Identification of novel innate immune mechanisms regulating oesophageal adenocarcinoma (OAC) progression and bacterial infection.

Thesis submitted to the University of Dublin, Trinity College for the Degree of Doctor of Philosophy

By

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I. Declaration of Authorship

I, Ewelina Flis-Jankowska, 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 (Except **Figure 5.10 B** which was added to support results in Chapter 5).

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II. Abstract

Inflammation is an essential immune system response to pathogens, damaged cells and stress stimuli and has an essential role in tissue repair and regeneration. The inflammatory response is the coordinated activation of signalling pathway leading to immune cell recruitment into the site of infection and production of inflammatory mediators. In response to the recognition of microorganisms and sterile stressors, a multiprotein complex, called the inflammasome is formed. The inflammasome activates the highly pro-inflammatory cytokines IL-1β and IL-18 and induced programmed cell death-pyroptosis thus inducing inflammation. Despite the undeniable protective role of inflammation, increasing evidence shows that that deregulation in immune response or prolonged inflammation causes and advances many common diseases.

Chronic inflammation plays a very important role in oesophageal adenocarcinoma (OAC) and its only known precursor, Barrett's oesophagus (BO). Recent studies suggest that microbial dysbiosis in oesophagus could contribute to BO and increase the risk of OAC development. TLR2 is involved in the innate immune response to microbial pathogens and host-derived molecules.We assume that TLR2 upregulation will increase the sensitivity of oesophageal cells to bacteria thus leading to chronic inflammation.. We show that BO and early-stage OAC cells were responsive to TLR2 stimulation and TLR2 neutralising antibody successfully inhibits TLR2-mediated chemokine production. Factors secreted from TLR2-activated oesophageal cells induce TLR2-mediated differentiation of murine macrophages into M2-like/TAM phenotype. We identify High Mobility Group Box 1 protein (HMGB1) as one of the factors secreted from TLR2-stimulated oesophageal adenocarcinoma cells. We show that extracellular HMGB1 can efficiently prime macrophages for inflammasome activation, upregulating caspase-11 and IL-1B. Findings suggest that HMGB1 is a potential target for early-stage OAC, and that blocking TLR2 signalling may limit HMGB1 release, inflammatory cell infiltration and inflammation during OAC progression.

Inflammation is critical for tuberculosis (TB) pathogenesis. Numerous host innate immune responses are induced upon *M.tuberculosis (Mtb),* although their mechanisms and impact on mycobacterium are not well understood. It is estimated that about one-third of the global population is infected with *Mtb*. When the infection is not cleared it remains

in a latent form for a long time and only 5-10% of infected individuals will develop active disease at some stage of their life. The inhibition of inflammation is the main survival strategy of *Mtb*. Nitric oxide and IL-1β play an important role in the host resistance to *Mtb*. Here we investigated the role of caspase-11 in *Mtb* infection. We first show that STAT1 activation, nitric oxide production and IL-1β expression in macrophages is mediated by caspase-11. The iNOS-induced nitric oxide production is regulated through IFNAR/JAK/STAT1 pathway. We also determined that Caspase-11 is required for restriction of *Mtb* proliferation in murine macrophages. As nitric oxide is well known to confine the growth of *Mtb* we hypothesise that *Mtb*-induced caspase-11 increases iNOS expression and nitric oxide production and is a crucial protein in the innate immune response to TB-induced pathogen. Inhibition of caspase-11 by mycobacteria could be a potential mechanism allowing *Mtb* proliferation in the host.

Peptic ulcers are another example of the inflammation-related condition, caused by the interaction between bacterial and host factors and are often influenced by the presence of *Helicobacter pylori* infection or use of NSAIDs. This study demonstrates enhanced expression of caspase-4 in peptic ulcer patient biopsies, indicating that pyroptosis and non-canonical inflammasome activity may be processes involved in peptic ulcer disease. We show that primary murine macrophages infected with *H. pylori* upregulate caspase-11 (the orthologue of human caspase-4), activate caspase-1 and secrete IL-1β. Prostaglandin E_2 inhibits caspase-4 mediated and indirectly caspase-1 driven pyroptosis probably through the limitation of DAMPs production. Overall, evidence is provided for a pathological role of caspase-4/11 in peptic ulcer disease and proposes that targeting caspase-4 or inhibiting pyroptosis may have therapeutic potential in the management of peptic ulcers.

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I dedicated this thesis to my mother.

She would never believe that I wrote a PhD thesis!

IV. Thesis output

Publications

First author:

- Caspase-4- a therapeutic target for peptic ulcer disease. E. Flis*, Z. Zaslona*, C.Nulty, J. Kearney, R. Fitzgerald, A. R. Douglas, D. McNamara, S. Smith, L.A.J O'Neill, E. M. Creagh. Journal of Immunology. *Under review.* ImmunoHorizons October 1, 2020, 4 (10) 627-633; DOI: https://doi.org/10.4049/immunohorizons.2000080
  *These authors contributed equally to this work
- HMGB1-mediates TLR2 inflammatory pathways during oesophageal adenocarcinoma. E. Flis, G. Barber, B. Keogh, A. Anand, M. Quante, J. O'Sullivan, E.M. Creagh. *Manuscript submitted to Cancer Immunology Research.*
- Caspase-11 regulates Nitric Oxide-mediated host responses to *Mycobacterium tuberculosis*. E. Flis, C. Nulty, E. Hackett, A. Agnew, F. Sheedy, E.M. Creagh. *In preparation.*

Contributing author:

- Caspase-11 regulates the tumour suppressor function of STAT1 in a murine model of colitis-associated carcinogenesis. B. Flood, J. Manils, C. Nulty, E. Flis, S. Kenealy, G. Barber, J. Fay, K. H. G. Mills, E. W. Kay & E. M. Creagh. *Oncogene*. 2019;38(14):2658-2674. doi: 10.1038/s41388-018-0613-5.
- Caspase-11 promotes allergic airway inflammation. Z Zaslona, E. Flis , M. Wilk, R. Carroll , E. Palsson-McDermott , M. Hughes ,C. Diskin , K. Banahan , D. Ryan, A. Hooftman, A. Misiak, J. Kearney, G. Lochnit, W. Bertrams, T. Greulich, B. Schmeck, O. McElvaney, K. Mills, E. Lavelle, M. Wygrecka, E.M. Creagh, LAJ O'Neill. *Nat Commun.*2020; 11(1):1055. doi: 10.1038/s41467-020-14945-2.
- Characterizing caspase-1 involvement during esophageal disease progression. G. Barber, A. Anand, K. Oficjalska, J. J. Phelan, A. B. Heeran, E. Flis, N. E. Clarke, J. A. Watson, J. Strangmann, B. Flood, H. O'Neill, D. O'Toole, F. MacCarthy, N. Ravi, J. V. Reynolds, Elaine W. Kay, M. Quante, J. O'Sullivan, E. M. Creagh. *Cancer Immunology Immunotherapy*, 2020. doi: 10.1007/s00262-020-02650-4.

Presentation

March 2017, Dublin, Ireland Oral presentation	TRACT Kick-off meeting
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September 2019, Dublin, Ireland Oral presentation	TBSI Research Symposium 2019
January 2020, Dublin, Ireland Poster presentation	Tipton PhD Award 2020
February 2020, Galway, Ireland Poster presentation	Irish Association for Cancer Research

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VIII. Abbreviation

Ab	Antibody
AIM2	Absent in melanoma
ASC	Apoptosis associated speck-like protein containing a CARD
ATP	Adenosine tri-phosphate
ANOVA	Analysis of variance
BO	Barrett's oesophagus
BM	Bone marrow
BMDM	Bone marrow derived macrophage
BMI	Body mass index
BSA	Bovine Serum albumine
CAPS	Cryopyrin associated periodic syndrome
CARD	Caspase activation and recruitment domain
cDNA	Complementary DNA
COX	Cyclooxygenase
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DISC	Death-induced signalling complex
DMSO	Dimethyl sulphoxide
DMEM	Dulbecco's modified eagle media
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSDMD	Gasdermin D
GORD	Gastro-oesophageal reflux disease
H&E	Haemotoxylin & eosin
HEK	Human embryonic kidney
HGD	High grade dysplasia
HMGB1	High mobility group box one
HKLR	Heat-killed *Lactobacillus rhamnosus*
HKST	Heat-killed *Salmonella Typhimurium*
HRP	Horse radish peroxidase
HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
IRAK	IL-1R-associated kinase

IκB	Inhibitor of κB
IKK	IκB kinase
iNOS	Inducible nitric oxide synthase
JNK	C-Jun-N-terminal kinase
LDH	Lactate dehydrogenase
LGD	Low-grade dysplasia
LP	Lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAL	MyD88 adaptor like
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage-colony stimulating factor
MMP	Matrix metalloproteinase
mRNA	messenger RNA
MyD88	Myeloid differentiating factor 88
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B
NK	Natural Killer
NLR	Nod-like receptor
NLRP	Nod-like receptor protein
NO	Nitric oxide
OAC	oesophageal adenocarcinoma
OMV	Outer membrane vesicle
OSCC	oesophageal squamous cell carcinoma
Pam2CSK4	Dipalmitoylated lipopeptide CysSerLys4
Pam3CSK4	Tripalmitoylated lipopeptide CysSerLys4
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
PRR	Pathogen recognition receptor
PYD	Pyrin domain
RAGE	Receptors for advanced glycation end-products
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel
SEAP	Secreted embryonic alkaline phosphatase
STAT	Signal transducer and activator of transcription
TBST	Tris buffered saline with Tween
TCD	Trinity College Dublin
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TIR	Toll/interleukin-1 receptor
TRAF	TNFR-associated factor
TRAM	TRIF related adaptor molecule

TRIF	TIR domain-containing adaptor inducing IFN-β
V	Volts
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
WT	Wild type

Chapter 1: General Introduction

1.1. Innate immunity

1.1.1. Innate immunity and inflammation

Immunity is a complex system in the body, composed of several alternative defences against infection that can be activated directly after recognition of an invading pathogen. Immunity serves as a barrier to block invasion and spread of viruses, bacteria, parasites and other pathogens, and to protect organisms against disease. There are two types of immunity. First, innate immunity is a highly conserved mechanism and acts as the first line of defence to particular microorganisms. Innate mechanisms include physiological barriers such as epithelial surfaces and innate immune cells which recognise foreign material in the body [1]. Second, adaptive immunity refers to antigen specific-immune responses. Adaptive immunity evolved in early vertebrates to generate more specific and stronger responses using immunological memory [2].

Innate immune cells, such as macrophages, mast cells, fibroblasts, dendritic cells, can recognise pathogen invasion by their expression of various pathogen recognition receptors (PRR) [3]. All microorganisms generate pathogen-associated molecular patterns (PAMP) which are specific to microbes. Recognition of PAMPs by PRRs leads to inflammatory responses that help to eliminate the invading pathogens. Commonly encountered bacterial PAMPs are derived from bacterial cell walls, such as lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria, flagellin, found in bacterial flagella, peptidoglycan (PGN), lipoproteins and nucleic acids from bacteria, fungi and viruses. [4] [5]. PRRs also recognise damage-associated molecular patterns (DAMP), which are released or exposed by living cells under stress, dying or dead cells. Common examples of DAMPs include reactive oxygen species (ROS), ribonucleic acids (RNA), deoxyribonucleic acids (DNA), adenosine triphosphate (ATP), high-mobility group box 1 protein (HMGB1) and endogenous heat shock proteins (HSPs) [6] [7]. Upon recognition of PAMPs and DAMPs, the innate immune system triggers the inflammatory response through secretion of cytokines and chemokines, which initiate recruitment of leukocytes to the site of infection [3].

1.1.1. Role of macrophages in innate immunity

Macrophages are important components of innate cellular immunity presenting distinct biological functions, mainly involved in host defence and immunity against foreign microorganisms, such as bacteria, viruses, fungi and parasites. Macrophages were first described by Elie Metchnikoff in 1882 in larvae of starfish inserted with thorns from the tangerine tree and in Daphnia Magna infected with fungal spores as cells responsible for the process of phagocytosis of foreign particles. Macrophages are the first innate immune cells and Ellie Metchnikoff for this discovery was awarded the Nobel Prize in 1908 [8]. Macrophages consist of heterogeneous cell populations. They arise from circulating monocytes or from the local proliferation of tissue-resident macrophage colony-forming unit cells. Monocytes migrate from blood vessels and differentiate into macrophages in the peripheral tissue at the site of inflammatory lesions [9]. When the monocyte reaches the extravascular tissue, they transform into larger phagocytic cells, called macrophages. In addition to phagocytic activity, macrophages can be activated, which results in increased cell size, increased production of lysosomal enzymes and higher ability to phagocytose and kill intracellular bacteria [10]. Depending on their polarization status macrophages can be divided into M0, M1 (classically activated) and M2 (alternatively activated) macrophages (**Figure 1.1**). M0 macrophages are naïve macrophages which have not been exposed to any pro- or anti-inflammatory stimuli. When M0 macrophages are exposed to a pathogen or cytokine stimulation, they can be polarised into pro-inflammatory (‘classically’ activated, M1) and anti-inflammatory (‘alternatively’ activated, M2) macrophages [11]. The term macrophage activation (classically, M1) was introduced by Mackaness in the 1960s to describe antigen-dependent, non-specific enhanced, microbicide activity of macrophages in BCG (bacillus Calmette-Guerin) and Listeria infections [12]. The enhancement was later linked with cytotoxic and antitumor activity [13]. In the 1990s, Stein and Doyle with colleagues, after the observation that IL-4 and IL-13 increased major histocompatibility complex (MHC II) class II antigen expression and reduced pro-inflammatory cytokine secretion, proposed alternative activation (M2) phenotype for mouse macrophages [14], [15].

1.1.1.1. M1 macrophages

Classically activated M1 macrophages are developed when M0 macrophages are stimulated by microbial substrates like lipopolysaccharide (LPS) and Th1 cytokines including IFNγ, IL-2, IL-12, IL-18, TNF-β and GM-CSF [16][17]. Three of the main M1 stimulators can be distinguished [18]. The first of them is IFNγ, which can polarise M1 alone or in combination with LPS [19]and other cytokines such as TNF and GM-CSF [20]. IFNγ is the main Th1 product and is produced by natural killer (NK) cells and macrophages. IFNγ signalling involves Janus kinase (Jak) 1 and Jak2 adaptors, which activates Signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factor (IRF). Proteins such as regulators cytokine-inducible SH2-containing protein (CISH), suppressor of cytokine signalling 1 (SOCS1), N-myc-interactor (NMI), protein tyrosine phosphatase, receptor type, C PTPRC and protein tyrosine phosphatase, receptor type, O (PTPRO) are controlled by IFNγ [21]. The best studied M1 activator is LPS, which is recognised by TLR4. LPS stimulation is associated with MyD88 and MAL/Tirap-dependent pathway which increases the production of pro-inflammatory cytokines: IFN-β, IL-8, TNFα, pro-IL-1β and chemokines: chemokine [C-C motif] ligand 2 CCL2, chemokine [C-X-C motif] ligand 10 [CXCL10], and CXCL11. The profiles are controlled by NF-κB, IRF, STAT1, EGR (early growth response) and AP-1 [21]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is another M1 activator. GM-CSF is produced by macrophages at low basal levels and is often elevated during inflammatory/immune reaction [22]. After macrophage activation, Jak2 kinase is recruited, what induces activation of STAT5, NF-κB, IRF5, ERK (extracellular signal activated kinase) and AKT (V-akt murine thymoma viral oncogene homolog 1) [23]. GM-CSF induces cytokine production: IL-8, IL-6, G-CSF, M-CSF, IL-1β and TNFα. M1 macrophages are involved in Th1-type responses, responsible for killing intracellular parasites and for perpetuating autoimmune responses. Activated M1 macrophages are characterised by their ability to secrete the large amount of pro-inflammatory cytokines: IL-6, TNFα, IL-1β and they have an IL-12^{high}, IL-23^{high} and IL-10^{low} phenotype. They are characterized by high production of effector molecules: nitric oxide (NO) and reactive oxygen intermediates (ROI), higher expression of major histocompatibility complex class II and costimulatory molecules and efficient antigen presentation. M1 macrophages exhibit cytotoxic function and anti-tumour activity [24] [25].

1.1.1.2. M2 macrophages

In contrast, the alternative, M2 form of macrophage activation is the term used for various forms of non-classically activated macrophages. M2 macrophages can be activated by fungal cells, parasites, immune complexes, complement, apoptotic cells, macrophage-colony stimulating factor (M-CSF), interleukin-4 (IL-4), IL-13, IL-10 and tumour growth factor-beta (TGF-β) [26]. M2 macrophages share IL-10^{high} and IL-12^{low} phenotype, generally have high levels of a scavenger, mannose [15] and galactose-type receptors (MGL-1, Galactose-Type C-Type Lectin) [27]. Other very common M2 markers are Ym1 (Chitinase-3-Like Protein), CD206, FIZZ1 (Found in Inflammatory Zone 1), CD209 (Dendritic Cell Specific ICAM-3 Grabbing Nonintegrin), Dectin-1, Neurotransmitters, Hormones and Growth Factors [28].

Activation of macrophages with IL-4/IL-13, activate Jak1 and Jak3 kinases, which leads to STAT6 activation. STAT6 coordinates with Krüppel-like factor 4 (KLF4) to induce M2 genes such us Arg-1, Ym1, Fizz1 and PPARγ. KLF4 provides also a molecular mechanism to restrict M1 pathway, and inhibit genes such as TNFα, Cox-2, CCL5 and NOS2 [29]. IL-4 induces macrophage fusion and decrease phagocytosis. Previous studies have shown that in IL-4 KO animals, the immune response against nematodes and some viral infections were impaired [30] [31]. In humans, a polymorphism in IL-4R has been correlated with the development of asthma and atopy [32] [33].

After activation of macrophages with IL-10, autophosphorylation of the IL-10 receptor leads to the activation of the transcription factor STAT3, which mediates inhibition of pro-inflammatory cytokine production. The binding of STAT3 up-regulates the expression of SOCS3, which mediates the suppression of pro-inflammatory cytokine signalling pathway [34].

Glucocorticoids (GCs) are generated from the glucocorticoid hormones as a result of metabolism by cellular enzymes in macrophages. Glucocorticoids are lipophilic and can diffuse through membranes, bind the glucocorticoid receptor (GCR) alpha and translocate into the nucleus as a complex. The GCR complex binds DNA and through interactions with NF-κB and AP-1, can promote/repress gene transcription of anti-inflammatory genes such as IL-10, IL1R2, CD163, TSC22 domain family, member 3 (DSIPI), MRC1 and thrombospondin 1 (THBS1) [35]. In monocytes treated with GCs (Fluticasone propionate), significant down-regulation of genes involved in the immune response, cell communication, cell motility, adhesion and activation was observed [35].

It has been shown that in human blood mononuclear cells, GCs strongly reduced production of IL-1β, TNFα, IL-2, IL-3, IL-4, IL-5, IL-10, IL-12, IFNγ, IL-6 and IL-8 [36]. GCs are very widely used immunosuppressive agents for the treatment of inflammatory disorders and autoimmune disease [37]. M-CSF is another M2 stimulus. The M-CSF receptor is a tyrosine kinase transmembrane receptor. M-CSF binding induces receptor dimerization, autophosphorylation and activates ERK, PI3K and phospholipase 3. M-CSF stimulation induces upregulation of cell cycle genes (cyclins A2, B1, D1, and E1) and downregulation of human leukocyte antigen (HLA) members [20]. M2 macrophages are further divided into subsets: M2a (induced by IL-4 or IL-13), M2b (induced by IL-1β or LPS), M2c (induced by IL-10, TGF-β or glucocorticoids) and M2d (induced by IL-6 and adenosines) based on their specific gene expression profiles [38] [20] [39] [40].

M2 are crucial for Th2 immune response including humoral immunity, tissue remodelling and wound healing [41]. M2 macrophages produce anti-inflammatory cytokines such as IL-10, IL-13 and TGF-β to promote tumour progression.

Macrophages express a wide range of receptors, which once activated, trigger innate immune responses and antimicrobial defences. These sensors are recognised as pattern recognition receptors (PRRs).

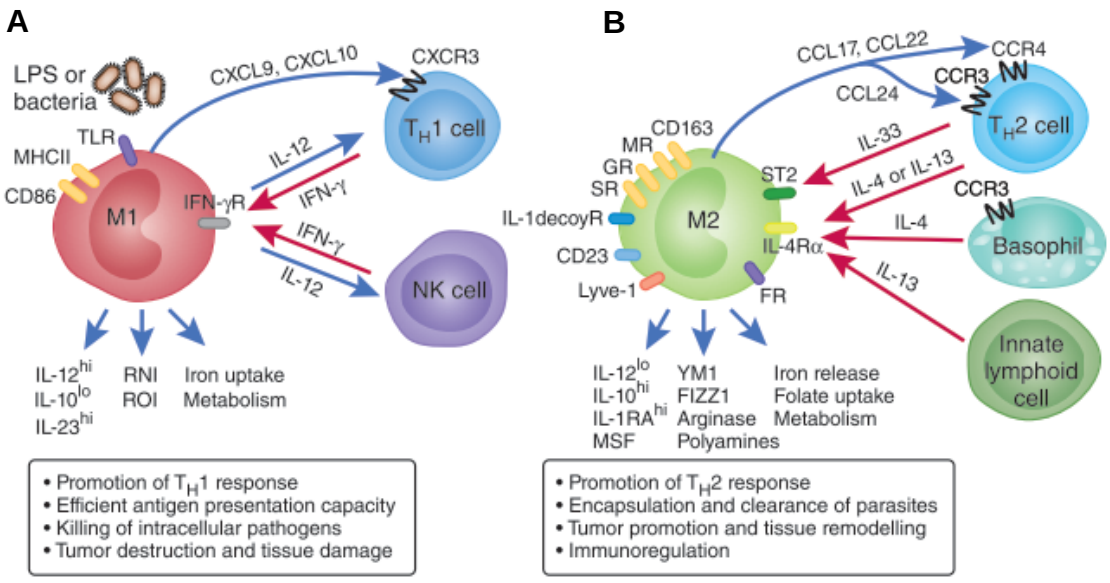


Figure 1.1 Inducers and features of polarised macrophages.

A After exposure of macrophages to IFNγ and/or LPS they are polarised into M1, with potential cytotoxic and anti-tumour functions. M1 macrophages are polarised by IFNy produced by Th1 and NK cells. M1 are characterised by IL-12^{high}, Il-10^{low}, IL-23^{high} phenotype. **B** M2 are 'alternatively' activated macrophages, which promote cancer development, tissue remodelling and immunoregulation. M2 are activated by IL-33, IL-4, IL-13 and are characterised by IL-12^{low}, IL-10^{high}, IL-$1RA^{high}$ phenotype. Typical M2 markers are YM1, FIZZ1 and Arginase. Picture taken from Biswas & Mantovani, *Nat. Immunol*. 2010 [17].

1.1.2. Pattern recognition receptors

Pattern recognition receptors (PRRs) are responsible for recognizing DAMPs and PAMPs. Currently, five classes of PRRs have been identified based on their protein homology, Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding domain, leucine-rich repeat (LRR)–containing (or NOD-like) receptors (NLRs), RIG-I-like receptors (RLRs), and the AIM2-like receptors (ALRs) [42]. These PRR classes can be further subdivided into two main types, according to the cellular localisation. The first group, TLRs and CLRs, are membrane-bound receptors found on the cell surface or on endocytic compartments. The second group, NLRs, RLRs, and ALRs are located in the cytoplasm, where they recognize intracellular pathogens [43].

The TLRs were the first PRRs identified in 1985 and are the best characterised PRRs of innate immunity [44].

1.1.3. Toll-like receptors and signalling

TLRs are essential components of innate immunity and play key roles in recognizing and mounting responses to PAMPs including flagellin, lipoproteins, LPS, and viral or bacterial nucleic acids. Activation of TLRs by a variety of bacterial products mediates cellular activation, which includes the expression of pro-inflammatory cytokines. Cytokines are small secreted proteins released by cells, they play a very important role in communication and interaction between the cells. Cytokine is a general name and specific cytokine types can be distinguished by: lymphokine (cytokines released by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities) and interleukin (cytokine interacting between leukocytes). Cytokines released from a cell can interact on the same cell (autocrine action), on the cells located nearby (paracrine action), or on distant cells (endocrine action). Cytokines are produced mainly by immune cells and stromal cells such as fibroblasts and endothelial cells. Cytokines regulate proliferation, differentiation, cell survival, immune cell activation, death and cell migration [45]. Cytokines are can be further categorised into sub-families of anti-inflammatory and pro-inflammatory cytokines. Previous studies have shown that expression of the pro-inflammatory cytokines, correlated with increased invasiveness and poor prognosis in cancers [46]. There is significant evidence to support the hypothesis that the pro-inflammatory cytokines IL-6 and IL-8 are involved in cancer development [47] [48].

IL-6 is a pro-inflammatory cytokine with typical pro-tumorigenic effects. IL-6 is produced by T cells, macrophages, fibroblasts, synovial cells, hepatocytes, endothelial cells and keratinocytes. IL-6 can regulate several cellular functions including cell proliferation, differentiation and activation and has been proposed as a predictor for malignancy [49]. IL-6, after binding with its receptor (IL-6Rα) and co-receptor pg130, activates the JAK/STAT signalling pathway of the Janus kinases (JAK) and activators of transcription, mainly STAT3. STATs are a family of transcription factors associated with tumorigenic processes [50]. IL-6/JAK signalling can also lead to the activation of MAPK and Akt signalling pathways. It has been shown that IL-6 with another pro-inflammatory cytokine TNFα, can promote tumour development by conversion of non-cancer cells into tumour stem cells [51].

IL-8, alternatively known as CXCL8 is a pro-inflammatory chemokine. Expression of IL-8 is regulated by activator protein and/or NF-κB-mediated transcriptional activity. The effects of IL-8 are mediated through the binding of this chemokine to the two cell-surface G protein-coupled receptors: CXCR1 and CXCR2 [52]. After activation of G protein, IL-8 signalling promotes activation of the primary effectors phosphatidyl-inositol-3-kinase (PI3K), phospholipase C (PLC), promoting the activation of Akt, PKC, calcium mobilization and/or MAPK signalling pathway [53]. Many studies have shown overexpression of IL-8 by tumour cells. IL-8 is known to promote angiogenesis and activate matrix metalloproteinase (MMP) which is involved in metastasis-related tissue remodelling [54] [55]. High IL-8 has been detected in the serum of cancer patients and levels of this chemokine correlates with tumour size, stage and prognosis [56] [57] [58].

TLRs are type I transmembrane glycoproteins which are structurally characterized by extracellular leucine-rich repeats (LRRs), which mediate PAMP recognition, a single transmembrane domain, and conserved intracellular Toll/IL-1 receptor (TIR) domain which mediates intracellular signalling [59][60]. The extracellular domain has 16-28 LRR modules [61]. The LRR modules contain 20~30 amino acid residues with conserved ‘‘LxxLxLxxN’’ motifs [62] [63]. To date, 10 TLRs have been characterised in humans, TLR1-TLR10; and 12 in mouse (TLR1–TLR9, TLR11–TLR13). TLR1-TLR9 are conserved in both species [64]. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the cell surface and they recognize mainly microbial membrane components like LPS, lipopeptides and microbial proteins (**Figure 1.2**). In contrast, TLR3, TLR7, TLR8 and TLR9 are expressed on the intracellular membranes of the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes [64].

TLR signalling pathways are mediated by MyD88-dependent pathways, which induce activation of transcriptions factors, NF-κB and AP-1; and/or TRIF-dependent pathways, which lead to the activation of IRF3. These pathways lead to the production of pro-inflammatory cytokines and type I IFNs.

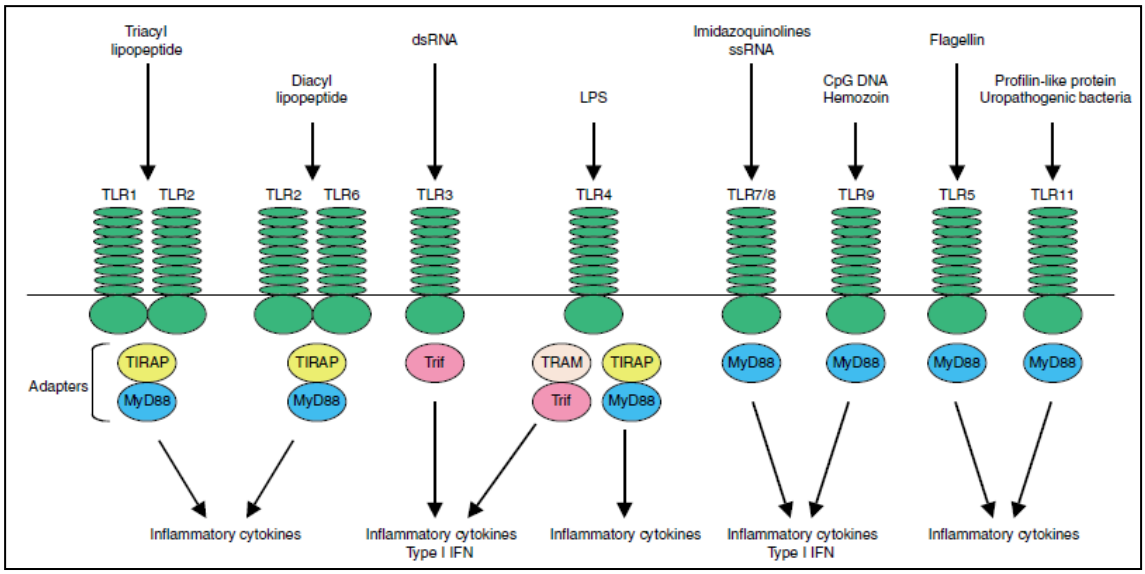


Figure 1.2 Toll-like receptor signalling pathways.

TLR2 after dimerization with TLR1 or TLR6 can be activated by triacyl lipopeptides and diacyl lipopeptides, respectively. TLR3 recognizes dsRNA. TLR4 is stimulated by bacterial LPS. TLR7/8 recognizes imidazoquinolines ssRNA. TLR9 is activated by bacterial and viral CpG DNA. TLR5 mediates recognition of flagelin and mouse TLR11 is activated by profiling-like protein uropathogenic bacteria. TLR1, TLR2, TLR6, TLR7/8, TLR9, TLR5 and TLR11 require MyD88 to activate inflammatory signalling. In contrast TLR3 requires only TRIF protein. In TLR4 signalling, both MyD88 and TRIF take part in signalling. Images taken from Kawai *et al. Cell Death and Differentiation*, 2006 [65].

1.1.3.1. MyD88 - dependent signalling pathway

With the exception of TLR3, all TLRs require MyD88 to activate inflammatory signalling pathways (**Figure 1.3**). After receptor engagement with the appropriate ligand, TLRs recruit MyD88, which is a proximal cytoplasmic adaptor protein. MyD88 subsequently recruits IL-1 receptor-associated kinases (IRAKs). IRAKs are a family of serine/threonine kinases which are required to activate nuclear factor kappa-B (NF-κB). There are four IRAKs: IRAK4, IRAK1, IRAK2 and IRAK-M. After stimulation of the TLR, IRAK1 is recruited by MyD88 and is activated by phosphorylation through IRAK4. Activation of IRAK1 induces recruitment of TRAF6, an E3 ubiquitin ligase. TRAF6 together with ubiquitin-conjugating enzymes UBC13 and UEV1A, induce K63-linked polyubiquitination of TRAF6 and recruitment of the TAK1 protein kinase complex. TAK1 forms a complex with TAB1, TAB2 and TAB3, which induce

activation of this protein. Activated TAK1 binds to the IKK complex, inducing phosphorylation of IKKB, activation of NF-κB and subsequent degradation of IκB proteins. TAK1 simultaneously activates the MAPKs ERK1, ERK2, p38 and JNK by inducing the phosphorylation of MAPK kinases, which leads to activation of various transcription factors, including AP-1 [66][60] [64]. For TLR2 and TLR4 signalling, activation of MyD88-dependent pathways requires a secondary adapter, TIRAP/Mal. It has been shown that mice deficient in TIRAP/Mal protein cannot induce inflammatory responses after stimulation of TLR1/2, TLR2/6 and TLR4 [67, 68]. Moreover, TLR4 is the only receptor which activates both MyD88 and TRIF-dependent pathways. TLR4 initially recruits MyD88 to trigger activation of NF-κB and MAPK. TLR4 subsequently undergoes endocytosis and, in endosomes forms a signalling complex with TRAM and TRIF to activate the IRF3 transcription factor [69].

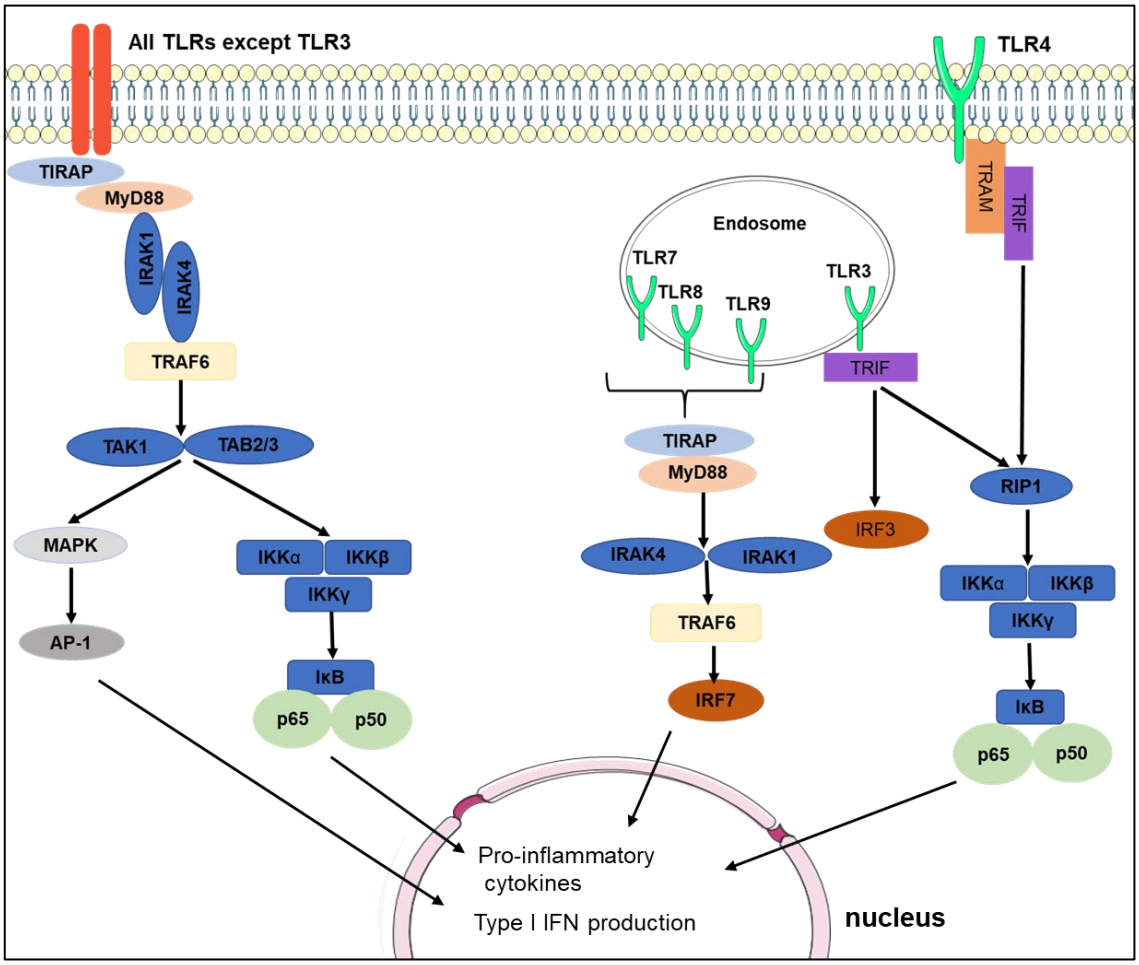


Figure 1.3 MyD88-dependent TLRs signalling pathway.

TLR 1, 2, 4, 5 and 6 are located on the cell membrane. MyD88 signalling pathway is used by all TLRs except TLR3. Upon recognising PAMPs, MyD88 forms complex with IRAK-4, leading to phosphorylation of IRAKs and interaction with TRAF6. MyD88-dependent signalling leads to activation of MAPK and IKK complexes, effecting activation of AP-1 and NF-κB, respectively and their subsequent translocation to the nucleus. TLR4 is the only TLR capable of signalling through both MyD88 and TRIF-dependent pathways. Activation of TLRs 3, 7, 8 and 9, localised on the endosome, leads to recruitment of MyD88, IRAK-1, IRAK-4, TRAF6 and the translocation of IRF7. TLR3 uses TRIF as an adaptor, resulting in RIP1 polyubiquitination and translocation of the NF-κB to the nucleus. Diagram based on Takeda & Akira, *Semin Immunol*. 2004; Zheng *et al. Front. Mol. Neurosci* 2020 [59] [70].

1.1.3.2. TRIF - dependent pathway

TIR domain-containing adaptor protein inducing IFN-β (TRIF), also known as Toll/IL-1R domain containing adaptor molecule 1 (TICAM1), is another adaptor for TLR3 and TLR4. TRIF is recruited to the intracellular TLR4 domain by TRIF related adaptor molecule (TRAM) also known as TICAM2 [71]. In contrast, TLR3 does not require TRAM protein and TRIF is the only adaptor used by this receptor to activate IRF-3. After TLR activation, TRIF combines with TRAF6 and TRAF3. TRAF3 recruits the IKK-related kinases, TBK1 and IKKi, for IRF3 phosphorylation. Phosphorylated IRF3 dimerises and translocate into the nucleus, to induce the expression of type I IFN genes. In contrast, TRAF-6 recruits the kinase RIP-1, which activates the TAK1 complex and induces activation of NF-κB and MAPKs [72].

1.1.4. Toll-like Receptor 2

TLR2 is expressed on the surface of many cell types and has several reported ligands. TLR2 was found to be expressed in immune tissue: spleen, lymph node, bone marrow and peripheral blood leukocytes [73]. Studies have revealed that TLR2 is expressed by monocytes, macrophages [74], [73], endothelial [75] and epithelial cells [76], dendritic [77] and murine T-cells [78]. TLR2 has the most complicated ligand-binding specificity in the TLR family and to be activated needs to dimerize [79]. TLR2 dimerizes with TLR1 or TLR6 and can recognize bacterial lipoproteins, lipopeptides, lipoarabinomannan (LAM) and zymosan. TLR2 ligand specificity is determined by its dimerization partner. Stimulation with diacyl lipopeptides from gram-positive bacteria induces binding with TLR6 [80], whereas triacyl lipopeptides from gram-negative

bacteria promote dimerization with TLR1 [81]. Both crystal structures for binding Pam_3CSK4 (N-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine) and Pam_2CSK4 (S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine) by TLR1/2 and TLR2/6, respectively, have been determined (**Figure 1.4**). Several interactions between ligands and TLR1/2 and TLR2/6 can be distinguished.

The main driving force for TLR2 binding is the hydrophobic synergy between the two ester-bound lipids and TLR2 pockets. The second is hydrogen bonds created between glycerol and the peptide backbone of the ligand and the LRR11 loops of TLR. This binding, bridging two TLRs, interacts with ligands and arranges the confirmation of the hydrophobic residues located in the centre of the dimerization interface. Third, characteristic only for TLR1-TLR2 is the interaction between the amine-bound lipid chain and the TLR1 channel and is essential for response to Pam_3CSK4. Pam_2CSK4 which does not have amide-bound lipid has low affinity to TLR1-TLR2 and is not able to induce stable heterodimerization of Pam_2CSK4. The last one is the peptide side chains (except for the first cysteine) which have a minor role in binding ligands to TLR1-TLR2 and TLR2-TLR6 [82].

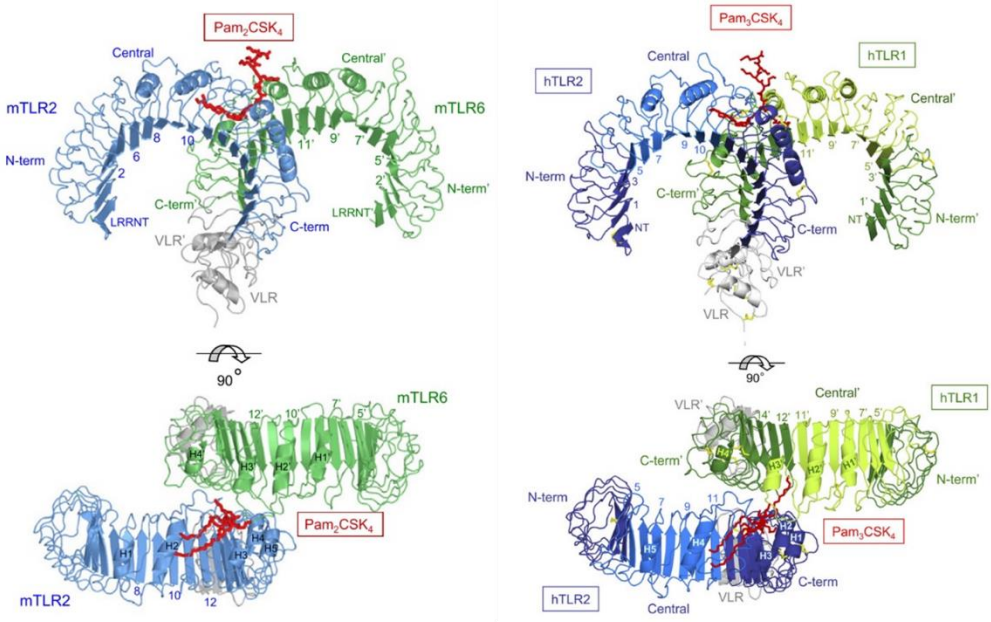


Figure 1. 4 Overall structure of the mouse TLR2-TLR6-Pam_2CSK4 (A) and human TLR1-TLR2-Pam_3CSK4 (B) structure.

A TLR6 and TLR2 are shown schematically in green and blue, respectively. Central domain is shown with light green or light blue. Pam_2CSK4 lipopeptide is red. Figure represents side (top structure) and top (bottom structure) view. Binding of Pam_2CSK4 induces the formation of 'm' shaped heterodimer of TLR2 and TLR6 in which C-terminal tails converge in the middle and N-terminal tails expand outside. The two ester-bound lipids of Pam_2CSK4 are inserted into internal hydrophobic pocket of TLR2. **B** TLR1 and TLR2 are shown schematically in green and blue, respectively. Central domain is shown with light green or light blue. Pam_3CSK4 lipopeptide is red. Figure represents side (top structure) and top (bottom structure) views. A single Pam_3CSK4 ligand is bound by the TLR1 and TLR2. Two of the three lipid chains of the ligand interact with a pocket in TLR2 and one amide-bound lipid chain is inserted into channel in TLR1. The TLR1 channel and TLR2 pocket form the lipid binding site. The C-terminal domains converge in the middle. TLR1 and TLR2 create 'm' shaped dimeric structure. Image taken from Kang *et al.* Immunity, 2009; Jin *et al.* Cell, 2007[82]; [79].

TLR2 is a plasma membrane-bound receptor. Heterodimers of TLR2 with TLR1 and TLR6 pre-exist and are not induced by the ligands. It was determined that after TLR1/2 and TLR2/6 stimulation with triacylated and diacylated lipoproteins, respectively, heterodimers were recruited into lipid rafts. TLR2/6 heterodimers were associated with lipid raft-resident proteins CD14 and CD36, whereas TLR1/2 heterodimers were associated just with CD14. Heterodimers once they are within lipid-rafts and engaged by appropriate ligands, internalize as a complex and are conducted to the Golgi apparatus for further processing. Selective recruitment of receptors within lipid rafts, triggered by specifics ligands, determines innate immune responses [83]. TLR2 can be also activated by endogenous ligands released from injured/inflamed cells. Endogenous activators are mainly extracellular matrix components [84] [85] such as fibronectin [86], heparan sulphate [87], biglycan [88], fibrinogen [89] and hyaluronan breakdown fragments [90] [91]. Other ligands such as high-mobility group box 1 (HMGB1) [92], heat shock proteins (HSP60, HSP70), endoplasmin [93][94][95] [96]) and S100 [97] proteins have been determined as endogenous TLR ligands. These endogenous TLR ligands and their receptors are in different cellular compartments and cannot bind under normal physiological conditions [98][99].

1.1.5. High-mobility group box 1 protein

High-mobility group box 1 (HMGB1) was discovered in 1973 as abundant non-histone DNA-binding protein in calf thymus and is named for its electrophoretic mobility on polyacrylamide gels. HMGB1 is a 25 kDa protein which consist of two DNA-binding HMG-box domains, N-terminal A and central B, and acidic C-terminal tail [100]. Under normal condition, HMGB1 is mainly located in the nucleus, where regulates DNA repair and genome stability. In addition to the role in the nucleus HMGB1 also functions as a damage-associated molecular pattern (DAMP). It has been shown that, after pathogen invasion, HMGB1 is produced by inflammatory cells and secreted as a cytokine [101]. During activation and cell death, HMGB1 can translocate from the nucleus to the cytoplasm and is release via two major pathways, passive and active. Hyper-acetylation of lysine AA residues, localized on two nuclear localizing sequences (NLSs) of HMGB1, enables the protein to be translocated from nucleus to cytoplasm. Hyperacetylation of the NLS-located lysines prevents the nuclear re-entry of HMGB1 [102]. HMGB1 is released passively from necrotic cells as membrane permeability breaks down and actively during apoptosis from multiple cell types including macrophages, monocytes, NK cells, DCs, endothelial cells and platelets [103] [102]. HMGB1 is actively secreted from inflammatory cells in response to tissue injury, oxidative stress, exogenous and endogenous stimuli of host origin including TNF-α, IFN-α, IFN-β, IFN-γ, hydrogen peroxide, nitric oxide and peroxynitrite [104] [105] [106] [107]. Additionally, it has been shown that HMGB1 is released during pyroptosis, an inflammatory form of cell death induced by caspases-1, -4 and -5 [108]. Once released, HMGB1 can bind to several cell membrane receptors to induce an inflammatory response. It has been determined that HMGB1 is able to engage the receptor for advanced glycation end products (RAGE), TLR2, TLR4, TLR9, macrophage antigen-1, syndecan-3, CD24-Singlec-10, CXCR4 and T cell Ig mucin-3 [109] [110] [102].

HMGB1 has been associated with many diseases, including sepsis [101], ischemia-reperfusion [111], Huntington's disease [112], Alzheimer's disease [113] and arthritis [114]. Many researchers presented the major role of HMGB1 in number of cancers, including colorectal [115], breast [116], lung [117], prostate [118], cervical [119] and oesophageal cancer [120]. HMGB1, can interact with TLR2 and stimulate tumour growth, progression and metastasis [121]. However it has been shown that HMGB1

released from glioblastoma multiform (GBM) primary brain tumour cells can activate TLR2 expressed on tumour-infiltrating myeloid DCs to mediate an effective anti-GBM immune response. Supernatants from drug-treated tumour cells activate TLR2 signalling, and blocking of HMGB1 with specific anti-HMGB1 antibody or glycyrrhizin (a specific HMGB1 inhibitor) inhibits tumour regression [122].

1.2. Caspases

Caspases are a family of cysteine proteases that consist of 13 members in humans (**Figure 1.5**). They are involved in maintaining homeostasis through regulating inflammation and cell death mechanisms, particularly apoptosis and pyroptosis [123]. Additionally, they are involved in other cellular processes including proliferation, differentiation, tumour suppression, neural development and ageing [124]. All caspases are synthesized as inactive single chain zymogens (pro-caspases) that are cleaved and activated following their dimerization or oligomerization. Additional signals are required to initiate caspase activation pathways. Pro-caspases have an N-terminal prodomain and C-terminal protease domain with a large and small subunit that contains the catalytic cysteine residues [125]. The caspases have been functionally subdivided into two groups: apoptotic and inflammatory caspases. Caspases involved in apoptosis are classified into two groups: initiator and effector caspases. Initiator caspases contain a long prodomain with protein interaction domains: CARDs in caspases -2, -9, -11, -12 (and inflammatory: caspase-1,-4,-5) and DEDs in caspases-8 and -10. These protein interaction domains trigger recruitment of initiator caspases into activation complexes such as apoptosomes, death-induced signalling complexes (DISC) and inflammasomes [126]. Initiator caspases are dimerized and activated in the multiprotein complexes. Active initiator caspases can subsequently cleave and directly activate downstream executioner (effectors) caspases (Caspases-3, -6 and -7) which process hundreds of cellular substrates to initiate apoptosis [127]. Effector caspases have small prodomains and after they are synthetized they exist as inactive homodimers until they become cleaved and activated by specifics initiator caspases [126]. The inflammatory caspases are: caspase-1 (human and mouse), -4, -5 (human), -12 (human and mouse) and -11 (mouse). During infection and cellular stress, inflammasome complexes assemble and induce the activation of caspase-1, and under certain conditions, such as Gram-negative

bacterial infection, this also involves the upregulation and activation of caspases -4 and -5 (or caspase-11 in mice), which is known as the noncanonoical inflammasome [128].

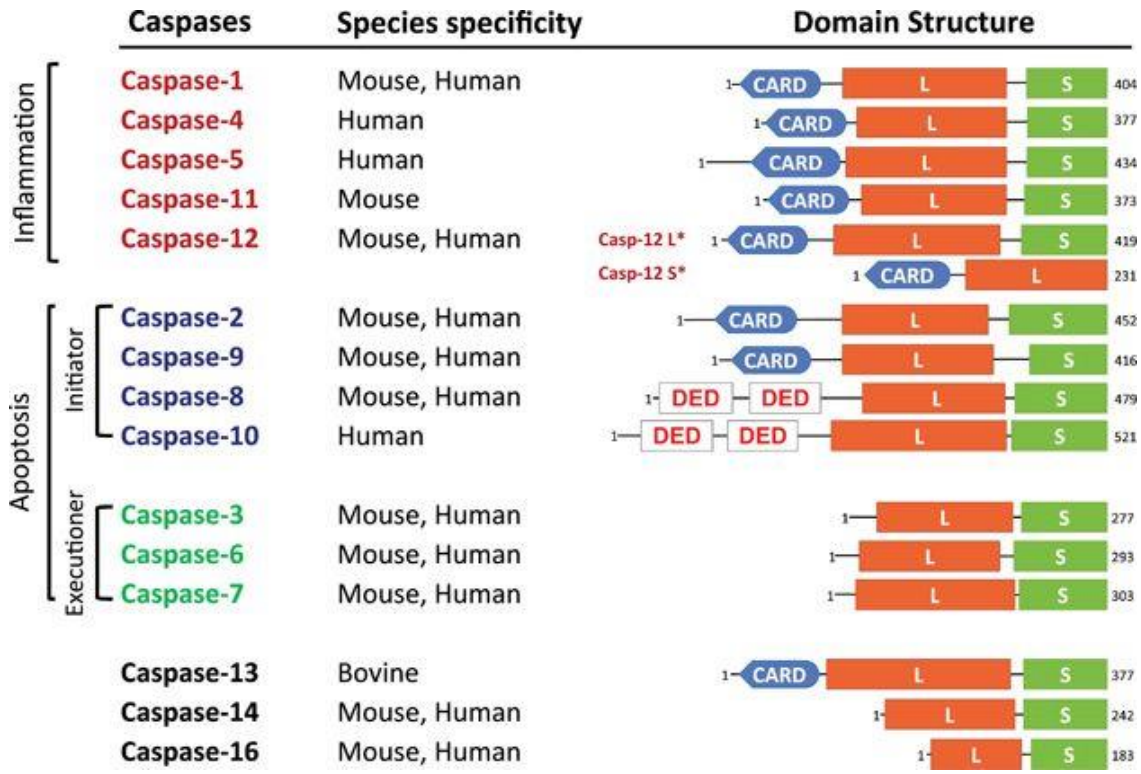


Figure 1.5 Domain structure of caspases.

Inflammatory caspases include caspases-1, -4, -5, -12 in humans and -11 in mice**.** All pro-caspases consist of an N-terminal pro-domain and C-terminal protease domain, with large and small subunits that contains the catalytic cysteine residue. Inflammatory caspase -1, -4, -5, -11, -12, and apoptotic caspase -2 and -9, consist of protein interaction domain CARD, whereas caspase -8 and -10 consist of the protein interaction domain, DED. These protein interaction domains facilitate the recruitment of initiator caspases into multiprotein activation complexes. Effector caspases -3, -6, and -7 have minimal prodomains and exist as inactive homodimers, until they are cleaved and activated by specific initiator caspases. Image taken from Shalini *et al. Cell Death and Differentiation*, 2014 [124].

1.2.1. Caspase-1 and canonical inflammasome

Caspase-1 is the best characterised inflammatory caspase. The first mammalian caspase-family cysteine protease was identified in 1992 by Thornberry, Tocci *et al.* as IL-1β-converting enzyme (ICE) for its ability to convert pro-IL-1β into its bioactive form [129] [130]. Caspase-1 is produced as an inactive zymogen that is recruited to the inflammasome which promotes autoactivation of the caspase-1 molecule [131]. Caspase-1 gene expression has been shown to be induced by IFNγ, IFNα and TNFα [132] [133]. Activated caspase-1 enables processing of pro-IL-1β and pro-IL-18 into their active forms. IL-1β and IL-18 are two closely related members of the interleukin-1 family and are potent molecules of the innate immune response. IL-18, formerly called Interferon-γ (IFNγ) inducing factor, induces the production of IFNγ by activated T cells and natural killers (NK) cells and plays a crucial role in epithelial cell proliferation [134] [135]. Pro-IL-1β is biologically inactive and needs to be processed to the mature 17 kDa IL-1β in order to function. IL-1β regulates response to infection, injury and immunological challenges by generating fever, activating lymphocytes and promoting leukocyte migration into sites of injury or infection [136]. Overproduction of IL-1β is associated with diseases, including rheumatoid arthritis, neuropathic pain, inflammatory bowel disease (IBD), multiple sclerosis (MS) and Alzheimer's disease [137] [138]. In terms of expression profiles, the main difference between IL-18 and IL-1β is that pro-IL-18 is present in blood monocytes from healthy patients and in the epithelial cells of the gastrointestinal tract. Pro-IL-18 is presents also in peritoneal macrophages and mouse spleen from healthy subjects. In contrast, pro-IL-1β expression is inducible, it is not constitutively present in epithelial cells and pro-IL-1β is absent in blood mononuclear cells and hematopoietic cells of healthy subjects [134]. Activation of caspase-1 also results in the cleavage of Gasdermin D (GSDMD) and leads to pyroptosis, a form of programmed cell death, characterised by plasma membrane rupture which eliminates the replicative niche of intracellular pathogen and releases inflammatory cytokines [139].

Early reports regarding caspase-1 have shown that monocytes from caspase-1 deficient mice did not produce IL-1β and IL-1α after LPS administration [140]. Caspase-1 deficient mice had a major defect in the production of mature IL-1β and were resistant to endotoxic shock [141]. A later study revealed that the 129 murine strain which was

used in previously published caspase-1 studies was deficient in both caspase-1 and caspase-11 genes.

Kayagaki *et al.* presented in 2011 that Casp1 and Casp11 genes are too close in the genome to enable their segregation by recombination. It brings new light on the role of caspase-11 independent from caspase-1 and highlights a crucial need to revisit previous studies analysing the role of caspase-1 using caspase-1 deficient mice, instead of caspase-1/-11 double knockout mice [142].

Canonical inflammasomes are protein complexes consisting of, at minimum, a sensor protein, adaptor protein apoptosis-associated speck-like protein with CARD (ASC) and pro-caspase-1 [143]. It has been reported that caspase-1 can be activated by different inflammasome complexes, including NLRP1, NLRP3, NLRC4, AIM2 and NLRP6 which assemble upon recognition of DAMPs and PAMPs in intracellular compartments. ASC contains two death fold-domains: pyrin domain (PYD) and caspase activation and recruitment domain (CARD). Upon activation, the NLRP3 protein interacts with ASC through the PYD domain and the CARD domain of ASC recruits pro-caspase-1 and initiates caspase-1 self-cleavage and the formation of the active heterotetrameric caspase-1 [144]. Two signals are required for NLRP3 inflammasome activation in macrophages (**Figure 1.6**). Due to the low expression level of NLRP3 in macrophages, a priming signal is firstly required to promote the NF-κB dependent transcriptional upregulation of NLRP3 and pro-IL-1β. This signal is received through various receptors including TLR, IL-1R, TNFR and NOD1. The Signal 2, which activates the NLRP3 sensor protein, is provided by bacterial, viral and fungi pathogens, intracellular PAMPs, DAMPs, ATP or pore-forming toxins [145]. Due to the diversity of NLRP3-stimulators, it is unlikely that NLRP3 directly interacts with its activators. NLRP3 is probably activated through cellular signals induced by NLRP3 stimulators such as K^+ efflux [146], production of mitochondrial reactive oxygen species (ROS) [147], release of mitochondrial DNA or the mitochondrial phospholipid cardiolipin [148] [149], Ca^{2+} signalling, mitochondrial dysfunction and lysosomal rapture [150]. Several regulators of NLRP3 such as double-stranded RNA-dependent protein kinase (PKR) [108], guanylate-binding protein 5 (GBP5) [151] and NEK7 [152], have been reported. NEK7 is required for NLRP3 inflammasome activation induced by all NLRP3 stimuli. NEK7 controls NLRP3 oligomerization, ASC speck formation and caspase-1 activation mediated by K^+ efflux [150]. Signal 2 initiates the oligomerisation and activation of the NLRP3 inflammasome by recruiting ASC and pro-caspase-1. NLRP3 recruits ASC, via

interaction of their respective PYD motifs. Pro-caspase-1 is subsequently recruited to the inflammasome sensor protein via the CARD-domain containing protein, ASC (**Figure 1.6**). The recruitment of multiple caspase-1 molecules into close proximity within the inflammasome complex enables their auto-processing, and activation of the inactive form of caspase-1 to its catalytically active species, p46 and p33/p10 subunits [153]. Active caspase-1 can subsequently process IL-1β, IL-18 and Gasdermin D (GSDMD) into their mature, active forms [150]. Following cleaveage of IL-1β, mature form of IL-1β is released outside the cells. In contrast to most of the cytokines which have a signal sequence to direct them into endoplasmic reticulum, IL-1β do not have the signal sequence and needs to be secreted through an unconventional pathway. A few unconventional secretion of IL-1β has been suggested. It has been shown that following TLRs activation, both pro-IL-1β and IL-1β are sequestrated into autophagosomes for autophagic degradation [154]. In addition, IL-1β is also passively released during cell death. In macrophages, activation of inflammasome leads to the cleveage of GSDMD into a N-terminal and C-terminal fragments. N-terminal GSDMD fragments triggers the formation of pores in the cell membrane and drives an osmotic cell lysis, called pyroptosis. [155]. It has been shown that formation of pores in the cell membrane is temporally correlated with IL-1β release in macrophages, indicating that secretion of that cytokine occure through these pores [139]

Moreover, early reports showed that in myeloid cells, in contrast to early IL-1β secretion, later IL-1β release is independent of GSDMD and IL-1β maturation is sufficient for slow IL-1β secretion from resting, non-pyroptotic macrophages. IL-1β [156].

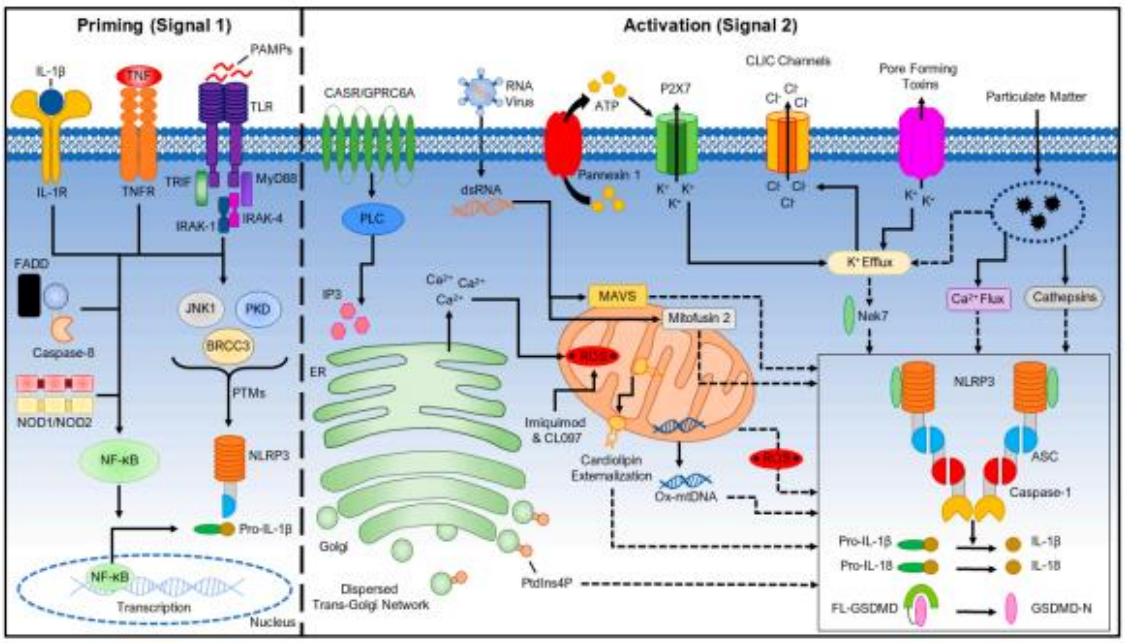


Figure 1.6 Schematic model of the canonical inflammasome activation.

Signal 1 (left) is provided by activation of receptors such as TLR, IL-1R and TNFR, and leads to the upregulation of NLRP3, pro-IL-1β and pro-IL-18, through the activation of the transcription factor NF-κB. Signal 2 (right) is provided by many common cellular events, including production of reactive oxygen species and mitochondrial dysfunction, changes in intracellular calcium levels, the formation of large nonspecific membrane pores, potassium efflux, extracellular ATP, RNA viruses. Picture taken from Kelley *et al. Int J Mol Sci,* 2019 [157].

1.2.2. Caspase-11 and non-canonical inflammasome.

Caspase-11 is a key mediator of the murine innate immune response, recognising gram-negative bacterial pathogens through binding LPS [158]. Caspase-11 was discovered in 1993 by Yuan *et al*. based on its similarity to caspase-1 (43%) [159]. The caspase formally is known as ich-3, is synthesised as a 43-kDa and 38-kDa precursor which can be activated as a direct response to gram-negative bacteria, but not gram-positive bacteria [160]. The caspase-11 promoter region contains several transcription factors binding sites, including NF-κB and signal transducer and activator of transcription (STAT1). TLRs or IFNs induce NF-κB and STAT1 activation, leading to upregulation of pro-caspase-11 [161]. Two orthologs of caspase-11 exist in primates, caspase-4 and caspase-5 [162] [163] [164].

In addition to the canonical inflammasome pathway described above, a non-canonical inflammasome, which requires the upregulation and activation of inflammatory

caspase-11 in mice and caspases-4 and -5 (functional orthologues of Caspase-11) in humans, has also been determined [165] (**Figure 1.7**). Although LPS is an important molecule in the activation of the non-canonical inflammasome, intracellular LPS alone is not sufficient for its activation. Similar to the canonical inflammasome, the non-canonical inflammasome also requires priming and activation steps [166] [167]. Levels of pro-caspase-11 are very low in macrophages and DCs and its upregulation occurs during priming by LPS, IFNγ and TNFα in MyD88/TRIF partially dependent manner [168] [166]. In contrast, activation of the non-canonical inflammasome in humans does not require a priming step, due to the high level of caspase-4 [169]. When pro-caspase-11 is upregulated in cells, the bacterial cell-wall component lipopolysaccharide (LPS) binds directly to caspase-11 in the cytosol, leading to oligomerization and activation of caspase-11. LPS binding is mediated by the CARD domain which is necessary for caspase-11 oligomerization [169]. Vanaja *et al*. reported that Outer Membrane Vesicles (OMVs) produced by extracellular bacteria allows the translocation and recognition of bacterial LPS by caspase-11 [170]. In contrast to the caspase-1, activated via canonical and non-canonical inflammasomes, caspase-11 cannot directly cleave pro-IL-1β and pro-IL-18 [142]. It was demonstrated that due to different crystal structures of caspase-1 and caspase-11 it is unlikely that caspase-11 can directly cleave IL-1β [161]. Secretion of pro-inflammatory cytokines after activation of the non-canonical inflammasome is observed as activation of caspase-11 triggers the assembly of NLRP3 inflammasome [142] [171]. It has been shown that caspase-11 activation leads to a drop in intracellular K^+ concentration thus inducing the assembly of NLRP3-ASC complexes [172]. The first studies using proper $Casp1^{-/-}$ and $Casp11^{-/-}$ knockouts have shown that Caspase-11 is critical for Caspase-1 activation and IL-1β production in macrophages infected with *E.coli*, *Citrobacter rodenium* and *Vibrio cholera*. Caspase-11 triggers caspase-1-dependent IL-1β and IL-18 production in response to non-canonical activators. Moreover, Caspase-1 deficient macrophages failed to IL-1β production following non-canonical inflammasome activation, confirming an essential role of Caspase-1 in IL-1β maturation. During murine endotoxin challenge caspase-11 rather than caspase-1 induces macrophage cell death and absence of caspase-11 protects mice from a lethal dose of LPS [142]. Activated caspase-4/-5/-11 induces pyroptosis through proteolytic cleavage of GSDMD. The cleaved N-terminal fragment of GSDMD subsequently leads to the formation of a plasma membrane pores [173]. Recent reports have shown the regulatory role of the non-canonical inflammasome in the pathogenesis of inflammatory

diseases [174]. It has been reported that caspase-11 has a protective role during dextran sulphate sodium (DSS)–induced colitis and in colitis-associated carcinogenesis (CAC). During CAC, caspase-11 induces IL-1β production via non-canonical inflammasome activation and mediates the tumour suppressor activities of STAT1 [175] [176]. Moreover, a recent report reveals for the first time the role of caspase-11 and caspase-11-dependent pyroptosis in the ovalbumin model of allergic airway inflammation. Caspase-11 was shown to be elevated in the lungs of mice with allergic airway inflammation. PGE_2, which is protective for asthma, was demonstrated to inhibit caspase-11-driven pyroptosis, thus suggesting inhibition of caspase-4/-11 as a therapeutic strategy in asthma [177].

There are still many unknowns about non-canonical inflammasome activation. In contrast to caspase-1, which activation requires recruitment to multiprotein platforms formed by sensor and adaptor proteins, no similar platform has been identified in the content of caspase-11 activation. It has been proposed that caspase-11 and human analogues caspase-4 and caspase-5 directly bind to lipid A moiety of LPS through their CARD domain resulting in their oligomerization and activation [169]. However, recent study published in 2020 by Shi *et al*. brings a new light on LPS binding mechanism and non-canonical inflammasome activation. The study showed the new role of interferon-induced guanylated–binding proteins (GBPs) in LPS-mediated non-canonical activation. Following Gram-negative bacteria infection GBP1 is triggered to bacteria, recruits GBP2-4 and forms GBP coat on cytosolic bacteria. As a consequence, GBP coat facilitiate recruitment and activation of caspase-4. It is still not clear how GBP recruits caspase-4 but it could suggest that GBPs function as a direct innate immune receptors for LPS and are responsible for non-canonical inflammasome activation [178].

