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A Toll-like receptor 7/8 ligand secreted by a myeloma cell line induces primary bone marrow stromal cells to produce survival promoting cytokines

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Abstract

Multiple myeloma (MM) is a cancer of plasma cells, which accumulate in the bone marrow (BM). The BM milieu provides MM cells with survival and proliferation signals. Toll-like receptors (TLRs) are immune receptors that mediate inflammatory cytokines upon recognition of molecules associated with infection, danger or stress. Tumor cells have been reported to secrete TLR-activating components, activating immune cells to release inflammatory cytokines, which in turn drives tumorigenesis. This study sought to investigate if myeloma cell lines release TLR-activating components that stimulate TLR-expressing cells in the BM to produce inflammatory cytokines that promote MM survival. Conditioned medium (CM) from MM cells was assayed for TLR activating ability using a TLR driven NF-κB reporter system. CM from the MM cell line U266 was found to activate TLR7 and TLR8. TLR7 and TLR8 are known to recognize RNA, however, RNase treatment of the U266 CM did not abolish the TLR7/8 activation. Extracellular vesicles (ECVs) were isolated from U266 to determine if the RNA ligand may be protected from RNase in ECV. U266-derived ECVs were found to mediate some TLR7 activation. Bone marrow stromal cells (BMSCs) were further found to up-regulate CCL3, CXCL1 and CXCL5 in response to TLR8 ligands, but not TLR7, indicating presence of TLR8 in these cells, which was confirmed at the mRNA level. Macrophages had a similar cytokine profile as BMSCs when stimulated with TLR ligands. However, only 3% of the BMSCs stained positive for the macrophage marker CD14. The results indicate that $CD14^-$ cells, in addition to $CD14^+$ cells, in BMSCs respond to TLR8 stimulation. Furthermore, BMSCs treated with U266 CM resulted in induction of CXCL1 and CXCL5. Collectively, U266 secreted a TLR7/8-activating component able to induce cytokine response in primary BMSCs. Taken together, this study provides further insight into the interplay between MM and BMSCs and a potential role for TLR8 to promote MM.

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Abbreviations

BM	Bone marrow
BMM	Bone marrow microenvironment
BMSC	Bone marrow stromal cells
CM	Conditioned medium
CpG	Deoxycytidyl deoxyguanosine
DAMP	Damage – associated molecular pattern
DC	Dendritic cell
ECM	Extracellular matrix
ECV	Extracellular vesicle
EV	Empty vector
FSL-1	Fibroblast stimulating factor-1
HEK	Human embryonic kidney
HMGB1	High-mobility group protein B1
HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
IκB	Inhibitor of kappa B
IKK	IκBα kinase complex
IMiD	Immunomodulatory drug
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon Regulatory Factor
LPS	Lipopolysaccharide
MGUS	Monoclonal gammopathy of undetermined significance
MM	Multiple myeloma
mDC	Myeloid dendritic cell

moDC	Monocyte – derived dendritic cells
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa-light chain-enhancer of activated B cells
PAMP	Pathogen – associated molecular pattern
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell
poly I:C	Polyionsine polycytidylic acid
PRR	Pathogen associated molecular patterns
R848	Resiquimod
siRNA	Small interfering RNA
SMM	Smoldering multiple myeloma
TAB	TGF-β-activated kinase 1 (TAK1) – binding proteins
TAK	TGF-β-activated kinase 1
TBK	TANK-binding kinase
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRAM	TRIF related adaptor protein
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction

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1 Introduction

1.1 Multiple Myeloma

Multiple myeloma (MM) is a hematological cancer of plasma cells infiltrating the bone marrow (BM). Myeloma is the second most common hematological cancer [1] and on a world basis, approximately 86 000 are diagnosed annually, accounting for 0.8% of all cancers. The median age at diagnosis is 66 years with a 4 year survival rate [2], and 63 000 myeloma patients die each year [3].

Multiple myeloma arises in malignant plasma cells derived from the germinal center homing to the bone marrow (BM)[4]. The bone marrow niche interacts with the myeloma cells, supporting their growth and proliferation through adhesion and cytokines. The bone marrow microenvironment (BMM) produces cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) favoring the malignant cells [5]. Consequently, the BMM promotes survival, progression and protection against drug-induced apoptosis of myeloma cells [6].

1.1.1 Diagnosis and disease progression

Multiple myeloma is preceded by the premalignant condition monoclonal gammopathy of undetermined significance (MGUS). MGUS is present in 1% of adults over the age of 25 [4]. MGUS is characterized by low levels of clonal plasma cells (>10%) and M protein in the serum (>3g/dl) [4]. About 1% of MGUS patients progress to MM per year [6], through the intermediate condition smoldering myeloma. Smoldering myeloma (SMM) has no clinical myeloma-related symptoms but is characterized by high levels of M protein (<3g/dl) and more than 10% of the bone marrow consists of clonal plasma cells. Further progression to multiple myeloma is diagnosed when clinical manifestations are evident in addition to high levels of M protein (<3g/dl) and plasma cells in the bone marrow (<10%) [7].

Clinical symptoms of myeloma are elevated calcium levels, renal failure, anemia and bone lesions. About 80% of all myeloma patients suffer from bone lesions[7]. These occur due to imbalance between the osteoblasts and osteoclasts leading to resorption of bone. In turn,

bone resorption lead to increased release of calcium into the blood stream causing hypercalcemia [2]. As a result of high amounts of proteins and calcium in the blood, many patients suffers from renal failure [2]. In addition, patients are in risk for opportunistic infections of bacteria and fungi due to a weakened immune system [1].

1.1.2 Treatment

MM has a high level of molecular heterogeneity, therefore many therapies are aimed to disrupt the interaction between myeloma cells and the bone marrow milieu [4]. Patients under 65 years old are eligible for stem cell transplantation, which is currently the most effective treatment [8]. However, as the median age of diagnosis is 66 years, the majority of MM patient are not eligible. Therefore, development of new and improved therapies is needed. In recent years, immunomodulatory drugs and proteasome inhibitors have been used in the clinic with positive effects.

Immunomodulatory drugs (IMiDs) have pleiotropic properties against MM such as cytokine regulation, inflammation and angiogenesis. IMiDs target the bone marrow milieu by inhibiting bone lesions [1] and adhesion to BM cells [9]. Thalidomide is an anti-inflammatory and anti-angiogenetic agent which induces apoptosis in myeloma cells. Its derivatives, lenalidomide and pomalidomide, both induce the same effects on MM cells [1].

Myeloma cells constantly produce M proteins making them vulnerable to ER stress due to unfolded proteins. Therefore, proteasome inhibitors have yielded good response in myeloma patients [10]. Bortezomib and Carfilzomib both target the 26S proteasome complex leading to apoptosis of MM cells [2]. In addition, Bortezomib blocks NF-κB activation and inhibits adhesion between MM cells and the BM cells [9, 10]. Despite these advances in treatment, multiple myeloma remains incurable.

1.1.3 The bone marrow milieu in MM

The bone marrow is the interior part of the bone and is made up of cellular and non-cellular components. The non-cellular component consists of extracellular matrix (ECM) proteins, cytokines and growth factors. The cellular part consists of hematopoietic and mesenchymal stem cells, endothelial cells, fibroblasts, immune cells, osteoclasts and stromal cells. Bone marrow stromal cells (BMSC) are characterized as a heterogeneous population with fibroblast-like morphology [11]. Myeloma cells rely on the interactions with the non-malignant BMSCs for survival and proliferation [12].

Although BMSCs are not malignant per se, they differ from their healthy counterparts [13]. The transformation to a myeloma supportive niche is thought to be driven by plasma cells re-structuring the BMM by direct interactions and crosstalk [12, 14]. MM-BMSCs have increased expression of IL-6 and vascular endothelial growth factor (VEGF) [5], a different gene expression profile [15], and are less immunosuppressive than normal BMSCs [13]. Collectively, these alterations yield better conditions for the malignant plasma cells.

Myeloma cells adhere to BMSCs via very late antigen 4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1), respectively. Adhesion initiates BMSCs secretion of cytokines implicated in MM survival and disease progression. IL-6, TNF-α, VEGF and CCL3 are induced by adhesion and have a major role in myeloma pathogenesis [6, 9]. In turn, secreted cytokines mediate a stronger adhesion between myeloma cells and BMSCs. This vicious interplay leads to a better adaptation of the BM microenvironment for MM cells to survive [12].

The strong bi-directional relationship between the MM cells and the BM niche influences myeloma progression and proliferation. The interplay protects MM cells from chemotherapy via adherence to the BMSCs, accumulate plasma cells and increase the tumor-initiating cancer cell population by secreting growth factors and cytokines [5] .

1.2 TLRs in innate immunity

The immune system consists of the innate and the adaptive immunity. The innate immune system compromises of macrophages, dendritic cells, and neutrophils providing a rapid, short-term response to pathogens. The adaptive immunity is activated later creating a long-lasting immunological memory. The innate immune system recognizes structures conserved among microbes, termed pathogen-associated molecules patterns (PAMPs). These exogenous molecules are detected by pattern recognition receptors (PRRs) expressed on a vast number of cell types particularly immune cells [16]. PRRs can also detect endogenous molecules, which are called damage associated molecular patterns (DAMPs). These self – derived molecules are associated with inflammation, autoimmune disease and malignancy[17].

PRRs are germline encoded receptors classified into four families. These families include retinoic acid inducible gene 1 – like receptors (RLRs), nucleotide oligomerization domain – like receptors (NLRs), C-type lectin receptors (CLRs) and toll – like receptors (TLRs) [18].

TLRs are type I transmembrane proteins part of the IL-1R/TLR superfamily. The TLRs share a common structure consisting of a leucine rich repeat (LRR) domain in the extracellular region and an intracellular region with the conserved Toll/IL-1 receptor (TIR) domain. The ligand binds to the LRR domain mediating downstream signaling through the TIR domain. TLR activation results in production of pro – inflammatory cytokines and type I interferons (IFNs) [18].

1.2.1 TLR expression and ligands

To date, ten functional TLRs have been identified in humans [18]. These receptors are expressed at different cellular localizations functioning as immune sentinels. TLRs are divided into two subgroups based on localization; TLRs on the plasma membrane and TLRs expressed on intracellular vesicles (Figure 1.1).

1.2.1.1 Cell surface TLRs

TLRs expressed on the plasma membrane recognize mainly microbial membrane components. TLR1 forms heterodimers with TLR2 to recognize triacylated lipopetides and the synthetic ligand Pam_3CSK_4, while TLR2/TLR6 recognizes diacylated lipopeptides. TLR4, together with co-receptors CD14 and MD-2, recognizes lipopolysaccharides (LPS), a membrane component on Gram - negative bacteria. TLR5 recognizes flagellin, a component of the bacterial flagella [19]. The ligand for TLR10 is yet to be determined [18].

1.2.1.2 Endosomal TLRs

TLR3 and TLR7 – 9 are located on intracellular vesicles and recognize nucleic acids originating from pathogens. Endosomal TLRs traffic between various intracellular components [20]. In unstimulated cells, these TLRs are sequestered at the endoplasmic reticulum (ER). Upon stimulation, the TLR traffics to the endosomes or endolysosomes where potential ligands are located [20]. Trafficking to the endosomes is a prerequisite for induction of immune response by endosomal TLRs [19, 20].

TLR9 mediates responses to unmethylated CpG DNA motifs originating from microbes. TLR3 recognizes viral double – stranded (ds) RNA and the synthetic dsRNA analog, polycytidylic acid (poly I:C). TLR7 was first shown to respond to imidazoquinoline derivates such as R848 [21]. The natural ligand was later found to be single – stranded (ss) RNA originating from viruses and bacteria [22, 23]. In addition, TLR7 senses small interfering RNA (siRNA) [24] and self-RNA in immune complexes [25].

Like TLR7, TLR8 also sense ssRNA and short dsRNA [26]. In addition, TLR8 mediates responses to the synthetic agonists R848 and CL075 [26]. TLR8 is phylogenetically similar to TLR7, however, TLR7 and TLR8 are expressed in different types of cells and mediate different cytokine profiles [27]. TLR7 is expressed in plasmacytoid dendritic cells (pDCs) and mediates a strong IFN-α secretion, while TLR8 is expressed in macrophages and myeloid dendritic cells (mDCs) and mediates strong pro-inflammatory responses, including TNF-α and CCL3 expression [27].

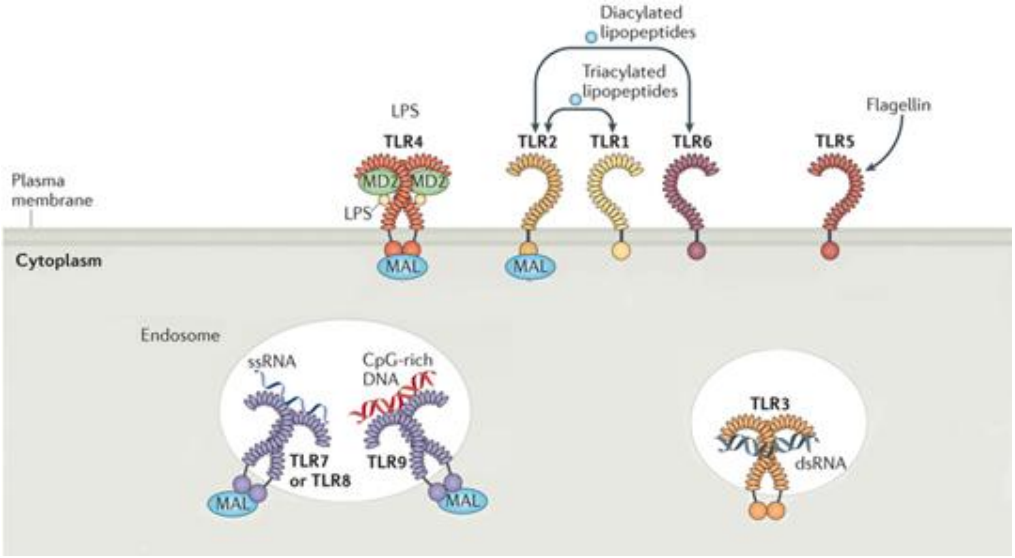


Figure 1.1 TLR ligands and localization.TLR1, TLR2, TLR4-6 are localized on the plasma membrane recognizing microbial PAMPs. TLR3, TLR7-9 are localized on endosomes recognizing nucleic acids. Figure adapted from Gay et al. [28]

1.2.1.3 Endogenous ligands

TLRs are best known to recognize exogenous ligands associated with microorganisms and mediate inflammatory responses that protects the host during the early phases of infection. In recent years, host molecules (DAMPs) are also reported to activate TLRs. DAMPs are cell components released from the host during stress, damage or other conditions disrupting homoeostasis. Endogenous ligands are believed to sense ongoing infections, mediate tissue repair and play a role in autoimmunity and tumorigenesis [29].

Endogenous molecules like peptides, polysaccharides, and nucleic acids are normally compartmentalized from TLRs hindering their activation. These molecules can, however, be released from inflamed or injured cells, thereby activating both plasma membrane and endosomal TLRs [29]. ECM products, heat shock proteins (HSPs) and HMGB1 (high – mobility group box 1) are reported to stimulate TLR2 and TLR4 [30]. Self-derived nucleic acids are able to activate endosomal TLRs [29].

Self - derived nucleic acids interact with host proteins with such as HMGB1, ribonucleoproteins or self-antibodies. These complexes facilitates entry into the cell and activate intracellular TLRs. For instance, self RNA can aggregate with LL37, an antimicrobial peptide mediated into the endosomes via endocytosis, where the aggregate activates TLR7 [19]. Immune complexes containing self nucleic acids are internalized via Fc receptors and

initiates TLR signaling [19]. Consequently, TLR activation by DAMPs may play a role in inflammatory diseases.

1.2.2 TLR signaling pathways

Activation of TLRs results in a cascade of signaling pathways resulting in induction of pro-inflammatory cytokines like IL-1β, CCL3 and TNF-α. Moreover, endosomal TLRs also mediate the induction of type I interferons in response to nucleic acids. In addition, co-stimulatory molecules are up-regulated on antigen-presenting cells, linking the innate and the adaptive immune system [31]. The biological response is dependent on TIR domain – containing adaptor molecules. There are four positive adaptors; myeloid differentiation factor 88 (MyD88), TIR domain-containing adaptor inducing interferon-β (IRF-β) (TRIF), TRIF-related adaptor molecule (TRAM) and TIR domain containing adapter (TIRAP). TRAM and TIRAP are sorting adaptors linking the signaling adaptors, MyD88 and TRIF, to TLR2 and TLR4 [19]. Consequently, the TLR signaling pathways can be divided into MyD88 – and TRIF – dependent signaling.

1.2.2.1 MyD88 - dependent signaling

All TLRs, except TLR3, utilize the MyD88 adaptor activating NF-κB and MAPK to induce pro-inflammatory cytokines [19]. MyD88 recruits Interleukin-1 receptor-associated kinases 4 and 1 (IRAK4/1). Activated IRAK1 recruits TNF receptor-associated factor 6 (TRAF6) by binding to its TRAF domain. TRAF6, an E3 ligase, disengages from the receptor and associates with the TGF-β-activated kinase 1 (TAK1)/TAK1-binding proteins (TAB)2/3 complex. TRAF6 and TAB proteins activate TAK1, which subsequently activates components of the IκB kinase (IKK) complex; IKKα and IKKβ. The IKK complex catalyzes the subsequent phosphorylation and degradation of the inhibitory protein IκB, enabling NF-κB to translocate to the nucleus (Figure 1.2) [32]. TLR7 – 9 bifurcate into the NF-κB and the interferon regulatory factor (IRF) pathway downstream of MyD88. MyD88 interacts with IRAK4, IRAK1 and TRAF3/6 to induce activation of IRF5 and IRF7 [33].

1.2.2.2 TRIF - dependent signaling

TRIF is recruited by TLR3 and TLR4 to induce activation of NF-κB and IRF3 resulting in pro-inflammatory cytokines and type I interferons. TLR4 interacts with TRIF through the bridging adaptor TRAM, while TLR3 interacts with TRIF directly through the TIR domains. TRIF recruits TRAF6. TRAF6 activates TAK1 which subsequently activates the IKK complex resulting in the translocation of NF-κB [34]. TRIF can also recruit TRAF3, which in turn associates with IKKε and TANK-binding kinase 1 (TBK1). IKKε/TBK1 complex activates IRF3 and IRF7 through phosphorylation. IRF3 and IRF7 translocate to the nucleus regulating transcription of target genes [35].

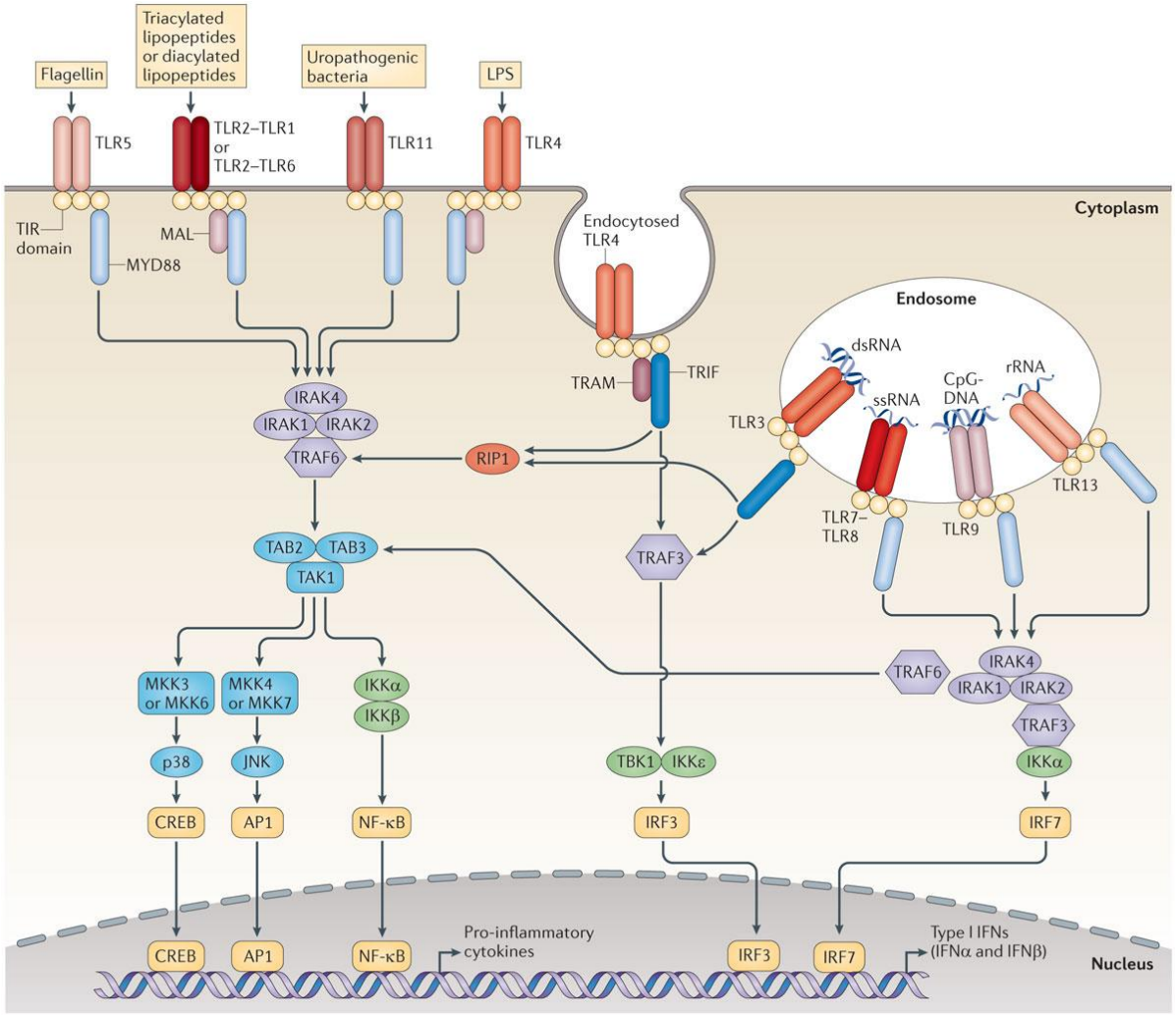


Figure 1.2 Overview of TLR downstream signaling pathways. Upon activation, all TLRs, except TLR3, signal via the adaptor molecule MyD88. Engagement of adaptor molecule stimulate downstream signaling involving IRAKs and TRAFs leading to activation of transcription factors. The activated transcription factors, NF-κB and IRFs, translocates to the nucleus where they regulate target gene transcription. These cascades lead to induction of pro-inflammatory cytokines and type I IFNs. Figure is from O'Neill et al. [36]

1.2.3 Transcription factors

1.2.3.1 NF-κB

NF-κB is the most prominent transcriptional response for TLR activation as it is a downstream target for TLRs [37]. The NF-κB family of transcription factors consists of five members; RelA (p65), RelB, c-Rel, p105 (precursor of p50) and p100 (precursor of p52). The members form homo – or heterodimers to regulate transcription of target genes [34]. The most common dimer is p65/p50 [38]. The NF-κB dimer is bound to IκB and as a result, sequestered in the cytoplasm. Stimulation with TLR ligands induce upstream kinases to phosphorylate IκB marking it for degradation, enabling the NF-κB dimer to translocate to the nucleus [34]. NF-κB binds to κB sites in the promoter of a large number of genes [39]. These genes are involved in innate and adaptive immunity, inflammation, adhesion, and proliferation [34].

1.2.3.2 IRFs

Interferon Regulatory Factors regulates gene expression of type I IFNs. There are 9 IRFs in humans where IRF1, 3, 5 and 7 regulate IFN gene expression. IRFs reside in the cytoplasm, but upon activation, IRFs are phosphorylated leading to dimerization and nuclear translocation where they bind to target promoters [40].

IRF5 is activated downstream of MyD88 – mediated signaling leading to transcription of pro-inflammatory cytokines like IL-6, IL-12 and TNF-α [41]. TLR7 and TLR8 activation induce IRF5 in immune cells leading to induction of both cytokines and type I interferons, specifically IFN-β [26, 40]. IRF5 is expressed in B cells, dendritic cells (DCs) [40], and peripheral blood lymphocytes [42].

IRF7 is expressed at low levels by most cells [35] acting as a strong inducer of type I IFNs. IRF7 is a lymphoid-specific factor constitutively expressed in B cells, pDC and monocytes [43, 44]. TLR7 and TLR8 initiated IRF7 signaling yields a strong induction of IFN-α and IFN-β in monocytes [26, 40].

1.3 The role of Toll-like receptors in Multiple Myeloma

Although TLRs are vital for immunity against microbial pathogens, TLRs are also associated with chronic inflammation, inflammatory disease and tumorigenesis. TLRs are shown to be expressed on various cancer cells implying a potential role for TLRs in cancer [29, 45]. Additionally, host molecules known as DAMPs, thought to originate from necrotic, damaged or malignant host cells, can engage TLRs. Inflammatory cells sustain proliferation and survival of many tumor cells [46].

Endogenous TLR ligands can promote MM cell proliferation directly, but can also activate TLRs on BMSCs to, in turn, produce cytokines subsequently promoting myeloma. Thus, TLRs and NF-κB may be the link between inflammation and cancer progression in multiple myeloma [46, 47].

1.3.1 TLR expression and function in MM cells

Myeloma cells express a different set of TLRs compared to healthy plasma cells. Normal B cells express several TLRs, including TLR7, 9 and 10 which are strongly expressed [47]. Myeloma cell lines and primary cells express TLR1-4, as well as TLR7-9 [48]. However, the TLR expression is uncertain partly due to the heterogeneous distribution among cell lines and primary cells. Overall, MM cells have a stronger expression of TLRs than healthy plasma cells [47].

TLR activation on MM cells induces diverse responses including tumor growth, apoptosis, immune evasion and survival [47]. TLR2/TLR1 enhances adhesion to BMSCs [46]. TLR4 and TLR9 induce pro-tumoral effects mainly by activating an autocrine IL-6 loop [49]. IL-6 induction promotes growth and shield myeloma cells from apoptosis [50]. TLR7 and TLR9 stimulation protect MM cells from drug-induced apoptosis [49]. In addition to stimulating growth, TLR signaling may also protect MM cells against the immune system. TLR2, 4 and 9 activation lead to evasion of the immune surveillance of MM cells by up-regulating B7 homolog-1 (B7-H1) inhibiting cytotoxic T cell generation [49]. Thus, MM cells can impair the functions of immune cells to evade the immune surveillance [47].

The majority of TLR responses in myeloma favor the cancer progression. However, TLR3 and TLR9 activation can induce both anti-apoptotic and pro-apoptotic effects. TLR3-induced IFN-α inhibits MM growth [49], while TLR3-mediated NF-κB activation promotes proliferation [47]. This dual effect makes targeting TLR signaling a challenge.

1.3.2 TLR expression in BMSCs

Bone marrow stromal cells consist of a heterogeneous cell population expressing TLRs [51]. BMSCs in MM patients are reported to express TLR1-6, while expression of TLR7-9 is more controversial [51]. Low mRNA expression of TLR8 and TLR9 have been reported in human MM-BMSCs [52]. BMSCs from healthy individuals are reported to express TLR3 and TLR4 strongly, as well as TLR5, TLR6 and TLR9 [53]. However, since BMSCs are a heterogeneous population, expression of TLRs might vary between donors [51].

Activation of TLRs on BMSCs in MM may lead to cytokine secretion, angiogenesis, chemotaxis and promotion of bone lesions. BMSCs have shown to induce various cytokines as response to TLR activation [54]. TLR4 activation induces secretion of a variety of cytokines and growth factors such as VEGF and insulin-like growth factor 1 (IGF-1) [51]. These cytokines are associated with MM pathogenesis. TLR2, 3, and 4 activation increases IL-6 expression [52], and IL-6 has been reported to be secreted in high levels in MM-BMSCs compared to normal BMSCs [13]. TLR3 or TLR4-mediated activation of BMSCs were shown to up-regulate expression of CXCL8, CCL5/RANTES and IL-1β suggesting a role for BMSCs to mediate an inflammatory milieu [53].

As illustrated in Figure 1.3, DAMPs or PAMPs may activate TLRs, which may result in an inflammatory milieu, immune tolerance or tumor angiogenesis. Overall, TLR-induced responses may in many cases promote cancer progression.

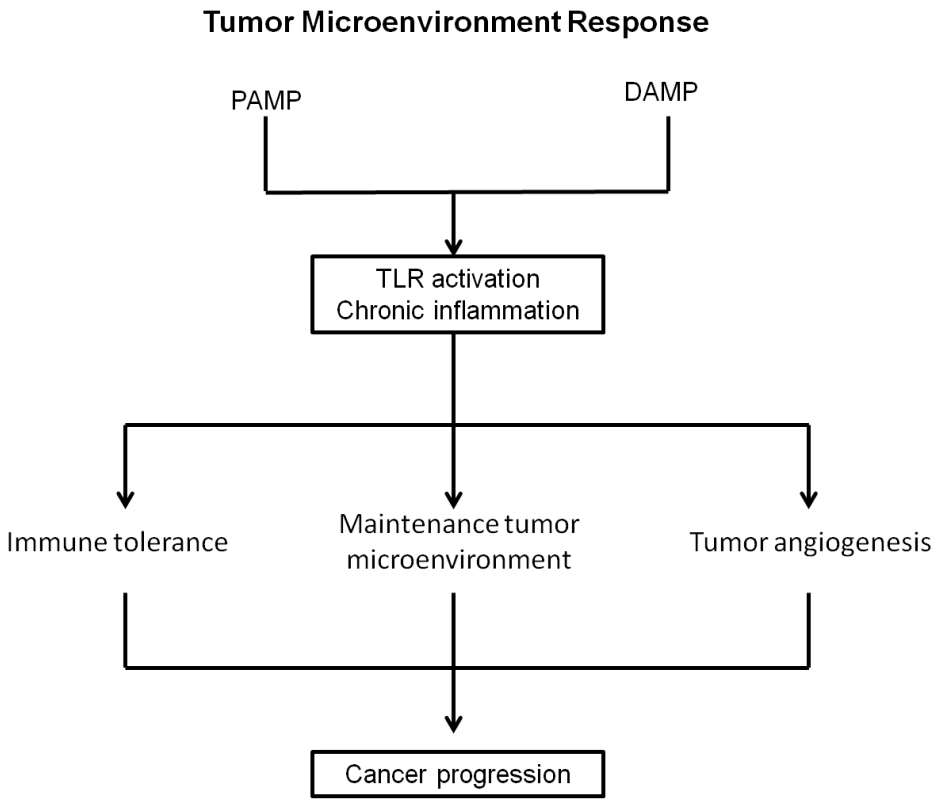


Figure 1.3 Overview of how the bone marrow microenvironment may respond to TLR activation mediating progression of multiple myeloma. DAMP or PAMP activation of TLRs results in chronic inflammation by induction of inflammatory cytokines. Chronic inflammation may lead to constitutive expression of cytokines, further leading to immune tolerance of the cancer cells, maintenance of the niche and tumor angiogenesis. The consequence is cancer progression. Figure adapted from Sato et al. [55]

2 Aims of study

The main goal of this study was to assess if myeloma cells secrete endogenous ligands for TLRs and examine the role of TLR7 and TLR8 in BMSCs.

Tumor cells are reported to secrete TLR-activating components able to activate immune cells [56]. Activation of TLRs is reported to modulate inflammation and drive tumorigenesis [45]. MM pathophysiology is strongly dependent on the interplay between plasma cells and the bone marrow niche for proliferation, survival and drug resistance. TLRs are reported to drive the immune modulating response of BMSCs [57]. BMSCs secrete a variety of cytokines such as IL-6 and IL-8 [53] mediating MM proliferation. A better understanding of the cytokine milieu in the bone marrow of multiple myeloma patients is needed for understanding MM disease progression [6]. This study sought to further unravel the role of TLRs in MM. This was executed by screening MM conditioned medium for TLR-activating components and further examining TLR responses in BMSCs by stimulating primary BMSCs with a panel of TLR ligands.

The study had the following objectives:

1. Investigate if myeloma cell lines release TLR-stimulating components
2. Characterize cytokine responses in bone marrow stromal cells from myeloma patients upon TLR stimulation
3. Examine TLR expression in BMSCs
4. Investigate gene expression in primary BMSCs in response to TLR stimulation
5. If relevant, determine if primary stroma cells are activated by myeloma cell conditioned medium

3 Materials and Methods

3.1 Long-term Culture of Multiple Myeloma cells

3.1.1 MM culture conditions

The MM cell lines used were ANBL-6 (a gift from D. Jelinek, Mayo Clinic, Rochester, MN, USA), INA-6 (a gift from Dr. M. Gramatzki, Erlangen, Germany), JJN-3 (a gift from J. Ball, University of Birmingham, United Kingdom), IH-1, KJON-1, OH-2 (established at St. Olavs Hospital, Trondheim University Hospital, Norway), RPMI-8226 and U266 (American Type Culture Collection, Rockville, MD, USA).

3.1.1.1 Reagents used in cell culturing

RPMI 1640 and L-glutamine were from Sigma-Aldrich (Schnelldorf, Germany). Fetal calf serum (FCS) from Gibco (cat.no. 10270-106, Life Technologies), human serum (HS v.i.) was obtained from the Department of Immunology and Transfusion Medicine at St. Olavs Hospital, Trondheim. Gentamicin purchased from Sanofi-Aventis (Norway), rhIL6 from Gibco (cat. no. PHC0061), and IL-6 sup was isolated from LPS – stimulated peripheral blood mononuclear cells (in-house).

3.1.2 Isolation of MM supernatant

All myeloma cell lines were cultured at 37 ^{o}C humidified atmosphere containing 5% CO_2. Cells were grown in RPMI 1640 supplemented with L-glutamine (100 µg/ml) and gentamicin (20 µg/ml) (referred to as RPMI) and supplemented with fetal calf serum (FCS), heat-inactivated human serum (HS i.v.), or IL-6 (Table 3.1).

