# *The regulation of dendritic cell maturation and survival by tumour necrosis factor receptors 1 and 2*


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## Abstract

Dendritic cells (DC) are potent antigen presenting cells which have been implicated in a number of autoimmune diseases. Tumour necrosis factor (TNF) is a key mediator of inflammatory diseases such as rheumatoid arthritis (RA) and plays a central role in DC biology. My aim was to identify the individual contributions of the two TNF receptors (TNFR1 and TNFR2) in regulating the maturation and survival of human inflammatory monocyte-derived (moDC) and steady-state myeloid DC. To address this, I have made use of TNFR-selective ligands in order to dissect the individual contributions of the two receptors.

In moDC, TNFR1-selective, but not TNFR2-selective stimulation resulted in increased expression of DC maturation markers CD83 and CD86, and enhanced T cell stimulatory capacity. A DNA binding assay was used to demonstrate that in moDC TNFR1, but not TNFR2, activates the classical p65 NFκB pathway whereas both TNFR1 and TNFR2 activate the alternative p100/p52 NFκB pathway, highlighting differences in signalling downstream of the receptors. Furthermore, moDC survival was prolonged by selective stimulation of either TNFR1 or TNFR2 as shown by reduced intracellular levels of active caspase-3, indicating that innate signals can promote DC survival in the absence of DC maturation. Accordingly, the p65 NFκB pathway was involved in the pro-survival effect of TNFR1 whereas the Bcl-2/Bcl-xL pathway (identified by the use of small molecule inhibitors) was essential to survival mediated by both TNFR.

In contrast, in myeloid DC, maturation was mainly mediated through TNFR1, whereas TNFR2 was superior in protecting DC from cell death. Antagonistic TNFR1-specific antibodies were used to confirm that cell death protection via TNFR2 was independent of TNFR1-mediated signalling and vice versa confirming that the two receptors can act independently of one another. Understanding the immunoregulatory properties of signalling through these two TNF receptors is important for the design of more targeted anti-TNF therapy.

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## List of abbreviations

| | |
|---|---|
| ACPA | Anti-citrullinated peptide antibody |
| AICD | Activation induced cell death |
| ALPS | Autoimmune lymphoproliferative syndrome |
| Apaf-1 | Apoptotic protease-activating factor-1 |
| APC | Antigen presenting cell |
| ATP | Adenosine triphosphate |
| Bcl-2 | B-cell lymphoma 2 |
| BCR | B cell receptor |
| BDCA | Blood dendritic cell antigen |
| BM | Bone marrow |
| BrdU | Bromodeoxyuridine |
| BSA | Bovine serum albumin |
| CARD | Caspase recruitment domain |
| cDC | Conventional or tissue resident DC |
| CIA | Collagen induced arthritis |
| cIAP | Cellular inhibitor of apoptosis proteins |
| CLIP | Class II associated Ii peptide |
| CRD | Cysteine rich domain |
| CTLA4 | Cytotoxic T lymphocyte-associated protein 4 |
| DAMP | Damage associated molecular pattern |
| DC | Dendritic cell |
| DC-SIGN | Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin |
| DED | Death effector domain |
| DF4 | DharmaFECT 4 |
| DISC | Death-inducing signalling complex |
| DMARD | Disease-modifying anti-rheumatic drugs |
| EAE | Experimental autoimmune encephalomyelitis |
| EBV | Epstein Barr virus |
| ER | Endoplasmic reticulum |
| FADD | Fas-associating protein including death domain |
| FBS | Foetal bovine serum |
| Flt3 | Fms-related tyrosine kinase |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GPI | Glucose-6-phosphate isomerise |
| HIV-1 | Human immunodeficiency virus type 1 |
| HLA | Human leukocyte antigen |
| hsp | Heat shock protein |
| HVEM | Herpes virus entry mediator |
| ICAM | Intercellular adhesion molecules |
| IFN | Interferon |
| IKK | IκB kinase |
| IPEX | Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome |
| JAK | Janus kinase |
| LC | Langerhans cell |
| LPS | Lipopolysaccharide |
| LRS | Leukocyte reduction system |
| LTα | Lymphotoxin alpha |
| MAb | Monoclonal antibody |
| MAPK | Mitogen-activated protein kinase |
| MCP | Monocyte chemotactic protein |
| M-CSF | Macrophage colony stimulating factor |

| | |
|---|---|
| MF | Murine fibroblast |
| MFI | Median fluorescence intensity |
| MHC | Major histocompatibility complex |
| MIF | Macrophage migration inhibitory factor |
| moDC | Monocyte-derived dendritic cell |
| MOMP | Mitochondrial outer membrane permeabilisation |
| MS | Multiple sclerosis |
| mTNF | Membrane-bound TNF |
| MyD88 | Myeloid differentiation primary response gene 88 |
| Nec-1 | Necrostatin-1 |
| NIK | NFκB inducing kinase |
| NK cell | Natural killer cell |
| OPG | Osteoprotegerin |
| PAMP | Pathogen associated molecular pattern |
| PBMC | Peripheral blood mononuclear cells |
| pDC | Plasmacytoid dendritic cell |
| PD-L1 | Programmed death-ligand 1 |
| $PGE_2$ | Prostaglandin $E_2$ |
| PGIA | Proteoglycan induced arthritis |
| PI3K | Phosphatidylinositol 3-kinase |
| PKR | Protein kinase R |
| PLAD | Pre-ligand binding assembly domain |
| PRR | Pattern recognition receptor |
| PS | Phosphatidylserine |
| PTPN22 | Protein tyrosine phosphatase-22 |
| RA | Rheumatoid arthritis |
| RANKL | Receptor activator of nuclear factor κB ligand |
| RF | Rheumatoid factor |
| RHD | Rel homology domain |
| RIP | Receptor interacting protein |
| ROS | Reactive oxygen species |
| SCID | Severe combined immunodeficiency |
| SF | Synovial fluid |
| siRNA | Small interfering RNA |
| STAT | Signal transducer and activator of transcription |
| sTNF | Soluble TNF |
| TACE | TNF alpha converting enzyme |
| TAP | Transporter associated with antigen processing |
| TCR | T cell antigen receptor |
| TGFβ | Transforming growth factor β |
| Th | T helper |
| TIMP | Tissue inhibitors of metalloprotinases |
| TipDC | TNF and nitric oxide (iNOS)-producing DC |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |
| TNFR | Tumour necrosis factor receptor |
| TRADD | TNF receptor-associated death domain |
| TRAF | TNF receptor-associated factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| TRANCE | TNF-related activation-induced cytokine |
| $T_{reg}$ | Regulatory T cell |
| TRID | TNFR1 internalisation domain |
| VCAM-1 | Vascular cell adhesion molecule-1 |
| VEGF | Vascular endothelial growth factor |

# 1. Introduction

## *1.1. Dendritic cells*

The term ‘dendritic cell’ (DC) was first proposed by Steinman and Cohn in 1973 (Steinman & Cohn 1973) in reference to the morphological features of a novel stellate cell population identified in mouse lymphoid organs. These cells had previously been identified in the epidermis by Paul Langerhans in 1868 and were initially termed Langerhans cells. DC have since been proven to function as potent antigen presenting cells (APC) (Sunshine et al. 1980) which can initiate and regulate antigen specific immune responses by activating naïve T cells thus providing a key link between innate and adaptive immunity (reviewed in (Banchereau & Steinman 1998; Steinman 2007)). Research into DC initially proved difficult until a method of culturing DC from mouse bone marrow in the presence of GM-CSF was discovered in 1992 (Inaba et al. 1992). This was followed by simultaneous studies showing that human DC could be cultured from monocytes in the presence of IL-4 and GM-CSF (Sallusto & Lanzavecchia 1994; Romani et al. 1994) therefore allowing large quantities to be generated for use in *in vitro* studies.

DC initially play a sensory role in the immune system and are specialised for the capture and processing of antigens. They reside in an immature state with high endocytic activity in areas of the body which are vulnerable to infection such as the skin, nasal passages, gastrointestinal tract and genitals, where they constantly sample their environment in search of pathogens. In the event that a pathogen associated molecular pattern (PAMP) or damage associated molecular pattern (DAMP) such as a toll-like receptor (TLR) ligand e.g. lipopolysaccharide (LPS) (Matzinger 2002) is identified, DC undergo a complex process of maturation into APC with a high capacity to stimulate naive T cells. DC maturation is characterised by the upregulation of MHC class II molecules, co-stimulatory molecules (such as CD80, CD83 and CD86), and chemokine receptors (e.g. CCR7), which allow migration into the lymph nodes where DC can interact with other immune cells. DC have also been shown to be 100 times more powerful at activating T cells than other types of APC such as macrophages (Steinman & Witmer 1978).

### *1.1.1. Origins and subsets*

DC are heterogeneous and exist in several subsets varying in origin and function within the immune system (mouse and human subsets have been reviewed extensively (Geissmann et al. 2010; Haniffa et al. 2013; Merad et al. 2013)). The phenotype of these subsets plays an important role in T cell activation, polarisation and the induction of tolerance. All human DC subsets are capable of antigen uptake and presentation to naive T cells, and are therefore capable of linking the innate and adaptive immune systems. For example, DC can detect pathogens through pattern recognition receptors (PRR) (see chapter 1.1.2) which provides an immediate defence against infection (innate immunity) but they can also process antigens and activate naïve T cells leading to long-lasting immunity (adaptive immunity). DC constitutively express MHC II molecules (HLA-DR) and lack the lineage markers CD3 (T cells), CD19/CD20 (B cells) and CD56 (NK cells). There is currently evidence for two DC developmental lineages; DC can develop from lymphoid-committed progenitors which also give rise to T cells, or myeloid-committed progenitors which are shared with phagocytes. The majority of DC are derived from $CD34^+$ haematopoietic stem cells and are continually produced in the bone marrow (BM) (Figure 1). Studies have suggested that Flt3L is required for human DC differentiation as administration of Flt3L has been shown to expand DC populations (Maraskovsky et al. 2000). Different tissues contain multiple subsets of DC with different functions and origins. However, more research is needed to determine whether specific subsets are specialised for the induction of different forms of tolerance.

DC can be divided into subsets based on location and surface phenotype (Figure 1) as well as their cytokine profile. In humans two of the main subtypes of DC are plasmacytoid (pDC) and conventional DC (cDC), each of which have specific functions within the immune system. Human pDC express CD123 and are characterised as CD303/BDCA-$2^+$ and CD304/BDCA-$4^+$ (Reizis et al. 2011), whereas human cDC are $CD11c^+$, although CD11c is also expressed on monocytes/macrophages. The pDC population produces high amounts of type I interferons (IFN) and is specialised in mediating anti-viral responses (reviewed in (Colonna et al. 2004)). Two cDC subsets (often referred to as myeloid DC) are present in the blood; CD1c/BDCA-$1^+$ DC are the most common and CD141/BDCA-$3^+$ DC form a small population (Dzionek et al. 2000; Macdonald et al. 2002). Similar CD1c/BDCA-$1^+$ and CD141/BDCA-$3^+$ subsets are also found in lymphoid tissues such as the spleen and tonsils and are referred to as lymph node-resident cDC (Segura et al. 2012). Lymph nodes also include migratory Langerhans cells (LC), migratory $CD1a^+$ DC and $CD14^+$ DC (Segura et al. 2012; Haniffa et al. 2012). The skin contains epidermal langarin$^+$ Langerhans cells (LC), two

subsets of dermal DC ($CD1a^+/CD14^-$ DC and $CD1a^-/CD14^+$ DC) (Nestle et al. 1993) and a small subset of CD141/BDCA-$3^+$ DC (Zaba et al. 2007; Haniffa et al. 2012). Migratory DC sample their environment before migrating to draining lymph nodes in order to present tissue-derived antigen to T cells whereas lymphoid tissue DC develop within the tissue and reside there throughout their lifespan. LC are langarin$^+$, $CD1a^+$, $CD45^+$ and $EpCAM^+$ and are found in skin and mucosa but are most prominent within stratum spinosum layer of the epidermis (Merad et al. 2008).

In general, myeloid DC capture antigens in the periphery, migrate to lymphoid organs where they can initiate an immune response, whereas lymphoid DC are found in the lymph nodes and may be responsible for inducing tolerance. Plasmacytoid DC are less efficient at antigen presentation but have the capacity to rapidly produce type I interferon (IFN) for example, in response to viruses. Both pDC, whose immediate precursor remains unknown, and preDC, the precursor of cDC, arise from a common lymphoid or myeloid progenitor which can no longer form monocytes (Liu et al. 2009) (Figure 1). Lymphoid tissue resident DC develop from the common DC progenitor which responds to Fms-related tyrosine kinase (Flt3) ligand and expresses a receptor for the stem cell factor c-Kit (Onai et al. 2007). Alternatively, the common macrophage DC precursor can give rise to cDC and macrophages but not pDC *in vitro* (Fogg et al. 2006).

To date, the relationship between mouse and human DC remains unclear. This is partially to do with the low frequencies of DC in human peripheral blood and also involves a difference in surface marker expression. In mice, lymphoid organs e.g. the spleen and lymph nodes contain cDC along with pDC which can be further divided into $CD8^+$ and $CD8^-$ pDC, whereas in non-lymphoid organs such as the skin, lung and intestine DC can be further divided into migratory DC, Langerhans cells, interstitial DC and dermal DC (Merad & Ginhoux 2007). In mice the common DC progenitor gives rise to pDC in the spleen and lymph node but cannot develop into macrophages (Shortman & Naik 2007). Unlike mouse DC, human DC do not express CD8 which has led to difficulties in defining different subsets. However, recent studies have suggested that human $CD141^+$ DC may represent $CD8^+$ cDC in mice and that human $CD1c^+$ DC may represent mouse $CD11b^+$ DC (Villadangos & Shortman 2010). For example, one of the hallmarks of $CD141^+$ DC is their ability to cross-present (Bachem et al. 2010) and in mice cross-presentation is exclusive to $CD8^+$ DC (den Haan et al. 2000; Schulz & Reis e Sousa 2002).

The phenotypical and functional overlap between DC subsets and also the overlap with other myeloid cell types has made the DC lineage and its subsets difficult to distinguish. It has been suggested that epigenetic systems such as chromatin

regulation may be responsible for plasticity between DC subsets, as varying levels of accessible chromatin may enable flexibility in gene expression depending on the microenvironment (Paul & Amit 2014). Additionally, the presence of transcription factors has been shown to have a direct effect on lineage plasticity between different cell types including the reprogramming of committed T cell progenitors to a DC phenotype (Laiosa et al. 2006; Graf & Enver 2009) as well as affecting plasticity between DC subsets. Blood myeloid DC and blood pDC have been shown to have distinct migratory capacities in response to chemokines. Myeloid DC have been shown to migrate in response to MCP-1/CCL-2, RANTES/CCL-5 and CXCL-12 whereas pDC only migrated in response to CXCL-12 (Penna et al. 2002). CD1c/BDCA-$1^+$ DC are specialised for the presentation of antigens to $CD4^+$ T cells, have the capacity to produce high levels of IL-12p70 following TLR activation (Nizzoli et al. 2013) and have been shown to have a higher capacity to stimulate allogenic T cells than CD141/BDCA-$3^+$ DC and pDC (Macdonald et al. 2002). DC are essential for cross priming of $CD8^+$ T cells *in vivo* (Jung et al. 2002). The CD141/BDCA-$3^+$ subset of DC have been shown to have a higher ability to cross-present cell-associated antigens than CD1c/BDCA-$1^+$ DC and pDC (Bachem et al. 2010) and are found in tissues such as the skin, lung and liver as well as peripheral blood (Haniffa et al. 2012). TLR9 (which detects unmethylated CpG DNA) is mainly restricted to pDC in humans (which also express high levels of TLR7 which detects ssRNA), suggesting that pDC may be more specialised in the detection of intracellular viral/bacterial infections than other DC subsets (Crozat et al. 2009).

#### *1.1.1.1. Monocyte-derived DC and inflammatory DC*

Monocytes found in the blood can differentiate into monocyte-derived DC (moDC), and have a high capacity to present antigen to T cells. MoDC are not normally found in healthy tissues although low numbers can be found in draining lymph nodes during the steady state and moDC numbers increase during inflammation (i.e. after stimulation with TLR ligands such as LPS). The differentiation of moDC from monocytes and subsequent recruitment into lymph nodes has been demonstrated in mice (Cheong et al. 2010) although more work is needed in order to define this subset in humans. In mice, monocytes which express GR-1 (a myeloid differentiation antigen; also known as Ly6G) have been shown to infiltrate inflamed skin and differentiate into Langerhans cells *in vivo* (i.e. under inflammatory conditions) (Ginhoux et al. 2006) and may also be the precursor to inflammatory TNF and nitric oxide (iNOS)-producing (Tip)DC (Serbina et al. 2003).

In humans, inflammatory DC exist in different subsets and have been described in synovial tissues and tumour ascites (Segura & Amigorena 2013). In addition, inflammatory TipDC and slan DC (6-sulfo LacNAc expressing DC) (both of which are $CD14^-$ $CD1c^-$) have been identified in inflamed skin (Gunther et al. 2012), although slan DC and $CD16^+$ monocytes are difficult to distinguish from eachother (but are distinct to blood DC) (Haniffa et al. 2012). Other populations of inflammatory DC have been described in both inflamed synovial tissues and malignant ascites which express $CD206^+$/$CD1a^+$/$CD11b^+$/SIRP-$\alpha^+$/Fc$\varepsilon$R1$^+$ but unlike TipDC/slanDC they are $CD14^+$ $CD1c^+$ (Segura & Amigorena 2013). Human inflammatory DC have also been shown to drive Th17 polarisation (Segura, et al. 2013).

MoDC can be generated *in vitro* from human peripheral blood in large quantities therefore they are the most extensively studied subset of DC in humans. Although generated in vitro, MoDC are considered to resemble inflammatory DC i.e. DC derived from monocytes under inflammatory conditions (as reviewed in mice (Shortman & Naik 2007) and humans (Segura & Amigorena 2013)). Inflammatory DC are thought to be derived from monocytes during cases of chronic inflammation such as RA therefore moDC may not represent all steady state (i.e. absence of inflammation) DC subsets *in vivo*.

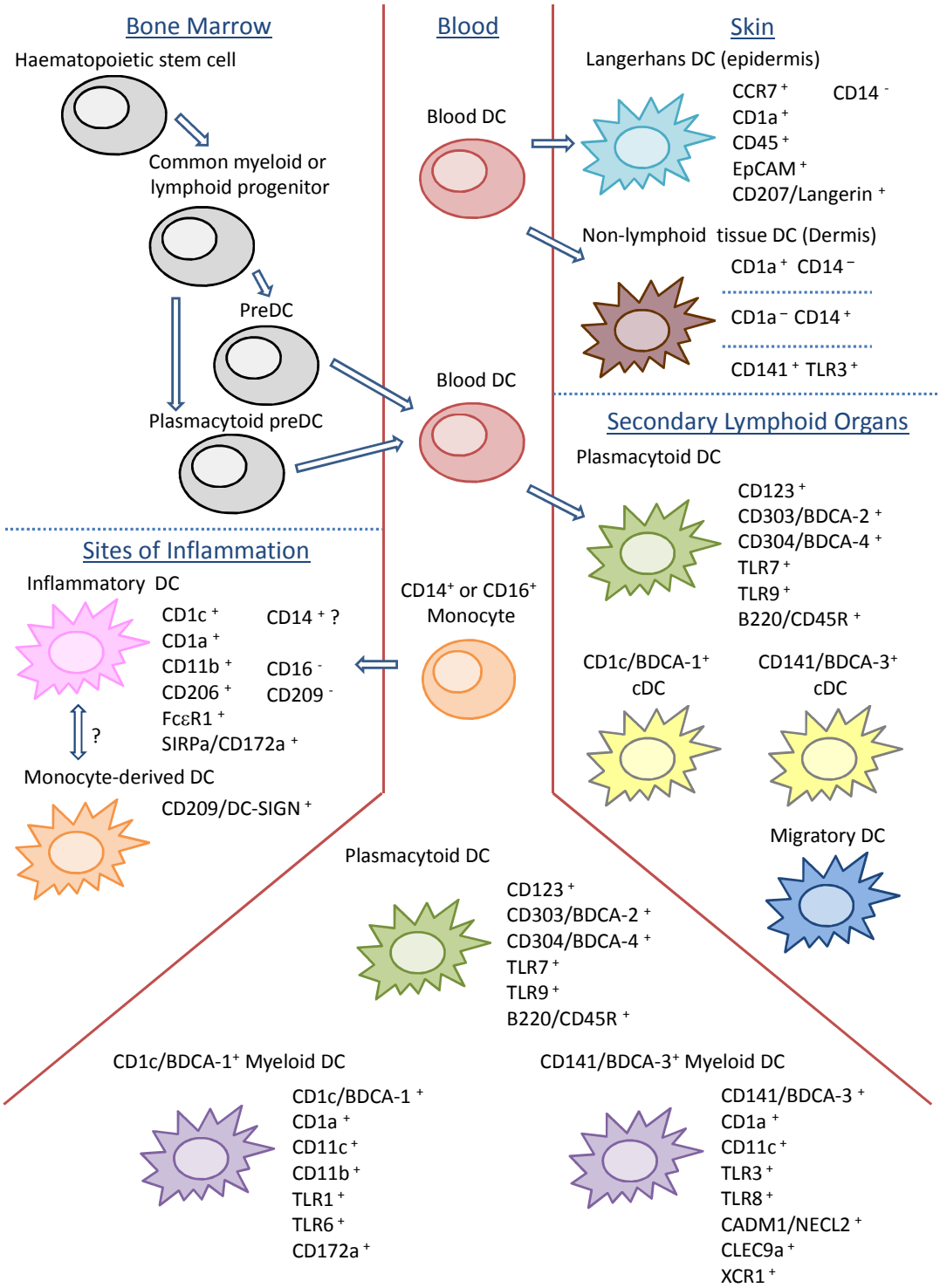


**Figure 1: Human DC subsets.** *DC originate from the bone marrow and can be divided into different subsets based on their precursors, location and surface phenotype as indicated. Two of the main human blood borne subtypes are plasmacytoid (pDC; CD123$^+$) and conventional DC (cDC; CD1c/BDCA-1$^+$ or CD141/BDCA-3$^+$). Similar cDC subsets are found in lymphoid tissues and the lymph nodes include a number of migratory subsets. The skin contains epidermal langarin$^+$ Langerhans cells (LC) and the dermis contains 3 subsets of dermal DC (CD1a$^+$/CD14$^-$ DC, CD1a$^-$/CD14$^+$ DC and CD141/BDCA-3$^+$ DC). Monocytes may also differentiate into inflammatory DC at sites of inflammation. See section 1.1.1 for further information.*

### *1.1.2. DC function and pattern recognition*

DC initiate and regulate immune responses, are highly specialised for antigen presentation and can polarise immune responses towards a Th1, Th2, Th17 or regulatory T cell ($T_{reg}$) phenotype. Subsets of pDC and monocyte-derived DC have a tolerogenic phenotype and can induce $T_{reg}$ cells in the periphery (Kuwana 2002). It has been shown in mouse models that constitutive depletion of DC or defects in DC apoptosis induced autoimmunity (Ohnmacht et al. 2009; Chen et al. 2006). Alternatively, the removal of DC in another mouse model was shown to abolish T cell priming and therefore inhibit autoimmunity (Jung et al. 2002) showing that DC are important at several stages of the immune response.

DC identify danger signals (PAMP or DAMP) through PRR on their surface. It was first proposed that the immune system senses bacterial products in order to distinguish self from non-self by Janeway in 1989 (Janeway 1989). This was further developed into the 'danger hypothesis' (Matzinger 2002) whereby immune cells also identify alarm signals from cells undergoing injury or stress e.g. heat shock proteins (Breloer et al. 2001).

#### *1.1.2.1. Toll-like receptors*

PRR are a primitive part of the immune system which evolved prior to the adaptive immune system however, they amount to the first line of defence for many types of infection and can recognise products from bacteria (such as lipopolysaccharide (LPS)), fungi (dectin-1 ligands) and viral particles (CpG). PRR include toll-like receptors (TLR) which recognise conserved sequences found throughout many bacterial species which are not usually found on self-cells. TLR were first discovered in *Drosophila melanogaster*. There are 10 TLR genes in humans which recognise different PAMP (Table 1), are highly evolutionarily conserved and are found in all vertebrates (Gay et al. 2006). Although the specific TLR-10 ligand remains unknown it has recently been shown the TLR-10 on macrophages and epithelial cells is activated and drives an inflammatory response to *Listeria monocytogenes* (Regan et al. 2013). Upon activation, all TLR except TLR-3 activate transcription factors (e.g. NFκB) via the adapter protein myeloid differentiation primary response gene 88 (MyD88).

The range of TLR expressed by each subset of DC can determine the cell's response to a particular pathogen and can also differentially regulate DC biology (for example, the cytokine profile produced by the DC) (Zanoni & Granucci 2010). For example, $CD11c^+$ lamina propria cells in the intestinal lumen of mice express high levels of TLR-5 and low levels of TLR-4 which allows a response to pathogens but maintains tolerance to commensal gut flora (Uematsu et al. 2006). TLR-2 is highly expressed on

tolDC but not mature DC (Harry et al. 2010). TLR-1-6, 8 and 10 are expressed by cDC whereas pDC respond strongly to viral infection and release high amounts of IFN as they express high levels of TLR-7 and TLR-9 which are stimulated by ssRNA and ssDNA, respectively.

Although TLR serve to protect the body by recognising pathogens they may also play a role in disease. It has been shown that the stimulation of multiple TLR pathways can synergistically enhance the production of inflammatory mediators by DC and that TLR-2 and TLR-4 mediated stimulation of moDC from RA patients resulted in higher production of inflammatory cytokines than moDC from healthy controls (Roelofs et al. 2005). This suggests that DC TLR signalling can lead to a pro-inflammatory environment and may play a role in the breakthrough of tolerance leading to RA. It has also been shown that the human immunodeficiency virus type 1 (HIV-1) requires signalling by TLR-8 and DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) for replication in DC (Gringhuis et al. 2010).

| Toll-like receptor | Ligand | Location of expression |
|---|---|---|
| TLR-1 dimer<br>TLR-2/TLR-6 dimer | Peptidoglycan<br>Lipoproteins<br>Lipoarabinomannan<br>GPI<br>Zymosan | Extracellular |
| TLR-3 | dsDNA | Intracellular |
| TLR-4 dimer (+CD14) | LPS | Extracellular |
| TLR-5 | Flagellin | Extracellular |
| TLR-7 | ssRNA | Intracellular |
| TLR-8 | | |
| TLR-9 | Unmethylated CpG (ssDNA) | Intracellular |
| TLR-10 | Unknown | Extracellular |

*Table 1: Toll-like receptor recognition molecules. Humans express 10 different toll-like receptors which recognise a range of different ligands as indicated. Adapted from* (Kanzler et al. 2007).

### *1.1.3. Antigen uptake and presentation by DC*

Immature DC constantly sample and process antigens from their environment. Antigens are processed into peptide fragments and are presented to T cells via the MHC complex. MHC class I molecules are made of an $\alpha$ chain and a $\beta$2-microglobulin chain, are found on almost all nucleated cells and present peptides from cytosolic proteins (such as viral proteins) to $CD8^+$ T cells. For the activation of T cells via the

MHC I pathway cytosolic proteins are degraded by the proteosome into peptides which are typically 7-9 residues long. Peptides are then transported into the endoplasmic reticulum (ER) by a transporter associated with antigen processing (TAP) in an ATP dependent manner (Chapman & Williams 2010).

Alternatively MHC class II molecules are made of an $\alpha$ and $\beta$ chain, are found on APC (such as DC) and present antigens from extracellular proteins to $CD4^+$ T cells (Jensen 2007). For the activation of T cells via MHC II molecules antigens are first taken up by endocytosis (including macropinocytosis and receptor-mediated endocytosis via the mannose receptor) (Sallusto et al. 1995). Following this the endosome fuses with a lysosome whose mildly acidic pH (4.5 to 5.5) allows optimal protease activity which degrades the proteins. In the ER, three invariant chains (Ii) form a complex with three MHC II molecules ($\alpha$ chains and $\beta$ chains). Ii prevents inappropriate loading of the MHC II molecule by occupying the peptide binding groove (Gautam et al. 1997). The complex then leaves the ER and fuses with the peptide-containing endosomes. The Ii chain is cut by cathespins to produce the class II associated Ii peptide (CLIP) which then dissociates from the MHC II complex and is replaced by the peptide with the assistance of the chaperone protein HLA-DM. MHC II-peptide complexes can then be presented on the APC membrane.

Antigen cross presentation describes the ability of DC and other APC to present extracellular antigens derived from the MHC II molecules of infected APC, on their own MHC I molecules to $CD8^+$ T cells without infection (reviewed in (Joffre et al. 2012)). Some DC subsets such as $CD8\alpha^+$ DC in the mouse (Hildner et al. 2008) or $BDCA3^+$ (Cohn et al. 2013) and $CD103^+$ DC (Bedoui et al. 2009) in humans are specialised in cross presentation which is important in both viral and anti-tumour responses.

### *1.1.4. Maturation of DC*

Under steady state conditions DC typically reside in an immature state where they have little ability to activate an effector T cell response and instead induce tolerance. The maturation state of DC is critical in determining their immunomodulatory role as DC play a key role in instigating effective immunity and in maintaining self-tolerance. Upon activation by PAMP or inflammatory cytokines, DC mature and express high levels of MHC II-peptide complexes, co-stimulatory molecules (e.g. CD86) and cytokines (such as IL-12). This drives the expansion of antigen specific T cells and can polarise the response depending on the nature of the maturation stimulus (Diebold 2008). Inflammatory mediators (such as TNF) alone are not sufficient to fully mature DC and induce effector T cell responses (Spörri & Reis E Sousa 2005) as full DC

maturation requires the recognition of PAMP such as a TLR agonist. The effect of TNF on DC maturation will be discussed in more detail later in this chapter (section 1.3.1).

In mouse models an increase in maturation signals through the overexpression of TLR-7 has been shown to increase DC proliferation and also systemic autoimmunity (Deane et al. 2007). It has been shown that NFκB1 (p105) is crucial in maintaining DC in an immature state and prevents the spontaneous production of TNF (Dissanayake et al. 2011). Another study has shown that dimethyl fumarate, a potential new treatment for multiple sclerosis (MS), inhibits the maturation of murine DC by suppressing p65 NFκB signalling and therefore prevents Th1 and Th17 cell differentiation (Peng et al. 2012).

The maturation state of DC also determines their sensitivity to different death inducing ligands. It has been shown that immature DC are sensitive to Fas (CD95) induced apoptosis but not apoptosis via MHC class II cross-linking whereas mature DC are not as sensitive to apoptosis via Fas but are sensitive to apoptosis induced by MHC class II cross-linking (McLellan et al. 2000). Also, mature human DC were more resistant to FasL (also known as Apo-1L or CD95L) and TRAIL mediated killing than immature DC (Leverkus et al. 2000).

### *1.1.5. DC survival and lifespan*

The regulation of DC survival is important in determining DC function including both immunity and tolerance. Prolonging the lifespan of DC has been shown to break tolerance and result in autoimmunity (Wang et al. 1999; Chen et al. 2006), whereas a decrease in DC survival reduces immune protection from pathogens and inhibits autoimmunity (Jung et al. 2002; Whartenby et al. 2005). Thus, the lifespan of DC is extremely important in both health and disease. Impaired apoptosis is not only associated with autoimmunity but is involved in cancer development. DC have been studied in relation to cancer immunotherapy and the development of DC-based vaccines (Palucka et al. 2007; Palucka et al. 2011). The effectiveness of these vaccines would depend on the survival potential of the DC.

Previous research suggests that DC have a short lifespan which is dependent on both the acquired immune system and on their environment (including pro-inflammatory cytokines). They undergo apoptosis shortly after maturation to prevent excessive T cell activation and subsequent autoimmunity ((McLellan et al. 2000) and reviewed in (Zanoni & Granucci 2010)). DC co-cultured with peptide specific T cells *in vitro* undergo rapid apoptosis through the ligation of Fas (on DC) with FasL (on T cells) (Matsue et al. 1999). However not all DC die upon T cell activation (Matsue et al. 1999) therefore surviving cells may have a role in the regulation or termination of immune responses.

Other groups have suggested that the upregulation of Bcl-xL by mature DC may be responsible for the protection of DC from Fas-mediated killing (Lundqvist et al. 2002).

Bromodeoxyuridine (BrdU) staining has been used to show that the half-life of DC in mice varies between different populations, for example the time taken to replace most DC is approximately 3 days in the spleen, 4 days in the mesenteric lymph nodes and 9 days in the thymus and skin-draining lymph nodes (Kamath et al. 2002). DC with longer half-lives due to an inherited mutation in caspase-10 have been identified in human patients with autoimmune lymphoproliferative syndrome (ALPS) which can result in a number of autoimmune effects (Wang et al. 1999). Other studies have shown that the type of T cell that DC interact with may also determine DC fate as memory $CD8^+$ T cells can protect DC from apoptosis induced by cytotoxic $CD8^+$ T cells (Watchmaker et al. 2008). In addition, the location of DC also plays a role as it has been shown that DC in proximity to stromal cells are long lived and have a regulatory phenotype (Zhang et al. 2004). LPS (Franchi et al. 2003), prostaglandin $E_2$ ($PGE_2$) (Vassiliou et al. 2014), and CD40 ligation (Haenssle et al. 2008; Caux et al. 1994) are known DC survival factors. TNF also promotes DC survival (Ludewig et al. 1995; Lehner et al. 2012) however the effect of TNF on DC survival will be discussed in more detail later in this chapter (section 1.3.1).

The regulation and expression of B-cell lymphoma 2 (Bcl-2) family members can also regulate DC survival (see chapter 1.4.2.1 for more detail on this pathway). DC expression of Bcl-2 itself can inhibit pro-apoptotic molecules such as Bim, Bax and Bad (Hou & Parijs 2004). Activation and maturation of DC through TLR-stimulation has been shown to upregulate Bcl-xL and enhance survival (Chen et al. 2007).

### *1.1.6. Human DC deficiencies*

DC are important in maintaining the balance between immunity and tolerance, however defects in DC apoptosis or the enhancement of DC survival may break tolerance and lead to autoimmunity. Several DC deficiencies have recently been described in humans and illustrate the importance of DC in regulating the immune system. For example, DC, monocyte, B and NK lymphoid deficiency (DCML) is caused by a heterozygous mutation in GATA-binding factor 2 (GATA2) and is associated with a complete lack of blood DC, pDC, tissue cDC, circulating monocytes, B cells and NK cells but does not affect granulocytes or platelets (Bigley et al. 2011). It has been discovered that 1 in 4 patients with DCML develop autoimmunity (Collin et al. 2011). Furthermore, two disease-causing mutations affecting the transcriptional activity of interferon regulatory factor 8 (IRF8) have been identified which show that IRF8 is critical for the development of DC and monocytes (Hambleton et al. 2011). The K108E variant was a

severe autosomal recessive immunodeficiency and patients had a complete lack of circulating DC and monocytes. The T80A variant had an autosomal dominant immunodeficiency which was less severe and patients showed a selective depletion of $CD11c^+/CD1c^+$ circulating DC (Hambleton et al. 2011). In addition, patients with reticular dysgenesis (an inherited immunodeficiency) lack Langerhans cells, blood monocytes, neutrophils and have low lymphocyte counts (Emile et al. 2000).

### *1.1.7. DC interaction with T cells*

Upon pathogenic infection, relevant information is obtained from affected cells or other immune cells e.g. NK or macrophages leading to DC activation and subsequent activation of naïve T cells. Activation of T cells requires three signals, firstly the recognition of peptide-MHC complexes via the T cell receptor (TCR), secondly an interaction between the co-stimulatory molecules such as CD80 and CD86 on the APC with CD28 on the T cell and thirdly pro-inflammatory cytokines. These signals can convey information on the identity of the pathogen, the antigenic structure and its pathogenicity as well as leading to T cell polarisation (see chapter 1.1.7.1), meaning that T cells in lymph nodes can be primed without experiencing the pathogen directly. If any of these signals are absent then the T cell is not activated and may undergo cell death or become anergic.

Immature DC express the chemokine receptors CCR1, CCR2, CCR5 and CXCR1 which recognise inflammatory chemokines and therefore recruit DC to inflamed tissues. Following DC maturation the expression of these chemokine receptors is reduced and the expression of CCR7 is increased (Sallusto et al. 1998) allowing the homing of DC to lymphoid organs. CCR7 deficient mice show impaired migration of DC to the lymph nodes (Förster et al. 1999). DC then localise to the T cell areas of the lymph nodes where they interact with naive T cells.

The formation of an immunological synapse allows cross-talk between DC and T cells. This involves the interaction of DC-derived cytokines or DC surface molecules with the corresponding receptor on the surface of naïve T cells and can influence T cell proliferation and differentiation (Figure 2 (A)). Both CD28 and CTLA-4 can bind to CD80 and CD86, however CD28 ligation stimulates the T cell whereas CTLA-4 transmits an inhibitory signal to the T cell and antagonises CD28 binding (Freeman et al. 1993). CD83 is also upregulated on DC during maturation and although its direct ligand is unknown, it is thought to dimerise and enhance T cell activation as prevention of dimerisation in an EAE model decreased T cell activation (Zinser et al. 2004). The ligation of cell surface molecule ICAM-1 (on DC) or the cytokine IL-12 (released by DC) to their respective receptors on naïve T cells results in Th1 polarisation, whereas

OX40-L ligation to OX40 (on T cells) results in Th2 polarisation. In addition, IL-23/IL-23R ligation results in Th17 polarisation and IL-10/IL-10R ligation results in $T_{reg}$ polarisation. CD80 and CD86 are co-stimulatory molecules which can bind to either CD28 and induce T cell activation or CTLA-4 which downregulates T cell responses. PD-L1/PD-1 ligation reduces T cell proliferation (Figure 2 (A)). Although mature DC can mediate antigen-specific T cell expansion and direct effector T cell responses, these interactions are not unidirectional. In addition, T cells can activate DC via CD40/CD40L interactions which induce DC to prime $CD8^+$ cytotoxic T cells, thus explaining the mechanism by which T helper cells are also involved in the cytotoxic T cell response (Schoenberger et al. 1998).

#### *1.1.7.1. T cell polarisation*

DC can adopt different Th1- or Th2-promoting effector functions (i.e. via the production of cytokines) depending on the context of their activation, thereby polarising the T cell response to a variety of pathogens. This is often referred to as the 'third signal' resulting in the antigen-specific activation of T cells (Kaliński et al. 1999). Immature DC are influenced by the presence of different factors such as inflammatory cytokines, whereas as they mature DC become less susceptible thereby the acquisition of polarizing signals is restricted to the site of pathogen entry. T cell lineages are shown in Figure 2 (B) and described below.

T helper 1 (Th1) cells are critical for the clearance of intracellular pathogens such as bacteria, parasites, yeast and viruses and is characterised by high IFNγ production (Zhu & Paul 2008). The presence of IFNγ during DC maturation increases their ability to produce IL-12. NK cells, cytotoxic T cells and complement fixing $IgG_{2A}$ antibodies act alongside the Th1 response in order to efficiently clear pathogens (O'Garra & Arai 2000). Th1 responses are induced by the cytokines IFNγ, IL-12 and IL-18, which activate transcription factors such as STAT4 and T box expressed in T cells (T-bet). The release of IL-12 by DC following PRR ligation is important in promoting Th1 lineage commitment from naive $CD4^+$ T cells. Binding of DC-derived IL-12 to its receptor (IL-12Rβ2 on T-cells) leads to the activation of STAT4 which affects transcriptional regulation and can upregulate expression of IL-18Rα. IL-18 and IL-12 then function to enhance IFNγ production and perpetuate the Th1 phenotype (Tominaga et al. 2000). T-bet is an essential regulator of the Th1 phenotype which is induced independently of STAT4, via IFNγ-mediated activation of STAT1 and induces IFNγ production and represses IL-4 expression (Afkarian et al. 2002).

The signature cytokine of Th1 cells is the pro-inflammatory cytokine IFNγ, but Th1 cells also produce IL-2, IL-3 and GM-CSF (Mosmann & Coffman 1989). Alternatively, Th1 cells also produce anti-inflammatory IL-10 in order to minimise tissue damage (O'Garra & Vieira 2007). IL-10 inhibits the ability of DC to produce IL-12 and reduces DC stimulatory capacity thereby inducing a tolerogenic phenotype. The balance between IL-10 and IFNγ production are important in determining whether an infection is cleared efficiently. The production of IFNγ from Th1 cells upregulates the IL-12 receptor on DC which in turn increases the production of IL-12 and amplifies the Th1 response and inhibits the development of Th2 cells (Gajewski & Fitch 1988). Th1 cells also produce IL-10 to limit the immune response and prevent excessive tissue damage (Gazzinelli et al. 1996). However IL-10 must be tightly regulated otherwise its production may lead to chronic infection (Ejrnaes et al. 2006; Brooks et al. 2006). In addition, the induction of T-bet in Th2 cells induces a Th1 cell phenotype showing plasticity between Th subsets (Szabo et al. 2000).

