*Sveriges lantbruksuniversitet*
Fakulteten för Veterinärmedicin och husdjursvetenskap
Institutionen för biomedicin och veterinär folkälsovetenskap

# Inflammatory cytokines induced by Bovine Viral Diarrhoea Virus (BVDV) in Peripheral Blood Mononuclear Cell (PBMC) subsets

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“I declare that this work has been composed entirely by myself based on my own data. Where I have mentioned materials or results generated by fellow researchers, I have acknowledged them appropriately”

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## ABSTRACT

Bovine viral diarrhoea virus (BVDV) is the causative agent of a complex of disease syndromes in cattle with high economical and welfare impacts. BVDV occurs as two biotypes; cytopathic (BVDVcp) and noncytopathic (BVDVncp) determined by differential effects on cultured cells and can also be divided into two genotypes (BVDV1 and BVDV2) on the basis of genomic diversity. The interaction between BVDV and the host's immune system is regarded a key aspect in the sequel of BVDV infection. Infection with BVDV normally causes an acute transient infection, with mild to subclinical signs, but occasionally results in severe and even fatal disease. More importantly, BVDVncp virus can cause persistent infections, evading both the adaptive immunity as well as important mechanisms of the innate immunity. In the present study, attempts were made to compare the effect of a BVDVncp infection on bovine monocyte-derived dendritic (mDCs) cells on their ability to produce IFNα/β, IL-10 and IL-12. To potentially assess strain-variabilities, two different type 1 BVDVncp strains were used, one associated with mild acute disease and the other one with severe acute disease. This study confirms previously published data demonstrating that mDCs are susceptible to BVDVncp infection which implicates the potential of BVDVncp to affect the functions of this important antigen presenting cell. Unfortunately no conclusive data was obtained from quantitative polymerase chain reaction for mRNA expression levels of IL-10 and IL-12. Production of IFNα/β was measured in supernatants from stimulated mDCs and consistent with existing data no notable levels of IFNα/β could be detected. To further investigate how BVDVncp interact with the IFNα/β response in mDCs, protein levels of the transcription factors interferon regulatory factor 3 (IRF3) and interferon regulatory factor 7 (IRF7) were investigated by Western blotting. Here, the strain causing a more severe disease form seemed to be able to interfere with IRF3 protein levels differentially compared to the strain inducing a mild disease form. Thus, the data indicate that BVDVncp may interact in more than one way with the IFNα/β pathway, potentially by inhibiting IRF3 activity, and that these differences could be strain-specific and potentially linked to disease severity.

## SAMMANFATTNING

Bovint virus diarré virus (BVDV) orsakar ett komplext sjukdomssyndrom hos boskap världen över med stora ekonomiska förluster och negativ inverkan på djurvälfärden som följd. BVDV delas in i två biotyper, cytopatogen typ och icke-cytopatogen typ (BVDVcp/BVDVncp). Detta är grundat på virusets effekt på celler odlade i cellkultur. Viruset kan även delas in i genotyper, BVDV1 och BVDV2, baserat på genomisk diversitet. Interaktionen mellan BVDV och värdens immunförsvar är avgörande för infektionens slutresultat. BVDV orsakar vanligen en akut, övergående infektion med inga eller milda kliniska symtom men kan i vissa fall orsaka allvarlig sjukdom med dödlig utgång. BVDVncp kan även orsaka persisterande infektioner där viruset undviker upptäckt och eliminering av värdens immunförsvar. I denna studie utfördes försök för att undersöka effekterna av BVDVncp infektion på bovina monocytderiverade dendritiska celler (mDC) med avseende på produktion av IFNα/β, IL-10 och IL-12. För att utröna potentiella skillnader mellan stammar jämfördes två BVDV1ncp stammar, den ena associerad med milda sjukdomssytom vid akut infektion och den andra associerad med allvarliga akuta sjukdomssymtom. Denna studie konfirmerar tidigare publicerade data som visar att mDC är mottagliga för infektion av BVDVncp vilket innebär att viruset potentiellt sett skulle kunna påverka funktionen hos denna viktiga antigen presenterande cell. Olyckligtvis erhölls inga konklusiva data från kvantitativ PCR för IL-10 och IL-12. Produktion av IFNα/β undersöktes i supernatanter från stimulerade mDC och i linje med tidigare publicerade resultat kunde inget IFNα/β uppmätas. För att ytterligare utforska hur BVDVncp interfererar med produktionen av IFNα/β undersöktes proteinnivåer av transkriptionsfaktorerna interferon regulatory factor 3(IRF3) och 7 (IRF7) med Western blot. Resultaten visar att virusstammarna interagerar olika med IRF3 och att det virusisolat som förknippas med allvarliga sjukdomssymtom ger ökade proteinnivåer av IRF3 i mDC. Detta indikerar att BVDVncp kan interferera med IFNα/β-signalvägen på mer än ett sätt, potentiellt genom att blockera aktiviteten hos IRF3 och att dessa skillnader kan vara stamspecifika med en potentiell koppling till sjukdomsframkallande förmåga.

## ABBREVIATIONS

Ab- Antibody

ACD-buffer- Acid Citrate Dextrose

APC- Antigen Presenting Cell

B- cell- B lymphocyte

BVDV- Bovine Viral Diarrhoea Virus

BVDVcp- Bovine Viral Diarrhoea Virus cytopathogenic biotype

BVDVncp- Bovine Viral Diarrhoea Virus non cytopathogenic biotype

BDV- Border Disease Virus

BSA- Bovine Serum Albumine

CAT- Chloramphenicol Acetyl Transferase

CD- Cluster of Differentiation

cDC- conventional Dendritic Cell

cDNA- complementary Deoxyribonucleic Acid

CSFV- Classical Swine Fever Virus

Cp- Cytopathogenic

CpG- Cytosine-phosphodiester bond- Guanosine

CpG- ODN- Cytosine-phosphodiester bond- Guanosine Oligodeoxynucleotides

DC- Dendritic Cell

DMEM- Dulbecco's modified Earl's medium

DNA- Deoxyribonucleic Acid

dNTP- Deoxyribonucleotide Triphosphates

ds- double stranded

dsRNA- double stranded Ribonucleic Acid

DTT- dithiothreitol

eIF2- eukaryotic translation-Initiation Factor 2

ELISA- Enzyme Linked Immunosorbant assay

FACS- Fluorescent Activated Cell Sorting

FBS- Foetal Bovine Serum

FSC- Forward Scatter

GADPH- Glyceraldehyde-3-phosphate dehydrogenase

GI-tract- Gastro Intestinal tract

GM-CSF- Granulocyte-Macrophage Colony-Stimulating Factor

HCV- Hepatitis C virus

HS- Haemorrhagic Syndrome

IAH- Institute of Animal Health

IL-1β- Interleukin 1β

IL-4- Interleukin 4

IL-10- Interleukin 10

IL-12- Interleukin 12

IFA- Immunofluorescent assay

IFN- Interferon

IFNR-Interferon Receptor

IκB- Inhibitor of NFκB

IKK- Inhibitor of NFκB Kinase

IPX staining- Immunoperoxidase staining

IRAK- Interleukin-1 Receptor-Associated Kinase

IRF- Interferon Regulatory Factor

ISGF3- Interferon Stimulated Gene Factor 3

ISG- Interferon Stimulated Genes

ISP-1- Interferon-β Promoter Stimulator-1

ISRE- Interferon Stimulated Response Element

JAK1- Janus Kinase 1

LDL-R- Low Density Lipoprotein Receptor

MACS- Magnetic Activated Cell Sorting

MDA5- Melanoma-Differentiation-Associated gene 5

MDBK- Mardin-Darby Bovine Kidney cells

MDBKt2- Transfected Mardin-Darby Bovine Kidney cells

MEM- Modified Earl's Media

mDC- monocyte derived Dendritic Cell

MAPK- Mitogen Activated Protein Kinase

MD- Mucosal Disease

MHC- Major Histocompatibility Complex

mRNA- messenger Ribonucleic Acid

MOI- Multiplicity of Infection

Mx- Myxovirus resistance gene

Ncp- Non cytopathogenic

NFκB- Nuclear Factor kappa B

NK-cell- Natural Killer cell

NRS- Normal Rabbit Serum

OAS- 2'5'-Oligoadenylate Synthetase

OD- Optical Density

ODN- Oligodeoxynucleotides

ORF- Open Reading Frame

PAMP- Pathogen Associated Molecular Pattern

PBMC- Peripheral Blood Mononuclear Cell

PBS- Phosphate Buffered Saline

PBS-T- Phosphate Buffered Saline- Tween

PCR- Polymerase Chain Reaction

pDC- plasmacytoid Dendritic Cell

Pen/Strept- Penicillin/Streptomycin

P.I.- Post Infection

PI- Persistently Infected

PKR- Protein Kinase R

Poly(I:C)- Polyinosinic:polycytidylic acid

PRRs- Pattern Recognition Receptor

qPCR- quantitative Polymerase Chain Reaction

rbo- recombinant bovine

rhu- recombinant human

RIG-1- Retinoic-acid-inducible Gene I

RIP1- Receptor Interacting Protein 1

RNA- Ribonucleic Acid

RPLPO- Ribosomal Protein Large PO

RT- Room Temperature

RVC- Royal Veterinary College

SD- Standard Deviation

SDS-Sodium Dodecyl Sulfate

SDS-PAGE- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

ss- single stranded

SSC- Side Scatter

STAT- Signal Transducer and Activator of Transcription

T-cell- T lymphocyte

TBK1- TRAF-family-member-associated NFκB activator Binding Kinase 1

Th-cell- T helper cell

TIR-domain- Toll/Interleukin-1 Receptor domain

TNFα- Tumour Necrosis Factor α

TRAF6- Tumour necrosis factor Receptor-Associated Factor 6

TRIF- TIR domain-containing adapter inducing IFN

TLR- Toll-Like Receptor

TYK2- Tyrosine Kinase 2

UK- United Kingdom

USA- United Sates of America

5'UTR- 5' Untranslated Region

WB- Western Blot

WNV- West Nile Virus

# INTRODUCTION

## 1.1 Bovine viral diarrhoea virus (BVDV)

BVDV is an endemic viral disease affecting both economical and welfare aspects of the cattle industry globally. Despite major control efforts this disease continues to cause problems, mainly due to the ability of BVDV to cause persistent infections *(Houe 1999).* The way BVDV interacts with the innate immune system is critical both for the pathogenesis of the BVDV disease complex and for the development of immunity. Knowledge of these processes is absolutely vital when it comes to developing efficacious vaccines.

### *1.1.1 Classification and characterisation*

BVDV belongs to the genus *Pestivirus* within the family *Flaviviridae* (Table 1.1). BVDV has a single stranded positive sense ribonucleic acid (ssRNA) genome approximately 12.5 Kb in length with a single open reading frame (ORF). The genome is translated to a polyprotein which is cleaved into individual viral proteins by host cell and viral proteases (Figure 1.1) *(Meyers and Thiel 1996).* The virus replicates in the cytoplasm and obtains the outer envelope from host membranes before being released by exocytosis *(Ridpath and Goyal 2005).*

*Tabel 1.1 Taxonomy of the Flaviviridae family*

| Family | Genus | Virus of importance to veterinary medicine* | Virus of importance to human medicine* |
|---|---|---|---|
| Flaviviridae | | | |
| | Pestivirus | | |
| | | Bovine viral diarrhoea virus 1 (BVDV1) | |
| | | Bovine viral diarrhoea virus 2 (BVDV2) | |
| | | Classical swine fever virus (CSFV) | |
| | | Border disease virus (BDV) | |
| | Flavivirus | | |
| | | Louping ill virus | Tick borne encephalitis virus |
| | | West Nile virus (WNV) | Dengue virus |
| | | Japanese encephalitis | Yellow fever virus |
| | Hepacivirus | | |
| | | | Hepatitis C virus (HCV) |

*Taxonomy of the Flaviviridae family (adapted from the database of the International Committee on Taxonomy of Viruses http://www.ncbi.nlm.nih.gov/ICTVdb/). * Not a complete list for the genus Flavivirus which contains a number of arthropod borne viruses causing important diseases, many with zoonotic potential.*

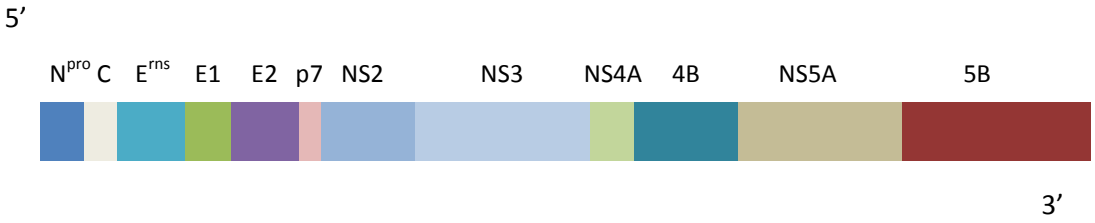



*Figure 1.1 Organisation of the BVDV genome (adapted from Meyers and Thiel 1996). The structural proteins building up the virion are the nuclocapsid protein C and the envelope associated glycoproteins $E^{rns}$, E1 and E2 (Meyers and Thiel 1996). E2 is the major target for neutralizing antibodies resulting in protective immunity (Bolin and Ridpath 1996 and Bruschke et al 1997). The nonstructural proteins governing viral functions are $N^{pro}$, P7, NS2-3, NS4A, NS4B, NS5A and NS5B. In the cytopathic biotype of BVDV NS2-3 is cleaved and NS3 is expressed alone at the same level as NS2-3. This makes NS3 a marker for BVDVcp (Meyers and Thiel 1996).*

The cellular receptor for BVDV is thought to be ubiquitously expressed, such as CD46 or the low density lipoprotein receptor (LDL-R), with uptake mediated by clathrin coated pits *(Agnello et al 1999, Maurer et al 2004, Blanchard et al 2006).*

#### *1.1.1.1 Genotypes*

Genomic and antigenic diversity is well documented among BVDV isolates. BVDV is divided into two genotypes; BVDV1 and BVDV2 on the basis of phylogenetic studies of the 5’ untranslated region (5’ UTR) (Figure 1.2) *(Ridpath et al 1994)*. BVDV1 is predominant in the UK but BVDV2 has been isolated *(Wakeley et al 2004).*

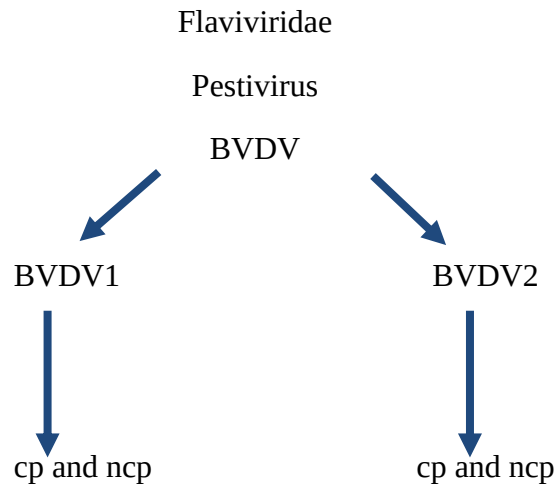



*Figure 1.2 Schematic illustration of the general categorisation of bovine viral diarrhea virus. BVDV can be subdivided into two genotypes; BVDV1 and BVDV2 (in each of these genotypes further subdivision can be made, 11 subgenotypes have recently been described within BVDV1 (Vilcek et al 2001). Regardless of genotype BVDV also exists in two different biotypes; non-cytopathogenic (ncp) or cytopathogenic (cp).*

#### *1.1.1.2 Biotypes*

All pestiviruses occur in two different biotypes depending on their ability to either induce visible cytopathic cell death or not: the former is the cytopathogenic biotype (BVDVcp) whilst the other is the non-cytopathogenic biotype (BVDVncp). The molecular basis remains to be elucidated. At the molecular level, BVDVcp but not BVDVncp cleaves NS2-3 to produce NS3 which can be used as a marker for infection *(Meyers and Thiel 1996, Kümmerer et al 2000).*

### *1.1.2 The Disease spectrum*

BVDV is responsible for a complex of diseases in cattle ranging from mild transient acute infections to fatal mucosal disease. The different aspects of the disease and the main concepts of the pathogenesis revealing the interesting nature of this virus have been reviewed by many *(Stöber 1984, Radostits and Littlejohns 1988, Brownlie 1991, Brownlie et al 2000).* Clinical disease can be divided into acute disease, *in utero* infection and mucosal disease (MD) (Figure 1.3).

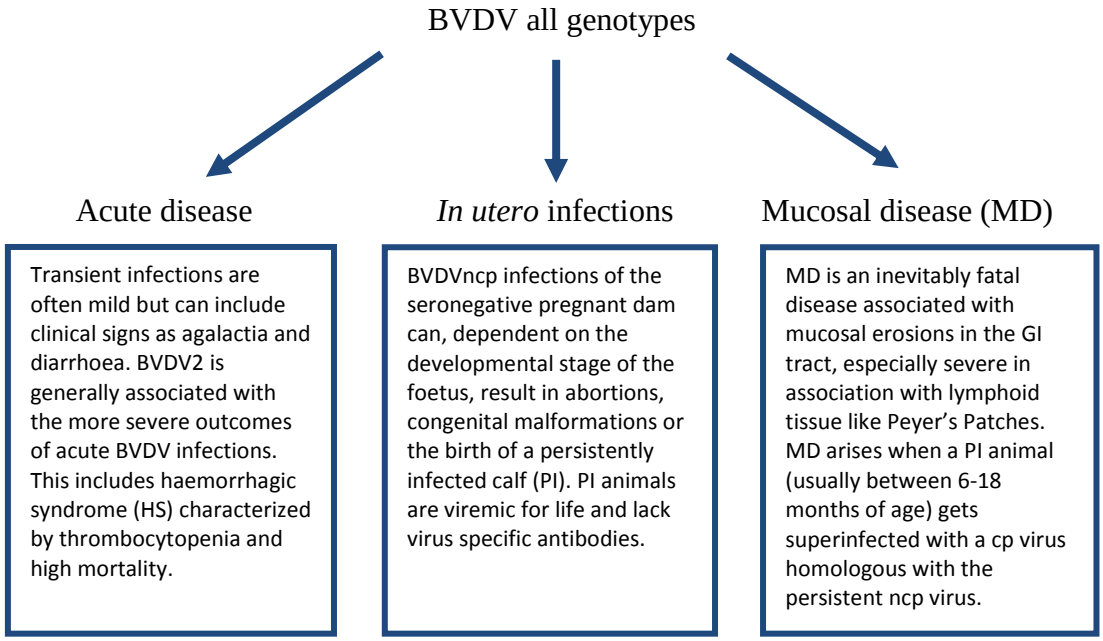



*Figure 1.3 Overview of the BVDV disease complex (Stöber 1984, Radostits and Littlejohns 1988, Brownlie 1991, Brownlie et al 2000)*

## 1.2 Recognition of viral infection by the innate immune system

One way in which viral infection can be detected by the innate immune system is through pattern recognition receptors (PRRs) recognizing pathogen associated molecular patterns (PAMPs). Recognition of PAMP by PRRs activates cellular signalling resulting in production of pro-inflammatory cytokines and interferon-α/β (IFNα/β also known as type I interferon) but also maturation of dendritic cells (DCs). PRRs can be classified into several families. One such family is the toll-like receptor (TLR) family which consists of membrane bound receptors primarily present in immune cells but also in other cell types like epithelial cells. Virus infection can also be sensed by cytosolic receptors such as the RIG-I-like family and the IFNα/β inducible protein protein kinase R (PKR) *(Haller et al 2007, Hornung et al 2008).*

### *1.2.1 TLRs*

Expression of TLRs varies dependent upon the cell subset and in some cases stimulation. Some TLRs are more ubiquitously expressed whereas other TLRs can be restricted to one cell type *(Janssens and Beyaert 2003).* Up to 14 TLRs *(Werling and Coffey 2007)* have been described in different species and those with known functions can be divided into two main groups; TLRs recognizing PAMPs made up by lipids and TLRs recognizing PAMPs made up by nucleic acid *(Brikos and O'Neill 2008).*

Viruses exploit the cellular machinery for their replication and dissemination thus lacking many PAMPs, therefore detection is dependent upon recognition of viral nucleic acids. So far TLR3, 7, 8 and 9 (Table 1.2) have been described to be involved and are strategically located on the endosomal membrane utilising a common route of viral infection through the endosomal pathway.