Myeloma cells were incubated at 37 ^{o}C, 5% CO_2 for a predetermined number of days. Cells cultured longer than 14 days were supplemented with 5 ml culture medium 1-2 times a week. After culturing, cells were transferred to a tube and spun at 2000 rpm for 8 minutes to eliminate cells and cellular debris. The supernatant was harvested and aliquoted before stored at -20 ^{o}C.

Table 3.1 Overview of medium for cell lines. Overview of growth medium used in the experiments.

Cell line	Growth medium
ANBL-6	10% FCS/RPMI with rhIL6 (1 ng/ml)
INA6	10% FCS/RPMI with rhIL6 (1 ng/ml)
JJN-3	10% FCS/RPMI
IH-1	10% HS v.i./RPMI with IL-6 sup
KJON-1	5% HS v.i./RPMI with IL-6 sup
OH-2	10% HS v.i./RPMI with IL-6 sup
RPMI-8226	20% FCS/RPMI
U266	15% FCS/RPMI

3.1.3 Luciferase Reporter assay to Determine if MM conditioned medium can Activate TLRs

The NF-κB luciferase reporter assay was utilized to screen for TLR-activating components secreted by myeloma cells.

3.1.3.1 The principle of NF-κB luciferase Reporter assay

Human embryonic kidney (HEK) cells lack most TLRs, except TLR1, 5, and 6 [58, 59]. Upon transfection of TLRs, HEK293 gain the ability to respond upon TLR activation. TLR-mediated pathway leads to activation of the transcription factor NF-κB. The NF-κB luciferase reporter plasmid consists of the promoter Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1) containing five NF-κB sites fused to the Firefly luciferase gene. Upon activation of the NF-κB pathway, NF-κB subunits will bind to the κB promoter sites activating transcription of the luciferase gene. The luciferase enzyme fluoresces upon addition of the substrate luciferin, as illustrated in Figure 3.1. Renilla luciferase is constitutively expressed and functions as an internal control for normalization of technical variability such as transfection efficiency. NF-κB activation is directly proportional to measured fluorescence.

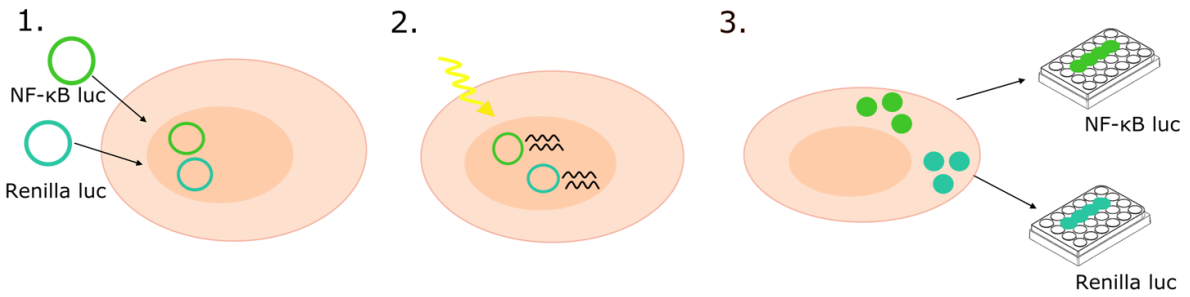


Figure 3.1 The principle of NF-κB luciferase reporter assay. 1) Cells are co-transfected with plasmids for NF-κB luciferase, Renilla luciferase and TLRs. 2) TLR ligands activate transfected TLRs that stimulate NF-κB to bind to κB sites in the promoter of the NF-κB luciferase vector and the reporter gene is transcribed. 3) Luciferase protein is synthesized, luciferase detection reagent is added and luciferase activity is measured. Renilla luciferase is independent of experimental modulation.

3.1.3.2 Reagents used in Luciferase Reporter assay

The TLR2Flag plasmid was provided by Dr. C. Kirschning (Technical University of Munich, Germany), TLR3Flag was a gift from Dr. Akira (Osaka University, Osaka, Japan), pFLAG-CMV1-TLR7 was made by Schoenemeyer et al [40], TLR8pUNO was purchased from InvivoGen, TLR9HA was a gift from Dr. Gregers (University of Oslo, Norway), and pcDNA3 plasmid was purchased from Invitrogen (CA, USA). The reporter plasmid ELAM luciferase was a gift from Prof Douglas Golenbock (UMass Medical School, MA, USA) and prhNull Renilla was purchased from Promega (Madison, WI, USA).

Pam3CysSK$_4$ was purchased from EMC microcollections GmbH (Tübingen, Germany), polyionsine polycytidylic acid (poly I:C (HMW)), R848 and CL075 were purchased from InvivoGen while CpG was from TIB MolBiol (Berlin, Germany).

GeneJuice® Transfection Reagent was from Novagen, Firefly substrate luciferin was prepared in-house (20 mM Tricine, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 530 µM ATP, 270 µM Acetyl CoEnzyme A, Luciferin (30 mg), 2M NaOH (570 µl), 50 mM Magnesium Carbonate Hydroxide (1.21 ml) and H_20 up to 228 ml), and colentrerazine (Renilla substrate) was from Sigma.

Table 3.2 TLR ligands used in NF-κB luciferase reporter assay

Ligand	Concentration	Target receptor
Pam3Cys	200 ng/ml	TLR2/1
Poly I:C	10 μg/ml	TLR3
R848	1 μg/ml	TLR7 and TLR8
CL075	1 μg/ml	TLR8
CpG	15 μM	TLR9

3.1.3.3 Procedure

HEK293 (American Type Culture Collection, Rockville, MD, USA) were cultured at 37 ^{o}C humidified atmosphere containing 5% CO_2. HEK293 was grown in 10% FCS/RPMI in a T75 flask and subcultured twice a week. During splitting, old medium was removed and cells were washed with DPBS (Sigma) before detachment with trypsin/EDTA (BioWhittaker®, Lonza). Trypsin was neutralized with growth medium and cells were transferred to a new T75 flask or plated out.

HEK293 cells were plated in 96-well plates in 10% FCS/RPMI. At 50 – 70% confluence, cells were transiently co-transfected with ELAM luciferase reporter (25 ng/well), Renilla luciferase control reporter (0.5 ng/well) and TLR expression plasmids or empty vector (pcDNA3) (10 ng/well) using GeneJuice (3 μl/μg plasmid) and RPMI with no additives. The cells were incubated for 24 hours at 37 ^{o}C, 5% CO_2. The supernatants were carefully removed and the cells were treated with conditioned medium from MM cell lines or TLR ligands for 48 hours at 37 ^{o}C, 5% CO_2. Cells were lysed using 1x Passive Lysis Buffer (50 μl/well, Promega) and stored at - 20 ^{o}C to enhance cell lysis. The lysate was thawed at room temperature for a minimum of 2 hours. To determine the NF-κB activity, the lysates (15 μl/well) were distributed to two separate white 96-well plates (OptiPlate^{TM}, Perkin Elmer). Luciferase substrate (35 μl/well) was added and luciferase activity measured using Walla Victor3^{TM} 0420 Multilabel Counter (Perkin Elmer^{TM}).

3.1.3.4 RNase and Heat treatment of MM conditioned medium

To determine the substance class of the stimuli in the conditioned medium, the conditioned medium was treated with either RNase A or heated. RNase A is an enzyme degrading RNA in the sample while heat causes proteins to denature.

Validation of RNase A by gel electrophoresis

RNase A functionality was validated by treating isolated U266 RNA and polyU with RNase and assayed RNA degradation by gel electrophoresis. RNA (100 – 200 ng) or polyU (5 µg/ml) were treated with RNase A (50 µg/ml) for 1,5 hour at 37 ^{o}C. The samples were applied to an 1% agarose gel (Seakem® Le Agarose, Lonza) containing GelRed^{TM} (Biotium) with TAE buffer and run for 40 minutes at 90 V. An 1kb ladder (N3232, New England Biolabs) was applied. The bands were visualized using Bio Doc-H Imaging System from UVP.

RNase A and Heat Treatment of U266 conditioned medium

U266 conditioned medium was treated with RNase A (50 µg/ml) for 1,5 hour at 37 ^{o}C or heated at 95 ^{o}C for 10 minutes. The treated conditioned medium were tested using the luciferase reporter assay as described in 3.1.3.

3.1.4 Isolation of Extracellular Vesicles from MM conditioned medium

Extracellular vesicles from myeloma cell lines were isolated using the ultracentrifugation method to assay the capability of ECVs to activate TLRs.

3.1.4.1 Principle of ultracentrifugation

Ultracentrifugation is a method based on size differentiation were particles of different sizes will sediment at different forces. Ultracentrifugation is used to isolate exosomes from cell culture media by multiple centrifugation steps, each with a greater force.

The first centrifugation steps eliminate cells and cellular debris by successive centrifugation at increasing forces as illustrated in Figure 3.2. The supernatant is used for the following centrifugation steps. At 10,000 x g large apoptotic bodies and larger extracellular vesicles sediment [60]. Ultracentrifugation at 120,000 x g sediments smaller extracellular vesicles

corresponding to exosomes in size. The pellet is washed to remove contaminants like proteins that sediment at same force as exosomes before the ultracentrifugation step is repeated to obtain the final exosomes [61].

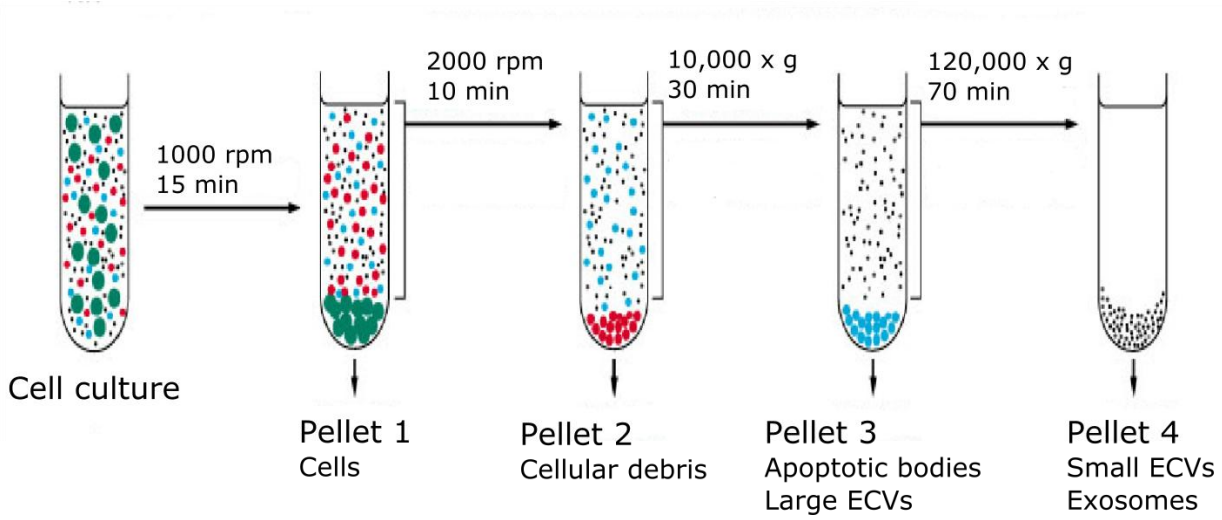


Figure 3.2 Flow chart of ultracentrifugation method. The cell culture is centrifuged to sediment cells (pellet 1) and cellular debris (pellet 2) before the supernatant is further centrifuged to sediment apopotic bodies and large extracellular vesicles (ECVs) (pellet 3). The supernatant is then centrifuged at 120,000 x g to sediment small extracellular vesicles corresponding to exosomes in size (pellet 4). Pellet 4 is washed with before the ultracentrifugation step is repeated and the pellet stored for further analysis. Figure adapted from UBC [62]

3.1.4.2 Procedure

FCS was centrifuged at 120 000 x g for 15 hours to remove serum-derived vesicles. Myeloma cells were centrifuged at 1500 rpm for 5 minutes, resuspended in vesicle-deprived 10% FCS/RPMI, and incubated in a 37 ^{o}C humidified atmosphere containing 5% CO_2. After 14 days culture, the cells were then centrifuged at 1000 rpm for 15 minutes at 4 ^{o}C to remove cells. The supernatant was transferred into a new tube and centrifuged at 2000 rpm for 10 minutes at 4 ^{o}C to remove remaining cell debris. Supernatant was transferred into 14 ml polypropylene tubes (cat.no. 352059, BD Falcon) before centrifuged at 10 000 x g for 30 minutes at 4 ^{o}C using a Sorvall RC 6+ centrifuge (Thermo Scientific) with a SS-34 rotor (Thermo Scientific). The supernatant was transferred to new tubes and placed in ice-water overnight at 4 ^{o}C. The following day, the supernatant was transferred to polyallomer centrifugation tubes (cat.no. 331372, Beckman Coulter) for ultracentrifugation at 120 000 x g for 70 minutes at 4 ^{o}C in SW 41 Ti swinging bucket rotor (Beckman Coulter) using Optima L-80 XP Ultracentrifuge (Beckman Coulter). Supernatant was removed and the pellet was

resuspended in RPMI 1640 and centrifuged again at 120 000 x g, 70 minutes at 4 ^{o}C to isolate the exosomes. Supernatant was eliminated and the pellets were stored at -80 ^{o}C.

3.2 Bone marrow stromal cells

BMSCs are a heterogeneous population of CD138^{-} cells isolated from the bone marrow of multiple myeloma patients. BMSCs from 10 different donors are mixed to obtain standardized BMSCs. More than 90 % of standard stromal cells were reported to be positive for CD90, CD44, CD73, CD105, CD29 and HLA-ABC by immunotyping using flow cytometry. A smaller fraction of cells were positive for CD14, CD45, CD209 and CD34. Therefore, BMSCs can be referred to as fibroblast-like cells [63].

3.2.1 Culture conditions

Standard stroma collected from myeloma patients were supplied by the National Biobank for Multiple Myeloma, St. Olavs, Norway and cultivated like described in Misund et al [63]. In brief, mononuclear cells after CD138 - positive selection were cultivated for three weeks. BMSCs from 10 patients were mixed to obtain standardized BMSC. The use of primary cells is approved by the Regional Committees for Medical and Health Research Ethics (REK 2011/2029).

3.2.2 Stimulation of BMSCs with TLR ligands

BMSCs from multiple myeloma patients were stimulated with TLR ligands to determine its effect on BMSCs. Cytokine induction was assayed by human cytokine proteome profiler and ELISA.

3.2.2.1 Reagents used for stimulation

poly I:C (HMW), lipopolysaccharide (LPS) K12 (E. *coli* K12), R837, CL264, R848, CL075 , polyU, polyU//LyoVec, and ssRNA41/LyoVec were purchased from InvivoGen. Fibroblast stimulating

factor-1 (FSL-1) was purchased from EMC microcollections GmbH (Tübingen, Germany). An overview of the TLR ligands, their targets receptors and concentrations used in this study is presented in Table 3.3.

Table 3.3 Overview of TLR ligands, their target receptor and concentrations used to stimulate BMSCs

Ligand	Concentration (µg/ml)	Target receptor
FSL-1	0.2	TLR2/6
Poly I:C	5 and 1	TLR3
LPS K12	0.2	TLR4
R837	5 and 1	TLR7
CL264	5	TLR7
R848	5 and 1	TLR7 and TLR8
CL075	5 and 1	TLR8
polyU	5	TLR8
polyU/LyoVec	5	TLR8
ssRNA41/LyoVec	5	Control

3.2.2.2 Procedure

BMSCs were obtained from the nitrogen tank and immediately thawed in water holding 37 ^{o}C. Cells were transferred to a tube with warm medium before centrifuged at 1500 rpm for 8 minutes. The pellet was resuspended in 2% A+ v.i./RPMI (1 ml) before counted using a Burker's chamber using Trypan blue (0.4%, Life Technologies) to eliminate nonviable cells during counting. The cells were seeded in a 96-well flat-bottom plate (2500 cells/well) and incubated at 37 ^{o}C/5% CO_2 overnight to adhere. Stimulations were prepared in RPMI with no additions before addition to the cells. Cells were stimulated for 72 hours in 2% A+ v.i./RPMI at 37 ^{o}C/5% CO_2. Following incubation the supernatant were harvested and stored at -20 ^{o}C.

3.2.3 Determining cytokine release in BMSCs using Human Cytokine Proteome profiler™

Proteome profiler™ was used to screen for cytokine expression of stimulated BMSCs.

3.2.3.1 The principle of proteome profiler™

A nitrocellulose membrane has been spotted with capture antibodies, in duplicates, for several cytokines and growth factors. Sample is added to the membrane overnight before the array is washed multiple times to remove unbound proteins. Detection antibodies and detection reagents are added to measure luminescent signal corresponding to the amount of protein bound [64].

3.2.3.2 Procedure

BMSCs had been stimulated like described in 3.2.2 with CL075 (5 µg/ml) for 72 hours in 37 ^{o}C, 5% CO_2. The supernatant was used to screen for 102 cytokines in a single sample. The human XL cytokine array kit assay (cat. no. ARY022, R&D systems) was performed according to manufacturer's protocol [64]. In short, the membrane was blocked with Array Buffer 6 before the supernatant was added. The membrane was incubated overnight at 4 ^{o}C on a shaker for optimal sensitivity. The membrane was washed three times with wash buffer before Detection Antibody cocktail was added and membrane incubated for 1 hour. The membrane was washed three times with wash buffer. Streptavidin-horseradish peroxidase (HRP) was added to the membrane for 30 minutes on a shaker before washing three times with wash buffer. Chemi Reagent Mix was pipetted evenly onto the membrane and incubated for 1 minute. The luminescence signal was detected with Li-COR Odyssey and analyzed using Image Studios v.3.1. The principle is illustrated in Figure 3.3.

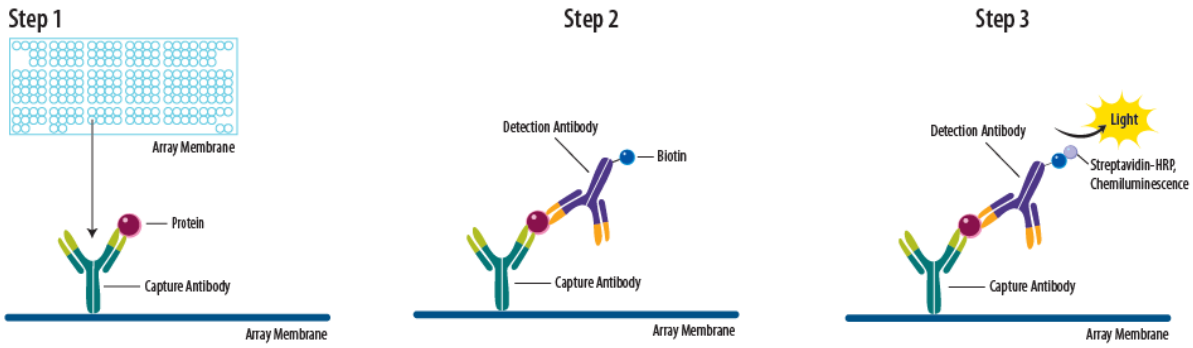


Figure 3.3 Proteome profiler procedure. Step 1) The membrane is pre-spotted with capture antibodies which target analytes in the cell supernatant bind to. Step 2) Detection antibody conjugated to biotin binds the protein-capture antibody complex. Step 3) Chemi Reagent mix reacts with Streptavidin-HRP creating a luminescence signal. Figure from R&D [65].

3.2.4 Enzyme-Linked Immunosorbent Assay - ELISA

ELISA was used to measure cytokine production by BMSCs, monocytes, macrophages and monocyte-derived dendritic cells after stimulation with TLR ligands.

3.2.4.1 The principle of ELISA

ELISA is a biochemical technique to detect the presence of a certain protein in a sample. The method uses a sandwich-like procedure where the protein of interest is between two antibodies specific for the target protein. One capture antibody binds the protein in the cell supernatant, while bound protein is detected by a second antibody.

The plate is coated with capture antibody for the analyte of interest and then blocked to hinder any nonspecific binding sites. Sample is added to the plate and the analyte of interest binds to the capture antibody. A detection antibody recognizes and binds to the analyte on a different epitope than the capture antibody. The detection antibody is biotinylated, which binds streptavidin-HRP through streptavidin. Between each addition of reagent and sample the plate is washed to remove any unbound reagents. HRP is an enzyme that upon addition of TMB substrate will yield a blue color. A stop solution is added to terminate the enzymatic reaction shifting the color from blue to yellow. The color intensity can be measured at an absorbance at 450 nm. The detected light intensity is proportional to the amount of analyte present in the sample.

The principle of sandwich ELISA is illustrated in Figure 3.4.

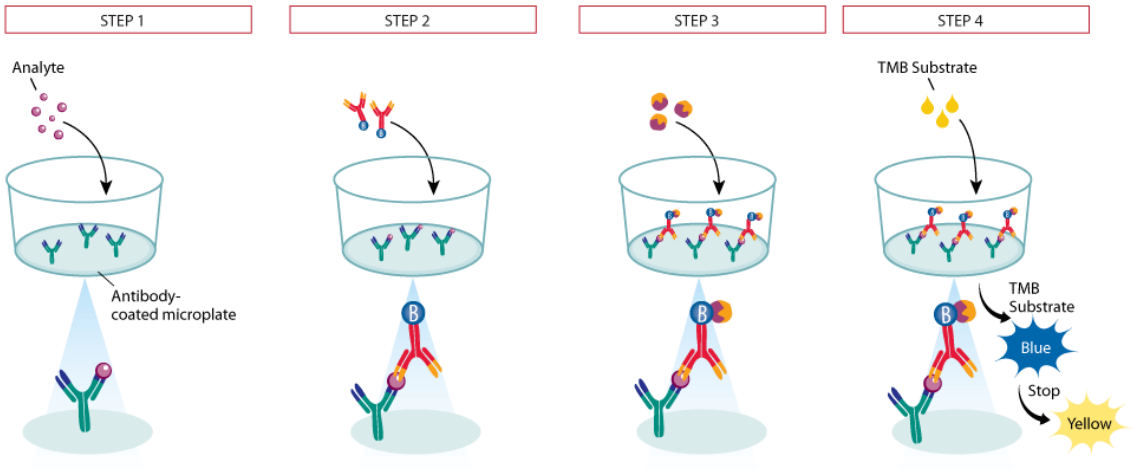


Figure 3.4 The principle of sandwich ELISA. (1) Before sample is added, the well is coated with capture antibody for analyte of interest, then blocked for any nonspecific binding sites. The analyte in the sample will bind to the capture antibody (2) Detection antibody conjugated to biotin binds to a different epitope on the analyte. (3) Streptavidin-HRP binds to the detection antibody through the streptavidin. (4) HRP converts the TMB substrate into a blue color. A stop solution terminates the enzymatic reaction and the color shift from blue to yellow. The color intensity can be measured at a 450 nm wavelength. Figure adapted from R&D [66].

3.2.4.2 Reagents used for ELISA

DuoSet® ELISA from R&D Systems® was used to detect the following cytokines in cell supernatant from cells stimulated with TLR ligands; human CCL3/MIP-1α (catalog number DY270), CXCL1 (cat.no. DY275-05), CXCL5 (cat. no. DY254), IL-1β (cat no. DY201) and TNF-α (cat. no. DY210). Verikine™ ELISA from PBL Assay Science was used to assess IFN-β (cat.no. 41410-1A).

Dulbecco's phosphate buffered saline (DPBS) and Bovine serum albumin (BSA, (cat. no. A7030-SOG)) were purchased from Sigma, substrate A (H_20_2) and B (TMB) were purchased from BioLegend.

3.2.4.3 Procedure

ELISA was performed according to manufacturer's recommendation. Briefly, a 96 half-well plate (Costar®, Corning, NY, USA) was coated with a known concentration of capture antibody (40 µl/well), sealed and incubated in room temperature overnight. The plate was

washed with wash buffer (0.05% Tween-20 in PBS, pH 7.2-7.4) three times using a Tecan automatic plate washer to remove excess capture antibody. Excess wash buffer was removed completely (referred to as wash step). The specified block buffer (100 µl/well) was applied to each well. The plate was sealed and incubated for at least 2 hours at room temperature. After incubation, the wash step was repeated. An 1:2 standard dilution series was prepared where the last well was left blank to create a standard curve. Samples (40 µl/well) were added, plate was sealed and incubated at room temperature overnight. The wash step was repeated before coating with detection antibody (40 µl/well) for 2 hours at room temperature. The wash step was repeated and streptavidin-HRP (40 µl/well) was diluted according to manufacturer's recommendation and added to all wells. The plate was covered with tin foil and incubated at room temperature for 20 – 45 minutes. Substrate was prepared by mixing equal volume of substrate A (H_2O_2) and B (TMB) immediately before addition to the plate (40 µl/well). The plate was covered with tin foil and incubated at room temperature until the standard had a clear blue gradient. Then, a stop solution (2M H_2SO_4) was added to terminate the enzymatic reaction. The samples' absorbance was measured at 450 nm using BioRad iMark™ Microplate Reader.

IFN-β ELISA

Detection of IFN-β expression was performed using Verikine™ Human IFN beta ELISA kit (cat.no. 41410) according to manufacturer's instruction (Appendix II). In brief, samples and standard curve were added to the plate for 1 hour before the wells were washed three times. Detection antibody, TMB and HRP was added before the samples' absorbance was measured at 450 nm using BioRad iMark™ Microplate Reader.

3.3 TLR responses in Monocytes, Macrophages and Monocyte-Derived Dendritic cells

Monocytes, macrophages and monocyte-derived dendritic cells were stimulated with TLR ligands and assayed for cytokine expression to compare with cytokine induction by TLR stimulated BMSCs.

3.3.1 Isolation of monocytes from peripheral blood mononuclear cells

A+ buffy coat was obtained from the Blood Bank at St. Olavs Hospital, Trondheim. The buffy coat was mixed with 100 ml DPBS and gently added to a tube with lymphoprep (Axis-Shield, Norway). The buffy coat was centrifuged at 800 x g for 20 minutes to isolate the peripheral blood mononuclear cells (PBMCs) by gradient sedimentation. The PBMCs were harvested from the distinct band in the interface between the sample and the lymphoprep using a sterile glass pipette. The cells were spun at 950 x g for 10 minutes and supernatant discarded. The cell pellet was resuspended in 20 ml Hanks' balanced salt solution (Sigma) and spun once more at 150 x g for 8 minutes. This washing step was repeated a total of four times. Cells were then resuspended in 10% HS/RPMI and counted using Countess^{TM} with Trypan blue as a dye exclusion method of non-viable cells. Cells were plated in 10% HS/RPMI and incubated at 37 ^{o}C, 5% $C0_2$.

3.3.2 Differentiation and Characterization of Macrophages and MoDCs

Macrophages can be differentiated from PBMCs by addition of serum while addition of granulocyte macrophage colony – stimulating factor (GM-CSF) and IL-4 induce differentiation to moDC [67, 68]. Differentiated cells express cellular markers, enabling to characterize them upon flow cytometry.

3.3.2.1 Differentiation procedure

PBMCs were isolated according to 3.3.1, plated in a 96-well plate at 0.5 - 0.7 mill cells/well in 10% HS/RPMI and incubated for 2 hours at 37 ^{o}C, 5% $C0_2$ for adherence of monocytes. After incubation, the cells were washed three times with Hanks' to remove non-adherent cells.

To differentiate cells into macrophages, 20% or 30% HS/RPMI was added on day 1 and changed on day 3 and day 5.

For differentiation to monocyte-derived dendritic cells, GM-CSF (100 ng/ml, cat. no. 215-GM/CF, R&D Systems®) and IL-4 (40 ng/ml, cat.no. 204-IL/CF, R&D Systems®) were added to 10% HS/RPMI. Medium was added on day 3 and day 5.

3.3.3 Characterization of Macrophages and Monocyte-derived dendritic cells by Flow Cytometry

Flow cytometry was used to assay differentiation of PBMCs to macrophages and monocyte-derived dendritic cells.

3.3.3.1 Principle of Flow Cytometry

Flow cytometry is a laser-based method to measure the properties of individual particles such as cells. Cells are focused in a single cell stream passing light beams to measure either light scattering or fluorescence emission. Cells can be labeled with fluorochrome–antibodies for cell surface receptors, which will emit fluorescence upon laser excitation at a specific wavelength. The light scatter hits a detector transforming the light scatter into digital signals for computer processing [69].

3.3.3.2 Antibody markers used

Control antibodies used were pFluor 450 lgG2a (eBioscience, 48-4724-82), APC lgG (R&D), FITC mouse IgGk1 and PE mouse lgG2a from Pharmingen.

Antibodies for macrophages were CD86 FITC (Pharmingen), CD83 PE (cat. no. PN IM2218, Immunotech, France), HLA-DR APC (eBioscience) and CD14 eFluor 450 (eBioScience). Antibodies for moDCs were the same as for macrophages except the addition of CD206 IgG2a (R&D).

3.3.3.3 Procedure for characterizing cells by Flow cytometry

At day 8, the macrophages were washed with warm DPBS before Accutase (0.5 ml) was added to detach the macrophages from the culture vessel. A cell scraper was used to ensure complete detachment. 1% FCS/DPBS was added to neutralize the enzyme and cells were spun down at 2000 rpm for 5 minutes at 4 ^{o}C, resuspended in 1% FCS/PBS (1 ml) and counted using Countess. Cells were distributed to a V – bottom 96 well plate and spun down at 2000 rpm for 5 minutes at 4 ^{o}C. Antibodies were added (50 µl/well) and cells incubated on

ice for 30 minutes. Cells were washed with 1% FCS/DPBS (150 µl) and centrifuged at 2000 rpm for 5 minutes to remove unbound antibodies, this was performed twice. Cells were then resuspended in 1% FCS/DPBS (350 µl) before transferred to small FACS tubes (MP biomedicals, LCC, France) and analyzed using a LSRII flow cytometer (BD Bioscience). Data were analyzed with FlowJo v10 software (Tree Star Inc, Ashland, OR).

Non-adherent moDCs were transferred into a tube before adherent cells were detached with warm DPBS. The cells were collected and stained using the same procedure as described above. A FcR inhibitor (eBioscience, 14-9161-73) was added to the cells for 10 minutes of incubation on ice before staining with antibodies. CompBeads were used for calculating compensation and functioning as a CD14 positive control. BD^{TM} Comp Beads (cat.no. 552843, BD Biosciences, CA, USA) were stained with antibodies with the same protocol as the cells. 1 drop of beads per well as recommended by the manufacturer was used.

3.3.4 Stimulation with TLR ligands of monocytes, macrophages and moDCs

TLR ligands used to stimulate the cells are listed in Table 3.3.

Monocytes and macrophages were stimulated in 2% HS/RPMI for 20 hours, incubated in 37 ^{o}C, 5% $C0_2$. Stimuli was added to moDCs in 10% FCS/RPMI with GM-CSF and IL-4 for 20 hours while incubated at 37 ^{o}C, 5% $C0_2$. Supernatant was harvested after stimulation and stored at -20 ^{o}C.

3.4 Determine NF-κB and IRF5 activation in response to TLR8 ligand CL075 in BMSCs using the ScanR system

Activation of TLR downstream signaling results in nuclear translocation of transcription factors to regulate expression of target genes. NF-κB is sequestered in the cytoplasm by IκB inhibitory proteins but upon activation, NF-κB is freed and translocates to the nucleus. IRF5, another transcription factor downstream of TLR signaling, also translocates to the nucleus upon activation where it binds the promoter of target genes. In this study, scanR was used to

screen BMSCs for nuclear translocation of transcription factor NF-κB and IRF5 after stimulation to determine activation of TLRs.

ScanR (Olympus, Heidelberg, Germany) is an automated fluorescence microscopy – based platform used for different screening assays by acquisition of multiple images per well. This enables the system to screen a large number of samples as well as quantification of fluorescent signal.

3.4.1 Reagents

10% saponin (1g to 10 ml deionized water, sterile filtrated) (Sigma Aldrich) diluted with PBS to yield an 1% PBS-S solution. Quench buffer (NH4CL (2.5 ml, 1M), saponin (250 µl, 10%), PBS), block buffer (20% A+/PBS-S), antibody buffer (1%A+/PBS-S), and PEM-buffer (100 mM K-Pipes, 5 mM EGTA, 2mM $MgCl_2$ and saponin (0.05%)) were made in-house.

Primary antibodies used were NF-κB p65 (cat. no. 8242S) and E1N9G (cat. no. 13496S) purchased from Cell Signaling Technology. CD14 antibody, α-CD14 18 D11 IgG was made in-house. Secondary antibodies were Alexa Fluor® 488 goat anti-mouse IgG (cat.no. A11001) and Alexa Fluor® 647 anti-rabbit IgG (cat.no. A21245) from Life Technologies.

3.4.2 Intracellular staining for ScanR

BMSCs were plated (1500 cells/well) in 10% HS v.i./RPMI. The 96-well glass plate (In Vitro Scientific, CA, USA), pre-coated with HS v.i. serum (40 µl/well), was incubated at 37 ^{o}C, 5% $C0_2$ for three hours to enhance adhesion of cells to the glass plate. Cells where then stimulated in 2% A+v.i./RPMI for 3 hours with TLR8 ligand CL075 (1 µg/ml).

After stimulation, the cells were fixated in 2% ice-cold paraformaldehyde (PFH) on ice for 15 minutes and washed with PBS for 5 minutes a total of three times. Cells were permeabilized with PEM-buffer for 10 minutes to enable primary antibodies to pass the cell membrane. Quenching was performed with NH_4Cl/PBS-S for 5 minutes to quench possible auto-fluorescence of the cells. 20% A+/PBS-S was added to the cells for 20 minutes to block any

auto - fluorescence. The cells were washed once with antibody solution (1% A+/PBS-S) before stained with primary antibody overnight at 4 ^{o}C.

