentinel that protects against infection and responds to changes in environmental stimuli. However, the role of P2X7R in IECs is poorly defined. Given that infection of pathogens often causes cellular damage and the released ATP may be sensed by P2X7R, we hypothesised that IECs initiate intestinal inflammation via activation of the P2X7R in response to infection. Thus, the aim of this thesis was to characterise the role of P2X7R in the initiation and development of small intestinal inflammation. In order to achieve this aim, we used two parasite-induced murine ileitis models, *Toxoplasma gondii* (T. gondii) and *Trichinella spiralis* (T. spiralis), which induce Th1 and Th2 immunity respectively.

In the *in vivo* model of *T. gondii* infection, we found that P2X7R deficiency was associated with less intestinal epithelial responsiveness to the infection. The P2X7R$^{-/-}$ IECs had reduced CCL5 and CCL20 chemokine expression which was associated with reduced recruitment of CD103$^+$CD11b$^-$ dendritic cells (DCs) to the small intestinal epithelium at day 1 post infection (p.i.). This finding was supported by infection of bone marrow chimeras showing a decrease in the recruitment of WT P2X7R$^{+/+}$ CD103$^+$ DCs to a P2X7R$^{-/-}$ epithelium. To address whether the reduced DC response impacted on development of adaptive immunity, we analysed serum IFN-$\gamma$ and the proportion of splenic IFN-$\gamma^+$CD4$^+$ T cells at day 8 p.i., and showed they were reduced in P2X7R$^{-/-}$ mice.
In the *in vivo* model of *T. spiralis* infection, P2X7R deficiency was also associated with reduced intestinal epithelial responsiveness to this infection characterised by lower CCL5 expression in IECs. A significant decrease in the recruitment of CD103+CD11b+ DCs at day 2 p.i. was noted in P2X7R−/− animals, and the importance of epithelial P2X7R in DC recruitment was confirmed using bone marrow chimeras. The P2X7R−/− mice, compared with the WT, had delayed progression of small intestinal inflammation, accompanied by a reduction in the percentage of IL-4+CD4+ T cells and IL-4 levels at day 8 p.i.. The reduced IL-4 response was associated with a delayed worm expulsion in the P2X7R−/− mice at day 12 p.i..

An *in vitro* study demonstrated that P2X7R blockade using the chemical inhibitor A-740003, significantly decreased CCL5, IL-6 and TNF-α secretion from mouse intestinal epithelial CMT-93 cells in response to *T. gondii* infection. A similar decrease in the level of CCL5 produced was also observed using primary P2X7R−/− intestinal crypt cells stimulated with lipopolysaccharide (LPS) compared with WT cells. This data indicates a proinflammatory role for P2X7R during infection. Although P2X7R signalling is known to induce the assembly of the inflammasome, IECs did not secrete the inflammasome-associated cytokines IL-1β and IL-18 in response to *T. gondii* infection. Moreover, P2X7R signalling had no effect on the induction of cell death in *T. gondii*-infected IECs. Interestingly, there was a novel finding that P2X7R antagonism inhibited *T. gondii* infectivity in CMT-93 cells.

In summary, we have shown that P2X7R signalling mediated CCL5 expression in IECs in response to infection. Epithelial chemokines are important for the initiation of small intestinal inflammation via recruitment of innate cells such as DCs which can then prime for protective adaptive immunity. These results in this thesis improve the understanding of the role of P2X7R in the intestinal immune system and reveal novel roles for epithelial P2X7R. The work suggests the potential of epithelial P2X7R as a target for pharmacological treatment of intestinal inflammatory disorders.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
Copyright Statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trade marks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DocuInfo.aspx?DocID=487), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.manchester.ac.uk/library/aboutus/regulations) and in The University’s policy on Presentation of Theses.
Acknowledgements

How time flies! Four years have passed in the blink of an eye. Finally it is the moment for me to achieve the goal of the PhD from the University of Manchester. I will never forget the hard time in the beginning of my PhD programme. During that time, I felt quite strange in Manchester, feeling lonely for having no friends. Thanks God for bringing so many good friends that helped me quickly get used to the unfamiliar environment. I am grateful to my supervisor Dr Sheena Cruickshank for her guidance and support over my PhD study. I’d like to thank my colleague, Michael Bramhall for being the first British friend I met here and his great help to my experimental work. I’d also like to thank Dr Joanne Pennock and my advisor Prof. Kathryn Else for their advice and help in my experiments. Many thanks to all the lab members including Larisa Logunova, Rowann Bowcutt, Ruth Forman, Becky Montacute, Tarfa Altorki, Maria Glymenaki, Helen Williams, Rachel Crompton and Gurdeep Singh. Thanks to all the friends of the fourth floor, especially Wesam Abdulaal and Soo Yee Tan, for their help. I’d also like to thank Dr Andrew Stagg and Dr Catherine Lawrence for being the examiners of my viva.

Thanks to the chaplains, Revd Shou-Hui Chung, Revd Dr Terry Biddington and Revd Jayne Prestwood as well as other church members, especially John Roussel and Catriona Roussel in St. Peter’s House, where I found a warm welcome and really feel like being at home. Massive thanks to all the members in the Mandarin Fellowship, such as Dr Wei-Hsiang Lin, Dr Yuchia Ko, Jinmei Mai, Dr Ka Kei Chan, Dr Ming-Tsung Lin, More, Dr Chris Tian, Joseph Yu, Angel Hsu, Minhan Kuo, Yipei Chen, Nana and Luke Lau, Noreena Liu, Dr I-Ling Tsai, Pju Huang, Dr Yifen Xu, Wenjiang Xu, Dr Owen Young, Alicia Feng, Ya Su, Dr Tommy Shyng, Dr Meilin Su, Jasmine Liu and Minerva Chung. I wholeheartedly mean that without you, I would never finish my PhD.

A big thank you to the friends I met here including Dr Jia Yu, Dr Zihui Xie, Dr Qian Wang, Ding Jin, Sally Liang, Dr Yi-Lien Yeh and Dr Nina Chen for fulfilling my life with a lot fun.

I’d like to thank Prof. Chun-Keung Yu and all the members in Yu’s lab such as Ya-Fang Wang, Yi-Ping Lee, Hsuang-Jung Huang, Sylvia Huang, Even Tsai, Wentz Yeh, Yiru Su, Wen-Hui Ma and Chun-Wei Chen and my colleagues in NLAC in Taiwan, especially Dr David Liang. You’re always my best friends and support.

A great thank to Sophie Hung for being my inequivalent support and the best audience during the four years of my study. Finally, I am grateful to my family, especially my parents, in Taiwan for providing me financial aid and incomparable love to sustain my study and my life in the UK.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAM</td>
<td>alternatively activated macrophage</td>
</tr>
<tr>
<td>ACK</td>
<td>ammonium chloride/potassium</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>APRIL</td>
<td>a proliferation-inducing ligand</td>
</tr>
<tr>
<td>ASC</td>
<td>apoptosis-associated speck-like protein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Arg-1</td>
<td>arginase-1</td>
</tr>
<tr>
<td>BAFF</td>
<td>B-cell activating factor</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BM</td>
<td>bone-marrow</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>BzATP</td>
<td>3'-O-4-benzoylbenzoyladenosine 5'-triphosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's disease</td>
</tr>
<tr>
<td>cDC</td>
<td>conventional dendritic cell</td>
</tr>
<tr>
<td>CDP</td>
<td>common dendritic cell precursor</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>DAMP</td>
<td>damage-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sulphate sodium</td>
</tr>
<tr>
<td>DT</td>
<td>diphtheria toxin</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Egr</td>
<td>early growth response protein</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>E/S</td>
<td>excretory/secretory</td>
</tr>
<tr>
<td>ES cell</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>FAE</td>
<td>follicle-associated epithelium</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Flt3L</td>
<td>Fms-like tyrosine kinase 3 ligand</td>
</tr>
<tr>
<td>GALT</td>
<td>gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GVHD</td>
<td>graft-versus-host disease</td>
</tr>
<tr>
<td>HBD2</td>
<td>human β-defensin 2</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered salt solution</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HEK293 cell</td>
<td>cell human embryonic kidney cell</td>
</tr>
<tr>
<td>HMGB-1</td>
<td>high mobility group box 1</td>
</tr>
<tr>
<td>Hepes</td>
<td>1% 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IBS</td>
<td>irritable bowel syndrome</td>
</tr>
<tr>
<td>IEC</td>
<td>intestinal epithelial cell</td>
</tr>
<tr>
<td>IEL</td>
<td>intestinal epithelial layer</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IFR</td>
<td>T-cell interfollicular region</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
</tr>
<tr>
<td>infDC</td>
<td>Inflammatory dendritic cell</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>IRG</td>
<td>immunity-related GTPases</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LD</td>
<td>lethal dose</td>
</tr>
<tr>
<td>LP</td>
<td>lamina propria</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>M cell</td>
<td>microfold cell</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAVS</td>
<td>mitochondrial antiviral-signalling</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MDA5</td>
<td>melanoma differentiation-associated gene 5</td>
</tr>
<tr>
<td>MDP</td>
<td>macrophage and DC precursor</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility class</td>
</tr>
<tr>
<td>M-MLV</td>
<td>Moloney Murine Leukemia Virus</td>
</tr>
<tr>
<td>MR</td>
<td>mannose receptor</td>
</tr>
<tr>
<td>NBF</td>
<td>neutral buffered formalin</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T-cell</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappaB</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NLR</td>
<td>nucleotide oligomerisation domain (NOD)-like receptors</td>
</tr>
<tr>
<td>NLRP</td>
<td>NLR sub-group protein</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting compound</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>P2XR</td>
<td>P2X receptor</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
</tbody>
</table>
pDC  plasmacytoid dendritic cell
PEST  penicillin/streptomycin
p.i.  post infection
PI-IBS  post-infectious irritable bowel syndrome
PP  Peyer's patch
PRR  pattern recognition receptor
PV  parasitophorous vacuole
qPCR  quantitative PCR
RIG-I  retinoic acid-inducible gene-I
RLR  retinoic acid-inducible gene-I (RIG-I)-like receptor
SED  subepithelial dome
sIgA  secretory immunoglobulin A
SPF  specific pathogen free
STAT  signal transducer and activator of transcription
Th  helper T cell
TLR  toll-like receptor
TNF  tumour necrosis factor
TM  transmembrane domain
Treg  regulatory T cell
TSLP  thymic stromal lymphopoietin
TUNEL  terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
T. gondii  Toxoplasma gondii
T. muris  Trichuris muris
T. spiralis  Trichinella spiralis
WT  wildtype
2-ME  beta-mercaptopoethanol
Cogito ergo sum

― René Descartes
Chapter One

General Introduction
1.1. Introduction

The gastrointestinal (GI) tract is a complex and dynamic network comprising a variety of interactions between epithelial cells, immune cells, food and a diverse commensal microbiota (Fig. 1.1). The GI tract is lined by epithelial cells which play a key role in nutrient absorption and surveillance of pathogenic invasion and thus, the balance of gut homeostasis. Under normal conditions, tolerogenic immune responses act to prevent inappropriate immune reactions to the commensal microbiota and food. In contrast, it is necessary to initiate protective immunity in response to pathogens including viruses, bacteria and parasites. Very little is known about how this switch from tolerance to gut inflammation is initiated in the response to infection. Epithelial cells that line the gut act as the first point of contact with tolerogenic versus inflammatory stimuli. Epithelial cells will be the first cells damaged or targeted in infection and are therefore important for inducing the inflammatory response. Moreover, intestinal epithelial cells (IECs) play a critical role in mediating the function of innate immune cells such as dendritic cells (DCs). DCs are indispensable for initiating the switch from tolerance to immunity via their ability to programme T cell function and bias. We propose that IECs sense the damage caused by infection and induce an early DC response which is critical in shaping the subsequent immune response and outcome to infection. The release of extracellular adenosine triphosphate (ATP) is a hallmark of tissue damage therefore we hypothesised that the interaction between IECs and DCs may be initiated by extracellular ATP released from dying cells. In order to define the role of IECs in sensing infection induced-extracellular ATP we used two parasite models to promote small intestinal inflammation and damage: *Toxoplasma gondii* (*T. gondii*), an obligate intracellular parasite that induces T helper (Th) 1 immunity and *Trichinella spiralis* (*T. spiralis*), a helminth parasite that induces Th2 immunity. These two ileitis models were investigated to determine whether innate immunity and/or Th1 and Th2 responses are initiated via similar or distinct mechanisms. The purinergic P2X7 receptor (P2X7R) is an important receptor for sensing extracellular ATP, therefore we used
P2X7R deficient (P2X7R\(^{-/-}\)) mice as a model to address the damage response of epithelial cells, recruitment and activation of DCs in the response to infection.

**Figure 1.1. The complex composition and environment of the GI tract.**

The structural, cellular and microbial compositions are diverse in the GI tract. The small intestine has millions of finger-like projections called villi which protrude from the epithelial lining to aid in absorption by increasing intestinal surface. Between the villi, the intervening intestinal epithelial glands are called crypts of Lieberkühn. In the small intestine, there are small lymphoid nodules termed Peyer’s patches, where microfold (M) cells reside that sample luminal antigens. The small intestine has antimicrobial peptide-secreting Paneth cells. The microbiota in the small intestine reside in the lumen and mucus layer with the density of bacteria increasing down the GI tract. There are abundant and diverse immune cell populations in the GI tract, including dendritic cells, CX3CR1\(^{+}\) macrophages, B cells, regulatory T cells and intraepithelial lymphocytes. The interaction between the microbiota, food, epithelium and immune cells determine the gut homeostasis. Cell populations are defined in the key. Adapted from\(^1\)

**1.2. Gastrointestinal inflammation**

Gastroenteritis is characterised by the inflammation of stomach and small or large intestine with symptoms including vomiting and diarrhoea\(^4\). Most gastroenteritis cases result from viral or bacterial infections\(^5\),\(^6\), and less commonly caused by parasites\(^4\). Non-infectious
causes of gastroenteritis include certain toxic ingestions (e.g., poisonous mushrooms\textsuperscript{7} and ciguatoxin\textsuperscript{8}), celiac disease\textsuperscript{9}, medications (e.g., non-steroidal anti-inflammatory drugs\textsuperscript{10} and some antibiotics\textsuperscript{11}) and autoimmune disorders such as inflammatory bowel disease\textsuperscript{12}. In a UK study, gastroenteritis accounted for 16\% of total medical presentations to a major paediatric emergency department\textsuperscript{13}. Severe gastroenteritis can cause dehydration which is fatal, especially for young children. An estimated 1.4 million children die each year from infectious gastroenteritis\textsuperscript{14}, although the incidence of global deaths has decreased because of improved water sanitation, proper use of rehydration therapy and rotavirus vaccination\textsuperscript{15-17}. The mortality in young children caused by infectious gastroenteritis in developing countries is much higher than in developed countries\textsuperscript{18}. Thus, more effort is needed to study the mechanisms underlying gastrointestinal inflammation to develop more efficient strategies to prevent mortality.

\subsection*{1.3. Gastrointestinal protozoan infection}
Numerous protozoa can infect the GI tract. Transmission is usually either through the direct faecal-oral route or via indirect transmission for example, contact with animals and livestock or consumption of contaminated food or water\textsuperscript{19}. Some of these enteric protozoa, such as \textit{Giardia lamblia}, cause acute self-limiting gastroenteritis but occasionally may lead to chronic diarrhoea and malnutrition.\textsuperscript{20} A few protozoan parasites like \textit{Entamoeba histolytica} which is a highly virulent and invasive pathogen, can causes amoebic dysentery, the third leading cause of death from parasitic disease. \textit{Entamoeba histolytica}, can sometimes also cause a lethal systemic invasion\textsuperscript{20}. Enteric protozoa-associated diarrhoea, especially in children, is frequently found in developing countries, where the poor sanitary environment and unclean water sources have sustained transmission of the disease\textsuperscript{21}. In many developed countries, enteric protozoa are not considered as a major cause of diarrhoeal diseases due to better hygiene conditions\textsuperscript{20}. However, the prevalence of these enteric protozoan is very
common in immunocompromised patients and is associated with life-threatening diarrhoea and other fatal complications in both developed and developing countries\textsuperscript{22}. Thus, enteric protozoan infection is still a great socioeconomic burden worldwide.

1.3.1. \textit{Toxoplasma gondii}

\textit{Toxoplasma gondii} (\textit{T. gondii}) is an obligate protozoan parasite belonging to the \textit{Toxoplasma} genus of \textit{Toxoplasmatinae} subfamily. \textit{T. gondii} is capable of infecting humans and all warm-blood animals including felids, bears, foxes and pigs\textsuperscript{23}. In the human population, the infection is normally through the ingestion of undercooked meat or contaminated food (section 1.3.2) and the prevalence of \textit{T. gondii} is differs from country to country (from 10 to 80\% approximately), which is influenced by both socioeconomic parameters and population habits\textsuperscript{24}. It is estimated that up to one third of the total human population in the world are infected by this parasite\textsuperscript{25}. Prevalence is lower in areas with dry and hot climates which are unfavourable for parasite oocyst survival in comparison to humid tropical areas\textsuperscript{26}. In healthy adults, the exposure of the parasite may only cause asymptomatic infection or mild flu-like illness, but in those individuals who are immunocompromised or immunosuppressed, \textit{T. gondii} infection can spread throughout the body and lead to a fatal disease. If infection occurs during pregnancy, it may pass to the foetus and cause spontaneous abortion or congenital defects in infants\textsuperscript{27}. In addition, reactivation of latent disease is associated with ocular disease and central nervous system (CNS) diseases such as schizophrenia\textsuperscript{28}

1.3.2. \textit{Life cycle of T. gondii}

\textit{T. gondii} was first discovered in an African rodent called the gundi in 1908, but its life cycle was finally defined in 1970 when it was determined that the cat plays a central role as the definitive host\textsuperscript{29}. Subsequent research has revealed that any felid species can act as a
definitive host. The parasite’s life cycle relies on a prey-predator system that alternates between definitive (sexual phase) and intermediate (asexual phase) hosts (Figure 1.2), where it can be transmitted not only between the intermediate and final hosts but also between intermediate hosts through carnivorism\textsuperscript{30}. The sexual phase of the life cycle takes place only in felids (both domestic and wild) which makes cats the primary definitive host. In the cat, ingested cysts release bradyzoites into the stomach and small intestine where they undergo the sexual cycle and form gametocytes. After fertilisation, oocysts formed within enterocytes are released and excreted in the cat faeces. Excreted oocysts undergo sporulation in the soil for a few days before they are infective to the intermediate hosts. Oocysts are remarkably robust and can persist for long periods in the environment and are resistant to changes in temperature, salt water and even many detergents\textsuperscript{31}. In contrast, the parasite undergoes only an asexual cycle in intermediate hosts. After oocyst ingestion, sporozoites are released and invade the intestinal epithelium of the intermediate hosts where they differentiate into tachyzoites. Tachyzoites rapidly replicate in epithelial cells and then disseminate throughout the body by infecting other nucleated cells. Finally, they differentiate into bradyzoites and form cysts in tissues (mainly in the brain, central nervous system and muscle). Ingestion of tissue cysts by other intermediate hosts will result in the asexual cycle being repeated again\textsuperscript{30}. 
Figure 1.2. The life cycle of Toxoplasma gondii.

The sexual phase occurs only in the intestine of the cat. Tachyzoites develop within enterocytes to form gametocytes (male and female) which fertilise to generate (a) oocysts that are shed in the faeces and undergo sporogony in the environment. Intermediate hosts can get infected by ingesting oocysts from the contaminated environment. During acute infection, (b) tachyzoites rapidly multiply and disseminate within the host within nucleated cells. Tachyzoites further convert into a semidormant stage of (c) bradyzoites which reside within tissue cysts and give rise to chronic infection. Cysts can transmitted by carnivorism between various hosts. Adapted from 30.

*T. gondii* tachyzoites can infect any nucleated cell and particularly target leukocytes including macrophages, monocytes, DCs and even plasmacytoid dendritic cells (pDC). Infected immune cells act as "Trojan horses" to enable the parasite to cross biological barriers and disseminate systemically whilst avoiding attack by the immune response 32, 33. After penetrating the host cells, the parasite drives the formation of a non-fusogenic compartment called the parasitophorous vacuole (PV), which is derived from both the parasite and the host cell. This invasion process is accompanied by the release of several excretory/secretory (E/S) proteins from specialised secretory organelles including
micronemes, rhoptries and dense granules\cite{34}. Proteins secreted from the micronemes interact with the host cell surface to facilitate cell invasion. Dense granule and rhoptry proteins promote a favourable environment in the host cell for parasite growth and development via modulation of a variety of host cellular processes\cite{35}.

### 1.3.3. Immunity in response to \textit{T. gondii} infection

The immune response against \textit{T. gondii} has been well studied and is typified by a strong Th1/cytotoxic T cell response. It has been reported that both interleukin (IL)-12 and interferon (IFN)-\(\gamma\) synergistically promote the differentiation of interferon (IFN)-\(\gamma\)-producing CD4\(^+\) Th1 cells and CD8\(^+\) T cells necessary for the Th1 immunity\cite{27,36}. The adaptive immune CD4\(^+\) and CD8\(^+\) T cells are a major source of IFN-\(\gamma\) in the response to \textit{T. gondii}, which protect mice from \textit{T. gondii}-induced encephalitis. IFN-\(\gamma\) has been reported to be the dominant factor that inhibits the multiplication of \textit{T. gondii} in infected-cells such as macrophages or monocytes\cite{37}. IFN-\(\gamma\) derived from natural killer (NK) and \(\gamma\delta\) T cells is shown to be indispensable for the early control of \textit{T. gondii} infection\cite{38}. Other non-T cells including macrophages and microglial cells are thought to be minor sources of IFN-\(\gamma\) in host-resistance against the parasite\cite{39}. In the response to \textit{T. gondii} infection, neutrophils attracted to the infected area are able to eliminate free parasites and may also contribute to IFN-\(\gamma\) secretion\cite{40,41}.

IL-12 produced by DCs via a MyD88-dependent mechanism in the initial phase of infection of \textit{T. gondii} is critical for driving IFN-\(\gamma\) production and differentiation of the Th1 cells\cite{42,43}. Moreover, other innate immune cells including macrophages and neutrophils also play a role as important IL-12 sources, which promotes NK cells to produce IFN-\(\gamma\)\cite{27}. The NK or CD4\(^+\)/CD8\(^+\) T cell-derived IFN-\(\gamma\) creates a positive feedback to promote classical activation
of macrophages, resulting in intracellular killing of *T. gondii* and the production of proinflammatory cytokines and nitric oxide (NO) as part of Th1 immune response\textsuperscript{27}.

As well as IL-12, another cytokine IL-18 was identified that stimulates IFN-\(\gamma\) production by T cells\textsuperscript{44}, induces cytotoxic activity of natural killer (NK) cells and CD8\(^+\) T cells\textsuperscript{45, 46} and enhances IL-12-mediated immunity against *T. gondii* infection\textsuperscript{47}. However, a previous study has reported that the increase of serum IL-18 is associated with lethality of virulent *T. gondii* RH strain infection, which results in severe Th1-induced immunopathology, and the abrogation of IL-18 using anti-IL-18 antibody prolonged mouse survival after lethal challenge\textsuperscript{48}. In a recent study, it was shown that the expression of IL-18 by IECs in response to *T. gondii* infection required an IL-10 family cytokine, IL-22, which is mainly produced by CD4\(^+\) T cells and innate lymphoid cells\textsuperscript{49}. Conversely, IEC-derived IL-18 can induce IL-22 production by innate lymphoid cells upon *T. gondii* infection as a positive feedback effect\textsuperscript{49}.

IL-23, an IL-12 family cytokine, which shares the IL-12p40 subunit with IL-12 has been reported to play an accessory protective role in the development of resistance to *T. gondii* infection\textsuperscript{50}. The mice infected by *T. gondii* had increased levels of IL-23 and the survival of the mice was enhanced by administration of IL-23 in the absence of IL-12\textsuperscript{50}. However, another *in vivo* study demonstrated that IL-23 up-regulated the production of matrixmetalloproteinase-2 which causes tissue damage in *T. gondii*-induced Th1 immunopathology\textsuperscript{51}. *In vitro* stimulation of human monocytes using live or dead *T. gondii* shows that both IL-23 and IL-12 were induced in a dose-dependent manner as early as 6 hr after infection but that live parasites induce significantly higher IL-23 than the dead parasites\textsuperscript{52}.  

30
Given that an over-reaction of Th1 immunity can result in immunopathology, the activity of the innate and adaptive immune cells involved must be tightly regulated in response to *T. gondii* infection\(^{53}\). During the mid-to-late phase of infection, the potential cytotoxic effects of Th1 immune response are controlled by IL-10 production at sites where the parasite highly replicates, for example, the liver and spleen\(^{54}\). Several groups have investigated the cell type that secrets IL-10 in response to *T. gondii* infection. A previous report shows that resident peritoneal macrophages appear to rapidly respond to intraperitoneal inoculation of *T. gondii* by producing IL-10\(^{55}\). However, it has been demonstrated that IL-10 derived from CD4\(^+\) Th1 cells producing both IFN-\(\gamma\) and IL-10, prevents immunopathology during toxoplasmosis\(^{53}\). The induction of IL-10 expression in Th1 cells is dependent on activation of the transcription regulator Blimp-1 which is induced by IL-27 and other factors such as Notch signalling\(^{56}\).

These findings suggest that the collaboration between the innate and adaptive immune cells in response to *T. gondii* plays a critical role in the determination of disease outcome and the development of protective immunity.

### 1.3.4. Virulent factors and immune regulation

Three major clonal lineages (types I, II, and III) of *T. gondii* have been isolated from humans and domestic animals in Europe and North America, and there is less than 1% genetic difference between these strains\(^{57}\). In human studies, type I strains are a major cause of ocular toxoplasmosis\(^{58}\). Maternal infection with type I strains is associated with an increased risk of schizophrenia and other psychoses in offspring, which suggests a higher neuropathogenic potential of this strain\(^{59}\). Type II strains are the most common *T. gondii* strain that causes human toxoplasmosis (approximately 70 to 80%) and the high prevalence is associated with a high infection rate in animals that are used as food such as pigs and
sheep\textsuperscript{60}. By contrast, type III strains rarely cause human toxoplasmosis but are largely found in animals albeit for unknown reasons\textsuperscript{60}.

The strain virulence of \textit{T. gondii} can be assessed according to pathology data obtained from mice infected with these parasites. Type I strains are the most virulent with the lowest lethal dose (LD\textsubscript{100} = 1), whereas type II (LD\textsubscript{50} = $\sim$10\textsuperscript{3}) and III strains (LD\textsubscript{50} = $\sim$10\textsuperscript{5}) are less virulent\textsuperscript{35}. Current genetic analysis of the virulence of \textit{T. gondii} has identified that genes coded for in E/S proteins including rhoptry protein ROP5, ROP16, ROP18, ROP38 and dense granular protein GRA15 determine the strain-dependent virulence\textsuperscript{61}.

Recent studies have clarified the functions of many of the E/S proteins. Briefly, the \textit{T. gondii} type I strains have a truncated non-functional GRA15 which causes a failure to activate nuclear factor-kappaB (NF-\kappaB) for inducing an early IL-12 response, allowing rapid parasite replication in the host\textsuperscript{62}. The cells infected by type I parasites have constitutive signal transducer and activator of transcription (STAT)3/6 activation which is promoted by ROP16, and this then inhibits the production of IL-1\beta, IL-6 and IL-12\textsuperscript{63}. In addition to the regulation of proinflammatory cytokines, the type I parasites block the immunity-related GTPases (IRG)-mediated parasite clearance via ROP18\textsuperscript{64} and ROP5\textsuperscript{65}, to promote parasite growth. In contrast, infection with type II parasites induces significant proinflammatory responses. These parasites express an intact form of GRA15 and efficiently activate NF-\kappaB in infected cells\textsuperscript{62}. The inactive ROP16 of the type II parasites does not activate STAT3/6 and induces Th1-associated cytokines\textsuperscript{63}. Unlike the type I parasites, the type II parasites express non-functional ROP5\textsuperscript{66} and ROP18\textsuperscript{64} and parasite replication is controlled by host immunity. Unlike the type II strains, the type III parasites express a truncated GRA5 which has no effect on NF-\kappaB activation but they do have active ROP16 for suppressing pro-inflammatory
However, the type III parasites, like the type II strain, have a non-functional ROP18 which makes the parasites vulnerable to IRG-mediated intracellular killing.

1.3.5. Mucosal responses

*T. gondii* infection in susceptible mice results in small intestinal inflammation and immunopathology. This immunopathology in the gut is characterised by a Th1-mediated increase in pro-inflammatory mediators such as IFN-γ, tumour necrosis factor (TNF)-α and NO. *In vitro* data by RNase protection assay demonstrates that expression of the chemokines CCL2, CCL3, CCL4, CCL5, CCL7, CXCL2 and CXCL10 in *T. gondii*-infected mouse small intestinal epithelial cells is enhanced. Consistent with this, a study using human IECs shows an early activation of mitogen-activated protein kinases (MAPK) and nuclear factor-kappaB (NF-κB) accompanied by up-regulation of IL-8, CCL3, CCL5, CCL7, CCL8, CXCL2 and CXCL10. The significant induction of IEC-derived chemokines and cytokines suggests IECs act as a sensor of *T. gondii* and an important initiator of small intestinal inflammation via chemoattraction of immune cells.

At the early stage of infection, *T. gondii* penetrates IECs and then disseminates via infected innate cells including macrophages and DCs. It has been reported that CCR2-dependent recruitment of Gr1+ inflammatory monocytes to the gut is required for controlling gut infection. A number of studies have also indicated the importance of intraepithelial lymphocytes in the maintenance of gut homeostasis and the control of infection. The interaction between IFN-γ- and TNF-α-producing lamina propria (LP) CD4+ T cells and enterocytes inhibits parasite replication in enterocytes and promotes proinflammatory responses. Many studies have demonstrated the importance of intraepithelial lymphocytes in the maintenance of IEC barrier and the generation of immune responses against intestinal infection. For example, intraepithelial CD8αβ+ lymphocytes from infected
mice produce IFN-γ in response to *T. gondii* stimulation and generate cytotoxicity to clear the parasite-infected IECs and macrophages. A previous study using adoptive transfer of intraepithelial lymphocytes has also shown that CD8+ αβ T cells and γδT cells synergistically confer *in vivo* protective immunity in response to *T. gondii* infection. In response to the chemokines CCL3 and CCL4, the expression of CCR5 is important for the migration of *T. gondii*-specific CD8+ lymphocytes to the inflamed gut. Innate lymphoid cells (ILCs) are also important in the parasite control. A recent study has shown NKp46+ ILC produce CCL3 in response to *T. gondii*-induced IEC-derived IL-18. The CCL3, in turn, recruits inflammatory monocytes via their CCR1 to the inflamed intestine.

Collectively these findings indicate a complex interplay between inflammatory monocytes, ILCs, LP CD4+ T cells, intraepithelial CD8+ T cells and IECs that provide mucosal protective responses against *T. gondii* infection (Figure 1.3).
In response to *T. gondii* infection, inflammatory monocytes are recruited for the control of infection by CCL2, derived from infected-IECs, and CCL3, secreted from the NKp46+ ILCs stimulated by IEC-derived IL-18. IFN-γ and TNF-α derived from CD4+ T cells synergistically inhibit parasite replication and stimulate IECs to produce chemokines including CCL2, CCL3, CCL4, CCL5, CXCL2 and CXCL10 for proinflammatory response. CCL3 and CCL4 derived from *T. gondii*-infected IECs attract CD8+ T cells to the inflamed epithelium for killing the infected-IECs and macrophages. The recruited CD8+ T cells can also produce IFN-γ to inhibit parasite replication in host cells.

### 1.3.6. *T. gondii*-induced murine ileitis model

Oral infection of a low dose (10 cysts/mouse) of type II strain *T. gondii* in C57BL/6 mice induces mucosal defences and the development of chronic infection. However, peroral infection using a high dose (100 cysts/mouse) of the type II strain parasite causes C57BL/6 mice to die from a lethal ileitis with massive mucosal necrosis during day 7 to day 13 post...
infection (p.i.)\textsuperscript{82}. By contrast BALB/c mice survive after the high dose challenge without marked necrosis of the villi and mucosal cell infiltration observed\textsuperscript{82}. The different susceptibilities of C57BL6 versus BALB/c strain mice to \textit{T. gondii}-induced ileitis are due to IL-10 which prevents mucosal destruction by the immunopathological responses in animals of resistant genetic backgrounds \textsuperscript{69}. The \textit{T. gondii} infection-induced ileal inflammation in C57BL/6 mice has been characterised as a Th1-mediated immunopathology\textsuperscript{69}. The development of necrosis in the gut depends on CD4\textsuperscript{+}\alpha\beta T cells, IFN-\gamma, TNF-\alpha and NO production\textsuperscript{69}. It has been further reported that NK cells, recruited via CCR5, contribute to intestinal pathology most likely through production of IFN-\gamma in response to \textit{T. gondii} infection\textsuperscript{83}. Moreover a recent study demonstrated that \textit{Escherichia coli} (\textit{E. coli}) and \textit{E. coli}-derived lipopolysaccharide (LPS) exacerbate this Th1-mediated immunopathology in the gut via the stimulation of toll-like receptor (TLR)4. This finding suggests a critical role of the microflora in the development of \textit{T. gondii}-induced ileitis.

The \textit{T. gondii}-triggered ileal inflammation has been shown to resemble ileal inflammation in inflammatory bowel disease (IBD)\textsuperscript{69}. IBD is defined as a group of GI tract inflammatory disorders including ulcerative colitis and Crohn's disease (CD)\textsuperscript{84}. The \textit{T. gondii}-induced ileitis in mice is similar to CD in humans. Histologically, \textit{T. gondii}-induced gut inflammation occurs in the ileum where CD lesions are also frequently found. The pathology in both \textit{T. gondii}-induced ileitis and CD are discontinuous with transmural damage but granulomatous inflammation can mostly be noted in CD, not in \textit{T. gondii}-induced ileitis\textsuperscript{85}. In line with the Th1-dominant immunopathology observed in \textit{T. gondii}-triggered ileitis, CD is reported to be driven by IL-12 and other Th1 cytokines (IFN-\gamma and TNF-\alpha)\textsuperscript{86, 87}. However, there is growing evidence showing that Th17 immune responses and IL-17 and IL-23, an important cytokine for Th17 polarisation, are also responsible for the pathogenesis of CD\textsuperscript{88}, whereas the \textit{T. gondii}-induced immunopathology in the ileum has been shown to be mediated by IL-23 but
independent of IL-17\textsuperscript{51}. However, given the similarities there may be some mechanisms in common in the initiation of inflammation between CD and \textit{T. gondii} that make \textit{T. gondii} a useful model of gut ileitis.

### 1.4. Gastrointestinal helminthic infection

Infection of helminthic parasites in the gut causes significant morbidity throughout the world\textsuperscript{89}. Infection by intestinal helminths rarely causes death but chronic infection can result in growth retardation in children, anaemia and some physical and cognitive problems\textsuperscript{90}. Collectively, gut parasite infections worsen the economic status of a country\textsuperscript{91}. To date, there still is no vaccine in widespread use for most helminths although a clinical trial for hookworm has started\textsuperscript{92}. Currently, clinical treatment depends on anthelmintic and protozoacide drugs\textsuperscript{93}. Although drugs such as mebendazole and metrozidazole help control disease symptoms, they do not prevent reinfection and thus the development of drug resistance is a concern\textsuperscript{94}. There is an urgent need to improve current therapeutics and develop new strategies against these intestinal helminth infections.

#### 1.4.1. \textit{Trichinella spiralis}

\textit{Trichinella spiralis} (\textit{T. spiralis}) is a parasitic nematode belonging to the \textit{Trichinella} genus of \textit{Trichinellidae} family\textsuperscript{95}. This parasite is widely prevalent in the world from the arctic to the tropics\textsuperscript{96}. Domestic and sylvatic swine, synanthropic animals living near the swine herd (e.g., rats, mustelides and foxes) as well as a broad range of sylvatic carnivores are considered as major reservoirs for \textit{T. spiralis} \textsuperscript{97}. Among these animals, swine are considered the most important reservoir for \textit{T. spiralis}. In pigs, the parasite can reach a high worm burden (~8000 larvae/g in the diaphragm) without causing illness\textsuperscript{98}. In the human population, the consumption of uncooked, or improperly handled, meat products from pigs or wild animals infected by \textit{T. spiralis} transmit infection (trichinosis). Trichinosis is most prevalent in
countries and areas such as China\textsuperscript{99}, Argentina\textsuperscript{100} and central and eastern Europe\textsuperscript{101} because of the habit of eating raw meat. There were also several human cases of trichinosis recently reported in Germany, Italy and United Kingdom\textsuperscript{102, 103}.

The symptoms of trichinosis in humans change along with the stage of the parasite life cycle (i.e., enteral or muscle phase)\textsuperscript{104}. Initially the parasite invades the gut and causes symptoms including mild gastroenteritis with diarrhoea and upper abdominal pain around day 2 p.i.\textsuperscript{104}. The pathology results from the invasion of the intestinal epithelium by the adult worms and the larvae released into the mucosa. After at least one week of infection, severe clinical features may arise due to the parenteral stage of infection (migratory newborn larvae and muscle invasion)\textsuperscript{104}. The main symptoms of trichinosis include fever, facial oedema, and diffuse myalgia, but occasionally there will be lethal complications such as myocarditis, thromboembolism, and encephalitis\textsuperscript{105, 106}. The duration and the severity of trichinosis is highly dependent on the number of larvae ingested by the host\textsuperscript{104} and the outcome of \textit{T. spiralis} infection may vary from asymptomatic to a fatal illness.

