The Role of Base Excision Repair in Regulating Endotoxin Induced Inflammation

A thesis submitted to The University of Manchester for the Degree of Doctor of Philosophy (PhD) in the Faculty of Medical and Human Sciences

2012

Alan Carter

School of Medicine

Health Sciences Research Group Occupational and Environmental Health
1. Introduction .................................................................................. 18
  1.1. Structure of Endotoxin ......................................................... 19
  1.2. Endotoxin Induced Inflammation ........................................... 21
    1.2.1. LPS Recognition .......................................................... 21
    1.2.2. Activation of the inflammatory response ...................... 21
    1.2.3. Inflammatory Signalling .............................................. 22
    1.2.3.1. Fibroblasts ............................................................. 24
    1.2.3.2. Human Polymophonuclear Leukocytes ....................... 25
  1.3. The Endotoxin Paradox .......................................................... 26
  1.4. Factors affecting the inflammation response to endotoxin ...... 27
  1.5. BER Proteins and Endotoxin Induced Inflammation ............... 31
  1.6. DNA Glycosylases and Endotoxin Induced Inflammation ...... 32
  1.7. Oxidative Damage ............................................................... 34
    1.7.1. Reactive Oxygen Species .............................................. 34
    1.7.2. Oxidative Adenine Damage .......................................... 39
    1.7.3. Oxidative Thymine Damage ......................................... 40
    1.7.4. Oxidative Cytosine Damage ......................................... 41
    1.7.5. Spontaneous Base Removal ......................................... 41
    1.7.6. DNA strand breaks .................................................... 42
    1.7.7. Lipid peroxidation ...................................................... 43
  1.8. DNA Repair ............................................................................ 44
    1.8.1. Base Excision Repair ................................................... 45
    1.8.2. DNA Glycosylases ....................................................... 47
      1.8.2.1. OGG1 ................................................................. 49
      1.8.2.2. NTH1 ................................................................. 52
      1.8.2.3. NEIL1 ............................................................... 54
      1.8.2.4. NEIL2 ............................................................... 56
  1.9. Hypothesis ............................................................................. 59
2. Materials and Methods ............................................................ 61
  2.1. Materials .............................................................................. 61
    2.1.1. Buffer composition ...................................................... 61
    2.1.2. Tissue culture media .................................................. 62
    2.1.3. PCR primers ............................................................... 62
    2.1.4. Molecular biology reagents ........................................ 65
    2.1.5. Protein analysis reagents ........................................... 65
    2.1.6. Equipment .................................................................. 66
    2.1.7. Transgenic Mouse and MEF sources ............................ 66
  2.2. Methods ................................................................................. 67
    2.2.1. Mouse Colony Management ....................................... 67
    2.2.2. Mouse Colony Characterisation .................................. 67
2.2.3. LPS Induced Organ Damage ....................................................... 68
2.2.4. Induction of Immune Response .............................................. 68
2.2.5. Establishment of MEF cultures from mouse embryos .............. 68
2.2.6. MEF culture ........................................................................... 70
2.2.7. Treatment of MEFs with LPS .................................................... 70
2.2.8. Genomic DNA extraction ....................................................... 71
2.2.9. Polymerase Chain Reaction (PCR) Genotyping ......................... 71
2.2.10. Agarose Gel Electrophoresis ................................................ 72
2.2.11. RNA extraction ...................................................................... 72
2.2.12. Reverse transcription (RT) .................................................. 73
2.2.13. Protein Analysis .................................................................... 73
  2.2.13.1. Protein extraction from mouse tissues .................................. 73
  2.2.13.2. Protein quantification ...................................................... 74
  2.2.13.3. Western blot ..................................................................... 74
  2.2.13.4. ELISA ............................................................................. 75
  2.2.13.5. Myeloperoxidase Assay ................................................... 76
  2.2.13.6. Malondialdehyde Assay ................................................. 76
  2.2.13.7. Glutathione Assay .......................................................... 77
2.2.14. Statistical Analysis ............................................................... 78

3. Mouse models: Generation and characterisation ...................... 79
3.1. Introduction ................................................................................ 79
  3.1.1. Aims ...................................................................................... 80
3.2. Results ...................................................................................... 81
  3.2.1. NEIL1 Mouse Colony ........................................................... 81
    3.2.1.1. Preparation of NEIL1 Knockout Mice .............................. 81
    3.2.1.2. Confirmation of Genotype .............................................. 82
    3.2.1.3. Viability and Mortality of NEIL1 mice ............................ 84
    3.2.1.4. NEIL1 Mouse Weights .................................................. 85
  3.2.2. NEIL2 Mouse Colony ........................................................... 91
    3.2.2.1. Genotyping ................................................................. 91
    3.2.2.2. Western and RT-PCR .................................................... 92
  3.2.3. OGG1 Mouse Colony ........................................................... 94
    3.2.3.1. OGG1 Mouse Genotyping ......................................... 94
3.3. Discussion ................................................................................ 95

4. Cytokine Output of DNA Glycosylase Disrupted Cells .......... 101
4.1. Introduction .............................................................................. 101
  4.1.1. Aims .................................................................................... 102
4.2. Results .................................................................................... 102
  4.2.1. TLR4 mRNA Transcription Analysis ................................... 102
  4.2.2. Cytokine Output from LPS Challenged MEF Cells ............... 103
4.3. Discussion ............................................................................... 108

5. Effects of NEIL1 Gene Knockout on Endotoxin Induced
   Inflammation .............................................................................. 113
5.1. Introduction ............................................................................. 113
  5.1.1. Aims ................................................................................... 114
5.2. Results ................................................................................... 114
  5.2.1. Cytokine Output in LPS Challenged NEIL1−/− Mice .......... 114
  5.2.2. Myeloperoxidase activity in LPS challenged NEIL1−/− mice .... 118
5.2.3. Malondialdehyde content in LPS challenged NEIL1<sup>−/−</sup> mice ....126
5.2.4. Glutathione levels in LPS challenged NEIL1<sup>−/−</sup> mice.................133
5.2.5. Age related cytokine output NEIL1<sup>−/−</sup> mice..........................141
5.3. Discussion.......................................................................................141

6. Effects of OGG1 Gene Knockout on Endotoxin Induced Inflammation
6.1. Introduction.......................................................................................153
6.1.1. Aims..............................................................................................154
6.2. Results ............................................................................................154
6.2.1. Cytokine Output in LPS Challenged OGG1<sup>−/−</sup> Mice..............154
6.2.2. Myeloperoxidase Activity in LPS Challenged OGG1<sup>−/−</sup> Mice.....159
6.2.3. Malondialdehyde Content in LPS Challenged OGG1<sup>−/−</sup> Mice....167
6.2.1. Glutathione Activity in LPS Challenged OGG1<sup>−/−</sup> Mice..........174
6.3. Discussion.......................................................................................182

7. Overall Discussion .............................................................................190

8. References..........................................................................................202

Main text word count including figures and tables : 41,001
Index of Figures

Figure 1.1: A schematic view of the structure of LPS 20
Figure 1.2: The Recognition of LPS and Activation of the Immune Response in Macrophages 22
Figure 1.3: Generation of Antimicrobial Reactive Oxygen and Reactive Nitrogen Species 26
Figure 1.4: Differences in Three Dimensional Shape of LPS from Different Sources 28
Figure 1.5: Hypothesis linking Lipid A configuration to Cytokine Output 29
Figure 1.6: Comparison of Changes to Cytokine Production after LPS Stimulation in PARP1 and OGG1 Knockout Mice 33
Figure 1.7: Production of Superoxide Radical and Hydrogen Peroxide by the Electron Transport Chain 35
Figure 1.8: The Fenton Reaction 37
Figure 1.9: Formation of Oxidised Guanine Products 38
Figure 1.10: 8-oxoG paired with cytosine and mispaired with adenine 39
Figure 1.11: Formation of Oxidised Adenine Products 39
Figure 1.12: Formation of Oxidised Thymine Products 40
Figure 1.13: Formation of Oxidised Cytosine Products 41
Figure 1.14: Formation of an AP site 42
Figure 1.15: Lipid Peroxidation 43
Figure 1.16: Composition of malondialdehyde and 4-hydroxyalkenal 44
Figure 1.17: Monofunctional DNA glycosylase BER (I), bifunctional APE1 dependant BER (II) and independent (III) pathways 46
Figure 1.18: Sequence alignment of critical domains of NEIL1 and NEIL2 with E. coli Nei/Fpg 54
Figure 3.1: Diagram of the NEIL1 Knockout Construct and Genotyping Results 81
Figure 3.2: Pedegree of NEIL1⁻/⁻ Mice 82
Figure 3.3: Confirmation of a NEIL1 Null Phenotype 83
Figure 3.4: The distribution of genotypes from HET x HET breeding pairs of NEIL1 Transgenic Mice
Figure 3.5: Average litter composition from NEIL1 colony
Figure 3.6: Mortality rates of Male and Female Neil1 Transgenic Mice
Figure 3.7: NEIL1 Transgenic male and female mouse weights over 12 Months
Figure 3.8: Length and BMI of NEIL1 Transgenic Mice
Figure 3.9: Male NEIL1 transgenic mouse organ weight comparisons
Figure 3.10: Female NEIL1 transgenic mouse organ weight comparisons
Figure 3.11: Diagram of the NEIL2 Knockout Construct and Genotyping Results
Figure 3.12: Confirmation of a NEIL2 Null Phenotype
Figure 3.13: Diagram of the OGG1 Knockout Construct and Genotyping Results
Figure 3.14: The creation of transgenic mice can result in linkage disequilibrium
Figure 4.1: RT-PCR for TLR4
Figure 4.2: IL-6 output of wildtype and knockout MEF cell lines
Figure 4.3: IL-10 output of wildtype and knockout MEF cell lines
Figure 4.4: MCP-1 output of wildtype and knockout MEF cell lines
Figure 5.1: IL-4 concentrations in blood serum taken from NEIL1 transgenic mice
Figure 5.2: IL-6 concentrations in blood serum taken from NEIL1 transgenic mice
Figure 5.3: IL-10 concentrations in blood serum taken from NEIL1 transgenic mice
Figure 5.4: IL-12 concentrations in blood serum taken from NEIL1 transgenic mice
Figure 5.5: MPO activity in heart tissues of NEIL1 transgenic mice exposed to LPS
Figure 5.6: MPO activity levels in lung tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.7: MPO activity in liver tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.8: MPO activity in kidney tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.9: MPO activity in ileum tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.10: MDA levels in heart tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.11: MDA levels in lung tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.12: MDA levels in liver tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.13: MDA levels in kidney tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.14: MDA levels in ileum tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.15: GSH levels in heart tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.16: GSH levels in lung tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.17: GSH levels in liver tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.18: GSH levels in kidney tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.19: GSH levels in ileum tissues of NEIL1 transgenic mice exposed to LPS

Figure 5.20: IL-4 concentrations in blood serum taken from NEIL1 transgenic mice

Figure 5.21: IL-6 concentrations in blood serum taken from NEIL1 transgenic mice
Figure 5.22: IL-10 concentrations in blood serum taken from NEIL1 transgenic mice 144
Figure 5.23: IL-12 concentrations in blood serum taken from NEIL1 transgenic mice 145
Figure 6.1: IL-4 concentrations in blood serum taken from OGG1 transgenic mice 155
Figure 6.2: IL-6 concentrations in blood serum taken from OGG1 transgenic mice 156
Figure 6.3: IL-10 concentrations in blood serum taken from OGG1 transgenic mice 157
Figure 6.4: IL-12 concentrations in blood serum taken from OGG1 transgenic mice 158
Figure 6.5: MPO activity in heart tissues of OGG1 transgenic mice exposed to LPS 161
Figure 6.6: MPO activity in lung tissues of OGG1 transgenic mice exposed to LPS 162
Figure 6.7: MPO activity levels in liver tissues of OGG1 transgenic mice exposed to LPS 163
Figure 6.8: MPO activity in kidney tissues of OGG1 transgenic mice exposed to LPS 164
Figure 6.9: MPO activity in ileum tissues of OGG1 transgenic mice exposed to LPS 165
Figure 6.10: MDA levels in heart tissues of OGG1 transgenic mice exposed to LPS 169
Figure 6.11: MDA levels in lung tissues of OGG1 transgenic mice exposed to LPS 170
Figure 6.12: MDA levels in liver tissues of OGG1 transgenic mice exposed to LPS 171
Figure 6.13: MDA levels in kidney tissues of OGG1 transgenic mice exposed to LPS 172
Figure 6.14: MDA levels in ileum tissues of OGG1 transgenic mice exposed to LPS 173
Figure 6.15: GSH levels in heart tissues of OGG1 transgenic mice exposed to LPS 177
Figure 6.16: GSH levels in lung tissues of OGG1 transgenic mice exposed to LPS 178
Figure 6.17: GSH levels in liver tissues of OGG1 transgenic mice exposed to LPS 179
Figure 6.18: GSH levels in kidney tissues of OGG1 transgenic mice exposed to LPS 180
Figure 6.19: GSH levels in ileum tissues of OGG1 transgenic mice exposed to LPS 181
Figure 6.20: Pathways involved in the generation and degradation of oxidants and the effects of OGG1 knockout at key endpoints 184
Figure 6.21: Proposed model of PARP-1 inhibition by oestrogen 188
Figure 7.1: Alterations in organ activity due to adrenaline and observed levels of GSH in NEIL1<sup>−/−</sup> mice 197
Index of Tables

Table 1.1: The generation of major ROS and major avenues of protection

Table 1.2: Human DNA glycosylases located in the nuclei and the DNA damage that they remove

Table 2.1: Molecular Biology Buffers
Table 2.2: Protein Analysis Buffers
Table 2.3: Tissue Culture Media
Table 2.4: PCR Genotyping Primers
Table 2.5: RT-PCR Primers

Table 3.1: Summary of Phenotypes of DNA Glycosylase Deficient Mice

Table 4.1: Comparison of IL-6 results between untreated and treated DNA glycosylase deficient MEF cells
Table 4.2: Comparison of IL-10 results between untreated and treated DNA glycosylase deficient MEF cells
Table 4.3: Comparison of MCP-1 results between untreated and treated DNA glycosylase deficient MEF cells
Table 4.4 Summary of Results from cytokine assays

Table 5.1: MPO activity in tissues of NEIL1 transgenic mice exposed to LPS
Table 5.2: MDA levels in tissues of NEIL1 transgenic mice exposed to LPS
Table 5.3: GSH levels in tissues of NEIL1 transgenic mice exposed to LPS
Table 5.4: Summary of NEIL1 transgenic mouse blood serum cytokine Results
Table 5.5: Summary of NEIL1 transgenic mouse organ damage results

Table 6.1: MPO activity in tissues of OGG1 transgenic mice exposed to LPS
Table 6.2: MDA levels in tissues of OGG1 transgenic mice exposed to LPS 168
Table 6.3: GSH levels in tissues of OGG1 transgenic mice exposed to LPS 175
Table 6.4: Summary of OGG1 transgenic mouse cytokine Results 182
Table 6.5: Summary of OGG1 transgenic mouse organ damage results 183
Table 7.1: Summary and comparison of NEIL1 and OGG1 cytokine output results when compared with those of WT animals 191
Table 7.2: Summary and comparison of NEIL1 and OGG1 organ damage results 192
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-OH-A</td>
<td>2-hydroxyadenine</td>
</tr>
<tr>
<td>5’dRP</td>
<td>5’-deoxyribose-5-phosphate</td>
</tr>
<tr>
<td>5,6-DHU</td>
<td>5,6-dihydouracil</td>
</tr>
<tr>
<td>5-FoU</td>
<td>5-formyluracil</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>5-HMU</td>
<td>5-hydroxymethyluracil</td>
</tr>
<tr>
<td>5-OHC</td>
<td>5-hydroxycytosine</td>
</tr>
<tr>
<td>5-OHU</td>
<td>5-hydroxyuracil</td>
</tr>
<tr>
<td>8-oxoA</td>
<td>7,8-dihydro-8-oxoadenine</td>
</tr>
<tr>
<td>8-oxoG</td>
<td>7,8-dihydro-8-oxoguanine</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APE1</td>
<td>Apurinic endonuclease 1</td>
</tr>
<tr>
<td>AP site</td>
<td>Apurinic/apyrimidinic sites</td>
</tr>
<tr>
<td>BPI</td>
<td>Bactericidal/permeability-increasing protein</td>
</tr>
<tr>
<td>Cg</td>
<td>Cytosine glycol</td>
</tr>
<tr>
<td>DEP</td>
<td>Diesel exhaust particles</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand breaks</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithio-bis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>Egr-1</td>
<td>Early growth response 1</td>
</tr>
<tr>
<td>ERα</td>
<td>Oestrogen receptor-α</td>
</tr>
<tr>
<td>FapyA</td>
<td>4,6-diamino-5-formamidopyrimidine</td>
</tr>
<tr>
<td>FapyG</td>
<td>6-diamino-4-hydroxy-5-formamidoguanine</td>
</tr>
<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione peroxidise</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HAE</td>
<td>4-hydroxyalkenals</td>
</tr>
<tr>
<td>HET</td>
<td>Heterozygous</td>
</tr>
</tbody>
</table>
HhH  Helix-hairpin-helix
IFN-γ  Interferon γ
IL-1  Interleukin 1
iNOS  inducible nitric oxide synthase
IRAK  Interleukin-1 receptor-associated kinase
KDO  2-keto-3-deoxooctonic acid
KO   Knockout
KPE  Potassium phosphate EDTA
LBP  LPS binding protein
LPS  Lipopolysaccharide
MAC  Membrane attack complex
MAPK1  Mitogen-activated protein kinase 1
MAPK8/JNK  Mitogen-activated protein kinase 8
MDA  Malondialdehyde
MIP1α  Macrophage inflammatory protein 1
MMR  Mismatch repair
MPG  N-methylpurine-DNA glycosylase
MPO  Myeloperoxidase
MyD88  Myeloid differentiation primary response gene 88
NADPH  Nicotinamide adenine dinucleotide phosphate
NER  Nucleotide excision repair
NEIL1/2  Nei endonuclease VIII-like 1/2
NFκB  Nuclear factor κB
NO  Nitric oxide
NOX  NADPH oxidase
NTH1  Nth endonuclease III-like
O2  Superoxide
OGG1  8-Oxoguanine glycosylase
PARP1  Poly (ADP-Ribose) Polymerase-1
PBS  Phosphate buffered saline
PCNA  Proliferating cell nuclear antigen
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PNK</td>
<td>Polynucleotide kinase</td>
</tr>
<tr>
<td>Pol β</td>
<td>DNA polymerase β</td>
</tr>
<tr>
<td>PUA</td>
<td>3'-phospho-α,β-unsaturated aldehyde</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase - PCR</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SMUG1</td>
<td>Single-strand selective Monofunctional Uracil DNA Glycosylase</td>
</tr>
<tr>
<td>SSB</td>
<td>Single strand breaks</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N-tetramethyl-ethylenediamine</td>
</tr>
<tr>
<td>Tg</td>
<td>Thymine glycol</td>
</tr>
<tr>
<td>T_{h1}</td>
<td>T-helper 1</td>
</tr>
<tr>
<td>T_{h2}</td>
<td>T helper 2</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll like receptor 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>Ug</td>
<td>Uracil glycol</td>
</tr>
<tr>
<td>UNG2</td>
<td>Uracil-DNA glycosylase 2</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
</tbody>
</table>
Abstract

Endotoxins, a component of the outer membrane of the cell wall of gram negative bacteria, stimulate the innate immune system to elicit an inflammatory response in mammals. Deletion of base excision repair (BER) genes has been reported to decrease the immune response to endotoxin in mouse models. It is currently unknown whether this role is limited to a few select proteins or a result of the general function of the BER pathway. The aim of this study was to identify if the loss of other BER proteins would trigger a similar response by measuring the levels of inflammatory cytokines produced and certain biomarkers of oxidative stress. To facilitate this, a new strain of NEIL1−/− mice was successfully created as well as a putative NEIL2−/− strain. A previous strain of NEIL1−/− mice displayed a sporadic obese phenotype, our NEIL1−/− mice showed no significant increase in bodyweight when compared to WT mice.

Whilst there were significant differences in the serum content of cytokines IL-6, IL-12, IL-10 and IL-4 between wildtype, NEIL1−/− and OGG1−/− mice challenged with lipopolysaccharide (LPS, the active component of endotoxin). When compared to wildtype animals both NEIL1−/− and OGG1−/− mice produced lower levels of the Th1 cytokine IL-6 (♂ 1 h; \( p<0.05 \) and ♀ 24 h; \( p<0.01 \)), and the Th2 IL-10 cytokine (♀ 6 and 24 h; \( p<0.01 \)) along with other sex and genotype specific differences.

When comparing LPS induced organ damage in NEIL1−/− and wildtype mice there were no significant differences in myeloperoxidase (MPO) activity or malondialdehyde (MDA) concentration due to genotype. However, there were significant differences observed in glutathione (GSH) levels in the heart (♂ \( p=0.01 \)), lung (♂ \( p=0.05 \)), liver (♂ \( p=0.05 \)) and ileum (♂ \( p=0.05 \)) that when considered alongside a significant increase in the weights of adrenal glands in NEIL1−/− knockout (♂ \( p=0.05 \), ♀ \( p=0.03 \)) mice were suggestive of a raised level of adrenaline.

The OGG1−/− mice displayed no significant genotype x treatment interaction in MPO activity, MDA levels or GSH levels. However, genotype x sex interactions were observed in the liver and lung tissues of OGG1−/− for MPO (lung ♀ \( p<0.01 \), liver ♀ \( p=0.02 \)), MDA (lung ♀ \( p<0.01 \)) and GSH (lung ♀ \( p=0.05 \), liver ♀ \( p=0.04 \)) indicating that female OGG1−/− mice had greater protection from the oxidative effects of LPS induced inflammation.

In conclusion whilst the knockout of OGG1 and NEIL1 genes had an effect on the inflammatory signalling response, this effect was not great enough to impact upon oxidative stress markers of inflammation within the tissues sampled. The mechanism of how this is accomplished is at present unclear and worthy of further study.
Declaration
“No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.”

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

(ii) Copies of this thesis, either in full or in extracts, may be made only in accordance with the regulations of the John Rylands University Library of Manchester. Details of these regulations may be obtained from the Librarian. This page must form part of any such copies made.

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

(iv) Further information on the conditions under which disclosure, publication and exploitation of this thesis, the Copyright and any Intellectual Property Rights and/or Reproductions described in it may take place is available from the Head of School of Medicine.
Acknowledgements

I would like to thank my supervisors Dr. Andrew Povey and Dr. Rhoderick Elder, for their guidance and support throughout the course of this research project, and my advisor Dr. Rachel Watson. I would also like to thank the British Cotton Growing Association for providing the funding to make this work possible.

I would also like to acknowledge the help I received from the members of staff at the Transgenic Animals Facility at The University of Manchester, especially Ian Townsend, Ruth Jones and Karen Fry. Also special thanks should go to those who helped me learn some of the finer techniques Dr. Mel Heeran and Brian A. Tefler.

A special mention should go to my colleagues in the Biomarkers Lab who in addition to giving me moral support made the atmosphere we worked in fun, a special thanks to W.M. Md. Saad whose work on the confirmation of the NEIL2 knockout is found within. Finally I would like to thank all my family and friends who supported me through their kind (and sometimes less than kind - but needed) advice, caring words and thoughtful actions.
1. Introduction

Cotton workers, and other workers exposed to organic dusts, have been observed to exhibit byssinosis (Mberikunash et al., 2010; McKERROW et al., 1958; ROACH and SCHILLING 1960). Symptoms include coughing, shortness of breath and difficulty breathing. These were more pronounced when the persons returned to work after the weekend, and then subsided the following day leading to the moniker “Monday Asthma” (Liu, 2007). Originally it was assumed that this was due to a build up of plant matter in the airways of these workers (ROACH and SCHILLING 1960), but later it was found to be due to the presence of endotoxins, potent inducers of neutrophilic airway inflammation, carried on the dust (Cavagna et al., 1969; Radon, 2006). Smokers can also exhibit similar symptoms due to endotoxin carried with the smoke particles from tobacco (Hasday et al., 1999). A person’s sensitivity to endotoxin induced inflammation has been shown to be affected by their genetic makeup (Eder et al., 2004).

Endotoxin is composed of complexes of lipooligosaccharides, lipoproteins and lipopolysaccharide (LPS), a substance found exclusively as part of the outer membrane of the cell wall of gram negative bacteria; indeed 3-10% of the dry weight of these bacteria is endotoxin (Fan and Cook 2004; Vaara, 1999). All sources of gram negative phospholipid contain this substance including blebs, vesicles and fragments of dead cells (Prins, 1996; Radon, 2006; Vaara, 1999). Hence humans can therefore be exposed to LPS in several ways including working in the presence of organic dust, smoking, exposure to invasive gram negative bacteria via injury, and exposure to intestinal bacteria during surgery (Beutler and Rietschel 2003; Hasday et al., 1999).

Endotoxin activates the innate immune system, that portion of the immune system which defends the host from organisms in a generic
manner, in an attempt to destroy any bacterial invaders. Once bacteria are killed, however, the endotoxins in their membrane continue to stimulate the immune system fuelling further response (Prins, 1996). This escalating response can lead to severe systemic inflammation, which manifests as fever, increased heart and respiratory rates, and is described as endotoxic (septic) shock (Beutler and Rietschel 2003). In order to understand why this occurs, it is important to further discuss the structure of endotoxin and the immune response to it, in further detail.

1.1. Structure of Endotoxin

Endotoxin refers to a mixture of bacterial cell surface molecules mainly consisting of LPS but including lipooligosaccharrides and lipoproteins. Alternatively, the term LPS refers to a pure substance which is not found in nature. The structure of LPS is shown in Figure 1.1. The molecule consists of four major domains, an O-antigenic polysaccharide, an outer core oligosaccharide, an inner core oligosaccharide and an acylated diglucosamine head group (lipid A) (Rietschel et al., 1994).

The O-antigenic polysaccharide consists of a repeating structure of one to eight glycosyl residues, the structure of which varies between serotypes. The size range of the O-antigenic polysaccharide chain is comparatively specific to a bacterial species although there is some heterogeneity, even within LPS from the same colony, in the length of these molecules. This is seen as an evolutionary measure in order to protect the bacterium from complement immune measures and phagocytosis by macrophages (Aspinall et al., 1996; Beutler and Rietschel 2003; Rietschel et al., 1994).

The outer core oligosaccharide consists of the common hexoses D-glucose, D-galactose, and N-acetyl-D-glucosamine. It is more uniform in composition than the O-antigenic polysaccharide and indeed only five different core types have been found in E. coli serotypes (Aspinall et al., 1996; Rietschel et al., 1994). The inner core oligosaccharide is composed
of two unusual sugars (heptose and 2-keto-3-deoxyoctonoic acid (KDO)), and it is important in the structure and functional viability of the outer membrane, and hence the viability of the bacterium (Aspinall et al., 1996; Rietschel et al., 1994).

![Figure 1.1: A schematic view of the structure of LPS](image)

Hep, L-glycerol-D-manno-heptose; Gal, galactose; Glc, glucose; KDO, 2-keto-3-deoxyoctonic acid; NGa, N-acetyl-galactosamine; NGc, N-acetyl-glucosamine. Adapted Magalhaes et al., 2007.

Lipid A is responsible for the toxic effects of the LPS chain and consists of two glucosamine units with fatty acid chains attached, each of these fatty acid chains normally contains a phosphate group (Raetz et al., 2009; Wang and Quinn 2010). When purified lipid A, elicits the same cytokine response as the whole molecule in animal models (Netea et al., 2002). All four of the LPS components are required to maintain the virulence of the bacterium, but only the inner core and lipid A are required for the viability of the organism (Vaara, 1999).
1.2. Endotoxin Induced Inflammation

1.2.1. LPS Recognition

The inflammatory response is an innate response generally to invasive pathogens, and occurs in two forms namely, an acute response which is direct response to a stimuli, e.g. tissue damage, and a chronic response which can lead to arthritis and the wasting associated with certain cancers (Gupta et al., 2011; Matsukawa et al., 1997).

Endotoxin is recognised by host cells after binding to recognition sites on their membranes, a process which requires the mediation of LPS-binding protein (LBP). In vitro, without LBP, large concentrations (>1mg/ml) of LPS are needed to activate macrophages. In vivo however, due to the presence of LBP, only nanogram amounts of LPS are required for cellular recognition, and an inflammatory response (Moore et al., 1976; Watson and Riblet 1974; Watson and Riblet 1975).

LBP shares many features with other phospholipid binding proteins, and works mainly as a transport protein. LBP first breaks down endotoxin from large complexes so that separated LPS molecules can be transported to target receptor, CD14/toll like receptor 4 (TLR4) as shown in Figure 1.2 (Paulos et al., 2007). Alternatively the LPS is transported by LBP to high-density lipoproteins where it becomes unable to activate the immune response and is removed from the system. Thus LBP is important for both the recognition and removal of LPS (Beutler and Rietschel 2003; Martin, 2000).

1.2.2. Activation of the inflammatory response

One of the primary signalling cells in the LPS inflammatory response is the monocyte which is present in all tissues (Butterfield et al., 2006). TLR4 having recognised LPS, then activates a number of proteins including NADPH oxidase (NOX) proteins which catalyse the formation of superoxide
When considering the inflammation reaction the major protein activator is nuclear factor κB (NF-κB) which is the key transcription factor in the expression of several pro-inflammatory proteins including cytokines, chemokines, inducible nitric oxide synthase (iNOS), the inducible form of cyclooxygenase and adhesion molecules (Gloire et al., 2006).

Figure 1.2: The Recognition of LPS and Activation of the Immune Response in Monocytes
The LPS/LBP complex is recognised by CD14/TLR4 which activate a series of proteins within the cell, beginning with myeloid differentiation primary response gene 88 (MyD88), causing an inflammatory activation cascade to proceed via IL-1R-associated kinase (IRAK) Adapted from (Buer and Balling 2003).

1.2.3. Inflammatory Signalling

Having been activated by LPB/LPS complexes, local cells (e.g. fibroblasts) and the more mobile leukocytes proceed to release various signalling proteins into the surrounding area including the primary inflammatory cytokines Interleukin 1 (IL-1), IL-6 and Tumour Necrosis Factor-α (TNF-α). These molecules act as stimulators of pro-inflammatory proteases (i.e. collagenase and elastase) to aid in tissue remodelling as well as many secondary messengers such as platelet activating factor (which induces
vasodilation and wound healing), prostaglandins (to induce vasodilation) and reactive oxygen species (ROS; increasing proinflammatory cytokine release) (Moller and Villiger 2006; Naik and Dixit 2011).

The release of the prostaglandins PGE2 and PGF2 influences the contraction and relaxation of the blood vessels leading to greater vascular permeability (vasodilation; Williams, 1979). The subsequent movement of fluids influences several physiological changes including a raise in local temperature, and the movement of neutrophils and macrophages from the blood stream to the local areas. These cells are drawn by chemotaxis (following concentrations of attractant proteins). Once at the source of the chemotactic agents the neutrophils, macrophages and monocytes continue releasing cytokoines and chemokines as well as beginning the process of phagocytosis (DiStasi and Ley 2009).

IL-6 is often used as a measure of immune response as it is induced in large quantities by LPS, and is released by fibroblasts (Van, 1990). Whilst increased IL-6 levels are used to denote a greater level of inflammation, it is in fact a pleiotropic cytokine as it acts as both a pro-inflammatory and anti-inflammatory signal. It increases inflammation by directly increasing macrophage activity. However, it also activates the release of adrenocorticotropic hormone which triggers the release of cortisol and IL-10, both of which lead to a reduction in inflammation (Moller and Villiger 2006).

IL-10 is also released from fibroblasts and activated macrophages, which also release IL-12. These Th2 cytokines modulate the production of cytokines from T lymphocytes and fibroblasts, including the chemoattractants IL-8 and MIP-1α which recruit more neutrophils to the surrounding area by chemotaxis (Maloney et al., 2005; Martinez et al., 2004; Reed and Milton 2001; Wan et al., 2000; Wang et al., 2005). IL-12 increases the effectiveness of T helper 1 (Th1) cells, modulators of the
innate immune system, in the area of endotoxin challenge by further promoting the production of lymphokines and interferon-γ, which trigger the proliferation of cytotoxic T-cells and further activate the macrophages so that they become more aggressive. These ‘angry’ macrophages also release proinflammatory molecules, such as IL-1 and TNF-α, at a much greater rate (Moller and Villiger 2006).

The differentiation of T helper 2 (Th2) cells is initiated by IL-4, and these cells then create more IL-4 in a self-promoting feedback loop (Takeda et al., 1996). The release of IL-10 increases the activity of Th2 cells which in turn activate B-cells increasing antibody production. IL-10 also inhibits the production of IL-12 by macrophages, thus ensuring that antibody production is kept at a maximum and that the Th1 cells are not over-stimulated into an auto-immune reaction (Wan et al., 2000).

1.2.3.1. Fibroblasts

Fibroblasts, connective tissue cells which secrete an extracellular matrix rich in collagen and other macromolecules had previously not been considered to produce inflammatory substances in significant amounts. More recently they have been shown to aid in the initiation of inflammatory cascades through the release of proinflammatory cytokines such as IL-6 (Van, 1990), but their major effects on the inflammatory response come via the induction of bone marrow derived immune cells, such as neutrophils and macrophages, to the area of infection through the release of chemokines such as IL-8, MCP-1 and MIP-1α (Maloney et al., 2005; Smith et al., 1997; Wang et al., 2005).