Caspase-4 and caspase-5 share approximately 55% protein identity to murine caspase-11 and are both consider as a human homologs of murine caspase-11 [160]. Where most of the studies analysing role of non-canonical inflammasome in human are focused on the role of caspase-4, less is known about caspase-5. Recent study reported by B. E. Bolivar *et al.* showed that excessive release of heme from red blood cells acts as a DAMP, inducing caspase-4-mediated but not caspase-5-mediated cell death. However, heme induced release of IL-1β and non-canonical activation of caspase-1 are mediated by both caspase-4 and caspase-5. The results indicate that caspase-5 and caspase-4

could have a distinct function under certain circumstances and more studies are needed to fully characterise their functions [179].

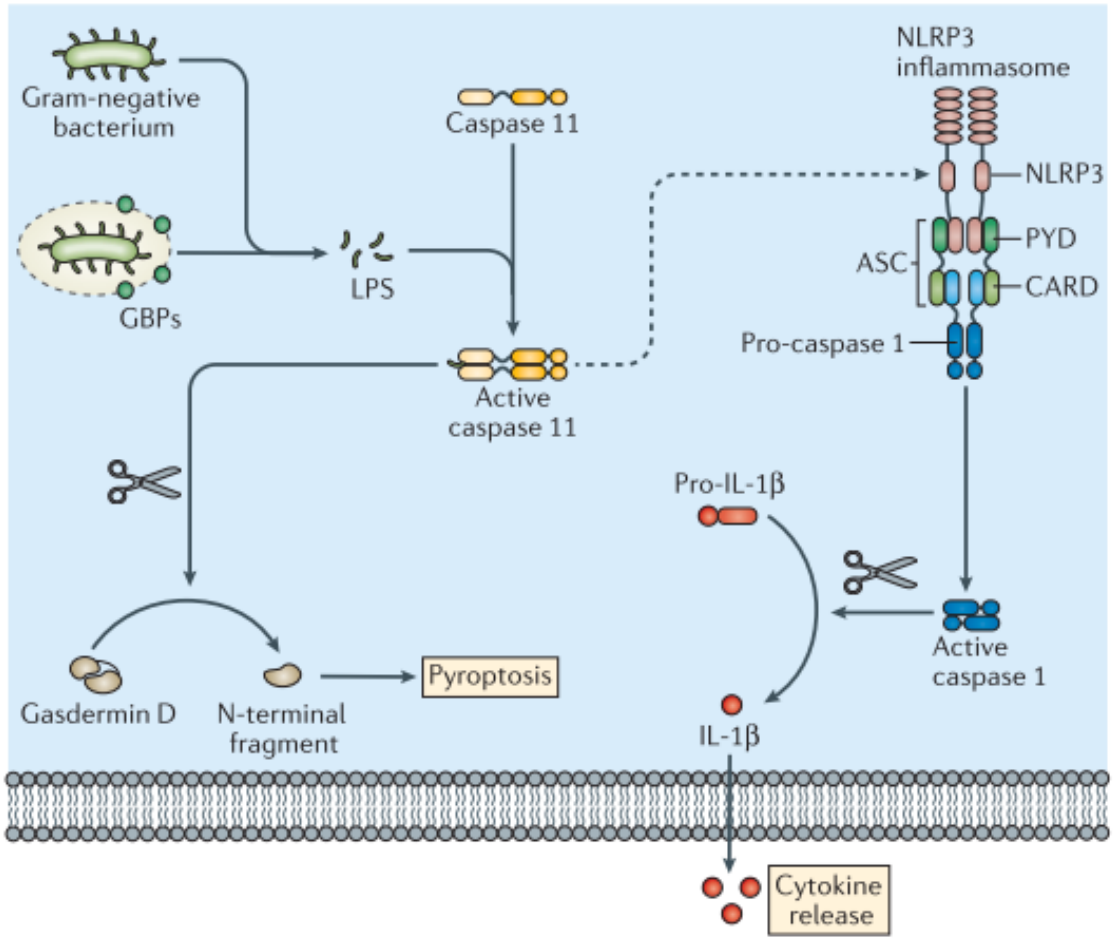


Figure 1.7 The non-canonical inflammasome activation.

Pro-caspase-11 (or human caspase-4/-5) directly binds intracellular LPS, leading to caspase-11 oligomerization and activation. Caspase-11 cleaves GSDMD to N-terminal GSDMD, which is able to create pores in the cell membrane and induces pyroptosis. Activation of caspase-11 triggers the assembly of NLRP3 inflammasome and indirectly activates caspase-1 and induces IL-1β maturation and secretion. Figure taken from Broz & Dixit, *Nat Rev Immunol*, 2016a [180].

1.2.3. Pyroptosis

Pyroptosis is a lytic type of programmed cell death, characterised by cell swelling, lysis and release of cytoplasmic content. Pyroptosis is one of the main effector mechanisms of pro-inflammatory caspases, induced by their activation [181] (**Figure 1.8**). The term 'pyroptosis' was introduced in 2001 an stems from the Greek *pyro* (fire or fever) and *ptosis* from fall [182]. In 2015, Kayagaki *et al.* and Shi *et al.* identified Gasdermin D (GSDMD) as a protein involved in caspase-1/-4/-5/-11- mediated pyroptosis [183] [184]. It has been shown that activated caspase-11 directly cleaves the 53-kDa inactive precursor of GSDMD to its mature 30 kDa N-terminal fragment and a 22 kDa C-terminal fragment. The N-terminal fragment triggers cellular membrane permeabilization. Cleaved GSDMD is essential for caspase-11-dependent pyroptosis and triggers NLRP3-dependent caspase-1 activation through a cell-intrinsic pathway, although the exact mechanism responsible for NLRP3 activation remains unclear.Mice deficient in GSDMD, similarly to mice deficient in caspase-11, were protected from LPS-induced lethal septic shock [184] [183]. Moreover, stimulation of BMDMs with canonical inflammasome activators revealed that GSDMD was only partially responsible for caspase-1-dependent pyroptosis. Caspase-1 is able to induce pyroptosis in the absence of GSDMD, suggesting the existence of other caspase-1 substrates responsible for pyroptosis [184]. Pyroptosis, due to creating pores in the membrane and releasing pro-inflammatory cytokines and DAMPs, leads to the local and systematic inflammatory response. Inflammation is associated with the generation and development of multiple disorders and disease. Pyroptosis has been implicated in Cryopyrin-associated periodic syndromes (CAPS) which due to mutation in NLRP3 induce increased inflammation activation and results in increased production of cytokines IL-1β, IL-18 and expanded pyroptosis. CAPS syndrome is characterised by fever, rashes, joint pain and conjunctivitis [185]. Inflammasome dysfunction plays an important role in neurodegenerative disease [186], cardiovascular disease [187] and AIDS [188]. Recent studies suggested that caspase-11 induced pyroptosis might be involved in allergic airway inflammation [177].

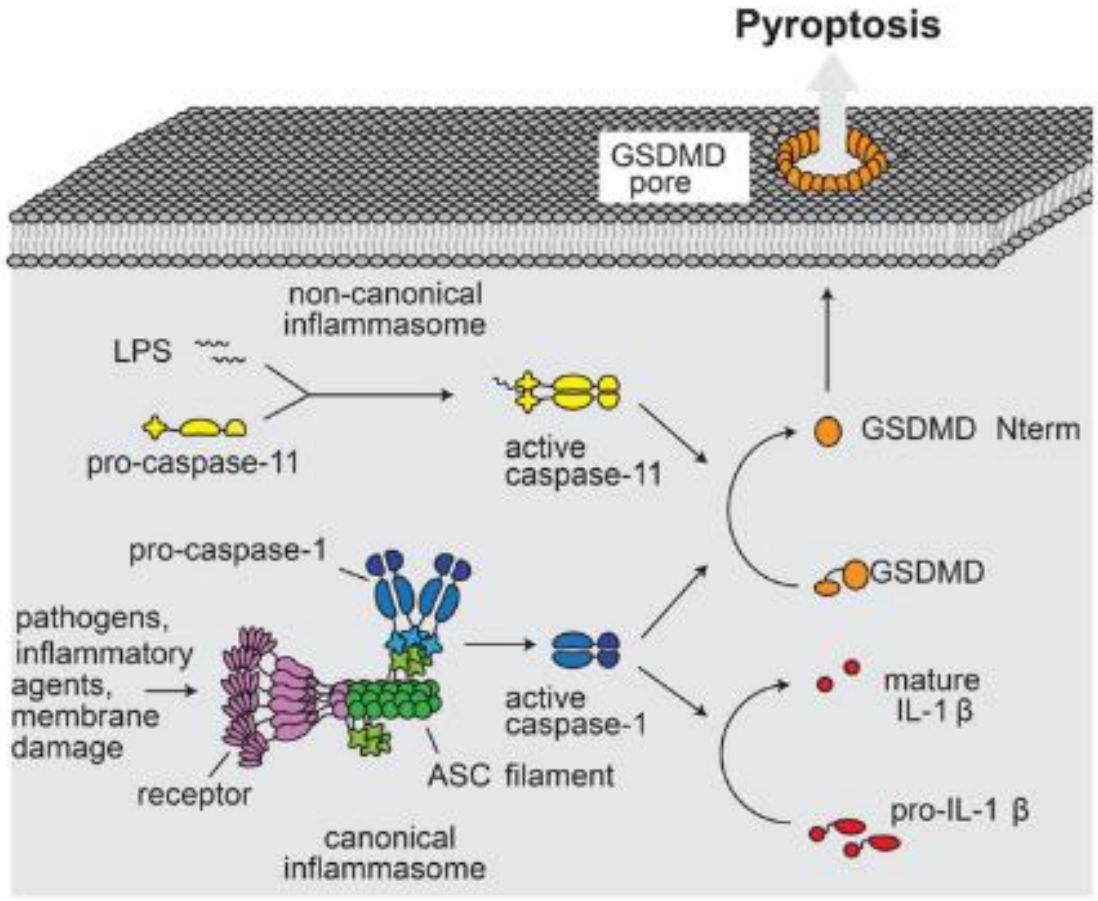


Figure 1.8 Mechanism of GSDMD-induced inflammation.

The non-canonical inflammasome pathway detects bacterial lipopolysaccharide (LPS) in the cytoplasm what leads to caspase-11 dimerization and activation. Caspase-11 cleaves Gasdermin D (GSDMD), releasing its amino-terminal domain from inhibitory carboxy-terminal domain. N-terminal GSDMD form pores in cell membrane and induce pyroptosis. The canonical inflammasome pathway triggers inflammasome assembly via detecting pathogens by sensor proteins (purple). These recruit pro-caspase-1 through adaptor protein ASC (green). Activated caspase-1 leads to IL-1β maturation, GSDMD cleavages and pyroptosis. Picture taken from Sborgi *et al.*, *The EMBO Journal*, 2016 [173].

1.3. Immune response to bacterial infection

1.3.1. Mycobacterium tuberculosis

1.3.1.1. Pathogenesis of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (*Mtb*) is the etiological agent of Tuberculosis (TB), one of the oldest human disease and leading bacterial cause of death worldwide. According to WHO, a total of 1.3 million people have died from TB every year, making this disease one of the top 10 causes of death and the leading cause of a single infectious agent [189]. *Mtb* is a highly well-adapted human parasite. TB appeared about 70,000 years ago. In the 18th century in Western Europe, had become an epidemic with the highest incidence among young people. TB incidence started to decrease in the 20th century due to the improvement of health, nutrition, and housing conditions. The introduction of the BCG vaccine in 1921 further reduced the incidence of TB. However, despite treatment improvement, TB incidence increased again in the 1980s when it was associated with the HIV pandemic and the appearance of antibiotic resistance [190].

1.3.1.2. Initial infection

TB is a contagious bacterial infection in which the lung represents the main entry site. The prognosis of the disease depends on the ability of the host to successfully eliminate the bacteria. The first line of defence against *Mtb* in the respiratory mucosa is airway epithelial cells (AECs) [191]. AECs are considered as a ‘non-professional’ immune cells [192]. AECs recognising *Mtb* through PRRs, such as TLRs, Dectin-1, C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain-containing protein 2 (NOD2), dendritic cell-specific intercellular adhesion molecule-3-grabbin non-integrin and mannose receptor [193]. PRR activation induces production of inflammatory cytokines and activation of mucosal-associated invariant T cells stimulating the production of IFNγ and TNFα [194].

While innate immunity is critical for early anti-mycobacterial responses it often creates niches for bacterial replication and *Mtb* is able to control host response to establish a chronic infection [195]. *Mtb* that successfully passes through the AECs will be delivered to the alveoli [196]. Macrophages are the first line of defence in the lower respiratory tract for *Mtb* infection. The lung compartment contains two ontologically

distinct populations of tissue resident macrophages. First, alveolar macrophages (AM) are derived from foetal monocytes during lung development and proliferate locally to maintain the population of the lung [197] and second, interstitial macrophages (IM) which are derived from circulating blood monocytes and are recruited to the interstitial space [198]. Inhaled droplets containing minute numbers of *Mtb* are engulfed by AM which are able to destroy them [199]. AM internalise *Mtb* through several phagocytic receptors, such as mannose receptor, scavenger receptor and complement receptor, which binds to nonopsonized or opsonized *Mtb* [199]. The ability of multiple receptors to internalize *Mtb* may be related to the complex structure of the cell surface of *Mtb* [200]. After internalisation, a phagosome is formed around the bacterium [201]. *Mtb* phagocytosis usually eradicates pathogens via fusion with the lysosome and acidification of the phagolysosomes. Generation of reactive oxygen and reactive nitrogen intermediates, particularly nitric oxide, are other mechanisms participating in bacterial clearance [202]. However, *Mtb* has evolved many mechanisms to evade clearance by the innate immune system. Some of these *Mtb* strategies include the inhibition of intracellular trafficking, the inhibition of autophagy, the acquisition of cytosol access, the neutralisation of toxic components as reactive oxygen species and toxic metals [196].

Dendritic cells (DCs) are also one of the first types of cells which respond to *Mtb* infection. DCs play a key role in the immune defence system due to antigen presentation, co-stimulatory activity and production of large amounts of cytokines [203]. DCs present antigens via Major Histocompatibility Complex (MHC) class I and II to T cells in the lymph node, thus DCs constitute a link between the innate and adaptive immune responses [196].

The role of neutrophils in *Mtb* is unclear. It has been shown that human neutrophils can either restrict [204] or favour [205] *Mtb* growth. Antimicrobial peptides and apoptotic neutrophils are phagocytised by *Mtb*-infected macrophages. Antimicrobial contents of the neutrophil fuse with *Mtb*-containing phagosomes in macrophages which improves bacterial killing [206]. After *Mtb* infection, neutrophils secrete chemokines and pro-inflammatory cytokines which lead to recruitment and activation of other immune cells [207].

These signalling events induce an immune response which subsequently leads to the formation of granulomas, the hallmark of tuberculosis. Bacteria are not eradicated and a balance between bacterial persistence and host defence develops. This balance might

last lifelong however less than 10% of infected individuals will develop active disease. Once disease develops, in untreated patients it is fatal in 50% cases [208]. As long as the immune response is competent the lesion will contain bacteria and infection is latent. Granuloma with pathogens and their microenvironment represents highly dynamic interaction between different T cell populations (CD4 T cell, CD8 T cell, γδ T cell, CD1 T cell), macrophages and dendritic cells. One of the reasons for *Mtb* success is its ability to control the phagosome that it resides in and to block phagosomal maturation into an acidic, hydrolytic compartment [201] [209]. The mycobacterial phagosome is characterised by low content of vacuolar ATPas, incomplete luminal acidification and low content of mature lysosomal enzymes [210]. Preventing phagolysosome formation creates a perfect environment for microbial survival and replication [211] [212]. Although, blocking of phagosome maturation by *Mtb* is not complete, and some bacteria are killed or prevented from replication through the production of antimicrobial effectors including reactive oxygen and nitrogen intermediates, produced by activated macrophages [213]. Upon infection, activated macrophages with pro-inflammatory phenotype produce pro-inflammatory mediators such as TNFα, IL-1, IL-6, IFN-I and IL-1β. Although effector functions are provided by macrophages, T cells are the major mediators of protection. The crosstalk between T cell and macrophages is facilitated by various cytokines. The production of cytokines is the main function of T helper cells (Th cells). Two subsets of Th cells can be distinguished, Th1 clones which produce Interleukin-2 (IL-2) and interferon-γ (IFNγ) and Th2 clones which secrete IL-4, IL-5, IL-6 and IL-10. CD4 T cells, CD8 T cells and γδT cells have also been shown to produce IFNγ and TNFα [214] [215] [216]. Th1 cytokines such as TNFα, IL-1β along with IFNγ produced by T lymphocytes, stimulate the expression of macrophage iNOS leading to NO production [217] [218] [219]. Th1 cytokines are essential for *Mtb* protective cellular immunity. It has been shown that IFNγ knockout mice were unable to contain, control and respond to a normally sublethal inoculum of *Mtb* [220]. These mice fail to produce reactive nitrogen intermediates and thus restrict the growth of the bacilli [221]. Similar observations, confirming the critical role of IFNγ in *Mtb* infection, was shown in PBMCs isolated from patients with a mutation in the IFNγ receptor ligand-binding chain (IFN-γR1) which had severe disseminated nontuberculous mycobacterial infection [222]. It has been shown that NO is a crucial molecule in the regulation of acute and chronic inflammation, host defence mechanisms and, together with related reactive nitrogen

intermediates (RNI), can kill or inhibit intracellular pathogens such as mycobacteria [210].

1.3.1.3. TLR activation in *Mycobacterium tuberculosis.*

Recognition of *Mtb* is a crucial step for an effective host response. When *Mtb* are internalized by AM or other immune cells, they activate many PRRs, including TLRs. It was found that *Mtb* activates cells in TLR-dependent manner. Responses to *Mtb* has been shown to be mediated through TLR2, TLR4 and TLR9 [223]. Unlike Gram-positive bacteria, activation of TLR by *Mtb* does not require the presence of CD14 [224]. Ligands for TLRs are lipoarabinomannan, the 19-kDa *M.tuberculosis* lipoprotein, lipomannan and phosphatidyl-myoinositol mannoside [225]. Following TLR activation, a TLR-signalling cascade activates the transcription factor NF-κB to translocate to the nucleus. In response, activated cells are secreting pro-inflammatory cytokines, including TNF and IL-12. Secreted TNF and IL-12 enhance antigen presentation and promote the production of anti-microbial molecules such as reactive nitrogen intermediates (RNI), reactive oxygen intermediates (ROI), and induce phagolysosome fusion, acidification and autophagy [226]. The production of RNI is a major defence mechanism of mammalian hosts against *Mtb*. It has been shown that TLR2 activation induces clearance of *Mtb* in both human and mice macrophages and it is mediated through distinct mechanisms. In mouse macrophages, TLR2 activation leads to nitric oxide-dependent *Mtb* killing, but in human monocytes and alveolar macrophages this effect is nitric oxide-independent [227]. TLR2 polymorphism is a risk factor for tuberculosis infection and no correlation between TLR4 polymorphism and *Mtb* incidence was observed [225]. Mice deficient in MyD88 are more susceptible to *Mtb* [228].

In contrast, Ch. Hölsher *et al*. presented that after *Mtb* aerosol infection, TLR2/4/9-deficient mice expressed pro-inflammatory cytokines and IFNγ at similar levels to wild-type, suggesting that TLR are dispensable for innate and Th1 responses in *Mtb* infection [229]. The differences observed between studies could be due to different *Mtb* strains and different housing condition of animals. Conversely to TLR-mediated elimination of bacteria, *Mtb* is able to modulate the innate immune response and promote survival. It has been shown that *Mtb* produce sulfoglycolipids which are competitive inhibitors for TLR1/2 or TLR2/6 and therefore inhibits NF-κB activation and cytokine production [230].

1.3.1.4. The role of inflammasome in *Mycobacterium tuberculosis* infection.

The pro-inflammatory cytokine IL-1β is a key mediator of inflammation and production of IL-1β is important for host immune defence against *Mtb* [231]. Expression of IL-1β is tightly controlled by a two step mechanism. Signal 1 induces the expression of pro-IL-1β and signal 2 which activates caspase-1 cause maturation and releasing of mature IL-1β [232]. It is well characterised that IL-1β, a component of the innate immune response, is an effective anti-tuberculosis molecule, either when induced by *Mtb* or when added exogenously [233] [234]. Mice deficient in IL-1β and in IL-1 receptor type I are more susceptible to *Mtb* infection [233] [231]. In macrophages and dendritic cells, the production of mature IL-1β and IL-18 is inflammasome-mediated [235]. It has been reported that both NLRP3 and AIM2 inflammasomes play a crucial role in *Mtb*-induced innate immunity [236] [237]. The NLRP3 Inflammasome stimulation and caspase-1 activation by *Mtb* is induced by type VII secretion system 6-kDa early secreted antigenic target (ESTAT)-6 secretion system-1, ESX-1 [238]. Through the ESX-1 secretion system bacteria release factors which breach the phagosomal membrane, enabling bacteria to access the cytosol [239]. *Mtb* mutants with defective ESTAT-6 are unable to disrupt the phagosome, activate NLRP3 and induce cell death [240].

Mtb has many mechanisms to cause persistent infection and evade bacterial clearance by the immune system [241]. Several reports have shown that *Mtb* actively suppresses inflammasome activation and IL-1β production thus promoting pathogen survival [242] [243]. S. Master *et al*. reported that *zmp1* gene in *Mtb* is responsible for blocking inflammasome activation and IL-1β production in host macrophages. Infection of mice macrophages with zmp1-deleted *Mtb* is able to activate inflammasome, enhance maturation of phagosomes, increase IL-1β production and cause more effective *Mtb* clearance in lungs [242]. It has been shown that during adaptive immunity, IFNγ produced by recruited lymphocytes, triggers induction of iNOS and NO production, which suppresses production of IL-1β by NLRP3 inflammasome. This suggests that adaptive immune responses, particularly NO production, can be responsible for suppressing innate inflammatory pathways [244].

1.3.1.5. Cell death in *Mycobacterium tuberculosis* infection.

Cell death is a fundamental event that helps maintain cellular homeostasis. *Mtb* has developed strategies to avoid both innate and adaptive immunity. Controlling macrophage cell death is one of the mechanisms which allows bacteria to evade these host defences [245]. The type of macrophage death induced by *Mtb* determines whether the host is able to successfully eliminate the bacteria. Two main types of cell death are observed during *Mtb* infection, necrosis, a form of cell injury which results in cell lysis and apoptosis, a form of programmed cell death that maintains an intact plasma membrane [246]. Induction of macrophage necrosis may facilitate bacterial transmission [247]. It has been shown that when infected macrophages contain more than 25 bacilli, the macrophages undergo necrosis [248]. The virulent *Mtb* strain, but not attenuated strain, induces necrosis in both human and murine macrophages thus allowing bacteria to evade host defence mechanisms by releasing bacteria and spreading the infection [249].

In contrast, apoptosis reduces *Mtb* survival and is a critical mechanism to protect against *Mtb* [246]. J. Keane *et al.* reported that at low MOI, attenuated *Mtb* strains induce TNFα-dependent apoptosis of human alveolar macrophages [250]. Moreover, attenuated bacilli induce significantly more apoptosis of AM than virulent strains. Low virulence strains, BCG and H37Ra are killed during TNFα-mediated apoptosis of macrophages, while the high virulence strains grow inside host macrophages [251].

1.3.2. Helicobacter pylori

1.3.2.1. Pathogenesis

Helicobacter pylori is a common gram-negative bacterium that infects approximately 50% of the world population [252]. *H. pylori* colonize and grow in human gastric epithelial tissue and mucus. After infection, *H.pylori* usually persists for life unless treated by antimicrobial therapy [253]. Colonization of stomach with *H. pylori* is associated with the development of the different gastrointestinal diseases, such as peptic ulcer, duodenal ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma and chronic gastritis, a risk factor for gastric cancer [254] [255] [256]. In 1994, *H.pylori* according to WHO was classified as a class 1 carcinogen [257]. It is recognized that *H. pylori* can be transmitted from one person to another by 3 described routes. The first

and the least common is through medical devices which had contact with the gastric mucosa of an infected person [258]. Other, more important ways of transmission are fecal-oral and oral-oral [259] [260]. Although *H.pylori* infection is very common, the bacterial transmission route is still not fully understood.

Many virulence factors have been reported to be involved in the pathogenic mechanism of infection by *H.pylori.* Various enzymes such as urease, catalase, lipase, phospholipase and protease; and toxins such as vacuolating cytotoxin and the immunogenic protein CagA, localised in the *H.pylori* pathogenicity island (PAI) can be identified. The PAI also incorporate other genes related to bacterial virulence, expression of pro-inflammatory cytokines in epithelial cells, and expression of protein forming a type 4 secretion system (T4SS) for the transport of CagA protein to eukaryotic cells [261].

H.pylori infection leads to inflammation through many various pathways, induced either in gastric epithelial cells which are the first contact with bacteria and in circulating immune cells infiltrated to the infection site. In *H. pylori*-infected patients levels of pro-inflammatory cytokines, IL-1, IL-6, IL-8 and TNFα are upregulated [262]. Epithelial gastric cells, as a consequence of *H.pylori*, upregulates pro-inflammatory cytokines and chemokines, such as IL-8, IL-1β, TNFα, IL-6, IL-12, CCL2-5, CCL20 and CXCL1-3 [263] [264]. The presence of chemokines induces the recruitment of immune cells, such as neutrophils, macrophages, DCs, NK cells and lymphocytes.

1.3.2.2. Inflammasome activation by *Helicobacter pylori*

H. pylori infection is accompanied by macrophage activation and IL-1β upregulation (Calam et al. 1997). IL-1β cytokine, product of caspase-1 activation, was significantly higher in gastric mucosa of *H.pylori*-infected patients than in uninfected (Yamaoka et al. 1997). Polymorphisms of Interleukin-1 which cause increased production of IL-1β are associated with increased risk of *H.pylori*-induced hypochlorhydria and gastric cancer (El-Omar et al. 2000). Stomach-specific overexpression of human IL-1β is sufficient to induce gastric inflammation in cancer in transgenic mice [265]. Although the role of inflammasomes in *H.pylori* infection in not well understood, Hitzler *et al*. demonstrated a dual function for caspase-1 in *H.pylori* infection. *H.pylori* mediated caspase-1 activation leads to IL-1β and IL-18 production. IL-1β has a crucial role in differentiation of Th1 and Th17 cell subsets, which are both essential for development

of protective immunity and gastric $CD4^+$ T cell-driven immunopathology. IL-18 regulates the proinflammatory activities of IL-1β, thereby preventing excessive T cell-driven response and gastric preneoplastic immunopathology [266].

1.4. Oesophageal cancer

Oesophageal cancer is the eighth most commonly occurring cancer in the world [267]. It is one of the most common cancers diagnosed, with an estimated global incidence of 455,800, with 400,200 deaths occurring in 2014 [268] [269]. The cancer is very aggressive and the prognosis still remains poor [270]. Despite advances in diagnosis and treatment, the overall five-year survival rate is still very low, at 15-20% worldwide [271]. There are two main types of oesophageal cancer: squamous cell carcinoma (SCC), located in the upper or middle third and adenocarcinoma (OAC), which develops at the junction of oesophagus and stomach. These two main subtypes account for more than 95% of malignant oesophageal tumours. Less than 1-2% of all oesophageal cancers represent sarcomas and small cell carcinomas. Rare subtypes of oesophageal cancer-like, lymphomas, melanomas, carcinoid tumours and sarcomas, may develop in oesophagus as well [270]. Squamous cell carcinoma is more common in non-industralised countries with the highest rates in Asia and Africa and main risk factors include smoking, alcohol use and achalasia. Adenocarcinoma occurs in developed nations and important risk factors are chronic gastroesophageal reflux, obesity and smoking. Although oesophageal SCC predominates worldwide, a marked rise in the incidence of OAC has been observed in Western nations [272].

1.4.1. Oesophageal adenocarcinoma progression

1.4.1.1. Barrett's metaplasia

Metaplasia refers to a process during which one fully differentiated cell type is replaced by another cell type, and it can occur as a consequence of chronic tissue injury [273]. Barrett's metaplasia is a condition wherein squamous epithelial cells that normally line the distal oesophagus are replaced by metaplastic columnar epithelial cells. It is not known why the damaged oesophageal tissue is replaced by columnar cells instead of

regenerated squamous cells. Barrett's Oesophagus (BO) is the only known precursor of OAC and the most important risk factor, with an estimated annual conversion rate of approximately 0.3 % [274]. BO develops in approximately 5-8% of patients with gastro-oesophageal reflux disease (GORD) and there is a significantly higher prevalence of BO in first-degree relatives of OAC patients [275].

A diagnosis of Barrett's oesophagus is made endoscopically, by confirmation that the columnar mucosa extends above the gastroesophageal junction, and lines the distal oesophagus. Barrett's columnar mucosa is red coloured and coarse, while oesophageal squamous mucosa is pale and glossy (**Figure 1.9**). The gastrointestinal junction is defined as being at the region which displays the highest extent of gastric folds (dashed white line, **Figure 1.10**). According to Prague classification [276], Barrett's metaplasia is characterized by the circumferential extent and maximum length of Barrett's epithelium, and is described in cm (**Figure 1.9B**). The distance between the gastroesophageal junction and the most proximal extent of Barrett's metaplasia determines whether long-segment (above 3 cm) or short-segment (below 3 cm) Barrett's oesophagus is present. Long-segment has a higher risk of OAC development [277] [278]. A positive Barrett's result is confirmed by the detection of columnar metaplasia with characteristic goblet cells in oesophageal biopsy tissue (**Figure 1.10**). Sometimes Barrett's biopsy specimens are composed of junctional epithelial cells, also termed cardiac mucosa, which is composed of mucus-secreting epithelial cells. Although cardiac mucosa can be metaplastic, has intestinal histochemical features and DNA abnormalities, it is not certain that cardiac mucosa has the same malignant predisposition as intestinal metaplasia [279] [280]. The main risk of Barrett's oesophagus is GORD. Obesity, especially an abdominal pattern of obesity [281] and cigarette smoking [282] is also associated with Barrett's metaplasia development.

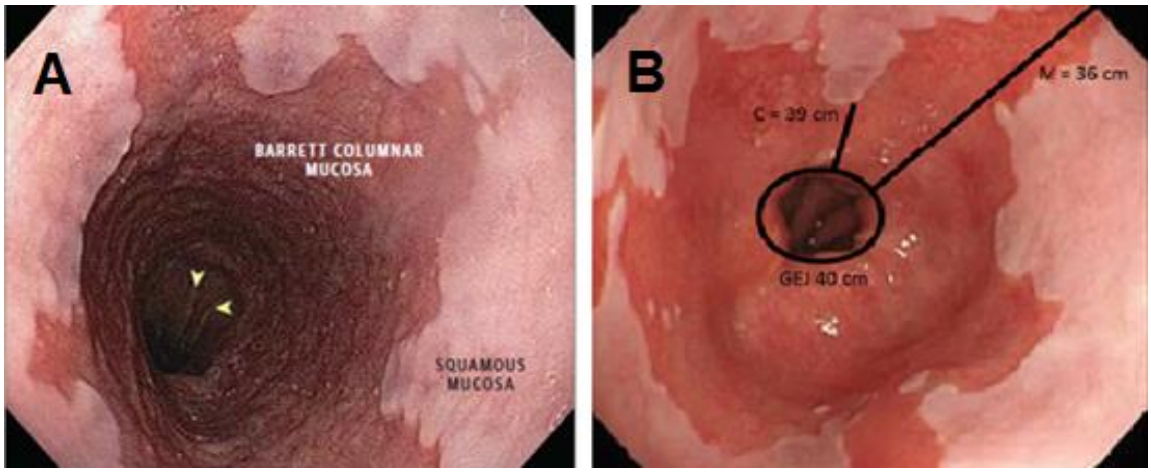


Figure 1.9 Endoscopic images of Barrett's oesophagus.

(A) Difference between Barrett's columnar mucosa and squamous mucosa. The yellow arrowheads indicate the top of the gastric folds, which determine the level of the gastroesophageal junction. **(B)** Prague classification of Barrett's oesophagus. -the C value is for a circumferential extent and the M value for maximal length. Image taken from Schoofs *et al. Annals of Gastroenterology*, 2017 [278].

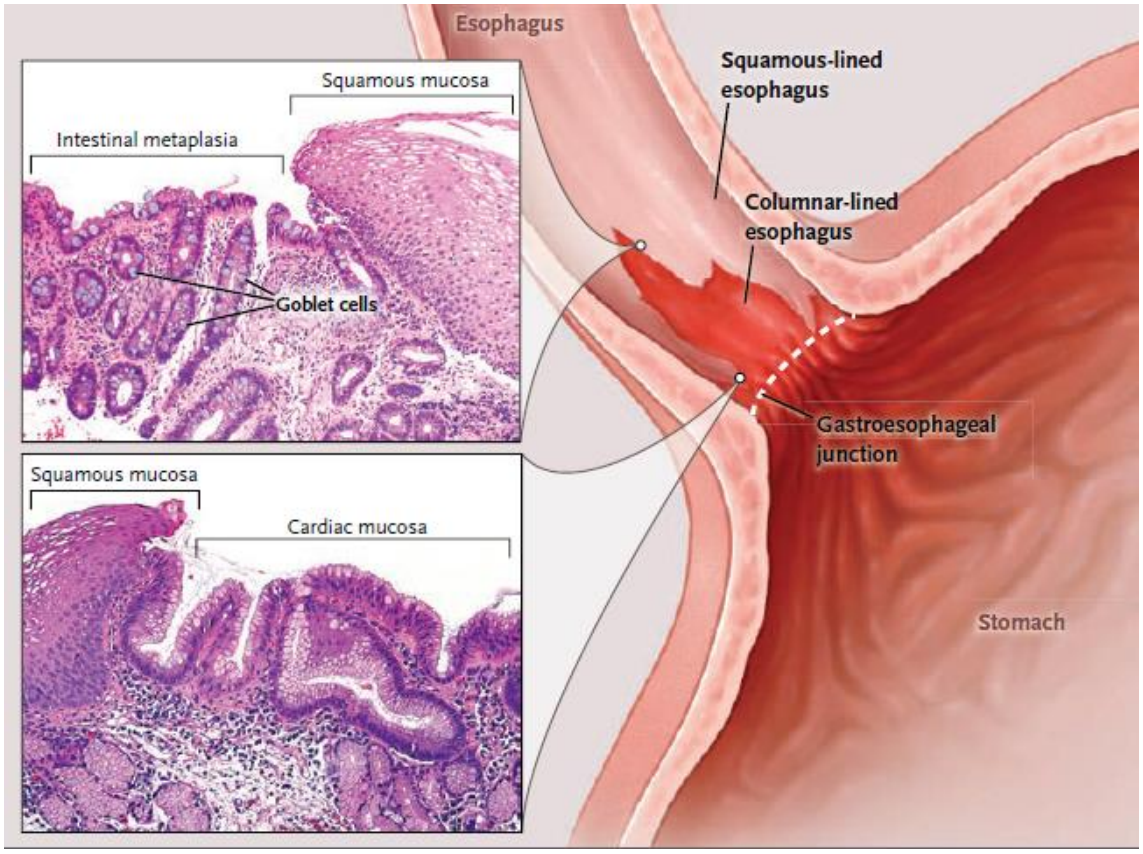


Figure 1.10 Haematoxylin & eosin staining for biopsy taken from distal oesophagus.

H&E staining reveals intestinal metaplasia with goblet cells, characteristic of Barrett's oesophagus. In the biopsy also cardiac mucosa may be identified within a Barrett's biopsy. The gastrointestinal junction is marked with a dashed white line. Image taken from Spechler *et al.New Engl J Med*, 2014 [283].

1.4.1.2. Dysplasia and Barrett's oesophagus

Malignant transformation of Barrett's oesophagus into OAC occurs through a series of histopathologic stages classified as metaplasia (no dysplasia), low-grade dysplasia (LGD), and high-grade dysplasia (HGD) (**Figure 1.11**). Dysplasia is characterised by abnormal epithelial cell development, growth and differentiation. Dysplasia is considered to be a pre-malignant condition with an increased risk of neoplastic development. LGD is a histologic diagnosis, characterised with pathological abnormalities: closely packed overlapping basal nuclei with hyperchromasia, basal stratification of nuclei, cytologic atypia extending to the mucosal surface, minimal or absent surface maturation, decreased goblet and columnar cell mucus [284]. HGD is considered to be a precursor of invasive adenocarcinoma. In HGD the cytological changes are severe with magnified nuclei at the surface, pronounced pleomorphism and focal loss of nuclear polarity. Changes in architecture are observed. Particularly, mild to marked architectural distortion, crowded glands, loss of lamina propria and cribriform or budding glands are detected [285]. A study on 210 patients with LGD has shown that the incidence of OAC was 0.44% per year. Progression to HGD was 1.6% per year. 5-year survival analysis showed that 97.4% of patients were free from cancer [286].

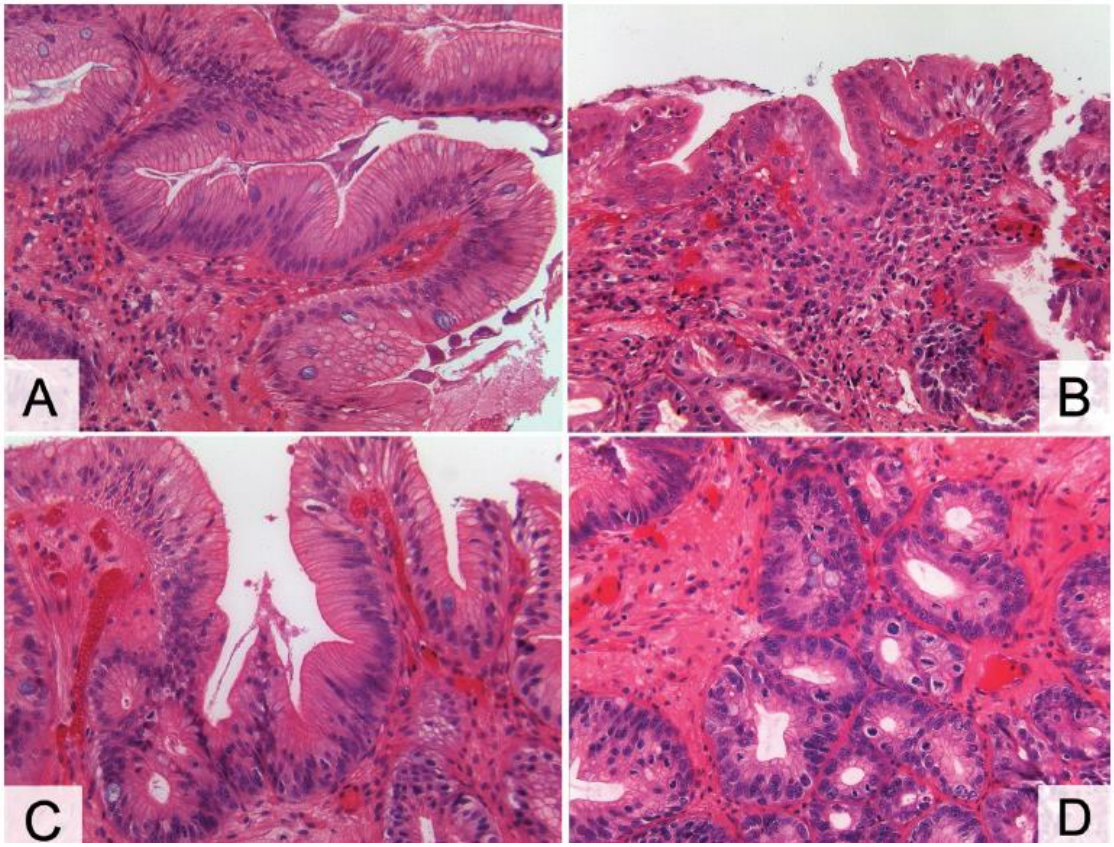


Figure 1.11 Immunohistochemical staining of Barrett's metaplasia and dysplasia using haematoxilin and eosin (H&E) staining.

A) Negative for dysplasia. Image shows columnar cell metaplasia with blue-tinted goblet cells. Nuclei are regular, smooth, and basally aligned. **B)** Dysplasia is present. Increased inflammation in lamina propria and epithelium. There is mild crowding of glands with mainly normal architecture. **C)** Low-grade dysplasia. Increased nuclear atypia with hyperchromasia, increased pleomorphism, and nuclear overlap. Distinct architectural changes with increased gland crowding. **D)** High-grade dysplasia. Cytological changes, nuclear enlargement, pleomorphism, hyperchromasia are observed. The glands are crowded with distinct decrease of lamina propria. Image taken from Booth and Thompson, *J. Gastrointestinal Oncology*, 2012 [285].

1.4.1.3. Oesophageal adenocarcinoma (OAC)

The mechanisms which induce malignant transformation in Barrett's oesophagus are still poorly understood, therefore understanding the links between inflammation and oesophageal carcinogenesis may provide important information on how to prevent cancer development. OAC is the predominant type of oesophageal cancer in Northern

America, Northern and Western Europe and the incidence of this cancer has increased sharply in the past few decades [287]. In 2012, 52,000 individuals with OAC were diagnosed worldwide, resulting in a global incidence rate of 0.7 per 100,000 persons per year (1.1 for men and 0.3 for women) [288]. Oesophageal adenocarcinoma is a highly lethal cancer, with a 5 year survival rate of 10% in most Western populations [289].

Risk factors

The main risk factors for OAC besides Barrett's oesophagus and gastro-oesophageal reflux are factors such as:

1. **Lower oesophageal sphincter relaxing drugs.** The studies were carried out in Sweden on patients using drugs associated with relaxation of the lower esophageal sphincter muscle (LES; muscle between the oesophagus and stomach). Results showed that daily use of these relaxing drugs (nitroglycerines, aminophyllines, β-receptor agonists, anticholinergics and benzodizapines) for more than 5 years increases the probability to develop OAC four-fold, compared to individuals who never used them [290].
2. **Body mass**. Many studies indicate the correlation between increasing body mass index (BMI) and risk of oesophageal adenocarcinoma, independent of reflux [291], [292]. Thrift *et al.* have shown that in people with BMI >30 (BMI for correct weight 18.5-24.9), the relative risk of developing OAC was increased by 16%, and BO risk increased by 12%, per 1 kg/m^2 increase in BMI [293].
3. **Tobacco smoking and alcohol drinking.** It is not clear whether smoking and alcohol drinking are risk factors for OAC. Some of the studies reported a moderately increased risk of OAC among tobacco smokers and people drinking alcohol [294], [295], whereas other shown no association between tobacco and alcohol and OAC development [296][297].
4. **Advancing age and male sex.** Men have approximately a 6-fold higher risk of developing OAC. This may be related to the more frequent use of tobacco and alcohol. Although the effect of BMI on the risk of OAC seems to be similar in women and men, abdominal obesity is more popular in men, which may be correlated with a higher risk of OAC [298].

Besides risk factors, some of the studies had shown inverse correlation between OAC development and *Helicobacter pylori* infection. Chow *et al*. showed that infection with $CagA^+$ strains of *Helicobacter pylori*, was significantly associated with a reduced risk of OAC and cancer by 60% [299]. Similar results were observed in a Swedish population, showing that relative risk for OAC associated with *H. pylori* infections was reduced by 50-80% [300].

Staging

The staging of OAC is based on the American Joint Committee on Cancer system, (**Table 1.1**) last updated in 2010. This was the first time when the staging of OAC was separated from squamous cell carcinoma. T1a cancers, also called intramucosal cancers, are confined within the mucosa and can invade the lamina propria. T1b can invade submucosa and has worse prognoses, similar to T2. T2 tumours invade the muscularis propria, T3 invade the adventitia and T4 can invade adjacent tissue [301].

Primary tumour	
Tis	High-grade dysplasia
T1a	Tumour invades lamina propria or muscularis mucosa (intramucosal)
T1b	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades adventitia
T4a	Resectable tumour invading pleura, pericardium, or diaphragm
T4b	Unresectable tumour invading other adjacent structures, such as aorta, vertebral body, trachea, etc.

Table 1.1 OAC staged, according to American Joint Committee on Cancer system, 2010.

Table taken from the American Joint Committee on cancer: the 7th edition of the AJCC Cancer Staging Manual and the future of TNM [302].

Treatment

Treatment approaches for OAC depend on the stage and grade of the tumour, location of the tumour and age of the patients. For superficially invasive adenocarcinoma (T1a, T1b) the most common treatments are endoscopic therapies, which have a high level of efficiency and low morbidity. Usually, it includes endoscopic resection followed by ablative therapy. For locally advanced OAC, in Ireland and the US, the most common

approach involves neoadjuvant chemotherapy and external beam radiotherapy, followed by oesophagostomy in a patient who are candidates for surgery. For patients with unrespectable or metastatic cancer, chemotherapy or chemoradiation therapy is recommended. In these patients, endoscopic therapy can offer significant palliation of dysphagia (difficulty swallowing). For patients with local advanced disease or with distant metastases, 5 year survival is less than 15% [301].

1.5. The role of inflammation and TLRs in cancer.

1.5.1. Innate immunity and inflammation in cancer.

Inflammation is a response of innate immunity to physical, physiological and oxidative stress and is associated with the release of a wide range of inflammatory cytokines and chemokines. In response to tissue injury, a cascade of cellular infiltration and cytokine release is observed. This involves activation and migration of immune cells, leukocytes (neutrophils, monocytes and eosinophils) to the place of damage. Neutrophils are the first recruited effectors in inflammation. Monocytes, which can differentiate into macrophages in tissue, are the next to migrate to the site of the tissue injury. After activation, macrophages can decrease immune reaction by releasing growth factors and cytokines which affect endothelial, epithelial and mesenchymal cells in the inflamed tissue [303]. There is a lot of evidence that many malignancies are induced by infections. The first time a connection between inflammation and cancer development was observed by Virchow in 1850s, who showed that chronic irritation can induce cancer and that inflammatory cells were present in clinical samples from tumours [304]. There are three mechanisms by which infections can cause cancer. Firstly, a factor causing persistent infection, and thus inducing chronic inflammation. Chronic inflammation contributes to cancer by creating a state of oxidative stress. Formation of reactive oxygen and nitrogen species can induce damage of DNA, proteins and cell membrane, can modify enzyme activities and gene expression, leading to carcinogenesis [305]. Secondly, infectious factors can directly transform cells, by inserting active oncogenes into the host genome and inhibiting tumour suppressors [306] and thirdly, viruses may contribute to the development of cancer by inducing

oncoproteins or by altering the expression of host cell proteins at the site of viral DNA integration [307].

1.5.2. Inflammation in the development of oesophageal adenocarcinoma

The main risk factors of oesophageal adenocarcinoma are gastroesophageal reflux disease and Barrett's oesophagus [308]. Both conditions are associated with inflammation of the oesophageal squamous epithelium. The molecular events that characterise the pathway from inflammation to metaplasia, dysplasia and then to adenocarcinoma are poorly understood. It was shown that chronic gastro-intestinal reflux induces oesophageal inflammation, which can progress to dysplasia and finally adenocarcinoma [309]. Other findings which confirm a link between inflammation and OAC is that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin can be correlated with decreased risk of cancer [310]. A better understanding of the inflammatory microenvironment will provide more evidence to confirm the role of inflammation in tumorigenesis. Prolonged inflammation may increase the release of pro-inflammatory mediators, like chemokines, cytokines, prostaglandins and relative oxygen/nitrogen species. Many of these mediators can promote cell growth, invasion and induce mutagenesis.

Analysis of the inflammatory profiles of oesophagitis (inflammation of the oesophagus), Barrett's oesophagus (BO), and normal oesophageal tissue have shown that pro-inflammatory cytokines IL-1β, IL-8 and IFNγ are significantly increased in oesophagitis compared to BO. It appears that, when BO develops, inflammatory profiles are altered. During oesophagitis, Th1-type (pro-inflammatory response) cytokines are mainly produced, whereas in BO Th-2 (anti-inflammatory response) type cytokines are predominant [311]. IL-10 levels are equally elevated in oesophagitis and BO, but only IL-4 in increased in BO [311]. Different studies have shown elevated levels of cytokines: IL-4, IL-6, IL-8, IL-1β and IFNγ in BO tissue compared to normal intestinal epithelium [312, 313]. Moreover, higher expression of IL-8 and IL-1β have been observed in OAC compared to dysplastic Barrett's epithelium [314].

1.5.2.1. Toll-like receptors in cancer

Increasing evidence shows that TLRs play important roles in cancer progression. To date, the different effects of TLRs on tumour have been reported in the literature. On

one hand, activated TLRs can suppress cancer progression, on the other hand, they can stimulate tumour progression, metastasis and influence tumour progression to chemotherapy [315]. Below, both types of action are described.

Toll-like receptors as a negative regulator of cancer

Currently, many TLRs ligands, which are inducing a protective immune response, are in clinical trials as antitumor drugs (**Table 1.2**).

It has previously been reported that TLR4 agonists LPS and OK-432, a *Streptococcus*-derived immunotherapeutic agent, possess high anti-tumour activity, after intratumoral administration, although they were not able to suppress tumour growth upon systemic administration [316]. Another study has shown that the TLR4 agonist OM-174 (lipid A analogue), increased absolute numbers of natural killer (NK), cytotoxic T lymphocyte (CTL) responses, increased numbers of CD4 and CD8 positive cells, and has anti-tumour activity [317]. To date, TLR9 is the only TLR for which systemic administration of a specificagonist has shown evidence of anti-tumour activity in clinical trials. It was shown that detection of the unmethylated CpG dinucleotides (CpG ODN) by this receptor, can suppress the growth of lymphomas, tumour of the brain and skin [318]. A possible mechanism underlying the anti-tumour activity of TLRs is their ability to activate tumour-specific immune responses by stimulating the migration of NK-cells and cytotoxic T-cells to the tumour site [319]. Another mechanism to explain the anti-tumour effect of TLRs is their conversion of M2 (tumour-stimulating) macrophages into M1 (tumour suppressing) macrophages and stimulation of the cellular anti-tumour immune response [27].

Type of cancer	**TLR**
Non-small cell lung carcinoma, late stage	TLR9
Melanoma, stage IV	TLR7
Melanoma, stage IIIb/c or IV	TLR9
Incompletely resectable pancreatic cancer	TLR2/6
Recidivism of non-Hodgkin’s lymphoma	TLR9
Recidivism of glioblastoma	TLR9
Chronic lymphocytic leukaemia	TLR7

Table 1. 2 TLRs in clinical trials.

Many TLR agonists are in clinical trials as anti-tumour agents. Both natural and synthetic agonists of TLR7 and TLR8 have demonstrated high activity against chronic lymphocytic leukaemia and tumours of the skin. The TLR9 ligands can supress the growth of lymphomas and tumours of the brain and skin. TLR2/6 ligands are used in resectable pancreatic cancer. Table taken from Shcheblyakov *et al*. *Acta Naturae*, 2010 [320]

Toll-like receptors induce progression of cancer

As previously discussed, inflammation and chronic infection are very common factors in cancer development. TLRs, which play an important role in innate immunity, are being studied in detail in the context of cancer development. A role for NF-κB in tumour development is well known [321] [322]. As TLR pathogen stimulation, can activate NF- κB and expression of NF- κB-dependent genes, a role for TLRs in tumour progression is being widely studied. Huang *et al*. have shown that TLR2 gene silencing in hepatocarcinoma cells results in reduced cell proliferation. Furthermore, they observed decreased secretion of IL-6 and IL-8, and showed that tumour volume was significantly reduced in mice that had been treated with the shTLR2 plasmid [323]. Other studies have shown that conditioned media from Lewis lung carcinoma (LLC) cells can activate macrophages through TLR2 stimulation. In $TLR2^{-/-}$ BMDMs, production of IL-6 and TNFα was significantly impaired. Consistent with this result, after LLC inoculation in $TLR2^{-/-}$ mice, they observed significantly decreased levels of TNFα, IL-1β, IL-6, Cxcl2 and Ccl4 [324]. Other studies have shown that TLR4 can also induce tumour progression. Sun *et al.* showed that TLR4 is highly expressed in oral squamous cell carcinoma and upon activation, NF-κB is activated, followed by the production of IL-6 and IL-8 [325]. Stimulation of TLRs with their ligands can activate myeloid cells and their progenitors, causing subsequent migration from the bone marrow into tissue [326] [324]. TLRs can promote cancer progression through stimulating angiogenesis by angiogenic factors like IL-8, VEGF and matrix metalloproteinase (MMP) [327]. Moreover, there is significant evidence that *H. pylori*, can bind to and signal through TLRs on gastric epithelial cells which induce inflammation and cancer progression [300, 328].

1.5.3. Toll-like receptor 2 in oesophageal adenocarcinoma progression

Recent studies have shown that in the human distal oesophagus, inflammation and metaplasia can be correlated with alteration of the microbiome and this change was observed during oesophagitis and BO [329]. There is some evidence showing that TLRs play an important role in OAC progression, however, role of TLR2 in OAC cancer is still not well characterised [330].

Previous studies have shown that TLRs are expressed in the normal oesophagus: oesophageal epithelial cells express TLRs 2, 3, 4, 7 [331] and in biopsies taken from normal oesophageal mucosa, TLR1, 2, 3 and 5 mRNA was detected [332]. Huhta *et al.* have shown expression of TLR1, 2, 4 and 6 in normal oesophageal squamous epithelium, columnar metaplasia, dysplasia of Barrett's oesophagus and oesophageal adenocarcinoma. Expression of these receptors was stepwise and increased in metaplasia, dysplasia and adenocarcinoma, compared to the normal epithelium [330]. Other studies have shown that TLR2 mRNA expression was significantly increased in OAC compared to the normal controls [333]. Immunocytochemistry reveals that in reflux esophagitis TLR2 was expressed around the papillae high up in the epithelium due to papillary hyperplasia, whereas in Barrett's oesophagus, TLR2 was localized to superficial epithelial cells, deeper crypts and lamina propria cells. In contrast, during OAC diffuse TLR2 expression was observed. Furthermore, activation of TLR2 in acid and bile salts-exposed Barrett's cells resulted in an increased number of lysosomes and mitochondria, lysosomal enzymes and induced the release of IL-8 chemokine [333]

1.6. Role of macrophages in cancer progression and metastasis

1.6.1. Tumour-associated macrophages (TAMs)

TAMs are an important link between inflammation and tumours. TAMs are key cell types in response to inflammation, function as scavengers, and are one of the most common immune cells in the tumour microenvironment [334]. The term TAM describes various macrophage subsets, which present different features, depending on the cytokine balance within the tumour microenvironment. Originally they were described as M2-like macrophages but follow-up studies showed that they can present both M1 and M2 polarisation hallmarks [335] [336]. TAMs exhibit a distinct transcriptional

profile from M1 and M2 macrophages and are described as a $CCL2^{high}CCL5^{high}IL\text{-}10^{high}$. Recent studies have shown that TAM can switch into M1 phenotype, which represents a very promising anti-cancer immunotherapeutic treatment strategy [337].

Many cancers are associated with inflammation that results in the recruitment of bone marrow-derived cells, which become circulating peripheral blood monocytes [338]. These monocytes are recruited to the tumour tissue and differentiate in response to specific cytokines, chemokines and growth factors produced by tumour cells in the tumour microenvironment. Hematopoietic cells (macrophages, neutrophils, mast cells) are recruited into many different tumours and the largest proportion of them has Tumour Associated Macrophages (TAMs) markers. However, in cancers such as gliomas and pancreatic cancer, TAM can originate from embryonic-derived tissue-resident macrophages [339]. Within the tumour site, they are differentiated in response to IL-4, IL-10, IL-13, and they promote tumour metastasis, angiogenesis and invasion [340] [341]. The direct stimulus for TAMs recruitment is provided by chemokines. The chemokine CCL2 was described in 1983 as the main chemotactic factor in tumorigenesis [342]. CCL2 is localized in epithelial areas of the tumour and [343] its level of expression is associated with a number of lymphocytes and macrophages localized in tumour [344]. Qian *et al*. showed that chemokine CCL2 and macrophage colony-stimulating factor (MCS-F) recruits inflammatory monocytes to pulmonary metastases. Other chemokines involved in monocyte recruitment are monocyte chemotactic protein-1 (MCP-1), and vascular endothelial growth factor (VEGF) [345], CCL5, CCL7, CXCL8 and CXCL12 [346]. Moreover, TAM can be recruited by other factors such as fibronectin, fibrinogen and products of ECM protein cleavage induced by macrophages or tumour cell-derived proteases [347]. Within the tumour site, TAMs accumulate into necrotic regions with low oxygen tension [348]. This localization is promoted by expression of HIF-1 dependent factors (VEGF, CXCL12 and its receptor CXCR4), which are induced in hypoxic regions [349] [350]. The most common markers for TAM are MGL-1, Dectin-1, CD68, CD206, VEGF-A, NOS2, CD81, MHCII and scavenger receptor A [351]. The presence of TAM has been shown to correlate with poor prognosis in breast, gastric, ovarian, oral, thyroid and bladder cancer, but not in colorectal cancer (CRC) [352]. Increase TAM density is usually correlated with advanced tumour progression and metastasis (80 % of all reports show a significant correlation between TAM density and poor prognosis; less than 10 % associate TAM density with a good prognosis) [353].

TAMs in angiogenesis

TAMs play important roles in angiogenesis by secreting growth and matrix remodelling factors such as vascular endothelial growth factor A (VEGF-A), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), urokinase-type plasminogen activator (uPA), metalloproteinase (MMP), which induce endothelial cell proliferation and facilitate their migration within the extracellular matrix. VEGF-A is the best-characterized angiogenesis factor and is recognized as a major pro-angiogenic cytokine realised by TAM [354]. VEGF-A binding with corresponding tyrosine kinase receptors, VEGFR-1 and VEGFR-2, and promote epithelial cell migration and proliferation [355]. TAMs also produce many angiogenesis modulating enzymes such as MMP-2, MMP-7, MMP-9, MMP-12, Cox-2, and chemokines: CXCL12, CCL2, CXCL8, CXCL1, CXCL13 and CXCL5 [347][353]. Many previous studies have shown the correlation between high numbers of TAM and high vascular grades in different cancers such as glioma, oesophageal squamous cell carcinoma, breast, bladder and prostate cancer [356], [357], [358], [359].

TAM in invasion and metastasis

TAM promote tumour invasion and metastasis by enhancing the invasion of malignant cells and by changing tumour microenvironment through secreting matrix proteins and several proteases such as serine proteases, MMP, cathepsins which can modify the ECM composition and promote basal membrane disruption. Previous studies have shown upregulation of MMP-9 during co-culture of macrophages with MCF-7 breast cancer cells [360], [361]. TNFα is produced by macrophages in response to pro-inflammatory signals. It has been shown that mice lacking the gene for TNFα are resistant to skin carcinogenesis [362]. Wyckoff *et al.* showed, with fluorescently labelled cells, that in mammary tumours, mobilization of malignant cells often occurs next to macrophages, which appear to assist tumour cells intravasation, directly on the vessels surface [363]. TAM produce IL-1β in pro-inflammatory response and it was shown that ablation of IL-1β in mice inhibits metastasis development in melanoma cell models, mammary and prostatic cancer cells. IL-1β is required for angiogenesis and invasiveness of tumour *in vivo* [353] (**Figure 1.12**).

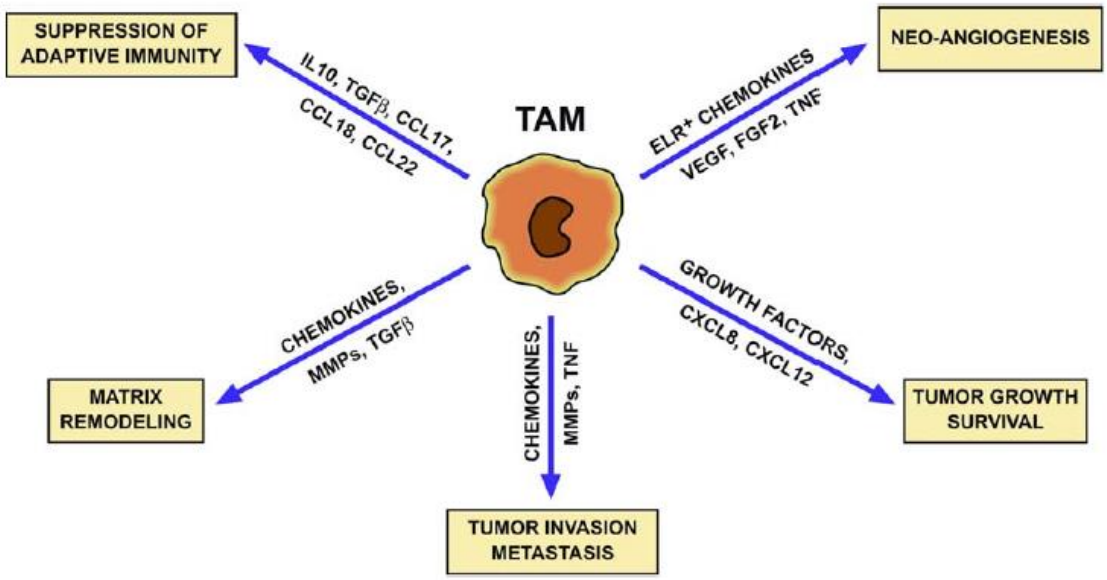


Figure 1.12 Tumour-associated macrophages (TAM) induced several pro-tumour functions.

TAMs macrophages can be polarised to acquire different pro-tumour functions. Tumour initiation is promoted through matrix remodelling, invasion, metastasis, tumour growth survival and angiogenesis. Each of these functions is promoted by different growth factors, chemokines and cytokines secreted from TAM in pro-inflammatory response. Picture taken from Sica *et al. ejconline,* 2006 [335]

1.6.2. Macrophages in oesophageal adenocarcinoma.

Evaluation of macrophage subtypes in adenocarcinoma tissues suggests that M1 macrophages are associated with the increased killing of tumour cells, while M2 macrophages correlate with tumourigenesis. As M1/M2 balance can be important for OAC progression, it is important to find the mechanisms which modulate polarisation of M1 and M2 macrophages located in the cancer microenvironment. It has been shown that in a rat model of gastric reflux, which is the main risk factor of Barrett's oesophagus, M1 macrophages infiltrate the oesophagus and activate pSTAT3 signalling in stromal cells and epithelium. Activation of this signalling promotes subsequent M2 macrophage polarisation and contributes to tumour development through regulatory T cells (Treg). The involvement of TAMs and Tregs in the inflammatory microenvironment promotes tumour development [364]. Cao W. *et al* demonstrate that

the number of M2-like macrophages was significantly increased in OAC patients with nodal spread, independent of locations. A higher M2/M1 ratio was associated with poor prognosis of OAC patients. Furthermore, they showed that OAC cells can polarise monocytes into M2-like macrophages, based on cytokine expression profile. Further studies showed that polarised M2-like macrophages subsequently promote OAC cell migration and invasion [365]. Interaction between macrophages and epithelial cells have a very important role in oesophageal cancer development [366]. Other studies from 2015, have shown that in OAC patients without neoadjuvant treatment, an increase of M2-like macrophages correlated with poor survival. The M2/M1 ratio was significantly higher in nodal positive OAC than in nodal-negative OAC. Studies also showed that conditioned media from OAC cell lines (SK-GT 4) can polarise THP-1 cells into TAM-like macrophages, which promote cancer cells migration and invasion [365]. Moreover, exosomes secreted from tumour microenvironment in OSCC are able to promote $PD1^+$ TAM expansion thus mediate cancer progression [120]. T. Yagi *et al.* reported that high density of TAM markers, CD163, CD204 was associated with poor prognosis. *In vitro* co-culture of oesophageal cancer cell lines with activated macrophages, significantly upregulate cancer cell invasion and migration [367].

1.7. Project hypothesis and aims

Overall hypothesis:

Targeting inflammatory response is a promising therapy in oesophageal adenocarcinoma and bacterial infection.

1. TLR2 presents a potential target to limit inflammation and hence disease progression of oesophageal adenocarcinoma.
2. Caspase-11 controls *Mycobacterial tuberculosis* infection in murine macrophages via its ability to regulate STAT1mediated nitric oxide production.
3. Caspase-4/-11 and non-canonical inflammasome activity are involved in peptic ulcer formation.

Specifically the main aims of the project are:

Chapter 1,

1. Determine TLR2 expression in a panel of oesophageal cell lines representing different disease stages.
2. Investigate whether a TLR2 neutralising antibody can inhibit TLR2 upregulation and activation in OAC cells.
3. Determine whether oesophageal cells release endogenous TLR2 ligands responsible for tumour-related inflammation and progression.
4. Identify the OAC secretory factor which is responsible for TLR2 acivation and its inflammatory effects.

Chapter 2,

1. Determine whether caspase-11 regulates iNOS expression and nitric oxide production in murine macrophages
2. Determine signalling pathway involved in caspase-11-mediated nitric oxide production
3. Investigate the importance of of caspase-11 during *Mycobacterial tuberculosis* infection.

Chapter 3,

1. Establish the expression levels of caspase-4/-11 during *H.pylori* infection and peptic ulcer disease.
2. Determine whether prostaglandin E2 (PGE_2) can inhibit caspase-11-mediated pyroptosis and inflammasome activation.