\subsection*{1.4.2. Life cycle of \textit{T. spiralis}}

The life cycle of \textit{T. spiralis} (Figure 1.4) begins with the enteral phase in which the first stage muscle larvae (L1) are liberated from Nurse cell-larva complexes in meat by digestive juices in the stomach (pepsin and hydrochloric acid)\textsuperscript{107}. The released larvae pass into the small intestine and invade the intestinal epithelium\textsuperscript{96, 108}. After four moult\textsuperscript{s} the worms are adult and this occurs within 10 to 28 hr post ingestion. About 30 hr post ingestion, the mature males and females mate and shed newborn larvae which migrate via the lymph and blood vessels. It is estimated that an adult female worm can shed 500 to 1,500 newborn larvae over its life span\textsuperscript{107}. The adult worms are then expelled from the gut within weeks\textsuperscript{107}. Worm expulsion in mice does not take place until approximately two weeks after
The newborn larvae migrate in blood and penetrate skeletal muscle in which they form nurse cells. After the muscle has been invaded, it usually takes two weeks to form a Nurse cell. By 20 days after penetrating the muscle, Nurse cell-larva complexes can form and the larvae become inactive for months or years.\textsuperscript{96}

\textbf{Figure 1.4. The Life Cycle of Trichinella spiralis.}
Starting with the enteral phase, the ingested first-stage larvae are released from the Nurse cells after which they enter the small intestine and invade the intestinal epithelium. After maturation the adult worms can mate and shed newborn larvae into the lymphatic system, which causes pathology in the host. In the parenteral stage, the newborn larvae can migrate throughout the host body via blood and penetrate muscle. After invading the muscle, Nurse cell-larva complexes are formed. Adapted from\textsuperscript{107}. 
1.4.3. **Immunomodulation**

The adaptive Th2 response, characterised by increased IL-4, IL-5, IL-10, and IL-13, has been widely accepted as the major immune response induced in gastrointestinal helminth infection\(^{110}\). However, *T. spiralis* infection elicits a complex immune response. It is reported that *T. spiralis* infection *in vitro* promotes CD4\(^+\) T cell polarisation towards a mixed Th1/Th2 response with elevated IL-4, IL-9, IL-13, IL-10 and IFN-\(\gamma\) production\(^{111}\). *In vivo* infection with *T. spiralis* is characterised by an early induction of the Th1 response during the enteral phase and a Th2 predominant response which is responsible for the parasite expulsion from the gut in the later migratory phase\(^{112}\). Furthermore mice infected by *T. spiralis* develop Th17-associated jejunal inflammation 2 weeks p.i. and IL-17A is reported to alter smooth muscle contractility, which may result in an irritable bowel syndrome (IBS)-like disorder\(^{113,114}\).

In order to inhabit the host for a long time, many parasites have their own ways to modulate host immunity\(^{112}\). *T. spiralis* can suppress or down-regulate host immune responses and generate a tolerogenic environment for survival. *T. spiralis* infection induces the differentiation of CD25\(^+\)Foxp3\(^+\) regulatory T cells (Tregs) that can help control and downregulate inflammatory responses\(^{112}\). DCs serve as a pivotal link between innate and adaptive immunity and act as a sensor for the detection of infection to initiate inflammation. Therefore the ability to regulate DC function is important for parasites to survive\(^{112}\). Exposure to *T. spiralis* and its E/S antigens, suppress LPS-induced DC maturation and alters the expression of TLR signalling genes\(^{115}\). DCs-stimulated by E/S antigens also produce IL-10 and TGF-\(\beta\) which may promote Treg differentiation and suppress the development of adaptive immunity\(^{116}\).
Recent studies have shown that *T. spiralis* infection is involved in the functional modulation of macrophages toward the alternative activation pathway (alternatively activated macrophage; AAM). E/S antigens from *T. spiralis* reduce the capacity of macrophages to express TNF-α, IL-1β, IL-6 and IL-12 after LPS challenge and E/S antigens alone can enhance the expression of arginase-1 (Arg-1), TGF-β and IL-10\textsuperscript{117}. In addition, IL-4 and IL-13 which are the dominant Th2 cytokines induced by *T. spiralis* infection are reported to mediate AAM differentiation\textsuperscript{118}. According to previous studies, the formation of AAM are noted not only in mice but also in guinea pigs in response to *T. spiralis* infection\textsuperscript{119}.

Collectively these observations demonstrate that *T. spiralis* modulates host immunity via suppression of proinflammatory mediators and eliciting regulatory cells to promote a more tolerogenic environment.

1.4.4. Mucosal responses in *T. spiralis* infection

*T. spiralis* infection initiates small intestinal inflammation by penetrating IECs. The innate mucosal response to infection is characterised by the infiltration of neutrophils, eosinophils, macrophages and mast cells into the LP\textsuperscript{120, 121}. Villous atrophy and crypt hyperplasia are often noted at day 8 p.i. followed by an increase in the number of mast cells and goblet cells\textsuperscript{122}. An *in vitro* study using human IEC cell lines has shown that larva migration and invasion into epithelial cells causes cell death and damage with elevation of IL-1β and IL-8\textsuperscript{123}. In the murine model *in vivo*, there is also an increase in the number of IL-1β\textsuperscript+ inflammatory cells noted in the LP at day 8 p.i.\textsuperscript{124}. The early stage of *T. spiralis* infection in the small intestine is reported to induce a rapid but transient expression of thymic stromal lymphopoietin (TSLP) which is critical for the development of anti-helminth Th2 immunity\textsuperscript{125}. 
1.4.5. *T. spiralis*-induced murine ileitis model

*T. spiralis* infection in murine animals has been proposed as a post-infectious irritable bowel syndrome (PI-IBS) model\(^{126, 127}\). *T. spiralis* infection-induced PI-IBS is characterised by hypersensitivity of the intestinal sensory nerves\(^{128}\), alteration of smooth muscle contractility\(^{129}\) and changes in IEC transport\(^{130}\). Interestingly, acute *T. spiralis*-induced small intestinal inflammation where T cell and IL-1\(\beta\) responses are invoked usually elicits long-term enteric dysfunction in mice or rats\(^{124, 127}\). Nevertheless, the susceptibility to *T. spiralis* is different between mouse strains\(^{131}\). The physiological changes and pathology induced by *T. spiralis* infection are seen in susceptible mice such as outbred NIH/Swiss and inbred C57BL/6, whereas BALB/c mice are generally resistant to the infection\(^{132, 133}\). Thus, genetic background also plays a crucial role in the phenotype of the *T. spiralis*-induced murine model.

1.5. Dendritic cell biology

Dendritic cells (DCs) are professional antigen presenting cells, which were identified by Ralph M. Steinman in the late 1970s and named due to their unusual shape with dendrite-like projections\(^{134}\). DCs are specialised at priming T cells to induce adaptive immune responses but DCs also play a central role in the maintenance of immune tolerance\(^{134}\). Immature DCs are found in tissue sites where they play a key role in monitoring the environment and they are phagocytic cells that can take up antigen via multiple routes such as pinocytosis and phagocytosis\(^{135}\). In response to inflammatory stimuli including ligands for TLRs and proinflammatory chemokines and cytokines\(^{136}\), immature DCs go through a maturation process whereby they up-regulate cell surface antigen presenting machinery including antigen peptide-loaded major histocompatibility complex (MHC) and co-stimulatory molecules (eg., CD40, CD80 and CD86)\(^{137}\) as well as chemokine receptors for lymphoid organ homing (eg., CCR7 and CXCR4)\(^{138}\). During maturation, DCs down-regulate
their phagocytic ability to prevent processing of new antigens and reduce chemokine receptors for local inflammatory responses. The mature DCs, then, migrate to draining lymph nodes, where they present T cells with processed antigens via their MHC molecules (signal-1) together of the appropriate co-stimulatory molecules (signal-2) and T cell-polarising cytokines (signal-3) such as IL-12. The combination of these three signals determines the differentiation of T cells into antigen-specific effector cells (eg., Th1, Th2 or Th17). By contrast, immature DCs are unable to provide all three signals to induce competent effector T cell responses, but they can present antigens to naïve T cells, resulting in T cell deletion or anergy. In the presence of tolerogenic factors including IL-10, vitamin D3 and retinoic acid, these immature or semi-mature DCs adopt a regulatory capacity to maintain immune tolerance by inducing Tregs via regulatory mediators (eg., IL-10 and TGF-β) and inhibitory signals by engagement of CTLA-4.

1.5.1. Murine intestinal DCs

Dendritic cells (DCs) in the intestine and gut-associated lymphoid tissues (GALT) are a heterogeneous population, which are able to initiate primary immune responses in the gut, and determine immunity towards either immunogenic or tolerogenic responses. Another important function of intestinal DCs is their ability to program gut-homing of lymphocytes within lymph nodes or other secondary lymphoid organs. The imprinting of gut-homing T cells is required for localisation of immune responses the appropriate tissues. Thus, intestinal DCs play a pivotal role in the maintenance of gut immune homeostasis.

1.5.2. The origins of murine intestinal DCs

A group of Lin−CD117−CX3CR1−CD115+ bone-marrow (BM) cells called the macrophage and dendritic cell precursor (MDP) is identified as the common progenitor of conventional DCs (cDCs), plasmacytoid DCs, Ly6C+ monocytes and macrophages. MDPs give rise to
another DC progenitor in BM called the common dendritic cell precursor (CDP) which is phenotypically similar to MDP but does not respond to macrophage colony-stimulating factor (M-CSF)\textsuperscript{143,144}. CDPs are reported to only generate pDCs and pre-DCs, a cDC restricted progenitor\textsuperscript{144-146}. Pre-DCs circulate through the blood and enter lymphoid and non-lymphoid organs where they differentiate into cDCs\textsuperscript{144,147}. In addition to pre-DCs, circulating Ly6C\textsuperscript{+} monocytes are recently suggested not only as the precursor of gut-resident macrophages under steady-state conditions\textsuperscript{148} but also migratory inflammatory DCs (infDC) in response to inflammatory stimuli\textsuperscript{149,150}. Collectively, these DC precursors contribute to the physiological homeostasis of DCs in different organs.

The origins of intestinal DC subsets have been previously characterised using adoptive transfer of DC precursors\textsuperscript{151-153}. In 2009, two independent studies demonstrated that MDPs give rise to both intestinal CD103\textsuperscript{+} and CD103\textsuperscript{-} DCs and the differentiation of CD103\textsuperscript{+} DCs is driven by Fms-like tyrosine kinase 3 ligand (Flt3L) while the differentiation of CD103\textsuperscript{-} DCs may require the combination of Flt3L and granulocyte-macrophage colony-stimulating factor (GM-CSF)\textsuperscript{151,152}. Moreover it has been shown that CDPs and pre-DCs differentiate into CD103\textsuperscript{+}CD11b\textsuperscript{-} DCs, CD103\textsuperscript{+}CD11b\textsuperscript{+} DCs and CD103\textsuperscript{-} DC subset whereas Ly6c\textsuperscript{hi} blood monocytes mainly contribute to the CX3CR1\textsuperscript{+} macrophages\textsuperscript{151,153}.

\subsection*{1.5.3. Murine intestinal DC subsets and their functions}

DCs are commonly characterised as CD11c\textsuperscript{+} and major histocompatibility class (MHC) II\textsuperscript{+} cells, but the CD11c\textsuperscript{-}MHCII\textsuperscript{+} population in the GI tract consists of not only DCs but also macrophages\textsuperscript{154}. This original definition of DCs as CD11c\textsuperscript{+} led to some controversies in the field as findings that were actually true of macrophages were attributed to DCs and vice versa. Recent studies demonstrated that Zbtb46, a zinc finger transcription factor, is selectively expressed by classical DC lineage and can be used to distinguish cDCs from
plasmacytoid DCs, monocytes/macrophages and other immune cell lineages\textsuperscript{155, 156}. Functionally, classical DCs are thought to be professional antigen-presenting cells which prime naïve T cells to elicit immunity or tolerance, whereas macrophages are often tissue-resident for housekeeping functions, such as clearance of dead cells and tissue remodelling, and have little or no T cell-priming capability\textsuperscript{157}.

There have been two major CD11c\textsuperscript{+} populations identified in the lamina propria (LP) of the murine gut as CD103\textsuperscript{+}CX3CR1\textsuperscript{-} (70\% of total CD11c\textsuperscript{+} cells in LP) cells and CD103\textsuperscript{-}CX3CR1\textsuperscript{+} cells (approximately 30\% of total CD11c\textsuperscript{+} cells in LP) respectively\textsuperscript{158}. The CD103\textsuperscript{+}CX3CR1\textsuperscript{-} (CD103\textsuperscript{+}) cells are characterised as genuine DCs for their ability to migrate from the LP to the mesenteric lymph nodes (MLNs) and present locally administered antigen to naïve T cells\textsuperscript{159}. In contrast the CD103\textsuperscript{-}CX3CR1\textsuperscript{+} cells are thought to be a mixed population containing macrophages and DCs\textsuperscript{160}.

CD103\textsuperscript{+} DCs produce retinoic acid (RA) which is proposed to be important for imprinting gut-homing T cells and generation of inducible Tregs\textsuperscript{141, 161}. There are two main subpopulations of CD103\textsuperscript{+} DCs as defined by their expression of the CD11b marker: CD103\textsuperscript{+}CD11b\textsuperscript{+} and CD103\textsuperscript{+}CD11b\textsuperscript{-} DCs\textsuperscript{154}. Many studies have highlighted the functions of CD103\textsuperscript{+}CD11b\textsuperscript{+} DCs including sampling and transportation of luminal antigens to MLN for priming T cells\textsuperscript{162} and the induction of Th17 immunity\textsuperscript{163}. However much less is known about the CD103\textsuperscript{+}CD11b\textsuperscript{-} DCs, although these cells express TLR3, TLR7, and TLR9 and produced IL-6 and IL-12p40 to promote Th1 immunity\textsuperscript{164}.

Intestinal CD103\textsuperscript{+}CX3CR1\textsuperscript{+} cells can be distinguished into two subsets using the macrophage markers CD64 and F4/80 and characterised as CD103\textsuperscript{+}CX3CR1\textsuperscript{+}F4/80\textsuperscript{+}CD64\textsuperscript{+} (CX3CR1\textsuperscript{+}) macrophages and CD103\textsuperscript{+}CX3CR1\textsuperscript{+}F4/80\textsuperscript{-}CD64\textsuperscript{-} (CD103\textsuperscript{+}) DCs\textsuperscript{153}. It has been shown that the
CX3CR1+ macrophages can extend processes through the mucosal epithelium taking up luminal antigens and they may also transfer antigens to CD103+ DCs for T cell priming. CD103- DCs are thought to have proinflammatory functions. Recently, two functionally distinct subpopulations were noted in the CD103- DC subset. They are defined by CCR2 expression as CCR2+ and CCR2-CD103- DCs. Among these two subpopulations, CCR2+CD103- DCs are proposed to express high levels of IL-12/IL-23p40 and be involved in the development of mucosal Th17 immunity.

Plasmacytoid DCs (pDCs) are a specialised DC subset. They arise from the same common DC progenitors as cDC but have an appearance and genetic profile that are more closely linked to lymphoid cells, and plasma cells. However, when activated, they have a dendritic morphology with some of the antigen presentation properties of cDC. Murine pDCs are identified as CD11clowB220Siglec-H+PDCA-1CCR9+ cells that constitute less than 0.2% of total spleen cells but are more predominant (1.1-1.3%) in GALT such as Peyer’s patches. Generally, pDCs are regarded as a proinflammatory mediator for their capacity of secreting large amounts of type-I IFN by sensing pathogenic and damage signals, which activate both innate and adaptive immune cells. However, some studies have suggested an immunoregulatory role of pDCs to promote the development of immune tolerance. For example, Mizuno et al. showed that CCR9+ pDCs recruited to the small intestine in a CCL25-dependent manner were capable of suppressing the Th1-mediated ileitis induced by adaptive transfer of effector CD4+ T cells.

Inflammatory DCs (infDCs) are MHCIICD11b-F4/80Ly6C+ cells, differentiated in situ from circulating monocytes which are recruited to the inflammation site in response to inflammatory stimuli including infection or colitis. Like cDCs which conventionally migrate from tissues to lymph nodes for priming T cells, infDCs are characterised by their
ability to migrate in the draining lymph nodes and induce T cell immune response\textsuperscript{150}. For example, the E-cadherin\textsuperscript{+} infDCs are reported to accumulate in the MLN and colon and exacerbate mucosal pathology in a T cell-induced colitis model\textsuperscript{172}. Moreover, recent studies also show that infDCs are recruited to the inflamed gut in response to the infection of intestinal pathogens such as \textit{T. gondii} and \textit{Salmonella typhimurium}\textsuperscript{173, 174}.

1.5.4. Murine intestinal DC migration

Briefly, DC migration includes: (1) newly formed DCs or their progenitors exit BM and enter the blood circulation; (2) the lymphoid or non-lymphoid tissue-targeting recruitment of the circulating DCs or progenitors; (3) interstitial migration and antigen sampling; (4) the ability to access lymphatic system to travel either to lymph nodes or to the blood; and (5) the capability to prime or stimulate migrating lymphocytes, allowing the exchange of crucial information regarding the nature of the antigens for directing the following immune response\textsuperscript{175}. Under steady-state conditions, DCs migrate regularly to the intestinal lamina propria and subsequently to the draining lymph nodes for the induction of immune tolerance in response to commensal microorganisms and food components\textsuperscript{161, 176}. During inflammation, both pre-DCs and monocytes are recruited to the site of inflammation and give rise to cDCs and inflammatory DCs/macrophages respectively\textsuperscript{150}. The collaboration of recruited cDCs and inflammatory DCs can efficiently prime naïve T cells or stimulate CD4\textsuperscript{+} and CD8\textsuperscript{+} tissue-resident memory T cells for the initiation of memory responses in the tissue\textsuperscript{150, 177}.

The primary sites for DCs to induce intestinal adaptive responses are Peyer’s patches (PPs) in the small intestine, isolated lymphoid follicles in the small and large intestine, and MLNs\textsuperscript{178}. In PPs, cDCs are predominantly classified as the CD11b\textsuperscript{+}CD8\textsuperscript{α}\textsuperscript{−}, CD11b\textsuperscript{−}CD8\textsuperscript{α}\textsuperscript{+} and CD11b\textsuperscript{−}CD8\textsuperscript{α} subtypes\textsuperscript{178}. The CD11b\textsuperscript{+CD8α}− DC subset of DCs has a higher capacity to
induce Th2 immunity, whereas the CD11bCD8α+ and CD11bCD8α− DCs are shown to produce IL-12 and drive Th1 immune responses. The CD11b+CD8α− DCs appear to be largely recruited to the sub-epithelial dome (SED) in response to CCL9 and CCL20 secreted by epithelial cells in the follicle-associated epithelium (FAE), whereas CD11b−CD8α+ DCs, constitutively express CCR7, mainly localised in the T-cell inter-follicular region (IRF) in response to CCL7. During infection, the CCR6-expressing CD11b+CD8α− and CD11b−CD8α+ subsets are capable of trafficking, in response to CCL20, from the SED to FAE where they activate pathogen-specific CD4+ T cells. Moreover, after maturation, the DCs in the SED can down-regulate CCR6, express more CCR7, and migrate to the IFR for T cell priming. In addition to the IRF DCs, DCs in the LP also express CCR7 which is required for migration toward CCL19 and CCL21, and the entry into the MLN for presenting IEC antigens. A number of studies have reported the mechanisms of how DCs migrate from intestinal LP toward draining lymph nodes. However, the exact mechanisms by which DCs exist in blood and access non-lymphoid tissues such as the intestine are not fully understood. It is proposed that circulating DCs and DC precursors respond to tissue-specific chemoattractant signals or adhesion molecules to enter specific organs for either maintenance of the physiological turnover of resident populations or activation in response to infection and/or inflammation. Indeed it has been revealed that the interaction between α4β7 integrin, a gut-homing receptor, and mucosal vascular adhesin, MAdCAM1, mediates the constitutive recruitment of pre-mucosal DCs into the LP, where they differentiate into CD103+CD11b+ and CD103+CD11b− DCs in a RA-regulated manner. During inflammation, proinflammatory chemokines, including CCL2, CCL3, CCL5, CCL20 and CXCL12, are produced by epithelial cells to attract circulating DCs and inflammatory monocytes to the inflamed intestinal mucosa. These inflammation-recruited monocytes may further differentiate into inflammatory DCs for initiation of immune response. The specific functions of chemokines and their corresponding receptors are summarised in Table A.1 (appendix).
1.5.5. DCs and *T. gondii*

Early DC responses are known to determine disease outcome in *T. gondii* infection. For example, a study by Tait *et al.* demonstrated that mice infected by the lethal virulent *T. gondii* strain (RH) had fewer infiltrating DC and pathogen-specific CD8+ T cells, whereas the avirulent parasites (PRU) induced robust DC infiltration at the infection site and draining lymph nodes, followed by a strong CD8+ T cell response. In addition, IL-12 derived from CD103+CD11b-CD8α+ DC is thought to be necessary to detect *T. gondii* infection and induce the production of IFN-γ by T cells or NK cells, which consequently reduces the parasite burden and protects mice from mortality. These findings show that the induction of DC responses is indispensable for the control of the infection. However, the recruitment of DCs in response to *T. gondii* infection may not be always beneficial. Indeed it has been reported that there is a preference for *T. gondii* to infect DCs over other blood cells and DCs have been identified as a carrier of the parasite for systemic dissemination. Moreover, *T. gondii* infection results in a hypermigratory phenotype in DCs, which favours parasite dissemination, and the type II strain appears to better exploit DC migration than the type I and type III parasites.

1.5.6. DCs and *T. spiralis*

DCs are crucial innate immune cells that generate appropriate protective immunities in response to many intestinal helminth infections. In murine *Trichuris muris* (*T. muris*) infection, an animal model for the human *Trichuris trichiura* infection, the worms stimulate IECs to secret TSLP which conditions intestinal DCs for the development of Th2 immunity, which determines host resistance to *T. muris*. Similarly, *T. spiralis* also induces IEC-derived TSLP production during intestinal infection. In addition, recognition of antigens such as heat shock protein-70 from *T. spiralis* by DCs favours the development of a mixed Th1/Th2
immunity, which largely reduces the number of muscle larvae in *T. spiralis*-infected mice\textsuperscript{111, 191}. These findings implicate the importance of DC in the development of immunity against *T. spiralis*. However, in order to survive in the host, *T. spiralis* modulates the host immune system. Recent studies have shown that *T. spiralis* infection generates Tregs that ameliorate inflammation and tissue damage in experimental models of autoimmune and allergic diseases including chemical-induced colitis and OVA-induced allergic airway inflammation\textsuperscript{192, 193}. Indeed it is believed that this immunosuppressive Treg response is mediated by trichinella E/S antigens-conditioned DCs\textsuperscript{116}. Trichinella E/S antigens stimulate DCs to produce IL-4, IL-10 and TGF-β, which favour the generation of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} Tregs and restrains the detrimental Th1/Th17 immunity in an experimental autoimmune encephalomyelitis model\textsuperscript{116}. In addition, another study also demonstrated that the E/S antigens-conditioned DCs can *in vivo* prime T cells, which results in a mixed Th1/Th2 immunity with an increase in the proportion of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} Tregs in the spleen\textsuperscript{194}.

Collectively, DC responses in the gut are crucial for the innate control of intestinal parasites and the development of adaptive immunity against infection. According to those previous findings, the detection of pathogenic invasion is critical for the intestinal DC recruitment and functions. It is believed that pattern recognition receptors for pathogens/damages and IEC-derived factors play a pivotal role in activating and directing DC functions. Thus, I will address the importance of pattern recognition of pathogens/damages and IECs in the following sections.

1.6. **Pattern recognition receptors and damage-and pathogen-associated molecular patterns**

Cells are equipped with receptors called pattern recognition receptors (PRRs) to sense pathogens and tissue damage\textsuperscript{195, 196}. PRRs include the toll-like receptors (TLRs), nucleotide
oligomerisation domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), C-type lectin receptors, and some unclassified receptors. PRRs recognise highly conserved molecular patterns on microbes and pathogens termed pathogen-associated molecular patterns (PAMPs) and can also recognise endogenous damage-associated molecular patterns (DAMPs)\textsuperscript{197}. PAMPs are molecular structures of pathogens such as LPS, oligodeoxynucleotides containing CpG motifs and viral double strand ribonucleic acid\textsuperscript{198}. DAMPs are derived from damaged cells releasing their cellular contents or components of the extracellular matrix (ECM) released during tissue damage. Examples of DAMPs include extracellular ATP, high mobility group box 1 (HMGB1), and uric acid\textsuperscript{199}. Sensing these DAMPs and PAMPs by PRRs is necessary for the initiation of innate immune responses against infection or trauma\textsuperscript{195}.

1.6.1. Infection and DAMPs/PAMPs

Exogenous PAMPs and their binding to PRRs is well-known to induce proinflammatory responses to eliminate pathogens\textsuperscript{197}. In addition, there are alarmins or DAMPs released in response to tissue injury, resulted from trauma, for example, chemical insults, radiation or the withdrawal of oxygen and/or nutrients\textsuperscript{195}.

Tissue injury is associated with cell death. Some types of cell death are reported to mediate the release of alarmins/DAMPs including necrosis, infection-induced cell lysis, apoptosis and autophagy\textsuperscript{200-202}. Necrosis describes the energy-independent degradative processes in response to cell death caused by non-programmed and accidental cell damage such as infection, toxin and trauma\textsuperscript{203}. In the processes of necrosis, dying cells undergo acute swelling and degeneration, nuclear pyknosis, karyorrhexis, karyolysis and loss of cell membrane integrity which results in the release of a large amount of intracellular contents into the surrounding environment, activating immune cells for inflammation\textsuperscript{204}. By contrast,
apoptosis is a caspase-3, 6 and 8-dependent programmed cell death, which occurs normally during development and aging. During apoptosis, there are morphological changes of cells such as cell shrinkage, nuclear pyknosis and karyorrhexis and separation of cell fragments into apoptotic bodies. Although apoptosis rarely induces inflammation or release of intracellular components, it has been known that apoptosis can lead to the release of some DAMPs like ATP and sphingosine-1-phosphate (S1P) as “find-me” signals to recruit and activate phagocytes for clearance of apoptotic cells. There are other programmed cell death mechanisms including pyroptosis and autophagy. The initiation of pyroptosis is inflammasome and caspase-1-dependent and, unlike apoptosis, pyroptosis is reported to be proinflammatory, following sensing of DAMPs by PRRs. The role of another programmed cell death, autophagy, is controversial. Physiologically, autophagy initiates via the formation of a double-membrane bound structure termed the autophagosome to eliminate unnecessary organelles for the promotion of cell survival in response to various stresses including starvation, hypoxia, mitochondrial damage, and pathogen infection. It is believed that autophagy-mediated cell death is the result of excessive levels of cellular autophagy.

Upon cellular stress resulting in tissue damage, cells may release alarmins which also can be recognised by PRRs including TLRs and the receptor for advanced glycation endproducts (RAGE). Theses alarmins are in fact endogenous molecules with the following characteristics:

1. Alarmins, such as the high mobility group box 1 (HMGB1), are rapidly released following non-programmed necrotic cell death.
2. Immune cells can also be induced to produce and release alarmins such as cathelicidins, defensins and S100 proteins without dying, generally via the endoplasmic reticulum (ER) to Golgi secretion pathway or other specialised non-classical pathways.
3. Alarmins, for example, adenosine triphosphate (ATP) recruit and activate receptor-expressing immune cells, including DCs, and are thus directly or indirectly involved in the induction of adaptive immunity.

4. Alarmins should also restore physiological homeostasis by mediating tissue regeneration after physical insult or inflammation as for example, IL-33, a tissue-derived nuclear alarmin. During homeostasis, IL-33 is constitutively expressed and localised in the nucleus in epithelial tissues, such as the lung, skin and stomach. The release of nuclear IL-33 upon tissue damage, cell death or cellular stress, following exposure to allergens or infection with viruses or parasites alerts the immune system by activating immune cells, such as mast cells and innate lymphoid cell (ILC)2s, to induce Th2 immunity\textsuperscript{209}. Moreover, in the gut, the expression of IL-33, by IECs is important to maintain Treg responses through activation of ST2 receptor in the inflamed gut to prevent pathology caused by uncontrolled inflammation\textsuperscript{210}.

Pathogen infection which often causes tissue damage, is the causative agent that not only results in the exposure of exogenous PAMPs but also causes the release of endogenous DAMPs or alarmins\textsuperscript{195}. During infection, there are a variety of PAMPs recognised by PRRs, for example, pathogen components such as lipopolysaccharide (recognised by toll-like receptor (TLR)4), β-glucan (recognised by dectin-1) and flagellin (recognised by TLR5)\textsuperscript{211}. Following tissue injury caused by infection, multiple DAMPs are released from dying cells, for example, nuclear proteins such as HMGB1 (recognised by TLR2 and TLR4)\textsuperscript{212, 213} and histones (recognised by TLR9)\textsuperscript{214} and purine metabolites including ATP (recognised by P2X receptors)\textsuperscript{215} and uric acid (recognised by inflammasome)\textsuperscript{216, 217}. Detection of these PAMPs and DAMPs by PRRs triggers proinflammatory effects, which is crucial for the eradication of pathogens, clearance of dead cells, and regeneration of injured tissue in response to infection\textsuperscript{218}.
1.6.2. Pattern recognition receptors and *T. gondii*

TLRs are important in the recognition of *T. gondii* infection and initiate early immune responses. In rodents, *T. gondii* profilin, which is found within the endolysosome, can be sensed by TLR11/TLR12. Other endosomal receptors TLR7 and TLR9 are also involved in host resistance to *T. gondii* by sensing parasite RNA and DNA. The *T. gondii* surface structure glycosylphosphatidylinositol (GPI) is a target for cell surface TLRs including TLR1/TLR2 and TLR4. As well as TLRs, the NLR NLPR1 plays a critical role in sensing *T. gondii* infection and mediates inflammasome formation to eliminate parasite load and dissemination in mice and rats. Another NLR family protein, NOD2, has also been shown to play a role in the development of the anti-*T. gondii* Th1 response in the production of IFN-γ. These findings suggest the importance of PRRs in the protective response to *T. gondii* infection.

1.6.3. Pattern recognition and *T. spiralis*

Unlike *T. gondii*, TLRs and NLRs have not been identified in the recognition of *Trichinella* components directly, but it is reported that TLR signalling may still play a role in response to *T. spiralis* infection. For example, the suppressive effect of E/S antigens from *T. spiralis* muscle larvae on DC maturation is mediated by interfering with TLR4 signalling. A C-type lectin PRR called mannose receptor (MR) that binds to microbial structures containing mannose, fucose and N-acetylglucosamine has been shown to play a role in the recognition of *T. spiralis* infection. The MR can recognise components of *T. spiralis* muscle larvae and potentially induce inflammation by inducing nitric oxide (NO) production in macrophages. In contrast, recent work demonstrated that *T. spiralis* larval E/S antigens can up-regulate MR expression on macrophages. *In vitro* studies showed that increased MR mediated IL-10 and TGF-β production and prevented the secretion of proinflammatory cytokines via inhibition of MyD88/NF-κB signalling. Thus, the role of MR in *T. spiralis* infection has not yet been fully determined.
1.7. **Intestinal epithelial cells and barrier function**

The epithelium forms a single celled layer covering the entire GI tract. There are four IEC lineages in the intestinal epithelium that are differentiated from common intestinal stem cells: absorptive columnar enterocytes, goblet cells, Paneth cells and enteroendocrine cells which produce hormone regulators for digestive function. This specialised single-layer network of IECs not only regulates absorption of nutrition but also forms a physiological barrier to commensal bacteria and pathogens. Epithelial cells directly contribute to host defence. Paneth cells are known to secret antimicrobial peptides such as defensins, lysozymes, cathelicidins and phospholipase A2. Mucosal secretory immunoglobulin A (sIgA) produced by plasma cells in the LP is transported through columnar enterocytes and limits microbial colonisation and opsonises bacteria in the lumen. Goblet cells produce mucus as a protective feature for the intestinal barrier. The glycoprotein-rich polymeric mucin can bind water for limiting or slowing down water diffusion in the gut. The antimicrobial peptides secreted by Paneth and goblet cells into the lumen are concentrated in the mucus near the epithelium and thus an antibacterial gradient is generated from the epithelium outward to the lumen that helps keep bacteria at a distance. In addition to anti-microbial roles, enterocytes produce a variety of chemokines and proinflammatory cytokines such as IL-6, TNF-α and IL-1α/β for the recruitment and activation of immune cells in response to injury or infection.

The passage of substances through the IEC barrier has been characterised as two distinct pathways, transcellular and paracellular. Nutritional absorption by enterocytes is mainly via the transcellular pathway followed by lysosomal degradation to convert the absorbed proteins into small, non-immunogenic peptides. The paracellular transport of macromolecules is regulated by the tight junction, an intercellular junctional complex that
seals adjacent IECs and maintains epithelial integrity. Tight junctions are reported to react to external stimuli like food components, pathogens or commensal microbiota\textsuperscript{238}. Alterations of IEC integrity and changes of microbiota/IEC crosstalk have been shown to cause intestinal inflammatory disorders including celiac disease\textsuperscript{236}, inflammatory bowel disease\textsuperscript{239}, and mucositis\textsuperscript{240} and increase susceptibility to infection\textsuperscript{241}. These findings support a critical role for IECs in the maintenance of gut immune homeostasis.

1.7.1. **Intestinal epithelial cells and immunosurveillance**

In spite of being the physiological barrier segregating the inner and outer environment, IECs also have functions in mediating the communication between the external environment and host immunity. A specialised IEC called the microfold cell (M cell) is found in the follicle-associated epithelium of the Peyer’s patches where it is mediates sampling of antigens and microorganisms in the gut lumen across the epithelium to the LP for immunosurveillance\textsuperscript{242}. As well as M cells, a recent report showed that goblet cells are also capable of delivering soluble antigens to LP CD103\textsuperscript{+} DCs for the development of immune responses\textsuperscript{243}. These pathways for sampling luminal antigens may direct host immunity toward tolerance or anti-pathogen responses.

1.7.2. **Intestinal epithelial cells and pattern recognition receptors**

As the first line facing a dynamic microbial environment, IECs express PRRs including TLRs, NLRs and RLRs to sense diverse microbial signals\textsuperscript{244}. As there are abundant symbiotic commensals in the GI tract, the recognition of microbial ligands in IECs in the steady state usually results in tolerance or hyporesponsiveness\textsuperscript{245}. Deficiency in MyD88 or TLR2 and TLR4 signalling results in significant loss of cytoprotective and reparative factors, which makes animals more vulnerable to dextran sulfate sodium (DSS) induced colitis\textsuperscript{246}. Moreover, another study demonstrated that prolonged stimulation of TLR2 and TLR4 in IECs leads to
a decrease in TLR expression and TLR-downstream signalling, which contributes to hyporesponsiveness. In spite of the maintenance of tolerance to commensals, IECs do respond differently to different commensal bacteria. For example, segmented filamentous bacteria preferentially condition IECs to produce antimicrobial peptides and develop Th17 immunity\textsuperscript{247}, whereas \textit{Bacteroides thetaiotaomicron}, a Gram-negative anaerobic commensal, negative regulates NF-κB signalling in IECs to exert anti-inflammatory responses\textsuperscript{248}. In addition, an \textit{ex vivo} work by Lan \textit{et al.} using primary colonic epithelial cells shows that stimulation of different commensal bacteria induces different cytokine and chemokine responses\textsuperscript{249}. These findings highlight the function of IECs in maintaining the balance between host and microbial symbiosis.

Despite the maintenance of hyporesponsiveness to the commensal microbiota, IECs, as the frontier sensor, must have mechanisms to distinguish commensal signals from pathogenic invasion for inducing appropriate inflammatory response. It has been reported that the polarised nature of IECs allows differential anatomical distribution of TLRs with differential responsiveness to apical versus basolateral stimulation\textsuperscript{250-252}. For example, apical stimulation of TLR9 leads to negative signals that inhibit the inflammatory responses induced by the activation of other TLRs, whereas basolateral exposure of TLR9 ligand results in canonical activation of the NF-κB pathway to promote intestinal inflammation\textsuperscript{250}. As well as TLRs, the activation of another PRR, NOD2, is also regulated by spatial segregation. A basolateral membrane scaffold protein, FERM and PDZ domain-containing 2 (FRMPD2), in IECs is reported to interact with the leucine-rich repeat (LRR) domain of NOD2, which localises the NOD2-signalling complex and restricts NOD2-mediating immune responses to the basolateral compartment of IECs\textsuperscript{253}. It is reported that mutations in the LRR domain, which impair the interaction with FRMPD2 and cause NOD2 dysfunction, are highly associated with the occurrence of CD\textsuperscript{254, 255}. Although spatial specific stimulation is crucial
for regulation of PRR functions, mechanisms by which IECs may use to switch the tolerogenic status to inflammation in response to infection or damage is still not well-understood.