As fibroblasts can be cultured relatively easily when compared to other immune cells, they have become a staple in in vitro experiments measuring cytokine secretion. For instance they have been used in experiments to define TLR activity and specificity (Kurt-Jones et al., 2004).
1.2.3.2. Human Polymorphonuclear Leukocytes

Human polymorphonuclear leukocytes (neutrophils, basophils and eosinophils) are important in the defence of the body from external pathogens such as bacteria and fungi, and are attracted to the site of invasion by macrophage release of IL-8, MCP-1 and Macrophage inflammatory protein 1 (MIP-1α) (Martinez et al., 2004). Once at the site of endotoxin challenge these cells attempt to remove bacterial invaders directly by phagocytosis, then releasing many cytotoxic granules which assist in the destruction and disposal of invasive organisms (Faurschou and Borregaard 2003). Of the many granules released, two interact directly with LPS; lysozyme which binds to LPS rendering it inactive, and bactericidal/permeability-increasing protein (BPI) which binds to the lipid A section of LPS, enabling other bactericides to reach the inner membrane (Faurschou and Borregaard 2003). During the course of phagocytosis the neutrophil produces ROS, including superoxide and hydrogen peroxide, and hypochlorite which are highly effective at killing the invading organism by directly damaging the cell (Figure 1.3) (Faurschou and Borregaard 2003).

As neutrophils are rapidly attracted to a site of bacterial invasion, great numbers of them can accumulate and this can lead to problems when they undergo necrotic lysis as this releases the ROS contained within the cell (Figure 1.3). Cytotoxic compounds in one area can cause a variety of problems including greater vascular permeability, oedema, oxidative stress and eventually apoptosis (Vernooy et al., 2001). However, not all cellular apoptosis is due to the action of neutrophils, as it has been shown that LPS can stimulate lung cells to undergo apoptosis in the absence of an inflammatory immune response. It has been proposed that the lipid A portion of the LPS molecule can activate the apoptosis pathways due to its
similarities with ceramide which has long been associated with programmed cell death (Pettus et al., 2002; Vernooy et al., 2001).

Figure 1.3: Generation of Antimicrobial Reactive Oxygen and Reactive Nitrogen Species
Within the neutrophil and macrophage several enzymes catalyse the formation of ROS from molecular oxygen. The superoxide anion can react with nitric oxide to form peroxynitrite or be oxidised into nitrogen dioxide. Adapted (Smith, 1994).

1.3. The Endotoxin Paradox

As early as 1966 it had been recognised that only mammals, and to a lesser extent avian species, are sensitive to endotoxin (Berczi et al., 1966). The question was asked, why if it causes such an exaggerated, and sometimes harmful, immune response, is endotoxin sensitivity a beneficial evolved characteristic? It has been shown that endotoxin insensitive mice were more susceptible to inner ear and other infections and that they also died at far lower doses of bacterial infection (Beutler, 2004). Sensitivity to LPS may allow animals to elicit an immune response to minor bacterial infections in order to prepare a response for times when the assault is greater (Beutler, 2004).

In humans, studies have shown an inverse relationship between endotoxin exposure and symptoms such as skin rash, shortness of breath and
coughing, and that in environments where endotoxin exposure occurs, cases of asthma are reduced (Braun-Fahrlander et al., 2002). Exposure to endotoxin and the subsequent release of cytokines may assist in the maturation of $T_{H1}$ modulated immunity reducing the risk of atopic sensitisation (Braun-Fahrlander et al., 2002; Douwes et al., 2000; McElvenny et al., 2011).

It has also been shown that cotton workers have less than the expected level of lung cancers (Enterline et al., 1985; McElvenny et al., 2011). In comparisons between animal farmers and crop farmers, it has been found that those working with animals, who in theory are exposed to greater quantities of endotoxin from bacteria in faeces, experience a reduced risk of lung cancer (Lange et al., 2003). There is also epidemiological evidence that endotoxin protects against the formation of cancer directly, those who give up smoking experience a short period of increased lung cancer risk (Lange et al., 2005), and it is believed that once smoking ceases this protective effect of endotoxin no longer acts on cancers already initiated by the carcinogenic compounds in cigarette smoke (Lange et al., 2005). As a result has been hypothesised that the direct or indirect activity of endotoxin reduces the ability of cancers to develop (Lange et al., 2005). In two studies, rabbits and guinea pigs were exposed to airborne endotoxins and the metastasis levels within the lungs were measured. In both cases the animals exposed to endotoxin had significantly reduced levels of cancer (Rylander, 2002).

1.4. Factors affecting the inflammation response to endotoxin.

The structure of endotoxin can vary between bacterial species and the inflammatory response does vary according to the strain of bacteria it comes from. Purified endotoxin from *E. coli* and *Salmonella typhosa* elicited the immune response similar to that detailed in section 1.2 at the greatest levels, followed by that from *Klebsiella pneumoniae* (K.
pneumoniae) and Pseudomonas aeruginosa (P. aeruginosa). Additionally, the response over time was significantly different according to which ‘species’ of LPS was administered. Specifically, treatment with endotoxin from K. pneumoniae caused a comparatively higher IL-1β, IL-10 and MCP-1 production, and a reduction in the amount of TNF-α secreted. More extremely, endotoxin from P. aeruginosa, only stimulated the release of MIP-1α and IL-1β after 4 and 8 hours respectively, whilst exhibiting almost no response in the release of TNF-α, interferon γ (IFN-γ) or IL-10 at any time point whilst all other LPSs tested initiated reactions after 4h (Mathiak et al., 2003; Schromm et al., 1998).

It is thought that the three dimensional shape of the lipid A portion of the LPS molecule could be responsible for this difference in response (Figure 1.4). By measuring the cytokine production in mice after treatment with various forms of LPS it was hypothesised that those LPS molecules with a more conical configuration were more readily detected by TLR4 whilst those of an intermediate configuration were detected more readily by a combination of TLR1/TLR2 and TLR4 although at a lesser efficiency, whilst those with a conical configuration were not detected by either (Figure 1.5)(Netea et al., 2002; Schromm et al., 1998).
Figure 1.5: Hypothesis linking Lipid A configuration to Cytokine Output

Lipid A in a conical configuration such as that from *E. coli* induces a strong proinflammatory response via TLR4, whilst those with a less conical form such as *P. gingivalis* bring about a smaller proinflammatory response via TLR2. Those with a cylindrical configuration activate neither, inducing minimal or no response.

As there is a smaller adaptive immune response to endotoxin as well as the larger innate, response the number of times a particular strain of bacterial endotoxin comes into contact with the immune system is also a factor. Antibodies are used to opsinise LPS for disposal by phagocytosis, and as the adaptive immune system comes into contact with specific strains of LPS it can more efficiently remove them in the future (Chaby, 1999).

If the endotoxins eliciting the immune response are part of a bacterial infection, the defence mechanisms of these invaders can play a part in how well the body can respond, for example the *E. coli* strain CFT073 has been found to release TIR domain containing–proteins which are taken in by macrophages *in vitro* then disrupt TLR4 signaling via the MyD88 signaling pathway (Cirl *et al.*, 2008).

The site of endotoxin exposure is also important factor in the immune response. Membrane attack complex (MAC) proteins are a response to bacterial invasion which destroy the invading organisms, releasing more endotoxins from their cell membranes. It was reported that the production of MAC proteins was reduced in lung tissues to reduce endotoxin exposure to the lung cells (Bolger *et al.*, 2007). It has also been reported that the
endotoxin induced response by lung wall epithelial cells produces a greater ratio of IL-10 to IL-12 when compared to similarly stimulated blood borne responses (Wan et al., 2000). This would suggest an effort from the lungs, which are exposed to endotoxin more regularly, to reduce harmful inflammation responses and promote the opsonisation of pathogens for disposal by phagocytosis (Bolger et al., 2007).

The subjects sex has an effect on the inflammatory response to endotoxin. It has been observed that females have a resistance to the effects of acute inflammation, whilst their prognosis in more long term inflammatory disorders is poorer (Lefévre et al., 2012).

In PARP-1 inhibited mice males responded less to endotoxin induced inflammation when compared to WT animals (measured by TNFα output), however the TNFα output of female WT mice began at a lower level and the inhibition of PARP-1 did not alter this output. In order to identify if this was due to the low output of TNFα in female mice further groups of WT and PARP-1 inhibited mice were treated with a more robust amount of LPS. Again no significant difference was observed between normal and PARP-1 inhibited serum TNFα concentrations (Mabley et al., 2005b).

There are two proposed reasons for this sex difference in inflammation, firstly that it is to do with hormones specifically oestrogen. Treatment of LPS treated tissues with oestrogen has been shown to reduce the production of MCP-1 from the brain, arteries and macrophages, IL-6 from the arteries and MIP2-α from the brain when compared to those treated with LPS alone (Nilsson, 2007). More recently it has also been described that X chromosome linked genes such as CD99, which plays a role in leukocyte diapedesis, may also contribute to the noted sex difference (Lefévre et al., 2012).
Other genetic factors also have a role in determining the magnitude of the endotoxin induced inflammatory response. It was shown that both C3H/HeJ and C57BL/10ScCr mice which exhibit endotoxic hyporesponsiveness both had mutations in the TLR4 gene (Qureshi et al., 1999). It was later reported that similar mutations in the human TLR4 gene also contribute to a lessened inflammatory response to endotoxin (Arbour et al., 2000). A host of gene deletions have also been recognised to effect endotoxin induced inflammation including signal transducer and activator of transcription 3 and Cyclooxygenase 1 and 2 (Kano et al., 2003; Martin et al., 2006).

1.5. BER Proteins and Endotoxin Induced Inflammation

Proteins not initially thought to be involved in modulating the inflammatory response have been discovered to have such a function. These include certain proteins linked with base excision repair. PARP1, a DNA damage sensor for single and double stranded breaks, was the first BER protein to be implicated in the inflammatory response. This was discovered through the use of PARP1⁻/⁻ knockout mice which showed a marked decrease in inflammation induced by caecal ligation and puncture, which results in the extrusion of gut content into the peritoneal cavity, when compared to wildtype mice. There was also a significant reduction in TNF-α (1059 ± 166 pg/ml vs. 1529 ± 122 pg/ml), IL-6 (approx. 28 ± 2 ng/ml vs. 40 ± 4 ng/ml) and IL-10 (4750 ± 2274 pg/ml vs. 6875 ± 2076 pg/ml) plasma concentrations when compared with wildtype mice 24 h after caecal ligation. Similarly MPO activity was also reduced in PARP1⁻/⁻ gut (approx. 3.2 ± 0.5 mU/m protein vs. 5.8 ± 0.4 mU/mg protein) and lung (approx. 180 ± 15 mU/m protein vs. 370 ± 30 mU/mg protein) tissues after 24 h. Survival rates of PARP1⁻/⁻ mice after caecal ligation were also significantly higher compared to PARP⁺/+ (20% vs. 4% survival) (Soriano et al., 2002; Virag and Szabo 2002).
This is probably due to the regulatory control PARP1 has on certain proteins, including iNOS. PARP1 also has been found to be a co-factor with NF-κB and AP-1, key inflammation transcription factors (Kiefmann et al., 2004). A suggested model for the role of PARP1 has been described by Virag and Szabo. The invasion of microbial particles activates neutrophils in the local area, leading to the release of ROS from these monocytes in order to damage, and kill the invaders. ROS release leads to DNA damage and causes the activation of PARP1, which in turn triggers chemokine and inflammatory cytokine production via NF-κB and AP-1 leading to the further recruitment of neutrophils to the area of invasion (Virag and Szabo, 2002).

APE1 is involved in redox reactions throughout the cell and is important in the regulation of many systems. Indeed APE1−/− mouse foetuses are unable to develop beyond implantation (Xanthoudakis et al., 1996). Expression of APE1 is activated by the presence of ROS (Yang et al., 2007). APE1 activates transcription factors NF-κB and AP-1 by the reduction of active cystine residues (Ando et al., 2008). Indeed the down regulation of APE1, via antisense cDNA and siRNA, attenuates NF-κB and AP-1 activation (Daily et al., 2001; Fung and Demple, 2005). Therefore it can be hypothesised that a reduction in APE1 activity within the cell would reduce cytokine and chemokine output from leukocytes, reducing the inflammation reaction in a similar fashion to that of PARP1.

1.6. DNA Glycosylases and Endotoxin Induced Inflammation

More specific base excision repair proteins, such as DNA glycosylases which remove modified DNA bases, have also been shown to alter the inflammatory response. It has been reported that 8-Oxoguanine glycosylase−/− (OGG1−/−) mice are resistant to the damage caused by endotoxin induced inflammation, indicating that this protein may also have a role in the regulation of inflammation (Mabley et al., 2005a). When given intraperitoneal injections of LPS (80 mg/kg) OGG1−/− mice showed a
significant decrease in the output of the chemokine MIP1-α (~60% decrease), and the cytokines TNFα (~31%) and IL-12 (~50%) when compared to WT controls, but a large increase in the Th2 cytokines IL-4 (~4.7 fold) and IL-10 (~2.4 fold). Th2 cytokines reduce IL-12 production and also signal for an increase in antibody production. Additionally, the MPO activity measured in lung (~60%) and heart (~50%) tissues was significantly lower in OGG1−/− mice than their OGG1+/+ counterparts: no difference was observed, however, in the liver, kidney or gut. Finally MDA content was significantly lower in the lung (~58%), heart (~55%) and liver (~72%) tissues of LPS treated OGG1−/− mice than those of OGG1+/+ mice, whilst there was no difference observed in kidney or gut (Mabley et al., 2005a). A comparison of OGG1 and PARP1 responses to LPS induced inflammation are found in Figure 1.6.

![Figure 1.6: Comparison of Changes to Cytokine Production after LPS Stimulation in PARP1 and OGG1 Knockout Mice](image)

The macrophage is activated when LBP binds with LPS and is presented to TLR4 creating an inflammatory signal cascade via MAL and MyD88 and ultimately nuclear factors such as NF-κB and AP-1. The down regulation of APE1 down regulates the activity of these nuclear factors as does the knockout of PARP-1 which has been shown to have an attenuating effect on IL-6, IL-10 and TNFα production. OGG1−/− mice have been shown to be protected against the negative effects of LPS induced inflammation, the probable mechanism of these differences is unknown but an increase in IL-4 and IL-10 production were observed whilst IL-12, MIP-1α and TNFα were reduced.
It has also been reported that the inflammatory response to *Helicobacter pylori*, a gram negative bacterium, was reduced in OGG1 deficient mice. OGG1<sup>-/-</sup> mice displayed less gut inflammation and histological lesions compared to WT mice, as well as a lowered tendency to recruit polymorphonuclear cells (neutrophils, basophils and eosinophils) to the gut tissue (OGG1<sup>-/-</sup> 33% vs. OGG1<sup>+/+</sup> 100%). In these mice a reduction in the mRNA expression of iNOS was also observed (Touati *et al.*, 2006). These results then suggest that OGG1 has a role in the expression of pro-inflammatory proteins in response to Gram-negative bacteria (Mabley *et al.*, 2005a; Touati *et al.*, 2006). In contrast it has been reported, that when exposed to diesel exhaust particles (DEP; inhaled 20 mg/m<sup>3</sup>) the difference between the macrophage and neutrophil population in BAL fluid between wildtype and OGG1<sup>-/-</sup> mice was negligible, although there was a ~58% reduction in IL-6 mRNA production in OGG1<sup>-/-</sup> lung tissue when compared to that of OGG1<sup>+/+</sup> (Risom *et al.*, 2007).

### 1.7. Oxidative Damage

The number of neutrophils and macrophages at the site of injury during inflammation increase dramatically. When these cells die they are subject to necrotic lysis and release reactive oxygen species into the area. The original purpose of these molecules was bactericidal, but they are also damaging to the host tissues and can damage anything they come into contact with (Smith, 1994). This includes DNA which has its own repair systems in place, the one most associated with oxidised bases is base excision repair (Slupphaug *et al.*, 2003).

#### 1.7.1. Reactive Oxygen Species

DNA damage is classified as any alteration to DNA structure or chemistry. The agents that react with DNA to cause this damage come from two sources, exogenous sources (*i.e.* outside of the body) including ionizing radiation, solar radiation and environmental carcinogens such as tobacco
smoke, and endogenous sources (i.e. inside the body) such as the by products of aerobic respiration (Droge, 2002). Perhaps the most important of these is oxidative stress due to both the metabolism of xenobiotic compounds and the ubiquitous nature of endogenous ROS (Hazra et al., 2002a).

Whilst each of us is dependent on oxygen for respiration, the reduction of oxygen in normal aerobic respiration causes ROS to be formed as energy is produced by the electron transport chain in the inner mitochondrial membrane (Figure 1.7) (Turrens, 2003). Additionally, as previously mentioned, ROS are also released in mammalian cells as part of the inflammatory response (Beutler, 2004).

There are many methods of reducing the amount of ROS within the cell, for example the enzymes catalase, superoxide dismutase and the glutathione S-transferase family of enzymes, as well as reducing agents like vitamins A, C and E (Oakley, 2005; Slupphaug et al., 2003).

![Diagram of Electron Transport Chain]

**Figure 1.7: Production of Superoxide Radical and Hydrogen Peroxide by the Electron Transport Chain**

Complexes I, II and III of the electron transport chain can leak electrons creating superoxide radical (O₂⁻). This can then aid in the production of hydrogen peroxide which can also be leaked into the cytosol of the cell (Turrens, 2003).
An overview of the main steps of generating and reducing ROS are found in Table 1.1. The formation of hydrogen peroxide (H$_2$O$_2$) is catalysed by iron (Figure 1.8), and to a lesser extent copper and nickel, into superoxide radicals via the Fenton reaction (Imlay et al., 1988; Lloyd and Phillips 1999). As a result there are defence mechanisms within the cell that sequester Fe$^{2+}$ ions to protect the cell from superoxide damage (Tenopoulou et al., 2005).

Some ROS such as the superoxide radical (O$_2^-$), and H$_2$O$_2$ are also key secondary messengers, important to the function of the cell and thus a balance must be struck between reducing oxidative damage, yet allowing cellular signalling to continue (Dalton et al., 2000). If the levels of these damaging molecules overwhelm the protections within the cell it can result in various types of damage including base damage and single- and double-strand breaks.

### Table 1.1: The generation of major ROS and major avenues of protection$^a$

<table>
<thead>
<tr>
<th>Generation of ROS</th>
<th>Protection against ROS</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O $\rightarrow$ H$_2$O$^+$ + e$^-$</td>
<td>2O$_2$$^{••}$ + 2H$^+$ $\rightarrow$ O$_2$ + H$_2$O$_2$ – catalysed by superoxide dismutase</td>
</tr>
<tr>
<td>H$_2$O$^+$ + H$_2$O $\rightarrow$ OH$^•$ + H$_3$O</td>
<td>2H$_2$O$_2$ $\rightarrow$ 2H$_2$O + O$_2$ – catalysed by catalase</td>
</tr>
<tr>
<td>OH$^•$ + OH$^•$ $\rightarrow$ H$_2$O$_2$</td>
<td></td>
</tr>
<tr>
<td>e$_{aq}$ + O$_2$ $\rightarrow$ O$_2$$^{••}$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Adapted from Slupphaug et al., 2003
\[ \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^* + \text{OH}^- \]

**Figure 1.8: The Fenton Reaction**

H\textsubscript{2}O\textsubscript{2} is formed by endogenous metabolism (Table 1.1) and is catalysed by Fe\textsuperscript{2+}, Cu\textsuperscript{2+} and Ni\textsuperscript{2+} to generate reactive oxygen species capable of damaging DNA.

DNA structural alterations that include the hydrolysis of the base to leave apurinic/apyrimidinic sites (AP sites), the formation of single-strand breaks and the addition of oxygen or alkyl groups can be termed minor damage as they do not halt the transcription process. This damage, however, if left unrepaired, can result in base mis-pairing during DNA synthesis leading to mutations, carcinogenesis and cell death. This damage is ongoing with in the cell and it has been calculated that DNA sustains approximately 800 alterations per hour (Vilenchik and Knudson, Jr. 2000).

Damage which if unrepaired can halt the transcription process can be termed major damage, and may cause apoptosis and premature aging. These types of damage include double-strand breaks, that are lethal to the cell if unrepaired and single-strand breaks which are converted to double-strand breaks during the process of DNA replication (Slupphaug *et al.*, 2003).

Base lesions are chemically altered forms of the four DNA bases (adenine, thymine, guanine and cytosine) produced by reactive oxygen species. If the base lesion halts transcription by blocking the progress of RNA polymerase II, then this a potentially lethal lesion (Wallace, 2002). Oxidative Guanine Damage

### 1.7.2. Oxidative Guanine Damage

Guanine is the most sensitive base target for ROS in DNA. The majority of the lesions formed are 7,8-dihydro-8-oxoguanine (8-oxoG) and 6-diamino-4-hydroxy-5-formamidoguanine (FapyG) (Bruskov *et al.*, 2002; Fromme
and Verdine 2004). Both are created when ROS react with carbon position 8, forming a double-bond by reducing or oxidising the bond with the adjacent nitrogen to a single-bond (Figure 1.9). Whilst 8-oxoG does not block DNA replication the resulting lesion has a high potential to be mutagenic as it can mispair with adenine (see Figure 1.10) and if left unrepaired will cause a GC → TA transversion following DNA replication leading to mutation (Fromme et al., 2004). As 8-OxoG is such a common lesion many studies use it as a marker of DNA damage (De Bont and van Larebeke 2004). FapyG in contrast can halt the replication process and is potentially mutagenic (Krishnamurthy et al., 2008; Wallace, 2002).

![Figure 1.9: Formation of Oxidised Guanine Products](image)

The reaction of ROS with Guanosine produces the unstable radical intermediate that can either be oxidized to 8-OxoGua or reduced to FapyG (adapted from Kalam et al., 2006).
1.7.3. Oxidative Adenine Damage

Similar to the oxidative products of guanine adenine is vunerable to oxidation at carbon position 8, resulting in 7,8-dihydro-8-oxoadenine (8-oxoA) and 4,6-diamino-5-formamidopyrimidine (FapyA) (Figure 1.11; (Kalam et al., 2006)). 8-oxoA does not block the replication process but it can pair with either thymine or guanine, which can result in AT → CG transversions but this happens comparatively rarely and upon most occasions it will pair with thymine. FapyA can halt the replication of DNA (Girard et al., 1998; Wallace, 2002).

Figure 1.10: 8-oxoGua paired with cytosine and mispaired with adenine adapted from Fromme et al., 2004

Figure 1.11: Formation of Oxidised Adenine Products.
ROS react with adenine to produce 8-oxoA or FapyA
1.7.4. Oxidative Thymine Damage

The major stable product of thymine oxidation \textit{in vitro} is 5,6-dihydroxy-5,6-dihydrothymine or thymine glycol (Tg). It is created in two forms but the \textit{cis} isomer is produced more often (Figure 1.12; (Brown \textit{et al.}, 2008)). It is formed when the double-bond between the fifth and sixth carbon is broken by ROS and an OH group is added to each carbon (Hazra \textit{et al.}, 2002b). Tg causes significant extrahelical distortions in a double DNA strand, and can block the progress of repair or replicative DNA polymerases along the DNA strand, and when left unrepaired this has been found to be lethal \textit{in vivo} (Aller \textit{et al.}, 2007).

It has been demonstrated that oxidation of thymine can lead to TA→CG transversions \textit{in vivo}. Thymine glycol can be bypassed by DNA polymerase I \textit{in vitro} and \textit{in vivo}, and the efficiency of this bypass depends on whether the \textit{cis} or \textit{trans} isoform is created (Hayes and LeClerc 1986). This suggests that the \textit{cis} form may be considered more mutagenic to the cell, and that mutagenesis is preferred to apoptosis (Wallace, 2002).

![Figure 1.12: Formation of Oxidised Thymine Products](image)

When thymine reacts with ROS both isomers of thymine glycol are created, although the \textit{cis} version is more common (adapted (Miller \textit{et al.}, 2004)).
1.7.5. Oxidative Cytosine Damage

Cytosine can form an oxidation product similar to thymine, cytosine glycol (Cg). However, Cg is unstable and will swiftly degrade \textit{in vivo} to either 5-hydroxycytosine (5-OHC) (by dehydration) or uracil glycol (by deamination) which will further be dehydrated to 5-hydroxyuracil (5-OHU) (Figure 1.13; (Tremblay et al., 2007)). 5-OHU will preferentially pair with adenine leading to CG → TA transitions. \textit{In vitro} it has been shown that 5-OHC has a chance of mispairing with cytosine resulting in CG → GC transversions (Purmal et al., 1994).

![Reaction of ROS with cytosine leads to cytosine glycol, which in turn can be dehydrated to 5-hydroxycytosine, deaminated to uracil glycol or deaminated and dehydrated to 5-hydroxyuracil (adapted from Tremblay et al., 2007).]

**Figure 1.13: Formation of Oxidised Cytosine Products**

The reaction of ROS with cytosine leads to cytosine glycol, which in turn can be dehydrated to 5-hydroxycytosine, deaminated to uracil glycol or deaminated and dehydrated to 5-hydroxyuracil (adapted from Tremblay et al., 2007).

1.7.6. Spontaneous Base Removal

Spontaneous depurination occurs when a purine is excised from the deoxyribose sugar by hydrolysis of the N-glycosidic link by ROS. It is estimated that 5000 purine bases are removed per cell, per day from DNA due to this process. The same process occurs with pyrimidines but at a much lower rate (Maynard et al., 2009). In double stranded DNA
Apurinic/apyrimidinic sites (AP sites) are efficiently repaired by BER, but in single strand DNA the missing base is replaced at random potentially leading to transitions and transversions. AP sites have also been shown to be cytotoxic as they block the activities of replicative DNA polymerases (Maynard et al., 2009).

**Figure 1.14: Formation of an AP site.**
The guanine is removed from the sugar phosphate backbone of the DNA strand by hydrolysis, leaving an AP site. Adapted from (Sheppard et al., 2000).

### 1.7.7. DNA strand breaks

Single-strand breaks (SSB) are caused by oxidative damage to the carbon bonds on the sugar phosphate backbone of the DNA strand. When these breaks are found opposite each other double-stranded breaks (DSB) are formed. DSBs are also formed when the replication fork encounters blocking lesions, including those formed by ROS, leading a strand break (Shrivastav et al., 2008)

Single strand breaks can be repaired by BER, but DSBs and SSBs in single stranded DNA are considered the most severe type of DNA damage (Shrivastav et al., 2008). Failure to repair, or misrepair of DSBs can lead to deletions, translocations, and chromosome fusions that enhance genome instability. These breaks can lead to mutations when breaks are
incorrectly repaired, and trigger apoptosis in order to prevent the propagation of mutant or cancerous cells (Shrivastav et al., 2008).

1.7.8. Lipid peroxidation

Lipids are readily oxidised by ROS, creating unstable molecules which after a series of reactions form lipid peroxides (Figure 1.15). These are a well established cause of cellular damage including that of DNA (Marnett, 1999).

Polyunsaturated fatty acid peroxides decompose to produce α,β-unsaturated aldehydes including malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) which react with DNA to form DNA adducts which are used as markers of lipid peroxidation (Marnett, 1999).

![Figure 1.15: Lipid Peroxidation](image)

A lipid peroxide is formed in three stages. During the initiation stage a free radical interacts with a hydrogen atom to form a lipid radical and water. In the propagation stage the unstable lipid radicals interact with other lipids at which point their radical interacts with a molecular oxygen to create a different fatty acid and a lipid peroxide. The process terminates when the radical interacts with another radical to produce a non-radical. Adapted from (Imlay, 2003).
Figure 1.16: Composition of malondialdehyde and 4-hydroxyalkenal

1.8. DNA Repair

Damage to bases which cause a disruption of DNA replication and RNA creation and/or RNA synthesis may result in cell death or base mis-pairing (mutation) (Michell et al., 2003). But as there are so many DNA damage causing agents within the cell it is important that mechanisms are in place to repair any damage that occurs. There are over 130 known genes involved in DNA repair and many of these are shared common co-factors from processes such as cell cycle regulation, transcription and DNA replication including Proliferating Cell Nuclear Antigen (PCNA) and replication protein A (RPA) (Slupphaug et al., 2003; Wood et al., 2001). Several DNA repair mechanisms have been identified including those that use the undamaged strand of the DNA molecule as a template for repair. These cut and patch type mechanisms include BER (Slupphaug et al., 2003), nucleotide excision repair (NER) (Mitchell et al., 2003) and mismatch repair (MMR) (Golyasnaya and Tsvetkova 2006). These pathways vary widely in the type of damage removed and the size of the excision made.

The importance of DNA repair systems is illustrated by the variety of diseases which are associated with their disruption. The first such disease was identified in 1968 when Cleaver established a link between NER and the skin disease xeroderma pigmentosum (Cleaver, 1968). Since this discovery connections have been formed between a lack of DNA repair and other diseases. Cockayne’s syndrome, characterised by a sensitivity to
light and the appearance of premature aging, and trichothiodystrophy, which causes mental and physical retardation, are also associated with NER (Lehmann, 2003) and Hereditary nonpolyposis colorectal cancer is associated with defects in MMR (Abdel-Rahman et al., 2005). Until 2003 it was thought that base excision repair was not linked to any inherited disease due to the large overlap in the function of BER proteins with other mechanisms (Krokan et al., 2000; Slupphaug et al., 2003). However, links between MUTYH and an autosomal recessive syndrome of adenomatous colorectal polyposis and very high colorectal cancer risk have been observed (Cheadle and Sampson 2003). Additionally, the single-strand breaks that are created as intermediates in the BER process have been implicated in the progression of neurodegenerative diseases such as spinocerebellar ataxia (Caldecott, 2003). Links have also been made between cancer (e.g. lung, skin, leukaemia and breast) and polymorphisms in BER proteins although these tend to be weak, such as that for OGG1 which has shown no consistent connections with any form of cancer (Hung et al., 2005). Polβ, however, has shown a strong connection with cancers, as ~30% of human tumours express Polβ variants (Starcevic et al., 2004).

1.8.1. Base Excision Repair

BER is the main mechanism used to remove non-helix distorting DNA base lesions (Slupphaug et al., 2003). However BER can also repair the consequences of base deamination, spontaneous hydrolysis of the \( N \)-glycosidic bond and SSBs (Fromme and Verdine 2004). Repair is initiated by one of a group of enzymes named DNA glycosylases, which remove damaged bases from DNA leaving the sugar phosphate backbone intact (Hazra et al., 2002a). Many different DNA glycosylases have been identified, and as a result there are several methods of excising damaged bases and repairing the subsequent AP site. These can be split into three major mechanisms of action: (i) monofunctional glycosylase APE1 dependent pathway, (ii) bifunctional glycosylase APE1 dependent pathway
and the third pathway is the (iii) APE1 independent pathway and is initiated by the NEIL1 and NEIL2 proteins (Figure 1.17) (Hazra et al., 2007).

There are two forms of BER, namely short- and long-patch repair. Short patch repair results in the removal of only a single damaged base whilst long patch repair as its name suggests repairs a longer section of DNA (2-15 nucleotides) and recruits many proteins active in DNA synthesis (Robertson et al., 2009).

**Figure 1.17: Monofunctional DNA glycosylase BER (I), bifunctional APE1 dependant BER (II) and independent (III) pathways**

* represents oxidised base. Adapted from Hazra et al., 2007.

All DNA glycosylases begin the reaction in a common manner (see Figure 1.17). The damaged base is located and the DNA is bent around the DNA glycosylase protein. The base is drawn into the active site of the protein where it is excised by a nucleophilic amino acid at the active site resulting in the formation of an AP site (Stivers and Jiang 2003). Monofunctional DNA glycosylases, such as Single-strand selective Monofunctional Uracil
DNA Glycosylase (SMUG1), then require APE1 to cleave the DNA strand 5’ to the AP site leaving a 5’-deoxyribose-5-phosphate (5’dRP) and 3’-OH (Christmann et al., 2003). This configuration blocks normal DNA polymerase activity. Poly (ADP-Ribose) Polymerase-1 (PARP1) then mediates the attachment of DNA polymerase β (Pol β) to the DNA strand, which adds a single nucleotide to the 5’ end of the nick. The dRP lyase activity of Pol β removes the sugar phosphate moiety and the process is completed by either DNA Ligases I or III, which repairs the sugar phosphate backbone of the DNA.

DNA glycosylases such as OGG1 and NTH1 act like monofunctional glycosylases but also have an AP lyase activity that incises the AP site via β-elimination, thus the term bifunctional DNA glycosylases. This process leaves a 5’-dRP and a 3’-phospho-α,β-unsaturated aldehyde (PUA). This is a substrate for APE1 which cleaves the 3’ PUA leaving a 3’-OH group (Figure 1.17) (Dianov et al., 2003; Izumi et al., 2003). Pol β and DNA ligase III/XRCC1 can seal the nick and the repair process is complete (Dianov et al., 2003).

The APE independent pathway is proposed to be used by bifunctional DNA glycosylases such as NEIL1 and 2. This takes advantage of the 5’ and 3’ phosphates produced during β,δ-elimination to utilise polynucleotide kinase (PNK) instead of APE1, to generate the 3’ OH group (Figure 1.17) (Hazra et al., 2007).