Th2 responses cells are required for the clearance of extracellular pathogens (including helminths) and are induced by the cytokines IL-4, IL-5, IL-9, and IL-13 (Paul & Zhu 2010), which activate the transcription factors STAT6 and GATA3. Th2 cells can activate mast cells and eosinophils and also provide B cell help resulting in the generation of $IgG_1$ and IgE antibodies which are important in immunity to parasites but are also the cause of type I hypersensitivity (allergic) reactions. IL-4/IL-4R mediated signalling can induce STAT6 activation and increases expression of the transcription factor GATA3 and c-Maf (O'Garra & Arai 2000) which may further increase the expression of IL-4R on the T cell surface. In addition, Th2 cells produce high levels of IL-4 which further induced GATA3 expression and provides a positive feedback loop (Ho et al. 2009). GATA3 inhibits Th1 differentiation via suppression of the IL-12Rβ2 thereby preventing STAT4 activation and subsequent Th1 cell responses (Ouyang et al. 1998). Also, GATA3 expression in Th1 cells can induce Th2 response genes (Zheng & Flavell 1997). $PGE_2$ is an inflammatory mediator produced by stromal fibroblasts. When present during DC maturation it reduces DC production of IL-12 thereby promoting the Th2 lineage (Kaliński et al. 1997) and is another example of a modulatory factor which can influence DC-mediated T cell polarisation.

Th17 cells produce large amounts of IL-17 and lead to a pro-inflammatory response as well as inducing neutrophil recruitment and providing immunity against extracellular bacteria and fungi. In humans, IL-6, IL-1β, TGFβ, and IL-23 regulate Th17 development (Acosta-Rodriguez et al. 2007; Manel et al. 2008; Volpe et al. 2008; Wilson et al. 2007; Segura, Touzot, et al. 2013). Th17 differentiation also requires activation of the transcription factor RAR-related orphan receptor (ROR)γt (Zhang et al. 2008). The

strength of T cell stimulation also plays a role in the Th17 response as low-strength but not high-strength T cell activation promoted Th17 responses (Purvis et al. 2010). The Th17 response has been shown to be important in the clearance of infections. For example, *B. Pertussis* can activate TLR4 on DC and induce the secretion of IL-1β, TNF and IL-23, thus enhancing T cell expression of IL-17 (Higgins et al. 2006). Th17 cells have also been widely implicated in the development of inflammatory diseases such as RA. For example, blocking IL-17 in a mouse experimental arthritis model decreased the severity of antigen-induced arthritis (Koenders et al. 2005). Also human inflammatory DC can induce the differentiation of Th17 cell from naive $CD4^+$ T cells (Segura, Touzot, et al. 2013). In addition, when TGFβ is absent and IL-12 is present, Th17 cells have been shown to switch to a Th1 cell phenotype which is again dependent on the Th1 transcription factors STAT4 and T-bet (Lee et al. 2009).

$T_{reg}$ cells have the ability to suppress the immune response to self and non-self antigens. Naturally occurring $T_{reg}$ cells are produced in the thymus during development (Ng 2001) and are $CD4^+FoxP3^+CD25^+$. FoxP3 is a transcriptional repressor of IL-12 (which is involved in T cell proliferation and B cell, NK cell, monocyte and macrophage growth). The expression of FoxP3 is essential for the suppressive function of naturally occurring $T_{reg}$ cells (Walker et al. 2003) and mutations in FoxP3 lead to severe autoimmune disease in both mice ('scurfy' mutant) and humans (Immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome) (Gambineri et al. 2003; Kim et al. 2007). Alternatively $T_{reg}$ cells can be induced in the periphery by cytokines such as IL-10 or TGFβ (Buckner & Ziegler 2004). $T_{reg}$ cells induced by IL-10 are $FoxP3^-CD4^+CD25^-$ (CD25 is the α chain of the IL-2R), whereas $T_{reg}$ cells induced by DC-derived TGFβ are $FoxP3^+CD4^+CD25^+$ (Marguti et al. 2009; Dumitriu et al. 2009). It has been shown that the regulation of Th17 and $T_{reg}$ cells is linked showing plasticity between T cell phenotypes. In the absence of IL-6, TGFβ induces FoxP3 expression which negatively effects Th17 differentiation. Alternatively, the presence of IL-6 prevents TGFβ-induced FoxP3 expression thus allowing Th17 induction (Zhang et al. 2008; Ichiyama et al. 2008).

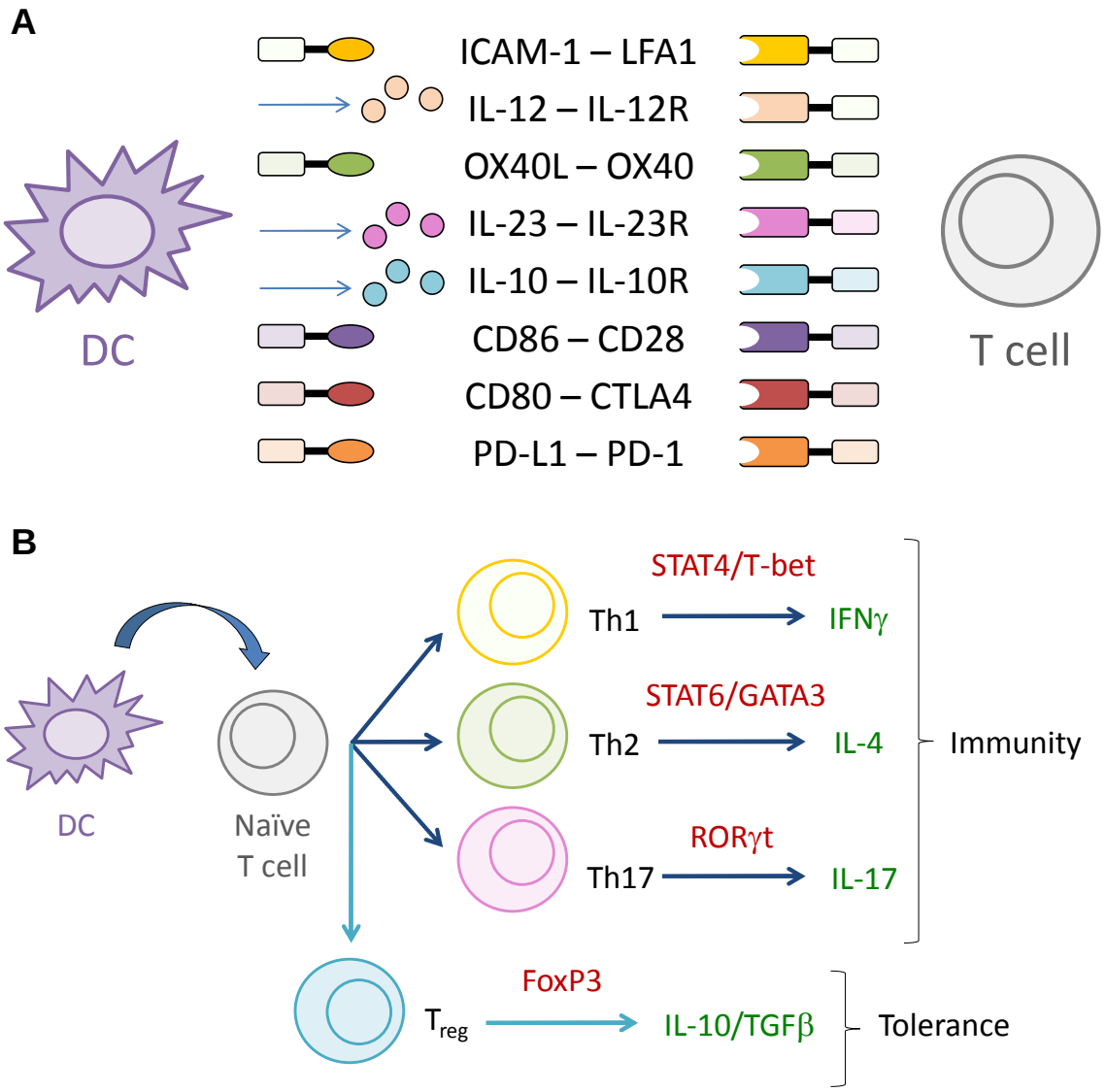


***Figure 2: Surface molecules, cytokines and transcription factors involved in T cell activation and polarisation by DC.*** DC can activate and polarise T cell responses. (A) Transmembrane surface molecules and cytokines (circles) involved in cross talk between DC and T cells. ICAM-1 or IL-12 ligation to their respective receptors results in Th1 polarisation, OX40-L ligation results in Th2 polarisation, IL-23 results in Th17 polarisation and IL-10 results in $T_{reg}$ polarisation. CD80 and CD86 are co-stimulatory molecules which can bind to either CD28 (T cell activation) or CTLA-4 (downregulates T cell responses). PD-L1/PD-1 ligation reduces T cell proliferation. (B) Upon activation by DC, naïve T cells differentiate into Th1, Th2 and Th17 lineages which result in immunity or DC may induce the production of $T_{reg}$ cells which induce tolerance. The transcription factors involved in each lineage are shown in red and the effector cytokines produced by the differentiated T cells are shown in green.

### *1.1.8. The balance between immunity and tolerance*

DC play a central role in inducing and maintaining both central and peripheral T cell tolerance. Central tolerance involves the presentation of self-antigens by thymic DC which have been shown to mediate the negative selection of developing thymocytes in vivo (Brocker et al. 1997). Peripheral tolerance develops upon antigen encounter in the absence of co-stimulatory signals, for example self-antigens generally do not induce DC activation therefore their presentation leads to T cell tolerance (Hawiger et al. 2001).

The balance between immunity and tolerance is determined by the maturation and activation status of DC. Immature DC are often described as having a tolerogenic phenotype as they express low levels of co-stimulatory molecules (CD80/CD86) and produce high levels of IL-10 and can induce tolerance in the periphery (reviewed in (Mahnke et al. 2002)). Alternatively mature DC express high levels of co-stimulatory molecules and produce cytokines such as IL-4, IL-12, IL-23 or IL-17 which can skew T cell responses towards a Th1, Th2 or Th17 phenotype. Autoreactive T cells are generally deleted within the thymus during development, however some T cells escape and can be found in the periphery (Walker & Abbas 2002) requiring a further level of regulation to ensure self-tolerance is maintained. This is achieved by the induction of T cell deletion (through an unknown mechanism), T cell anergy or through differentiation into $T_{reg}$ cells.

In the steady state immature DC constantly endocytose antigen (Sallusto et al. 1995). In the absence of co-stimulatory molecules DC do not become activated and recognition of the peptide-MHC complex leads to T cell anergy leaving the T cell unresponsive to any further activation, even by APC which do express the correct co-stimulatory molecules (Janeway & Bottomly 1994). DC endocytosis can be used to target antigens to DC and induce T cell anergy *in vivo* (Hawiger et al. 2001). In mouse models immature BM-DC generated *in vitro* and injected intravenously can also induce $CD4^+$ T cell anergy which has been shown to prolong the acceptance of allogeneic heart transplants (Fu et al. 1996; Lutz et al. 2000). However, it is not only immature DC which can induce tolerance as under certain circumstances, mature DC can also induce regulatory $CD4^+$ T cells and produce IL-10 *in vivo* (Akbari et al. 2001; McGuirk et al. 2002). Recent theories suggest that as only full maturation is enough to initiate an immune response whereas tolerance is seen when partial maturation of DC occurs (Frick et al. 2010; Lutz & Schuler 2002).

Silencing of RelB has been shown to prevent full maturation of DC and the resulting semi-mature DC inhibited the immune response, allowed the expansion of $T_{reg}$ cells

and prevented allograft rejection (Li et al. 2007). Blockade of TNF has also been shown to prevent the maturation of DC which prevents the initiation of a T cells response and could also prevent allograft rejection in half of the recipients (Wang et al. 2012). It has been suggested that DC may be used as a novel tolerance-inducing cellular therapeutic. The potential use of different subsets has been described (Lebre & Tak 2009) and it has been shown that clinical grade tolDC can be generated from RA patients (Harry et al. 2010). Two Phase I clinical trials using tolDC in type I diabetes or RA have been conducted recently and results showed that DC treatment was well tolerated with no major adverse effects (Giannoukakis et al. 2011; Thomas et al. 2011). Another Phase I tolDC trial studying autologous tolDC for the treatment of RA (AUTODECRA) is currently nearing completion (Hilkens & Isaacs 2013) and has also found that tolDC treatment is safe (C. Hilkens, personal communication). The potential for tolDC treatment in the prevention of transplant graft rejection has also been widely investigated (Ezzelarab & Thomson 2012; Svajger & Rozman 2014).

### *1.2. Rheumatoid arthritis*

The name rheumatoid arthritis (RA) was first coined in 1859 by Dr Alfred Baring Garrod, a British rheumatologist, although the disease had previously been described in 1800 by the French physician Dr Augustin Jacob Landré-Beauvais (Fraser 1982). RA is a chronic autoimmune disease which, along with many other autoimmune diseases, is the product of a breakdown in self-tolerance the outcome of which is the development of chronic inflammation and tissue destruction. RA is characterised by synovial inflammation of peripheral joints as well as cartilage loss and bone destruction (Firestein 2003), however its precise aetiology remains unknown. The pattern of joint involvement is often symmetrical and frequently affects the shoulders, wrists, knuckles, middle finger joints, knees, ankles, balls of feet and middle toe joints. Subtypes of RA and the assessment of disease progress are described by (Scott & Steer 2007).

#### *1.2.1. An overview of RA epidemiology, aetiology and pathology*

RA affects around 0.5 – 1% of the population (Silman & Pearson 2002) in total and over 400,000 people in the UK with onset most commonly between the ages of 40 and 50. RA affects pre-menopausal women 2 to 3 times more than men and its incidence increases with age. RA patients have an increased risk of comorbidity for example cardiovascular, infectious, haematologic, gastrointestinal and respiratory diseases (Gabriel & Michaud 2009). Overall life expectancy is lower in RA patients compared to the rest of the population and research suggests that this difference may be increasing (Gonzalez et al. 2007).

The disease can lead to severe disability therefore has a great impact on both the National Health Service as well as the economy, with around 30% of patients losing their jobs within 5 years due to disease severity (Sokka et al. 2010). The direct cost (medicines and care) of RA estimated by the UK National Audit Office is around £560 million and the total costs (including indirect costs such as unemployment) can be up to £4.8 billion a year (National Audit Office 2009). The development and manufacture of new therapeutics is expensive and prices can be over £10,000 per patient per year. Over time the benefit of such treatments may balance their cost (Schoels et al. 2010) although this is difficult to determine until treatments have been in use for several years.

Autoantibodies such as rheumatoid factor (RF) and anti-citrullinated peptide antibody (ACPA) are associated with RA diagnosis (Goëb et al. 2008) and can appear in the blood 10-15 years before the clinical onset suggesting that the process leading to RA starts long before symptoms occur. ACPA recognise citrullinated epitopes such as citrullinated fibrin, fibrinogen, collagen and vimentin whereas RF is an autoantibody specific for the Fc portion of IgG. IgM RF is present in up to 75% of RA patients and is associated with a more severe disease pathology although IgG and IgA RF are also been implicated in disease. It is important to also consider that RF is not RA specific may also be found in other autoimmune diseases, infections and malignancies. IgM RF and ACPA are highly associated with each other and with more aggressive RA. Other putative autoantigens include type II collagen (Kim et al. 1999), cartilage glycoprotein 39 (van Bilsen et al. 2004), aggrecan (Zou et al. 2003) and BiP (Bodman-Smith et al. 2004) to which reactivity has been shown in RA patients.

The synovial membrane lines non-weight bearing parts of the synovial cavity and contains two major cell types; type A synoviocytes (macrophage-like) and type B synoviocytes (fibroblast-like) (Iwanaga et al. 2000). During RA the synovial membrane becomes hyperplastic; neovascularisation occurs, the number of type A and type B synoviocytes increases and the sublining becomes infiltrated with immune cells. As the disease progresses synoviocytes migrate onto the articular cartilage forming the pannus and immune cells infiltrate the synovium. Articular cartilage is made up of chondrocytes within a matrix of collagen and proteoglycans. The breakdown of cartilage is mediated by metalloproteinases which are regulated by tissue inhibitors of metalloprotinases (TIMP) (Firestein 2003). Growth factors such as transforming growth factor $\beta$ (TGF$\beta$) and insulin-like growth factor-1 (IGF-1) stimulate cartilage growth however, in RA the increase in inflammatory cytokines (IL-1 and TNF) reduces synthesis and increases the breakdown of cartilage.

Pro-inflammatory cytokines are abundant in RA joints mainly including TNF, IL-1, and IL-6 but others are also present including IL-12, IL-15, IL-17 and IL-18. The RA joint also includes anti-inflammatory cytokines including IL-10, IL-13 and TGFβ as well as neutralising factors such as soluble TNFR and IL-1 receptor antagonist. Other inflammatory mediators such as vascular endothelial growth factor (VEGF) and vascular cell adhesion molecule-1 (VCAM-1) which stimulate endothelial cell growth may also be involved in RA. An imbalance between pro- and anti-inflammatory cytokine is known to play a role in autoimmunity and inflammation and is therefore an important pathogenic mechanism in RA (reviewed in (McInnes & Schett 2007)). In addition, the accumulation of reactive oxygen species (ROS) is attributed to reperfusion injury within the chronically inflamed joint. Consequent hypoxia contributes to the promotion of neovascularisation in rheumatoid synovitis.

Infectious triggers have been implicated in RA and a transient infection could trigger chronic inflammation. Epstein Barr virus (EBV) has been shown to enhance antibody production of $CD25^+$ B cells in RA patients which may contribute to inflammation (Brisslert et al. 2013). *Mycobacterium tuberculosis* and heat shock proteins have also been identified as possible triggers and Parovirus B19 is known to cause an illness in humans with similarities to RA (Colmegna & Alberts-Grill 2009). As RA is more common in women but improves during pregnancy, this suggests that sex hormones may be involved. Also, certain foods may trigger episodes of arthritis or may be protective (Di Giuseppe et al. 2013) and, although it seems to vary between patients, caffeine consumption and high protein diets have both been implicated. A flow diagram of the pathology of RA is shown in Figure 3.

### *1.2.2. Genetic involvement in the aetiology of RA*

Studies have shown that there is a genetic susceptibility to RA as disease concordance in monozygotic (identical) twins was around 15% and dizygotic (non-identical) twins was 3.6% (Silman et al. 1993). This shows that RA does have a genetic factor, however, other influences may also be involved in its aetiology.

The MHC genetic region has been linked to RA. This region includes both class I (HLA-A, HLA-B and HLA-C) and class II (HLA-DR, HLA-DQ and HLA-DP) genes. A degree of genetic association with the HLA-DR haplotypes can be seen as over 90% of RA patients possess a HLA-DRB1*04 subtype (DRB1*0401, *0404, *0405, *0408) whereas the frequency of these alleles in the average population is around 35% (Ebringer & Wilson 2000). The HLA-DRB1 allele includes a 5 amino acid sequence (position 67-74) in the third hypervariable region known as the ‘shared epitope’ which is strongly associated with RA pathogenesis (Gregersen et al. 1987). Although the autoantigen in

RA has not been definitively identified, it is possible that the shared epitope may bind an autoantigen-derived peptide with high affinity leading to an increased risk of RA. It is also possible that the shared epitope may be homologous to microbial antigens and bacterial heat shock proteins therefore T cells generated during an infection may cross react with autoantigens leading to disease.

Other genes associated with RA include protein tyrosine phosphatase-22 (PTPN22) which regulates lymphocyte activation by reducing TCR signalling (Fousteri et al. 2013; Begovich et al. 2004), cytotoxic T lymphocyte-associated protein 4 (CTLA-4) which downregulates T cell activation and STAT4 which is a signalling molecule downstream of the IL-12/IL-23 receptor. An interaction between the HLA-DRB1 shared epitope and the A allele of *PTPN22* polymorphism R620W are associated with ACPA positive RA (Kallberg et al. 2007).

A relationship between smoking and the shared epitope has been identified as important in RA aetiology. It has been shown that smoking is linked to RA in patients which carry a predisposing genotype (such as HLA-DRB1) and is potentially due to citrullination of proteins in the lung (Klareskog et al. 2006; Mahdi et al. 2009). This along with other genetic factors such as single nucleotide polymorphisms (Hussein et al. 2011) and non-genetic risk factors, suggest that the epidemiology of RA cannot be attributed to a single distinct cause (Silman & Pearson 2002).

### *1.2.3. The contribution of immune cells to RA pathogenesis*

During RA, immune cells such as $CD4^+$ T cells, B cells, macrophages and DC infiltrate the synovium where they can form highly organised structures (e.g. germinal centres) and interact with synoviocytes. Type B (fibroblast-like) synoviocytes are major effectors of cartilage destruction. They also secrete growth factors which inhibit apoptosis of lymphocytes such as BAFF which maintains B cell survival and IFN$\beta$ which prevents T cell apoptosis (Bartok & Firestein 2010). Receptor activator of nuclear factor $\kappa$B ligand (RANKL) is induced on type B synoviocytes and osteoblasts by pro-inflammatory cytokines and a soluble form is secreted by activated T cells. It interacts with RANK on osteoclast precursors resulting in their differentiation and activation leading to bone destruction (Zhang et al. 2001). Macrophage colony stimulating factor (M-CSF) is also required for osteoclast formation.

The vascular endothelium in rheumatoid synovium expresses a number of adhesion molecules (including E-selectin) and intercellular adhesion molecules (such as ICAM), the expression of which is stimulated by pro-inflammatory cytokines and results in the recruitment of inflammatory cells. Chemokines including monocyte chemotactic protein-1 (MCP-1), IL-8, RANTES, monocyte chemoattractant protein 4 (MCP-4) and CCL20

also recruit inflammatory cells (Szekanecz et al. 2003). Therefore although pro-inflammatory cytokines play a role in the pathology of RA (McInnes & Schett 2007), it is unlikely that they are the primary cause.

The role of B cells in RA (reviewed in (Marston et al. 2010)) involves the production of autoantibodies (RF and ACPA) and the secretion of pro-inflammatory cytokines (TNF and IL-6). B cells may contribute to RA through a number of different mechanisms including antigen presentation, T cell activation and DC modulation via the production of cytokines. B cells produce a wide range of cytokines (Pistoia 1997) and the cytokine profile produced is dependent on a range of different signals. For example B cells have been shown to produce pro-inflammatory cytokines (TNF, LT and IL-6) in response to B cell receptor (BCR) and CD40 stimulation but produce anti-inflammatory cytokines (IL-10) in response to CD40 stimulation alone (Duddy et al. 2004). Also it has been shown that RF specific B cells can be activated by immune complexes via synergistic activation of the B cell receptor and TLR (Leadbetter et al. 2002). B cell depletion (e.g. using Rituximab) has proved to be an effective therapy for RA and is usually used following the failure of at least one TNF antagonist (Edwards et al. 2004; Lee et al. 2011)(Lee et al. 2011).

Macrophages have also been implicated in RA pathogenesis, although they are unlikely to be the primary cause, as they are found in high numbers in synovial fluid and can produce pro-inflammatory cytokines. Macrophages produce a number of pro-inflammatory cytokines including TNF, IL-1, IL-6 and macrophage migration inhibitory factor (MIF) which is a potent pro-inflammatory cytokine (Morand et al. 2006) which can contribute to and amplify inflammation.

#### *1.2.3.1. DC involvement in RA pathogenesis*

DC have been suggested as the driving force behind RA and contribute to its pathogenesis in a variety of ways (Lutzky et al. 2007; Pettit & Thomas 1999). DC production of pro-inflammatory cytokines such as TNF may directly contribute to the pathogenesis of RA (Leung et al. 2002). In the rheumatoid joint the DC population increases in response to the inflammatory environment and may drive the development of $CD34^+$ progenitors to form CD14-derived DC (Santiago-schwarz et al. 2001). TNF in the joint may also induce differentiation of inflammatory DC from monocytes as data has shown that monocytes can differentiate into $CD70^+$ DC which induce Th1 and Th17 responses in the presence of TNF (Iwamoto et al. 2007). Inflammatory DC have been shown to act as effectors in cartilage destruction (characteristic of arthritis) via an indirect mechanism involving the production of TNF (Lakey et al. 2009).

Differentiated DC which are positive for nuclear RelB and have a high capacity to stimulate T cells are enriched in the synovial tissues of RA patients (Thomas et al. 1994; Pettit et al. 2000). DC are often found in close association with T cells and have the capacity to present autoantigens and activate T cells therefore may perpetuate the inflammatory response (Lutzky et al. 2007). It has therefore been suggested that in disease, peripheral DC may prime the immune system to respond to self-antigens as opposed to inducing tolerance.

Both myeloid and pDC are present within RA synovial tissues (Jongbloed et al. 2006). Myeloid DC having a more mature phenotype and express CD80, CD83, CD86 and DC-LAMP (Thomas & Quinn 1996; Thomas et al. 1994) whereas pDC appear more immature and have low or no expression of CD83, CD86 or DC-LAMP (which promotes Th1 responses) (Van Krinks et al. 2004). However, myeloid DC can respond to further stimulation *ex vivo* suggesting that they may not be fully mature within the synovial tissues (Jongbloed et al. 2006). Both myeloid DC and pDC have been shown to express IL-15, IL-18 and IFN$\alpha/\beta$ were mainly expressed by pDC and IL-12p70 and IL-23p19 were mainly expressed by myeloid DC (Lebre et al. 2008).

DC may also affect the initiation and propagation of RA by presenting arithrogenic antigens to T cells. It has also been shown that myeloid DC expressing high levels of heat shock protein (hsp)70 can be found in synovial fluid of RA patients and indicates that hsp70 may chaperone autologous antigens onto the surface of DC (Martin et al. 2014). This suggests that DC found in synovial fluid may be capable of presenting autoantigens within the joint. Synovial DC can induce Th1 responses *ex vivo* and therefore potentially also promote a Th1 response in vivo (Santiago-schwarz et al. 2001). It has been shown in a mouse model of arthritis that the expression of tenascin-C by DC is important in the DC-mediated polarisation of T cells to Th17 cells during inflammation (Ruhmann et al. 2012).

#### *1.2.3.2. T cell involvement in RA pathogenesis*

T cells play an important role in the immune system, are present in rheumatoid synovium (Bankhurst et al. 1976) and have been implicated in the pathogenesis of RA (Lundy et al. 2007). T cells develop in the thymus and can recognise a wide range of antigens due to gene rearrangement of the T cell antigen receptor (TCR). Any T cell which recognise self-antigens are usually clonally deleted before they can leave the thymus (von Boehmer et al. 1989), but a number of auto-reactive T cells escape negative selection and are released from the thymus. The effects of T cells in RA pathogenesis are reviewed by (Cope 2008).

It has been shown that $CD4^+$ T cells (also known as T helper cells) are essential for the transfer of collagen-induced arthritis (CIA) to severe combined immunodeficient (SCID) mice which have no B or T cells (Kadowaki et al. 1994). The K/BxN mouse model (100% of which develop spontaneous arthritis) shows that arthritis may result from a breakdown in the mechanism of self-tolerance as opposed to recognition of a specific autoantigen (Kouskoff et al. 1996). In this model arthritis is caused by an autoimmune systemic T cell-mediated response to the cytosolic enzyme glucose-6-phosphate isomerise (GPI) (Kamradt & Schubert 2005).

Regulatory T ($T_{reg}$) cells maintain tolerance to self-antigens in the periphery by suppressing the immune system. $CD4^+CD25^+$ $T_{reg}$ cells isolated from the peripheral blood of RA patients have been shown to be ineffective in the suppression of $CD4^+CD25^-$ effector T cells suggesting that their function is impaired. Treatment with anti-TNF resulted in an expansion of natural $T_{reg}$ cells and an increase in their suppressive activity (Ehrenstein et al. 2004). Also the success of CTLA4Ig treatment such as Abatacept which inhibits T cell co-stimulation and down regulates their activation shows that T cells are important in RA pathogenesis (Teng et al. 2005). Abatacept is composed of the Fc region of $IgG_1$ and the extracellular domain of CTLA4 which blocks co-stimulation between CD28 and CD80/CD86 (B7 molecules) and therefore blocks the co-stimulation of T cells (Buch et al. 2008). Effector T cell and $T_{reg}$ function can also be restored by anti-TNF therapy in RA patients (Maurice et al. 1999; Bryl et al. 2005).

RA is often considered a Th1-mediated disease however recent data also implicate Th17 cells in the pathogenesis of inflammatory diseases. IL-17 is highly produced in the RA synovium (Chabaud et al. 1999). Th17 cells have been shown to play a role in enhancing collagen induced arthritis (CIA) in mice (Murphy et al. 2003). Other mouse models have shown that inhibition of IL-17 in the joints suppresses inflammation and damage whereas overexpression increases joint damage (Lubberts et al. 2005). IL-17 activates endothelial cells and induces pro-inflammatory cytokines (TNF, IL-1, IL-6 and IL-8) and RANKL on chondrocytes and osteoblasts leading to osteoclast activation (Van bezooijen et al. 1999; Lubberts et al. 2005). These contribute to synovial inflammation and bone damage.

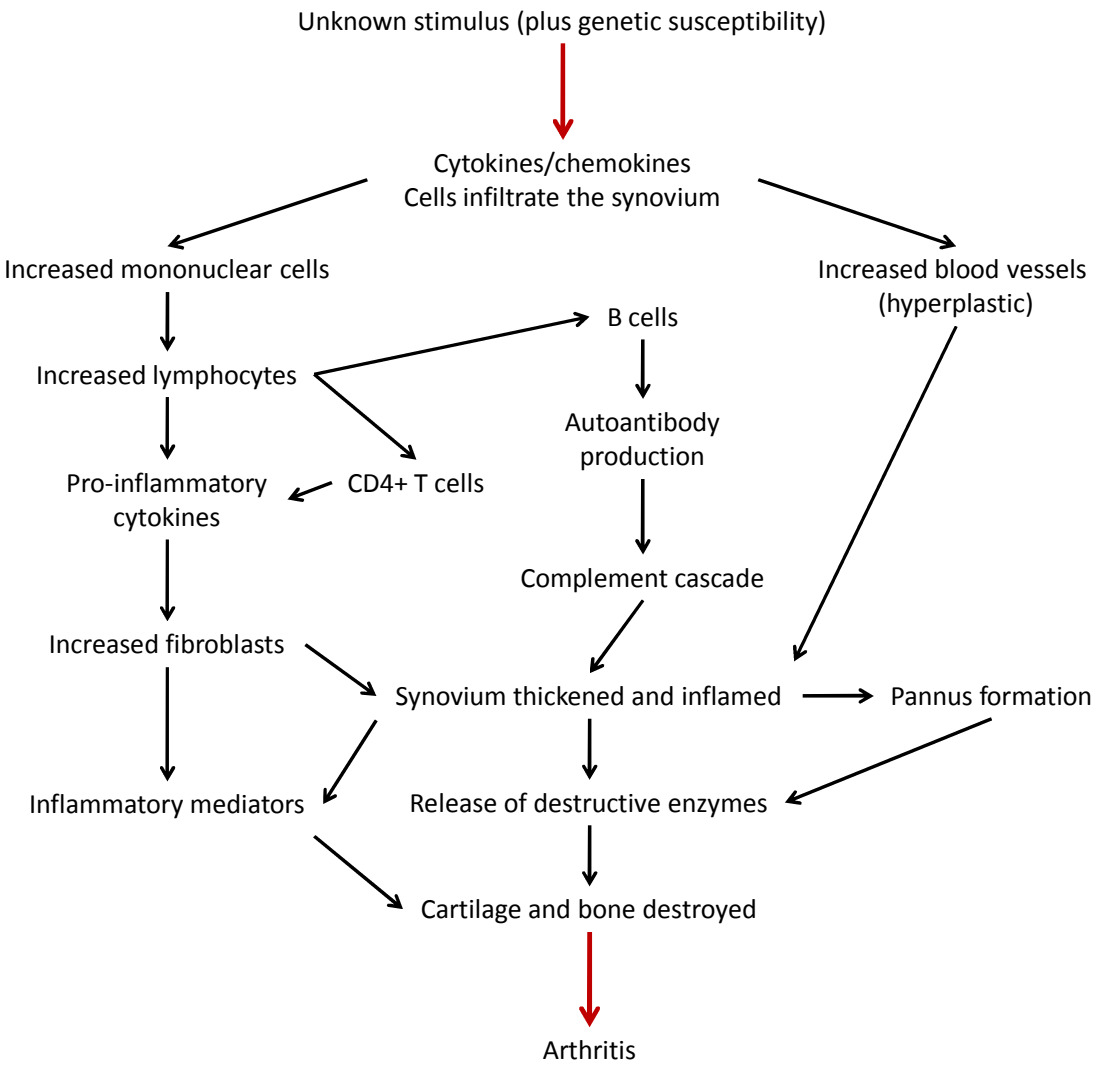


**Figure 3: Factors contributing to the development of RA.** *The primary trigger for RA remains unknown but may involve an infectious trigger, citrullination of proteins, a genetic predisposition or a combination of these factors. The infiltration of immune cells into the synovium leads to the initiation of an immune response and recruitment of immune cells. B cells may produce autoantibodies and T cells release pro-inflammatory cytokines. The following production of pro-inflammatory mediators and the release of enzymes leads to the destruction of bone and cartilage resulting in arthritis.*

### *1.3. Tumour necrosis factor*

Tumour necrosis factor (TNF) is a ubiquitously expressed cytokine with a diverse biological role and is a key mediator of inflammatory diseases such as RA. It was first described and named by Carswell et al. in 1975 (Carswell et al. 1975) and was later cloned and biochemicaly characterised (Pennica et al. 1985). The TNF superfamily contains 19 members, which signal through 29 membrane receptors. TNF is produced as a 26 kDa type II membrane protein (mTNF) (Kriegler et al. 1988) by many different cell types including activated immune cells such as DC. The transmembrane TNF protein forms stable homotrimers and can be cleaved by the metalloprotease TNF converting enzyme (TACE) to release a soluble form (sTNF) (Black et al. 1997; Wajant et al. 2003). Both mTNF and sTNF exist as homotrimeric bioactive forms and exert differential, but also overlapping functions. Transgenic mouse models have been used to show that sTNF is required for the development of chronic inflammation whereas mTNF can protect against chronic inflammation and autoimmunity (Apostolaki & Victoratos 2010).

#### *1.3.1. TNF in DC survival and maturation*

TNF also plays an important role in DC survival and function. It promotes both DC survival (Ludewig et al. 1995) and maturation and has been shown to increase the expression of MHCII and co-stimulatory molecules such as CD40 (Sallusto & Lanzavecchia 1994). Mice deficient in TNF fail to induce full DC maturation *in vivo* in response to viral challenge (Trevejo et al. 2001) and *ex vivo* generation and maturation of DC is also impaired but can be restored by the addition of exogenous TNF (Ritter et al. 2003; Brunner et al. 2014). Immature DC can induce T cell tolerance whereas mature DC activate T cells, therefore dysregulation of TNF in DC maturation may be linked to autoimmunity (Chen et al. 2006).

Neutralisation of autocrine TNF during DC maturation impaired the survival of DC and enhanced co-stimulatory molecule expression and T cell stimulatory capacity (Baldwin et al. 2010; van Lieshout et al. 2005; Lehner et al. 2012). The effect of blocking TNF during the maturation of monocyte-derived DC isolated from RA patients and healthy controls has also been studied. Results showed that although cell surface maturation markers such as CD86, CD83 and HLA-DR were not altered in either RA or control cells, the TNF blockade resulted in a semi-mature DC phenotype with reduced chemokine receptor expression and decreased IL-1$\beta$ and IL-6 secretion (van Lieshout et al. 2005). In addition, DC from RA patients receiving anti-TNF therapy display impaired upregulation of CD80 and CD86 after *ex vivo* LPS stimulation (Baldwin et al. 2010).

Autocrine TNF is essential for moDC survival and induces anti-apoptotic members of the Bcl-2 family (Lehner et al. 2012). Overexpression of Bcl-2 specifically in DC can prolong their survival in transgenic mice (Nopora & Brocker 2002) and upregulation of Bcl-xL is associated with a reduction in Fas-mediated apoptosis induced by DC activation (Lundqvist et al. 2002), thereby indicating the importance of Bcl-2/Bcl-xL in DC survival which will be discussed in more detail in chapter 1.4.2.1.

### *1.3.2. TNF receptors*

TNF exerts its bioactivity via two transmembrane receptors; TNF receptor (TNFR) 1 and TNFR2, however the individual functions of these receptors is not yet fully understood. TNFR1 is expressed ubiquitously at low levels whereas TNFR2 expression is highly regulated and restricted to subpopulations of immune cells (including DC), neuronal tissues and endothelial cells (Wajant et al. 2003) (Table 2). Both receptors are capable of binding mTNF and sTNF with high affinity. However, only sTNF leads to efficient activation of TNFR1, whereas mTNF is capable of activating both TNFR1 and TNFR2 (Grell et al. 1995). Although the molecular basis of this difference is not yet fully understood, it could be demonstrated that the difference in response of TNFR1 and TNFR2 to sTNF is instead controlled by the TNFR stalk region, which links the transmembrane region with the extracellular four cystein-rich regions of the TNFR (Richter et al. 2012), thereby affecting the cell surface distribution, pre-clustering and localisation of the TNFR and thus its responsiveness towards sTNF. The half-life of the TNF/TNFR complex seems to have less impact on sTNF responsiveness than originally thought (Krippner-Heidenreich et al. 2002; Richter et al. 2012).

| **TNFR1** | **TNFR2** |
|---|---|
| Ubiquitously expressed | Immune cell and neuron restricted |
| Responds strongly to both sTNF and mTNF | Responds strongly to mTNF but not sTNF |
| Mainly pro-inflammatory | Pro- and anti-inflammatory |
| Direct activator of cell death | Cooperates or counteracts TNFR1 signalling |

*Table 2: A comparison of TNFR1 and TNFR2. Differences in expression, TNF responsiveness and signalling between TNFR1 and TNFR2 are shown.*

Members of the TNFR superfamily are divided into two groups according to their intracellular domains; death domain containing receptors (e.g. TNFR1) and non-death domain containing receptors (e.g. TNFR2) which contain one or more TNF receptor associated factor (TRAF) binding sites. Receptor activation results in the recruitment of the adaptor molecule TRAF 2 via a consensus motif (Ye et al. 1999) and leads to the activation of the JNK and NFκB pathways (as described in chapter 1.4 and 1.4.1). As well as a death domain, TNFR1 also contains a TNFR1 internalisation domain (TRID) which includes an YXXW motif essential for receptor internalisation. A single amino acid substitution in this motif prevents internalisation and therefore prevents the formation of the death-inducing signalling complex (DISC) and TNF-induced apoptosis (Schneider-Brachert et al. 2004) indicating that TNFR1 internalisation is essential for the induction of apoptosis.

The extracellular domains of both TNFR contain four cystein rich domains (CRD) connected to a transmembrane domain by a stalk region. Each CRD contains two out of five structural molecules (A1, A2, B1, B2 and C2) as described by Naismith and Sprang (Naismith & Sprang 1998). The intracellular domain of TNFR1 includes a death domain whereas TNFR2 contains two TNF associated factor 2 (TRAF2) binding domains. Crystal structure analysis of the LTα/TNFR and the TNFR/TNF complex revealed that receptor subunits bind the grooves between TNF or LTα homotrimer subunits (Rodseth et al. 1994). The membrane distal CRD (CRD1) at the N-terminus of each receptor includes a conserved pre-ligand binding assembly domain (PLAD) which is distinct from the ligand binding domain and is involved in the formation of TNFR homodimers/homotrimers prior to ligand binding. Efficient binding of the TNF ligand by the TNFR has been shown to depend on receptor self-assembly and is abolished by the deletion of CRD1 (F. K.-M. Chan 2000), although this could also be caused by potential destabilization of the ligand binding domain of CRD2 (Branschädel et al. 2010). TNFR homodimerisation/pre-assembly does not in itself induce signalling but may function to enhance the efficiency and specificity of TNFR-signalling by avoiding heterodimerisation with other TNFR (i.e. TNF may otherwise recruit TNFR2 to a TNFR1 complex which would inhibit TNFR1-mediated signalling). Alternatively, this may not be the case as TRAIL receptors have been shown to form PLAD-mediated ligand-independent heterodimers (Neumann et al. 2014) and TNFR heterodimers may be prevented by different areas of receptor localisation.

### *1.4. Initiation of TNFR1- and TNFR2-mediated signalling*

Efficient signalling requires the formation of TNF-TNFR clusters. Binding of TNF is the prerequisite for receptor clustering, the formation of the signalling complex and finally

for signal initiation (Banner et al. 1993). The minimal cluster size required for efficient signalling is not known to date, however, in the case of the Fas/FADD complex biochemical and structural analyses reveal that higher order ligand/receptor clusters optimally position the FADD death effector domain (DED) to interact with the caspase-8 DED (Scott et al. 2009), thus suggesting 5-7 receptors complexed to two ligands as the minimal signalling competent ligand/receptor complex (Wang et al. 2010). As Fas and TNFR1 are both members of the TNFR superfamily which can recruit FADD and lead to DISC formatting, it is possible that TNF/TNFR complexes may follow a similar pattern.

