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# « The Metastatic Intestinal Epithelial cell line HT-29 secretes the chemokines CXCL10 and CXCL11 in a Toll-Like Receptor 3 dependent manner in response to double-stranded RNA »

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**Abstract**

Toll-like receptors (TLRs) are essential sensors of molecules associated with infection, danger, and stress by host innate immunity. This study investigated the properties of Toll-like receptor 3 (TLR3) in relation to colon cancer. TLR3 senses pathogen derived double stranded RNA (dsRNA), endogenous mRNA and the synthetic dsRNA analogue Poly I:C. The metastatic colon cancer cell line HT-29 was used to observe signalling outcomes and effects of receptor activation in response to dsRNA. Initially, a cytokine profiler kit was used in order to determine cytokine secretion in Poly I:C stimulated HT-29 cells. Stimulated HT-29 showed a potent induction of the chemokines CXCL10, CXCL11 and CCL20 secretion in this assay. CXCL10 and CXCL11 secretion in HT29 cells upon Poly I:C stimulation was found to be dependent on TLR3, and its adapter molecule TRIF, by silencing the expression of these proteins by siRNA. TLR3 typically induce potent IFNβ responses in most cells. Interestingly, HT-29 failed to induce IFNβ in response to added Poly I:C, although transfected Poly I:C induced a potent IFNβ response. TLR3 normally resides is the ER, and is dependent on the protein UNC93b1 for trafficking to the endosomes. It has also been reported to be trafficked to the cell surface in an UNC93b1 dependent manner. Knockdown of UNC93b1 with siRNA failed to affect CXCL10 and CXCL11 cytokine secretion, indicating that CXCL10 and CXCL11 secretion is not UNC93b1 dependent. Poly I:C has been reported to induce apoptosis in a TLR3 dependent manner in several cancer cell lines, but the viability of HT-29 cells was not affected even at concentrations of Poly I:C. Combined, these results provide insight into TLR3 expression and signalling in the metastatic intestinal epithelial cell line HT-29. Taken together, these results imply a possible role for TLR3 in cancer progression.

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## Abbreviations

| | |
|---|---|
| AP-1: | Activator protein 1 |
| CCL: | C-C ligand |
| CCR: | C-C receptor |
| CLRs: | C-type lectin receptors |
| CRC: | Colorectal cancer |
| CREB: | Cyclic AMP transcription binding protein |
| CXCL: | C-X-C ligand |
| CXCR: | C-X-C receptor |
| dsRNA: | double-stranded ribonucleic acid |
| DAMP: | Damage/danger associated molecular pattern |
| HMGB1: | High mobility group box 1 |
| IEC: | Intestinal epithelial cells |
| IFNs: | Interferons |
| IKK: | IκB kinase |
| IRAK: | IL-1 receptor associated kinase |
| IRF: | Interferon regulatory factor |
| LLR: | Leucine-rich-repeats |
| MAL: | Myeloid adapter protein |
| MyD88: | Myeloid differentiation primary response gene 88 |
| NEMO: | NFκB essential modulator |
| NFκB: | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NLRs: | NOD-like receptors |
| PAMP: | Pathogen associated molecular pattern |
| Poly I:C: | Polyinosinic:polycytidylic acid |
| PRRs: | Pattern recognition receptor |
| RHIM: | Receptor interactive protein homotypic interactive motif |
| RIP-1: | Receptor interacting protein 1 |
| RLRs: | Retinoic acid inducible gene RIG-I like receptor |
| RT: | Room temperatur |
| siRNA: | short/small interfering ribonucleic acid |
| ssRNA: | single-stranded ribonucleic acid |
| TAB: | TAK binding protein |
| TAK1: | TGF-β activated kinase 1 |
| TBK1: | Tank binding kinase 1 |
| TIR: | Toll/IL-1 receptor |
| TLRs: | Toll-like receptors |
| TRAM: | TRIF-related adapter molecule |
| TRIF: | TIR domain containing adapter protein inducing IFNβ |
| qRT-PCR: | quantitative real time polymerase chain reaction |

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# 1 Introduction

The immune system is the human body's defence mechanism against invading pathogens and altered self cells. It is a complex system that has developed several mechanisms to best protect the host. The immune system is divided into two branches: innate and adaptive immunity. Innate immunity is the body's first line of defence against pathogens. It provides a fast and somewhat nonspecific response in order to protect the host. Both humoral and cellular components participate in the response in order to clear pathogens. Pattern recognition receptors (PRRs) are cornerstones in innate immunity, and are pivotal for protection. Adaptive immunity is a later and more tailored response against pathogens, which relies on specialized immune cells. It provides a potent and long lasting defence through immunological memory[1].

## 1.1 Signalling Pattern Recognition Receptors (PRRs)

Pattern Recognition Receptors are critical components of the immune system. PRRs are constitutively expressed in a variety of cells, including immune cells such as macrophages, neutrophils and dendritic cells. Furthermore, they are also found in nonprofessional cells such as epithelial and endothelial cells. These receptors are responsible for recognising pathogen associated molecular patterns (PAMPs), molecules derived from pathogens, and are part of the immune system's first line of defence against pathogens. In addition, they are able to detect damage/danger associated molecular patterns (DAMPs), which are endogenous molecules the cells themselves express in reaction to stress or damage. The PRRs initiate signal transduction pathways upon recognition of a PAMP or DAMP, resulting in mechanisms that clear infection or act in response to endogenous danger, such as production of inflammatory cytokines or type 1 interferons. The nature of the response is dependant of the origin and localization of the PAMP/DAMP. The nature of the PRR is also essential to the response achieved. PRRs can be divided into three classes: secreted PRRs, endocytic PRRs and signalling PRRs. Secreted PRRs participate in the immune response by opsonising pathogens for phagocytosis or lysis. Endocytic PRRs encourage engulfment and lysosomal degradation of pathogens. Signalling PRRs are responsible for signal

transduction upon recognition of a PAMP/DAMP, and induce transcription of immune response genes [2].

The signalling PRRs can be divided into Retinoic acid inducible gene (RIG)-I like receptors (RLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). RLRs and NLRs are both cytoplasmic proteins, whereas CLRs and TLRs are transmembrane proteins[3]. **RLRs** are cytoplasmic receptors able to sense single stranded RNA (ssRNA) and double stranded RNA (dsRNA) associated with viruses, and induce type 1 interferon production[4]. **NLRs** partake in several processed upon activation, such as peptidoglycan recognition and inflammasome formation[5]. **CLRs** are transmembrane proteins capable of recognising carbohydrates of pathogens, and can induce production of pro-inflammatory cytokines[3]. **TLRs** are membrane bound proteins able to recognize PAMPs both on the cell surface and in endosomes[5].

## 1.2 Toll-Like receptors (TLRs)

Toll-like receptors are transmembrane proteins, key in innate immune responses. They are specialised proteins, able to recognize conserved features of microbial components. Upon pathogen recognition they mediate inflammatory responses, by inducing production of chemokines, cytokines and co-stimulatory molecules. The secretion of cytokines as a result of TLR activation bridges the innate and adaptive immunity as the secretions of these factors participate in initiating adaptive immune responses. The TLRs are single transmembrane proteins comprised of an exterior N-terminal leucine rich repeat (LLR) domain, followed by a transmembrane region and a cytoplasmic Toll/IL-1 receptor (TIR) domain. The exterior domain is responsible for ligand recognition and binding, whereas the cytoplasmic TIR domain is responsible for initiating signalling pathways through adapter molecule recruitment[2]. TLRs form homodimers or heterodimers upon ligand recognition, and it is the proximity of the TIR domains in the dimer that results in signalling. When binding a ligand the two TLRs are pulled together and their TIR domains are close enough to signal. Ten functional TLRs are found in humans, TLR1-TLR10, which are able to recognize a wide range of microbial derived molecules. Ligand recognition and accessibility is dependent on the cellular localization of the receptors[6].

The TLRs can roughly be divided into two groups; TLRs expressed on the cell surface and TLRs expressed in intracellular vesicles. TLR1, 2, 4, 5, 6, and possibly 10 are found on the cell surface, whilst TLR3, 7, 8, and 9 are found in intracellular compartments. Cell surface TLRs are mainly able to recognise PAMPs derived from microbial membranes, such as lipids and lipoproteins. TLRs localized in intracellular vesicles are able to recognize microbial derived nucleic acids. **See Figure 1.1.**

### 1.2.1 Cell surface TLRs and their ligands

**TLR2** is able to recognise a variety of PAMPs, including lipopeptides from bacteria, zymosan from fungi and lipoarabinomannam from mycobacteria. It can form heterodimers with **TLR1**, and the dimers are able to detect triactylated lipoproteins, lipopolysaccharides and peptidoglycan. **TLR2** is also able to dimerize with **TLR6**, and together they recognize diacylated lipoproteins. CD36 can act as a co-receptors for the TLR2/6 heterodimer and aid the recognition of PAMPs[7]. The immune response initiated by both TLR2/1 and TLR2/6 results mainly in the production of inflammatory cytokines[2].

**TLR4** forms homodimers and effectively recognises lipopolysaccharides (LPS) from gram-negative bacteria. Two accessory proteins, CD14 and LPS binding protein (LBP), extract LPS form the bacterial wall and transfer it to TLR4 and MD2. LPB binds LPS, transfers it to CD14 that in turn forms a complex with TLR4 and MD2. The accessory molecule MD2 is necessary for ligand binding. In addition to cell surface signalling, TLR4 is able to translocate to the endosomes and initiate signalling[8]. Induction of TLR4 signalling results in the secretion of inflammatory cytokines and type 1 IFN production.

**TLR5** is responsible for recognizing flagellin, the main component of bacterial flagellas. Several microbial pathogens use flagellas as their main motility apparatus, thereby making TLR5 an important tool in host defence. TLR5 acts as a homodimer, and recognizes a domain of flagellin conserved across several species. Signalling through TLR5 mainly induces the production of inflammatory cytokines [9].

Information regarding **TLR10** is lacking, as it has no mouse homologue. The ligand of the receptors is still unknown, however it is speculated to form homodimers and heterodimers with TLR1 and TLR2[10]. To current knowledge it lacks downstream signalling, and can potentially act in inhibitory ways[11].

### 1.2.2 Nucleic acid sensing TLRs and their ligands

The nucleic acid sensing TLRs are localized in intracellular compartments, such as endosomes and endolysosomes, and recognize microbial derived nucleic acids upon endocytosis. All form homodimers. TLR3 and TLR9 recognise dsRNA and DNA, respectively. TLR7 and TLR8 both recognize ssRNA. These TLRs are all able induce type 1 interferons (IFNs) in addition to inflammatory cytokines, when activated.

**TLR3** recognizes genomic RNA from retroviruses, dsRNA produced during the replication stage in ssRNA viruses and small interfering RNAs (siRNA). The receptor mounts a strong antiviral immune response by potently inducing type 1 INFs and inflammatory cytokines[12]. **TLR7** and **TLR8** both able recognize ssRNA, short dsRNA and bacterial RNA[4, 13]. **TLR9** is capable of recognizing both viral and bacterial DNA. Both bacterical and viral DNA has a high content of un-methylated CpG dinucleotides in a motif referred to as CpG-DNA, which distinguishes it from mammalian DNA that is highly methylated[14]. TLR9 recognizes this CpG motif and is able to mount an immune response upon stimulation[15].

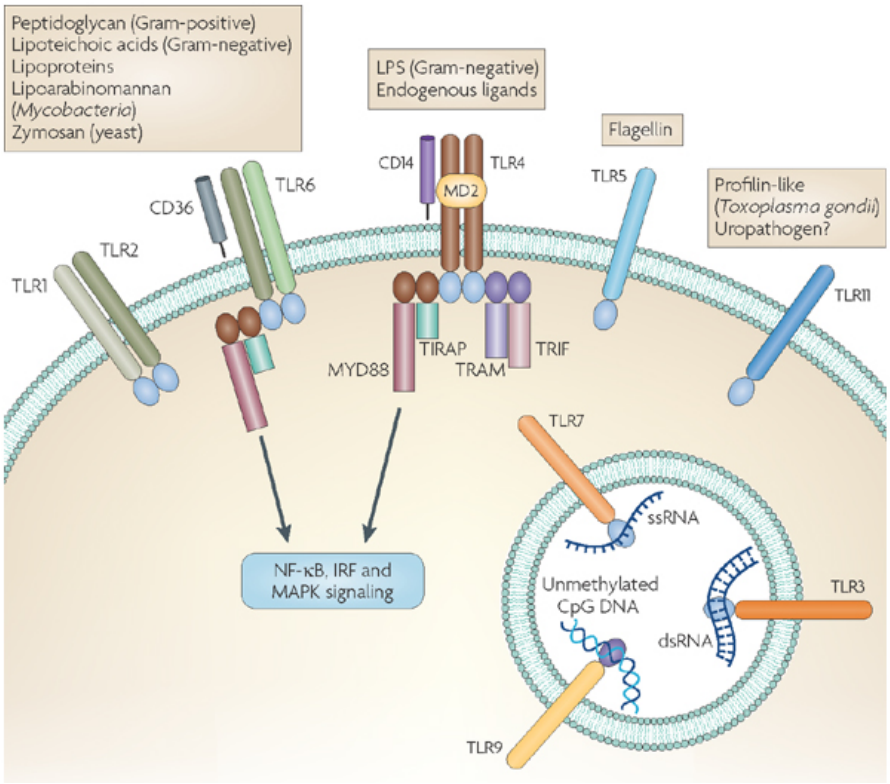



**Figure 1-1: Overview of TLR localization and ligand specificity.** Adapted from [16].

### 1.2.3 TLR signalling pathways

TLR signalling is the result of interaction between the TIR domains of two TLRs, either as homodimers or heterodimers. Upon ligand recognition, conformational change occurs and the TIR domains are pulled in close proximity, allowing signal transduction to transpire. TIR-domain-containing adapter molecules are recruited to the TIR domains of the activated TLR dimer. There are four TIR adapter molecules; Myeloid differentiation primary response gene 88 (MyD88), myeloid adapter-like protein (MAL/TIRAP), TIR-domain containing adapter protein inducing IFNβ (TRIF/TICAM), and TRIF-related adapter molecule (TRAM). MAL and TRAM acts as bridging molecules for MyD88 and TRIF, respectively. All TLRs are able to utilize MyD88 as an adapter molecule except TLR3, which signals trough TRIF exclusively[17]. TLR4 is unique as it is able to utilize both MyD88 and TRIF. Consequently, TLR signalling can be divided into MyD88 dependent-, and TRIF dependent signalling pathways[18].

#### *1.2.3.1 MyD88 dependent signalling*

The MyD88 dependent signalling pathways is initiated when the two TIR domains of a TLR undergoes conformational change upon activation of the TLR. MyD88 binds to the cytoplasmic TIR domain of the dimer via its own TIR domain. Consequently it recruits members of the IL-1 receptor associated kinase family (IRAK), namely IRAK4 through its death domain[3]. MyD88 is able to accomplish this without other adapter molecules in TLR5, TLR7/8, and TLR9, whilst TLR2/1, TLR2/6 and TLR4 is dependent on the adapter molecule MAL in addition to MyD88 to accomplish the same signal transduction[19, 20]. IRAK4, a serine/threonine kinase, is then able to recruit IRAK1 and IRAK2 through its own death domain[21]. The IRAK complex then disassociates from MyD88 and interacts with TNFR associated factor 6 (TRAF6). TRAF6 is an E3 ubiquitin ligase able to ubiquitinate itself and its targets. TRAF6 is then capable of interacting with downstream proteins TAK1 binding protein 2 and 3 (TAB2, TAB3), which together are capable of activating TGF-β activated kinase 1 (TAK1)[22, 23]. This complex can then activate several downstream signalling pathways **(Figure1.2)**. TAK1 can phosphorylate and activate two MAPK pathways, MKK3/6 and MKK4/7, which in turn lead to activation of p38 and JNK respectively. They successively activate transcription factors Cyclic AMP transcription binding protein (CREB) and Activator protein 1 (AP-1)[18]. TAK1 is also capable of phosphorylating and activating the IκB kinase (IKK) complex, which consists of IKKα, IKKβ and the scaffold protein NF-κB essential modulator (NEMO). The IKK complex phosphorylates IκBa, an NF-κB inhibitory molecule, and this leads to subsequent degradation. Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) is then free to translocate to the nucleus. The transcription of CREB, AP-1 and NF-κB leads to production of inflammatory cytokines.

The MyD88 dependent pathway is able to induce the production of type 1 IFNs in addition to inflammatory cytokines. This is possible when signalling through TLR7, TLR8 and TLR9 occurs. Activation of these receptors induce recruitment of MyD88, which in turn recruits IRAK1, IRAK2, IRAK4, TRAF3, TRAF6 and IKKα. This enables phosphorylation of IRF7, an interferon regulatory factor (IRF) family member. IRF7 is then free to translocate to the nucleus and transcription of type 1 IFN can begin[24].

### *1.2.3.2 TRIF dependent signalling*

The TRIF dependent signalling pathway is utilised by TLR3 and endocytosed TLR4. TLR3 is able to recruit TRIF directly upon ligand recognition, whereas TLR4 need a second adapter molecule, TRAM, in order to recruit TRIF[25]. TRIF then recruits TRAF3, which in turn is able to activate Tank binding kinase 1 (TBK1) and IKK-ε [26]. TBK1 and IKK-ε phosphorylates IRF3 or IRF7, enabling translocation to the nucleus that results in the production of IFN-β[27]. TRIF is also able to activate receptor-interacting protein-1 (RIP1), through its receptor interactive protein homotypic interactive motif (RHIM), which subsequently activates TRAF6 and allows for activation of NF-κB[28]. Translocation of CREB and AP-1 occurs trough this pathway, leading to the production of inflammatory cytokines[18]. The different downstream pathways are depicted in **Figure 1.2**.

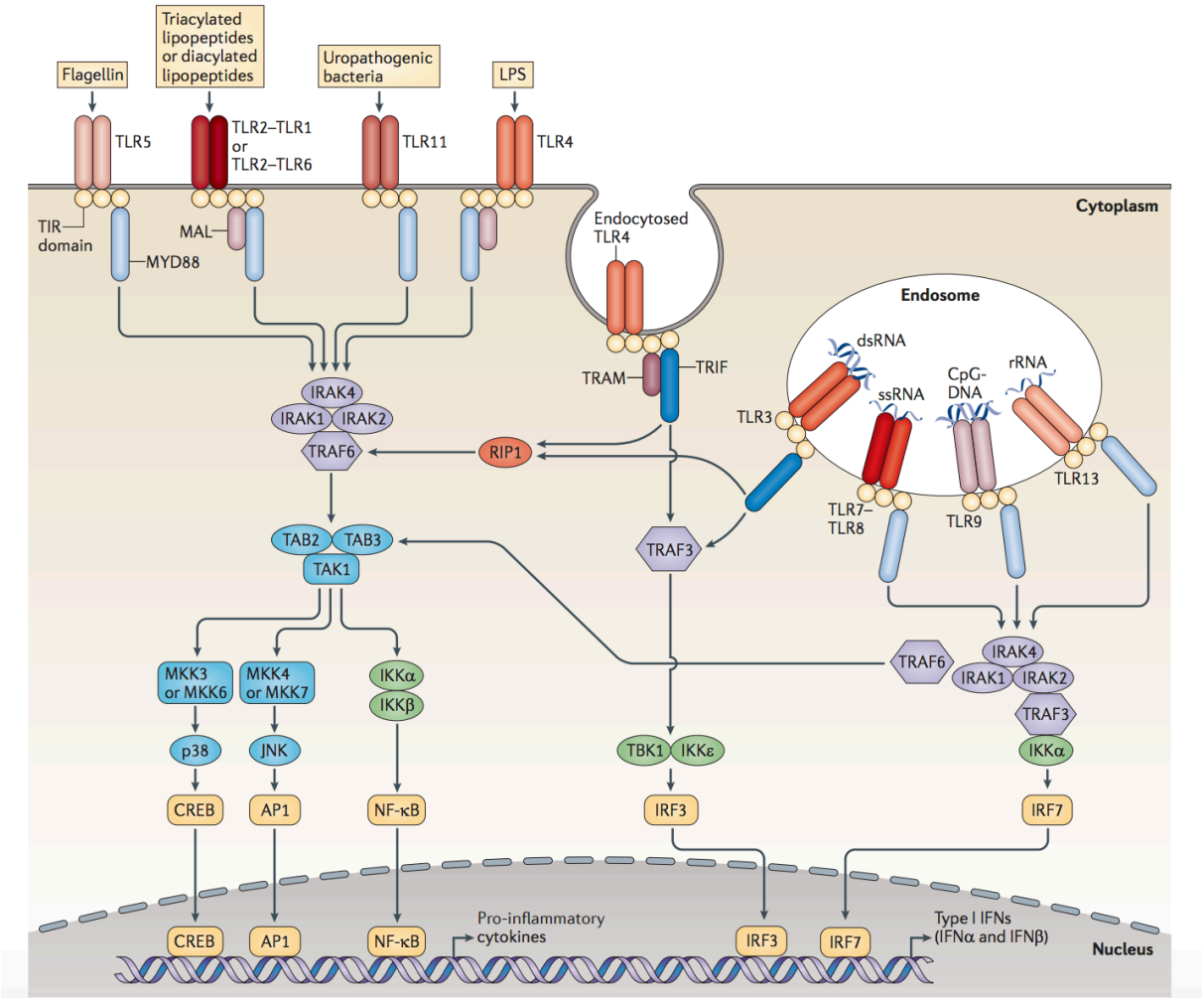



**Figure 1-2: TLR signalling pathways.** TLRs with their respective ligands and signalling pathways. TLR11 and TLR13 are murine TLR receptors not functionally present in humans. Figure adapted from [18].

### 1.2.4 Endogenous TLR ligands

In addition to initiating immune responses as part of the host defence against pathogens the TLRs are able to recognise a range of endogenous ligands collectively called danger associated molecular patterns (DAMPs). Being able to initiate an immune response as a result of endogenous danger signals further strengthens the efficiency of the host defence, as the activation of TLRs through endogenous ligands promotes tissue repair and damage control upon injury. Endogenous ligands are molecules derived from host cells or tissues, and can consist of cellular components or gene products[29]. The localization of the TLRs is of such nature that the host in a normal state cannot mount inappropriate immune responses upon DAMP recognition; hence the TLRs and the endogenous ligands are normally unable to interact. This situation changes drastically upon tissue injury or cellular damage, when cellular components can leak into compartments they usually are isolated from. Endogenous immune responses are initiated in order to promote tissue repair and restrict the extent of damage. Likewise, these responses can be initiated in concert with recognition of PAMPs during infection when host molecules are released upon cell lysis by for instance viruses[10].

Several endogenous ligands capable of activating TLR signalling have been identified. The nature of the ligand determines the TLR response, some ligands are exclusive to a specific TLR while others are able to activate several. Many are components of the extracellular matrix, such as; fibronectin, heparin sulphate, biglycan, and fibrinogen. Heat shock proteins and high mobility group box 1 (HMGB1), as well as human cardiac myosin, are potent endogenous protein ligands. DNA, RNA, small interfering RNAs (siRNA) and messenger RNA (mRNA) are also implicated as endogenous ligands[10, 30].

**TLR2** is able to recognize biglycan, HMGB1, and human cardiac myosin. **TLR4** is able to recognize the previously stated, and in addition heparin sulphate, fibronectin, fibrinogen and heat shock proteins. **TLR9** recognises DNA and HMGB1. **TLR7** can sense RNA and siRNA, and **TLR8** is also capable of detecting siRNA in addition to human cardiac myosin. **TLR3** can sense mRNA[10].

