Modulation of prostaglandin production by indole-3-pyruvate and 4-octyl itaconate in proinflammatory macrophages

Thesis submitted to the
University of Dublin
for the degree of
Doctor of Philosophy
by
Ciana Diskin

Supervisor: Professor Luke O’Neill
School of Biochemistry and Immunology
Trinity College Dublin
February 2022
Declaration

This thesis is submitted by the undersigned to the University of Dublin for the examination of Doctor of Philosophy. I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and is entirely my own work with the exception of the following figures:

Figure 3.7
Figure 3.16
Figure 3.17
This work was performed by Sarah Corcoran

Figure 3.12
Figure 3.13
This work was performed with Sarah Corcoran

Figure 3.2
Figure 3.3
Figure 4.3
Figure 4.4
Figure 4.16
Figure 4.17
Figure 4.23
This work was performed with Sarah Corcoran and Victoria Tyrrell

I agree to deposit this thesis in the University’s open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

________________________

Ciana Diskin
Acknowledgements

First and foremost, I want to thank Luke. Every meeting with you, both lab meetings and one-to-one, makes everyone more confident and excited about their work because your enthusiasm and scientific curiosity is so infectious. Not only are you an incredibly helpful and encouraging supervisor, but you also seem to have a knack for hiring people that make your lab into a really fun environment.

I also want to thank all the LON lab members, both past and present. Sarah, I have had so much fun with you throughout my PhD, from growing cute little trypanosomes together to me crashing in your tent at Body&Soul - I hope to be friends with you for a very long time. Z, you being biggest gossip in the lab (apart from maybe Alex) always made for very interesting coffee breaks and I’m very grateful for the huge amount of help you gave me at the start of my project. Dylan, I’m not sure if I will ever meet someone as enthusiastic as you are about science and life in general, your presence was sorely missed after you left the lab. Niamh, you were always my go-to for Bake Off discussions and a frequent instigator of Friday pints. Jamie, Mark and Richie were such good craic to have around the lab and always had me in stitches. Marah, you were always great for playing excellent music in the lab and keeping the LON lab playlist up to scratch. And to the current members of the lab - Anne you are one of the most helpful and generous people I have had the pleasure of meeting, as well as being such a calming presence whenever I was stressing over something. Kathy, I always had so much fun with you and we are all so lucky to have you running so many important aspects of lab. Eva, I have very much appreciated your gifts of baking supplies like apples from your garden and Scandinavian spices, as well as your sarcastic sense of humour. And a big thank you to Cait for taking care of so much behind the scenes. To Alex, the former Bradley Cooper lookalike, I still don’t quite believe that you’re leaving the lab at all. Although being the PhD student that came after you meant that I had a very tough act to follow, I couldn’t have asked for a better PhD mate. And to Christian, our other conference buddy, we’re going to have so much fun in Mexico. The other boyband members Shane, Tristram and Hauke have made the lab very fun with all the cans, extracurricular activities and lab pranks. Maureen, when I leave I will certainly miss your very funny (and sometimes rather graphic) drawings.
Alessia, you are one of the funniest people to ever work in Luke’s lab (which is a high bar), as well as one of the sweetest. I love that you always celebrate both big and small wins for everyone in the lab. The most recent people to join the LON lab, Emily and Juliana, you have been wonderful additions to the group and to the girlband. I love that you are both always up for trying new things (even the chilly Irish sea) and I have had so much fun with you guys since you joined. All I can do is hope that my next workplace is even half as much fun as the LON lab has been.

I also have to thank all of my family and friends who have provided me with many fun distractions during my PhD, from the best holidays and parties to Zoom murder mysteries and food/wine deliveries during lockdowns. A special mention has to go to my sister Ailbhe, who endured many graphic design queries every time I made figures, diagrams or posters over the last four years.

A huge thank you is owed to my partner Rob. I am so glad that we were able to coordinate starting and finishing our PhDs at the same time and therefore undergo the whole process together. Your support has been incredible and you are especially skilled in keeping me calm when things don’t go according to plan. I am really excited to see where we end up next.
# Table of Contents

Declaration..........................................................................................................................2
Acknowledgments.................................................................................................................3
Table of Contents..................................................................................................................5
List of Figures........................................................................................................................11
List of Tables..........................................................................................................................15
Abbreviations.......................................................................................................................16
Abstract.................................................................................................................................22

**Chapter 1: Introduction**..................................................................................................24

1.1 Macrophages and the innate immune system.................................................................25
1.2 The immune response to trypanosomes and other parasites.........................................26
1.3 TLR signalling and inflammation....................................................................................28
1.4 Prostaglandins..................................................................................................................32
1.5 Annexin A1.......................................................................................................................38
1.6 Metabolic reprogramming in macrophages.....................................................................43
1.7 Microbe-derived metabolites and immunomodulators....................................................48
1.8 Human African trypanosomiasis......................................................................................49
1.9 The aryl hydrocarbon receptor.......................................................................................53
1.10 Post-translational modification by metabolites in immunity.........................................56
1.11 Itaconate and its derivatives...........................................................................................58
1.12 Fumarate and DMF........................................................................................................63
1.13 NRF2 signalling..............................................................................................................66
1.14 Aims.................................................................................................................................69

**Chapter 2: Materials and Methods**...............................................................................70

2.1 Materials...........................................................................................................................71
2.1.1 Buffers..........................................................................................................................71
2.1.2 Animals........................................................................................................................71
2.1.3 Human blood samples.................................................................................................72
2.1.4 Cell culture reagents.................................................................................................72
2.1.5 Stimulants, metabolites and treatments.....................................................................72
2.1.6 Transfection reagents...............................................................................................73
2.1.7 Western blotting reagents.........................................................................................73
2.1.8 Antibodies..............................................................................73
2.1.9 PCR reagents........................................................................73
2.1.10 ELISA reagents.................................................................75
2.1.11 COX activity assay reagents..................................................75
2.1.12 Mass spectrometry reagents..................................................75
2.1.13 Software.............................................................................75

2.2 Methods..................................................................................76
2.2.1 L929 cell culture....................................................................76
2.2.2 BMDM generation.................................................................76
2.2.3 PBMC and primary human macrophage isolation....................77
2.2.4 Trypanosome culture and lysis...............................................77
2.2.5 siRNA knockdown in BMDMs...............................................78
2.2.6 Western blot.........................................................................78
2.2.7 RT-qPCR.............................................................................81
2.2.8 ELISA..................................................................................82
2.2.9 COX activity assay...............................................................83
2.2.10 Mass spectrometry..............................................................83
2.2.11 Statistical analysis..............................................................84

Chapter 3: Indole-3-pyruvate inhibits prostaglandin production in macrophages through inhibition of COX2...........................................................................85

3.1 I3P inhibits PG production in macrophages................................86
3.1.1. I3P inhibits LPS-induced PG secretion from BMDMs.............86
3.1.2 I3P inhibits LPS-induced PGD2 at six hours...........................86
3.1.3 I3P inhibits LPS-induced PGE2, PGD2, 15-deoxy-PGJ2 and TXB2 at 24 hours.................................................................87
3.1.4 Indomethacin and I3P inhibit all PGs detected by ELISA...........87
3.1.5 I3P inhibits LPS-induced PG secretion from primary human macrophages..........................................................92
3.1.6 I3P inhibits trypanosome lysate-induced PG production..........92

3.2 I3P boosts COX2 expression in macrophages............................95
3.2.1 I3P augments LPS-induced Ptgs2 mRNA expression.................95
3.2.2 I3P increases LPS-induced COX2 protein expression...............95
3.2.3 I3P increases LPS-induced COX2 protein expression at concentrations of 500 μM.

3.2.4 I3P increases LPS-induced COX2 protein expression in primary human macrophages.

3.2.5 I3P augments trypanosome lysate-induced COX2 expression.

3.3 The augmentation of COX2 expression by I3P is regulated by a PG feedback loop.

3.3.1 Addition of PGE$_2$ attenuates the I3P-induced boost in COX2 expression.

3.3.2 An EP2 antagonist enhances the I3P-induced increase in COX2 expression.

3.3.3 An EP4 antagonist enhances the I3P-induced boost in COX2 expression.

3.3.4 Addition of forskolin attenuates the I3P-associated boost in COX2 expression.

3.4 The augmentation of COX2 expression by I3P is partially dependent on AhR activation.

3.4.1 3MC increases COX2 expression in an AhR-dependent manner.

3.4.2 The I3P-induced boost in COX2 expression is blunted in $AhR^{-/-}$ macrophages.

3.4.3 AhR knockdown abolishes the capacity of I3P to augment COX2 expression.

3.4.4 AhR knockdown has no effect on the capacity of I3P to inhibit PG production.

3.5 I3P inhibits COX2 enzymatic activity.

3.5.1 Indomethacin and I3P increase LPS-induced COX2 expression.

3.5.2 Addition of AA does not overcome the inhibitory effect of I3P and PGs.

3.5.3 I3P directly inhibits COX2 enzymatic activity.

3.6 Discussion.

3.6.1 Rationale.

3.6.2 I3P inhibits PG production in macrophages.

3.6.3 I3P boosts COX2 through a PG feedback loop and AhR activation.

3.6.4 I3P inhibits COX activity.

3.6.5 Future directions.
Chapter 4: 4-Octyl itaconate inhibits COX2 expression and prostaglandin production in macrophages........128

4.1 4-OI inhibits Pam3CSK4-induced PG production..................................................129
  4.1.1 4-OI inhibits Pam3CSK4-induced PG production in BMDMs............................129
  4.1.2 4-OI impairs Pam3CSK4-induced PG secretion from human PBMCs.............129
  4.1.3 4-OI inhibits Pam3CSK4-induced PGD$_2$ at six hours.................................132
  4.1.4 4-OI inhibits Pam3CSK4-induced PGE$_2$, PGD$_2$, 15-deoxy-PGJ$_2$ and TXB$_2$
     at 24 hours.....................................................................................................................132
  4.1.5 Indomethacin and NS-398 inhibit all Pam3CSK4-induced PGs detected by
     ELISA............................................................................................................................132

4.2 4-OI inhibits Pam3CSK4-induced COX2 expression..............................................136
  4.2.1 4-OI inhibits Pam3CSK4-induced Ptgs2 mRNA levels at four
     and eight hours in BMDMs..........................................................................................136
  4.2.2 4-OI decreases Pam3CSK4-induced COX2 protein expression
     at 6 and 24 hours........................................................................................................136
  4.2.3 4-OI reduced Pam3CSK4-induced PTGS2 transcript levels
     in PBMCs......................................................................................................................136
  4.2.4 50 μM 4-OI is sufficient to reduce Ptgs2 transcript..............................................140
  4.2.5 100 μM 4-OI is sufficient to reduce COX2 protein expression........................140
  4.2.6 4-OI does not affect phospho-cPLA2 or total cPLA2 expression....................143
  4.2.7 4-OI does not alter Ptges mRNA levels..............................................................143

Selection of Pam3CSK4 stimulation as the experimental model.........................146
  4.3.1 4-OI increases LPS-induced COX2 protein levels while decreasing
     Pam3CSK4-induced COX2 at 24 hours.................................................................146
  4.3.2 4-OI, but not OMS, reduces COX2 expression in response to stimulation
     with most TLR ligands.................................................................................................146
  4.3.3 4-OI decreases LPS-induced COX2 expression at four hours....................147
  4.3.4 4-OI decreases LPS-induced PGD$_2$ at six hours............................................151
  4.3.5 4-OI lowers LPS-induced TXB$_2$ but does not affect LPS-induced PGE$_2$,
     PGD$_2$ and 15-deoxy-PGJ$_2$ at 24 hours.................................................................151
  4.3.6 4-OI decreases LPS-induced PG production in human PBMCs....................154
  4.3.7 4-OI decreases LPS-induced COX2 expression at 24 hours in
     human PBMCs and macrophages...............................................................................154

8
4.4 Endogenous itaconate does not affect COX2 expression of prostaglandin production

4.4.1 Endogenous itaconate does not affect Pam3CSK4-induced Ptgs2 mRNA levels

4.4.2 Endogenous itaconate does not affect COX2 protein expression

4.4.3 Endogenous itaconate has no effect on PG production measured by ELISA

4.4.4 Endogenous itaconate has no effect on PGE₂, PGD₂, 15-deoxy-PGJ₂ or TXB₂ measured by mass spectrometry

4.4.5 Exogenous unmodified itaconate moderately reduces PG production

4.4.6 Exogenous unmodified itaconate moderately reduces COX2 Expression

4.5 DMF and DEM also reduce COX2 expression and prostaglandin production

4.5.1 DMF reduces Ptgs2 mRNA levels

4.5.2 DMF decreases COX2 protein expression

4.5.3 DMF reduces PG production

4.5.4 DEM reduces Ptgs2 mRNA levels

4.5.5 DEM decreases COX2 protein expression

4.5.6 DEM reduces PG production

4.6 The capacity of 4-OI and DMF to reduce COX2 expression and prostaglandin production is NRF2-independent

4.6.1 4-OI stabilises NRF2 and activates NRF2-dependent genes

4.6.2 DMF and DEM activate NRF2-dependent genes

4.6.3 4-OI still reduces COX2 protein expression in the context of NRF2 knockdown

4.6.4 NRF2 KO BMDMs display no Nqo1 activation while KEAP1 knockdown BMDMs exhibit enhanced upregulation of Nqo1

4.6.5 4-OI, DMF and DEM maintain the capacity to impair Ptgs2 transcription in NRF2 knockout and KEAP1 knockdown cells

4.6.6 4-OI and DMF maintain the capacity to reduce COX2 protein in NRF2 knockout and KEAP1 knockdown cells
4.6.7 4-OI and DMF inhibit PG secretion from NRF2 knockout and KEAP1 knockout cells.................................................................178
4.6.8 4-OI does not affect p38, NF-kB p65 or ERK phosphorylation..............184
4.6.9 The capacity of 4-OI to inhibit COX2 expression is not dependent on ATF4......................................................................................184
4.6.10 The capacity of 4-OI to inhibit COX2 expression is not dependent on annexin A1..............................................................................184

4.7 4-OI induces annexin A1 secretion.................................................................188
4.7.1 4-OI induces annexin A1 secretion from BMDMs......................................188
4.7.2 DMF induces annexin A1 secretion............................................................188
4.7.3 DEM induces annexin A1 secretion............................................................189
4.7.4 Endogenous itaconate does not affect annexin A1 secretion....................193
4.7.5 Exogenous itaconate induces annexin A1 secretion.................................193
4.7.6 4-OI does not affect Anxa1 or Abca1 mRNA levels.................................196
4.7.7 Knockdown of NRF2 impairs annexin A1 secretion by 4-OI....................196
4.7.8 4-OI and DMF do not induce annexin A1 secretion from NRF2 knockout BMDMs..............................................................................196

4.8 Discussion....................................................................................................201
4.8.1 Rationale....................................................................................................201
4.8.2 4-OI impairs macrophage PG production..............................................201
4.8.3 4-OI decreases COX2 expression............................................................203
4.8.4 Endogenous itaconate does not affect COX2 or PG levels....................204
4.8.5 4-OI, DMF and DEM reduce COX2 expression and PG production in an NRF2-independent manner......................................................205
4.8.6 4-OI induces annexin A1 secretion............................................................209
4.8.7 Future directions........................................................................................215

Chapter 5: General Discussion.................................................................216
5.1 Endogenous versus derivatised metabolites..............................................217
5.2 Therapeutic applications...........................................................................220
5.3 Final remarks..............................................................................................222

Chapter 6: References..................................................................................225

Chapter 7: Publications...............................................................................252
List of Figures

Figure 1.1 TLR signalling pathways ................................................................. 31
Figure 1.2 Prostaglandin synthesis ................................................................. 33
Figure 1.3 EP receptors .................................................................................. 35
Figure 1.4 Effects of PGE$_2$ ........................................................................ 37
Figure 1.5 Annexin A1 secretion ................................................................. 40
Figure 1.6 Anti-inflammatory effects of annexin A1........................................ 42
Figure 1.7 Metabolic reprogramming of macrophages ................................. 46
Figure 1.8 *T. brucei* life cycle ................................................................... 50
Figure 1.9 cASAT transaminates amino acids to form keto acids ............... 52
Figure 1.10 AhR signalling ............................................................................ 55
Figure 1.11 Immunomodulatory effects 4-OI and itaconate ....................... 62
Figure 1.12 Immunomodulatory effects of DMF and fumarate ................. 65
Figure 1.13 NRF2 regulation by KEAP1 ....................................................... 68
Figure 3.1 I3P inhibits LPS-induced PG production in BMDMs ............... 88
Figure 3.2 I3P inhibits LPS-induced PGD$_2$ at six hours ......................... 89
Figure 3.3 I3P inhibits LPS-induced PGE$_2$, PGD$_2$, 15-deoxy-PGJ$_2$ and TXB$_2$ at 24 hours ......................................................................................... 90
Figure 3.4 Indomethacin and I3P inhibit all PGs detected by ELISA ........ 91
Figure 3.5 I3P inhibits LPS-induced PG production in primary human macrophages ................................................................................. 93
Figure 3.6 I3P inhibits trypanosome lysate-induced PG production in BMDMs . 94
Figure 3.7 I3P increases LPS-induced *Ptgs2* mRNA expression ............... 96
Figure 3.8 I3P increases LPS-induced COX2 protein expression ............... 97
Figure 3.9 I3P increases LPS-induced COX2 protein expression at concentrations of 500 μM ................................................................. 98
Figure 3.10 I3P increases LPS-induced COX2 expression in primary human macrophages ................................................................................. 100
Figure 3.11 I3P augments trypanosome lysate-induced COX2 expression in BMDMs ...................................................................................... 101
Figure 3.12 PGE$_2$ attenuates the I3P-induced boost in COX2 ................. 104
Figure 3.13 An EP2 antagonist enhances the I3P-induced increase in COX2.. 105
Figure 3.14 An EP4 antagonist enhances the I3P-induced boost in COX2 expression

Figure 3.15 Forskolin attenuates the I3P-associated boost in COX2

Figure 3.16 3MC increases COX2 expression in an AhR-dependent manner

Figure 3.17 The I3P-induced boost in COX2 expression is blunted in $AhR^{-/-}$ macrophages

Figure 3.18 AhR knockdown abolishes the capacity of I3P to augment COX2 expression

Figure 3.19 AhR knockdown has no effect on the capacity of I3P to inhibit PGs

Figure 3.20 Indomethacin and I3P increase LPS-induced COX2 expression

Figure 3.21 Addition of arachidonic acid does not overcome the inhibitory effect of I3P on prostaglandins

Figure 3.22 I3P directly inhibits COX2 enzymatic activity

Figure 3.23 I3P augments COX2 expression by two distinct mechanisms

Figure 3.24 Trypanosome-derived I3P inhibited COX2 activity and PG synthesis

Figure 4.1 4-OI inhibits Pam3CSK4-induced PG production in BMDMs

Figure 4.2 4-OI reduces Pam3CSK4-induced PG production in human PBMCs

Figure 4.3 4-OI inhibits Pam3CSK4-induced PGD$_2$ at six hours

Figure 4.4 4-OI inhibits Pam3CSK4-induced PGE$_2$, PGD$_2$, 15-deoxy-PGJ$_2$ and TXB$_2$ at 24 hours

Figure 4.5 Indomethacin and NS-398 inhibit all Pam3CSK4-induced PGs detected by ELISA

Figure 4.6 4-OI inhibits Pam3CSK4-induced induction of $Ptgs2$ transcript at four and eight hours in BMDMs

Figure 4.7 4-OI decreases Pam3CSK4-induced COX2 protein expression at 6 and 24 hours

Figure 4.8 4-OI inhibits Pam3CSK4-induced $PTGS2$ transcript levels in PBMCs

Figure 4.9 50 μM 4-OI is sufficient to lower $Ptgs2$ mRNA levels

Figure 4.10 25 μM 4-OI is sufficient to reduce COX2 protein expression at 24 hours
Figure 4.11 4-OI does not affect phospho-cPLA2 or total cPLA2 expression

Figure 4.12 4-OI does not alter Ptges mRNA levels

Figure 4.13 4-OI increases LPS-induced COX2 expression while decreasing Pam3CSK4-induced COX2 at 24 hours

Figure 4.14 4-OI, but not OMS, reduces COX2 expression upon stimulation with most TLR ligands

Figure 4.15 4-OI decreases LPS-induced COX2 expression at four hours

Figure 4.16 4-OI decreases LPS-induced PGD2 at six hours

Figure 4.17 4-OI lowers TXB2 but does not affect LPS-induced PGE2, PGD2, and 15-deoxy-PGJ2 at 24 hours

Figure 4.18 4-OI decreases LPS-induced PG production in human PBMCs

Figure 4.19 4-OI decreases LPS-induced COX2 expression at 24 hours in human PBMCs and macrophages

Figure 4.20 Endogenous itaconate does not alter Pam3CSK4-induced Ptgs2 mRNA levels

Figure 4.21 Endogenous itaconate does not affect COX2 protein expression

Figure 4.22 Endogenous itaconate has no effect on PG production

Figure 4.23 Endogenous itaconate does not alter PGE2, PGD2, 15-deoxy-PGJ2 or TXB2 secretion

Figure 4.24 Exogenous itaconate moderately reduces PG production

Figure 4.25 Exogenous itaconate moderately reduces COX2 expression

Figure 4.26 DMF reduces Ptgs2 mRNA levels

Figure 4.27 DMF attenuates COX2 protein expression

Figure 4.28 DMF lowers prostaglandin production

Figure 4.29 DEM reduces Ptgs2 mRNA levels

Figure 4.30 DEM decreases COX2 protein expression

Figure 4.31 DEM impairs prostaglandin production

Figure 4.32 4-OI stabilises NRF2 and activates NRF2-dependent genes

Figure 4.33 DMF and DEM activate NRF-dependent genes

Figure 4.34 4-OI still reduces COX2 protein expression when NRF2 is knocked down

Figure 4.35 NRF2 KO BMDMs display no Nqo1 activation while KEAP1 KD BMDMs exhibit enhanced upregulation of Nqo1
Figure 4.36 4-OI, DMF and DEM maintain the capacity to impair Ptgs2 transcription in NRF2 KO and KEAP1 KD cells.................................181
Figure 4.37 4-OI and DMF maintain the capacity to reduce COX2 protein in NRF2 KO and KEAP1 KD cells..................................................182
Figure 4.38 4-OI and DMF inhibit PG secretion from NRF2 KO and KEAP1 KD cells........................................................................183
Figure 4.39 4-OI does not inhibit p38, NF-κB p65 or ERK phosphorylation......185
Figure 4.40 The capacity of 4-OI to inhibit COX2 expression is not dependent on ATF4........................................................................186
Figure 4.41 The capacity of 4-OI to inhibit COX2 expression is not dependent on annexin A1.................................................................187
Figure 4.42 4-OI induces annexin A1 secretion in BMDMs......................190
Figure 4.43 DMF induces annexin A1 secretion........................................191
Figure 4.44 DEM induces annexin A1 secretion........................................192
Figure 4.45 Endogenous itaconate does not affect annexin A1 secretion.......194
Figure 4.46 Exogenous itaconate induces annexin A1 secretion.................195
Figure 4.47 4-OI does not alter Anxa1 or Abca1 mRNA levels......................198
Figure 4.48 Knockdown of NRF2 impairs annexin A1 secretion by 4-OI.....199
Figure 4.49 4-OI and DMF do not induce annexin A1 secretion from NRF2 KO BMDMs.................................................................200
Figure 4.50 4-OI, DMF and DEM modulate COX2 induction and PG production......................................................................................207
Figure 4.51 4-OI induces annexin A1 secretion..........................................211
Figure 4.52 Possible effects of 4-OI on annexin A1......................................214
Figure 5.1 General summary....................................................................223
List of Tables

Table 2.1 Buffer composition.................................................................................71
Table 2.2 Primer sequences......................................................................................74
Table 2.3 SDS-PAGE gel compositions...................................................................80
Table 2.4 Reverse transcription master mix components...........................................82
Table 2.5 qPCR master mix components...................................................................82
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>15d-PGJ₂</td>
<td>15-Deoxy-(\Delta^{12,14})-PGJ₂</td>
</tr>
<tr>
<td>3MC</td>
<td>3-Methylcholanthrene</td>
</tr>
<tr>
<td>4-OI</td>
<td>4-Octyl itaconate</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP binding cassette transporter A1</td>
</tr>
<tr>
<td>ACLY</td>
<td>ATP citrate lyase</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AhRR</td>
<td>Aryl hydrocarbon receptor repressor</td>
</tr>
<tr>
<td>AIP</td>
<td>Aryl hydrocarbon receptor interacting protein</td>
</tr>
<tr>
<td>AKA</td>
<td>Aromatic keto acids</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ANXA1</td>
<td>Annexin A1</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow-derived macrophage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Cis-aconitate decarboxylase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cASAT</td>
<td>Cytoplasmic aspartate aminotransferase</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>COVID-19</td>
<td>Coronavirus disease 19</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphate-guanine</td>
</tr>
<tr>
<td>cPLA2</td>
<td>Cytoplasmic phospholipase A2</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP responsive element binding protein</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
</tbody>
</table>
CSF  Cerebrospinal fluid
CYP450  Cytochrome P450
DAMP  Danger-associated molecular pattern
DEM  Diethyl maleate
DMEM  Dulbecco’s modified eagle medium
DMF  Dimethyl fumarate
DMI  Dimethyl itaconate
DMSO  Dimethyl sulfoxide
DP  D prostanoid receptor
DTT  1,4-Dithiothreitol
EAE  Experimental autoimmune encephalomyelitis
ECL  Enhanced chemiluminescence
EET  Epoxyeicosatrienoic acids
EGF  Epidermal growth factor
ELISA  Enzyme-linked immunosorbent assay
EP  E prostanoid receptor
ER  Endoplasmic reticulum
ERK  Extracellular signal-regulated kinase
ETC  Electron transport chain
FCS  Foetal calf serum
FDA  US Food and Drug Administration
FH  Fumarate hydratase
FICZ  6-Formylindolo(3,2-b)carbazole
FP  F prostanoid receptor
FPR  Formyl peptide receptor
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
GI  Gastrointestinal
GM-CSF  Granulocyte-macrophage colony-stimulating factor
GPCR  G-protein-coupled receptor
HAT  Human African trypanosomiasis
HDAC  Histone deacetylase
HIF-1α  Hypoxia-inducible factor 1α
HO-1  Heme oxygenase 1
HPP  4-Hydroxyphenylpyruvate
HSP90  Heat shock protein 90
I3P  Indole-3-pyruvate
IC50  Half maximal inhibitory concentration
ICAM-1  Intracellular adhesion molecule 1
ILC  Innate lymphoid cell
IFN  Interferon
Ig  Immunoglobulin
IL  Interleukin
iNOS  Inducible nitric oxide synthase
IP  I prostanoid receptor
IP₃  Inositol triphosphate
IRAK  Interleukin 1 receptor-associated kinase
IRF  Interferon regulatory factor
IRG1  Immune responsive gene 1
ISG  Interferon-stimulated gene
ITA  Itaconic acid
IkBζ  Nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor ζ
KEAP1  Kelch-like-ECH-associated protein 1
KD  Knockdown
KO  Knockout
LAT  Linker of activator for T cells
LC/MS/MS  Liquid chromatography with tandem mass spectrometry
LDHA  Lactate dehydrogenase A
LOX  Lipoxygenase
LPS  Lipopolysaccharide
LRR  Leucine-rich repeats
LTB₄  Leukotriene B₄
LXA₄  Lipoxin A₄
M-CSF  Macrophage colony-stimulating factor
MAL  MYD88 adaptor-like protein
MAPK  Mitogen-activated protein kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD2</td>
<td>Myeloid differentiation factor 2</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mechanistic target of rapamycin complex 1</td>
</tr>
<tr>
<td>MYD88</td>
<td>Myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>NEK7</td>
<td>NIMA related kinase 7</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κ-light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NOD-like receptor containing pyrin domain 3</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerisation domain</td>
</tr>
<tr>
<td>NQO1</td>
<td>NAD(P)H:quinone acceptor oxidoreductase 1</td>
</tr>
<tr>
<td>NRF2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>OMS</td>
<td>4-Octyl-2-methyl succinate</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>Pam</td>
<td>Pam3CSK4</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCG-1β</td>
<td>PPAR-γ coactivator 1β</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGDS</td>
<td>Prostaglandin D synthase</td>
</tr>
<tr>
<td>PGES</td>
<td>Prostaglandin E synthase</td>
</tr>
<tr>
<td>PGFS</td>
<td>Prostaglandin F synthase</td>
</tr>
<tr>
<td>PGIS</td>
<td>Prostaglandin I synthase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKM2</td>
<td>Pyruvate kinase isozyme M2</td>
</tr>
<tr>
<td>PP</td>
<td>Phenylpyruvate</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PSG</td>
<td>Phosphate-saline-glucose</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>R848</td>
<td>Resiquimod</td>
</tr>
<tr>
<td>RET</td>
<td>Reverse electron transport</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene I</td>
</tr>
<tr>
<td>RIP1</td>
<td>Receptor-interacting protein 1</td>
</tr>
<tr>
<td>RIPK</td>
<td>Receptor-interacting serine/threonine protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RPS18</td>
<td>40S ribosomal protein S18</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SARM</td>
<td>Sterile α and HEAT-Armadillo motifs-containing protein</td>
</tr>
<tr>
<td>SARS-CoV2</td>
<td>Severe acute respiratory syndrome coronavirus 2</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor-β-activated kinase 1</td>
</tr>
<tr>
<td>tBHQ</td>
<td>Tert-butylhydroquinone</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween 20</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-interleukin-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TP</td>
<td>Thromboxane prostanoid receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain-containing protein inducing IFN-β</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoi etin</td>
</tr>
<tr>
<td>TXAS</td>
<td>Thromboxane A synthase</td>
</tr>
<tr>
<td>TXB₂</td>
<td>Thromboxane B₂</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VSG</td>
<td>Variable surface glycoprotein</td>
</tr>
<tr>
<td>XRE</td>
<td>Xenobiotic response element</td>
</tr>
<tr>
<td>α-KG</td>
<td>α-Ketoglutarate</td>
</tr>
</tbody>
</table>
Abstract

The recent surge in immunometabolism research has revealed how specific changes in the level of metabolites can affect the immune response. One important feature of the immune response is the production of a class of lipid mediators termed prostaglandins (PGs). PGs exhibit a wide range of effector functions, many of which are proinflammatory. This is highlighted by the widespread and efficacious use of nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit PG production, to treat inflammation. In order to block PG synthesis, NSAIDs target the cyclooxygenase enzymes, of which there are two isoforms. COX1 is ubiquitously and constitutively expressed whereas COX2 is inducible by proinflammatory stimuli. Here I have investigated the impact of two metabolites with known immunomodulatory properties, indole-3-pyruvate (I3P) and 4-octyl itaconate (4-OI), on COX2 expression and PG production in macrophages.

I3P is one of several aromatic keto acids, which are derived from metabolism of amino acids and secreted by the bloodstream form of the protozoan parasite Trypanosoma brucei. T. brucei is the causative agent of the neglected tropical disease human African trypanosomiasis, also known as sleeping sickness. Trypanosomes have evolved many immune-evasion mechanisms and here I describe a novel immunomodulatory role for trypanosome-derived I3P. I have found that I3P inhibits the production of PGs from LPS-stimulated macrophages, while counterintuitively augmenting COX2 expression. I demonstrate that the I3P-mediated boost in COX2 levels is dependent on two distinct mechanisms. This increase is in part due to I3P relieving a negative feedback loop on COX2 by PGE\textsubscript{2}. Additionally, I3P activates the aryl hydrocarbon receptor, which can subsequently upregulate COX2 expression. However, the enhancement of COX2 expression is of little functionality as I also demonstrate that I3P directly inhibits COX2 activity to limit PG production. I3P thereby facilitates an evasion strategy by which T. brucei may modulate host PGs during infection, which is likely to be advantageous to the parasite.

I have also evaluated the impact that the itaconate derivative 4-OI has on COX2 and PG secretion. Itaconate synthesis occurs via conversion of the Krebs cycle intermediate cis-aconitate. This reaction is catalysed by the enzyme IRG1, which
is potently upregulated in macrophages in response to TLR ligands. Both itaconate, and its derivative 4-OI, are known to exhibit a wide range of anti-inflammatory effects, many of which are dependent on its capacity to modify cysteine residues on protein targets. Here I demonstrate that 4-OI decreases PG production in proinflammatory macrophages. This reduction is due to a robust suppression of COX2 expression, with both mRNA and protein levels affected. As there was no difference in COX2 expression and PG production between \( Irg1^{+/+} \) and \( Irg1^{-/-} \) macrophages, I have concluded that endogenous itaconate has no bearing on this pathway. Another Krebs cycle metabolite derivative, dimethyl fumarate (DMF), which shares a number of the immunomodulatory properties associated with 4-OI, also decreases COX2 expression and limits PG production. As both 4-OI and DMF are known to potently activate the master antioxidant transcription factor NRF2, I also demonstrate that the modulation of COX2 expression and PG synthesis by 4-OI and DMF is NRF2-independent. Furthermore, I have shown that both of these Krebs cycle derivatives induce the secretion of annexin A1 from macrophages, a protein that is known to elicit a plethora of anti-inflammatory functions.

Overall, I have identified novel metabolic modulators of PG production in macrophages. These findings have identified a potential immune evasion strategy by trypanosomes during infection, as well as providing novel insight into the potential of 4-OI as an anti-inflammatory agent.
Chapter 1: Introduction
In recent years, there has been a significant focus on immunometabolism and a plethora of metabolites that affect immune function in a variety of ways have been described. Prostaglandins (PGs) are a class of lipid mediators which play key roles in inflammation, but modulation of PG production by immunometabolites remains largely unexplored. This thesis will detail how a naturally occurring parasite-derived metabolite, indole-3-pyruvate (I3P), and two derivatives of Krebs cycle metabolites, 4-octyl itaconate (4-OI) and dimethyl fumarate (DMF), regulate PG production in macrophages. This introduction will firstly outline immune pathways that are important in macrophage function before introducing the metabolic pathways that are relevant for this thesis.

1.1 Macrophages and the innate immune system

The immune system is divided into two arms, innate and adaptive immunity. The innate immune system represents the first line of defence against infection. The innate immune system provides an immediate but less specific response to invading pathogens and is critical for mounting the more specific adaptive immune response [1]. Adaptive immunity emerges later during infection and targets the pathogen in an antigen-specific manner that requires gene rearrangement [2].

The innate immune system encompasses cell types such as neutrophils, natural killer (NK) cells, dendritic cells and macrophages. Macrophages are innate immune cells of myeloid lineage derived from haematopoietic stem cells present in the bone marrow. They are crucial effector cells in both host defence and tissue maintenance. Macrophages can be tissue-resident or derived from circulating monocytes that are extravasated into the surrounding tissues. Macrophages are often referred to as “professional phagocytes” due to their efficiency in this process. Phagocytosis is the recognition, engulfment and breakdown of particulates, which can be pathogen-derived or host-derived from apoptotic cells or cell debris [3, 4]. Macrophages can also influence the adaptive immune response by presenting antigens derived from phagocytosed material to lymphocytes on MHC class II molecules at the cell surface [5].
Macrophages respond to a wide array of external signals and therefore a great deal of heterogeneity in macrophage function and phenotype is observed [6]. Established nomenclature designates activated macrophages into one of two categories in vitro. Classically activated (M1) macrophages are generated by stimulating resting (M0) macrophages with lipopolysaccharide (LPS) and interferon gamma (IFN-γ) and are associated with a proinflammatory phenotype, whereas alternatively activated (M2) macrophages arise from stimulation with IL-4 and IL-13 in vitro and are associated with anti-inflammatory responses, resolution of inflammation and tissue repair. M1 macrophages produce a broad panel of proinflammatory mediators including cytokines such as interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α), various chemokines, reactive oxygen species (ROS), nitric oxide (NO) and lipid mediators such as PGs. While all of these effectors are important in pathogen clearance, they can also contribute to excessive inflammation and associated tissue damage. M2 macrophages secrete anti-inflammatory cytokines such as IL-10 and TGF-β which dampen inflammation and promote tissue repair [7]. While the binary M1/M2 classification system is useful for examining the phenotypic, functional and metabolic changes that occur with this skewing, it has become increasingly clear that this is not representative of the functional diversity in vivo. The vast array of macrophage activation statuses has been demonstrated in transcriptomics studies carried out on macrophages exposed to a wide range of stimuli [8]. In reality, most macrophages in vivo are likely to fall somewhere between the two extreme M1 and M2 phenotypes [9].

1.2 The immune response to trypanosomes and other parasites

There are three main classes of parasites; ectoparasites, metazoan parasites and protozoan parasites. Ectoparasites are arthropods that infect the superficial layers of skin, such as ticks that transmit *Borrelia burgdorferi*, the causative agent of Lyme disease [10]. Metazoan parasites, such as helminths, are multicellular organisms whereas protozoan parasites, including *T. brucei*, the parasite with which this thesis is concerned, are unicellular. The immune system possesses many strategies to target parasites and in turn, most parasites have evolved unique immune evasion mechanisms.
Helminths can infect many different tissues in mammalian hosts and tend to induce what is known as a type 2 immune response. This response is thought to be initiated by damage to epithelial cells during helminth infection, which causes them to secrete the alarmins IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) [11], which stimulate type 2 immunity though induction of type 2 innate lymphoid cells (ILC2s). ILC2s secrete IL-5 and IL-13 and thereby promote T helper 2 (Th2) cell differentiation [12]. Th2 cells produce cytokines such as IL-4, IL-5 and IL-13 which can activate other cell types to respond to the infection. Macrophages express IL-4 and IL-13 receptors on their surface and binding of these cytokines promotes the alternatively activated macrophage phenotype, which is associated with helminth killing and expulsion [13, 14]. ILC2- and Th2-derived IL-5 functions in the differentiation and recruitment of eosinophils, which are known to release cytotoxic mediators and enhance host resistance to helminth infection [15, 16]. The type 2 immune response associated with helminth infection is also characteristic of asthma and allergic disease [17].

A slightly different profile of immune responses is associated with host resistance to *T. brucei* and other protozoan parasites. Macrophages are among the most important innate cells that contribute to host immunity during trypanosomiasis. At early stages of the disease, classically activated (M1) macrophages contribute to trypanosome clearance through phagocytosis, particularly by Kuppfer cells in the liver [18] and phagocytosis is known to be enhanced by complement activation during trypanosomiasis [19]. These M1 macrophages also function in secretion of cytokines, such as IL-12 and TNF-α, as well as NO production [20]. NO synthesis is also known to be important for the control of *Leishmania major*, another protozoan parasite [21]. Although this initial M1 phenotype is associated with parasite clearance, alternative macrophage activation has been found to be enhance host survival at later stages of disease [22]. As trypanosomes are extracellular bloodborne parasites, they are significantly exposed to the humoral response and antibody-mediated clearance. Indeed, B-cell adoptive transfer has been shown to confer resistance to infection [23] and it has also been demonstrated that the antibody response to trypanosomiasis is dependent on the
crucial B cell adaptor protein Bam32 [24]. While some trypanosome-induced antibody production occurs in a T cell-independent manner, it has been shown that the humoral response during trypanosomiasis is enhanced by activation of CD4+ T cells [25]. Trypanosomes have evolved numerous strategies in order to evade various aspects the host immune response, some of which will be described in later sections.

Toll-like receptors (TLRs), are often associated with the recognition of bacterial and viral components during infection but TLRs are also known to function in the recognition of trypanosomes and other protozoa. It has been shown that the glycosylphosphatidylinositol (GPI) anchor of T. brucei can activate macrophages [26]. Although the TLR involved in recognition of T. brucei GPI was not identified, it has been demonstrated for other protozoan parasites, such as Trypanosoma cruzi and Plasmodium falciparum, that TLR2 is involved in GPI recognition [27, 28]. The activation of neutrophils by a T. brucei surface polymer called lipophosphoglycan has also been shown to be dependent on TLR2 and TLR4 [29]. Additionally, it has also been shown that trypanosomal DNA, which contains unmethylated CpG motifs, binds TLR9 [30]. TLR signalling will now be discussed more generally in the next section.

1.3 TLR signalling and inflammation

An infection is initially detected upon binding of pathogen-associated molecular patterns (PAMPs) to germline-encoded pattern recognition receptors (PRRs), which are expressed on macrophages and other innate immune cells. This broad class of receptors includes Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors and C-type lectin receptors [31]. PRRs can also be activated by another class of ligands termed danger-associated molecular patterns (DAMPs), which are host-derived and indicate endogenous damage or dysfunction. TLRs are of particular importance for this thesis.

TLR binding is one of the main ways in which macrophages are activated. Humans express ten TLRs while mice express twelve. Some TLRs are located at the cell surface whereas others are intracellular. All TLRs have an ectodomain which
contains leucine-rich repeats (LRRs) and orchestrates PAMP recognition, a transmembrane domain and a cytoplasmic Toll/IL-1 receptor domain that is involved in downstream signalling [32]. The TLRs are highly conserved between species and key pioneering discoveries regarding the TLRs were conducted using the model organism *Drosophila melanogaster* [33, 34]. When research in mammalian TLRs began shortly thereafter, it was discovered that activation of TLR4 could induce CD80 which provides co-stimulation to activate T cells. This finding provided one of the first pieces of evidence linking the innate and adaptive immune systems [35].

TLR4, which detects LPS derived from the wall of Gram-negative bacteria, was one of the first TLRs to be characterised due to the well-known role of LPS in sepsis [36, 37]. However, TLR4 itself lacks binding capacity for LPS and so LPS binds CD14 on the cell surface and is transferred to the TLR4-MD2 complex in order to initiate signalling [38]. TLR4 can induce a myeloid differentiation primary response protein 88 (MYD88)-dependent or -independent signalling cascade. In the MYD88-dependent pathway, MYD88 interacts with TLRs through a TIR domain and a bridging adaptor protein MYD88 adaptor-like protein (MAL) has been shown to facilitate this interaction for both cell surface and endosomal TLRs [39, 40]. IL-1 receptor associated kinases (IRAKs) are then recruited via death domain interactions, followed by TNF receptor-associated factor 6 (TRAF6). TRAF6 activates TAK1 which subsequently activates the IKK and mitogen-activated protein kinase (MAPK) pathways [41]. This signalling pathway leads to the activation of the transcription factors nuclear factor-κB (NF-κB), cyclic AMP responsive element binding protein (CREB) and activator protein 1 (AP1) which initiate the transcription of a number of proinflammatory genes [42]. In order for the MYD88-independent pathway to be initiated, the TLR4 complex is endocytosed following ligand binding and two other adaptors called TIR domain-containing adaptor protein inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) are recruited [43, 44]. This leads to the activation of the transcription factor interferon regulatory factor 3 (IRF3), which initiates the production of type I IFNs. NF-κB, CREB and AP-1 can also be activated through this pathway via receptor-interacting protein 1 (RIP1). A fifth TIR adaptor protein called sterile α and HEAT-
Armadillo motifs-containing protein (SARM) has been shown to negatively regulate TRIF signalling [45].

TLR2, which is also located at the cell surface, binds bacterial lipopeptides. It forms heterodimers with TLR1 and TLR6 to detect triacylated lipopeptides (such as Pam3CSK4) and diacylated lipopeptides (such as Pam2CSK4) respectively [46, 47]. The function of TLR10 was unclear until it was demonstrated to be the only anti-inflammatory TLR, exhibiting suppressive effects on TLR2-mediated cytokine production. TLR10 exerts these effects by also forming a heterodimer with TLR2 [48]. The other cell surface TLRs, TLR5 and TLR11 detect bacterial flagellin and a component of uropathogenic bacteria respectively [49, 50]. All the cell surface TLRs utilize MYD88 signalling but only TLR4 and TLR2 signal through MAL. Many of the endosomal TLRs detect viral components. TLR3 recognizes double-stranded RNA, which only exists in viruses [51] while TLR7 and TLR8 can sense signal-stranded viral RNA [52]. Endosomal TLRs can also recognize bacterial motifs. TLR9 detects CpG-rich unmethylated DNA that is common in bacteria [53] and TLR13 recognizes bacterial ribosomal RNA [54]. TLR3 signals through TRIF and TRAM while TLR7, TLR8, TLR9 and TLR13 signal via MYD88. The ligands and signalling pathways of TLRs are depicted in Figure 1.1.
Figure 1.1 TLR signalling pathways

The cell surface TLRs TLR4, TLR5, TLR11, TLR2-TLR1 and TLR2-TLR6 heterodimers detect various extracellular ligands. Upon binding of their respective ligands, these TLRs initiate MYD88 signalling via TIR domain interactions. TLR4 and TLR2 heterodimers also deploy the adaptor MAL. The resulting signal transduction eventually culminates in the activation of the transcription factors NF-κB, CREB and AP1 to induce transcription of various proinflammatory genes. TLR4 is the only cell surface TLR that can also be endocytosed. Endocytosed TLR4 activates a signalling pathway through the adaptor proteins TRIF and TRAM, finally leading to the activation of the transcription factor IRF3, which initiates transcription of type I IFNs. TRIF signalling can also activate NF-κB, CREB and AP1 via RIP3. The endosomal TLRs, TLR13, TLR9, TLR7, TLR8 and TLR3 detect ligands derived from viruses or intracellular bacteria. TLR3, like endocytosed TLR4, signals through TRIF and TRAM to activate IRF3, while the other endosomal TLRs signal via MYD88 to activate IRF7, which also induces transcription of type I IFNs.
**1.4 Prostaglandins**

Macrophages produce eicosanoids, which are a class of oxygenated lipid mediators that have many roles during inflammation. The first step in the synthesis of eicosanoids is the release of arachidonic acid from membrane phospholipids, which is catalysed by cytosolic phospholipase A2 (cPLA2). cPLA2 activity can be inhibited by a protein called annexin A1, which will be discussed in the next section. Once released into the cytosol, AA is metabolised by three main pathways to yield various eicosanoids. One pathway occurs via the lipoxygenase (LOX) enzymes which produce leukotrienes, such as leukotriene B\(_4\) (LTB\(_4\)), which acts as a potent chemoattractant for neutrophils and eosinophils [55] and lipoxins, such as LXA\(_4\) which exerts a wide range of anti-inflammatory effects [56, 57]. A second pathway involves cytochrome P450 (CYP450) enzymes which generate epoxyeicosatrienoic acids (EETs) [58], which have been demonstrated to impair vascular adhesion of leukocytes [59]. This thesis focuses on the third pathway whereby the COX enzymes convert AA into prostanoids, a collective term that includes prostaglandins and thromboxanes.

COX metabolises AA in two steps. Firstly, it catalyses the conversion of AA to PGG\(_2\) [60], before the peroxidase activity of the enzyme yields PGH\(_2\) [61, 62]. COX exists in two isoforms, COX1 which is ubiquitously and constitutively expressed, and COX2, which is inducible by proinflammatory stimuli such as LPS [63]. The induction of COX2 can occur through activation of various proinflammatory signalling cascades including NF-κB, CREB and p38 and extracellular signal-regulated kinase (ERK) MAPK pathways [64]. COX2 is also known to be transcriptionally induced by the aryl hydrocarbon receptor (AhR) [65, 66], which will be discussed later in detail. PGH\(_2\) is then converted by the synthases PGE synthases 1, 2 and 3 (PGES) [67], PGD synthase (PGDS) [68], PGF synthase (PGFS) [69], PGI synthase (PGIS) [70] and thromboxane A synthase (TXAS) [71] to produce PGE\(_2\), PGD\(_2\), PGF\(_{2\alpha}\), PGI\(_2\) and TXA\(_2\) respectively (see **Figure 1.2**). At nanomolar concentrations, each of these prostanoids can bind to their corresponding receptors and signal in an autocrine or paracrine manner. PGE\(_2\) binds to four E prostanoid receptors (EP1-4), PGD\(_2\) binds to two receptors DP1
and DP2 (also known as CRTH2), PGF$_{2\alpha}$ binds to the PGF receptor (FP), PGI$_2$ binds to the PGI receptor (IP) and TXA$_2$ binds the TXA receptor TP.

Figure 1.2 Prostaglandin synthesis
The first step in the synthesis of prostaglandins is the conversion of membrane phospholipids to arachidonic acid by cPLA2, which is negatively regulated by annexin A1. Arachidonic acid is then converted to PGH$_2$ by COX1, which is constitutively expressed, and COX2, which is inducible by inflammatory stimuli. PGH$_2$ can then be converted into PGE$_2$, PGI$_2$, PGD$_2$, PGF$_{2\alpha}$ or TXA$_2$ by specific synthases. PGD$_2$ can subsequently be converted non-enzymatically into J series PGs while TXA$_2$ is further metabolised to TXB$_2$, which also occurs in a non-enzymatic manner.
The role of various prostaglandins in the induction, regulation and resolution of inflammation has been well characterised. For example, PGD$_2$ has been reported to contribute to the allergic response. PGD$_2$ is predominantly secreted by mast cells but can also be produced by other innate immune cells including macrophages [72-74]. Engagement of PGD$_2$ with its receptors facilitates chemotaxis and activation of basophils, eosinophils and Th2 cells during allergic disease [75-77] and elevated PGD$_2$ levels were found to be associated with systemic mastocytosis [78] and correlated with asthma severity [79]. In the brain PGD$_2$ is involved in other processes, including regulation of the sleep cycle [80] and pain perception [81]. However, PGD$_2$ has also been reported to play some anti-inflammatory roles with PGD$_2$ attenuating inflammation in some models such as colitis [82] and dermatitis [83]. Furthermore, PGD$_2$ can also be converted non-enzymatically into the J series of prostaglandins, such as 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d-PGJ$_2$), which are widely considered to display anti-inflammatory properties [84, 85]. 15d-PGJ$_2$ has been reported to inhibit NF-$\kappa$B signalling [86, 87] and ameliorate several inflammatory disease models through its ability to activate peroxisome proliferator-activated receptor gamma (PPAR-$\gamma$) [88, 89]. TXA$_2$ has a short half-life and is non-enzymatically metabolised into TXB$_2$, which is biologically inactive. TXA$_2$ promotes platelet aggregation and thrombosis [90] and promotes endothelial expression of adhesion proteins such as intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [91].

PGE$_2$ is the most widely studied prostaglandin and exerts a vast array of biological effects. The complexity of its functions may be attributed to the fact that PGE$_2$ binds four different G-protein-coupled receptors (GPCRs), EP1-4, which vary in their tissue expression and downstream signalling. Ligation of PGE$_2$ to EP1 induces the breakdown of phosphatidylinositol, thereby triggering the formation of inositol triphosphate (IP$_3$) and elevation of intracellular calcium. Engagement of EP2 and EP4 activates adenylate cyclase, which leads to an increase in intracellular cyclic AMP and subsequent activation of protein kinase A (PKA). Binding of PGE$_2$ to EP3 causes an increase in intracellular calcium and downregulation of cAMP generation [92]. An overview of EP receptor signalling is displayed in Figure 1.3. BMDMs express all EP receptors to some extent, with EP2 the most highly expressed of the four [93].
Figure 1.3 EP receptors

PGE₂ binds to four different EP receptors, with expression of these receptors varying with cell type and tissue. When PGE₂ binds to EP1, phosphatidylinositol is metabolised to yield IP₃ and the level of intracellular calcium rises. Ligation of PGE₂ to the EP2 and EP4 receptors triggers an increase in intracellular cAMP which activates PKA to initiate the signalling cascade. When PGE₂ ligates the EP3 receptor the levels of intracellular cAMP fall and an elevation in calcium occurs. The differences between the receptors contribute to the capacity of PGE₂ to exert a vast range of effects, both pro- and anti-inflammatory.
The involvement of PGE₂ in the induction and exacerbation of inflammation is well-characterised, with PGE₂ having been shown to contribute to several of the hallmarks of inflammation, including fever and pain. PGE₂ is a potent pyrogen, with elevated levels of PGE₂ detected in the cerebrospinal fluid (CSF) of febrile patients [94]. EP3 in particular has been implicated in the pyrogenic effects of PGE₂ [95, 96]. The contribution of PGE₂ to pain responses is also well-documented [97-99]. PGE₂ also promotes vasodilation [100]. Evidence from experimental models lacking PGES or one or more of the EP receptors have implicated PGE₂ in various inflammatory disease models, such as rheumatoid arthritis [98, 101], psoriasis [102] and atherosclerosis [103]. PGE₂ signalling via EP2 was even recently implicated in the process of aging [104]. In macrophages, PGE₂ has been reported to augment the production of several proinflammatory cytokines such as IL-1β and IL-6 [105-107]. PGE₂ has also been shown to activate a variety of other immune cell types, including mast cells [108], T helper 1 (Th1) cells [109, 110] and T helper 17 (Th17) cells [102, 111].