1.8.2. DNA Glycosylases

The major DNA glycosylases specialised for removal of oxidised base lesions in BER can be split into three families. First are monofunctional glycosylases such as; N-methylpurine-DNA glycosylase (MPG) which removes alkylated bases from DNA, Uracil-DNA glycosylase 2 (UNG2) and SMUG1 which remove uracil residues from DNA. Second are bifunctional
DNA glycosylases, such as the mammalian DNA glycosylases OGG1 and NTH1 (Dou et al., 2003), that share features and methods of reaction with the *Escherichia coli* gene *nth*. Proteins of this family contain a helix-hairpin-helix motif and have an internal Lys residue that acts as an active site, which carries out β-elimination cleaving the DNA strand 3' to the oxidised base and leaving 5'-phosphate and 3' PUA termini (Hazra et al., 2002a). The third major class of DNA glycosylases share homology with the *E. coli* Nei (endonuclease VIII) including helix-2turn-helix motifs and a catalytic pro2 residue, and are described as NEI Like enzymes 1, 2 and 3 (NEIL1, NEIL2 and NEIL3).

### Table 1.2: Human DNA glycosylases located in the nuclei and the DNA damage that they remove

<table>
<thead>
<tr>
<th>Name</th>
<th>Lyase Activity</th>
<th>Known Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-OGG1</td>
<td>Yes</td>
<td>FaPyG:C &gt; 8-oxoG:C &gt; 8-oxoG:T, 8-oxoA:T</td>
</tr>
<tr>
<td>NTH1</td>
<td>Yes</td>
<td>5-OHC, Tg:A &gt; Tg:G, 5-OHU, 5,6-DHU, FaPyG:A/G/T, 5-FoU</td>
</tr>
<tr>
<td>NEIL1</td>
<td>Yes</td>
<td>FaPyA, FaPyG, 8-oxoG:C &gt; 8-oxoG:G &gt; 8-oxoG:T, Tg, 5-OHC, 5,6-DHU</td>
</tr>
<tr>
<td>NEIL2</td>
<td>Yes</td>
<td>5-OHU, 5,6-DHU, 5-OHC</td>
</tr>
<tr>
<td>NEIL3</td>
<td>Yes</td>
<td>FaPyA,FaPyG</td>
</tr>
<tr>
<td>SMUG1</td>
<td>No</td>
<td>ssU &gt; U:G &gt; ss5-HMU:G &gt; U:A &gt; 5-HMU:A &gt; eC:G &gt; 5-FU:A</td>
</tr>
<tr>
<td>UNG-2</td>
<td>No</td>
<td>ssU &gt; U:G &gt; U:A &gt; 5-FU</td>
</tr>
<tr>
<td>MUTYH</td>
<td>No</td>
<td>A:G, A:8-oxoG &gt; 2-OH-A:G &gt;&gt; C:A</td>
</tr>
<tr>
<td>MTH1</td>
<td>No</td>
<td>Cpg:T, CpG:U</td>
</tr>
<tr>
<td>MPG</td>
<td>No</td>
<td>3-methyladenine&gt;Adenine residues</td>
</tr>
<tr>
<td>TDG</td>
<td>No</td>
<td>U:G, T:G</td>
</tr>
<tr>
<td>MBD4</td>
<td>No</td>
<td>U:G, T:G</td>
</tr>
</tbody>
</table>

FaPyG, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-oxoG, 7,8-dihydro-8-oxoguanine; 5-OHC, 5-hydroxycytosine; Tg, thymine glycol; 5-FoU, 5-formyluracil; 5,6-DHU, 5,6-dihydrouracil; FaPyA, 4,6-diamino-5-formamidopyrimidine; 5-OHU, 5-hydroxyuracil; 5-HMU, 5-hydroxymethyluracil; 5-FU, 5-fluorouracil; 2-OH-A, 2-hydroxyadenine; ss, single-stranded. *adapted from Slupphaug et al., 2003
1.8.2.1. OGG1

OGG1 is the mammalian homologue of the MUTM gene in *E. Coli* (Arai *et al.*, 1997). The human DNA glycosylases α-OGG1 and β-OGG1 are produced by alternative splicing of the same DNA transcript found on chromosome 3p26. The full code for OGG1 consists of 8 exons, and α-Ogg1 is a result of the transcription of exons 1-7 which results in the formation of a protein of weight 39 KDa, whilst β-Ogg1 is formed when exons 1-6 and 8 are transcribed and produce a protein 47 KDa in weight. The structure indicates that OGG1 contains both a zinc finger and a helix-hairpin-helix (HhH) DNA binding site (Arai *et al.*, 1997). The OGG1 proteins are found ubiquitously throughout the body in the nucleus and mitochondrion. It is transcribed in greater amounts in thymus, testis, kidneys, intestine and brain tissues (Radicella *et al.*, 1997).

In its human form α-OGG1 is located in the nucleus and excises 8-OxoA opposite T, 8-OxoG opposite C and T and both FapyG and meFapyG opposite C (Table 1.2), whereas the mouse form only excises 8-oxoG lesions opposite C and T (Audebert *et al.*, 2000; Campalans *et al.*, 2007; Dherin *et al.*, 1999). The structure of the hOGG1 protein suggests that it makes contact with the base opposite 8-oxoG through hydrogen bonds to hydrogen acceptors of the paired base. This may explain the different levels of affinity it has for certain pairings (Banerjee and Verdine 2006).

Human β-OGG1 is located in the mitochondrial matrix, but its role in the repair of mtDNA is unknown as it does not have any detectable DNA glycosylase or AP lyase activity (Hashiguchi *et al.*, 2004). Indeed it has been suggested that while 8-oxoG glycosylase activity has been detected in the mitochondrial matrix, this is the result of α-OGG1 activity in the mitochondria rather than that of β-OGG1 (Bohr, 2002; Hashiguchi *et al.*, 2004). β-OGG1, however, is thought to play a role in the regulation of apoptosis within the cell, as an over expression of the protein was shown to have a protective effect when the cells were challenged with xanthine.
oxidase induced ROS (Dobson et al., 2002; Ruchko et al., 2005). Further studies have linked β-OGG1 with oxidant induced apoptosis, noting that its disruption increases programmed cell death (Panduri et al., 2009).

Whilst Ogg1 expression is not affected by the cell cycle, it has been proposed that Ogg1 expression can be induced by certain exogenous signals such as oxidative stress (Risom et al., 2007). There are many layers of regulation on the activity of OGG1 including product inhibition (Sidorenko et al., 2008), reversible phosphorylation and nitric oxide (NO) inactivation (Jaiswal et al., 2001). In the presence of APE1 the efficiency of OGG1 can improve up to 5 fold (Hill et al., 2001) and APE1 may thus have a role in facilitating product dissociation of OGG1. Phosphorylation has also been shown to regulate OGG1 activity and cellular localisation. The exact location of the phosphorylation site is yet to be confirmed, four have been identified, but preliminary in silico analysis has identified serine$^{326}$ as a likely target for protein kinase A (Smart et al., 2006; Svilar et al., 2011). The adjacent cysteine is able to form a thiolate anion that is susceptible to oxidation. The secondary messenger NO has been shown to disrupt the zinc finger motif through the process of thiol nitrosylation, which causes the ejection of the zinc ion and irreversible loss of catalytic activity (Jaiswal et al., 2001).

When there are mutations in the OGG1 gene, incidence of cancer and other age related diseases thought to have links with DNA damage are not clearly increased, but this thought to be due to the presence of other BER proteins acting as backups, or that the mutation has no functional effect (Osterod et al., 2001). When considering specific polymorphisms alterations at codon 326 (Ser/Cys and Cys/Cys) are the ones most documented. It has been shown in MEFs that these polymorphisms lead to the accumulation of FaPyG within the cell (Bravard et al., 2009; Smart et al., 2006). A higher risk of several cancers has been linked with this
polymorphism including lung (Kohno et al., 2006), gastric (Farinati et al., 2008), prostate (Chen et al., 2003), and orolaryngeal (Elahi et al., 2002). Conversely there have been several studies that have shown no links with this polymorphism and the risk of specific cancers including breast cancer (Vogel et al., 2003), colorectal (Hansen et al., 2005) and stomach (Hanaoka et al., 2001).

The mouse OGG1 gene is found on chromosome 6 and shares 83% amino identity with the human α-OGG1, evidence for OGG1 activity has been identified in mouse mitochondria. It has been suggested that mOGG1 is distributed throughout the cell, to where it is needed via active transport involving the cells microtubules (Conlon et al., 2004). Again OGG1 is found ubiquitously in the mouse but is at its highest levels in tissue extracted from the testes (Rosenquist et al., 1997).

In OGG1 knockout mice reported thus far no significant difference has been observed in the viability, appearance or survival when compared to wildtype mice. In the 12 month period that these mice were studied there was no increased incidence of cancer in the mice (Klungland et al., 1999). However, it has been reported, in a different strain of OGG1/− mice, that the incidence of lung cancer increased after 19 months of observation (Sakumi et al., 2003) It was also reported that the accumulation of 8-oxoG residues did increase when compared with wildtype mice. In nuclear DNA there was a tissue specific increase of 10 – 100 % (testes and liver respectively) in 8-oxoG residues. The removal of oxidised guanines from the DNA was not halted completely though, and there was significantly slower repair in the knockout line. In the mitochondria disruption of the OGG1 gene can cause 8-oxoG to accumulate to 20 times the amount found in normal cells it has been shown that this has no significant effect on the respiratory function of the mitochondria (Stuart et al. 2004, de Souza-Pinto et al., 2001).
1.8.2.2. NTH1

The mammalian homologue of *E. coli* nth, or endonuclease III, is NTH1. The gene encoding the human form of this protein is found on chromosome 16p13 and comprises 6 exons (Martin *et al.*, 2004). The NTH1 protein has a mass of 34 KDa and is expressed throughout the body within the nucleus of cells, although it is more highly expressed in the heart, liver and brain tissues, and least expressed in muscle tissue (Gros *et al.*, 2002; Hazra *et al.*, 2002b; Ikeda *et al.*, 2002). As well as the conserved HhH DNA binding motif present in other nth like DNA glycosylases, it also has a 4Fe-4S cluster loop motif which helps to locate the enzyme more accurately onto the DNA molecule (Gros *et al.*, 2002; Izumi *et al.*, 2003). The two iron-sulphur clusters form a pocket with the catalytically active Lys$^{120}$ and Asp$^{138}$ at its mouth.

NTH1 expression is cell cycle dependant, with expression low during the $G_0$-$G_1$ phases, but increased at the start of S-phase where it reached its maximum level. This suggests a link between NTH1 expression and DNA synthesis (Luna *et al.*, 2000). Like OGG1, NTH1 is also substrate inhibited and the addition of APE increases its efficiency at removing Tg from DNA ~3 fold (Marenstein *et al.*, 2003). NTH1 activity has been shown to be modulated by XPG, a structure-specific endonuclease involved in NER, and P53, thus connecting BER with NER and p53-dependent damage response pathways. Both of these interactions stimulate NTH1 activity; XPG at the RNA transcription complex and p53 during DNA damage sensing (Oyama *et al.*, 2004). The transcription factor YB-1 has also been shown to increase the rate of lyase activity *in vitro* (Marenstein *et al.*, 2003). NTH1 was shown to bind to PCNA, but was not stimulated as a result of such binding (Dou *et al.*, 2008). However it has been theorised that the interaction with PCNA, could play a coordinating role during S phase (Oyama *et al.*, 2004) and that PCNA in the replication machinery recruits
NTH1 to Tg lesion when the DNA replication machinery stalls (Oyama et al., 2004).

As a bifunctional DNA glycosylase NTH1 can remove specific base lesions via β-elimination (Dizdaroglu et al., 1999). The substrates that NTH1 removes most efficiently are the oxidised pyrimidines shown in Table 1.2. Of these products thymine glycol (Tg) is toxic, unlike 8-oxoG which is mutagenic. Tg halts the progress of replicative DNA polymerases and, if not removed, can lead to cell death (Izumi et al., 2003).

The mouse NTH1 gene is located on chromosome 17 and comprises 6 exons. The resulting protein has a mass of 37 kDa and shares 84% amino acid identity with hNTH1 and shares similar substrate specificity (Sarker et al., 1998). Whilst human NTH1 protein is exclusively transported to the nucleus, mouse NTH1 protein is transported to the mitochondria (Ikeda et al., 2002).

NTH1−/− mice do not show any phenotypical difference to wildtype mice in terms of viability, growth rate, age related differences and cancer risk (Parsons and Elder 2003). In the genomic DNA of NTH1−/− mice Tg seemed to be removed at normal rates suggesting a functional backup that repairs oxidised pyrimidines is involved. However, Tg was not observed to be removed at all from mitochondrial extracts (Karahanil et al., 2003; Parsons and Elder 2003). NTH1−/− cells showed no increased sensitivity to menadione, H₂O₂ or X-ray treatments, and still removed Tg lesions, although it was reported that they did so at a slower rate with substantial amounts of BER intermediates detected after treatment when none were detected in WT cells (Parsons and Elder 2003). In NTH1−/− mouse thymus, removal of Tg was observed, interestingly this cleavage was greater against Tg:G than Tg:A base pairs (Ocampo et al., 2002). However there was no Tg excision activity observed in liver mitochondrial extracts from a different strain of NTH1−/− mice (Karahanil et al., 2003).
1.8.2.3. NEIL1

The fact that NTH1 knockout mice appear phenotypically normal was crucial in the discovery of the NEIL1 and NEIL2 proteins, as it was discovered that in the absence of NTH1 other as yet unknown proteins were repairing Tg (Hazra et al., 2002a). Of these proteins NEIL1 was identified as a backup for NTH1, as the substrates it excises are similar (Takao et al., 2002a).

The NEIL1 gene is located on chromosome 15 at location q24.2 and comprises 10 exons, the subsequent protein has a mass of 44 KDa and is present in both the nucleus and the mitochondria of the cell (Hazra et al., 2002a; Vartanian et al., 2006; Zody et al., 2006).

As an APE independent enzyme (Figure 1.17), NEIL1 performs β,δ-elimination. Whilst it contains a DNA binding motif (a helix-2turn-helix), it does not contain the expected zinc finger DNA binding motif, although it does contain a ‘zincless finger’ which has been proposed to fill a similar role (Doublie et al., 2004). The suggested active site is a conserved Pro2 at the N-terminal (Figure 1.18) (Hazra et al., 2002a).

Figure 1.18: Sequence alignment of critical domains of NEIL1 and NEIL2 with E. coli Nei and Fpg
adapted from Hazra et al., 2007

NEIL1 is a bi-functional DNA glycosylase/AP lyase protein that removes a wide range of oxidised bases from DNA (Table 1.2) (Shinmura et al., 2004; Takao et al., 2002a). It has been suggested that as NEIL1 can excise the same substrates as OGG1 that it could be a functional back up.
However, it excises 8-oxoG from double-stranded DNA at a much slower rate than OGG1 (Hazra et al., 2002a; Morland et al., 2002).

Expression of human NEIL1 varies throughout the body with levels higher in the pancreas, liver and thymus than the testis and muscle (Hazra et al., 2002b). Significantly it is expressed 6-7 times more abundantly during the S-phase of the cell cycle than during the G0-G1 phase. This contrasts with that of other DNA glycosylases such as OGG1 whose expression is not significantly affected by the cell cycle (Hazra et al., 2002a). This co-ordinated change in expression and the wide range of lesions repaired, suggest that NEIL1 is involved in the repair of damaged bases at the replication fork (Takao et al., 2002a). This is further supported by the work of Dou et al. who showed that NEIL1 (and NEIL2) can function as DNA glycosylases in a bubble structure of DNA and on single- and double-stranded DNA, and indeed the catalytic activity of NEIL1 is greater when repairing single-stranded DNA (Dou et al., 2003).

FEN1, PCNA and CSB proteins have been shown to increase the activity of NEIL1 in vitro (Dou et al., 2003; Hegde et al., 2008; Muftuoglu et al., 2009). NEIL1 has also been shown to interact with DNA polβ and DNA ligase IIIα. This suggests that the enzyme may have a role in DNA repair co-ordination rather than being a less efficient backup for OGG1 (Dou et al., 2003). Interestingly, whilst no direct interaction has been observed between NEIL1 and OGG1, the presence of NEIL1 has been shown to increase the efficiency of OGG1 in vitro, and is due to the NEIL1 protein higher affinity for AP sites. It is also suggested that NEIL1 is acting as a backup for APE1 to stimulate 8-oxoG repair (Hazra et al., 2007; Mokkapati et al., 2004b). A study of various hNEIL1 polymorphisms as shown that a reduction in the efficiency of the NEIL1 protein may be involved in the pathogenesis of gastric cancers (Shinmura et al., 2004).
The mouse NEIL1 gene is located on chromosome 9 at location 9c and comprises 9 exons. The subsequent protein has a mass of 44 KDa and is present in the nucleus of the cell (Morland et al., 2002). Expression levels for NEIL1 vary between mouse tissues with levels highest in the prostate, ovary, brain, and spleen. Again the expression of the mouse NEIL1 protein is cell cycle dependant, with the greatest expression in S-phase (Hazra et al., 2002a). When compared with WT controls, the mortality rate of Neil1-deficient cell lines high in response to low doses of γ-irradiation (3 min in a Gammacell-40 Cesium irradiator at a dose rate of 0.75 Gy/mim) (Rosenquist et al., 2003).

Whilst NEIL1 knockout mice do not show any immediate effects on weight and viability, Vartanian et al. (2006) reported that a high proportion of mice within the colony had been found to exhibited symptoms similar to the human metabolic syndrome. This occurred in all of the male knockout mice in the study and a large proportion of the female mice, with the males being much more seriously obese than the females after around 8 months. Additionally serum levels of leptin were significantly higher in NEIL1−/− mice. It was suggested that the symptoms associated with metabolic syndrome may have been caused by a build up of DNA damage in the mitochondria disrupting metabolic processes (Vartanian et al., 2006). A further breeding pair from this colony was housed in a different animal facility but the resulting offspring did not display any of the symptoms previously documented (Chan et al., 2009). However more recently the link between NEIL1 and obesity has been renewed due to data showing a significantly greater susceptibility to obesity and reduction in voluntary exercise, and it is now thought that the disruption of the NEIL1 protein is a predisposing factor to obesity (Sampath et al., 2011).

1.8.2.4. NEIL2

The NEIL2 gene is located on chromosome 8 at position p23.1, it contains five exons and codes for a protein with a mass of 37 KDa and is located
mainly in the nucleus (Nusbaum et al., 2006). The protein sequence indicates several conserved sequences that are also found in the NEIL1 protein including a catalytic proline terminal, the catalytic lysine residue, the H2TH binding motif and additionally a zinc finger motif (Figure 1.18) (Hazra et al., 2007). Like NEIL1, NEIL2 can perform APE independant β/δ elimination and acts primarily on 5-OHU, as well as other oxidised cytosines (see Table 1.2) (Dou et al., 2003; Hazra et al., 2002b).

NEIL2 mRNA is found ubiquitously throughout the body, but at different levels depending on the tissues examined. High gene expression has been reported in skeletal muscle and testis, and low expression levels in lung, liver, spleen, thymus, prostate, and ovaries (Morland et al., 2002). The tissue specificity of this enzyme seems to compensate for NEIL1 and NTH1 as the latter two enzymes are expressed less in muscle and testis, whereas NEIL2 levels are higher in these two tissues. NEIL2 has a greater affinity with the replication bubble structure of DNA than NEIL1, but unlike NEIL1 its expression is not connected to that of the cell cycle. This suggests that NEIL2 may be involved in global base excision repair, but it can be inferred that it is connected with DNA replication and transcription due to its ability to bind with single- and double-stranded DNA (Dou et al., 2003; Hazra et al., 2002b; Takao et al., 2002b).

NEIL2 activity can be increased by interactions with YB-1, or decreased by interacting with pyridoxal-5′-phosphate (Das et al., 2007b; Grin et al., 2010). NEIL2 has also been shown to stably interact with the p300 transcriptional cofactor which acetylates Lys sites of proteins. The acetylation of Lys49 of NEIL2 is the most effective target which leads to inhibition of DNA glycosylase and AP lyase activity. Acetylation of other Lys residues does not produce an inhibitory effect, indicating that reversible modification of Lys49 could regulate the activity of NEIL2 (Bhakat et al., 2004).
The mouse NEIL2 gene is located on chromosome 14 D1. It contains five exons and codes for a protein with a mass of 37 KDa and is located mainly in the nucleus (Church et al., 2009). To date no NEIL2−/− mice have been reported.
1.9. Hypothesis

“If DNA glycosylases, specifically NEIL1, NEIL2, OGG1 and NTH1, are involved in the regulation of endotoxin induced inflammation and then the knockout of these genes, in murine models, will result in a reduction in the inflammatory response to endotoxin.”

In order to test this hypothesis the following objectives will be carried out:

I. The molecular characterisation of new NEIL1\(^{-/-}\) and NEIL2\(^{-/-}\) knockout mouse strains.

II. The identification of any phenotypes in the gross physiology of NEIL1\(^{-/-}\) and NEIL2\(^{-/-}\) knockout mouse strains.

III. The maintenance of colonies of NEIL1\(^{-/-}\), OGG1\(^{-/-}\) and NEIL2\(^{-/-}\) mice (Chapter 3).

IV. Creating murine embryonic fibroblasts (MEFs) from WT, OGG1\(^{-/-}\) NEIL1\(^{-/-}\) and NEIL2\(^{-/-}\) mouse strains.

V. Using the resulting MEF strains in conjunction with pre-existing MEF cultures (NTH1\(^{-/-}\) and [OGG1/NTH1] \(^{-/-}\)) as models of inflammation to identify the magnitude and the nature of this reduction in inflammatory response due to the disruption of base excision repair enzymes by measuring the levels of IL-6, IL-10 and MIP-1α released from the MEFs after exposure to LPS (Chapter 4).

VI. Identifying the extent to which the knockout of NEIL1 and OGG1 reduces the immune response to LPS induced inflammation in whole animal models by:-

   a. Establishing whether levels of cytokines IL-6, IL-12, IL-10 and IL-4 differ between NEIL1\(^{-/-}\), OGG1\(^{-/-}\) and WT mouse serum, at basal and LPS treated levels.
b. Establishing whether levels of MPO, MDA and GSH are altered between NEIL1−/−, OGG1−/− and WT mouse tissues, at basal and LPS treated levels (Chapters 5&6).
2. Materials and Methods

2.1. Materials

2.1.1. Buffer composition

The compositions of the various buffers and solutions used are contained in Tables 2.1 and 2.2. All buffers were made in double distilled water (ddH₂O).

<table>
<thead>
<tr>
<th>Use</th>
<th>Name</th>
<th>Compostion</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA electrophoresis</td>
<td>5 x TBE</td>
<td>0.45 M Tris-HCL pH 8.2, 0.45 M boric acid, 7.9mM EDTA.</td>
</tr>
<tr>
<td></td>
<td>6 x loading buffer</td>
<td>3.6 mM bromophenol blue, 1.2 M sucrose, 120mM EDTA.</td>
</tr>
<tr>
<td></td>
<td>Agarose gel</td>
<td>0.8 - 1.5% (w/v) agarose in 0.5 TBE, 0.8mM ethidium bromide.</td>
</tr>
<tr>
<td>Protein extraction</td>
<td>RIPA buffer</td>
<td>150 mM NaCl, 50 mM Tris-HCl pH 8.0, 12mM sodium deoxycholate, 0.1% (w/v) SDS, 1% (v/v) Triton x-100.</td>
</tr>
<tr>
<td></td>
<td>Cell lysis buffer</td>
<td>50mM Tris-HCl pH 8.0, 200mM NaCl, 1 mM EDTA, 1 mM DTT, 1/1,000 (v/v) protease inhibitor cocktail.</td>
</tr>
<tr>
<td>Protein electrophoresis</td>
<td>Loading gel</td>
<td>17% (v/v) Protogel, 125mM Tris-HCl pH 6.8, 0.1% (w/v) SDS, 2mM ammonium persulphate, 0.85mM N,N,N,N-tetramethyl-ethylenediamine (TEMED).</td>
</tr>
<tr>
<td></td>
<td>Resolving gel</td>
<td>40% (v/v) Protogel, 375mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 2mM ammonium persulphate, 0.85mM TEMED.</td>
</tr>
<tr>
<td></td>
<td>5 x SDS loading buffer</td>
<td>0.3 M Tris-HCl pH 6.8, 0.25 M DTT, 20% (v/v) glycerol, 10% (w/v) SDS, 0.05% (w/v) bromophenol blue.</td>
</tr>
<tr>
<td></td>
<td>10 x SDS running buffer</td>
<td>0.25 M Tris Base, 1.92 M glycine, 1% w/v SDS.</td>
</tr>
<tr>
<td>Western blot</td>
<td>Transfer buffer</td>
<td>25 mM Tris Base, 0.192 M Glycine, 20% (v/v) methanol.</td>
</tr>
<tr>
<td></td>
<td>10 x TBS</td>
<td>0.5 M Tris-HCl pH 7.5, 1.5 M NaCl.</td>
</tr>
<tr>
<td></td>
<td>TBS(T)</td>
<td>1 x TBS, 0.1% (v/v) Tween-20</td>
</tr>
<tr>
<td></td>
<td>Blocking solution</td>
<td>5% (w/v) skimmed milk powder (Marvel) in TBS(T).</td>
</tr>
<tr>
<td></td>
<td>Coomassie Blue stain</td>
<td>0.25% (w/v) Coomassie Blue R250, 40% (v/v) methanol, 10% glacial acetic acid.</td>
</tr>
<tr>
<td></td>
<td>Gel destain</td>
<td>20% (v/v) methanol, 5% (v/v) glacial acetic acid.</td>
</tr>
</tbody>
</table>
Table 2.2: Protein Analysis Buffers

<table>
<thead>
<tr>
<th>Use</th>
<th>Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 ELISA</td>
<td>Carbonate buffer</td>
<td>0.2 M sodium carbonate, 0.2 M sodium bicarbonate pH 9.6.</td>
</tr>
<tr>
<td></td>
<td>Amplex Red mastermix</td>
<td>25 µl of Amplex Red (25 mg/ml), 5.7 µl H₂O₂, ddH₂O to 10 ml.</td>
</tr>
<tr>
<td>MPO assay</td>
<td>MPO Working buffer</td>
<td>0.5% (w/v) hexadecyltrimethylammonium bromide, 10 mM 3-N-morpholinopropanesulfonic acid.</td>
</tr>
<tr>
<td></td>
<td>MPO Detection buffer</td>
<td>1.6 mM 3,3',5,5'-Tetramethylbenzidine, 1 mM H₂O₂.</td>
</tr>
<tr>
<td>MDA assay</td>
<td>MDA Working buffer</td>
<td>5 mM Tris pH 7.4, 1.15% (w/v) KCl.</td>
</tr>
<tr>
<td>GSH assay</td>
<td>Potassium phosphate</td>
<td>1.36% (w/v) KH₂PO₄</td>
</tr>
<tr>
<td></td>
<td>EDTA (KPE) buffer</td>
<td>solution A</td>
</tr>
<tr>
<td></td>
<td>KPE buffer solution B</td>
<td>1.7% (w/v) K₂HPO₄</td>
</tr>
<tr>
<td></td>
<td>KPE buffer</td>
<td>16% solution A, 84% solution B, 0.33% EDTA pH 7.5.</td>
</tr>
</tbody>
</table>

2.1.2. Tissue culture media
The compositions of the tissue culture media used are detailed in Table 2.3. All media was filter sterilised (0.22µm; Millipore, UK) before use.

Table 2.3: Tissue Culture Media

<table>
<thead>
<tr>
<th>Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF Media A</td>
<td>Dulbecco's Modified Eagle Medium : F12 Nutrient mixture (DMEM:F12), 10% (v/v) heat-inactivated fetal calf serum (PAA Biotechnology, UK), 2 mM L-glutamine, 0.11 M NaHCO₃, 1x Penicillin-streptomycin.</td>
</tr>
<tr>
<td>MEF Media B</td>
<td>Dulbecco's Modified Eagle Medium : F12 Nutrient mixture (DMEM:F12), 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.11 M NaHCO₃.</td>
</tr>
</tbody>
</table>

2.1.3. PCR primers
All primers were obtained from MWG, Germany.
Table 2.4: PCR Genotyping Primers

Shaded areas indicate combinations of primers that were not used.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Name</th>
<th>Position</th>
<th>Sequence (5' - 3')</th>
<th>WT Size (bp)</th>
<th>KO Size (bp)</th>
<th>Annealing Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEIL1</td>
<td>1907f</td>
<td>Exon 2</td>
<td>CAA CTA TCT GCG GGC AGA GAT</td>
<td>1108</td>
<td></td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>3015r</td>
<td>Intron between exons 5 and 6</td>
<td>GGA GAC AAC TCT GGA GTC AAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>807f</td>
<td>Intron between exons 1 and 2</td>
<td>GTT TAA ACT TTC ACC ACA TTG ATG ACG TGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nNEOr</td>
<td>Within Neo-TK cassette</td>
<td>GCA GCC TCT GTT CCA CAT ACA C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEIL2</td>
<td>410f</td>
<td>Intron between exons 1 and 2</td>
<td>CTT GGA CCA GAG ATA CTT CTC AG</td>
<td>553</td>
<td></td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>963r</td>
<td>Intron between exons 1 and 2</td>
<td>GGG TTA GTT AGA CTA CAG ACT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>-871f</td>
<td>Intron before exon 1</td>
<td>GCG AGT ACT CTC CCT TAT ACT G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nNEOr</td>
<td>Within Neo-TK cassette</td>
<td>GCA GCC TCT GTT CCA CAT ACA C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OGG1</td>
<td>6333f</td>
<td>Intron between exons 4 and 5</td>
<td>GTG GCT GAC TGC ATC TGC TT</td>
<td>323</td>
<td></td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>6656r</td>
<td>Intron between exons 5 and 6</td>
<td>GCA TAA GGT CCC CAC AGA TTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2372f</td>
<td>Exon 3</td>
<td>GCT TCC CAA ACC TCC ATG C</td>
<td></td>
<td></td>
<td>~1000</td>
</tr>
<tr>
<td></td>
<td>oNEOr</td>
<td>Within Neo-Pgk cassette</td>
<td>GCC GAA TAG CCT CTC CAC CCA AGC</td>
<td></td>
<td></td>
<td>58</td>
</tr>
<tr>
<td>NTH1</td>
<td>2956f</td>
<td>Exon 4</td>
<td>TGA GCT GGT AGC CTT GCC AGG TG</td>
<td>597</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>3530f</td>
<td>Intron between exons 4 and 5</td>
<td>GCA CAG TCA AGC ATG ATT ATA AG</td>
<td></td>
<td></td>
<td>~1800</td>
</tr>
<tr>
<td></td>
<td>ntNEOr</td>
<td>Within Neo-Pgk cassette</td>
<td>GCT CTG ATG CCG CCG TGT TCC G</td>
<td></td>
<td></td>
<td>60</td>
</tr>
</tbody>
</table>
Table 2.5: RT-PCR Primers

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Name</th>
<th>Position</th>
<th>Sequence (5' - 3')</th>
<th>Size (bp)</th>
<th>Annealing Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEIL1</td>
<td>328f</td>
<td>Exon 1</td>
<td>CTT GCC CTT TGC TTC GTA GAC ATC</td>
<td>217</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>545r</td>
<td>Exon 2</td>
<td>GGA TCT CTG CCC GCA GAT AGT T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>534f</td>
<td>Exon 4</td>
<td>GTT TAA ACT TTC ACC ACA TTG ATG ACG TGC GG</td>
<td>222</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>765r</td>
<td>Exon 5</td>
<td>GCA GCC TCT GTT CCA CAT ACA C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>12926</td>
<td>Exon 2</td>
<td>CAG TGG GTC AAG GAA CAG AAG C</td>
<td>540</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>13443</td>
<td>Exon 2</td>
<td>GAC AAT GAA GAT GAT GCC AGA GC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.1.4. Molecular biology reagents

All reagents obtained from Sigma-Aldrich except:-
- AMV reverse transcriptase (10 U/µl): Roche Applied Biosciences, UK.
- Reverse transcription random primers, RNasin (40 U/µl): Promega, UK.
- Restriction endonucleases Xmn1 (10 U/Xl) and associated buffers: New England Biolabs, UK.
- BIOTAQ polymerase (10 U/µl) and associated buffer, Bio-X-Act long DNA polymerase (10 U/µl) and associated buffer, dNTPs and HyperLadder I: Bioline, UK.
- Direct PCR (cell) and Direct PCR (ear) lysis buffer: Viagen Biotech, US.
- RNeasy minikit, DNeasy blood and tissue kit: Qiagen, UK.

2.1.5. Protein analysis reagents

All reagents obtained from Sigma-Aldrich except:-
- Precision plus protein-all blue standards and DC protein assay: Bio-Rad, UK.
- Protogel (30% Acrylamide / 0.8% bisacrylamide) solution: National Diagnostics, US.
- Enhanced chemiluminescent (ECL) advanced western blot detection kit, and HiBond 0.45 Xm ECL nitrocellulose membrane: Amersham, UK.
- Antibodies, Abcam, UK.
- Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies: Dakocytomation, Denmark.
- Nylon Membranes, positively charged: Roche Applied Sciences, UK.
- Labeling and Detection - DIG system for in situ hybridisation: Roche Applied Sciences, UK.
2.1.6. Equipment

The following equipment was used:
- Spectrophotometric plate reader: SPECTRAmax PLUS 384 (software: SOFTmax PRO): Molecular Devices, US.
- NanoDrop 1000 spectrophotometer: Thermo Fisher Scientific, UK.
- PAGE electrophoresis and western-blot: Mini-Protean 3 cell vertical electrophoresis system and Mini Trans-Blot cell blotting system: Bio-Rad, UK.
- Sonicator: Sonopuls HD2070 MS72 sonicator, Bandelin electronics, GmbH&Co., Germany.