#### *1.2.4.1 Unfavourable effects of endogenous ligand recognition*

Endogenous ligand recognition has also been shown to partake in prolonged inflammation, autoimmunity and tumourigenesis[10]. Endogenous ligand recognition has been implicated to be a major contributor to ischemia and reperfusion (RI) induced inflammation. TLR2, TLR4 or MyD88 deficient mice show attenuated myocardial infarctions, smaller infarction sizes, better preserved ventricular function and reduced ventricular remodelling after ischemia, compared to wild type mice [31]. Similar observations have been made in kidney ischaemia and liver injury[32-34]. TLR3 is a known sensor of RNA from necrotic cells, however this response may be adverse in acute inflammatory responses. Cavassani et al showed that TLR3 deficient mice experiencing acute polymicrobial peritonitis and ischemic gut injury in the absence of viral stimuli, mounted an immediate cytokine/chemokine response. This response however, quickly decline to baseline expression levels and the mice did not experience the lethal effects of prolonged inflammation[35].

Several TLRs have been identified as contributors in autoimmune diseases as well. Endogenous activation of TLR3 and TLR4 have been implicated to lead to disease progression in rheumatoid arthritis (RA), as they are capable of activating synovial fibroblasts, a major contributor to inflammation and bone erosion in RA[36]. TLR7 and TLR9 have both been connected to systemic lupus erythematous (SLE), as RNA and DNA in immune complexes are capable of activating the receptors. IFN-α production by plasmacytoid pre-dendritic cells ensues, and a high level of IFN-α is a characteristic of SLE[37].

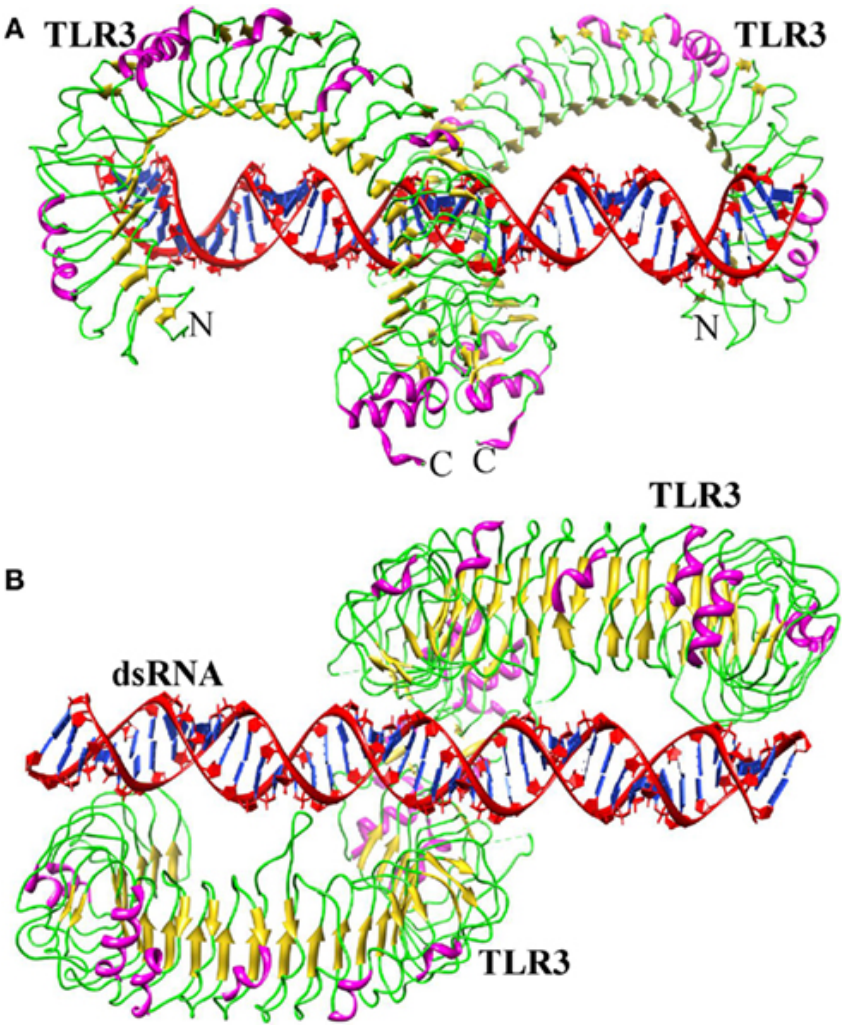
The role of TLRs in cancer progression is much disputed, as evidence supporting them as both positive and negative mediators of cancer progression have been uncovered. Activation of TLR signalling in TLR mediated chronic inflammation can induce an anti-tumour T-cell response. Research has demonstrated that dying tumour cells release HMGB1, and that its recognition through TLR4 and MyD88 dependent signalling triggers activation of cytotoxic T lymphocytes and promotes anti-tumour immunity[38]. TLR3 has also been shown to induce apoptosis in cancer cells through induction of IFNβ and IFN-inducible proteins, and have an anti-tumorigenic effect. TLR3 is able to

recognise endogenous RNA released by cancer cells and induce an immune response[39]. Contrary, up-regulation and expression of TLRs have been demonstrated in several cancers, and TLRs have been linked to tumour progression[40]. TLRs are primarily expressed on immune cells, however epithelial cells also express TLR to some extent. Up-regulation and expression of TLRs have been particularly observed in cancer of epithelial origin, likely due to the fact that epithelial cells are among the first to come in contact with pathogens and are therefore normally express TLRs that in turn are affected in a tumorigenic environment[40]. Cancer development has been linked to several inflammatory diseases and inflammatory states, where the inflammatory environment promotes cancer progression and tumorigenesis. This includes inflammatory bowel disease (IBD), chronic bronchitis, papilloma virus causing cervical inflammation, chronic pancreatis, and several more a likely to be identified in the future. Interestingly, TLR3 in particular has been connected to several of these.

## 1.3 TLR3

### 1.3.1 Structure

The structure of TLR3, like the TLRs in general, consists of a leucine rich repeat domain, known as the ectodomain, a transmembrane domain and a TIR domain. The ectodomain of TLR3 has the shape of a solenoid horseshoe, and consists of several leucine rich repeats (LLRs) similar to the other TLRs found in humans. It is this region of the protein that can bind and form dimers with other TLR3 proteins upon ligand recognition. The protein was first thought to be free of glycosylation, thus allowing more readily dimerization, however 3D crystallography has shown that TLR3 is a glycoprotein. TLR3 however, is still able to form dimers via its ectodomain, due to "naked" regions where it is able to bind its ligands and neighbouring TLR3 [41]. The protein forms homodimers when it is activated. The structure of TLR3 is illustrated in **Figure 1.3.**




**Figur 1-3: Structure of TLR3.** Front and side-view of the TLR3 receptors upon ligand binding. Adapted from [42].

### 1.3.2 Ligands

As preciously reviewed, TLR3 is one of the important TLRs for mediating innate immune responses toward viral infections. It acts as a sensor of dsRNA, which is a PAMP often associated with virus infection. TLR3 recognizes genomic dsRNA derived from retroviruses, or dsRNA generated in the cytosol during the replication stages of ssRNA viruses or DNA viruses[43]. In addition TLR3 has been found to recognise siRNA[44]. The receptor can also recognise the synthetic analogue of dsRNA known as polyinosinic:polycytidylic acid (Poly I:C), a compound commonly used in research to activate TLR3[45]. Several studies have demonstrated that TLR3 can act as an endogenous ligand receptor, and recognise host RNA[46]. RNA from or a associated with necrotic host cells have been demonstrated to activate TLR3, consequently inducing immune responses[35].

### 1.3.3 TLR3 signalling and responses

Upon recognition of dsRNA or endogenous RNA TLR3 transmits a signal trough TRIF, as described in section 1.2.3.2. Signalling through TLR3 induces immune responses through different transcription factors: IRF3/IRF7, AP1 and NFkB. The activation of IRF3/IRF7 leads to production of IFNβ, whereas activation of AP1 and NF-kB leads to induction of cytokines.

Type 1 Interferons, which IFNβ is a part of, have three main functions. They provide antiviral protection upon recognition of pathogens, both in infected and neighbouring cells. They promote antigen presentation and activation of natural killer cells, as well as chemokine production in innate immune cells. Type 1 IFNs are also capable of promoting adaptive immune responses, inducing antibody production and effector T cell responses[47]. Dendritic cell maturation is also induced in this process. It is important to note that TLR3 is not the only receptor able to induce IFN-B responses in cells, as TLR7-9 also have this ability[3].

Signalling through TLR3 also leads to cytokine production as a result of AP1 and NF-kB translocation to the nucleus. TLR3 is capable of inducing several cytokines, such as interleukin-8(IL-8), tumour necrosis factor α (TNF α), C-C ligand 20 (CCL20) and several C-X-C chemokines[48]. Examples of induced cytokines are C-X-C ligand (CXCL) 9, 10, and 11. CXCL9-11 are known angiostatic chemokines that can affect cell proliferation, direct migration and promote adhesion of T-cells and NK cells, when binding the receptor C-X-C receptor 3 (CXCR3)[49]. This receptor is expressed on a variety of cells, and as a result these chemokines are able to influence a broad range of cells[50].

### 1.3.4 TLR3 localization

TLR3, as previously mentioned, is primarily expressed and signals from intracellular compartments. Compartmentalization of nucleic acid sensing TLRs reduces the likelihood of endogenous ligand recognition and promotion of unwanted immune responses, as host RNAs are found in the cytosol of the cell[6]. It also provides the cell with a tool to recognise internalized ligands through phagocytosis, as some pathogen

components are dependent on degradation in order to trigger ligand recognition. This mainly occurs through the endocytic pathway, where pathogens and their components can be internalised through various mechanisms such as phagocytosis and endocytosis[51]. TLR3 is most abundantly found in the early endosomes, and ligand recognition through TLR3 is dependent on the acidity of the compartment for activation[52]. Trafficking from the endoplasmic reticulum (ER), where the nucleic acid sensing TLRs originate, to endosomes is necessary for recognition to occur[53]. The trafficking involves transport from the ER, possibly via the Golgi apparatus, and is dependent on the ER localized protein UNC93b1. Both pathogenic and endogenous ligands can be internalised by the cell and recognized by TLR3 through this mechanism[54]. TLR3 is primarily known to be located in endosomes, however recent research proposes that is can be also be found on the plasma membrane[53].

## 1.4 TLR3 and cancer

TLR3 expression was initially only reported in immune cells, however several studies have shown TLR3 expression and function in different cell types, including tumour tissue and cancer cell lines, indicating that TLR3 has a role in tumour biology[16]. TLR3 expression has been reported in several cancers, including lung cancer, breast cancer, melanoma, prostate cancer and colon cancer, however its role remains unclear[55, 56]. TLR3 appears to have anti-cancer properties in some cancers and in others it seems to promote tumour progression.

Strong TLR3 expression was observed in poorly differentiated tumours in head and neck cancer, where activation of the receptor was linked to increased migration of cancer cells[57]. Increased expression of TLR3 has also been observed in melanoma, where activation of the receptor led to cytokine secretion and cell migration[58, 59]. TLR3 expression has also been linked to increased risk of metastasis in breast cancer[60]. Activated TR3 and consequent secretion of chemokines has also been increasingly linked to cancer cell migration and metastasis in colon cancer[61, 62].

TLR3 has been reported to induce apoptosis in cancer cells, through activation of IRF3 and production of type 1 IFNs[39]. Research has indicated that administration of Poly

I:C is able to induce apoptosis in several types of cancer, such as breast cancers and prostate cancers, through the activation of the extrinsic and intrinsic apoptotic pathways[55]. Activation of TLR3 in melanoma cells led to pro-apoptotic and anti-proliferative signalling, resulting in cancer cell death[63]. Administration of Poly I:C,, leading to activated TLR3, was also found to enhance CD8 T-cell responses and promote anti-tumour immunity[64].

As a result, Poly I:C and other TLR3 agonists have been proposed for use in cancer therapy and are under clinical trials. Ampligen is a Poly I:C derivate that acts on dendritic cells, promoting tumour regression. Hiltonol is another Poly I:C derivate that has been implicated to boost anti-tumour immune responses[65].

The cytokine products of TLR3 activation, CXCL9-11, are capable of attracting anti-tumour T-lymphocytes, and are implicated to inhibit tumour growth and promote regression[49]. The use of CXCL10 has been suggested to aid commercial chemotherapy, as it has proven a potent inhibitor of angiogenesis, and is shown to reduce tumour growth. C-X-C chemokines have also been reported as chemo-protectants of hematopoietic cells, and suggested to aid the survival of these during chemotherapy[66]. The effects of CXCL9-11 are in part due to the properties of their receptor, CXCR3. Three splice variant of the CXCR3 gene has been established, CXCR3-A, CXCR3-B, and CXCR3-alt. Differing roles have been assigned to the different variant of the gene, where CXCR3-A and CXCR3-B have opposing roles. CXCR3-A is proposed to promote proliferation and migration of cells, whereas CXCR3-B inhibits this and promotes apoptosis. Signalling outcome of TRL3 activations can therefore have varying effects depending on presence of CXCLR3 variants on the neighbouring cells.

### 1.4.1 TLR3 and colon cancer

Colorectal cancer (CRC) is the third most common cancer worldwide, and is ranked fourth in relation to cancer-related deaths. The disease originates from epithelial cells that line the colon and rectum of the gastrointestinal tract. Distant metastasis is the major contributor to mortality in the disease. Patients with local CRC have a 5-year survival rate of 80-90%, whereas patients with CRC and distant metastasis have a 5-

year survival rate of 10-20%. Distant metastasis is linked to poor survival[67]. In spite of improved diagnosis and treatment, CRC is still a major contributor to the cancer burden worldwide.

Inflammation as a result of microbial infections and chronic inflammatory diseases has been firmly liked to carcinogenesis[68]. Tissues subjected to prolonged inflammatory responses are more likely to develop cancer. The microenvironment that arises in the tissue upon prolonged inflammatory responses promotes consecutive cell proliferation as a result of continued tissue damage, predisposing the affected cells to form neoplasms[69]. Chronic inflammatory states in the bowel, such as in inflammatory bowel disease (IBD), demonstrate a higher likelihood of development of colon cancer compared to normal bowel[70]. TLR3 has been suggested as an important mediator in cancer progression and tumourigenesis of colon cancer, mainly through its signalling products and their effects on other cells.

CCL20 is a chemokine that binds the receptor CCR6. Expression of both CCL20 and CCR6 have been linked to CRC, where both have been found up-regulated in cancer cells compared to normal colon mucosa[62]. Stimulation of CRC cells with CCL20 was shown to promote adhesion, proliferation and migrations of the cancer cells. Expression of CCR6 has also been linked to liver metastasis of CRC[71].

CXCL9, CXCL10 and CXCL11 are ligands for CXCR3, a receptor that in CRC is linked cancer progression; hence these cytokines are therefore potentially liked to cancer metastasis. CXCR3 has been increasing proven an important mediator of metastasis and tumour progression in the recent years. CXCR3 expression has been reported up-regulated in metastatic colon cancer cells, whereas in primary lesions CXCR3 expression was normal. Up-regulation of CXCR3 was also reported in metastatic sites, such as liver metastasis[67]. Patients with up-regulated CXCR3 were more frequently liked to metastasis to the lymph nodes and other organs, with a poorer survival rate[72]. CXCL10 in particular has been associated with CXCR3 and cancer progression, as the chemokine has been reported to promote migration and adhesion in

metastatic CRC cells[61]. Taken together, the interplay between TLR3, secretion of chemokines and activation of CXCR3 appears to have an important role in CRC.

# 2 Aims of Study

TLRs and their secreted end products have proven exceedingly important in inflammation and cancer progression. Research has proposed both cancer promoting and anti-tumour properties of TLRs in cancer progression[16]. Recently, TLR3 expression has been reported in colon cancer cells, with an up-regulation of expression in more differentiated cells[73]. HT-29 is a cell line derived from colorectal adenocarcinoma, and is known to exhibit metastatic properties. This study aimed to investigate a potential role of TLR3 and its signalling outcomes in relation to the metastatic intestinal epithelial cell line HT-29, and ultimately form a better understanding of TLR3 in relation to colon cancer.

The objective of this study was:

1. Perform an initial screen of cytokine secretion induced in HT-29 upon stimulation of TLR3 pathway.
2. Confirm any secreted cytokines from HT-29.
3. Establish the role of TLR3 and TRIF in relation to the cytokine secretion.
4. Investigate the role of the trafficking protein UNC93b1 in relation to TLR3 and secreted cytokines in HT-29 cells.

# 3 Materials and Methods

## 3.1 Cell culture

The human intestinal epithelial cell line HT-29 was used in this study. It was purchased from the American Type Cell Collection (ATCC). HT-29 was derived from cells in a colorectal adenocarcinoma of a 44-year-old female. The cell line is known to have metastatic properties[74].

### 3.1.1 Reagents

RPMI 1640 medium and L-glutamine were obtained from Sigma Aldrich. Foetal Calf Serum (FCS) was purchased from Gibco by Life Technologies. Gentamycin was obtained from Sanofi Aventis (Norway). Trypsin/EDTA was purchased from Lonza/Bio-Whittaker®. Corning® cell culture flasks with vented caps were purchased from Sigma Aldrich.

### 3.1.2 Cell culture conditions

The cell line was cultured in T75 corning cell culture flasks. It was cultured in RPMI medium supplemented with 10% Foetal Calf Serum (FCS), 0,31% glutamine and 0,05% gentamycin. HT-29 cells were incubated at 37°C, 5% $CO_2$. The cells were passaged twice each week, before full confluence occurred.

### 3.1.3 Cell passaging procedure

For cell passaging, old culture medium was removed. The cells were then washed with 5ml DPBS to remove the old medium completely, as the medium contains trypsin inhibitor. DPBS was removed and 2ml of trypsin/EDTA was added to detach the cells. In order to inactivate the trypsin once the cells were detached, fresh medium was added and the cells were split/alequoted in appropriate amounts in new flasks.

## 3.2 Proteome Profiler Array

A Proteome Profiler Array was used to determine the cytokine, chemokine and growth factor secretion from the HT-29 cells. The array used in this study was the Human XL Cytokine Array Kit from R&D Systems. It gives relative expression levels of 102 soluble proteins.

### 3.2.1 The principle behind the array:

A nitrocellulose membrane has been spotted with capture and control antibodies for soluble proteins. Each cytokine on the membrane is represented in duplicates. The membrane is incubated over night with the sample of choice. This could be cell culture supernatant, cell lysate, urine, saliva, serum, plasma, human milk, or tissue lysate. This is followed by membrane washing to remove excess and unbound sample. A mixture of detection antibodies is then added, followed by Streptavidin-HRP and chemiluminescent detection reagents. This produces a signal in each spot on the membrane equivalent to the amount of bound protein.

### 3.2.2 Reagents used:

Proteome Profiler™ Array, Human XL Cytokine Array Kit was purchased from R&D Systems. Poly I:C was purchased from Invivogen. A LI-COR Odyssey Fc machine and Image Studio™ 3.1 software from LI-COR bioscience were used for analysis.

### 3.2.3 Proteome Profiler Array procedure

The Proteome Profiler Array was conducted on HT-29 cells that had been treated with 5µg/ml Poly I:C or only treated with medium. HT-29 cells were harvested from stock and plated in a 24 well plate, 300 000 cells/well. The 24 well plate was incubated in 37°C, 5%$CO_2$ for 24 hours. Old medium was removed from all the wells, and fresh was added. 12 wells in the plate were stimulated with 5µg/ml TLR3 ligand Poly I:C and 12 were treated only with normal medium. The plate was incubated in 37°C, 5%$CO_2$ for 20 hours. The supernatant from the stimulated and un-stimulated cells was harvested after 20 hours in separate tubes. The rest of the experiment was conducted as described in the protocol given by R&D systems for the Human XL Cytokine Array Kit **(see Appendix I)**. In order to develop the membrane an alternative method than described in the R&D protocol was used. A LI-COR Fc machine was used to develop the membrane, and Image Studio™ 3.1 software was used to analyse and quantify.

## 3.3 Stimulation experiments

HT-29 cells were stimulated with TLR3 ligand polyinosinic:polycytidylic acid (Poly I:C) to determine the stimulation effects in HT-29. A time dependent experiment with different Poly I:C concentrations was carried out in order to establish the optimal Poly

I:C concentration to stimulate HT-29 cells with. ELISA and qRT-PCR was carried out in order to assess the cytokine secretion at the different time-points and concentrations. A second experiment was carried out in order to assess direct simulation and endosomal simulation of TLR3. HT-29 cells were stimulated both on the surface and via transfection in a time dependant manner. ELISA and qRT-PCR were performed to assay potential cytokine induction in added and transfected Poly I:C at the different time-points.

### 3.3.1 Reagents used during stimulation with Poly I:C

Poly I:C was obtained from Invivogen. Lipofectamine/RNAiMAX was purchased from Invitrogen.

### 3.3.2 Poly I:C dose and time experiment

HT-29 cells were harvested and counted on the Countess™, thereafter spun down at 1500 rpm for 8 minutes. They were then suspended in IEC medium and plated 200 000 cells/well in three 24 well plates. The plates were incubated at 37°C, 5%$CO_2$ overnight. The cells were stimulated with the following concentrations of Poly I:C ; 10µg/ml, 5µg/ml, 2,5µg/ml, 1µg/ml and 0,5µg/ml, at 25-, 20-,10-, 5-, 2,5- and 1 hours. The setup of the experiment is illustrated in **Figure 3.1**. Tubes with medium and the mentioned concentrations were prepared. For the first time point, medium was removed from all the wells and 300µl of fresh medium was added, followed by addition of 100µl of the different stimuli concentrations according to setup in **Figure 3.1**. This was performed in the same fashion for all the other time-points. Supernatant was harvested after 25 hours, and stored at -20°C. Cells were lysed with RA1 buffer and stored at -80°C.

| Time (Hours): | Poly-IC (µg/ml): 10 | 5 | 2,5 | 1 | 0,5 | Medium |
|---|---|---|---|---|---|---|
| 25 | | | | | | |
| | | | | | | |
| 20 | | | | | | |
| | | | | | | |

| | 10 | 5 | 2,5 | 1 | 0,5 | Medium |
|---|---|---|---|---|---|---|
| 10 | | | | | | |
| | | | | | | |
| 5 | | | | | | |
| | | | | | | |

| | 10 | 5 | 2,5 | 1 | 0,5 | Medium |
|---|---|---|---|---|---|---|
| 2,5 | | | | | | |
| | | | | | | |
| 1 | | | | | | |
| | | | | | | |

**Figure 3-1: Experimental setup for time and Poly I:C dose experiment.**

### 3.3.3 Added and transfected Poly I:C stimulation procedure

HT-29 cells were harvested and counted on the Countess™, thereafter spun down at 1500 rpm for 8 minutes. They were then suspended in IEC medium and plated 200 000 cells/well in two 24 well plates, one designated for direct simulation and one for transfection stimulation. The cells were allowed to attach before starting stimulations. A mastermix for the two stimuli was made before stimulations started. The mix for added Poly I:C consisted of 2mL 10%FCS/RPMI and 5µl Poly I:C. The mix for tansfected Poly I:C was prepared by adding 50µl RPMI with no additives and 10µl Lipofectamine to an eppendorf tube. A second eppendorf tube with 50µl RPMI with no additives and 5µl Poly I:C was also prepared. The contents of the two eppendorf tubes

were mixed and incubated at RT for 15 minutes. 400µl of stimuli was added to the designated wells after removing old medium. The time-points chosen were 24 hours, 20 hours, 10 hours, 6 hours, 3 hours, and 0 hours.