However, PGE₂ has also been shown to exert a number of anti-inflammatory functions, particularly in the environment of the lung where PGE₂ has been shown to be protective during allergic airway inflammation [112-114]. PGE₂ has also been shown to mediate immune suppression during liver injury in both human patients and mouse models [115]. PGE₂ has additionally been demonstrated to enhance production of the anti-inflammatory cytokine IL-10 [116, 117] and impair chemokine production in macrophages [118]. Some of the effects of PGE₂ are displayed diagrammatically in Figure 1.4.
PGE$_2$ is known to function as a potent pyrogen and is also involved in the pain response. In addition, PGE$_2$ can induce vasodilation. PGE$_2$ can also augment cytokine production in macrophages, as well as activating other immune cell types such as mast cells and T cells. While many of the functions of PGE$_2$ are proinflammatory, it can also elicit anti-inflammatory effects in certain settings. One prominent example of this is during airway inflammation.
Although PGs have been demonstrated to play various anti-inflammatory roles, their contribution to the induction and exacerbation of inflammation is undeniable given the widespread and successful use of NSAIDs. Based on salicylic acid, which was isolated from willow bark and known to have anti-inflammatory properties, aspirin has been used for over a century in the treatment of acute inflammation, with other NSAIDs developed in later years [119]. NSAIDs, including indomethacin and aspirin, inhibit COX activity [120, 121] and are still commonly used today to counteract pain and fever during inflammation, infection or injury. However, due to some prostanoids also playing homeostatic roles, NSAID use is not without adverse effects. For example, NSAIDs can contribute to gastrointestinal (GI) symptoms [122], which is thought to be because of the housekeeping functions that COX1 mediates in the GI tract.

1.5 Annexin A1

Annexin A1, formerly known as lipocortin-1, is a 37 kDa protein that belongs to the annexin superfamily of proteins and it is widely considered to be anti-inflammatory. Like all annexin proteins, it consists of a unique N-terminal domain and a conserved domain that facilitates binding to phospholipids in a calcium-dependent manner [123]. Annexin A1 is known to be induced by glucocorticoids [124] and one of its best characterised anti-inflammatory effects is the suppression of eicosanoid synthesis. Annexin A1 has been shown to function as a negative regulator of cPLA2 activity and thereby limits AA production [125]. This is believed to occur through direct interaction with cPLA2 [126, 127]. There is also some evidence to indicate that annexin A1 may reduce expression of cPLA2, in addition to blocking its activity [128, 129]. The same studies suggested that annexin A1 negatively regulated COX2 expression as well.

However, control of eicosanoid production is by no means the only anti-inflammatory function associated with annexin A1. Annexin A1 has been shown to assume a wide array of immunomodulatory roles in a variety of immune cell types. While some effects, such as cPLA2 inhibition, are dependent on cytosolic annexin A1, it is also well-documented that annexin A1 can be secreted [130]. Once
secreted, annexin A1 can bind to a family of GCPRs named formyl peptide receptors (FPRs), thereby giving rise to a plethora of autocrine and paracrine effects. A depiction of annexin A1 secretion and FPR binding is displayed in **Figure 1.5**. Although annexin A1 has been shown to bind to other FPRs such as FPR1 [131], most of the biological effects associated with annexin A1 have been attributed to ligation of FPR2, also known as ALX. In addition to recognition of annexin A1, FPR2 also binds damage-induced peptides and LXA₄, a well-characterised anti-inflammatory lipid mediator [132]. While the downstream effects of secreted annexin A1 binding to FPRs have been well studied, our understanding of the process of annexin A1 secretion is limited. Although annexin A1 is secreted by a number of different cell types in response to various stimuli, it does not contain a canonical signal sequence for secretion [133]. ATP-binding cassette transporter A1 (ABCA1), which is highly expressed in macrophages and functions in cholesterol efflux [134], has been implicated in the secretion of annexin A1. ABC transporters were first associated with annexin A1 secretion when it was demonstrated that various inhibitors of ABC transporters blocked annexin A1 secretion in inflamed rat mucosa [135]. It was also shown using both pharmacological and genetic methods to target ABCA1 that annexin A1 secretion by pituitary folliculostellate cells was ABCA1-dependent [136]. A more recent study showed that secretion of annexin A1 via ABCA1 ameliorated retinal inflammation in a murine model of glaucoma. Importantly, the authors also showed colocalisation of ABCA1 and annexin A1 by immunofluorescence, as well as direct interaction between the two proteins by co-immunoprecipitation [137]. A different mechanism of annexin A1 secretion was shown in neutrophils whereby annexin A1 was stored in gelatinase granules and externalised upon adhesion to endothelial cells [138]. Annexin A1 has also been reported to be cleaved intracellularly into a 33 kDa fragment, which can be secreted [139, 140]. However, much remains to be clarified in relation to the regulation of annexin A1 secretion.
Annexin A1 can be secreted from immune cells such as macrophages. Secretion of annexin A1 from other cell types occurs via the ABC transporter ABCA1. Although it is known that this transporter is abundant in macrophages, it has not been definitively demonstrated that annexin A1 secretion from macrophages requires ABCA1. Once annexin A1 is secreted, it can bind to FPRs, most commonly FPR2, either on the same cell to signal in an autocrine manner or on another cell to signal in a paracrine manner. Many immune cells, such as neutrophils, express this receptor.

**Figure 1.5 Annexin A1 secretion**

Annexin A1 can be secreted from immune cells such as macrophages. Secretion of annexin A1 from other cell types occurs via the ABC transporter ABCA1. Although it is known that this transporter is abundant in macrophages, it has not been definitively demonstrated that annexin A1 secretion from macrophages requires ABCA1. Once annexin A1 is secreted, it can bind to FPRs, most commonly FPR2, either on the same cell to signal in an autocrine manner or on another cell to signal in a paracrine manner. Many immune cells, such as neutrophils, express this receptor.
Annexin A1 has been shown to mediate immunomodulatory functions, mostly anti-inflammatory, in a variety of immune cells (summarised in Figure 1.6). Studies examining the roles of annexin A1 often make use of Anxa1−/− mice, Fpr2−/− mice and the addition of exogenous annexin A1 protein or Ac2-26, a synthetic mimetic of the annexin A1 N-terminal fragment [130]. Rather extensive literature exists on the impact that annexin A1 has on neutrophil functions. Annexin A1 has been shown to limit neutrophil migration [141], extravasation [142] and adhesion to the endothelium [143]. Annexin A1 has also been shown to promote apoptosis in neutrophils [144], as well as in monocytes [145]. Annexin A1 was also shown to hinder adhesion of monocytes to the endothelium through downregulation of α4β1 integrin [146]. Annexin A1 is known to negatively regulate mast cell degranulation, activation and cytokine secretion, meaning that it has protective effects in models of mast cell-driven diseases such as allergic conjunctivitis [147] and atopic dermatitis [148]. Annexin A1 is also known to have many anti-inflammatory roles in macrophages. It was shown to augment IL-10 production and limit NO synthesis in macrophages [149]. It has also been reported that apoptotic neutrophils release annexin A1, which functions as an “eat me” signal and stimulates phagocytosis by macrophages [150]. Recently, it was demonstrated that annexin A1 drives muscle regeneration through binding to FPR2 on macrophages and subsequent activation of AMPK. This induces an anti-inflammatory pro-repair phenotype, which promotes muscle repair [151]. Engagement of FPR2 by annexin A1 was also recently reported to enhance GM-CSF production and increase numbers of alveolar macrophages, which provided a protective effect in an influenza A murine model [152].

Post-translational modifications (PTMs) have been shown to regulate the function of annexin A1. Tyrosine phosphorylation of annexin A1 in hepatocytes was shown to occur via epidermal growth factor (EGF) signalling and resulted in the modulation of cPLA2 activity [153]. Serine phosphorylation of annexin A1 was found to be required for its translocation to the membrane in pituitary folliculostellate cells [154, 155]. Annexin A1 has also been found to be modified by itaconate [156] but the consequence of this modification, if any, is currently unknown. Itaconate is a metabolite that is increased in LPS-activated macrophages with numerous functions that largely drive an anti-inflammatory
Itaconate is synthesised as a result of metabolic reprogramming in macrophages, which will now be described.

Figure 1.6 Anti-inflammatory effects of annexin A1
Annexin A1 is highly expressed in various innate immune cells and plays a range of anti-inflammatory roles. Annexin A1 limits the activation and degranulation of mast cells. Annexin A1 is known to impair adhesion and migration of both neutrophils and monocytes. Annexin A1 also promotes apoptosis in both neutrophils and monocytes. Apoptotic neutrophils also release annexin A1, which facilitates their phagocytosis by macrophages. Annexin A1 is known to induce the production of the anti-inflammatory cytokine IL-10 in macrophages, while inhibiting NO generation.
1.6 Metabolic reprogramming in macrophages

In recent years there has been a considerable surge in research into immunometabolism, with many new discoveries revealing how immune effector functions such as cytokine production and killing of pathogens are directly coupled to specific changes in cellular metabolism. Immune cell activation usually causes drastic metabolic changes and additionally, many metabolic enzymes have been demonstrated to exhibit moonlighting activity, meaning they perform functions outside of their canonical enzymatic activity [157]. Metabolic reprogramming has proven to be of vast importance in a plethora of immune cell types, with macrophages being at the forefront of immunometabolism research.

When macrophages are stimulated with LPS, glycolysis is potently upregulated, an observation that was made several decades ago [158, 159] but more recently the mechanisms and functional consequences of this metabolic shift have been elucidated. Glycolysis is a less efficient method of producing ATP, but a potent upregulation of glycolysis can be achieved much more rapidly than an increase in oxidative phosphorylation (OXPHOS), which would require mitochondrial biogenesis. Glycolysis also provides biosynthetic intermediates to be used in other cellular pathways, such as the pentose phosphate pathway (PPP) [160]. Glycolysis has been shown to be required for essential macrophage functions including the generation of ROS [161] and opsonin-induced phagocytosis [162]. The elevated rates of glycolysis that are observed in proinflammatory macrophages are heavily dependent on hypoxia-inducible factor 1 alpha (HIF-1α). HIF-1α is induced by LPS and regulates the transcription of several glycolytic genes [163], as well as the key proinflammatory cytokine IL-1β [164]. Several glycolytic enzymes have also been found to carry out moonlighting functions in macrophages. Pyruvate kinase M2 (PKM2) functions as a glycolytic enzyme in tetrameric form but as an enzymatically inactivate dimer, it can translocate to the nucleus and serve as a co-activator of HIF-1α to promote the transcription of glycolytic and proinflammatory genes [165]. Another glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can act as an RNA-binding protein and in this manner GAPDH has been shown to modulate the translation of the cytokines IFN-γ [166] and TNF-α [167].
Fatty acid metabolism is also altered by macrophage activation. LPS-treated macrophages display an increase in fatty acid synthesis and it was shown that carbons from glucose breakdown in these cells were preferentially incorporated into fatty acids and sterols [168]. On the other hand, upregulation of β-oxidation of fatty acids is associated with IL-4-treated macrophages. This metabolic shift in M2 macrophages occurs via STAT6 and PCG-1β and has been found to promote an anti-inflammatory phenotype by reducing ROS production and ER stress [169]. Amino acid metabolism can also regulate macrophage effector function, particularly with regards to arginine. Proinflammatory macrophages use arginine as a substrate for inducible nitric oxide synthase (iNOS) to generate NO [170], whereas M2 macrophages convert arginine to citrulline via arginase, resulting in a less inflammatory phenotype [171].

Macrophages also undergo changes in oxidative metabolism depending on their activation status. Activation of macrophages with IL-4 causes an upregulation of OXPHOS. M2 macrophages use OXPHOS as their primary source of ATP and therefore the tricarboxylic acid (TCA) cycle, also known as the Krebs cycle, remains intact. Conversely, stimulation of macrophages with LPS results in downregulation of OXPHOS and a Krebs cycle that is broken in two places, resulting in an accumulation of citrate [172] and succinate [164]. It was shown that succinate accumulated in LPS-treated macrophages, leading to the stabilisation of HIF-1α and the transcription of IL-1β, among other proinflammatory genes [164]. It has also been demonstrated that the accumulation of succinate in LPS-stimulated macrophages accelerates succinate dehydrogenase (SDH) activity, the only enzyme that plays a role in both the TCA cycle and the electron transport chain. This surge in SDH activity, in addition to an increase in mitochondrial membrane potential (MMP), generates mitochondrial ROS, seemingly through reverse electron transport at complex I of the electron transport chain [173]. The accumulation of citrate fuels fatty acid synthesis, which is crucial for membrane biogenesis. The first step in this process is the conversion of citrate to acetyl CoA, which is carried out by ATP citrate lyase (ACLY) [174]. Acetyl CoA also plays a key role in epigenetic modifications, as it is required for acetylation of histones and other cellular proteins [175]. In addition, citrate can be used to fuel production of itaconate, which will be discussed in detail in subsequent sections. The key
metabolic differences between proinflammatory and anti-inflammatory macrophages are highlighted in Figure 1.7.

It is common to use derivatives of metabolites as experimental tools when studying immunometabolism, as the non-derivatised form of the metabolite of interest might not be cell-permeable or there may be stability issues. There is also growing interest in using metabolite derivatives therapeutically. An example of a metabolite derivative that is already approved for clinical use is dimethyl fumarate (DMF), which is commonly used for treatment of multiple sclerosis (MS) [176].
Figure 1.7 Metabolic reprogramming of macrophages

LPS-activated (M1) macrophages have a very different metabolic phenotype to IL-4-activated (M2) macrophages. LPS-activated macrophages potently upregulate glycolysis and GLUT1 expression for glucose uptake. The transcription factors NF-κB and HIF-1α are activated and induce transcription of many proinflammatory genes. The Krebs cycle is broken at two points, resulting in the accumulation of citrate and succinate. Much of the respiratory function of the mitochondria is lost. Macrophages stimulated with IL-4 and IL-13 also upregulate glycolysis, but not to the same extent as M1 macrophages. The transcription factors STAT6 and PGC-1β are activated and transcribe a variety of anti-inflammatory genes. The Krebs cycle is intact and there is a substantial boost in oxidative phosphorylation. β-Oxidation of fatty acids is also upregulated in M2 macrophages.
1.7 Microbe-derived metabolites as immunomodulators

In the previous section, the effect that endogenous metabolites can have on the function of macrophages and other immune cells was discussed. However, there is also a growing literature on how exogenous, microbe-derived metabolites can influence specific immune functions during host-pathogen or commensal interactions. Extensive literature exists on the metabolic interactions between commensal bacteria in the gut and the host immune system. For example, commensal bacteria are known to secrete large quantities of the short chain fatty acid n-butyrate, which was demonstrated to attenuate LPS-induced IL-6, IL-12 and NO production through inhibition of histone deacetylase (HDAC) activity [177]. Butyrate was also shown to enhance anti-microbial functions of macrophages, again through HDAC inhibition [178]. Intestinal commensal bacteria also produce acetate, which has been reported to modulate production and reactivity of IgA [179]. In addition to commensal-derived metabolites, pathogen-derived metabolites can also have profound effects on host immunity. *Streptococcus pneumoniae* was found to secrete a metabolite termed 2-pentylfuran, which augments cytokine production, ROS generation and phagocytosis in microglia [180]. The protozoan parasite *Plasmodium*, the causative agent of malaria, infects red blood cells and metabolises host hemoglobin to yield the metabolite hemozoin. It has been reported that hemozoin, in a similar manner to urate crystals, activates the NOD-like receptor containing pyrin domain 3 (NLRP3) inflammasome and thereby increase IL-1β secretion [181].

The protozoan parasite *Trypanosoma brucei* (*T. brucei*) is the etiological agent of human African trypanosomiasis (HAT), more commonly known as sleeping sickness. *T. brucei* is known to secrete a class of metabolites termed aromatic keto acids, AKAs, which includes indole-3-pyruvate (I3P), a key focus of this thesis. Recent reports have described how AKAs can impair proinflammatory macrophage functions. I3P was found to decrease HIF-1α levels in LPS-stimulated macrophages, thereby impairing glycolysis and production of the proinflammatory cytokine IL-1β [182]. Several *T. brucei*-derived AKAs, including I3P, were also shown to activate the NRF2 antioxidant pathway and reduce secretion of IL-6 from
microglia [183]. I3P has also been shown to activate the aryl hydrocarbon receptor (AhR) [184-186], which has multiple immunological roles.

1.8 Human African trypanosomiasis

Human African trypanosomiasis (HAT) is one of the world's neglected tropical diseases. *T. brucei*, an extracellular parasitic protozoan of the genus *Trypanosoma*, is the causative agent. The disease is commonly known as sleeping sickness in humans and can be fatal if left untreated. Humans can be infected with two sub-species of the parasite, *T. brucei gambiense*, which cause a more chronic disease progression with slower onset, and *T. brucei rhodesiense*, which causes a more acute form of disease associated with rapid onset [187]. Early-stage symptoms include fever, weight loss and general malaise but rare cases of myocarditis can also occur. In the second stage of HAT progression the parasite may cross the blood brain barrier, yielding more serious neurological symptoms which can potentially be fatal. Late-stage symptoms include mental disturbances, behavioural changes and sensory and motor system issues, as well as the sleep cycle disturbances that lend the disease its name [188]. In addition to causing human disease, *T. brucei* can also infect livestock with the disease in domestic animals such as cattle termed Nagana. This can have a devastating economic impact in impoverished communities. Treatments for HAT remain limited and unfortunately drug resistance is also an increasing feature [189].

*T. brucei* is transmitted through the bite of a tsetse fly. When an uninfected tsetse fly takes a blood meal from an infected mammalian host it can ingest trypanosomes that have assumed the short and stumpy trypomastigote form, which is optimal for uptake by the fly [190]. In the midgut of the tsetse fly, the trypanosomes then differentiate into procyclic trypomastigotes and subsequently migrate to the salivary glands of the fly. There they differentiate into the epimastigote form and proliferate to much greater numbers. The trypanosomes then differentiate into the metacyclic trypomastigote form, which possess the ability to infect prospective mammalian hosts when the tsetse fly takes a blood meal. Once the metacyclic trypomastigotes have entered the bloodstream of the mammalian host, they differentiate into long, slender trypomastigotes and proliferate rapidly in the blood, as well as the lymph and CSF. A certain proportion of these trypanosomes will
differentiate into the stumpy trypomastigote form, which is the only form that is viable in the fly midgut and thus the life cycle of *T. brucei* begins again [191]. The life cycle of the *T. brucei* is visually represented in Figure 1.8.

Figure 1.8 *T. brucei* life cycle
The extracellular protozoan parasite *T. brucei* infects two types of hosts during its life cycle, the tsetse fly and mammals, including humans. When an uninfected tsetse fly takes a blood meal from an infected mammal it can ingest the stumpy form of the bloodstream trypomastigotes. These bloodstream trypomastigotes then differentiate into procyclic trypomastigotes in the fly midgut, before migrating up to the salivary glands of the fly. There, the procyclic trypomastigotes transform into epimastigotes in order to proliferate. They subsequently differentiate into metacyclic trypomastigotes and can be injected into the mammalian host upon the tsetse fly’s next blood meal. The metacyclic trypomastigotes differentiate into slender bloodstream trypomastigotes and rapidly proliferate by binary fission in the mammalian bloodstream and CSF. It is at this point in the *T. brucei* life cycle that large amounts of aromatic keto acids such as I3P are secreted. A certain proportion of the parasites then differentiate into the stumpy form, which is optimal for uptake by a tsetse fly to continue the life cycle.
A key molecular feature of the bloodstream stage of HAT is a perturbation of aromatic amino acid metabolism. Levels of the amino acids tryptophan, phenylalanine and tyrosine in infected animals are significantly decreased relative to healthy controls [192-195]. This decrease is inversely linked to a simultaneous increase in AKAs, such as I3P as mentioned above, as well as phenylpyruvate (PP) and 4-hydroxyphenylpyruvate (HPP), in the serum and urine of infected animals [182, 196-198]. These AKAs are formed by the transamination of amino acids by the parasitic enzyme cytoplasmic aspartate aminotransferase (cASAT) [199, 200], as per Figure 1.9. Interestingly, the abnormal levels of AKAs in the blood and urine of infected animals, detectable by a pungent odour and red or brown colour of the urine, has traditionally been used as a diagnostic tool in order to identify infected livestock [201]. As previously mentioned, I3P has emerged as having immunomodulatory properties in recent years [182, 183].
The *T. brucei* enzyme cASAT converts the amino acids phenylalanine, tyrosine and tryptophan to the aromatic keto acids phenylpyruvate, 4-hydroxyphenylpyruvate and indole-3-pyruvate respectively. α-Ketoglutarate (α-KG) is utilised as a cofactor for this reaction, yielding glutamate.

Figure 1.9 cASAT transaminates amino acids to form keto acids
During the course of infection, trypanosomes proliferate to extremely high numbers in the blood of infected individuals, at times reaching up to $0.2-1 \times 10^9$ cells per mL of blood [202]. The constant exposure of the parasite to the host immune system places the parasite under unique evolutionary pressure to both evade these unfavourable immune responses while also prolonging host survival to increase the likelihood of disease dissemination to other hosts via the tsetse fly. The most notable mechanism of immune evasion by trypanosomes is the rapid antigenic variation of the variable surface glycoproteins (VSGs) that entirely cover the surface of the trypanosome, which enables *T. brucei* to evade host antibodies [203, 204]. The manner in which VSGs are densely packed on the surface of the trypanosome also confers shielding of other parasitic PAMPs from innate immune recognition. Detection of VSGs by the innate immune system has been shown to be dependent on MYD88 signalling and a role for TLR9 in the detection of *T. brucei* DNA has been observed [30]. Although *T. brucei* evasion strategies that target host PGs have not yet been described, alterations in PG secretion have been demonstrated during infection with *T. brucei* in mice. Macrophages isolated from infected mice during the first peak of parasitaemia displayed enhanced secretion of PGE$_2$, whereas macrophages taken at a later stage of infection had reduced capacity for both basal and LPS-induced PGE$_2$ secretion [205]. Inhibiting PG production could promote host survival by limiting the febrile response and furthermore, certain PGs have been shown to be directly trypanocidal [206, 207]. However, the mechanism of PG modulation by *T. brucei* was uncharacterised prior to this study.

1.9 The aryl hydrocarbon receptor

As stated above, I3P has been shown to activate the aryl hydrocarbon receptor (AhR), which is a ligand-activated transcription factor that functions as a xenobiotic sensor. Prior to its activation, AhR is maintained in the cytosol as part of a chaperone complex consisting of the AhR-interacting protein (AIP), the 90 kDa heat shock protein (HSP90), the kinase Src and the co-chaperone p23 [208]. The formation of this complex stabilises cytosolic AhR and maintains it in a conformation that displays high affinity for its agonists [209]. Upon ligand binding,
AIP dissociates from the complex and allows AhR to interact with importin-β, which mediates its nuclear import. In the nucleus, AhR then heterodimerises with its binding partner, AhR nuclear translocator (ARNT), which is also known as HIF-1β. The AhR-ARNT complex can then bind to xenobiotic response elements (XREs) in the promoters of relevant genes and induce their transcription. The AhR repressor (AhRR) negatively regulates AhR transcriptional activity by competing for interaction with ARNT in order to limit its availability [210]. It has also been shown that HIF-1α can compete for ARNT binding in a similar manner [211]. Classic AhR genes include Cyp1a1, Cyp1b1 and Ahrr, which yields a negative feedback loop. Many AhR target genes, such as the CYP enzymes, are involved in the detoxification of xenobiotics and therefore function in the breakdown of AhR ligands themselves [212]. XREs have also been identified in the promoter regions of a number of immune genes, including COX2 [65, 66]. An overview of AhR signalling is provided in Figure 1.10.
Figure 1.10 AhR signalling

Under homeostatic conditions, AhR is maintained in its inactive state bound to AIP, HSP90, Src and p23. Upon binding of a xenobiotic ligand to AhR, this chaperone complex dissociates and AhR can translocate to the nucleus. There AhR can interact with its binding partner ARNT, a process that is inhibited by AhRR. The AhR-ARNT heterodimer can then bind to XREs in the promoters of target genes and upregulate their transcription.
The AhR is often referred to as a promiscuous receptor given the wide range of ligands it binds. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is widely considered to be the prototypic xenobiotic agonist and the most potent AhR activator. This is a major constituent of Agent Orange, a herbicide which was used as a chemical weapon during the Vietnam war [213]. However, many non-xenobiotic physiological AhR ligands have also been identified, including many tryptophan breakdown products, such as I3P [184-186]. Cruciferous vegetables are also known to serve as a source of AhR-activating compounds such as indole-3-acetonitrile and indole-3-carbinol [214].

AhR has been demonstrated to play many roles in immunity. AhR has been implicated in both proinflammatory and anti-inflammatory pathways, likely due to differences in cell type and the AhR ligand in question. For example, TCDD was found to induce regulatory T (T_{reg}) cells that were protective during experimental autoimmune encephalomyelitis (EAE) in mice whereas another AhR ligand 6-formylindolo(3,2-b)carbazole (FICZ) enhanced Th17 differentiation and cytokine production, thereby exacerbating disease [215]. Use of the AhR ligand VAF347 was shown to attenuate inflammatory functions of dendritic cells and thereby promote graft tolerance [216] and ameliorate allergic lung inflammation [217]. AhR deficiency has been shown to aggravate a murine model of colitis and disrupt intestinal epithelium integrity [218].

1.10 Post-translational modification by metabolites in immunity

It has already been discussed how changes in metabolite concentrations in immune cells contribute to their effector functions. However, in recent years post-translational modifications of proteins has emerged as a key means by which intracellular metabolites can modulate immunity [219]. For instance, I have previously described how many immune cells undergo a potent upregulation of glycolysis upon activation. This metabolic shift means that large amounts of lactate are produced and secreted and recently, lactate was shown to modify histones in a process termed lactylation. In proinflammatory macrophages, lactylation of histones at promoter regions of genes increases their expression and genes
associated with wound healing, such as Arg1, were found to be particularly upregulated [220]. This metabolite-derived PTM therefore provides a mechanism by which proinflammatory macrophages can adopt a more homeostatic phenotype at later time points in order to facilitate the resolution of inflammation.

Changes in the lipid metabolism of immune cells can also lead to protein modification. Acetyl CoA is converted to malonyl CoA for the purpose of fatty acid and cholesterol synthesis. The concentration of malonyl CoA increases upon stimulation of resting macrophages with LPS and it was reported that in these proinflammatory macrophages the glycolytic enzyme GAPDH becomes malonylated. This lysine PTM enhances its glycolytic activity, leading to production of IL-1β, while limiting the RNA-binding capacity of GAPDH, which allows TNF-α to be translated [221]. Fatty acid synthesis is upregulated in M1 macrophages [168], activated dendritic cells [222], effector CD4+ T cells [223] and activated B cells [224] and is considered to be crucial for membrane biogenesis and the production of some proinflammatory mediators. However, the fatty acids palmitate and myristate can also regulate immune effector functions through PTMs termed palmitoylation and myristoylation, which occur on cysteine and glycine residues respectively. TLR2 has been shown to undergo palmitoylation, which regulates its cell surface expression and signalling capacity [225]. Palmitoylation has also been reported to modulate the localisation of linker of activation for T cells (LAT) during T cell receptor (TCR) engagement [226, 227]. In macrophages, myristoylation of TLR signalling adaptor TRAM facilitates its association with TLR4 upon LPS stimulation and is required for downstream signalling and activation of the transcription factors NF-κB and IRF3 [228]. In T cells, it was demonstrated that myristoylation of AMPK mediates its activation and translocation to the lysosome where it can inhibit mTORC1 activation [229].

As previously mentioned, an accumulation of succinate occurs in proinflammatory macrophages due to breakpoints in the Krebs cycle. This can give rise to a PTM on lysine residues termed succinylation. Succinylation of PKM2 at K311 has been shown to impair its glycolytic activity and induce its nuclear translocation with a concomitant increase in IL-1β production. Succinylation of PKM2 was also shown to contribute to inflammation during a murine model of colitis [230]. Two further
PTMs mediated by Krebs cycle-derived metabolites, itaconate and fumarate, will be discussed in the following sections.

1.11 Itaconate and its derivatives

The accumulation of citrate that occurs as a result of the disrupted Krebs cycle in proinflammatory macrophages gives rise to itaconate synthesis. In the next step of the Krebs cycle, aconitase converts citrate into isocitrate via cis-aconitate, an intermediate which can be diverted away from the canonical Krebs cycle in order to form itaconate. In LPS-stimulated BMDMs itaconate is the most abundant metabolite, reaching concentrations of up to 5 mM [156]. The conversion of cis-aconitate to itaconate is mediated by the enzyme cis-aconitate decarboxylase (CAD, or IRG1), which is encoded by the gene Immune responsive gene 1 (Irg1). This gene was so named prior to its function being deciphered when it was discovered to be one of the most highly upregulated gene in macrophages in response to LPS [231]. The link between IRG1 and itaconate production was only made in 2013 when Michelucchi et al. demonstrated that IRG1 was the aconitate decarboxylase responsible for this reaction [232]. Preceding the elucidation of its function, IRG1 was shown to be localised to the mitochondria [233].

The role of itaconate in immune modulation has only recently been a focus but its antibacterial effects have been studied since the 1970s. Itaconate was demonstrated to exert bacteriostatic effects on Pseudomonas indigofera via inhibition of the bacterial enzyme isocitrate lyase, which is involved in the glyoxylate cycle [234]. Itaconate has also been shown to inhibit the growth of Mycobacterium tuberculosis (Mtb), Salmonella enterica [232, 235] and Legionella pneumophila [236]. Furthermore, a number of bacterial species including Yersinia pestis and Pseudomonas aeruginosa express genes encoding three separate enzymes capable of degrading itaconate and it was found that the expression of these genes was critical for the survival of these bacteria in macrophages [237], possibly indicating evolutionary pressure. In vivo, Irg1−/− mice exhibit increased susceptibility to Mtb infection as they succumbed more rapidly to the disease and had higher mortality rates than wild type controls [238].
A variety of experimental tools have been used in the studies that have illuminated the ways in which itaconate regulates immune function. A key method for revealing the effects of endogenous itaconate is the use of \textit{Irg1}\textsuperscript{-/-} mice and macrophages isolated from these animals. Exogenous itaconate is also used to treat macrophages and other cells but little is currently understood about the transport of itaconate across the plasma membrane or if a receptor exists for which itaconate is a ligand. Several derivatives of itaconate have been developed as way of boosting intracellular itaconate. One such derivative, dimethyl itaconate, DMI, was used in several studies [239, 240] but it was later demonstrated using carbon tracing that DMI cannot be metabolised to itaconate intracellularly [241]. The most commonly used itaconate derivative is 4-octyl itaconate (4-OI), which has been shown to be converted into itaconate intracellularly in macrophages [242]. While derivatives are undoubtedly a very useful tool, it is important to bear in mind that they may be more reactive and therefore effects observed with a metabolite derivative may not always be indicative of the action of the corresponding endogenous metabolite. However, the potential of itaconate derivatives based on 4-OI for clinical use is currently being explored. Recently there have also been several studies conducted using specific thiol-reactive probes in the study of itaconate, which have revealed many protein targets of itaconate which were previously unknown [243, 244].

More recently, many studies have shed light on the immunomodulatory properties of itaconate, in addition to its anti-bacterial functions. An early indication of the anti-inflammatory effects of itaconate was the implication of IRG1 in implantation during pregnancy, a process generally considered to involve immune suppression or tolerance [245]. More recently, itaconate has been shown to limit production of a number of proinflammatory cytokines including IL-1\textbeta, IL-18 and IL-6 and also impair NO synthesis [156, 239, 240]. One mechanism by which itaconate exerts its immunomodulatory effects is through SDH inhibition. Itaconate was first demonstrated to inhibit SDH in 1949 [246] but more recently it was confirmed that SDH inhibition by itaconate contributes to the accumulation of succinate observed in proinflammatory macrophages [247]. Another study also confirmed these results and demonstrated that itaconate lowers proinflammatory cytokines and NO through inhibition of SDH activity [239]. It has also been reported that itaconate can
drive immune tolerance in human monocytes [248]. β-Glucan, a fungal cell wall component, is known to induce trained immunity in monocytes and counteract immune tolerance and it was found that β-glucan mediated these effects through inhibition of IRG1 expression. These observations were found to be dependent on SDH inhibition as the addition of fumarate to bypass this inhibitory activity reversed the effects. Furthermore, single nucleotide polymorphisms (SNPs) in the genes encoding both IRG1 and SDH which were associated with sepsis were identified in humans [248], further highlighting the link between itaconate and SDH. While much of the research on itaconate has been limited to immune cells, an effect of itaconate in neurons was also observed. It was shown that during a murine model of Zika virus infection neurons upregulated IRG1 via RIPK signalling and that this metabolic shift induced an antiviral state and conferred resistance to infection. The authors also showed that the restriction of Zika virus replication was dependent on the inhibition of SDH activity [249].

However, not all of the reported effects of itaconate are due to SDH inhibition. Itaconate can be transported to the cytosol by the mitochondrial oxoglutarate, dicarboxylate and citrate transporters [156] and can thereby exert immunomodulatory functions elsewhere in the cell. An important aspect of itaconate biology is that both the endogenous metabolite and 4-OI have been shown to function as cysteine modifiers [156]. This cysteine alkylation was originally termed 2,3-dicarboxypropylation and is also sometimes referred to as itaconation. This PTM is the basis of many of the anti-inflammatory functions associated with itaconate and 4-OI. Both itaconate [250] and 4-OI [156], has been shown to modify cysteine residues on kelch-like ECH associated protein 1 (KEAP1) which functions as a negative regulator of the master antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (NRF2). This means that 4-OI is a potent activator of NRF2, which will be discussed in more detail in subsequent sections. The ability of 4-OI to activate NRF2 has been implicated in several of its anti-inflammatory and protective functions. It was firstly shown that activation of NRF2 by 4-OI decreases proinflammatory cytokine production [156] as NRF2 inhibits transcription of genes such as Il1b and Il6 [251]. The capacity of 4-OI to suppress stimulator of interferon genes (STING) [252] and to restrict SARS-CoV2 replication and pathology [253] are NRF2-dependent effects. Furthermore,
Itaconate has been found to suppress abdominal aortic aneurysm formation through its ability to activate NRF2 [254]. Therefore activation of NRF2 by itaconate and 4-OI, through modification of KEAP1, appears to contribute significantly to its anti-inflammatory actions.

However, KEAP1 is not the only protein that is modified by itaconate. The study which identified the KEAP1 PTM also discovered many more protein targets of both endogenous itaconate and 4-OI, such as lactate dehydrogenase A (LDHA) and annexin A1, which will be described in detail in a later section. The functional consequence of these modifications have not yet been elucidated but several more accounts of immunomodulatory PTMs by itaconate have emerged over the last few years, including modification of several enzymes. GAPDH was shown to be modified by 4-OI, which reduced the activity of GAPDH and thereby decreased aerobic glycolysis and associated proinflammatory cytokine production [255]. The activity of another glycolytic enzyme, aldolase A, was also shown to be impaired by 2,3-dicarboxypropylation [243]. Modification of receptor interacting protein kinase 3 (RIPK3) at cysteine 360 (C360) by itaconate was found to promote its phosphorylation and activation [244], indicating that itaconate may potentially play a role in necroptosis. It was also shown that itaconate can form adducts with glutathione and trigger the electrophilic stress response via activating transcription factor 3 (ATF3) and inhibit a secondary wave of proinflammatory cytokine transcription [240]. Furthermore, itaconate and 4-OI have been shown to modulate NLRP3 inflammasome activation and pyroptosis through two distinct protein modifications. Hooftman et al. showed that 4-OI modified C548 on NLRP3 which disrupted the interaction between NLRP3 and NEK7, thereby impairing inflammasome assembly [242]. Bambouskova et al. showed that itaconate generation limits caspase 1 activation and processing of gasdermin D, which the authors demonstrate to be modified by itaconate at C77 [256]. A number of the immunomodulatory effects of itaconate are displayed diagrammatically in Figure 1.11.
Figure 1.11 Immunomodulatory effects of 4-OI and itaconate

Itaconate is synthesised from the Krebs cycle metabolite cis-aconitate in a reaction catalysed by the enzyme IRG1. Itaconate can inhibit activity of SDH, which functions as complex II of the electron transport chain. Once synthesised, itaconate can be transported out of the mitochondrion and exert immunomodulatory effects in the cytosol. The cell-permeable derivative 4-OI shares a number of targets with itaconate. 4-OI modifies and impairs the activity of two glycolytic enzymes, GAPDH and aldolase. Itaconate and derivatives also activate the transcription factor ATF3, which is involved in the integrated stress response. Itaconate and 4-OI impair NLRP3 inflammasome assembly through 2,3-dicarboxypropylation of NLRP3, which disrupts its direct interaction with NEK7. Itaconate can also modify gasdermin D and hinder its processing by caspase 1. Both of these modifications can lead to decreased secretion of IL-1β and IL-18, as well as reduced pyroptosis. 4-OI and itaconate also modify crucial cysteines on KEAP1, which enables NRF2 to translocate to the nucleus. In the nucleus, NRF2 induces
genes involved in the antioxidant response and also downregulates certain proinflammatory cytokines.

Itaconate has also been implicated in the IFN response but the interplay between itaconate and IFN signalling is somewhat complex. Type I IFNs are critical for antiviral defence but dysregulation of the IFN response has also been linked to several chronic inflammatory diseases. Firstly, it was found that IFNs induce transcription of \( lrg1 \) [236] and additionally, interferon regulatory factor 1 (IRF1), a transcription factor that is activated by type I IFNs, was identified as a potential regulator of \( lrg1 \) [257]. It was later demonstrated that the induction of \( lrg1 \) by LPS and the TLR3 agonist Poly(I:C) occurred in an IFN-dependent fashion and that 4-OI potently blocked type I IFN production [156]. However, it was later shown that both exogenous and endogenous itaconate boosted type I IFN secretion, as well as the induction of interferon-stimulated genes (ISGs) [258], which indicates that itaconate and 4-OI or other derivatives can have very different effects on some biological processes. The mechanisms by which 4-OI downregulates and itaconate boosts type I IFNs have not yet been elucidated.

**1.12 Fumarate and DMF**

Fumarate is another Krebs cycle metabolite that has been shown to modify cysteine residues on target proteins in a modification termed succination (not to be confused with succinylation) [259]. Although this modification has been more extensively studied in the context of cancer and diabetes [260, 261], it has recently been shown to regulate certain immune processes. Similar to itaconate biology, several derivatives of fumarate have been developed, including dimethyl fumarate (DMF), which is already used clinically in the treatment of MS and psoriasis [176]. The action of DMF as an anti-inflammatory drug was discovered serendipitously but more insights into its mechanisms of action are emerging all the time. DMF is known to succinate key cysteine residues on KEAP1 [262] and can therefore activate NRF2 in a similar manner to 4-OI. Endogenous fumarate has also been shown to modify these cysteine residues on KEAP1 to activate NRF2 [263]. More recently, DMF was shown to modify C150 on GAPDH in murine macrophages and T cells, as well as the corresponding active site cysteine (C152) for human GAPDH.
in PBMCs from MS patients treated with DMF [264]. The authors also show that GAPDH could be modified by endogenous fumarate in both murine and human immune cells and that this PTM impaired glycolytic flux and lowered proinflammatory cytokine production. It was also reported that both DMF and endogenous fumarate succinate gasdermin D [265]. This PTM was found to impair the interaction between gasdermin D and caspases, thereby limiting pyroptosis. It is quite striking that itaconate and fumarate share several protein targets for modification, including KEAP1, GAPDH and gasdermin D. DMF has also been shown to modify NF-κB p65 and thereby abrogate NF-κB signalling [266]. Figure 1.12 displays several targets of succination that have immunomodulatory consequences.
Both DMF and endogenous fumarate succinate KEAP1, which enables NRF2 to translocate to the nucleus. There NRF2 upregulates genes involved in the antioxidant response and downregulates some proinflammatory cytokines. DMF and fumarate also modify GAPDH, which impairs glycolysis, which is required for the production of pro-inflammatory cytokines such as IL-1β. Finally, DMF and fumarate have been shown to succinate gasdermin D, which limits pyroptosis and cytokine release.
1.13 NRF2 signalling

NRF2 is a transcription factor that is considered to be the master regulator of the antioxidant response. Under basal conditions, NRF2 is maintained in an inhibited state in the cytosol bound to its negative regulator KEAP1 and the ubiquitin ligase Cullin 3, which targets it for degradation. KEAP1 is a cysteine-rich protein, which allows it to function as a sensor of oxidative and electrophilic stress. Under conditions of increased ROS or cellular stress, cysteine residues on KEAP1 become oxidised and this prevents the proteasomal degradation of NRF2 [267]. As previously discussed, 4-OI and DMF have been shown to activate NRF2 through modification of these key cysteines on KEAP1. Some other pharmacological activators of NRF2, such as diethyl maleate (DEM) are also known to function through these KEAP1 cysteines [268]. NRF2 can then accumulate in the cytosol and translocate to the nucleus, where it upregulates a number of genes involved in the antioxidant response. NRF2 binds to promoter regions that contain an antioxidant response element (ARE) and induces transcription of these genes [269]. One gene induced by NRF2 is Hmox1 encoding heme oxygenase 1 (HO-1), which is responsible for the oxidative cleavage of heme, thus generating biliverdin, carbon monoxide and ferrous iron [270]. NRF2 also upregulates NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1) which reduces quinones to hydroquinones in order to relieve oxidative stress [271]. NRF2 also modulates both the synthesis and the redox state of intracellular glutathione levels through induction of glutamate-cysteine ligase and glutathione reductase [272, 273]. An overview of NRF2 signalling is provided in Figure 1.13.

NRF2 has been recognised as having a protective role in several inflammatory models, including airway inflammation [274] and sepsis [275]. At a molecular level, NRF2 has also been shown to regulate proinflammatory mediators. As well as dampening inflammation through redox control, NRF2 was also discovered to directly bind to the promoters of the proinflammatory cytokines IL-1β and IL-6 and block their transcription by inhibiting RNA polymerase II recruitment. It was also demonstrated that the control of these cytokines by NRF2 was independent of both ROS levels and the classic ARE sequence [251]. More recently, activation of NRF2 was shown to induce COX2 expression in melanoma cells. However, the authors
showed that NRF2 does not bind to the promoter of *Ptgs2* (the gene encoding COX2) but instead upregulates ATF4, which directly induces transcription of *Ptgs2* [276]. It remains to be clarified if this effect is also true for macrophages or other immune cells.
Under basal conditions, NRF2 is bound to KEAP1 and the ubiquitin ligase Cullin 3, which targets NRF2 for proteasomal degradation. Under conditions of cellular oxidative stress, crucial cysteines on KEAP1 such as Cys151, are modified. This permits newly synthesised NRF2 to translocate to the nucleus and bind to AREs in promoters of various genes. NRF2 upregulates transcription of genes involved in the antioxidant response such as Hmox1 and Nqo1. NRF2 also binds to the promoters of genes encoding proinflammatory cytokines such as IL-1β and IL-6 and thereby inhibits their transcription.
1.14 Aims

This project concerns the regulation of PG synthesis by metabolites. The overall aim of the project was to determine if the parasite-derived metabolite I3P and the host metabolite itaconate, as well as the itaconate derivative 4-OI, modulate PG production in macrophages. Due to reports in the literature of perturbations in PG production during *T. brucei* infection [205], I hypothesised that a trypanosome-derived secreted factor may influence PG synthesis. As I3P had already been demonstrated to play immunomodulatory roles during infection [182, 183], it seemed like an attractive candidate to explore. As annexin A1, which can limit PG synthesis, had been shown to undergo 2,3-dicarboxypropylation [156, 243, 244, 250], I hypothesised that itaconate and 4-OI might impact on PG production in macrophages. I sought to test these hypotheses with the specific aims as follows;

- To determine the effect of I3P on PG production and related enzyme expression
- To establish a mechanism for the effect of I3P on PG production
- To determine the effect of the Krebs cycle metabolite derivates 4-OI and DMF on PG production and related enzyme expression
- To investigate if endogenous itaconate modulates PG production
- To determine if itaconate and 4-OI affect other aspects of annexin A1 biology, separate to the regulation of PG synthesis

Overall I have found that I3P inhibits PG production via COX inhibition. I have demonstrated that both 4-OI and DMF impair transcription of COX2 and downstream PG production in an NRF2-independent manner. I have also identified a difference between the effect of endogenous itaconate and 4-OI on this pathway. My work therefore provides new insights into the immunomodulatory properties of I3P, 4-OI and DMF, that could have therapeutic consequences in the effort to develop novel anti-inflammatory agents.
Chapter 2:  
Materials and Methods
2.1 Materials

2.1.1 Buffers

Composition of the various buffers used is provided in Table 2.1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffered saline (PBS) (10X)</td>
<td>1.45 M NaCl, 39 mM NaH₂PO₄, 22.7 M Na₂HPO₄</td>
</tr>
<tr>
<td>Phosphate-saline-glucose (PSG) buffer</td>
<td>3 mM NaH₂PO₄, 57 mM Na₂HPO₄, 44 mM NaCl, 5 mM KCl, 1 mM MgCl₂, (pH 8.0)</td>
</tr>
<tr>
<td>Sample lysis buffer</td>
<td>0.125 M Tris pH 6.8, 10% glycerol, 0.02% SDS, 5% DTT. DTT is added immediately before use.</td>
</tr>
<tr>
<td>SDS-PAGE running buffer (10X)</td>
<td>25 mM Tris, 192 mM glycine, 0.1% SDS</td>
</tr>
<tr>
<td>SDS-PAGE transfer buffer (10X)</td>
<td>0.25 M Tris, 1.9 M glycine, 35 mM SDS</td>
</tr>
<tr>
<td>Tris-buffered saline tween (TBST) (10X)</td>
<td>12.11g Tris, 87.6g NaCl, 10 mL Tween-20, distilled H₂O to 1 L.</td>
</tr>
</tbody>
</table>

Table 2.1 Buffer composition

2.1.2 Animals

BMDMs were generated from the bone marrow of C57BL/6 mice (Harlan UK). The animals were housed under specific pathogen-free conditions in accordance with Irish and European Union regulations. All experiments were subject to prior ethical approval by Trinity College Dublin Animal Research Ethics Committee and Health Products Regulatory Authority (HPRA). *Irg1*⁻/⁻ mice (named C57BL/6N-Acod1<sup>em1(IMPC)</sup>/J) were generated by CRISPR-targeted deletion of exon 4 of *Irg1* and were purchased from the Jackson Laboratory. Age-matched wild-type mice were used as controls. Bones from NRF2 knockout, KEAP1 knockdown and wild-
type controls were kindly provided by Professor Albena Dinkova-Kostova (University of Dundee, UK).

2.1.3 Human blood samples

PBMCs were isolated from buffy coats obtained from the Irish Blood Transfusion Service. All experiments performed on human samples were subject to prior approval from the School of Biochemistry and Immunology Research Ethics Committee (TCD).

2.1.4 Cell culture reagents

DMEM, RPMI and T175 flasks were purchased from Thermo Fisher Scientific. 12-well and 24-well plates were purchased from Corning. Foetal calf serum (FCS) was purchased from Biosera. Penicillin/streptomycin, trypsin-EDTA, sterile PBS and red cell lysis buffer were purchased from Sigma Aldrich. Lymphoprep was purchased from Stemcell Technologies. The human CD14+ Positive Selection kit was purchased from Miltenyi Biotec. Recombinant human M-CSF was purchased from Immunotools.

2.1.5 Stimulants, metabolites and treatments

LPS from *E.coli* (serotype EH100) was purchased from Enzo Life Sciences. Pam3CSK4 and Poly(I:C) were purchased from Invivogen. CpG (ODN 1826) was purchased from Miltenyi Biotec. R848 was purchased from Sigma Aldrich. 4-Octyl itaconate was initially supplied by Professor Richard Hartley (University of Glasgow) and results were later confirmed with commercially available 4-OI (Sigma Aldrich). 4-Octyl-2-methyl succinate was also synthesised by Professor Richard Hartley. Dimethyl sulfoxide (DMSO), indole-3-pyruvate (I3P), itaconic acid, dimethyl fumarate (DMF), diethyl maleate (DEM), arachidonic acid, indomethacin, NS-398, AH6809, PGE2, forskolin, 3-methylcholanthrene (3MC) were purchased from Sigma Aldrich. Murine recombinant IFN-γ was purchased from Immunotools. GW 627368X was purchased from Cayman Chemical.
2.1.6 Transfection reagents

RNAiMAX was purchased from Thermo Fisher Scientific. Predesigned Silencer Select siRNAs were also purchased from Thermo Fisher Scientific. The Silencer Select siRNAs used were as follows; Negative Control (4390844), AhR (4390771/s62162), NRF2 (4390815/s70522), ATF4 (4392421/s62689), Annexin A1 (4390771/s69299).

2.1.7 Western blotting reagents

The gel running apparatus was purchased from Bio-Rad. Acrylamide:bisacrylamide, sodium dodecyl sulphate (SDS), ammonium persulphate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), Tris and HCl were purchased from Sigma Aldrich. StrataClean Resin was purchased from Agilent. The Spectra Multicolor Broad Range Protein Ladder was purchased from Thermo Fisher Scientific. PVDF membrane was purchased from Merck Millipore. Milk powder was purchased from Tesco. WesternBright ECL was purchased from Advansta and SuperSignal West Femto Maximum Sensitivity Substrate was purchased from Thermo Fisher Scientific. Western blots were visualised using the ChemiDoc™ MP Imaging System from Bio-Rad.

2.1.8 Antibodies

Anti-β-actin was purchased from Sigma Aldrich. Anti-COX2 was purchased from Abcam. Anti-NRF2, anti-KEAP1, anti-IRG1, anti-α-tubulin, anti-ATF4, anti-annexin A1, anti-phospho-cPLA2 (Ser505), anti-cPLA2, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-p38 MAPK, anti-phospho-NF-κB p65 (Ser 536), anti-NF-κB p65, anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) and anti-p44/42 MAPK (ERK1/2) were purchased from Cell Signaling. Anti-mouse IgG and anti-rabbit IgG secondary horseradish peroxidase-conjugated antibodies were purchased from Jackson Immunoresearch.

2.1.9 PCR reagents
The Ambion RNA extraction kits were purchased from Thermo Fisher Scientific. The High-Capacity cDNA Reverse Transcription kit, 8-strip PCR tubes, 96-well qPCR plates, plate seals and SYBR green master mix were purchased from Applied Biosystems. Primers were designed in house and purchased from Eurofins Genomics. The sequences of all primers used are given in Table 2.2. cDNA synthesis was carried out using a Veriti 96-well fast thermal cycle and qPCR was carried out using a 7500 fast real-time PCR system, both purchased from Applied Biosystems.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abca1</td>
<td>5'-CAG CAC CGT GTC TTG TCT GA-3'</td>
<td>5'-GAG ACA TCG ATG GTC AGC GT-3'</td>
</tr>
<tr>
<td>Ahr</td>
<td>5'-GGC AGG ATT TGC AAG AAG GAG-3'</td>
<td>5'-TGG ATG ACA TCA GAC TGC TGA A-3'</td>
</tr>
<tr>
<td>Anxa1</td>
<td>5'-ACA ACC ATC GTG AAG TGT GC-3'</td>
<td>5'-ATT TCC GAA CGG GAG ACC AT-3'</td>
</tr>
<tr>
<td>Cd86</td>
<td>5'-TCT CCA CGG AAA CAG CAT CT-3'</td>
<td>5'-CTT ACG GAA GCA CCC ATG AT-3'</td>
</tr>
<tr>
<td>Hmox1</td>
<td>5'-CCT CAC AGA TGG CGT CAC TT-3'</td>
<td>5'-GCT GAT CTG GGG TTT CCC TC-3'</td>
</tr>
<tr>
<td>Il1b</td>
<td>5'-GGA AGC AGC CCT TCA TCT TT-3'</td>
<td>5'-TGG CAA CTG TTC CTG AAC TC-3'</td>
</tr>
<tr>
<td>Il6</td>
<td>5'-CCA CAG TCC TTC AGA GAG ATA CA-3'</td>
<td>5'-CCT TCT GTG ACT CCA GCT TAT C-3'</td>
</tr>
<tr>
<td>Nqo1</td>
<td>5'-GCT GCA GAC CTG GTG ATA TT-3'</td>
<td>5'-ACT CTC TCA AAC CAG CCT TT-3'</td>
</tr>
<tr>
<td>Ptges</td>
<td>5'GGA AGA AGG CTT TTG CCA ACC-3'</td>
<td>5'-CGA AGC CGA GGA AGA GGA AA-3'</td>
</tr>
<tr>
<td>Ptgs2</td>
<td>5'CGG ACT GGA TTC TAT GGT GAA A-3'</td>
<td>5'CTT GAA GTG GGT CAG GAT GTA G-3'</td>
</tr>
<tr>
<td>Rps18</td>
<td>5'-GGA TGT GAA GGA TGG GAA GT-3'</td>
<td>5'-CCC TCT ATG GGC TCG AAT TT-3'</td>
</tr>
</tbody>
</table>
Table 2.2 Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>5′-CGA AGC GGA CTA CTA TGC TAA A-3′</th>
<th>5′- TCC CGA ATG TCT GAC GTA TTG-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgfβ1</td>
<td>5′-TGC GCC TTT TCA AGG ATG GA-3′</td>
<td>5′-CCC CAC AGC AAA CCG TAG AT-3′</td>
</tr>
<tr>
<td>PTGS2</td>
<td>5′- TCA CCG TTT GGC TCG ATA TT-3′</td>
<td>5′-GGC AGA GGC TGT AGA TGA TT-3′</td>
</tr>
</tbody>
</table>

2.1.10 ELISA reagents

The PGE\(_2\) ELISA kit was purchased from Enzo Life Sciences. Although this item is sold as a PGE\(_2\)-specific ELISA kit, our data suggested that other COX-derived oxylipins were also detected with this kit. Therefore measurements carried out using this ELISA kit are referred to as quantifying PGs instead of PGE\(_2\).