Several PRRs in IECs have been shown to be important to initiate host defence in response to infection. For example, rotavirus infection activates RIG-I, melanoma differentiation-associated gene 5 (MDA5) and mitochondrial antiviral-signalling (MAVS) pathways to induce type-I IFN response in IECs\textsuperscript{256}. NOD2 signalling in IECs is critical for recruitment of CD103\(^+\) DC and the development of protective Th2 immunity in response to \textit{Trichuris muris} infection\textsuperscript{257}. A recent study demonstrated that the early activation of NLRP3-dependent inflammasome response in IECs protects mice from \textit{Citrobacter rodentium} infection\textsuperscript{258}.

### 1.7.3. Intestinal epithelial cells and immune homeostasis

In order to regulate gut immune homeostasis, IECs produce numerous immunomodulators that are required for the differentiation and function of innate and adaptive immune cells\textsuperscript{245}. IECs are reported to attract CCR9\(^+\) intraepithelial lymphocytes via production of CCL25\textsuperscript{259}. IEC-derived IL-15 and IL-7 have been shown to be required for the development of intraepithelial NK cells, \(\alpha\beta\)T cells and \(\gamma\delta\)T cells\textsuperscript{260-262}. In response to LPS stimulation, IECs produce CCL20 and CCL28 to recruit IgA-producing B cells which are subsequently stimulated by IEC-derived TNF family ligands, a proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF) to promote IgA2 class-switching\textsuperscript{263}. The lumen antigen-sampling of epithelium-associated CX3CR1\(^+\) macrophages is controlled by IECs via the expression of CX3CL1\textsuperscript{264}. Moreover, IECs produce semaphorin-7A to condition CX3CR1\(^+\) macrophages towards a tolerogenic phenotype by secreting IL-10\textsuperscript{265} which promotes the expansion of LP Tregs\textsuperscript{266}.  

Many studies have indicated the importance of IEC/DC crosstalk in the modulation of gut immune responses. For example, DCs, conditioned by IEC-derived IL-10, are capable of inducing regulatory T cells or Th2 responses\(^{267}\). IEC-produced TSLP can suppress IL-12 expression by DCs and the conditioned DCs express OX40 ligand and produce IL-10 favouring the development of Th2 immunity\(^{268}\), whereas unconditioned DCs highly express IL-12 and may induce Th1 or Th17 immunity\(^{267, 269}\). TGF-\(\beta\) and retinoic acid (RA) expression by IECs are crucial factors for CD103\(^{+}\) DC conditioning\(^{270}\). It has been reported that conditioned migratory CD103\(^{+}\) DCs possess a Treg-promoting phenotype and the RA-derived from these CD103\(^{+}\) DCs imprint gut-homing in T cells and B cells\(^{141}\).

In addition to innate immune cells such as DCs and macrophages, the recently identified innate lymphoid cells (ILCs) are also partially regulated by IECs for the maintenance of gut immune homeostasis\(^{245}\). Three groups of ILCs, the ILC1s, ILC2s and ILC3s, have been categorised by their cytokine expression and the transcription factors that regulate their development and function\(^{271}\). ILC1s produce IFN-\(\gamma\) and TNF-\(\alpha\) for inducing intestinal inflammation in response to IL-15 and IL-12\(^{271}\), which is indirectly suppressed by IEC-derived IL-25\(^{2}\). The proliferation and activation of IL-5/IL-13-producing ILC2 cells is supported by IECs via secretion of IL-25, IL-33 and TSLP to promote Th2 immunity in the gut\(^{2}\). The function of IL-23-responsive ILC3 cells are complicated but similar to Th17/Th22 cells which secrete IL-17A\(^{272, 273}\) or IL-22\(^{274, 275}\) for respectively inducing intestinal inflammation or protecting tissue from immune-mediated damage\(^{271}\). Indeed IECs are reported to either suppress ILC3-secreted IL-22 or promote IL-22 production in response to symbiotic microbial stimulation\(^{276, 277}\). These studies indicated that IECs play a pivotal role in the balance between inflammation and protective functions and targeting differential ILC3 cells by IECs leads to distinct immune responses.
Collectively, IECs serve as a mediator linking the microbiota and the host immune system, which contributes to the maintenance of gut immune homeostasis. In addition, the cross-talk between IECs and innate/adaptive immune cells is critical for directing the appropriate intestinal immune response. In the following section, I would like to introduce a DAMP receptor, P2X7 receptor that IECs may use to sense tissue damage and initiate intestinal inflammation in response to infection.

1.8. Purinergic P2X7 receptor

Extracellular ATP, as a DAMP molecule, has been shown to be recognised by the ATP–gated purinergic receptor P2X7 receptor (P27R). The P2X7R is a member of the P2X receptor (P2XR) family which are extracellular ATP-gated ion channels with similar structures: an extracellular ligand-binding site for a native agonist and an ion channel formed by transmembrane domains (TM). The P2XR family is extensive with seven genes encoding seven P2X subunits (P2X1-7). The majority of the P2X subunits have 55% homology, but the P2X7 receptor (P2X7R) is the least like the others with approximately 43% homology.

Each P2XR subunit has a large extracellular nucleotid-binding domain flanked by two hydrophobic regions (TM domains: TM1 and TM2), and cytoplasm-located N and C termini. Functional receptors are formed from both homotrimers and heterotrimers. All P2XRs except P2X6R can form homotrimers and there have been six functional P2XR heteromeric receptors characterised: P2X1/2R, P2X1/4R, P2X1/5R, P2X2/3R, P2X2/6R, and P2X4/6R. P2X7R is the only one that does not hetero-polymerise with the other P2XR.

A basic structure of P2X7R is summarised in Fig. 1.5.

P2X7R is ubiquitously expressed in various cell types including immune cells, neuron cells, endothelial cells and epithelial cells. Activation of P2X7R, by its native agonist ATP, induces influx of Ca$^{2+}$ and Na$^{+}$ accompanied by efflux of K$^{+}$ which results in membrane
depolarisation. Many studies have reported that P2X7R plays a modulatory role in the initiation of inflammatory responses in DCs and macrophages to allergen or intracellular infection. In addition, P2X7R is known to be involved in chronic neuropathic pain, inflammatory disorders, and autoimmune diseases.

Figure 1.5. The basic structure of P2X7R.
A cartoon representation of the subunit arrangement of mammalian P2X7Rs. Each subunit has two hydrophobic, transmembrane domains (TM1 and TM2) separated by an extracellular domain (EC). S-S: disulphide bonds.

1.8.1. P2X7R signalling pathways
P2X7R action and intracellular signalling can be mediated by several transcription factor networks (summarised in Fig. 1.6) including mitogen-activated protein kinase (MAPK) pathway, activator protein-1 (AP-1) family, early growth response protein (EGR), cAMP
response element-binding protein (CREB), NF-κB and nuclear factor of activated T-cells (NFAT).

**AP-1**

Extracellular ATP stimulation activates multiple downstream signalling events including the AP-1 transcription factors such as FosB, c-Fos, and JunB in human lymphoblast cell lines, RAW 264.7 murine macrophages and in human peripheral blood monocytes\(^{289, 290}\). A role for P2X7R-induced FosB also revealed that a P2X7R agonist could induce the FosB-dependent expression of pro-inflammatory proteins such as cyclooxygenase-2 (COX-2), the rate-limiting enzyme in the biosynthesis of prostaglandins such as PGE\(_2\)\(^{290}\).

**EGR family**

The EGR family, which includes Egr-1, Egr-2, Egr-3, and Egr-4, are zinc-finger transcription factors that play important roles in immunity\(^{291}\). There is abundant evidence indicating that stimulation of P2X7R can influence EGR gene expression. Stefano et al. demonstrated that stimulating P2X7R expressing human embryonic kidney 293 (HEK293) cells with 3′-O-4-benzoylbenzoyladenosine 5′-triphosphate (BzATP) could enhance the up-regulation of Egr-1 via the ERK pathway and the Elk-1 transcription factor\(^{292}\). P2X7R stimulation has also been shown to induce Egr-2 and Egr-3 production in microglial cells, with knock-down of EGR resulting in attenuated P2X7R-mediated IL-6 and TNF-α production\(^{293}\).

**CREB**

CREB is a transcription factor, which binds to a specific DNA sequence called the cAMP response element (CRE: 5’TGACGTCA3’). P2X7R, in response to the agonist BzATP, can induce CREB phosphorylation in both murine and human macrophages as well as microglial
cells. P2X7R signalling can also activate CREB via the extracellular-signal-regulated kinase (ERK)-dependent pathway, which is thought to be involved in the neuroprotective effect of P2X7R.

NF-κB

P2X7R stimulation by its agonist can promote NF-κB translocation and DNA binding in mouse microglial cells, mouse osteoclasts, murine macrophages, human T cells, and human peripheral blood mononuclear cells (PBMCs). Activated NF-κB translocates to the nucleus and induces the expression of cytokines such as TNF-α and IL-6 as well as chemokines.

NFAT

Several groups have shown that P2X7R activation is also relevant to the nuclear factor of activated T-cells (NFAT) transcription factor family. The NFAT family are important mediators of inflammation as well as the cardiovascular and musculoskeletal development.
Figure 1.6. A summary of P2X7R activation-mediated intracellular signalling and control of transcriptional regulators.

ATP stimulation induces efflux of potassium and influx of calcium and sodium, which may lead to MAPK activation, NFAT and NF-κB nuclear translocation, formation of reactive oxygen species (ROS), and other signalling events such as phospholipase D (PLD). MAPK activation is directly linked to the activation of CREB and EGR transcriptional factors. The AP-1 family includes cFos, FosB, JunB, cJun; Elk-1: Ets-like gene-1 is linked to EGR expression; CBP is CREB binding protein required for CREB-mediated gene expression; P is phosphate. Adapted from 263

1.8.2. P2X7R in immune cells

P2X7R is highly expressed in immune cells, such as monocytes/macrophages, DCs and lymphocytes, and has been shown to have multiple functions in the regulation of immune responses 297. The best known function of P2X7R is to trigger the assembly of the NLRP3 inflammasome, a multiprotein complex containing NLRP3, an adaptor apoptosis-associated speck-like protein (ASC) and procaspase-1 297. This inflammasome complex initiates a series of proteolytic events processing the secretion of proinflammatory cytokines IL-1β and IL-18 298, 299. Many publications have demonstrated the P2X7R-mediated release of IL-1β and
IL-18 from macrophages\textsuperscript{300-303}. Those data suggest that P2X7R is important for monocyte and macrophage initiation of the inflammatory response.

Recent investigations on P2X7R in DCs have shown that extracellular ATP stimulation causes human and murine DC maturation, activation and apoptosis\textsuperscript{304-306}. Furthermore, P2X7R deficient DCs cannot induce Th2 immunity in a murine asthma model\textsuperscript{285}, and blockade of P2X7R in a graft-versus-host disease (GVHD) model resulted in increased numbers of Tregs and reduced fatality\textsuperscript{307}. P2X7R is also involved in mast cell function with recent studies demonstrating that extracellular ATP stimulation promoted mast cell death and concomitantly the expression of several proinflammatory cytokines, such as IL-4, IL-6, IL-13, and TNF-\(\alpha\)\textsuperscript{308}. Other studies show that P2X7R mediates superoxide generation in neutrophils\textsuperscript{309}, suppresses the function and development of Tregs\textsuperscript{310} and the homing of lymphocytes in chronic lymphocytic leukaemia\textsuperscript{311}. Collectively these studies not only implicate a role for P2X7R in innate immunity but also in the modulation of adaptive immunity, allergy, and tolerance.

**1.8.3. P2X7R-mediated cell death**

P2X7R stimulation by the agonist BzATP mediates the formation of plasma membrane pores which cause cytolysis of macrophages\textsuperscript{312, 313}. P2X7R has also been reported to mediate cell death in human embryonic kidney cells, human cervical epithelial cells and human intestinal epithelial cells\textsuperscript{284, 314, 315}. Moreover, in vivo studies in rats demonstrated that peritraumatic neuron death is associated with activation of P2X7R by extracellular ATP\textsuperscript{316}. There have been several studies reporting the mechanism of P2X7R-induced apoptosis in different cell types. Although overexpression of P2X7R in HEK293 cells was found to promote cell growth, sustained high levels of extracellular ATP stimulation caused rapid mitochondrial depolarisation, increased mitochondrial Ca\(^{2+}\), mitochondrial fragmentation
and cell death\textsuperscript{314}. Similar results were reported by Garcia-Marcos et al. using rat submandibular glands, but they found that Na\textsuperscript{+}, not Ca\textsuperscript{2+} induced mitochondrial membrane depolarisation in response to ATP stimulation\textsuperscript{317}. Apparently, P2X7R is a bifunctional receptor in the regulation of mitochondrial integrity.

1.8.4. P2X7R-mediated killing of intracellular pathogens

Activation of P2X7R has also been described as inducing intracellular killing of \textit{Mycobacterium tuberculosis} (\textit{M. tuberculosis}) and \textit{T. gondii} in macrophages\textsuperscript{318, 319}. The P2X7R–mediated killing of \textit{M. tuberculosis} and \textit{T. gondii} in macrophages is proposed to be mediated both by apoptosis of infected cells and/or promoting phagosome-lysosome fusion\textsuperscript{286, 318-320}. Activation of P2X7R was reported to trigger IL-1\beta secretion from macrophages in the presence of LPS\textsuperscript{300}. In addition, IL-1\beta can induce apoptosis or necrosis in several kinds of cell including epithelial, pancreatic beta and neuron cells\textsuperscript{321-323}. It was speculated that the main cause of macrophage apoptosis and killing of intracellular pathogens may be the P2X7R–induced release of IL-1\beta. However, the murine macrophage cell line, RAW 264.7, which does not secrete functional IL-1\beta, is susceptible to ATP-induced macrophage apoptosis and can drive concomitant killing of intracellular pathogen\textsuperscript{303, 324}.

1.8.5. P2X7R-mediated mucosal inflammatory responses

The importance of mucosal P2X7R in the development of intestinal inflammation has been previously demonstrated. \textit{In vitro} experiments using mouse intestinal epithelial cells, Mode-K, demonstrated that activation of P2X7R enhanced the production of CXCL1 and IL-6 in response to TLR2/4 stimulation\textsuperscript{325}. \textit{In vivo} intrarectal injection of ATP also induced the expression of CXCL1 and IL-6 in IEC\textsuperscript{325}. In a recent study, the histological analysis of colon specimens from CD patients showed an over-expression of P2X7R in the epithelium and LP as well as the infiltrated DCs and macrophages\textsuperscript{326}. Blocking P2X7R in a mouse model of
colitis, not only efficiently decreased cytokine production of IL-1β and TNF-α, but also protected mice from the induction of colitis. These findings suggest an important role of P2X7R in mucosal IECs and immune cells in the modulation of intestinal inflammatory responses.

1.8.6. **P2X7R-/-** animal models

There are two gene-knockout mouse models widely used for studying P2X7R function: one is the P2X7R-/- Pfizer (The Jackson Laboratory) and the other is the P2X7R-/- Glaxo (Glaxo-Smith-Kline, UK). These two P2X7R-/- mice were developed using different constructs (Fig. 1.7). For the construct used in P2X7R-/- Pfizer mice, the targeted P2X7R gene was disrupted at the carboxyl-terminal coding region by a targeting vector containing a neomycin resistance gene driven by the mouse phosphoglycerate kinase promoter. The 129P2/OlaHsd-derived E14Tg2a embryonic stem (ES) cells were transformed using this construct and correctly targeted ES cells were injected into C57BL/6 blastocysts. The chimeric animals derived from targeted ES cells were backcrossed to C57BL/6 mice for 7 generations and named as B6.129P2-P2rx7tm1Gab/J (The Jackson Laboratory). For the construct used in P2X7R-/- Glaxo mice, gene targeting was performed in 129 ES cells. A targeting vector containing the LacZ reporter gene and neomycin-resistance cassette was used to locate immediately downstream of the translation codon in exon 1 of the P2rx7 gene. The gene targeting resulted in the disruption of exon 1 by insertion of the LacZ gene and neomycin-resistance cassette without deletion of any of the P2rx7 sequence. The chimeric animals-derived from targeted 129 ES cells were maintained on a C57BL/6 background.

Although both the Pfizer and Glaxo mice are functionally P2X7R deficient, several conflicting results have been reported using these mice. An *in vivo* study of osteoclasts using the Pfizer
P2X7R knockout mice showed that the knockouts have brittle bones characterised by increased bone absorption and deficiency in periostal bone formation\textsuperscript{330}. However, conflicting observations were found using the P2X7R\textsuperscript{-/-} Glaxo mice which demonstrated no significant skeletal problems and the retention of ability of precursors to form multinucleated osteoclasts\textsuperscript{331}. Chessel \textit{et al.} found that the P2X7R\textsuperscript{-/-} Glaxo mice have enhanced IL-6 secretion following intraplantar Freund's complete adjuvant injection\textsuperscript{287}. In two studies of multiple sclerosis, the P2X7R\textsuperscript{-/-} Glaxo mice had reduced axonal damage with lower incidence of experimental autoimmune encephalomyelitis\textsuperscript{332}, whereas axonal damage was exacerbated in the P2X7R\textsuperscript{-/-} Pfizer mice\textsuperscript{332, 333}. The main reason as to why the immunophenotypes of these two knockout mice are different is unexplained, but it is believed that the gene disruption methods used in making the mice may cause the different phenotypes. Several reports have shown that P2X7R\textsuperscript{-/-} Pfizer mice are a useful tool for studying ATP/P2X7R interaction in intestinal immune cells and revealed the importance of P2X7R in the development of intestinal inflammation\textsuperscript{310, 334}. Thus, we used P2X7R\textsuperscript{-/-} Pfizer mice to study the role of P2X7R in the initiation of small intestinal inflammation in response to parasitic infection.
Figure 1.7. The constructs of P2X7R+/− Pfizer and P2X7R+/− Glaxo mice. 
(a) The Pfizer mice were constructed by a deletion in the region containing amino acid 506–532, followed by a neomycin cassette inserted in a 3’ to 5’ direction, whereas (b) the Glaxo mice had an insertion of lacZ gene at the exon 1 region of the P2X7R gene. Adapted from310.

1.9. Hypothesis and aims

The importance of P2X7R as a sensor of damage to trigger the proinflammatory responses has been well-established. However, most of the work that has been done is focused on immune cells and very little is known about the role of P2X7R in epithelial cells, especially the IECs. IECs are important for the modulation of intestinal immune homeostasis and the initiation of immunity. Furthermore, the interaction between IECs and DCs determines DC function and can impact on the outcome to infection. We hypothesised that P2X7R in IECs may therefore play a role in IECs/DCs crosstalk for the initiation of intestinal inflammation and protective immunity in response to infection.
Thus, my hypothesis is that P2X7R in IECs plays a critical role in recognising and responding to infection which promotes recruitment of intestinal DCs, initiation of inflammation and the subsequent generation of adaptive immunity in response to infections.

The aims of this thesis are:

(a) To investigate the initiation of immune response and the IEC-derived chemokine factors in response to *T. gondii* infection.

This aim was addressed in Chapter 2 using the type II *T. gondii* PRU strain to infect wildtype (WT) and P2X7R$^{-/-}$ mice littermate controls. The *in vivo* effect of P2X7R deficiency was assessed by analysing the early infiltration of immune cell populations and comparing the early IEC-derived chemokine response between WT and P2X7R$^{-/-}$ mice. In addition, the development of protective Th1 immunity against *T. gondii* infection was investigated by analysis of splenic IFN-$\gamma^+$ CD4$^+$ T cells and serum IFN-$\gamma$ levels in WT and P2X7R$^{-/-}$ mice after infection.

(b) To investigate the initiation of immune response and the IEC-derived chemokine factors in response to *T. spiralis* infection.

This aim was addressed in Chapter 3 using *T. spiralis* to infect WT and P2X7R$^{-/-}$ mice littermate controls. The *in vivo* effect of P2X7R deficiency was assessed by analysing the early infiltration of immune cell populations and comparing the early IEC-derived chemokine response between WT and P2X7R$^{-/-}$ mice. In addition, the development of protective Th2 immunity against *T. spiralis* infection was investigated by analysing worm burden, IL-4 production and the proportion of IL-4$^+$ CD4$^+$ T cells in the spleen and mesenteric lymph node (MLN).
(c) To clarify the mechanism of how P2X7R regulates IEC-derived chemokine production in response to T. gondii infection.

This aim was addressed in Chapter 4 using mouse colorectal carcinoma cells as an in vitro model for T. gondii infection and the P2X7R antagonist A-740003 to block P2X7R function. To investigate TLR signalling in the P2X7R-dependent regulation of chemokine response, primary WT and P2X7R\(^{-/-}\) crypt organoids were also established and stimulated by LPS and their cytokine responses analysed. In addition, the traditional P2X7R functions including induction of apoptosis and NF-κB activation were assessed to see which pathway plays a major role in the P2X7R-dependent chemokine response in IECs in response to T. gondii infection.

References


73


45. Zhang T, Kawakami K, Qureshi MH, Okamura H, Kurimoto M, Saito A. Interleukin-12 (IL-12) and IL-18 synergistically induce the fungicidal activity of murine peritoneal exudate cells against Cryptococcus neoformans through production of gamma interferon by natural killer cells. *Infect Immun* 1997; **65**(9): 3594-3599.


70. Schreiner M, Liesenfeld O. Small intestinal inflammation following oral infection with Toxoplasma gondii does not occur exclusively in C57BL/6 mice: review of 70 reports from the literature. *Mem Inst Oswaldo Cruz* 2009; 104(2): 221-233.


Chapter Two

A Novel Role for P2X7 Receptor in Promoting Recruitment of CD103+ Dendritic Cells and Regulating Immunity to *Toxoplasma gondii* infection
A Novel Role for P2X7 Receptor in Promoting Recruitment of CD103+ Dendritic Cells and Regulating Immunity to *Toxoplasma gondii* infection

Szu-Wei Huang1, Catherine Walker,1 David Brough1, Werner Muller1, Gloria Lopez-castejon2, Sheena Cruickshank1

1Faculty of Life Sciences, The University of Manchester, Manchester, UK, 2Manchester Collaborative Centre for Inflammation Research, University of Manchester, UK.

**AUTHOR CONTRIBUTION**

Szu-Wei Huang carried out all experimental work and analysis.

Catherine Walker helped with the primary culture of small intestinal crypt cells

David Brough provided scientific input.

Gloria Lopez Castejon provided scientific input.

Werner Muller helped with the primary culture of small intestinal crypt cells

Sheena Cruickshank is the main supervisor of this project.
ABSTRACT

The purinergic P2X7 receptor (P2X7R) recognises ATP released by cell damage. We investigated the role of the P2X7R in initiation of gut inflammation and immunity against the common enteric pathogen *Toxoplasma gondii*. *T. gondii* infection *in vivo* induced early epithelial cell death in wildtype (WT) mice which was significantly reduced in littermate P2X7R⁻/⁻ mice. Analysis of dendritic cell subsets in the gut revealed a rapid infection-induced recruitment of CD11c⁺CD103⁺ CD11b⁻ dendritic cell (DC) subsets into the epithelial layer of WT but not P2X7R⁻/⁻ mice. To address the effect of impaired DC migration in P2X7R⁻/⁻ mice on immunity we analysed T cell responses at day 8 post-infection and found reduced IFN-γ⁺ CD4⁺ T cells and serum IFN-γ levels. *In vitro* assays showed normal DC migration and antigen presenting cell function suggesting that epithelial expression of P2X7R was important for DC recruitment. Indeed, experiments using bone marrow chimeras demonstrated the importance of epithelial P2X7R to the DC response. Compared with WT, P2X7R⁻/⁻ epithelial cells had reduced CCL5 and CCL20 expression in response to infection. Thus, P2X7R signalling is required for chemokine-mediated early recruitment of DCs in the small intestine and protective T-cell immunity against *T. gondii* infection.
INTRODUCTION

Toxoplasma gondii, is an obligate protozoan parasite that infects humans and warm-blooded animals via undercooked contaminated meat and oocyst-contaminated food\(^1\). T. gondii infection is controlled by an interleukin (IL)-12-dependent Th1 and IFN-\(\gamma\) dominated immune response\(^2\). Dendritic cells (DCs) are one of the major immune cells that produce IL-12 and initiate protective immunity against T. gondii\(^3,4\). The intestine has a diverse DC population with the majority expressing CD103, which plays a crucial role in immune tolerance and generation of adaptive immunity\(^5\). During inflammation, CCR2-dependent monocyte recruitment to the inflamed site results in their differentiation into macrophages or inflammatory DCs (InfDCs)\(^6\). There have been several studies indicating the importance of InfDCs in the control of intracellular pathogens including Leishmania major, Trypanosoma brucei, Mycobacterium tuberculosis and herpes simplex virus-2 (HSV-2)\(^7\).

Numerous chemokines, including CCL2, CCL3, CCL5, CCL20 and CXCL8, promote recruitment of InfDCs to sites of infection\(^8\). Previously, we demonstrated that colonic epithelial derived chemokines promote rapid recruitment of DCs into the large intestine after Trichuris muris (T. muris) infection\(^9,10\). A role for CCR5, the receptor for CCL3, CCL4, and CCL5 is implicated in Toxoplasma infection\(^11\). Although CCR5 expression on DCs is important for antigen delivery\(^12\), it not understood how the DC are recruited to the site of infection. Given that the intestinal epithelium is the major entry site of pathogens, and produces infection-induced chemokines and cytokines\(^13\), it seems probable that epithelial cells act as an infection-sensor of infections. Indeed, epithelial cells can be infected and lysed by T. gondii, and epithelial cells can promote DC polarisation\(^14\). These data support the hypotheses that infection induced cell injury initiates epithelial responsiveness, and the interaction between epithelial cells and DCs is required for DC recruitment.
Following cell damage from necrotic death a number of damage-associated molecular pattern molecules (DAMPs) are released. Many DAMPs are cytosolic or nuclear components such as adenosine 5′-triphosphate (ATP). Extracellular ATP is sensed by the ATP–gated purinergic P2X receptors (P2XRs). There are seven different mammalian genes encoding for P2X receptor subunits, namely, P2X1-P2X7, which trimerically assemble to form the P2XRs. Activation of P2X7R by ATP induces Ca\textsuperscript{2+} and Na\textsuperscript{+} influx and K\textsuperscript{+} efflux resulting in alteration of cell permeability by membrane pore formation, and cell death. Under pathophysiological conditions, accumulation of ATP released from lysed cells can enhance P2X7R activation and trigger activation of the NLRP3 inflammasome promoting IL-1β and IL-18 secretion. P2X7R is expressed by a variety of cell types including immune and epithelial cells. However, little is known about P2X7R activation in epithelial cells.

In this study, we investigated the role of P2X7R in the initiation of inflammation and the development of protective immunity to *T. gondii* infection. We found that P2X7R\textsuperscript{-/-} mice had a significant reduction in early infiltration of CD103\textsuperscript{+} DCs to the small intestinal epithelium in response to infection and a subsequent reduction in Th1 immunity. Reduced DC recruitment was attributed to a defective responsiveness of intestinal epithelial cells (IECs) to infection, with reduced chemokine production. Our data indicate a novel role for P2X7R in the initiation of small intestinal inflammation through chemotactic recruitment of DCs and establishment of protective Th1 immunity.

**METHODS**

**Mice.** C57BL/6 (WT) and P2X7R\textsuperscript{-/-} littermate controls were generated from breeding heterozygous Pfizer P2X7R\textsuperscript{-/-} mice (The Jackson Lab, USA). All mice were maintained by the Biological Services Unit (BSU), University of Manchester, UK, and kept in individually ventilated cages and fed a standard chow. Experiments were performed in accordance with
the Home Office Animals (Scientific Procedures) Act (1986). Three to six mice per group were used per study and each infection was repeated two to three times.

Parasites and cells. The type-II Prugniaud (PRU) expressing tandem dimers of tomato red (tdTomato) fluorescent protein *Toxoplasma gondii* strain was used in our experiments. Tachyzoites were harvested by serial 4-5 day passage in human foreskin fibroblast (HFF) cultured in DMEM (Sigma-Aldrich) with 10% FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (PEST) (Sigma-Aldrich). Each mouse was orally infected with $1 \times 10^6$ of tachyzoites in 0.1 ml PBS in the morning. The mouse colonic epithelial cell line, CMT-93, was maintained in DMEM (Sigma-Aldrich) media with 10% FBS (Sigma-Aldrich), 2mM L-glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin (PEST) (Sigma-Aldrich). CMT-93 cells were infected with PRU at a ratio of 1:1 for 24 hr and treated with the selective P2X7R antagonist, A-740003 (500µM; Sigma-Aldrich) to block P2X7R at the same time of infection.

Isolation of small intestinal cells. Small intestine cells were isolated as described previously. Briefly, Peyer’s patches (PP) were removed, and the remaining tissue cut into smaller pieces and transferred into HBSS supplemented with 1% Hepes and 2mM ethylenediaminetetraacetic acid (EDTA). The tissue pieces were incubated at 37° C with shaking for three sets of 15 minutes and the cells collected and pooled. The leukocytes in the intestinal epithelial layer (IEL) were isolated by Percoll density gradient isolation (Scientific Laboratories Supplies, Yorkshire, UK) and resuspended in RPMI-1640 (Sigma-Aldrich) with 10% FBS and 1% Hepes. The remaining tissue fragments were incubated in RPMI media supplemented with collagenase VIII and CaCl$_2$ at 37° C for 60 min to collect cells in lamina propria layer (LPL). All the IEL and LPL cells were resuspended at $1 \times 10^7$ cells/ml in FACS buffer (PBS plus 2% FBS with 0.1% sodium azide) for flow cytometry analysis.
**Flow cytometry analysis.** Cells were incubated with Fc receptor blocker, anti-CD16/32 antibody (eBioscience). After washing, cells were incubated with FITC-anti-CD45 (eBioscience), Pacific Blue-anti-MHCII (BioLegend), APC-anti-F4/80 (eBioscience), Alex Fluor 700-anti-CD11c (eBioscience), APC-Cy7-anti-11b (BD) and PerCP/Cy5.5-anti-CD103 (BioLegend) antibodies. For assessing intracellular cytokine expression, cells were labelled with FITC-anti-CD3 (BD) and PerCP-anti-CD4 (BD) antibodies, fixed and permeabilised using FOXP3 Fix/Perm buffer set (BioLegend) followed by staining with PE/Cy7-anti-CD25, Alexa Fluor 647-anti-FOXP3, BV-421-anti-IFN-γ, PE-IL-4 and BV-605 anti-IL-17 antibodies (All from BioLegend). Cells were acquired by flow cytometry on the BD LSRII and the data analysed using FlowJo flow cytometry software (Tree Star Ashland, OR).

**ELISA and cytokine analysis.** The CCL5 levels in mouse serum and supernatants from CMT-93 and crypt organoids were measured using ELISA duoset kit (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. Levels of IL-4, IL-10, IL-6, IL-9, IL-13, interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and IL-12p70 in serum were determined using a cytometric bead array according to the manufacturer’s instructions (CBA, Becton Dickinson (BD), Oxford, UK) and analysed using BD Cytometer and FCAP Array software.

**Histology.** Ileums and liver were dissected and fixed in neutral buffered formalin for 24 hr, routinely processed, and embedded in paraffin wax. Then, 5μm sections were dewaxed, rehydrated, and stained using a standard hematoxylin and eosin (H&E). All pathology was done in a blinded fashion. Ileum sections were examined for histopathological changes including necrosis, inflammatory infiltration and villus atrophy by measuring the length of 10 to 15 villi per specimen using WCIF ImageJ software (http://imagej.nih.gov/ij). The liver
sections were examined for liver pathology and the numbers of inflammatory foci were measured from 4 different fields of views in a specimen under microscopy.

**Immunofluorescence.** Ileum snips taken at autopsy were prepared as described previously in infection. Briefly, tissue was frozen in OCT and 5μm sections stained frozen in OCT embedding matrix (Thermo Fisher Scientific, Cheshire, UK) were fixed in 4% paraformaldehyde at 4 °C and blocked using tryamide blocking reagent (PerkinElmer, Cambridge, UK) avidin/biotin blocking kit (Vector Lab, Peterborough, UK). For analysis of CD103+CD11c+ DCs, slides were stained with CD103 (Beckon Dickinson, Oxford, UK) and CD11c-biotin antibodies (eBioscience, Hatfield, UK), Alexa Fluor 488-conjugated goat anti-rat antibody (Invitrogen, Life Technologies Ltd, UK), streptavidin–horseradish peroxidase for 30 min and Tyramide Cy3 detection antibody and mounted with vector shield containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Lab). Slides were viewed blinded for counting the number CD11c+CD103+ DCs in villi per field of view. Three to four fields of view were counted per section and the average count taken per mouse. The average number of the intracellular fluorescent tachyzoites per villus in small intestine was determined by directly counting the tachyzoites present in 10 to 15 villi per section of DAPI stained tissue. The *in situ* cell death in small intestine in response to infection was assessed using TUNEL assay (In situ cell death detection kit, Roche Diagnostics GmbH, Germany) following the manufacturer’s instructions. Cell death was determined by the average number of TUNEL-positive cells viewed from 3 fields per frozen section.

**Mouse bone marrow-derived dendritic cells.** Bone marrow cells from C57BL/6 and P2X7R-/- mice were cultured in bone marrow derived cell (BMDC) culture media containing RPMI-1640 (Sigma-Aldrich) with 10% FBS (Gibco Life Technologies, Paisley, UK), 1% PEST, 1% L-glutamine, 50μM beta-mercaptoethanol (2-ME) and 4% granulocyte-macrophage colony-
stimulating factor (GM-CSF). After 6 days of culture, the phenotype of BMDCs was confirmed by staining with PE-Cy7-anti-CD45, Alex Fluor 700-anti-CD11c (eBioscience) and Pacific Blue-anti-MHCII (BioLegend) antibodies and the purity of MHCII^+CD45^+CD11c^+ BMDCs was >90%.

**Chemokine migration assay.** BMDCs from C57BL/6 or P2X7R^-/- mice were added to the upper well of transwell plates (Fisher Scientific, Loughborough, UK) at 1 × 10^6 per well and 0.1% bovine serum albumin (BSA), CCL5 or CCL20 chemokine (both from R&D Systems) added to the bottom well in a two-fold serial dilution (1000 to 250 pg/ml). After incubating for 3 hr at 37 °C, the number of cells in the bottom well was counted in triplicate using a CASY TT cell counter (Roche Innovatis AG, Germany). The percentage of migrated cells was calculated by subtracting background migration from the BSA only data and calculating the proportion of migrated cells.

**OT-II cell and splenocyte stimulation for intracellular cytokine staining.** Splenocytes (10^6 cells/ml) from C57BL/6 OT-II mice with IL-10-green fluorescence protein (GFP) reporter122 (RAG-1-OT-II x Vert-X F1 cross) were co-cultured with OVA-pulsed BMDCs from WT or P2X7R^-/- mice at a ratio of 10:1 in a 48-well plate (BD Falcon, Oxford, UK) for 72 h. Splenocytes were harvested and stimulated with phorbol 12-myristate 13-acetate (PMA, 5 ng/ml, Sigma-Aldrich) and ionomycin (500 ng/ml, Sigma-Aldrich) for 1h followed by 3 h of incubation with a Brefeldin A (BFA, 1µg/ml, BD). For parasite infected mice, splenocytes were harvested at day 8 p.i. and stimulated with PMA, ionomycin and BFA.

**Small intestinal epithelial cell isolation and qPCR.** Tissue pieces of small intestine were incubated in HBSS supplemented with 1% Hepes and 2mM EDTA at 37° C with shaking for 30 min and the epithelial cells collected. Epithelial purity was confirmed by flow cytometry
using CD326 (Ep-CAM, Cambridge Bioscience, Cambridge, UK) and CD45-FITC (eBioscience) and preparations were >95% pure. Total RNA was isolated from cells by homogenising in TRIsure (Bioline, London, UK) and chloroform/isopropanol isolation (Sigma-Aldrich) and the concentration measured on a nanodrop-1000 spectrophotometer (Labtech International, East Sussex, UK). cDNA was prepared using Bioscript-MMLV kit (Bioscript, London, UK). Quantitative PCR was performed using KAPA SYBR FAST qPCR Kit (Kapa Biosystems Inc., MA) and an Opticon quantitative PCR thermal cycler (Bio-Rad, Hemel Hempstead, UK). The CCL5 and CCL20 expression ratios of samples were calculated by normalising to the reference gene (YWHAZ). The primer sequences for CCL5: GGGTACCATGAAGATCTCTGCA (forward) and TTGGCGGTTCCTTCGAGTGA (reverse); For CCL20: AATGGCCTGCGGTGGCAA (forward) and CATCGGCCATCTGTCTTGTA (reverse); For YWHAZ: TTCTTGATCCCCAATGCTTC (forward) and TTCTTGTCATCACCAGCAGC (reverse).