## 3.4 MTT assay

An MTT assay was preformed in order to determine cell viability in HT-29 cells after stimulation with different concentrations of added Poly I:C.

### 3.4.1 The principle

An MTT assay is a colorimetric assay that determines cell viability. The assay utilises the yellow tetrazole 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), as metabolically active cells can reduce it to its insoluble form Formazan, which has a purple colour. This coloured product can be measured by optical density and cell viability can be determined.

### 3.4.2 Reagents used

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide and $NH_4OH$ were purchased from Sigma Aldrich. Isopropanol was purchased from Kremyl Norge. Poly I:C was purchased from Invivogen.

### 3.4.3 MTT assay procedure

20 000 cells/well were plated in a 96 well plate. The plate was incubated over night, 37°C, 5%$CO_2$. A stimulation mix for each concentration of Poly I:C to be assayed was prepared in an eppendorf tube. The stimulation mix consisted of 10%FCS/RPMI and Poly I:C. The concentrations of Poly I:C used were 50µg/ml, 25µg/ml, 10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml, 0.625µg/ml, 0.31µg/ml and 0.15µg/ml. Medium was used as an un-stimulated control. 100µl of designated stimuli mix was added to the wells, and the plate was incubated at 37°C, 5%$CO_2$ for 20 hours. The supernatant was harvested in a 96 well plate and frozen at -20°C. Normal growth medium with MTT (1:10) was added to the remaining cells in the wells. The plate was incubated at 37°C, 5%$CO_2$ for 2 hours, allowing the cells to take up and metabolize the MTT. The supernatant was removed and isopropanol with 25% $NH_4OH$ was added. The plate was placed on a shaker protected form light for 30 minutes. Absorbance was measured at 570nm.

## 3.5 Enzyme-linked Immunosorbent Assay (ELISA)

ELISAs are biochemical assays used to detect and quantify substances such as proteins or peptides in a sample. The technique uses the properties of antibodies in order to specifically target the substance of interest. The cytokines CXCL10, CXCL11 and IFNβ were assayed in samples form various experiments in this study. A "sandwich" ELISA assay method was used.

### 3.5.1 The principle:

In a "sandwich" ELISA the substance of interest is bound between two antibodies, a capture and a detection antibody, hence the name. **Figure 3.2** illustrates the principle. A known concentration of the capture antibody is coated on the surface of a plastic microwell. The well is then washed to remove any unbound capture antibody. This is followed by buffer addition, in order to ensure that any remaining protein binding sites are blocked. The well is washed again to remove any unbound substances. The sample of interest is then added. If the sample contains the substance to which the capture antibody is specific, it will bind to the immobilized antibody. The well is yet again washed to remove unbound sample. A detection antibody is then added, which also has an epitope specific to the substance of interest. The substance of interest is now "sandwiched" between the capture and detection antibodies. The detection antibody is also bound to biotin, a compound that enables the detection antibody to link to an enzyme. The enzyme, Horseradish peroxidase, is conjugated to Streptavidin, which has a high affinity for biotin, thus enabling the linking. Once bound, a substrate solution, Tetramethylbenzidine (TMB), is added, and colour change occurs as a result of the reaction between HRP and the substrate turning the solution blue. The colour change is proportional to the amount of substance bound by the antibodies. The reaction is stopped by adding 2M $H_2SO_4$, which changes the colour from blue to yellow. The colour reaction is then measured in absorbance on a plate reader at 450 nm and 570nm.

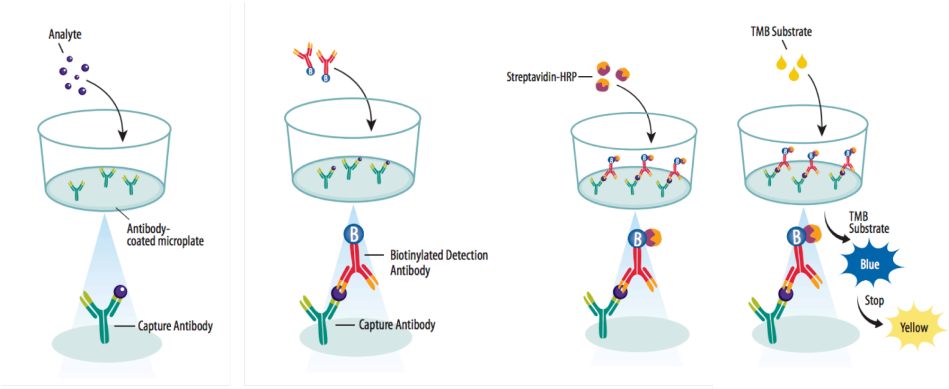



**Figure 3-2 Sandwich ELISA assay principle:** Step one describes the binding of capture antibody and analyte to the microwell. In step two the addition and binding of detection antibody is illustrated. Step three and four depict the addition of Streptavidin-HRP and the colour change induced when adding TMB and $H_2SO_4$[75].

### 3.5.2 Reagents used

The CXL10 and CXCL11 Duo®Set ELISA kits used in this study was purchased from R&D Systems. Phosphate Buffered Saline (Dulbeccos A) tablets to make wash buffer were obtain from OXID. Tween and Bovine Serum Albumine (BSA) were purchased from Sigma. TMB substrate solution A and B was obtained from BioLegend. The VeriKine™ Human IFN Beta ELISA kit was from pbl Assay Science.

### 3.5.3 Assay procedure

CXCL10 and CXCL11 ELISA Assay procedure:

Half of the volumes recommended by R&D Systems were used per well.

1. Capture antibody was diluted to the recommended working concentration in PBS. 50µl was added to each well of the 96 microwell plate, and then sealed with an adhesive strip to prevent any evaporation. The plate was incubated at RT over night.
2. The plate was washed three times on an automated plate washer with 0,05% PBS/TWEEN solution.
3. 150µl of reagent diluent (1%PBS/BSA) was added to each well for blocking. An adhesive strip was added and the plate was incubated at RT for 1 hour.
4. The plate was washed, see step 2.
5. 50µl of sample and 50µl of standard (diluted in reagent diluent) was added to designated wells, the plate was sealed and incubated at RT over night.

a. Samples assayed for CXCL10 were diluted 1:10 or 1:20 in reagent diluent
b. Samples assayed for CXCL11 were diluted 1:4 or 1:7 in reagent diluent

6. The plate was washed, see step 2.
7. Detection antibody was diluted in reagent diluent according to the recommended working concentration by R&D systems. The plate was coated, 50µl detection antibody solution in each well, sealed and incubated at RT for two hours.
8. The plate was washed, see step 2.
9. 50µl of Streptavidin-HRP diluted to the recommended working concentration (1:40 or 1:200) was added to each well. The plate was sealed and incubated for 45 minutes at RT, protected from light.
10. The plate was washed, see step 2.
11. 50µl of the substrate solution TMB was added to each well. The TMB was mixed 1:1 of colour reagent A and B. The plate was incubated at RT, protected from light, until a sufficient colour change was observed, and no longer than 20 minutes.
12. 25µl of $H_2SO_4$ was added to each well in order to stop the colour reaction.
13. The plate was analysed on a BioRad microplate reader at wavelengths 450nm and 570nm.

IFNβ ELISA Assay Procedure:

The experiment was conducted as described in the protocol given by pbl Assay Science for The VeriKine™ Human IFN Beta ELISA kit. The procedure is attached in the **Appendix II**. The plate was analysed on a BioRad microplate reader at wavelengths 450nm and 570nm

## 3.6 Quantitative real time PCR

Quantitative real-time Polymerase Chain Reaction (qRT-PCR) was used in this study to assess the mRNA expression of several genes in the HT-29 cell line after various experiments. The TaqMan qRT-PCR method was used. The gene expression of CXCL10, CXCL11, TLR3, TICAM-1 (TRIF), IFNβ1 and UNC93b1 was assessed using qRT-PCR.

### 3.6.1 The principle of qRT-PCR

qRT-PCR is a technique that allows simultaneous amplification and quantification of a given cDNA target. The principle is illustrated in **Figure 3.3**. The TaqMan qRT-PCR method utilizes the TaqMan chemistry. Taq polymerase 5′→3′ exonuclease activity and fluorescence resonance energy transefer (FRET) are key principles in this method. The assay contains two target specific primers, and a probe containing a fluorescent reporter dye on the 5′end and a quencher on the 3′ end. The probe sits between the two primers. The reporter dye generates a fluorescent signal when light from the real time instrument is shone onto it. The quencher absorbs this signal generated by the reporter when the two are in close proximity. In a TaqMan qRT-PCR assay the signal from the reporter dye is only registered when the reporter and quencher are far apart. This occurs when the target of interest is present in the sample, and is amplified. The primers and probes attach to the cDNA target of interest, and the Taq polymerase amplifies from the 5′→3′ direction. When it meets the probe it is able to cleave it by its endonuclease activity. This separates the reporter and quencher and a signal is detectable. The fluorescent signal from the reporter grows proportionally to the amplification of the product.

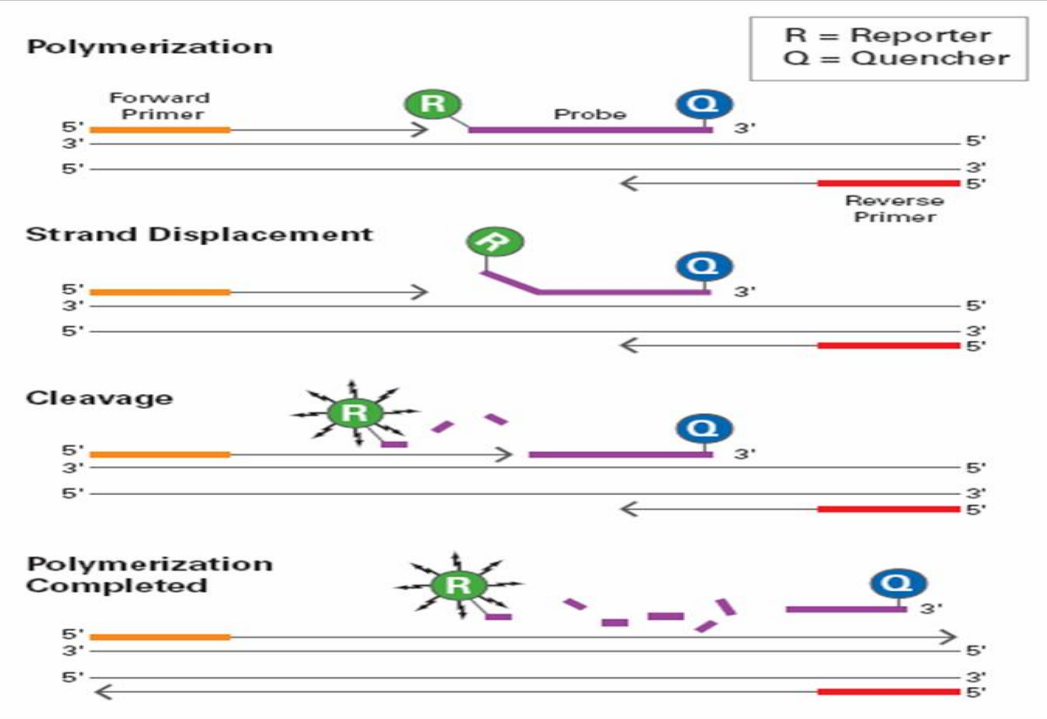



**Figure 3-3 The principle of TaqMan qRT-PCR.** The strands are extended by the Taq polymerase in a 5′→3′ direction from the primer sites. The Taq polymerase displaces and cleaves the probe containing the reporter and quencher. The reporter is no longer quenched by the quencher, and can signal freely. The strand polymerization competed, one amplification cycle is concluded[76].

### 3.6.2 Reagents used

RNA was isolated using a NucleoSpin® 8/96 RNA kit from Macherey-Nagel. cDNA was synthesised using High Capacity RNA- to cDNA kit from Applied Bioststems on a Bio-Rad S1000 Thermal Cycler. A Nanodrop N.D. 1000 specrophotometer from Saveen Werner was used to measure RNA concentration in the samples. TaqMan gene expression assays; GAPDH (Hs99999905_m1), TLR3 (Hs01551078_m1), TICAM-1 (Hs00706140_s1), CXCL10 (Hs01124251_g1), CXCL11 (Hs04187682_g1) and IFNβ1(Hs01077958_s1), were used with the TaqMan Fast Real-Time PCR Universal PCR Master Mix (FASTA mix) in the PCR reaction setup. The qRT-PCR was performed on an Applied Biosystems StepOne PLUS qRT-PCR machine and software.

### 3.6.3 RNA isolation

RNA was isolated from HT-29. After harvesting medium from the well in the 24 well plate the cells were cultured in, 300µl of RA1 lysis buffer was added in order to lyse the cells. The plates were incubated at RT for 15 minutes, then frozen at -80°C in order to help the lysis process and to store the lysate stably. The lysate was then thawed on a

shaker at RT. When defrosted, 300µl of RA4 buffer was added to each well, and mixed thoroughly. The 600µl from each well were then transferred to tubestrips, and then onto the 96 well silica membrane of the RNA binding plate from the NucleoSpin® kit. The NucleoVac 96 manifold vacuum system was used instead of a centrifuge to pull the lysate and buffer solutions through the silica. A minimum of -0,2 bar was applied to the vacuum steps. The lysate was bound to the silica membrane using vacuum for 1 minute. 500µl of RA3 buffer was added to each well and vacuum was applied for 3 minutes. This was followed by addition of 95µl/well rDNase reaction mixture, incubated at RT for 15 minutes. Vacuum was applied for 1 minute to pull through the solution. Subsequently 500µl RA2/well and 800µl RA3/well was added, with 1 minute vacuum after each addition. 500µl RA4/well was then added, and a high vacuum of -0,6 bar was applied for 1 hour. The wash-plate was then removed and a 96 well elution plate was placed under the RNA binding plate for elution of the RNA sample. 75µl of RNase free $H_2O$ was added to each well and RNA was eluted at vacuum for 1 minute. The plate containing RNA was immediately set on ice to prevent degradation. The concentration of each RNA sample was measured on the NanoDrop N.D. 1000 spectrophotometer.

### 3.6.4 cDNA synthesis

cDNA synthesis was performed using the Applied Biosystems High Capacity RNA- to cDNA kit, by reverse transcription of the RNA samples to cDNA. Each RNA sample and reaction mix was prepared for cDNA synthesis as described in **Table 3.1** below. A master mix of RT buffer and enzyme mix was prepared in an eppendorf tube, volumes appropriate to the number of RNA sample to be converted. 11µl of the master mix was aliquoted to tube-strips or plates, followed by addition of 9µl RNA sample. The tube-strips were sealed with caps and spun down in order to remove air bubbles and bring the contents to the bottom of the tube. The tube-strips were then placed in the thermal cycler. The samples were incubated at 37°C for 60 minutes, then brought to 95°C for 5 minutes in order to stop the reaction. The samples were then held at 4°C, and either used directly for PCR or stored at -20°C.

Table 3-1 Overview of components and volumes used in cDNA synthesis reaction.

| Component | Volume/reaction +RT | Volume/reaction -RT |
|---|---|---|
| 2xRT buffer | 10µl | 10µl |
| 20xEnzyme mix | 1µl | - |
| RNA sample | 9µl | 9µl |
| Sterile Ion filtered water (SIW) | To a total of 20µl | To a total of 20µl |
| Total volume per reaction | 20µl | 20µl |

### 3.6.5 qRT-PCR

The cDNA samples were diluted in sterile ion filtered water (SIW) to a concentration of 5ng/ul, giving 50ng in total/PCR reaction. The PCR reagents and sample was mixed as in the table below **Table 3.2**. A master mix was prepared containing the primer and FASTA mix, and 11µl was distributed to each well on a 96 well PCR plate. 9µl of sample was then added to the designated wells. The master mix contained all the components necessary for a successful qRT-PCR run. The components include Taq polymerase, Mg2Cl2, deoxynucleoside triphosphates (dNTP), ROX™ reference dye and stabilizers. Once administered to the PCR plate, it was sealed with an adhesive cover and spun down at 1500rpm for 5 minutes to remove air bubbles. The plate was then assayed using the Applied Biosystems StepOne PLUS PCR system and software.

Table 3-2 Components and volumes/reaction used in the TaqMan fast real time qRT-PCR assay

| Component | Volume/reaction |
|---|---|
| FASTA mix | 10,0µl |
| Primer | 1,0µl |
| cDNA sample | 9,0µl |
| Total volume | 20,0µl |

## 3.7 siRNA knockdown experiments

Knockdown experiments using short interfering RNA (siRNA), also known as silencing RNA, were conducted in order to assess the components of the TLR3 signalling pathway. siRNAs against TLR3, TRIF and UNC93b1 were used in this study.

### 3.7.1 The principle of siRNA knockdown

RNA interference (RNAi) is the phenomenon in which post-translational silencing of gene expression occurs in response to the introduction of double stranded RNA to the cell. The process is illustrated in **Figure 3.4**. Double stranded RNA is cleaved by an enzyme known as Dicer into short RNAs (siRNA), 21-23 nucleotides in length. The siRNAs triggers the activation of RNA-induced-silencing-complex (RISC). RISC uses the anti-sense strand of the siRNAs to guide single stranded RNA cleavage, such as cleavage of mRNA, therefore promoting mRNA degradation. The mechanism is exploited in siRNA knockdown experiments, in which siRNA specific to a target is introduced in order to knock down a cell response[77]. Different administration systems are available, but in this study the lipid-based system Lipofectamine was used.

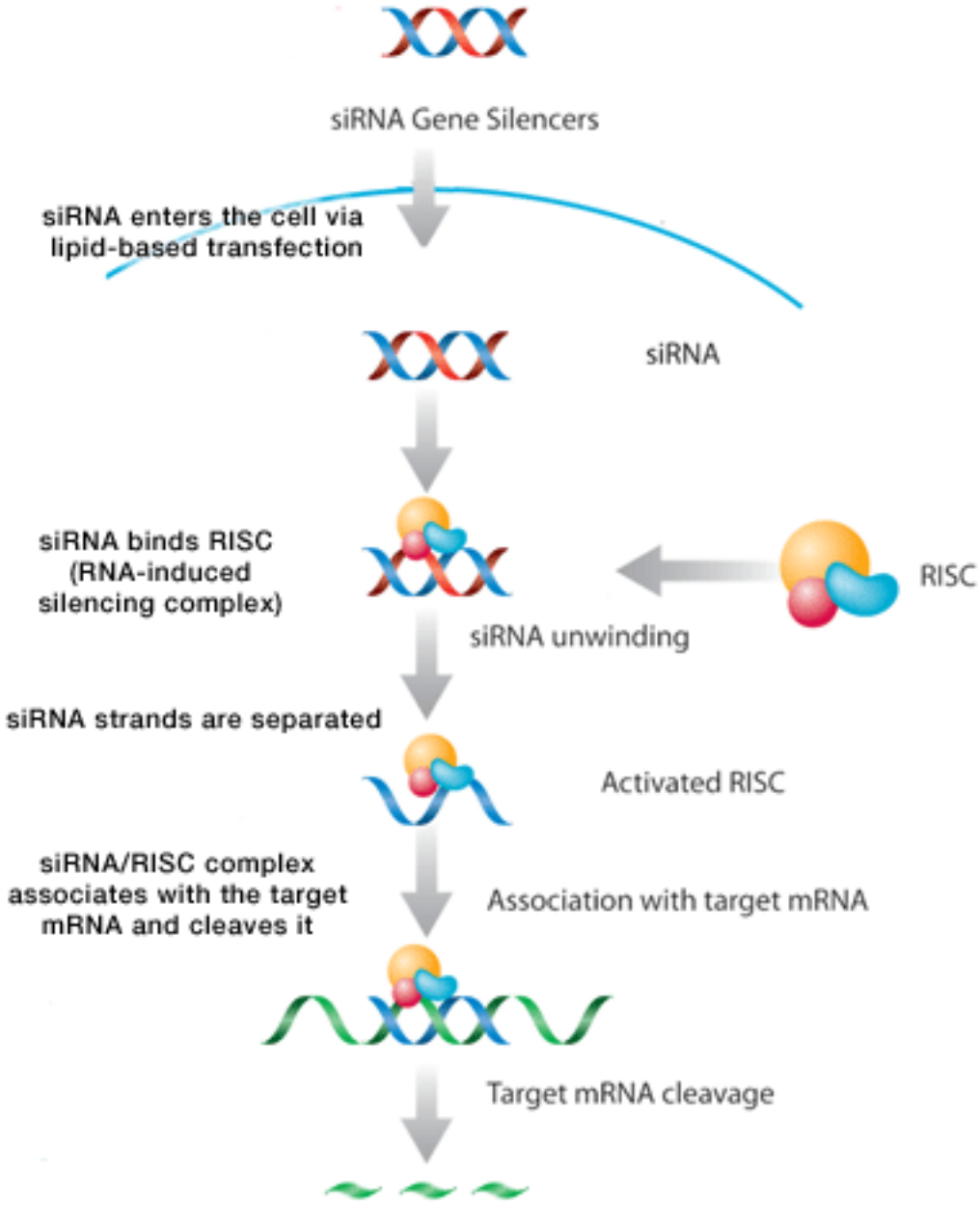



**Figure 3-4 Illustration of siRNA process inducing RNAi:** siRNA is detected by- and binds to RISC upon administration to the cell. The siRNA strands are separated and RISC uses the anti-sense strand of the siRNA strand to guide single-stranded RNA cleavage. The target mRNA is degrade as a result.

### 3.7.2 Reagents used

Lipofectamine® RNAiMAX was purchased from Invivogen™. siTLR3 , siUNC93b1-1, siUNC93b1-5 and Allstars Negative control siRNA (siCTR) were purchased from Quiagen. siTICAM-1 was purchased from Ambion® by Life Technologies. Poly I:C was obtained from Invivogen. Opti-MEM® medium was purchased from Gibco by Life technologies.

### 3.7.3 Procedure

The protocol consists of several parts; to prepare transfection reagents and to harvest cells and plate them in 24 well plates, followed by addition of transfection reagents. The transfection reagents were prepared by the protocols described below. Preparing transfection reagents was the first step in the protocol, as the reagents need to incubate with the transfection mediator for a minimum of 30 minutes at RT before addition to the cells. HT-29 cells were harvested and counted on the Countess™, then spun down at 1500rpm for 8 minutes. The supernatant was discarded and the cell pellet was re-suspended in 10% FCS/RPMI, containing no L-glutamine or gentamycin. The cells were then plated 300µl/well, 200 000 cells/well in a 24 well plate. 100µl of transfection medium with Lipofectamine RNAiMAX and siRNA against the target of interest was then added to the designated wells. siTLR3, siTICAM-1, siUNC93b1-1, and siUNC93b1-5 were used in this study. siCTR and was used as a negative control in all siRNA experiments. Medium with only Lipofectamine RNAiMAX was also used as a control. The plate was incubated at 37°C, 5%$CO_2$ for 24 hours. Two tubes containing fresh 10%FCS/RPMI with L-glutamine and gentamycin were prepared. Poly I:C was added to one of the tubes, giving a concentration of 2,5µg/ml or 5µg/ml. Old medium as removed from the 24 well plate, and medium with/without stimuli was added to the wells, as seen in **Figure 3.5**. The plate was then incubated for 20 hours, 37°C, 5%$CO_2$. Supernatant was harvested and frozen at -20°C. 300ul RA1 buffer was added to each well, and the plate was frozen at -80°C.

| | Unstimulated | | | Poly I:C stimulated | | |
|---|---|---|---|---|---|---|
| Medium | | | | | | |
| siRNA | | | | | | |
| siCTR | | | | | | |
| RNAiMAX | | | | | | |

Figure 3-5 siRNA experiment setup.