TNFR exist as pre-formed complexes on the cell surface (involving the PLAD domain within CRD1), which is thought to be essential for ligand binding (Chan 2000; Branschädel et al. 2010). Interestingly, soluble PLAD proteins can block the effects of TNFR1 and TNFR2 mediated signalling *in vitro* and in a collagen induced arthritis (CIA) mouse model (Deng et al. 2005) indicating the importance of PLAD in ligand binding and/or higher molecular TNF/TNFR complexes. Upon TNF binding the PLAD interaction is replaced by a more stable receptor-ligand interaction and therefore free to associate with other TNF/TNFR trimers. This could be the link to higher order molecular TNF/TNFR complexes such as a dimer- (Figure 4) or trimer-based model. However, it currently remains unclear whether this is indeed the case.

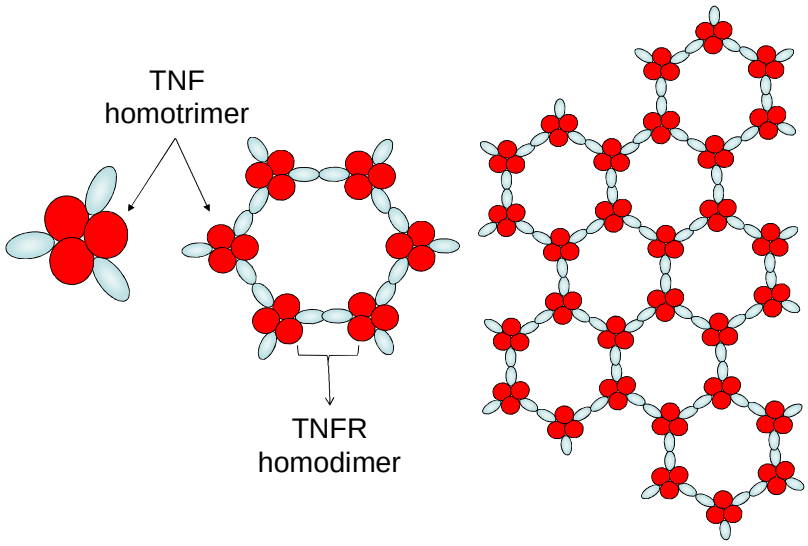


**Figure 4: Dimer model of a ligand-bound receptor complex.** *It is possible that three pre-assembled receptor dimers may bind one TNF homotrimer therefore aggregation of dimers is required to maintain the threefold symmetry of the ligand-bound receptor complex (Krippner-Heidenreich et al. 2002; Schneider-Brachert et al. 2004; Chan 2007).*

TNFR-mediated signalling can activate a number of different signalling pathways for example; the NFκB pathway (chapter 1.4.1), the mitogen-activated protein kinase-dependent (MAPK) pathway and the PI3K pathway. The MAPK/extracellular signal-regulated kinases (ERK) pathway (also known as the Ras-Raf-MEK-ERK pathway) is pro-apoptotic and involves a chain reaction of protein phosphorylation which acts as an on/off switch to downstream proteins. After membrane receptor activation the GTPase Ras activates Raf (MAP3K) which activates MEK1 and MEK2 turn activate a MAPK which can then activate various transcription factors such as AP-1. The PI3K pathway (summarized in (Cantley 2002)) is important in the inhibition of apoptosis. PI3K activation leads to the activation and phosphorylation of Akt (a serine/threonine protein kinase), which can activate proteins involved in protein synthesis, cell proliferation, cell cycle entry and apoptosis.

Intracellular signalling mechanisms of TNFR1 and TNFR2 are summarised in Figure 5. Briefly, TNFR1 activates the anti-apoptotic classical NFκB pathway and the pro-apoptotic caspase and MAPK pathways. Unlike the NFκB and MAPK pathways which occur after TNFR1 complex formation at the cell surface, apoptosis induction is dependent on TNFR1 endocytosis (Schneider-Brachert et al. 2004). Notably, TNFR1 can also be involved in superoxide production, lipid metabolism and activation of JNK, p38 and Akt pathways although these are not the focus of my work. TNFR2 can provide cell protective functions through the activation of NFκB and can also mediate MAPK activation (Zhao et al. 2007) as well as influencing apoptosis in T cells (Lin et al. 1997). Although TNFR2 does not contain a death domain and is therefore not capable of directly activating caspases, it may indirectly provide apoptosis inducing functions, but this is dependent on the cell type and its environment. For example, using blood from type I diabetes patients, TNFR2 agonism resulted in the selective death of insulin-autoreactive T cells but not other activated or memory T cells (Ban et al. 2008). TNFR2 can also induce activation induced cell death (AICD) in $CD8^+$ T cells as activated $TNFR2^{-/-}$ $CD8^+$ T cells were resistant to AICD (Twu et al. 2011). In contrast, TNFR1 showed enhanced classical NFκB activation and subsequent pro-survival signals which limited TNFR2-dependent AICD (Twu et al. 2011).

TRAF2 interacts directly with TNFR2 whereas it is recruited to TNFR1 via the adapter protein TRADD. TRADD also recruits the protein kinase RIP (receptor interacting protein) to TNFR1 which associates with TRAF2 and is essential for the activation of p65 NFκB. The poly-ubiquitination of RIP1 is mediated by cellular inhibitor of apoptosis proteins (cIAP) 1 and 2 which are constitutively bound to TRAF2. This induces the assembly of IKK complexes (Varfolomeev & Vucic 2008) which are essential for the phosphorylation and degradation of the inhibitory IkBα protein (described in more detail

in chapter 1.4.1). The TRAF2-cIAP1/2 complex promotes NFκB activation and caspase-8 inhibition. It therefore leads to TNF stimulated expression of pro-survival genes e.g. transcription factors which inhibit the activation of JNK and anti-apoptotic factors such as c-FLIP, cIAP1/2 and Bcl-xL.

The efficacy of TNF-mediated signalling may also depend on the concentration of soluble TNFR (both receptors can be cleaved from the cell surface as observed in chronic inflammation where high amounts of particularly soluble TNFR2 is found in the serum). Soluble TNFR1 and soluble TNFR2 can inhibit the effects of TNF by reducing its availability to bind with signalling competent receptors. Also, TNFR2 activation can lead to TRAF depletion thereby limiting its availability for recruitment to TNFR1, reducing the activation of the classical NFκB pathway and leading to apoptosis (Fotin-Mleczek et al. 2002; Rauert et al. 2011).

### *1.4.1. The NFκB pathway*

Nuclear factor (NF)κB is a transcription factor which controls cell proliferation and survival and is widely implicated in both pro- and anti-inflammatory processes (Bonizzi & Karin 2004). Defects in the NFκB pathway contribute to inflammatory disease pathology and NFκB is constitutively active in many autoimmune diseases therefore targeting the NFκB pathway is considered a promising potential target for RA therapeutics.

In mammalian cells the NFκB family contains five members; p65 (RelA), RelB, c-Rel, p50/p105 (NFκB1) and p52/p100 (NFκB2) which can form homo- and heterodimers with each combination exerting a different function in gene regulation. The inhibitor of κB (IκB) family are characterised by 6 or 7 ankyrin repeats in the C-terminus and include IκBα, IκBβ, IκBε and Bcl-3 which interact with (and inhibit) NFκB proteins. NFκB family members all contain a Rel homology domain (RHD) in their N-terminus which allows dimerisation, nuclear translocation and IκB interaction (Perkins 2007). In the cytoplasm of unstimulated cells NFκB dimers are held in an inactive state by IκB proteins which mask the NFκB nuclear localization sequence located within the RHD. IκBα is only partially effective as when dimerised with NFκB the complex may still enter the nucleus. However, the IκBα/NFκB complex is quickly exported due to a nuclear export sequence in IκBα (Hayden & Ghosh 2004).

NFκB signalling involves 2 major pathways; the classical (cannonical) pathway and the alternative (non-canonical) pathway (Figure 5). The classical pathway is induced by inflammatory stimuli and can be activated by both TNFR1 and TNFR2 (Wajant &

Scheurich 2011; Lawrence 2009). It involves the activation of the IKK complex leading to rapid phosphorylation of IκBα at Ser32 and Ser36 causing its degradation by the 26S proteosome. This most commonly results in the activation of the p50/p65 heterodimer and its translocation into the nucleus. Classical NFκB activation results in the transcription of cytokines, chemokines and adhesion molecule receptors (Vallabhapurapu & Karin 2009) and is therefore a key component in the pro-inflammatory effects of TNF. The alternative pathway can be activated by non-inflammatory stimuli and can result in the inhibition of pro-inflammatory cytokines and the induction of T cell tolerance (Zhu & Fu 2010). It can be initiated by TNFR2 but not TNFR1 (Rauert et al. 2010) and involves the activation of IKKα (but not IKKβ or NEMO) by NFκB inducing kinase (NIK). It is characterised by the induction of p100 processing and the formation of p52. Heterodimers of p52 and RelB then enter the nucleus where they bind specific κB elements and induce genes involved in proliferation and the adaptive immune response (Perkins 2007). Defects in the alternative NFκB pathway have been shown to result in defects in immune development (Caamaño et al. 1998; Franzoso et al. 1998) and lead to autoimmunity (Cheema et al. 2001).

The classical (p65) NFκB pathway NFκB pathway can be activated by both TNFR1 and TNFR2 (Lawrence 2009; Wajant & Scheurich 2011) and has been shown to be essential for DC survival (Sánchez-Sánchez et al. 2004; Kim & Joo 2009). TNF-induced phosphorylation of p65 at serine-276 is required for p65/RelB complex formation and in the absence of ser-276 phosphorylation, TNF stimulation leads to a strong increase in RelB controlled genes such as pro-survival Bcl-xL (Jacque et al. 2005). Some research indicates that TNF may activate the classical but not the alternative NFκB pathway (Derudder et al. 2003), however this result appears to depend on both the cell type and the type of stimulation used as the alternative (p100/p52) pathway has been shown to be activated by TNFR2 but not TNFR1 in primary T cells (Rauert et al. 2010).

Although there has been a lot of research into the signalling mechanisms of TNFR1, much less is known about TNFR2. TNFR2 was described to activate both the classical and non-classical NFκB pathways (Krippner-Heidenreich et al. 2002; Rauert et al. 2010), although the classical NFκB pathway was activated to a lesser extent (McFarlane et al. 2002). Upon TNFR2 stimulation TRAF2 recruitment to the receptor is required for the activation of JNK and NFκB. Activation of TNFR2 can lead to the production of sTNF which results in the activation of TNFR1 in an autocrine/paracrine fashion resulting in a feedback loop. It has been shown that blocking TNFR2 on activated T cells inhibited TNF production which suggested that mTNF on the surface of monocytes may mediate signalling (Rossol et al. 2007). TNFR2 was also shown to

activate NFκB via the activation of PI3K and Akt in mouse neurons (Fontaine et al. 2002). However, more research is needed to determine whether these mechanisms are also activated on DC. Also, DC expression of NIK (involved in the alternative NFκB pathway) is required to promote Th1 and Th17 responses (Hofmann et al. 2011) whereas NIK is not required for the activation of the classical NFκB pathway by TNF (Yin et al. 2001). During alternative NFκB signalling RelB complexes with p52 and translocates to the nucleus. RelB deficient mice have defects in DC cross-priming (Castiglioni et al. 2002) indicating its importance in the immune response.

### *1.4.2. Caspases and apoptotic cell death*

Caspases are a family of cysteine proteases that play an essential role in cell death (both apoptosis and necrosis). To date, 14 caspases have been identified in mammals, 11 or 12 of which are found in humans; caspase-1 to caspase-10, caspase-14 and caspase-12 (depending on hereditary polymorphisms) (Saleh et al. 2004). Caspases exist in the cell as inactive pro-enzymes (pro-caspases) which are found in all nucleated mammalian cells and are regulated at a post-translational level ensuring that they can be rapidly activated. Cleavage activates the caspase molecule which contains an N-terminal pro-domain, a large subunit containing the active site and a small C-terminal subunit. There are two types of apoptotic caspases; initiator caspases e.g. caspase-2, -8, -9 and -10 which activate effector caspases e.g. caspase-3, -6 and -7 thus leading to apoptosis. Other caspases such as caspase-4 and -5 are inflammatory enzymes (reviewed in (Venero et al. 2013).

The initiation of apoptosis can occur via a cell intrinsic or extrinsic pathway. The intrinsic pathway requires the release of cytochrome c from mitochondria (Figure 6) and involves members of the Bcl-2 family (as described in more detail in chapter 1.4.2.1). The extrinsic pathway involves the binding of an external ligand to a 'death receptor' (e.g. TNFR1 or Fas) on the plasma membrane. This allows the recruitment of proteins (such as FADD) to the intracellular portion of the receptor and promotes the activation of initiator caspases and subsequent effector caspases resulting in apoptosis (Parrish et al. 2013). When pro-caspase-8 is cleaved to its active form (caspase-8), it can lead to the activation of the extrinsic (type I) or intrinsic (type II) signalling pathway (Figure 5). High levels of activated caspase-8 initiate the type I pathway by mediating the activation of procaspase-3 to caspase-3 resulting in apoptosis (Schütze et al. 2008). Alternatively, low amounts of caspase-8 result in the type II pathway where the low signal is amplified via mitochondria resulting in the activation of caspase-3 via caspase-9.

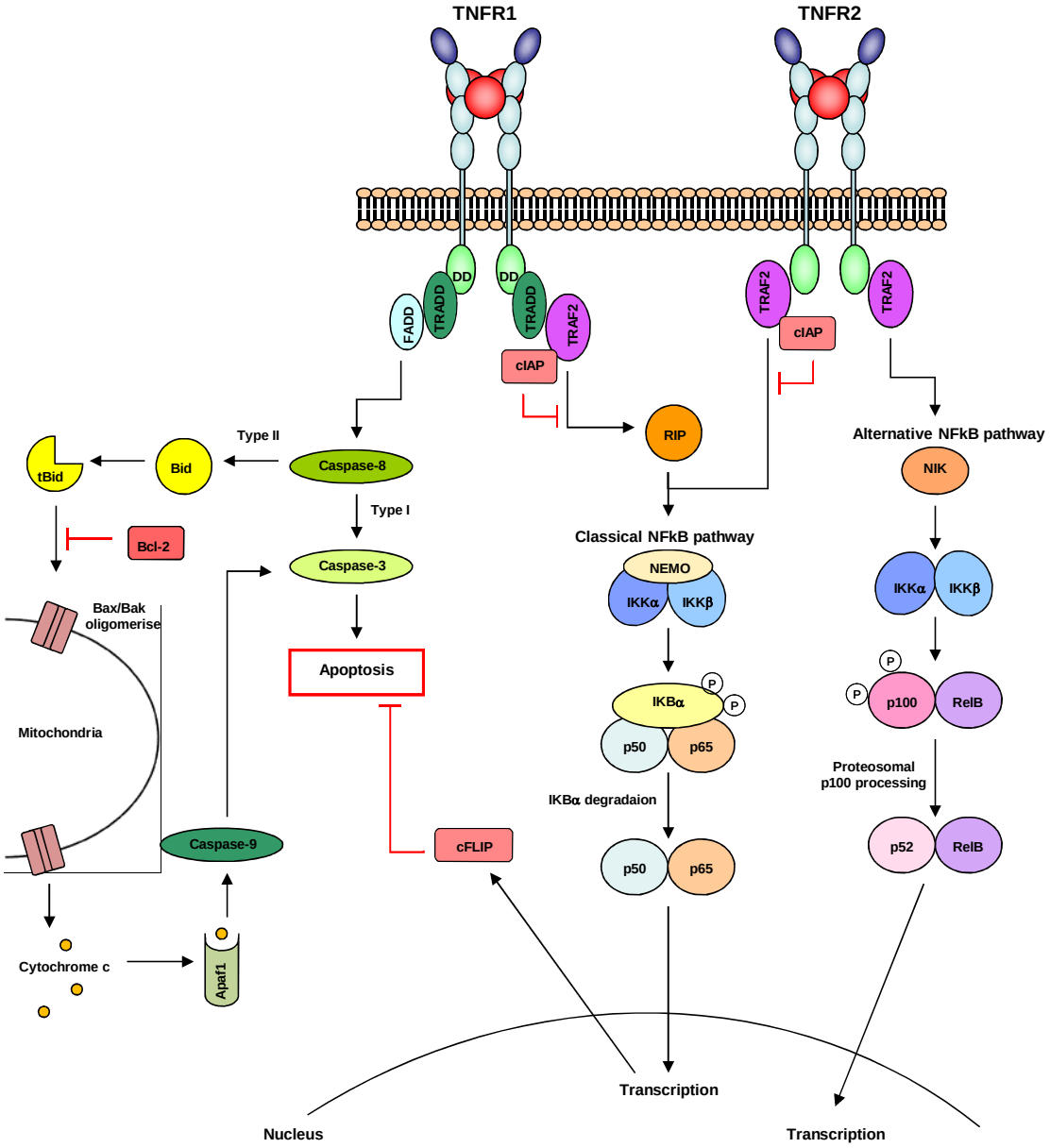


**Figure 5: TNFR1 and TNFR2 signalling pathways.** *TNFR-mediated signalling can activate caspases leading to cellular apoptosis (via either the intrinsic/mitochondrial pathway or the extrinsic/death receptor-mediated pathway) or can activate NFκB thereby inducing the expression of anti-apoptotic molecules and preventing apoptosis. TNFR1 and TNFR2-signalling can activate the classical NFκB pathway (including the phosphorylation and translocation of p65) which usually exerts anti-apoptotic signals. Whereas TNFR1 generally only activates the classical NFκB pathway, TNFR2-mediated signalling can activate both the classical and the non-classical NFκB pathways. The non-classical NFκB involves the processing of p100 to p52 and its translocation to the nucleus. Signalling pathways described in detail in section 1.4. Adapted from (Schütze et al. 2008; Perkins 2007).*

### *1.4.2.1. The Bcl-2 family*

The mitochondrial pathway of apoptosis (type II pathway) is shown in Figure 6 and involves members of the B cell lymphoma (Bcl-2) family. The Bcl-2 family play a major role in both positively and negatively regulating mitochondria-dependent apoptosis. Pro-apoptotic family members such as Bax and Bak can indirectly activate pro-caspases by inducing the release of cytochrome c whereas anti-apoptotic members including Bcl-2 itself and Bcl-xL inhibit cytochrome c release, therefore preventing apoptosis. Both p65 NFκB and p52 NFκB can bind to the Bcl-xL promoter therefore both the classical and alternative NFκB pathways may play a role in the regulation of its expression (Marinari et al. 2004; Tamatani 1999).

Other members of the Bcl-2 family include BH3-domain only proteins such as tBid, Bad and Bik, which consist only of the BH3 death domain. These proteins can initiate apoptosis by activating pro-apoptotic members or inactivating anti-apoptotic members. For example, the activation of pro-apoptotic members can be mediated by an apoptotic stimulus which can then lead to the cleavage of Bid to tBID which translocates into the mitochondria. The translocation of tBID to the mitochondria causes Bax and/or Bak (pro-apoptotic members of the Bcl-2 family) to oligomerise and form a pore in the outer mitochondrial membrane (Ow et al. 2008). Oligomerisation of Bax and/or Bak may be inhibited by Bcl-2 itself which is an anti-apoptotic family member. This pore formation allows the release of cytochrome c which binds the adapter protein apoptotic protease-activating factor-1 (Apaf-1) and along with the co-factor ATP, catalyzes its oligomerisation forming a complex called the apoptosome (Solary et al. 2008). A caspase recruitment domain (CARD) motif in Apaf-1 recruits procaspase-9 via a CARD motif in its prodomain (Chen & Wang 2002) resulting in auto-processing to caspase-9, which activates caspase-3. Unlike executioner caspases, caspase-9 is an initiator caspase and must be bound to Apaf1 as well as being cleaved to allow its activation (Zanoni & Granucci 2010).

### *1.4.2.2. TNFR-mediated caspase activation*

TNFR1 mediates both pro- and anti-apoptotic actions. Following classical NFκB pathway activation through TNFR1 there is an increase in expression of anti-apoptotic genes, resulting in enhanced cell survival. Alternatively, when the classical NFκB pathway is not fully activated, TNFR1 can mediate a pro-apoptotic signal via activation of the caspase cascade (Festjens et al. 2007). Upon TNF binding, TNFR-associated death domain (TRADD) is recruited to the TNFR1 death domain. This leads to recruitment of Fas-associating protein including death domain (FADD) and

procaspase-8 which form the death-inducing signalling complex (DISC) required for apoptosis (Fesik 2000). Death receptor mediated apoptosis can be directly inhibited by cFLIP which modulates caspase-8 activation (Schütze et al. 2008) (Figure 5).

TNFR1 may mediate pro- and anti-apoptotic responses by the formation of two separate protein complexes after TNF binding and caspase-8 activation (Micheau & Tschopp 2003). Complex I forms within minutes at the plasma membrane and may lead to NFκB activation without affecting apoptosis, whereas complex II is formed in the cytoplasm after around 2 hours. Caspase-8 and FADD are recruited to complex II and initiates apoptosis unless NFκB signalling has induced the appropriate anti-apoptotic proteins. TNFR2 can also activate the caspase cascade for example caspase-3 is activated during the selective death of autoreactive T cells following TNFR2 agonism in blood from type I diabetes patients (Ban et al. 2008).

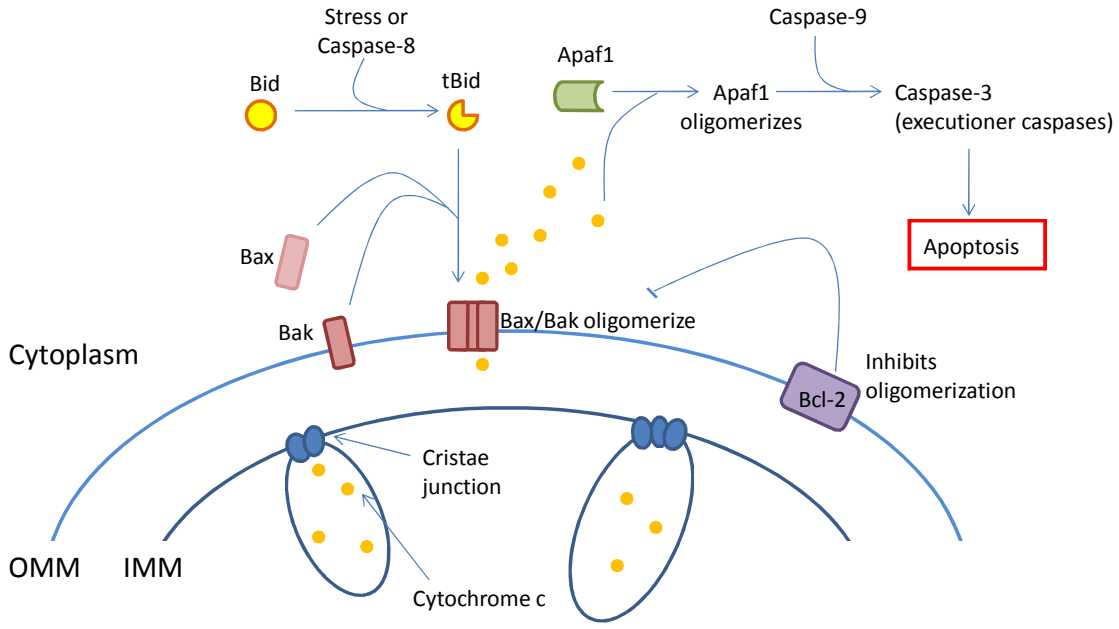


**Figure 6: The intrinsic (mitochondrial) pathway of apoptosis.** *A proposed mechanism by which Bcl-2 protein family members contribute to cytochrome c release and apoptosis. Cytochrome c is sequestered in cristae junctions in the inner mitochondrial membrane (IMM). The cleavage and subsequent activation of Bid occurs via stress signals or the extrinsic pathway of apoptosis and induces pore formation by Bax/Bak oligomerisation in the outer mitochondrial membrane (OMM). Bax/Bak oligomerisation can be inhibited by anti-apoptotic proteins such as Bcl-2 thereby preventing apoptosis. Once released, cytochrome c associates with Apaf1 and activates the caspase cascade resulting in apoptosis. Adapted from (Fraser 1982; Lutzky et al. 2007).*

### *1.5. Differential functions of TNFR1 and TNFR2 in disease models*

TNFR1 can mediate inflammatory actions (Chen & Goeddel 2002) but has also been shown to be involved in the negative regulation of pro-inflammatory IL-12p70 and IL-23 (Zakharova & Ziegler 2005). A number of studies have utilised TNFR-deficient mouse models to elucidate the role of TNFR1 and TNFR2 in disease. The data from a wide range of disease models indicate that the two TNFR can mediate opposing effects and point to a pro-inflammatory, destructive role for TNFR1 and a protective, anti-inflammatory role for TNFR2 (Masli & Turpie 2009).

In the murine antigen-induced experimental autoimmune encephalomyelitis (EAE) model TNFR1$^{-/-}$ deficient mice showed disease suppression whereas TNFR1$^{-/-}$/TNFR2$^{-/-}$ double-deficient mice showed an increase in symptoms (Kollias 2005). Another group showed that EAE was enhanced in TNFR2 deficient mice whereas TNFR1 deficient mice were resistant to EAE (Suvannavejh et al. 2000). Also in EAE, it has been shown that TNF1 is required for the detrimental effects of TNF during the acute phase of disease but is not required for TNF-mediated immunosuppression and subsequent disease remission (Kassiotis & Kollias 2001). In addition, the absence of TNFR1 has been shown to reduce the severity of induced arthritis whereas the absence of TNFR2 increased arthritis development (Blüml et al. 2010) and TNFR2 has been shown to play a protective role in bone resorbtion in mouse models (Nagano et al. 2011). These studies suggest a role for TNFR2 in suppressing inflammatory responses. Furthermore, TNFR1 has been shown to mediate cardiac dysfunction whereas TNFR2 plays a cardioprotective role in TNF-induced myocardial infarction (Higuchi et al. 2004; Monden et al. 2007) and pulmonary inflammation following bacterial challenge is increased in TNFR2 deficient mice but attenuated in TNFR1 deficient mice (Peschon et al. 2014). Also, in a murine model of CIA, a TNFR1-selective antagonist showed anti-inflammatory effects in established disease and did not result in the reactivation of viral infections unlike Etanercept (Shibata et al. 2009) suggesting that TNFR1 mediates pro-inflammatory effects.

A lack of TNFR2 has been shown to enhance neurodegeneration in a retinal ischemia mouse model whereas a lack of TNFR1 reduced the neurodegeneration (Fontaine et al. 2002). In addition, *in vitro* culture of mouse microglia have shown that TNFR2 promotes anti-inflammatory pathways (Veroni et al. 2010). In a mouse model of polymicrobial sepsis, mice lacking TNFR1 had enhanced survival whereas mice lacking TNFR2 had shortened survival and enhanced symptoms (Ebach et al. 2005). In mice with traumatic brain injury, a TNFR2 agonist reduced the inflammatory response after trauma by inhibiting the activation of the NFκB and p38MAPK pathways (Wang et al. 2013). Moreover, in human autoimmune diseases such as type 1 diabetes it has been

shown that TNFR2 agonists can selectively target and destroy autoreactive T cells from isolated human blood (Chen et al. 2010), further suggesting a protective role for TNFR2. In addition, TNFR2 has been shown to be preferentially expressed by $T_{reg}$ cells and promotes their survival and expansion (Chen & Oppenheim 2011).

This array of previous research shows opposing roles for TNFR1 and TNFR2 in different diseases, including both mouse and human models. Of particular interest is the anti-inflammatory function of TNFR2 as although TNF is primarily thought of as a pro-inflammatory cytokine, it has also long been known for its puzzling anti-inflammatory functions. For example inflammatory IL-12 production is reduced in TNF-treated murine macrophages and DC (Zakharova & Ziegler 2005) and although anti-TNF treatment can improve the disease state of RA patients, in MS anti-TNF exacerbated the disease (Robinson et al. 2001) and it may also result in demyelination and lupus (Mohan et al. 2001). The actions of TNFR2 may therefore be disease-protective and provide a potential avenue for the development of more targeted therapies i.e. involving the specific blockade of TNFR1 or specific activation of TNFR2.

### *1.6. The role of TNF in RA and the rational for anti-TNF therapies*

TNF has been associated with a range of autoimmune diseases such as RA, diabetes, multiple sclerosis (MS) and Crohn's (inflammatory bowel) disease (Feldmann et al. 2005). Synovial fluid (SF) from RA patients contains detectable levels of TNF (Saxne et al. 1988). Also, TNF has been shown to induce collagenase production by synovial cells (Dayer et al. 1985) as well as stimulating bone resorption *in vitro* (Bertolini et al. 1986) suggesting that TNF is intricately involved in the pathogenesis of RA. Furthermore, a direct involvement of TNF in arthritis was shown using a mouse model expressing human TNF (Keffer et al. 1991). Blocking TNF in the collagen induced arthritis (CIA) mouse model, which is the most commonly studied autoimmune model of RA, showed a reduction in disease severity whether administered before or after the onset of clinical arthritis (Williams et al. 1992). As in other autoimmune diseases the role of TNFR1 and TNFR2 in arthritis was originally investigated using mouse models. TNFR1 is generally considered as the driving force in arthritis development. The absence of TNFR1 has been shown to suppress induced arthritis whereas the absence of TNFR2 increased arthritis development (Blüml et al. 2010). Another group has also shown that TNFR1 is essential for the development of arthritis in mice transgenic for human TNF (Armaka et al. 2008). Furthermore, reintroduction of TNFR1 in mesenchymal cells in this model was sufficient for the development of arthritis (Armaka et al. 2008). As in other disease models, this data suggests a mainly pro-inflammatory, destructive role for TNFR1 and a protective, anti-inflammatory role for TNFR2 however,

there are also studies which show opposing data. For example, the role of TNFR1 in collagen induced arthritis (CIA), which is often used as a model for RA, appears to be more complicated. One study has shown that a lack of TNFR1 on haematopoietic cells increased the severity of CIA suggesting that TNFR1 may mediate an anti-inflammatory response in these cells. Alternatively, in another study, TNFR1 deficient mice were used to show that TNFR1 was essential for the proinflammatory signal in CIA whereas TNFR2 was found to be involved during CIA onset even in the absence of TNFR1 (Tada et al. 2001). These studies suggest that the roles of TNFR1 and TNFR2 are not as simple as 'disease-enhancing' and 'disease-protective', but vary according to cell type and environment therefore it is important to take into account the two TNFR when studying different cell types and also different diseases.

Therapeutic blockade of TNF is a very successful treatment for RA with five TNF antagonists currently approved for use in the UK; infliximab (a mouse-human chimera monoclonal antibody), adalimumab (a fully humanised monoclonal antibody) golimumab (human monoclonal antibody), certolizumab (PEGylated Fab' fragment a humanised monoclonal antibody) and etanercept (a soluble TNFR Fc-fusion protein) (Tracey et al. 2008). These have been shown, from phase III clinical trials onwards, to provide significant benefits even in patients who do not respond to other disease-modifying anti-rheumatic drugs (DMARD) (Maini et al. 1999; Feldmann et al. 2005). However, 30-40% of patients still do not respond to anti-TNF treatment and a significant number who do respond later relapse. Also, these TNF antagonists are associated with a number of side effects such as reactivation of latent infections e.g. tuberculosis or an increased risk of serious infections and malignancies e.g. lymphoma (Bongartz et al. 2006). This shows both the importance of TNF in RA and the need for improved and more targeted therapies.

Another member of the TNF superfamily is lymphotoxin (LT)$\alpha$ which is a close homologue to TNF and can bind both TNF receptors (TNFR1 and TNFR2) (Medvedev et al. 1996) along with its own receptor herpesvirus entry mediator (HVEM) (reviewed in (Remouchamps et al. 2011)). LT$\alpha$ can be detected in the joints of RA patients. In addition, LT$\alpha$ was shown to be preferentially expressed on Th1 and Th17 cells in a murine model of RA, and its removal inhibited arthritis (Chiang et al. 2009). LT$\alpha$ was as effective as TNF at activating type B (fibroblast-like) synoviocytes and, therefore, could contribute to RA pathogenesis (Calmon-Hamaty et al. 2010). Etanercept (a TNFR2-Fc fusion protein) also blocks LT$\alpha$ and was shown in one patient to induce clinical remission after failure of anti-TNF infliximab therapy (Buch et al. 2004), which may have been due to the different ligand specificities of infliximab and etanercept. Therefore, the role of LT$\alpha$ in arthritis is still unclear (Calmon-Hamaty et al. 2011).

### *1.7. Hypothesis*

TNF is known to play an important role in the maturation and survival of DC, but the individual roles of the two TNFR remain unknown. The majority of previous research has focussed on mouse models where either TNFR1 and/or TNFR2 have been knocked out, thus allowing the effects mediated by the two TNFR to be studied individually. While the human TNFR system is relatively understudied, the use of mouse models indicates that the two TNFR can mediate opposing effects in disease models. I hypothesise that TNFR1- and TNFR2-mediated signalling will exert different effects on human DC maturation and survival, and that these effects may differ between inflammatory and steady-state DC subsets.

### *1.8. Aims*

1) To utilise previously defined TNFR-selective ligands in order to dissect the roles of TNFR1 and TNFR2 in DC.
2) Identify the expression of TNFR1 and TNFR2 on human DC subsets (moDC, pDC and blood myeloid $CD1c^+$ DC).
3) Determine the competency of each TNFR for the activation of downstream NFκB signalling in moDC.
4) Investigate the involvement of TNFR1 and TNFR2 in the maturation of moDC and blood myeloid $CD1c^+$ DC.
5) Determine the individual roles of TNFR1 and TNFR2 in moDC and blood myeloid $CD1c^+$ DC survival and identify the pathways involved in the regulation of DC lifespan.
6) Determine the effect of TNFR1 and TNFR2 on the maturation and survival of myeloid $CD1c^+$ DC isolated from synovial fluid in order to compare steady state DC and DC from an inflammatory environment.

# 2. Materials and Methods

## *2.1. Cell isolation and culture*

The use of leukocyte reduction system (LRS) cones from platelet donations from healthy volunteers was approved by the Newcastle and North Tyneside Research Ethics Committee 2.

### *2.1.1. PBMC isolation*

LRS cones were diluted 1:2 (or peripheral blood 1:1) with room temperature Hanks balanced salt solution (HBSS; $Ca^{2+}$ and $Mg^{2+}$ free; Sigma-Aldrich, St Louis, USA, H9394) + 2mM EDTA. Peripheral blood mononuclear cells (PBMC) were isolated from LRS cones by density gradient centrifugation on Lymphoprep (Axis-Shield Diagnostics; Oslo, Norway). 20ml blood was layered onto 15ml Lymphoprep and centrifuged for 30 min at room temperature (895 g). PBMC were recovered from the interface and washed in cold Hanks + 1% FBS (Gibco; Paisley, UK), 600 g, 7 min, 4°C to remove any lymphoprep. Cells were then washed again in cold Hanks 1% FCS (250 g, 7 min, 4°C) to remove any platelets which may activate monocytes. Cells were filtered through a 70μm nylon filter to remove any debris and counted using a Burker counting chamber.

### *2.1.2. Monocyte-derived DC*

To generate immature monocyte-derived DC (moDC) as previously described (Anderson et al. 2008), $CD14^+$ monocytes were isolated from PBMC by positive magnetic selection using anti-CD14 magnetic microbeads (Miltenyi Biotec; Cologne, Germany). PBMC were resuspended in ice-cold MACS buffer (PBS, 2mM EDTA + 0.5% FCS) at 800μl per 100 x $10^6$ PBMC and microbeads (coupled to anti-CD14 monoclonal antibodies) at 100μl per 100 x $10^6$ PBMC and incubated on ice for 20 min. PBMC were then washed in MACS buffer (400g, 7 min, 4°C) to remove any unbound microbeads and the pellet was resuspended in 3ml MACS buffer. PBMC were added to an LS MACS column pre-rinsed with MACS buffer where $CD14^+$ cells are attracted to the magnetic beads within the column and are therefore retained in the column whereas $CD14^-$ cells flow through into the waste tube. The column was washed 3 times with 3ml MACS buffer, then it was removed from the magnet and the $CD14^+$ cells were flushed out with 3ml MACS buffer. The PBMC population generally contained 15-30% $CD14^+$ monocytes.

Cells were then washed in Hanks 1% FBS, plated at 0.5 x $10^6$/ml in RPMI-1640 (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (all Sigma-Aldrich) in a 24 well plate and cultured for 6 days in the presence of IL-4 and GM-CSF (50 ng/ml each, Immunotools, Friesoythe, Germany) at 37°C, 5% $CO_2$ to generate immature monocyte-derived (mo)DC. On day 3 media was refreshed by removing 480µl media and adding 500µl warm RF10 containing 50ng/ml IL-4 and GM-CSF. All further moDC cultures were carried out at 37°C, 5% $CO_2$, in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.

### *2.1.3. Myeloid blood DC*

Blood myeloid DC were separated from PBMC using an immunomagnetic negative selection kit (EasySep human myeloid DC enrichment kit; StemCell Technologies; Vancouver, Canada). Up to a maximum of 4 x $10^8$ PBMC were placed in a 14 ml (17 x 100 mm) polystyrene tube (BD Biosciences; Oxford, UK) at a concentration of 5 x $10^7$ cells/ml in PBS, 2% FBS, 1 mM EDTA ($Ca^{2+}$ and $Mg^{2+}$ free). The anti-human CD32 (Fcγ RII) Blocker was added at 15 µl per 1 ml, the EasySep™ Human Myeloid DC Enrichment Cocktail was added at 50 µl per 1 ml of cells and cells were incubated at room temperature for 30 min.

EasySep™ D Magnetic Particles were vortexed for 30 seconds to ensure that the particles are in a uniform suspension with no visible aggregates prior to use. The EasySep™ D Magnetic Particles were added to the cells at 100 µl per 1 ml of cells and were incubated at room temperature for 10 minutes. The cell suspension was brought to a total volume of 10 ml with PBS, 2% FBS, 1 mM EDTA ($Ca^{2+}$ and $Mg^{2+}$ free) and were mixed gently by pipetting up and down. Then the tube was placed into the Silver EasySep™ Magnet (without the tube cap) and incubated at room temperature for 5 min. Then the EasySep™ Magnet (and tube) was picked up and inverted in one continuous motion, pouring off the desired fraction into a new 14 ml polystyrene tube. The magnet and tube were left inverted for 2 - 3 seconds, then return to upright position being careful not to shake or blot off any drops that may remain hanging from the mouth of the tube. The magnetically labelled unwanted cells remained bound inside the original tube, held by the magnetic field of the EasySep™ Magnet. The original tube was removed from the EasySep™ Magnet and replaced with the new tube, incubated for another 5 min before pouring off the desired fraction as previously (for a total of 2 separations). 10 ml PBS, 2% FBS, 1 mM EDTA ($Ca^{2+}$ and $Mg^{2+}$ free) was added to the original tube and mixed gently, then the 5 min incubation and pouring off

of the desired fraction was repeated (another total of 2 separations). The negatively selected, enriched cells in the final tube (20 ml) were washed once (400g, 7 min) and then cultured in a 96-well plate at 6.5 x $10^4$ cells/200 µl.

### *2.1.4. T cells*

$CD4^+$ T-cells were isolated from LRS cones using a human $CD4^+$ T-cell enrichment cocktail (RosetteSep; StemCell Technologies). RosetteSep Human $CD4^+$ T Cell Enrichment Cocktail was added at 75 µl per ml whole blood, mixed well and incubated for 20 min at room temperature. The sample was diluted 1:2 with PBS + 2% FBS, mixed gently and enriched cells were separated by density gradient centrifugation on Lymphoprep (Axis-Shield Diagnostics) (895 g, 30 min, room temperature). Cells were recovered from the interface and washed in PBS + 2% FBS (600g, 7 min, room temperature) and counted using a Burker counting chamber.

## *2.2. Cell lines*

The MF TNFR1-Fas and MF TNFR2-Fas cells are chimaeric constructs of TNFR-Fas stably expressed in immortalized fibroblasts derived from double-receptor knock-out mice (Krippner-Heidenreich et al. 2002). The CD40L (J558L) cells (Lane et al. 1995) are mouse B cell myeloma cells which have been transfected so they express mouse CD40L (which cross reacts with human CD40) and were a kind gift from Peter Lane (Birmingham).