**Primary culture of mouse small intestinal crypt organoids.** Organoids were prepared using published protocols. Briefly, fragments of small intestine were transferred into crypt isolation buffer (2mM EDTA in PBS) under gentle agitation at 4°C for 30 min. The released crypts were resuspended in Matrigel™ (BD) (300-500 crypts/50ml) and cultured in Advanced DMEM/F12 + GlutaMAX™ (Life Technologies) supplemented with 10mM Hepes (Sigma-Aldrich), 1% PEST (Sigma-Aldrich), 1% N2 supplement (Life Technologies), 2% B27 supplement (Life Technologies), murine recombinant epidermal growth factor (50ng/ml), murine recombinant Noggin (100ng/ml) (PeproTech EC Ltd., London, UK) and Human recombinant R-spondin1 (1µg/ml) (R&D). Well-differentiated crypt organoids were split into 30 organoids/50µl Matrigel™/well and used for LPS stimulation.

**Statistics.** Statistical analyses were performed using Student’s t-test, one-way or two-way ANOVA and Kruskal–Wallis test for the proportion of PRU⁺ cells in the MLN and spleen. The
P-values of < 0.05 were considered significant. All statistical analyses were carried out using GraphPad Prism for windows, version 5 (La Jolla, CA).

RESULTS

Early *T. gondii* induced-cell death is P2X7R dependent.

We hypothesised that P2X7R would be important in recognising parasite induced damage and therefore we assessed early damage to infection. Ileal tissue from C57BL/6 (WT) and P2X7R⁻/⁻ mice pre and day one post-infection (p.i.) was analysed by histology and for cell death by TUNEL. Despite there being no overt pathology at day 1 p.i. (data not shown), there was significant cell death in WT mice (Figure 2.1a and b). Most of the cell death in the ileum at the early stage of infection was observed in epithelial cells (Figure 2.1a). Analysis of P2X7R⁻/⁻ mice revealed a significant decrease in cell death post-infection compared with WT (4 ± 1.3 cells/field of view (P2X7R⁻/⁻ mice) vs. 21.6 ± 3 cells per field/view (WT mice), $P = 0.0004$).
Figure 2.1. P2X7R promotes infection induced-cell death.

Cell death in the small intestine pre and post-infection with *T. gondii* (1x10^6 tachyzoites/mouse) was analysed by TUNEL assay (a) Representative images of section of ileum with TUNEL+ dead cells (green) from naïve mice and P2X7R^-/- and littermate C57BL/6 mice day 1 p.i.. Sections were counterstained with DAPI (blue). (b) Quantification of cell death at day 0 and day 1 p.i. in C57BL/6 (open bars) and P2X7R^-/- mice (black bars). Data shown are means ± SD pooled from two independent experiments. n= 6 per group. Statistical difference was measured using two-way ANOVA with Bonferroni post-hoc test. ***P < 0.001. Scale bar: 20µm.

Delayed recruitment of CD103^+ DCs to the small intestinal epithelium in P2X7R^-/- mice.

DC responses during acute infection of *T. gondii* have been shown to determine disease outcome and parasite burden. To assess whether P2X7R signalling plays a role in recruitment of DCs to the intestinal epithelium, we investigated small intestinal DCs and macrophages following *T. gondii* infection (*Figure 2.2a-c, Figure S2.1*). The percentages of CD103^+ DC subtypes in intestinal epithelial layers (IEL) were similar between naïve WT (CD103^+CD11b^-DC: 5.3 ± 1.6% and CD103^+CD11b^+ DC: 5.8 ± 4% of CD45^+ population) and naïve P2X7R^-/- (CD103^+CD11b^- DC: 5.8 ± 1.5% and CD103^+CD11b^+DC: 6.8 ± 0.4% of CD45^+ population) mice (*Figure 2.2b* and c). At day 1 p.i., control WT mice had a six-fold increase in the proportion of CD103^+CD11b^- DCs (32.68 ± 4.9%) in the IEL, compared with P2X7R^-/- animals in which there was only a threefold increase (17.28 ± 1.7%, P = 0.002) (*Figure 2.2b*). The frequency of IEL CD103^+CD11b^- DCs decreased to levels similar to naïve WT and P2X7R^-/- animals at day 5 p.i.. In contrast, there was a significant increase in the proportions of
intraepithelial CD103^+CD11b^+ DCs in WT and P2X7R^-/^- mice at day 5 p.i. (Figure 2.2c). The proportion of CD103^- DCs were similar in the IEL compartment of naïve mice. In response to infection there was a significant decrease in CD103^- DCs in the IEL compartment at day 1 and day 5 p.i. and a decrease in the LPL compartment at day 5 p.i.. However, the proportion of CD103^- DCs showed no difference between WT and P2X7R^-/^- mice in the IEL or LPL compartment (Figure S2.1a and b). There was not a general reduction of phagocyte recruitment into the IEL of the P2X7R^-/^- intestine as the proportions of F4/80^-CD11c^+ macrophages were similar between the WT and P2X7^-/- before and after infection (Figure S2.1c and d).

In order to validate the flow cytometry and better define DC localisation, frozen ileum sections were analysed by immunohistochemistry for CD103^+CD11c^+ DCs (CD103^+ DCs) (Figure 2.2d and e). The number and localisation of CD103^+ DCs were unchanged between naïve WT and P2X7R^-/^- mice. Post-infection, most of the DCs localised to the subepithelial areas of villi (Figure 2.2d). Consistent with the flow cytometry data, there was an increase in the CD103^+ DC population in the villi of WT mice at day 1 p.i. which was reduced in magnitude in P2X7R^-/^- mice (17.2 ± 2.9% in control vs. 7.3 ± 0.9% in P2X7R^-/^-; \( P = 0.0173 \)). A similar reduction in recruitment of CD103^+ DCs in P2X7R^-/^- mice was also observed at day 5 p.i. (35.7 ± 4% in control vs. 18.7 ± 3.1% in P2X7R^-/^-; \( P = 0.0042 \)) (Figure 2.2e).
Figure 2.2. Impaired early recruitment of CD11c+CD103+CD11b- dendritic cells (DCs) in P2X7R−/− mice after infection.

C57BL/6 and P2X7R−/− mice were orally infected with T. gondii (1x10^6 tachyzoites/mouse) and sacrificed at day 0, 1, and 5 p.i. (a) Gating strategy for distinction between small intestinal CD11c+ cell populations identified as CD11c+CD103+CD11b- DC, CD11c+CD103+CD11b+ DC, CD11c+CD103-CD11b+ DC and CD11b+CD11c+F4/80+ macrophages. Representative FACS plots are shown in Figure A.2. The frequency of intraepithelial CD11c+CD103+CD11b- DC (b) and CD11c+CD103+CD11b+ DC (c) were calculated as the percentage of CD45+MHCII+ cell population in C57BL/6 (open bars) and P2X7R−/− mice (black bars). Representative image of frozen ileum sample from C57BL/6 mouse at day 1 p.i. stained for CD11c (red), CD103 (green), and counterstained with DAPI (blue) with co-localised cells shown in yellow (arrow) (d) and the numbers of CD11c+CD103+ cells per field of view quantified (e). Scale bar: 50µm. n= 6 per group from two experiments (means ± SD). Statistical difference was measured using two-way ANOVA with Bonferroni post-hoc test. *P < 0.05 and **P < 0.01 and ***P < 0.001. a: compared with other timepoint groups.
**P2X7R−/− animals have reduced development of Th1 immunity.**

To assess whether the delayed recruitment of CD103+ DCs influenced the development of Th1 immunity against *T. gondii*, splenocytes were collected from WT and P2X7R−/− mice at day 8 p.i., and the T cell responses to infection were analysed. Splenic CD4+ T cells were assessed for intracellular cytokine production of IFN-γ+, IL-17+, and IL-4+ (Figure 2.3a, b and d). There was a significant increase in the proportions of IFN-γ+ cells in WT mice, compared to P2X7R−/− (Figure 2.3a, 19.8 ± 5.3% in control vs. 1.5% ± 0.3 in P2X7R−/−; *P* = 0.0098). In contrast, *T. gondii*-infected mice developed very few IL-17+ Th17 cells (Figure 2.3b). Interestingly, we also noted that the proportion of IL-4+ cells in WT mice was lower than P2X7R−/− mice (Figure 2.3c, *P*=0.02). As an important regulator in the inflammatory response to *T. gondii* the proportion of CD25+Foxp3+ regulatory T cells (Tregs) expressing IL-10 were also assessed following infection. There were significantly fewer IL-10 producing Tregs in P2X7R−/− compared to WT mice post infection (9.9 ± 1.8% in WT vs. 2.9 ± 0.6% in P2X7R−/−, *P* = 0.0093) (Figure 2.3d).

To confirm whether the delayed recruitment of DCs in P2X7R−/− animals altered the adaptive immune response systemically, serum cytokine levels during infection were analysed. P2X7R−/− mice had significantly less IFN-γ in their serum compared with control mice at day 8 p.i. (Figure 2.3e, *P* < 0.05), consistent with the reduction in the splenic IFN-γ+ CD4+ T cell population. Significantly higher serum IL-6 was also observed in WT compared to P2X7R−/− mice at day 5 p.i. (Figure 2.3f, *P* < 0.05). Taken together, these findings indicate that P2X7R−/− mice have a delayed Th1 adaptive immune response with less systemic inflammation in response to *T. gondii* infection.
Figure 2.3. P2X7R deficiency impaired development of Th1 immunity against infection. Splenocytes from C57BL/6 and P2X7R-/- mice (n=3 to 4 per group) at day 8 p.i. with *T. gondii* were analysed by flow cytometry for the percentage of CD4 cells from C57BL/6 (open bars) and P2X7R-/- mice (black bars) expressing IFN-γ (a), IL-17 (b), IL-4 (c) and (d) the percentage CD4+CD25+Foxp3+ Tregs. Representative FACS plots are shown in Figure A.4. (e) Serum IFN-γ and (f) IL-6 levels of C57BL/6 and P2X7R-/- mice at day 8 p.i. were analysed by CBA. n= 6 per group. Data shown are means ± SD pooled from two independent experiments. Statistical difference was measured using two-way ANOVA with Bonferroni post-hoc test. *P < 0.05 and **P < 0.01. a: compared with other time-point groups.

P2X7R-/- mice have a higher parasite burden post-infection.

The observation that there was a reduced immune response to *T. gondii* in P2X7R-/- mice suggested that parasite burden and pathology may also be altered. Therefore parasite burden in the ileum villi was enumerated. Compared with WT mice, P2X7R-/- mice had a trend for a higher parasite burden at day 1 p.i. (8 ± 4.3 tachyzoites/villus in P2X7R-/- vs. 5 ± 2.3 tachyzoites/villus in WT) and significantly higher burden at day 5 p.i. (11 ± 0.5 tachyzoites/villus in P2X7R-/- vs. 5 ± 2.3 tachyzoites/villus in WT)
tachyzoites in P2X7R\(^{-/-}\) vs. 8 ± 2.0 tachyzoites in WT, \(P = 0.0178\) (Figure 2.4a and b). These data implicate a role for P2X7R\(^{-/-}\) in controlling the local infection of *T. gondii*.

**P2X7R deficiency has no effect on small intestinal pathology after infection.**

The possibility that a higher parasite burden in the mouse would correlate with worsened pathology was investigated. Small intestinal villus height in WT and P2X7R\(^{-/-}\) mice was measured at different time-points following infection. WT and P2X7R\(^{-/-}\) mice developed moderate villus atrophy at day 5 p.i. (Figure 2.4c) but were not significantly different to each other (Figure 2.4d). This finding indicated that P2X7R\(^{-/-}\) mice were not more susceptible to acute *T. gondii* infection despite the higher parasite burden. Indeed, WT and P2X7R\(^{-/-}\) mice had a similar weight loss in response to the infection (data not shown). Intriguingly, analysis of liver pathology showed significantly more inflammatory foci in WT mice at day 8 p.i., compared with P2X7R\(^{-/-}\) mice, implying a more rapid dissemination of tachyzoites out of the gut in the WT mice (Figure S2.2a and b). Given the parasite is thought to invade MLN to systemically disperse via infected phagocytes\(^{24, 25}\), we investigated parasite burden in the MLN and spleen day 1 p.i.. *T. gondii* preferentially infected CD11b\(^{+}\)CD103\(^{+}\) DC and CD11b\(^{+}\) macrophages rather than CD11b\(^{-}\)CD103\(^{+}\) DCs and that this was not significantly different between WT and P2X7R\(^{-/-}\) mice (0.29 ± 0.06% (CD11b\(^{+}\)CD103\(^{+}\) DC) vs. 0.14 ± 0.05% (CD11b\(^{-}\)CD103\(^{+}\) DC) in WT MLN, \(P < 0.05\); 0.3 ± 0.07% (CD11b\(^{+}\)CD103\(^{+}\) DC) vs. 0.08 ± 0.11% (CD11b\(^{-}\)CD103\(^{+}\) DC) in P2X7R\(^{-/-}\) MLN, \(P < 0.05\)). In the spleen, the data showed that macrophages were the major phagocytes infected by *T. gondii* (0.43 ± 0.27% (macrophage) vs. 0.03 ± 0.01% (CD11b\(^{+}\)CD103\(^{+}\) DC) in WT spleen, \(P < 0.05\); 0.48 ± 0.33% (macrophage) vs. 0.04 ± 0.01% (CD11b\(^{+}\)CD103\(^{+}\) DC, \(P < 0.05\)) in P2X7R\(^{-/-}\) spleen) (Figure S2.2c and d). These data suggest that a defect in early DC infiltration does not prevent parasite dissemination in P2X7R\(^{-/-}\) animals.
Figure 2.4. Acute *T. gondii* induced pathology is not dependent on P2X7R. C57BL/6 and P2X7R<sup>−/−</sup> mice were orally infected with *T. gondii* (1x10<sup>6</sup> tachyzoites/mouse) and sacrificed at day 0, 1, 5 and 8 p.i.. Parasite load was confirmed by counting the tomato-red expressing parasites in the ileum by microscopy. (a) Representative images of ileum with red *T. gondii* parasites (arrows) and a DAPI (blue) counterstain and (b) quantification of parasite load per field of view in C57BL/6 (open bars) and P2X7R<sup>−/−</sup> (black bars) mice. (Scale bar: 20 µm). (c) Pathology of the gut was assessed by analysing villus atrophy in small intestine by H&E. Representative images of H&E stained small intestine sections at day 0 and day 5 p.i. (Scale bar: 50 µm) and (d) Villus heights at day 0, 1, 5 and 8 p.i. from C57BL/6 (open bars) and P2X7R<sup>−/−</sup> (black bars) mice were measured showing no difference between C57BL/6 and P2X7R<sup>−/−</sup> mice. Data shown are means ± SD pooled from two independent experiments. n= 6 per group. Statistical difference was measured using two-way ANOVA with Bonferroni post-hoc test.*P < 0.05.

**P2X7R<sup>−/−</sup> DCs can migrate and present antigen normally in vitro.**

P2X7R is expressed by DCs and epithelial cells. To dissect whether the reduction of early CD103<sup>+</sup> DC infiltration to the small intestine in P2X7R<sup>−/−</sup> mice was because of a defect on DC migration we generated bone-marrow derived dendritic cells (BMDCs) from WT and P2X7R<sup>−/−</sup> mice and monitored BMDC migration in response to CCL5 or CCL20, chemokines...
previously reported to promote DC recruitment to the epithelium \(^9, \!10\). Both CCL5 and CCL20 induced a two- to three-fold increase of BMDC migration and there was no difference of the migratory response between WT and P2X7R\(^{-/-}\) BMDCs (Figure 2.5a and b).

The possibility that P2X7R\(^{-/-}\) DCs might be able to effectively present antigen was next investigated. WT or P2X7R\(^{-/-}\) BMDCs that had been pulsed with ovalbumin (OVA) were cocultured with OVA peptide-specific OT-II splenocytes at a ratio of 1:10 for 48 hr. Data showed that both WT and P2X7R\(^{-/-}\) BMDCs were able to present OVA antigen and induced the development of IFN-\(\gamma^+\) CD4 T cells (2.9 \pm 0.5% in WT vs. 2.8\pm 0.3 \% in P2X7R\(^{-/-}\)) to equivalent levels (Figure 2.5c). Overall, these data indicate that P2X7R\(^{-/-}\) DCs are able to respond normally to chemokines and there was no difference in antigen presentation between WT and P2X7R\(^{-/-}\) BMDCs.

**Impaired chemotactic function in P2X7R\(^{-/-}\) intestinal epithelial cells.**

Since the *in vitro* migration function of P2X7R\(^{-/-}\) DCs was normal, we hypothesised that the defect in migration *in vivo* was due to impaired epithelial chemokine responses. To investigate this possibility, we generated bone marrow chimeras. WT and P2X7R\(^{-/-}\) mice were irradiated and WT mice were reconstituted with P2X7R\(^{-/-}\) bone marrow and P2X7R\(^{-/-}\) mice with WT bone marrow (referred as C57BL/6\(^{P2X7R^{-/-}}\) and P2X7R\(^{-/-}\)-C57BL/6). Successful reconstitution was confirmed by genotyping the peripheral blood mononuclear cells (PBMCs) from chimeras (data not shown). Following infection, CD103\(^+\)CD11b\(^-\) and CD103\(^+\)CD11b\(^+\) DCs were recruited to the small intestinal epithelium at day 1p.i.. WT mice that were reconstituted with P2X7R\(^{-/-}\) bone marrow (C57BL/6\(^{P2X7R^{-/-}}\)) responded robustly to infection with DC recruitment into the IEL. In contrast the P2X7R\(^{-/-}\) mice (i.e. those with a P2X7R null epithelium) that had been reconstituted with WT bone marrow (P2X7R\(^{-/-}\)-C57BL/6) had a significant reduction in the magnitude of the DC response (CD103\(^+\)CD11b\(^-\) DCs: 11.5%
± 0.8 in C57BL/6P2X7R/- vs. CD103+CD11b+ DCs: 7.6% ± 0.8 in P2X7R/-C57BL/6, P = 0.0092; CD103+CD11b+ DCs: 15.5% ± 0.8 in C57BL/6P2X7R/- vs. CD103+CD11b+ DCs: 8.3% ± 0.6 in P2X7R/-C57BL/6, P = 0.0001) (Figure 2.5d). These data further support the observation in vitro that P2X7R/- DCs have normal in vivo migration ability. Taken together these data indicate a role P2X7R in the epithelial cell-derived chemokine response to infection.

Figure 2.5. Epithelial P2X7R is necessary for the intraepithelial recruitment of dendritic cells (DCs).
Bone marrow derived dendritic cell (BMDCs) from C57BL/6 (open bars) and P2X7R/- (black bars) mice were stimulated with (a) CCL5 and (b) CCL20 and migration was assessed using a transwell migration assay. (c) OT-II splenocytes stimulated with ovalbumin (OVA)-pulsed BMDCs from C57BL/6 (open bars) or P2X7R/- (black bars) mice for 48 hr, were analysed by flow cytometry for intracellular IFN-γ and quantified by flow cytometry. Statistical difference was measured using two-way ANOVA with Bonferroni post-hoc test. *P < 0.05 and **P < 0.01 as compared with BSA treatment or no OVA group. Bone marrow chimeras C57BL/6P2X7R/- (grey bars) and P2X7R/-C57BL/6 (hatched bars) were generated and orally infected with T. gondii PRU (1x10⁶ tachyzoites/mouse) and sacrificed at day 1 p.i.. Intraepithelial cells were isolated from the small intestine and stained for CD45, MHCII, F4/80, CD11b, CD11c, and CD103. (d) The graph shows the percentage of CD11c+CD103+CD11b- and CD11c+CD103+CD11b+ DCs of the CD45+MHC+ cells (n= 6) with mean value per group, and are pooled from two independent experiments (means ± SD), Statistical difference was measured using Student’s t-test. **P < 0.01.
Epithelial Responses to Infection.

To study the early chemokine response, intestinal epithelial cells (IECs) from WT and P2X7R−/− mice at day 0 and day 1 p.i. were analysed by qPCR. The qPCR data showed increased expression of both CCL5 and CCL20 in WT IECs at day 1 p.i. as compared with day 0 (Figure 2.6a and b, CCL5: \( P < 0.05 \); CCL20: \( P < 0.01 \)). In contrast, the CCL5 and CCL20 expression levels at day 1 p.i. remained low in P2X7R−/− IECs (Figure 2.6a and b). Since there was significantly less early epithelial chemokine expression in P2X7R−/− mice, the serum chemokine levels of the animals at day 1 p.i. were assessed. Consistent with the qPCR data, P2X7R−/− mice had significantly lower serum CCL5 levels when compared to WT mice (57 ± 9.7 pg/ml in WT vs. 26 ± 5.2 pg/ml in P2X7R−/−) (Figure 2.6c, \( P = 0.0178 \)).

Further investigation of ex vivo CCL5 levels used primary small intestinal crypt organoid cells in culture. P2X7R deficiency did not influence the basal levels of CCL5 secretion from crypt organoids (30 ± 6.8 pg/ml (WT) vs. 28 ± 6.0 pg/ml (P2X7R−/−)). However, WT organoids secreted significantly more CCL5 than P2X7R−/− organoids in response to lipopolysaccharide (LPS) stimulation (Figure 2.6d, \( P < 0.01 \)). To mimic infection in vitro, we treated mouse colonic epithelial cells, CMT-93, with *T. gondii* plus or minus a selective P2X7R inhibitor A-740003 for 24 hr. A-740003 treatment did not alter basal CCL5 levels (Figure 2.6e) but blocking P2X7R by A-740003 in CMT-93 cells decreased CCL5 production in response to *T. gondii* infection (Figure 2.6e). Collectively, these data indicate that P2X7R deficiency impairs the epithelial chemokine response to *T. gondii* infection and, therefore, results in delayed early recruitment of DCs.
Figure 2.6. P2X7R promotes CCL5 production in intestinal epithelial cells in response to infection.

Epithelial cells from the small intestines of C57BL/6 and P2X7R\(^{-/-}\) mice at day 0 and 1 post-infection with *T. gondii* (1x10\(^6\) tachyzoites/mouse) were collected at autopsy and analysed by qPCR for expression of (a) CCL5 and (b) CCL20 at day 0 and 1 p.i.. (c) Serum CCL5 was analysed by cytokine bead array (CBA) at day 0 and 1 p.i. (C57BL/6 are open bars and P2X7R\(^{-/-}\) are filled bars). Data shown are for individual mice (n= 3 to 6), with mean value per group, and are pooled from two independent experiments (means ± SD). *P < 0.05 and **P < 0.01. Crypt cells were isolated from C57BL/6 (open bars) or P2X7R\(^{-/-}\) mice (black bars) and cultured as ex vivo crypt organoids and treated with or without LPS (20\(\mu\)g/ml) for 6 hr. (d) CCL5 production of crypt organoid was analysed by ELISA (means ± SEM, n= 3). The mouse intestinal epithelial cell line CMT-93 was treated with (hatched bars) or without the P2X7R inhibitor (grey bars), A-740003 (500\(\mu\)M) and infected by *T. gondii* for 24 hr. (e) The supernatants from uninfected control and infected cells were collected for CCL5 analysis by ELISA (means ± SEM, n= 4). Statistical difference was measured using two-way ANOVA with Bonferroni post-hoc test. *P < 0.05, **P < 0.01 and ***P < 0.001.
Discussion

In this study, *T. gondii* infection in P2X7R−/− mice resulted in a delayed infiltration of CD103+ DCs. Early DC responses are known to alter disease outcome to intracellular pathogen infections including *T. gondii*, *Listeria monocytogenes* and malaria4, 26. Thus as we had a reduced DC response we investigated whether P2X7R−/− mice would have a worse outcome to infection. Surprisingly, there was no clear difference in intestinal pathology and survival rate between WT and P2X7R−/− mice. However, the decreased infiltration of CD103+ DCs into the intestinal epithelium of P2X7R−/− mice was associated with impaired development of Th1 immunity and reduced early local control of *T. gondii* replication in the small intestine.

Our data suggest that in vivo P2X7R might involve in sensing infection-induced damage or by inducing cell death. P2X7R−/− mice developed less intestinal epithelial cell (IEC) death in response to infection, and had a higher parasite gut burden. Previous studies in murine fibroblasts and splenocytes in vitro show that inhibition of host cell apoptosis correlates with improved *T. gondii* survival via inactivation of caspase-3, -8 and -9 and induction of anti-apoptotic NF-κB signalling27, 28. In addition, P2X7R, as a pro-apoptotic receptor, has been reported to be important for clearing intracellular toxoplasma infection in human and murine macrophages in vitro by mediating host cell apoptosis or necrosis29. Consistent with these previous studies, our data suggest that P2X7R deficiency makes IECs insensitive to extracellular ATP released from damaged infected-cells30. Further, the lack of functional P2X7R may impair the ability induce a cytolytic effect and kill intracellular parasites. P2X7R−/− mice, therefore, become unable to control early *T. gondii* replication in the gut. Although WT mice were able to develop early apoptosis against intracellular *T. gondii*, the parasite load kept increasing suggesting the apoptosis induced in the presence of functional P2X7R was insufficient to control *T. gondii* infection in the small intestine. This might be explained by the previous finding that C57BL/6 and DAB mice have a single amino acid mutation
(P451L) in the C’-terminal tail of the P2X7R\(^{31}\) which is thought to reduce ATP-mediated apoptosis and membrane pore formation. However, the impairment of cell death by the P451L mutation does not affect the activation of Ca\(^{2+}\)-dependent phosphatidylcholine-hydrolysing phospholipase D (PC-PLD). Therefore, the intact Ca\(^{2+}\)-dependent downstream intracellular signalling may be necessary for activation of inflammation-associated genes such as CCL5 and CCL20 described here.

IECs are important sources of cytokines and chemokines. Among these IEC-derived CC chemokines, CCL2, CCL5 and CCL20 are potential chemoattractants for intestinal DC homing\(^9,32\). Oral inoculation of \textit{T. gondii} has been reported to elevate chemokine expression including CCL2, CCL3, CCL5, CXCL2, CXCL9 and CXCL10 in IECs\(^{33}\). Our \textit{in vivo} and \textit{in vitro} data shows that the absence of P2X7R results in impaired chemokine production, including CCL5 and CCL20. Our \textit{ex vivo} data have showed that CCL5 production in response to LPS treatment in C57BL/6 organoids was attenuated in P2X7R\(^{-/-}\) organoids. This result suggests that blocking P2X7R signalling attenuates TLR activation-induced chemokine production. The importance of TLR signalling in sensing pathogen-associated molecular patterns (PAMPs) has been demonstrated, and TLR11/12 are required for recognising toxoplasma profilin to regulate DC-derived IL-12 in response to \textit{T. gondii} infection\(^{34}\). Furthermore, TLR2 and TLR-adaptor MyD88 knock-out mice are more susceptible to \textit{T. gondii} with decreased chemokines production\(^{35,36}\). Our observation that epithelial responses to infection, and \textit{in vitro} to LPS in the absence of P2X7R, were reduced\(^{37}\), suggests that P2X7R may have a regulatory role in pathogen-pattern recognition receptor-mediated pro-inflammatory signalling.

Intestinal CD103\(^+\) DCs can be divided into two main subsets, CD103\(^+\)CD11b\(^+\) and CD103\(^+\)CD11b\(^-\) DCs, which differ in terms of distribution, requirement for transcription
factors and in vivo functions\textsuperscript{38}. CD103\(^{+}\)CD11b\(^{-}\) DCs are dominant in colon LP and extra-intestinal tissue\textsuperscript{39}, whereas CD103\(^{+}\)CD11b\(^{+}\) DCs are the major subset in the small intestinal LP and villus\textsuperscript{38}. Additionally, the CD103\(^{+}\)CD11b\(^{-}\)(CD8\(\alpha\)\(^{+}\)) DC subset in the small intestine expresses several different TLRs and promotes Th1 immunity and CTL activity\textsuperscript{40}. In the T. gondii-infected mouse ileitis model, we noted that blocking P2X7R delayed the recruitment of CD103\(^{+}\)CD11b\(^{-}\) DCs. CD103\(^{+}\)CD11b\(^{+}\) DCs are thought to be the major IL-12-producing immune cells against acute T. gondii infection\textsuperscript{41} and play an important role in cross-presentation\textsuperscript{42}. Thus, the intraepithelial recruitment of CD103\(^{+}\)CD11b\(^{-}\) DC observed in WT mice is arguably necessary for early control of T. gondii infection and the development of Th1 immunity. The in vivo role of CD103\(^{+}\)CD11b\(^{+}\) DCs in small intestinal inflammation is not yet clear but selective reduction of CD103\(^{+}\)CD11b\(^{+}\) DCs by depleting interferon regulatory factor 4 (IRF4) has been shown to impair Th17 differentiation in MLN but not in spleen where we saw no change in Th17 cells either\textsuperscript{43}. Thus, it is still unclear which CD103\(^{+}\) DC subset plays a more important role against T. gondii infection.

Collectively, our findings identify a novel role for small-intestinal epithelial P2X7R in the induction of early DC infiltration in response to protozoan parasite infection. Given the pro-inflammatory role as a potential tissue damage-sensor to initiate inflammation, P2X7R is likely to be an important pharmaceutical target for development of new therapies for inflammatory disorders in the gut.

ACKNOWLEDGEMENTS

This work was funded by a scholarship from the Ministry of Education, Taiwan awarded to Szu-Wei Huang and BBSRC Grant BBG018839/1 for Catherine Walker and Werner Muller.
REFERENCES


3. Aliberti J, Jankovic D, Sher A. Turning it on and off: regulation of dendritic cell function in *Toxoplasma gondii* infection. *Immunological reviews* 2004; 201: 26-34.


Supplementary Figure S2.1. P2X7R deficiency does not affect the recruitment of small intestinal CD103−DCs and F4/80+CD11c+ macrophages in response to infection.

C57BL/6 (open bars) and P2X7R−/− mice (black bars) were orally infected with T. gondii (1x10⁶ tachyzoites/mouse) and sacrificed at day 0 and 1 p.i.. Small intestinal IEL and LPL cells from T. gondii-infected mice were collected and analysed by flow cytometry. Representative FACS plots are shown in Figure A.2 and A.3. The frequency of small intestinal (a) IEL and (b) LPL compartment of CD11c+CD103−CD11b+ DC and (c) IEL and (d) LPL compartment of CD11b+CD11c+F4/80+ macrophage were calculated as the percentage of CD45+MHCII+ cell population. n= 6 per group, pooled from two independent experiments (means ± SD). Statistical difference was measured using two-way ANOVA with Bonferroni post-hoc test. *P < 0.05 and ***P < 0.001 as compared with other timepoint groups.
Supplementary Figure S2.2. P2X7R<sup>−/−</sup> mice had significantly fewer inflammatory foci in the liver at day 8 post infection.

C57BL/6 (open bars) and P2X7R<sup>−/−</sup> mice (black bars) were orally infected with *T. gondii* (1x10<sup>6</sup> tachyzoites/mouse) and liver pathology analysed at day 0, 5 and 8 p.i. by H&E. The number of inflammatory foci in the liver were enumerated (a) Representative image of liver inflammatory foci (arrow) by the portal vein (Scale bar: 20µm). (b) The numbers of inflammatory foci per field of view under microscope were quantified in C57BL/6 (open bars) and P2X7R<sup>−/−</sup> (black bars) mice. Data shown are means ± SD pooled from two independent experiments. n= 6 per group. Statistical difference was measured using two-way ANOVA with Bonferroni post-hoc test. *P* < 0.05. The frequencies of PRU-infected macrophages, CD103<sup>+</sup>CD11b<sup>+</sup> DC and CD103<sup>+</sup>CD11b<sup>−</sup> DC at day 1 p.i. in the MLN (c) and spleen (d) in C57BL/6 (open bars) and P2X7R<sup>−/−</sup> (black bars) mice were analysed by flow cytometry. Data shown are means ± SD. n= 5 per group. Statistical difference was measured using Kruskal–Wallis test. *P* < 0.05.
Chapter Three
Epithelial Expression of P2X7 Receptor Promotes DC Recruitment in *Trichinella spiralis*-induced Intestinal Inflammation
Epithelial Expression of P2X7 Receptor Promotes DC Recruitment in
*Trichinella spiralis*-induced Intestinal Inflammation

Szu-Wei Huang¹, Joanne Pennock², Kathryn Else¹, David Brough¹, Gloria Lopez Castejon¹,
Sheena Cruickshank¹

¹Faculty of Life Sciences, The University of Manchester, Manchester, UK
²Faculty of Medical and Human Sciences, The University of Manchester, Manchester, UK

**AUTHOR CONTRIBUTION**

Szu-Wei Huang carried out all experimental work and analysis.

Joanne Pennock helped with *in vivo* parasite infection.

Kathryn Else helped prepare infectious larvae recovered from digestion of infected-mice.

David Brough and Gloria Lopez Castejon helped with technical and scientific advice.

Sheena Cruickshank is the main supervisor of this project.
ABSTRACT

ATP released from damaged cells can be recognised by the purinergic sensor P2X7 receptor (P2X7R) to induce inflammatory responses but its potential role in initiating protective immunity is not well understood. We investigated the role of P2X7R in small intestinal inflammation using the nematode worm *Trichinella spiralis* (*T. spiralis*). Compared with littermate wildtype (WT) mice, P2X7R−/− mice had delayed worm expulsion from the gut and had worsened gut pathology. Establishment of the early innate response and recruitment of dendritic cells (DCs) to the site of infection is important for protective immunity, thus we analysed DC recruitment into the gut. WT mice had a robust, early recruitment of the CD11c+CD103+CD11b+ DC subset into the villi in response to infection. In contrast, P2X7R−/− mice had a significantly reduced recruitment of the CD11c+CD103+CD11b+ DCs. As DCs are important for T cell priming, we investigated effector T cell responses to *T. spiralis* and found reduced Th2 cytokine (IL-4) responses to antigen stimulation. The defect in T cell priming in P2X7R−/− mice was not due to abnormal DC function suggesting that it was driven by the reduced availability of DCs. Epithelial cells are important sensors of infection that recruit DCs to the site of infection. qPCR analysis showed a reduction in epithelial CCL5 production from P2X7R−/− mice. The importance of epithelial P2X7R in DC migration was confirmed using bone marrow chimeras. Thus, P2X7R signalling in epithelial cells promotes chemokine-mediated recruitment of DCs in the small intestine and initiation of protective immunity.
INTRODUCTION

Cell damage or death, e.g. necrosis, results in the release of a number of cytosol or nuclear contents- the so-called damage-associated molecular pattern molecules (DAMPs). These endogenous molecules, include extracellular matrix components, calcium-binding proteins, and nucleotides. DAMPs are normally recognised by Toll-like receptors (TLRs) and other cellular receptors as danger signals for the immune system. During cell damage, induction of DAMP signalling causes the release of pro-inflammatory cytokines and chemokines. Damage induced cytokines promote the activation of antigen-presenting cells (APCs) including macrophages and dendritic cells to prime adaptive immune responses. Indeed, DAMP-mediated inflammation is involved in numerous inflammatory disorders such as sepsis, rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis.

Damaged cells can release extracellular adenosine 5'-triphosphate (ATP) which acts as a DAMP. Extracellular ATP is recognised by the ATP-gated purinergic P2X receptor 7 (P2X7R). P2X7R is expressed by a large variety of cell types including immune cells and epithelial cells. Activation of P2X7R signalling induces influx of Ca$^{2+}$ and Na$^+$ accompanied by efflux of K$^+$, which leads to membrane pore formation and induction of cell apoptosis. Additionally, ATP released from dying cells in inflammation or infection can stimulate P2X7R and enhance the secretion of IL-1β and IL-18 via activation of NLRP3 inflammasome in immune cells. Although many studies have revealed the role of P2X7R in immune system, very little is known about P2X7R in epithelial cells.

Epithelial cells that line the gut are a critical interface acting as a barrier protecting the underlying lamina propria from the external environment. As well as barrier function, intestinal epithelial cell (IEC) signalling contributes to intestinal homeostasis by regulation of dendritic cell (DC) and T cell responses in the gut. IEC-derived inflammatory cytokines...
and chemokines are important for initiation of gut inflammation and recruitment of inflammatory immune cells such as neutrophils and DCs\textsuperscript{7}. Indeed we have previously demonstrated that colonic epithelium-derived CCL2, CCL5 and CCL20 are required for the early recruitment of DCs to the site of infection in response to the parasite \textit{Trichuris muris} (\textit{T. muris}). Resistance to \textit{T. muris} infection is associated with rapid mobilisation and activation of intestinal DCs to the epithelial layer\textsuperscript{8,9}. These findings suggest IECs have a pivotal role in early sensing of parasite invasion and induction of protective immunity.

As we have previously found that epithelial P2X7R senses the damage caused by the protozoan \textit{Toxoplasma gondii} (\textit{T. gondii}) infection, initiates inflammation and promote Th1 immunity (Chapter two), we wished to study whether the P2X7R-mediated epithelial response is a Th1 specific or a general mechanism against infection. In order to address this question, we decided to use a Th2-dominant parasitic ileitis model: \textit{Trichinella spiralis} (\textit{T. spiralis}). \textit{T. spiralis} is a widespread helminthic parasite that causes Trichinosis and is well-known to induce Th2 immune response in animals and humans\textsuperscript{10}. During \textit{T. spiralis} infection, the worms penetrate the intestinal epithelium, lamina propria and musculature causing damage as they migrate\textsuperscript{11}. In line with the data in Chapter two we hypothesised that IECs could sense damage caused by the worm migration via P2X7R as well. Notably, a previous study has shown that mice lacking P2X7R impaired the development of intestinal inflammation in response to \textit{T. spiralis} infection\textsuperscript{12}. Therefore we would like to further clarify the role of P2X7R in IECs in the initiation of inflammation and the development of Th2 immunity against \textit{T. spiralis}.