2.1.11 COX activity assay reagents

The COX activity assay kit was purchased from Cayman Chemical and the recombinant human COX2 was purchased from R&D Systems.

2.1.12 Mass spectrometry reagents

RRHD Eclipse Plus C18 Columns were purchased from Agilent. PGE\(_2\)-d4, PGD\(_2\)-d4, 20-HETE-d6, 5-HETE-d8, 12-HETE-d8, 15-HETE-d8, 13-HODE-d4 and TXB\(_2\)-d4 standards were purchased from Cayman Chemical.

2.1.13 Software

Graphpad Prism version 9 was used to format the data and carry out statistical analysis. ImageLab 6.0 was used to analyse Western blots by densitometry. For mass spectrometry, chromatographic peaks were integrated using Multiquant 3.0.2 software (Sciex). Adobe Illustrator was used to design schematics.
**2.2 Methods**

**2.2.1 L929 cell culture**

L929 cells were cultured in DMEM containing 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin. The cells were washed with PBS before trypsin-EDTA was used to detach them and they were resuspended in fresh media. The cells were then seeded into T175 tissue culture flasks and incubated at 37°C and 5% CO2 for seven days following the point at which confluence was reached. The resulting supernatant was then harvested and utilised as a source of M-CSF for BMDM differentiation.

**2.2.2 BMDM generation and culture**

C57BL/6J mice aged between six and ten weeks were euthanised in a CO2 chamber and death was subsequently confirmed by cervical dislocation. The tibia, femur and hip were extracted and all fat and muscle removed from around the bones. The bone marrow was then flushed from these bones into a 50 mL conical tube using a 23 gauge needle and a syringe containing DMEM. The bone marrow was centrifuged at 453 x g for five minutes and the resulting pellet was then resuspended in 3 mL red cell lysis buffer. After five minutes incubation, the red cell lysis buffer was diluted with DMEM and again centrifuged at 453 x g for five minutes. Cells were resuspended in 30 mL DMEM containing 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin and 20% (v/v) L929 supernatant. The cell suspension was filtered through a cell strainer and divided between three non-coated sterile 10 cm dishes at a volume of 10 mL per dish. The cells were incubated at 37°C with a humidified atmosphere of 5% CO2 for six days.

After this incubation period, the media was replaced with PBS and cells were lifted off the plates using a cell scraper. The cells were counted and plated at a density of 0.5 x 10^6 cells per mL in DMEM containing 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin and 10% (v/v) L929 supernatant, unless otherwise stated.

Concentrations up to 1 mM I3P were used to treat BMDMs, with a previous study from our lab showing that this concentration did not affect cell viability as measured
by an LDH assay [182]. BMDMs were treated with 4-OI at concentrations up to 200 μM, with a previous study from our lab having demonstrated that 4-OI concentrations of up to 500 μM did not cause cytotoxicity as measured by LDH release [156]. BMDMs were treated with a maximal concentration of 25 μM DMF, with concentrations of up to 100 μM having been reported to cause no cytotoxicity [277]. BMDMs were treated with 100 μM DEM, which has been demonstrated to have no effect on cell viability [278].

2.2.3 PBMC and primary human macrophage isolation

The blood was diluted 1:1 with PBS and 30 mL was gently layered over 20 mL Lymphoprep in a 50 mL conical tube. The tube was then centrifuged at 400 x g for 30 minutes with the brake off. The topmost plasma layer was removed and discarded and the middle layer of PBMCs was transferred to a clean tube and washed twice with PBS. PBMCs were counted and seeded at a density of 1 x 10^6 cells per mL in RPMI containing 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin.

If human macrophages were required, CD14⁺ monocytes were isolated from the PBMCs using anti-CD14-labelled magnetic beads, according to manufacturer’s instructions. The human monocytes were then seeded at 0.5 x 10^6 cells/mL and maintained in RPMI containing 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin and 50 ng/mL recombinant human M-CSF. After six days the monocytes had differentiated into macrophages and were ready for use in experiments.

2.2.4 Trypanosome culture and lysis

Monomorphic MITat 1.1 bloodstream form trypanosomes were cultured in HMI9 medium that contained 10% FCS. Cells in the log phase of growth were centrifuged at 1500 x g for 5 min and subsequently lysed using both osmotic shock and sonication. After centrifugation the cell pellet was resuspended at 1 x 10^9 cells/mL in PSG buffer and thereafter diluted 1 in 10 with sterile water. The resulting suspension was sonicated (30% power, 50% duration, Bandelin Sonopuls) for 1 minute before being incubated at room temperature for 5 minutes. The lysate was
centrifuged at 14,000 x g for 10 minutes to yield a soluble lysate fraction. This soluble lysate fraction was subsequently used to stimulate BMDMs.

2.2.5 siRNA knockdown in BMDMs

Lipofectamine RNAiMAX was preincubated with the relevant siRNA, diluted in DMEM containing no FCS or penicillin/streptomycin. This mixture was then used to treat BMDMs so that the final concentrations were 5 μL/mL Lipofectamine RNAiMAX and 50 nM siRNA. The cells were incubated for eight hours before the media containing the transfection reagents was replaced with DMEM containing 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin. The cells were left for 24-96 hours (depending on the turnover of the protein being targeted) and were then used for experimental treatments as required.

2.2.6 Western blot

Cell lysis

The supernatant was removed from cells and the cells were lysed in 40-80 μL sample lysis buffer. The plates were carefully agitated to ensure the complete coverage of the base of each well with lysis buffer. The cell lysates were then transferred to 1.5 mL microcentrifuge tubes and incubated at 95°C for five minutes in order to denature the proteins.

Concentration of supernatants

1 μL of StrataClean Resin was added for every 100 μL of supernatant prior to a shaking incubation step for two minutes at 4°C. The supernatants were then centrifuged at 210 x g for two minutes at 4°C. The supernatants were discarded and 20 μL sample lysis buffer was added to the pellet and mixed by gentle flicking of the tubes. The supernatant proteins were eluted from the StrataClean Resin by heating the samples to 95°C for five minutes, followed by centrifugation at 210 x g for two minutes.

SDS-PAGE
SDS polyacrylamide gel electrophoresis was used to resolve proteins according to their molecular weight. Samples are loaded into wells of a 5% stacking gel, which sits on a resolving gel. A molecular weight marker was also loaded on one side of the gel to provide reference for protein sizes. The percentage of the resolving gel depended on the molecular weight of the proteins of interest, with higher percentage gels being used for smaller proteins and vice versa. Gel compositions are provided in Table 2.3. The Bio-Rad gel running apparatus was used, whereby the gels were fastened into electrode in a tank and submerged in SDS-PAGE running buffer. The samples were run through the stacking gel at 80 V and at 110 V thereafter.
Table 2.3 SDS-PAGE gel compositions

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stacking Gel</th>
<th>8% Resolving Gel</th>
<th>10% Resolving Gel</th>
<th>12% Resolving Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>2 mL</td>
<td>3.5 mL</td>
<td>3 mL</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>500 μL</td>
<td>2 mL</td>
<td>2.5 mL</td>
<td>3 mL</td>
</tr>
<tr>
<td>Tris 1 M pH 6.8</td>
<td>375 μL</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tris 1.5 M pH 8.8</td>
<td>-</td>
<td>1.9 mL</td>
<td>1.9 mL</td>
<td>1.9 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>30 μL</td>
<td>75 μL</td>
<td>75 μL</td>
<td>75 μL</td>
</tr>
<tr>
<td>10% APS</td>
<td>30 μL</td>
<td>75 μL</td>
<td>75 μL</td>
<td>75 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>3 μL</td>
<td>3 μL</td>
<td>3 μL</td>
<td>3 μL</td>
</tr>
</tbody>
</table>

Electrophoretic transfer of proteins

The proteins in the gel were then transferred to PVDF membrane using the Bio-Rad wet transfer system. To set up the transfer system, a sponge was first placed on the black, cathode side of the cassette. Two pieces of filter paper than had been soaked in transfer buffer were then placed on top of the sponge, after which the resolving gel was carefully placed on top of the filter paper. The PVDF membrane (which was previously activated in 100% methanol) was then added on top of the gel, followed by two more pieces of filter paper and another sponge. A roller was used to remove air bubbles between the layers after each addition. The cassette was then closed, secured inside the transfer tank and submerged in transfer buffer. The transfer was run at either 200 mA per tank for a duration of two hours or 40 mA per tank if left overnight.

Incubation with antibody and protein visualisation

Upon completion of the transfer, the PVDF was removed from the tank and incubated for one hour in 5% (w/v) milk powder solution made up in TBST. This
was in order to block any parts of the membrane that were not already covered by protein from the transfer. The primary antibody solution was made up in 5% (w/v) bovine serum albumin (BSA) at a dilution of 1 in 1000-5000, depending on the sensitivity of the antibody. The membrane was then incubated in primary antibody solution overnight at 4°C while continuously rolling. The membrane was then washed three times in TBST for ten minutes each time prior to incubation with a secondary antibody (diluted at 1 in 2000 in 5% (w/v) milk powder) for one hour. The membrane was then washed in TBST another three times. Chemiluminescent substrate was then added to the membrane immediately prior to protein visualisation using the Bio-Rad ChemiDoc MP™ Imaging System. Densitometry was subsequently performed using ImageLab software. All proteins were normalised to β-actin.

2.2.7 RT-qPCR

RNA extraction

RNA was extracted from cell lysates using the Ambion RNA Extraction kit. Cells were lysed in 350 μL RNA lysis buffer and further processed according to manufacturer’s instructions. 30 μL RNAse-free water was used to elute the RNA and the RNA concentration was subsequently quantified using a NanoDrop 2000 spectrophotometer. The RNA concentration of all samples was normalised to that of the lowest in each experiment using RNAse-free water.

Reverse transcription

The normalised RNA samples were converted into cDNA using the High-Capacity cDNA Reverse Transcription kit. All samples and reagents were kept on ice during preparation. 10 μL of RNA was combined with 10 μL reverse transcription master mix in 8-strip PCR tubes. The composition of the reverse transcription master mix are provided in Table 2.4. The samples were incubated in a thermal cycler at 25°C for five minutes, 42°C for 30 minutes, 85°C for five minutes followed by holding at 4°C. The resulting cDNA was diluted 1 in 6 with RNAse-free water and stored at -20°C until further use.
Reagent & Volume per sample (μL) 
--- & --- 
10X RT buffer & 2 
10X random primers & 2 
25X deoxyribonucleoside triphosphates (dNTPs) & 0.8 
Reverse transcriptase enzyme & 1 
RNAse- and DNAse-free water & 4.2 

**Table 2.4 Reverse transcription master mix components**

**qPCR**

Real-time quantitative PCR was carried out using cDNA and primers as listed in **Table 2.2**. Keeping all samples and reagents on ice during preparation, 4.8 μL of cDNA was mixed with 5.2 μL of qPCR master mix in a 96 well PCR plate. The composition of the qPCR master mix is provided in **Table 2.5**. The reaction was carried out using a 7500 Fast Real-Time PCR machine and the first time each primer was used, a melt curve was also generated. The relative expression of each gene for each sample was calculated using the $2^{-\Delta\Delta CT}$ from the CT values generated.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per sample (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>0.1</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.1</td>
</tr>
<tr>
<td>SYBR green</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table 2.5 qPCR master mix components**

**2.2.8 ELISA**

Cell supernatants were collected and PG concentrations were measured using the PGE₂ ELISA kit, according to the manufacturer’s instructions. Although this item is sold as a PGE₂-specific ELISA kit, our data suggested that other COX-derived oxylipins were also detected with this kit. Therefore measurements carried out using this ELISA kit are referred to as quantifying PGs instead of PGE₂.
2.2.9 COX activity assay

COX activity was measured using the COX Activity Assay Kit, according to manufacturer’s instructions. 1 μg of recombinant human COX2 was used per well and for these experiments DMSO was used as the solvent for I3P, rather than cell culture media. COX activity was measured by reading absorbance at 590 nm.

2.2.10 Mass spectrometry

Cell supernatants were collected and snap frozen in liquid nitrogen immediately. Samples were spiked with 2.1-2.9ng of PGE$_2$-d4, PGD$_2$-d4, 20-HETE-d6, 5-HETE-d8, 12-HETE-d8, 15-HETE-d8, 13-HODE-d4 and TXB$_2$-d4 standards prior to extraction. Lipids were extracted by adding a 2.5 ml solvent mixture (1 M acetic acid/isopropanol/hexane; 2:20:30, v/v/v) to 1 ml supernatants in a glass extraction vial and vortexed for 30 sec. 2.5ml hexane was added to samples and after vortexing for 30 seconds, tubes were centrifuged (500 g for 5 min at 4 °C) to recover lipids in the upper hexane layer (aqueous phase), which was transferred to a clean tube. Aqueous samples were re-extracted as above by addition of 2.5 ml hexane, and upper layers were combined. Lipid extraction from the lower aqueous layer was then completed according to the Bligh and Dyer technique. Specifically, 3.75ml of a 2:1 ratio of methanol:chloroform was added followed by vortexing for 30 secs. Subsequent additions of 1.25ml chloroform and 1.25ml water were followed with a vortexing step for 30 seconds, and the lower layer was recovered following centrifugation as above and combined with the upper layers from the first stage of extraction. Solvent was dried under vacuum and lipid extract was reconstituted in 100μl HPLC grade methanol. Lipids were separated by liquid chromatography (LC) using a gradient of 30-100% B over 20 minutes (A: Water:Mob B 95:5 + 0.1% Acetic Acid, B: Acetonitrile: Methanol – 80:15 + 0.1% Acetic Acid) on an Eclipse Plus C18 Column, and analysed on a Sciex QTRAP® 6500 LC-MS/MS system. Source conditions: temperature 475°C, ion spray voltage -4500, gas 1 60, gas 2 60, curtain gas 35. Lipid were detected using multiple reaction monitoring with the following precursor to product ion transitions: PGE$_2$ and PGD$_2$ [M-H]$^-$ 351.2/271.1, 15-deoxy-PGJ$_2$ 315.2/271.1, TXB$_2$ 369.2/169.1, 5-HETE 319.2/115.1, 8-HETE 319.2/155.101, 9-HETE 319.2/167.1, 11-HETE
319.2/167.102, 12-HETE 319.2/179.1, 15-HETE 319.2/219.1, 5-HEPE 317.2/115.1, 8-HEPE 317.2/155.1, 9-HEPE 317.2/167.1, 11-HEPE 317.2/167.101, 12-HEPE 317.2/179.1, 15-HEPE 317.2/219.1, 18-HEPE 317.2/259.1, 4-HDOHE 343.2/101.1, 7-HDOHE 343.2/141.1, 8-HDOHE 343.2/189.1, 10-HDOHE 343.2/133.101, 11-HDOHE 343.2/121.1, 13-HDOHE 343.2/193.1, 14-HDOHE 343.2/205.1, 16-HDOHE 343.2/233.101, 20-HDOHE 343.2/241.101, 9-ODE 295.2/171.1, 13-ODE 295.2/195.1, 5-HETrE 321.2/115.1, 15-HETrE 321.2/221.1, 9,10-DiHOME 313.2/201.1, 12,13-DiHOME 313.2/183.1, 8,9-DiHETrE 337.2/127.1, 11,12-DiHETrE 337.2/167.1, 14,15-DiHETrE 337.2/207.1, 14,15-DiHETE 335.201/207.1, 17,18-DiHETE 335.2/247.1, Deuterated internal standards were monitored using precursor to product ions transitions of: TXB2-d4 [M-H]+ 373.2/173.1, PGE2-d4 and PGD2-d4 355.2/275.1, 5-HETE-d8 327.2/116.1, 12-HETE-d8 327.2/184.1, 15-HETE-d8 327.2/226.1, 13-ODE-d4 299.2/198.1 and 20-HETE-d6 325.2/281.1. Chromatographic peaks were integrated using Multiquant 3.0.2 software (Sciex). The criteria for assigning a peak was signal:noise of at least 5:1 and with at least 7 points across a peak. The ratio of analyte peak areas to internal standard was taken and lipids quantified using a standard curve made up and run at the same time as the samples.

2.2.11 Statistical analysis

Statistical significance was established by the one-way or two-way ANOVA methods as indicated in the figure legends. For the one-way ANOVA, data was analysed with no matching or pairing. Gaussian distribution and equal standard deviations were assumed. Multiple comparisons were performed comparing the mean of each column to the mean of every other column. The Tukey test was used for correction for multiple comparisons. For the two-way ANOVA, multiple comparisons were performed comparing each cell mean with the other cell mean in that row. The Šidák test was used for correction for multiple comparisons. Data are expressed as mean ± standard error of the mean. Significance was designated as follows: *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001. GraphPad Prism version 9 software was used for statistical analysis.
Chapter 3:
Indole-3-pyruvate inhibits prostaglandin production in macrophages through inhibition of COX2
3.1 I3P inhibits PG production in macrophages

I3P is a metabolite secreted by *T. brucei*, the etiological agent of human African trypanosomiasis, otherwise known as sleeping sickness. I3P has recently been shown to elicit immunomodulatory properties such as reducing cytokine production in macrophages [182, 183]. Although perturbations in PG secretion have been reported during trypanosomiasis [205], the role of I3P in PG modulation was previously uncharacterised. This chapter of the thesis therefore explores the effect of I3P on COX2 expression and PG production in LPS-activated macrophages.

3.1.1 I3P inhibits LPS-induced PG secretion from BMDMs

In order to investigate if I3P played a role in the modulation of macrophage PG production, BMDMs were treated with various concentrations of I3P prior to stimulation with the TLR4 agonist LPS for 24 hours. The maximum concentration used was 1 mM I3P, which has been demonstrated to be non-toxic in a previous study from our lab [182]. LPS, although derived from Gram negative bacteria, is relevant in the study of *T. brucei* infection. It has been reported that the LPS concentrations in the circulation are considerably elevated during trypanosomiasis [279-281] and it has also been suggested that the presence of LPS enhances the host inflammatory response and thereby contributes to disease pathology [279]. PGE$_2$ was measured using an ELISA and as can be seen in Figure 3.1, LPS induced a robust increase in PGE$_2$, which was potently inhibited by I3P pretreatment, at concentrations as low as 62.5 μM.

3.1.2 I3P inhibits LPS-induced PGD$_2$ at six hours

As lipid-based ELISAs are known to exhibit a certain degree of non-specific binding, the ELISA results were confirmed using mass spectrometry, a more specific and sensitive method for the detection of lipids. BMDMs were pretreated with I3P prior to stimulation with LPS for six hours and at this timepoint, PGD$_2$ was the only PG detected. As can be seen in Figure 3.2, LPS caused upregulation of PGD$_2$, which was potently blocked by I3P.
3.1.3 I3P inhibits LPS-induced PGE\(_2\), PGD\(_2\), 15-deoxy-PGJ\(_2\) and TXB\(_2\) at 24 hours

In response to a longer LPS stimulation of 24 hours, the oxylipins PGE\(_2\) (**Figure 3.2A**), PGD\(_2\) (**Figure 3.2B**), 15-deoxy-PGJ\(_2\) (**Figure 3.2C**) and TXB\(_2\) (**Figure 3.2D**) were all detected by LC/MS/MS as being strongly upregulated by LPS. Furthermore, all were potently blocked by I3P pretreatment, which confirmed and extended the ELISA result. However, the levels of PGE\(_2\) detected by LC/MS/MS were considerably lower compared to detection by ELISA in **Figure 3.1**, which is likely due to lower specificity of antibody-based methods for lipid quantitation. Nonetheless, all eicosanoids downstream of COX activity appeared to be inhibited by I3P.

3.1.4 Indomethacin and I3P inhibit all PGs detected by ELISA

Since the ELISA reported higher levels of PGE\(_2\) compared to those detected by LC/MS/MS, we considered that the ELISA was reporting on COX-derived PGs more broadly. Therefore, throughout this thesis, results obtained using this ELISA will be referred to as measuring PGs rather than PGE\(_2\), even though the kit is sold as a PGE\(_2\)-specific ELISA. In order to confirm that only COX-derived oxylipins were detected by this method, BMDMs were pretreated with the pan-COX inhibitor indomethacin, which completely blocked generation of lipids detected by the ELISA, as seen in **Figure 3.4**.
Figure 3.1 I3P inhibits LPS-induced PG production in BMDMs

BMDMs were treated with various concentrations (31.25 μM to 1000 μM) of I3P or control media for one hour prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell supernatants were subsequently analysed for PG concentration by ELISA. Data are mean ± S.E.M. for n=5. **p < 0.005, ***p < 0.0005, ****p < 0.0001, ns (non-significant) p > 0.05 by one-way ANOVA.
Figure 3.2 I3P inhibits LPS-induced PGD$_2$ at six hours

BMDMs (1 x 10$^6$) were pretreated with 1 mM I3P or control media for one hour prior to stimulation with LPS (100 ng/mL) for six hours. Tandem mass spectrometry (LC/MS/MS) was performed to determine concentrations of PGD$_2$. Data are mean ± S.E.M. for n=4. ****p < 0.0001 by one-way ANOVA.
BMDMs (1 x 10^6) were pretreated with 1 mM 3-InsP or control media for one hour prior to stimulation with LPS (100 ng/mL) for 24 hours. Tandem mass spectrometry (LC/MS/MS) was performed to determine concentrations of PGE_2 (A), PGD_2 (B), 15-deoxy-Δ12,14-PGJ_2 (C) and TXB_2 (D). Data are mean ± S.E.M. for n=4. ****p < 0.0001 by one-way ANOVA.
Figure 3.4 Indomethacin and I3P inhibit all PGs detected by ELISA

BMDMs were treated with indomethacin (50 or 100 μM), I3P (0.5 or 1 mM), or the appropriate vehicle control (DMSO or media) for one hour prior to stimulation with LPS (100 ng/mL) for 24 hours. The resulting supernatants were analysed for PG concentration by ELISA. Data are mean ± S.E.M. for n=3. ****p < 0.0001 by one-way ANOVA.
3.1.5 I3P inhibits LPS-induced PG secretion from primary human macrophages

In order to investigate if I3P would also inhibit prostaglandin production in a human system, primary human macrophages isolated from CD14\(^+\) peripheral blood monocytes were used. LPS stimulation for 24 hours induced PG secretion by these macrophages, which was blocked by I3P (Figure 3.5), thereby indicating a potential role during human disease.

3.1.6 I3P inhibits trypanosome lysate-induced PG production

Although LPS is considered to be relevant in the context of trypanosomiasis, I also wanted to investigate if \textit{T. brucei}-derived material would induce PG production in BMDMs and if I3P would still inhibit PGs under these conditions. It has previously been reported that for macrophages to respond to trypanosomes, they require stimulation with IFN-\(\gamma\) [26, 30, 182]. Therefore BMDMs were co-treated with trypanosome lysate and IFN-\(\gamma\), with or without I3P pretreatment. As can be seen in Figure 3.6, stimulation with IFN-\(\gamma\) and trypanosome lysate induced PG production in BMDMs and this was again inhibited by I3P pretreatment. This mirrors the effect that I3P has on LPS-induced PGs.
Figure 3.5 I3P inhibits LPS-induced PG production in primary human macrophages

Primary human macrophages were pretreated with I3P (1 mM, 0.5 mM or 0.25 mM) or control media for one hour before they were treated with LPS (100 ng/mL) for 24 hours. The supernatants were analysed for PG concentration by ELISA. Data are mean ± S.E.M. for n=4. ****p < 0.0001 by one-way ANOVA.
Figure 3.6 I3P inhibits trypanosome lysate-induced PG production in BMDMs

BMDMs were pretreated with 1 mM I3P or control media for 30 minutes before they were treated with 100 ng/mL IFN-γ and 25 or 100 µg/mL trypanosome lysate for 24 hours. The supernatants were analysed for PG concentration by ELISA. Data are mean ± S.E.M. for n=3. ****p < 0.0001 by one-way ANOVA.
3.2 I3P boosts COX2 expression in macrophages

3.2.1 I3P augments LPS-induced Ptgs2 mRNA expression

I next investigated if the changes we had observed in PG production were due to changes in expression of the enzymes in the PG synthesis pathway with I3P. As COX2 is known to be potently upregulated by LPS stimulation [282] and is often referred to as the rate-limiting enzyme for this pathway, COX2 expression was measured in BMDMs with I3P treatment. LPS increased expression of Ptgs2, the gene that encodes COX2, but somewhat unexpectedly, pretreatment with I3P boosted LPS-induced Ptgs2 transcript (Figure 3.7A). As was reported in a previous study from our lab [182], I3P reduced Il1b transcript (Figure 3.7B) while Il6 and Tnf mRNA levels (Figure 3.7C and Figure 3.7D) were unaffected.

3.2.2 I3P increases LPS-induced COX2 protein expression

Protein expression of COX2 was next examined and a similar trend was observed. As can be seen in Figure 3.8A, I3P further augmented LPS-induced COX2 expression at both 6 hours (lane 4 compared to lane 3) and 24 hours (lane 6 compared to lane 5). As was reported in a previous study from our lab [182], I3P reduced IL-1β protein expression (lanes 4 and 6 compared to lanes 3 and 4). These results were confirmed by performing densitometric analysis on COX2 (Figure 3.8B) and IL-1β (Figure 3.8C).

3.2.3 I3P increases LPS-induced COX2 protein expression at concentrations of 500 μM

Although concentrations as low as 62.5 μM were sufficient for I3P to impair PG production (Figure 3.1), lower concentrations of I3P had no effect on COX2 induction. As seen in Figure 3.9A, concentrations of 500 μM were required for the I3P-induced increase in COX2 expression. This has been quantified using densitometry in Figure 3.9B. The observation of this I3P-induced boost in COX2 expression was unexpected given that I3P had greatly reduced the secretion of PGs.
Figure 3.7 I3P increases LPS-induced Ptgs2 mRNA expression while reducing Il1b mRNA

BMDMs were pretreated with 1 mM I3P for one hour prior to stimulation with LPS (100 ng/mL) for eight hours (A) or six hours (B-D). The cells were lysed, mRNA was extracted and the expression of Ptgs2 (A), Il1b (B), Il6 (C) and Tnf (D) was quantified by qPCR. All genes were normalised to Rps18. Data are mean ± S.E.M. for n=3. ****p < 0.0001, ns (non-significant) p > 0.05 by one-way ANOVA.
Figure 3.8 I3P increases LPS-induced COX2 protein expression while reducing pro-IL-β

BMDMs were treated with 1 mM I3P for one hour prior to stimulation with LPS (100 ng/mL) for 6 and 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=4. (B) Western blots were quantified by densitometry. Data are mean ± S.D. for n=3. **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
Figure 3.9 I3P increases LPS-induced COX2 protein expression at concentrations of 500 μM

BMDMs were treated with various concentrations (31.25 μM to 1000 μM) of I3P or control media for one hour prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=5. (B) Western blots were quantified by densitometry. Data are mean ± S.D. for n=5. **p < 0.005, ****p < 0.0001 by one-way ANOVA.
3.2.4 I3P increases LPS-induced COX2 protein expression in primary human macrophages

As I3P inhibited PG production in human macrophages in the same manner as BMDMs, it was next investigated if the capacity of I3P to boost COX2 expression was also true for human macrophages. As can be seen in Figure 3.10A, 500 μM of I3P or higher further enhanced LPS-induced COX2 expression (lanes 4 and 5 compared to lane 3). This has been quantified using densitometry in Figure 3.10B.

3.2.5 I3P augments trypanosome lysate-induced COX2 expression

As I3P inhibited trypanosome lysate-induced PG production, it was tested if I3P would also increase trypanosome lysate-induced COX2 induction. As can be seen in Figure 3.11A, I3P further boosted COX2 expression in BMDMs stimulated with *T. brucei* lysate and IFN-γ (lanes 8 and 9 compared to lanes 5 and 6). This has been quantified using densitometry in Figure 3.11B.
Figure 3.10 I3P increases LPS-induced COX2 expression in primary human macrophages

Primary human macrophages were pretreated with I3P (1 mM, 0.5 mM or 0.25 mM) or control media for one hour before they were treated with LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=4. (B) Western blots were quantified by densitometry. Data are mean ± S.D. for n=4.
Figure 3.11 I3P augments trypanosome lysate-induced COX2 expression in BMDMs

BMDMs were pretreated with 1 mM I3P or control media for 30 minutes before they were treated with 100 ng/mL IFN-γ and 25 or 100 μg/mL trypanosome lysate for 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=3. (B) Western blots were quantified by densitometry. Data are mean ± S.D. for n=3. ***p < 0.0005 by one-way ANOVA.
3.3 The augmentation of COX2 expression by I3P is regulated by a PG feedback loop

3.3.1 Addition of PGE\(_2\) attenuates the I3P-induced boost in COX2

One hypothesis considered was that perhaps the I3P-mediated inhibition of PG secretion was part of a negative feedback loop whereby decreased levels of PGs would lead to an upregulation of COX2 transcription. To test this hypothesis, exogenous PGE\(_2\) was added to BMDMs and as can be seen in Figure 3.12A, the addition of PGE\(_2\) attenuated the ability of I3P to augment LPS-induced COX2 protein (lanes 7 and 8 compared to lanes 3 and 4). This has been quantified using densitometry in Figure 3.12B.

3.3.2 An EP2 antagonist enhances the I3P-induced increase in COX2

PGE\(_2\) binds to four different EP receptors (EP1-4) [283]. EP2 expression has been suggested to be particularly important for macrophage function [93, 284]. As can be seen in Figure 3.13A, treatment with the EP1/2 antagonist AH6809 amplified the increase in COX2 with I3P treatment (lanes 7 and 8 compared to lanes 3 and 4). This has been quantified using densitometry in Figure 3.13B.

3.3.3 An EP4 antagonist enhances the I3P-induced boost in COX2 expression

EP4 has also been reported to modulate macrophage function [285, 286]. Treatment of BMDMs with the antagonist GW 627368X, which targets EP4, also enhanced the increase in COX2 with I3P treatment (Figure 3.14A, lanes 7 and 8 compared to lanes 3 and 4). This has been quantified using densitometry in Figure 3.14B.

3.3.4 Addition of forskolin attenuates the I3P-associated boost in COX2

EP2 and EP4 share similarities in their signalling pathways in that binding of PGE\(_2\) to the EP2 or EP4 receptor causes an increase in cAMP levels [283, 287]. Forskolin elevates intracellular cAMP levels and thereby mimics EP2 and EP4 signalling. As
can be seen in Figure 3.15A, treatment of BMDMs with forskolin yielded decreased COX2 protein levels in response to both LPS and I3P (lanes 7 and 8 compared to lanes 3 and 4). Collectively, these data indicate the possibility of a feedback loop whereby lower PG concentrations and hence lack of EP2/EP4 ligation may cause an increase in Ptgs2 transcription. This has been quantified using densitometry in Figure 3.15B.
Figure 3.12 PGE₂ attenuates the I3P-induced boost in COX2

BMDMs were treated with 10 μM PGE₂ for one hour and subsequently with 1 mM I3P for one hour further, prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=3. (B) Western blots were quantified by densitometry. Data are mean ± S.D. for n=3. *p < 0.05, ***p < 0.0005 by one-way ANOVA.
Figure 3.13 An EP2 antagonist enhances the I3P-induced increase in COX2

BMDMs were treated with 5 μM of the EP2 antagonist AH6809 for one hour and subsequently with 1 mM I3P for one hour further, prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell lysates were subsequently harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=4. (B) Western blots were quantified by densitometry. Data are mean ± S.D. for n=4. *p < 0.05, ***p < 0.0005 by one-way ANOVA.
Figure 3.14 An EP4 antagonist enhances the I3P-induced boost in COX2 expression

BMDMs were treated with 10 μM of the EP4 antagonist GW 627368X for thirty minutes and subsequently with 0.5 mM I3P for one hour further, prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=4. (B) Western blots were quantified by densitometry. Data are mean ± S.D. for n=4. **p < 0.005, ***p < 0.0005 by one-way ANOVA.
Figure 3.15 Forskolin attenuates the I3P-associated boost in COX2

BMDMs were treated with 10 μM forskolin for thirty minutes and subsequently with 1 mM I3P for one hour further, prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell lysates were subsequently harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=4. (B) Western blots were quantified by densitometry. Data are mean ± S.D. for n=4. ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
3.4 The augmentation of COX2 expression by I3P is partially dependent on AhR activation

3.4.1 3MC increases COX2 expression in an AhR-dependent manner

Due to previous reports of I3P activating the AhR [184-186], I next investigated if AhR was playing a role in the I3P-mediated modulation of COX2 and PG production. It has also been demonstrated that the COX2 promoter contains an XRE [65, 66], so therefore it seemed plausible that the I3P-induced boost in COX2 expression could be AhR-dependent. To investigate, the AhR agonist 3-methylcholanthrene (3MC) was used. As can be seen in Figure 3.16A, treatment of BMDMs with 3MC further increased LPS-induced COX2 expression in a similar manner to I3P (lane 6 compared to lane 4). However, 3MC had no effect on COX2 protein expression in AhR-/BMDMs (lane 12 compared to lane 10), confirming that the increase in COX2 mediated by 3MC is via AhR activation. This has been quantified using densitometry in Figure 3.16B.

3.4.2 The I3P-induced boost in COX2 expression is blunted in AhR-/ macrophages

It was also tested if I3P would maintain the ability to augment COX2 expression in AhR-/BMDMs. As can be seen in Figure 3.17A, the capacity of I3P to increase LPS-induced COX2 expression was partially impaired in the AhR-/BMDMs, compared with the induction of COX2 observed in the wild-type BMDMs (lanes 11 and 12 compared to lanes 5 and 6). This suggests that the boost in COX2 expression by I3P is at least partially dependent on activation of the AhR receptor. This has been quantified using densitometry in Figure 3.17B.
Figure 3.16 3MC increases COX2 expression in an AhR-dependent manner

BMDMs from AhR<sup>+/+</sup> and AhR<sup>−/−</sup> mice were pretreated with 10 μM 3MC for 30 minutes prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 levels were measured by Western blotting. (A) One representative image is shown for n=3. (B) Western blots were quantified by densitometry. Data are mean ± S.D. for n=3. ****p < 0.0001, ns (non-significant) p > 0.05 by one-way ANOVA.
Figure 3.17 The I3P-induced boost in COX2 expression is blunted in AhR\textsuperscript{-/-} macrophages

BMDMs from AhR\textsuperscript{+/+} and AhR\textsuperscript{-/-} mice were pretreated with I3P (1 mM) for one hour prior to stimulation with LPS (10 or 100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 levels were measured by Western blotting. (A) One representative image is shown for n=3. (B) Western blots were quantified by densitometry. Data are mean ± S.D. for n=3. *p < 0.05, ****p < 0.0001, ns (non-significant) p > 0.05 by one-way ANOVA.
3.4.3 AhR knockdown abolishes the capacity of I3P to augment COX2 expression

The capacity of I3P to further increase LPS-induced COX2 expression was also examined in BMDMs where siRNA was used to knockdown AhR. As per Figure 3.18A, this knockdown was successful in decreasing Ahr mRNA levels. As can be seen in Figure 3.18B, I3P did not boost LPS-induced Ptgs2 transcript under conditions of AhR knockdown, again indicating that this process involves AhR activation by I3P.

3.4.4 AhR knockdown has no effect on the capacity of I3P to inhibit PG production

The capacity of I3P to further inhibit PG production in BMDMs was also examined in the context of AhR knockdown. Although silencing of AhR gave rise to reduced PG levels, I3P still significantly inhibited PG production (Figure 3.19). This indicates that the enhancement of LPS-induced COX2 expression by I3P is AhR-dependent but inhibition of PG secretion by I3P occurs via an alternative mechanism.
Figure 3.18 AhR knockdown abolishes the capacity of I3P to augment COX2 expression

BMDMs were transfected with 50 nM control siRNA or Ahr siRNA for 48 hours. The cells were pretreated with I3P (0.5 or 1 mM) for one hour prior to stimulation with LPS (100 ng/mL) for 24 hours. The cells were lysed, mRNA was extracted and the expression of (A) Ahr and (B) Ptgs2 was quantified by qPCR. Both genes were normalised to Rps18. Data are mean ± S.E.M. for n=4. *p < 0.05, **p < 0.005, ****p < 0.0001 by one-way ANOVA.
Figure 3.19 AhR knockdown has no effect on the capacity of I3P to inhibit PGs

BMDMs were transfected with 50 nM control siRNA or Ahr siRNA for 48 hours. The cells were pretreated with I3P (0.5 or 1 mM) for one hour prior to stimulation with LPS (100 ng/mL) for 24 hours. The concentration of PGs in the supernatants was quantified by ELISA. Data are mean ± S.E.M. for n=4. *p < 0.05, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
3.5 I3P inhibits COX2 enzymatic activity

3.5.1 Indomethacin and I3P increase LPS-induced COX2 expression

Given the powerful inhibitory effect that I3P had on LPS-induced PGs and thromboxanes, we wondered whether I3P could be directly inhibiting COX activity. Indomethacin, a well-characterised COX inhibitor, is also built on an indole-3-acetic acid framework and hence shares structural similarities with I3P (Figure 3.20A). As already shown in Figure 3.4, I3P inhibited PGs in a similar manner to indomethacin. As had been previously shown [288, 289] and like I3P, indomethacin also increased LPS-induced COX2 expression (Figure 3.20B, lanes 5 and 6 compared to lane 4), albeit to a lesser extent than I3P (lanes 11 and 12 compared to lane 10). This has been quantified using densitometry in Figure 3.20C. Due to these similarities to the archetypal COX inhibitor indomethacin, the hypothesis that I3P was directly inhibiting COX2 was considered.

3.5.2 Addition of arachidonic acid does not overcome the inhibitory effect of I3P on PGs

In order to elucidate if COX2 activity was indeed the point of regulation by I3P, exogenous AA, the substrate of the COX enzymes (see Figure 3.21A), was added to BMDMs. As can be seen in Figure 3.21B, although addition of AA greatly increased PG production, I3P could still potently impair LPS-induced PG secretion. This served as indirect evidence that COX2 activity was inhibited by I3P, as the addition of COX substrate, AA, was insufficient to overcome the inhibitory effect.

3.5.3 I3P directly inhibits COX2 enzymatic activity

In order to definitively ascertain if I3P could directly inhibit COX2, an enzymatic activity assay was carried out. Varying concentrations of I3P were added to recombinant human COX2 and COX activity was assayed. As can be seen in Figure 3.22, I3P potently inhibited COX activity with an IC50 of 68.97 μM, thereby confirming that I3P functions as a COX2 inhibitor.
Figure 3.20 Indomethacin and I3P increase LPS-induced COX2 expression

(A) Schematic showing the structures of indole-3-acetic acid, indomethacin and indole-3-pyruvate. (B and C) BMDMs were treated with indomethacin (50 or 100 μM), I3P (0.5 or 1 mM), or the relevant vehicle control (DMSO or media) for one hour prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 levels were measured by Western blotting. (B) One representative image is shown for n=3. (C) Western blots were quantified by densitometry. Data are mean ± S.D. for n=3. *p < 0.05, ****p < 0.0001 by one-way ANOVA.
Figure 3.21 Addition of arachidonic acid does not overcome the inhibitory effect of I3P on prostaglandins

(A) Schematic depicting hypothesised inhibition of COX2 by I3P. (B) BMDMs were treated with 1 mM I3P for 45 mins, followed by addition of 5 μM arachidonic acid or vehicle (DMSO) for a further 15 minutes, prior to stimulation with LPS (100 ng/mL) for 24 hours. The concentration of PGs in the supernatants was quantified by ELISA. Data are mean ± S.E.M. for n=5. *p < 0.05, ****p < 0.0001 by two-way ANOVA.
Figure 3.22 I3P directly inhibits COX2 enzymatic activity

COX activity was measured after incubation of 1 μg recombinant human COX2 with various concentrations (31.25 μM to 1000 μM) of I3P or vehicle control (DMSO). Data are mean ± S.E.M. for n=3.
3.6 Discussion

3.6.1 Rationale

In this chapter, I have investigated the role of the trypanosome-derived metabolite I3P in modulation of COX2 expression and PG production. Previous work from our group and others had established an immunomodulatory role for I3P. McGettrick et al. demonstrated that I3P induces the hydroxylation and subsequent degradation of HIF-1α in LPS-activated macrophages. The decrease in HIF-1α levels result in impairment of LPS-induced glycolysis and a concomitant decrease in IL-1β production [182]. A recent study also showed that I3P, as well as another trypanosome-secreted aromatic keto acid, HPP, stabilised NRF2 levels [183], which downregulates the pro-inflammatory cytokines IL-1β and IL-6. Prior to the beginning of this project, I3P was also known to activate the AhR [184-186], which has been implicated in a number of immunological processes, such as regulation of T cell differentiation [215, 216] and skewing dendritic cells to a less inflammatory phenotype [217]. I3P was also shown to ameliorate inflammation in a murine colitis model, an effect shown to be via AhR activation [185]. However PG synthesis was an important inflammatory pathway that had thus far not been associated with I3P or other trypanosome-secreted factors. Although no precise mechanisms had been elucidated, perturbations to PG production in response to trypanosomes had been observed in vivo. Peritoneal macrophages isolated from mice at an early stage of T. brucei infection exhibited augmented secretion of PGE₂, whereas macrophages isolated at a later stage of infection secreted lower levels of PGE₂ basally and in response to LPS [205]. Therefore, due to the previously characterised immunomodulatory properties of I3P and the observation that PG secretion was altered during T. brucei infection, I intended to evaluate the potential of I3P as a modulator of PG production.

Over the course of this study, I have demonstrated that I3P potently inhibits prostanoid secretion from LPS-stimulated macrophages. Contrary to this I3P-induced decrease in PGs, I have shown that I3P boosts LPS-induced COX2 expression, an effect that seems to be the result of two distinct mechanisms, as depicted in Figure 3.23. I have demonstrated that the increase in COX2 expression
is partially dependent on limiting a feedback loop involving PGE\textsubscript{2} acting via EP2 and EP4 receptors. Additionally, I have provided evidence that this effect is partially dependent on AhR activation. I have also clarified that the reason I3P downregulates LPS-induced PG production despite enhancing COX2 expression is that I3P functions as a direct COX2 inhibitor. These results provide novel insight into the interactions between \textit{T. brucei} and the host immune system.

3.6.2 I3P inhibits PG production in macrophages

My results suggested that I3P potently inhibits LPS-induced PG production in macrophages. Previous work from our group [182] established that in the serum of rats infected with \textit{T. brucei}, concentrations of AKAs reach up to 0.5 mM. Although the method of detection used cannot distinguish between I3P, HPP or PP, there are reasons to believe that I3P is the predominant constituent. The kinetic properties of cASAT indicate that tryptophan is significantly preferred over tyrosine or phenylalanine as an amino acid donor [200] and furthermore, tryptophan is the amino acid most markedly reduced in infected mammals [192]. My data shows that PG secretion is reduced by I3P at concentrations as low as 62.5 \(\mu\)M, a concentration that seems very reasonable to expect to be reached during infection, based on previous data [182]. One notable observation is that PG detection by ELISA yielded much higher concentrations compared to similar experiments conducted using mass spectrometry as a more precise method of detection, although the same trends were observed regardless of method of quantification. The lower specificity of antibody-based methods for lipid detection are known to be problematic, with one study demonstrating major cross-reactivity between PGs and cannabinoid metabolites, with which they share a lipid backbone [290]. However, the result that I3P inhibited PGE\textsubscript{2} production was in agreement with the study showing that in late-stage infection macrophages secrete less PGE\textsubscript{2} [205]. Furthermore, the lipidomic screen conducted using tandem mass spectrometry showed that I3P was inhibiting all COX-derived lipids, which indicated that I3P was not targeting one of the specific PG synthase enzymes. Since the ELISA detected higher levels of PGE\textsubscript{2}, I considered it to be reporting on COX-derived PGs more broadly. In order to confirm that the ELISA detected COX-derived lipids only, I used
the pan-COX inhibitor indomethacin. Treatment with indomethacin completely blocked generation of LPS-induced lipids detected by ELISA, verifying that only COX-derived oxylipins were quantified with this method. Although the ELISA kit used for detection of PGs is sold as a PGE$_2$-specific kit, I therefore refer to measurements made using this ELISA as measuring PGs instead of PGE$_2$.

Of course, it is interesting to speculate why _T. brucei_ would have evolved to inhibit host PGs. One possibility could be that it could provide an evolutionary advantage to limit the febrile effects of PGE$_2$, which are well characterised [291-293]. It may be beneficial for the parasite to dampen fever in order to prolong host survival, which may allow further dissemination of disease. In this way _T. brucei_ would be mirroring pharmacological use of NSAIDs during infection. Another potential reason could be to limit levels of PGD$_2$. PGD$_2$ and the PGD$_2$-derived J series PGs have been shown to induce programmed cell death in trypanosomes in a manner dependent on reactive oxygen species [206]. Therefore, inhibition of PGs may be a method of self-preservation for _T. brucei_. A point that may also be of interest is that PGD$_2$ functions as a sleep-regulating hormone when secreted in the CSF [80], given that human African trypanosomiasis is associated with sleep disturbances [188]. I3P also inhibited generation of 15dPGJ$_2$, which is known to activate NRF2 [294]. In spite of this, I3P has been shown to stabilise NRF2 [183], although the mechanism by which this occurs has not been fully elucidated. I3P may also disrupt certain anti-inflammatory effects of PGs that have been reported but as is the case with NSAIDs, I3P is likely to be anti-inflammatory on balance. I3P inhibited TXB$_2$ production, the inactive breakdown product of TXA$_2$, which we can assume is also decreased by I3P. TXA$_2$ promotes platelet aggregation and coagulation [90] and interestingly, coagulation was shown to be impaired during _T. brucei_ infection in mammals [295].

For many of the experiments in this chapter I have used LPS as a stimulus. LPS, although derived from Gram negative bacteria, is relevant in the study of _T. brucei_ infection. It has been demonstrated that the concentration of LPS in the circulation is substantially elevated during trypanosomiasis [279-281] and it has been suggested that the presence of LPS contributes to disease pathology through enhancement of the host proinflammatory response [279]. However, I was also
able to show that I3P inhibited PG production induced by trypanosome lysate, when the cells were co-stimulated with IFN-γ. The majority of experiments were conducted using BMDMs but in order to evaluate translation to human infection, I also demonstrated that I3P blocked PG secretion from human macrophages. These two observations together suggest that the inhibition of PGs we have observed could hold true for trypanosomiasis in humans.

3.6.3 I3P boosts COX2 through a PG feedback loop and AhR activation

I also found that I3P yields a strong enhancement of LPS-induced COX2 expression in BMDMs, an unexpected result given I had obtained results showing potent inhibition of PG production by I3P. This result was also mirrored with LPS-induced COX2 in human macrophages and trypanosome lysate-induced COX2 in BMDMs. However, one point to note is that while concentrations of I3P as low as 62.5 μM were sufficient to block PG production, concentrations of 500 μM were required to drive a considerable increase in COX2 levels. As this is at the high end of the range of the concentrations of keto acids detected in serum in vivo [182], this may call into question the physiological relevance of this observation during infection. Nonetheless I aimed to decipher the mechanism by which I3P augmented LPS-induced COX2 expression.

One hypothesis stemmed from the idea that these two observations were linked; that the inhibition of PGs somehow gave rise to the boost in COX2 expression. This formed the concept that perhaps some type of feedback loop exists such that when low amounts of PGs are being produced, this would drive the macrophages to upregulate COX2 transcription to restore the level of PG secretion required. I found that the addition of PGE₂ reduced LPS- and I3P-induced COX2 expression, which suggested the involvement of the EP receptors in this process. PGE₂ binds four different EP receptors (EP1-4), all of which are expressed in macrophages to some extent [93] but the expression of EP2 has been particularly implicated in macrophage function [93, 284]. EP2 shares a signal transduction pathway with EP4, with engagement of both receptors leading to an activation of adenylate cyclase to generate cAMP, which leads to the subsequent activation of PKA [92].
The use of antagonists of both EP2 and EP4 augmented COX2 expression, suggesting that PGE\(_2\) binding to these receptors is the event that triggers upregulation of COX2 transcription. The observation that reduction of COX2 expression was also seen upon treatment with forskolin, which elevates intracellular cAMP concentrations, thereby mimicking ligation of EP2 and EP4, provides additional evidence to implicate EP2 and EP4 in this proposed feedback loop. However, I cannot definitively rule out the potential involvement of EP1 and EP3 without having tested antagonists or agonists for these receptors. These results may also have been strengthened by the use of an EP2 agonist, such as butaprost [104] and an EP4 agonist, such as KAG-308 [296], in addition to the antagonists used. Additionally, I did not evaluate if the exogenous addition of another PG, PGD\(_2\) for example, would exhibit the same effect. Nonetheless, the fact that I and others [288, 289] have shown that indomethacin, which inhibits PG synthesis, also boosts COX2 expression further supports the existence of a feedback loop whereby the absence of PGE\(_2\) and thereby lack of EP receptor engagement induces COX2 upregulation.

A second hypothesis for the mechanism by which I3P augments COX2 expression was that it occurred via AhR activation. I3P had previously been reported to activate the AhR [184-186] and it was also known that an XRE is present in the Ptgs2 promoter [65, 66], two findings which warranted investigation into the role AhR might play in this observed effect. My results showed that another AhR activator 3MC enhanced COX2 expression in wild-type macrophages but failed to exert the same effect in AhR\(^{-/-}\) cells. The capacity of I3P to augment COX2 expression was blunted but not entirely overcome in AhR\(^{-/-}\) BMDMs, which is likely due to the PG feedback loop described above also participating in the effect. Interestingly, there is a report in the literature of I3P attenuating ultraviolet B-induced COX2 expression in keratinocytes [297]. A potential explanation for the discrepancies between cell types is the strong degree of variation associated with AhR activation responses between different cell types and ligands [212]. However, it is also important to note that PG secretion was not measured in this study [297] as my data demonstrates that the I3P-induced increase in COX2 expression is AhR-dependent but the inhibition of PGs by I3P is not. It has also been reported that the canonical AhR ligand TCDD induces COX2 expression in fibroblasts,
which was accompanied by a concomitant increase in PG secretion [298], again suggesting that the decrease in PGs I have observed with I3P treatment is independent of AhR activation. Therefore I explored a separate potential mechanism by which I3P could block PG production. The mechanisms by which I3P boosts COX2 transcription are shown diagrammatically in Figure 3.23.