The cells were washed with PBS-S twice to remove unbound primary antibody before washed briefly with antibody solution. The cells were stained with secondary antibody for 30 minutes at room temperature. After staining, the cells were washed three times with PBS-S before a second fixation in 4% PFH for 20 minutes at room temperature. A final wash with PBS-S for 5 minutes was performed before nuclear staining with Hoechst 33342 (Life Technologies). The cells were analyzed using scanR performed by Nadra Nilsen. Acquired data were analyzed using scanR analysis software v1.3.

3.5 Quantitative Real Time PCR

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) was used to determine the mRNA expression of TLRs in BMSCs. In addition, qRT-PCR was used to optimize stimulation concentration and time-point prior to Nanostring gene expression analysis of BMSCs.

qRT-PCR is a method to amplify and quantify a predetermined DNA sequences in a sample using two primers complementary to target. Primers bound to targets provide a 3' – hydroxyl terminus for the DNA polymerase. PCR is a cyclical process in three reoccurring steps: denaturation, annealing and elongation. The DNA is denatured to single strands in order for the primers to anneal to the target sequence. The DNA polymerase elongates the primers creating a double stranded DNA molecule of the target gene. This cycle is repeated where the newly synthesized DNA strands function as templates for the subsequent rounds of PCR. This enables an exponential amplification of the target sequence.

3.5.1 Principle of qRT-PCR

TaqMan assay

TaqMan probe has a fluorochrome at the 5’ end and a quencher at the 3’ end of the probe. The probe hybridizes to an internal region of the target, and during elongation, the DNA polymerase digests (5’-3’ exonuclease) the probe, separating the fluorochrome and the quencher enabling the fluorescent signal to be detected. The emitted fluorescence is continuously measured. The change in target concentration is reflected in the change in intensity of the measured fluorescence [70].

SYBR Green assay

SYBR Green is an intercalating fluorescent dye used as a fluorescent reporter molecule in qRT-PCR. The dye inserts between the two strands of the DNA duplex. SYBR Green is more sensitive than TaqMan making it suitable for detecting low DNA concentrations. SYBR Green binds any non-specific DNA duplexes co-amplified during the PCR thermocycling detecting unspecific products. Therefore, a post-PCR melt curve analysis is executed to check for unspecific amplified products and primer dimers [70].

3.5.2 Procedure of qRT-PCR

In order to perform qRT-PCR, RNA must be extracted and synthesized to cDNA.

RNA isolation

RNA isolation was performed using the RNeasy Mini kit from Qiagen (cat.no. 74104). Cells were stimulated and supernatant discarded and cells were washed with DPBS. Lysis buffer was added and stored at – 80 ^{o}C to enhance lysis. β-mercaptoethanol (1 %, Merck – Schuchardt, Germany) was added to the lysis buffer to stabilize the RNA by eliminating ribonucleases released during cell lysis. The RNA was extracted according to manufacturers protocol. In brief, 1 volume of 70% ethanol was added to the lysate before loaded onto the membrane in the spin column provided in the kit. RNA bound the column and contaminants were washed away before RNA was eluted using RNase – free water. The RNA quality and concentration was measured using NanoDrop ND-1000 before stored at -80 ^{o}C.

cDNA synthesis

cDNA was made from extracted RNA using High Capacity RNA-to-cDNA kit from Applied Biosystems (cat. no. 4387406). For each sample, buffer mix (10 µl) and enzyme mix (1 µl) was added to a tube before 9 µl of RNA was added. The strip was sealed and briefly centrifuged to eliminate any air bubbles. The samples were incubated at 37 ^{o}C for 60 minutes, heated up to 95 ^{o}C for 5 minutes to terminate the enzymatic reaction and held on 4 ^{o}C for infinity using 2720 Thermal Cycler from Applied Biosystems.

qRT – PCR

TaqMan gene expression assays were purchased from Applied Biosystems; CCL3 (Hs00234142) and TBP (Hs00427620). Primers used in the SYBR Green assay was designed by Guro Stødle and had the following sequences; TLR2 forward (tgactcccaggagctcttag), TLR2 reverse (cttccttggagaggctgatg), TLR3 forward (gccttctgcacgaatttga), TLR3 reverse (tccagctgaacctgagttcc), TLR4 forward (cctggacctgagctttaatc), TLR4 reverse (aaaggctcccagggctaaac), TLR7 forward (gtttctgtgcacctgtgatg), TLR7 reverse (tgtggccaggtaaggaatag), TLR8 forward (gttggaactacacggaaacc), TLR8 reverse (ggactggcacaaatgacatc), TBP forward (ttgctgcggtaatcatgagg), and TBP reverse (gccagtctggactgttcttc).

cDNA samples were diluted with sterile ion filtered water (SIW) to a concentration of 40 – 50 ng/µl before mixed as described in Table 3.4 for the TaqMan assays or as in Table 3.5 for the SYBR Green assay. The Perfecta® qPCR FastMix® (cat.no. 95077-012, Quanta Biosciences inc, MD, USA) contains AccuStart™ Taq DNA polymerase, dNTPs, $MgCl_2$, ROX (reference dye) and stabilizers. The Power SYBR® Green PCR Master mix (cat.no. 4367659, Applied Biosystems by Life Technologies) contains SYBR® Green I Dye, AmpliTaq Gold® DNA polymerase, dNTPs, ROX and optimized buffer components.

The samples were distributed in a 96 well PCR plate sealed with tape and spun at 1500 rpm for 5 minutes to eliminate air bubbles. The samples were analyzed on the StepOnePlus™ Real Time PCR machine from Applied Biosystems using StepOne software v2.2 and v2.3. The cycling parameters for TaqMan assay were; 2 minutes at 50 ^{o}C, 95 ^{o}C for 20 seconds for

initial denaturation, then 95 ^{o}C for 1 second and 60 ^{o}C for 20 seconds for a total of 40 cycles. The cycling parameters for SYBR Green assay were; 5 minutes at 95 ^{o}C, then a total of 40 cycles of 5 seconds at 95 ^{o}C, 10 seconds at 60 ^{o}C and 10 seconds for 72 ^{o}C. In addition, a melting curve was generated with the temperature range from 50 ^{o}C to 95 ^{o}C with read at every 0.6 ^{o}C. The SYBR Green qRT-PCR set-up and experiment was performed by Ingrid Kjønstad.

Table 3.4 Reagents used per sample in the TaqMan PCR

Reagent	**Volume (µl)**
Fast RealTime PCR reaction mix	10.0
Primer	1.0
cDNA sample	9.0
Total volume	20.0

Table 3.5 Reagents used per sample in the SYBR Green PCR

Reagent	**Volume (µl)**
Power SYBR Green PCR Master mix	10.0
Forward primer 300 nM	0.12
Reverse primer 300 nM	0.12
cDNA sample	9.0
SIW	
Total volume	20.0

3.6 Determine mRNA expression in BMSCs with the nCounter™ Gene expression analysis from nanostring technologies

The nCounter™ Gene expression assay was used to determine mRNA expression of genes expressed in BMSCs upon stimulation with the TLR8 ligand CL075.

3.6.1 Principle of nCounter™ Gene expression analysis

nCounter™ Gene expression analysis is an assay to count the number of mRNA transcripts of a target in a given sample.

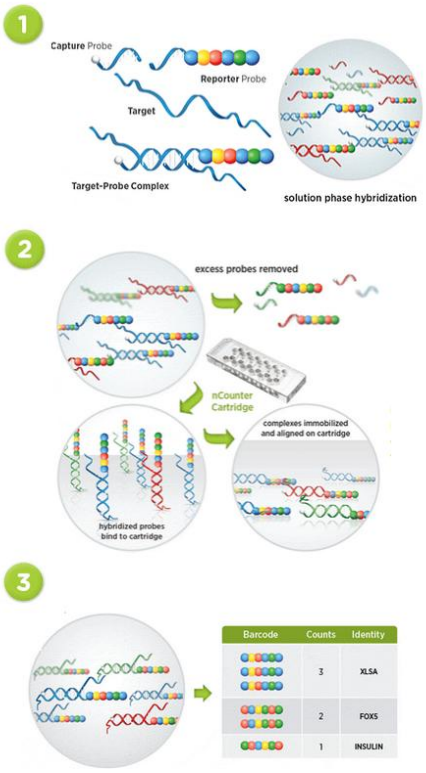


Figure 3.5. Principle of nCounter™ Gene expression assay technology. 1) Target is hybridized to probes before 2) immobilized on a cartridge and 3) quantified by Data Analyzer.

The technology is based on color-coded barcodes attached to a target-specific probe corresponding to a gene of interest. Thus, one color-coded barcode represent a single target gene. The process consists of three major steps; hybridization, immobilization and detection. During hybridization, one capture probe and one reporter probe hybridize to the target molecule in the sample. The reporter probe contains the color-coded barcode, while the capture probe ensures immobilization. After hybridization, unbound probes are washed away and the complex of probes and target binds to a cartridge. The complex binds randomly on the cartridge surface before the probes are counted. As each probe has an unique barcode, the digital analyzer count the number of times a particular barcode is detected which equals the number of transcripts of the target molecule in the sample [71].

3.6.1.1 nCounter Nanostring procedure

The gene expression panel used in this study was PanCancer Immune Profiling for human cells (cat. no. GXA-PATH1-12, Nanostring Technologies).

RNA from BMSCs was isolated as described in 3.5.2 after stimulation with TLR8 ligand CL075 (1 µg/ml) for 6 hours. Samples were diluted with RNase free H_20 to obtain 100 ng RNA in 5 µl. The samples were mixed with CodeSet reagents from the nCounter kit as listed in Table 3.6. A master mix containing Reporter Codeset, hybridization buffer and RNase free H_2O was prepared before aliquoted to tubes. The RNA samples were added for a total of 100 ng RNA and the Capture Probeset was added immediately before a quick spin down and placing the samples in a thermal cycler. The samples were incubated at 65 ^oC over night for hybridization with the probes.

The cartridge was prepared by centrifugation at 2000 x g for 2 minutes and placed in the nCounter Prep station. The samples were added to the cartridge and sealed with adhesive tape before processed. The cartridge was then transferred to the nCounter Digital Analyzer for data collection. The data was analyzed using nSolver Analysis Software v.2.5.

Table 3.6 Reagents for hybridization in nCounter gene expression analysis

Reagent	Volume (µl)
Reporter CodeSet	5
Hybridization buffer	5
RNase free H_2O	12.5
Sample RNA	5
Capture ProbeSet	2.5
Total volume	30

4 Results

4.1 Screening conditioned MM medium for TLR-activating components

TLRs are reported to sense endogenous ligands released by host cells during stress, injury or in conditions such as malignancy [29]. TLR2 and TLR4 detect proteins and membrane components [30], while host nucleic acids activate the endosomal TLRs [25]. TLR activation induces inflammation, which is reported to have a vital role in tumorigenesis and cancer progression [55], may be a driving force in MM. Tumor cells can release endogenous TLR ligands activating adjacent immune cells, which may create an inflammatory metastasis promoting environment [56, 72]. Versican, a proteoglycan, is linked to metastasis through activation of macrophages. Versican is reported to stimulate TLR2 in myeloma-associated macrophages [56].

4.1.1 Optimization of NF-κB reporter assay for detecting Toll-like receptor ligands

This study sought to assess if myeloma cells release TLR-activating components into the milieu, which may activate bone marrow cells expressing TLRs. A TLR-NF-κB reporter assay was utilized to detect any TLR activating components in conditioned medium (CM) from MM cells. The TLR-NF-κB reporter assay consists of HEK293 cells expressing different TLRs and a NF-κB luciferase reporter. The NF-κB luciferase plasmid consists of a promoter with five NF-κB binding sites fused to the Firefly luciferase gene. HEK293 do not normally express TLRs, except for TLR1, 5 and 6 [58, 59], so overexpression of a given TLR allows for detection of any NF-κB activation observed in response to CM from MM cells to that single TLR. Upon activation of a given TLR, HEK293 initiate downstream signaling, activating NF-κB to bind to the κB promoter sites upstream of the luciferase gene. Measured fluorescence from the expressed luciferase is directly proportional to NF-κB activation. To test and optimize this system, HEK293 were transfected with TLR3, 7, and 8 in addition to a NF-κB driven luciferase plasmid. A Renilla luciferase, which is constitutively active, was co-transfected into the HEK293 cells to function as an internal control for cell number and technical variability.

The TLR-NF-κB reporter system was tested for specificity and optimized for appropriate TLR plasmid amount by co-transfecting HEK293 with the NF-κB driven luciferase plasmid and different amounts (5 – 20 ng/well) of empty vector (EV), TLR3, TLR7, or TLR8 for 24 hours. Cells were also transfected with a constitutively active Renilla luciferase control plasmid. Cells were subsequently stimulated with the TLR ligands poly I:C (TLR3), R848 (TLR7/8) or CL075 (TLR8) for 24 hours before cells were lysed and assayed for Firefly luciferase and Renilla luciferase activity.

The results show a strong fold-induction of NF-κB activation for all transfected TLRs stimulated with ligands (Figure 4.1). The TLRs responded to its specific TLR ligand indicating the specificity of the assay. A doubling of plasmid amount from 10 ng/well to 20 ng/well gave incremental or no increase in measured activity. Therefore, the optimal amount was found to be 10 ng per well. This plasmid amount was used in NF-κB reporter assays to screen for TLR-activating components released by MM cells.

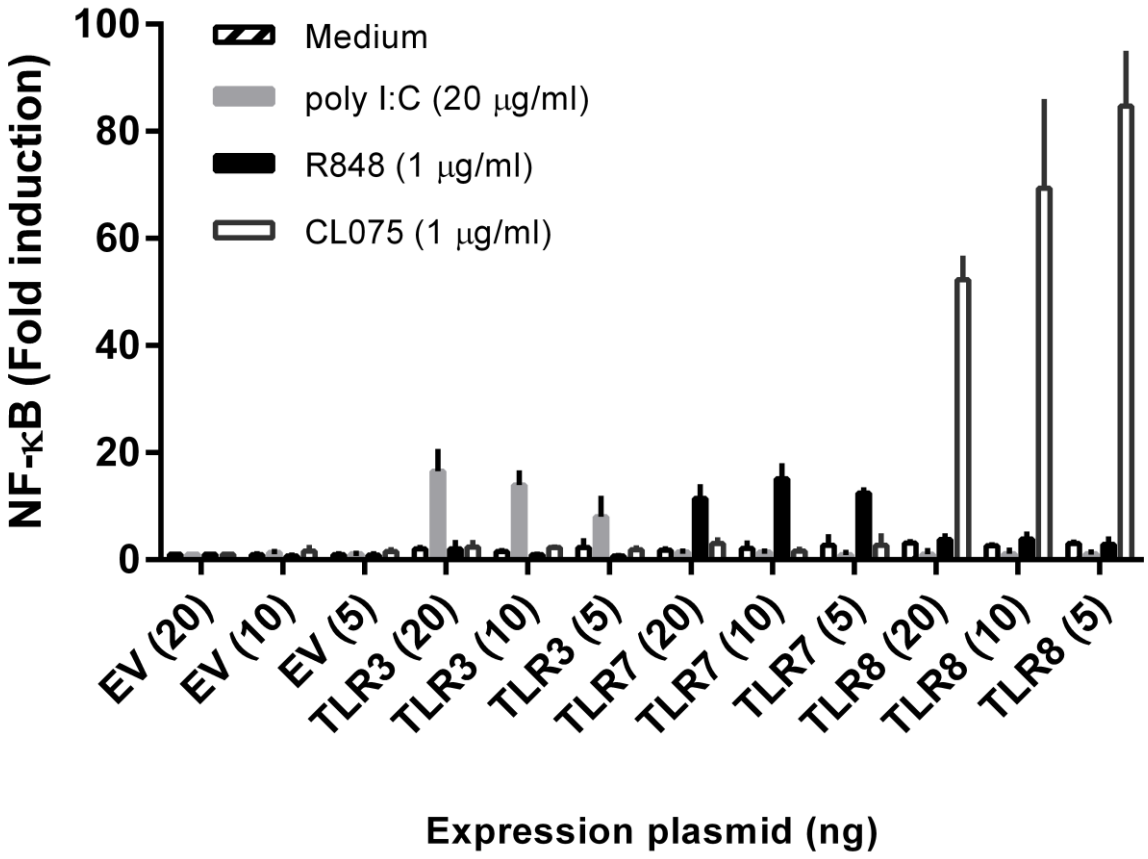


Figure 4.1 Testing and optimization of a TLR-driven NF-κB reporter assay for detection of TLR ligands. HEK293 cells in 96-well plates were transfected with empty vector (EV), TLR3, TLR7, or TLR8 (5, 10 or 20 ng/well) in addition to the NF-κB luciferase reporter and a constitutively active Renilla luciferase plasmid for 24 hours at 37 ^{o}C/5% CO_2. Cells were subsequently stimulated with TLR3 ligand poly I:C (20 μg/ml), TLR7/8 ligand R848 (1 μg/ml) and TLR8 ligand CL075 (1 μg/ml) for 24 hours before cells were lysed and assayed for luciferase activity. The data were normalized against Renilla luciferase and are presented as fold-induction to medium. The results show mean and range of duplicates and are representative of two independent experiments.

4.1.2 The MM cell line U266 activates TLR7 and TLR8

Conditioned medium from seven MM cell lines was prepared to test if MM cells release TLR-activating components. Cultures of seven different MM cell lines were incubated for a long-term period (18 days) in an attempt to concentrate any TLR-activating components present in the MM CM. Cell viability and cell number was assessed in MM cell cultures, before conditioned medium from these cell lines was harvested by centrifugation. The lowest viability was measured for INA-6 (30%) and RPMI-8226 (42%), while OH-2 and IH-1 had the highest viability with 84% and 66%, respectively (Table 4.1).

Table 4.1 Cell viability for 18 days long-term MM cell cultures.

Cell line	Total live cells (x10^7)	Total dead cells (x10^7)	Viability (%)	Volume (ml)
ANBL-6	1,08	1,27	46	32
IH-1	1,67	0,86	66	27
INA-6	1,92	4,48	30	34
JJN-3	1,90	2,33	45	28
OH-2	1,06	0,20	84	59
RPMI-8226	1,92	2,65	42	30
U266	1,90	1,16	62	26

Conditioned medium from 18 day cultures were then assayed for TLR-activating components using the NF-κB luciferase reporter assay. HEK293 cells were transiently transfected with empty vector, TLR2, TLR3, TLR7, TLR8 or TLR9 and co-transfected with NF-κB and Renilla luciferase reporter plasmid for 24 hours. The transfected cells were then treated with MM CM for 48 hours before luciferase activity was measured as a read-out for any induced NF-κB activation by conditioned MM medium.

Interestingly, U266 CM was found to activate TLR7 and TLR8, but not any of the other TLRs (Figure 4.2 A). TLR7-mediated NF-κB activation in response to U266 CM was as strong as the TLR7 ligand R848, implying a potent TLR7 induction. A small induction of TLR8-mediated NF-κB activation was also observed (Figure 4.2 A). Among the tested MM CM, only conditioned medium from U266 were able to induce detectable levels of TLR-mediated NF-κB activation. Positive controls demonstrated that Pam3Cys activated TLR2, poly I:C activated TLR3, R848 activated TLR7, CL075 activated TLR8, and CpG activated TLR9 (Figure 4.2 A).

TLR7 and TLR8 are known to be stimulated by ssRNA and short dsRNA, which could be released from dying cells in long-term culture. The observed TLR7 and TLR8 activation could thus be a result of high levels of cell death and extracellular RNA in the U266 CM. U266 CM, which activated TLR7 and TLR8, had a viability of 62% at the time of harvest and a far lower number of dead cells compared to INA-6 cells in INA-6 CM (Figure 4.2 B). This suggests that the TLR activation is not due to components released from dead cells alone. Moreover, none of the cell lines with high number of dead cells were able to activate TLRs in the NF-κB reporter assay (Figure 4.2). Hence, these results indicate no connection between cell death and TLR activation.

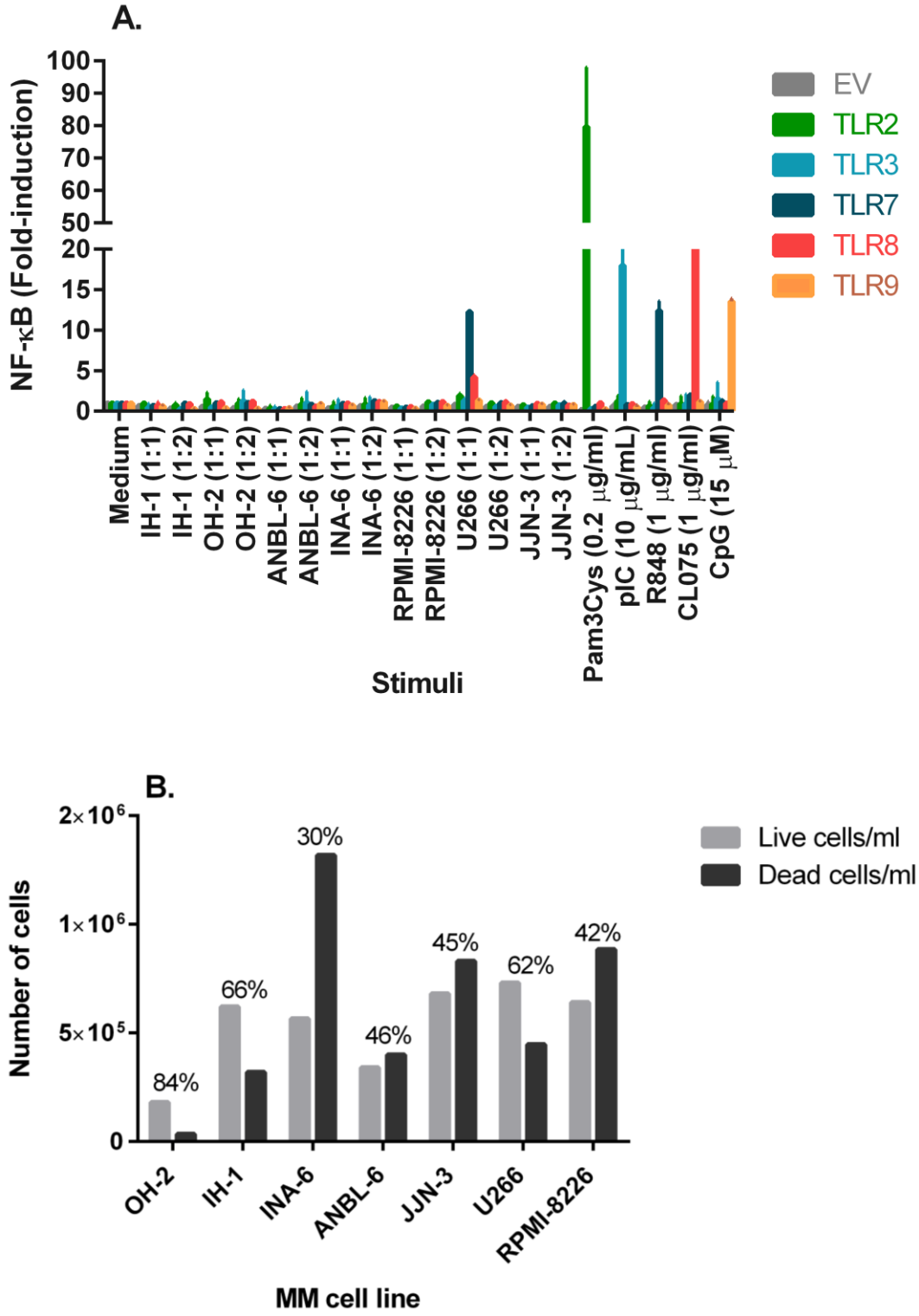


Figure 4.2 U266 is the only MM cell line to activate TLRs. A) HEK293 in 96-well plate were transiently co-transfected with NF-κB luciferase (25 ng), Renilla luciferase (0.5 ng) and TLRs (10 ng). 24 hours post transfection, HEK293 were stimulated with MM conditioned medium from seven different MM cell lines for 48 hours at 37 ^{o}C/5% CO_2. The data were normalized against Renilla luciferase and are presented as fold-induction to medium. Results show mean and range of duplicates. B) The viability and cell counts of MM culture used to harvest MM CM from 18 days of culture. The results show live and dead cells per ml, and viability as percentage of the total cell population.

4.1.3 U266 long-term culture conditioned medium activates TLR7 and TLR8

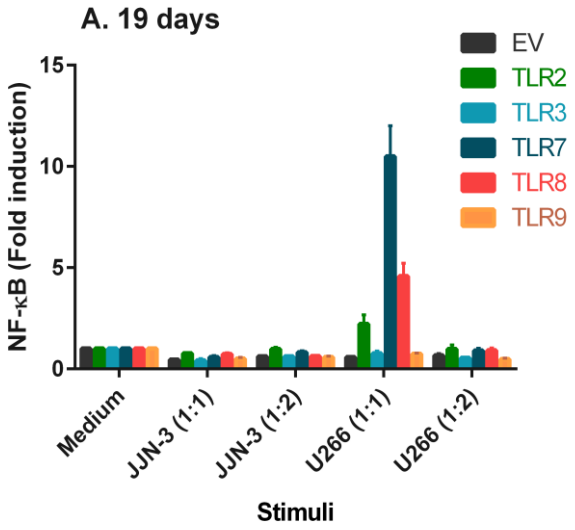
To verify the initial findings in Figure 4.2, three batches of MM CM was prepared from the different cell lines following incubation for 19, 21 and 22 days respectively. Cell viability and cell number was determined to evaluate whether viability associated with TLR activation.

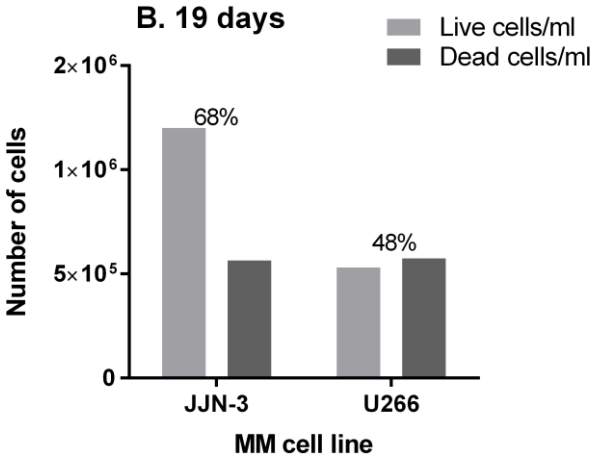
The viability for U266 was above 60% except for 19 days culture with a viability of 48% (Table 4.2). JJN-3 had lower cell viability than U266 except for 19 days culture, thereby functioning as a good control regarding viability (Table 4.2). The conditioned medium were subsequently assayed for TLR-activating components using the NF-κB luciferase reporter system as previously described (4.1.2).

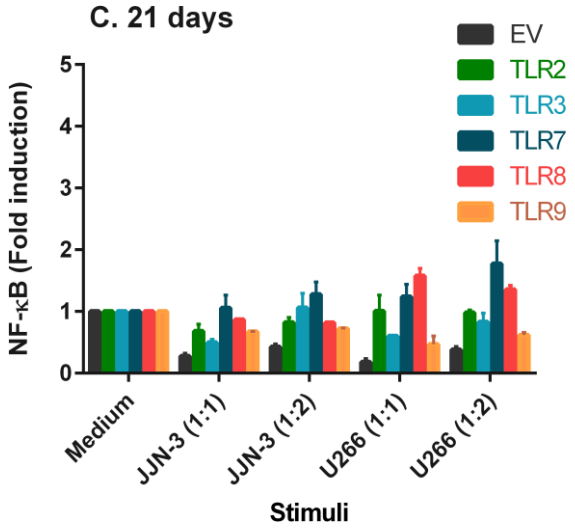
Table 4.2 Viability for U266 and JJN-3 long-term cultures.

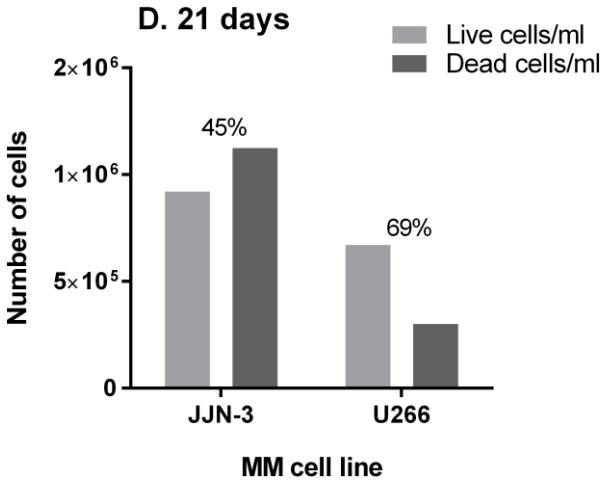
Cell culture (days)	Total live cells (x10^7)	Total dead cells (x10^7)	Viability (%)	Volume (ml)
JJN-3 (19)	1,93	0,91	68	16
U266 (19)	1,11	1,20	48	21
JJN-3 (21)	3,47	4,24	45	37
U266 (21)	2,48	1,11	69	37
JJN-3 (22)	3,13	1,22	72	28
U266 (22)	3,14	0,83	79	36

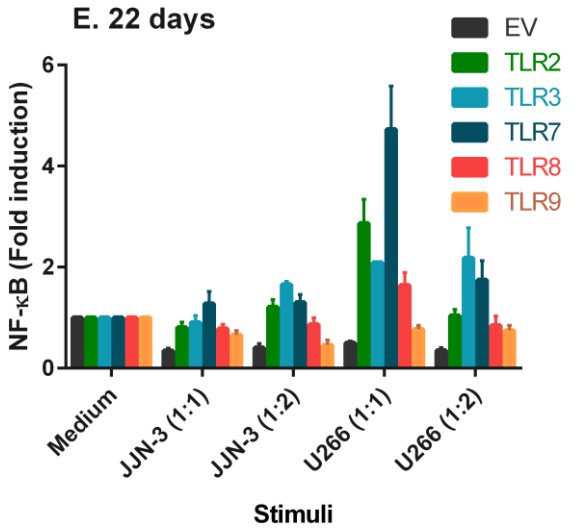
U266 conditioned medium from 19 and 22 day cultures were consistently able to activate TLR7 and TLR8 (Figure 4.3 A and E), although no induction was observed for U266 from 21 day culture (Figure 4.3 C). A small induction of TLR2 by U266 was observed, but was found not to be significant (Figure 4.3 A). JJN-3 CM from all cultures did not activate TLRs, despite higher levels of cell death (Figure 4.3), in accordance with previous observations (Figure 4.2 A). Absence of TLR activation by JJN-3 suggests that high cell death is not associated with TLR activation and indicates that the ability to stimulate TLRs may be specific for U266 cell line, or U266 secretes higher amounts of the TLR-activating component compared to JJN-3. Diluted U266 CM did not activate any TLRs indicating a low concentration of the TLR-activating component. No other MM cell line was able to activate TLRs (Appendix III), in accordance with previous results (Figure 4.2).


A. 19 days
EV
TLR2
TLR3
TLR7
TLR8
TLR9
NF-κB (Fold induction)
15
10
5
0
Medium
JJN-3 (1:1)
JJN-3 (1:2)
U266 (1:1)
U266 (1:2)
Stimuli


B. 19 days
Live cells/ml
Dead cells/ml
Number of cells
2×10⁶
1×10⁶
5×10⁵
0
68%
48%
JJN-3
U266
MM cell line


C. 21 days
EV
TLR2
TLR3
TLR7
TLR8
TLR9
NF-κB (Fold induction)
5
4
3
2
1
0
Medium
JJN-3 (1:1)
JJN-3 (1:2)
U266 (1:1)
U266 (1:2)
Stimuli


D. 21 days
Live cells/ml
Dead cells/ml
Number of cells
2×10⁶
1×10⁶
5×10⁵
0
45%
69%
JJN-3
U266
MM cell line


E. 22 days
EV
TLR2
TLR3
TLR7
TLR8
TLR9
NF-κB (Fold induction)
6
4
2
0
Medium
JJN-3 (1:1)
JJN-3 (1:2)
U266 (1:1)
U266 (1:2)
Stimuli

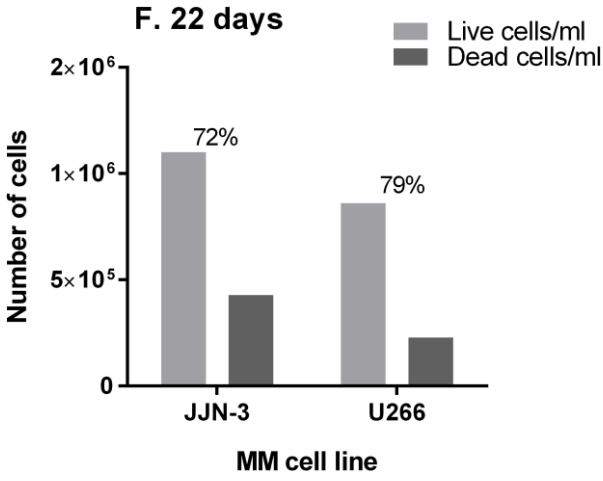

F. 22 days
Live cells/ml
Dead cells/ml
Number of cells
2×10⁶
1×10⁶
5×10⁵
0
72%
79%
JJN-3
U266
MM cell line

Figure 4.3 Long-term U266 conditioned medium activated TLR7 and TLR8. U266 and JJN-3 harvested from A) 19 days, C) 21 days and E) 22 days culture at 37 ^{o}C/5%C CO_2. HEK293 were plated in 96-well plate and tranfected with a given TLR (10 ng), NF-κB luciferase (25 ng) and Renilla luciferase (0.5 ng) plasmid for 24 hours at 37 ^{o}C/5% CO_2. U266 and JJN-3 CM were added to transfected HEK293 for 48 hours at 37 ^{o}C/5% CO_2 before subsequent lysis. The data were normalized against Renilla luciferase and are presented as fold-induction to medium. Results show mean and range of duplicates and are representative of two independent experiments. B, D and F show the viability and cell counts of U266 and JJN-3 culture used to harvest MM CM from. Data are presented as live and dead cells per ml and viability as percentage of the total cell population.