*Table 1.2 Toll-like receptors sensing viral nucleic acid*

| Toll like receptor | Ligand | General expression |
|---|---|---|
| TLR3 | Viral dsRNA and the synthetic dsRNA mimic Poly(I:C) | The cell membrane or endosomal membrane depending on cell type and species |
| TLR7 and TLR8 | ssRNA and synthetic imidazoquinoline-like molecules imiquimod (R-837) and resiquimod (R-848) | The endosomal membrane in immune cells |
| TLR9 | Unmethylated CpG sequences in DNA and synthetic CpG-ODN | The endosomal membrane in plasmacytoid dendritic cells, species dependent |

*Overview of toll like receptors (TLR) involved in viral recognition, their ligand specificity and general expression pattern in the human and murine system (Alexopoulou et al 2001,* Gorden *et al 2005, Kawai and Akira 2006, Barton 2007, Müller et al 2008) Poly (I:C): Polyinosinic:polycytidylic acid, CpG-ODN: Cytosine-phosphodiester bond- Guanosine Oligodeoxynucleotides*

TLR3 not only recognises viruses with a double stranded RNA (dsRNA) genome but also ssRNA viruses since dsRNA intermediates are produced during replication. TLR9 recognises unmethylated CpG sequences in DNA which occur in a much higher frequency in bacterial and viral DNA in comparison to vertebrate DNA. TLR7 and TLR8 are stimulated by ssRNA viruses but what motifs they recognise is unknown (Alexopoulou et al 2001, Kawai and Akira *2006, Barton 2007, Müller et al 2008).*

#### *1.2.1.1 TLR signalling*

Cellular signalling is generally very complex with different pathways being integrated or connected, therefore TLR signalling still contains aspects yet to be discovered, however this has been reviewed recently *(Kawai and Akira 2006, Brikos and O'Neill 2008, Randall and Goodbourn 2008).*

Ligand binding to its specific TLR induces receptor dimerisation bringing together a TLR/IL-1 (TIR) signalling domain capable of binding adaptor proteins MyD88 or TRIF relaying the signal intracellularly towards the nucleus, ultimately activating various transcription factors including NFkB, and ATF2/c-Jun complex (Figure 1.4)*(Brikos and O'Neill 2008)*. These transcription factors are central in regulating expression of pro-inflammatory cytokines such as IL-6, IL-1β, TNFα and IL-12. Signalling through TLRs also activate interferon regulatory factors (IRFs), mainly IRF3 and IRF7, which along with NFkB and ATF2/c-Jun regulate transcription of IFNα/β allowing an anti-viral state to be created *(Kawai and Akira 2006).* The TLRs' involvement in induction of IFNα/β expression and induction of DC maturation is of utmost importance in the antiviral immunity *(Parker et al 2007, Barton 2007).*

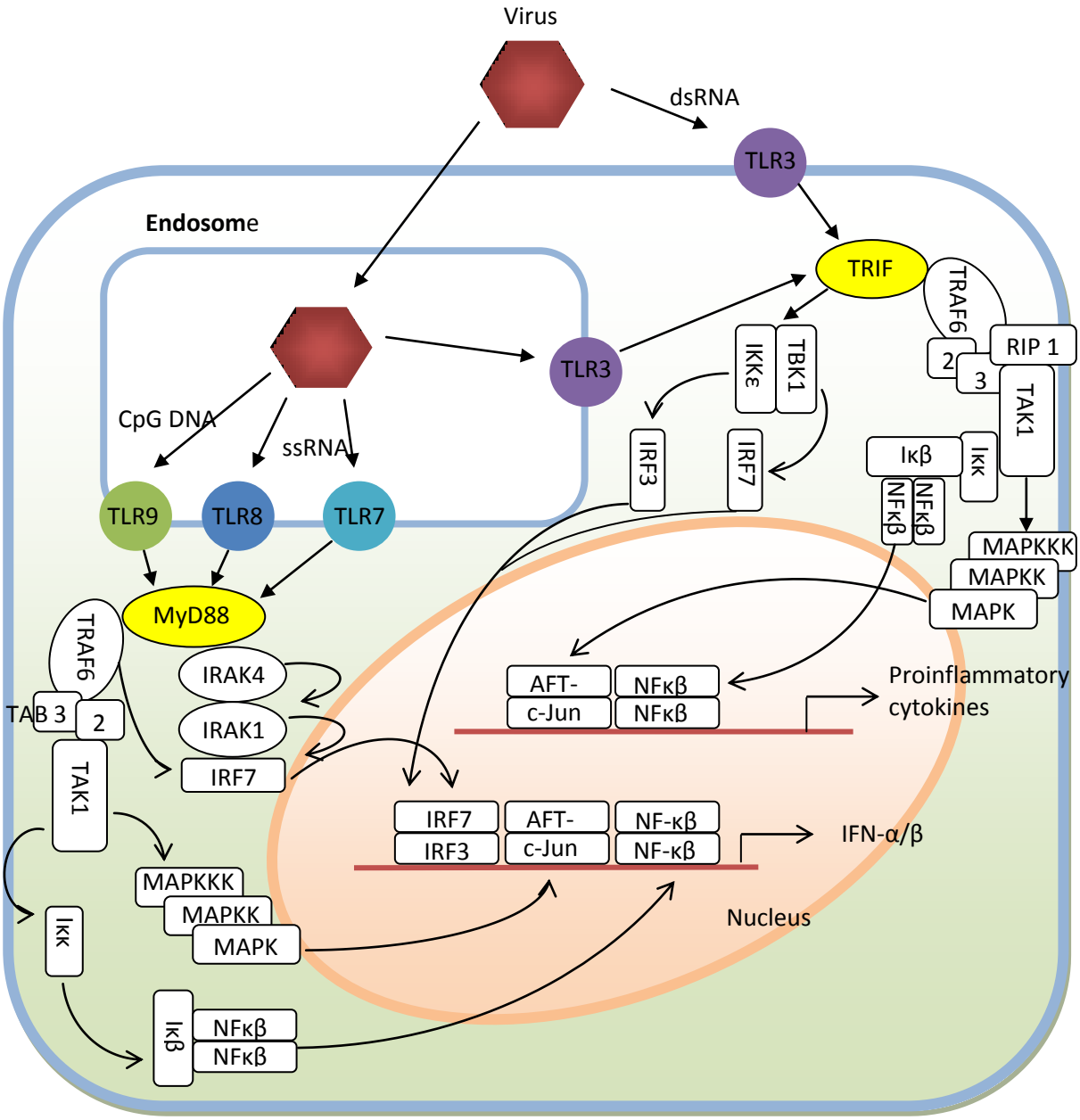



*Figure 1.4 Schematic representation showing an outline of TLR-signalling pathways (adjusted from Kawai and Akira 2006, Randall and Goodbourn 2008, Brikos and O'Neill 2008) MyD88 is used as an adaptor by the IL-1 receptor and most TLRs except TLR-3. Binding of MyD88 to the receptor leads to employment of interleukin-1 receptor-associated kinases (IRAKs) which bind tumour necrosis factor receptor-associated factor 6 (TRAF6) to the receptor signalling complex. IRAKs and TRAF-6 will activate interferon regulatory factor 7 (IRF7) which translocates to the nucleus to stimulate IFNα/β production. TRAF6 interacts with an additional complex consisting of TAK1-binding proteins (TAB2/3) and transforming growth factor-β-activated kinase 1 (TAK1). TAK-1 is the key kinase passing on the phosphorylation cascade to the inhibitor of NFκB kinase (IKK) complex. This complex phosphorylates inhibitor of NFκB (IκB) leading to degradation of this protein and dissociation from nuclear factor-kappaB (NFκB). Subsequently NFκB translocates to the nucleus and stimulates transcription of proinflammatory cytokines. In addition to activatining IKK TAK-1 also activates the mitogen activated protein kinase (MAPK) cascade which results in activation of AFT-2/c-Jun, transcription factors stimulating expression of IFNα/β and pro-inflammatory cytokines.*

*In TLR3 signalling the adaptor protein TIR domain-containing adapter inducing IFN (TRIF) is employed instead of MyD88. To transmit the stimulatory signal TRIF binds a kinase called TRAF-family-member-associated NFκB activator binding kinase 1 (TBK1). TBK1 activates interferon regulatory factor 3 (IRF3) and a homologue to IKK named IKKε. NFκB activation via TLR3 requires assembly of TRAF6 and receptor interacting protein 1 (RIP1) to recruit the IKK complex.*

### *1.2.2 Cytosolic receptors sensing dsRNA*

TLR expression is limited dependent upon cell type, however all cells have the ability to sense and respond to dsRNA via cytosolic receptors (Figure 1.5). RIG-1 and MDA5 are ubiquitously expressed receptors which activate NFkB and IRF3 and IRF7 upon binding dsRNA resulting in the transcription of IFNα/β and pro-inflammatory cytokines *(Meylan et al 2006, Randall and Goodbourn 2008).* Interferon inducible protein kinase R (PKR) is activated by dsRNA resulting in further amplifying IFNα/β production and the shut down of protein synthesis through the inactivation of protein translation initiation factor eIF2 *(Kumar et al 1994, Zamanian-Daryoush et al 2000).*

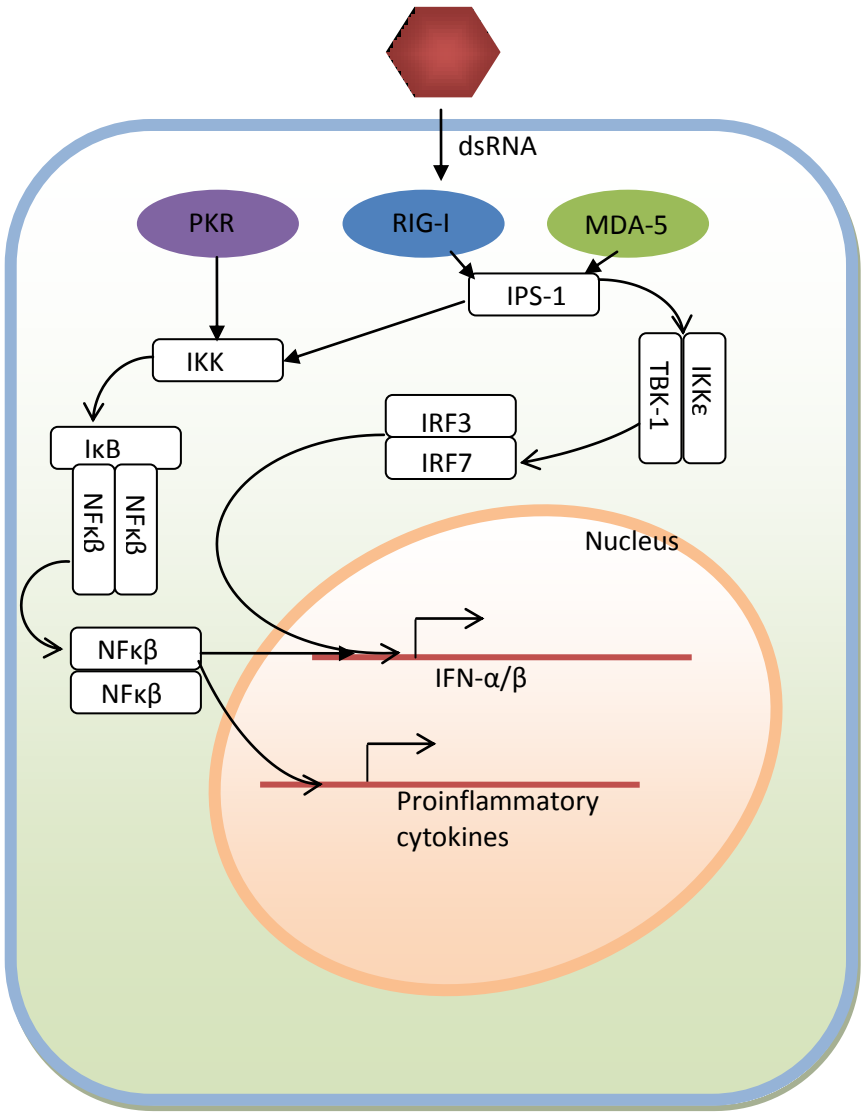



*Figure 1.5 Different intracellular receptors sensing dsRNA (adapted from Haller 2007). Retinoic-acid-inducible gene I (RIG-I)-I and melanoma-differentiation-associated gene 5 (MDA5) elicit activation of NFκB and IRF3 and IRF7 when stimulated by dsRNA. Signalling is similar to the pathway previously described for TLR3. Activated MAD5 and RIG-I activate TBK1 and IKKε via the adaptor protein interferon-β promoter stimulator-1 (ISP-1) (Kawai et al 2005, Meylan et al 2006). Protein kinase R (PKR) has been shown to activate NFκβ via activation of inhibitor of NFκB (IKK) which inactivates inhibitor of NFκB (IκB) (Kumar et al 1994, Zamanian-Daryoush et al 2000, Hovanessian 2007).*

### *1.2.3 The IFNα/β response*

IFNα/β is a group of cytokines integral to the anti-viral immune response capable of inducing an anti-viral state in an autocrine and paracrine manner signalling through IFNα/β receptor (Figure 1.6). As described above, all cell types can mount an IFNα/β response; however the exact mechanism of induction is diverse and dependent upon cell type and viral recognition receptors. IFNα/β anti-viral actions include activation of NK-cells and DCs, promotion of cross-presentation and production of IFNγ from DCs and T-cells, favouring a Th1-cell mediated immune response *(Haeryfar 2005).*

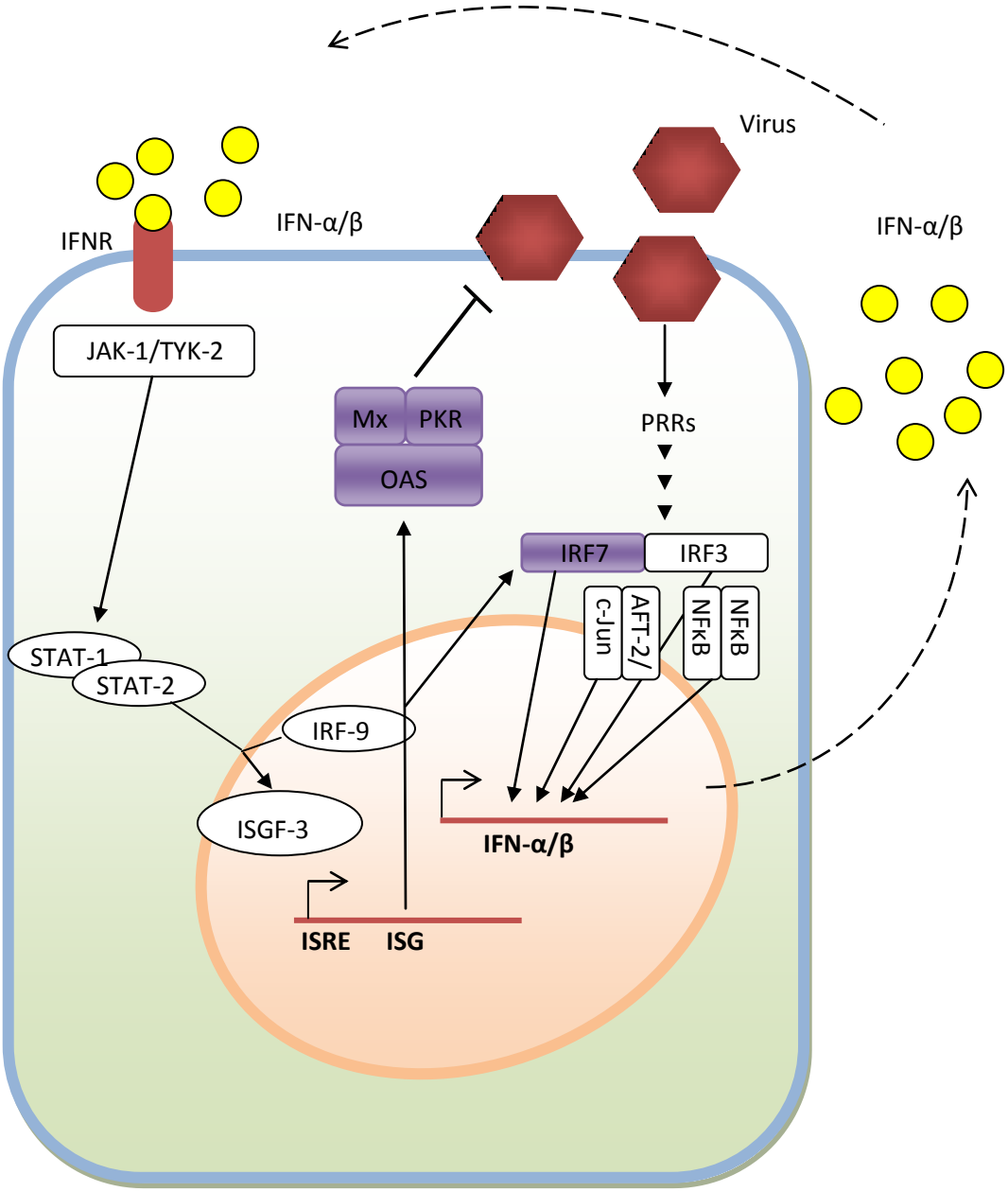



*Figure1.6 Schematic picture of IFN signalling (adapted from Haller et al 2007). Signalling via the IFNα/β receptor (IFNR) employs the JAK/STAT pathway. This pathway includes Janus kinase (JAK1), tyrosine kinase 2 (TYK2) and signal transducer and activator of transcription (STAT1 and STAT2). Activation of the receptor leads to dimerisation of STAT1 and STAT2 and subsequent translocation to the nucleus where the heterodimer interacts with interferon regulatory factor 9 (IRF9). The complex formed is called interferon stimulated gene factor 3 (ISGF3) and binds to interferon stimulated response element (ISRE) in promoters of IFN stimulated genes (ISG). The result is upregulation of hundreds of genes responsible for creating an antiviral sate in the cell. The functions of a few of these genes have been investigated in detail. Important mediators of the cellular antiviral response are protein kinase R (PKR), 2'5'-oligoadenylate synthetase (OAS) and the Mx protein. The result of IFNα/β stimulation includes shutting down cellular protein synthesis, arresting the cell cycle and establishing a pro-apoptotic state in the cell (Randall and Goodbourn 2008). In contrast to the*

*ubiquitously expressed IRF3, expression of IRF7 is normally very sparse (except in plasmacytoid dendritic cells also called natural interferon cells). IFNα/β upregulate IRF7 expression which amplifies the interferon response in a positive feedback loop (Paun and Pitha 2007).*

## 1.3 Dendritic cells (DCs)

DCs were first described in 1973 by Steinman and Cohnin. It is now clear that DCs compromise a very heterogeneous population of cells with different functions and specific expression patterns of cell surface markers. The bovine system does not seem to be an exception when it comes to heterogeneity in DC populations. At least two subsets of DCs that show functional differences *in vitro* have been differentiated in bovine afferent lymph *(Howard et al 1997, Howard et al 1999, Stephens et al 2003)*. All DCs are however derived from a common multipotent haematopoietic stem cell and can be generated from either myeloid or lymphoid precursors *(Shortman and Liu 2002, Fitzgerald-Bocarsly and Feng 2007)*. A broad, commonly encountered division of DC populations is conventional DCs (cDCs) versus plasmacytoid DCs (pDCs). The latter of these two is at present under characterisation in the bovine system *(unpublished data, A. Stalker personal communication)*. The major task of pDCs during viral infections is production of vast amounts IFNα/β and less so antigen presentation *(Carbone et al 2003, Haeryfar 2005).* The following general description of DC function applies to the cDC.

### *1.3.1 Functions of immature DCs- antigen uptake*

In their immature state DCs act as sentinels of the immune system. Strategically scattered in close proximity with epithelium outlining body surfaces they constantly sample the environment for antigen. DCs are extremely efficient in antigen uptake by a number of different mechanisms such as constitutive macropinocytosis *(Sallusto et al 1995)* (this has not been demonstrated in bovine monocyte derived DCs (mDCs) *(Werling et al 1999)*), receptor mediated endocytosis *(Sallusto and Lanzavecchia et al 1994, Arnold-Schild et al 1999)*, phagocytosis *(Reis e Sousa et al 1993)* and caveaolae formation *(Werling et al 1999)*. In addition to antigen uptake, innate properties of DCs include cytokine production and production of anti-microbial proteins and peptides such as defensins and members of the complement system. Production of interleukin-12 (IL-12) and IFNα/β provides innate resistance, activating natural killer cells (NK-cells) and IFNα/β, stimulating antiviral defenses. The mobilised NK-cells and the produced cytokines (e.g. IL-12 and IFNα/β) act back on the DCs to drive their maturation but also to influence the subsequent adaptive immune response *(Andoniou et al 2005, Steinmann and Hemmi 2006).*

### *1.3.2 Functions of mature DCs- antigen presentation*

Recognition of PAMPs via PRRs along with stimulation by pro-inflammatory cytokines (such as TNFα and IL-1β) leads to activation and maturation of DCs. During maturation DCs change from being a specialised phagocyte in the periphery to becoming a professional antigen presenting cell (APC) able to activate antigen specific T-cell clones in secondary lymphoid tissues. En route, DCs acquire the characteristic dendritic shape, increase expression of surface co-stimulatory molecules (such as CD40, CD80 and CD86), process and present

antigen on major histocompatibility complexes (MHC I and II) allowing efficient contact with, and activation of, naïve T-cells upon reaching the T-cell paracortex. Depending on the stimuli initiating DC maturation the DC will initiate different T-cell responses, in that way shaping the adaptive response to suit the specific pathogen *(Pulendran et al 2001).* The nature of the adaptive response is determined firstly by the peptide-MHC complex presented (endogenous antigen on MHC I and exogenous antigen on MHC II presented to $CD8^+$T-cells and $CD4^+$T-cells respectively) and secondly by the cytokines present in the microenvironment, for instance production of IL-12 and IFNγ will result in a Th1-biased cell mediated immune response *(Mellman and Steinman 2001, Steinmann and Hemmi 2006)*. Furthermore, DCs seem to be the cell type with the most evolved cross-presenting abilities *(Hotta et al 2005)*. Cross-presentation of antigen to CD8+T-cells is important when APCs are resistant to the infecting virus enabling extracellular antigen to be presented on MHC class I. This allows uninfected APCs to present antigen from engulfed virus infected cells on MHC class I stimulating $CD8^+$T-cells *(Brode and Macary 2004)*.

### *1.3.3 Bovine mDCs*

The most common procedure for generating DCs is culturing the easiest accessible DC precursor; the blood monocyte with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (IL-4) *(Romani et al 1994)*. This procedure has also been described for production of bovine mDCs *(Werling et al 1999)*. Bovine DCs derived from blood monocytes express moderate to high levels of costimulatory molecules and MHC class II molecules and have a down regulated expression of the myeloid cell differentiation molecule CD14, commonly used as a marker for monocytes *(Beekhuizen et al 1991, Bajer et al 2003,)*. Bovine mDCs provide a good tool for studying DC function *in vitro*.

## 1.4 BVDV and immunity

The interaction between BVDV and the host's immune system is a key feature in the BVDV disease complex. Establishment of persistent infections in the foetus is the most evident outcome of the ability of BVDV to subvert the immune system. Another effect of BVDV on the immune system is transient immunosuppression associated with acute infections. The mechanism behind this effect is under investigation, for a good review see *Chase et al 2004.* BVDV has been shown to infect many immune cells including T-cells, B-cells, monocytes, macrophages and DCs *(Sopp et al 1994, Glew et al 2003)*. Infecting APCs (macrophages, DCs and B-cells) has the potential to strongly affect the immune response since they have a key role in inducing and shaping the adaptive immune response.

So far most research has focused on how BVDV affects bovine monocyte and macrophage function. BVDV has been shown to alter function of TLRs, expression of cytokines and costimulatory molecules in bovine monocytes and macrophages *(Franchini et al 2006, Lee et al 2007)* resulting in an adverse affect on their ability to stimulate Th-cells *(Glew et al 2003)*. No impairment of APC-function has however been demonstrated for BVDV infected DCs.

### *1.4.1 In Utero Infections and Generation of PIs*

Both BVDV biotypes interact differently with the immune system, with the most documented distinction being the capacity to stimulate IFNα/β production (Table 1.3).

*Table 1.3 IFNα/β production in response to BVDV infection*

| BVDV biotype | Effect on type I interferon production |
|---|---|
| Cytopathogenic | IFNα/β↑*in vitro* and *in vivo* including the early foetus |
| Non-cytopathogenic | IFNα/β↓*in vitro* in most cell types and *in vivo* in the early foetus<br>IFNα/β↑*in vivo* apart from in the early foetus |

*Differential effects of BVDVcp and BVDVncp on IFNα/β production (adapted from Chase et al 2004). In vitro studies have shown that BVDVcp but not BVDVncp induces IFNα/β production (Adler et al 1997, Peterhans et al 2003, Glew et al 2003). It has also been demonstrated that BVDVcp induces IFNα/β production in the early foetus whereas BVDVncp does not (Charlston et al 2001) thus, the incapability of cp virus to establish persistent infections. Infection of immunocompetent calves with BVDVncp on the other hand results in production of IFNα/β (Charleston et al 2002, Müller-Doblies et al 2004). This difference in interferon response to BVDVncp infection in the foetus and the immunocompetent calf may be dependent on the developmental stage of the innate immune system. The cellular source of IFNα/β in BVDV infections could be the bovine equivalent to plasmacytoid dendritic cells which have not yet been fully characterized in cattle. Identification of this interferon producing cell explains why BVDVncp does not induce any detectable interferon response in other cell types in vitro in contrast to the interferon levels detected in vivo following infection with BVDVncp (Brackenbury et al 2005).*

Unlike BVDVcp, BVDVncp evades the IFNα/β response in the early foetus enabling the establishment of persistent infection (PI). Both biotypes can cross the placenta to initiate foetal infection; however the outcome is dependent upon both the developmental stage of the foetus and the biotype (Table 1.4).

*Table 1.4 Outcomes of in utero infections with BVDVncp*

| Time of infection | Consequence |
|---|---|
| First trimester (0 to 110 days) | Abortions, foetal mummification, congenital damage in organs with active cell division at the time of infection (lungs, skin, eyes, thymus and CNS are the organs most commonly affected)<br>Establishment of persistent infections |
| Second trimester (110 to 180/200 days) | Foetal loss and congenital damage |
| Third trimester (200 and onwards) | The immune system is mature enough to mount a protective response |

*Outcomes of in utero infections with BVDVncp (adapted from Brownlie et al 2000)*

Whereas BVDVcp infection seems to be cleared by the foetus, BVDVncp infection of a seronegative dam results in a high frequency of abortions, congenital damage and if infection occurs in the first trimester might result in the birth of a PI animal *(Brownlie 1989, Brownlie 1991)*. At this stage, the immune system of the foetus is not yet developed and cannot clear the virus, resulting in tolerance to the infecting virus. PI animals are the main source of virus within a herd, constantly excreting large amounts of virus, creating an important transmission route during movement *(Brownlie et al 1986, Houe 1999)*. A further complication with PI animals is the development of inevitably fatal Mucosal Disease (MD) associated with severe lesions within the mucosa of the gastrointestinal tract. Experimentally, MD can be induced in PIs from seronegative dams infected with BVDVncp in the first trimester and subsequently superinfected with antigenically homologous BVDVcp or through spontaneous conversion of BVDVncp to BVDVcp *(Brownlie et al 1984, Bolin. et al 1985, Brownlie 1991, Brownlie and Clarke 1993)*. The exact pathogenesis behind lesions observed in MD and the mechanisms for conversion of BVDVncp to BVDVcp is largely unknown.