Chapter 2: Materials and methods

Materials

Table 2.1 Materials

Product	Source
Agarose (Electrophoresis grade)	Invitrogen
Ammonium Persulfate (APS)	Sigma Aldrich
Aprotinin	Sigma Aldrich
Bicinchoninic acid (BCA) assay	Thermo Scientific
Bovine Serum Albumine (BSA)	Sigma Aldrich
BEGM BulletKit	Lonza
Cell culture plastics	Cruinn Ltd.
Chemiluminescent	Millipore
Corning ®Costar® Not Treated Multiple Well Plates	Sigma Aldrich
Dimethyl Sulphoxide (DMSO)	Sigma Aldrich
Dithiothreitol (DTT)	Sigma Aldrich
DNA Ladder, Quick-Load 2-log	New England BioLabs
Dulbecco's Modified Eagles Medium (DMEM)	Biosciences
Ethanol	TCD Biohazard Stores
EDTA	Sigma-Aldrich
EGTA	Sigma-Aldrich
Foetal Bovine Serum (FBS)	Labtech
Fluorescent Mounting Medium	DAKO
GAPDH forward primer	MWG
GAPDH reverse primer	MWG
GoTaq® Hot Start Green Master Mix	Sigma-Aldrich
Glycerol	Sigma Aldrich
Glycine	Sigma Aldrich
Goat anti-Mouse IgG (H+L) Superclonal™ 2nd Ab, Alexa Fluor 647	ThermoFisher Scientific
Griess assay	Enzo
GoTaq Hot start green master mix	Promega
Heat-killed Lactobacillus rhamnosus	InvivoGen
Heat-killed Salmonella typhimurium	InvivoGen
Heat-killed Listeria monocytogenes	InvivoGen
High-binding-96-well plate	Cruinn Diagnostics
Human Serum	Sigma-Aldrich

Quick Load® 2-Log DNA Ladder	New England BioLabs
mIFNγ	PeproTech
Leupeptin	Sigma Aldrich
Lipofectamine™ 2000	Invitrogen
Lipopolysaccharide (LPS) from E. coli O111:B4	Sigma Aldrich
Lipopolysaccharide (LPS) from E.coli, R515	Alexis
Methanol	TCD Biohazard Stores
Nitrocellulose membrane 0.45 μm	Sigma Aldrich
Nuclease free water	Sigma Aldrich
NP-40	Sigma Aldrich
TLR2 mAb; T2.5	Invivogen
Opti-MEM	Invitrogen
Pam3CSK4 - Synthetic triacylated lipoprotein	Invivogen
Pam2CSK4- Synthetic diacylated lipoprotein	InvivoGen
Penicillin Streptomycin (Pen-Strep)	InvitroGen
Phenylmethylsulphonyl fluoride (PMSF)	Sigma Aldrich
Phosphate Buffered Saline (PBS)	Biosciences
PJK (mutant reverse primer)	MWG
Proteinase K	Sigma Aldrich
Rotor Stator Homogeniser	Janke and Kunkel
Ruxolitinib	Selleckchem
Sodium azide (NaN_3)	Sigma Aldrich
Sodium Chloride	Sigma Aldrich
Sodium deoxycholate	Sigma Aldrich
Sodium dodecyl sulphate (SDS)	Sigma Aldrich
SY21 (WT forward primer)	MWG
SY22 (WT reverse primer)	MWG
TEMED	Sigma Aldrich
TNFα	InvivoGen
Trizma base (TRIS)	Sigma Aldrich
TrypLE	Biosciences
Virkon tablets 5g	VWR
Water, DNAase/RNAse free	Biosciences

Table 2.2 Buffers and Solutions

Name	Composition
10X Phosphate-Buffered Saline (PBS)	• 1.45 M NaCl • 39 mM NaH_2PO_4 (Sigma-Aldrich) • 227 mM $NaHPO_4$ (Sigma-Aldrich) • pH 7.2
10X Tris-Buffered Saline (TBS)	• 10 mM Trizma Base (Tris) • 150 mM NaCl • pH 8.0
10X WB transfer buffer	• 0.25 M Trizma Base • 1.9 M Glycine
10X WB running buffer	• 0.25 M Trizma base • 1.9 M Glycine • 35 mM SDS
Wash buffer (TBST)	• 0.1 % (v/v) Tween-20/PBS (1X)
Resolving gel buffer	• 1.5 M Trizma base • pH 8.8
Stacking gel buffer	• 1 M Trizma base • pH 6.8
1X Laemmli buffer (Laemmli buffer was used as a 2X and 5X stock)	• 10% (v/v) Glycerol • 10% (w/v) SDS • 0.1% (w/v) Bromophenol Blue (Sigma-Aldrich) • 62.5 mM Tris-HCl • 150 mM DTT • pH 6.8
RIPA lysis buffer	• 50 mM Tris • 150 mM NaCl • 0.02% NaN_3 • 0.1% SDS • 1% NP-40 • 0.5% Sodium Deoxycholate Supplemented with: • 1mM PMSF • 1 µg/µl leupeptin • 1 µg/µl aprotinin
Subcellular fractionation buffer	• 20 mM HEPES (pH 7.4) • 10 mM KCl • 2 mM $MgCl_2$ • 1 mM EGTA • 1 mM EDTA Supplemented with: • 1mM PMSF

	• 1 µg/µl leupeptin • 1 µg/µl aprotinin

Methods

Table 2.3 Cell lines

Name	Histology	Cancer stage	Media	Source
GO	Human, oesophageal columnar epithelium, high grade dysplasia		BEGM + supplied supplements 10% (v/v) FBS 1% (v/v) Pen-Strep	American Type Culture Collection (ATCC)(LGC Standards, Middlesex, UK)
SK-GT 4	Distal oesophagus adenocarcinoma, well-differentiated	IIB	RPMI 1640 GlutaMAX™ 10% (v/v) FBS 1% (v/v) Pen-Strep	American Type Culture Collection (ATCC)(LGC Standards, Middlesex, UK)
OE33	Distal oesophagus adenocarcinoma, poorly-differentiated	IIA	RPMI 1640 GlutaMAX™ 10% (v/v) FBS 1% (v/v) Pen-Strep	European Collection of Authenticated Cell Cultures (ECACC) (Sailsbury)
FLO-1	Distal oesophagus adenocarcinoma	III	RPMI 1640 GlutaMAX™ 10% (v/v) FBS 1% (v/v) Pen-Strep	European Collection of Authenticated Cell Cultures (ECACC) (Sailsbury)
OACM5.1C	Lymph node metastases of Distal Oesophageal Adenocarcinoma	IV	RPMI 1640 GlutaMAX™ 10% (v/v) FBS 1% (v/v) Pen-Strep	European Collection of Authenticated Cell Cultures (ECACC) (Sailsbury)
OACP 4 C	Gastric cardia adenocarcinoma	IV	RPMI 1640 GlutaMAX™ 10% (v/v) FBS 1% (v/v) Pen-Strep	European Collection of Authenticated Cell Cultures (ECACC) (Sailsbury)
THP1	Human Monocyte cell line. Derived from acute monocytic		RPMI 1640 GlutaMAX™ 10% (v/v) FBS 1% (v/v) Pen-Strep	American Type Culture Collection (ATCC) (LGC Standards, Middlesex, UK)

	leukaemia patient			
THP1-XBlue-CD14	Derived from THP-1 cell line		RPMI 1640 GlutaMAX™ 10% (v/v) FBS 1% (v/v) Pen-Strep Selective antibiotics: 200 µg/ml of Zeocin™ 250 µg/ml of G418	InvivoGen
HEK-293T	Cells derived from human embryonic kidney		DMEM GlutaMAX™ 10% (v/v) FBS 1% (v/v) Pen-Strep	American Type Culture Collection (ATCC) (LGC Standards, Middlesex, UK)

2.1. Cell culture

Cell lines and primary cells were cultured in level II laminar flow cabinets and maintained within aseptic conditions. Cells were monitored daily under a bright light microscope (Leica DMIL) to ensure no overt contamination/infection within the flasks. To count cells, a haemocytometer (under 10X magnification) was used. Cells were grown in appropriate media as described in **Table 2.3.** All cell line media was supplemented with 10% of foetal bovine serum (FBS) with ultra-low endotoxin levels and 1% of penicillin-streptomycin (Pen-Strep, 100 U/ml and 100 µg/ml, respectively). OE33, SK-GT 4, FLO-1, OACM5.1C, OACP 4C, THP1, THP1-XBlue-CD14 were grown in Roswell Park Memorial Institute (RPMI) 1640 GlutaMAX^{TM}. GO cells were cultured in BEGM^{TM} Bronchial Epithelial Cell Growth Medium Bullet kit with provided kit supplements (2 ml BPE, 0.5 ml hydrocortisone, 0.5 ml hEGF, 0.5 ml epinephrine, 0.5 ml transferrin, 0.5ml insulin, 0.5 ml retinoic Acid, 0.5 ml triiodothyronine, 0.5 ml GA-1000 per 500 ml media). Cultured cells were grown in sterile T25, T75 or T175 flasks, in an incubator maintained at 37 °C and 5% CO_2, 95% O_2.

2.1.1. OAC and Barrett's dysplasia cell lines

OAC and Barrett's dysplasia cell lines were used: OE33, SK-GT4, FLO-1, OACM5.1, OACP4 C, and GO. All OAC cells grown in RPMI medium with 10 % FBS, 1 % Pen-Strep. All oesophageal cell lines were passaged two-three times per week, depending on when cells reached approximately 80% confluency. Cells were subcultured by initial washing with 5 ml Phosphate Buffer Saline (PBS) followed by incubation with 2 ml TrypLe Express until cells had detached sufficiently from the plate. The TrypLe Express was deactivated by adding 10 ml media and cells were transferred into 50 ml falcons and centrifuged at 400 g for 5 min. The cell pellet was resuspended in 1 ml media and cells were split in a 1:5 and 1:4 ratio for OAC cell lines and GO, respectively.

2.1.2. HEK293 cell lines

Human embryonic kidneys 293T cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, 1% Pen-Strep. Cells were maintained at 2 x 10^5 cells/ml and sub-cultured when cells reached 80-90% confluency. Cells were passaged as described for OAC and GO cells lines, above.

2.1.3. THP-1 cell line

The human monocytic cell line, THP-1, were originally derived from an acute monocytic leukaemia patient. THP-1 cells are suspension, grown in RPMI, medium with 10% FBS, 1% Pen-Strep. Cultures were maintained by the addition of fresh medium or replacement of medium every 2 to 3 days. Cells were sub-cultured when cells concentration reached 8 x 10^5 cells/ml and never grown beyond 1 x 10^6 cells/ml.

2.1.4. THP1-XBlue-CD14

THP-1-XBlue-CD14 are derived from human monocytic THP-1 cells. Cells stably express NF-κB- and AP-1- inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. SEAP was described for the first time in 1988 as a novel reporter gene. As alkaline phosphatase (AP) is not normally secreted from eukaryotic cells, the SEAP gene that encodes a secreted form of human placental AP was generated. For this purpose, SEAP expression vectors under the control of the RSV or HIV-I LTR promoters were generated and transfected into monkey cell lines and two distinct assays

were employed to reveal that it can be successfully used for experiments to accurately measurement of reporter gene expression [368]. Upon TLR activation in THP1-XBlue-CD14 cells, transcription factors are activated, and SEAP is secreted. SEAP is detectable with QUANTI-Blue solution. QUANTI-Blue is a colorimetric enzyme assay developed to determine alkaline phosphate (AP) activity in a biological sample. QUANTI-Blue solution changes from pink to purple-blue in the presence of AP. THP1-XBlue-CD14 express all TLRs. To maintain selection pressure, selective antibiotics: 200 µg/ml Zeocin and 250 µg/ul G418 were added at every passage. Cells were passaged every 3 days by inoculating 5 x 10^5 cells/ml and were not grown beyond 2 x 10^6 cells/ml.

2.1.5. Isolation and culture of Bone Marrow-Derived Macrophages

Bone Marrow Derived Macrophages (BMDM) are primary macrophage cells derived from the femurs and tibiae of C57BL/6J wild-type, Caspase-11-, TLR2-, NLRP3-, IFNR1- or IFNy-deficient mice during this study. TLR2 and NLRP3, IFNR1, IFNγ deficient mice were kindly provided from Dr Sarah Doyle and Prof. Ed Lavelle Labs, respectively. Mice were housed in a sterile pathogen free facility in Trinity College Dublin and humanely sacrificed by CO_2 inhalation. Following euthanasia, mice were sprayed with 70% ethanol, dissected and leg bones were removed for cell isolation. In a tissue culture hood, cleaned femurs were flushed to extrude bone marrow using sterile 25G needles and a syringe filled with PBS. Bone marrow cells were centrifuged at 400 g for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 3 ml red blood cell lysis buffer for 2 min. To neutralise the lysis buffer, 7 ml DMEM, 10 % FBS, 1 % Pen-Strep was added and cells were centrifuged at 400 g for 5 min. If red blood cells were still evident in the pellet, this step was repeated. Isolated cells were seeded on T175 flask in 20 ml DMEM, supplemented with 20 % L929 media, and cultured for 7 days, with a change in media after 3 days. Macrophage Colony Stimulating Factor (M-CSF) is present in L929 media (**see 2.1.6**).

2.1.6. L929 cell line & Generation of L929 conditioned media.

L929 cells are a mouse subcutaneous connective tissue cell line derived from a 100-day old male C3H/An mouse. L929 cells constitutively secrete M-CSF and to a lesser extent, GM-CSF, which can augment the effects of M-CSF on monocytes and macrophages [369] [370]. Cells were cultured in DMEM GlutaMAX, 10 % FBS, 1%

Pen-Strep. L929 cells were seeded at 2 x 10^7 cells in a T175 flask containing 40 ml medium. Cells were grown in a humidified incubator with 5% CO_2 at 37°C for 10 days. The resulting supernatants were filtered through a 0.45-µm filter, aliquoted and stored at -20°C for use as L929 conditioned media.

2.1.7. Isolation of human monocytes and differentiation into macrophages

Primary human monocytes derived macrophages (MDM) were isolated from Buffy packs by density gradient centrifugation and adherence purification. Buffy packs were obtained from the Irish Blood Transfusion Services (Dublin, Ireland). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. Blood from buffy packs were transferred into a sterile 100 ml container and blood was diluted 1:1 with sterile phosphate buffered saline (PBS). 25 ml of diluted blood was gently layered on top of 15 ml Lymphoprep (Stemcell) in a 50 ml tube and the mixture was centrifuged at 400 g for 25 min at room temperature. The buffy coat layer of PBMC was transferred with Pasteur pipette to a fresh 50 ml falcon. The volume in the PBMC falcon tube was brought up to 50 ml with PBS and the falcon was spun at 300 xg for 10 min. Supernatant was discarded and the PBMC pellet was resuspended in 1 ml of red cell lysis buffer (Sigma) for 1 min to lyse erythrocytes. The red cell lysis buffer was neutralised with 50 ml PBS and the falcon was centrifuged at 300 g for 10 min. The cell pellet was resuspended in 5 ml RPMI media supplemented with 10 % human serum.
Cells were counted and seeded at 2 x 10^6 cells/well in 12-well non-coated adherent tissue plates (Sigma, CLS3737-100EA). Cells were cultured in RPMI-1640 supplemented with 10 % human AB serum (Sigma-Aldrich) on adherent plates. Non-adherent cells were removing by washing every 2-3 days. Monocytes are highly adherent to plastic and thus the purification of monocytes was based on this attribute. Cells were cultured for a total of 7 days to allow differentiation to macrophages.

2.1.8. Generation of OAC conditioned media

Conditioned media was collected from TLR2-stimulated GO, SK-GT4 and OE33 cell lines in the presence/absence of the TLR2 neutralising antibody (αTLR2, T2.5) as follows: Cells were seeded at 2 x 10^5 (GO), 1.6 x 10^5 (SK-GT 4), 2 x 10^5 (OE33) and cultured for 24 h. The following day, cells were washed with PBS and pre-incubated for 1 h in the presence/absence of 10 µg/ml αTLR2. Cells were subsequently stimulated with 0.05 µg/ml Pam_3CSK4 (P3C), 0.05 µg/ml Pam_2CSK4 (P2C), 1 µg/ml LPS or 10^7

cells/ml HKLM for 4 h. Following 4 h stimulation, media with synthetic ligands was removed, cells were washed 3X with PBS and fresh 2 ml RMPI 1640 (10% FBS, 1% P/S) was added for a further 24 h incubation. After 24 h, conditioned media from GO (GO CM), SK-GT 4 (SK-GT 4 CM) and OE33 (OE33 CM) were collected and 50 CM was directly added to monocytes/macrophages.

2.1.9. Cell counting

Cell counting was performed using a bright light haemocytometer. To count cells, 10 µl of cell suspension (diluted 20X) was added between the haemocytometer and coverslip using a P20 pipette. The haemocytometer was placed under a bright light microscope with 10X objective. The number of cells was counted in the 4 corner squares and then number was divided by 4 to obtain average number. Value was multiplied by the dilution factor and then by 10^4 to give the number of cells per millilitre. The appropriate number of cells, diluted in appropriate culture media, was seeded in tissue culture plates for each relevant experiment. Seeded cells were allowed ~18 h to adhere on the plate.

2.1.10. Cryopreservation of cells

Low passage number stocks of each cell line were cryopreserved in liquid nitrogen (-180°C). Cells were frozen down for long term storage and stored in liquid nitrogen with a cryoprotective agent, Dimethylsulfoxide (DMSO). DMSO is a widely used cryoprotectant as it applies a slower cooling rate by reducing the freezing point of the media, thereby preventing ice crystal formation which would damage the cells. Freezing medium was prepared with 40 % culture medium, 50 % FBS and 10 % DMSO. Cell pellets (10^7cells) were resuspended in 1 ml of freezing medium and transferred to 1.5 ml cryovials where they were immediately transferred to isopropanol in Mr. Frosty™ freezing container and stored at -80°C for 24 h, before transferring to liquid nitrogen for long term storage. The freezing container allows for gentle freezing of cells, at a rate of 1°C per/min, to minimise cold shock.

2.1.11. Thawing of cells

Cryovials were removed from liquid nitrogen and rapidly thawed in a 37°C water bath (ensuring the rim of the cryovial does not to go below the water surface). The cells were then immediately transferred into 10 ml pre-warmed culture medium and centrifuged

for 5 min at 300 xg. Cells were gently resuspended in 5 ml complete culture medium, transferred into a 25 cm^2 T25 flask and grown at 37°C in a 5% CO_2 (v/v), 95% O_2 (v/v) incubator until confluent. After 24 h the growth media was completely replaced to minimise the presence of trace amounts of DMSO.

2.1.12. Mycoplasma detection in cell cultures

Mycoplasma is one of the main problems in cell culture. It can affect cell proliferation, cell agregation and morphological and physiological changes. In addition to monitoring cell morphology under microscop, cell lines were tested for mycoplasma every 5-6 months using polymerase chain reaction (PCR) as described by Young *et al.* [371]

To detect if mycoplasma is present, media (1mL) was collected from a confluent flask of each cell lines and transferred to eppendorfs. To remove cell debris, eppendorfs were centrifuged at 400 x g for 1 min at RT. A PCR reaction MIX was made up as described in **Table 2.5** and primers decribed in **Table 2.4** were used. As negative control water and as positive control, supernatants containing mycoplasma were used. The PCR reaction was performed in a PTC-200 Peltier Thermal Cycler (Bio-Rad, **Table 2.6**). Following PCR reaction, DNA samples were run on a 2% agarose gel by DNA electrophoresis. DNA bands were visualised using a GelDoc-it System (UVP, LLC, CA, USA) under ultra-violet light.

Table 2.4 Mycoplasma primers for PCR

Primer	**Sequence**
Forward	5'-TGCACCATCTGTCACTTCTGTTAACCTC-3'
Reverse	5'-GGGAGCAAACAGGATTAGATACCCT-3'

Table 2.5 PCR reaction components

Component	**Volume (µL)**
DNA (isolated from cell culture supernatants)	0.5
Forward primer (10 µM stock)	0.5
Reverse primer (10 µM stock)	0.5
GoTaq ® Hot Start Master Mix (2X)	12.5
Nuclease free water	11
Total reaction volume	25

Table 2.6 PCR reaction conditions

Temperature (°C)	Time (cycles)
95	5 min (1)
94	30 sec (40)
55	30 sec (40)
72	1 min (40)
72	10 min (1)
4	∞

2.2. Cell stimulations

2.2.1. OAC cell stimulations

The OAC cell lines were seeded at 2 x 10^5 (GO), 1.6 x 10^5 (SK-GT 4), 2 x 10^5 (OE33), 2x10^5 (FLO-1) on 12 well plates. Following day, cells were treated with either 1 μg/ml LPS, 20 ng/ml TNFα or TLR2 ligands (**Table 2.7**) for 24 h. After stimulation, supernatants were collected, and cells were lysed with RIPA buffer supplemented with protease inhibitors (10 μg/ml leupeptin, 1.7 μg/ml aprotinin and 0.1 mM PMSF).

2.2.2. THP1-XBlue-CD14 stimulations

THP1-XBlue-CD14 were seeded at 4 x 10^6 cells/ml in 96 well plate. Cells were stimulated with 50 % OAC conditioned media (CM) (**see 2.1.8**), TLR ligands (**Table 2.7**), and IFNγ (negative control) at 37°C in a 5 % CO_2 incubator.

Table 2.7 TLRs agonists

Name	Specificity	Used concentration	Stock concentration
Pam_3CSK4- Synthetic triacylated lipoprotein	TLR1/2	0.05 μg/ml	1 mg/ml
Pam_2CSK4- Synthetic diacylated lipoprotein	TLR2/6	0.05 μg/ml	1 mg/ml
HKLM-Heat Killed *Listeria monocytogenes*	TLR2	10^7	10^{10}
HKST-Heat Killed *Salmonella typhimurium*	TLR2	10^6	10^{10}

HKLR-Heat Killed *Lactobacillus rhamnosus*	TLR2	10^7	10^{10}
LPS (Sigma)	TLR4	1 µg/ml	0.1 mg/ml
LPS (Alexis)	TLR4	1 µg/ml	0.1 mg/ml

2.2.3. MDM & BMDMs stimulation

BMDMs and MDM were seeded at 1 x 10^6 cells/ml. After 24 h, cells were washed with PBS before stimulating with 50% SK-GT 4 conditioned media (SK-GT 4 CM), 0.05 µg/ml Pam_3CSK4, 0.05 µg/ml Pam_2CSK4 or 1µg/ml LPS. After 24 h stimulation, supernatants and lysates were collected for further analysis. For ELISA/nitric oxide measurements, BMDMs were stimulated with LPS (1 µg/ml), IFNy (10;40;100 ng/ml), Pam_3CSK4 (0.05µl/ml), Pam_2CSK4 (0.05µl/ml)or with 50% SK-GT 4 CM; and inhibitors, Ruxolitinib (0.1; 0.5 µM) and Fludorabine (5-50 µm) added 1 h prior stimulation. To collect lysates, medium was removed, wells were washed with PBS and lysed using RIPA buffer, supplemented with protease inhibitors (10 µg/ml leupeptin, 1.7 µg/ml aprotinin and 0.1 mM PMSF).

2.2.4. TLR2 inhibition

To analyse the role of TLR2 in OAC progression, cell lines or primary cells were pretreated for 1 h with anti-TLR2 neutralising antibody (T2.5, InvivoGen; 10 µg/ml) prior to stimulation with TLR ligands. This commercially available antibody was obtained from, Opsona Therapeutics Ltd. during the project secondment. MAb-mTLR2 (T2.5) is a monoclonal antibody that reacts with mouse TLR2 (CD282). MAb-mTLR2 is an antagonistic antibody. The antibody is cross reactive with human TLR2.

2.2.5. Human data

The ulcer study was conducted after ethical approval was granted from the SJH/AMNCH Research Ethics Committee in accordance with European communities (Clinical Trials on Medicinal Products for Human Use) regulations 2004 & ICH GCP

guidelines. Patients participating in the study were between 18 – 80 years of age referred by their treating clinician for a gastroscopy based on standard clinical assessment. Two additional peptic biopsies, one at the side of any ulcer or inflammation and one from adjacent non-affected tissue were taken. One antral and one corpus biopsy was taken from unaffected control patients. Patients with a known history of peptic malignancy (carcinoma or lymphoma), stromal tumour, or previous upper gastrointestinal surgery, as well as those with the endoscopic description of a mass, polyp, nodule, or lump anywhere in the oesophagus, stomach, or small intestine were excluded from the study. Biopsies were anonymous and stored in -80 in AMNCH and TBSI until analysis. Our procedures had no effect on patients care and reports to their primary consultants which were carried out as normal.

2.3. Western Blot analysis

2.3.1. Preparation of lysates

To harvest cells, culture plates were placed directly on ice. Supernatants were transferred into 1.5 ml eppendorfs, and cells were washed with cold PBS. Ice cold RIPA buffer (to analyse whole cell lysates) or subcellular fractionation buffer (to analyse cytoplasmic fraction) (**Table 2.2**), supplemented with protease inhibitors (10 μg/ml leupeptin, 1.7 μg/ml aprotinin and 0.1 mM PMSF), were added into each well (10^7 cells/ml lysis buffer). Cells in RIPA buffer were incubated 5 min on ice and were transferred to 1.5 ml eppendorfs and centrifuged at 20,000 g for 10 min, 4°C in an Eppendorf 5417R refrigerated centrifuge. The protein lysates were transferred into new Eppendorf tubes and stored at -20°C for next use. To prepare cytoplasmic fractions, subcellular fractionation buffer was added, cells were scraped, collected into 1.5 ml eppendorfs and incubated for 30 min on ice. Samples were centrifuged at 720 xg for 5 min. Supernatants which contained cytoplasm, mitochondria and membrane fractions were transferred into new 1.5 ml eppendorfs (the pellet contains nuclei). Supernatants were centrifuged at 10,000 xg for 5 min to remove the mitochondrial fraction. Following that, supernatants were transferred into new 1.5 ml Eppendorf tubes and protein concentration was determined using BCA assay.

2.3.2. Preparation of lysates from OVA-induced allergic airway inflammation

The whole lungs were isolated from indomethacin and misoprostol treated mice which had been subjected to OVA-induced allergic airway inflammation. The lungs were placed into 500 µl RIPA and homogenisation was performed using a bench-top rotor-stator homogeniser (Ultra-Turrax, Janke & Kunkel). Samples were centrifuged at 20,000 for 10 min at 4°C in an Eppendorf 5417R refrigerated centrifuge. The protein lysates were transferred into new Eppendorf tubes and protein concentration was determined using BCA assay. Western Blot samples were prepared using 2x Laemmli sample buffer with 150 mM DTT to obtain final concentration 1 µg/µl. Samples were boiled at 90°C for 5 min and stored at -20°C for further use.

2.3.3. Preparation of lysates from peptic biopsies.

Biopsies from ulcer and from adjacent non-affected tissue were placed in 200 µl RIPA buffer and homogenised using 5mm Stainless Steel Beads (200, QIAGEN). Following homogenisation, samples were centrifuged at 20,000 xg for 10 min at 4°C in an Eppendorf 5417R refrigerated centrifuge. Protein lysates were collected and transferred to new Eppendorf tubes and protein concentration was determined using BCA assay. Western Blot samples were prepared using 2x Laemmli buffer with 150 mM DTT to obtain final concentration 1 µg/µl. Samples were boiled at 90°C for 5 min and stored at -20°C for further use.

2.3.4. Protein precipitation from cell-conditioned media using StrataClean™ beads.

StrataClean TM resin (Agilent) is a phenol-free technique for DNA purification. The solid phase silica-based resin contains hydroxyl groups that react with proteins in a similar manner as a hydroxyl group of phenol. Before use, resins were vortexed until the slurry was completely resuspended. Resin was added at 10% of total sample volume and conditioned media was agitated on a rotator for 1 h at 4 °C. Samples were centrifuged for 5 min at 1000 xg (4°C) and supernatants were discarded. Resin beads were resuspended in 1X Laemmli buffer with 150 mM DTT and boiled at 90°C for 5 min. Prepared samples were stored at -20°C for further use.

2.3.5. Determination of protein concentration

The Bicinchoninic Acid (BCA) Protein Assay was used to quantitate total protein concentration. The principle of this method is that proteins reduce Cu^{2+} to Cu^{1+} in alkaline solution, resulting in a purple colour formation by bicinchoninic acid. The purple reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentration over a broad working range (20-2000 µg/ml).

For this assay a standard curve between 2.5-320 µg/ml of bovine serum albumin (BSA) was prepared by two-fold serial dilutions. 50 µl of each sample was added in duplicate to a 96 well plate. 50 µl of Micro BCA™ Working Reagent (25-parts Reagent MA, 24-parts Reagent MB and 1-part Reagent MC) was added to each sample and plate was incubated at 36°C for 40 min in the dark. Absorbance at 562 nm was acquired using the Spectra Max PLUS340 Microplate Reader (Molecular Devices). Protein concentration was determined relevant to the BSA standard curve and samples with normalized protein concentration were prepared.

To prepare samples for Western blot, 1X Laemmli buffer (**Table 2.2**) with 150 mM DTT were added and samples were boiled at 90°C for 5 min. Ready to use samples were stored at -20°C for further use.

2.3.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used method for separating proteins by electrophoresis, using a discontinuous polyacrylamide gel and sodium dodecyl sulphate (SDS) to denature proteins. SDS is an anionic detergent which disrupts the tertiary structure of proteins. Proteins unfold into linear chains with negative charges, causing them to migrate to the anode when an electrical charge is applied to the gel.

Polyacrylamide gels were prepared as described in **Table 2.8**. Firstly, clean glass plates were assembled. Resolving gel was prepared, mixed and poured into the gap between glass plates, leaving space for stacking gel. H_2O was added on the top of the gel to eliminate air bubbles. After 20 min H_2O was removed and stacking gel was poured onto resolving gel and the appropriate comb was immediately inserted. After 30 min, gels were polymerised and ready to use. Gels in plates were then placed in a Biorad gel

electrophoresis rig, situated in a tank and filled with 1X Running Buffer (**Table 2.2**). Combs were removed and equal amount of total proteins (4-20 µg) were loaded into wells along with molecular weight marker (SeeBlue Plus2 pre-stained protein ladder). Gels were run at 90V until the dye moved into resolving gels and then voltage was increased into 120V until the dye front runs off the bottom of the gels.

Table 2.8 Components of polyacrylamide gels

Reagent	15% Resolving	12% Resolving	10% Resolving	5% Stacking
dH_2O	2.4 ml	3.4 ml	4.1 ml	3.4 ml
Protogel (30 % acrylamide, 0.8 % bisacrylamide)	5 mL	4 mL	3.3 mL	1 mL
1.5 M Tris-HCl (pH 8.8)	2.5 ml	2.5 ml	2.5 ml	-
1 M Tris-HCl (pH 6.8)	-	-	-	1.5 ml
10% SDS	100 µl	100 µl	100 µl	60 µl
10% APS	50 µl	50 µl	50 µl	60 µl
TEMED	5 µl	5 µl	5 µl	1.5 µl

2.3.7. Nitrocellulose wet transfer

Directly after SDS-PAGE, gels were removed from the glass plates and stacking gel was removed and discarded. A Bio-Rad wet transfer system was used to transfer proteins from the gel onto Protran BA 85 Nitrocellulose membrane (GE Healthcare Life Science). The nitrocellulaose membrane, filter paper and sponges were soaked in 1X Transfer buffer (**Table 2.2**). The transfer sandwich was then set up in the transfer cassette in the following order: sponge, two filter papers, gel, nitrocellulose membrane, two filter papers, and sponge. Air bubbles were removed and the gel-membrane 'sandwich' was placed between two electrodes in the transfer apparatus, submerged in a 1X Transfer buffer. When the electric field is applied, the protein from the gel will migrate from cathode towards anode to the membrane where the proteins become tightly attached. Protein from the gel was transferred into nitrocellulose membrane for 40-80 min (depending on the protein size) at 200 mA or overnight at 40mA.

2.3.8. Blocking, immune-blotting and visualisation of protein

Following protein transfer to nitrocellulose membrane, non-specific binding sites on the membrane were blocked by incubation in 5 % non-fat dried milk (Marvel) in 1X TBST (**Table 2.2**) for 1 h at room temperature (RT). Following blocking, membrane was probed with the appropriate primary antibody (**Table 2.9**) diluted in 5 % milk in TBST overnight at 4°C (or 2 h at RT). The following day, the membrane was washed in TBST (3x10 min) and then incubated with relevant secondary antibody (**Table 2.10**) conjugated to horse radish peroxidase (HRP) for 1 h at RT. Membrane was washed 3 x 10 min with TBST, transferred to a new container and then enhanced chemiluminescent (ECL) substrate was added on the membrane to detect antibody-bound proteins. The ECL substrate contains Luminol Enhancer Solution and Peroxidase Solution. In the presence of HRP, Luminol oxidises and forms an excited-state product that emits lights as it decays to the ground state.

This results in a release of light appearing as a luminescent band on the membrane. The ECL was added to the membrane and placing in a BioRad Gel DocTMXR+System to develop. For analysis BioRad ChemiDocTM MP Imaging System was used.

Table 2.9 Primary antibodies for Western Blotting

Name (anti-)	Features	Sources
HMGB1 (3935S)	Rabbit polyclonal (3935)	Cell Signaling
TLR2	Goat polyclonal (AF2616)	R&D system
mNLRP3 (15101S)	Rabbit monoclonal (clone D4D8T)	Cell Signaling
hNLRP3 (13158)	Rabbit polyclonal (Clone: D2P5E)	Cell Signaling
Caspase 11 (C1354)	Rat monoclonal (clone 17D9)	Sigma
iNOS (13120S)	Rabbit monoclonal (clone D6B6S)	Cell Signalling
Stat1 (#9172)	Rabbit polyclonal (9172)	Cell Signalling
Phospho-Stat1(#7649)	Rabbit monoclonal (Tyr701)	Cell signalling
RAGE (sc-74473)	Mouse monoclonal (Clone E-1)	Santa Cruz
Lamin B (12586)	Rabbit monoclonal (D4Q4Z)	Cell Sinaling
mCaspase-1 p10 (sc-514)	Rabbit polyclonal (Clone: M-20)	Santa Cruz
Caspase-4/Tx (M029-3)	Mouse monoclonal (Clone: 4BD)	MBL
Caspase-5 (M060-3)	Mouse monoclonal (Clone: 4F7)	MBL
IL-1β	Goat polyclonal (Clone: AF-401)	R&D System

β-actin (A3854)	Mouse monoclonal (clone: AC-15)	Sigma Aldrich
GAPDH	Mouse monoclonal (clone 6C5)	Merck

Table 2.10. Secondary antibody for Western Blotting

Name (anti-)	**Sources**
HRP conjugated anti-rat IgG	Jackson Immunolabs
HRP conjugated anti-rabbit IgG	Jackson Immunolabs
HRP conjugated anti-goat IgG	Jackson Immunolabs
HRP conjugated anti-mouse IgG	Jackson Immunolabs

2.3.9. Densitometry analysis of protein expression

In order to compare target protein expression levels between different samples on the same blot, loading control was used to normalize data. Each blot was probed with housekeeping gene, β-actin (42 kDa) or GAPDH (36 kDa), to measure general expression of protein in samples.

To get an objective measure of the signal generated on a Western blot, densitometry analysis of Western blots was performed with Bio-Rad Image Lab software. The relative density of each band of interest was normalised to the density of the relative control. Densitometry analysis was performed on blots from three independent experiments and results were presented as a mean ± SEM to obtain statistical significance for densitometry graphs.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Cytokine concentrations were determined using commercially available ELISA kits (**Table 2.11**) according to the manufacturer guidelines. All ELISAs were performed in high binding 96 well plates (Greiner MICROLON®). Briefly, the ELISA plate was coated with capture antibody (50 µl per well) and plate was incubated at 4°C overnight. Next day, plate was washed 4 times with PBST (1xPBS with 0.05% Tween). All washes were removed by tipping the plate over the sink. The remaining drops were removed by gently patting the plate onto a paper towel. In the next step, 100 µl Assay Diluent (1% BSA in PBS) was incubated at RT for 1 h to reduce non-specific binding. The plate was washed 4 times with PBST before adding 50 µl samples/standards to wells at the appropriate dilutions and incubating at RT for 2 h or at 4°C overnight. Washes were repeated and 50 µl of biotinylated detection antibody diluted in Assay Diluent (200X) was incubated at RT for 1 h. Washes were repeated (3 x PBST) and 50 µl of HRP-conjugated Streptavidin diluted in Assay Diluent (1000X) was applied to the plate and incubated for 30 min at RT. The plate was then washed x5 in PBST with 30 second incubations to reduce background. Next, 50 µl of TMB solution was added to each well and the plate was incubated for 15-30 min at RT in darkness. 20 µl 2N H_2SO_4 was added to stop the reaction. To determine optical density, the plate was read at 450 nm using a Spectra Max Microplate Reader (Molecular Devices). Unknown cytokine concentrations were determined by plotting a standard curve of the known concentrations for each ELISA plate.

Table 2.11. Commercial ELISA kits

Cytokine	Supplier	Top working standard (pg/ml)	Species
IL-6	Biolegend/MSC	2,000	Human
TNFα	Biolegend/MSC	500	Human
IL-8	Biolegend/MSC	1,000	Human
IL-10	Biolegend/MSC	2,000	Mouse
IL-6	Biolegend/MSC	500	Mouse
TNFα	Biolegend/MSC	500	Mouse
IL-1α	Biolegend/MSD	125	Mouse
IL-β	Meso Scale Discovery (V-PLEX kit).	1030	Mouse

2.5. Real-Time Polymerase Chain Reaction (RT-PCR)

2.5.1. RNA isolation

RNA was isolated using Isolate II RNA Mini kit (Bioline) by following the protocol as issued by the manufacturer. To isolate RNA from the cells, indicated cells were seeded for specific experiments on 24 well plates. After stimulations, supernatants from the cells were removed, wells were washed with PBS and lysis buffer RLY (350 µl). Plate was incubated for 10 min on ice to lyse cells. After this time lysis buffer RLY was transferred to appropriate tubes and filtered through columns to remove viscosity and cell debris. An equal amount of 70 % ethanol was added into flow-through to precipitate nucleic acid before filtering through a blue mini column. Lysates were loaded onto RNA binding membrane. To digest DNA, 95 µl of DNase I mix, was added directly onto to centre of the RNA binding membrane in collection tube. In the next step, columns were washed once with wash buffer RW1 (to inactivate the DNase I), and then twice with RW2 buffer. Between every wash, columns were spun down at 11,000 xg for 30 seconds. Finally, RNA was eluted into nuclease-free 1.5 ml collection tubes, using 40 µl RNase free water and centrifugation at 11,000 xg for 1 min.

2.5.2. RNA quantification

Quantification and purity of RNA samples were assessed with the NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The NanoDrop® ND-1000 consists of an upper and lower measurement pedestal. RNA samples were applied onto the lower measurement pedestal. The sample is then automatically drawn between the upper and lower measurement pedestals to facilitate spectral measurement. Nuclease free water was used for blank measurement. Between each measurement, old samples were wiped from the upper and lower pedestals. The NanoDrop® calculates absorbance ratios of 260:280 nm and 260:230 nm to measure purity of each sample. For 260:280, RNA is considered as a 'pure' when ratio is around 2. If the ratio is appreciably lower, it may indicate presence of proteins or phenols that absorbs strongly at or near 280 nm. In the secondary measure, 260:230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower, it may indicate the presence of contaminants (EDTA, phenol, carbohydrates) which absorb at 230 nm.

2.5.3. Reverse transcription

Reverse transcription was carried out using High Capacity cDNA Reverse transcription kit (Applied Biosystem) by following the standard protocol as issued by the manufacturer.

The kit was allowed to thaw, before preparing mix on RNase free bench. 5.8 µl Master Mix was added into each PCR tube, following addition of 14.2 µl of mix samples and RNase free water (to get 1 µg of RNA in total per reaction) (**Table 2.12**). Reverse transcription was performed using a PTC-200 Peltier Thermal Cycler (Bio-Rad, Dublin, Ireland) according to **Table 2.13.** Following reverse transcription, samples were diluted 20X in nuclease free water, and stored -20°C until further use.

Table 2.12 Reverse transcription Master Mix

Reagent	Volume (µl)
10x Reverse Transcription (RT) buffer	2
25x deoxyribose nucleoside triphosphate (dNTP) Mix	0.8
10x RT random primers	2
Reverse transcriptase	1
Nuclease free water + RNA	14.2

Table 2.13 Thermal cycler program conditions

Settings	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

2.5.4. Quantitative Polymerase Chain Reaction (qPCR)

A Real-Time PCR also known as quantitative polymerase chain reaction (qPCR) of cDNA samples was carried out using SYBR^{TM} Green PCR Core Reagents (Invitrogen). Amplifications were performed in 1 µg cDNA diluted with nuclease free water. Appropriate primers were designed **(Table 2.15)**, using the Primer designing tool-NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All primers were checked with Standard Nucleotide Blast, to identify any unspecified primer targets. PCR reaction mix of each particular gene of interest was prepared on ice in 1.5 ml eppendorfs as outlined in **Table 2.14.** 4.7 µl cDNA was added in duplicate to each well of a 96-well PCR MicroAmp plate (Biosciences). 5.3 µl of the relative gene expression mix was added to each well. Reaction was carried out with One Step Plus v2.0 (Applied Biosystem). Data were normalized to GAPDH and mRNA expression fold-change relative to controls was calculated using the $2^{-\Delta\Delta ct}$ method using forward and reverse primers (Eurofins).

Table 2.14 PCR Reaction MIX

Reagent	Volume (µl)
SYBR™Green PCR Core Reagents (2x)	5
Primers (forward + reverse)	0.3

Table 2.15 Primers used in PCR reaction

Gene	Forward primers (5'->3')	Reverse primers(5'->3')
TLR1	GCC CAA GGA AAA GAG CAA AC	AAG CAG CAA TAT CAA CAG GAG
TLR2	ATC CTC CAA TCA GGC TTC TCT	ACA CCT CTG TAG GTC ACT GTT G
TLR6	CAG TTA ATA CTT TAG GGT GC	CGT TTC TAT GTG GTT GAG GG
Arg1	CTC CAA GCC AAA GTC CTT AGA G	AGG AGC TGT CAT TAG GGA CAT C

2.6. QUANTI Blue™ assay

To analyse whether conditioned media from OAC cell lines were secreting TLR ligands, THP1-XBlue-CD14 cells were used. THP1-XBlue-CD14 stably express an NF-κB- and AP-1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. Upon TLR stimulation, THP1-XBlue-CD14 cells activate transcription factors and subsequently the secretion of SEAP which can be detected in supernatants with QUANTI Blue™, by a spectrophotometric change in colour to purple/blue.

For experiments, 50 % conditioned media from SK-GT 4, OE33 and GO were added (**see 2.1.7**) to THP1-XBlue-CD14 (seeded at $4x10^6$ /ml) (50 µl CM added to 50 µl THP1-XBlue-CD14 cells) and incubated for 24h. After this time 160 µl QUANTI-Blue™ was added into each well and incubated with 40 µl supernatants from THP1-XBlue-CD1 for 2 hours. SEAP levels were determined with the Spectra Max PLUS340 Microplate Reader (Molecular Devices) at 650 nm. THP1-XBlue-CD14 cells were stimulated directly with TLR ligands and IFNγ as positive and negative controls, respectively.

2.7. Determination of TLR2 expression using Flow Cytometry.

TLR2 expression in the panel of OAC cell lines was determined using flow cytometric analysis, performed using a BD Accuri C6 Plus (BD Biosciences). OAC, THP1, and HEK 293 cells used at 2 x 10^5 cells in 200 µl PBS per experiment. Two samples were generated per cell line, one incubated with primary and secondary antibody and the second, control sample, with secondary antibody only, to identify any non-specific binding. Cells were washed with PBS and centrifuged at 400 xg for 5 min. Cells were blocked with 5% BSA (200 µl/sample) for 15 min, to reduce non-specific binding. Cells were subsequently centrifuged at 400 g, 5 min and incubated on ice with 10 µg/ml of αTLR2 for 15 min. After this time, cells were washed with PBS, centrifuged and incubated on ice with 2 µg/ml of secondary anti-mouse antibody Alexa Fluor 647 for 30 min in the dark. Finally, cells were washed with PBS, centrifuged as usual, and resuspended in 200 µl 5 % BSA in PBS for analysis.

2.8. Griess assay

The Griess assay detects the presence of nitrite (NO_2^-) in solution. NO_2^- is one of two primary, stable and non-volatile breakdown products of NO. Griess reagent contains sulphanilamide and N-alpha-naphthyl-ethylenediamine dihydrochloride (NED), the sulphanilamide binds to nitrite forming a diazonium derivative which then binds to N-alpha-naphthyl-ethylenediamine dihydrochloride to form an Azo compound with an absorbance at 540 nm. The detection of nitrite in solution is an indicator of NO production as NO is quickly metabolised to nitrite in cells (therefore the higher the concentration of nitrite, the higher the rate of NO production).

Serial dilutions of sodium nitrite solution in PBS of concentrations ranging from 100 µM to 1.56 µM were prepared in duplicate on a 96-well plate (50 µl standard solutions were added to the plate). 50 µl sample supernatants in duplicate were added to the 96-well plate and 50 µl Griess reagent was added to each sample well. Following incubation for 2-5 min at RT, absorbance at 540 nm was measured using the Spectra Max PLUS340 Microplate reader. The total nitrite concentration of each sample supernatant was determined using the sodium nitrite standard curve.

Figure 2.1 Chemical reactions involved in the measurement of NO2 – using the Griess Reagent System.

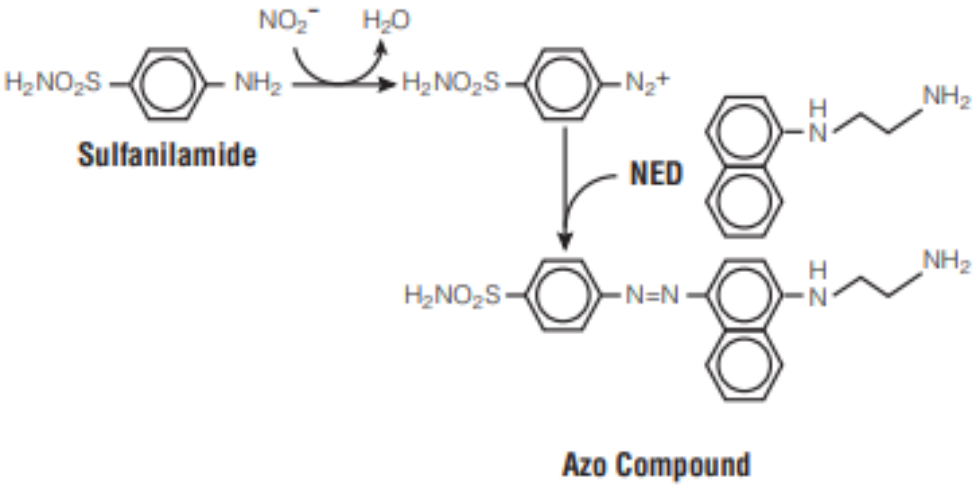


Image taken from: *worldwide.promega.com; technical bulletin*

2.9. Genotyping

2.9.1. Genomic DNA isolation and extraction

Ear punches were retrieved from all mice born to the caspase-11 weaning. Each punch was placed in 500 µl DNA extraction buffer (100 mM Tris-HCl; 5 mM EDTA; 0.2% (w/v) SDS; 200 mM NaCl, 500 µg/ml Proteinase K) and incubated in a 55°C waterbath overnight. Following complete digestion, the samples were centrifuged at 20,000 xg for 10 min at 4°C 475 µl supernatants were transferred into 950 µl ice cold 100% ethanol. Samples were vortexed to aid DNA precipitation, centrifuged at 20,000 xg for 10 min at 4°C and DNA pellets were allowed to air dry for approximately 1 h. Pellets were resuspended in 35 µl nuclease free water and stored at -20°C.

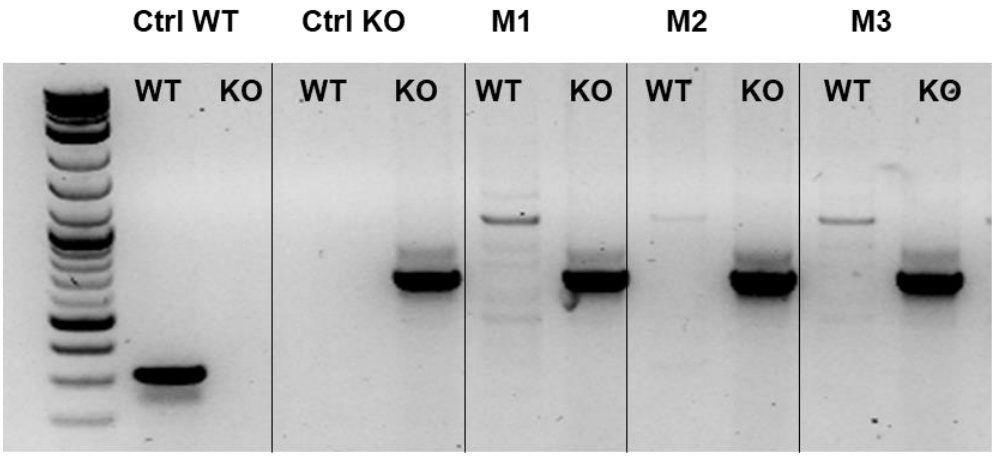


Figure 2.2 Agarose gel electrophoresis of PCR products.

Genomic DNA was isolated from wild-type and caspase-11 deficient mice ear punches and amplified via PCR. Samples with loading buffer were loaded on 2% agarose gel and resolved according to molecular size. Lane 1 100 bp marker ledder (Quick-Load® 2-Log DNA Ladder (0.1-10 kb), lanes 1-2 wild-type control mice, lane 3-4 Caspase-11 control mice, lane 5-6; 7-8; 9-10 represent mice 1 (M1), 2 (M2) and 3 (M3) respectively.

2.9.2. Total DNA quantification

DNA concentration and quality were assessed using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.,Waltham, MA, USA). The NanoDrop® ND-1000 is a full-spectrum (220-750 nm) spectrophotometer that measures 1-1.5 µl samples with high accuracy and reproducibility.

The concentration of DNA was calculated by the software based on the absorbance at 260 and analysis constant, which for DNA is 50 ng/µl. The ratio of absorbance at 260 and 280 nm is used to access the purity of DNA. The ration of ~1.8 is generally accepted as a 'pure' for DNA. If the ratio is significantly lower, it may indicate presence of protein, phenol or other contaminants with absorbance near to 280 nm.

2.9.3. Polymerase chain reaction (PCR)

The genomic DNA isolated from caspase-11 colony mice was amplified via PCR to differentiate between mice containing the WT and mutant caspase-11 genes using the primers listed in **Table 2.16**

The PCR reaction was carried out using the GoTaq® Hot Start Green Master Mix which consistent of a 2X ready-to-use-solution of GoTaq® Hot Start Polymerase, dNTPs (400 μM dATP; 400 μM dGTP; 400 μM dCTP; 400 μM dTTP) and 4 mM MgCl2. The volumes of each PCR reaction component are shown in **Table 2.17.** PCR conditions were: 1 cycle of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and finishing with 10 min incubation at 72°C (**Table 2.18**). The PCR was performed using PTC-200 Peltier Thermal Cycler (Bio-Rad, Dublin, Ireland).

Table 2.16 Primers used for PCR.

Primer	Sequence
SY21 (WT forward primer)	5'GGCATGGAGTCAGAGATGAAAGAC 3'
SY22 (WT reverse primer)	5'GCCCATGTGGCATTACCTGCCAGC 3'
SYKO (mutant forward primer)	5'AGATCTACACCTCGCACAACTGG 3'
PJK (mutant reverse primer)	5'TGGCGCTACCGGTGGATGTGGAATTGT 3'
GAPDH forward primer	5'TTCACCACCATGGAGAAGGC 3'
GAPDH forward primer	5'GGCATGGACTGTGGTCATGA 3'

Table 2.17 PCR reaction components.

Component	Volume (μl)
Template DNA (100 ng)	2
GoTaq® Hot Start Master Mix (2X)	10
Forward primer	0.4
Reverse primer	0.4
Nuclease free water	7.2
Total volume	20

Table 2.18 PCR reaction conditions

Temperature (°C)	Time (Cycles)
94	3 min (1)
94	1 min (35)
55	1 min (35)
72	1 min (35)
72	10 min (1)
4	∞

2.9.4. DNA gel electrophoresis

DNA agarose gels (2% (w/v)) were prepared by heating electrophoresis grade agarose in 1 X TAE buffer (40 mM Tris-acetate; 1 mM EDTA) until fully dissolved. Following the addition of 1X SYBR® Safe DNA gel stain to the solution, it was placed in a Horizon® 11.14 Gel Electrophoresis Apparatus (Life Technologies, Dublin, Ireland) and left to set. Once set, it was submerged in 1X TAE buffer and 20 µl of each sample was loaded into the wells together with size marker, Quick-Load® 2-Log DNA Ladder (0.1-10 kb) (New England BioLabs® inc., UK). Gels were run at 120 V for 40 min. Results were visualised and imaged under UV light using a GelDoc-itTM System (UVP, LLC, CA, USA).

2.10. Statistical analysis

Data were analysed using Excel Microsoft Office 365 ProPlus and Prism 5 Software (GraphPad). Error bars indicate SEM as indicated. The unpaired two-tailed Student *t*-test was used to compare the mean values between two groups. When three or more variables were present, the two-way analysis of variance (ANOVA) followed by Bonferroni post-test was used. p values less than 0.05 were considered significant, *$p<0.05$; **$p<0.01$; $p<0.001$.

2.11. Supplementary methods

The following methods were performed by another individual and analysis was carried out by Ewelina Flis. Therefore, these have been included in a supplementary section.

2.11.1. *Mycobacterium tuberculosis* infection

Mycobacterium tuberculosis (Mtb) infection was carried out by Emer E. Hackett from Frederick J. Sheedy's Lab, Trinity College Dublin.

Mtb strain H37Ra was obtained from ATCC and prepared according to the manufacturer's instructions. H37Ra stocks were made in 1 ml aliquots and stored at -80°C. Before use, aliquots were thawed, resuspended in 5 ml sterile PBS and sonicated for 15 min. Falcons were centrifuged at 3800 xg for 10 min. This process was repeated 10 times. The supernatant was removed, the pellet was resuspended in 2 ml DMEM and sonicated for 15 min. Cell suspension was syringed through a series of needles of decreasing gauge size down to 25G until the solution passed through the needle with resistance. Cells were grown to log phase in Middlebrook 7H9 broth (Difco) supplemented with ADC enrichment medium (Becton Dickinson) and 0.05 % Tween 80 (Difco) made in sterile water. *Mtb* was then sub-cultured weekly for four to five weeks. On the day of infection, bacteria in log-phase were centrifuged at 1,600 xg for 10 min, pallet was collected, and cells were resuspended in DMEM. To de-clump bacteria, cells were passed through a 25G needle several times. To obtain single cell suspension the bacterial suspension was centrifuged at 70 xg for 3 min. After centrifugation, the supernatant of this spin was quantified by spectrophotometry (OD_{600nm}) and used to infect macrophages. Macrophages were infected at a MOI of 5 bacilli per cell (MOI 5) for 3 h. After 3h media with bacteria were collected and bacteria were removed by centrifugation at 18,000 g for 10 min. Macrophages were washed with DMEM to remove remaining bacteria and bacteria-free media was returned to the wells. Macrophages and supernatants were collected after 3 h, 24 h and 72 h for analysis. Baseline growth was assessed by lysing 3 h time-point in 0.1% Triton-X for 10 min. Serial dilutions were plated on 7H10 Middlebrook Agar plates in triplicate and colony-forming units enumerated after incubation at 37°C for 14 – 21 days after plating. For later growth measurements this lysate was combined with pelleted extracellular bacteria, obtained by centrifugation of supernatant.

2.11.2. *Helicobacter pylori* infection.

Helicobacter pylori infection was carried out by Rebecca Fitzgerald, Sinead Smith's Lab, Trinity College Dublin.

Helicobacter pylori strain NCTC 11638 was obtained from NCTC collection. Bacterial biomass was obtained by growth of the clinical *H. pylori* strain NCTC 11638 on Colombia blood agar under microaerophilic conditions at 37°C. Bacteria were inoculated into Brucella broth with 10% FCS and grown under microaerophilic conditions at 37°C overnight with shaking. Bacteria were washed in PBS (pH 7.4) and resuspended in antibiotic-free culture medium (DMEM) before the infection of cell cultures. Macrophages were infected with *H. pylori* strain NCTC11638 for 6 h, 24 h and 48 h at a MOI of 1:50 and 1:100.

2.11.3. OVA-induced allergic airway inflammation.

Induction of OVA-induced allergic airway inflammation was carried out by Dr Zbigniew Zaslona, Luke O'Neil's Lab, Trinity College Dublin.

Allergic airway inflammation was induced by intraperitoneal injection of 20 μg antigen OVA (chicken leg ovalbumin) mixed with 2 mg of adjuvant aluminum hydroxide (alum), followed 7 days later by two airway challenges with 1% OVA. Allergic airway inflammation was characterised by eosinophilic inflammation and induction of TH2 cytokines in bronchoalveolar lavage fluid (BALF). Murine lungs were collected 24 h after the last airway challenge.

2.11.4. *In vivo* misoprostol and indomethacin treatment.

Misoprostol is a stable methyl ester of a PGE_1 analogue [372] and indomethacin is a nonsteroidal anti-inflammatory drug which mediates its effects via targeting cyclooxygenase (COX) to inhibit prostaglandin production [373]. To determine the effect of PGE_2 and indomethacin on allergic airway inflammation, mice were injected subcutaneously with 200 μg saline containing 2 mg/kg of misoprostol or indomethacin in 0.5% DMSO, 2 h before intraperitoneal sensitization and airway challenge with OVA. Control mice were treated with 200 μg saline containing 0.5% DMSO alone.

2.11.5. Preparation of microglial cells from WT and Caspase-11-deficient mice

Brains were isolated from WT and Caspase-11 deficient mice and homogenized using the gentle-MACS Dissociator (Miltenyi Biotec, Woking, UK) in combination with the AdultBrain Dissociation Kit (Miltenyi Biotec, Wokin, UK), according to the manufacturer's instructions. Homogenized tissue was washed in cold PBS and centrifuged 300 xg for 10 min at 4°C. The resulting pellet containing microglia was resuspended in 90 µl buffer (provided in the kit) per 10^7 total cells., stained with CD11b microbeads (10 µl of CD11b MicroBeads per 10^7 total cells; Miltenyi Biotec, Woking, UK) and magnetically separated using the QuadroMACS separator (Miltenyi Biotec, Woking, UK) according to the manufacturer's instructions. Cells were seeded in a 24 well plate at 1.6 x 10^5 cells/well (final volume 500 µl). Cells were cultured in DMEM (10 % FBS, 1% pen-strep, supplemented with M-CSF (100 ng/mL, R&D system, Abingdon, UK) and GM-CSF (100 ng/mL, R&D system, Abingdon, UK)) in a humidified incubator, 5% CO_2 at 37°C for 7 days and subsequently used for experiments.

Chapter 3: HMGB1-mediates TLR2 inflammatory pathways during oesophageal adenocarcinoma.

3.1. Introduction

Persistent, sustained inflammation can predispose cellular tissue to the development of malignancy and the tumour microenvironment is necessary for the tumour progression process [338]. In the oesophagus, long standing inflammation caused by acid reflux and bile acids causes the replacement of squamous cells with more intestinal-like, columnar epithelial cells containing goblet cells (known as Barrett's metaplasia). Over the course of time and disease progression, Barrett's metaplasia may begin to develop features of dysplasia, which is defined by abnormal epithelial cell development, growth or differentiation [374]. Dysplasia is not malignant, however is it considered a pre-malignant condition with an increased risk of developing cancer [375]. Oesophageal cancer represents the sixth most common form of cancer worldwide [376], and its increasing incidence and poor prognosis make it a significant global public health problem. The majority of oesophageal cancer is oesophageal squamous cell carcinoma (OSSC) but incidence of oesophagous adenocarcinoma (OAC) is rising rapidly in Western world [377]. Most cases are detected very late, with a fatality rate of 90% [378]. The current standard of care for OAC patients is resective surgery, which is often preceded by chemoradiation therapy. Pre-operative therapy is considered to be better tolerated than post-operative chemoradiotherapy, permits downstaging and better resectability in the subsequent surgery, and may eradicate occult disease [379]. However, the majority of OAC patients at advanced levels of disease fail to benefit from current clinical therapies, as the 5 years survival rate remains at <20%. Therefore, a significant proportion (50-60%) of patients do not benefit from pre-operative therapy [380]. Although there is a clear clinical need for OAC patient stratification, based on predicted response to therapy, currently no predictive markers have been successfully applied for individual patient treatment (i.e. guidance of patients to NAT or direct surgery).

It has been postulated that conversion of squamous cells into intestinal-like epithelial cells during Barrett's progression protects the oesophagus from acid and bile induced tissue damage. This mechanism is observed in the gut where intestinal epithelial cells (IECs) provide protection by allowing colonization of commensal bacteria which aid in digestion in the intestinal tract [381]. The gut microorganisms participate also in synthesis of certain vitamins, promoting the development of the gastrointestinal immune system, regulating metabolism and preventing pathogen invasion. Disruption of

relationships between host and commensal bacteria may lead to gastrointestinal carcinogenesis by disrupting epithelial barriers, triggering inflammation and inducing subsequent DNA damage or pro-oncogenic signalling. The role of microbiota in the oesophagus is not as well-known as in the distal gastrointestinal tract, however several studies suggest that it may have an important role in Barrett's oesophagus and oesophageal adenocarcinoma development [366]. Alteration of the microbiota results in dysregulation of innate immune signalling and may trigger chronic inflammation and breakdown of intestinal homeostasis. [382, 383]. Microbes or their products could activate some receptors on the epithelial cell membrane and lead to the production and release cytokines, chemokine and other inflammatory factors involved in inflammation [384]. TLRs recognize molecules derived from many types of microbes [385] and have a well-known role in carcinogenesis [382]. It has been demonstrated that significant differentials in oesophageal microbiome composition between healthy subjects and those with oesophagitis or Barrett's oesophagus [386] exist, therefore there is rationale to explore the potential role of TLRs in BO and OAC progression.

TLRs are critical regulators of innate immunity, and their dysregulation is associated with a number of inflammation-associated malignancies [64]. TLR engagement on cancer cells can stimulate the release of chemokines and danger-associated molecular patterns (DAMPs), signals which can promote cancer survival, tumour progression and regulate immune responses within the tumour microenvironment [387]. Importantly NF-κB, which is activated through TLRs, has been identified as a key transcription factor that may be involved in transition from epithelial inflammation to cancer [388]. TLR1, 2, 3 and 5 are expressed in human oesophageal epithelial cells and in normal human oesophageal mucosa. It has been shown that although TLR3 is the most abundantly expressed TLR in the oesophageal epithelium, activation of this receptor upregulates the expression and ligand responsiveness of TLR2 [389]. Verbeek *et al.* demonstrated that TLR2 is expressed in normal squamous oesophagus and its mRNA increased in reflux esophagitis, Barrett's oesophagus and is the most abundantly observed TLR in OAC biopsies [333]. More specifically, Huhta *et al*. reported in 2016 that TLR2, and TLR1 and TLR6, which are necessary for TLR2 activation, are expressed in normal oesophageal squamous epithelium and their expression increases in patients during progression to BO and OAC [330].

TLR2, is the only receptor so far, which to be activated needs to form heterodimers with TLR1 or TLR6 and then triggers inflammatory signalling pathways in response to a

range of stimulants. TLR2 ligands are represented by diacyl and triacylglycerol moieties, proteins and polysaccharides [390]. In addition to recognition of microbial ligands, TLR2 can be activated by several endogenous activators derived from altered or damaged host tissue or cells, which are therefore termed 'alarmins' [64][391][392][393]. Endogenous TLR2 ligands are either derived from host tissue and cells, components of the cells or induced gene products under specific conditions [394]. Endogenous activators of TLR2 include cardiac myosin [99], hyaluronan fragments [395], heat shock proteins [93], biglycan [88], endoplasmin [95] and High Mobility Group Box 1 (HMGB1) [396]. In pathological condition, endogenous ligands are released passively from injured/inflamed tissue and cells or actively secreted via lysosomes [394]. Sterile inflammation and signalling events triggered by endogenous ligands for TLR2 have been implicated in pathologies which include carcinogenesis and chemoresistance [324][397].

There is much evidence showing that the tumour microenvironment contains many different cell populations that interact with cancer cells and participate in all stages of tumour formation [366]. It has been shown that oesophageal cancer induces antitumor immunity in early stage of development [398]. Recruitment of immune cells and their response against oesophageal cells in the early stage of cancer prevents and controls its development, although when cancer cells battle with the immune system, oesophageal cells modulate immune cells response and inhibit the antitumor immunity [399]. Tumour escape from antitumor immunity is critical for tumour survival and progression. Tumour cells can suppress the antitumor immune response by recruitment of various immune cell populations, including myeloid-derived suppressor cells, regulatory T cells, Th17 cells and tumour-associated macrophages, which establish the tumour inflammatory microenvironment [366]. It has been suggested that immune cells recruited to the tumour site, produce a wide range of cytokines that promote the survival, proliferation, invasion and migration of tumour cells [400, 401]. Macrophages are the major component of immune cells in tumours. Macrophages present remarkable plasticity and can exist as 'classically' activated macrophages that produce type I proinflammatory cytokines and have antitumor qualities, called M1 and M2 'alternatively' activated macrophages which produce type II cytokines and have pro-tumorigenic properties [17]. After M2 polarization by tumour microenvironment, TAMs produce growth factors and proteases which increase tumour invasion, angiogenesis, metastasis and immunosuppression [402].

A large volume of studies suggest that TAMs participate in tumour progression and cancer metastasis [403]. It has been reported that high TAM density correlates with poor prognosis in breast cancer [404], lung [405], gastric cancer, and head and neck cancer [406]. Tumour macrophage infiltration has a key role in tumour proliferation and is associated with poor survival in OAC patients [407], although very few studies have been done on the role of TAMs in OAC progression and prognosis. It has been shown that during reflux esophagitis M1 macrophages infiltrate to the oesophagus and activate the Stat3 pathway in stromal cells and epithelium. Following M1 macrophage infiltration, M2-like TAMs macrophages are recruited to oesophagus where together with Treg contribute to tumour development [364]. The M2/M1 ratio was increased in OAC with nodal spread and highly associated with lymph node metastasis [365].

As it is well known that Barrett's oesophagus is highly associated with chronic inflammation induced by infiltrating cells, and resulting alterations leads to oesophageal adenocarcinoma, a study to investigate whether TLR2 expression might be involved in tumour progression has been designed. The study aimed to analyse if TLR2-mediated signalling could be involved in macrophage infiltration, thus promoting cancer development.

In Brief:

Hypothesis and aims of Chapter 3

Hypothesis: TLR2 represents a potential target to limit inflammation and disease progression in oesophageal adenocarcinoma.

Specific aims for Chapter 3:

- Determine the expression of TLR2 in panel of oesophageal cell lines representing different stages of oesophageal adenocarcinoma progression.
- Determine the effect of a TLR2 neutralising antibody on TLR2 expression and chemokine production.
- Determine effect of secreted factors from oesophageal cancer cells on monocyte/macrophage activation.
- Identify the potential TLR2 ligand being secreted from oesophageal adenocarcinoma cells.
- Characterise the phenotype of macrophages stimulated with conditioned media from oesophageal adenocarcinoma cells.

3.2. Results

3.2.1. Public datasets reveal upregulated TLR2 expression in OAC biopsies.

Oesophageal adenocarcinoma (OAC) can develop through a metaplasia-dysplasia-carcinoma sequence. Chronic inflammation and exposure to bile acids are the main pathogenic factors causing transformation of normal epithelium to Barrett's oesophagus (BO) and OAC [408]. The main challenge associated with OAC is the fact that detection of BO and early stage of OAC is very limited, which causes a high mortality rate. There is a growing interest in developing techniques to detect oesophageal disease at early stages or find biomarkers which could indicate pathogenic changes [409]. Huhta *el al*. demonstrated that TLR2 expression increases from squamous oesophagus towards intestinal metaplasia, dysplasia and OAC [330].

Upon initiation of this study, further investigation of recent reports of TLR2 overexpression during OAC was determined [330] [333]. Analysis of publicly available datasets confirmed that TLR2 was significantly increased in OAC, compared to adjacent normal oesophageal tissue (**Figure 3.1**) [410]. Therefore hypothesised that TLR2 would be strongly expressed in OAC cell lines and may be associated with progression of OAC from BO was made.

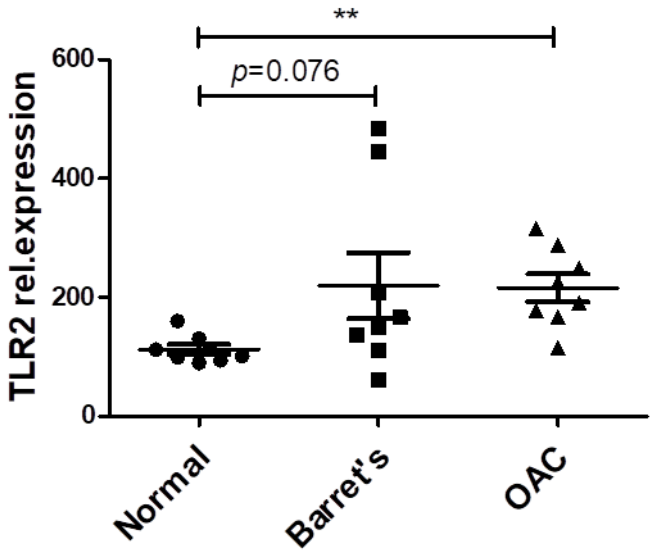


Figure 3.1. Analysis of public data sets reveal that TLR2 expression increase in Barrett's oesophagus and oesophageal adenocarcinoma tissues.