4.1.4 RNA content does not correlate with U266's ability to activate TLR7/8

U266 conditioned medium activated TLR7 and TLR8 as observed in the NF-κB luciferase reporter assay (Figure 4.3). TLR7 and TLR8 recognize ssRNA and short dsRNA [23, 25], which could be released from necrotic cells in the MM long – term cultures.

Although the ability to activate TLRs did not correlate with the cultures' viability, the observed stimulation could be due to a particular high RNA content in U266 compared to other MM cell lines. Total RNA was therefore isolated from all tested MM cell lines described as in section 3.5.2 to determine if high RNA content in U266 could explain the observed TLR7 and TLR8 activation induced by U266 CM.

Figure 4.4 shows that INA-6 had the highest RNA content while KJON-1 had the lowest. U266 did not have a high RNA content compared to other MM cell lines. Therefore, neither low viability, nor high RNA content are associated with activation of TLRs by U266 conditioned medium. The RNA content and viability of U266 is comparable to JJN-3. Therefore, JJN-3 was used as a control cell line for subsequent experiments.

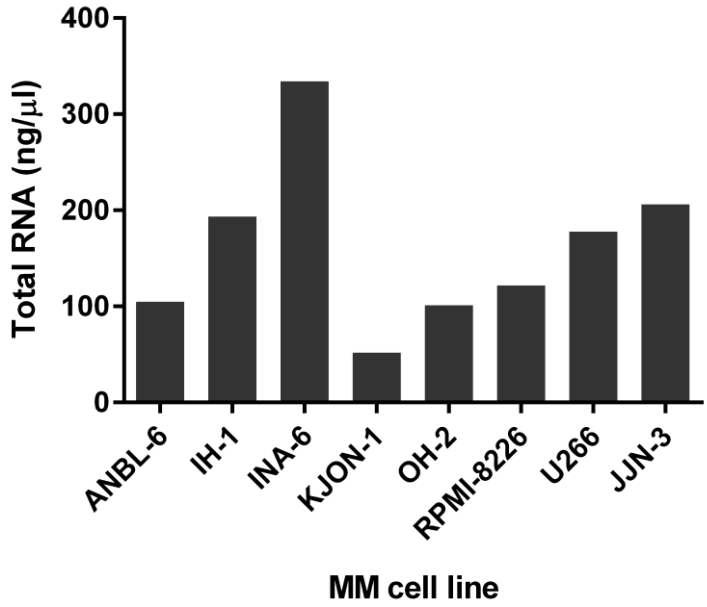


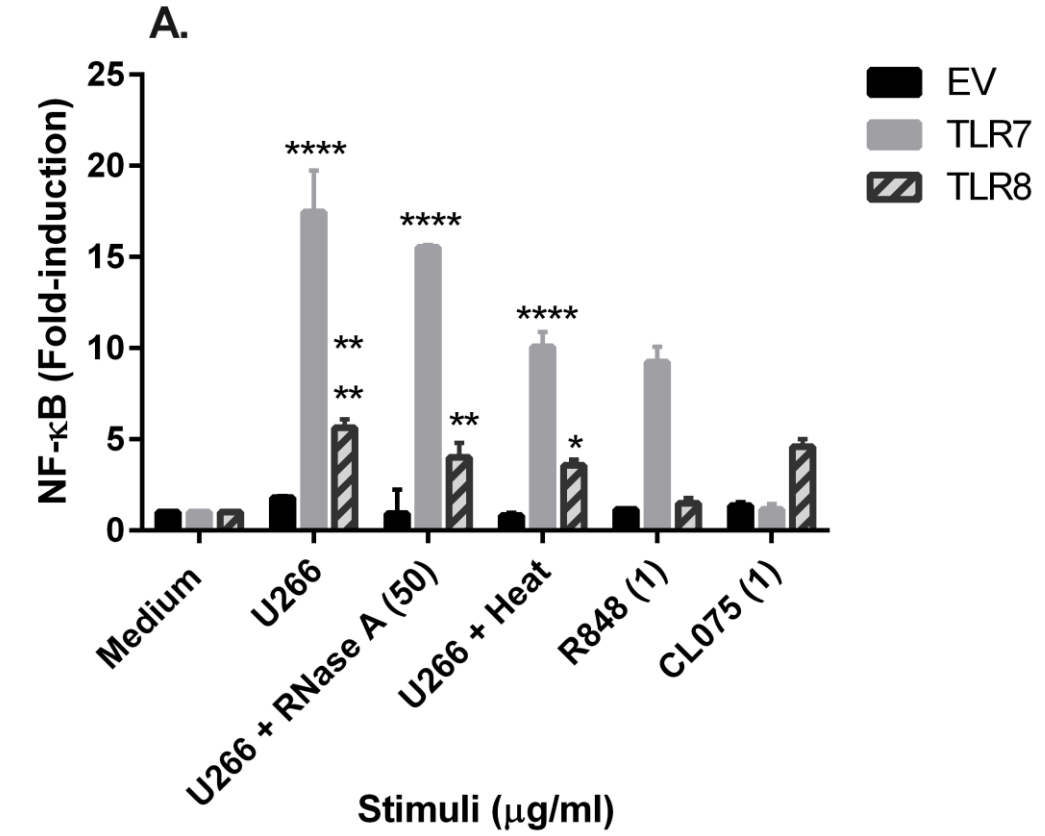
Figure 4.4 Total RNA extracted from MM cell lines. Total RNA was isolated from myeloma cells (0.5 million cells) and concentration measured on Nanodrop.

4.2 RNase A does not abolish TLR activation by U266 conditioned medium

In order to assess if the TLR7/8 - activating component in U266 was a RNA or protein–based molecule, U266 CM was treated with RNase or heat. RNase A is an enzyme degrading, while heat causes proteins to denature. The treated CM was assessed for its capability to activate TLRs by the TLR-NF-κB reporter assay.

U266 19 days conditioned medium was treated with either RNase (50 μg/ml, 1,5 hour at 37 ^{o}C) or heated (10 min, 96 ^{o}C) before assayed using the NF-κB luciferase reporter assay. Untreated U266 CM yielded a strong TLR7 activation and a weaker TLR8 activation (Figure 4.5 A). Surprisingly, RNase-treated CM activated TLR7 and TLR8 as potent as TLR7/8 – mediated NF-κB activation by untreated CM in the reporter system. The results suggest that the TLR7/8 activating component is RNase resistant. TLR activation of the heat – treated CM was detected but weaker than both untreated and RNase – treated CM, indicating that the component is partially heat sensitive. These results indicate either that the TLR-activating component is not RNA, RNA is a short RNA strand, or that RNA in U266 CM is protected from the RNase. Positive controls demonstrated that R848 activated TLR7 and CL075 activated TLR8 (Figure 4.5 A).

In order to validate the functionality of RNase A, RNA isolated from U266 was treated with RNase for 1,5 hour at 37 ^{o}C before assayed on an agarose gel. As shown in Figure 4.5 B, untreated RNA (lane 2 and 6) shows two distinct bands that disappear with RNase – treated RNA (lane 3 and 7) verifying the functionality of RNase A. The TLR8 ligand polyU was also tested, however, due to its small size it was undetectable (lane 4 and 5). Based on the results the RNase A is functional.



B.

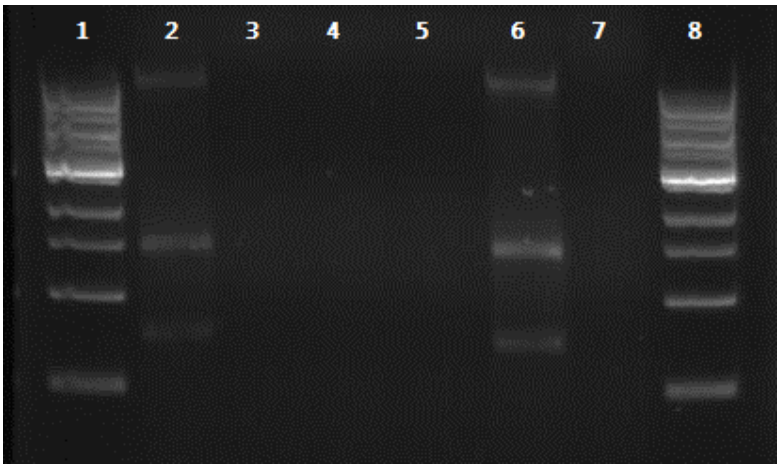


Figure 4.5 RNase treated U266 conditioned medium activates TLR7. A) HEK293 cells were transiently transfected with either empty vector (EV), TLR7 or TLR8 (10 ng), NF-κB luciferase (25 ng) and Renilla luciferase (0.5 ng) at 37 ^{o}C/5% CO_2 for 24 hours. Untreated, RNase- treated (50 μg/ml, 1.5 hour, 37 ^{o}C), or heat-treated (10 min, 95 ^{o}C) U266 19 days CM was added to transfected HEK293 for 48 hours at 37 ^{o}C/5% CO_2 before subsequent cell lysis and measurement of fluorescent activity. The data were normalized against Renilla luciferase and are presented as fold-induction to medium. Results show mean and range of duplicates. P-values calculated using One-way ANOVA with Dunnett's post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. B) The following samples were run on an 1% agarose gel for 40 minutes at 90V: 1) 1kb ladder, 2) 100 ng RNA U266, 3) 100 ng RNA treated with RNase (50 μg/ml), 4) polyU (5 μg/ml), 5) polyU (5 μg/ml) treated with RNase (50 μg/ml), 6) 200 ng RNA U266, 7) 200 ng RNA treated with RNase (50 μg/ml), 8) 1kb ladder.

4.3 U266 14 days conditioned medium activates TLR7

The two MM cell lines, U266 and JJN-3, were cultured short – term (7 and 14 days) to determine if TLR-activating components could be detected at an earlier time point with the NF-κB reporter system. U266 and JJN-3 were cultured for either 7 or 14 days before the conditioned medium was harvested. Cell viability and cell number was determined using the cell counter Countess^{TM} using Trypan blue to eliminate non-viable cells. Both JJN-3 cultures had lower viability and higher number of dead cells than U266 cell cultures (Table 4.3), indicating JJN-3 as an appropriate control for viability.

Table 4.3 Viability for U266 and JJN-3 short-term cell cultures.

Cell culture (days)	Total live cells (x10^7)	Total dead cells (x10^7)	Viability (%)	Volume (ml)
JJN-3 (7 days)	2,0	0,74	73	15
U266 (7 days)	1,6	0,3	84	20
JJN-3 (14 days)	1,76	0,65	73	13
U266 (14 days)	1,53	0,33	82	18

HEK293 were transiently transfected with empty vector, TLR2, TLR3, TLR7, TLR8 or TLR9, NF-κB and Renilla luciferase plasmids for 24 hours. The transfected HEK cells were then treated with MM CM for 48 hours before subsequently lysed and luciferase activity was measured.

The results show that 14 days CM strongly activated TLR7 (Figure 4.6 A and B), and the observed TLR7 activation by U266 CM (Figure 4.6 B) was as potent as the TLR7 ligand R848 (Figure 4.6 C). The U266 7 days CM was not able to induce any TLRs. The results suggest that at least a 14-days culture period is needed for accumulation of detectable levels of TLR-activating components in the U266 CM. Positive controls demonstrated specificity of the TLR response (Figure 4.6 C). There was a minor difference in viability between 7 days and 14 days U266 cultures (Figure 4.6 D) indicating the TLR-activating component in U266 CM is not associated with number of dead cells, in line with previous results (Figure 4.2 and 4.3).

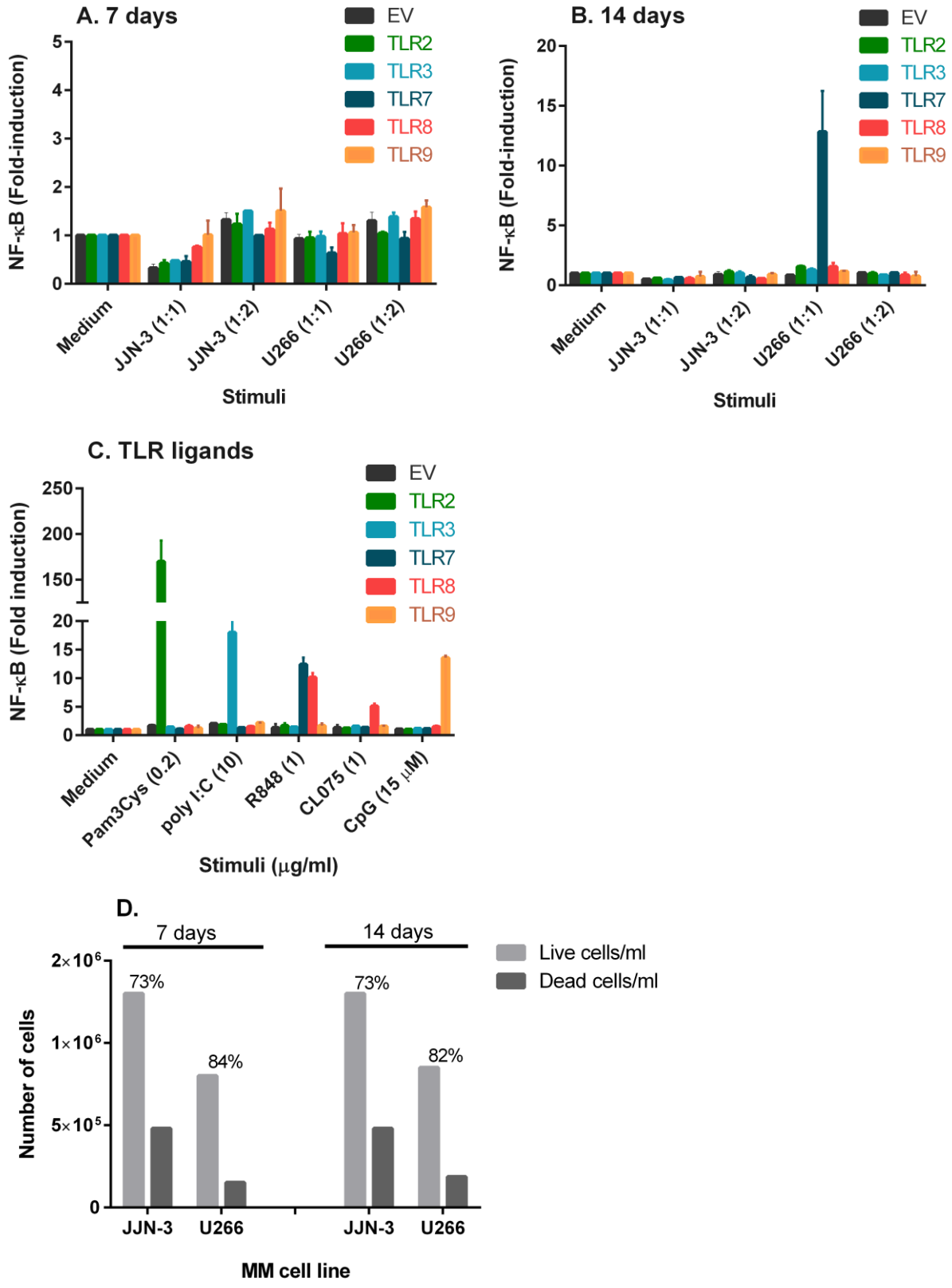


Figure 4.6 U266 14 days conditioned medium activates TLR7.HEK293 cells were transfected with either empty vector (EV), TLR2, TLR3, TLR7, TLR8 or TLR9 (10 ng), NF-κB luciferase (25 ng) and Renilla luciferase (0.5 ng) plasmid for 24 hours at 37 ^{o}C/5% CO_2. HEK293 were treated with A) 7 day MM CM, B) 14 day MM CM, or C) TLR ligands for 48 hours at 37 ^{o}C/5% CO_2. The data were normalized against Renilla luciferase and are presented as fold-induction to medium. Results show mean and range of duplicates. D) MM cell culture viability presented as live and dead cells per ml and viability percentage of the total cell population.

4.4 Extracellular vesicles from MM cells activate TLR7

MM conditioned medium treated with RNase did not abolish TLR7/8 activation (Figure 4.5). This indicates that the TLR ligand either is not RNA, that the ligand is short RNA not degraded efficiently by RNase A e.g. miRNA [73], or that the RNA is protected from degradation in exosomes [74]. Extracellular vesicles (ECVs) encapsulate nucleic acids and thereby protect them from the extracellular environment before delivering its cargo to the target cell. One type of ECVs are exosomes. Exosomes are vesicles of 30 to 100 nm in size secreted by cells, including myeloma cells [75]. A strong TLR7 and TLR8 activation by RNase-treated supernatant could be due to vesicle encapsulation of the RNA [76].

ECVs from U266 and JJN-3 were isolated to assess if the vesicles were capable of activating TLR7/8. U266 and JJN-3 were cultured in ECV-deprived medium for 14 days as previous results indicate that U266 14 days-culture was able to activate TLRs (Figure 4.6). The viability in MM cultures was assessed, where U266 had 78% viability, while JJN-3 had a lower viability at 63% (Table 4.4).

Table 4.4 Viability for U266 and JJN-3 exosome-derived cultures

Cell culture	Total live cells ($x10^{10}$)	Total dead cells ($x10^{10}$)	Viability (%)	Volume (ml)
JJN-3	2,05	1,2	63	90
U266	0,99	0,28	78	100

Extracellular vesicles from U266 and JJN-3 were then isolated using ultracentrifugation to isolate exosomes. In brief, supernatant was isolated from cells by centrifugation at 1000 rpm for 15 minutes, then at 2000 rpm for 10 minutes to eliminate cellular debris. The supernatant was further centrifuged at 10 000 x g for 30 minutes for sedimentation of apoptotic bodies and large ECVs. The supernatant was finally centrifuged at 120 000 x g for 70 minutes to isolate the exosomes. The exosome pellets were resuspended in 20 ml ECV-deprived medium. Supernatant after each centrifugation step was assayed together with the isolated ECVs to determine the TLR-activation properties of supernatant from each centrifugation.

Figure 4.7 shows that ECVs isolated from both U266 and JJN-3 were able to activate TLR7 but not TLR8. In addition, the JJN-3 supernatant after the first 120,000 x g centrifugation yielded a small TLR7 activation. Only U266 ECV TLR7-activation was found to be significant. None of the U266 fractions from the early ultracentrifugation steps activated either TLR7 or TLR8. The TLR responses observed for isolated extracellular vesicles were markedly lower (Figure 4.7) than for isolated conditioned medium (Figure 4.6). Collectively, TLR7 activation may partially be mediated by exosomes originating from U266 cells.

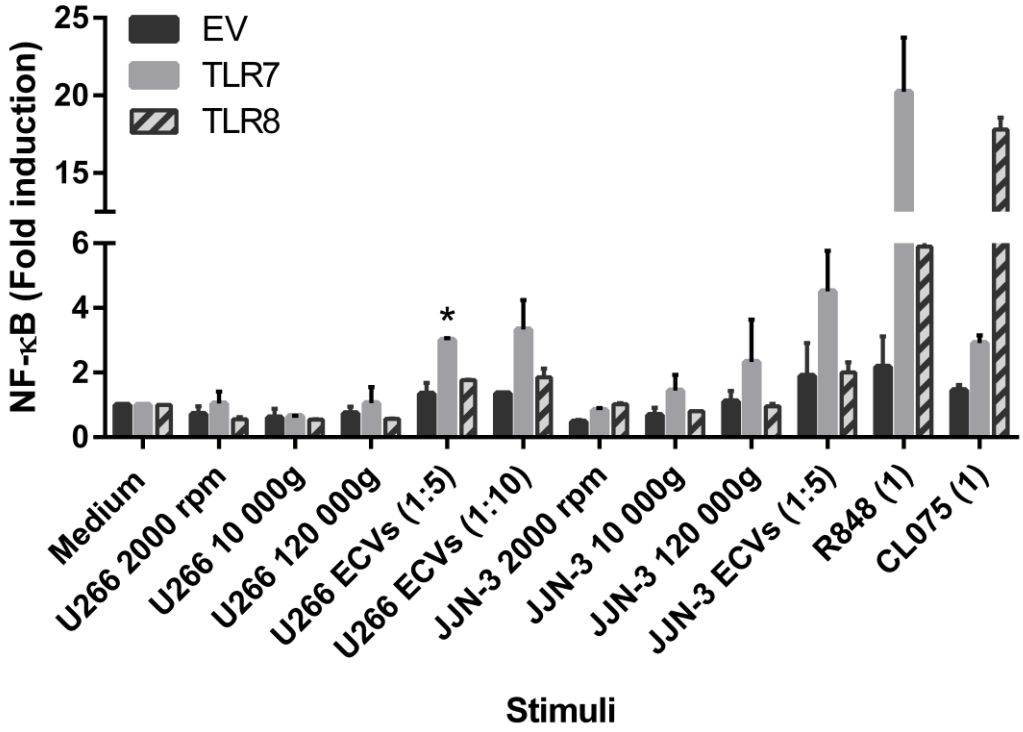


Figure 4.7 Extracellular vesicles isolated from MM supernatant activate TLR7. Extracellular vesicles (ECVs) were isolated by ultracentrifugation from U266 and JJN-3 14 days-culture and assayed for the ability to activate TLRs using the TLR-NF-κB reporter assay. HEK293 were transfected with either empty vector (EV), TLR7, or TLR8 (10 ng) and NF-κB (25 ng) and Renilla luciferase (0.5 ng) plasmids for 24 hours at 37 ^{o}C/5% CO_2. HEK293 were treated with MM fractions, ECVs or TLR ligands (1 μg/ml) for 48 hours at 37 ^{o}C/5% CO_2. The data were normalized against Renilla luciferase and are presented as fold-induction to medium. Results are shown as mean and range of duplicates. P-values calculated using unpaired two-tailed t-test with Welch's correction. * $p < 0.05$

Taken together, the MM cell line U266 was found to secrete a TLR7/8 – activating component. TLR7/8 recognize RNA, however, the secreted component's ability to activate TLR7/8 was not affected by RNase treatment, hence the RNA may be protected from the RNase by being encapsulated in a vesicle. Extracellular vesicles isolated from U266 CM

mediated some TLR7 activation, implying that TLR activation may partly be due to extracellular vesicles. The TLR7/8 –activating component may activate TLR – expressing cells in the bone marrow.

4.5 Bone marrow stromal cells from myeloma patients induce cytokine responses upon stimulation with TLR ligands

A previous study showed that bone marrow stromal cells (BMSCs) from multiple myeloma patients responded to TLR stimulation by inducing cytokines like IL-8 and CCL3 (Kjønstad & Nilsen, unpublished data). U266 cells released TLR-activating components (Figure 4.3) with the potential to activate TLRs on cells in the BM milieu. Therefore, elucidating TLR responses in BMSCs is of great interest as U266 could potentially activate BMSCs.

4.5.1 BMSCs induce CCL3 in response to TLR8 ligands

To assess TLR response in MM-BMSCs, a standard stromal batch (BMSC19) was stimulated with the following TLR ligands; FSL-1 (TLR2/6), poly I:C (TLR3), LPS K12 (TLR4), R837 (TLR7), CL264 (TLR7), R848 (TLR7/8), CL075, polyU, polyU/LyoVec (TLR8) and ssRNA41/LyoVec (control) for 72 hours. BMSC19 are a mixture of BMSCs originating from 10 myeloma patients [63]. CCL3 induction in response to TLR ligands was assayed by ELISA to determine if TLR ligands induced cytokine response in these cells. CCL3 is a chemokine induced upon NF-κB activation [77]. CCL3 stimulates proliferation and survival of plasma cells [78], and high CCL3 levels in MM patients are associated with poor prognosis [77].

Figure 4.8 shows that BMSC19 induce CCL3 in response to ligands for various TLRs. BMSC19 responded strongly to TLR7/8 (R848) and TLR8 (CL075) ligands, but not to the TLR7 specific ligands (R837 and CL264), indicating that these cells may express TLR8. A small, but not significant induction of CCL3 was observed in response to TLR2 (Pam3Cys), TLR3 (poly I:C) and TLR4 (LPS K12) ligands (Figure 4.8).

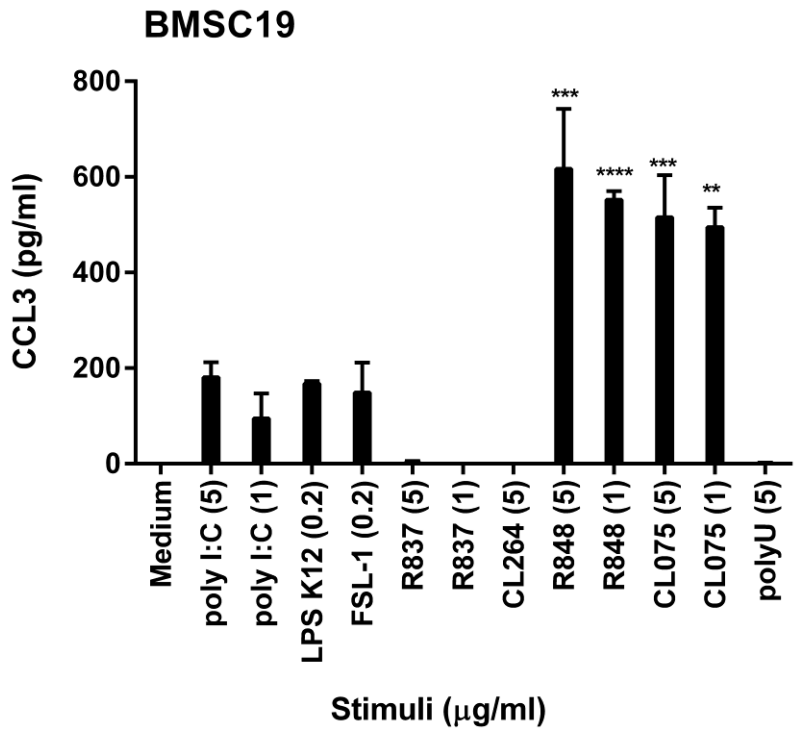


Figure 4.8 CCL3 induction in R848 and CL075 stimulated BMSCs. BMSC9 were stimulated with TLR ligands in 2% A+ v.i./RPMI for 72 hours at 37 ^{o}C/5% CO_2. CCL3 induction was assayed by ELISA. Detection limit was 8 pg/ml. Results shown mean and standard deviation for triplicates. P-values calculated using One-way ANOVA with Dunnett's post-hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

4.5.2 Macrophages express CCL3 upon TLR stimulation

BMSCs are a heterogeneous $CD138^-$ cell population, including a small population of macrophages and possibly monocyte-derived dendritic cells (moDCs). Macrophages and moDCs express high levels of TLRs and respond potently to TLR ligands [79]. Since TLR8 responses have previously been observed in primary MM-BMSCs and not in the stromal cell lines HS-5 and HS-27a (Kjønstad & Nilsen, unpublished data), the observed TLR8 response could be due to macrophages or moDCs present in the primary BMSCs population.

To determine if the CCL3 response induced by TLR8 could be due to macrophages or moDCs, monocytes, macrophages or moDCs were stimulated with TLR ligands and assayed for CCL3 induction. In addition, to confirm the CCL3 induction observed in BMSC19 (Figure 4.8), BMSC20 were stimulated with the same panel of TLR ligands.

Monocytes were isolated from healthy donors and differentiated to either macrophages (20% FCS/RPMI) or moDCs (IL-4 (40 ng/ml), GM-CSF (100 ng/ml)) for 7 days. Differentiated cells were characterized by flow cytometry (Appendix VI). Monocytes, macrophages or moDCs were stimulated with the same panel of TLR ligands as BMSCs (4.5.1) for 20 hours. CCL3 induction was assessed by ELISA.

Figure 4.9 D shows that BMSC20 induced a significant CCL3 up-regulation in response to TLR7/8 ligands (R848 and CL075). The responses in BMSC20 are concurrent with observations for BMSC19 (Figure 4.8). Monocytes induced strong cytokine expression in response to R848 and CL075 (TLR7/8), while a strong, but lower response was observed to LPS (TLR4) (Figure 4.9 A). Macrophages strongly induced CCL3 upon TLR2/6, TLR4 and TLR7/8 stimulation (Figure 4.9 B), while the highest CCL3 induction in moDCs was seen upon TLR4 (LPS K12) and TLR3 (poly I:C) stimulation. Elevated CCL3 was also observed for the ligand R848 but not for the TLR8 ligand CL075 (Figure 4.9 C). The results indicate that it is unlikely that moDCs induced CCL3 in response to TLR8 ligands in BMSCs (Figure 4.9 C). Macrophages responded to the same TLR ligands as both BMSC19 and BMSC20, although the response in macrophages were higher than observed in BMSCs (Figure 4.9). Taken together, macrophages could be a potential source of CCL3 in BMSCs upon TLR stimulation.

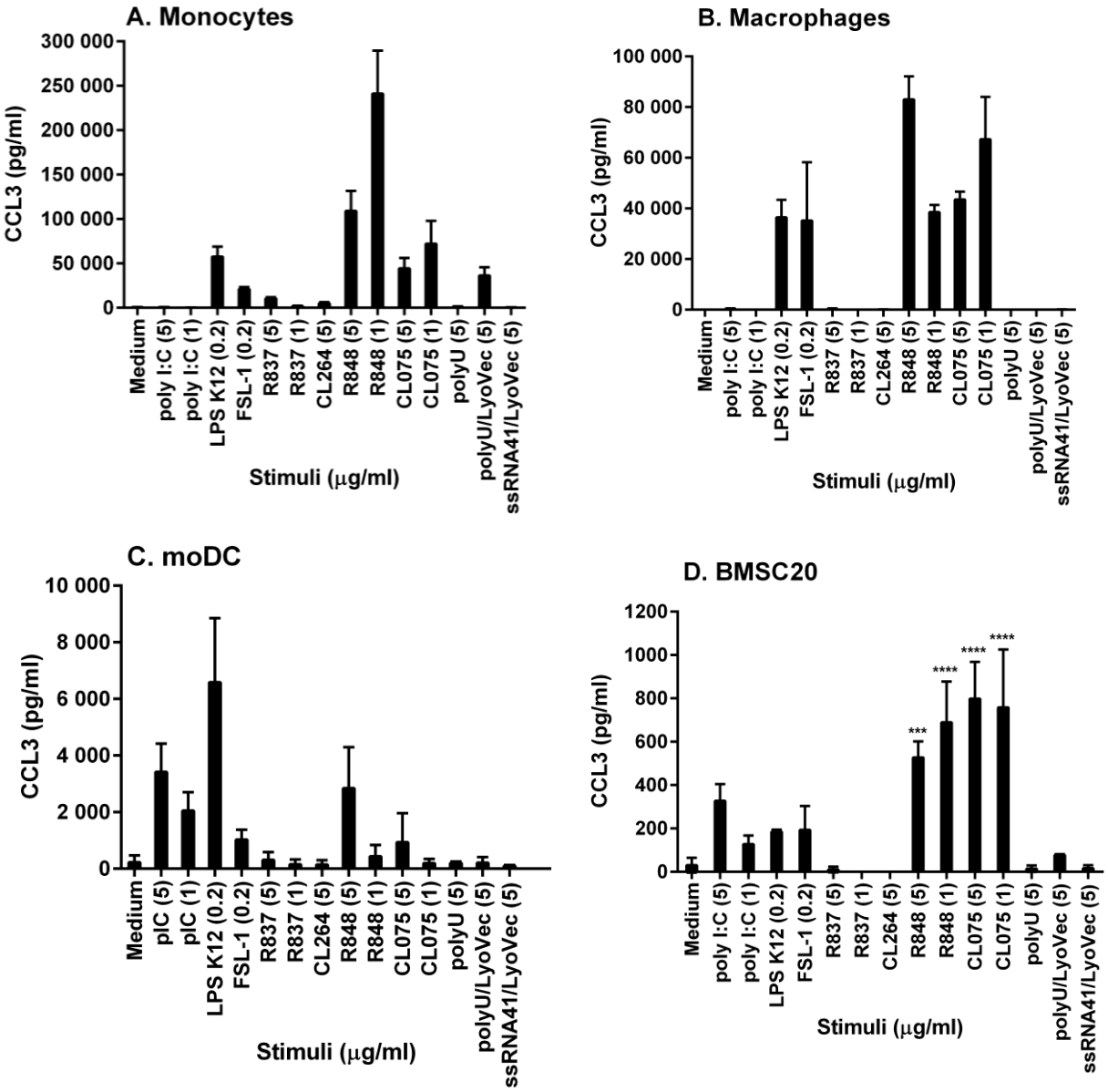


Figure 4.9 CCL3 induction in monocytes, macrophages, moDCs and BMSC20 upon TLR stimulation. A) Monocytes and B) macrophages were stimulated with TLR ligands for 20 hours in 2% HS/RPMI at 37 ^{o}C/5% CO_2. C) MoDCs were stimulated with TLR ligands in 10% HS/RPMI (with GM-CSF and IL-4) for 20 hours at 37 ^{o}C/5% CO_2. D) BMSC20 were stimulated with TLR ligands in 2% A+ v.i./RPMI for 72 hours at 37 ^{o}C, 5% CO_2. The supernatant from stimulated cells were assayed for CCL3 expression using ELISA. The results are presented as mean and standard deviation of triplicates. The data are representative for three individual experiments and three donors. Macrophages and moDCs originated from the same donor.

4.6 Proteome profiling of BMSCs reveals up-regulation of several cytokines upon TLR8 stimulation

Next, a proteome profiler kit was used to screen BMSCs supernatant for cytokine expression. The kit screened for 102 cytokines and other soluble proteins to assess cytokine expression induced by TLR8 ligand CL075 in BMSCs to identify other cytokines that were induced in these cells in response to TLR8 activation.