2.1.7. Transgenic Mouse and MEF sources

NEIL1<sup>−/−</sup> and NEIL2<sup>−/−</sup> knockout mice were generated by Dr. Rhod Elder using published protocols (Perez-Campo et al., 2007). OGG1<sup>−/−</sup> mice had been designed previously (Klungland et al., 1999) and were obtained from embryos stored at the Patterson Institute. All MEFs used of these genotype
were derived from these mice and in addition from from NTH1\(^{-/-}\) (Elder and Dianov 2002) and [NTH1/OGG1]\(^{-/-}\) (Karahalil et al., 2003) mice.

2.2. Methods

2.2.1. Mouse Colony Management

Heterozygous (HET) NEIL1, putative NEIL2 and OGG1 female mice were backcrossed to a C57Bl/6J male. A HET male from the resulting offspring was selected at random to breed to further C57B1/6J females for a further five generations. Various HET mice were intercrossed and produced mice for initial phenotypic characterisation. Knockout x knockout (KO x KO) and wildtype x wildtype (WT x WT) mice were interbred to produce mice for experimental purposes such as the creation of MEFs. All mice were housed in solid floored cages where food and water were provided \textit{ad libitum}.

2.2.2. Mouse Colony Characterisation

The offspring of HET X HET crosses were housed in boxes separated by sex and litter, and these became the characterisation group. Mouse body weight was then measured at monthly intervals and mice were also examined to detect outward phenotypic changes. After 12 months the NEIL1 mice were sacrificed by cervical dislocation and scalp to tail length of the mouse measured. Major organs were also taken and the weights of the heart, lungs, liver, kidney, spleen, omentum fat, reproductive organs (testes and both ovaries and uterus in male and female mice respectively) and adrenal glands measured. Six male and six female mice of each genotype had blood samples taken by cardiac puncture whilst under terminal anaesthetic (isoflurane) to be used with cytokine ELISA’s.
2.2.3. LPS Induced Organ Damage

For MPO, MDA and GSH assays, the wildtype, NEIL1\(^{+/−}\) and OGG1\(^{−/−}\) mice to be treated with LPS were first weighed individually, then injected \(i.p.\) with the appropriate amount of LPS (20 mg/kg) in phosphate buffered saline (PBS; pH 7.4). After 12 h the mice were sacrificed by cervical dislocation and the heart, lungs, liver, kidneys, and a section of ileum were removed and snap frozen in liquid nitrogen. These were then stored at -80°C.

2.2.4. Induction of Immune Response

For cytokine assays the wildtype, NEIL1\(^{+/−}\) and OGG1\(^{−/−}\) mice to be treated with LPS were first weighed individually, then injected \(i.p.\) with the appropriate amount of LPS (2 mg/kg) in PBS. After 1, 6 or 24 h the mice were placed under terminal anaesthetic (isoflurane) and exsanguinated by cardiac puncture after which they were killed by cervical dislocation. The blood was stored on ice with heparin (0.05 ml/ml blood). Blood samples were then centrifuged for 15 min at 8000 \(g\) and the supernatant removed to a separate Eppendorf. Both cells and supernatant were then stored at -80°C.

2.2.5. Establishment of MEF cultures from mouse embryos

In order to establish wildtype, NEIL1\(^{+/−}\) and OGG1\(^{−/−}\) MEF cell lines WT x WT and KO x KO pairings were set up in order to produce the required offspring. The females of these pairings were checked daily for vaginal plugging that would indicate that mating had occurred. Thirteen days after plugging was observed the pregnant mouse was killed by cervical dislocation, the uterus removed, and placed in filtered phosphate buffered saline (PBS).

The individual embryos were then removed from the uterus and separated from the placenta. The head was cut off and stored for genotyping.
Additionally the umbilical chord and internal organs (heart, liver, kidney & gut) were excised from the embryo, and the embryo was then washed in PBS to remove any remaining debris. Using a scalpel blade the remaining tissue was minced finely, and 1 ml of 0.5 µg/ml trypsin added. To ensure sufficient primary breakdown of the embryo, the trypsin/embryo mixture was aspirated through a 1000 µl pipette tip. The mixture was then incubated in a water bath for 30 min at 37°C, mixing at 10 min intervals.

In order to wash out the trypsin the mixture was diluted in 6 ml of MEF media A, and was then centrifuged for 5 min at 126 g. At this point a jelly like substance may have formed in the supernatant and this was kept and the rest of the supernatant discarded, and cells from the pellet and jelly resuspended in 1 ml MEF media A. The cells were then plated onto 25 cm² (T25) vented cell culture flasks and incubated with 3 ml of MEF media A in a humidified atmosphere containing 5% CO₂ and 3% O₂. After a day, when the cells had reached confluence, they were washed with PBS, 250 µl of Trypsin added and the cells incubated at 37°C. When all cells had detached from the flask, 2 ml of MEF media A was added and the mixture transferred to a sterile centrifuge tube. Cells were centrifuged for 5 min at 126 g, the supernatant discarded and the pellet was resuspended in 1 ml of MEF media A. This was then placed in a new T25 and 3 ml of primary MEF culture medium A added. When the cells in this flask had grown to confluence they were removed from the flask by trypsinisation and after centrifugation in MEF media A, the cells were resuspended in media containing 10% dimethyl sulfoxide (DMSO), transferred to a labelled cryotube and placed on ice for 30 min before being placed in storage in a -80°C freezer.
2.2.6. MEF culture

NTH1\(^{+/}\) and [OGG1/NTH]\(^{+/}\) MEFs were taken from stocks held in liquid N\(_2\). Cells were thawed rapidly in a 37°C water bath, and 6 ml of MEF media B, held at room temperature, added slowly. To wash out the DMSO, the cells were centrifuged at 126 \(g\) for 5 min, the supernatant discarded and the cells resuspended in MEF media B. All MEFs were incubated at 37°C in 75 cm\(^2\) (T75) vented flasks in a humidified atmosphere containing 5% CO\(_2\) and 3% O\(_2\).

Cells were passaged upon reaching confluence by washing cells in PBS, trypsinising them with 750 µl of Trypsin-EDTA and incubating at 37°C. When all the cells had been detached from the flask, 6 ml of MEF media B was added and the mixture transferred to a sterile centrifuge tube. Cells were centrifuged for 5 min at 126 \(g\), the supernatant discarded and the pellet was resuspended in 1 ml of MEF media B. The MEFs were then counted using a haemocytometer and 6 \(\times\) 10\(^4\) cells plated in a new T75 flask containing 10 ml of medium B.

At regular stages, stocks of MEFs were frozen for future use; cells were pelleted by centrifugation and the supernatant discarded. The cells were then resuspended in 1 ml of MEF media B containing 10% DMSO, and transferred to a labelled cryotube, which was placed on ice for 30 min before storage in a -80°C freezer.

2.2.7. Treatment of MEFs with LPS

Duplicate samples of 2 \(\times\) 10\(^5\) MEF cells of each genotype WT, OGG1\(^{+/}\), NTH1\(^{+/}\), [OGG1/NTH1]\(^{+/}\) and NEIL1\(^{+/}\) were plated in separate wells in a 24-well plate. Controls were incubated with 1 ml of MEF media B only, while the
experimental samples were treated with 1 ml of MEF media B containing 0.25 µg/ml purified LPS from *Escherichia coli* serotype 127:B8. Plates were then incubated for 18 h before the supernatant was aspirated into clean Eppendorf tubes and frozen.

### 2.2.8. Genomic DNA extraction

DNA was extracted from a variety of sources, including earpunches, heads of embryos and MEF cells. Such material was placed into a clean 0.5 ml Eppendorf tube. One hundred and fifty microlitres of the appropriate DirectPCR Lysis Reagent was added (Direct PCR ear for earpunch and head pieces, Direct PCR cell for cells). The samples were then incubated for 5 – 6 h at 55°C with occasional agitation (~ every 2 h) to ensure all the material came into contact with the lysis solution. At the end of the incubation, samples were examined visually to ensure that complete lysis of the material had occurred. If not the mixture was then left until lysis had been completed (up to overnight). After lysis, the temperature was increased to 85°C for 45 min to inactivate the Proteinase K. DNA concentration was determined prior to storage by measuring the absorbance of the solution at 260 nm using a Nanodrop spectrophotometer. For quality control purposes, the ratio of absorbance at λ = 260 and 280 nm and at λ = 260 and 230 nm were monitored and values from 1.8 to 2.2 were accepted as good quality indicators.

### 2.2.9. Polymerase Chain Reaction (PCR) Genotyping

For each 50 µl reaction, 1.5 µl of the appropriate DNA solution (section 2.2.8) was used, to which was added; 5µl of 10x Taq buffer, 0.5 µl of dNTPs (25 mM), 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), 0.2 µl of Taq DNA polymerase and 38.8 µl of H₂O. The PCR
amplification cycle was as follows; 95°C for 1 min; 95°C for 30 s (denaturing), ~60°C (varies according to primer used; Table 2.4) for 30 s (annealing), and 72°C for 1 min (elongation) for 30 cycles; 72°C for 5 min and 25°C for 1 s.

2.2.10. Agarose Gel Electrophoresis

Agarose gels were prepared by melting agarose in 0.5 x TBE in a microwave. 6 µl of loading buffer was added to each 50 µl PCR reaction and 15 µl of this was then added into each well. In order to estimate fragment size, one well was loaded with 3 µl of a DNA marker (Hyper Ladder I). For DNA fragments below 1 kb a 1.5% agarose gel was used whilst for those fragments above 1 kb a 0.8% agarose gel was required. Electrophoresis was performed in 0.5x TBE at 90 V for approximately 45 min. Results were visualised by using a Typhoon™ laser, scanning at 532 nm.

2.2.11. RNA extraction

For total RNA extraction, 2 x 10^5 MEF cells were trypsinised (section 2.2.6) and pelleted in a centrifuge at 8000 g for 10 min. A RNeasy mini prep kit was used according to manufacturer’s instructions. Cells were disrupted by adding 600 µl of buffer RLT and the mixture homogenised by passing the lysate at least 5 times through a blunt 20–gauge needle attached to a syringe. 600 µl of 70% ethanol was added to the homogenised lysate which was mixed by pipetting and 700 µl of the resulting mixture added to an RNeasy spin column placed in a 2 ml collection tube. This was centrifuged at 8000 g for 15 s and the flow through discarded. Seven hundred microlitres of buffer RW1 was added to the spin column, the mixture centrifuged at 8000 g for 15 s and the flow through discarded. The column membrane was washed by adding 500 µl of buffer RPE and centrifuging at 8000 g for 15 s and discarding the flow through; this step was repeated but with this centrifugation step lasting.
2 min in order to dry the membrane. The spin column was placed in a clean 1.5 ml collection tube and 30 µl of RNase free water was added to the column. This was centrifuged at 8000 $g$ for 2 min to elute the RNA. Total RNA concentration was determined by measuring the optical density of the solutions at $\lambda = 260$ nm using an ND-1000 spectrophotometer (NanoDrop Technologies).

2.2.12. Reverse transcription (RT)

1.5 µg of RNA was dissolved in 11.75 µl of RNase free water and incubated for 10 min at 70°C. The mRNA was reverse-transcribed into first strand cDNA by adding 4 µl MgCl$_2$ (25 mM), 2 µl 10x reverse transcriptase buffer, 1 µl dNTPs (10 mM), 1 µl random primers and 0.25 µl AMV reverse transcriptase. The samples were then incubated at the following temperatures: 25°C for 10 min, 42°C for 1 h, 95°C for 5 min and 0°C for 10 mins. The PCR step was performed with Taq DNA polymerase as previously described (section 2.2.9) using 1 µl of cDNA with an annealing temperature of 60°C.

2.2.13. Protein Analysis

2.2.13.1. Protein extraction from mouse tissues

Approximately 5 mg of mouse tissue was added into a clean mortar, which was kept cool over ice, 300 µl cold RIPA buffer added and the organ was disrupted using a pestel. The solution was transferred to a 1.5 ml Eppendorf tube and agitated for 2 h on ice. The sample was centrifuged at 8000 $g$ for 20 min at 4°C, and the supernatant was removed. 20 µl of the supernatant was used for protein quantification (see 2.2.13.2). An additional supernatant aliquot, to be used for the western blot, was diluted in 1 x SDS-PAGE loading buffer and denatured by heating to 95°C for 5 min.
2.2.13.2. Protein quantification

Diluted protein samples (10 µl, 1/5 - 1/25 dilution) and a range of bovine serum albumin (BSA) protein standards (10 µl, 0 – 2.0 µg/µl) were mixed with 200 µl of Bio-Rad DC protein assay reagent, in triplicate, in a 96-well plate. The absorbance at 595 nm was measured in each well using a plate reader. Protein concentration in the supernatant was determined using the standard curve.

2.2.13.3. Western blot

Up to 50 µg of cell lysate (see 2.2.13.1) in 1 x SDS buffer was separated on a 12 or 16 % polyacrylamide gel (Protogel) by electrophoresis for 1 h at 200 V, in SDS running buffer. A blue pre-stained molecular weight marker was included in each gel. A positive control in the form of protein from a WT animal was also included. The proteins were then transferred onto a nitrocellulose membrane by electro-blotting for 1 h at 100 V, in ice cold western blotting transfer buffer. A blue pre-stained molecular weight marker was included in each gel. A positive control in the form of protein from a WT animal was also included. The proteins were then transferred onto a nitrocellulose membrane by electro-blotting for 1 h at 100 V, in ice cold western blotting transfer buffer. The membrane was then incubated in blocking solution for 1 h at RT and then incubated with primary antibody in blocking solution (dilution: 1/2000) for 1 h at room temperature. After three washes in TBS(T), the membrane was incubated with HRP conjugated secondary antibody in blocking solution (dilution: 1/1,000) for 1 h at RT. The blot was then washed three times in TBS(T) prior to ECL detection according to the manufacturer’s instructions. Briefly, the membrane was drained and 1 ml of a 50:50 mixture of reagent A and reagent B was poured over the membrane and incubated for 5 min at room temperature. The membrane was drained of the excess ECL reagent and exposed to ECL-sensitive film.
2.2.13.4. ELISA

To each well of a Maxisorp 96-well plate, 50 µl of carbonate buffer containing 25 µg/ml rat monoclonal anti-(mouse) interleukin-6 IgG was added, and incubated overnight at 4°C. Each well was washed twice with PBS and blocked with 100 µl of PBS containing 5% (w/v) non-fat milk (NFM) and incubated at room temperature for a further 2 h. Each well was washed twice with PBS before triplicate samples of 50 µl of either, increasing concentrations of IL-6 in tissue culture medium, or each sample was added to a well. Following incubation for 2 h at room temperature, the plate was washed three times in PBS and 50 µl of rabbit polyclonal anti-(mouse) interleukin-6 IgG diluted 1:2000 in PBS containing 0.5% (w/v) NFM was then added to each well. After incubation at room temperature for 2 h, the plate was washed 3 times with PBS-Tween 20, 50 µl of goat anti-rabbit IgG conjugated with horseradish peroxidase diluted 1:2000 in PBS containing 0.5% (w/v) NFM added, and the plate incubated at 4°C for a further 16 h.

Plates were then washed a further 3 times with PBS containing 1% Tween 20 and 50 µl of Amplex Red mastermix was added. After 5 min the plate was transferred to a Typhoon™ laser system, and fluorescence visualised by scanning at 580 nm. A standard curve was produced using the results from the known levels of IL-6 and this was used to determine the levels of IL-6 in the cell supernatants.

All other ELISAs were obtained from eBioscience: the is described in brief. 50 µl of sample was placed into each well of a 96 well plate along with a standard curve of the protein to be examined, and incubated overnight at 4°C. The wells were then emptied and washed 3 times in PBS-T, 100 µl of blocking solution added, and incubated for 1 h at RT. Again the wells were emptied, washed 5 times with PBS-T, 50 µl of primary antibody solution added and incubated for 2 h at RT. After another 5 washes with PBS-T, 50 µl
of the detection antibody solution was added to the wells and incubated for a further 30 min. Again the wells were washed 5 times with PBS-T and 50 µl of the developer solution was added. The development was halted after 10 min by the addition of 25 µl of 2N H₂SO₄. The absorbance was then read at 650 nm and compared with the standard curve to identify the protein content of the original samples.

2.2.13.5. Myeloperoxidase Assay

The protocol was adapted from Graff et al., 1998. ~25 mg tissue samples were weighed and placed in 1.5 ml Eppendorf tubes containing 5 µl/mg MPO working buffer. This was sonicated over ice for 2 s and the resulting solution centrifuged at 8000 g for 15 min. After discarding the supernatant the pellet was resuspended in 500 µl of MPO working buffer (Graff et al., 1998).

The resulting mixture was sonicated over ice for 20 s and then snap frozen, after which it was thawed. This procedure was repeated twice. The resulting solution was centrifuged at 8000 g for 10 min and stored on ice.

An aliquot of the supernatant was diluted 1:2 in working buffer and a DC protein assay was performed (Section 2.2.13.2). To 10 µl of diluted supernatant 190 µl of detection buffer was added and the absorbance read at 650 nm every 30 s for 2 mins. This was compared to the protein result from the DC assay to give a result measured in mU(MPO)/mg protein.

2.2.13.6. Malondialdehyde Assay

The protocol was adapted from Mabley et al., 2005a. Tissue samples were weighed (~20 mg) and placed in MDA working buffer (100mg/ml) inside a 1.5 ml Eppendorf tube. This was then sonicated for 5 s. In a separate 1.5 ml Eppendorf, 75 µl of homogenate was placed to which was added 546 µl 0.8%
(w/v) thiobarbutic acid, 75 µl 8.1% (v/v) SDS, 546 µl 20% (v/v) acetic acid (pH 3.5) and 225 µl distilled H₂O. An external standard was created using 1,1,3,3-tetramethoxypropane (0 – 14 nmol). All solutions were heated to 90°C for 45 min and the absorbance measured on a microplate reader at 532 nm. Protein in each sample was measured using a DC protein assay (see 2.2.12.2). MDA were adjusted to give a result measured in nMol(MDA)/mg protein (Mabley et al., 2005a).

**2.2.13.7. Glutathione Assay**

The protocol was adapted from Rahman et al., 2006. Tissue samples (~20 mg) were weighed and placed in 1.5 ml Eppendorf tubes containing 5 µl/ml 0.6% (w/v) sulfosalyclic acid solution (if assaying liver 5% (w/v) metaphosphoric acid was added as well). The mixture was sonicated over ice for 5 s and centrifuged at 3000 g at 4°C for 10 min. The supernatant was then removed and stored at 4°C up to 1 h before use. Samples were diluted 1/20 in ddH₂O immediately before use (Rahman et al., 2006).

20 µl of the diluted samples were added to wells on a 96 well plate. 2 mg of both 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB) and Nicotinamide adenine dinucleotide phosphate (NADPH), and 40 µl of glutathione reductase (GR) were each added to separate vials of 3 ml KPE buffer. DTNB and GR solutions were mixed together equally and 120 µl added to each well. The samples were incubated at room temperature for 30 s and 60 µl of NADPH solution was added. Absorbance was read at 412 nm immediately and every 30 s for 2 min. Results were compared to a standard curve of glutathione (GSH). Protein content was measured using a DC protein assay (2.2.13.2) and GSH levels reported as nmol(GSH)/mg protein.
In order to compare numbers of pups of each genotype in each litter a chi squared test was performed. To compare survival rates of each genotype a log-rank analysis was performed.

When comparing cell cytokine production and mouse cytokine production one-way ANOVAs were performed. ANOVAs were performed when comparing weights, and as there were 3 groups of mice (WT, HET and KO) a LDS post hoc test was performed. When comparing organ damage results (MPO, MDA and GSH) factorial analyses were performed using a 3-way ANOVA. Sample size was calculated using a statistical power of 0.8. A $p<0.05$ was considered statistically significant.
3. Mouse models: Generation and characterisation

3.1. Introduction

The first NEIL1\(^{--}\) mice were reported in 2006; there was no immediate difference observed in weight or viability of the mice when compared to NEIL1\(^{+/+}\) mice. However, after 6-10 months all the male mice in the third generation became significantly overweight when compared to the WT mice (mean weight 50 g vs. 28 g), and the female mice were also overweight, but to a less severe degree (mean 32 g vs. 28 g). The abdominal cavity of both male and female NEIL1\(^{--}\) mice was found to contain extensive fat deposits, and their livers were significantly larger due to fat storage. The plasma of the NEIL1\(^{--}\) mice also contained elevated concentrations of leptin, which is usually noted with elevated fat deposits, but not insulin (at fasting and fed conditions) which would also be expected (Hoffler \textit{et al.}, 2009). Increased fat deposits and hyperleptinemia were consistent with the human disease metabolic syndrome. It is suggested by Vartanian \textit{et al.} that the build up of DNA damage in the mitochondria of the NEIL1\(^{--}\) mice was disrupting the metabolic processes. Other intermittent phenotypes observed included reduced subcutaneous fat deposits, skin ulcerations, joint inflammation, infertility and cancers (Vartanian \textit{et al.}, 2006). However when mice taken from this colony were bred in a different animal facility, no obese phenotype was observed in any of the mice bred there despite several years of continuous breeding (Chan \textit{et al.}, 2009). This suggested that there may have been another reason for the increased fat content in this strain of NEIL1\(^{--}\) mice. In a follow up paper the same group, at Oregon Health and Science University, have shown that with an increase in oxidative damage (caused by a high fat diet) a greater percentage of male and female mice exhibit markers the metabolic syndrome, such as increased weight and hyperleptinemia (Sampath \textit{et al.}, 2011). This supports the mitochondrial damage model.
previously mentioned which suggested that the metabolic syndrome phenotype is not caused wholly by the absence of the NEIL1 gene, but its removal does increase the mouse’s susceptibility to the symptom. The removal of any protein which repairs oxidative damage in the mitochondria could then contribute to the condition (Sampath et al., 2011). Indeed, in one strain of OGG1<sup>-/-</sup> mice it has been reported that male OGG1<sup>-/-</sup> mice were significantly heavier than OGG1<sup>+/+</sup> mice after 24-28 weeks (Arai et al., 2006).

More work has been done to identify the effects of the knockout of OGG1 in vivo. An OGG1<sup>+/+</sup> mouse was first documented in 1999, and there were no significant differences in viability, and they were indistinguishable from OGG1<sup>-/-</sup> and OGG1<sup>+/+</sup> mice up to 18 months of age (Klungland et al., 1999). In a different strain of OGG1<sup>-/-</sup> mice it was noted that after 19 months the incidence of cancer in the animals increased (Sakumi et al., 2003).

In contrast to this work on NEIL1 and OGG1, very little work has been done on the effects of NEIL2 in vivo, and whilst a few binding partners have been identified to this date no NEIL2<sup>-/-</sup> mice have been reported (Bhakat et al., 2004; Das et al., 2007b; Grin et al., 2010).

### 3.1.1. Aims

The objectives of this chapter are to:

I. Confirm that the disruption of the NEIL1 and NEIL2 genes have resulted in the absence of NEIL1 and NEIL2 proteins respectively in newly developed strains of mice.

II. Identify any phenotype in the gross physiology of these NEIL1<sup>-/-</sup> and NEIL2<sup>-/-</sup> mice, especially pertaining to symptoms similar to the human metabolic syndrome.
III. Confirm the presence of the OGG1 knockout construct in the genomic DNA of OGG1\(^{-/-}\) mice.

3.2. Results

3.2.1. NEIL1 Mouse Colony

3.2.1.1. Preparation of NEIL1 Knockout Mice.

The NEIL1 mouse colony was created from two NEIL1\(^{+/+}\) chimeras created from ES cells prepared by Dr. Rhod Elder (Perez-Campo et al., 2007). Animals in this colony were genotyped by PCR (Figure 3.1) and so identified as either wildtype (NEIL1\(^{+/+}\), WT), heterozygous (NEIL1\(^{+/+}\), HET) and knockout (NEIL1\(^{-/-}\), KO) mice. The appropriate mice identified from these PCRs were then used in the selective breeding of the NEIL1\(^{-/-}\) line and backcrossing to a C57/B16J background (Figure 3.2).

Figure 3.1: Pedegree of NEIL1 Transgenic Mice

♂ Chimera 83 was crossed with a ♀ C57/B16J mouse to produce a litter of WT and HET animals. Then a ♀ C57/B16J and HET ♀ 29 were bred together to produce a backcross, from the resulting litter ♀ 130 was used to further backcross the animals. HET male 20 was crossed with HET ♀ (22 and 25) to produce a litter containing WT, HET and KO animals. These mice were then be used to produce further WT (140 x 121), KO (139 x 146) and HET (138 x 119 & 120) litters.
3.2.1.2. Confirmation of Genotype.

In order to confirm the disruption of the NEIL1 gene at the transcription level RT-PCR was performed on cDNA isolated from liver tissue excised from NEIL1\(^{-/-}\) (KO) and NEIL1\(^{+/+}\) (wildtype) mice (Figure 3.3A). The PCR primers were designed to amplify a section of DNA 449bp in length spanning exons 1 – 3. This amplicon was only observed in the PCR using the NEIL1\(^{+/+}\) sample for its source cDNA. This confirmed that there was no complete NEIL1 cDNA in samples created from RNA extracted from tissues taken from NEIL1\(^{-/-}\) mice, whilst it was detected in the cDNA created from WT animals. A PCR spanning exons 4 and 5 of the GAPDH gene was used as a control, this fragment was predicted to be 222 bp in length. Its presence in both WT and KO samples confirmed that the samples contained viable cDNA.

![Diagram of the NEIL1 Knockout Construct and Genotyping Results](image)

(A) To test for the WT gene, the primers 1907 – 3015 were used, giving rise to a band of 1108 bp. Primer 1907 was designed to anneal to the region in the NEIL1 gene to be deleted. For the KO allele the primers used were 201 – 807 (1226 bp) where the 201 primer is complementary to a sequence in the TK/Neo cassette. (B) The PCR genotyping results for offspring of NEIL1 HET x HET crosses. For mice 2 and 3, only the 1108 bp bands (left lane) are obtained and it is thus a WT, whilst for mouse 4 only the 1226 bp band (right lane) is obtained identifying it as a KO mouse. However, mouse 1 shows both bands and is therefore a HET.
A Western blot was then used to show the presence or absence of NEIL1 protein in samples taken from NEIL1 WT and KO mice. A band indicating a protein ~44 KDa (NEIL1 weighs 43.7 KDa) was detected in the samples taken from male and female NEIL1 WT animals. In the samples taken from NEIL1 KO animals no band was observed (Figure 3.3).

(A) RT-PCR of RNA extracted from liver of NEIL1 WT and KO mice. PCR was performed using primers 328 – 545 (exon 1-2, 217 bp) for NEIL1 and primers 534-765 (exon 4-5, 222 bp) for GAPDH (picture courtesy of Dumax-Vorzet). The absence of a band in the NEIL1 lane of the NEIL1 KO indicates that NEIL1 RNA is not being produced in the liver of these mice. (Bii) 10 µg of liver protein from male and female Wt and Neil1<sup>+/−</sup> were separated on a 12% SDS-PAGE gel. (Bi) The proteins were transferred onto nitrocellulose membrane and the membrane probed with Neil1<sup>+/−</sup> anti-serum. A band corresponding to NEIL1 (43.7 KDa) is evident in the WT lane of males and females only, indicating that the NEIL1 protein is not produced in the knockout animal.
3.2.1.3. Viability and Mortality of NEIL1 mice.

The number of pups produced from NEIL1 HET X HET crosses were produced in a ratio of 31:47:20 (WT:HET:KO). This was not significantly different from the expected ratio of 1:2:1 ($p=0.27$ by Chi-squared test; Figure 3.4).

![Bar chart showing genotype distribution](image)

**Figure 3.4: The distribution of genotypes from HET x HET breeding pairs of NEIL1 Transgenic Mice.**

There was no difference between the genotype distribution (1:1.5:0.6) and the expected result (1:2:1; $p=0.27$).

To further examine the viability of the mice a comparison of the litter sizes from WT X WT, HET X HET and KO X KO pairs was performed. No significant difference in litter size was observed ($p=0.18$). Neither were there any significant differences in the number of males or female mice produced per litter ($p=0.28$ and 0.54 respectively; Figure 3.5).

To compare mortality rates HET X HET litters were separated into males and females and fed *ad libitum*, dates of death were recorded and used to create the Kaplan-Meier survival curves. Of the mice only 5 males died during the course of the experiment (2 WT, 1 HET, 2 KO), and 3 females (2 WT, 0 HET, 1 KO), and all deceased mice were found dead in their cages. No significant
difference in mortality was observed between WT, HET and KO mice in either male ($p=0.92$) or female ($p=0.20$) animals (Figure 3.6).

![Figure 3.5: Average litter composition from NEIL1 colony](image)

(A) Combined litter sizes, (B) amount of females per litter, (C) amount of males per litter. Litter size and composition did not differ significantly with genotype ($n=16$ (WT), 10 (HET), 13 (KO)).

### 3.2.1.4. NEIL1 Mouse Weights

Mice from HET X HET crosses were housed by sex in their original litters and fed *ad libitum*. Their weights were measured every month for twelve months. There was no evidence that the weight of the animals varied by genotype at any time point (Figure 3.7).

After twelve months the animals were sacrificed, measured from scalp to tail and organs collected and weighed. When examined several mice had
symptoms of disease. A single male NEIL1\(^{+/+}\) mouse had a splenic growth and a NEIL1\(^{-/-}\) male mouse had a cyst on its gut, that was not connected to any abnormal growth. One NEIL1\(^{-/-}\) female mouse had cataracts over each eye, one female NEIL1\(^{+/+}\) mouse had a large cyst connected to a tumour on its stomach, and a NEIL1\(^{+/+}\) mouse had many smaller cancers along the length of its intestines.

(A) Male

(B) Female

**Figure 3.6: Mortality rates of (A) Male and (B) Female Transgenic Mice**

Survival curves for NEIL1 mice over the course of 370 days, Log-Rank analysis indicates no significant difference in the mortality rate of male \((p=0.92; n=53)\) or female \((p=0.20; n=43)\) WT, HET and KO mice.
Figure 3.7: NEIL1 transgenic male and female mouse weights over 12 months
Mean values and standard error of mean for 54 mice split between (A) male (n=30) and (B) female mice (n=48); two way ANOVA indicates no significant difference at any time point (Male p>0.06, Female p>0.35)

The length, BMI, and organ weights of the mice are shown in Figures 3.8-10. No significant differences were observed due to genotype in BMI or scalp to tail lengths (Figure 3.8).

In male mice, a significant difference was observed in the total weight of the sex organs between NEIL1\(^{+/-}\) and NEIL1\(^{+/+}\) mice (KO 209.6 ±13.7 mg; HET 244.9 ±7.9 mg; p=0.03). This was also shown when adjusted as a proportion of total bodyweight (KO 0.5 ±0.0 %; HET 0.6 ±0.0 %; p=0.06). In female
mice, evidence of a significant difference in the weight of the sex organs was observed between HET and KO when adjusted for length (KO 5.8 ±0.9 mg/mm; HET 4.4 ±0.4 mg/mm; \( p=0.08 \)).

![Graphs showing scalp to tail length and BMI for WT, HET, and KO mice](image)

**Figure 3.8: Length and BMI of NEIL1 Transgenic Mice**

Mice were sacrificed at 12 months and weight and length measured (male n=19 WT, 22 HET, 7 KO; female n=9 WT, 19 HET, 9 KO). A – B show mean weights and standard error and C – D shows mean organ size (± SEM) as a percentage of bodyweight. No significant difference due to genotype was detected in scalp to tail measurement (male \( p=0.51 \); female \( p=0.07 \)) or BMI (male \( p=0.27 \); female \( p=0.41 \)).

There was a significant difference in male mouse omentum weight with KO mice (1124.3 ±102.7 mg) having significantly less omentum fat than WT mice (1537.5 ±56.6 mg; \( p=0.03 \)). This was also the case when considering the spleen as a percentage of total bodyweight (KO 2.7 ±0.2 %; WT 3.4 ±0.1 %; \( p=0.02 \)) and the scalp to tail length of the animal (KO 20.5 ±1.6 mg/mm; WT 17.2 ±0.6 mg/mm; \( p=0.02 \); Figure 3.9).
Mice were sacrificed at 12 months and their organs removed and weighed (male n=19 WT, 22 HET, 7 KO). Panel A shows total tissue weights by genotype, panel B tissue weight as a percentage of body weight by genotype and panel C organ weight to length by genotype. There was evidence of a difference between WT and KO in the weight of adrenal glands and kidneys \((p=0.07)\). There was a significant difference between HET and KO in the weights of the mouse sex organs \((p=0.03)\) and evidence of a difference when seen as a percentage of bodyweight \((p=0.06)\). There was a significant difference between WT and KO for omentum fat in each case \((p<0.03)\).
Figure 3.10: Female NEIL1 transgenic mouse organ weight comparisons

Mice were sacrificed at 12 months and their organs removed and weighed (female n=9 WT, 19 HET, 9 KO). Panel A shows total tissue weights by genotype, panel B tissue weight as a percentage of body weight by genotype and panel C organ weight to length by genotype. In each case there were significant differences between HET and KO for adrenal glands (p<0.03). There was also evidence of a difference in spleen weights between WT and KO in each case (p<0.09). Evidence of a significant difference was observed between HET and KO in the weight of the sex organs when considering length (p=0.08).
In female mice the adrenal glands were significantly heavier in NEIL1<sup>+/−</sup> mice (27.8 ±7.2 mg) than in NEIL1<sup>+/+</sup> mice (17.3 ±1.4 mg; \(p=0.03\)), this was born out when considering the weight as a proportion of body weight (KO 0.8 ±0.2 %; HET 0.5 ±0.1 %; \(p=0.01\)) and when compared to length (KO 0.3 ±0.1 mg/mm; HET 0.2 ±0.0 mg/mm; \(p=0.02\)). There was also evidence of a difference in male adrenal gland weight, where the NEIL1<sup>+/−</sup> mice (27.8 ±7.2 mg) were heavier than the NEIL1<sup>+/+</sup> mice (17.3 ±1.4 mg; \(p=0.03\)).