In this study, we investigated the role of P2X7R in small intestinal inflammation and the development of protective immunity using a \textit{T. spiralis}-induced ileitis model. We found that P2X7R\textsuperscript{-/-} mice had a significant decrease of CD103\textsuperscript{+}CD11b\textsuperscript{+} DC infiltration to the epithelial
layer in response to infection. P2X7R−/− mice had a reduced Th2 response with a concomitant delay of parasite expulsion. The reduction of CD103+CD11b+ DCs recruitment was due to a defect in IEC responsiveness to T. spiralis. Our data suggest a novel role of P2X7R in IECs for triggering small intestinal inflammation through DC recruitment and establishment of protective Th2 immune response.

METHODS

Mice. Specific-pathogen-free, 8 to 10 week-old heterozygous breeders were crossed with C57BL/6J (WT) with Pfizer P2X7R−/− mice (The Jackson Lab, USA)13 to generate heterozygote breeders to yield WT and P2X7R−/− littermate controls. All mice were maintained by the Biological Services Unit (BSU), University of Manchester, UK, and kept in individually ventilated cages and fed a standard chow. Animals were treated and experiments performed in accordance with the Home Office Animals (Scientific Procedures) Act (1986).

Trichinella spiralis infection. The maintenance, recovery and infection of T. spiralis was described previously14. Age matched male mice were infected with 400 larvae by oral gavage and the daily body weight change was monitored. Worm burden was assessed by longitudinal section of the small intestine followed by incubation in PBS at 37°C for 4 hr.

Isolation of small intestinal cells. Following removal of Peyer’s patches, the small intestines were cut into pieces and incubated in Hank’s buffered salt solution (HBSS, Sigma-Aldrich, Gillingham, UK) supplemented with 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, Sigma-Aldrich) and 2mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) at 37°C with shaking before being collected. Leukocytes from the collected intestinal epithelial layer (IEL) cells were isolated by Percoll gradient separation (Scientific Laboratories Supplies, Yorkshire, UK) and resuspended in R10 media containing RPMI-1640 (Sigma-Aldrich) with
10% FBS and 1% Hepes. The remaining tissue fragments were further digested using R10 media supplemented with collagenase VIII and CaCl₂ at 37°C for 60 min. The digested contents were filtered to collect lamina propria cells (LPL cells). All the collected cells were resuspended in FACS buffer (PBS plus 2% FBS with 0.1% sodium azide) before antibody labelling for flow cytometry analysis.

**Flow cytometry.** Small intestinal cells were prepared in single-cell suspension at 1 × 10⁷ cells/ml and after blocking in anti-CD16/32 (Fc receptor) antibody (eBioscience, Hatfield, UK), cells were incubated with FITC-anti-CD45 (eBioscience), Pacific Blue-anti-MHCII (BioLegend), APC-anti-F4/80 (eBioscience), Alex Fluor 700-anti-CD11c (eBioscience), APC-Cy7-anti-11b (BD) and PerCP/Cy5.5-anti-CD103 (BioLegend) antibodies. For assessing intracellular cytokine expression, stimulated OT-II cells, splenocytes and MLN cells from *T. spiralis*-infected mice were labelled with FITC-anti-CD3 (BD) and PerCP-anti-CD4 (BD) antibodies. These cells were then fixed and permeabilised followed by staining with PE/Cy7-anti-CD25, Alexa Fluor 647-anti-FOXP3, BV-421-anti-IFN-γ and PE-IL-4 (All from BioLegend). All the cells were acquired by flow cytometry on the BD LSRII and the data analysed using FlowJo flow cytometry software (Tree Star Ashland, OR).

**Cytokine and chemokine analysis.** The CCL5 levels in mouse serum were measured using ELISA duoset kit (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. Levels of IL-4, IL-6, IL-13, IFN-γ, TNF-α and CCL2 were determined using a cytometric bead array according to the manufacturer’s instructions (CBA, Becton Dickinson (BD), Oxford, UK) and analysed using a BD FacsAria cytometer and FCAP Array software.
**Histology.** 5 μm-ileum sections were dewaxed, rehydrated, and stained using hematoxylin and eosin (H&E). All pathology was done in a blinded, randomised fashion. The ileum sections were examined for histopathological changes and the villous length by measuring 10 to 15 villi per specimen using WCIF ImageJ software (http://imagej.nih.gov/ij). The inflammation was graded according to **Table 3.1**.

**Table 3.1 Inflammatory Scoring for Assessment of Ileal Damage**

<table>
<thead>
<tr>
<th>Score</th>
<th>Histological findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal mucosa</td>
</tr>
<tr>
<td>1</td>
<td>Slight inflammation: mucosal oedema with intestinal lacteal dilation</td>
</tr>
<tr>
<td>2</td>
<td>Intermediate inflammation: mild crypt hyperplasia and neutrophil infiltration in epithelium</td>
</tr>
<tr>
<td>3</td>
<td>Moderate inflammation: marked villous blunting and crypt hyperplasia; necrotic debris noted in the lumen; significant increase in polymorphonuclear and mononuclear infiltration in epithelium</td>
</tr>
<tr>
<td>4</td>
<td>Severe inflammation: loss of architecture with erosion or ulceration; massive inflammatory cell infiltration</td>
</tr>
</tbody>
</table>

**Immunofluorescence.** Fixed frozen ileum sections were blocked by tryamide blocking reagent (PerkinElmer, Cambridge, UK) and avidin/biotin blocking kit (Vector Lab, Peterborough, UK). For analysis of CD103^+CD11c^+ DCs, the sections were stained with rat anti-mouse CD103 (Beckon Dickinson, Oxford, UK) and anti-mouse CD11c-biotin antibodies (eBioscience) for overnight incubation at 4 °C. Then, sections were incubated with Alexa Fluor 488-conjugated goat anti-rat antibody (Invitrogen, Life Technologies Ltd, UK) for 1 hr and subsequently with streptavidin–horseradish peroxidase. Sections were incubated with Tyramide Cyanine3 reagent (PerkinElmer) for 10 min, washed and mounted with vector shield containing 4’,6-diamidino-2-phenylindole (DAPI) (Vectashield, Vector Laboratories, Peterborough, UK). Slides were viewed blinded for counting the number of CD11c^+CD103^+ DCs in villi from 3 fields of view per section. The cell death in small intestine was assessed by TUNEL assay (*In situ* cell death detection kit, Roche Diagnostics GmbH, Germany) following the manufacturer’s instructions.
**Mouse bone marrow-derived dendritic cells.** After lysis of red blood cells, bone marrow cells were cultured in culture media containing RPMI-1640 (Sigma-Aldrich) with 10% FBS (Gibco Life Technologies, Paisley, UK), 1% PEST, 1% L-glutamine, 50µM beta-mercaptoethanol (2-ME) and 4% granulocyte-macrophage colony-stimulating factor (GM-CSF). After 6 days of culture, the purity of MHCII⁺CD45⁺CD11c⁺ BMDCs was >90%.

**Stimulation of cells for cytokine staining.** OT-II splenocytes (10⁶ cells/ml) from C57BL/6 OT-II mice with IL-10-green fluorescence protein (GFP) reporter122 (RAG-1-OT-II x Vert-X F1 cross) were co-cultured with OVA-pulsed BMDCs from WT or P2X7R⁻/⁻ mice at a ratio of 10:1 (BD Falcon, Oxford, UK). After 72 hr, the splenocytes harvested were stimulated with phorbol 12-myristate 13-acetate (PMA, 5 ng/ml, Sigma-Aldrich) and ionomycin (500 ng/ml, Sigma-Aldrich) for 1 hr followed by another 3 hr with Brefeldin A (BFA, 1µg/ml, BD). For parasite-infected mice, cells from spleen and MLN were harvested at day 8 p.i. and stimulated following the same protocol as OVA-stimulated OT-II splenocytes. After stimulation, these cells were ready for cell-marker and intracellular cytokine staining.

**qPCR.** The tissue pieces of small intestine were collected by filtering through 70µm nylon mesh (Fisher Scientific, Loughborough, UK). Epithelial purity was confirmed by flow cytometry using antibodies against CD326 (Ep-CAM, Cambridge Bioscience, Cambridge, UK) and CD45-FITC (eBioscience) showing they were >95% pure. Total RNA was isolated from cells by homogenising in TRIsure (Bioline, London, UK). After phase separation using chloroform (Sigma-Aldrich), RNA was precipitated in isopropanol (Sigma-Aldrich). RNA concentration was measured on a nanodrop-1000 spectrophotometer (Labtech International, East Sussex, UK). The complementary DNA conversion from RNA was done using Bioscript-MMLV kit (Bioscript, London, UK). Quantitative PCR was performed using
KAPA SYBR FAST qPCR Kit (Kapa Biosystems Inc., MA) and an Opticon quantitative PCR thermal cycler (Bio-Rad, Hemel Hempstead, UK). The CCL5 expression ratios of samples were determined by normalising to the reference gene (YWHAZ). The primer sequences for CCL5: GGGTACCATGAAGATCTCTGCA (forward) and TTGGCGGTTCTCTCGAGTGA (reverse); For YWHAZ: TTCTGTGCCCAATGCTTC (forward) and TTCTGTGATCACCAGCACG (reverse).

**Statistics.** Statistical analyses were performed using a Student’s t-test, one-way or two-way ANOVA. The $P$-values of $< 0.05$ were considered significant. All statistical analyses were carried out using GraphPad Prism for windows, version 5 (La Jolla, CA).

**RESULTS**

**P2X7R mice have reduced inflammation in response to infection is critical to protect animals from *T. spiralis* infection.**

To determine the role of P2X7R in nematode infection, we infected P2X7R$^{-/-}$ mice and C57BL/6 wildtype (WT) littermate controls with *T. spiralis* and assessed daily body weight change and ileac pathology. In response to infection, the villi were slightly hypertrophic in both WT and P2X7R$^{-/-}$ mice at day 8 post infection (p.i.) but there was significant shortening of villi length in P2X7R$^{-/-}$ mice compared with WT mice at day 12 p.i.. (470 ± 76$\mu$m in WT vs. 384 ± 48$\mu$m in P2X7R$^{-/-}$, $P = 0.0421$) (**Figure 3.1a**). There was no difference in small intestine muscle wall thickness between the strains. Body weight monitoring showed a significant decrease in the weight of WT mice at day 8 p.i. compared with P2X7R$^{-/-}$ (10.82 ± 0.7% in WT vs. 7.51 ± 1.0% in P2X7R$^{-/-}$, $P = 0.0253$) (**Figure 3.1b**). In line with the body weight changes, histopathologically moderate ileitis was noted in both WT and P2X7R$^{-/-}$ animals after day 8 p.i. but WT mice had more severe tissue damage with more infiltration of polymorphonuclear leukocytes (PMN) and mononuclear inflammatory cells (**Figure 3.1c** and d, inflammatory score: 2.2 ± 0.4 in WT vs. 1.5 ± 0.4 in P2X7R$^{-/-}$, $P < 0.05$). However, there
was a mild remission of inflammation at day 12 p.i. in WT mice (inflammatory score: 2.2 ± 0.4 day 8 p.i. to 1.7 ± 0.5 day 12 p.i.). In contrast, the inflammatory score increased in P2X7R\(^{-/-}\) mice showing more lymphocyte infiltration at day 12 p.i. (inflammatory score: 1.5 ± 0.4 day 8 p.i. vs. 2.3 ± 0.8 day 12 p.i., \(P < 0.05\)) (Figure 3.1c and d). These data indicate an earlier onset of intestinal inflammation in WT compared with P2X7R\(^{-/-}\) mice.

![Graph showing villus heights and body weight change](image)

**Figure 3.1.** P2X7R\(^{-/-}\) mice have delayed gut inflammation in response to infection.

WT and P2X7R\(^{-/-}\) mice were orally inoculated with *T. spiralis* (400 larva/mouse) and sacrificed at day 0, 2, 8 and 12 p.i.. (a) Villus heights and (b) body weight change in WT and P2X7R\(^{-/-}\) mice at each time-point was monitored. (c) Ileac inflammation at day 0, 2, 8 and 12 was scored from 0 to 4 as summarised in Table 3.1. (d) Representative images of small intestinal pathology (H&E staining) in WT (upper) and P2X7R\(^{-/-}\) (lower) at day 0, 8 and 12 p.i. (Scale bar: 50\(\mu\)m). Inset box shows higher magnification of small box area. Yellow arrows indicate the infiltration of PMN leukocytes. Data shown are means ± SD (n= 6 per group), pooled from two independent experiments. Statistical difference was measured using Student’s t-test. *\(P < 0.05\).
Delayed recruitment of CD103⁺CD11b⁺ DCs to the small intestinal epithelium in P2X7R⁻/⁻ mice.

The early innate response and specifically DC recruitment in nematode infections have been shown to determine disease outcome⁹. We therefore investigated whether P2X7R signalling was promoting DC responses in the intestine. We analysed the antigen-presenting cells in the small intestine following *T. spiralis* infection (Figure 3.2a-c, Figure S3.1). The percentages of CD103⁺ DC subtypes were similar between naive WT and P2X7R⁻/⁻ mice in the intestinal epithelial layer (IEL CD103⁺CD11b⁻ DC: 5.4 ± 1.3% (WT) vs. 7.1 ± 1.2% (P2X7R⁻/⁻) of CD45⁺ population; CD103⁺CD11b⁺DC: 5.7 ± 1.7% (WT) vs. 6.4 ± 1.2% (P2X7R⁻/⁻) of CD45⁺ population) and lamina propria layer (LPL CD103⁺CD11b⁻ DC: 1.6 ± 0.6% (WT) vs. 2.3 ± 0.7% (P2X7R⁻/⁻) of CD45⁺ population; CD103⁺CD11b⁺DC: 9.0 ± 2.8% (WT) vs. 8.8 ± 4.6% (P2X7R⁻/⁻) of CD45⁺ population) (Figure 3.2b and c, Figure S3.1a and b). At day 2 p.i., WT mice had a six-fold increase in the proportion of CD103⁺CD11b⁺ DCs in IEL as well as a three-fold increase in LPL, compared with P2X7R⁻/⁻ animals in which the proportion of CD103⁺CD11b⁺ DCs remained low and similar to day 0 (IEL: 32.5 ± 14.7% in WT vs. 10 ± 8.5% in P2X7R⁻/⁻, *P* = 0.0088; LPL: 31.2 ± 7.0% in WT vs. 16.6 ± 9.0% in P2X7R⁻/⁻, *P* = 0.0121) (Figure 3.2b and c).

Both WT and P2X7R⁻/⁻ had an increase in the IEL and LPL CD103⁺CD11b⁺ DCs at day 8 p.i., but again the proportion of IEL CD103⁺CD11b⁺ DCs in the WT IEL was significantly higher than P2X7R⁻/⁻ (89.4 ± 5.3% in WT vs. 70.6 ± 12.8% in P2X7R⁻/⁻, *P* < 0.05). The proportion of CD103⁺CD11b⁻ DCs and CD103⁺CD11b⁺ DCs subsets in the IEL remained constant after infection whereas they increased in the LPL at day 2 p.i. (Figure S3.1a-d, *P* < 0.001 as compared with naive group). However, there was no significant difference in the proportion of CD103⁺CD11b⁻ DCs and CD103⁺CD11b⁺ DCs in IEL and LPL between WT and P2X7R⁻/⁻ mice (Figure S3.1a-d). In contrast to the DC subset responses to infection, the F4/80⁺CD11c⁺ macrophage subset in the IEL and LPL compartments of the P2X7R⁻/⁻ small intestine were similar to WT with both strains showing a moderate increase in macrophage numbers at day 8 p.i. in the IEL and LPL (Figure S3.1e and f).
To confirm the flow cytometry results and better define DC localisation, frozen ileum sections were analysed by immunohistochemistry for CD103⁺CD11c⁺ DCs (Figure 3.2d and e). The number and localisation of CD103⁺CD11c⁺ DCs were similar between naive WT and P2X7R⁻/⁻ mice. In response to infection, most of the CD103⁺CD11c⁺ DCs were noted in the villi. The majority of DCs were in the villus lamina propria region but DCs were also observed adjacent or attached to the basal layer of the intestinal epithelial cells (Figure 3.2d). In agreement with the flow cytometry data, there were increased numbers of CD103⁺CD11c⁺ DCs in the villi of WT mice compared with P2X7R⁻/⁻ mice at day 2 p.i. (9.9 ± 1.8/villus in WT vs. 3.5 ± 0.9/villus in P2X7R⁻/⁻, P < 0.001) (Figure 3.2e).
Figure 3.2. Impaired recruitment of CD11c+CD103+CD11b+ dendritic cells in P2X7R−/− mice in response to infection.

WT and P2X7R−/− mice were orally inoculated with *T. spiralis* (400 larva/mouse) and sacrificed at day 0, 2 and 8 p.i.. (a) Gating strategy for distinction of small intestinal CD11c+ cell populations as CD103+CD11b− DC, CD103+CD11b+ DC, CD103−CD11b+ DC and F4/80+CD11b+CD11c+ macrophages. Representative FACS plots are shown in Figure A.5 and A.6. The frequencies of CD11c+CD103+CD11b− DC in (b) IEL and (c) LPL were calculated as the percentage of CD45+MHCII+ cell population in WT (white bars) and P2X7R−/− mice (black bars). (d) Representative image of frozen ileum samples from WT and P2X7R−/− at day 2 p.i. stained for CD11c (red), CD103 (green), and counterstained with DAPI (blue) with co-localised cells shown in yellow (white arrow). IEC border is indicated by a white dashed line. The colocalised cells are highlighted by the inserts. (e) The numbers of CD11c+CD103+ cells per villus quantified. Scale bar: 50µm (insert: 25µm). n= 6 per group from two experiments (means ± SD). Statistical difference was measured using two-way ANOVA. *P < 0.05 and **P < 0.01 and ***P <0.001.
P2X7R\(^{-/-}\) intestinal epithelial cells have impaired chemokine responses.

We hypothesised that the reduction in DC recruitment was due to reduced epithelial chemokine responses since we had previously observed an epithelial dysfunction in *T. gondii* infection in P2X7R\(^{-/-}\) mice. We investigated this hypothesis by generating bone marrow chimeras. WT and P2X7R\(^{-/-}\) mice were irradiated. WT mice were reconstituted with P2X7R\(^{-/-}\) bone marrow to generate mice with WT epithelium and P2X7R\(^{-/-}\) DCs (referred as C57BL/6\(^{P2X7R^{-/-}}\)) and P2X7R\(^{-/-}\) mice were reconstituted with WT bone marrow mice to generate mice with P2X7R\(^{-/-}\) villus epithelial cells and WT bone marrow (P2X7R\(^{-/-}\)-C57BL/6). Successful reconstitution was confirmed by analysing IL-6 secretion from P2X7R agonist, 3’-O-(4-benzoyl)benzoyl-ATP, (BzATP)-stimulated splenocytes (Figure 3.3a). The P2X7R\(^{-/-}\)-C57BL/6 splenocytes produced significantly higher levels of IL-6 in response to BzATP treatment as compared with the C57BL/6\(^{P2X7R^{-/-}}\) cells (596 ± 158 pg/ml in P2X7R\(^{-/-}\)-C57BL/6 vs. 235 ± 125 pg/ml in C57BL/6\(^{P2X7R^{-/-}}\), \(P< 0.001\)). Following infection, we assessed CD103\(^{+}\)CD11b\(^{+}\) DCs recruitment to the small intestinal epithelium at day 2 p.i.. WT mice that were reconstituted with P2X7R\(^{-/-}\) bone marrow (C57BL/6\(^{P2X7R^{-/-}}\)) responded robustly to infection with DC recruitment into the IEL and LPL. In contrast the P2X7R\(^{-/-}\) mice that had been reconstituted with WT bone marrow (but had a P2X7R null epithelium, P2X7R\(^{-/-}\)-C57BL/6) had a significant reduction in the magnitude of the DC response in both IEL and LPL compartments (IEL CD103\(^{+}\)CD11b\(^{+}\) DCs: 60.5 ± 14% in C57BL/6\(^{P2X7R^{-/-}}\) vs. 40 ± 5.1% in P2X7R\(^{-/-}\)-C57BL/6, \(P = 0.013\); LPL CD103\(^{+}\)CD11b\(^{+}\) DCs: 68 ± 11% in C57BL/6\(^{P2X7R^{-/-}}\) vs. CD103\(^{+}\)CD11b\(^{+}\) DCs: 45.5 ± 11% in P2X7R\(^{-/-}\)-C57BL/6, \(P = 0.014\)) (Figure 3.3b). Thus, these data indicate that the in vivo migration ability of P2X7R\(^{-/-}\) DCs is normal in the gut and implicate P2X7R on epithelial cells in mediating DC recruitment in response to infection-induced injury.
Figure 3.3. Epithelial P2X7R is required for CD103⁺CD11b⁺ dendritic cell recruitment.

Bone marrow chimeras C57BL/6\(^{P2X7R^{-/-}}\) (grey bars) and P2X7R\(^{-/-}\)-C57BL/6 (black bars) were orally infected with *T. spiralis* (400 larva/mouse) and sacrificed at day 2 p.i. for isolation of splenocytes and small intestinal cells. \(n = 5\) per group, pooled from two different experiments (means ± SD). Statistical difference was measured using two-way ANOVA. \(***P < 0.001\) (a). Chimerism was confirmed by stimulating splenocytes with BzATP (100\(\mu\)M) and IL-6 secretion assessed using ELISA. The IEL and LPL were stained for CD45, MHCII, F4/80, CD11b, CD11c, and CD103. (b) The graph shows the percentage of CD45⁺MHCII⁺F4/80⁻CD11c⁻CD103⁺CD11b⁺ DCs out of the CD45⁺MHCII⁺ cells with means ± SD (\(n = 5\) per group, pooled from two different experiments). Statistical difference was measured using Student’s t-test. \(*P < 0.05\).

**Reduced Epithelial Responses to Infection.**

To study the epithelial response in P2X7R\(^{-/-}\) mice, ileum samples and small intestinal epithelial cells (IECs) from WT and P2X7R\(^{-/-}\) mice were collected at day 0 and day 2 p.i.. The analysis of ileum sections using TUNEL assay showed a robust increase in the numbers of epithelial cell death in both WT and P2X7R\(^{-/-}\) mice at day 2 p.i. but P2X7R\(^{-/-}\) mice had significantly less cell death in response to infection as compared with WT (10 ± 2.0 in WT vs. 5 ± 1.5 in P2X7R\(^{-/-}\), \(P < 0.001\)) (Figure 3.4a and b). Then, we analysed WT and P2X7R\(^{-/-}\) IECs by qPCR. Both WT and P2X7R\(^{-/-}\) mice had increased epithelial CCL5 expression following infection however this was significantly reduced in P2X7R\(^{-/-}\) IEC (Figure 3.4c, 10 ± 3.3 in WT vs. 4.5 ± 4.0 in P2X7R\(^{-/-}\), \(P = 0.042\)). We also investigated the levels of serum CCL5 at day 2 p.i.. Consistent with the qPCR data, P2X7R\(^{-/-}\) mice had significantly lower serum CCL5 levels.
when compared to WT mice (79 ± 21 pg/ml in WT vs. 55 ± 8.5 pg/ml in P2X7R−/−) (Figure 3.4d, $P = 0.03$). Collectively, these results indicate that P2X7R deficiency impairs epithelial responses to *T. spiralis* infection by reducing chemotactic function and cell death induction, which results in delayed recruitment of DCs.

Figure 3.4. Reduced epithelial CCL5 production in P2X7R−/− mice.
(a) Representative images of TUNEL stained sections of ileum from WT (left) and P2X7R−/− (right) mice day 2 p.i. with the TUNEL+ dead cells in green. Sections were counterstained with DAPI (blue). Epithelium border is indicated by white dashed lines. Scale bar: 20µm. (b) Quantification of cell death (TUNEL+ cells) at day 0 and 2 p.i. in WT (white bars) and P2X7R−/− mice (black bars). n= 6 per group, pooled from two independent experiments (means ± SD). Statistical difference was measured using two-way ANOVA. ***$P < 0.001$. Epithelial cells from the small intestines of *T. spiralis*-infected WT (white squares) and P2X7R−/− mice (black squares) at day 0 and day 2 p.i. were isolated and analysed for the expression of (c) CCL5 by qPCR. (f) Serum CCL5 was assessed by CBA day 1 p.i.. Data are means ± SD (n= 3-6 per group), pooled from two independent experiments. Statistical difference was measured using Student’s t-test. *$P < 0.05$.

**P2X7R deficiency impairs the development of Th2 immunity.**

DCs are critical cells for priming adaptive immunity. Given the reduction in the magnitude of DC migration we hypothesised this would impact on T cell responses in infection. First,
we investigated whether there were differences in the ability of DCs from WT versus P2X7R−/− to prime Th2 responses (Figure S3.2). We analysed the antigen presentation ability of DCs by co-culturing ovalbumin (OVA)-pulsed WT or P2X7R−/− bone marrow-derived DCs (BMDCs) with OVA peptide-specific OT-II splenocytes at a ratio of 1:10 for 48 hr. We found that both WT and P2X7R−/− BMDCs were able to present OVA antigen and equivalently stimulate OT-II splenocytes to express IL-4 (frequency of IL-4+CD4+ OT-II cells: 6.6 ± 2.0% in WT vs. 5.2 ± 1.0% in P2X7R−/− of total CD4+ OT-II cells, P= 0.344). These data suggest that P2X7R−/− DCs are functional.

Splenocytes were collected from WT and P2X7R−/− mice at day 8 p.i., and stimulated with trichinella antigen for 24 hr and cytokine production was analysed. Our results showed a significant reduction of IL-4 in P2X7R−/− mice (Figure 3.5a, P< 0.05). Surprisingly there was also a significant reduction in TNF-α production (Figure 3.5b, P< 0.05). However, the levels of other cytokines and chemokine produced by the splenocytes including IL-6, IL-13, interferon (IFN)-γ and CCL2 were similar between WT and P2X7R−/− animals (Figure 3.5c-f) suggesting there was no generalised impairment of cytokine responses. We further assessed the populations of IL-4+, IFN-γ+ CD4+ T cells and CD25+Foxp3+CD4+ regulatory T cells (Tregs) in mesenteric lymph nodes (MLN) and spleen of mice at day 8 p.i.. The frequencies of Tregs and IFN-γ+ expressing cells were low and there was no difference between WT and P2X7R−/− mice. In contrast, there was a significant increase in the proportions of IL-4+ cells in WT mice, compared to P2X7R−/− mice from both MLN (18.2 ± 7.0% (WT) vs. 9.8 ± 2.4% (P2X7R−/−) of total CD4+ T cells, P= 0.0195) and spleen; (12.3 ± 1.8% (WT) vs. 7.5 ± 0.8% (P2X7R−/−) of total CD4+ T cells, P< 0.001)) (Figure 3.5g and h). Collectively, these results indicate that P2X7R−/− animals have reduced Th2 responses to T. spiralis infection thus we investigated worm burden. P2X7R−/− mice, as expected, had a significantly higher worm burden (105 ± 48 in WT vs. 257 ± 80 in P2X7R−/−, P= 0.048) day 12 p.i. (Figure
3.5i). The enhanced worm burden in P2X7R−/− animals suggests enhanced susceptibility to 
*T. spiralis* infection.
Figure 3.5. Cytokine Responses and worm burden in P2X7R−/− mice.

Supernatants from trichinella antigen-stimulated WT and P2X7R−/− splenocytes taken at day 8 p.i. were collected for analysis of (a) IL-4, (b) TNF-α, (c) IL-6, (d) IL-13, (e) CCL5 and (f) IFN-γ by CBA. Cells from (g) MLN and (h) spleen of WT and P2X7R−/− mice day 8 p.i. were analysed by flow cytometry for the percentage of CD4+ cells from WT (white bars) and P2X7R−/− mice (black bars) expressing IFN-γ or IL-4 and CD4+CD25+Foxp3+ Tregs. Data shown are means ± SD (n= 6 per group), pooled from two independent experiments. Statistical difference was measured using Student’s t-test. *P < 0.05 and ***P < 0.001. Representative FACS plots are shown in Figure A.7. (i) WT and P2X7R−/− mice were orally inoculated with T. spiralis (400 larva/mouse). Worm burdens at day 8 and 12 p.i. were quantified and measured using two-way ANOVA. Data shown are means ± SD (n= 6 per group), pooled from two independent experiments. *P < 0.05 and **P < 0.01.
DISCUSSION

Since there have been many studies focusing on the in vitro functions of P2X7R in murine and human macrophages in clearing intracellular pathogens\textsuperscript{15}, our recent work has demonstrated the in vivo function of P2X7R in the initiation of intestinal inflammation and DC recruitment in response to intracellular pathogen, \textit{T. gondii} (Chapter 2). Similarly, a previous work by Miller et al. using P2X7R-deficient mice has indicated P2X7R as a regulator of systemic inflammation in response to \textit{T. gondii} infection\textsuperscript{16}. However, there is only one recent study showed P2X7R to initiate inflammation-associated post-infectious visceral hypersensitivity in \textit{T. spiralis} infection\textsuperscript{12}, and the relationship between P2X7R and enteric helminth infection is not well known.

In our study, we have found a novel role for P2X7R in epithelial cell responses to infection. Although lack of functional P2X7R was associated was less early inflammation and cell damage, the mice were less able to generate IL-4 and expel worms from the gut. Numerous studies have indicated the importance of DCs in the development of anti-helminthic responses. Our group has established that rapid mobilisation of colonic DCs to intestinal epithelium is associated with the resistance to \textit{T. muris}\textsuperscript{9} in mice. Other studies have also reported, a correlation between the expansion of CD11c\textsuperscript{+}B220\textsuperscript{-} DC in MLN and worm expulsion along with the increase of Th2 cytokines in response to \textit{T. muris}\textsuperscript{17}. Consistent with previous findings, our results reveal that P2X7R\textsuperscript{-/-} mice with impaired DC recruitment have attenuated anti-helminthic immunity characterised by reduced IL-4 production and increased worm burden. Collectively, our data presents a possible role of P2X7R in the initiation of small intestinal inflammation by recruiting immune antigen presenting cells against \textit{T. spiralis}. 
Burrowing parasites and their products can cause traumatic damage to intestinal epithelium, where enteric bacteria may further invade, resulting in mucosal inflammatory responses. A number of studies have reported P2X7R as a pro-apoptotic receptor by sensing extracellular ATP. Moreover, P2X7R-induced apoptosis in immune cells has been implicated in the elimination of intracellular pathogens such as Mycobacterium tuberculosis and T. gondii. Regarding the role of P2X7R in epithelial cells, P2X7R activation has been reported to down-regulate Wnt/β-catenin signalling which results in alveolar epithelial type I cell death in lipopolysaccharide (LPS)-induced acute lung injury. Another study also demonstrated that extracellular ATP induced P2X7R dependent apoptosis in human intestinal epithelial cells (IECs). Accordingly, our data shows that P2X7R-/- mice had less intestinal epithelial cell death at day 2 p.i., which suggests epithelial P2X7R is involved in mediating epithelial cell death in response to infection. In addition, the increased epithelial cell death in WT animals could result in the release of more chemoattractants including fractalkine, lysophosphatidylcholine, sphingosine-1-phosphate, and ATP, which may further induce chemokine production and recruit more immune cells to the infection site. Indeed, the WT mice, compared with the P2X7R-/- mice, earlier developed worsened gut inflammation and robust immune response to T. spiralis infection. Our data implicate a possibility that P2X7R-induced cell death may induce intestinal inflammatory response.

The local release of monocyte or PMN-associated chemokines during T. spiralis infection is important for host to initiate inflammation and the subsequent anti-parasitic immunity. Previous studies have shown that increased CCL2 and CXCL2 in the diaphragm and intercostal muscles promote inflammation in late T. spiralis infection. The interaction between CCR3 and its ligands, CCL11 and CCL24, is necessary for gastrointestinal eosinophilia in both T. spiralis and T. muris infection. According to our data, T spiralis infection resulted in an early increase of CCL5 production in WT mice in the small intestinal
epithelium and serum. This finding is consistent with a previous study showing that *T. spiralis* infection can significantly up-regulate CCL5 mRNA expression in the jejunum. A number of studies have revealed the role of P2X7R signalling in the production of chemokines and cytokines. In microglial cells, P2X7R stimulation by BzATP resulted in the release of IL-6, TNF-α and CCL2. Another study showed that ATP can effectively condition murine IEC, largely through P2X7R, to produce CXCL1, IL-6, TGF-β and thymic stromal lymphopoietin (TSLP) in response to toll-like receptor (TLR) stimulation. Thus, P2X7R is pivotal in regulating pro-inflammatory mediators. Indeed, P2X7R−/− mice, compared with WT, had significantly reduced CCL5 production in our ileitis model. Previous studies have demonstrated that mucosal epithelium-derived CCL5 is involved in the recruitment of dendritic cells in response to helminthic infection. In light of our data showing a reduction in the IEC-derived CCL5 in P2X7R−/− mice, it is not surprising that a chemotactic defect in the DC recruitment to the gut epithelium was noted.

Mouse intestinal conventional DC populations can be defined into two major DC subtypes: CD103+ and CD103−CD11b+ DCs. Published studies indicate that CD103−CD11b+ DCs efficiently induce Th1 immune response by producing IL-12 and IL-23. The CD103+ DC subtype is further categorised as CD103+CD11b+ DC and CD103+CD11b− DC in terms of distribution and function. CD103+CD11b− DCs are a major subset in colon LP while CD103−CD11b+ DCs are dominant in the small intestinal LP and villus. Although both CD103+ subsets are able to induce T helper cell immunity, the CD103−CD11b+ DCs are thought to be critical in both tolerogenic and inflammatory responses. Our data indicate that P2X7R−/− mice, compared with WT, had a significant delay in the recruitment of small-intestinal dominant CD103−CD11b+ DCs into the small intestine. A previous report shows that defective chemoattraction of small intestinal CD103+ DCs in neonatal mice impairs the development of protective immunity against *Cryptosporidium parvum* infection. Thus the
delayed chemoattraction of this important CD103+ DC subset may correlate with the impaired Th2 immunity seen in P2X7R−/− mice. However, there is still a possibility that Th2 polarisation of DC may be altered by changed profile of conditioning cytokines such as TSLP in P2X7R−/− IEC.

In summary, our findings suggest a novel role for mucosal epithelial P2X7R in the induction of early DC recruitment in response to nematode parasite infection. As an important tissue damage-sensor to initiate inflammation, P2X7R is a potential pharmaceutical target for development of new therapies for intestinal inflammatory disorders.

ACKNOWLEDGEMENTS

This work was funded by a scholarship from the Ministry of Education, Taiwan awarded to Szu-Wei Huang.

REFERENCES

1. Piccinini AM, Midwood KS. DAMPenning inflammation by modulating TLR signalling. *Mediators of inflammation* 2010; **2010**.


Supplementary Figure S3.1. The recruitment of CD103-CD11b+ DCs, CD103+CD11b- DCs and F4/80+CD11c+ macrophages were unaffected in P2X7R-/- mice.

WT and P2X7R-/- mice were orally inoculated with *T. spiralis* (400 larva/mouse) and sacrificed at day 0, 2 and 8 p.i.. Small intestinal IEL and LPL leukocytes were analysed using flow cytometry. Representative FACS plots are shown in Figure A.5 and A.6. The frequencies of small intestinal antigen presenting cells including CD11c+CD103-CD11b+ DCs (a and b), CD11c+CD103+CD11b+ DCs (c and d) and CD11c+F4/80+ macrophages (e and f) were calculated as the percentage of CD45+MHCII+ cell population. n= 6 per group, pooled from two independent experiments (means ± SD). Statistical difference was measured using two-way ANOVA. ***P < 0.001 as compared with naive control. a: compared with other timepoint groups.
Supplementary Figure S3.2. P2X7R−/− BMDCs are functional.
The expression of IL-4 in OT-II splenocytes stimulated with OVA-pulsed BMDCs from WT or P2X7R−/− mice was quantified using flow cytometry. Data shown are means ± SEM (n=3) **p < 0.01 and *** P < 0.001 as compared with BSA treatment (no OVA).
Chapter Four

_In vitro_ P2X7 Receptor-mediated Promotion of Chemokine and Pro-inflammatory Cytokine Responses in Intestinal Epithelial Cells
In vitro P2X7 Receptor-mediated Promotion of Chemokine and Pro-inflammatory Cytokine Responses in Intestinal Epithelial Cells

Szu-Wei Huang¹, David Brough¹, Gloria Lopez-Castejon², Catherine Walker¹, Werner Muller¹, Sheena Cruickshank¹

¹Faculty of Life Sciences, The University of Manchester, Manchester, UK
²Manchester Collaborative Centre for Inflammation Research, University of Manchester, UK.

AUTHOR CONTRIBUTION

Szu-Wei Huang carried out all experimental work and analysis.

David Brough provided ATP for P2X7R stimulation and scientific input.

Gloria Lopez Castejon provided IL-18 ELISA kit and scientific input.