4.6.1 Up-regulation of CCL3 and other cytokines

Supernatant from BMSCs stimulated with CL075 (5 µg/ml) for 72 hours was assayed using a human XL proteome profiler kit (R&D) designed to detect soluble proteins and developed according to manufacturer's instructions. In brief, capture antibodies were spotted in duplicate on a membrane and target proteins were detected and visualized using chemiluminescent detection reagents. Luminescent signal corresponding to the amount of protein bound was measured and quantified using Image analyzer software.

Figure 4.10 shows bound cytokines on the developed membranes for both unstimulated and TLR8 stimulated BMSCs supernatant, where several cytokines were found to be up-regulated in stimulated BMSCs. In agreement with results in Figure 4.8, CCL3/CCL4 were induced by the TLR8 ligand CL075. The induction in stimulated BMSCs was found to be 13-fold compared to unstimulated BMSCs (Figure 4.10 C). In addition, IL-6 and IL-8 were strongly constitutively expressed (Figure 4.10 A). Interestingly, the chemokines CXCL1 and CXCL5 were found to be strongly up-regulated in response to stimulation, even more potently induced than CCL3 (Figure 4.10 B). TLR8 stimulation of BMSC19 led to an 11-fold induction of CXCL1 while CXCL5 was induced 7-fold (Figure 4.10 C). Induction of other inflammatory cytokines such as IL-1β and TNF-α was not observed in BMSCs in response to CL075 using the proteome assay. Positive references were detected (upper left and right corner) on both membranes ensuring correct exposure.

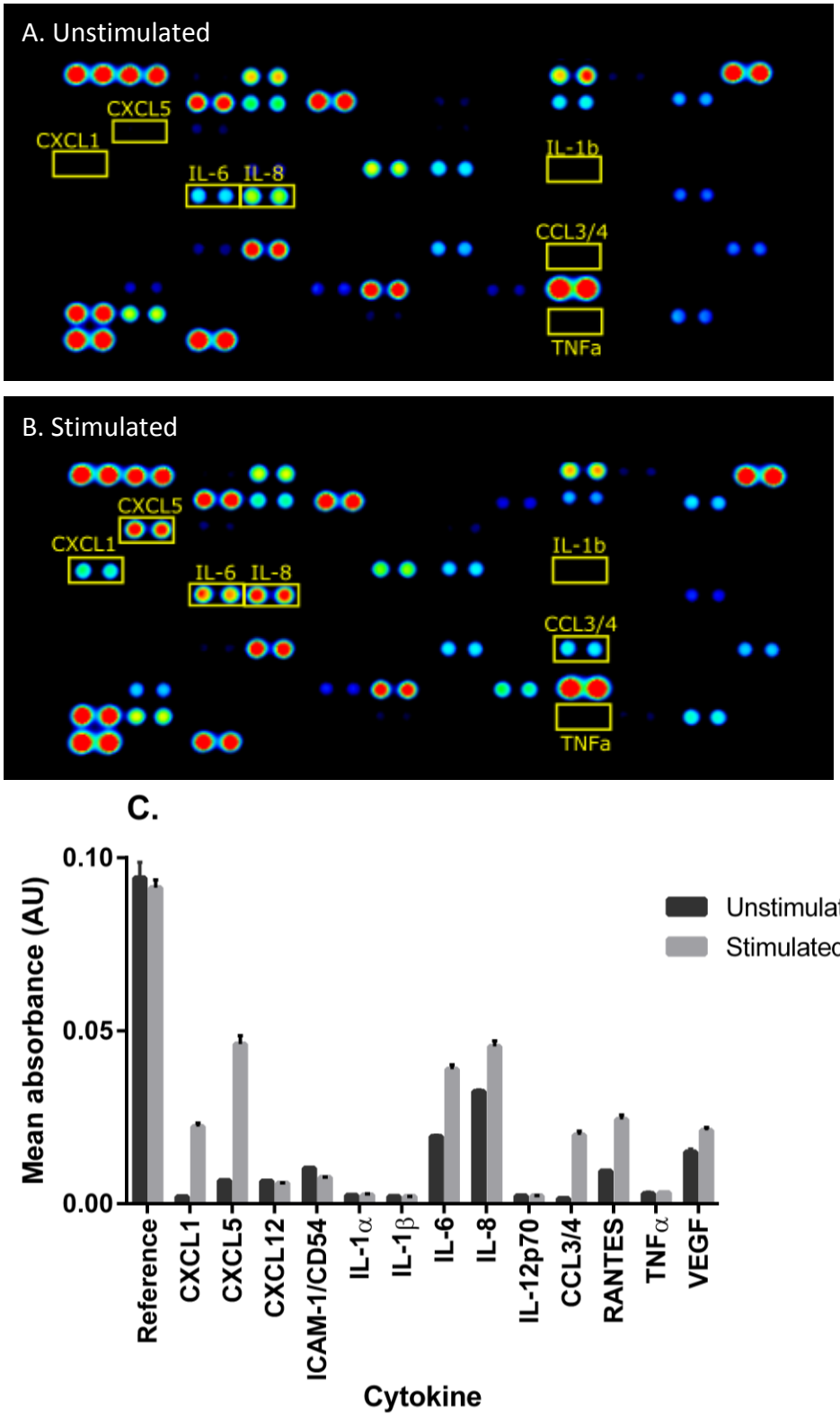


Figure 4.10 Cytokine induced in BMSCs upon TLR8 stimulation. BMSC19 were either A) unstimulated or B) stimulated with CL075 (5 µg/ml) for 72 hours in 2% A+ v.i./RPMI at 37 ^{o}C/5% CO_2. The supernatant was screened for cytokine expression using the Proteome profiler XL cytokine array kit. Signal absorbance detected after 20 minutes exposure using Li-COR Odessay. C) Mean signal absorbances (arbitrary unit, AU) were measured using Image Analyzer. The data are representative of mean and range of duplicates.

4.6.2 CXCL1 and CXCL5 induced by TLR8 stimulation in bone marrow stromal cells

The proteome profiler kit indicated that CXCL1 and CXCL5 were up-regulated in MM-BMSCs in response to TLR8 stimulation (Figure 4.10). CXCL1 and CXCL5 are chemokines associated with advanced tumor stages, invasion and metastasis when overexpressed [80]. High expression is observed in MM patients compared to healthy individuals [81], and both CXCL1 and CXCL5 concentration increases with the MM disease stage [82]. Both chemokines are reported to stimulate cell migration and proliferation of myeloma cells [15, 81].

Given the role of CXCL1 and CXCL5 in MM and the high induction observed in the proteome profiler (Figure 4.10 B and C), their expression levels were quantified utilizing ELISA. Both CXCL1 and CXCL5 were induced upon CL075 stimulation (Figure 4.11). CXCL1 and CXCL5 induction were also observed in BMSC20 (Figure 4.11).

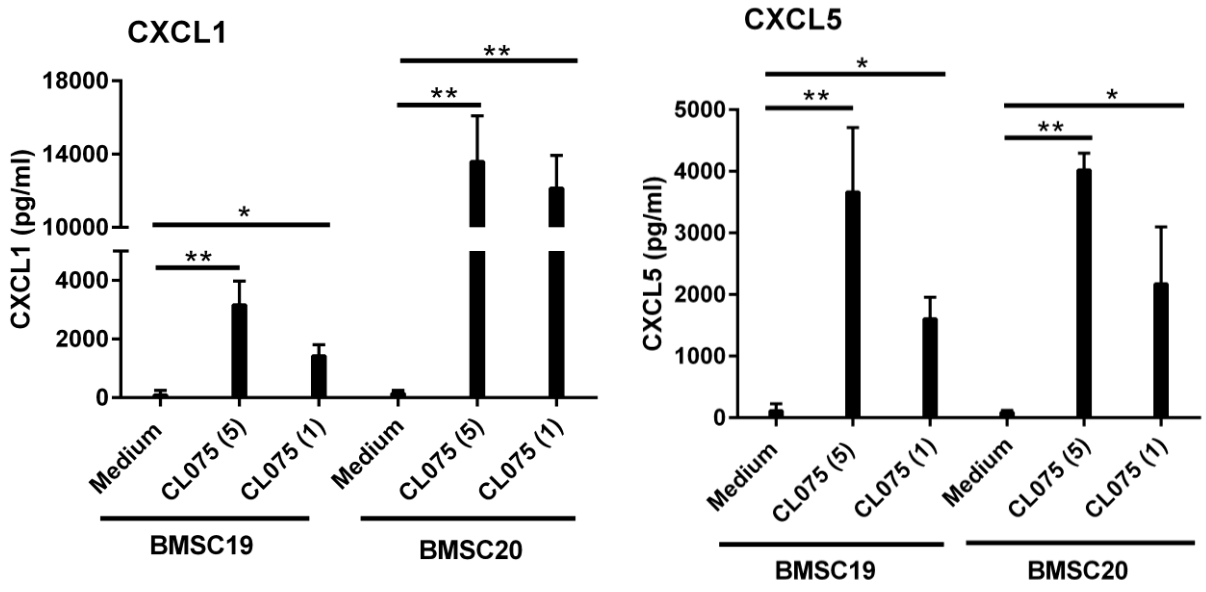
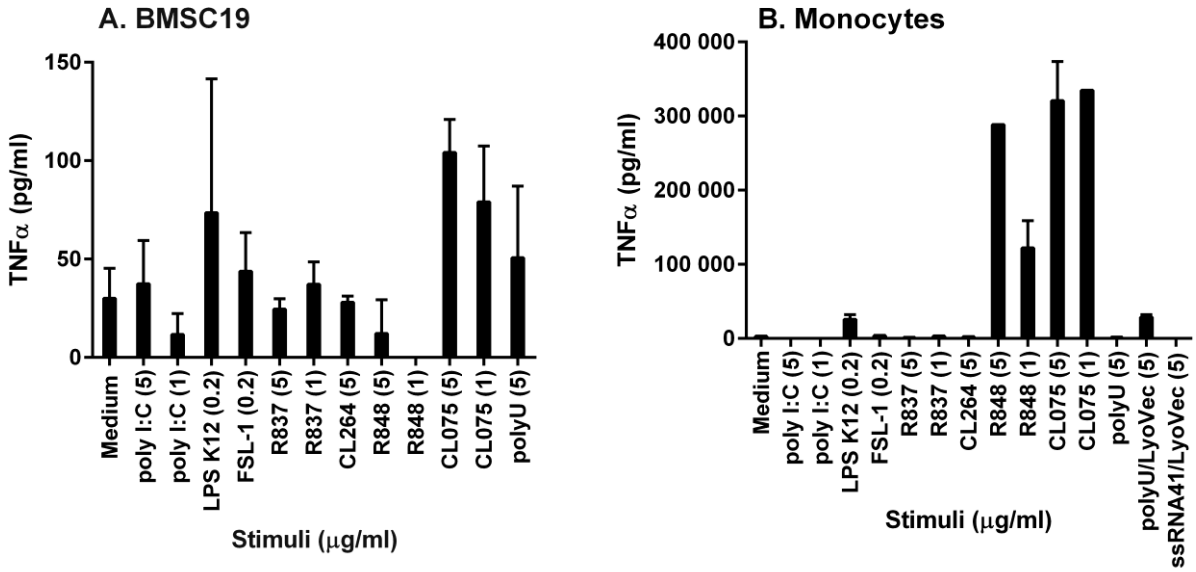


Figure 4.11 CXCL1 and CXCL5 are induced in TLR8 stimulated BMSCs. BMSC19 and BMSC20 were stimulated for 72 hours with the TLR8 ligand CL075 (5 or 1 µg/ml) in 2% A+v.i./RPMI at 37 ^{o}C, 5% CO_2. The supernantants were assayed for CXCL1 and CXCL5 induction by ELISA. Data were normalized to the respective medium sample. Results show mean and standard deviations for triplicates. P-values calculated using One-way ANOVA with Dunnett's post-hoc test. * $p < 0.05$; ** $p < 0.01$.

4.6.3 Bone marrow stromal cells express low levels of TNF-α

BMSCs failed to induce TNF-α in response to CL075 in the proteome profiler kit (Figure 4.12), suggesting that TLR8 responses in BMSCs may not be due to macrophages. Macrophages are known to potently induce TNF-α in response to TLR stimulation [83]. Given the potential presence of macrophages in BMSCs, TNF-α secretion was assessed by ELISA from TLR stimulated BMSCs, monocytes, macrophages and moDCs to compare the cytokine induction profile.

Figure 4.12 shows that both monocytes and macrophages induced TNF-α strongly upon stimulation with TLR7/8 (R848) and TLR8 (CL075) ligands (Figure 4.12 B and C). In addition, a response to the TLR4 ligand LPS K12 was also observed. MoDCs yielded a strong response upon stimulation of TLR4 (LPS K12), while a lower response was observed for TLR3 (poly I:C) (Figure 4.12 D). BMSCs induced TNF-α at low levels upon stimulation with TLR ligands compared to monocytes and macrophages (Figure 4.12 A). A significant difference between BMSC19 and macrophage induced TNF-α was found for stimulation with R848 (5 µg/ml) ($p<0,05$) and CL075 (5 µg/ml) ($p<0,01$) using a two-tailed unpaired t-test with Welch's correction. The results indicate that TLR8 responding cells in BMSCs are not macrophages, however, macrophage contribution to TNF-α expression cannot be eliminated.



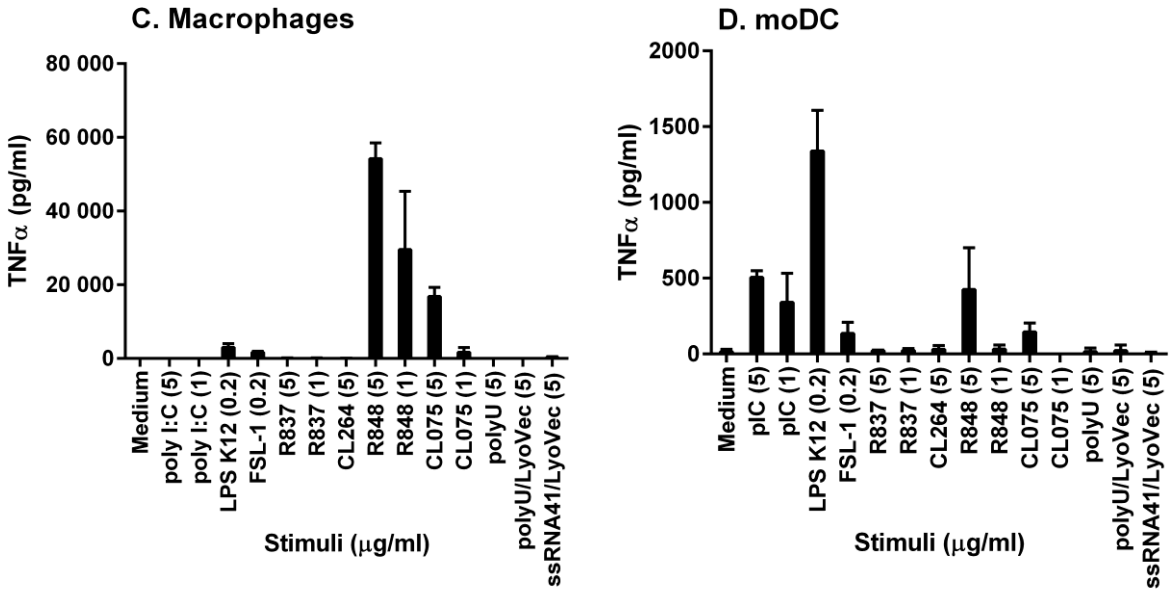


Figure 4.12 TNF-α expression upon TLR stimulation. A) BMSC19 were stimulated with TLR ligands in 2% A+v.i./RPMI for 72 hours at 37 ^{o}C/5% CO_2. R848 (1) was not determined. B) Monocytes and C) Macrophages were stimulated with TLR ligands in 2% HS/RPMI for 20 hours at 37 ^{o}C/5% $C0_2$ while D) moDCs were stimulated for the same duration in 10% HS/RPMI with IL-4 (40 ng/ml) and GM-CSF (100 ng/ml). The supernatants were assayed for TNF-α by ELISA .Detection limit was 3,9 pg/ml. Data were normalized against medium sample. The results are presented as mean and standard deviation of triplicates. Data for the immune cells are representative for three individual experiments.

4.7 Gene expression analysis of BMSCs stimulated with CL075

Previous assays to determine cytokine induction showed that BMSCs responded to the TLR ligands R848 and CL075 (Figure 4.8 and 4.10), indicating that BMSCs may express TLR8. To validate these findings and to investigate if other cytokine responses could be induced by the TLR8 ligand CL075, gene expression analysis was performed on BMSCs stimulated with CL075. The PanCancer Immune Profiling gene expression kit consisted of a panel of 770 genes related to cancer and inflammation. In addition, the kit detected genes encoding for cell markers, which may indicate presence of immune cells and BMSC markers in the heterogeneous stromal cell population.

4.7.1 Optimization of stimulation for BMSCs

In order to determine a concentration and time-point suitable to assay gene expression, BMSCs were stimulated with the TLR8 ligand CL075 with concentrations ranging from 0,1 µg/ml to 5 µg/ml for 72 hours, and a time study from 3 to 72 hours with CL075 (1 µg/ml). CCL3 was used as a reporter for gene induction upon stimulation.

Figure 4.13 shows that the highest fold – induction of CCL3 mRNA occurred in BMSCs after stimulation with 1 µg/ml CL075 for 6 hours. Therefore, this concentration and time-point was chosen for the nCounter gene expression analysis.

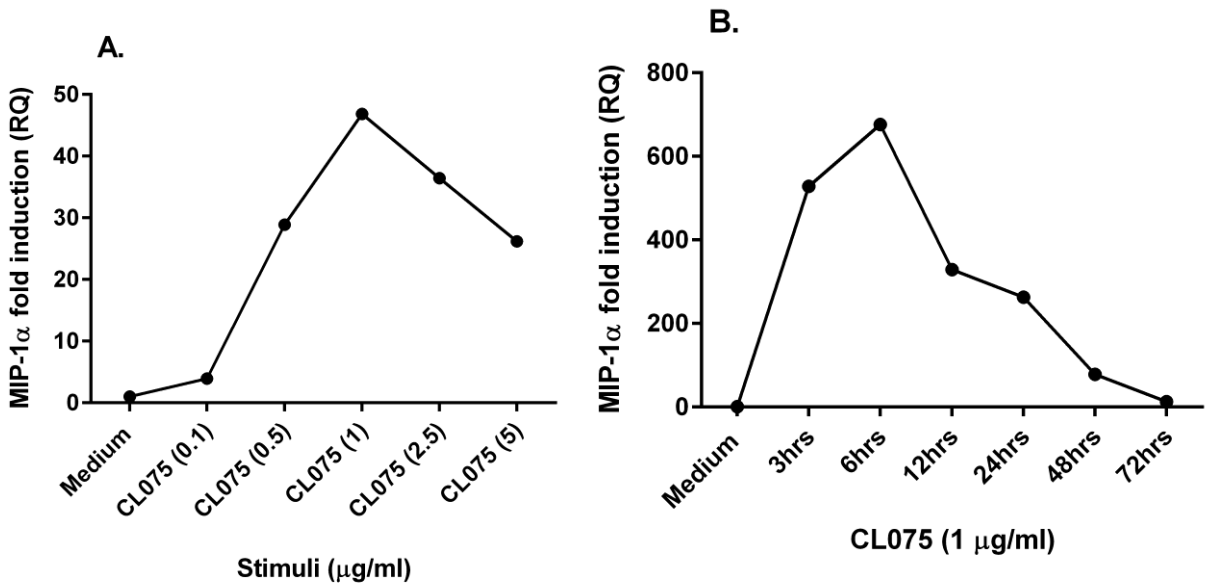


Figure 4.13 Optimization for gene expression assay.A) Dose and B) time study was performed on BMSC20 stimulated with the TLR8 ligand CL075 in 2% A+v.i./RPMI at 37 ^{o}C/5% CO_2. In the dose study (A) cells were stimulate for 72 hours. RNA was isolated from BMSC20 cells after stimulation. The RNA was reverse transcribed to cDNA before measured for CCL3 expression with qRT-PCR. The data were normalized to the housekeeping gene TBP. The results are presented as fold-induction (RQ) in relation to medium sample.

4.7.2 Gene expression analysis of TLR8 stimulated BMSCs

The PanCancer Immune Profiling kit was used to assess gene expression in BMSCs upon stimulation with CL075. BMSC19 were stimulated with CL075 (1 µg/ml) for 6 hours at 37 ^{o}C, 5% CO_2 before total RNA was isolated and analyzed with Nanostring. Selected genes are presented below, while remaining data are listed in Appendix V.

TLR mRNA expression in BMSCs

BMSCs responded to ligands for TLR2/6, TLR3, TLR7/8, and TLR8 (Figure 4.8), indicating expression of TLR2, TLR3, TLR6 and TLR8. Therefore, TLR gene expression in BMSCs was assessed. Surprisingly, the results show that BMSCs expressed mainly TLR4 mRNA (Table 4.5). Remaining TLRs were either expressed at low levels or barely detectable.

Table 4.5 TLR mRNA expression in BMSC19. BMSC19 were stimulated with CL075 (1 µg/ml) for 6 hours. Data were normalized against housekeeping genes and positive controls.

Gene	BMSC19 Medium	BMSC19 CL075
TLR1	14	3
TLR2	14	3
TLR3	7	3
TLR4	268	284
TLR5	2	7
TLR6	9	10
TLR7	2	3
TLR8	2	3
TLR9	2	3

Cytokine expression in BMSCs

Although, TLR expression on BMSCs were low, previous results have detected up-regulation of cytokines in response to stimulation with the TLR8 ligand CL075 (Figure 4.8, 4.10 and 4.11). These cytokines were assessed with the gene expression analysis (Table 4.6). CCL3

mRNA was highly up-regulated (397-fold), correlating with the observation in Figure 4.8. TNF-α mRNA was also highly up-regulated, while protein expression was detected at lower concentrations (Figure 4.12). The chemokines CXCL1 and CXCL5 had also higher mRNA expression upon stimulation, in accordance with previous results detecting the chemokines at protein level (Figure 4.11).

Multiple cytokines were observed to be elevated in response to the TLR8 ligand CL075, and the most regulated genes encoding for cytokines are shown (Table 4.6). IL-6 and IL-8 were expressed in unstimulated BMSCs and slightly induced upon TLR8 stimulation, in agreement with protein expression (Figure 4.10). Other pro-inflammatory cytokines, IL-1β and CSF2 (GM-CSF) were strongly up-regulated. Furthermore, RANTES/CCL5 and IL-32, both implicated in inflammation [84, 85], were induced at mRNA level. These results indicate that BMSCs stimulated with the TLR8 ligand CL075 contributes to an inflammatory milieu.

Table 4.6 mRNA expression of cytokines genes in BMSC19 after stimulation with TLR8 ligand CL075 (1 µg/ml) for 6 hours. Data were normalized against housekeeping genes and positive controls

Gene	BMSC medium	BMSC CL075	Fold change
IL-1β	2	1427	602,06
CCL3	2	942	397,61
TNF	2	785	331,35
CCL3L1	2	728	307,38
CCL4 (MIP-1β)	2	652	274,95
IL32	5	585	123,11
IL8	377	12 748	33,79
CSF2 (GM-CSF)	2	77	32,43
CCL5 (RANTES)	2	53	22,56
CXCL1	55	1089	19,96
IL6	541	1888	3,49
CXCL5	95	257	2,71
IFNB1	2	3	1,5

Cell marker expression in BMSCs

The PanCancer Immune Profiling kit also detected genes encoding for cell surface markers to indicate cell types present in BMSC19. As mentioned, BMSCs are a heterogeneous cell population aspirated from the bone marrow. Therefore, gene expression of cell markers were assessed. Misund et al. [63] characterized MM-BMSCs with flow cytometry. BMSCs should express CD44, CD90, CD73, CD105, CD29 and HLA-ABC. In addition, BMSCs are negative for CD14, CD45, CD209 and HLA-DR [63].

As shown in Table 4.7, BMSC19 expressed high levels of CD44 and HLA-ABC mRNA in accordance with previous results [63]. mRNA levels of CD45, CD209 and CD34 were barely detectable indicating absence of hematopoietic and dendritic cells. Macrophages are known to express CD14, CD86 and HLA-DR. BMSC19 expressed CD14 and HLA-DR, albeit at low levels. CD86 was not part of the profiling kit. Therefore, in accordance with Misund et al. [63], BMSCs may contain a small population of macrophages.

Table 4.7 Cell marker gene expression in BMSCs. BMSC19 were stimulated with CL075 (5 µg/ml) for 6 hours. Data are normalized against housekeeping genes and positive controls

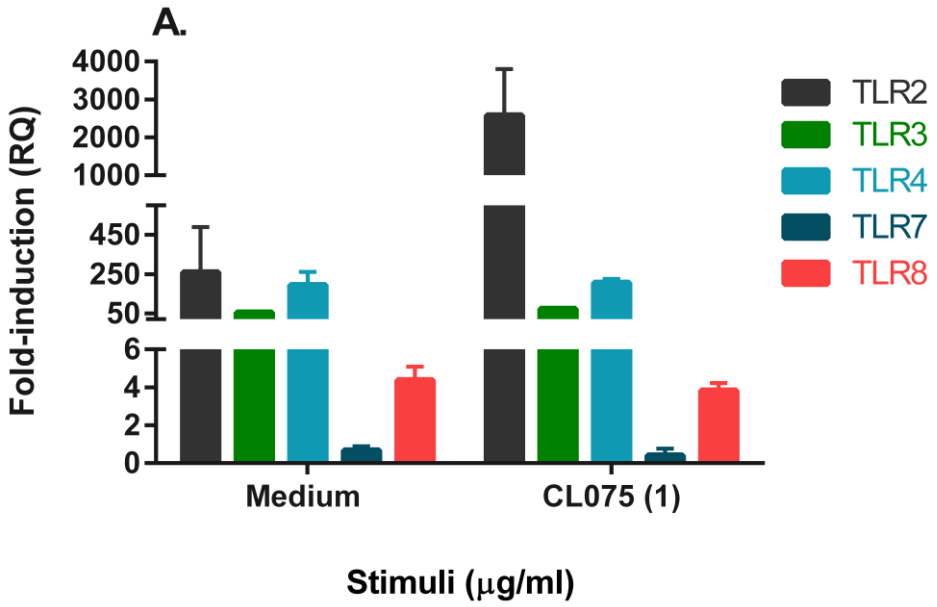
Gene	BMSC medium	BMSC CL075	Fold change
CD44	7355	7482	1,02
HLA-A	6389	7051	1,10
HLA-B	5203	5083	0,98
HLA-C	2190	2476	1,13
CD45	2	3	1,41
CD209	2	3	1,14
CD34	2	3	1,14
CD14	47	7	0,14
HLA-DRA	19	27	1,41
HLA-DRB3	28	53	1,88
HLA-DRB4	2	3	1,14

4.7.3 BMSCs express TLR2, TLR3, TLR4 and TLR8

The Nanostring results revealed very low mRNA expression of all TLRs (1-9) except TLR4 (Table 4.5). Nanostring is a non-amplified system that may be unable to detect very low expression levels of TLRs. Therefore, the TLR expression in BMSCs was investigated using qRT-PCR.

BMSC19 were either unstimulated or stimulated with CL075 for 6 hours before RNA was isolated and assayed for TLR2, 3, 4, 7, and 8 expression by qRT-PCR. RNA from HEK293 cells transfected with TLR2, TLR3, TLR4, TLR7, TLR8 or control vector was assayed by qRT-PCR using the same primers to assess functionality and specificity of the TLR primers.

qRT-PCR revealed that BMSCs expressed TLR2, TLR3, TLR4 and low expression of TLR8, but not TLR7 (Figure 4.14 A) compared to HEK293 expressing control vector. TLR expressions were not induced by the TLR8 ligand CL075 except TLR2, which was strongly induced. Due to lack of detection of positive control for TLR2, this detection could be unspecific. The TLR primers detected TLR expression in HEK293 cells overexpressing the receptors except TLR2 (Figure 4.14 B), implying that the primers detect TLR mRNA except for TLR2.



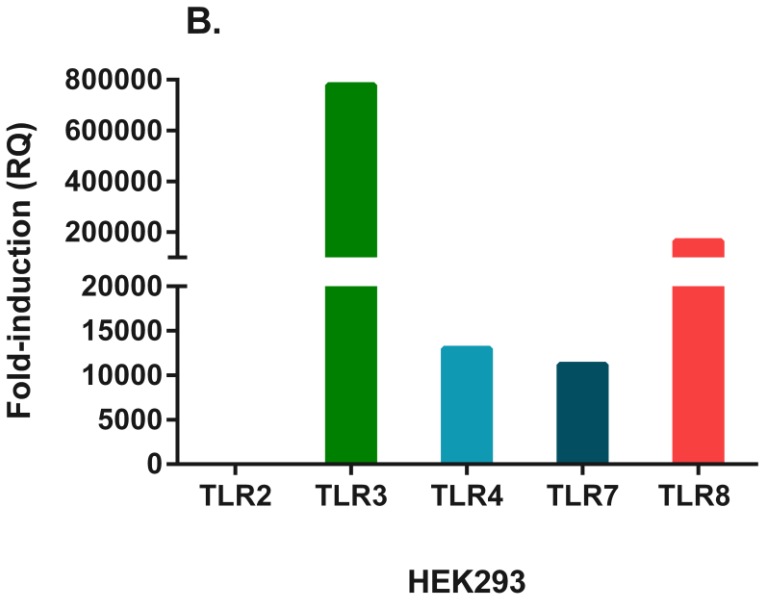


Figure 4.14 TLR expression in BMSC19 using in-house designed primers. A) BMSC19 were treated with CL075 (1 µg/ml) for 6 hours at 37°C/5% CO_2 before total RNA was isolated. RNA concentration was measured and reversely transcribed to cDNA before measured for TLR transcripts by qRT-PCR. B) HEK293 cells transfected with a given TLR (2 µg/ml) were used as a positive control for TLR primers. BMSCs and HEK293 were assayed for expression of TLR2, TLR3, TLR4, TLR7 and TLR8 with TBP as endogenous control. Results show fold induction of biological triplicates of BMSCs relative to the expression of the respective TLR receptor in HEK293 transfected with empty vector. BMSCs data are presented as mean and standard deviation while HEK293 data are presented for one sample.

4.7.4 IL-1β mRNA is induced but not secreted by BMSCs

A strong induction of IL-1β mRNA, in response to the TLR8 ligand CL075, was observed in the gene expression analysis (Table 4.6). However, no IL-1β secretion was observed with the proteome profiler (Figure 4.10). To confirm presence or absence at protein level, IL-1β expression in BMSCs was assessed by ELISA. In addition, monocytes, macrophages and moDCs were also assessed for IL-1β expression to evaluate the immune cells' cytokine profile to BMSCs.

BMSCs, monocytes, macrophages and moDCs were stimulated with a panel of TLR ligands (Table 3.3) before supernatant was harvested and analyzed for IL-1β protein by ELISA. BMSCs were stimulated for 72 hours, while immune cells were stimulated for 20 hours.

Figure 4.15 shows that BMSC19 did not express IL-1β in response to CL075 stimulation, which concurs with results from proteome profiler (Figure 4.10). These results suggest that BMSCs fail to secrete IL-1β, although these cells were previously observed to induce a strong up-regulation of IL-1β at the mRNA level in response to CL075 (Table 4.6). None of the

assessed TLR ligands were found to induce IL-1β in BMSCs (Figure 4.15 A). In contrast to BMSCs, monocytes induced high levels of IL-1β in response to R848 and CL075 (TLR7/8) stimulation, while a weaker response was observed upon TLR4 stimulation (Figure 4.15 B). Macrophages expressed low levels of IL-1β in response to TLR stimulation (Figure 4.15 C), while moDCs expressed IL-1β upon stimulation with the TLR7/8 ligand R848 and TLR8 ligand CL075 (Figure 4.15 D). Hence, IL-1β secretion is detected in monocytes, and to some extent in macrophages and moDCs upon stimulation with TLR7/8 ligand R848 and TLR8 ligand CL075, while no induction in observed in BMSCs.