In female mice there was evidence that the spleens in NEIL1 KO (139.3 ±14.4 mg) mice were significantly lighter than the NEIL1<sup>+/+</sup> mice (230.4 ±43.7 mg; \(p=0.08\)). This was also the case when considering the spleen as a percentage of total bodyweight (KO 0.4 ±0.0 %; WT 0.7 ±0.2 %; \(p=0.08\)) and the scalp to tail length of the animal (KO 1.7 ±0.2 mg/mm; WT 2.8 ±0.6 mg/mm; \(p=0.09\); Figure 3.10).

### 3.2.2. NEIL2 Mouse Colony

#### 3.2.2.1. Genotyping

The NEIL2 mouse colony was bred from NEIL2<sup>+/−</sup> chimeras created from constructed ES cells prepared by Dr. Rhod Elder (Perez-Campo et al., 2007). Animals in this colony were genotyped by PCR (Figure 3.11) and so identified as either wildtype (NEIL2<sup>+/+</sup>, WT), heterozygous (NEIL2<sup>+/−</sup>, HET) and knockout (NEIL2<sup>−/−</sup>, KO) mice. The appropriate mice identified from these PCRs were then be used in the selective breeding of the NEIL2<sup>−/−</sup> line and backcrossing to a C57/B16J background.
Figure 3.11: The NEIL2 Knockout Construct and Genotyping Results.

(A) Diagram of NEIL2 construct. To test for the WT gene, the primers 410 – 963 were used, giving rise to a band of 553 bp. Primer 410 was designed to anneal to the region to be deleted in the NEIL2 gene. For the KO allele the primers used were -871 – 201 (1.3 kb) where the 201 primer is complementary to a sequence in the TK/Neo cassette. (B) PCR genotyping results for offspring of NEIL2 HET x HET crosses. For mouse 3, only the 553 bp band (left lane) is obtained and it is thus a WT, while for mouse 1 only the 1.2 kb band (right lane) is obtained and is thus a KO mice. However, mouse 2 shows both bands and is therefore a HET.

3.2.2.2. Western and RT-PCR

The confirmation of genotype was performed by a colleague (Saad, W M), the results of which are given in brief below.

In order to confirm the disruption of the NEIL2 gene at the transcription level RT-PCR was performed on cDNA produced from mRNA obtained from liver, testes and skeletal muscle tissue excised from NEIL2−/− (KO) and NEIL2+/+ (wildtype) mice (Figure 3.12A). The PCR primers were designed to amplify a section of DNA ~600bp in length. This amplicon was observed in all the PCRs using animals identified as NEIL2+/+ and NEIL2−/− by genotyping when compared to a positive contro, although there were variances in the strength of the band produced. Specifically the bands for WT skeletal muscle and KO
liver were much lighter than others produced from the same amount of source cDNA. This indicated that NEIL2 mRNA was still being produced in the putative NEIL2<sup>−/−</sup> mouse.

(A) RT-PCR

![RT-PCR image](image_url)

(Bi)

![Western blot image](image_url)

**(Figure 3.12: Confirmation of a NEIL2 Null Phenotype)**

(A) RT-PCR of RNA extracted from NEIL2 WT and putative KO mice. PCR was performed on tissues taken from liver, testes and skeletal muscle. There is a band present in each lane at ~600 bp indicating the presence of cDNA corresponding to NEIL2 mRNA. (Bi) 10µg of protein extracts from WT and Neil2<sup>−/−</sup> livers (male) and protein control (+ve) were separated on a 12% SDS-PAGE gel. (Bii) The protein was transfer onto a nitrocellulose membrane and the membrane were probed with Neil2<sup>−/−</sup> primary antibody, a band corresponding to NEIL1 (36.9 kDa) is evident in the WT, KO and positive control lanes, indicating that the NEIL1 protein is produced in the putative knockout animal (data courtesy of Saad W M).

A Western blot was used to identify the absence of the NEIL2 protein in samples taken from NEIL2 WT and KO mice. A band indicating a protein ~37 KDa (NEIL2 weighs 36.9 KDa) was shown in the samples taken from NEIL2<sup>+/+</sup> and NEIL2<sup>−/−</sup> mice as well as the positive control. This indicated that the NEIL2 protein was still being produced in its undisrupted form in the putative NEIL2<sup>−/−</sup> mice (Figure 3.12).
3.2.3. OGG1 Mouse Colony

3.2.3.1. OGG1 Mouse Genotyping

OGG1<sup>−/−</sup> embryos were removed from storage in liquid nitrogen and thawed and implanted into a female mouse to be brought to term. These animals were then bred with WT (C57/B16J) mice to produce OGG1<sup>+/−</sup> mice. Animals from these litters were interbred to produce wildtype (OGG1<sup>+/+</sup>, WT), heterozygous (OGG1<sup>+/−</sup>, HET) and knockout (OGG1<sup>−/−</sup>, KO) mice. Animals in this colony were genotyped by PCR (Figure 3.17) and so identified as WT, HET and KO mice. The appropriate mice identified from these PCRs were then used in the selective breeding of OGG1<sup>+/+</sup> and OGG1<sup>−/−</sup> for experimentation.

![Diagram of the OGG1 Knockout Construct and Genotyping Results](image)

**Figure 3.13: Diagram of the OGG1 Knockout Construct and Genotyping Results**

(A) Diagram of OGG1 knockout construct. To test for the WT gene, the primers 2327 – 663 were used, giving rise to a band of 323 bp. Primer 1907 was designed to anneal to the deleted region in the NEIL1 gene. For the KO allele the primers used were NEO – 2327 (~1000 bp) where the NEO primer is complementary to a sequence in the TK/Neo cassette. (B) PCR genotyping results for offspring of NEIL1 HET x HET crosses. For animal 2, only the 323 bp band is obtained and it is thus a WT, whilst for animal 3 only the 1000 bp band is obtained identifying it as a KO mouse. However, animal 1 shows both bands and is therefore a HET.
3.3. Discussion

In order to identify phenotypes displayed by DNA glycosylase deficient mice, a new strain of NEIL1\(^{-/-}\) mice has been created. PCR, RT-PCR and western blot procedures indicate that disruption of the NEIL1 gene has proved a success. The PCR test to identify changes in genomic mouse DNA of the mice has proved the existence of both the WT gene and the correctly placed KO cassette within the colony (Figure 3.1). RT-PCR has also indicated the lack of NEIL1 mRNA production within the tested animals (Figure 3.3).

Furthermore, the western blotting indicates that the NEIL1 protein, whilst present in NEIL1\(^{+/+}\) animals, is not detected in the tissues of the NEIL1\(^{-/-}\) mice. The band produced from samples taken from WT animals is at the expected position on the blot to be identified as the NEIL1 protein (Figure 3.3; 43.7 kDa), but there is also a lighter band at ~28 kDa. When the NEIL1 protein was first reported the western blot identifying it had several bands one of which was around 25 kDa (Takao et al., 2002a).

When considering the mean weights of other C57BL/6 mice colonies in which the mouse weights rarely exceed 35 g (males) and 30 g (females) (Arai et al., 2006; Goodrick et al., 1990), compared to this the weights of the mice in this colony are very high, especially those of WT males who at 12 months have a mean weight of 47.7 g. So whilst this does not directly support the theory that the lack of the NEIL1 protein leads to the onset of metabolic syndrome, these animals are already very large, a factor that perhaps eclipses any differences that would be shown from the knockout of the NEIL1 gene.

The NEIL1\(^{-/-}\) mice reported here did not display a comparatively obese phenotype when compared to their WT siblings, nor was the extensive abdominal fat described by Vartanian et al., (2006) found in the NEIL1\(^{-/-}\) mice. Indeed, the omentum fat content of the NEIL1\(^{-/-}\) was significantly lighter than that of the NEIL1\(^{+/+}\) mice. Previous observations of NEIL1\(^{-/-}\) mice have also described enlarged livers and kidneys due to fatty deposits within them.
This was not observed in the newly developed NEIL1−/− mice. Indeed there was a suggestion that the kidneys of the male NEIL1−/− mice were lighter than those of the NEIL1+/− mice although this was found to be non-significant after adjustment for weight and length.

Significant differences were observed between weights of reproductive tissues and adrenal glands. Changes in the weights of reproductive tissues are often synonymous with changes in the output of the hormones associated with them, (eg. testosterone in males) or a sign of greater hormone output from other sources (eg. FSH in females; (Hewitt and Korach 2003; Jin et al., 2008)). The increase in the size of the adrenal glands is suggestive of an increase in adrenalin production. All of these hormones affect behaviour, and in the recent paper by Sampath et al. (2011) small changes in behaviour were noted to the mouse circadian rhythms. The mice seemed to be active for more of the light dark cycle, but they did not engage in much exercise during this time, preferring to eat.

One reason for the apparent differences in the two NEIL1−/− strains could be the genes foreign to C57BL/6 mice flanking the NEIL1 gene. This problem stems from the fact that the ES cells that are used to create the chimera for the knockout are usually from a different strain of mice (Tc-1) to the background that the KO gene will be backcrossed onto (C57BL/6). Thus, not only the disrupted gene will be transmitted to their progenitors but any genes that are on the same allele. When comparing the knockout animals created with this method with wild-type animals, most of these transmitted genes will not pose a problem, due to their independence from the knockout gene (Wolfer et al., 2002).

However, for any genes linked to the targeted gene a “linkage disequilibrium” will be created, because the null mutation will be flanked by two foreign genes, and the wild-type locus flanked by two C57BL/6 genes. This could cause an effect that would be confused for an overt phenotype. This can be
overcome by using C57BL/6 to create the ES cells, or by breeding an extensively backcrossed knockout (10 generations) with the strain the ES cells were created from and comparing the phenotype of the resulting mice to that of those being investigated (Wolfer et al., 2002). The ES cells for our NEIL1\textsuperscript{-/-} mice were taken from TC-1 mice, and those from the previously reported NEIL1\textsuperscript{-/-} colony used ES cells from CJ7 mice which could account for the difference in phenotype.

![Diagram](A) X (B) → (C)

**Figure 3.14: The creation of transgenic mice can result in linkage disequilibrium**
The cloned disrupted C57BL/6 gene of interest is inserted into the ES cell DNA (A), and the resulting ES cells are combined with blastocysts to create chimeras containing the disrupted gene. The resulting chimera is bred with a C57BL/6 mouse (B) and through this and successive backcrosses the allele containing the disrupted gene is integrated into the C57BL/6 DNA (C). However, the flanking genes on the Tc-1 allele will always be associated with the knockout.

The success of the production of the NEIL1 knockout model was tempered by the conflicting results in the NEIL2 genotyping, RT-PCR and western blot. Indeed, both the NEIL1 and NEIL2 strains were created by the same method outlined by Perez-Campo et al., (2007) and as one of the strains has achieved the knockout of its target gene it is unlikely to be a flaw in the technique. As the gene was inherited normally (i.e. the ratio of WT:HET:KO mice was within expected amounts, data not shown) it indicates that at whatever point the Neo/Tk insert has been placed in the genome it has not caused reduced viability in the “knockout” mice, which is further supported by the lack of
significant difference observed in the mortality of NEIL2+/+, NEIL2+-/- or the putative NEIL2-/- mice.

Several reasons for the failure of the knockout can be postulated: (i) the Tk/Neo insert is located in the wrong position, (ii) there could be a duplicate NEIL2 gene, (iii) conserved portions of the NEIL2 gene are being spliced from another region of the genomic DNA. None of these explain fully why PCR genotyping gives the expected sized bands for the primers used and why apparently KO, HET and WT NEIL2 animals are created. In order to determine the actual positioning of the Tk/Neo insert the technique fluorescent in situ hybridization could be used which would at least confirm that the insert was on the correct chromosome. A further assay that could be performed would be an activity assay on purified “NEIL2” protein obtained from putative NEIL2-/- mice. This would confirm that the protein identified on the western blot was a functional NEIL2, rather than a similar sized, and shaped protein that the antibody recognized.

The lack of a strong observable phenotype in the NEIL1 knockout animals is hardly surprising, as in most cases the deletion of a DNA glycosylase results in very little overt phenotypic change (Table 3.1). This is theorized to be due to the overlapping functions of the DNA glycosylases meaning that they act as functional backups for each other (Parsons and Elder 2003). These backups mean that BER is available to the cell even if there are dysfunctional DNA glycosylases, as knockouts of more downstream BER proteins which cannot be functionally backed up, such as APE1, Pol β, DNA ligase I & III and XRCC1 are lethal to the cell (Larsen et al., 2007). There is one exception to this trend, TDG, which has been found to be embryonic lethal when disrupted in the mouse genome. This is suggested to be due to TDG’s activity in maintaining chromatin throughout cell development, and that the knockout of this gene results in the loss of epigenetic control (Cortazar et al., 2011). In most cases it takes a double knockout of two DNA glycosylases which excise similar lesions to cause a major response such as that of [OGG1/MUTYH]-/- which causes the
50% survival age to drop to 10.3 months, when compared to \( \text{OGG1}^{+/+} \), \( \text{MUTYH}^{+/+} \) and WT animals whose 50% survival age was never below 18 m. Increased tumor incidence in the \( \text{[OGG1/MUTYH]}^{+/+} \) mice from two months of age was suggested to be due to the build up in mutations in the codon 12 region of the K-Ras oncogene (Xie et al., 2004).

This implies the question which two DNA repair genes would be best to knock out in a mouse model. Future work could be best served by considering the properties of NEIL1, in order to test the hypothesis that it is damage within the mitochondria that causes the symptoms of metabolic syndrome, another DNA glycosylase that operates in the mitochondria, such as OGG1 in which one strain of knockout mice has also shown increased weight of KO mice (Arai et al., 2006), could be knocked out. When a working NEIL2 model is presented it would be good to create a \( \text{[NEIL1/NEIL2]}^{+/+} \) to study the effects of the loss of single strand BER within the cell.
Table 3.1: Summary of Phenotypes of DNA Glycosylase Deficient Mice.

<table>
<thead>
<tr>
<th>DNA Glycosylase</th>
<th>Type</th>
<th>Gross Phenotype</th>
<th>Molecular Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTH1</td>
<td>Monofunctional</td>
<td>Increased levels of lung, liver and stomach cancers (16.1% vs. WT 4.4%)</td>
<td>Non-significant increase in 8-OxoG</td>
<td>(Sakumi et al., 2003; Tsuzuki et al., 2001)</td>
</tr>
<tr>
<td>MPG</td>
<td>Monofunctional</td>
<td>No overt phenotype at 24 months</td>
<td>Increased alkylation sensitivity</td>
<td>(Elder et al., 1998; Engelward et al., 1997)</td>
</tr>
<tr>
<td>UNG</td>
<td>Monofunctional</td>
<td>After 24 months increased disposition to B-cell lymphoma (28% vs. 1.3%)</td>
<td>12 months 1.5 fold increase in C:G → T:A transitions</td>
<td>(Nilsen et al., 2003; Nilsen et al., 2000)</td>
</tr>
<tr>
<td>MUTYH</td>
<td>Monofunctional</td>
<td>After 18 months increased lung, liver and stomach cancers (59.5 % vs. 34.9%)</td>
<td>Increased 8-oxo-dGTP in the nucleotide pool</td>
<td>(Tsuzuki et al., 2001)</td>
</tr>
<tr>
<td>SMUG1</td>
<td>Monofunctional</td>
<td>No overt phenotype</td>
<td>Increased mutation rate in ES cells</td>
<td>(Hirano et al., 2003)</td>
</tr>
<tr>
<td>TDG</td>
<td>Monofunctional</td>
<td>Embryonic lethal</td>
<td>Lethal</td>
<td>(Cortazar et al., 2011)</td>
</tr>
<tr>
<td>MBD4</td>
<td>Monofunctional</td>
<td>No overt phenotype</td>
<td>Increased Mutation frequency</td>
<td>(Millar et al., 2002)</td>
</tr>
<tr>
<td>Ogg1</td>
<td>Bifunctional/monofunctional</td>
<td>Greater incidence of lung cancers after 19 months (48% vs. 11.1%)</td>
<td>Increased levels of 8-OxoG in genomic and mtDNA</td>
<td>(Klungland et al., 1999)</td>
</tr>
<tr>
<td>OGG1/MTH1</td>
<td>Bifunctional</td>
<td>Reduced tumorigenesis compared to OGG1&lt;sup&gt;−/−&lt;/sup&gt; mice (0% vs. 14.3%)</td>
<td>Increased 8-OxoG in genomic DNA</td>
<td>(Sakumi et al., 2003)</td>
</tr>
<tr>
<td>OGG1/MUTYH</td>
<td>Bifunctional/monofunctional</td>
<td>Increased tumour incidence after 2 months (31.4% vs. 4.3%)</td>
<td>No significant difference in repair efficy noted</td>
<td>(Xie et al., 2004)</td>
</tr>
<tr>
<td>NTH1</td>
<td>Bifunctional</td>
<td>No overt phenotype</td>
<td>No significant difference in repair efficy noted</td>
<td>(Elder and Dianov 2002; Ocampo et al., 2002; Takao et al., 2002b)</td>
</tr>
<tr>
<td>OGG1/NTH1</td>
<td>Bifunctional/bifunctional</td>
<td>No overt phenotype</td>
<td>No significant differences noted</td>
<td>(Elder et al., 2004)</td>
</tr>
<tr>
<td>NEIL1</td>
<td>Bifunctional</td>
<td>Sporadic symptoms of human metabolic syndrome</td>
<td>Increased mtDNA Damage and Deletions</td>
<td>(Vartanian et al., 2006)</td>
</tr>
<tr>
<td>NEIL1/NTH1</td>
<td>Bifunctional/bifunctional</td>
<td>Increase in tumours, especially lung (74%) and liver (43%) after 24 months</td>
<td>Similar DNA damage patterns as NEIL1</td>
<td>(Chan et al., 2009)</td>
</tr>
<tr>
<td>NEIL2</td>
<td>Bifunctional</td>
<td>No known knockout available</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>NEIL3</td>
<td>Bifunctional</td>
<td>No known knockout available</td>
<td>n/a</td>
<td></td>
</tr>
</tbody>
</table>
4. Cytokine Output of DNA Glycosylase Disrupted Cells

4.1. Introduction

The creation of a new strain of NEIL1^{-/-} mice has been described and no overt phenotype was found in unchallenged animals (Chapter 3). Other mouse knockout models of the bifunctional DNA glycosylases OGG1 and NTH1 have already been created and no observable overt phenotype was also observed in these animals (Elder and Dianov 2002; Klungland et al., 1999). Neither was there any overt phenotype described in the double knockout of [OGG1/NTH1] (Parsons and Elder 2003).

Several proteins linked to BER have been identified as having links with the inflammatory response including PARP-1, APE-1, OGG1 and MUTYH. PARP-1 and APE-1 knockouts reduce the inflammatory response via an attenuation of the redox sensitive transcription factor NF-κB transcription factor (Ando et al., 2008; Virag and Szabo 2002). There is also a reduction in inflammation noted in the OGG1^{-/-} models of LPS induced inflammation and Helicobacter Pylori induced gut inflammation (Mabley et al., 2005b; Touati et al., 2006).

Previously MEF’s have been used in the study of the DNA repair/damage response of KO mice, although no in vitro studies have been carried out to identify any alterations in MEF response to LPS in any DNA glycosylase deficient models. (Elder and Dianov 2002; Le Page F. et al., 2000). Additionally fibroblasts are a good model when studying the inflammatory response, as they participate in the inflammatory and wound healing processes by producing various cytokines and immune mediators in response to LPS and other antigens (Ivor, 1994). The inflammatory response to LPS is initiated through the TLR4 receptor, the cellular response includes the release of various signalling molecules including IL-1, IL-6, IL-8, IL-10, MCP-1, MIP-
1α, TNF-α and NO (Kurt-Jones et al., 2004; Shirota et al., 2006; Tominaga et al., 1997).

### 4.1.1. Aims

To identify if the disruption of BER enzymes (specifically the bifunctional DNA glycosylases OGG1, NTH1 and NEIL1), affects the release of inflammatory cytokines and chemokines from MEF's by measuring the levels of IL-6, IL-10 and MIP-1α.

### 4.2. Results

#### 4.2.1. TLR4 mRNA Transcription Analysis

To confirm that the MEFs would be responsive to LPS challenge via the TLR4/MyD88 pathway, total mRNA was extracted and an RT-PCR reaction was performed to test for TLR4 gene expression, using the primers designed by Kurt-Jones et al. (2004) both of which are found in exon 2 and give rise to a band of 540 bp (Figure 4.1). This band is present in each cell line indicating the presence of TLR4 transcription.

![Figure 4.1: RT-PCR for TLR4](image)

The 540 bp fragment indicating TLR4 transcription was identified in WT, NTH1+/-, OGG1+/-, NTH/OGG1+/- and NEIL1+/- MEFs.
4.2.2. Cytokine Output from LPS Challenged MEF Cells

Figures 4.2 – 4.4 show the concentration of the cytokines IL-6, IL-10 and MIP-1α in the supernatant taken from MEFs cultured with and without LPS. WT (324.2 ± 31.7 pg/ml) cells have the greatest basal IL-6 output (Figure 4.2A). NTH1⁻/⁻ and NEIL1⁻/⁻ MEFs showed significantly lower levels of IL-6 within the supernatant, with NEIL1⁻/⁻ having the lowest level (71.6 ± 16.5 pg/ml, p=0.01), 22% that of WT MEFs, followed by NTH1⁻/⁻ (194.3 ± 34.4 pg/ml, p=0.03). The supernatant of OGG1⁻/⁻ MEFs (232.6 ± 59.5 pg/ml) and \([\text{OGG1}/\text{NTH1}]⁻/⁻\) MEFs (257.4 ± 64.9 pg/ml) had lower IL-6 content but this was not significantly different to that of the WT cells (p=0.12 and p=0.25 respectively). There were no significant differences in treated IL-6 levels between WT and KO MEFs (Figure 4.2B; p>0.29), although all but one result is between 43 - 53% (NEIL1⁻/⁻ 1407.9 ± 488.4 pg/ml - OGG1⁻/⁻ 1726.3 ± 827.8 pg/ml) of the WT (3267.6 ± 606.7 pg/ml). Levels using \([\text{OGG1}/\text{NTH1}]⁻/⁻\) MEFs were higher (4563.3 ± 1681.9 pg/ml) and were 139% of the WT result. The increase in IL-6 levels following LPS treatment was approximately 10 fold using WT MEFs (10.1 fold), slightly lower using OGG1⁻/⁻ (7.4 fold) and NTH1⁻/⁻ MEFs (8.5 fold), and was much greater using \([\text{OGG1}/\text{NTH1}]⁻/⁻\) (17.7 fold) and NEIL1⁻/⁻ (19.7 fold) cell lines (Table 4.1).

Basal IL-10 levels differed significantly from WT (131.7 ± 22.1 pg/ml) only for OGG1⁻/⁻ MEFs (203.7 ± 44.8 pg/ml; Figure 4.3A; p<0.01). For the other cell lines, the mean IL-10 concentration was 65 - 93% of the wildtype concentration \(([[\text{OGG1}/\text{NTH1}]]⁻/⁻, 85.3 ± 16.0 \text{ mg/ml} - \text{NTH1}⁻/⁻, 123.0 ± 21.6 \text{ pg/ml respectively} ; p>0.30). IL-10 levels after LPS treatment were similar in the knockout cell lines when compared to the WT cells. OGG1⁻/⁻ MEFs produced more IL-10 than WT cells and each of the other cell lines was 60 - 87% lower. The increases in IL-10 after LPS treatment can be split between two general categories (Table 4.2), WT (10.1 fold), OGG1⁻/⁻ and NEIL1⁻/⁻ (7.1
and 7.2 fold respectively), and NTH1/− (4.8 fold) and [OGG1/NTH1]/− (4.4 fold).

Basal MIP-1α levels in knockout MEFSs were significantly lower than those in the spermatant of WT MEFs (262.95 ± 21.7 pg/ml; Figure 4.4A). Of the KO MEFs, the cell line with the highest output was [OGG1/NTH1]/− (77.1 ± 21.1 pg/ml), followed by NTH1/− (53.7 ± 15.3 pg/ml), NEIL1/− (29.9 ± 6.3 pg/ml) and OGG1/− cells (25.3 ± 5.4 pg/ml; p<0.02). After LPS treatment, MIP-1α levels in the supernatant of KO MEFs were significantly different from WT. NTH1/− MEFs (1651.1 ± 346.8 pg/ml) had the highest concentration of MIP-1α followed by [OGG1/NTH1]/− (1455.7 ± 466.4 pg/ml), OGG1/− (1223.4 ± 561.7 pg/ml) and NEIL1/− cells (360.5 ± 180.9 pg/ml; p<0.03; Figure 4.4B). In WT cells, the increase from basal rate to treated level of cytokine was 12.9 fold (Table 4.3) but all of the other increases, except for that of NEIL1, was much higher, the greatest of which was produced by OGG1/− MEFs (48.5 fold), followed by NTH1/− (18.9 fold), [OGG1/NTH1]/− (18.9 fold) and NEIL1/− (12.1 fold).
Figure 4.2: IL-6 output of wildtype and knockout MEF cell lines
Cells were treated with 0.1mg/ml LPS and incubated for 18 h, and the supernatant was then assayed for IL-6 by ELISA. Panels A-B show levels of IL-6 released by the different cell lines tested (A) without LPS treatment and (B) with LPS treatment (+/− SEM, N=5). Significant results are noted for NEIL1+/− (p<0.01) and NTH1+/− (p=0.03) basal results when compared to wildtype.

Table 4.1: Comparison of IL-6 results between untreated and treated DNA glycosylase deficient MEF cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Untreated (pg/ml)</th>
<th>Treated (pg/ml)</th>
<th>Mean increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>324.1 ± 31.7</td>
<td>3267.6 ± 606.8</td>
<td>10.1</td>
</tr>
<tr>
<td>OGG1+/−</td>
<td>232.6 ± 59.5</td>
<td>1726.3 ± 827.8</td>
<td>7.4</td>
</tr>
<tr>
<td>NTH1+/−</td>
<td>194.3 ± 34.1</td>
<td>1657.9 ± 969.2</td>
<td>8.5</td>
</tr>
<tr>
<td>[OGG1/NTH1]+/−</td>
<td>257.4 ± 64.9</td>
<td>1407.9 ± 488.4</td>
<td>17.7</td>
</tr>
<tr>
<td>NEIL1+/−</td>
<td>71.6 ± 16.5</td>
<td>1407.9 ± 488.4</td>
<td>19.7</td>
</tr>
</tbody>
</table>

*Mean +/- SEM, N=5.
Cells were incubated with 0.1mg/ml LPS for 18 h, and the supernatant was then assayed for IL-10 by ELISA. Panels A-B show levels of IL-10 released by the different cell lines tested (A) without LPS treatment and (B) with LPS treatment (+/- SEM, N=5). There was a significant difference in basal results between WT and OGG1⁻/⁻ (p<0.01).

Table 4.2: Comparison of IL-10 results between untreated and treated DNA glycosylase deficient MEF cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>IL-10 (pg/ml)</th>
<th>Mean Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>WT</td>
<td>906.7 ± 192.5</td>
<td>10.1</td>
</tr>
<tr>
<td>OGG1⁻/⁻</td>
<td>1319.0 ± 432.9</td>
<td>7.1</td>
</tr>
<tr>
<td>NTH1⁻/⁻</td>
<td>789.9 ± 315.7</td>
<td>4.8</td>
</tr>
<tr>
<td>[OGG1/NTH1]⁻/⁻</td>
<td>541.6 ± 239.0</td>
<td>4.4</td>
</tr>
<tr>
<td>NEIL1⁻/⁻</td>
<td>724.0 ± 374.8</td>
<td>7.2</td>
</tr>
</tbody>
</table>

\(^a\) Mean +/- SEM, N=5.
Figure 4.4: MIP-1α output of wildtype and knockout MEF cell lines

Cells were incubated with 0.1mg/ml LPS for 18 h, and the supernatant was then assayed for MIP-1α by ELISA. Panels A-B show levels of MIP-1α released by the different cell lines tested (A) without LPS treatment and (B) with LPS treatment (+/- SEM, N=5). Basal MIP-1α levels were reduced when compared to WT in each case (p<0.02). In treated levels MIP-1α was significantly lower in each case (p<0.03).

Table 4.3: Comparison of MIP-1α results between untreated and treated DNA glycosylase deficient MEF cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MIP-1α (pg/ml)</th>
<th>Mean Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>WT</td>
<td>263 ± 21.7</td>
<td>3400 ± 556.4</td>
</tr>
<tr>
<td>OGG1−/−</td>
<td>25.3 ± 5.4</td>
<td>1223.4 ± 561.7</td>
</tr>
<tr>
<td>NTH1−/−</td>
<td>53.7 ± 15.3#</td>
<td>1651.1 ± 346.8</td>
</tr>
<tr>
<td>OGG1/NTH1−/−</td>
<td>77.1 ± 21.1</td>
<td>1455.7 ± 466.4</td>
</tr>
<tr>
<td>NEIL1−/−</td>
<td>29.9 ± 6.3</td>
<td>360.5 ± 180.9</td>
</tr>
</tbody>
</table>

*aMean +/- SEM, N=5.
4.3. Discussion

Fibroblast cells are good indicators of level of inflammatory response, although they are only one cell type and as such are limited. Disruption of bifunctional DNA glycosylases reduces the basal and LPS treated output of MIP-1α from all KO MEFs when compared to WT MEFs. Additionally, OGG1−/− cells had higher basal IL-10 levels, NTH1−/− cells had lower basal IL-6 levels as did NEIL1−/− cells (Table 4.4).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Untreated</th>
<th>LPS</th>
<th>Untreated</th>
<th>LPS</th>
<th>Untreated</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>OGG1−/−</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>NTH1−/−</td>
<td>↓</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>[OGG1/NTH1]−/−</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>NEIL1−/−</td>
<td>↓</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

↑ = Significant increase compared to WT
↓ = Significant Decrease compared to WT

To explore possible causes for the IL-6 results it is best to identify possible connections between those MEFS which showed marked reductions in output of IL-6 namely NTH1−/− and NEIL1−/−. NEIL1 and NTH1 both remove oxidised cytosines and Tg from DNA, but in experiments to detect these oxidative compounds in liver tissue, there was no significant difference between either NTH1−/−, NEIL1−/− or [NTH1/NEIL1]−/− and the control mice (Chan et al., 2009). It was suggested that this might have been due to a complementary enzyme, namely NEIL2, removing the oxidised cytosine products. Additionally, as the NER system has been shown in vivo to remove Tg at a biologically significant rate, it may compensate for the lack of BER Tg removal (Reardon et al., 1997). Even with these backup repair systems in place [NTH1/NEIL1]−/− mice had a higher incidence of both lung and liver tumours, which was seen as evidence of a previously unknown base damage type that was not being excised due to the absence of these genes (Chan et al., 2009). It was also
surmised that this unidentified damage type would be highly damaging as it caused the initiation of cancers in the normally resistant liver tissues (Chan et al., 2009). If there is such an oxidised base it may be that its reduced excision from the DNA causes a reduction in the basal release of the proinflammatory cytokine IL-6. Both NTH1 and NEIL1 are capable of β-elimination in the BER process, although NEIL1 is also thought to be capable of APE1 independent β,δ-elimination (Elder and Dianov 2002; Takao et al., 2002b), but there are no protein intermediates that would be specific to these two proteins in the BER system to account for why only cells from these strains produced a reduced basal IL-6 level.

PCNA is a protein which binds to both NEIL1, increasing NEIL1 activity, and NTH1, the activity of which remains unchanged (Dou et al., 2008; Oyama et al., 2004). This protein links both NEIL1 and NTH1 to the S-phase as PCNA plays a role in DNA replication at this point (Kisielewska et al., 2005). Additionally, due to the positive correlation between PCNA concentration and severity of inflammation, PCNA has been used as a marker for inflammation in inflammatory bowel disease and several types of cancer including liver (Ding et al., 2005; Harrison et al., 1993). As basal IL-6 is also linked to embryonic development and organ regeneration (Chan et al., 2009; Lee, 1992), this could indicate that a disruption in the NTH1 and NEIL1 genes may have a deleterious effect at this stage, although our own and previous work with NTH1 and NEIL1 mice does not suggest one (Chapter 3) (Chan et al., 2009; Elder and Dianov 2002; Ocampo et al., 2002; Takao et al., 2002a; Vartanian et al., 2006).

When considering the output of IL-6 it is interesting that the double knockout of [OGG1/NTH1]−/− showed a greater fold increase (17.5) in output than both OGG1−/− (8.5) and NTH1−/− (7.4) cells (Table 4.1). It would be logical to conclude that as the OGG1−/− and NTH1−/− cells both displayed reductions in
IL-6 output compared to WT cells, that the double knockout of these proteins would show a similar, if not addative effect. However there is a precedent for double knockouts of DNA glycosylases leading to unexpected results. A strain of OGG1+/− mice was reported to exhibit increased carcinogenesis after 19 months; the double [OGG1/MTH1] knockout was expected to increase the mouse susceptibility to cancer. However, whilst the double knockout did increase the occurrence of oxidised base lesions significantly no cancers found in the animals (Sakumi et al., 2003). The reasoning for this unexpected result was suspected to be either (i) a build up of oxidised purines suppressing tumourgenesis by interfering with oncogenic processes or that (ii) a tumour suppressor gene was transferred from the ES cells on the same allele as the disrupted MTH1 gene, and was not removed through backcrossing ((Sakumi et al., 2003); See comments on linkage disequilibrium, section 3.5)).