### *1.4.2 Acute BVDV Infections*

Acute infections can be caused by both biotypes of BVDV where the outcome is highly variable mostly due to virulence of the individual strain (Table 1.5).

*Table 1.5 Outcomes of acute transient BVDV infections*

| Virulence | Clinical signs and findings | Viral spread |
|---|---|---|
| Low virulence | No evident clinical signs<br>Mild pyrexia, mild leucopenia and decreased milk production | Virus mainly confined to lymphoid organs but also detected in the intestinal mucosa |
| High virulence | Severe pyrexia, anorexia, depression, diarrhoea, occulo-nasal discharge, respiratory distress, haemorrhage, severe lymphopenia and thrombocytopenia, high mortality in all age groups<br>Lesions include cell depletion in lymphoid organs, epithelial necrosis in the GI tract and pneumonia | Predominantly in lymphoid tissues, mucosa of the GI tract, respiratory tract, endocrine organs, bone marrow but eventually general spread to most organs |

*Overview of the most frequently seen outcomes of acute BVDV infections caused by strains of high and low virulence. (Development of clinical signs and distribution of the virus have been documented in field cases and experimental infections Pritchard et al 1989, Sol et al 1989, Wilhelmsen et al 1990, Hibberd et al 1993, David et al 1994,* Marshall et al 1996, *Spagnuolo-Weaver et al 1997, Bruschke et al 1998, Carman et al 1998, Odeón et al 1999, Hamers et al 2000, Stoffregen et al 2000, Muskens et al 2004, Dabak et al 2007) Hypervirulent strains predominantly belong to the BVDV2 genotype and are largely confined to North America (Pellerin et al 1994). However all BVDV2 strains are not hypervirulent (Ridpath et al 2000) and outbreaks of severe acute BVDV infections have also been caused by strains identified as BVDV1 (Hibbered et al 1993, Muskens et al 2004 and Hamers et al 2000) Both low and high virulent strains initially replicates in the nasal mucosa (Bruschke et al 1998) followed by spread of the virus to various tissues. Strains of high virulence show a more widespread distribution than low virulent strains. Severe clinical signs are correlated with a higher level of viremia (Walz 2001) but specific virulence factors for BVDV have not yet been characterized. The pathogenesis behind the lymphocyte depletion, thrombocytopenia and mucosal lesions is unknown.*

Most acute infections are mild or subclinical; however the incidence of severe acute BVDV infections is increasing *(Pritchard et al 1989, Sol et al 1989, Hibberd et al 1993, David et al 1994, Carman et al 1998, Muskens et al 2004).* Mild infections should not be neglected, BVDV infections are suspected to cause immunosuppressive effects *(Brownlie 1991)* and if concurrent with insemination, result in reduced conception and pregnancy rates in heifers *(McGowan et al 1993).* Acute infections are cleared however, with protective antibodies generally being detected 2-3 weeks post infection for which CD4+T-cells are required *(Howard et al 1992).* Adaptive immune responses to BVDV also differs between biotypes, with BVDVncp inducing a stronger, faster humoral Th2-biased response

than BVDVcp which mostly results in a Th1-biased, cell mediated immune response *(Lambot et al 1997, Rhodes et al 1999, Collen et al 2000).*

## 1.5 Aims and hypothesis of the project

Until recently most research has focused on the differences between BVDVncp and BVDVcp, however the increase in incidence of severe type 1 BVDVncp infections asks for more knowledge about BVDVncp strain virulence. Knowledge about how ncp strains of different virulence interact with the immune system may explain how BVDVncp infections can result in such an array of diverse syndromes as persistent infection, subclinical transient infections and fatal disease.

The aim of this project is to compare cytokine production from bovine mDCs stimulated with the BVDVncp type 1 strains, Ho916ncp and Ky1203ncp. Ho916ncp was originally isolated from a severe fatal outbreak of BVDV in a dairy herd 1993 *(Hibberd et al 1993).* Experimental infection of gnotobiotic calves with Ho916ncp resulted in prolonged viremia (more than 2 weeks, to be compared with 7-10 days for mild transient infections) and marked thrombocytopenia which is rare for type 1 isolates *(unpublished data, J. Brownlie, personal communication).* In contrast to Ho916ncp which can be described as causing acute severe disease, Ky1203ncp (originally isolated from a field case of MD) is associated with mild acute disease *(J. Brownlie personal communication).*

The hypothesis is that these two ncpBVDV type 1 strains associated with different clinical outcomes also will differ in the cytokine response they induce from mDCs.

## MATERIAL AND METHODS

### 2.1 Experimental outline

Peripheral blood mononuclear cells (PBMCs) were isolated from 3 animals. CD14+ cells were enriched by magnetic cell sorting (MACS) and cultured in the presence of recombinant bovine (rbo) IL-4 and rboGM-CSF for 6 days to differentiate into mDCs (Figure 2.1). mDCs were infected with virus strains Ho916ncp and Ky1203ncp, respective mock and control ligands. After 48 hrs cells were harvested, isolating supernatants and cell lysates. To analyse supernatants for IFNα/β production a chloramphenicol acetyltransferase (CAT) enzyme reporter gene assay was employed. Protein and RNA were extracted from cell lysates and cDNA synthesised to asses expression levels of IL-10 and IL-12 by quantitative polymerase chain reaction (qPCR). Protein extracts were analysed by Western blotting for IRF3 and 7. In addition, the ability of the BVDV strains to infect mDCs was examined by immunoperoxidase (IPX) staining. Unstimulated mDCs and transfected Mardin-Darby bovine kidney cells (MDBKt2) cells were screened for BVDV and TLR-expression by reverse transcriptase PCR. Expression of CD14 was assessed by flow cytometry on isolated CD14+ cells and compared with the same cells after 6 days culturing with rboIL-4 and rboGM-CSF. All cell culturing and virus work was carried out in class II safety hoods.

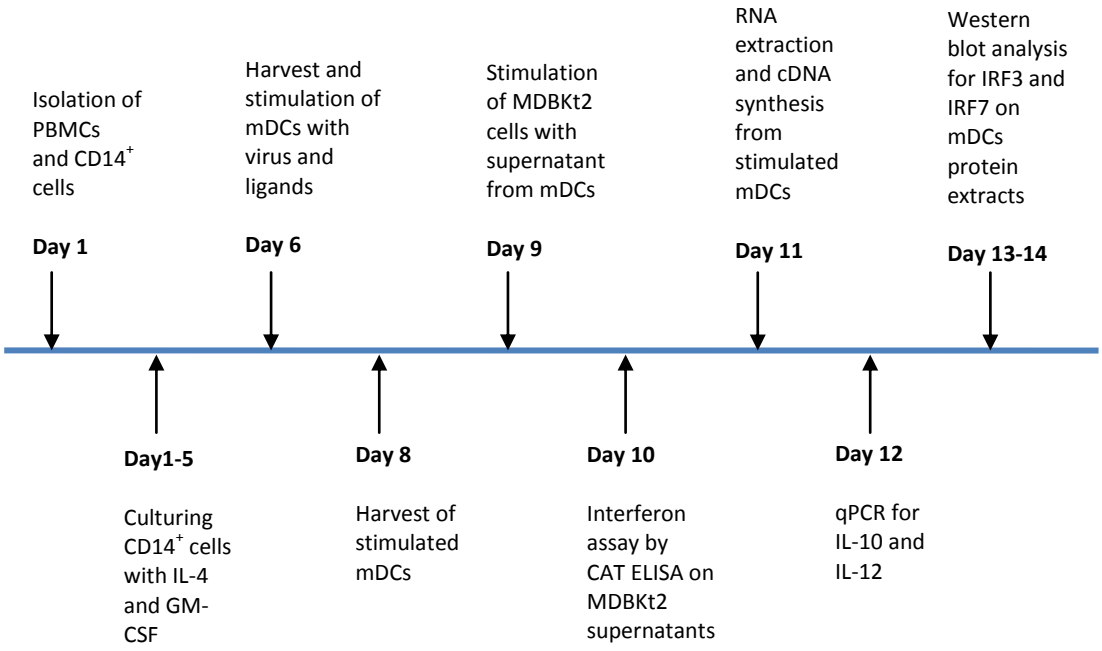



*Figure 2.1 Experimental outline and timeline showing the hypothetical minimal time required for processing and analyzing one set of samples. Due to a number of practical issues like limited access to lab facilities and restricted time points for bleeding animals this hypothetical timeline could not be followed. After day 8, harvesting of the stimulated mDCs cell lysates and supernatants were stored at -20° until following processing of samples could be performed. mDCs were isolated from 3 animals and stimulated with virus but due to the time limit only the interferon assay was carried out for all 3. Western blot and qPCR analysis were only completed for 2 animals.*

## 2.2 Animals and blood collection

Blood was collected from 3 Holstein-Friesian bullocks between 6 and 8 months of age housed at Bolton Farm at the Royal Veterinary College, UK. All animals were tested for BVDV antibodies and presence of viral nucleic acid by the Veterinary Laboratories Agency, UK. Blood was collected from the jugular vein into sterile glass bottles using 10% acid citrate dextrose (ACD) buffer as anticoagulant (Appendix 1).

## 2.3 mDCs

### *2.3.1 Isolation of monocytes and generation of mDCs*

PBMCs were isolated from whole blood as previously described *(Werling et al 1999)* with minor modifications. Blood was added to 50ml Falcon tubes and buffy coat isolated by centrifugation (1500 g, 20 min). The buffy coat was washed with phosphate buffered saline (PBS) (Sigma Aldrich, UK) (300 g for 10 min at 4°C). Red blood cells were lysed by incubation in ammonium-chloride lysis buffer for 10 min (Appendix 1) and white blood cells washed three times with PBS (600 g for 10 min at 4°C). The cell pellet was resuspended in 30 ml PBS and under-laid with 15 ml Histopaque (density=1.083 g $ml^{-1}$, Sigma Aldrich, UK). After 45 min centrifugation at 1200 g at room temperature (RT) PBMCs were isolated from the interface layer. After two more washes in PBS the PBMCs were counted and their viability assessed by Tryphan Blue exclusion (0.1% Sigma Aldrich).

To isolate monocytes, PBMCs were incubated with mouse anti human CD14 coated paramagnetic microbeads and separated using a MidiMacs column (Miltenyi Biotech, Germany) according to manufacturer's instructions. To generate DCs, $CD14^+$ enriched cells were cultured at 2 x $10^6$ cells per well in 6 well low-binding plates (Corning, Sigma Aldrich, UK) using mDC media (Appendix 1) containing rboIL-4 and rboGM-CSF *(Glew et al 2003, Werling et al 2006)*. Cells were cultured for 6 days, and every second day 2 ml media was replaced. On day 6, media was removed and mDCs harvested using 1 ml Accutase (PAA, Austria) per well to dislodge cells from the plastic.

### *2.3.2 Flow cytometric analysis of CD14 expression on monocytes and mDCs*

Monocytes and mDCs were adjusted to a concentration of 8 x $10^6$cells $ml^{-1}$ in PBS 1%BSA 0.01%$NaN_3$ (Appendix 1). 25 µl of the cell suspension was added to labeled wells on a 96 well U-bottom plate (Greiner, Germany). After 2 min centrifugation 170 g for 2 min at 4°C, discarding supernatant the cells were resuspended in 25 µl sterile filtered goat serum (Sigma-Aldrich, UK) and incubated 10 min at RT. Cells were stained with 25 µl FITC-conjugated mouse antihuman CD14 TÜK4 clone antibody (Ab) (Serotec, UK) diluted 1:10 in PBS 1%BSA 0.01%$NaN_3$. 25 µl PBS 1%BSA 0.01%$NaN_3$ was added to negative control wells. After 15 min incubation in darkness cells were centrifuged as above before resuspended in 400 µl PBS and analysed by a BD FACSAria Flow Cytometer (Immunocytometry Systems, BD Biosciences, UK). Generated data was further analyzed in FlowJo (Version 7.2.1 TreeStar, USA).

## 2.4 Susceptibility of mDCs to BVDV infection

### *2.4.1 Infection of mDCs with BVDV*

mDCs cultured in 24 well plates (Greiner, Germany) at 1 x $10^5$ cells per well in mDC media were infected with the BVDV strains Ho916ncp and Ky1203ncp at a multiplicity of infection (MOI) of 0.1. The virus stocks were kindly supplied by J. Brownlie, RVC, UK, grown and titred as described *(Brownlie et al 1984)* with a determined titre of 3.65 x $10^5$ $TCID_{50}$ $ml^{-1}$ and 1.12 x $10^6$ $TCID_{50}$ $ml^{-1}$ respectively. BVDV strain NADL was used as a positive control to generate distinctive IPX staining results. The NADL BVDV strain as well as MDBK-cells were included as positive controls for staining procedures and infectivity assays, respectively *(Deregt D. and Prins S. 1998)*. MDBK-cells were cultured to 80% confluency in MDBK growth media (Appendix 1) at 37˚C, 5% $CO_2$ and in MDBK assay media (Appendix 1) at 1 x $10^5$ per well for the infection assay. Infected cells (summarized in Table 2.1) were incubated 24 hrs at 37˚C, 5% $CO_2$.

*Table 2.1 Conditions used to study susceptibility of mDCs to Ho916ncp and Ky1203ncp*

| Cell type | Virus |
|---|---|
| mDC | - |
| mDC | NADL |
| mDC | Ho916ncp |
| MDBK | Ho916ncp |
| mDC | Ky1203ncp |
| MDBK | Ky1203ncp |

*Each condition was set up in duplicate. The virus strains were separated on different plates to minimize the contamination risk. This experiment was set up in 3 individual repeats with mDCs isolated from different animals.*

### *2.4.2 IPX staining*

IPX staining was used to identify BVDV infected cells by light microscopy (Ward and Kaeberle 1984). Cells were fixed in 80% ice cold acetone (BDH, VWR, UK) for 30 min at -20˚C. IPX staining was performed incubating cells with hyperimmune BVDV serum (V182, J. Brownlie), diluted 1:100 in PBS-Tween (PBS-T) 5% normal rabbit serum (NRS) (Appendix 1) for 20 min at 37˚C, followed by four times washing in PBS-T. Thereafter, cells were incubated with anti-bovine horseradish peroxidase conjungate Ab (Sigma Aldrich, UK; diluted 1:2000 in PBS-T 5% NRS) as described above. Cells were washed as described, and Ab binding was visualised by incubation with di-amino benzidine (DAB) substrate (Sigma Aldrich, UK) in darkness for 20-30 min. Cells were examined using an inverted microscope. Brown, cytoplasmic staining indicated presence of BVDV.

## 2.5 Stimulation of IFNα/β production in mDCs

mDCs were plated at 5 x $10^5$ cells per well in flat bottom 96well plate (Greiner, Germany) in mDC media (Appendix 1). Plates were incubated at 37˚C, 5% $CO_2$ to let cells adhere before stimulated with ligands, 4 wells per ligand (Table 2.2, Figure 2.2).

*Table 2.2 Stimulation ligands*

| Ligand | Sequence/Details | Concentration |
|---|---|---|
| Poly(I:C) | polyinosinic: polycytidylic acid | 10 µg ml-1 |
| CpG 2336 | G*G*GGACGACGTCGTGG*G*G*G*G*G | 50 µg ml-1 |
| CpG 2243 | G*G*G*GGAGCATGCTGG*G*G*G*G*G | 50 µg ml-1 |
| BVDV Ho916ncp | Ho916ncp infected MDBK cell lysate supernatants | MOI of 0.1 |
| Mock Ho916ncp | Mock infected MDBK cell lysate supernatants | MOI of 0.1 |
| BVDV Ky1203ncp | Ky1203ncp infected MDBK cell lysate supernatants | MOI of 0.1 |
| Mock Ky1203ncp | Mock infected MDBK cell lysate supernatants | MOI of 0.1 |

*Ligands used to stimulate mDCs. * Denotes phosphorothioate bond. Poly(I:C) from Alexis Biochemical, UK and CpG from Coley Pharmacueticals, USA. CpG 2243 is a scrambled control sequence.*

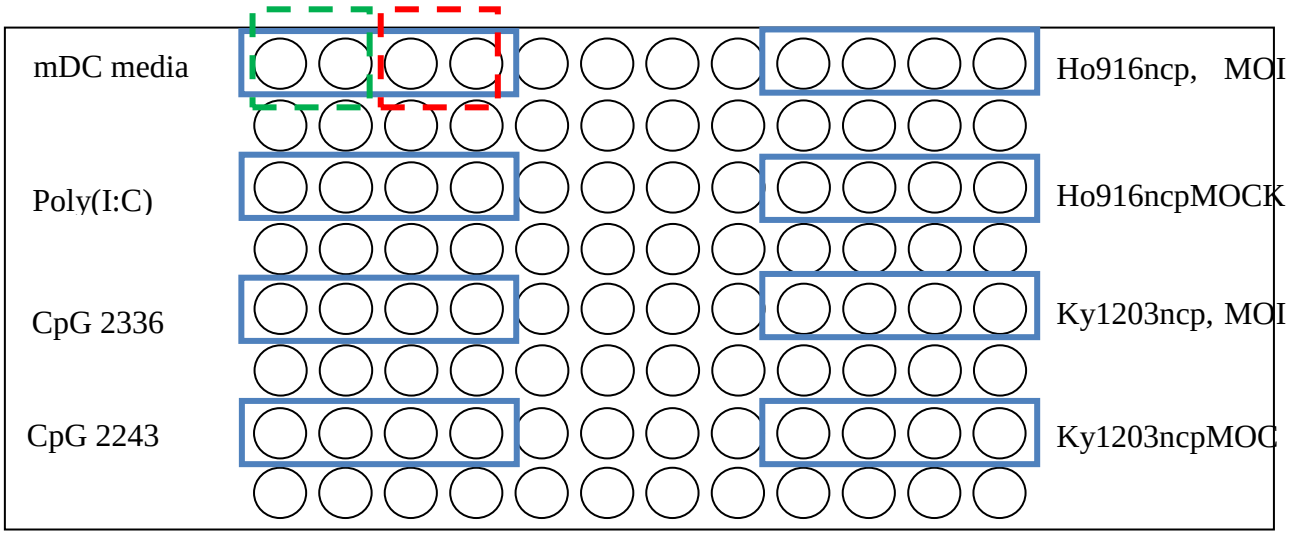



*Figure 2.2 Plate layout for stimulation of mDCs. Plates were harvested after 48 hrs incubation at 37˚C, 5% $CO_2$. Supernatants were collected from the first two wells of each condition (green) and RNA extracted from the cells. From the second two wells (red) the supernatant was discarded and protein extracted.*

The total volume in each well was adjusted to 200 µl with mDC media and plates incubated 48 hrs at 37˚C, 5% $CO_2$. Supernatant from 2 wells of each condition was collected for IFNα/β analysis. Cells were washed once with PBS and thereafter prepared for RNA extraction or protein extraction using RLT buffer

(QIAGEN, UK) or Mammalian Protein Extraction Reagent (MPER) mixed with Halt protease inhibitor (Thermo Scientific, UK) according to manufacturers' protocol. Samples were stored at -20˚C.

## 2.6 Detection of IFNα/β

### *2.6.1 Sub-culturing MDBKt2-cells*

MDBKt2-cells were cultured until 80% confluent at 37˚C, 5% $CO_2$ in MDBKt2 media (Appendix 1). MDBKt2-cells were used for IFNα/β detection in supernatants from stimulated mDCs using a CAT enzyme reporter gene assay as previously described *(Fray et al 2001)*. The MDBKt2-cells are transfected with a plasmid containing the human *Mx* gene promoter coupled with a gene encoding CAT-enzyme. IFNα/β stimulate the *Mx* promoter inducing transcription of CAT enzyme. The amount of CAT-enzyme can be quantified by a commercially available enzyme linked immunosorbant assay-kit (CAT-ELISA; Roche Diagnostics, Germany).

For the CAT reporter gene assay MDBKt2-cells were plated in 24 well plates (Greiner, Germany) at 2.5 x $10^5$cells per well in 2% MDBKt2 assay media (Appendix 1). After 24 hrs in culture, media was removed and cells washed with PBS before adding 400 µl MDBKt2 2% assay per well. 100 µl of either mDC-supernatant, recombinant human (rhu)IFNα (NIBSC, UK), or rboIFNα (Molecular Immunology, RVC) was used to create a standard curve as well as internal reference. To do so, 250 international units (IU) of rhuIFN were serial diluted 1:2, whereas rboIFN was serial diluted 1:4. Plates were incubated 24 hrs at 37˚C, 5% $CO_2$ and thereafter stored at -20˚.

### *2.6.2 Stimulation of MDBKt2-cells*

To assess the ability of MDBKt2-cells to directly respond to IFNα/β-inducing ligands or BVDV virus, MDBKt2-cells were stimulated as described in 2.5 and cell lysates analysed by the CAT-ELISA kit.

### *2.6.3 CAT-ELISA*

The CAT-ELISA was performed as according manufacturer's protocol, and the principle of this sandwich ELISA is shown in Figure 2.3. and Figure 2.4.

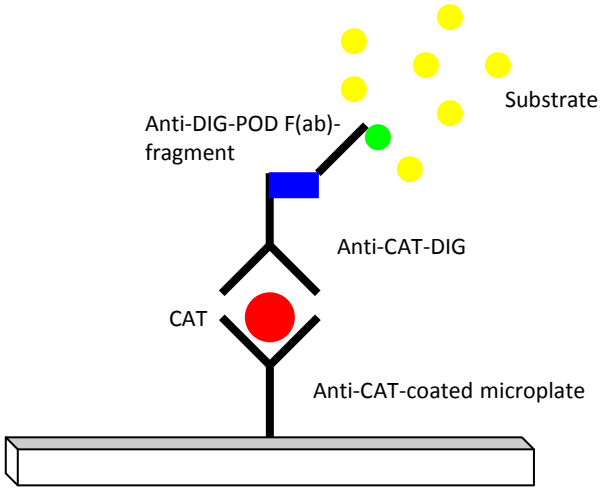



*Figure 2.3 Principle for sandwich ELISA detecting CAT-enzyme. The ELISA plate is coated with anti-CAT antibodies. The secondary anti-CAT antibody is labelled with digoxigenin. A F(ab)-fragment against digoxigenin conjugated with peroxidase will visualize antibody binding when peroxidase substrate is added. The reaction between substrate and peroxidase yields a coloured product.*

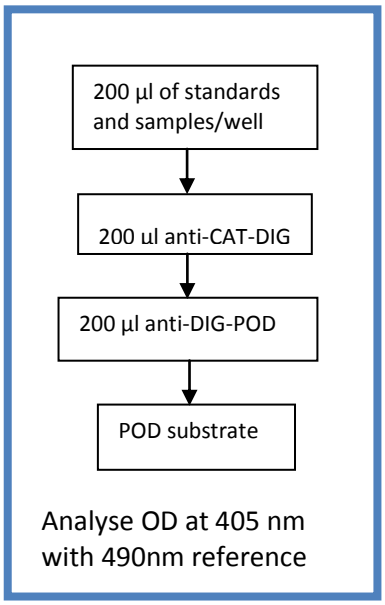



*Figure 2.4 Schematic Flow Chart of CAT-ELISA protocol. 200 µl per well of samples and standards diluted 1:2 was added to the ELISA plate. After 1 hr incubation at 37˚C and x5 washing with washing buffer 200 µl digoxigenin labelled anti-CAT antibody (anti-CAT-DIG) was added per well. Incubation and washing was repeated before adding 200 µl peroxidase labelled anti-digoxigenin antibody (anti-DIG-POD) per well. After a final incubation and x5 wash 200 µl peroxidase substrate was added. The optical density (OD) of individual wells was read at 405 nm with 490 nm reference (SpectraMax M2 microplate reader, Moleculer Devices, USA).*

## 2.7 RNA extraction

Total RNA from mDC stimulated as described in section 2.5 was extracted using the RNeasy mini kit (QIAGEN, UK) according to manufacturer's protocol. Extracted RNA was treated with RNase-free DNase I (Ambion (Europe) Ltd., UK) at 37°C for 20 min to remove contaminating genomic DNA. RNA yields and quality were determined using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA) (260/280 ratio >1.9 regarded satisfactory).