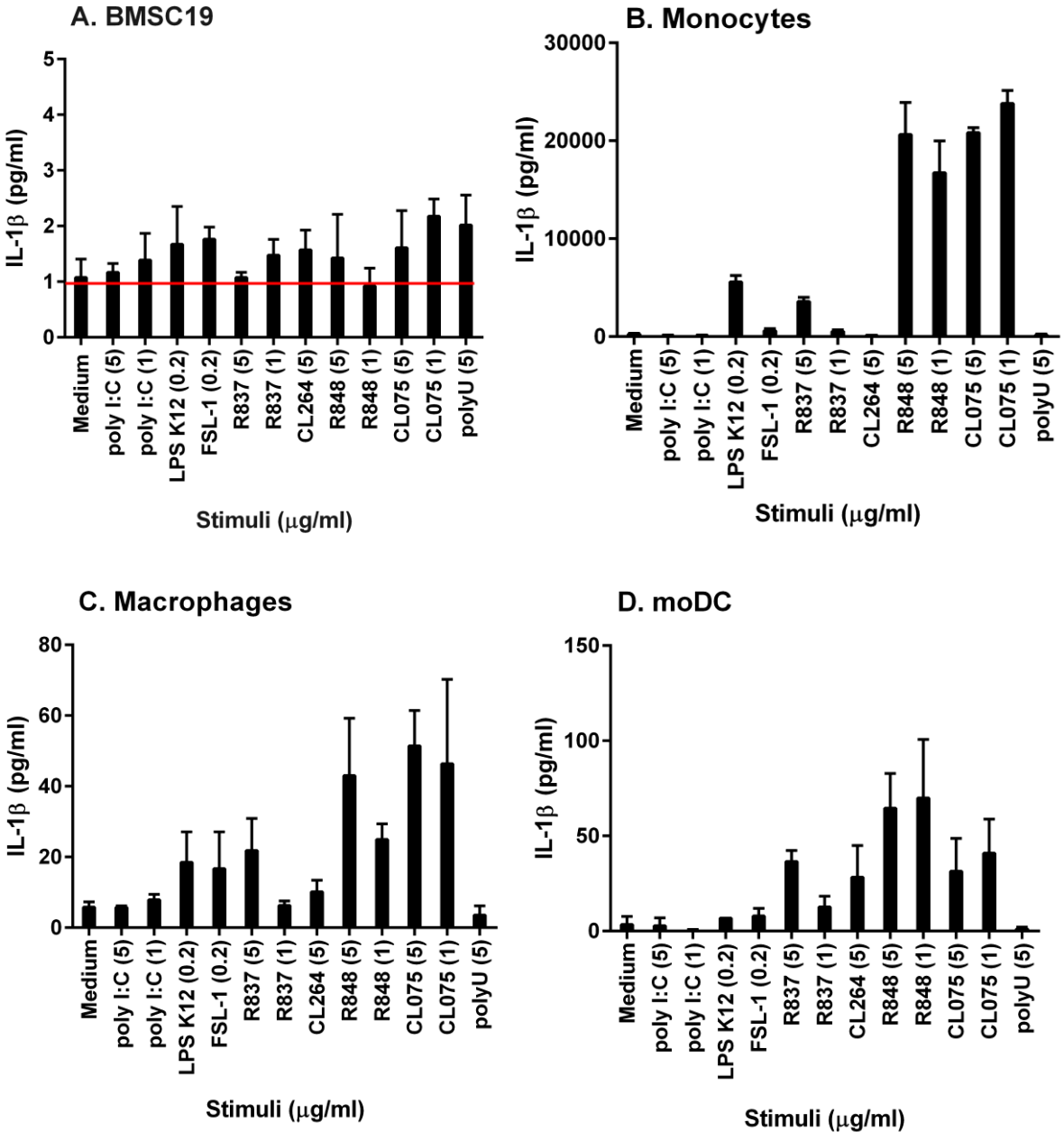


Figure 4.15 IL-1β induction in response to TLR ligands.A) BMSC19 were stimulated with TLR ligands in 2% A+v.i./RPMI for 72 hours at 37 ^{o}C/5% CO_2. B) Monocytes and, C) Macrophages were stimulated with TLR ligands in 2% HS/RPMI for 20 hours at 37 ^{o}C/5% $C0_2$, while D) moDCs were stimulated for the same duration in 10% HS/RPMI with IL-4 (40 ng/ml) and GM-CSF (100 ng/ml). The supernatants were assayed for IL-1β by ELISA. Data were normalized against medium sample. The results are presented as mean and standard deviation of triplicates. Data for the immune cells are representative for three individual experiments.

4.7.5 IFN-β is not secreted by TLR stimulated BMSCs

Endosomal TLRs (TLR3, TLR7-9) are able to induce type 1 interferons, IFN-α and IFN-β, as part of the antiviral response. These interferons are regulated by the transcription factors IRF5 and IRF7 downstream of TLR7 and TLR8 [40]. IFN-β expression in TLR stimulated BMSC19 was determined with ELISA to examine if these cells induce IFNs in response to TLR ligands.

As observed in Figure 4.16, IFN-β was not detected in BMSCs upon TLR stimulation. This is in agreement with the gene expression analysis (Table 4.6).

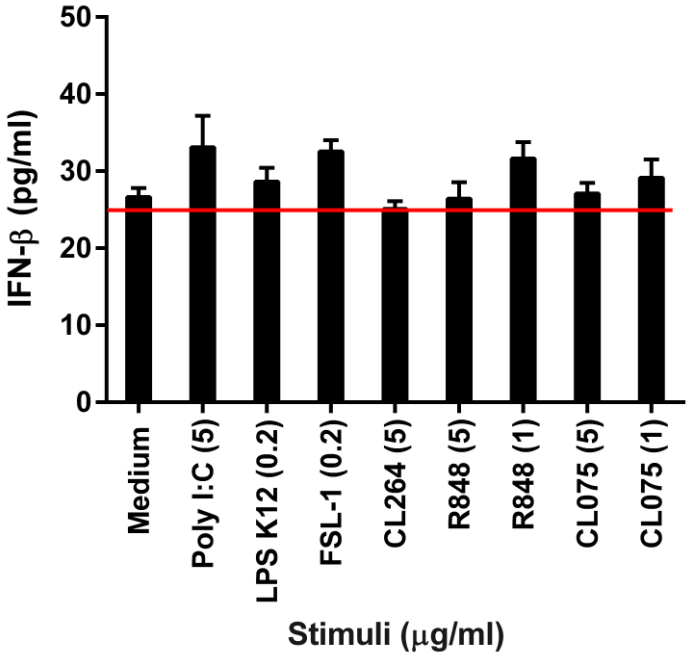


Figure 4.16 IFN-β is not secreted by BMSC19. BMSC19 were stimulated with TLR ligands for 72 hours in 2% A+ v.i./RPMI at 37 ^{o}C/5% CO_2. The supernatant was assayed for IFN-β using ELISA. Detection limit was 25 pg/ml as indicated by the red line. Results show mean and standard deviation for triplicates.

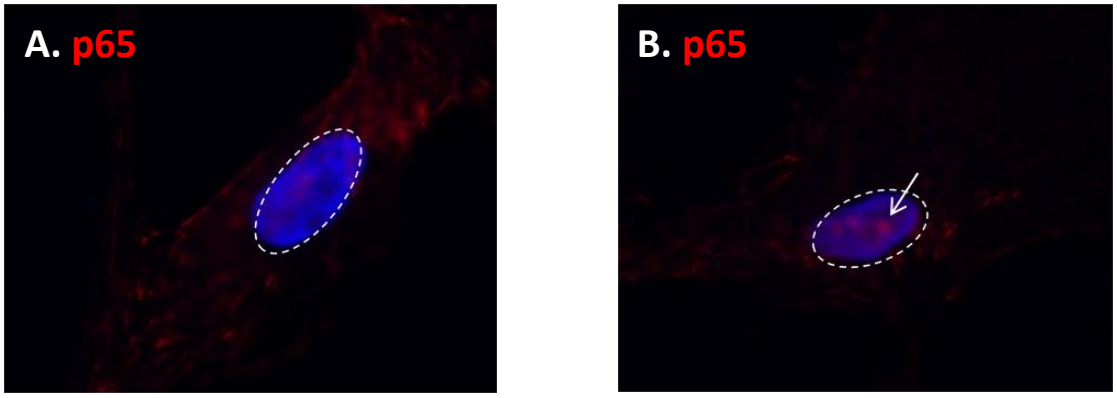
4.8 Bone marrow stromal cells signal through NF-κB and IRF5 upon TLR8 stimulation

Activation of TLR downstream signaling results in nuclear translocation of transcription factors such as NF-κB and IRF5, which bind to promoters of target genes and regulate gene expression. BMSCs were found to express TLR8 and respond to TLR8 activation by up-regulating numerous cytokines (Figure 4.10 and Table 4.6).

To study the role of NF-κB and IRF5 in BMSCs, BMSC19 were stimulated with the TLR8 ligand CL075 for 3 hours and stained with antibodies against the NF-κB subunit p65 and IRF5 as well as the nuclear stain Hoechst 33342. Nuclear translocation of p65 and IRF5 was determined by high-throughput confocal microscopy using an Olympus ScanR. In addition, BMSCs were stained for CD14 to determine presence of the macrophage marker CD14. The results were analyzed and expression of the antibodies against p65, IRF5 and CD14 were quantified using ScanR.

Staining of BMSC19 revealed p65 in the cytoplasm of unstimulated cells (Figure 4.17 A) and translocation to the nucleus upon stimulation of TLR8, as indicated by the arrow (Figure 4.17 B). A strong IRF5 staining was observed in both unstimulated and stimulated cells (Figure 4.17 C and D), suggesting that IRF5 is constitutively expressed and not induced by TLR8 stimulation. Positive staining for CD14 were observed, and upon TLR8 stimulation, IRF5 staining was detected in the nucleus of CD14 positive cells; indicated by the arrows (Figure 4.17 E and F).

Unstimulated **Stimulated (CL075)**



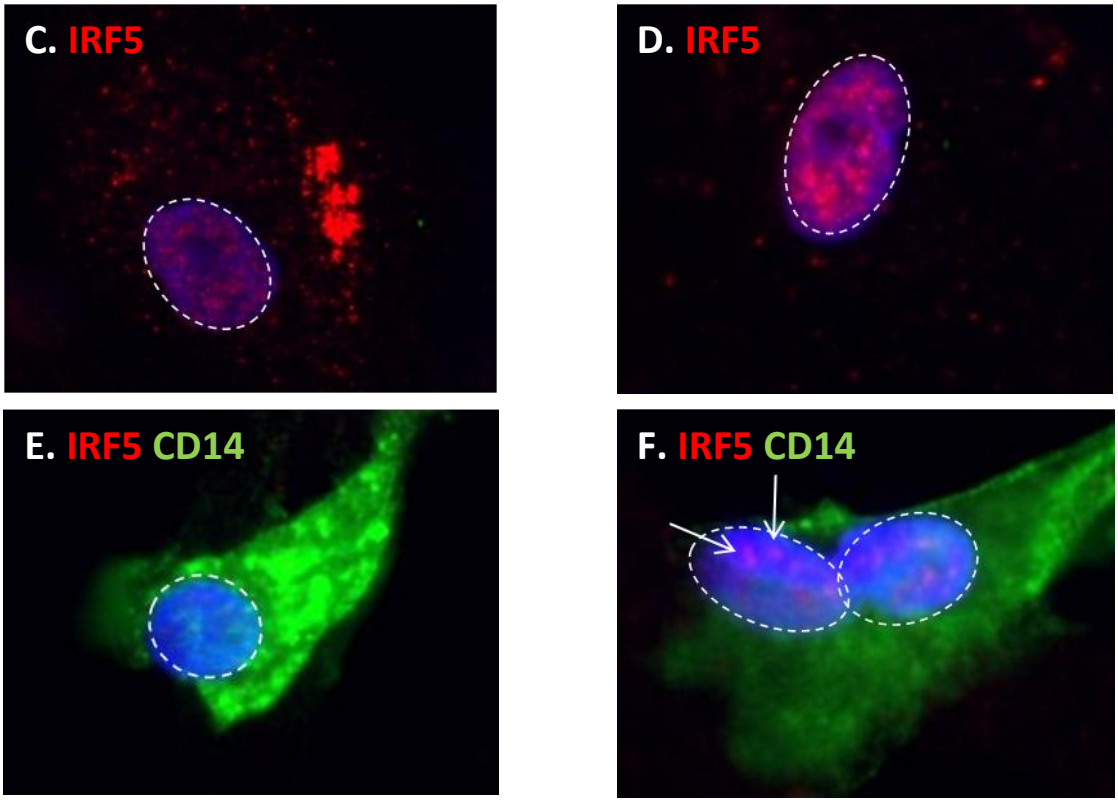


Figure 4.17 p65, IRF5, and CD14 staining in BMSCs upon TLR8 stimulation. BMSC19 were either unstimulated or stimulated with the TLR8 ligand CL075 (1 µg/ml) for 3 hours at 2% A+v.i./RPMI at 37 ^{o}C/5% CO_2. BMSC19 were stained with antibodies against p65 (Alexa 647), IRF5 (Alexa 647), or CD14 (Alexa 488) as well as the nuclear stain Hoechst 33342. A) Unstimulated BMSC19 stained against p65. B) Stimulated BMSC19 stained against p65. C) Unstimulated BMSC19 stained against IRF5. D) Stimulated BMSC19 stained against IRF5. E) Unstimulated BMSCs stained against IRF5 and CD14. F) Stimulated BMSCs stained against IRF5 and CD14.

In unstimulated cells, 6.8% of the cell population stained positive for p65 in the nucleus. Upon TLR8 stimulation, cells positive for nuclear p65 increased to 32.5% of BMSCs (Figure 4.18 A). These observations indicate that NF-κB was activated in BMSCs downstream of TLR signaling stimulated with the TLR8 ligand CL075. Furthermore, the observed strong staining against IRF5 (Figure 4.17 C and D) was confirmed, as 66% of BMSCs were positive for nuclear IRF5, both in unstimulated and TLR8 stimulated cells (Figure 4.18 A). This suggests that IRF5 is constitutively activated and not induced by TLR8 stimulation in BMSCs.

The results demonstrated that almost 3% of the unstimulated BMSCs stained positive for CD14 (Figure 4.18 B). Surprisingly, less than 1% of the population stained positive for both p65 and CD14, whereas slightly more than 2% were positive for both nuclear IRF5 and CD14 (Figure 4.18 B). Collectively, the results suggest that the majority of BMSCs consist of $CD14^{-}$ cells responding to TLR8 stimulation (Figure 4.18 A). Furthermore, the small population of

$CD14^+$ cells positive for nuclar NF-κB and IRF5, indicate presence of $CD14^-$ cells expressing TLR8 and responding to TLR8 stimulation in BMSCs. Still, signaling $CD14^+$ cells upon TLR8 stimulation in BMSCs cannot be eliminated.

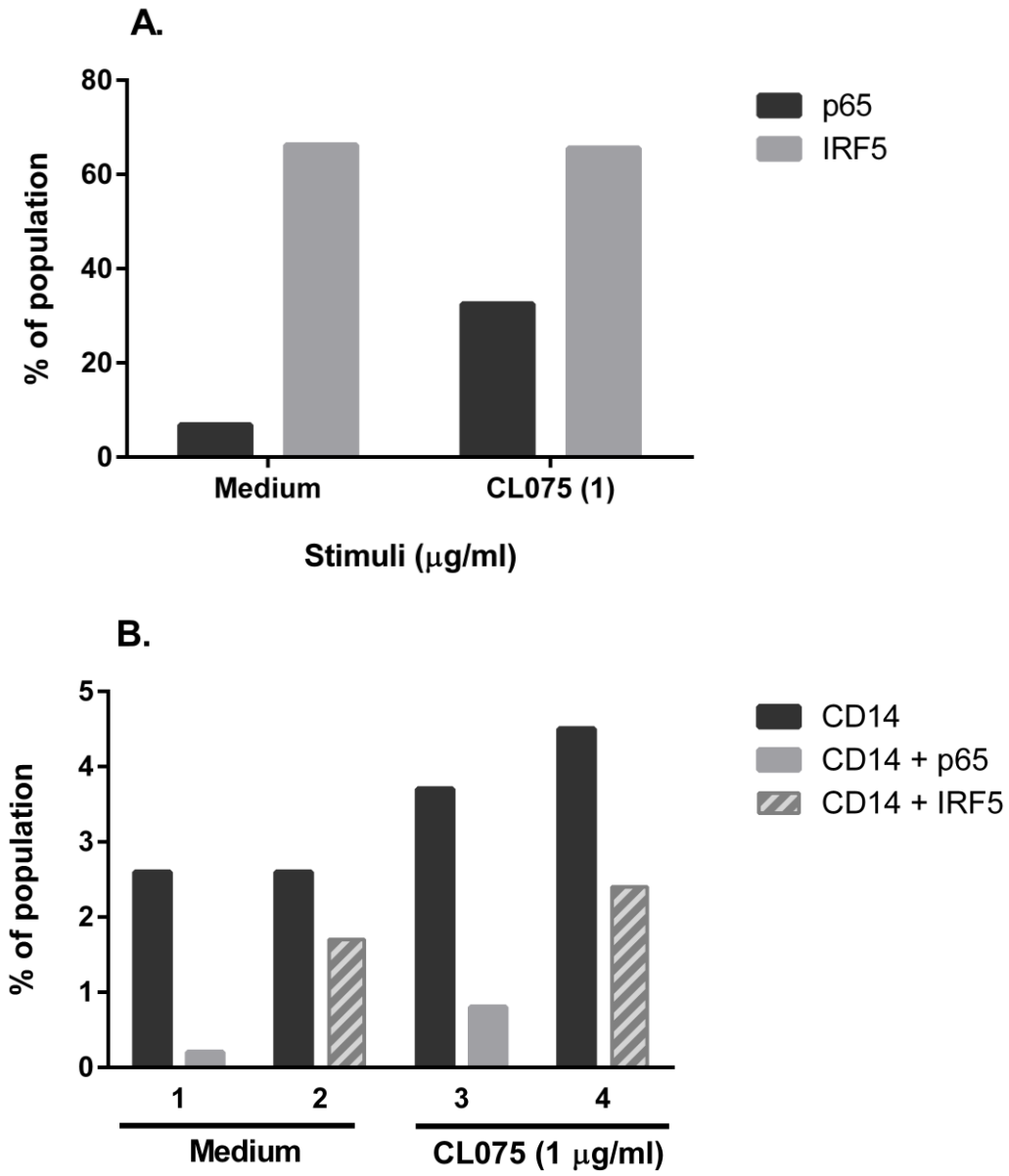


Figure 4.18 CD14, nuclear p65 and IRF5 quantification in BMSC19 upon TLR8 stimulation. BMSC19 were either unstimulated or stimulated with TLR8 ligand CL075 (1 μg/ml) for 3 hours at 2%A+v.i./RPMI at 37 ^{o}C/5% CO_2. BMSC19 were stained with antibodies against p65 (Alexa 647), IRF5 (Alexa 647) and CD14 (Alexa 488) and nucleus marked with Hoechst 33342. A) Quantification of BMSC19 with positive nuclear staining against p65 and IRF5, B) Percentage of $CD14^+$ cells with p65 or IRF5 in the nucleus. Results are presented as percentage of the total cell population in unstimulated BMSC19 (well 1 and 2) and TLR8 stimulated BMSC19 (well 3 and 4). More than 1500 cells per well were counted with scanR.

4.9 BMSCs secrete cytokines upon stimulation with MM conditioned medium

Myeloma cells are reported to shape their environment creating a supportive niche [4] where BMSCs support MM cell proliferation and protects the cells from drug-mediated apoptosis [5]. U266 CM was found to activated TLR7 and TLR8 in the NF-κB reporter assay (Figure 4.3 and 4.6). The TLR-activating components released by U266 could potentially stimulate TLR8 on BMSCs (Figure 4.14) leading to induction of TLR-mediated cytokines. This was examined by stimulating BMSCs with MM CM to assess if cytokine induction could be induced in BMSCs in response to U266 CM.

4.9.1 CCL3 is secreted by MM cells

To assess if BMSCs were activated by MM CM, BMSC19 were stimulated with U266 and ANBL-6 CM, before the BMSCs supernatant was assessed for cytokine expression. ANBL-6 had previously not activated TLRs (Appendix III) and therefore used as a control. Previous results have shown induction of CCL3 upon activation of TLR7/8 on BMSCs (Figure 4.8). Therefore, CCL3 induction in BMSCs was assessed by ELISA after stimulation with U266 and ANBL-6 CM. CCL3 protein expression in MM CM was also assessed to determine any presence of CCL3 in CM.

Results show that CCL3 was secreted at high levels from U266 cells, and expressed at lower concentration in ANBL-6 CM (Figure 4.19). Therefore, CCL3 expression in the supernatant of U266 CM treated BMSCs may be due to CCL3 secretion by the myeloma cells and not a response to MM CM treatment of BMSCs. Consequently, CCL3 is not an appropriate cytokine to determine stimulation of BMSCs by U266 CM.

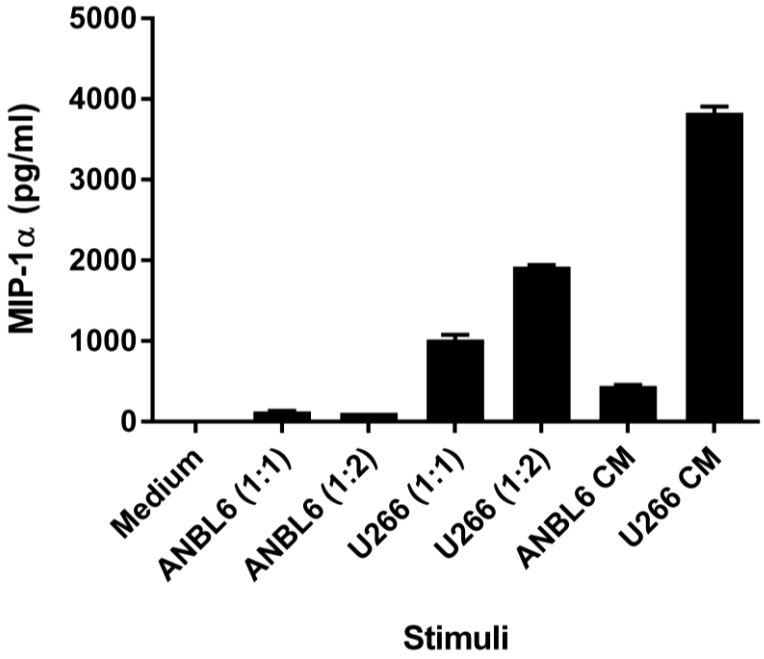


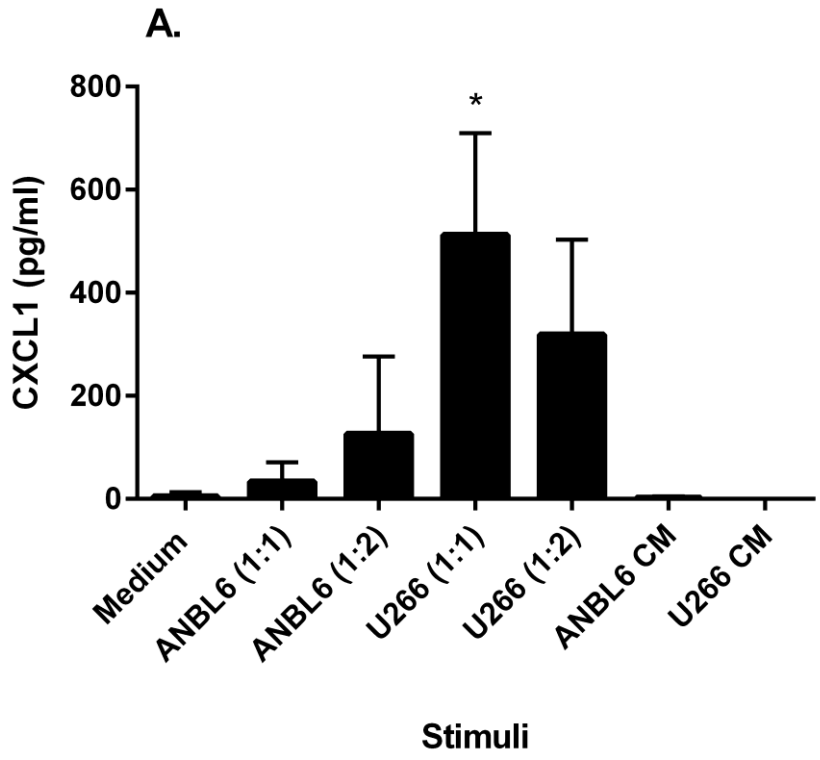
Figure 4.19 MM cells secrete high levels of CCL3. BMSC19 were plated in a 96-well plate and treated with ANBL-6 and U266 19 days conditioned medium for 72 hours at 37 ^{o}C/5% CO_2. BMSC19 supernatant stimulated with ANBL-6 (1:1 and 1:2) or U266 (1:1 and 1:2), in addition, ANBL-6 CM and U266 CM were assayed for CCL3 by ELISA. The detection limit was 8 pg/ml. Results show mean and standard deviation of triplicates.

4.9.2 BMSCs induce CXCL1 and CXCL5 expression upon stimulation with U266 conditioned medium

CXCL1 and CXCL5 are chemokines not previously reported to be expressed by B cells or malignant plasma cells [81]. Previous results indicated that BMSCs expressed the chemokines CXCL1 and CXCL5 upon TLR8 ligand stimulation (Figure 4.10). Hence, BMSCs treated with U266 and ANBL-6 CM were assessed for CXCL1 and CXCL5 expression, in order to determine any induction of these chemokines that may not be constitutively expressed by MM cells.

U266 nor ANBL-6 CM did secrete either CXCL1 or CXCL5, so these chemokines was used to evaluate BMSCs induction upon treatment with MM CM (Figure 4.20). Undiluted U266 CM induced a significant response of the chemokines in BMSCs, while diluted supernatant induced a lower response not found to be significant. U266 nor ANBL-6 CM did contain detectable levels of CXCL1 or CXCL5, implying that the observed CXCL1 and CXCL5 induction in U266 CM – treated BMSCs was due to CXCL1 and CXCL5 induction in these cells (Figure 4.20). No significant induction of the chemokines was observed for ANBL-6 CM treated

BMSCs. The results indicate that the TLR7/8-activating components secreted from U266 can induce cytokine response in primary BMSCs.



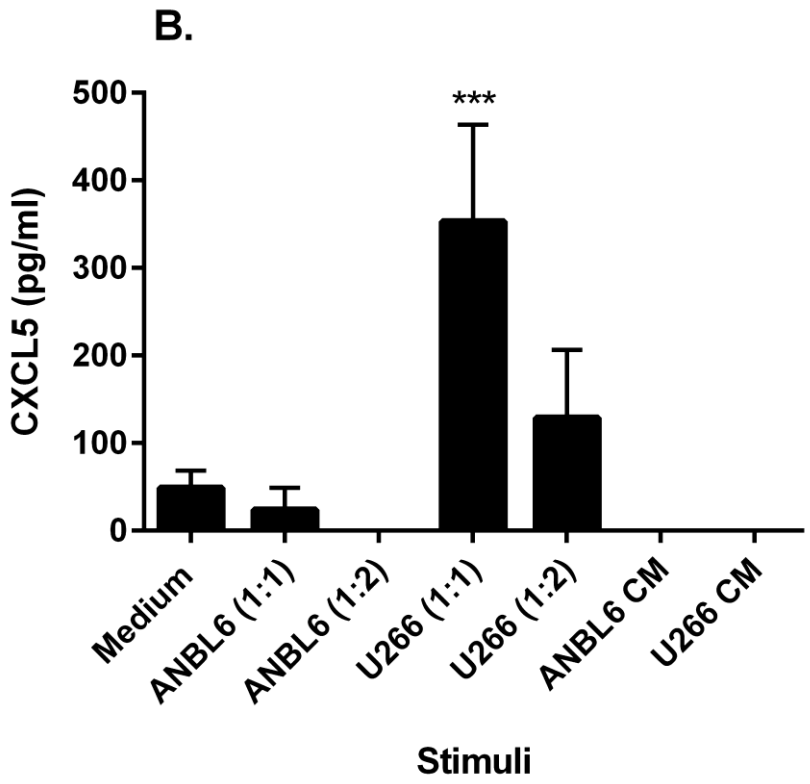


Figure 4.20 BMSCs induce CXCL1 and CXCL5 upon stimulation with MM conditioned medium. BMSC19 were plated in a 96-well plate and stimulated with ANBL-6 and U266 19 days conditioned medium for 72 hours at 37 ^{o}C/5% CO_2. BMSC19 supernatant stimulated with ANBL-6 (1:1 and 1:2) or U266 (1:1 and 1:2), in addition, ANBL-6 CM and U266 CM were assayed for A) CXCL1 and B) CXCL5 by ELISA. Results show mean and standard deviation of triplicates. P-values calculated using One-way ANOVA with Dunnett's post-hoc test. * $p < 0.05$; *** $p < 0.001$.

5 Discussion

TLRs mediate inflammatory signals that may play a role in tumorigenesis. Cancer cells are found to secrete endogenous TLR ligands activating TLRs on immune cells or other non-malignant cells in the microenvironment [56, 76]. BMSCs are reported to express functional TLRs, and activation of BMSCs by TLR ligands may lead to inflammation and enhanced survival of MM cells [86]. This study sought to investigate if myeloma cell lines release TLR-stimulating components and examine cytokine response in BMSCs upon stimulation with TLR ligands and MM conditioned medium.

In this study, the MM cell line U266 was found to secrete a TLR7/8 – activating component, which may activate TLR7/8 expressing cells in the BM, creating an inflammatory milieu. MM-BMSCs were found to express TLR8 and respond to TLR8 ligands by secreting various cytokines implicated in MM pathogenesis. CD14 staining of BMSCs revealed that a minor population stained positive for CD14, implying that the majority of the BMSC population are CD14 negative cells expressing TLR8 and responding upon TLR8 stimulation. Furthermore, primary BMSCs treated with U266 CM resulted in induction of chemokines, suggesting that BMSCs may respond to the TLR8-activating component in U266 CM. U266 CM may also induce responses in other TLR7/8 expressing cells in the BM as illustrated in Figure 5.1.

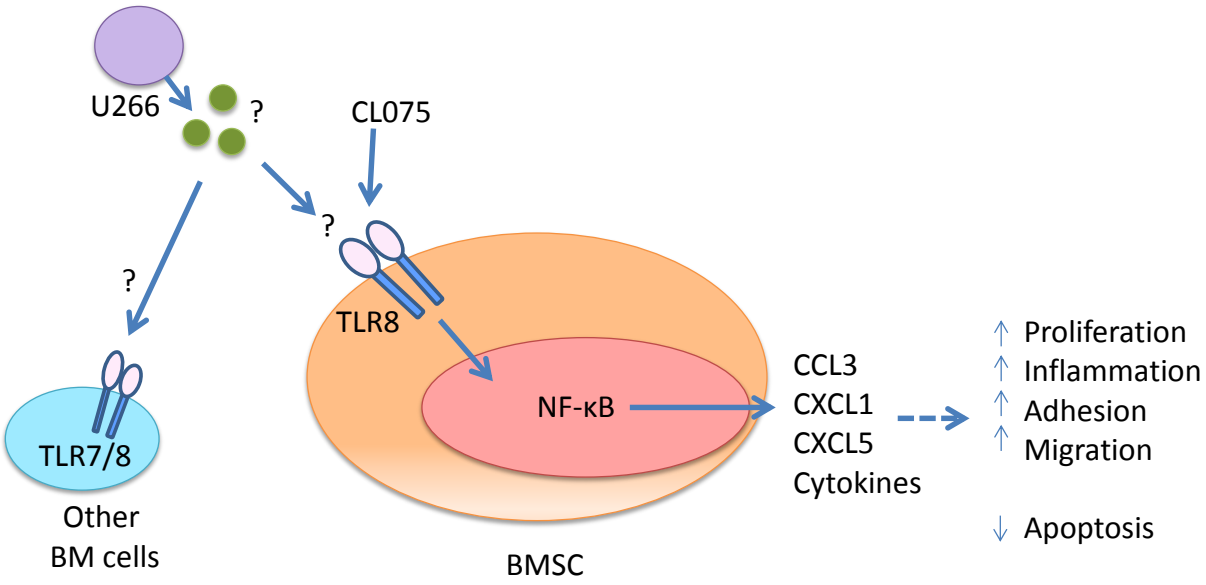


Figure 5.1 A schematic overview of the findings in this study. U266 secretes an unknown TLR-activating component with the ability to stimulate MM-BMSCs. CL075 stimulated BMSCs induced NF-κB activation leading to secretion of CCL3, CXCL1, CXCL5 and other various cytokines. These cytokines are reported to induce proliferation, inflammation, adhesion and migration. In addition, U266 CM could potentially activate other cells in the BM milieu expressing TLR7 or TLR8, or both.

5.1 U266 secreted a TLR-activating component

MM cells were assayed for the ability to activate TLRs by utilizing a TLR-NF-κB luciferase reporter system. Assessed by NF-κB activation, U266 was the only cell line able to induce TLRs (Appendix III). U266 has constitutive active STAT3, protecting cells from apoptosis [87], and are reported to shed high levels of syndecan [88]. One could speculate if these characteristics can explain why U266 was the only cell line to activate TLR7 and TLR8. However, syndecan shedding are reported for other MM cell lines [89] not found to activate TLRs in the TLR-NF-κB reporter system.

U266 activated mainly TLR7 and sometimes TLR8 (Figure 4.3 and 4.6). Overall, the TLR8 response was weaker compared to the TLR7 response. This weak activation of TLR8 may be due to different affinity, as the TLR-stimulating component may have a higher affinity for TLR7 compared to TLR8. Previous studies have observed that ligands stimulate the two closely related TLRs with different affinity [23]. Furthermore, empty vector control in the NF-κB luciferase reporter system was pcDNA while TLR8 had a pUNO vector backbone. This

difference might yield a higher background masking some of the TLR8 response, hence further studies should include pUNO empty vector.

5.1.1 U266 CM's ability to activate TLRs is not associated with viability

Endogenous TLRs are demonstrated to originate from necrotic cells releasing their intracellular contents [30]. Research has shown that mRNA and siRNA from necrotic cells stimulate TLR3 [90], while TLR2 and TLR4 recognize host proteins [30]. Apoptotic cells bud off blebs and apoptotic cell bodies are endocytosed by cells. If apoptotic cell bodies are not phagocytosed, they can become secondary necrotic cells, thus stimulating endosomal TLRs. U266 CM was found to only activate TLR7 and TLR8, and not TLR2 or TLR3 (Figure 4.3), which may reflect absence of necrotic cells. U266 cultured for either 7 or 14 days was determined to have similar viability, but only CM isolated from 14 days culture activated TLR7 (Figure 4.6). Consequently, it is unlikely that necrotic or apoptotic cells mediate the observed TLR activation. This suggests that live U266 cells secrete TLR-activating components to induce TLR-responses in the BM milieu to favor MM proliferation.

Necrosis [91] or apoptosis [92] could be induced in MM cells to assess release of TLR7/8-activating components by necrotic or apoptotic cells. However, TLR7/8 induction by necrotic or apoptotic cells might not be due to the similar TLR-activating component observed from U266 CM. Hence, concentrating CM from U266 short-term culture with high viability might up-concentrate the TLR-activating component enabling detection by the TLR-NF-κB luciferase reporter system.