As both single knockout cell types used in this study showed a decreased IL-6 response it would suggest that the unexpected result may be due to increased oxidised bases in the DNA. It has already been reported that an increase in oxidised lipids in an area can stimulate an immune response of increased IL-8 and MIP-1α (Berliner and Watson 2005). However, if a similar effect was in effect here it would most probably also result in a significant increase in MIP-1α output which is not shown here. It would still be interesting however, to identify if there was a change in immune response from cells with greater levels of DNA base oxidation.

Another potential reason for this unexpected result may be is due to the difference in passage number between the [OGG1/NTH1]+/+ stock that were thawed from stocks of immortalised cells and the WT, OGG1−/− and NEIL1−/− MEFs which were all created from primary cells; and the NTH1+/− which were thawed from stocks at passage 10. The immortalised cells may have
spontaneously mutated to increase IL-6 production, especially as they are from a model that is already prone to mutation due to the disruption of BER processes.

The increased output of basal IL-10 from the OGG1\(^{−/−}\) mice is interesting as IL-10 is most often seen as an anti-inflammatory cytokine, it is a sign of a concerted anti-inflammatory response from any of the cells rather than a blanket effect reduction seen in other cell lines such as the treated NTH1\(^{−/−}\) cells.

The reduction in MIP-1α outputs of both basal and treated DNA glycosylase knockout cells was reduced significantly in all cases. A reason for the seemingly reduced levels of MIP-1α could be that the WT cells that the DNA glycosylase knockout cells are compared to are producing an abnormally large amount of MIP-1α. However, other work with LPS as a ligand has shown similar levels of MIP-1α being produced from WT MEF’s (Kurt-Jones et al., 2004). A reduction in MIP-1α would indicate that less neutrophils would be recruited to the area of inflammation. This would cause a net reduction in the amount of ROS accumulated in the region and thus less MPO, MDA and more GSH to be observed indicating, less DNA damage occurring.

The IL-6 released from OGG1\(^{−/−}\), NTH1\(^{−/−}\) and NEIL1\(^{−/−}\) cells after stimulation also seemed less than that of WT cells, although not significantly. This could be explained by a suppression of NF-κ\(\beta\), but, this would result in a global suppression of cytokine production (Donadelli et al., 2000). The rise in IL-10 output in OGG1\(^{−/−}\) cells would suggest a controlled change in inflammatory response.

NEIL1\(^{−/−}\) cells had the lowest output of MIP-1α both at basal and LPS treated levels, indicating that the neutrophil response and thus ROS levels in the
inflammatory response would be lower than in other strains of animals. Whilst the effectiveness of MEF’s as a model of immune reactions has already been discussed (section 4.1), the fact remains that they are colonies of a single cell type and that as such only the accumulation of the measured cytokines can be known, there are no mechanisms in place for the dispersal and clearing of unwanted cytokines. *In vivo* experiments will be able to give more detail, on the cytokine response in the body at specific time points and give more details on how this response relates to neutrophil infiltration and oxidative damage.
5. Effects of NEIL1 Gene Knockout on Endotoxin Induced Inflammation

5.1. Introduction

The first protein linking BER to the inflammatory response was PARP-1 in a model of septic shock. Septic shock was induced via caecal ligation and puncture, which resulted in the extrusion of gut contents into the peritoneal cavity inducing a multi-bacterial response (Soriano et al., 2002; Virag and Szabo 2002). In PARP1/− mice there was a reduction in the plasma concentration of TNF-α, IL-6 and IL-10 cytokines when compared to that of PARP-1+/+ mice. There was also a reduction in MPO activity and MDA levels in PARP-1/− mice indicating that there was a protective effect against the deleterious effects of inflammation in the PARP1 knockout (Soriano et al., 2002). OGG1/− mice have also shown resistance to the deleterious effects of inflammation induced by LPS and Helicobacter Pylori, with animals displaying decreased mortality and reduced gut inflammation respectively (Mabley et al., 2005a; Touati et al., 2006).

NEIL1/− mice have been described as having symptoms of metabolic syndrome, a human disease which has been associated with raised serum levels of the inflammatory markers IL-6, TNFα and fibrinogen (Mabley et al., 2005a; Touati et al., 2006). However in later studies this phenotype has been shown to be inconsistent and it has been suggested that the absence of NEIL1 increases susceptibility to increased weight (metabolic syndrome; (Chan et al., 2009; Sampath et al., 2011)). A new NEIL1/− mouse strain has recently been developed (Chapter 3), and these animals will thus be used to identify if the NEIL1 protein modifies LPS induced inflammation in order to further study the links between LPS induced inflammation and the BER pathway.
5.1.1. Aims

To identify the extent to which NEIL1 determines the immune response to LPS by:

1) Establishing whether serum levels of IL-6, IL-12, IL-10 and IL-4 differ between NEIL1\(^{-/-}\) and NEIL1\(^{+/+}\) mice, at baseline or after LPS treatment.

2) Identifying whether any LPS induced changes in MPO activity, GSH levels and MDA levels differ between NEIL1\(^{-/-}\) and NEIL1\(^{+/+}\) mice.

3) Determining if the effects of oxidative damage caused by age are altered in NEIL1\(^{-/-}\) mice when compared to NEIL1\(^{+/+}\) mice.

5.2. Results

5.2.1. Cytokine Output in LPS Challenged NEIL1\(^{-/-}\) Mice

Figures 5.1 – 5.4 present interleukin concentrations present in the blood serum of mice injected with LPS (2 mg/kg \textit{i.p.}) after 0, 1, 6 and 24 h (mean \pm SEM; n=6). IL-4 concentrations were not significantly different at any time point when comparing male NEIL1\(^{+/+}\) and NEIL1\(^{-/-}\) mice (Figure 5.1A). There was, however, a significant difference between WT and KO female mice at 6 h; here the NEIL1\(^{+/+}\) mice had more IL-4 within the serum than NEIL1\(^{-/-}\) mice (216.9 \pm 37.0 pg/ml vs. 63.9 \pm 15.9 pg/ml; Figure 5.1B; \(p<0.01\)).

IL-6 concentrations were significantly different between male WT and KO mice at baseline (WT 340.8 \pm 12.0 pg/ml vs. KO 7.3 \pm 5.6 pg/ml; \(p<0.01\)), 1 h (WT 5383.5 \pm 378.2 pg/ml vs. KO 6690.0 \pm 492.7 pg/ml; \(p=0.04\)) and 24 h (WT 390.6 \pm 458.6 pg/ml vs. KO 29.0 \pm 17.7 pg/ml; \(p<0.01\)). At 0 and 24 h the WT concentration of IL-6 was higher than KO levels, whilst at 6h the KO levels were higher than WT levels (Figure 5.2A). There were significant
differences between concentrations of IL-6 in the serum of female WT and KO mice at 1 (WT 7143.1 ± 605.3 pg/ml vs. KO 5518.7 ± 504.0 pg/ml; \(p=0.05\)) and 24 h (WT 2684.6 ± 819.4 pg/ml vs. KO 72.4 ± 16.4 pg/ml; \(p<0.01\)). In both of these cases IL-6 levels were greater in WT serum than KO serum (Figure 5.2B)

(A) Male IL-4 levels

(B) Female IL-4 Levels

**Figure 5.1: IL-4 concentrations in blood serum taken from NEIL1 transgenic mice**

Mice were treated with LPS (2 mg/kg i.p.), sacrificed at the appropriate time and blood removed by cardiac puncture for analysis by ELISA (mean ± SEM; n=6). Panels A and B show serum IL-4 levels at 0, 1, 6 and 24 h in male and female mice respectively. (* - \(p<0.05\); ** - \(p<0.01\)). (A) No significant difference was observed between WT and KO ♂ mice (\(p>0.14\)). (B) There was a significant difference in IL-4 levels between ♀ WT and KO mice at 6 h (\(p<0.01\)).
(A) Male IL-6 Levels

![Graph showing IL-6 levels for male WT and KO mice.](image)

(B) Female IL-6 Levels

![Graph showing IL-6 levels for female WT and KO mice.](image)

**Figure 5.2: IL-6 concentrations in blood serum taken from NEIL1 transgenic mice**

Mice were treated with LPS (2 mg/kg i.p.), sacrificed at the appropriate time and blood removed by cardiac puncture for analysis by ELISA (mean ± SEM; n=6). Panels A and B show serum IL-6 levels at 0, 1, 6 and 24 h in male and female mice respectively. (* - \( p \leq 0.05; ** - \( p \leq 0.01)\). (A) A significant difference in IL-6 levels was observed between WT and KO ♂ mice at 0 (\( p < 0.01 \)), 1 h (\( p = 0.04 \)) and 24 h (\( p < 0.01 \)). (B) There was a significant difference in IL-6 levels between ♀ WT and KO mice at 1 (\( p = 0.05 \)) and 24 h (\( p < 0.01 \)).

IL-10 concentrations did not differ significantly between male WT and KO mice (\( p > 0.11 \)). There were significant differences between concentrations of IL-10 in the serum of female WT and KO mice at 6 h (WT 446.2 ± 185.4 pg/ml vs. KO 138.2 ± 57.7 pg/ml; \( p = 0.02 \)) and 24 h (WT 34.3 ± 9.3 pg/ml vs. KO 24.7 ± 1.6 pg/ml; \( p < 0.01 \)). In both of these cases IL-10 levels were greater in WT serum than KO serum (Figure 5.3B).
IL-10 levels were measured in blood serum taken from OGG1 transgenic mice. Mice were treated with LPS (2 mg/kg i.p.), sacrificed at the appropriate time, and blood removed by cardiac puncture for analysis by ELISA. Panels A and B show serum IL-10 levels at 0, 1, 6, and 24 h in male and female mice, respectively. Male mice did not show significant differences between WT and KO mice (p>0.11). Female mice showed a significant difference in IL-10 levels between WT and KO mice at 6 h (p=0.02) and 24 h (p<0.01).

IL-12 concentrations were significantly different between male WT and KO mice at 6 h (WT 23.8 ± 3.8 pg/ml vs. KO 36.0 ± 0.7 pg/ml; p<0.01). At this time point, the KO concentration of IL-12 was higher than WT levels (Figure 5.4A). There was no significant difference between NEIL1^+/+ and NEIL1^-/- in IL-12 levels at any time point in female mice (p>0.67).
Figure 5.4: IL-12 concentrations in blood serum taken from NEIL1 transgenic mice

Mice were treated with LPS (2 mg/kg i.p.), sacrificed at the appropriate time and blood removed by cardiac puncture for analysis by ELISA (mean ± SEM; n=6). Panels A and B show serum IL-12 levels at 0, 1, 6 and 24 h in male and female mice respectively. (* - \( p \leq 0.05 \); ** - \( p \leq 0.01 \)). (A) A significant difference was observed between WT and KO \( \text{♂} \) mice at 6 h (\( p < 0.01 \)). (B) There was no evidence of a significant difference in IL-12 levels between \( \text{♀} \) WT and KO mice (\( p > 0.67 \)).

5.2.2. Myeloperoxidase activity in LPS challenged NEIL1\(^{-/-}\) mice

The MPO activity in various tissues of male and female WT and NEIL1 KO mice following treatment with a vehicle or LPS is shown in Table 5.1. Figures 5.5 – 5.9 present MPO activity in the heart, lung, liver, kidney and ileum
respectively. Panel A of these figures shows the main effects of comparing treatment (vehicle vs. LPS), genotype (WT vs. NEIL1 KO) and sex (male vs. female). Panels B - D show the interactions of (B) treatment and genotype, (C) treatment and sex and (D) genotype and sex. Panel E shows the three way interaction between treatment, genotype and sex.

In the heart there were no three- or two-way interactions in MPO activity. There was an overall sex difference with the tissues from female mice (2.62 ±0.58 mU/mg protein) having much lower MPO activity than those from the males (12.06 ±1.16 mU/mg protein; Figure 5.5A; \( p=0.01 \)).

Similarly in the lung tissues there were no three- or two-way interactions. Treated animals (118.08 ±17.08 mU/mg protein) had greater MPO activity than those of the vehicle treated animals (70.84 ±11.09 mU/mg protein; Figure 5.6A; \( p=0.02 \)).

Whilst there was no three-way interaction observed in the liver tissues, there was a significant treatment x sex interaction. Both male and female mouse liver tissues have similar levels of MPO activity when challenged with LPS there were radically different levels of MPO activity at basal levels meaning that whilst activity increased in female mice (5.14 fold), levels actually decreased in male mice (0.87 fold; Figure 5.7C; \( p=0.05 \)). Evidence of a significant treatment x genotype interaction was observed showing that while treatment caused an increase in MPO activity, this increase was greater in NEIL1\(^{+/+}\) mice (1.9 fold) than NEIL1\(^{-/-}\) mice (1.5 fold; Figure 5.7B; \( p=0.08 \)).

There were no significant differences observed in kidney tissues (Figure 5.8). The three-way interaction in ileum tissues was significant. In vehicle treated female animals and vehicle treated and LPS treated male animals
Table 5.1: MPO activity in tissues of NEIL1 transgenic mice exposed to LPS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Vehicle</th>
<th>Treated</th>
<th>Treatment x Sex x Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male WT</td>
<td>Female WT</td>
<td>Male KO</td>
</tr>
<tr>
<td>Heart</td>
<td>10.28 (±0.80)</td>
<td>12.20 (±0.72)</td>
<td>1.31 (±0.41)</td>
</tr>
<tr>
<td>Lung</td>
<td>76.41 (±23.19)</td>
<td>130.70 (±24.68)</td>
<td>35.67 (±3.91)</td>
</tr>
<tr>
<td>Liver</td>
<td>17.00 (±2.23)</td>
<td>18.85 (±5.21)</td>
<td>1.76 (±0.40)</td>
</tr>
<tr>
<td>Kidney</td>
<td>16.18 (±4.84)</td>
<td>13.47 (±3.10)</td>
<td>8.49 (±2.94)</td>
</tr>
<tr>
<td>Ileum</td>
<td>40.17 (±22.39)</td>
<td>19.85 (±5.09)</td>
<td>26.71 (±3.54)</td>
</tr>
</tbody>
</table>

Mice were treated with LPS (20 mg/kg i.p.) or a vehicle (PBS), sacrificed after 12 hours and tissues removed for analysis. Results are shown as tissue MPO activity (mU/mg protein; mean ± SEM; n=6).
Figure 5.5: MPO activity in heart tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. MPO activity was significantly higher in ♀ animals (vs. ♂; Panel A; p<0.01). No other significant effects observed.
Figure 5.6: MPO activity levels in lung tissues of NEIL1 transgenic mice exposed to LPS
Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. MPO activity were significantly higher in vehicle treated animals (vs. treatment; Panel A; p<0.01) no other significant effects were observed.
Figure 5.7: MPO activity in liver tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg _i.p._), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. An treatment X sex interaction was observed (Panel C; _p_=0.05). MPO activity was significantly higher in ♀ animals (_vs._ ♂; Panel A; _p_<0.01). No other significant effects were observed.
Figure 5.8: MPO activity in kidney tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. No significant effects were observed.
Figure 5.9: MPO activity in ileum tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. A treatment X sex interaction was observed (Panel C); a significantly lower increase in MPO activity in LPS treated KO ♀’s (vs. treated WT ♀’s; *p=0.04). MPO activity was significantly higher in vehicle treated animals (vs. treatment; *p=0.03). No other significant differences were observed.
there was no significant difference in MPO activity due to genotype, however LPS treated female NEIL1+/+ mice (84.5 ± 21.8 mU/mg) had greater MPO activity than NEIL1−/− mice (39.0 ± 4.5 mU/mg; Figure 5.9E; *p*=0.02).

5.2.3. Malondialdehyde content in LPS challenged NEIL1−/− mice

The MDA levels in various tissues of male and female WT and NEIL1 KO mice following treatment with a vehicle or LPS are shown in Table 5.3. Figures 5.10 - 5.14 present MDA levels in the heart, lung, liver, kidney and ileum respectively. Panel A of these figures shows the main effects of treatment (vehicle vs. LPS), genotype (WT vs. NEIL1 KO) and sex (male vs. female). Panels B - D show the main effect interactions of (B) treatment and genotype, (C) treatment and sex and (D) genotype and sex. Panel E shows the three way interaction between treatment, genotype and sex.

There were no significant three- or two-way interactions in heart tissues. There was a sex difference in that female mouse heart tissues had more MDA (6.71 ±0.98 nmol/mg protein) than male mice (2.99 ±0.99 nmol/mg protein; Figure 5.10A; *p*=0.01). There were no significant differences found in lung tissue (Figure 5.11).

In the liver there was no significant three-way interaction. However there was a treatment x genotype interaction observed. Both genotypes had increases in MDA levels when treated with LPS, the MDA content of NEIL1+/+ (1.1 fold) increased less than NEIL1−/− (1.6 fold; Figure 5.12B; *p*<0.01). There was also a sex difference observed between MDA levels in which male liver MDA levels (0.78 ±0.15 nmol/mg protein) were lower than those in the liver of female mice (1.21 ±0.15 nmol/mg protein; Figure 5.12A; *p*=0.02).
Table 5.2: MDA levels in tissues of NEIL1 transgenic mice exposed to LPS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Vehicle</th>
<th>Treated</th>
<th>Treatment x Genotype x Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
<td>WT</td>
</tr>
<tr>
<td>Heart</td>
<td>3.10 (±0.54)</td>
<td>2.64 (±0.61)</td>
<td>4.45 (±0.71)</td>
</tr>
<tr>
<td>Lung</td>
<td>4.78 (±0.47)</td>
<td>4.25 (±0.63)</td>
<td>5.37 (±0.53)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.51 (±0.11)</td>
<td>0.27 (±0.03)</td>
<td>1.14 (±0.35)</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.34 (±0.55)</td>
<td>2.42 (±0.28)</td>
<td>1.79 (±0.45)</td>
</tr>
<tr>
<td>Ileum</td>
<td>7.02 (±1.65)</td>
<td>7.28 (±0.85)</td>
<td>6.75 (±1.02)</td>
</tr>
</tbody>
</table>

Mice were treated with LPS (20 mg/kg i.p.) or a vehicle (PBS), sacrificed after 12 hours and tissues removed for analysis. Results are shown as tissue MDA levels (μM/mg protein; mean ± SEM; n=6).
Figure 5.10: MDA levels in heart tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg _i.p._), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. MDA levels were significantly higher in ♂ (vs. ♀; Panel A _p_<0.01). No other significant effects were observed.
Figure 5.11: MDA levels in lung tissues of NEIL1 mice exposed to LPS
Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. No significant effects were observed.
Figure 5.12: MDA levels in liver tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. A treatment x genotype interaction was observed (Panel B; p<0.01). MDA levels were significantly higher in treated animals (vs. vehicle treated; Panel A p<0.01), and ♀ (Panel A vs. ♂). No other significant effects were observed.
Figure 5.13: MDA levels in kidney tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. MDA levels were significantly higher in treated animals (vs. vehicle treated; Panel A; p<0.01). No other significant effect was observed.
Figure 5.14: MDA levels in ileum tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. No significant effect was observed.
In the kidney and liver tissues there were no significant three- or two-way interactions. In the kidney there was a significant difference observed between vehicle treated and LPS treated mouse tissues as vehicle treated animals (2.22 ±0.02 nmol/mg protein) have lower MDA levels than LPS treated animals (3.12 ±0.37 nmol/mg protein; Figure 5.13A; p=0.03). There were no significant interactions or differences observed in MDA levels in ileum tissues (Figure 5.14).

### 5.2.4. Glutathione levels in LPS challenged NEIL1\(^{-/-}\) mice

The GSH levels in various tissues of male and female WT and OGG1 KO mice following treatment with a vehicle or LPS are shown in Table 5.3. Figures 5.15 – 5.19 present GSH levels in the heart, lung, liver, kidney and ileum respectively. Panel A of these figures shows the main effects comparing treatment (vehicle vs. LPS), genotype (WT vs. NEIL1 KO) and sex (male vs. female). Panels B - D show the interactions of (B) treatment and genotype, (C) treatment and sex and (D) genotype and sex. Panel F shows the three-way interaction between treatment, genotype and sex.

There were no three-way interactions observed in the results for any tissue. There was a genotype x sex interaction in the heart. Whilst GSH levels were lower in NEIL1\(^{-/-}\) animals than NEIL1\(^{+/+}\) animals there was a greater reduction in the female animals (0.6 fold) than the males (0.8 fold; Figure 5.15D; p<0.01). There was also a decrease in GSH levels observed due to treatment, with vehicle treated mice (0.34 ±0.03 nmol/mg protein) having greater GSH levels than LPS treated mice (0.27 ±0.04 nmol/mg protein; Figure 5.15A; p=0.01).
Table 5.3: GSH levels in tissues of NEIL1 transgenic mice exposed to LPS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Vehicle Male</th>
<th>Female</th>
<th>Treated Male</th>
<th>Female</th>
<th>Genotype x Treatment x</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
<td>WT</td>
<td>KO</td>
<td>WT</td>
</tr>
<tr>
<td>Heart</td>
<td>0.22</td>
<td>0.23</td>
<td>0.56</td>
<td>0.35</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>(±0.01)</td>
<td>(±0.02)</td>
<td>(±0.04)</td>
<td>(±0.03)</td>
<td>(±0.03)</td>
</tr>
<tr>
<td>Lung</td>
<td>4.13</td>
<td>3.53</td>
<td>5.12</td>
<td>4.27</td>
<td>4.52</td>
</tr>
<tr>
<td></td>
<td>(±0.65)</td>
<td>(±0.45)</td>
<td>(±0.38)</td>
<td>(±0.40)</td>
<td>(±1.51)</td>
</tr>
<tr>
<td>Liver</td>
<td>3.18</td>
<td>1.25</td>
<td>4.60</td>
<td>4.10</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>(±1.09)</td>
<td>(±0.32)</td>
<td>(±1.04)</td>
<td>(±0.92)</td>
<td>(±0.00)</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.24</td>
<td>0.43</td>
<td>0.23</td>
<td>0.09</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>(±0.06)</td>
<td>(±0.06)</td>
<td>(±0.14)</td>
<td>(±0.03)</td>
<td>(±0.06)</td>
</tr>
<tr>
<td>Ileum</td>
<td>0.97</td>
<td>1.84</td>
<td>0.68</td>
<td>0.83</td>
<td>1.40</td>
</tr>
<tr>
<td></td>
<td>(±0.35)</td>
<td>(±0.80)</td>
<td>(±0.10)</td>
<td>(±0.18)</td>
<td>(±0.41)</td>
</tr>
</tbody>
</table>

Mice were treated with LPS (20 mg/kg i.p.) or a vehicle (PBS), sacrificed after 12 hours and tissues removed for analysis. Results are shown as mean tissue GSH levels (mU/mg protein; ± SEM; n=6).
Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. (Panel A) An genotype X sex interaction was observed (Panel D; p<0.01). GSH levels were significantly lower (p<0.01) in vehicle KO ♂’s (vs. vehicle wt ♂’s) and in treated KO ♂’s and ♀’s (vs. treated WT ♂’s and ♀’s). Panel A: GSH levels were significantly higher (p<0.01) in vehicle treated animals (vs. treatment), WT (vs. KO) and ♀ (vs. ♂).
Figure 5.16: GSH levels in lung tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. GSH levels were significantly higher (Panel A; $p<0.05$) in vehicle treated animals (♂ vs. treatment), and ♂ (♀ vs. ♀). No other significant effect was observed.
Figure 5.17: GSH levels in liver tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. GSH levels were significantly higher (Panel A; p<0.05) in vehicle treated animals (vs. treatment), WT (vs. KO) and ♀ (vs. ♂). No other significant effect was observed.
Figure 5.18: GSH levels in kidney tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. A treatment X sex interaction was observed (Panel C; \( p=0.05 \)). GSH levels were significantly lower in ♀ (vs. ♂; Panel A; \( p<0.01 \)). No other significant effect was observed.
Figure 5.19: GSH levels in ileum tissues of NEIL1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. GSH levels were significantly lower (Panel A; p<0.05) in WT animals (♂ vs. KO) and ♀ (♀ vs. ♂). No other significant effect was observed.
In lung tissue there were no two-way interactions but there was a difference observed upon treatment and with genotype. LPS treated mice (2.95 ±0.44 nmol/mg protein) had lower GSH levels than vehicle treated mice (3.12 ±0.30 nmol/mg protein; Figure 5.16A; \( p=0.01 \)) and NEIL1\(^{-/-}\) mice (3.12 ±0.37 nmol/mg protein) had lower GSH levels than NEIL1\(^{+/+}\) mice (4.08 ±0.42 nmol/mg protein; Figure 5.16A; \( p=0.05 \)).

In liver tissue there were again no significant two-way interactions. However, there were significant differences with treatment, genotype and sex (Figure 5.17A). There was a significantly lower level of GSH in LPS treated mouse liver (3.28 ±0.48 nmol/mg protein) when compared to the liver of vehicle treated mice (0.87 ±0.19 nmol/mg protein; \( p=0.01 \)). There was significantly less GSH in the liver of NEIL1\(^{-/-}\) (1.63 ±0.38 nmol/mg protein) mice when compared to NEIL1\(^{+/+}\) mice (2.52 ±0.48 nmol/mg protein; \( p=0.04 \)), and male mouse livers (1.18 ±0.36 nmol/mg protein) had less GSH than female livers (2.97 ±0.44 nmol/mg protein; \( p=0.01 \)).

The treatment x sex interaction was significant in kidney. Whilst there was an increase in GSH levels in male mice (1.6 fold) due to LPS treatment, there was no real change in female mice (0.93 fold; Figure 5.18C; \( p=0.05 \)).

In the ileum there were no significant two-way interactions, but there were significant differences with genotype and sex. There was significantly greater GSH in the ileum of NEIL1\(^{-/-}\) (1.41 ±0.29 nmol/mg protein) mice when compared to NEIL1\(^{+/+}\) mice (0.83 ±0.15 nmol/mg protein; \( p=0.05 \)), and male mouse ileal tissues (1.64 ±0.29 nmol/mg protein) had greater GSH levels than female tissues (0.59 ±0.06 nmol/mg protein; \( p=0.01 \)).
5.2.5. Age related cytokine output NEIL1⁻/⁻ mice

Figures 5.20 – 5.23 detail IL concentrations in mouse blood of animals sacrificed at 6-8 weeks and 12 months. Panel A shows the age x genotype x sex interaction, panels B – D detail age x genotype, age x sex and genotype x sex interactions respectively and panel E shows the main effects.

There were no significant differences observed in IL-4 concentrations although there was evidence of a significant effect in the sex x age interaction as female mouse IL-4 levels increased with age (2.20 fold), whilst male IL-4 levels decreased slightly (0.78 fold; $p=0.07$).

There was no evidence of an age x genotype x sex interaction in IL-6 levels. The genotype x age interaction was significant in that whilst the blood IL-6 concentration was similar at 12 months, it was significantly higher at 6-8 weeks in WT mice (28.14 ± 7.54 pg/ml) than NEIL1 KO mice (15.88 ± 9.78 pg/ml), meaning that the reduction due to age was considerably greater in WT mice ($p>0.01$). There is a significant genotype x sex interaction observed in IL-10 levels as whilst IL-10 levels reduced due to disruption of the NEIL1 gene in female mice (0.54 fold) levels it actually increased in male mice (1.73 fold; $p=0.02$). There were no significant differences in IL-12 concentration.

5.3. Discussion

Previous work has identified several BER related genes that are associated with modulating the inflammatory response (Ando et al., 2008; Mabley et al., 2005a; Touati et al., 2006; Virag and Szabo 2002). Here we show that the disruption of the NEIL1 gene reduces initial IL-6 production, but levels of pro- and anti-inflammatory cytokines indicate an overall increase
Figure 5.20: IL-4 concentrations in blood serum taken from NEIL1 transgenic mice
Mice were sacrificed at the appropriate time and blood removed by cardiac puncture for analysis by ELISA (mean ± SEM; n=6). Panel A shows 3-way interactions between age, genotype and sex, panels B–D show interactions between age x genotype, age x sex and genotype x sex respectively and panels E shows main effects of age, genotype and sex. No significant interactions were observed in the IL-4 results although there was evidence of a sex x age interaction (Panel C; p=0.07)
Figure 5.21: IL-6 concentrations in blood serum taken from NEIL1 transgenic mice
Mice were sacrificed at the appropriate time and blood removed by cardiac puncture for analysis by ELISA (mean ± SEM; n=6). Panel A shows 3-way interactions between age, genotype and sex, panels B–D show interactions between age x genotype, age x sex and genotype x sex respectively and panels E shows main effects of age, genotype and sex. There was a significant age x genotype interaction ($p<0.01$), there were also significant differences in the main effects of age ($p<0.01$) and genotype ($p=0.03$).
Mice were sacrificed at the appropriate time and blood removed by cardiac puncture for analysis by ELISA (mean ± SEM; n=6). Panel A shows 3-way interactions between age, genotype and sex, panels B–D show interactions between age x genotype, age x sex and genotype x sex respectively and panels E shows main effects of age, genotype and sex. There was a significant genotype and sex interaction ($p=0.02$).
Mice were sacrificed at the appropriate time and blood removed by cardiac puncture for analysis by ELISA (mean ± SEM; n=6). Panel A shows 3-way interactions between age, genotype and sex, panels B–D show interactions between age x genotype, age x sex and genotype x sex respectively and panels E shows main effects of age, genotype and sex. No significant differences were observed.
inflammation after 6 h. There are tissue specific differences in tissue GSH content: in the heart, lung and liver of NEIL1−/− mice there is less GSH implying more ROS are produced, but in the ileum tissues GSH levels rise (Table 5.5).

<table>
<thead>
<tr>
<th><strong>Table 5.4 Summary of NEIL1 transgenic mouse blood serum cytokine results</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>IL-4</td>
</tr>
<tr>
<td>IL-6</td>
</tr>
<tr>
<td>IL-10</td>
</tr>
<tr>
<td>IL-12</td>
</tr>
</tbody>
</table>

** = p≤0.01, * = 0.05≤p>0.01, - = p≥0.10, ↓/↑ = comparison to WT.

The detection of LPS by monocytes initiates an immune signalling cascade; this is made up of many cytokines, and other signalling proteins (Moller and Villiger 2006). The upregulation of IL-6 is one of the primary elements in this cascade which goes on to stimulate increased serum levels of other cytokines including the T_{h1} cytokine, IL-12, and the T_{h2} cell stimulating anti-inflammatory cytokines, IL-4 and 10 (Moller and Villiger 2006; Reed and Milton 2001; Wan et al., 2000). Here we have found that the basal levels of IL-6 are significantly lower in male NEIL1−/− mice, and that in response to the LPS challenge the level of IL-6 at 1 h in the serum was lower in NEIL1−/− female mice, indicating a slower initial output of IL-6. 6 h after treatment the levels of IL-12 (pro-inflammatory) in male animals had increased but levels of the anti-inflammatory cytokines (IL-4 and 10) in female mice had decreased. These results may indicate differing immune responses to compensate for the initially slowed IL-6 signalling. These differences in T_{h1}/T_{h2} responses between male and female mice can be explained by oestrogens ability to modulate the immune response reducing T_{h1} activity and increasing that of T_{h2} (Salem, 2004). Alternatively, the disruption of the NEIL1 protein could be modulating the
Table 5.5: Summary of NEIL1 organ damage results

<table>
<thead>
<tr>
<th>Primary interactions</th>
<th>Secondary Interactions</th>
<th>Tertiary Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment (LPS Treated vs. Vehicle)</td>
<td>Genotype (KO vs. WT)</td>
</tr>
<tr>
<td>Heart</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>*↑</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ileum</td>
<td>*↑</td>
<td>-</td>
</tr>
<tr>
<td>Heart</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td><strong>↑</strong></td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>*↑</td>
<td>-</td>
</tr>
<tr>
<td>Ileum</td>
<td>-</td>
<td>~↑</td>
</tr>
<tr>
<td>Heart</td>
<td><strong>↓</strong></td>
<td><strong>↓</strong></td>
</tr>
<tr>
<td>Lung</td>
<td><em>^↑</em></td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td><strong>↓</strong></td>
<td><em>^↓</em></td>
</tr>
<tr>
<td>Kidney</td>
<td>~↑</td>
<td>~↑</td>
</tr>
<tr>
<td>Ileum</td>
<td>*↑</td>
<td>*↑</td>
</tr>
</tbody>
</table>

** = p≤0.01, * = 0.05≥p>0.01, ~ = 0.10>p>0.05, - = p≥0.10, ↓/↑ = comparison.
activation of transcription factors such as NF-κβ and AP-1, maybe via PARP1 or APE1. In order to discern if this is the case electrophoretic mobility-shift assays could be performed using cell extracts from cultured cells (Abdel-Latif et al., 2004).