## 2.8 cDNA synthesis

For each sample, 10 ng of RNA was transcribed to cDNA using the Supercript II reverse transcription system (Invitrogen, UK). Briefly, reverse transcription was performed using 200 units SuperScript II reverse transcriptase (Invitrogen, UK), 500 ng oligo dT, 10 mM deoxyribonucleoside triphosphate (dNTPs) mix, 10 mM dithiothreitol (DTT), 5× first-strand buffer and 40 units RNAsin (Promega, UK). The reverse transcription reaction was allowed to proceed at 42°C for 50 min, followed by 70°C for 15 min. Synthesised cDNA was incubated at 37°C for 20 min with 2 units RNase H (Promega) to remove original RNA templates. cDNA yields and quality were determined using a NanoDrop® ND-1000 Spectrophotometer. The integrity of the cDNA was assessed by PCR for the

constitutively expressed gene β-actin (see 2.9 and Table 2.3). cDNA was stored at -20°C until required.

To detect BVDV nucleic acid in potentially infected cells reverse transcription was performed as above but using 500 ng random hexamer primers (GE Healthcare, UK) instead of oligo dT primers.

## 2.9 PCR

PCR amplification of cDNA adjusted to 50ng $\mu l^{-1}$ was carried out using primers listed in Table 2.3. A 50 µl reaction was used containing 50 ng cDNA template, 10 pmol $\mu l^{-1}$ sense and antisense primers, 10 mM dNTPs 10 mM, (Bioline, UK), 1x GoTaq green reaction buffer (Promega, UK) and 1.25 units GoTaq DNA Polymerase (Promega, UK). Molecular Biology Water (Sigma Aldrich, UK) was used as negative control and plasmid preps (0.5 µl per reaction) as positive controls (Appendix 2). The reaction was heated to 94°C for 2 min, followed by 30 cycles of 94°C for 40 sec, 55°C for 1 min, 72 °C for 1 min, and a final extension step of 72°C for 7 min. PCR was performed on a G-STORM thermocycler (GRI systems, UK).

*Table 2.3 PCR primers*

| Gene (Accession Number) | Forward Primer | Reverse Primer | Expected size |
|---|---|---|---|
| Bovine β-actin (AY141970) | CCA GAC AGC ACT GTG TTG GC | GAG GAA GCT GTG CTA CGT CGC | 300 bp |
| Bovine TLR1 (AY634638) | TTC CAG AGC TGC CAG AAG AT | GAG ATT GTG GTG GGC AAA GT | 627 bp |
| Bovine TLR2 (AY634629) | CAG CAA CTG AAG ACG TTG GA | CAC CAC TCG CTC TTC ACA AA | 571 bp |
| Bovine TLR3 (AY812026) | CCC CAG TCT CAC AGA GAA GC | CCT GTG AGT TCT TGC CCA AT | 645 bp |
| Bovine TLR4 (AY634630) | TGC TGG CTG CAA AAA GTA TG | TCT GCA GGA CGA TGA AGA TG | 335 bp |
| Bovine TLR5 (AY634631) | TGC ATC CAG ATG CTT TTC AG | CCT TCA GCT CCT GGA GTG TC | 618 bp |
| Bovine TLR6 (AY487803) | AGG CCA AGT ATC CAG TGA CG | GAG ATT GTG GTG GGC AAA GT | 538 bp |
| Bovine TLR7 (AY487802) | GGA AAT TGC CCT CGT TGT TA | TGC AGT GTT TCA AGG ACC TG | 620 bp |
| Bovine TLR8 (AY642125) | TTG ATG ACG ATG CTG CTT TC | GGG TTA CCC CCT AGT TCC AA | 665 bp |
| Bovine TLR9 (AY859726) | CAA GTG CTC GAC CTG AGT GA | CCA TGG TAC AGG TCC AGC TT | 690 bp |
| Bovine TLR10 (AY634632) | CAC CTG ACA TCT TTG CGA GA | TTC CCT CAT GAA GGC AAA TC | 602 bp |
| BVDV 5' UTR (M31182) | ATG CCC ATA GTA GGA CTA GCA | TCA ACT CCA TGT GCC ATG TAC | 287 bp |

PCR products were analysed by electrophoresis (100 V, 30 min) on a 1% agarose gel containing 1 µl Safe View (NBS Biologicals, UK) loading 5 µl of each PCR product and 5 µl 100 bp DNA Ladder (Fermentas, Germany). DNA bands were visualised by ultraviolet transillumination.

## 2.10 *Taq*Man® qPCR

mRNA expression of IL-10 and IL-12 from mDCs stimulated as described in (Section 2.5) was quantified using *Taq*Man® qPCR techonolgy. Primers and *Taq*Man® probes were previously designed at Institute of Animal Health (IAH), Compton, UK using primer Express software (Applied Biosystems, Foster City, CA, USA) (Table 2.4). Primers and probes were used at a concentration of $3x10^{-7}$ M and $1x10^{-7}$ M respectively. Probes were labelled at the 5' end with the reporter dye FAM (6-carboxyfluorescein) and at the 3' end with the quencher dye TAMARA (6-carboxytetramethyl-rhodamine). qPCR was carried out using *Taq* Man® Universal PCR Mastermix (Applied Biosystems), with 100 ng cDNA as a template, on ABI 7500 Fast sequence detection system (Applied Biosystems). The amplification cycle consisted of an initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Results were quantified by comparison with standard curves of known copy numbers of

plasmid DNA containing targeted gene sequences. To ensure that each sample contained cDNA of the same amount and quality (testing efficacy of RNA extraction and cDNA synthesis), expression of the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GADPH) and the ribosomal protein RPLPO was measured in each sample. Samples, standards and no template controls were set up in triplicate. Data analysis was carried out using Microsoft® Excel 2007 (Microsoft Co, USA).

*Table 2.4 Primers and probes for Taq Man® qPCR*

| Target | Forward primer | Reverse primer | Probe |
|---|---|---|---|
| GADPH | CAT GTT CCA GTA TGA TTC CAC CC | GAG CTT CCC GTT CTC TGC C | CGG CAA GTT CAA CGG CAC AGT CA |
| RPLPO | GCA CAA TTG AAA TCC TGA GTG | GGG TTG TAG ATG CTG CCA TT | AGC GAA GCC ACG CTG CTG AA |
| IL-10 | GGT GAT GCC ACA GGC TGA G | AGC TTC TCC CCC AGT GAG TTC | CAC GGG CCT GAC ATC AAG GAG CA |
| IL-12p40 | CCA AAG TCA CAT GCC ACA AGG | CTG TAG TAG CGG TCC CGG G | TGC CAA CGT CCG CGT GCA A |

## 2.11 Western blotting (WB)

Protein concentrations were determined using a NanoDrop® ND-1000 Spectrophotometer. Samples were prepared for reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by adding 5x Laemmli buffer (Appendix 1) and 8 min boiling. After brief re-heating, 10 µl sample per well (5 µg protein) was loaded to a 10% Tris-HCl gel (Bio-Rad, UK), ran at 30 mA per gel for 90 min. The proteins separated by size were transferred to 0.2 µm nitrocellulose membranes (Amersham Biosciences, Germany). PBS-T/5% non-fat milk powder (Appendix 1) was used for blocking membranes (1 hr at RT with gentle agitation), diluting Ab and washing off unbound Ab. Membranes were incubated with Abs listed in Table 2.5. ß-actin was used as a protein loading control on membranes treated with stripping buffer (Thermo Sceintific, UK) according to manufacturer's prtocol. Ab binding was visualised using the ECL system (Amersham Biosciences, Germany) according to manufacturer's protocol and a Curix 60 processor (Agfa-Gevaert N.V., Mortsel-Belgium).

*Table 2.5 WB Antibodies and Conditions*

| Antibody | Concentration and incubation | Specificity | Molecular Weight |
|---|---|---|---|
| IRF3 | 1:200, over night | Polyclonal rabbit anti-Human IRF3 (FL-425)* | ~50 kDa |
| IRF7 | 1:200, over night | Polyclonal rabbit anti-Human IRF7 (H-246)* | ~50 kDa |
| β-actin | 1:5000, 1 hr | Monoclonal mouse anti-Actin Clone C4** | ~42 kDa |

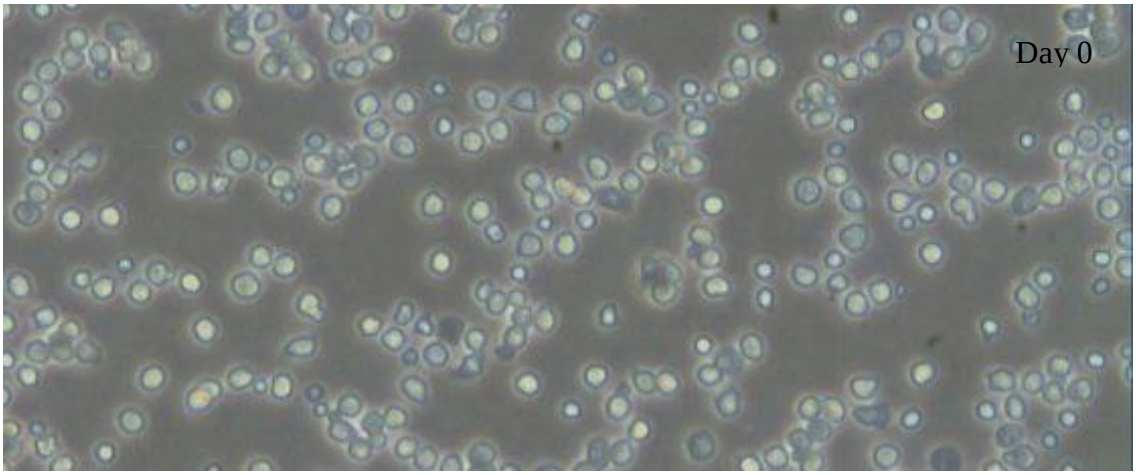
** Santa Cruz Biotechnologies Inc, USA) ** Millipore (Chemicon), UK. See Appendix 4 for further details.*

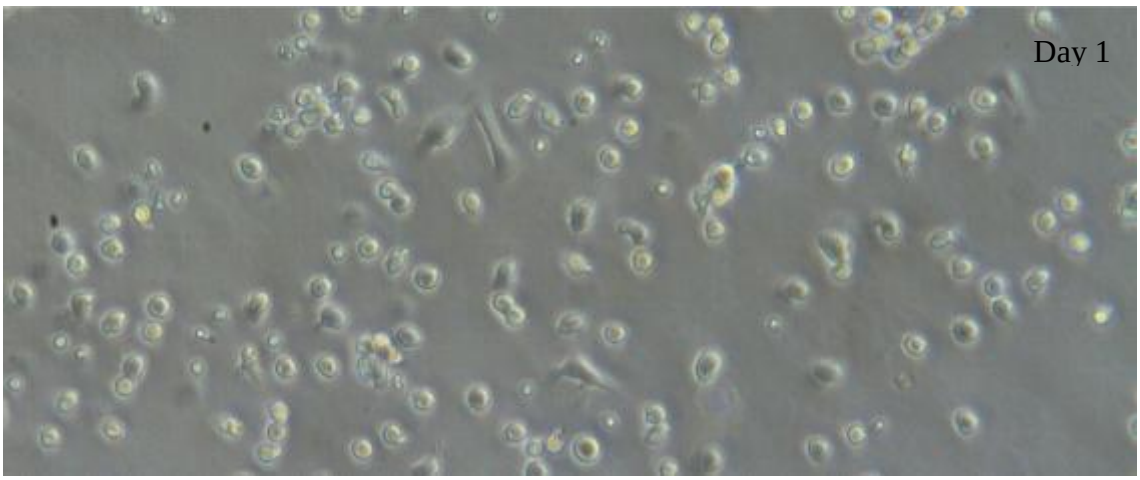
# RESULTS

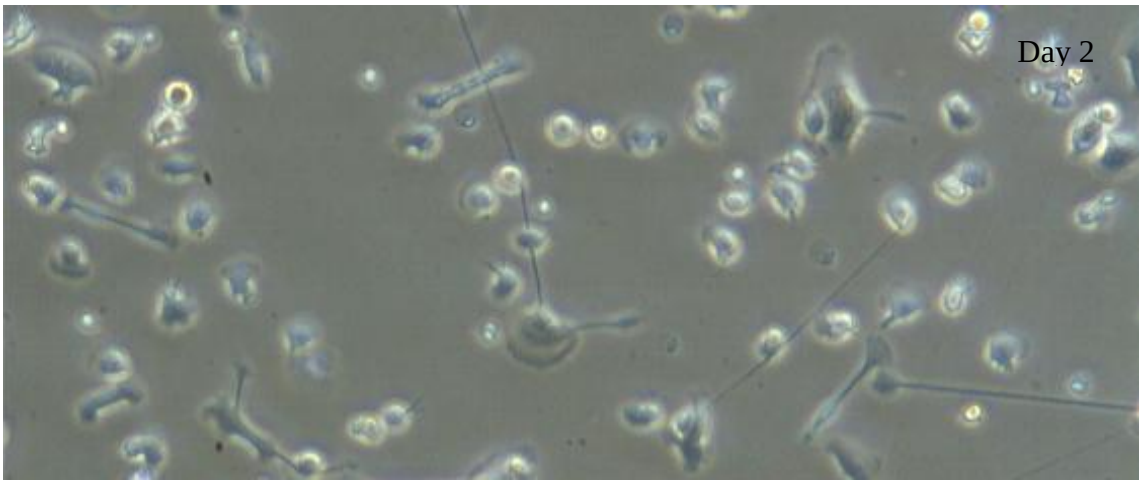
## 3.1 Generation of mDCs

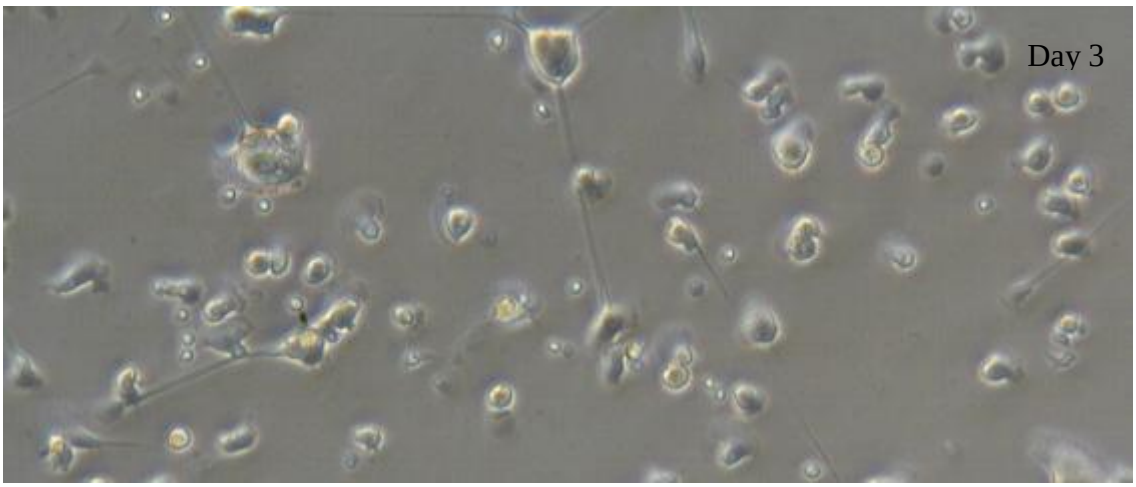
### *3.1.1 Morphology*

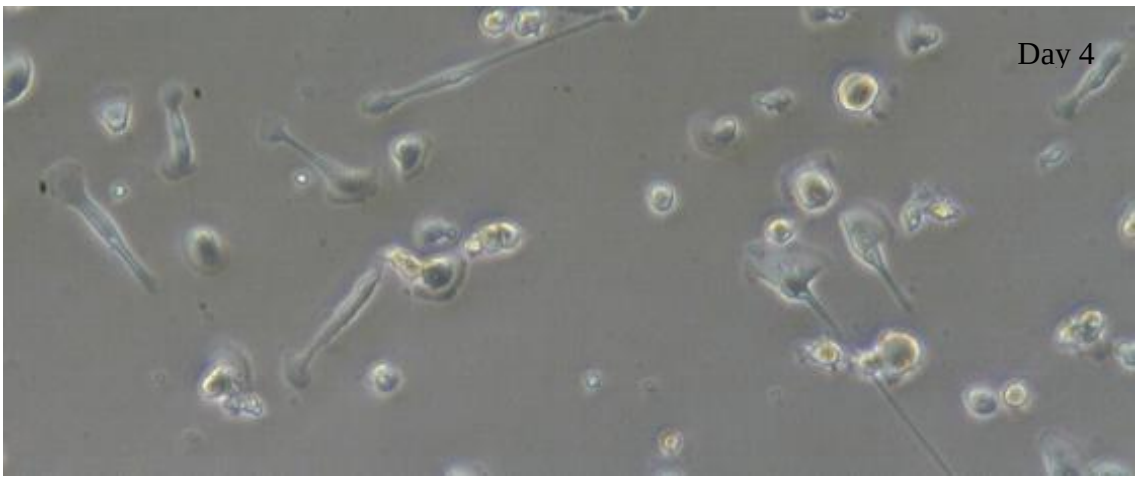
mDCs were generated by culturing $CD14^+$ cells for 6 days with rboIL-4 and rboGM-CSF. By day 6 cells were adhering to the plastic and an irregularly large cytoplasm with projections could be observed by light microscopy (Figure 3.1).




Day 2


Day 3


Day 4

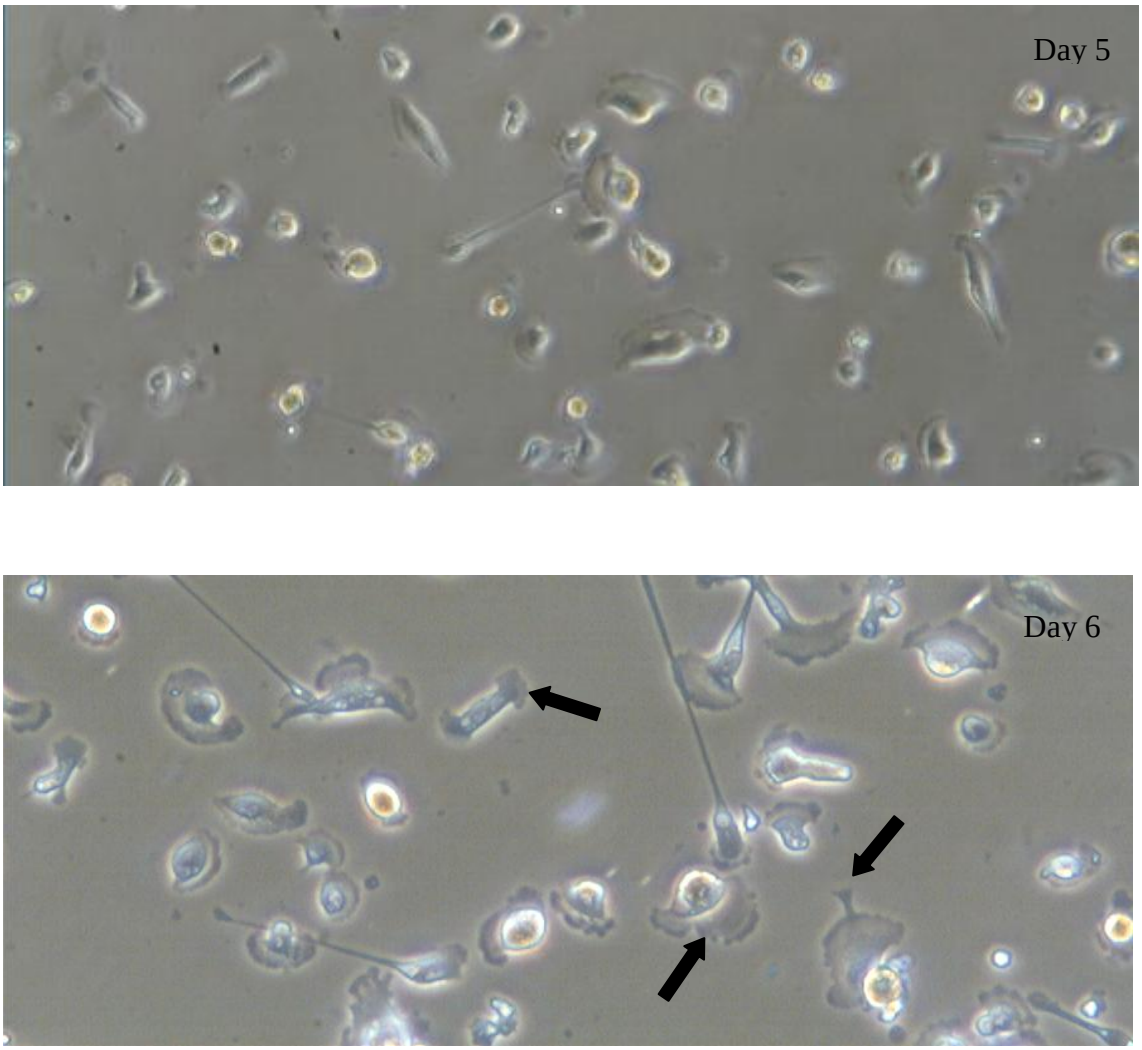
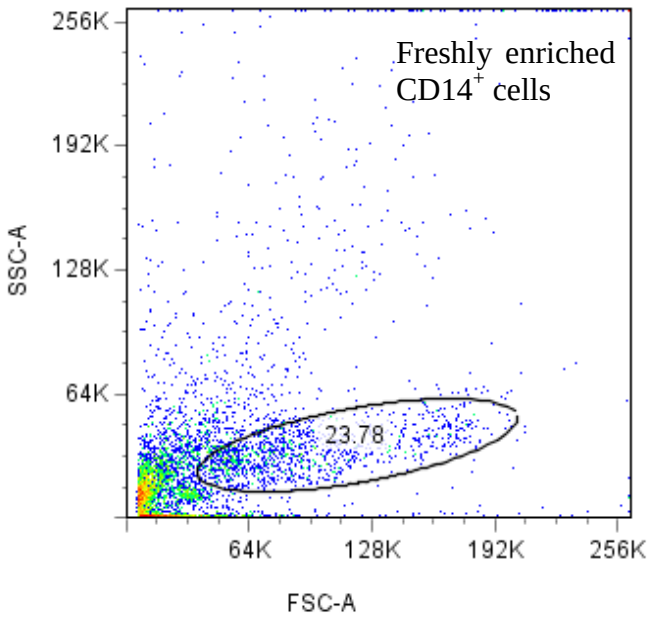
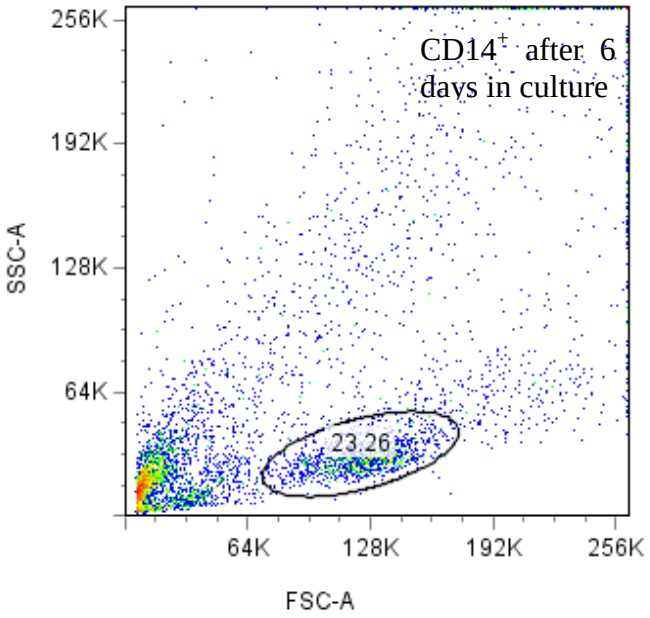



*Figure 3.1 Differentiation of monocytes to mDCs. Representative pictures showing culturing of CD14$^+$ cells for 6 days in culture media supplemented with IL-4 and GM-CSF. Magnification 100x (Olympus CK2 inverted microscope). Arrows indicate cytoplasmic protrusions, a characteristic feature of DC morphology.*

Forward/Side Scatter analysis by flow cytometry of enriched CD14$^+$ cells before and after 6 days culturing with rboIL-4 and rboGM-CSF also illustrates a change in morphology (Figure 3.2). Disregarding cellular debris and big granular dying cells sticking together, the Forward/Side scatter analysis of cultured cells show a fairly dense, homogenous cell population with a slight increase in size.