5.1.2 TLR7/8 ligand is protected from RNase degradation

The natural ligand for TLR7 and TLR8 is ssRNA and short dsRNA [93]. Therefore, the U266 CM was treated with RNase to assess if the TLR7/8 response abolished, indicating RNA as the TLR-activating component. Interestingly, RNase - treated U266 CM activated TLR7 as potent as untreated supernatant (Figure 4.5 A). Based on these findings, the RNA may be protected from the RNase; dsRNA is resistant to certain nucleases [24], or RNA is encapsulated into a vesicle [74].

Heat – treated U266 CM induced a weaker TLR7 activation. This suggests that the RNA may be bound to a protein [94] or encapsulated in a vesicle [74] since proteins are degraded at high temperatures. Exposing the vesicle or protein-RNA complex to high temperatures could have degraded the protein or increased the permeability of the membrane, or both, resulting in the observed loss of TLR7 activation (Figure 4.5). Furthermore, TLR7/8 activation requires endosomal entry of the ligand [20] and this entry may be facilitated by the vesicle or protein-RNA complex. Moreover, RNases are naturally found in the cellular environment and cells release RNases [95], therefore free RNA in the conditioned medium would be rapidly degraded. Evidently, if RNA mediates the TLR7/8 activation, the RNA must be protected from the environment by either being conjugated to a protein or encapsulated into a vesicle.

Heat – treated supernatant was still able to activate TLR7 (Figure 4.5) which may be due to inefficient or too short incubation of heat treatment to degrade the proteins or vesicles completely. Treating U266 CM with RNases targeting short dsRNA or proteinase - treatment may result in further loss of TLR7/8 activation. Inhibiting endocytosis to interfere with endosome acidification could block the process, confirming TLR7/8 activation. In addition, these results must be confirmed in several U266 CM for validation. Furthermore, the TLR7/8 – activating component could be isolated and identified by RNA immunoprecipitation.

5.1.3 Extracellular vesicles may mediate part of the TLR7 activation

Based on the observation that the TLR-activating component was protected from RNase, extracellular vesicles were isolated from U266 and JJN-3 cultures. Tumor-derived extracellular vesicles, in particular exosomes, are reported to mediate intercellular signaling [96]. A recent report demonstrated bi-directional communication between BMSCs and MM through exosomes [97]. ECVs isolated from U266 and JJN-3 were able to activate a low response of TLR7, but only U266 induction was found significant (Figure 4.7). Exosomes may partly mediate the TLR7 activation, however, the ECVs were observed to have an apoptotic effect on transfected HEK293 impeding the ability to detect TLR-activating components.

ECVs were isolated by the ultracentrifugation method based on the concept of different sedimentation speeds. However, vesicles of similar size to exosomes (30 to 100 nm), are not

separated by this method [60]. Apoptotic cells release apoptotic blebs, even though commonly larger than exosomes, these blebs may be co-isolated with exosomes [61]. To eliminate such contaminations, confirmation of vesicle size should be performed by nanosight [60]. In addition, AnnexinV/PI can be utilized to determine if the isolation contains apoptotic bodies [75]. Furthermore, co-isolation of proteins and other impurities may have been present even though the isolated exosomes were washed with PBS. Further studies should characterize the U266 - derived ECVs before further assessing ECVs potential to activate TLRs. Moreover, ECVs treated with either a combination of a detergent and RNase or RNase alone would indicate presence of RNA in the vesicle.

5.2 BMSCs induced cytokines implicated in MM survival and progression

Primary BMSCs responded to multiple TLR ligands – especially ligands for TLR7 and TLR8 (Figure 4.8). Several cytokines were up-regulated in response to the TLR8 ligand CL075 such as CCL3, CXCL1 and CXCL5. CCL3 was highly up-regulated both at the protein (Figure 4.8) and at the mRNA level (Table 4.6). These observations are in accordance with the previous study (Kjønstad and Nilsen, unpublished data).

The proteome profiler array revealed a high induction of the chemokines CXCL1 and CXCL5 upon TLR8 stimulation of BMSCs (Figure 4.11), which was confirmed by ELISA (Figure 4.11) and gene expression (Table 4.6). These results are consistent with reports that have found that CXCL1 is induced upon stimulation in MSCs [81], while another group reported of CXCL5 elevated in BM fibroblasts from MGUS patients [98]. The results suggest a role for CXCL1 and CXCL5 in MM. CXCL5 is detected in several solid cancers, and are associated with invasion, proliferation and angiogenesis [80]. Furthermore, CXCL1 elevation is associated with invasion and inflammation [98]. CXCL1 and CXCL5 signal through the same receptor as IL-8, and the receptor is expressed on myeloma cells [99]. Hence, secreted CXCL1 and CXCL5 from BMSCs may stimulate MM proliferation [15]. CXCL1 and CXCL5 secretions was not investigated in monocytes, macrophages or moDCs in this study, but could be determined with ELISA.

TNF-α was detected in TLR7/8 stimulated BMSCs (Figure 4.12 A). Although protein secretion of TNF-α was low (Figure 4.10 and 4.12 A), TNF-α mRNA was highly induced by TLR8

stimulation with a 330 – fold induction (Table 4.6). TNF-α is highly regulated post-transcriptionally [100] which might account for the discrepancy. In addition, TNF-α may be a rapid, quickly down-regulated and degraded, hence after 72 hours protein expression has decreased.

IL-1β mRNA was highly elevated in BMSCs upon stimulation with the TLR8 ligand CL075 (Table 4.6), however no IL-1β protein secretion was detected by either ELISA (Figure 4.15) or proteome profiler (Figure 4.10). IL-1β is reported to be secreted by BMSCs in MM [6] and previous studies have detected IL-1β in response to TLR3 and TLR4 stimulation [57]. The discrepancy between high mRNA levels and no secreted IL-1β could be due to an inactive inflammasome. The inflammasome mediates cleavage of pro-IL-1β to mature IL-1β via capsase-1 [101]. BMSCs expressed PYCARD and NLRP3, both inflammasome components, but caspase-1 was barely detectable (Appendix V). This indicates that TLR8-stimulated BMSCs may require a second signal to activate the inflammasome to mediate secretion of IL-1β.

BMSCs responded to several TLR ligands (Figure 4.8), indicating expression of TLR2, TLR3, TLR4, and TLR8. Gene expression analysis detected only TLR4 mRNA (Table 4.5). Since this is a non-amplified system, low expression of TLR receptors may not be detectable. With qRT-PCR, TLR4 expression was confirmed, and in addition, TLR2, TLR3 and TLR8 was detected (Figure 4.14). TLR2, TLR3, and TLR4 was highly expressed while TLR8 was expressed at low levels. TLR7 mRNA was not expressed by BMSCs. HEK293 overexpressing TLR2 was not detected by the TLR2 primers, thus TLR2 expression in BMSC19 requires confirmation as the TLR2 primers could amplify an unspecific product.

Previous studies confirm the observations that BMSCs express TLR3, TLR4 and lack of TLR7 expression [51], however, TLR8 mRNA expression are not in concordance with most reports. The finding of TLR8 mRNA is supported by one study that detected low quantities of TLR8 mRNA [52]. The variation between previous research and TLR expression found in this study may be explained by a heterogeneous cell population, different primers or detection method. The presence of TLR8 suggests that the cytokine response to TLR7/8 and TLR8 ligands is mediated by TLR8. The TLR expression must be examined in other batches of BMSCs such as BMSC20 to confirm TLR8 expression. In addition, TLR protein expression

should be investigated using methods like Western blotting, flow cytometry or fluorescent staining with TLR antibodies to confirm TLR8 protein expression.

5.3 NF-κB and IRF5 activation in BMSCs

The TLR8 ligand CL075 activates downstream signaling resulting in elevation of cytokines. TLR signaling pathways converge at NF-κB, and in addition, TLR8 can activate the transcription factors IRF5 and IRF7 [40]. Therefore, scanR was utilized to determine nuclear translocation of NF-κB and IRF5 upon stimulation with the TLR8 ligand CL075. Results showed a high constitutive nuclear expression of IRF5 regardless of stimulation with CL075, while NF-κB was induced upon stimulation in 32,5% of the cell population (Figure 4.17).

IRF5 is required for expression of pro-inflammatory genes such as IL-12, IL-23 [102] and contributes to IL-6, TNF-α expression with NF-κB [41]. In addition, IRF5 mediates IFN-β but not IFN-α [41]. Based on the high constitutive IRF5 expression, these cytokines were anticipated to be highly expressed. Neither IL-12 nor IL-23 are detect in BMSCs (Appendix V). Moreover, IFN-β was not detected at mRNA (Table 4.6) or protein level (Figure 4.16), indicating absence IRF5 activation. Recently, IRF5 was found to signal through a TLR8 – TAK1 – IKKβ pathway in monocytes and macrophages [103]. BMSCs express both TAK1 (MAP3K7 gene) and IKKB (IkBkB gene), while IRF5 mRNA was barely detectable (Appendix V). The IRF5 staining detected by scanR was performed without control antibodies; hence, unspecific staining is not accounted for. Combined, these results indicate absence of IRF5 mediated signaling. The elicited response upon TLR activation is dependent on the nature of the ligand as well as the cell type [33], thus the reported IRF5 mediated cytokines may not be induced in BMSCs. However, IRF5 expression should be examined at RNA and protein level to further enlighten the discrepancy between the strong IRF5 staining and the lack of expression of IRF5 targets. In addition, the specificity of the IRF5 fluorescent staining of BMSCs should be further assessed.

5.4 BMSCs are TLR8 responding cells

A previous study observed a discrepancy between the stromal cell lines HS-5 and HS-7a and primary BMSC, as the primary BMSCs responded to TLR7/8 ligands whereas stromal cell lines did not respond (Kjønstad & Nilsen, unpublished data). BMSCs have been characterized as fibroblast-like cells with presence of monocytes and macrophages [63]. In order to identify the TLR8 responding cells, monocytes, macrophages and moDCs were stimulated with the same panel of TLR ligands as BMSCs to observe similarities in cytokine profile. Further, BMSCs were characterized for mRNA expression of cell markers by Nanostring. Moreover, BMSCs were stained for CD14, a marker for both monocytes and macrophages, to assess if $CD14^+$ cells expressed p65 and IRF5 in the nucleus.

Overall, the cytokine profile of TLR stimulated BMSCs was similar to that of macrophages, implying a possible role for macrophages to partly mediate the observed cytokine inductions. CCL3 is inducible in most hematopoietic cells [104] and both macrophages and moDCs secreted high levels of CCL3 upon TLR8 stimulation (50 ng/ml and 800 pg/ml, respectively) compared to BMSCs (600 pg/ml). Hence, CCL3 secretion in BMSCs might be due to macrophages. Macrophages yielded a strong up-regulation of TNF-α for TLR4 (LPS K12) and TLR2/6 (FSL-1) ligands (Figure 4.9 B). The results are in accordance with the literature reporting TNF-α to be overexpressed in bone marrow $CD14^+$ cells compared to BMSCs [53, 56]. IL-1β was not detected in BMSCs, while low expression was found in macrophages (Figure 4.15). In agreement with the literature, macrophages expressed low levels of IL-1β, while monocytes expressed high levels [105]. Collectively, the cytokine profile of BMSCs compared to immune cells had the same overall features. However, macrophages generally had a stronger cytokine response than BMSCs.

BMSCs were further stained for CD14 as well as the transcription factors NF-κB and IRF5 to assess if $CD14^+$ cells were activated by TLR8 ligand CL075. CD14 staining revealed a small positive population of $CD14^+$ cells in the BMSCs. On average, 3,35% of the cell population was positive for CD14 (Figure 4.17 C). Upon stimulation, NF-κB was detected in the nucleus of 0.8% of $CD14^+$ cells, while 2.4% of $CD14^+$ cells stained positive for IRF5 in the nucleus. The results indicate a small population of $CD14^+$ cells capable of signaling upon TLR8 stimulation. Moreover, cell marker expression revealed that BMSCs expressed low levels of CD14 and

HLA-DR mRNA (Table 4.7), both markers for macrophages. Acknowledging the possibility of CD14 negative or dim monocytes [106] and macrophages in the bone marrow, the overall results indicate that cytokine induction observed in BMSCs are most likely not due to macrophages. However, macrophages contribution to cytokine induction cannot be eliminated. Future study on homogeneous cell populations is needed for further assessment. Furthermore, TLR8 expression along with NF-κB activation, and the TLR8-responding cells should be further characterized.

5.5 BMSCs stimulated by U266 CM induced MM associated chemokines

The myeloma cell line U266 secreted an unknown component capable of activating TLR7 and TLR8 as observed in NF-κB luciferase reporter assay (Figure 4.2). BMSCs responded upon stimulation with the TLR8 ligand CL075. Therefore, BMSCs were stimulated with U266 conditioned medium to assess the potential activation of BMSCs.

BMSCs stimulated with U266 CM up-regulated secretion of both CXCL1 and CXCL5 indicating a response towards U266 CM. A study conducted by Kaiser et al. [81] found elevated CXCL1 secretion from healthy BMSCs upon co-culture with myeloma cells, while another group reported of both induced CXCL1 and CXCL5 in co-cultures [15]. It is tempting to speculate if the interaction between U266 CM and BMSCs is mediated via TLR8. Further studies may use siRNA and knockout studies to investigate if the TLR-activating component in U266 CM activates BMSCs via TLR8. In addition, immune cells expressing TLR7 and TLR8, e.g. pDCs express TLR7 and are reported to be abundant in the bone marrow, hence, pDCs could be stimulated with U266 CM to study potential activation of pDCs and other TLR-expressing cells found in the BM microenvironment.

6 Conclusion

In this study, U266 conditioned medium was found to activate TLR7 and TLR8, which could potentially activate TLR-expressing cells in the bone marrow milieu. TLRs are a promising link between inflammation and tumorigenesis, since inflammation may promote MM progression. U266 CM was found to strongly activate TLR7 and less potently activate TLR8. The TLR-activating component was protected from RNase, likely by being bound to a protein or encapsulated in a vesicle. Extracellular vesicles isolated from U266 CM induced some TLR7 activation observed in response to U266 CM. Furthermore, primary MM-BMSCs secreted several cytokines in response to TLR activation. In particular, a strong expression was observed for TLR7/8 ligands. BMSCs were found to express TLR3, TLR4, and low levels of TLR8 mRNA. The results suggest BMSCs have an important role in the MM pathogenesis by secreting cytokines implicated in survival, proliferation, invasion and bone resorption. U266 CM was able to stimulate BMSCs to secrete chemokines implicated in tumor survival and progression. This activation may be mediated in part through TLR8. The observed response implies MM cells potential to alter the bone marrow, creating an inflammatory milieu optimized for tumor growth.

Taken together, the results indicate the ability of MM cells to shape its environment through TLRs and their inflammatory response. Further studies should focus on identifying the TLR-activating component and elucidate its implications in the bone marrow.

7 Literature

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Appendice

Appendix I: DuoSet® ELISA Development kit

Human CCL3/MIP-1α

Catalog number DY270

Materials provided with the kit

Capture Antibody (part 840313, 3 vials) – 72 ug/mL of mouse anti-human MIP-1α antibody when reconstituted with 1.0 mL PBS. Dilute to the working concentration of 0.4 ug/mL in PBS.

Detection Antibody (part 840314, 3 vials) – 36 ug/mL of biotinylated goat anti-human MIP-1α when reconstituted with 1.0 mL Reagent Diluent. Dilute to a working concentration of 200 ng/mL in Reagent Diluent.

Standard (part 840422, 3 vials) – 90 ng/mL of recombinant human MIP-1α when reconstituted with 0.5 mL deoinized or destilled water. A high standard of 500 pg/mL is recommended.

Human CXCL1/ GROα

Catalog number DY275-05

Materials provided with the kit

Capture Antibody (part 840255, 1 vial) – 480 ug/mL of mouse anti-human GROα antibody when reconstituted with 0.5 mL PBS. Dilute to the working concentration of 4.0 ug/mL in PBS.

Detection Antibody (part 840256, 1 vial) – 2.4 ug/mL of biotinylated goat anti-human GROα when reconstituted with 1.0 mL Reagent Diluent. Dilute to a working concentration of 40 ng/mL in Reagent Diluent.

Standard (part 840257, 1 vial) – 90 ng/mL of recombinant human GROα when reconstituted with 0.5 mL Reagent Diluent. A high standard of 2000 pg/mL is recommended.

Human CXCL5/ ENA-78

Catalog number DY274

Materials provided with the kit

Capture Antibody (part 840468, 1 vial) – 360 ug/mL of mouse anti-human ENA-78 antibody when reconstituted with 1.0 mL PBS. Dilute to the working concentration of 2.0 ug/mL in PBS.

Detection Antibody (part 840469, 1 vial) – 18 ug/mL of biotinylated goat anti-human ENA-78 when reconstituted with 1.0 mL Reagent Diluent. Dilute to a working concentration of 100 ng/mL in Reagent Diluent.

Standard (part 840470, 3 vials) – 90 ng/mL of recombinant human ENA-78 when reconstituted with 0.5 mL Reagent Diluent. A high standard of 1000 pg/mL is recommended.

Human IL-1β/IL-1F2

Catalog number DY201

Materials provided with the kit

Capture Antibody (part 840168, 3 vials) – 480 ug/mL of mouse anti-human IL-1β antibody when reconstituted with 0.5 mL PBS. Dilute to the working concentration of 4.0 ug/mL in PBS.

Detection Antibody (part 840169, 3 vials) – 12 ug/mL of biotinylated goat anti-human IL-1β when reconstituted with 1.0 mL Reagent Diluent. Dilute to a working concentration of 200 ng/mL in Reagent Diluent.

Standard (part 840170, 3 vials) – 90 ng/mL of recombinant human MIP-1α when reconstituted with 0.5 mL deoinized or destilled water. A high standard of 250 pg/mL is recommended.

Human TNF-α

Catalog number DY210

Materials provided with the kit

Capture Antibody (part 840119, 1 vial) – 720 ug/mL of mouse anti-human TNF-α antibody when reconstituted with 1.0 mL PBS. Dilute to the working concentration of 4.0 ug/mL in PBS.

Detection Antibody (part 840120, 1 vial) – 90 ug/mL of biotinylated goat anti-human TNF-α when reconstituted with 1.0 mL Reagent Diluent. Dilute to a working concentration of 500 ng/mL in Reagent Diluent.

Standard (part 840121, 3 vial) – 370 ng/mL of recombinant human TNF-α when reconstituted with 0.5 mL Reagent Diluent. A high standard of 1000 pg/mL is recommended.

Appendix II: IFN-β Verikine™ ELISA Assay Procedure

An extract from the VeriKine™ Human IFN Beta ELISA Kit (cat. no. 41410) protocol .

All reagents and incubations should be at room temperature.

1. Standards and test samples

Add 50 µl sample diluent to all wells, then add 50 µl of the prepared, diluted standard curve, blanks or test samples. Cover with plate sealer and incubate for 1 hour. After 1 hour, empty the wells and wash the wells three times with diluted wash buffer.

2. Antibody solution

Add 100 µl of diluted antibody solution to each well, cover the plate and incubate for 1 hour. After one hour, empty the wells and wash the wells three times with diluted wash buffer.

3. HRP

Add 100 µl of diluted HRP solution to each well, cover the plate and incubate for 1 hour. After one hour, empty the wells and wash the wells three times with diluted wash buffer.

4. TMB substrate

Add 100 µl of the TMB substrate solution to each well, do not cover the plate with tape, but incubate the plate in the dark for 15 minutes.

5. Stop solution

Add 100 µl of stop solution to each well.

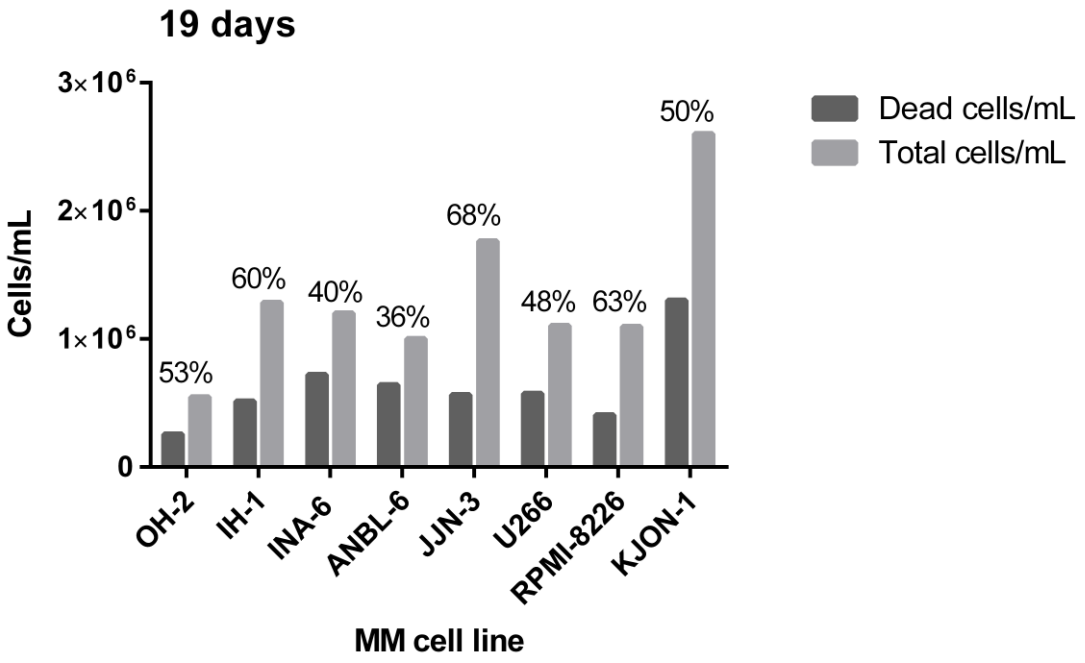
6. Read

Determine absorbance at 450 nm within 5 minutes after addition of the stop solution.

Appendix III: Luciferase reporter assay to determine the ability of conditioned media from MM cells to activate TLRs

Myeloma cell lines were cultured for a predetermined number of days before the conditioned medium were isolated. Cell viability and cell number was determined using the cell counter Countess™ using Trypan blue to eliminate non-viable cells.

Conditioned medium from long-term cultures were assayed for TLR activation using the NF-κB luciferase reporter gene assay. HEK293 cells were transiently transfected with TLR expression plasmids and reporter plasmids before stimulated for 48 hours in 37 ^{o}C/5% CO_2. NF-κB expression was assayed by measuring the luciferase activity. Results are presented as mean and standard deviation of duplicates.



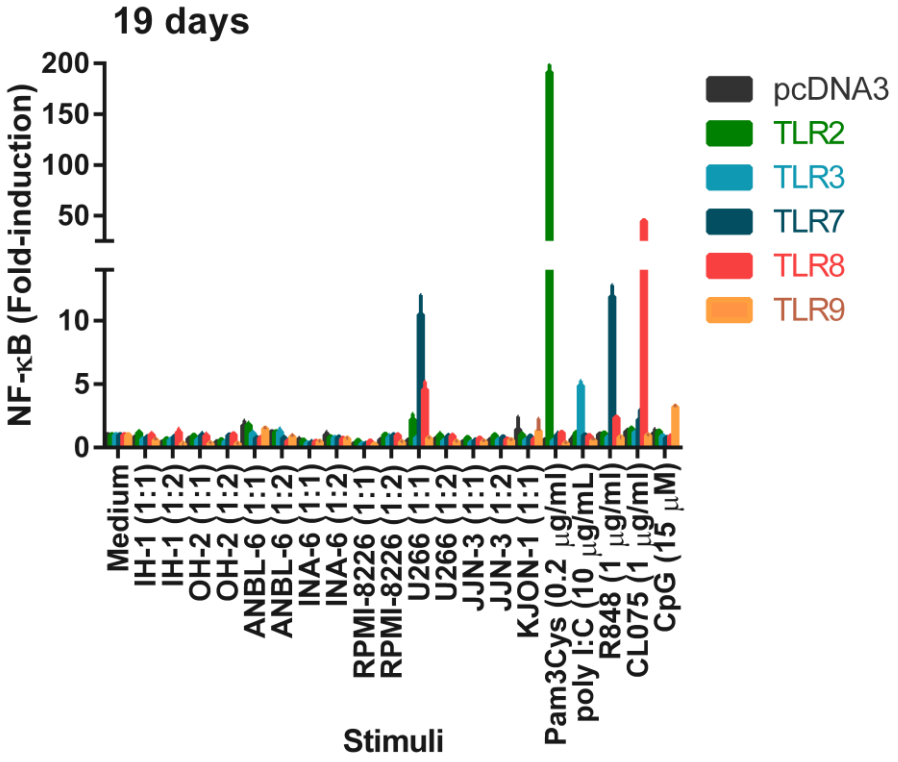


Figure 1. A) Viability data and B) NF-κB reporter assay results for 19 days MM conditioned medium.

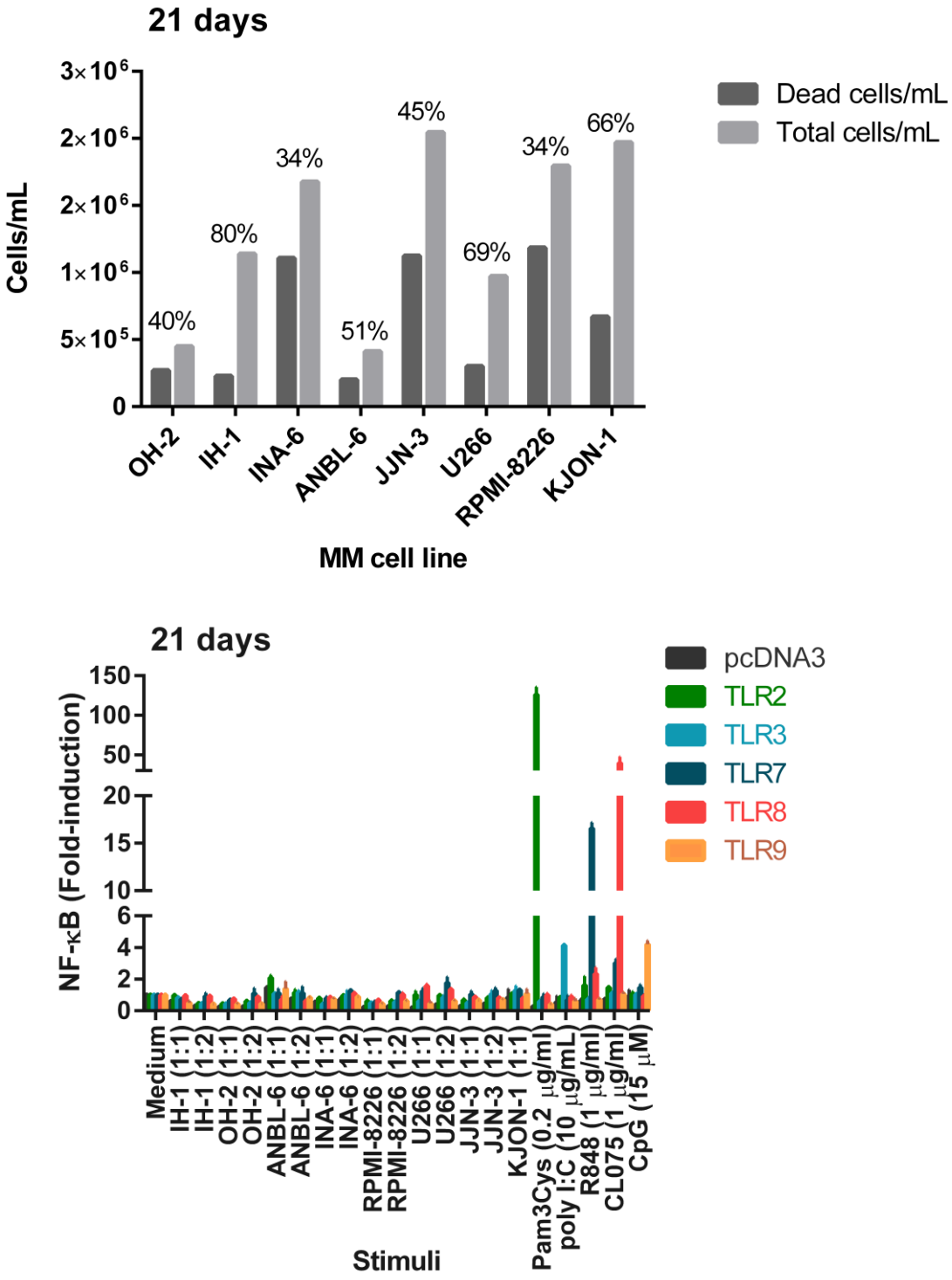


Figure 2. A) Viability data and B) NF-κB reporter assay results for 21 days MM conditioned medium.

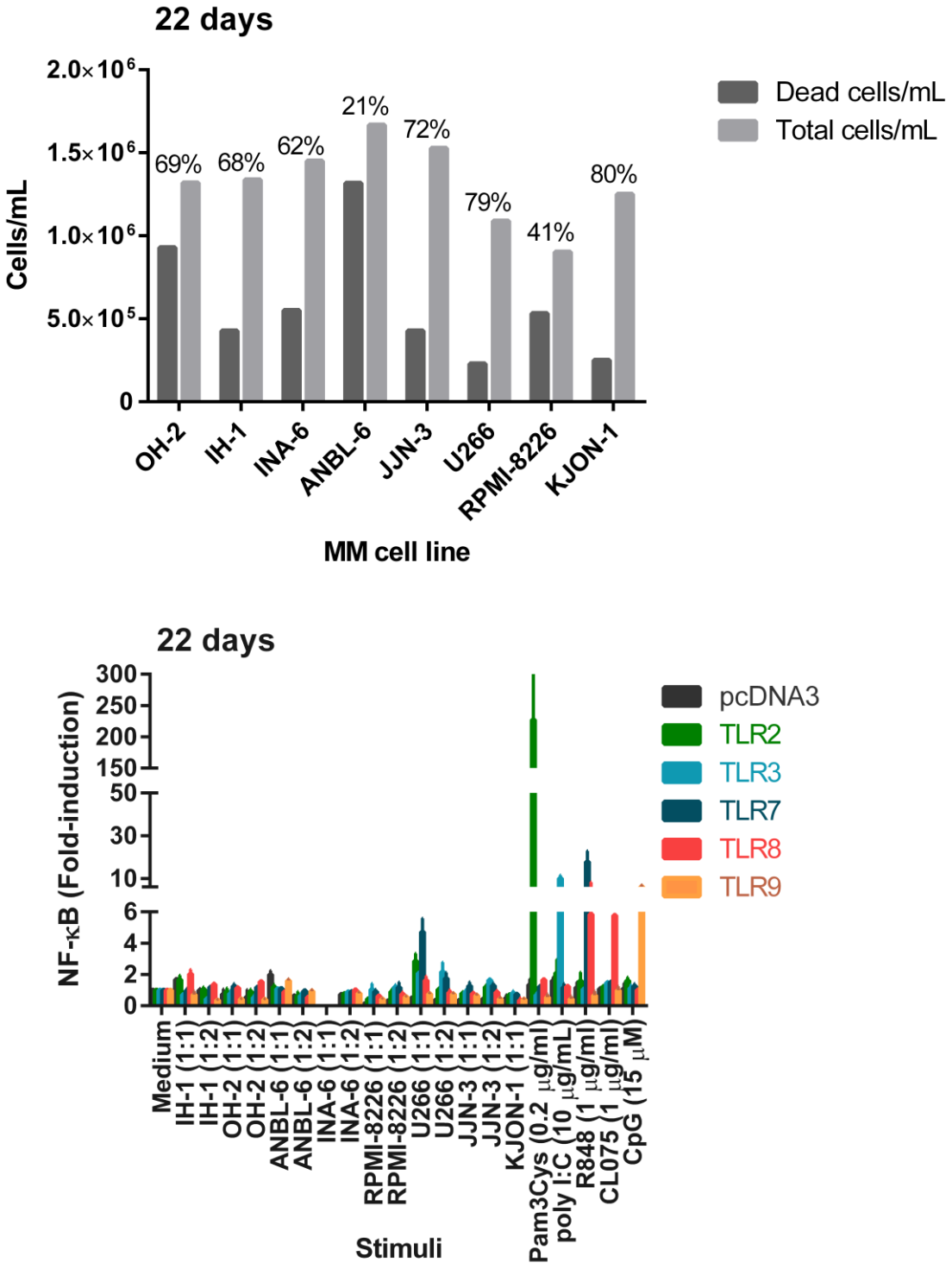
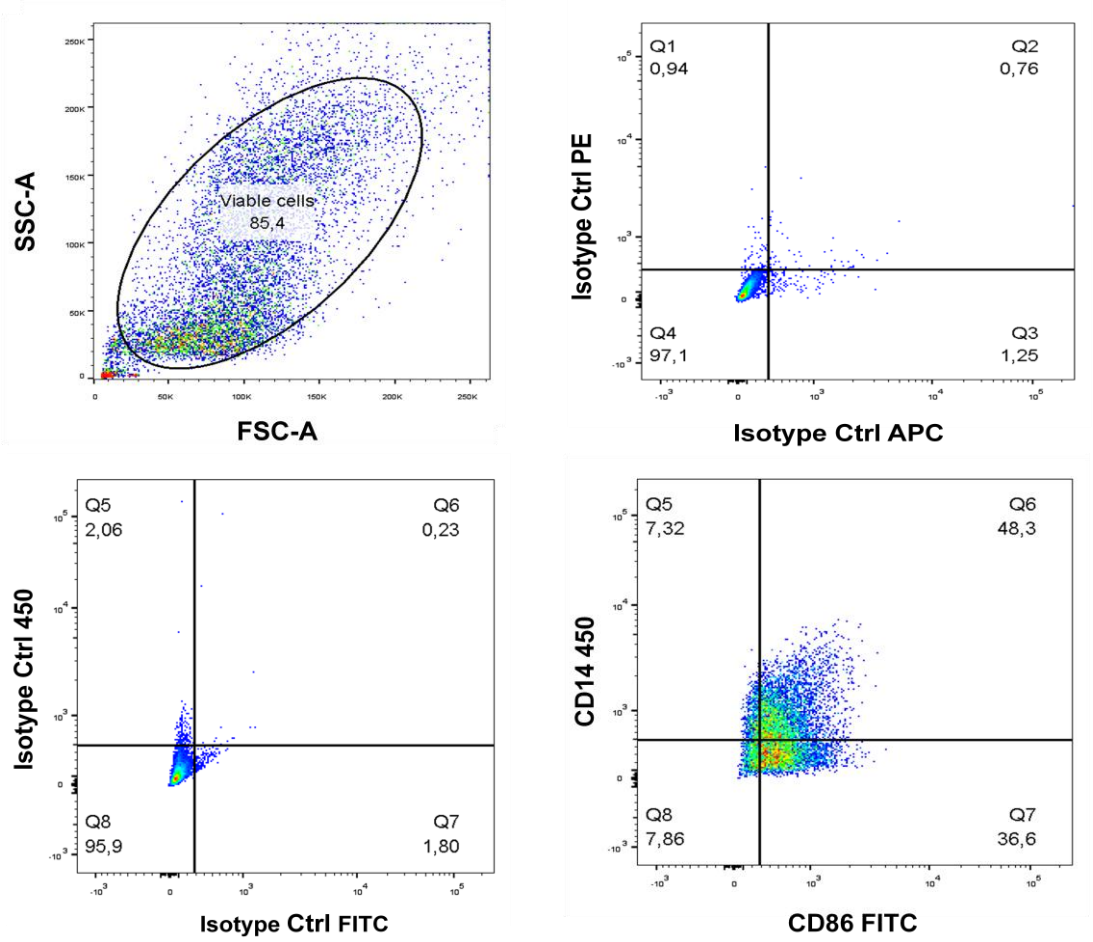


Figure 3. A) Viability data and B) NF-κB reporter assay results for 22 days MM conditioned medium.