**Figure 3.23 I3P augments COX2 expression by two distinct mechanisms**

As I3P inhibits COX2 activity to block PG synthesis, PGE₂ will no longer signal in an autocrine manner via EP2 and EP4. This decrease in cAMP levels appears to upregulate the transcription of Ptgs2, the gene encoding COX2, although the mechanism by which this occurs is not fully discerned. I3P also activates AhR, which causes AhR to dissociate from its chaperone complex and translocate to the nucleus. There it will bind to and induce the transcription of genes containing an XRE in their promoter. Ptgs2 contains an XRE and therefore its transcription, and subsequent translation to protein, is boosted by I3P via AhR activation.
3.6.4 I3P inhibits COX activity

Due to the exclusion of AhR as a potential mechanism, the fact that I3P inhibited all PGs downstream of COX activity that were detected in our screen, and taking into account the structural similarities between the archetypal COX inhibitor indomethacin and I3P, I considered that I3P might be directly inhibiting COX2 enzymatic activity. Additionally, the observation that I3P could still inhibit PGs with AA supplementation suggested that I3P was not targeting the pathway upstream of COX, for instance inhibiting cPLA2 activity. This evidence was indirect but conclusive evidence was subsequently obtained using a COX activity assay. In this assay, incubation of concentrations of I3P inhibited recombinant human COX2, with an IC50 value of 69 μM. While this IC50 value is higher than that of most pharmacologically developed NSAIDs [299], it is sufficiently low such that it is likely to have functional consequences in vivo. The inhibition of COX2 activity and downstream PG production is displayed in Figure 3.24. From the data in this chapter alone we cannot conclude if I3P also inhibits the COX1 isoform. Given its structural similarity to indomethacin, it is likely that I3P inhibits COX1 as well as COX2 but in macrophages this is difficult to dissect. It has been reported that PGs in macrophages are more dependent on COX2 than COX1 [300] and indeed my data shows very little, if any, PG secretion without stimulation of the macrophages to induce COX2 upregulation. Therefore in order to determine if I3P also inhibits COX1, the enzymatic activity assay would have to be repeated using recombinant COX1 protein.

An aspect of trypanosome biology that is intriguing in this context is the fact that trypanosomes make their own PGs [301]. Counterintuitively, trypanosomes have been shown to synthesise PGD2 among their PGs, even though PGD2 has been shown to induce programmed cell death in T. brucei [206, 207]. Perhaps it is advantageous for them to inhibit host-derived PGs so that they alone can control the levels of PGD2 and other PGs, potentially as a form of population control or quorum sensing. Another notable feature of trypanosome PG synthesis is that they also secrete PGF2α. While this PG was not detected in my experiments, it is known to act as an abortifacient [302] and could potentially contribute to the clinical observation of increased risk of miscarriage during trypanosomiasis [303].
Another potential reason why inhibition of host COX activity could be beneficial to the parasite could be to indirectly regulate IFN-γ production. PGE$_2$ has been reported to downregulate type I IFN production [304, 305]. Type I IFNs have been shown to negatively regulate IFN-γ [306], which is associated with host resistance during trypanosomiasis and for this reason type I IFNs are linked to host susceptibility to trypanosomes. Therefore, inhibition of PGE$_2$ could be advantageous for the parasite as it could interfere with IFN-γ-mediated trypanosome clearance.
Figure 3.24 Trypanosome-derived I3P inhibits COX2 activity and PG synthesis

The bloodstream form of *T. brucei* secretes several AKAs, including I3P. I3P directly inhibits the enzymatic activity of COX2 in both murine and human macrophages. This leads to a dramatic decrease in the PGs and thromboxanes that are synthesised and secreted by the macrophage. Decreasing PGD₂ synthesis may be advantageous to the parasite as PGD₂ is known to be trypanocidal. Inhibiting PGE₂ production may be beneficial to *T. brucei* in order to combat the pyrogenic effects associated with PGE₂ in order to prolong host survival to ensure dissemination of the disease. Lowering PGE₂ levels may also augment type I IFNs, which is associated with trypanosome persistence.
3.6.5 Future directions

Although these data certainly provide insight into the molecular mechanisms by which I3P modulates COX2 and PGs, a weakness of this work is the lack of *in vivo* evidence. It would strengthen the ability to translate these findings to human infection if serum PGs could be measured during murine infection with wild-type and perhaps with cASAT-deficient trypanosomes. If cASAT-deficient trypanosomes did not inhibit serum PGs to the same extent as wild-type *T. brucei* but this effect could be rescued by injection of I3P, this would confirm our proposed model. A simpler experiment could be to inject mice intraperitoneally with LPS with and without I3P to evaluate if I3P can also inhibit LPS-induced PG generation *in vivo*.

It would also be informative to test if other trypanosome-secreted AKAs have similar impact on PG synthesis and COX2 transcription, as there can be overlap in the immunomodulatory roles they play. It was demonstrated that HPP and PP did not impair glycolysis as I3P did [182] and only I3P of the three AKAs tested dampened inflammation during a model of colitis [185]. On the other hand, HPP (but not PP) was found to stabilise NRF2 [183] and activate AhR [184, 185] as I3P was shown to do. Therefore it would be useful to test if HPP and PP inhibit COX activity, reduce PG secretion and augment COX2 expression in the same manner as I3P.

It would also be interesting to assess if I3P modulates other AA-derived oxylipins such as leukotrienes, as NSAIDs have been reported to enhance leukotriene synthesis in some settings [307]. When measuring PGs by tandem mass spectrometry, LOX-derived and CYP450-derived oxylipins were detected in our supernatants, however due to some background levels of these oxylipins in tissue culture serum, elevations in response to LPS were not clearly seen. For this reason, these results are inconclusive and therefore have not been included in this thesis. It would be useful to repeat these experiments under low serum conditions so that the modulation of other oxylipins by I3P can be assessed.
Chapter 4:
4-Octyl itaconate inhibits COX2 expression and prostaglandin production in macrophages
4.1 4-OI inhibits Pam3CSK-induced PG production

Itaconate is a metabolite that is highly upregulated in proinflammatory macrophages and has been shown to drive an anti-inflammatory phenotype [239, 242]. 4-OI is a derivative of itaconate which is presently under investigation as an anti-inflammatory agent. 4-OI was originally developed as an experimental tool to replicate the effects of endogenous itaconate but it is now clear that some of its functions diverge from those of the endogenous metabolite [258]. DMF is a derivative based on fumarate, another Krebs cycle metabolite. DMF is already used clinically for the treatment of multiple sclerosis and significant overlap appears to exist between the effects of 4-OI and DMF. While many immunomodulatory properties of itaconate and derivatives have been recently identified, the effect of itaconate and 4-OI on PG production had not been previously studied. This chapter of the thesis therefore investigates the effect of itaconate, 4-OI and DMF on COX2 expression and PG production in macrophages. I also explore the impact of 4-OI and DMF on secretion of annexin A1, a well-characterised anti-inflammatory effector protein.

4.1.1 4-OI inhibits Pam3CSK4-induced PG production in BMDMs

In order to investigate if 4-OI might play a role in modulation of PGs, BMDMs were pretreated with various concentrations of 4-OI prior to stimulation with the TLR1/2 agonist Pam3CSK4 for 24 hours. The highest concentration used in this experiment was 200 μM and 4-OI concentrations of up to 500 μM have been demonstrated to be non-toxic in a previous study from our lab [156]. As can be seen in Figure 4.1, Pam3CSK4 strongly upregulated PG production as measured by ELISA, which was blocked by 4-OI at concentrations as low as 25 μM.

4.1.2 4-OI impairs Pam3CSK4-induced PG secretion from human PBMCs

In order to investigate if 4-OI had a similar effect on PG secretion in human cells, human PBMCs were pretreated with 4-OI prior to stimulation with Pam3CSK4 for 24 hours. As can be seen in Figure 4.2, 4-OI also impaired PG production in human PBMCs.
Figure 4.1 4-OI inhibits Pam3CSK4-induced PG production in BMDMs

BMDMs were pretreated with various concentrations of 4-OI (25-200 μM) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA. Data are mean ± S.E.M. for n=4. ****p < 0.0001 by one-way ANOVA.
Figure 4.2 4-OI reduces Pam3CSK4-induced PG production in human PBMCs

Human PBMCs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (1 μg/mL) for 24 hours. Supernatants were analysed for PG concentration by ELISA. Data are mean ± S.E.M. for n=5 and statistically analysed by one-way ANOVA.
4.1.3 4-OI inhibits Pam3CSK4-induced PGD$_2$ at six hours

As discussed in Chapter 3 of this thesis, lipid-based ELISAs are believed to exhibit some non-specificity. Therefore, the ELISA results obtained with 4-OI were again confirmed using mass spectrometry, more specific method of detection for oxylipins. BMDMs were pretreated with 4-OI prior to stimulation with Pam3CSK for six hours and as before, PGD$_2$ was the only PG detected at this timepoint. As can be seen in Figure 4.3, Pam3CSK4 caused a boost in PGD$_2$ levels, which was decreased by 4-OI.

4.1.4 4-OI inhibits Pam3CSK4-induced PGE$_2$, PGD$_2$, 15-deoxy-PGJ$_2$ and TXB$_2$ at 24 hours

Following a longer stimulation of 24 hours with Pam3CSK4, the oxylipins PGE$_2$ (Figure 4.4A), PGD$_2$ (Figure 4.4B), 15-deoxy-PGJ$_2$ (Figure 4.4C) and TXB$_2$ (Figure 4.4D) were all detected by LC/MS/MS as being strongly upregulated by Pam3CSK. All were potently inhibited by 4-OI, which mirrors the result obtained by ELISA in Figure 4.1. As in Chapter 3, the levels of PGE$_2$ detected by LC/MS/MS were considerably lower compared to detection by ELISA, which is likely due to lower specificity of antibody-based methods for lipid quantitation. Nonetheless, all eicosanoids downstream of COX activity appeared to be potently reduced by 4-OI.

4.1.5 Indomethacin and NS-398 inhibit all Pam3CSK4-induced PGs detected by ELISA

It was already shown in Figure 3.4 that all LPS-induced PGs detected by the ELISA are inhibited by the pan-COX inhibitor indomethacin. As can be seen in Figure 4.5A, indomethacin also suppressed all Pam3CSK4-induced PGs that were detected by the ELISA. In addition, the COX2-specific inhibitor NS-398 also inhibited virtually all Pam3CSK4-induced PGs detected by the ELISA (Figure 4.5B). This provides further evidence that PG secretion from BMDMs is almost entirely dependent on the COX2 isoform.
BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. Supernatants were subsequently analysed by tandem mass spectrometry in order to determine PGD$_2$ concentrations. Data are mean ± S.E.M. for n=4. **$p < 0.005$ by one-way ANOVA.
Figure 4.4 4-OI inhibits Pam3CSK4-induced PGE₂, PGD₂, 15-deoxy-PGJ₂ and TXB₂ at 24 hours

BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. Supernatants were subsequently analysed by tandem mass spectrometry in order to determine (A) PGE₂, (B) PGD₂, (C) 15-deoxy-PGJ₂ and (D) TXB₂ concentrations. Data are mean ± S.E.M. for n=4. ****p < 0.0001 by one-way ANOVA.
Figure 4.5 Indomethacin and NS-398 inhibit all Pam3CSK4-induced PGs detected by ELISA

BMDMs were pretreated with (A) indomethacin (50 μM or 100 μM) or (B) NS-398 (10 μM or 20 μM) for one hour prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA. Data are mean ± S.E.M. for n=3. ****p < 0.0001 by one-way ANOVA.
4.2 4-OI inhibits Pam3CSK-induced COX2 expression

4.2.1 4-OI inhibits Pam3CSK4-induced Ptgs2 mRNA levels at four and eight hours in BMDMs

As 4-OI suppressed all detected oxylipins downstream of COX activity, we next investigated if 4-OI had an effect on COX2 expression. PG secretion from BMDMs appeared to be dependent on COX2 (Figure 4.5B) and it is potently upregulated by TLR ligands such as Pam3CSK4, as can be seen by the induction of Ptgs2 in Figure 4.6A. At the timepoints where Ptgs2 induction is highest, at four and eight hours, 4-OI potently reduces transcript levels. As was reported in a previous study from our lab [156], 4-OI reduced transcript levels of the cytokines Il1b (Figure 4.6B) and Il6 (Figure 4.6C). Other genes, such as Tgfb1 (Figure 4.6D) and Cd86 (Figure 4.6E) were unaffected by 4-OI.

4.2.2 4-OI decreases Pam3CSK4-induced COX2 protein expression at 6 and 24 hours

The effect of 4-OI on COX2 protein levels were next examined. As can be seen in Figure 4.7A, 4-OI also suppressed Pam3CSK4-induced COX2 protein expression at both 6 hours (lane 4 compared to lane 2) and 24 hours (lane 8 compared to lane 6). This has been quantified using densitometry in Figure 4.7B.

4.2.3 4-OI reduces Pam3CSK4-induced PTGS2 transcript levels in PBMCs

As 4-OI inhibited PG production in human PBMCs in a similar manner as BMDMs, it was next investigated if the capacity of 4-OI to attenuate COX2 expression also occurred in human PBMCs. As can be seen in Figure 4.8, 4-OI reduced PTGS2 mRNA levels in human PBMCs.
Figure 4.6 4-OI inhibits Pam3CSK4-induced induction of *Ptgs2* transcript at four and eight hours in BMDMs

(A) BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 2, 4, 8, 24 or 48 hours. The cells were lysed, mRNA was extracted and *Ptgs2* expression was measured by qPCR. *Ptgs2* was normalised to *Rps18*. (B-E) BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. The cells were lysed, mRNA was extracted and *Il1b* (B), *Il6* (C), *Tgfb1* (D) and *Cd86* (E) expression was measured by qPCR. Data are mean ± S.E.M. for n=4 (A) or n=5 (B-E). *p < 0.05, ***p < 0.0005, ****p < 0.0001 by two-way ANOVA (A) or one-way ANOVA (B-E).
Figure 4.7 4-OI decreases Pam3CSK4-induced COX2 protein expression at 6 and 24 hours

BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 6 hours or 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=3. (B) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=3. ****p < 0.0001 by two-way ANOVA.
Figure 4.8 4-OI inhibits Pam3CSK4-induced *PTGS2* transcript levels in PBMCs

Human PBMCs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (1 μg/mL) for six hours. The cells were then lysed, mRNA was extracted and *PTGS2* expression was measured by qPCR. *PTGS2* was normalised to *RPS18*. Data are mean ± S.E.M. for n=6. *p < 0.05 by one-way ANOVA.
4.2.4 50 μM 4-OI is sufficient to reduce Ptgs2 transcript

It was next evaluated if lower concentrations of 4-OI could reduce COX2 expression, as concentrations as low as 25 μM were demonstrated to impair PG production (Figure 4.1). Ptgs2 transcript levels were measured and as can be seen in Figure 4.9, concentrations of 50 μM were sufficient to significantly reduce Ptgs2 mRNA levels at six hours following Pam3CSK stimulation.

4.2.5 100 μM 4-OI is sufficient to reduce COX2 protein expression

COX2 protein expression was next examined and as can be seen in Figure 4.10A, 100 μM 4-OI was sufficient to significantly reduce Pam3CSK4-induced COX2 protein at 24 hours (lanes 4-8 compared to lane 3). This has been quantified using densitometry in Figure 4.10B.
Figure 4.9 50 μM 4-OI is sufficient to lower Ptgs2 mRNA levels

BMDMs were pretreated with various concentrations of 4-OI (25-200 μM) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. After cell lysis, mRNA was extracted and Ptgs2 expression was measured by qPCR. Ptgs2 was normalised to Rps18. Data are mean ± S.E.M. for n=4. **p < 0.005, ****p < 0.0001, ns (non-significant) p > 0.05 by one-way ANOVA.
Figure 4.10 100 μM 4-OI is sufficient to significantly reduce COX2 protein expression at 24 hours

BMDMs were pretreated with various concentrations of 4-OI (25-200 μM) prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=4. (B) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=4. *p < 0.05, **p < 0.005 by one-way ANOVA.
4.2.6 4-OI does not affect phospho-cPLA2 or total cPLA2 expression

Although the 4-OI-mediated suppression of COX2 expression seemed to be the most likely reason for the capacity of 4-OI to inhibit PG production, other enzymes involved in the PG synthesis pathway were also examined. One such enzyme was cPLA2, which catalyses the conversion of membrane phospholipids to arachidonic acid. As can be seen in Figure 4.11A, 4-OI did not affect the phosphorylation or total expression of cPLA2 at shorter timepoints (lanes 7-12 compared to lanes 1-6). This has been quantified using densitometry in Figure 4.11B. In addition, 4-OI did not alter total cPLA2 at a longer timepoint of six hours (Figure 4.11C, lanes 3 and 4 compared to lanes 1 and 2). This was quantified using densitometry in Figure 4.11D.

4.2.7 4-OI does not alter Ptges mRNA levels

The impact of 4-OI on expression of PGES, which catalyses the conversion of PGH₂ to PGE₂, was also assessed. As can be seen in Figure 4.12, 4-OI did not alter transcript levels of Ptges, the gene that encodes PGES. Therefore 4-OI seems to specifically affect the transcription of COX2 to target PG production.
Figure 4.11 4-OI does not affect phospho-cPLA2 or total cPLA2 expression

(A and B) BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for various timepoints (10-120 minutes). Cell lysates were harvested and phospho-cPLA2 and total cPLA2 expression were analysed by Western blotting. (A) One representative image is shown for n=3 (B) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=3.

(C and D) BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. cPLA2 expression was analysed by Western blotting. (C) One representative image is shown for n=3. (D) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=3.
Figure 4.12 4-OI does not alter Ptges mRNA levels

BMDMs were pretreated with 200 μM 4-OI prior to stimulation with Pam3CSK4 (100 ng/mL) for four hours. After cell lysis, mRNA was extracted and Ptges expression was measured by qPCR. Ptges was normalised to Rps18. Data are mean ± S.E.M. for n=3. Ns (non-significant) p > 0.05 by one-way ANOVA.
4.3 Selection of Pam3CSK4 stimulation as the experimental model

4.3.1 4-OI increases LPS-induced COX2 protein levels while decreasing Pam3CSK4-induced COX2 at 24 hours

COX2 and downstream PGs are induced by various TLR ligands [63]. As is seen in Figure 4.13A, stimulation with both LPS and Pam3CSK4 induced COX2 expression but 4-OI yielded opposite effects depending on which TLR agonist was used as a stimulus. In cells stimulated with LPS for 24 hours, 4-OI increased COX2 (lane 5 compared to lane 2) whereas in Pam3CSK4-stimulated cells 4-OI reduced COX2 expression (lane 6 compared to lane 3). This was quantified using densitometry in Figure 4.13B.

4.3.2 4-OI, but not OMS, reduces COX2 expression in response to stimulation with most TLR ligands

Due to the anomaly between LPS- and Pam3CSK4-treated cells, the response to two additional TLR ligands, the TLR9 ligand CpG and the TLR7/8 ligand R848, was evaluated. As before, 4-OI augmented the expression of LPS-induced COX2 (lane 7 compared to lane 2) but decreased the expression of COX2 in cells stimulated with Pam3CSK4, CpG and R848 (lanes 8-10 compared to lanes 3-5) (Figure 4.14A). In order to account for any potential off-target effects that 4-OI may exert due to its octyl tail, the control compound 4-methyl-2-succinate, OMS, that contains the same octyl tail as 4-OI, was used. As can be seen in Figure 4.14B, OMS had no effect on COX2 expression in cells stimulated with Pam3CSK4, CpG or R848 (lanes 8-10 compared to lanes 3-5). However, OMS caused an enhancement of LPS-induced COX2 expression in a similar manner to 4-OI (lane 7 compared to lane 2). Thus LPS was the only TLR agonist out of the four tested that resulted in an increase in COX2 expression by 4-OI, rather than a decrease. In addition, only the 4-OI-mediated boost in COX2 in LPS-treated cells was recapitulated by the control compound OMS. This was quantified using densitometry in Figure 4.14C and Figure 4.14D.
4.3.3 4-OI decreases LPS-induced COX2 expression at four hours

The effect of 4-OI on COX2 expression on an earlier timepoint was then examined. As can be seen in Figure 4.15, at 24 hours 4-OI again caused a decrease in Pam3CSK4-induced COX2 (lane 12 compared to lane 9) but an increase in LPS-induced COX2 (lane 11 compared to lane 8). However at the earlier timepoint of four hours post-stimulation, treatment with 4-OI caused a reduction in COX2 expression in both LPS-stimulated cells and Pam3CSK4-stimulated cells (lanes 5 and 6 compared to lanes 2 and 3). This was quantified using densitometry in Figure 4.15B and Figure 4.15C. Therefore the effect of 4-OI on LPS-induced COX2 seems to vary with timing.
Figure 4.13 4-OI increases LPS-induced COX2 expression while decreasing Pam3CSK4-induced COX2 at 24 hours

BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) or LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=3. (B) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=3. *p < 0.05, ****p < 0.0001 by one-way ANOVA.
Figure 4.14 4-OI, but not OMS, reduces COX2 expression upon stimulation with most TLR ligands

BMDMs were pre-treated with either 200 μM 4-OI (A and C) or 200 μM OMS (B and D) for two hours followed by a 24 hour treatment with LPS (100 ng/mL), Pam3CSK4 (100 ng/mL), CpG (1 μg/mL), R848 (1 μg/mL). Cell lysates were harvested and COX2 was measured by Western blotting. (A and B) One representative image is shown for n=3. (C and D) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=3. **p < 0.005 by one-way ANOVA.
Figure 4.15 4-OI decreases LPS-induced COX2 expression at four hours

BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) or LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=3. (B and C) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=3. **p < 0.005, ***p < 0.0005 by one-way ANOVA.
4.3.4 4-OI decreases LPS-induced PGD$_2$ at six hours

Similar to previous results, after six hours stimulation with LPS, PGD$_2$ was the only PG detected by LC/MS/MS. As can be seen in Figure 4.16, LPS induced robust elevation of PGD$_2$ concentrations, which were potently inhibited by 4-OI. This observation is perhaps expected, given that four hours following LPS stimulation 4-OI had decreased LPS-induced COX2 expression (Figure 4.15).

4.3.5 4-OI lowers LPS-induced TXB$_2$ but does not affect LPS-induced PGE$_2$, PGD$_2$ and 15-deoxy-PGJ$_2$ at 24 hours

Following a longer stimulation with LPS for 24 hours, the oxylipins PGE$_2$ (Figure 4.17A), PGD$_2$ (Figure 4.17B), 15-deoxy-PGJ$_2$ (Figure 4.17C) and TXB$_2$ (Figure 4.17D) were all detected by LC/MS/MS as being strongly upregulated by LPS. 4-OI decreased LPS-induced TXB$_2$ (Figure 4.17D) but did not affect concentrations of PGE$_2$, PGD$_2$ or 15-deoxy-PGJ$_2$ (Figure 4.17A-C). This observation is possibly due to the fact that 4-OI reduces LPS-induced COX2 levels at earlier timepoints yet augments LPS-induced COX2 expression at later timepoints, additively resulting in the overall concentrations of secreted PGs being unchanged.
Figure 4.16 4-OI decreases LPS-induced PGD$_2$ at six hours

BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with LPS (100 ng/mL) for six hours. Supernatants were subsequently analysed by tandem mass spectrometry in order to determine PGD$_2$ concentrations. Data are mean ± S.E.M. for n=4. ***p < 0.0005 by one-way ANOVA.
Figure 4.17 4-OI lowers TXB$_2$ but does not affect LPS-induced PGE$_2$, PGD$_2$, and 15-deoxy-PGJ$_2$ at 24 hours

BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with LPS (100 ng/mL) for 24 hours. Supernatants were subsequently analysed by tandem mass spectrometry in order to determine (A) PGE$_2$, (B) PGD$_2$, (C) 15-deoxy-PGJ$_2$ and (D) TXB$_2$ concentrations. Data are mean ± S.E.M. for n=3. ***p < 0.0005 by one-way ANOVA.
4.3.6 4-OI decreases LPS-induced PG production in human PBMCs

It was next investigated if the effects of 4-OI on LPS-induced COX2 and PGs were also true in human cells. Human PBMCs were pretreated with 4-OI prior to stimulation with LPS for 24 hours. As can be seen in Figure 4.18, 4-OI potently inhibited LPS-induced PG secretion from human PBMCs.

4.3.7 4-OI decreases LPS-induced COX2 expression at 24 hours in human PBMCs and macrophages

The effect of 4-OI on human COX2 expression was also assessed. As can be seen in Figure 4.19A, 4-OI attenuated the expression of LPS-induced COX2 at 24 hours (lane 8 compared to lane 2), although COX2 induction by the other TLR ligands was not detected in this experiment. This was quantified using densitometry in Figure 4.19B. The effect of 4-OI on COX2 protein was also examined using primary human macrophages. In these cells treatment with 4-OI reduced COX2 expression induced by both LPS and Pam3CSK 24 hours post-stimulation (Figure 4.19C, lanes 5 and 6 compared to lanes 2 and 3). This was quantified using densitometry in Figure 4.19D.

Therefore it seems as though a discrepancy exists between the effect of 4-OI on LPS-induced COX2 in mouse and in human. Another inconsistency was also observed in that the effect of 4-OI on COX2 expression seems to heavily depend on timing (Figure 4.15). Furthermore, it was only in cells stimulated with LPS that 4-OI increased COX2 levels as a decrease was observed with all other TLR ligands tested (Figure 4.14A). It is also concerning that the control compound OMS mimicked the effect of 4-OI on LPS-induced COX2 (Figure 4.14B). No such discrepancies were observed when using Pam3CSK4 to stimulate the macrophages and Pam3CSK4 still yields a robust induction of COX2 and downstream PG production. It was also recently shown that stimulation with LPS and Pam3CSK4 yielded similar levels of itaconate [308]. For these reasons, Pam3CSK4 stimulation was selected as the main experimental model for studying the modulation of COX2 and PG production by 4-OI. The basis for the curious effect of 4-OI on LPS-induced COX2 in BMDMs remains unknown.
Figure 4.18 4-OI decreases LPS-induced PG production in human PBMCs

Human PBMCs were pretreated with 200 μM 4-OI for two hours prior to stimulation with LPS (100 ng/mL) for 24 hours. Supernatants were analysed for PG concentration by ELISA. Data are mean ± S.E.M. for n=3. *p < 0.05 by one-way ANOVA.
Figure 4.19 4-OI decreases LPS-induced COX2 expression at 24 hours in human PBMCs and macrophages

(A and B) Human PBMCs were pretreated with 200 μM 4-OI for two hours prior to stimulation with LPS (100 ng/mL), Pam3CSK4 (100 ng/mL), Poly (I:C) (1 μg/mL), CpG (1 μg/mL) or R848 (1 μg/mL) for 24 hours. (B and D) Primary human macrophages were pretreated with either 200 μM 4-OI for two hours prior to stimulation with LPS (100 ng/mL) or Pam3CSK4 (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A and C) One representative image is shown for n=3. (B and D) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=3. *p < 0.05, ****p < 0.0001 by one-way ANOVA.
4.4 Endogenous itaconate does not affect COX2 expression or prostaglandin production

4.4.1 Endogenous itaconate does not affect Pam3CSK4-induced Ptgs2 mRNA levels

It was next investigated if endogenous itaconate would impact COX2 expression and PG synthesis in a similar manner to the derivatised 4-OI. For this BMDMs lacking \( \textit{lr}g1 \), the gene that encodes the enzyme responsible for itaconate synthesis, were used. However, when \( \textit{Ptgs2} \) transcript levels between \( \textit{lr}g1^{+/+} \) BMDMs and \( \textit{lr}g1^{-/-} \) BMDMs were compared, there was no difference in COX2 induction by Pam3CSK4 at two, four or six hours post-stimulation (Figure 4.20).

4.4.2 Endogenous itaconate does not affect COX2 protein expression

It was also tested if endogenous itaconate affected COX2 protein levels. As can be seen in Figure 4.21A and by densitometry in Figure 4.21B, it was firstly confirmed that the \( \textit{lr}g1^{-/-} \) BMDMs do not express any IRG1. COX2 protein was induced by the TLR agonists LPS, Pam3CSK4, Poly (I:C) and R848. However, no difference in COX2 protein expression was observed comparing \( \textit{lr}g1^{+/+} \) BMDMs and \( \textit{lr}g1^{-/-} \) BMDMs (Figure 4.21A, lanes 7-10 compared to lanes 2-5 and Figure 4.21C).
Figure 4.20 Endogenous itaconate does not alter Pam3CSK4-induced \textit{Ptgs2} mRNA levels

BMDMs from \textit{Irg1}^{+/+} and \textit{Irg1}^{-/-} mice were stimulated with Pam3CSK4 (100 ng/mL) for two, four or six hours. The cells were lysed and mRNA extracted in order to quantify \textit{Ptgs2} by qPCR. \textit{Ptgs2} was normalised to \textit{Rps18}. Data are mean ± S.E.M. for n=4.
Figure 4.21 Endogenous itaconate does not affect COX2 protein expression

BMDMs from \(\text{Irg}^{+/+}\) and \(\text{Irg}^{-/-}\) mice were stimulated with LPS (100 ng/mL), Pam3CSK4 (100 ng/mL), Poly (I:C) (1 μg/mL) or R848 (100 ng/mL) for 24 hours. Cell lysates were harvested and IRG1 and COX2 were measured by Western blotting. (A) One representative image is shown for \(n=4\). IRG1 (B) and COX2 (C) expression were quantified by densitometry. Data are mean ± S.E.M. for \(n=4\).
4.4.3 Endogenous itaconate has no effect on PG production measured by ELISA

The effect of endogenous itaconate on PG production was next explored. Measuring PG secretion by ELISA, no differences were detected between $lrg1^{+/+}$ BMDMs and $lrg1^{-/-}$ BMDMs (Figure 4.22).

4.4.4 Endogenous itaconate has no effect on PGE$_2$, PGD$_2$, 15-deoxy-PGJ$_2$ or TXB$_2$ measured by mass spectrometry

PG secretion from $lrg1^{+/+}$ BMDMs and $lrg1^{-/-}$ BMDMs was also measured using LC/MS/MS. As can be seen in Figure 4.23, the production of Pam3CSK4-induced PGE$_2$ (Figure 4.23A), PGD$_2$ (Figure 4.23B), 15-deoxy-PGJ$_2$ (Figure 4.23C) and TXB$_2$ (Figure 4.23D) is unaffected by endogenous itaconate. Therefore we can conclude that loss of IRG1 has no effect on COX2 expression or PG production.
Figure 4.22 Endogenous itaconate has no effect on PG production

BMDMs from *Irg1*+/+ and *Irg1*−/− mice were stimulated with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the supernatants were subsequently quantified by ELISA. Data are mean ± S.E.M. for n=4.
Figure 4.23 Endogenous itaconate does not alter PGE$_2$, PGD$_2$, 15-deoxy-PGJ$_2$ or TXB$_2$ secretion

BMDMs from $lrg1^{+/+}$ and $lrg1^{-/-}$ mice were stimulated with Pam3CSK4 (100 ng/mL) for 24 hours. The cell supernatants were then analysed by tandem mass spectrometry in order to determine (A) PGE$_2$, (B) PGD$_2$, (C) 15-deoxy-PGJ$_2$ and (D) TXB$_2$ concentrations. Data are mean ± S.E.M. for n=4.
4.4.5 Exogenous unmodified itaconate moderately reduces PG production

It was then examined if exogenous addition of itaconate would mimic the effect of 4-OI on COX2 expression and PG production. BMDMs were treated with itaconic acid (adjusted to pH 7.4) for 12 hours and subsequently stimulated with Pam3CSK4 for 24 hours. As can be seen in Figure 4.24, treatment with exogenous itaconic acid significantly reduced PG secretion, although to a lesser extent than 4-OI.

4.4.6 Exogenous unmodified itaconate moderately reduces COX2 expression

The effect of exogenous itaconate on COX2 expression was next evaluated. As can be seen in Figure 4.25A, the TLR ligands LPS, Pam3CSK4, R848 and Poly (I:C) caused an upregulation of COX2 protein and treatment with itaconic acid moderately reduced COX2 levels (lanes 7-10 compared to lanes 2-5), again mirroring the effect of 4-OI but to a lesser degree. This was quantified by densitometry in Figure 4.25B.
Figure 4.24 Exogenous itaconate moderately reduces PG production

BMDMs were pretreated with 5 mM itaconic acid (at pH 7.4) for 12 hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA. Data are mean ± S.E.M. for n=3. *p < 0.05 by one-way ANOVA.
Figure 4.25 Exogenous itaconate moderately reduces COX2 expression

BMDMs were pretreated with 5 mM itaconic acid for 12 hours prior to stimulation with LPS (100 ng/mL), Pam3CSK4 (100 ng/mL), R848 (1 μg/mL) or Poly (I:C) (1 μg/mL) for 24 hours. Cell lysates were harvested and COX2 was measured by Western blotting. (A) One representative image is shown for n=3. (B) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=4. *p < 0.05 by one-way ANOVA.
4.5 DMF and DEM also reduce COX2 expression and prostaglandin production

4.5.1 DMF reduces Ptgs2 mRNA levels

Given that there is a great deal of overlap in the anti-inflammatory effects of 4-OI and DMF, it was next explored if DMF would also affect COX2 induction and downstream PG synthesis. As can be seen in Figure 4.26A, pretreatment of BMDMs with concentrations of DMF as low as 5 μM impaired induction of Ptgs2 transcript by Pam3CSK4. The highest concentration used was 25 μM, which has been shown to cause no cytotoxicity [277]. As can be seen in Figure 4.26B, induction of Ptgs2 is highest at four hours and eight hours and at these timepoints DMF potently inhibited Ptgs2 transcription. Figure 4.26C shows that DMF pretreatment also reduced transcript levels of il1b, as has been previously reported in the literature [264].

4.5.2 DMF decreases COX2 protein expression

The effect of DMF on COX2 protein levels were next examined. As can be seen in Figure 4.27A, DMF also significantly suppressed Pam3CSK4-induced COX2 protein expression, at concentrations as low as 10 μM (lanes 4-8 compared to lanes 3). This was quantified by densitometry in Figure 4.27B.

4.5.3 DMF reduces PG production

It was also assessed if DMF, like 4-OI can inhibit PG production in BMDMs. As can be seen in Figure 4.28, pretreatment of BMDMs with concentrations of DMF as low as 5 μM reduced PG secretion.
Figure 4.26 DMF reduces *Ptgs2* mRNA levels

(A) BMDMs were pretreated with various concentrations of DMF (5-25 μM) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for four hours. (B) BMDMs were pretreated with 25 μM DMF for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 2, 4, 8, 24 or 48 hours. (C) BMDMs were pretreated with 25 μM DMF for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for four hours. After cell lysis, mRNA was extracted and *Ptgs2* (A and B) and *Il1b* (C) levels were quantified by qPCR. *Ptgs2* and *Il1b* were normalised to *Rps18*. Data are mean ± S.E.M. for n=4 (A and B) or n=6 (C). *p < 0.05, ****p < 0.0001 by one-way ANOVA.
Figure 4.27 DMF attenuates COX2 protein expression

BMDMs were pretreated with various concentrations of DMF (5-25 μM) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 expression was analysed by Western blotting. (A) One representative image is shown for n=4. (B) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=4. *p < 0.05, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
BMDMs were pretreated with various concentrations of DMF (5-25 μM) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA. Data are mean ± S.E.M. for n=4. ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
4.5.4 DEM reduces *Ptgs2* mRNA levels

4-OI and DMF are both known to be potent NRF2 activators through their ability to modify crucial cysteine residues on KEAP1, a negative regulator of NRF2. Therefore the effect of another NRF2 activating compound, DEM, on COX2 expression and PG production was next investigated. As can be seen in Figure 4.29A, pretreatment of BMDMs with DEM inhibited induction of *Ptgs2* transcript by Pam3CSK4. 100 μM DEM was used, which has been demonstrated to cause no cytotoxicity [278]. Figure 4.29B shows that DEM pretreatment also reduced transcript levels of *Il1b*, as has been previously reported in the literature [251].

4.5.5 DEM decreases COX2 protein expression

The effect of DEM on COX2 protein levels were next examined. As can be seen in Figure 4.30A, DEM also decreased Pam3CSK4-induced COX2 protein expression (lane 4 compared to lane 2). This has been quantified by densitometry in Figure 4.30B.

4.5.6 DEM reduces PG production

It was also assessed if DEM, like 4-OI and DMF, would impair PG production in BMDMs. As can be seen in Figure 4.31, pretreatment of BMDMs with DEM reduced PG secretion. These data collectively indicated that NRF2 might play a role in the capacity of 4-OI to attenuate COX2 expression and thereby lower PG secretion.
Figure 4.29 DEM reduces Ptgs2 mRNA levels

(A and B) BMDMs were pretreated with 100 μM DEM for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for four hours. After cell lysis, mRNA was extracted and Ptgs2 levels were quantified by qPCR. Ptgs2 (A) and Il1b (B) were normalised to Rps18. Data are mean ± S.E.M. for n=6. ****p < 0.0001 by one-way ANOVA.
BMDMs were pretreated with 100 μM DEM for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. Cell lysates were harvested and COX2 expression was analysed by Western blotting. (A) One representative image is shown for n=3. (B) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=3. ****p < 0.0001 by one-way ANOVA.
Figure 4.31 DEM impairs prostaglandin production

BMDMs were pretreated with 100 μM DEM for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA. Data are mean ± S.E.M. for n=4.
4.6 The capacity of 4-Oi and DMF to reduce COX2 expression and prostaglandin production is NRF2-independent

4.6.1 4-Oi stabilises NRF2 and activates NRF2-dependent genes

It has been previously reported that 4-Oi stabilises NRF2 and thereby gives rise to induction of NRF2-dependent genes [156]. Figure 4.32A shows that treatment with 4-Oi increases expression of NRF2 in BMDMs (lanes 3 and 4 compared to lanes 1 and 2). This was quantified by densitometry in Figure 4.32B. 4-Oi also drives transcription of the NRF2-dependent genes \( Nqo1 \) (Figure 4.32C) and \( Hmox1 \) (Figure 4.32D).

4.6.2 DMF and DEM activate NRF-dependent genes

DMF and DEM are also well characterised as NRF2 activators [262, 309]. DMF induces transcription of the NRF2-dependent genes \( Hmox1 \) (Figure 4.33A) and \( Nqo1 \) (Figure 4.33B). DEM also drives induction of \( Hmox1 \) (Figure 4.33C) and \( Nqo1 \) (Figure 4.33D).
Figure 4.32 4-OI stabilises NRF2 and activates NRF2-dependent genes

(A) BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. Cell lysates were harvested and NRF2 expression was analysed by Western blotting. (A) One representative image is shown for n=3. (B) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=3. (C and D) BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. After cell lysis, mRNA was extracted and Hmox1 levels (C) and Nqo1 levels (D) were quantified by qPCR. Nqo1 and Hmox1 were normalised to Rps18. Data are mean ± S.E.M. for n=3 for (C), n=5 for (D). **p < 0.005, ***p < 0.0005 by one-way ANOVA.
BMDMs were pretreated with 25 μM DMF (A and B) or 100 μM DEM (C and D) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for four hours. After cell lysis, mRNA was extracted and Hmox1 (A and C) and Nqo1 levels (B and D) were quantified by qPCR. Nqo1 and Hmox1 were normalised to Rps18. Data are mean ± S.E.M. for n=3 (A and C) and n=6 (B and D). *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.

Figure 4.33 DMF and DEM activate NRF-dependent genes
4.6.3 4-Oi still reduces COX2 protein expression in the context of NRF2 knockdown

In order to elucidate if the effect that 4-Oi (as well as DMF and DEM) had on COX2 and PGs was via NRF2 activation, knockdown of NRF2 using siRNA was performed. As can be seen in Figure 4.34A, the silencing of NRF2 was successful (top panel, lanes 5-8 compared to lanes 1-4). However, 4-Oi still decreased COX2 expression under conditions of NRF2 knockdown (lane 8 compared to lane 6). This was quantified by densitometry in Figure 4.34B and Figure 4.34C.

4.6.4 NRF2 knockout BMDMs display a lack of Nqo1 activation and augmented Il1b transcription while KEAP1 knockdown BMDMs exhibit enhanced of Nqo1 and reduced Il1b transcription

As knockdown using siRNA does not completely abolish the target protein, the hypothesis was then tested in cells completely lacking NRF2. BMDMs from wild-type, NRF2 knockout and KEAP1 knockdown mice were utilised. The KEAP1 knockdown cells provided an experimental set up with enhanced NRF2 activation, in addition to a set up where NRF2 was absent. Firstly, characterisation in terms of NRF2 activation in these macrophages was carried out. As expected, expression of the NRF2-dependent gene Nqo1 was completely ablated (even in response to 4-Oi, DMF and DEM) in the NRF2 knockout BMDMs whereas the KEAP1 knockdown BMDMs displayed enhanced Nqo1 transcription compared to wild type cells (Figure 4.35A-C). Il1b is known to be transcriptionally regulated by NRF2 [251] and therefore the result that Il1b expression is augmented in NRF2 knockout BMDMs and reduced in KEAP1 knockdown cells is expected (Figure 4.35D-F). However, all three compounds were still able to impair Il1b transcription, implying that this effect is not completely NRF2-dependent.

4.6.5 4-Oi, DMF and DEM maintain the capacity to impair Ptgs2 transcription in NRF2 knockout and KEAP1 knockdown cells

The effect of 4-Oi, DMF and DEM on Ptgs2 in NRF2 knockout and KEAP1 knockdown BMDMs was next explored. As can be seen in Figure 4.36A, 4-Oi still
reduced Ptgs2 mRNA levels in wildtype, NRF2 knockout and KEAP1 knockdown cells. Similarly, DMF and DEM maintained the capacity to impair induction of Ptgs2 transcript in all three genotypes (Figure 4.36B and Figure 4.36C respectively).

4.6.6 4-OI and DMF maintain the capacity to reduce COX2 protein in NRF2 knockout and KEAP1 knockdown cells

Levels of COX2 protein were next examined. As expected, treatment with 4-OI (Figure 4.37A, lanes 4, 8 and 12 compared to lanes 2, 6 and 10) and DMF (Figure 4.37C, lanes 4, 8 and 12 compared to lanes 2, 6 and 10) still decreased COX2 protein levels in wildtype, NRF2 knockout and KEAP1 knockdown cells. This was quantified by densitometry in Figure 4.37B and Figure 4.37D. Therefore we can conclude that the effect of 4-OI and DMF on COX2 expression is independent of NRF2 activation.

4.6.7 4-OI and DMF inhibit PG secretion from NRF2 knockout and KEAP1 knockdown cells

Finally, the effect of 4-OI and DMF on PG production in NRF2 knockout and KEAP1 knockdown BMDMs was evaluated. 4-OI maintained the capacity to inhibit PG production in NRF2 knockout and KEAP1 knockdown macrophages (Figure 4.38A), as did DMF (Figure 4.38B). This result was somewhat unsurprising given that 4-OI and DMF reduced COX2 expression regardless of genotype (Figure 4.36 and Figure 4.37). It is also important to note that cells lacking NRF2 displayed attenuated COX2 expression and PG production. If the hypothesis was correct, that 4-OI and DMF impaired COX2 transcription and PG production through NRF2 activation, then enhanced COX2 expression in the NRF2 knockout cells compared to wild type controls would have been expected. Therefore the trend of COX2 expression in the wild type, NRF2 knockout and KEAP1 knockdown cells that is seen in Figure 4.37 is another indication that the effect of 4-OI and DMF on COX2 is entirely separate to their NRF2 activating function.
Figure 4.34 4-OI still reduces COX2 protein expression when NRF2 is knocked down

BMDMs were transfected with 50 nM control siRNA or NRF2 siRNA for 24 hours. The cells were then pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. Cell lysates were harvested and NRF2 and COX2 expression were analysed by Western blotting. (A) One representative image is shown for n=3. COX2 (B) and NRF2 (C) were quantified by densitometry. Data are mean ± S.E.M. for n=3. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
Figure 4.35 NRF2 knockout BMDMs display a lack of *Nqo1* activation and augmented *Il1b* transcription while KEAP1 knockdown BMDMs exhibit enhanced *Nqo1* and reduced *Il1b* transcription.

BMDMs from wild-type, NRF2 knockout and KEAP1 knockdown mice were pretreated with 200 μM 4-OI (A and D), 25 μM DMF (B and E) or 100 μM DEM (C and F) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. The cells were lysed, mRNA was extracted and *Nqo1* (A-C) and *Il1b* (D-F) levels were quantified by qPCR. *Nqo1* and *Il1b* were normalised to *Rps18*. Data are mean ± S.E.M. for n=3. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
Figure 4.36 4-OI, DMF and DEM maintain the capacity to impair $Ptgs2$ transcription in NRF2 KO and KEAP1 KD cells

BMDMs from wild-type, NRF2 knockout and KEAP1 knockdown mice were pretreated with 200 μM 4-OI (A), 25 μM DMF (B) or 100 μM DEM (C) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. The cells were lysed, mRNA was extracted and $Ptgs2$ expression was quantified by qPCR. $Ptgs2$ was normalised to $Rps18$. Data are mean ± S.E.M. for n=3 (A-C). *p < 0.05, **p < 0.005, ****p < 0.0001 by one-way ANOVA.
Figure 4.37 4-OI and DMF maintain the capacity to reduce COX2 protein in NRF2 KO and KEAP1 KD cells

BMDMs from wild-type, NRF2 knockout and KEAP1 knockdown mice were pretreated with 200 μM 4-OI (A and B), 25 μM DMF (C and D) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. Cell lysates were harvested and NRF2, KEAP1 and COX2 expression were analysed by Western blotting. (A and C) One representative image is shown for n=3. (B and D) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=3. *p < 0.05, ****p < 0.0001 by one-way ANOVA.
Figure 4.38 4-OI and DMF inhibit PG secretion from NRF2 KO and KEAP1 KD cells

BMDMs from wild-type, NRF2 knockout and KEAP1 knockdown mice were pretreated with 200 μM 4-OI (A), 25 μM DMF (B) or 100 μM DEM (C) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the supernatants were subsequently quantified by ELISA. Data are mean ± S.E.M. for n=3. *p < 0.05, **p < 0.005, ***p < 0.0005, by one-way ANOVA.
4.6.8 4-OI does not affect p38, NF-κB p65 or ERK phosphorylation

As NRF2 activation had been ruled out as a mechanism to explain the effect of 4-OI on COX2 and PG secretion, alternative mechanisms were then considered. NF-κB, p38 and ERK signalling pathways are all known to induce *Ptgs2* transcription. Therefore the effect of 4-OI on the phosphorylation of p38, NF-κB and ERK was examined, with phosphorylation being indicative of activity for all each of these pathways. As can be seen in Figure 4.39A, none of these signalling pathways appeared to be affected by 4-OI (lanes 7-12 compared to lanes 1-6). This was quantified by densitometry in Figure 4.39B-D. Taking these data into account, it is unlikely that any of these signalling pathways are involved in the capacity of 4-OI to attenuate COX2 expression.

4.6.9 The capacity of 4-OI to inhibit COX2 expression is not dependent on ATF4

Recently, ATF4 was shown to bind to the *Ptgs2* promoter and induce its transcription [276]. Therefore it was investigated if the effect of 4-OI on COX2 could potentially be through ATF4. However, as can be seen in Figure 4.40A, 4-OI actually increased ATF4 expression and ATF4 knockdown had no effect on the inhibition of COX2 by 4-OI (lanes 6 and 8 compared to lanes 2 and 4). This was quantified by densitometry in Figure 4.40B and Figure 4.40C.

4.6.10 The capacity of 4-OI to inhibit COX2 expression is not dependent on annexin A1

As previously discussed, annexin A1 is modified by itaconate and itaconate derivatives including 4-OI [156, 243, 244, 250]. This was the initial reason for studying the effect of 4-OI on PG production, as annexin A1 is known to function as a negative regulator of cPLA2, which catalyses the first step of PG biosynthesis. Therefore it was tested if the effect of 4-OI on PG production was dependent on annexin A1. As can be seen in Figure 4.41, silencing annexin A1 did not alter the inhibition of PG production by 4-OI, indicating that the effect of 4-OI on PG secretion is independent of any effect 4-OI may have on annexin A1.
Figure 4.39 4-OI does not inhibit p38, NF-κB p65 or ERK phosphorylation

BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for various timepoints (10-120 minutes). Phospho-p38, total p38, phospho-NF-κB p65, total NF-κB p65, phospho-ERK and total ERK expression were analysed by Western blotting. (A) One representative image is shown for n=3. (B) Western blots were quantified by densitometry. Data are mean ± S.E.M. for n=3.
Figure 4.40 The capacity of 4-OI to inhibit COX2 expression is not dependent on ATF4

BMDMs were transfected with 50 nM control siRNA or ATF4 siRNA for 48 hours. The cells were then pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. Cell lysates were harvested and ATF4 and COX2 expression were analysed by Western blotting. (A) One representative image is shown for n=3. ATF4 (B) and COX2 (C) expression were quantified by densitometry. Data are mean ± S.E.M. for n=3. *p < 0.05, **p < 0.005 by one-way ANOVA.
Figure 4.41 The capacity of 4-OI to inhibit COX2 expression is not dependent on annexin A1

BMDMs were transfected with 50 nM control siRNA or Anxa1 siRNA for 96 hours. The cells were then pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the supernatants were subsequently quantified by ELISA. Data are mean ± S.E.M. for n=3. ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
4.7 4-OI induces annexin A1 secretion

4.7.1 4-OI induces annexin A1 secretion in BMDMs

The capacity of 4-OI to modulate PG production was shown to be independent of annexin A1 (Figure 4.41) but we began to wonder if 4-OI may regulate another function of annexin A1, as itaconate and related derivatives had been shown to modify annexin A1 in multiple reports [156, 243, 244, 250]. It has been demonstrated that annexin A1 can be secreted [135, 137], although current knowledge of how this process is regulated is limited. To assess if 4-OI affected annexin A1 secretion, BMDM supernatants were concentrated and used for Western blotting. As can be seen in Figure 4.42A, 4-OI induced the presence of annexin A1 in the cell supernatant (lanes 6-10 compared to lanes 1-5). Both lysate and supernatant levels of annexin A1 protein were quantified by densitometry in Figure 4.42B and Figure 4.42C respectively. It is worth noting that the annexin A1 detected in the supernatant is around 33kDa whereas in the lysate the band is present at the expected size of 37 kDa. There are reports in the literature of a cleaved form of annexin A1 measuring 33 kDa being secreted [139, 140]. It is also notable that while 4-OI does induce a low level of annexin A1 secretion in unstimulated cells, this effect is far more pronounced in the macrophages that had been stimulated with various TLR ligands (lanes 7-10 compared to lane 6).

4.7.2 DMF induces annexin A1 secretion

As the effects of DMF have been shown to overlap with those of 4-OI, including the effect of 4-OI and DMF on COX2 discussed earlier in this chapter, it was next tested if DMF would induce annexin A1 secretion. As can be seen in Figure 4.43A, DMF treatment yielded increased expression of the 33 kDa form of annexin A1 in the cell supernatants (lanes 2 and 4 compared to lanes 1 and 3). The lysate expression of annexin A1 seems to be inversely correlated with the amount of annexin A1 secreted from the BMDMs and the effect of DMF on annexin A1 secretion was much more marked in LPS-stimulated cells. Both lysate and
supernatant levels of annexin A1 protein were quantified by densitometry in Figure 4.43B and Figure 4.43C respectively.

4.7.3 DEM induces annexin A1 secretion

As DEM was found to reduce COX2 and PG production in a manner similar to 4-OI and DMF earlier in this chapter, it was next evaluated if DEM could modulate annexin A1 secretion too. As can be seen in Figure 4.44A, DEM induced the presence of annexin A1 in the macrophage supernatants (lanes 2 and 4 compared to lanes 1 and 3). As before, the effect of DEM on annexin A1 secretion was far more pronounced in LPS-stimulated cells. Both lysate and supernatant levels of annexin A1 protein were quantified by densitometry in Figure 4.44B and Figure 4.44C respectively.
Figure 4.42 4-OI induces annexin A1 secretion in BMDMs

BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with LPS (100 ng/mL), Pam3CSK4 (100 ng/mL), Poly (I:C) (1 μg/mL) or R848 (500 ng/mL) for 24 hours. Cell lysates were harvested and annexin A1 expression was analysed by Western blotting. Cell supernatants were concentrated and also analysed by Western blotting. (A) One representative image is shown for n=4. Annexin A1 expression in both the lysate (B) and supernatant (C) was quantified by densitometry. Data are mean ± S.E.M. for n=4. *p < 0.05 by one-way ANOVA.
Figure 4.43 DMF induces annexin A1 secretion

BMDMs were pretreated with 25 μM DMF for two hours prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and annexin A1 expression was analysed by Western blotting. Cell supernatants were concentrated and also analysed by Western blotting. (A) One representative image is shown for n=3. Annexin A1 expression in both the lysate (B) and supernatant (C) was quantified by densitometry. Data are mean ± S.E.M. for n=3.
BMDMs were pretreated with 100 μM DEM for two hours prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and annexin A1 expression was analysed by Western blotting. Cell supernatants were concentrated and also analysed by Western blotting. (A) One representative image is shown for n=3. Annexin A1 expression in both the lysate (B) and supernatant (C) was quantified by densitometry. Data are mean ± S.E.M. for n=3. *p < 0.05 by one-way ANOVA.
4.7.4 Endogenous itaconate does not affect annexin A1 secretion

It was next investigated if endogenous itaconate would impact annexin A1 secretion in a similar manner to 4-OI. As can be seen in Figure 4.45A, there was no observed difference in terms of annexin A1 secretion into the supernatant between \( lrg1^{+/+} \) and \( lrg1^{-/-} \) BMDMs. This was quantified by densitometry in Figure 4.45B-D.