## *2.3. TNF ligands, antibodies and inhibitors*

The TNF ligands used were recombinant sTNF (50 ng/ml; 2 x $10^7$ units/mg; provided by Knoll AG, Ludwigshaven, Germany), recombinant $CysTNF_{wt}$ and mutants derived thereof (TNFR1-selective CysTNF32W/86T, $CysTNF_{R1}$; TNFR2-selective CysTNF143N/145R, $CysTNF_{R2}$; all at 50 ng/ml) (Krippner-Heidenreich et al. 2002; Bryde et al. 2005). The mutations that provide selectivity for the two TNFR were originally selected by a mutagenesis screen and were analysed for selective binding to either TNFR1 or TNFR2 in competition with wild type sTNF (Loetscher et al. 1993). It was found that a combination of the mutations $Arg^{32}$ to Trp and $Ser^{86}$ to Thr showed high affinity binding to TNFR1 comparable to sTNF, but over 6000-fold lower affinity to TNFR2. Whereas the mutation $Asp^{143}$ to Asn combined with $Ala^{145}$ to Arg showed low affinity to TNFR1 (over 2500-fold lower than sTNF), however, binding to TNFR2 was reduced by 5-10-fold (Loetscher et al. 1993). The cloning, expression and purification of the CysTNF variants were described previously (Krippner-Heidenreich et al. 2002; Bryde et al. 2005). $CysTNF_{wt}$, $CysTNF_{R1}$ and $CysTNF_{R2}$ contain a free Cys residue at

their N-terminus which leads to oligomerisation of the TNF monomers, primarily to hexamers. Additionally, each of the CysTNF variants contain an N-terminal His-tag thereby facilitating their purification. It has been demonstrated that sTNF and the TNF variants possess similar bioactivities when used at saturating concentrations of 50 ng/ml (which is at least 50-fold in excess of their ED50). The ED50 of sTNF and $CysTNF_{R1}$ = 0.1-0.3 ng/ml and the ED50 of $CysTNF_{wt}$ and $CysTNF_{R2}$ plus MAb 80M2 = 0.3-1 ng/ml as determined by a cellular *in vitro* system (see method 2.4, Figure 8 and (Krippner-Heidenreich et al. 2002)).

| Ligand | TNFR activation |
|---|---|
| sTNF | TNFR1 |
| $CysTNF_{wt}$ | TNFR1 and TNFR2 |
| $CysTNF_{R1}$ | TNFR1 |
| $CysTNF_{R2}$ | TNFR2 |

The antibodies used include MAb 80M2; a mouse anti-human TNFR2-specific monoclonal antibody which was a kind gift from Peter Scheurich, IZI Stuttgart, Germany; (Grell et al. 1995). The antagonistic TNFR-specific antibodies are TNFR1-specific H398 (Hbt Hycult) and TNFR2-specific MR2-1; (Hbt Hycult for moDC) or clone 22221; (R&D Systems, for blood DC). The small molecule inhibitors used were BAY 11-7082 (Cell Signalling), Abt-737 (Selleck Chemicals), LY294002 (Cell Signaling #9903), U0126 (MEK1/2, Cell Signaling #9901).

### *2.3.1. Antibodies for flow cytometry*

| Antibody | Fluorophore | Clone | Company |
|---|---|---|---|
| Active Caspase-3 | PE | C92-605 | BD Biosciences |
| CD1c | PE-Cy7 | L161 | Biolegend |
| CD3 | FITC | UCHT1 | BD Biosciences |
| CD11c | FITC | Lot 5130228177 | Miltenyi |
| CD11c | V450 | B-ly6 | BD Biosciences |
| CD14 | APC-H7 | MΦPg | BD Biosciences |
| CD16 | APC | B73.1 | BD Biosciences |
| CD19 | FITC | HIB19 | BD Biosciences |
| CD20 | FITC | 2H7 | BD Biosciences |
| CD45 | V500 | H130 | BD Biosciences |
| CD54 (ICAM-1) | APC | Lot 74900 | BD Biosciences |
| CD56 | FITC | B159 | BD Biosciences |
| CD83 | APC | HB15e | BD Biosciences |
| CD86 | FITC | 2331 | BD Biosciences |
| CD123 | PerCP-Cy5.5 | 6H6 | eBiosciences |
| CD274 (PD-L1) | FITC | MIH1 | BD Biosciences |
| HLA-DR | AF700 | G46-6 | BD Biosciences |
| HLA-DR | Per-CP | L203 | R&D Systems |
| Anti-mouse $IgG_{2A}$ | Biotin | R19-15 | BD Biosciences |
| Mouse $IgG_{2A}$ Isotype | PE | Lot LHC0809031 | R&D Systems |

### *2.4. Cytotoxicity assay (activity test for TNF and TNF variants)*

Cells were seeded at 2 x $10^4$ (MF TNFR1-Fas or MF TNFR2-Fas) per well of a 96 well plate in RPMI 1640 + 10% FCS + 2mM L-Glutamine and incubated overnight at 37°C. The next day MF TNFR-Fas cells were left untreated or treated in triplicates with increasing concentrations of TNF, CysTNF or variants thereof with subsequent 3 fold dilutions (0.0153 - 100 ng/ml) for 6 hours at 37°C.

Supernatants were discarded and cells were washed with PBS. Any residual PBS was removed by tapping the plate upside down on a dry paper towel. Cells were then incubated for 15 min with 50 µl/well crystal violet solution (0.5% w/v crystal violet powder, 20% v/v methanol in 1000 ml $dH_2O$). The residual stain was washed away with running water (whilst being careful not to splash water directly onto the cells). Plates were allowed to dry overnight then 100 µl methanol was added to each well and results were read on a TECAN microplate reader at λ=550nm.

### *2.5. Stimulation of DC with TNF*

Immature moDC were harvested on ice, washed three times in HBSS (Sigma-Aldrich) containing 1% FBS then re-plated in a 24 well plate at either 2.5 x $10^5$ cells/ml (for flow cytometric analyses) or 5 x $10^5$ cells/ml (for all other assays). MoDC or myeloid blood DC were left untreated or were stimulated with recombinant sTNF or with recombinant $CysTNF_{wt}$ and mutants derived thereof (TNFR1-selective $CysTNF_{R1}$; TNFR2-selective $CysTNF_{R2}$), all at 50 ng/ml. As a positive control, DC were treated with standard-grade *E. coli* LPS (100 ng/ml; Sigma-Aldrich). For TNFR2-selective stimulation, DC were pre-treated with 80M2 MAb (2 µg/ml) for 5 min prior to the addition of $CysTNF_{R2}$.

Where indicated, DC were incubated for 30 min with or without TNFR1- and/or TNFR2-specific antagonistic antibodies H398 (Hbt Hycult) and 22221 (R&D Systems), respectively; 10 µg/ml each, prior to the addition of TNF ligands. Alternatively, DC were incubated for 30 min with or without 10 µM of signaling pathway small molecule inhibitors BAY 11-7082 (Cell Signalling), Abt-737 (Selleck Chemicals), LY294002 (Cell Signaling #9903) or U0126 (MEK1/2, Cell Signaling #9901) (all resuspended in DMSO), prior to the addition of TNF ligands.

### *2.6. Flow Cytometry*

All flow cytometry experiments were analysed using FlowJo (TreeStar) software.

#### *2.6.1. Cell surface protein expression*

DC were harvested 24 h after stimulation and re-suspended in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline (PBS) containing 3% FBS, 0.1% sodium azide and 2 mM EDTA). Cells were incubated for 30 mins on ice with human IgG (a kind gift of Sophie Hambleton, UK) and selected MAbs. For moDC the following MAbs were used as indicated; HLA-DR-PerCP, CD83-APC, CD86-FITC, ICAM-1-APC and PD-L1-FITC. For blood myeloid DC, purity was checked with CD11c-FITC and CD1c-PE-Cy7. Cells were then washed twice and resuspended in a final volume of 200 μl FACS buffer and data were acquired on a FACSCanto II flow cytometer (Becton Dickinson). Alternatively, PBMC were incubated with CD3-FITC, CD19-FITC, CD20-FITC, CD56-FITC, CD123-PerCP-Cy5.5, CD1c-PE-Cy7, CD16-APC, HLA-DR-AF700, CD14-APC-H7,CD11c-V450 and CD45-V500 and acquired on a Fortessa X20.

#### *2.6.2. TNFR expression*

For the detection of cell surface TNFR1 and TNFR2 a 3-step amplification system was used. First, DC were re-suspended in 100μl PBA (PBS containing 0.5% w/v bovine serum albumin (BSA) and 0.01% w/v sodium azide) and incubated with anti-TNFR1 MAb (H398, 100-150 μg/ml) or anti-TNFR2 MAb (MR2-1; 50 μg/ml; Hbt Hycult for moDC; clone 22221; 150 μg/ml for blood DC) in the presence of 2% human IgG (to block non-specific binding to Fc receptors) and incubated for 1 h on ice in the dark. Surface protein MAb (as described previously) may also be added at this point for multiple surface staining of PBMC. Cells were then washed twice in PBA and blocked in 2% rat serum (Sigma) in PBA for 10 min on ice prior to incubation with biotinylated rat anti-mouse $IgG_{2A}$ antibodies (eBioscience, final dilution 1/50) for 20 min. Cells were then washed twice in PBA and incubated with APC- (moDC; eBioscience) or PE- (blood DC; Beckton Dickinson) coupled streptavidin (BD Biosciences, final dilution 1/10) for another 20 min on ice. Cells were then washed twice in PBA and then resuspended in a final volume of 200μl PBA and acquired on a FACSCanto II flow cytometer.

#### *2.6.3. Annexin V/ViaProbe*

For Annexin V/ViaProbe detection DC were washed twice with cold PBS and resuspended in 100μl 1x Binding Buffer (0.01M Hepes (pH7.4), 0.14M NaCl, 2.5mM

$CaCl_2$). Then 5 μl of AnnexinV and/or 5 μl ViaProbe (BD Biosciences) was added to the cells and incubated for 15 min at room temperature in the dark before 1x binding buffer was added to a final volume of 200μl.

### *2.6.4. Intracellular active caspase-3*

For caspase-3 staining moDC or myeloid blood DC were harvested after 48 or 24 h of stimulation, respectively, fixed with 100 μl Cytofix/Cytoperm buffer for 20 min on ice then washed twice in 2x Perm/Wash buffer (both from BD Biosciences). Cells were incubated with 2% (v/v) rabbit serum (Sigma) for 15 min at 4°C prior to the addition of MAb specific for active caspase-3-PE (C92-605; BD Biosciences) for 20 min at room temperature in the dark. Cells were then washed twice in Perm/Wash buffer and resuspended in a final volume of 200μl FACS buffer. Data were acquired on a FACSCanto II flow cytometer.

### *2.6.5. PhosFlow*

Freshly isolated blood myeloid DC were resuspended at 250,000 cells/ml in 'v' bottomed eppendorf tubes and stimulated with sTNF or the TNFR-selective ligands for 5, 15 or 30 min in a pre-heated 37°C water bath. Cells were centrifuged (400 g, 5 min), the cell pellet was resuspended in 100μl PBS (containing $Ca^{2+}$ and $Mg^{2+}$) + 0.2% BSA + 0.01% azide (PBA) and 1 ml BD PhosFlow Fix Buffer I was added. Tubes were incubated in a water bath for 12 min at 37°C before centrifugation (600 g, 8 min, room temperature). Supernatants were aspirated, tubes were vortexed and cells were washed in 1 ml PBA (including $Ca^{2+}$ and $Mg^{2+}$) (600 g, 8 min, room temperature). Cells were then resuspended in 1 ml pre-chilled (-20°C) Perm Buffer III and stored overnight at -80°C. Once cells had thawed they were transferred into FACS tubes and washed twice in 3 ml PBA. To block the cells 50μl 4% mouse serum was added and tubes were incubated for 15 min at room temperature (final volume will be 100μl with 2% block). 50μl Ab solution was added (phosho-p65 PEcy7, 1:50), tubes were vortexed and incubated at room temperature for 1 hour (protected from the light). Cells were then washed (3 ml PBA, 600 g, 6 min at room temperature), resuspended in 200 μl and data were acquired on a FACSCanto II flow cytometer.

## *2.7. Cell death analysis (colorimetric assay)*

MoDC were harvested, washed once and collected by centrifugation at 250 x g for 10 minutes. The supernatant was gently removed and discarded while the cell pellet was lysed by the addition of 25 μl of cold Lysis Buffer (R&D Systems) per 1 x $10^6$ cells. The

cell lysate is incubated on ice for 10 minutes and then centrifuged at 10,000 x g for 3 minutes and the supernatant was transferred to a new tube and kept on ice. The protein content of the lysates was measured using a Bradford assay and the caspase-3 colorimetric assay was performed on 100 µg total protein from lysed moDC in a flat bottomed 96 well plate. Prior to use, 10 µl of fresh DTT stock was added per 1 ml of 2X Reaction Buffer 3, which was then added to the lysates to a final volume of 50 µl. Next, 5 µl of the *p*-nitroaniline-coupled Caspase-3-specific tetrapeptides (Asp-Val-Glu-Asp(DVED)-*p*NA) substrate (R&D Systems) was added and the plate was incubated at 37°C for 2 hours. Absorbance was measured at 405 nm on a microplate reader (Sunrise; Tecan Ltd.).

### *2.8. Bradford assay*

The Bradford assay was used to determine protein concentrations. When coomassie dye is bound to protein it results in a colour change (red to blue) and an absorbance shift from 470 to 590 nm. The change in fluorescence is proportional to the amount of protein present. A standard dilution of BSA was prepared; 2000 µg/ml, 750 µg/ml, 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml and 0µg/ml ($H_20$) in order to give a standard curve. 10 µl/well of each standard was added to a well of a flat bottomed 96 well plate in duplicate along with 10µl for the blank (e.g. of lysis buffer). Samples were diluted in $dH_2O$ as required (e.g. 5µl sample + 5µl $dH_2O$). 150 µl/well of room temperature Bradford reagent was added to each well and samples were read at $\lambda$ = 595nm on a microplate reader (Sunrise; Tecan Ltd.).

### *2.9. NFκB activation*

Reagents were prepared according to manufacturer's instructions (Active Motif) as follows (volume for 1 well, multiplied according to the number of wells required):

| | | |
|---|---|---|
| Complete Lysis buffer | DTT | 0.11 µl |
| | Protease inhibitor cocktail | 0.23 µl |
| | Lysis buffer AM2 | 22.2 µl |
| Complete Binding buffer | DTT | 0.07 µl |
| | Herring sperm DNA | 0.34 µl |
| | Binding buffer AM3 | 33.4 µl |
| 1x Wash buffer | dH2O | 2.025 µl |
| | 10x Wash buffer AM2 | 225 µl |
| 1x Ab binding buffer | dH2O | 202.5 µl |
| | 10x Ab Binding buffer AM2 | 22.5 µl |

### *2.9.1. Preparation of nuclear extracts*

Nuclear extracts were prepared from moDC which had been stimulated with TNF, variants thereof or LPS for 15 min (for p65) or 24 h (for p52) using an Active Motif Nuclear Extract Kit. DC were harvested and washed in ice-cold PBS then re-suspended in ice-cold 500 μl 1x hypotonic buffer and incubated on ice for 15 min. 25 μl detergent was added and the cells were vortexed for 10 seconds before centrifugation at 4°C, 14,000 g for 1 min. The supernatant (cytoplasmic fraction) was removed and stored at -80°C. The nuclear pellet was re-suspended in 50 μl complete lysis buffer (10 mM DTT, lysis buffer AM1, protease inhibitor cocktail), vortexed for 10 sec and incubated for 30 min on ice on a rocking platform set at 150 rpm. Extracts were vortexed for 30 sec, centrifuged at 4°C, 14,000 g for 10 min and supernatants (nuclear fraction) were stored at -80°C.

### *2.9.2. Oligonucleotide binding assay*

The oligonucleotide binding capability of p65 and p52 NFκB was assessed using TransAM transcription factor kits specific for p65 or p52 (Active Motif). 5 μg of total nuclear protein per DC sample was used (except in titration experiments), as determined by Bradford assay.

First 30 μl of complete binding buffer was added to each well, then 5 μg of each sample of nuclear extract was diluted to a volume of 20 μl and added to the wells before the plate was sealed with an adhesive cover and incubated at 100 rpm on a rocking table for 1 h at room temperature. Wells were washed 3 times with 200 μl 1x Wash Buffer, 100 μl of the NFκB antibody (p65 or p52) diluted in 1x antibody binding buffer (1;1000) was added per well and the plate was incubated for one hour at room temperature without agitation. Wells were washed 3 times as previously, 100 μl of HRP-conjugated antibody diluted in 1x antibody binding buffer (1:1000) was added per well and the plate was incubated at room temperature for 1 h. wells were then washed 4 times as previously, 100 μl room temperature developing solution was added and wells were incubated for 5 min at room temperature protected from direct light. 100 μl Stop Solution was added to each well and the NFκB-binding to the oligonucleotide-coated 96-well plates was determined at 450 nm on a microplate reader (Sunrise; Tecan Ltd.).

### *2.10. Mixed lymphocyte reaction*

The principle of an MLR is that the T cell receptor on allogeneic T cells will react to the mismatched MHC molecules present on the DC resulting in an alloresponse. MoDC were stimulated with TNF, variants thereof or LPS for 24 h, harvested and washed thoroughly in HBSS containing 1% FBS. 1 x $10^4$ DC were co-cultured in triplicate with 1 x $10^5$ allogeneic $CD4^+$ T-cells (isolated from LRS cones using a human $CD4^+$ T-cell enrichment cocktail (RosetteSep; StemCell Technologies)) in a 96-well plate (total volume 200 µl/well,). After 3 days supernatants (100 µl/well) were harvested and IL-10 and IFN-γ levels were determined using a specific sandwich ELISA (BD Biosciences). To measure T-cell proliferation 10 kBq $^3$H-thymidine (Perkin-Elmer, Shelton, CT) was added to each well for 8 h and radioactivity was quantified using a beta-scintillation counter (Perkin-Elmer Microbeta Trilux).

### *2.11. ELISA*

| ELISA | Capture Antibody | Top Standard | Detection Antibody | Develop | Read |
|---|---|---|---|---|---|
| IL-6 | 1 µg/ml | 2000 pg/ml | 1 µg/ml | 30 min | 490 nm |
| IL-10 | 2 µg/ml | 2000 pg/ml | 1 µg/ml | 40 min | 490 nm |
| IL-12 | 4 µg/ml | 4000 pg/ml | 1 µg/ml | 30 min | 490 nm |
| IFNγ | 1 µg/ml | 2000 pg/ml | 1 µg/ml | 30 min | 490 nm |
| TNFα | 2 µg/ml | 2000 pg/ml | 0.5 µg/ml | 30 min | 490 nm |
| IL-23 | 1/250 | 2000 pg/ml | 1/250 | 15 min | 450 nm |

The capture antibody was diluted to the required concentration (see table above) in coating buffer (0.06 M $Na_2HPO_4$ (4.35g), 0.08 M $NaH_2PO_4.H_2O$ (5.37g)) and 50 µl was added to each well, the plate was covered with adhesive plate sealer and incubated in a moist box at 4°C overnight. The next day the capture antibody was discarded, the plate was washed once with wash buffer (PBS, 0.1% Tween-20), and 100 µl block (PBS, 1% BSA) was added to each well. The plate was sealed and incubated at room temperature for 1-2 hours. The plate was washed 3 times as previously and 50 µl standards or samples diluted in diluent (PBS, 1% BSA, 0.1% Tween-20) was added to each well. The plate was sealed and incubated overnight at 4°C. The plate was washed 4 times as previously, 50 µl biotinylated detection antibody (diluted in diluent) was added per well, the plate was sealed and incubated at room temperature for 1 h. Next the plate was washed 4 times as previously, the streptavidin-horseradish peroxidase conjugate was diluted 1/1000 in diluent, 50 µl was added per well, the plate was sealed and incubated at room temperature for 30 min. The plate was washed 5 times as previously and the substrate; one OPD tablet, 13ml citrate phosphate buffer

(0.03 M citric acid (2.55g), 0.05 M $Na_2HPO_4$ (3.66g), 0.03 M $Na_2HPO_4.2H_2O$ (4.58g)) and $H_2O_2$ (6 μl 30% stock, added immediately prior to use) for the appropriate development time. The reaction was stopped by the addition of 50 μl 3M $H_2SO_4$ and the absorbance was read at the appropriate wavelength on a microplate reader (Sunrise; Tecan Ltd.).

### *2.12. Stimulation of DC and preparation of cell lysates*

DC were harvested, washed in HBBS 1 % FCS and 2 x $10^6$ cells per condition were added to 250 μl RF10. For MAb 80M2 treatment cells were pre-incubated with MAb 80M2 (2 μg/ml) for 30 minutes, then stimulated with the appropriate ligand (sTNF, $CysTNF_{wt}$, $CysTNF_{R1}$, $CysTNF_{R2}$ 100ng/ml) and incubated at 37ºC for specific time points. Stimulation of DC was stopped by the addition of ice-cold DPBS and centrifuging for 30 s at 400 g, 4˚C then DPBS was aspirated leaving the cell pellet. MoDC lysed on ice in high salt buffer (250 mM Tris HCl, pH 7.5, 375 mM NaCl, 2.5% (w/v) Deoxycholate (SOC), 1% (v/v) Triton X-100) supplemented with protease inhibitors (Roche Applied Science) and 0.5 mM PMSF. Cell lysates were sonicated and centrifuged at 14,500g for 10 min. Protein concentrations of supernatants were determined by Bradford assay.

### *2.13. Western blotting*

Prior to use, 80 µg of total protein was denatured at 96°C for 3 min in Laemmli buffer (62.5 mM Tris HCl pH 6.8, 10 % (v/v) glycerol, 4% (w/v) sodiumdodecylsufate (SDS), 0.1% w/v bromophenol blue, 5% (v/v) β-mercaptoethanol). Proteins were separated along with a pre-stained protein standard (NEB broad range 15-175 kDa) by SDS-PAGE (12% Acrylamide). Proteins were then transferred onto hydrophobic polyvinylidene fluoride (PVDF) membrane (pre-soaked in methanol prior to use) in transfer buffer (0.025 M Tris base, 0.2 M glycine diluted with 20% v/v methanol and $H_2O$) at 100 V for 80 min at 1.4 mA/$cm^2$. After the transfer, the membrane was blocked with 5% non fat milk powder in PBS-T (PBS plus 0.05% Tween-20) for 1 hour. The membrane was washed 4 times for 7 min in PBS-T before adding the primary antibodies; with Bcl-xl (#2762, Cell Signaling) and GAPDH (6C5, abcam) MAbs overnight at 4ºC. The membrane was then washed again in PBS-T and incubated at room temperature in the appropriate HRP-coupled secondary antibody; either goat anti-rabbit IgG-HRP (Santa Cruz) or anti-mouse IgG-HRP (Dianova) antibodies for 2 hours. Chemiluminescence substrate SuperSignal West (Thermo Scientific/Pierce) was used, and chemiluminescence was captured on Kodak autoradiography film.

### *2.14. siRNA*

DC were harvested, washed and re-plated at 0.5 x $10^6$ in a 24 well plate at a final volume of 1 ml antibiotic-free media plus IL-4 and GM-CSF (50 ng/ml each, Immunotools, Friesoythe, Germany). The transfection reagent DharmaFECT 4 (0.5 - 5 μl; Thermo Scientific) was added to TNFR1- or TNFR2-specific siRNA (ON-TARGETplus SMARTpool siRNA, Dharmacon) or non-sense siRNA (all 5 - 50 nM) and incubated for 20 min (room temperature) while keeping light exposure to a minimum, then the solution was added to the moDC and incubated at 37°C, 5% $CO_2$ for 24 h.

### *2.15. Statistics*

Statistics were performed using Prism 5.0 (GraphPad Software) and a general linear model was performed using SPSS (Version 19) software. Differences between the treatment groups were tested after adjusting for subject-to-subject variability and the assumptions underlying the model confirmed by residual analysis. An F-test was performed to test for differences between the treatment groups and contrasts formed to compare the groups stimulated with TNFR-selective ligands with and without inhibitors.

# 3. TNF-induced maturation of monocyte-derived DC

## *3.1. Introduction*

Dendritic cells (DC) are professional antigen presenting cells (APC) which play a key role in initiating and regulating immune responses and can be divided into several subsets, the main two in humans being conventional/myeloid DC ($CD1c^+$ or $CD141^+$) and plasmacytoid (p)DC ($CD123^+$). Under steady state conditions DC typically reside in an immature state where they have little ability to activate an effector T cell response and may instead induce T cell tolerance (Banchereau & Steinman 1998). Upon activation by PAMP or inflammatory cytokines DC mature and express high levels of MHC II-peptide complexes, co-stimulatory molecules and cytokines (Matzinger 2002; Breloer et al. 2001). This drives the expansion of antigen specific T cells and can control T cell polarisation depending on the nature of the maturation stimulus (Diebold 2008; Kaliński et al. 1999). The maturation state of DC is critical in determining their immunomodulatory role and in maintaining the balance between immunity and tolerance, whereas dysregulation of DC function can lead to autoimmunity.

TNF is a cytokine predominantly known for its pro-inflammatory actions and can promote DC maturation (Sallusto & Lanzavecchia 1994). Previous studies have shown that mice deficient in TNF fail to induce full DC maturation in response to viral challenge *in vivo* (Trevejo et al. 2001) and that maturation of BM-derived DC from these mice is impaired (Ritter et al. 2003). More specifically, murine knock-out models have shown that without TNFR1, DC maturation is impaired in response to pathogens (Ding et al. 2011; Sundquist & Wick 2005). In human cells the importance of TNF in DC activity can be demonstrated by the effect of TNF neutralisation which has been shown to reduce the T cell stimulatory capacity of DC (van Lieshout et al. 2005; Baldwin et al. 2010). Furthermore patients on anti-TNF therapy display impaired co-stimulatory molecule upregulation after LPS stimulation (Baldwin et al. 2010).

As TNF plays such an important role in DC, dissecting the role of its two receptors (TNFR1 and TNFR2) is vital in the development of new DC modulating drugs. For example, TNFR2 polymorphisms or upregulation of TNFR2 have been associated with several autoimmune diseases (Barton et al. 2001; Holtmann et al. 2002; Komata et al. 1999) and in a transgenic mouse model, high levels of either TNFR resulted in severe inflammatory diseases regardless of the level of TNF (Douni & Kollias 1998). However, it has been shown that in type I diabetes a TNFR2 agonist selectively killed autoreactive T cells in isolated human blood (Ban et al. 2008). Furthermore, TNFR-deficient mouse models have shown that TNFR2 plays a protective role and TNFR1 plays a destructive role in EAE (Suvannavejh et al. 2000), neurodegeneration

(Fontaine et al. 2002) and cardiac dysfunction (Higuchi et al. 2004; Monden et al. 2007), thus illustrating the need for the development of specific therapies which could selectively target individual TNFR.

To date the contributions of the two TNF receptors to human DC survival and immunostimulatory function are not fully understood. The majority of studies investigating the individual roles of TNFR1 and TNFR2 have been conducted in mouse models whereas the human TNFR system is relatively understudied. There is no known natural TNFR2 ligand which does not bind to TNFR1 making human cell studies difficult as any effects of TNFR2 may be masked or counteracted by TNFR1. For this reason, much of the previous work has made use of mouse models where either TNFR1 and/or TNFR2 has been knocked out thus allowing the effects mediated by the two TNFR to be studied individually. To counteract this problem, I have made use of previously described TNFR-selective ligands and a TNF mutein mimicking mTNF bioactivity (Krippner-Heidenreich et al. 2002; Bryde et al. 2005) in order to dissect the roles of TNFR1 and TNFR2.

### *3.2. Specific aims*

1) Determine the expression of TNFR1 and TNFR2 on human moDC.
2) Determine the competency of each TNFR for the activation of downstream NFκB signalling in moDC.
3) Determine the involvement of TNFR1 and TNFR2 in the maturation and subsequent T cell stimulatory capacity of moDC.

### *3.3. Experimental Approach*

To address these aims I used monocyte-derived (mo)DC which are differentiated *in vitro* and can be used as a model for inflammatory DC. MoDC are not normally found in healthy tissue but are thought to differentiate from monocytes during cases of chronic inflammation such as RA (as reviewed in (Shortman & Naik 2007; Segura, Durand, et al. 2013)). MoDC were treated with sTNF, $CysTNF_{wt}$ and TNFR-selective variants $CysTNF_{R1}$ and $CysTNF_{R2}$. As $CysTNF_{R2}$ was used in combination with the TNFR2-specific MAb 80M2, which facilitates efficient stimulation through pre-clustering of TNFR2, 80M2 alone was used as a control in all experiments. MoDC were washed extensively after harvesting prior to stimulation with TNF in order to remove any cytokines (i.e. IL-4 and GM-CSF) from the culture medium following their differentiation from monocytes. This is necessary because GM-CSF may enhance DC survival (Wan et al. 2013) and IL-4 can reduce the expression of TNFR2 in DC (Lutz et al. 2002)

which could affect my studies. An oligonucleotide binding assay was used to determine NFκB activation as this method detects phosphorylation at multiple sites compared to antibodies used in Western blotting or flow cytometry. A small number of experiments were only conducted once in order to optimise the experimental system and maximise the amount of TNFR-specific data, for example, the time course and amount of nuclear extract used in the NFκB activation assays. All key experiments were repeated three to six times.

## *3.4. Results*

### *3.4.1. Characterisation of moDC*

The characterisation of moDC is outlined in Figure 7. CD14 is a myeloid cell-specific glycoprotein expressed highly on the surface of monocytes and can be used as a control for the positive selection of monocytes from peripheral whole blood. Results show that human PBMC contain a small population of these $CD14^+$ cells (10-20%) whereas cells separated by positive selection are 99.9% $CD14^+$ and no $CD14^+$ cells can be found in the flow through (which includes all cells that were not bound to the anti-CD14 magnetic microbeads (Miltenyi Biotec)). Once the monocytes differentiate into moDC (via the addition of IL-4 and GM-CSF) the expression of CD14 is abolished. Resulting immature moDC expressed the appropriate surface markers; high CD1a (a transmembrane glycoprotein which can present lipid/glycolipid antigens to T cells and is commonly used as a marker for moDC cultures), low CD83 (involved in regulating antigen presentation and T cell co-stimulation), low CD86 (a T cell co-stimulatory molecule) and high HLA-DR (an MHC class II molecule involved in antigen presentation). Upon stimulation with LPS (100 ng/ml) for 24 h, the expression of CD83, CD86 and HLA-DR increase indicating phenotypical moDC maturation. This confirms that the standard method of *in vitro* moDC generation used previously by our group (Anderson et al. 2009; Baldwin et al. 2010) and by others (Delirezh & Shojaeefar 2012) is appropriate for use in further experiments.

### *3.4.2. The bioactivity of TNF and TNFR receptor-selective ligands*

TNF ligands had been generated prior to this project with specific mutations providing specificity for one receptor or the other ($CysTNF_{R1}$ or $CysTNF_{R2}$) (Krippner-Heidenreich et al. 2002). $CysTNF_{wt}$ has membrane-bound TNF-like activity due to its oligomerised form generated by a free Cys residue at the N-terminus which leads to oligomerisation, primarily to hexamers. The monoclonal antibody (MAb) 80M2 facilitates the clustering of TNFR2 without causing its activation (Grell et al. 1995). To determine the bioactivity of the various ligands (sTNF, $CysTNF_{wt}$, $CysTNF_{R1}$, $CysTNF_{R2}$) a cytotoxicity assay was performed using murine fibroblast (MF) TNFR1-Fas and MF TNFR2-Fas cell lines as generated previously (Krippner-Heidenreich et al. 2002). Murine fibroblasts were originally derived from TNFR1/TNFR2 double knock-out mice and were stably transfected with hybrid constructs consisting of the N-terminal, extracellular and transmembrane part of TNFR1 or TNFR2 fused to the cytoplasmic death domain containing region of Fas, the prototype of apoptosis inducing receptors of the TNFR

superfamily. This allowed me to determine the ED50 (effective dose which produces a response in 50% of the cells) of each ligand.

Figure 8 shows that the various TNF ligands are all highly bioactive. The cytotoxic effects of both $CysTNF_{wt}$ and $CysTNF_{R1}$ are comparable to that of sTNF in MF TNFR1-Fas cells (Figure 8 (A) and Figure 8 (B), respectively). The ED50 of these ligands is around 1 ng/ml. As expected due to the established inability of sTNF to activate TNFR2, Figure 8 (C) shows no cytotoxic response of MF TNFR2-Fas to sTNF, whereas the ED50 after treatment with $CysTNF_{wt}$ is 1 ng/ml (similar to that seen in MF-TNFR1-Fas cells). Figure 8 (D and F) shows that MF TNFR2-Fas cells do not respond to $CysTNF_{R2}$ alone apart from a minimal effect at very high concentrations of 100 ng/ml. However, in combination with MAb 80M2 $CysTNF_{R2}$ gains full bioactivity with an ED50 = 1ng/ml which is comparable to that of $CysTNF_{wt}$ (Figure 8 (D)). Importantly, 80M2 alone does not have any effect on cell viability (purple unfilled diamond, one point). This shows that the $CysTNF_{R2}$ ligand is potent to initiate signalling, however, it is only efficient when used in combination with 80M2. Notably, the activity of $CysTNF_{wt}$ cannot be increased by co-treatment with 80M2 (Figure 8 (E)) suggesting that the low bioactivity of $CysTNF_{R2}$ is due to the two point mutations introduced to give its receptor selectivity. In addition, $CysTNF_{R1}$ has no effect on MF TNFR2-Fas cells (Figure 8 (F)) confirming that this ligand is not cross-reactive with TNFR2. Alternatively, recent data from another member of the lab confirms these results and also shows that 100ng/ml $CysTNF_{R2}$ plus 80M2 has no effect on MF TNFR1-Fas cells (Etherington 2014). Results confirm that the TNF ligands have comparable bioactivities and can therefore be used in further experiments.

### *3.4.3. TNFR1 and TNFR2 are expressed on immature moDC*

Prior to performing experiments examining the role of individual TNF receptor stimulation in moDC it was necessary to confirm the expression of TNFR1 and TNFR2 on moDC. Flow cytometry was used to detect TNFR expression on the surface of moDC (Figure 9). Results show that immature moDC do express both TNFR1 and TNFR2 and that expression is reduced when cells are matured with LPS. As DC are important in the induction of immune tolerance I also analysed the expression of each TNFR on tolerogenic moDC (differentiated using Vitamin D3 and Dexamethasone) (Anderson et al. 2008; Anderson et al. 2009). Results showed that both TNFR1 and TNFR2 are expressed at higher levels on tolerogenic moDC than on immature DC although donor to donor variability was also greater. TNFR1 and TNFR2 were detected using H398 (an $IgG_{2A}$ antibody) and MR2-1 (an $IgG_1$ antibody) respectively, and the same biotin and streptavidin conjugated antibodies. As different subtypes of primary

antibodies are used, it is difficult to accurately compare the relative amounts of each TNFR. However, as saturating concentrations of the antibodies are used results generally suggest that moDC express higher levels of TNFR2 as compared to TNFR1. My data confirm that both TNFR1 and TNFR2 are present on the surface of moDC and leads on to the question, 'are the two TNFR signalling competent?' which will be addressed in the following section.

### *3.4.4. TNFR1- but not TNFR2-mediated signalling activates the classical p65 NFκB pathway in moDC*

As described previously both TNFR1 and TNFR2 are capable of activating the classical NFκB pathway (Wajant & Scheurich 2011; Lawrence 2009). It has been shown that TNFR1 can activate the classical p65 NFκB pathway in DC (Peng et al. 2012) but to my knowledge there are no studies published demonstrating TNFR2-mediated p65 activation in DC. In order to determine if the two TNFR expressed on the surface of moDC were capable of initiating downstream signalling pathways I investigated the potential activation of classical and alternative NFκB pathways. To investigate the capability of TNFR1 and TNFR2 to induce classical NFκB (p65) signalling a preliminary Western blot was conducted using moDC lysates and a phospho-specific p65 antibody. Figure 10 shows that p65 phosphorylation is induced by stimulation with sTNF and $CysTNF_{R1}$ after 30 min when compared to unstimulated cells. However it was difficult to determine if any signalling was induced by TNFR2-stimulation.

The phospho-p65 antibody used in the Western blot only recognises p65 when it is phosphorylated at Ser536, whereas it may alternatively be phosphorylated at other sites including Ser276 and Ser468. The population of modified subunits is not uniform and different stimuli may activate different pools therefore in order to allow for the different phosphorylation sites and to increase accuracy an oligonucleotide binding assay was used. The TransAM NFκB assay kit recommends using between 2 and 20 μg nuclear extract per well. Due to the limited amount of reagents and the use of primary cells it was not possible to attain 20 μg of nuclear extract per condition therefore the assay was optimised by using varying amounts of nuclear extract (Figure 11 (A)). As the difference in p65 activation between immature moDC and TNFR1-stimulated moDC was greater when using 5 μg nuclear extract per well, this amount was chosen and used for all future experiments. Furthermore, in all p65 assays moDC were stimulated with TNF and its variants for 30 min as according to the Western blot (Figure 10) 15 min was not enough time to allow phosphorylation and nuclear translocation but after 60 min the signal was slightly decreased (Figure 10 and confirmed in Figure 11 (B)).

Results in Figure 12 (A and B) show that all of the TNFR1-stimulating ligands (sTNF, $CysTNF_{wt}$ and $CysTNF_{R1}$) activate the classical p65-mediated NFκB pathway whereas no activation was detected via TNFR2. Figure 12 (A) includes the controls $CysTNF_{R2}$ and 80M2 alone which have no effect on p65 activation. Figure 12 (B) includes an increased number of donors and shows that $CysTNF_{R1}$ but not $CysTNF_{R2}$ plus 80M2 significantly activates the p65 NFκB pathway. The activation of p65 by TNFR1 and not TNFR2 was also confirmed using flow cytometry (Figure 13). This technique only requires p65 phosphorylation at Ser529 and not nuclear translocation therefore I performed a time course of moDC stimulation between 5 and 30 min (Figure 13 (A)). This experiment showed that the optimum time point for p65 phosphorylation was 5 min not 30 min as in the previous assays (Figure 10 and Figure 11). My results confirm that TNFR1 on moDC is signalling-competent, however no TNFR2-mediated p65 activation was detected. The potential activation of other pathways is therefore addressed in the following section.

### *3.4.5. TNFR1- and TNFR2-mediated signalling activates the alternative p52 NFκB signalling pathway*

In contrast to TNFR1, TNFR2 was described to activate the alternative NFκB signalling pathway in primary T cells (Rauert et al. 2010). To assess whether this is also the case in moDC I used a TransAM p52 oligonucleotide binding assay as the processing of p100 to p52 is characteristic of the alternative NFκB pathway activation. Longer time points were predicted as cleavage of p100 is a slower process than that of p65-phosphorylation (Naude et al. 2011), therefore, I stimulated moDC for 12, 24 and 48 hours (Figure 14). Results show that after 12 hours there is low activation of p52 by $CysTNF_{R1}$ but no activation by $CysTNF_{R2}$ plus MAb 80M2, after 24 hours there is activation through both receptors, however, activation via TNFR2 was diminished by 48 hours suggesting that signalling through TNFR2 may be more transient than signalling via TNFR1. For all further experiments 24 hours was chosen as the optimum time point.

Figure 15 (A) includes the controls $CysTNF_{R2}$ and 80M2 alone which have no effect on p52 activation. Figure 15 (B) shows that activation of the alternative NFκB signalling pathway is significantly induced by both TNFR1 and TNFR2 stimulation. To confirm that the effect of TNFR1-stimulation is in fact occurring via TNFR1 I used the TNFR1-specific antagonistic antibody H398 (Figure 16). Results show that blocking TNFR1 partially decreased the activation of p52 when used in combination with all of the TNFR1-stimulating ligands. In order to account for a possible increase in cell death in unstimulated moDC at the 24 hour time point GM-CSF was added to enhance cell

survival (Wan et al. 2013). Figure 17 shows that the addition of GM-CSF did not alter the activation of p52 compared to unstimulated cells and activation was still increased by TNFR-selective stimulation. Also, as TNF is not necessarily a full maturation factor for DC, I stimulated moDC with CD40L expressing J558L cells (mouse B cell myeloma cells transfected to express CD40L), which resulted in a 2-fold increase in p52 activation compared to moDC stimulated with TNF (Figure 17).

Previous data suggest that high levels of the adapter kinase RIP1 may mediate the activity of the classical NFκB pathway and that RIP1 can suppress TNFR1-mediated activation of the alternative NFκB pathway (Kim et al. 2011; Gentle et al. 2011). As TNFR1-selective stimulation activates both the classical and alternative NFκB pathways it is possible that RIP is not essential for activation of the classical pathway in my system and that it may be absent, therefore allowing activation of the alternative pathway. However, preliminary results suggest that this is not the case as RIP is expressed in moDC and its expression is not reduced or absent during stimulation with sTNF (Figure 18) therefore it remains unclear under which conditions TNFR1 induces alternative NFκB signalling (this will be discussed further in chapter 3.5.2). In summary, my data show that both TNFR1 and TNFR2 are signalling competent in moDC and that they differentially activate the classical and alternative NFκB pathways.

### *3.4.6. TNFR1- but not TNFR2-mediated signalling enhances the expression of surface molecules associated with moDC maturation*

Maturation of DC can be characterised by an increase in the expression of surface molecules such as CD83 (a regulator of antigen presentation), the co-stimulatory molecule CD86 and the MHC class II molecule HLA-DR. MoDC were stimulated with increasing concentrations of sTNF from 0.1 to 100 ng/ml and the expression of these maturation-associated markers was assessed by flow cytometry. Results (Figure 19) confirm that sTNF is capable of inducing phenotypical maturation of moDC and that 50 ng/ml is a suitable concentration for use in future experiments as this maximally induced maturation marker expression. However, as the increase in marker expression induced by sTNF is much lower than that of LPS I also investigated the addition of cytokines which may potentially amplify the sTNF-mediated signal and allow a more clear dissection of the roles of each TNFR.

Figure 20 shows the expression of maturation-associated markers following a titration of both IL-1β and sTNF (0.4 to 50 ng/ml of each). Without the addition of IL-1β, there is an increase in expression of CD83, CD86 and HLA-DR after treating the cells with 10 or 50 ng/ml of sTNF (as shown previously in Figure 19). However, there is also an increase in the expression of these molecules when IL-1β alone is added even at low

concentrations of 0.4 ng/ml. Also, IL-1 concentrations higher than 2 ng/ml may mask the effect of sTNF. Although 0.4 ng/ml of IL-1β alone resulted in increased marker expression this expression was further increased in combination with 10 or 50 ng/ml of sTNF when compared to sTNF alone. I also investigated the addition of TNFR1- and TNFR2-selective ligands (Figure 21). However, the effect of TNFR1- or TNFR2-selective stimulation was not affected by the addition of IL-1β therefore there was no advantage to the addition of IL-1β in any future experiments.