The chemoattractants such as MIP-1α released as part of this signaling cascade induce leukocytes, in particular neutrophils, to the area of invasion. These cells produce an array of bactericidal compounds, including ROS and RNS. These oxidizing compounds are produced via a network of enzymes including superoxide dismutase (SOD) and myeloperoxidase (MPO), the activity of which can be measured as an indication of neutrophil infiltration. In mice treated with LPS (80 mg/kg i.p.) a significant increase in MPO activity can be measured after 4 h and this activity continues to increase up to 24 h (Kabir et al., 2002; Yamashita et al., 2000). In the case of these NEIL1<sup>−/−</sup> mice there was no significant difference in MPO levels compared to NEIL1<sup>+/+</sup> mice, this indicated a reduced chemoattractant activity as suggested in the results from the NEIL1<sup>−/−</sup> cells (section 4.2).

ROS produced by neutrophils, of course, are not specifically bactericidal and a build up of these compounds can cause cellular damage. One measure of this damage is MDA levels. Previous studies have shown that when mice are treated with LPS the MDA concentrations in the liver, kidney, lung, gut and heart tissues are raised significantly, although not significantly so in brain tissue (Mabley et al., 2005a; Sebai et al., 2010) accumulation of ROS within the system, which are then neutralised via the GSH pathway. Interestingly there have been cases observed when the immune response has shown markers of oxidative damage to increase without an increase in neutrophil aggregation, in these cases it is thought to be due to an increase in NO secondary messengers released as part of inflammation (Pheng et al., 1995).
Raised levels of NO have been shown to increase the creation of Tg in replicated DNA significantly. TG can block the progress of repair or replicative DNA polymerases along the DNA strand (Aller et al., 2007). NEIL1 has been implicated in the S-phase and repair within the replication fork. Thus the reduced release of Tg from the ssDNA, due to the knockout of NEIL1, could potentially be extending the S-phase and disrupting production of both cytokines and neutrophils (Orr et al., 2007; Orren et al., 1997).

NEIL1 has also been shown to interact with several proteins that have no effect on the activity of NEIL1 including DNA ligase IIIα, polβ and OGG1 (Mokkapati et al., 2004a). DNA ligase and polβ both interact with XRCC1, and whilst XRCC1 has no catalytic properties of its own it does interact with both PARP-1 and APE1 (Mutamba et al., 2011). Mutations in XRCC1 have been shown to be associated with various inflammation-associated malignancies including an increase the prevalence of breast cancer (Duell et al., 2001) and reduced susceptibility to oesophageal (Lee et al., 2001), lung (Ratnasinghe et al., 2001) and bladder cancers (Stern et al., 2001).

Additionally these proteins also interact with PARP-1 (Mutamba et al., 2011), the knockout of which has been shown to reduce the immune response to endotoxin (Virag and Szabo 2002). However, this inhibition is thought to be due to a blanket reduction in all cytokine activity due to the inhibition of NF-κβ and AP-1 with which it has been shown to be a co-factor with (Kiefmann et al., 2004). This was not observed in the developed NEIL1−/− mice which exhibited both increases and decreases in cytokine production.

A number of proteins have been shown to interact with NEIL1 increasing its activity including FEN-1, PCNA, CSB and WRN (Das et al., 2007a; Dou et al., 2008; Hegde et al., 2008; Muftuoglu et al., 2009). WRN has also
been shown to be linked to inflammation; siRNA inhibition of this protein has been shown to increase both IL-6 production and the activation of NF-κβ (Turaga et al., 2009). As NEIL1 is activated by WRN and there was no overall increase in inflammatory damage it is probable that this is not the pathway by which inflammation is modulated.

FEN-1 as with many of these proteins does also interact with PARP-1 (Bouchard et al., 2003) and has been implicated in autoimmunity disorders, chronic inflammation and cancer formation (Kucherlapati et al., 2002; Lam et al., 2006; Zheng et al., 2007). More recently it was suggested that the upregulation of this protein was responsible for the increased inflammatory mediated cancer risk. The mechanism implicated in this rise in inflammatory signals was the hypomethylation of key promoter signals, leading to the hyperexpression of genes (Singh et al., 2008). The lack of NEIL1 protein to bind with may therefore increase free FEN-1 proteins within the cell reducing promoter methylation, and thus increasing these inflammatory signals.

PCNA interacts with NEIL1 during the S-phase as it plays a role in DNA replication at this point (Kisielewska et al., 2005). Additionally, due to the positive correlation between PCNA concentration and severity of inflammation, PCNA has been used as a marker for inflammation in inflammatory bowel disease and several types of cancer including liver (Ding et al., 2005; Harrison et al., 1993).

Little is known how the CSB protein may influence inflammatory response but in an experiment in which CSB$^{-/-}$ mice were exposed to ozone, the basal IL-6 levels in these mice were higher and the IL-6 response of the mice in question was accelerated whilst the accumulation of oxidative damage was decreased (Kooter et al., 2008). Whilst there was an increased basal IL-6 level in the male NEIL1$^{-/-}$ mice, the IL-6 production was not accelerated as it was in the CSB$^{-/-}$ mice. Although no mechanism
for CSB affecting the immune response has been forthcoming, the removal of the NEIL1 protein may result in excess “free” CSB within the cell.

Each of these proteins, including NEIL1 itself, can be linked to inflammation via APE1. This enzymes expression is activated by the presence of ROS such as those released by the innate immune response (Yang et al., 2007). APE1 activates transcription factors NF-κB and AP-1 by the reduction of active cystine residues (Ando et al., 2008). The down regulation of the disruption of APE1 activity has been shown to decrease the activation of transcription factors NF-κB and AP-1 (Daily et al., 2001; Fung and Demple 2005).

As the mice got older, basal levels of the cytokine IL-6 decreased, however aging is more often cited as causing increased IL-6 production (Daynes et al., 1993; Ershler et al., 1993), but there is controversy on this point as cases exist where no change (Beharka et al., 2001) or a decrease is observed (Boehmer et al., 2004; Goodman and Stein 1994; Sharman et al., 2001). These NEIL1+/− mice had initially reduced basal levels of IL-6 which reduced to similar levels as WT mice at 12 months. A decrease in basal IL-6 is thought to be due to reduced efficiency in the transcription pathways, particularly the JNK and p38 pathways (Boehmer et al., 2004; Goodman and Stein 1994) or due to the deregulation of IL-6 with age, due to inhibition of NF-κB by an increase in Th2 activity (Ye and Johnson 2001). Another hypothesis is that in younger mice, IL-6 plays a role in developmental processes such as osteoclastogenesis, vascular development and the development of the brain and that the basal level of IL-6 reduces when it is no longer needed (Sharman et al., 2001).

The large numbers of proteins that may link through NEIL1 to inflammation indicate that the BER repair system and inflammation may be linked at several points, however there are many points where the BER
system “bottlenecks” i.e. after APE1 has cleaved the 3’ PUA leaving a gap that can be repaired by Pol β and DNA ligase III/XRCC1.

In conclusion, the knockout of the NEIL1 gene seems to slow the initiation of the immune response, although this is compensated for by an increase in the proinflammatory cytokine, IL-12, in males and a decrease in the anti-inflammatory ILs 10 and 4 in the blood serum. Additionally there was no difference in levels of MPO or MDA between NEIL1<sup>−/−</sup> and NEIL1<sup>+/+</sup> mice, although there was differences observed between overall GSH, indicating a mild ROS increase. The results shed more light on how previously identified BER proteins may have modulated inflammation but more work need to be done to identify the specific pathway.
6. Effects of OGG1 Gene Knockout on Endotoxin Induced Inflammation

6.1. Introduction

As outlined previously base excision repair proteins such as PARP-1, APE-1 and MUTYH have been implicated in the regulation of the immune system in response to endotoxin (Sections 1.3.1. & 1.3.2.) (Casorelli et al., 2010; Virag and Szabo 2002). There is also evidence that another base excision repair protein, OGG1, may have potentially protective effects against endotoxin or helicobacter pylori induced inflammation (Mabley et al., 2005a; Touati et al., 2006). This was first reported in 2005 when it was shown that after LPS treatment (55 mg/kg i.p.) the mortality in OGG1−/− mice was lower than that in wildtype animals. The levels of the IL-12 and TNF-α in blood plasma were reduced in OGG1−/− mice when compared with OGG1+/+ mice as were levels of MIP-1α. Additionally, levels of anti-inflammatory cytokines IL-4 and IL-10 were increased in the plasma of the OGG1−/− mice when compared to OGG1+/+ mice, indicating an overall suppression of the immune system. MPO activity in heart and lung tissues was significantly reduced in OGG1−/− mice, compared to OGG1+/+ mice after the mice were challenged with 80 mg/kg LPS (i.p.). Additionally MDA levels were also reduced in the lung, heart and liver of OGG1−/− mice when compared to their OGG1+/+ counterparts. These results suggest that the reduction in levels of pro-inflammatory cytokines and chemokines, and the increase in anti-inflammatory cytokines reduced neutrophil recruitment and activation, which in turn attenuated the production of MDA (Mabley et al., 2005a).

The same strain of OGG1−/− mice, this time bred onto a Big blue mouse background useful for the observation of mutations (Hill et al., 1999), infected with the gram-negative bacteria helicobacter pylori (H. pylori) exhibited less severe histological lesions of the gastric mucosa than those of OGG1+/+ mice. Additionally, infiltration of polymorphonuclear cells
(neutrophils, basophils and eosinophils) was observed in the gut tissue of 33% of OGG1−/− mice treated with *H. Pylori*, compared to 100% of the OGG1+/+ mice, indicating that the recruitment of these cells was also attenuated (Touati *et al.*, 2006).

These studies support the hypothesis that there is a link between OGG1 and the inflammatory response, but this evidence has not been confirmed by other sources.

**6.1.1. Aims**

To identify the extent to which OGG1 determines the immune response to LPS induced inflammation by:

1. Establishing whether levels of IL-6, IL-12, IL-10 and IL-4 differ between OGG1−/− and OGG1+/+ mouse serum, at baseline and after LPS treatment.
2. Identify whether changes in MPO activity, GSH levels and MDA levels induced by LPS differ between OGG1−/− and OGG1+/+ mice.

**6.2. Results**

**6.2.1. Cytokine Output in LPS Challenged OGG1−/− Mice**

Figures 6.1 – 6.4 present interleukin concentrations measured in blood serum of mice after 0, 1, 6 and 24 h after injection with LPS (2 mg/kg *i.p.*; mean ± SEM; n=6). There was a significant difference in IL-4 levels between WT and KO male mice at 6 h, here the OGG1+/+ (18.1 ± 0.9 pg/ml) mice having more IL-4 in their serum than OGG1−/− mice (12.9 ± 2.4 pg/ml; Figure 6.1A; *p*=0.05). IL-4 concentrations were not significantly different at any time point when comparing female OGG1+/+ and OGG1−/− mice (*p*>0.55).

Serum IL-6 concentrations were significantly different between male WT and KO mice at baseline (WT 5.2 ± 1.4 pg/ml vs. KO 1.8 ± 0.6 pg/ml;
\( p < 0.01 \), at which point the KO concentration of IL-6 was higher than WT levels (Figure 6.2A).

(A) Male IL-4 levels

(B) Female IL-4 Levels

**Figure 6.1: IL-4 concentrations in blood serum taken from OGG1 transgenic mice**

Mice were treated with LPS (2 mg/kg i.p.), sacrificed at the appropriate time and blood removed by cardiac puncture for analysis by ELISA (mean ± SEM; n=6). Panels A and B show serum IL-4 levels at 0, 1, 6 and 24 h in male and female respectively. (*) - \( p \leq 0.05 \); ** - \( p \leq 0.01 \). A significant difference was observed between WT and KO ♂ mice at 1 (\( p = 0.04 \)) and 6 h (\( p = 0.05 \)). There was no significant difference in IL-4 levels between ♀ WT and KO mice (\( p > 0.55 \)).

There were significant differences between concentrations of IL-6 in the serum of female WT and KO mice at 1 h (WT 414.3 ± 46.5 pg/ml vs. KO 311.2 ± 23.1 pg/ml; \( p < 0.01 \)) at which point the IL-6 levels in WT serum was higher than that of the KO mice. After 24 h (WT 2.6 ± 2.5 pg/ml vs. KO 23.8 ± 1.2 pg/ml; \( p < 0.01 \); Figure 6.2B).
Figure 6.2: IL-6 concentrations in blood serum taken from OGG1 transgenic mice

Mice were treated with LPS (2 mg/kg i.p.), sacrificed at the appropriate time and blood removed by cardiac puncture for analysis by ELISA (mean ± SEM; n=6). Panels A and B show serum IL-6 levels at 0, 1, 6 and 24 h im male and female mice respectively. (*) - *p*≤0.05; ** - *p*≤0.01). A significant difference was observed between WT and KO ♂ mice at 0 h (*p*<0.01). There was also a significant difference in IL-6 levels between ♀ WT and KO mice at 1 (*p*<0.01) and 24 h (*p*<0.01).

There were significant differences between concentrations of IL-10 in the serum of WT and KO male mice at 6 h (WT 2356.6 ± 414.7 pg/ml vs. KO 3730.1 ± 381.9 pg/ml; *p*=0.02) and 24 h (WT – 60.2 ± 52.7 pg/ml vs. KO 1498.2 ± 228.9 pg/ml; *p*<0.01). In both of these cases IL-10 levels were greater in KO serum than WT serum (Figure 6.3A). Similarly there were significant differences between concentrations of IL-10 in the serum of female WT and KO mice at 6 h (WT 5447.7 ± 103.8 pg/ml vs. KO 4323.5
± 321.1 pg/ml; $p<0.01$) and 24 h (WT 63.1 ± 1.8 pg/ml vs. KO 45.7 ± 1.9 pg/ml; $p<0.01$). However, in these cases the levels of IL-10 in KO mice were lower than those of the WT mice (Figure 6.3B).

![Graph A: Male IL-10 Levels](graph_a.png)

![Graph B: Female IL-10 Levels](graph_b.png)

**Figure 6.3: IL-10 concentrations in blood serum taken from OGG1 transgenic mice**

Mice were treated with LPS (2 mg/kg i.p.), sacrificed at the appropriate time and blood removed by cardiac puncture for analysis by ELISA (mean ± SEM; n=6). Panels A and B show serum IL-10 levels at 0, 1, 6 and 24 h in male and female mice respectively. (* - $p \leq 0.05$; ** - $p \leq 0.01$). A significant difference was observed between WT and KO $\delta$ mice at 6 ($p=0.02$) and 24 h ($p<0.01$). There was a significant difference in IL-10 levels between $\varphi$ WT and KO mice at 6 and 24 h ($p<0.01$).

Whilst there was no significant differences in serum IL-12 concentrations between OGG1$^{+/+}$ and OGG1$^{-/-}$ mice in either male or female mice, there was evidence of a significant difference between OGG1$^{+/+}$ and OGG1$^{-/-}$ in
IL-12 levels at 1 h in female mice (WT 77.4 ± 68.2 pg/ml vs. KO 50.7 ± 24.9 pg/ml; Figure 6.4B; p=0.06).

(A) Male IL-12 Levels

(B) Female IL-12 Levels

Figure 6.4: IL-12 concentrations in blood serum taken from OGG1 transgenic mice

Mice were treated with LPS (2 mg/kg i.p.), sacrificed at the appropriate time and blood removed by cardiac puncture for analysis by ELISA (mean ± SEM; n=6). Panels A and B show serum IL-12 levels at 0, 1, 6 and 24 h in male and female mice respectively. (* - p≤0.05; ** - p≤0.01). No significant difference was observed between WT and KO ♂ mice at any time point (p>0.42). There was evidence of a significant difference in IL-12 levels between ♀ WT and KO mice at 1 h (p=0.06).
6.2.2. Myeloperoxidase Activity in LPS Challenged OGG1\(^{-/-}\) Mice

MPO activity in various tissues of male and female OGG1\(^{+/+}\) and OGG1\(^{-/-}\) mice following treatment with a vehicle or LPS are shown in Table 6.2. Figures 6.5 – 6.9 present MPO activity levels in the heart, lung, liver, kidney and ileum respectively. Panel A of these figures shows the main effects of treatment (vehicle vs. LPS), genotype (WT vs. OGG1 KO) and sex (male vs. female). Panels B - D show the interactions of (B) treatment and genotype, (C) treatment and sex and (D) genotype and sex. Panel E shows the three way interaction between treatment, genotype and sex.

There was no significant treatment x genotype x sex, genotype x sex or treatment x genotype interaction in determining heart MPO activity (Figure 6.5). There was evidence of a treatment x sex interaction (Figure 6.5C; \(p=0.08\)) in that treatment decreased MPO activity in female mice from 28.1 (± 5.3) mU/mg protein to 15.8 (±2.2) mU/mg protein whereas there was no effect of treatment on MPO activity in male mice (i.e. both WT and Ogg1 KO mice combined). MPO activity in the heart did not vary with genotype.
Table 6.1: MPO activity in tissues of OGG1 transgenic mice exposed to LPS

<table>
<thead>
<tr>
<th>Tissue</th>
<th></th>
<th>Vehicle</th>
<th>Treated</th>
<th>Treatment X Genotype X Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WT</td>
<td>KO</td>
<td>WT</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td>18.71 (±1.06)</td>
<td>16.54 (±2.32)</td>
<td>25.48 (±6.78)</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td>29.75 (±2.96)</td>
<td>45.01 (±4.49)</td>
<td>33.10 (±7.04)</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>10.75 (±1.93)</td>
<td>13.13 (±1.76)</td>
<td>9.66 (±2.06)</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>6.69 (±1.91)</td>
<td>5.29 (±1.33)</td>
<td>9.20 (±1.95)</td>
</tr>
<tr>
<td>Ileum</td>
<td></td>
<td>11.05 (±1.04)</td>
<td>12.94 (±2.77)</td>
<td>28.33 (±8.82)</td>
</tr>
</tbody>
</table>

Mice were treated with LPS (20 mg/kg i.p.) or vehicle (PBS), sacrificed after 12 hours and tissues removed for analysis. Tissue MPO activity (mU/mg protein) results are shown as mean ± SEM (n=6).
Figure 6.5: MPO activity in heart tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.) or vehicle only, sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. No significant effect were observed.
Figure 6.6: MPO activity in lung tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.) or vehicle only, sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. A significant genotype X sex interaction was observed. MPO activity was significantly higher in treated WT ♂ (vs. vehicle treated WT ♂; p<0.02). MPO activity was significantly lower (p<0.03) in vehicle treated animals (vs. treatment), WT (vs. KO) and ♀ (vs. ♂).
Figure 6.7: MPO activity levels in liver tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.) or vehicle only, sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. Significant group X sex and genotype X sex interactions (p<0.05) were observed. MPO activity was significantly lower (p=0.01) in vehicle treated mice (♀) than treated (♂). No other significant interactions were observed.
Figure 6.8: MPO activity in kidney tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.) or vehicle only, sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. MPO activity was significantly lower in vehicle treated animals (vs. treatment; \( p<0.01 \)). No other significant effect was observed.
Figure 6.9: MPO activity in ileum tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.) or vehicle only, sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. A significant treatment X sex interaction was observed in (\(p=0.04\)). MPO activity was significantly higher in \(♀\) (vs. \(♂\); \(p=0.03\)). No other significant effect was observed.
In the lung there was no significant three way interaction observed in MPO activity, although, there was a significant interaction between genotype and sex (Figure 6.6D; $p=0.01$). Whilst there was an increase in MPO activity in male animals due to the knockout of the Ogg1 gene (1.6 fold) there was no significant difference in MPO activity in female mouse lung tissue (1.1 fold). The treatment x genotype (Figure 6.6B; $p=0.08$) and treatment x sex (Figure 6.6D; $p=0.08$) interactions both approached significance in that whilst in each case MPO activity increased due to treatment that increase was greater in Ogg1$^{-/-}$ (4.7 fold vs. 3.9 fold) animals and male mice (4.3 fold vs. 4.0 fold).

In the liver there was a significant three way interaction between treatment, genotype and sex in determining MPO activity (Figure 6.7E; $p=0.05$). In male and female mice, treatment resulted in a significant increase in MPO activity in both Ogg1$^{+/+}$ and Ogg1$^{-/-}$ mice. Whereas in both male and female WT mice this increase was similar (4.3 fold in male and 4.7 in female), in male Ogg1$^{-/-}$ mice this increase was 6.7 fold and in female mice only 3.7 fold.

In the kidney there was no significant three way interaction between treatment, genotype and sex (Figure 6.8E). There was an interaction approaching significance between treatment and sex (Figure 6.8C; $p=0.07$) in that whilst treated animals had greater MPO activity, these levels rose more in male mice (2.8 fold) when compared to female mice (1.5 fold). Interestingly whilst the female mice initially had lower levels of MPO activity, after treatment MPO activity levels were higher than those of the males (Figure 6.8C).

In the ileum there was no significant three way interaction, although it did approach significance (Figure 6.9E; $p=0.07$). The treatment x sex interaction was significant (Figure 6.9C; $p=0.04$), indicating that whilst MPO activity of LPS treated male (18.1 ±3.5 mU/mg protein) and female
(20.3 ±4.6 mU/mg protein) mice were not significantly different, vehicle treated female mice had significantly higher MPO activity levels (27.0 ±4.8 mU/mg protein) when compared to that of the males (12.0 ±1.4 mU/mg protein) leading to a drop in MPO activity levels when treated with LPS. Mean MPO activity was significantly higher in female mice (22.0 ±3.3 mU/mg protein) than males (15.0 ±1.9 mU/mg protein; Figure 6.9A; p=0.03).

6.2.3. Malondialdehyde Content in LPS Challenged OGG1⁺/- Mice

The MDA levels in various tissues of male and female OGG1⁺/+ and OGG1⁻⁻ following treatment with a vehicle or LPS is shown in Table 6.3. Figures 6.10 – 6.14 present MDA levels in the heart, lung, liver, kidney and ileum respectively. Panel A of these figures shows the main effects of treatment (vehicle vs. LPS), genotype (WT vs. OGG1 KO) and sex (male vs. female). Panels B - D show the interactions of (B) treatment and genotype, (C) treatment and sex and (D) genotype and sex. Panel E shows the three way interaction between treatment group, genotype and sex.

There were no three way interactions observed in the results of the MDA assays, neither were there many two way interactions. Within the heart there were no significant differences at all (Figure 6.10). In the lung there were significant differences in MDA levels observed between vehicle treated animals (5.5 ±0.6 nmol/mg protein) and those treated with LPS (7.4 ±0.8 nmol/mg protein; Figure 6.11A; p=0.05). There was also a significant difference observed between MDA levels in male (5.0 ±0.4 nmol/mg protein) and female mice (7.7 ±0.8 nmol/mg protein; Figure 6.11A; p=0.01).
Table 6.2: MDA levels in tissues of OGG1 transgenic mice exposed to LPS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Vehicle</th>
<th>Treated</th>
<th>Treatment X Genotype X</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
<td>WT</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>0.40 (±0.11)</td>
<td>0.94 (±0.37)</td>
<td>1.08 (±0.21)</td>
</tr>
<tr>
<td>Lung</td>
<td>6.48 (±1.71)</td>
<td>6.02 (±1.68)</td>
<td>5.16 (±0.95)</td>
</tr>
<tr>
<td>Liver</td>
<td>2.11 (±0.16)</td>
<td>2.87 (±0.60)</td>
<td>1.93 (±0.32)*</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.20 (±0.14)</td>
<td>1.11 (±0.18)</td>
<td>1.10 (±0.14)</td>
</tr>
<tr>
<td>Ileum</td>
<td>3.21 (±0.75)</td>
<td>6.25 (±2.12)</td>
<td>5.54 (±0.48)</td>
</tr>
</tbody>
</table>

Mice were treated with LPS (20 mg/kg i.p.) or a vehicle (PBS), sacrificed after 12 hours and tissues removed for analysis. Tissue MPO activity (mU/mg protein) results are shown as mean ± SEM (n=6).
Figure 6.10: MDA levels in heart tissues of OGG1 transgenic mice exposed to LPS
Mice were treated with LPS (20 mg/kg i.p.) or vehicle only, sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. No significant effects were observed.
Figure 6.11: MDA levels in lung tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.) or vehicle only, sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. MDA levels were significantly higher ($p<0.01$) in treated animals ($\boldsymbol{\text{v}}$s. vehicle treated), and $\boldsymbol{\text{♀}}$ ($\boldsymbol{\text{v}}$s. $\boldsymbol{\text{♂}}$). No other significant effects were observed.
Figure 6.12: MDA levels in liver tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.) or vehicle only, sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. A significant treatment x sex and genotype X sex interactions were observed (p<0.01). Significantly lower levels of MDA were detected in vehicle treated WT ♀’s (vs. treated WT ♀’s; p<0.01). MDA levels were significantly lower in ♀ (vs. ♂; p<0.01).
Figure 6.13: MDA levels in kidney tissues of OGG1 mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.) or vehicle only, sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. MDA levels were significantly higher in vehicle treated animals (vs. treatment; p<0.01). No other significant effects were observed.
Figure 6.14: MDA levels in ileum tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.) or vehicle only, sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. MDA levels were significantly higher in treated animals (vs. vehicle treated; p<0.01). No other significant effects were observed.
In liver tissues significant treatment x sex (Figure 6.12C; \( p=0.01 \)) and genotype x sex (Figure 6.12D; \( p=0.01 \)) interactions were observed. The treatment x sex interaction reflects that whilst the male animals responded to the LPS treatment with an increase in MDA (1.6 fold), there was a decrease observed in the female animals (0.5 fold). Similarly whilst there was an increase in MDA levels (1.4 fold) in liver tissues from male mice due to the knockout of the OGG1 gene, the female animals seemed to have a decrease in MDA levels (0.4 fold) in the liver.

In the kidney, there was only one significant difference and that was between the two treatment groups (Figure 6.13A), the vehicle treated animals had significantly lower levels of MDA (1.3 ±0.1 nmol/mg protein) than those of the animals treated with LPS (2.2 ±0.3 nmol/mg protein; \( p=0.01 \)). Similarly this was the only difference in the ileum tissues showing again that vehicle treated animals had lower levels of MDA (4.9 ±1.0 nmol/mg protein) than those treated with LPS (7.2 ±0.6 nmol/mg protein; Figure 6.14A; \( p=0.01 \)). There were no other significant differences at any level in both organs.

### 6.2.1. Glutathione Activity in LPS Challenged OGG1\(^{-/-}\) Mice

The GSH levels in various tissues of male and female OGG1\(^{+/+}\) and OGG1\(^{-/-}\) mice following treatment with a vehicle or LPS is shown in Table 6.4. Figures 6.15 – 6.19 present GSH activity levels in the heart, lung, liver, kidney and ileum respectively. Panel A of these figures shows the main effects of treatment (vehicle vs. LPS), genotype (WT vs. OGG1 KO) and sex (male vs. female). Panels B - D show the interactions of (B) treatment and genotype, (C) treatment and sex and (D) genotype and sex. Panel E shows the three way interaction between treatment, genotype and sex.
Table 6.3: GSH levels in tissues of OGG1 transgenic mice exposed to LPS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Vehicle</th>
<th>Male</th>
<th>Female</th>
<th>Treated</th>
<th>Male</th>
<th>Female</th>
<th>Treatment X Genotype X Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT</td>
<td>KO</td>
<td>WT</td>
<td>KO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td>0.49 (±0.04)</td>
<td>0.29 (±0.04)</td>
<td>0.44 (±0.05)</td>
<td>0.48 (±0.01)</td>
<td>1.10 (±0.77)</td>
<td>0.50 (±0.36)</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td>4.90 (±0.98)</td>
<td>6.74 (±1.13)</td>
<td>5.11 (±0.38)</td>
<td>4.27 (±0.40)</td>
<td>2.44 (±1.18)</td>
<td>4.00 (±0.48)</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>4.01 (±1.56)</td>
<td>4.72 (±0.57)</td>
<td>4.55 (±0.32)</td>
<td>3.32 (±0.44)</td>
<td>2.44 (±0.62)</td>
<td>2.55 (±0.53)</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>0.47 (±0.10)</td>
<td>0.59 (±0.11)</td>
<td>1.14 (±0.06)</td>
<td>1.11 (±0.05)</td>
<td>0.47 (±0.05)</td>
<td>0.86 (±0.17)</td>
</tr>
<tr>
<td>Ileum</td>
<td></td>
<td>1.35 (±0.07)</td>
<td>2.21 (±0.32)</td>
<td>1.19 (±0.37)</td>
<td>1.70 (±0.65)</td>
<td>1.29 (±0.08)</td>
<td>0.97 (±0.09)</td>
</tr>
</tbody>
</table>

Mice were treated with LPS (20 mg/kg i.p.) or vehicle (PBS), sacrificed after 12 hours and tissues removed for analysis. Tissue MPO activity (mU/mg protein) results are shown as mean ± SEM (n=6).
There were no three way interactions detected when comparing GSH levels in the mice. Indeed, in the heart there were no significant differences identified at any level (Figure 6.15). In the lung treatment x genotype and treatment x sex were not significant (Figures 6.16B & C). There was, however, a significant genotype x sex interaction, as whilst GSH levels in female mice remained similar in WT and Ogg<sup>−/−</sup> lung tissues (0.9 fold), it increased significantly in male tissues (1.5 fold; Figure 6.16D). There was also a difference observed between the treatment groups as vehicle treated mice (5.3 ±0.4 nmol/mg protein) had higher GSH levels than those of LPS treated mice (3.0 ±0.4 nmol/mg protein; Figure 6.16A; ρ=0.01).

There was an interaction between genotype x sex, as whilst GSH levels in female mice were reduced when comparing WT and Ogg<sup>−/−</sup> lung tissues (0.6 fold) it remained similar when comparing male tissues (1.1 fold; Figure 6.17D). Within the liver treatment x genotype and treatment x sex interactions were not significant. There was a difference between the treatment groups as vehicle treated mice (4.2 ±0.4 nmol/mg protein) had higher GSH levels in their liver tissues than those of LPS treated mice (2.7 ±0.3 nmol/mg protein; Figure 6.17A; ρ=0.01).

In the kidney, there was only one significant difference found, male mice had lower GSH levels within their kidney (0.6 ±0.1 nmol/mg protein) than the female mice (1.1 ±0.1 nmol/mg protein; Figure 6.18A; ρ=0.01). In the ileum tissues assayed there was a treatment x genotype interaction which approached significance (Figure 6.15B; ρ=0.06). The reduction in GSH levels due to treatment was greater in Ogg<sup>−/−</sup> animals (0.7 fold) than those of their WT counterparts (0.9 fold). There were no other significant differences observed.
Figure 6.15: GSH levels in heart tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. GSH levels were significantly higher in vehicle treated WT ♂ (vs. treated KO ♀; p<0.01). No other significant effects were observed.
Figure 6.16: GSH levels in lung tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. A genotype X sex interaction was observed in (p=0.05). GSH levels were significantly higher in vehicle treated animals (vs treatment; p<0.01). No other significant effects were observed.
Figure 6.17: GSH levels in liver tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. A genotype X sex interaction was observed (p<0.01). GSH levels were significantly higher in vehicle treated animals (vs. treatment; p<0.01). No other significant effects were observed.
Figure 6.18: GSH levels in kidney tissues of OGG1 transgenic mice exposed to LPS

Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. A genotype X sex interaction was observed (p<0.01). GSH levels were significantly higher in ♀ animals (vs. ♂; p<0.01). No other significant effects were observed.
Figure 6.19: GSH levels in ileum tissues of OGG1 mice exposed to LPS
Mice were treated with LPS (20 mg/kg i.p.), sacrificed after 12 hours and tissues removed for analysis (mean ± SEM; n=6). Panel A Main effects of treatment, genotype and sex, panels B – D Interactions between treatment and genotype, treatment and sex and genotype and sex respectively. Panel E shows 3-way interactions between treatment, genotype and sex. GSH levels were significantly higher in vehicle treated animals (vs. treated; p<0.01).
6.3. Discussion

Previous work has indicated that the disruption of the OGG1 gene in mice had a protective effect against high dose LPS induced inflammation (Mabley et al., 2005a). Here we show that at lower levels of LPS toxicity, OGG1 disruption can vary levels of cytokine production and other markers of endotoxin induced inflammation in lung and liver tissues, but whether it is a protection from the damaging effects of inflammation varies according to the endpoint measured. Furthermore, there is a strong genotype x sex interaction indicating that whilst many endpoints in male mice indicate increased oxidative damage due to inflammation, female animals showed little or no change (Figure 6.20). As the OGG1⁻/⁻ mice that were previously reported to have been protected from inflammation have been male this contradicts the previously published data (Mabley et al., 2005a; Touati et al., 2006). These results suggest that OGG1 may play a role in cytokine transcription or production, but that this effect may be tempered by a sex linked effect.

