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Investigating the Role of Inflammatory Caspases during Disease-Associated Inflammation

Katarzyna Oficjalska M.Sc.

A PhD thesis submitted to Trinity College Dublin
as completion of the degree of Doctor of Philosophy

Supervisor: Dr. Emma Creagh

School of Biochemistry and Immunology
Trinity College Dublin
2015
Declaration of authorship

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work. I agree to deposit this thesis in the University’s open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.
Acknowledgements

First and foremost, I would like to sincerely thank my supervisor, Dr. Emma Creagh whose support during this PhD study was invaluable. I’m very grateful for the opportunity to have worked on this very interesting project and for all her help and valuable guidance throughout the whole PhD process. I’m extremely grateful that Emma afforded me so much great advice and was always willing to discuss all the details of any experiments, no matter how big or small. I really appreciate Emma’s guidance and willingness to direct me down scientific pathways that facilitated the findings we obtained on this fascinating study throughout the four years. I also really appreciate her patience and assistance during the writing of this thesis. I feel very lucky that I could be a part of her research group and I can’t imagine having a better supervisor than her!

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Moreover, I would like to thank my parents who have always encouraged me to study and they were proud of my decision when I decided to do a PhD. I also want to
thank my best friend Martynka for her uplifting and positive advice and also for the fact that I can always count on her in any kind of life circumstances.

Last but not least, I would like to thank my boyfriend Brian for his constant encouragement, extreme patience, motivational and inspirational talks throughout this study. He has always been a great partner, perfect soulmate and a true best friend!
Abstract

Caspases are a group of proteolytic enzymes involved in the co-ordination of cellular processes such as inflammation and apoptosis. Functional mechanisms surrounding the activation and signalling pathways mediated by inflammatory caspases (such as caspase-1, -4 and -5) are being intensively researched at present. It is well established that canonical inflammasome mediated caspase-1 activation promotes the maturation and secretion of pro-inflammatory cytokines such as IL-1β and IL-18, which further amplifies the pro-inflammatory immune response. However, activation of the non-canonical inflammasome (mediated by human caspase-4 and -5 or their murine homolog, caspase-11), serves as an additional pathway for enhancing canonical inflammasome activation in response to Gram-negative bacterial infection. This highlights the crucial importance of these caspases during the host immune response and caspase-1 regulation. As canonical activation of inflammasome signalling is a critical modulator of immune system homeostasis, its dysregulation leads to the development of a multitude of inflammatory disorders and also inflammatory associated cancers. Altered activity of caspase-1 and the canonical inflammasome has been implicated in the development of intestinal inflammatory diseases such as inflammatory bowel disease (IBD) and colitis associated cancer (CAC). However, there is still little known regarding the role of murine caspase-11 and human caspase-4 and -5 during acute and chronic disease associated inflammation of the gastrointestinal tract (GI).

Herein the first part of this study, the involvement of murine caspase-11 and the non-canonical inflammasome during acute intestinal inflammation was investigated using a mouse model of IBD - the acute dextran sodium sulfate (DSS)-induced colitis model. It was found that caspase-11-mediated non-canonical inflammasome is activated in mice during acute DSS-induced intestinal inflammation. Caspase-11 knockout mice displayed enhanced susceptibility to DSS colitis, which was associated with impaired IL-18 production. The attenuated caspase-11-mediated IL-18 production was linked with reduced intestinal epithelial cell proliferation and increased cell death, thus identifying the importance of the non-canonical inflammasome in the maintenance of epithelial barrier integrity. Moreover, examination of the signalling events that led to the upregulation and activation of caspase-11 in vivo revealed a novel requirement for type II IFNs during experimentally induced acute colitis. Collectively our data indicates
that type II IFN-mediated caspase-11 upregulation plays a key role in maintaining intestinal epithelial barrier in vivo during acute colitis.

The second part of this project was focused on interrogating the role of human inflammatory caspases-1, -4 and -5 in chronic inflammatory diseases, such as Inflammatory bowel disease (IBD) and Barrett’s oesophagus (BO), and their contribution to the progression of these diseases to colorectal and oesophageal cancers, respectively. IHC analysis of caspase expression revealed that caspase-4 and -5 contribute significantly to the inflammatory status of IBD patients, implicating these caspases as novel modulators of intestinal inflammation. This study also demonstrates epithelial caspase-4 and -5 expression exclusively in neoplastic tissue of CAC patients, identifying caspases-4 and -5 as potentially specific diagnostic biomarker of colorectal carcinoma. This study also revealed significant alterations in caspase-1 and -5 expression levels in Barrett’s oesophagus patients that developed oesophageal adenocarcinoma (OAC), suggesting that caspases-1 and -5 are involved in pathogenesis of oesophageal inflammation, metaplasia and the cancer development. It would appear that caspase-1, -4 and -5 may play a crucial role in invasion of poorly differentiated adenocarcinomas of the oesophagus. To our knowledge, this is the first report which has examined caspase-4 and -5 expression levels in the context of these chronic inflammatory diseases and their progression to cancers.

Our data strongly indicates the crucial involvement of inflammatory caspases during acute and chronic disease associated inflammation of the gastrointestinal tract (GI).
Publications


* These authors contributed equally to this work.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Acidic transactivation domain</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AIM2</td>
<td>Absent in melanoma 2</td>
</tr>
<tr>
<td>A1M2</td>
<td>Absent in melanoma 2</td>
</tr>
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<td>AIP1</td>
<td>Actin-interactin protein 1</td>
</tr>
<tr>
<td>AOM</td>
<td>Azoxymethan</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APS</td>
<td>Adenosine-5'-phosphosulfate</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis speck-like protein containing a CARD</td>
</tr>
<tr>
<td>ASP</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>ATG16L1</td>
<td>Autophagy related 16-like 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-triphosphate</td>
</tr>
<tr>
<td>B cell</td>
<td>Hematopoietic stem cells that matured in the bone-marrow</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculoviral inhibitory repeat</td>
</tr>
<tr>
<td>BMDMs</td>
<td>Bone marrow derived macrophages</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone morphogenetic protein 4</td>
</tr>
<tr>
<td>BO</td>
<td>Barrett's oesophagus</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>CAC</td>
<td>Colitis-associated cancer</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CCR6</td>
<td>Chemokine (C-C motif) receptor 6</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's disease</td>
</tr>
<tr>
<td>CDX</td>
<td>Caudal-type homeodomain transcription factor</td>
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<tr>
<td>cIAP1</td>
<td>Cellular inhibitor of apoptosis protein-1</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclo-oxygenase-2</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CRI</td>
<td>Cancer related inflammation</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera toxin B</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>DAI</td>
<td>Disease activity index</td>
</tr>
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<td>DAMP</td>
<td>Danger-associated molecular pattern</td>
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<td>Description</td>
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<td>--------------</td>
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<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
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<tr>
<td>DED</td>
<td>Death effector domain</td>
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<td>Defcr</td>
<td>Defensin-related cryptdin</td>
</tr>
<tr>
<td>dH2O</td>
<td>Distilled Water</td>
</tr>
<tr>
<td>DKO</td>
<td>Double knockout</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran sodium sulfate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM1</td>
<td>Extracellular matrix protein 1</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EHEC</td>
<td>Enterohaemorrhagic Escherichia coli</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FGF2</td>
<td>Fibroblast growth factor 2</td>
</tr>
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<td>FITC-dextran</td>
<td>Fluorescein isothiocyanate–dextran</td>
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<tr>
<td>GERD</td>
<td>Gastroesophageal reflux disease</td>
</tr>
<tr>
<td>GBPs</td>
<td>Guanylate-binding proteins</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HD-5</td>
<td>Defensin, alpha 5</td>
</tr>
<tr>
<td>HD-6</td>
<td>Defensin, alpha 6</td>
</tr>
<tr>
<td>HGD</td>
<td>High grade dysplasia</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-mobility group box 1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>HSP</td>
<td>Heat-shock protein</td>
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<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleukin 1beta converting enzyme</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<td>Ich-3</td>
<td>ICE and CED homolog-3</td>
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<td>IEC</td>
<td>Intestinal epithelial cells</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IgA</td>
<td>Immunoglobulin A</td>
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<td>IGF-1</td>
<td>Insulin like growth factor-1</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intestinal metaplasia</td>
</tr>
<tr>
<td>i.p injection</td>
<td>Intraperitoneal injection</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>IRGM</td>
<td>Immunity-related GTPase family, M</td>
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<tr>
<td>IVC</td>
<td>Individually ventilated cage</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>C-Jun-N-terminal kinase</td>
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<tr>
<td>LGD</td>
<td>Low grade dysplasia</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeat</td>
</tr>
<tr>
<td>LUBAC</td>
<td>Linear ubiquitination assembly complex</td>
</tr>
<tr>
<td>MAL</td>
<td>MyD88 adaptor-like protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>Max</td>
<td>Myc-associated factor X</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
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<tr>
<td>MDP</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
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<td>MST1</td>
<td>Macrophage stimulating 1 (hepatocyte growth factor-like)</td>
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<tr>
<td>MSU</td>
<td>Monosodium urate monohydrate</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>Myc</td>
<td>Myelocytomatosis oncogene</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NACHT</td>
<td>NAIP, CIITA, HET-E and TP1</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NAIP</td>
<td>NLR family, apoptosis inhibitory protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NAT</td>
<td>Neoadjuvant therapy</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
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<td>NLRC4</td>
<td>NLR family CARD domain-containing 4</td>
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<td>NLRP</td>
<td>NACHT, LRR and PYD domains-containing protein</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NLR family, pyrin domain-containing 3</td>
</tr>
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<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
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<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory</td>
</tr>
<tr>
<td>OAC</td>
<td>Oesophageal adenocarcinoma</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PARP1</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>Polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin domain</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-like receptor</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNI</td>
<td>Reactive nitrogen intermediates</td>
</tr>
<tr>
<td>RONS</td>
<td>Reactive oxygen and nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>SARM</td>
<td>Sterile-α and armadillo repeat containing molecule</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium-dodecyl sulphate</td>
</tr>
<tr>
<td>SNP</td>
<td>Single-nucleotide polymorphism</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol Regulatory Element Binding Protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T cell</td>
<td>Hematopoietic stem cells that matured in the thymus</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris base, acetic acid and EDTA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor beta 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper type 2 cells</td>
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<tr>
<td>TICAM</td>
<td>Toll-IL-1-homology domain-containing adaptor molecule</td>
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<td>TIR</td>
<td>Toll/Interleukin-1 receptor</td>
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<td>TIRAP</td>
<td>TIR domain containing adaptor protein</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNBS</td>
<td>Trinitrobenzene sulfonic acid</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
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<td>TNM</td>
<td>Tumour-nodes-metastasis</td>
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<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
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<td>TRIF</td>
<td>TIR domain containing adaptor inducing interferon-β</td>
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<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
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<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labelling</td>
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<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultraviolet B</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>XBPI</td>
<td>X box binding protein</td>
</tr>
<tr>
<td>YVAD-fmk</td>
<td>Carbobenzoxy-tyrosyl-valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone</td>
</tr>
</tbody>
</table>
# Table of contents

Declaration of authorship........................................................................................................ i  
Acknowledgements.................................................................................................................. ii  
Abstract.................................................................................................................................. iv  
Publications.............................................................................................................................  vi  
Abbreviations............................................................................................................................ vii  

**Chapter 1: Introduction**

1.1 Immunity (Innate Immunity) .......................................................................................... 1  
  1.1.1 Pattern recognition receptors (PRRs) ................................................................. 2  
  1.1.2 Toll like receptors (TLRs) .................................................................................... 2  
  1.1.3 NOD like receptors (NLRs) .................................................................................. 6  
1.2 Caspases........................................................................................................................... 9  
  1.2.1 Gene orthology and chromosomal localization of inflammatory caspases ......... 12  
1.3 Inflammatory Caspase-1 .............................................................................................. 15  
  1.3.1 Caspase-1 and the canonical NLRP3 inflammasome ............................................ 16  
1.4 Murine caspase-11 and the non-canonical inflammasome ......................................... 20  
1.5 Inflammatory caspase-4 and -5 .................................................................................. 25  
1.6 Intestinal inflammation ................................................................................................. 27  
1.7 Inflammatory Bowel Disease (IBD) .......................................................................... 28  
1.8 Mouse models of IBD (DSS-induced colitis model).................................................. 32  
1.9 Role of the innate immune system in the development of IBD................................. 33  
  1.9.1 Role of TLRs in IBD ............................................................................................ 36  
  1.9.2 Role of NLRs in IBD .......................................................................................... 37  
  1.9.3 NLRP3 in IBD ..................................................................................................... 39  
  1.9.4 Role of inflammatory caspases in IBD ................................................................. 41  
1.10 IBD chronic inflammation – a cause of colitis associated cancer (CAC) development.................................................................................................................. 42  
1.11 From Barrett’s oesophagus to OAC – a role for inflammation ................................ 45  
1.12 Project aims................................................................................................................... 50  

xii
Chapter 2: Materials and Methods

2.1 Animals ........................................................................................................................... 51
  2.1.1 Genotyping of Casp11−/−, Casp11+/− and Casp11+/+ C57BL/6J mice ......................... 52
    2.1.1.1 Isolation of genomic DNA from mouse ear punches ...................................... 52
    2.1.1.2 Polymerase Chain Reaction (PCR) ................................................................. 52
    2.1.1.3 DNA Gel Electrophoresis ................................................................................ 54
  2.1.2 Induction, treatment and assessment of DSS-induced colitis ........................................ 54
    2.1.2.1 DSS-induced colitis in WT and Casp11−/− mice ............................................. 54
    2.1.2.2 Exogenous IL-18 and IFN-γ treatment ........................................................... 55
  2.1.3 Histology ................................................................................................................... 56
    2.1.3.1 Tissue processing ............................................................................................... 56
    2.1.3.2 Hematoxylin and eosin (H&E) staining .......................................................... 57
    2.1.3.3 H&E histological analysis ................................................................................ 57
  2.1.4 Immunohistochemistry ............................................................................................ 58
    2.1.4.1 Immunofluorescence staining ........................................................................... 58
    2.1.4.2 In situ intestinal proliferation assay ................................................................. 58
  2.1.5 Cell death assay (TUNEL staining) ........................................................................ 59
  2.1.6 FITC-dextran assessment of intestinal permeability ............................................. 59
  2.2 Measurement of cytokine concentration by ELISA .................................................. 60
    2.2.1 Preparation of samples from colon homogenates ................................................ 60
    2.2.2 BCA protein assay ................................................................................................ 60
    2.2.3 ELISA assay ........................................................................................................ 61
  2.3 Western blotting ............................................................................................................. 63
    2.3.1 Preparation of samples from colon homogenates ................................................ 63
    2.3.2 SDS-Polyacrylamide Gel Electrophoresis ............................................................. 63
    2.3.3 Transfer of proteins to blotting membrane ............................................................ 64
    2.3.4 Antibody blotting ................................................................................................ 64
  2.4 Quantitative PCR ........................................................................................................... 66
    2.4.1 RNA isolation from colon tissue ........................................................................... 66
    2.4.2 mRNA purification from colon tissue ................................................................... 67
    2.4.3 Reverse transcription and real time-PCR reaction ............................................. 67
Chapter 3:
Investigating the role of Caspase-11 in a murine model of DSS-induced colitis

3.1 Introduction........................................................................................................... 76
3.2 Results.................................................................................................................... 78
  3.2.1 Genotyping of Casp11\(^{+/+}\), Casp11\(^{-/-}\) and Casp11\(^{+/+}\) C57BL/6J mice ...... 78
  3.2.2 Characterisation of caspase-11 knockout mice and the canonical inflammasome pathay............................................................. 79
  3.2.3 Increased susceptibility of caspase-11\(^{-/-}\) mice to DSS induced colitis .......... 80
  3.2.4 Analysis of cytokine levels in colon homogenates of 2\% DSS treated WT and Casp11\(^{-/-}\) mice by ELISA .................................................. 84
  3.2.5 IL-18 rescues the colitis susceptibility phenotype of caspase-11\(^{-/-}\) mice ....... 85
3.3 Discussion............................................................................................................. 88

Chapter 4:
Functional and mechanistic insights into the regulation of caspase-11 in a DSS-induced mouse model of colitis

4.1 Introduction........................................................................................................... 111
4.2 Results.................................................................................................................... 113
  4.2.1 Caspase-11 is required for preservation of epithelial barrier integrity after DSS administration ................................................................. 113
4.2.2 Caspase-11 mediated IL-18 production results in IEC proliferation .......... 113
4.2.3 Increase in epithelial barrier damage in DSS treated Casp11−/− mice is associated with an increase in cell death ................................................................. 115
4.2.4 Increased epithelial barrier damage of Casp11−/− is not associated with IL-22 mediated induction of anti-microbial peptides during colitis (such as Reg3β and Reg3γ) ......................................................................................................................... 116
4.2.5 DSS-colitis induces caspase-11 upregulation and is independent of TRIF and type I IFNs ............................................................................................................. 116
4.2.6 A potentially important role for Mal signalling in caspase-11 upregulation during DSS colitis ........................................................................................................... 118
4.2.7 Type II IFN signalling mediates caspase-11 upregulation during DSS colitis. 119
4.3 Discussion ............................................................................................................... 121

Chapter 5:
Investigation of inflammatory caspase expression patterns in inflammatory diseases including IBD, Barrett’s oesophagus (BO) and inflammatory associated cancers
5.1 Introduction ............................................................................................................. 138
5.2 Results .................................................................................................................... 142
5.2.1 Inflammatory caspase gene expression in IBD (Belgian cohort) ................. 142
5.2.2 Inflammatory caspase expression in IBD (Irish cohort) ............................... 142
5.2.2.1 Correlation between clinical disease and inflammation scores in UC patients ................................................................. 142
5.2.2.2 IHC analysis of inflammatory caspase expression in UC patients ........ 143
5.2.3 Caspase inhibition reduces inflammatory cytokine secretion from ulcerative colitis (UC) patient biopsies ................................................................. 144
5.2.4 Inflammatory caspase expression in colitis associated cancer (CAC) tissues (Irish cohort) ................................................................. 145
5.2.5 Inflammatory caspase expression in tissues of Barrett’s metaplasia and oesophageal adenocarcinoma (OAC) patients (Irish cohort) ................. 146
5.2.6 Analysis of the inflammatory caspase expression in OAC tumour tissues ..... 147
5.3 Discussion ............................................................................................................. 150
Chapter 1
Introduction
1.1 Immunity (Innate Immunity)

The human immune system consists of two distinct branches - innate and adaptive immunity. Coherent cooperation between both is crucial to eliminate infections that occur after breaching of the anatomic and physiological barriers by pathogens. The innate immune system, the evolutionarily older defence system acts as a first line of defence by sensing and eliminating pathogens during infection. However, when the innate immune system is overwhelmed, it triggers and directs the adaptive arm, thus activating specific B and T cells for pathogen clearance (Turvey and Broide, 2010).

The innate immune system makes use of several mechanisms to counter microbial invasion including: (i) the anatomical barriers such as the skin and mucous membranes that mechanically prevent dispersion throughout the body; and (ii) opsonisation and removal of the invading factor by the complement system and pattern recognition receptors (PRR). PRRs are expressed by many cell types such as macrophages, monocytes, dendritic cells (DC), neutrophils and epithelial cells. They facilitate early detection of a wide range of pathogens (directly at the site of infection) by recognising conserved microbial components called pathogen-associated molecular pattern (PAMPs). PAMPs are small molecular motifs conserved within classes of microbes which are crucial for their survival and pathogenicity. PAMPs can be of diverse origins including: cell wall components such as peptidoglycan (PGN) and lipopolysaccharide (LPS), flagellin, lipoproteins, RNA and DNA of bacteria, fungi and viruses (Takeuchi and Akira, 2010). However, the activation of innate immunity is not only based on the recognition of PAMPs but also relies on the presence of danger signals or danger-associated molecular patterns (DAMPs) released by injured cells. DAMPs are a set of a host-derived molecules that signal cellular stress, damage or non-physiological cell death and examples include DNA-binding proteins such as High-mobility group box 1 (HMGB1), heat-shock proteins (HSPs), extracellular ATP, and uric acid crystals (Strowig, Henao-Mejia et al. 2012). Sensing DAMPs by the innate immunity not only allows the recognition of an ongoing infection/injury and subsequent recruitment of more immune cells, but also can initiate the repair of the damaged tissue.

Overall, upon recognition of diverse PAMPs and DAMPs, the innate immune system initiates the inflammatory response by secreting cytokines and chemokines and recruits immune cells to the site of infection to clear and trigger the immune responses.
1.1.1 Pattern recognition receptors (PRRs)

PRRs are essential for detecting invading pathogens and initiating the innate and adaptive immune responses. There are four main classes of PRRs recognized to date including: (i) Toll-like receptors (TLRs), (ii) C-type lectin receptors (CLRs); (iii) NOD-like receptors (NLRs); and (iv) RIG-like receptors (RLRs) (Martinon and Tschopp, 2005). Since a single pathogen can simultaneously activate multiple PRRs, crosstalk between different receptors may also play a role in enhancing or inhibiting the immune response. Therefore, tight regulation of PRR signalling is required in order to eliminate infectious pathogens and at the same time, prevent aberrant or excessive PRR activation, which can lead to the development of inflammatory and autoimmune disorders. This research project focuses on inflammatory caspase activation, which is mediated via TLR and NLR signalling events. Therefore more detailed discussion of these, most relevant PRRs is also included.

1.1.2 Toll like receptors (TLRs)

TLRs are a class of functional type-I transmembrane glycoproteins expressed by a wide spectrum of cells ranging from myelomonocytic cells to endothelial and epithelial cells. They are involved in regulation of innate and adaptive immune responses and have an essential role in inflammation. The TLRs contain extracellular leucine-rich repeats (LRRs) which mediate ligand binding, and a cytoplasmic Toll/IL-1R (TIR) domain, which facilitates the docking of adapter molecules. TLRs have been identified and sub-categorized based on their cellular location (Li et al., 2012). Some of the TLRs such TLR1/2/4/5/6/11 are expressed on the plasma membrane of innate cells such as dendritic cells (DCs), neutrophils and macrophages where they recognise microbial derived membrane components. Another member of the TLR family, TLR10, is expressed in tissues rich in immune cells (such as spleen, lymph node, thymus, tonsil, and lung). TLR10 appears to be structurally related to TLR1 and TLR6, however, as yet, no ligands for TLR10 have been discovered (Takeuchi and Akira, 2010). Others, such as TLR3/7/8/9/13 are expressed exclusively in intracellular vesicles such as endosomes, where they mainly function in the recognition of microbial nucleic acid (Kawai and Akira, 2010). Upon binding with their ligands on extracellular LRRs, TLRs form homo- or hetero-dimers, recruit TIR domain-containing adaptor proteins and
activate downstream signalling pathways. TLRs have five TIR-containing adaptor proteins: Myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (Mal, also known as TIRAP), TIR domain containing adaptor inducing interferon-β (TRIF also known as TICAM-1), TRIF-related adaptor molecule (TRAM, also known as TICAM2), and sterile a and heatarmadillo motifs (SARM) which negatively regulates TRIF signalling (Jenkins and Mansell, 2010; O'Neill and Bowie, 2007). Engagement of the signalling adaptor molecules stimulates downstream signalling pathways that comprise interactions between IL-1R-associated kinases (IRAKs) and the adaptor molecules TNF receptor-associated factors (TRAFs). This leads to the activation of the mitogen-activated protein kinases (MAPKs), JUN N-terminal kinase (JNK) and p38, and to the activation of transcription factors. The transcription factors activated downstream of TLR signalling include nuclear factor-κB (NF-κB), the interferon-regulatory factors 3/7 (IRF-3/-7), cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1). A major consequence of TLR signalling cascades via NF-κB, mitogen activated protein kinase (MAPK), CREB and AP1 is the transcriptional activation of various pro-inflammatory cytokines, chemokines and adhesion molecules. However, activation of the endosomal TLRs results in stimulation of the interferon regulatory factor 3/7 (IRF-3/-7) signaling pathway which in turn stimulates the production of type-I Interferons (IFNα/β) (Fig. 1.1) (McGettrick and O’Neill, 2010).

For example, TLR2 recognises its ligands (bacterial and mycoplasmal lipoproteins) by forming a heterodimer complex with either TLR1 or TLR6. The resulting heterodimers detect distinct lipoproteins i.e. the TLR1/TLR2 complex ligates with triacyl lipoprotein while the TLR6/TLR2 complex ligates with diacyl lipoprotein. TLR2 ligand stimulation results in engagement of the signalling adaptor molecules such as (Mal and MyD88) which leads to MAPK, CREB and AP1 activation and pro-inflammatory cytokines production (Fig. 1.1). However, TLR4 primarily detects lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria known to induce septic shock, following complex formation with other cell surface molecules such as myeloid differentiation factor-2 (MD2). Upon ligation, symmetrical interaction of two TLR4-MD2-LPS complexes occurs to form a TLR4 homodimer (Park et al., 2009). The TLR4 homodimer at the cell membrane firstly triggers MAL-MyD88-mediated signalling pathway resulting in activation of NF-κB,
API1 and CREB transcription factors and production of pro-inflammatory cytokines. TLR4 then interacts with TRAM and is relocated to the endosomal compartment. In the endosome TLR4-TRAM interacts with TRIF and triggers signalling pathway leading to activation of IRF3 and production of type I IFNs (Fig. 1.1) (McGettrick and O’Neill, 2010; O’Neill et al., 2013). Activation of TLR 3, 7, 8, 9 and 13 in response to bacterial and viral nucleic acids, and endogenous ‘pathogenic scenario’ nucleic acids, results in type I IFNs and pro-inflammatory cytokine production. In the case of TLR 7, 8, 9 and 13, MyD88-mediated activation of TRAF6 additionally results in binding and activation of TRAF3. This is followed by IRAK and IKKα activation and results in phosphorylation of IRF7. Activated IRF7 is translocated to the nucleus where it induces type I IFN responses. Interestingly, TLR3 ligation with viral double-stranded RNA (dsRNA) results in both induction of type I IFN and NF-κB pathways (Alexopoulou et al., 2001). TLR3 by interaction with TRIF and TRAF3 leads to activation of TBK1/IKKε complex which then phosphorylates IRF3 and results in its activation in the translocation to the nucleus and induction of type I IFN. TLR3-dependent NF-κB activation is mediated by interaction of TRIF with RIP1 and TRAF6/IRAK complex. This multi-protein complex promotes IKK activation through TAK1 complex (consisting of TAK1 and TAB2/3 proteins) what leads to NF-κB dependent pro-inflammatory cytokines production (Fig. 1.1).

Notably, important interactions occur between TLRs and certain NLRs for inducing certain pro-inflammatory cytokines (such as pro-IL-1β and pro-IL-18) through NF-κB stimulation and ‘priming’ caspase-1 activating complexes (termed inflammasomes). Both inflammasome-mediated caspase-1 activation and pro-IL-1β and pro-IL-18 production are highly dependent on ‘priming’ with TLR agonists (e.g. LPS stimulation via TLR4). Priming is necessary for pro-IL-1β and pro-IL-18 production and also potentiates induction of NLRP3. Therefore, the TLR and NLR pathways are clearly integrated for producing pro-inflammatory cytokines which play a crucial role in the initiation of inflammatory responses (Bauernfeind et al., 2009; Creagh and O'Neill, 2006).
Figure 1.1: TLR signalling pathways

TLR signalling is initiated by ligand-induced dimerization of receptors. Engagement of the signalling adaptor molecules (MyD88 and Mal or TRIF and TRAM) stimulates downstream signalling pathways that involve interactions with IL-1R associated kinases (IRAKs) or TNF receptor associated factors (TRAFs). This results in the activation of MAPKs; JUN N-terminal kinase (JNK) and p38. The subsequent activation of transcription factors such as NF-κB, interferon-regulatory factors (IRFs), cyclic AMP-responsive element-binding protein (CREB) and activator protein 1 (AP1) results in the induction of pro-inflammatory cytokines and type 1 IFNs. (dsRNA, double-stranded RNA; IKK, inhibitor of NF-κB kinase; LPS, lipopolysaccharide; MKK, MAP kinase kinase; RIP1, receptor-interacting protein 1; rRNA, ribosomal RNA; ssRNA, single-stranded RNA; TAB, TAK1-binding protein; TAK, TGFB-activated kinase; TBK1, TANK-binding kinase 1). Figure adapted from (O'Neill et al., 2013).
1.1.3 NOD like receptors (NLRs)

The NOD-like receptors (NLRs) are a specialized group of intracellular receptors that represent a key component of the host innate immune system. Most NLRs are expressed in the cytosol and constitute a big family consisting of 23 members in humans and at least 34 are described in mice (Kumar et al., 2011). Each NLR contains three characteristic domains: (i) C-terminal leucine-rich repeat (LRR) domain responsible for ligand sensing and plays a regulatory role in innate immune signaling through protein-protein interactions; (ii) central nucleotide binding and oligomerization domain (NACHT or NOD) and (iii) N-terminal effector domain responsible for signal transduction and activation of the inflammatory response (Franchi et al., 2009b). The N-terminal domain varies between NLRs dividing them into 5 main subfamilies: NLRAs with an acidic transactivation domain (AD); NLRBs with a baculoviral inhibitor repeat domain (BIR). This domain contains two major groups: inhibitor of apoptosis proteins (IAPs) and neuronal apoptosis inhibitor proteins (NAIPs); NLRCs with a caspase activation and recruitment domain (CARD). The CARD domain is involved in the interaction with CARD-containing proteins such as caspase-1 and ASC (adapter protein apoptosis speck protein with caspase recruitment) in inflammasome activation; NLRPs with a pyrin domain (PYD). This domain is homologous to CARD and interacts with other PYD-containing proteins during signalling transduction. The CARD, PYD or BIR domains of NLRs are mainly involved in mediating signalling pathways during apoptosis and inflammation; NLRX encompassing those members of the NLRs family whose domains have not yet been identified. Domain structures of human NLRs are shown in (Fig 1.2). NLRs are expressed in various cell types and tissues, their expression patterns are different. For example, NLRX1 and NLRP1 are widely expressed in many tissues or cell types, while NOD1, NOD2, NLRP3 and NAIP are highly expressed in immune cells and epithelial cells. NLRP4, NLRP5, NLRP7, NLRP8, NLRP10 and NLRP11 are strongly expressed in germ cells or specific tissues. (Kumar et al., 2011; O'Neill and Bowie, 2007; Yeretssian et al., 2008). NLRs “sense” infection through the recognition of a variety of PAMPs and DAMPs in the cytosol and consequently trigger inflammatory responses (i.e. through the activation of NF-κB and MAPK pathways) (Kofoed and Vance, 2011; Krishnaswamy et al., 2013). As described in the subsequent section, activation of some NLRs also leads to the formation of multi-protein inflammasome complexes that serve as platforms for the
cleavage and activation of Caspase-1. Caspase-1 promotes the maturation and secretion of IL-1β and IL-18, which further amplifies the pro-inflammatory immune response (Bryant and Fitzgerald, 2009). It has been also shown that NLRs play an important role in cell death regulation and the development of adaptive immunity (Ting et al., 2010).
<table>
<thead>
<tr>
<th>NLR Family</th>
<th>Symbol</th>
<th>Structure</th>
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<tbody>
<tr>
<td>NLRA</td>
<td>CIITA</td>
<td><img src="image" alt="Card AD Nacht NAD LRRs" /></td>
</tr>
<tr>
<td>NLRB</td>
<td>NAIP</td>
<td><img src="image" alt="BIR BIR BIR Nacht LRRs" /></td>
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<td></td>
<td>NOD1</td>
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<tr>
<td>NLRC</td>
<td>NOD2</td>
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<td></td>
<td>NLRC4 (IPAF)</td>
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<td>NLRP</td>
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<td>NLRP2-9; NLRP11-14</td>
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<td>NLRP10</td>
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<td>NLRX</td>
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Figure 1.2: Domain structures of human NLRs

The human NLR family is divided into five subfamilies including NLRA, NLRB, NLRC, NLRP and NLRX. The symbols and domain structures of the human NLRs are shown. Domains: AD acidic transactivation domain; BIR baculoviral inhibition of apoptosis protein repeat domain; CARD caspase recruitment domain; FIIND domain with function to find; LRR leucine-rich repeat; NACHT nucleotide-binding and oligomerization domain; NAD, NACHT-associated domain; PYD pyrin domain; ? is the undefined domain.
1.2 Caspases

Caspases, or cysteine-aspartic proteases are a family of cysteine proteases that play essential roles in apoptosis (programmed cell death), necrosis, and inflammation (Alnemri et al., 1996). They are widely expressed in almost all cells as inactive zymogens consisting of three domains: the N-terminal propeptide (prodomain) of variable length, a large subunit (~20 kDa) and a small subunit (~10 kDa) (Nunez and del Peso, 1998). The caspase catalytic region is composed of both large and small subunits with a conserved pentapeptide ‘QACXG’ active site (where X can be arginine (R), glutamine (Q) or glycine (G)) found in the large subunit. Caspases recognize a tetrapeptide motif in their substrates and have an absolute specificity for an aspartic acid (Asp) residue at the scissile bond. Proteolysis is required to activate the pro-enzyme to its active form. This occurs at specific aspartate cleavage sites which are found between the pro-domain and the large subunit as well as within the interdomain linker within the pro-enzyme. This allows the large and small subunits to assemble into functionally active heterodimers (Regula and Kirshenbaum, 2005).

The caspase family can be divided into three groups: two groups of apoptotic caspases including initiator and effector caspases and one group of inflammatory caspases (Fig. 1.2). Initiator/upstream caspases (caspase-2, -8, -9, and -10) are thought to be primarily responsible for initiating caspase-activation cascades (via cleaving inactive pro-forms of effector caspases, thereby activating them). They posses long N-terminal prodoms that contain protein-protein interaction motifs (caspase recruitment domains, CARDs or death effector domains, DEDs). Interestingly, recent reports revealed that caspase-8 also plays a role in inciting inflammation through different mechanisms (i.e. by direct cleavage of pro-IL-1β to its active form, by direct activation of caspase-1 or by its incorporation into inflammasome complexes) (Antonopoulos et al., 2013; Gurung et al., 2014; Philip et al., 2014). Effector/downstream caspases (caspase-3, -6 and -7) are responsible for the actual demolition of cells during apoptosis and they have short or absent pro-domains. Inflammatory caspases (caspase-1, -4, -5, -11 and -12) are involved in regulation of inflammatory responses. Similar to initiator caspases, they contain long CARD-containing pro-domains. In humans, a polymorphism in caspase-12 results in production of either a truncated CARD-only protein (Casp12-S) or a full-length caspase molecule (Casp12-L), which appears to be enzymatically inactive (Saleh et al., 2004). The full-length variant molecule is entirely
confined to populations of African descent and confers hypo-responsiveness to LPS-mediated production of cytokines such as IL-1β and IFN-γ. Therefore, caspase-12 in human appears to have a negative regulatory function on proinflammatory signalling pathways (including NF-κB, IL-1 pathways) and has been also implicated as a repressor of caspase-1 activity (Yeretssian et al., 2008). Furthermore, caspase-12 was originally proposed to mediate ER-stress induced apoptosis in mice (Nakagawa et al., 2000). However, some other studies have shown a lack of difference in ER-stress mediated apoptosis between caspase-12 deficient and proficient cells (Obeng and Boise, 2005).
Figure 1.3: The caspase family structure

The figure outlines the three major groups of the caspase family. **Group I**: Inflammatory caspases; **Group II**: Apoptosis initiator caspases and **Group III**: Apoptosis effector caspases. The **CARD**, the **DED**, and the large (p20) and small (p10) catalytic subunits are indicated.
1.2.1 Gene orthology and chromosomal localization of inflammatory caspases

Inflammatory caspases (also known as group I caspases) are encoded by four genes in humans: *caspase-1, -4, -5* and *-12* and three main genes in the mouse: *caspase-1, -11* and *-12*. In mammals, these caspases have a CARD domain at the N-terminus (Fig. 1.4A). Human and mouse inflammatory caspases share significant similarities and are organised in a single locus. Phylogenetic analysis of the conserved CARD domain suggests that the inflammatory caspases can be separated into evolutionary related clusters (Martinon and Tschopp, 2007). Human inflammatory caspases are clustered together on chromosome 11q22 in the following order from the telomere to centromere: *caspase-1, -5, -4* and *-12*. Mouse inflammatory caspases are clustered on a syntenic region on chromosome 9A1 and they are arranged in a similar order to humans, except that *caspase-4* and *caspase-5* genes are replaced by the *caspase-11* gene (Fig. 1.4B). Sequence comparison of caspase domains and pro-domains suggests that human caspase-4 and caspase-5 have originated from a duplication of caspase-11 (Martinon and Tschopp, 2004). There is a lack of a precise human orthologue of murine caspase-11 and both caspase-4 and -5 are considered to be it (showing 68% and 47% amino acid sequence identity with murine caspase-11, respectively) (Fig. 1.5). Interestingly, inhibitors of caspase-1, ICEBERG and COP, which are CARD only proteins, are also found in the same locus in humans but not in the murine locus. However, as it has been previously mentioned, the caspase-12 sequence is present in the human genome but it doesn’t encode an active enzyme in most humans (only people of African descent express full length caspase-12, and these individuals appear to be more susceptible to inflammatory diseases) (Fischer et al., 2002; Saleh et al., 2004).
Figure 1.4: Structural and gene arrangement of the inflammatory caspases

(A) Domain structure of human and murine inflammatory caspases. CARD, caspase-recruitment domain; (B) Chromosomal organization of inflammatory caspases in humans and mice. Adapted from Martinon et al., 2004.
Figure 1.5: Amino acid sequence alignment of murine caspase-11 and human caspase-4 and -5.

Murine caspase-11 and human caspase-4 and-5 amino acid sequences were obtained from Uniprot website (http://www.uniprot.org/). Caspase accession codes: caspase-4, P49662; caspase-5, P51878 and caspase-11, P70343. Amino acid sequences were aligned using Vector NTI software (Invitrogen, Life Sciences). The amino acid homology between caspases is: human caspase-4 versus human caspase-5: 63%; human caspase-4 versus murine caspase 11: 68%; human caspase-5 versus murine caspase 11: 47%. Amino acids highlighted in yellow indicate identical amino acids; amino acids highlighted in green indicate conserved amino acids; amino acids highlighted in blue indicate that two amino acids are identical and the non-highlighted black amino acids indicate a lack of similarity.
1.3 Inflammatory Caspase-1

To date, caspase-1 is one of the best characterized inflammatory caspases and is activated within multi-protein complexes known as inflammasomes (Martinon et al., 2002). It has been shown that caspase-1 is activated in different inflammasome complexes (including NLRP1, NLRP3, NLRC4, AIM2 and NLRP6) which assemble in response to a variety of pathogen, or danger-associated molecular patterns (PAMPs and DAMPs) (Fig. 1.6) (Martinon et al., 2009). Caspase-1 is recruited to the inflammasome through the caspase recruitment domain (CARD) or pyrin domain (PYD), resulting in proximity-induced autoactivation. For example, NLRC4 can bind and activate caspase-1 directly via a CARD/CARD mediated interaction. However, NLRP1 and NLRP3 require CARD/PYD containing inflammasome adapter ASC (apoptosis-associated speck-like protein containing a CARD) for caspase-1 recruitment (Agostini et al., 2004; Martinon et al., 2002; Poyet et al., 2001).

Activated caspase-1 processes the pro-forms of IL-1β and IL-18, leading to their maturation and secretion. IL-1β and IL-18 are two closely related IL-1 family cytokines that function as key mediators of host immune response. These inflammatory cytokines are commonly recognized for their ability to cause a wide variety of biological effects associated with infection, inflammation and autoimmunity (Sims and Smith, 2010). They induce inflammation and participate in epithelial repair and the healing process through the recruitment and activation of immune cells and by inducing the secretion of other pro-inflammatory cytokines, chemokines and growth factors (Dinarello, 2009). IL-1β regulates systemic and local responses to infection, injury and immunological challenges by generating fever, activating lymphocytes and promoting leukocyte migration into sites of injury or infection. Although IL-18 lacks the pyrogenic activity of IL-1β, it induces interferon-γ (IFNγ) production by activated T cells and natural killers (NK) cells and plays a crucial role in epithelial cell proliferation (Dinarello, 2009; Reuter and Pizarro, 2004).

In addition to the proteolytic activation of IL-1β and IL-18, inflammasome activation also leads to pyroptosis. Pyroptosis is a caspase-dependent form of cell death that acts as an innate immune effector mechanism against intracellular bacteria. Morphologically, pyroptotic cells are characterised by cytoplasmic swelling, membrane rupture, nuclear condensation and DNA fragmentation (Fink and Cookson, 2006). In
addition, disproportionate caspase-1 activation in damaged neurons and in pathogen infected myeloid cells can lead to the induction of pyroptosis (Denes et al., 2012). It has been known for a while that pyroptosis is induced by canonical activation of caspase-1 by NLRP3 and NLRC4 inflammasomes. However, it has been also proposed that caspase-11 regulates pyroptosis (Kayagaki et al., 2011), which is further described in section 1.4.

Caspase-1 is also believed to play a protective role against pathogenic bacteria. It has been reported to protect host cells by repairing the damage caused by bacterial pore-forming toxins that are released by pathogenic bacteria (such as S. aurerus and Aeromanas hydrophila) (Gonzalez et al., 2008). The toxin-induced membrane permeabilization leads to a decrease in cytoplasmic potassium, which promotes the formation of the NLRP3 and NLRC4 inflammasome, and the activation of caspase-1. Therefore, in this context caspase-1 induces the activation of the central regulators of membrane biogenesis, the Sterol Regulatory Element Binding Proteins (SREBPs) and release of growth factors such as fibroblast growth factor 2 (FGF2), thus, promoting host cell survival upon toxin challenge by facilitating membrane repair (Gurcel et al., 2006). However, caspase-1 mediated activation of SREBPs is thought to be indirect and the caspase-1 substrates which drive this pathway still remain unknown.

Caspase-1 activity also cleaves DNA damage repair enzyme, poly (ADP-ribose) polymerase 1 (PARP1) and glycolysis enzymes (e.g. fructose-bisphosphate aldolase), possibly to preserve energy stores in order to allow for proper dismantling of the cell (Malireddi et al., 2010; Shao et al., 2007). Lastly, caspase-1 is also involved in activation of caspase-7 leading to restriction of Legionella replication by targeting the infected cells to lysosome (Akhter et al., 2009). A proteome-wide screen identified caspase-7 as a direct substrate of caspase-1, and biochemical studies confirmed that caspase-7 is cleaved by caspase-1 after the canonical activation (Lamkanfi et al., 2008).