Alternatively, another group has described synergy between IL-4 and TNF in murine bone marrow derived DC (Lutz et al. 2002). Figure 22 shows that in moDC IL-4 alone has no effect but can enhance the effect of sTNF by 38% (CD83), 30% (CD86) and 16% (HLA-DR). In order to dissect the roles of each individual TNFR, I then repeated this experiment with the TNFR-selective ligands both in the absence (Figure 23 (A)) or presence (Figure 23 (B)) of IL-4. Results show that all TNFR1-stimulating ligands (sTNF, $CysTNF_{wt}$ and $CysTNF_{R1}$) but not the TNFR2-selective ligands ($CysTNF_{R2}$ plus MAb 80M2) increased the surface expression of CD83, CD86 and HLA-DR. The increase in CD83 and CD86 expression levels could be further enhanced by co-treatment of sTNF, $CysTNF_{wt}$ or $CysTNF_{R1}$ with the cytokine IL-4, which by itself did not affect DC maturation. However, even in presence of IL-4, TNFR2-selective stimulation did not significantly enhance expression of CD83, CD86 or HLA-DR. Similar results were also seen with the expression of the surface markers PD-L1 and ICAM-1 where expression was increased by LPS and TNFR1- but not TNFR2-selective stimulation (Figure 24). PD-L1 plays a role in suppressing the immune system and is essential for peripheral T cell tolerance (Keir et al. 2006; Latchman et al. 2001), whereas ICAM-1 is an adhesion molecule which facilitates leukocyte endothelial transmigration and pro-inflammatory effects (Yang et al. 2005). Results therefore indicate that TNFR1 may affect both suppression and enhancement of immune responses thereby highlighting the importance of TNFR-mediated responses.

Another indicator of DC maturation is immune cell-modulatory cytokine production. In a preliminary experiment I looked at TNF production by moDC in response to LPS. Figure 25 (A) shows that immature (unstimulated) moDC cultured for 24 h produce very low levels of TNF which is comparable to that of moDC cultured with MAb 80M2 alone indicating that the MAb 80M2 alone does not induce endogenous TNF production. As no effect on maturation was seen upon TNFR2-selective stimulation, levels of TNF in supernatants from $CysTNF_{R2}$ (50 ng/ml) plus MAb 80M2 cultures was also measured. The high amounts of TNF in these supernatants confirmed that the TNFR2-selective ligand was indeed added to each of the cultures. Supernatants from the same cell cultures were also analysed for the production of IL-6, IL-10, IL-12 and IL-23 (Figure 25

(B)) and results show that neither TNFR1- or TNFR2-mediated signalling induces detectable levels of these cytokines within 24 h of TNF treatment.

Data therefore show that TNFR1 but not TNFR2 induces moDC maturation in terms of enhanced surface marker expression and leads to the question, 'does an increase in surface marker expression also translate to a functional effect?' which will be addressed in the following section.

### *3.4.7. MoDC matured in the presence of TNFR1-stimulating TNF ligands have enhanced T cell stimulatory capacity*

In order to assess whether the enhancement of maturation-associated surface marker expression correlated with a functional effect, it is important to determine if moDC can activate a T cell response. To investigate this, a mixed lymphocyte reaction was performed in which moDC were stimulated with LPS, sTNF, $CysTNF_{wt}$, $CysTNF_{R1}$ or $CysTNF_{R2}$ + 80M2 for 24 h. MoDC were then washed thoroughly to remove any remaining stimulus (e.g. TNF) before they were cultured with allogeneic T cells and the incorporation of $^{3}$H-thymidine was detected. Immature moDC cultured with sTNF or $CysTNF_{wt}$ (both of which can act via TNFR1) displayed a significantly increased ability to induce T cell proliferation (Figure 26 (A-D)) but not IFN-$\gamma$ production at either 3 or 6 days of co-culture (Figure 26 (E-F)). However, the TNFR1-selective ligand $CysTNF_{R1}$ did not induce a significant T cell response (Figure 26 (A-F)). In addition, TNFR2-stimulated DC did not enhance T cell stimulatory capacity as there was no significant increase in T cell proliferation (Figure 26 (A-D)) and no IFN-$\gamma$ cytokine production (Figure 26 (E-F)) at either day 3 or day 6. This is in keeping with the lack of a significant increase in maturation marker expression by $CysTNF_{R2}$ + 80M2 stimulated moDC. Figure 26 (A and B) also shows that T cells cultured alone (without moDC) have very low levels of proliferation. As there was not a significant increase in T cell proliferation at day 6 following LPS stimulation of the moDC (Figure 26 (B)), a time course was performed showing that T cell proliferation peaked at 5 day after which proliferation declined (Figure 26). Results suggest that TNFR1 but not TNFR2 induce moDC maturation in terms of phenotype (enhanced maturation marker expression) and function (enhanced T cell stimulatory capacity).

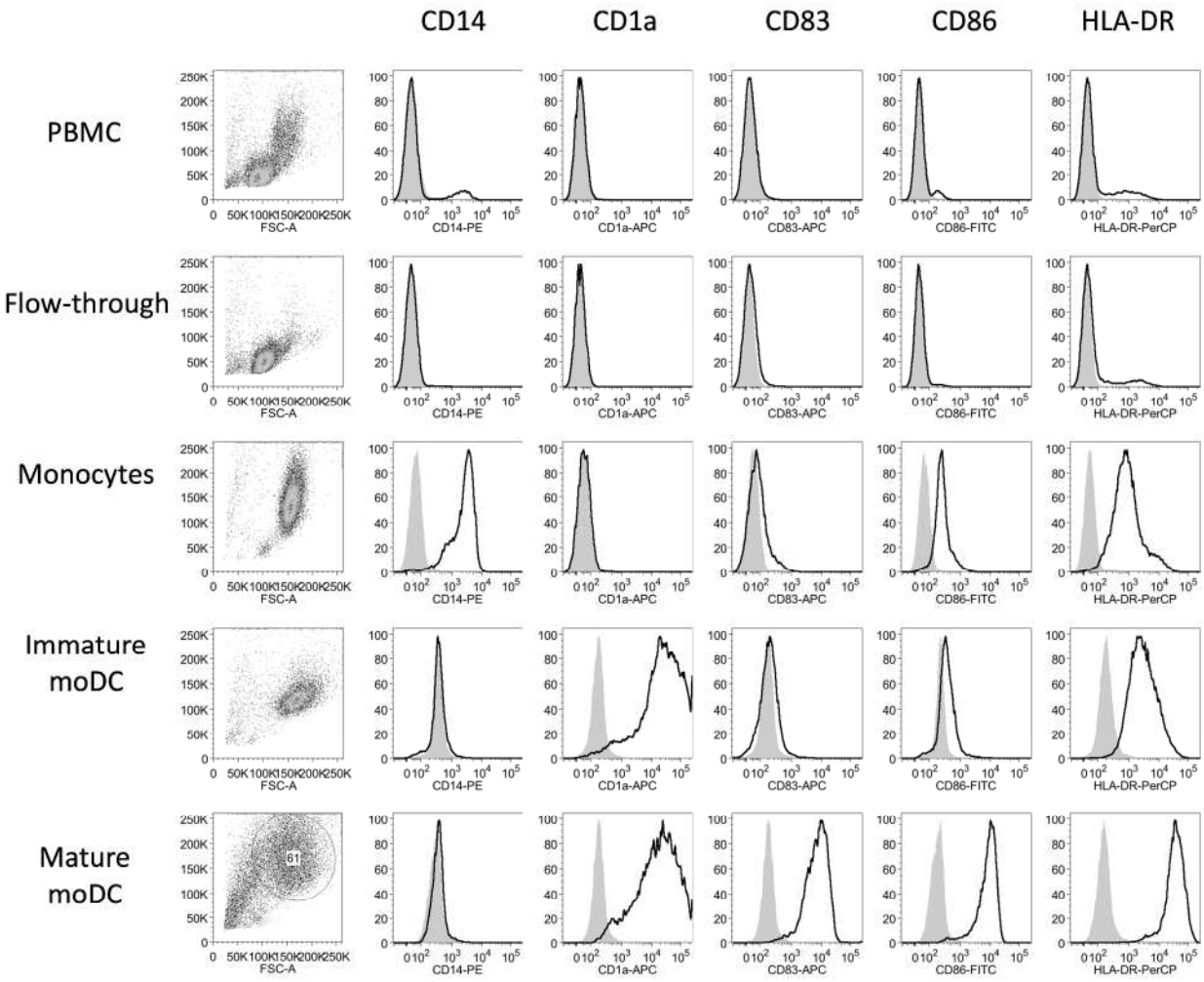


**Figure 7: Characterisation of moDC surface marker expression.** *PBMC were isolated from peripheral whole blood and CD14$^+$ monocytes were separated by positive selection. Immature moDC differentiated using IL-4/GM-CSF were harvested at day 6 or matured by the addition of LPS for 24 h. Cells at each stage of isolation and differentiation were analysed by flow cytometry. The first column shows side scatter (y axis representing granularity) versus forward scatter (x axis indicating size). The other columns show analysis of monocyte specific CD14, DC specific glycoprotein CD1a, regulatory molecule CD83, co-stimulatory molecule CD86 and APC marker HLA-DR (black lines) and are shown in relation to unstained cells (grey shaded). These data are representative of at least three independent experiments.*

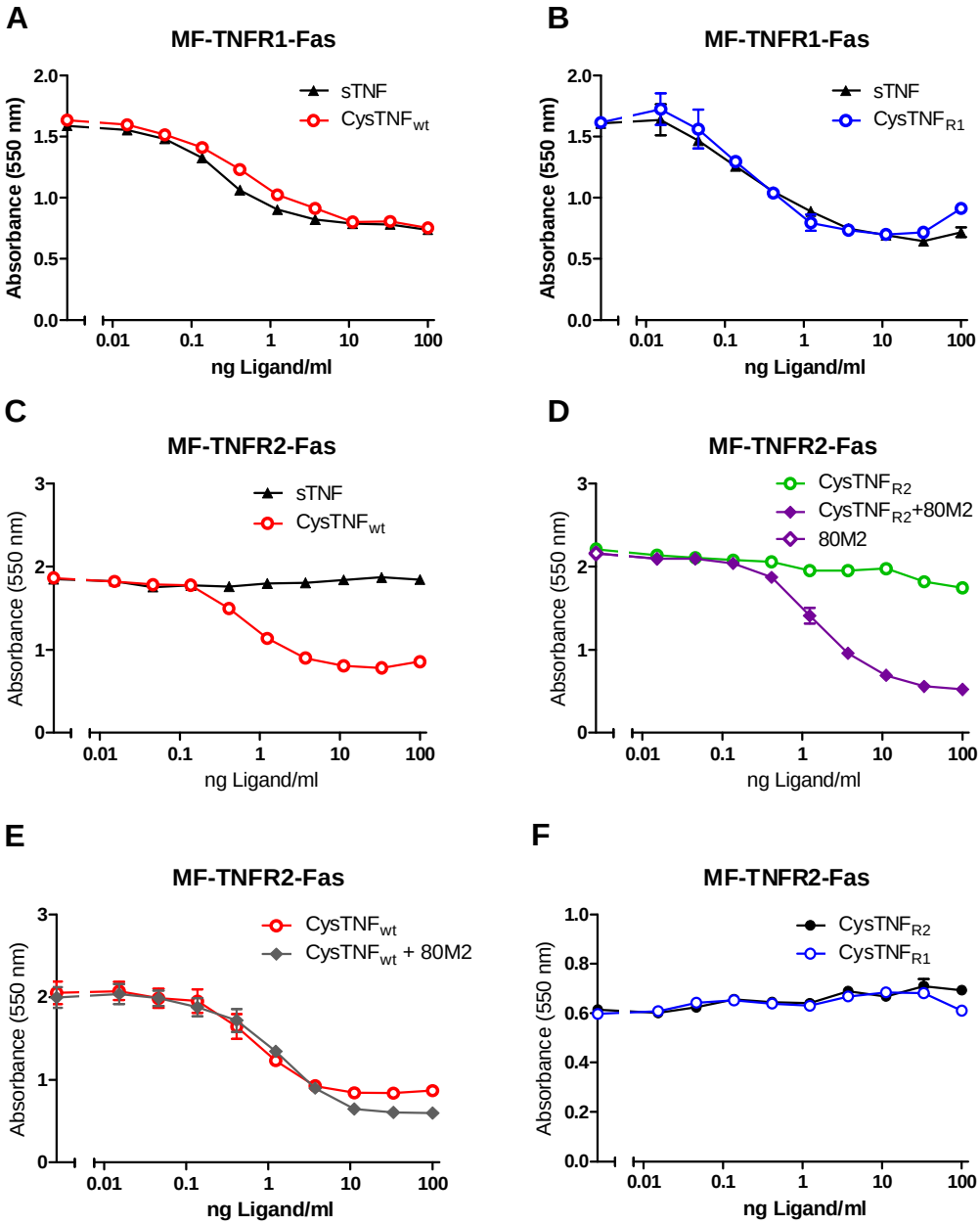


**Figure 8: Determination of the bioactivity of TNF variants using MF-TNFR1-Fas and MF-TNFR2-Fas cell lines.** *MF stably transfected with expression constructs encoding TNFR1-Fas (A and B) or TNFR2-Fas (C-F) chimeras remained untreated or were stimulated with increasing concentrations (0.015-100 ng/ml) of sTNF, CysTNF$_{wt}$, CysTNF$_{R1}$ or CysTNF$_{R2}$ +/- 80M2 (2 μg/ml) as indicated, in triplicate for 6 hours. Adherent cells were stained using crystal violet. Corresponding absorbances at 550 nm were determined for quantitative analysis of cell viability. (A and B) represent four independent experiments (C-E) represent two independent experiments (F) is reproduced with permission from* (Maney 2010) *and represents one experiment.*

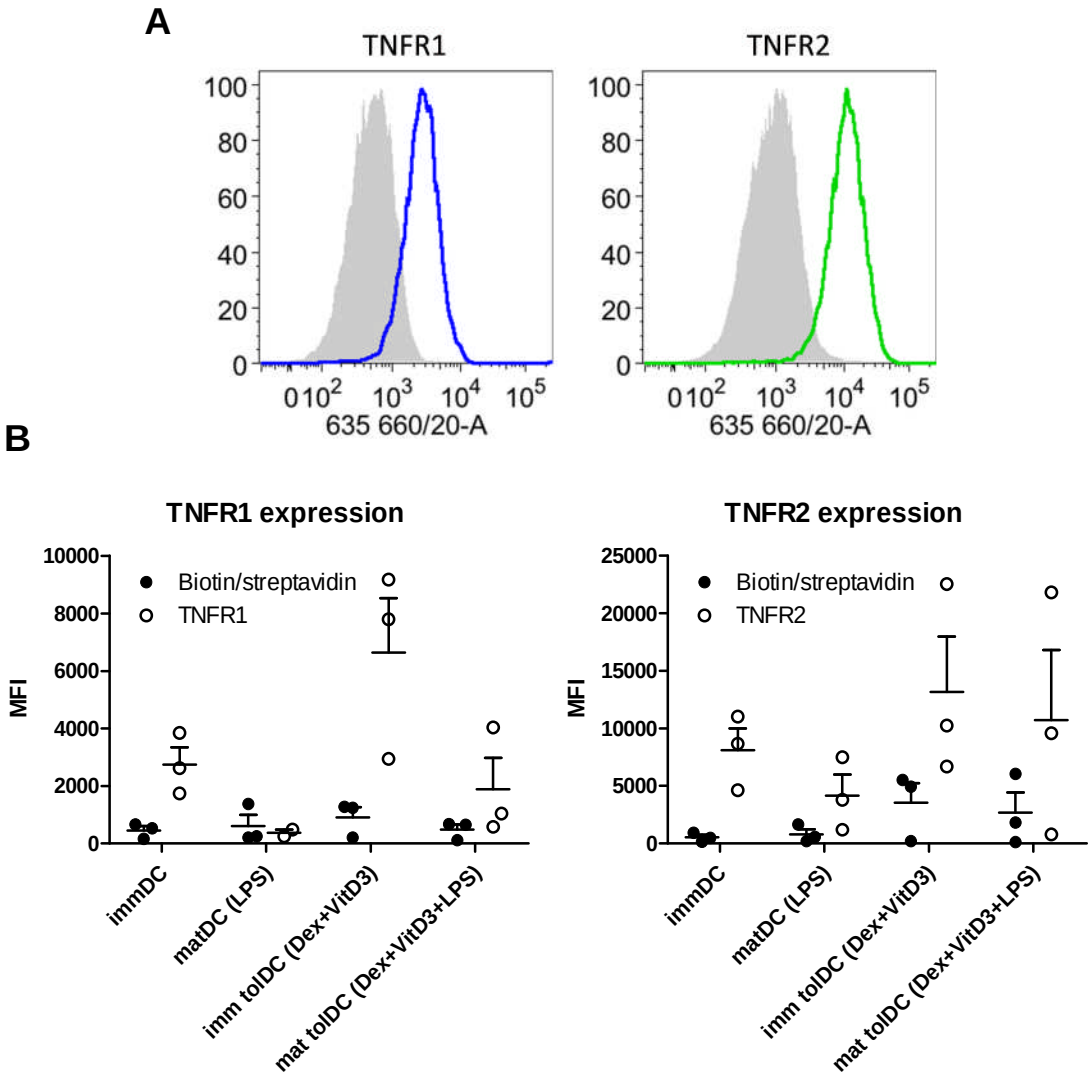


**Figure 9: Expression of TNFR1 and TNFR2 by moDC.** *(A and B) Surface expression of TNFR1 and TNFR2 on (A and B) immature (unstimulated) and (B) mature (LPS treated, 100 ng/ml for 24 h) moDC was analysed by flow cytometry. TNFR were detected using a biotin/streptavidin-based amplification protocol; i.e. using a secondary anti-mouse antibody labelled with biotin and HRP-conjugated streptavidin. (A) TNFR1-specific antibody H398 (blue line, left) or TNFR2-specific antibody MR2-1 (green line, right) are compared with biotin/streptavidin alone (grey shaded). Data are representative of three independent donors. (B) Mean of the median fluorescence intensity and the SEM of three independent donors including that shown in (A).*

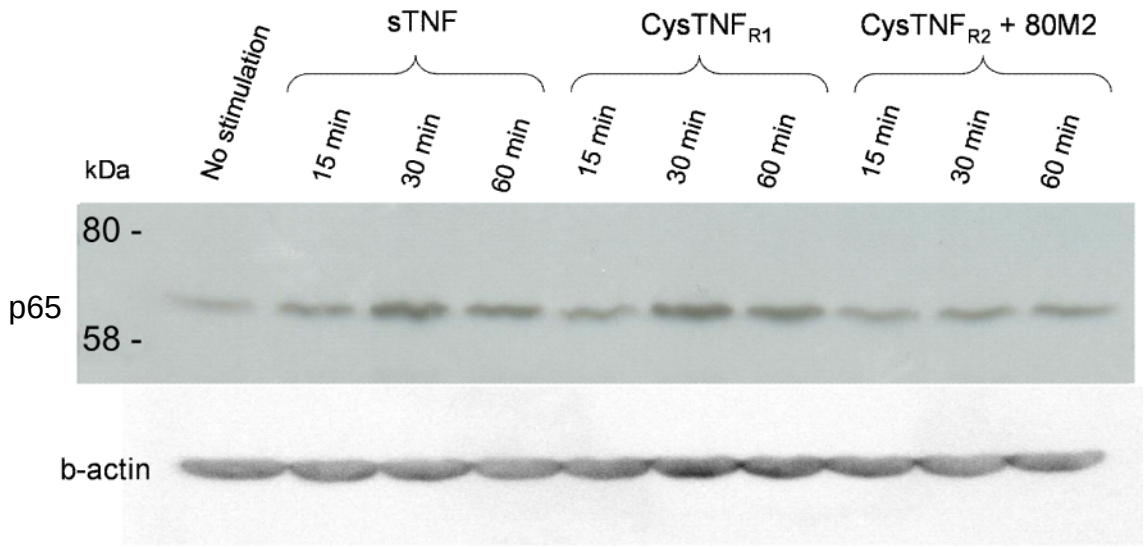


**Figure 10: Time course analysis of TNF-mediated p65 NFκB activation in moDC.** *Immature moDC were stimulated as indicated with 50 ng/ml sTNF, CysTNF$_{R1}$ or CysTNF$_{R2}$ plus MAb 80M2 for 15, 30 or 60 min. Cell extracts were first analysed for the expression of phospho-p65 (Ser536) (top) by Western blot analysis and the membrane was then re-probed for beta-actin (bottom). Results are representative of three individual experiments.*

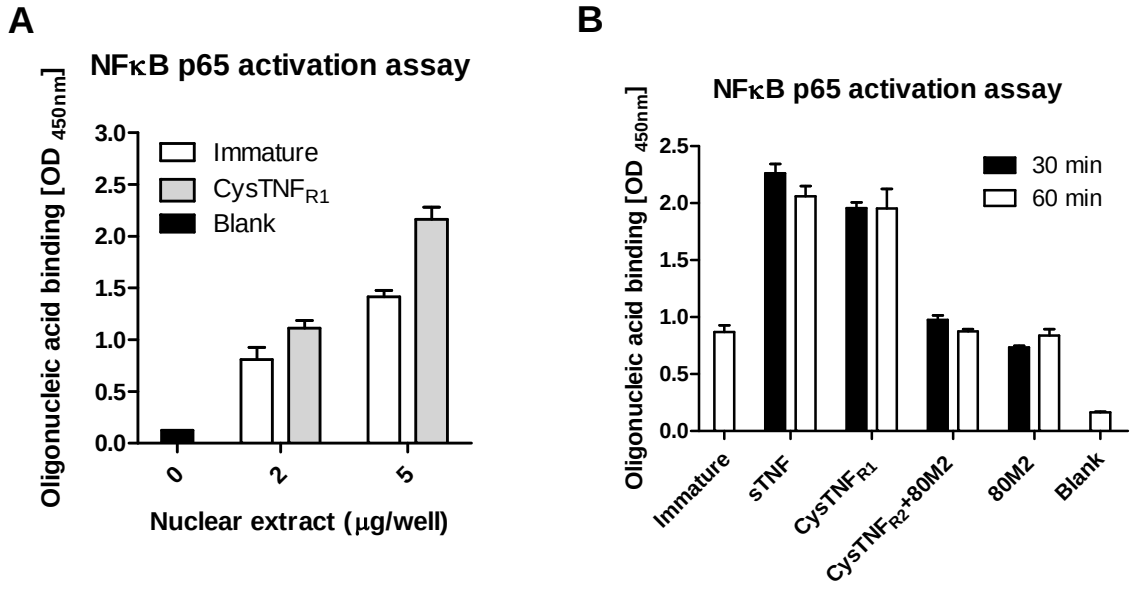


**Figure 11: Optimisation of the oligonucleotide binding assay to assess TNFR-mediated p65 NFκB activation in moDC.** *Immature moDC were left untreated or stimulated with 50 ng/ml sTNF, CysTNF$_{R1}$ or CysTNF$_{R2}$ +/- MAb 80M2 for 30 min (A, B) or 60 min (B) as indicated. Nuclear proteins were extracted and either 2 μg (A) or 5 μg (A, B) was used to determine the translocation and DNA-binding capability of p65 using a colorimetric oligonucleotide binding assay. Results represent one individual experiment and the mean +/- SEM of duplicate wells are shown.*

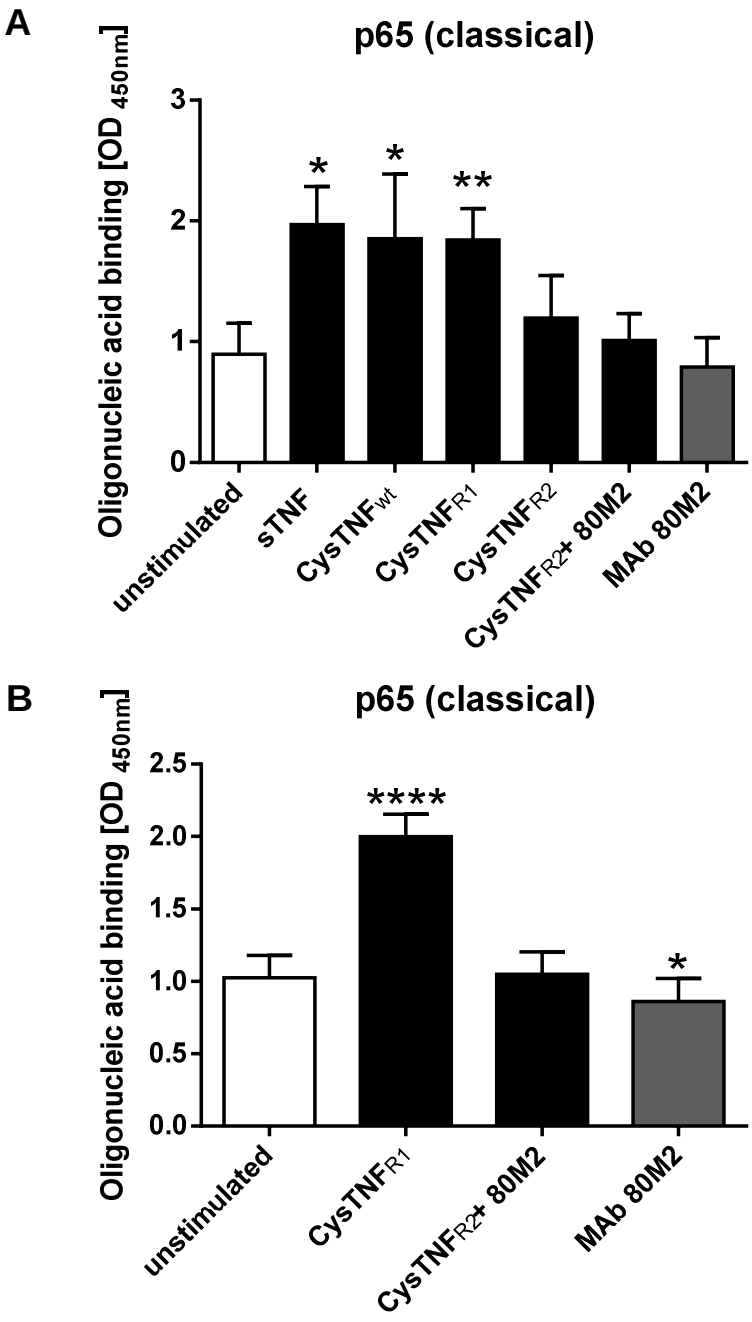


**Figure 12: TNFR1- but not TNFR2-mediated signalling activates the p65 classical NFκB pathway in moDC.** *Immature moDC were stimulated as indicated with 50 ng/ml sTNF, CysTNF$_{R1}$ or CysTNF$_{R2}$ +/- MAb 80M2 for 30 min. Nuclear proteins were extracted and the translocation and DNA-binding capability of p65 was determined using a colorimetric oligonucleotide binding assay. The mean and SEM from 3 (A) and 6 (B) independent donors are shown. Significance is shown in relation to immature moDC and is determined using a student's t test; *P≤0.05 **P≤0.01 ***P≤0.001 ****P≤0.0001.*

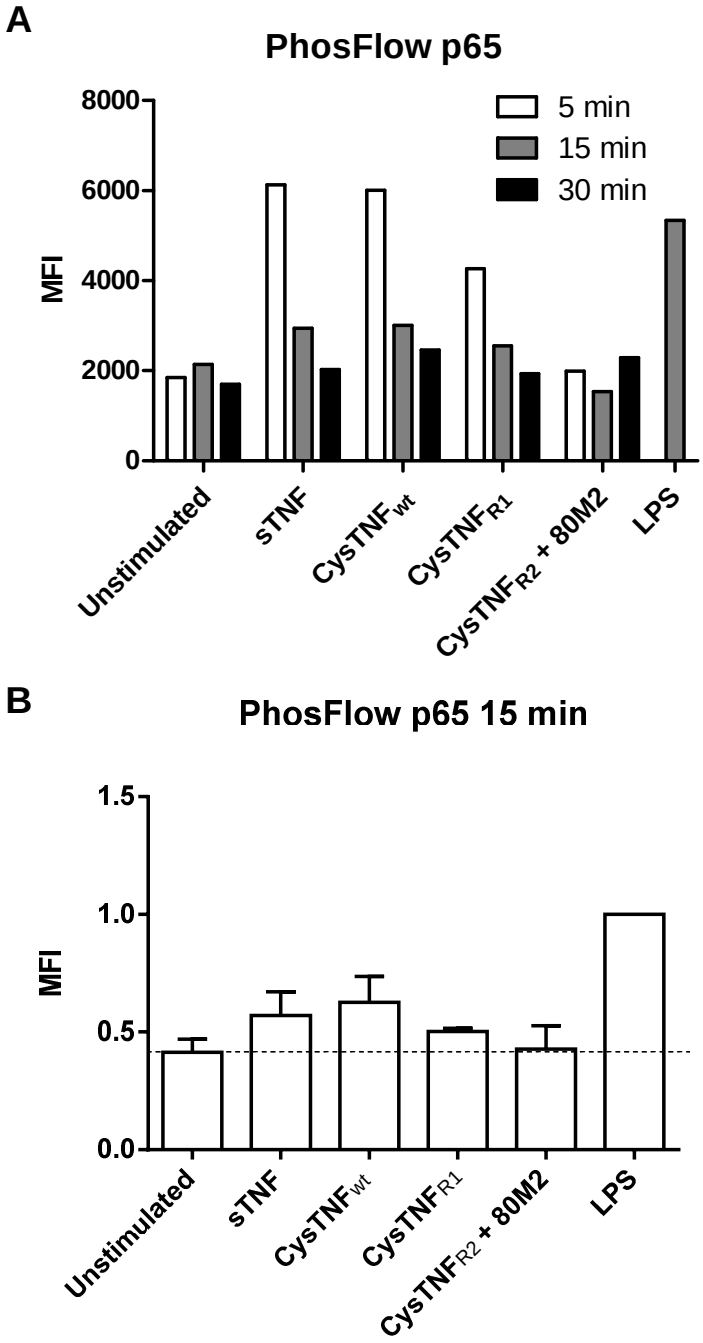


**Figure 13: Stimulation of TNFR1 but not TNFR2 induces the phosphorylation of p65 (Ser529).** *Immature moDC were left untreated or stimulated with 50 ng/ml TNF-selective ligands +/- MAb 80M2 for 5, 15 or 30 min (A) or for 15 min (B) as indicated. Phosphorylation of p65 was detected using phospho-specific antibodies which recognise phosphorylation at Ser529 conjugated to PEcy7 and detected by flow cytometry. (A) Results represent one donor. (B) Data are normalised to LPS and represents the median +/- SEM of 3 individual experiments with different donors (none of which are significant).*

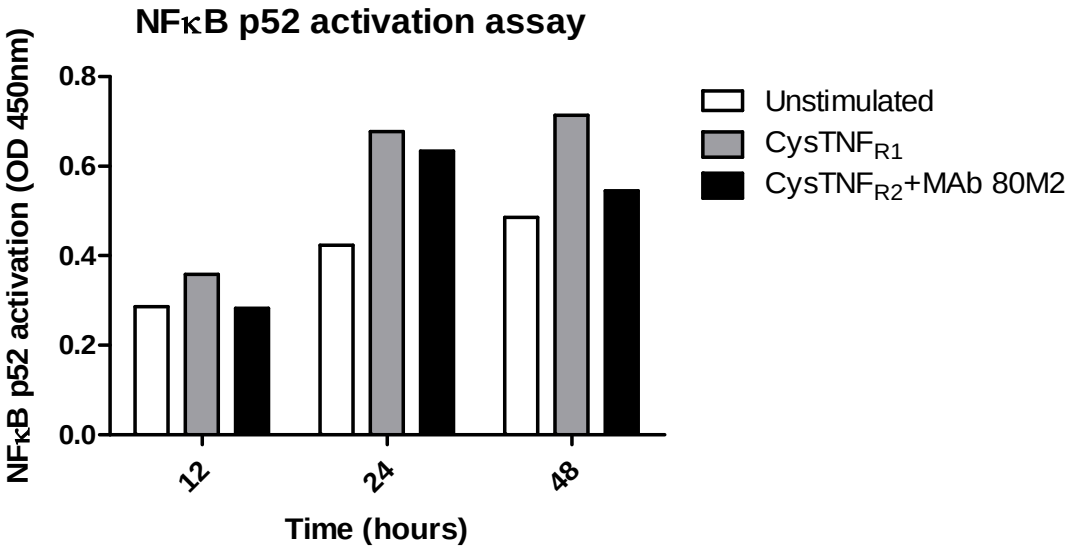


**Figure 14: Time course of TNFR-mediated p52 NFκB activation in moDC.** *Immature moDC were left untreated or stimulated with 50 ng/ml TNF-selective ligands +/- MAb 80M2 for 12, 24 or 48 h as indicated. Nuclear proteins were extracted and 5 μg was used to determine the translocation and DNA-binding capability of p52 using a colorimetric oligonucleotide binding assay. Results represent one individual experiment.*

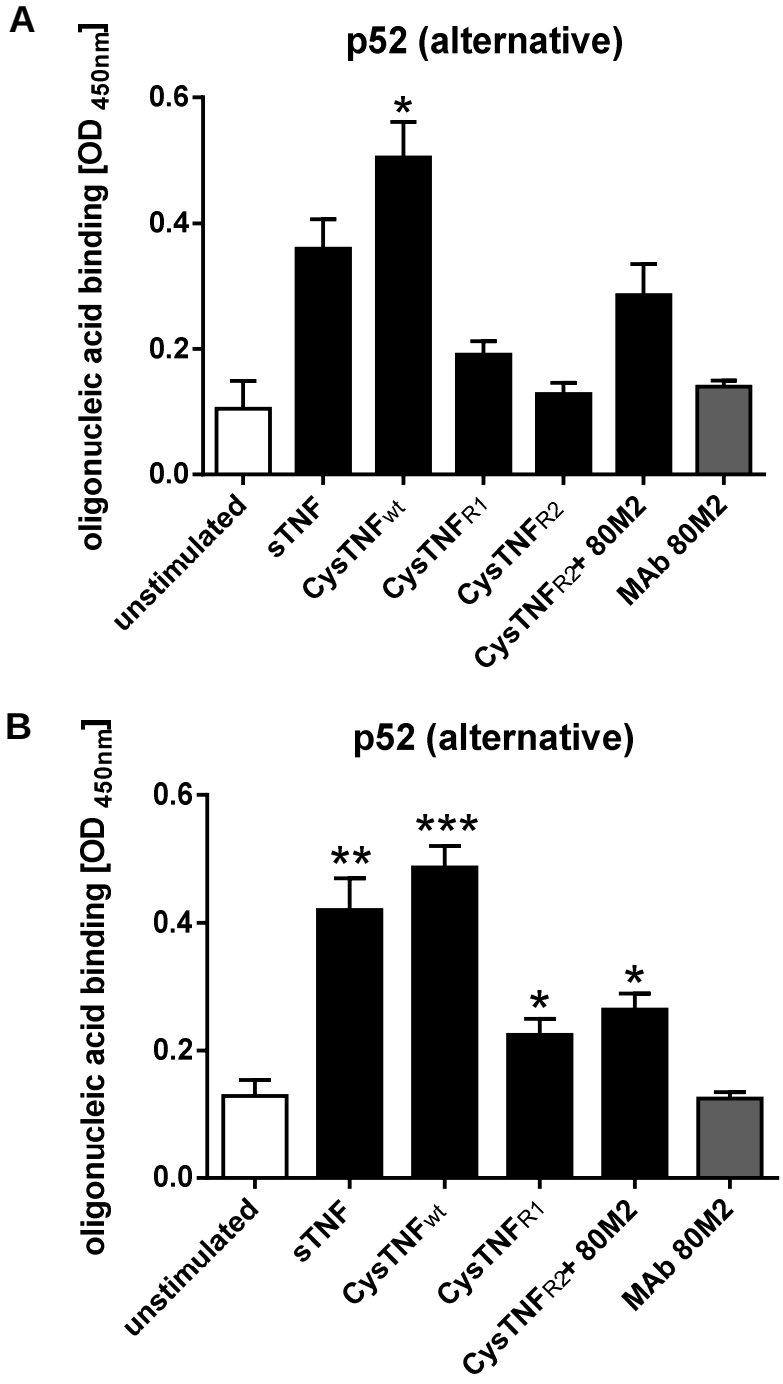


**Figure 15: TNFR1 and TNFR2 are signalling competent in moDC and both activate the alternative p52 NFκB pathway.** *Immature moDC were stimulated as indicated with 50 ng/ml of the TNF ligands +/- MAb 80M2 for 24 h. Nuclear proteins were extracted and the translocation and DNA-binding capability of p52 was determined using a colorimetric oligonucleotide binding assay. The mean and SEM from 3 (A) or 6 (B) independent donors are shown. Significance is shown in relation to immature (unstimulated) moDC and is determined using a student's t test; *P≤0.05 **P≤0.01 ***P≤0.001.*

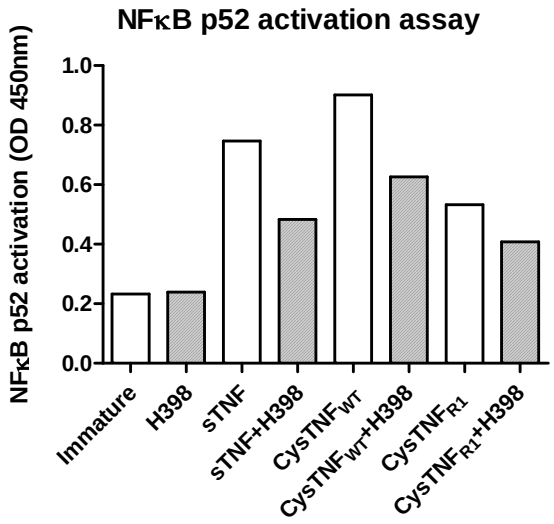


**Figure 16: The activation of p52 by TNFR1 can be prevented by the addition of a TNFR1-specific antagonistic antibody.** *Immature moDC were left untreated or treated with the TNFR1-specific antagonistic antibody H398 (shaded bars) for 30 min prior to stimulation with 50 ng/ml TNF-selective ligands +/- MAb 80M2 for 24 hours. Nuclear proteins were extracted and 5 μg was used to determine the translocation and DNA-binding capability of p52 using a colorimetric oligonucleotide binding assay. Results represent one individual experiment.*

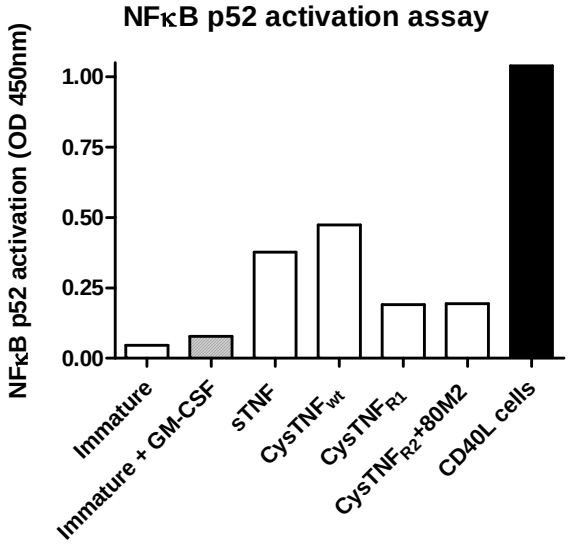


**Figure 17: GM-CSF does not increase the basal level of p52 activation in moDC and TNF is less efficient at activating p52 in moDC than CD40L cells.** *Immature moDC (white) were left untreated, treated with GM-CSF (50 ng/ml, shaded bar) or stimulated with 50 ng/ml TNF-selective ligands +/- MAb 80M2 or CD40L cells (grey) for 24 h. Nuclear proteins were extracted and 5 μg was used to determine the translocation and DNA-binding capability of p52 using a colorimetric oligonucleotide binding assay. Results represent one individual experiment.*

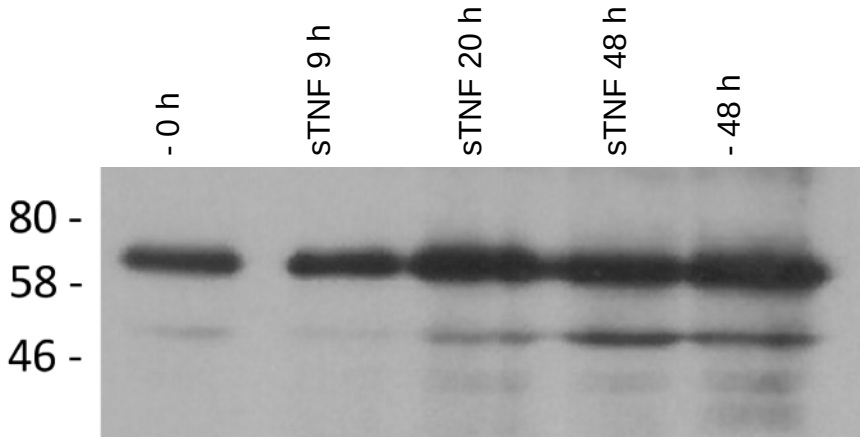


**Figure 18: MoDC express RIP both with and without stimulation with sTNF.** *Immature moDC were left untreated or were stimulated as indicated with 50 ng/ml sTNF for 9, 20 or 48 h. Cell extracts were analysed for the expression of RIP (60 kDa) by Western blot analysis. Results represent one individual experiment.*