**A**




**B**

*Figure 3.2 Dot plot showing CD14$^+$ cells before and after 6 days culturing with rboIL-4 and rboGM-CSF analysed for size (Forward Scatter FSC-A) and granularity (Side Scatter- SSC-A) by flow cytometry. 5000 cells were recorded using a FACSAria and data analysed in FlowJo (TreeStar, California, USA). A) CD14$^+$ cells enriched by MACS, B) The same cells after 6 days in culture with IL-4 and GM-CSF.*

### *3.1.2 CD14 expression*

After 6 days culturing of $CD14^+$ enriched cells with rboIL-4 and rboGM-CSF FACS analysis showed a down regulation of CD14 expression (Figure 3.3). The staining picture varied between individual animals (3 individual repeats) but all showed the same trend of CD14 down regulation.

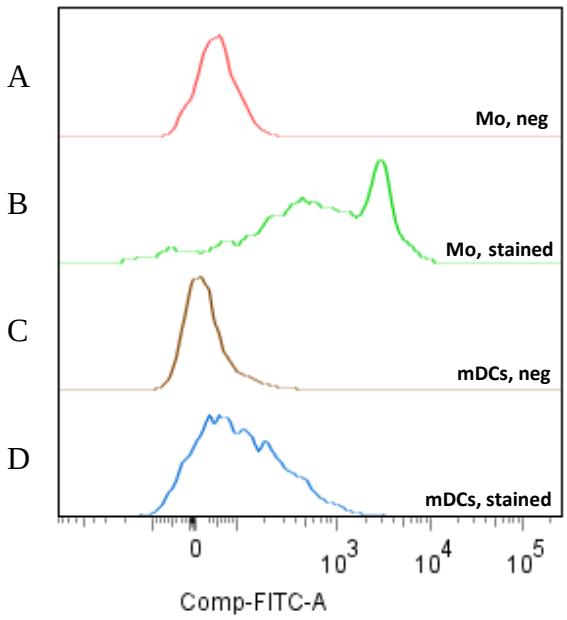


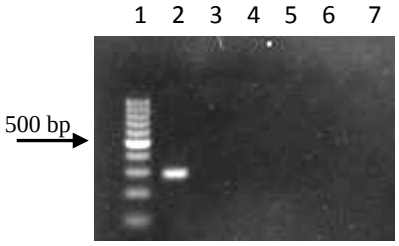

*Figure 3.3 FITC labeled CD14 antibody staining of monocytes (Mo) enriched by $CD14^+$ MACS before and after 6 days culturing with rboIL-4 and rboGM-CSF. Representative data from flow cytometric analysis of 5000 cells (A and B) and 30 000 cells (C and D) are presented as histograms. Data presented are based on uniformly gated cell populations as shown in Figure 3.2. A) monocytes, unstained B) monocytes, FITC CD14 staining, C) mDCs, unstained D) FITC CD14 staining, mDCs*

Taken together, the changes in morphology and decreased CD14 expression demonstrate that isolated monocytes resemble mDCs after 6 days in culture.

## 3.2 Reverse transcriptase PCR for TLR expression and presence of BVDV

### *3.2.1 BVDV*

To ensure that cells were free from BVDV infection prior to stimulation with Ho961ncp and Ky1203ncp, cells were screened by reverse transcriptase PCR. mDCs isolated from the 3 animals used for the stimulation assay and MDBKt2-cells used for the CAT-enzyme reporter gene assay all screened negative (Figure 3.4).




1) ladder
2) plasmid containing 5'UTR sequence from BVDV type 1 strain NADL
3) negative control
4) cDNA from MDBKt2 cells
5) cDNA from mDCs animal 1
6) cDNA from mDCs animal 2
7) cDNA from mDCs animal 3

*Figure 3.4 Screening for presence of BVDV nucleic acid in mDCs and MDBKt2 cells by reverse transcriptase PCR using primers described in Table 2.3. PCR products were analysed by gel electrophoresis on a 1% agarose gel containing 1 µl Safe View (NBS Biologicals, UK) and visualized by ultraviolet transillumination. A plasmid control cointaining 5'UTR sequence from the BVDV strain NADL (Appendix 2) was loaded next to a 100 bp ladder (Fermentas, Germany).*

### *3.2.2 mRNA expression of TLR3, 7, 8, 9 in mDCs and MDBKt2-cells*

To investigate the ability of mDCs to sense viral infection through TLR recognition, mRNA expression of TLR3, 7, 8 and 9 in unstimulated mDCs from 3 animals was determined by reverse transcriptase PCR. This was also performed for MDBKt2-cells to explore their capability of TLR-dependent IFNα/β production.

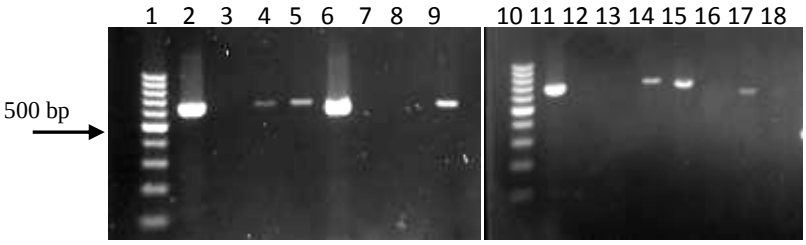



*Figure 3.5 Representative result of reverse transcriptase PCR for mRNA expression of viral TLRs in mDCs and MDBKt2 -cells using primers described in Table 2.3. Each TLR set include a positive plasmid control containing bovine TLR constructs (Appendix 2) loaded next to a negative control*

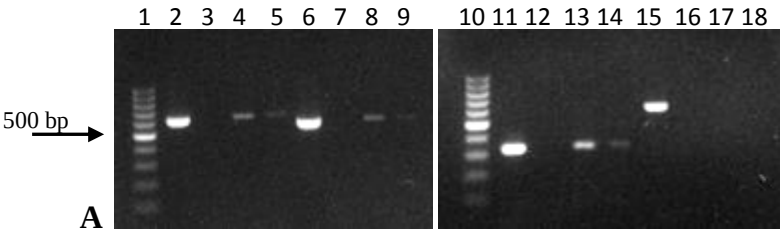
1) Ladder
2) plasmid containing bovine TLR3
3) negative control TLR3
4) cDNA from MDBKt2 cells, TLR3
5) cDNA from mDCs animal 2, TLR3
6) plasmid containing bovine TLR7
7) negative control TLR7
8) cDNA from MDBKt2 cells, TLR7
9) cDNA from mDCs animal 2, TLR7
10) Ladder
11) Plasmid containing TLR8
12) Negative control TLR8
13) cDNA from MDBKt2 cells, TLR8
14) cDNA from mDCs animal 2, TLR8
15) plasmid containing bovine TLR9
16) negative control TLR9
17) cDNA from MDBKt2 cells, TLR9
18) cDNA from mDCs animal 2, TLR9

MDBKt2-cells were shown to express TLR3 and TLR9 (Figure 3.5 lane 4 and 17). TLR expression in mDCs was largely consistent between the 3 animals (Table 3.1). PCR products of the right sizes (Table 2.3) were generated using

primers for TLR3, 7 and 8 (Figure 3.5 lane 5, 9 and 14). TLR9 primers gave rise to a faint band only in mDCs from animal 2 (Figure 3.5 lane 18).

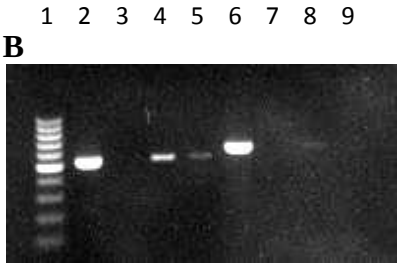
### *3.2.3 mRNA expression of TLR1, 2, 4, 5, 6, 10 in mDCs*

mRNA expression of TLR1, 2, 4, 5, 6, and 10 in mDCs from 3 animals was investigated as a part of general characterisation.




10) ladder
11) plasmid containing bovine TLR1
12) negative control TLR1
13) cDNA from mDCs animal 2, TLR1
14) cDNA from mDCs animal 3, TLR1
15) plasmid containing bovine TLR2
16) negative control TLR2
17) cDNA from mDCs animal 2, TLR2
18) cDNA from mDCs animal 3, TLR2

1) ladder
2) plasmid containing bovine TLR1
3) negative control TLR1
4) cDNA from mDCs animal 2, TLR1
5) cDNA from mDCs animal 3, TLR1
6) plasmid containing bovine TLR2
7) negative control TLR2
8) cDNA from mDCs animal 2, TLR2
9) cDNA from mDCs animal 3, TLR2




1) ladder
2) plasmid containingl bovineTLR6
3) negative control TLR6
4) cDNA from mDCs animal 2, TLR6
5) cDNA from mDCs animal 3, TLR6
6) plasmid containing bovine TLR10
7) negative control TLR10
8) cDNA from mDCs animal 2, TLR10
9) cDNA from mDCs animal 3, TLR10

*Figure 3.6 Representative result of reverse transcriptase PCR for mRNA expression of TLR1, 2, 4, 5 (A) and TLR6 and 10 (B) in mDCs cells using primers described in Table 2.3. Each TLR set include a positive plasmid control containing bovine TLR constructs (Appendix 2) loaded next to a negative control.*

Expression of TLR1, 2, 4, 5, 6 and 10 was largely consistent between the 3 animals (Figure 3.6 A and B) only diverging in expression of TLR 10 for which 1 out of 3 was positive. For an overview of TLR expression see Table 3.1.

*Table 3.1 Overview of reverse transcriptase PCR results for TLR expression in mDCs from 3 animals.*

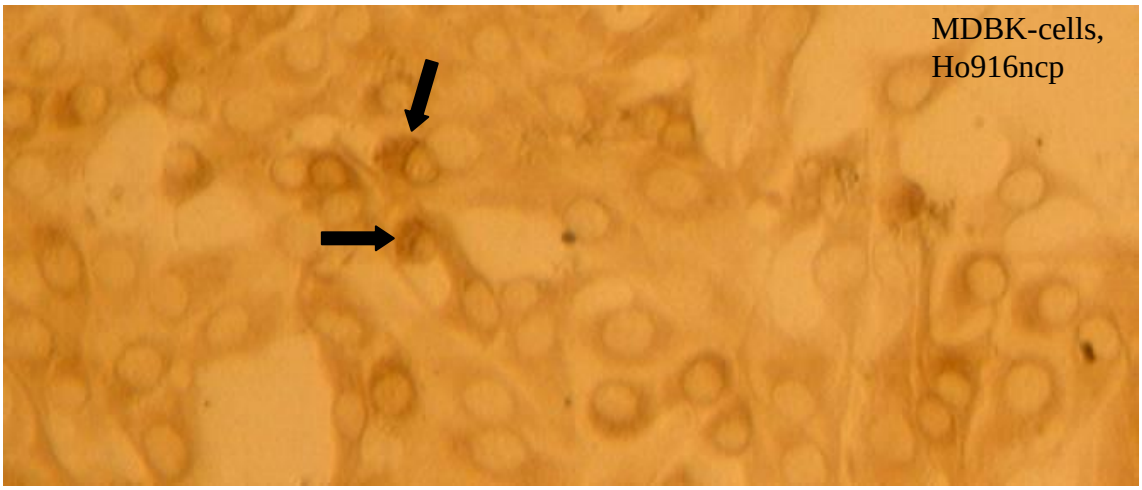
| TLR | Presence in mDC |
|---|---|
| TLR1 | + |
| TLR2 | + |
| TLR3 | + |
| TLR4 | + |
| TLR5 | - |
| TLR6 | + |
| TLR7 | + |
| TLR8 | + |
| TLR9 | -/+ |
| TLR10 | -/+ |

## 3.3 Susceptibility of mDCs to BVDV infection

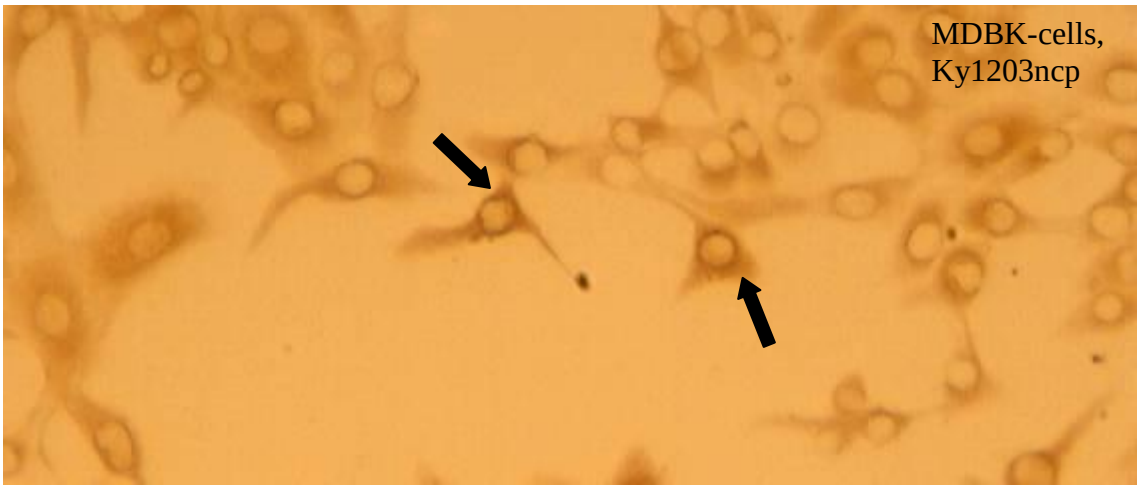
### *3.3.1 IPX staining*

To investigate if isolated mDCs were susceptible to infection with either Ho916ncp or Ky1203ncp, mDCs were infected at a MOI of 0.1 and stained by IPX (48 hrs p.i. using BVDV hyperimmune serum). Brown cytoplasmic staining indicates presence of BVDV (Figure 3.7). Positive staining for BVDV infection was achieved for mDCs from 2 out of 3 animals. A result from the third repeat could not be determined due to staining difficulties. As a positive control, results of MDBK-cells infected with either strain are shown for comparative reasons.

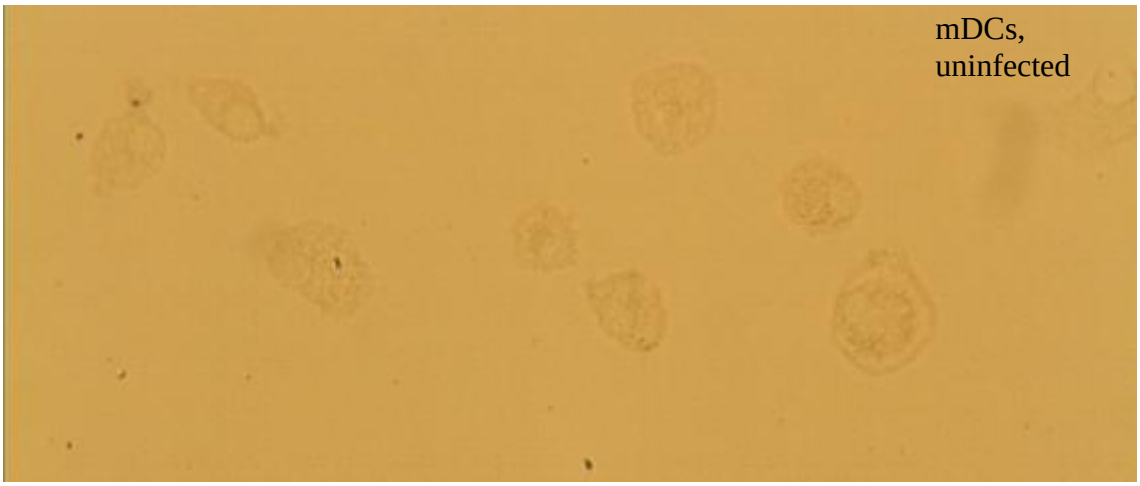
**A**



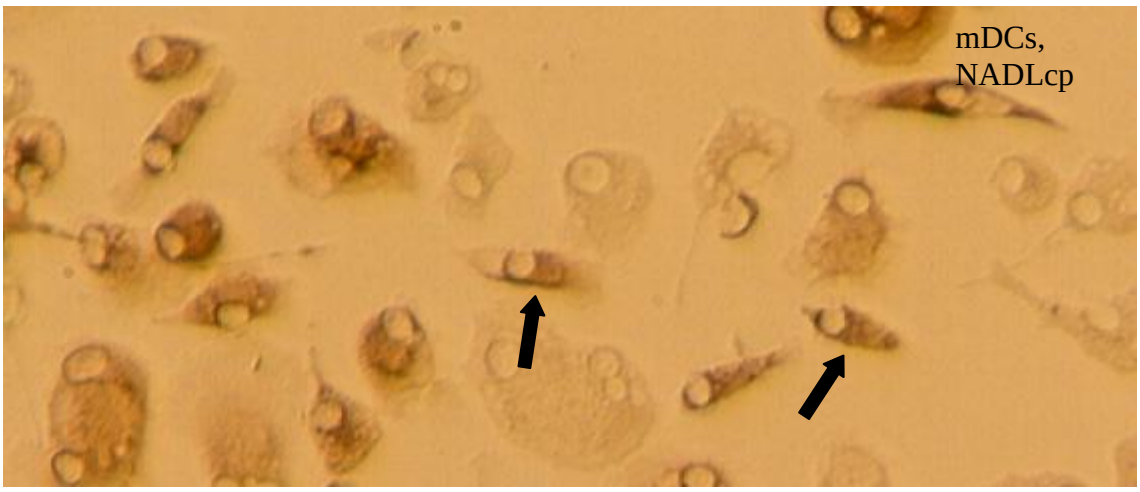
**B**



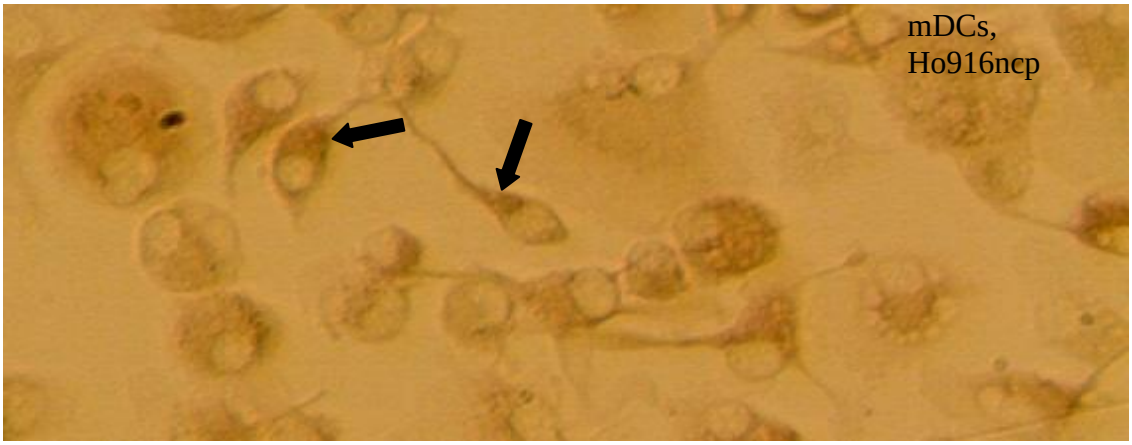

**C**




D



**E**




**F**

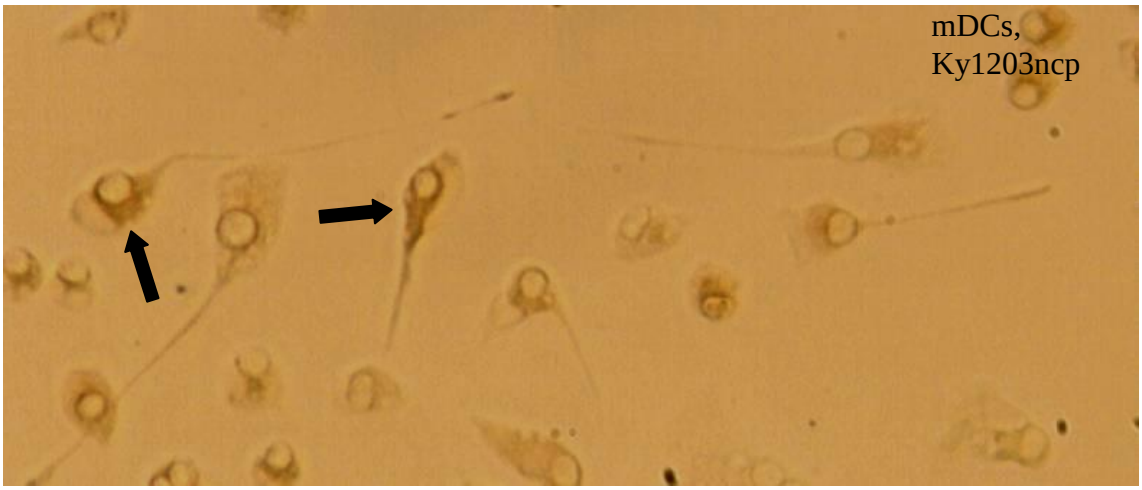



*Figure 3.7 Representative results from IPX staining determined by light microscopy, magnification 100x (Motic AE20 binocular inverted microscope) A) and B) MDBK-cells infected with Ho916ncp and Ky1203 respectively. Infection of MDBK-cells was included to generate expected positive staining results as these cells are known to be susceptible to BVDV. C) Stained uninfected mDCs as a negative control D) mDCs, infected with NADL, a BVDVcp strain frequently used in the lab as a positive staining control. E) mDCs infected with Ho916ncp MOI 0.1, F) mDCs infected with Ky1203ncp, MOI 0.1.*

## 3.4 Detection of IFNα/β production from stimulated MDBKt2-cells

MDBKt2-cells were employed to detect IFNα/β in culture supernatants by a CAT-enzyme reporter gene assay described in section 2.6. Due to the possible remaining presence of IFNα/β stimulating ligands after 48 hrs with mDCs an

experiment was designed to assess the ability of MBDKt2-cells to induce IFNα/β directly. MBDKt2-cells were therefore directly stimulated with IFNα/β-inducing ligands (Table 2.2) and virus at a MOI of 0.1 and 0.01. An IFNα/β response was observed from poly(I:C) stimulated cells only (Figure 3.8).