Expression profile of adjacent normal oesophageal epithelium, Barrett's metaplasia, and Barrett's-associated adenocarcinoma, matched tissues from individual patients (n=8). Tissues were obtained by transhiatal esophagectomy from patients with known Barrett's oesophagus and oesophageal adenocarcinoma. TLR2 expression levels were analysed using the following published dataset: GDS1321 (Kimchi ET *et al*. (2005) Cancer Res.). Graph shows the gene expression value in each individual sample and the mean ± SEM in each experimental group. Statistical analysis was performed using unpaired two-tailed student *t*-test to compare mean values between groups, ** $p<0.01$

3.2.2. Screening of oesophageal cell lines to determine TLR2 expression by Flow cytometry

Oesophageal epithelial cells have been shown to express TLRs [331]. Normal human oesophageal epithelial cell lines TE-1 and EPC-2 express TLR-2, -3, - 4, -7 and TLRs1-10 (but no TLR4), respectively. In EPC-2 cell lines, activation of these TLRs induce upregulation of the chemokine, IL-8. Furthermore, biopsies taken from oesophageal mucosa express TLR-1, -2, -3, and -5 mRNA [332]. Previous studies have shown that TLR2 is upregulated in Barrett's metaplasia, dysplasia and OAC tissue. A graduated increase was found through normal epithelium-metaplasia-dysplasia disease sequence [330].

A panel of OAC cell lines (**Table 3.1**) were subsequently screened for TLR2 expression by flow cytometry, to identify cell lines with potential to respond to TLR2 agonists. TLR2 receptor expression was analysed by flow cytometry, using the αTLR2 antibody (T2.5, Invitrogen). HEK 293T cells, which should be deficient in TLR2 mRNA expression [411]; and THP1 - human monocytic cells, which express high level of TLR2 [412], were used as negative and positive controls, respectively.

Flow cytometry analysis shows that the OAC cell lines SK-GT 4 (78.3% TLR2 expression), FLO-1 (82%), and OE33 (93.5%) and the Barrett's dysplasia cell line, GO (68.4%) express high levels of the TLR2 receptor, compared to HEK 293T cells (12.1%) (**Figure 3.2).** In contrast, OACM 5.1C cells (8.5%) do not appear to express the TLR2 receptor on the membrane and OACP4 C shows quite low TLR2 expression (39.5%) compared to positive cells. The cells with the lowest TLR2 expression: OACM5.1C and OACP4 C represent more advanced, IV grade cancer, while SK-GT 4, OE33 and represent earlier stage, II grade cancer. More specifically, SK-GT 4 represent

stage IIB and represent well-differentiated cancer, while OE33 cell lines are stage IIA and they are poorly differentiated. GO cells which are high-grade dysplastic, pre-cancerous cells also have high TLR2 expression. This suggests that during OAC progression TLR2 can be upregulated in Barrett's-OAC cells sequence and then OAC cells may lose TLR2 expression during malignant progression. In light of these results, hypothesis that the OAC cell lines SK-GT 4, FLO-1 and OE33 would respond to TLR2 agonists, such as Pam2CSK4 (P2C; TLR2/6 agonist) and Pam3CSK4 (P3C; TLR1/2 agonist) was made and these cell lines were used for further analysis.

Name	Histology	Cancer stage
GO	Human, oesophageal columnar epithelium, high grade dysplasia	Pre-cancer
SK-GT 4	Distal oesophagus adenocarcinoma, well-differentiated	IIB
OE33	Distal oesophagus adenocarcinoma, poorly-differentiated	IIA
FLO-1	Distal oesophagus adenocarcinoma	III
OACM5.1C	Lymph node metastases of Distal Oesophageal Adenocarcinoma	IV
OACP 4 C	Gastric cardia adenocarcinoma	IV

Table 3.1. The panel of dysplastic/OAC cell lines used in experiments.

For experiments, dysplastic and OAC cell lines from different cancer stages were used. II-stage: SK-GT 4 -well-differentiated oesophageal adenocarcinoma (IIB); OE33-poorly differentiated oesophageal adenocarcinoma (IIA). III-stage: FLO-1-esophageal adenocarcinoma; IV stage: OACM5.1C- Lymph node metastases of Distal Oesophageal Adenocarcinoma; OACP 4C- Gastric cardia adenocarcinoma

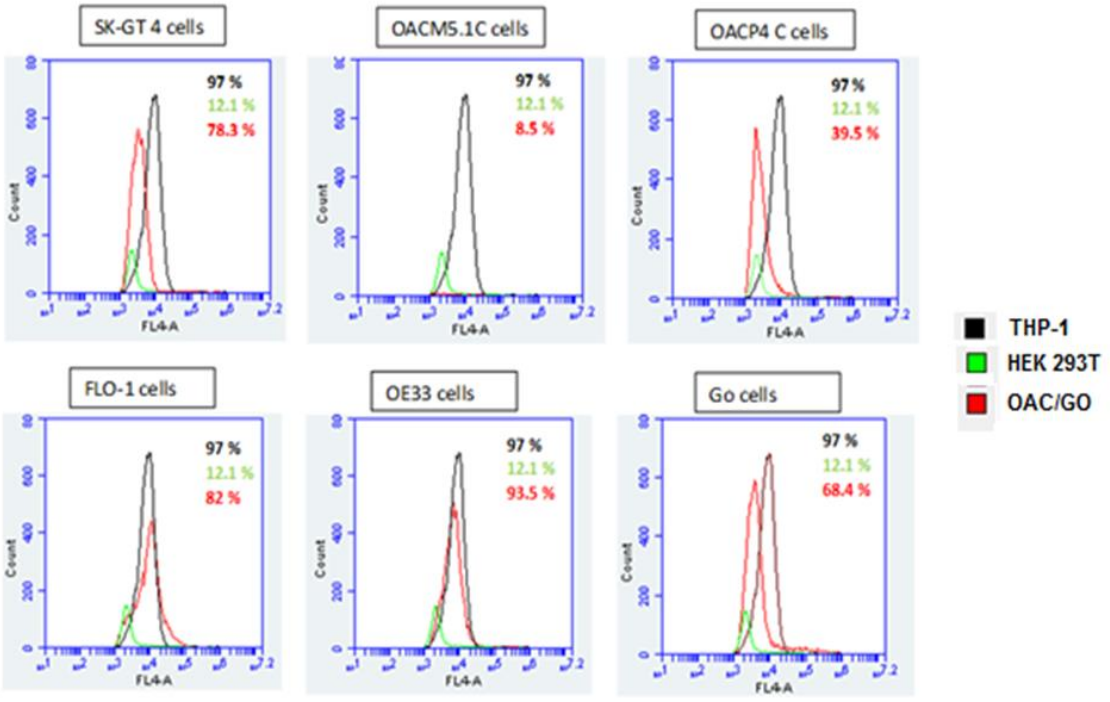


Figure 3.2. TLR2 expression in oesophageal cell lines. Flow cytometry analysis (gated for TLR2 positive cells) using αTLR2 neutralising antibody.

Histogram plots of indicated oesophageal cell lines (red colour) were overlaid with positive (THP1, black colour) and negative (HEK 293T, green colour) controls for TLR2 expression. All oesophageal cells were incubated with αTLR2 Ab (2 µg/ml) primary antibody and anti-mouse Alexa Fluor 647 secondary antibody (2 µg/ml). Cells were analysed with BD Accuri™ C6. The experiment was done in triplicate. Each graph presents one representative result.

3.2.3. Determining mRNA levels of TLR-2, -1 and -6 by Real-Time PCR

Following TLR2 analysis by FACS, further investigation of TLR2 activation in oesophageal cells was made. As TLR2 forms heterodimers with TLR1 or TLR6 to mediate intracellular signalling [413], levels of TLR1 and TLR6 were determined by Real-Time PCR. Neither binding of Pam3CSK4 (P3C; triacylated lipopeptide, TLR1/2 ligand) or Pam2CSK4 (P2C; diacylated lipopeptide, TLR2/6 ligand) is able to induce TLR2 signalling without TLR1 or TLR6, respectively [79]. The mRNA of TLR1, -2 and -6 was determined in the oesophageal cells with the highest level of TLR, as determined by FACS, (**Figure 3.2**) and THP-1 and HEK293 cells were used as a positive and negative control, respectively. **Figure 3.3A** shows that TLR2 mRNA expression increased from Barrett's cell line, GO to oesophageal carcinoma cells, stage IIB, SK-GT 4 and the highest level is observed in OE33 cells, which represents stage IIA OAC. Surprisingly no TLR2 mRNA was detected in FLO-1 cells, representing stage III OAC and which according to FACS analysis, 82% of cells had TLR2 protein. Levels of TLR1 and TLR6 mRNA were very low in all analysed oesophageal cells (**Figure 3.3A**). The positive and negative control cell lines, THP1 and HEK293T, were confirmed to have high and low levels of TLR2 mRNA, respectively (**Figure 3.3A**). To analyse whether stimulation of cells with TLR2 ligands would increase TLR2 expression, oesophageal cells GO, SK-GT 4 and OE33 were stimulated with the synthetic TLR1/2 lipoprotein, Pam3CSK4 (P3C) for 2, 4 and 6 h, and TLR2 mRNA was analysed. No significant difference in TLR2 mRNA was observed after P3C stimulation, although TLR2 mRNA levels slightly increased in all stimulated cell lines when compared to unstimulated (**Figure 3.3B**).

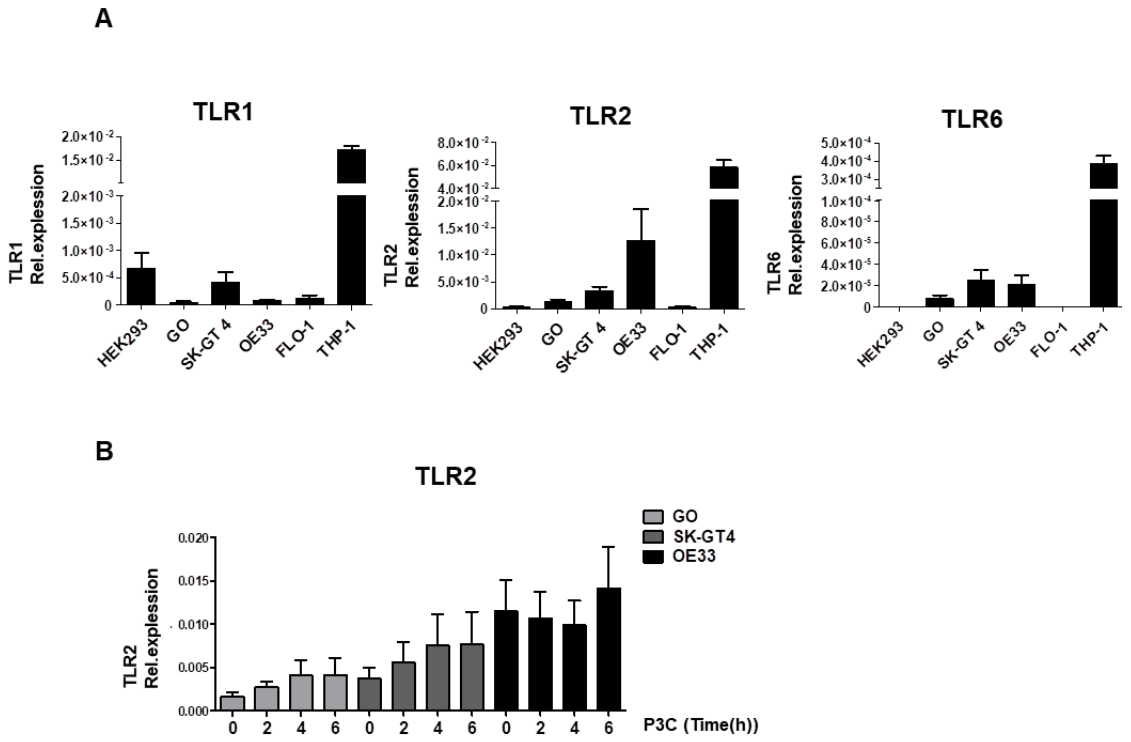


Figure 3.3. Trend towards increased TLR2 mRNA expression following TLR2 stimulation of Barrett's and early-stage OAC cell lines, SK-GT 4 and OE33.

A) Expression of TLR1, TLR2 and TLR6 in OAC cell lines (SKGT 4, OE33 and FLO-1) and Barrett's dysplasia (GO) was measured and quantified by quantitative polymerase chain reaction (qPCR). TLR mRNA expression levels were measured at basal levels, with no cell stimulations. Values represent the mean ± S.E.M of three independent experiments run in duplicate. Data are normalized to the stably expressed reference gene, GAPDH. HEK293 and THP-1 were used as a negative and positive control, respectively. **B)** GO, SK-GT 4 and OE33 cells were seeded on 24 well plate 24 h prior stimulation. Cells were left untreated or treated with P3C (0.05 µg/ml) for 2 h, 4 h or 6 h and RNA was collected for analysis. Expression of mRNA TLR2 was determined by qPCR. Values represent the mean ± S.E.M of three independent experiments. Data are normalized to the stably expressed reference gene, GAPDH.

3.2.4. Barrett's dysplasia and early oesophageal adenocarcinoma cell lines are responsive to TLR2.

Results obtained from FACS and mRNA revealed that the highest level of TLR2 was observed in GO, SK-GT 4 and OE33 cell lines and results suggest that TLR2 expression very likely increases from Barrett's dysplasia to early stage OAC, while no TLR2 mRNA was observed in FLO-1 cells from the most advanced cancer stage. To assess TLR2 protein levels in response to TLR2 stimulation, the oesophageal cell lines were stimulated with the synthetic TLR2 ligands, Pam3CSK4 (P3C) and Pam2CSK4 (P2C), which activate TLR1/2 and TLR2/6, respectively. Western blot reveals that GO, SK-GT 4 and OE33 cells express basal levels of TLR2, which are upregulated following stimulation with either TLR2 ligand (**Figure 3.4**). Similar to TLR2 mRNA observations, no TLR2 protein expression was detected in FLO-1 by Western blot (**Figure 3.3; Figure 3.4**).

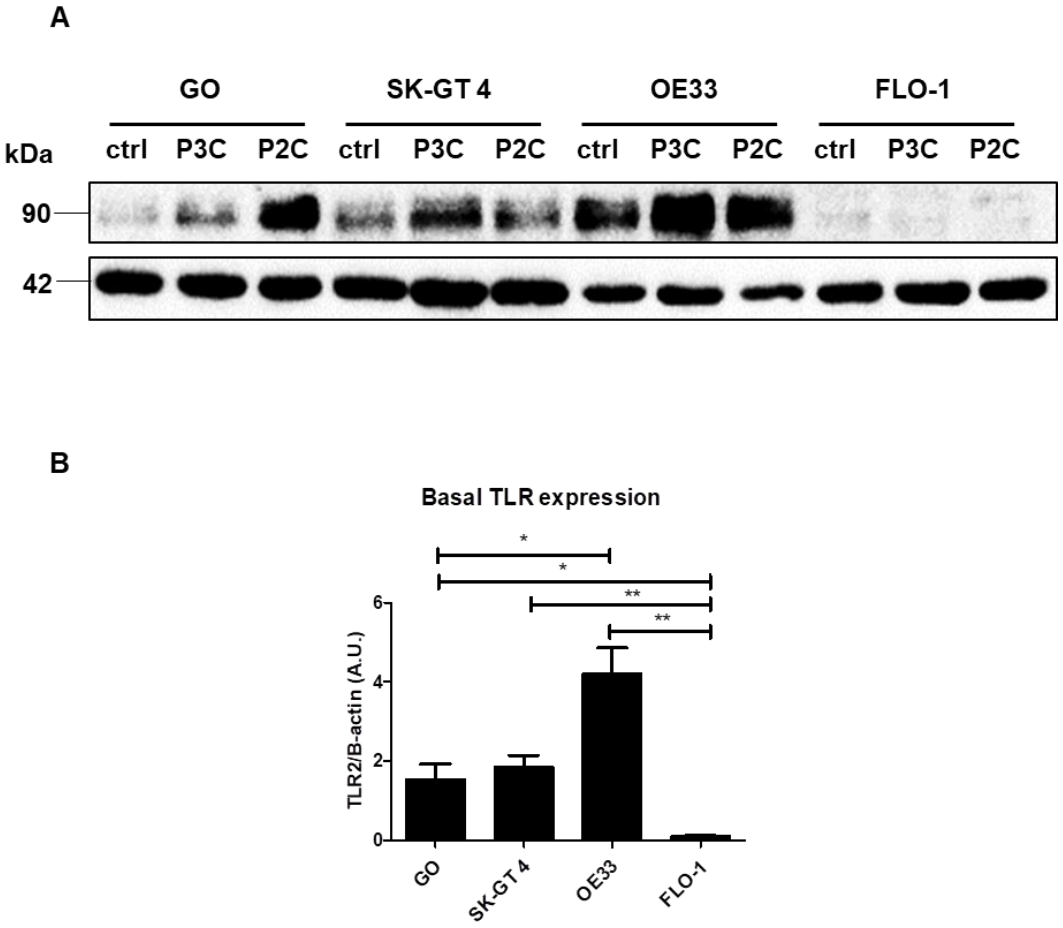


Figure 3.4. TLR2 ligands induce TLR2 protein expression in Barrett's and early-stage oesophageal cell lines.

Barrett's cell line: GO and oesophagous adenocarcinoma cell lines: SK-GT4, OE33 and FLO-1 were seeded (GO,OE33 and FLO-1 at $2x10^5$ cells/well; SK-GT 4 at $1,6 x10^5$ cells/well) 24 hours prior to stimulation. Cells were stimulated for 24 h with P3C (0.05 μg/ml) or with P2C (0.05 μg/ml). Protein lysates (10 μg) from 3 independent experiments were resolved by SDS-PAGE, wet transferred to nitrocellulose and probed for TLR2 (90 kDa) and loading control, β-actin (42 kDa). **A)** Representative immunoblots of TLR2; **B)** Densitometry analysis showing basal expression levels of TLR2 protein in cell lines (untreated).; (A.U. refers to Arbitrary Unit).

3.2.5. TLR2 activation induces IL-8 chemokine release in GO and early stage OAC cell lines

Inflammation is considered as the seventh hallmark of cancer [414]. The inflammatory response in tumour environment is characterised by infiltration of immune cells and alterations in cytokines and chemokines [415]. Chronic inflammation plays a role in OAC pathogenesis and contributes to the initiation or progression of OAC from BO. Cytokines, chemokines and growth factors are the main molecules in inflammation and cancer, promoting proliferation, angiogenesis and carcinogenesis through direct effects, or through recruitment of immune cells [415]. TLRs participate in innate immune responses to microbial pathogens. Activation of TLR proteins by a variety of bacterial products mediates cellular activation, including the expression of pro-inflammatory cytokines. Previous studies have shown that expression of pro-inflammatory cytokines correlates with increased invasiveness and poor prognosis in cancers [46]. There is strong evidence that the pro-inflammatory cytokines IL-6 and IL-8 are involved in cancer development [47] [48].

In this study, the role of TLR2 signalling in the production of pro-inflammatory cytokines: IL-6 and IL-8 was investigated. Having established that TLR2 expression was the highest in GO, SK-GT 4 and OE33 cells, the ability of TLR2 ligands to stimulate these cells was analysed. IL-6 and IL-8 was also assessed in FLO-1 to confirm that they do not express TLR2. As a ligand, synthetic tri-acylated lipopeptide (LP) (Pam3CSK4) that mimics the acylated amino terminus of bacterial LPs was used.

Pam3CSK4 is a potent activator of NF-κB, and activation is mediated by heterodimeric TLR2/TLR1, which recognises LPs with three fatty acids, a structural characteristic of bacterial LPs. Other TLR2 agonists used were synthetic di-acylated lipopeptide (Pam2CSK4), which recognise TLR2/TLR6 heterodimers and Heat-Killed *Listeria Monocytogenes* (HKLM), a facultative intracellular Gram-positive bacterium. Infection of cells with HKLM induces a strong nonspecific response with release of proinflammatory cytokines. This response is mediated by the activation of TLRs, mainly TLR2 and MyD88 [416] [417].

The effect of the TLR2 agonists: Pam3CSK4 (P3C; TLR1/2 agonist), Pam2CSK4 (P2C; TRL2/6) and HKLM (TLR1/2 agonist) on the secretion of pro-inflammatory cytokines IL-6 and IL-8 was assessed. All cells were stimulated also with the TLR4 ligand, LPS as a control (**Figure 3.5, Figure 3.6**).

GO, which are high-grade dysplasia cells, show markedly increased IL-6 release after stimulation cells with P2C. Other ligands such as P3C, HKLM and LPS increased IL-6 release but levels were much lower than in P2C stimulated (**Figure 3.6**). Stimulation of GO cells with TLR2 ligands P3C and P2C show significantly increased IL-8 release (**Figure 3.5)**. SK-GT 4 cells show significantly increased IL-8 release after stimulation with TLR2 agonists, although the secreted IL-6 level was very low (~70 pg/ml) (**Figure 3.6).** Stimulation OE33 with TLR2 ligands induced high levels of IL-8 secretion, although no IL-6 was detected in supernatants. Despite the high TLR2 expression levels measured in FLO-1 cells by flow cytometry, TLR2 agonists did not induce significant secretion of IL-6 and IL-8 in this OAC cell line (**Figure 3.5; Figure 3.6**). These observations suggest that Barrett's dysplasia and early stage OAC cells are responsive to TLR2 stimulation, which may be important during cancer progression.

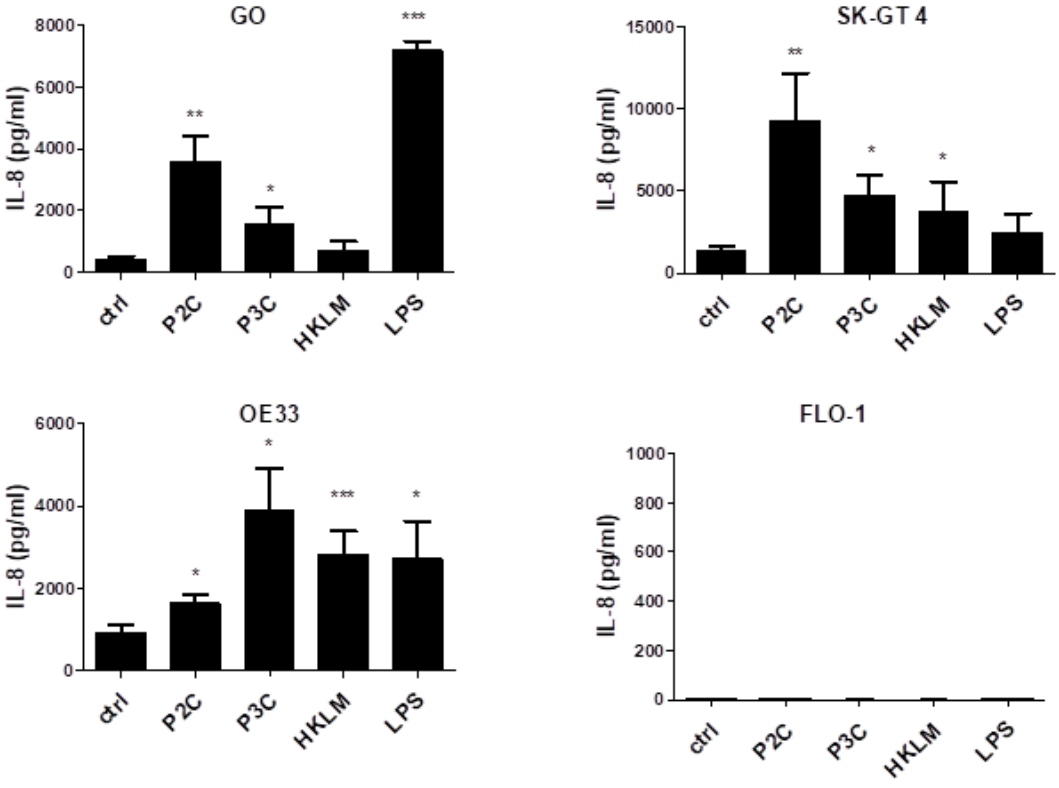


Figure 3.5. High-grade dysplasia and early stage cancer oesophageal cell lines release IL-8 in response to TLR2 ligands.

The OAC cell lines: SK-GT 4, OE33, FLO-1 and Barrett's cell line: GO were left untreated or stimulated for 24 h with TLR2 agonists: P3C (0.05 µg/ml), P2C (0.05 µg/ml), HKLM (10^7 cells/ml), LPS (1 µg/ml). Levels of IL-8 in the supernatants were determined by ELISA. Values represent the mean ± S.E.M of four independent experiments. Statistical analysis was performed using unpaired two-tailed student *t*-test to compare mean values between treated and untreated cells, *p<0.05; ** p<0.01; ***p<0.001.

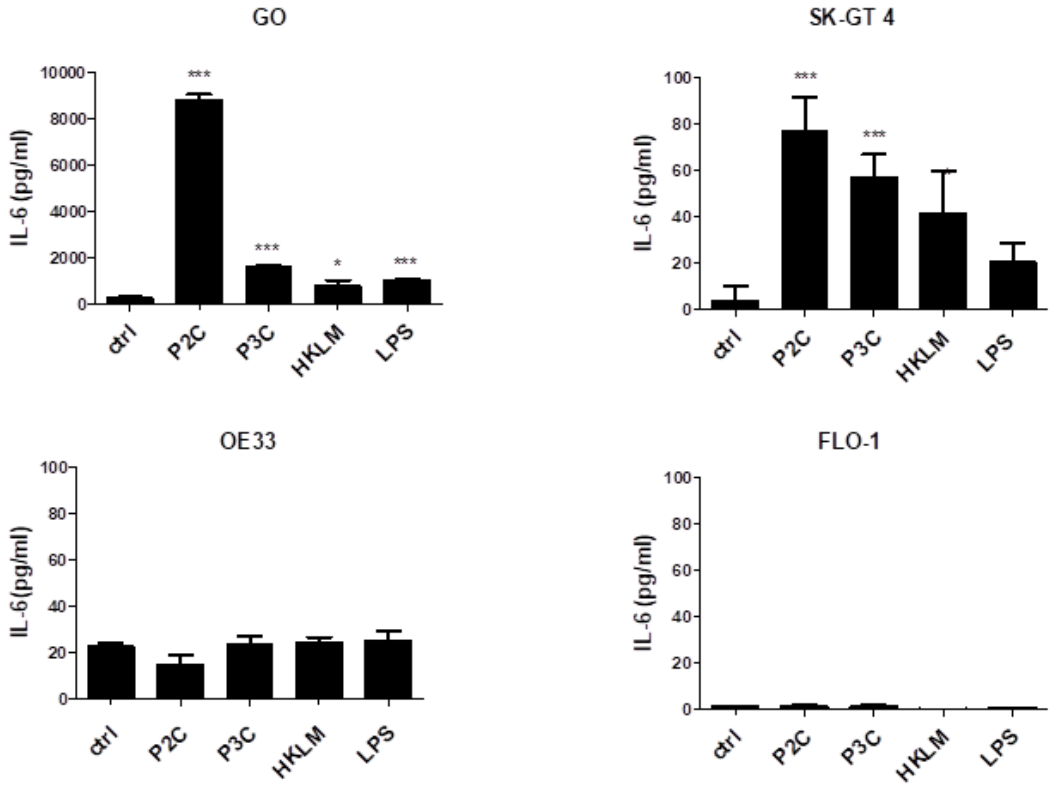


Figure 3.6. High-grade dysplasia, GO and early stage cancer oesophageal cell line, SK-GT 4 release IL-6 in response to TLR2 ligands.

The Barrett's cell line: GO and OAC cell lines: SK-GT 4, OE33, FLO-1 were left untreated or stimulated for 24 h with TLR2 agonists: P3C (0.05 µg/ml), P2C (0.05 µg/ml), HKLM (10^7 cells/ml) and TLR4 ligand, LPS (1 µg/ml). Levels of IL-6 in the supernatants were determined by ELISA. Values represent the mean ± S.E.M of four independent experiments. Statistical analysis was performed using unpaired two-tailed student *t*-test to compare mean values between treated cells, *$p<0.05$; ** $p<0.01$; ***$p<0.001$.

3.2.6. IL-8 secretion and TLR2 upregulation are inhibited by a TLR2 neutralising antibody.

TLR2 expression in oesophageal cells and their ability to induce pro-inflammatory cytokines, suggest that TLR2 may play an integral role in the pathogenesis of OAC and therefore TLR2 inhibition might present a rational target for therapeutic intervention.

Chemokine production is an essential factor in inflammation-mediated tumour progression, and high IL-8 expression in oesophageal squamous cell carcinoma (OSCC) has been shown to correlate with distant metastasis and a poor prognosis [418]. IL-8 receptor inhibition decreased the invasiveness of OAC-derived cells, suggesting this pathway as a potential therapeutic target [419].

Anti-TLR2 neutralising antibody (αTLR2, T2.5, Invivogen) is a monoclonal anti-TLR2 antibody which has binding specificity for TLR2. It is believed to function through targeting the ligand-binding site on the receptor and prevent heterodimerization with co-receptors TLR1 or TLR6. αTLR2 is a mouse antibody and is cross reactive with human TLR2. αTLR2 have been used successfully in several animal models such as ischemia/reperfusion injury in mice [420]. A previous study has shown that αTLR2 significantly inhibits spontaneous release of IL-8, TNFα, IFNγ and IL-1β in rheumatoid arthritis (RA) pathogenesis [421]. It has also been shown that in gastric tumorigenesis, αTLR2 significantly suppressed the TLR2-dependent induction of Cxcl2 and TNFα genes. Treatment of $gp130^{F/F}$ mice with αTLR2 reduced the size, overall tumour burden and suppressed both initiation and growth of gastric tumours. This study confirmed that blocking TLR2 signalling can inhibit tumour growth by acting directly on the tumour cells to promote apoptosis and suppress proliferation [422].

Based on the above results, study to analyse whether the anti-TLR2 antibody (αTLR2) can inhibit release of IL-8 in oesophageal cell lines was designed. SK-GT 4 and GO cells were pre-treated with increasing concentrations of TLR2α (10 µg/ml, 20 µg/ml, 40 µg/ml) for one hour before stimulation with TLR2 agonists (P3C and P2C). Measurement of IL-8 secretion after 24 h revealed that αTLR2 significantly inhibits TLR2-mediated IL-8 production in P2C (**Figure 3.7A,C**) and P3C (**Figure 3.7B,D**) stimulated SK-GT 4 and GO cells.

Western blot analysis showed that TLR2 expression is upregulated in SK-GT4 and GO cells following stimulation with TLR2 ligands, and that inhibiting TLR2 signalling with TLR2α (10 ug/ml) blocks TLR2 upregulation in both cell lines (**Figure 3.8A,B**). This

data reveals that αTLR2 limits both TLR2-mediated chemokine secretion and TLR2 upregulation in both high grade dysplastic and early stage cancer oesophageal cell lines.

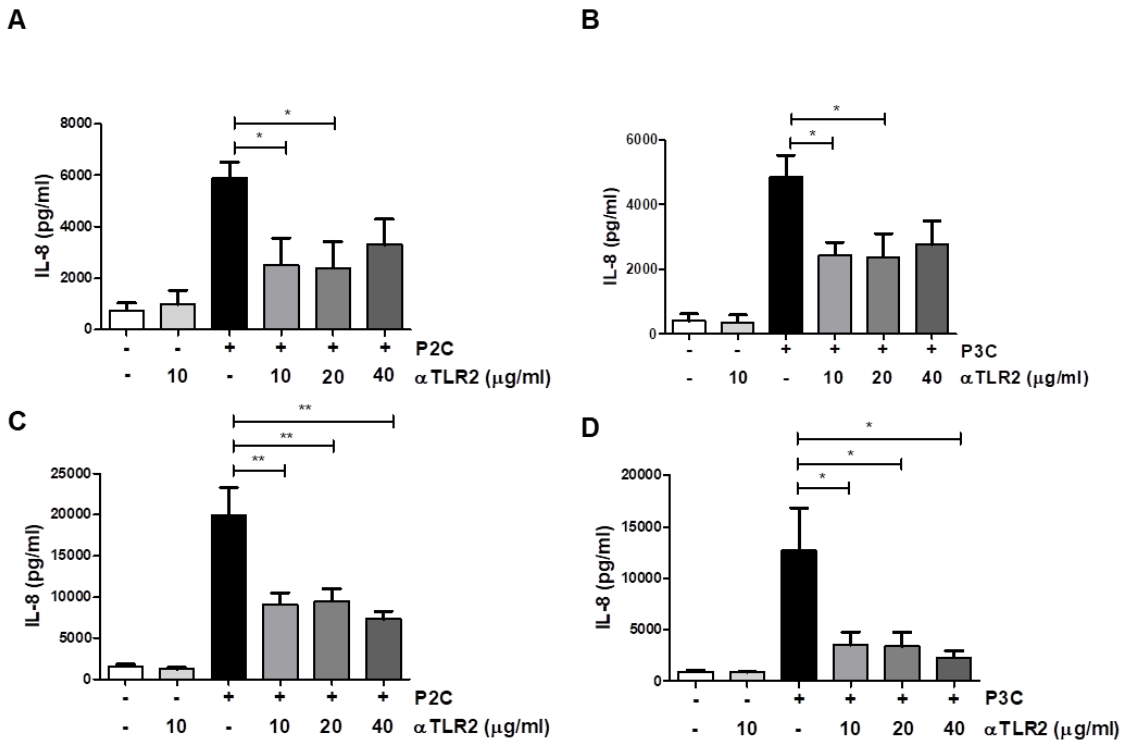


Figure 3.7. A TLR2 neutralising antibody inhibits IL-8 production in oesophageal cell lines.

GO **(A-B)** and SK-GT 4 **(C-D)** cells were left untreated or pre-treated with αTLR2 Ab (10 μg/ml; 20 μg/ml; 40 μg/ml) for 1 h, prior to stimulation with: (**B-D)** P3C (0.05 μg/ml) or (**A-C)** P2C (0.05 μg/ml) for 24 h. Culture supernatants were analysed for IL-8 secretion by ELISA. Values represent the mean ± S.E.M of n=5 for SK-GT 4 and n=3 for GO. Statistical analysis was performed using unpaired two-tailed student *t*-test to compare mean values between αTLR2 treated and untreated cells, **p*<0.05; ** *p*<0.01; ****p*<0.001

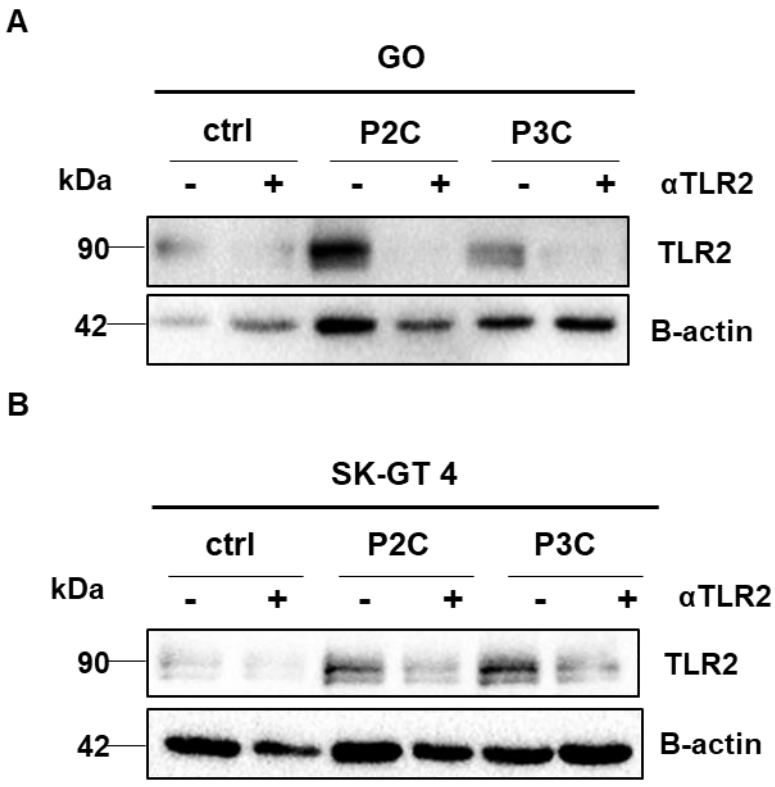


Figure 3.8. A TLR2 neutralizing antibody inhibits the upregulation of TLR2 in oesophageal cell lines.

GO **A)** and SK-GT 4 **B)** cells were seeded at 2 x 10^5 cell/ml, 1.6 x 10^5 cell/ml, respectively. After 24 h cells were left untreated or pre-treated with αTLR2 (10 μg/ml) for 1 h, prior to stimulation with P2C (0.05 μg/ml) or P3C (0.05 μg/ml) for 24 h. Lysates were collected and samples (10 μg of protein) were run on SDS-PAGE gel and wet transferred to nitrocellulose. Immunoblots were probed for TLR2 (90 kDa) and β-actin (42 kDa) as a loading control. Figure presents representative blot of three independent experiments.

3.2.7. Oesophageal cell lines release endogenous TLR2 ligands.

Many endogenous molecules can also act as potent inflammatory mediators through activation of TLRs. Endogenous molecules activate TLRs and induce inflammatory response in many pathogeneses. During inflammation, endogenous ligands are released passively from injured/inflamed tissue, dying cells or actively secreted by activated cells through a non-conventional lysosomal route. Most endogenous TLR ligands can bind TLR2 and TLR4 [394]. It has been reported that dying tumour cells release endogenous TLR ligands [423] [122], which induce solid tumour progression [424] [425] and leukemic cell growth [426].

Therefore study to determine whether TLR-stimulated oesophageal cell lines may release endogenous TLR ligands, which could contribute to the increased TLR2 expression observed during OAC development [330], were designed. For this objective a THP1-XBlue-CD14 reporter cell line were employed. This THP1 reporter cell line stably expresses an NF-κB- and AP-1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. The cells also stably express CD14, a macrophage-specific differentiation antigen that interacts with several TLRs [427]. Thus, the reporter cells will secrete SEAP following their stimulation by TLR ligands, which can be spectrophotometrically detected using QUANTI-Blue reagent. Direct stimulation of THP1-XBlue-CD14 cells with a range of TLR agonists was initially performed to confirm the validity of the reporter system (**Figure 3.9).** Results show that the undifferentiated THP-1 cell line is highly responsive to heat killed bacteria (HKLM, HKST, HKLR), unpurified LPS (Sigma), and the TLR2 ligands, P2C and P3C. Furthermore, TLR-mediated THP1-XBlue-CD14 activation can be successfully inhibited by αTLR2 (**Figure 3.9**).

To determine whether TLR2 activating factors are secreted from stimulated oesophageal cell lines, GO, SK-GT 4 and OE33 cells were left untreated or pre-treated with αTLR2 prior to TLR stimulation with P3C, P2C, HKLM and LPS (Sigma). Following 4 h TLR stimulation, media was removed and cells were washed three times with PBS before the addition of fresh media, which was further incubated with cells for 24 h, to generate conditioned media (CM). CM was added to THP1-XBlue-CD14 cells at 50% final volume and incubated for 24 h before measuring SEAP activity (**Figure 3.10**, Schematic representation of the experiment). Results show that CM from oesophageal cell lines stimulated with direct TLR2 agonists, P2C and P3C, induced the

activation of TLRs in THP1-XBlue-CD14 cells, which was inhibited in cells that were pre-treated with αTLR2 (**Figure 3.11 A-C**). This result suggests that TLR2 engagement on dysplastic oesophageal and OAC cell lines induces their secretion of endogenous TLR2 ligands. In contrast, no TLR activation was observed in THP1-XBlue-CD14 cells incubated with CM from LPS and HKLM-stimulated oesophageal cell lines, which may be due to lower cellular responses to that ligand (**Figure 3.5, 3.6**). To confirm that endogenous TLR ligands were being secreted, and rule out the possibility that synthetic ligands remaining in the CM may be responsible for the activation of THP1-XBlue-CD14 cells, a time-course (1 min - 24 h) to generate CM at different incubation times was performed, and the resulting CM was added to THP1-XBlue-CD14 cells before measuring SEAP activity (**Figure 3.12**). Results reveal that secretion of TLR ligands from oesophageal cell lines increase over initial timepoints, with the highest levels of SEAP activation being observed from CM collected following 4 h incubation. Results also confirm that CM collected directly (1 min) after synthetic ligands were removed was unable to activate SEAP in THP1-XBlue-CD14 cells (**Figure 3.12 A--C**). To determine the extent of TLR2-specific endogenous ligands being secreted from oesophageal cells, THP1-XBlue-CD14 cells were pre-treated with a concentration range of αTLR2 (0.4, 4 and 40 μg/ml) before incubation with CM from each of the cell lines GO, OE33 and SK-GT 4. Results reveal that even low αTLR2 concentrations (0.4 μg/ml) inhibit SEAP release from THP1-XBlue-CD14 cells, suggesting that TLR2 activating factors within the CM are fully responsible for this signal (**Figure 3.13 A-C**). Moreover, heat-inactivation of CM completely inhibits their ability to activate TLRs, what suggests that factor(s) responsible for TLR2 activation is peptide origin (**Figure 3.14**).

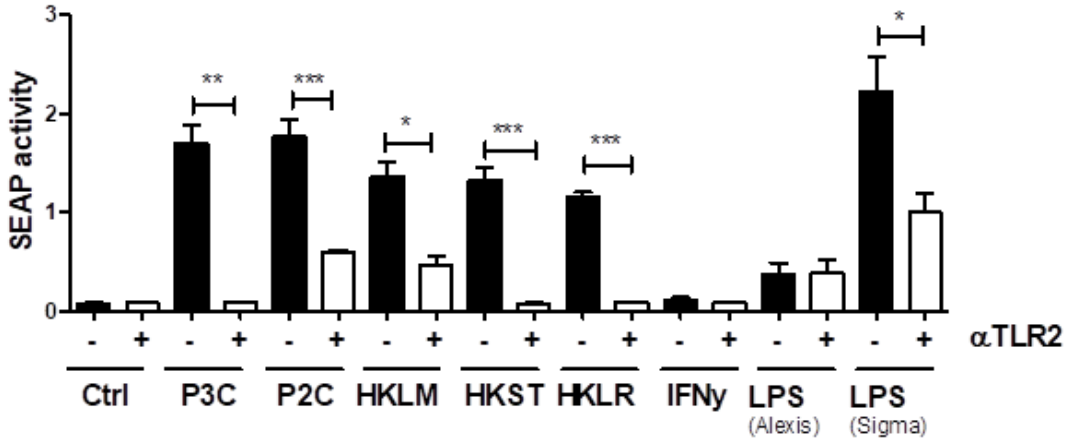


Figure 3.9. Direct TLR2 stimulation of the TLR reporter cell line, THP1-XBlue-CD14, is inhibited by the αTLR2 neutralising antibody.

THP1-XBlue-CD14 were seeded at 4 x 10^6 cells/ml prior stimulation. 24 h later cells were left untreated or pre-treated for 1 h with αTLR2 prior to stimulation with P3C (0.05 μg/ml); P2C (0.05 μg/ml); heat-killed bacteria: *Listeria Monocytogenes* (HKLM, 10^7 cells/ml); *Salmonella Typhimurium* (HKST, 10^6 cells/ml); *Lactobacillus Rhamnosus* (HKLR, 10^7 cells/ml); IFNγ (40 ng/ml); LPS (Alexis, Ultrapure, 1 μg/ml) or LPS (Sigma, 1 μg/ml) for 24 h. TLR stimulation was assessed by measuring the levels of SEAP in the supernatants by using QUANTI-Blue reagent. Values represent the mean ± S.E.M of three independent experiments. Statistical analysis was performed using unpaired two-tailed student *t*-test to compare mean values between αTLR2-treated and untreated cells, *p<0.05; ** p<0.01; ***p<0.001

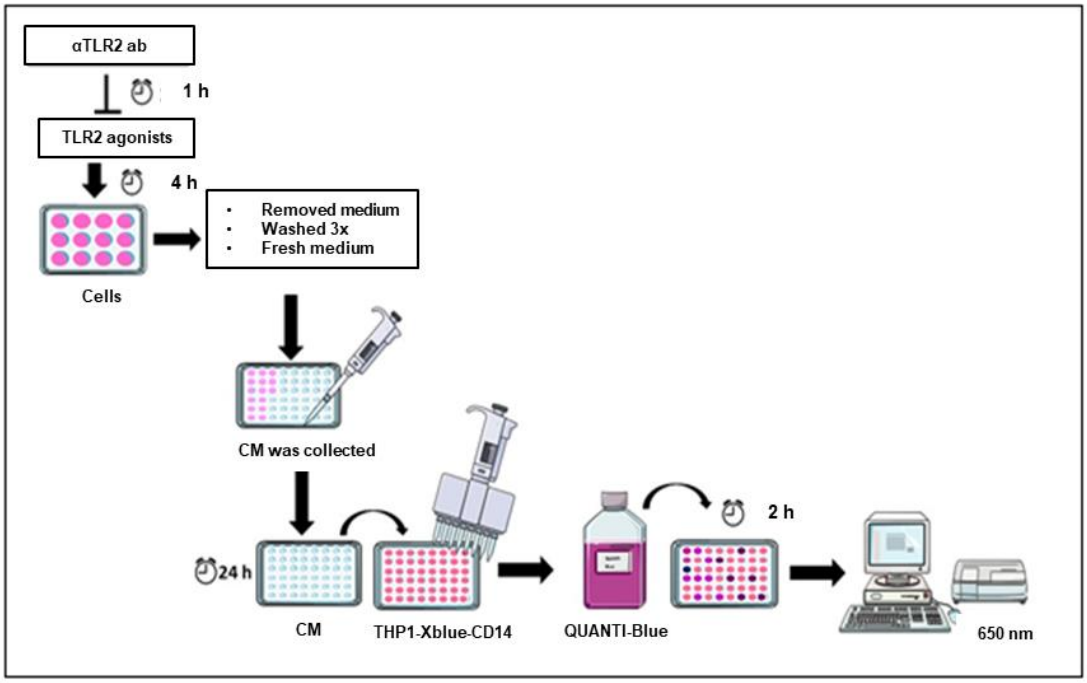


Figure 3.10. Experimental scheme. Collection of Conditioned Media from TLR2-prestimulated esophgaeal cells.

Oesophageal cell lines (GO, SK-GT 4 and OE33) (seeded at $2x10^5$; $1.6x10^5$; $2x10^5$, respectively) were left unstimulated or stimulated with αTLR2 (10µg/ml) for 1 h prior to stimulation with P3C (0.05 µg/ml), P2C (0.05 µg/ml) or LPS (1 µg/ml) for 4 h. Then, media were removed, cells were washed x3 with PBS and fresh media were added/well. After 24 h, 50 µl of CM were collected and added to 50 µl of THP1-XBlue-CD14 (seeded at 4 x 10^6 cells/ml) for 24 h. Plate was incubated 24 hours in 37°C. 40 µl supernatant was added to 160 µl QUANTI-Blue™ and incubated for 2 h at RT in a 96 well plate prior to absorbance was measurement at 650 nm.

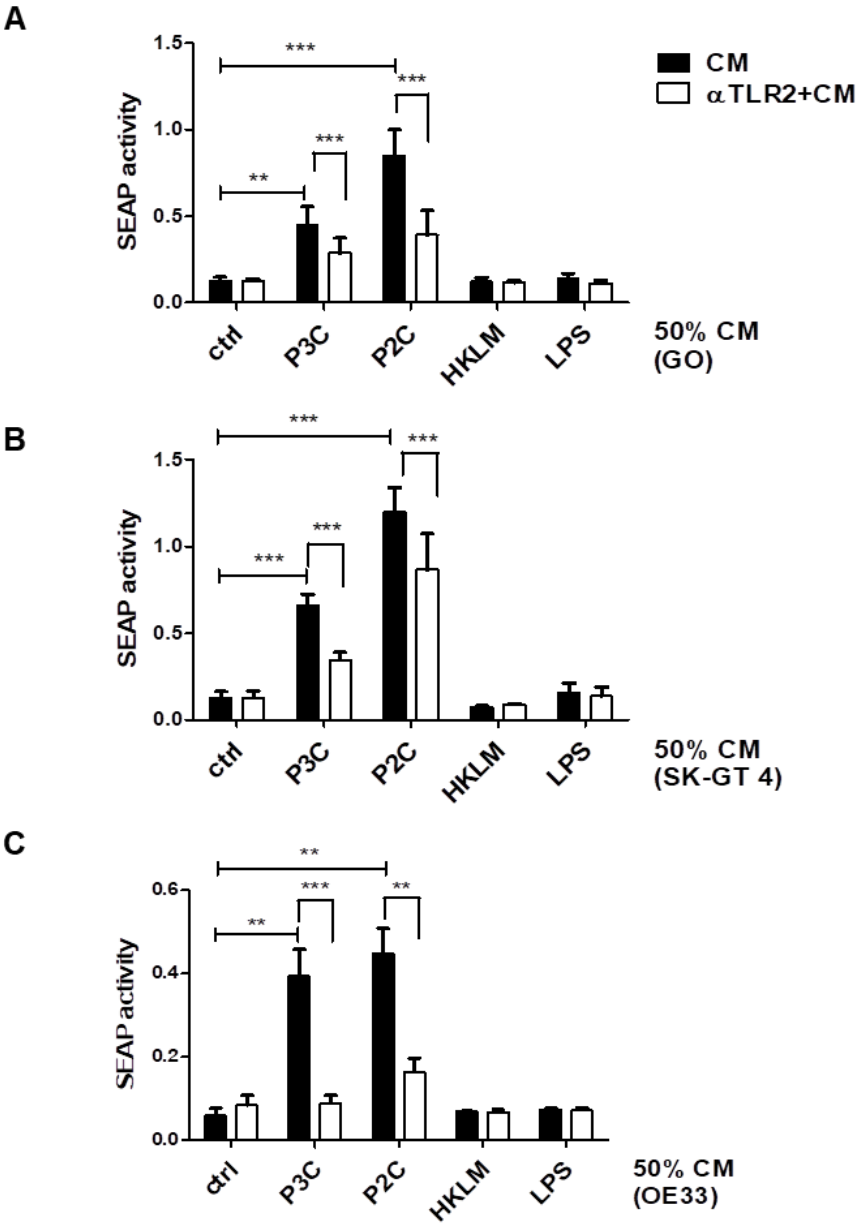


Figure 3.11. TLR2-stimulated dysplastic/OAC cell lines secrete factors which activate THP1-Blue-CD14 cells.

Oesophageal cells; GO, SK-GT 4 and OE33, were seeded 2 x 10^5 cells/ml; 1.6 x 10^5; 2 x 10^5 cells/ml, respectively. Following 1 h αTLR2 pre-treatment (10 µg/ml), cells were stimulated with P3C (0.05 µg/ml), P2C (0.05 µg/ml), HKLM (10^7cells/ml) or LPS (1 µg/ml) for 4 h. Media was removed, cells were washed x3 with PBS and incubated in fresh media for 24 h. Conditioned media (CM) was collected from cells and added (50% final conc.) to the TLR reporter cell line THP1-XBlue-CD14 (seeded at 4x10^6cells/ml) for 24 h. TLR stimulation was assessed by measuring supernatant SEAP levels, using QUANTI-Blue reagent. Statistical analysis was performed using 2way *ANOVA* test to compare mean values between untreated and αTLR2 treated cells and unpaired student *t*-test to compare ctrl CM to P3C CM and P2C CM, *p<0.05; **p<0.01; ***p<0.001.

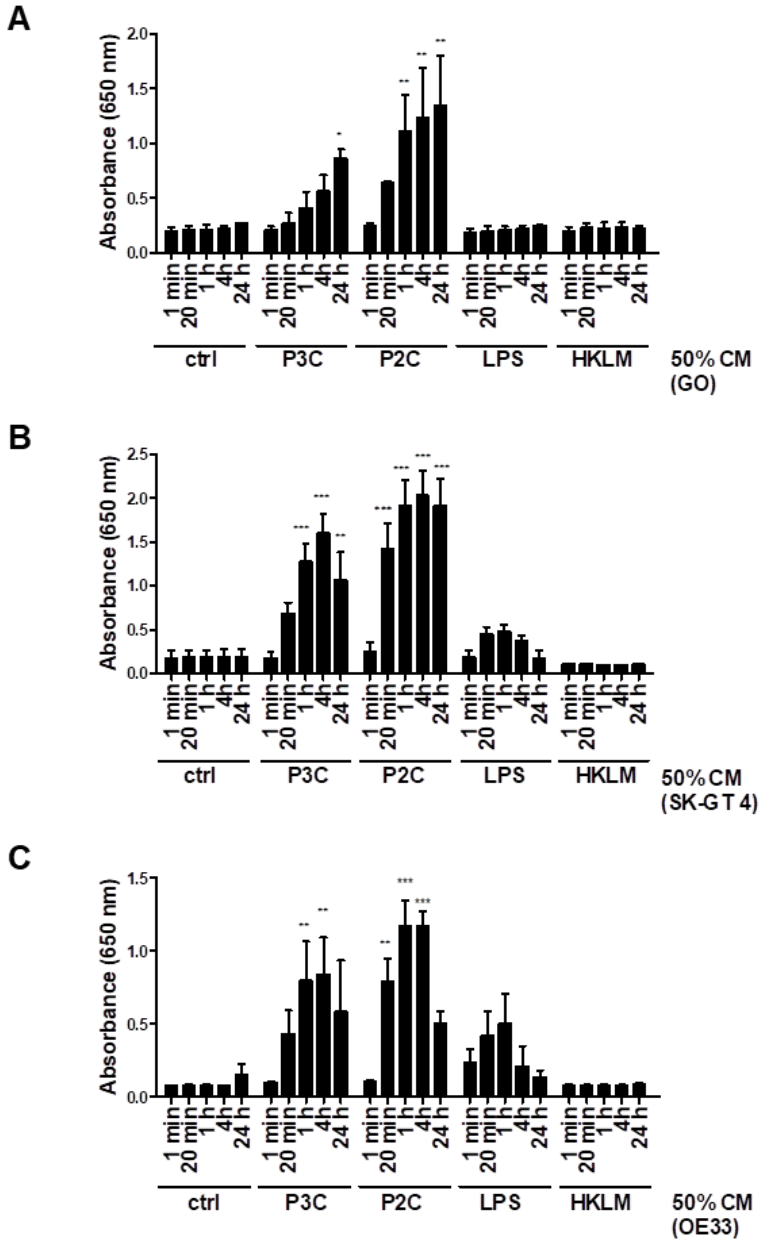


Figure 3.12. Factors secreted gradually from stimulated OAC cell lines activate THP1-Blue-CD14 cells.

Oesophageal cells (GO, SK-GT 4 and OE33) were seeded 2 x 10^5 cells/ml; 1.6 x 10^5; 2 x 10^5 cells/ml, respectively. Following 1 h αTLR2 Ab pre-treatment (10 µg/ml), cells were stimulated with P3C (0.05 µg/ml), P2C (0.05 µg/ml), HKLM (10^7cells/ml) or LPS (1 µg/ml) for 4 h. Media was removed, cells were washed x3 with PBS and new media was added. Conditioned media (CM) was collected from cells at 1 min, 20 mins, 1 h, 4 h and 24 h and added (50% final conc.) to the TLR reporter cell line THP1-XBlue-CD14 (seeded at 4x10^6cells/mL) for 24 h. TLR stimulation was assessed by measuring supernatant SEAP levels, using QUANTI-Blue reagent. Statistical analysis was performed using 2way *ANOVA* test to compare mean ±S.E.M values between ctrl CM and P3C-, P2C-, HKLM- and LPS CM *p<0.05; **p<0.01; ***p<0.001.

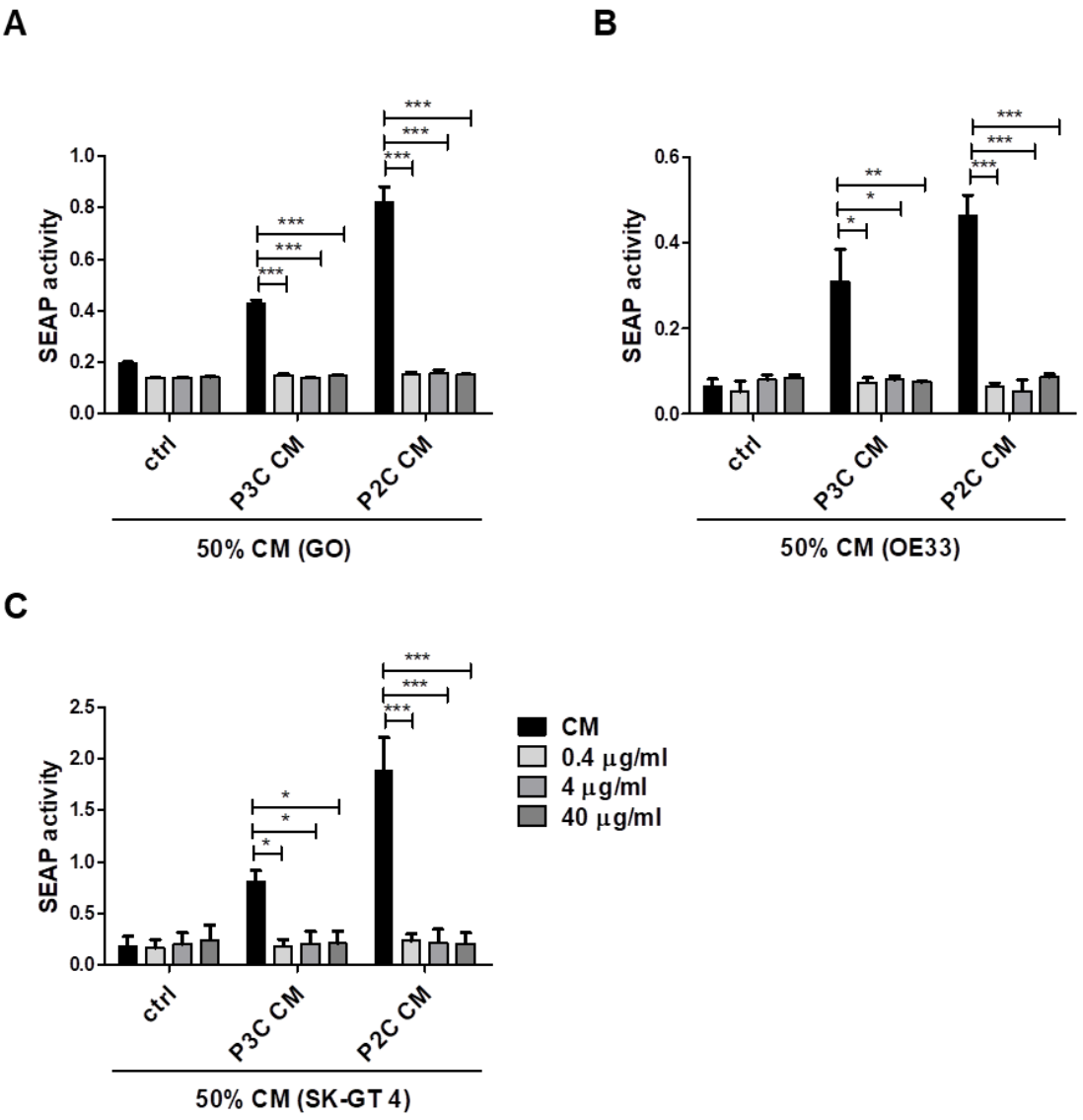


Figure 3.13. Factors secreted from OAC cell lines activate THP1-XBlue-CD14 via the TLR2 receptor.

Oesophageal cells GO, SK-GT 4 and OE33 were seeded 2 x 10^5 cells/ml; 1.6 x 10^5 cells/ml; 2 x 10^5 cells/ml, respectively. Cells were stimulated with P3C (0.05 µg/ml), P2C (0.05 µg/ml) for 4 h. Media was removed, cells were washed x3 with PBS and incubated in fresh media for 24 h. THP1-XBlue-CD14 (seeded at 4x10^6 cells/mL) were pre-treated 1 h with αTLR2 (0.4; 4; 40 µg/ml) or left untreated and then stimulated for 24 h with 50 % CM (ctrl CM, P3C CM, P2C CM). TLR stimulation was assessed by measuring supernatant SEAP levels, using QUANTI-Blue reagent. Statistical analysis was performed using 2way ANOVA to compare mean ±S.E.M values between untreated and αTLR2-treated cells, *p<0.05; **p<0.01; ***p<0.001.

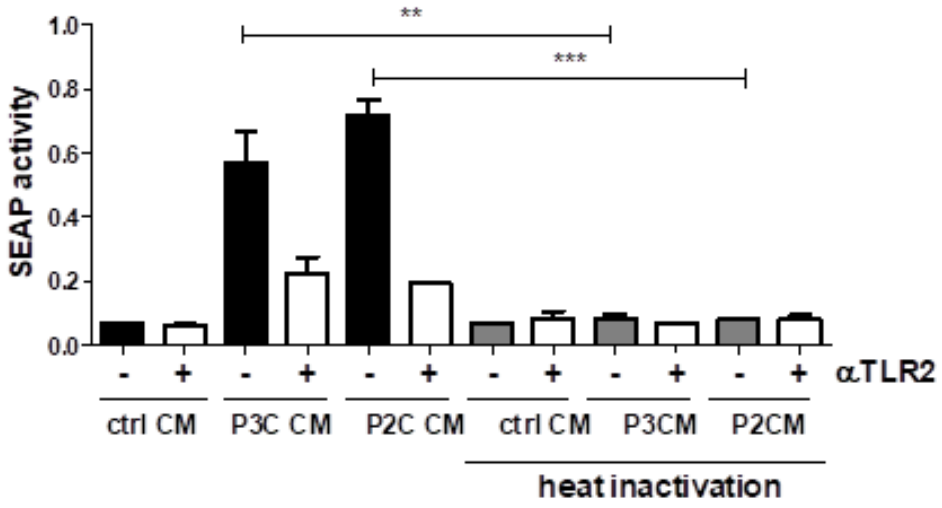


Figure 3.14. Heat-inactivation of conditioned media from OAC cells decreases its ability to activate TLRs.

CM was collected from unstimulated or αTLR2-stimulated SK-GT 4 (ctrl CM), P2C-pretreated SK-GT 4 (P2C CM) and P3C-pretreated SK-GT 4 (P3C CM). Half of the collected CM were heat-inactivated by incubating for 1 h at 75 °C and then cooled to 37°C. 50 µl of ctrl CM, P3C CM, P2C CM and heat-inactivated ctrl CM, P3C CM and P2C CM were added to THP1-XBlue-CD14 and incubated for 24 h at 37 °C. 40 µl supernatant was added to 160 µl QUANTI-Blue™ and incubated for 2 h at RT in a 96 well plate prior to absorbance was measurement at 650 nm. Values represent the mean ± S.E.M of three independent experiments (run in duplicates). Statistical analysis was performed using 2way *ANOVA* test to compare mean ±S.E.M values between CM and heat-inactivated CM, **$p<0.01$; ***$p<0.001$.

3.2.8. Tumour cells release HMGB1 following TLR2 stimulation.

Our next endeavour was to determine the oesophageal cell-derived factors responsible for the activation of TLR2 in THP1-XBlue-CD14 cells. As it was mentioned before, TLRs can recognise damage-associated molecular patterns (DAMPs), which are released from stressed or dying cells. HMGB1 is one of the several 'alarmins' that have been reported to signal via TLR2 [428]. HMGB1 is passively released from necrotic cells or is actively secreted by inflammatory cells [429].

Therefore, HMGB1 expression following TLR2 stimulation of the OAC cell line, SK-GT 4 was analysed. TLR2 stimulation induced increased expression levels of both TLR2 and HMGB1 in SK-GT 4 cells over 24 h, which were inhibited in the presence of the TLR2 neutralising antibody (**Figure 3.15 A**). TLR2 stimulation also caused the upregulation of RAGE, another HMGB1 receptor, suggesting that HMGB1 is being released from the cells [430]. However, RAGE expression was not inhibited by αTLR2, suggesting that secreted factors additional to HMGB1 contribute to its upregulation (**Figure 3.15 A**). One of the protein types which activate RAGE are S100 proteins, which are released under cell stress or inflammation and induce cancer cell growth through RAGE [431, 432]. To determine whether translocation from the nucleus and cellular release of HMGB1 occurs following TLR2 stimulation, HMGB1 levels were measured in cytoplasmic extracts and CM from SK-GT 4 cells over a P2C-stimulation time-course. Results show that HMGB1 translocation into the cytoplasm occurs within 4 h and peaks at 20 h, following P2C stimulation (**Figure 3.16 A, C**). To confirm that the analysed cytoplasmic fraction does not contain nuclear proteins, Lamin B1 expression was also determined. Lamins are critical for structural properties of the nucleus and among them Lamin B1 is most commonly used as a nuclear loading control [430]. Lamin B1 was not detected in cytoplasmic fraction (**Figure 3.16 A**) and expression of both β-actin (cytoplasmic loading control) and Lamin B1 was detected in the whole cell lysate (**Figure 3.16 B**). Cellular release of HMGB1 into supernatants is initially detectable when cytoplasmic HMGB1 levels are highest, 20 h following P2C stimulation, with maximum HMGB1 secretion occurring at 36 h and returning to undetectable levels by 72 h (**Figure 3.16 A**). Translocation of HMGB1 from cytoplasm to media was verified using densitometry analysis (**Figure 3.16 C, D**).

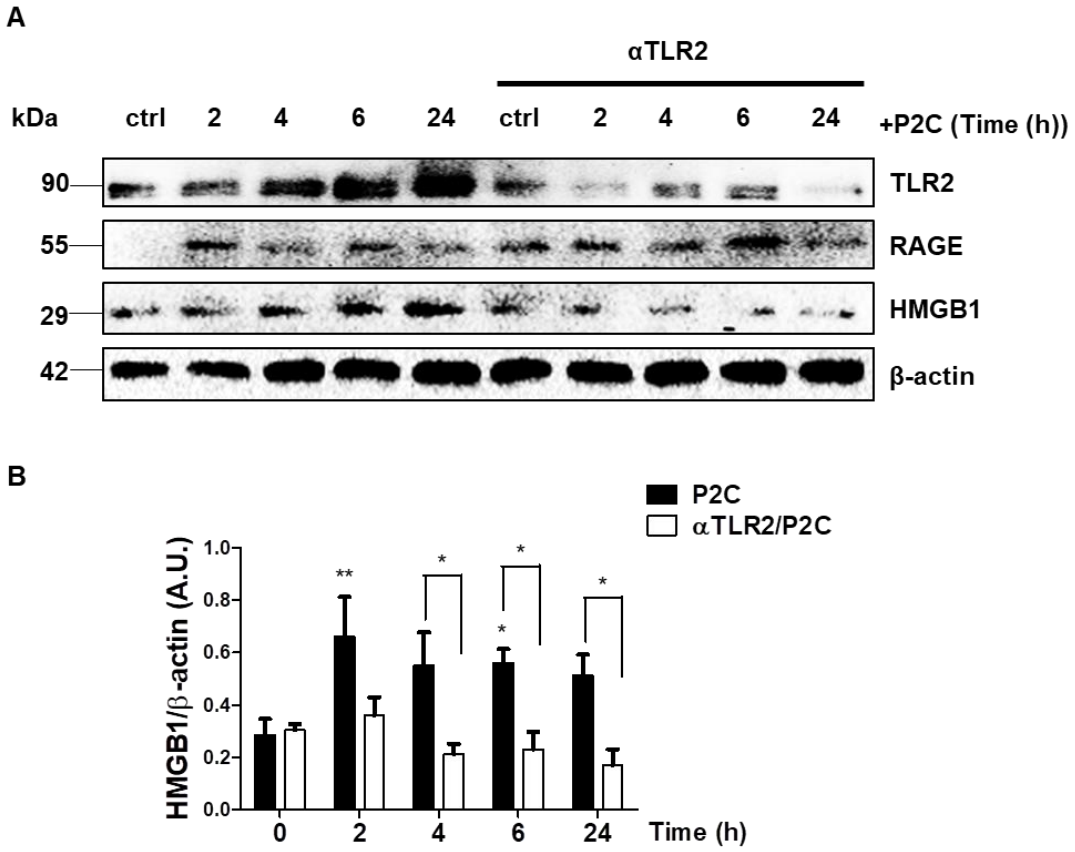


Figure 3.15. TLR2-stimulation of oesophageal cancer cells induces HMGB1 upregulation

A SK-GT 4 cells were seeded at 1.6 x10^5 cells/ml in 12 well plate 24 h prior stimulation. Cells were stimulated with P2C (0.05 μg/ml) for the indicated times. Protein lysate (10 μg) was resolved by SDS-PAGE, wet transferred to nitrocellulose and probed for TLR2 (90 kDa), HMGB1 (27 kDa), RAGE (55 kDa) and loading control, β-actin (42 kDa). Immunoblot is representative of three independent experiments. **B** Densitometric analysis of HMGB1 in SK-GT 4 cells. Data represents mean ±S.E.M of three independent experiments. Statistical analysis was performed using 2way *ANOVA* test to compare mean values between untreated and αTLR2 treated cells and unpaired two-tail student *t*-test to compare mean values between control (0 h) and P2C treated (2;4;6;24 h) cells, *p<0.05; **p<0.01; (A.U. refers to Arbitrary Unit).