1.3.1 Caspase-1 and the canonical NLRP3 inflammasome

The NLRP3 inflammasome is the most characterized inflammasome, consisting of the NLRP3 scaffold, the ASC (PYCARD) adaptor, and caspase-1. Numerous chemically and structurally diverse stimuli are now known to trigger formation of this inflammasome. These include viruses (e.g. Sendai, Influenza), fungi (e.g. Candida
*albicans, Saccharomyces cerevisiae* and bacteria (e.g. *Staphylococcus aureus* and *Listeria monocytogenes*) (Bryant and Fitzgerald, 2009). Diverse PAMPs (e.g. microbial derivatives such as MDP, bacterial pore-forming toxins) and endogenous DAMPs (e.g. extracellular ATP) or various insoluble molecules (silica, urea crystals) may also activate this inflammasome (Dostert et al., 2008; Mariathasan et al., 2006; Martinon et al., 2006). The exact mechanism by which these stimuli activate NLRP3 remains unclear, however, reports have revealed that NLRP3 can be activated through distinct canonical and non-canonical pathways. In this section, the canonical pathway will be described, and the non-canonical mechanism will be discussed in greater detail in the following section.

In general it has been shown that canonical NLRP3 activation requires two signals. Signal 1, known as priming, is represented by stimulation of TLRs, IL-1R, TNFR1 and TNFR2 by various ligands, which results in the activation of the transcription factor NF-κB (Bauernfeind et al., 2009; Franchi et al., 2009a). The activation of NF-κB is critical for upregulating the transcription of both pro-IL-1β and NLRP3, as pro-IL-1β is not constitutively expressed and basal levels of NLRP3 are inadequate for efficient inflammasome formation. Signal 2 activates the NLRP3 inflammasome machinery resulting in activation of caspase-1, which culminates in secretion of IL-1β and IL-18 and also induces pyroptosis. A number of mechanisms have been proposed that trigger the canonical NLRP3 activation during activation by signal 2, however, the most commonly known and also some of the most recently characterised pathways will be described here. It was proposed that sensing extracellular ATP stimulates the purogenic P2X7 ATP-gated ion channel, triggering K⁺ efflux and inducing gradual recruitment of the pannexin-1 membrane pore. This model postulates that pore formation allows extracellular NLRP3 agonists to access the cytosol and directly activate NLRP3 (Kanneganti et al., 2007). Also, phagocytosed extracellular material such as monosodium urate (MSU) crystals, silica or alum can trigger NLRP3 activation. In addition the release of lysosomal enzymes, such as cathepsin B, into the cytosol following degradation of the lysosome, has been implied in NLRP3 activation (Hornung et al., 2009). NLRP3 inflammasome activation also occurs following the release of reactive oxygen species (ROS) from damaged mitochondria via mitochondrial voltage-dependent ion channels. It has been proposed that oxidised mitochondrial DNA (mtDNA), released from damaged mitochondria following bacterial
infection, binds and activates the NLRP3 inflammasome (Schroder and Tschopp, 2010; Zhou et al., 2011). In addition, post-translational protein modifications such as phosphorylation and ubiquitination have also been proposed to play crucial role in NLRP3 inflammasome activation. For example, it has been shown that Syk- and Jnk-dependent manner ASC phosphorylation is required for NLRP3 activity in murine macrophages (Hara et al., 2013). It has also been recently demonstrated that the linear ubiquitination assembly complex (LUBAC) is an essential regulator of the activation of the NLRP3/ASC inflammasome in primary bone marrow–derived macrophages (BMDMs) independently of NF-κB activation (Rodgers et al., 2014). Furthermore, it has been shown that caspase-8 and its adapter Fas-associated death domain (FADD) are required for both priming and activation of both canonical and non-canonical NLRP3 inflammasomes in macrophages. This study revealed that FADD and caspase-8 drive NLRP3 activation and IL-1β secretion at two regulatory checkpoints. At the transcriptional level, FADD and caspase-8 trigger NF-κB dependent transcriptional upregulation of proIL-1β and NLRP3 as well as TRIF dependent procaspase-11 induction to prime canonical and non-canonical NLRP3 inflammasome for activation by their respective stimuli. However, at the posttranslational level, FADD acts as a platform for procaspase-8 activation, and they both interact with core components of the NLRP3 inflammasome to trigger stimulus-dependent caspase-1/11 maturation and IL-1β secretion (Gurung et al., 2014). Moreover, in an additional study, Man et al. using confocal and super resolution microscopy approaches revealed that active forms of both caspase-1 and caspase-8 are present within the core of the multi-protein inflammasome complex, surrounded by NLRP3, NLRC4, and ASC in macrophages infected with Salmonella Typhimurium. Thus, the authors suggest that the inflammasome is a dynamic macromolecular protein complex capable of recruiting different NLRs and multiple caspases to coordinate inflammasome responses to infection (Man et al., 2014).
Figure 1.6: The different types of inflammasomes activating caspase-1

The NLRs NLRP1, NLRC4, NLRP3, NLRP6, and the HIN200 proteins AIM2 and IFI16 build up the inflammasome upon sensing different PAMPs or DAMPs. Whereas AIM2 and IFI16 assemble through binding of pathogen-derived dsDNA, more indirect mechanisms induce formation of the other types of inflammasomes. NLRC4 is activated by binding to NAIPs, NAIP5 and NAIP6 sense flagellin, and NAIP2 the T3SS rod protein. The NLRP3 inflammasome is activated by many different DAMPs and PAMPs. Here, generation of ROS, efflux of K⁺ or cathepsin B release upon lysosomal damage may trigger complex formation. The stimulus that results in assembly of the NLRP6 inflammasome remains to be identified. All inflammasomes recruit Asc and caspase-1, what induces activation of the protease, subsequent activation of proIL-1β and proIL-18, and, finally, secretion of the active cytokines (Sollberger et al., 2013).
1.4 Murine caspase-11 and the non-canonical inflammasome

Caspase-11, originally named *Ich-3*, is a key member of the caspase-1 subfamily of cysteine proteases and is considered as a homolog of human caspase-4 or caspase-5 (which still remains a matter of debate). Caspase-11 is synthesized as 43 and 38 kDa precursors and its expression is barely detectable under normal conditions but highly inducible by proinflammatory or cytotoxic stimuli (Kang et al., 2000; Wang et al., 1996). It was first reported that proinflammatory mediators like lipopolysaccharide (LPS) and interferon-γ (IFN-γ) induce expression of caspase-11 by activating nuclear factor (NF-κB) and signal transducer and activator of transcription 1 (STAT1), respectively (Schauvliege et al., 2002).

Caspase-11 plays an important role in the regulation of inflammation and apoptosis. Initially, a crucial involvement of caspase-11 has been shown in many mouse models associating it with pathological apoptosis and inflammation such as brain ischemia, LPS-induced septic shock, experimental autoimmune encephalomyelitis (EAE) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson’s disease (Furuya et al., 2004; Hisahara et al., 2001; Wang et al., 1996). Moreover, increased levels of caspase-11 have been observed in some of these pathological conditions including induced cerebral ischemia (MCAO) and EAE in mice. It was believed that higher expression of caspase-11 leads to its autoactivation. Kang et al. and Hisahara et al. have shown that caspase-11 deficient mice had a reduced number of apoptotic cells and defect in caspase-3 activation compared to control mice during MCAO and EAE course. These findings further revealed that caspase-11 can directly activate effector caspase-3 and -7 to regulate apoptosis which implicate caspase-11 to play a pivotal role in the progression of these diseases (Hisahara et al., 2001; Kang et al., 2000).

The signalling pathway leading to caspase-11 induction has been also studied in the murine models of septic shock. An extensive number of recent studies have focused on its role in bacterial infection as assessed using various *in vitro* models. Early work by the group of Junying Yuan (1996) demonstrated a critical role for caspase-11 in caspase-1 activation and also reported that both caspase-1 and caspase-11 were crucial for the induction of endotoxic shock *in vivo*. In their study caspase-1 and caspase-11 deficient mice were resistant to endotoxic shock induced by bacterial LPS and failed to produce mature IL-1β after LPS stimulation (Wang et al., 1996). More recently,
Kayagaki et al. reported that all Casp1<sup>−/−</sup> mice also lack caspase-11, due to the generation of the Casp1<sup>−/−</sup> line in the 129 mouse strain background, which express a mis-spliced and truncated version of the Casp11 messenger RNA (Kayagaki et al., 2011). Furthermore, they have reported that caspase-11 rather than caspase-1 is the main effector of LPS induced lethal septic shock, which is contrary to the previous thinking that both caspases were implicated in the aforementioned mouse model. Therefore, this suggested that earlier findings could be misleading due to the fact that Casp1<sup>−/−</sup> mice might have also been deficient in caspase-11 making them Casp1<sup>−/−</sup>/Casp11<sup>−/−</sup> double knockout mice (Kayagaki et al., 2011). It also questions the reliability of the Casp11<sup>−/−</sup> data, as littermate controls used in experiments may also have had mutated caspase-11 gene (from 129 background).

An examination of the inactivating caspase-11 mutation in Casp1<sup>−/−</sup> mice revealed that LPS/cholera toxin B (CTB) or Gram-negative bacteria infection require caspase-11 to induce caspase-1 NLRP3 inflammasome (Kayagaki et al., 2011). The new caspase-11 dependent inflammasome was called the non-canonical inflammasome and subsequent to this work the mechanism of the non-canonical pathway has been progressively elucidated. Studies have shown that this pathway is activated in response to a broad number of Gram-negative (but not Gram-positive) bacteria and engages caspase-11 to trigger caspase-1 activation, production of IL-1β and IL-18 and induced caspase-11 dependent pyroptosis (accompanied by release of IL-1α and the high mobility group box1, (HMGB1) (Kayagaki et al., 2011; Rathinam et al., 2012). It has been shown that caspase-11 dependent IL-1β and IL-18 processing requires, caspase-1, as well as NLRP3 and ASC), however caspase-1 is dispensable for caspase-11 mediated pyroptosis (Kayagaki et al., 2011; Kayagaki et al., 2013). Notably, caspase-11 does not participate in other inflammasome pathways such as NLRC4 (flagellin), AIM2 (dsDNA) or the canonical NLRP3 (ATP) (Kayagaki et al., 2011).

As caspase-11 activation was significantly delayed after infection with extracellular bacteria compared to intracellular L. pneumophila (Case et al., 2013; Kayagaki et al., 2011; Rathinam et al., 2012), and in addition, bacteria that escape the phagosome and replicate in the cytosol such as B. thailandensis and B. pseudomallei induced rapid caspase-11-dependent pyroptosis, it was hypothesized that a conserved microbial ligand within the cytosol triggers caspase-11 production. Following on from these findings two independent studies identified that LPS as being the key bacterial
component which trigger caspase-11 activation during the non-canonical inflammasome pathway. It has been discovered that, caspase-11 activation was induced by the acylated lipid A component of LPS in most Gram-negative bacteria, thus, explaining why caspase-11 specifically reacted to intracellular Gram-negative pathogens. Interestingly, synthetic lipid A and LPS isolated from E. coli- and S. Typhimurium-activated caspase-11 independently of the established membrane-bound LPS receptor TLR4 (Hagar et al., 2013; Kayagaki et al., 2013). Instead, caspase-11 activation required LPS to be present in the cytosolic compartment (Hagar et al., 2013; Kayagaki et al., 2013). This suggests the presence of an intracellular LPS-sensor that triggers caspase-11 activation. In agreement, a recent study by Shi et al, revealed that the CARD motif of recombinant caspase-11 (and also caspase-4 and -5) directly binds LPS with high affinity, suggesting that these caspases act both as the LPS-sensor and -effector in infected macrophages (Shi et al., 2014). Moreover, caspase-11 activation contributed to LPS-induced lethality of TLR4-deficient mice that had been pre-stimulated with non-lethal doses of the TLR3 agonist polyinosinic:polycytidylic acid (polyI:C) to induce caspase-11 expression (Hagar et al., 2013; Kayagaki et al., 2013). In contrast, most of the CaspIL^{-} mice survived this treatment regimen. Therefore, this suggests that caspase-11 contributes to LPS-induced lethality independently of TLR4 and caspase-1.

It has been proposed that the upstream mechanism governing activation of the non-canonical pathway involves TLR4-TRIF mediated recognition of extracellular LPS, which induces type I interferon signalling to engage caspase-11 expression (Broz et al., 2012a; Gurung et al., 2012; Rathinam et al., 2012). Although NF-κB can induce caspase-11 expression, type I IFN-mediated activation of STAT1-IRF9 has been implied as the main mechanism upregulating caspase-11 expression in physiologically relevant conditions such as bacterial infection (Rathinam et al., 2012). Recent in vivo findings also highlight the importance of type I IFNs for the caspase-11 pathway. Pretreatment with polyI:C ligand, which triggers type I IFN production via TLR3 was required for caspase-11 activation in response to cytosolic LPS in TLR4-deficient mice (Hagar et al., 2013; Kayagaki et al., 2013). In addition to this priming mechanism, it has been shown that type I IFN modulates the caspase-11 pathway via its effects on guanylate-binding proteins (GBPs), a family of type I IFN-inducible proteins involved in host immunity and antimicrobial defences. Two parallel studies have shown involvement of type I IFN inducible GBPs in caspase-11 activation (Meunier et al.,
2014; Pilla et al., 2014). One study reported that GBPs disrupt the pathogen-containing vacuole, thus enabling bacteria to enter the cytosol and activate caspase-11 (Meunier et al., 2014). However, the other report proposed that GBPs have no effect on vacuolar integrity but instead act downstream to activate caspase-11 (Pilla et al., 2014), leaving the mechanism of GBPs in licensing the cytosolic LPS-driven caspase-11 pathway open to debate. The current model of caspase-11 activation is shown in (Fig.1.7).

While it is clear that caspase-11 coordinates cell death and caspase-1 maturation downstream of the NLRP3 inflammasome, how exactly caspase-11 mediates these events remains unclear. Some reports indicate that caspase-11 forms a complex together with NLRP3/ASC and procaspase-1 (Kayagaki et al., 2011; Rathinam et al., 2012), while others suggest that caspase-11 acts upstream of NLRP3 activation (Broz et al., 2012b). Interestingly, a most recent study by Broz et al showed that caspase-11 acts upstream of NLRP3 and controls the assembly of NLRP3–ASC complexes (ASC specks) in a cell-autonomous process in LPS transfected BMDMs. In addition, it has been shown that caspase-11 activation by LPS transfection causes a drop of intracellular K⁺ levels which is essential to activate NLRP3. Thus, these findings indicate that caspase-11 activates NLRP3 inflammasome through controlling K⁺ efflux (Ruhl and Broz, 2015).
Non-canonical NLRP3 inflammasome activation is induced by Gram-negative bacteria. Extracellular LPS induces the expression of pro-IL-1β and NLRP3 via the TLR4-MyD88-dependent pathway and type I interferon via the TLR4-TRIF-dependent pathway. Type 1 interferon provides a feedback loop and activates type I interferon receptor (IFNAR) to induce caspase-11 expression. Cytosolic Gram-negative bacteria deliver LPS into the cytosol when they escape the vacuole. Vacuolar Gram-negative bacteria release their LPS into the cytosol through a mechanism that requires vacuolar rupture mediated by interferon-inducible guanylate-binding proteins (GBPs). Caspase-11 is proposed to activate following its binding to cytosolic LPS. Caspase-11 then drives pyroptosis and activation of the non-canonical NLRP3 inflammasome. Adapted from (Man and Kanneganti, 2015).
1.5 Inflammatory caspase-4 and -5

To date, the roles of caspase-4 and caspase-5 during inflammation are still not well understood, mainly due to the lack of a direct murine homolog. However, it is widely accepted that murine caspase-11 is a functional orthologue of caspase-4 and -5 as they have arisen from a caspase-11 gene duplication event (Martinon and Tschopp, 2007). Similar to caspase-4, caspase-5 expression is induced by IFNγ but also LPS (which is a characteristic shared with caspase-11) (Ahn et al., 2002; Lin et al., 2000). Early reports demonstrated that caspase-4 initiated apoptosis following activation during endoplasmic reticulum (ER) stress (Hitomi et al., 2004). In addition, it has been shown that both caspases mediate inflammasome activation. Caspase-4 has been proposed to be required for efficient caspase-1 activation and IL-1β secretion in UVB-irradiated human keratinocytes, suggesting its essential role in NLRP3 inflammasome activation (Sollberger et al., 2012). Another, more recent study revealed that transgenic mice expressing human caspase-4 exhibited enhanced endotoxin sensitivity following LPS challenge. It has also been shown that caspase-4 mediates caspase-1 activation and IL-1β and IL-18 secretion following TLR2 or TLR4 priming signals in BMDMs from these mice. These findings highlight the importance of caspase-4 in licensing TLR mediated inflammasome activation (Kajiwara et al., 2014). On the other hand, caspase-5 was originally identified as a component of the NLRP1 inflammasome, which was based on the immunoprecipitation experiments revealing that caspase-1 and -5 are co-immunoprecipitated with several other proteins, forming the NLRP1 inflammasome. This study also revealed that caspase-5 participated in IL-1β processing, because pro-IL-1β processing occurred most efficiently when both caspase-1 and caspase-5 are co-activated in a cell-free system, indicating a role for caspase-5 in IL-1β maturation (Martinon et al., 2002). Interestingly, a recent study by Sanders et al demonstrated that caspase-4 and -5 can be recruited into different inflammasomes under certain circumstances, thus suggesting their independent roles in inflammasome regulation. It has been shown that caspase-4 directly interacts with NLRP1, NLRP3 and NLRC4, whereas, caspase-5 interacts with NLRP1. Moreover, it has been determined that definite inflammasome proteins induce heterodimerization of caspase-1 with caspase-4 or -5. Therefore, this may represent an alternative regulating mechanism of caspase-1 activation or alternatively these heterodimers may function as distinct enzymatic complexes (Sanders et al., 2015).
Further, a study by Shi et al has shown that, in addition to murine caspase-11, both human caspase-4 and -5 directly bind to LPS (through the lipid A moiety - CARD domain interaction). Although, both caspses are identified to bind LPS, only endogenous caspase-4 has been found to induce pyroptosis in human cells (Shi et al., 2014). These findings suggest that pyroptosis induced by cytosolic LPS is not initiated by a traditional inflammasome but mainly through a single caspase that acts as both, the receptor and pyroptotic initiator.

There is a recent growing body of evidence supporting caspase-4 and -5 involvement in the non-canonical inflammasome in human myeloid and epithelial cells. For example, a study by Schmidt-Burgk et al revealed that caspase-4 mediates caspase-1 activation, pyroptosis and IL-1β production in human THP1 cells in response to cytosolic LPS in a TLR4-independent manner. Interestingly, caspase-4 mediated IL-1β maturation required potassium efflux-mediated NLRP3 activation for caspase-1 maturation and subsequent IL-1β secretion (Schmid-Burgk et al., 2015). However, a follow up study by Baker et al showed that both caspase-4 and -5 are crucial in mediating pyroptosis and IL-1β release in response to Salmonella infected in human monocytic cell lines (Baker et al., 2015). With regard to the epithelial non-canonical inflammasome, the emerging role of caspase-4 was highlighted by discovery of a Shigella flexneri effector molecule, OspC3, which inhibits caspase-4 activity therefore delaying epithelial cell death and promoting infection (Kobayashi et al., 2013). Also, E. coli and S. typhimurium induced caspase-4-dependent but caspase-1-independent pyroptotic cell death of human IEC (Kobayashi et al., 2013). In line with these findings, another study by Knodler et al revealed that caspase-4 is required for IL-18 processing and pyroptosis (again independent of caspase-1) in S Typhimurium infected human IEC in order to extrude and remove infected cells from the polarized cell layer (Knodler et al., 2014). In addition, examination of a panel of epithelial cell lines (such as HT-29, colorectal adenocarcinoma; HCT-8, ileocecal colorectal adenocarcinoma and Caco-2, colorectal adenocarcinoma) revealed that all these cell lines exhibit high mRNA and protein expression of caspase-4 and moderate expression of caspase -5. However, expression pattern of these caspases in epithelial cells wasn’t examined (Knodler et al., 2014).
Caspase-4 has also been identified as a new target gene of NF-κB and mediator of Fas-induced apoptosis in neuroblastoma cells, thus, implicating a crucial role for caspase-4 in neurodegenerative disease (Yang et al., 2015). Moreover, caspase-5 frameshift mutations are frequently found in endometrial carcinoma (Martinon and Tschopp, 2004) and caspase-5 has been shown to cleave Max, a component of the Myc/Max/Mad network of transcription factors which is often deregulated in tumours, suggesting a regulatory role of caspase-5 in tumourigenesis (Krippner-Heidenreich et al., 2001). Increased caspase-5 mRNA expression levels were found in skin biopsies of patients with psoriasis, thus indicating its role in the inflammatory processes occurring during chronic inflammatory skin diseases. This study suggested that caspase-5 upregulation was mediated via NF-κB pathway (Salskov-Iversen et al., 2011).

However, a role for these caspases in diseases in which pathogenesis is caused by chronic inflammation such as IBD and Barrett’s oesophagus, or their progression to colorectal and oesophageal adenocarcinomas, respectively, has yet to be established.

1.6 Intestinal inflammation

Today, the innate immune system is recognised as the major contributor to acute inflammation induced by microbial infection or tissue damage (Akira et al., 2006; Beutler et al., 2006). It is well accepted that physiological inflammation is beneficial in the intestine and diverse innate immune compartments in the gut encompass many innate leucocyte populations, as well as several types of intestinal epithelial cells (IEC) which act together to maintain a balanced immune response to microbiota (Artis, 2008). Importantly, the intestinal epithelium seems to play a crucial role in regulation of intestinal homeostasis. It consists of a single layer of columnar epithelial cells that provide an effective physical barrier separating the vast bacterial load of the intestinal flora from cells of the host immune system. The continuous crypts and villi that make up the intestinal epithelium possess several physical, biochemical and immunological mechanisms ensuring intestinal homeostasis, i.e. mutualistic interactions with commensal microbes contrasted by protective immunity to invasive pathogens. Actin-rich microvillar protrusions from the apical IEC surface form a mechanical brush border, which, in combination with goblet cell-secreted mucins, comprise a sterile barrier that is impermeable to most intestinal microbes. The mucus layer of the intestine
consists of an inner glycocalyx of membrane-anchored mucins, covered by an outer layer of secreted mucins, which, in addition to being a viscous barrier to microbes, forms a matrix loaded with high concentrations of IEC-derived antimicrobial peptides and secretory IgA (Hooper and Macpherson, 2010). IEC are permanently in contact with the intestinal lumen contents and, therefore, ideally located to undertake immunosurveillance of commensal and pathogenic populations within the intestinal microbiota. Microbe-associated molecular pattern triggering of PRRs classically drives a nuclear factor-κB (NF-κB)-dependent pro-inflammatory response and initiation of both innate and adaptive immune responses to the invading microbe. Importantly, triggering of PRR signalling within IEC is critical for a broad spectrum of host-protective responses to pathogenic species in the intestine (Lavelle et al., 2010).

1.7 Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) refers to chronic and relapsing inflammatory disorders affecting the gastrointestinal tract and is most commonly diagnosed in patients in the second to the fourth decades of life, affecting men and women equally. IBD is more prevalent in Caucasians and in those living in northern hemisphere-industrialized countries. However, rates are also on the rise in non-whites and in southern, non-industrialized nations. IBD affects over 2 million people in Europe and more than 15,000 in Ireland. IBD patients typically suffer from recurrent intestinal inflammation, diarrhea, abdominal pain, rectal bleeding, weight loss and anaemia; all of which have a dramatic impact on the patient’s quality of life. There are two main clinical forms of IBD – the first is Crohn's disease (CD), traditionally described as patchy inflammation affecting any part of the gastrointestinal tract from mouth to anus, although it usually starts in the terminal ileum. Pathological lesions in CD are mostly characterised with trans-mural inflammation and granulomas, high infiltration of macrophages and T cells with a Th1/Th17 profile. The second, Ulcerative colitis (UC), is generally localised to the rectum and colon where mucosal inflammation and high infiltration of neutrophils and T cells with an atypical T helper (Th2) phenotype being observed (Kaser et al., 2010). IBD is considered as a multifactorial disorder and its precise aetiology still remains unclear, however, several factors have been identified which make a major contribution to its pathogenesis. These include genetic susceptibility of the host,
environmental factors (e.g. intestinal microflora) and the alterations in the host immune system. The cross-regulation of these key factors in multiple ways, leads to development of IBD (Kaser et al., 2010).

The importance of innate and adaptive immune pathways in intestinal homeostasis is underscored by genome-wide association studies (GWAS) that have identified polymorphisms in many innate and adaptive immunity genes that influence susceptibility to IBD. This intensive research has identified 99 unique loci associated to IBD, including 71 loci specific for CD and 47 loci specific for UC, where 28 loci are shared between both diseases (Anderson et al., 2011; Franke et al., 2010). However, loci identified to date represent only 10% of the overall variance of potential disease risk (Barrett et al., 2008), suggesting that the phenotype of IBD may be due to the interaction between hundreds or thousands of common single nucleotide polymorphisms of minor biological impact or/ and that the phenotype is attributed to the effects of rare variants with profound impact (Goldstein, 2009). Most of the identified loci are involved in regulation of innate and adaptive immunity (IL-23R, IL-10, STAT, JAK2), regulation of inflammation (CCR6, MST1) and regulation of endoplasmic reticulum (ER) stress and autophagy (X box binding protein 1 (XBP1), ATG16L1, IRGM). All these pathways seem to affect the regulation of the immune system and its response to commensal bacteria and particularly the function of Paneth cells and the presentation of peptides by innate immune cells to adaptive immune cells such as T lymphocytes. Interestingly, autophagy genes (e.g. ATG16L1), NOD-like receptors (e.g. NOD2/CARD15) and intelectins have been related to CD whereas loci related to regulatory pathways (e.g. IL-10, ARPC2) and intestinal epithelial cell function (e.g. ECM1) are more specific for UC (Karantanos and Gazouli, 2011). Table 1.1 summarizes some the gene regions that are proven to be associated with Crohn's disease or Ulcerative colitis. Moreover, it has been widely accepted that aberrant recognition of commensal bacteria in the gut by series of pattern recognition receptors (PRRs) crucially underlines pathogenesis of IBD (Abreu, 2010; Cario, 2010), which will be further discussed in section 1.9.

Nowadays, the goal of inflammatory bowel disease treatment is to reduce the inflammation that triggers the disease symptoms. In the best cases, this may lead not only to symptom relief but also to long-term remission. IBD treatment usually involves either drug therapy or surgery. IBD is currently treated with steroids, azathioprine,
methotrexate, tacrolimus. These agents suppress the response of the host against bacteria that have penetrated the epithelium, thus reducing secondary inflammation. However, they are all accompanied by several potentially serious side effects, including opportunistic infections (Toruner et al., 2008). Recent advances in the treatment of inflammatory bowel disease have focused on the development of biologic agents (e.g., monoclonal antibodies, recombinant proteins or peptides, antisense oligonucleotides) targeted at neutralizing specific pro-inflammatory proteins. Specifically, monoclonal antibodies targeting tumour necrosis factor (TNF)-alpha (such as infliximab, adalimumab or certolizumab pegol) have proven to be highly effective in patients with moderate to severe Crohn's disease and Ulcerative colitis (Colombel et al., 2009; Rivkin; Sandborn et al., 2009).
<table>
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<th>Chromosome</th>
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<th>Associated with Ulcerative colitis</th>
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</table>

Table 1.1 Gene associations in Crohn’s disease and Ulcerative colitis

ATG16L1, autophagy related 16-like protein 1; IL12B, interleukin-12; IL23R, interleukin-23 receptor; IRGM, immunity-related GTPase family, M; NKX2-3, NK2 transcription factor related, locus 3; NOD2, nucleotide-binding oligomerization domain protein 2; PTGER4, prostaglandin receptor, EP4; PTPN2, protein tyrosine phosphatase, non-receptor type 2; SLC22A5, solute carrier family 22, member 5; STAT3, signal transducer and activator of transcription 3; ZNF365, zinc-finger protein 365. Adapted and modified from (Cho, 2008).
1.8 Mouse models of IBD (DSS-induced colitis model)

In the past few decades, dozens of different murine models have been established to study the pathology of IBD and to help to develop effective therapy for this disease. The mouse models of IBD are classified into 6 major groups: chemically induced, bacterially induced, cell-transfer, spontaneous, congenital (spontaneous gene mutation), and genetically engineered models (Mizoguchi, 2012). Although these animals model don’t fully mirror human IBD, they do provide us with an opportunity to gain a significant understanding of the complex mechanism of IBD. Moreover, they have aided the development of novel therapeutic strategies for the treatment of IBD (Boismenu and Chen, 2000; Mizoguchi, 2012). To date, a number of murine IBD models have been developed, each displaying varying strengths and limitations (e.g. ease and length of experiments, type of inflammation induced (acute or chronic), spontaneous versus chemically induced onset and subtle versus severe phenotypes) (Mizoguchi, 2012). Despite some limitations, the murine IBD model(s) have contributed greatly to our understanding of this complex disease. It has been commonly accepted that chemically induced murine models of IBD are one of the most commonly used models because of their simplicity and wide applicability. Today, the most commonly used chemical models are the dextran sodium sulfate (DSS) and trinitrobenzene sulfonic acid (TNBS) induced colitis. Moreover, combining azoxymethane (AOM) pre-treatment with multiple cycles of DSS administration can be used as a robust model of colitis-associated-carcinogenesis (CAC) (Elson et al., 1995).

In the DSS model, oral administration of heparin-like polysaccharide DSS in drinking water induces colitis. The exact mechanism through which DSS initiates colitis is unknown, however the literature suggests that direct epithelial cell toxicity, increased intestinal permeability and macrophage activation are the major factors that contribute to the pathology of this form of colitis (Dieleman et al., 1994; Kim et al., 2010; Perse and Cerar, 2012). It has also been proposed that the imbalance between increased apoptosis and decreased proliferation causes relevant leaks in the epithelium barrier and thus, facilitates the mucosal invasion of intraluminal microorganisms during the acute phase of DSS colitis (Araki et al., 2010). The typical onset of DSS-induced colitis is characterised by diarrhea, bloody faeces, weight loss and the histological features of inflammation. As the acute inflammatory response is independent of T- and B-cells, the model is particularly useful to study the contribution of innate immune mechanisms in
intestinal inflammation. The acute dextran sodium sulfate (DSS) colitis mouse model was chosen to examine the role of caspase-11 in innate immune response during intestinal inflammation in this study. This model is simple to perform; the onset, duration, and severity of inflammation are immediate and controllable. Importantly, the acute DSS-induced colitis model mimics the clinical and histological features of IBD, that have characteristics of UC (Egger et al., 2000; Okayasu et al., 1990), making this murine model highly relevant. To date, the murine IBD DSS-induced model has been widely used to understand inflammation signalling pathways and it has been performed in various knockout mice such as NLRP3, Caspase-1, ASC, and other inflammasome components (Allen et al., 2010; Araki et al., 2005; Takagi et al., 2003). Thus, we believe it would facilitate us with a relevant model to investigate the putative role of caspase-11 in such processes.

1.9 Role of the innate immune system in the development of IBD

The human intestine is constantly being exposed to an enormous number of microorganisms. However, in most cases, infection is prevented because of a highly developed immune system which forms an immediate line of response to immunologic challenges presented by bacteria, viruses, and fungi (Eckburg and Relman, 2007; Lavelle et al., 2010). The mucosal immune system has evolved to balance the need to respond to pathogens while co-existing with commensal bacteria and food antigens. In inflammatory bowel disease (IBD), this hyporesponsiveness or tolerance breaks down and inflammation supervenes, driven by the intestinal microbial flora (Fig. 1.8).

As previously described, bacteria contain compounds that are recognized by a variety of receptors, including TLRs and NLRs and are potent stimuli of innate immune responses. These aforementioned multi-receptors are expressed on epithelial and immune cells in the gastrointestinal tract and various mutations in these receptors have been associated with development of IBD. Numerous PRR gene knockout mice have been generated and have provided important information pertaining to individual PRRs regarding their intestinal phenotypes and susceptibility to colitis, suggesting their important role during intestinal inflammation (Table 1.2).
Figure 1.8: Host - microbe interactions in the gut and development of IBD

The intestinal epithelial barrier protects underlying mucosal tissues from commensal bacteria present in the gut lumen. In healthy individuals, a state of immune tolerance exists that allows non-pathogenic microbes to live in the gut without any detrimental immune response. Dendritic cells residing in the intraepithelial spaces and lamina propria sample commensal bacteria and induce a regulatory immune response, which provides tolerance to commensal flora. In susceptible hosts, the epithelial barrier is compromised and allows commensal bacteria to invade the lamina propria and mucosa. Infiltrated bacteria interact with macrophages, dendritic cells and neutrophils via innate recognition receptors such as TLRs and NLRs. Activation of innate immune receptors induces the production of proinflammatory cytokines and chemokines that further recruit myeloid-derived immune cells to the infected tissue, which accelerates the inflammatory response and leads to the development of IBD. Adapted from Zaki et al., 2011.
<table>
<thead>
<tr>
<th>PRR target</th>
<th>Gene modification</th>
<th>Intestinal phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TLRs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>Knockout</td>
<td>• Increased susceptibility to DSS colitis (Cario et al., 2007)</td>
</tr>
<tr>
<td>TLR4</td>
<td></td>
<td>• Increased susceptibility to DSS colitis, exacerbate IL-10−/− colitis (Fukata et al., 2005; Matharu et al.)</td>
</tr>
<tr>
<td>TLR5</td>
<td>Knockout</td>
<td>• Spontaneous colitis (25%), increased susceptibility to DSS colitis (Vijay et al., 2007; Carvalho et al., 2012)</td>
</tr>
<tr>
<td>TLR9</td>
<td></td>
<td>• Increased susceptibility to acute DSS colitis but resistant to chronic DSS colitis (Rachmilewitz et al., 2004)</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td></td>
<td>• Increased susceptibility to DSS colitis (Fukata et al., 2005)</td>
</tr>
<tr>
<td>TRIF−/−</td>
<td></td>
<td>• Reduced or lack of contribution to susceptibility to acute and chronic DSS (Breglio et al., 2000; Brandt et al., 2000)</td>
</tr>
<tr>
<td><strong>NLRs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD1</td>
<td>Knockout</td>
<td>• Increased susceptibility to DSS colitis (Goh et al., 2008)</td>
</tr>
<tr>
<td>NOD2</td>
<td>Knockout</td>
<td>• Increased susceptibility to DSS colitis and TNBS colitis (Kobayashi et al., 2005; Petnicki-Ocwieja et al., 2009)</td>
</tr>
<tr>
<td>NOD2</td>
<td>Knock-in of human NOD2-3020incC mutation</td>
<td>• Increased susceptibility to bacterial-induced intestinal inflammation (Petnicki-Ocwieja et al., 2009)</td>
</tr>
<tr>
<td>NLRC4</td>
<td>Knockout</td>
<td>• Similar susceptibility to DSS colitis as WT mice (Hu et al., 2010)</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Knockout</td>
<td>• Increased or reduced susceptibility to DSS colitis (depending on the reports). Increased or reduced susceptibility to TNBS colitis (Zaki et al., 2010; Dupon-Chicoine et al., 2010; Bauer et al., 2010; Zaki et al., 2011)</td>
</tr>
<tr>
<td>NLRP6</td>
<td>Knockout</td>
<td>• Spontaneous colitis, increased susceptibility to TNBS colitis (Goh et al., 2011; Normand et al., 2011)</td>
</tr>
</tbody>
</table>

Table 1.2 Intestinal phenotype and their susceptibility to colitis in PRR knockout mice. Adapted and modified from (Fukata and Arditi, 2013).
1.9.1 Role of TLRs in IBD

Differential alteration of TLR expression in inflammatory bowel disease (IBD) was first described 13 years ago (Cario and Podolsky, 2000). Since then, studies from numerous groups have led to the current concept that TLRs represent key mediators of innate host defence in the intestine and are involved in maintaining mucosal as well as commensal homeostasis. Failure of this regulatory mechanism mediated via TLRs in the gut is postulated to contribute to the development of IBD (Abraham and Medzhitov, 2011). The role of TLRs in IBD has been previously extensively studied (Abreu and Arditi, 2004; Cario, 2010), and only the most relevant one (TLR4) for this study will be summarized in the following section.

TLR4 in IBD

TLR4 is a membrane bound PRR which can stimulate an innate immune response by recognition of bacterial lipopolysaccharide (LPS). TLR4 is also expressed intracellularly, in the Golgi apparatus of IECs (Hornef et al., 2003) and in CD4+ CD25+ T-regulatory (Tregs) cells (Caramalho et al., 2003). There is a large body of literature indicating a role for TLR4 in the inflammatory process in IBD and colitis-associated cancer, CAC (Abreu and Arditi, 2004; Cario, 2010). Luminal LPS is usually non-immunogenic within the healthy intestine most likely due to low TLR4 expression on IECs. TLR4 up-regulation may alter this balance from tolerance to a pro-inflammatory state (Cario and Podolsky, 2000). Indeed, TLR4 has been shown to be upregulated in both CD and UC (Hausmann et al., 2002). A possible “gain of function” hyperactivity mutation has also been described for TLR4, where up-regulation of TLR4 on IECs due to long lasting disease may lead to increased LPS sensitivity and heightened pro-inflammatory response (Melgar and Shanahan, 2010). Notably, the cellular distribution of TLR4 differs between CD and UC, with TLR4+ cells localised near the mucosal surface, thereby supporting the superficial inflammation observed in UC (Hausmann et al., 2002). Accumulating data has also implicated genetic variants of TLR4 contributing to the IBD phenotype. Importantly, the TLR4 gene is found on chromosome 9, a region containing a CD susceptibility gene (Cho et al., 1998). Two main single nucleotide polymorphisms (SNPs) of TLR4 have been observed, namely the co-segregating, missense mutations Asp299Gly and Thr399Ile, with the Asp299Gly mutation resulting
in a hypo-responsive phenotype to LPS (Arbour et al., 2000). Indeed, another report has shown that C3H/HeJ mice strain which have a single point mutation in TLR4, were more susceptible to developing DSS induced colitis (Elson et al., 2005).

The crucial role not only for TLR4 but also for TLR2 and their adapter protein MyD88 in the gut environment relates to their protective role in epithelial barrier repair which has been well demonstrated by several groups (Araki et al., 2005; Rakoff-Nahoum et al., 2004). For instance, in a DSS-induced colitis model it has been shown that TLR2<sup>-/-</sup>, TLR4<sup>-/-</sup> and MyD88<sup>-/-</sup> mice exhibited more severe disease symptoms compared to WT mice. Moreover, it has been also reported that intestinal epithelial cells proliferation in these knockout mice were markedly decreased, suggesting the importance of MyD88-dependent TLR signalling in protection against DSS damage (Fukata et al., 2005; Rakoff-Nahoum et al., 2004). However, the role of TRIF (another TLR4 adaptor) during acute-induced colitis in mice still remains unresolved. Results to date are highly contradictory based on differing susceptibility of TRIF knockout mice to DSS treatment. Brandi <i>et al</i> showed that TRIF signalling makes no contribution to protection against DSS-induced colitis (Brandl et al., 2010), while in contrast, Breglio <i>et al</i> showed that TRIF protects the mice against the development of DSS induced colitis in both acute and chronic models (Breglio et al., 2008). Given, this data further investigations into the role of TRIF in colitis is required.

1.9.2 Role of NLRs in IBD

As previously mentioned, NLRs have been referred to as the cytosolic guards of the innate immune system, recognising divergent molecular motifs and subsequently modulating the immune response whilst also functioning to maintain the reliability of the intestinal barrier against luminal microbes (Rosenstiel et al., 2007). The profound biological consequences of NLR-ligand recognition coupled with the evident association of polymorphisms in NLR genes in a multitude of polygenic, chronic inflammatory diseases (including CD), has implicated dysregulated NLR innate signalling as a contributor to disease pathogenesis (Shaw et al., 2011). The role of two crucial NLRs family members (NOD2 and NLRP3) in the pathogenesis of IBD will be summarised in this section.
NOD2 in IBD

NOD2 (CARD15) polymorphisms were the first recognized definitive risk factors for Crohn’s disease (Cho, 2008). NOD2 is a pattern recognition receptor which senses muramyl-dipeptide (MDP) a constituent of bacterial cell walls (in both Gram-positive and Gram-negative bacteria). Activation of NOD2 by MDP results in the activation of nuclear factor-κB (NF-κB) and mitogen-activated protein (MAP) kinase signalling pathways (Kobayashi et al., 2005). These pathways culminate in the transcriptional activation of genes encoding cytokines, chemokines and a variety of pro-inflammatory mediators that activate cells of the innate and adaptive immune system. It has been reported in many studies that the CD associated NOD2 mutations (which are primarily located in the leucine-rich-repeat region that mediates sensing of MDP) result in a functionally inactive NOD2 protein that leads to defective activation of NF-κB and antibacterial defences thus favoring a loss of function phenotype (Brain et al., 2010; Cario, 2005). In contrast, increased activation of NF-κB and overproduction of proinflammatory cytokines such TNF-α, IL-1β or IL-6 was observed in the lamina propria of patients with Crohn’s disease, providing evidence for gain of function phenotype (Schreiber et al., 1998).

The precise mechanism by which NOD2 mutations contribute to CD remains largely controversial and reflects the complex contributions to NF-κB regulation in this setting. Loss of function mutations may result in altered host-microbial interactions such as altered tolerance to chronic bacterial stimulation, impaired clearance of pathogens or decreased production of anti-microbial products such as β-defensins. For example, mice lacking NOD2 showed impaired antibacterial responses after challenge with Listeria monocytogenes, which was paralleled by diminished expression of at least two Paneth cell-derived antimicrobial peptides, Defcr4 and Defcr-rs10 (Kobayashi et al., 2005). CD patients with mutant NOD2 were shown to have decreased expression of the human Paneth cell α-defensins HD-5 and HD-6 in the small intestine (Wehkamp and Stange, 2005), suggesting that CD-associated NOD2 mutations may be functionally equivalent to the loss of function phenotype. In addition, another study demonstrated that dendritic cells expressing Crohn’s disease mutant forms of NOD2 and ATG16L1 showed reduced autophagy in response to MDP and this led to impaired antigen presentation and bacterial clearance (Rioux et al., 2007; Travassos et al., 2010).
In line with gain of function phenotype Maeda et al showed that NOD2fs knock-in mice showed enhanced NF-κB activity and IL-1β secretion in response to MDP as well as increased susceptibility to DSS induce intestinal inflammation (Maeda et al., 2005). Moreover, in both chemical and antigen-driven colitis models, NOD2^{+/−} mice were more susceptible and demonstrated excessive intestinal inflammation compared to the wild type mice (Barreau et al., 2007; Watanabe et al., 2008; Watanabe et al., 2006). In pathogen driven colitis, again NOD2^{+/−} mice were also more susceptible to *Helicobacter hepaticus* infection which correlated with increased intestinal inflammation (Biswas et al., 2010). In short, it is clear that NOD2 is a very important sensor of bacteria, especially in intestinal epithelial cells and structural changes of this protein may influence the innate immune response to intestinal bacteria and contribute to the pathogenesis of IBD.