Transfection reagent preparation using RPMI:

Transfection mixes were made according to **Table 3.3** below. The volumes were up-scaled according to the number of wells to be transfected. RPMI medium with no additives, Lipofectamine RNAiMAX and siRNA were added to a falcon tube, and incubated at RT for 30 minutes. RNAiMAX control was made by adding Lipofectamine 2 x the volume of siRNA to RPMI. After incubation, 100ul of the mixes were added to the designated wells in the setup.

**Table 3-3: Components and volumes used per well in siRNA knockdown experiments**

| Components | siTLR3 (10nM) | siTICAM-1 (10nM) | siUNC93b1-1 (10nM) | siUNC93b1-5 (10nM) | siCTR (10nM) |
|---|---|---|---|---|---|
| siRNA | 0,2µl | 0,4µl | 0,4µl | 0,4µl | 0,2µl |
| Lipofectamine RNAiMAX | 0,4µl | 0,8µl | 0,8µl | 0,8µl | 0,4µl |
| RPMI (no additives) | 99,4µl | 98,8µl | 98,8µl | 98,8µl | 99,4µl |
| Total volume | 100µl | 100µl | 100µl | 100µl | 100µl |

Transfection reagent preparation using Opti-MEM®:

Transfection mixes were made according to **Table 3.4** below. The volumes were up-scaled according to the number of wells to be transfected. The mixes were incubated at RT for 5-10 minutes. Mix 1 and Mix 2 were then mixed and incubated for 30 minutes at RT. Cells were harvested, re-suspended in Opti-MEM medium and plated 100 000 cells/well in a 24 well plate. 100µl of transfection medium with Lipofectamine RNAiMAX and siRNA against the target of interest was then added to the designated wells. The plate was incubated at 37°C, 5%$CO_2$ for 48 hours. Two tubes containing fresh 10%FCS/RPMI with L-glutamine and gentamycin were prepared. Poly I:C was added to one of the tubes, giving a concentration of 2,5µg/ml or 5µg/ml. Old medium as removed form the 24 well plate, and medium with/without stimuli was added to the wells, as seen in **Figure 3.5**. The plate was then incubated for 20 hours, 37°C, 5%$CO_2$.

Supernatant was harvested and frozen at -20°C. 300ul RA1 buffer was added to each well, and the plate was frozen at -80°C.

**Tabell 3-4 Components and volumes used per well in siRNA experiments with Opti-MEM medium**

| | Components | siUNC93b1-1 (50nM) | siUNC93b1-5 (50nM) | siCTR (50nM) | siUNC93b1-1 (25nM) | siUNC93b1 5 (25nM) | siCTR (25nM) |
|---|---|---|---|---|---|---|---|
| **Mix 1:** | Opti-MEM | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl |
| | siRNA | 2µl | 2µl | 1µl | 1µl | 1µl | 0,5µl |
| **Mix 2:** | Opti-MEM | 50µl | 50µl | 50µl | 50µl | 50µl | 50µl |
| | RNAiMAX | 4µl | 4µl | 2µl | 2µl | 2µl | 1µl |

# 4 Results

## 4.1 Screening of metastatic IEC supernatant for overview of cytokine secretion

The cytokine release from cells is crucial in regulating processes and shaping the environment around them. Cancerous and metastatic cells are known to secret cytokines in a fashion differing from normal cells, which can positively or negatively affect the normal cellular processes and environment surrounding them [78, 79]. The expression and role of TLR3 in relation to colon cancer is debated, and this study sought to explore the TLR3 pathway and its secreted products. A proteome profiler array was preformed in order to determine the cytokine secretion of metastatic IEC. The IEC cell line HT-29 cells were chosen for the cytokine screening, as it is known to exhibit metastatic properties[67]. Supernatant from un-stimulated and Poly I:C stimulated HT-29 was assayed using the Human XL Cytokine Array Kit from R&D System, screening for 102 cytokines.

### 4.1.1 Poly I:C stimulated Metastatic IEC HT-29 secrete CXCL10 and CXCL11

The proteome profile of un-stimulated and Poly I:C stimulated HT-29 cells was determined by the Proteome profiler, as described in section 3.2. Initial evaluation of the developed membranes illustrated a clear difference in certain cytokines absent in the un-stimulated sample and present in the stimulated sample, as depicted in **Figure 4.1**. Quantitative analysis by comparing the un-stimulated and stimulated membranes in **Figure 4.1.C and D** indicated that CXCL10, CXCL11, and CCL20 were highly expressed in supernatant of Poly I:C stimulated HT-29 compared to un-stimulated. **Figure 4.1** illustrates the difference in relative mean absorbance and fold induction between un-stimulated and stimulated samples of the array. Several cytokines were present at similar levels in the un-stimulated and stimulated supernatant, however 3 cytokines in particular stood out. The quantification exhibited that CXCL10, CXCL11 and CCL20 were most potently induced in stimulated supernatant, and nearly absent in the un-stimulated supernatant.

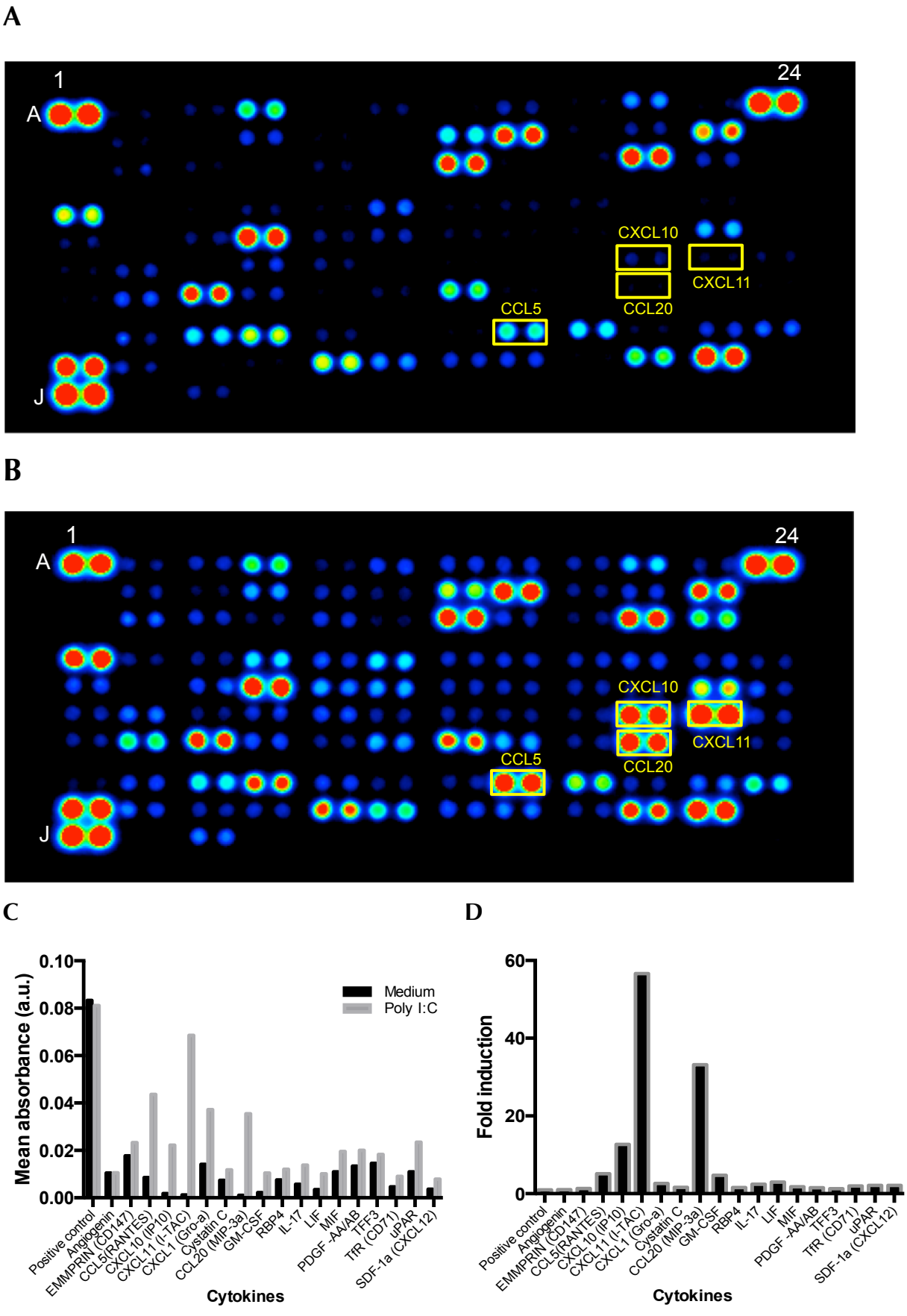



**Figure 4-1: Un-stimulated and Poly I:C stimulated HT-29 cells exhibit different cytokine profiles.** HT-29 cells were either left untreated or stimulated with 5µg/ml Poly I:C and incubated at 37°C, 5%CO2 for 20 hours before

supernatant was harvested for the proteome profiler array. (A) The figure illustrates pixel intensity of the membrane incubated with supernatant from un-stimulated HT-29 cells. (B) Illustrates the membrane incubated with supernatant from Poly I:C stimulated HT-29 cells. (C) Relative mean absorbance of un-stimulated and Poly I:C treated supernatant from HT-29 cells was measured using Image Studio™ 3.1 software. (D) Fold induction of Poly I:C stimulated HT-29 supernatant was plotted against the baseline un-stimulated supernatant cytokine response. Results demonstrate a mean of duplicate cytokine spots from the membrane.

## 4.2 Stimulation with Poly I:C does not impair viability in HT-29 cells

Poly I:C was found to induce potent cytokine responses in HT-29 cells **(Figure 4.1)**. As Poly I:C is known to induce apoptosis in several types of cancer cells, including prostate cancer cells and breast cancer cells, it was appropriate to investigate if Poly I:C had any toxic effect on the HT-29 cells as well[39, 55]. A dose response experiment followed by an MTT assay was performed in order to establish the viability of HT-29 cells when stimulated with different concentrations of Poly I:C. **Figure 4.2** shows that the viability of HT-29 cells was not affected when the cells were treated with Poly I:C. A slight decrease in viability was detected at stimulation with 50µg/ml Poly I:C indicating that concentrations higher than this may affect cell viability.

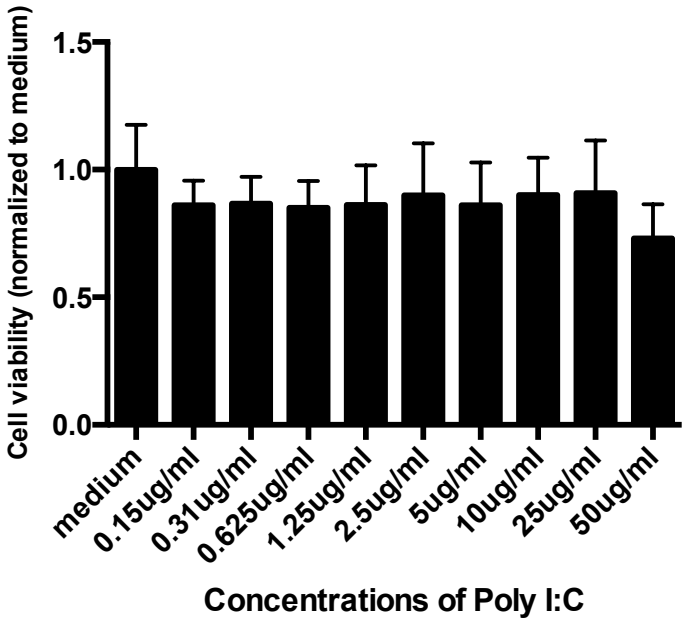



**Figure 4-2: Stimulation with Poly I:C does not impair cell viability in HT-29 cells.** Cells were plated 20 000cells/well in a 96 well plate. The plate was incubated for 24 hours at 37°C, 5%$CO_2$. Stimulation with Poly I:C concentrations 0,15µg/ml, 0,31µg/ml, 0,625µg/ml, 1,25µg/ml, 2,5µg/ml, 5µg/ml, 10µg/ml, 25µg/ml and 50 µg/ml was performed. An MTT assay was performed after 20 hours incubation at 37°C, 5%$CO_2$. The figure represents cell viability normalized to the medium control. The results are presented as mean and standard deviations of four biological replicates and are representative of one experiment.

## 4.3 CXCL10, CXCL11 and IFN-β secretion in response to Poly I:C stimulation

This study wanted to further investigate CXCL10 and CXCL11 secretion in HT-29 cells upon Poly I:C administration, as observed in the initial experiment **(Figure 4.1)**. Therefore, optimal Poly I:C stimulation concentration needed to be determined for future stimulation experiments. A dose response experiment was conducted in order to assess the optimal stimulation concentration of Poly I:C. The experiment was also conducted in a time dependant manner to determine when cytokine secretion occurred. CXCL10 was chosen for an initial experiment, as previous studies on colon cancer cells reports CXCL10 secretion, and since it is an end-product of the TLR3 signalling pathway[80]. CXCL10 cytokine secretion was measured with ELISA. **Figure 4.3** illustrates CXCL10 secretion at different time-points and doses of Poly I:C stimuli. CXCL10 secretion occurs after 5 hours, and decreases after 20 hours of stimulation with Poly I:C. The lowest and most potent dose of Poly I:C for stimulations was 2,5 µg/ml.

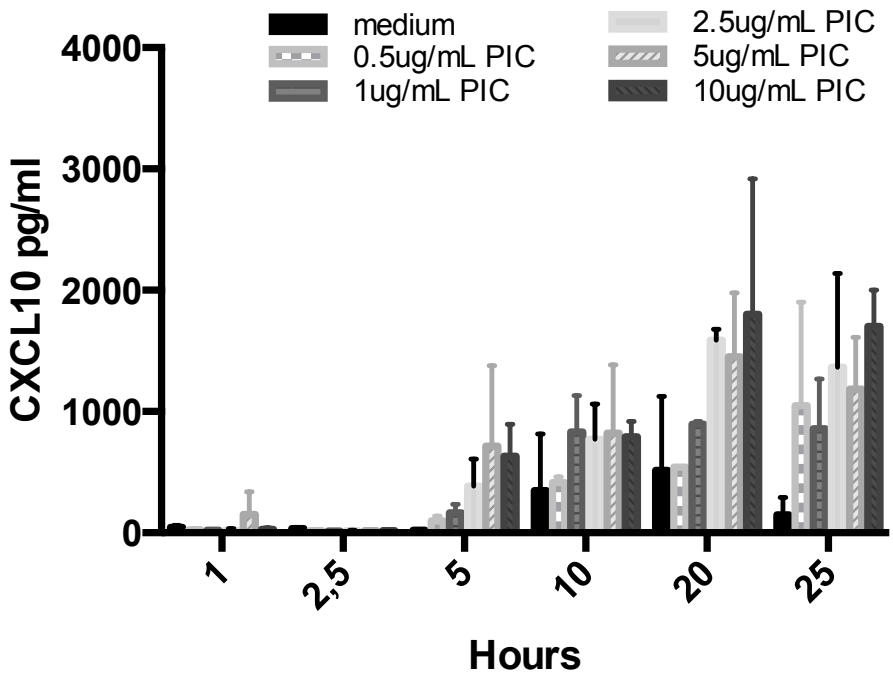



**Figure 4-3: HT-29 cell secrete CXCL10 after 5 hours and responds optimally to 2,5 Poly I:C.** HT-29 cells were plated, 200 000 cells/well, and incubated overnight at 37°C, 5%CO. The cells were stimulated with 0,5µg/ml, 1µg/ml, 2,5µg/ml, 5µg/ml and 10µg/ml Poly I:C in 10%FCS/RPMI. The stimulations were preformed in a time dependant manner, with stimulation times: 25 hours, 20 hours, 10 hours, 5 hours, 2,5 hours and 1 hour. CXCL10 secretion was measured with ELISA. Results are presented as mean and standard deviations of duplicates for each sample.

qRT-PCR was performed in order to determine CXCL10, CXCL11 and IFNβ1 expression on mRNA level. The cytokines were chosen, as they represent different signalling outcomes of the TLR3 signalling pathway. Cytokine expression was assessed at the 5 hour time-point for all concentrations. A constitutive low expression of IFNβ1 was detected, whereas CXCL10 and CXCL11 expression increased with increasing doses of Poly I:C, as depicted in **Figure 4.4**. The three highest doses of Poly I:C; 2,5µg/ml, 5µg/ml and 10µg/ml, gave no great difference in induction. 2,5µg/ml is sufficient to induce a cytokine response in HT-29 cells. **Figure 4.5** illustrates cytokine expression on mRNA level for CXCL10, CXCL11 and IFNβ1 in a time dependent manner. The cells were stimulated with 2,5µg/ml and 10µg/ml Poly I:C. The cytokine expression at these two concentrations was assessed in order to determine if a similar expression was elicited with the higher and lower doses. A similar trend was observed between the two, stimulations with 2,5µg/ml yielded a slight lower response compared to stimulation with 10µg/ml. Hence, stimulations with 2,5µg/ml was deemed sufficient to elicit cytokine secretion in HT-29 cells. Cytokine expression on mRNA level started at 5 hours, peaked at 10 hours, and was declining at 20 hours of Poly I:C stimulation.

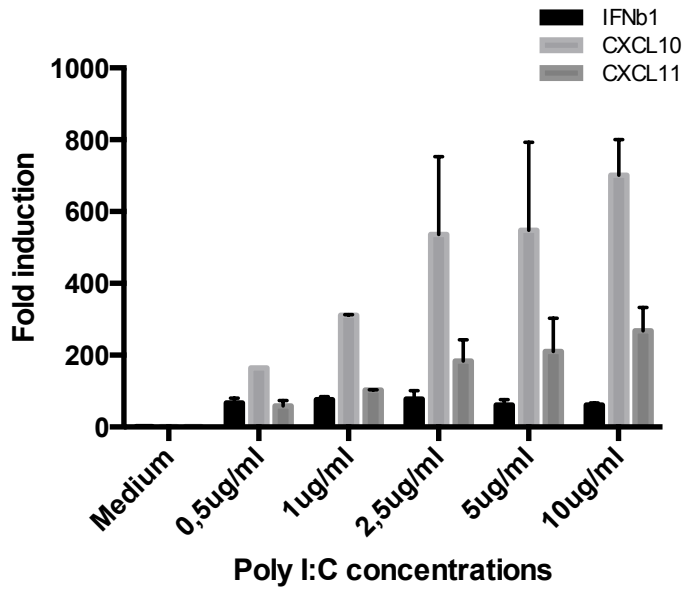



**Figure 4-4: IFNβ1, CXCL10 and CXCL11 fold induction after 5 hours of Poly I:C stimulation indicates that IFNβ1 is not affected by stimulation.** HT-29 cells were plated, 200 000 cells/well, and incubated overnight at 37°C, 5%CO2. The cells were stimulated with 0,5µg/ml, 1µg/ml, 2,5µg/ml, 5µg/ml and 10µg/ml Poly I:C in 10%FCS/RPMI. qRT-PCR was performed in order to determine mRNA levels of IFNβ1, CXCL10 and CXCL11. The 5 hour stimulation is depicted in this result. The results are presented as mean and standard deviations of two biological replicates and are representative of one experiment.

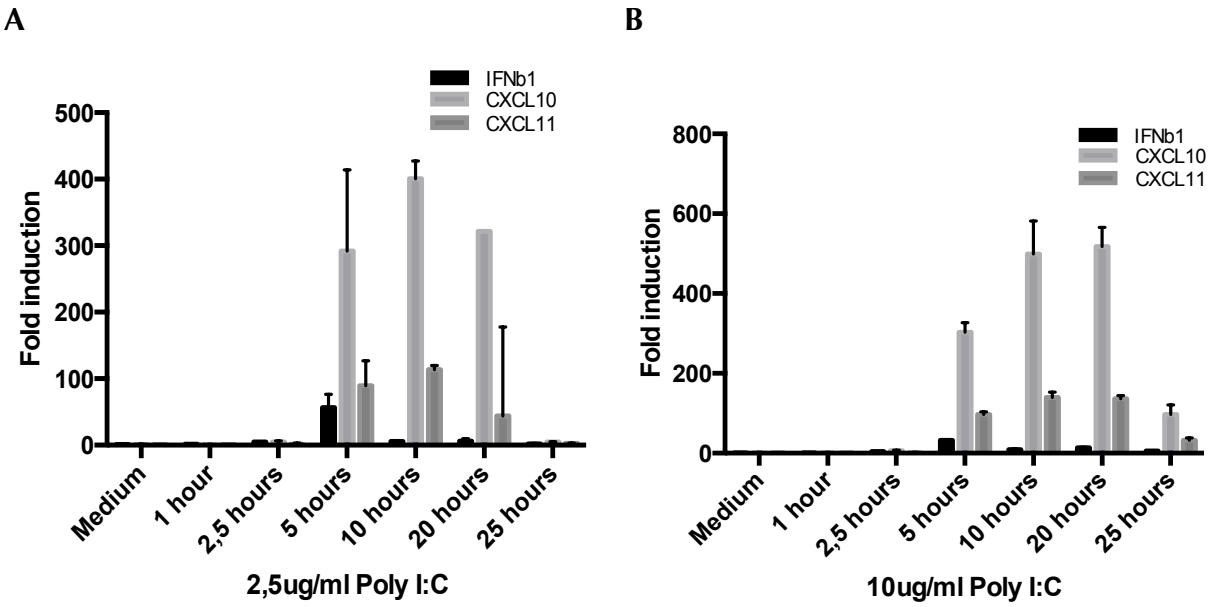
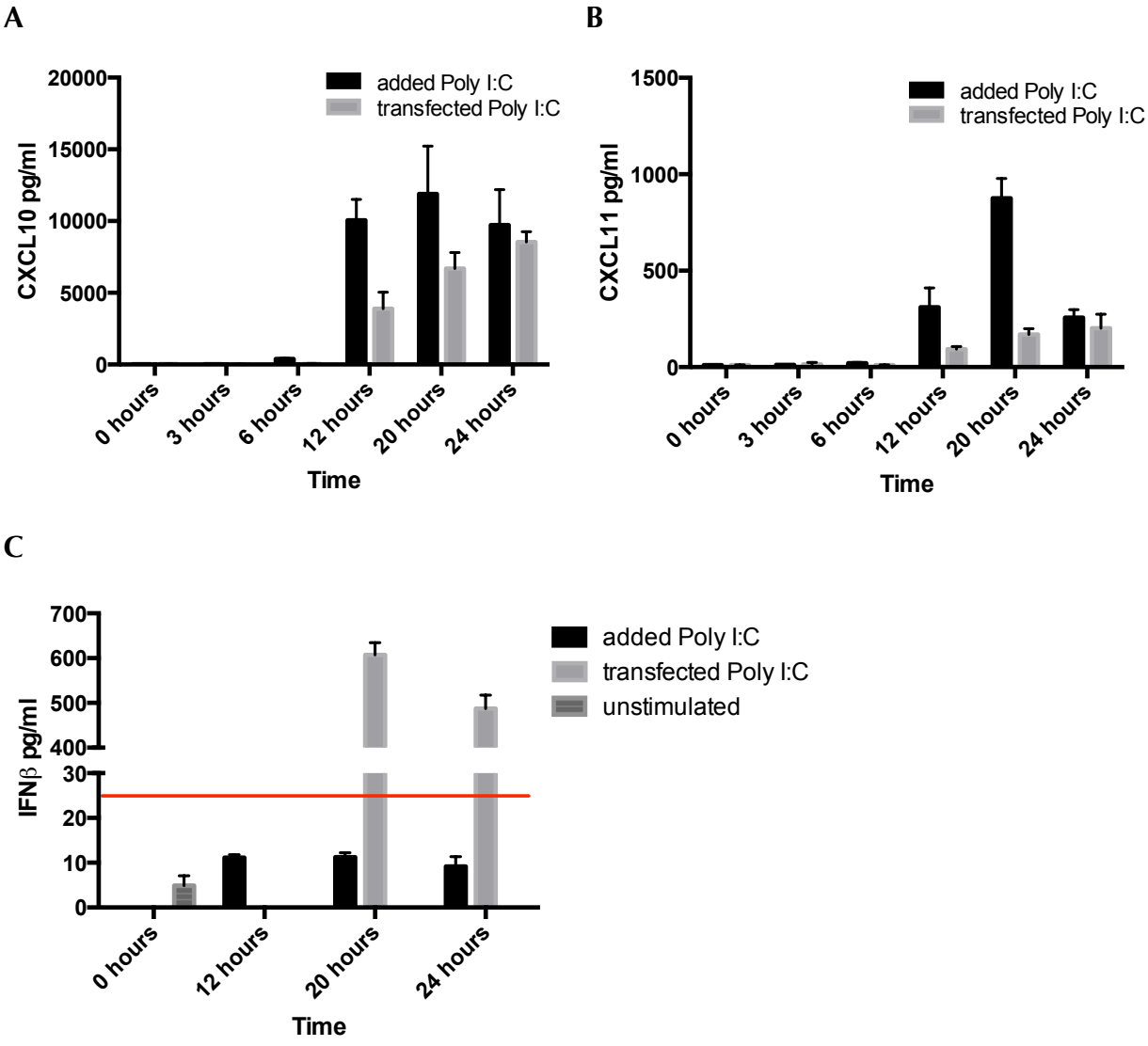



**Figure 4-5: Stimulation with (A) 2,5µg/ml Poly I:C and (B) 10µg/ml Poly I:C shows CXCL10 and CXCL11 induction, but no prominent IFNβ1 induction.** HT-29 cells were plated, 200 000 cells/well, and incubated overnight at 37°C, 5%CO2. The cells were stimulated with 2,5µg/ml or 10µg/ml Poly I:C in 10%FCS/RPMI. The stimulations were preformed in a time dependant manner, with stimulation times: 25 hours, 20 hours, 10 hours, 5 hours, 2,5 hours and 1 hour. qRT-PCR was performed in order to assess mRNA levels of the cytokines. (A) Depicts IFNβ1, CXCL10 and CXCL11 expression at the different time point when stimulated with 2,5µg/ml Poly I:C. (B) depicts I IFNβ1, CXCL10 and CXCL11 expression at the different time point when stimulated with 10µ g/ml Poly I:C.T he results are presented as mean and standard deviations of two biological replicates and are representative of one experiment.