4.7.5 Exogenous itaconate induces annexin A1 secretion

It was then examined if exogenous addition of itaconate would mimic the effect of 4-OI on annexin A1 secretion. As can be seen in Figure 4.46A, treatment with exogenous itaconic acid induced annexin A1 secretion approximately in a dose-dependent manner. Similar to previous results, the observed effect was far more potent in LPS-stimulated BMDMs. This was quantified by densitometry in Figure 4.46B and Figure 4.46C.
Figure 4.45 Endogenous itaconate does not affect annexin A1 secretion

BMDMs from Irg1\textsuperscript{+/+} and Irg1\textsuperscript{-/-} mice (1 x 10\textsuperscript{6} cells) were stimulated with LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and IRG1 and annexin A1 expression were analysed by Western blotting. Cell supernatants were concentrated and also analysed by Western blotting. One representative image is shown for n=3. Annexin A1 expression in both the lysate (B) and supernatant (C) was quantified by densitometry. Data are mean ± S.E.M. for n=3.
Figure 4.46 Exogenous itaconate induces annexin A1 secretion

BMDMs were treated with itaconic acid (5-15 mM, at pH 7.4) and Pam3CSK4 (100 ng/mL) for 24 hours. Cell lysates were harvested and IRG1 and annexin A1 expression were analysed by Western blotting. Cell supernatants were concentrated and also analysed by Western blotting. (A) One representative image is shown for n=3. Annexin A1 expression in both the lysate (B) and supernatant (C) was quantified by densitometry. Data are mean ± S.E.M. for n=3. *p < 0.05, **p < 0.005 by one-way ANOVA.
4.7.6 4-OI does not affect Anxa1 or Abca1 mRNA levels

It was next examined if 4-OI modulated the transcription of Anxa1, the gene that encodes annexin A1. As can be seen in Figure 4.47A, 4-OI had no effect on induction of Anxa1. This result, coupled with the observation that intracellular annexin A1 tends to be inversely correlated with secreted annexin A1, indicates that 4-OI regulates the process of secretion but not the overall expression of annexin A1. It was also assessed if 4-OI regulated the transcription of Abca1, which encodes a transporter that has been reported to facilitate annexin A1 secretion [137]. As can be seen in Figure 4.47B, 4-OI did augment Abca1 transcription in unstimulated cells but had no effect in LPS-stimulated cells. Given that the effect of 4-OI on annexin A1 secretion is considerably more potent in LPS-stimulated cells, this difference in Abca1 induction in unstimulated cells is unlikely to account for the modulation of annexin A1 secretion by 4-OI.

4.7.7 Knockdown of NRF2 impairs annexin A1 secretion by 4-OI

As 4-OI, DMF and DEM had all been shown to induce annexin A1 secretion (Figures 4.42-4.44) and are all known to activate NRF2, it was investigated if NRF2 was involved in this effect. In order to elucidate if the effect was NRF2-dependent, knockdown of NRF2 using siRNA was performed. As can be seen in Figure 4.48A, knockdown of NRF2 impaired the 4-OI-induced secretion of annexin A1 (lanes 6 and 8 compared to lanes 2 and 4). This has been quantified by densitometry in Figure 4.48B-D.

4.7.8 4-OI and DMF do not induce annexin A1 secretion from NRF2 KO BMDMs

As a knockdown using siRNA does not completely abolish the target protein, the hypothesis was then tested in BMDMs isolated from NRF2 knockout mice and matched wild type controls. As can be seen in Figure 4.49A, 4-OI and DMF induced annexin A1 secretion, particularly in LPS-stimulated cells in wild type BMDMs but no annexin A1 secretion was observed in NRF2 knockout cells (lanes 7-12 compared to lanes 1-6). This has been quantified by densitometry in Figure...
4.48B-D. This observation provides compelling evidence that the capacity of 4-OI and DMF to induce annexin A1 is NRF2-dependent but the specific link between NRF2 and annexin A1 secretion is currently unclear.
Figure 4.47 4-OI does not alter *Anxa1* or *Abca1* mRNA levels

(A and B) BMDMs were pretreated with 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for four hours. After cell lysis, mRNA was extracted and *Anxa1* (A and C) and *Abca1* levels (B and D) were quantified by qPCR. *Anxa1* and *Abca1* were normalised to *Rps18*. Data are mean ± S.E.M. for n=3 (A and B). **p < 0.005 by one-way ANOVA.
BMDMs were transfected with 50 nM control siRNA or NRF2 siRNA for 48 hours. The cells were then pretreated with 200 μM 4-OI for two hours prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and NRF2 and annexin A1 expression were analysed by Western blotting. Cell supernatants were concentrated and also analysed by Western blotting. (A) One representative image is shown for n=2. NRF2 expression (B), as well as annexin A1 expression in both the lysate (C) and supernatant (D) was quantified by densitometry. Data are mean ± S.E.M. for n=2.
Figure 4.49 4-OI and DMF do not induce annexin A1 secretion from NRF2 KO BMDMs

BMDMs from wild-type and NRF2 knockout mice were pretreated with 200 μM 4-OI or 25 μM DMF for two hours prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell lysates were harvested and NRF2 and annexin A1 expression were analysed by Western blotting. Cell supernatants were concentrated and also analysed by Western blotting. (A) One representative image is shown for n=3. NRF2 expression (B), as well as annexin A1 expression in both the lysate (C) and supernatant (D) was quantified by densitometry. Data are mean ± S.E.M. for n=3. **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
4.8 Discussion

4.8.1 Rationale

In this chapter, I have explored the role of itaconate and its derivative 4-OI in regulation of COX2 expression and PG production. Itaconate and derivatives have emerged in recent years as exerting multiple anti-inflammatory effects such as modulation of proinflammatory cytokines [156, 239, 240], inflammasome activation [242, 256] and LPS-driven glycolysis [243, 255]. However, the impact of itaconate and related derivatives on proinflammatory lipid mediators had not yet been probed. Multiple studies had demonstrated that annexin A1 underwent 2,3-dicarboxypropylation mediated by both endogenous itaconate [156] as well as itaconate-derived probes [243, 244]. While annexin A1 is associated with a number of anti-inflammatory processes, one that was of particular interest to our group was the negative regulation of PG synthesis. Annexin A1 directly inhibits cPLA2 activity to limit AA production [125], therefore I hypothesised that the modification by itaconate may enhance this negative regulation by annexin A1 to impair PG production. I also considered that the modification by itaconate might influence one or more of the other anti-inflammatory properties of annexin A1, some of which are mediated by secreted annexin A1.

4.8.2 4-OI impairs macrophage PG production

As in the case of I3P in the preceding chapter, I found that the itaconate derivative 4-OI, at concentrations as low as 25 μM, decreased PG production in stimulated macrophages. As before, the results obtained using mass spectrometry mirrored the trend observed with the ELISA, although the PGE$_2$ concentrations detected by mass spectrometry were lower than those measured by ELISA. I confirmed that the Pam3CSK4-induced PGs were dependent on COX activity using the pan-COX inhibitor indomethacin. I also used a COX2-specific inhibitor, which demonstrated that almost all Pam3CSK4-induced PGs are dependent on COX2 activity in macrophages. The lipidomic screen conducted using tandem mass spectrometry showed that 4-OI was inhibiting all oxylipins downstream of COX, which may have
various effects. The involvement of PGE$_2$ as a pyrogen and an effector of the pain response is well-characterised [95, 97] and therefore this 4-OI-mediated decrease in PGE$_2$ is likely to contribute to the anti-inflammatory effects of 4-OI. Given that PGE$_2$ has been shown to display anti-inflammatory effects in certain contexts, such as in the environment of the lung [112-114], it might be expected that 4-OI could have detrimental effects in these circumstances. However, inhalation of itaconate was shown to protect mice from lung fibrosis [310], although this route of administration has not yet been tested for 4-OI. PGD$_2$ is known to exacerbate allergic inflammation [75], although it can also be converted non-enzymatically to the J series PGs, which generally exert anti-inflammatory effects [85, 86]. Interestingly, 15d-PGJ$_2$, which is downstream of COX activity and therefore would be reduced by 4-OI, has been shown to activate NRF2 [311], although treatment with 4-OI also leads to NRF2 activation. 4-OI was also shown to inhibit TXB$_2$ and likely also reduces the transient TXA$_2$, which means that 4-OI might impair platelet aggregation and thrombosis [90].

It is also possible that the effect of 4-OI on PG production will contribute to the attenuation of other inflammatory markers. Macrophages are known to express EP receptors [93] and PGE$_2$ can thereby signal in an autocrine manner. A study from our group has previously demonstrated that endogenously produced PGE$_2$ ligating the EP2 receptor is essential for induction of pro-IL-1β [105]. Therefore the impairment of this PG signalling loop could potentially contribute to the 4-OI-induced decrease in pro-IL-1β that has been reported [156]. PGE$_2$ has also been reported to enhance IL-6 production in macrophages [106, 107], another proinflammatory cytokine that is inhibited by 4-OI [156]. Hence, suppression of PGE$_2$ by 4-OI could conceivably be a factor in the decrease of IL-6. Treatment of macrophages with PGE$_2$ was also demonstrated to enhance production of IL-10 [117], an anti-inflammatory cytokine that has also been reported to be inhibited by 4-OI. It is also plausible that an impairment of PG secretion could contribute to the anti-inflammatory effects of 4-OI during in vivo models [156, 242, 255]. Conversely, the capacity of 4-OI to attenuate the production of other proinflammatory mediators could potentially affect PG synthesis. For example, IL-1β and NO, both of which are inhibited by 4-OI [156, 239], have been reported to contribute to PG production through induction of COX2 [312, 313].
4.8.3 4-OI decreases COX2 expression

Given the observation that 4-OI reduced PG production, it seemed likely that the 2,3-dicarboxypropylation of annexin A1 might be modulating cPLA2. However, phosphorylation of cPLA2, which is indicative of its activity [314], was unchanged in response to treatment with 4-OI, whereas a strong reduction in COX2 mRNA and protein levels was observed. Although 4-OI had yielded similar results to those obtained using I3P such that all prostanoids downstream of COX activity were reduced, the attenuation of COX2 expression indicated that a separate mechanism was at play. The observation that COX2 levels are downregulated by 4-OI explains the suppression of PG production so it seemed most likely that 4-OI was affecting transcription of *Ptgs2* only, rather than inhibiting enzymatic activity like I3P.

One gap in these data is that I did not assess if 4-OI has any impact on COX1 expression. It is probably not very likely as the induction of COX1 and COX2 are controlled by different signals and transcription factors, although there is some overlap in the regulation of the two isoforms [315]. Most NSAIDs do not discriminate between the isoforms and hence inhibit both COX1 and COX2. One common side effect of NSAID use is the onset of gastrointestinal symptoms, which is thought to be stem from toxicity associated with inhibition of COX1 in the gut, where COX1 is known to play important housekeeping roles [316]. Another reason why it may be beneficial for 4-OI to suppress COX2 expression only is that COX1-derived PGE\textsubscript{2} was found to mediate resolution and immune suppression at later timepoints during zymosan-induced inflammation [317]. Therefore if 4-OI were to modulate the expression of COX2 only, this could potentially give it an advantage over archetypal NSAIDs as an anti-inflammatory agent.

The discrepancy between stimulation with LPS and with the other TLR ligands in murine macrophages is intriguing. After initially observing this difference between LPS and Pam3CSK4, I considered that perhaps it was a difference between MYD88-dependent and MYD88-independent signalling but because stimulation using Poly(I:C) yielded the same result as Pam3CSK4, this hypothesis was discounted. Due to the fact that 4-OI does decrease LPS-induced COX2 at earlier
timepoints it is likely that the same mechanism by which 4-OI downregulates Pam3CSK4-induced COX2 occurs in these cells. However, it appears that an additional mechanism transpires at a later stage post-LPS stimulation that somehow increases COX2. These two distinct mechanisms seem to counteract each other at a certain stage, considering that most LPS-induced prostanoids were unchanged by 4-OI at 24 hours. Given that the control compound OMS had the same effect as 4-OI in LPS-stimulated cells, coupled with the fact that the result obtained with murine BMDMs does not match the observations in primary human cells, I must conclude that the effect is an artefact of some kind that we cannot presently explain.

**4.8.4 Endogenous itaconate does not affect COX2 or PG levels**

In view of the fact that there was no difference in COX2 expression or PG secretion between *Irg1*+/+ and *Irg1*−/− BMDMs, this work highlights the importance of bearing in mind that metabolite derivatives do not always truly represent the action of the corresponding endogenous metabolites. In the case of 4-OI, treatment with this derivative replicates the biological effects of endogenous itaconate in a number of studies, for example the inhibition of LPS-driven glycolysis [255] and impairment of NLRP3 inflammasome activation [242]. Nonetheless, there are cases whereby 4-OI and endogenous itaconate do not elicit the same biological effects. Probably the most dramatic example of this is in the case of type I IFNs, which are elevated by endogenous itaconate [258] but suppressed by 4-OI [156]. While both 4-OI and endogenous itaconate have been shown to mediate 2,3-dicarboxypropylation, the protein targets are not always the same. A previous study from our group showed that while there were some overlapping proteins modified by both endogenous itaconate and 4-OI, many targets were mutually exclusive [156]. It is conceivable that differences in the relative electrophilicities between itaconate and 4-OI or other derivatives could contribute to this divergence. It may be that 4-OI can modify a wider range of targets due to a higher degree of electrophilicity. Although 4-OI was shown to be converted to itaconate intracellularly, there was also uncleaved 4-OI detected in these cells [242], so the observations described in this chapter could potentially be a 4-OI specific effect.
However, it is interesting that exogenous unmodified itaconate seemed to have a similar impact to 4-OI on COX2 expression and PG production, albeit to a lesser extent. As mentioned already, this difference could be due to increased electrophilicity of 4-OI such that much higher concentrations of unmodified itaconate are required to elicit the same effect. Although intracellular concentrations of itaconate have been reported to reach up to 5 mM in LPS-stimulated BMDMs [156] and it was shown that Pam3CSK4 stimulation induced similar levels of itaconate to that of LPS-stimulated cells [308], I cannot be sure of exact concentrations during my experiments using Irg1+/+ and Irg1−/− BMDM as itaconate was not quantified. Another possibility is the existence of a receptor for itaconate that has yet to be identified. A receptor exists for succinate, termed GPR91, which elicits many of the physiological effects of succinate [318] so it is plausible that a receptor may also exist for itaconate. The fact that DMI was demonstrated to boost intracellular itaconate despite not being converted to itaconate itself [241] might also be the result of a receptor-mediated effect.

4.8.5 4-OI, DMF and DEM reduce COX2 expression and PG production in an NRF2-independent manner

The observation that endogenous itaconate did not have the same effect on COX2 induction as 4-OI did guided me to consider that NRF2 could possibly be involved in this process, as the role of endogenous itaconate in NRF2 activation is much less clear than that of 4-OI. One study showed that LPS-induced NRF2 levels were lower in lrg1−/− BMDMs compared to wild-type cells [240]. However, a later study from the same group showed no changes in NRF2 activation with exogenous itaconate up to 7.5 mM [258] and another group published that there was no difference in LPS-induced NRF2 stabilisation between lrg1+/+ and lrg1−/− BMDMs [319]. Despite some discrepancies in the literature, it is clear that 4-OI activates NRF2 and downstream gene expression much more potently than endogenous itaconate. This is why I decided to evaluate if two other NRF2-activating compounds would elicit similar effects on COX2 and PG production. My results showed that both DMF and DEM decreased Ptgs2 transcription and impaired PG secretion, as 4-OI had done (see Figure 4.50). This further suggested that NRF2
might be involved in this effect. Although it has been demonstrated that NRF2 does not bind to the promoter of *Ptgs2* [276] and therefore would not be able to suppress its transcription in the same manner as *Il1b* [251], I considered that it could be affecting COX2 levels indirectly. For example, NRF2 has been shown to upregulate COX2 in melanoma cell lines through induction of ATF4, which directly promotes transcription of *Ptgs2* [276].
Figure 4.50 4-OI, DMF and DEM modulate COX2 induction and PG production

4-OI, DMF and DEM all reduce transcription of Ptgs2, the gene that encodes COX2. Although the mechanism of this process is not fully elucidated, it is likely to be a shared mechanism between the three compounds. The subsequent decrease in COX2 protein limits the conversion of AA into PGH$_2$ and thereby reduces downstream synthesis of PGs and thromboxanes.
However, using both an NRF2 knockout, NRF2 knockdown and KEAP1 knockdown system it became clear that the effect of 4-OI, DMF and DEM on COX2 and PG production was independent of the capacity of these compounds to activate NRF2. Although DMF is known to be a potent NRF2 activator [262, 320], NRF2-independent anti-inflammatory functions of DMF have begun to emerge, such as the blunting of glycolysis through GAPDH succination [264] and inhibition of pyroptosis via succination of gasdermin D [265]. It is also true for 4-OI that a number of its anti-inflammatory effects have been found to be independent of NRF2 activation, such as inhibition of inflammasome activation [242] and impairment of aerobic glycolysis [255], which occur via 2,3-dicarboxyproplyation of NLRP3 and GAPDH respectively. Although there is less known about NRF2-independent functions of DEM, there are some reports of protein targets other than KEAP1 being modified by DEM. For example, DEM was shown to modify cysteines on the *Schizosaccharomyces pombe* transcription factor Pap1, thereby blocking its nuclear export and facilitating its activation [321]. Therefore it is possible that 4-OI, DMF and DEM all share a target protein that is currently unidentified and when modified, somehow contributes to the downregulation of Ptgs2 transcription.

Due to the observation that cPLA2 phosphorylation was unaffected by 4-OI, I already suspected that the effect on COX2 was not dependent on annexin A1. However, there were some reports in the literature suggesting that annexin A1 could modulate COX2 expression [128, 129, 322]. Therefore I tested if 4-OI could still impair PG production in an annexin A1-deficient system, which confirmed that annexin A1 was not involved in this process. Although it has been implicated in several of the anti-inflammatory properties of itaconate [239, 248], SDH inhibition was not considered as a potential mechanism for my results because while non-derivatised itaconate has been demonstrated to inhibit SDH activity [247] and contribute to succinate accumulation, 4-OI was found to be unable to recapitulate this effect of itaconate [258, 323].

As mentioned previously, it is possible that the inhibition of other pro-inflammatory mediators, such as IL-1β [312] and NO [313], could be responsible for the suppression of Ptgs2 transcription by 4-OI. Another hypothesis that perhaps merits closer examination is the impairment of NF-κB signalling. From my results and
work previously published by our group [156], 4-OI does not affect phosphorylation of p65, which is indicative of NF-κB activity [324]. However, DMF has been found to inhibit NF-κB nuclear translocation and DNA binding through modification of p65 and importantly, this was independent of phosphorylation status [266]. DEM has also been reported to attenuate nuclear translocation of NF-κB [325]. Although the phosphorylation status of p65 has been deemed unchanged by 4-OI, the nuclear translocation of NF-κB was shown to be reduced by 4-OI treatment [255]. Another report demonstrated that 4-OI inhibited p65 DNA binding in both LPS-stimulated THP-1 cells and in PBMCs isolated from patients with systemic lupus erythematosus [326]. However, in this study they show that the inhibition of NF-κB activity is compromised in NRF2-deficient cells whereas my observed effect of 4-OI on COX2 expression is NRF2-independent. Nonetheless, it is conceivable that the suppression of COX2 expression and PG synthesis by 4-OI could possibly be due to impaired NF-κB signalling.

4.8.6 4-OI induces annexin A1 secretion

As my data had clearly shown that the modulation of PG production by 4-OI was not dependent on annexin A1, I began to explore the possibility that 4-OI could be altering another aspect of annexin A1 biology. As the modification on Cys189 of annexin A1 by itaconate and related derivatives had been shown on multiple counts [156, 243, 244, 250], I assumed that it must be affecting annexin A1 in some way. Although it lacks a classical secretory signal sequence [133], annexin A1 can be secreted from multiple cell types [136-138] and elicit effects through binding to FPRs, especially FPR2. By concentrating supernatants and blotting for annexin A1, I demonstrated that 4-OI treatment increased the presence of annexin A1 in the supernatants. Although there was no difference between Irg1+/+ and Irg1−/− BMDMs in terms of annexin A1 secretion, the addition of exogenous itaconate did induce the presence of annexin A1 in the supernatants, although rather high concentrations (5-15 mM) of itaconate were used. As already discussed for a similar observation with COX2 expression, this could potentially be due to differences in electrophilicity between 4-OI and itaconate meaning that higher concentrations of itaconate are required in order to elicit the same effects, or this
might possibility suggest the involvement of a receptor-mediated effect. As before, DMF and DEM yielded similar results to 4-OI, perhaps indicating that a cysteine modification could be responsible for this observation. Furthermore, the same cysteine residue, Cys189, has been shown to be modified by DMF in neurons and astrocytes [327] and low annexin A1 levels were found to correlate with severity of relapsing-remitting MS [328], a disease for which DMF is used as treatment. Secreted annexin A1 has the potential to elicit many possible effects, both autocrine and paracrine (see Figure 4.51).
Figure 4.51 4-OI induces annexin A1 secretion

4-OI induces annexin A1 secretion from macrophages, which may occur via ABCA1. Once secreted, annexin A1 can bind to formyl peptide receptors, particularly FPR2 either on the same cell to exert autocrine effects or another cells to exert paracrine effects. FPRs are expressed on a variety of immune cells and annexin A1 has been shown to elicit many different anti-inflammatory effects.
An important detail to note is that while the full-sized annexin A1 protein of 37 kDa was always detected in the cell lysates, the bands detected in the supernatants were at around 33 kDa. Annexin A1 has been reported to be cleaved into a 33 kDa peptide by elastase and proteinase 3 in neutrophils and this fragment has been shown to be secreted [139, 140]. Bands of this size were not clearly identified in the lysates, which might suggest an active process of annexin A1 cleavage that is associated with its secretion. An interesting point is that although LPS or other TLR ligands do not induce annexin A1 secretion (they in fact can slightly reduce secretion), the effect of 4-Oi and other compounds on annexin A1 secretion are far more pronounced in stimulated cells. Perhaps this could mean that an effector required for annexin A1 secretion, such as a protease or a transporter, is LPS-inducible.

A rather unexpected result was that 4-Oi- and DMF-induced annexin A1 secretion was almost entirely ablated in NRF2 knockout BMDMs, in addition to being attenuated with NRF2 knockdown. Literature that links annexin A1 and NRF2 is somewhat lacking so it is not entirely clear how NRF2 activation by 4-Oi and DMF may lead to secretion of annexin A1. However, there are reports of NRF2 activating compounds increasing ABCA1 expression in macrophages [329, 330]. These studies are primarily concerned with the facilitation of cholesterol efflux by ABCA1, which is the best-known function of this transporter, but ABCA1 has also been shown to mediate annexin A1 secretion in multiple cell types [136, 137]. Although the dependency of annexin A1 secretion on ABCA1 has yet to be definitively proven for macrophages, it seems likely due to the high expression of ABCA1 in macrophages [134]. One study showed that curcumin treatment increased ABCA1 expression in a manner that was dependent on HO-1 upregulation driven by NRF2 activation [329]. Another study demonstrated that the NRF2 activator tert-butylhydroquinone (tBHQ) also augmented ABCA1 expression, an effect that was abrogated upon NRF2 or HO-1 knockdown or inhibition [330]. Although I tested if mRNA levels of Abca1 were altered with 4-Oi and observed only a modest increase in unstimulated cells, the timepoint of four hours may have been too early given that 4-Oi- and DMF-induced annexin A1 secretion is seen at 24 hours. The increase in ABCA1 expression by curcumin and tBHQ was also seen at later timepoints of 12-24 hours [329, 330]. Importantly, tBHQ did not have an effect on
Abca1 mRNA levels but nonetheless increased ABCA1 at the protein level. The authors showed that the tBHQ-mediated increase was through inhibition of calpain-mediated proteolysis of ABCA1 [330]. Hence, 4-OI and DMF may have a more significant impact on protein levels of ABCA1, which I have not yet examined. If 4-OI did promote annexin A1 secretion via NRF2 activation, the trend I have observed in the unstimulated compared to the LPS-stimulated cells might make sense. The expression of NRF2 in response to LPS and 4-OI is usually higher compared to treatment with 4-OI alone, which could potentially explain why the effect of 4-OI on annexin A1 secretion is stronger in LPS-stimulated cells. Another possible reason to favour NRF2 involvement as a mechanism is that NRF2 activation by 4-OI, DMF and DEM is far more potent than endogenous itaconate. Even though endogenous itaconate was shown to mediate the 2,3-dicarboxypropylation of annexin A1 [156], I saw no difference in secretion between Irg1+/+ and Irg1−/− BMDMs.

However, the hypothesis that the modification of annexin A1 by 4-OI and possibly DMF affects secretion of annexin A1 still warrants further investigation. Given the consistency of the modification both between a number of studies by different groups [156, 243, 244, 250] and also between 4-OI and DMF [327], it is difficult to envisage that this modification has no bearing whatsoever on function. It has already been reported that annexin A1 phosphorylation regulates trafficking of annexin A1 to the plasma membrane [154, 155] so perhaps the PTM by 4-OI might also influence membrane translocation to facilitate secretion. Another possibility is that the PTM might affect cleavage of annexin A1, which is known to occur prior to its externalisation and secretion [139, 140]. As 2,3-dicarboxypropylation of NLRP3 was shown to inhibit the direct interaction between NLRP3 and NEK7 [242], the possibility that modification of annexin A1 may disrupt its interaction with ABCA1 should perhaps be considered. If its transpires that the secretion of annexin A1 is independent of this PTM then perhaps the likelihood that the modification may alter binding of annexin A1 to the FPRs could also be examined. Although the mechanism by which 4-OI induces annexin secretion has not yet been discerned, my speculation as to how 4-OI may regulate annexin A1 secretion is outlined in Figure 4.52.
Figure 4.52 Possible mechanisms of 4-Ol-induced annexin A1 secretion

The mechanism by which 4-Ol induces annexin A1 secretion from BMDMs has not yet been elucidated. One possibility is that HO-1 induction by 4-Ol, via NRF2 activation, causes an increase in ABCA1 expression at the cell surface. ABCA1 is a transporter that has been reported to facilitate annexin A1 secretion. It is also known that annexin A1 can be post-translationally modified at cysteine 189 by itaconate and its derivatives. The functional consequence of this modification is currently unknown but we may speculate that it could potentially impact cleavage of annexin A1 to its 33 kDa fragment. Alternatively, this modification could strengthen the interaction between annexin A1 and ABCA1, or it could possibly induce the trafficking of annexin A1 to the plasma membrane. Further work is required in order to clarify which of these mechanisms might be at play.
4.8.7 Future directions

Because I have shown that COX2 expression and downstream PGs are attenuated by 4-OI and other compounds tested, it would also be valuable to evaluate if 4-OI would drive AA into the synthesis of other eicosanoids such as leukotrienes. Similarly to the experiments performed with I3P, LPS- and Pam3CSK4-induced increases in LOX-derived and CYP450-derived oxylipins were not observed due to interference by tissue culture serum and therefore the results were inconclusive (data not shown). It would be interesting to repeat these experiments with 4-OI under low serum conditions so that other branches of eicosanoids could be assessed. It would also be useful to determine more definitively if 4-OI modulates NF-κB signalling. This could be done by measuring its translocation to the nucleus with 4-OI, or using a p65 DNA binding assay in order to obtain a clearer answer as to whether NF-κB is involved in the process.

In terms of investigating annexin A1 secretion by 4-OI and other compounds, there remains a great deal of work to be done. Firstly, it would be useful to evaluate if 4-OI and DMF increase protein levels of ABCA1 and if this effect is dependent on NRF2 and HO-1. It would also be beneficial to test if knockdown of KEAP1 induces annexin A1 secretion, which would activate NRF2 without relying on compounds that could potentially target other cysteines in the cell and thereby yield a wide array of effects. In order to investigate if the modification of annexin A1 by 4-OI and possibly DMF plays a role in annexin A1 secretion, the relevant cysteine (Cys189) could be mutated. If 4-OI could induce secretion of wild-type annexin A1 but this effect was abrogated when Cys189 was mutated to another residue such as alanine, this would implicate the PTM in annexin A1 secretion. Using this proposed mutant annexin A1, cleavage, subcellular localisation and interaction with ABCA1 could also be examined. Many studies have been published in which 4-OI and DMF were found to reduce inflammation in vivo during a variety of disease models. It would be intriguing to use Anxa1−/− mice or a neutralising antibody against annexin A1 in order to elucidate if annexin A1 secretion contributes to the anti-inflammatory effects of 4-OI or DMF in any of these models.
Chapter 5:
General Discussion
5.1 Endogenous versus derivatised metabolites

It is very common in the study of immunometabolism to make use of derivatives as experimental tools but much of the research conducted in recent years, including the data presented in this thesis, has demonstrated that metabolite derivatives do not always represent the action of the corresponding endogenous metabolite. For the project investigating immunomodulatory properties of I3P it was important that I use the unmodified metabolite because the aim was to investigate the host-pathogen interactions during trypanosomiasis and a derivative may not portray this. This work also importantly adds to the rather limited but growing body of knowledge of how pathogen-derived metabolites alter the immune response, as the vast majority of immunometabolism research focuses on the roles of host metabolites. In the context of itaconate research, the potential use of derivatives as pharmacological anti-inflammatory agents has recently garnered much interest, even if some functions of itaconate derivatives are not truly representative of endogenous itaconate. My work has highlighted key differences between the actions of the itaconate derivative 4-OI and that of endogenous itaconate in the context of PG modulation and annexin A1 secretion. Across the current literature on itaconate biology, there are many more examples of divergence between itaconate and its derivatives.

One example of these differences is SDH inhibition. It is well documented that itaconate functions as an inhibitor of SDH and thereby contributes to the accumulation of succinate observed in proinflammatory macrophages [239, 247, 258]. On the other hand, 4-OI has been shown not to inhibit SDH activity and induce succinate build-up [258, 323]. Another itaconate derivative, DMI, was initially reported to inhibit SDH [240]. However, in a later publication by the same group the authors reported that DMI, in addition to 4-OI, failed to stimulate intracellular succinate accumulation which indicates SDH inhibition [258]. Hence, it would seem as though only non-derivatised itaconate is capable of inhibiting SDH activity. Additionally, as previously discussed, the evidence that endogenous itaconate activates NRF2 is far less persuasive than the proven NRF2-activating capacities of 4-OI and DMI. While the derivatives DMI and 4-OI reduced expression of IκBζ, a transcription factor that regulates a secondary transcriptional response
to TLR agonists, the addition of exogenous itaconate was found to have no effect [240, 258]. Perhaps the strongest example of a key difference between itaconate and its derivatives is their impact on type I IFNs, which are crucial effectors of the antiviral response. Both endogenous and exogenous unmodified itaconate were found to enhance the production of type I IFNs [258]. This indicates the existence of a positive feedback loop as type I IFN signal transduction has been reported to induce transcription of *Irg1* [156, 236, 257], thereby leading to a boost in itaconate production. Conversely, the itaconate derivatives 4-OI [156] and DMI [258] have been shown to inhibit type I IFN secretion. Modulation of the IFN response is a rare example of itaconate and its derivative exerting completely opposite effects.

However, there are also cases whereby itaconate derivates mimic the action of endogenous itaconate. A good example of this is in relation to NLRP3 inflammasome activation and pyroptosis, which are negatively regulated by both endogenous itaconate and 4-OI [242, 256]. There are also some proteins that we know are modified by both 4-OI and endogenous itaconate, LDHA being one example [156]. However the functional consequences of the modification of LDHA and other overlapping targets have not yet been elucidated. Collectively these data provide compelling evidence that itaconate derivatives should be considered separately to endogenous itaconate, despite some overlap in their functions.

In this thesis I have also employed the use of the fumarate derivative DMF. Importantly, both 4-OI and DMF activate NRF2 through modification of key cysteines on KEAP1 but they also share additional targets such as GAPDH [255, 264] and gasdermin D [256, 265]. Although as of yet I have not determined if endogenous fumarate has a similar effect on COX2 induction and annexin A1 secretion, it would be interesting to do so. While the role of endogenous itaconate is usually investigated using mice or cells deficient in IRG1, the enzyme that catalyses synthesis of itaconate, endogenous fumarate is usually examined by manipulation of fumarate hydratase (FH), the enzyme that facilitates metabolism of fumarate. Experiments are usually conducted using cells from homozygous or heterozygous FH-deficient mice or using an inhibitor of FH. Several of the immunomodulatory effects of DMF have been shown to also hold true for endogenous fumarate, including modulation of glycolysis [264] and inhibition of
pyroptosis [265]. Hence, it might be worth exploring the effect of endogenous fumarate on COX2 expression and annexin A1 expression, despite having shown that endogenous itaconate does not regulate these processes.

A likely explanation for observed differences between itaconate and related derivatives is differences in electrophilicity, which would correlate with the relative ability of a compound to modify cysteines. This could mean that the naturally occurring process of 2,3-dicarboxypropylation of protein targets may be amplified by 4-OI due to increased electrophilicity and therefore cysteine reactivity. This could explain why endogenous itaconate sometimes elicits the same effects as 4-OI, but not in all instances. Even aside from contrasting effects of derivatives, there appears to be some differences between endogenous and exogenous itaconate. My data shows discrepancies in terms of both PG modulation and annexin A1 secretion between results comparing $Irg1^{+/+}$ and $Irg1^{-/-}$ macrophages and those testing the effect of exogenous itaconic acid supplementation. A potential issue with treating cells with exogenous itaconate is that little is known about how itaconate is taken up by cells from the external environment. Although several reports have suggested that uptake of itaconate occurs [258, 331, 332], the results from these studies are in arbitrary units instead of actual concentrations. Therefore we still do not know the full extent of this uptake. We perhaps should consider the possibility of a plasma membrane transporter for itaconate, as we know that three mitochondrial transporters (the dicarboxylate, citrate and oxoglutarate carriers) are essential for transportation of itaconate to the cytosol [156]. As previously discussed, the possibility of a receptor for itaconate should also be considered. GCPRs for other Krebs cycle metabolites such as succinate and α-ketoglutarate have been identified [333] and the observation that DMI boosts intracellular itaconate although it is not converted to itaconate may also indicate the presence of a receptor for itaconate [241].

While the itaconate derivates 4-OI and DMI have proven effective across a range of inflammatory disease models [156, 240, 242], there is less evidence of the anti-inflammatory role of endogenous itaconate in similar models, although it was shown to be of importance in a murine model of psoriasis [256]. However, even if more in vivo data using $Irg1^{-/-}$ mice is obtained, it may not be readily translatable.
to humans. Although it has been shown in both murine and human immune cells that IRG1 is massively upregulated in response to stimulation with TLR agonists [156, 232], the relative intracellular concentrations may differ. While concentrations of itaconate in the millimolar range have been reported for LPS-stimulated murine macrophages [156, 232], itaconate concentrations in human macrophages were an order of magnitude lower at around 60 μM [232]. Additional evidence for enhanced itaconate production in mice is that the catalytic rate of murine IRG1 was found to be around three-fold higher than that of human IRG1 [334]. Therefore it is possible that endogenous itaconate might play an enhanced anti-inflammatory role in mice compared to humans. However the identification of SNPs in human IRG1 that mediate immune tolerance in monocytes indicates potential importance of endogenous itaconate production in humans. Furthermore, enhanced expression of IRG1 was observed in PBMCs isolated from septic patients [335]. Additional evidence for the anti-inflammatory role of endogenous itaconate in human disease arises from two studies showing that decreased itaconate levels are associated with disease severity in rheumatoid arthritis [336] and COVID-19 [337]. Although this correlation has been established, additional experiments would be required in order to prove causality.

5.2 Therapeutic applications

An examination of new medicines approved between 1981 and 2010 by the US Food and Drug Administration (FDA) found that around one third of small molecule drugs were either natural products or derivatised versions of natural products [338, 339]. Coupled with the surge in immunometabolism research over the last decade, this will surely result in the harnessing of metabolites for clinical use in inflammatory disease and infections. While reviewing FDA approval of natural products over this timeframe, Newman et al. also advocate for research into microbe-derived products as drug leads [339], which is interesting in the context of the recently discovered immunomodulatory properties of I3P [182, 183].

In this thesis, I have identified two novel ways of targeting PG production. However, the widespread use of NSAIDs, an extensive class of drugs that function by
inhibiting one or both isoforms of COX, calls into question the need for novel agents that target PG synthesis. So what advantages might the therapeutic use of I3P, 4-OI or DMF have over existing NSAIDs? Firstly, 4-OI and DMF may only regulate the COX2 isoform, which might mitigate side effects from COX1 inhibition, such as GI-related symptoms [316]. However, COX2-specific inhibitors have been developed and are associated greater risk of cardiovascular side effects [340]. I also have not tested if 4-OI and DMF downregulate COX2 in cell types other than macrophages. If the effect of 4-OI and DMF on PG production was restricted to immune cells, this could represent a notable advantage over classical NSAIDs that function systemically, leading to possible side effects. Of course another key difference between I3P, 4-OI and DMF compared to NSAIDs is that the compounds I have tested exhibit a plethora of immunomodulatory effects. All three have been shown to reduce LPS-driven glycolysis [182, 255, 264], downregulate production of proinflammatory cytokines [156, 182, 183, 264] and activate the antioxidant response via NRF2 [156, 183, 262], in addition to inhibition of inflammasome activation and pyroptosis by 4-OI and DMF [242, 256, 265]. Therefore we would expect them to play a much more general anti-inflammatory role that would include, but not be limited to, the inhibition of PG synthesis.

Of course, a huge advantage of DMF is that it is already clinically approved for use in humans [176] so we know about its toxicity, stability and safety in humans. Such information is not yet known for 4-OI so it is possibly that alternatives based on 4-OI would have to be developed for use in human disease. Despite the fact that DMF is already used therapeutically, new insights into its mechanisms of action are emerging all the time. Previously thought to function predominantly via activation of NRF2, a number of NRF2-independent effects of DMF have come to light over the last few years [264, 265], including its effects on Ptgs2 transcription presented in this thesis.

Although itaconate derivatives have not yet been tested in humans, 4-OI [156, 242] and DMI [240, 341] have shown efficacy in a variety of murine models of inflammatory disease. Reports of using non-derivatised itaconate in vivo are scarce but one study used inhaled itaconate, which was shown to ameliorate all markers of pulmonary fibrosis in mice [310]. This suggests that derivatisation of
Itaconate is perhaps not always required for therapeutic application. One benefit of the clinical use of itaconate over 4-OI could be the anti-bacterial effects that have been demonstrated for itaconate [234, 235]. Although anti-viral roles have been described for 4-OI [253], it is also known to inhibit the production of type I IFNs [156], which would be detrimental during viral infection. Therefore the clinical use of unmodified itaconate, which boosts type I IFN production [258], could possibly be explored in this context. The use of non-derivatised itaconate may also indicate that other unmodified metabolites, such as I3P, could potentially be used therapeutically. McGettrick et al. injected mice intraperitoneally with I3P [182], which alleviated acute IL-1β production in response to LPS. However, whether I3P is sufficiently stable in vivo for longer timeframes has yet to be explored.

In addition to the modulation of PGs, I have also shown that 4-OI and DMF induce annexin A1 secretion. This means that they could potentially mimic the action of glucocorticoids in some respects, in addition to their similarities with NSAIDs. Glucocorticoids are used in the treatment in a wide variety of anti-inflammatory diseases, including COVID-19 [342], and they induce the expression of annexin A1, as well as the expression of its receptor FPR2 [343]. However it is yet to be tested if 4-OI and DMF have significant effects on PG production and annexin A1 secretion in vivo.

5.3 Final remarks

It seems as though metabolite derivatives should possibly be considered completely separately to their corresponding endogenous metabolite. The effects observed with these derivatives are still of great interest, but from a pharmacological point of view rather than providing insight into the role of the endogenous metabolite. In many instances, it seems that derivatives mimic the action of endogenous metabolites, but enhance or amplify the effects so that they are much more potent effectors. The clinical potential of both microbe- and host-derived metabolites, as well as their derivatives, remains to be seen in the context of human disease.

The main findings of this thesis are summarised in Figure 5.1.
Figure 5.1 General summary
The protozoan parasite Trypanosoma brucei produces a metabolite termed I3P as a result of the breakdown of host tryptophan. I3P directly inhibits COX2 activity and thereby limits PG production. Impairment of the pyrogenic effects of PGE$_2$ and the trypanocidal capacity of PGD$_2$ may overall increase trypanosome survival during infection. Upon stimulation with TLR ligands, macrophages produce large amounts of a metabolite called itaconate. Itaconate is considered to elicit largely anti-inflammatory effects and therefore derivatives of itaconate, such as 4-OI, are being studied with potential therapeutic use in mind. 4-OI decreases COX2 expression, which leads to a reduction in downstream PG synthesis. It is likely that this will drive an anti-inflammatory phenotype and therefore both of these compounds display potential as anti-inflammatory agents.
Chapter 6:
References


106. Hinson, R.M., J.A. Williams, and E. Shacter, Elevated interleukin 6 is induced by prostaglandin E2 in a murine model of inflammation: possible


Chapter 7: Publications

First author publications


Other publications


The Trypanosome-Derived Metabolite Indole-3-Pyruvate Inhibits Prostaglandin Production in Macrophages by Targeting COX2


*J Immunol* published online 11 October 2021
http://www.jimmunol.org/content/early/2021/10/11/jimmunol.2100402

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2021/10/11/jimmunol.2100402.DCSupplemental

**Why The JI?** Submit online.
- **Rapid Reviews! 30 days**\(^*\) from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

\(^*\)average

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Trypanosome-Derived Metabolite Indole-3-Pyruvate Inhibits Prostaglandin Production in Macrophages by Targeting COX2

Ciana Diskin,*1 Sarah E. Corcoran,*1 Victoria J. Tyrrell, † Anne F. McGettrick,* Zbigniew Zaslona,* Valerie B. O’Donnell, † Derek P. Nolan,* and Luke A. J. O’Neill*

The protozoan parasite *Trypanosoma brucei* is the causative agent of the neglected tropical disease human African trypanosomiasis, otherwise known as sleeping sickness. Trypanosomes have evolved many immune-evasion mechanisms to facilitate their own survival, as well as prolonging host survival to ensure completion of the parasitic life cycle. A key feature of the bloodstream form of *T. brucei* is the secretion of aromatic keto acids, which are metabolized from tryptophan. In this study, we describe an immunomodulatory role for one of these keto acids, indole-3-pyruvate (I3P). We demonstrate that I3P inhibits the production of PGs in activated macrophages. We also show that, despite the reduction in downstream PGs, I3P augments the expression of cyclooxygenase (COX2). This increase in COX2 expression is mediated in part via inhibition of PGs relieving a negative-feedback loop on COX2. Activation of the aryl hydrocarbon receptor also participates in this effect. However, the increase in COX2 expression is of little functionality, as we also provide evidence to suggest that I3P targets COX activity. This study therefore details an evasion strategy by which a trypanosome-secreted metabolite potently inhibits macrophage-derived PGs, which might promote host and trypanosome survival. *The Journal of Immunology*, 2021, 207: 1–10.

H

uman African trypanosomiasis (HAT) is one of the world’s neglected diseases. *Trypanosoma brucei*, an extracellular parasitic protozoan of the genus *Trypanosoma*, is the causative agent. The disease is commonly known as sleeping sickness in humans and nagana in animals and can be fatal if left untreated. These diseases remain a major burden in sub-Saharan Africa for humans and livestock. Treatments remain limited, and drug resistance is an increasing feature (1).

A molecular aspect of the bloodstream stage of HAT is a perturbation of aromatic amino acid metabolism. Serum levels of tryptophan in infected animals are significantly decreased relative to healthy controls (2–5). This decrease is inversely linked to a simultaneous increase in aromatic keto acids, such as indole-3-pyruvate (I3P), phenylpyruvate, and hydroxyphenylpyruvate in the serum and urine (6–9). These keto acids are formed by the transamination of tryptophan by the parasitic enzyme aspartate aminotransferase (10, 11). The abnormal levels of aromatic keto acids in the blood and urine of infected animals, detectable by a pungent odor and red or brown color of the urine, have traditionally been used as a diagnostic tool to identify infected livestock (12).

During the course of infection, trypanosomes proliferate to extremely high numbers in the blood of infected individuals, at times reaching up to $0.2 \times 10^9$ cells/ml blood (13). The constant exposure of the parasite to the host immune system places the parasite under unique evolutionary pressure to both evade these unfavorable immune responses while also prolonging host survival to increase the likelihood of disease dissemination to other hosts via the tsetse fly. The most notable mechanism of immune evasion by trypanosomes is the rapid antigenic variation of the variable surface glycoproteins that entirely cover the surface of the trypanosome, which enables *T. brucei* to evade host Abs (14, 15). This interplay between host defense and parasite-evasion strategies gives rise to waves of parasitemia as the numbers of trypanosomes rise and fall cyclically, meaning that the concentrations of I3P will also fluctuate over the course of infection.

Macrophages are also known to be key effectors of host immune defense during infection. Recent reports have described how the keto acids that *T. brucei* secrete impair proinflammatory macrophage functions. I3P was found to decrease HIF-1α levels in LPS-stimulated macrophages, thereby impairing glycolysis and the proinflammatory cytokine IL-1β (9). Several *T. brucei*-derived keto acids, including I3P, were also shown to activate the NRF2 anti-oxidant pathway and reduce secretion of IL-6 from microglia (16). I3P has also been shown to activate the aryl hydrocarbon receptor (AhR) (17–19), which has multiple immunological roles.

Production of eicosanoids is another crucial arm of the innate immune response, and eicosanoids are divided into three classes: PGs, thromboxanes, and leukotrienes. These are lipid mediators, many of which have potent proinflammatory functions (20). PGs and thromboxanes are synthesized downstream of cyclooxygenase pathways.

Address correspondence and reprint requests to Prof. Luke A. J. O’Neill, Inflammation Research Group, Trinity College, Dublin 2, Ireland. E-mail address: laonell@tcd.ie

The online version of this article contains supplemental material.

Abbreviations used in this article: AA, arachidonic acid; AhR, aryl hydrocarbon receptor; BMDM, bone marrow–derived macrophage; COX, cyclooxygenase; EP, E prostanoid; HAT, human African trypanosomiasis; I3P, indole-3-pyruvate; LC-MS/MS, liquid chromatography–tandem mass spectrometry; 3MC, 3-methylcholanthrene; qPCR, quantitative PCR; siRNA, small interfering RNA; TXB2, thromboxane B2;

Copyright © 2021 by The American Association of Immunologists, Inc. 0022-1767/21/$37.50
(COX) activity, the enzyme that nonsteroidal anti-inflammatory drugs, such as indomethacin and aspirin, target. There are two isoforms of COX. COX1 is ubiquitous and constitutively expressed, whereas COX2 is inducible by inflammatory stimuli, such as LPS. In particular, myeloid-derived PGE2 has been shown to be proinflammatory, promoting fever and even recently being implicated in aging-associated inflammation (21). PGE2 can bind to four different E prostanoid receptors (EP1–4) and through engagement of these receptors can activate other immune cells, including mast cells (22), Th1, and Th17 cells (23), thereby enabling PGE2 to both initiate and prolong inflammation. The significance of the proinflammatory impact of PGE2 and other PGs is highlighted by the widespread use of nonsteroidal anti-inflammatory drugs in the treatment of inflammatory diseases (24).

Alterations in PG secretion have been demonstrated during infection with T. brucei in mice. Macrophages isolated from infected mice during the first peak of parasitemia displayed enhanced secretion of PGE2, whereas macrophages taken at a later stage of infection had reduced capacity for both basal and LPS-induced PGE2 secretion (25). Inhibiting PG production could promote host survival by limiting the febrile response. In addition, PGs have been shown to be directly trypanocidal (26, 27). However, the mechanism of PG modulation by T. brucei has not yet been elucidated.

In this study, we describe a potential role for I3P during trypanosomiasis involving inhibition of PG production. Somewhat counterintuitively, I3P increases COX2 mRNA and protein, in a manner that is partially AhR-dependent and may also be the result of a feedback loop induced by low PG concentrations. However, this transcriptional boost of COX2 is of little functional consequence given that we also provide evidence that indicates that I3P blocks COX2 activity. We also show that this could have relevance for trypanosomiasis, as I3P also inhibits induction of PGs by T. brucei lysates, as well as inhibiting PG production in primary human macrophages. Our study therefore describes a potential immune-evasion mechanism by which a T. brucei–secreted metabolite suppresses host macrophage PG synthesis, possibly promoting survival of host and parasite.

Materials and Methods

Reagents

LPS derived from Escherichia coli, serotype EH100 (Enzo Life Sciences), I3P, 3-methylcholanthrene, indomethacin, arachidonic acid (AA), AH6809, forskolin (Sigma-Aldrich), murine recombinant IFN-γ (Immunotools), and GW 627368X (Cayman Chemical) were used. Silencer Select control small interfering RNA (siRNA), Silencer Select AhR siRNA (assay ID s62162), and Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) were also used. Abs used were anti-COX2 (Abcam) and anti-β-actin (Sigma-Aldrich), as well as anti-mouse IgG and anti-rabbit IgG secondary HRP-conjugated Abs (Jackson ImmunoResearch Laboratories). A PGE2 ELISA kit was also used (Enzo Life Sciences).

Mice and bone marrow–derived macrophage generation

Bone marrow–derived macrophages (BMDMs) were isolated from C57BL/6J mice (Harlan UK). Bones from AhR−/− mice and their matched wild-type mice were kindly provided by Prof. Brigitta Stockinger (Francis Crick Institute, London, U.K.). All animals were maintained under specific pathogen-free conditions in accordance with Irish and European Union regulations. All experiments were subject to prior ethical approval by Trinity College Dublin Animal Research Ethics Committee and the Health Products Regulatory Authority. Mice were euthanized in a carbon dioxide chamber, followed by cervical dislocation as confirmation of death. Bone marrow cells were flushed from the tibia, femur, and hip of the mice and differentiated in DMEM, which contained 1% penicillin/streptomycin, 10% FCS, and 20% L929 supernatant. After 6 d, the cells were counted and replated for experiments.

Primary human macrophage culture

Human PBMCs were isolated from buffy coats from healthy donors using Lymphoprep (Axis Shield). The blood was diluted 1:1 with PBS, and then 30 ml was layered on 20 ml Lymphoprep and spun for 20 min at 2000 rpm with no brake on. The middle layer of PBMCs was then transferred to a new tube and washed in PBS. To isolate the monocytes from these PBMCs, positive selection of CD14+ cells was carried out using anti-CD14–labeled magnetic beads (Miltenyi Biotec), according to the manufacturer’s instructions. The monocytes were then maintained in RPMI 1640 media, which contained 1% penicillin/streptomycin, 10% FCS, and 50 ng/ml M-CSF (Immunotools). After 6 d, the monocytes had differentiated into macrophages and were then used for experiments.