Appendix IV: Characterization of macrophages and moDCs differentiated from PBMCs

The phenotypic profiles of differentiated macrophages and moDCs were analyzed by flow cytometry staining with fluorochrome-labeled antibodies against cellular markers. Macrophages are known to express CD14, CD86 and HLA-DR. The following antibodies were used for characterizing macrophages: CD14, CD86, HLA-DR and CD19. CD19 is a marker for B cell lineage while CD14, CD86 and HLA-DR are macrophage markers [107].

Based on the results, part of the cell population express CD14, CD86 and HLA-DR (Figure 4). Results show the majority of the population is either $CD86^{+}$ or $CD86^{+}CD14^{+}$, while a smaller part are positive for HLA-DR. None of the cells expresses CD19, eliminating B cells in the population. The results indicate a heterogeneous population exhibiting macrophage properties.



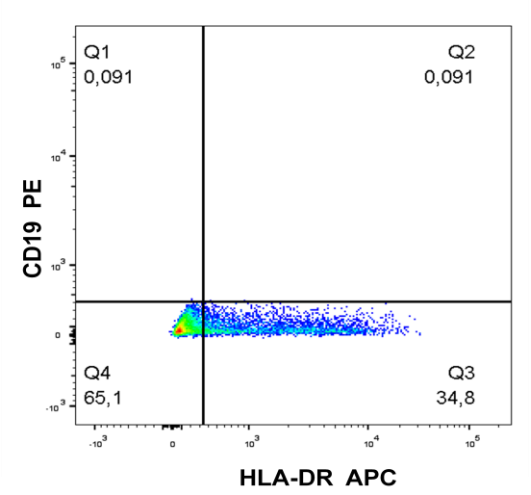


Figure 4. Characterization of differentiated macrophages. Macrophages were differentiated from PBMCs with 20% FCS/RPMI for 8 days. The cells were stained and assessed for expression of the following cellular markers; CD86, CD19, HLA-DR and CD14 using flow cytometry. Viable cells were gated and used for further analysis. Isotype control antibodies were used to determine negative staining allowing maximum 2% leakage. This gate was used to assess positive expression of the antibodies.

Markers for moDC were CD83, CD86, HLA-DR and CD206. In addition, these cells were also stained for CD14. CD83 is a marker for dendritic cells and activated B and T cells while CD206 is a marker for human monocytes derived dendritic cells. CD14 expression is lost upon differentiation from monocytes [68].

A fraction of the cell population expressed CD86, CD206 and HLA-DR, while none expressed CD83 (Figure 5). The cell population was negative for CD14 indicating absence of macrophages. A shift in CD83 expression in moDCs was observed compared to macrophages; although the shift was small in unstimulated cells.

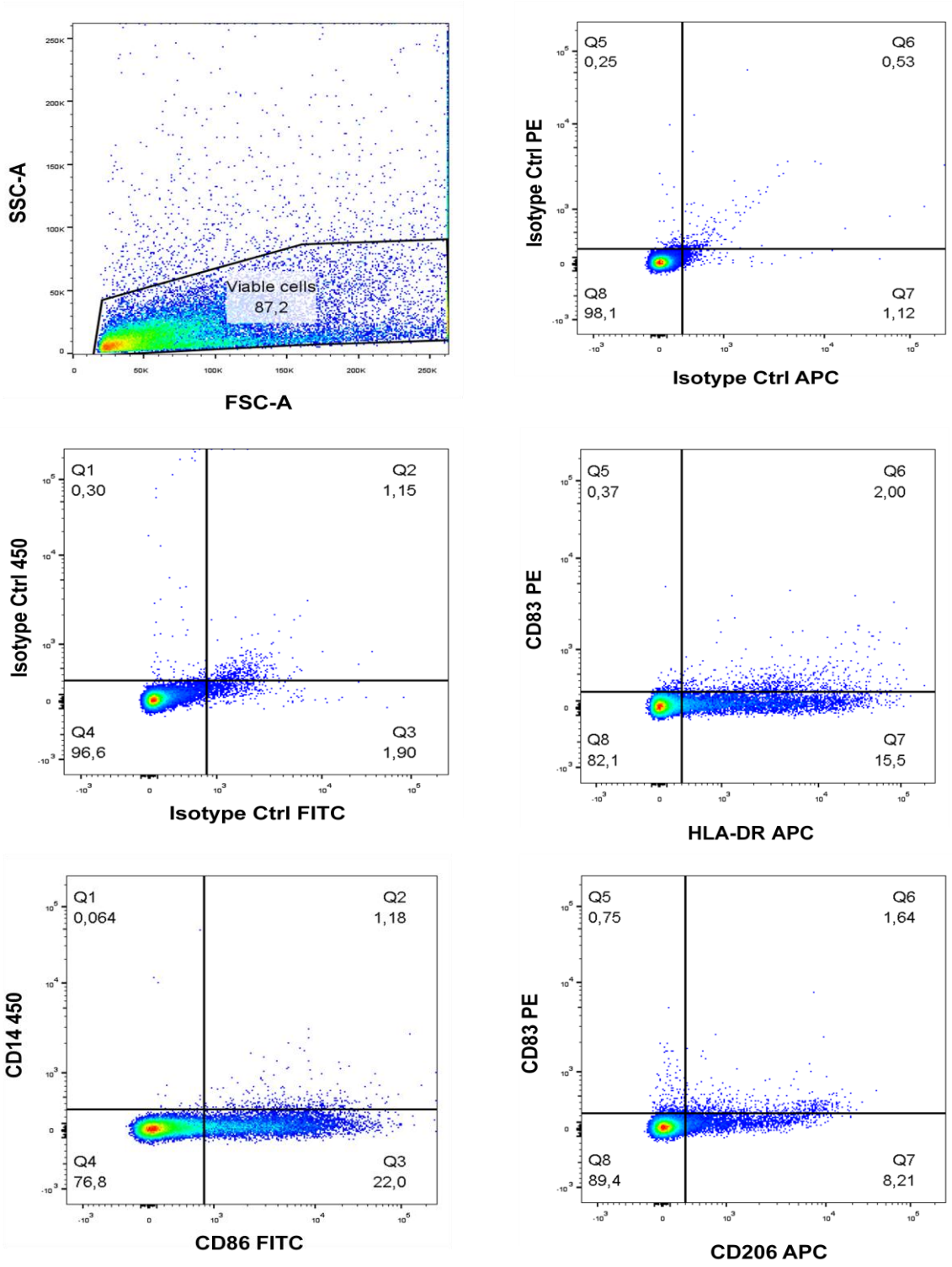


Figure 5. Characterization of monocyte-derived dendritic cells. MoDCs were differentiated from PBMCs with 10% FCS/RPMI with GM-CSF (100 ng/ml) and IL-4 (40 ng/ml) for 8 days. The cells were stained and assessed for expression of the following markers; CD86, CD83, CD14, HLA-DR and CD206 using flow cytometry. Gated viable cells were used for further analysis. Isotype control antibodies were used to determine negative expression allowing maximum 2% leakage.

Appendix V: Gene expression analysis using the nCounter Nanostring technology

The nCounter™ Gene expression assay was used to determine expression of genes in primary BMSCs upon stimulation with the TLR8 ligand CL075.

BMSC19 were either unstimulated or stimulated with CL075 (1 µg/ml) for 6 hours before RNA was isolated. Gene expressions were analyzed using a PanCancer Immune Profiling kit from Nanostring technologies. Data were normalized against 40 housekeeping genes and 6 positive controls before the data were analyzed. The highest value for a negative control was 1, so results above 1 was regarded as positive.

The results were analyzed using nSolver Analysis Software and are presented in table 1.

Table 1. Gene expression of BMSCs

Gene	Medium	CL075 (1 µg/ml)	Fold change
A2M	33	43	1,31
ABCB1	2	3	1,41
ABL1	700	752	1,07
ADA	74	53	0,73
ADORA2A	2	3	1,41
AICDA	2	3	1,41
AIRE	2	3	1,41
AKT3	391	361	0,92
ALCAM	1397	1437	1,03
AMBP	5	3	0,70
AMICA1	2	3	1,41
ANP32B	1051	992	0,94
ANXA1	18585	15328	0,82
APOE	339	344	1,01
APP	4835	4147	0,86
ARG1	2	3	1,41
ARG2	26	10	0,38
ATF1	107	120	1,13
ATF2	116	140	1,21
ATG10	36	47	1,31
ATG12	33	13	0,40
ATG16L1	116	107	0,92
ATG5	180	194	1,07

ATG7	114	80	0,70
ATM	2	3	1,41
AXL	2940	2670	0,91
BAGE	2	3	1,41
BATF	2	3	1,41
BAX	1727	1460	0,85
BCL10	1578	1537	0,97
BCL2	5	3	0,70
BCL2L1	754	675	0,89
BCL6	152	254	1,67
BID	152	254	1,67
BIRC5	899	932	1,04
BLK	17	13	0,80
BLNK	2	3	1,41
BMI1	373	311	0,83
BST1	228	160	0,70
BST2	114	70	0,62
BTK	9	3	0,35
BTLA	2	3	1,41
C1QA	36	23	0,66
C1QB	12	37	3,10
C1QBP	2337	2182	0,93
C1R	591	491	0,83
C1S	503	545	1,08
C2	2	3	1,41
C3	5	13	2,81
C3AR1	2	3	1,41
C4B	2	13	5,64
C4BPA	2	3	1,41
C5	5	7	1,41
C6	2	3	1,41
C7	2	7	2,82
C8A	2	3	1,41
C8B	2	3	1,41
C8G	14	3	0,23
C9	2	3	1,41
CAMP	2	3	1,41
CARD11	7	3	0,47
CARD9	14	3	0,23
CASP1	5	3	0,70
CASP10	2	3	1,41
CASP3	171	327	1,92
CASP8	107	90	0,84
CCL1	2	3	1,41

CCL11	2	3	1,41
CCL13	2	3	1,41
CCL14	2	3	1,41
CCL15	2	3	1,41
CCL16	2	3	1,41
CCL17	2	3	1,41
CCL18	2	3	1,41
CCL19	2	3	1,41
CCL2	617	7428	12,04
CCL20	21	505	23,63
CCL21	2	3	1,41
CCL22	2	3	1,41
CCL23	2	3	1,41
CCL24	2	3	1,41
CCL25	2	3	1,41
CCL26	349	271	0,78
CCL27	2	3	1,41
CCL28	2	3	1,41
CCL3	2	942	397,61
CCL3L1	2	728	307,38
CCL4	2	652	274,95
CCL5	2	53	22,56
CCL7	12	33	2,82
CCL8	2	10	4,23
CCND3	482	531	1,10
CCR1	2	3	1,41
CCR2	2	7	2,82
CCR3	2	3	1,41
CCR4	2	3	1,41
CCR5	2	3	1,41
CCR6	2	3	1,41
CCR7	2	3	1,41
CCR9	2	3	1,41
CCRL2	2	7	2,82
CD14	47	7	0,14
CD160	2	3	1,41
CD163	26	3	0,13
CD164	31	391	12,68
CD180	2	3	1,41
CD19	2	10	4,23
CD1A	2	3	1,41
CD1B	2	3	1,41
CD1C	2	3	1,41
CD1D	2	3	1,41

CD1E	2	3	1,41
CD2	2	3	1,41
CD200	43	13	0,31
CD207	2	3	1,41
CD209	2	3	1,41
CD22	2	3	1,41
CD24	2	3	1,41
CD244	2	3	1,41
CD247	2	3	1,41
CD27	2	3	1,41
CD274	21	30	1,41
CD276	1836	1778	0,97
CD28	2	3	1,41
CD33	2	3	1,41
CD34	2	3	1,41
CD36	173	100	0,58
CD37	2	3	1,41
CD38	2	3	1,41
CD3D	2	3	1,41
CD3E	2	3	1,41
CD3EAP	252	227	0,90
CD3G	2	3	1,41
CD4	21	20	0,94
CD40	33	87	2,62
CD40LG	2	3	1,41
CD44	7355	7482	1,02
CD45R0	2	3	1,41
CD46	603	498	0,83
CD47	937	899	0,96
CD48	2	33	14,10
CD5	2	10	4,23
CD53	21	30	1,41
CD55	2	3	1,41
CD58	97	177	1,82
CD59	4964	5651	1,14
CD6	2	3	1,41
CD63	30128	25995	0,86
CD68	484	414	0,86
CD7	2	3	1,41
CD70	2	3	1,41
CD74	50	77	1,54
CD79A	2	3	1,41
CD79B	2	3	1,41
CD80	2	3	1,41

CD81	9132	7599	0,83
CD83	36	154	4,32
CD84	9	3	0,35
CD86	2	3	1,41
CD8A	2	3	1,41
CD8B	2	7	2,82
CD9	550	595	1,08
CD96	2	3	1,41
CD97	1704	1377	0,81
CD99	6605	5457	0,83
CDH1	2	33	14,10
CDH5	7	3	0,47
CDK1	693	578	0,83
CDKN1A	1632	2122	1,30
CEACAM1	2	3	1,41
CEACAM6	2	3	1,41
CEACAM8	2	3	1,41
CEBPB	9	140	14,79
CFB	7	47	6,57
CFD	17	3	0,20
CFI	45	40	0,89
CFP	2	3	1,41
CHIT1	119	134	1,13
CHUK	308	261	0,85
CKLF	546	508	0,93
CLEC4A	21	3	0,16
CLEC4C	2	3	1,41
CLEC5A	2	3	1,41
CLEC6A	2	3	1,41
CLEC7A	2	3	1,41
CLU	28	20	0,70
CMA1	2	3	1,41
CMKLR1	47	17	0,35
COL3A1	27375	24668	0,90
COLEC12	40	27	0,66
CR1	2	3	1,41
CR2	2	3	1,41
CREB1	292	234	0,80
CREB5	40	77	1,91
CREBBP	2	3	1,41
CRP	2	3	1,41
CSF1	2	57	23,97
CSF1R	2	3	1,41
CSF2	2	77	32,43

CSF2RB	2	3	1,41
CSF3	2	3	1,41
CSF3R	2	3	1,41
CT45A1	2	3	1,41
CTAG1B	2	3	1,41
CTAGE1	2	3	1,41
CTCFL	2	3	1,41
CTLA4_all	2	3	1,41
CTSG	2	3	1,41
CTSH	26	17	0,64
CTSL	693	752	1,09
CTSS	43	43	1,02
CTSW	2	3	1,41
CX3CL1	2	3	1,41
CX3CR1	2	3	1,41
CXCL1	55	1089	19,96
CXCL10	2	3	1,41
CXCL11	2	3	1,41
CXCL12	85	77	0,90
CXCL13	2	3	1,41
CXCL14	2	3	1,41
CXCL16	17	23	1,41
CXCL2	5	67	14,07
CXCL3	38	565	14,88
CXCL5	95	257	2,71
CXCL6	330	922	2,80
CXCL9	2	3	1,41
CXCR1	2	3	1,41
CXCR2	2	3	1,41
CXCR3	2	3	1,41
CXCR4	9	3	0,35
CXCR5	2	3	1,41
CXCR6	2	3	1,41
CYBB	2	3	1,41
CYFIP2	14	13	0,94
CYLD	266	418	1,57
DDX43	2	3	1,41
DDX58	142	327	2,30
DEFB1	2	3	1,41
DMBT1	2	3	1,41
DOCK9	24	17	0,70
DPP4	93	70	0,76
DUSP4	88	27	0,30
DUSP6	408	454	1,11

EBI3	2	10	4,23
ECSIT	66	77	1,16
EGR1	19	84	4,40
EGR2	2	3	1,41
ELANE	2	3	1,41
ELK1	159	110	0,69
ENG	3065	2533	0,83
ENTPD1	2	3	1,41
EOMES	2	3	1,41
EP300	244	211	0,86
EPCAM	2	3	1,41
ETS1	225	421	1,87
EWSR1	894	936	1,05
F12	2	3	1,41
F13A1	2	3	1,41
F2RL1	83	60	0,72
FADD	389	418	1,07
FAS	66	70	1,06
FCER1A	2	3	1,41
FCER1G	40	80	1,99
FCER2	2	3	1,41
FCGR1A/B	2	3	1,41
FCGR2A	26	27	1,02
FCGR2B	2	7	2,82
FCGR3A	12	3	0,28
FEZ1	128	100	0,78
FLT3	2	3	1,41
FLT3LG	2	10	4,23
FN1	57750	54907	0,95
FOS	142	77	0,54
FOXJ1	2	3	1,41
FOXP3	2	3	1,41
FPR2	2	3	1,41
FUT5	2	3	1,41
FUT7	2	3	1,41
FYN	418	481	1,15
GAGE1	2	3	1,41
GATA3	2	3	1,41
GNLY	2	3	1,41
GPI	3049	2596	0,85
GTF3C1	289	317	1,10
GZMA	2	3	1,41
GZMB	2	3	1,41
GZMH	2	3	1,41

GZMK	2	3	1,41
GZMM	2	3	1,41
HAMP	2	3	1,41
HAVCR2	21	3	0,16
HCK	2	23	9,87
HLA-A	6389	7051	1,10
HLA-B	5203	5083	0,98
HLA-C	2190	2476	1,13
HLA-DMA	12	7	0,56
HLA-DMB	2	3	1,41
HLA-DOB	2	3	1,41
HLA-DPA1	28	13	0,47
HLA-DPB1	14	20	1,41
HLA-DQA1	2	3	1,41
HLA-DQB1	2	3	1,41
HLA-DRA	19	27	1,41
HLA-DRB3	28	53	1,88
HLA-DRB4	2	3	1,41
HLA-E	1300	1243	0,96
HLA-G	17	642	38,63
HMGB1	574	1063	1,85
HRAS	247	264	1,07
HSD11B1	5	3	0,70
ICAM1	28	391	13,73
ICAM2	38	40	1,06
ICAM3	332	314	0,95
ICAM4	2	3	1,41
ICOS	2	3	1,41
ICOSLG	2	3	1,41
IDO1	7	3	0,47
IFI16	451	541	1,20
IFI27	71	100	1,41
IFI35	12	37	3,10
IFIH1	2	33	14,10
IFIT1	31	100	3,25
IFIT2	5	30	6,33
IFITM1	26	107	4,10
IFITM2	1447	1360	0,94
IFNA1/13	2	3	1,41
IFNA17	2	3	1,41
IFNA2	2	3	1,41
IFNA7	2	3	1,41
IFNA8	2	3	1,41
IFNAR1	33	104	3,12

IFNAR2	199	351	1,76
IFNB1	2	3	1,41
IFNG	2	3	1,41
IFNGR1	356	458	1,29
IGF1R	218	184	0,84
IGF2R	327	378	1,15
IGLL1	2	3	1,41
IKBKB	185	134	0,72
IKBKE	81	97	1,20
IKBKG	346	331	0,96
IL10	2	3	1,41
IL10RA	2	7	2,82
IL11	202	177	0,88
IL11RA	59	40	0,68
IL12A	2	3	1,41
IL12B	2	3	1,41
IL12RB1	2	3	1,41
IL12RB2	2	3	1,41
IL13	2	3	1,41
IL13RA1	868	862	0,99
IL13RA2	50	50	1,01
IL15	17	57	3,42
IL15RA	5	43	9,15
IL16	7	3	0,47
IL17A	2	3	1,41
IL17B	2	3	1,41
IL17F	2	3	1,41
IL17RA	55	74	1,35
IL17RB	2	3	1,41
IL18	2	7	2,82
IL18R1	2	3	1,41
IL18RAP	2	3	1,41
IL19	2	3	1,41
IL1A	2	40	16,92
IL1B	2	1427	602,06
IL1R1	349	331	0,95
IL1R2	2	3	1,41
IL1RAP	123	90	0,73
IL1RAPL2	2	3	1,41
IL1RL1	45	20	0,44
IL1RL2	2	13	5,64
IL1RN	19	154	8,10
IL2	2	3	1,41
IL21	2	3	1,41

IL21R	2	3	1,41
IL22	2	3	1,41
IL22RA1	2	3	1,41
IL22RA2	2	3	1,41
IL23A	2	3	1,41
IL23R	2	3	1,41
IL24	2	3	1,41
IL25	2	3	1,41
IL26	14	13	0,94
IL27	2	3	1,41
IL28A	9	3	0,35
IL29	2	3	1,41
IL2RA	2	3	1,41
IL2RB	2	3	1,41
IL2RG	2	3	1,41
IL3	2	3	1,41
IL32	5	585	123,11
IL34	2	3	1,41
IL3RA	2	3	1,41
IL4	2	3	1,41
IL4R	66	77	1,16
IL5	2	3	1,41
IL5RA	2	3	1,41
IL6	541	1888	3,49
IL6R	2	3	1,41
IL6ST	1919	1988	1,04
IL7	2	3	1,41
IL7R	66	150	2,26
IL8	377	12748	33,79
IL9	2	3	1,41
ILF3	1369	1170	0,85
INPP5D	2	3	1,41
IRAK1	804	775	0,96
IRAK2	66	281	4,23
IRAK4	85	63	0,74
IRF1	119	301	2,54
IRF2	33	63	1,91
IRF3	93	147	1,59
IRF4	2	3	1,41
IRF5	2	3	1,41
IRF7	81	127	1,57
IRF8	2	3	1,41
IRGM	2	3	1,41
ISG15	154	184	1,19

ISG20	7	47	6,57
ITCH	354	317	0,90
ITGA1	173	157	0,91
ITGA2	819	916	1,12
ITGA2B	2	3	1,41
ITGA4	166	70	0,42
ITGA5	6997	6931	0,99
ITGA6	159	174	1,09
ITGAE	195	277	1,43
ITGAL	2	3	1,41
ITGAM	2	3	1,41
ITGAX	28	20	0,70
ITGB1	15944	16682	1,05
ITGB2	24	40	1,69
ITGB3	114	104	0,91
ITGB4	2	3	1,41
ITK	2	3	1,41
JAK1	1473	1233	0,84
JAK2	112	97	0,87
JAK3	19	27	1,41
JAM3	738	575	0,78
KIR3DL1	14	10	0,70
KIR3DL2	2	3	1,41
KIR3DL3	2	3	1,41
KIR_Activating_Subgroup_1	2	3	1,41
KIR_Activating_Subgroup_2	2	3	1,41
KIR_Inhibiting_Subgroup_1	2	3	1,41
KIR_Inhibiting_Subgroup_2	2	3	1,41
KIT	2	3	1,41
KLRB1	2	3	1,41
KLRC1	2	3	1,41
KLRC2	2	3	1,41
KLRD1	2	3	1,41
KLRF1	2	3	1,41
KLRG1	2	3	1,41
KLRK1	2	3	1,41
LAG3	2	3	1,41
LAIR2	2	3	1,41
LAMP1	5559	4825	0,87
LAMP2	750	886	1,18
LAMP3	2	3	1,41

LBP	2	3	1,41
LCK	2	3	1,41
LCN2	2	3	1,41
LCP1	62	47	0,76
LGALS3	930	966	1,04
LIF	135	444	3,29
LILRA1	2	3	1,41
LILRA4	2	3	1,41
LILRA5	2	3	1,41
LILRB1	2	3	1,41
LILRB2	12	23	1,97
LILRB3	2	3	1,41
LRP1	633	795	1,26
LRRN3	9	10	1,06
LTA	2	3	1,41
LTB	2	3	1,41
LTBR	1606	1233	0,77
LTF	7	3	0,47
LTK	2	3	1,41
LY86	2	13	5,64
LY9	2	3	1,41
LY96	95	120	1,27
LYN	38	84	2,20
MAF	12	3	0,28
MAGEA1	2	3	1,41
MAGEA12	2	3	1,41
MAGEA3	2	3	1,41
MAGEA4	2	3	1,41
MAGEB2	2	3	1,41
MAGEC1	2	3	1,41
MAGEC2	2	3	1,41
MAP2K1	652	612	0,94
MAP2K2	2126	1828	0,86
MAP2K4	100	120	1,21
MAP3K1	43	23	0,55
MAP3K5	40	50	1,24
MAP3K7	854	708	0,83
MAP4K2	47	37	0,77
MAPK1	698	478	0,69
MAPK11	19	30	1,58
MAPK14	332	321	0,97
MAPK3	494	468	0,95
MAPK8	323	314	0,97
MAPKAPK2	823	855	1,04

MARCO	2	3	1,41
MASP1	2	3	1,41
MASP2	9	3	0,35
MAVS	211	197	0,93
MBL2	2	3	1,41
MCAM	261	291	1,11
MEF2C	12	20	1,69
MEFV	2	3	1,41
MERTK	2	3	1,41
MFGE8	2567	2282	0,89
MICA	444	471	1,06
MICB	76	67	0,88
MIF	3656	4702	1,29
MME	112	114	1,02
MNX1	2	3	1,41
MPPED1	2	3	1,41
MR1	2	3	1,41
MRC1	17	3	0,20
MS4A1	2	3	1,41
MS4A2	2	3	1,41
MSR1	26	3	0,13
MST1R	2	3	1,41
MUC1	17	20	1,21
MX1	26	127	4,87
MYD88	339	297	0,88
NCAM1	5	3	0,70
NCF4	2	3	1,41
NCR1	2	3	1,41
NEFL	2	3	1,41
NFATC1	40	70	1,74
NFATC2	88	23	0,27
NFATC3	97	107	1,10
NFATC4	180	177	0,98
NFKB1	57	187	3,29
NFKB2	339	1086	3,20
NFKBIA	199	1885	9,46
NLRC5	36	77	2,16
NLRP3	2	27	11,28
NOD1	47	47	0,99
NOD2	2	3	1,41
NOS2A	2	3	1,41
NOTCH1	36	60	1,69
NRP1	2892	2115	0,73
NT5E	1331	1661	1,25

NUP107	299	291	0,97
OAS3	5	33	7,04
OSM	2	3	1,41
PASD1	2	3	1,41
PAX5	36	17	0,47
PBK	12	47	3,94
PDCD1	2	3	1,41
PDCD1LG2	76	194	2,55
PDGFC	688	812	1,18
PDGFRB	1039	745	0,72
PECAM1	2	20	8,46
PIK3CD	59	150	2,53
PIK3CG	2	3	1,41
PIN1	486	521	1,07
PLA2G1B	2	3	1,41
PLA2G6	9	3	0,35
PLAU	1229	942	0,77
PLAUR	807	1150	1,42
PMCH	2	3	1,41
PNMA1	479	424	0,89
POU2AF1	2	3	1,41
POU2F2	12	30	2,54
PPARG	24	7	0,28
PPBP	2	17	7,05
PRAME	2	3	1,41
PRF1	2	3	1,41
PRG2	2	3	1,41
PRKCD	114	107	0,94
PRKCE	17	27	1,61
PRM1	2	3	1,41
PSEN1	591	485	0,82
PSEN2	62	30	0,49
PSMB10	133	150	1,13
PSMB7	918	1216	1,32
PSMB8	159	134	0,84
PSMB9	62	154	2,49
PSMD7	1125	1069	0,95
PTGDR2	2	3	1,41
PTGS2	133	327	2,46
PVR	726	772	1,06
PYCARD	33	17	0,50
RAG1	21	13	0,63
REL	17	33	2,01
RELA	332	354	1,07

RELB	50	244	4,90
REPS1	263	160	0,61
RIPK2	190	271	1,43
ROPN1	2	3	1,41
RORA	52	63	1,22
RORC	2	3	1,41
RPS6	26158	22860	0,87
RRAD	147	364	2,48
RUNX1	114	144	1,26
RUNX3	52	43	0,83
S100A12	2	3	1,41
S100A7	2	3	1,41
S100A8	2	3	1,41
S100B	2	3	1,41
SAA1	138	144	1,04
SBNO2	256	261	1,02
SELE	2	3	1,41
SELL	2	3	1,41
SELPLG	5	20	4,22
SEMG1	2	3	1,41
SERPINB2	204	334	1,64
SERPING1	726	545	0,75
SH2B2	2	3	1,41
SH2D1A	2	3	1,41
SH2D1B	2	3	1,41
SIGIRR	2	3	1,41
SIGLEC1	2	3	1,41
SLAMF1	2	3	1,41
SLAMF6	2	3	1,41
SLAMF7	2	87	36,66
SLC11A1	7	13	1,88
SMAD2	28	84	2,93
SMAD3	280	304	1,09
SMPD3	2	3	1,41
SOCS1	17	20	1,21
SPA17	202	144	0,71
SPACA3	2	3	1,41
SPANXB1	2	3	1,41
SPINK5	2	3	1,41
SPN	2	3	1,41
SPO11	2	3	1,41
SPP1	1447	1076	0,74
SSX1	2	3	1,41
SSX4	2	3	1,41

ST6GAL1	2	30	12,69
STAT1	1044	909	0,87
STAT2	413	331	0,80
STAT3	883	902	1,02
STAT4	14	7	0,47
STAT5B	187	150	0,80
STAT6	1129	889	0,79
SYCP1	9	3	0,35
SYK	2	3	1,41
SYT17	2	3	1,41
TAB1	161	100	0,62
TAL1	2	3	1,41
TANK	104	144	1,38
TAP1	93	241	2,60
TAP2	278	368	1,32
TAPBP	491	739	1,50
TARP	9	3	0,35
TBK1	270	261	0,96
TBX21	2	3	1,41
TCF7	147	147	1,00
TFE3	282	284	1,01
TFEB	2	3	1,41
TFRC	1711	1567	0,92
TGFB1	3998	3840	0,96
TGFB2	119	94	0,79
THBD	24	3	0,14
THBS1	25921	20294	0,78
THY1	6565	5557	0,85
TICAM1	159	187	1,18
TICAM2	190	244	1,29
TIGIT	2	7	2,82
TIRAP	14	10	0,70
TLR1	14	3	0,23
TLR10	2	3	1,41
TLR2	14	3	0,23
TLR3	7	3	0,47
TLR4	268	284	1,06
TLR5	2	7	2,82
TLR6	9	10	1,06
TLR7	2	3	1,41
TLR8	2	3	1,41
TLR9	2	3	1,41
TMEFF2	2	3	1,41
TNF	2	785	331,35

TNFAIP3	610	2038	3,34
TNFRSF10B	800	999	1,25
TNFRSF10C	2	3	1,41
TNFRSF11A	24	3	0,14
TNFRSF11B	857	1140	1,33
TNFRSF12A	415	792	1,91
TNFRSF13B	2	3	1,41
TNFRSF13C	2	3	1,41
TNFRSF14	7	3	0,47
TNFRSF17	2	3	1,41
TNFRSF18	2	10	4,23
TNFRSF1A	2150	1885	0,88
TNFRSF1B	21	20	0,94
TNFRSF4	7	3	0,47
TNFRSF8	2	3	1,41
TNFRSF9	2	7	2,82
TNFSF10	2	3	1,41
TNFSF11	2	3	1,41
TNFSF12	157	140	0,90
TNFSF13	2	3	1,41
TNFSF13B	2	3	1,41
TNFSF14	2	3	1,41
TNFSF15	2	10	4,23
TNFSF18	2	3	1,41
TNFSF4	24	30	1,27
TNFSF8	2	3	1,41
TOLLIP	311	237	0,76
TP53	529	792	1,50
TPSAB1	2	3	1,41
TPTE	2	3	1,41
TRAF2	152	254	1,67
TRAF3	149	264	1,77
TRAF6	62	74	1,19
TREM1	26	3	0,13
TREM2	12	20	1,69
TTK	62	53	0,87
TXK	2	3	1,41
TXNIP	764	585	0,77
TYK2	228	174	0,76
UBC	17216	22506	1,31
ULBP2	45	84	1,85
USP9Y	2	3	1,41
VCAM1	242	1845	7,62
VEGFA	2093	1357	0,65

VEGFC	1284	1490	1,16
XCL2	2	3	1,41
XCR1	2	3	1,41
YTHDF2	71	144	2,02
ZAP70	2	3	1,41
ZNF205	52	37	0,70
NEG_A	1	1	
NEG_B	1	1	
NEG_C	1	1	
NEG_D	1	1	
NEG_E	1	1	
NEG_F	1	1	
NEG_G	1	1	
NEG_H	1	1	
POS_A	8257	5227	
POS_B	1057	859	
POS_C	332	234	
POS_D	251	151	
POS_E	26	21	
POS_F	4	3	