## 4.4 Mode of ligand administration affects cytokine expression in metastatic IEC

Mode of ligand administration was assessed in order to investigate if direct addition of Poly I:C would lead to a different ligand response compared to transfection of the ligand. Supernatant from HT-29 cells treated with either directly added Poly I:C, or Poly I:C administered through transfection was assayed for CXCL10, CXCL11 and IFNβ content by ELISA. The experiment was performed in a time dependent manner in order to investigate when the different cytokines were secreted. **Figure 4.6** illustrates the cytokine content of CXCL10, CXCL11 and IFNβ in response to added and transfected Poly I:C. HT-29 cells stimulated with added Poly I:C gave higher CXCL10 and CXCL11 response compared to stimulation with transfected Poly I:C **(Figure 4.6.A and B)**. This study indicated a later cytokine secretion, occurring after 6 hours of Poly I:C stimulation, compared to the previous dose response experiment conducted **(see figure**

**4.3)**. A slight increase in both CXCL10 and CXCL11 can be observed at the 6 hour time point for added Poly I:C. The response is prominent at the 12 hour time-point and reaches it's peak at the 20 hour time-point. This is followed by a decline at the 24 hour time-point. This indicates that stimulation for 20 hours is optimal, and that stimulation exceeding 20 hours will be on the declining side of the response. CXCL10 and CXCL11 secretion in response to stimulation with transfected Poly I:C displays a later response compared to the directly added poly I:C. The response is observed at the 12 hour time-point and gradually increases toward the 24 hour time-point. An increase in cell death was observed by microscopy after incubation with transfected Poly I:C for the 20 and 24 hour time-points compared to the other time-points. IFNβ secretion displayed no detectable secretion in samples treated with added Poly I:C. IFNβ secretion in transfected samples were only measured at the 20 and 24 hour time-point, where secretion was highest at 20 hours before decreasing.




**Figur 4-6: Stimulation with added Poly I:C yield a more potent induction of CXCL10 (A) and CXCL11 (B) compared to its transfected counterpart. IFNβ secretion(C) presents the opposite trend.** HT-29 cells were plated, 200 000 cells/well, and incubated overnight at 37°C, 5%CO2. The cells were stimulated with 2,5µg/ml Poly I:C directly added in 10%FCS/RPMI, or transfected in using Lipofectamine. The stimulations were preformed in a time dependant manner, with stimulation times: 24 hours, 20 hours, 12hours, 5 hours, 2,5 hours and 1 hour. Cytokine levels in supernatant were assayed with ELISA. Due to assay limitations, the IFNβ secretion in response to transfected Poly I:C was only assayed for the 20 hour and 24 hour time-point. IFNβ secretion in response to added Poly I:C was assyed for 12-, 20-, and 24 hours. Detection limit of the IFNβ ELISA, marked with a red line, was 25pg/ml. Results show mean and standard deviation of biological triplicates.

mRNA expression of CXCL10 and IFNβ1 was examined by qRT-PCR, in order to compare mRNA levels to secreted cytokine. Expression was assessed for both stimulation with added and transfected Poly I:C. **Figure 4.7** illustrates CXCL10 expression in HT-29 cells when stimulated with added or transfected Poly I:C. CXCL10

expression in the added Poly I:C samples displays a similar trend to the secreted CXCL10 **(Figure 4.6.A and 4.7.A)**. The expression can be observed at an earlier time-point on mRNA level, compared to protein expression. The results for CXCL10 with added Poly I:C displays a response in correspondence with a bell shaped curve; an increase, a peak and a decrease in expression. A more potent expression is observed in the samples stimulated with transfected Poly I:C. CXCL10 expression with transfected Poly I:C does not yield the same bell shaped curve response, as a decrease is observed at the 20 hour time-point before another increase in response is seen after 24 hours.

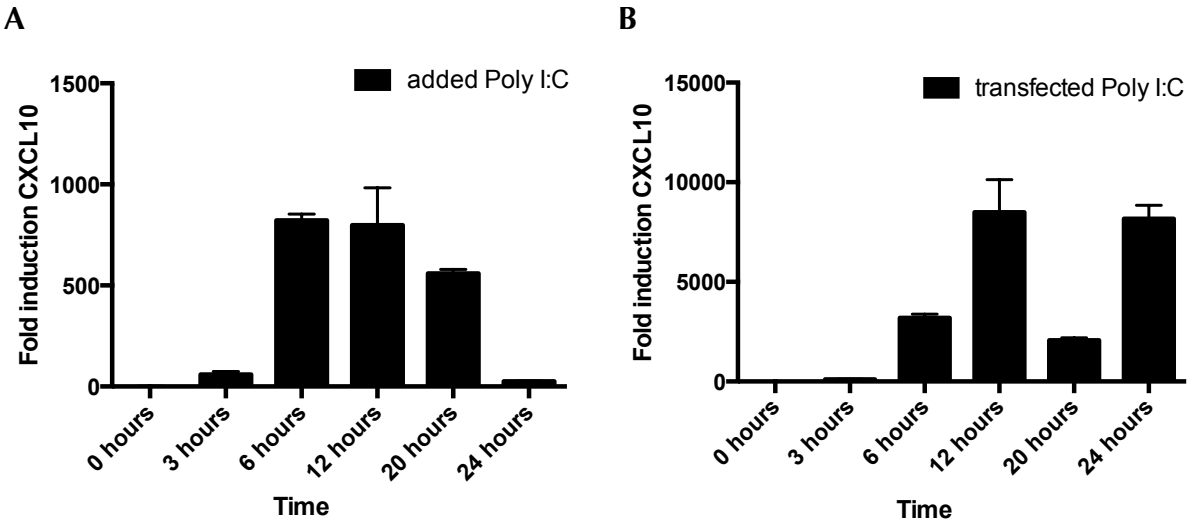



**Figure 4-7: Stimulation with transfected Poly I:C yield a more potent induction of CXCL10 expression on mRNA level compared to stimulation with directly added Poly I:C.** HT-29 cells were plated, 200 000 cells/well, and incubated overnight at 37°C, 5%CO2. The cells were stimulated with 2,5µg/ml Poly I:C directly added in 10%FCS/RPMI, or transfected in using Lipofectamine. The stimulations were performed in a time dependant manner, with stimulation times: 24 hours, 20 hours, 12hours, 5 hours, 2,5 hours and 1 hour. Real time qRT-PCR was performed to assess CXCL10 expression on mRNA level in response to (A) added Poly I:C, and (B) transfected Poly I:C. Results show mean and standard deviation of biological triplicates.

IFNβ1 expression on mRNA level was assessed. HT-29 cells experience a significant expression of IFNβ1 when stimulated with transfected Poly I:C compared to stimulation with directly added Poly I:C **(Figure 4.8)**. qRT-PCR results display a peak of IFNβ1 expression at 3 hours of added Poly I:C stimulation, and subsequent decrease of expression at the following time-points. IFNβ1 expression in response to transfected Poly I:C resembles a bell curve, where peak expression is at the 12 hour time-point followed by a subsequent decrease at the 20 and 24 hours time-points.

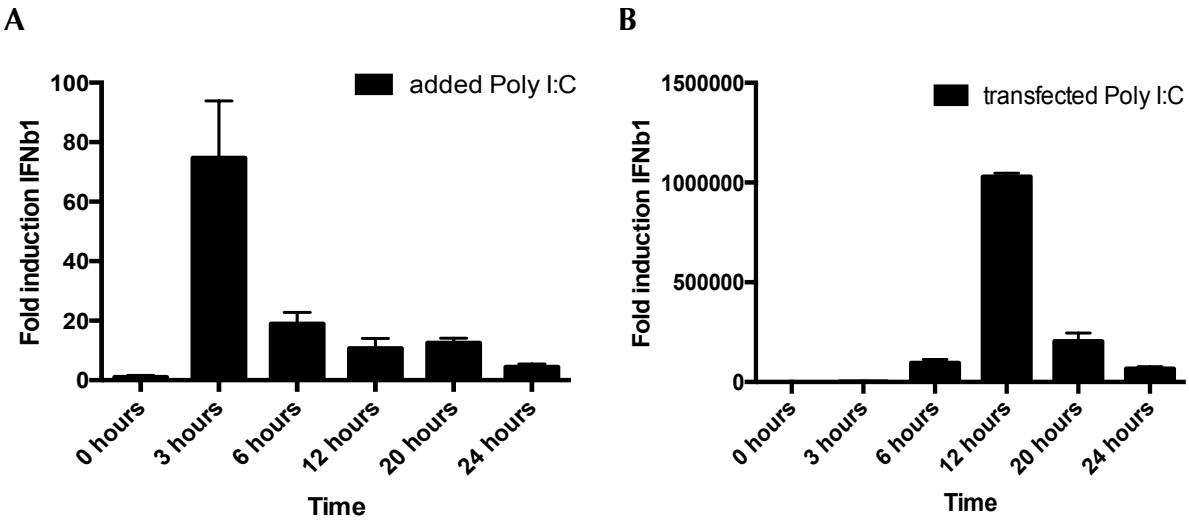



**Figure 4-8: Stimulation with transfected Poly I:C yield a more potent induction of IFNβ1 expression on mRNA level compared to stimulation with directly added Poly I:C.** HT-29 cells were plated, 200 000 cells/well, and incubated overnight at 37°C, 5%CO2. The cells were stimulated with 2,5µg/ml Poly I:C directly added in 10%FCS/RPMI, or transfected in using Lipofectamine. The stimulations were preformed in a time dependant manner, with stimulation times: 24 hours, 20 hours, 12hours, 5 hours, 2,5 hours and 1 hour. Real time qRT-PCR was performed to assess CXCL10 expression on mRNA level in response to (A) added Poly I:C, and (B) transfected Poly I:C. Results show mean and standard deviation of biological triplicates.

## 4.5 CXCL10 and CXCL11 expression in HT-29 is dependant on TLR3

CXCL10 and CXCL11 are known to be secreted as a result of TLR3 activation, through late NF-κB signalling, but can also be induced by cytosolic receptors RIG-I and MDA5[81]. It was therefore necessary to establish which receptor induced the observed cytokine responses. HT-29 cells were treated with siRNA in order to confirm that the CXCL10 and CXCL11 response observed in previous experiments was produced by the TLR3 signalling pathway. siRNA against TLR3 was administered to the cells as described in section 3.7. The amount of CXCL10 and CXCL11 in the supernatant of untreated, siTLR3-, siCTR- and RNAiMAX treated cells were measured with ELISA. **Figure 4.9. A and B** illustrates the CXCL10 and CXCL11 secretion, respectively. In both the CXCL10 and CXCL11 ELISA assays there is a significant reduction of secreted cytokines in the siTLR3 treated samples compared to the medium controls. This indicates that the secretion is TLR3 dependent. In order to confirm that the siRNA against TLR3 successfully knocked down the receptor, a qRT-PCR analysis of the cells collected in the experiment was carried out. Data in **Figure 4.9.C** illustrates that TLR3 had indeed been significantly knocked down in the experiment. qRT-PCR was performed to confirm that TLR3 induced the CXCL10 and CXCL11 secretion, and in the

absence of the receptor the response was lost. Several controls were included, as siRNA can stimulate the TLR3 pathway and potentially mask or prevent knockdown. These controls include non-silencing siRNA (siCTR) and transfecting agent alone (RNAiMAX). A slight induction of siCTR and RNAiMAX compared to the medium sample was observed, hence these may affect the TLR3 induction slightly.

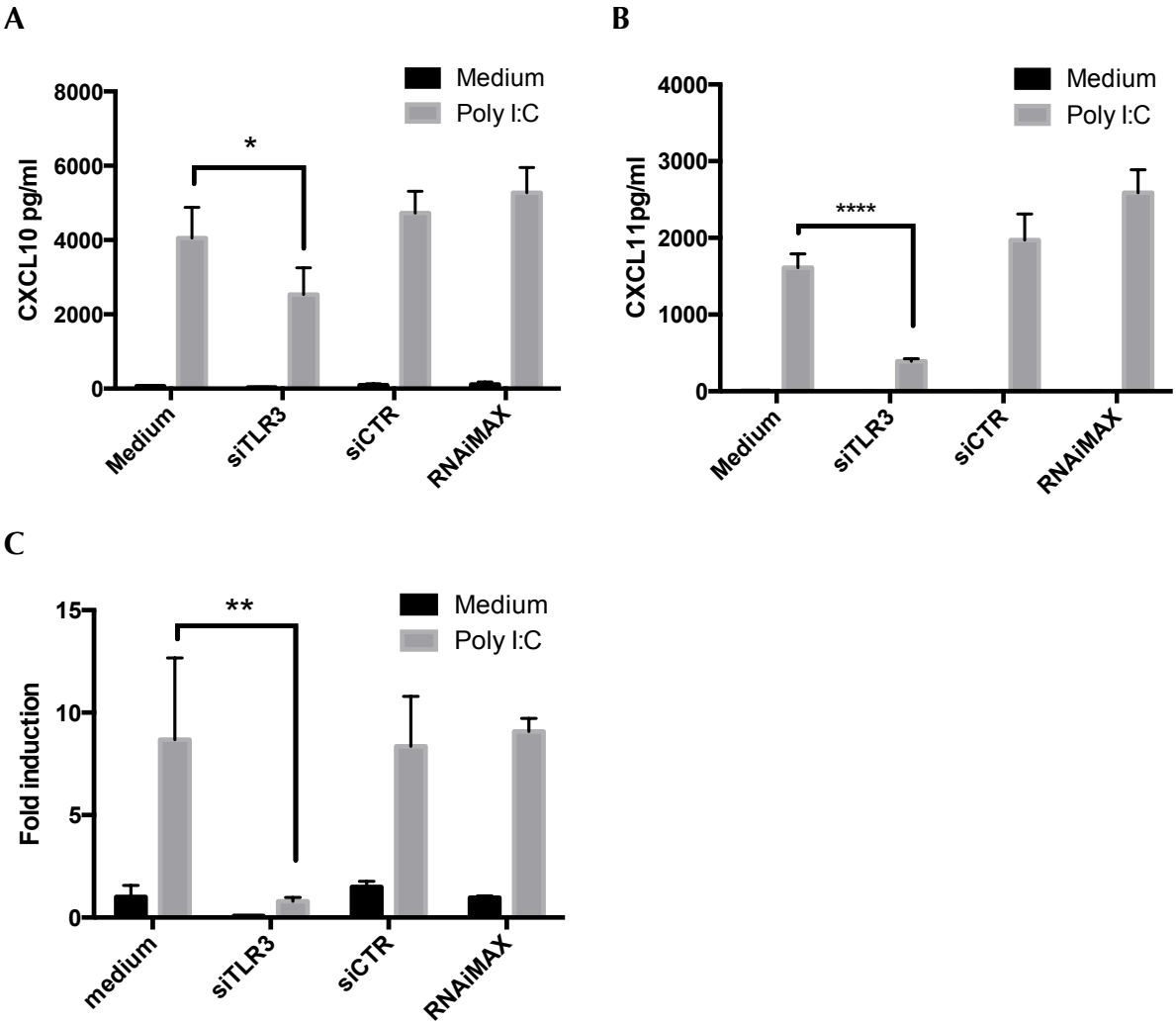



**Figure 4-9: CXCL10 and CXCL11 secretion in HT-29 is TLR3 dependent.** HT-29 cells were plated in a 24 well plate and treated with siTLR3 (10nM) , siCTR (10nM) or RNAiMAX. The plate was then incubated for 24 hours at 37°C, 5%$CO_2$. The plate was stimulated with 5µg/ml Poly I:C or left untreated. The plate was incubated for 20 hours 37°C, 5%$CO_2$. Supernatant was collected, and (A) CXCL10 and (B) CXCL11 were assayed by ELISA. HT-29 cells from siRNA TLR3 knockdown experiment were harvested. RNA was isolated using a NucleoSpin® 8/96 RNA kit by vacuum and cDNA was synthesised. (C) TLR3 cDNA present in the samples was assayed by qRT-PCR using Taqman GAPDH (housekeeping gene) and TLR3 (target gene). All stimulated samples were significantly ($P<0,05$) up regulated in TLR3 compared to their un-treated counterparts with the exception of siTLR3 treated samples. One Way ANOVA with Sidak's multiple comparisons test gave a significant difference between Poly I:C stimulated medium samples and siTLR3 samples for both CXCL10 and CXCL11 assayed by ELISA: (A) $P<0,05$, and (B) $P<0,01$. There was a significant difference been all un-stimulated samples and their stimulated counterparts. There was no significant difference between the un-stimulated medium sample and the TLR3 knockdown sample. Results show mean and

standard deviation of biological triplicates. In the qRT-PCR assay untreated medium was set as the control, and fold induction was calculated accordingly.

## 4.6 TRIF is necessary to produce CXCL10 and CXCL11 in HT-29

TRIF (TICAM-1) is an essential adapter molecule solely found in the TLR3 and TLR4 signalling pathways[2], and knockdown of the adapter molecule was performed in order to gain insight on its role in the secretion of CXLC10 and CXCL11. HT-29 cells were treated with siRNA against TRIF, as well as siCTR, and RNAiMAX as controls. The amount of CXCL10 and CXCL11 in the supernatant of untreated, siTICAM-1-, siCTR- and RNAiMAX treated cells were measured with ELISA. Knockdown yielded the same decrease in secreted CXCL10 **(Figure 4.10.A)** and CXCL11 **(Figure 4.10.B)** as with TLR3 knockdown **(Figure 4.9)**. qRT-PCR was performed on the cells collected from the experiment to confirm knockdown of TRIF on mRNA level. TRIF was significantly knocked down in the Poly I:C treated samples **(Figure 4.10.C)**. The un-treated samples were too low to detect any significant increase or decrease compared to the medium sample.

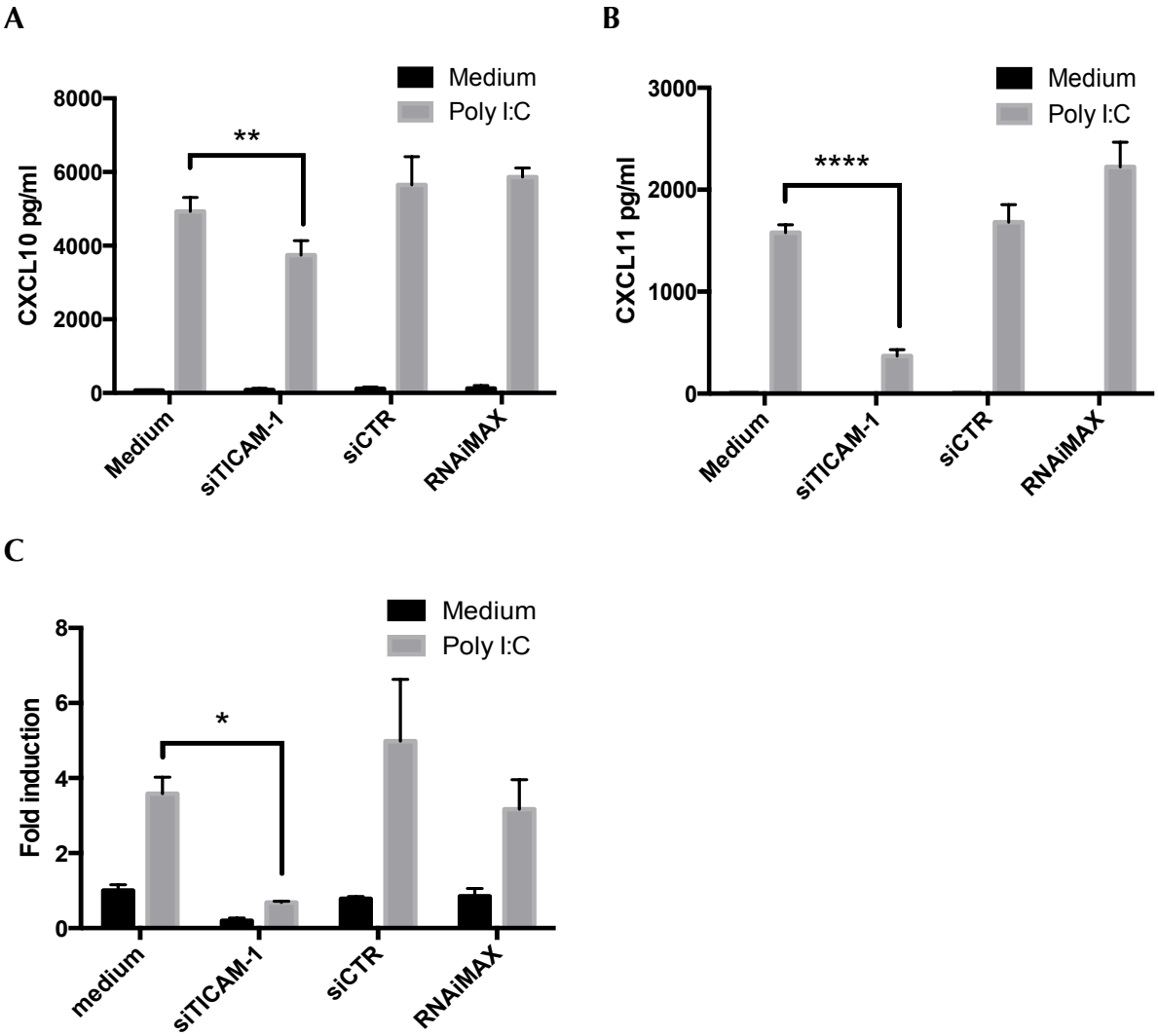



**Figure 4-10: TRIF is necessary for CXCL10 and CXCL11 secretion in HT-29 cells**. HT-29 cells were plated in a 24 well plate and treated with siTICAM-1 (10nM), siCTR (10nM) or RNAiMAX.. The plate was then incubated for 24 hours at 37°C, 5%$CO_2$. The plate was stimulated with 5µg/ml Poly I:C or left untreated. The plate was incubated for 20 hours 37°C, 5%$CO_2$. Supernatant was collected, and (A) CXCL10 and (B) CXCL11 were assayed by ELISA. HT-29 cells from siRNA TICAM-1 knockdown experiment were harvested. RNA was isolated using a NucleoSpin® 8/96 RNA kit by vacuum and cDNA was synthesised. (C) TICAM-1 cDNA present in the samples was assayed by qRT-PCR using Taqman GAPDH (housekeeping gene) and TICAM-1 (target gene). One Way ANOVA with Sidak's multiple comparisons test gave a significant difference between Poly I:C stimulated medium samples and siTICAM-1 samples, $P<0,05$. There was no significant increase between the untreated and treated siTICAM-1 samples. One Way ANOVA with Sidak's multiple comparisons test gave a significant difference between Poly I:C stimulated medium samples and siTICAM-1 samples for both CXCL10 and CXCL11 assayed by ELISA: (A) $P<0,05$, and (B) $P<0,01$. There was a significant difference been all un-stimulated samples and their stimulated counterparts. There was no significant difference between the un-stimulated medium sample and the TICAM-1 knockdown sample. Results show mean and standard deviation of biological triplicates. In the qRT-PCR assay untreated medium was set as the control, and fold induction was calculated accordingly.