Catherine Walker and Werner Muller helped with the primary culture of small intestinal crypt cells

Sheena Cruickshank is the main supervisor of this project.
The infection of *Toxoplasma gondii* (*T. gondii*) in intestinal epithelial cells (IECs) is known to initiate proinflammatory responses in the gut. However, the detail of how *T. gondii* induces gut inflammation is unclear. Given that the purinergic P2X7 receptor (P2X7R) is an inflammation mediator, we looked at how P2X7R functions in response to *T. gondii* infection. Blocking P2X7R using A-740003, caused significantly decreased levels of CCL5, IL-6 and TNF-α secretion by mouse intestinal carcinoma CMT-93 cells in response to *T. gondii* infection. A similar reduction of CCL5 was noted in primary P2X7R−/− intestinal crypt cells stimulated with lipopolysaccharide. This data suggests that P2X7R promotes proinflammatory responses against infection. P2X7R signalling is known to be involved in the inflammasome assembly. In response to infection, epithelial cells did not secrete the inflammasome-associated cytokines IL-1β and IL-18. Furthermore, P2X7R signalling had no effect on induction of cell death in *T. gondii*-infected IECs. Notably, blockade of P2X7R reduced parasite burden, which is a novel finding linking P2X7R signalling in epithelial cells with *T. gondii* infectivity. In sum our results demonstrate that P2X7R plays a pivotal role in epithelial recognition of infection.
INTRODUCTION

Toxoplasma gondii (T. gondii) is an obligate protozoan parasite that is mainly acquired through ingestion of contaminated uncooked meat or oocyst-contaminated food\(^1\). Following ingestion, the released parasites penetrate intestinal epithelial cells (IECs) and further disseminate to multiple tissues, forming cysts in the skeletal muscle, central nervous system, brain and eyes\(^2\). In most cases, the invasion of T. gondii into the gut elicits a rapid mucosal immune response to control parasite replication and initiation of adaptive immunity against the infection. However, the induction and regulation of the mucosal immune responses are highly dependent on the host genetic background and parasite virulence\(^3\).

The host defence against infection in the gut is first provided by IECs, a single-layer barrier that protects the underlying gastrointestinal (GI) tissue from the entry of pathogens. As the first cell layer of the mucosal surface, IECs physiologically act as a sensor of invasive microbial pathogens. Epithelial cells express a variety of pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMPs). These PRRs, including toll-like receptors (TLRs) and nucleotide oligomerisation domain proteins-1 and -2 (NOD1 and NOD2), have been reported to play a crucial role in the initiation of gut inflammation by sensing infection or tissue damage\(^4\).

During intracellular multiplication (endodyogeny), T. gondii multiplies and causes cell lysis as it exits the host cells\(^5\). Cell death and lysis are well-known to cause release of DAMP molecules such as the high-mobility group protein B1 (HMGB1), DNA and extracellular ATP\(^6\). Extracellular ATP can be sensed by a purinergic ATP-gated receptor P2X7 (P2X7R). Activation of P2X7R mediates apoptosis, inflammasome formation and inflammatory responses\(^7\). Indeed, our in vivo data have shown that P2X7R\(^{-/-}\) mice had reduced IEC apoptosis in
response to *T. gondii* and *Trichinella spiralis* (*T. spiralis*) infection (Chapter two and Chapter three). A number of studies have also revealed the importance of P2X7R in the development of intestinal inflammation\(^8,\,9\). Consistent with these observations, our data have indicated a role for P2X7R in mediating chemokine production by small intestinal epithelial cells. CCL5 is an important chemokine for the recruitment of T cells, natural killer (NK) cells, DCs and monocytes via binding to its receptors CCR1, CCR3, CCR4 and CCR5\(^10\). Our findings also imply that the reduction of CCL5 in the gut impairs early recruitment of dendritic cells (DCs) in response to infection (Chapter two and Chapter three). These findings suggest a pivotal role of P2X7R in the initialisation of intestinal inflammation during infection by promotion of IEC chemokine responses.

Many published studies have focused on the role of P2X7R in the mechanism of regulating the functions of immune cells. One of the best described functions of P2X7R in immune cells is the promotion of interleukin (IL)-1\(\beta\) and IL-18 secretion\(^11\). This process is mediated by P2X7R activation of inflammasome assembly. The inflammasome-dependent proteolytic activity cleaves pro-IL-1\(\beta\) and pro-IL-18 by caspase-1 to form the mature cytokines\(^12\). In addition, P2X7R signalling is also involved in the transcriptional regulation of immune cells. Previous studies have indicated that P2X7R activation can mediate IL-6 and tumour-necrosis factor (TNF)-\(\alpha\) production through activation of the early growth response protein (Egr) in murine microglial cells\(^13\). Exposure of benzoylbenzoyl-ATP (BzATP, P2X7R agonist) results in activation of mitogen-activated protein kinases (MAPK) and nuclear factor-kappaB (NF-\(\kappa\)B), which in turn increases the expression of cyclooxygenase-2 (COX-2) in human monocytes\(^14\) and the production of IL-2 in T cells\(^15\). P2X7R activation has been shown to induce apoptosis and autophagy in human IECs through the production of reactive oxygen species (ROS)\(^16\), however, very little is known about P2X7R signalling in IEC function and the epithelial response to infection.
As we have revealed the importance of P2X7R signalling in the \textit{in vivo} production of CCL5 in IECs during \textit{T. gondii} infection, we wanted to further clarify the mechanism of how epithelial P2X7R regulates the production of chemokines and proinflammatory cytokines. Thus, we investigated P2X7R signalling and cytokine production using \textit{in vitro} models of mouse colorectal carcinoma CMT-93 cells and primary small intestinal crypt cells. We found that P2X7R signalling regulates the CCL5 and proinflammatory cytokine production in IECs possibly via the enhancement of TLR downstream signalling in response to \textit{T. gondii} infection.
**METHODS**

**Parasites and cells.** *Toxoplasma gondii* type-I RH strain expressing yellow fluorescence protein (YFP)\(^{17}\) and type-II Prugniaud (PRU) strain expressing tandem dimers of tomato red (tdTomato) fluorescent protein\(^{18}\) were used in our experiments. Tachyzoites were harvested by serial 4-5 day passage in human foreskin fibroblast (HFF) with culture media containing DMEM (Sigma-Aldrich) with 10% FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (PEST) (Sigma-Aldrich). The parasite count and the infection rate of RH or PRU parasites in cells was measured by flow cytometry. The mouse colonic epithelial cell line, CMT-93, was maintained in DMEM (Sigma-Aldrich) media with 10% FBS (Sigma-Aldrich), 2mM L-glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin (PEST) (Sigma-Aldrich). CMT-93 cells were infected with PRU or RH parasites at a ratio of 1:1 for 24 or 48 hr. The expression of P2X7R in CMT-93 cells was assessed by intracellular staining of P2X7R using FITC-conjugated rat anti-mouse P2X7R antibody (Aviva System Biology, CA) and analysed by flow cytometry.

**Cell treatments.** CMT-93 cells were treated with the selective P2X7R antagonist, A-740003 (500µM; Sigma-Aldrich) to block P2X7R at the same time as infection with parasite. The pan-caspase inhibitor Z-VAD (100µM; Sigma-Aldrich) was used to inhibit infection induced-apoptosis in CMT-93 cells. NF-κB inhibitor Bay117082 (10µM; Sigma-Aldrich) was used to block parasite-induced NF-κB activation. To simulate pathogen-induced TLR activation, we used LPS (20µg/ml; Sigma-Aldrich) from *Salmonella enterica* serotype enteritidis to stimulate CMT-93 cells and primary crypt organoids for 6 hr. Purified ATP (5mM; Sigma-Aldrich) was used to induce P2X7R activation.

**Primary culture of mouse small intestinal crypt organoids.** We prepared the organoids following published protocols\(^{19}\). The small intestine was cut into small pieces and...
transferred into crypt isolation buffer (2mM EDTA in PBS) under gentle agitation at 4°C for 30 min. The isolated crypts were resuspended in Matrigel™ (BD) (300-500 crypts/50µl) and cultured in Advanced DMEM/F12 + GlutaMAXTM (Life Technologies) supplemented with 10mM HEPES (Sigma-Aldrich), 1% PEST (Sigma-Aldrich), 1% N2 supplement (Life Technologies), 2% B27 supplement (Life Technologies), murine recombinant epidermal growth factor (50ng/ml), murine recombinant Noggin (100ng/ml) (PeproTech EC Ltd., London, UK) and human recombinant R-spondin1 (1µg/ml) (R&D). Well-differentiated crypt organoids were split into 30 organoids/50µl Matrigel™/well.

Cytokine and chemokine analysis. The IL-1β, IL-18 and CCL5 levels in supernatants from CMT-93 and crypt organoids were measured using an ELISA duoset kit (R&D Systems, Abingdon, UK) and the concentrations of IL-6 and TNF-α in CMT-93 supernatant were measured using a mouse quantitative ELISA kit (QuantiKine ELISA, R&D) according to the manufacturer’s instructions.

Cell death analysis. The percentage of dead CMT-93 cells in response to *T. gondii* infection was measured using the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen). Briefly cells were harvested and washed in cold PBS followed by incubation with FITC-annexin V (20x dilution) and propidium iodide (PI) (100x dilution) in annexin-binding buffer at room temperature for 15 min. Then, the annexin V/PI-labelled cells were washed using annexin-binding buffer twice and analysed immediately by flow cytometry. Annexin V and PI double positive cells were counted as dead, apoptotic cells.

Statistics. Statistical analyses were performed using a Student’s t-test, one way ANOVA and two-way ANOVA with Bonferroni post-hoc test. The *P*-values of < 0.05 were considered
significant. All statistical analyses were carried out using GraphPad Prism for windows, version 5 (La Jolla, CA).

RESULTS

P2X7R signalling promotes CCL5 and proinflammatory cytokine production in response to *T. gondii* infection.

Previously our *in vivo* data showed a significant reduction of proinflammatory cytokines and chemokine production in P2X7R<sup>−/−</sup> mice and a decrease of CCL5 expression in P2X7R<sup>−/−</sup> gut epithelial cells in response to *T. gondii* and *T. spiralis* infection. Thus, we further investigated the mechanism of how P2X7R signalling promotes epithelial CCL5 expression in response to *T. gondii* infection. We used a mouse colorectal epithelial cancer cell, CMT-93, as an *in vitro* model cell. To confirm CMT-93 cells were a suitable model, we analysed expression of P2X7R by flow cytometry and showed the cells expressed P2X7R (Figure 4.1a). We then measured the production of CCL5 and the pro-inflammatory cytokines TNF-α and IL-6 in naïve CMT-93 cells or cells infected with *T. gondii*. Levels of CCL5, TNF-α and IL-6 were similar between naïve CMT-93 and CMT-93 cells treated with the selective P2X7R antagonist A-740003 (CMT-93+A-740003). In response to infection, there was a robust up-regulation of CCL5 (25-fold increase), TNF-α (2-fold increase) and IL-6 (3-fold increase) (Figure 4.1c and d). Inhibition of P2X7R down-regulated the production of CCL5 by CMT-93 cells (255.2 ± 20.87 pg/ml in CMT-93 vs. 162.8 ± 7.19 pg/ml in CMT-93+A-740003, *P* < 0.001). The proinflammatory cytokines TNF-α (37.6 ± 1.9 pg/ml in CMT-93 vs. 19.1 ± 0.7 pg/ml in CMT-93+A-740003, *P* < 0.001) and IL-6 (83.5 ± 10.4 pg/ml in CMT-93 vs. 18.5 ± 1.7 pg/ml in CMT-93+A-740003, *P* < 0.001) were also down-regulated in *T. gondii*-infected CMT-93 cells in response to P2X7R inhibition (Figure 4.1b, c and d). These *in vitro* results confirm our previous findings that P2X7R signalling promotes epithelial chemokines in *T. gondii* infection.
P2X7R stimulation is important for capase-1 activation and the assembly of the inflammasome\textsuperscript{20}. Therefore, we tested whether P2X7R in epithelial cells promoted production of the inflammasome associated cytokines IL-1\(\beta\) and IL-18 in response to \textit{T. gondii} infection. However, we found no detectable IL-1\(\beta\) production by CMT-93 cells (data not shown). Surprisingly, \textit{T. gondii} infection caused a significant decrease of IL-18 secretion from CMT-93 cells (\textbf{Figure 4.1e}). Furthermore, the P2X7R antagonist (A-740003) had no effect on the production of IL-1\(\beta\) and IL-18 (\textbf{Figure 4.1e}) by epithelial cells before and after infection. This finding suggests that the P2X7R-dependent regulation of chemokines and proinflammatory cytokine production in response to \textit{T. gondii} infection is inflammasome independent.
Figure 4.1. Inhibition of P2X7R down-regulates *T. gondii*-induced chemokine and pro-inflammatory cytokine responses.

P2X7R expression is confirmed in CMT-93 epithelial cells by flow cytometry. (a) Representative histogram of isotype control stain (red) and CMT-93 cells stained with P2X7R (blue) is shown. CMT-93 epithelial cells treated with (black bars) or without (white bars) the P2X7R antagonist A-740003 (500µM) were infected with *T. gondii* (PRU strain) in a ratio of 1:1 for 24 hr. The concentrations of (b) CCL5, (c) TNF-α (d) IL-6 and (e) IL-18 in the supernatant from naive and infected cells were analysed by ELISA. The data are means ± SEM pooled from 3 independent experiments and analysed using two-way ANOVA with Bonferroni post-hoc test. **P < 0.01 and ***P < 0.001 indicate significant difference between groups.

**Apoptosis is not required for P2X7R-dependent regulation of CCL5.**

P2X7R activation is known to induce the assembly of the inflammasome and caspase-8-dependent cell death\(^{21}\). As we did not see evidence of epithelial production of the inflammasome-associated cytokines IL-1β and IL-18, we investigated whether epithelial
P2X7R is involved in cell death. Thus, we analysed the proportion of apoptotic cells in CMT-93 infected by *T. gondii* for 24 h. Compared with naive cells, *T. gondii* infection caused a significant increase in cell death in CMT-93 cells (the proportion of apoptotic cells: 3.7 ± 0.3% in naive CMT-93 versus 21.6 ± 0.9% in infected CMT-93) (Figure 4.2a and b). However, inhibition of P2X7R did not alter the percentage of dead cells in response to infection (Figure 4.2b), which suggests that P2X7R is not involved in *T. gondii*-induced cell death.

Although blocking P2X7R signalling did not inhibit infection-induced apoptosis, we could not exclude the possibility that cell death would influence the CCL5 responses in *T. gondii* infection. Because the link between apoptosis and CCL5 was unknown, we investigated if inhibition of apoptosis would affect epithelial CCL5 production. Our findings show that inhibition of apoptosis using the pan-caspase inhibitor Z-VAD (100µM) dramatically elevated the CCL5 levels in naive CMT-93 cells (45.4 ± 2.4 pg/ml (media) vs. 216.7 ± 6.8 pg/ml (Z-VAD), *P* < 0.05) but had little effect in *T. gondii*-infected CMT-93 cells (Figure 4.2c). This data implicates that the less apoptosis happens, the more CCL5 increases in naive cells. However, inhibition of apoptosis did not protect cells from *T. gondii*-induced cell death and thus did not affect CCL5 production in response to the parasite infection.
Figure 4.2. Inhibition of P2X7R has no effect on infection-induced cell death.
Following infection by PRU (in a ratio of 1:1) for 24 hr, CMT-93 cells with or without the P2X7R antagonist A-740003 treatment (500µM) were collected for analysis of cell death by Annexin-V/PI staining. Apoptotic cells were identified as Annexin-V and PI double positive cells (a) Representative flow cytometry graphs are shown and (b) the data was quantified indicating that A-740003 treatment had no effect on infection-induced cell death. Naive or PRU-infected CMT-93 cells were treated with media or an apoptosis inhibitor Z-VAD (100µM) and (c) the CCL5 concentrations in the supernatant were measured by ELISA. The data are means ± SEM pooled from 3 independent experiments. Data was analysed using two-way ANOVA with Bonferroni post-hoc test. *P < 0.05 and ***P < 0.001.

P2X7R regulates TLR signalling-associated CCL5 response.
The TLR adaptor MyD88 has been reported to be required for innate sensing of *T. gondii* and the production of chemokines and cytokines\(^2\), but the role of P2X7R in this TLR signalling-dependent response is unknown. Thus, we investigated whether TLR-MyD88 downstream signalling was involved in P2X7R-dependent regulation of CCL5. We used primary small intestinal crypt organoids from littermate WT and P2X7R\(^{-/-}\) mice and stimulated the primary organoids with LPS (20µg/ml) and analysed CCL5, IL-1\(\beta\) and IL-18.
secretion. Our data revealed that LPS enhanced CCL5 and IL-1β production but decreased IL-18 production in both WT and P2X7R−/− organoids. There was no difference in the response of WT versus P2X7R−/− cells to LPS stimulation in terms of IL-1β or IL-18 levels. However, CCL5 production was significantly reduced in LPS stimulated P2X7R−/− organoids (100.4 ± 7.6 pg/ml in WT vs. 52.5 ± 15.2 pg/ml in P2X7R−/−, P < 0.01) (Figure 4.3a-c). This data suggests that P2X7R is involved in the regulation of TLR4 signalling in primary organoids possibly by modulating the magnitude of the TLR mediated response. To look at this further, we stimulated our CMT-93 model using LPS for 6 hr. However, we did not see any up-regulation of CCL5 in CMT-93 in response to LPS stimulation even in the presence of ATP (5mM) (data not shown). This finding suggests that CMT-93 cells may not respond to LPS treatment or may not respond as efficiently as primary crypt cells and thus need a longer incubation.
**Figure 4.3. Inhibition of P2X7R down-regulates LPS-induced CCL5 response.**

Small intestinal crypt organoids isolated from C57BL/6 (WT, grey bars) or P2X7R\(^{-/-}\) (black bars) mice were stimulated by LPS (20µg/ml) for 6 hr. The concentrations of (a) CCL5, (b) IL-1β and (c) IL-18 in the supernatant were analysed by ELISA. The LPS-induced CCL5 production was blocked by P2X7R inhibition. The data are means ± SEM pooled from 3 independent experiments and analysed using two-way ANOVA with Bonferroni post-hoc test. *\(P < 0.05\) and **\(P < 0.01\). N.D not detected.

**NF-κB signalling is involved in the CCL5 production in response to infection.**

The NF-κB pathway is involved both in TLR signalling and NOD signalling-dependent CCL5 expression\(^{23}\). In addition, activation of P2X7R signalling can induce NF-κB activation\(^{11}\). Thus, we investigated the relationship between CCL5 and NF-κB signalling in IECs. According to previous studies NF-κB, is activated by type-II *T. gondii* strains but not by the type I and type III strains\(^{24}\), we tested whether there was strain-dependent CCL5 production in CMT-93 cells in response to *T. gondii* infection. Interestingly, we found type I RH toxoplasma parasites were unable to induce CCL5 production (Figure 4.4). This finding implied a possible involvement of NF-κB signalling in the *T. gondii*-induced CCL5 response.
Figure 4.4. Infection of type I toxoplasma RH strain does not induce CCL5 production in IECs.

Following infection by RH or PRU parasites (in a ratio of 1:1) for 24 hr, the supernatant from the control (white bar) and infected CMT-93 cells (RH: grey bar and PRU: hatched bar) were collected for analysis of CCL5 production. The data are means ± SEM pooled from 3 independent experiments. Data was analysed using one-way ANOVA with Turkey post-hoc test. ***P < 0.001.

In order to further clarify the role of NF-κB in *T. gondii*-induced epithelial CCL5 production, we treated parasite-infected CMT-93 cells with the NF-κB inhibitor Bay117082 and analysed CCL5 levels in response to treatment. Inhibition of NF-κB significantly decreased CCL5 production in response to infection (591.6 ± 50.4 pg/ml in CMT-93+PRU vs. 445.9 ± 35.7 pg/ml in CMT-93+PRU with Bay117082, P < 0.05) (Figure 4.5). In sum, these findings suggest that NF-κB signalling is involved in *T. gondii* induced epithelial CCL5 production. Nevertheless neither NF-κB inhibitor nor P2X7R antagonist could totally block the *T. gondii*-induced CCL5 response (CCL5 in Bay117082 group: 34.6 ± 3.5 pg/ml (CMT-93) vs. 445 ± 35.7 pg/ml (CMT-93+PRU), P < 0.001). Thus, the data suggests the regulation of CCL5 in response to *T. gondii* infection is not only dependent on NF-κB and P2X7R signalling but involves other transcription factors or receptors.
Figure 4.5. The infection-induced CCL5 production is partially via activation of NF-κB signalling.

Non-infected (grey bars) and PRU-infected CMT-93 cells (hatched bars) were treated with media only, the P2X7R inhibitor A-740003 (500μM) or the NF-κB inhibitor Bay117082 (10μM). The concentrations of CCL5 in the supernatant were measured by ELISA. This result shows a partial suppression of *T. gondii*-induced CCL5 response as NF-κB activation is inhibited. The data are means ± SEM pooled from 3 independent experiments and were analysed using two-way ANOVA with Bonferroni post-hoc test. *P < 0.05, **P < 0.01 and ***P < 0.001.

The Kinetics of *T. gondii* infection are altered by P2X7R inhibition.

As *T. gondii* infection induces CCL5 in intestinal epithelial cells, we investigated whether blocking P2X7R also inhibited parasite infection and our data could be attributed to a reduction in infectivity. We first investigated whether the P2X7R antagonist A-740003 was toxic to *T. gondii*. *T. gondii* were pre-treated with A-740003 for 4 hr, washed to remove all the inhibitor and then used to infect CMT-93 cells for 24 hr. We found no difference in the percentage of parasite-infected epithelial cells if we used *T. gondii* that had been pre-treated with P2X7R inhibitor (Figure 4.6a). This finding indicates that the P2X7R inhibitor, A-740003, is not toxic to the parasite. In contrast though, blockade of P2X7R in CMT-93 cells, caused a significant decrease in the proportion of parasite infected cells compared with untreated cells (24 hr post infection (p.i.): 17.5 ± 0.4% in CMT-93 vs. 1.9 ± 0.1% in CMT-93+A-740003, *P < 0.001) (Figure 4.6b and c). Nevertheless, the percentage of parasite
infected cells treated with A-740003 gradually increased by 48 hr p.i. (CMT-93+A-740003: 1.9 ± 0.1% (24 hr p.i.) vs. 9.2 ± 1.6% (48 hr p.i.), \( P < 0.01 \)) (Figure 4.6c). The data thus suggest that inhibition of P2X7R impacts on parasite infectivity or replication in intestinal epithelial cells. The reduction in parasite burden may therefore contribute to the reduced chemokine and cytokine response by CMT-93 cells.
Figure 4.6. Inhibition of P2X7R delays *T. gondii* infection in CMT-93 cells.

To assess whether A-740003 affected parasite viability, PRU were pre-treated with A-740003 (500µM) for 4 hr, washed and used to infect CMT-93 cells. (a) The percentage of parasite infected cells were quantified by flow cytometry showing that A-740003 did not alter parasite viability. CMT-93 cells with or without A-740003 treatment (500µM) were collected 24 and 48 hr post infection and analysed for the proportion of PRU-infected cells. (b) Representative histograms (CMT-93 cells only shown red; CMT-93 cells infected with PRU shown blue) at 24 hr p.i. are shown and (c) the data combined from 3 independent experiments was quantified indicating a significant reduction in the infectivity of PRU at 24 and 48 hr p.i. in A-740003 treated CMT-93 cells. The data are means ± SEM pooled from 3 independent experiments and analysed using two-way ANOVA with Bonferroni post-hoc test. **P < 0.01 and ***P < 0.001.

**DISCUSSION**

This present study provides evidence that P2X7R signalling promotes *T. gondii*-induced CCL5 and pro-inflammatory cytokine responses in IECs and this process is unlikely to be dependent on the traditional P2X7R functions including the assembly of inflammasome and the induction of cell death. We show that P2X7R has a novel function in the control of the parasite infectivity in IECs. Additionally there is possibly an involvement of P2X7R in the modulation of TLR-mediated CCL5 production. Finally, NF-κB contributes to the induction of CCL5 in IECs in response to *T. gondii* infection.
Several studies have investigated the responsiveness of IECs in *T. gondii* infection. Infection of murine IECs with *T. gondii* showed increased CCL2, CCL3, CCL5, CXCL2, CXCL9 and CXCL10\(^{25}\). Recently Ju *et al.* investigated *T. gondii* infection in human enterocytes and found a series of chemotactic and proinflammatory mediators were induced including CCL3, CCL5, CCL8, CCL7, CCL15, CCL20, CCL24, CXCL2, CXCL10 and IL-8\(^{26}\). Herein our *in vitro* and *in vivo* data now demonstrate the involvement of P2X7R signalling in the induction of CCL5.

In this study, our data shows that the P2X7R antagonist influenced the infectivity of *T. gondii* in IECs. This novel and surprising finding indicates that P2X7R signalling may be involved in the process of the *T. gondii* life cycle in host cells. According to our data, the P2X7R antagonist was not toxic to *T. gondii* (*Figure 4.6a*). Moreover the antagonist did not affect parasite-induced cell death in the first 24 hr of the infection (*Figure 4.2a*) which encompasses the time (18-24 h) needed for parasite multiplication and cell lysis\(^{27}\). Accordingly, because we collected the cells at 24 hr p.i., most of the initially infected CMT-93 cells were believed to be lysed by that time-point and the released parasites were able to re-invade the surrounding naive cells. Thus, the reduced infection rate noted in P2X7R-blocked cells at 24 hr p.i. shows a reduction in the number of released parasites, which may implicate a delay or decrease of intracellular parasite multiplication (*Figure 4.6c*).

Several innate factors may affect *T. gondii* replication in host cells. For example, cytokines such as IL-1β and TNF-α have been shown to inhibit *T. gondii* replication in endothelial cells\(^{28}\). However according to our results, there was no detectable IL-1β in response to infection and the P2X7R antagonist reduced the TNF-α levels in the parasite infected-CMT-93 cells. Thus, IL-1β and TNF-α are not involved in the mechanism of P2X7R-mediated *T. gondii* growth. A previous report demonstrated that IL-6 treatment enhanced *T. gondii* replication and impaired IFN-γ-mediated parasite killing\(^{29}\). As we also see reduced IL-6 in
the absence of P2X7R in our study, the decreased parasite infectivity by P2X7R blockade may be due to deprivation of IL-6 or other factors which are necessary for parasite replication.

Multiple TLRs are involved in sensing *T. gondii* infection and initiating proinflammatory responses. In rodents, *T. gondii* profilin is the major target for TLR11/TLR12\(^{30, 31}\). Other endosomal receptors TLR7 and TLR9 are also reported to induce host resistance to *T. gondii* by sensing parasite RNA and DNA in the absence of TLR11\(^{32}\). In addition cell surface TLRs including TLR1/TLR2 and TLR4 are involved in the recognition of *T. gondii* surface structure glycosylphosphatidylinositol (GPI)\(^{33}\). To study whether TLR signalling is involved in the P2X7R-dependent CCL5 response, we used LPS to stimulate TLR4 in primary crypt organoids. We found that LPS-induced CCL5 production in primary organoids but the CCL5 up-regulation was reduced in the absence of P2X7R. However, when we used CMT-93 cells for LPS stimulation, we found that CMT-93 cells acted very differently from the primary organoids in response to LPS: there was no CCL5 induced in CMT-93 cells stimulated by LPS. Some studies may explain why the difference in response to LPS occurs between primary and carcinoma cells. Carcinoma cells are known to have alterations in function due the fact they have been immortalised and maintained in culture. It has been previously shown that LPS activated NF-κB but had little effect on the activation of p38 and c-Jun N-terminal kinase (JNK) in CMT-93 cells and some human colon carcinoma cells\(^{34, 35}\). Given that p38 and JNK are involved in constitutive transcription of CCL5\(^{36}\), these findings imply that there is a suppressed LPS-induction of CCL5 in intestinal carcinoma cells. Taken together, intestinal carcinoma cells may not be a good model for LPS induced-CCL5 production due to their hyporesponsiveness to LPS. Alternatively, whereas LPS increases CCL5 transcription in bovine\(^{37}\) and murine\(^{38}\) epithelial cells by 6 hr of stimulation, CCL5 expression does peak
until 24 hr after stimulation\textsuperscript{38}. Thus, there is a possibility that our 6 hr-LPS stimulation was too short to see a significant CCL5 response in CMT-93 cells.

NF-κB, as one of the major downstream transcription factors of TLR signallng is crucial for chemokine and proinflammatory cytokine production\textsuperscript{39}. Previous studies have reported that only \textit{T. gondii} type II strain (eg. PRU) can significantly induce early cytokine and DC responses\textsuperscript{24}. Consistently we found that \textit{T. gondii} type I RH strain infection was unable to induce CCL5 (\textbf{Figure 4.4}), whereas the infection using the type II PRU strain induced significantly higher amounts of CCL5, IL-6 and TNF-α in CMT-93 cells (\textbf{Figure 4.1}). This finding supports a role for NF-κB signalling in the induction of CCL5 in response to PRU infection. However, our data shows that blocking NF-κB signalling did not totally down-regulate \textit{T. gondii}-induced CCL5 production. Thus, not only NF-κB but also other signalling pathways are involved to induce CCL5 production in response to Type II \textit{T. gondii} infection in epithelial cells. A previous study has indicated that the p38/MAPK signalling is required for eliciting CCL5 in epithelial cells in the infection of respiratory syncytial virus\textsuperscript{40}. Moreover \textit{T. gondii} infection can induce a rapid phosphorylation of p38/MAPK in IECs\textsuperscript{26}. Thus, p38/MAPK may be another important pathway in the production of epithelial CCL5 in response to intracellular pathogens. However, further studies will be needed to reveal the detailed mechanism of \textit{T. gondii} infection-induced CCL5 production in IECs.

In conclusion, P2X7R is not involved in the induction of cell death and the inflammasome assembly in IECs in response to \textit{T. gondii} infection. Instead, P2X7R signalling promotes chemokine and cytokine production and may play a novel role in the control of parasite infectivity.
ACKNOWLEDGEMENTS

This work was funded by a scholarship from the Ministry of Education, Taiwan awarded to Szu-Wei Huang and BBSRC Grant BBG018839/1 for Catherine Walker and Werner Muller.

REFERENCES


Chapter Five
Overall Discussion
5.1 Key findings

1. P2X7R deficient IECs have reduced CCL5 expression in response to infection.

2. P2X7R deficient mice have a reduced intestinal DC recruitment in the early response to infection.

3. P2X7R deficient mice developed reduced adaptive T immunity in response to infection.

4. P2X7R modulates LPS-induced CCL5 response in IECs in an inflammasome independent manner.

5. P2X7R antagonism reduces the infectivity of *Toxoplasma gondii* in IECs.

5.2 P2X7 receptor as a potential initiator of intestinal inflammation?

A lot of literature has suggested a mechanism in which some endogenous intracellular molecules of the host can be released into the extracellular environment during pathological conditions that can then control the activity of immune cells in response to harmful stimuli such as trauma or infection. Extracellular ATP as one of the released endogenous alarmins following cellular stress or cell death is reported to drive systemic inflammation and tissue damage via activation of the membrane-bound purinergic P2X7 receptor (P2X7R). Once activated by extracellular ATP, the P2X7R signalling subsequently opens the pannexin-I hemichannel to release ATP for amplification of the extracellular ATP-induced responses. Continuous stimulation of P2X7R causes apoptosis in some immune cells such as macrophages and non-immune cells such as intestinal epithelial cells (IECs). Despite a variety of downstream events that have been noted following P2X7R activation by its ligand, P2X7R activation is best known for its role in the assembly of the NOD-like receptor protein 3 (NALP3) inflammasome, promoting the secretion of proinflammatory cytokines including interleukin (IL)-1β and IL-18, and activating nuclear factor kappa B (NF-κB). Given that the P2X7R is expressed ubiquitously in almost all tissues and cell types,
many studies have found the involvement of the P2X7R in the development of inflammatory disorders in body systems such as neurons, kidneys, lungs and immune system\textsuperscript{12}. Work to date shows that released extracellular ATP can activate the P2X7R on infiltrating immune cells such as macrophages, dendritic cells (DCs) and T cells and that ATP acts as a natural adjuvant to drive the production of proinflammatory cytokines, antigen-presentation and T cell differentiation\textsuperscript{12-14}. In addition, P2X7R\textsuperscript{-/-} animals, compared with their wildtype (WT) counterparts, not only have less production of inflammatory cytokines but also have decreased or delayed early infiltration of immune cells including neutrophils and macrophages in response to pathogenic infection\textsuperscript{15, 16}. These findings implicate a chemotactic defect of recruitment of immune cells to initiate inflammation in P2X7R\textsuperscript{-/-} animals.

By using \textit{in vivo} and \textit{in vitro} parasite infection models, our data propose a mechanism whereby the early recruitment of immune cells by epithelial cells is impaired in P2X7R\textsuperscript{-/-} mice. From our \textit{in vivo} data, we found that P2X7R deficiency causes reduced intestinal epithelial responsiveness with impaired chemokine expression of CCL5 and CCL20 in response to protozoan parasite \textit{Toxoplasma gondii} (\textit{T. gondii}) and helminth \textit{Trichinella spiralis} (\textit{T. spiralis}) infection. This defect in epithelial responses is associated with delayed recruitment of CD103\textsuperscript{+} DCs to the infection site which may then impair the subsequent adaptive immune response. In our \textit{in vitro} studies in IEC, stimulation of P2X7R ligand ATP did not induce CCL5 production on its own but in conjunction with a second signal from infection or LPS, did induce a robust response with increased production of CCL5, IL-6 and TNF-\alpha. Thus P2X7R signalling may not directly cause the production of CCL5, IL-6 and TNF-\alpha, but instead enhances the development of proinflammatory chemokine and cytokine responses in IECs. Collectively, these data indicates that P2X7R plays an important role to initiate intestinal inflammation in response to pathogens via increasing epithelial
chemokine and cytokine production to enable the recruitment of innate immune cells such as DCs in the early phase of infection (Figure 5.1).

**Figure 5.1. The mechanism of P2X7R-dependent initiation of inflammation in response to intestinal infection.**

(a) Intestinal infections stimulate pathogen-associated molecular pattern (PAMP) receptors (e.g. TLRs) and their down-stream signals in intestinal epithelial cells (IECs), which is enhanced by P2X7R signalling to express CCL5, CCL20, IL-6 and TNF-α perhaps via NF-κB or MAPK pathways. (b) The CCL5 further attracts CD103+ dendritic cells (DCs) to the infection site. Then the infiltrated CD103+ DCs can migrate to secondary lymphoid organs to induce protective T cell immunity.

### 5.3 How important are DCs in the initiation of immunity to intestinal infections?

The importance of DCs in the initiation of immunity against intestinal infections including parasites, bacteria and viruses has been previously revealed. Conditional ablation of DCs using a diphtheria toxin (DT)/diphtheria toxin receptor (DTR) inducible system, targeting CD11c+ cells, allowed researchers to further study the functions of DCs in response to intestinal infection in intact animals. For example, the absence of CD103+CD11b- and CD103+CD11b+ migratory DCs in the small intestine decreased T cell
activation with lower Th1 immunity in response to *Citrobacter rodentium* (*C. rodentium*) infection in the small intestine. In the small intestinal infection of *Heligmosomoides polygyrus* (*H. polygyrus*), depletion of CD11c^hi^ DCs (predominantly CD103^+^ DCs) resulted in the dramatic inhibition of Th2 effector response, favouring the development of chronic infection with an immunosuppressive phenotype^24^. However, the authors also showed that depletion of CD11c^hi^ DCs did not alter the innate type 2 response and the induction of alternative activated macrophages, which means that, despite DCs, other innate immune cells, such as basophils^25^ and nuocytes^26^ can play a role as type 2 responders to intestinal parasite infection^26^. Interestingly, a similar result was found in the large intestinal infection of *Trichuris muris* (*T. muris*), a well-characterised *in vivo* model of Th2 immunity, showing that depletion of CD11c^+^ DCs had no effect on Th2 cytokine production or worm burden^27^. These findings suggest that DCs may not be essential for the intestinal type 2 response. However, our data showed the reduced DC response was associated with a lower Th2 response in *T. spiralis* infection. Thus, the role of DC in the initiation of Th2 immunity in response to infection is still unclear. In the context of parasite-induced Th2 immunity, Cook et al. demonstrated that IL-4-induced alternative activation of DCs and the secretion of RELMα from alternative activated DC promotes Th2 responses *in vitro* and *in vivo*^28^. A recently published paper by the same group further shows that the DC-directed Th2 immunity in response to *Schistosoma mansoni* parasite and house dust mite allergen is epigenetically controlled by methyl-CpG-binding protein Mbd2^29^. Thus, the initiation of Th2 by DCs may depend on the context of infection or stimulation such as exposure of IL-4 or induction of Mbd2-dependent gene expression including *Jak2* and *Irf4*^29^.

The role of DCs in the development of Th1 immunity in response to intestinal parasite infection has also been studied. In the small intestinal infection with protozoan *Cryptosporidium parvum* (*C. parvum*), depletion of DCs significantly increased the
susceptibility of mice to the infection; reconstitution of DCs rescued the mortality with increased numbers of IFN-γ-producing CD4+ and CD8+ T cells in the spleen and MLN30. In our toxoplasma infection-induced ileitis model, the Th1 response is impaired by the delayed recruitment of DCs. Our finding is supported by a previous study using DC-depleted mice showing that DCs are indispensable for the development of Th1-dependent antigen specific CD8+ response against T. gondii, especially the type II strains31. Thus, DCs may be essential for the development of Th1 immunity in response to intestinal protozoan parasite infection.