### 1.9.3 NLRP3 in IBD

Previous studies in relation to the gut-microbiota interplay related to IBD pathogenesis have raised the possibility that NLRP3 inflammasome plays a crucial role in the development of pathological intestinal inflammation during this disease. It has been found that SNPs as regulatory elements of NLRP3 are strongly associated with increased susceptibility to CD development in humans (Villani et al., 2009). In this regard, NLRP3 SNPs were associated with lower NLRP3 expression and IL-1β secretion from LPS-activated monocytes that were homozygous for the risk alleles (Villani et al., 2009). In contrast, another study has shown that NLRP3 mutations in monocytic cells displayed enhanced caspase-1 activation and IL-1β production resulting in enhanced pro-inflammatory responses (Dowds et al., 2004). Similarly, increased expression of IL-1β in the inflamed mucosa of patients with CD and UC has been also observed (Papadakis and Targan, 2000). Moreover, elevated levels of IL-18 in cells of the intestinal mucosa were also detected in affected regions of the gut of CD patients (Monteleone et al., 1999; Pizarro et al., 1999). Therefore, it appears that homeostasis of the intestinal epithelium is highly sensitive to the levels of expression of the inflammasome effectors IL-1β and IL-18, and deregulated expression (culminating in either increased or decreased protein levels) of these cytokines might severely affect the susceptibility of the gastrointestinal tract to IBD.

39
The key role of the NLRP3 inflammasome in regulating gut homeostasis was supported by several in vivo studies examining the molecular mechanisms by which NLRP3, ASC and caspase-1 control integrity of the intestinal epithelium and modulate immune responses to microbiota in the gut during experimental colitis. In the context of the DSS colitis models, NLRP3^{-/-} and ASC^{-/-} mice were more susceptible in both the acute and chronic phases of colitis (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). NLRP3^{-/-} mice were also more susceptible to TNBS-induced colitis than WT mice (Hirota et al., 2011; Zaki et al., 2010a). This data supports a critical role of inflammasome signalling in protection against pathological intestinal inflammation. In addition NLRP3^{-/-} mice exhibited increased mucosal permeability accompanied by impaired epithelial proliferation during DSS colitis (Zaki et al., 2010a). Moreover, Liu et al using the C. rodentium-induced colitis model, demonstrated that NLRP3^{-/-} mice could not properly control bacterial infection as increased pathogen translocation to other organs and also impaired histopathology in colon were observed (Liu et al., 2012). However, Bauer et al reported the opposite result, showing that NLRP3^{-/-} mice developed less severe colitis than wild type mice producing lower levels of proinflammatory cytokines in colonic tissue.

In the context of DSS colitis models the role of IL-18 is still under debate. Some reports have shown that IL-18 and IL18R knockout mice develop more severe colitis compared to WT mice, suggesting a beneficial role for IL-18 by contributing to intestinal epithelial cell regeneration during acute DSS colitis (Ishikura et al., 2003; Takagi et al., 2003). In contrast, other studies have shown that IL-18 neutralisation with anti-IL-18 antibodies reduced the severity of colitis in mice accompanied with IFN-γ and TNF-α production suggesting a detrimental role for IL-18 during the intestinal inflammation (Siegmund et al., 2001). Furthermore, Lebeis et al showed that IL-1R knockout mice infected with C. rodentium exhibited increased mortality together with severe colitis characterized by intramural colonic bleeding and intestinal damage, suggesting that IL-1R signalling protects mice following infection with C. rodentium (Lebeis et al., 2009).
1.9.4 Role of inflammatory caspases in IBD

Deregulation of caspase activity has been associated with a variety of human diseases, including inflammatory bowel disease (IBD) and colorectal cancer (van der Woude et al., 2004). It has long been declared that increased secretion of the main substrates of caspase-1 (IL-1β and IL-18) is associated with intestinal inflammation and the increased risk of developing IBD (Pizarro et al., 1999). Aside from caspase-1 and caspase-12 as detailed in the paragraph below, there are no reports to suggest the involvement of caspase-4 and -5 in the regulation of intestinal inflammation during IBD. In these reports, a role of the aforementioned caspases and other inflammasome components have been examined using various mouse models of IBD, including the dextran sulfate sodium (DSS) induced colitis model.

Many reports have demonstrated that caspase-1 has a protective role during intestinal inflammation. For example, Dupaul-Chicoine, Zaki and colleagues reported that Caspase-1 protects the intestine from excess injury by promoting tissue repair (Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). They have shown that caspase-1 deficient mice develop more severe colitis than control mice after administration of dextran sulfate sodium (DSS), presumably because of defects in mucosal repair. Moreover, administration of IL-18 rescued the mice, from colitis demonstrating that caspase-1-induced maturation of this cytokine which controls tissue damage in the intestine. Furthermore, mice deficient in caspase-12 (a repressor of caspase-1), were resistant to colitis, secreted more cytokines, including IL-1β, and had increased tissue repair compared with control mice (Dupaul-Chicoine et al., 2010). Together with their increased inflammatory response, the enhanced repair response of Casp-12^-/- mice rendered them more susceptible to colorectal cancer induced by azoxymethane (AOM) + DSS. These findings revealed that inflammatory caspases induce inflammation in the intestine after injury but are also required for tissue repair and maintenance of intestinal immune homeostasis.

Despite these fascinating insights into the crucial role for inflammatory caspase-1 and -12 and other inflammasome components during the intestinal inflammation, the involvement and regulation of human caspase-4 and -5 (and their murine homolog caspase-11) has yet to be determined.
1.10 IBD chronic inflammation – a cause of colitis associated cancer (CAC) development

The link between the tumour and its environment has been well established and is assumed to be driven by chronic inflammation, considered now to be the seventh hallmark of cancer (Hanahan and Weinberg, 2000). Tumour cells rely on their environment for development and progression (Allen and Louise Jones, 2011; Gribben et al., 2010). The inflammatory response in the tumour microenvironment is characterised by infiltration of immune cells, alterations in cytokines and chemokines and vascular changes. Chronically inflamed tissues, in which these changes often already exist, are susceptible to tumour formation. Many studies show that tumours develop and progress within inflammatory diseases. For example, patients with long standing inflammation of the oesophagus caused by acid reflux known as Barrett’s oesophagus are at increased risk of developing oesophageal adenocarcinoma (OAC) (Hvid-Jensen et al., 2011). Patients with a history of persistent inflammation of the stomach lining, known as gastritis, are at increased risk of developing gastric cancer (Vannella et al., 2012). Patients with gallbladder inflammation, caused by infection or gallstones, are at increased risk of developing gallbladder cancer (Stinton and Shaffer, 2012). Notably, the chronic inflammation highlighting the pathogenesis of inflammatory bowel diseases (IBD) is an important risk factor for the development of colon cancer called colitis-associated cancer (CAC) (Feagins et al., 2009; Lakatos and Lakatos, 2008; Saleh and Trinchieri, 2011). It has been shown that UC increases the cumulative risk of CAC by up to 18-20%, while CD up to 8% after of 30 years of disease (Canavan et al., 2006; Eaden et al., 2001). The overall exact increase in prevalence of CAC in IBD patients depends on disease severity and duration, patient groups analyzed, accessibility to preventive colonoscopies in normal cohorts and efficacy of anti-inflammatory therapies and IBD management (Herrinton et al., 2012; Jess et al., 2012; Rubin et al., 2008). Interestingly, in murine models of CAC, only single injection of carcinogen azoxymethane (AOM) give rise to multiple colonic tumours, when coupled to the induction of chronic colitis (Neufert et al., 2007; Okayasu et al., 1996). However, it takes multiple carcinogen injections and longer periods of time for tumours to progress when there is a lack of inflammation. These clinical and experimental observations clearly identify CAC as classical inflammation-driven cancer and make mouse models of CAC extremely valuable for our understanding of general
mechanisms which link inflammation and cancer (Grivennikov et al., 2010; Trinchieri, 2012).

The molecular mechanisms by which inflammation promotes cancer development are still being uncovered and may differ between CAC and other forms of colorectal cancer. However a few mechanisms have been identified explaining how sustained inflammation in the colon can lead to development of CAC. Firstly, involvement of reactive oxygen and nitrogen species (RONS) has been implied to play a crucial role (Coussens and Werb, 2002). It appears that during chronic inflammation, inflammatory cells, such as activated macrophages and neutrophils, are potent sources of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), (highly reactive and mutagenic agents) which cause DNA damage and mutations (Hussain et al., 2003; Meira et al., 2008), therefore facilitating tumour development. It is also worth noting that DNA damaging agents can be produced not only by immune cells but also by epithelial and pre-malignant cells, and the latter can be primed to produce ROS and RNI by inflammatory cytokines, such as TNF and IL-1 (Goodman et al., 2004; Shaked et al., 2012). Further acceleration of CAC tumourigenesis by inflammation and inflammation-derived endogenous DNA damaging agents can be associated with oxidative inactivation of genes encoding DNA repair components, such as mismatch repair (MMR) (Colotta et al., 2009; Hussain et al., 2003).

The importance of inflammation in the development of CAC is highlighted by the dependence of tumour growth and progression on the activation of NF-κB. NF-κB is a key player in inflammation whose activity is triggered in response to infectious agents and proinflammatory cytokines via the IκB kinase (IKK) complex (Karin and Greten, 2005). Using a colitis-associated cancer mouse model, Greten et al showed that IKKβ/NF-κB pathway in epithelial cells contributes to tumour promotion by suppressing apoptosis through the the mitochondrial pathway. However, in myeloid cells, IKKβ/NF-κB was involved in production of inflammatory mediators that promote tumour growth. These results collectively implied that NF-κB driven inflammatory responses play a role in survival and proliferation of pre-malignant cells and served as proof for the connection between inflammation and cancer (Greten et al., 2004). Notably, during IBD and also as well in many types of cancer, including CAC and CRC (colorectal cancer), NF-κB is hyperactivated, suggesting its important role in driving inflammation in these diseases (Greten et al., 2004; Karin and Greten, 2005). It has been
also shown that the levels of NF-κB activating cytokines, such as TNF and IL-1, are elevated in IBD and CAC (Atreya and Neurath, 2008; Karin, 2006; Murch et al., 1993). Today, inhibition of these cytokines is currently clinically used to treat IBD and may represent potential attractive targets for CAC treatment in the future.

There have been many murine models developed for the study of colon carcinogenesis and examples include: (i) genetic models, involving the development of genetically engineered mice deficient in the adenomatosis polyposis coli (APC) gene or mismatch repair genes (such as Msh2, Msh3, Msh6). These models are focused on mimicking immune dysregulation, inflammation and epithelial barrier dysfunction as seen in human disease; (ii) xenoplant models, involving development of tumours after subcutaneous or intravenous injection of human tumour cells in immunodeficient mouse strains (e.g. SCID mice). These models are mainly used to supply human tumours in an experimental in vivo environment for the assessment of cytostatic therapeutic compounds; or chemically induced models, involving injections of chemical carcinogens (e.g. dimethylhydrazine or azoxymethan (AOM)) in mice to induce colon lesions similar to human malignancy. These models can recapitulate in a highly reliable way the tumour phases of initiation and progression (De Robertis et al., 2011; Kanneganti et al., 2011).

To date, it is well established that inflammasome activation has been shown to play an important role in inflammation associated CAC tumourigenesis. The role of the inflammasome in colorectal cancer tumourigenesis has been mainly explored using the chemically induced mouse model, the AOM/DSS induced associated colorectal cancer model. The AOM/DSS model involves initial administration of the DNA methylating agent AOM followed by repeated cycles of DSS which is an inflammatory agent that disrupts the epithelial lining of the colon (De Robertis et al., 2011). It appears that AOM/DSS induced tumourigenic potential is mouse strain dependent. For example, a study compared Balb/c, C3H/HeN, C57BL/6N and DBA/2N mouse strains using a single AOM intraperitoneal injection followed by 1% (w/v) DSS for 4 days and then no further treatment for 18 weeks. Results showed that there was 100% tumour frequency in Balb/c mice and 50% in C57BL/6N. The C3H/HeN and DBA/2N mice only developed some adenomas but no adenocarcinomas (Suzuki et al., 2006). There are also other factors, which can affect induction and severity of the AOM/DSS model, including DSS source, molecular weight, numbers of DSS cycles, AOM doses or
imprecise injection techniques (Neufert et al., 2007). Therefore optimising experiments are usually required before achieving a successful development of AOM/DSS induced tumours in mice.

Using optimised AOM/DSS mouse models, it has been demonstrated that IL-18 knockout mice, exhibited increased inflammation and tumour development compared to wild type mice (Salcedo et al., 2010), thus suggesting a protective role for IL-18 in carcinogenesis and tumour progression. In addition, it has been also shown that IL-18 can affect epithelial growth by regulating the production of additional interleukins. In this regard, activation of NLRP3 or NLRP6 inflammasomes led to IL-18-dependent downregulation of IL-22 binding protein (IL-22bp) and higher expression of IL-22. This IL-22-IL-22bp axis was shown to critically regulate intestinal tissue repair and tumourigenesis in the colon (Huber et al., 2012). Moreover, mice lacking NLRP3 were shown to be more susceptible to tumourigenesis in the AOM-DSS model which was exhibited by increased inflammation, higher tumour burden and reduced colonic IL-18 levels compared to control mice (Allen et al., 2010; Zaki et al., 2010b). Recombinant IL-18 was successfully used to rescue the mice and was able to inhibit tumour progression, suggesting a protective role for IL-18 in inflammation-promoted tumourigenesis in the colon (Zaki et al., 2010b). Interestingly, caspase-1 deficient mice were also shown to be more susceptible to AOM/DSS induced colitis associated tumourigenesis, which was exhibited by enhanced tumour formation. CaspL^-^ mice showed increased colonic epithelial cell proliferation in the early stages of tumour development and reduced apoptosis in advanced tumours. Surprisingly, this suggested a role for proinflammatory caspase-1 in tumourigenesis not through regulation of inflammation but mainly through regulation of colonic epithelial cell proliferation and apoptosis (Hu et al., 2010). Despite the depth of knowledge accumulated to date regarding the important role of inflammation and inflammasome components in developing CAC cancer, the role of inflammatory caspase-4 and -5 (and their murine homolog caspase-11) in CAC development has yet to be documented.

1.11 From Barrett's oesophagus to OAC - a role for inflammation

Barrett's oesophagus (BO) occurs in the distal oesophagus and is defined as the replacement of the normal stratified squamous epithelium with columnar epithelium
containing mucin-producing goblet cells. The process in which one kind of fully differentiated (adult) cell replaces another is called metaplasia (Spechler, 1993). Metaplasia is commonly a consequence of chronic inflammation and Barrett’s metaplasia mainly results from chronic inflammation of the oesophagus caused by acid reflux, called gastroesophageal reflux disease (GERD). To date, it has been broadly established that GERD plays a central role in the pathogenesis of BO metaplasia (Fitzgerald et al., 2002b; Monkemuller et al., 2012). However, there are many other factors which also contribute to its occurrence including central obesity, which causes increased intragastric pressure that enhances reflux. Patients with obesity also have elevated levels of insulin like growth factor-1 (IGF-1) which promote cell proliferation and determine cell differentiation (McElholm et al., 2010). Genetic and epigenetic studies have shown modifications of intestinal-specific transcription factors (CDX1 and CDX2) and protein (BMP4), that regulate the development and differentiation of the intestinal columnar epithelium (Lord et al., 2005; Silberg et al., 1997; Zhou et al., 2009). Additional risk factors include a diet low in fruit and vegetables, cigarette smoking, elevated levels of nitrites and bile acids (Iijima et al., 2002; Kubo et al., 2010; Lubin et al., 2012).

Barrett’s oesophagus is a premalignant condition which results in an increased risk of progressing to oesophageal adenocarcinoma (OAC) in the setting of chronic inflammation (Hameeteman et al., 1989). It is established that Barrett’s epithelium progress sequentially from intestinal metaplasia (IM) to low grade dysplasia (LGD), high grade dysplasia (HGD) and eventually to oesophageal adenocarcinoma (OAC) (Hvid-Jensen et al., 2011; Ronkainen et al., 2005). Although BO is associated with a low (0.5%) annual incidence of HGD or OAC, a four-fold increase in incidence of oesophageal cancer has been noted in certain patient populations (Brown et al., 2008; Sharma et al., 2006; Spechler et al., 2010). Despite the extensive studies associated with gene expression profiles of BO and OAC (van Baal et al., 2008; van Baal et al., 2005), the knowledge regarding processes involved in the progression to OAC in patients with BO is still incomplete.

As pathogenesis in many human cancers has been linked to inflammation, it is proposed that the inflammatory microenvironment may play a crucial role in BO progression to OAC. In fact a number of different types of immune cells have been shown to play a key role in this process. Immune cells can facilitate tumour
development through the secretion of factors that promote carcinogenesis and help the tumour to evade the host response by creating an immunosuppressive environment (Gribben et al., 2010). Although immune cell infiltration is observed during all stages of the progression from BO to dysplasia to OAC, there is a lack of significant research data outlining the exact roles they play in oesophageal disease. However, it has been shown that dendritic cells (DCs) levels are increased in BO tissue compared to normal oesophageal tissue (Bobryshev et al., 2009). Although the role of DCs remains unclear, it was proposed that DCs in the oesophageal microenvironment may activate dormant stem cells, identified by the stem cell marker Musashi-1, causing the development of BO and OAC (Bobryshev et al., 2010). Another type of inflammatory cell, T-helper cells have been identified in both squamous epithelium and in Barrett’s epithelium, while precursor (CD7+) T cells were located in adenocarcinoma tissue. Interestingly, NF-κB activation, a marker of inflammation, as well as apoptosis and caspase activity was observed in such T cells in Barrett’s and OAC (Berndt et al., 2010). Infiltration of eosinophils has been also shown in the mucosa of a subset of BO patients, associated with cell hyperplasia (Ravi et al., 2011). Increased number of macrophages producing the angiogenic factor vascular endothelial growth factor (VEGF) were also found in OAC patients (McDonnell et al., 2003).

Signalling molecules, including cytokines, chemokines and growth factors, clearly play key roles in the development of both inflammation and cancer. This is predominantly through the direct effects of promoting proliferation, angiogenesis and carcinogenesis as well as through the recruitment of immune cells (Grivennikov et al., 2010). Therefore, it is not surprising that a multitude of immune mediators present in the microenvironment of Barrett’s metaplasia facilitate its further progression to dysplasia and adenocarcinoma. The elevated levels of IL-1β, IL-6 and IL-8 were found in reflux injury-induced chronic inflammation of BO patients suggesting that these cytokines can contribute to the metaplastic and dysplastic conversion of BO (Fitzgerald et al., 2002a). It has been also shown that TGF-β1 expression, (which is an important promotor of tumour growth) was significantly increased in OAC tissue compared with Barrett’s tissue and its overexpression was associated with advanced tumour stage (von Rahden et al., 2006). Moreover, an increase in TGF-β migration and invasion in OAC cells by causing failure of cell-cycle arrest resulted in increased proliferation (Onwuegbusi et al., 2007). BO is also associated with cyclo-oxygenase-2 (COX-2)
upregulation (a key inflammatory molecule implicated in many cancers and linked with NF-κB activation) and its expression is further increased in dysplasia and adenocarcinoma (Lagorce et al., 2003; Morris et al., 2001). The transcription factor NF-κB has been also been found to be upregulated in progression from BO to OAC in tissue samples, along with one of its target molecules, IL-8 (Jenkins et al., 2007; O'Riordan et al., 2005). Interestingly, Quante et al revealed an important role for IL-1β in induction of BO and its progression to OAC. Using a transgenic mouse model of oesophageal inflammation, in which IL-1β expression was driven from Epstein-virus promoter, it was shown that mice developed metaplasia within a year and later progressed to dysplasia and OAC. Lastly, it has been demonstrated that IL-1β mediates its carcinogenic effect partially through IL-6-dependent activation and migration of gastric cardia progenitor cells, suggesting the presence of a tumour-promoting IL-1β-IL-6-pSTAT3 signalling cascade in mouse OAC (Quante et al., 2012).

Of note, overexpression of a number of TLRs have also been implicated in the metaplasia-dysplasia-adenocarcinoma sequence. In particular, TLR4 expression was found to be gradually increased in normal, Barrett’s metaplasia and OAC patient tissues, suggesting that this immune receptor may be involved in BO and OAC development. Interestingly, LPS/TLR4 activation in BO ex vivo culture biopsies led to increased COX-2 expression, implicating its contribution to malignant transformation (Verbeek et al., 2014). Increased expression of TLR5 and TLR9 have also been identified in OAC tissues. Elevated levels of TLR9 were linked to metastasis, poor grade of differentiation and poor diagnosis in OAC (Helminen et al., 2014; Kauppila et al., 2011). Overall, little information exists that documents the role of TLRs in OAC development or the role of inflammatory caspases in both BO and OAC pathogenesis.

Over recent decades, there has been a dramatic epidemiological shift in the incidence of oesophageal adenocarcinoma (OAC), which is now the dominant subtype of oesophageal cancer in many western countries (Devesa et al., 1998; Pera et al., 2005). Current treatment is based on the cancer's stage and location, together with the person's general condition and individual preferences. In most cases, chemotherapy with or without radiation therapy is used along with surgery (Stahl et al., 2013). Chemotherapy may be given after surgery (adjuvant, i.e. to reduce risk of recurrence) or before surgery (neoadjuvant). Photodynamic therapy has also been used to treat OAC. This therapy involves the use of drugs that are absorbed by cancer cells (when exposed
to a special light, the drugs become active and destroys the cancer cells) (Lagergren and Lagergren, 2013; Rees et al., 2010). However, despite improved therapies, the prognosis for patients with advanced OAC is still poor, with a 5-year survival rate of less than 20% (Hirst et al., 2011), mostly due to lack of obvious symptoms. By the time the first symptoms (such as difficulty swallowing) appear, the cancer has already well progressed. The diagnosis of BO, which is only one well-recognized premalignant condition in OAC development also appears to be problematic as Barrett's oesophagus have no specific clinical symptoms (despite that BO have symptoms of GERD such as frequent and longstanding heartburn, trouble swallowing, vomiting blood). Today, only endoscopy permits identification of different degrees of dysplasia in BO patients. In addition, there is a lack of biomarkers established to predict BO progression to OAC, thus dysplasia remains the main practical factor useful for identifying patients at increased risk for the development of oesophageal adenocarcinoma in clinical practice. Thus, there is a critical need for the identification of new biomarkers and new therapeutic targets which would facilitate the recognition of BO progression to OAC and allows for successful treatment. To this end, the elucidation of the roles played by caspase-1, -4 and -5 (and their murine homolog caspase-11) in this project will help provide a broader understanding pertaining to their function in modulating inflammation in inflammatory diseases such as IBD and Barrett’s oesophageal and also in inflammatory associated cancers.
1.12 Project aims

The overall aim of this project is to gain an enhanced understanding of the role of inflammatory caspases during disease-associated inflammation.

More specifically the three main aims of the project are:

1. To investigate the role of caspase-11 (the murine ortholog of human caspases-4 and -5) in an experimental murine model of acute colitis.

2. To determine the functional implication of caspase-11 activation during experimental colitis; and to gain mechanistic insight into the signalling pathways governing intestinal caspase-11 regulation.

3. To determine if there is an inflammatory role for human caspases-1, -4 and -5 in chronic inflammatory diseases, such as Inflammatory bowel disease (IBD) and Barrett’s oesophagus (BO), and to assess their involvement in the progression of these diseases to colorectal and oesophageal cancers, respectively.
Chapter 2
Materials and Methods
2.1 Animals

The caspase-11 knockout mice (on a C57BL/6J background strain) were obtained from Junying Yuan’s laboratory, Harvard Medical School, USA. Casp11⁻/⁻ mice where generated by targeting the casp-11 gene using 129-derived ES cells, as described (Wang et al., 1998). Briefly, the mutation in the casp-11 locus was created by using a replacement type targeting vector, designed to select homologous recombination events in the casp-11 locus. In this construct a 1.5 kb casp-11 genomic DNA fragment, containing the coding region for the active site (which is the pentapeptide QACRG encoded in exon 5), was replaced with the gene encoding neomycin phosphotransferase (neo cassette). This insertion resulted in deletion of 16 amino acids from the coding region of casp-11 in exon 5, including the active site, thus resulting in the elimination of functional caspase-11 protein expression. The created Casp-11 mutant ES cell clones were injected to blastocysts of C57BL/6J female mice to create chimeric mice which were further mated with C57BL/6J wild type female mice in order to obtain germline transmission of the casp-11 mutant alleles (Wang et al., 1998). Of the progeny from such crosses the segregation of the mutant casp-11 alleles agrees with the Mendelian inheritance.

Since obtaining the Casp11⁻/⁻ mice, they were re-crossed with wild-type (WT) C57BL/6J mice (purchased from Harlan, UK Ltd). Heterozygous and homozygous breeding pairs are being used to generate caspase-11 knockout (Casp11⁻/⁻) and their WT littermate controls for experimental trials in the Trinity College Dublin BioResource Unit. The mice are housed in individually ventilated cages (IVCs) in Trinity College’s Bioreources Unit, so the environment in which they are raised is strictly controlled, free of disease and fed a uniform diet. All experiments were performed under license from the HPRA (Health Products Regulatory Authority) with the approval from the Trinity College Dublin BioResources Ethics Committee. All mice were 8-12 weeks old and they were body weight and aged matched for every experiment. In the co-housing experiment 6 week old wild type and Casp11⁻/⁻ mice were co-housed for 2 weeks, after which they were challenged with DSS.

The IFNy⁻/⁻, IFNAR⁻/⁻, TRIF⁻/⁻ and Mal⁻/⁻ mice were kindly provided by Dr. Rachel McLoughlin, Prof. Kingston Mills, Prof. Padraic Fallon and Dr Ed Lavelle, respectively, Trinity College Dublin.
2.1.1 Genotyping of Casp11<sup>−/−</sup>, Casp11<sup>+/+</sup> and Casp11<sup>+/−</sup> C57BL/6J mice

2.1.1.1 Isolation of genomic DNA from mouse ear punches

Ear punches were taken from 21 day old mice to obtain a tissue sample from which genomic DNA was isolated. Tissue samples were transferred into a 1.5 ml tube and digested immediately or stored in -20°C. To each ear punch, 500 µl of extraction buffer (100 mM Tris-HCl, 5 mM EDTA, 0.2% (w/v) SDS, 200 mM NaCl, 500 µg/ml Proteinase K) was added and samples were incubated at 55°C o/n. The following day, samples were vortexed and checked for tissue degradation. Degraded samples were centrifuged at 20,900 g for 10 min and supernatants were transferred into fresh tubes containing 950 µl of ice-cold ethanol (100% v/v). Samples were vortexed until DNA precipitation was evident (white DNA fibres became visible). The DNA was then pelleted by centrifugation at 20, 900 g for 10 min and ethanol was aspirated. The DNA pellet was allowed to air dry for 40-60 min before resuspending in 35µl nuclease free water and stored at -20°C until use. DNA concentration was determined spectrophotometrically using a Nanodrop (Thermo Scientific) according to the manufacturer’s instructions.

2.1.1.2 Polymerase Chain Reaction (PCR)

Genomic DNA isolated from C57BL/6J mice (as described in section 2.1.1.1) was amplified via PCR to differentiate between mice containing the wild type- and mutant- caspase-11 genes, using primers adapted from (Hisahara et al., 2001). The PCR components are shown in Table 2.1 and Table 2.2. The conditions used are shown in Table 2.3. The PCR was performed using PTC-200 Peltier Thermal Cycler (MJ Research).
Table 2.1 Reagents used for PCR

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoTaq hotstart green mastermix, 2x</td>
<td></td>
<td>MSC</td>
</tr>
<tr>
<td>SY21 (WT forward primer)</td>
<td>5'GGCATGGAGTCAGAGATGAAAGAC3'</td>
<td>MWG</td>
</tr>
<tr>
<td>SY22 (WT reverse primer)</td>
<td>5'GCCCATGTGGCATTACCTGCCAGC3'</td>
<td>MWG</td>
</tr>
<tr>
<td>SYKO (mutant forward primer)</td>
<td>5'AGATCTACACCTCTGCACAACTGG 3'</td>
<td>MWG</td>
</tr>
<tr>
<td>PJK (mutant reverse primer)</td>
<td>5'TGGCGCTACCGTGATGAAAGGC 3'</td>
<td>MWG</td>
</tr>
<tr>
<td>GAPDH forward primer</td>
<td>5'TTCACCACCATGGAGAAGGC 3'</td>
<td>MWG</td>
</tr>
<tr>
<td>GAPDH reverse primer</td>
<td>5'GGCATGGACTGTGGTCATGA 3'</td>
<td>MWG</td>
</tr>
<tr>
<td>Ni-NTA agarose</td>
<td>polysaccharide obtained from agar that is used for gel electrophoresis</td>
<td>Qiagen</td>
</tr>
<tr>
<td>SYBR Safe DNA gel stain</td>
<td>highly sensitive stain for visualization of DNA in agarose or acrylamide gels</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

Table 2.2 PCR reaction components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA (100ng)</td>
<td>2</td>
</tr>
<tr>
<td>GoTaq Master Mix, 2x</td>
<td>10</td>
</tr>
<tr>
<td>Forward primer (10 μM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Reverse primer (10 μM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>7.2</td>
</tr>
<tr>
<td>Total Volume</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 2.3 PCR conditions

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>94</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>1 min</td>
<td>35</td>
</tr>
<tr>
<td>72</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

2.1.1.3 DNA Gel Electrophoresis

Agarose gels (1 - 2 % (w/v)) were made in 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The gel mixture was heated in a microwave until the agarose had fully dissolved, cooled and 1 x Sybr Safe was added. The gel was allowed to set for 30 min after which it was placed in an electrophoresis tank (BioRad, Ireland) and submerged in 1 x TAE buffer. 20 µl of PCR reaction mixture as well as DNA size ladders (Invitrogen) were loaded on the agarose gel. The gel was run at 120 Volts for 40 min. After electrophoresis the gel was photographed under a UV light to visualise for the presence of the PCR product with the expected bands sizes.

2.1.2 Induction, treatment and assessment of DSS-induced colitis

2.1.2.1 DSS-induced colitis in WT and Casp11−/− mice

Experimental colitis was induced in C57BL/6J Casp11−/−, IFNγ−/−, IFNAR−/−, TRIF−/− and Maf−/− mice and their WT (wild type) littermates. Animals were given 2% and 3% (w/v) dextran sodium sulfate (DSS) in sterile drinking water from two different suppliers (Fisher Scientific, MW 500,000 and MP Biomedicals, MW 36,000-50,000) for various numbers of days (3-11 days, as indicated). Fresh DSS solution was made every 3 days. The mice were scored daily for their disease activity index (DAI) by their weight loss, stool consistency and rectal bleeding. The DSS-induced colitis scoring system is shown in Table 2.4. Occult bleeding was detected based on the peroxidise
activity of haem in the stool using Hemoplus Kit (Sarstedt). On the last day of the experiment mice were sacrificed (by exposure to carbon dioxide, followed by cervical dislocation) and colons were removed and distance from the ceco-colonic junction to the rectum was measured. Colon length was measured as an indication of colonic inflammation. The colons were cleaned of fecal material with chilled PBS and sections of the middle colon (0.5cm) were collected into 0.5 ml 1 x lysis buffer (PBS, 1% (v/v) NP40 and proteinase inhibitor cocktail, Roche) and stored at -20°C for ELISA analysis. Additional sections of the middle colon were collected into 0.5 ml of RIPA buffer (50 mM TRIS; 150 mM NaCl; 0.02% (w/v) NaN₃; 0.1% SDS; 1% NP-40; 0.5% sodium deoxycholate) containing 5 mM EDTA, 1 µg/ml leupeptin, 1.7 µg/ml aprotinin and 0.1 mM PMSF and stored at -20°C for Western Blotting analysis. Sections of the distal colon (0.5cm) were fixed in 10% (v/v) buffered formalin (Sigma) for histological assessment of colitis.

Table 2.4  Scoring system for the disease activity index (DAI)

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight loss (%)</th>
<th>Stool consistency (Diarrhea score)</th>
<th>Occult/gross blood in stool (Bleeding score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>Well formed pellets</td>
<td>No blood-negative</td>
</tr>
<tr>
<td>1</td>
<td>1-5%</td>
<td>Changed formed pellets</td>
<td>Blood-positive trace</td>
</tr>
<tr>
<td>2</td>
<td>5-10%</td>
<td>Loose stool</td>
<td>Positive, moderate trace</td>
</tr>
<tr>
<td>3</td>
<td>10-15%</td>
<td>Diarrhea/No stool</td>
<td>Positive, gross trace</td>
</tr>
<tr>
<td>4</td>
<td>&gt;15%</td>
<td></td>
<td>Gross bleeding anus</td>
</tr>
</tbody>
</table>

2.1.2.2 Exogenous IL-18 and IFN-γ treatment

C57BL/6J WT and Casp11⁻/⁻ mice were treated with 2% (w/v) DSS (MP Biomedicals) for 6 days. For the IL-18 rescue experiments, Casp11⁻/⁻ mice were intraperitoneally (i.p) injected with PBS or 0.05 µg rIL-18 (R&D Systems)/mouse in 100 µl PBS for the first 3 and 7 consecutive days of two independent experiments. Alternatively, for the IFNγ rescue experiment, WT mice were i.p injected with PBS or 200IU rIFNγ (Immunotools) /mouse in 100µl of PBS for the 8 consecutive days.
2.1.3 Histology

2.1.3.1 Tissue processing

After fixation for 24 h, in 10% buffered formalin (v/v), colon sections for histological analysis were placed in 70% ethanol for long term storage. Prior to paraffin embedding all tissue samples were processed using the TP1020 Automatic Tissue Processor (Leica®, Microsystems, Ireland) which is a fully automated instrument capable of subjecting tissue samples to 10 individual treatments (Table 2.5). Tissue processing resulted in dehydrated, clarified and paraffin infiltrated colon samples.

Table 2.5 Tissue processing treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol</td>
<td>1</td>
</tr>
<tr>
<td>80% ethanol</td>
<td>1</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>1</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1</td>
</tr>
<tr>
<td>50% ethanol / 50% xylene</td>
<td>1</td>
</tr>
<tr>
<td>100% xylene</td>
<td>1</td>
</tr>
<tr>
<td>100% xylene</td>
<td>1</td>
</tr>
<tr>
<td>100% paraffin</td>
<td>1</td>
</tr>
<tr>
<td>100% paraffin</td>
<td>1</td>
</tr>
</tbody>
</table>

Following processing each colon sample was individually embedded in paraffin using the Leica EG1150H Heated Paraffin Embedding Module (Leica®, Microsystems, Ireland). When completely set, 5 μm sections of both proximal and distal colon samples were cut using the Leica RM2235 Manual Rotary Microtome (Leica®, Microsystems, Ireland). Sectioned tissue was mounted on glass slides in a water bath, placed in a slide rack and left at 55°C overnight to dry.
2.1.3.2 Hematoxylin and eosin (H&E) staining

The standard H&E histological stain uses two separate dyes to stain the nucleus and cytoplasm of connective tissue. Hematoxylin is a dark purple dye that stains chromatin within the nucleus leaving it a dark purple/blue colour. Eosin is a dark orange-red dye that stains eosinophilic cytoplasmic material, such as connective tissue and collagen, leaving them various shades of red, pink and orange. The tissue sections were deparaffinised in Xylene (2 x 10 dips), rehydrated in 100%, 75%, 50% ethanol (each % x 10 dips) and washed in running tap water (10 dips). Next, the slides were incubated in Sellafield’s hematoxylin (Sigma) for 5 min, washed in running tap water (30-40 dips), incubated in standard eosin (Sigma) for 2 min and washed again in running tap water (30-40 dips). Slides were then dehydrated in 50%-75%-100% ethanol (each % x 10 dips) and cleared in Xylene (2 x 10 dips). The tissue slides were mounted using a Surgipath® Sub-X® mounting medium (Leica®, Microsystems, Ireland).

2.1.3.3 H&E histological analysis

H&E slides of distal colon sections were viewed using the Olympus BX51 research microscope (Olympus Inc., Tokyo, Japan). Colitis severity was assessed by a combined score of colon inflammatory cell infiltration (0-3) and tissue disruption (0-3) as outlined in Table 2.6. The histological scoring was performed in a blinded fashion by Dr. Mathilde Raverdeau from Prof. Kingston Mills lab.

Table 2.6 Colitis severity scoring system

<table>
<thead>
<tr>
<th>Score</th>
<th>Infiltration (Inflammation score)</th>
<th>Tissue disruption (Crypt damage score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Occasional infiltration in lamina propria</td>
<td>No mucosal damage</td>
</tr>
<tr>
<td>1</td>
<td>Increased infiltrate in lamina propria (predominantly at base of crypts)</td>
<td>Partial (&lt;50%) loss of crypts in large areas</td>
</tr>
<tr>
<td>2</td>
<td>Extension of infiltrate into mucosa</td>
<td>Partial to total (50-100%) loss of crypts in large areas</td>
</tr>
<tr>
<td>3</td>
<td>Transmural extension of infiltrate</td>
<td>Total loss of crypts in large areas and epithelial loss</td>
</tr>
</tbody>
</table>
2.1.4 Immunohistochemistry

2.1.4.1 Immunofluorescence staining

The distal colon sections were deparaffinized in Histoclear (National Diagnostics) for 5 min, progressively rehydrated in decreasing concentrations of ethanol (100%, 90%, and 70% for 5 min each), and finally incubated in water for 5 min. The retrieval process was performed by incubating the slides in boiling 0.01 M sodium citrate buffer (pH 6.0) for 10 min. The sections were then washed in PBS containing 0.1% Tween 20 and incubated overnight at 4°C with rabbit primary Ab proliferating cell nuclear antigen (PCNA; Abcam) at a dilution of 1:100. After several washes, the slides were incubated for 45 min with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) at a dilution of 1:500. The sections were mounted using fluorescence mounting medium (DakoCytomation) containing DAPI for DNA staining and viewed on a point-scanning confocal microscope (FV1000; Olympus). Images were obtained and analyzed using Olympus FV-10 ASW viewer software. Quantitative fluorescence intensity measurement of PCNA-positive epithelial cells was analyzed in three specifically selected areas of colonic tissue per mouse using Imaris software.

2.1.4.2 In situ intestinal proliferation assay

The presence of proliferating cells in mouse colonic tissues was detected by immunoperoxidase staining for 5-bromo-2-deoxyuridine (BrdU, a thymidine analogue) incorporation into DNA, using an in situ BrdU staining kit (BD Biosciences). On the last day of the experiment (day 5), BrdU (BD Biosciences) was dissolved in PBS at 1 mg/ml and mice were i.p injected at 50 mg/ml body weight. Distal colon tissues were collected from mice 3 h later, fixed in 10% (v/v) neutral-buffered formalin for 24 h, and embedded in paraffin. Immunohistochemistry was performed according to the BrdU staining kit manufacturer guidelines. The tissue sections were deparaffinised in Xylene (2 x 5 min) and rehydrated in 100% and 95% ethanol (each % x 3 min). To block endogenous peroxidase activity, tissue sections were treated with 3% hydrogen peroxidise (Sigma) for 10 min and then washed in PBS (3x5min). The antigen retrieval process was performed by incubating the slides in boiling BD™ Retrievagen A (pH 6.0) solution for 10 min. Sections were further incubated with the biotinylated anti-BrdU
antibody (1:10 dilution) for 1 h. After 1 hour incubation tissue sections were washed in PBS (3 x 2 min) and ready to use Strepavidin-HRP was applied into each tissue for 30 min. Next, the tissue sections were washed again in PBS (3 x 2 min). One drop of DAB (3,3-diaminobenzidine) chromogenic substrate was added onto each tissue section and incubated for 5 min or less until the desired colour intensity was developed. The slides were then counterstained with Hemotoxylin (Sigma) for 60 s, washed under running water for 10 min. Next the slides were dehydrated in 95%, 95%, 100%, 100% ethanol for 5 min each and cleared in Xylene (3 x 5 min). The tissue slides were mounted using a Surgipath® Sub-X® mounting medium (Leica®, Microsystems, Ireland). Images were taken using the Olympus BX51 research microscope (Olympus Inc., Tokyo, Japan). The number of BrdU⁺ cells per 50 well-oriented crypts/mouse were analysed.

2.1.5 Cell death assay (TUNEL staining)

Apoptosis in distal colonic tissue sections was analysed by fluorescence microscopy using an In situ Cell Death Detection Kit Fluorescein (Roche) according to the manufacturer’s protocol. This kit is based on the detection of single- and double-stranded DNA breaks that occur at the early stages of apoptosis and it’s relies on the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technology. Images of stained tissue sections were taken using the Olympus BX51 research microscope (Olympus Inc., Tokyo, Japan). For analysis, five random optical fields per colon were taken for the water treated and 2% (w/v) DSS-treated animals.

2.1.6 FITC-dextran assessment of intestinal permeability

C57BL/6J WT and Casp11⁻/⁻ mice were treated for 6 days with 2% DSS, followed by 1 day with regular drinking water. On the last day of the experiment mice were gavaged with permeability tracer FITC-dextran (molecular mass, 4 kDa; FD4; Sigma) at a concentration 60 mg/100 g body weight. Four hours after gavage, blood was collected by cardiac puncture. 250 µl of blood/mouse was immediately transferred into the tube containing 50µl of acid-citrate dextrose ((ACD), 20 mM citric acid, 110
mM sodium citrate, 5 mM dextrose, water) anticoagulant and kept on ice. Blood samples were centrifuged at 1000 x g for 12 min at 4°C and serum supernatants were transferred into a dark tube. Fluorescence of FITC-dextran in serum was measured on a FLUOstar OPTIMA Microplate Reader (BMG Labtech) at 490 nm excitation and 520 nm emission wavelengths. FITC-dextran concentration was determined from a standard curve generated by 2-fold serial dilutions of FITC-dextran (top standard, 800 ng/mL).

2.2 Measurement of cytokine concentration by ELISA

2.2.1 Preparation of samples from colon homogenates

To measure the cytokine amounts in colon tissue, middle sections of the colons were homogenized in lysis buffer (1 x PBS, 1% (v/v) NP40 and protease inhibitor cocktail from Roche) using a benchtop rotor-stator homogeniser. Colon homogenates were then centrifuged at 14,000 g for 10 min at 4°C. 50 µl of each sample supernatant was used for micro-BCA protein quantification (Thermo Scientific, Fisher). The rest of the sample supernatants were stored at -80°C until further use in ELISA assay.