Trypanosome culture and lysis

Monomorphic MITat 1.1 bloodstream forms were cultured in HMI9 medium containing 10% FCS. Cells in the log phase of growth were harvested by centrifugation at 1500 × g for 5 min and lysed by a combination of osmotic shock and sonication. After centrifugation, the cells were resuspended at 1 × 106 cells/ml in PBS glucose buffer (3 mM NaH2PO4, 57 mM Na2HPO4, 44 mM NaCl, 5 mM KCl, and 1 mM MgCl2 [pH 8]) and subsequently diluted 1 in 10 with sterile water. The resulting suspension was subjected to sonication (30% power, 50% duration; Bandelin Sonopuls) for 1 min before being incubated at room temperature for 5 min. The lysate was centrifuged at 14,000 × g for 10 min to yield a soluble lysate fraction. This soluble lysate fraction was subsequently used to stimulate BMDMs.

siRNA transfection of BMDMs

Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) was preincubated with the relevant siRNA and diluted in DMEM media containing no FCS or penicillin/streptomycin. This mixture was then used to treat BMDMs, yielding final concentrations of 5 μM Lipofectamine RNAiMAX Transfection Reagent and 50 nM siRNA. The cells were then left for 8 h, before this media was replaced with DMEM containing 1% penicillin/streptomycin and 10% FCS. At 48 h posttransfection, the cells were used for experiments as required.

Western blotting

Cells were lysed in sample buffer (0.125 M Tris [pH 6.8], 10% [v/v] glycerol, and 0.20% SDS) and heated to 95°C for 5 min. The protein samples and Spectra BR protein ladder (Thermo Fisher Scientific) were then resolved on SDS-polyacrylamide gels, before being transferred to polyvinylidene fluoride membrane. Membranes were blocked for 1 h in 5% (w/v) dried milk in TBST before being incubated overnight at 4°C with the primary Ab. Following incubation for 1 h with the secondary Ab, the blots were developed using chemiluminescent substrate (Thermo Fisher Scientific).

Real-time PCR

Cells were lysed and RNA extracted using the PureLink RNA Minikit (Ambion). cDNA was then prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer’s instructions. Real-time quantitative PCR (qPCR) was then carried out on the resulting cDNA using a 7500 Fast Real-Time PCR System with PowerUp SYBR Green Master Mix (Applied Biosystems). The primer pair sequences resulting cDNA using a 7500 Fast Real-Time PCR System with PowerUp SYBR Green Master Mix (Applied Biosystems). The primer pair sequences used were as follows: Rps15, 5′-CCCTCTATGCGTCAATGTTGTTT-3′ (forward) and 5′-CCCTCTATGCGTCAATGTTGTTT-3′ (reverse); Pigs2, 5′-CGGTTCTGACCAACATGTTGCAAAA-3′ (forward) and 5′-CTTGAATGATGGTGAGTTGAGTTGCAAAA-3′ (reverse); Pgts, 5′-TCACTAGCCACATGTTGCAAAA-3′ (forward) and 5′-CGAAGGCAAAATGCTGCAAA-3′ (reverse); Tbxas1, 5′-CAGGTTGGTGTTGGAACACTT-3′ (forward) and 5′-ACTGAACCTATGGTACATGGAATGCGGTATG-3′ (reverse); Ahr, 5′-GGCAAGGCAAAATGCTGCAAA-3′ (forward) and 5′-TGGAGGCAAAATGCTGCAAA-3′ (reverse). The cycling threshold method (2−ΔΔCT) was used to calculate relative quantification after normalization of each gene to murine Rps18. The calibrator used was the sample treated with neither LPS nor I3P. For knockdown experiments, the calibrator used was the sample treated with control siRNA but neither LPS nor I3P.

ELISA

Cell supernatants were collected, and PG concentrations were measured using an ELISA kit for PGE2 (Enzo Life Sciences), according to the manufacturer’s instructions. Although this item is sold as a PGE2-specific ELISA kit, our data suggested that other COX-derived oxylipins were also detected.
performed to determine concentrations of the PGs PGE2 (\(\text{PGE}_2\)), PGD2 (\(\text{PGD}_2\)) (for 1 h prior to stimulation with LPS (100 ng/ml) for 24 h. LC-MS/MS was analyzed for PG concentration by ELISA (\(\text{PG}_2\)), PGD2-d4, and thromboxane B2 (TXB2-d4) standards (Cayman Chemical) prior to extraction. Lipids were extracted by adding a 2.5-ml solvent mixture (1 M acetic acid/isopropanol/hexane/acetone 2:20:30 [v/v/v]) to 1 ml supernatant in a glass extraction vial and vortexed for 30 s. A total of 2.5 ml hexane was added to samples, and after vortexing for 30 s, tubes were centrifuged (500 × g for 5 min at 4°C) to recover lipids in the upper hexane layer (aqueous phase), which was transferred to a clean tube. Aqueous samples were re-extracted as above by addition of 2.5 ml hexane, and upper layers were combined. Lipid extraction from the lower aqueous layer was then completed according to the Bligh and Dyer technique. Specifically, 3.75 ml of a 2:1 ratio of methanol/chloroform was added followed by vortexing for 30 s. Subsequent additions of 1.25 ml chloroform and 1.25 ml water were followed with a vortexing step for 30 s, and the lower layer was recovered following centrifugation as above and combined with the upper layers from the first stage of extraction. Solvent was dried under vacuum, and lipid extract was reconstituted in 100 μl HPLC-grade methanol. Lipids were separated by liquid chromatography (LC) using a gradient of 30–100% B over 20 min (A: water/Mob B 95:5 plus 0.1% acetic acid; B: acetonitrile/methanol 80:15 plus 0.1% acetic acid) on an Eclipse Plus C18 Column (Agilent Technologies) and analyzed on a Sciex QTRAP 6500 LC-MS/MS system. Source conditions were: temperature 475°C, ion spray voltage±4500, gas 1 60, gas 2 60, and curtain gas 35. Lipids were detected using multiple-reaction monitoring with the following precursor to product ion transitions: \(\text{PG}_2\) and \(\text{PGD}_2\) \([\text{M}-\text{H}]\) 351.2/271.1, 15-deoxy-\(\text{PGJ}_2\) 315.2/271.1, \(\text{TXB}_2\) 369.2/169.1, 5-HETE 319.2/151.1, 8-HETE 319.2/155.101, 9-HETE 319.2/167.1, \(\text{PGJ}_2\) 319.2/167.102, 12-HETE 319.2/179.1, 15-HETE 319.2/191.1, 5-HEPE 317.2/115.1, 8-HEPE 317.2/151.5, 9-HEPE 317.2/157.1, 11-HEPE 317.2/171.1, 12-HEPE 317.2/179.1, 15-HEPE 317.2/191.1, 18-HEPE 317.2/259.1, 4-HDOHE 343.2/101.1, 7-HDOHE 343.2/141.1, 8-HDOHE 343.2/189.1, 10-HDOHE 343.2/133.101, 11-HDOHE 343.2/121.1, 13-HDOHE 343.2/193.1, 14-HDOHE 343.2/205.1, 16-HDOHE 343.2/233.101, 20-HETE 343.2/281.1. Chromatographic peaks were integrated using Multiquant 3.0.2 software (Sciex). The criteria for assigning a peak were signal/noise of at least 5:1 and with at least seven points across a peak. The ratio of analyte peak areas to internal standard was taken and lipids quantified using a standard curve made up and run at the same time as the samples.

**Oxylipin analysis**

Cell supernatants were collected and snap frozen in liquid nitrogen immediately. Samples were spiked with 2.1–2.9 ng \(\text{PGE}_2\)-d4, \(\text{PGD}_2\)-d4, 20-HETE-d6, 5-HETE-d8, 12-HETE-d8, 15-HETE-d8, 13-HODE-d4, and thromboxane B2 (TXB2)-d4 standards (Cayman Chemical) prior to extraction. Lipids were extracted by adding a 2.5-ml solvent mixture (1 M acetic acid/isopropanol/hexane/acetone 2:20:30 [v/v/v]) to 1 ml supernatant in a glass extraction vial and vortexed for 30 s. A total of 2.5 ml hexane was added to samples, and after vortexing for 30 s, tubes were centrifuged (500 × g for 5 min at 4°C) to recover lipids in the upper hexane layer (aqueous phase), which was transferred to a clean tube. Aqueous samples were re-extracted as above by addition of 2.5 ml hexane, and upper layers were combined. Lipid extraction from the lower aqueous layer was then completed according to the Bligh and Dyer technique. Specifically, 3.75 ml of a 2:1 ratio of methanol/chloroform was added followed by vortexing for 30 s. Subsequent additions of 1.25 ml chloroform and 1.25 ml water were followed with a vortexing step for 30 s, and the lower layer was recovered following centrifugation as above and combined with the upper layers from the first stage of extraction. Solvent was dried under vacuum, and lipid extract was reconstituted in 100 μl HPLC-grade methanol. Lipids were separated by liquid chromatography (LC) using a gradient of 30–100% B over 20 min (A: water/Mob B 95:5 plus 0.1% acetic acid; B: acetonitrile/methanol 80:15 plus 0.1% acetic acid) on an Eclipse Plus C18 Column (Agilent Technologies) and analyzed on a Sciex QTRAP 6500 LC-MS/MS system. Source conditions were: temperature 475°C, ion spray voltage±4500, gas 1 60, gas 2 60, and curtain gas 35. Lipids were detected using multiple-reaction monitoring with the following precursor to product ion transitions: \(\text{PG}_2\) and \(\text{PGD}_2\) \([\text{M}-\text{H}]\) 351.2/271.1, 15-deoxy-\(\text{PGJ}_2\) 315.2/271.1, \(\text{TXB}_2\) 369.2/169.1, 5-HETE 319.2/151.1, 8-HETE 319.2/155.101, 9-HETE 319.2/167.1, \(\text{PGJ}_2\) 319.2/167.102, 12-HETE 319.2/179.1, 15-HETE 319.2/191.1, 5-HEPE 317.2/115.1, 8-HEPE 317.2/151.5, 9-HEPE 317.2/157.1, 11-HEPE 317.2/171.1, 12-HEPE 317.2/179.1, 15-HEPE 317.2/191.1, 18-HEPE 317.2/259.1, 4-HDOHE 343.2/101.1, 7-HDOHE 343.2/141.1, 8-HDOHE 343.2/189.1, 10-HDOHE 343.2/133.101, 11-HDOHE 343.2/121.1, 13-HDOHE 343.2/193.1, 14-HDOHE 343.2/205.1, 16-HDOHE 343.2/233.101, 20-HETE 343.2/281.1. Chromatographic peaks were integrated using Multiquant 3.0.2 software (Sciex). The criteria for assigning a peak were signal/noise of at least 5:1 and with at least seven points across a peak. The ratio of analyte peak areas to internal standard was taken and lipids quantified using a standard curve made up and run at the same time as the samples.

**COX activity assay**

COX activity was measured using the COX Activity Assay Kit (Cayman Chemical) according to the manufacturer’s instructions. A total of 1 μg recombinant human COX2 (R&D Systems) was used per well, and for this experiment, DMSO was used as the solvent for I3P rather than cell culture media.

15-deoxy-Δ12,14-PGJ2 (D), and TXB2 (E) (n = 4 from three independent experiments). (F) BMDMs were treated with indomethacin (50 or 100 μM), I3P (0.5 or 1 mM), or the appropriate vehicle control (DMSO or media) for 1 h prior to stimulation with LPS (100 ng/ml) for 24 h. The supernatants were analyzed for PG concentration by ELISA (n = 3 from three independent experiments). Data are mean ± SEM. A one-way ANOVA was performed. The data show the adjusted \(p\) value obtained from multiple comparisons, corrected for using the Tukey test.

**\(p < 0.005, ***p < 0.0005, ****p < 0.0001.**

\(\text{I3P inhibits PG and thromboxane production by LPS-stimulated macrophages. (A) BMDMs were treated with various concentrations (31.25 μM to 1000 μM) of I3P or control media for 1 h prior to stimulation with LPS (100 ng/ml) for 24 h. Cell supernatants were subsequently analyzed for PG concentration by ELISA (n = 5 from three independent experiments). (B–E) BMDMs were treated with 1 mM I3P or control media for 1 h prior to stimulation with LPS (100 ng/ml) for 24 h. LC-MS/MS system was performed to determine concentrations of the PGs PGE2 (B), PGD2 (C), and with this kit. Therefore, we refer to measurements carried out using this ELISA kit throughout the article as quantifying PGs instead of PGE2.**
Statistical analysis

Statistical significance was determined by the one-way or two-way ANOVA methods as described in the figure legends. For the one-way ANOVA, data were analyzed with no matching or pairing. Gaussian distribution and equal SDs were assumed. Multiple comparisons were performed comparing the mean of each column to the mean of every other column. The Tukey test was used for correction for multiple comparisons. For the two-way ANOVA, multiple comparisons were performed comparing each cell mean with the other cell mean in that row. The Sidak test was used for correction for multiple comparisons. Data were expressed as mean ± SEM. Significance was defined as follows: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. GraphPad Prism v9 software was used for statistical analysis.

Results

I3P inhibits LPS-induced PG and thromboxane secretion by macrophages

To investigate if I3P would modulate macrophage PG production, we treated murine BMDMs with I3P before stimulation with the TLR4 agonist LPS for 24 h. LPS, although derived from Gram-negative bacteria, is relevant in the study of T. brucei infection. It has been demonstrated that the concentration of LPS in the circulation is substantially elevated during trypanosomiasis (28–30), and it has been suggested that the presence of LPS contributes to disease pathology through enhancement of the host proinflammatory response (30). We first measured PGE2 using an ELISA and found a robust increase in response to LPS, which was potently inhibited by I3P pretreatment at concentrations as low as 62.5 μM (Fig. 1A). To confirm this result, a lipidomic screen of oxylipins was carried out using quantitative LC–tandem mass spectrometry (LC-MS/MS). In this study, LPS stimulation yielded a robust increase in PGE2 (Fig. 1B), although the levels were considerably lower for LC-MS/MS due to lower specificity of Ab-based methods for lipid quantitation. Using LC-MS/MS, we also found that LPS stimulated elevations in PGD2 (Fig. 1C), 15-deoxy-PGJ2 (Fig. 1D), and TXB2 (Fig. 1E). Generation of all of these was potently inhibited by I3P.
pretreatment, confirming and extending the ELISA result. Using LC-MS/MS, we also measured lipoxygenase-derived and cytochrome P450-derived oxylipins in our supernatants; however, due to some background levels of these in tissue culture serum, elevations in response to LPS were not clearly seen, and no consistent impact of I3P was observed (Supplemental Figs. 1–3). Thus, we concluded that I3P was blocking eicosanoids specifically at the level of COX activity, likely through preventing generation of PGs and TXB2. Because the ELISA is reporting ~10-fold higher levels of PGE$_2$ than LC-MS/MS, we considered it was reporting on COX-derived PGs more broadly. As confirmation, preincubation of BMDMs with the pan-COX inhibitor indomethacin completely blocked generation of lipids detected using this method (Fig. 1F).

**I3P augments LPS-induced COX2 expression**

We next investigated if the changes we had observed in PG production were due to changes in expression of any of the enzymes in the pathway. As COX2 is known to be potently upregulated by LPS stimulation (31) and is often referred to as the rate-limiting enzyme for this pathway, we first measured COX2 expression in BMDMs with I3P treatment. Although LPS increased expression of *Ptgs2*, the gene that encodes COX2, pretreatment with I3P further boosted *Ptgs2* transcript (Fig. 2A). When protein levels of COX2 were examined by Western blot, a similar trend was observed with I3P further increasing LPS-induced COX2 expression at both the 6-h (lane 4 compared with lane 3) and 24-h time points (lane 6 compared with lane 5) (Fig. 2B, 2C). This I3P-induced increase in COX2 expression was seen at 1000 μM and 500 μM, but not at lower concentrations of I3P (Fig. 2D, 2E). The observation of this I3P-induced increase in COX2 expression was unexpected given that I3P had greatly reduced the secretion of PGs, which are dependent on COX2 expression in macrophages. We also measured the expression levels of a number of other enzymes involved in PG/thromboxane synthesis. We saw no changes with I3P treatment in the transcript levels of *Ptges* (Fig. 2F), which encodes PGE$_2$ synthase, nor did we observe any changes in *Pgis* (Fig. 2G), which encodes PGI$_2$ synthase. I3P caused a significant decrease in the gene encoding thromboxane A synthase, *Tbxas1*, at the 8-h time point (Fig. 2H). However, this modest reduction is unlikely to account for the potent decrease in TXB$_2$ observed with I3P (Fig. 1E), nor could it account for the inhibition of PGs by I3P. We also tested whether I3P would affect intracellular PG levels in the same manner as secreted PGs. I3P reduced both intracellular and extracellular concentrations of PGs (Fig. 2I), implying that I3P is unlikely to affect PG transport. Thus, in terms of expression of enzymes in the PG synthesis pathway, I3P appears to significantly affect COX2 levels only, boosting its expression while inhibiting PG production.

**FIGURE 3.** I3P inhibits COX2 activity. (A) Schematic showing the structures of indole-3-acetic acid, indomethacin, and I3P. (B) BMDMs were treated with indomethacin (50 or 100 μM), I3P (0.5 or 1 mM), or the relevant vehicle control (DMSO or media) for 1 h prior to stimulation with LPS (100 ng/ml) for 24 h, and COX2 levels were measured by Western blotting (n = 3 from three independent experiments). (C) Quantification by densitometry is shown. (D) Schematic depicting hypothesis of I3P inhibition of COX2. (E) BMDMs were treated with 1 mM I3P for 45 min, followed by addition of 5 μM AA or vehicle (DMSO) for a further 15 min, prior to stimulation with LPS (100 ng/ml) for 24 h. The concentration of PGs in the supernatants was quantified by ELISA (n = 5 from three independent experiments). (F) COX2 activity was measured after incubation of 1 μg recombinant COX2 with various concentrations (31.25 μM to 1000 μM) of I3P or vehicle control (DMSO) (n = 3 from three independent experiments). Densitometry analysis is presented as mean ± SD. All other data are mean ± SEM. A one-way ANOVA was performed for (C). A two-way ANOVA was performed for (E). The data show the adjusted p value obtained from multiple comparisons, corrected for using the Tukey test for one-way ANOVA or the Sidák test for two-way ANOVA. *p < 0.05, ****p < 0.0001.
I3P targets COX2 activity

Given the potent inhibitory effect that I3P had on LPS-induced PGs and thromboxanes, we wondered whether I3P may be a direct COX inhibitor. One of the best-characterized COX inhibitors, indomethacin, is also built on an indole-3-acetic acid framework and therefore shares structural similarities to I3P (Fig. 3A). As already shown, I3P was able to elicit a similar inhibitory effect compared with indomethacin, measuring PGs by ELISA (Fig. 1F). As had been previously reported in the literature (32, 33) and in a similar manner to I3P, indomethacin also boosted LPS-induced COX2 expression (Fig. 3B, 3C, lanes 5 and 6 compared with lane 4), albeit to a lesser degree than I3P (lanes 11 and 12 compared with lane 10). Due to these similarities to the archetypal COX inhibitor indomethacin, we hypothesized that I3P may be inhibiting COX2. First, we supplemented the media with AA, the substrate of COX2 (Fig. 3C), to elucidate if COX2 was the point of modulation. When exogenous AA was added, I3P could still potently inhibit LPS-induced PGs (Fig. 3E), which indirectly suggested that COX2 activity was being blocked by I3P, as the addition of AA was insufficient to overcome the effect. We next tested if I3P could directly inhibit COX2 activity, by adding varying concentrations of I3P to recombinant COX2 and assaying COX activity. We found that I3P potently inhibited COX activity with an IC50 of 68.97 μM (Fig. 3F), thus confirming that I3P functions as a COX2 inhibitor.

The increase in COX2 expression by I3P may depend on a PG feedback loop

We hypothesized that perhaps the I3P-mediated inhibition of COX2 activity and downstream reduction in PGs was part of a negative-feedback loop by which decreased levels of PGs would cause an upregulation of COX2 transcription. We found that the addition of exogenous PGE2 modestly attenuated the ability of I3P to boost LPS-induced COX2 expression (Fig. 4A, 4B). PGE2 can bind to four different EP receptors (EP1–4). The expression of EP2 has been particularly implicated in macrophage function (34, 35), and binding of PGE2 to the EP2 receptor or the EP4 receptor causes an increase in cAMP production (36). Treatment with the EP2 antagonist AH6809 augmented the increase in COX2 with I3P treatment (Fig. 4C, 4D, lanes 7 and 8 compared with lanes 3 and 4). Treatment with the EP4 antagonist GW 627368X also amplified the increase in COX2 with I3P treatment (Fig. 4E, 4F, lanes 7 and 8 compared with lanes 3 and 4). Furthermore, pretreatment with forskolin, which raises intracellular cAMP levels and thereby mimics engagement of the EP2 and EP4 receptors, gave rise to decreased COX2 expression in response to LPS and I3P (Fig. 4G, 4H, lanes 7 and 8 compared with lanes 3 and 4). These data suggest the possibility of a feedback loop by which low concentrations of PGs and lack of EP2 and EP4 engagement cause a transcriptional increase in COX2 expression.
The boost in COX2 expression by I3P is also partially AhR-dependent.

As I3P has previously been shown to activate the AhR (17–19) and it is known that the COX2 promoter contains a corresponding xenobiotic-responsive element (39, 40), we reasoned that the AhR may also play a role in the I3P-induced increase in COX2 that we observed. Treatment with the AhR agonist 3-methylcholanthrene (3MC) increased LPS-induced COX2 expression in a similar manner to I3P (Fig. 5A, 5B, lane 4 compared with lane 6). However, 3MC had no effect on COX2 levels in AhR−/− BMDMs (Fig. 5A, 5B, lane 10 compared with lane 12). Similarly, the capacity of I3P to augment LPS-induced COX2 expression was partially impaired in the AhR−/− BMDMs, compared with the induction of COX2 observed in the wild-type BMDMs (Fig. 5C, 5D, lanes 11 and 12 compared with lanes 5 and 6). These data suggest that the increase in COX2 expression by I3P is partially dependent on activation of the AhR receptor. To test if AhR was also involved in PG inhibition by I3P, we knocked down AhR in BMDMs using siRNA, which successfully lowered transcript levels of AhR (Fig. 5E).

When AhR was silenced, I3P could no longer induce a boost in PGs (Fig. 5G), suggesting that the augmentation of COX2 expression by I3P is AhR-dependent but inhibition of PG production by I3P is not.

I3P inhibits trypanosome lysate-induced PGs and LPS-induced PGs in human macrophages

To demonstrate relevance during trypanosomiasis, we next investigated the effect of I3P on PGs induced by T. brucei lysate. It has previously been reported that for macrophages to respond to trypanosomes, they require stimulation with IFN-γ (9, 41), hence we cotreated the BMDMs with trypanosome lysate and IFN-γ, with or without I3P pretreatment. The cells that were pretreated with I3P prior to stimulation with IFN-γ and trypanosome lysate displayed enhanced levels of COX2 protein (Fig. 6A, 6B, lanes 8 and 9 compared with lanes 5 and 6) and secreted much less PGs (Fig. 6C). This mirrors the effects we have seen with I3P on LPS-induced COX2 and PGs. To investigate relevance of these findings for HAT, we tested if I3P had the same effects on human macrophages. The cells pretreated with I3P prior to stimulation with LPS displayed enhanced COX2 expression (Fig. 6D, 6E, lanes 4–6 compared with lane 3) and reduced PG production (Fig. 6F), thereby indicating that these effects may play a role in human infection. The secretion of I3P by T. brucei and the impact it has on PG/thromboxane production in macrophages through COX inhibition is depicted in Fig. 6G.

Discussion

Recently, the T. brucei–secreted metabolite I3P has emerged as an important immunomodulator, particularly regarding the regulation of host macrophage function (9, 16). In this study, we describe another mechanism by which I3P dampens the host immune response. We have provided evidence that I3P inhibits PG and thromboxane secretion from macrophages. We also have shown that I3P increases COX2 expression levels beyond those of LPS stimulation alone, contrary to the decrease in COX-dependent PGs. Our data indicate that the I3P-induced boost in COX2 may be mediated by two distinct mechanisms. We show that this COX2 boost is partially

![Image](http://www.jimmunol.org/)

**FIGURE 5.** The augmentation of COX2 expression by I3P is partially AhR-dependent. (A) BMDMs from AhR+/+ and AhR−/− mice were pretreated with 10 μM 3MC for 30 min prior to stimulation with LPS (100 ng/ml) for 24 h. (B) COX2 levels were measured by Western blotting (n = 3 from one independent experiment), and quantification by densitometry is shown. (C) BMDMs from AhR+/+ and AhR−/− mice were pretreated with I3P (1 mM) for 1 h prior to stimulation with LPS (10 or 100 ng/ml) for 24 h. (D) COX2 was quantified by Western blotting (n = 3 from one independent experiment), and quantification by densitometry is shown. (E–G) BMDMs were transfected with 50 nM control siRNA or AhR siRNA for 48 h. The cells were pretreated with I3P (0.5 or 1 mM) for 1 h prior to stimulation with LPS (100 ng/ml) for 24 h. The cells were lysed, mRNA was extracted and the expression of AhR (E) and Ptg2 (F) was quantified by qPCR (n = 4 from three independent experiments). All genes were normalized to rps18. (G) The concentration of PGs in the supernatants was quantified by ELISA (n = 4 from three independent experiments). Densitometry analysis is presented as mean ± SD. All other data are mean ± SEM. A one-way ANOVA was performed. The data show the adjusted p value obtained from multiple comparisons, corrected for using the Tukey test. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.
dependent on limiting a feedback loop involving endogenous PGE\textsubscript{2} acting via EP2 and EP4 receptors. In addition, we provide evidence that the effect is, at least partially, dependent on AhR activation. However, it would seem that this increase in COX2 expression by I3P has little functional effect, as I3P dramatically reduces PGs and thromboxanes downstream of COX2, despite this transcriptional increase. In addition, the fact that higher concentrations of I3P are required to increase COX2 expression may call into question the physiological relevance of this observation during infection in vivo. In contrast, I3P inhibits PG production at concentrations as low as 62.5 μM and therefore is likely to yield similar effects in vivo. At these higher concentrations, the increase in COX2 expression might have other functional consequences because COX2 has been shown to interact with and inhibit p53, affecting cell survival (42). This requires further investigation in the context of trypanosomiasis. Despite this COX2 induction, we provide evidence showing that I3P inhibits COX2 activity and have demonstrated that this inhibition of PG production is also observed when macrophages are stimulated with trypanosome-derived lysates. Furthermore, we have shown that the effects of I3P on COX2 expression and PG production are also true for primary human macrophages.

It was reported several decades ago that PG secretion was perturbed during trypanosomiasis. Macrophages taken from mice during an early stage of a T. brucei infection model displayed enhanced secretion of PGE\textsubscript{2} compared with uninfected controls, whereas at later stages of infection, the isolated macrophages secreted reduced 

---

**FIGURE 6.** I3P blocks trypanosome lysate-induced PG production and LPS-induced PG production in human macrophages. (A–C) BMDMs were pre-treated with 1 mM I3P or control media for 30 min before they were treated with 100 ng/ml IFN-γ and 25 or 100 μg/ml trypanosome lysate for 24 h. COX2 expression was measured by Western blotting (n = 3 from three independent experiments) (A), and quantification by densitometry is shown (B). (C) PGs were measured by ELISA (n = 3 from three independent experiments). (D–F) Primary human macrophages were pre-treated with I3P (1 mM, 0.5 mM, or 0.25 mM) or control media for 1 h before they were treated with LPS (100 ng/ml) for 24 h. COX2 expression was measured by Western blotting (n = 4 from three independent experiments) (D), and quantification by densitometry is shown (E). (G) PGs were measured by ELISA (n = 4 from three independent experiments). (G) Schematic representation of inhibition of COX2 by I3P. Densitometry analysis is presented as mean ± SD. All other data are mean ± SEM. A one-way ANOVA was performed. The data show the adjusted p value obtained from multiple comparisons, corrected for using the Tukey test. ***p < 0.0005, ****p < 0.0001.
concentrations of PGE2, and their ability to respond to LPS was not impaired (25). Our findings may shed light on the mechanism by which macrophage PGE2 secretion is hindered later in the course of disease. As the parasites proliferate to great numbers in the bloodstream (13), they will metabolize tryptophan to generate increasing concentrations of I3P (8), a metabolite that we have shown blocks COX2 activity and thereby lowers PGs. However, this proposed mechanism may in reality be complicated by the fact that trypanosomes have been reported to produce PGs of their own, which are known to interfere with host responses (27, 44).

Naturally, these data pose the question: why would T. brucei have evolved to inhibit host PGs? One possibility could be that it is beneficial to the parasite to inhibit the pyrogenic effects of PGE2, which are known to interfere with host responses (27, 44).

Our results reveal a potential mechanism of immunomodulation by T. brucei, mediated by one of the trypanosome-secreted keto acids, I3P. These data provide insight into the interaction between T. brucei and the host immune system and could potentially inform new therapeutic strategies to limit trypanosomiasis via modulation of I3P.

Acknowledgments
We thank Professor Brigitte Stockinger (Francis Crick Institute, London, U.K.) for providing legs from AhR+/− and AhR−/− mice.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. I3P has no consistent effect on the production of the LOX-derived HEPE and HDOHE oxylipins. BMDMs were treated with 1 mM I3P or control media for one hour prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell supernatants were subsequently analysed by tandem mass spectrometry (LC/MS/MS) to determine the concentration of the oxylipins above (n=4 from three independent experiments). Data are mean ± S.E.M. A one-way ANOVA was performed. The data show the adjusted p-value obtained from multiple comparisons, corrected for using the Tukey test. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.
Supplementary Figure 2. I3P has no consistent effect on the production of the LOX-derived HETE, HODE and HETrE oxylipins. (A-J) BMDMs were treated with 1 mM I3P or control media for one hour prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell supernatants were subsequently analysed by tandem mass spectrometry (LC/MS/MS) to determine the concentration of the oxylipins above (n=4 from three independent experiments). Data are mean ± S.E.M. A one-way ANOVA was performed. The data show the adjusted p-value obtained from multiple comparisons, corrected for using the Tukey test. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.
Supplementary Figure 3. I3P has no consistent effect on the production of the cytochrome P450-derived DiHOME, DiHETE and DiHETE oxylipins. (A-G) BMDMs were treated with 1 mM I3P or control media for one hour prior to stimulation with LPS (100 ng/mL) for 24 hours. Cell supernatants were subsequently analysed by tandem mass spectrometry (LC/MS/MS) to determine the concentration of the oxylipins above (n=4 from three independent experiments). Data are mean ± S.E.M. A one-way ANOVA was performed. The data show the adjusted p-value obtained from multiple comparisons, corrected for using the Tukey test. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001.
4-Octyl-Itaconate and Dimethyl Fumarate Inhibit COX2 Expression and Prostaglandin Production in Macrophages


*J Immunol* published online 11 October 2021
http://www.jimmunol.org/content/early/2021/10/11/jimmunol.2100488

Supplementary Material
http://www.jimmunol.org/content/suppl/2021/10/11/jimmunol.2100488.DCSupplemental

Why *The JI*? Submit online.

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
4-Octyl-Itaconate and Dimethyl Fumarate Inhibit COX2 Expression and Prostaglandin Production in Macrophages


PGs are important proinflammatory lipid mediators, the significance of which is highlighted by the widespread and efficacious use of nonsteroidal anti-inflammatory drugs in the treatment of inflammation. 4-Octyl itaconate (4-OI), a derivative of the Krebs cycle–derived metabolite itaconate, has recently garnered much interest as an anti-inflammatory agent. In this article, we show that 4-OI limits PG production in murine macrophages stimulated with the TLR1/2 ligand Pam3CSK4. This decrease in PG secretion is due to a robust suppression of cyclooxygenase 2 (COX2) expression by 4-OI, with both mRNA and protein levels decreased. Dimethyl fumarate, a fumarate derivative used in the treatment of multiple sclerosis, with properties similar to itaconate, replicated the phenotype observed with 4-OI. We also demonstrate that the decrease in COX2 expression and inhibition of downstream PG production occurs in an NRF2-independent manner. Our findings provide a new insight into the potential of 4-OI as an anti-inflammatory agent and also identifies a novel anti-inflammatory function of dimethyl fumarate. The Journal of Immunology, 2021, 207: 1–9.

Postaglandins are key lipid mediators, which exert a wide variety of roles in physiology. Their synthesis begins with the release of arachidonic acid by cytoplasmic phospholipase A2 (cPLA2) from membrane phospholipids, after which it is converted to PGH2 by the cyclooxygenase (COX) enzymes. COX1 is constitutively and ubiquitously expressed and is known to play several homeostatic roles, whereas COX2 is inducible by inflammatory stimuli, including TLR ligands and cytokines. PGH2 can then be converted into various PGs and thromboxanes by a range of synthase enzymes (1).

The widespread and effective use of nonsteroidal anti-inflammatory drugs, which inhibit COX enzymes, highlights the clinical importance of blocking PG production in inflammation (2). The proinflammatory effects of PGE2 in particular are well characterized. The capacity of PGE2 to induce a wide range of physiological and often pathological phenotypes is largely due to its binding to four different E prostanoid receptors (EP1–4), which vary in their tissue expression and downstream signal transduction pathways (3). PGE2 has been shown to activate mast cells (4), Th1 cells (5, 6), and Th17 cells (7–9). PGE2 has been implicated in inflammatory diseases, such as psoriasis (9) and rheumatoid arthritis (10), in pain responses (11, 12), and recently in aging (13). However, PGE2 has also been shown to exert anti-inflammatory functions, particularly in the environment of the lung (14–16). Another PG, PGD2, has been reported to contribute to the allergic response. Engagement of PGD2 with its receptors facilitates chemotaxis and activation of eosinophils and Th2 cells during allergic disease (17–19). Thromboxanes, which are also downstream of COX activity, promote vasoconstriction and platelet aggregation (20).

Itaconate is a metabolite that has emerged in recent years as an important immunomodulator (21). It is synthesized via the decarboxylation of the Krebs cycle intermediate cis-aconitate by the enzyme aconitate decarboxylase 1 (also known as IRG1), encoded by immune responsive gene 1 (22). The expression of IRG1 is predominantly restricted to macrophages and several other immune cell types and is markedly upregulated on stimulation with TLR ligands (23), thereby leading to an increase in intracellular itaconate. 4-Octyl itaconate (4-OI) is a cell-permeable derivative that is commonly used in the study of itaconate and has been shown to be converted into itaconate intracellularly in macrophages (24).

An important aspect of itaconate biology is that both the endogenous metabolite and 4-OI have been shown to function as cysteine modifiers (25). This cysteine alkylation was originally termed 2,3-dicarboxypropylation and is also referred to as itaconation. This posttranslational modification is the basis of many of the anti-inflammatory functions associated with itaconate and 4-OI (24, 26–30). 4-OI has been shown to modify cysteine residues on KEAP1 (25), which functions as a negative regulator of the master antioxidant transcription factor NRF2. Modifications of these cysteine residues cause KEAP1 to be degraded, which liberates NRF2 and permits translocation to the nucleus (31), where NRF2 induces transcription of antioxidant genes (32) and inhibits transcription of certain proinflammatory cytokines (33). The ability of 4-OI to activate NRF2 has been...
implicated in several of its anti-inflammatory and protective functions (25, 34–38).

Dimethyl fumarate (DMF) is a derivative of another Krebs cycle metabolite, fumarate, that is clinically approved for the treatment of multiple sclerosis (39). Like 4-OI, DMF is also a potent cysteine modifier (cysteine alkylation by fumarate is termed succination) (40), and DMF shares some of the same targets as 4-OI, such as GAPDH (27, 41), gasdermin D (28, 30, 42), and importantly, KEAP1 (25, 43), meaning that DMF is also a potent NRF2 activator. Therefore, these two metabolite derivatives often have similar effects on biological pathways.

The effect of itaconate and its derivatives on PG production has not been studied to date. In this study, we show that 4-OI greatly reduces PG production in proinflammatory macrophages through transcriptional suppression of COX2. We demonstrate that PG production and COX2 transcription are unchanged by the deletion of IRG1, indicating a difference with endogenous itaconate. However, DMF replicates the decrease in COX2 expression and PG secretion observed with 4-OI. Finally, we show that 4-OI and DMF reduce COX2 expression in an NRF2-independent manner. We have therefore uncovered a novel anti-inflammatory role of 4-OI and DMF in macrophages.

Materials and Methods

Reagents

4-OI was initially supplied by Prof. Richard Hartley, and results were later confirmed with commercially available 4-OI (Sigma-Aldrich). Pam3CSK4 (InvivoGen) was also supplied. Abs were used without prior application of the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to the manufacturer’s instructions. Immunoassays (mouse or human) were performed with a 7500 Fast Real-Time PCR System with PowerUp SYBR Green Master Mix (Applied Biosystems). All experiments carried out required prior ethical approval by Trinity College Dublin on October 26, 2021.
vial and vortexed for 30 s. A total of 2.5 ml hexane was added to samples and after vortexing for 30 s, tubes were centrifuged (500 × g for 5 min at 4°C) to recover lipids in the upper hexane layer (aqueous phase), which was transferred to a clean tube. Aqueous samples were reextracted as described earlier by addition of 2.5 ml hexane, and upper layers were combined. Lipid extraction from the lower aqueous layer was then completed according to the Bligh and Dyer technique. Specifically, 3.75 ml of a 2:1 ratio of methanol:chloroform was added followed by vortexing for 30 s. Subsequent additions of 1.25 ml chloroform and 1.25 ml water were followed with a vortexing step for 30 s, and the lower layer was recovered after centrifugation as described earlier and combined with the upper layers from the first stage of extraction. Solvent was dried under vacuum, and lipid extract was reconstituted in 100 µHPLC-grade methanol. Lipids were separated by liquid chromatography (LC) using a gradient of 30–100% B over 20 min (A: Water:Mob B 95:5 + 0.1% acetic acid; B: acetonitrile: methanol, 80:15 + 0.1% acetic acid) on an Eclipse Plus C18 Column (Agilent) and analyzed on a Sciex QTRAP 6500 LC tandem mass spectrometry (LC-MS/MS) system. Source conditions were temperature 475°C, ion spray voltage –4500, gas 1 60, gas 2 60, curtain gas 35. Lipids were detected using multiple reaction monitoring with the following parent-to-daughter ion transitions: PGE2 and PGD2 [M-H]-351.2/271.1, 15-deoxy-PGJ2 [M-H]-315.2/271.1, and TXB2 [M-H]-369.2/169.1. Deuterated internal standards were monitored using precursor to product ions transitions of TXB2-d4 [M-H]-373.2/173.1, PGE2-d4 and PGD2-d4 [M-H]-355.2/275.1, and 20-HETE-d6 [M-H]-325.2/281.1. Chromatographic peaks were integrated using Multiquant 3.0.2 software (Sciex). The criteria for assigning a peak were signal-to-noise ratio of at least 5:1 and with at least 7 points across a peak. The ratio of analyte peak areas to internal standard was taken, and lipids were quantified using a standard curve made up and run at the same time as the samples.

Statistical analysis

Statistical significance was established by the one-way or two-way ANOVA methods as indicated in the figure legends. Data are expressed as mean ± SEM. Significance was designated as follows: *p < 0.05, **p < 0.005, ***p < 0.0005, and ****p < 0.00005, and ****p < 0.0001. GraphPad Prism version 9 software was used for statistical analysis.

Results

4-OI inhibits Pam3CSK4-induced PG and thromboxane secretion by macrophages

To investigate whether 4-OI might play a role in modulation of PGs, we treated murine BMDMs with 4-OI before stimulation with the TLR1/2 agonist Pam3CSK4 for 24 h, which strongly induces PGs. We first used a PGE2 ELISA and observed a strong upregulation of cell supernatant PGs by Pam3CSK4, which was inhibited by 4-OI at concentrations as low as 25 µM (Fig. 1A). 4-OI also reduced Pam3CSK4-induced PG secretion by human PBMCs (Fig. 1B). To validate this result, we performed a lipidomic screen of oxylipins downstream of COX activity. As a confirmation that the ELISA was detecting COX-derived PGs in general, we found that the COX inhibitor indomethacin completely inhibited the Pam3CSK4-induced lipids from BMDMs detected by the ELISA (Fig. 1G). In addition, we used the COX2-specific inhibitor NS-398, which also inhibited all Pam3CSK4-induced lipids detected by the ELISA (Fig. 1H), indicating that PG secretion from BMDMs is almost entirely dependent on COX2.
4-OI suppresses Pam3CSK4-induced COX2 expression

Because we had observed that 4-OI suppressed all detected oxylipins downstream of COX activity, we next investigated whether 4-OI had an effect on COX2 expression. COX2 is potently upregulated by TLR ligands, such as Pam3CSK4, as seen by upregulation of Ptgs2 transcript, the gene that encodes COX2, as well as a strong boost in COX2 protein levels. 4-OI (200 μM) potently blocked Pam3CSK4-induced Ptgs2 levels at 4 and 8 h, the time points with the greatest induction of Ptgs2 (Fig. 2A). 4-OI also greatly decreased COX2 transcript levels at both 6 and 24 h (Fig. 2B). Concentrations as low as 25 μM 4-OI reduced COX2 protein at 24 h (Fig. 2C), whereas concentrations of 50 μM or higher decreased Ptgs2 transcript at 6 h (Fig. 2D). 4-OI also reduced transcript levels of PTGS2 in human PBMCs (Fig. 2E). The reduction of COX2 expression with 4-OI is likely the reason that 4-OI inhibits PG secretion, because COX2 is often referred to as the rate-limiting enzyme for PG synthesis. 4-OI did not alter the phosphorylation levels of cPLA2, nor did it affect total cPLA2 (Fig. 2F). 4-OI also had no significant effect on the mRNA levels of Ptges (Fig. 2E), the gene that encodes PGE2 synthase, the enzyme that catalyzes conversion of PGH2 to PGE2. Therefore, on the PGE2 biosynthetic pathway, 4-OI seems to specifically suppress COX2 expression, which leads to a decrease in downstream PG production.

As has been previously reported (25, 27), we observed that 4-OI modulated the mRNA expression of several cytokine genes in BMDMs and PBMCs that were induced by Pam3CSK4 stimulation (Supplemental Fig. 1A–F, 1J–N), such as yielding a reduction in Il1b, Il6, and Il10 transcripts. 4-OI also reduced mRNA levels of Nos2, which encodes NO synthase (Supplemental Fig. 1G), and the chemokine Ccl2 (Supplemental Fig. 1H). Cd86 levels were unchanged (Supplemental Fig. 1I). As expected, 4-OI increased transcript levels of the NRF2-dependent gene Hmox1 in both BMDMs and PBMCs (Supplemental Fig. 1F, 1O, respectively).

**FIGURE 2.** 4-OI inhibits Pam3CSK4-induced COX2 expression. (A) BMDMs were pretreated with 200 μM 4-OI before stimulation with Pam3CSK4 (100 ng/ml) for 2, 4, 8, 24, or 48 h. The cells were lysed, mRNA was extracted, and Ptgs2 expression was measured by qPCR (n = 4). (B) BMDMs were pretreated with 200 μM 4-OI before stimulation with Pam3CSK4 (100 ng/ml) for 6 or 24 h. COX2 expression was analyzed by Western blotting (n = 6). (C) BMDMs were pretreated with various concentrations of 4-OI (25–200 μM) before stimulation with Pam3CSK4 (100 ng/ml) for 24 h. COX2 expression was analyzed by Western blotting (n = 4). (D) BMDMs were pretreated with various concentrations of 4-OI (25–200 μM) before stimulation with Pam3CSK4 (100 ng/ml) for 6 h. After cell lysis, mRNA was extracted, and Ptgs2 expression was measured by qPCR (n = 4). (E) PBMCs were pretreated with 200 μM 4-OI for 2 h before stimulation with Pam3CSK4 (1 μg/ml) for 6 h. The cells were then lysed, mRNA was extracted, and Ptgs2 expression was measured by qPCR (n = 6). (F) BMDMs were pretreated with 200 μM 4-OI before stimulation with Pam3CSK4 (100 ng/ml) for various time points (10–120 min). Phospho-cPLA2 and total cPLA2 expression was analyzed by Western blotting (n = 3). (G) BMDMs were pretreated with 200 μM 4-OI before stimulation with Pam3CSK4 (100 ng/ml) for 4 h. After cell lysis, mRNA was extracted, and Ptges expression was measured by qPCR (n = 3). Data are mean ± SEM. *p < 0.05, **p < 0.0005, ****p < 0.0001, ns, not significant, by one-way ANOVA.
Endogenous itaconate does not affect COX2 expression or PG production

We next tested whether endogenous itaconate, as well as the derivatized 4-OI, would impact COX2 expression and PG synthesis. For this we used BMDMs lacking Irg1, the gene that encodes the enzyme responsible for itaconate synthesis. However, when Ptgs2 transcript levels between Irg1+/+ BMDMs and Irg1−/− BMDMs are compared, there is no difference in COX2 induction by Pam3CSK4 (Fig. 3A). In addition, there were no changes in the COX-derived oxylipins PGE₂ (Fig. 3B), PGD₂ (Fig. 3C), 15-deoxy-PGJ₂ (Fig. 3D), and TXB₂ (Fig. 3E) when measured by MS/MS. This shows that endogenous itaconate does not affect COX2 expression or PG production.

DMF also reduces Pam3CSK4-induced COX2 expression and PG production

We next tested whether DMF might have similar effects on COX2 and PG production to 4-OI. Pretreatment of BMDMs with concentrations as low as 5 μM DMF decreased levels of Ptgs2 transcript (Fig. 4A). DMF significantly downregulated Ptgs2 mRNA levels at 4 and 8 h (Fig. 4B), similar to 4-OI, COX2 protein levels were also attenuated by DMF, using concentrations as low as 5 μM (Fig. 4C). DMF also decreased Pam3CSK4-induced PG secretion by BMDMs (Fig. 4D). Given that 4-OI and DMF are both potent cysteine modiﬁers and have very similar effects regarding COX2 expression and PG synthesis, it is likely that they are acting through a shared mechanism.

The Journal of Immunology 5

FIGURE 3. Endogenous itaconate does not affect COX2 expression or PG production. (A) BMDMs from Irg1+/+ and Irg1−/− mice were stimulated with Pam3CSK4 (100 ng/ml) for 2, 4, or 6 h. The cells were lysed and mRNA extracted to quantify Ptgs2 by qPCR (n = 4). (B–E) BMDMs from Irg1+/+ and Irg1−/− mice were stimulated with Pam3CSK4 (100 ng/ml) for 24 h. The cell supernatants were then analyzed by MS/MS to determine (B) PGE₂, (C) PGD₂, (D) 15-deoxy-PGJ₂, and (E) TXB₂ concentrations (n = 4). Data are mean ± SEM. Data were analysed using one-way ANOVA or two-way ANOVA for (A).
FIGURE 4. DMF decreases COX2 expression and PG production. (A) BMDMs were pretreated with various concentrations of DMF (5–25 μM) for 2 h before stimulation with Pam3CSK4 (100 ng/ml) for 4 h. After cell lysis, mRNA was extracted, and Ptgs2 levels were quantified by qPCR (n = 4). (B) BMDMs were pretreated with 25 μM DMF for 2 h before stimulation with Pam3CSK4 (100 ng/ml) for 2, 4, 8, 24, or 48 h. After cell lysis, mRNA was extracted, and Ptgs2 levels were quantified by qPCR (n = 4). (C) BMDMs were pretreated with various concentrations of DMF (5–25 μM) for 2 h before stimulation with Pam3CSK4 (100 ng/ml) for 24 h. COX2 expression was analyzed by Western blotting (n = 4). (D) BMDMs were pretreated with various concentrations of DMF (5–25 μM) for 2 h before stimulation with Pam3CSK4 (100 ng/ml) for 24 h. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA (n = 4). Data are mean ± SEM. *p < 0.05, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.

of 4-OI to reduce COX2 expression (Supplemental Fig. 3A). Furthermore, 4-OI and DMF both blocked PG production in all genotypes (Fig. 5G, 5H, respectively). It is also worth noting that cells lacking NRF2 have lower levels of COX2 and PGs, whereas the KEAP1 knockdown cells, which exhibit augmented NRF2 activation, display elevated COX2 expression and PG production (Fig. 5E–H). Interestingly, DEM, which is considered to be a well-characterized NRF2 activator, could also still block Ptgs2 transcription in NRF2 knockout and KEAP1 knockdown BMDMs (Supplemental Fig. 2E), indicating that its ability to reduce COX2 is separate to its NRF2-activating function. We also observed that 4-OI had no effect on NF-kB p65 phosphorylation, p38 phosphorylation, or ERK phosphorylation (Supplemental Fig. 3B), all of which are signaling pathways that are known to modulate Ptgs2 transcription. We also investigated whether ATF4 was involved in the observed effect, given that ATF4 was recently shown to bind directly to the Ptgs2 promoter and induce its transcription (45). However, 4-OI actually increased ATF4 expression, and ATF4 silencing had no effect on the inhibition of COX2 by 4-OI (Supplemental Fig. 3C). We also tested whether the effect of 4-OI on COX2 might be dependent on annexin A1, which has been shown to be modified by itaconate and itaconate derivatives (25, 28, 29) and is known to function as a negative regulator of cPLA2, which catalyzes the first step of PG biosynthesis. However, silencing annexin A1 did not alter the inhibition of PG production by 4-OI (Supplemental Fig. 3D). These results indicate that the capacity of 4-OI and DMF to suppress COX2 expression and downstream PG production is independent of NRF2 activation and other known signals that regulate COX2.

Discussion

The itaconate derivative 4-OI has recently garnered much attention as an immunomodulator. The findings of this study suggest a role for 4-OI in the inhibition of PG production in macrophages activated with the TLR1/2 ligand Pam3CSK4. We show that 4-OI potently reduces several COX-derived oxylipins and provide evidence that 4-OI suppresses COX2 transcription. We demonstrate that although endogenous itaconate derived from IRG1 activity does not affect COX2 levels, DMF attenuated COX2 expression and PG secretion in a similar manner to 4-OI. This implies that a cysteine modification may be involved, but we also provide evidence that suggests that the effect of 4-OI and DMF on PG production is not via KEAP1 degradation and NRF2 activation. Further work is required to fully elucidate the mechanism by which 4-OI and DMF impair COX2 transcription.

Our knowledge of how Krebs cycle activity impacts inflammation has been rapidly expanding over the last decade. Some Krebs cycle intermediates are known to exert proinflammatory actions, such as succinate, which has been shown to stabilize HIF-1α, thereby upregulating IL-1β transcription (46). Succinate has also been reported to exacerbate certain inflammatory diseases, such as arthritis (47) and type 2 diabetes (48). Another Krebs cycle intermediate, citrate, has also been shown to alter the inflammatory response. The mitochondrial export, in addition to the breakdown of citrate, has been reported to be essential for NO and PG production (49, 50). Several anti-inflammatory roles have recently been described for both itaconate (25, 30, 51) and fumarate (41, 42), and in this article we report a novel function for derivatives of these two Krebs cycle metabolites in macrophages.

This work also highlights the importance of bearing in mind that metabolite derivatives do not always truly represent the action of the corresponding endogenous metabolites. In the case of 4-OI, the use of this derivative replicates the biological effects of endogenous itaconate in a number of cases, such as the inactivation of the NLRP3 inflammasome (24) and impairment of glycolysis (27). However, there are also incidences where 4-OI and endogenous itaconate differ in their effects, such as type I IFN production, which is boosted by endogenous itaconate (52) but inhibited by 4-OI (25). Although itaconate has also been shown to modify proteins in the same manner as 4-OI, the targets are not always the same. Our previous study (25) found that, although there were some overlapping proteins modified by both 4-OI and endogenous itaconate, many targets were mutually exclusive. This could potentially be because of differences in electrophilicity between itaconate and 4-OI.
Although DMF is known to be a potent NRF2 activator (43, 53), NRF2-independent anti-inflammatory functions of DMF have begun to emerge, such as the impairment of glycolysis through GAPDH succination (41) and inhibition of pyroptosis via gasdermin D (42). It is also true for 4-OI that a number of its currently known anti-inflammatory effects are NRF2 dependent (25, 34, 35), although others are not (24, 27, 30, 51). Interestingly, DEM, which is predominantly used as an experimental tool to pharmacologically activate NRF2, also suppressed COX2 expression in an NRF2-independent manner. Because DEM has been shown to modify cysteines on KEAP1 in a similar way to 4-OI and DMF (44), perhaps DEM also possesses the capacity to modify other reactive cysteines in the cell with further reaching implications. An observation that adds complexity to this system is that 15-deoxy-Δ12,14-PGJ2.