The murine intestinal classical DCs are a mixed population composed of CD103+ and CD103- DC subsets which are both derived from committed DC precursors32, 33. The intestinal CD103+ DC subset has been shown to be an important regulator of gut immune homeostasis32. These cells are required for the maintenance of gut tolerance through promoting the differentiation and gut-homing of regulatory T cells (Tregs)34. However, in a murine T cell-transfer model of colitis, the CD103+ DC taken from the mesenteric lymph nodes (MLNs) of colitic mice failed to induce Treg differentiation and instead favoured the generation of interferon (IFN)-γ-producing CD4+ T cells35. This result indicates that the phenotype of CD103+ DCs can be changed from tolerogenic under steady-state conditions to immunogenic in response to environmental stimuli. The intestinal CD103+ DCs can be further divided into two subpopulations: CD103+CD11b+ and CD103+CD11b- DCs34. Both of them potentially initiate active immunity in response to pathogen-association molecular patterns. For example, it is reported that stimulation of toll-like receptor (TLR)5 in CD103+CD11b+ DCs with bacterial flagellin increases their ability to produce IL-23 and promotes the differentiation of IFN-γ- and IL-17-producing T cells36, 37. Similarly, stimulation of TLR7/8 increases the cross-presentation ability of CD103+CD11b- DCs to induce Th1 responses and cytotoxic T lymphocyte (CTL) activity38. In vivo studies using pathogens also reveal the importance of CD103+ DCs in the development of protective immunity in animals.
It is reported that notch-2-dependent CD103\(^+\)CD11b\(^+\) DCs are a critical source of IL-23 which is required for the survival of mice infected with \textit{C. rodentium}\textsuperscript{39}. In response to infection with the \textit{C. parvum}, both intestinal CD103\(^+\)CD11b\(^+\) and CD103\(^+\)CD11b\(^-\) DCs contribute to the production of IL-12 and IFN-\(\gamma\) for the development of protective immunity\textsuperscript{18}. A study using another protozoan \textit{T. gondii} shows that Baft3\(^-/-\) mice were highly susceptible to the acute infection because of a lacking of CD103\(^+\)CD11b\(^-\) DCs derived IL-12. Indeed, the importance of CD103\(^+\)CD11b\(^-\) DCs is also supported by our work showing that P2X7R\(^-/-\) mice, which had delayed recruitment of CD103\(^+\)CD11b\(^-\) DCs had reduced Th1 cytokines in response to \textit{T. gondii} infection. However, regardless of the reduction of Th1 immunity, P2X7R\(^-/-\) mice did not develop more severe gut pathology by day 8 post \textit{T. gondii} infection. The Th1 response is necessary for the conversion of tachyzoites to the stable bradyzoite chronic infection therefore a reduced Th1 response may impact on the development of a stable chronic infection. Thus, the greatest impact of the decreased CD103\(^+\)CD11b\(^-\) DCs may not be on acute infection but chronic infection.

As well as gut epithelial cells responding to bacteria and protozoan parasites, previously published papers by our group demonstrate a crucial role of epithelial NOD-2-dependent chemoattraction of CD103\(^+\) DCs in the initiation of T cell responses against the nematode parasite \textit{Trichuris muris} (\textit{T. muris})\textsuperscript{40}. Similarly, according to our data, the absence of P2X7R alters the early recruitment of intestinal CD103\(^+\)CD11b\(^+\) DCs, which may make P2X7R\(^-/-\) mice more susceptible to \textit{T. spiralis} infection. Notably, our previous work on \textit{T. muris} showed that the reduced CD103\(^+\) DC recruitment in NOD2\(^-/-\) mice decreases the activation and gut-homing of T cells but did not affect the Th2-associated IL-13 production at day 21 p.i..

Moreover, NOD2\(^-/-\) mice had delayed worm expulsion but were able to ultimately expel the worms. These findings, together with the observations using DC-depleted mice (earlier mentioned in section 5.4), suggest that CD103\(^+\) DCs are not essential for the development of Th2 immunity\textsuperscript{40}. However, in our \textit{T. spiralis} infection model, we measured the production
of antigen-specific IL-4 and IL-13 in splenocytes at day 8 p.i. which is an early time point for the development of the Th2 response, and we found a reduction of IL-4 followed by delayed onset of worm expulsion in P2X7R−/− animals. Our findings in T spiralis and T. muris infection suggest that the early recruitment of CD103+ DCs may promote an earlier initiation of protective responses in WT mice, compared with NOD2−/− and P2X7R−/− mice. However, our work suggests CD103+ DCs are not essential for fully development of Th2 immunity, which means, as well as CD103+ DCs, other DC subsets or innate immune cells may also contribute the development of Th2 immunity in response to infection. Indeed numerous publications have shown that innate cells such as basophils and ILC2s are involved in the development of Th2 response. It is reported that basophils stimulated by TSLP and IL-25 promote Th2 differentiation. ILC2s, primed by the type-2-inducing cytokines IL25 and IL33, produce large amounts of IL-5 and IL-13 - cytokines which are required for the initiation of Th2 immunity.

Many previous publications have revealed how important the intestinal CD103+ DCs are in the initiation of immunity, but very little is known about the function of intestinal CD103− DCs during infections. A recent report by Scott et al. shows that CCR2+CD103+CD11b+ DCs in vitro highly express IL-12/IL-23p40 in response to stimulation of TLR-4 and efficiently drive the differentiation of interferon-γ and interleukin-17-producing effector T cells. However, in our study, there was no difference in the frequency of CD103− DCs between WT and P2X7R−/− mice in response to T. gondii and T. spiralis infections, so we conclude that epithelial cell P2X7R is not involved in the recruitment of the CD103− DC population, at least in our ileitis models. Thus, due to the lack of other in vivo studies, the exact role of CD103− DCs in response to infection are still unclear.

Given that mucosal DC are not functionally hardwired but are influenced by conditioning
signals secreted from epithelial cells, constitutively or in response to commensal or pathogen signals\textsuperscript{45}, the interaction between IECs and DCs also determines the phenotypes of DCs in response to infection. For example, in infection with \textit{T. muris} and \textit{T. spiralis}, the worms stimulate IECs to secret thymic stromal lymphopoitin (TSLP) which conditions intestinal DCs toward a Th2 pathway\textsuperscript{46, 47}. In addition to TSLP, other IEC-derived factors for DC conditioning include transforming growth factor (TGF)-\(\beta\), IL-10, retinoic acid (RA) and prostaglandin E (PGE)\textsubscript{2}\textsuperscript{48, 49}. These factors control the development of DC-dependent gut homeostasis and gut-tropic immune responses\textsuperscript{48}. In our study, we did not look at whether DC conditioning was altered in P2X7R\textsuperscript{−/−} mice and it would be interesting to investigate the role of P2X7R in IEC-dependent DC conditioning and influences on the development of T cell immune responses.

In sum, our work highlights a role of CD103\textsuperscript{+} DCs in the initiation of immunity in response to the intestinal infection. However, we were only able to demonstrate that there was a decrease in the frequency of intestinal CD103\textsuperscript{+} DCs and the reduction in T cell responses in P2X7R\textsuperscript{−/−} mice in response to infection, and we did not investigate whether reconstituting the number of CD103\textsuperscript{+} DCs in P2X7R\textsuperscript{−/−} mice could rescue the reduced T cell immune response by examining later time-points.

5.4 How does P2X7R affect the chemoattraction of immune cells?

P2X7R has been shown to be associated with a variety of inflammatory disorders in humans and rodents by many groups\textsuperscript{50}, and some studies have investigated the role of P2X7R in the chemotaxis of immune cells. Kataoka \textit{et al.} demonstrated that P2X7R stimulation rapidly (less than one hour) induced the activation of nuclear factor of activated T-cells (NFAT) in mouse microglial cells and promoted the release of CCL3, which may contribute to the inflammation in the central nervous system (CNS)\textsuperscript{51}. In a murine model of epileptic seizure,
P2X7R signalling promoted CCL2 (released from microglial cells) and CXCL2 (released from astrocytes) in the induction of epileptic seizure, which mediated the infiltration of monocytes and neutrophils into the frontoparietal cortex. Aside from the inflammatory infiltration in the CNS, it has been shown that the ATP-induced neutrophil recruitment mediated via the CXCL2 produced by peritoneal exudate macrophages is P2X7R-dependent. Indeed, a recent study using a model of irritant contact dermatitis demonstrated that P2X7R deletion or blocking in vivo reduced neutrophil infiltration and in vitro prevented the increase of IL-1β in macrophages and DCs. According to these studies, it is obvious that the P2X7R in immune cells promotes chemoattraction of proinflammatory cells and, thus, possibly further exaggerates inflammatory disorders. In the experiments focusing on non-immune cells and tissues, knock-down or blocking P2X7R in the kidney efficiently attenuated lupus nephritis with significantly reduced infiltration of immune cells in mice. It was previously demonstrated that ATP acted primarily on P2X7R to enhance the secretion of CXCL1 from IECs in response to the stimulation of TLR1/2. This data implicates P2X7R activation as a secondary signal to enhance chemokine production in response to first signals such as TLR stimulation. All of these previous findings support the role of epithelial P2X7R as a chemotaxis promoter of immune cells during inflammation.

In our study, we found that P2X7R deficiency reduced CCL5 and CCL20 expression in IECs and possibly delayed the recruitment of CD103+ DCs, instead of macrophages. Because we looked at the very early time point of infection and infiltration of macrophages the hallmark of chronic inflammation, it is reasonable that we did not find increased macrophages in our infection models. However, CCL5 and CCL20 chemoattract a variety of immune cells apart from DCs and macrophages. It has been known that CCL5 targets immune cells with the corresponding receptors CCR1, CCR3 or CCR5, including lymphocytes, macrophages, DCs, monocytes and neutrophils; CCL20 is strongly chemotactic for lymphocytes and
DCs by targeting CCR6 receptor\textsuperscript{60, 61}. In our study, we did not look at the early infiltration of monocytes and neutrophils although they respond to CCL5 and are reported to be attracted via chemokine responses mediated by P2X7R stimulation\textsuperscript{52}. Since our data from \textit{T. spiralis} infection shows a marked infiltration of polymorphonuclear leukocytes in WT mice as compared with P2X7R\textsuperscript{−/−} mice by day 8 p.i., it is possible that P2X7R signalling also mediates the recruitment of monocytes and neutrophils. However, further study may be needed to clarify the possibility.

\textbf{5.5 How does CCL5 expression up-regulate in IECs in response to infection?}

CCL5 is a chemokine relevant to a number of inflammatory diseases and shown to be released from a variety of cell types including T cells, monocytes, endothelial cells and epithelial cells\textsuperscript{62}. The expression of CCL5 is associated with proinflammatory stimuli and infection with pathogens\textsuperscript{62}. It has been reported that stimulation with the proinflammatory cytokine TNF-\(\alpha\) and IL-1\(\beta\) induce the expression of CCL5 in human fibroblasts and keratinocytes within hours\textsuperscript{63, 64}. Infections are also a strong trigger of the CCL5 response, via activation of pattern recognition receptors such as TLRs and downstream cellular signalling including NF-\(\kappa\)B and mitogen-activated protein kinases (MAPK) pathways.\textsuperscript{65} TLR4 activation can induce CCL5 expression via transactivation of epidermal growth factor receptor (EGFR) and phosphoinositide 3-kinase (PI3K)/Akt pathway which subsequently activate NF-\(\kappa\)B in rat smooth muscle cells\textsuperscript{66}. In a murine \textit{Leishmania major} infection model, the TLR4-deficient mice failed to express CCL2, CCL3, CCL5 and CXCL10\textsuperscript{67}. In viral infection, the cooperation of IFN-regulatory factor (IRF) and NF-\(\kappa\)B pathways is necessary for the induction of CCL5\textsuperscript{68}. Work by Thomas \textit{et al.} demonstrated that the respiratory syncytial virus (RSV) infection induced CCL5 expression in bronchial epithelial cells in a NF-\(\kappa\)B-dependent manner\textsuperscript{69}. In addition, another later study showed that activation of mitogen-activated protein kinases (MAPK) was also involved in the induction of CCL5 in alveolar epithelial cells in response to RSV infection\textsuperscript{70}. Oral infection of \textit{T. gondii} is reported to
significantly increase the expression of CCL5 in IECs. *T. spiralis* infection is associated with up-regulated CCL5 mRNA expression in the jejunum. Collectively, these findings indicate that infections and TLR downstream signalling pathways including NF-κB and MAPK are involved in the induction of CCL5.

In our study, we found that only infection with type II *T. gondii* (PRU) *in vitro* induced CCL5 production in IECs but the type I RH strain did not. According to previous studies, type I *T. gondii* strains transiently block NF-κB nuclear translocation in murine macrophages and human fibroblasts but do not inhibit IKK activation and IκB degradation. In contrast, type II strains efficiently drive NF-κB activation and trigger strong NF-κB-associated proinflammatory responses including the production of TNF-α, IL-6, IL-1β, IL-12 and IL-18 in murine splenocytes and bone-marrow-derived macrophages. Thus, we blocked NF-κB signalling and as expected, the type II *T. gondii* strain induced increase of CCL5 was attenuated although not to the basal levels secreted by control cells. This result suggests that, as well as NF-κB, there may be another signalling pathway such as MAPK involved in the induction of CCL5 in response to *T. gondii* infection. However, we did not investigate whether the MAPK pathway contributes to the CCL5 response, so the role of MAPK in the *T. gondii*-induced increase of CCL5 in IECs is still unclear. In order to establish the missing link between MAPK and the induction of CCL5 in IECs, we could block the MAPK pathway using a p38 inhibitor such as SB203580. Furthermore, the combination of MAPK inhibition and NF-κB blocking may be needed to see if the CCL5 can be reduced to basal levels.

5.6 How does P2X7R modulate CCL5 induction in response to *T. gondii* infection?

So far, there are not any published studies directly showing how P2X7R signalling modulates the production of CCL5 chemokine, despite the fact that extracellular ATP is able to act on P2 receptors (mainly P2Y1R) to up-regulate IFN-γ-stimulated chemokine expression.
including CCL2, CCL5 and CXCL8 in human keratinocytes. Thus, this is the first study demonstrating a pivotal role of P2X7R in the modulation of CCL5 chemokine production in IECs in response to infection. However, our data did not reveal the detailed pathways in which P2X7R signalling promotes the CCL5 production. In order to clarify how P2X7R signalling is involved in the regulation of CCL5, we could try the following strategies: Given that TLR downstream MyD88-dependent signalling is indispensable for the induction of proinflammatory response and the resistance of *T. gondii* infection, and P2X7R deficiency alters the TLR-dependent CCL5 production, these results implicate a possibility that TLR downstream signalling is tuned by P2X7R. Thus, we could use MyD88 deficient (MyD88^-/-) IECs infected by *T. gondii* to see if CCL5 production will be reduced and determine which cellular pathway (NF-κB or MAPK) plays the most important role in the induction of CCL5. Extracellular ATP is able to activate NF-κB and MAPK via P2X7R, and the NF-κB and MAPK pathways are crucial for the induction of CCL5, so we may be able to analyse whether P2X7R deficiency or treatment using a P2X7R antagonist would result in reduced nuclear translocation of NF-κB and less p38/MAPK activation.

5.7 How does P2X7R antagonism cause the reduction in *T. gondii* infectivity?

The infectivity of *T. gondii* is highly strain-dependent. In line with the difference in the *in vivo* virulence of strains, the type I parasites have a significantly higher replication rate than the Type II and III strains, which is correlated with a detrimental effect on host innate defence and loss of early control of the infection. *In vitro* work by Morampudi *et al.* showed that the type I RH strain inhibited the expression of the antimicrobial peptide β-defensin 2 (HBD2) in human IECs at an early time-point of infection, whereas the type II and type III *T. gondii* stains induced HBD2. Consistently, the expression of HBD2 is mediated through the activation of NF-κB signalling which is also suppressed by the type I parasites. In addition, analysis of the expression of innate immune genes in human IECs...
following infection with the type I, II or II strains shows that the type I parasites do not induce early proinflammatory cytokine and chemokine responses including IL-1β, IL-6, IL-8 and CCL20 which are critical for the initiation of host immunity to control *T. gondii* infection and parasite replication. Thus, the infectivity of *T. gondii* is generally influenced by the host innate antimicrobial and proinflammatory responses. However, the production of proinflammatory cytokines does not always benefit the host but sometimes benefits the parasite. A previous study by Beaman et al. demonstrated that pretreatment of murine peritoneal macrophages with IL-6 enhanced the replication of *T. gondii* in a dose-dependent manner. Moreover, the IL-6 treatment even impaired the IFN-γ-mediated intracellular killing of *T. gondii*. This report suggests that the induction of proinflammatory cytokine IL-6 by *T. gondii* infection may promote the replication of the parasite.

In our study, we found that the P2X7R antagonism significantly reduced the percentage of *T. gondii*-infected CMT-93 cells by the first 24 hours post-infection (p.i.) although the infection rate would restore in the following days. Since the pretreatment of P2X7R antagonist to *T. gondii* showed no parasite toxicity of the antagonist and the decreased infection rate under P2X7R antagonism significantly restored in 24 hours, the antagonist-mediated decrease in the percentage of parasite-infected cells may be due to the reduction of parasite replication or prevention of the parasites from infecting the cells. There is a previous study demonstrating that IL-6 benefits *T. gondii* replication. According to our data, we did find a decrease in the production of IL-6 from *T. gondii*-infected CMT-93 cells in response to P2X7R antagonist treatment, so the reduced parasite infectivity may therefore result from this decreased IL-6. A rescue experiment whereby exogenous IL-6 is added to the CMT-93 cells (+/- P2X7R inhibition) may help to characterise the role of IL-6 in the infectivity of *T. gondii*. Although P2X7R antagonism may reduce *T. gondii* infectivity through inhibition of IL-6 production, there is still a possibility that P2X7R antagonism
inhibits *T. gondii* infection. The invasion of *T. gondii* into cells is a complex process of multiple steps consisting of parasite-cell contact, close attachment, gliding motility, and penetration\(^83\) and any defect of the invasion steps can affect the ability of the parasite to infect. However, there have not been any published studies demonstrating that P2X7R is involved in the invasion of *T. gondii* into cells. Interestingly though mutations in P2X7R are associated with increased susceptibility to *T. gondii* infection although current studies have focused on the role of P2X7R in promoting phagolysosome formation\(^84\). Future experiments may be needed to clarify whether P2X7R antagonism affects the crucial invasion steps of the parasite such as the major surface protein (SAG-1)-dependent parasite-cell binding\(^85\) and the early parasite invasion facilitated by host microtubule activity\(^86\). Regardless, the mechanism of how P2X7R antagonism affects the *T. gondii* infectivity is still unclear, this is still a novel finding that has not been published before.

**5.8 Does P2X7R signalling plays a protective role in intestinal infection?**

In our model of *T. gondii* infection, despite the fact that both WT and P2X7R\(^-/-\) mice developed similar inflammatory pathology in the gut after day 5 p.i., our data showed a pro-inflammatory role of P2X7R in the initiation of innate immune response and the development of T cell immunity. By contrast, a previous study by Miller *et al.* suggests P2X7R has a role as an immuno-regulator in response to *T. gondii* infection and the absence of P2X7R caused uncontrolled nitric oxide (NO) production and delayed IL-10 responses\(^87\). Given that both Miller and our work used the same Pfizer P2X7R\(^-/-\) mice and we infected the mice with type II strain parasites (Miller used ME49; we used PRU)\(^87\), the reason why we get conflicting results is unclear. A possible reason is the difference in the route of parasite infection: Miller infected mice with *T. gondii* by intraperitoneal injection (IP) and our mice were orally inoculated (PO) with the parasites. In fact, the disease outcomes of *T. gondii* via different routes of infection (IP versus PO) are different. In response to the
challenge of the same dose of the type II ME49 strain, more C57BL/6 mice survived after IP infection as compared with PO infection\textsuperscript{88}. The increased mortality by PO infection resulted from INF-\(\gamma\)-mediated necrotic ileitis in C57BL/6 mice\textsuperscript{89}. This finding suggests that the outcomes of the systemic immune response versus local immune response induced by type II strains in C57BL/6 mice are different. Given that P2X7R\textsuperscript{-/-} mice respond differently to IP and PO infection of type II \textit{T. gondii}, it may imply that P2X7R plays different role in systemic immune response and local immune response. In our study, the mortality of WT and P2X7R\textsuperscript{-/-} animals showed no difference by day 8 p.i which is still in the acute stage of infection, but it was demonstrated by Miller \textit{et al.} that P2X7R\textsuperscript{-/-} mice succumbed to \textit{T. gondii} infection earlier than C57BL/6 mice by day 18 p.i.\textsuperscript{87}, which corresponds to chronic infection. In addition, our data shows that P2X7R\textsuperscript{-/-} mice had a reduced IFN-\(\gamma\) response, which is known to lead to chronic \textit{T. gondii} infection\textsuperscript{90}. Thus, it would be interesting to look at later time-points to clarify whether P2X7R deficiency increases the susceptibility of animals to \textit{T. gondii} infection. Intriguingly, our WT mice, compared with P2X7R\textsuperscript{-/-} mice, had more inflammatory foci in their livers at day 8 p.i., regardless of their having a stronger Th1 immune response. DC can play a role as a carrier of \textit{T. gondii} tachyzoites for systemic dissemination\textsuperscript{91} and infection is associated with hypermobile DCs.\textsuperscript{92} In our data we saw a reduced early DC recruitment caused by P2X7R deficiency thus this may result in reduced early dissemination of the parasite and may explain why P2X7R\textsuperscript{-/-} animals had fewer inflammatory foci in the livers at day 8. Examination of parasite burden in the different organs would be interesting to assess. Furthermore, with luciferase tagged parasite strains available it is possible to track parasite dissemination over the time course of infection by multispectral analysis\textsuperscript{93} and this would be interesting to do also. Collectively, our data suggest that P2X7R is strongly associated with the development of inflammation and protective immunity but further study will be needed to determine the role of P2X7R in the disease outcome in response to \textit{T. gondii} infection.
In our model of *T. spiralis* infection, P2X7R deficiency resulted in reduced IEC responsiveness to the infection and delayed DC recruitment which consequently attenuated the intensity of adaptive Th2-associated immunity followed by delayed worm expulsion. Histologically, WT mice, compared with P2X7R<sup>−/−</sup> mice, developed more severe inflammatory pathology in the gut with marked infiltration of polymorphonuclear cells including neutrophils and some eosinophils in the lamina propria at day 8 p.i., which is consistent with the finding that the WT mice lost more body weight by day 8 p.i.. The intestinal inflammatory pathology in both WT and P2X7R<sup>−/−</sup> mice became pronounced with lymphomononuclear infiltration by day 12 p.i., but WT mice expelled significantly more parasites at the same time-point. Our data clearly demonstrate that P2X7R plays a proinflammatory and protective role in response to *T. spiralis* infection in our model. The proinflammatory role of P2X7R in our model is supported by a previous study by Keating *et al.*, showing that P2X7R deficiency resulted in an attenuation of the innate inflammatory response to *T. spiralis* infection<sup>16</sup>. However, they found that P2X7R<sup>−/−</sup> mice had early decreased IL-1β in the gut and serum at day 2 p.i.<sup>16</sup>, whereas there was no detectable IL-1β noted in our WT and P2X7R<sup>−/−</sup> mice during the infection. The difference in the secretion of IL-1β may be due to the different P2X7R knockout mice used for the experiments: Keating used P2X7R<sup>−/−</sup> Glaxo (Glaxo-Smith-Kline) and we used P2X7R<sup>−/−</sup> Pfizer (The Jackson Laboratory), and many studies have indicated the conflicting immunophenotypes of these two P2X7R<sup>−/−</sup> mice<sup>50</sup>. Regardless of the difference in the release of IL-1β, both Keating and our work suggest P2X7R as an initiator of proinflammatory responses and our data further demonstrate the importance of P2X7R in the development of protective T cell immunity against *T. spiralis* infection. Furthermore, the delayed worm expulsion noted in our P2X7R<sup>−/−</sup> mice suggest a delay in the initiation of protective immunity, which may be resulted from reduced gut inflammation caused by P2X7R deficiency.
5.9 Conclusions

In conclusion, the research described in this thesis has suggested that P2X7R is associated with the development of intestinal inflammation and the development of adaptive immunity in response to intestinal parasite infections. Our data demonstrate that infection of intestinal parasites activates P2X7R and increases intestinal epithelial responsiveness to the tissue injury caused by infection with *T. gondii* and *T. spiralis*. The activation of epithelial P2X7R subsequently promotes local proinflammatory responses including the expression of CCL5 chemokine in IECs at an early time-point of infection and drives the recruitment of DCs. In response to *T. gondii* infection, P2X7R−/− animals have delayed DC recruitment in the gut, attenuated systemic inflammation and reduced T cell immunity. Similar results are found in P2X7R−/− animals infected by *T. spiralis* as well. The *in vivo* data attribute an important role of P2X7R as the initiator of intestinal inflammation and immune cell infiltration, which promotes DCs and the development of Th1 or Th2 immunity and determines the susceptibility of the animals to the infection. We have also investigated the mechanism of how P2X7R signalling regulates the production of CCL5 using *in vitro* murine IEC models. Our data shows that the P2X7R signalling is involved in the modulation of TLR signalling to enhance cytokine and chemokine responses. We also demonstrate that the production of CCL5 in IECs in response to *T. gondii* infection is partially NF-κB-dependent but that an additional intracellular signalling pathway, for example, MAPK may be involved. However, the details of how P2X7R signalling may regulate the translocation of NF-κB subunits or phosphorylation of MAPK pathways have not yet been established. Finally, in addition to the P2X7R-associated CCL5 induction, we demonstrate a novel finding that P2X7R antagonism reduces *T. gondii* infectivity in IECs through an unknown mechanism.
5.10 Future work and significance

This thesis has addressed the importance of P2X7R in the increase of IEC responsiveness, the initiation of intestinal inflammation and the development of adaptive immunity to *T. gondii* and *T. spiralis* respectively. However, there are still some details that may need to be clarified and some questions that remain unanswered.

In our *in vivo* infection models, we have demonstrated that there was delayed recruitment of CD103+ DCs in P2X7R⁻/⁻ mice and these mice had reduced Th1 or Th2 immunity. However, we did not provide direct evidence revealing whether it is the recruited CD103+ DCs that contribute to the development of the adaptive T cell responses. In order to answer this question, an experiment could be done by adoptively transferring of CD103+ DCs to see if this replenishment of CD103+ DCs could rescue the phenotype of impaired T cell immunity in P2X7R⁻/⁻ mice. However, there are issues in transfer of DCs to ensure they home to the right area and do not become activated during preparation. Moreover, the recruitment of DCs may depend on IEC-derived CCL5, so it is possible that the transferred CD103+ DCs would fail to be attracted to the gut due to the defect of CCL5 expression in P2X7R⁻/⁻ IECs. These are the difficulties that may make the adoptive transfer of DCs problematic. Although it is hard to properly reconstitute CD103+ DCs in P2X7R⁻/⁻ mice, a short-term conditional depletion of CD103+ DCs in WT mice using a DT/CD103-DTR system at the early time point of infection to may be considered as another approach to investigate if the early recruited CD103+ DCs really contribute the initiation of T cell immunity. Alternatively, we could repeat the chimeras and investigate later timepoints.

We have shown that P2X7R deficiency results in the reduction of CCL5 expression in IECs, but we have not provided direct evidence that the recruitment of CD103+ DCs mainly depends on IEC-derived CCL5. In order to answer this question, we could use blocking
antibodies \textit{in vivo} as we have done previously\textsuperscript{17}. Another option would be to generate conditional knockout mice lacking IEC-derived CCL5 (Vil-cre-CCL5\textsuperscript{-/-}) by crossing Vil-cre transgenic mice with the mice containing a \textit{loxP} site-flanked \textit{Ccl5} (CCL5\textit{f/f}). Through using the Vil-cre-CCL5\textsuperscript{-/-} mice in our parasite infection models, we could understand more clearly about the role of CCL5 in the chemoattraction of CD103\textsuperscript{+} DCs. Although our bone marrow chimera experiments have demonstrated that WT P2X7R\textsuperscript{+/+} CD103\textsuperscript{+} DCs were not recruited to the P2X7R\textsuperscript{-/-} epithelium, we cannot exclude the contribution of other cells types in the gut. Thus, it would be interesting to conditionally knockout the \textit{p2rx7} gene in IECs to generate Vil-cre-P2X7R\textsuperscript{-/-} mice and then use these mice to study the influence of P2X7R signalling on the production of IEC-derived CCL5 and DC recruitment.

In the \textit{in vitro} infection model, we showed that P2X7R antagonist A-740003 reduced the infectivity of \textit{T. gondii} in CMT-93 cells through an unknown mechanism. Given that both parasite replication rate and parasite-cell binding determine the parasite infectivity, it is possible that P2X7R antagonism may reduce the parasite infectivity through interfering either with parasite replication or parasite attachment to host cells. In regards of a previous finding that IL-6 benefits \textit{T. gondii} replication\textsuperscript{82} and there is reduced IL-6 production caused by the P2X7R antagonist in our \textit{in vitro} model, we hypothesise that the IL-6 deprivation resulted from P2X7R antagonism inhibits the parasite infectivity. In order to answer this question, we could add exogenous IL-6 to the cells treated with or without the P2X7R antagonist and see if infection rate could be restored. Additionally knockdown of IL-6 in CMT-93 cells may be another approach to confirm the role of IL-6 in the replication of \textit{T. gondii}. On the other hand, several lectin-like proteins of \textit{T. gondii} including surface antigen (SAG)-1 and micronemal protein (MIC)-1, are critical for the binding of \textit{T. gondii} to host cells\textsuperscript{85, 94}, so there is a possibility that the P2X7R antagonist, A-740003, interferes in the interaction between the lectin-like proteins and the membrane-bound sugars of CMT-93.
cells. In order to clarify the interaction between A-740003 and the membrane-bound sugars, a competitive binding assay could be performed to see if A-740003 will compete the sugar-binding sites with the lectins and the lectin-like proteins of *T. gondii*. Another route would be to use mutant forms of *T. gondii* that lack the lectin proteins or have altered intracellular replication to see if A-740003 treatment can decrease parasite invasion.

According to our data, P2X7R signalling enhanced the proinflammatory cytokine and chemokine responses induced by stimuli such as *T. gondii* infection or TLR4 stimulation. However, it is still unknown exactly how the intracellular signalling pathway activated by these stimuli is modulated by P2X7R signalling. In the *ex vivo* model using the primary WT and P2X7R-/- crypt cells, we have demonstrated that P2X7R signalling promoted LPS-induced CCL5 production, which implicates a possible involvement of P2X7R signalling in the downstream signalling pathways of TLRs. Thus, we can stimulate different TLRs with TLR ligands and block P2X7R to see which TLR responses are enhanced by P2X7R signalling. Then we can investigate if the TLR downstream signalling pathways, such as NF-κB and MAPK, can be activated by ATP or suppressed by P2X7R antagonist. These additional experiments may help to understand the detailed mechanism of P2X7R signalling-dependent initiation of inflammation in response to pathogen infection.

The main aim of this thesis was to characterise the role of P2X7R in small intestinal inflammation. Although many previous studies have shown the importance of the activation of P2X7R on immune cells in the development of inflammation, our data indicate that in reaction to infection and tissue injury in the gut, the P2X7R of IECs perhaps plays an even more important role in the early initiation of inflammation by recruiting DCs to the infection site. Thus, our findings may expand the knowledge of how intestinal inflammation starts and how danger signals act as alarmins to alert immune cells. It would
be interesting to define alarmin production in the context of infection-induced injury in more detail. As well as the proinflammatory role of P2X7R in murine infection-induced intestinal inflammation\textsuperscript{95, 96}, a recent study has proposed the relationship between P2X7R activation and inflammatory bowel disease (IBD) in human patients\textsuperscript{97}. Thus, P2X7R is a potential target for pharmacological treatment of intestinal inflammatory disorders. Our findings suggesting P2X7R of IECs as the initiator of intestinal inflammation could have an important impact on the development of new medicines or strategies to prevent intestinal inflammation.

References


21. Fleeton MN, Contractor N, Leon F, Wetzel JD, Dermody TS, Kelsall BL. Peyer's patch


26. Smith KA, Harcus Y, Garbi N, Hammerling GJ, MacDonald AS, Maizels RM. Type 2 innate immunity in helminth infection is induced redundantly and acts autonomously following CD11c(+) cell depletion. *Infection and immunity* 2012; **80**(10): 3481-3489.


30. Bedi B, McNair NN, Mead JR. Dendritic cells play a role in host susceptibility to Cryptosporidium parvum infection. *Immunology letters* 2014; **158**(1-2): 42-51.


34. Ruane DT, Lavelle EC. The role of CD103(+) dendritic cells in the intestinal mucosal


47. Giacomini PR, Siracusa MC, Walsh KP, Grencis RK, Kubo M, Comeau MR et al. Thymic stromal lymphopoitetin-dependent basophils promote Th2 cytokine responses
following intestinal helminth infection. *J Immunol* 2012; 189(9): 4371-4378.


62. Krensky AM, Ahn YT. Mechanisms of disease: regulation of RANTES (CCL5) in renal


90. Sturge CR, Yarovinsky F. Complex immune cell interplay in the gamma interferon response during *Toxoplasma gondii* infection. *Infection and immunity* 2014; **82**(8): 3090-3097.


Chapter Six
Supplementary Materials and Methods
6.1 Animals and cell culture

6.1.1 Animals

Specific-pathogen-free (SPF) Pfizer P2X7R⁻/⁻ mice, bought from The Jackson Laboratory (The Jackson Lab, Bar Harbor, USA), were crossbred with SPF C57BL/6JNCrl mice (Charles River, Margate, UK) to generate heterozygous F1 (P2X7R⁻/⁺) mice. Then we further generated F2 mice from breeding P2X7R⁻/⁻ F1 parental mice. These F2 mice were genotyped and the 10 to 12 week-old male F2 C57BL/6 (WT) and P2X7R⁻/⁻ littermate controls were chosen for experimental use. All mice were maintained by the Biological Services Unit, University of Manchester, UK, and kept in individually ventilated cages and fed a standard chow. Experiments were performed in accordance with the Home Office Animals (Scientific Procedures) Act (1986). Three to six mice per group were used per study and each infection was repeated two to three times.

6.1.2 Cell culture

Both human foreskin fibroblasts and mouse colorectal carcinoma cell line, CMT-93 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich, Gillingham, UK) with 10% foetal bovine serum (FBS, Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin (PEST, Sigma-Aldrich) at 37°C supplied with 5% CO2. Human foreskin fibroblast cells were passaged weekly and used before the 10th passage. CMT-93 cells were passaged twice a week and used between passages 2 to 20.

6.2 Parasite specific techniques

6.2.1 Culture of Toxoplasma gondii in human foreskin fibroblasts

Transgenic Toxoplasma gondii (T. gondii) parasites engineered to express fluorescent reporter genes were provided by Dr Boris Striepen (University of Georgia, USA). Two strains of T. gondii were used: virulent type I T. gondii RH strain expressing yellow fluorescent
protein (YFP-RH)\(^1\) and avirulent type II Prugniaud (PRU) strain expressing ovalbumin (OVA) with tandem dimeric tomato red fluorescent protein (PRU-OVA-tdTomato)\(^2\). The tachyzoites were maintained by passage every 4-5 days in human foreskin fibroblasts cultured in DMEM (Sigma-Aldrich) with 10% FBS (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich) and 1% PEST (Sigma-Aldrich). The harvested tachyzoites were passed through nylon mesh (Fisher Scientific, Loughborough, UK) and suspended in phosphate-buffered saline (PBS) or culture media before being used for *in vivo* or *in vitro* infection respectively.

### 6.2.2 Maintenance and recovery of *Trichinella spiralis*

*Trichinella spiralis* (*T. spiralis*) was maintained in adult male C57BL/6JNCrl (Charles River, UK) as stock mice by oral inoculation. Larvae were recovered from stock mice by digestion of the skeletal muscle using pre-warmed phosphate buffered saline (PBS) containing pepsin (0.5%) and hydrochloric acid (0.5%) at 37°C. The recovered larvae were washed and condensed in PBS. After condensation, the larvae were suspended in 0.2% agar solution. 50µl suspension of larvae in agar was taken for counting in triplicate. Then the larvae were adjusted to the appropriate concentration using 0.2% agar solution for oral inoculation. For antigen preparation, larvae were suspended in PBS.

### 6.2.3 Preparation of *T. spiralis* antigen

The recovered muscle larvae were resuspended in 2 ml of ice-cold PBS and lysed using FastPrep™ Lysing Matrix D (MP Biomedicals, Santa Ana, USA). Then the larvae lysate were centrifuged at 13,000xg for 20 min at 4°C to collect the supernatant containing the soluble antigen. The supernatant was transferred for dialysis at 6 to 8°C overnight with 3 PBS changes. The next day, the prepared antigen solution was filtered through a 0.22µm filter (Millipore Corporation, MA, USA) and aliquoted and stored at -80°C until required. Protein
concentration was determined by a Nanodrop ND-1000 spectrophotometer (Labtech international, UK).