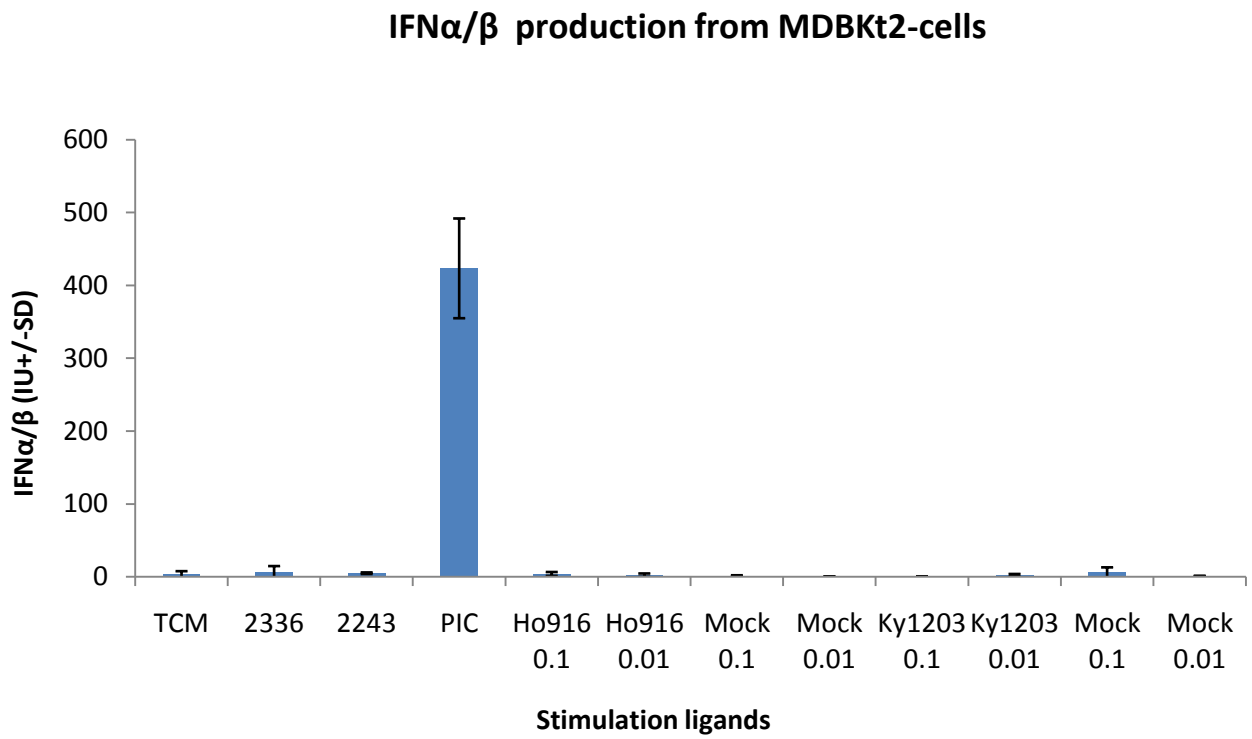


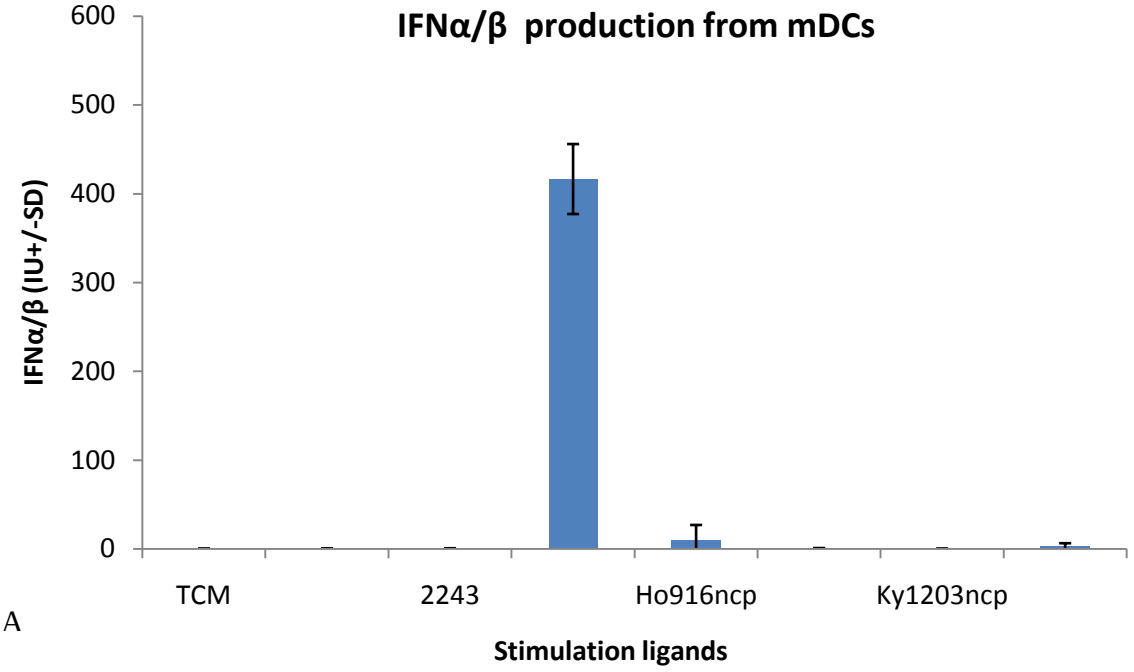

*Figure 3.8 Histogram showing production of IFNα/β in from MDBKt2-cells in response to stimulation with ligands and virus as described in section 2.5. TCM: tissue culture media (Appendix 1 MDBKt2 growth media), 2336: CpG2336, 2243: CpG2243 scrambled control sequence, PIC: poly(I:C), Ho916: Ho916ncp, Ky1203: Ky1203ncp. Details for stimulation ligands are described in table 2.2.*

Since MDBKt2-cells express TLR3, the response to the synthetic TLR3-ligand poly(I:C) is not surprising and has to be taken into consideration when analyzing IFNα/β data from stimulated mDCs. However, no production of IFNα/β in response to stimulation with Ho916ncp, Ky1203ncp or any other IFNα/β-inducing ligand could be detected by this test.

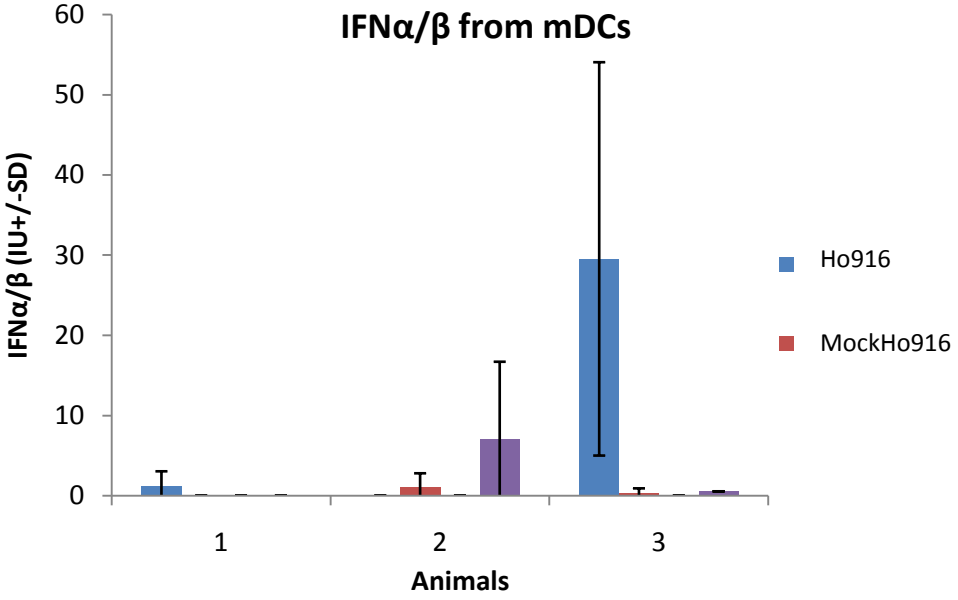
## 3.5 Detection of IFNα/β production from stimulated mDCs

mDCs from 3 animals were stimulated with Ho916ncp and Ky1203ncp at a MOI of 0.1 and control ligands as described in section 2.6. Supernatants from

stimulated cells were analysed for presence of IFNα/β. The results were largely consistent, high IFNα/β levels were only generated from stimulation with the positive control ligand poly(I:C) as can be seen in Figure 3.9 A showing pooled data from the three animals. A low level of IFNα/β compared with the response to poly(I:C) was however detected from 1 animal when stimulated with Ho916ncp (Figure 3.9 B). The response to Ho916ncp seen in animal 3 is due to a response from only one of the duplicates in the experimental set up. Furthermore, mDCs generated from animal 2 seemed to respond to the mock controls, but not to any ligand or either virus (Figure 3.9 B).




A




B

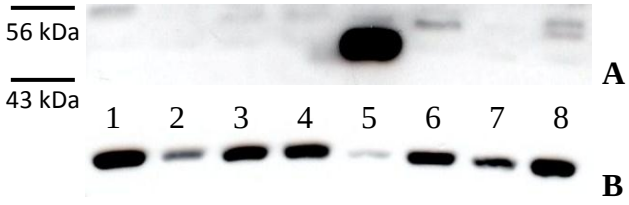
*Figure 3.9 Histogram showing production of IFNα/β in from mDCs cells in response to stimulation with ligands and virus as described in section 2.6. TCM: tissue culture media (Appendix 1, mDC media), 2336: CpG2336, 2243: CpG2243, PIC: poly(I:C), Ho916: Ho916ncp, Ky1203: Ky1203ncp. Details for stimulation ligands are described in Table 2.2. A) Mean values for IFNα/β production from 3 individual experiments. B) Individual data for animal 1, 2 and 3 only showing IFNα/β production in response to stimulation with Ho916ncp, Ky1203ncp and respective mock.*

### 3.6 Western blot for IRF3 and IRF7

The results shown in Figure 3.9 indicate the possibility that mDCs may respond to at least Ho916ncp with the production of IFNα/β. To further investigate the IFNα/β response in mDCs stimulated with Ho916ncp and Ky1203ncp, protein expression of IRF3 and IRF7 was analysed by WB for animal 2 and 3.

Bands of the expected size for IRF3 (about 50 kDa, Appendix 4) were detected in both animals and can be seen as a doublet (as described by others *(La Rocca et al 2005)*) (Figure 3.10 A, lane 1, 3, 4, 5, 6 and 8). The relative staining intensity of these bands were notably strong in protein extracts from mDCs stimulated with Ho916ncp (Figure 3.10 A lane 5) and faint from mDCs stimulated with poly(I:C) and Ky1203ncp (Figure 3.10 A lane 2 and 7). A band of the expected size for IRF7 (about 54 kDa (Swiss-Prot Acc. No. Q92985)) was only detected for animal 2 in mDCs stimulated with Ho916ncp (Figure 3.11 A lane 5).

Despite the fact that all samples were adjusted to the same protein-concentration, bands for the loading control β-actin (about 40 kDa, Appendix 4) were remarkably fainter for mDC protein extract treated with either poly(I:C), Ho916 or Ky1203ncp (Figure 3.10 B and 3.11 B lane 2, 5, 7).




1. TCM (mDC media, appendix 1)
2. Poly(I:C)
3. CpG2336
4. CpG2243
5. Ho916ncp, MOI 0.1
6. MOCK
7. Ky1203ncp, MOI 0.1
8. MOCK

*Figure 3.10 Results from Western blot analysis of IRF3 and β-actin representative for animal 2 and 3. A) Protein extracts from stimulated mDCs probed for IRF3, B) The membrane was stripped and re-probed for β-actin to ensure that the same amount of protein was loaded in each well.*


1. TCM (mDC media, appendix 1)
2. Poly(I:C)
3. CpG2336
4. CpG2243
5. Ho916ncp, MOI 0.1
6. MOCK
7. Ky1203ncp, MOI 0.1
8. MOCK

*Figure 3.11 Results from Western blot analysis of IRF7 and β-actin for animal 2. A) Protein extracts from stimulated mDCs probed for IRF7, B) The membrane was stripped and re-probed for β-actin to ensure that the same amount of protein was loaded in each well.*

### 3.7 qPCR for IL-10 and IL-12

A preliminary study of how infection with Ho916ncp and Ky1203ncp affect expression of the cytokines IL-10 and IL-12 in mDCs was performed using *Taq*Man®qPCR techonolgy for relative quantification of mRNA expression levels.

A relative standard curve method was used to quantify expression of targeted genes IL-10 and IL-12 and the endogenous control genes GADPH and RPLPO. No data was obtained from IL-12 due to contamination of no template controls.

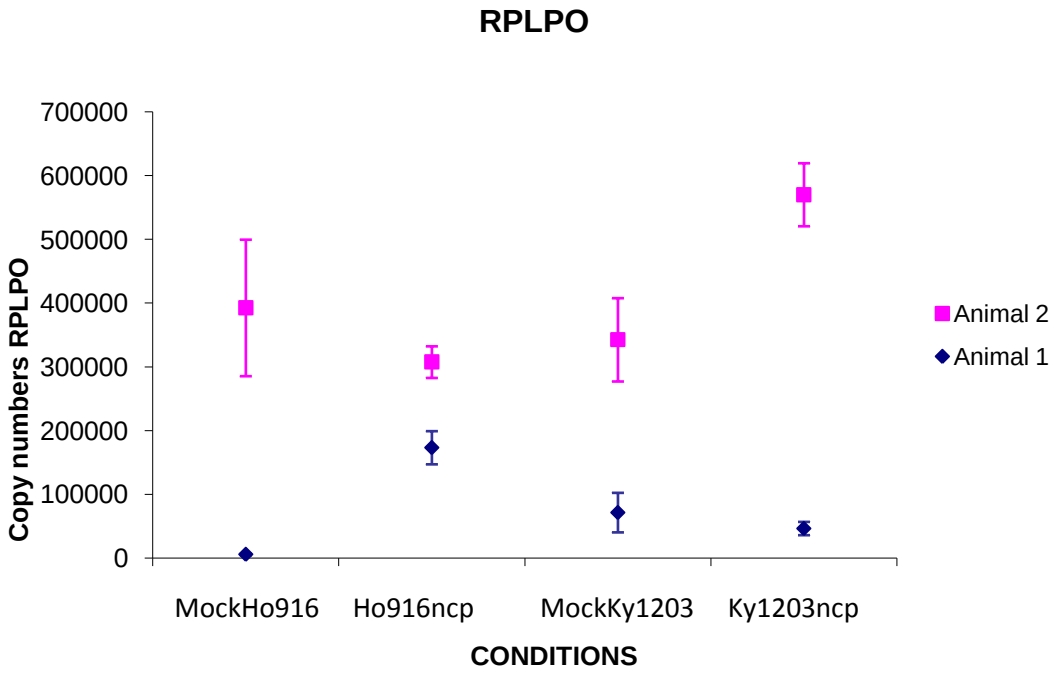



*Figure 3.12 Results from qPCR for the housekeeping gene RPLPO displayed as a dot plot diagram showing mean cDNA copy numbers from triplicates of each condition. MockHo916: mock Ho916ncp, MockKy1203: mock Ky1203ncp. Error bars represent standard deviations(SD).*

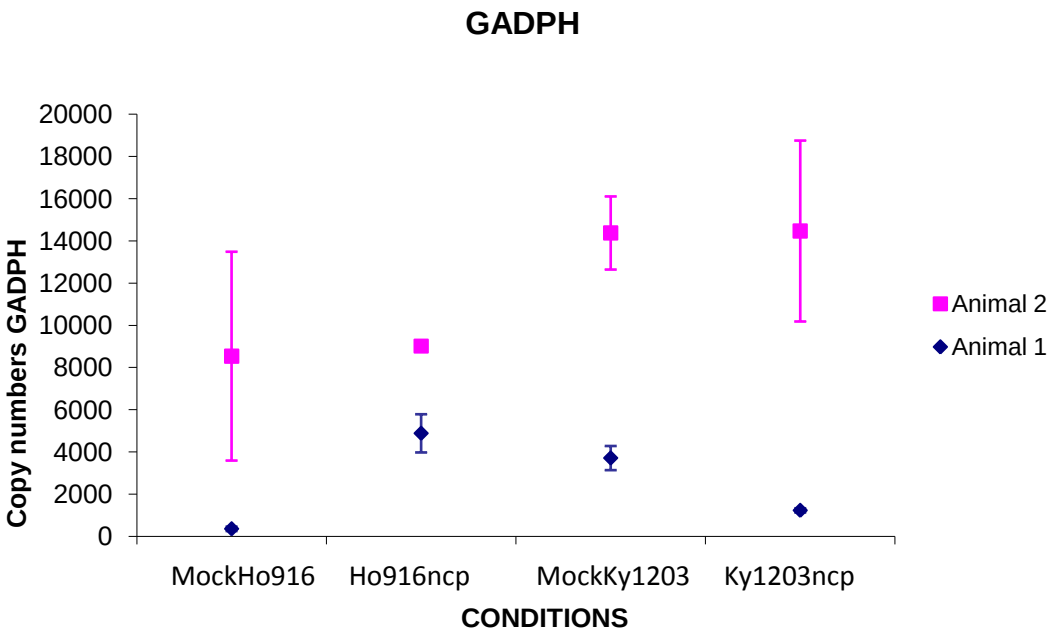



*Figure 3.13 Results from qPCR for the housekeeping gene GADPH displayed as a dot plot diagram showing mean cDNA copy numbers from triplicates of each condition. MockHo916: mock Ho916ncp, MockKy1203: mock Ky1203ncp. Error bars represent standard deviations (SD).*

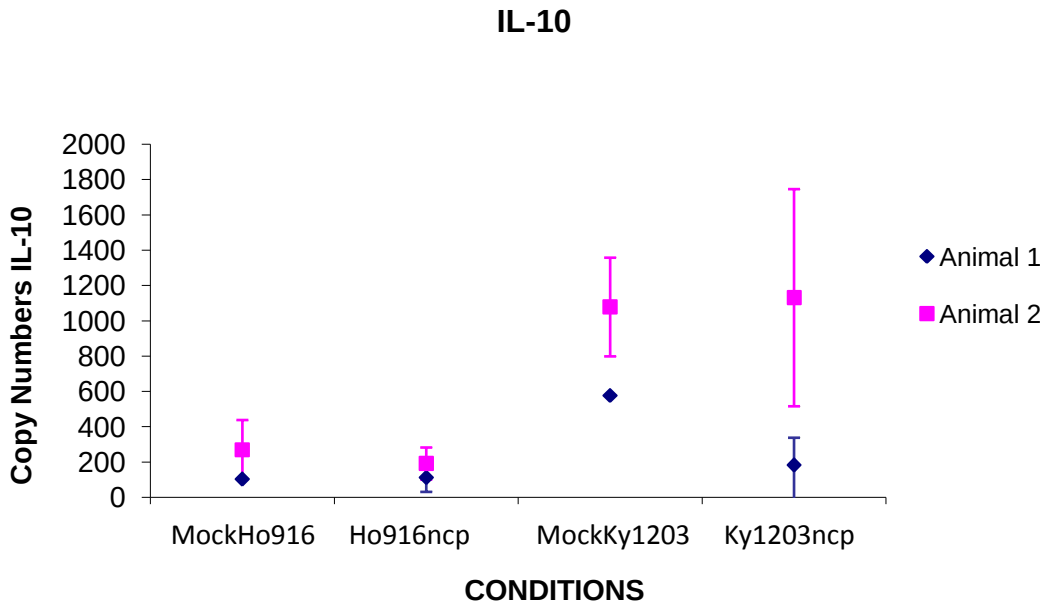



*Figure 3.14 Results from qPCR for IL-10 displayed as a dot plot diagram showing mean cDNA copy numbers from triplicates of each condition. MockHo916: mock Ho916ncp, MockKy1203: mock Ky1203ncp. Error bars represent standard deviations (SD). cDNA copy numbers could only be determined in one of the triplicates for Animal 1 mock Ho916ncp and mock Ky1203ncp, hence absence of error bars.*

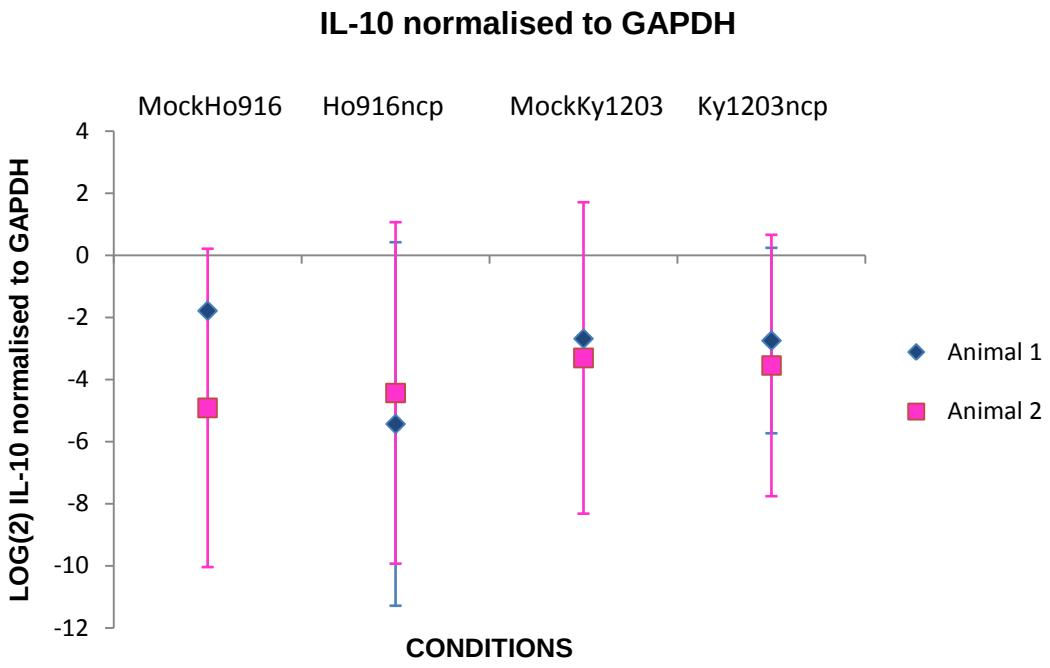



*Figure 3.15 Dot plot showing mean quantities of IL-10 cDNA normalised to mean quantities of GADPH to correct results skewed by differing amounts of input cDNA. MockHo916: mock Ho916ncp, MockKy1203: mock Ky1203ncp. Error bars represent standard deviations. cDNA copy numbers could only be determined in one of the triplicates for Animal 1 mock Ho916ncp and mock Ky1203ncp, hence absence of error bars.*

Any relative difference in mRNA expression levels of IL-10 between mDCs stimulated with Ho916ncp compared with Ky1203ncp could not be accurately assessed due to the high level of variability in the assay.

# DISCUSSION

Little is known about why clinical signs following transient acute infection with BVDVncp type 1 vary between strains. The interaction between a virus and the host's immune system is clearly of great importance for the outcome of infection. Thus, it is interesting to investigate how BVDV strains associated with different clinical signs influence early immune responses. BVDV has a predilection for infecting cells of the immune system including APCs *(Sopp et al 1994 and Glew et al 2003)*. DCs are considered the most important APC *in vivo* due their efficacy in stimulating naïve T-cells *(Steinmann and Hemmi 2006)*. The key role of this cell type in shaping the subsequent adaptive immune response makes studying viral interactions with DCs most important.