## 4.7 UNC93b1 and metastatic IEC

UNC93b1 is proposed to have an essential role in TLR3 trafficking from ER to the endosomal compartment, allowing TLR3 ligand recognition to occur[54]. Knockdown of UNC93b1 was performed in order to determine if CXCL10 and CXCL11 secretion through TLR3 is UNC93b1 dependent. Two different UNC93b1 siRNAs were used in separate experiments referred to as siUNC93b1-1 and siUNC93b1-5 (**Figure 4.11 and figure 4.12**), to ensure knockdown occurred and results were not an artefact of treatment. HT-29 cells were treated with siRNA against UNC93b1, as well as siCTR, and RNAiMAX as controls. The amount of CXCL10 and CXCL11 in the supernatant of untreated, siUNC93b1-, siCTR- and RNAiMAX treated cells were measured with ELISA. qRT-PCR was performed on cells from the knockdown experiment, in order to assess mRNA expression of CXCL10, CXCL11 and UNC93b1. ELISA results from knockdown with siUNC93b1-1 displayed no knockdown trends for CXCL10 and CXCL11 **(Figure 4.11)**. CXCL10 levels were so high that they were close to exceeding the limits of the assay, and no clear trend could be observed. CXCL11 secretion was increased in the siUNC93b1 treated sample, whereas medium, siCTR and RNAiMAX treated samples gave the same level response. qRT-PCR of CXCL10, CXCL11 and UNC93b1 on mRNA level was assessed in order to see if secreted protein correlated with mRNA levels, and to confirm if UNC93b1 was indeed knocked down. Knockdown of UNC93b1 in the untreated samples was successful, but could not be confirmed for the Poly I:C treated samples, likely due to large standard deviations. mRNA expression of CXCL10 and CXCL11 correlated with results from the ELISA assay, giving an increased response in the siUNC93b1 treated samples. UNC93b1 was knocked down on mRNA level, however this did not affect the secretion of CXCL10 and CXCL11.

A second siRNA against UNC93b1, siUNC93b1-5, was also used, as different siRNAs may yield different results. Both ELISA and qRT-PCR were performed as mentioned above. The results of knockdown with siUNC93b1 5 **(Figure 4.12)** display the same trends as for knockdown with with siUNC93b1 1 **(Figure 4.11)**. A slight decrease of CXCL10 secretion in the siUNC93b1 sample assayed on ELISA was observed, however no decrease was observed on mRNA level. CXCL11 levels in siUNC93b1 samples increased in both ELISA and qRT-PCR compared to the medium control. UNC93b1

however displayed a significant decrease, P<0,05, in siUNC93b1 treated samples, both for medium and Poly I:C stimulations. UNC93b1 was indeed knocked down, however CXCL10 and CXCL11 secretion did not display the same trend.

Finally an alternative transfection method using Optimem medium was performed (data not shown) in order to assess if the transfection affected the results, but the same trends were observed as in figure (4.11 and 4.12).

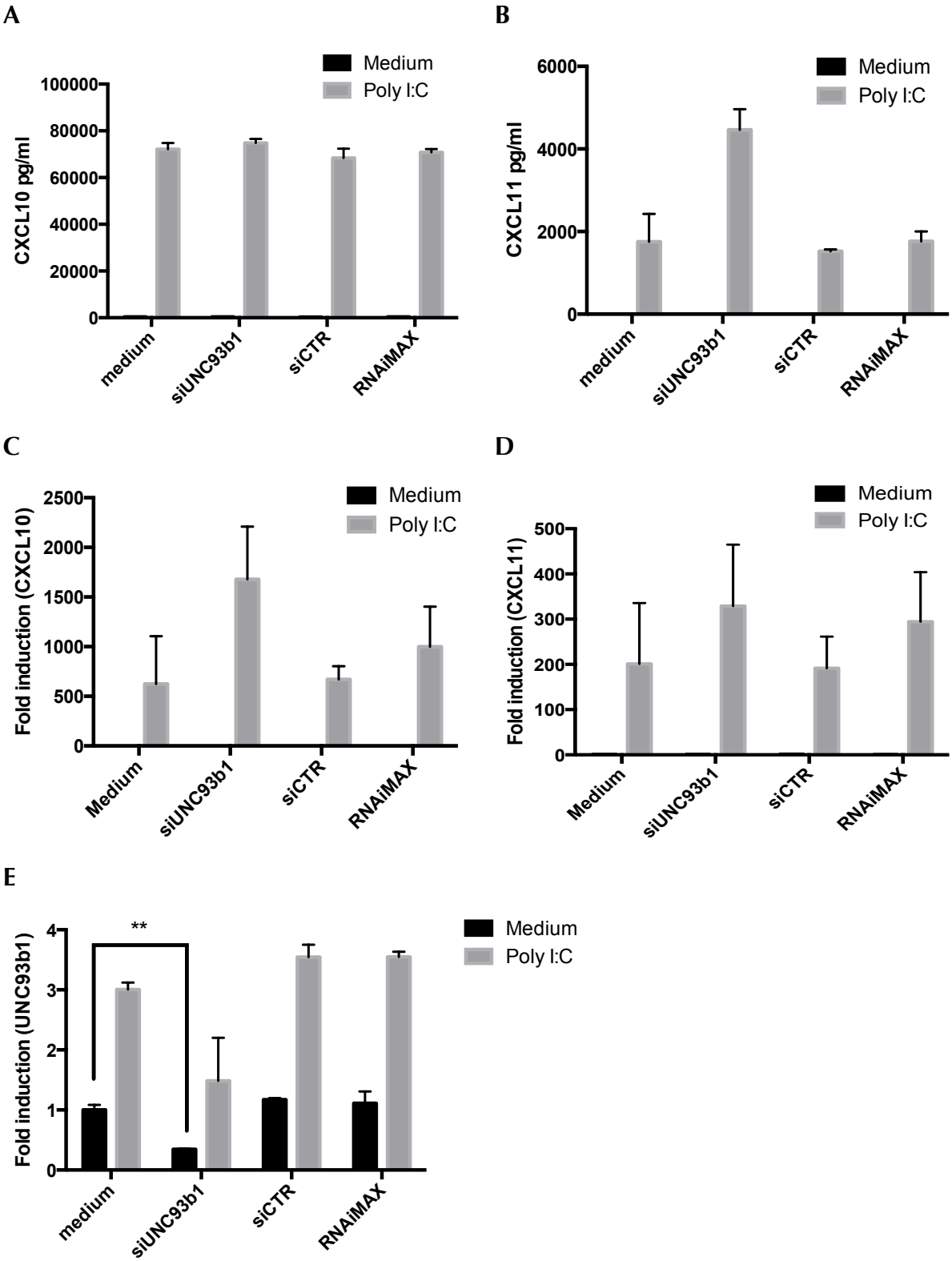



**Figure 4-11: CXCL10 and CXCL11 secretion is not affected by absent UNC93b1 when knocked down using siUNC93b1-1.** HT-29 cells were plated in a 24 well plate and treated with siUNC93b1-1 (10nM), siCTR (10nM) or RNAiMAX. The plate was then incubated for 24 hours at 37°C, 5%$CO_2$. The plate was stimulated with 5µg/ml Poly I:C or left untreated. The plate was incubated for 20 hours, 37°C, 5%$CO_2$. Supernatant was collected, and (A) CXCL10 and (B) CXCL11 were assayed by ELISA. HT-29 cells from siRNA UNC93b1 knockdown experiment were

harvested. RNA was isolated using a NucleoSpin® 8/96 RNA kit by vacuum and cDNA was synthesised. CXCL10 (C), CXCL11 (D) and UNC93b1 (E) cDNA present in the samples were assayed by qRT-PCR using Taqman GAPDH (housekeeping gene) and CXCL10, CXCL11 and UNC93b1 (target genes). Un-paired two- tailed t-test with Welch's correction gave a significant difference between untreated medium sample and untreated siUNC93b1 sample, $P<0,05$ (E). There was no significant difference between the Poly I:C treated medium sample and Poly I:C treated siUNC93b1 sample. No statistics were performed for the samples assayed for CXCL10 and CXLC11 by ELISA and qRT-PCR, as they all displayed an increase in siUNC93b1 treated samples compared to the medium control. Results show mean and standard deviation of biological triplicates. In the qRT-PCR assay untreated medium was set as the control, and fold induction was calculated accordingly.

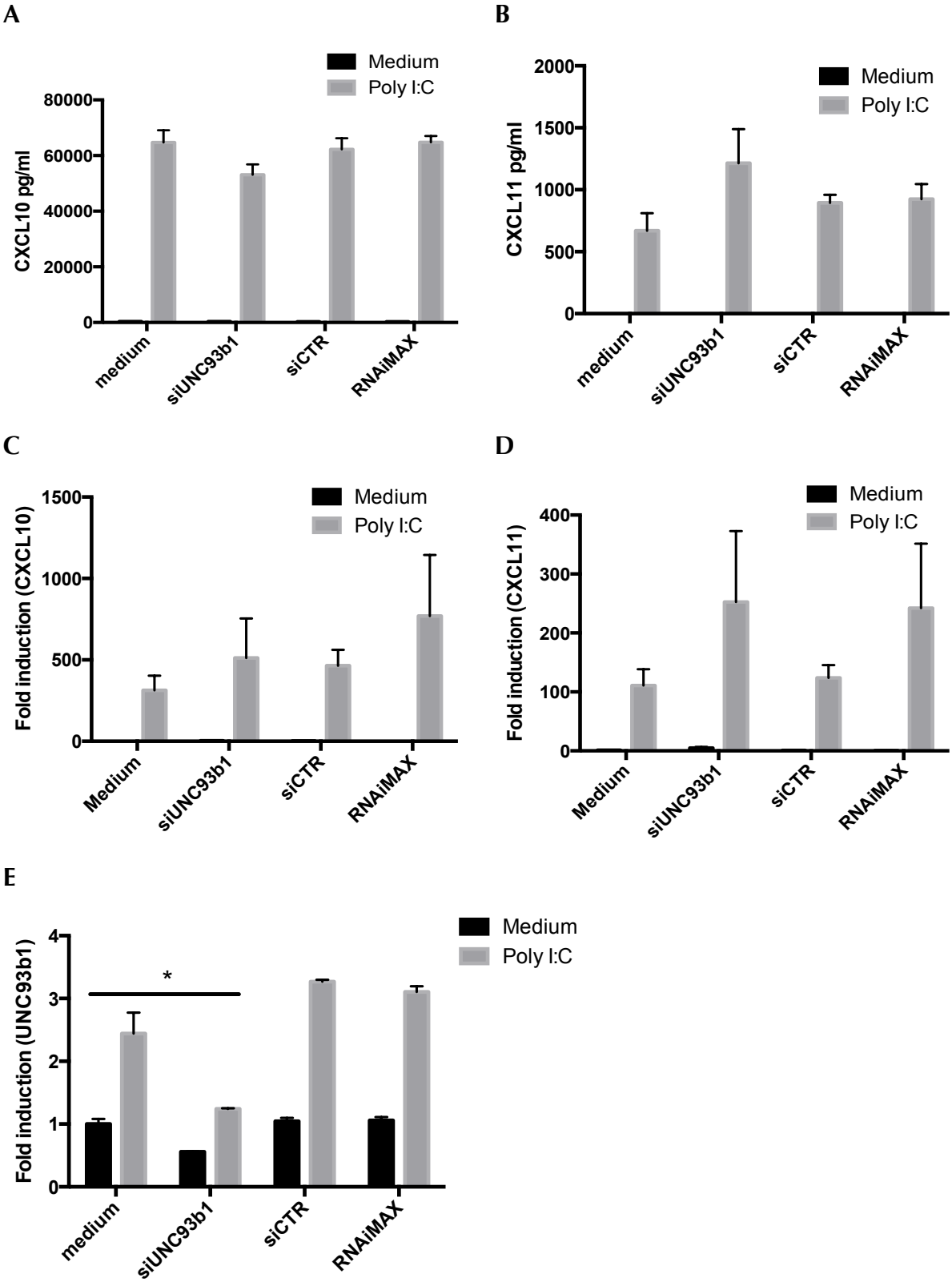



**Figure 4-12: CXCL10 and CXCL11 secretion is not affected by absent UNC93b1 when knocked down using siUNC93b1-5.** HT-29 cells were plated in a 24 well plate and treated with siUNC93b1-5 (10nM), siCTR (10nM) or RNAiMAX. The plate was then incubated for 24 hours at 37°C, 5%CO2. The plate was stimulated with 5µg/ml Poly I:C or left untreated, and incubated for 20 hours, 37°C, 5%CO2. Supernatant was collected, and (A) CXCL10 and (B)

CXCL11 were assayed by ELISA. HT-29 cells from siRNA UNC93b1 knockdown experiment were harvested. RNA was isolated using a NucleoSpin® 8/96 RNA kit by vacuum and cDNA was synthesised. CXCL10 (C), CXCL11 (D) and UNC93b1 (E) cDNA present in the samples were assayed by qRT-PCR using Taqman GAPDH (housekeeping gene) and CXCL10, CXCL11 and UNC93b1 (target genes). Un-paired two- tailed t-test with Welch's correction gave a significant difference between untreated medium sample and untreated siUNC93b1 sample, $P<0,05$ (E). A significant decrease was also observed between Poly I:C treated medium sample and Poly I:C treated siUNC93b1 sample, $P<0,05$. No statistics were performed for the samples assayed for CXCL10 and CXLC11 by ELISA and qRT-PCR, as they all displayed an increase in siUNC93b1 treated samples compared to the medium control. Results show mean and standard deviation of biological triplicates. In the qRT-PCR assay untreated medium was set as the control, and fold induction was calculated accordingly.

# 5 Discussion

TLRs have been increasingly linked to prolonged inflammation, cancer progression and metastasis. Up-regulation of TLRs in several cancers, when compared to expression in healthy cells, has previously been reported[82]. The secreted products of TLR signalling have also been linked to cancer promotion[55]. Contrary, activation of TLRs has also been shown to have cancer modulatory properties, and agonists of TLRs have been proposed as therapy in several cancers[82, 83]. TLR3 and its products have recently been reported expressed in different cancers, and display ambiguous roles. Recent studies have uncovered TLR3 to have an emerging role in relation to colon cancer progression, with a particular emphasis on the function of its secreted cytokines[61, 73]. This study investigated the properties of TLR3 in relation to colon cancer, using the metastatic colon cancer cell line HT-29 to observe signalling outcomes and effects of receptor activation.

## 5.1 CXCL10, CXCL11 and IFN-β in response to Poly I:C

The HT-29 cell line was screened using a human XL cytokine profiler kit to determine basal cytokine secretion and to investigate cytokine induction in response to the TLR3 ligand Poly I:C. Interestingly, un-stimulated HT-29 cells secreted a number of cytokines **(Figure 4.1.A)**. Cancer cells are known to modulate their surroundings, and HT-29 cells have previously been shown to secrete cytokines capable of this on a basal level[84]. Poly I:C stimulated HT-29 cells demonstrated potent secretion of CXCL10, CXCL11 and CCL20 **(Figure 4.1.B)**. Quantifying the mean absorbance of the samples on the membrane demonstrated the same trend, where CXCL11, CCL20 and CXCL10 yielded the highest inductions, respectively **(Figure 4.1.C and D)**. HT-29 cells are known to secrete CXCL10 upon Poly I:C stimulation[85]. CCL20 secretion has also been demonstrated in HT-29, but not directly in relation to Poly I:C stimulation[86].

CXCL10 and CXCL11 both belong to the same family of chemokines, and bind to the same receptor CXCR3, whereas CCL20 binds to the receptor CCR6[87, 88]. CCL20 and its receptor have been demonstrated in colon cancer, with a definite up-regulation in colon cancer samples compared to normal healthy controls[62]. This study found no

basal secretion of CCL20 from the colon cancer cell line HT-29 **(Figure 4.1.A)**, however the chemokine was highly up-regulated upon Poly I:C stimulation **(Figure 4.1.B-D)**. This suggests that activation of dsRNA receptors in cancer cells may be a potential source of CCL20 induction in colon cancer. CXCL10 and CXCL11 have also been increasingly liked to CRC progression and metastasis, mainly by activating their receptor CXCR3[49]. No basal secretion of CXCL10 and CXCL11 was detected in HT-29 cell **(Figure 4.1.A)**, however both chemokines were potently induced upon Poly I:C stimulation **(Figure 4.1.B-D)**. HT-29 cells have previously been demonstrated to secrete CXCL10 upon TLR3 stimulation[85]. This implies that dsRNA sensors, such as TLR3, are capable of inducing secretion of these chemokines in colon cancer. This study chose to further focus on chemokines CXCL10 and CXCL11, as they belong to the same sub-family of cytokines and share the same receptor.

A dose response experiment performed in a time-dependent manner was conducted in order to investigate optimal stimulation concentration with Poly I:C in addition to when cytokine secretion occurred. This experiment found that CXCL10 secretion occurred after 5 hours of Poly I:C stimulation, and that the response declined after 20 hours of stimulation. A Poly I:C stimulation with 2,5 µg/ml was found to be optimal **(Figure 4.3)**. The results indicate that the cytokine production is intermediate or late, since the cytokine secretion started as late as 5 hours, and peaked at 20 hours **(Figure 4.3)**. This may be a result of late NF-κB induction and IRF3 or IRF7 induction. IRF3 alone is known to directly induce CXCL10[89], however, a stronger induction of the cytokine could be expected with activation of both NF-κB and IRF3[90]. This may explain why the peak secretion is as late as 20 hours, if IRF translocation initiates the initial CXCL10 secretion, and late NF-κB may contribute to a more potent secretion once activated. Translocation of IRF3 can also lead to production of IFNβ, which may also act in an autocrine manner and stimulate further production of CXCL10 upon ligand binding to IFNAR[91].

Further investigation of CXCL10 and CXCL11 on mRNA level showed that CXCL10 and CXCL11 expression gradually increased with time and peaked at 10 and 20 hours respectively, and expression was almost lost at 25 hours **(Figure 4.4 and 4.5)**. This

supports the findings on protein level. Increasing concentrations of Poly I:C produced a more prominent cytokine expression, and CXCL10 expression was overall higher than CXCL11 expression. This suggests that CXCL10 is more potently induced in response to Poly I:C than CXCL11.

IFNβ is a known product of TLR3 activation and was therefore also assessed. IFNβ levels were constitutively low compared to CXCL10 and CXCL11, and increasing doses of Poly I:C did not affect IFNβ expression **(Figure 4.4 and 4.5)**. This was of interest as IFNβ is one of the end-products in TLR3 signalling though IRF3 and/or IRF7, and the expression of IFNβ was expected[89, 92]. This suggests that expression of IFNβ is not potently induced through TLR3 in HT-29 cells. As a result, the signalling pathways induced upon Poly I:C stimulation should be further explored. A possible way to investigate the pathways could be to use ScanR and stain NF-κB, IRF3, and IRF7 in order to determine nuclear translocation, to assess which pathways are more induced upon stimulation. IFNβ is only produced by IRF activation[93], however as both IRF3 and IRF7, alone or as heterodimers, are capable of producing IFNβ it would beneficial to assess all three transcription factors[89]. Alternatively a NF-κB luciferase reporter assay could be used to assess NF-κB activation. This study further investigated if induction of CXCL10, CXCL11 and IFNβ cytokines could be affected by the mode of ligand administration to the cells.

## 5.2 Mode of ligands administration affects cytokine secretion

Different modes of Poly I:C administration were assessed to establish if it affected CXCL10, CXCL11 and IFNβ secretion. Poly I:C stimulation was performed by either directly adding or transfecting Poly I:C into the cells in order to assess the outcome this had on cytokine release by HT-29 cells. In order to detect when cytokine secretion occurred, the experiment was conducted in a time dependent manner.

Added Poly I:C was found to induce CXCL10 secretion more potently compared to transfected Poly I:C **(Figure 4.6)** on protein level. CXCL10 secretion in response to added Poly I:C was first detected after 6 hours of stimulation, reached its peak at 20

hours of stimulation and started to declined after 24 hours of stimulation. CXCL10 secretion in response to transfected Poly I:C was detected later, at 12 hours, and had not started to decline at 24 hours of stimulation. This suggests that the CXCL10 response to transfected Poly I:C is later than the response to added Poly I:C. This may be due to the way the ligand is introduced. Previous studies have found that transfected Poly I:C activates several receptors, such as RIG-I and MDA5 in addition to TLR3[81] This may effect the CXCL10 secretion, as the pathways involved may have differing effects. Recent studies have reported TLR3 on the cell surface, and this may in part explain the more rapid induction of CXCL10 in the response to addition of Poly I:C[94]. Surface TLR3 may be more readily available for ligand binding, and a synergistic effect of endosomal and surface TLR may strengthen the induction of CXCL10. Inhibition of endocytosis could assess if the HT-29 cells are dependent on ligand internalization in order to produce a TLR3 response.