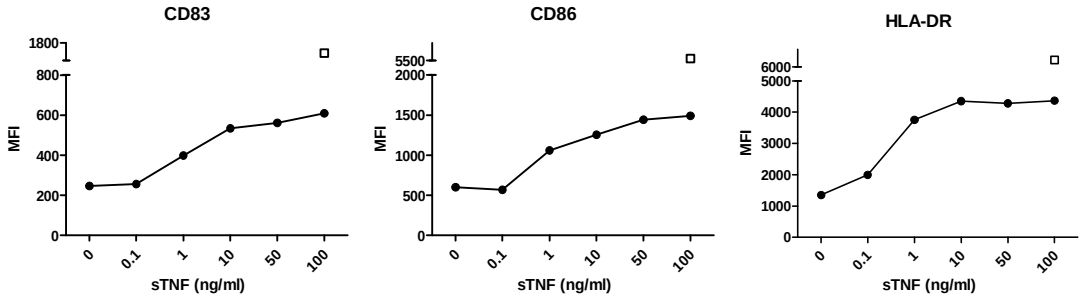


**Figure 19: Stimulation of moDC with sTNF enhances the expression of surface proteins associated with moDC maturation in a dose dependent manner.** *Immature moDC were treated as indicated with increasing concentrations of sTNF (0.1 - 100 ng/ml) or with 100 ng/ml LPS (open square) for 24 h. The expression of CD83, CD86 and HLA-DR was determined by flow cytometry. Data represent one independent experiment.*

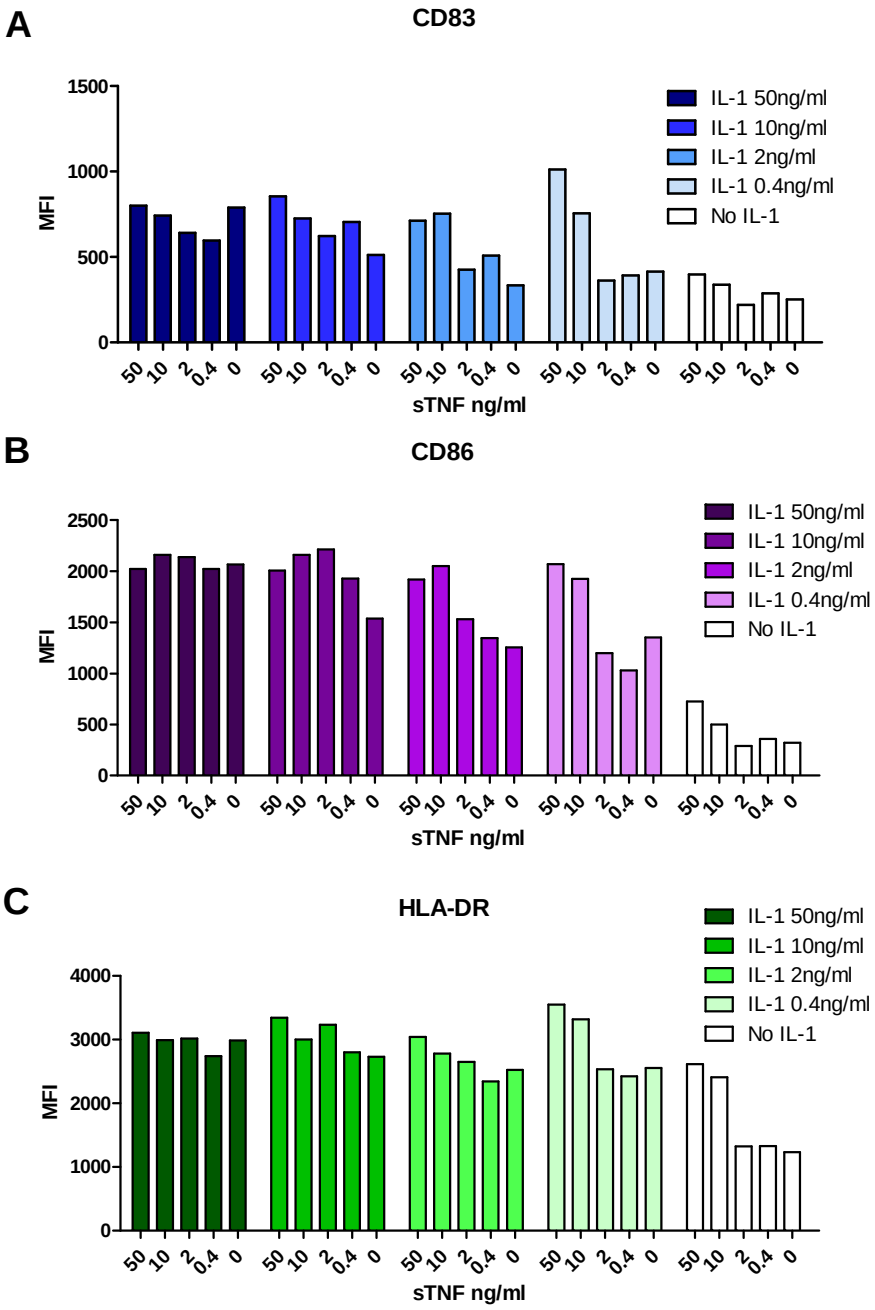


**Figure 20: IL-1 induces an increase in moDC maturation marker expression which is independent to the addition of sTNF.** *Immature moDC were treated as indicated with increasing concentrations of sTNF (0.1 - 100 ng/ml) and also increasing concentrations of IL-1 (0.1 - 100 ng/ml) for 24 h. The expression of CD83 (A), CD86 (B) and HLA-DR (C) was determined by flow cytometry and the median fluorescence intensity (MFI) is shown. Data represent one independent experiment.*

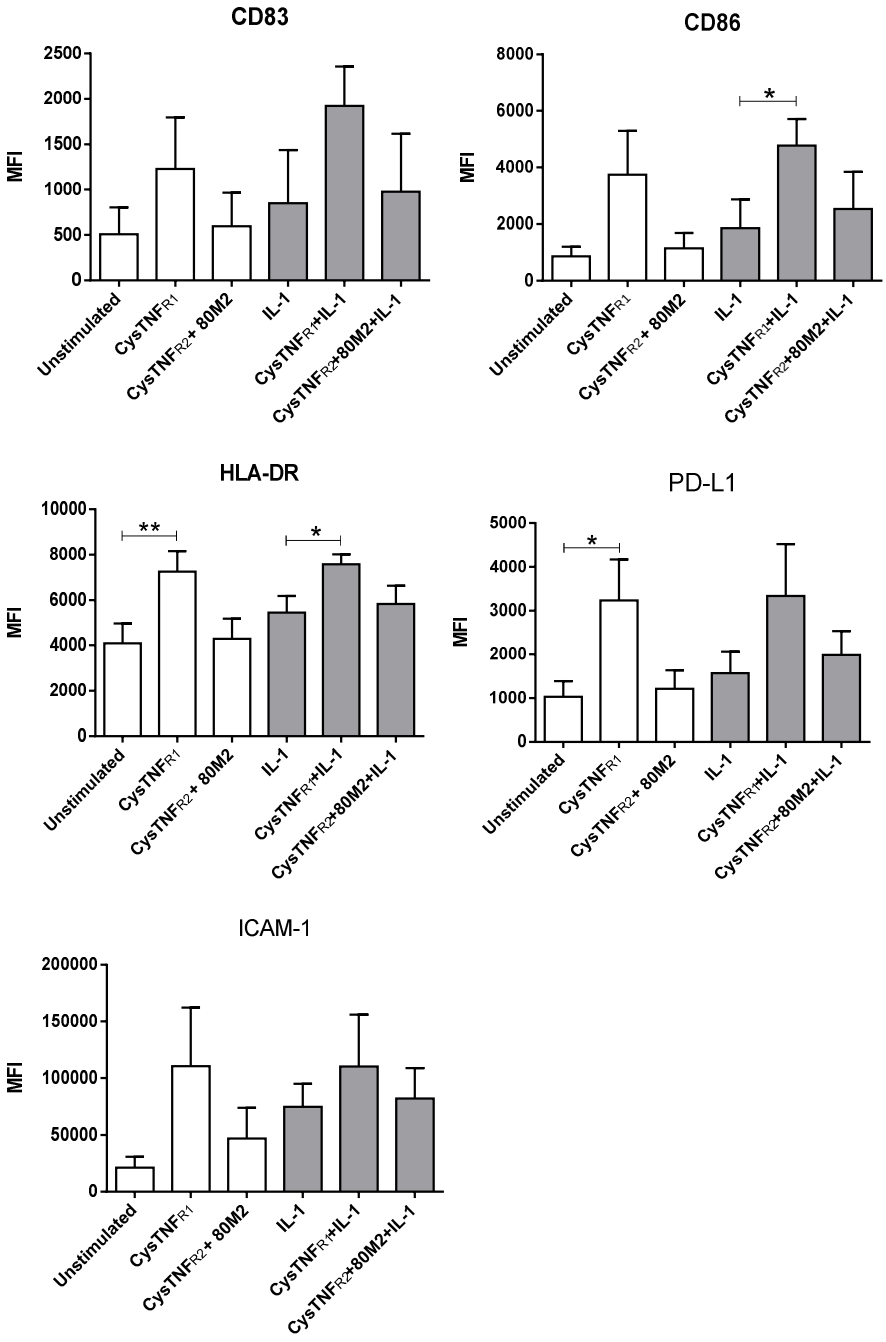


**Figure 21: IL-1 does not enhance moDC maturation marker expression mediated by TNFR2.** *Immature moDC were treated as indicated with 50 ng/ml of $CysTNF_{R1}$ or $CysTNF_{R2}$ plus MAb 80M2, with (grey) or without (white) 0.4 ng/ml IL-1 for 24 h. The expression of CD83, CD86, HLA-DR, PD-L1 and ICAM-1 was determined by flow cytometry. MFI indicates the median fluorescence intensity, error bars show the mean of the MFI +/- SEM. Data represent 4 independent experiments. Statistical differences were determined in relation to un-stimulated (immature) moDC using a student's t test; *P≤0.05 **P≤0.01 ***P≤0.001.*

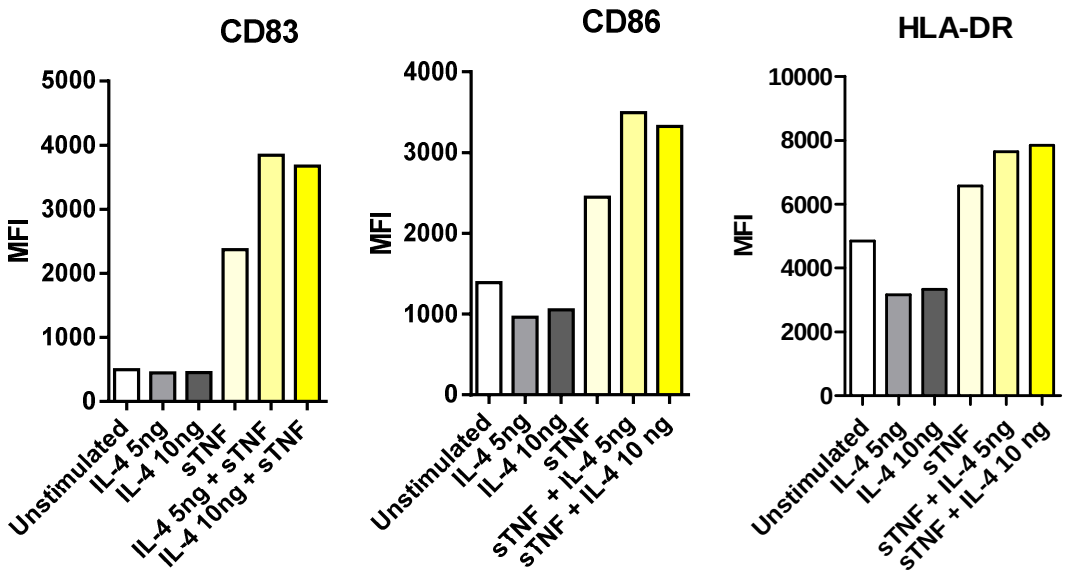


**Figure 22: IL-4 enhances sTNF-induced expression of moDC maturation markers.** *Immature moDC were treated as indicated with sTNF (50 ng/ml) in the presence or absence of IL-4 (5 or 10 ng/ml) for 24 h. The expression of CD83, CD86 and HLA-DR was determined by flow cytometry. Data represent one independent experiment.*

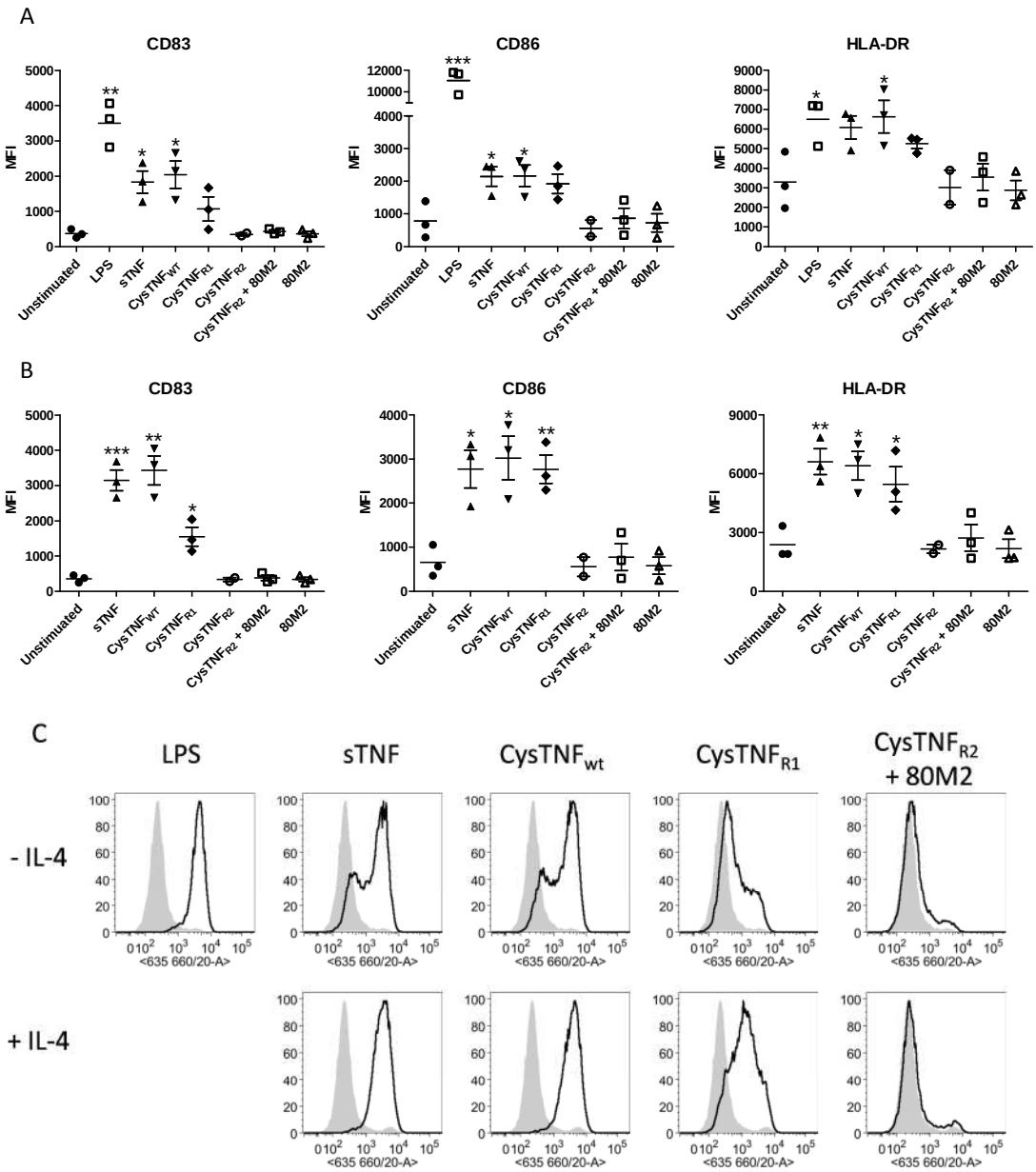


**Figure 23: TNFR1- but not TNFR2-mediated signalling results in enhanced expression of moDC surface proteins associated with moDC maturation.** *Immature moDC were treated as indicated with LPS (100 ng/ml), TNF ligands (50 ng/ml) +/- MAb 80M2 (2 μg/ml) for 24 h in the absence (A) or presence (B) of IL-4 (10 ng/ml). The expression of CD83, CD86 and HLA-DR was determined by flow cytometry. (A and B) MFI indicates the median fluorescence intensity, error bars show the mean of the MFI +/- SEM. Statistical differences were determined in relation to un-stimulated (immature) moDC using a student's t test; *P≤0.05 **P≤0.01 ***P≤0.001. (C) CD83 expression by ligand-treated moDC (black line) is shown in relation to expression by immature moDC (grey shaded). Data are representative of 3 independent experiments with moDC from different donors.*

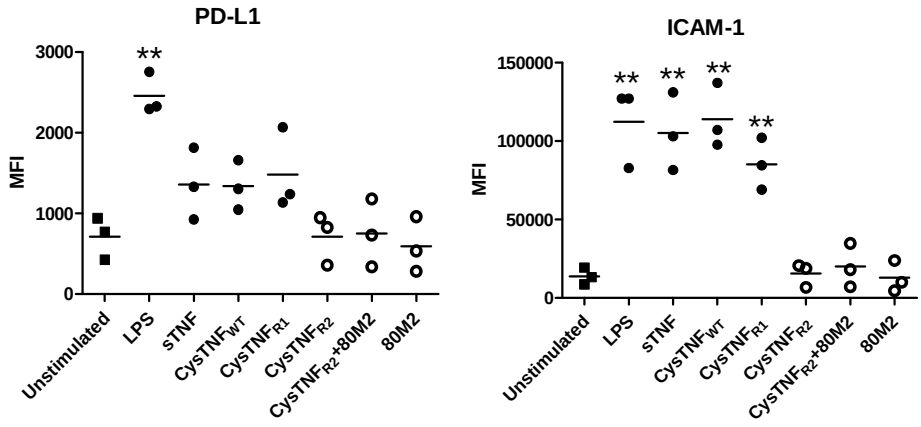


**Figure 24: TNFR1- but not TNFR2-mediated signalling results in enhanced expression of additional surface proteins associated with moDC maturation.** *Immature moDC were treated as indicated with LPS (100 ng/ml), TNF ligands (50 ng/ml) +/- MAb 80M2 (2 μg/ml) for 24 h. The expression of PD-L1 and ICAM-1 was determined by flow cytometry. (A and B) MFI indicates the median fluorescence intensity; error bars show the mean of the MFI +/- SEM. Statistical differences were determined in relation to un-stimulated (immature) moDC using a student's t test; *P≤0.05 **P≤0.01 ***P≤0.001. Data are representative of 3 independent experiments with moDC from different donors.*

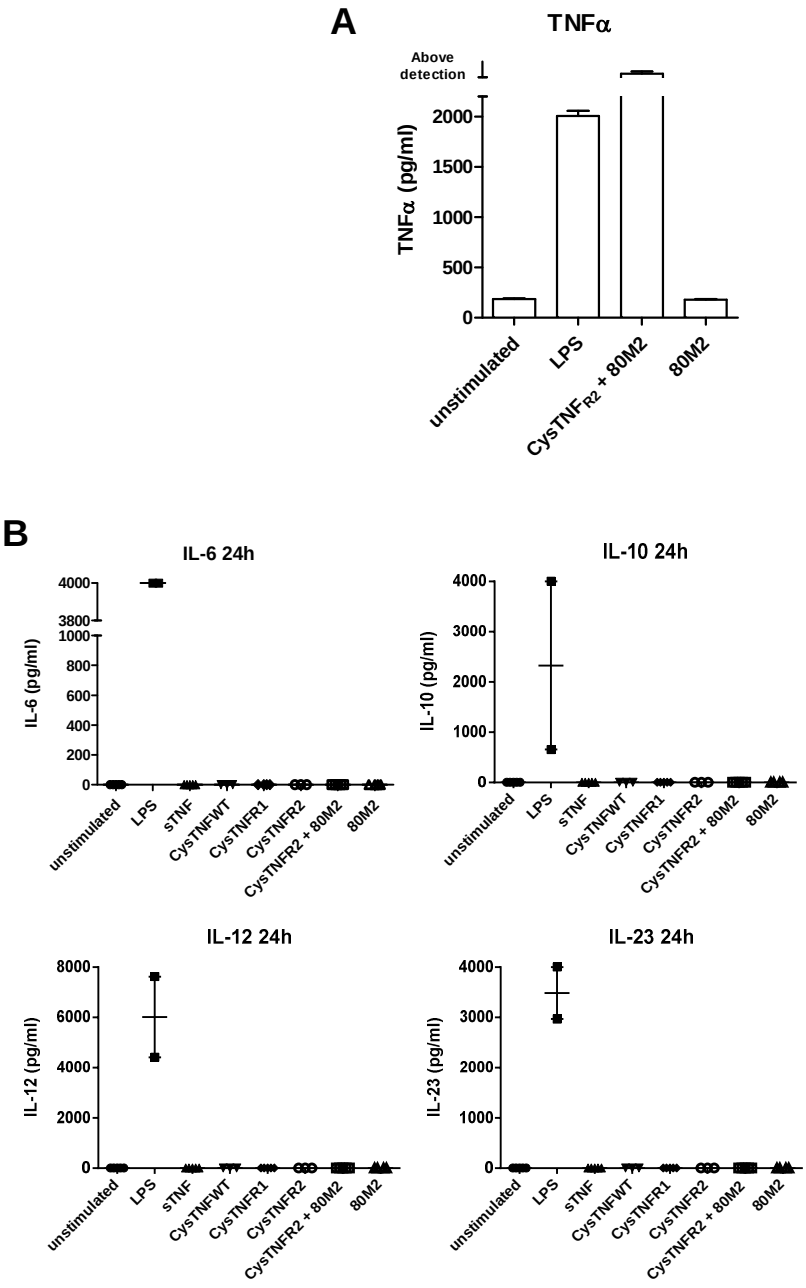


**Figure 25: TNF-mediated maturation does not induce cytokine production in moDC.** *Immature moDC were treated with LPS (100 ng/ml), sTNF or TNFR-selective ligands (50 ng/ml) +/- MAb 80M2 for 24 h. Supernatants were harvested and cytokine concentrations (TNF (A), IL-6, IL-10, IL-12 or IL-23 (B)) were determined using duplicate wells in a sandwich ELISA. Results represent (A) 4 independent donors and (B) 2 (LPS), 3 ($CysTNF_{wt}$ and $CysTNF_{R2}$) or 5 (unstimulated, sTNF, $CysTNF_{R1}$, $CysTNF_{R2}$+80M2 and 80M2) independent donors.*

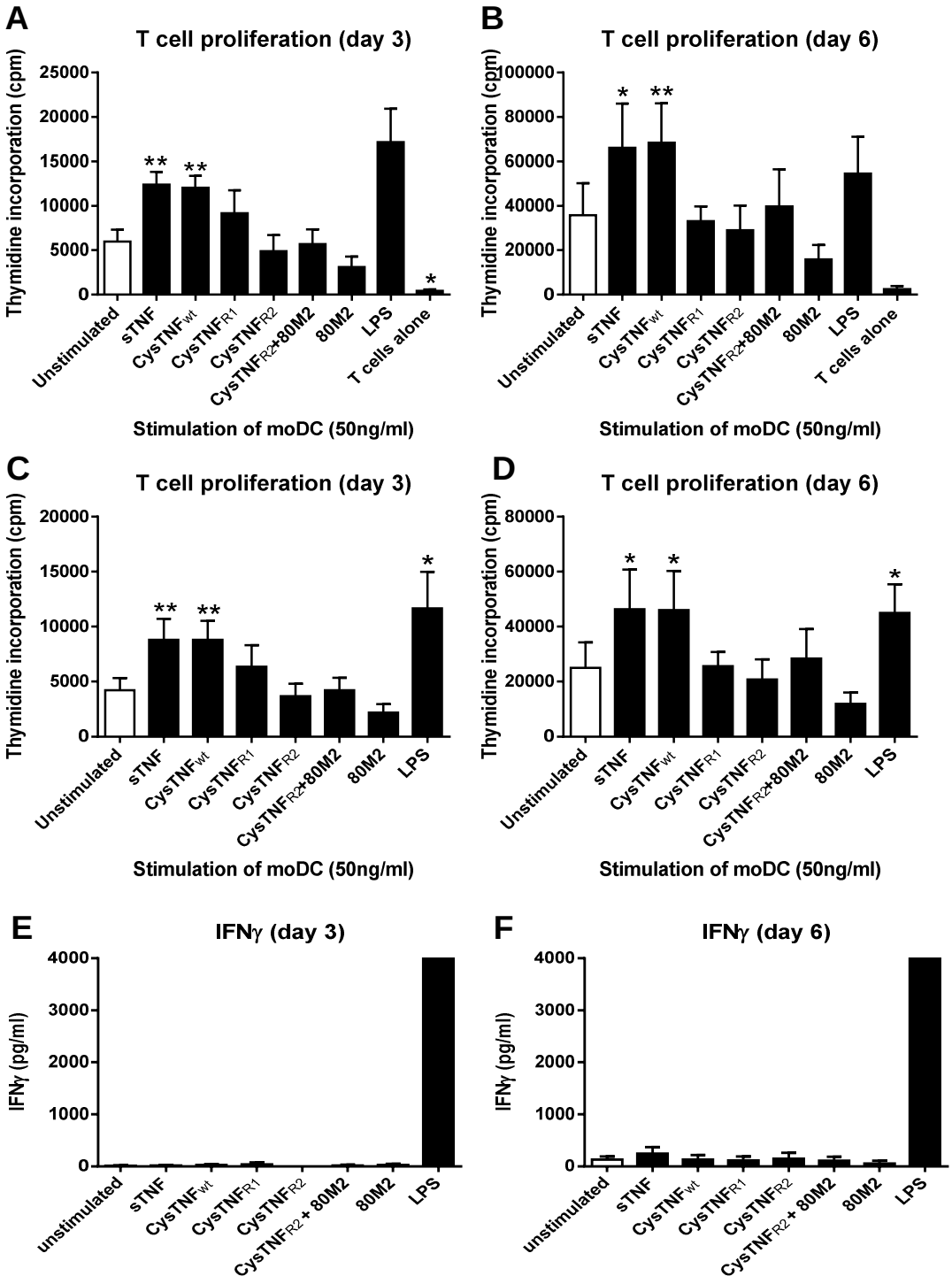


**Figure 26: Signalling via sTNF and CysTNF$_{wt}$ enhances the T cell stimulatory capacity of moDC.** *Immature moDC were left untreated or were treated with LPS (100 ng/ml), TNF or TNFR-selective ligands (50 ng/ml) +/- MAb 80M2 as indicated for 24 h, washed thoroughly and co-cultured with allogeneic CD4$^+$ T cells at a ratio of 2 x 10$^4$ moDC to 1 x 10$^5$ T cells for 3 (A, C) or 6 (B, D) days. T cell proliferation after incubation with an excess of $^3$H-thymidine for 8 h was determined by quantification of incorporated $^3$H-thymidine (A-D). Supernatants were harvested at day 3 (E) or day 6 (F) and IFNg concentrations were determined using an IFNg-specific sandwich ELISA. Results represent 4 (A, B), 6 (C-E) or 3 (F) independent donors.*

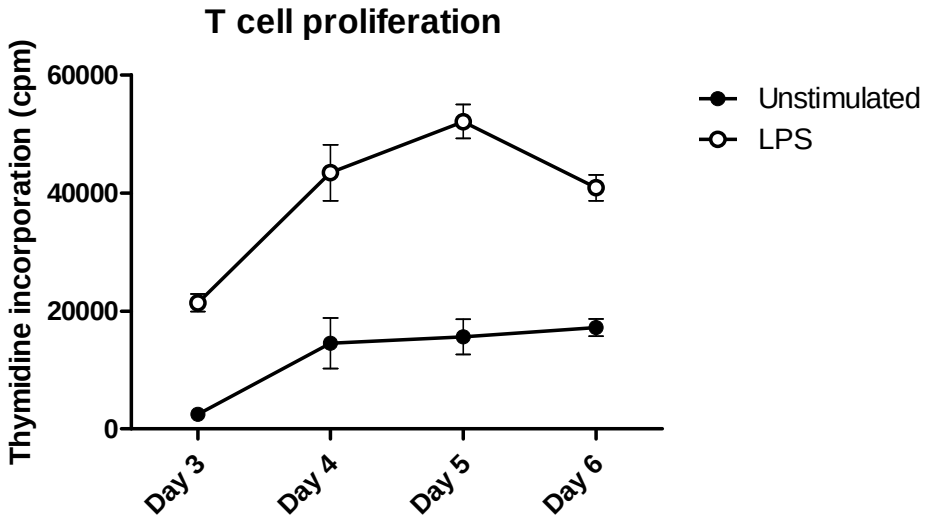


**Figure 27: T cell proliferation after co-culture with LPS-stimulated moDC is highest after 5 days.** *Immature moDC were left untreated or were treated with LPS (100 ng/ml) for 24 h, washed thoroughly and co-cultured with allogeneic $CD4^+$ T cells at a ratio of 2 x $10^4$ moDC to 1 x $10^5$ T cells for 3 to 6 days. T cell proliferation after incubation with an excess of $^3$H-thymidine for 8 h was determined by quantification of incorporated $^3$H-thymidine. Results represent one independent experiment.*

### *3.5. Discussion*

This study is the first to use TNFR-selective ligands to investigate the roles of TNFR1 and TNFR2 in regulating human moDC maturation. I have made use of previously developed and well defined TNFR-selective ligands to exclude knock-out induced side effects and to directly investigate each receptor in human cells. I have also used $CysTNF_{wt}$ which mimics the activity of mTNF as using sTNF alone will only efficiently activate TNFR1 (but not TNFR2), whereas mTNF activates both TNFR and thus resembles cell-to-cell interaction induced signalling.

The aims of this chapter were to investigate the expression and signalling competence of TNFR1 and TNFR2 on human moDC, to explore the effect of TNFR signalling on moDC maturation and to determine their ability to stimulate T cell proliferation. I have shown that moDC do express signalling competent TNFR1 and TNFR2 and that the downstream signalling of each receptor is different but overlapping. I have also shown that TNFR1- but not TNFR2-mediated signalling results in moDC maturation as defined by enhanced expression of maturation markers. However TNF-induced signalling alone (when compared to the TLR ligand LPS) is not sufficient to induce full maturation as shown by a lack of cytokine production by the moDC and by subsequently activated T cells.

#### *3.5.1. MoDC expression of TNFR and TNFR2*

I have shown that both TNFR1 and TNFR2 are expressed on human immature moDC and that their expression is reduced following moDC maturation (Figure 9). Although a similar reduction in TNFR expression is seen in all samples, the variability in expression on immature moDC between each donor is high (almost 3-fold in some samples). This difference in receptor expression may account for donor to donor variability in other assays i.e. some donors appear to have a greater response to TNF than others.

#### *3.5.2. NFκB pathway activation and DC maturation*

NFκB transcription factors regulate a range of genes involved in the inflammatory response and NFκB signalling is widely implicated in inflammatory diseases, both in pro- and anti-inflammatory processes (Bonizzi & Karin 2004). The regulation of the classical and alternative NFκB pathways is crucial in the balance between inflammation and tolerance. My findings that the classical (p65) and alternative (p52) NFκB signalling pathways in moDC are differentially activated by TNFR1 and TNFR2 may partially explain the opposing functions of TNF in disease (see chapter 1.5). The lack of

activation of p65 via TNFR2 observed in my data is most likely to be moDC-specific as others have shown that the classical NFκB pathway is activated by TNFR2, albeit to a lower extent compared to TNFR1 for example in cell lines (McFarlane et al. 2002) and in mouse neurones which lack TNFR1 (Marchetti et al. 2004).

In contrast to my data, other groups have shown that TNFR2 can activate the classical NFκB pathway. In a mouse model with neurones lacking TNFR1, TNFR2-mediated signalling activated the PI3K pathway leading to IκBα phosphorylation, degradation and subsequent classical p65 NFκB pathway activation (Marchetti et al. 2004). Additionally, human KYM-1 rhabdomyosarcoma cells which express TNFR1 and high levels of endogenously expressed TNFR2 or HeLa cells generated to over-express exogenous TNFR2, can both activate the classical NFκB pathway via TNFR1 and TNFR2 as measured by IκBα degradation and NFκB gene activity, although the effect of TNFR2 was much weaker than TNFR1 (McFarlane et al. 2002). This suggests that although TNFR1 is not responsible for all TNF-mediated classical NFκB activation, it is predominantly responsible, as even when over-expressed TNFR2 is not efficient in the activation of p65 NFκB. Therefore it is possible that TNFR2 may mediate some activation of the classical NFκB pathway in moDC but may still be too weak to be detected by the DNA-binding ELISA used in this study.

The classical p65 NFκB pathway is essential for DC maturation as suppression of p65 NFκB signalling inhibits DC maturation in a mouse model (Peng et al. 2012). Depending on the cell type and environment TNFR1 and TNFR2 can both independently activate the classical NFκB pathway, however they may differ in the kinetics of this activation; in primary cortical neurones from TNFR knock-out mice, TNFR1 induced transient p65 NFκB activation with an optimum activation after 1 hour of TNF treatment, whereas TNFR2 induced weaker but more long term p65 NFκB signalling for up to 4 hours (Marchetti et al. 2004). This partially fits with my data as although no p65 activation could be detected via TNFR2-stimulation, the oligonucleotide binding assay (Figure 11) showed that TNFR1-mediated signalling activated the p65 NFκB pathway optimally around 30 min with activity decreasing slightly by 60 minutes.

Although not studied here, the NFκB protein RelB has also been shown to be essential for DC maturation (Zanetti et al. 2003). RelB is generally known as an effector of the alternative NFκB pathway (through a direct interaction with p52), but it has also been shown to act as an effector of the classical NFκB pathway through the formation of a RelB-p50 dimer (Shih et al. 2012). Unlike the RelB-p52 dimer, the formation of the RelB-p50 dimer is affected by the presence of IκB molecules allowing rapid activation

of the classical NFκB pathway leading to DC maturation in response to pathogens (Shih et al. 2012). Therefore the dimerisation partner of RelB may be the crucial switch in determining the cellular response to TNFR-mediated signalling. It would be interesting to investigate whether TNFR1-stimulation leads to DC maturation via RelB-p50-supported p65 actiavtion, thereby contributing to the lower activation of the alternative NFκB pathway by TNFR1 than TNFR2.

My data also show that the alternative NFκB pathway is activated by both TNFR1 and TNFR2. TNFR1- and TNFR2-mediated p52 activation was increased at 24 h and the TNFR1-mediated response remained the same after 48 h, whereas TNFR2-mediated activation was transient and reduced after 24 h (Figure 14). It is not clear whether this is a clear difference between TNFR1- and TNFR2-mediated p52 activation as this may also be due to the effect of point mutations in the TNFR1- and TNFR2-selective ligands which may alter the stability of TNF/TNFR complexes and therefore may affect their signalling capabilities. CD40L (J558L) cells are mouse B cell myeloma cells which have been transfected to express CD40L and were used as a positive control for p52 activation in moDC (Figure 17). Generally, this classical p52 activator showed only around 2 fold higher activation of p52 compared to mTNF suggesting that mTNF is quite efficient at activating p52. However, it was not possible to fully remove all the CD40L cells from the moDC cultures before lysis therefore it cannot be excluded that these cells also contribute to the p52 activation detected in my assay. Also, the inclusion of some CD40L cells may decrease the amount of moDC nuclear protein included in the assay leading to an underrepresentation of CD40L cell-mediated p52 activation.

In addition, the blocking effect of H398 (a TNFR1-specific antagonistic antibody) was not complete for any of the TNFR1-stimulating ligands. This may be due to sub-optimal conditions as H398 was not titrated or sTNF may have bound TNFR1 before the antagonistic antibody had bound to all the TNFR1 molecules, although this is unlikely as cells were incubated with H398 for 30 min prior to the addition of TNF and also a high amount of H398 was used (10 μg/ml). Alternatively, H398 has weak agonistic activity and although the use of H398 alone has no effect (suggesting that this is not the case), the agonistic effect may only occur in combination with sTNF or when the ligands are used in combination. An alternate method for blocking individual TNFR would be to use the monovalent Fab fragment of the antibodies which bind to TNFR1 or TNFR2 but are not able to crosslink and rearrange TNFR on the cell surface preventing TNFR pre-clustering. These antagonists would also have the advantage that they have no Fc portion and therefore cannot bind non-specifically to Fc receptors

on the DC which may affect DC maturation, but have the disadvantage that due to the low affinity of these Fab fragments, high amounts would be needed.

The strong activation of p52 by sTNF in moDC (Figure 15 (B)) is of particular interest as this has not been reported to date, although more work is needed to identify the full process as whether the increase in p52 upon sTNF stimulation is the result of active p100 processing or due to a strong induction of p100 expression is not clear. However, sTNF seems to act through TNFR1 as its activation of p52 can be partially blocked by antagonising TNFR1 (Figure 16). The reason that the effect is not fully abolished may be due to the weak agonistic activity of the H398 antagonisitc antibody.

### *3.5.3. TNFR signalling and DC maturation*

The majority of previous work on the maturation of human DC has been performed using sTNF giving no answer to the question of which TNFR is involved in DC maturation. The use of murine knock-out models however, has shed some light on the requirement of TNFR-signalling for DC maturation. Whereas mice deficient in TNFR1 show impaired DC maturation in response to attenuated mouse hepatitis virus, the lack of TNFR2 does not affect DC maturation (Ding et al. 2011). Another study also shows that in TNFR1 knockout mice DC maturation is impaired in response to pathogens, but did not investigate the effect of TNFR2 (Sundquist & Wick 2005). Furthermore, TNFR1-stimulation can also activate DC in an autoimmune context. For example, in a rat model a retinal autoantigen (S-antigen) activated and induced the maturation of DC via TNFR1 and these DC could then activate an antigen-specific B cell response *in vivo* (Liversidge et al. 2000). Again, TNFR2 was not investigated in this study. In addition, these studies do not address whether the TNFR1-mediated response is due to an inability of TNFR2 to induce maturation or whether the pathogenic stimuli used do not result in the availability of a TNFR2 ligand (e.g. mTNF).

My data show that TNFR1-mediated signalling increased the expression of surface markers involved in the co-stimulation and activation of T cells. DC stimulated with sTNF (which acts via TNFR1) also enhanced $CD4^+$ T cell proliferation, however the resulting T cells failed to produce an IFN$\gamma$ response (Figure 26 (E and F)). The effect of TNF-stimulated DC on T cell proliferation was much lower than that of LPS-stimulated DC, which consequently were capable of inducing T cell IFN$\gamma$ production. This supports previous work which shows that inflammatory mediators (such as TNF) are not sufficient to fully mature DC and induce effector T cell responses (Spörri & Reis E Sousa 2005). Full DC maturation requires a PAMP (e.g. a TLR agonist) which can induce the production of inflammatory cytokines (Roelofs et al. 2005) as well as enhancing maturation marker expression.

The role of TNF-induced phenotypical maturation of DC is uncertain. It is possible that TNF may serve to sensitise cells of the immune system in order to enhance the release of pro-inflammatory molecules. However, *in vivo* it is unlikely that DC would be exposed to TNF alone as during infection or inflammation the cytokine milieu is broad and can induce multiple overlapping signalling pathways. Furthermore, inhibition of TNF during LPS maturation reduces stimulatory capacity (Baldwin et al. 2010), thus, although TNF in itself is not enough for DC maturation, it may cooperate with PAMPS to induce full maturation. As it is possible that other cytokines may be involved in the effect of TNFR1- or TNFR2-stimulated DC on T cell function more experiments are needed to analyse a wider range of cytokines and to determine the potential cytokine profile. Also, DC can produce a range of chemokines which may also differ depending on TNFR1- or TNFR2-signalling. It would also be interesting to analyse the effect of DC on T cells in the presence of T cell polarising cytokines such as IL-12 or IL-4 which may differentiate the T cell response.

My data also show that TNFR1- but not TNFR2-mediated signalling induces moDC maturation and sTNF/CysTNF$_{wt}$ induces a T cell stimulatory capacity. The failure of TNFR2 to induce maturation in moDC could be caused by the failure of this receptor to induce detectable activation of the classical p65 NFκB pathway whereas TNFR1 was capable of activating the classical NFκB pathway via p65. Importantly, TNFR2-selective stimulation does not appear to suppress TNFR1-mediated DC maturation as CysTNF$_{wt}$, mimicking mTNF action and thereby triggering both TNFR, potently induced DC maturation to a similar extent as the TNFR1-stimulating ligands, sTNF and CysTNF$_{R1}$. These data indicate that DC maturation is independent of TNFR crosstalk indicating different functions for the two TNFR as described previously (Apostolaki & Victoratos 2010; Blüml et al. 2012).