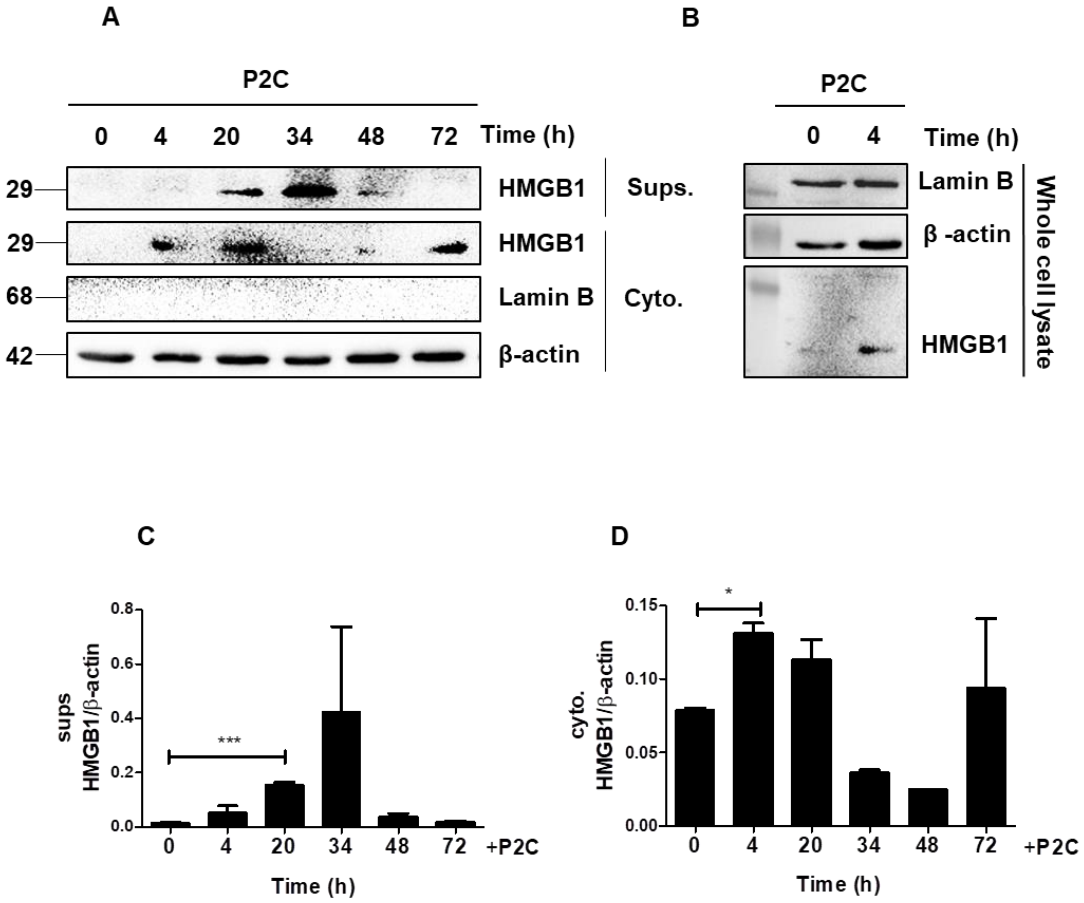


Figure 3.16. TLR2-stimulation of oesophageal cancer cells induces HMGB1 secretion.

SK-GT 4 cells were seeded at 5 x 10^5 cells/well in 6 well plate 24 h prior stimulation. Cells were stimulated with P2C (0.05 μg/ml) for the indicated times. **A)** HMGB1 levels in supernatants were analysed by Western blot using Strata Clean beads. To analyse HMGB1 in cytoplasm, media was removed, cells were washed with PBS and subcellular fraction buffer was added. Protein lysate (20 μg) was resolved by SDS-PAGE, wet transferred to nitrocellulose and probed for HMGB1 (27kDa), Lamin B (nuclear protein, 68kDa) and β-actin (cytoplasmic protein, 42 kDa) **B)** Expression of Lamin B, β-actin and HMGB1 were analysed in whole cell lysates. **C)** Densitometry of HMGB1 levels in supernatants and **D)** cytoplasm based on blots, values represent the mean ±S.E.M. Statistical analysis was performed using unpaired two-tail student *t*-test to compare mean values between untreated and P2C-treated cells, *p<0.05, **p<0.05

3.2.9. The endogenous TLR2 ligand, HMGB1 activates primary macrophages.

Results presented thus far suggest that autocrine signalling is responsible for the upregulation of TLR2 during oesophageal disease. Dysplastic and OAC cell lines respond to TLR2 stimulation by secreting inflammatory chemokines (**Figure 3.5**) and HMGB1 (**Figure 3.16**) which are potent activators of inflammation and proliferation within the tumour microenvironment [433] [92]. CM collected from oesophageal cells is able to activate TLR2 on monocytic cells (**Figure 3.11**). Recent studies have shown that oesophageal squamous cell carcinoma (OSCC)-derived exosomal HMGB1 can differentiate monocytes into tumour-associated macrophages (TAM) by upregulating expression of PD1, CD206, and IL-10 thus mediate OSCC progression [120]. When HMGB1 is released into tumor microenvironment, it induces inflammatory responses. Once released, HMGB1 can bind to receptors including RAGE, TLR2 or TLR4 and mediate cellular responses such as chemotaxis and release of proinflammatory cytokines [434].

Based on the above information whether recombinant protein HMGB1 participates in macrophage activation was analysed. Murine macrophages (BMDM) were stimulated with different concentrations of rHMGB1 (10, 40, 60, 100 ng/ml) for 24 h. Results revealed that rHMGB1 was most effective at 40 ng/ml and 60 ng/ml and upregulated expression of caspase-11 and pro-IL-1β, important components of inflammasome. Upregulation of both proteins was blocked by αTLR2, suggesting that the signal is mediated by TLR2 receptor (**Figure 3.17**). When human macrophages (MDMs) were stimulated with rHMGB1, similar results were observed for pro-IL-1β but upregulation of caspase-4, the human homolog of Caspase-11, was not inhibited in αTLR2 pretreated cells, suggesting that rHMGB1 activates human macrophages via other receptors additional to TLR2 (**Figure 3.18**).

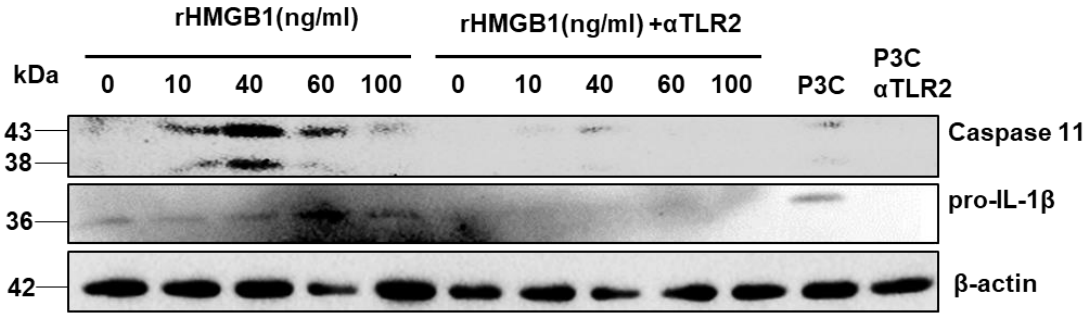


Figure 3.17. HMGB1 induces upregulation of Caspase-11 and pro-IL-1β in BMDM via TLR2 signalling.

Murine BMDMs were seeded at 1 x 10^6 cells/ml in 24 well plate, 24h prior stimulation. Cells were left untreated or incubated for 1h with 10 μg/ml αTLR2 Ab and then stimulated for 24h with; recombinant HMGB1 (10; 40; 60; 100 ng/ml) or directly with P3C (0.05 μg/ml) as a positive control. Lysates (10 μg) were collected and samples were run on 12% SDS-PAGE gel. BMDM expression of Caspase-11 (38, 43 kDa), pro-IL-1β (36 kDa) and β-actin (42 kDa; loading control) were determined by Western blot. Blots are representative of three independent experiments. Figure represents one representative result.

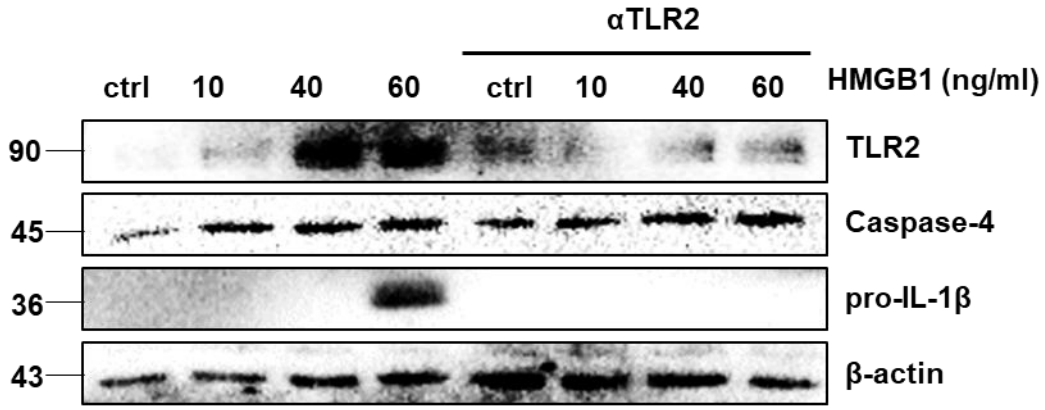


Figure 3.18. HMGB1 induces upregulation of pro-IL-1β in MDM via TLR2 signalling.

Human MDMs were seeded at 2 x 10^6 cells/ml in 12 well plate, 24h prior stimulation. Cells were left untreated or incubated for 1 h with 10 μg/ml αTLR2 and then stimulated for 24 h with; HMGB1 (10; 40; 60 ng/ml). Lysates (10 μg) were collected and samples were run on 12% SDS-PAGE gel. BMDM expression of Caspase-11 (38, 42 kDa) , pro-IL-1β (36 kDa) and β-actin (43kDa; loading control) were determined by Western blot. Blots are representative of three independent experiments. Figure represents one representative result.

3.2.10. CM from oesophageal cancer cells activates macrophages through TLR2.

Previous results demonstrated that rHMGB1 can upregulate the inflammatory proteins, caspase-11 and pro-IL-1β, in murine macrophages through TLR2. Based on information that SK-GT 4, upon TLR2 activation, releases HMGB1 into media, study to analyse whether CM from oesophageal adenocarcinoma cells is able to activate macrophages in a similar way to rHMGB1 was conducted.

As detailed in the introduction section, there is significant evidence which identifies an association between chronic inflammation and the development of oesophageal adenocarcinoma. Infiltrating immune cells and cytokines are key contributors to the local microenvironment during chronic inflammation, and can have crucial roles in tumorigenesis and metastasis [435]. Macrophages play an important role in coordinating inflammation and are known to stimulate key steps in tumour progression [436]. The diverse functional effects of macrophages are attributed to their ability to polarize into a range of phenotypes (M1, M2a, M2b, M2c) in response to their microenvironment [19]. In addition to the production of proinflammatory mediators such as TNFα, IL-1β or IL-6, macrophages activated by TLRs also produce anti-inflammatory cytokines such as IL-10, polarising them into an immunoregulatory M2b phenotype, which drives Th2 responses [19].

Based on the above information the influence of OAC CM on macrophages was analysed. Murine macrophages were incubated with CM from unstimulated, P2C and P3C-stimulated SK-GT 4 cells, prepared in the same manner as for THP1-XBlue-CD14 stimulations (**Figure 3.10**). After 24 h incubation, supernatants, protein and RNA lysates from BMDMs were analysed by ELISA, Western blot and qPCR analysis, respectively (**Figure 3.19; Figure 3.20**). Results show that, similar to direct stimulation with P2C and P3C, CM from both P2C and P3C-stimulated SK-GT 4 cells induce the secretion of IL-6, TNFα and IL-10 (**Figure 3.19, A-C**). The qPCR analyses reveal that P3C CM and P2C CM upregulate expression of Arginase-1, a typical marker of M2 macrophages (**Figure 3.19 D**). Western blot results reveal that SK-GT 4 CM induces the upregulation of NLRP3, caspase-11 and pro-IL-1β (**Figure 3.20**). Similar results were observed in monocyte-derived macrophages (MDMs) in which stimulation with CM from P3C and P2C stimulated SK-GT 4 upregulates expression of NLRP3, pro-IL-

1β and the caspase-11 human homologs, caspase-4 and caspase-5 (**Figure 3.21**). As previous results demonstrated that rHMGB1 activates BMDMs through TLR2, and hypothesis that HMGB1 is one of the proteins in OAC CM was made, further study to see whether the similar activation of BMDM will be observed with OAC CM was designed. Before OAC CM was added to the BMDMs, cells were preincubated with αTLR2. Results revealed that αTLR2 significantly impaired the upregulation of inflammatory proteins, NLRP3, caspase-11 and pro-IL-1β by OAC CM, confirming the presence of TLR2 ligands in the CM (**Figure 3.22**). Analysis of cytokines characteristic for M2/TAM, IL-6, TNFα and IL-10 revealed that similar to inflammasome-related protein expression, production of these cytokines was induced through TLR2 activation. Preincubation of BMDMs with αTLR2 inhibit production of IL-10, TNFα and IL-6 in P3C-/P2C-OAC CM stimulated BMDMs (**Figure 3.23**). To support hypothesis that inflammasome priming of BMDM cytokine production is occurring via TLR2, experiments were repeated in WT and TLR2-deficient BMDM. Results reveal that significantly less IL-6 and TNFα secretion occurred in *Tlr2*$^{-/-}$ BMDM, when compared to WT (**Figure 3.24**). These results confirm that ligands present in OAC CM activate macrophages specifically through TLR2.

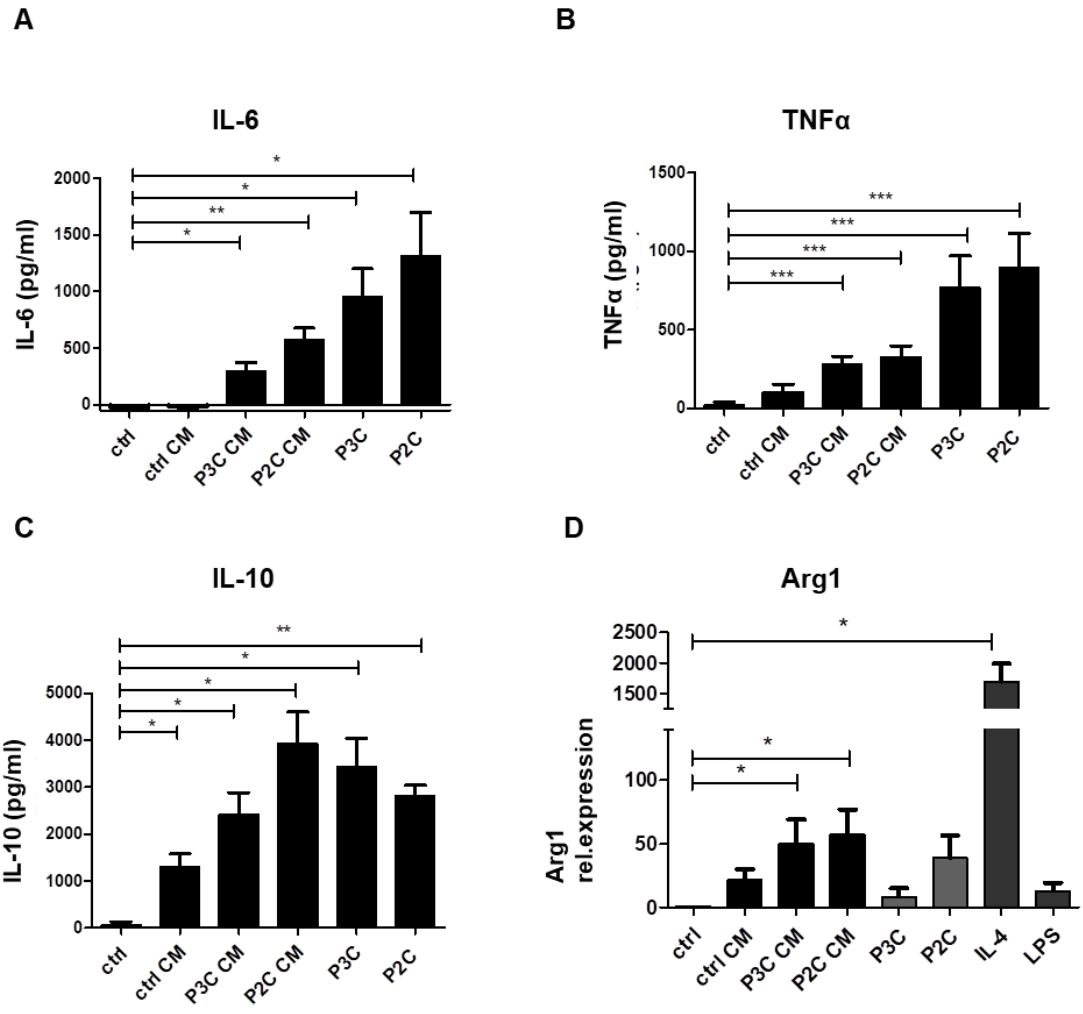


Figure 3.19 Conditioned media from OAC cells induce cytokine secretion and Arginase-1 expression in BMDM.

Murine BMDM were seeded at 1 x 10^6 cells/ml for 24 h prior to stimulation. BMDMs were left untreated or treated with: 50% CM collected from unstimulated, P3C- or P2C- stimulated (0.05 µg/ml) SK-GT 4 cells; or directly with P3C or P2C (0.05 µg/ml) as a positive control for 24 h. Levels of IL-6 **(A)**, TNFα **(B)** and IL-10 **(C)** in supernatants were determined by ELISA. **D)** Arginase-1 expression was measured and quantified by quantitative polymerase chain reaction (qPCR). Data are normalized to the stably expressed reference gene, GAPDH. BMDMs were stimulated with IL-4 as positive control for M2 phenotype and LPS as a control for M1 phenotype. Values represent the mean ±S.E.M of three independent experiments. Statistical analysis was performed using unpaired two-tail student *t*-test to compare mean values between treated and untreated cells, *p<0.05; **p<0.01; ***p<0.001.

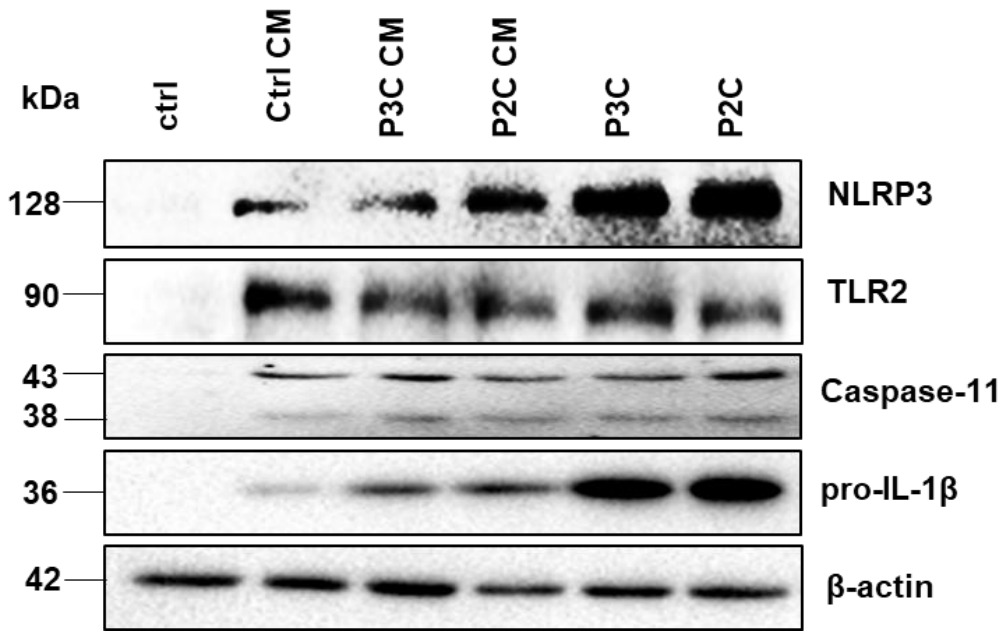


Figure 3.20 Conditioned media from OAC cells induces the upregulation of inflammasome-related proteins in primary murine macrophages.

BMDM were seeded at 1 x 10^6 cells/ml 24 h prior stimulation. Next day, BMDMs were left untreated or incubated for 24 h with: 50% CM collected from unstimulated, P3C- or P2C- stimulated (0.05 μg/ml) SK-GT 4 cells; or directly with P3C or P2C (0.05 μg/ml) as a positive control. Protein lysate (10 μg) was resolved by 12% by SDS-PAGE, wet transferred to nitrocellulose and probed for NLRP3 (128 kDa), TLR2 (90 kDa), Caspase-11 (38, 42 kDa), pro-IL-1β (36 kDa) and loading control, β-actin (43 kDa). Immunoblot is representative of three independent experiments. Figure represents one representative result.

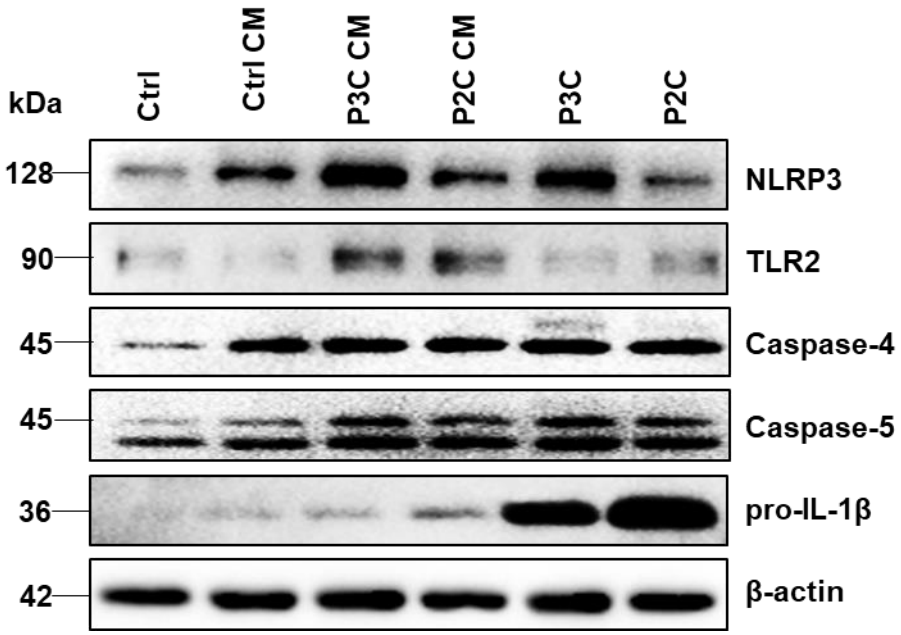


Figure 3.21. Conditioned media from OAC cells induces the upregulation of inflammasome-related proteins in primary human macrophages.

Human Monocyte-derived Macrophages (MDMs) were left untreated or incubated for 24 h with: 50% CM collected from unstimulated, P3C or P2C stimulated (0.05 μg/ml) SK-GT 4 cells; or directly with P3C or P2C (0.05 μg/ml) as a positive control. Protein lysate (10 μg) was resolved by 12% by SDS-PAGE, wet transferred to nitrocellulose and probed for NLRP3 (128 kDa), TLR2 (90 kDa), Caspase-4 (45 kDa), Caspase-5 (45 kDa), pro-IL-1β (36 kDa) and loading control, β-actin (42 kDa). Immunoblot is representative of three independent experiments. Figure represents one representative result.

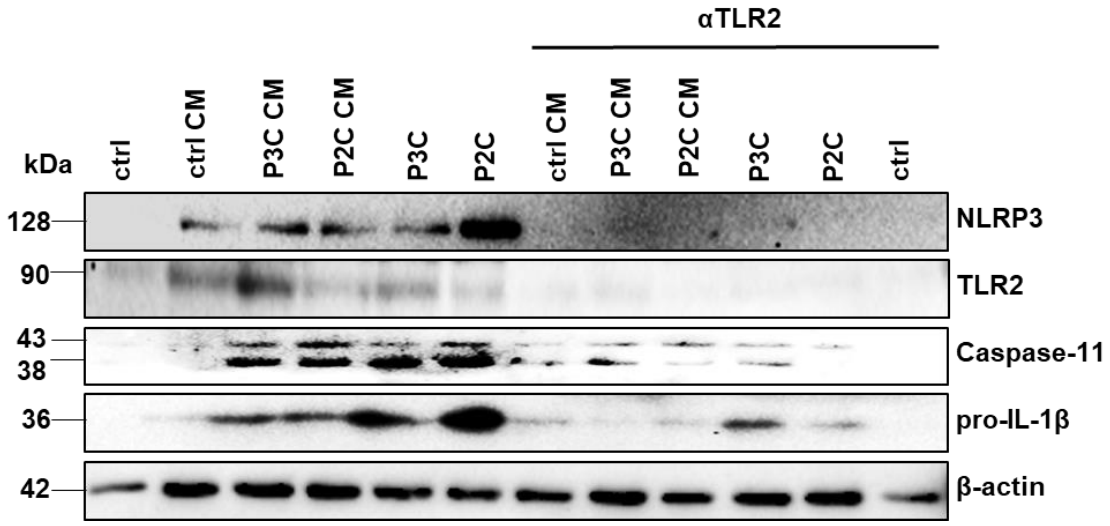


Figure 3.22. Conditioned media from OAC cells induce the upregulation of inflammasome-related proteins in BMDM via TLR2.

BMDMs were seeded at 1 x 10^6 cells/ml in 12 well plate, 24h prior stimulation. Cells were left untreated or incubated for 1h with 10 μg/ml αTLR2 Ab and then stimulated for 24 h with: 50% CM collected from unstimulated, P3C or P2C pre-treated (0.05 μg/ml) SK-GT 4 cells; or directly with P3C or P2C (0.05 μg/ml) as a positive control. BMDM expression of NLRP3 (128 kDa), TLR2 (90 kDa), Caspase-11 (38, 42 kDa) , pro-IL-1β (36 kDa) and β-actin (43kDa; loading control) were determined by Western blot. Blots are representative of three independent experiments. Figure represents one representative result.

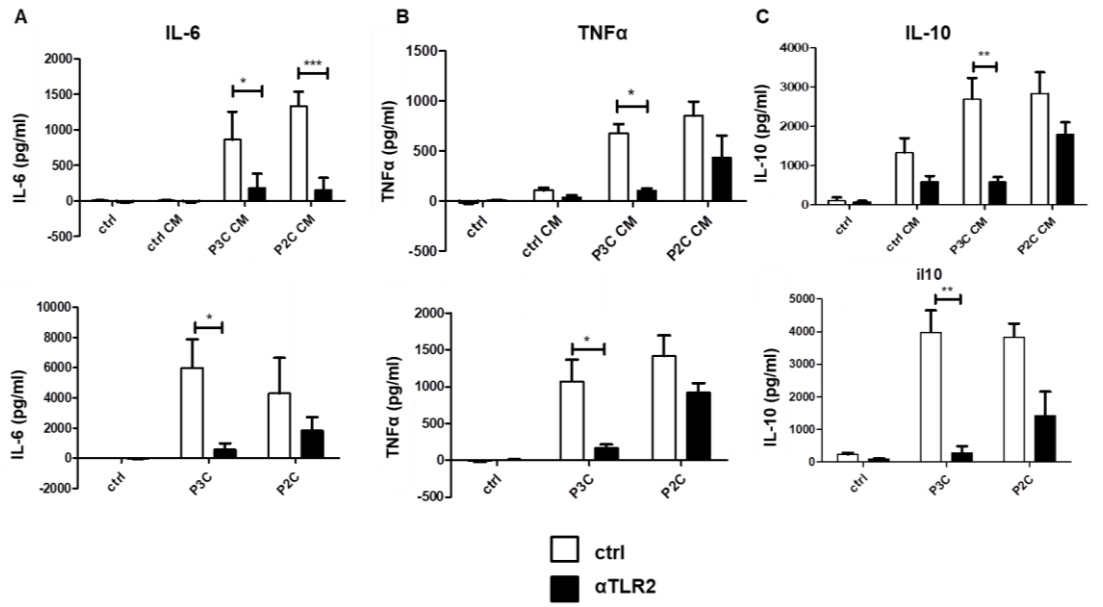


Figure 3.23. Conditioned media from OAC cells induce the upregulation of cytokines in BMDM via TLR2.

BMDMs were seeded at 1 x 10^6 cells/ml in 12 well plate, 24 h prior stimulation. Cells were left untreated or incubated for 1 h with 10 µg/ml αTLR2 Ab and then stimulated for 24 h with: 50% CM collected from unstimulated (ctrl CM), P3C or P2C pre-treated (0.05 µg/ml) SK-GT 4 cells (top panel); or directly with P3C or P2C (0.05 µg/ml) as a positive control (bottom). Levels of **A)** IL-6, **B)** TNFα and **C)** IL-10 were determined by ELISA. Values represent the mean ±S.E.M of three independent experiments (in duplicates). Statistical analysis was performed using unpaired two-tail student *t*-test to compare mean values between untreated and αTLR2 Ab treated BMDMs; *p<0.05; **p<0.01; p***<0.001.

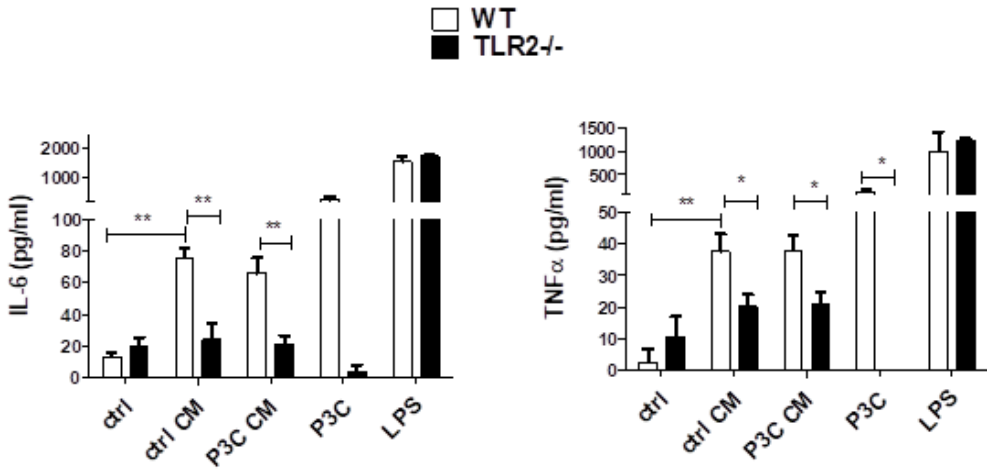


Figure 3.24. Conditioned media from OAC cells induces pro-inflammatory cytokine secretion via TLR2 signalling.

WT and TLR2 KO BMDMs were seeded at 1 x 10^6 cells/ml in 24 well plate, 24h prior stimulation. Cells were left untreated or incubated for 24h with: 50% CM collected from unstimulated, P3C- or P2C- stimulated (0.05 μg/ml) SK-GT 4 cells; or directly with P3C or P2C (0.05 μg/ml) as a positive control. Levels of IL-6 and TNFα were determined by ELISA. Values represent the mean ±S.E.M of two independent experiments (in duplicates). Statistical analysis was performed using unpaired two-tail student *t*-test to compare mean values between WT and TLR2 KO stimulations and treated and untreated WT BMDMs, *$p<0.05$; **$p<0.01$;

Summary of main findings in Chapter 3

- Screening a panel of oesophageal cell lines, the highest TLR2 expression levels are observed in Barrett's oesophagus and early-stage OAC cells.
- Activation of TLR2 signalling induces production of the chemokine IL-8 and release of HMGB1 protein into the cell culture microenvironment.
- Exogenous HMGB1 and CM-containing HMGB1 caused the TLR2-dependent upregulation of inflammasome-related proteins in primary murine macrophages.
- CM from TLR2-activated SK-GT 4 cells causes the polarisation of primary murine macrophages into an M2/TAM-like phenotype.

3.3. Discussion

Results from this chapter demonstrate that TLR2 expression increased in cells derived from Barrett's oesophagus cells and early stage OAC disease stages, represented by SK-GT 4 and OE33 cell lines. TLR2 signalling induces production of the chemokine IL-8 and 'alarmin' protein HMGB1. HMGB1 released from oesophageal cells results in the activation of primary macrophages through TLR2. Blocking TLR2 with a neutralising antibody successfully inhibits TLR2-mediated IL-8 and HMGB1 production and could be a promising therapeutic target for Barrett's oesophagus and the prevention of OAC development.

Chronic inflammation plays a very important role in oesophageal adenocarcinoma and its precursor lesion, BO. There is substantial evidence which shows a clear correlation between the upregulation of TLR expression and cancer development. TLR2 plays a key role in the activation of inflammatory responses via its ability to recognise PAMPs during host defence against invading pathogens [390]. Based on previous reports, showing that TLR2 is increased through the OAC disease sequence of reflux esophagitis, Barrett's oesophagus and adenocarcinoma [333], the role of TLR2 in OAC development and progression was investigated.

This study used a panel of oesophageal cell lines to generate data which suggests that TLR2 mRNA and protein expression is high in dysplastic and early stage adenocarcinoma oesophageal tissue, whereas advanced oesophageal adenocarcinoma has low TLR2 expression. GO cells are dysplastic (Barrett's high-grade dysplasia) pre-cancerous cells and OE33 and SK-GT 4 represent poorly-differentiated, stage IIA and well-differentiated, stage IIB cancer cells, respectively, therefore suggesting that TLR2 signalling is only relevant in dysplastic and early stages of OAC disease, and that TLR2 expression and signalling is downregulated during malignant progression, as no TLR2 expression was observed in the stage III cancer cells, FLO-1. In support of our findings, a recent report by Huhta and colleagues identified the expression of TLR1, 2, and 6 in oesophageal tissue by immunohistochemistry, and showed that TLR expression level increased in Barrett's metaplasia and dysplasia, corresponding with increased TLR expression from gastric to intestinal-type epithelia. However, they did not observe significant increases in TLR2 expression in the subsequent progression to adenocarcinoma, with TLR1 and TLR6 showing decreased expression levels in carcinomas as compared with premalignant epithelium [330].

TLR2 is activated by lipoproteins/peptidoglycans from gram-positive and gram-negative bacteria and endogenous ligands released during tissue stress [80][81][437]. Activation of TLR2 leads to downstream NF-κB activation-mediated inflammatory signalling. Although no active NF-κB is detectable in the normal oesophagus, high levels of active NF-κB have been detected in Barrett's and OAC tissue [314] [437]. Obtained results showed that TLR2 expression is upregulated on SK-GT4 and GO cells following TLR2 stimulation, and that the TLR2 neutralising antibody, αTLR2, can block both TLR2 upregulation and TLR2-mediated IL-8 production.

Interleukin IL-8 is a cytokine from the CXC chemokine family, originally classified as a neutrophil chemoattractant. Expression of IL-8 is regulated by activator protein and/or NF-κB-mediated transcriptional activity. The effect of IL-8 is mediated through the binding of this chemokine to the two cell-surface G protein-coupled receptors: CXCR1 and CXCR2 [52]. After activation of G proteins (CXCR1 and CXCR2), IL-8 signalling promotes activation of the primary effectors phosphatidyl-inositol-3-kinase (PI3K), phospholipase 3 (PLC), promoting the activation of Akt, PKC, calcium mobilization and/or MAPK signalling pathway [53]. Chemokines play dual roles in tumour progression. On the one hand, chemokines recruit immune cells which promote anti-tumour activities and eliminate tumour cells. Conversely, attraction of immune cells by chemokines may facilitate the increase of tumour growth and deliver survival factors and angiogenic mediators for the tumours [438] [304]. At present, many reports are focused on the important role of IL-8 in tumour progression and metastasis in a variety of human cancers, including breast cancer [439], lung cancer [440], colorectal cancer [441] and oesophageal cancer [419]. High IL-8 has been detected in the serum of pancreatic and lung cancer patients and levels of this chemokine correlate with tumour size, stage and prognosis [56] [57] [58]. High expression of IL-8 in human oesophageal cancer tissue was correlated with poor prognosis of patients [418]. IL-8 expression in oesophageal tissue has been shown to correlate with histopathologic inflammation in Barrett's oesophagus patients and is strongly expressed in OAC patients, suggesting a role for IL-8 in oesophageal carcinogenesis [442]. It has been shown that increased circulating proinflammatory cytokines, including IL-8, participates in BO development [443].

Results therefore suggest that αTLR2 may represent a potential therapeutic to limit TLR2-mediated auto-upregulation and NF-κB-mediated inflammation during Barrett's and progression to OAC. Although OAC cells have been reported to express elevated

levels of TLR2, there is no real understanding of why oesophageal cancer cells upregulate this receptor, or the consequence of TLR2 expression in these cells. The hypothesis that since TLR2-activated oesophageal cells produce chemokines, which promote the trafficking of leukocytes into tumour microenvironment, they also induce tumour progression via macrophage differentiation was made.

Monocytes and macrophages play a very important role in tissue homeostasis and immunity. It is well known that monocytes are recruited from the circulation by various chemoattractants released by cancer cells [444]. Infiltration of peripheral monocytes into the tumour microenvironment induces their differentiation into tissue macrophages, which can be phenotypically classified as a pro-inflammatory, classically activated M1 macrophages or anti-inflammatory M2 macrophages [445]. Our results indicate that factors secreted from TLR2-activated dysplastic and oesophageal adenocarcinoma cells are able to activate TLRs in human monocytic cells. Conditioned media (CM) collected from unstimulated oesophageal cells does not activate TLRs on THP1 cells, which suggests that upon TLR2 activation, oesophageal cells start secreting endogenous TLRs ligands into the tumour microenvironment. To support our hypothesis, pre-incubation of OAC cells with αTLR2 before TLR2 activation, decreased their ability to activate TLR2 on THP-1 cells, again suggesting that upon TLR2 stimulation OAC cells starts secreting factors which could induce inflammation. As pre-incubation of THP-1 cells with αTLR2 totally blocks the ability of OAC CM to activate TLRs, the hypothesis that OAC CM contains endogenous TLR2 ligands, which are released upon TLR2 stimulation was made. In support of our findings, Lewis lung carcinoma (LLC) cells secrete factors which activate TLR2 in murine macrophages and induce IL-6 and TNFα production and lung inflammation [324]. As heat-inactivation of OAC CM totally decreased its ability to activate TLR2 on THP-1, results suggest that factors in CM are of protein origin.

One of the proteins which has been reported to be released from the cancer cells and is a TLR2 endogenous ligand is HMGB1 [92]. Recently, many studies have focussed on the role of HMGB1 during tumour progression [101] [446]. HMGB1 is a nonhistone DNA-binding nuclear protein which promote the assembly of nucleoprotein complexes [447]. HMGB1 can be passively released from dying cells during necrosis or actively from immune or cancer cells after exogenous and endogenous stimuli [448]. Once released into the tumour microenvironment, HMGB1 binds with high affinity to several receptors including the receptor for advanced glycation end products (RAGE) [449],

TLR2 [450], TLR4 [451] and TLR9 [452]. Intracellular and extracellular forms of HMGB1 have been correlated with tumour formation, progression, and metastasis. Increased HMGB1 expression was detected in several solid tumours including breast cancer [116], colorectal cancer [453], lung cancer [117] and oesophageal cancer [454].

In the context of oesophageal cancer, most of the studies are focused on the role of HMGB1 in oesophageal squamous cell carcinoma (OSCC). High levels of HMGB1 were associated with poor clinical prognosis in OSCC patients and knockout of HMGB1 inhibits proliferation and increases radiosensitivity of oesophageal cancer cells [454]. In patients with locally advanced OSCC, HMGB1 expressed in OSCC tissue associated with recurrence after postoperative radiotherapy [455]. However, until our study the effect of HMGB1 in OAC was unclear. A recent report has shown that HMGB1 has dynamic subcellular expression; with nuclear HMGB1 expression decreasing from normal squamous epithelium towards metaplasia, and an opposite trend was observed in cytoplasm, where HMGB1 expression increased during BO progression. Interestingly, no further increase of HMGB1 expression was observed in OAC tissue, suggesting that HMGB1 is particularly relevant in pre-malignant oesophageal pathology and progression towards malignancy [456].

Results demonstrate that, upon TLR2 stimulation, OAC cancer cells upregulate HMGB1, which translocates to the cytosol before being released extracellularly. As blocking TLR2 signalling causes decreased HMGB1 expression in SK-GT 4, combined with our observation that αTLR2 pretreatment inhibits the ability of OAC CM to activate TLRs on monocytic cells, we hypothesise that HMGB1 is the main OAC secreted factor responsible for TLR2 activation on monocytic cells. Activation of TLR2 also induces upregulation of RAGE expression, which is not inhibited by αTLR2. This suggests that, together with HMGB1 release, other endogenous factors are released and activate RAGE. RAGE is transmembrane receptor which binds to diverse range of endogenous extracellular ligands, including HMGB1, S100 and Beta-Amyloid [457].

Bin Li *et al.* reported that exosomal HMGB1 obtained from OSCC cells, similar to recombinant HMGB1, can differentiate monocytes into Tumour-associated Macrophages (TAMs) [120]. Our results revealed that both recombinant HMGB1 and OAC CM are capable of upregulating the expression of caspase-4/-11 and pro-IL-1β in human and murine primary macrophages. In murine macrophages, upregulation of caspase-11 and pro-IL-1β by recombinant HMGB1 was mediated by TLR2. In human macrophages pro-IL-1β expression was TLR2-dependent, however expression of

caspase-4 was not inhibited by αTLR2, suggesting that in human macrophages HMGB1 activates caspase-4 via receptors additional to TLR2.

TLR2-activated OAC CM induces the production of cytokines from macrophages which have characteristics of both M1 and M2 subsets - IL-6, TNFα and IL-10. Furthermore results indicated that OAC CM upregulated expression of Agrinase 1, a typical marker of M2 macrophages. Several studies indicate that CM from tumour cells can influence differentiation into a M2/Tumour associated macrophage (TAM)-like phenotype. Evaluation of macrophage subtypes in adenocarcinoma tissues suggest that M1 macrophages are associated with increased killing of tumour cells, while M2 macrophages correlate with tumourigenesis. For example, elevated M2/M1 ratios in OAC patient resection tissues correlate with lymph node metastasis and poor patient survival rates. Cao *et al.* demonstrated that co-culturing SK-GT 4 with THP-1 monocyte cells resulted in increased IL-10 and polarisation into M2d-like macrophages. The M2d-like macrophages stimulated OAC cell migration and increased cell invasion [365]. It has been shown that autocrine IL-6 production by macrophages can skew them into a M2d subtype [39].

Our results therefore suggest that CM from TLR2-stimulated oesophageal cells can differentiate macrophages into M2/TAM-like macrophages and promote cancer progression. As the effects of CM are blocked by αTLR2 and production of IL-6 and TNFα was significantly inhibited in TLR2-deficient BMDMs it confirms that endogenous TLR2 ligands present in OAC CM are responsible for these effects. Supporting our findings, conditioned media collected from radiated pancreatic cancer cells was shown to significantly promote cell migration through TLR2. Silencing the TLR2 endogenous ligand, HMGB1 [450], attenuated cancer cell ability to promote cell migration, suggesting that HMGB1 is one of the secreted factors in CM responsible for TLR2 activation [428].

To summarise, data generated during this study identifies that TLR2 is upregulated during BO and early stage of cancer development, the most inflammatory stages of OAC disease TLR2 stimulated BO and OAC cells respond to TLR2 agonists by secreting chemokines and tumour-secretory factors which have TLR2 signalling capability. Obtained results identified HMGB1 as a factor secreted upon TLR2 stimulation which is responsible for macrophage activation. Tumour secreted factors, including HMGB1, have the ability to polarise macrophages into a pro-tumourigenic

M2-like phenotype via TLR2, suggesting that TLR2 signalling is involved in the polarising effects of the tumour microenvironment. This suggests that, once TLR2 signalling is initiated during early stage adenocarcinoma, the positive amplification of TLR2-mediated inflammation and TLR2 expression which occurs during disease progression may be controlled by αTLR2. Above results have shown that TLR2 neutralisation limits the ability of OAC cells to secrete these TLR2 activating factors and could represent a promising target for BO or early-stage OAC patients.

Chapter 4: Caspase-11 regulates Nitric Oxide-mediated host responses to *Mycobacterium tuberculosis*

4.1. Introduction

Mycobacterium tuberculosis is the etiological agent of tuberculosis (TB) and leading cause of death due to a single infectious agent. It is estimated that about 2 billion people might have latent *Mtb* infection [458]. Currently there are no effective vaccines against infection and no biomarkers for protective immunity [459] [460]. Tuberculosis is acquired through the inhalation of droplets with live *Mtb* released by contagious individuals, and the lung is the major site of infection. The mechanism responsible for early control of bacterial proliferation is regulated by cell activation. The major players of innate immunity in *Mtb* infection are dendritic cells (DC), mast cells, natural killer cells (NK), macrophages and complement [192]. Macrophages are phagocytes at the frontline of host immune response against pathogens, including *Mtb* [461] [462]. Recognition of *Mtb* by macrophages induces their activation and production of antimicrobial effectors, such as reactive nitrogen intermediates, including nitric oxide (NO). The production of reactive nitrogen intermediates is an important host defence mechanism during responses to bacterial pathogen infections. It has been shown that NO is a crucial molecule in the regulation of acute and chronic inflammation and host defence mechanisms, and together with related reactive nitrogen intermediates (RNIs), can kill or inhibit intracellular pathogens such as mycobacteria [210].

NO is synthetized by nitric-oxide synthases (NOS): neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Both nNOS and eNOS are low–output Ca^{2+} -dependent enzymes producing NO in a pulsatile manner and are basally expressed in neurons and endothelial cells, respectively [463]. In contrast, iNOS is a high-output Ca^{2+}-independent (iNOS activity does not respond to changes in Calcium levels in the cells) synthase. iNOS expression is induced in a wide range of cells including macrophages, neutrophils, epithelial cells and hepatocytes and is upregulated during infection and inflammation [464] [465]. nNOS and eNOS are constitutively expressed while iNOS is expressed only in activated cells. Both nNOS and eNOS are producing low fluxes of NO for short periods of time. At this low concentration (<1 µM), NO acts as an intracellular signal which activate or inhibit different proteins. Unlike nNOS and eNOS, which are tightly regulated, iNOS produces high amounts of NO after stimulation with proinflammatory cytokines such as TNF, IFNγ and IL-1 or by hypoxia and LPS [466] [467]. NO produced at high concentrations (>1µM) is able to perform

nitrosation, nitration and oxidation reactions. iNOS expression is regulated at multiple levels from transcription, synthesis, stability, activity and degradation [468].

NO is synthetized universally from L-arginine (as a substrate) and molecular oxygen in reactions catalysed by NOS and utilizing electrons donated by NADPH (co-substrate). Amino acid L-arginine is obtained from exogenous and endogenous sources, including protein degradation and also *de novo* synthesis from citrulline by renal arginosuccinate synthase [469]. Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R-)5,6,7,8-tetrahydro-l-biopterin (BH4) are cofactors of all isozymes [470] [471]. All NOS isoforms are homodimers. NOS transfers electrons supplied by NADPH via the flavins FAD and FMN in the carboxy-terminal reductase domain, to the heme in the amino-terminal oxygenase domain. The oxygenase domain binds molecular oxygen, cofactor BH_4 and the substrate L-arginine, necessary for reaction. At the heme site O_2 is reduced and activated and L-arginine is oxidized to L-citruline and NO via the intermediate N-hydroxy-L-arginine [472]. NO has a short half-life and remains between 3 s to 20 s in aqueous and oxygen-containing solutions [473]. Under normoxia, NO is rapidly oxidized to nitrite (NO2-) and nitrate (NO3-). The bactericidal effect of NO in human tissue macrophages could be direct or indirect through RNS [462].

Reduction products of oxygen are called Reactive Oxygen Intermediates (ROI) and include superoxide ($O2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (●OH). These reactive products will quickly react with NO, leading to production of peroxynitrite (ONOO-) [474].

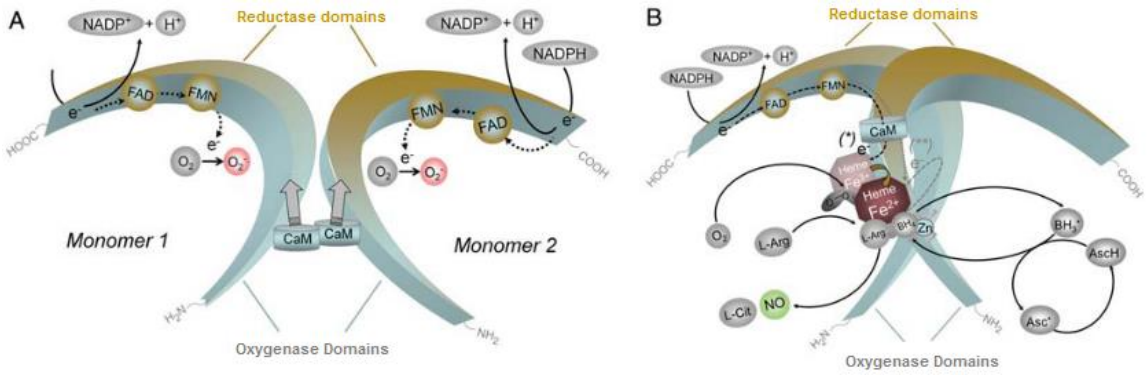


Figure 4.1 Structure of functional NOS and nitric oxide production

A) A functional NOS transfer electrons from reduced NADPH to FAD and FMN and reduce molecular oxygen to superoxide ($O_2^{-\bullet}$). Reductase domains bind calmodulin (CaM),increasing electron transfer within the reductase domain. NOS monomers are not able to bind co-factor or the substrate L-arginine.NOS monomers must homodimerize to gain enzymatic activity and produce NO **B)** In the presence of heme, which is essential for interdomain electron transfer from the flavins to the heme of the opposite monomers, NOS can form a functional dimer. In nNOS and eNOS calmodulin binds only when Ca^{2+} is elevated whereas calmodulin binds to iNOS even in the absence of Ca^{2+}.When substrate L-arginine and cofactor BH_4 are present, NOS dimers couple their heme and O_2 reduction and oxidize L-arginine to L-citruline and NO. NOS conduct two separate oxidation steps. At first, NOS hydroxylates L-arginine to Nω-hydroxy-L-arginine and in the second step, it converts this intermediate to NO and L-citruline. Picture taken from Förstermann & Sessa. *European Heart Journal*.2012 [472].

Signalling pathways involved in the expression of inducible nitric-oxide synthase

The expression of iNOS is regulated by cytokines and synthetized *de novo* in response to different stimuli [475]. Cell wall components of bacteria and fungi induce innate immune signalling cascades and promote iNOS expression. Two major signalling pathways are involved in induction of iNOS gene expression, the mitogen-activated protein kinase (MAPK) pathway and the Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) pathway, known as the JAK/STAT pathway. LPS and TNFα activate the MAPK pathway which subsequently leads to activation of nuclear factor kappa-B (NF-κB) and activator protein-1 (AP-1) [476]. iNOS expression may also be induced by IFNγ. Upon binding to the Interferon Receptor 1 (IFNR1) and

IFNR2 complex, IFNγ activates kinases of the JAK family and phosphorylates the substrate protein STAT1, signal transducers and activators of transcription 1. The phosphorylated STAT1 dimerises and translocates to the nucleus where it binds specific DNA elements and induces transcription of IRF-1 [477][478]. Both of these pathways are able to individually induce iNOS expression but their interaction greatly amplifies the response [479]. Simultaneous binding of transcription factors IRF-1 and NF-κB promote their interaction and increased iNOS expression. Following macrophage stimulation with IFNγ and TNFα, IRF-1 and NF-κB co-localize in the nucleus and bend the DNA of the iNOS promoter region [480]. IRF-1 directly interacts with the p50 subunit of NF-κB *in vitro* [481].

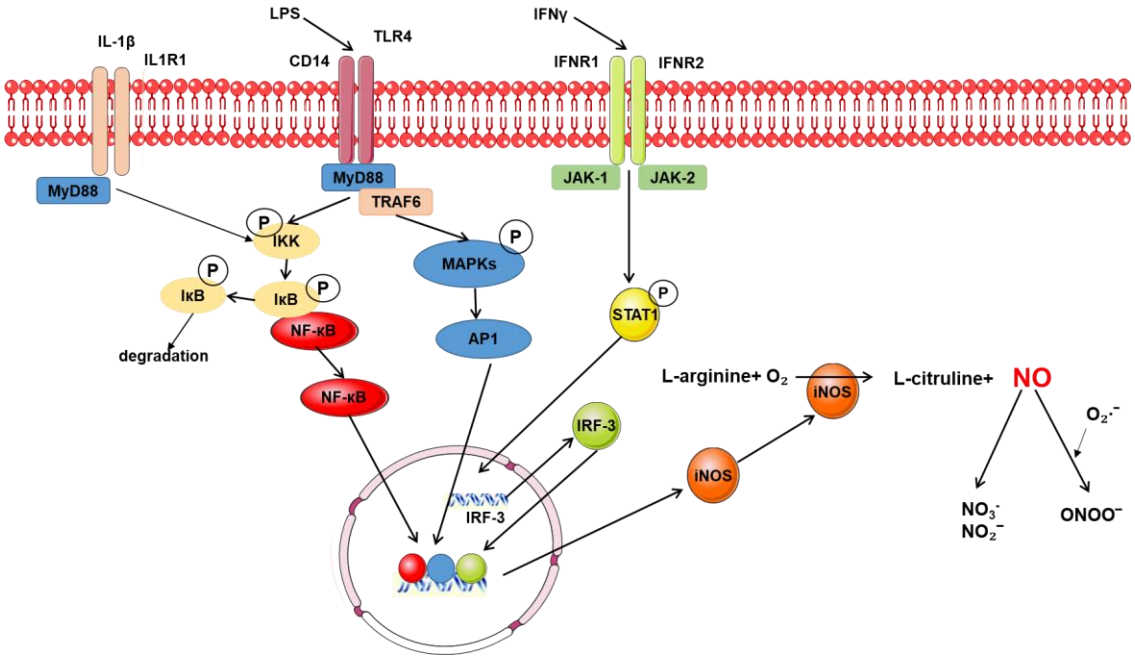


Figure 4.2. Inducible nitric oxide synthase activated by LPS and IFNγ.

Transcription of iNOS is induced by bacterial endotoxin (LPS) or by cytokines such as IFNγ and IL-1β. The iNOS expression can by regulated by AP-1, NF-κB and STAT1 transcription factors. IFNγ interacts with the IFNR1 and IFNR2 complex, which activates kinases of the JAKs family and subsequently induces phosphorylation of signal transducers and activators of transcription (STAT) pathways. Phosphorylated STAT dimerises and translocates to the nucleus where it activates transcription by binding to specific response elements in gene promoters [482]. LPS binds to CD14 on the cell membrane and activates NF-κB and AP1. IκB-NF-κB complex is inactivated in the cytosol. Free NF-κB translocates to the nucleus and induces expression of iNOS. IL-1β induces iNOS expression through NF-κB [483]. The biosynthesis of NO is carried out from L-arginine and molecular oxygen. NO is converted into highly Reactive Nitrogen Species (RNS) such as NO_3^- and NO_2^-.

Oxygen free radicals react with NO resulting in the formation of peroxynitrite anion ($ONOO^-$). Produced NO and RNI can directly kill intracellular *Mtb* in infected macrophages [462].

The role of Nitric Oxide in Mycobacterium

Activation of iNOS in activated macrophages leads to production of large amounts of NO which has antimicrobial activity [484]. The lipoarabinomannan cell-wall components of mycobacteria have been shown to directly induce NO production in murine and human macrophages [485] [486]. Due to high affinity of NO to protein-bound iron, NO is able to inhibit key enzymes, such as iron-sulfur cluster-dependent enzymes (complexes I and II) involved in mitochondrial electron transport and cis-aconitase, an important enzyme in the citric acid cycle [472]. In addition, high concentrations of NO directly interfere with the DNA of target cells, leading to the generation of abasic sites and DNA strand breaks [487]. Other potential killing mechanisms by NO include interaction with heme groups, thiols, aromatic or phenolic residues, tyrosyl radicals, and amines [488]. A combination of these effects is likely to explain the basis of NO- induced cytotoxicity on bacterial pathogens.

Many reports have shown that production of reactive oxygen and nitrogen oxide species such as superoxide ($O2^-$) and nitric oxide (NO) by macrophages is a very effective host defence mechanism against bacterial, viral, parasitic and fungal infections [202]. NO plays a key role in innate immunity and host defence against mycobacteria. Chan J and colleagues reported in 1992 that murine activated macrophages, through an L-arginine-dependent cytotoxic pathway, effectively inhibit growth and kill *Mtb*. Moreover iNOS inhibitors increased the mortality of mice infected with *Mtb*, bacterial burden and pathological tissue damage, thus confirming the role of NO in resistance against *Mtb* [489][490]. It has been reported that *NOS2 (*iNOS*)* locus is necessary to control primary tuberculosis in mice. iNOS deficient mice were more susceptible to *Mtb* and allowed *Mtb* to growt faster in the lungs compared to WT [491]. Exogenous NO delivered even at very low concentrations (<100 ppm) present powerful dose- and time dependent mycobacteriocidal action. This effect was observed for both H37Rv laboratory strain and multidrug resistant (isoniazid and rifampin) wild strains of *Mtb*, suggesting that NO action is independent of pharmacologic action [492].

The role of NO in human antimicrobial mechanisms remains controversial. A report from 1998 has shown that, in contrast to murine macrophages, IFN-γ failed to enhance *Mtb* growth inhibition by human alveolar macrophages and peripheral blood monocytes [493]**.** NOS2 mRNA and NO were not detectable in human macrophages stimulated with LPS, IFNγ or *Mtb*, suggesting that human alveolar macrophages have NO-independent mycobacteriostatic activity in *Mtb* infection [493]. However, supportive evidence for the importance of NO in human *Mtb* infection has been growing in recent years. Several studies reported that LPS from *S.typhimurium, Ch.pneumoniae, P.aeruginosa* induce NOS expression (iNOS, eNOS) and NO production in human monocyte-derived macrophages [494][495]. iNOS expression and nitrite levels were increased in alveolar macrophages and PBMCs obtained from TB patients compared to controls [496] [219] [218] [497]. Moreover it has been shown that NO plays a regulatory role in IL-1β and TNFα production, which are important in host defence against mycobacterial infection [219] [218]. Taken together, these data suggest that NO plays an important role in both experimental murine models of *Mtb* and in human *Mtb* patients.

Role of Nitric Oxide in inflammasome regulation.

Nitric oxide has many roles in immune responses, including regulation of signalling cascades, transcription factors, migration, cytokine production and T-cell differentiation [498] [499] [500]. The inflammasome is a multiprotein complex that mediates the activation of caspase-1 which leads to maturation and secretion pro-inflammatory cytokines IL-1β and IL-18. Recently, several studies have focussed on the role of inflammasomes during chronic inflammation in *Mtb*. Mishra *et al.* showed that mice lacking iNOS (*Nos2*$^{-/-}$), IFNγ or T and B cells (*Rag2*$^{-/-}$) all have enhanced production of IL-1β. Nos2$^{-/-}$ mice had more severe disease and higher IL-1β production than wild-type mice under conditions where both strains had similar levels of *Mtb* uptake, suggesting that generated NO inhibits the inflammasome. Mishra *et al.* suggested that when adaptive immunity is activated, IFNγ produced by newly recruited lymphocytes, activates macrophages and triggers expression of iNOS. iNOS-mediated NO production causes S-nitrosylation of NLRP3 thus prevents oligomerization of ASC and maturation of IL-1β [244]. It has been shown that type I interferons, such as IFNβ also negatively regulate NLRP3 [501]. The iNOS deficiency promotes accumulation of damaged

mitochondria and increases susceptibility to LPS-induced septic shock in mice. Presented results confirm that NO inhibits NLRP3 inflammasome-mediated IL-1β production and caspase-1 activation but also inhibits, to a lesser extent, NLRC4- and AIM-2 inflammasome formation. Inhibition of continuous IL-1β production by the NLRP3 inflammasome inhibits persistent neutrophil recruitment and prevents tissue damage that results from excessive inflammasome signalling [502]. Above results suggest that the NO produced as a result of *Mtb* infection is indispensable in modulating adaptive immunity and protects against a destructive inflammatory response.

As both IFN-β and IFN-γ prime the activation of caspase-11 and the noncanonical inflammasome, there is rationale to analyse the role of nitric oxide production and caspase-11 during *Mtb* infection.

In Brief:

Hypothesis and aims of Chapter 4

Hypothesis: Caspase-11, through its regulation of iNOS expression and nitric oxide production, attenuates intracellular *Mycobacterium tuberculosis* (*Mtb*) infection in murine macrophages.

Specific aims of Chapter 4:

1. Determine iNOS expression and nitric oxide production levels in LPS- and *Mtb*-stimulated wild-type and caspase-11 deficient primary murine macrophages.
2. Determine the ability of caspase-11 to regulate NF-κB nuclear translocation and STAT1 activation, two of the main transcription factors involved in iNOS induction.
3. Examine the role of caspase-11 in *Mtb* invasion.
4. Determine whether NLRP3 has a role in iNOS expression and nitric oxide production.
5. Compare the production levels of nitric oxide in male and female murine macrophages.

4.2. Results

4.2.1. Caspase-11 regulates LPS- and IFNγ-induced iNOS expression in BMDMs.

Caspase-11 and its human orthologues Caspase-4 and Caspase-5 are important components of the innate immune system in response to bacterial infection [503]. Increased caspase-11 expression was observed in lymphocytes, macrophages and hepatocytes stimulated with LPS and IFNγ *in vitro* [504] [505]. LPS, the major component of the outer membrane of gram-negative bacteria, activates Caspase-11-dependent non-canonical inflammasome to avoid excessive inflammatory response [506]. Nitric oxide (NO) is a well-known inflammatory mediator, generated by inducible NO synthase (iNOS) in response to danger or foreign signals. Constant production of NO assures cytostatic and cytotoxic activity against viruses, bacteria, fungi and protozoa [475]. A previous observation has shown that stimulation of intestinal epithelial cells with IFNγ and LPS activated iNOS expression through a JAK/STAT1 signalling pathway [507]. As previous findings from our lab have shown that caspase-11 regulates STAT1 activation in LPS-stimulated BMDMs [175], experiments to analyse whether caspase-11 also regulates iNOS expression, and subsequently NO production, were carried out.

BMDMs were isolated from WT and Caspase-11 deficient mice. Following 7 days of differentiation, BMDMs were stimulated with LPS and IFNγ for 24 h. Results confirmed that both LPS and IFNγ are capable of stimulating caspase-11 upregulation in WT BMDM. LPS-stimulated WT BMDM expresses high levels of iNOS, which is significantly lower in caspase-11 deficient BMDM (**Figure 4.1A**). NO production in BMDMs was determined as nitrite released into cell supernatant using Griess assay, which is a colorimetric method. After iNOS upregulation, BMDMs produce NO, concentrations of which were significantly higher in WT, compared to caspase-11 deficient, BMDMs (**Figure 4.1B**). Both iNOS expression and NO production were higher in LPS-stimulated than in IFNγ treated BMDMs (**Figure 4.1 A-B**). Similar observations were reported by using immunofluorescence staining. After 24 h stimulation with LPS, iNOS expression was induced in BMDMs and higher levels were detected in WT, compared to caspase-11 deficient, BMDMs (**Figure 4.2**).

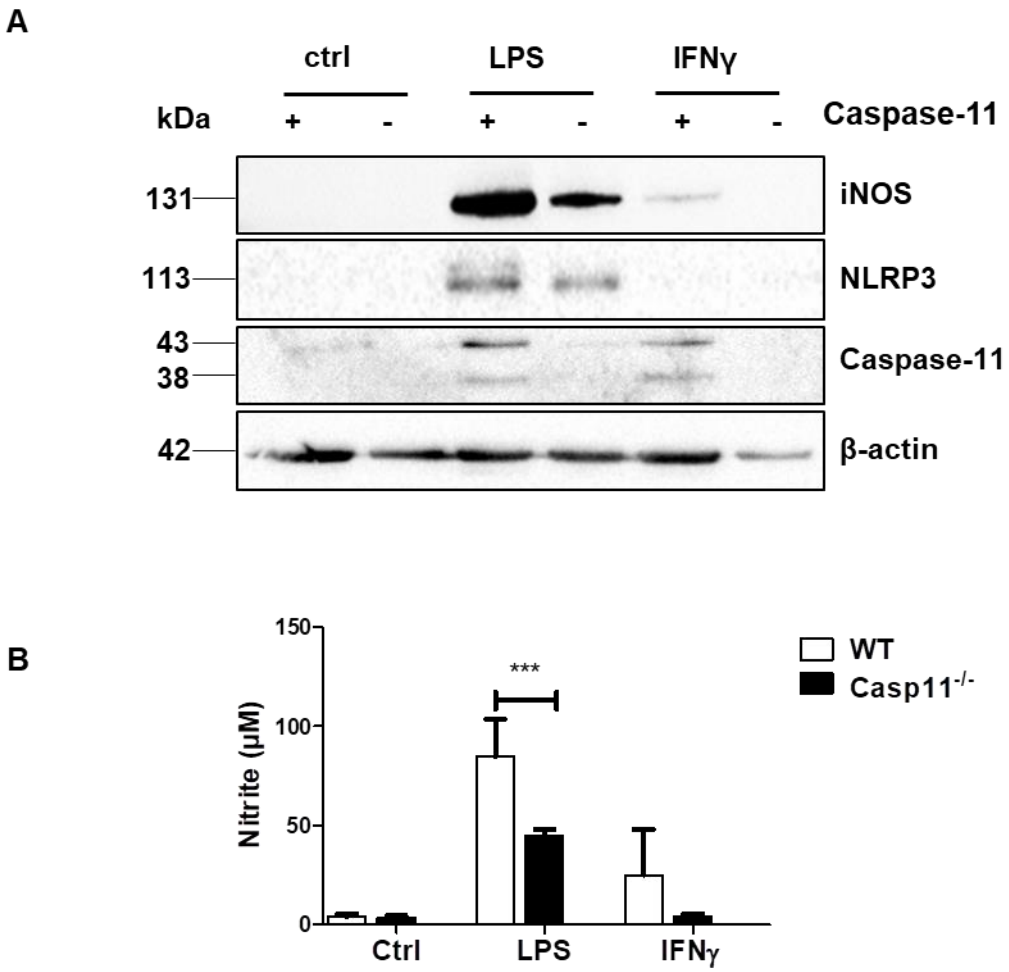


Figure 4.1. Nitric Oxide production and iNOS expression is significantly impaired in Caspase-11-deficient LPS-stimulated BMDMs.