## 4.1 Generation of mDCs

Due to the small number of circulating DCs, *(Averill et al 2007)* methods to derive DCs from more accessible precursors have been developed. One such method is isolating monocytes from peripheral blood and culturing them in the presence of IL-4 and GM-CSF to differentiate them into mDCs *(Sallusto and Lanzavecchia 1994)*. mDCs generated from monocytes in peripheral blood offers a well established *in vitro* model for DC function *(Glew et al 2003, Barnes et al 2008)* and was therefore used in the present study.

Enrichment of monocytes by sorting $CD14^+$ cells from PBMCs did not generate a cell population of similar purity as previously achieved in the lab using the same technique. The reduction in purity could depend on the microbeads used for the sorting process since they had reached the end of their shelf-life. However, even though the FSC/SSC analysis presented as a dot plot shows a rather non-homogenous cell population, a proportion of cells were indeed CD14 positive as demonstrated by FITC-CD14 staining. To generate mDCs $CD14^+$ cells were cultured for 6 days in the presence of rboIL-4 and rboGM-CSF. However, despite the fact that low binding plates were used, cells adhering to the plastic could be seen by day 1. These cells are most likely monocytes since lymphocytes do not adhere when grown in culture. Indeed, another method to enrich monocytes from PBMCs is to isolate adherent cells by removing cells in suspension by washing *(Werling et al 1999)*. Some contaminating non-adherent cells (different subsets of lymphocytes) can therefore be presumed to be lost during the culturing procedure which includes exchange of culture media.

Light microscopy was used to study changes in cell morphology during culturing with cytokine enriched medium. The morphology of the majority of cells by day 6, characterised by a typical large cytoplasm with cytoplasmic protrusions, was consistent with previous descriptions of bovine mDC features *(Yamakawa 2007).*

High levels of CD14 expression is commonly used as a marker for monocytes. In contrast, CD14 expression is considerably lower on mDCs *(Beekhuizen et al 1991, Werling et al 1999, Bajer et al 2003, Yamakawa et al 2008)*. Consistent

with this, FACS analysis regarding CD14 expressions on cells after 6 days in culture with rboIL-4 and rboGM-CSF showed reduced CD14 expression compared to freshly enriched monocytes. It would have been desirable to have analysed a higher and more uniform number of cells but due to the limited amount of mDCs recovered this was not possible.

Taken together, the phenotype of cultured cells analysed by studying morphology and CD14 expression is comparable with the phenotype previously described for bovine mDCs.

## 4.2 TLR expression

Expression of TLRs on mRNA and protein level varies dependent upon the cell subset and stimulation. Some TLRs are more ubiquitously expressed whereas other TLRs can be restricted to one cell type *(Janssens and Beyaert 2003).*

The result from screening mRNA expression of TLRs in mDCs was largely consistent between the 3 animals tested but differs somewhat from pre-existing data. In contrast to the results in the present study, bovine mDCs have previously been reported to express TLR5 but not TLR3 and TLR7 *(Werling et al 2006).* However, little is still known about the regulation of TLR expression. Thus, differences described for bovine mDCs may be due to differential exposure to factors influencing TLR expression levels, including animal housing, age, breed and also differences in how the cells were treated during culturing. It should also be kept in mind that the absence of mRNA expression at a certain time point may not necessarily indicate that the corresponding protein is not expressed. Discrepancy in results for expression of a specific TLR could therefore also be explained by a difference in transcription levels for the targeted TLR at the time point analysed *(D. Werling, personal communication).*

Examining the expression of TLRs at the mRNA level in mDCs aids the understanding of how these cells can sense and respond to different pathogens. Presence of TLR3, TLR7 and TLR8 in mDCs indicates that viral infection has the potential to activate antiviral responses via TLR-dependent signalling pathways.

## 4.3 Susceptibility to infection

mDCs were infected with Ho916ncp and Ky1203ncp at a MOI of 0.1 and infection detected by IPX staining for BVDV. Positive cytoplasmic staining examined by light microscopy was observed in mDCs infected with both Ho916ncp and Ky1203ncp. Not all cells were infected as seen in Figure 3.7 and no obvious difference between the relative numbers of cells infected was observed by visual inspection.

Due to staining difficulties a result (positive versus negative) could only be read from 2 out of 3 repeats. The third repeat had a high level of background staining. Cells stained rather intensively could be seen in the periphery of the wells.

However, this staining had a less distinct cytoplasmic confinement. This staining pattern could be due to inadequate washing of the wells leaving residual secondary antibody along the edges. It would have been desirable to repeat the study for this animal but due to the time limit this was not an option.

Anti-BVDV hyperimmune serum is routinely used in the lab for examining presence of virus in buffy coat samples by an immunofluorescence assay (IFA) analysed by fluorescent microscopy. This method is not more specific since it still utilises polyclonal antibodies but the fluorescent staining with a secondary anti-bovine Cy3 labelled antibody might be easier to read than the brown staining generated from IPX. IFA may therefore be a better option than IPX. To reduce unspecific staining a method using monoclonal antibodies against a specific viral protein could also be employed.

The results of experimental infection of mDCs with Ho916ncp and Ky1203ncp are consistent with previously published data *(Glew et al 2003)* indicating that mDCs are susceptible to BVDVncp infection. It would be interesting to quantify and compare the magnitude of infection and viral replication between Ho916ncp and Ky1203ncp. A study comparing the level of infection of mDCs between BVDV strains belonging to genotype 1 and 2 revealed a higher magnitude of infection for the type 2 strain (a field isolated called KE13) *(Glew 2000).* It is possible that this differential ability of BVDV strains of different genotypes to infect mDCs also applies to strains within the BVDV 1 group. Theoretically, the ability of a strain to infect mDCs at a higher degree could coincide with a greater impact on mDC function. To identify a potential difference between BVDVncp strains in their capability to infect mDCs, BVDV infected cells could be stained with a fluorochrome conjungated antibody followed by flow cytometric analysis to compare the relative numbers of cells infected by Ho916ncp and Ky1203cp. To investigate any difference in viral replication an assay could be set up where mDCs infected with Ho916ncp and Ky1203ncp are lysed, or the supernatant collected at set time points p.i. (for example 24, 48, 72 and 96 hrs) with the purpose of comparing the virus titre in cell lysates/supernatants from each time point.

Susceptibility of mDCs to BVDV implicates that BVDV has the potential to modify the functions of this important immune cell. Infection and dysregulation of DC functions has been shown for many viruses able to establish chronic infections or with known immunosuppressive effects, for example measles virus *(Fugier-Vivier et al 2005)*, HCV *(Kanto et al 1999, Averill et la 2007)* and human immunodeficiency virus *(Macatonia et al 1990)*. mDCs infected with BVDVncp have however recently been shown not to be compromised in their ability to present antigen and induce BVDV specific $CD4^+$ and $CD8^+$ T-cell proliferation *(Glew et al 2001)*. To date, other than IFNα/β, cytokine data from mDCs infected with BVDVncp is lacking.

## 4.4 IFNα/β production

IFNα/β is a key component in the first line of defense against viral infections, inducing an anti viral state in cells subject to infection and affecting the development of adaptive immunity. Mammalian cells have evolved a number of mechanisms whereby viral infection can be sensed and activate cellular signalling to stimulate production of IFNα/β, but at the same time many viruses have evolved counter mechanisms to subvert these defences *(Garcia-Sastre and Brion 2006).*

Neither Ho916ncp nor Ky1203ncp induced strong production of interferon from mDCs. Small amounts of IFNα/β, less than a tenfold of the amount of IFNα/β detected for the positive control poly(I:C), were detected from animal 3 in response to Ho916ncp and in mock treated cells for animal 2 but only in one of the duplicates for each condition (Figure 3.9 B). One possible explanation would be a cross-contamination from a sample with poly(I:C) stimulated cells. Care was however taken to minimize this risk by setting up the plates with a row of empty wells separating each condition. To find out if Ho916ncp does indeed stimulate IFNα/β production from mDCs the number of technical as well as biological repeats would need to be increased.

The amount of IFNα/β in cell lysates from mDCs 48 hrs p.i. was analysed by a CAT assay *(Fray et al 2001).* Due to the possible remaining presence of IFNα/β stimulating ligands in mDC supernatants, an experiment was designed to assess the ability of MBDKt2-cells to induce IFNα/β in response to the ligands. MDBKt2-cells did respond to direct stimulation with poly(I:C) but not to any other ligand.

Since some data report rapid degradation of poly(I:C) in serum *(De Clercq 1979),* poly(I:C) was pre-incubated for 48 hrs at 37°C, 5% $CO_2$ in MDBKt2 culture media (containing foetal calf serum) before addition to the MDBKt2-cells to mimic the conditions for the mDC stimulation assay. The response to poly(I:C) was however not affected by this (data not shown).

The amount of IFNα/β produced from MDBKt2-cells in response to poly(I:C) correlates with the amounts registered for mDCs stimulated with poly(I:C). It is therefore not possible to determine if any of the IFNα/β detected by the assay originated from the mDCs or was in fact due to poly(I:C) in the supernatant from mDC cultures. A well designed positive control is of particular importance when results are negative as in this case. However, since WB data confirm mDC responsiveness to viral infection and the CAT-ELISA did pick up presence of interferon from poly(I:C) stimulation, the negative results for IFNα/β production from mDCs stimulated with Ho916ncp and Ky1203ncp can be regarded as real. It is therefore crucial for subsequent experiments to identify a positive control which induces IFNα/β in mDCs but not in MDBKt2-cells. Such a control could be based on the differences in PRR expression between mDC and MDBKt2-cells, which seem to lack TLR7 and TLR8 expression. Another approach would be to choose a different cell line for transfection with the plasmid expressing the human *Mx* promoter linked to the CAT reporter gene (e.g. human embryonic kidney cells

which have a very limited TLR expression). Even so, in both cases it is not known whether other intracellular PRRs may compensate for a lack of TLR expression. However, the most promising alternative would be the development of an IFNα/β ELISA to directly analyse IFNα/β production in the supernatants.

The lack of IFNα/β production from BVDVncp stimulated mDCs is consistent with previously published results *(Glew et al 2003)*. Since mDCs have been shown to be susceptible to infection and to express TLRs capable of recognising viral nucleic acids (TLR3, 7 and 8), the lack of IFNα/β can be presumed to depend on the virus actively evading TLR-dependent as well as TLR-independent induction of, or an active suppression of IFNα/β production. Several viruses belonging to the *Flavivridae* family (CSFV, WNV and HCV) are known to interfere with the IFNα/β response by affecting the functions of IRF3, a transcription factor pivotal for inducing IFNα/β production *(Baigent et al 2002, Fredricksen et al 2004, La Rocca et al 2005, Li et al 2005, Hilton et al 2006).* Exactly how this effect is mediated for BVDVncp is not known, but inhibition of IFNα/β has been shown to depend on proteins coded by the viral $E^{rns}$ and $N^{pro}$ genes *(Meyers et al 2007)*. The product of $E^{rns}$ is a secreted glycoprotein with the ability to bind and degrade dsRNA thereby preventing it from activating dsRNA-induced signalling *(Iqbal et al 2004)* and the protease encoded by $N^{pro}$ has been shown to block the function of IRF3 *(Hilton et al 2006, Gil et al 2006)*.

## 4.5 Regulation of IFNα/β production through IRF3 and IRF7

IRF3 is a pivotal transcription factor for IFNα/β expression and is constitutively expressed in an inactive form. In contrast to IRF3, IRF7 is only expressed at very low levels in most cell types (IRF7 expression in pDCs is however constitutively high) but is induced by IFNα/β or TNFα. IRF3 and IRF7 are activated by phosphorylation, an event induced by signalling through TLRs recognising viral nucleic acid or the cytosolic dsRNA sensors RIG-I and MDA5 (Figure 1.4 and 1.5). Upon activation IRF3 and IRF7 forms homo- or heterodimers and translocate to the nucleus where they bind DNA. IRF3 is only able to stimulate expression of a limited number of type I IFN genes whereas IRF7 induces expression of a broader spectrum of type I IFN isotypes *(Lin et al 1998, Paun A. and Pitha P. M. 2007)*. To further investigate how Ho916ncp and Ky1203ncp interact with the IFNα/β response in mDCs protein levels of IRF3 and IRF7 were investigated by WB.

The results in Figure 3.10 A and 3.11 A show a difference in IRF3 and IRF7 protein concentration after incubation of mDC with either Ho916ncp or Ky1203ncp. WB results from mDCs treated with Ho916ncp show intense bands of the correct sizes for IRF3 and IRF7 compared to Ky1203ncp and controls.

The high protein levels of IRF3 in mDCs stimulated with Ho916ncp indicating cytoplasmic or nuclear accumulation could be due to a specific effect of Ho916ncp or be dependent on a difference in kinetics between the strains, with Ky1203ncp causing the same accumulation at another time point. Based on this a

time course study of IRF3 protein levels in mDCs infected with Ho916ncp and Ky1203ncp should be undertaken.

In contrast to the results obtained from mDCs stimulated with Ho916ncp, others have demonstrated a gradual decrease in IRF3 protein levels (inline with results for Ky1203ncp in the present study) in calf testis cells from 6 hrs to 72 hrs p.i. *(Hilton et al 2006)* with the BVDVncp strain pe515. The protein level of IRF3 was not shown to be upregulated at any time point in this study.

However, the turnover of IRF3 could be different in mDCs in response to BVDVncp infection than in cells previously studied. A study comparing the effect of Newcastle virus infection on IRF3 and IRF7 protein stability in immortalized embryo fibroblasts and lymphoid tissue (thymus and spleen) from mice show a decrease in IRF3 and IRF7 stability in the fibroblasts whereas IRF7 protein stability was increased (shown to be partly IFNα/β-independent) in lymphoid tissue and IRF3 stability was unaffected *(Parkash and Levy 2006)*. This suggests a potential difference in the affect of viral infection on IRF3 and IRF7 turnover between different cell types.

It would therefore be interesting to perform a parallel time course study for IRF 3 in non-immune cells infected with Ho916ncp and Ky1203ncp to see if the effect on IRF3 levels is a feature of mDCs.

The decrease in IRF3 has been suggested to be a part of the mechanism whereby BVDVncp blocks IRF3 function. $N^{pro}$ has been shown to enhance proteosomal degradation of IRF3 and to inhibit IRF3 from binding DNA. Published data is however inconsistent regarding the latter of these two effects *(Baigent et al 2002, Hilton et al 2006, Chen et al 2007)*. In view of the fact that no considerable amounts of IFNα/β could be detected in mDC supernatants 48 hrs p.i. with Ho916ncp despite high IRF3 protein levels, degradation of IRF3 is unlikely to be the only means whereby IFNα/β production is inhibited by this BVDV strain.

In conclusion, the data of the present study indicate that BVDVncp may interact in more than one way with the IRF3 signalling pathway, leading even to its stabilisation and that this interaction may depend on cell type and/or strain of BVDVncp studied.

IRF7 expression is normally very low in most cell types *(Paun A. and Pitha P. M. 2007)* and accordingly no IRF7 was detected in stimulated mDCs with one exception; mDCs stimulated with Ho916ncp from animal 2. Since IRF7 expression is induced by IFNα/β a possible explanation for this could be that Ho916ncp does stimulate production of IFNα/β but at levels too low to allow detection by the CAT-ELISA used. However, if this was the case one would expect IRF7 expression in animal 3 for which a small amount IFNα/β indeed was detected (Figure 3.9B) but no IRF7 expression was detected in these mDCs. Due to differences in results between animal 2 and 3 the effect of Ho916ncp on IRF7 is uncertain and the experiment should be repeated, ideally coupled with a more sensitive IFNα/β assay.

β-actin was used as a loading control to visualise that the same amount of protein was loaded in each well, but the WB results showed variable band-intensities, as can be seen in Figure 3.10 B and 3.11 B. Interestingly, lower levels of β-actin were present in samples generated from mDCs treated with either poly(I:C), Ho916ncp and Ky1203ncp. These discrepancies can potentially be explained through either loading errors or an effect of these three agents on β-actin protein levels. Whereas the first effect potentially can be excluded, as all samples were analysed and adjusted to the same concentration, the second possibility can only be excluded through use of another loading control with stable protein levels in all conditions. It is striking however that poly(I:C), Ho916ncp and Ky1203ncp all share the potential to activate cellular responses to dsRNA. Recently, poly(I:C) has been shown to induce IFNα/β-induced apoptosis in bovine turbinate cells *(Schweizer and Peterhans 2001)*, which potentially could explain the reduced β-actin levels seen. In contrast, BVDVncp strains have been shown to protect cells from IFNα/β-mediated apoptosis induced by dsRNA dependent signalling *(Schweizer and Peterhans 2001)*. Moreover, no notable amount of IFNα/β was detected from mDCs stimulated with Ho916ncp and Ky1203ncp. Thus, the reduced β-actin content in samples prepared from mDC incubated with either BVDVncp strain in present study can not be explained with cellular apoptosis induced by IFNα/β.

Another possibility would be an IFNα/β-mediated down regulation of protein expression caused by autocrine actions of IFNα/β produced by stimulated mDCs. A IFNα/β response was however only detected for poly(I:C). However, it cannot be excluded that mDCs stimulated with Ho916ncp and Ky1203ncp did induce IFNα/β at levels at levels too low to allow detection by the IFNα/β detection assay used. The only possibility to explore this further would be by using an assay with a higher sensitivity compared to the CAT-ELISA used in the present study.

Even if β-actin levels are considered as a true indicator of the amount protein loaded, the increase of IRF3 and IRF7 in mDCs stimulated with Ho916ncp is still convincing since the amount of protein seems lower compared to mock infected mDCs. The apparent decrease in IRF3 in Ky is however more uncertain since total protein levels also appear lower. Another loading control for which the protein expression level is stable in all conditions has to be employed to asses these results.

## 4.6 IL-10 and IL-12

IL-10 and IL-12 are two cytokines secreted by mDCs which have important effects on the developing adaptive immune response. IL-10 supports the development of a Th2-response, leading potentially to an increased humoral response. Furthermore, IL-10 impedes the development of a Th1-type of response by blocking IL-12 supported IFNγ production, as well as down regulates production of proinflammatory cytokines such as TNF-α, IL-1 and IL-6. IL-10 also inhibits APC maturation *(Dumoutier and Renauld 2002)*. IL-12 is a heterodimeric cytokine composed of the subunits p35 and p40 with a key role in

the development of Th1-responses by increasing production of cytokines such as IFNγ *(Airoldi et al 2002)*. To date, no data are available regarding the effect of BVDVncp on expression of IL-10 and IL-12.

Unfortunately no reliable data were obtained in the present study to assess the effect of BVDVncp on relative mRNA expression levels for IL-10 and IL-12 by qPCR. Neither of the BVDVncp strains seems to induce IL-10 mRNA expression levels but due to the great assay variability, these results are highly questionable.

The no template control for IL-12 was contaminated and gave a signal higher than the signal detected for several samples. The experiment is therefore invalid and has to be repeated taking greater care to avoid contamination. The variation between triplicates in all parametes analysed (IL-10, IL-12, GADPH, RPLPO) was also greater than acceptable. Here, identical triplicates should have a $C_T$ SD <0.3 according to manufacturer's guide (Applied Biosystems, USA). Undetermined and variable values suggest lack of/variable amounts of cDNA template or failure of the PCR reaction to proceed due to presence of inhibitory factors. Though possible to reduce, inhibitory factors are inherently difficult to eliminate completely *(Nolan et al 2006 a)*. Pipetting accuracy can however be increased by frequent calibration of pipettes, practise and by increasing the volumes pipetted.

To acquire accurate relative quantification of the cDNA target between conditions the quantity of the targeted cDNA must be normalised to correct for potential differences in total cDNA amount. One way of doing this is by normalising target cDNA levels to the levels of a stable endogenous control gene (commonly referred to as housekeeping genes). The quantity of these should be relative to the input of cDNA and hence not be affected by differences in treatment between conditions. Appropriate endogenous controls must therefore be selected and tested for each study *(Nolan et al 2006 b)*. Stability of GADPH and RPLPO had not been tested in advance for this specific study and expression were found to be highly variable between animals and conditions. However, since levels of GADPH and RPLPO expression are strongly variable between the two animals for the same condition (Figure 3.12, 3.13), the main determinant for variability in expression levels might not be the treatment, but may be due to different amounts of cDNA. In an attempt to investigate this, the cDNA concentration was re-evaluated using the NanoDrop® ND-1000 Spectrophotometer as well as the Eppendorf Biophotometer. Results (Appendix 3) were quite variable between samples compared to the expected and originally adjusted concentration of 100 ng $\mu l^{-1}$.

In conclusion the experiment regarding the impact of a BVDVncp infection on cytokine expression of mDC must be repeated with appropriate stable reference genes, more uniform amounts of cDNA and avoiding contamination of samples. Since qPCR is an extremely sensitive technique relying on many steps which can create variability a good normalisation strategy must be followed and results should ideally be backed up by another assay, such as ELISAs for IL-10 and IL-12 quantifying protein levels of each cytokine in the supernatant.

## 4.7 Finial conclusions and future work

The aim of this project was to compare cytokine production from bovine mDCs stimulated with the BVDVncp type 1 strains Ho916ncp and Ky1203ncp with the hypothesis that these two strains associated with different clinical outcomes would also differ in the cytokine response they induce from mDCs.

mDCs did not produce any notably levels IFNα/β at 48hrs p.i. in response to Ho916ncp and Ky1203ncp and hence no convincing difference between the viruses could be demonstrated. This could be due to a viral block of the IFNα/β response in mDCs but since only one time point was investigated this can not be deduced from this study. The lack of IFNα/β at 48hrs p.i. is consistent with existing data *(Glew et al 2003)* but a time course study is yet to be undertaken.

A difference between the strains was however detected in their interaction with IRF3. mDCs infected with Ho916ncp showed an accumulation of IRF3 in contrast to Ky1203ncp which seemed to decrease IRF3 protein levels. This indicates that BVDVncp may interact in more than one way with the IRF3 signalling pathway, leading even to stabilisation of IRF3 and that this interaction may depend on cell type and/or strain of BVDVncp studied.

The ability of BVDVncp to subvert IFNα/β production is regarded central for establishment of persistent infection in the early foetus. In contrast to *in vitro* studies and BVDVncp infection of the early foetus, BVDVncp infection of calves, pregnant cows and foetuses in late gestation results in production of IFNα/β *(Charlston B. et al 2002, Smirnova et al 2008).* This contradicting data *in vivo* and *in vitro* can be explained by the presence of a cell subset *in vivo* in which BVDVncp does not block IFNα/β production. Some studies describe cDCs as an important source of IFNα/β in response to viral infection *(Diebold et al 2003).* However, the data presented in this study do not suggest that cDCs are the cell type responsible for IFNα/β production in response to BVDV *in vivo*. The cell type responsible for IFNα/β production demonstrated *in vivo* is more likely the not yet fully characterised bovine equivalent of pDCs, as postulated by *Brackenbury et al 2005*. In parallel to experiments performed with mDCs in this study, monocytes and lineage negative cells ($LIN^-$cells, the potential bovine equivalent of pDCs) generated from the same animals have been treated as described for mDCs. Indeed, stimulation of $LIN^-$cells with BVDVncp did result in production of IFNα/β detected at 48 hrs *(A. Stalker, personal communication),* with Ho916ncp inducing more IFNα/β compared to Ky1203ncp.

BVDVncp strains of high virulence show a more widespread distribution than low virulent strains and severe clinical signs have been shown to correlate with a higher level of viremia *(Walz 2001).* It can be hypothesized that the outcome of BVDVncp acute infections is determined by a battle between viral spread and the host's immune reaction. An initial difference in how BVDVncp strains interact with innate immune functions might therefore be central in determining the outcome of infection. The difference in the IFNα/β response between Ho916ncp and Ky1203ncp which has been demonstrated for pDCs now asks for further characterisation before one can speculate about potential consequence on

subsequent immune events and ultimately systemic effects explaining differences in clinical signs.