Interestingly, the up-regulation of CD83, the activation of the p65 NFκB pathway and the induction of T cell proliferation following stimulation with the TNFR1-selective ligand is less prominent than that of sTNF or CysTNF$_{wt}$. The reason for this remains unclear as all three ligands have similar bioactivities and have comparable effects on CD86 and HLA-DR expression therefore it seems unlikely to be due to the ligand concentration used. It is possible that the effect of CysTNF$_{R1}$ may be slower to initiate the phosphorylation of p65 than sTNF or CysTNF$_{wt}$ rather than the signal being weaker although the PhosFlow time course in Figure 13 suggests that this is also not the case. Furthermore, in the p52 NFκB oligonucleotide binding assay the activation by sTNF is again greater than that of CysTNF$_{R1}$. It is possible that these differences are due to the effects of the point mutations which give the ligand its receptor-selectivity.

### *3.5.4. Conclusion*

In conclusion, the classical and alternative NFκB signalling pathways are differentially activated by TNFR1 and TNFR2. TNFR1 activates the classical NFκB pathway and has the capacity to induce phenotypical maturation in moDC. TNFR2-mediated stimulation did not produce any detectable activation of the classical NFκB pathway, however both TNFR can activate the alternative NFκB pathway. As TNFR2-mediated signalling results in p52 activation, but not in maturation, the function of the alternative NFκB pathway is unclear but may play a role in other pathways such as DC survival. The activation of TNFR-mediated signalling pathways may involve differences in signalling kinetics; TNFR1-induced classical NFκB activation can occur in minutes and be transient whereas TNFR2 may take hours to promote NFκB activation but signalling may remain active for longer. Since NFκB signalling is not only involved in maturation but also plays a key role in cell survival, I explored the effect of TNFR-signalling on DC survival which will be discussed in my next chapter.

# 4. TNF induced protection of moDC from cytokine deprivation-induced cell death

## 4.1. Introduction

Maintaining the balance between immunity and tolerance is an important function of DC and is controlled not only by their maturation state (as discussed in the previous chapter), but also by their longevity. Prolonging the lifespan of DC has been shown to break tolerance and result in autoimmunity (Wang et al. 1999; Chen et al. 2006), whereas a decrease in DC survival reduces immune protection from pathogens as well as inhibiting autoimmunity (Jung et al. 2002; Whartenby et al. 2005).

Previous research suggests that DC have a relatively short lifespan and undergo apoptosis shortly after maturation in order to prevent excessive T cell activation and subsequent autoimmunity (McLellan et al. 2000). Although TNF is known to promote DC survival (Ludewig et al. 1995; Lehner et al. 2012), the contribution of the two TNF receptors to the lifespan of DC is poorly understood. TNFR1, but not TNFR2, contains an intracellular death domain, suggesting that they may play different roles in cell survival. It has been shown that bone marrow-derived DC from TNFR1$^{-/-}$ deficient mice have increased resistance to Fas-mediated apoptosis whereas TNFR2$^{-/-}$ deficient mice do not (Funk et al. 2000). It has also been shown that during TNF-mediated signalling, p65 NFκB plays a crucial role in protection from apoptosis (Beg et al. 1995). As I have previously shown TNFR1 and TNFR2 differentially activate this pathway (chapter 3.4; Figure 12 and Figure 15), it is possible that TNFR1 and TNFR2 also differentially regulate DC survival.

## *4.2. Specific aims*

1) Investigate the individual roles of TNFR1 and TNFR2 in moDC survival.
2) Identify the pathways involved in TNFR1- and TNFR2-mediated regulation of moDC lifespan.

### *4.3. Experimental Approach*

To address the question of whether TNF-induced enhancement of moDC survival is mediated through one or both TNFR, monocytes were differentiated into immature moDC and extensively washed to remove cytokines (i.e. IL-4 and GM-CSF) from the culture medium. I took this approach as 'cytokine deprivation' leads to cell death in moDC (Baldwin et al. 2010) and I also treated the cells with sTNF, $CysTNF_{wt}$ or the TNFR-selective ligands as described previously. A number of methods were chosen for cell death analysis. The detection of intracellular active caspase-3 was chosen as caspase-3 is central to the execution phase of apoptosis and is activated by both the extrinsic (death ligand) and intrinsic (mitochondrial) pathways. The enzymatic activity of caspase-3 was also studied to confirm that caspase-3 was not only processed but was also active. Additionally, Annexin V and ViaProbe were analysed as they can be used to detect early and late stages of cell death, respectively. Annexin V detects the externalization of phosphatidylserine (PS) which is located on the cytoplasmic surface of normal viable cells, but is translocated to the outer leaflet of the membrane early in apoptosis where it can be detected (and is therefore present before and after the cell membrane becomes compromised). ViaProbe (7-AAD) recognises and binds to double stranded DNA and cannot cross the cell membrane therefore indicating membrane compromised (i.e. 'dead') cells and emits a fluorescent signal which can be detected by flow cytometry.

## *4.4. Results*

### *4.4.1. Selective stimulation of TNFR1 or TNFR2 reduces cytokine deprivation-induced cell death in moDC*

It has previously been shown that a high proportion of moDC die after 48 hours of culture without the addition of exogenous cytokines (e.g. IL-4 and GM-CSF which are known survival factors) and that LPS-induced autocrine TNF can protect immature moDC from cell death (Baldwin et al. 2010). I could confirm these published data by detecting increasing levels of active caspase-3 (i.e. an increase in cell death) over time in response to the removal of IL-4 and GM-CSF (Figure 28 (A)). Figure 28 (B) shows that exogenously given sTNF prevents cytokine deprivation-induced cell death in a concentration dependant manner. Unstained cells were classed as caspase-3 negative and used for the gating of caspase-3 positive cells. The addition of an isotype control showed no deviation from the unstained cells (Figure 28 (C)). As the observed effect of sTNF on DC survival is most likely to be mediated via TNFR1, TNFR1 may have the ability to rescue moDC from cytokine withdrawal-induced cell death. However, the effect of TNFR2 has not been addressed thus far, therefore TNFR-selective ligands are required to assess how this effect is mediated.

To further study the regulation of moDC survival by TNF, moDC induced to undergo cell death via cytokine-deprivation, were treated with the TNFR-selective ligands. Cell death was determined by measuring intracellular active caspase-3 levels by flow cytometry (Figure 29 (A, B)), and a colorimetric assay to measure caspase-3 enzymatic activity via cleavage of a *p*-nitroaniline-coupled DEVD substrate (Figure 29 (C)). Results in Figure 29 show that sTNF (which acts primarily through TNFR1) and $CysTNF_{wt}$ (which acts through both TNFR) significantly rescued moDC from cell death after cytokine withdrawal (from 61.3 ± 6.8% caspase-3 positive in unstimulated moDC, to 19.3 ± 4.4%; p=0.0002 with sTNF and 17.5 ± 5.1%; p=0.0008 with $CysTNF_{wt}$). Data also show that both TNFR1- and TNFR2-selective ligands significantly reduced moDC cell death although this was to a slightly lesser degree than sTNF and $CysTNF_{wt}$ (from 61.3 ± 6.8% caspase-3 positive in unstimulated moDC, to 29.8 ± 5.2%; p=0.0034 with $CysTNF_{R1}$ and 31.77 ± 4.15%; p=0.0029 with $CysTNF_{R2}$ + 80M2). As expected, $CysTNF_{R2}$ alone or MAb 80M2 alone had no effect on moDC survival (Figure 29). Similar results showing that TNFR1- and TNFR2-mediated signalling enhances moDC survival were also shown using Annexin V/ViaProbe detection by flow cytometry (Figure 30).

To obtain a better understanding of the type of cell death moDC undergo after cytokine withdrawal, I used a number of inhibitors for pathways associated with cell death. The

inhibitors were added immediately after cytokine withdrawal and the degree of cell death was determined after 48 h using Annexin V and ViaProbe (as previously). Necrostatin-1 (Nec-1), an inhibitor of necroptosis, did not inhibit cell death whereas $zVAD_{fmk}$, an irreversible pan caspase inhibitor which is cell-permeable, did partially inhibit cell death as shown by an increase in live cells from 21% to 44% (Figure 31 (A, B)), suggesting that DC were undergoing, at least in part, apoptosis. In addition, a caspase-8 specific inhibitor ($zIETD_{fmk}$) also reduced apoptosis as shown by an increase in live cells corresponding to an increase in the inhibitor (from 21% to 50% at 30 μM $zIETD_{fmk}$), suggesting that moDC cell death after cytokine withdrawal involves the extrinsic (death receptor-mediated) pathway (Figure 31 (A)). Previous research has suggested that inhibiting caspase-8 may induce necroptosis (O'Donnell et al. 2011) therefore I also used a combination of both $zVAD_{fmk}$ and Nec-1. This increased moDC survival from 25 ± 2.4% (unstimulated cells) to 51 ± 1.3%; p=0.004 ($zVAD_{fmk}$ and Nec-1) compared to 39 ± 2.9%; p=0.023 ($zVAD_{fmk}$ alone) (Figure 10 (B)) suggesting that necroptosis does occur following caspase inhibition but not following cytokine deprivation alone.

Notably, none of the inhibitors fully prevented cell death indicating that the timing of the addition of inhibitors or the type of inhibitors used was not optimal. It is possible that without an activation signal (i.e. TLR ligation) DC may undergo cell death via multiple mechanisms and the inhibition of one may enhance others. As inhibitors were dissolved in DMSO which can be toxic to cells, the same DMSO concentration used in the inhibition experiments (0.01%) was used as a control and showed no difference in the amount of cell death when compared to unstimulated moDC, thereby excluding an effect on survival mediated by the solvent (Figure 31 (A)).

The TNF superfamily also includes TNF-related apoptosis-inducing ligand (TRAIL) which binds to the death receptors TRAIL R1 (DR4) and TRAIL R2 (DR5) and induces the caspase-8-dependent extrinsic pathway of apoptosis. TRAIL can also bind TRAIL R3 (DcR1) and TRAIL R4 (DcR2) which function as decoy receptors and protect cells from apoptosis by neutralizing TRAIL. Mature DC have previously been shown to be resistant to TRAIL-mediated apoptosis whereas immature DC were partially sensitive (Leverkus et al. 2000). In line with this TRAIL R1-4 are expressed by immature moDC and their expression is reduced as moDC mature (Figure 32) thereby they could potentially mediate the extrinsic pathway of apoptosis in DC.

Results therefore show that both TNFR1 and TNFR2 can enhance moDC survival and that the mechanism involved in moDC cell death after cytokine withdrawal is, at least in part, the extrinsic (death receptor-mediated) pathway of apoptosis.

### *4.4.2. Antagonisitc TNFR-specific antibodies block TNFR1- and TNFR2-mediated protection from cell death in moDC*

In order to investigate the possibility that enhanced moDC survival mediated by TNFR1- and TNFR2-selective ligands may be induced by co-operation between the two receptors or by the indirect activation of TNFR1 via TNFR2-mediatied production of sTNF (Grell et al. 1999), I used TNFR-specific antagonistic antibodies which were added directly after washing the moDC, i.e. after cytokine withdrawal, but 30 min prior to TNFR-stimulation. I confirmed that neither of the TNFR antagonists had any effect on moDC survival when used alone as compared to unstimulated cells. The antagonistic TNFR1-specific antibody completely blocked the $CysTNF_{R1}$-mediated survival of moDC but did not affect the response to $CysTNF_{R2}$ plus 80M2 suggesting that TNFR2 acts in a TNFR1-independent manner (Figure 33). In addition, the anti-TNFR2 antibody inhibited the response to $CysTNF_{R2}$ plus 80M2 but had no effect on the TNFR1-mediated response (Figure 33). This suggests that both TNFR1 and TNFR2 can independently contribute to the survival of moDC. As different numbers of donors were used for each condition, Appendix A shows Figure 33 divided into corresponding donors.

In line with previous results (chapter 3.4.2; Figure 8) and other research (Grell et al. 1995; Richter et al. 2012; Krippner-Heidenreich et al. 2002), sTNF acts primarily through TNFR1. Figure 33 shows the pro-survival effect of sTNF (from 66 ± 3.4% caspase-3 positive in unstimulated moDC, to 18 ± 2.4%; $p<0.0001$ with sTNF) was blocked by anti-TNFR1 (from 18 ± 2.4% caspase-$3^+$ cells to 51 ± 0.9%; $p<0.0001$), but was not affected by anti-TNFR2 indicating that the sTNF-mediated rescue from cell death in moDC is mediated through TNFR1, but not TNFR2. Furthermore, the pro-survival effect of $CysTNF_{wt}$ (from 66 ± 3.4% caspase-3 positive cells in unstimulated moDC, to 16 ± 1.7%; $p<0.0001$ with $CysTNF_{wt}$) was only partially blocked by antagonising either TNFR1 (from 16 ± 1.7% to 35 ± 3.9%; $p=0.0004$) or TNFR2 (from 16 ± 1.7% to 20 ± 2.1%; $p=0.14$) alone, which may be due to the strong avidity of the pre-oligomerised ligand or its ability to signal through both TNFR. Moreover, the effect of $CysTNF_{wt}$ could be fully blocked by antagonising both TNFR (from 16 ± 1.7% caspase-3 positive cells with $CysTNF_{wt}$ to 64 ± 5.8%; $p<0.0001$ with both TNFR antagonists) (Figure 33), indicating that mTNF enhances moDC survival through both TNFR1- and TNFR2-mediated signalling.

Additionally, human IgG was used to block Fc receptors which confirmed that the effect of the antagonistic antibodies was not due to Fc receptor mediated signalling (Figure 34). These data indicate an important role for TNFR2 in DC survival and demonstrate that TNFR1 and TNFR2 independently mediate the rescue of moDC from cell death.

Data also show that as expected, sTNF cannot act through TNFR2 whereas mTNF activates both TNFR1 and TNFR2 in moDC.

### *4.4.3. Optimisation of TNFR knock down in moDC using siRNA*

To further investigate the individual effects of TNFR1 and TNFR2 on moDC maturation and survival and to exclude any potential cross-reaction between the receptors I attempted to knock-down TNFR expression by RNA interference. Figure 35 (A) shows that TNFR1-specific siRNA reduced TNFR1 expression by 30%, whereas TNFR1 expression was not affected by TNFR2-specific siRNA. Alternatively, Figure 35 (A) also shows that TNFR2-specific siRNA reduced TNFR2 expression by 47% which was not affected by TNFR1-specific siRNA. Data indicate that both TNFR can be partially knocked-down by the use of TNFR-specific siRNA and that there is no cross reactivity of the siRNA between the two receptors. However, the non-sense siRNA also showed a reduction in expression of both TNFR1 and TNFR2 (expression was reduced by 19% and 35%, respectively). Furthermore, the expression of surface maturation markers CD83, CD86 and HLA-DR were increased by the addition of non-sense siRNA and to a lesser extent by the TNFR-specific siRNA (Figure 35 (B)). As I have shown previously (chapter 3.4.3, Figure 9 (B)), that the maturation of moDC reduced the expression of TNFR1 and TNFR2, this may be the cause of the reduction in TNFR expression in response to non-sense siRNA. I also cannot exclude that the TNFR expression in response to TNFR-specific siRNA may be affected by the increase in maturation.

In order to reduce the effect of maturation on the expression of TNFR1 and TNFR2 I titrated both the DF4 reagent (used for siRNA transfection) and the siRNA concentration. Figure 36 shows that 0.5 μl DF4 has almost no effect on moDC maturation regardless of the concentration of siRNA. However 2.5 μl and 5 μl DF4 showed an increase in all the maturation markers (CD83, CD86 and HLA-DR). Figure 37 shows the expression of TNFR1 and TNFR2 in response to TNFR-specific siRNA; Figure 37 (A) showed a reduction in TNFR1 expression of 43% by 50 nM TNFR1-specific siRNA whereas TNFR1 expression was not affected by the same concentration of TNFR2-specific siRNA (Figure 37 (B)). Similarly, Figure 37 (D) showed a reduction in TNFR2 expression of 43% by 50 nM TNFR2-specific siRNA whereas TNFR2 expression was not reduced by the same concentration of TNFR1-specific siRNA (Figure 37 (B)). Lower concentrations (25 nM) of TNFR1- or TNFR2-specific siRNA had no effect on either receptor indicating that no less than 50 nM siRNA should be used in future experiments. Although the expression of both TNFR1 and TNFR2 is decreased more by 2.5 μl and 5 μl DF4 Figure 37 (A-D), this may be due

to increased moDC maturation (as shown in Figure 36) and, therefore, these volumes cannot be used.

Together, I found conditions (50 nM siRNA and 0.5 μl DF4) that would allow a significant knockdown of TNFR1 and TNFR2, however, the effects of siRNA itself on moDC maturation are not desirable. Overall, the use of siRNA was less effective than the antagonistic TNFR-specific antibodies, therefore, this line of research was not continued.

### *4.4.4. The Bcl-2/Bcl-xL pathway mediates pro-survival effects of both TNFR1 and TNFR2 whereas p65 NFκB signaling is involved in TNFR1- but not TNFR2-mediated moDC survival*

In order to further study the pathways downstream of TNFR1 and TNFR2 and to dissect their involvement in regulating the lifespan of moDC, I used a number of commonly used small molecule inhibitors to target different signalling pathways; the classical NFκB pathway, the PI3K pathway, the MAPK/ERK pathway and the Bcl-2/Bcl-xL mitochondrial apoptosis pathway. All inhibitors were added to DC cultures for 30 min prior to the addition of sTNF, $CysTNF_{wt}$ or the TNFR-selective ligands. Both the p65 NFκB pathway and the Bcl-2/Bcl-xL pathway have previously been shown to be essential for DC survival (Lehner et al. 2012; Kimberley & Screaton 2004; Kim & Joo 2009; Mattioli et al. 2009; Sánchez-Sánchez et al. 2004). I have shown that the classical p65 NFκB pathway is activated by TNFR1 but not TNFR2 (chapter 3.4.4; Figure 12 and Figure 15), therefore, I hypothesised that inhibition of this pathway would only affect TNFR1-mediated survival. In accordance with this, the BAY-11-7082 compound, which inhibits phosphorylation of IκBα and prevents its degradation (Richter et al. 2001), therefore blocking the classical NFκB pathway, impaired the enhancement of moDC survival mediated by TNFR1 (Figure 38 (A)) but not TNFR2 (Figure 38 (B)). With the addition of Bay-11-7082 alone, there appeared to be a slight reduction in Caspase-$3^+$ cells to 62 ± 4.6% compared to 74 ± 4.6% in unstimulated cells, which was not statistically significant. $CysTNF_{R1}$ reduced the percentage of active caspase-3 from 74 ± 4.6% to 40 ± 4.6% indicating enhanced survival. This was then blocked by the addition of Bay-11-7082 as shown by an increase in caspase-3 from 40 ± 4.6% ($CysTNF_{R1}$) to 67 ± 6.1%; p=0.017 (Figure 38 (A)). This confirms a pro-survival role for the classical NFκB pathway in moDC which is activated by TNFR1- but not TNFR2-selective stimulation.

Alternatively, the BH3 mimetic ABT-737 leads to the induction of apoptosis via the mitochondrial pathway (described in chapter 1.4.2.1). Briefly, ABT-737 inhibits the

heterodimerisation of Bcl-xL and Bcl-2 with members of the pro-apoptotic machinery such as Bax and Bak, thereby reducing the activity of pro-survival proteins (Bcl-xL and Bcl-2) and allowing Bax and/or Bak to form pores in the mitochondrial membrane, releasing cytochrome c and initiating to apoptosis. ABT-737 did not significantly affect the percentage of caspase-3 compared to that of unstimulated cells (75 ± 5.6% and 71 ± 7.5%, respectively) (Figure 38 (C)). However, ABT-737 significantly blocked the TNFR1-mediated pro-survival effect as the level of caspase-3 is increased from 43 ± 5.7% with $CysTNF_{R1}$ to 67 ± 5.6%; p=0.0082 with $CysTNF_{R1}$ plus ABT-737 (Figure 38 (C)). The effect of TNFR2-stimulation on moDC survival is also significantly inhibited by ABT-737 (from 47 ± 15.5% with $CysTNF_{R2}$ + 80M2 to 68 ± 8.1% with $CysTNF_{R2}$ + 80M2 plus ABT-737 (Figure 38 (D)). This suggests that both TNFR act via the Bcl-2/Bcl-xL pathway. In accordance, stimulation of either TNFR1 or TNFR2 results in upregulation of Bcl-xL expression in moDC at the protein level (Figure 38 (E)). GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) is an enzyme involved in the breakdown of glucose which is expressed at relatively constant levels in all cells and is required for the maintenance of basic cellular function. The level of GAPDH is consistent between all treatment groups indicating that the amount of protein loaded from each sample was equal (Figure 38 (E)).

LY294002 is an inhibitor of the phosphatidylinositol 3-kinase (PI3K) pathway and prevents subsequent Akt phosphorylation. This pathway is hyperactive in many cancers where downstream signaling can reduce apoptosis and allow cell proliferation (Workman et al. 2010), demonstrating the importance of the PI3K pathway in the regulation of cell survival. LY294002 did not significantly affect the active caspase-3 levels compared to that of unstimulated cells (83 ± 5.1% and 79 ± 5.9%, respectively) (Figure 39 (A)). However, LY294002 significantly blocked the TNFR1-mediated pro-survival effect as the level of caspase-3 is increased from 42 ± 4.0% with $CysTNF_{R1}$ to 63 ± 5.2%; p=0.0093 with $CysTNF_{R1}$ plus LY294002 (Figure 39 (A)). The TNFR2-mediated pro-survival effect was also significantly blocked as the level of caspase-3 increased from 39 ± 9.8% with TNFR2-stimulation to 61 ± 6.6%; p=0.0313 with TNFR2-stimulation plus LY294002 (Figure 39 (B)). Inhibition of the PI3K pathway, therefore, significantly prevents both TNFR1- and TNFR2-mediated enhancement of moDC survival (Figure 39 (A and B)) suggesting that the PI3K pathway is used by both receptors to support moDC survival.

The MAPK/ERK pathway is also known to be involved in a range of human cancers and participates in cross-talk with the PI3K pathway (Britten 2013). U0126 is an inhibitor of MEK1 and MEK2 kinase activity that blocks activation of the MAPK/ERK pathway by preventing the transcriptional activity of AP-1. U0126 did not significantly

affect the percentage of caspase-$3^+$ cells compared to that of unstimulated cells (74 ± 7.1% and 74 ± 7.0%, respectively) (Figure 39 (C)). However, inhibition of MEK1/2 significantly blocked the TNFR1-mediated pro-survival effect of $CysTNF_{R1}$-mediated signalling as the level of caspase-3 is increased from 35 ± 5.3% with $CysTNF_{R1}$ to 61 ± 4.2%; p=0.0359 with $CysTNF_{R1}$ plus U0126 (Figure 39 (C)). The effect of TNFR2-stimulation on moDC survival was not significantly inhibited by U0126 even though the mean percentage of caspase-3 increases from 35 ± 11.0% with $CysTNF_{R2}$ + 80M2 to 60 ± 8.0% with $CysTNF_{R2}$ + 80M2 plus U0126 (Figure 39 (D)). Thus, my data suggest that TNFR1-mediated enhancement of survival may involve the MAPK/ERK pathway as well as the PI3K pathway (summarised in Figure 41 (A)).

Data from this chapter and the previous chapter are summarised in Figure 41 (B); TNFR1-signalling activates both the p65 and the p52 NFκB pathway resulting in moDC maturation and survival via the Bcl-2/Bcl-xL pathway. Alternatively TNFR2-signalling activates the p52 but not the p65 NFκB pathway and does not result in moDC maturation but does lead to moDC survival via the Bcl-2/Bcl-xL pathway.

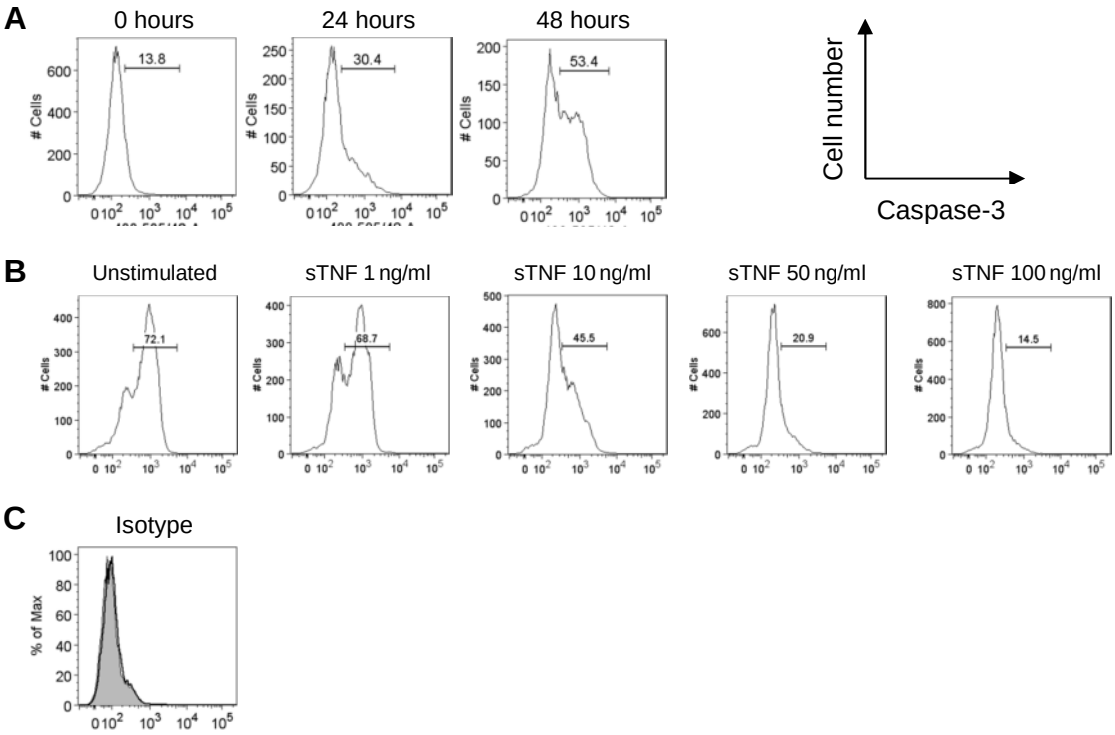


***Figure 28: sTNF protects moDC from cytokine deprivation-induced cell death.*** Immature moDC were left untreated for 0, 24 or 48 h (A) or were treated as indicated with varying concentrations of sTNF (1-100 ng/ml) for 48 h (B). Cell viability was assessed by the presence of intracellular active caspase-3 determined by flow cytometry (A, B). The percentages of cells gated positive for active caspase-3 are indicated. Data represents two individual experiments with different donors. (C) Isotype control (black line) compared to unstained moDC (grey shaded). Data represents at least 3 independent experiments.

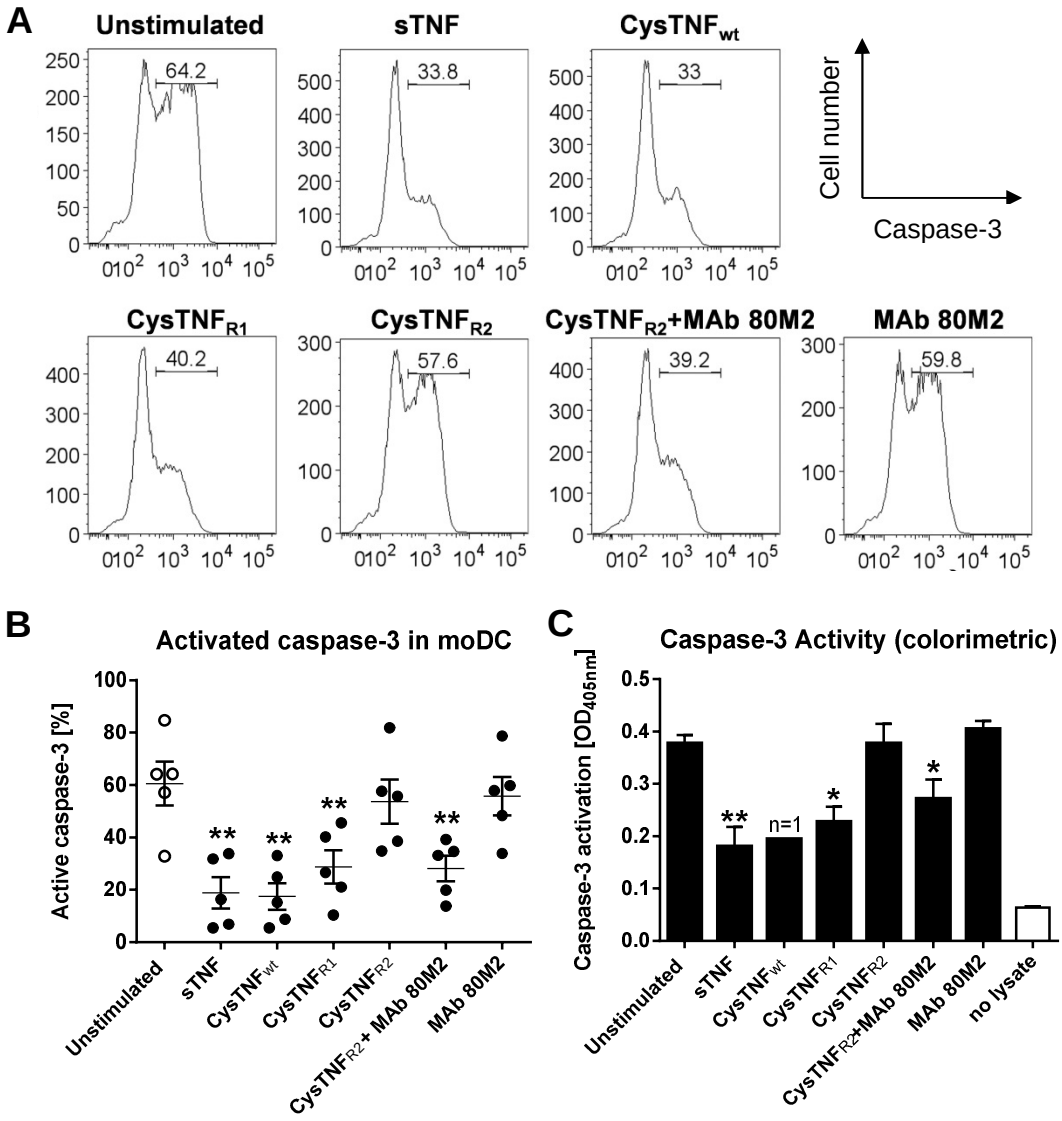


**Figure 29: TNFR1- and TNFR2-mediated signalling protects moDC from cytokine deprivation-induced cell death.** *Immature moDC were left untreated or were treated as indicated with sTNF, CysTNF$_{wt}$, CysTNF$_{R1}$ or CysTNF$_{R2}$ (50 ng/ml) +/- MAb 80M2 for 48 h. Cell viability was assessed by the presence of intracellular active caspase-3 determined by flow cytometry (A, B) or a colorimetric caspase-3 activity assay (C). (A) Cells were stained with anti-human active caspase-3 antibodies. The percentages of cells gated positive for active caspase-3 are indicated and data shown are representative of 5 independent experiments. (B) Data from 5 independent experiments with different donors are shown (mean +/- SEM) including that shown in (A). (C) Colorimetric assessment of caspase-3 activity in whole cell lysates using p-nitroaniline-coupled DEVD as substrate ('no lysate' contains the substrate only). The mean and SEM of 4 independent experiments are shown except CysTNF$_{wt}$ which shows one donor. (B and C) Statistical differences were determined in relation to unstimulated (immature) moDC using a student's t test; *P<0.05 **P<0.01 ***P<0.001.*

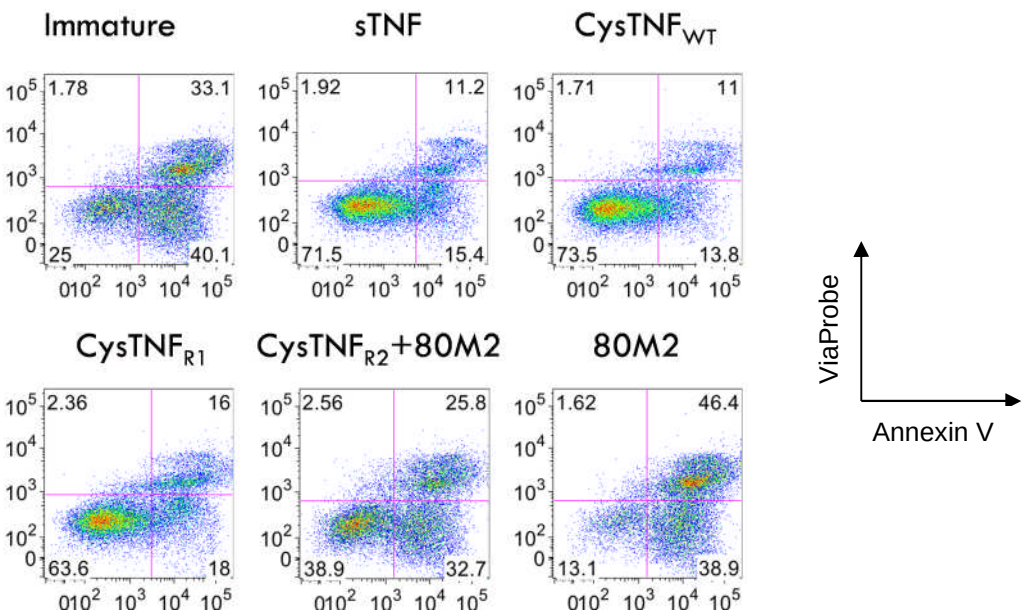


**Figure 30: TNFR1- and TNFR2-mediated signalling protects moDC from apoptosis following cytokine deprivation.** *Immature moDC were left untreated or were treated as indicated with sTNF, CysTNF$_{wt}$ or the TNFR-selective ligands (50 ng/ml) +/- MAb 80M2 for 48 h. Cell viability was assessed by ViaProbe and/or Annexin V detection by flow cytometry. Results represent three independent experiments.*

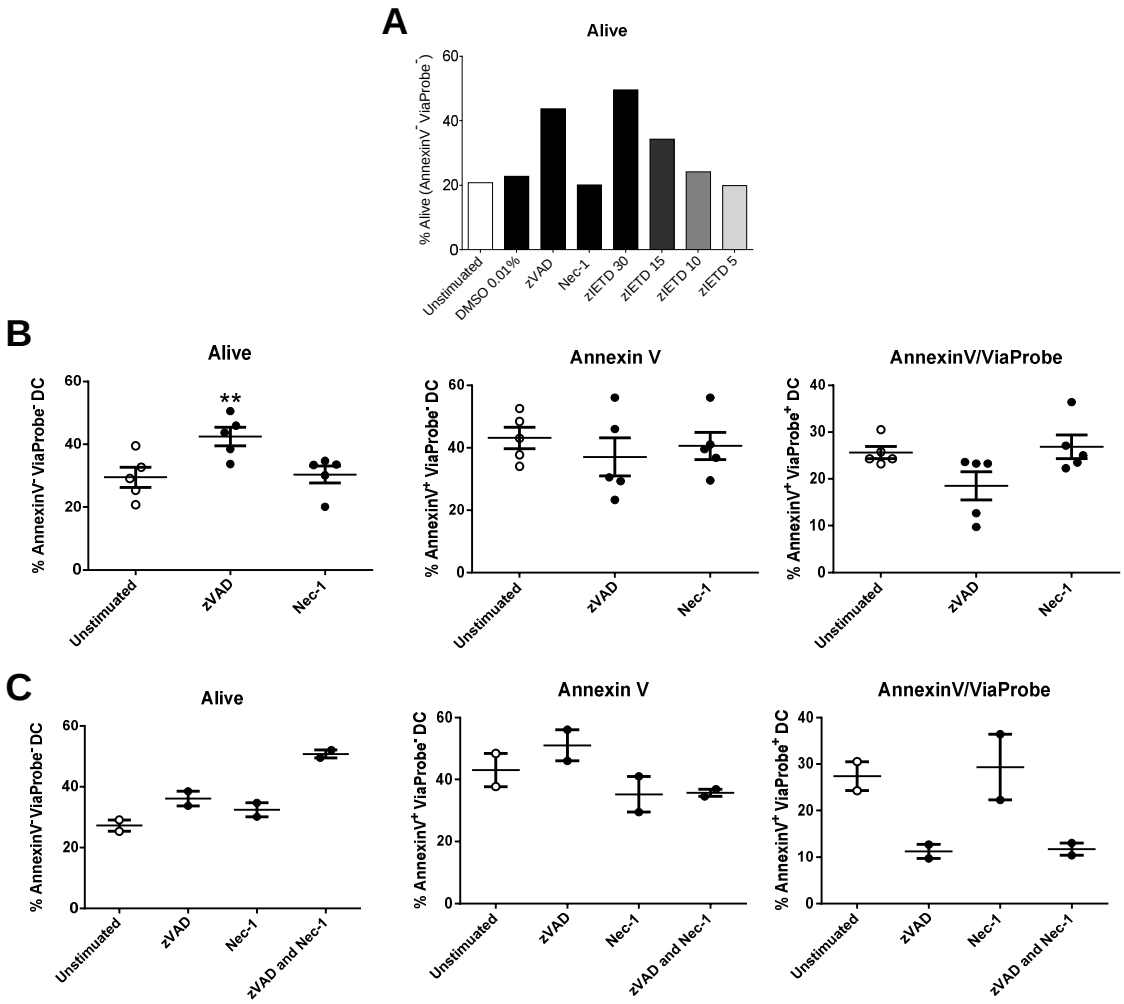


**Figure 31: Cytokine withdrawal induces moDC apoptosis potentially via a caspase-dependent pathway.** *Immature moDC were washed to remove cytokines and cells were treated with a pan-caspase inhibitor ($zVAD_{fmk}$; 20 μM), a caspase-8 inhibitor ($zIETD_{fmk}$; 5-30 μM), a necroptosis inhibitor (Nec-1; 30 μM) or DMSO (0.01%) as a control, for 48 h. (A-C) Cell viability was assessed by Annexin V and/or ViaProbe detection by flow cytometry. (A) One experiment showing a titration of $zIETD_{fmk}$ (zVAD and Nec-1 are also included in (B)). Data show 1 (A), 5 (B) or 2 (C) independent experiments with different donors and the mean +/- the SEM is shown (B, C). Statistical differences were determined in relation to unstimulated (immature) moDC using a student's t test; *P<0.05 **P<0.01 ***P<0.001.*

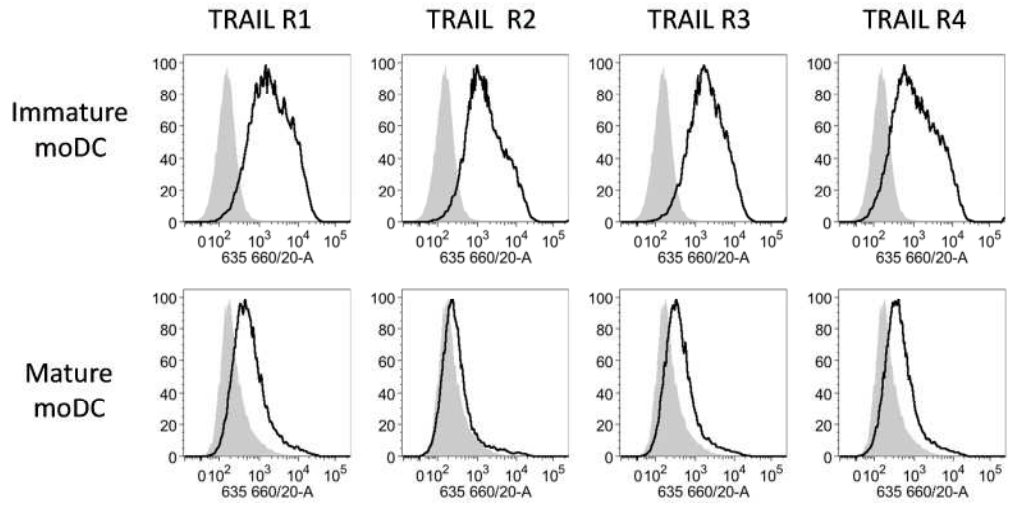


**Figure 32: Cell surface expression of TRAIL receptors by moDC.** *The cell surface expression of TRAILR1, TRAILR2, TRAILR3 and TRAILR4 (black line) compared to unstainted moDC (grey shaded) on immature and mature moDC was analysed by flow cytometry. Results represent two independent experiments with different donors.*