**FIGURE 5.** The capacity of 4-OI and DMF to reduce COX2 expression and PG production is not NRF2 dependent. (A-D) BMDMs from wild-type, NRF2 knockout, and KEAP1 knockdown mice were pretreated with 200 μM 4-OI (A and C) or 25 μM DMF (B and D) for 2 h before stimulation with Pam3CSK4 (100 ng/ml) for 6 h. The cells were lysed, mRNA was extracted, and Nqo1 expression (A and B) and Ptgs2 expression (C and D) were quantified by qPCR (n = 3). (E-H) BMDMs from wild-type, NRF2 knockout, and KEAP1 knockdown mice were pretreated with 200 μM 4-OI (E and G) or 25 μM DMF (F and H) for 2 h before stimulation with Pam3CSK4 (100 ng/ml) for 24 h. COX2 expression was analyzed by Western blotting (E and F) (n = 3). The supernatants were analyzed for PG concentration by ELISA (G and H) (n = 3). Data are mean ± SEM. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
downstream of COX activity and therefore would be reduced by 4-OI, has been shown to activate NRF2 (54). Nonetheless, our data reveal that endogenous PGE2 acting via the EP2 receptor is required for induction of pro-IL-1β (56). Hence the inhibition of PGE2 secretion from the macrophage and subsequent binding to EP2 could potentially contribute to the attenuation of other proinflammatory mediators that could potentially affect the induction of COX2. For example, IL-1β and NO, both of which are inhibited by 4-OI (25), have been reported to contribute to the attenuation of other inflammatory mediators.

We thank Prof. Richard Hartley (University of Glasgow) for the synthesis of 4-OI. We thank Prof. Albeena Dinkova-Kostova (University of Dundee) for providing bones from NRF2 knockout, KEAP1 knockdown, and matched wild-type mice.

Acknowledgments

The authors have no financial conflicts of interest.

References


14. Gauvreau, G. M., R. M. Watson, and M. O. Myllyla. 1999. cytotoxic T lymphocytes (CTL) contribute to the attenuation of other proinflammatory effects of 4-OI. We thank Prof. Albeena Dinkova-Kostova (University of Dundee) for providing bones from NRF2 knockout, KEAP1 knockdown, and matched wild-type mice.

Disclosures

The authors have no financial conflicts of interest.


Supplemental Figure 1. 4-OI alters proinflammatory gene expression. (A–I) BMDMs were pretreated with DMSO or 4-OI (100 μM or 200 μM) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. (A) Il1b, (B) Il6, (C) Tnf, (D) Il10, (E) Tgfb1, (F) Hmox1, (G) Nos2, (H) Ccl2 and (I) Cd86 expression were quantified by qPCR (n=5). (J–O) Human PBMCs were pretreated with DMSO or 200 μM 4-OI for two hours prior to stimulation with Pam3CSK4 (1 μg/mL) for six hours. (J) Il1b, (K) Il6, (L) Tnf, (M) Il10, (N) Tgfb1 and (O) Hmox1 expression were quantified by qPCR (n=6).
Supplemental Figure 2. DEM reduces COX2 expression and prostaglandin production in an NRF2-independent manner. (A) BMDMs were pretreated with 100 μM DEM for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. COX2 expression was analysed by Western blotting (n=4). (B) BMDMs were pretreated with 100 μM DEM for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for four hours. After cell lysis, mRNA was extracted and Ptgs2 levels were quantified by qPCR (n=6). (C) BMDMs were pretreated with 100 μM DEM for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA (n=4). (D and E) BMDMs from wild-type, NRF2 knockout and KEAP1 knockdown mice were pretreated with 100 μM DEM for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. The cells were lysed, mRNA was extracted and Nqo1 expression (D) and Ptgs2 expression (E) were quantified by qPCR (n=3). Data are mean ± S.E.M. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
Supplemental Figure 3. The capacity of 4-OI to inhibit COX2 expression and prostaglandin production is not via NRF2, NF-kB, p38, ERK, ATF4 or annexin A1. (A) BMDMs were transfected with 50 nM control siRNA or NRF2 siRNA for 24 hours. The cells then were treated with 200 μM 4-OI prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. NRF2 and COX2 expression were analysed by Western blotting (n=5). (B) BMDMs were pretreated with 200 μM 4-OI prior to stimulation with Pam3CSK4 (100 ng/mL) for 10, 20, 40, 60 or 120 minutes. Phospho and total p38, p65 and ERK levels were measured by Western blotting (n=3). (C) BMDMs were transfected with 50 nM control siRNA or ATF4 siRNA for 48 hours. The cells then were treated with 200 μM 4-OI prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. ATF4 and COX2 expression were analysed by Western blotting (n=3). (D) BMDMs were transfected with 50 nM control siRNA or anxa1 siRNA for 96 hours. The cells then were treated with 200 μM 4-OI prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA (n=3). Data are mean ± S.E.M. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.
Modification of Proteins by Metabolites in Immunity

C. Diskin,1 T.A.J. Ryan,1 and L.A.J. O’Neill1,*
1School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Ireland
*Correspondence: laoneill@tcd.ie
https://doi.org/10.1016/j.immuni.2020.09.014

SUMMARY

Immunometabolism has emerged as a key focus for immunologists, with metabolic change in immune cells becoming as important a determinant for specific immune effector responses as discrete signaling pathways. A key output for these changes involves post-translational modification (PTM) of proteins by metabolites. Products of glycolysis and Krebs cycle pathways can mediate these events, as can lipids, amino acids, and polyamines. A rich and diverse set of PTMs in macrophages and T cells has been uncovered, altering phenotype and modulating immunity and inflammation in different contexts. We review the recent findings in this area and speculate whether they could be of use in the effort to develop therapeutics for immune-related diseases.

The recent growth in the field of immunometabolism has revealed how specific changes in the concentrations of metabolites in immune cells can affect the immune response (Makowski et al., 2020; O’Neill et al., 2016). The consequences of these metabolic alterations go beyond the traditional role of metabolism in bioenergetics and biosynthesis. Immunologists have instead revealed roles for specific metabolites in targeting processes that have impact on immune cell function in more specific ways. Post-translational modification of proteins is emerging as a key means by which intracellular metabolites can modulate immunity. In this review, we focus on recent developments in this specific area and speculate on their importance as a critical functional readout for the metabolic changes that are happening in immune cells. A wealth of information is emerging that could prove useful in the search for immunomodulatory therapeutics for immune and inflammatory disease.

LACTYLATION CONTRIBUTES TO RESOLUTION OF INFLAMMATION

The renaissance of interest in metabolism in immune cells can be traced to the observation that inflammatory cells, such as classically activated macrophages and T helper-17 (Th17) cells, display the so-called Warburg effect (Krawczyk et al., 2010; Masters et al., 2010; Shi et al., 2011). This phenomenon was originally described in cancer, where glycolysis is potently upregulated, even when oxygen is readily available, and is a major generator of adenosine triphosphate (ATP) in tumors (Vander Heiden et al., 2009; Warburg, 1956). Blocking glycolysis in immune cells was shown to impact on interleukin-1β (IL-1β) production from macrophages (Mills et al., 2016; Tannahill et al., 2013), Th17 cell differentiation (Shi et al., 2011) and dendritic cell (DC) activation (Krawczyk et al., 2010). High rates of glycolysis mean that large amounts of lactate are produced and secreted. Previously, lactate was considered to be little more than a waste by-product of this metabolic process but emerging evidence indicates that it has a number of notable physiological roles, such as fueling Krebs cycle (Faubert et al., 2017; Hui et al., 2017).

A recent contribution to what Warburg metabolism might mean for macrophages has been made when lactate was shown to modify histones in a process termed lactylation. This post-translational modification (PTM) was shown to occur in several cancer cell lines under conditions of hypoxia and is highly prominent in proinflammatory classically activated macrophages (Zhang et al., 2019a). Lactylation of histones at promoter regions of genes increases their expression and genes associated with wound healing, such as Arg1, have been found to be particularly upregulated. Standard proinflammatory genes, such as those encoding Tnf and Il6, are unaffected. This study revealed a mechanism by which proinflammatory macrophages could adopt a more homeostatic alternatively activated phenotype at later time points, in order to facilitate the resolution of inflammation and was consistent with previous studies linking lactate to alternative macrophage polarization (Colegio et al., 2014). Importantly, the authors demonstrated that lactylation could be derived from both exogenous and glucose-dependent endogenous lactate. Histone lactylation may also contribute to the alternatively activated profile that is typical of tumor-associated macrophages (TAMs). As tumors are often hypoxic environments, this leads to lactate accumulation, which may alter the epigenetic landscape of the resident macrophages, giving rise to the typical TAM phenotype that is observed. High lactation in TAMs has also been observed in two mouse models of cancer (Zhang et al., 2019a). This study therefore reveals another important aspect of glycolysis in macrophages. At early time points, glycolysis is required for proinflammatory gene expression, but at later time points the main product of glycolysis in Warburg metabolism, lactate, promotes an anti-inflammatory phenotype by lactylating histones on specific target genes important for resolution of inflammation (see Figure 1). This PTM would therefore...
appear to be critical for an anti-inflammatory pro-repair response in macrophages. Further PTMs mediated by glycolytic intermediates may well emerge.

**ACETYLATION OF HISTONES AND OTHER IMMUNE TARGETS**

Moving on from glycolysis, the next major metabolic pathway is Krebs cycle, the central hub of all metabolism. It takes place in the mitochondrial matrix and the main role ascribed to it is to generate nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂), which deliver electrons to the electron transport chain, leading to ATP production in the process called oxidative phosphorylation (OXPHOS). Krebs cycle is fueled by acetyl-CoA derived from glucose or fatty acids, beginning a series of eight oxidizing reactions. Alterations in Krebs cycle have been reported to occur in various immune cell types upon their activation. Krebs cycle and oxidative phosphorylation are functional in most T cell subsets, with particular importance in memory CD8⁺ T cells (van der Windt et al., 2013), but alterations have been studied most extensively in macrophages (Jha et al., 2015; Ryan and O'Neill, 2020). Alternatively activated M2 macrophages possess an intact Krebs cycle coupled to OXPHOS, which is thought to facilitate production of UDP-GlcNAc intermediates for the glycosylation of C-type lectins and mannose receptors, which are both important for the role of alternatively activated macrophages in the recognition of specific pathogens, including fungi and parasites. Classically activated macrophages, on the other hand, potently upregulate glycolysis as their predominant ATP-generating pathway as stated above, and their Krebs cycle becomes disrupted at two distinct breakpoints. This results in the accumulation of succinate and citrate, which in turn leads to accumulation of itaconate. Succinate and itaconate have both been shown to modify proteins, as have two other Krebs cycle intermediates, acetyl-CoA and fumarate.

Acetyl-CoA represents the entry point into Krebs cycle. The tricarboxylic acid (TCA) intermediate citrate can be exported from the mitochondria to the cytosol and converted to acetyl-CoA by ATP citrate lyase (ACLY), which leads to acetylation (Loftus and Finlay, 2016). Acetylation and deacetylation of lysine residues are the most broadly studied and extensively characterized epigenetic modifications of histones (Chen et al., 2020). Two types of key regulators, histone acetyltransferases (HATs) and histone deacetylases (HDACs), dynamically control the acetylation state of histones. The antagonistic actions of these enzymes on histones serve as an important mechanism for the epigenetic regulation of gene expression (Wang et al., 2009). HATs promote acetylation, which is related to transcriptional activity, whereas HDACs catalyze the removal of acetyl groups from histone tails, which is linked to transcriptional inhibition (Sterner and Berger, 2000).

The acetylation state of proteins, which is reversible, is highly relevant to their stability and activity in cells. Defects in protein acetylation frequently result in severe abnormalities of development and physiology due to the dysregulation of gene expression and protein function in animal models, and are implicated in the pathogenesis of many human diseases (Haberland et al., 2009; Johnstone, 2002; Wang et al., 2014; Zhou et al., 2011). Histone acetylation is promoted by the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS), which leads to acetylation (Lauterbach et al., 2019). It has been recently shown that CD8⁺ T cells in low glucose tumor microenvironments can utilize acetate as a fuel source (Qiu et al., 2019). The uptake of acetate by these T cells also contribute to increased acetylation of histones and upregulation of genes such as IFN-γ for immune activation.
HDACs play an important role in macrophage activation and have been shown to regulate production of multiple inflammatory genes (Das Gupta et al., 2016; Nguyen et al., 2018). Deacetylation, which is controlled by sirtuins, is important in M2 macrophage gene expression (Park et al., 2017). Moreover, the interplay between acetylation and deacetylation in macrophage activation is highlighted by the recent discovery that NLRP3, a pattern recognition receptor (PRR), is modified by acetylation in macrophages, which leads to its activation (Zhang et al., 2011). Similar to malonylation, this modification alters the positively charged lysine to have a net charge of -1, and while no succinylating enzyme has been thus far identified, desuccinylation is catalyzed by SIRT5, as mentioned above (Du et al., 2011). A report of elevated succinate concentrations in proinflammatory macrophages also demonstrated an enrichment in global protein succinylation, including the identification of several metabolic enzymes that were succinylated, such as GAPDH and malate dehydrogenase (MDH), but the functional consequence of succinylation of these proteins has not been further investigated. There is also a decrease in the expression of SIRT5 when macrophages were stimulated with LPS (Tannahill et al., 2013), which would skew proteins toward succinylation. As shown in Figure 1, pyruvate kinase 2 (PKM2) is an important target for succinylation in macrophages. In LPS-activated macrophages, the glycolytic enzyme PKM2 has been shown to translocate to the nucleus, where it can form a complex with hypoxia inducible factor -1α (HIF-1α) and promote the transcription of HIF-dependent genes, including that encoding interleukin-1β (IL-1β) (Palsson-McDermott et al., 2015). Furthermore, nuclear translocation of PKM2 in T cells has been found to promote Th1 and Th17 cell polarization (Angiari et al., 2020), with nuclear PKM2 having been shown to induce STAT3 phosphorylation and thereby enhance IL-17 transcription (Pucino et al., 2019). Succinylation of PKM2 at K311 has been shown to impair its glycolytic activity and induce its nuclear translocation with a concomitant increase in IL-1β production (Wang et al., 2017). In addition, Sirt5-deficient mice exhibit increased susceptibility to dextran sulfate-induced colitis due to hypersuccinylation of PKM2, leading to elevated IL-1β (Wang et al., 2017). Interestingly, in the context of cancer, succinylation of PKM2 has been shown to increase its enzymatic activity but here a different succinylated lysine residue (K498) has been reported (Xiangyun et al., 2017), indicating that succinylation may give rise to contrasting effects on the same protein target, depending on which lysine residue is modified. This reveals the complexity of succinylation as a process requiring further analysis in an immune context.

Fumarate has been shown to modify cysteine residues on target proteins in a modification termed succinylation (not to be confused with succinylation) (Ali et al., 2006). Although this modification is better studied in cancer and diabetes (Blažnik et al., 2008; Yang et al., 2014), it has recently been shown to occur on GAPDH in immune cells, as shown in Figure 1. A fumarate derivative, dimethyl fumarate (DMF), that is currently used in the treatment of multiple sclerosis (MS) was found to modify cysteine 150 on GAPDH in murine macrophages and T cells, as well as the corresponding active site cysteine (C152) for mTORC1 activity, which has been found to contribute to defects in angiogenesis in endothelial cells (Bruning et al., 2018a). Malonylation of mTOR may well play a notable role in macrophage, T cell, and B cell activation, given its central signaling role in these cell types, but this has yet to be examined.

**MALONYLATION DRIVES PROINFLAMMATORY MACROPHAGES**

Acetyl-CoA can be converted to malonyl CoA by acetyl-CoA carboxylase (ACC) for the synthesis of fatty acids or cholesterol. Malonyl-CoA can also promote malonylation, a recently identified, evolutionarily conserved PTM (Peng et al., 2011; Xie et al., 2012), wherein malonyl-CoA is used as a substrate to add a malonyl group to lysine residues (Bowman et al., 2017; Colak et al., 2015), changing its charge from +1 to -1 (Galván-Peña et al., 2019). This change is predicted to disrupt electrostatic interactions with other amino acids and alter protein conformation and binding to targets (Peng et al., 2011). The only known regulator of malonylation is SIRT5, which acts not only as a deacetylase, but can also remove other acylations, such as succinylation and glutarylation (Du et al., 2011; Tan et al., 2014).

GAPDH becomes malonylated (Figure 2) in pro-inflammatory macrophages, which enhances its glycolytic activity and reduces its RNA-binding capacity, allowing TNF-α to be translated, and enabling the production of further pro-inflammatory cytokines, including IL-1β and IL-6 (Galván-Peña et al., 2019). Malonylation was therefore proposed as a mechanism by which macrophages can control the production of proinflammatory cytokines through GAPDH.

mTOR has also been shown to undergo malonylation (Figure 2) when fatty acid synthase (FASN) is inhibited or silenced. This reduces mTORC1 activity, which has been found to contribute to defects in angiogenesis in endothelial cells (Bruning et al., 2018a). Malonylation of mTOR may well play a notable role in macrophage, T cell, and B cell activation, given its central signaling role in these cell types, but this has yet to be examined.

**SUCCINYLATION, SUCCINATION, AND 2,3-DICARBOXYPROPYLATION TARGET GLYCOLYSIS IN IMMUNE CELLS**

Succinate accumulation can give rise to a PTM termed succinylation, which involves the transfer of a succinyl-CoA moiety to a lysine residue (Zhang et al., 2011). Similar to malonylation, this modification alters the positively charged lysine to have a net charge of -1, and while no succinylating enzyme has been thus far identified, desuccinylation is catalyzed by SIRT5, as mentioned above (Du et al., 2011). A report of elevated succinate concentrations in proinflammatory macrophages also demonstrated an enrichment in global protein succinylation, including the identification of several metabolic enzymes that were succinylated, such as GAPDH and malate dehydrogenase (MDH), but the functional consequence of succinylation of these proteins has not been further investigated. There is also a decrease in the expression of SIRT5 when macrophages were stimulated with LPS (Tannahill et al., 2013), which would skew proteins toward succinylation. As shown in Figure 1, pyruvate kinase 2 (PKM2) is an important target for succinylation in macrophages. In LPS-activated macrophages, the glycolytic enzyme PKM2 has been shown to translocate to the nucleus, where it can form a complex with hypoxia inducible factor -1α (HIF-1α) and promote the transcription of HIF-dependent genes, including that encoding interleukin-1β (IL-1β) (Palsson-McDermott et al., 2015). Furthermore, nuclear translocation of PKM2 in T cells has been found to promote Th1 and Th17 cell polarization (Angiari et al., 2020), with nuclear PKM2 having been shown to induce STAT3 phosphorylation and thereby enhance IL-17 transcription (Pucino et al., 2019). Succinylation of PKM2 at K311 has been shown to impair its glycolytic activity and induce its nuclear translocation with a concomitant increase in IL-1β production (Wang et al., 2017). In addition, Sirt5-deficient mice exhibit increased susceptibility to dextran sulfate-induced colitis due to hypersuccinylation of PKM2, leading to elevated IL-1β (Wang et al., 2017). Interestingly, in the context of cancer, succinylation of PKM2 has been shown to increase its enzymatic activity but here a different succinylated lysine residue (K498) has been reported (Xiangyun et al., 2017), indicating that succinylation may give rise to contrasting effects on the same protein target, depending on which lysine residue is modified. This reveals the complexity of succinylation as a process requiring further analysis in an immune context.

Fumarate has been shown to modify cysteine residues on target proteins in a modification termed succinylation (not to be confused with succinylation) (Ali et al., 2006). Although this modification is better studied in cancer and diabetes (Blažnik et al., 2008; Yang et al., 2014), it has recently been shown to occur on GAPDH in immune cells, as shown in Figure 1. A fumarate derivative, dimethyl fumarate (DMF), that is currently used in the treatment of multiple sclerosis (MS) was found to modify cysteine 150 on GAPDH in murine macrophages and T cells, as well as the corresponding active site cysteine (C152) for mTORC1 activity, which has been found to contribute to defects in angiogenesis in endothelial cells (Bruning et al., 2018a). Malonylation of mTOR may well play a notable role in macrophage, T cell, and B cell activation, given its central signaling role in these cell types, but this has yet to be examined.
human GAPDH in PBMCs from MS patients treated with DMF (Kornberg et al., 2018). The authors also show that GAPDH could be modified by endogenous fumarate in both murine and human immune cells. Succination of GAPDH has been shown to inhibit its enzymatic activity and curtail glycolytic flux. In macrophages, succination of GAPDH is required for DMF-induced inhibition of IL-1β and in CD4+ T cells for the ability of DMF to impair Th1 and Th17 cell differentiation and cytokine production. This study is crucial as it demonstrates that metabolic PTMs can be exploited as a clinical intervention.

Itaconate is another metabolite linked to Krebs cycle that can modify proteins. The accumulation of citrate can lead to the production of itaconate, via cis-aconitate, which is diverted away from Krebs cycle by the enzyme cis-aconitate decarboxylase (CAD), which is encoded by the gene termed Immune responsive gene 1 (IRG1). IRG1, or CAD, is induced in classically activated macrophages by LPS, via type I interferons (IFNs) (Naujoks et al., 2016). Prior to the functional characterization of IRG1, this gene is known to be potently upregulated in LPS-activated macrophages (Lee et al., 1995) before it was later identified as the enzyme responsible for the synthesis of itaconate (Michelucci et al., 2013). Itaconate is one of the most abundant metabolites in classically activated macrophages (Jha et al., 2015; Mills et al., 2018), and recent evidence indicates that it is an anti-inflammatory metabolite. Itaconate has been shown to exert some of these effects through succinate dehydrogenase (SDH) inhibition (Cordes et al., 2016; Daniels et al., 2019; Lampropoulou et al., 2016) and activating transcription factor 3 (ATF3) activation (Bambouskova et al., 2018). However, itaconate has also been identified as a cysteine modifier (Mills et al., 2018), as shown in Figure 1. The modification is called 2,3-dicarboxypropylation, which is a form of cysteine alkylation. It has been found to occur on several cysteine residues on KEAP1 in response to the cell-permeable itaconate derivative, 4-octyl itaconate (4-OI) (Mills et al., 2018), including C151, which has been previously reported to play a role in KEAP1-dependent regulation of NRF2 expression (Zhang and Hannink, 2003). Under basal conditions, KEAP1 associates with NRF2 and induces its degradation but modification of these key cysteine residues on KEAP1 by 4-OI results in the destabilization of KEAP1 and accumulation of NRF2. This prompts the initiation of the NRF2 antioxidant response, which in turn inhibits transcription of some proinflammatory cytokines, including IL-1β and IL-6. IRG1-deficient macrophages are impaired in the induction of NRF2 by LPS (Bambouskova et al., 2018). Modification of KEAP1 by endogenous itaconate has not been demonstrated...
In macrophage lysates (Qin et al., 2019). In this screen, several of the proteins previously shown to be 2,3-dicarboxypropylated were confirmed, including LDHA and annexin A1, in addition to numerous additional protein targets being identified. The authors selected aldolase A, a glycolytic enzyme, as the focus for further validation and investigation. They showed that aldolase A is modified by endogenous itaconate on two cysteine residues and that this greatly impairs its glycolytic activity and contributes to the capacity of itaconate to downregulate IL-1β production, which is known to require glycolysis (Tannahill et al., 2013). It has also been shown that modification of LDHA by itaconate hinders its enzymatic activity but interestingly this does not affect IL-1β production, suggesting that glycolysis per se is not required for IL-1β production. More recently, Qin et al. have developed a biorthogonal itaconate-alkyne probe in order to assess targets of 2,3-dicarboxypropylation in living macrophages (Qin et al., 2020). This study identifies many additional immune targets that undergo 2,3-dicarboxypropylation in Raw264.7 macrophages, such as NLRP3, AIM2, IRAK4, and gasdermin D. Further functional characterization of one of the targets, RIPK3, has determined that modification of C360 on RIPK3 by itaconate promotes phosphorylation and activation of RIPK3 (Qin et al., 2020), suggesting that itaconate may play a role in necroptosis.

Modification of another glycolytic enzyme, GAPDH, by itaconate has also been reported in this study (Qin et al., 2019), and this has been confirmed in another study, where cysteine 22 of GAPDH has been shown to be 2,3-dicarboxypropylated by 4-Oi (Liao et al., 2019). This modification reduces enzymatic activity of GAPDH, decreasing aerobic glycolysis and lowering proinflammatory cytokine production (Liao et al., 2019). Interestingly, in another study, 2,3-dicarboxypropylation of GAPDH on cysteine 245 has been reported to have no effect on its glycolytic activity (Qin et al., 2019), again demonstrating that a metabolic PTM on different residues of the same protein can induce contrasting effects. However, it is important to recognize that metabolite derivatives may give rise to off-target effects. A recent study has shown that itaconate derivatives are not always converted to itaconate intracellularly and has also demonstrated that not all of the effects of 4-Oi could be recapitulated by itaconate (Swain et al., 2020). Nonetheless, there is evidence that the same proteins, LDHA for example, can be modified by both derivatives and endogenous itaconate (Mills et al., 2018).

These studies indicate that glycolysis is another target for itaconate, acting via modification of aldolase A and GAPDH and limiting the inflammatory response. It is also quite striking that succinate, itaconate, and fumarate can target enzymes in glycolysis, which could be a broad method of negative feedback in activated macrophages in order to avoid excessive inflammation (see Figure 1). These findings point to a potentially important crosstalk from Krebs Cycle to glycolysis. The observation that the therapeutic agent DMF and itaconate can both modify GAPDH perhaps suggests that DMF has fortuitously been found as an anti-inflammatory agent that is carrying out the same natural process as itaconate to regulate inflammation. Certainly, the clinical use of DMF highlights the importance of PTMs for future therapeutic development.

**IMMUNE TARGETS OF PALMITOYLETION**

Similar to glycolysis and Krebs cycle, lipid metabolism also undergoes profound alteration during immune cell activation. Fatty acid oxidation is utilized for energy generation mainly by anti-inflammatory or tolerogenic immune cells such as M2 macrophages (Vats et al., 2006), memory T cells (van der Windt et al., 2012), and regulatory T cells (Michalek et al., 2011). Fatty acid synthesis, on the other hand, is generally associated with classically activated macrophages (Feingold et al., 2012), activated dendritic cells (Everts et al., 2014), effector CD4+ T cells (Berod et al., 2014), and activated B cells (Duft et al., 2014). Fatty acid synthesis is considered to be essential for membrane biogenesis and the synthesis of some proinflammatory mediators. The fatty acids palmitate and myristate, however, can also modify proteins and thereby alter their function.

Palmitic acid is a common long-chain saturated fatty acid that is incorporated into phospholipids, sphingolipids, and triglycerides, but can also modify cysteine (or less frequently serine and threonine) residues in a process termed palmitoylation. GAPDH perhaps suggests that DMF has fortuitously been found as an anti-inflammatory agent that is carrying out the same natural process as itaconate to regulate inflammation. Certainly, the clinical use of DMF highlights the importance of PTMs for future therapeutic development.

**IMMUNE TARGETS OF PALMITOYLETION**

Similar to glycolysis and Krebs cycle, lipid metabolism also undergoes profound alteration during immune cell activation. Fatty acid oxidation is utilized for energy generation mainly by anti-inflammatory or tolerogenic immune cells such as M2 macrophages (Vats et al., 2006), memory T cells (van der Windt et al., 2012), and regulatory T cells (Michalek et al., 2011). Fatty acid synthesis, on the other hand, is generally associated with classically activated macrophages (Feingold et al., 2012), activated dendritic cells (Everts et al., 2014), effector CD4+ T cells (Berod et al., 2014), and activated B cells (Duft et al., 2014). Fatty acid synthesis is considered to be essential for membrane biogenesis and the synthesis of some proinflammatory mediators. The fatty acids palmitate and myristate, however, can also modify proteins and thereby alter their function.

Palmitic acid is a common long-chain saturated fatty acid that is incorporated into phospholipids, sphingolipids, and triglycerides, but can also modify cysteine (or less frequently serine and threonine) residues in a process termed palmitoylation. This lipid modification has been studied for around 40 years (Schmidt and Schiesinger, 1979). Palmitoylation of proteins often facilitates docking to membranes and many transmembrane proteins also undergo palmitoylation. Both palmitoylation and de-palmitoylation are enzymatically controlled, with the DHHC protein family functioning as palmitoyltransferases (Korycka et al., 2012) and enzymes such as the acyl protein thioesterase and alpha beta hydrolase-domain containing protein 17 (ABHD17) protein family exhibiting de-palmitoylase activity (Duncan and Gilman, 1998; Lin and Conibear, 2015).

A number of proteins important for innate immune signaling pathways have been shown to undergo palmitoylation. The lipopeptide-sensing pattern recognition receptor TLR2 undergoes palmitoylation, which regulates its cell surface expression and ability to signal (Chesarino et al., 2014). The adaptor protein MyD88, which is involved in signaling by the IL-1 receptor and TLR family, has also been demonstrated to undergo palmitoylation, which is required for IRAK4 kinase binding and downstream signal transduction in response to TLR4 stimulation with LPS (Kim et al., 2019). MyD88 palmitoylation has been found to be mediated by the palmitoyltransferase ZDHHC6 but is also dependent on fatty acid synthase (FASN) activity.

The peptidoglycan-sensing PRRs nucleotide oligomerisation domain-like receptors 1 (NOD1) and NOD2 have been shown to undergo palmitoylation in primary monocytes and macrophages and a variety of cell lines (Lu et al., 2019). This
palmitoylation has been found to be dependent on the palmitoyltransferase ZDHHC5, which is recruited to phagosomes in a model of *Salmonella typhimurium* infection. The palmitoylation of NOD1 and NOD2 is required for their membrane localization to bacteria-containing vacuoles, without which downstream NF-κB and MAPK activation was abrogated.

The P2X7 receptor also undergoes palmitoylation (McCarthy et al., 2019). P2X7 detects extracellular ATP, which acts as a danger-associated molecular pattern (DAMP) and can activate the NLRP3 inflammasome, promoting caspase-1 activation and leading to the production of IL-1β and IL-18, and driving a type of cell death termed pyroptosis via the cleavage of gaskersmins (Piccini et al., 2008). P2X7, unlike the other P2X receptor family members, displays a lack of receptor desensitization, which has been found to be due to the palmitoylation of a cytoplasmic cysteine-rich site at the end of one of its transmembrane domains. Mutation or deletion of this site resulted in complete channel desensitization, indicating that palmitoylation protects P2X7 from desensitization. The TNF receptor TNF-R1 has also been found to be constitutively palmitoylated but rapidly depalmitoylated in response to ligand binding in a monocytic cell line. Mutation of the palmitoylated residue impaired TNF-R1 localization to the plasma membrane and downstream signaling (Zingler et al., 2019).

Stimulator of interferon genes (STING) is another important innate immune protein that undergoes palmitoylation (Mukai et al., 2016). STING is activated by the second messenger cGAMP, which is generated by the DNA sensor cGAS. STING in turn activates TBK1, which activates interferon regulatory factor 3 (IRF3), leading to the production of type I IFNs. Both pharmacological inhibition of palmitoylation and mutation of the palmitoylated cysteine residues (Cys88 and Cys91) on STING impaired its ability to induce type I IFNs during viral infection. Palmitoylation of STING may facilitate its anchoring to lipid rafts at the trans-Golgi network, as perturbing Golgi lipid composition also represses the type I IFN response.

All of these palmitoylations are therefore needed for these pathways to be triggered, pointing to palmitoylation as a key event in innate immunity. Figure 2 outlines the numerous immune effector proteins that are modulated by palmitoylation.

Palmitoylation has also been shown to play a role in adaptive immunity. It modulates the localization of linker of activation for T cells (LAT) during T cell receptor (TCR) engagement (Hundt et al., 2006; Zhang et al., 1998). The important T cell checkpoint regulator programmed death-ligand 1 (PD-L1) has been demonstrated to be palmitoylated, and this modification inhibits its ubiquitination and subsequent lysosomal degradation, thereby stabilizing PD-L1 (Yao et al., 2019). Inhibition of PD-L1 palmitoylation by 2-bromopalmitate or genetic silencing of the palmitoyltransferase ZDHHC3 augments T cell anti-tumor responses, both in vitro and in vivo. Blocking the palmitoylation of PD-L1 could potentially be explored as a therapeutic measure, since PD-L1 is commonly targeted by anti-PD-L1 antibodies for treatment of many cancers. However, one issue with this treatment is that the antibodies can only target PD-L1 on the cell surface. Intracellular PD-L1 can be stored and later redistributed to the plasma membrane to overcome immune checkpoint blockade therapy. However, Yao and colleagues have developed a peptide that specifically abolished PD-L1 palmitoylation, destabilizing it. This approach greatly enhances anti-tumor T cell immunity, which could have therapeutic potential.

**MYRISTOYLATION MODULATES T CELL FUNCTION**

Another long-chain saturated fatty acid, myristic acid, can be attached to N-terminal glycine residues to give rise to another modification termed myristoylation. Similar to palmitoylation, myristoylation often induces trafficking to and association with membranes in the cell or lipid rafts. Myristoylation is catalyzed by the ubiquitously expressed N-myristoyltransferase 1 (NMT1) and NMT2 (Zha et al., 2000). Myristoylation has been shown to play a critical role in the initiation of the innate and adaptive immune responses.

In macrophages, myristoylation of TRIF-related adaptor molecule (TRAM) facilitates its association with TLR4 upon LPS stimulation and is required for downstream signaling and activation of NF-κB and IRF3 (Rowe et al., 2006). In T cells, in conjunction with palmitoylation, myristoylation contributes to the regulation of TCR signaling and in the absence of myristoylation the subcellular localization of myristoylated alanine-rich C-kinase substrate (MARCKS), Lck and Fyn is perturbed (Alland et al., 1994; Graff et al., 1989; Rampoldi et al., 2015). In CD4+ T cells isolated from PBMCs of rheumatoid arthritis (RA) patients a reduced expression of NMT1 has been observed, but not in CD4+ T cells of patients with other autoimmune diseases (Wen et al., 2019). This observation was somewhat surprising as a lack of myristoylation leading to T cell hyperactivation is unexpected. Restoring the expression of NMT1 in the RA patient CD4+ T cells diminishes Th1 and Th17 cell development, whereas genetic silencing of NMT1 in healthy donor CD4+ T cells augments Th1 and Th17 cell differentiation. NMT1 has also shown to protect against synovial inflammation in a murine model of arthritis. The authors found AMP-activated protein kinase (AMPK) was a key target here. It had previously been demonstrated that the b-subunit of AMPK undergoes myristoylation (Oakhill et al., 2010) and that this modification induces its activation and translocation to the lysosome, where it can inhibit mTORC1 activation (Zhang et al., 2014). The authors showed that NMT1 deficiency in RA CD4+ T cells resulted in impaired AMPK trafficking to the lysosome and hyperactivation of mTORC1, thereby ultimately promoting inflammation. In this case therefore, myristoylation is needed to limit T cell activation and control inflammation. Boosting myristoylation of AMPK would therefore have an anti-inflammatory effect. The role of both palmitoylation and myristoylation in immunity are summarized in Figure 2.

One aspect of lipid PTMs that has not been thoroughly examined is the source of the fatty acids used for palmitoylation and myristoylation. Several studies have used labeled palmitate or myristate to demonstrate the incorporation of exogenous fatty acids into lipid PTMs (Hundt et al., 2006; Kim et al., 2019; Mukai et al., 2016; Rowe et al., 2006; Wen et al., 2019) but few have determined whether endogenous processes such as fatty acid synthesis or triglyceride breakdown are utilized for these PTMs. One recent report has shown that inhibition or genetic silencing of FASN impaired MYD88 palmitoylation (Kim et al., 2019), in addition to demonstrating that exogenous palmitate contributes to this modification. An earlier study has found that while both exogenous and endogenous sources of palmitate
and myristate are used for protein acylation, the ratio of endogenous to exogenous fatty acid incorporation varies between cell lines (Towler and Glaser, 1986). Therefore, the requirement of de novo lipogenesis for lipid-derived PTMs may well vary between immune cell type or depend on the activation state of the cell. It is also unclear if fatty acid binding proteins play a role in this process, although it was reported that the acyl-CoA binding protein ACBD6 sequesters palmitoyl-CoA to prevent it from binding and inhibiting NMT2, thereby allowing myristoylation to occur (Soupena et al., 2016). Although the evidence is limited, it is possible that fatty acid or acyl-CoA binding proteins are somehow involved in palmitoylation and myristoylation, as a large proportion of intracellular fatty acids are bound to these proteins (Resh, 2016).

**AMINO ACID PTMs REGULATE IMMUNE FUNCTION**

Amino acids can also modify proteins in immune cells. These PTMs occur at distinct amino acid side chains or peptide linkages and are most often mediated by enzymatic activity and can have various consequences. Glutamate can modify proteins by glutamylation, also termed polyglutamylation. This PTM generates lateral acidic glutamate side chains onto the γ-carboxyl groups of glutamic acid residues in the sequence of target proteins (Garnham et al., 2015; Rogowski et al., 2010; van Dijk et al., 2008). Glutamylation is highly conserved in all metazoans and protists, exerting critical roles in many physiological and pathological processes (Garnham and Roll-Mecak, 2012). Recent studies have highlighted the role of glutamylation in regulating immune effector mechanisms (see Figure 3).

Glutamylation of cGAS impairs its ability to bind cytosolic DNA and its capacity to synthesize cGAMP to initiate antiviral responses via STING (Xia et al., 2016). The α-chain of the IL-7 receptor undergoes glutamylation, which has been shown to increase the expression of transcription factor Sall3 via STAT5 activation. IL-7 is an important regulator of type 3 innate immune responses via STING (Xia et al., 2016). Thus, in macrophages, metabolic switching between oxidative phosphorylation and glycolysis supports divergent functional states stimulated by activation signals (Puleston et al., 2019). In macrophages, metabolic switching between oxidative phosphorylation and glycolysis supports divergent functional states stimulated by activation signals (Puleston et al., 2019). Thus, in macrophages, hypusination of eIF5A appears to be dynamically regulated after activation, with hypusinated eIF5A being critical for IL-4-driven macrophages and being required for anti-parasitic responses to *Heligmosomoides polygyrus*. Puleston et al. further described a role for hypusination of eIF5A in T cells. T cells deficient in enzymes involved in hypusine synthesis failed to differentiate into functionally distinct subsets (Puleston et al., 2020). Further characterization of hypusination across different immune cell types is warranted.

Another amino acid-based PTM is citrullination. This modification occurs when peptidylarginine deiminases (PADs) catalyze the conversion of arginine residues to citrulline, an amino acid that is not conventionally present in proteins (Vossenaar et al., 2003). Autoantibodies against citrullinated proteins are a hallmark of rheumatoid arthritis, and dysregulated citrullination is implicated in the development of this disease (Schellekens et al., 1998). Interestingly, the presence of anti-citrullination antibodies can be used as a biomarker for rheumatoid arthritis and is associated with more severe disease (Lundberg et al., 2005).

**CONCLUDING REMARKS AND THERAPEUTIC IMPLICATIONS**

How metabolic PTMs regulate innate and adaptive immune function is a rapidly developing area of research. Our current knowledge already demonstrates the striking impact that the metabolic dynamics of an immune cell can have on the function of immune effector proteins. The PTMs described in this review are shown in Table 1. These modifications regulate immunological processes by a wide variety of mechanisms. They can alter enzyme activity, facilitate or disrupt protein-protein interactions, determine subcellular localization through membrane anchoring and govern gene expression, through modification of transcription factors or histones to alter chromatin accessibility.

It is extremely likely that additional metabolic PTMs and further mechanisms by which these modifications shape the immune system will be identified in the future.
A number of immune effector proteins undergo glutamylation. IL-7Rα glutamylation induces STAT5 activation and subsequent expression of Sall3, an important ILC3 transcription factor. Glutamylation of cGAS impairs both its DNA-sensing and cGAMP synthesis activity. BAP1 also undergoes glutamylation, which induces its ubiquitination and degradation, preventing the expression of genes necessary for HSC self-renewal. Hypusination mediated by spermidine occurs only on the translation initiation factor eIF5A, which impacts on protein expression in various immune cell types. eIF5A hypusination is required for the differentiation of CD4+ effector subsets. It is also required for CD83 expression, which is necessary for dendritic cell maturation. Hypusination of eIF5A is critical for TFEB expression in B cells, which induces autophagy and prevents B cell senescence. Furthermore, eIF5A hypusination is required for the mitochondrial metabolism associated with alternative macrophage activation.
response in health and disease will emerge over the course of the next few years. Recent advances in mass spectrometry techniques have made these discoveries possible but the ability to manipulate these modifications, in addition to their detection and identification by proteomics, is crucial for the understanding of the effects they have on the immune response. When an enzyme is known to catalyze the addition or removal of a modification, manipulation of the system is considerably simpler. For example, when investigating palmitoylation both genetic silencing and pharmacological inhibition of the ZDHHC enzymes using 2-bromopalmitate and cerulenin can be employed (Resh, 2006). While recent research has highlighted the importance of metabolic PTMs in the context of immunity, many more questions remain to be answered, as outlined in the Outstanding Questions box.

Metabolic PTMs can therefore potently modulate various immune functions and processes. Can these discoveries be harnessed therapeutically? One possibility is to further explore how these metabolic PTMs may influence drug binding, which could affect drug efficacy (Su et al., 2017). Monoclonal antibodies specific for a protein target with or without a PTM are already commonly used in research but the clinical implications of this have not yet been thoroughly explored. A recent study has shown that glycosylation of PD-L1 impairs antibody binding, which would lead to a poorer clinical outcome (Lee et al., 2019). Several PTMs have been reported to alter the activity of enzymes, several of which are discussed above. If it were possible to develop an inhibitor that bound the modified form of the enzyme this could potentially be an effective way to target enzymes only in certain cell types that undergo certain metabolic changes. For example, targeting modified glycolytic enzymes such as malonylated GAPDH (Galván-Peña et al., 2019) or succinylated PKM2 (Wang et al., 2017) could inhibit the hyperactivation of immune cells such as macrophages in inflammatory disease without affecting glycolysis in other cell types.

---

### Table 1. Metabolic PTMs in Immunity

<table>
<thead>
<tr>
<th>Name of modification</th>
<th>Metabolite</th>
<th>Residue modified</th>
<th>Protein targets</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycolysis-derived PTMs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactylation</td>
<td>Lactate</td>
<td>Lysine</td>
<td>Histones</td>
<td>(Zhang et al., 2019a)</td>
</tr>
<tr>
<td><strong>Krebs cycle-derived PTMs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylation</td>
<td>Acetyl CoA</td>
<td>Lysine</td>
<td>Histones, cGAS</td>
<td>(Chen et al., 2020)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NLRP3, GAPDH</td>
<td>(Dai et al., 2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(He et al., 2020)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Baimer et al., 2016)</td>
</tr>
<tr>
<td>Malonylation</td>
<td>Malonyl CoA</td>
<td>Lysine</td>
<td>GAPDH, mTOR</td>
<td>(Galván-Peña et al., 2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Bruning et al., 2018a)</td>
</tr>
<tr>
<td>Succinylation</td>
<td>Succinyl CoA</td>
<td>Lysine</td>
<td>PKM2</td>
<td></td>
</tr>
<tr>
<td>Succination</td>
<td>Fumarate</td>
<td>Cysteine</td>
<td>GAPDH</td>
<td>(Kornberg et al., 2018)</td>
</tr>
<tr>
<td>Dicarboxypropylation</td>
<td>Itaconate</td>
<td>Cysteine</td>
<td>NRF2, Aldolase,</td>
<td>(Mills et al., 2018)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GAPDH, RIPK3</td>
<td>(Qin et al., 2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Liao et al., 2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Qin et al., 2020)</td>
</tr>
<tr>
<td><strong>Lipid-derived PTMs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoylation</td>
<td>Palmitic acid</td>
<td>Cysteine, Serine</td>
<td>TLR2, MYD88,</td>
<td>(Chesarino et al., 2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NOD1/NOD2, P2X7,</td>
<td>(Kim et al., 2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TNF-R1, STING,</td>
<td>(Lu et al., 2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LAT, PD-L1</td>
<td>(McCarthy et al., 2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Zingler et al., 2019)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Mukai et al., 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Hundt et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Yao et al., 2019)</td>
</tr>
<tr>
<td>Myristoylation</td>
<td>Myristic acid</td>
<td>Glycine</td>
<td>TRAM, MARCKS,</td>
<td>(Rowe et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lck, Fyn, AMPK</td>
<td>(Graft et al., 1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Rampoldi et al., 2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Alland et al., 1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Ven et al., 2019)</td>
</tr>
<tr>
<td><strong>Amino acid-derived PTMs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamylation</td>
<td>Glutamate</td>
<td>Glutamate</td>
<td>cGAS, IL-7Rx,</td>
<td>(Xia et al., 2016)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BAP1</td>
<td>(Liu et al., 2017)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Xiong et al., 2020)</td>
</tr>
<tr>
<td>Hypusination</td>
<td>Spermidine</td>
<td>Lysine</td>
<td>eIF5A</td>
<td>(Zhang et al., 2019b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Puleston et al., 2019)</td>
</tr>
</tbody>
</table>
Another prospect that could be explored is the pharmacological induction of metabolic modifications. We have already seen evidence of this approach from the study which showed that DMF mediates succination of GAPDH in immune cells, which contributes to its anti-inflammatory effects (Kornberg et al., 2018). This report indicates that it may be possible to use metabolite derivatives, including 4-OI or molecules based on it, to induce certain metabolic PTMs in the treatment of autoimmune disorders. It may also be possible to competitively inhibit the modification of a particular protein target. This was undertaken in the study that demonstrated that blocking PD-L1 palmitoylation improved anti-tumor T cell responses (Yao et al., 2019).

We expect that more metabolic PTMs will emerge and provide insight into immunomodulation in health and disease. We anticipate that these metabolic PTMs may be exploited for the design of future therapeutics for immune and inflammatory disease.

REFERENCES


**Metabolic Modulation in Macrophage Effector Function**

**Ciana Diskin and Eva M. Pålsson-McDermott**

School of Biochemistry and Immunology, Trinity College Dublin, Trinity Biomedical Science Institute, Dublin, Ireland

Traditionally cellular respiration or metabolism has been viewed as catabolic and anabolic pathways generating energy and biosynthetic precursors required for growth and general cellular maintenance. However, growing literature provides evidence of a much broader role for metabolic reactions and processes in controlling immunological effector functions. Much of this research into immunometabolism has focused on macrophages, cells that are central in pro- as well as anti-inflammatory responses—responses that in turn are a direct result of metabolic reprogramming. As we learn more about the precise role of metabolic pathways and pathway intermediates in immune function, a novel opportunity to target immunometabolism therapeutically has emerged. Here, we review the current understanding of the regulation of macrophage function through metabolic remodeling.

**Keywords:** macrophage, immunometabolism, glycolysis, tricarboxylic acid cycle, electron transport chain

**INTRODUCTION**

All living cells rely on an organized sequence of anabolic and catabolic reactions to produce a steady supply of energy and biosynthetic precursors. In order to optimize functionality, enzymes that control these tightly regulated metabolic pathways are compartmentalized into specific organelles within the cells. Immune cells such as macrophages are no different in this aspect; however, their efficient operation is especially important in the context of immune responses. The ability to rapidly shift from one metabolic state to another, depending on the immune challenge, allows macrophages to be effective both in combating pathogens and in fostering tissue repair.

**Abbreviations:** ACC, acetyl-CoA carboxylase; ACLY, ATP citrate lyase; ACO2, aconitase 2; AOX, alternative oxidase; BCAA, branched-chain amino acid; BCKA, branched-chain keto acid; BMDM, bone-marrow-derived macrophage; CAD, cis-aconitate decarboxylase; CARKL, carbohydrate kinase-like protein; CIC, citrate carrier; CPT1, carnitine palmitoyltransferase 1; DGAT2, diacylglycerol O-acyl transferase 2; DMI, dimethyl itaconate; DMM, dimethyl malonate; ECAR, extracellular acidification rate; ECSIT, evolutionarily conserved signaling intermediate in toll pathway; EIF2AK2, eukaryotic translation initiation factor-2 alpha kinase 2; ETC, electron transport chain; FAS, fatty-acid synthase; FIH, factor-inhibiting HIF; GATL, gamma-interferon-activated inhibitor of translation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT1, glucose transporter 1; GM-CSF, granulocyte-macrophage colony-stimulating factor; GPCR, G-protein-coupled receptor; GPT3, glutamic-pyruvic transaminase; HIF, hypoxia-inducible factor; HMG CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HO-1, heme oxygenase 1; HRE, hypoxia response element; IDH, isocitrate dehydrogenase; IFN-γ, interferon gamma; IL, interleukin; iNOS, inducible nitric oxide synthase; LAL, lysosomal acid lipase; LDH, lactate dehydrogenase; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide; MCAD, medium-chain acyl coenzyme A dehydrogenase; M-CSF, macrophage colony-stimulating factor; MDH, malate dehydrogenase; mTOR, mechanistic target of rapamycin; NLRP3, nod-like receptor family pyrin domain containing 3; NO, nitric oxide; NOX4, NADPH oxidase 4; OAR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PBMC, peripheral blood mononuclear cells; PDH, pyruvate dehydrogenase; PKC-β, PPAR gamma coactivator 1β; PGE2, prostaglandin E2; PKM2, pyruvate kinase M2; PPP, pentose phosphate pathway; PRK, pattern recognition receptor; RA, rheumatoid arthritis; RET, reverse electron transfer; ROS, reactive oxygen species; SDH, succinate dehydrogenase; STAT, signal transducer and activator of transcription; SUCNR1, succinate receptor 1; TAM, tumor-associated macrophage; TCA, tricarboxylic acid cycle; TLR, toll-like receptor; TNF-α, tumor necrosis factor-alpha; UCP, uncoupling protein; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau protein; 2-DG, 2-deoxyglucose; 3′UTR, 3′ untranslated region.
recent studies now reveal that immune effector functions such as cytokine production in response to pathogens are directly coupled to specific changes in cellular metabolism. This metabolic reprogramming of immune cells is required for both inflammatory and anti-inflammatory responses.

Macrophages are found in almost every tissue in our body, and along with dendritic cells they are at the forefront of initiating an innate immune response through phagocytosis and cytokine release, as well as an adaptive immune response through antigen presentation. Recognized nomenclature divides activated macrophages into two subgroups in vitro: the classically activated macrophages (M1) associated with inflammatory responses, which in vitro are generated by typically stimulating the resting macrophages with lipopolysaccharide (LPS) and interferon gamma (IFN-γ). Secondly, the alternatively activated macrophages (M2) are associated with tissue remodeling, resolution of inflammation, and anti-inflammatory responses, and are generated in vitro using anti-inflammatory stimuli including IL-4. We now know that this is an oversimplification of the actual functional diversity occurring in vivo. The vast spectrum of different macrophage activation statuses was clearly demonstrated in a transcriptomics study by Xue et al. who stimulated human macrophages with a range of stimuli (1). In addition, gene-set enrichment analysis was applied to sample groups from smokers and COPD patients. The data set generated, and other transcriptional studies published since, proposes a spectrum model of macrophage activation rather than the dichotomous M1/M2 classification system. While useful in mapping the metabolic pathways of differentially activated macrophages, and although many of the studies described here classify macrophages as M1 or M2, we now view macrophage polarization differently. Evidenced primarily in vivo, macrophages respond to specific external stimuli, resulting in unique sets of macrophage phenotypes that fall between the two extremes of M1 and M2. Hence, manipulating or skewing the different macrophage phenotypes in clinical settings such as asthma, sepsis, tumor, atherosclerosis, infectious disease, and metabolic disorders may provide us with a novel therapeutic approach.

Here, we review current literature on how macrophages utilize metabolic pathways in order to generate adequate energy and biosynthetic macromolecules to meet the fluctuating needs involved in host immune responses.

**GLYCOLYSIS**

**Overview**

Glucose, fructose, pyruvate, and other small carbohydrates play key roles in energy metabolism as well as provide carbon skeletons for the synthesis of other macromolecules. Glycolysis, the process which involves the breakdown of six-carbon glucose to three-carbon pyruvate, is central in generating ATP without requiring oxygen, where the reverse process, gluconeogenesis, consumes ATP while generating polysaccharides for storage. Glycolysis involves 10 enzymatically regulated steps, overall generating two molecules of pyruvate per molecule of glucose, with a net energy gain of two ATP and two NADH (Figure 1).

Although often illustrated as a linear reaction, in fact glycolysis branches off in order for intermediate metabolites to proceed along other metabolic pathways. These include the first intermediate of glycolysis, glucose-6-phosphate, which is required for glycogen synthesis and the pentose phosphate pathway (PPP), as well as the glycolytic intermediate glyceraldehyde-3-phosphate, which through glycerol generates triglycerides and fatty acids (Figure 1).