To begin with, it must be investigated if the difference in amount of IFNα/β from pDCs lies in a different capability of the two strains to induce IFNα/β or in different kinetics of IFNα/β induction. Is Ky1203ncp able to produce the same amount of IFNα/β at another time point and if so, which strain is able to delay IFNα/β production in comparison to the other? A time course study needs to be undertaken to address these questions and ideally, such a study should be set up for a greater number of animals. It would also be interesting to distinguish how pDCs differ in their interaction with BVDVncp compared with other cell types, enabling them to produce IFNα/β in response to stimulation.

Finally, with respect to the key role of IFNα/β in the innate antiviral immunity it is important to further distinguish the interactions of Ho916ncp and Ky1203ncp with the IFNα/β response. Nonetheless, development of an appropriate adaptive immune response leading to efficient viral clearance is complex and influenced by several factors including interaction of additional cytokines produced by APCs. To create a somewhat more complete picture of how BVDVncp strains affect early immune events their affect on additional cytokine responses need to be investigated. As a first step towards this, IL-10 and IL-12 production from mDCs could be reassessed.

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## APPENDIX 1

### *Buffers and Reagents*

*Acid Citrate Dextrose (ACD)*

| | |
|---|---|
| Citric Acid Monohydrate (Sigma Aldrich UK) | 4.0 g |
| Sodium Citrate (Sigma Aldrich, UK) | 11.3 g |
| D(+)-Glucose (Sigma Aldrich, UK) | 11.0 g |

Dissolved in 500 ml of $ddH_20$ and sterile filtered (0.22 μm)

*D (+) Glucose 0.3 mM stock*

| | |
|---|---|
| D (+) Glucose (Sigma Aldrich, UK) | 29.73 g |

Dissolved in 500ml $ddH_20$ and stored at 4˚C for 1 month.

*Ammonium-Chloride Lysis Buffer*

| | |
|---|---|
| Ammonium Chloride (BDH, UK) | 8.29 g |
| Potassium Hydrogen Carbonate (Sigma Aldrich, UK) | 1.0 g |
| EDTA disodium salt (Sigma Aldrich, UK) | 0.037 g |

Dissolved in 1 L of $ddH_20$, adjusted to pH 7.2 and sterile filtered (0.22 μm)

*PBS/1% BSA*

| | |
|---|---|
| 1X PBS w/o $MgCl_2$ and $CaCl_2$ pH 7.2 (Sigma Aldrich, UK) | 500 ml |
| Bovine Serum Albumin Fraction V (PAA, Austria) | 5.0 g |

Dissolved together with gentle heating (30˚C) and mixing, sterile filtered (0.22 μm)

*FACSFlow/1%BSA*

| | |
|---|---|
| BD FACSFlow™ Sheath Fluid (BD Biosciences, UK) | 500 ml |
| Bovine Serum Albumin Fraction V (PAA, Austria) | 5.0 g |

Dissolved together with gentle heating (30˚C) and mixing, sterile filtered (0.22 μm)

*mDC differentiation media*

| | |
|---|---|
| RPMI 1640 + Glutamax I + 25 mM HEPES (Invitrogen, UK) | 90 ml |
| FBS (PAA, Austria) | 10 ml |
| Gentamycin (10 mg $ml^{-1}$) (Sigma Aldrich) | 0.5 ml |
| Sodium Pyruvate (1 M) (Sigma Aldrich) | 0.1 ml |
| β-mercaptoethanol (0.005 M) (Sigma Aldrich) | 0.1 ml |
| Recombinant bovine IL-4 | 1.0 ml |
| Recombinant bovine GM-CSF | 1.0 ml |

Bovine recombinant GM-CSF and IL-4 were cloned in house and subsequently expressed in Chinese hamster overy (CHO) cells produced at the Moredun Research Institute, Penicuik, UK and the Institute for Animal Health, Compton, UK respectively.

Prepared in laminar flow in sterile 100ml glass bottles

*MDBKt2 Growth Media*

| | |
|---|---|
| DMEM + Glutamax I + Pyruvate (Invitrogen, UK) | 90 ml |
| FBS (PAA., Austria) | 10 ml |
| Pen/Strep (Sigma Aldrich, UK) (5000IU $ml^{-1}$/ 5mg $ml^{-1}$) | 2.0 ml |
| Blasticidin S HCl (Invitrogen, UK) (10mg $ml^{-1}$) | 0.1 ml |

Prepared in laminar flow in sterile 100 ml glass bottles

*MDBKt2 Assay Media*

| | |
|---|---|
| DMEM + Glutamax I + Pyruvate (Invitrogen, UK) | 98 ml |
| FBS (PAA, Austria) | 2.0 ml |
| Pen/Strep (Sigma Aldrich, UK) (5000 IU $ml^{-1}$/ 5mg $ml^{-1}$) | 2.0 ml |
| Blasticidin S HCl (Invitrogen, UK) (10 mg $ml^{-1}$) | 0.1 ml |

Prepared in laminar flow in sterile 100 ml glass bottles

*10% Growth Medium MDBK*

| | |
|---|---|
| MEM (Invitrogen, UK) | 500 ml |
| FBS (PAA, Austria) | 50 ml |
| L- Glutmamine (200 mM) (PAA, Austria) | 5 ml |
| Pen/Strep (5000 IU $ml^{-1}$/5 mg $ml^{-1}$)(Invitrogen, UK) | 5 ml |

*2% Maintenance Medium MDBK*

| | |
|---|---|
| MEM (Invitrogen, UK) | 500 ml |
| FBS (PAA, Austria) | 5 ml |
| L- Glutmamine (200 mM) (PAA, Austria) | 5 ml |
| Pen/Strep (5000 IU $ml^{-1}$/5 mg $ml^{-1}$)(Invitrogen, UK) | 5 ml |

*PBS-T (0.05%) stock*

| | |
|---|---|
| 10X PBS in ddH20 (Invitrogen, UK) | 1000ml |
| Tween20 | 0.5 ml |

*PBS-T 5%NRS*

500 ml PBS-T stock as above with addition:

| | |
|---|---|
| Normal Rabbit Serum (NRS) (Invitrogen, UK) | 25 ml |

Aliquoted to 50 ml and stored at -20˚C

*PBS/1% BSA/0.1% $NaN_3$*

| | |
|---|---|
| 1X PBS w/o $MgCl_2$ and $CaCl_2$ (Sigma Aldrich, UK) | 500 ml |
| Bovine Serum Albumin Fraction V (PAA, Austria) | 5.0 ml |
| Sodium Azide (10%) in $ddH_20$ (Sigma Aldrich) | 5.0 ml |

BSA dissolved in PBS with gentle heating (30˚C) prior to addition of Sodium Azide

*5X Laemmli Sample Buffer*

| | |
|---|---|
| Tris/Glycine Running Buffer 1X (Bio-Rad, UK) | 6.25 ml |

| | |
|---|---|
| 30% Glycerol (Sigma Aldrich, UK) | 16.5 ml |
| 10% SDS (Fluka, Sigma Aldrich, UK) | 20 ml |
| B-mercaptoethanol (Sigma Aldrich, UK) | 5 ml |
| 1% Bromophenol Blue (Sigma Aldrich, UK) | 1 ml |

*30% Glycerol*

| | |
|---|---|
| Glycerol (100%) (Sigma Aldrich) | 3 ml |
| Molecular Biology Grade $H_20$ (Sigma Aldrich) | 7 ml |

*PBS-T/5% non-fat milk*

| | |
|---|---|
| 10X PBS w/o $MgCl_2$ and $CaCl_2$ (Invitrogen, UK) | 50 ml |
| dd$H_20$ | 450 ml |
| Non-fat dried milk powder (Marvel, UK) | 5 g |
| Tween20 (Sigma Aldrich, UK) | 0.25 ml |

## APPENDIX 2

### *Plasmid details*

*TLR1*

Bovine TLR1 full length construct (Accession Number: AY634638) in pCR2.1 TOPO (Invitrogen, UK) from Tracey Coffey, Institute of Animal Health, Compton as part of the Genesis Faraday TLR Consortium.

*TLR2*

Full length bovine TLR2 construct (Accession Number: AY634629) in pCR2.1 TOPO (Invitrogen, UK) by D. Werling, Royal Veterinary College, UK.

*TLR3*

Full length bovine TLR3 construct (Accession Number: AY821206) in pCR2.1 TOPO (Invitrogen, UK) from Tracey Coffey, Institute of Animal Health, Compton as part of the Genesis Faraday TLR Consortium.

*TLR4*

Full length bovine TLR4 construct (Accession Number: AY634630) in pcDNA3.1/V5-his (Invitrogen, UK) by D. Werling, Royal Veterinary College, UK.

*TLR5*

Bovine TLR5 full length construct (Accession Number: AY634631) in pCR2.1 TOPO (Invitrogen, UK) from Tracey Coffey, Institute of Animal Health, Compton as part of the Genesis Faraday TLR Consortium.

*TLR6*

Full length bovine TLR6 (Accession Number: AY487803) in pCR2.1 TOPO (Invitrogen, UK) from Tracey Coffey, Institute of Animal Health, Compton as part of the Genesis Faraday TLR Consortium.

*TLR7*

Bovine TLR7 full construct (Accession Number: AY487802) in pCR2.1 TOPO (Invitrogen, UK) from Tracey Coffey, Institute of Animal Health, Compton as part of the Genesis Faraday TLR Consortium.

*TLR8*

Bovine TLR8 full length construct (Accession Number: AY642125) in pCR2.1 TOPO (Invitrogen, UK) from Tracey Coffey, Institute of Animal Health, Compton as part of the Genesis Faraday TLR Consortium.

*TLR9*

Full length bovine TLR9 (Accession Number: AY859726) in modified pASK-IBA1 (893plasmid) from Hans-Martin Seyfert.

*TLR10*

Full length bovine TLR10 (Accession Number: AY634632) in pCR2.1 TOPO (Invitrogen, UK) from Tracey Coffey, Institute of Animal Health, Compton as part of the Genesis Faraday TLR Consortium.

*β-actin*

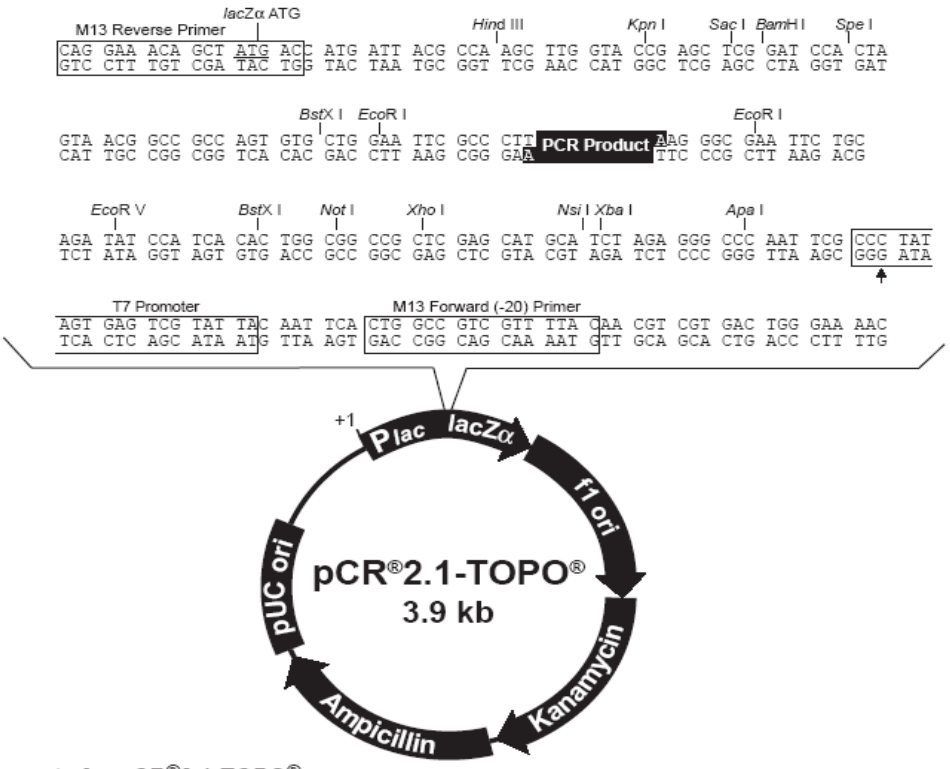
Bovine β-actin full length construct (Accession Number: AY141970) in pCIneo (Promega, UK) by D. Werling, Royal Veterinary College, UK.

*5'UTR BVDV*

5'UTR sequence from BVDV type 1 strain NADL (Accession Number: M31182) in pGEM-T (Promega, UK) from Carole Thomas, Royal Veterinary College, UK.

***Plasmid Maps***

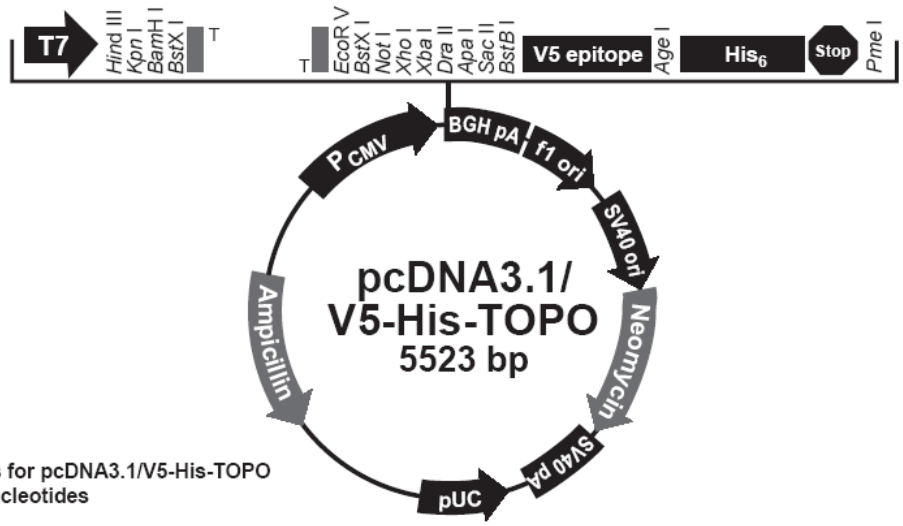
*pCR2.1 TOPO*




**Comments for pCR®2.1-TOPO®**
**3931 nucleotides**

*LacZα* fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

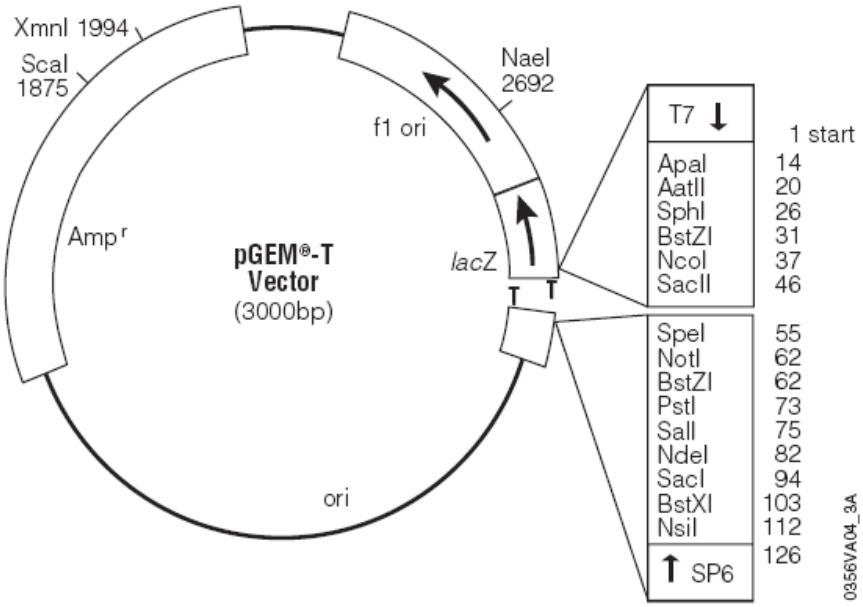
*pcDNA3.1V5-His*



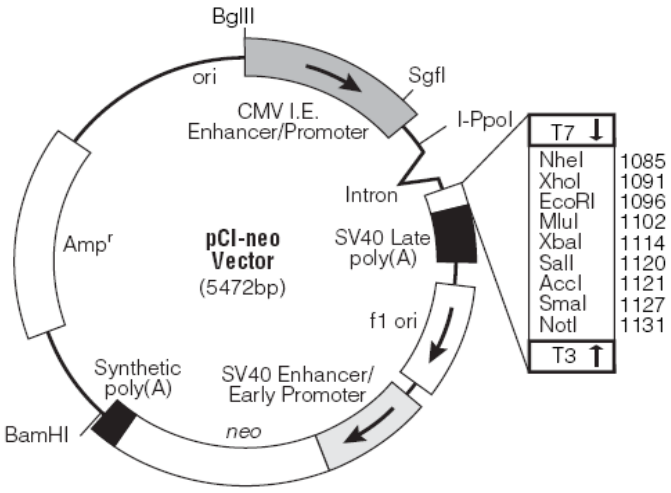

**Comments for pcDNA3.1/V5-His-TOPO**
**5523 nucleotides**

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 902-1019
TOPO® Cloning site: 953-954
V5 epitope: bases 1020-1061
Polyhistidine tag: bases 1071-1088
BGH reverse priming site: bases 1111-1128
BGH polyadenylation signal: bases 1110-1324
f1 origin of replication: bases 1387-1800
SV40 promoter and origin: bases 1865-2190
Neomycin resistance gene: bases 2226-3020
SV40 polyadenylation signal: bases 3039-3277
pUC origin: bases 3709-4382
Ampicillin resistance gene: bases 4527-5387

*pGEM-T plasmid*




| | |
|---|---|
| T7 RNA polymerase transcription initiation site | 1 |
| multiple cloning region | 10–113 |
| SP6 RNA polymerase promoter (–17 to +3) | 124–143 |
| SP6 RNA polymerase transcription initiation site | 126 |
| pUC/M13 Reverse Sequencing Primer binding site | 161–177 |
| *lac*Z start codon | 165 |
| *lac* operator | 185–201 |
| β-lactamase coding region | 1322–2182 |
| phage f1 region | 2365–2820 |
| *lac* operon sequences | 2821–2981, 151–380 |
| pUC/M13 Forward Sequencing Primer binding site | 2941–2957 |
| T7 RNA polymerase promoter (–17 to +3) | 2984–3 |

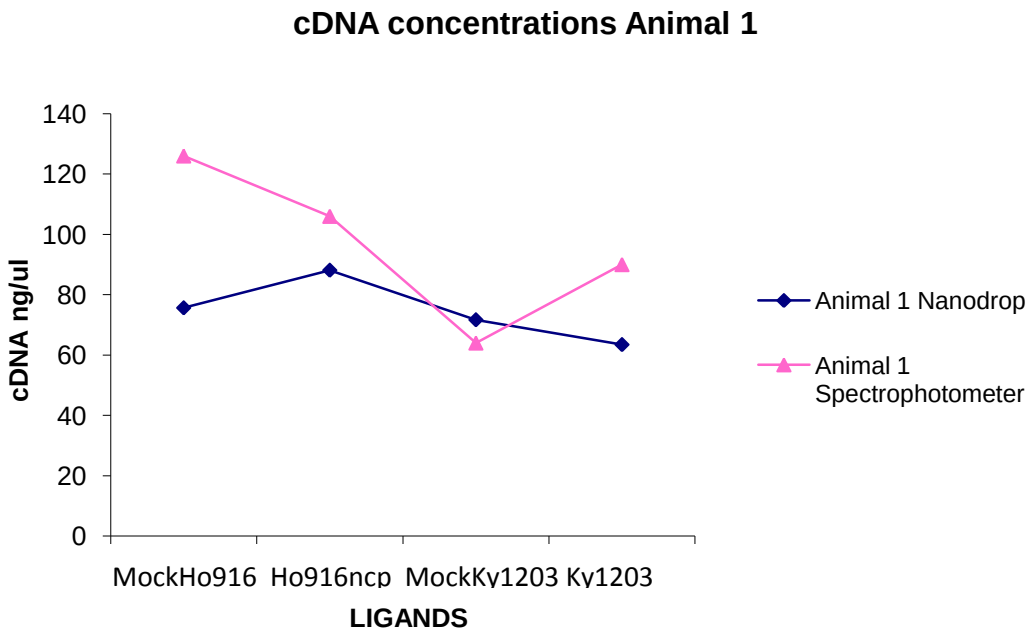


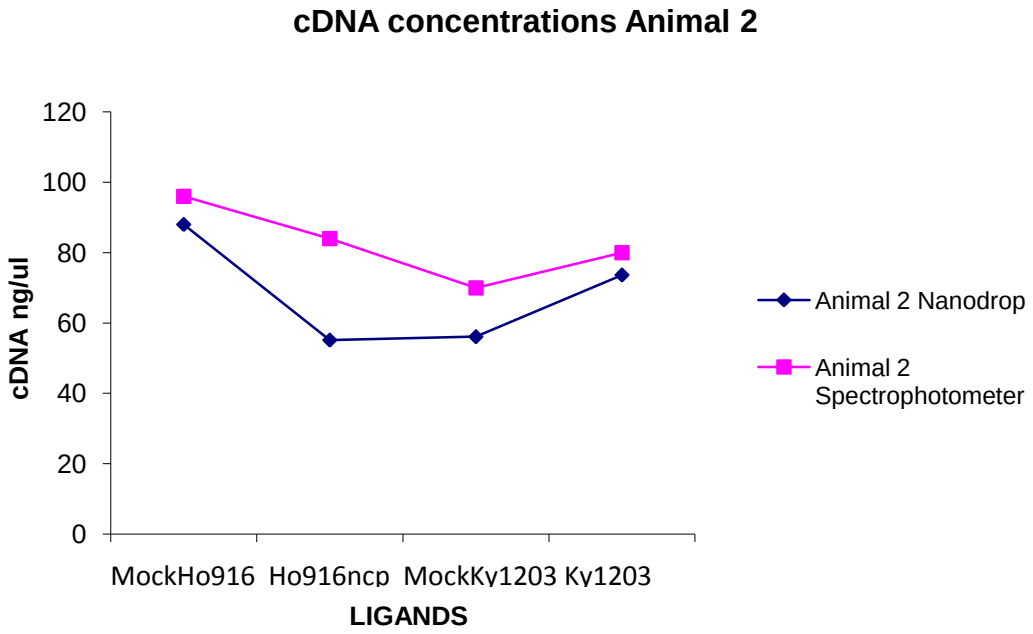

**pCI-neo Sequence Reference Points:**

| | |
|---|---|
| CMV immediate-early enhancer/promoter region | 1–750 |
| Chimeric intron | 890–1022 |
| T7-EEV sequencing primer binding region | 1053–1074 |
| T7 RNA polymerase promoter (–17 to +2) | 1067–1085 |
| Multiple cloning region | 1085–1137 |
| T3 RNA polymerase promoter (–17 to +3) | 1140–1158 |
| SV40 late polyadenylation signal | 1167–1388 |
| Phage f1 region | 1483–1938 |
| SV40 enhancer and early promoter | 2000–2418 |
| SV40 minimum origin of replication | 2316–2381 |
| Coding region of neomycin phosphotransferase | 2463–3257 |
| Synthetic polyadenylation signal | 3321–3369 |
| β-lactamase ($Amp^r$) coding region | 3780–4640 |

## APPENDIX 3

Spectrophotometric analysis of cDNA concentrations




cDNA concentration (ng $\mu l^{-1}$) measured by NanoDrop® ND-1000 Spectrophotometer (Nandrop) and Eppendorf Biophotometer (Spectrophotometer) synthesised from mDCs stimulated with Ho916ncp and Ky1203ncp and respective mock (MockHo916ncp and MockKy1203).

## APPENDIX 4

Datasheets for antibodies used in Western blot for detection of β-actin, IRF3 and IRF7.