BMDMs were seeded at 10^6 cells/ml on a 24 well plate. After 24 h cells were stimulated with LPS (1 μg/ml) or IFNγ (10 ng/ml) for 24 h. **A** Protein lysates (10 μg) analysed by western blot for iNOS (131 kDa), Caspase-11 (38, 42 kDa) and β-actin (43 kDa) as a loading control. Blots shown are representative of 3 independent experiments. **B** Nitrite concentration in the supernatants was measured using the Griess assay. Statistical analysis was performed using two-tailed *ANOVA* test to compare WT and Casp11$^{-/-}$ mean ± SEM values from 3 independent experiments, ***p<0.001

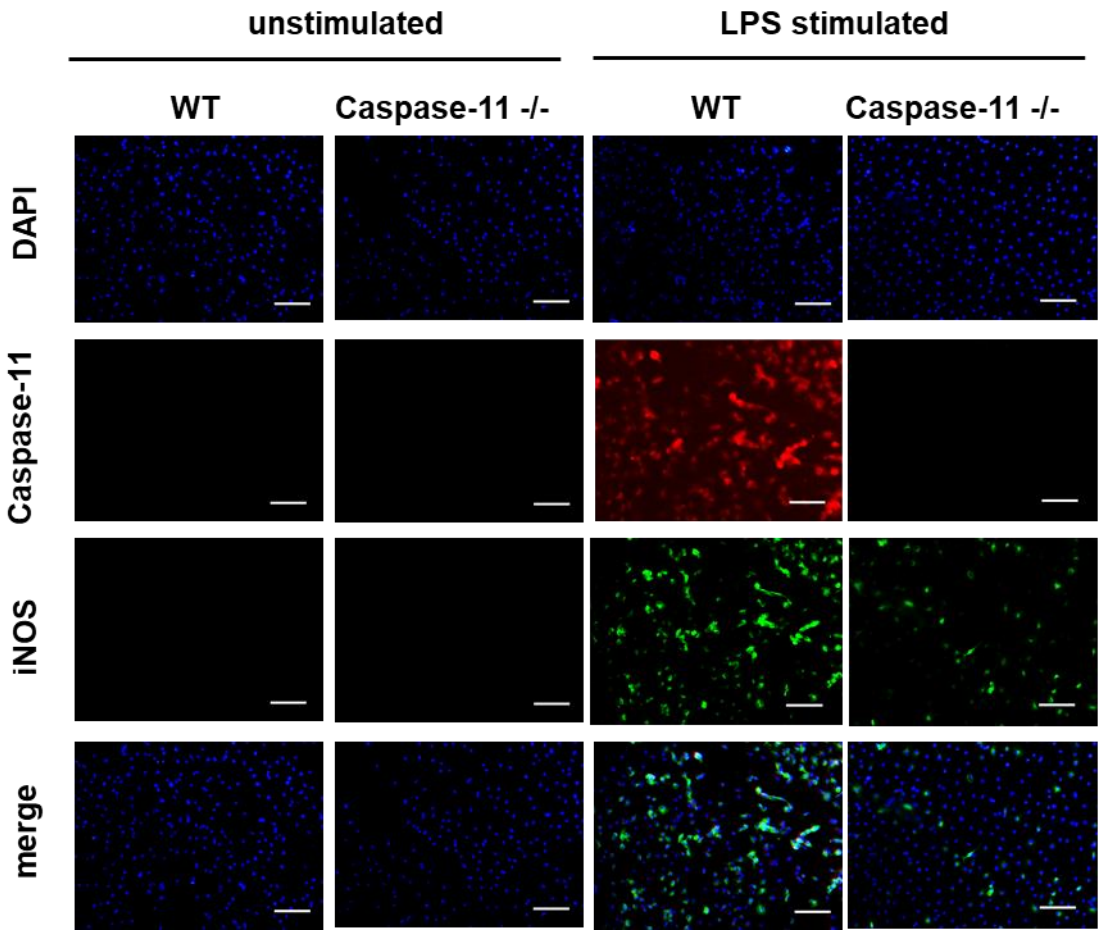


Figure 4.2. iNOS expression is impaired in Caspase-11 deficient BMDMs.

WT and Caspase-11$^{-/-}$ BMDMs were seeded 5 x 10^5 cells on coverslips in a 24-well plate. Cells were stimulated with LPS (1 μg/ml) where indicated, and fixed with 4% paraformaldehyde 24 h later. Cells were permeabilised with 0.2% Triton X-100 and stained for Caspase-11 (red) and iNOS (green). Nuclei were stained with DAPI. Immunofluorescence was viewed on Olympus BX51 and images were taken at 10X magnification. Scale bars=100μm

4.2.2. STAT1 activation in primary murine macrophages is regulated by Caspase-11.

iNOS activity in macrophages is regulated and modulated by Toll-like receptors and CD14 [508]. CD14 is the receptor for LPS and plays an important role in anti-inflammatory responses via activation of the NF-κB pathway [509]. Following LPS stimulation, the IκB-NF-κB complex is phosphorylated by IκB kinase (IKK) and free NF-κB is translocated from cytosol to the nucleus and subsequently induce iNOS gene expression [510] [511]. Another transcription factor which regulates iNOS is STAT1. Following TLR4 activation by LPS, IFNβ is induced and secreted from the cell, where it then binds to IFNAR1 and IFNAR2 receptors, activating the classical Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway [512]. Activated STAT1 translocate to the nucleus and increases iNOS induction and NO production [513]. LPS-stimulated IFNAR$^{-/-}$ BMDMs were unable to produce nitric oxide or express iNOS, indicating an essential role of type I IFNs in LPS-stimulated NO-production [514]. Inhibition of both transcription factors NF-κB and STAT1, decreased NO production [515] [507].

Based on **Figures 4.1** and **4.2,** which show that iNOS induction and NO production are impaired in caspase-11$^{-/-}$ BMDMs, we next sought to analyse the activation status of NF-κB and STAT1 in Caspase-11$^{-/-}$ BMDMs. To analyse NF-κB activation, expression of the IκB protein, IκBα was determined. NF-κB exists in the cytoplasm in an inactive complex binds with IκB (IκB-NF-κB complex), which block NF-κB translocation to nuclei. After LPS stimulation, the complex NF-κB- IκB is disrupted by IκB kinase complex which mediated IκBα phosphorylation and targets IκBα for ubiquitination and proteasomal degradation [516] [517]. Results revealed that after LPS stimulation, IκBα was degraded at 15 min and then newly-synthetized at 30 min. However, no difference in the kinetics of IκBα degradation or expression was observed between WT and caspase-11 deficient BMDMs (**Figure 4.3**). The next experiment confirmed that STAT1 phosphorylation is impaired in Caspase-11$^{-/-}$ BMDMs, which affects iNOS induction. iNOS expression was upregulated in WT BMDMs after 4 h with the highest level at 24 h after LPS administration. In Caspase-11$^{-/-}$ BMDM, iNOS was detected only at 6 h after LPS treatment and its expression levels were significantly lower compared to iNOS expression in WT BMDMs (**Figure 4.4**). This experiment confirms previous

observations, showing that caspase-11 regulates LPS mediated STAT1 activation in murine BMDMs [175].

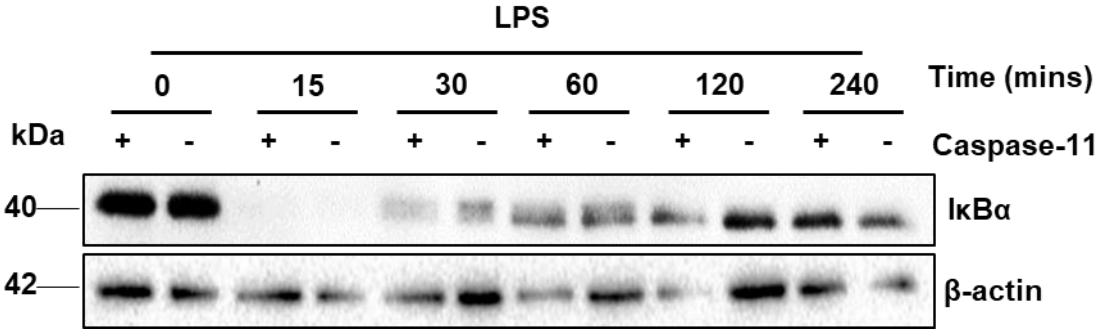


Figure 4.3. The kinetics of LPS-induced IκBα degradation is similar in WT and Caspase-11 deficient BMDMs.

WT and Caspase-$11^{-/-}$ BMDMs were seeded at 10^6 cells/ ml in 24 well plate 24 h prior treatment. Cells were stimulated with LPS (1 ug/ml) over a 4 h timecourse, as indicated. Lysates (10 µg) was resolved by SDS-PAGE, wet transferred to nitrocellulose and probed for Ikkα (40 kDa) and β-actin (43 kDa) as a loading control. Blots are representative of three independent experiments.

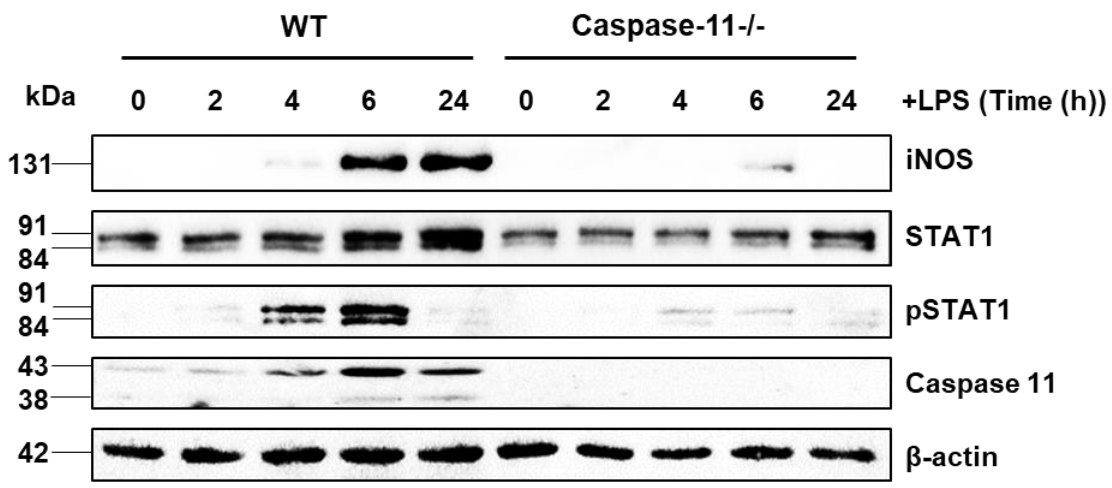


Figure 4.4. Caspase-11 regulates LPS-mediated STAT1 activation and iNOS expression in BMDMs.

WT and Caspase-$11^{-/-}$ BMDMs were seeded at 10^6 cells/ml on 24 well plate. 24 h later, cells were stimulated with LPS (1 ug/ml) over a 24 h timecourse, as indicated. Protein lysate (10 µg) was run on western blot and probed for iNOS (131 kDa), STAT1 (87 kDa), pSTAT1 (87 kDa), caspase-11 (38,43 kDa) and β-actin (42 kDa) as a loading control. Blots are representative of three independent experiments.

4.2.3. STAT1 activation regulates iNOS induction and Caspase-11 upregulation.

Based on previous observations showing that caspase-11 is required for LPS-mediated pSTAT1 activation and iNOS induction studies to confirm that STAT1 regulates iNOS expression in macrophages was carried out. The STAT1 signalling pathway plays an essential role in iNOS activation [507], although the signalling events involved in regulating iNOS expression are not well understood. It has been shown that JAK2, which mediates activation of STAT1, is involved in iNOS induction in IFNγ/LPS stimulated glial cells [518]. Moreover, STAT1 acetylation leads to decreased STAT1 binding to the iNOS promoter, subsequently inhibiting iNOS induction [519]. Nitric oxide is involved in various physiological functions and is an important mediator in many biological processes. iNOS is absent in resting cells and the gene is expressed in response to stimuli such as proinflammatory cytokines. The role of iNOS is not explicit. On the one hand, iNOS inhibits a large variety of microbes and can control intracellular growth of bacteria but on the other, upregulation of iNOS leads to tissue damage, inflammatory neurodegeneration and sepsis [520] [521] [522]. As the cytotoxic role of reactive nitrogen species in many intracellular bacteria is well known, understanding of signalling pathways which induce iNOS expression could be very important for improving available treatments.

To confirm that LPS-mediated iNOS expression in BMDMs is mediated by STAT1 phosphorylation, the specific STAT1 inhibitor, Fludarabine was used, at different concentrations ranging from 5 µM to 50 µM, and at different time-points, 2 h and 24 h before LPS administration (Parameters were chosen based on publicly available data). **Figure 4.5** revealed that a 2 h pre-treatment with Fludarabine was not able to inhibit STAT1 phosphorylation and therefore did not effect iNOS induction or NO production. In turn, 24 h pre-incubation with Fludorabine was toxic for cells and induced cell death at higher concentrations (**Figure 4.5 A, B, E, F**). Following the observation that effective doses of Fludarabine are toxic to BMDM, another inhibitor, Ruxolitinib was tested. Ruxolitinib is a potent and selective JAK1/2 inhibitor [523] and currently clinically used in the treatment of JAK2 V617F-positive myeloproliferative neoplasms, including intermediate or high risk myelofibrosis and polycythemia vera [524] [525]. Ruxolitinib selectively inhibits STAT1 and STAT3 phosphorylation in BMDMs. It has

been shown that Ruxolitinib abrogates TLR4/TRIF/IFNβ signalling pathway in LPS-stimulated human macrophages and inhibits STAT1 activation [526].

To determine whether STAT1 phosphorylation is involved in iNOS induction and NO production, BMDMs were pre-incubated with Ruxolitinib before stimulation with LPS. Results revealed that 0.5 μM Ruxolitinib significantly inhibits pSTAT1 activation simultaneously with iNOS inhibition. The use of 0.1 μM Ruxolitinib downregulated pSTAT1 levels after 2 h LPS stimulation but no difference was observed after 4 h LPS-treatment. 0.5 μM Ruxolitinib downregulated caspase-11 expression when compared to vehicle-treated (0.5 μM DMSO) LPS-stimulated BMDM (**Figure 4.6 A**). Similar to iNOS inhibition, NO production in Ruxolitinib pre-treated, LPS-stimulated BMDMs was significantly impaired at both concentrations, 0.5 and 0.1 μM (**Figure 4.6 B**). Type I interferons (IFNα/β) bind to IFNAR cell surface receptors (IFNAR1 and IFNAR2) and induce JAK/STAT signalling pathway [527]. To confirm that NO production is regulated by STAT1, $IFNAR^{-/-}$ and $IFN\gamma^{-/-}$ BMDM were stimulated with LPS and NO production was analysed in supernatants. Griess assay analysis revealed that NO production was significantly impaired in IFNAR deficient BMDMs compared to WT (**Figure 4.7A**). To confirm findings, pSTAT1 activation was determined. Western Blot analysis of lysates from $IFNAR^{-/-}$ and $IFN\gamma^{-/-}$ BMDMs revealed that STAT1 activation was impaired in $IFNAR^{-/-}$ but not in $IFNy^{-/-}$ BMDMs, suggesting again that NO production is regulated by STAT1 signalling pathway **(Figure 4.7 B, C).**

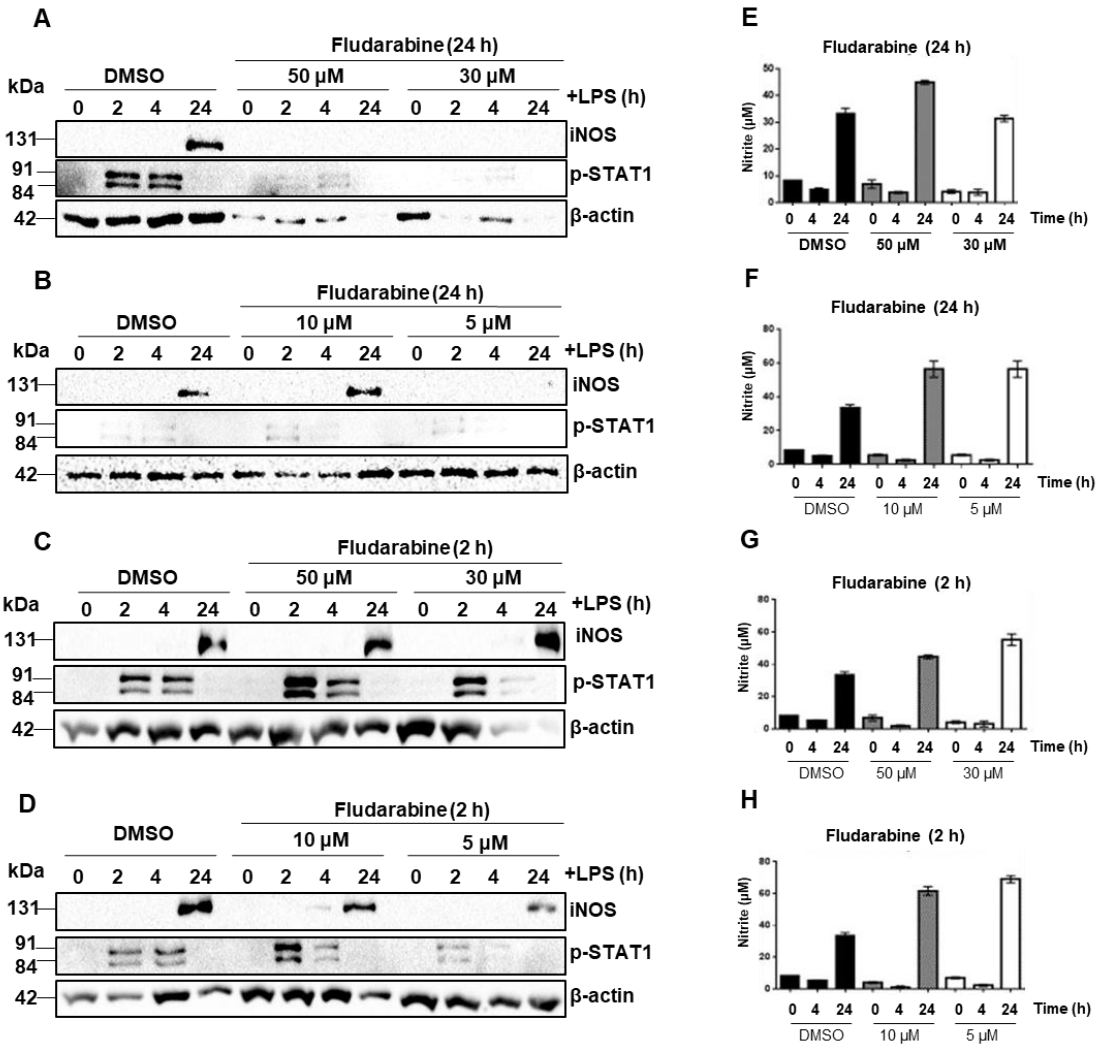


Figure 4.5. The STAT1 inhibitor, Fludorabine, is toxic to BMDMs.

BMDMs were seeded at 10^6 cells/ml on a 24-well plate. Following day, cells were pre-treated for 24 h or 2h with Fludarabine (5 μM, 10 μM, 30 μM, 50 μM) or with DMSO as a vehicle and then stimulated with LPS (1 ug/ml) over a 24 h timecourse, as indicated. Lysates (**A-D**) and supernatants (**E-H**) were collected for further analysis. **A, B, C, D** Lysates (10 μg) were prepared for Western blot and expression of iNOS (131 kDa), p-STAT1 (87 kDa) and β-actin (42 kDa) as a loading control were analysed. The figure is representative of two independent experiments. **E, F, G, H** Nitrite levels in supernatants were determined by Griess assay. Values represent the mean ± SEM of two independent experiments.

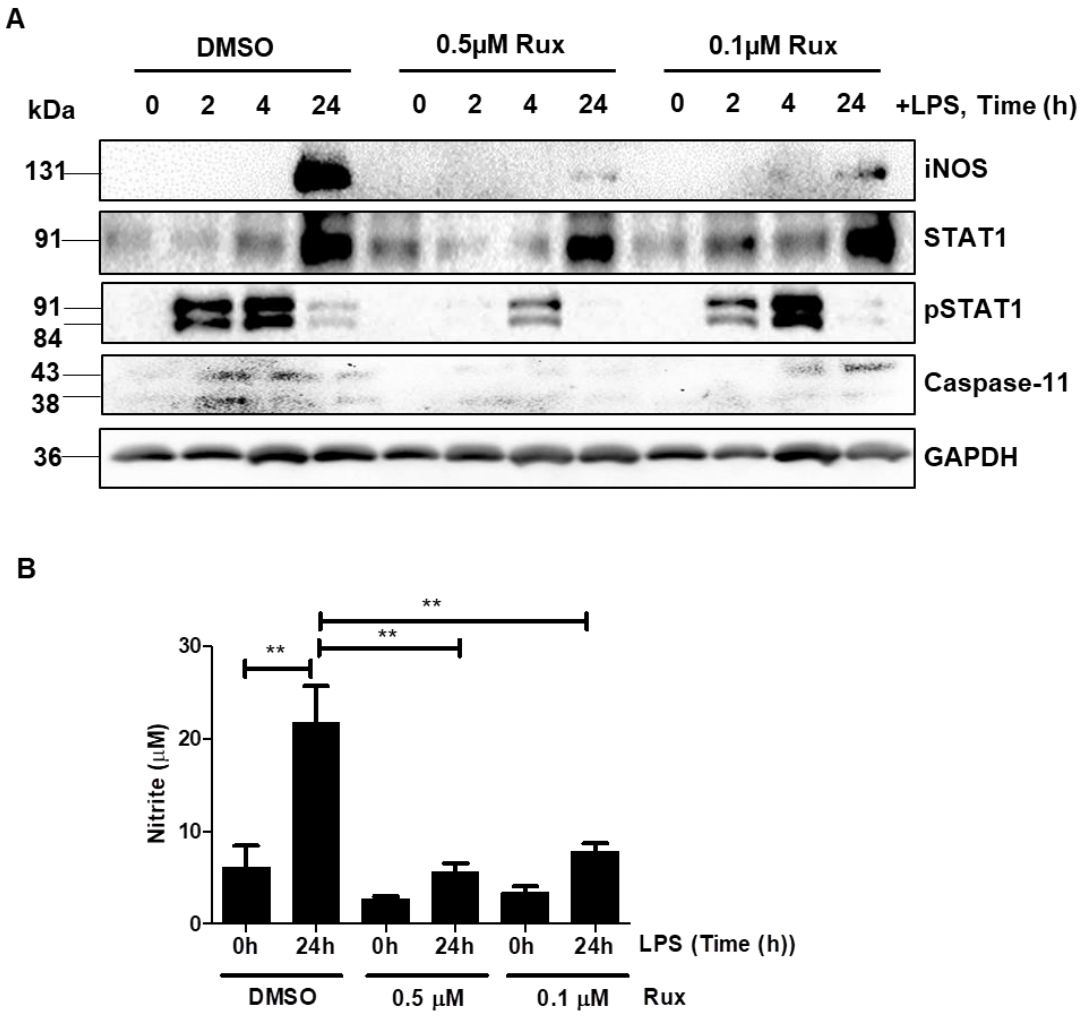


Figure 4.6. Treatment with the JAK1/2 inhibitor, Ruxolitinib, inhibits STAT1 activation, iNOS upregulation and NO.

BMDMs were seeded at 10^6 cells/ml on a 24-well plate. The following day, cells were pre-treated for 1 h with Ruxolitinib (Rux 0.5 μM; 0.1 μM) or with DMSO (vehicle control), followed by stimulation with LPS (1 ug/ml) for 2, 4 and 24 h. **A** Lysates (10 μg) were prepared for Western blot and expression of iNOS (131 kDa), p-STAT1 (87 kDa), STAT1 (87 kDa) caspase-11 (38, 42 kDa) and GAPDH (36 kDa) were analysed. **B** Nitrite levels in supernatants were determined by Griess assay. Values represent the mean ± SEM of three independent experiments. Statistical analysis was performed using the 1way *ANOVA* test to compare mean values; **$p<0.001$.

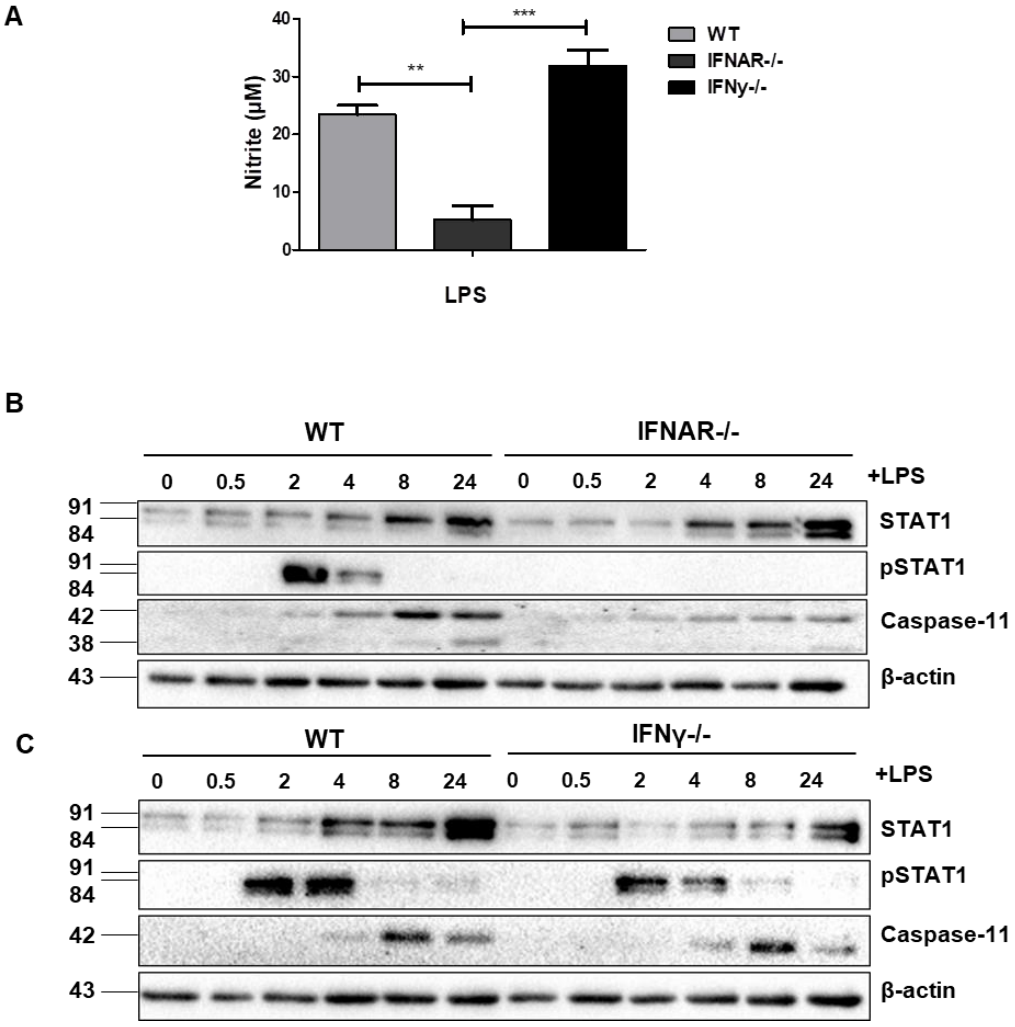


Figure 4.7. Impaired nitric oxide production in LPS-stimulated BMDM deficient in the Type I interferon-receptor, IFNAR.

WT, IFNAR$^{-/-}$ and IFNy$^{-/-}$ BMDMs were seeded at 10^6 cells/ml on a 24-well plate. The following day cells were stimulated with LPS (1μg/ml) for 24 h. **A** Nitrite levels in supernatants were determined by Griess assay. Values represent the mean ± SEM of three independent experiments. Statistical analysis was performed using the 1way *ANOVA* test to compare mean values; **p<0.001; ***p<0.0001 **B, C** Lysates from LPS-stimulated WT, IFNAR$^{-/-}$ and IFNy$^{-/-}$ BMDMs were prepared for Western blot and expression of STAT1 (91, 84 kDa), pSTAT1 (91, 84 kDa), Caspase-11 (38, 41 kDa), β-actin (43 kDa). The figure is representative of two independent experiments.

4.2.4. Caspase-11 regulates iNOS, pro-IL-1β expression and NO production in *Mtb* infected murine primary macrophages.

Macrophages are the first line of host defence against *Mtb*. Once pathogens enter the host, macrophages can phagocytose and eliminate intracellular microbes by different bactericidal mechanisms such as, acidification of the phagosomes and triggering phagosome fusion to lysosomes, producing bactericidal free radicals and activating programmed cell death [528]. However, when *Mtb* are phagocytise into macrophages they block the maturation of the phagosome thus preventing phagosome-lysosome fusion [201] [529]. The role of caspase-11 in innate immune defences against bacterial pathogens it is not fully understood. Most of the recent work describing the role of caspases has focused on their role in anti-inflammatory cytokine production and cell death with less consideration on emerging ancillary function [530]. Li *et al.* demonstrated an unexpected role for caspase-11 in regulating of cell migration through promoting cofilin-mediated actin depolymerization [531]. Based on information that proper dynamic actin polymerization and depolymerisation is required for phagosome maturation [532] and Ahter *et al*. have shown that caspase-11 contributes to phagosome-lysosome fusion [533] hypothesise that caspase-11 could have biological function during *Mtb* infection was made.

Inducible nitric oxide synthase (iNOS) is a cytoplasmic protein involved in NO production in macrophages. It is well characterised that NO has an antimicrobial role in a mouse model of Tuberculosis and that *Mtb*-induced NO production kills mycobacteria in macrophages *in vitro* [227] [534] [490]. Mice deficient in iNOS were more susceptible to *Mtb* infection, confirming that the NOS2 locus is necessary to control primary tuberculosis in mice [491].

To determine whether *Mtb* infection of BMDMs induces caspase-11-dependent iNOS expression and NO production, macrophages were infected with H37Ra *Mtb* (MOI 5) over a 72 h timecourse. Expression of caspase-11 and iNOS was evaluated 24 h after infection by immunoblot. Results revealed that, similar to LPS stimulation, *Mtb* infection induced iNOS expression which was impaired in caspase-11 deficient BMDM (**Figure 4.8 A;** densitometry analysis **Figure 4.8 B**). BMDMs from Casp11$^{-/-}$ mice also showed major defects in pro-IL-1β expression in response to *Mtb* infection. (**Figure 4.8 A**; densitometry analysis **Figure 4.8 C**). Analysis of NO production by Griess Assay

revealed that NO is produced 24 h after *Mtb* infection and levels increased in time dependent manner with ~8.5-fold increase at 72 h compared to 24 h. NO production was significantly impaired in Caspase-11 deficient macrophages at both indicated times (**Figure 4.9 A**). As IFNγ is a cytokine which is elevated following bacterial infection and enhances the LPS-induced production of antibacterial mediators by macrophages [535], and NO production in 24 h infected BMDMs was very low (~15μM) (**Figure 4.9 A**), we wanted to see effect of 1 h IFNγ-priming on NO production. IFNγ priming accordingly increased NO production in *Mtb* infected BMDMs, and similar to *Mtb* infection alone, NO production was significantly inhibited in Caspase-11 deficient BMDMs (**Figure 4.9 B**).

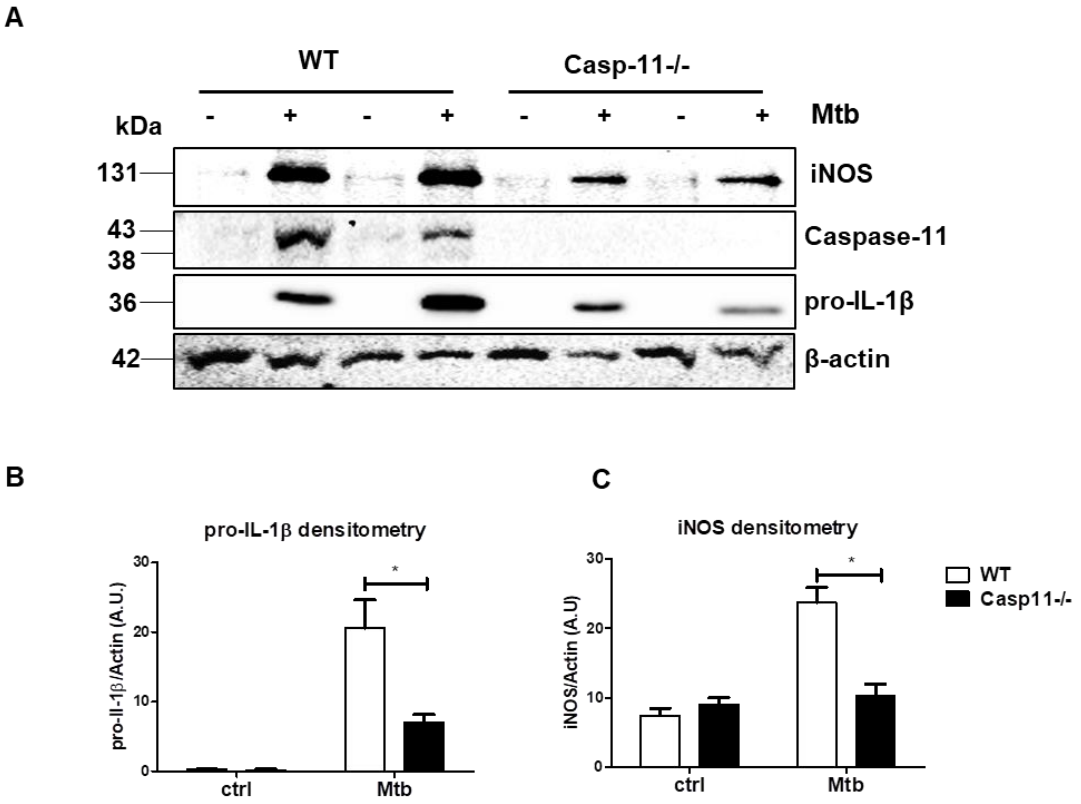


Figure 4.8. Caspase-11 regulates iNOS expression during *Mtb* infection.

WT and Casp-11$^{-/-}$ BMDMs were seeded at 10^6 cells/ml (in antibiotic-free DMEM supplemented with 10% FBS). After 24 h, BMDMs were infected with *Mycobacterium tuberculosis* (*Mtb*) strain H37Ra (MOI 5). **A)** 24 h after infection cell lysates (10 μg) were analysed by Western (10% gel) for expression of iNOS (131 kDa), caspase-11 (38, 43 kDa), pro-IL-1β (36 kDa) and β-actin (42 kDa). The blot represent 2 mice per group, n=4. **B** Densitometry analysis of pro-IL-1β and iNOS. Values represent the mean ± SEM of three independent experiments. Statistical analysis was performed using the 2way *ANOVA* test to compare mean values; *p<0.05.

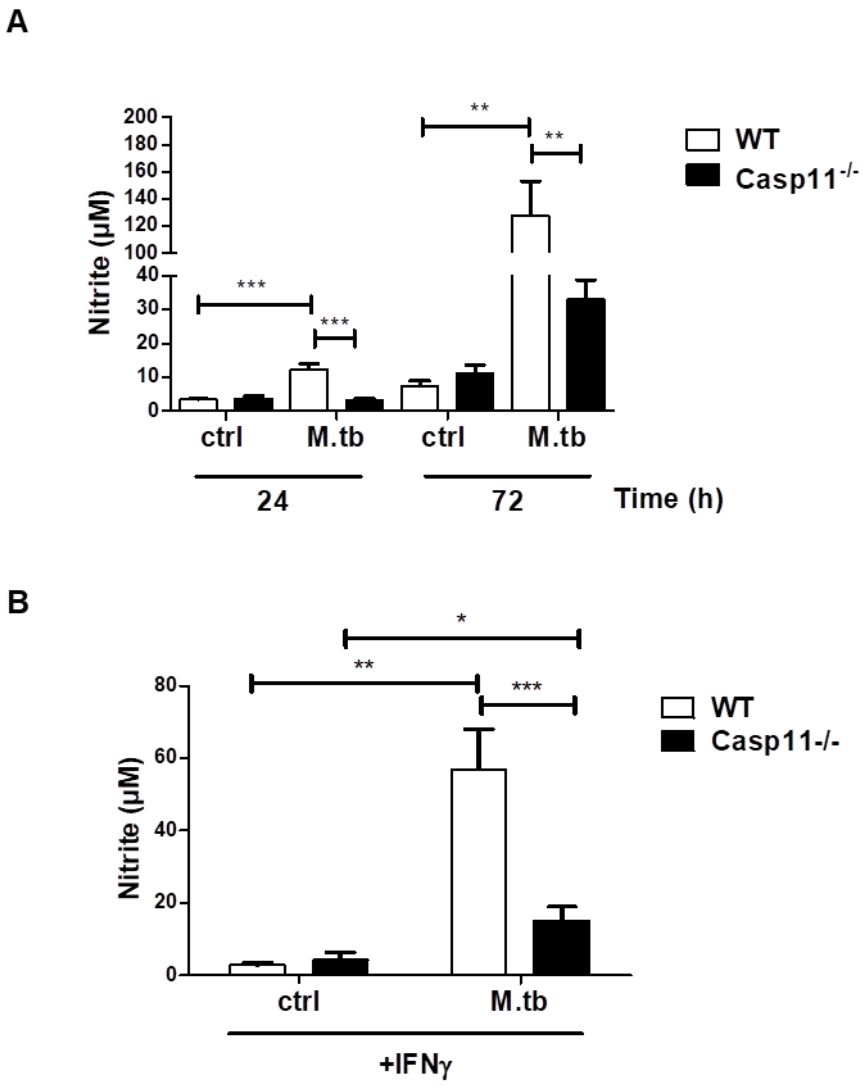


Figure 4.9. Caspase-11 regulates NO production during *Mtb* infection.

WT and Casp-11$^{-/-}$ BMDMs were seeded at 10^6 cells/ml (in antibiotic-free DMEM supplemented with 10% FBS). After 24 h, BMDMs were infected with *Mycobacterium tuberculosis* (*Mtb*) strain H37Ra (MOI 5). **A)** 24 h and 72 h after infection, supernatants were collected and nitrite was determined with Griess assay. Statistical analysis was performed using unpaired student *t*-test to compare mean ± SEM of *Mtb*-treated and untreated cells and the 2way *ANOVA* test to compare mean ± SEM of WT and Casp11-/- BMDMs, n=3; **p<0.01, ***p<0.001. **B)** Before *Mtb* infection, BMDMs were pre-treated for 1 h with IFNy (10 ng/ml). Nitrite concentration was detected in the media 24 h after *Mtb* infection. Statistical analysis was performed using student *t*-test to compare mean ± S.E.M values of *Mtb*-stimulated and unstimulated cells and 2way *ANOVA* test to compare mean ±S.E.M values of WT and Casp-11$^{-/-}$ BMDMs;*p<0.05 **p<0.01; ***p<0.001.

4.3. Caspase-11 promotes bacterial clearance but not cell death.

Experiments presented thus far have shown that *Mtb* H37Ra infected macrophages release high levels of nitric oxide inducing by iNOS expression. In activated macrophages, nitric oxide is rapidly oxidised to reactive nitrogen oxide species (RNOS) which plays an important role in the clearance of intracellular bacteria due to its cytotoxic effects [491]. In addition to the role of nitric oxide in bacterial pathogenesis, it has been shown that activation of caspase-11 plays a protective role during gram-negative bacterial infection. Caspase-11 deficient mice are more susceptible to *legionella pneumophila, Escherichia coli, Salmonella typhimurium* and *Klebsiella pneumonia* suggesting that Caspase-11 plays an important role in defending against bacterial infections [533] [536] [537]. To determine the role of Caspase-11 in the pathogenesis of TB, the replication of *Mtb* in WT and Caspase-$11^{-/-}$ murine macrophages was assessed using two alternative assays. Baseline growth and bacterial uptake was assessed by lysing 3 h time-point bacteria. CFUs were determined 14-21 days after plating bacteria collected from stimulated WT and Caspase-$11^{-/-}$ BMDM. During *Mtb* infection, caspase-11 deficient BMDMs exhibited more bacterial replication at 24 h and bacterial numbers were significantly higher at 72 h after infection, compared to WT. (**Figure 4.10 A**). The difference in intracellular bacterial replication between WT and Caspase-11-deficient macrophages was not due to differential uptake of *Mtb* because at 3 h post infection, the number of mycobacteria in WT and Caspase-$11^{-/-}$ BMDMs was comparable (**Figure 4.10 A**). To confirm the results obtained from CFU counts, bacterial viability in WT and Caspase-$11^{-/-}$ BMDMs was measured by detection of bacterial IS1160 genomic DNA (custom designed probe from Frederick Sheedy's Lab) by qPCR. *Mtb* genomic IS6110 is an insertion sequence presents exclusively in the *Mtb* genome [538]. The Sequence IS6110 is present only in *Mtb* bacteria and detection of this sequence is widely used as a tool for TB diagnosis in biological samples [539, 540]. Caspase-$11^{-/-}$ BMDMs present significantly higher expression of bacterial IS1160 compared to WT at 72 h after *Mtb* infection (**Figure 4.10 B**), indicating again that Caspase-$11^{-/-}$ macrophages have reduced ability to clear *Mtb* bacteria than WT.

Macrophages infected with *Mtb* have been reported to undergo apoptotic and necrotic cell death *in vitro* [249] [541]. As Caspase-11 is responsible for pyroptotic cell death a lactate dehydrogenase (LDH)-release assay was used to measure cell death induced by *Mtb*. However, the absence of Caspase-11 had no impact on cell death upon infection with *Mtb* indicating that *Mtb*-induce cell death is Caspase-11 independent (**Figure 4.11**). Infected and uninfected BMDM from WT and Caspase-$11^{-/-}$ mice had about 20% cell lysis relative to the lysis control, which was set to 100% lysis (**Figure 4.11**).

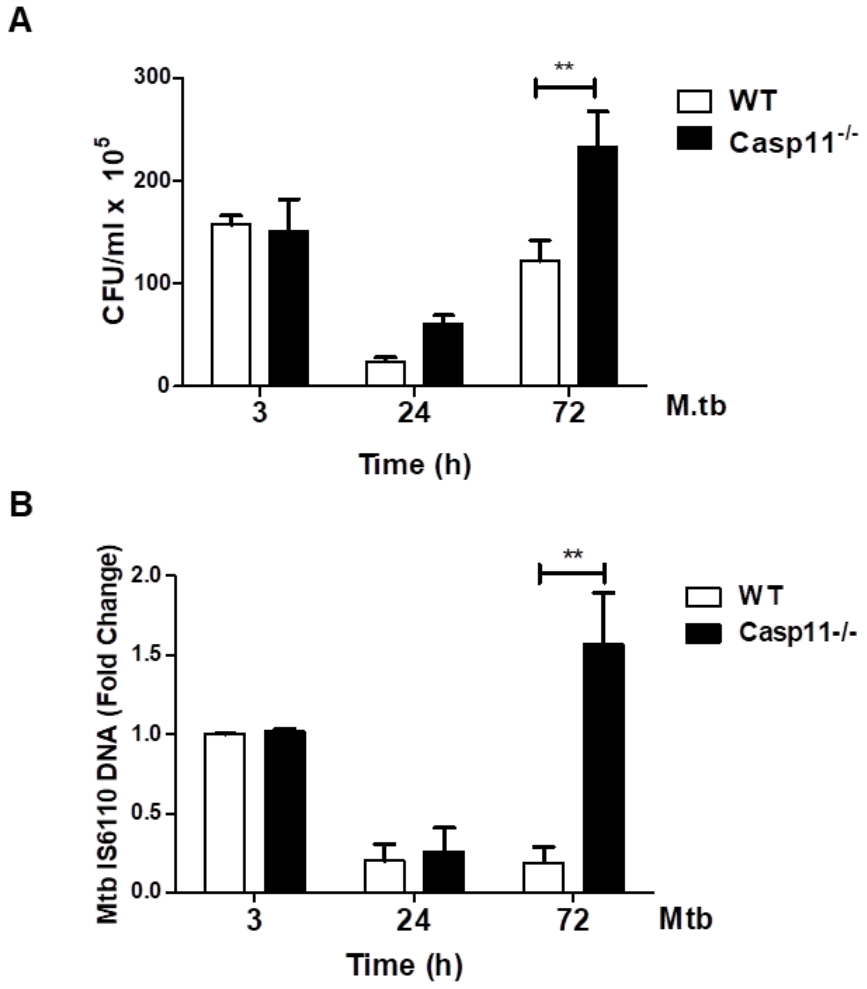


Figure 4.10. Caspase-11 attenuates intracellular pathogen invasion in BMDMs during *Mtb* infection.

WT and Casp-11$^{-/-}$ BMDMs were seeded at 10^6 cells/ml and cultured in antibiotic-free DMEM for 24 h prior *Mtb* infection. Macrophages were infected wit *Mtb* strain H37Ra at an MOI of 5 bacilli per cell for 3 h. Then extracellular bacteria were removed by collecting the supernatants and centrifugation to pellet extracellular bacteria. Bacteria-free media were returned to macrophages and cells were incubated for 24 h and 72 h. **A)** Baseline growth was assessed by lysing 3 h time-point in 0.1 % Triton-X for 10 min. Serial dilution were plated on 7H10 Middlebrook Agar in triplicate and colony-forming units (CFU) were analysed after incubation at 37°C for 14-21 days after plating. **B)** For measurement of mycobacterial viability by qPCR, RNA was isolated after 3 h, 24 h and 72 h *Mtb* infection and expression of Mtb IS6100 was analysed. For quantification, Mtb IS6110 expression in genomic DNA was normalized to bacterial housekeeping 16 s rRNA and macrophage 18 s rRNA expression in RNA samples and expressed relative to 3h post-infection using the ΔΔCT method. Values represent the mean ±S.E.M of three independent experiments. Statistical analysis was performed using 2way *ANOVA* test to compare mean values WT and Casp-11$^{-/-}$ BMDMs; **p<0.01;

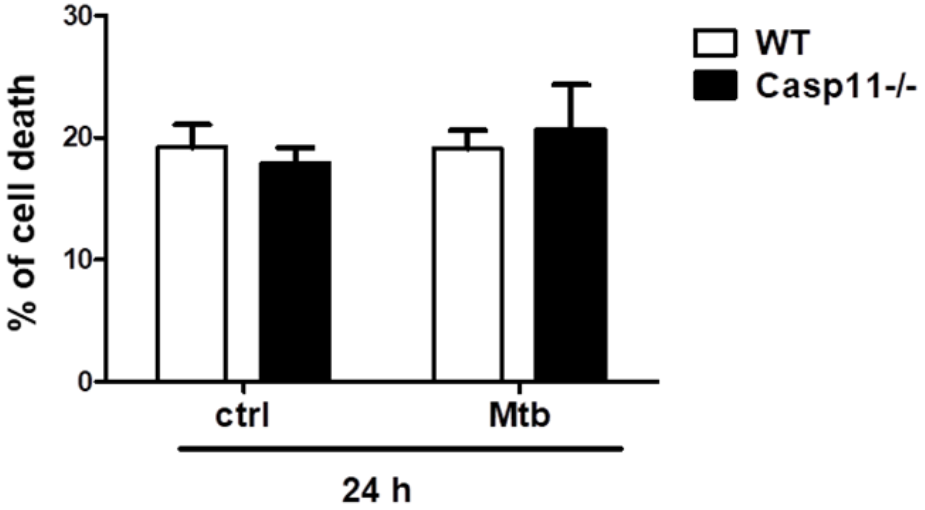
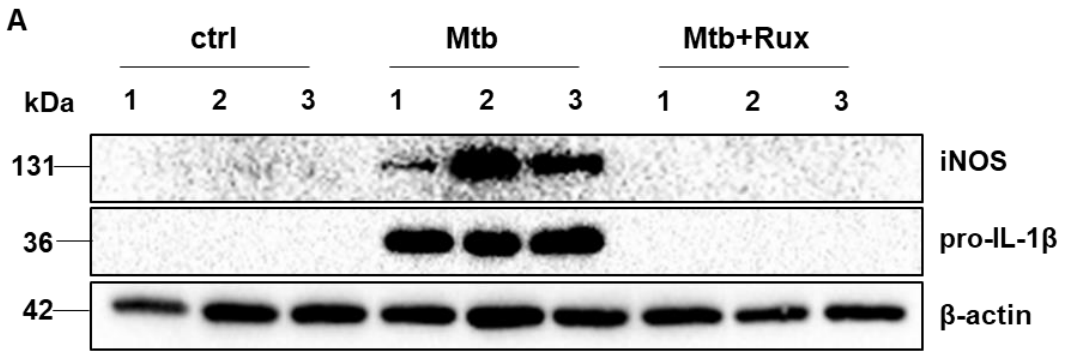


Figure 4.11. *M.tuberculosis* infection does not induce LDH release

WT BMDMs were seeded at10^6 cells/ml (in antibiotic-free DMEM supplemented with 10% FBS). After 24 hours, the indicated BMDMs were infected with *Mtb* strain H37Ra at an MOI of 5 bacilli. Bacteria-free supernatants were collected after 24 h and analysed for cell death using LDH assay. The results shown are from 3 independent experiments. Error bars represents mean ±S. E. M.

4.3.1. iNOS expression and NO production in *Mycobacterium tuberculosis*-infected murine macrophages is regulated by JAK1/2.

Previous observations of this study have shown that blocking STAT1 phosphorylation inhibits iNOS induction and NO production in LPS-stimulated BMDMs (**Figure 4.6**). To determine whether similar mechanisms occur upon *Mtb* infection, murine macrophages were pre-treated (1 h) with the JAK1/2 inhibitor, Ruxolitinib (Rux) before their exposure to H37Ra *Mtb*. Western Blot analysis revealed that Rux pre-treatment blocked iNOS induction and NO production in *Mtb*-infected macrophages. (**Figure 4.12 A, B**). Similarly, pro-IL-1β expression was upregulated in *Mtb*-infected macrophages and subsequently downregulated under conditions of JAK1/JAK2 inhibition (**Figure 4.12 A**).



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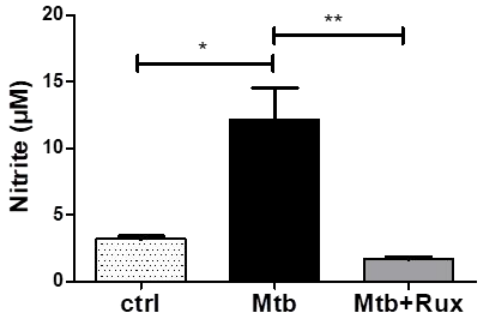


Figure 4.12. Ruxolitinib treatment inhibits iNOS and pro-IL-1β upregulation, and NO production, in *Mtb*-infected BMDMs

WT BMDMs were seeded at10^6 cells/ml (in antibiotic-free DMEM supplemented with 10% FBS). After 24 hours, the indicated BMDMs were pre-treated with Ruxolitinib (Rux, 0.5 μM) for 1 h prior to *Mtb* infection (H37Rv, MOI 5) for 24 h. Nitrite concentration in supernatants were determined using the Griess Assay. Values represent the mean ±S.E.M of n=3. Statistical analysis was performed using one-way *ANOVA* test; *p<0.05, **p<0.01

4.3.2. NLRP3 is not involved in caspase-11 mediated nitric oxide production in LPS-stimulated murine macrophages.

NLRP3 is an intracellular sensor protein for inflammasome activation in response to PAMP or DAMP exposure. A two-step process is required for NLRP3 inflammasome activation. The first, priming signal leads to the activation of NF-κB and promotes expression of NLRP3 and pro-IL-1β in cells. The second, activating signal, induced by a range of stimulants including ATP and bacterial pore-forming toxins, directly activates inflammasome formation, which causes caspase-1 activation and the resulting maturation of IL-1β, IL-18 and processing of GSDMD, leading to pyroptosis [542]. In addition activated caspase-11 regulates NLRP3 activation via the non-canonical inflammasome. Caspase-11 controls IL-1β secretion through NLRP3-dependent caspase-1 activation [543]. In the resting state, without a priming step, cellular NLRP3 levels are low and prevent inflammasome assembly and activation. Activation of inflammasomes is critical for the maintenance of homeostasis during pathogen invasion. Although NLRP3 inflammasome activation is required for host immune defence during pathogenic infection, extensive NLRP3 inflammasome activation also leads to tissue damage and it is potentially hazardous for the host [544].

Given the important roles of both iNOS and NLRP3 in host defences against bacterial infectiuons, next study to analysed whether the NLRP3 protein is able to control iNOS expression and NO production was conducted. WT and $NLRP3^{-/-}$ BMDMs were stimulated with LPS and IFNγ for 24 h. Results confirmed that LPS stimulation induces high NLRP3 expression in WT BMDMs. However, iNOS expression was higher in

NLRP3 deficient BMDMs compared to WT (**Figure 4.13 A)**. Similar results were observed for NO production, as the concentration of nitrite in the supernatants from LPS-stimulated BMDMs was significantly higher in NLRP3$^{-/-}$ macrophages, compared to WT (**Figure 4.13 B**). The increased levels of iNOS may be a compensatory mechanism for the absence of the NLRP3 sensor protein in these cells. iNOS induction was not detected after IFNγ stimulation, and no differences in caspase-11 expression were observed, suggesting that caspase-11-dependent iNOS expression is independent of NLRP3 (**Figure 4.13 A)**.

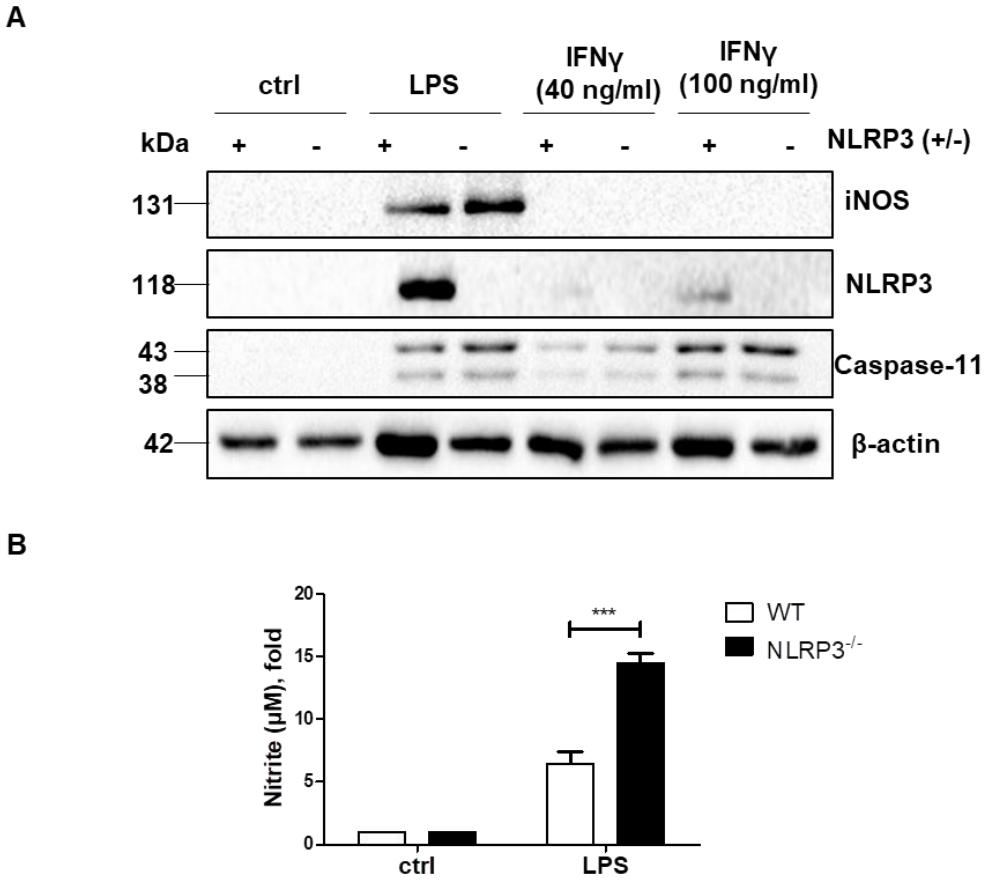


Figure 4.13. LPS stimulated Nitric oxide production and iNOS upregulation is higher in NLRP3$^{-/-}$ BMDM, compared to WT.

WT and NLRP3 deficient mice BMDMs were seeded at 10^6 cells/ml and stimulated with LPS (1 ug/ml) or IFNγ (40 ng/ml; 100 ng/ml) for 24 h. **A** Protein lysates (10 μg) were analysed by western blot and probed for iNOS (131 kDa), NLRP3 (118 kDa), Caspase-11 (38,42 kDa) and loading control β-actin (43 kDa). Immunoblot is representative of three independent experiments. **B** Nitrite levels in supernatants from LPS-stimulated WT and NLRP3-/- BMDMs were measured using Griess assay. Results were measured with spectrophotometer at 480 nm. Data is expressed as a mean ±S.E.M and presented as a fold of three independent experiments, ***p<0.001.

4.3.3. An inverse correlation between iNOS expression and STAT1 activation is observed in NLRP3 deficient murine macrophages.

STAT1 activation is mediated by its phosphorylation, leading to its dimerization and nuclear translocation, to enable its transcription factor function [545]. It has been shown that type I IFN inhibits NLRP3 inflammasome activation and caspase-1 activation through STAT1-dependent activation, by an as yet undefined mechanism [546]. Previous observations with WT and Caspase-11 deficient BMDMs showed that caspase-11 prevents pSTAT1 activation, which subsequently leads to iNOS inhibition (**Figure 4.4**). As NLRP3 protein, similarly to Caspase-11, is upregulated through LPS/NF-κB signalling, analysis whether iNOS expression is also regulated by STAT1 activation in WT and NLRP3$^{-/-}$ BMDMs was carried out. Results revealed that, iNOS expression was upregulated in NLRP3$^{-/-}$ BMDMs compared to WT at 6 and 24 h LPS treatment (**Figure 4.14**). NLRP3 appears to have some regulatory control on STAT1 activation, as reduced STAT1 phosphorylation was observed in NLRP3 deficient macrophages compared to WT, and less expression of total STAT1 was observed in NLRP3$^{-/-}$ BMDM (**Figure 4.14**). Expression of pSTAT1 negatively correlates with iNOS expression in NLRP3$^{-/-}$ BMDM, which is in contrast to results observed with Casp11$^{-/-}$ BMDM. No difference in Caspase-11 expression was observed between WT and NLRP3$^{-/-}$ BMDMs (**Figure 4.14**).

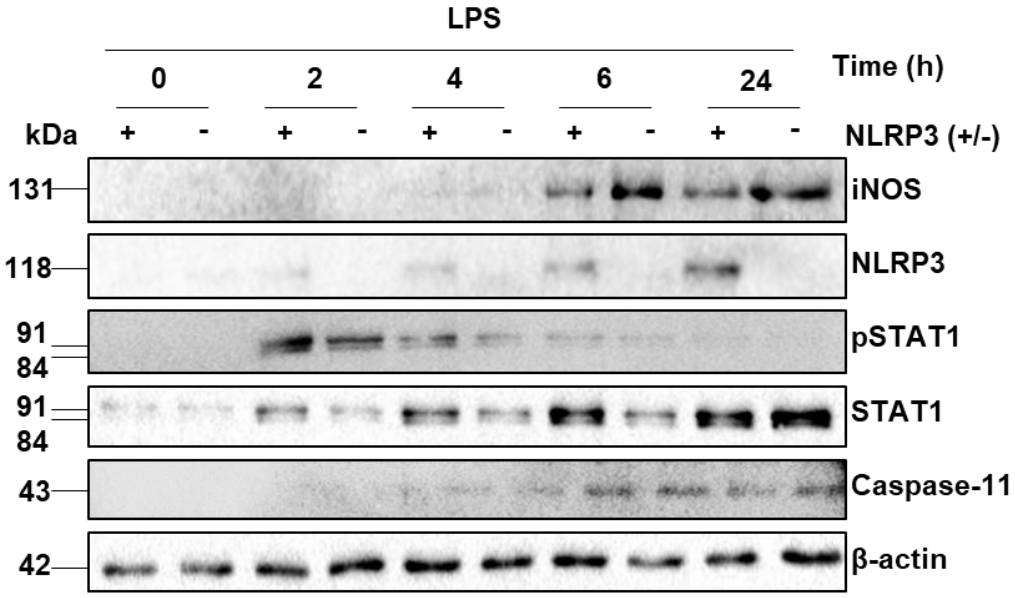


Figure 4.14. NLRP3 appears to positively regulate LPS-mediated STAT1 activity, but negatively regulate iNOS upregulation in BMDMs.

BMDMs from WT and NLRP3 deficient mice were seeded at 10^6 cells/ml and stimulated with LPS (1 ug/ml) over a 24 h timecourse. Protein lysates (10 μg) were analysed by western blot and probed for iNOS (131 kDa), NLRP3 (118 kDa), Caspase-11 (38,42 kDa), pSTAT1 (87 kDa), pSTAT1 (87 kDa) and loading control β-actin (43 kDa). Immunoblot is representative of three independent experiments.

4.3.4. iNOS expression and NO production is sex-independent in Glial cells and BMDMs.

Nitric oxide is involved in various physiological and pathological processes in many human organs, such as brain and spinal cord. Over expression of iNOS has been linked to many human disorders such as, Parkinson's disease, multiple sclerosis, Alzheimer's disease [547], which are connected with glial cell activation [548]. Glial cells, together with neurons, are the major cell types in the central nervous system. They are essential for neuronal protection, survival and regeneration during neurological disorders. It has been shown that LPS alone is able to activate iNOS in murine glial cells [549] [550]. For several neurological disorders, sex difference in their incidence, severity and progression are observed. It has been shown that iNOS expression differs in male and female mice and can be important is susceptibility to neurodegenerative disorders. In murine models of Parkinson's disease, which is found more frequently in the men than in women, increase of iNOS protein expression occurred more rapidly in male than in female mice [551]. It has been shown that in murine Parkinson's disease model, inhibition of NOS protected mice from severe injury and neuron degeneration [552]. Another neurodegenerative disease, Alzheimer's disease is more common in women than in men and nitric oxide production is considered as a major contributor to oxidative stress-associated neurodegeneration [553].

To determine whether iNOS expression and NO production are sex-dependent, glial cells and BMDMs from the same 3 month old WT and Caspase-11 deficient mice were isolated. Glial cells and BMDMs were stimulated with LPS for 24 h and results were analysed by Western Blot and Griess assay. In glial cells, similar to BMDMs, levels of iNOS expression and NO production were significantly higher in WT compared to

Caspase-11 deficient cells. (**Figure 4.15 A, B**; **Figure 4.16 A, B**). No differences in iNOS expression or NO production were observed between WT cells of males and females. Interestingly, in Caspase-11 deficient BMDM, levels of iNOS upregulation and nitrite in supernatants are significantly more impaired in BMDM from females, compared to males. (**Figure 4.15 A, B**).

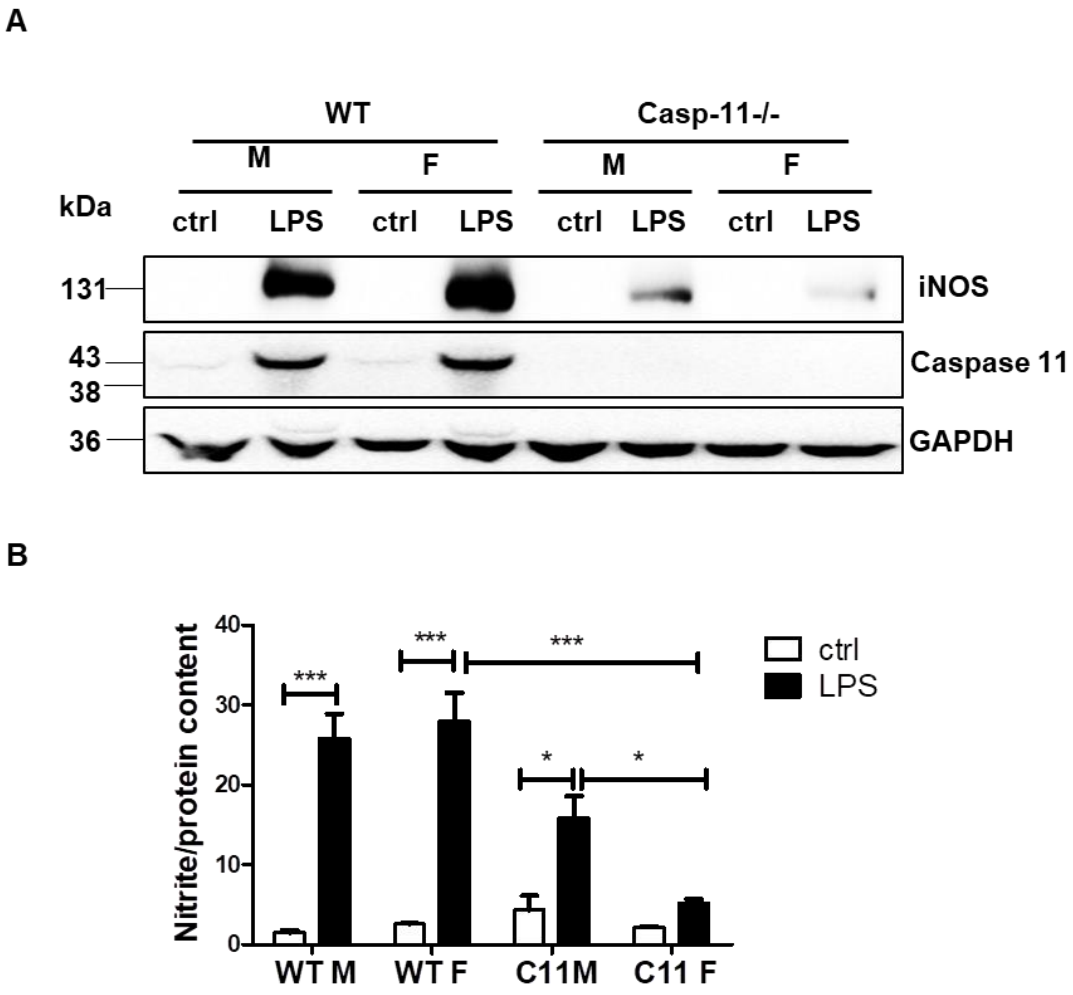


Figure 4.15. Similar levels of NO production and iNOS expression in LPS-stimulated BMDMs from male and female mice.