2.2.2 BCA protein assay

Thermo Scientific Micro BCA™ kit (Thermo Fischer) was used to determine the protein concentrations in cell culture lysates and colon homogenates in prior to ELISA and Western blotting analyses. The BCA protein assay is based on the ability of bicinechonic acid (BCA) to detect cuprous cations (Cu⁺) which are formed when peptides of three or more amino acids chelates cupric ions (Cu²⁺). The resulting chelation produces a strong purple colour which is detected spectrophotometrically at 562 nm. A strong linear relationship between protein concentration and BCA/Cu⁺ complex formation exists over a large protein concentration range (Smith et al., 1985). A 2.5 – 320 µg/ml bovine serum albumin (BSA) serial dilution was prepared with PBS. 50 µl of each BSA dilution was added to a 96 well plate in triplicate. Each sample lysate was diluted 1:6.25, 1:12.5, 1:25, 1:50, 1:100 and 1:200 in a separate 96 well plate with PBS. 50 µl of each sample lysate dilution was added to the same 96 well plate which contained the BSA dilutions. An appropriate volume of Thermo Scientific Micro BCA™ Working Reagent (WR) was prepared comprising 25 parts Micro BCA Reagent
MA and 24 parts Micro BCA Reagent MB with 1 part Micro BCA Reagent MC (25:24:1, Reagent MA:MB:MC). 50 μl of Micro BCA WR was added to each sample lysate dilution well (and each BSA standard dilution well) and the plate was incubated at 37°C in the dark for 30 min. The absorbance was then read at 595 nm using the Spectra Max 340 PC Microplate Reader (Molecular Devices, LLC, CA, US). The BSA protein standard curve was used to determine the sample lysate protein concentrations from the BCA absorbance change.

2.2.3 ELISA assay

Cytokine concentrations were determined using commercially available ELISA kits according to the manufacturer guidelines. Antibody dilutions and incubation times were different between manufacturers and are detailed below. High binding 96-well plates (Greiner, Cruinn) were coated overnight at 4°C with 50 μl per well of capture antibody diluted in 1xPBS. Plates were washed 4 times in ELISA wash buffer (PBS/0.05% (v/v) Tween-20) and non-specific binding sites were blocked by incubation with 200 μl of assay diluent (1% (w/v) BSA in 1x PBS) for 2 h at room temperature. Plates were washed again 4 times before the addition of 50 μl (120 μg) per well of sample supernatant. A two-fold dilution series for each protein standard (using the recommended protein concentration) diluted in assay diluent buffer was also added to each ELISA plate and plates were incubated overnight at 4 °C. After washing, 50 μl of specific biotinylated detection antibody diluted in assay diluent was added per well and incubated at room temperature. Plates were washed 4 times and incubated with 50 μl/well horseradish peroxidase (HRP)-conjugated streptavidin in a dilution of 1:1000 (Biolegend) or 1:200 (R&D Systems) for 30 min at room temperature in the dark. The wells were washed 5 times with washing buffer, soaking the wells in buffer for 30 s to 1 minute for each wash to minimize the background. 50 μl of the substrate solution (TMB, Pierce) was added to each well and incubated for 20 minutes at room temperature, out of direct sunlight. To stop developing colour 50 μl of 1 M H₂SO₄ was added to each well and OD values were determined by measuring absorbance at 450 nm using a Spectramax Microplate Reader (Molecular Devices). Cytokine concentrations were calculated using the standard curve prepared using the recombinant cytokines.
Table 2.7 ELISA kits

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Supplier</th>
<th>Top Working Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>Biolegend/MSC</td>
<td>125 ng/ml</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Biolegend/MSC</td>
<td>2000 pg/ml</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Biolegend/MSC</td>
<td>1000 pg/ml</td>
</tr>
<tr>
<td>IL-17A</td>
<td>Biolegend/MSC</td>
<td>1000 pg/ml</td>
</tr>
<tr>
<td>IL-6</td>
<td>Biolegend/MSC</td>
<td>500 pg/ml</td>
</tr>
<tr>
<td>IL-18</td>
<td>R&amp;D Systems</td>
<td>4000 pg/ml</td>
</tr>
<tr>
<td>CXCL2/MIP-2</td>
<td>R&amp;D Systems</td>
<td>1000 pg/ml</td>
</tr>
<tr>
<td>IL-10</td>
<td>R&amp;D Systems</td>
<td>2000 pg/ml</td>
</tr>
<tr>
<td>IL-22</td>
<td>R&amp;D Systems</td>
<td>2000 pg/ml</td>
</tr>
</tbody>
</table>

ELISA assay details

**R&D systems**

Capture antibody dilution: 1/180;
Assay diluent: 1% (w/v) BSA in PBS;
Detection antibody dilution: 1/180, incubation time 2 h;
Streptavidin dilution: 1/200;

**BioLegend**

Capture antibody concentration: 1/200;
Assay diluent: 1% (w/v) BSA in PBS;
Detection antibody: 1/200; incubation time 1h;
Streptavidin dilution: 1/1000
2.3 Western blotting

2.3.1 Preparation of samples from colon homogenates

Middle sections of the colons were homogenized in RIPA lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate and 1% (v/v) NP-40) supplemented with 5 mM EDTA and proteinase inhibitors: aprotinin, phenylmethansulfonyl and leupeptin (as shown in Table 2.8) using a benchtop rotor-stator homogeniser. Next colon homogenates were centrifuged at 14,000 g for 10 min at 4 °C. 50 µl of each sample supernatant was used for micro-BCA protein quantification (Thermo Scientific, Fisher). The rest of the sample supernatants were stored at -20°C until further use.

Table 2.8 Small molecule inhibitors

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
<th>Supplier</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete Ultra Tablets</td>
<td>Protease inhibitor cocktail</td>
<td>Roche</td>
<td>1 tablet/5ml</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Serine protease inhibitor</td>
<td>Sigma</td>
<td>1.7 µg/ml</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>Serine and Cysteine proteases inhibitor</td>
<td>Sigma</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td>PMSF</td>
<td>Serine protease inhibitor</td>
<td>Sigma</td>
<td>1 µg/ml</td>
</tr>
</tbody>
</table>

2.3.2 SDS-Polyacrylamide Gel Electrophoresis

Directly before electrophoresis the protein lysates (normalised via micro-BCA assay) were diluted with 2 x SDS loading buffer (2.5 mM Tris-HCl, pH 6.8, 11% (v/v) Glycerol, 10% (w/v) SDS, 0.1% (w/v) bromophenol blue, dH2O and 50 mM dithiothreitol (DTT) - only added before use) and boiled at 95 °C for 5-10 min. Typically, 40 µg of each sample was then resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Mini-PROTEAN electrophoresis cell. Samples were run on 5% and 12% (v/v) polyacrylamide gels (as shown in Table 2.9) at 100V alongside with pre-stained protein markers (SeeBlue Plus2, Invitrogen) as molecular weight standards in 1x running buffer (2.5 mM Tris-base, 25 mM Glycine, 0.01% (w/v) Sodium dodecyl sulphate (SDS)).
Table 2.9 Components of polyacrylamide gel

<table>
<thead>
<tr>
<th>12% (v/v) Resolving gel (15ml)</th>
<th>5% (v/v) Stacking gel (5ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>dH₂O</td>
</tr>
<tr>
<td>4.9 ml</td>
<td>3.4 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCL (pH 8.8)</td>
<td>1 M Tris-HCL (pH 6.8)</td>
</tr>
<tr>
<td>3.8 ml</td>
<td>0.63 ml</td>
</tr>
<tr>
<td>Acrylamide:bisacrylamide (30% (v/v) ProtoGel, National Diagnostics)</td>
<td>Acrylamide:bisacrylamide (30% (v/v) ProtoGel, National Diagnostics)</td>
</tr>
<tr>
<td>6 ml</td>
<td>0.83 ml</td>
</tr>
<tr>
<td>10% (w/v) SDS (Sigma)</td>
<td>10% (w/v) SDS (Sigma)</td>
</tr>
<tr>
<td>0.15 ml</td>
<td>50 µl</td>
</tr>
<tr>
<td>10% (w/v) Ammonium persulfate (APS) (Sigma)</td>
<td>10% (w/v) Ammonium persulfate (APS) (Sigma)</td>
</tr>
<tr>
<td>0.15 ml</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED (Sigma)</td>
<td>TEMED (Sigma)</td>
</tr>
<tr>
<td>6 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

2.3.3 Transfer of proteins to blotting membrane

Directly after SDS-PAGE proteins were transferred to Protran nitrocellulose membrane (VWR) using a wet transfer system. Nitrocellulose membrane, gel, and filter paper were soaked in 1 x transfer buffer, pH 8.3 (48 mM Tris-HCl, 39 mM Glycine, 20% (v/v) Methanol, 0.037% (w/v) SDS). The membrane was placed on top of two pieces of filter paper followed by the gel and then two other sheets of filter paper to make a layered sandwich. Air bubbles were removed and the sandwich was placed between soft pads in the transfer cassette. The transfer was carried out at 200 mA for 60 mins or at 30 mA o/n.

2.3.4 Antibody blotting

The blotting membrane was blocked to prevent non-specific binding by incubation in blocking solution (5% (w/v) NFDM (non-fat dried milk) powder in 1 x TBST) for 1 hr at RT on a rocker. Next the membrane was incubated with the primary antibody (as listed in Table 3.0) for 2 hr at RT or at 4°C overnight on a rocker. The membrane was then washed 3 x 10 min with 1 x TBST (10 mM Tris-HCl, pH 8, 15 mM NaCl, 0.05 % (w/v) Tween-20) and incubated with the secondary horseradish
peroxidase (HRP) linked antibody (as listed in Table 3.0) for 1 hr at RT. Again the blot was washed 3 x 10 min with 1 x TBST and developed using the ECL Chemiluminescent Substrate (Millipore). The membrane was placed between two sheets of transparent acetate in a cassette; photographic film was placed on the top of the membrane and exposed for the required amount of time. The film was processed using a Fuji X-ray developer. Alternatively, the BioRad ChemiDoc™ MP Imaging System was used to obtain western blot images directly from the nitrocellulose membrane.

**Table 3.0 Antibodies used in Western blotting**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Type</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-Casp-1</td>
<td>goat polyclonal</td>
<td>Primary</td>
<td>Santa Cruz</td>
<td>1:1000</td>
</tr>
<tr>
<td>(M19)</td>
<td>detects p20 subunit and precursor of mouse caspase-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Casp-1</td>
<td>rabbit polyclonal</td>
<td>Primary</td>
<td>Santa Cruz</td>
<td>1:1000</td>
</tr>
<tr>
<td>(M20)</td>
<td>detects p10 subunit and precursor of mouse caspase-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Casp1</td>
<td>rabbit polyclonal</td>
<td>Primary</td>
<td>Santa Cruz</td>
<td>1:1000</td>
</tr>
<tr>
<td>(A19)</td>
<td>detects caspase-1 precursor, CARD17 and COP of human origin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Casp-11</td>
<td>rat monoclonal</td>
<td>Primary</td>
<td>Sigma</td>
<td>1:1000</td>
</tr>
<tr>
<td>(17D9)</td>
<td>detects full length and p10 and p30 subunits of mouse caspase-11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Casp4</td>
<td>mouse monoclonal</td>
<td>Primary</td>
<td>MBL</td>
<td>1:1000</td>
</tr>
<tr>
<td>(4B9)</td>
<td>detects caspase-4 epitope TX of human origin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Casp5</td>
<td>mouse monoclonal</td>
<td>Primary</td>
<td>MBL</td>
<td>1:1000</td>
</tr>
<tr>
<td>(4F7)</td>
<td>detects caspase-5 epitope at the C-terminal of human origin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-IL-18</td>
<td>chicken polyclonal</td>
<td>Primary</td>
<td>Santa Cruz</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-IL-1β</td>
<td>goat polyclonal</td>
<td>Primary</td>
<td>R&amp;D Systems</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

65
anti-Stat1: rabbit polyclonal Primary Cell signalling 1:500, 1:1000
detects endogenous levels of total Stat1 protein

anti-pStat1: rabbit polyclonal Primary Cell signalling 1:500, 1:1000
(Tyr701) detects endogenous levels of human Stat1 when phosphorylated at tyrosine 701

anti-cytokeratin-18: goat polyclonal Primary Santa Cruz 1:500, 1:1000
(N16) detects an epitope at the N-terminal of cytokeratin-18

anti-β-actin: mouse monoclonal Primary/Secondary Sigma 1:10000 detects an epitope located on the N-terminal of β-actin

anti-mouse IgG conjugated to horseredish peroxidase Secondary Jackson IR 1:1000

anti-rabbit IgG conjugated to horseredish peroxidase Secondary Jackson IR 1:1000

anti-goat IgG conjugated to horseredish peroxidise Secondary Jackson IR 1:1000

anti-rat IgG conjugated to horseredish peroxidise Secondary Jackson IR 1:1000

2.4 Quantitative PCR

2.4.1 RNA isolation from colon tissue

Total RNA was extracted from colon tissue using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Typically, 25 mg of colon tissue was removed from the RNAlater regent and homogenised in 600 µl of RLT buffer containing 14.3 M β-mercaptoethanol (Sigma) using a benchtop rotor-stator homogeniser. The colon homogenates were centrifuged at 14,000 g for 3 min and sample supernatants were removed to new sterile RNase-free tubes. One volume of 70% (v/v) ethanol was added to the sample lysate, mixed and applied into RNease Mini spin column placed in a 2ml collection tube and centrifuged at 10,000 g for 30 s (at this stage RNA binds to the membrane of the column). The RNease spin column was washed 3
times with the supplied wash buffers following a short spin after each wash (at 10,000 g for 30 s). Next, the RNA was eluted from the spin column by applying 30 μl of RNase-free water and centrifuged at 14,000 g for 1 min. The RNA concentration was determined spectrophotometrically using a Nanodrop (Thermo Scientific) according to the manufacturer’s instructions.

2.4.2 mRNA purification from colon tissue

DSS, the chemical used to induce colitis in mice interferes with the analysis of gene expression by inhibiting polymerase activity at various stages of the process (Kerr et al., 2012). Therefore, to reduce the contamination with DSS, mRNA was purified from total RNA isolated from colon tissue using Dynabeads® mRNA Purification Kit according to manufacturer instructions. The magnetic Dynabeads were coupled with Oligo(dT)_{25} that specifically capture poly(A)-tails of mRNA molecules. The assay was performed on flat-bottom RNase-free 96-well plates, and during buffer changes the magnetic beads were immobilised with the help of a magnet designed for the purpose of the assay. First beads were equilibrated with Binding Buffer, and then mixed with equal volume (25 μl) of purified total RNA (10 μg per sample). The beads and RNA were incubated with gentle mixing for 5 min at room temperature. The beads were then washed twice with wash buffer to remove contaminating RNA species. Finally the interaction between mRNA and the beads was disrupted by heating at 65 °C for 2 min and purified mRNA was eluted into 15 μl of RNase-free H₂O. The samples were processed immediately after isolation.

2.4.3 Reverse transcription and real time-PCR reaction

1 μg (tissue) of each RNA sample was reverse-transcribed into cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems), according to the manufacturer’s protocol, and the cDNA reverse transcriptase product was diluted 1:8 with nuclease free water. Transcripts were quantified by real time quantitative PCR on an ABI 7500 Fast Real Time PCR System with Applied Biosystems predesigned Taqman Gene Expression Assays and reagents according to the manufacturer’s instructions. For each sample, mRNA abundance was
normalised to the amount of 18S ribosomal RNA (rRNA) and is expressed as fold difference compared to control mice.

Table 3.1 Primers used in quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Product Code</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>18S rRNA</td>
<td>4319413E</td>
<td>ABI</td>
</tr>
<tr>
<td>Reg3β</td>
<td>RegIIβ</td>
<td>Mm00440616_gl</td>
<td>ABI</td>
</tr>
<tr>
<td>Reg3γ</td>
<td>RegIIγ</td>
<td>Mm00441127_ml</td>
<td>ABI</td>
</tr>
</tbody>
</table>

2.5 Cell culture

2.5.1 Generation of bone marrow derived macrophages (BMDMs) cells

Bone marrow derived macrophages were generated from C57BL/6J WT and Casp11 patents. Mice were sacrificed by exposure to carbon dioxide and their femurs and tibiae were removed and the surrounding muscle and tissue was cleaned. The bone marrow was then flushed from the bones using a 25 gauge needle attached to a 10 ml syringe containing cell culture media (DMEM, 10% (v/v) FBS, 1% (w/v) pen/strep) into a 10 cm² culture dish. A 5 ml plastic pipette was used to gently homogenize the bone marrow cell clusters. The single cell suspension was centrifuged at 600 g for 5 min and cells were re-suspended in 5 ml of Red Blood Cell Lysis buffer (Sigma) for 3-4 min to lyse contaminating red blood cells. Cells were washed in culture medium and pelleted by centrifugation at 600 g for 5 min. The fresh bone marrow cells were then re-suspended in 15 ml of cell culture media containing 20% (v/v) of L929 cell line supernatant (L929 medium is a source of macrophage-colony stimulating factor (M-CSF) that turns the progenitor cells into macrophages). The cells were cultured in a T175cm² tissue culture flask (Greiner, Cruinn) at 37°C, 5% CO₂. After 3-days the medium was replaced with 15 ml fresh medium containing 20% (v/v) of L929 cell line supernatant and cells were then cultured for another 6-8 days.
2.5.2 Stimulation of BMDMs with TLR ligands

BMDMs from WT and Casp11\(^{/-}\) mice were plated at the concentration of 2 \(\times 10^5\) cells/ml in 12-well tissue culture plates (Greiner, Cruinn) and cultured o/n. Next day BMDMs were primed with LPS (1\(\mu\)g/ml) for 4 h, followed by ATP (5mM) stimulation for 30 min. Cells were harvested for RT-PCR and WB assays. For the BMDMs obtained from the TRIF\(^{-/-}\) deficient versus WT mice, different sets of stimulations were performed. BMDMs were plated as described above and cultured o/n. Next day cells were pre-treated with LPS (200 ng/ml, Sigma) for 3 h and then stimulated with IFN-\(\gamma\) (60 ng/ml, R&D Systems) and IFN-\(\beta\) (500 IU/ml, PBL IFN Source) for 16 h. The following day cell lysates were harvested for Western Blotting.

2.5.3 Preparation of samples from BMDMs for Western blotting

BMDMs were seeded at a density of 2 \(\times 10^5\) cells/ml, 1 ml per well in 12-well tissue culture plates (Greiner, Cruinn). After the appropriate stimulation (as described in section 2.4.2) the supernatants were removed, cells were washed with PBS and lysed in 60 \(\mu\)l of ice-cold RIPA buffer. Samples were clarified by centrifugation at 10,000 g for 10 min at 4\(^\circ\)C. 50 \(\mu\)l of each sample supernatant was used for micro-BCA protein quantification (Thermo Scientific, Fisher). The rest of the sample supernatants were stored at -20\(^\circ\)C until further use.

2.5.4 Isolation of colonic epithelial cells

Colonic epithelial cells were isolated using the BD Cell Recovery Solution (VWR), as previously described (Perreault and Beaulieu, 1998) The colons from WT mice were longitudinally sectioned, washed in ice-cold PBS, and cut into 3-cm-long fragments. Colon segments were incubated in a 5 ml ice-cold Cell Recovery Solution at 4\(^\circ\)C for 4 h without agitation. Samples were then vortexed, centrifuged at 350g for 5 min at 4\(^\circ\)C, and washed twice in ice-cold PBS. Cell pellets were resuspended in RPMI 1640 medium (Invitrogen) and filtered through 100-\(\mu\)m cell strainers. Cell pellets were lysed in ice-cold RIPA buffer and analysed by Western blotting. Enrichment for colonic epithelial cells was determined by probing membranes with the epithelial cell–specific marker, cytokeratin-18 (Santa Cruz).
2.6 Clinical samples

Belgian IBD patient population: This study was approved by the regional ethics committee (EC 2000/242), and all participating patients signed informed consents. In total, 103 biopsies were retrieved from controls (n=23; 10 males, 13 females, average age 51 (range 22-69)), and patients with CD (n=54; 27 males, 27 females, average age 37 (range 8-72)) or UC (n=16; 8 males, 8 females, average age 38 (range 7-61)). All patients within this cohort were medication free or on 5-ASA. In addition, the majority of patients have previously been included in a genome-wide scan (Barrett et al., 2008).

Irish UC patient population: This study was approved by the Research and Ethics committee of St. Vincent’s University Hospital. All subjects provided written informed consent. Intestinal biopsies were retrieved from 36 patients with a diagnosis of UC, with prospective Mayo scores of endoscopic activity: Mayo 0 (inactive, n=11; 3 males, 8 females, average age 46 (range 19-60)), Mayo 1 (minimal, n=7; 6 males, 1 female, average age 41 (range 34-64)), Mayo 2 (mild, n=8; 8 males, average age 37 (range 22-76)) and Mayo 3 (moderate to severe, n=10; 7 males, 3 females, average age 40 (range 29-61)). Inflammation score assigned by a pathologist to UC patient was graded on a scale: 0 (no activity, n=11); 1 (mild activity, n=7); 2 (moderate activity, n=9) and 3 (severe activity, n=9). All but 4 patients in this cohort were receiving medication including aminosalicylates, corticosteroids, immunomodulators or biological therapy (adalimumab or infliximab) according to best clinical practice for treatment of their disease.

Irish OAC patient population: This study was approved by the Research Ethics committee (AMNCH) of St. James’s University Hospital. All subjects provided written informed consent. Oesophagectomy tissues were retrieved from 32 oesophageal adenocarcinoma (OAC) patients and tissue microarrays (TMA) were constructed from formalin-fixed paraffin-embedded pre-treatment tumour biopsies. To account for heterogeneity, three cores were sampled from different areas of adjacent normal, Barrett’s oesophagus (metaplasia) and oesophageal adenocarcinoma (tumour) patient resection tissues. Defined areas of tumour grades were assigned by a pathologist according to the 7th Edition of the American Joint Committee on Cancer (AJCC) TNM (Tumour-Node-Metastasis) staging system as outlined below (Figure 2.1 and Table 3.2),
(Rice, 2015). 13 patients in this cohort received a course of neoadjuvant therapy according to best clinical practice for treatment of their disease.

![Figure 2.1: The seventh edition of the American Joint Committee on Cancer (AJCC) TNM staging system.](image)

T is classified as follows: Tis, high-grade dysplasia (HGD); T1, cancer invades lamina propria, muscularis mucosae, or submucosa; T2, cancer invades muscularis propria; T3, cancer invades adventitia; T4a, resectable cancer invading adjacent structures such as pleura, pericardium, or diaphragm; and T4b, unresectable cancer invading other adjacent structures, such as the aorta, vertebral body, or trachea. The N classifications are as follows: N0, no regional lymph node metastasis; N1, regional lymph node metastases involving one to two nodes; N2, regional lymph node metastases involving three to six nodes; and N3, regional lymph node metastases involving seven or more nodes. M is classified as follows: M0, no distant metastasis; and M1, distant metastasis. Adapted from (Rice, 2015).
Table 3.2 The seventh edition of the American Joint Committee on Cancer (AJCC) TNM staging system.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Contents</th>
</tr>
</thead>
</table>
| Primary tumour (T)      | TX: primary tumour cannot be assessed  
|                         | T0: no evidence of primary tumour  
|                         | Tis: high-grade dysplasia (*)  
|                         | T1: tumour invades lamina propria, muscularis mucosae, or submucosa  
|                         | T1a: tumour invades lamina propria or muscularis mucosae  
|                         | T1b: tumour invades submucosa  
|                         | T2: tumour invades muscularis propria  
|                         | T3: tumour invades adventitia  
|                         | T4: tumour invades adjacent structures  
|                         | T4a: resectable tumour invading pleura, pericardium, or diaphragm  
|                         | T4b: unresectable tumour invading other adjacent structures, such as aorta, vertebral body, trachea, etc. |
| Regional lymph nodes    | NX: regional lymph nodes cannot be assessed  
| (N) **                   | N0: no regional lymph node metastasis  
|                         | N1: regional lymph node metastases involving 1 to 2 nodes  
|                         | N2: regional lymph node metastases involving 3 to 6 nodes  
|                         | N3: regional lymph node metastases involving 7 or more nodes |
| Distant metastasis (M)  | M0: no distant metastasis  
|                         | M1: distant metastasis |
| Histopathologic type    | Squamous cell carcinoma  
|                         | Adenocarcinoma |
| Histologic grade (G)    | GX: grade cannot be assessed-stage grouping as G1  
|                         | G1: well differentiated  
|                         | G2: moderately differentiated  
|                         | G3: poorly differentiated  
|                         | G4: undifferentiated-stage grouping as G3 squamous |
| Location ***            | Upper or middle-cancers above lower border of inferior pulmonary vein  
|                         | Lower-below inferior pulmonary vein |

* Includes all non-invasive neoplastic epithelium that was previously called carcinoma in situ. Cancers stated to be non-invasive or in situ are classified as Tis; ** Number must be recorded for total number of regional nodes sampled and total number of reported nodes with metastases; *** Location (primary cancer site) is defined by position of upper (proximal) edge of tumour in oesophagus.
2.6.1 Biopsy explant cultures

Colon specimens were obtained from patients undergoing colonoscopy either for UC or for surveillance investigation (controls). Four patients were recruited prior to endoscopy and informed consent was obtained. At the time of endoscopy, two biopsies were taken from one healthy patient; one non-adjacent normal and one mild inflamed biopsy were taken from each of two patients; and four biopsies were taken from severe inflamed tissues from each of two patients. Tissue fragments were placed directly in ice-cold buffer (PBS containing 100 U/mL penicillin, 100 μg/mL streptomycin, 4 μg/mL fungizone, 30 μg/mL gentamicin (Invitrogen)) and washed three times before dividing into equal (~5mm) sections and transferring into 96 well flat-bottomed plate (1 section per well). Colon sections were covered with media (RPMI with penicillin/streptomycin and 20% FBS) (Invitrogen) supplemented with caspase inhibitor Z-YVAD-FMK (10μM R&D Systems) or vehicle control (DMSO). Samples were incubated at 37°C for 18 hours before harvesting supernatants and assaying for IL-1β and IL-6 production (eBiosciences/Biolegend ELISA kits) according to the manufacturer guidelines. Colonic biopsies were homogenised in RIPA buffer (50 mM Tris, pH8, 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate and 1% (v/v) NP-40) supplemented with protease inhibitors (Sigma). 10μg protein lysate was analyzed by Western blotting for expression of capase-1 (Santa Cruz), caspase-4 and -5 (MBL) and β-actin (Sigma). Colonic biopsies were cultured and samples supernatants were harvested by a staff member of Dr. Elizabeth Ryan’s group in St. Vincent’s University Hospital.

2.6.2 Immunohistochemistry of UC patient biopsies

Immunohistochemistry (IHC) was performed using formalin-fixed, paraffin-embedded (FFPE) tissues obtained from UC patients. A routine three-stage immunoperoxidase labelling technique incorporating avidin-biotin immunoperoxidase complex (DAKO, Glostrup, Denmark) was used. The tissue sections were deparaffinised by heating at 90°C for 30 min in an oven to melt the wax and cleared in Xylene (3 x 10 min) and rehydrated in 100% - 75% - 50% ethanol (each % x 5 min) to ensure that primary antibodies have complete access to the tissue. Epitope recognition by the antibodies can be affected following formaldehyde fixation, due to methylene
bridges forming between proteins. These bridges were removed using a heat-induced epitope removal (HIER) method by incubating the slides in boiling 0.01 M sodium citrate buffer (pH 6.0) for 10 min. To reduce any non-specific background binding the slides were blocked with (1x Casein, Vector Labs) for 20 min and then washed with PBS/0.05% (v/v) Tween-20 for 5 min. Next tissue sections were treated with 3% hydrogen peroxidise (Sigma) for 7 min and then washed with PBS/0.05% (v/v) Tween-20 for 5 min. Sections were further incubated with the primary anti-caspase-1 (Santa Cruz, 1:150 dilution.), anti-caspase-4 (MBL, 1:25 dilution) and anti-caspase-5 (MBL, 1:75 dilution) antibodies for 1 h. At the same time, extra sections were also incubated with appropriate isotype matched mouse/rabbit monoclonal antibodies (negative controls). After 1 hour incubation tissue sections were washed with PBS/0.05% (v/v) Tween-20 for 5 min and 100 µl of the secondary antibody (anti rabbit/mouse polyclonal IgG, conjugated to a horseradish peroxidise, Dako EnVision) was applied for 30 min incubation and washed again with PBS/0.05% (v/v) Tween-20 for 5 min. 100 µl of DAB (3,3-diaminobenzidine) chromogenic substrate for peroxidise was added onto each tissue and incubated until a brown reaction product was observed. The slides were then counterstained with Hemotoxylin (Sigma) and washed under running water for 5 min. Slides were dehydrated in 50%-75%-100% ethanol (each % x 5 min) and cleared in Xylene (3 x 5 min) The tissue slides were mounted using a DPX mountant (Sigma). Images were captured using Olympus DP50 light microscope and AnalySIS software (Soft Imaging System Corporation, Lakewood, Colorado, USA). Caspase expression was assessed by two blinded reviewers using a validated semi-quantitative scoring method. All IHC stained cells were assessed by a combined score of intensity and percentage of nuclear and cytoplasmic staining. Intensity of staining was graded using a scale of 0 - 3 where 0 = negative, 1 = weak, 2 = moderate and 3 = strong. Percentage positivity was graded using a scale of 0 - 4 where 0 = no stained cells, 1 = 1 - 25% stained cells, 2 = 25 - 50% stained cells, 3 =50 - 75% stained cells and 4 = 75 - 100% stained cells (Biniecka et al., 2011).

2.6.3 Immunohistochemistry of OAC patient biopsies

Immunohistochemical staining for the expression of inflammatory caspase-1, -4 and -5 in human TMAs of adjacent normal, Barrett's oesophagus (metaplasia) and
oesophageal adenocarcinoma patient resection tissues was carried out by Joanna Fay, of Prof. Elaine Kay’s group at the Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin, using the Leica Bond-III fully automated tissue stainer (Leica Biosystems, Newcastle, UK). The Bond-III system dewaxed slides before pre-treatment with Bond Epitope Retrieval Solution 1. Primary antibodies anti-caspase-1 (Santa Cruz, 1:250 dilution), anti-caspase-4 (MBL, 1:400 dilution) and anti-caspase-5 (MBL, 1:100 dilution) were diluted in Bond Primary Antibody Diluent. Detection and visualisation of stained cells was achieved using the Bond Polymer Refine Detection Kit, using DAB as the chromagen. Tissues were counterstained with hematoxylin and coverslipped. Appropriate negative controls (omission of primary antibodies) were used in all assays. Caspase expression was assessed by two blinded reviewers using a validated semi-quantitative scoring method. All IHC stained cells were assessed by multiplying the percentage of positive cells (P) by the intensity (I) of nuclear and cytoplasmic staining. Percentage of positive cells was assessed using 6 categories (0%, 10%, 25%, 50%, 75%, 90% and 100%). Intensity of staining was graded using a scale of 0 – 3 where 0 = negative, 1 = weak, 2 = moderate and 3 = strong staining (Phelan et al., 2014).

2.7 Statistical analysis

Data were analysed using Prism 5 software (GraphPad). Error bars indicate SEM as indicated. For in vitro and in vivo experiments, the unpaired two-tailed Student t test was used to compare the mean values between two groups. Statistical differences in mean values between more than two experimental groups were determined by Two-way Analysis of Variance (ANOVA) followed by Bonferroni post-test. For human clinical data differences in parameters between two groups were performed using the non-parametric Kruskal-Wallis test (with Dunn’s post hoc comparison to control) for qPCR analysis and unpaired Mann-Whitney U-test or one-way ANOVA for IHC expression data. Correlation analysis was undertaken by calculating the Spearman’s correlation coefficient, abbreviated r_s. p values of less than 0.05 were considered statistically significant.
Chapter 3

Investigating the role of Caspase-11 in a murine model of DSS-induced colitis
3.1 Introduction

The innate immune system represents the first line of defence against infectious agents, and co-ordinates cellular and molecular mechanisms that result in effective inflammatory and anti-microbial responses against pathogens. Infection and cellular stress trigger assembly of canonical and non-canonical inflammasome complexes that activate the pro-inflammatory caspases, caspase-1 and -11, respectively. Activation of caspase-1 by canonical inflammasome complexes results in the cleavage and secretion of IL-1β and IL-18 (Mariathasan et al., 2006; Martinon et al., 2006). However, activation of the non-canonical inflammasome, mediated by caspase-11, serves as an additional regulatory pathway for caspase-1 dependent production of the pro-inflammatory cytokines IL-1β and IL-18 in response to Gram-negative bacteria (Kayagaki et al., 2011; Rathinam et al., 2012). Caspase-11 activation has recently been shown to have a key role in pyroptotic cell death, activated in response to intracellular pathogens (Kayagaki et al., 2011; Kayagaki et al., 2013). Although pyroptosis was initially identified as a caspase-1 dependent process, caspase-11 is capable of inducing it independently of the canonical inflammasome (Aachoui et al., 2013; Case et al., 2013). These recent reports attributing caspase-11 with inflammatory and pyroptotic functions highlight the emerging importance of this inflammatory caspase during the innate immune response. Although most of the studies elucidating the mechanism of non-canonical inflammasome activation have been carried out in vitro using bone marrow derived macrophages (BMDMs), murine models of septic shock have confirmed that the non-canonical inflammasome drives lethal sepsis in vivo (Hagar et al., 2013; Kayagaki et al., 2013). In addition, a study with the Gram-negative bacterium, Citrobacter Rodentium, also reveals a role for the non-canonical inflammasome in vivo (Gurung et al., 2012).

In contrast to the non-canonical inflammasome, the role of the canonical inflammasome in intestinal inflammation has been well characterized. As canonical activation of inflammasome signalling is an essential modulator of immune system homeostasis and epithelial barrier function in the gut, its dysregulation can result in a multitude of immune disorders, including inflammatory bowel disease (IBD) (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Elinav et al., 2011). Our understanding of the innate immune mechanisms (including canonical inflammasome) contributing to
intestinal inflammation has markedly evolved in recent years through the analysis of genetic and chemically induced models in rodents.

Today, the dextran sodium sulfate (DSS) model is one of the most widely used mouse models of chemically induced colitis, which mimics symptoms of human Ulcerative colitis (Kitajima et al., 1999; Okayasu et al., 1990). Oral administration of DSS in drinking water results in disruption of the murine intestinal epithelial barrier, exposing cells of the lamina propria to commensal bacteria and their products, such as LPS and peptidoglycan (PGN) resulting in extensive inflammation (Kitajima et al., 1999). Due to its simplicity and reproducibility this mouse model has been extensively used in many studies, including those investigating the role of canonical inflammasome in intestinal inflammation. Several reports have demonstrated that NLRP3\textsuperscript{-/-}, Casp1\textsuperscript{-/-} and ASC\textsuperscript{-/-} mice were hypersensitive to DSS colitis (Allen et al., 2010; Zaki et al., 2010a). Similarly, mice lacking the inflammasome substrate IL-18 (IL-18\textsuperscript{-/-} mice), its receptor (IL-18R\textsuperscript{-/-} mice) or the IL-1 receptor (IL-1R\textsuperscript{-/-} mice) exhibited higher mortality rates, more severe histopathological changes and increased cytokine production compared to their wild-type counterparts, therefore, confirming the critical role of NLRP3 for the preservation of intestinal homeostasis and protection against excessive inflammation in the gut (Lebeis et al., 2009; Takagi et al., 2003).

Interestingly, the recent characterization of the non-canonical inflammasome has led to the discovery that previously generated caspase-1 null mice (generated on the 129 strain) also lack a functional caspase-11 gene, making them Casp1Casp11 double knockout (DKO) mice (Kayagaki et al., 2011). Subsequent studies utilizing Casp11\textsuperscript{-/-} and Casp1Casp11 DKO mice revealed that caspase-11 modulates many biological functions that were previously considered to be solely caspase-1 dependent and have also been shown to have caspase-1-independent functions (Aachoui et al., 2013; Broz and Monack, 2013; Kayagaki et al., 2011; Rathinam et al., 2012). Based on these revelations, this study was designed to evaluate the involvement of murine caspase-11 in the regulation of intestinal inflammation \textit{in vivo} using the acute DSS-induced colitis mouse model.

Overall, the main focus of this chapter was to understand the contribution of caspase-11 to inflammatory processes that occur in the murine intestine \textit{in vivo}, during DSS-induced acute colitis trials.
3.2 Results

3.2.1 Genotyping of Casp11\(^{+/+}\), Casp11\(^{-/-}\) and Casp11\(^{+/-}\) C57BL/6J mice

The caspase-11 knockout mice (Casp11\(^{-/-}\)), on a C57BL/6J background strain, were generated and obtained from Junying Yuan’s laboratory, Harvard Medical School (Wang et al., 1998). Caspase-11 knockout mice were imported into TCD in June 2010 where they were backcrossed onto the C57BL/6J background. Since then heterozygous and homozygous breeding pairs are being used to generate Casp11\(^{-/-}\) and their WT littermate controls for experimental trials in the BRU in Trinity College Dublin. The 21-day offspring of the breeding pairs are routinely genotyped in order to differentiate between the three different caspase-11 genotypes (as shown in Fig. 3.1): (i) (+/+ ) wild type caspase-11 (Casp11\(^{+/+}\)) - both alleles contain the wild type version of caspase-11 gene; (ii) (-/-) homozygous caspase-11 (Casp11\(^{-/-}\)) - both alleles contain the mutated caspase-11 gene and; (iii) (+/-) heterozygous caspase-11 (Casp11\(^{+/-}\)) - one allele contains the mutated caspase-11 gene and the other allele contains the wild type caspase-11 gene. Genomic DNA was isolated from ear punches obtained from the offspring of the Caspase-11 knockout mice to confirm genotypes. The genotyping PCR reaction was performed with a number of specific primers namely SY21 & SY22 (see material and methods 2.1.1.2) for amplification of the wild type caspase-11 gene and SYKO & PJK (see material and methods 2.1.1.2) for amplification of the mutated caspase-11 gene. The primer sequences and PCR conditions were adapted from (Hisahara et al., 2001). The results (Fig. 3.1) show three types of genotype: (i) (+/+ ) one PCR product with expected size of ~ 200 bp was amplified with the SY21 & SY22 primers; (ii) (-/-) one PCR product with the expected size of ~ 600 bp was amplified with the SYKO & PJK primers; and (iii) (+/-) two PCR products were amplified with the expected sizes of ~ 200 bp and ~ 600 bp with both SY21 & SY22 and SYKO & PJK primers. GAPDH (P) gene was used as a positive control and the GAPDH PCR product was successfully amplified with expected size of ~180bp. Omission of DNA in the PCR reaction was used as the negative control (N) with no PCR product amplified as expected.
3.2.2 Characterisation of caspase-11 knockout mice and the canonical inflammasome pathway

In a relatively recent study, Kayagaki et al. reported that ES (embryonic stem) cell lines derived from 129 murine strains carry an inactivating mutation (5 bp deletion) within the Casp11 gene locus. The ES cells from the 129 murine strains are commonly used to generate genetically modified mice including Casp11⁻/⁻, Casp3⁻/⁻ Casp11⁻/⁻ or cIAP1⁻/⁻ knockout mice (Simpson et al., 1997; Wang et al., 1998). Caspase-1, -3 and cIAP1 null mice, despite backcrossing into a C57BL/6 mouse strain background, are also deficient in caspase-11 (Berghe et al., 2013; Kayagaki et al., 2011; Kenneth et al., 2012).

For targeted genes which are located in proximity to the Casp11 gene locus, such as Casp1 or cIAP1, the recombination frequency for allelic exchange during backcrossing is extremely low. Therefore, this may be the reason why the caspase-11 passenger mutation was maintained in these knockout mice. Nevertheless, the Casp3 gene locus is located on a different chromosome than the Casp11 gene and the reason for maintaining the caspase-11 passenger mutation in Casp3⁻/⁻ mice still remains unclear. However, it has been assumed that Caspase-3 null mice were bred without additional backcrossing which may have influenced the maintenance of the caspase-11 mutation (Berghe et al., 2013). Although, the Casp11⁻/⁻ mice used in our experiments were extensively backcrossed onto C57BL/6J, based on these revelations it was important to characterise the Casp11⁻/⁻ and WT mice bred in the BRU or Comparative Medicine animal units in Trinity College Dublin and used for the experimental trials in this study.

To characterise the mice, BMDMs from C57BL/6J WT and Casp11⁻/⁻ mice were cultured, harvested and primed with LPS (1 µg/ml) for 4 h, followed by ATP (5 mM) stimulation for 30 min. LPS is a known inducer of Caspase-11 and Caspase-1 expression in macrophages (Kahlenberg and Dubyak, 2004; Wang et al., 1996). Stimulation with LPS in the presence of ATP was used in our experiments for more robust induction and activation of caspase-1 in macrophages (LPS+ATP activate the canonical NLRP3 inflammasome pathway). Cells were harvested for RT-PCR (Fig. 3.2 A) and WB (Fig. 3.2 B) analysis. The results of the RT-PCR assay confirmed that caspase-11 transcripts were expressed only in WT mice in both unstimulated and stimulated (LPS and LPS + ATP) samples (which are visible as 1121 bp products on
agarose gels). As expected, there were no caspase-11 transcripts detected in BMDMs of Casp11"−" mice. Product for GAPDH transcripts was observed and visible at the expected size of 400 bp in each sample which served as positive controls for the experiment. The WB results (Fig. 3.2 B) augments the findings for the RT-PCR assay, showing production of the pro-caspase-11 form in BMDMs of WT mice only (visible as two bands at the expected sizes of 43 kDa and 38 kDa). No bands were detected for the Casp11 knockout samples, as expected.

In addition, production of the pro-caspase-1 (45 kDa), pro-IL-1β (31 kDa) cleaved caspase-1 (20 kDa) and IL-1β (17 kDa) were observed in the lysates (Fig. 3.2C) and in the supernatants (Fig. 3.2D) of LPS (1 μg/ml) primed BMDMs for 8 h, followed by ATP (5 mM) stimulation for 30 min in both wild type and caspase-11-deficient BMDMs. This confirms that the canonical NLRP3 inflammasome pathway (following stimulation with canonical stimuli such as LPS and ATP) is fully functional in WT and Casp11"−" mice. Results for the β-actin loading controls (bands for β-actin observed at expected size of 42 kDa) in each sample indicated approximate equal loadings for each protein sample.