CXCL10 expression on mRNA level was in discrepancy with results observed on protein level. CXCL10 expression was higher for stimulation with transfected Poly I:C compared to added Poly I:C **(Figure 4.7.)**. This may be due to un-translated CXCL10 mRNA, the mRNA may be degraded or not all translated into protein. Transfection with Poly I:C is also known to simultaneously induce apoptosis and induce IFNβ expression, through RIG-I, MDA5 and TLR3 activation[81]. This may explain why the protein was not expressed. CXCL11 secretion on protein level displayed the same trends as CXCL10, however to a much lesser extent. This was consistent with earlier findings in this study where CXCL11 levels overall were lower than CXCL10 levels.

Interestingly, although IFNβ induction was not observed upon addition of Poly I:C, as previously discussed, transfection of Poly I:C into the cells induced strong IFNβ responses in HT-29 cells.

Due to assay limitations, not all time points were assessed for IFNβ, and this should be performed in further studies to better understand IFNβ secretion in HT-29 cells. Stimulation with added Poly I:C gave no prominent secretion of IFNβ on protein level **(Figure 4.6)**. However, all values for secreted IFNβ when stimulated with added Poly

I:C were below the detection limit of the assay, and should be repeated with a ELISA with better sensitivity. Nonetheless, this indicates that little or no IFNβ is produced in response to added Poly I:C in HT-29 cells. The two time-points assessed for IFNβ secretion in response to transfected Poly I:C both indicated a substantial secretion of IFNβ **(Figure 4.6)**. Similar trends were observed on mRNA level, where the IFNβ were constitutively low in added Poly I:C stimulated samples compared to transfected Poly I:C stimulated samples **(Figure 4.8)**. One outlier value was observed for the 3 hour time-point with added Poly I:C, where IFNβ levels were more prominently induced. This could indicate an initial response to added Poly I:C, that rapidly declined. This time-point was not assayed on ELISA for IFNβ secretion in this study and therefore no basis to explore this result is available. In order to better understand the IFNβ secretion in HT-29 cells, this experiment could be repeated with additional time-points and assayed both on ELISA and qRT-PCR.

The difference in IFNβ secretion observed with different modes of ligand administration suggests that different pathways are activated or modulated upon stimulation. Previous studies in prostate cancer cells have reported that transfected Poly I:C induce apoptosis in these cells alongside a strong IFNβ induction. [81]. This is in concordance with the findings in this study, where potently induced IFNβ and cells death was observed in samples treated with Poly I:C **(Figure 4.6 and 4.8)**. Palchetti et al found that added Poly I:C did not induce the same apoptotic pathways as transfected Poly I:C in prostate cancer cells. Interestingly, they found that transfected Poly I:C induced apoptosis by two distinct pathways. One apoptotic pathway was activated trough TLR3, and was IRF-3 independent, and the other induced apoptosis through MDA5, RIG-I and IRF3, accompanied by potent IFNβ secretion[81]. The same pathways should be explored in relation to HT-29 and transfected Poly I:C induced responses. siRNA knockdown experiments of TLR3, RIG-I and MDA5 should be performed to assess their role in relation IFNβ production and possible cell death in HT-29 cells. Stimulation of HT-29 cells with added Poly I:C did not give IFNβ production. IFNβ would be expected upon TLR3 activation, suggesting that the pathways leading to IFNβ production is not potently induced by added Poly I:C or is alternatively modulated resulting in only CXCL10 and CXCL11 secretion. The TLR3 pathway should be further explored in order

to understand the events leading to CXCL10 and CXCL11 secretion, but not IFNβ secretion. This study further investigated if the observed CXCL10 and CXCL11 secretion was dependent on TLR3.

## 5.3 TLR3 and TRIF are essential in CXC10 and CXCL11 secretion

CXCL10 and CXCL11 are known products of TLR3 signalling, however other pathways can also induce them upon Poly I:C stimulation, such as the RLRs RIG-I and MDA5[95]. In order to determine if the cytokine secretion observed was induced through TLR3, knockdown of the receptor was performed **(Figure 4.9)**. Significant reduction of secreted CXCL10 and CXCL11 was observed in TLR3 knockdown samples on protein level. These results are consistent with the observed knockdown of TLR3 on mRNA level **(Figure 4.9)**. Knockdown of TRIF was also preformed in order to confirm dependence on TLR3, as it is an essential adapter molecule to ensure signalling[3]. Knockdown of TRIF was confirmed on mRNA level, and was consistent with significant reduction of CXCL10 and CXCL11 secretion on protein level **(Figure 4.10)**. Taken together: knockdown of TLR3 and TRIF displayed a significant reduction of CXCL10 and CXCL11 **(figure 4.9 and 4.10)**, showing that they are indeed dependent on TLR3 and TRIF in order to be secreted. This consisted with other studies indicating that CXCL10 secretion is TLR3 dependent in HT-29 cells[85]. It would be beneficial to establish if this signalling through TLR3 occurred from endosomes of from TLR3 located on the cell surface. Future studies could assess TLR3 localization using confocal microscopy and stain for the receptor in both a Poly I:C stimulated situation and in normal conditions. This would provide information on the nature of TLR3 in different conditions.

## 5.4 CXL10 and CXCL11 secretion in relation to UNC93b1 is unclear

UNC93b1 knockdown was preformed in order to assess if CXCL10 and CXCL11 secretion was dependent on the protein. UNC93b1 is linked to trafficking of TLR3 to endosomes, and implied to be required for ligand recognition [96]. As CXCL10 and CXCL11 secretion was demonstrated dependent on TLR3 in this study **(Figure 4.9 and 4.10)**, naturally UNC93b1 dependence was also investigated. CXCL10 and CXCL11 levels in UNC93b1 knockdown samples were assayed both on protein and mRNA level

with ELISA and qRT-PCR respectively **(Figure 4.11 and 4.12)**. Knockdown was performed with two different siRNA against UNC93b1 in separate experiments to ensure that the observed response is not an artefact of treatment, but indeed knockdown of the protein.

Results of knockdown with siUNC93b1-1 and siUnc93b1-5 on mRNA level showed that the protein had been significantly knocked down in medium samples for both siRNA, whereas only UNC93b1-5 showed significant knockdown in Poly I:C stimulated samples **(Figure 4.11.E and 4.12.E)**. CXCL10 and CXCL11 levels for the same UNC93b1 knockdown samples were measured on ELISA **(Figure 4.11.A-B and 4.12.A-B)**. CXCL11 levels were similar for the Poly I:C stimulated samples in the medium, siCTR and RNAiMAX samples, whereas a clear increase was observed in the siUNC93b1 sample. This indicated an up-regulation of CXCL11, rather than knockdown. Similar trends were observed on protein level when using siUNC93b1-5 **(Figure 4.12)**. The transfection agent RNAiMAX gave a slight increase of CXCL10 and CXCL11 compared to medium and siCTR samples, hence in this case the transfection agent alone might stimulate the pathway itself, or the increase can be a result of general treatment. Despite diluting the sample 1:20 and increasing the standard 2 fold, **(Figure 4.11.A and 4.12.A)**, CXCL10 levels nearly exceeded the limits of the assay, and any trend was hard to observe. Severe dilution of the sample could affect the observed results. The experiment should therefore be repeated, preferably with a different ELISA system that can handle such high values. qRT-PCR of siUNC93b1-5 samples for CXCL10 and CXCL11 **(Figure 4.12. C and D)** displayed a similar trend to CXCL10 and CXCL11 in siUNC93b1-1 knockdown samples **(Figure 4.11. C and D)**, where both CXCL10 and CXCL11 was induced in the siUNC93b1 samples. siUNC93b1-1 may affect the induction of CXCL10 and CXCL11 more potently compared to siUNC93b1-5, as levels of the cytokines were overall higher in the siUNC93b1-1 experiment both for ELISA and qRT-PCR results.

ELISA and qRT-PCR of CXCL10 and CXCL11 **(Figure 4.11, 4.12)** displayed no decrease in cytokine secretion when UNC93b1 was knocked down. However, the results of UNC93b1 knockdown on qRT-PCR showed a significant knockdown of the UNC93b1

**(Figure 4.11.E and 4.12.E)**. Several factors may explain these results. If TLR3 surface expression is present in HT-29 cells, the components necessary for signalling may already be present, and UNC93b1 may have no functional role in this scenario. Recent studies have reported cell surface localization of UNC93b1 alongside TLR3[96], and this should be further explored as the role of UNC93b1 in CXCL10 and CXCL11 secretion remained undetermined in this study. Confocal microscopy with fluorescent staining of the TLR3 receptor could investigate the localization of the receptor in HT-29 cells, and characterize expression of the receptors in these cells. An interesting option to pursue would be to assess both un-stimulated and Poly I:C stimulated cells with confocal microscopy and note the expression and possible differences between the two. If receptor expression increases when Poly I:C is administered, the receptor would likely be dependent on some form of trafficking to achieve transport from the ER to the cell membrane. However, if baseline expression on the cell surface is high regardless, this may explain the inconclusive results in relation to UNC93b1 dependence. The increase of CXCL10 and CXCL11 secretion observed in UNC93b1 knockdown samples **(Figure 4.11 and 4.12)** may be explained by the use of siRNA itself, as siRNA has been reported to activate TLR3 in some cases [97]. siRNA is double stranded and is supposed to trigger RNAi when administered to cells, however TLR3 is a sensor for dsRNA, and as a result the siRNA can trigger TLR3 activation. Subsequently, increased production of CXCL10 and CXCL11 may occur.

Other aspects of the TLR3 pathway, such as TBK1, STAT1, P38, JNK and ERK1/2 should also be explored in order to form a better understanding of the pathway and its related components in HT-29 cells.

## 5.5 Poly I:C does not impair viability in HT-29 cells

Poly I:C is known to induce apoptosis in several cell lines. Dying cells can release endogenous ligands and trigger TLR3 activation[98]. In order to ensure that the results observed were produced by Poly I:C administration alone, and not dying cells, the cell viability of HT-29 cell in response to Poly I:C stimulation was assessed. No notable reduction in cell viability was observed when cells were subjected to Poly I:C stimulation **(Figure 4.2)**. Flow cytometry with AnnexinV/PI staining could determine if

apoptosis of cells had indeed occurred. Several studies have reported that administration of Poly I:C leads to rapid apoptosis, in a TLR3 and TRIF dependent manner[39, 55, 83]. Induction of apoptosis in different cancers has also been linked to CXCL10, through its receptor CXCR3-B[99]. However reduction in cell viability was not observed in the conducted experiment. This implies that HT-29 cells do not experience cell death upon Poly I:C administration. CXCL10 can also induce apoptosis through CXCR3, and despite observed CXCL10 secretion in HT-29 cells, cell viability was not affected. This could suggest that HT-29 cells either do not express CXCR3 receptors or that the alterative splice form of the receptor, CXCR3-A is present. Previous studies have investigated CXCR3 expression in several colon cancer cell lines, HT-29 among them, and found that HT-29 indeed expressed CXCR3[72]. CXCR3 activation was found to have anti-apoptotic properties, and is indicated to have an important role in tumour progression by promoting survival and migration[100]. This implicates the anti-apoptotic splice form of the CXCR3 receptor is present, CXCR3-A, and not the apoptosis inducing receptor, CXCR3-B. This study did not further investigate presence of CXCR3, but future studies could confirm the presence of the receptor using confocal microscopy with fluorescent staining or flow cytometry. qRT-PCR for the receptor could be used to confirm presence on mRNA level.

## 5.6 TLR3 and colon cancer

Prolonged and chronic inflammation increases the risk of developing cancer. Several inflammatory diseases have been liked to increased risk of cancer development[68]. A well-documented example is the association between IBD and increased risk of developing CRC. The risk of developing CRC has been shown to increase with both the severity and the duration of inflammation. Prolonged inflammation, leading to tissue damage and detrimental immune responses, has been linked to the transition of normal cells to cancer cells. Use of anti-inflammatory drugs have shown to lower this risk[101]. TLR3 agonists have been proposed for use as adjuvants in cancer treatment, as activation of TLR3 have been demonstrated to induce apoptosis in several cancers[39, 65, 81]. In this study, activation of TLR3 was not found to impair cell viability, but was able to induce potent cytokine responses upon activation. The results

in this study indicate that the use of TLR3 agonists in therapy may have detrimental effects in certain tissues, as TLR3 activation could lead to induction of potent pro-inflammatory cytokines, that could further drive inflammatory responses and promote cancer progression.

# 6 Conclusion

In this study HT-29 cells were found to secrete the chemokines CXCL10 and CXCL11 upon stimulation with added Poly I:C. Stimulation with added Poly I:C did not affect the viability of HT-29 cells. Cytokine secretion was found to vary dependent on mode of ligand administration. Stimulation with added Poly I:C gave a prominent secretion of CXCL10 and CXCL11, on both gene and protein level. No prominent induction of IFNβ was observed on protein level, however gene expression was present. Stimulation with transfected Poly I:C gave CXCL10 and CXCL11 induction to a lesser degree compared to added Poly I:C. Stimulation with transfected Poly I:C gave a prominent IFNβ secretion observed both on gene and protein level. Collectively these results imply that different mechanisms or pathways are activated depending on the mode of ligand administration. Knockdown of TLR3 and TRIF established that CXCL10 and CXCL11 secretion upon added Poly I:C stimulation was TLR3 dependent. UNC93b1 displayed no clear role in secretion of CXCL10 and CXCL11, but should be further explored.

Activation of TLR3 has shown to induce apoptosis in several cancers, and as a result TLR3 agonists have been proposed as adjuvant in chemotherapy. In context with the results of this study, where TLR3 activation was found to not impair cell viability and instead induce potent pro-inflammatory cytokines, TLR3 agonists in therapy may have detrimental effects in certain cancers. The results in this study suggest that activation of TLR3 and resulting secretion of chemokines may participate in driving inflammatory response and promote in colon cancer progression.

# 7 References


1. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity*. Cell, 2006. **124**(4): p. 783-801.
2. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors*. Nat Immunol, 2010. **11**(5): p. 373-84.
3. Takeuchi, O. and S. Akira, *Pattern Recognition Receptors and Inflammation*. Cell, 2010. **140**(6): p. 805-820.
4. Broz, P. and D.M. Monack, *Newly described pattern recognition receptors team up against intracellular pathogens*. Nat Rev Immunol, 2013. **13**(8): p. 551-65.
5. Rasmussen, S.B., L.S. Reinert, and S.R. Paludan, *Innate recognition of intracellular pathogens: detection and activation of the first line of defense*. APMIS, 2009. **117**(5-6): p. 323-37.
6. Barton, G.M. and J.C. Kagan, *A cell biological view of Toll-like receptor function: regulation through compartmentalization*. Nat Rev Immunol, 2009. **9**(8): p. 535-42.
7. Hoebe, K., et al., *CD36 is a sensor of diacylglycerides*. Nature, 2005. **433**(7025): p. 523-7.
8. Park, B.S., et al., *The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex*. Nature, 2009. **458**(7242): p. 1191-5.
9. Hayashi, F., et al., *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5*. Nature, 2001. **410**(6832): p. 1099-103.
10. Yu, L., L. Wang, and S. Chen, *Endogenous toll-like receptor ligands and their biological significance*. J Cell Mol Med, 2010. **14**(11): p. 2592-603.
11. Stappers, M.H., et al., *Genetic Variation in the Inhibitory Toll-Like Receptor 10 Influences Susceptibility to Complicated Skin and Skin Structure Infections*. J Infect Dis, 2015.
12. Kawai, T. and S. Akira, *Toll-like receptor and RIG-I-like receptor signaling*. Ann N Y Acad Sci, 2008. **1143**: p. 1-20.
13. Cervantes, J.L., et al., *TLR8: the forgotten relative revindicated*. Cell Mol Immunol, 2012. **9**(6): p. 434-8.
14. Haas, T., et al., *The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation*. Immunity, 2008. **28**(3): p. 315-23.
15. Latz, E., et al., *TLR9 signals after translocating from the ER to CpG DNA in the lysosome*. Nat Immunol, 2004. **5**(2): p. 190-8.
16. Rakoff-Nahoum, S. and R. Medzhitov, *Toll-like receptors and cancer*. Nature Reviews: Cancer, 2009. **9**: p. 57-63.
17. Oshiumi, H., et al., *TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction*. Nat Immunol, 2003. **4**(2): p. 161-7.
18. O'Neill, L.A., D. Golenbock, and A.G. Bowie, *The history of Toll-like receptors - redefining innate immunity*. Nat Rev Immunol, 2013. **13**(6): p. 453-60.
19. Fitzgerald, K.A., et al., *Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction*. Nature, 2001. **413**(6851): p. 78-83.

20. O'Neill, L.A., et al., *Mal and MyD88: adapter proteins involved in signal transduction by Toll-like receptors.* J Endotoxin Res, 2003. **9**(1): p. 55-9.
21. Kawagoe, T., et al., *Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2.* Nat Immunol, 2008. **9**(6): p. 684-91.
22. Yamamoto, M., et al., *Key function for the Ubc13 E2 ubiquitin-conjugating enzyme in immune receptor signaling.* Nat Immunol, 2006. **7**(9): p. 962-70.
23. Rahighi, S., et al., *Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation.* Cell, 2009. **136**(6): p. 1098-109.
24. Honda, K., et al., *Regulation of the type I IFN induction: a current view.* Int Immunol, 2005. **17**(11): p. 1367-78.
25. Seya, T., et al., *TICAM-1 and TICAM-2: toll-like receptor adapters that participate in induction of type 1 interferons.* Int J Biochem Cell Biol, 2005. **37**(3): p. 524-9.
26. Hacker, H., et al., *Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6.* Nature, 2006. **439**(7073): p. 204-7.
27. Lazear, H.M., et al., *IRF-3, IRF-5, and IRF-7 coordinately regulate the type I IFN response in myeloid dendritic cells downstream of MAVS signaling.* PLoS Pathog, 2013. **9**(1): p. e1003118.
28. Meylan, E., et al., *RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation.* Nat Immunol, 2004. **5**(5): p. 503-7.
29. Erridge, C., *Endogenous ligands of TLR2 and TLR4: agonists or assistants?* J Leukoc Biol, 2010. **87**(6): p. 989-99.
30. Piccinini, A.M. and K.S. Midwood, *DAMPening inflammation by modulating TLR signalling.* Mediators Inflamm, 2010. **2010**.
31. Chao, W., *Toll-like receptor signaling: a critical modulator of cell survival and ischemic injury in the heart.* Am J Physiol Heart Circ Physiol, 2009. **296**(1): p. H1-H12.
32. Wu, H., et al., *TLR4 activation mediates kidney ischemia/reperfusion injury.* J Clin Invest, 2007. **117**(10): p. 2847-59.
33. Tsung, A., et al., *The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion.* J Exp Med, 2005. **201**(7): p. 1135-43.
34. Zhai, Y., et al., *Evidence for the pivotal role of endogenous toll-like receptor 4 ligands in liver ischemia and reperfusion injury.* Transplantation, 2008. **85**(7): p. 1016-22.
35. Cavassani, K.A., et al., *TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events.* J Exp Med, 2008. **205**(11): p. 2609-21.
36. Ospelt, C., et al., *Overexpression of toll-like receptors 3 and 4 in synovial tissue from patients with early rheumatoid arthritis: toll-like receptor expression in early and longstanding arthritis.* Arthritis Rheum, 2008. **58**(12): p. 3684-92.
37. Barrat, F.J., et al., *Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus.* J Exp Med, 2005. **202**(8): p. 1131-9.
38. Apetoh, L., et al., *Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy.* Nat Med, 2007. **13**(9): p. 1050-9.
39. Salaun, B., et al., *TLR3 can directly trigger apoptosis in human cancer cells.* J Immunol, 2006. **176**(8): p. 4894-901.

40. Yu, L. and S. Chen, *Toll-like receptors expressed in tumor cells: targets for therapy*. Cancer Immunol Immunother, 2008. **57**(9): p. 1271-8.
41. Wang, Y., et al., *Dimerization of Toll-like Receptor 3 (TLR3) Is Required for Ligand Binding*. J Biol Chem, 2010. **285**(47): p. 36836-41.
42. Manavalan, B., S. Basith, and S. Choi, *Similar Structures but Different Roles - An Updated Perspective on TLR Structures*. Front Physiol, 2011. **2**: p. 41.
43. Tatematsu, M., T. Seya, and M. Matsumoto, *Beyond dsRNA: Toll-like receptor 3 signalling in RNA-induced immune responses*. Biochem J, 2014. **458**(2): p. 195-201.
44. Kawai, T. and S. Akira, *The roles of TLRs, RLRs and NLRs in pathogen recognitionInt Immunol. 21:313*. Int Immunol, 2009. **21**(4): p. 317-37.
45. Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-[kappa]B by Toll-like receptor 3*. Nature, 2001. **413**: p. 732-738.
46. Kariko, K., et al., *mRNA is an endogenous ligand for Toll-like receptor 3*. J Biol Chem, 2004. **279**(13): p. 12542-50.
47. Ivashkiv, L.B. and L.T. Donlin, *Regulation of type I interferon responses*. Nat Rev Immunol, 2014. **14**(1): p. 36-49.
48. Lundberg, A.M., et al., *Key differences in TLR3/poly I:C signaling and cytokine induction by human primary cells: a phenomenon absent from murine cell systems*. Blood, 2007. **110**(9): p. 3245-52.
49. Billottet, C., C. Quemener, and A. Bikfalvi, *CXCR3, a double-edged sword in tumor progression and angiogenesis*. Biochim Biophys Acta, 2013. **1836**(2): p. 287-95.
50. Dufour, J.H., et al., *IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking*. J Immunol, 2002. **168**(7): p. 3195-204.
51. Blasius, A.L. and B. Beutler, *Intracellular toll-like receptors*. Immunity, 2010. **32**(3): p. 305-15.
52. de Bouteiller, O., et al., *Recognition of double-stranded RNA by human toll-like receptor 3 and downstream receptor signaling requires multimerization and an acidic pH*. J Biol Chem, 2005. **280**(46): p. 38133-45.
53. Pohar, J., et al., *The role of UNC93B1 protein in surface localization of TLR3 receptor and in cell priming to nucleic acid agonists*. J Biol Chem, 2013. **288**(1): p. 442-54.
54. Kim, Y.M., et al., *UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes*. Nature, 2008. **452**(7184): p. 234-8.
55. Gambara, G., et al., *TLR3 engagement induces IRF-3-dependent apoptosis in androgen-sensitive prostate cancer cells and inhibits tumour growth in vivo*. J Cell Mol Med, 2015. **19**(2): p. 327-39.
56. Galli, R., et al., *Toll-like receptor 3 (TLR3) activation induces microRNA-dependent reexpression of functional RARβ and tumor regression*. Proc Natl Acad Sci U S A, 2013. **110**(24): p. 9812-7.
57. Chuang, H.C., et al., *Toll-like receptor 3-mediated tumor invasion in head and neck cancer*. Oral Oncol, 2012. **48**(3): p. 226-32.
58. Goto, Y., et al., *Activation of toll-like receptors 2, 3, and 4 on human melanoma cells induces inflammatory factors*. Mol Cancer Ther, 2008. **7**(11): p. 3642-53.
59. Sato, Y., et al., *Cancer Cells Expressing Toll-like Receptors and the Tumor Microenvironment*. Cancer Microenviron, 2009. **2**(Suppl 1): p. 205-14.