6.2.4 In vivo and in vitro Infections

In order to establish the in vivo T. gondii -induced ileitis, WT and P2X7R⁻/⁻ mice were orally infected with 1 x 10⁶ of T. gondii tachyzoites (PRU-OVA-tdTomato) in 0.1 ml PBS per mouse. To establish T. spiralis-induced ileitis model, WT and P2X7R⁻/⁻ mice were orally inoculated with 400 larvae per mouse. After infection with T. gondii or T. spiralis, daily body weight change of each mouse was monitored for 8 to 12 days. The T. gondii infected mice were sacrificed at day 0, 1, 5 and 8 p.i., while the T. spiralis-infected mice were sacrificed at day 0, 2, 8 and 12 p.i.. For in vitro T. gondii infection, PRU or RH tachyzoites were seeded onto confluent CMT-93 cells, treated with or without selective P2X7R antagonist, A-740003 (500µM, Sigma-Aldrich) in a ratio of 1:1 for 24 or 48 hr. After infection, the supernatant was collected by centrifugation at 1000xg for 10 minutes and stored at -20°C until analysis. As a negative control, media was harvested from CMT-93 cells at the same density, treated with or without A-740003, but in the absence of T. gondii infection.

6.2.5 Reagents used in in vitro cell line model

Selective P2X7R antagonist, A-740003 (500µM) was used to block P2X7R in CMT-93 cells at the same time as infection with T. gondii. The pan-caspase inhibitor Z-VAD (100µM, Sigma-Aldrich) was used to inhibit infection induced-apoptosis in CMT-93 cells. NF-κB inhibitor Bay117082 (10µM, Sigma-Aldrich) was used to block T. gondii infection (PRU)-induced NF-κB activation. To simulate pathogen-induced TLR activation, we used LPS (20µg/ml, Sigma-Aldrich) from Salmonella enterica serotype enteritidis to stimulate CMT-93 cells and primary crypt organoids for 6 hr. Purified ATP (5mM, Sigma-Aldrich) was used to induce P2X7R activation. All reagents were bought from Sigma-Aldrich.
6.3 Ex vivo analysis

6.3.1 Assessment of T. spiralis burden in the gut

The entire small intestine was harvested in warm PBS at day 8 and day 12 p.i.. Then the lumen of the small intestine was opened longitudinally and the whole intestinal tissue was wrapped in a piece of clean gauze and soaked in a 50-ml Falcon tube containing 30 ml of 37°C PBS. The tube with the intestinal tissue was incubated in a 37°C water bath for 4 hr. After incubation, the wrapped intestinal tissue was removed from the tube and PBS containing the worms was poured into a transparent petri-dish for parasite counting using an inverted microscope.

6.3.2 Mouse bone marrow-derived dendritic cells

Long bones (femur and tibia) were harvested from WT C57BL/6JNCrI and P2X7R<sup>−/−</sup> mice. The bone marrow cells were flushed out and collected in cold Hank's buffered salt solution (HBSS, Sigma-Aldrich). Red blood cells (RBC) were lysed using ammonium chloride/potassium (ACK) lysis buffer containing NH₄Cl (0.15M, Sigma-Aldrich), KHCO₃ (10mM, Sigma-Aldrich) and disodium ethylenediaminetetraacetate (EDTA, 0.1mM, Sigma-Aldrich). The cells were cultured in bone marrow-derived dendritic cell (BMDC) culture media containing RPMI-1640 (Sigma-Aldrich) with 10% FBS (Gibco, Life Technologies, Paisley, UK), 1% PEST (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), 50µM beta-mercaptoethanol (2-ME, Sigma-Aldrich) and 4% granulocyte-macrophage colony-stimulating factor (GM-CSF, 20ng/ml, Biolegend, London, UK). After 6 days of culture, the well-differentiated BMDCs were used for migration assays and OT-II cell stimulation.
6.3.3 Isolation of the small intestinal cells

Immediately following autopsy, small intestines were maintained in cold HBSS with 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, Sigma-Aldrich). All the Peyer’s patches (PP) were removed. Then, the PP-excised small intestine tissue was cut into smaller pieces and transferred into pre-warmed HBSS supplemented with 1% Hepes and 25mM EDTA for separation of the intestinal epithelial layer (IEL) which contained most of the villi and crypts\(^3\). The tissue pieces were incubated at 37°C with shaking for 15 min after which cells were collected by filtering through 70\(\mu\)m nylon mesh (Fisher Scientific). This incubation was repeated a further two times with the cells collected after each 15 min incubation and the resultant cells were pooled together. The cells collected using EDTA method were defined as IEL cells. Leukocytes from the pooled IEL cells were isolated using Percoll density gradient isolation (Scientific Laboratories Supplies, Yorkshire, UK). Briefly the cells were resuspended in 4 ml of 40% Percoll (Scientific Laboratories Supplies) and overlaid on 3 ml of 80% Percoll (Scientific Laboratories Supplies). After centrifugation at 1000xg for 25 min with no brake, the cells were collected and resuspended in R10 media containing RPMI-1640 (Sigma-Aldrich) with 10% FBS and 1% Hepes. The remaining tissue fragments from the original incubations were further digested using R10 media supplemented with collagenase VIII and CaCl\(_2\) at 37°C. After 60 minutes, the digested contents were filtered through a nylon mesh to collect the cells in the lamina propria layer (LPL cells). All the intestinal cells were resuspended in FACS buffer (PBS plus 2% FBS with 0.1% sodium azide) before antibody labelling for flow cytometry analysis.

6.3.4 Isolation and culture of the primary small intestinal crypt cells

Organoids were prepared using published protocols\(^4\). During autopsy, the proximal half of the small intestine was harvested. The fat of the intestinal tissue was removed and the intestine was opened longitudinally and flattened out. The mucus and villi in the lumen was
carefully scraped off and washed in ice-cold PBS. After washing, the small intestine was cut into 4mm fragments and washed in ice-cold PBS 3 to 5 times until the supernatant was almost clear. The intestinal tissue was transferred into crypt isolation buffer (2mM EDTA in PBS) under gentle agitation at 4°C for 30 min. After allowing the fragments to settle, the supernatant was removed and the remained tissue resuspended in ice-cold PBS and pipetted up-and-down to release crypts. The released crypts were resuspended in 20 ml of ice-cold PBS and passed through a 70µm nylon mesh. After centrifugation at 300xg for 3 min, the pellet was transferred into a 15-ml Falcon tube and resuspended in 10 ml of the basal culture media containing Advanced DMEM/F12 + GlutaMAX™ (Life Technologies). After two washes using 10 ml of the basal culture media, the cell number was calculated and the crypts were resuspended in Matrigel™ (Becton Dickinson (BD), Oxford, UK) at 300-500 crypt cells/50µl and cultured in Advanced DMEM/F12 + GlutaMAX™ (Life Technologies) supplemented with 10mM Hepes (Sigma-Aldrich), 1% PEST (Sigma-Aldrich), 1% N2 supplement (Life Technologies), 2% B27 supplement (Life Technologies), murine recombinant epidermal growth factor (50ng/ml), murine recombinant Noggin (100ng/ml) (PeproTech EC Ltd., London, UK) and Human recombinant R-spondin1 (1µg/ml) (R&D Systems, Abingdon, UK). Well-differentiated crypt organoids were passaged into 30 organoids/50µl Matrigel™/well and used for LPS stimulation.

6.3.5 Cytokine and chemokine analysis

Cytometric Bead Array

The levels of IL-4, IL-6, IL-10, IL-13, IL-12p70 IFN-γ, TNF-α and CCL2 in the serum from the infection experiments and the supernatants harvested from T. spiralis antigen-stimulated splenocytes were determined using the Beckton Dickinson Cytometric Bead Array (CBA) Mouse/Rat Soluble Protein Flex Set system (BD). The lyophilised cytokine standard of each cytokine and chemokine were pooled together to reconstitute a standard mix using assay
diluent equilibrated for 15 min at room temperature. A blank (assay diluent only) and a series of two-fold dilutions of the standard mix (from 1:2 to 1:256) were prepared. The Protein Flex Set Capture Bead mix and Protein Flex Set Detection Reagent mix were prepared by 1-in-55 dilution in the capture bead diluent or detection bead diluent respectively; the beads of each analyte were pooled allowing 0.3μl of each bead per well and reconstituted (16.5μl/well) in the total volume needed in capture bead or detection reagent diluent. 16.5μl of capture bead mix and 16.5μl of standard or sample was added to each well of 96-well plates and incubated for 1 hr at room temperature. Then 16.5μl of detection bead mix was added to each well, incubated for 1 hr, washed in 200μl of wash buffer and resuspended in 200μl of wash buffer/well. Samples were then analysed using MACSQuant Analyzer (Miltenyi Biotec Ltd., Bisley, UK) and FCAP Array software (BD).

**Enzyme-linked immunosorbent assay**

The levels of TNF-α, IL-1α/β, IL-6, IL-18 and CCL5 from the supernatant of in vitro *T. gondii* infection in intestinal carcinoma cells and ex vivo LPS stimulation in crypt organoids were analysed by sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well ELISA microplates (R&D) were coated with capture antibody (100μl/well) and left overnight at 4°C. The next day, the pre-coated microplates were washed 3 times using wash buffer containing 0.05% Tween-20 in PBS. All subsequent steps were performed at room temperature. Non-specific binding was blocked using 200μl/well of blocking solution (3% BSA in PBS) for 3 hr. The standard was prepared by two-fold serial dilutions using assay diluent (1% BSA in PBS) according to the manufacturer’s instructions. After blocking, 100μl/well of standard/sample were added to the plates and incubated for 2 hr. After washing, 100μl of detection antibody, diluted in assay diluent to the working concentration, was added to each well and incubated for 2 hr. After washing, plates were incubated with 100μl/well of Streptavidin-HRP diluted in assay diluent, was incubated for 30 min in the dark. After
washing, plates were incubated with 100µl/well of TMB substrate solution (R&D) in the dark for 15 to 20 min. Finally, the substrate reaction was stopped using 50µl/well of stop solution (0.16M sulphuric acid) and the optical density of each well was determined using a Dynex MRX II microplate reader (Dynex Technologies, VA, USA) set to 450nm.

6.4 Histology

6.4.1 Tissue processing

On autopsy, ileum snips (approximately 3mm in length) were fixed in NBF (4% neutral buffered formalin (Sigma-Aldrich) in PBS) for 24 hr. The formalin-fixed samples were placed in histology cassettes and transferred into 70% ethanol for processing using a Microm STP 120 Tissue Processor (Mircom International, Germany). Briefly, tissues were placed in 70% ethanol for 15 min, 90% ethanol for 30 min, 95% ethanol for 30 min, twice in 100% ethanol for 60 min, 100% ethanol for 20 min, xylene for 15 min, twice in xylene for 30 min (all at 40°C), and twice in frigowax pastillated wax (BD) for 1 hr at 60°C. The processed samples were then mounted in wax blocks using a Micro EC-350 embedding system (Microm, Germany) and 5µm serial-sections were cut using a Mircom HM325 microtome (Mircom, Germany). The sections were floated on warm water and transferred to glass slides and dried for further staining.

6.4.2 Haematoxylin and eosin staining

The formalin-fixed tissue sections were dewaxed in Citroclear (HD supplies, Bucks, UK) for 15 min and rehydrated through absolute alcohol to 70% alcohol (1 min in each concentration of alcohol). The sections were briefly washed in distilled water and stained in Harris Hematoxylin solution (Sigma-Aldrich) for 4 min. After staining, the sections were washed in running tap water for 1 min followed by differentiation in 1% acid alcohol for 10 seconds. The sections went through washing and bluing in running tap water for 5 min.
before eosin staining. After staining using eosin solution (Sigma-Aldrich) for 30 seconds, the sections were washed in tap water for 1 min. Dehydration was performed whereby the sections were taken through 70% alcohol to absolute alcohol. Before mounting the sections were immersed in Citroclear (HD supplies) for 30 seconds. The sections were finally mounted with DPX mounting media (Thermo Fischer Scientific, Hemel, UK) and sealed. Slides were viewed using Nikon Eclipse E400 microscope (Nikon, Japan) equipped with lenses at x20 and x40 magnification. The average of villus heights (µm) was determined by measuring the length of 10 to 15 villi from three different fields of view (20X) per specimen using NIH Image software (ImageJ, version 1.45s). All slides were analysed in a blinded fashion.

6.5 Immunohistochemistry

6.5.1 Tissue processing

The ileum samples were snap frozen in optimal cutting compound (OCT, Raymond A Lamb, Eastbourne, UK) and stored at -80°C at least 24 hr. The OCT embedded tissue was cut into sections at 5 µm thickness using a Leica CM-1100 cryostat (Leica Biosystems, Linford Wood, UK) placed onto charged slides (Thermo Scientific West Sussex, UK) and stored at -80°C until assayed.

6.5.2 TUNEL staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (In situ cell death detection kit, Roche Diagnostics GmbH, Germany) was used for assessing in situ intestinal cell death in response to infection. The cryo-preserved tissue-section slides were fixed using 4% paraformaldehyde solution for 15 min followed by wash in PBS for 30 min. The sections went through 2 min of permeabilisation using 0.1% Triton X-100 (Sigma-Aldrich) with 0.1% sodium citrate in ice-cold PBS. 50µl of TUNEL reaction mixture was added
and incubated with the sections for 60 min at 37°C in the dark. After 3 washes in PBS, the slides were analysed under an Olympus BX51 fluorescence microscope equipped with lenses at x20 and x40 magnification (Olympus, Japan). Dead cells positive for TUNEL signal were counted in a blind and randomised order. Three fields/view were counted per section.

6.5.3 Staining of CD11c⁺CD103⁺ dendritic cells

Cryo-preserved tissue sections on slides were fixed using 4% paraformaldehyde solution for 15 min followed by washing in PBS for 5 min. The tissue sections were blocked using tryamide blocking reagent (PerkinElmer, Cambridge, UK) at room temperature for 30 min, washed twice in PBS, blocked again using avidin/biotin blocking kit (Invitrogen) at room temperature for 30 min, washed twice in PBS, followed by incubation with CD103 (diluted, 10µg/ml, BD) and CD11c-biotin antibodies (diluted, 2.5µg/ml, eBioscience, Hatfield, UK) overnight at 4°C. After washing thoroughly in PBS with 0.05% BSA, the sections were stained with Alexa Fluor 488-conjugated goat anti-rat antibody (diluted, 10µg/ml, Invitrogen), streptavidin–horseradish peroxidase (HRP, diluted, 1.5µg/ml, Invitrogen) for 1 hr at room temperature in the dark. After washing thoroughly, the sections were incubated with cyanine3 Tyramide Amplification System (PerkinElmer) for 5 min at room temperature in the dark. After a final wash, the tissue sections were mounted using a mounting media containing DAPI (Vectashield, Vector Laboratories, Peterborough, UK) and then sealed until viewing. Slides were analysed under an Olympus BX51 fluorescence microscope equipped with lenses at x20 and x40 magnification (Olympus, Japan). Cells were counted per field of view in a blind and randomised order. Three fields/view were counted per section. Control sections were prepared without CD103 and CD11c-biotin antibodies but secondary antibodies instead.
6.6 Flow cytometry

6.6.1 FACS analysis for small intestinal cells

Small intestinal cells isolated from the intestinal epithelial layer (IEL) and lamina propria layer (LPL) were counted using an automated cell counter (Casy® 1 Schärfe, Germany), and were resuspended in the appropriate volume of FACS buffer (5% FBS in PBS). The cells were then incubated with 50 µl of Fc receptor blocker, anti-CD16/32 antibody (diluted, 2.5 µg/ml, eBioscience) for 20 min on ice. After washing in FACS buffer, the cells were stained with a mixture of fluorescence-labelled antibodies including FITC-anti-CD45 (diluted, 2.5 µg/ml, eBioscience), Pacific Blue-anti-MHC-II (diluted, 2.5 µg/ml, BioLegend), APC-anti-F4/80 (diluted, 1 µg/ml, eBioscience), Alexa Fluor 700-anti-CD11c (diluted, 1 µg/ml, eBioscience), APC-Cy7-anti-11b (diluted, 1 µg/ml, BD) and PerCP/Cy5.5-anti-CD103 (diluted, 2 µg/ml, BioLegend) antibodies for 30 min on ice. The stained cells were acquired on an LSR II flow cytometer (BD) using FACSDiva software (BD). Data analysis was performed using FlowJo software (Tree Star inc., Oregon, US). Negative controls were prepared using splenocytes only without antibody staining. Splenocytes were collected and stained by individual fluorescence-labelled antibody as positive controls for gate setting (Figure A.1, appendix): FITC-anti-CD45 (diluted, 2.5 µg/ml, eBioscience), Pacific Blue-anti-MHCII (diluted, 2.5 µg/ml, BioLegend), APC-anti-MHC-II (diluted, 2.5 µg/ml, eBioscience), Alexa Fluor 700-anti-MHC-II (diluted, 2.5 µg/ml, BioLegend), APC-Cy7-anti-11b (diluted, 1 µg/ml, BD) and PerCP/Cy5.5-anti-CD4 (diluted, 2.0 µg/ml, BioLegend).

6.6.2 FACS analysis for intracellular cytokine expression

Splenocytes or MLN cells harvested at day 8 p.i. were suspended in lymphocyte culture medium (RPMI-1640 (Sigma-Aldrich) plus 10% FBS (Gibco, Life Technologies), 1% PEST, 1% L-glutamine, 50 µM 2-ME) and stimulated with phorbol 12-myristate 13-acetate (PMA; 5 ng/ml, Sigma-Aldrich) and ionomycin (500 ng/ml, Sigma-Aldrich) for 1 hr followed by 3 hr
of incubation with Brefeldin A (BFA; 1µg/ml, BD). After stimulation, cells were washed twice using FACS buffer followed by Fc receptor blocking with anti-CD16/32 antibody (eBioscience) for 20 min on ice. Surface staining was carried out using FITC-anti-CD3 (BD) and PerCP-anti-CD4 (BD) antibodies for 30 min on ice in the dark. After two washes using FACS buffer, cells were fixed and permeabilised using FOXP3 Fix/Perm buffer set (BioLegend). Briefly, cells were fixed using the Biolegend’s FOXP3 fixing buffer for 20 min at room temperature followed by wash using the BioLegend’s FOXP3 permeabilisation buffer and permeabilised by incubation in the permeabilisation buffer for 15 min. Then intracellular staining was done by staining with PE/Cy7-anti-CD25 (diluted, 2µg/ml), Alexa Fluor 647-anti-FOXP3 (diluted, 4µg/ml), BV-421-anti-IFN-γ (diluted, 4µg/ml), PE-IL-4 (diluted, 4µg/ml) and BV-605 anti-IL-17 (diluted, 4µg/ml) antibodies (All from BioLegend). The stained cells were acquired on an LSR II flow cytometer (BD) using FACSDiva software (BD). Data analysis was performed using FlowJo software (Tree Star Inc., Oregon, US). Negative controls were prepared using cells only without surface and intracellular staining.

6.7 Molecular Biology

6.7.1 Isolation of small intestinal epithelial cells for RNA extraction

Small intestines were harvested in cold HBSS with 1% Hepes followed by removal of all PPs. Then, the PP-excised small intestine tissue was cut into smaller pieces and transferred into pre-warmed HBSS supplemented with 1% Hepes and 25mM EDTA. The tissue pieces were incubated at 37°C with shaking for 15 min after which cells were collected by filtering through 70µm nylon mesh (Fisher Scientific). This incubation was repeated a further two times with the cells collected after each 15 min incubation and the resultant cells were pooled together. The cell pellets were then collected by centrifugation (at 300g for 3 min) and washed twice using ice-cold PBS, followed by centrifugation at 400xg for 5 min to remove the supernatant and resuspended in 1ml TRIsure (Bioline, London, UK) ready for
RNA extraction. Epithelial purity was confirmed by flow cytometry using CD326 (Ep-CAM, Cambridge Bioscience, Cambridge, UK) and CD45-FITC (eBioscience) and preparations were > 95% pure.

6.7.2 RNA extraction and Preparation

Total RNA was isolated from cells by homogenising using TRIsure. The homogenised samples were placed into a fresh tube and added two 0.2ml of chloroform (Sigma-Aldrich), vigorously shaken for at least 15 sec and incubated at room temperature for 3 min. The samples were then centrifuged for phase separation at 12,000xg for 15min at 4°C. The aqueous phase with RNA in it was collected and mixed with 0.5ml of isopropanol (Sigma-Aldrich) and incubated for 10 min at room temperature for RNA precipitation. Then RNA was centrifuged at 12,000xg for 10 min at 4°C and the supernatant removed. RNA pellets were washed once using 1ml of ice-cold 75% ethanol followed by centrifugation at 7500g for 5 min at 4°C. Ethanol was carefully removed, the pellets air-dried and resuspended in 25μl of nuclease free water (Promega, Southampton, UK). The RNA concentration was determined using a nanodrop-1000 spectrophotometer (Labtech international).

Extracted RNA was suspended in nuclease free water at a concentration of 1μg/μl. As 2μg of total RNA was needed for cDNA conversion, 2μl of RNA was used and added in 8μl of DNase-I working solution (DNase-I + 10x DNase reaction buffer + diethylpyrocarbonate (DEPC)-treated H₂O, Sigma-Aldrich) for removal of contaminated genomic DNA. The RNA/DNase-I suspension was incubated at 37°C, for 30 min. Then DNase-I reaction was inactivated by 1μl of EDTA stop solution (Sigma-Aldrich) and incubated for 10 min at 65°C.
6.7.3 cDNA conversion

Bioscript Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase kit (Bioline, London, UK) was used for cDNA synthesis. Briefly, the genomic DNA-free RNA suspension (described in Section 6.7.2) was added with an oligo-dT primer (0.5µg/µl, Promega), deoxynucleotides (dNTPs; 10mM, Bioline) and RNase inhibitor (Promega). Then the RNA suspension was incubated for 5-min at 70°C, and cooled on ice for at least 1 min. After the ice-cold incubation, a reverse transcription mixture prepared from the Bioscript kit (Bioline), containing reverse transcription buffer, M-MLV reverse transcriptase, RNase inhibitor and DEPC-treated H₂O, were added to the RNA suspension. Then the reverse transcription was carried out by incubating with M-MLV reverse transcriptase at 37°C for 1 hr. The reaction was stopped by incubating at 85°C for 5 min. cDNA was prepared and frozen in -20°C freezer until needed.

6.7.4 Quantitative PCR

Quantitative PCR (qPCR) was performed using the SYBR green I core kit (Eurogentec, Southampton, UK) with an Opticon quantitative PCR thermal cycler (Bio-Rad, Hemel Hempstead, UK). Each sample was serially two-fold diluted, and expression ratios were normalized to the mean of the reference gene (YWAHZ). Primer sequences used are described in Table 6.1.
### Table 6.1: Primer sequences for qPCR analysis of small intestinal epithelial cells (shown from 5’ to 3’).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>YWAHZ</td>
<td>TTCTTGATCCCCAATGCTTC</td>
<td>TTCTTGTCATCACCAGCAGC</td>
</tr>
<tr>
<td>CCL5</td>
<td>GGGTACCATGAAGATCTCTGCA</td>
<td>TTGGCGGTTCCTCGAGTGA</td>
</tr>
<tr>
<td>CCL20</td>
<td>AATGGCCTGCGGTGCA</td>
<td>CATCGGCCATCTGTCTTGTGA</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGTAATGAAAGACGGCACACC</td>
<td>TCTTCTTGGATGTATTGCTTG</td>
</tr>
</tbody>
</table>

### 6.7.5 Genotyping of wildtype and P2X7R⁻/⁻ mice

The ear tissue collected from wildtype (WT) or P2X7R⁻/⁻ (KO) littermate mice was digested in 350µl digestion buffer containing 100mM Tris-HCl, 5mM EDTA, 200mM NaCl and 0.2% sodium dodecyl sulphate (Sigma-Aldrich) with 15µl of proteinase K (10mg/ml, Sigma-Aldrich). After digestion at 54°C for 30 min on a shaker, the suspension was centrifuged at 10,000xg for 10 min and the supernatant was added to 300µl of isopropanol and centrifuged at 10,000xg for 5 min. The resultant pellet was resuspended in 500µl 70% ethanol and centrifuged at 10,000xg for 1 min. After air-drying for 10 min at 54°C the pellet was resuspended in 25µl of nuclear free water and stored at 4°C.

The targeted sequences on DNA templates were amplified by polymerase chain reaction (PCR). The PCR mixture, in a volume of 50µl, consisted of 2µl of DNA templates, 5µl of 2.5mM dNTPs (Bioline), 5µl of 25mM MgCl₂ (Promega), 10µl of 5X GoTaq® PCR buffer (Promega), 0.5µl of WT and knockout (KO) forward primers (10μM), 0.5µl of WT and KO reverse primers (10μM), 0.25µl of GoTaq® DNA polymerase (Promega) and 26µl of nuclear free water (Promega). PCR was performed as follows: 95°C for 2 min, 95°C for 30 seconds, 59°C for 45 seconds, 72°C for 1 min, and then 35 cycles at 95°C for 30 seconds, 59°C for 45...
seconds, and 72°C for 1 min, ended up with a final extension 72°C for 10 min. The PCR product of the WT gene is 363 base pairs (bp), the P2X7R KO one is 286 bp and the heterozygous one 363 and 286 bp (Figure 6.1). The primers used for defining P2X7R−/− (KO) and WT genes are listed in Table 6.2.

**Table 6.2: Primer sequences for genotyping** (shown from 5’ to 3’).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>TGGACTTCTCCGACCTGTCT</td>
<td>TGGCATAGCACCTGTAAGCA</td>
</tr>
<tr>
<td>KO</td>
<td>CTTGGGTGGAGAGGCTATTC</td>
<td>AGGTGAGATGACAGGAGATC</td>
</tr>
</tbody>
</table>

*Figure 6.1. Genotyping for WT and P2X7R−/− mice.*

The ear tissue samples used for PCR reaction were collected from littermate mice of three genotypes: C57BL/6 (WT), P2X7R−/− (KO) and heterozygote (H). The PCR product of WT is 363 bp; the KO is 280 bp; the heterozygote have both 280 bp and 363 bp products.

### 6.8. Generation of bone-marrow chimeras

Recipient mice were placed into rodent irradiation cage and irradiated at 2x5Gy doses for 4 hr. Donor mice were sacrificed and both legs harvested at the hip joint, muscle removed and the bones transferred in ice-cold HBSS (Sigma-Aldrich). In a sterile laminar-flow hood, the tibia was removed from the femur and the bone marrow was flushed out using HBSS. In order to create a single cell suspension, the bone marrow clumps were dispersed by passing back through a needle. Bone marrow cells were then counted and re-suspended in sterile PBS to a concentration of 27 million cells/ml. After irradiation, the recipient mice were placed into a heated cage until they had recovered and were more active. Mice were
inoculated intravenously via the tail vein with 5 million donor cells in a volume of 200μl. Recipient mice were given mash and jelly to eat with antibiotic water containing 2mg/ml of neomycin sulphate (Sigma-Aldrich) and used for experiments after 6 weeks of bone marrow reconstitution.

In order to check if the bone marrow reconstitution was successful, chimera mice were tail-bled for collecting peripheral blood and DNA was harvested. The collected blood was centrifuged to separate the serum and peripheral blood cells. Red blood cells were removed by incubating in ACK lysis buffer at room temperature for 5 min. The resultant peripheral leukocytes were incubated in 1 ml of TRIsure (Bioline) at room temperature for 5 min, added to 0.2 ml of chloroform (Sigma-Aldrich) and centrifuged at 12,000xg for 15 min for phase separation. The aqueous phase was discarded and the remained DNA/protein mixture was added with 0.3 ml of ethanol, incubated at room temperature for 2 to 3 min, centrifuged at 12,000xg for 5 min. The phenol/ethanol supernatant was discarded and DNA pellets were collected, washed twice using 1 ml of 0.1M sodium citrate, incubated at room temperature for 30 min. After incubation, the pellets were spun down at 10,000xg for 5 min, resuspended in 75% ethanol, incubated at room temperature for 10 min, centrifuged at 12,000xg for 5 min. Then the DNA pellets were collected by removing the supernatant, air-dried and resuspended in 15μl of nuclear free water for genotyping following the PCR procedure mentioned before.

An additional method was used to confirm the bone marrow reconstitution after experiments. Splenocytes were harvested on autopsy of the chimeric mice. One million splenocytes per mouse were co-cultured with BzATP (100μM) for 24 hr in 300μl of lymphocyte cell culture media containing RPMI-1640 (Sigma-Aldrich) plus 10% FBS (Gibco,
Life Technologies), 1% PEST, 1% L-glutamine and 50µM 2-ME. The supernatant was then collected for analysis of IL-6 levels by ELISA (Section 6.3.5)

References


Appendix
<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Receptor</th>
<th>Function</th>
</tr>
</thead>
</table>
| CCL2      | CCR2/CCR4        | 1. Induction by *T. gondii* infection.  
                        | 2. Attracting Gr1+ inflammatory monocytes.  
| CCL3      | CCR5/CCR1        | 1. Induction by *T. gondii* infection.  
                        | 2. Gut migration of CD8 T cells in response to *T. gondii* infection.  
| CCL4      | CCR5/CCR1        | 1. Induction by *T. gondii* infection.  
                        | 2. Gut migration of CD8 T cells in response to *T. gondii* infection.  |
| CCL5      | CCR5/CCR4/CCR1   | 1. Induction by *T. gondii* infection.  
                        | 2. Gut migration of CD8 T cells in response to *T. gondii* infection.  
| CCL7      | CCR7/CCR2/CCR1   | 1. Induction by *T. gondii* infection.  
                        | 2. Localisation of CD8α+ DCs in the IFR of Peyer’s patches.             |
| CCL8      | CCR5/CCR1        | Induction by *T. gondii* infection.                                                          |
| CCL9      | CCR1             | 1. Induction by *T. gondii* infection.  
                        | 2. Recruitment of CD11b+ DCs to the SED of Peyer’s patches.             |
| CCL11     | CCR3/CCR2/CCR5   | Gastrointestinal eosinophilia in response to *T. spiralis* and *T. muris* infection.        |
| CCL15     | CCR3/CCR1        | Induction by *T. gondii* infection.                                                          |
| CCL19     | CCR7             | MLN migration of DCs                                                                         |
| CCL20     | CCR6             | 1. Recruitment of CD11b+ DCs to the SED of Peyer’s patches.                                  
                        | 2. Gut recruitment of DCs/monocytes.  
                        | 3. Mucosa recruitment of IgA+ B cells.                                   |
| CCL21     | CCR7             | MLN migration of DCs                                                                         |
| CCL24     | CCR3             | 1. Induction by *T. gondii* infection.  
<pre><code>                    | 2. Gastrointestinal eosinophilia in response to *T. spiralis* and *T. muris* infection. |
</code></pre>
<p>| CCL25     | CCR9             | Gut recruitment of CCR9+ pDCs.                                                               |</p>
<table>
<thead>
<tr>
<th></th>
<th>CCL28</th>
<th>CCR10/CCR3</th>
<th>Mucosa recruitment of IgA+ B cells.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXC</td>
<td>CXCL1</td>
<td>CXCR2</td>
<td>Secreted from IECs in response to the stimulation of TLR1/2.</td>
</tr>
<tr>
<td></td>
<td>CXCL2</td>
<td>CXCR2</td>
<td>Induction by <em>T. gondii</em> infection.</td>
</tr>
<tr>
<td></td>
<td>CXCL10</td>
<td>CXCR3</td>
<td>Induction by <em>T. gondii</em> infection.</td>
</tr>
<tr>
<td></td>
<td>CXCL12</td>
<td>CXCR4</td>
<td>Gut recruitment of DCs/monocytes.</td>
</tr>
<tr>
<td>CX3C</td>
<td>CX3CL1</td>
<td>CX3CR1</td>
<td>Secreted by IECs to control the lumen antigen sampling of CX3CR1+ macrophages.</td>
</tr>
</tbody>
</table>
Figure A.1. Gate setting of FACS analysis for small intestinal cells.
Splenocytes collected from C57BL/6 and P2X7R<sup>-/-</sup> mice were resuspended in appropriate volume of FACS buffer and stained by fluorescence-conjugated antibodies for FACS gate setting. Representative histograms are shown. Unstained cells were used as negative control (blue curve) and antibody-labelled cells were used as positive controls (red curves). Cells were stained by (a) FITC-anti-CD45 (positive control for CD45-FITC), (b) pacific blue (PB)-anti-MHC-II (positive control for MHC-II-PB), (c) APC-anti-MHC-II (positive control for F4/80-APC), (d) Alexa Fluor-700 (AF-700)-anti-MHC-II (positive control for CD11c-AF-700), (e) APC-Cy7-anti-CD11b (positive control for CD11b-APC-Cy7) and (f) PerCP/Cy5.5-anti-CD4 (positive control for CD103-PerCP/Cy5.5).
Figure A.2. Representative plots of small intestinal DC and macrophages population in IEL in response to *T. gondii* infection.

C57BL/6 and P2X7R<sup>-/-</sup> mice were orally infected with *T. gondii* (1x10<sup>6</sup> tachyzoites/mouse) and sacrificed at day 0, 1, and 5 p.i.. Small intestinal cells in intestinal epithelial layer were collected and analysed by FACS. (a) CD45<sup>+</sup>MHC-II<sup>+</sup>F4/80<sup>-</sup>CD11c<sup>+</sup> cells were gated and further identified as CD103<sup>+</sup>CD11b<sup>-</sup> DC, CD103<sup>+</sup>CD11b<sup>+</sup> DC, CD103<sup>-</sup>CD11b<sup>+</sup> DC. (b) CD45<sup>+</sup>MHC-II<sup>+</sup>F4/80<sup>+</sup> cells were identified, from which CD11c<sup>-</sup>(CD11b<sup>+</sup>) macrophages were gated. The frequencies (%) of the gated populations were calculated as the percentages of CD45<sup>+</sup>MHC-II<sup>+</sup> cells.
Figure A.3. Representative plots of small intestinal DC and macrophages population in LPL in response to *T. gondii* infection.

C57BL/6 and P2X7R−/− mice were orally infected with *T. gondii* (1x10⁶ tachyzoites/mouse) and sacrificed at day 0, 1, and 5 p.i.. Small intestinal cells in lamina propria were collected and analysed by FACS. (a) CD45⁺MHC-II⁺F4/80⁺CD11c⁺ cell were gated and further identified as CD103⁺CD11b⁻ DC, CD103⁺CD11b⁺ DC, CD103⁻CD11b⁺ DC. (b) CD45⁺MHC-II⁺F4/80⁺ cells were gated, from which CD11c⁺(CD11b⁺) macrophages were identified. The frequencies (%) of the gated populations were calculated as the percentages of CD45⁺MHC-II⁺ cells.
Figure A.4. Representative FACS plots showing P2X7R deficiency impairs Th1 response to *T. gondii* infection.

Splenocytes from C57BL/6 and P2X7R^{-/-} mice at day 8 p.i. with *T. gondii* were analysed by FACS for the intracellular cytokine expression and the proportion of CD4^{+}CD25^{+}Foxp3^{+} regulatory T cells (Tregs). (a) Gating strategy for distinction of CD3^{+}CD4^{+} T cells. (b) Representative plots of the expression of IFN-γ, IL-4, IL-17 and the percentage of Tregs. % shown as the percentage of CD4^{+} T cells.
Figure A.5. Representative plots of small intestinal DC and macrophages population in IEL in response to *T. spiralis* infection.

C57BL/6 and P2X7R−/− mice were orally infected with *T. spiralis* (400 larva/mouse) and sacrificed at day 0, 2, and 8 p.i.. Small intestinal cells in intestinal epithelial layer were collected and analysed by FACS. (a) CD45+MHC-II+F4/80−CD11c+ cell were gated and further identified as CD103+CD11b− DC, CD103+CD11b+ DC, CD103−CD11b+ DC. (b) CD45+MHC-II+F4/80+ cells were identified, from which CD11c+(CD11b+) macrophages were gated. The frequencies (%) of the gated populations were calculated as the percentages of CD45+MHC-II+ cells.
Figure A.6. Representative plots of small intestinal DC and macrophages population in LPL in response to *T. spiralis* infection.

C57BL/6 and P2X7R−/− mice were orally infected with *T. spiralis* (400 larva/mouse) and sacrificed at day 0, 2, and 8 p.i.. Small intestinal cells in lamina propria were collected and analysed by FACS. (a) CD45⁺MHCII⁺F4/80⁻CD11c⁺ cell were gated and further identified as CD103⁺CD11b⁻ DC, CD103⁺CD11b⁺ DC, CD103⁻CD11b⁺ DC. (b) CD45⁺MHCII⁺F4/80⁺ cells were gated, from which CD11c⁺(CD11b⁺) macrophages were identified. The frequencies (%) of the gated populations were calculated as the percentages of CD45⁺MHC-II⁺ cells.
Figure A.7. Representative FACS plots showing P2X7R deficiency impairs Th2 response to *T. spiralis* infection.

Splenocytes from C57BL/6 and P2X7R−/− mice at day 8 p.i. with *T. spiralis* were analysed by FACS for the intracellular cytokine expression and the proportion of CD4+CD25+Foxp3+ regulatory T cells (Tregs). (a) Gating strategy for distinction of CD3+CD4+ T cells. (b) Representative plots of the expression of IFN-γ, IL-4 and the percentage of Tregs. % shown as the percentage of CD4+ T cells.