3.2.3 Increased susceptibility of caspase-11"−" mice to DSS induced colitis

In order to evaluate the involvement of murine caspase-11 in the regulation of intestinal inflammation in vivo, the acute dextran sodium sulfate (DSS)-induced colitis mouse model was employed. There are numerous factors which can affect and influence induction and severity of the DSS colitis in mice including: DSS concentration, molecular weight, duration of exposure, manufacturer or intestinal microflora of the animals (Bamba et al., 2012; Kitajima et al., 2000; Perse and Cerar, 2012; Zijlstra et al., 2003). Based on the preceding reports and from personal communication with Dr Sinead Corr (TCD) pertaining to the preferred DSS vendor and optimal in vivo concentration to ensure for successful induction of colitis in mice, DSS was incorporated with different molecular weights from two different suppliers (DSS from Fisher Scientific, Mw = 500 kDa and DSS from MP-Biomedicals with a range of Mw between 36 - 50 kDa).

The first experiment was performed by giving mice 2% DSS (Fisher) for 11-consecutive days in drinking water and the mice were scored for their disease activity
index (DAI) (which is a mean of individual scores of weight loss, stool consistency and bleeding) for the duration of this period (Fig. 3.3). Administration of DSS resulted in the loss of 4% of initial body weight, for both Casp11<sup>−/−</sup> and WT mice by day 11 (Fig. 3.3 A). Caspase-11 null mice exhibited higher bleeding (2 ± SEM vs 0 ± SEM) and diarrhoea (2.4 ± SEM vs 1.4 ± SEM) scores compared to their wild type littermates (Fig. 3.3 B and 3.3 C). Caspase-11<sup>−/−</sup> mice also exhibited a significantly higher reduction of the colon length (an indication of inflammation) compared to the wild type group (Fig 3.3 E). The clinical score (DAI) was also higher for the Casp11<sup>−/−</sup> mice compared to the WT group (1.6 ± SEM vs 0.5 ± SEM) (Fig. 3.3 D). Taken together, the data suggested that Casp11<sup>−/−</sup> mice appeared to be more susceptible to DSS-induced colitis than their wild type littermates. However, the low percentage loss in body weight, as well as the lack of difference in % body weight loss between both groups, together with the low clinical scores reached by both WT vs Casp11<sup>−/−</sup> suggested that the experiment was in need of further optimisation. Areas for optimisation that were considered included % DSS and exposure time (days) to DSS during the trial.

Initial optimisation efforts focused on treating the mice with a higher dose of DSS (3%) from Fisher for 9 instead of 11 days (Fig. 3.4). A number of interesting observations were made in this colitis trial. Firstly, Casp11<sup>−/−</sup> mice appeared to lose more body weight than their wild type littermates (4.5% vs 2%, respectively) by day 9, however these changes weren’t significant (Fig. 3.4 A). Bleeding scores for both groups were very low during the 9-day trial and there was no significant difference observed between both groups (Fig.3.4 B). Diarrhea scores steadily increased during the trial for both groups, with a significant higher score for Casp11<sup>−/−</sup> mice over WT littermates being observed on day 9 (Fig.3.4 C). The clinical score was also increasing during the trial for both groups, reaching a higher score for Casp11 mice over the WT littermates on day 9 (1.8 ± SEM vs 1.0 ± SEM, respectively), however this difference wasn’t significant (Fig 3.4 D). Interestingly, and in agreement with the first DSS-induced mouse colitis model, the Casp11<sup>−/−</sup> mice exhibited a significantly shorter colon length compared to their wild type littermates (Fig. 3.4 E). In summary this trial revealed only a slight difference in percentage of body weight loss and colon length between the two tested mice groups. Akin to the first trial, the mice didn’t reach high scores for clinical symptoms of colitis and therefore the trial wasn’t considered optimal.
To further optimise our mouse model of colitis it was decided to use the DSS from MP-BioMedicals which consisted of DSS with a range of lower molecular weights (36-50 kDa) compared to Fisher DSS (500 kDa). During this trial, animals were fed with 3% DSS in drinking water for 7 days. This time animals from both groups (WT vs Casp11\(^{-/-}\)) exhibited extremely severe symptoms of colitis (Fig 3.5). During the 7-day trial both groups reached the maximum scores for their disease activity index with no major difference between WT vs Casp11\(^{-/-}\) mice being observed (Fig. 3.5. A and D). Due to very severe inflammation, mice exhibited drastic colon shortening in both groups (but as observed previously the Caspase-11 null mice had significantly shorter colons compared to the wild type group (3.6 mm ± SEM vs 4.3mm ± SEM, respectively) (Fig.3.5E). This experiment revealed that the percentage of DSS used this time was more than likely too high and caused the colitis to be too severe making it difficult to observe any significant changes between both groups, excluding the difference in colon shortening length.

Following those results the % DSS was dropped from 3% to 2% (again from MP BioMedicals) for the next DSS-colitis trial. During the trial, animals were fed with 2% DSS in drinking water for 6 days. In this trial the DSS conditions appeared to be optimised in that the observed form of mouse colitis was consistent with what has been reported in the literature (Melgar et al., 2005; Perse and Cerar, 2012). Animals from both groups exhibited severe signs of colitis, reaching high scores in their DAI system, and at the same time a clear difference between Casp11\(^{-/-}\) and WT mice being observed. (Fig.3.6). Caspase-11 null mice exhibited a significantly higher percentage (21%) body weight loss compared to their wild type littermates (14%) by day 8 (Fig. 3.6 A). Moreover Casp11\(^{-/-}\) mice developed more severe bleeding and diarrhea scores compared to the wild type group (Fig. 3.6 B, C). All these clinical symptoms of induced colitis were associated with significant shortening of the colons in DSS treated mice compared to the healthy control animals (treated with water only), with up to 50% reduction of the colon length being observed. Similarly, but even more conspicuous to the previous DSS trials, was that Caspase-11 null mice had significantly shorter colon lengths compared to the wild type group (3.3mm ±SEM vs 4.4mm ±SEM, respectively) (Fig.3.6 E).

In agreement with previous studies (Takagi et al., 2003; Zaki et al., 2010a), DSS treatment triggers marked histopathological changes in the colons of DSS treated mice characterised by increased inflammatory cell infiltration and mucosal crypt damage.
Colitis severity in this study was assessed using the histological scoring system (as described in 2.1.3 section of materials and methods). H&E stained distal colon sections of 2% DSS (MP BioMedicals) treated mice evaluated on day 8 indicated significantly higher histological scores for Casp11−/− mice compared to the WT animals (Fig.3.7A). Distal tissues of Casp11−/− mice displayed total loss of crypts and epithelial loss in large areas with transmural extension of infiltrates. On the other hand, distal tissues of WT mice exhibited partial to total (50-100%) loss of crypts in large areas with extension of infiltrates into mucosa only (Fig.3.7 B, C). Consistent with the absence of disease in animals that were not fed with DSS (water only), no signs of inflammation or tissue damage were observed (Fig.3.7 A, B, C).

WB analysis was also performed on colon samples from wild-type mice subjected to the optimised DSS-induced colitis model, to examine for caspase-11 expression levels during the duration of the trial. Interestingly, our results revealed that caspase-11 levels were upregulated and activated in a time dependent manner during DSS exposure (Fig. 3.8), therefore demonstrating that caspase-11 is involved and contributes to protection against acute DSS colitis.

Although the WT and Casp11−/− mice were bred as littermates (from heterozygous pairs) as much as possible, WT and Casp11−/− homozygous pairs were also set-up to increase animal numbers. Therefore, it was important for us to determine whether all differences observed during the course of DSS colitis between Casp11−/− and WT mice were due to the altered microbiota composition or rather caspase-11 deficiency. To address this question, cohorts of WT and Casp11−/− mice were co-housed for 2 weeks before treatment with 2% DSS for 6 days. The results from this trial revealed that Casp11−/− mice still retained their increased susceptibility to colitis, as reflected by their significant increased body weight loss (Fig. 3.9A) and colon shortening (Fig.3.9 E). However, it appeared that cohousing of Casp11−/− mice affected their previously observed significant differences compared to WT mice in regards to diarrhea, bleeding and clinical scores (Fig.3.6 B, C, D and Fig.3.9 B, C and D).

Taken together, our results indicate that we had found the optimal conditions of DSS treatment to successfully induce acute colitis in C57BL/6J WT and Casp11−/− mice (2% DSS (MP BioMedicals) treatment for 6 days, followed by 2 days with regular drinking water)). Furthermore our study reveals a protective role in vivo for caspase-11
during DSS-induced acute colitis, as we have shown that Casp11\(^{-}\) mice are more susceptible to DSS-induced colitis than their WT littermates.

3.2.4 Analysis of cytokine levels in colon homogenates of 2% DSS treated WT and Casp11\(^{-}\) mice by ELISA

Multiple cytokines have been shown to modulate the severity of DSS induced colitis in mice (Demon et al., 2014; Elinav et al., 2011; Rakoff-Nahoum et al., 2004; Zaki et al., 2010a). To address whether the hyper-susceptible phenotype of Casp11\(^{-}\) mice was associated with deregulated inflammatory cytokine production, the cytokine expression profiles from untreated and DSS-treated WT and Casp11\(^{-}\) mice were measured by ELISA using colon homogenates. The colons were harvested either on day 5 or day 6 after DSS administration (Fig.3.10 and Fig 3.11). The inflammatory cytokine levels in colon homogenates harvested on day 5 after DSS treatment were slightly elevated in DSS treated WT and Casp11\(^{-}\) compared to water treated controls (as expected). However there was no major difference in cytokine levels between DSS treated WT and Casp11\(^{-}\) groups (Fig.3.10). In contrast, the data obtained from the colon homogenates obtained on day 6 revealed a significant difference in a certain cytokine levels between the two DSS-treated mice groups. A major reduction in cytokine levels of IL-18, IL-1\(\alpha\) and IL-22 were observed in Casp11\(^{-}\) mice compared to their wild type littermates. Additionally, a modest reduction in IL-6 in the colons of Caspase-11 null mice was also detected compared to the wild type group. However, for the cytokines IL-1\(\beta\), IFN-\(\gamma\), IL-17A, IL-10 and MIP-2 no significant difference was observed between both DSS treated groups (Fig.3.11).

It has been reported that IL-18 contributes to epithelial cell regeneration and has an important role in intestinal inflammation during IBD (Dupaual-Chicoine et al., 2010). Furthermore in response to DSS-induced injury, IL-18 deficiency has been shown to result in a severe form of colitis (Pizarro et al., 1999). Therefore, the reduced levels of IL-18 observed in the DSS-treated Casp11\(^{-}\) mice suggested it may be making a significant contribution to the severe phenotype of colitis that was observed in these mice. It has been also reported that caspase-11 and the non-canonical inflammasome are required for caspase-1 activation (and production of IL-1\(\beta\) and IL-18) in response to specific Gram-negative bacterial infections (Kayagaki et al., 2011; Rathinam et al., 84
2012). Thus, the significantly low levels of IL-18 in DSS treated Casp11-/- mice which were consistently observed during numerous DSS trials, strongly suggests that caspase-11 is an important regulator of IL-18 during the intestinal inflammation (Fig. 3.11).

It has been also shown that IL-1α and the IL-18 precursor are both expressed constitutively in intestinal epithelial cells (IECs) throughout the gastrointestinal tract (Pizarro et al., 1999), whereas expression of the IL-1β precursor requires NF-κB-mediated priming, which may explain decreased levels of IL-1α and IL-18, but not IL-1β, detected in colons from Casp11-/- mice. Notably, IL-18 levels are elevated in Casp11-/- mice compared with WT mice at steady state (Fig. 3.10). This might suggest that a caspase-11 independent mechanism, responsible for enhancing basal IL-18 expression, is activated in Casp11-/- mice.

We further examined whether IL-18 serum levels were also affected in Casp11-/- mice. However, there was no significant difference in systemic release of IL-18 in serum of water treated controls) and DSS-treated WT and Casp11-/- mice, suggesting that the observed differences in IL-18 expression were confined to the intestine (Fig. 3.12). IL-22, which has been shown to have a beneficial effect on maintaining the integrity of the colonic mucosa (Zenewicz et al., 2008) was also reduced in DSS treated Casp11-/- mice. As Caspase-11 null mice also exhibited more severe clinical features of colitis than the WT mice, this result suggests that the lower levels of this cytokine may have also contributed to the development of a more severe form of colitis in Casp11-/- mice.

Lastly, we also found lower IL-1α levels in Casp11-/- mice versus WT mice, indicating that caspase-11 may play a regulatory role in IL-1α secretion during the acute colitis in mice. Similarly, in one of the first published papers on caspase-11, Wang et al revealed that Casp11-/- mice have reduced plasma levels of IL-1α after LPS stimulation compared to their wild type littermates. This suggests that caspase-11 could be involved in the regulation of IL-1α secretion (Wang et al., 1998).

### 3.2.5 IL-18 rescues the colitis susceptibility phenotype of caspase-11-/- mice

Inflammasome-mediated IL-18 production has previously been shown to have a crucial role in the mucosal barrier repair and protection against DSS-induced colitis. It
has also been observed that the enhanced susceptibility to colitis in Casp1^-/-, ASC^-/- and NLRP3^-/- mice could be reversed by the administration of recombinant IL-18 (rIL-18) (Dupaul-Chicoine et al., 2010; Zaki et al., 2010a).

To determine the functional significance of decreased levels of IL-18 production in DSS treated Casp1^-/- mice (as discussed in section 3.2.4) the following experiments were performed. Two groups of Casp1^-/- mice were treated with 2% DSS for 6 days. One cohort of mice received a daily injection of 0.05 µg (i.p) recombinant IL-18/mouse for the first 3 consecutive days whereas the control group was treated with DSS only (Casp1^-/- mice). Based on the DAI scores from this trial (Fig. 3.13) it was observed that exogenous administration of IL-18 transiently rescued Casp1^-/- mice from DSS-induced colitis. Casp1^-/- mice injected with IL-18 maintained a more stable body weight, lower bleeding and diarrhea scores compared to the control group for 3 days post IL-18 injection (Fig 3.13 A, B, C). However, on day 6 of the experiment both cohorts (Casp1^-/- and Casp1^-/+ + IL-18) similarly reached severe symptoms of colitis (Fig.3.13 D). The Casp1^-/+ + IL-18 mice more than likely reached this phenotype as IL-18 was only exogenously administered for 3-days. Thus, this suggested to prevent severe colitis symptoms from re-occurring, extended exogenous administration of IL-18 would be required.

Following those results, it was decided to further evaluate the role of IL-18 in protection against DSS-induced colitis in Casp1^-/- mice by administering exogenous rIL-18 for the duration of the model as opposed to only 3-days. This time, one cohort of 2% DSS treated (6-day DSS treatment, followed by 2 days with water) Casp1^-/- mice received a daily (i.p) injection of 0.05 µg recombinant IL-18/mouse for 7 consecutive days of the experiment whereas the control group was (i.p) injected with PBS only (Fig.3.14). Interestingly, this IL-18 administration regimen reversed the Casp1^-/- mice susceptibility to colitis showing that rIL-18-injected Casp1^-/- animals exhibited a significant lower percentage of body weight loss compared to the DSS treated Casp1^-/- cohort injected with PBS (8% vs 25%, respectively) by day 8 and also lower bleeding and diarrhea scores (Fig. 3.14 A, B, C). Moreover, colons harvested from rIL-18-injected Casp1^-/- animals showed longer lengths compared to the PBS injected Casp1^-/- controls (3.9mm ±SEM vs 3.1mm ±SEM) (Fig.3.14 E). These findings suggest that the IL-18 deficiency observed in colon homogenates from Casp1^-/- mice contributes to their enhanced susceptibility to colitis.
Histological analysis of H&E stained colons from the above described *in vivo* DSS trial (Fig.3.15) revealed significant protection of crypt architecture in IL-18-compared with PBS-treated Casp11\(^{-/-}\) mice with DSS-induced colitis. Casp11\(^{-/-}\) +IL-18 displayed only partial (≤50%) loss of crypts in large areas compared to PBS injected mice, which mainly exhibited total loss of crypt in large areas and epithelial loss (Fig. 3.15 B). Interestingly, there was no observed difference in the levels of inflammatory cell infiltration between PBS and IL-18 injected mice. Both groups exhibited a similar increase of transmural extension of infiltrates. Consistent with the absence of disease in animals that were not fed with DSS (water only), no signs of tissue damage or inflammation were observed (Fig.3.15). Overall, these findings are consistent with a specific role for IL-18 in intestinal epithelial regeneration and repair in this disease model.

Cytokine levels from colon homogenates of DSS treated Casp11\(^{-/-}\) vs Casp11\(^{-/-}\) + IL-18 administered mice and water treated Casp11\(^{-/-}\) mice (used as healthy controls) were also analysed by ELISA (day 8). In contrast to our expectations, administration of IL-18 led to a lack of significant difference in cytokine production (including IL-18, IL-1\(\alpha\) and IL-6 levels) between those two DSS treated groups (Fig.3.16). The reason for this may be related to the fact that the colon samples were taken at day 8, when any IL-18 regulatory effects on endogenous cytokine levels had diminished (as the last IL-18 i.p injection was performed 24 hours earlier). This is consistent with a previous report where it has been shown that the half-life of exogenous administrated IL-18 into mice is 16 hours (Hosohara et al., 2002). Thus, the major protective effect of IL-18 on disease deterioration appears to be at the level of the epithelial barrier.

Taken together, this data clearly demonstrates that daily exogenous IL-18 administration rescued the Casp11\(^{-/-}\) mice from DSS-induced colitis, suggesting a protective *in vivo* role for non-canonical inflammasome-mediated IL-18 production during DSS-induced intestinal inflammation.
3.3 Discussion

The relatively recent research pertaining to caspase-11 has focused on its role in: (i) non-canonical inflammasome activation in response to *Gram-negative* and certain pore forming toxins (Gurung et al., 2012; Kayagaki et al., 2011); (ii) in host defence in response to bacterial infections (Aachoui et al., 2013; Case et al., 2013) and (iii) in murine models of acute septic shock (Hagar et al., 2013; Kayagaki et al., 2013). Despite these new and fascinating insights into caspase-11 mediated signalling events, the involvement and regulation of murine caspase-11 during intestinal inflammation has only been recently explored by us and others (Demon et al., 2014; Williams et al., 2015). Results from this chapter revealed a crucial role for caspase-11 in the regulation of intestinal inflammation during acute DSS-induced murine colitis, and the identification of IL-18 as one of the key cytokines involved in these processes. These findings, taken together, strongly suggest a protective role for caspase-11 during acute colitis.

Initial experiments set out to characterise the caspase-11 mice being used, following a recent report by Kayagaki *et al* which revealed that a mice strain 129, (which was also used for the generation of Caspase-11 knockout mice that were used in this study) harbour a mutation in the *caspase-11* locus that attenuates caspase-11 expression and results in the production of a non-functional caspase-11 protein. RT-PCR and WB analyses in this study clearly confirmed the presence of caspase-11 transcripts and pro-caspase-11 protein expression in BMDMs isolated (+ induced with LPS/ATP) from WT mice in contrast to those from Caspase-11 null mice, thus confirming the validity of the Casp11<sup>−/−</sup> mice (Fig.3.2 A, B). The possibility also exists that the inactivating caspase-11 mutation within the *caspase-11* gene in Casp11<sup>−/−</sup> mice may have been present but didn’t affect C57BL/6J WT alleles during extensive crossing with Caspase-11 knockout mice, allowing for successful (correct genotype) offspring breeding. In the past, it has been commonly assumed that a minimum of 10 backcross generations are required to replace the genome in congenic strains (Markel et al., 1997). However, it is now commonly accepted that no matter how many generations are backcrossed, backcrossing is insufficient to segregate flanking chromosomal DNA from the ablated gene target. Hence, there is a significant risk of “carrying-over” donor genetic backgrounds and flanking genes which can be responsible for observed phenotypes (Eisener-Dorman et al., 2009; Vanden Berghe et al., 2015). In addition, WB
analysis revealed that the canonical inflammasome activation is intact in the in-bred Casp11−/− mice housed at our facility, as evidenced by the activation of caspase-1 and maturation of IL-1β following NLRP3 activation (LPS and ATP stimulation) as shown in (Fig. 3.2 C,D).

To investigate a role for caspase-11 in intestinal inflammation, in-bred cohorts of WT and Casp11−/− mice were used in acute DSS-induced colitis trials. The results from DSS trials revealed that optimising the DSS conditions (such as molecular weight, concentration or manufacturer brand) played a major role in facilitating the successful induction of colitis in mice. The first two DSS trials were performed using 2% and 3% DSS from Fisher Scientific (500 kDa), however the desired colitis type in these mice wasn’t induced effectively (Fig. 3.3 and Fig. 3.4). Although Casp11−/− mice appeared to be slightly more susceptible to colitis compared to their wild type littermates, most of these changes weren’t significant. In contrast, data obtained from DSS trials using 2% and 3% DSS from MP-BioMedicals (36 - 50 kDa) was more positive in terms of inducing the typical symptoms of murine colitis. In mice treated with 3% DSS it was hard to distinguish differences between WT and Casp11−/− susceptibility to the acute form of colitis observed, as it was too severe (Fig. 3.5). Finally, induction of the desired form of DSS colitis was performed successfully using 2% DSS from MP-BioMedicals. Both groups of mice developed the major manifestations of colitis within 6 days, followed by 2 days with drinking water. Notably, Caspase-11 null mice were significantly more susceptible to colitis than their wild type littermates (Fig. 3.6).

Taken together, the results demonstrate major differences in DSS responsiveness due to different molecular masses of DSS and percentage concentrations used in experiments. It has been previously reported that different molecular weights of DSS could possibly influence induction of colitis. For example, Kitajima et al reported that the severity and primary location of colitis differs with the administration of DSS of different molecular weights in BALB/c mice (Kitajima et al., 2000) Administration of DSS at 5 kDa and 40 kDa induced severe symptoms of colitis however no colitis was observed in mice administered with 500 kDa DSS. There is a possibility that the high molecular weight of DSS prevents passage of the molecule through the mucosal membrane. Their findings nicely support and confirm our data obtained from the first two DSS trials which would suggest that DSS (Fisher) at high molecular weight does not induce acute colitis effectively in C57B/L6J mice. Data obtained from the 2% DSS
trial (MP-BioMedicals) clearly demonstrates a higher susceptibility of Casp11⁻/⁻ mice to DSS-induced colitis compared to their wild type littermates. This was borne out by the fact that the Casp11⁻/⁻ developed more severe clinical symptoms of colitis, more drastic shortening of the colon (which is a sign of severe inflammation) and also more severe histological scores with significantly increased cell infiltration and tissue damage (Fig.3.6 and Fig.3.7). In addition, data obtained from WB analysis of DSS treated mice, revealed that DSS up-regulates expression of caspase-11 in inflamed colons during the colitis trial, thus highlighting its involvement in acute colitis (Fig 3.8). Our results are consistent with two very recent reports, in which Casp11⁻/⁻ mice were also hyper-susceptible to acute experimental colitis (when treated with DSS from BioMedicals) (Demon et al., 2014; Williams et al., 2015). Taken together, these findings reveal a protective role for caspase-11 in the regulation of intestinal inflammation during acute induce colitis. Moreover, it might also suggest that the reported hyper-susceptible phenotype of Casp1Caspi11 mice to DSS-induced colitis (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Zaki et al., 2010a) may be in part due to deficient caspase-11 expression.

It has also been reported that several genetically altered mice that present with increased or reduced susceptibility to colitis have an altered gut microbiota composition (Brinkman et al., 2013; Chassaing and Darfeuille-Michaud, 2011; Elinav et al., 2011). To evaluate if the hyper-susceptible phenotype of Casp11⁻/⁻ mice to DSS colitis was a result of caspase-11 deficiency rather than their specific gut microbiota composition, the optimised DSS-colitis model was performed using co-housed Casp11⁻/⁻ and WT mice. Results from this trial confirmed increased susceptibilities of co-housed Casp11⁻/⁻ mice to colitis in regards to their body weight and colon shortening scores (Fig. 3.9 A, E). However, there was no apparent differences in regards to diarrhea, bleeding and clinical scores observed between co-housed Casp11⁻/⁻ and WT mice. Thus, these findings suggest a possibility that gut microflora of Casp11⁻/⁻ mice may also contribute to their enhanced susceptibility to DSS-colitis. Our observations are in contrast to a report which showed that despite the fact that caspase-11 plays a role in modulating the gut microbiota composition, co-housed Casp11⁻/⁻ mice retain their enhanced susceptibility to DSS-induced colitis (Demon et al., 2014).

In this study release of crucial proinflammatory cytokines from the inflamed colon tissues was also examined. Results clearly showed decreased levels of IL-18, IL-
22, IL-1α and IL-6 in Caspase-11 null mice, suggesting that caspase-11 is involved in regulating these cytokines during inflammation in acute colitis.

Interestingly, IL-18 and IL-22 contribute to intestinal epithelial cell regeneration by promoting epithelial cell proliferation. It has been reported that in response to DSS-induced injury mice deficient in IL-18 and IL-22 develop severe symptoms of colitis (Coruh et al., 2001; Zenewicz et al., 2008). Moreover, it has been shown that IL-18 has a protective role in the early acute phase of mucosal immune responses when IEC are the primary source of IL-18 (Takagi et al., 2003).

All these reports strongly support our findings and suggest that caspase-11 mediated down regulation of IL-22 and IL-18 could contribute to the severe colitic phenotype observed in these mice. Similar to our study, Williams et al also observed attenuated levels of IL-18 in colon tissues harvested from Casp11−/− mice (Williams et al., 2015). This suggests that caspase-11 contributes to the function of the canonical inflammasome in the experimental colitis model. However, in contrast to our findings and Williams’s observations, the study of Demon et al detected elevated levels of IL-1β and IL-18 in the colons of caspase-11 deficient mice, thus, suggesting that DSS sensitivity in the Casp11−/− mice is independent of caspase-1 and the canonical inflammasome (Demon et al., 2014). It should be noted here that even small design differences (DSS%, exposure time to DSS), environmental variability, difference in microflora, diet and other vivarium-specific considerations (Whittem et al., 2010) could highly impact the induced DSS pathogenesis and significantly influence caspase-11 activation and its interaction between the canonical and non-canonical inflammasomes.

Furthermore, decreased levels of IL-1α in Casp11−/− mice observed in our study is supported by findings of Casson et al where they have shown that caspase-11 mediated IL-1α production was required for neutrophil recruitment to the site of infection in vivo during bacterial infection models (Casson et al., 2013). Therefore, this may suggest that caspase-11 may also be responsible for recruiting neutrophils to sites of intestinal damage in the DSS-induced colitis model, although this was not examined during our study. The exact mechanism of caspase-11 mediated regulation of the aforementioned cytokines in our model of DSS-induced colitis remains unknown and merits further investigation.
Moreover, a study by Grivennikov et al has demonstrated that the DSS treated IL-6^- mice were more susceptible to acute colitis compared to WT mice. DSS-exposed IL-6^- mice exhibited elevated levels of apoptosis and decreased levels of IEC in the inflamed colons (Grivennikov et al., 2009). This data suggests that the attenuated levels of IL-6 observed in DSS treated Casp11^- mice can be linked to its role in the maintenance of epithelial integrity.

The functional significance of decreased IL-18 production in DSS treated Casp11^- mice was assessed in IL-18 rescue experiments (Fig. 3.13 and Fig. 3.14). Data from these experiments revealed that only daily exogenous administration of IL-18 significantly decreased the severity of colitis in Casp11 null mice. In agreement with our findings Dupaul-Chicoine et al showed that the severe phenotype of colitis in Caspase-1 null mice which were also deficient in IL-18 production was completely reversed by exogenous administration of this cytokine (Dupaul-Chicoine et al., 2010). It is important to highlight that in Chicoine's et al study, phenotypic Caspase-1 null mice were rescued after 3-day administration of IL-18, however in this study the intermittent exogenous IL-18 administration only transiently attenuated the symptoms of colitis in Caspase-11 null mice. Thus, these results would suggest that caspase-11-induced production of IL-18 has a cytoprotective role during colitis, albeit in a less robust manner to that of Caspase-1. Interestingly and akin to our findings, the parallel study performed by Williams et al revealed that DSS treated Casp11^- injected daily with rIL-18 exhibited significant improvements in clinical and histopathological features associated with acute colitis. Overall, these findings support our data and implicate the importance of non-canonical inflammasome in DSS mediated intestinal inflammation.

Surprisingly, no significant increase of colon IL-18 levels in IL-18 rescued Casp11^- mice was observed (Fig. 3.16), which may be related to the short half-life of IL-18 in vivo. It has also been demonstrated that IL-18^- and IL-18R^+ mice have enhanced susceptibility to acute DSS colitis, thus highlighting the crucial role of this cytokine in intestinal regeneration and tissue repair (Ishikura et al., 2003; Takagi et al., 2003). In contrast, another study has revealed a detrimental rather than protective role for IL-18 during DSS-induced colitis. In this model, reducing IL-18 with neutralizing antibodies was protective and linked to a reduction of IFNγ levels (Siegmund et al., 2001). Furthermore blocking IL-18 with the IL-18 binding protein (IL-18BP) also reduced colitis induced by antigen sensitization (Ten Hove et al., 2001). To reconcile
these data, the hypothesis has been made that IL-18, being constitutive in the intestinal epithelium, has a protective role and it contributes to maintaining the intestinal barrier. However, once the barrier has been damaged, IL-18 becomes pro-inflammatory and its hyperactivation is detrimental, therefore its inhibition at this stage would be beneficial (Siegmund, 2010).

In summary, our findings reveal a protective role for caspase-11 in vivo during acute DSS-induced intestinal inflammation, and demonstrate the involvement of caspase-11 in IL-18 production, suggesting an important role in the maintenance of epithelial barrier integrity. Our results are consistent with two recently published reports which document that caspase-11 null mice are also highly susceptible to acute DSS-induced colitis (Demon et al., 2014; Williams et al., 2015).
Figure 3.1: Genotyping of Casp11<sup>+/+</sup>, Casp11<sup>-/-</sup> and Casp11<sup>+/+</sup> C57BL/6J mice

Genomic DNA was isolated from the ear punches of 21-day old C57BL/6J mice and used in a PCR with specific primers to differentiate between mice containing the wild type- (A) and mutant- (B) caspase-11 genes. 100 ng of PCR product was loaded onto a 1.5% (w/v) agarose gel and stained with SYBR Safe for UV-visualisation. (+/+) mouse indicates the presence of WT Casp11 only (expected size of ~ 200 bp); (-/-) mouse indicates the presence of the mutant caspase-11 gene (expected size of ~ 600 bp); and (+/-) mouse indicates a heterozygous genotype with both WT- and mutant- caspase-11 genes present. The gene GAPDH (P) was amplified as an internal positive control (expected size of ~ 180 bp). Omission of DNA in the PCR reaction (N) served as the negative control.
Figure 3.2: Examination of the canonical inflammasome pathway in Caspase-11 knockout mice

BMDMs (2 x 10^6 cells/ml) from C57BL/6J mice were primed with LPS (1 µg/ml) for 4 h, followed by ATP (5 mM) stimulation for 30 mins. Cells were harvested for: (A) RT-PCR: RNA was extracted from cells and reverse transcribed into cDNA. The Casp11 gene was amplified from the cDNA with specific primers (Forward: 5'ATGGCTGAAAACAAACACCGC3' and Reverse: 5'TCAGTTGCCAGGAAAGAG3') and 10 µl of PCR product (1121bp) was loaded on a 1% (w/v) agarose gel. (B) Western blotting: 40 µg of cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose and probed for pro-caspase-11 (43 and 38 kDa). (C) Western Blot detection of procaspase-1 (45 kDa), pro-IL-1β (31 kDa), cleaved caspase-1 (20 kDa) and IL-1β (17 kDa) in the lysates, and (D) in the supernatants of LPS (1 µg/ml) primed BMDMs for 8 h, followed by ATP (5 mM) stimulation for 30 mins. For (A) GAPDH (200 bp) and for (B, C and D) β-actin (42 kDa) was used as loading control. Results are representative of two independent experiments.

For (A) data was obtained by E. C. Murphy and for (C, D) data was obtained by S. Wade.
Figure 3.3: DSS Optimisation: Colitis trial of WT and Casp11⁻/⁻ mice treated with 2% DSS (Fisher)

C57BL/6J WT and Casp11⁻/⁻ mice were treated with 2% (w/v) DSS (Fisher) and monitored on a daily basis for 11 consecutive days. (A) Body weight loss of WT and Casp11⁻/⁻ mice; (B) Intestinal bleeding was graded on a scale of 0-4 (with zero indicating no bleeding and 4 indicating severe bleeding); (C) Diarrhea was graded on a scale of 0-3 (with zero indicating no diarrhea and 3 indicating severe diarrhea); (D) Disease activity index (DAI) which measured a combination of body weight loss, stool consistency and intestinal bleeding; (E) Colon length measurements. Data represent mean ±SEM of n=5 mice. P values were calculated for (A-D) using the Two-way ANOVA followed by the Bonferroni post-test; ***p<0.001, **p<0.01, *p<0.05; and for (E) with the Student's t-test * p<0.05.
Figure 3.4: DSS Optimisation: Colitis trial of WT and Casp11−/− mice treated with 3% DSS (Fisher)

C57BL/6J WT and Casp11−/− mice were treated with 3% (w/v) DSS (Fisher) and monitored on a daily basis for 9 consecutive days. (A) Body weight loss of WT and Casp11−/− mice; (B) Intestinal bleeding was graded on a scale of 0-4 (with zero indicating no bleeding and 4 indicating severe bleeding); (C) Diarrhea was graded on a scale of 0-3 (with zero indicating no diarrhea and 3 indicating severe diarrhea); (D) Disease activity index (DAI) which measured a combination of body weight loss, stool consistency and intestinal bleeding; (E) Colon length measurements. Data represent mean ±SEM of n=5 mice. P values were calculated for (A-D) using the Two-way ANOVA followed by the Bonferroni post-test; ***p<0.001, ** p<0.01, *p<0.05; and for (E) with the Student's t-test * p<0.05.
Figure 3.5: DSS Optimisation: Colitis trial of WT and Casp11\textsuperscript{−/−} mice treated with 3% DSS (MP BioMedicals)

C57BL/6J WT and Casp11\textsuperscript{−/−} mice were treated with 3% (w/v) DSS (MP BioMedicals) and monitored on a daily basis for 7 consecutive days. (A) Body weight loss of WT and Casp11\textsuperscript{−/−} mice; (B) Intestinal bleeding was graded on a scale of 0-4 (with zero indicating no bleeding and 4 indicating severe bleeding); (C) Diarrhea was graded on a scale of 0-3 (with zero indicating no diarrhea and 3 indicating severe diarrhea); (D) Disease activity index (DAI) which measured a combination of body weight loss, stool consistency and intestinal bleeding; (E) Colon length measurements. Data represent mean ±SEM of n=5 mice. P values were calculated for (A-D) using the Two-way ANOVA followed by the Bonferroni post-test; ***p<0.001, ** p<0.01, *p<0.05; and for (E) with the Student's t-test * p<0.05.
C57BL/6J WT and Casp11−/− mice were treated for 6 consecutive days with 2% (w/v) DSS (MP BioMedicals) and for 2 subsequent days with regular drinking water. (A) Body weight loss of WT and Casp11−/− mice; (B) Intestinal bleeding was graded on a scale of 0-4 (with zero indicating no bleeding and 4 indicating severe bleeding); (C) Diarrhea was graded on a scale of 0-3 (with zero indicating no diarrhea and 3 indicating severe diarrhea); (D) Disease activity index (DAI) which measured a combination of body weight loss, stool consistency and intestinal bleeding; (E) Colon length measurements. The experiment was repeated three times with similar results. Data represent mean ±SEM of n=5 mice and n=3 for the control group. P values were calculated for (A-D) using the Two-way ANOVA followed by the Bonferroni post-test; ***p<0.001, ** p<0.01, *p<0.05 and for (E) p values were calculated with Student's t-test; * p<0.05.
Figure 3.6 continued
Figure 3.7: Increased intestinal inflammation and crypt damage in Casp11−/− mice, as revealed by histological analysis in WT and Casp11−/− mice treated with 2% DSS.

Distal colons of C57BL/6J WT and Casp11−/− mice treated for 6 days with 2% DSS, followed by 2 days with regular drinking water and control group (water only) were harvested and analyzed using hematoxylin and eosin (H&E) staining. Colitis severity of H&E stained colonic tissues was assessed by: (A) combined histological score of (B) tissue disruption (crypt damage score; 0–3, according to the severity of mucosal and crypts damages) and (C) colon cellular infiltration (inflammation score; 0–3, according to the extent of inflammation throughout the intestinal wall). (D) Representative microscopic pictures of (H&E) stained colon sections of water and 2% DSS treated WT and Casp11−/− mice on the last day of the experiment (day 8); (magnification 200x). Data represent mean ±SEM, (2% DSS, n=6; control, n=2). P values were calculated with Student's t-test; * p<0.05, ***p<0.001.
Figure 3.8: Pro-caspase-11 expression and activation is enhanced during DSS-induced colitis

C57BL/6J WT (wild-type mice) were treated with 2% (w/v) DSS from MP BioMedicals for 3- and 7-days. After DSS treatment colons were harvested, homogenized and 40 ug of each protein lysate was resolved by SDS-PAGE, transferred to nitrocellulose and probed for pro-caspase-11 (43 and 38 kDa), active caspase-11 (30 kDa) and β-actin (42 kDa) (which served as a loading control). Each lane represents an individual mouse. Results are representative of three independent experiments.
Figure 3.9: The microbiota of Casp11⁻/⁻ mice does not account for their hypersensitivity to DSS-induced colitis

C57BL/6J WT and Casp11⁻/⁻ mice were co-housed for 2 weeks before treatment with 2% (w/v) DSS (MP BioMedicals) for 6 days, followed by 2 days with regular drinking water. (A) Body weight loss of WT and Casp11⁻/⁻ mice; (B) Intestinal bleeding was graded on a scale of 0-4 (with zero indicating no bleeding and 4 indicating severe bleeding); (C) Diarrhea was graded on a scale of 0-3 (with zero indicating no diarrhea and 3 indicating severe diarrhea); (D) Disease activity index (DAI) which measured a combination of body weight loss, stool consistency and intestinal bleeding; (E) Colon length measurements. The experiment was repeated twice with similar results. Data represent mean ±SEM of n=5 mice and n=3 for the control group. P values were calculated for (A-D) using the Two-way ANOVA followed by the Bonferroni post-test; ***p<0.001, ** p<0.01, and for (E) p values were calculated with Student's t-test; * p<0.05, ** p<0.01, ***p<0.001.
Figure 3.10: Cytokine levels in the colon homogenates of WT and Casp11−/− mice treated with 2% DSS (MP BioMedicals) for 5 days

Distal colons of WT and Casp11−/− mice were harvested 5 days post treatment with 2% (w/v) DSS. Protein lysates from the colons were prepared, quantified and normalised (110 µg/50 µl) for the determination of colon cytokine (IL-1α, IL-1β, IL-18, IL-6, IL-22, IFN-γ, IL-17A, IL-10 and MIP-2) levels by ELISA. Protein lysate readings were applied to a standard protein concentration curve for each cytokine to determine actual cytokine levels present in the colon lysate. Data represent mean ±SEM of n=5 mice and n=2 for the control group. P values were calculated using Student’s t-test; *p<0.05.
Figure 3.11: Cytokine levels in the colon homogenates of WT and Casp11^{-/-} mice treated with 2% DSS (MP BioMedicals) for 6 days

Distal colons of WT and Casp11^{-/-} mice were harvested 6 days post treatment with 2% (w/v) DSS. Protein lysates from the colons were prepared, quantified and normalised (120 µg/50 µl) for the determination of colon cytokine (IL-1α, IL-1β, IL-18, IL-6, IL-22, IFN-γ, IL-17A, IL-10 and MIP-2) levels by ELISA. Protein lysate readings were applied to a standard protein concentration curve for each cytokine to determine actual cytokine levels present in the colon lysate. The experiment was repeated three times with similar results. Data represent mean ±SEM of n=5 mice and n=2 for the control group. P values were calculated using Student’s t-test; *p<0.05.
Figure 3.12: Levels of IL-18 in serum during DSS-induced colitis

WT and Casp11^−/− mice were treated with 2% (w/v) DSS (MP BioMedicals) for 6 consecutive days. Blood was collected by cardiac puncture on the last day of the experiment and serum from the blood was obtained. Protein amounts were quantified and normalised (40 μg/50 μl) for the determination of systemic release of IL-18 in serum by ELISA. Protein readings were applied to a standard protein concentration curve to determine actual cytokine level present in the serum. Data represent mean ±SEM of n=6 mice and n=2 for the control group. P values were calculated using Student’s t-test.
Casp11<sup>−/−</sup> mice were treated for 6 days with 2% (w/v) DSS and injected intraperitoneally on days 0, 1 and 2 with 0.05 μg of recombinant IL18 (rIL18). (A) Body weight loss of Casp11<sup>−/−</sup> and Casp11<sup>−/−</sup> + IL18 mice; (B) Intestinal bleeding was graded on a scale of 0-4 (with zero indicating no bleeding and 4 indicating severe bleeding); (C) Diarrhea was graded on a scale of 0-3 (with zero indicating no diarrhea and 3 indicating severe diarrhea); (D) Disease activity index (DAI) which measured a combination of body weight loss, stool consistency and intestinal bleeding; (E) Colon length measurements. Data represent mean ±SEM of n=3 mice and n=2 for the control group. P values for (A-D) were calculated using the Two-way ANOVA followed by the Bonferroni post-test; ***p<0.001, ** p<0.01, *p<0.05 and for (E) p values were calculated with Student's t-test; ** p<0.01 and ns (not significant).
Fig. 3.14: Daily exogenous IL-18 administration significantly decreases severity of DSS-induced colitis in Casp11⁻/⁻ mice

Casp11⁻/⁻ mice were treated for 6 days with 2% (w/v) DSS, followed by 2 days with regular drinking water and injected intraperitoneally for 7 days of the experiment with 0.05 μg of recombinant IL18 (rIL18). (A) Body weight loss of Casp11⁻/⁻ and Casp11⁻/⁻ + IL18 mice; (B) Intestinal bleeding was graded on a scale of 0-4 (with zero indicating no bleeding and 4 indicating severe bleeding); (C) Diarrhea was graded on a scale of 0-3 (with zero indicating no diarrhea and 3 indicating severe diarrhea); (D) Disease activity index (DAI) which measured a combination of body weight loss, stool consistency and intestinal bleeding; (E) Colon length measurements. This experiment was repeated twice with similar results. Data represent mean ±SEM of n=5 mice and n=3 for the control group. P values for (A-D) were calculated using the Two-way ANOVA followed by the Bonferroni post-test; ***p<0.001, **p<0.01, *p<0.05 and for (E) p values were calculated with Student's t-test; *p<0.05.
Figure 3.15: IL-18 administration significantly attenuates histological colonic crypt damage in Casp11+/− mice treated with 2% DSS

Distal colons of Casp11+/− ± IL-18 mice treated for 6 days with 2% DSS, followed by 2 days with regular drinking water and control group (water only) were harvested and analyzed using hematoxylin and eosin (H&E) staining. Colitis severity of H&E stained colonic tissues was assessed by: (A) combined histological score of (B) tissue disruption (crypt damage score; 0–3, according to the severity of mucosal and crypts damages) and (C) colon cellular infiltration (inflammation score; 0–3, according to the extent of inflammation throughout the intestinal wall). (D) Representative microscopic pictures of (H&E) stained colon sections of water and 2% DSS treated Casp11+/−±IL18 mice on the last day of the experiment (day 8); (magnification 200x). Data represent mean ±SEM, (2% DSS, n=6; control, n=3). P values were calculated with Student's t-test; * p<0.05, **p<0.01.
Figure 3.16: IL-18 administration does not cause detectable differences in colon cytokine levels of Casp11⁻/⁻ mice treated with 2% DSS

Distal colons from the mice groups treated with 2% (w/v) DSS (Casp11⁻/⁻ ± IL18) and water only (control group) were harvested after 8 days treatment. Protein lysates from colons were prepared, quantified and normalised (120 µg / 50 µl) for the determination of colon cytokine (IL-1α, IL-1β, IL-18, IL-6, IL-22, IFN-γ, IL-17A, IL-10 and MIP-2) levels by ELISA. Protein lysate readings were applied to a standard protein concentration curve for each cytokine to determine actual cytokine levels present in the colon lysate. The experiment was repeated twice with similar results. Data represent ±SEM of n=5 mice and n=3 for the control group. P values were calculated using Student’s t-test; *p<0.05.
Chapter 4

Functional and mechanistic insights into the regulation of caspase-11 in a DSS-induced mouse model of colitis
4.1 Introduction

Significant progress has been made in understanding the fundamental importance of both innate immunity and epithelial barrier dysfunction in the pathogenesis of intestinal inflammation during inflammatory bowel disease (IBD) (Turner, 2009). In the gastrointestinal tract, a single layer of intestinal epithelial cells (IECs) prevents entry of commensal bacteria and luminal antigens, whereas the underlying lamina propria and lymphoid tissue, rich in professional immune cells, are considered to be mainly involved in systemic immune response to pathogenic challenge (Artis, 2008). In order to gain access to the host, pathogens must first breach the intestinal epithelial barrier, thus the mechanisms regulating this barrier play an essential role in prevention of many diseases, including IBD.