Table 6.4 Summary of OGG1 transgenic mouse blood serum cytokine results

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Time (h)</th>
<th>0</th>
<th>1</th>
<th>6</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>IL-4</td>
<td>-</td>
<td>-</td>
<td>*↓</td>
<td>-</td>
<td>*↓</td>
</tr>
<tr>
<td>IL-6</td>
<td>**↓</td>
<td>-</td>
<td>*↓</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>*↑</td>
</tr>
<tr>
<td>IL-12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

** = p≤0.01, * = 0.05≥p>0.01, - = p≥0.10, ↓/↑ = comparison to WT.

Whilst these results are being compared to previous mouse studies using LPS to induce inflammation it is important to note the dose of LPS given to the mice in previous studies. Previous work on OGG1⁻/⁻ animals report administering LPS at 80 mg/kg and incubating for 12 h as a model of septic shock (Mabley et al., 2005a). When trying to duplicate these conditions a large proportion of the mice dosed with these levels of LPS
Table 6.5: Summary of OGG1 organ damage results

<table>
<thead>
<tr>
<th>Primary interactions</th>
<th>Secondary Interactions</th>
<th>Tertiary Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (LPS Treated vs. Vehicle)</td>
<td>Genotype (KO vs. WT)</td>
<td>Sex (♀ vs. ♂)</td>
</tr>
<tr>
<td>Heart</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td><strong>↑</strong></td>
<td><em>↑</em></td>
</tr>
<tr>
<td>Liver</td>
<td><strong>↑</strong></td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td><strong>↑</strong></td>
<td>-</td>
</tr>
<tr>
<td>Ileum</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MPO</td>
<td>Heart</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>*↑</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>~↑</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td><strong>↑</strong></td>
<td>-</td>
</tr>
<tr>
<td>Ileum</td>
<td><strong>↑</strong></td>
<td>-</td>
</tr>
<tr>
<td>MDA</td>
<td>Heart</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td><strong>↓</strong></td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td><strong>↓</strong></td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ileum</td>
<td><strong>↓</strong></td>
<td>-</td>
</tr>
<tr>
<td>GSH</td>
<td>Heart</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td><strong>↓</strong></td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td><strong>↓</strong></td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ileum</td>
<td><strong>↓</strong></td>
<td>-</td>
</tr>
</tbody>
</table>

** = p ≤ 0.01, * = 0.05 ≤ p > 0.01, ~ = 0.10 > p > 0.05, - = p > 0.10, ↓/↑ = comparison.
died before the treatment period was complete, and the remaining animals exhibited signs of severe hypothermia. Furthermore, it has been reported elsewhere that this dose of LPS was fatal in 90% of C57BLK/6 mice, although in this study no mice died before 12 h (Yamashita et al., 2000). To ensure that mortality did not confound the results of the experiment a lower dose of LPS was used. In order to identify levels of LPS toxicity that altered levels of cytokine production, it was found that much lower (~1 mg/kg) levels elicited a response in cytokine production (Hasko et al., 1996; Izeboud et al., 1999).

**Figure 6.20 Pathways involved in the generation and degradation of oxidants and the effects of OGG1 knockout at key endpoints**

In normal mice a rise in myeloperoxidase indicates increased phagocyte activation which release ROS into the affected area. This rise in ROS leads to a drop in GSH as the cell attempts to lessen the oxidative load. Increased ROS leads to the formation of more oxidised lipids in the cell membrane. Key results from lung and liver tissues taken from new OGG1−/− mice coloured blue. Here this model is not followed by either the male or female mice. Phagocyte enzymes coloured red. GSH, Glutathione; GSSG, Glutathione Disulphide; GPX, Glutathione peroxidase. Adapted from (Jaber et al., 2005)

In order to identify changes in MPO, MDA and GSH dose of 20 mg/kg LPS was considered an appropriate amount as it had been used previously to obtain a significant difference in MPO and MDA levels, between treated and untreated animals (Koizumi et al., 2003; Li et al., 2005). This use of a
lower dose of LPS may have contributed to the lack of a treatment effect in the heart, kidney and ileum that had been noted in a previous study of OGG1+/− mice (Mabley *et al.*, 2005a), and may explain why less significant differences were observed overall.

In OGG1+/− male mice the observed significant decrease in IL-6 levels at 1 and 6 h indicate an increase Th1 response, whilst increased levels of the anti-inflammatory cytokine between 6 and 24 h would suggest a reduced inflammatory response, the reduction in IL-4 would suggest a lessened anti-inflammatory response. The increase in MPO activity and MDA also suggests that a larger number of neutrophils were attracted to the lung and liver. These endpoints together indicate that whilst there was an overall reduction in measured pro-inflammatory signals, larger amounts of neutrophils were being attracted to the area. Furthermore, greater amounts of GSH were present where suggesting that either (i) less ROS were being neutralised by (GPX) or SOD (ii) more GSH was being produced within the tissues. As in all tissues except the heart, basal levels of GSH were higher in OGG1+/− male mice than OGG1+/+ male mice (Figures 6.16E – 6.19E; non-significant) hypothesis (ii) seems plausible.

Alternatively in female mouse serum samples, the low basal levels of IL-6 and a slower initial build up of this cytokine leads to reduced levels of both IL-4 and 10, signifying that the initiation of the inflammatory response would be depressed but the decrease in the regulatory anti-inflammatory cytokines IL-4 and IL-10 would suggest that overall innate inflammatory response may be unchanged. The lack of significant change in MPO activity does indeed indicate this (Figure 6.6D & 6.7D). A decrease in GSH however does suggest that either (i) more ROS are being neutralised by glutathione peroxidise (GPX) or that (ii) less glutathione is being produced within the tissues. Basal levels of GSH are lower in all tissues from female mice except the heart (Figures 6.16E – 6.19E; non-significant), leading to a hypothesis that the differing male and female
GSH levels are an adaptation to the scale of the immune response within the animals. Indeed oxidative stress has been shown to up regulate GPX mRNA transcription and activity (Esposito et al., 1999). The lowered MDA levels in the lung and liver tissues from female mice would then be another indication of an increase in ROS neutralisation through the GSH/GSSG pathway.

Oestrogen can act as an antioxidant and this has been shown ex vivo and in vivo (Leal et al., 1998; Prokai et al., 2003; Sugioka et al., 1987; Zhang et al., 2007). During pregnancy, where oestrogen production is increased IL-10 and IL-4 (Th2) activity is observed (Ito et al., 2001). After intratracheal LPS administration hypothermia and airway hyper responsiveness are greater in male mice than female mice (Card et al., 2006). The response markers to LPS induced inflammation (TNFα and MIP1α) in female mice are reduced when compared to those in male mice (Mabley et al., 2005b). Additionally it has been shown that the removal of the ovaries in WT mice nullifies this protection with cytokine activity becoming comparable to that of their male counterparts (Speyer et al., 2005). In our study we see that the knockout of the OGG1 gene seems to be causing increased oxidative damage in males, but this is reduced in females due to the protective effects detailed.

In order to identify mechanisms that may be causing this differing effect of the OGG1 knockout it is important to note that during a state of inflammation the activity of OGG1 is subject to NO mediated inhibition, whilst there is a simultaneous increase in cellular 8-OxoG (Jaiswal et al., 2001). This is due to the redox mediated down regulation of OGG1, and it may be that under the duress of inflammation the cell prioritises the accumulation of 8-OxoG to OGG1 activity (Jaiswal et al., 2000; Jaiswal et al., 2001). One reason proposed for this by Radek and Bodogh (2010) is that in the process of DNA repair OGG1 removes a base causing the creation of single stranded gaps and nicks in the DNA backbone. These
gaps and nicks may then be recognised by DNA-damage-dependent kinases, which can trigger an inflammatory response. Thus in OGG1−/− mice there are fewer gaps created and inflammation may be curtailed. Indeed in the pancreatic islet cells of patients with the inflammatory disease, type II diabetes, there is reduced OGG1 activity in the nucleus whilst mitochondrial DNA repair continues as normal (Radak and Boldogh 2010; Tyrberg et al., 2002).

It has been argued that the increase of 8-oxodG within the cell can reduce inflammation by inhibiting Rac1/2, subsequently regulating the redox-sensitive NF-κβ pathway by the activation of the NADPH oxidase complex (Ock et al., 2011b). Additionally it was shown that the inhibition of Rac1 can affect the STAT3 signalling pathway which is associated with chronic inflammatory diseases (Kim et al., 2006; Ock et al., 2011a).

It has already been shown that female mice are more resistant to oxidative stress than those of males (Du et al., 2004). Here we report that female OGG1−/− mice are more resistant to LPS induced inflammation than male OGG1−/− mice. Could the reduced inflammatory response that is reported in other female mice be reducing the oxidative damage to the cell further so that there are fewer strand breaks, reducing the feed back from gap dependant kinases and thus reducing inflammation more? In order to identify if inflammation increased in the presence of increased single strand breaks, SSB’s could be induced in vivo utilising increased concentrations of LPS or with cells in vitro using H2O2 or UV radiation to induce the breaks (Frankenberg-Schwager et al., 2008).

Additionally this observed effect could be the result of OGG1 interacting with other proteins as gender differences have also been observed in the protective effect of the PARP-1 inhibite mice. Whilst PARP-1 inhibited male mice showed a marked decrease in TNFα output (~50%) after LPS challenge (1 mg/kg i.p.) compared to non-inhibited animals (~1025 vs.
~450 pg/ml). The TNFα output of female mice that were not PARP-1 inhibited began at a lower level and the inhibition of PARP-1 did not change this level (both ~4000 pg/ml). In order to identify if this was a due to the low uninhibited amount of TNFα a further set of female mice were treated with a more robust amount of LPS (30 mg/kg i.p.). Again no significant difference was observed between normal and PARP-1 inhibited serum TNFα concentrations (Mabley et al., 2005b).

In gel shift assays it was further shown that PARP-1 cooperatively interacts with oestrogen receptor-α (ERα), this cooperation is further enhanced in the presence of oestrogen. A model was proposed in which the PARP-1 – ERα – oestrogen complex inhibits the activity of PARP-1 (Figure 6.21; (Mabley et al., 2005b).

![Figure 6.21 Proposed model of PARP-1 inhibition by oestrogen](image)

**Figure 6.21 Proposed model of PARP-1 inhibition by oestrogen**

Whilst the PARP-1 and ERα proteins interact stably on single stranded DNA breaks (top), the addition of oestrogen causes a conformational change in ERα which binds PARP-1 more tightly causing the zinc-finger locating portion of the protein to become less able to locate on the DNA strand reducing the efficiency of DNA repair. Adapted from (Mabley et al., 2005b).

To date no physical interaction between OGG1 and PARP-1 has been identified, but OGG1 acts in the base excision repair pathway ahead of
PARP-1. If the inflammatory effect of PARP-1 is triggered by its detection of DNA breaks then the removal of the OGG1 protein and the subsequent reduction in these breaks would reduce its inflammatory effect. In order to identify if this were the case PARP-1 activity could be assessed by the quantity of poly (ADP-ribose) (PAR) polymers produced in OGG1−/− extracts or in peripheral blood mononuclear cells from OGG1−/− mice (Tentori et al., 2003). If no link were found between OGG1 and PARP-1 it may be interesting to determine if transcription factor activation, such as NF-κB, was altered in OGG1−/− mice, and proceed to identify the process by which inflammation is mediated by OGG1.

If proved to be a modifying factor in inflammation the OGG1 protein could conceivably be targeted as a therapy for combating inflammation based disease. It has the advantage that (in mice) OGG1s disruption has few short term side effects (Klungland et al., 1999). Care would have to be taken however in long term use as older mice experienced higher rates of cancer (Sakumi et al., 2003) and polymorphisms of the gene in human populations have shown variable links with various cancers including lung (Kohno et al., 2006), gastric (Farinati et al., 2008) and prostate cancers (Chen et al., 2003).
7. Overall Discussion

Previously documented literature has demonstrated that the disruption of certain genes connected with the base excision repair pathway reduce the inflammatory response to endotoxin induced inflammation (Mabley et al., 2005a; Virag and Szabo 2002). Here we have investigated the hypothesis that if DNA glycosylases, specifically NEIL1, NEIL2, OGG1 and NTH1, are involved in the regulation of endotoxin induced inflammation, then the knockout of these genes, in murine models, will result in a reduction in the inflammatory response to endotoxin measured by cytokine response and certain oxidative stress markers. In order to investigate this hypothesis a new strain of NEIL1\(^{-/}\) mouse was created. Using MEFs derived from these NEIL1\(^{-/}\) mice, and other DNA glycosylase deficient mice, a reduction in MIP-1\(\alpha\) production was observed in both untreated and LPS treated NEIL1\(^{-/}\), OGG1\(^{-/}\), NTH1\(^{-/}\) and [OGG1/NTH1]\(^{-/}\) cells. Additionally, OGG1\(^{-/}\) cells had higher basal IL-10 levels, and NEIL1\(^{-/}\) cells had lower basal IL-6 output.

*In vivo* at lower levels of LPS toxicity (20 mg/kg), OGG1 disruption varied cytokine production (as measured in blood serum), but this difference differed according to sex: basal IL-6 levels and LPS-induced IL-10 levels were higher but LPS-induced IL-4 levels lower in male OGG1\(^{-/}\) mice. In female OGG1\(^{-/}\) mice there was a decrease in LPS treated levels of IL-10, and in the rate of IL-6 release. There was no significant genotype x treatment interaction in MPO, MDA and GSH results for OGG1\(^{-/}\) mice (Table 7.2). However there was a strong genotype x sex interaction in lung and liver indicating that whilst many endpoints on male OGG1\(^{-/}\) mice indicate increased oxidative damage due to inflammation, female OGG1\(^{-/}\) animals showed little or no change. Male NEIL1\(^{-/}\) mice had higher basal levels of IL-6, and although IL-6 production was slowed, its maximum level was similar to that of WT animals, additionally, after LPS treatment serum IL-12 levels were increased. There were no significant differences
Table 7.1: Summary and comparison of NEIL1 and OGG1 cytokine output results when compared with those of WT animals

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEIL1/−</td>
<td>OGG1/−</td>
<td>NEIL1/−</td>
<td>OGG1/−</td>
<td>NEIL1/−</td>
<td>OGG1/−</td>
<td>NEIL1/−</td>
<td>OGG1/−</td>
<td>NEIL1/−</td>
<td>OGG1/−</td>
<td>NEIL1/−</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>*↓</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

** = p≤0.01, * = 0.05≥p>0.01, ~ = 0.10>p>0.05, - = p≥0.10, ↓↑ = comparison.
Table 7.2: Summary and comparison of NEIL1 and OGG1 organ damage results

<table>
<thead>
<tr>
<th>Primary interactions</th>
<th>Secondary Interactions</th>
<th>Tertiary Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong> (LPS Treated vs. Vehicle)</td>
<td><strong>Genotype</strong> (KO vs. WT)</td>
<td><strong>Sex</strong> (♀ vs. ♂)</td>
</tr>
<tr>
<td>NEIL1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>OGG1&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>NEIL1&lt;sup&gt;+/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heart</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>*↑</td>
<td>**↑</td>
</tr>
<tr>
<td>Liver</td>
<td>-</td>
<td>**↑</td>
</tr>
<tr>
<td>Kidney</td>
<td>-</td>
<td>**↑</td>
</tr>
<tr>
<td>Ileum</td>
<td>*↑</td>
<td>-</td>
</tr>
<tr>
<td><strong>MPO</strong></td>
<td>Heart</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>-</td>
<td>*↑</td>
</tr>
<tr>
<td>Liver</td>
<td>**↑</td>
<td>~↑</td>
</tr>
<tr>
<td>Kidney</td>
<td>*↑</td>
<td>**↑</td>
</tr>
<tr>
<td>Ileum</td>
<td>*↑</td>
<td>-</td>
</tr>
<tr>
<td><strong>MDA</strong></td>
<td>Heart</td>
<td>**↓</td>
</tr>
<tr>
<td>Lung</td>
<td>**↓</td>
<td>*↓</td>
</tr>
<tr>
<td>Liver</td>
<td>**↓</td>
<td>**↓</td>
</tr>
<tr>
<td>Kidney</td>
<td>~↑</td>
<td>-</td>
</tr>
<tr>
<td>Ileum</td>
<td>**↓</td>
<td>*↑</td>
</tr>
</tbody>
</table>

** = p≤0.01, * = 0.05≤p>0.01, ~ = 0.10>p>0.05, - = p≥0.10, ↓/↑ = comparison.
with genotype in the MPO and MDA levels of NEIL1\(^{-/-}\) mice although there was an indication that GSH levels were raised in the heart, lung and liver tissues and decreased in the ileum. These \textit{in vivo} results indicate that at this level of LPS there was a variable change in cytokine production, but that this change is not great enough to change neutrophil recruitment enough to reduce oxidative damage markers.

When cytokine release between OGG1\(^{-/-}\) and NEIL1\(^{-/-}\) mice was compared (Table 7.1) it can be seen that the deletion of DNA glycosylases NEIL1 and OGG1 has similar effects reducing the concentration of the cytokines in IL-6 (male 1 h and female 24 h) and IL-10 (female 6 and 24 h) in serum samples. This indicates that both T\(_{h1}\) and T\(_{h2}\) responses were affected by the knockout of these DNA glycosylases. The differences in male and female responses shown are probably due to the differences in oestrogen levels, as higher physiological levels of oestrogen modulate the immune from a T\(_{h1}\) mediated response to that of T\(_{h2}\) mediated response (Gilmore \textit{et al.}, 1997; Salem, 2004; Whitacre, 2001).

Additionally the interaction between genotype x sex was only observed strongly in the OGG1\(^{-/-}\) mice. There was however, no genotype x treatment interaction observed in the case of either NEIL1\(^{-/-}\) or OGG1\(^{-/-}\) mice, indicating that whilst there was a general change in cytokine activity, neutrophil aggregation and ROS production were not affected significantly. Perhaps this can be explained somewhat by the reduction in MIP-1\(\alpha\) production noted in all knockout MEF cell types, as a reduction in the production of this chemokine would reduce the accumulation of leukocytes.

The pathway by which the NEIL1 and OGG1 proteins modulate the immune system can be surmised to be a different from that of PARP1 as while PARP1 knockout resulted in a reduced output of all measured cytokines, this was not apparent in OGG1\(^{-/-}\) and NEIL1\(^{-/-}\) mice as cytokine
output also increased. The effect of PARP1 may be due to it’s interaction with NF-κβ (Virag and Szabo 2002). Similarly APE1 can also be discounted as it activates NF-κβ via redox reactions meaning a lack of this enzyme would result in a complete reduction in all cytokines (Ando et al., 2008).

It would be interesting to study if the NEIL1 protein is inhibited during inflammatory processes as is OGG1 by NO (Jaiswal et al., 2000; Jaiswal et al., 2001), but currently there is no literature on the subject. If it were found to be so it would add weight to the theory that damage dependant kinases are responsible for the increase in inflammation (Hofseth et al., 2003). The disruption of the OGG1 gene has previously been reported to reduce the inflammatory response (Mabley et al., 2005a; Touati et al., 2006). OGG1 is inhibited in the inflammatory reaction by NO, it is thought that this inhibition is preferable to the creation of gaps in the DNA strand due to the removal of oxidised bases (Jaiswal et al., 2001). These gaps have been implicated in the inflammatory response as their presence actives DNA damage dependent kinases which can trigger inflammation through the activation of p53 (Gudkov et al., 2011). As NEIL1 has been shown to interact with OGG1, indeed it is suggested that OGG1 activity is stimulated in the presence of NEIL1 (Mokkapati et al., 2004a), then the disruption of the NEIL1 gene may reduce inflammatory activity in a similar manner to NO, but on a permanent basis. This may come someway to explaining the change in IL-6 basal and 1 h levels due to reduced initial immune signalling. It may also explain the reduction in MPO activity as there would be less MIP-1α produced during the immune response.

Disruption of bifunctional DNA glycosylases was also found to reduce the basal and LPS induced output of MIP-1α from all BER KO MEFs when compared to WT cells. As MIP-1α is a potent chemokine that attracts neutrophils to the area of infection, this would suggest a decrease in neutrophil activity in vivo. It is interesting to note that basal and LPS stimulated MPO activity was not affected by genotype in either the
NEIL1\textsuperscript{−/−} or OGG1\textsuperscript{−/−} mouse models. It could be the case that in the \textit{in vivo} model there are other regulatory systems available to overcome the effects a potential loss in MIP-1\textalpha output. Additionally, OGG1\textsuperscript{−/−} cells had higher basal IL-10 levels, whilst in the whole animal model this was also not observed. Indeed NEIL1\textsuperscript{−/−} cells had lower basal IL-6 output, whilst the male animals showed a marked increase in basal rates of IL-6. In line with previously published material detailing the use of MEF’s, the cells were not used after passage 10 so as to reduce the risk of possible mycoplasma infections and mutations being the cause of any reported effects (Khobta \textit{et al.}, 2009).

During the course of this study two new strains of mice were created, namely a NEIL1\textsuperscript{−/−} mouse and a putative NEIL2\textsuperscript{−/−} mouse. Assays examining the NEIL1\textsuperscript{−/−} genomic DNA (PCR), mRNA (RT-PCR) and protein (western blot) have confirmed the success of this model. As a previous colony of NEIL1\textsuperscript{−/−} mice had displayed a sporadic phenotype that shared similar symptoms with the human metabolic syndrome, a colony of these animals was kept for an extended period of time in order to discern if a similar phenotype would be observed (Vartanian \textit{et al.}, 2006). Whilst there was no significant difference in overall weight between the NEIL1\textsuperscript{+/+} and NEIL1\textsuperscript{−/−} mice, a significant difference was observed in the weight of ommentum fat surrounding the intestinal tract of the NEIL1\textsuperscript{−/−} male mice. This indicates less overall fat in these animals, showing that the obese phenotype did not appear in these NEIL1\textsuperscript{−/−} mice. There is however data indicating that the size of the adrenal glands was increased in NEIL1\textsuperscript{−/−} mice and that the reproductive organs of the NEIL1\textsuperscript{−/−} female mice was increased whilst the testes size of male NEIL1\textsuperscript{−/−} mice was decreased. This suggests that there may be hormonal differences in the mice including raised oestrogen levels in the NEIL1\textsuperscript{−/−} female knockout animals and lowered testosterone levels in the NEIL1\textsuperscript{−/−} male mice as changes in the production of the appropriate hormone effects the size of these organs (Kenagy and Trombulak 1986; Wood, 2000). Interestingly it has been
observed that in cases of obesity in humans the concentration of blood born testosterone decreases and oestrogen increases (Kley et al., 1979). It was not discerned in this study whether the increase in adrenal gland size raised adrenaline output, as some diseases such as Phenochromocytoma do increase adrenal size and output (Strong et al., 2008), whereas Addison’s disease does not but it increases the cortex layer around the gland reducing the size of the adrenaline producing medulla (Zelissen et al., 1995).

There were overall differences in the tissue GSH content in that levels were lower in the heart, lung and liver of NEIL1−/− mice, but elevated in the ileum. In order to investigate why there could be changes in the GSH levels but not in MPO or MDA levels, other known differences in the NEIL1−/− mice were examined. As both male and female animals produce both testosterone and oestrogen, the reduction in male testes weight and increase in female sex organ weight suggest a shift in the ratio of testosterone:oestrogen toward a larger proportion of oestrogen in both male and female NEIL1 mice. However as oestrogen has been shown to be an antioxidant this would not account for lowered levels of GSH (Leal et al., 1998; Prokai et al., 2003; Sugioka et al., 1987; Zhang et al., 2007).

It is also possible that a change in the levels of serum adrenaline, suggested by the increased adrenal gland size, could alter the levels of ROS and therefore GSH within the tissues mentioned. As adrenaline affects the fight or flight response an increase in its output affects different organs and systems in different ways. Heart rate and lung respiration increase, causing a raised generation of ROS in those organs (Sarker et al., 2009). In the liver an increase in adrenaline stimulates glycogenolysis, and increases mitochondrial ROS creation via NADPH oxidase (Diaz-Cruz et al., 2007) The presence of adrenaline additionally reduces the metabolic activity in ileum tissues by restricting blood flow to the area potentially reducing the creation of ROS (Konstantinidis et al.,
This suggests GSH levels may be altered by changes in metabolism due to an increased production of adrenaline (Figure 7.1). If an overproduction of adrenaline is the reason for the changes in GSH levels, it does not explain a lack in an increase in MDA. Perhaps the increase of adrenaline is great enough to elicit a change in GSH levels, but that this level of oxidation is neutralised by cellular antioxidants before significant lipid oxidation can occur.

![Adrenaline Diagram]

**Figure 7.1: Alterations in organ activity due to adrenaline and observed levels of GSH in NEIL1−/− mice**

An increase in adrenaline activates the fight or flight responses in mammalian systems, triggering increased activity in organs such as the heart, lung and liver and reducing activity in areas that are not required for immediate gain such as the gut. In organs where activity is increased one would expect an increase in ROS production as a by product of metabolism, resulting in a reduction in cellular GSH. Conversely in the gut where activity is decreased one may expect oxidative stress to be reduced due to a reduction in metabolism.

Classically, raised adrenalin levels lead to hypertension and eventually kidney failure (Franco-Morselli et al., 1977), Phenochromocytoma, a rare cancer of the adrenal gland, which also causes raised adrenaline levels results in an increased heart rate, increased blood glucose and weight loss (Strong et al., 2008). In order to identify if there are raised adrenalin
levels in the NEIL1<sup>−/−</sup> mice serum levels of adrenaline should be measured. Alternatively studies could be performed around the previously mentioned symptoms, namely the measurement of the mouse’s blood pressure, blood glucose and serum creatine (to measure kidney function) which would all be good measures of systemic damage due to over production of adrenaline (Dunn <i>et al.</i>, 2004). Our NEIL1<sup>−/−</sup> mice have shown no overt change in weight, though previously described NEIL1<sup>−/−</sup> mice have shown increases in weight and additionally the previous mice were noted to be less active, spending fewer hours each day in voluntary exercise (Sampath <i>et al.</i>, 2011; Vartanian <i>et al.</i>, 2006).

If the absence of the NEIL1 protein does indeed cause an increase in adrenal output it could suggest links with the activation of adrenocorticotropic hormone (ACTH), implicating a connection with the central nervous system (Wilson III and McNeill 2007). NEIL1 mRNA is transcribed in brain tissue in a moderate amount when compared to other tissues (Hazra <i>et al.</i>, 2002a). It has been reported that whilst the expression of other BER proteins, OGG1 and APE-1, decline by 40 - 50% in rat brain from the embryonic (1 day prior to birth) to the young adult stage (3 months), in the same period NEIL1 and NEIL2 expression increased (1.5 and 2.5 fold respectively (Englander and Ma 2006). Additionally in mice, mitochondrial NEIL1 expression in the cortex increased 1.7 fold between adulthood (5 months) and middle age (10 months), whilst there was no increase in its expression in the hippocampus over the same time period, indicating that if there were some link between NEIL1 and brain activity it would most likely be connected to the cortex region. This was not specific to NEIL1 and in the same mice similar increases were detected in NEIL2, NTH1, OGG1 and APE-1 expression. These changes in expression have been suggested as being a response to a build up in oxidative damage as the brain ages, and could indicating why mRNA instability and neurodegenerative diseases are more common in the hippocampus (Gredilla <i>et al.</i>, 2010). Whilst the first
of the two studies implicates that NEIL1 and NEIL2 are important in the mature brain, the second study indicates that the specific difference is not in the mitochondria of either the cortex or hippocampus, suggesting that the ability to repair within the bubble structure genomic DNA is important to the mature brain. It would be interesting to identify which areas of the brain, rather than just general brain tissue, show marked difference in BER protein expression, and how the disruption of DNA glycosylases affects their function.

When attempting to confirm the success of the NEIL2 knockout only the PCR of genomic DNA suggested that NEIL2\(^{-/-}\) mice had been generated and that the Tk/Neo cassette was present in the genomic DNA, and in the correct position. However, RT-PCR indicated that undisrupted mRNA was being produced and western blots indicated that a protein of the appropriate size was present that bound to anti-NEIL2 IgG. This is a discrepancy that at the moment we are unable to explain with any certainty. Further investigation into the positioning of the Tk/Neo cassette could be performed using techniques such as fluorescence \textit{in situ} hybridization, this may not result in this NEIL2 model being validated as the western blot and RT-PCR confirm the presence of NEIL2 protein, but it would confirm the chromosomal positioning of the insert perhaps leading to greater understanding of the reason that the knockout was unsuccessful.

Further understanding of the role of DNA glycosylases in the inflammatory response to endotoxin could be elucidated by the performing of further experiments upon the organs that had already been analysed for MPO, MDA and GSH. Portions of these organs have been fixed in formalin solution which would make it possible for histological analysis of the samples to be performed. This would be useful in identifying and phenotyping the infiltrating cells to the areas of inflammation.
In order to further examine if a DNA glycosylase affects the inflammation response to endotoxin it may be useful to perform some additional work on double DNA glycosylase knockout mice. This is due to the redundancy built into the base excision repair system. Interesting pairings to look at may would be those with similar substrates such as [OGG1/NEIL1]^{+/-} and [NEIL1/NTH1]^{+/-}. A [NEIL1/NTH1]^{+/-} mouse has already been produced and has shown particular susceptibility to pulmonary and hepatic tumours (Chan et al., 2009).

As a new NEIL1^{-/-} model has been described that does not seem to exhibit the same phenotype as previously presented models it may be interesting to identify if the DNA damage response is effected in a similar fashion. In MEF cells treated with DNA damaging agents such as H2O2 this could be examined using comet assays. To compare the accumulation of oxidised bases, such as FapyA, FapyG and 8-oxo-G, over the course of a lifetime gas chromatography/mass spectrometry could be used.

In conclusion we have found that the deletion of DNA glycosylases has led to reduction in cytokine outputs (IL-6 and IL-10) when challenged with LPS but also had very little effect on the physiological markers MPO, MDA and GSH. This indicates that whilst the disruption of these genes may reduce inflammatory signalling this reduction is not great enough to lessen the oxidative stress caused during the inflammatory response. This could be due to the levels of LPS used to elicit the response in our experiments. Indeed the sex of the subjects seemed to have a more protective effect on inflammatory response. These results point to DNA glycosylases being a poor target for anti-inflammatory therapies in all but the severest of cases.

Furthermore the previously reported obesity phenotype in the NEIL1^{-/-} mice was not noted in this strain of mice, although there is evidence of possible hormonal changes, specifically increased oestrogen and
adrenaline, in the animals. The results for tissue GSH in NEIL1−/− mice we observed a pattern similar to that of raised adrenal output.
8.0 References


Arai T, Kelly VP, Minowa O, Noda T and Nishimura S (2006) The study using wild-type and Ogg1 knockout mice exposed to potassium bromate shows no tumor induction despite an extensive accumulation of 8-hydroxyguanine in kidney DNA. *Toxicology.* **221** (2-3) 179 - 186


Bruskov SVI, Malakhova LV, Masalimov ZK and Chernikov AV (2002) Heat-induced formation of reactive oxygen species and 8-oxoguanine, a biomarker of damage to DNA. *Nucleic Acids Research.* **30** (6) 1354 - 1363


Conlon KA, Zharkov DO and Berrios M (2004) Cell cycle regulation of the murine 8-oxoguanine DNA glycosylase (mOGG1): mOGG1 associates with microtubules during interphase and mitosis. *DNA Repair (Amst).* **3** (12) 1601 - 1615


damage by the DNA glycosylase NEIL1. *J. Biol. Chem.* **282** (36) 26591 - 26602


Droge W (2002) Free radicals in the physiological control of cell function. *Physiol Rev.* **82** (1) 47 - 95


Girard PM, D'Ham C, Cadet J and Boiteux S (1998) Opposite base-dependent excision of 7,8-dihydro-8-oxoadenine by the Ogg1 protein of Saccharomyces cerevisiae. *Carcinogenesis.* **19** (7) 1299 - 1305


Harrison RF, Reynolds GM and Rowlands DC (1993) Immunohistochemical evidence for the expression of proliferating cell nuclear antigen (PCNA) by non-proliferating hepatocytes adjacent to metastatic tumours and in inflammatory conditions. *J. Pathol.* **171** (2) 115 - 122

Hasday JD, Bascom R, Costa JJ, Fitzgerald T and Dubin W (1999) Bacterial endotoxin is an active component of cigarette smoke. *Chest.* **115** (3) 829 - 835

Hashiguchi K, Stuart JA, de Souza-Pinto NC and Bohr VA (2004) The C-terminal alphaO helix of human Ogg1 is essential for 8-oxoguanine DNA glycosylase activity: the mitochondrial beta-Ogg1 lacks this domain and does not have glycosylase activity. *Nucleic Acids Res.* **32** (18) 5596 - 5608


Jaiswal M, LaRusso NF, Burgart LJ and Gores GJ (2000) Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. *Cancer Res.* 60 (1) 184 - 190


Kley HK, Solbach HG, McKinnan JC and Kruskemper HL (1979) Testosterone decrease and oestrogen increase in male patients with obesity. *Acta Endocrinol. (Copenh).* 91 (3) 553 - 563


Lee FD (1992) The role of interleukin-6 in development. Dev. Biol. 151 (2) 331 - 338


Lloyd DR and Phillips DH (1999) Oxidative DNA damage mediated by copper(II), iron(II) and nickel(II) fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-


Martin SP, Hortelano S, Bosca L and Casado M (2006) Cyclooxygenase 2: understanding the pathophysiological role through genetically altered mouse models. Front Biosci. 11 2876 - 2888


Matsukawa A, Yoshimura T, Miyamoto K, Ohkawara S and Yoshinaga M (1997) Analysis of the inflammatory cytokine network among TNF alpha, IL-1 beta, IL-1 receptor antagonist, and IL-8 in LPS-induced rabbit arthritis. Lab Invest. 76 (5) 629 - 638

Maynard S, Schurman SH, Harboe C, de Souza-Pinto NC and Bohr VA (2009) Base excision repair of oxidative DNA damage and association with cancer and aging. Carcinogenesis. 30 (1) 2 - 10


Moller BS and Villiger PM (2006) Inhibition of IL-1, IL-6, and TNF-alpha in immune-mediated inflammatory diseases. *Springer Semin. Immunopathol.* **27** (4) 391 - 408


Vaara M (1999) Lipopolysaccharide and the Permeability of the Bacterial Outer Membrane. **10** (2) 31 - 38


Ye SM and Johnson RW (2001) An age-related decline in interleukin-10 may contribute to the increased expression of interleukin-6 in brain of aged mice. *Neuroimmunomodulation.* **9** (4) 183 - 192