60. González-Reyes, S., et al., *Study of TLR3, TLR4 and TLR9 in breast carcinomas and their association with metastasis*. BMC Cancer, 2010. **10**: p. 665.
61. Zipin-Roitman, A., et al., *CXCL10 promotes invasion-related properties in human colorectal carcinoma cells*. Cancer Res, 2007. **67**(7): p. 3396-405.
62. Frick, V.O., et al., *CCR6/CCL20 chemokine expression profile in distinct colorectal malignancies*. Scand J Immunol, 2013. **78**(3): p. 298-305.
63. Salaun, B., et al., *Toll-like receptor 3 expressed by melanoma cells as a target for therapy?* Clin Cancer Res, 2007. **13**(15 Pt 1): p. 4565-74.
64. Salem, M.L., et al., *Defining the antigen-specific T-cell response to vaccination and poly(I:C)/TLR3 signaling: evidence of enhanced primary and memory CD8 T-cell responses and antitumor immunity*. J Immunother, 2005. **28**(3): p. 220-8.
65. Seya, T., M. Azuma, and M. Matsumoto, *Targeting TLR3 with no RIG-I/MDA5 activation is effective in immunotherapy for cancer*. Expert Opin Ther Targets, 2013. **17**(5): p. 533-44.
66. Li, G., et al., *Improved therapeutic effectiveness by combining recombinant CXC chemokine ligand 10 with Cisplatin in solid tumors*. Clin Cancer Res, 2005. **11**(11): p. 4217-24.
67. Murakami, T., et al., *The role of CXCR3 and CXCR4 in colorectal cancer metastasis*. Int J Cancer, 2013. **132**(2): p. 276-87.
68. Rakoff-Nahoum, S., *Why Cancer and Inflammation?* Yale J Biol Med, 2006. **79**(3-4): p. 123-30.
69. Lu, H., W. Ouyang, and C. Huang, *Inflammation, a key event in cancer development*. Mol Cancer Res, 2006. **4**(4): p. 221-33.
70. Triantafillidis, J.K., G. Nasioulas, and P.A. Kosmidis, *Colorectal cancer and inflammatory bowel disease: epidemiology, risk factors, mechanisms of carcinogenesis and prevention strategies*. Anticancer Res, 2009. **29**(7): p. 2727-37.
71. Ghadjar, P., et al., *The chemokine CCL20 and its receptor CCR6 in human malignancy with focus on colorectal cancer*. Int J Cancer, 2009. **125**(4): p. 741-5.
72. Kawada, K., et al., *Chemokine receptor CXCR3 promotes colon cancer metastasis to lymph nodes*. Oncogene, 2007. **26**(32): p. 4679-88.
73. Nojiri, K., et al., *The expression and function of Toll-like receptors 3 and 9 in human colon carcinoma*. Oncol Rep, 2013. **29**(5): p. 1737-43.
74. Price, J.E., et al., *Organ distribution of experimental metastases of a human colorectal carcinoma injected in nude mice*. Clin Exp Metastasis, 1989. **7**(1): p. 55-68.
75. Systems, R.D. *ELISA Reference Guide & catalog*. Available from: http://www.rndsystems.com/resources/images/6836.pdf.
76. Johnson, L. *Diagnostic Parasitology*. 2009; Available from: http://www.isu.edu/mls/shields09wikis/parasitology.html.
77. Mocellin, S. and M. Provenzano, *RNA interference: learning gene knock-down from cell physiology*. J Transl Med, 2004. **2**(1): p. 39.
78. Landskron, G., et al., *Chronic inflammation and cytokines in the tumor microenvironment*. J Immunol Res, 2014. **2014**: p. 149185.
79. Yamamoto, T., et al., *Characteristic cytokine generation patterns in cancer cells and infiltrating lymphocytes in oral squamous cell carcinomas and the influence*

*of chemoradiation combined with immunotherapy on these patterns*. Oncology, 2003. **64**(4): p. 407-15.
80. Liu, M., S. Guo, and J.K. Stiles, *The emerging role of CXCL10 in cancer (Review)*. Oncol Lett, 2011. **2**(4): p. 583-9.
81. Palchetti, S., et al., *Transfected poly(I:C) activates different dsRNA receptors, leading to apoptosis or immunoadjuvant response in androgen-independent prostate cancer cells*. J Biol Chem, 2015. **290**(9): p. 5470-83.
82. So, E.Y. and T. Ouchi, *The application of Toll like receptors for cancer therapy*. Int J Biol Sci, 2010. **6**(7): p. 675-81.
83. Harashima, N., et al., *Roles of the PI3K/Akt pathway and autophagy in TLR3 signaling-induced apoptosis and growth arrest of human prostate cancer cells*. Cancer Immunol Immunother, 2012. **61**(5): p. 667-76.
84. Bru, A., et al., *Tumour cell lines HT-29 and FaDu produce proinflammatory cytokines and activate neutrophils in vitro: possible applications for neutrophil-based antitumour treatment*. Mediators Inflamm, 2009. **2009**: p. 817498.
85. Ostvik, A.E., et al., *Enhanced expression of CXCL10 in inflammatory bowel disease: potential role of mucosal Toll-like receptor 3 stimulation*. Inflamm Bowel Dis, 2013. **19**(2): p. 265-74.
86. Brand, S., et al., *Cell differentiation dependent expressed CCR6 mediates ERK-1/2, SAPK/JNK, and Akt signaling resulting in proliferation and migration of colorectal cancer cells*. J Cell Biochem, 2006. **97**(4): p. 709-23.
87. Schutyser, E., S. Struyf, and J. Van Damme, *The CC chemokine CCL20 and its receptor CCR6*. Cytokine Growth Factor Rev, 2003. **14**(5): p. 409-26.
88. Muller, M., et al., *Review: The chemokine receptor CXCR3 and its ligands CXCL9, CXCL10 and CXCL11 in neuroimmunity--a tale of conflict and conundrum*. Neuropathol Appl Neurobiol, 2010. **36**(5): p. 368-87.
89. Honda, K. and T. Taniguchi, *IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors*. Nat Rev Immunol, 2006. **6**(9): p. 644-58.
90. Brownell, J., et al., *Direct, interferon-independent activation of the CXCL10 promoter by NF-kappaB and interferon regulatory factor 3 during hepatitis C virus infection*. J Virol, 2014. **88**(3): p. 1582-90.
91. Yamamoto, M., K. Takeda, and S. Akira, *TIR domain-containing adaptors define the specificity of TLR signaling*. Mol Immunol, 2004. **40**(12): p. 861-8.
92. Vareille, M., et al., *The Airway Epithelium: Soldier in the Fight against Respiratory Viruses*. Clin Microbiol Rev, 2011. **24**(1): p. 210-29.
93. Kawai, T. and S. Akira, *TLR signaling*. Semin Immunol, 2007. **19**(1): p. 24-32.
94. Murakami, Y., et al., *Roles of the cleaved N-terminal TLR3 fragment and cell surface TLR3 in double-stranded RNA sensing*. J Immunol, 2014. **193**(10): p. 5208-17.
95. Barral, P.M., et al., *Functions of the cytoplasmic RNA sensors RIG-I and MDA-5: key regulators of innate immunity*. Pharmacol Ther, 2009. **124**(2): p. 219-34.
96. Pohar, J., et al., *The Ectodomain of TLR3 Receptor Is Required for Its Plasma Membrane Translocation*. PLoS One, 2014. **9**(3).
97. Kariko, K., et al., *Exogenous siRNA mediates sequence-independent gene suppression by signaling through toll-like receptor 3*. Cells Tissues Organs, 2004. **177**(3): p. 132-8.

98. Paone, A., et al., *Toll-like receptor 3 triggers apoptosis of human prostate cancer cells through a PKC-alpha-dependent mechanism.* Carcinogenesis, 2008. **29**(7): p. 1334-42.
99. Balan, M. and S. Pal, *A novel CXCR3-B chemokine receptor-induced growth-inhibitory signal in cancer cells is mediated through the regulation of Bach-1 protein and Nrf2 protein nuclear translocation.* J Biol Chem, 2014. **289**(6): p. 3126-37.
100. Cambien, B., et al., *Organ-specific inhibition of metastatic colon carcinoma by CXCR3 antagonism.* Br J Cancer, 2009. **100**(11): p. 1755-64.
101. Munkholm, P., *Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease.* Aliment Pharmacol Ther, 2003. **18 Suppl 2**: p. 1-5.

# Appendix

## Appendix I: Human XL Cytokine Array Kit

ARRAY PROCEDURE:

Bring all reagents to room temperature before use. Keep samples on ice. To avoid contamination, wear gloves while performing the procedures.

Note: High levels of some proteins are found in saliva. It is recommended that a mask and gloves be used to protect kit reagents from contamination.

1. Prepare all reagents and samples as directed in the previous sections.
2. Pipet 2.0 mL of Array Buffer 6 into each well of the 4-Well Multi-dish. Array Buffer 6 serves as a block buffer.
3. Place each membrane in a separate well. The number on the membrane should be facing upward.

Note: Upon contact with Array Buffer 6, the blue dye from the spots will disappear, but the capture antibodies are retained in their specific locations.

4. Incubate for one hour on a rocking platform shaker. Orient the 4-Well Multi-dish so that each membrane rocks end to end in its well
5. While the arrays are blocking, prepare samples by diluting the desired quantity to a final volume of 1.5 mL with Array Buffer 6.
6. Aspirate Array Buffer 6 from the wells of the 4-Well Multi-dish and add the prepared samples. Place the lid on the 4-Well Multi-dish.
7. Incubate overnight at 2-8 °C on a rocking platform shaker.

Note: A shorter incubation time may be used if optimal sensitivity is not required.

8. Carefully remove each membrane and place into individual plastic containers with 20 mL of 1X Wash Buffer. Rinse the 4-Well Multi-dish with deionized or distilled water and dry thoroughly.
9. Wash each membrane with 1X Wash Buffer for 10 minutes on a rocking platform shaker. Repeat two times for a total of three washes.

10. For each array, add 30 µL of Detection Antibody Cocktail to 1.5 mL of 1X Array Buffer 4/6. Pipette 1.5 mL per well of diluted Detection Antibody Cocktail into the 4-Well Multi-dish.

11. Carefully remove each array from its wash container. Allow excess Wash Buffer to drain from the array. Return the array to the 4-Well Multi-dish containing the diluted Detection Antibody Cocktail, and cover with the lid.

12. Incubate for 1 hour on a rocking platform shaker.

13. Wash each array as described in steps 8 and 9.

14. Pipette 2.0 mL of 1X Streptavidin-HRP into each well of the 4-Well Multi-dish.

15. Carefully remove each membrane from its wash container. Allow excess Wash Buffer to drain from the membrane. Return the membrane to the 4-Well Multi-dish containing the 1X Streptavidin-HRP. Cover the wells with the lid.

16. Incubate for 30 minutes at room temperature on a rocking platform shaker.

17. Wash each array as described in steps 8 and 9.

Note: Complete the remaining steps without interruption.

18. Carefully remove each membrane from its wash container. Allow excess Wash Buffer to drain from the membrane by blotting the lower edge onto paper towels. Place each membrane on the bottom sheet of the plastic sheet protector with the identification number facing up.

19. Pipette 1.0 mL of the prepared Chemi Reagent Mix evenly onto each membrane.

Note: Using less than 1.0 mL of Chemi Reagent Mix per membrane may result in incomplete membrane coverage.

20. Carefully cover with the top sheet of the plastic sheet protector. Gently smooth out any air bubbles and ensure Chemi Reagent Mix is spread evenly to all corners of each membrane. Incubate for 1 minute.

21. Position paper towels on the top and sides of the plastic sheet protector containing the membranes and carefully squeeze out excess Chemi Reagent Mix.

22. Leaving membranes on the bottom plastic sheet protector, cover the membranes with plastic wrap taking care to gently smooth out any air bubbles. Wrap the excess plastic wrap around the back of the sheet protector so that the membranes and sheet protector are completely wrapped.

23. Place the membranes with the identification numbers facing up in an autoradiography film cassette.

Note: Use an autoradiography cassette that is not used with radioactive isotope detection.

24. Expose membranes to X-ray film for 1-10 minutes. Multiple exposure times are recommended.

Refer to the table below for the Human XL Cytokine Array coordinates.

| Coordinate | Analyte/Control | Entrez Gene ID | Alternate Nomenclature |
|---|---|---|---|
| A1, A2 | Reference Spots | N/A | RS |
| A3, A4 | Adiponectin | 9370 | Acrp30 |
| A5, A6 | Aggrecan | 176 | Aggrecan 1 |
| A7, A8 | Angiogenin | 283 | _____ |
| A9, A10 | Angiopoietin-1 | 284 | Ang-1, ANGPT1 |
| A11, A12 | Angiopoietin-2 | 285 | Ang-2, ANGPT2 |
| A13, A14 | BAFF | 10673 | BLyS, TNFSF13B |
| A15, A16 | BDNF | 627 | Brain-derived Neurotrophic Factor |
| A17, A18 | Complement Component C5/C5a | 727 | C5/C5a |
| A19, A20 | CD14 | 929 | _____ |
| A21, A22 | CD30 | 943 | TNFRSF8 |

| | | | |
|---|---|---|---|
| A23, A24 | Reference Spots | N/A | RS |
| B3, B4 | CD40 ligand | 959 | CD40L, TNFSF5, CD154, TRAP |
| B5, B6 | Chitinase 3-like 1 | 1116 | CHI3L1, YKL-40 |
| B7, B8 | Complement Factor D | 1675 | Adipsin, CFD |
| B9, B10 | C-Reactive Protein | 1401 | CRP |
| B11, B12 | Cripto-1 | 6997 | Teratocarcinoma-derived Growth Factor |
| B13, B14 | Cystatin C | 1471 | CST3, ARMD11 |
| B15, B16 | Dkk-1 | 22943 | Dickkopf-1 |
| B17, B18 | DPPIV | 1803 | CD26, DPP4, Dipeptidyl-peptidase IV |
| B19, B20 | EGF | 1950 | Epidermal Growth Factor |
| B21, B22 | EMMPRIN | 682 | CD147, Basigin |
| C3, C4 | ENA-78 | 6374 | CXCL5 |
| C5, C6 | Endoglin | 2022 | CD105, ENG |
| C7, C8 | Fas Ligand | 356 | TNFSF6, CD178, CD95L |
| C9, C10 | FGF basic | 2247 | FGF-2 |
| C11, C12 | FGF-7 | 2252 | KGF |
| C13, C14 | FGF-19 | 9965 | _____ |
| C15, C16 | Flt-3 Ligand | 2323 | FLT3LG |

| | | | |
|---|---|---|---|
| C17, C18 | G-CSF | 1440 | CSF3 |
| C19, C20 | GDF-15 | 9518 | MIC-1 |
| C21, C22 | GM-CSF | 1437 | CSF2 |
| D1, D2 | GRO-α | 2919 | CXCL1, MSGA-α |
| D3, D4 | Growth Hormone | 2688 | GH, Somatotropin |
| D5, D6 | HGF | 3082 | Scatter Factor, SF |
| D7, D8 | ICAM-1 | 3383 | CD54 |
| D9, D10 | IFN-γ | 3458 | IFNG |
| D11, D12 | IGFBP-2 | 3485 | _____ |

| Coordinate | Analyte/Control | Entrez Gene ID | Alternate Nomenclature |
|---|---|---|---|
| D13, D14 | IGFBP-3 | 3486 | _____ |
| D15, D16 | IL-1α | 3552 | IL-1F1 |
| D17, D18 | IL-1β | 3553 | IL-1F2 |
| D19, D20 | IL-1ra | 3557 | IL-1F3 |
| D21, D22 | IL-2 | 3558 | _____ |
| D23, D24 | IL-3 | 3562 | _____ |
| E1, E2 | IL-4 | 3565 | _____ |
| E3, E4 | IL-5 | 3567 | _____ |

| | | | |
|---|---|---|---|
| E5, E6 | IL-6 | 3569 | _____ |
| E7, E8 | IL-8 | 3576 | CXCL8 |
| E9, E10 | IL-10 | 3586 | _____ |
| E11, E12 | IL-11 | 3589 | _____ |
| E13, E14 | IL-12 p70 | 3593 | _____ |
| E15, E16 | IL-13 | 3596 | _____ |
| E17, E18 | IL-15 | 3600 | _____ |
| E19, E20 | IL-16 | 3603 | _____ |
| E21, E22 | IL-17A | 3605 | IL-17, CTLA8 |
| E23, E24 | IL-18 BPa | 10068 | _____ |
| F1, F2 | IL-19 | 29949 | _____ |
| F3, F4 | IL-22 | 50616 | IL-TIF |
| F5, F6 | IL-23 | 51561 | IL-23A, SGRF |
| F7, F8 | IL-24 | 3627 | C49A, FISP, MDA-7, MOB-5, ST16 |
| F9, F10 | IL-27 | 246778 | _____ |
| F11, F12 | IL-31 | 386653 | _____ |
| F13, F14 | IL-32α/β/γ | 9235 | _____ |
| F15, F16 | IL-33 | 90865 | C9orf26, DVS27, NF-HEV |
| F17, F18 | IL-34 | 146433 | C16orf77 |

| | | | |
|---|---|---|---|
| F19, F20 | IP-10 | 3627 | CXCL10 |
| F21, F22 | I-TAC | 6373 | CXCL11, SCYB9B |
| F23, F24 | Kallikrein 3 | 354 | PSA, KLK3 |
| G1, G2 | Leptin | 3952 | OB |
| G3, G4 | LIF | 3976 | _____ |
| G5, G6 | Lipocalin-2 | 3934 | NGAL, LCN2, Siderocalin |
| G7, G8 | MCP-1 | 6347 | CCL2, MCAF |
| G9, G10 | MCP-3 | 6354 | CCL7, MARC |
| G11, G12 | M-CSF | 1435 | CSF1 |
| G13, G14 | MIF | 4282 | _____ |
| G15, G16 | MIG | 4283 | CXCL9 |

| Coordinate | Analyte/Control | Entrez Gene ID | Alternate Nomenclature |
|---|---|---|---|
| G17, G18 | MIP-1α/MIP-1β | 6348/6351 | CCL3/CCL4 |
| G19, G20 | MIP-3α | 6364 | CCL20, Exodus-1, LARC |
| G21, G22 | MIP-3β | 6363 | CCL19, ELC |
| G23, G24 | MMP-9 | 4318 | CLG4B, Gelatinase B |
| H1, H2 | Myeloperoxidase | 4353 | MPO, Lactoperoxidase |
| H3, H4 | Osteopontin | 6696 | OPN |

| | | | |
|---|---|---|---|
| H5, H6 | PDGF-AA | 5154 | _____ |
| H7, H8 | PDGF-AB/BB | 5154/5155 | _____ |
| H9, H10 | Pentraxin-3 | 5806 | PTX3, TSG-14 |
| H11, H12 | PF4 | 5196 | CXCL4 |
| H13, H14 | RAGE | 177 | _____ |
| H15, H16 | RANTES | 6352 | CCL5 |
| H17, H18 | RBP4 | 5950 | _____ |
| H19, H20 | Relaxin-2 | 6019 | RLN2, RLXH2 |
| H21, H22 | Resistin | 56729 | ADSF, FIZZ3, RETN |
| H23, H24 | SDF-1α | 6387 | CXCL12, PBSF |
| I1, I2 | Serpin E1 | 5054 | PAI-I, PAI-1, Nexin |
| I3, I4 | SHBG | 6462 | ABP |
| I5, I6 | ST2 | 9173 | IL-1 R4, IL1RL1, ST2L |
| I7, I8 | TARC | 6361 | CCL17 |
| I9, I10 | TFF3 | 7033 | ITF,TFI |
| I11, I12 | TfR | 7037 | CD71, TFR1, TFRC, TRFR |
| I13, I14 | TGF-α | 7039 | TGFA |
| I15, I16 | Thrombospondin-1 | 7057 | THBS1, TSP-1 |
| I17, I18 | TNF-alpha | 7124 | TNFSF1A |

| | | | |
|---|---|---|---|
| I19, I20 | uPAR | 5329 | PLAUR |
| I21, I22 | VEGF | 7422 | BEGFA |
| J1, J2 | Reference Spots | N/A | RS |
| J5, J6 | Vitamin D BP | 2638 | VDB, DBP, VDBP |
| J23, J24 | Negative Controls | N/A | Control (-) |

## Appendix II: IFNβ ASSAY PROCEDURE

All incubations should be performed at room temperature (RT), 22-25°C, keeping the plate away from drafts and other temperature fluctuations. Use plate sealers to cover the plate as directed. During all wash steps remove contents of plate by inverting and shaking over a sink and blotting the plate on lint- free absorbent paper; tap the plate. All wells should be filled with a minimum of 250 µl of diluted Wash Buffer at each wash step. Refer to Preparation of Reagents for dilution of concentrated solutions.

1. **Standards and Test Samples:** Determine the number of microplate strips required to test the desired number of samples plus the appropriate number of wells needed to run blanks and standards. We recommend running the Human IFN Beta Standard, blanks and samples in duplicate or triplicate (see Figure 2 for example plate setup). A standard curve is required for each assay. Remove extra microtiter strips from the frame, seal in the foil bag provided and store at 2-8°C. Unused strips can be used in later assays.

Add 50 µl Sample Diluent to the wells. Add 50 µl of the diluted Standard Curve, blanks or test samples. Cover with plate sealer and incubate for 1 hour.

After 1 hour, empty the contents of the plate and wash the wells three times with diluted Wash Buffer (refer to Preparation of Reagents).

2. **Antibody Solution:** Add 100 µl of diluted Antibody Solution (refer to Preparation of Reagents) to each well. Cover with plate sealer and incubate for 1 hour.

After 1 hour, empty the contents of the plate and wash the wells three times with diluted Wash Buffer.

3. **HRP:** Add 100 µl of diluted HRP Solution (refer to Preparation of Reagents) to each well. Cover with plate sealer and incubate for 1 hour. During this incubation period, warm the TMB Substrate Solution to RT (22-25°C).

After 1 hour, empty the contents of the plate and wash the wells three times with diluted Wash Buffer.

4. **TMB Substrate:** Add 100 µl of the TMB Substrate Solution to each well. Incubate, in the dark, at RT (22-25°C), for 15 minutes. Do not use a plate sealer during the incubation.

5. **Stop Solution:** After the 15 minute incubation of TMB, DO NOT EMPTY THE WELLS AND DO NOT WASH. Add 100 µl of Stop Solution to each well.

6. **Read:** Using a microplate reader, determine the absorbance at 450 nm within 5 minutes after the addition of the Stop Solution.

## Appendix III: DuoSet ELISA Development kit

**Human CXCL10/IP-10 DuoSet ELISA development kit**

Catalogue number: DY266

**Capture Antibody:** 240 µg/ml of mouse anti-human IP-10 antibody when reconstituted with 0.5ml PBS. Diluted to a working concentration of 2 µg/ml in PBS.

**Detection Antibody:** 0.75µg/ml of biotinylated goat anti-human IP-10 antibody when reconstituted with 1ml reagent diluent. Diluted to a working concentration of 12,5ng/ml in reagent diluent.

**Standard:** 130ng/ml of recombinant human IP-10 when reconstituted with 0.5ml reagent diluent. A standard of 2000pg/ml was used.

**Human CXCL11/I-TAC DuoSet ELISA development kit**

Catalogue number: DY672

**Capture Antibody:** 180µg/ml of mouse anti-CXCL11 when reconstituted with 1ml PBS. Diluted to a working concentration of 1µg/ml in PBS.

**Detection Antibody:** 108µg/ml of biotinylated goat anti-human CXCL11 when reconstituted with 0.5ml of reagent diluent.

**Standard:** 15ng/ml of recombinant CXCL11when reconstituted with 0.5ml of reagent diluent. A standard of 500pg/ml was used.