Accumulating evidence demonstrates that microbial sensing through pattern recognition receptors (PRRs) drives complementary functions in IECs and haematopoietic cells, which together control intestinal homeostasis and prevent against bacterial invasion (Abreu, 2010; Asquith et al., 2010; Cario, 2010). In the healthy intestine, basal PRR activation maintains barrier function and commensal composition, but aberrant PRR signalling may be a key contributor to the pathophysiology of IBD (Cario, 2010; Maloy and Powrie, 2011).

The importance of TER and NLR signalling in regulating epithelial barrier function has been widely reported using the DSS-colitis mouse model, in which DSS administration effects in chemical destruction of the IEC layer and penetration of commensal bacteria, leading to acute colitis followed by restitution and repair of the epithelial barrier. Interestingly, mice that lack specific TLRs, (including Tlr2 and Tlr4) or that are deficient in the TLR signalling adaptor proteins such as MyD88 or Mal, display increased susceptibility to DSS colitis, characterized by defective tissue repair and/or increased mortality (Araki et al., 2005; Aviello et al., 2014; Rakoff-Nahoum et al., 2004). In addition, some studies have reported that NLR-mediated inflammasome activation also contributes to protection after damage of the epithelium, because mice deficient in NLRP3, its adaptor ASC or caspase-1 exhibited enhanced colitis and mortality after DSS administration (Allen et al., 2010; Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). Furthermore, caspase-1 mediated IL-18 production by the NLRP3 canonical inflammasome in colonic epithelial cells was identified as a crucial mediator
of repair of the mucosal barrier and protection against DSS induced colitis (Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). Interestingly, non-canonical inflammasome activation of caspase-11 (caspase-4) has been also shown to play a critical role in antimicrobial defence within the epithelial barrier in the gut (Knodler et al., 2014). Moreover, two recent reports have also indicated an important role for caspase-11 in host protection against intestinal inflammation and bacterial infection (Demon et al., 2014; Williams et al., 2015).

The majority of earlier studies evaluating caspase-11 function strongly support a synergistic model where the non-canonical inflammasome effectively modulates canonical signalling and cytokine maturation following Gram-negative bacterial infection (Kayagaki et al., 2013; Rathinam et al., 2012). However, the role of caspase-11 and its proposed upstream activating mechanism (via TLR4-TRIF-IFN-I signalling pathway) occurring during host defence against Gram-negative bacteria (Broz et al., 2012b; Gurung et al., 2012; Rathinam et al., 2012) has not been fully elucidated in the context of intestinal inflammation.

Therefore, having demonstrated (in chapter 3) an important role for caspase-11 in regulating IL-18 levels in colonic mucosa (thus, implicating its role in the restitution of gut epithelia after DSS injury), we wanted to further define its involvement in maintaining epithelial barrier integrity. Moreover, we also wanted to investigate the mechanistic signalling events which trigger the non-canonical and caspase-11 activation during intestinal inflammation in the DSS mouse model of acute colitis.
4.2 Results

4.2.1 Caspase-11 is required for preservation of epithelial barrier integrity after DSS administration

Previous reports demonstrated that NLRP3\(^{-/-}\), Casp1\(^{-/-}\) and Asc\(^{-/-}\) mice were more susceptible to DSS-induced colitis when compared with WT mice, partially due to enhanced epithelial barrier disruption (Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). Given this, and the fact that in our study the Casp11\(^{-/-}\) mice were more susceptible to colitis, we therefore investigated the role of caspase-11 in maintaining epithelial integrity in the gut during DSS induced acute colitis in vivo. 2% DSS treatment of Caspase-11 null mice confirmed their enhanced susceptibility to experimental colitis, as demonstrated by a significantly higher percentage of body weight loss and more severe bleeding and diarrhea scores compared to the wild type mice (Fig. 4.1 A,B,C).

We next examined epithelial barrier damage levels in water and DSS treated Casp11\(^{-/-}\) versus WT mice using a permeability assay with FITC-dextran (as previously described in materials and methods section 2.1.6). As anticipated, in water treated Casp11\(^{-/-}\) and WT mice, there was little FITC-dextran observed in the serum with no notable differences between both groups, confirming a functional intestinal barrier in the steady state. There was, however, significantly more FITC-dextran present in the serum of DSS-treated Casp11\(^{-/-}\) compared to WT mice (p<0.01), (Fig. 4.1D). These results strongly suggest that caspase-11 is important for regulation of gastrointestinal permeability and maintenance of epithelial barrier integrity during DSS induced colitis.

4.2.2 Caspase-11 mediated IL-18 production results in IEC proliferation

It has previously been shown that impaired epithelial barrier function in NLRP3\(^{-/-}\) and Casp-1\(^{-/-}\) mice during DSS induced colitis is associated with decreased epithelial cell proliferation (Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). Based on our data showing defective epithelial barrier integrity in Casp11\(^{-/-}\) mice undergoing acute colitis, we next assessed IEC proliferation levels in murine colonic tissues of water and DSS treated mice.

The number of proliferating cells in the intestinal epithelium was detected by immunohistochemical staining of colonic tissues with two classic proliferation markers:
5-bromo-2-deoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA), which are successfully applied to label actively growing and dividing cells in cell culture or paraffin embedded tissues (Muskhelishvili et al., 2003). The in vivo labelling of DNA with BrdU (a thymidine analog) allows its incorporation into nuclei during the DNA synthesis phase of the cell cycle (S-phase only) and is further detected by IHC with an anti-BrdU antibody (Gratzner, 1982). However, PCNA is an auxiliary protein of DNA polymerases δ and is critical for DNA synthesis. Expression of PCNA increases during the G1-phase, peaks at the S-phase, and declines during G2/M-phases of the cell cycle, thus, PCNA immunostaining allows the identification of cells in the different phases of the cycle (Foley et al., 1993).

Analysis of BrdU-positive intestinal epithelial cells revealed a significantly decreased number (p<0.001) of proliferating cells in Casp11−/− colons compared to WT mice after 5 days of DSS treatment (Fig. 4.2 A, B). Similarly, quantification of PCNA positive epithelial cells also indicated a significantly impaired amount (p<0.001) of IEC proliferation in Casp11+ compared to WT mice after 8 days DSS treatment (Fig.4.3 A, B). Interestingly, untreated WT and Casp11−/− mice showed comparable amounts of BrdU and PCNA staining in colonic crypts compared to DSS treated mice, indicating a specific role for caspase-11 in epithelial cell proliferation after DSS-induced injury (Fig.4.2 A, B and 4.3 A, B).

In addition, to determine the involvement of IL-18 in driving IEC proliferation in the Casp11−/− colitis model, colon sections from the IL-18 rescue experiment were also stained for BrdU and PCNA quantification after 5 and 8 days of DSS treatment, respectively (Fig.4.2 C, D and 4.3 C, D). Our findings revealed that the IL-18 rescue phenotype of DSS treated Casp11−/− mice correlated with increased IEC proliferation, exhibited by elevated levels (p<0.05) of BrdU and PCNA positive cells in colonic mucosa compared to the PBS treated mice (Fig. 4.2 C, D and 4.3 C, D). The data presented here suggest that caspase-11 mediated IL-18 production controls mucosal integrity by increasing epithelial cell proliferation after acute DSS-induced intestinal injury.
4.2.3 Increase in epithelial barrier damage in DSS treated Casp11<sup>+/−</sup> mice is associated with an increase in cell death

Epithelial cell death induction has been identified as a crucial mechanism contributing to disease severity in DSS-induced colitis (Qiu et al., 2011). To investigate if decreased barrier function in the absence of caspase-11 could be explained by increased cell death of epithelial cells, we characterised the extent of cell death using the technique of terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labelling (TUNEL)-staining. This assay is a common method for detecting DNA fragmentation that results from apoptotic signalling cascades. It relies on the ability of the TdT enzyme to incorporate labeled dUTP into free 3'-hydroxyl termini generated by the fragmentation of genomic DNA into low molecular weight double-stranded DNA and high molecular weight single-stranded ("nicks") DNA (Gavrieli et al., 1992).

The distal colon samples of water and 2% DSS-fed WT, Casp11<sup>+/−</sup>± IL-18 mice were collected at day five post-DSS treatment. Cell death was quantified by the number of TUNEL-positive cells on histological sections (as described in the materials and methods section 2.1.5). As anticipated, colonic epithelium of untreated WT and Casp11<sup>+/−</sup> mice contained a low number of TUNEL-positive cells (Fig. 4.4). In contrast, there was a significantly higher level (p<0.05) of epithelial cell death in Casp11<sup>+/−</sup> mice compared to WT littermates at steady stage (Fig. 4.4 A, B). DSS treatment significantly increased the number of TUNEL-positive cells detected in colons of DSS treated WT and Casp11<sup>+/−</sup> mice compared to the water treated controls. (Fig. 4.4 B, D). Notably, the extent of epithelial cell death was also significantly elevated (p<0.01) in colonic epithelium of Casp11<sup>+/−</sup> compared to WT mice (Fig.4.4 A, B), suggesting that caspase-11 might be involved in controlling cell death during colitis. Interestingly, exogenous IL-18 administered Casp11<sup>+/−</sup> mice exhibited lower levels of epithelial cell death compared the PBS injected mice (Fig.4.4 C, D). This result confirms a crucial role of caspase-11 mediated IL-18 production in maintenance of epithelial barrier integrity. Collectively, these data suggest that enhanced epithelial barrier damage in DSS treated Casp11<sup>+/−</sup> mice is associated with increased epithelial cell death during this course of disease.
4.2.4 Increased epithelial barrier damage of Casp11⁻/⁻ is not associated with IL-22 mediated induction of anti-microbial peptides (such as Reg3β and Reg3γ) during colitis

Previous reports have demonstrated a beneficial role for IL-22 in intestinal epithelial barrier function through enhancing epithelial cell proliferation migration, and mucus production (Brand et al., 2006; Sugimoto et al., 2008). Moreover, in mouse models of colitis induced by *Citrobacter rodentium* or DSS, IL-22 was required for enhanced antimicrobial peptide production (including Reg3β and Reg3γ) to promote bacterial clearance or restore the barrier integrity following DSS injury (Mielke et al., 2013; Zheng et al., 2008; Zindl et al., 2013). Therefore, to determine the functional significance of decreased IL-22 levels in DSS treated Casp11⁻/⁻ mice (Fig.3.11 in chapter 3) we hypothesised that enhanced epithelial barrier damage in Casp11⁻/⁻ might be related to impaired IL-22-mediated Reg3β and Reg3γ production. The antimicrobial peptides expression levels in colons of untreated and DSS-fed WT and Casp11⁻/⁻ mice were measured by quantitative RT-PCR (as described in the materials and methods section 2.4). As expected, colonic epithelium of untreated WT and Casp11⁻/⁻ mice expressed low levels of Reg3β and Reg3γ transcripts (Fig. 4.5). However, there was no significant difference in antimicrobial peptides expression between DSS treated WT and Casp11⁻/⁻ groups. Overall, these data demonstrate that enhanced epithelial barrier damage in Casp11⁻/⁻ mice is not associated with decreased expression of IL-22-responsive antimicrobial peptides.

4.2.5 DSS-colitis induces caspase-11 upregulation and is independent of TRIF and type I IFNs

A number of previous reports have shown that non-canonical inflammasome activation following exposure to *Gram-negative* bacteria is mediated by TRIF and type I IFNs (Gurung et al., 2012; Rathinam et al., 2012). More recent data suggests that it may be the LPS-mediated priming of caspase-11, rather than its activation, that is dependent on TRIF and type I IFN signalling (Casson et al., 2013; Kayagaki et al., 2013). Based on these studies, and having demonstrated in chapter 3 of this thesis, a crucial role for DSS treatment in upregulating caspase-11 expression during acute colitis (Fig.3.8), we decided to further explore the mechanism of caspase-11 regulation.
Because DSS administration causes epithelial damage and infiltration of commensal bacteria, a proportion of which are Gram-negative, we hypothesized that TRIF and type I IFNs may also be responsible for the upregulation of caspase-11 in the colitis model.

To address this hypothesis, wild type and TRIF-/- knockout mice were kindly provided by the laboratory of Prof. Padraic Fallon. The DSS trial (as advised by K. Oficjalska) was performed by Gabriella Aviello, a member of the Prof. Fallon group. The mice were treated with water only on day 0 (healthy controls) or 2% DSS for 3 and 7 days. Western blotting analysis of mouse colon homogenates was performed to check expression levels of caspase-11. Unexpectedly, we saw no differences in caspase-11 upregulation between the DSS treated WT and TRIF-/- mice groups (Fig 4.6A). Surprisingly, there was also a significant enhancement in caspase-11 activation in TRIP knockout mice. These findings suggest that TRIP is not required for caspase-11 upregulation or activation in vivo during DSS-colitis. In addition, we did not observe any significant differences in the susceptibilities of WT and TRIF deficient mice to DSS-induced colitis (Fig. 4.6 B). However, in the absence of TRIF, caspase-11 appears to be constitutively activated, suggesting that TRIF mediated signalling events may be involved in limiting caspase-11 and non-canonical inflammasome activation in vivo during DSS colitis.

This data is in contrast to findings reported from other in vitro studies, which used LPS-primed BMDMs infected with Gram-negative bacteria to demonstrate a clear requirement of TRIF for caspase-11 upregulation (Broz et al., 2012b; Rathinam et al., 2012). It has also been previously reported that caspase-11 was transcriptionally upregulated in EHEC-infected macrophages in a TRIF-dependent manner, however, stimulation of TRIF deficient macrophages with IFN-β and IFN-γ alone restored their ability to up-regulate caspase-11 expression (Rathinam et al., 2012). Moreover, another study has revealed that IFN-γ restored the ability of the TRIF deficient macrophages to induce pro-inflammatory responses to Salmonella infections in vitro (Talbot et al., 2009).

Based on these aforementioned reports, we decided to test and confirm the TRIF dependency and IFNs restoring capabilities for caspase-11 upregulation in vitro. WT and TRIF-/- BMDMs were either untreated or primed with LPS before stimulation with IFN-γ or IFN-β, as indicated (Fig.4.6C). Results shown in Fig.4.6C reveal that TRIF has
a significant role in caspase-11 upregulation following stimulation of BMDM with LPS, IFN-γ or IFN-β. LPS primed macrophages in combination with IFN stimulation appears to restore the ability of TRIF<sup>−/−</sup> BMDM to induce high caspase-11 expression levels. This confirms a crucial role for LPS priming in caspase-11 upregulation, and suggests that co-stimulation with LPS and Type I or Type II IFN relieves the requirement for TRIF. Collectively, this data strongly suggests that LPS, TRIF and IFNs are important mediators of caspase-11 production in BMDMs, however, there are additional, and as of yet still unknown, signalling pathways which contribute to its regulation.

Next, we evaluated the dependency of caspase-11 expression on type I IFN signalling during DSS-induced colitis. To investigate this, WT and IFNAR<sup>−/−</sup> mice were treated with water only on day 0 (healthy controls) or 2% DSS for 3 and 7 days. Western blotting analysis of mouse colon homogenates was performed to check expression levels of caspase-11 in all animals (Fig.4.7A). Similar to our observations with TRIF<sup>−/−</sup> mice, there was no apparent difference in caspase-11 expression between colons of WT and IFNAR<sup>−/−</sup> mice. However, caspase-11 was similarly upregulated in DSS treated WT and IFNAR<sup>−/−</sup> mice, which nicely correlated with the longer exposure time to DSS treatment, confirming that DSS up-regulates caspase-11 during colitis. Surprisingly, caspase-11 (p30) activation was observed in DSS treated IFNAR<sup>−/−</sup> mice at day 7. Contrary to previous reports (Katakura et al., 2005), we found no significant difference between the susceptibilities of IFNAR<sup>−/−</sup> and WT mice to DSS-colitis, as mice exhibited similar weight loss profiles (Fig.4.7B). This data suggest that an alternative pathway to type I IFNs mediates the induction and activation of caspase-11 in vivo during this model of intestinal inflammation.

### 4.2.6 A potentially important role for Mal signalling in caspase-11 upregulation during DSS colitis

As TRIF and IFNAR appeared to be dispensable for caspase-11 upregulation during colitis, we further examined the dependency of caspase-11 expression on another specific adaptor for TLR4 signalling, namely MyD88 adaptor-like (Mal/TIRAP). Mal is commonly considered an important component in the signalling machinery required for the host response to LPS (Fitzgerald et al., 2001; Miggin et al., 2007). In addition and similar to caspase-11, Mal has been shown to have a protective role during colitis
(Aviello et al., 2014) and also has an essential role in maintaining epithelial barrier integrity during bacterial infections (Corr et al., 2014). Therefore, we hypothesized that Mal might be involved in caspase-11 upregulation in vivo during acute colitis. Interestingly, our results revealed that caspase-11 expression was completely abolished in Mal^−/− mice prior to and after DSS treatment compared to WT littermates, as indicated (Fig. 4.8A). In agreement with previous reports (Aviello et al., 2014), Mal^−/− mice were also more susceptible to DSS-colitis, as exhibited by their higher percentage of weight loss compared to WT mice (Fig. 4.8B). Overall these data suggest that Mal signalling might be critical in caspase-11 upregulation during acute colitis, however, additional studies need to be performed to further investigate these preliminary findings.

4.2.7 Type II IFN signalling mediates caspase-11 upregulation during DSS colitis

As caspase-11 has been shown to be responsive to both type I and type II IFNs (Fig. 4.6C) and (Rathinam et al., 2012; Schauvliege et al., 2002)), the possibility that IFN-γ may be involved in caspase-11 upregulation in mice during acute DSS-colitis was further explored. WT and IFN-γ^−/− mice were subjected to 2% DSS treatment for 3 and 7 days. Western blotting analysis of mouse colon homogenates was performed to check expression levels of caspase-11 and STAT1 in these animals (Fig. 4.9A). Interestingly, our data revealed that caspase-11 expression and activation was partially abolished in DSS treated IFN-γ knockout mice following exposure to DSS (Fig. 4.9A-C). Similar to caspase-11, STAT1 expression and phosphorylation levels were enhanced in the colon of WT mice during DSS colitis. As expected, STAT1 levels were significantly reduced in IFN-γ^−/− mice, suggesting that caspase-11 regulation during colitis may occur via a type II IFN-STAT1 signalling pathway. However, Caspase-11 levels appear to be normal in untreated IFN-γ^−/− mice (Fig. 4.9 A-C), supporting the evidence that there are also alternative pathways that control intestinal caspase-11 transcription, in response to distinct stimuli. In addition, we also observed that, similar to Casp11^−/− mice, IFN-γ^−/− mice were more susceptible to DSS-colitis, as exhibited by their significant weight loss compared to WT mice, indicating an important role for type II IFN during acute colitis (Fig. 4.9D).
Further, to gain mechanistic insight into the regulation of caspase-11 in the acute DSS model, we attempted to identify the cell types responsible for upregulating caspase-11 in the colon. Separation of epithelial and lamina propria cell layers from DSS-treated WT mice demonstrated that caspase-11 upregulation occurs in the intestinal epithelial cell (IEC) fraction following 3 and 6 days DSS treatment (Fig. 4.9E). Collectively, these findings reveal a protective role for the non-canonical inflammasome and caspase-11 in IECs during acute colitis.

Despite our findings, a role for type II IFN in various mouse models of colitis still remains a controversial subject. Some reports support its proinflammatory function (Ito et al., 2006; Takagi et al., 2003) but others suggest a lack or anti-inflammatory role in colitis (Sheikh et al., 2010; Tozawa et al., 2003). Given these conflicting reports, it was important for us to confirm the previously observed protective features of IFN-γ (Fig. 4.9) in our acute DSS colitis model. To examine this, two groups of WT mice were treated with 2% DSS for 6 days followed by water for 2 days. One cohort of mice received a daily injection of 200 IU (i,p) recombinant IFN-γ/mouse for 8 consecutive days whereas the control group was (i,p) injected with PBS only. Based on the DAI scores from this trial (Fig. 4.10) we observed that exogenous administration of IFN-γ attenuated the DSS-induced colitis phenotype, confirming its protective role in this mouse model.
4.3 Discussion

Results from the first chapter of this thesis revealed not only a protective role for caspase-11 during intestinal inflammation in acute DSS-induced murine colitis, but also implicated IL-18 as a key cytokine involved in this process. To build on this data we performed additional studies to provide further functional and mechanistic insights into the role of non-canonical inflammasome in this mouse model of colitis.

As IL-18 has been associated with repair and restitution of ulcerated epithelium and as colitis was also shown to be more severe under conditions in which epithelial cell integrity is defective (Coruh et al., 2001; Reuter and Pizarro, 2004; Zaki et al., 2010a), we decided to further investigate whether deficiency in IL-18 production in DSS treated Casp11−/− was associated with impaired epithelial barrier integrity. Comparison of FITC-Dextran uptake in DSS-fed WT and Caspase-11−/− mice revealed decreased barrier integrity in DSS-fed Casp11−/− mice, indicating a crucial role for caspase-11 in regulation of gastrointestinal permeability (Fig.4.1). These findings were in agreement with previous reports which also implicated components of the canonical inflammasome in maintaining epithelial integrity in the gut (Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). As defects in barrier function after DSS injury might be explained by increased apoptosis and/or decreased epithelial cell proliferation (Araki et al., 2010; Reuter and Pizarro, 2004), we also identified an association between IEC proliferation and cell death after DSS injury. Our results revealed that colons from DSS treated Casp11−/− mice displayed significantly less IEC proliferation that those of WT mice (Fig.4.2 and 4.3). Similar results have been previously shown for DSS treated NLRP3−/−, Casp1−/− mice and thus the canonical inflammasome (Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). The enhanced IEC proliferation in above reports was mainly linked to IL-18 properties, which nicely supports our findings and therefore explains a functional role for caspase-11 mediated IL-18 production in intestinal regeneration and barrier repair. Further support for the importance of IL-18 in protection and maintenance of epithelial barrier, has also been previously documented in that NLRP6-inflammasome mediated IL-18 secretion by IECs prevents gut colonization by colitogenic microbiota (Elinav et al., 2011).

Following on from these results, analysis of epithelial cell death measured by TUNEL staining showed significantly increased number of TUNEL-positive cells in
colons of DSS treated Casp11−/− mice compared to WT littermates (Fig. 4.4). This might suggest that caspase-11 may have a role in inhibiting cell death in acute colitis. However, in contrast to our results, Demon et al observed no differences in TUNEL-positive cells in the colons of DSS treated WT, Casp11+/− and Casp1+/−/Casp11−/− mice, thus, suggesting that increased susceptibility of Casp11−/− to DSS is not associated with deregulated apoptosis (Demon et al., 2014). This discrepancy between both studies might be explained by different experimental designs as in Demon’s et al study, the epithelial cell death was assessed during either early onset (day 3 after 4% DSS treatment) or recovery phase (day 10 after 5 days 2% DSS treatment followed by 2 days of water) of the disease. However, in our study TUNEL analysis was performed at moderate stage of disease progression (day 5 after 2% DSS treatment). Our findings suggest that caspase-11 might serve as a negative regulator of epithelial cell death during colitis. However, this contradicts with previous reports which mainly implicate caspase-11 in induction of apoptosis (through caspase-3 activation) under certain pathological conditions (such us cerebral ischemia or EAE) and in methamphetamine-induced neuron apoptosis (Hisahara et al., 2001; Huang et al., 2015; Kang et al., 2000). In addition caspase-11 is also considered as a key driver of pyroptosis in response to non-canonical stimuli, implicating its crucial role in antimicrobial defence (Hagar et al., 2013; Kayagaki et al., 2011; Kayagaki et al., 2013). Recently, Demon et al also implicated caspase-11 in driving pyroptosis during colitis (Demon et al., 2014), however, this was solely based on the reduced systemic HMGB1 levels in Casp11−/− mice. Unluckily, this marker is not only associated with pyroptosis but also with necrotic cell death (Lamkanfi, 2011; Lu et al., 2013) suggesting that caspase-11 may either mediate pyroptosis and/or necrosis. Unfortunately, there is a lack of specific in vivo markers for pyroptosis restricting evaluation of its role in vivo. It is commonly considered that the TUNEL staining is broadly used for detecting DNA fragmentation of apoptotic cells, however, DNA fragmentation also occurs during pyroptosis and it has been reported that pyroptotic cells that incur DNA damage also become positive in the TUNEL assay (Bergsbaken and Cookson, 2007; Mariathasan et al., 2005). As our results seen to be in contrast to other published findings and at odds with the accepted role for caspase-11 in driving pyroptosis, further experiments are required to elucidate the precise role of caspase-11 in regulating apoptosis/pyroptosis during colitis. To confirm our results an alternative method of apoptosis detection should be used, for
example immunofluorescent staining of active caspase-3, cleaved PARP (poly-ADP-ribose polymerase) or anti-apoptotic proteins (such as Bcl-xl) to assess their levels in WT vs Casp11\(^{-/-}\) colonic sections. Furthermore, if these readouts were to confirm our prior findings it would be worth performing a broad range of \textit{in vitro} experiments using caspase-11 (and its human homologs caspase-4 and -5) knockdowns in epithelial cell lines stimulated with various non-canonical stimuli to assess their roles in driving apoptosis and pyroptosis \textit{in vitro}.

To further evaluate mechanistic insights into the role of non-canonical inflammasome in controlling mucosal integrity during acute colitis, we hypothesized that, similar to what has been shown following \textit{Gram-negative} infection in mice, the TRIF-IFN-1 signalling axis plays a crucial role in the upregulation and/or activation of caspase-11 in this model. In conflict with our expectations, our results revealed that TRIF signalling makes no contribution to the pro-caspase-11 upregulation during colitis \textit{in vivo}, as Western blotting analysis showed no differences in caspase-11 expression between TRIF and WT mice (Fig.4.6 A). In support of previous findings (Broz et al., 2012b; Rathinam et al., 2012), LPS stimulated BM-derived macrophages from the same TRIF\(^{-/-}\) mice had impaired caspase-11 expression levels, highlighting the signalling complexities between \textit{in vivo} and \textit{in vitro} studies.

Nevertheless, the role of TLR4-TRIF signalling axis in caspase-11 expression and activation reported in a number of different studies still remains a topic of great debate. For example, some studies demonstrated that caspase-11 upregulation was reduced in TRIF\(^{-/-}\) macrophages infected with \textit{C. rodentium}, \textit{E. coli} and \textit{EHEC} (Gurung et al., 2012; Rathinam et al., 2012). However, other studies revealed that although caspase-11 induction was delayed in macrophages after \textit{Salmonella} infection, the protein levels of Caspase 11 remained the same (Broz et al., 2012b; Case et al., 2013). Moreover, Gurung \textit{et al} showed importance of the TLR4-TRIF pathway in pro-caspase-11 upregulation and activation during infection of macrophages with non-canonical stimuli (such \textit{C. rodentium} and \textit{E. coli}). However, the relevance of these findings were lacking \textit{in vivo} as \textit{C. rodentium} infected TLR4\(^{-/-}\), TRIF\(^{-/-}\) and Casp11\(^{-/-}\) mice displayed only slightly increased bacterial burdens in the stool and none of the infected mice succumbed to infection, suggesting that the non-canonical inflammasome plays relatively modest role during \textit{C. rodentium in vivo} (Gurung et al., 2012).
Interestingly and in agreement with previous reports (Rathinam et al., 2012, Talbot), our *in vitro* studies also revealed that TRIF dependency for LPS mediated upregulation of caspase-11 can be overcome by the presence of IFN-γ and IFN-β (Fig 4.6 C). These data implicate an important role for both type I and II IFNs in caspase-11 regulation *in vitro*. In contrast, our *in vivo* studies revealed a lack of difference in caspase-11 expression in the colons of DSS treated mice (WT versus IFNAR'^'') (Fig.4.7 A), suggesting that Type I IFN signalling is dispensable for DSS mediated caspase-11 upregulation *in vivo*. This was also in contrast with previous findings implicating Type 1 IFN as a key regulator of caspase-11 in response to Gram-negative bacterial infection (Broz et al., 2012b; Gurung et al., 2012; Rathinam et al., 2012). Therefore, these results highlight additional signalling complexities between *in vitro* and *in vivo* studies.

However, a lack of involvement of TRIF-type I IFN pathway in caspase-11 regulation *in vivo* in our study is nicely supported by other reports (Hagar et al., 2013; Kayagaki et al., 2013). These authors discovered the existence of new TLR4-independent mechanism for innate immune recognition of LPS. Kayagaki *et al* showed that the entire TLR4-TRIF-type-I IFN pathway is dispensable for intracellular LPS to trigger the non-canonical inflammasome engaging pro-caspase-11 induction and activation in BMDMs. They also further extended their *in vitro* study to a mouse model of acute septic shock which revealed that both TLR4-dependent and -independent mechanisms for sensing LPS contribute to the model of lethal sepsis *in vivo* (Kayagaki et al., 2013).

As a requirement of TRIF/IFN-1 axis in caspase-11 upregulation appears to be dispensable during acute colitis *in vivo*, we further hypothesized that Mal (another signalling adaptor for LPS/TLR4 sensing) may serve as upstream regulator of caspase-11 expression *in vivo*. In agreement with other study (Aviello et al., 2014), we also showed enhanced susceptibility of Mal'' mice to colitis. Intriguingly, we observed a lack of caspase-11 expression in colons from water and DSS treated Mal'' mice (Fig 4.8). To support the above findings, caspase-11 expression was also completely lost in LPS stimulated BMDMs from Mal deficient mice (data not shown). Therefore we hypothesised that there are two possibilities which may explain an absence of caspase-11 expression in these mice. The first one indicates that Mal is critical for induction of caspase-11 during colitis. If this scenario is true, this would suggest that LPS sensing...
though TLR4/MyD88/Mal/NF-κB pathway is essential for transcriptional caspase-11 production. To further elucidate this, it would be worth performing a set of experiments using WT BMDMs, in which NF-κB activation is inactivated by specific MAL inhibitors (which block Mal association to TLR4) therefore inhibiting Mal-dependent LPS-induced NF-κB activation. Cell lysate readouts from those experiments would further examine if Mal is crucial in mediating caspase-11 expression in vitro. Alternatively, another explanation may be that Mal^−/− mice (which were generated on the 129 strain) also lack a functional caspase-11 gene, similar to other 129 derived knockout mice such as Casp1, cIAP1 and Casp3 (Berghe et al., 2013; Kayagaki et al., 2011; Kenneth et al., 2012) suggesting that they are doubly deficient Mal^−/−/Casp11^−/− mice. This would fully explain a lack of caspase-11 in untreated and DSS treated Mal^−/− mice. However, further analysis of the presence an inactivating caspase11 gene mutation (5bp deletion in exon 7) (Kayagaki et al., 2011) in Mal^+/+ mice is required. Those two possibilities: either Mal is critical for caspase-11 or the Mal^−/− mice are Mal^−/−/Casp11^−/−/DKOs - currently being addressed in ongoing studies in Dr. Creagh’s lab.

Furthermore, given the fact that type II IFN is a commonly known transcriptional inducer of caspase-11 expression in vitro (Schauvliege et al., 2002), we next assessed the involvement of IFN-γ in caspase-11 regulation in vivo. Our data revealed that caspase-11 expression and activation in DSS treated IFN-γ^−/− mice were partially impaired compared to WT controls during colitis (Fig. 4.9 A-C). In addition, STAT1 and pSTAT1 levels were also highly reduced in IFN-γ^−/− mice. Therefore our data indicate that caspase-11 regulation during DSS-induced colitis may occur via a type II IFN-STAT1 pathway. Moreover, due to the fact that the caspase-11 promoter region contains a number of putative transcription factor binding sites, including κB and STAT sites, which were earlier shown to synergistically regulate caspase-11 expression via LPS and IFNγ in vitro (Schauvliege et al., 2002). We have also seen a synergistic upregulation of caspase-11 by IFN-γ and LPS in WT and TRIF^−/− BMDMs (Fig. 4.6C). Interestingly, recent findings by Qiao et al revealed that IFN-γ-activated STAT1 synergistically enhances TLR induced transcription of proinflammatory cytokines through epigenetic priming mechanisms. This study revealed that IFN-γ induces sustained occupancy of transcription factor STAT1 what is associated with increased histone acetylation and IRF-1 recruitment at promoters and enhancers of genes that are synergistically activated by IFN-γ and LPS. However, this priming of chromatin does
not activate transcription but significantly increases and prolongs recruitment of TLR4-induced transcription factors to gene promoters and enhancers, thus creating a primed chromatin environment to enhance TLR induced gene transcription. Therefore, above findings might suggest a direct role for IFN-γ in priming the caspase-11 gene before its enhanced upregulation occurs in response to microbial patterns (such as LPS or other TLR ligands) during DSS induced inflammation in vivo. If this assumption is correct, it suggests that JAK-STAT1 pathway may be a potential target in the management of colitis.

Another interesting observation revealed during the project was that caspase-11 upregulation occurs in the intestinal epithelial cells during acute colitis, thus indicating a protective role for the non-canonical inflammasome in IECs during acute colitis (Fig 4.9E). This finding was partially consistent with another study where it was demonstrated that caspase-11 is also expressed in epithelial cells, however, they linked its protective role with induction of pyroptosis as a protection against intestinal inflammation (Demon et al., 2014). In contrast, Williams et al demonstrated that caspase-11 expresses and functions in both hematopoietic- and non-hematopoietic-derived cells (including macrophages and epithelial cells) to attenuate intestinal inflammation in acute colitis. However this group did not report on how the loss of non-canonical inflammasome in both compartments results in exaggeration of colitis (Williams et al., 2015). As we failed to isolate the pure lamina propria fraction to assess caspase-11 expression of the DSS treated mice, the aforementioned data suggests that caspase-11 is expressed in both IEC and immune cells, is most likely (Williams et al., 2015). In agreement with these findings, we can hypothesise that similar to the reported contrasting roles for IL-18 (protective in IEC, but inflammatory at the level of lamina propria) (Reuter and Pizarro, 2004; Siegmund et al., 2001; Takagi et al., 2003), caspase-11 may also play a dual function (depending on the site of its expression) during colitis. Our data strongly support the first part of our hypothesis, suggesting that caspase-11 once expressed in IEC contributes to the preservation of the intestinal barrier, mainly by inducing proliferation and enhancing the regeneration of the damaged epithelium through modulating IL-18 production. On the other hand, activation of the non-canonical inflammasome in the lamina propria may initially recruit infiltration of neutrophils and macrophage to help eliminate the initial cause of DSS-induced intestinal damage. However, during increasing sustained inflammation it may lead to enhanced
inflammatory mediator production, therefore, resulting in colitis pathology. To test the second part of the hypothesis, it would be worth first confirming and further characterising what immune cell types express caspase-11 and also investigate the role of caspase-11 in the recruitment of immune cells to site of intestinal damage during DSS damage. This could be achieved by performing immunohistochemical staining with an anti-caspase-11 antibody and specific markers for neutrophil (Ly6G) and macrophage (F4/80) presence. In addition, it would be most interesting to evaluate the role of caspase-11 in a chronic model of DSS colitis and test if caspase-11 plays a pro- or anti-inflammatory role in this context.