For many years, the general school of thought has been that pyruvate generated through glycolysis enters the mitochondria where it undergoes oxidative decarboxylation by pyruvate dehydrogenase (PDH) and thereby serves as the major source of acetyl-CoA, the starting point of the tricarboxylic acid (TCA) cycle (see below). During periods of high energy demand or low oxygen supply, pyruvate can instead be converted to lactate, another potential nutrient, by lactate dehydrogenase (LDH). However, new data by Hui et al. beg us to rethink the role for lactate in fueling the TCA cycle (2). Instead of pyruvate feeding the TCA cycle, circulating lactate was instead shown to be the primary source of carbon for the TCA cycle in all tissues except for brain.

**Glycolysis and Macrophage Function**

The upregulation of glycolysis in activated macrophages was first observed several decades ago (3–5), but it is only in recent years that the mechanisms governing the process and the functional significance of this metabolic shift have been unearthed. A sharp increase in the rate of glycolysis is now closely associated with an inflammatory phenotype in macrophages. It has been demonstrated that administration of 2-deoxyglucose (2-DG), a derivative of glucose that is taken up by the cell but cannot be further processed, blocks many aspects of the classical M1 inflammatory phenotype including opsonin-mediated phagocytosis (6, 7), secretion of pro-inflammatory cytokines, and production of reactive oxygen species (ROS) (8). In contrast, alternatively activated M2 macrophages or those associated with immune tolerance, such as alveolar macrophages, employ oxidative phosphorylation (OXPHOS) over glycolysis as their main source of ATP (5). The rapid increase in glucose uptake by classically activated M1 macrophages is facilitated by upregulation of glucose transporter 1 (GLUT1) expression (8, 9) (Figure 1).

It may seem counterintuitive that activated macrophages utilize glycolysis as their main source of energy, as OXPHOS generates 36 molecules of ATP compared with glycolysis, which produces a mere two molecules of ATP per molecule of glucose. However, a substantial boost in glycolysis can be achieved more rapidly than in OXPHOS, which would require concomitant mitochondrial biogenesis. Not only does glycolysis confer an advantage in terms of the speed at which it can be upregulated, but also it provides biosynthetic intermediates to be used in the PPP, among other processes, which are hugely important for classical macrophage activation and effector functions [reviewed in Ref. (10, 11)].

Multiple studies using murine and human macrophages have demonstrated that classically activated M1 macrophages are heavily dependent on glycolysis. Rodriguez-Prados et al. used a glucose tracer-based metabolomics approach to show that
Figure 1 | Overview of macrophage metabolic pathways, including glycolysis (1), pentose phosphate pathway (2), tricarboxylic acid (TCA) cycle (3), electron transport chain and oxidative phosphorylation (4), fatty-acid synthesis (5), and beta (fatty acid) oxidation (6).
activation of murine peritoneal macrophages through various toll-like receptor (TLR) pathways all resulted in a highly glycolytic phenotype (12). It was also determined using intracellular flux analysis to study metabolic features of murine M1 and M2 macrophages, that M2 macrophages display enhanced mitochondrial OXPHOS, whereas M1 macrophages predominantly use glycolysis to generate ATP (13). Human studies seem to mimic observations in mice, with the leukocytes of patients suffering from sepsis undergoing a shift to aerobic glycolysis, which is reversed upon patient recovery (14). In contrast to LPS and other pro-inflammatory stimuli enhancing aerobic glycolysis in macrophages, anti-inflammatory signals have been shown to exert the opposite effect on macrophage glucose metabolism. It was recently established that interleukin (IL)-10 suppresses glycolysis in LPS-stimulated wild-type bone-marrow-derived macrophages (BMDMs). Furthermore, in contrast BMDMs derived from Ifnar−/− mice exhibit elevated rates of glycolysis (15). The effect of IL-10 on glycolysis may be dependent on nitric oxide (NO) (16).

The elevated glycolysis associated with inflammatory macrophages is heavily dependent on hypoxia-inducible factor-1 (HIF-1α). When oxygen levels are low, HIF-1α no longer undergoes prolyl hydroxylation, leading to a decreased binding of the interacting partner von Hippel–Lindau protein (VHL), and reduced proteosomal degradation of HIF-1α. As a result, stabilized HIF-1α can bind the constitutively expressed HIF-1β subunit, initiating the transcription of hypoxic genes, including glucose transporters and glycolytic enzymes (17–23). Blouin et al. were first to show that stimulation of macrophages with LPS increased HIF-1α protein levels, leading to a functional HIF-1 complex that bind to hypoxic response elements (HREs) in target genes (24). It was later determined that the induction of HIF-1α in the context of inflammation was dependent on NF-κB, which acts as a transcriptional activator of HIF-1α (25). HIF-1α was also found to play a role in trained immunity, which involves epigenetic remodeling of myeloid cells in response to stimuli such as β-glucan (26). β-Glucan derived from Candida Albicans plays a central role in the induction of innate immune memory and is known to confer protection against a range of infections. It was also found that the HIF-1α glycolytic reprogramming of activated macrophages played a significant role in monocyte-derived macrophage migration into tissues (27). HIF-1α also induces the transcription of the key pro-inflammatory cytokine IL-1β (28).

Although a Warburg-like phenomenon is predominantly associated with M1 macrophages, alternatively activated macrophages also display an upregulated rate of glycolysis in addition to augmented mitochondrial metabolism. Huang et al. found that both IL-4 and macrophage colony-stimulating factor (M-CSF) drive mechanistic target of rapamycin complex 2 (mTORC2) activation, which in turn induces interferon regulatory factor 4 (IRF4) expression, contributing to the upregulation of glycolysis (29). Another study found that M-CSF, which is associated with M2 polarization, instigated a similar expression of glucose transporters, a higher lactate production rate, and increased expression of several glycolytic enzymes in macrophages, compared with granulocyte-macrophage colony-stimulating factor (GM-CSF), which is typically associated with M1-like phenotype (30). Tan et al. found that administration of 2-DG in addition to IL-4 reduced the expression of early M2 activation markers (31). As more studies like these emerge, it may transpire that glycolysis could play a more important role in M2 macrophages than previously considered.

Multiple Roles of Glycolytic Enzymes

Specific roles for several of the glycolytic enzymes have been identified in macrophages. Some of these enzymes “moonlight” by carrying out functions in immunity separate to their glycolytic activity. One example includes hexokinase, which has been found to not only function as a pattern recognition receptor (PRR), but also, together with mTORC1, plays a critical role for nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome assembly (32). Furthermore, a component of bacterial peptidoglycan, N-acetylg glucosamine, can bind to hexokinase, resulting in its inhibition and subsequent dissociation from the outer membrane of the mitochondria, culminating in NLRP3 activation (33).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can bind to AU-rich RNA sequences in its Rossmann fold, the site that typically binds NAD+ (34). GAPDH takes part in formation of the gamma-interferon-activated inhibitor of translation (GAIT) complex. Upon assembly in murine macrophages, the GAIT complex binds to a specific element in the 3′ untranslated region (3′UTR) of several inflammatory mRNAs and inhibits their translation. Its targets include vascular endothelial growth factor (VEGF), several chemokines, and corresponding chemokine receptors (35). GAPDH alone can bind directly to mRNA to inhibit translation of IFN-γ in T cells via 3′UTR binding (36) and also block translation of tumor necrosis factor-α (TNF-α) mRNA in human macrophages (37). α-enolase is another glycolytic enzyme that was found to display non-glycolytic functions in macrophages. Bae et al. found that monocytes and macrophages in the inflamed synovium of rheumatoid arthritis (RA) patients and in a mouse model of arthritis expressed surface α-enolase. Antibodies against enolase, previously reported in RA patients, were shown to increase the production of pro-inflammatory cytokines and prostaglandins from enolase-expressing macrophages and therefore may contribute to the pathogenesis of the disease (38).

Another glycolytic regulator that is of great importance in macrophage effector functions is pyruvate kinase M2 (PKM2). PKM2 is an HIF-1α target gene that was originally found to promote the Warburg effect in tumor cells. It was also found to interact directly with HIF-1α in the nucleus and enhance the transcription of HIF-1α-responsive genes (39, 40). PKM2 was later found to play a significant role in LPS-activated macrophages. Dimeric enzymatically inactive PKM2 translocates to the nucleus where it acts as a coactivator of HIF-1α, promoting expression of pro-inflammatory as well as pro-glycolytic genes. Nuclear PKM2 together with HIF-1α binds directly to HRE sites in the IL-1β promoter in LPS-stimulated macrophages (41). Using small-molecule activators that promote a tetrameric form of PKM2, the pyruvate kinase enzymatic activity can be restored while simultaneously preventing nuclear translocation. PKM2 activators impaired M1 macrophage polarization, promoting expression of M2 genes while reducing LPS-induced glycolysis. Furthermore, these activators diminished IL-1β production in vivo in response
to Salmonella typhimurium or LPS alone and increased levels of anti-inflammatory IL-10. PKM2 was also found to play a role in NLRP3 and absent in melanoma 2 (AIM2) inflammasome activation. It was demonstrated that PKM2-dependent glycolysis promotes the phosphorylation of eukaryotic translation initiation factor-2 alpha kinase 2 (EIF2AK2, also called PKR), which was previously shown to be necessary for inflammation activation and secretion of IL-1β, IL-18, and high-mobility group box 1 protein (HMGB1) from macrophages (42, 43). PKM2 may also contribute to the pathogenesis of coronary artery disease. Peripheral circulating monocytes differentiated ex vivo, as well as macrophages from the atherosclerotic plaques of patients suffering from coronary artery disease exhibit increased expression of dimeric PKM2, augmented glycolytic flux, and upregulated ROS production. PKM2 translocates to the nucleus and phosphorylates signal transducer and activator of transcription 3 (STAT3), contributing to the increase in IL-1β and IL-6 associated with these patients (44). Very recently, PKM2 was shown to regulate the expression of the checkpoint programmed death-ligand 1 (PD-L1), a ligand for the immune checkpoint receptor PD-1, in macrophages as well as other immune cells and cancer cells. Both pharmacological intervention and genetic silencing of PKM2 inhibited LPS-induced expression of PD-L1. Furthermore, PKM2 and HIF-1α bind to two HRE sites in the promoter of PD-L1 (45). This observation could have therapeutic potential as targeting immune checkpoints such as PD-L1 and PD1 has proven successful clinically [reviewed in Ref. (46)].

**Tumor-Associated Macrophages (TAMS)**

In cells undergoing Warburg metabolism, pyruvate resulting from glycolysis is diverted away from the TCA cycle and instead becomes converted to lactate by LDH. In addition to macrophages producing lactate, extracellular lactate from surrounding tissues also impact on macrophage function. Lactate secreted from tumor cells was found to drive M2 polarization in TAMs, which facilitated tumor growth (47). Although TAMs are often considered to be more M2-like, we now know that they have a high glycolytic rate similar to M1 macrophages; however, the effect of this on tumor progression is somewhat unclear (48). Murine TAMs exhibit diminished glycolysis through expression of REDD1, an mTORC1 inhibitor. This decrease in glycolysis is thought to facilitate metastasis and aberrant angiogenesis in tumors (49). However, a study carried out using TAMs generated in vitro from human monocytes yielded quite different results. They found an elevated glycolytic flux in TAMs to be associated with angiogenesis and metastasis in pancreatic cancer and showed that treatment with 2-DG was sufficient to reverse this effect (50). TAM metabolism is undoubtedly complicated and this area of research was extensively reviewed recently (51).

**THE PENTOSE PHOSPHATE PATHWAY (PPP)**

**Overview**

Glucose-6-phosphate from glycolysis feeds the anabolic PPP, which not only generates pentoses and 5-ribose phosphate for nucleic acid production but also serves as our major source of NADPH (Figure 1). NADPH provides the reducing power required for a range of synthetic reactions and anabolic pathways. NADPH offers reducing equivalents for generation of the antioxidant glutathione, thereby allowing for clearance of harmful ROS as well as being responsible for the respiratory burst in neutrophils and macrophages generating H₂O₂ to aid bacterial killing.

Like glycolysis, the PPP takes place in the cytosol and can be divided into an initial oxidative phase during which NADPH is generated, and a later non-oxidative phase where five-carbon sugars are synthesized.

**PPP and Macrophage Function**

The PPP has been shown to be upregulated in M1 macrophages (28, 52). NADPH is likely to be of great importance for M1 macrophages as it is required by the enzyme NADPH oxidase which catalyzes the generation of ROS. As mentioned, NADPH is also used for the production of antioxidants, which may be important in the resolving phase of inflammation (53). Production of nucleotides is likely to be essential for activated macrophages. Although they display a reduced rate of proliferation, nucleotides are required for miRNAs involved in gene regulation (11). M2 macrophages, on the other hand, appear to suppress the PPP. Haschemi et al. demonstrated that regulation of the PPP in macrophages is under the control of the carbohydrate kinase-like protein (CARKL), a sedoheptulose kinase. CARKL was found to be upregulated in response to IL-4 but suppressed in response to LPS, resulting in an inhibition of the PPP in M2 macrophages (54). This conclusion was drawn from experiments using primary murine macrophages, human peripheral blood mononuclear cells (PBMC), and the macrophage cell line RAW 264.7. Employing overexpression and genetic silencing in RAW 264.7 cells, the authors found that the loss of CARKL mimicked the increase in extracellular acidification rate (ECAR) and decrease in oxygen consumption rate (OCR) that is seen upon LPS stimulation, while overexpression attenuated the effect that LPS has on the ECAR and OCR. Therefore, the downregulation of CARKL seems to be important for the redirection of glucose from aerobic metabolism to glycolysis and the PPP that is seen in pro-inflammatory macrophages.

**THE TRICARBOXYLIC ACID (TCA) CYCLE**

**Overview**

When oxygen is readily available, glycolysis becomes the initial stage of glucose catabolism. Once pyruvate and lactate are generated, three further metabolic processes occurring in the mitochondria become responsible for potentially generating a further 36 molecules of ATP per glucose molecule. Firstly, pyruvate is oxidized through a series of reactions termed the TCA cycle. This is followed by the electron transport chain (ETC), and lastly the OXPHOS of ADP to ATP, a process that is driven by the proton gradient resulting from electron transport (Figure 1).

The point of entry for pyruvate formed during glycolysis into the TCA cycle comes when pyruvate is decarboxylated...
to acetyl CoA by the PDH complex. Acetyl CoA then enters a series of eight enzymatically regulated oxidizing reactions where each acetyl CoA is converted into two molecules of water and carbon dioxide. Pyruvate loses one-carbon and the two-carbon acetyl group of acetyl CoA condenses with the acceptor compound oxaloacetate resulting in six-carbon citrate. In a cyclic, carefully regulated series of reactions citrate is decarboxylated and oxidized resulting in malate from which the starting oxaloacetate is regenerated, completing the cycle (Figure 1). Only one single ATP is directly generated by one lap around the TCA cycle (two per molecule of glucose); however, most of the energy produced is stored in the form of the reduced coenzymes NADH and FADH2 which can drive the production of large amounts of ATP in the subsequent reactions of the ETC and OXPHOS (see below). In contrast to glycolysis the TCA cycle requires oxygen.

TCA Cycle and Macrophage Function

In addition to the increased glycolytic flux and reduced oxygen consumption that have been extensively studied in inflammatory macrophages for several decades (55), significant changes are also known to occur in the TCA or Krebs cycle. As with glycolysis, key intermediates of the TCA cycle serve as precursors in biosynthetic pathways. Citrate plays an important role here, fueling not only fatty-acid synthesis and histone acetylation but also acts as a precursor of itaconate, one of the most highly induced metabolites in LPS-activated macrophages. Citrate is firstly converted into cis-aconitate by mitochondrial aconitate 2 (ACO2), which is turned into itaconate by immune-responsive gene 1 (IRG1), also known as cis-aconitate decarboxylase (CAD) (Figure 1).

Resting macrophages and M2-like macrophages are considered to utilize an intact TCA cycle in conjunction with OXPHOS in order to generate ATP. An intact TCA cycle is thought to be important for the UDP-GlcNAc-mediated glycosylation of lectin and mannose receptors that are highly expressed on M2 macrophages (52). Pyruvate generated in M1 macrophages is converted to acetyl CoA by PDH, which is later converted to citrate. PDH activity was found to be intact in M1 macrophages, even though HIF-1α can potentially induce pyruvate dehydrogenase kinase 1 (PDK-1), an inhibitor of PDH (56). Interestingly, as mentioned, recent data have established that glucose fuels the TCA cycle indirectly via circulating lactate. Using 13C-labeled lactate and other metabolites, the authors show that the carbons in TCA cycle intermediates in most tissues arose from circulating lactate, instead of directly from glycolysis (2). As inflammatory macrophages are known to produce large quantities of lactate, it is plausible that this lactate could be important for use in the TCA cycle in surrounding tissues or even other immune cells. However, it remains to be confirmed if these new data have any implications for macrophage metabolism.

When macrophages are stimulated with LPS or another inflammatory signal, their TCA cycle becomes disrupted at distinct points in the cycle (52, 57). Therefore, an accumulation of certain metabolites such as citrate, itaconate, and succinate occurs in M1 macrophages.

Citrate

M1 macrophages display increased levels of citrate giving a first indication to its importance in macrophage effector functions. This accumulation in citrate is likely due to a downregulation of isocitrate dehydrogenase (IDH), the enzyme that catalyzes the conversion of isocitrate to α-ketoglutarate (52). An early study also reported enhanced activity of citrate synthase, the enzyme that catalyzes the formation of citrate from acetyl CoA and oxaloacetate (3). Both mRNA levels and protein expression of the mitochondrial citrate carrier (CIC, also termed Slc25a1) were found to be elevated in LPS-stimulated macrophages. CIC exports citrate from the mitochondrial matrix while importing cytosolic malate (58). In addition, the same group later demonstrated that CIC is upregulated in response to the pro-inflammatory cytokines TNF-α and IFN-γ (59). Once in the cytosol, citrate can be used for de novo lipogenesis (discussed below), which is important for membrane biogenesis. This involves the conversion of citrate to acetyl CoA by ATP citrate lyase (ACLY) (60). Interestingly, ACLY activity was found to be regulated by IL-4 via Akt-mTORC1 signaling. This alters histone acetylation and therefore regulates the expression of a subset of M2-associated genes (61). Citrate also appears to be critical for prostaglandin production, as both pharmacological inhibition and knockdown of CIC markedly reduced prostaglandin E2 (PGE2) levels (59). Disrupting ACLY activity also led to a significant decrease in PGE2 (60). In addition to acting as a pro-inflammatory mediator itself, PGE2 was also recently shown to be essential for LPS-induced expression of pro-IL-1β (62). Perturbing the activity and/or expression of CIC or ACLY also negatively affects NO and ROS production (58–60). Genetic silencing of ACLY and the use of three different inhibitors all reduce levels of ROS and NO in the human cell line U937. Similarly, knockdown or chemical inhibition of CIC also significantly reduced levels of ROS and NO. Although the mechanism is not yet fully elucidated, authors propose that ACLY activity may play a role in ROS and NO production through indirectly boosting NADPH supplies.

Citrate accumulation in macrophages can also lead to changes in gene expression. Citrate-derived acetyl CoA is critical for histone acetylation, as this process was found to be impaired upon siRNA-induced silencing of ACLY. These epigenetic changes could be of great importance in the context of inflammation; for example, IL-6 expression has been shown to be regulated by histone acetylation in macrophages (63). Non-histone protein acetylation can also impact cytokine expression, as microtubule acetylation was found to modulate IL-10 production (64). Citrate has also been identified as an inhibitor of HIF asparaginyl hydroxylase (FIH), which acts as a negative regulator of HIF-1α activity. Hence, citrate could potentially indirectly regulate HIF-1α targeted genes (65). However, none of the studies linking citrate to acetylation of proteins have yet been carried out in macrophages.

Citrate-derived acetyl CoA can also be converted to malonyl CoA, which acts as a cofactor for a lysine modification dubbed malonylation. This modification changes a positively charged residue into a negative charge (66, 67). This modification, although only recently discovered, has already been implicated in
type-2 diabetes (68). Malonylation has not yet been documented in macrophages or other immune cells but could potentially play a role, given the accumulation of citrate observed in M1 macrophages.

**Itaconate**

Citrate-derived cis-aconitate can be converted to itaconate, one of the most highly induced metabolites in activated macrophages (52, 56, 69). Although itaconate has been gaining more interest in recent years, its antibacterial effects have been recognized since the 1970s when it was shown to inhibit the growth of *Pseudomonas indigofera* by targeting isocitrate lyase—an important enzyme in the glyoxylate cycle in bacteria (70). More recently, itaconate has been shown to exert bacteriostatic effects on *Mycobacterium tuberculosis*, *S. enterica* (71), and *Legionella pneumophila* (72). There is also evidence of bacteria evolving to combat the action of this immunometabolite, as *P. aeruginosa* and *Yersinia pestis* were both found to express three separate enzymes that function in the degradation of itaconate. These three genes were found to be critical for the pathogenicity and survival of these bacteria (73). Itaconate was also implicated in a pro-inflammatory setting in two metabolic screens: one carried out in mice infected with *M. tuberculosis* (74) and the other in the macrophage-like cell line RAW 264.7 stimulated with LPS (75).

Immune-responsive gene 1, also later known as CAD/ACOD1, was previously known to be induced upon stimulation with LPS (76), but its function was not elucidated until Michelucci et al. demonstrated that it was the enzyme responsible for catalyzing the decarboxylation of cis-aconitate to produce itaconate. As expected, knocking down IRG1 in a macrophage cell line resulted in impaired antibacterial activity, due to a significant drop in itaconate levels (71). In addition to studies showing an increase in IRG1 expression in murine M1 macrophages, it has also been shown to be upregulated in humans during sepsis (77). Although the bactericidal effects have been well characterized, the immunomodulatory function of itaconate is a more recent area of study. Itaconate is generally thought of as being anti-inflammatory and was shown to inhibit the production of several TLR-induced pro-inflammatory cytokines by augmenting the expression of A20, a negative regulator of NF-κB (77). IRG1 was also found to be induced by the activity of heme oxygenase 1 (HO-1), which is expressed in the lungs and associated with LPS tolerance. Induction of HO-1 with carbon monoxide was found to decrease TNF-α levels and inhibition of HO-1 had the opposite effect, providing further evidence for the anti-inflammatory effects of IRG1 (78). IRG1 has also been shown to play a role in implantation, a process generally associated with immune tolerance or immune suppression. The expression of IRG1 was demonstrated to be regulated by the progesterone receptor (79) and leukemia inhibitory factor (LIF) (80), both of which are heavily involved in the implantation process.

Itaconate was found to associate with the mitochondria (81) and was shown to reduce mitochondrial substrate-level phosphorylation, an effect that was abrogated upon siRNA-mediated silencing of IRG1 (82). Itaconate has been proposed to contribute to the second breakpoint in the TCA cycle that occurs in M1 macrophages. Through its ability to inhibit succinate dehydrogenase (SDH), the enzyme that catalyzes the oxidation of succinate to fumarate, overproduction of itaconate leads to an accumulation of succinate (83, 84). This increase in succinate levels was abolished in *Irg1*<sup>−/−</sup> macrophages, whereas treatment with exogenous itaconate in the form of dimethyl itaconate (DMI) was found to enhance succinate levels (83). Furthermore, by using exogenous itaconate, as well as using mice lacking IRG1, Lampropoulou et al. confirmed that itaconate acts in an anti-inflammatory manner by inhibiting SDH-mediated oxidation of succinate, impacting on mitochondrial respiration and production of pro-inflammatory cytokines in macrophages in vitro and in vivo (84). However, another study employing radiolabeling suggests that exogenous DMI does not get taken up by the cell nor processed to itaconate intracellularly. Nonetheless, the presence of DMI in the media still leads to an increase in intracellular levels of itaconate so the authors have postulated that the effects may be due to an unidentified receptor. Therefore, any results obtained using DMI as a source of exogenous itaconate must be interpreted with caution.

**Succinate**

Succinate was identified as an oncometabolite before its importance in macrophage metabolism became clear. In 2005, succinate was found to drive the Warburg effect by activating HIF-1α through the inhibition of cytosolic prolyl hydroxylases (85). However, the role of succinate in inflammatory macrophages did not present itself until Tannahill et al. demonstrated that the accumulation of succinate in LPS-stimulated macrophages induced HIF-1α stabilization and activation which in turn leads to an upregulation of pro-inflammatory IL-1β as a target gene (28). This study established that cytosolic HIF-1 α prolyl hydroxylases are inhibited upon the accumulation of succinate in normoxic inflammatory macrophages, and confirmed that the IL-1β gene contains HREs in its promoter. The authors also observed a boost in global LPS-induced succinylation, a protein modification akin to malonylation, although the functional significance of the modification is unclear (28).

Another important discovery regarding succinate was the identification of the succinate receptor GPR91, which was previously considered an orphan G-protein coupled receptor (GPCR). A study conducted by He et al. assigned the TCA cycle intermediate succinate and α-ketoglutarate to the orphan GPCRs GPR91 and GPR99, respectively (86). GPR91, which has since been renamed the succinate receptor (SUCNR1), was found to be expressed in a wide variety of tissues (86). SUCNR1 has since been shown to be expressed on macrophages and to play a role in many inflammatory diseases (87–89). Activated M1 macrophages secrete succinate into the extracellular space while also upregulating expression of SUCNR1. This way succinate signals in an autocrine and paracrine manner to stimulate the release of IL-1β. High levels of succinate were found in synovial fluid taken from RA patients and using a *Sucnr1<sup>−/−</sup>* mouse model of arthritis, this succinate feed forward loop was confirmed by impaired macrophage activation as well as reduced levels of IL-1β (87). In contrast, *Sucnr1<sup>−/−</sup>* mice were found to exhibit exacerbated allergic contact dermatitis and in this same study the authors
also reported that SUCNR1 deficiency improved arthritis in the mouse model (88). The authors proposed that the increased severity of allergic contact dermatitis observed in the Sucnr1<sup>−/−</sup> mice was due to abnormal mast cell development, which leads to mast cell hyperactivation. Recently, the succinate receptor has been implicated in type-2 diabetes and the associated adipose tissue inflammation (89). Succinate levels were raised in both type-2 diabetic patients and mice fed a high-fat diet compared with healthy controls. Sucnr1<sup>−/−</sup> mice displayed improved glucose tolerance and had significantly fewer macrophages present in their adipose tissue. In addition, macrophages from the Sucnr1<sup>−/−</sup> mice exhibited impaired chemotaxis toward apoptotic adipocytes (89). With more research currently ongoing into the effects of succinate and its receptor in inflammation, the therapeutic potential of targeting SUCNR1 for inflammatory diseases may soon be defined.

While most work has focused on citrate, itaconate, and succinate, other TCA cycle intermediates have also been found to play significant roles in macrophages. Arts et al. demonstrated that an accumulation of fumarate in M1 macrophages, which was dependent on glutaminolysis, was of great importance in trained immunity of macrophages. Fumarate alone induced epigenetic changes akin to those observed in response to β-glucan and also augmented pro-inflammatory cytokine production upon restimulation with LPS (90). This was the first piece of evidence that an accumulation of TCA cycle intermediates can alter the macrophage epigenome but more recently α-ketoglutarate has also been shown to play a part in epigenetic reprogramming in macrophages. It was discovered that α-ketoglutarate, again produced via glutaminolysis, is crucial for full M2 activation and drives epigenetic changes in M2-associated genes in a Jumonji Domain Containing Protein 3 (JMJD3)-dependent manner (91). Furthermore, treatment of BMDMs with an inhibitor of glutaminolysis boosted pro-inflammatory cytokine secretion. Ratios of α-ketoglutarate to succinate in an M1 macrophage versus M2 macrophage differ. There is a larger succinate to α-ketoglutarate ratio in M1 macrophages due to partial blockade of succinate oxidation by SDH. However, in M2 macrophages succinate oxidation proceeds as normal and α-ketoglutarate becomes more important for processes such as epigenetic changes. As these findings are still recent, more data will no doubt emerge detailing how TCA cycle intermediates and other metabolic changes sculpt the macrophage epigenome.

**ELECTRON TRANSPORT CHAIN (ETC) AND OXIDATIVE PHOSPHORYLATION**

**Overview**
As for the TCA cycle, the reactions of the ETC occur in the mitochondria. The mitochondria have an outer permeable membrane and an inner membrane with extensive folds called cristae. Large electron-carrier complexes in the inner membrane of mitochondria reoxidize NADH and FADH<sub>2</sub> generated from the TCA cycle, and in the process electrons are passed stepwise to molecular oxygen. During this process protons are taken up from the mitochondrial matrix space and transferred to the intermembrane space. The potential energy of the NADH and FADH<sub>2</sub> generated during glycolysis and the TCA cycle is thereby used to drive the synthesis of large amounts of ATP as an electrochemical potential gradient for protons is created across the inner mitochondrial membrane (Figure 1). Flow of protons back into the matrix through ATP synthase drives synthesis of ATP. Four large complexes, except cytochrome c and ubiquinone, contain the electron carriers that make up the electron transfer chain. Complex I is the largest of the complexes and contains NADH dehydrogenase responsible for oxidizing NADH. Complex II is composed of four subunits of SDH, the only enzyme that participates in both the TCA cycle and the ETC. SDH is localized on the inner face of the mitochondrial inner membrane where it oxidizes succinate to fumarate through binding of succinate to SDHA, a reaction that is coupled to the reduction of ubiquinone to ubiquinol. As electrons move down the respiratory chain through complex III and complex IV to O<sub>2</sub>, H<sup>+</sup> ions are transferred to the matrix creating the proton gradient required by the coupled reaction of F<sub>0</sub>F<sub>1</sub> ATP synthase complex (Figure 2).

**ETC and Macrophage Function**
While classically activated macrophages are known to produce most of their ATP via glycolysis, alternatively activated macrophages have been shown to utilize OXPHOS. Therefore, M2 macrophages maintain forward electron flow through the ETC and predominantly generate ATP via ATP synthase (52). Treatment of macrophages with IL-4 was shown to upregulate OXPHOS via the transcription factor STAT6 and PPARγ coactivator-1α (PGC-1α). Overexpression of PGC-1α in BMDMs reduced the production of pro-inflammatory cytokines and PGC-1α knockdown impaired traits of alternative activation such as the promotion of fatty-acid oxidation and arginase activity (92). This effect on OXPHOS is not exclusive to IL-4, as IL-25 was also found to promote M2 polarization and elevate macrophage mitochondrial respiratory capacity (93). IL-10 also stimulates OXPHOS, one way in which the anti-inflammatory cytokine opposes M1 polarization (15).

However, in M1 macrophages OXPHOS is abated and the ETC becomes dysregulated (52). In one study, the downregulation of OXPHOS and the concomitant increase in mitochondrial fragmentation in response to pro-inflammatory stimuli was shown to be under the control of a microRNA–mi-R125b (94). Pro-inflammatory macrophages modify the ETC so that its primary function is ROS production, which is critical in the defense against infection. It is well established that phagosomal ROS is vital for M1 macrophages but the importance of mitochondrial ROS (mROS) has only been appreciated more recently. It was demonstrated that several TLR receptors signal through TRAF6 and evolutionarily conserved signaling intermediate in toll pathway (ECSIT) (which associates with complex I of the ETC) to promote mROS production and the recruitment of the mitochondria to the phagosomes. Perturbation of this signaling pathway was shown to impair bacterial killing by macrophages (95). These findings are not restricted to mice as immune cells isolated from patients suffering from TNF receptor-associated periodic syndrome (TRAPS) were found to have elevated mROS (96). In addition to a more direct role in bacterial killing (95), mROS was also demonstrated to contribute to NLRP3...
**FIGURE 2** Diagram depicting the flow of electrons in anti-inflammatory macrophages (top) versus the reverse electron transport (RET) phenomenon observed in lipopolysaccharide (LPS)-stimulated macrophages (bottom).
inflammasome activation in macrophages (97). Further evidence for the importance of mROS in macrophages arises from the role that uncoupling protein 2 (UCP2) appears to play. UCP2 is located at the inner mitochondrial membrane and unlike its homolog UCP1, which is expressed mainly in brown adipose tissue (98), UCP2 is highly expressed in macrophages along with other immune cells. UCP2 is believed to mitigate ROS levels in macrophages through uncoupling of OXPHOS. UCP2 expression in other immune cells. UCP2 is believed to mitigate ROS levels in tissue (98), UCP2 is highly expressed in macrophages along with its homolog UCP1, which is expressed mainly in brown adipose tissue (98). UCP2 is highly expressed in macrophages along with other immune cells. UCP2 is believed to mitigate ROS levels in macrophages through uncoupling of OXPHOS. UCP2−/− mice were found to be more resistant to Toxoplasma gondii infection and macrophages from these knockout mice were found to have elevated levels of ROS compared with wild-type controls (99).

As expected, IL-10 was found to exert the opposite effect on mROS compared with LPS and other TLR agonists. Treatment of macrophages with IL-10 promoted the abolition of dysfunctional mitochondria (characterized by elevated levels of ROS and lower mitochondrial membrane potential) through induction of mitophagy (15). The complexes of the ETC except for complex II (SDH) are known to be able to form supercomplexes in the mitochondrial inner membrane, which seems to change how the electrons are processed depending on the carbon source (100). Supercomplex formation is also thought to restrict mROS production (101). Macrophages were shown to disassemble these supercomplexes in response to bacterial detection, a process which was found to be dependent on TLR signaling and NLRP3 activation (102). Although complex II cannot form supercomplexes, it has been shown that phosphorylation of complex II by Fgr kinase is important in this disassembly (103).

In recent years, it has come to light that mROS in pro-inflammatory macrophages may be generated via reverse electron transport (RET) (Figure 2). The first evidence of RET giving rise to mROS was in oxygen sensing (104) and aging in Drosophila melanogaster (105). RET at complex I was also demonstrated to drive mROS production in reperfusion injury due to the accumulation of succinate and the elevated activity of SDH (106). Later RET was found to occur in macrophages when Mills et al. established that the build-up of succinate in LPS-stimulated macrophages and the oxidation of this succinate by SDH resulted in the production of mROS, seemingly from RET at complex I (107) (Figure 2). Definite roles have emerged for complex I and complex II in ROS production in M1 macrophages through studies in which inhibitors of these complexes were used. For example, Kelly et al. used metformin and rotenone to inhibit complex I in LPS-treated BMDMs, which markedly reduced mROS production as well as decreased IL-1β and increased IL-10 (108). However, another study used imiquimod and a similar molecule called CL097 to inhibit complex I and NAD(P)H dehydrogenase, quinone 2 (NQO2) and observed a boost in ROS production and NLRP3 activation (109). Inhibiting complex II (SDH) with dimethyl malonate (DMM) inhibits IL-1β production and raises IL-10 levels both in BMDMs and in vivo. Expression of alternative oxidase (AOX), which provides a different route for excess electrons so that ROS are not formed, prevents the inflammatory phenotype (107). M1 macrophages also produce NO, which is induced upon HIF-1α activation (110) and contributes to the bactericidal and antitumor capacity of macrophages (111). NO is known to inhibit mitochondrial respiration via complex IV (cytochrome c oxidase) (112, 113), and it may also inhibit complex I through S-nitrosylation of thiol groups on the enzyme (114). This inhibition of mitochondrial OXPHOS has been found to prevent the repolarization of M1 macrophages to an anti-inflammatory M2 phenotype, although the reverse is possible. Inhibition of NO production was shown to significantly improve this repolarization (115). This observation could be clinically relevant as it may be desirable to repolarize M1 macrophages to M2 in the case of inflammatory diseases.

**FATTY-ACID SYNTHESIS AND BETA OXIDATION**

**Overview**

The synthesis and degradation of fatty acids occur by two separate processes in different parts of the cell. Fatty-acid synthesis takes place in the cytosol, using citrate from the TCA cycle as a substrate in a series of reactions catalyzed by fatty-acid synthase (FAS). Citrate leaves the mitochondria and the TCA cycle in exchange for malate with the help of the mitochondrial CIC. Cytosolic citrate is then broken down by ACLY into acetyl-CoA and oxaloacetate. While oxaloacetate can be converted back into malate by malate dehydrogenase (MDH) and re-enter the mitochondria, acetyl-CoA on the other hand is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA can then be polymerized by FAS in a series of repetitive reactions, growing by two carbons with each reaction until it reaches the 16 carbon length of palmitic acid. In addition, acetyl-CoA plays a central role in cholesterol synthesis through the cytosolic mevalonate pathway. Three molecules of acetyl-CoA are condensed to form 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), which in turn is converted into mevalonate by HMG-CoA reductase, followed by a series of reactions leading to cholesterol.

As beta oxidation or fatty-acid degradation takes place within the mitochondria, the first step involves transport of free cytosolic fatty acids across the mitochondrial membranes. This process starts when fatty acids are converted to acyl-CoA by acyl synthetase, and aided by carnitine palmitoyltransferase I (CPT1) in the inner membrane of the mitochondria, acyl then becomes bound to carnitine. The carnitine–acyl-CPT1 complex enters the mitochondrial matrix aided by carnitine translocase and acyl is finally released with the help of carnitine palmitoyltransferase II (CPT-2), resulting in mitochondrial acyl-CoA. Acyl-CoA can then be oxidized by FAS in a series of repetitive cyclic reactions with a net yield of each oxidation cycle being one NADH, one FADH2, and one molecule of acetyl-CoA.

Malonyl-CoA generated during fatty-acid synthesis serves as a key regulatory feedback loop for beta oxidation as it inhibits the rate-limiting enzyme CPT1, thereby preventing cytosolic fatty acids from binding carnitine and entering the mitochondria where beta oxidation takes place.

**Fatty-Acid Synthesis and Macrophage Function**

Overall, fatty-acid synthesis is closely linked to pro-inflammatory effector functions of macrophages. We and others have shown an
increase in citrate and fatty acids in LPS-activated macrophages (28, 52). Feingold et al. demonstrated that glucose-derived carbons generated through an increased rate of glycolysis in LPS-activated macrophages were preferentially incorporated into fatty acids and sterols (116). In addition, LPS-activated macrophages, as well as macrophages associated with atherosclerosis, so-called foam cells, display an increased accumulation of triglycerides and cholesterol esters which may contribute to the pathogenesis of chronic inflammatory diseases (117–119). This accumulation is in large due to increased de novo synthesis of fatty acids, coupled to a robust increase in several of the key enzymes involved in glycerol lipid synthesis including glutamic-pyruvic transaminase (GPT3), Lipin 1, and diacylglycerol O-acyl transferase 2 (DGAT2). Paired with the marked increase in fatty-acid synthesis observed in LPS-stimulated macrophages is a marked decrease in fatty-acid oxidation, linked with suppressed expression of CPT1 (116). Differentiation of monocytes is linked with an M-CSF-stimulated upregulation of genes required for fatty-acid synthesis, and a switch in major lipid synthesis class from cholesterol in monocytes to phosphatidylcholine in macrophages. This induction of fatty-acid synthesis is critical for monocyte differentiation and phagocytic activity of macrophages. A newly identified protein named FAMIN was found to associate with FAS on peroxisomes and regulate de novo lipogenesis. Interestingly, FAMIN was identified through single-nucleotide polymorphisms (SNPs) associated with inflammatory diseases and was found to be essential for the production of pro-inflammatory cytokines and ROS, as well inflammasome activation in LPS-stimulated macrophages and in a murine model of sepsis (120).

**Beta Oxidation and Macrophage Function**

In a similar manner to fatty-acid synthesis being coupled to pro-inflammatory macrophages, beta oxidation is synonymous with anti-inflammatory macrophages. Lipolysis liberates free fatty acids, which are taken up by the macrophage by fatty-acid transporters such as CD36, thereby fueling mitochondrial OXPHOS. In IL-4-stimulated macrophages, this metabolic switch is largely mediated through STAT6 and PGC1β (92). Alternatively activated anti-inflammatory M2 macrophages display increased expression of CPT-1, CD36, and medium-chain acyl coenzyme A dehydrogenase (MCAD) (92). M2 polarization depends on lysosomal acid lipase (LAL)-mediated lipolysis as demonstrated by a blocked protective M2 response during parasitic helminth infection (121).

Malonyl-CoA from the TCA regulates fatty-acid oxidation by binding to CPT1, thereby making this the rate-limiting step in beta oxidation. Inflammatory macrophages of adipose tissue contribute to obesity-induced insulin resistance triggered by fatty acids and a range of other stimuli including ROS and pro-inflammatory cytokines. Promoting increased fatty-acid oxidation by over expressing CPT-1 in human adipose tissue macrophages promoted fatty-acid oxidation causing reduced inflammatory responses, as well as improved insulin sensitivity of adipocytes, reduced endoplasmic reticulum stress and less ROS damage in macrophages (122).

Taken together, this indicates that boosting fatty-acid oxidation in inflammatory macrophages would have beneficial anti-inflammatory effects. However, recent studies suggest that the assumption that fatty-acid oxidation is purely anti-inflammatory may be an over simplification. NLRP3 is an important component of one of the large multiprotein inflammasomes. Assembly of the NLRP3 inflammasome occurs in response to a range of different stimuli including viruses, components of bacteria as well as bacterial toxins, liposomes, and cholesterol crystals (123–128). Interestingly, NLRP3 is also activated by palmitate, which through oxidation via CPT1, fuels mitochondrial respiration, subsequent production of ROS, and activation of NLRP3 (129–131). Hence, in addition to LPS-activated macrophages requiring fatty-acid synthesis and FAS for adequate activation of NLRP3, oxidation of palmitate is also required for mitochondrial ROS activation of NLRP3. In addition, NLRP3 activation can be inhibited by modulating the activity of NADPH oxidase 4 (NOX4) (129). NOX4 regulates CPT1A activity and fatty-acid oxidation, and inhibition of NOX4 leads to suppressed NLRP3 activity and reduced secretion of IL-1β and IL-18 in vitro as well as in vivo (132).

Furthermore, as expected, macrophages generated from mice lacking CPT2 displayed impaired fatty-acid oxidation. Surprisingly, however, this did not affect their response to IL-4 polarization (133), implying that fatty-acid oxidation is not required for differentiation of M2 macrophages and that the role for fatty-acid oxidation here is more complex than originally proposed.

**AMINO-ACID METABOLISM**

**Overview**

The metabolism of amino acids plays an important role in many cellular processes where free amino acids are used as building blocks for not only protein synthesis but also for de novo synthesis of branched chain fatty acids, as in the case of valine and leucine, while glutamine and aspartate are used for purine and pyrimidine synthesis. Furthermore, cellular as well as dietary amino-acid catabolism can be used to support ATP production or as a source of citrate for fatty-acid synthesis. An initial step of transamination, resulting in α-ketoacids, allows for the carbon skeleton of the amino acid to enter the TCA cycle at one of multiple points such as α-ketoglutarate, succinyl-CoA, fumarate, oxaloacetate, pyruvate, or acetyl-CoA, thereby providing fuel in times of superfluous cellular amino acids.

**Amino-Acid Metabolism and Macrophage Function**

Cells, including macrophages, of higher vertebrates can synthesize 11 of the 20 amino acids. Dietary intake and protein salvage pathways are the only source of the remaining nine essential amino acids. Three of the essential amino acids, leucine, valine, and isoleucine, are the so-called branched-chain amino acids (BCAAs) with diverse roles outside of nutrition, such as regulation of protein degradation and synthesis in skeletal muscle, as well as regulation of synthesis of neurotransmitters such as serotonin in the brain, thereby affecting behavior. BCAAs also facilitate glucose uptake by the liver and skeletal muscles as well as enhance glycogen synthesis.
The first evidence that amino-acid metabolism can regulate macrophage effector function came with the discovery that macrophages block tumor growth through the consumption of arginine leading to the production of NO (134–138). Since then we have learned that availability and metabolism of several other amino acids such as glutamine and tryptophan also regulate macrophage immune function. The serine/threonine kinase mTOR forms two complexes, mTORC1 and mTORC2, and has been shown to be an important regulator in both innate and adaptive immune cells (139). mTOR plays a key role in macrophages, providing a link between amino-acid availability, coupling this to growth, proliferation, and protein synthesis. Branched-chain ketoacids (BCKAs), a product of BCAA catabolism, has been shown to directly regulate macrophage function by reducing the phagocytic ability of TAMs (140). Further evidence for a role for BCAA comes from data demonstrating that BCAT1, the enzyme responsible for the first step in BCAA catabolism, has been shown to directly regulate macrophage function by reducing the phagocytic ability of TAMs (140). Further evidence for a role for BCAA comes from data demonstrating that BCAT1, the enzyme responsible for the first step in BCAA catabolism, regulates the metabolic reprogramming in human macrophages. Inhibition of BCAT1 results in decreased glycolysis, oxygen consumption, IRG1 expression, as well as itaconate levels (141). In the interest of space, we will here briefly discuss the role of arginine in macrophage function [amino-acid metabolism in immunity has been reviewed in Ref. (142, 143)].

Arginine

L-arginine has several key roles in macrophage function. During inflammation, macrophages are responsible for the majority of ROS and nitrogen species produced including NO. This NO production in macrophages, in response to LPS and IFN-γ as well as ILs such as IL-13, requires extracellular L-arginine which enters the cells through specific transmembrane transporters (144–152). Arginine uptake is regulated by pro-inflammatory signals such as IL-1β (149). Once inside the cell, apart from being a precursor in protein synthesis, arginine also acts as a substrate for multiple enzymes including inducible nitric-oxide synthase (iNOS) and arginase, resulting in the production of NO and citrulline, respectively. Both of these metabolic pathways are utilized in macrophages with great opposing effects on immune function, with M1 macrophages utilizing arginine through iNOS resulting in pro-inflammatory NO, and M2 macrophages fluxing arginine via arginase resulting in citrulline and a tolerant phenotype associated with wound healing (153). Furthermore, arginase expression in macrophages is linked to limiting inflammatory effector T cell function, as well as correlating with disease severity in visceral leishmaniasis and HIV infection (154, 155). Arginine supplies required for efficient NO output can be restricted by arginase activity, though macrophages can circumvent this by converting L-citrulline to L-arginine, thereby restoring intracellular availability of arginine. However, L-arginine generated in this manner is less effective as a substrate for arginase-derived L-ornithine production of the urea cycle compared with L-arginine originating from the extracellular milieu (156). Arginine metabolism in myeloid cells has been reviewed in depth by Rodriguez et al. (157).

CONCLUDING REMARKS

The ever-growing literature on immunometabolism demonstrates new roles for metabolic pathways as well as specific pathway intermediates in the metabolic reprogramming of macrophages, leading to profound changes in immune effector functions (Figure 3). In order to simplify and provide an overview of our
current understanding of immunometabolism in macrophages, we have here described each pathway as a separate entity; however, in reality these processes are intimately linked. In addition, using a simplified interpretation of macrophage activation gives us a general view of metabolism in inflammatory versus anti-inflammatory macrophages, while reality proves more complex with a spectrum of macrophage subsets occurring in disease. Many questions are still outstanding; however, by its very nature immunometabolism has already firmly established itself as a field, which will provide us with future therapeutic targets for the treatment of immune disorders.

REFERENCES


AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work was funded by Science Foundation Ireland, and Wellcome Trust, UK. Wellcome Trust (Award number(s): 205455/14107); Science Foundation Ireland (Award number(s): 12/IA/1531).
through HIF-1alpha. Nature (2013) 496(7444):238–42. doi:10.1038/nature12186


Diskin and Pålsson-McDermott

Immunometabolism in Macrophage Function

104. Fernandez-Aguera MC, Gao L, Gonzalez-Rodriguez P, Pintado CO,
et al. Mitochondrial ROS produced via reverse electron transport extend
2016.03.009
106. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL,
et al. Ischaemic accumulation of succinate controls reperfusion injury
nature13909
doi:10.1016/j.cell.2016.08.064
108. Kelly B, Tannahill GM, Murphy MP, O’Neill LA. Metformin inhibits the
production of reactive oxygen species from NADH:ubiquinone oxidoreductase to limit induction of interleukin-1beta (IL-1beta) and boosts
interleukin-10 (IL-10) in lipopolysaccharide (LPS)-activated macrophages.
et al. K+ Efflux-independent NLRP3 inflammasome activation by small
doi:10.1016/j.immuni.2016.08.010
110. Takeda N, O’Dea EL, Doedens A, Kim JW, Weidemann A, Stockmann C,
111. Lorsbach RB, Murphy WJ, Lowenstein CJ, Snyder SH, Russell SW.
Expression of the nitric oxide synthase gene in mouse macrophages activated
112. Brown GC, Cooper CE. Nanomolar concentrations of nitric oxide
reversibly inhibit synaptosomal respiration by competing with oxygen at
113. Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AH.
Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the
114. Clementi E, Brown GC, Feelisch M, Moncada S. Persistent inhibition of cell
respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial
complex I and protective action of glutathione. Proc Natl Acad Sci U S A
den Berg SM, et al. Mitochondrial dysfunction prevents repolarization of
celrep.2016.09.008
116. Feingold KR, Shigenaga JK, Kazemi MR, McDonald CM, Patzek SM,
stimulation of RAW 264.7 macrophages induces lipid accumulation and
118. Lopes-Virella MF, Klein RL, Stevenson HC. Low density lipoprotein meta­
bolism in human macrophages stimulated with microbial or microbialrelated products. Arteriosclerosis (1987) 7(2):176–84. doi:10.1161/01.ATV.
7.2.176
119. Oiknine J, Aviram M. Increased susceptibility to activation and increased
uptake of low density lipoprotein by cholesterol-loaded macrophages.
120. Cader MZ, Boroviak K, Zhang Q, Assadi G, Kempster SL, Sewell GW,
et al. C13orf31 (FAMIN) is a central regulator of immunometabolic function.
Nat Immunol (2016) 17(9):1046–56. doi:10.1038/ni.3532
121. Huang SC, Everts B, Ivanova Y, O’Sullivan D, Nascimento M, Smith AM,
et al. Cell-intrinsic lysosomal lipolysis is essential for alternative activation

Frontiers in Immunology | www.frontiersin.org

doi:10.1152/ajpendo.00362.2014
The intracellular sensor NLRP3 mediates key innate and healing responses
to influenza A virus via the regulation of caspase-1. Immunity (2009)
124. Duncan JA, Gao X, Huang MT, O’Connor BP, Thomas CE, Willingham SB,
et al. Neisseria gonorrhoeae activates the proteinase cathepsin B to mediate
the signaling activities of the NLRP3 and ASC-containing inflammasome.
Differential activation of the inflammasome by caspase-1 adaptors ASC and
et al. NLRP3 inflammasomes are required for atherogenesis and activated
nature08938
127. Rajamaki K, Lappalainen J, Oorni K, Valimaki E, Matikainen S, Kovanen PT,
et al. Cholesterol crystals activate the NLRP3 inflammasome in human
128. McGettrick AF, O’Neill LA. NLRP3 and IL-1beta in macrophages as critical
regulators of metabolic diseases. Diabetes Obes Metab (2013) 15(Suppl
129. Moon JS, Nakahira K, Chung KP, DeNicola GM, Koo MJ, Pabon MA,
et al. NOX4-dependent fatty acid oxidation promotes NLRP3 inflammasome
nm.4153
Immunoresponsive gene 1 augments bactericidal activity of macrophagelineage cells by regulating beta-oxidation-dependent mitochondrial ROS
NLRP3-ASC inflammasome activation interferes with insulin signaling.
fatty acid synthase promotes NLRP3 inflammasome activation during
133. Nomura M, Liu J, Rovira II, Gonzalez-Hurtado E, Lee J, Wolfgang MJ,
17(3):216–7. doi:10.1038/ni.3366
134. Currie GA. Activated macrophages kill tumour cells by releasing arginase.
135. Currie GA, Gyure L, Cifuentes L. Microenvironmental arginine depletion
bjc.1979.112
137. Mills CD. Molecular basis of “suppressor” macrophages. Arginine meta­
2719–23.
15.1.323
139. Powell JD, Pollizzi KN, Heikamp EB, Horton MR. Regulation of immune
annurev-immunol-020711-075024
140. Silva LS, Poschet G, Nonnenmacher Y, Becker HM, Sapcariu S, Gaupel AC,
et al. Branched-chain ketoacids secreted by glioblastoma cells via MCT1
doi:10.15252/embr.201744154
141. Papathanassiu AE, Ko JH, Imprialou M, Bagnati M, Srivastava PK, Vu HA,
8:16040. doi:10.1038/ncomms16040

16

February 2018 | Volume 9 | Article 270




Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2018 Diskin and Pålsson-McDermott. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.