BMDMs isolated from WT and Caspase-11 deficient C57BL/6J mice were seeded at 10^6 cells/ml in 24 well plate. Following day, BMDMs were left untreated or stimulated with 1 μg/ml LPS for 24 h. **A** Cell lysates were generated in RIPA buffer for WB analysis of iNOS (131 kDa), Caspase-11 (38, 42 kDa) and GAPDH (36 kDa) as a loading control. The figure represents two independent experiments (two mice per group). **B** Nitrite levels in supernatants were determined by Griess assay. Values represent the mean ± SEM of two independent experiments (two mice per group). Statistical analysis was performed using *ANOVA* test to compare mean values **p<0.01;***p<0.001

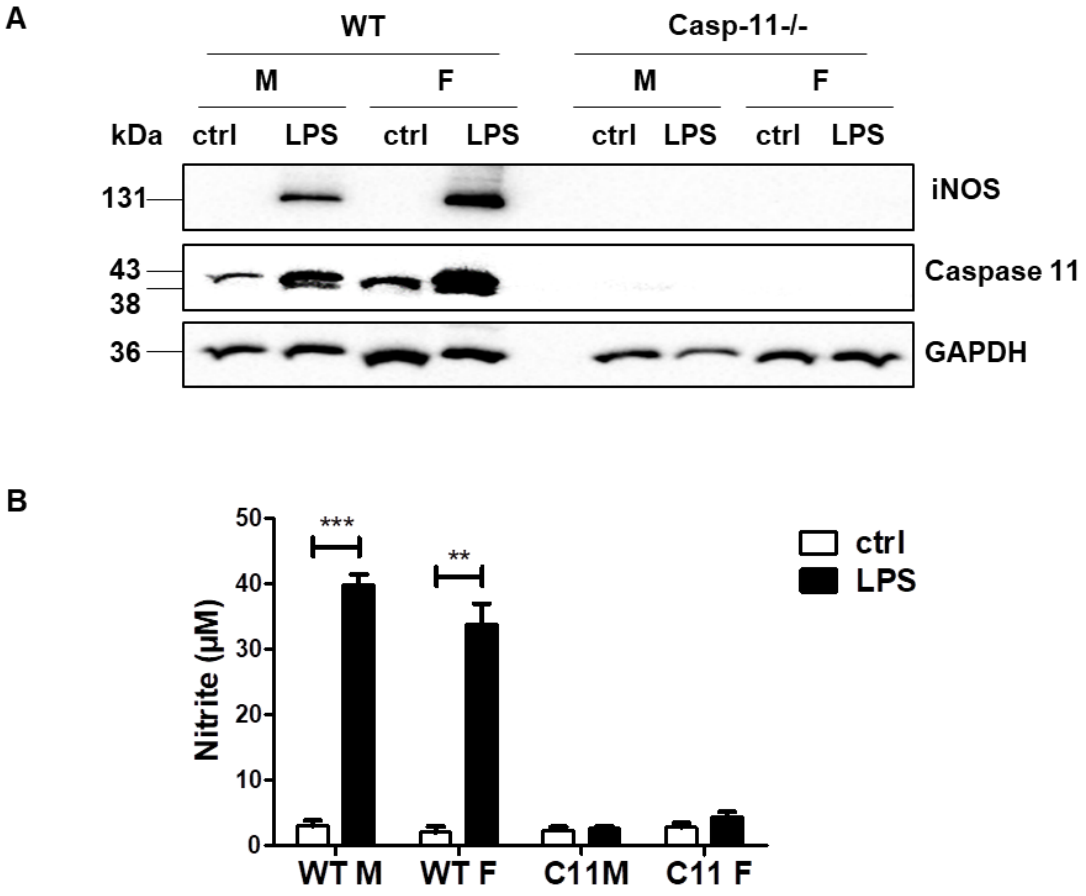


Figure 4.16. Caspase-11 regulates NO production and iNOS expression in LPS-stimulated Glial cells, in both male and female mice.

Glial cells were isolated from brain of WT and Caspase-11 deficient C57BL/6J mice and seeded at 1.6 x 10^5 cells/well in 24 well plate. Cells were cultured for 7 days in DMEM supplemented with GM-CSF and M-CSF(1000x) was changed at day 4. Glial cells were left untreated or stimulated with 1 μg/ml LPS for 24h. **A** Protein lysates (4 μg) were analysed by western blot (10 %) for iNOS (131 kDa), Caspase-11 (42;38 kDa) and GAPDH (36 kDa) as a loading control. The figure represents two independent experiments (two mice per group). **B** Nitrite levels of supernatants were determined by Griess assay. Values represent the mean ± SEM of two independent experiments (two mice per group). Statistical analysis was performed using *ANOVA* test to compare mean values *$p<0.05$; ***$p<0.0001$.

Summary of the main findings of Chapter 4

- Caspase-11 regulates iNOS expression and NO production in LPS- and *M. tuberculosis* H37Ra- stimulated primary murine macrophages
- Caspase-11 regulates NO production indirectly, via its ability to regulate type I IFN-mediated STAT1 activation.
- In *M. tuberculosis*-infected murine macrophages, caspase-11 regulates pro-IL-1β expression through the JAK/STAT1 signalling pathway.
- Caspase-11 contributes to host defence and cellular clearance of intracellular *M. tuberculosis* in primary murine macrophages.
- In contrast to caspase-11, NLRP3 appears to have a negative effect on iNOS expression and NO production in LPS-stimulated primary murine macrophages.
- Caspase-11 regulates iNOS expression and NO production in primary murine glial cells.
- The iNOS-mediated NO production in murine macrophages and murine microglial cells is not sex-dependent, however caspase-11 appears to have greater regulatory control on iNOS and NO production in BMDM from female mice, compared to males.

4.4. Discussion

Although *Mtb* was identified as the causative agent of tuberculosis (TB) nearly 130 years ago, TB still remains the leading cause of death from a single infectious disease agent and the main cause of death in HIV-infected patients [554]. Despite TB vaccine and antituberculosis drugs being discovered more than 60 years ago, tuberculosis still killed around 1.3 million HIV-negative individuals in 2018 (World Health Organization [WHO] TB Report 2019). As recent *Mtb* strains are becoming resistant to nearly all effective drug therapies, it highlights the need for finding a new effective strategy to control TB [555].

Recent data published from our lab revealed that caspase-11 regulates STAT1 activation in murine macrophages [175]. As the JAK/STAT1 signalling pathway plays an essential role in the expression of inducible Nitric-Oxide Synthase (iNOS) and nitric oxide production [507] [556], an experiment to analyse the role of caspase-11 in iNOS-dependent nitric oxide production in response to lipopolysaccharide (LPS) stimulation and *Mtb* infection was conducted. Results from this chapter demonstrated that iNOS expression and nitric oxide production are regulated by caspase-11 in both LPS stimulated and *Mtb*-infected murine macrophages. Using a specific JAK1/JAK2 inhibitor results have shown that the STAT1 pathway augmented LPS- and *Mtb*-mediated induction of NO accumulation by increasing iNOS protein expression in murine macrophages. Inhibition of STAT1 phosphorylation decreased caspase-11 and iNOS expression suggesting a correlation between these proteins. In *Mtb*-infected caspase-11 deficient BMDMs expression of iNOS and pro-IL-1β are downregulated compared to WT, and they are regulated by JAK/STAT signalling pathway. Caspase-11 deficient BMDMs permitted significantly more *Mtb* growth compared to WT. Altogether, our data suggest that caspase-11 modulates STAT1 activation, which regulates pro-IL-1β expression, iNOS expression and NO production. The present study demonstrated for the first time that caspase-11 regulates, via STAT1 phosphorylation, *Mtb*-induced expression of iNOS and controls *Mtb* growth in murine macrophages. As nitric oxide is a key anti-mycobacterial molecule, it suggests that caspase-11 is a very important molecule in host response to *Mtb* infection.

Innate and adaptive immunity are relatively efficient in protecting host and killing *Mtb*. It is estimated that only about 5-10% of infected individuals will develop active TB

during their lifetime [557]. Host cells that play a main role in protection against TB are macrophages, dendritic cells, T cells and airway epithelial cells [490] [489]. Exposure of host cells to *Mtb* results in their activation and microbial killing. Host cells are able to directly kill *Mtb* by the production of antimicrobial cytokines, reactive oxygen species and phagocytosis. Macrophages are the main effector cells that eliminate mycobacteria. *Mtb*-infected macrophages produce cytokines such as tumor necrosis factor (TNF)-α and interleukin IL-1β, which along with interferon (IFN)-γ produced by T lymphocytes, induce NO production in macrophages [558]. Despite the induction of a robust host immune response following *Mtb* infection, mycobacterium develops several mechanisms to successfully evade host immunity and establishes a persistent infection. The mechanisms by which *Mtb* circumvents the innate immune host defense is still not clearly understood [559].

iNOS is a critical regulator of susceptibility to *Mtb* infection in mice. Effect of iNOS in *Mtb* is associated with the production of nitric oxygen which is converted into reactive nitrogen intermediates (RNI) such as NO_2^-, NO_3^- and peroxynitrite ($ONOO^-$) which have been thoroughly studied *in vitro* using murine macrophages [475] [462]. It has been shown that L-arginine dependent production of RNI is responsible for killing and inhibiting the growth of virulent *Mtb* [490]. Exogenous NO has dose-and time-dependent mycobacteriocidal action [492]. iNOS deficient mice are more susceptible to *Mtb* growth. Inhibition of iNOS via administration of N6-(1-iminoethyl)-L-lysine (L-NMMA) accelerates chronic tuberculosis in wild-type mice [491]. In contrast to NO role in *Mtb*-infected mice, the biology of iNOS and NO in human tuberculosis is still controversial [560]. This is due to the fact that NO production levels are much more robust in mice than in humans [202].

Presented results showed that LPS stimulation and *Mtb*-infection leads to iNOS upregulation and excessive production of nitric oxide. Although 24 h after *Mtb* infection, nitrite concentration detected in supernatants was quite low (~ 15 μM), priming murine macrophages with IFNγ significantly increased production of nitric oxide by *Mtb* (~55 μM). It was confirmed that in LPS-stimulated murine macrophages, nitric oxide production is mediated by type I IFN associated receptor, IFNAR and STAT1 phosphorylation. This is consistent with previous observation showing that JAK/STAT1 signaling is essential for iNOS expression and STAT1 is required for

iNOS activation in murine fibroblast and macrophages [561] [556]. S.Shi *et al.* studies report that iNOS mRNA expression was significantly impaired in IFN-αβR$^{-/-}$ and STAT1$^{-/-}$ *Mtb* infected macrophages suggesting that *Mtb* induces a signal transduction pathway that depends on IFNAR. Bacterial survival was increased in STAT1$^{-/-}$ *Mtb* infected-BMDMs, implying STAT1 and iNOS in bactericidal activity in macrophages [562]. It has been shown that STAT1 phosphorylation and NO production were impaired in macrophages obtained from patients with active tuberculosis. Whether this is the reason for tuberculosis predisposition or it is a consequence of disease is still not clear[563], athough sequencing of the STAT1 gene in 2 patients who developed mycobacterial infection in their childchood, revealed a L706S mutation, suggesting a correlation between STAT1 heterozygosity and predisposition to *Mtb* infection [564]. In clinical studies, TB patients with inhaled IFNα enhance their response against *Mtb*. [565]. Moreover, *Mtb* is able to inhibit NO production, through inhibition of type I IFN signalling and STAT1/STAT2 phosphorylation, in order to evade host defense mechanism. IFNAR signalling is required for IFNβ induced NO production and *Mtb* killing [566]. In caspase-11 deficient murine macrophages STAT1 phosphorylation is inhibited which correlates with downregulation of iNOS expression and nitric oxide production, suggesting that caspase-11 controls iNOS expression and NO production through the STAT1-mediated pathway. Inhibition of STAT1 phosphorylation with JAK1/JAK2 specific inhibitor blocks iNOS induction and NO production but also leads to the downregulation of pro-caspase-11. Previous studies have shown that IFNAR$^{-/-}$ and STAT1$^{-/-}$ macrophages infected with *Salmonella* do not activate caspase-11 or inflammasome-mediated cell death. Exogenous IFNβ restored cell death and caspase-11 processing confirming the important role of type I IFN in caspase-11 activation [166]. Moreover, it has been shown that the promoter region of caspase-11 contains several transcription factor binding sites including the STAT1-binding site [567].

LPS induces the degradation of IκBα which leads to nuclear translocation of NF-κB and transcription of NF-κB-related genes. No difference in IκBα expression and degradation was observed in wild-type and caspase-11 deficient BMDMs. This result suggests that caspase-11 mediated iNOS expression is not regulated by NF-κB. It is consistent with previous reports by others showing that LPS-induced iNOS expression is not necessarily mediated by NF-κB transcription factors in macrophages and glial cells In microglial cells, the flavonoid, Oroxylin A supresses LPS-mediated NO production via

blocking iNOS expression in an NF-κB-independent manner [568]. Moreover, inhibition of iNOS signalling by green tea and ginseng was regulated by STAT1 but not NF-κB in human epithelial cell lines and murine macrophages, respectively [569] [570].

Inflammasomes are key components of cytosolic surveillance for microbial infection. Recognition of pathogens by cytosolic pattern recognition receptors which are part of canonical inflammasomes promote the activation of caspase-1 and its subsequent processing of GSDMD, IL-18 and IL-1β. So far NLRP3 and AIM2 inflammasomes have been described to play a critical role in *Mtb*-induced immunity. It has been shown that *Mtb* causes cell membrane damage-mediated K^+ efflux, NLRP3 activation and subsequently caspase-1 mediated IL-1β production in macrophages [571] which restrict bacteria growth [572]. However, several studies presented no difference in susceptibility to *Mtb* infection in NLRP3-deficient mice [236] [234] [573]. In contrast, AIM2 deficient mice were highly sensitive to *Mtb* infection presenting AIM2 as an important molecule for activation of the inflammasome in *Mtb* infection. Genomic DNA extracted from *Mtb* induce caspase-1 activation, IL-1β and IL-18 secretion in AIM2-dependent manner [237].

In contrast, noncanonical inflammasome is regulated by murine caspase-11. Both caspase-1 and caspase-11 (canonical and noncanonical inflammasomes) activate GSDMD which when cleaved, forms pores in the cell membrane and induces cell death, called pyroptosis [184] [183].

Caspase-11 has become of specific interest in cellular microbiology due to the ability to become activated in response to LPS, the main component of the gram-negative bacteria membrane. Activation of caspase-11 dependent non-canonical inflammasome requires 2 steps. Intitially, gram-negative bacteria leads to upregulation of pro-caspase-11 which in resting cells is expressed at very low level. Once pro-caspase-11 is synthesized, intracellular LPS derived from bacteria which escape from phagosome, activate noocanonical inflammasome [574]. In opposition to gram-negative bacteria, gram-positive bacteria activate caspase-11 through IFN I signalling and lead to caspase-11 autoactivation. It has been shown that gram-positive bacteria, such as Group B S*treptococcus* (GBS), *Streptococcus pneumoniae (Pneumococcus), Staphylococcus aureus, and Bacillus subtilis* activate NLRP3 inflammasome independently of IFNAR-STAT1-caspase-11 axis [575]. Our results showed that *Mtb* infection increase caspase-11 expression. Infecting wild-type and caspase-11 deficient murine macrophages with

Mtb, revealed that caspase-11 is necessary to protect macrophages from *Mtb* invasion. Caspase-11 deficient murine macrophages presented a significantly higher number of invaded mycobacteria compared to wild-type. Following *Mtb* infection no LDH release was observed, confirming that lower intracellular numbers of *Mtb* in WT BMDMs is not due to macrophage necrotic cell death. It has been shown that *Mtb* H37Rv infection does not lead to disruption of the cell membrane and LDH release in wild-type and caspase-1/11$^{-/-}$ dendritic cells [576] suggesting that another type of cell death is involved in *Mtb* infection. As *Mycobacterium tuberculosis* resembles both gram-negative and gram-positive characteristics [577] it brings a new role for caspase-11 outside of gram-negative bacterial infection, as it has been considered so far. How caspase-11 binds cytosolic *Mtb* which is not a typical gram-negative bacteria is not clear. Hara *et al.* reported a novel signalling pathway induced by *Listeria monocytogenes*. *L. monocytogenes*, gram-positive bacteria, activates NLRP6 which then recruits caspase-11 and mediate caspase-11-dependent, caspase-1 activation and IL-1β maturation in macrophages [578]. In addition, it has been shown that guanylated-binding proteins (GBPs; IFN-inducible GTPases) present in mouse chromosome 3 are involved in recognition of *B.abortus* LPS by caspase-11 and contribute to non-canonical inflammasome activation. In the absence of GBPs, caspase-11 could not be activated by *B.abortus* as observed in WT macrophages, indicating GBPs as essential for *B.abortus*-induced caspase-11-and subsequently non-canonical inflammasome activation [579]. Above findings could suggest that Caspase-11 interact with mycobacterium through upregulation of iNOS-dependent NO production or through unknown mediators/proteins.

Activation of noncanonical inflammasome can indirectly promote IL-1β production through triggering the NLRP3 canonical inflammasome [142]. LPS priming of WT and NLRP3$^{-/-}$ BMDMs revealed that iNOS expression and NO production was significantly upregulated in NLRP3$^{-/-}$ BMDMs, suggesting that regulation of iNOS and pro-IL-1β by caspase-11 is independent of an NLRP3 inflammasome.

Broz P. *et al.* demonstrated that upon *S.tymphimurium* infection first, the level of pro-IL-1β maturation is reduced in caspase-11 deficient BMDMs and then totally abrogated in the absence of caspase-1 [166]. IL-1β is a pro-inflammatory cytokine which along with IL-1α shares the signalling receptor IL-1R1. It has been shown that IL-1β activates

innate antimicrobial activity and supresses the growth of intracellular *Mtb* in both human and murine macrophages [580]. Mice deficient in IL-1, IL-18 and IL-1 receptor type 1 (IL-1R1) are more susceptible to *Mtb* infection [231] [581] [582]. IL-1αβ$^{-/-}$ mice are extremely susceptible to low dose aerosol *Mtb* infection suggesting a very important role of this cytokine in bacterial clearance and in promoting initial resistance to *Mtb* [583]. IL-1β directly kills *Mtb* in both murine and human macrophages and is able to recruit other antimicrobial effector molecules [580]. Although the importance of IL-1β, the main product of inflammasomes, in TB control has been widely described, less is known about the regulation of this process during *Mtb* infection [583] [233]. Following *Mtb* infection results indicated that similar trend to iNOS expression was observed in pro-IL-1β expression. Pro-IL-1β was downregulated in caspase-11 deficient BMDMs and inhibition of JAK1/JAK2 pathway abrogated pro-IL-1β expression. As it has been reported that caspase-11 controls IL-1β secretion by potentiating caspase-1 activation [543], the reduced pro-IL-1β suggests that caspase-11 may regulate IL-1β both at the level of processing and at a level before processing. It has been shown that IL-1β induces iNOS gene expression, *de novo* synthesis of iNOS and stimulates NO production in rat vascular endothelial cells [584]. Previous studies have shown that caspase-11 regulates IL-1β-mediated STAT1 activation in murine macrophages [175]. Based on above results the hypothesis that caspase-11 regulates STAT1-mediated IL-1β production which leads to NO production and mycobacteria killing can be made.

After macrophages internalize mycobacterium, pathogens are trapped in the vacuole called a phagosome, which undergoes fusion with the lysosome, enables phagosomal maturation and pathogen degradation [458]. The main strategy of mycobacterium to survive in the host macrophages is inhibition of phagosome-lysosome fusion, which enables bacteria to survive within the immature phagosomal compartment [585]. Akhter *et al.* showed that Caspase-11, independently of the inflammasome, promotes pathogen-containing phagosome-lysosome fusion, thus contributing to bacterial clearance. In caspase-11$^{-/-}$ macrophages, the fusion of the *L.pneumophila*-containing phagosome with lysosome was defective what allowed pathogen to evade degradation within macrophages and induce bacteria proliferation in the host. Another study showed that in *B. cenocepacia*- infected Caspase-11$^{-/-}$ macrophages restriction of bacteria is impaired compare to WT cells [586]. A similar effect could be observed in *Mtb* infection. Defect in phagosome containing *Mtb*-lysosome fusion in caspase-11 deficient BMDMs could explain the reason why caspase-11$^{-/-}$ BMDMs has significantly higher *Mtb* invasion

compare to wild-type. Caspase-11 could be responsible for a specific mechanism regulating anti-microbial autophagy independently of caspase-1.

As it has been shown that opposite to caspase-11$^{-/-}$ BMDMs, in NLRP3$^{-/-}$ BMDMs production of iNOS-dependent NO production increased compared to WT BMDMS, it could suggest that NLRP3 has a role separate to caspase-11 during bacterial infection. It has been shown that the duration of LPS priming before inflammasome activation signal, is very important for Caspase-1 activation and production of mature IL-1β. When priming lasts for 12 h, a high level of iNOS is detected, Caspase-1 activation is blocked and IL-1β production is inhibited compare to 6 h priming when iNOS expression in undetectable [587]. These results suggest that in the absence of NLRP3 protein, macrophages produced a higher level of nitric oxide which then inhibits inflammasome and enables *Mtb* to survive in the host. It suggests that the priming signal, which induces expression of NLRP3, is detrimental for nitric oxide-induced suppression of NLRP3 inflammasome.

High levels of nitric oxide may play a role in tissue damage. Nitric oxide can be cytotoxic for the cells which produce it and for neighboring cells and induce tissue injury [588]. It can suggest that NLRP3 is necessary for fine-tuning nitric oxide levels in the cells and protects against harmful effects of nitric oxide. Mishra *et al*. reported that *Mtb*-induced NO production negatively regulates NLRP3 inflammasome and decreases IL-1β production thus reducing persistent neutrophil recruitment and tissue damage during bacterial infection. NO causes S-nitrosylation of NLRP3 and prevents the oligomerization of the ASC adaptor which is essential for Caspase-1 activation [244]. No difference in pro-caspase-11 expression was observed in WT and NLRP3$^{-/-}$ BMDMs suggesting again that different mechanisms are involved in regulating iNOS expression by caspase-11 and NLRP3. More experiments are needed to confirm the role of NLRP3 protein expression in regulating nitric oxide production, however these results do suggest that caspase-11 is functioning independently of the non-canonical inflammasome.

In addition to the protective role of nitric oxide in bacterial pathogenesis, an imbalance in nitric oxide metabolism has been linked to neurodegenerative diseases [589]. Since the physiological level of nitric oxide appears to be neuroprotective and extensive nitric oxide production is decidedly neurotoxic, understanding of mechanism regulating nitric

oxide production in the central nervous system (CNS) is necessary for better therapeutic treatment of neurodegenerative diseases [590].

It has been reported that alteration in nitric oxide signalling may be related to the pathogenesis of various neurodegenerative disorders including Parkinson's, Alzheimer's diseases and other disorders of the CNS [547]. Activated glial cells express iNOS and produce more nitric oxide which in consequence triggers calcium mobilization from the endoplasmic reticulum, release of vesicular glutamate from astroglial cells and leads to neuronal cell death. This change in microglia potentially contributes to neurodegeneration [547].

Presented results have shown that similar to macrophages, iNOS expression and nitric oxide production is regulated by caspase-11 in murine glial cells. As the incidence of Alzheimer's disease is strongly associated with sex experiment to determine the production of nitric oxide in glial cells isolated from male and female was conducted. No significant differences between male and female cells were observed. This suggests that increased nitric oxide production in neurodegenerative disease could be rather induced by an increased number of glial cells than by higher production of nitric oxide by them. Interestingly, although in Caspase-$11^{-/-}$ macrophages nitric oxide production was significantly lower than in WT, in male Caspase-$11^{-/-}$ BMDMs iNOS-mediated NO production was higher than NO in female. Taking into consideration that one of the most common neurodegenerative disease, Parkinson's disease occur more commonly in men than in women, evidence can suggest that caspase-11-mediated NO regulation is altered in men, making them more susceptible to disease. More experiments are needed to further investigate this hypothesis.

Chapter 5: Caspase-4- a therapeutic target for inflammation-associated diseases: peptic ulcer and asthma.

5.1. Introduction

Inflammation is linked with development of many diseases. There are increasing evidences showing that chronic inflammation is associated with development of peptic ulcer [591] and asthma [592] and inhibition of resolved inflammation could be protective in these diseases.

Peptic ulcers are acid-induced lesions localised in gastric and duodenal tissues. Gastric ulcers are a consequence of mucosal injury in patients with normal or decreased acid secretion, whereas duodenal ulcer patients secrete about twice as much acid as controls [593]. Gastric ulcers are localised close to the mucosal boundary, between corpus with high local acid concentration and little inflammation; and antrum which is the major site of inflammation. The majority of gastric ulcers are localised in the gastric antrum [594]. Infection with *H.pylori* results in chronic inflammation and alongside with using NSAIDs, is considering as a one of the main risk factor for peptic ulcer development [254] [256]. *H.pylori* has many virulence factors allowing it to adhere to and create inflammation in gastric mucosa. The *H.pylori* surface protein urease has chemotactic activity for monocytes and neutrophils. The urease protein was identified in the lamina propria of gastric antrum from patients with *H.pylori*-associated gastritis but not in uninfected patients [595]. Although around 50% of the world's population have chronic *H.pylori* infection, only 5-10 % will develop ulcers [596]. However, *H.pylori* is responsible for 90 % of duodenal ulcers and 70-90 % of peptic ulcers [597]. Symptoms of peptic ulcers are variable and the most common are abdominal pain, nausea, vomiting, weight loss, bleeding and perforation in more advanced stages of disease [254].

H.pylori is a gram-negative bacterium and is able to activate the inflammasome in human and mouse immune cells through the TLR-NLRP3-caspase-1 axis [598].

In response to *H.pylori* infection, neutrophils and macrophages are recruited to gastric tissue, leading to macrophage activation and increased IL-1α, TNFα, IL-6, IL-8, MIP-1α and GRO-α production [599][600][601][602]. Several studies have shown that *H.pylori* infection induces inflammasome activation and IL-1β production [603][604][605]. Production of these chemokines and inflammatory molecules might be important in the regulation, recruitment and activation of inflammatory cells, including macrophages themselves, at the gastric mucosa [602].

There are no studies showing the role of non-canonical inflammsome in peptic ulcer formation. Study from 2009 linked upregulation of caspase-4 to decreased adenosine deaminase activity and implicated caspase-4 as a factor inducing gastric ulcers [606]. Peptic ulcer and long-standing inflammation can lead to gastric cancer [607]. It has been shown that pyroptosis is associated with gastric cancer and can influence the proliferation, metastasis and tumour invasion [608]. As it is well known that Caspase-11 triggers the activation of pyroptosis, it brings new light on the role of Caspase-11 and non-canonical inflammasone in peptic ulcer and gastric cancer development.

As mentioned before, the main risk factors for peptic ulcer development are *H.pylori* infection and NSAIDs. NSAIDs, such as aspirin and indomethacin, are widely used to help reduce pain and inflammation and have proven effectiveness in the treatment of arthritis and other musculoskeletal disorders. Unfortunately, their use has been associated with mucosal injury, peptic ulcer disease and related complications such as upper gastrointestinal haemorrhage and perforation [609][610]. Several mechanisms showing how NSAIDs could damage the mucosa have been presented. NSAIDs can directly damage gastric mucosa [611]. NSAIDs with a carboxylic acid residue enter epithelial cells and within a neutral intracellular environment the drugs are converted to an ionized state and are not able to diffuse out. The drugs accumulate in epithelial cells, the osmotic movement of water into the cells causes swelling of the cells and leads to lysis [612]. NSAIDs could damage the gastric epithelial cells via uncoupling of mitochondrial respiration, depletion of ATP, therefore reducing the ability to maintain intracellular pH [613]. The third mechanism of NSAID action could be through reducing hydrophobicity of the mucus gel layer in the stomach [614][615][616]. In addition to damaging gastric mucosa through direct irritation, NSAIDs suppress the synthesis of gastric prostaglandins [614]. NSAIDs act through the cyclo-oxygenase (COX) pathway and inhibit cyclooxygenase 1 and/or 2 (COX-1/COX-2). Two isoforms of COX exist, COX-1 and COX-2 [254]. COX-1 is constitutively expressed in most tissues including the stomach and is involved in maintaining gastric mucosal integrity. The COX-2 enzyme is mainly responsible for causing inflammation and in the human stomach, little or no COX-2 protein and activity were demonstrated [617][618]. Despite different properties of enzymes, Wallace *et al.* reported in 2000, that inhibition of both COX-1 and COX-2 is required for gastric ulceration to occur [619]. These proteins are the main enzymes which take part in the prostaglandin (PG) synthesis pathway [620][621]. PGs are synthesized in cells from different essential fatty acid precursors,

including arachidonic acid (AA). Physiological and pathological stimuli activate enzyme phospholipase A_2 (PLA_2) which liberate AA from membrane phospholipids into the cytoplasm. Upon release, AA is converted into prostaglandin H_2 (PGH_2) by COX [622]. PGH_2 is an unstable molecule that is transformed into several biologically active prostaglandins [623]. PGs which were derived from AA are classified into series-2 PGs and prostaglandin E2 (PGE_2), prostaglandin D2 (PGD_2), prostaglandin I2 (PGI_2), prostaglandin F2α and thromboxane A2 (TXA_2) can be distinguish [624].

Prostaglandins represent an important part of the gastric mucosal barrier through regulating processes such as submucosal blood flow, effective mucosal adaptation to initial tissue damage and rapid recovery after tissue damage [625]. Robert *et al.* reported in 1976 that PGE_2 inhibits gastric acid secretion and prevents peptic ulcer formation, suggesting PGE_2 as a potent treatment of peptic ulcer [626]. Following their synthesis, PGs are immediately released outside the cells, enabling their regulation of biological functions through specific receptors. PGE_2 acts by binding four types of G protein-coupled membrane receptors EP1, EP2, EP3 and EP4 [627]. Among them, EP2 is responsible for inhibiting macrophage function and inhibiting gastric irritant-induced apoptosis [628][629]. In addition to PGs role in gastric protection, during acute inflammation PGE_2 acts as a vasodilator and promotes tissue influx of neutrophils, macrophages and mast cells [630][631][632]. PGE_2 also has immunosuppressive properties and takes part in tissue regeneration and homeostasis regulation [633][634]. Recent reports have shown that PGE_2 inhibits NLRP3 inflammasome activation and IL-1β production [635][636].

However, prostaglandins are not the only endogenous substance involved in regulating mucosal defence. There is evidence showing the important role of nitric oxide (NO) as a mediator of mucosal defence. Both prostaglandins and NO possess cytoprotective effects. Suppression of nitric oxide, similar to prostaglandin synthesis inhibition, increases the susceptibility of gastric to injury [637][638]. NO is synthesized by iNOS and is an important intracellular and intercellular signalling molecule involved in many biological and pathological functions [639]. The main roles of NO are maintaining mucosal microcirculation homeostasis and inhibition of inflammation through the downregulation of the surface expression of adherent molecules [640]. Although NO is required for normal gastrointestinal function, there are several reports showing that a large excess of NO in gastric tissue may have detrimental effects on gastrointestinal mucosa integrity [641]. In the gastrointestinal tract, elevated iNOS expression was

detected during *H.pylori* infection, inflammatory diseases and NSAID-induced ulcerogenesis [642][643][644]. The iNOS expression was upregulated in mouse and rat models of stomach ulcer, but iNOS deficiency-induced larger ulcers and more severe inflammation during the healing process, suggesting the key role of iNOS activity in ulcer repair processes [645]. The expression of iNOS is increased in duodenal ulcer patients and may contribute to the pathogenesis of the disease [645]. *H.pylori* infection leads to upregulation of iNOS in gastric epithelial cells and increased NO levels. *H.pylori* infection, during the development of chronic gastritis, induces infiltration of gastric mucosa with macrophages and lymphocytes. Expression of iNOS was induced in immune cells and accumulation of free radicals led to destructive changes in gastric mucosa, erosions and regeneration disorders [646].

Although PGE_2 is widely considered as a proinflammatory mediator and has been implicated in several inflammatory diseases, PGE_2 also has protective effects in different organs, as already described for the gastrointestinal tract and respiratory system [647]. PGE_2 is the most abundantly produced prostanoid in the body and a major metabolite in the lower respiratory tract. PGE_2 is produced by several cells in human airways, including airway epithelium, smooth muscle and macrophages [648].

Asthma is a multifactorial and complex chronic inflammatory disorder of the lung, characterised by epithelial disruption, airway smooth muscle hypertrophy, increased mucus secretion and cytokine production and infiltration with immune cells [649][650]. Factors which trigger asthma include allergens, exercise, cold exposure, air pollutants and respiratory viral infections [651]. The most effective drugs used in asthma are inhaled corticosteroids [652]. Several studies have shown a protective role for PGE_2 and suggested that PGE_2 action was mediated mainly through EP2 receptors [653][654]. The protective role of PGE_2 could be provided by a direct effect on airway smooth-muscle relaxation but also through the inhibition of inflammatory signalling [655]. Both inhaled and endogenous PGE_2 protects against airway inflammation and bronchoconstriction, and deletion of genes encoding COX isoenzymes cause increased allergic inflammation in a mouse model of asthma [656][657][658]. Around 10-20 % of asthmatic patients are sensitive to aspirin or other NSAIDs and ingestion of these drugs provokes bronchoconstriction and exacerbates bronchospasms with attacks of asthma and rhinitis [659]. It has been shown that all NSAIDs that inhibit both COX-1 and COX-2, induce aspirin-induced asthma [660]. Moreover, inhaled PGE_2 prevents aspirin-

induced bronchoconstriction and can be highly effective in preventing aspirin-induced asthma [661].

In Brief:

Hypothesis and aims of Chapter 5

Hypothesis: Caspase-4/-11 is a therapeutic target for two inflammation-associated diseases: *Helicobacter pylori*-induced peptic ulcer and allergen-induced asthma.

Specific aims of Chapter 5:

1. Determine caspase-4 expression levels in human peptic ulcer biopsies.
2. Investigate the role of caspase-11 in *Helicobacter pylori* infected murine macrophages.
3. Determine the role of prostaglandin E2 (PGE_2) in caspase-11 driven pyrotosis.
4. Determine caspase-11 expression levels in a murine model of asthma.
5. Analyse the role of misoprostol and indomethacin on caspase-11 expression in a murine model of asthma.

5.2. Results

5.2.1. Caspase-4 expression is increased in patient ulcer biopsies.

Treatment of the OVA mouse model of allergic inflammation with misoprostol and indomethacin revealed that misoprostol successfully inhibits caspase-11 upregulation where indomethacin, one of the NSAIDs, further increased caspase-11 expression. As it is well known that using high doses of NSAIDs is one of the main risk factor for peptic ulcer development [662] and misoprostol is preventing and healing gastric ulcer inuced by NSAIDs in patients [663], following experiments were focused on the role of caspase-11/-4 in the formation of peptic ulcers..

To analyse the expression of caspase-1 and caspase-4, the main components of the canonical and non-canonical inflammasome, respectively, biopsies were isolated from peptic ulcer patients. From each patient, two biopsies were taken, one at the side of antral ulcer and one from corpus region. As a control, antrum and corpus biopsies were collected from unaffected patients. Western blot analysis revealed that caspase-4 was upregulated in the antrum region of peptic ulcer patients compared to healthy controls (**Figure 5.1 A, B**). In contrast, within the corpus region, caspase-4 was upregulated in one of the peptic ulcer patients, although no significant difference was observed overall between healthy and peptic ulcer patients (**Figure 5.2 A, B**). No differences in caspase-1 expression levels were observed between healthy and peptic ulcer patients (**Figure 5.1 A, C; Figure 5.2 A, C**). These results confirm previously reported findings, suggesting that caspase-4 might be involved in inflammation and mucosal damage which occur during ulcer formation [606].

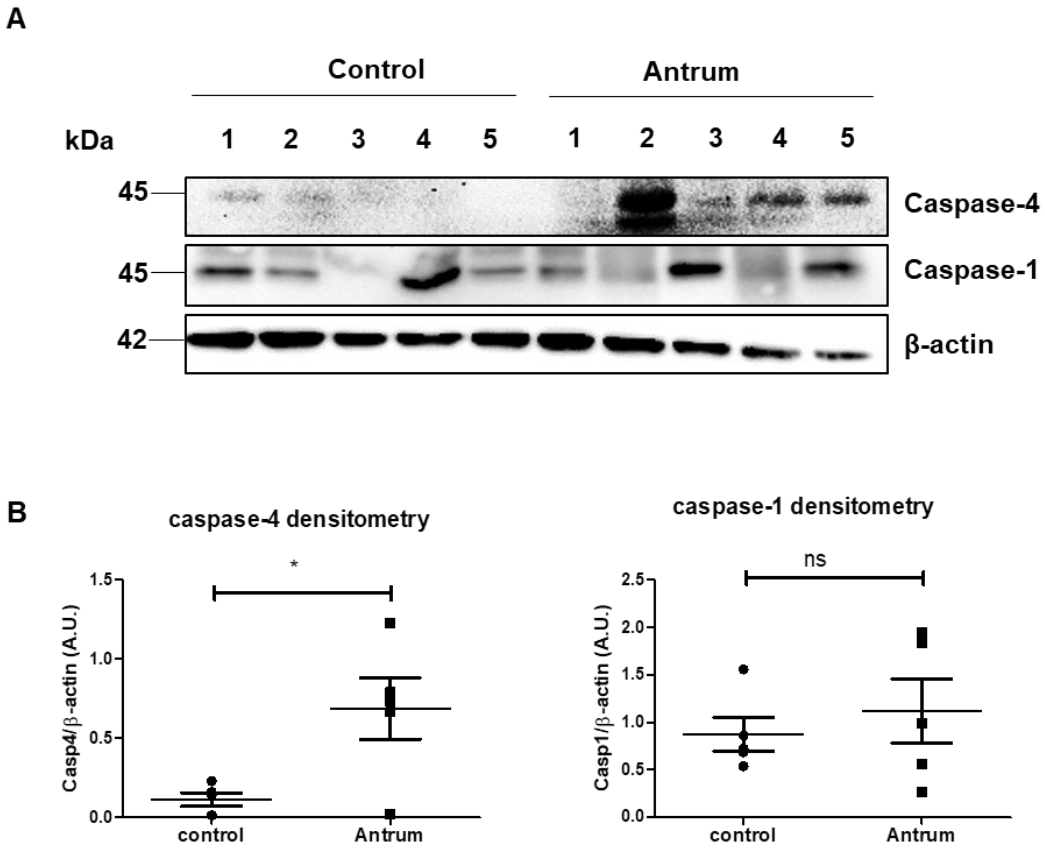


Figure 5.1. Caspase-4 expression is increased in ulcerated, compared to healthy, gastric antrum tissue.

Peptic biopsies of gastric antrum tissue were taken from peptic ulcer patients. Control biopsies were collected from antrum tissue of non-affected patients. **A)** Tissue lysates (20 µg) were probed by Western blot for caspase-4 (45 kDa), caspase-1 (45 kDa) and β-actin (42 kDa) as a loading control. **B)** Densitometric analysis of caspase-4 and caspase-1 in antrum biopsies. Each dot represents an individual patient. Error bars represent mean ±SEM. (A.U refers to Arbitrary Unit). Statistical analysis was performed using paired student's *t*-test; *$p<0.05$.

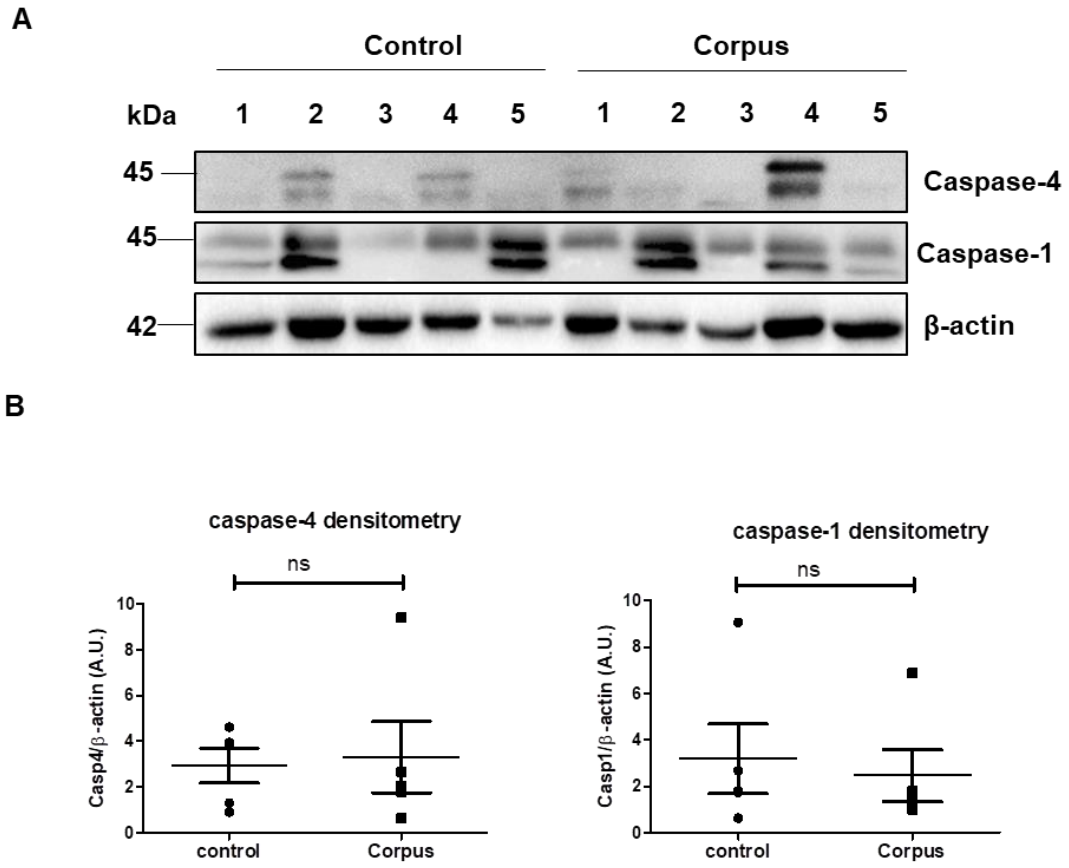


Figure 5.2. Expression of caspase-1 and caspase-4 are not elevated in gastric corpus tissue from peptic ulcer patients.

Peptic biopsies of gastric corpus tissue were taken from peptic ulcer patients. Control biopsies were collected from corpus tissue of non-affected patients. **A)** Tissue lysates (20 μg) were probed by Western blot for caspase-4 (45 kDa), caspase-1 (45 kDa) and β-actin (42 kDa) as a loading control. **B)** Densitometric analysis of caspase-4 and caspase-1 in gastric corpus biopsies. Each dot represents an individual patient. Error bars represent mean ±SEM; A.U refers to Arbitrary Unit. Statistical analysis was performed using paired student's *t*-test; *p<0.05.

5.2.2. Caspase-11 regulates Caspase-1 activity and IL-1β production in *H.pylori*-infected macrophages.

Analysis of human biopsies reveals that caspase-4 is upregulated in antrum biopsies suggesting that noncanonical inflammasome might be involved in peptic ulcer development (**Figure 5.1**). Based on this result, experiments to determine the role of caspase-11 (in human caspase-4) in *H.pylori* infected macrophages were conducted. WT and Caspase-11 deficient macrophages were infected with *H.pylori* (strain NCTC 11638, MOI 50 and 100) for 6, 24 and 48 h. Activation of caspase-1 was observed in WT BMDMs at 24 h, confirmed by detection of the autocatalytically activated p10-kDa subunit of caspase-1 by Western blotting (**Figure 5.3 A**). *H.pylori* also upregulated the expression of pro-IL-1β at 6, 24 and 48 h. Moreover, caspase-1 activation and pro-IL-1β expression are regulated by caspase-11 (**Figure 5.3 A, B**). Autocatalytically activated caspase-1 induces the maturation of IL-1β and IL-18, which are subsequently released to initiate inflammation and host defence mechanisms [266]. To confirm caspase-1 activation, the level of IL-1β in supernatants was determined. Similar to western blot results, IL-1β production was shown to be regulated by caspase-11, as IL-1β production was significantly decreased in caspase-11 deficient BMDMs at 24 h (MOI 50 and MOI 100), compared to WT BMDMs (**Figure 5.4 A**). The observation that mature IL-1β is secreted following *H.pylori* infection suggests that *H.pylori* is able to provide both signals, priming and second signal, necessary for inflammasome activation. In contrast, no difference in IL-1α production in WT and Casp11$^{-/-}$ BMDMs was observed (**Figure 5.4 B**), even though it has been reported that caspase-11 activation leads to NLRP3 inflammasome activation with IL-1β and IL-1α release [142]. No differences in the inflammasome-independent cytokines, TNFα and IL-6, were observed in *H.pylori*-infected WT and Caspase-11$^{-/-}$ BMDMs (**Figure 5.4 C, D**).

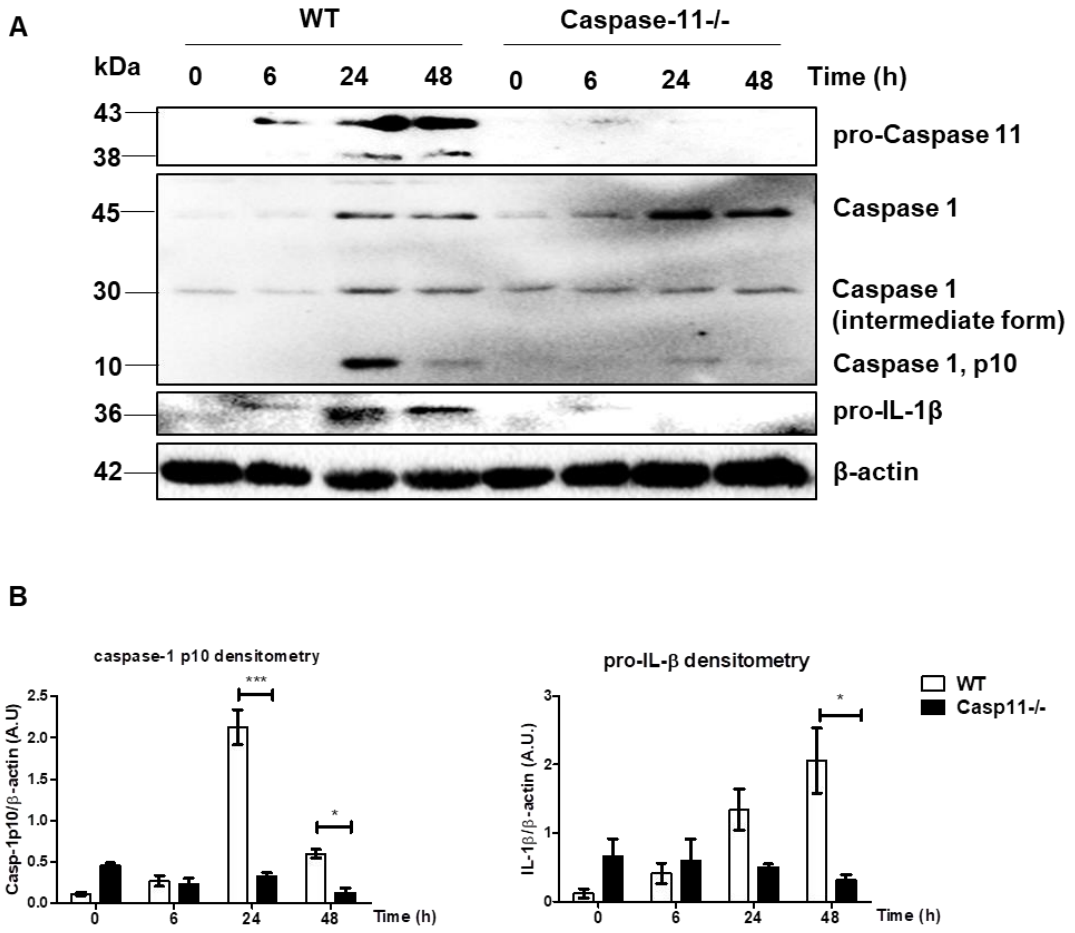


Figure 5.3. Caspase-11 regulates pro-IL-1β expression and caspase-1 activation in *H.pylori*-infected BMDMs

WT and Caspase-11 deficient BMDMs were infected with *H.pylori* strain NCTC 11638 (MOI 100) for 6, 24 or 48 h. **A)** Protein lysates were analysed by western blot for Caspase-11 (38; 43 kDa), pro-caspase-1, intermediate form of caspase-1 and cleaved caspase-1 p10 (45, 30 and 10 kDa, respectively), pro-IL-1β (36 kDa) and β-actin (42 kDa) as a loading control. Blots shown are representative of 3 independent experiments. **B)** Densitometric analysis of caspase-1 p10 and pro-IL-β relative expression; A.U refers to Arbitrary Unit. Statistical analysis was performed using 2way *ANOVA* test to compare WT and Casp11-/- mean ± SEM values from 3 independent experiments; *p<0.05; ***p<0.001; (A.U refers to Arbitrary Unit).

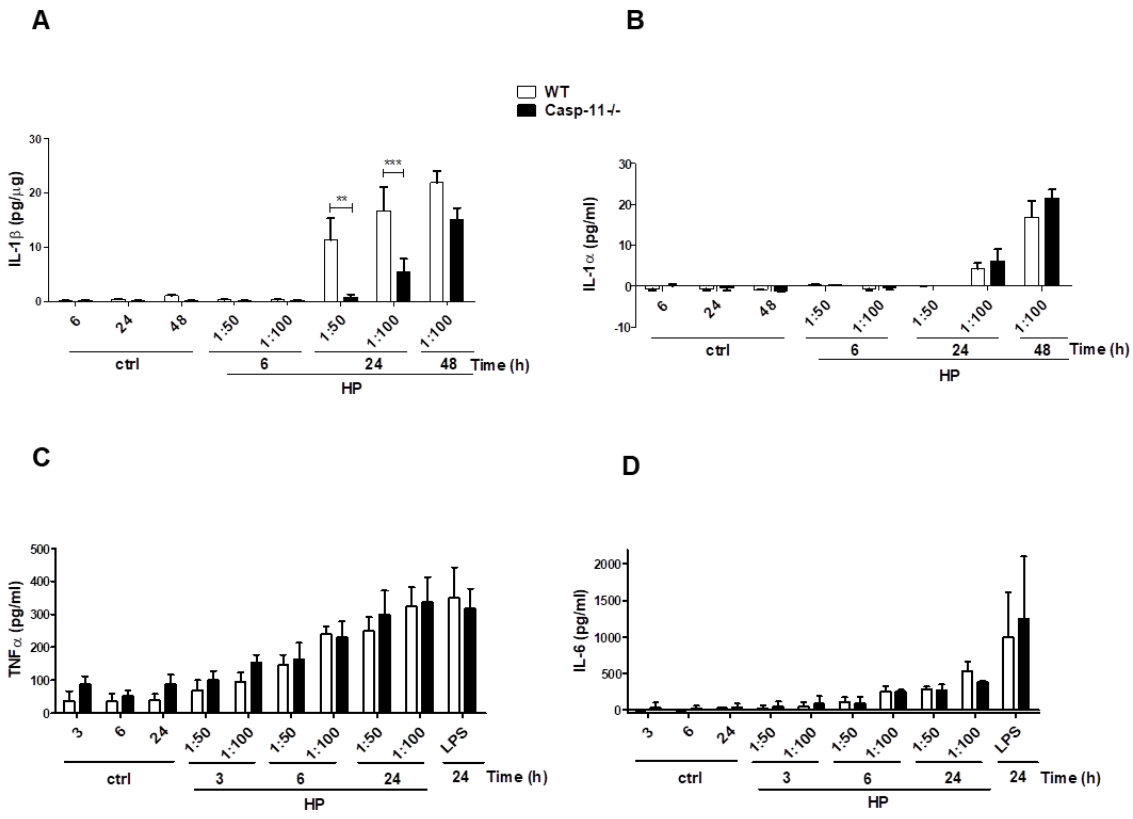


Figure 5.4. Caspase-11 regulates IL-1β, but not IL-1α, IL-6 and TNFα secretion in *H.pylori*-infected BMDMs

BMDM were infected with *H.pylori* strain NCTC 11638 at MOI 50 (1:50) or MOI 100 (1:100) for 6, 24 or 48 h. Supernatants were subsequently measured for levels of secreted cytokines **A)** IL-1β (V- PLEX, MSD plate, concentration of IL-1β in pg/ml was divided by protein concentration in μg/ml) **B)** IL-1α (BioLegend ELISA kit) **C)** TNFα (BioLegend ELISA kit) and **D)** IL-6 (BioLegend ELISA kit).
Data represents mean ± SEM values from 3 independent experiments for 6 and 24 h stimulation and 2 independent experiments for 48 h stimulation. 2way *ANOVA* test was used to compare WT and Casp11-/- BMDMs. **p<0.01; ***p<0.001.

5.2.3. *H.pylori* infection does not induce pyroptosis in murine macrophages.

The results presented have shown that *H.pylori* infection induces caspase-11-dependent caspase-1 activation and IL-1β production. Activation of both canonical and noncanonical inflammasomes result in the induction of inflammatory programmed cell death, pyroptosis [664]. However, BMDMs infected with *H.pylori* did not secrete more LDH than uninfected cells suggesting that, although caspases-1 and -11 are activated, *H.pylori* does not induce cell death at the time points investigated (**Figure 5.5**).

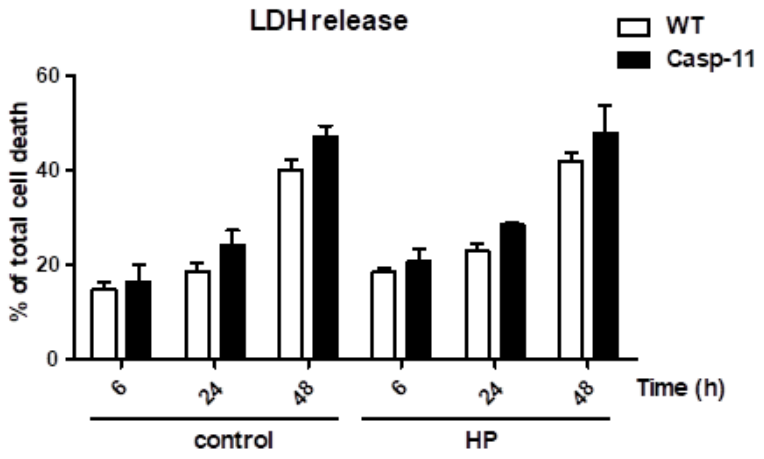


Figure 5.5. *H.pylori* does not induce cell death in BMDMs

BMDMs were seeded at 10^6 cells/ml (in antibiotic-free DMEM) for 24 h. Next day, cells were infected with *H.pylori* strain NCTC 11638 (HP) at MOI 100 for 6, 24 or 48 h. Supernatants were subsequently measured for levels of secreted LDH. Data represents mean ± SEM values of n=3 for 6 h and 24 h timepoints and n=2 for 48 h timepoints.

5.2.4. *H.pylori*-induced iNOS expression is regulated by caspase-11.

Under normal conditions, NO protects the gastrointestinal mucosa from injuries caused by ethanol, mineral acids and bile-acids, ischemia/reperfusion injuries and early endotoxin-induced damage [665]. NO is involved in the modulation of the gastric mucosal integrity [666]. However, high concentrations may have harmful effects on the gastrointestinal tract. Increased iNOS activity has been detected in *H.pylori*-induced gastritis, inflammatory bowel disease and NSAID-induced ulcerogenesis [644][642][643].

As previousl results determined that caspase-11 regulates iNOS expression and NO production in LPS- and *Mtb*-stimulated BMDMs (Chapter 4, **Figure 4.7**) iNOS expression was analysed in *H.pylori*-infected BMDMs. iNOS expression was upregulated in WT BMDMs at 24 h and subsequently decreased 48 h post *H.pylori* infection (**Figure 5.6 A**). Similar to the previous observation, iNOS induction was regulated by caspase-11, as iNOS upregulation was not observed in caspase-11 deficient BMDMs. Despite iNOS induction, NO was not detectable in BMDM supernatants (**Figure 5.6 C**).

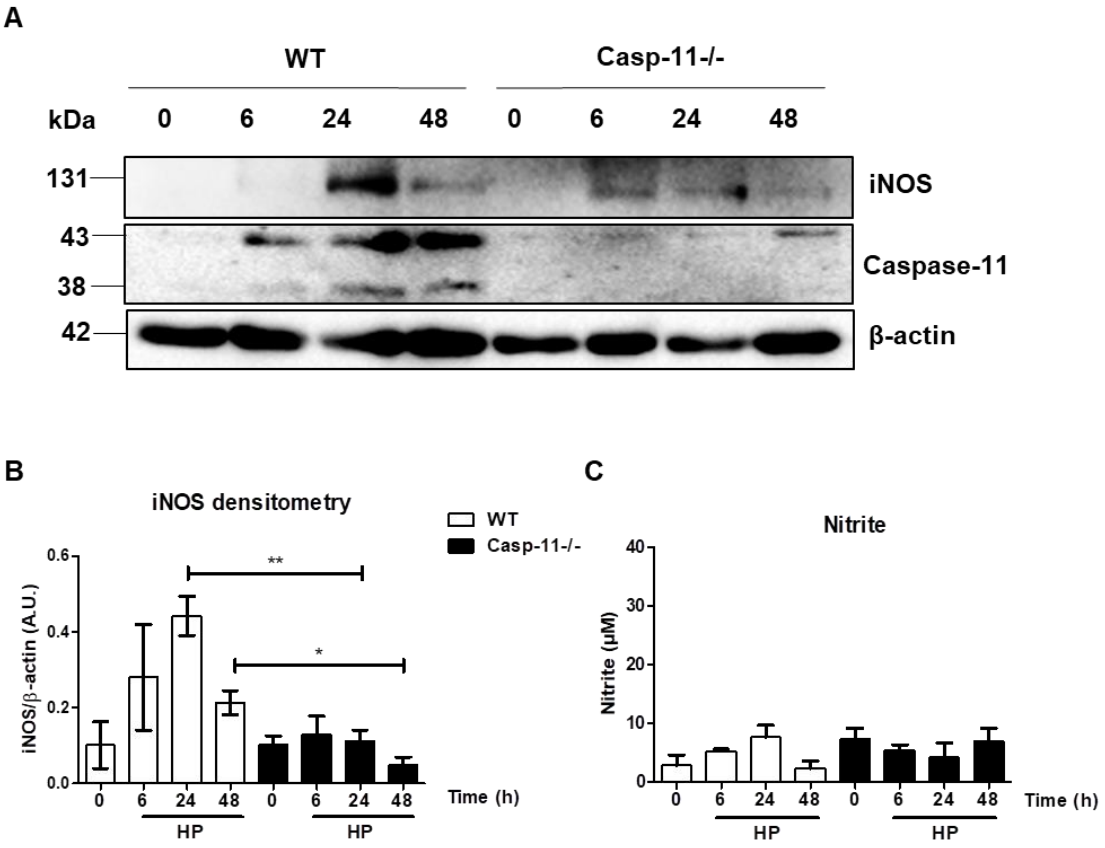


Figure 5.6. Caspase-11 regulates iNOS expression in *H.pylori*-infected BMDMs

WT and Caspase-11 deficient BMDMs were infected with *H.pylori* strain NCTC 11638 at MOI of 100 for 6, 24 or 48 h. **A)** Protein lysates analysed by western blot for Caspase-11 (38; 43 kDa), iNOS (131 kDa) and β-actin (42 kDa) as a loading control. Blots shown are representative of 3 independent experiments. **B)** Densitometric analysis of iNOS relative expression; A.U refers to Arbitrary Unit. Statistical analysis was performed using student's t-test to compare mean ± SEM values from 3 independent experiments, *$p<0.05$.**C)** Nitrite concentration in the media was determined using the Griess assay. Values represent the mean ± SEM of n=3 for 6 and 24 timepoints and n=2 for 48 h timepoint.

5.2.5. Prostaglandin E2 inhibits caspase-4/-11 and caspase-4-driven pyroptosis in LPS-primed cells.

Prostaglandin E2 (PGE_2) is produced and stored in the gastric and duodenal mucosa and is an important component of the gastric mucosal barrier [667]. It has been shown that decreased levels of mucosal PGE_2 represent a contributory factor for gastric ulcer formation [668] and the stable PGE1 analogue, misoprostol, has a clinically proven protective role in peptic ulcers [669, 670].

Based on information that caspase-4 is upregulated in peptic ulcer biopsies and the main risk factor for peptic ulcers, *H.pylori* causes caspase-11 upregulation in macrophages, experiments to analyse whether PGE_2 could control caspase-11 expression were conducted. BMDMs were pre-treated with PGE_2 and subsequently primed with LPS. mRNA analysis reveals that PGE_2 inhibits caspase-11 transcription (**Figure 5.7 A**), resulting in the inhibition of caspase-11 protein expression (**Figure 5.7 C, D**). Four specific G protein-coupled subtypes EP1-EP4 are recognized as PGE2 receptors [671]. The EP2 receptor is the most abundantly expressed PGE_2 receptor on bone marrow cells and maturing macrophages and plays a role in the suppression of macrophage maturation [629]. The preliminary data show that EP2 receptor-deficient BMDMs expressed higher levels of caspase-11 than wild-type cells (**Figure 5.7 B**). To exclude that PGE_2 could inhibit caspase-11 through blocking TLR4 priming, PGE_2 was added after 4 h LPS priming and 30 min before LPS transfection. Results revealed that caspase-11 expression was unchanged (**Figure 5.8 A, B**) however, it has been shown that pyroptosis is significantly inhibited under these conditions [177]. To confirm the above findings in humans, the effects of PGE_2 on caspase-4 in primary monocyte-derived macrophages (MDM) was analysed. PBMCs were obtained from healthy volunteers, and cells were differentiated into macrophages using adherence method. Pre-treatment with PGE_2, before 4 h LPS stimulation, significantly decreased caspase-4 expression in MDMs (**Figure 5.9 A, B**). However, PGE_2 added either, before or after LPS priming is able to inhibit cell death in LPS transfected MDMs (**Figure 5.9 C**).

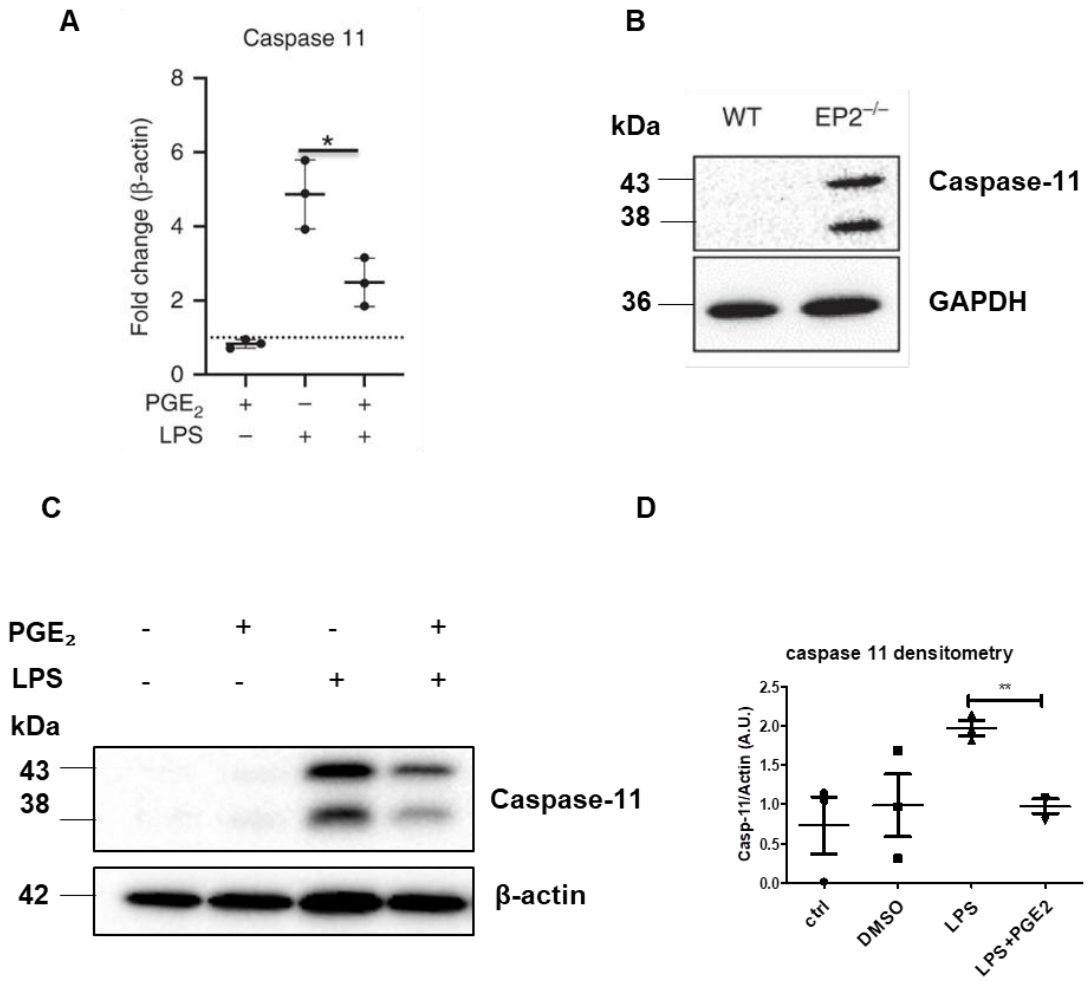


Figure 5.7. Prostaglandin E2 inhibits LPS-mediated caspase-11 upregulation.

A) Murine BMDMs were pretreated with 1 μg PGE_2 or DMSO for 30 min followed by 4 h stimulation with 100 ng/ml of LPS. RNA was isolated and subjected to qPCR for caspase-11 mRNA expression. The results shown are from a single experiment, with three mice in each group, and are representative of three independent experiments. Values represent the mean ± SEM of duplicates Student's two-tailed t-test was used to compare treated and untreated cells, *$p < 0.01$ **B)** BMDMs from WT or from EP2-deficient mice were assessed for caspase-11 expression by western blot. The results shown are representative of BMDMs prepared from three mice of each genotype in a single experiment. **C**) BMDMs were treated with 1 μg PGE_2 for 30 min followed by 4 h stimulation with 100 ng/ml of LPS. Protein lysates (20 μg) were analysed by western blot for caspase-11 (38;43 kDa) and β-actin (42 kDa) expression. **D)** Densitometric analysis of caspase-11 representing the mean ± SEM values from 3 independent experiments; A.U refers to Arbitrary Unit. Statistical analysis was performed using student's t-test, **$p<0.01$.