In summary, our study to date reveals a link between caspase-11 mediated IL-18 production to the increased susceptibility of Casp11^−/− mice to colitis, suggesting the involvement of non-canonical inflammasome in induction of IEC proliferation, inhibition of cell death and epithelial barrier function during acute colitis. We also propose a novel type II IFN-mediated signalling pathway leading to the upregulation and activation of intestinal caspase-11 in IECs in vivo. These findings highlight the emerging importance of non-canonical inflammasome activation in innate immunity and chronic inflammatory diseases, such as inflammatory bowel disease (IBD).
C57BL/6J WT and Casp11−/− mice were treated for 6 days with 2% DSS, followed by 1 day with regular drinking water and control group (water only) were monitored daily for: (A) Body weight loss; (B) Intestinal bleeding (graded on a scale of 0-4, with 0 indicating no bleeding and 4 indicating severe bleeding); (C) Diarrhea (graded on a scale of 0-3, with 0 indicating no diarrhea and 3 indicating severe diarrhea); (D) Plasma FITC-dextran concentrations of water and 2% DSS treated WT and Casp11−/− mice, 4 hours after oral gavage of FITC-dextran (60 mg/100 g) on the last day of the experiment (day 7). The experiment was repeated twice with similar results. Data represent mean ±SEM, (2% DSS, n=6; control, n=3). P values were calculated for (A-C) using the Two-way ANOVA followed by the Bonferroni post-test; ***p<0.001, ** p<0.01, *p<0.05; and for (D) with the Student's t-test * p<0.05, ** p<0.01.
Figure 4.2: The enhanced severity of DSS-induced colonic damage in Casp11" mice is associated with decreased IEC (Intestinal Epithelial Cell) proliferation (BrdU)

IEC proliferating cells in colonic sections of water and DSS treated WT and Casp11" mice and Casp11" +/- IL18 were detected by immunoperoxidase staining of the proliferation marker (BrdU). Mice were i.p. injected with BrdU (50mg/ml) and distal colon tissues were harvested 3 hours later to obtain formalin-fixed, paraffin-embedded (FFPE) tissue blocks. (A) Representative microscopic pictures of immunohistochemical staining of the proliferation marker (BrdU) from distal colon tissue sections of water and DSS-treated WT and Casp11" mice and (C) Casp11" +/- IL18 (day 5); (magnification 200x). (B) and (D) Quantification of BrdU positive cells shown in (A) and (C). On average, 50-well oriented crypts were scored per mouse. Data represent mean ±SEM, (2% DSS, n=6; control, n=4). P values were calculated with the Student's t-test; * p<0.05; ***p<0.001.
Figure 4.3: The enhanced severity of DSS-induced colonic damage in Casp11^−/− mice is associated with decreased IEC (Intestinal Epithelial Cell) proliferation (PCNA)

(A) Representative microscopic pictures of immunofluorescence staining of the proliferating cell nuclear antigen (PCNA) marker from distal colon tissue sections of water and DSS-treated WT and Casp11^−/− mice and (C) Casp11^−/+ IL18 (day 8); (magnification 400x). (B) and (D) Fluorescence quantification of PCNA^+ cells shown in (A) and (C). On average, 30 crypts (3 fields of 10 crypts) were scored per mouse. Data represent mean ±SEM, (2% DSS, n=6; control, n=3). P values were calculated with the Student's t-test; * p<0.05; *** p<0.001.
Figure 4.4: Increased susceptibility of Casp11" mice to DSS colitis is associated with enhanced cell death

(A) Representative microscopic pictures of the fluorescence staining of the TUNEL (TdT-mediated dUTP nick end labeling)-positive cells from distal colon tissue sections of water and DSS-treated WT and Casp11" mice and (C) Casp11" +/− IL18 (day 5); (magnification 200x). (B) and (D) Quantification of TUNEL positive cells per field shown in (A) and (C). On average, 5 random optical fields were scored per mouse. Data represent mean ±SEM, (2% DSS, n=6; control, n=4). P values were calculated with the Student's t-test; * p<0.05, **<0.01, ***p<0.001.
Figure 4.5: The enhanced severity of DSS-induced colonic damage in Casp11⁺/⁻ mice is not associated with impaired antimicrobial production

Colonic sections of water and DSS treated WT and Casp11⁺/⁻ harvested at day 6 were homogenized and RNA was extracted. Further the quantitative RT-PCR reaction was performed to determine relative expression of (A) Reg3β and (B) Reg3γ transcripts in mouse colons. Relative levels of expression were determined by normalization to 18S rRNA. Data represent mean ±SEM of n=5 mice and n=2 for the control group. P values were calculated with the Student's t-test; ns (non-significant).
Figure 4.6: DSS-induced caspase-11 upregulation in the colon is independent of TRIF-mediated signalling

C57BL/6J WT (wild-type mice) and TRIF^−/− (TIR-domain-containing adapter-inducing interferon-β knockout mice) were treated with 2% (w/v) DSS for 0, 3 and 7-days. After DSS treatment colons were harvested, homogenized and 40 µg of each protein lysate was resolved by SDS-PAGE, transferred to nitrocellulose and probed for: (A) pro-caspase-11 (43 and 38 kDa), cleaved caspase-11 (30 kDa) and β-actin (42 kDa) (which served as a loading control). Each lane represents an individual mouse. (B) Body weight loss of WT and TRIF^−/− mice treated with 2% DSS in drinking water for 7 days and weighed daily. Data represent mean ±SEM, (2% DSS, n=4). (C) Western blot detection of pro-caspase-11 in WT and TRIF^−/− BMMDMs cell lysates stimulated as indicated. Results are representative of two independent experiments.
Figure 4.7: DSS-induced caspase-11 upregulation in the colon is independent of type-I IFN signalling

C57BL/6J WT (wild-type mice), IFNAR^-^ (type I IFN receptor knockout mice) and Casp11^-^ (Caspase-11 knockout mice) were treated with 2% (w/v) DSS for 0, 3 and 7 days. After DSS treatment colons were harvested, homogenized and 40 μg of each protein lysate was resolved by SDS-PAGE, transferred to nitrocellulose and probed for (A) pro-caspase-11 (43 and 38 kDa), cleaved caspase-11 (30kDa) and β-actin (42 kDa) (which served as a loading control). Each lane represents an individual mouse. (B) Body weight loss of WT and IFNAR^-^- mice treated with 2% DSS in drinking water for 7 days and weighed daily. Data represent mean ±SEM, (2% DSS, n=4). Results are representative of two independent experiments.
Figure 4.8: Mal signalling may be crucial for caspase-11 upregulation in the colon during DSS colitis

C57BL/6J WT (wild-type mice) and Mal\textsuperscript{-/-} (MyD88 adaptor-like knockout mice) were treated with 2% (w/v) DSS in drinking water for 0, 3 and 7 days. After DSS treatment colons were harvested, homogenized and 20 µg of each protein lysate was resolved by SDS-PAGE, transferred to nitrocellulose and probed for (A) pro-caspase-11 (43 and 38 kDa) and β-actin (42 kDa, which served as a loading control). Each lane represents an individual mouse. (B) Body weight loss of WT and Mal\textsuperscript{-/-} mice treated with 2% DSS in drinking water for 7 days and weighed daily. Results are representative of two independent experiments. Data represent mean ±SEM, (2% DSS, n=4).
C57BL/6J WT (wild-type mice) and IFNγ−/− (IFNγ knockout mice) were treated with 2% (w/v) DSS in drinking water for 0, 3 and 7 days. After DSS treatment colons were harvested, homogenized and 40 μg of each protein lysate was resolved by SDS-PAGE, transferred to nitrocellulose and probed for (A) pro-caspase-11 (43 and 38 kDa), cleaved caspase-11 (30 kDa), STAT1 (91kDa), phospho STAT1 (91 and 84 kDa) and β-actin (42 kDa, which served as a loading control). Each lane represents an individual mouse. Results are representative of two independent experiments. (B and C) Relative densitometric analysis of individual bands were performed using ImageJ software. (D) Body weight loss of WT and IFNγ−/− mice treated with 2% DSS in drinking water for 7 days and weighed daily. Results are representative of two independent experiments. Data represent mean ±SEM, (2% DSS, n=8); p values were calculated with the Student’s t-test **p<0.01. (E) Western blot showing pro-caspase-11 and cytokeratin-18 expression in epithelial cells isolated from WT mice treated with 2% DSS in drinking water for 0, 3 and 6 days.
Figure 4.10: A protective role for IFNγ in the acute induced DSS-colitis

C57BL/6J WT mice were treated for 6 days with 2% DSS, followed by 2 days with regular drinking water and control group (water only). Mice were injected daily either with recombinant IFNγ (200IU)/mouse or PBS and monitored for: (A) Body weight loss; (B) Intestinal bleeding (graded on a scale of 0-4, with 0 indicating no bleeding and 4 indicating severe bleeding); (C) Diarrhea (graded on a scale of 0-3, with 0 indicating no diarrhea and 3 indicating severe diarrhea) and (D) Colon length measurements. Data represent mean ±SEM, (2% DSS, n=5; control, n=2). P values were calculated for (A) using the Two-way ANOVA followed by the Bonferroni post-test; ** p<0.01; and for (D) with the Student's t-test * p<0.05, ***p<0.001.
Chapter 5
Investigation of inflammatory caspase expression patterns in inflammatory diseases including IBD, Barrett’s oesophagus (BO) and inflammatory associated cancers
5.1 Introduction

Inflammatory bowel disease (IBD) represents major remitting and relapsing inflammatory disorders of the gastrointestinal tract, which are characterized by chronic recurrent intestinal inflammation. IBD comprises two main subtypes: Crohn's disease (CD) and Ulcerative colitis (UC). The prevalence of IBD in Western countries is estimated at 1/1,000 inhabitants (Baumgart et al., 2011), with worldwide prevalence increasing over the last 50 years (Molodecky et al., 2012). Despite the dramatic advances made in IBD treatment over the last decade, no therapies currently exist to effectively cure IBD. This is mainly due to its complex pathogenesis involving multiple factors such as genetics, environment and alterations in immune responses to the commensal bacteria of the intestine (Chandel et al., 2015; Cho, 2008). It has been reported that persistent and sustained intestinal inflammation occurring during IBD is associated with a risk of developing colorectal cancer (CRC) (Dyson and Rutter, 2012; Eaden et al., 2001; Rubin et al., 2013). This is not surprising as cancer related inflammation (CRI) has been proposed as the seventh hallmark of cancer development and numerous epidemiological reports suggest that up to 15% of human cancer data incidence is associated with chronic inflammation (Kuper et al., 2000; Mantovani et al., 2008). With IBD-related neoplastic progression, malignancy is thought to follow a chronic inflammation-dysplasia-adenocarcinoma sequence (Hamilton, 2012). Dysplasia is the earliest recognisable precursor to CRC and, at the moment, is the most reliable marker of the risk of colitis tissue becoming cancerous (Mescoli et al., 2013). Current chemoprevention strategies for IBD patients include maintenance anti-inflammatory medications to prevent dysplasia/cancer development. In addition, regular surveillance colonoscopy allows for the identification and treatment of premalignant CAC lesions. However, dysplastic lesions associated with IBD-CAC are difficult to grade, and might be easily misplaced with epithelial aberrations caused by colonic chronic inflammation (Lim et al., 2003; Ullman et al., 2009). Therefore, there is a need for identification of new specific biomarkers which would facilitate a clear recognition between dysplastic/neoplastic and chronically inflamed tissue.

Barrett's oesophagus (BO) is another example of a chronic inflammatory disorder which is associated with a risk of developing oesophageal adenocarcinoma (OAC). BO has also been considered as the most important precursor lesion of OAC (Hameeteman et al., 1989; Sharma et al., 2006). Although the overall risk of a patient
with BO developing OAC remains small at approximately 0.3%, the risk of OAC development in a patient with BO is 50 times that of the general population (Desai et al., 2012). BO occurs in the in the distal oesophagus in the setting of chronic gastroesophageal reflux disease (GERD) and is histopathologically defined as the replacement of the normal squamous epithelium with an intestinalized (metaplastic) columnar epithelium (Falk et al., 2011). It is well established that BO metaplasia progresses sequentially through low grade dysplasia (LGD), high grade dysplasia (HGD) and eventually leads to oesophageal adenocarcinoma (OAC) development (Hvid-Jensen et al., 2011; Ronkainen et al., 2005). Nowadays, endoscopy usually permits the identification of different degrees of dysplasia in BO patients and histopathology is considered the gold standard for diagnosis of dysplasia, which still remains the best biomarker for estimation of the risk of OAC development (Werner and Lassmann, 2012). In addition, it has been reported that numerous genetic molecular changes have been correlated with metaplasia-dysplasia-carcinoma sequence. Some of these genetic changes have been proposed as an adjunct to morphology for the screening and surveillance of patients with Barrett’s oesophagus (Rustgi, 1997; Zeki and Fitzgerald, 2015). In particular, p53 immunohistochemistry has been shown to be useful clinically as an inter-observer agreement for reporting dysplasia, particularly low grade dysplasia (Fitzgerald et al., 2014; Kaye et al., 2009). It has been also proposed that p53 can serve as an indicator of progression in metaplasia-dysplasia-adenocarcinoma sequence, although this was not clinically approved (Sikkema et al., 2009; Skacel et al., 2002). Therefore, there is a clear need for the determination of new markers of BO progression to OAC, which would facilitate the diagnosis of patients who are at a high risk of developing cancer.

It has been broadly reported that the inflammatory microenvironment plays a key role in BO and OAC development, which was covered in detail in the introduction section 1.11. In addition, the risk of cancer development in BO has been shown to be reduced with the use of anti-inflammatory and non-steroidal anti-inflammatory (NSAIDs) drugs, therefore implicating inflammation as a causative factor in OAC development (Sadeghi et al., 2008; Wang et al., 2011). Currently, there are few reports which link elevated levels of IL-1β (a key inflammatory cytokine activated in caspase-1 mediated inflammasome complexes) with the stepwise progression from Barrett’s metaplasia to invasive cancer (Fitzgerald et al., 2002a; Quante et al., 2012). There is
also a paucity of studies regarding inflammasome activation or inflammatory caspase involvement in the pathogenesis of these diseases. However, with regard to pathological intestinal inflammation occurring during IBD, excessive production of the inflammatory cytokines, IL-1β and IL-18 has been observed in the colons of IBD patients (Mahida et al., 1989; Papadakis and Targan, 2000; Pizarro et al., 1999). IL-1β expression has also been found to be upregulated during colorectal cancer and has been identified as a key factor in tumour progression and metastasis (Apte et al., 2006; Vidal-Vanaclocha et al., 2000). Therefore, it is likely that caspase-1 may have a role in driving intestinal inflammation during IBD and colorectal cancer (Becker et al., 2013). The roles of caspases-4 and -5 during inflammation are less characterised compared to caspase-1, due to the lack of direct homolog of caspase-4 or -5 in a mouse. To date, no physiological substrates of caspase-4 or -5 have been verified in vivo, although there is a growing body of evidence to support their pro-inflammatory involvement in the non-canonical inflammasome (Kajiwara et al., 2014; Sollberger et al., 2012). Recently, it has been demonstrated that human caspase-4 and -5 mediate non-canonical NLRP3 activation to drive IL-1β production in human myeloid cells (Baker et al., 2015; Schmid-Burgk et al., 2015). It has been also reported that caspase-4 is required for IL-18 processing and pyroptosis in *S. typhimurium* and *E. coli* infected human IEC (Knodler et al., 2014; Kobayashi et al., 2013). Thus, these findings highlight the importance of these caspases and the non-canonical inflammasome during the innate immune response in humans. However, to date, most reports have focused predominantly on elucidating a role of caspase-11 (murine homolog of caspase-4 and -5) and the non-canonical inflammasome activation in murine bacterial infection and acute septic shock models (Creagh, 2014; Man and Kanneganti, 2015). In addition, we and others have also demonstrated a role for the non-canonical inflammasome and caspase-11 during acute colitis in mice (Williams et al., 2015).

Collectively, these new revelations have prompted us to investigate whether the functional homologs of caspase-11, human caspases-4 and -5, are involved in mediating inflammation during human inflammatory diseases and cancer. Therefore the main aim of this chapter was to examine inflammatory caspases expression patterns in tissues of patients with IBD and Barrett’s metaplasia and their progression to inflammatory associated cancers such as CAC and OAC. In addition, we set out to determine whether
our findings would reveal a potential role for caspases 4 or 5 to serve as diagnostic biomarkers of these diseases and/or their progression to cancer.
5.2 Results

5.2.1 Inflammatory caspase gene expression in IBD (Belgian cohort)

Preliminary analysis of CASP1, CASP4 and CASP5 gene expression in colonic tissue samples from IBD patients was carried out in collaboration with the laboratory of Prof. Debbie Laukens (Ghent University, Belgium). cDNA was prepared from 103 colon biopsies of 53 CD patients, 26 UC patients and 24 healthy controls and real time PCR was performed to quantify caspase expression levels in intestinal biopsies from those patients. UC/CD healthy or inflamed tissue was obtained from UC/CD patients with and without endoscopic evidence of inflammation. Control tissue was obtained from endoscopically normal biopsies from non-IBD control individuals. Mean relative expression values ($\log_{10}$) were graphed for each of the inflammatory caspases (Fig. 5.1). As expected, caspase-1 expression was significantly elevated in colonic tissue samples from inflamed UC/CD patients compared to healthy tissue from non-IBD controls and IBD healthy patients (Fig. 5.1A). Both caspase-4 and caspase-5 expression was also significantly increased in inflamed colonic tissue, when compared to non-IBD controls and IBD healthy patient tissue (Fig. 5.1 C, E). These data indicate that, in addition to caspase-1, caspases-4 and -5 may also have a role during intestinal inflammation. Interestingly, the data shown in (Fig. 5.1 C, D) also revealed that caspase-4 expression is higher in non-inflamed tissue from IBD patients (in both ileum and colon), suggesting that it may be an early marker of IBD. In contrast, both caspase-1 and -5 expression was significantly higher in inflamed colonic, not ileal, tissue (Fig. 5.1 A, B, E and F), suggesting their potential involvement with colitis, rather than ileitis (inflammation of the ileum). Taken together, these data support the hypothesis that inflammatory caspases-1, -4 and -5 may have an important role in intestinal inflammation observed during IBD.

5.2.2 Inflammatory caspase expression in IBD (Irish cohort)

5.2.2.1 Correlation between clinical disease and inflammation scores in UC patients

To follow on from the aforementioned results and to confirm our hypothesis that inflammatory caspase-1, -4 and -5 are involved in intestinal inflammation, we examined
the relationship between inflammatory caspase expression and both inflammation and disease activity in UC patients from an Irish cohort. Disease activity (Mayo) scores were prospectively assigned to IBD patients by the physician at the time of endoscopy, and biopsies from 36 UC patients with Mayo scores ranging from 0 to 3 (i.e. normal/inactive disease to severe disease) were coded and sectioned for histological assessment. The inflammation score was assigned by a pathologist and graded on the basis of immune cell activity: (0) no activity; (1) mild activity; (2) moderate activity; and (3) strong activity. The Mayo scores were graphed against inflammation scores, and the data obtained from this analysis clearly demonstrate a strong correlation \( (r_s=0.636, p<0.0001) \) between the clinical Mayo scores and the histological inflammation scores (Fig.5.2), confirming the validity of both scoring systems.

5.2.2.2 IHC analysis of inflammatory caspase expression in UC patients

To successfully perform the IHC staining, it was important to determine an optimal antibody dilution which would result in specific staining with the strongest possible intensity, without generating background staining. Therefore, there was a need to optimise the selected anti-caspase antibodies prior to staining the IBD patient samples. All IHC working antibody dilutions were optimised using a routine three-stage immunoperoxidase labelling technique incorporating avidin-biotin immunoperoxidase complex (Dako) (as described in materials and methods section 2.6.2). IHC antibody concentration optimisation was carried out on colorectal cancer test tissue blocks. The following anti-caspase dilutions: (anti-caspase-1, 1:150; anti-caspase-4, 1:25 and anti-caspase-5, 1:75) were chosen and resulted in successful staining of IBD patient samples (Fig.5.3). IHC stained tissue samples (using the above optimised antibody dilutions) from UC patients were double scored blindly (by Dr. Liz Ryan and I) to assess caspase-1, -4 and -5 expression patterns using a semi-quantitative scoring system (as outlined in materials and methods section 2.6.2). The results obtained from this study indicated that although caspase-1 was expressed at low levels in non-inflamed tissue, it was strongly expressed in both epithelial cells and infiltrating immune cells (both in nuclei and cytoplasm) of the lamina propria in inflamed patient tissue (Fig. 5.4A). A comparison of caspase-1 IHC scores revealed a weak correlation with inflammation \( (r_s=0.294, p<0.0001) \) and endoscopic Mayo \( (r_s=0.294, p<0.0001) \) scores, revealing that caspase-1
appears to be maximally expressed once inflammation begins and is sustained during moderate and strong inflammation (Fig. 5.4B and C). In contrast to caspase-1, caspase-4 and -5 were only expressed in the infiltrating immune cells (cytoplasm) within the lamina propria. Neither caspase-4 or -5 were detectable within epithelial cells of patients with UC, regardless of the degree of inflammation (Fig. 5.5A and 5.6A). Expression of caspase-4 in the lamina propria clearly correlated with the extent of inflammation ($r_i: 0.502, p<0.0003$) (Fig. 5.5B) and Mayo score ($r_i: 0.504, p<0.0002$) (Fig. 5.5B,C), with maximum caspase-4 expression present in sections with inflammatory and Mayo scores from moderate to strong (scores of 2 and 3). Caspase-5 IHC staining intensity and percentage expression within the lamina propria correlated with inflammatory scores up to moderate levels ($r_i: 0.422, p<0.003$) (Fig. 5.6B) and minimal to mild Mayo scores ($r_i: 0.473, p<0.0007$) (Fig. 5.6C). In addition, caspase-4 and -5 IHC positivity was identified in macrophage, neutrophil and lymphocyte populations within the lamina propria of UC patients, by an experienced clinical histopathologist. Overall, these data indicate that inflammatory caspase-1, -4 and -5 may have a role in IBD-associated intestinal inflammation.

5.2.3 Caspase inhibition reduces inflammatory cytokine secretion from ulcerative colitis (UC) patient biopsies

To further support our hypothesis that inflammatory caspases have a direct role in the intestinal inflammation observed during UC, biopsy tissue from healthy or UC patients with varying degrees of inflammation were *ex vivo* incubated in the presence or absence of the caspase-4 inhibitor, YVAD.fmk. Western blotting revealed that caspase-1 expression was increased gradually from healthy and normal tissues with the strongest expression in patient samples with mild disease. This observation nicely supports our previous results (Fig. 5.4C) where caspase-1 expression across the Mayo scores showed a similar pattern. Interestingly, Western blotting analysis showed low caspase-4 levels in normal, healthy and mild biopsy tissue, with significantly elevated caspase-4 expression in UC patients with severe disease (Fig. 5.7A). However, minimal caspase-5 expression was detected in all UC biopsy tissues, suggesting its low expression in these samples. It has been reported that enhanced levels of proinflammatory cytokines such as IL-6 and IL-1β in colonic biopsies of UC and CD patients play important roles in
exacerbating intestinal inflammation during IBD (Reinecker et al., 1993). Therefore, we set out to further assess if caspase inhibition can affect production of these cytokines in IBD patients biopsies. ELISAs from biopsy culture supernatants showed that caspase inhibition resulted in reduced levels of IL-1β and IL-6 production from the healthy and severe biopsy tissues (Fig. 5.7B and 5.7C). Thus, data from this study further reveal an involvement for caspase-4 and, to a lesser extent, caspases-5 and -1 in UC-associated inflammation. However, additional studies need to be performed to further investigate these preliminary findings.

5.2.4 Inflammatory caspase expression in colitis associated cancer (CAC) tissues (Irish cohort)

There is a strong body of evidence that long standing ulcerative colitis carries a significant risk for the development of colitis associated colorectal cancer (CAC) (Rubin et al., 2013). Having demonstrated the upregulation of expression of inflammatory caspases during chronic inflammation in UC patients in this study, we wanted to further determine whether their expression is also elevated in CAC patient tissues. Therefore, a set of UC-associated CRC patient resections were examined in areas of normal adjacent, inflamed and tumour tissue for inflammatory caspase expression. Scoring of the lamina propria in tissue from these patients had similar expression profiles to those previously observed in UC patients (Fig. 5.5-5.7). Furthermore, in CAC patients, there was a significant increase of caspases-4 and -5 expression in the lamina propria of both inflamed and tumour tissue compared to normal adjacent tissue (Fig.5.8A and C). Strikingly, epithelial expression of caspases-4 and -5 were dramatically restricted to neoplastic tissue, even within areas of severely inflamed tissue. Areas of normal and inflamed tissue from CAC patient resections all remained completely negative for epithelial caspase-4 and -5 expression (Fig. 5.8B and D). In contrast, caspase-1 was expressed at high levels in epithelium and the lamina propria of normal, inflamed and tumour tissue, making it less specific to the inflammatory/malignant status of the tissue (Fig. 5.8C and D). Similar to observations from UC patients, caspase-4 and -5 staining was identified (by a clinical pathologist) in infiltrating macrophages and lymphocytes in areas of inflamed/dysplastic tissue. These findings indicate that caspases-4 and -5 are discriminators between inflamed and
neoplastic tissue, and suggests that there may be significant scope for the detection of epithelial caspase-4 and -5 as determinants of malignancy within colorectal mucosa.

The above IHC analysis of inflammatory caspase expression in tissue samples from an Irish cohort of CAC patients was carried out by Brian Flood (Emma Creagh Laboratory, TCD) in collaboration with Prof. Elaine Kay, Drs. Tony O'Grady, Katherine Sheehan and Joanna Fay (Beaumont Hospital Dublin and Royal College of Surgeons in Ireland).

5.2.5 Inflammatory caspase expression in tissues of Barrett's metaplasia and oesophageal adenocarcinoma (OAC) patients (Irish cohort)

So far, our results have demonstrated: (i) a clear correlation between inflammatory caspase-4 and -5 expression in the lamina propria and the level of tissue inflammation and the grade of disease activity in UC patients, thus indicating their potential role in disease pathogenesis; (ii) significant overexpression of caspase-4 and -5 in the epithelia of the neoplastic tissue of CAC patients, implicating them as potential specific biomarkers for early detection of premalignant changes in the colon. To determine whether these observations may also be valid in other tissue within the GI tract, and owing to the fact that there are no reports of studies examining the role of inflammatory caspases in pathogenesis of BO and OAC, we hypothesised that caspase-1, -4 and -5 may also play a role in inflammation associated with BO and OAC development. We also wanted to assess if the specific epithelial caspase expression occurring in CAC tissue, may also appear during BO progression to OAC and serve as potential diagnostic biomarker for these diseases in the future.

In order to test our hypotheses, tissue microarrays (TMA) of adjacent normal, Barrett's oesophagus (metaplasia) and oesophageal adenocarcinoma (tumour) patient resection tissues were obtained from the oesophagectomy tissue of 32 patients diagnosed with oesophageal adenocarcinoma (OAC). The TMAs were IHC stained with optimised anti-caspase antibodies (as outlined in materials and methods section 2.6.3). After successful IHC staining, the TMAs were scored to examine caspase expression patterns using a validated semi-quantitative scoring method (as outlined in 2.6.3 section). Similar to UC and CAC findings (demonstrated in previous sections), the results obtained from this study revealed that caspase-1 is also expressed in both
epithelial cells and infiltrating immune cells (both in nuclei and cytoplasm) of the lamina propria in normal, Barrett's metaplasia (inflamed) and tumour tissue. As expected, caspase-1 was expressed at low levels in non-inflamed tissue and at marked increased levels in metaplastic and tumour tissues (in both stromal and epithelial cells), suggesting its involvement in BO and OAC associated inflammation (Fig.5.9). Contrary to our expectations and previous UC and CAC findings, caspase-4 and -5 were expressed in both epithelial and stromal cells within normal, metaplastic and OAC tumour tissues (Fig.5.10 and 5.11). Moreover, caspase-4 levels appeared to be elevated in Barrett’s metaplasia tissues compared to normal and tumour tissues in both epithelial and stromal cells, however, these changes were not significant, suggesting that caspase-4 plays a minor role in driving BO associated inflammation. Strikingly, epithelial and stromal caspase-5 expression was increased from normal, through metaplastic tissues, reaching its highest levels in tumour tissue (Fig.5.11). Despite the similar trends in caspase-5 expression patterns in both epithelial and stromal cells only epithelial caspase-5 levels were significant (5.11 B), suggesting its involvement and function in the pathogenesis of BO and OAC are more specifically within epithelial cells rather than stromal cells. In addition, these data indicate that caspase-1 and -5, rather than caspase-4 may play important roles during BO-associated chronic inflammation and its progression to OAC.

5.2.6 Analysis of the inflammatory caspase expression in OAC tumour tissues

After analysing caspase expression patterns across normal-metaplasia adenocarcinoma disease sequence in OAC patients, and also having access to the characteristics of OAC patients and tumours as outlined in Table 5.1, we wanted to further investigate caspase expression profiles more specifically: (i) within different stages of tumour progression; (ii) within various differentiation stages of oesophageal adenocarcinoma; and (iii) in patients that underwent neoadjuvant therapy.

Firstly, the caspase expression patterns were examined within the various tumour stages in OAC patient cohort, assigned by a pathologist according to the AJCC TNM staging system (Rice, 2015). The TNM staging system is most often used by doctors to stage cancer in order to assess patient prognosis and choose the appropriate
treatment. One of the key criteria of this system to be examined is the T category, which gives information about aspects of the original (primary) tumour, such as its size, how deeply it has grown into the organ it started in, and whether it has grown into nearby tissues. Most oesophageal cancers start in the innermost lining of the oesophagus (the epithelium) and then grow into deeper layers over time. In this study the T categories were assigned to OAC patient cohort as follows: T1 - cancer invades layers under the epithelium such as lamina propria, muscularis mucosae or submucosa; T2 - cancer invades the thick muscle layer, the muscularis propria and T3 - cancer invades the outer layer of the oesophagus, the adventitia. IHC analysis of stromal caspase levels in those three categories revealed no major difference in caspase-1, -4 and -5 expression across T1, T2 and T3 stages (Fig. 5.12). Although there was a lack of difference in epithelial caspase-1 and -4 levels within all T categories, epithelial caspase-5 levels were significantly reduced in advanced adenocarcinomas (T3) compared to less advanced (T1, T2) tumours (Fig. 5.12). The reason for the significant reduction of caspase-5 expression in T3 tumours remains unclear and this observation needs to be further confirmed in a bigger patient cohort. However, due to the fact that most of patients with T3 tumours have been undergoing chemoradiotherapy treatment, the assumption can be made here that the reduced caspase-5 levels of patients in advanced stage adenocarcinomas could be due to positive response to cancer therapy.

We also assessed caspase expression patterns in defined differentiation grades of OAC patient tissues (assigned by a pathologist, using the AJCC TNM staging system mentioned above). Differentiation or grade of the cancer is based on how abnormal the cells and tissue look under the microscope and can be a useful indicator in predicting how aggressive the cancer is (how fast it may grow and spread) and it also facilitates accurate treatment choice. According to the AJCC system, oesophageal adenocarcinomas are graded as well differentiated (G1 or low-grade), moderately differentiated (G2 or intermediate-grade) and poorly differentiated (G3 or high-grade) based on the degree of glandular differentiation. In general, well differentiated adenocarcinomas (G1) are less likely to spread and invade surrounding tissue and they are more responsive to cancer treatment. This is mainly due to the fact that the cells are well differentiated in an early stage. However, poorly differentiated adenocarcinomas (G3) are more likely to be more aggressive and tend to divide rapidly and invade surrounding tissues and organs. In this study, IHC analysis of caspase-1, -4 and -5

148
patterns in stromal cells of OAC patients revealed that there was a dramatic significant increase in all inflammatory caspase expression levels in invasive poorly differentiated adenocarcinoma tissues compared to poorly differentiated adenocarcinoma samples (Fig. 5.13, D-F and G-L ). Caspase levels in stroma appeared to be also elevated in invasive moderately differentiated adenocarcinomas compared to moderately differentiated adenocarcinoma tissue of OAC patients, however, these observations were lacking in significance values (Fig 5.13, D-F). In contrast, there were no meaningful changes in caspase expression across the low-intermediate-high adenocarcinoma grades in epithelial cell layers (Fig.5.13, A-C). Overall, these findings might suggest that proinflammatory caspases expressed in stromal cells may facilitate invasion of high grade adenocarcinomas, however further studies are required to authenticate this hypothesis.

Lastly, we examined whether OAC patients in receipt of neoadjuvant (NAT) chemoradiation therapy showed any alterations in caspase expression levels. The main aim of combining neoadjuvant chemotherapy and radiotherapy is to exploit the radiosensitising effects of chemotherapy, to reduce the tumour size and maximise local control (Herskovic et al., 1992). Neoadjuvant chemoradiation therapy followed by surgery is increasingly the standard of care for oesophageal cancer patients treated with curative intent (Shapiro et al., 2015). Examination of both the underlying molecular mechanisms and biomarkers of resistance to therapy is central to improving the efficacy of treatment and survival of OAC patients. Analysis of IHC staining revealed that epithelial caspase-1 and -5 levels were significantly elevated in OAC patients after receiving NAT therapy compared to OAC patients without NAT treatment. In contrast, stromal caspase-1 and -5 expression levels appeared to be reduced in patients with NAT treatment compared to those without NAT treatment. Interestingly, both epithelial and stromal caspase-4 expression patterns remained unchanged before and after NAT treatment (Fig.5.14). Overall, these findings revealed that NAT treatment appears to effect caspase-1 and -5 expression levels in stromal and epithelial cells, suggesting their sensitivity to the treatment.
5.3 Discussion

Results from this chapter clearly demonstrate significantly enhanced expression of inflammatory caspases-1, -4 and -5 in patient biopsies of IBD and Barrett’s oesophagus; and also in tissue samples of CAC and OAC patients, implicating a role for the pro-inflammatory caspases in pathogenesis of these diseases. To our knowledge, this is the first study to examine caspase-4 and -5 expression levels in the context of these chronic inflammatory diseases and their progression to cancers.

Initial data obtained from a pilot study of Belgian IBD patients demonstrated that inflammatory caspase-1, -4 and -5 mRNA levels were elevated in inflamed UC and CD patient biopsies compared to healthy tissue samples (Fig.5.1). In addition there was greater caspase-4 expression in non-inflamed IBD patient biopsies compared to healthy tissue which suggests that caspase-4 may be an early marker for this disease (5.1C). The preliminary data from the Belgian study was supported by results obtained from IHC analysis of tissue biopsies from Irish UC patients, which also showed enhanced caspase expression levels in inflamed UC tissues (Fig.5.4-5.6). However, the observation showing elevated caspase-4 mRNA levels in healthy colon tissue from IBD patients (Fig. 5.1C) was not supported by IHC scoring of caspase-4 protein levels in UC patients (Fig. 5.5C), thus, whether basal caspase-4 expression is elevated in healthy IBD mucosa compared with that of healthy, disease free patients’ needs be clarified in future experiments. In agreement with our findings revealing elevated caspase-4 and -5 levels in IBD patients, a recent study by Williams et al based on gene expression metadata analysis showed that CASP4 and CASP5 expression is significantly upregulated in colon biopsies of UC patients with active disease compared to those in remission (Williams et al., 2015). Hence, our combined findings highlight the potential importance of these caspases in regulating intestinal inflammation in UC patients. Furthermore, in our study, caspase expression, particularly caspase-4 and -5, correlated very closely with the level of tissue inflammation and the grade of disease activity in UC patients, suggesting that their enhanced expression may play a role in UC pathogenesis (Fig.5.5 and 5.6). Based on these findings we propose that caspase 4 and 5 expression show potential to serve as good markers of the UC disease progression.

To further extend the preliminary data, it would be worth elucidating caspase-4 and -5 expression profiles (via RT-PCR) in blood or stool samples of healthy and UC
patient cohorts (with various grades of inflammation and Mayo scores). If these results were to confirm our present observations, this data could help facilitate the design of a diagnostic test for the UC disease progression. Moreover, IHC analysis of UC cohort also revealed that inflammatory caspases are highly expressed within infiltrating immune cells in the stroma of IBD patients. In particular, macrophages, neutrophils, lymphocytes and plasma cells were all identified as being caspase-4 and -5 positive. A strong correlation between inflammation score and caspase-4 and -5 expression observed in UC patient biopsies, supports the hypothesis that caspases-4 and -5 contribute to intestinal inflammation, most probably through activation of the non-canonical inflammasome, leading to increased IL-1β and IL-18 production (Kayagaki et al., 2011; Rathinam et al., 2012). To support this hypothesis, we have seen that in UC colonic explants biopsy cultures inhibition of human caspase-4 led to decreased IL-1β levels in those samples. In addition, we also noted attenuated levels of IL-6, which may suggest that caspase-4 can mediate IL-6 production during intestinal inflammation. This observation can be linked to a previously reported role for caspase-4 in mediating LPS-induced NF-κB dependent cytokine production (including IL-6) in vitro (Lakshmanan and Porter, 2007). However, some researchers argue that caspase-4 specific properties of the Z-YVAD inhibitor are inaccurate as it has been previously been reported that this inhibitor also blocks caspase-1 activity (Bian et al., 2009).

In order to further investigate these preliminary results, it would be worth performing extra studies using a bigger number of patients; secondly-it would be worth optimising the efficient transfection technique for using anti-caspase-4 and -5 siRNAs in UC explant cultures. These future mechanistic studies may provide more specific data on caspase-4 and -5 functions during pathologic inflammation in UC patients. Although evidence for the importance of caspase-4 and -5 mediated non-canonical inflammasome activation during human inflammation is emerging (Baker et al., 2015; Schmid-Burgk et al., 2015; Sollberger et al., 2012), the majority of studies to date characterizing non-canonical inflammasome activation have been carried out in mice. Recent reports have demonstrated a protective role for the non-canonical inflammasome during a murine model of acute colitis (Demon et al., 2014; Oficjalska et al., 2015; Williams et al., 2015). Here, we provide evidence that increased levels of human caspase-4 and -5 mediated non-canonical inflammasome activation during chronic intestinal inflammation may contribute to the disease pathogenesis/progression. To
further support our findings regarding a potential deleterious role for caspase-4 and -5 during inflammatory human disease, Simpson et al have implicated increased caspase-4 and -5 mRNA levels in regulation of pathological inflammation, possibly through the overproduction of IL-1β in patients with neutrophilic asthma (Simpson et al., 2014). Moreover, enhanced caspase-5 mRNA expression was found in skin biopsies of patients with psoriasis, suggesting its role in the inflammatory processes occurring during chronic inflammatory skin diseases (Salskov-Iversen et al., 2011).

The majority of the aforementioned conclusions implicating a pro-inflammatory role for caspase-4 and -5 during intestinal inflammation in IBD patients in this study are mainly based on expression pattern analysis. However, it is also worth mentioning that observed enhanced caspase-4 and -5 levels in IBD patient tissues may be a result of their involvement in negative regulatory responses during intestinal inflammation. Today, it appears that dysregulation of expression of TLR negative regulators play a dominant role during inflammation associated with IBD. To date, several of the TLR inhibitory proteins (such as SIGIRR, A20, PPARγ, IRAK-m and Tollip) have been found to be strongly expressed in the GI tract and have been shown, predominantly using murine models, to functionally suppress both TLR responses and intestinal inflammation (Shibolet and Podolsky, 2007). In support of the important role of TLRs inhibitors in intestinal inflammation, it has been shown that SIGIRR and A20 deficient mice exhibited increased susceptibility to colitis (Vereecke et al., 2010; Xiao et al., 2007). Moreover, a recent study by Fernandes et al revealed significantly increased expression patterns of some of the TLR negative regulators in the active IBD population. In this study, it was shown that expression of A20 and SOCS1 was increased in colonic tissues of UC patients while expression of IRAK-m and Bcl-3 was enhanced in both UC and CD patients (Fernandes et al., 2015). These proteins are known to be induced by transcription factors, including NF-κB, which are activated by inflammatory signal transduction pathways. Thus, it was speculated that the upregulation of inhibitory proteins is a positive feedback mechanism to reduce the IBD-related inflammation. Therefore, it can be hypothesised here that enhanced caspase-4 and -5 expression observed in this study may also be a result of a positive feedback response to the dampening down of inflammatory responses during inflammation in IBD.
Collectively, these findings highlight the significance of caspases 4 and 5 in regulation of inflammation and underscore the importance of additional studies to better elucidate their functions in human inflammatory diseases.

Having demonstrated the upregulation of caspase-4 and -5 levels in IBD patients, we further evaluated the involvement of these caspases in colitis associated cancer. Similar to observations in IBD patients, increased stromal caspase-4 and -5 levels were also detected in inflamed sections of CAC tissues (Fig. 5.8). An absence of epithelial caspase-4 and -5 positive staining in normal and inflamed sections of CAC tissue was also observed, which was again in agreement with the IBD study (Fig. 5.6 and 5.7). In addition, a recent study by Williams et al revealed that CASP4 and CASP5 levels were significantly upregulated in biopsies from CAC patients (Williams et al., 2015). Therefore, these findings give strong evidence for the involvement of caspase-4 and -5 during colorectal inflammation and carcinoma. Furthermore, in our study, we also found a dramatic transition in the expression patterns of caspase-4 and -5 in epithelial cells, from no expression in normal and inflamed tissues to strong expression in neoplastic tissue of CAC patients (Fig. 5.8). The same observation was also noted for sporadic CRC patient tissues. These results are demonstrated in our recently published report, which is also attached to this thesis (Flood et al., 2015). The mechanistic details of how and when the switch to epithelial expression of caspase-4 and -5 occurs are still unclear and need further examination. The fact that caspase-4 and -5 were found to be overexpressed in epithelia of both CAC and CRC tissue but not in inflamed tissue suggests these epithelial-expressed caspases may serve as potential biomarkers for early detection of colorectal carcinoma.

As previously mentioned, dysplastic lesions associated with IBD-CAC are difficult to grade, and distinguishing between neoplastic and chronically inflamed tissue represents a major challenge for pathologists, thus our findings may be relevant for the developing a diagnostic test for CAC in the future. Our studies have shown that within tumour areas, stromal staining for caspases-4 and -5 was even stronger than that observed in inflamed tissue, and epithelial cells became strongly positive for caspase-4 and -5 (Fig.5.8 A,C). This suggests that inflammatory cells may have a role in inducing the expression of caspase-4 and -5 in malignant epithelial cells. This concept is plausible since it’s widely accepted that infiltrating immune cells are involved in the development of epithelial-originated tumours and can also promote tumour growth,
particularly during inflammation associated cancer (Lu et al., 2006; Smyth et al., 2004). However, caspase-4 and -5 expressed in stromal infiltrating immune cells may have a different role in epithelial cells. Previous analysis of the role of NF-κB, in a mouse model of CAC revealed that this inflammatory transcription factor can drive tumourigenesis via two distinct mechanisms involving stimulation of pro-inflammatory cytokines by infiltrating cells and prevention of death of IECs with tumourigenic potential (Greten et al., 2004). Caspase-4 has been shown previously to have a role in LPS-mediated NF-κB activation (Lakshmanan and Porter, 2007). Thus, it is possible that caspases-4 and -5 may be responsible for driving tumour initiation by preventing the death of IECs during pathogenesis of CAC and CRC. However, extra mechanistic studies are required to evaluate the exact roles for these caspases in the colorectal cancer development. For example it would worth performing a panel of caspase-4 and -5 knockdowns *in vitro* studies using various colon adenocarcinoma cell lines, to determine if/how these caspases have an effect on the apoptosis/cell death or growth in these cells.

In light of these results, which imply the inflammatory caspases as specific biomarkers with diagnostic and therapeutic potential in IBD and CRC, we hypothesised that similar observations could be made in BO and OAC patients. Contrary to our expectations, all three caspases were expressed in both epithelial and stromal cells of normal, Barrett's metaplasia and oesophageal adenocarcinoma (Fig.5.9-5.11). Therefore, these results highlight unique caspase-4 and -5 expression status (stromal cells-normal/inflamed tissue; and epithelial cells- tumour tissue) for UC/CAC/CRC patients. IHC analysis of the oesophagectomy tissues of OAC patients revealed increased epithelial and stromal caspase-1 expression in metaplastic and tumour biopsies compared to normal adjacent tissue, indicating a role for caspase-1 in pathogenesis of BO and OAC (Fig.5.9). The exact role of caspase-1 during development of these diseases still remains unknown and merits further investigation. However, it has been reported that IL-1β is highly upregulated in human BO and OAC (Fitzgerald et al., 2002a). Moreover, overexpression of IL-1β in a mouse model of oesophageal inflammation led to the development of oesophageal metaplasia, dysplasia and eventually OAC (Quante et al., 2012). Thus, we can hypothesise that increased caspase-1 expression observed in BO and OAC patient biopsies in this study may contribute to the pathogenesis of these diseases by enhancing IL-1β activation.
Among all three inflammatory caspases, epithelial caspase-5 expression appeared to be significantly enhanced from normal, through metaplastic tissues with the highest levels in tumour samples (Fig.5.11), suggesting that this caspase may play an important role in the BO development and its progression to OAC cancer. It has been shown that cell hyperproliferation is a key characteristic during the neoplastic transformation of Barrett’s mucosa. Numerous studies have shown that increased cell proliferation status in oesophageal biopsies is routinely processed by IHC staining with the proliferation markers such as PCNA and Ki67 (Hong et al., 1995; Illueca et al., 2000; Volkweis et al., 2012). Moreover, we have shown that caspase-11 (murine, homolog of caspase-4 and -5) is involved in induction of cell proliferation in vivo. Therefore, we can hypothesise that caspase-5 may enhance cell proliferation and facilitate the neoplastic development of Barrett’s oesophagus. To assess this hypothesis, it is planned to perform Ki67 IHC staining on oesophagectomy tissues of OAC patients (which were previously stained with anti-caspase antibodies) and check for a positive correlation between caspase-5 expression and cell proliferation status in these tissues. In addition, it may also be worth performing in vitro studies using a panel of various oesophageal adenocarcinoma cell lines to examine whether caspase-5 can influence cell proliferation when overexpressed in these cells. With regard to caspase-4, it appears that this caspase may not play a major role in BO and OAC, as the majority of data obtained was insignificant (Fig 5.10).

We also examined caspase expression within different types of oesophageal adenocarcinoma. IHC staining analysis revealed dramatically elevated caspase-1, -4 and -5 expression levels in stromal cells of invasive poorly differentiated adenocarcinomas (Fig.5.13). It has been reported that caspase-1, -4 and -5 are involved in IL-1β activation (Baker et al., 2015; Dinarello, 2009), thus it might be assumed here that these caspases can facilitate invasion of high grade adenocarcinomas of the oesophagus through enhanced IL-1β activation. To support this hypothesis, it has been reported that IL-1β promotes tumour invasiveness and facilitates metastasis through mechanisms including tumour angiogenesis promotion and induction of inflammatory molecules such as matrix metalloproteinases (MMPs) and adhesion molecules on malignant cells or endothelial cells, leading to tumour dissemination and metastasis (Apte et al., 2006; Petrella and Vincenti, 2012; Vidal-Vanaclocha et al., 2000). Thus, inflammatory caspases may enhance expression of MMPs or members of the vascular endothelial
growth factor (VEGF) family, whose overexpression patterns have already been shown to correlate with cancer invasion in OAC patients (McDonnell et al., 2003). Hence, it would be prudent to stain a bigger cohort of TMAs invasive/non-invasive poorly differentiated adenocarcinomas tissues with anti-IL-1β, anti-VEGF, anti-MMPs and see how their expression correlate with the caspase levels in those tissues.

Interestingly, we also observed that both caspase-1 and -5 have similar expression patterns in response to NAT therapy. Both caspase levels were elevated in the epithelium but attenuated in the stroma of tumour tissue after NAT treatment (Fig.5.14). This could suggest that these caspases may have different functions within stromal and epithelial cells. The typical neoadjuvant treatment for oesophageal cancer is based on chemotherapeutic drugs (e.g. fluoroaracil and cisplatin), whose main anticancer/cytotoxic effect involves induction of apoptosis in tumour cells (Campbell and Villaflor, 2010; Jordan and Carmo-Fonseca, 2000). It has been demonstrated that caspase-1 can act as an efficient chemo-sensitiser to cisplatin treatment by enhancing the cisplatin-induced mitochondrial apoptotic pathway activation in vitro (following caspase-1 transfection in HeLa cells). It has been also shown that caspase-1 is a good chemo- and radio-sensitiser in cervical carcinomas (HeLa-induced tumours) in vivo (Martin-Duque et al., 2006). Therefore, we can hypothesise that increased levels of caspase-1 and -5 in epithelial cells may be due to their induction by cisplatin to enhance apoptosis in tumour cells. In our recent published report we suggested that increased caspase-4 and -5 expression in infiltrating cells of CAC patients can contribute to tumour development (Flood et al., 2015). Therefore, it can be conjectured here that decreased levels of caspase-1 and -5 in stromal cells may be associated with caspase sensitiveness to the NAT chemoradiation therapy. However, a role for caspase in chemo/radio resistance and sensitiveness in OAC cancer needs to be further investigated.

Overall, our findings from the IBD/CAC study demonstrate that increased expression of caspase-4 and -5 may indicate their role in inflammation of IBD patients, implicating these caspases as potential modulators of intestinal inflammation. Moreover, we have also shown that specific epithelial caspase-4 and -5 expression in neoplastic tissue can serve as highly specific biomarker of colorectal carcinoma. In regards to Barrett’s oesophagus and OAC study we have shown that caspase -1 and -5 are involved in pathogenesis of BO and OAC. It has been also demonstrated that
caspase-1, -4 and -5 may play a role in the invasion of poorly differentiated adenocarcinoma of the oesophagus.
Figure 5.1: Increased inflammatory caspase gene expression in IBD patient biopsies from a Belgian cohort

Relative expression of (A, B) CASP1, (C,D) CASP4 and (E,F) CASP5 in biopsy specimens from non-IBD healthy control (n=21), active ulcerative colitis (UC) patients in endoscopically healthy (n=10) and inflamed (n=11) areas, and active Crohn’s disease (CD) patients in healthy (n=38) and inflamed (n=21) colon and ileum as quantified by qPCR. Data are normalized to the median of 3 stably expressed reference genes and represent median and range; *p < 0.05; **p < 0.01; ***p < 0.001 (Kruskal-Wallis test with Dunn’s post hoc comparison to control).
Figure 5.2: Strong correlation ($r_s = 0.636$, $p < 0.0001$) between clinical Mayo and pathological inflammation scores in ulcerative colitis (UC) patient biopsies.

Intestinal biopsies were retrieved from 36 patients with a diagnosis of UC, with prospective Mayo score of endoscopic activity assigned by the physician at time of endoscopy: Mayo 0 (inactive, $n=11$); Mayo 1 (minimal, $n=7$); Mayo 2 (mild, $n=8$) and Mayo 3 (moderate to severe, $n=10$). Inflammation score assigned by a pathologist to UC patient was graded on a scale: 0 (no activity, $n=11$); 1 (mild activity, $n=7$); 2 (moderate activity, $n=9$) and 3 (severe activity, $n=9$). Correlation between Mayo and Inflammation scores were undertaken by calculating the nonparametric Spearman’s correlation coefficient, abbreviated $r_s$. Data represent mean ±SEM. $p$ values were calculated using the one-way ANOVA followed by Tukey post-test; *$p < 0.05$; ***$p < 0.001$ and ns (not significant).
Figure 5.3: Optimisation of anti-caspase-1, 4 and-5 antibody concentrations in immunohistochemical (IHC) staining

A routine three-stage immunoperoxidase labelling technique incorporating avidin–biotin immunoperoxidase complex (Dako) was used for IHC antibody optimisation. Representative images of optimised antibody concentrations of colorectal cancer test tissue blocks via IHC staining. (A) anti-caspase-1 (Santa Cruz, 1:150 dilution); (B) anti-caspase-4 (MBL, 1:25 dilution); (C) anti-caspase-5 (MBL, 1:75 dilution) and (D) negative staining was observed for isotype IgG control (negative control). Magnification x200.
Figure 5.4: Inflammatory caspase-1 expression shows a weak correlation with clinical disease ($r_s=0.294$, $p<0.0001$) and inflammation ($r_s=0.226$, $p<0.0001$) scores in ulcerative colitis (UC) patient biopsies from an Irish cohort.

IHC staining was performed using formalin-fixed, paraffin-embedded (FFPE) tissues obtained from 36 UC patients. All IHC-stained cells were assessed by a combined score of intensity and percentage of nuclear and cytoplasmic staining. (A) Representative images of caspase-1 expression pattern in epithelial cells (top panel) and stromal cells (bottom panel) from non-inflamed and inflamed UC biopsy sections. Negative staining was observed for isotype IgG control (negative control). (B) Caspase-1 expression levels versus histological inflammatory score for UC colonic biopsies. Inflammation score was graded on a scale: 0 (no activity, $n=11$); 1 (mild activity, $n=7$); 2 (moderate activity, $n=9$) and 3 (severe activity, $n=9$). (C) Caspase-1 expression levels versus endoscopic Mayo score. Disease activity (Mayo score) was graded as follows: 0 (inactive, $n=11$), 1 (minimal, $n=7$), 2 (mild, $n=8$) and 3 (moderate to severe, $n=10$). Magnification $\times 200$. Data represent mean $\pm$SEM. P values were calculated using the one-way ANOVA, followed by the Tukey post-test; **$p<0.01$; ***$p<0.001$. 


IHC staining was performed using formalin-fixed, paraffin-embedded (FFPE) tissues obtained from 36 UC patients. All IHC-stained cells were assessed by a combined score of intensity and percentage of nuclear and cytoplasmic staining. (A) Representative images of caspase-4 expression pattern in stromal cells from non-inflamed and inflamed UC biopsy sections. Negative staining was observed for isotype IgG control (negative control). (B) Caspase-4 expression levels versus histological inflammatory score for UC colonic biopsies. Inflammation score was graded on a scale: 0 (no activity, n=11); 1 (mild activity, n=7); 2 (moderate activity, n=9) and 3 (severe activity, n=9). (C) Caspase-1 expression levels versus endoscopic Mayo score. Disease activity (Mayo score) was graded as follows: 0 (inactive, n=11), 1 (minimal, n=7), 2 (mild, n=8) and 3 (moderate to severe, n=10). Magnification x200. Data represent mean ±SEM. P values were calculated using the one-way ANOVA, followed by the Tukey post-test *p < 0.05; **p < 0.01.
Figure 5.6: Inflammatory caspase-5 expression correlates with clinical disease ($r_s=0.473$, $p<0.0007$) and inflammation ($r_s=0.422$, $p<0.003$) scores in ulcerative colitis (UC) patient biopsies from an Irish cohort.

IHC staining was performed using formalin-fixed, paraffin-embedded (FFPE) tissues obtained from 36 UC patients. All IHC-stained cells were assessed by a combined score of intensity and percentage of nuclear and cytoplasmic staining. (A) Representative images of caspase-5 expression pattern in stromal cells from non-inflamed and inflamed UC biopsy sections. Negative staining was observed for isotype IgG control (negative control). (B) Caspase-5 expression levels versus histological inflammatory score for UC colonic biopsies. Inflammation score was graded on a scale: 0 (no activity, $n=11$); 1 (mild activity, $n=7$); 2 (moderate activity, $n=9$) and 3 (severe activity, $n=9$). (C) Caspase-1 expression levels versus endoscopic Mayo score. Disease activity (Mayo score) was graded as follows: 0 (inactive, $n=11$), 1 (minimal, $n=7$), 2 (mild, $n=8$) and 3 (moderate to severe, $n=10$). Magnification ×200. Data represent mean ±SEM. P values were calculated using the one-way ANOVA, followed by the Tukey post-test *$p<0.05$; ***$p<0.001$. 

*Figure 5.6: Inflammatory caspase-5 expression correlates with clinical disease ($r_s=0.473$, $p<0.0007$) and inflammation ($r_s=0.422$, $p<0.003$) scores in ulcerative colitis (UC) patient biopsies from an Irish cohort.*
Figure 5.7: Caspase inhibition attenuates inflammatory cytokine secretion from ulcerative colitis (UC) patient biopsies

Colonic explants biopsy cultures from healthy and UC patients [non-adjacent normal and inflamed tissue (mild and severe)] were incubated with caspase inhibitor YVAD.fmk [Z-YVAD-FMK (Z-Tyr-Val-Ala-Asp (OMe)-fluoromethylketone)] (10 μM) or vehicle control [dimethylsulphoxide (DMSO)] for 18 h. (A) 10 μg of protein lysate was probed for caspases-1 (45 kDa), caspase-4 (43 kDa) and caspase-5 (47 kDa) expression by Western blotting. Human acute monocytic leukaemia (THP1) cell lysate was used as a positive control for caspase expression. β-actin (42 kDa) served as a loading control. Secreted levels of (B) IL-1β and (C) IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) in culture supernatants from colonic explants. Results are representative of two independent experiments. Data represent mean ±SEM. P values were calculated with Student’s t-test; * p<0.05, ***p<0.001.
Figure 5.8: IHC staining of colitis-associated CRC (CAC) patient resection tissue confirms tumour-specific epithelial expression of caspases-4 and -5

Representative images of caspases-1, -4 and -5 expression patterns in normal, inflamed and tumour (A) stromal and (B) epithelial tissue from CAC patient biopsies. Defined areas of: normal (N) (n=6); inflamed (I) (n=7); and tumour (T) (n=8) tissue from CAC patient biopsies were immunohistochemistry (IHC) scored for caspases-1, -4 and -5 expression in (C) stromal; and (D) epithelial regions. Data represent mean ± SEM. P values were calculated using the one-way ANOVA followed by the Tukey post-test *p < 0.05, ***p < 0.001; scale bar = 50 μm. This work was carried out by B. Flood.
Figure 5.9: Increased caspase-1 expression in areas of inflammation (metaplasia) and tumour in oesophagectomy tissue from adenocarcinoma (OAC) patients

Representative images of caspase-1 expression pattern in epithelial cells (top panel) and stromal cells (bottom panel) from within defined areas of adjacent normal, Barrett's oesophagus (metaplasia) and oesophageal adenocarcinoma (tumour) patient resection tissues. Defined areas of adjacent normal, intestinal metaplasia and tumour tissue from OAC patient sections were IHC scored for caspase-1 expression in epithelial (B) and (C) stromal cells using a validated semi-quantitative scoring method. Data represent mean ± SEM. Magnification ×200. P values were calculated using the one-way ANOVA followed by the Tukey post-test; *p < 0.05, ***p < 0.001. Caspase-1 (n=32 patients per each group).
Figure 5.10: Increased caspase-4 expression in inflamed (metaplasia) areas of oesophagectomy tissue from oesophageal adenocarcinoma (OAC) patients

Representative images of caspase-4 expression pattern in epithelial cells (top panel) and stromal cells (bottom panel) from within defined areas of adjacent normal, Barrett's oesophagus (metaplasia) and oesophageal adenocarcinoma (tumour) patient resection tissues. Defined areas of adjacent normal, intestinal metaplasia and tumour tissue from OAC patient sections were IHC scored for caspase-4 expression in epithelial (B) and (C) stromal cells using a validated semi-quantitative scoring method. Data represent mean ± SEM. Magnification ×200. Caspase-4 (n=32 patients per each group).
Figure 5.11: Increased caspase-5 expression in inflamed (metaplasia) and tumour areas of oesophagectomy tissue from oesophageal adenocarcinoma (OAC) patients

Representative images of caspase-5 expression pattern in epithelial cells (top panel) and stromal cells (bottom panel) from within defined areas of adjacent normal, Barrett's oesophagus (metaplasia) and oesophageal adenocarcinoma (tumour) patient resection tissues. Defined areas of adjacent normal, intestinal metaplasia and tumour tissue from OAC patient sections were IHC scored for caspase-1 expression in epithelial (B) and (C) stromal cells using a validated semi-quantitative scoring method. Data represent mean ± SEM. Magnification ×200. P values were calculated using the one-way ANOVA followed by the Tukey post-test; *p < 0.05, ***p < 0.001. Caspase-5 (n=32 patients per each group).
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>NAT (Y/N)</th>
<th>Differentiation</th>
<th>Grade</th>
<th>Lymph node</th>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>pT1aN0Mx</td>
<td>17 nodes negative</td>
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</tbody>
</table>

Table 5.1: Characteristics of oesophageal adenocarcinoma (OAC) patient cohort and tumours examined for caspase-1, 4 and -5 expression by immunohistochemistry (IHC)
Figure 5.12: Inflammatory caspase expression analysis across tumour grades reveals reduced epithelial caspase-5 levels in advanced stage tumours

Tumour tissue from OAC patient sections were IHC scored for caspase-1, -4, -5 in epithelial (A, B, C) and stromal (D, E, F) regions were graphed according to their tumour stage. Tumour stages were assigned by a pathologist with TNM (Tumour-Node-Metastasis) clinical staging classification (Rice, 2015). T refers to the size or direct extent of the primary tumour and was further categorised as follows: (T1-tumour has grown no further than the layer of supportive tissue, n=6; T2-tumour has grown into the muscle layer of the wall of the oesophagus, n=4 and T3 –tumour has grown into the membrane covering the outside of the oesophagus, n=14). Data represent mean ± SEM. P values were calculated using the one-way ANOVA followed by the Tukey post-test; *p < 0.05.
Figure 5.13: Increased stromal caspase-1, -4 and -5 expression in invasive versus non-invasive, poorly differentiated adenocarcinoma tissue of OAC patients

Tumour tissues from OAC patient sections were IHC scored for caspase-1, -4, -5 expression in epithelial (A, B, C) and stromal (D, E, F) regions. Scores were graphed according to their defined OAC differentiation status: WD (Well differentiated adenocarcinoma, n=4); M (Moderately differentiated adenocarcinoma, n=7); IM (Moderately invasive differentiated adenocarcinoma, n=4); P (Poorly differentiated adenocarcinoma, n=5) and IP (poorly invasive differentiated adenocarcinoma, n=5). Representative images of stromal caspase-1,-4 and-5 expression patterns within (P) poorly differentiated (G, H, I) and (IP) invasive poorly differentiated (J, K, L) adenocarcinoma tissues from OAC patients. Magnification ×200. Data represent mean ± SEM. P values were calculated using the one-way ANOVA followed by the Tukey post-test; ***p<0.00.
Figure 5.14: Caspase-1 and -5 expression levels are altered in tumour tissues from OAC patients with/without neoadjuvant therapy

Tumour tissue from OAC patients with (+) and without (-) neoadjuvant therapy (NAT) were IHC scored for caspase-1, -4, -5 expression in epithelial (A, B, C) and stromal (D, E, F) regions using a validated semi-quantitative scoring method. Data represent mean ± SEM. (+NAT, n=13 and -NAT, n=19). P values were calculated using the Mann-Whitney U-test; *p < 0.05.
Chapter 6
General Discussion
6. General discussion

Major progress has been made in understanding the fundamental importance of murine inflammatory caspase-11 in experimental in vivo and in vitro models of acute septic shock, bacterial infection, and various neurodegenerative diseases (Jimenez Fernandez and Lamkanfi, 2015; Uchiyama and Tsutsui, 2015). In particular, there is a growing body of evidence supporting the involvement of murine caspase-11 and human caspase-4 and -5 in non-canonical inflammasome activation, thus, highlighting a crucial role for these caspases in innate immune responses (Man and Kanneganti, 2015; Stowe et al., 2015). Caspase-4/-5 and -11 have also been identified to directly bind to cytosolic LPS resulting in the triggering of pyroptosis and anti-bacterial defenses (Baker et al., 2015; Kayagaki et al., 2013; Shi et al., 2014), suggesting that they may play a key role in driving Gram-negative sepsis. Despite the growing body of literature elucidating function and signalling pathways governing caspase-4, -5 and -11 regulation, there is still little known regarding the role of these caspases and the non-canonical inflammasome during acute/chronic inflammation. To address this deficit, in this report we assessed: (i) the involvement of caspase-11 during intestinal inflammation using a mouse model of acute colitis; and (ii) the contribution of human inflammatory caspase-1, -4 and -5 during chronic inflammation in the colon and oesophagus of IBD/CAC and BO/OAC patients.

Interestingly, our findings from the in vivo animal studies clearly revealed a protective role for caspase-11 during colitis. Casp11−/− mice were hyper-susceptible to acute experimental colitis and exhibited pronounced clinical symptoms over the duration of the disease course and exhibited severe mucosal damage of the colonic tissue compared to WT littermate controls. In prominent agreement with our results, two recent studies (Demon et al., 2014; Williams et al., 2015) have also revealed a protective role for caspase-11 during acute colitis, thus highlighting the reliability of our data and indicating a crucial role of caspase-11 in regulating intestinal inflammation. From our studies, it was observed that the DSS treated caspase-11 deficient mice displayed significant defects in IL-18, IL-22 and IL-1α production in colonic homogenates, indicating that caspase-11 is involved in regulation of these cytokines during acute colitis. However, to our surprise there was no difference in IL-1β production between Casp11−/− and WT mice. In contrast to our data, Williams et al study revealed that both IL-18 and IL-1β levels were reduced in colons of DSS treated
Casp11\(^{-}\) mice (Williams et al., 2015), thus implicating a crucial role for non-canonical inflammasome during acute colitis. A possible explanation for the observed differences between our two studies could be due to experimental design differences; we evaluated cytokine levels in colon homogenates, which allows for evaluation of both pro- and cleaved cytokine levels. However, Williams et al incorporated a culture biopsy method which estimated only secreted (active) cytokine levels in colonic tissue. Thus, it would be worth confirming our observations obtained from the colon homogenates in the setting of an organ culture method.

Previous data has shown that IL-22 and IL-18 play important roles in IEC proliferation and epithelial barrier repair (Siegmund, 2010; Sonnenberg et al., 2010). Interestingly, their reduced levels observed in DSS treated Casp11\(^{-}\) mice are consistent with increased barrier permeability and mucosal damage observed in the mice used in this study. IL-18 and IL-22 knockout mice are also reported to be more susceptible to acute colitis (Coruh et al., 2001; Zenewicz et al., 2008). These findings support our data and indicate that caspase-11 mediated down-regulation of IL-18 and IL-22 contributes to the hyper-susceptible phenotype of caspase-11 deficient mice and to the development of colitis. Moreover, our data clearly revealed that the enhanced epithelial barrier damage in Casp11\(^{-}\) was due to decreased IL-18 mediated IEC proliferation. A previously reported role for IL-22 in maintaining epithelial barrier integrity via antimicrobial peptide production (such as Reg3\(\beta\) and Reg3\(\gamma\)) following DSS injury (Mielke et al., 2013; Zheng et al., 2008; Zindl et al., 2013) was not confirmed/observed in our study. However, this does not exclude the possibility that the protective function of IL-22 can be mediated by its induction of other Reg family members (e.g. Reg1, RegII) (Zheng et al., 2008). Hence, based on our results it appears likely that IL-18 is a crucial cytokine involved in the maintenance of epithelial barrier integrity in Casp11\(^{-}\) mice during DSS acute colitis.

Our demonstration that the DSS susceptibility phenotype of Casp11\(^{-}\) mice can be rescued by exogenous administration of IL-18 is in agreement with other studies which link caspase-1 and NLRP3 deficiency with decreased IL-18 activity in the intestine (Dupaul-Chicoine et al., 2010; Zaki et al., 2010a). These findings indicate the importance of the non-canonical inflammasome in DSS mediated intestinal inflammation. Intriguingly, excessive epithelial cell death has been identified as a key feature contributing to the severity of DSS induced colitis in mice and pathology of UC
patients (Dirisina et al., 2011; Qiu et al., 2011). In this study, we have linked increased level of epithelial cell death with enhanced epithelial barrier damage observed in DSS treated Casp11^/- mice, suggesting that caspase-11 may contribute to the inhibition of cell death in acute colitis. However, this hypothesis contradicts current knowledge and published reports which implicate caspase-11 in apoptosis and pyroptosis induction rather than inhibition (Hagar et al., 2013; Hisahara et al., 2001; Kayagaki et al., 2011) and requires further investigation.

In terms of our investigations into the signalling events that lead to upregulation and activation of caspase-11 in vivo we observed a requirement of the type II IFN-STAT1 mediated pathway during acute colitis. To gain a mechanistic insight into the regulation of caspase-11 in this model, we identified that caspase-11 upregulation occurs in the IEC fraction. However, it has been shown by others that caspase-11 is expressed also in the colonic immune cells (Williams et al., 2015). Based on this report and as we failed to isolate the lamina propria fraction, we do not exclude the possibility that type II IFN-STAT1 pathway can also drive induction of caspase-11 in the lamina propria fraction of colonic mucosa. Some interesting follow on experiments that could be considered include the culturing of primary IEC and lamina propria cells from mice, which could be further stimulated with type II IFNs (+/- STAT1 inhibitors) so to examine the effects on caspase-11 expression.

To our surprise, the TRIF-IFN-I signalling pathway, which was previously reported as a crucial caspase-11 upstream upregulating mechanism occurring during host defence against Gram-negative bacterial infection in BMDMs (Broz et al., 2012b; Gurung et al., 2012; Rathinam et al., 2012) appeared to be fully dispensable for caspase-11 upregulation and activation in the context of intestinal inflammation during acute colitis in vivo. Interestingly, the observation of increased caspase-11 expression and activity in TRIF deficient mice indicating that TRIF can act as negative regulator of caspase-11 during colitis prompted us to further examine a role of TRIF in regulating human caspase-4 and -5 in vitro. In addition, caspase-4 and -5 levels have been shown to be elevated in human IBD patients (Flood et al., 2015), analogously to caspase-11 in mice during colitis, thus, it was hypothesised that TRIF may act as a negative regulator of caspase-4/-5 in human colons in IBD. Unfortunately, this hypothesis was not supported by data (unpublished) obtained by Louise McAteer (a student in Dr. Creagh's lab) who showed in a series of in vitro experiments that TRIF does not interact directly
or via adaptor TRAF6 with caspase-4/-5, and also has no effect on caspase-4 and -5 expression. Overall, our findings highlight the signalling complexities and discrepancies which occur between mouse-human homologs (such as caspase-11 versus caspase-4 and -5) in the settings of *in vivo* and *in vitro* studies.

The interesting data generated from our studies of caspase-11 in murine colitis model was then extended to the analysis of caspase-4 and -5 expression in samples from patients with inflammatory diseases. Examination of human caspase-1, -4 and -5 expression patterns in inflammatory diseases (IBD and Barrett’s oesophagus) and inflammatory associated cancers (CAC and OAC) revealed increased expression of the pro-inflammatory caspases in these diseases. Firstly, we showed that proinflammatory caspase-1, -4 and -5 expression patterns were significantly enhanced in IBD patients biopsies in two independent patient cohorts (Belgium and Irish cohort) as evaluated by two different techniques. Furthermore, in particularly caspase-4 and -5 expression correlated very closely with the level of tissue inflammation and the grade of disease activity in UC patients (in the Irish cohort) indicating that their increased expression has a role in UC pathogenesis. Caspase-1 is the only inflammatory caspase that previously been linked to the pathogenesis of UC (Mahida et al., 1989; Papadakis and Targan, 2000). Our data clearly indicates that this role should now also be extended to caspase-4 and -5 during intestinal inflammation. To support our findings, there is also a growing body of literature which has implicated the importance of caspase-4/-5 in non-canonical inflammasome activation to drive IL-1β and IL-18 production *in vitro* (Baker et al., 2015; Schmid-Burgk et al., 2015 Knodler et al., 2014; Kobayashi et al., 2013), highlighting the emerging significance for these caspases during inflammation. Furthermore, a recent study linked elevated caspase-4 and -5 levels with the overproduction of IL-1β in patients with neutrophilic asthma, suggesting an important role for these caspases in inflammatory diseases in tissues other than the GI tract (Simpson et al., 2014).

The major findings obtained from our analysis of caspase-4 and -5 expression patterns in CAC patient biopsies were: (i) increased stromal caspase-4 and -5 levels detected in inflamed tissue biopsies, which nicely corroborates our observations from UC patient samples; (ii) their unique expression switch in epithelial cells, from a lack of expression in normal/inflamed tissues to robust expression in neoplastic tissue. The later finding is particularly interesting as it identifies caspase-4 and -5 specific epithelial
expression as potential biomarkers for colorectal carcinoma. As dysplastic lesions associated with IBD-CAC are difficult to grade and distinguishing between neoplastic and chronically inflamed tissue is still a major challenge for pathologists (Lim et al., 2003; Ullman et al., 2009), our findings may be relevant for the development of a diagnostic test to aid early CAC diagnosis in the future. In order to develop such a test, it will be necessary to determine the stage at which epithelial expression of caspase-4/-5 occurs. To address this, it is planned to perform caspase-4 and -5 IHC staining using patient tissues with low and high grade dysplasia as well as with the early T stages (T1-T2) of tumour progression in a larger CAC patient cohort. In parallel, it is also planned to perform caspase-4 and -5 IHC staining of patient tissues with other adenocarcinoma type cancers such as prostate, gastric or breast cancer to investigate if the specific expression of caspase-4/-5 also occurs. Such observations in different cancers would facilitate broader clinical usage of the diagnostic test based on specific epithelial caspase-4/-5 patterns.

Furthermore, based on the fact that there are no reports of studies investigating the role of inflammatory caspases in pathogenesis of BO and OAC, we were prompted to determine whether the observed specific epithelial caspase expression occurring in CAC tissue may also be valid in OAC patient tissues. Contrary to expectation, specific expression of caspase-4 and -5 in epithelial cells in CAC patient tissues was not observed in OAC patient tissues. In addition, based on IHC expression analysis, caspase-1 and caspase-5 seem to be more involved in the pathogenesis of BO and OAC compared to caspase-4. This observation is in contrast to our UC/CAC data, which revealed a crucial involvement of caspase-4/-5 rather than caspase-1 in pathogenesis of these diseases. This data highlight that despite commonly proposed structural and functional similarities for caspase-4/-5, they actually may have different functions within distinct diseases. To support the above statement, some early reports also suggest different roles for these caspases under specific circumstances. Caspase-5 has been implicated as an NLRP1 inflammasome component, indicating its role in IL-1β maturation in vitro (Martinon et al., 2002). On the other hand, caspase-4 has been shown to be required for efficient caspase-1 activation and IL-1β secretion in UVB-irradiated human keratinocytes, indicating its key role in NLRP3 inflammasome activation (Sollberger et al., 2012). Moreover, caspase-4 has been shown to be involved in induction of apoptosis following activation during endoplasmic reticulum (ER) stress.
Thus, collectively these studies indicate that caspases may mediate different signalling pathways which might contribute to disease pathogenesis.

Another interesting finding from the caspase-1, -4 and -5 IHC analysis of OAC patients biopsies is that they exhibit significantly enhanced expression in invasive high grade oesophageal adenocarcinomas indicating their role in tumour invasion. However, how these caspases may contribute to the mechanisms of tumour invasion is unknown. It can be hypothesised here that they may facilitate tumour invasion by enhancing IL-1β production which has been broadly reported to play an important role in tumour invasiveness (Apte et al., 2006; Petrella and Vincenti, 2012; Vidal-Vanaclocha et al., 2000). However, it would be prudent to see if our initial observations can be extended to a larger patient number prior to future investigation on their involvement in tumour invasion. Interestingly, caspase IHC staining of OAC tissues also revealed that NAT chemoradiation treatment appears to effect caspase-1 and -5 expression levels in stromal and epithelial cells, suggesting that their expression is induced following the treatment. Currently neoadjuvant chemoradiation therapy followed by surgery is becoming a standard approach of care in advanced oesophageal cancer patients (Shapiro et al., 2015). However, significant numbers of treated patients do not respond well to treatment (Blencowe et al., 2012; Dittrick et al., 2012). Thus, there is a critical need to evaluate the molecular mechanisms and biomarkers of resistance to therapy in order to improve the effectiveness of OAC patient treatment. Our preliminary data indicate that NAT treatment affects caspase-1 and -5 expression levels in epithelial and stromal cells, however, if these changes are absolutely linked to their chemo/radio resistance or sensitivity to therapy requires further study. To further investigate if the alterations in caspase expression are associated with radio-resistance or -sensitivity it is planned to perform a set of in vitro experiments using well established isogenic cell line models of radioresistant OAC (Lynam-Lennon et al., 2010). In addition, to further assess if caspase levels are associated with response to neoadjuvant chemoradiation in OAC patient tumours, the correlation between caspase expression patterns and tumour regression grading (TRG) scores is also under investigation in Dr. Creagh’s group.

Overall, the data generated in this study on the role of inflammatory caspases in the in vivo transgenic mouse model of colitis and clinical human UC studies led to some very interesting conclusions. Firstly, both murine caspase-11 and human caspase-4 and -5 have been upregulated in colons of DSS treated mice and UC patients, respectively,
indicating their involvement in mediating intestinal inflammation. Similarly, we have also shown contrasting colonic expression patterns between murine caspase-11 and human caspase-4 and -5. It has now been demonstrated by us and other groups that murine caspase-11 is expressed in both IEC and immune cells of mouse colonic tissue during colitis, however, human caspase-4 and -5 would appear only to be expressed in human infiltrating immune cells in UC patients (Flood et al., 2015; Oficjalska et al., 2015; Williams et al., 2015). Why these commonly considered homologs show different expression patterns in the colonic tissue remains unclear. However this observation may shed potential doubt on a commonly proposed model of orthologility between murine caspase-11 and human caspase-4 and -5 (Martinon and Tschopp, 2007) and suggests further investigation is merited. It is well established that the DSS acute colitis mouse model mimics the clinical and histological features of UC (Egger et al., 2000; Okayasu et al., 1990). It is important to highlight here that despite the multitude of animal models of IBD (Mizoguchi, 2012), there is no perfect experimental model, because patients with IBD present a heterogeneous spectrum of pathological features that reflect the participation of a diverse range of innate and adaptive immune effectors. The DSS acute colitis model (which was also used in this study) is particularly useful for investigating the contribution of the innate immune system to the development of UC pathological intestinal inflammation, and it has been broadly used in various knockout mice such as NLRP3, Caspase-1, ASC, and other inflammasome components (Dupaul-Chicoine et al., 2010; Takagi et al., 2003; Zaki et al., 2010a). However, like all animal models, the DSS acute mouse model has also its own limitations including reliability of translating mouse data to human. Using the aforementioned mouse model, we have revealed a significant role for caspase-11 and mechanistic insights into its regulation during acute colitis in mice (Oficjalska et al., 2015). Hence, it is not a surprise that all our findings in this model of acute inflammation did not directly translate to the data obtained from chronically inflamed UC human tissues, (which are the only clinically available samples which can be obtained from UC patients). Therefore, a worthwhile pursuit for future studies would be to examine a role of caspase-11 during intestinal inflammation in parallel sets of different models of colitis such as the DSS-induced chronic model or Gram-negative bacteria induced colitis model (e.g. Citrobacter rodentium-induced colitis). Undertaking an orthogonal disease model approach may facilitate a better
understanding of how murine-11 and human caspase-4/-5 compare and function during colitis and IBD.

Other interesting observations of note from our studies regarding the human clinical samples was the detection of contrasting caspase-4 and -5 expression patterns between normal adjacent/inflamed UC/neoplastic CAC and normal adjacent/inflamed BO/neoplastic CAC tissues. It is likely that multiple factors contribute to the presence of specific epithelial caspase-4 and -5 expression in normal squamous and columnar epithelium cells in the oesophagectomy tissue from OAC patients. It is well established that columnar epithelium cells form the lining of the colon, however, the oesophagus is lined by stratified squamous epithelium cells. Multi-layered (stratified) epithelia differ from one-layered (simple) epithelia by various architectural and functional properties (Herrington, 2014). Thus, the possible reasons for the contrasting caspase-4 and -5 expression patterns we observe is due to different types of epithelial cells present in the colon and the oesophagus. It can be also hypothesised that epithelial caspase-4 and -5 expression patterns observed in Barrett’s metaplasia could be due to their different cell of origin. There are several hypotheses proposed to explain the cell of origin of Barrett’s oesophagus. These include: ‘transdifferentiation’ of squamous epithelial cells into columnar BE cells, migration upward of suboesophageal gland cells, migration of an embryonic population residing at the squamo-columnar junction, migration of columnar epithelia cells from the gastric cardia and migration in of bone marrow progenitors (Kapoor et al., 2015). Given such a diversity of cell of origins of BO it may be associated with observed epithelial caspase expression in BO tissues. Another possibility which may explain the presence of epithelial caspase-4 and -5 expression in normal adjacent and in intestinal metaplasia tissues of BO patients could be also linked to the earlier presence of gastro-oesophageal reflux (GERD) in these patients, which plays a central role in the pathogenesis of BO metaplasia (Fitzgerald et al., 2002b; Monkmuller et al., 2012). It has been reported that chronic GERD is usually the underlying cause of the repetitive mucosal injury and also provides an abnormal environment during the healing process that predisposes to the development of intestinal metaplasia of the oesophagus (Souza, 2010). Interestingly, it has been also proposed that the refluxate stimulates oesophageal squamous epithelial cells to secrete overwhelming quantities of chemokines, which attract tissue-damaging inflammatory responses (Souza et al., 2009). This is a novel perspective considering the common
belief that refluxate causes direct mucosal damage. Therefore, it could be hypothesised that the pre-existence of GERD in Barrett’s oesophagus patients may have an impact on proinflammatory caspase expression in these tissues.

Another potentially important readout from this study was that proinflammatory caspases can have cell type specific roles during inflammation of the gastrointestinal tract. Historically, IEC has been primarily considered as a mechanical barrier against invading pathogens, whereas the underlying lamina propria and lymphoid tissues, rich in professional immune cells were considered the main immunological responders in the gut to pathogenic challenge and drivers of intestinal inflammation (Artis, 2008). But now it is well recognised that IECs also play a key role in the innate immune defence in the gut (Maloy and Powrie, 2011). In our studies, we have shown that human caspase-4 and -5 and murine caspase-11 non-canonical inflammasomes can be involved in regulation of intestinal inflammation (in both protective or detrimental ways), depending on the site of their expression (IEC or immune cells) and the stage of inflammation (acute or chronic). It appears likely that a lack of caspase-11 during acute colitis is associated with exacerbated intestinal inflammation in response to induced colonic epithelial damage. We have also implicated caspase-11 and the non-canonical inflammasome with protective functions within IEC during acute colitis. In support of our findings, it has been also shown that epithelial non-canonical inflammasome activation of caspase-4 and -11 promotes host defence against \textit{S. Typhimurium} (which is a Gram-negative bacteria involved in pathogenesis of IBD), thus indicating a protective role for these caspases during bacterial infections of the gut (Knodler et al., 2014). Intriguingly, IHC analysis of human caspase-4 and -5 expression patterns revealed that these caspases are only expressed in infiltrating immune cells of UC patients. In addition, increased expression of inflammatory caspases-4 and -5 was significantly correlated with inflammation activity scores of UC patients, thus indicating their potential contribution to IBD pathogenesis presumably via enhancing inflammatory processes during chronic intestinal inflammation. Recent reports have also implicated caspase-4 involvement in caspase-1 activation, pyroptosis and IL-1β production using a human monocytic cell line (THP1) (Schmid-Burgk et al., 2015, Baker et al., 2015). Thus, we can hypothesize that caspase-4 and -5 overexpression in infiltrating cells of UC patients may enhance non-canonical inflammasome activation and lead to progression of pathological inflammation as observed in IBD patients. Our observations
are also in agreement with a previously stated role for IL-18 (protective role in IEC during acute colitis or destructive role in intestinal immune cells, during chronic colitis) (Reuter and Pizarro, 2004; Siegmund et al., 2001; Takagi et al., 2003). Moreover, given that increased IL-1β and IL-18 levels are frequently detected in inflamed mucosa of IBD patients (Mahida et al., 1989; Papadakis and Targan, 2000; Pizarro et al., 1999), adds more evidence that increased canonical or non-canonical inflammasome activation is involved in triggering chronic inflammation during IBD. Overall, it appears that intestinal homeostasis is highly sensitive to the expression levels of the canonical and non-canonical inflammasome components and also suggests cell-type-specific inflammasome function. Thus, deregulated expression of inflammasome components may significantly affect the susceptibility of the gastrointestinal tract to IBD development. Currently, inflammasomes and their components are emerging as targets for the development of novel anti-inflammatory drugs for the treatment of various diseases including chronic inflammatory diseases (Ozaki et al., 2015). Therefore, our findings reveal a fascinating insight into the contribution of human caspase-4 and -5 into IBD pathogenesis and may be of benefit for aiding future therapeutics that aim to reduce intestinal inflammation.

Alternatively, there is also a possibility that the enhanced caspase-4 and -5 expression observed in the tissues of IBD patients in this study, may be associated with their potential role as negative regulators that function to dampen inflammatory responses during inflammation. Many negative regulators have been implicated in attenuating TLRs and NLRs signalling to prevent overactive inflammatory responses and to maintain the immunological balance (Creagh and O'Neill, 2006). For TLRs, these include soluble decoy TLR receptors (e.g. TLR2 and TLR4), which are naturally produced in blood and tissues during bacterial infection and appear to inhibit the interactions between bacterial ligands and their TLRs. Also, certain negative regulators of TLR signalling are upregulated following TLR stimulation, such as: ST2 (sequesters MyD88 or Mal); MyD88s (displaces MyD88); interleukin-1 receptor associated kinase (IRAK)-M, a suppressor of cytokine signalling-1 (SOCS1) (mediates Mal degradation); and A20 (de-ubiquitinates TNF receptor-associated factor 6 (TRAF6) to block NF-κB activation) (Liew et al., 2005; Mansell et al., 2006). These aforementioned regulators reduce the TLR response through a negative feedback loop. Other negative regulators that target TLRs attain their effects by down regulating the transcription or translation
of TLR genes or by the degradation of TLR proteins. For example: TRIAD3A (ubiquitinates TLRs, targeting them for degradation) or Toll-interacting protein (TOLLIP) (controls the extent of responses to IL-1 and LPS) (Bulut et al., 2001; Chuang and Ulevitch, 2004; Zhang and Ghosh, 2002). Also several negative regulators of NLRs have been identified, including: ErbB2-interacting protein (ERBIN), a member of the LRR and PDZ-domain-protein family, which appears to be a negative regulator of NOD2-dependent NF-κB and MAPK signalling (Martinon and Tschopp, 2004; McDonald et al., 2005); caspase-1 inhibitors, such as CARD-only protein (COP) and ICEBERG, that appear to act by preventing inflammasome-mediated oligomerization of caspase-1 or caspase-5; proteinase inhibitor 9 (PI-9), which is induced by LPS and directly blocks the active site of caspase-1 and caspase-12, which directly suppresses caspase-1 (Saleh et al., 2006).

Negative regulators of TLRs such as A20, PPARγ, IRAK-m and TOLLIP have been also found to be essential in the regulation of TLRs in the GI tract (Shibolet and Podolsky, 2007). Recently, alterations in expression patterns of these proteins have been linked with the pathogenesis of IBD (Fernandes et al., 2015). Interestingly, the zinc finger protein A20 (an ubiquitin-modifying enzyme), which inhibits tumour necrosis factor (TNF)-induced NF-κB activity (Beyaert et al., 2000) has been broadly reported to play important role during pathological intestinal inflammation. For example, a genome wide association study has identified A20 as a susceptibility gene for Crohn's Disease (2007; Barmada et al., 2004). Also, significantly enhanced A20 expression was detected in the tissue biopsies of UC patients (Fernandes et al., 2015). This protein is known to be induced by transcription factors, (e.g. NF-κB), which is activated by inflammatory signal transduction pathways. Thus, it appears that its upregulation is likely a positive feedback mechanism to limit the inflammation seen in IBD. Furthermore, a study by Oshima, et al revealed that this protein is an early response negative regulator of TLR-5 signalling in IECs that functions during intestinal inflammation. It has been shown that flagellin-mediated TLR-5 signalling induces abundant expression of A20 rapidly in IECs, which was also confirmed in vivo in a DSS induced acute colitis model. Thus, these findings indicate the importance of A20 upregulation in the early phase of intestinal inflammation (Oshima et al., 2010).

Another negative regulator which has been linked with pathological intestinal inflammation is TOLLIP. Various IEC lines constitutively (or by induction) express
large amounts of the inhibitory molecule TOLLIP \textit{in vitro} and \textit{in vivo} (Melmed et al., 2003; Otte et al., 2004). Inhibition of TLR signaling by TOLLIP appears to be mediated through its ability to suppress the activity of IL-1 receptor-associated kinase (IRAK) after TLR2/4 activation (Burns et al., 2000; Zhang and Ghosh, 2002). It has been also reported that prior exposure of human IEC to a TLR ligand (such as LPS) induces a hypo responsive state to a second challenge with the same or another TLR ligand by selectively limiting proinflammatory responses through upregulation of TOLLIP and subsequent suppression of IRAK \textit{in-vitro} (Otte et al., 2004). Therefore, it has been suggested that negative regulation of TLR signalling by TOLLIP may limit the production of proinflammatory mediators during intestinal inflammation.

Overall, the above findings suggest that the regulation of TLR inhibitory proteins expression has an important role during inflammation associated with IBD. Thus, it can be speculated here, that enhanced caspase-4 and -5 expression in colonic tissues of IBD patients noted in this study, may be due to their potential function as negative regulators in reducing the production of proinflammatory mediators during intestinal inflammation. As caspase-4 and -5 expression (similarly to A20), may be induced via the NF-κB pathway, it can be hypothesised here, that caspase-4 and -5 upregulation in UC patients may act as a positive feedback loop to reduce the intestinal inflammation in IBD patients. However, further studies are required to fully elucidate the role of these proteins in the pathogenesis of IBD.

To conclude, this study has identified a potential role for human caspases-4 and -5 during IBD. Altered/enhanced expression of caspase-4 and -5 has also been demonstrated in CRC and the BO-OAC progression, suggesting their involvement in these additional diseases. Murine studies have revealed a protective role for caspase-11 during experimental colitis, confirming a requirement for caspase-11 for IL-18 production and epithelial barrier repair. Results from this study have led us to question whether the proposed functional homology between human caspases-4 and -5 and murine caspase-11 really exists in the context of intestinal disease.
Chapter 7
References


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