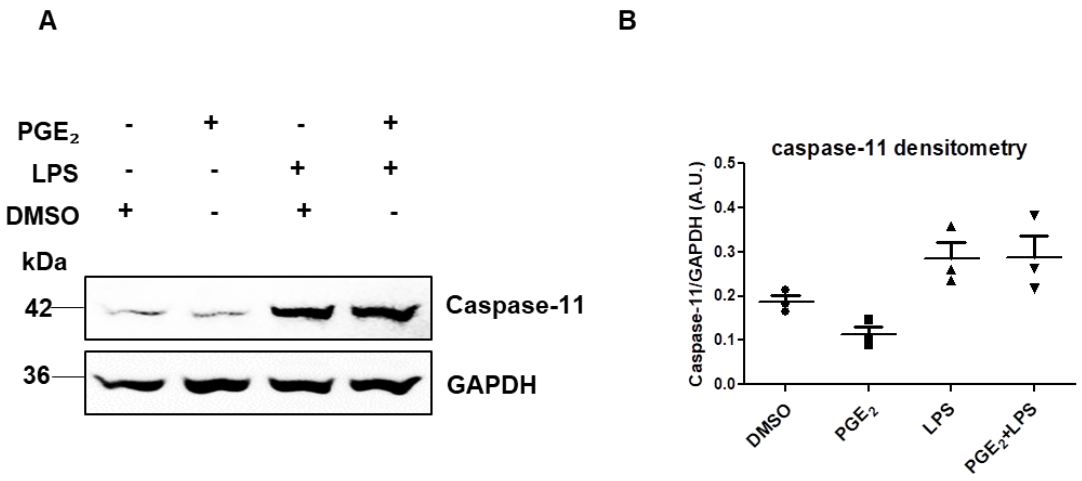


Figure 5.8. Prostaglandin E2 does not inhibit caspase-11 expression when added after LPS priming

A) Murine BMDMs were stimulated with 100 ng/ml of LPS for 4 h and then treated with 1 μg of PGE_2 for 30 min. Protein lysates (20 μg) analysed by western blot for caspase-11 (38;43 kDa) and GAPDH (36 kDa) as a loading control. Blots shown are representative of 3 independent experiments. **B)** Densitometric analysis represents the mean ±SEM values from 3 independent experiments; A.U refers to Arbitrary Unit.

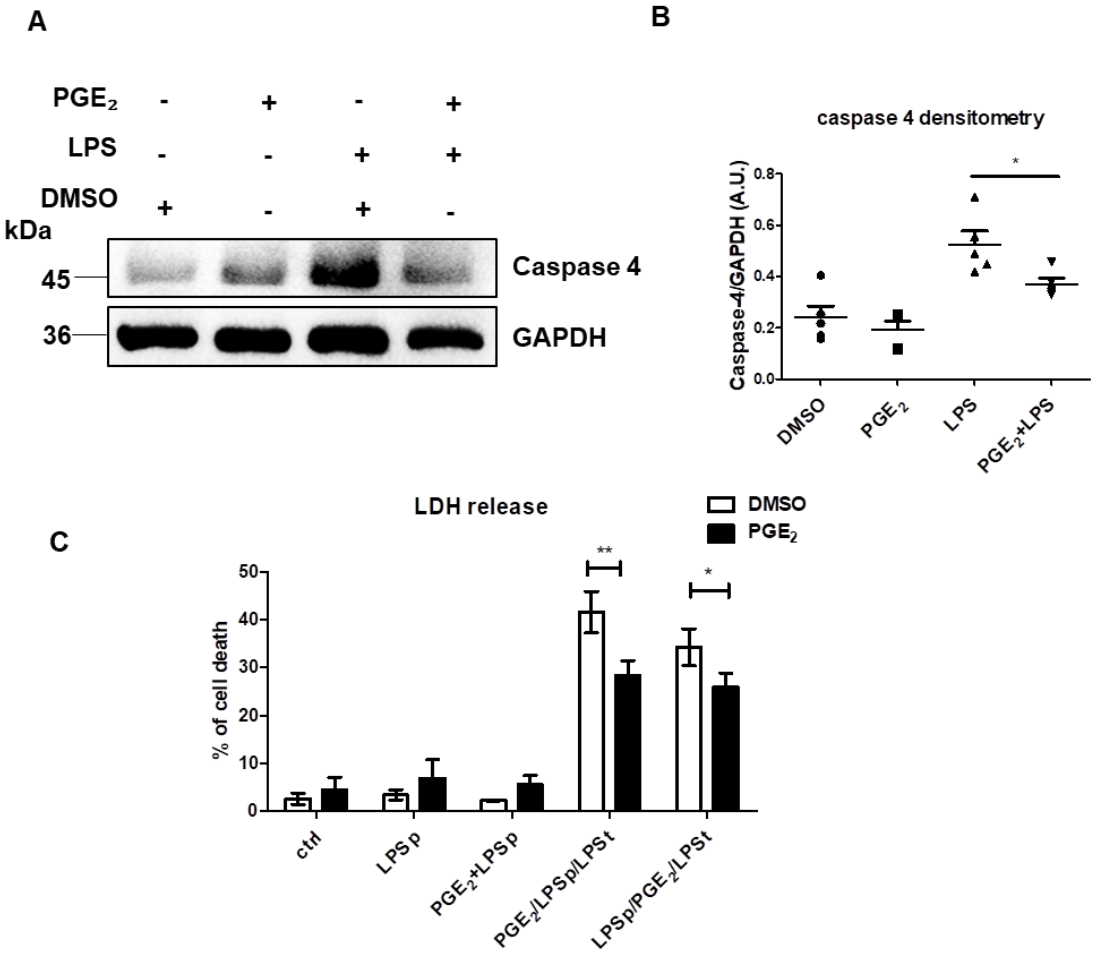
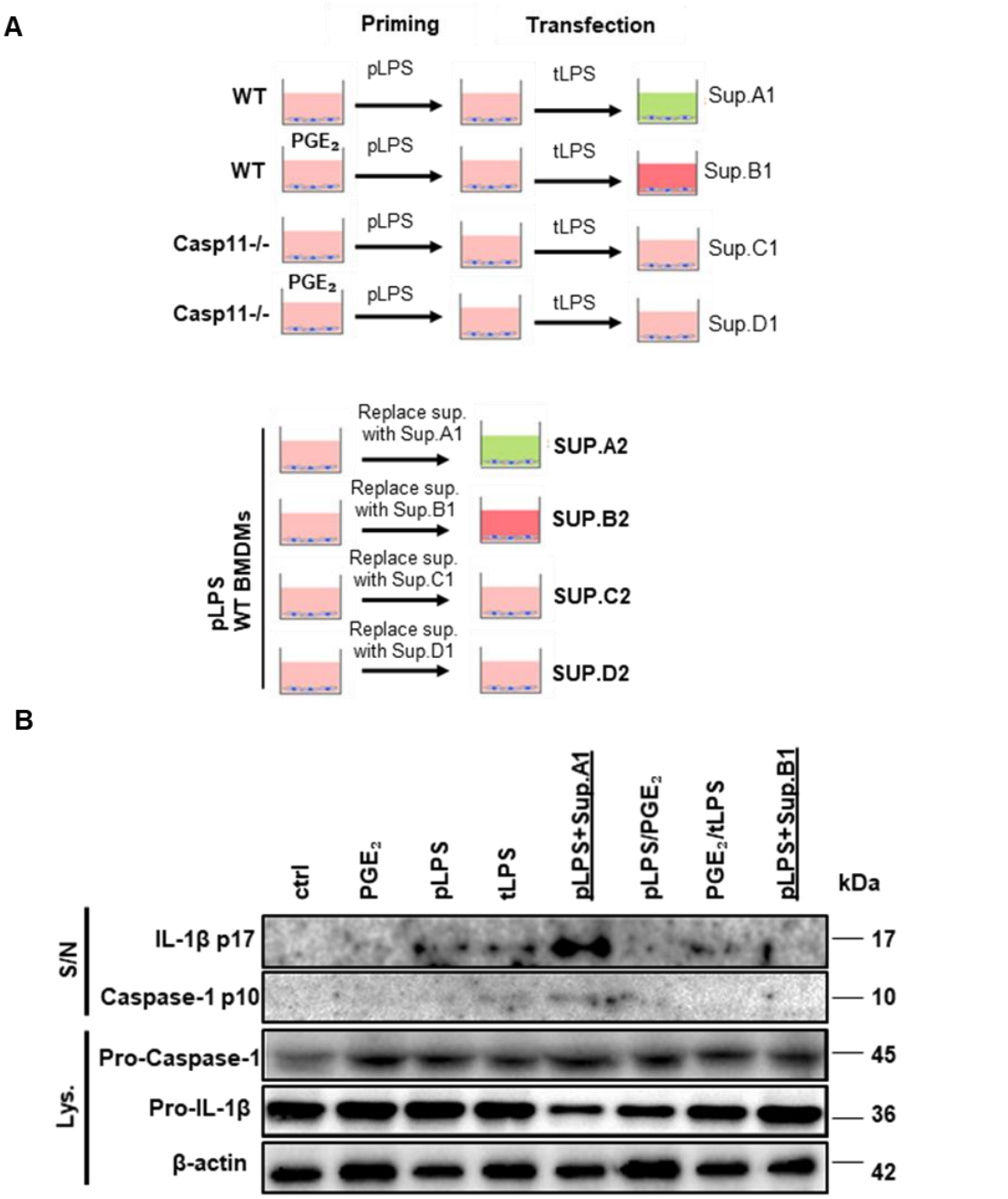


Figure 5.9. Prostaglandin E2 inhibits caspase-4 expression and cell death in LPS-primed macrophages.

A) Human primary monocyte-derived macrophages were treated with 1 µg PGE_2 or DMSO for 30 min followed by 100 ng/ml LPS for 4 h. Protein lysates (20µg) analysed by western blot for caspase-4 (45 kDa) expression and GAPDH (36 kDa) as a loading control. Blots shown are representative of 4 independent experiments. **B)** Densitometric analysis of caspase-4 expression; A.U refers to Arbitrary Unit. Statistical analysis was performed using unpaired student's t-test to compare mean ± SEM values,n=4; *$p<0.05$. **C)** Human monocyte-derived macrophages were treated with 1 µg PGE_2 for 30 mins before priming with 100 ng/ml of LPS for 4 h (PGE_2 + LPSp); followed by 2 µg/ml LPS transfection (PGE_2/LPSp/LPSt); or priming with LPS (100 ng/ml) for 4 h and treating with 1 µg PGE_2 for 30 mins before 2 µg/ml LPS transfection (LPSp/PGE_2/LPSt). Supernatants were analysed for LDH secretion as a measure of cell death. Data from 3 independent experiments are shown. Each experiment represents data from one patient. Statistical analysis was performed using 2way ANOVA test to compare mean ± SEM values from PGE_2 treated and untreated cells (DMSO); *$p<0.05$; **$p<0.01$.

5.2.6. PGE_2 indirectly regulates caspase-1 activity, via its inhibition of caspase-11

Caspase-11 mediated pyroptosis induces the release of a number of DAMPs and ATP, which have the capacity to trigger NLRP3 and caspase-1 and further boost pyroptosis levels [672]. PGE_2 has a well-established protective role in the gastric mucosa from a different form of damage, including these caused by stress, NSAIDs abuse or bile acids [673]. Based on that information an experiment to analyse whether PGE_2 could limit DAMP production, thus subsequently indirectly inhibiting caspase-1 activation and caspase-1 dependent pyroptosis was designed. WT BMDMs were pretreated with PGE_2 or DMSO, primed with LPS and subsequently transfected with LPS to induce non-canonical, caspase-11 dependent pyroptosis (donors). To support the hypothesis that PGE_2 inhibits pyroptosis through caspase-11 inhibition, caspase-11 deficient BMDMs were similarly treated to compare their response with PGE_2-treated WT BMDMs. *In vitro*, canonical inflammasome requires LPS priming followed by addition of ATP or nigericin to imitate DAMPs. To induce canonical inflammasome-dependent pyroptosis, media collected from PGE_2 pre-treated, LPS primed and LPS transfected WT and Casp-$11^{-/-}$ BMDMs (donors) were collected and added to LPS primed WT BMDMs (recipients) (**Figure 5.10 A;** schematic representation of experiment). Western blotting revealed that supernatants from PGE_2 pre-treated, LPS primed and transfected WT BMDM (donors), incubated with LPS primed WT BMDM (recipients), caused significantly lower secretion levels of mature IL-1β and processed caspase-1 (p10), when compared with donor supernatants from similarly stimulated WT BMDM that received no PGE_2 pre-treatment (**Figure 5.10 B**, compare pLPS+SupA1 to pLPS+SupB1). Moreover, analysis of IL-1β production revealed inhibitory effect of PGE_2 on IL-1β (**Figure 5.12 B**). Results showed that media collected from donor cells are able to induce cell death in LPS primed BMDMs and PGE_2 significantly inhibits DAMP release following non-canonical inflammasome activation (**Figure 5.11 A**; compare A2 to B2). A similar effect was observed in Caspase-$11^{-/-}$ BMDMs, showing that the absence of caspase-11 can indirectly inhibit caspase-1 dependent cell death (**Figure 5.11 A**; compare A2 to C2).



Western blot run by Z.Zaslona (Z Zaslona *et al. Immunohorizons*, 2020, [674])

Figure 5.10. Prostoglandin E2 inhibits canonical inflammasome activation.

A) Schematic representation of the experiment designed to explore paracrine signalling mechanisms for pyroptosis. **B)** WT and Caspase-11 deficient BMDMs were pretreated with PGE_2 or vehicle (DMSO), primed with 100 ng/ml LPS for 4 h and subsequently transfected with 2 μg LPS, to generate supernatants (donors, Sup A1-D1). A separate plate of LPS primed (4 h) WT BMDMs (recipients) were treated with supernatants A1-D1. Western blot analysis of recipient BMDMs was performed to determine the levels of pro-caspase-1, pro-IL-1β and β-actin (loading control) in lysates from LPS-primed WT BMDM treated with donor supernatants A1 or B1, alongside relevant control treatments (PGE2, pLPS, tLPS treatments alone or combined). Levels of mature IL-1β (p17) and caspase-1 (p10) in supernatants from the same experiment. Blots shown are representative of 3 independent experiments.

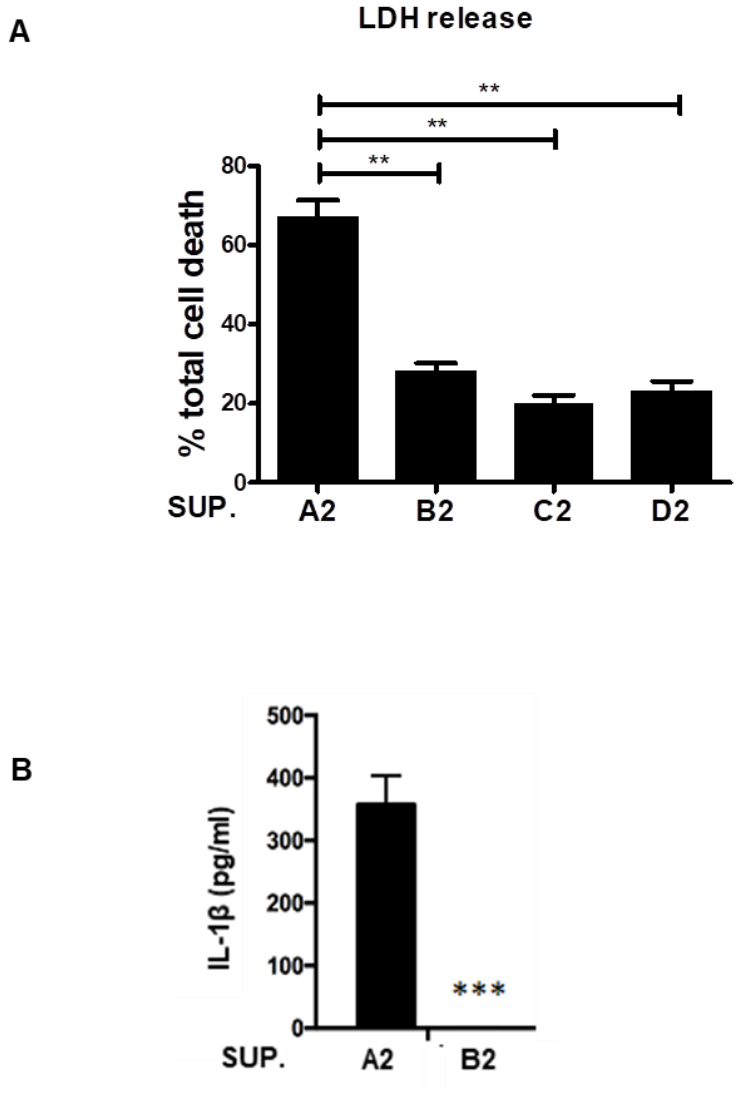


Figure 5.11. Prostaglandin E2 inhibition of non-canonical Caspase-11 activation blocks classical caspase-1 dependent IL-1β secretion and cell death.

WT and Caspase-11 deficient BMDMs were pretreated with PGE_2 or vehicle (DMSO), primed with 100 ng/ml LPS for 4 h and subsequently transfected with 2 μg LPS, to generate supernatants (donors, Sup A1-D1). A separate plate of LPS primed (4 h) WT BMDMs (recipients) were treated with supernatants A1-D1. Resulting lysates were assayed by ELISA and LDH assay. Data represents mean ± SEM values of n=3. Statistical analysis was performed using student's *t*-test to compare mean values between cells, **p<0.01; ***p<0.001.

5.2.7. Caspase-11 expression is regulated by PGE_2 in allergic lung inflammation.

It has been shown that PGE_2 has a protective role in asthma disease [675]. PGE_2 is produced in the lung and, when inhaled, attenuates allergen-induced airway responses, hyperresponsiveness and inflammation, suggesting a protective role of endogenous PGE_2 [648, 657]. To analyse the role of PGE_2 in asthma an OVA mouse model of allergic airway inflammation (AAI) was used. AAI was induced by intraperitoneal injection of OVA (chicken leg ovalbumin) and the adjuvant aluminium hydroxide (alum), followed 7 days later by two airway challenges with 1% OVA. This well-established protocol [676] [677] results in eosinophilic inflammation and induction of Th2 cytokines in bronchoalveolar lavage fluid (BALF). The lungs were collected 24 h after the last airway challenge. Samples were homogenised and protein expression level was determined by western blotting. Caspase-11 expression was significantly up-regulated during AAI, compared to healthy mice **(Figure 5.12 A**). Expression of pro-IL-1β was also up-regulated in 3 from 4 AAI biopsies (**Figure 5.12 A**; lane 5-8) but this result was not shown to be statistically significant (**Figure 5.12 A, C**) according to densitometry analysis. It has been shown that treatment OVA mouse model with the exogenous PGE1 analogue, misoprostol, inhibits allergic inflammation [658]. To determine whether PGE_2 regulates caspase-11 expression during AAI, mice were treated with misoprostol before sensitization and each airway challenge. Misoprostol is a stable, synthetic PGE_1 analogue which closely resembles naturally occurring prostanoids, including PGE_2. Misoprostol regulates inflammatory cytokines and immune function *in vitro,* similarly to PGE_2 [372]. Results revealed that expression of caspase-11 and pro-IL-1β are both inhibited in the lung tissues of misoprostol-treated mice (**Figure 5.12 A**).

The nonsteroidal anti-inflammatory drug (NSAID), indomethacin blocks PGE_2 biosynthesis via inhibition of cyclooxygenase (COX) [373][621]. To analyse the role of NSAID on caspase-11 in AAI, mice were treated with indomethacin before intraperitoneal sensitization and each airway challenge with OVA. Protein analysis by western blot showed that caspase-11 is significantly increased in the lung of indomethacin treated AAI mice compared to the lung from indomethacin untreated AAI (**Figure 5.13 A;** densitometric analysis **Figure 5.13 B**).

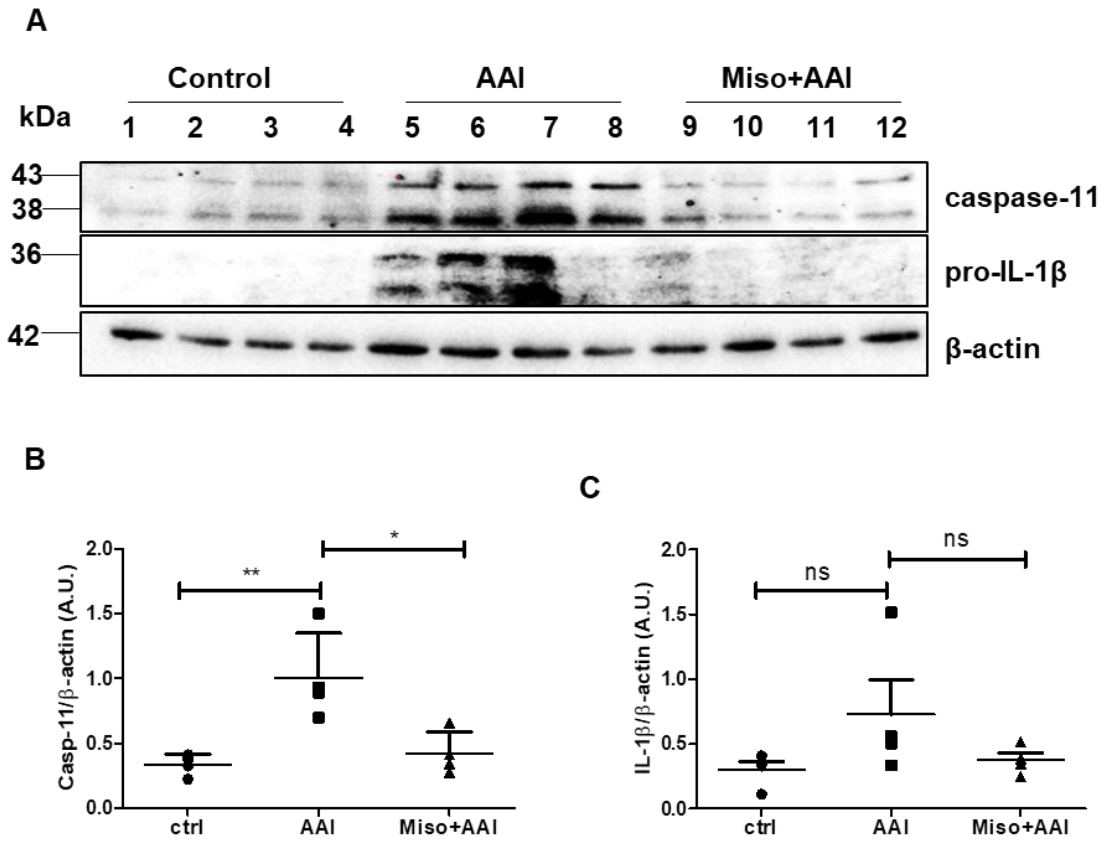


Figure 5.12. Misoprostol dampens caspase-11 expression during allergic airway inflammation.

Murine allergic airway inflammation (AAI) was induced by intraperitoneal injection of 20 µg OVA emulsified in 200 µl of sterile phosphate buffered saline (PBS) containing 2 mg of aluminium hydroxide, followed 7 days later by two airway challenges with 1% OVA. In misoprostol stimulated AAI mice, 200 µl of PBS containing 2 mg/kg of misoprostol in 0.5% DMSO as given 2 h before sensitization and 2 h before each airway challenge. Control mice received 200 µl of PBS containing 0.5% DMSO alone. Lungs were collected 24 h after the final airway challenge. The whole-lung lobes were homogenised and samples for western blot were prepared. A) Protein lysates (20µg) analysed by western blot for caspase-11 (38; 43 kDa), pro-IL-1β (36 kDa) and β-actin (42 kDa) as a loading control. There were four mice in each group, and analysis of samples from each mouse is shown. B, C) Densitometric analysis of caspase-11 and pro-IL-1β expression, relative to β-actin. (A.U refers to Arbitrary Unit). Statistical analysis was performed using student's t-test to compare mean ± SEM values, *$p<0.05$.

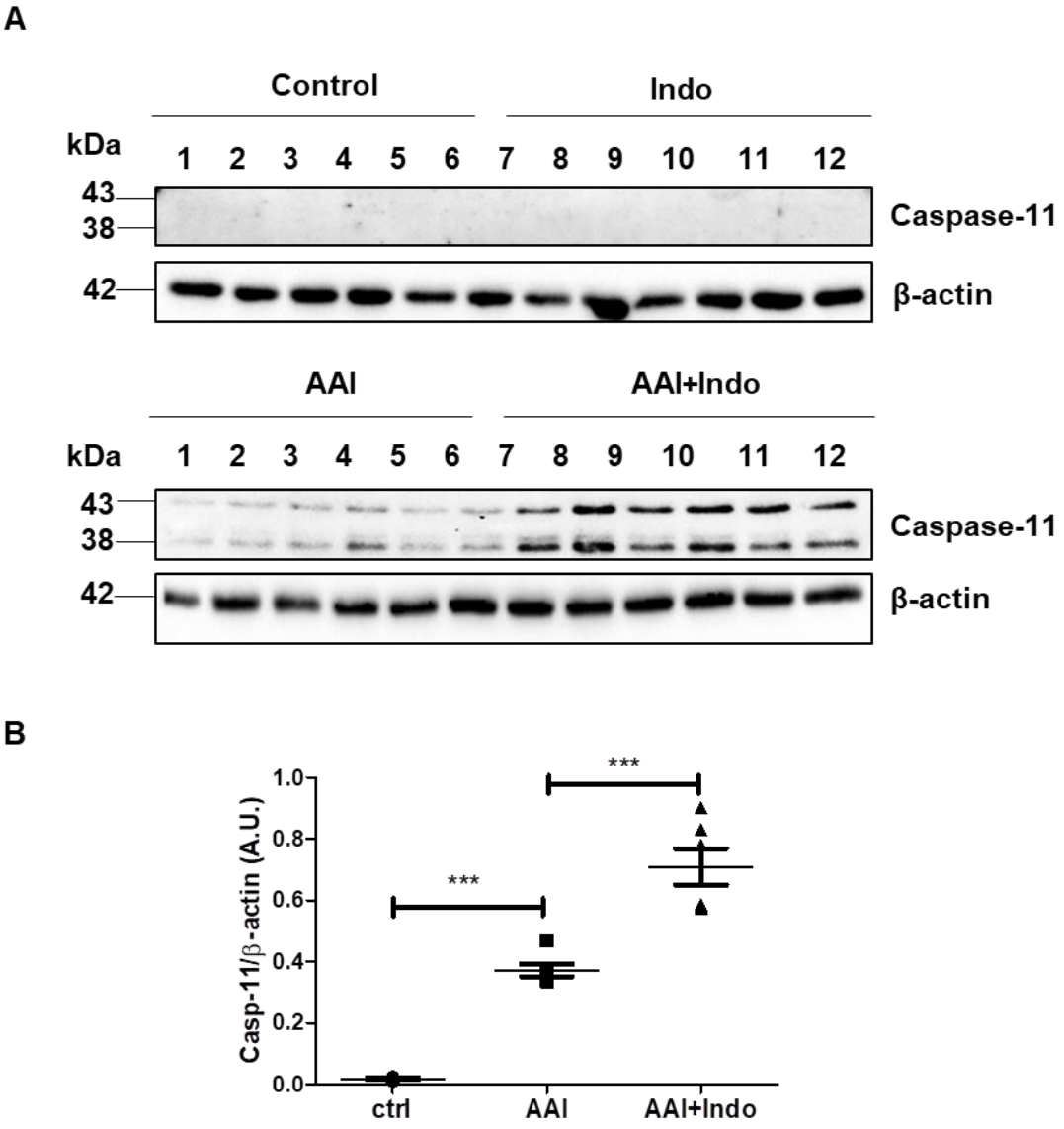


Figure 5.13. Indomethacin increases caspase-11 expression during allergic airway inflammation.

Murine allergic airway inflammation (AAI) was induced by 20 µg OVA emulsified in 200 µl of sterile phosphate buffered saline (PBS) containing 2 mg of aluminium hydroxide, followed 7 days later by two airway challenges with 1% OVA. In indomethacin stimulated AAI mice, 200 µl of PBS containing 2 mg/kg of indomethacin in 0.5% DMSO was given 2 h before sensitization and 2 h before each airway challenge. Control mice received 200 µl of PBS containing 0.5% DMSO alone. Lungs were collected 24 h after the final airway challenge. The whole-lung lobes were homogenised and samples prepared for western blot. A) Protein lysates (20µg) analysed by western blot for caspase-11 (38; 43 kDa) and β-actin (42 kDa) as a loading control. There were six mice in each group, and analysis of samples from each mouse is shown. B) Densitometry analysis of caspase-11 expression relative to actin (A.U refers to Arbitrary Unit). Statistical analysis of caspase-11 was performed using student's t-test to compare mean ± SEM values, ***$p<0.001$.

Summary of the main findings of Chapter 5

- Patient antrum ulcer biopsies have elevated caspase-4 expression levels.
- *H. pylori*-one of the main risk factor for ulcer formation, induces caspase-11 and caspase-1 activation and IL-1β release in murine macrophages.
- PGE_2 inhibits caspase-4/-11 mediated pyroptosis and regulates caspase-1 activity.
- Caspase-11/-4 is upregulated in murine model of asthma
- The PGE_1 analog-misoprostol inhibits, and indomethacin upregulates, caspase-11 expression in murine model of asthma.

5.3. Discussion

In this chapter the importance of caspase-4/-11 in two diseases induced by chronic inflammation, peptic ulcer and asthma disease was analysed.

Results from the first part have shown that Caspase-4 was upregulated in antrum peptic ulcer biopsies. Furthermore, *H.pylori*-infection, which is the main risk factor for peptic ulcer formation, induce caspase-11 expression in murine macrophages. Caspase-4/-11 is the main regulatory component of the non-canonical inflammasome, suggesting the involvement of this inflammasome in peptic ulcer development. In *H.pylori*-infected BMDMs, caspase-1 activation and IL-1β production are regulated by caspase-11.

In the second part, results demonstrate that caspase-11 is upregulated in a mouse model of allergic airway inflammation and NSAID treatment, which is associated with an increase risk of asthma exacerbation, further upregulates caspase-11 expression. The PGE_1 analogue, misoprostol inhibits caspase-11 expression in a mouse model of allergic airway inflammation.

Demonstrated results showed that PGE_2 blocks the LPS-mediated induction of caspase-4/-11 and limits caspase-4-dependent cell death in macrophages. As obtained results revealed that misoprostol inhibits also caspase-11 expression in mouse model of asthma, it therefore suggest that inhibition of caspase-4/-11 and the non-canonical inflammasome may be a therapeutic strategy to prevent peptic ulcer formation and inhibit allergic airway inflammation.

Misoprostol is a well-known medication used to prevent ulcers in patients taking NSAIDs, the main risk factor of ulcer disease. Analysis of human biopsies from corpus and antrum regions of peptic ulcer indicated that caspase-4 is upregulated in antrum peptic ulcer. No difference in caspase-1 was observed between healthy and peptic ulcer biopsies, which can suggest that caspase-4 and possibly the non-canonical inflammasome are involved in peptic ulcer formation. Caspase-4 upregulation was observed only in the antrum region which is the main site of *H. pylori* infection and peptic ulcer development. It was reported in 1959 by Oi *et al*. that gastric ulcers are located close to the mucosal boundary between corpus and antrum, and always developing at the antral side of the mucosal margin [594]. Gastritis is usually more severe in the antrum and only a little or no inflammation is observed in the gastric corpus [254].

Regulation of inflammasome activation provides the correct balance between the production of anti- and pro-inflammatory cytokines [138]. It has been presented by Zhang *et al.* that expression of NLRP3 protein was elevated and caspase-1 cleavage was observed in the mouse model of acute gastric injury. Pre-treatment with caspase-1 inhibitor, AC-YVAD-CMK, protects against acute gastric ulceration by reducing elevated levels of IL-1β and IL-18. Additionally, cold-restraint stress-and ethanol-induced gastric mucosal histopathological changes were alleviated following AC-YVAD-CMK administration [678]. Caspase-4 has previously been linked to decreased adenosine deaminase activity and implicated as a factor including peptic ulcer formation [606].

Caspase-4/-11 activation and function are mainly studied in the context of gram-negative bacterial infection and LPS recognition [167]. *H.pylori* infection along with NSAID use is well-known risk factors in peptic ulcer development. Higashimori *et al.* reported that NSAID-induced intestinal damage and gastric inflammation are mediated by NLRP3 inflammasome, caspase-1 activation and IL-1β production [679]. *H.pylori* causes an inflammatory response through the activation of immune cells in gastric mucosa, leading to epithelial cell degeneration and injury [254]. It has been shown that infection with *H.pylori* induces inflammasome activation and IL-1β production in an NLRP3- and ASC-dependent manner [603]. The mature IL-1β is involved in the recruitment of leukocytes and production inflammatory mediators [680]. Although is not reported yet, this suggests that *H.pylori* induces pyroptosis in damaged and infected gastric mucosa, causing ulceration. The role of the pro-inflammatory cytokine IL-1β has been extensively studied in *H.pylori* infections, and studies reveal that elevated IL-1β production is associated with *H.pylori*-induced gastric pathology. Inhibiting inflammatory cascades which lead to abdominal and systematic inflammation is very important, as untreated peptic ulcers cause complications which can lead to peritonitis and often sepsis. In the current study, evidence show that IL-1β secretion induced by *H.pylori* in murine macrophages depends on inflammasome activation which is regulated by caspase-11. It suggests that *H.pylori* activates the non-canonical inflammasome, causing pyroptosis and IL-1β production. The release of DAMPs and ATP following pyroptosis can subsequently activate the canonical inflammasome in cells of the surrounding environment, further boosting the inflammatory response. NLRP3 can be activated by a broad spectrum of stimuli and is considered to be a sensor of the disruption of host physiology. Semper *et al.* presented that blocking of typical

NLRP3 stimuli, such as potassium efflux, lysosomal destabilization and ROS generation, reduced IL-1β secretion upon *H.pylori* infection [603]. Caspase-11 needs two signals to be activated. First IFN-dependent priming, to upregulate pro-caspase-11 and second, recognition of intracellular LPS derived from bacteria, which leads to non-canonical inflammasome activation [574]. Infection of BMDMs with *H.pylori* alone induces caspase-11-dependent, caspase-1 activation and IL-1β production, suggesting that *H.pylori* provides both signals. However previous reports suggested that tetra-acylated lipid A from *H.pylori* neither stimulate TLR4 nor caspase-11 [681], suggesting that *H.pylori* LPS needs to be accompanied by other virulence factors to activate inflammasome. It has been presented that cag Pathogenicity island is the main virulence factor responsible for inflammasome activation in murine BMDCs and human PBMCs [603]. Another study has shown that LPS *H.pylori* upregulate pro-IL-1β through LPS/TLR4/MyD88 axis while urease B subunit (UreB) acts through TLR2 and is necessary for NLRP3 inflammasome activation and IL-1β processing in BMDCs [682].

PGE_2 is an important contributory factor for tissue homeostasis and was determined as a negative regulator of inflammatory responses activated by DAMPs [683]. Although *H.pylori* inflammation itself does not induce pyroptosis in murine macrophages, results demonstrated that PGE_2 pre-treatment attenuates caspase-11 expression and caspase-11 driven pyroptosis and has the capacity to indirectly inhibit caspase-1 activation by preventing the release of DAMPs in LPS transfected macrophages. Taking into consideration that PGE_2 is able to limit LPS responsiveness through inhibition of TLR4 [684], we speculated that PGE_2 could indirectly inhibit NLRP3 in neighbouring cells. PGE_2 ability to inhibit caspase-11-dependent pyroptosis could explain the cytoprotective effect of PGE_2 in ulcer treatment [685]. These observations suggest that inhibition of caspase-11-mediated pyroptosis could be important for tissue-protective effects.

In addition to inflammasome activation in *H.pylori*-infected macrophages, upregulation of iNOS expression was observed. However, in *H.pylori*-infected BMDMs no nitrite production, the stable metabolite of NO, was detected expression of synthase which is necessary for NO production was upregulated. This could be due to low levels of iNOS stimulation by *H.pylori* and undetectable nitrite levels using the Griess assay technique. iNOS expression is regulated by caspase-11, further supporting a role for caspase-11 in host defence responses to *H.pylori*. Under normal conditions, NO together with PGs play a key role in the maintenance of the gastrointestinal mucosa and both share

similarities in gastrointestinal functions. Constitutive NO maintains the integrity of the gastric epithelium and the mucus barrier [641]. A physiological level of NO regulates vascular tone and neurotransmission and is cytoprotective by interacting with sensory neuropeptide and endogenous prostaglandins [686]. Bacterial pathogens lead to upregulation of iNOS which produce a large amount of NO and may lead to tissue damage and induce pathologic conditions [687] [688]. In the gastrointestinal tract, iNOS is activated in *H.pylori*-induced gastritis, inflammatory bowel disease, and NSAIDs induced ulcerogenesis [644][642][643]. In addition, inhibition of iNOS activity decreases colitis and ileitis in an animal model [689]. There are several studies showing molecular cross-talk between NO and PGs which together may regulate tissue homeostasis or when their expression is disturbed, they can contribute to pathophysiological processes. NO has been demonstrated to modulate COX expression and PG production. However, how NO regulates PG production is not clear. Clancy *et al.* reported that NO activated COX-1 but inhibited COX-2-derived PGE_2 production and indicated that inhibition of PGE_2 synthesis by NO is accompanied by nitration of COX-2 [690]. Considering that overproduction of iNOS [666] and PGE_2 inhibition [691] are involved in peptic ulcer formation,hypothesisthat caspase-11mediates these mechanism and may be an important target for the treatment of gastric inflammation-related diseases was made [692].

To strengthen the hypothesis regarding a protective role for PGE_2 in inflammatory disease, the effect of PGE_2 on caspase-11 expression in the OVA mouse model of allergic airway inflammation, which mimics acute asthma characteristics [693], was analysed. Asthma is a chronic inflammatory disease of the airways characterised by inflammatory cell infiltration, including monocytes which promote early events in allergic lung inflammation [676]. PGE_2 is one of the most abundant COX products synthesised in airway epithelium and smooth muscle and is considered to be an important inhibitory modulator in inflammatory processes of the airway [694][695]. Analysis of murine lung tissue from this model of airway inflammation revealed that caspase-11 upregulation occurs. Blocking PGE_2 with the NSAID indomethacin enhances, while the prostaglandin E_1 analogue misoprostol decreases, caspase-11 expression in the lungs. Further upregulation caspase-11 in indomethacin treated mice, may explain how NSAIDs exacerbate asthma, which is observed in 7% of adults with this disease [660]. Inhibition of the COX pathway, which is induced by NSAIDs, leads to increased leukotriene synthesis and risk of bronchospasm or asthma exacerbation

[696, 697]. We do not know how caspase-11 is upregulated in the allergic airway inflammation model but it has been reported that LPS exacerbates airway inflammation in an animal model [698][699, 700]. The lungs harbour distinct bacteria, including LPS-containing microbes [701] and exposure to LPS plays a major role in asthma development [702]. It is possible that the protective role of PGE_2 in the model of allergic airway inflammation is caused by the inhibition of LPS-induced caspase-11 expression.

Results from above chapter demonstrated that *in vitro* PGE_2 inhibits caspase-4-dependent pyroptosis and through inhibition of DAMPs release, inhibits NLRP3 activation and caspase-1 dependent pyroptosis. Allergen-induced airway inflammation leads to epithelial damage which may enhance susceptibility to allergens and sensitivity of the airways, which subsequently leads to persistent asthma and airway remodelling [703][704][705]. Cell death in lung tissue leads to permanent structural changes because of its fragile structure [706]. Recent studies have shown that epithelial pyroptosis plays a key role in asthma pathogenesis and airway remodelling [707]. Based on these results, a hypothesis that the protective role of PGE_2 might be due to blocking caspase-4/-11 expression was made.

Our findings define the role of caspase-4/11 in peptic ulcer and asthma disease and support inhibition of caspase-4 as a therapeutic strategy in these diseases.

Chapter 6: General discussion and future plans

6.1. General disscussion

In 2000, Hanahan and Weinberg published 'The Hallmarks of Cancer' describing the six hallmark capabilities acquired during multistep development of cancer that enable tumour growth and metastatic dissemination [414]. A decade later, they revisited the hallmarks of cancer review and updated it, by adding two emerging hallmarks, genome instability and mutation, and tumour-promoting inflammation [708][709]. Although inflammation is controlled by several mediators and pathways, most of these are initiated by key sensors, pattern recognition receptors (PRR) which sense danger signals but also participate in tissue damage, bacterial infections and metabolic disorders leading to chronic inflammation [710]. Toll-like receptors are the best characterised PRRs for the detection of pathogen-associated molecular patterns (PAMPs). Several studies have shown that significantly higher expression of TLRs are associated with poor prognosis and increased probability of lymph node metastasis [711]. Oesophageal cancer has been described as an 'exemplar model' of inflammation-related cancer (Picardo et al., 2012). The incidence of OAC has markedly increased in the past decades and BO is considered as the only known precancerous lesion of OAC. OAC develops through the metaplasia-dysplasia-carcinoma sequence and it is believed that normal and inflamed tissue transforms to Barrett's oesophagus (BO) in response to chronic reflux of bile and acid from the stomach and duodenum. The development of BO is strongly related to gastroesophageal reflux disease (GERD) [712]. Although the progression rate from BO to OAC is quite low, there is an increase in cancer rate due to poor diagnostic methods. Only around 10% of oesophageal cancers are identified at early stage (T1) using available diagnostic tools [713]. As BO does not produce any symptoms, besides the one related with GERD, there is a high need to find biomarkers which will help to identify BO and early stage OAC. Finding biomarkers that participate in initiation, development and progression of OAC will help predict the prognosis of OAC, improve available treatments and increase the survival rate.

TLR2 expression is has been reported to increase during the development of Barrett's to high-grade dysplasia and oesophageal adenocarcinoma [330]. Results in Chapter 3 support this observation, showing that TLR2 is expressed in a Barrett's dysplasia cell line and increased in early-stage oesophageal adenocarcinoma cell lines. Our findings suggest that TLR2-related inflammation is most relevant in the early stages of OAC and TLR2 upregulation could be involved in the development of BO and progression to

OAC. Detection of TLR2 in BO patients could provide a novel biomarker for predicting the prognosis of OAC. Recent report by Wang *et al.* presented that TLR2 expression was higher in breast cancer tissue compared to normal and correlated with tumour size, tumour subtype and TNM stage. Patients with high TLR2 expression had a poorer prognosis than patients with low TLR2 expression, indicating elevated TLR2 as a prognostic biomarker [714].

Recently many studies are focusing on the role of the microbiome in OAC pathogenesis [383]. Zaidi *et al.* studied the association between TLR signalling pathways and microbiome changes during OAC progression. His studies revealed that TLR2 was upregulated in the rat model of OAC, which correlates with the prevalence of *E.Coli* in BO and OAC [382]. It has been shown that oesophageal bacteria differ among normal oesophagus, Barrett's oesophagus and OAC, supporting the hypothesis that OAC progression is related to microbiome changes, possibly through the innate immune system [386]. Upregulation of TLR2 would enable enhanced recognition of altered microbiome by pre-cancerous cells thus leading to enhanced inflammation and progression to cancer.

Following TLR2 activation in BO and early-stage OAC cells increased production of chemokine IL-8 was observed. High serum IL-8 levels correlated with poor prognosis in many cancers and promote resistance to treatments, stemness and recruitment of immune suppressive cells to the tumour site. IL-8 mediates the activation and chemotaxis of immune cells, leading to infiltration into tumour site and promoting cancer progression and metastasis [715]. It has been suggested that IL-8 upregulation may occur early during cancer development and has the potential to serve as a biomarker of disease and be a useful tool to screen patients with BO for cancer progression [716]. As it has been shown that inhibition of specific IL-8 receptor, CXCR-2 reduced invasiveness of OAC cells [419], it is likely that targeting of TLR2 in BO represents a novel strategy to prevent early-stage tumour development. TLRs play a key role in activating immune response. The emerging evidence suggests that TLRs are activated by PAMPs and endogenous ligands derived by cancer cells thus leading to activation of immune cells, increased levels of pro-inflammatory cytokines and chemokines and further boosting inflammation.

It has been shown that many endogenous ligands released from tumour cells into their microenvironment can trigger TLR immune responses and increase TLR expression [717]. Since TLR2-activated oesophageal cells produce chemokine IL-8 which

promotes leukocyte trafficking to the tumour microenvironment, they can also induce tumour progression via macrophage activation. Our studies revealed that TLR2 activation of OAC in addition to increased secretion of IL-8, induce translocation of 'alarmin' HMGB1 protein from nucleus to cytoplasm and releasing HMGB1 into cell culture. Since it has been discovered by Wang *et al.* that HMGB1 can be released from cultured macrophages upon cytokine stimulations, the role of HMGB1 in inflammation and tumour-related inflammation has been widely analysed [101]. Under this specific condition, HMGB1 is released from necrotic cells and secreted from inflammatory cells. HMGB1 released by tumour cells can promote TAM differentiation [718]. OAC usually requires multidisciplinary care. At present, chemoradiotherapy is accepted as a standard treatment for locally advanced oesophageal cancer [719]. However, resistance to available treatment might result in treatment failure and cancer relapse. In oesophageal cancer survival rates beyond 5 years are around 20 % or less [720]. He *et al.* presented that HMGB1 is released from irradiated colon and cervical cancer cells and through binding to the RAGE receptor, promotes cancer cell repopulation. A positive correlation between the high expression of HMGB1 and malignant phenotype in CRC patients was observed. As inhibition of HMGB1 inhibits HMGB1-mediated tumour cell proliferation, they suggested HMGB1 as a novel target for cancer treatment [430]. Inhibition of HMGB1 release during chemoradiotherapy could improve the treatment of OAC cancer and increase the sensitivity of cells to treatment.

Approximately 50% of oesophageal cancer patients present with distant lymph nodes at the time of presentation [721]. TAMs are the main promoters of tumour growth and metastases. They induce tumour related-angiogenesis, invasion, and proliferation [722] [401]. Monocytes produced from bone marrow hematopoietic stem cells are infiltrate into the tumour site and polarise to the TAM-like phenotype [723]. A meta-analysis of 16 studies revealed that CD163 M2-like TAM macrophages are a potential risk factor for oesophageal cancer [334]. Moreover, HMGB1 released from oesophageal squamous cell carcinoma triggers the differentiation of monocytes into M2-like TAMs. Polarised macrophages create the perfect condition for oesophageal cancer progression [120]

Our results revealed that OAC conditioned media which contains HMGB1 activates primary murine macrophages and induces the production of cytokines typical for M2-like TAM macrophages. As OAC CM-mediated the upregulation of caspase-11 in murine macrophages, but not caspase-4 in human macrophages, was inhibited by αTLR2, it suggests that factors additional to TLR2 ligands can mediate human

macrophage activation. As differences between human and murine responses to OAC CM was observed, it suggests that more experiments should be performed in human cells to confirm finding observed in murine macrophages. Differences between human and murine primary macrophages could be due to interspecies differences. Previous studies have shown differences in polarising human and murine macrophages. Stimulation with IL-4 induces TAM markers, Arginase-1 and Ym1 expression in murine macrophages but not in human macrophages [724]. For human studies, blood circulated monocytes were used whereas for murine studies cells were obtained from bone marrow, which could be another reason for observed differences between species.

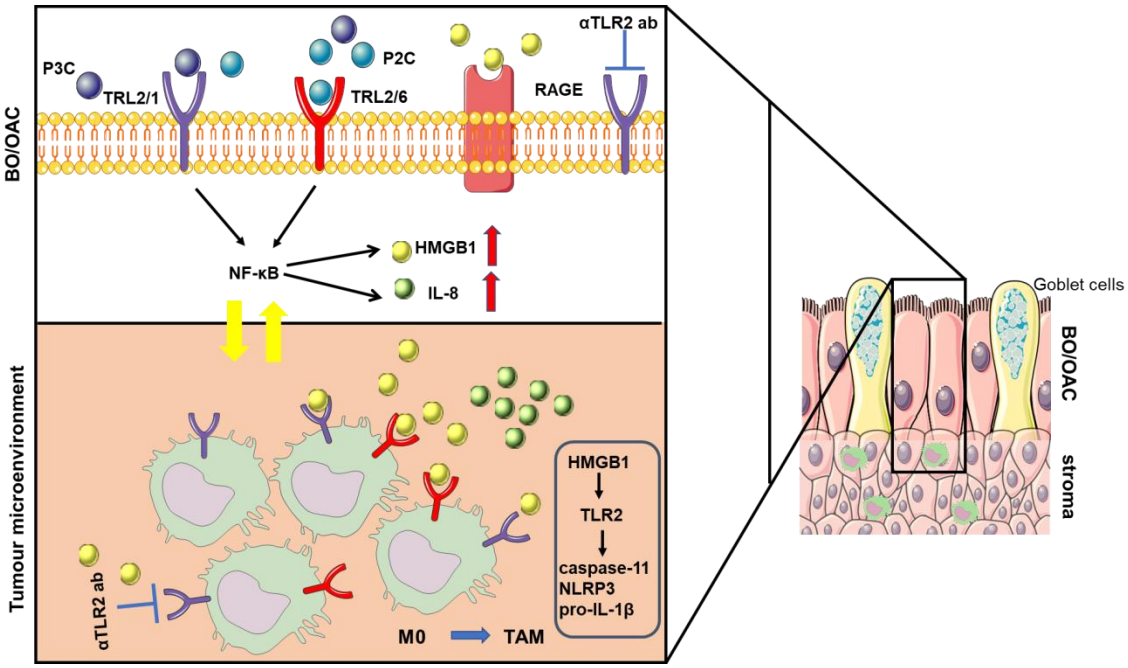


Figure 6.1. Schematic representation of the main findings of Chapter 3 study.

Upregulation of TLR2 in early-stage OAC cells leads to increased production of chemokine IL-8 and release of HMGB1. IL-8 attracts macrophages to the tumour site and in tumour microenvironment macrophages are polarised into M2-like TAM macrophages. HMGB1 released from OAC cells causes the TLR2-dependent upregulation of inflammasome-related proteins in macrophages. Treatment of OAC cells with αTLR2 neutralising antibody decreases IL-8 production and HMGB1 upregulation and supports targeting TLR2 as a potential therapeutic agent for the treatment early-stage of OAC patients.

In addition to the important role of the immune system in cancer, innate immunity is the primary host defense against invading microorganisms. Here the role of caspase-11 during *M.tuberculosis* and *H.pylori* infection was analysed.

Inflammatory caspases function as central adapters of innate immunity which through regulation of the assembly of multiprotein complexes, called inflammasomes, control host defense against pathogens [123]. Caspase-11 and its human orthologs caspase-4 and caspase-5 expression and function are studied in the context of bacterial infection and LPS recognition [503]. Many studies have shown that caspase-11-mediated inflammatory response plays an important role in gram-negative bacterial infections including, *Escherichia Coli, Salmonella typhimurium, Legionella pneumophila, Shigella flexneri, Citrobacter rodentium, Burkholderia* spp [166][725][142][575][726][727]. Our results obtained in Chapter 4 have shown that caspase-11 is induced upon *Mtb* infection in primary murine macrophages and leads to caspase-11 mediated iNOS induction and nitric oxide production. *Mtb* is a causative agent of Tuberculosis (TB), the disease which, despite widely used vaccines and many available treatment methods, is still a major cause of mortality [728]. Caspase-11 deficient BMDMs are not able to restrict pathogen invasion and proliferation, resulting in a significantly higher number of bacilli contained in the caspase-11 deficient, compared to wild-type, cells. Proposed work demonstrated an additional mechanism by which caspase-11 promotes anti-mycobacterial responses, through the regulation of nitric oxide production.

To date, many studies have reported a key role for nitric oxide in *Mtb*-infected murine macrophages [490][489]. The role of nitric oxide production in human macrophages is still controversial. The first reports showed that human macrophages failed to secrete nitric oxide following stimulation with endotoxin, IFNγ, TNFα, GM-CSF and *L. monocytogenes* [729]. However, peripheral blood monocyte-derived macrophages obtained from healthy donors usually do not express iNOS. However, many studies have reported that macrophages obtained from patients with inflammation or other inflammatory conditions have upregulated iNOS [729]. NO is a key molecule involved in many biological functions [730]. In addition to TB, both excessive and lack of nitric oxide production participates in many other diseases, including neurodegenerative diseases [731], ischemic brain injury [732], sepsis and other infectious diseases [733]. Presented experiments showed that caspase-11 regulates nitric oxide production in *E.coli* LPS- and *Mtb*-stimulated murine macrophages. As *E.coli* LPS represents typical gram-negative bacteria and *Mtb* has the chemical characteristics of either gram-negative

or gram-positive bacteria, it suggests that caspase-11-mediated nitric oxide production might be relevant in a wide range of diseases induced by both gram-positive and-negative bacteria and targeting caspase-11/-4 in diseases caused by high nitric oxide production could be a promising treatment. The limitation to current knowledge on nitric oxide inhibition is that most of the studies were performed in animal models. More studies are needed to understand the specifics correlation between caspase-4 and nitric oxide.

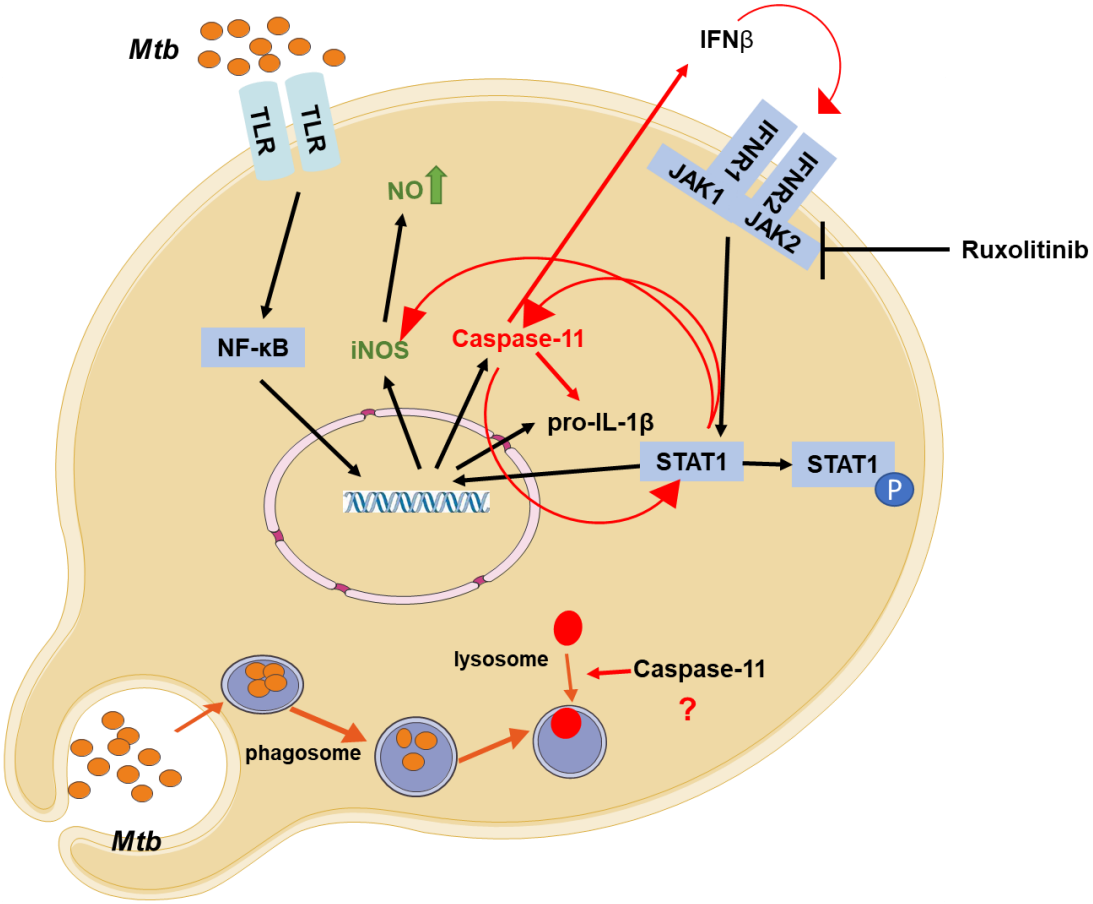


Figure 6.2. Schematic representation of the main findings of Chapter 4 study.

Surface TLRs recognise *Mtb* infection and induce caspase-11 upregulation. Caspase-11 through IFNAR/JAK/STAT1 signalling pathway leads to iNOS upregulation and NO production. Inhibition of STAT1 phosphorylation inhibits caspase-11 expression, suggesting positive feedback loop between caspase-11 and STAT1. Caspase-11 mediates pro-IL-1β expression. Blocking JAK/STAT1 signalling with Ruxolitinib leads to downregulation of caspase-11, pro-IL-1β and iNOS. Caspase-11 through actin polymerization could be involved in phagosome-lysosome fusion thus help in bacteria emilination. More studies are required to analyse this role.

In Chapter 5 the role of caspase-11 during *H.pylori* infection was analysed. *H.pylori* is a gram-negative gastric bacterium present in over half of the world's population during their lifetime. *H.pylori* is able to evade innate immune responses *in vivo* and is the main risk factor for the development of gastritis, peptic ulcer disease and gastric cancer [734]. Analysis of human peptic ulcer biopsies revealed that caspase-4 is upregulated in gastric tissue, suggesting the involvement of the non-canonical inflammasome and pyroptosis in ulcer formation. Here evidence demonstrated that caspase-4 upregulation can be induced by a combination of two factors: exposure to LPS from *H.pylori*; and NSAIDs, which block the production of Prostaglandin E_2 (PGE2), an endogenous negative regulator of caspase-4/-11 [677]. It has been shown that *H.pylori* infection leads to caspase-1 upregulation, IL-1β secretion and pyroptosis in gastric epithelial cells [735]. Although pyroptosis was not detected in *H.pylori* stimulated murine macrophages, it is likely that *H.pylori* activates pyroptosis in ulcers of the damaged and infected gastric mucosa. Pyroptosis results in the release of intracellular inflammatory contents which induce additional inflammatory signalling pathway, boost existing inflammation, subsequently lead to tissue damage and inflammatory diseases [736][737][738]. It has been shown that pyroptosis is critical in neurodegenerative diseases, which are mainly caused by mass mortality of neurons. Mass mortality of endothelial cells undergoing pyroptosis reduces vascular endothelial functions leading to cardiovascular diseases [739]. Excessive inflammasome activation leads to various diseases, including diabetes, atherosclerosis and metabolic syndrome. By far, most of the studies analysing the role of inflammasome in diseases are focused on inhibiting NLRP3 inflammasome components. In recent years several inhibitors of NLRP3 inflammasome pathway have been reported. Inhibition of NLRP3 inflammasome has been obtained by targeting the NLRP3 ATPase domain, inhibiting ATP-sensitive K^+ channels or blocking caspase-1 cleavage and IL-β maturation [740]. Our results revealed that caspase-11 regulates caspase-1 activation and IL-1β production in *H.pylori* infected murine macrophages. This suggests that instead of blocking NLRP3 inflammasome and its downstream signals, inhibition of caspase-11 could be a promising treatment target in inflammatory diseases. The role of caspase-11 beyond gram-negative infection has not been extensively explored and very little is known about the negative regulation of caspase-11. A recent report presented by Zaslona *et al.* showed for the first time that

upregulation of caspase-11 could be involved in asthma disease and targeting noncanonical inflammasome represents a promising treatment method in aspirin-induced asthma. Our data present that PGE_2 attenuates caspase-4 driven pyroptosis and has the capacity to indirectly inhibit caspase-1-dependent pyroptosis, preventing the release of DAMPs following non-canonical activation. As PGE_2 has strong cytoprotective effects and a well known role in maintaining tissue homeostasis, it supports our hypothesis that inhibition of caspase-11 could block inflammasome-induced pyroptosis and protect host tissue from pyroptosis-mediated damage.

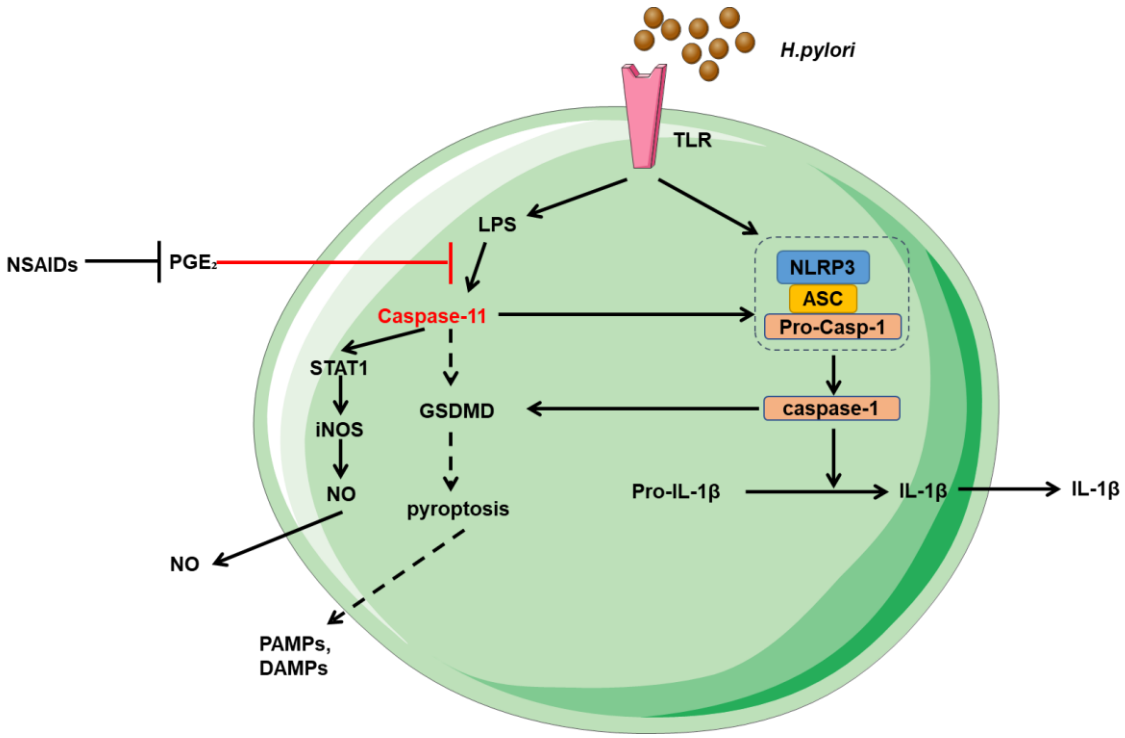


Figure 6.3. Schematic representation of the main findings of Chapter 5 study.

Intracellular LPS from *H.pylori* leads to caspase-11 activation which mediates caspase-1 activation and IL-1β production. *H.pylori* infection induces caspase-11-regulated iNOS expression. Pyroptosis is likely activated by caspase-11 and participate in damage of human gastric tissue. PGE_2 can inhibit caspase-11 transcription and caspase-11 activation by LPS and limits pyroptosis.

6.2. Future directions

Findings from the first chapter revealed that TLR2 expression is highest in Barrett's oesophagus and early-stage OAC cell lines. Activation of TLR2 leads to the release of HMGB1 from OAC cells which potentially could activate macrophages, differentiate them into M2/TAM-like macrophages and promote tumour progression. We suggest inhibition of TLR2 with a neutralising antibody as a potential treatment in Barrett's oesophagus and early-stage OAC patients. It would be interesting to further investigate the role of HMGB1 in OAC progression. As obtained results show that TLR2 signalling may be most relevant during BO and early-stage OAC, the next step would be to check expression of HMGB1 in the panel of OAC cell lines representing different stages of disease progression. As it has been shown that stress-induced released of HMGB1 in the extracellular matrix can regulate metastasis [741], angiogenesis [742] and immune response [743] the next step would be to analyse the influence of HMGB1 on OAC cells proliferation, migration and invasion using the appropriate cell assays. The selective knockout of HMGB1 and addition of exogenous HMGB1 to cells in culture could provide insights into the effect of this protein in BO and OAC cells. Results indicated that conditioned media collected from TLR2-activated OAC cells polarize murine macrophages towards an M2/TAM-like phenotype. M2 macrophages are further subdivided into M2a, M2b, M2c, and M2d types based on their cytokine expression profile. More specific analysis is needed to characterise the precise type of M2-like TAM like macrophages being induced by secreted factors from OAC. The next step, to determine the TAM subtype being induced, would be to screen the expression of all typical TAM markers.

As evidence determined that upregulation of inflammasome-related proteins and production of cytokines in OAC CM-treated primary murine macrophages is TLR2-dependent, future experiments should determine whether OAC CM induced expression of M2-like TAM markers is also mediated by TLR2, and whether inhibition of TLR2 with a neutralising antibody would block macrophage polarisation into TAM phenotype. Treatment of macrophages with αTLR2 ab blocks upregulation of caspase-11 by OAC CM but not caspase-4, suggesting differential responses between human and murine macrophages. More experiments should be done in human macrophages to analyse whether our findings can be transferred to human models.

Since TLR2 signalling in BO and OAC cells increased the production of chemokine IL-8 and HMGB1, whose expression in cancers correlate with their metastatic potential, the next step would be to analyse the influence of TLR2 expression on OAC progression in a mouse model. Micheal Quante's Lab (TUM, Germany) generated a transgenic BO and OAC mouse model which expressed an Epstein-Barr virus (EBV) ED-L2 IL-1β transgene that specifically targets stratified epithelium to express IL-1β, including that of the oesophageal and squamous forestomach mucosa. These pL2-IL-1β transgenic mice progress to BO in 12 months and subsequently to adenocarcinoma in the following months [744]. Other ongoing research in our lab revealed that in Barrett's organoids taken from pL2-IL-1β mouse model, TLR2 activity mediates secretion of IL-8 orthologs, MIP-2 and CXCL-1. Having collaborative access to this BO and OAC mouse model, it would be worth analysing expression of TLR2 and HMGB1 at different stages of disease using immunocytochemistry staining.

In Chapter 4 results demonstrated that caspase-11 regulates nitric oxide production and attenuated *Mtb* invasion in murine macrophages. As clearance of mycobacterium in caspase-11 deficient macrophages is delayed it suggests that that caspase-11 is necessary for the bactericidal effects of nitric oxide. Recent studies, published by Akhter *et al.* presented that caspase-11 is not essential for caspase-1 activation in response to bacterial infection, but is responsible for the fusion of *L. pneumophila* bacteria-containing phagosomes and lysosomes, thus leading to bacterial elimination. Caspase-11 promotes that fusion event through modulating actin polymerization [533]. As similar processes could be observed in *Mtb*-infected murine macrophages, in the next step it would be worth using *Mtb* bacteria-containing phagosomes acquiring lysotracker red, a dye that traffic to acidic vacuoles and analyse differences in fusion to lysosomes between WT and Caspase-11 deficient macrophages. To exclude the protective role of NLRP3 inflammasome, *Mtb* infection using caspase-1 deficient BMDMs or inhibition caspase-1 with specific inhibitor and then CFU counting in these cells should be perform. After obtaining successful *in vitro* results the next step should be analysing the role of caspase-11 in *Mtb* infection using a mouse model. As caspase-11 is necessary to protect murine macrophages from invading *Mtb*, higher bacteria burdens in lungs and spleen of infected caspase-11 deficient mice should be observed.

In the last Chapter 5, results showed that caspase-4 is upregulated in human peptic ulcer biopsies suggesting that non-canonical inflammasome and pyroptosis might play an

important role in peptic ulcer formation. As caspase-4 expression was determined in a small cohort of patients (5 controls and 5 peptic ulcer biopsies), further exploration of this association with a larger sample size is needed. *H.pylori* upregulates caspase-11 in macrophages and the NSAID, indomethacin, upregulates caspase-11 in murine allergic airway inflammation [177]. *H.pylori* infection and the use of NSAIDs are two known risk factors for the development of peptic ulcers [745]. During analysing caspase-4 expression in peptic ulcer biopsies it should be taken into consideration if patients had *H.pylori* infection or regularly used NSAIDs. Previous study suggested that tetra-acylated lipid A from *H.pylori* neither stimulates TLR4 nor caspase-11 [681]. As differences could be due to different *H.pylori* strains, it would be worth extracting LPS *H.pylori* and repeating experiments to determine if LPS or other virulence factors such as cag pathogenicity island [603] are responsible for inflammasome activation. Following that, the effect of *H.pylori* infection on caspase-4 and caspase-1 expression in *H.pylori* LPS-infected human monocytic cells should be established.

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