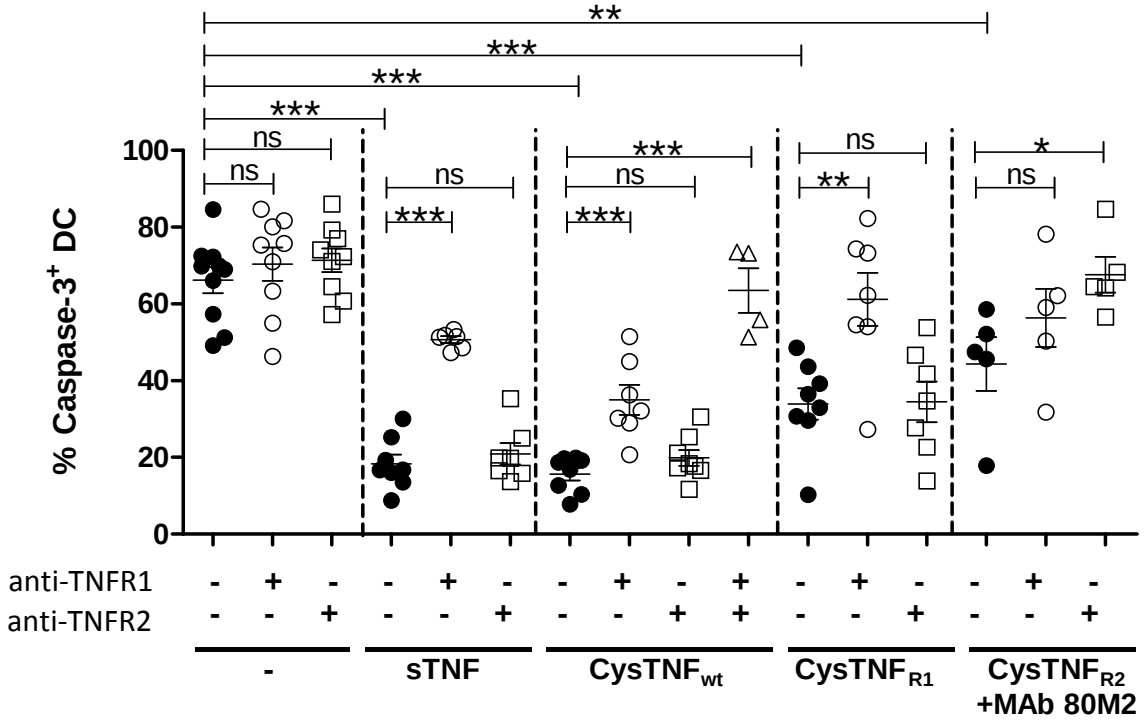


**Figure 33: TNFR1-antagonism blocks TNFR1-mediated but not TNFR2-mediated protection from cell death and vice versa.** *Immature moDC were left untreated or were treated with antagonistic TNFR1- or TNFR2-specific antagonistic antibodies (clone H398 or 22221 respectively) for 30 min prior to stimulation with sTNF, CysTNF$_{wt}$, CysTNF$_{R1}$ or CysTNF$_{R2}$ (50 ng/ml) +/- MAb 80M2 for 48 h. Cell viability was assessed by the presence of active caspase-3 determined by flow cytometry. The percentages of cells gated positive for active caspase-3 are indicated. Results represent a minimum of 4 independent experiments with different donors. The mean +/- SEM are shown and statistical differences were determined in relation to unstimulated (immature) moDC using a student's t test; *P<0.05 **P<0.01 ***P<0.001.*

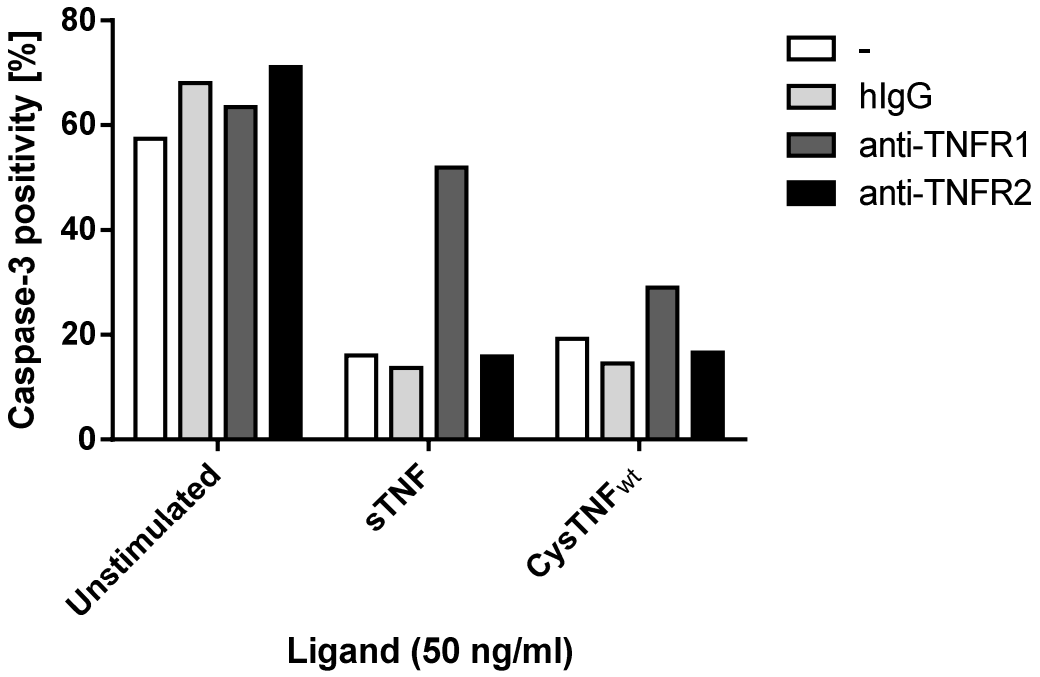


**Figure 34: Blocking Fc receptors with human IgG does not affect caspase-3 processing upon TNFR stimulation.** *Immature moDC were left untreated or were treated with 10 μg/ml human IgG, TNFR1- or TNFR2-specific antagonistic antibodies for 30 min prior to stimulation with sTNF or CysTNFwt (50 ng/ml) for 48 h. Cell viability was assessed by the presence of intracellular active caspase-3 determined by flow cytometry. The percentages of cells gated positive for active caspase-3 are indicated. Results represent 2 independent experiments with different donors.*

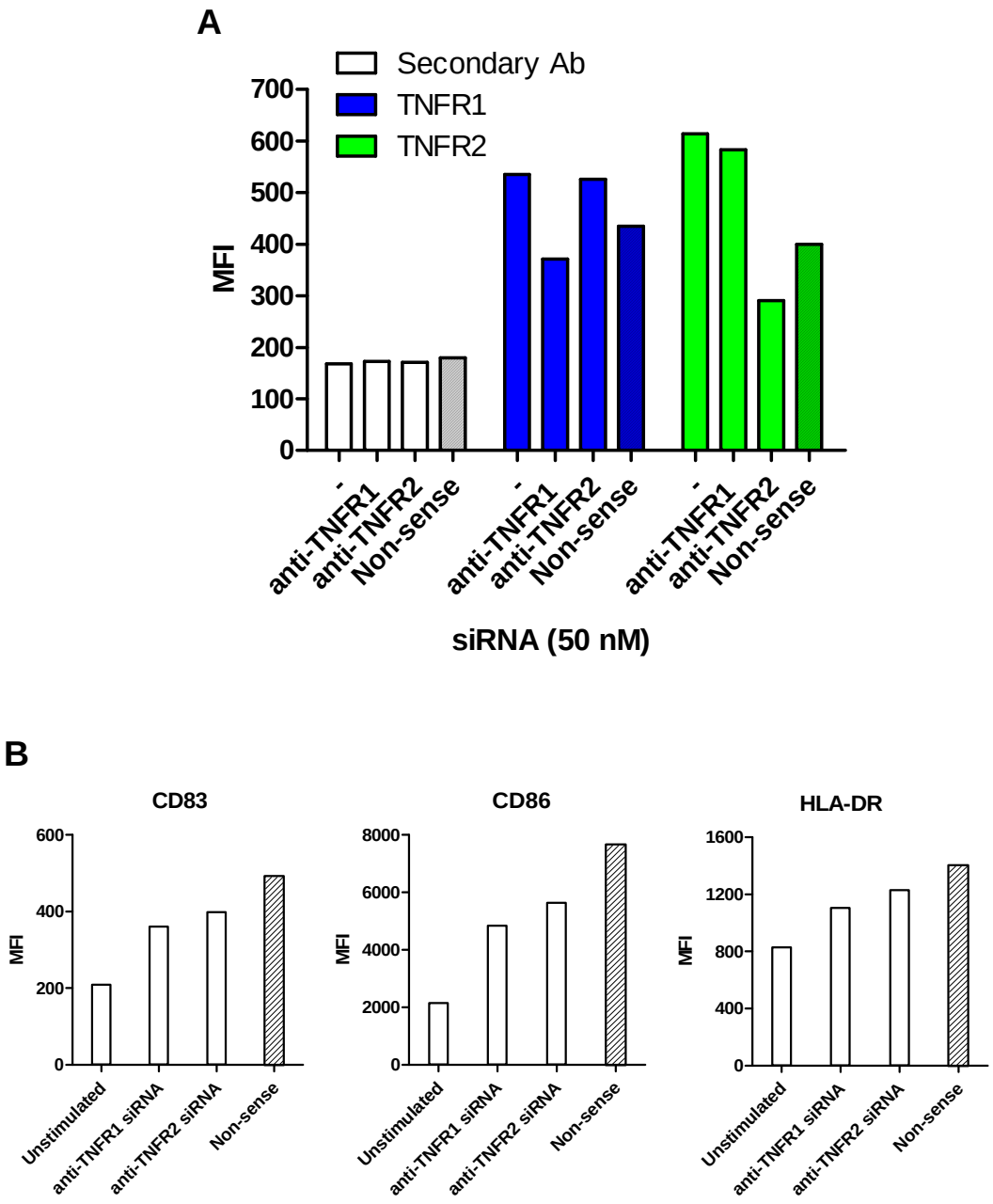


**Figure 35: TNFR expression following treatment of moDC with TNFR specific siRNA.** *Immature moDC were cultured in antibiotic free media in the presence of the DF4 transfection reagent (Dharmacon) alone or DF4 plus 50 nM anti-TNFR1 siRNA, anti-TNFR2 siRNA or non-sense siRNA (shaded bars) for 24 h. Expression of (A) TNFR1 (blue) and TNFR2 (green) or (B) CD83, CD86 and HLA-DR was analysed by flow cytometry. The median fluorescence intensity (MFI) is shown and results represent 2 independent experiments.*

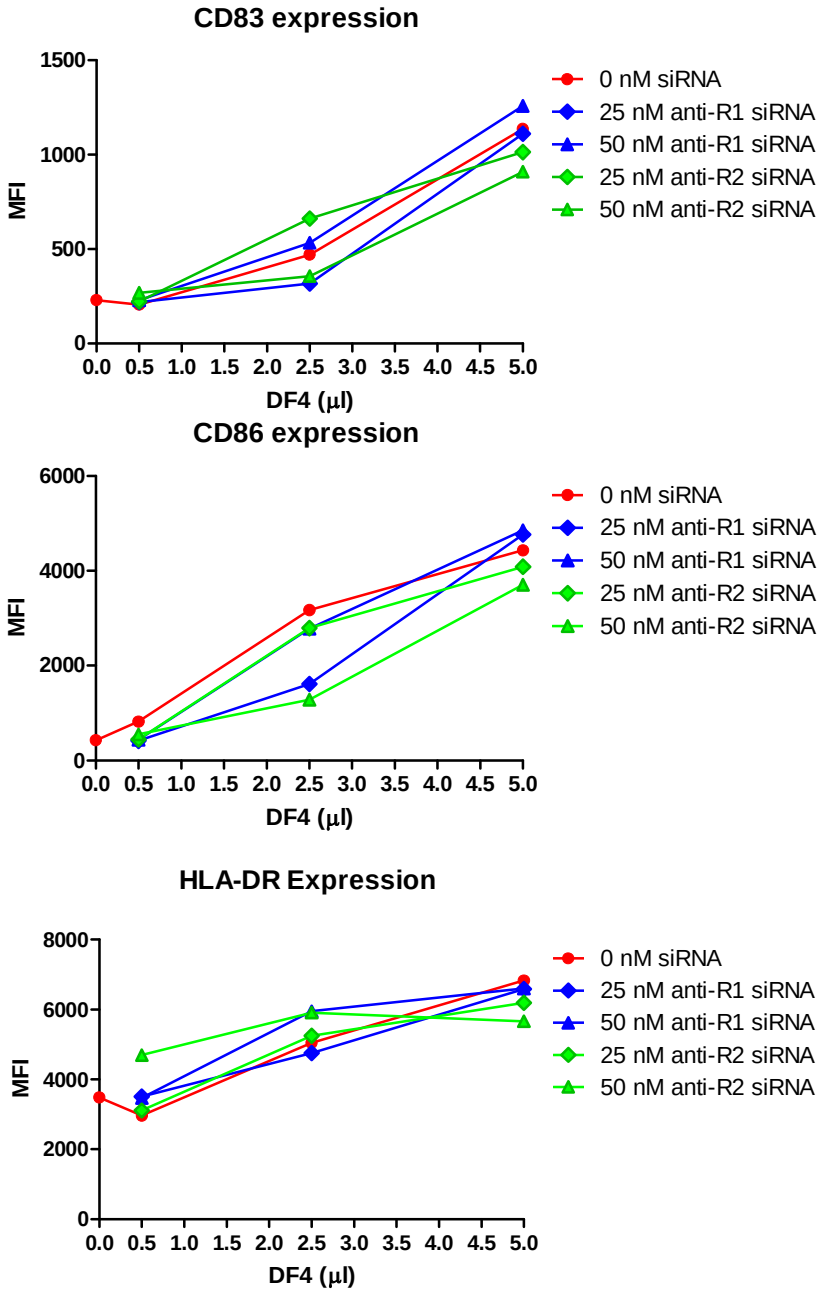


**Figure 36: Titration of siRNA and transfection reagent DF4 and the effect on moDC maturation marker expression.** *Immature moDC were cultured in antibiotic free media and treated with increasing amounts of the transfection reagent DF4 (O.5 to 5 µl) and 25 nM or 50 nM anti-TNFR1 siRNA or anti-TNFR2 siRNA for 24 h. Cell surface expression of CD83, CD86 or HLA-DR was determined by flow cytometry. The median fluorescence intensity (MFI) is shown and results represent 2 independent experiments.*

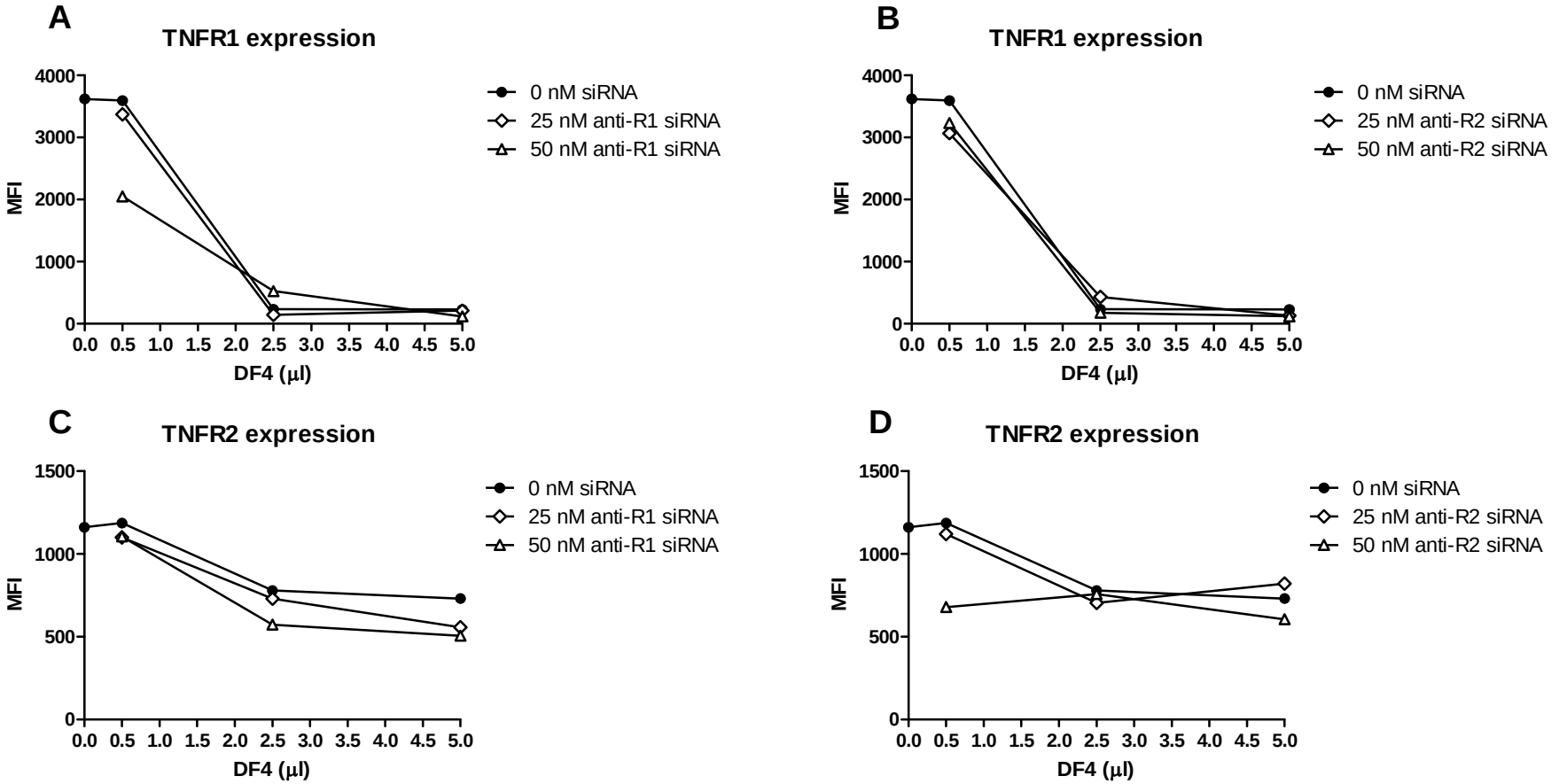


**Figure 37: Titration of siRNA and transfection reagent DF4 and the effect on moDC TNFR expression.** *Immature moDC were cultured in antibiotic free media and treated with increasing amounts of the transfection reagent DF4 (Dharmacon) and 25 nM or 50 nM anti-TNFR1 siRNA or anti-TNFR2 siRNA for 24 h. Cell surface expression of TNFR1 or TNFR2 was determined by flow cytometry. The median fluorescence intensity (MFI) is shown and results show one experiment (also shown in Figure 36).*

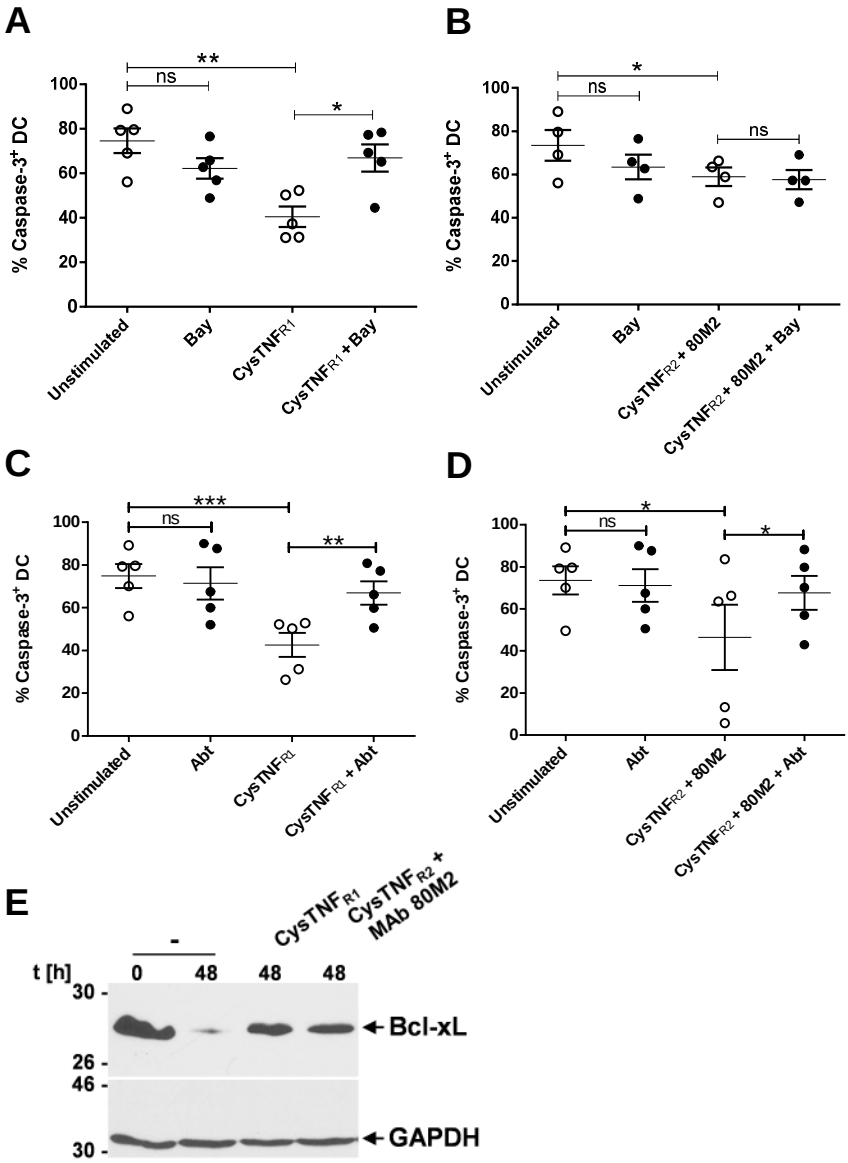


**Figure 38: Cell death protection mediated by both TNFR1 and TNFR2 is dependent on the Bcl-2/Bcl-xL pathway, and in the case of TNFR1, also requires p65 NFκB signalling.** *(A-D) Immature moDC were left untreated or were treated with 50 ng/ml CysTNF$_{R1}$ (A, C) or CysTNF$_{R2}$ plus MAb 80M2 (B, D) in the presence or absence of 10 μM of the chemical compounds Bay 11-7082 (A, B) or ABT-737 (C, D). Cell viability was assessed by the presence of intracellular active caspase-3 determined by flow cytometry. The percentages of cells gated positive for active caspase-3 are indicated. The mean and SEM of 5 (A, C, D) or 4 (B) independent donors are shown and statistical differences were determined in relation to unstimulated moDC using a student's t test; *P<0.05 **P<0.01 ***P<0.001. (E) TNFR1- and TNFR2-mediated signalling up-regulates Bcl-xL. Immature moDC were harvested immediately or after 48 h with or without stimulation (50 ng/ml CysTNF$_{R1}$ or CysTNF$_{R2}$ plus MAb 80M2). Whole cell extracts were analysed for the expression of Bcl-xL (top, #2762, Cell Signalling) and re-probed for GAPDH (bottom; 6C5, abcam) by Western Blot analysis. The blots shown are representative of three independent experiments.*

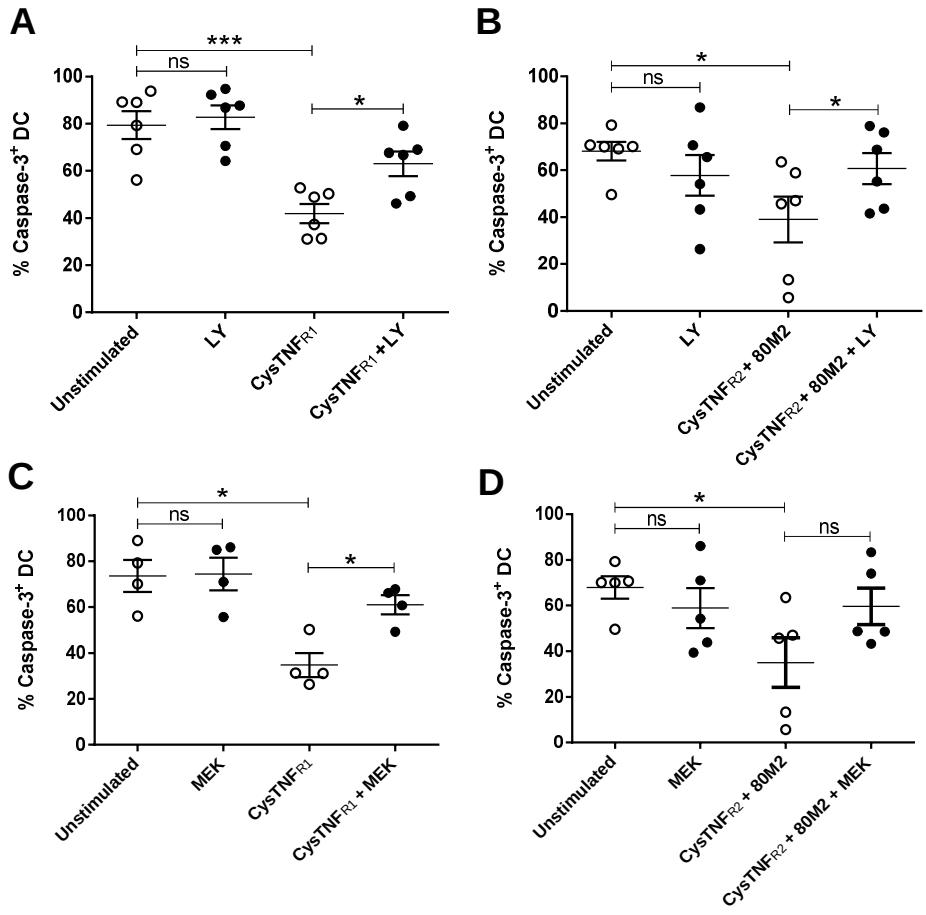


**Figure 39: Cell death protection mediated by both TNFR1 and TNFR2 is dependent on the PI3K pathway, and in the case of TNFR1, also involves the MAPK pathway.** *(A-D) Immature moDC were left untreated or were treated with 50 ng/ml CysTNF$_{R1}$ (A, C) or CysTNF$_{R2}$ plus MAb 80M2 (B, D) in the presence or absence of 10 μM of the chemical compounds LY294002 (A, B) or MEK (U0126) (C, D). Cell viability was assessed by the presence of intracellular active caspase-3 determined by flow cytometry. The percentages of cells gated positive for active caspase-3 are indicated. The mean and SEM of 6 (A, B), 4 (C) or 5 (D) independent experiments with different donors are shown. Statistical differences were determined in relation to unstimulated (immature) moDC using a student's t test; *P<0.05 **P<0.01 ***P<0.001.*

### *4.5. Discussion*

The main aims of this chapter were to investigate the individual roles of TNFR1 and TNFR2 in moDC survival and to identify the signalling pathways involved. In the previous chapter (3.4) I showed that TNFR1- but not TNFR2-mediated signalling activated the classical NFκB pathway whereas both TNFR activated the alternative NFκB pathway suggesting that the two receptors may mediate different but overlapping functions. I also discussed data showing that TNFR1- but not TNFR2-signalling induced phenotypical maturation of moDC. In contrast, in this chapter, I show data demonstrating that moDC survival can be enhanced independently by both TNFR1- and TNFR2-mediated signalling, indicating that some innate signals may promote DC survival even in the absence of maturation. I have also shown that moDC survival can be enhanced by both TNFR1- and TNFR2-mediated signalling and that both TNFR are dependent on the Bcl-2/Bcl-xL pathway. Only the TNFR1-, but not TNFR2-mediated protection depends on the activation of the p65 NFκB pathway. These data may, at least in part, underline the differences in the functional effects mediated by the two receptors demonstrating their distinct roles in regulating DC maturation and survival.

#### *4.5.1. TNFR-mediated moDC survival and TNFR antagonsim*

The survival of DC is a key factor in the regulation of adaptive immune responses. Similarly to my data using cytokine withdrawal, other research has shown that withdrawing plasma from the culture medium of mature human moDC reduced their survival and that this effect was prevented by the addition of TNF (Um et al. 2004), thereby confirming that TNF is important in moDC survival.

More specifically, my data show that both TNFR1- and TNFR2-selective stimulation enhance moDC survival. Both $CysTNF_{R1}$ and $CysTNF_{R2}$ + 80M2 were less effective at enhancing moDC survival than sTNF or $CysTNF_{wt}$. As discussed in the previous chapter (3.5.3), the reason for this remains unclear. One option may involve the introduction of point mutations which give the ligands receptor selectivity, however, all four ligands have similar bioactivities (when $CysTNF_{R2}$ is used in combination with 80M2) suggesting that this is not the case. Furthermore, the blocking effect of the TNFR1-specific antagonistic antibody H398 was only complete for $CysTNF_{R1}$, but not for sTNF or $CysTNF_{wt}$, whereas the TNFR2-specific antagonistic antibody 22221 fully blocked $CysTNF_{R2}$ + 80M2 but showed almost no effect on $CysTNF_{wt}$. This may be due to sub-optimal conditions as H398 and 22221 were not titrated, or sTNF and $CysTNF_{wt}$ may have bound to the TNFR before the antagonistic antibody had bound to all the TNFR molecules, although these reasons are unlikely as cells were incubated with the

antagonistic antibodies for 30 min prior to the addition of TNF and high concentrations were used (10 μg/ml). Also, the pro-survival effect of $CysTNF_{wt}$ was only partially blocked by antagonising either TNFR1 or TNFR2 alone, which may be due to the strong avidity of the pre-oligomerised ligand or its ability to signal through both TNFR as the effect of $CysTNF_{wt}$ could be fully blocked by antagonising both TNFR.

In addition, my results show that there is high variability between donors (Figure 33), firstly in the amount of cell death following cytokine withdrawal; for example, in unstimulated cells the percentage of caspase-$3^+$ cells ranges from 49% to 85%, and secondly in the degree to which TNFR-selective stimulation protect the cells; for $CysTNF_{R1}$ the lowest reduction in active caspase-3 compared to unstimulated cells from the same donor was 17% and the highest was 60%, whereas for $CysTNF_{R2}$ + 80M2 the lowest was 11% and the highest was 57%. The reason for this may be the high variability in TNFR1 and TNFR2 expression between donors as I discussed in the previous chapter (chapter 3.4.3; Figure 9).

The use of siRNA to knockdown TNFR in moDC proved difficult due to off target effects such as the induction of moDC maturation. The use of siRNA has been shown to result in activation of the dsRNA recognition protein PKR (protein kinase R) in mammalian cell lines which resulted in IFN-mediated activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and global upregulation of IFN-stimulated genes (Sledz et al. 2003; Sledz & Williams 2004). PKR can also activate NFκB signalling via the phosphorylation of IKBα *in vitro* (Kumar et al. 1994) which may then regulate processes such as cell proliferation. It is therefore extremely difficult to prevent siRNA from mediating effects on moDC survival and maturation beyond the silencing of specific target genes (i.e. TNFR) rendering this method unsuitable for use in any further experiments.

### *4.5.2. Overlapping signalling mechanisms involved in TNF-mediated DC survival*

Activation of p65 and p52 NFκB has been shown to confer resistance to both the intrinsic and extrinsic apoptosis pathways in lymphoma cell lines (Bernal-Mizrachi et al. 2006). I have shown that TNFR1-mediated signalling can activate both NFκB pathways and enhance moDC survival whereas TNFR2-mediated signalling also enhances moDC survival, but only activates the alternative NFκB pathway. My data also show that cytokine withdrawal induces moDC death, at least in part, via the external pathway of apoptosis and that this is reduced by TNFR1- or TNFR2-mediated signalling. TNFR2 enhanced moDC survival potentially via activation of the alternative NFκB pathway, whereas the TNFR1-mediated enhancement of survival may be due to activation of the classical and/or alternative NFκB pathway (as summarised in Figure 41 (B)).

Different mechanisms of cell death are described by (Galluzzi et al. 2012). Intrinsic apoptosis can be caspase-dependent (involving caspase-9 and -3) or caspase-independent, involving mitochondrial outer membrane permeabilisation (MOMP) and is triggered by intracellular stress such as DNA damage or oxidative stress. Alternatively, extrinsic apoptosis refers to a caspase-dependent apoptotic cell death induced by extracellular signals. It can be initiated by the binding of ligands to their death domain-containing receptors i.e. TNF and TNFR1, FasL (CD95L) and Fas (CD95), TRAIL and TRAILR1 or TRAILR2, although activation of a death receptor does not always lead to cell death as shown by my data regarding TNFR1 and discussed by (Schütze et al. 2008). My data show that TNFR1, TRAILR1 and TRAILR2 are all expressed on the surface of moDC and therefore may interact during the induction of cell death. For example, TNFR1 stimulation in human multiple myeloma cell lines increased cell death induced by FasL, but reduced TRAIL-mediated cell death (Rauert et al. 2011).

My results show that upon cytokine withdrawal, moDC undergo cell death, at least in part, via apoptosis and not necroptosis as cell death can be inhibited by a caspase-inhibitor ($zVAD_{fmk}$) but not a necroptosis inhibitor (Nec-1). Necroptosis (programmed necrosis) can also be triggered by the ligation of death receptors under certain circumstances for example, inhibition of caspases prevents degradation of RIP1 and/or RIP3 which suppresses apoptotic signalling leading to necroptosis (Cho et al. 2010; He et al. 2009). My data support this finding as the combinational use of both $zVAD_{fmk}$ and Nec-1 further increased moDC survival. However, the rescue from cell death was not complete and even when blocking caspase-mediated apoptosis and necroptosis, moDC still showed 49% dead cells after 48 h. The underlying mechanism for this remains unknown. It is possible that cell death has already been initiated in some cells i.e. during the harvesting and washing of the moDC, or that the inhibitors were not fully optimised, or that another pathway is also involved (for example caspase-independent intracellular apoptosis).

#### *4.5.2.1. The NFκB signalling pathway and DC survival*

Both the classical and alternative NFκB pathways are known to enhance APC survival. Contrary to the results presented in this thesis, previous research indicates that TNF activates the classical but not the alternative NFκB pathway (Derudder et al. 2003). My data has shown that only TNFR1- but not TNFR2-mediated signalling activates the classical NFκB pathway but that both TNFR1 and TNFR2 activate the alternative NFκB pathway. Generally, TNFR1-mediated activation of the alternative NFκB pathway is suppressed by the adapter kinase RIP1 through the inhibition of TNF-mediated TRAF2 and cIAP1 degradation (Kim et al. 2011; Gentle et al. 2011). As my results show that

TNFR1-selective stimulation activates both the classical and alternative NFκB pathways, it is possible that RIP is absent in my system and that its absence may allow activation of the alternative NFκB pathway. However, preliminary results suggest that this is not the case as RIP is expressed in moDC and its expression is not reduced or absent during stimulation with sTNF (Figure 18), therefore, it remains unclear under which physiological or pathological conditions TNFR1 can mediate the induction of the alternative NFκB pathway.

It has been previously reported that TNF fails to activate p100 processing and therefore alternative NFκB pathway (Coope et al. 2002). More recent data has demonstrated that in a number of cell lines and also in primary T cells that TNFR2 but not TNFR1 activated the alternative NFκB pathway (Rauert et al. 2010). Also, in the same study, sTNF trimers failed to activate the alternative NFκB pathway showing that the alternative NFκB pathway involves not only TNFR2, but also mTNF (Rauert et al. 2010), therefore the lack of TNF-mediated alternative NFκB pathway activity in previous studies may be due to the use of sTNF which does not activate TNFR2. However, both of these studies are inconsistent with my data which shows that both TNFR1 and TNFR2 activate the alternative NFκB pathway in moDC suggesting that the alternative NFκB pathway can be differentially activated by TNF depending on the cell type.

The alternative NFκB pathway involves the release of NIK from its constitutive degradation allowing NIK to accumulate before it can facilitate the phosphorylation and activation of IKKα, which in turn targets p100 for phosphorylation and partial degradation releasing p52. The p52 molecule can then form a complex with RelB and translocate to the nucleus (Figure 40 (B)). Previous data shows that NIK was required for TNF-mediated activation of the alternative but not the classical NFκB pathway in lymphoblastoid cells, whereas NIK was required for CD40-mediated activation of both NFκB pathways (Ramakrishnan et al. 2004). Other data confirm that *de novo* synthesis of NIK is required for the activation of alternative NFκB signalling mediated by CD40 (Qing et al. 2005). Authors suggest that this may account for the delay in alternative NFκB pathway activation (compared to the classical NFκB pathway) and may explain why TNF fails to induce p100 processing. My data however, shows that both TNFR1- and TNFR2-mediated signalling in moDC activated the alternative NFκB pathway (albeit to a lower extent than CD40-stimulation). Defects in the regulation of NIK can result in the uncontrolled growth of immune cells, for example constitutive activation of p100 processing mediated through the over-expression of NIK has been shown to result in B cell hyperplasia and autoimmunity (Sasaki et al. 2008). NIK over-expression

also resulted in increased levels of Bcl-xL but not Bcl-2 (Sasaki et al. 2008), although it remains unclear if RelB/p52 dimers are directly responsible for the activation of Bcl-xL.

#### *4.5.2.2. The PI3K/Akt and MAPK/ERK pathways*

A number of other pathways are also implicated in DC survival. The PI3K pathway (summarised in (Cantley 2002)) is important in the inhibition of apoptosis and is linked to a number of cancers (Workman et al. 2010) as well as inflammatory and autoimmune diseases. Therefore, proteins within the PI3K pathway provide potential therapeutic targets (Foster et al. 2012) in a range of diseases. PI3K activation leads to the activation and phosphorylation of Akt (a serine/threonine protein kinase), which can activate proteins involved in protein synthesis, cell proliferation, cell cycle entry and apoptosis. For example, Akt can inhibit pro-apoptotic Bax thereby preventing pore formation in the mitochondrial outer membrane and inhibiting apoptosis (Yamaguchi & Wang 2001), as well as being able to activate the MAPK pathway. My data show that inhibition of the PI3K pathway significantly prevents both TNFR1- and TNFR2-mediated enhancement of moDC survival (Figure 39 (B)) suggesting that the PI3K pathway is used by both receptors to support moDC survival. In line with this, previous studies have shown human CD34-derived myeloid DC required PI3K-Akt-mTOR signalling for proliferation and survival during differentiation, but not for the surface expression of co-stimulatory molecules (van de Laar et al. 2010). In addition, BM-derived DC from $TNF^{-/-}$ mice have defects in maturation in response to adenovirus infection which has been shown to be dependent on the autocrine production of TNF via the PI3K pathway (Philpott et al. 2004).

The MAPK/ERK pathway is involved in cross-talk with the PI3K pathway and is also known to be involved in a range of human cancers (Britten 2013). Both the PI3K and MAPK/ERK pathways have been shown to be differentially involved in the regulation of DC IL-12, IL-23 and IL-27 cytokine production (Jackson et al. 2010). My data suggest that both TNFR1- and TNFR2-mediated enhancement of survival may involve the PI3K pathway (Figure 39 (A, B)), however, only TNFR1-mediated survival significantly involved the MAPK/ERK pathway (Figure 39 (C)). The results for TNFR2 (Figure 39 (D)) were not significant, possibly due to the high donor-to-donor variation (which may involve differences in TNFR expression between donors). Additionally, it has been shown that moDC from elderly donors are phenotypically comparable but functionally different (including reduced Akt phosphorylation and therefore reduced PI3K pathway activation) when compared to those from young donors (Agrawal et al. 2014). As the age of donors in my experiments is unknown, this may also contribute to donor variability.

### *4.5.3. Regulation of moDC survival by Bcl-2 family members*

Bcl-2 family proteins play a major role in both positively and negatively regulating mitochondria-dependent apoptosis. In addition to the differential activation of classical and alternative NFκB signalling by TNFR1 and TNFR2, I have also shown that both TNFR induced the upregulation of anti-apoptotic Bcl-xL (Figure 38 (E)). In support of this, it has been shown that both p65 and p52 are capable of binding to the Bcl-xL promoter and may play a role in the regulation of its expression (Tamatani 1999; Marinari et al. 2004).

The ratio of pro- and anti-apoptotic members of the Bcl-2 family is important in regulating the lifespan of DC and may be responsible for the longevity of different DC populations. It has been shown that shorter lived myeloid DC have a lower ratio of anti-apoptotic Bcl-2/Bcl-xL to pro-apoptotic Bax/Bak compared to longer lived plasmacytoid DC (Chen et al. 2007). Also, autocrine TNF is essential for moDC survival and induces different members of the Bcl-2 family involved in protection from both the intrinsic and extrinsic apoptosis pathways (Lehner et al. 2012). *In vivo* transgenic mouse studies have shown that the overexpression of the anti-apoptotic protein Bcl-2 specifically in DC prolonged mouse survival and increased immunogenicity (Nopora & Brocker 2002). In addition, up-regulation of anti-apoptotic Bcl-xL is associated with the reduction of Fas-mediated apoptosis in human LPS-matured DC (Lundqvist et al. 2002). This suggests that the independent functions of TNFR1 and TNFR2 in DC may converge at the level of Bcl-2/Bcl-xL which is already known to play a central role in DC survival and function (Hou & Van Parijs 2004; Lehner et al. 2012).

### *4.5.4. Conclusion*

In conclusion, both TNFR1 and TNFR2 are able to independently enhance moDC survival. As TNFR2 had no effect on the maturation of moDC (chapter 3.4.6) but significantly enhanced moDC survival this indicates that TNFR2 can promote DC survival even in the absence of maturation. In addition my data show that in order to enhance moDC survival, both TNFR are dependent on the Bcl-2/Bcl-xL pathway whereas only TNFR1, but not TNFR2 depends on the activation of the p65 NFκB pathway. Therefore my data suggest a possible mechanism for the differences in the functional effects mediated by the two receptors.

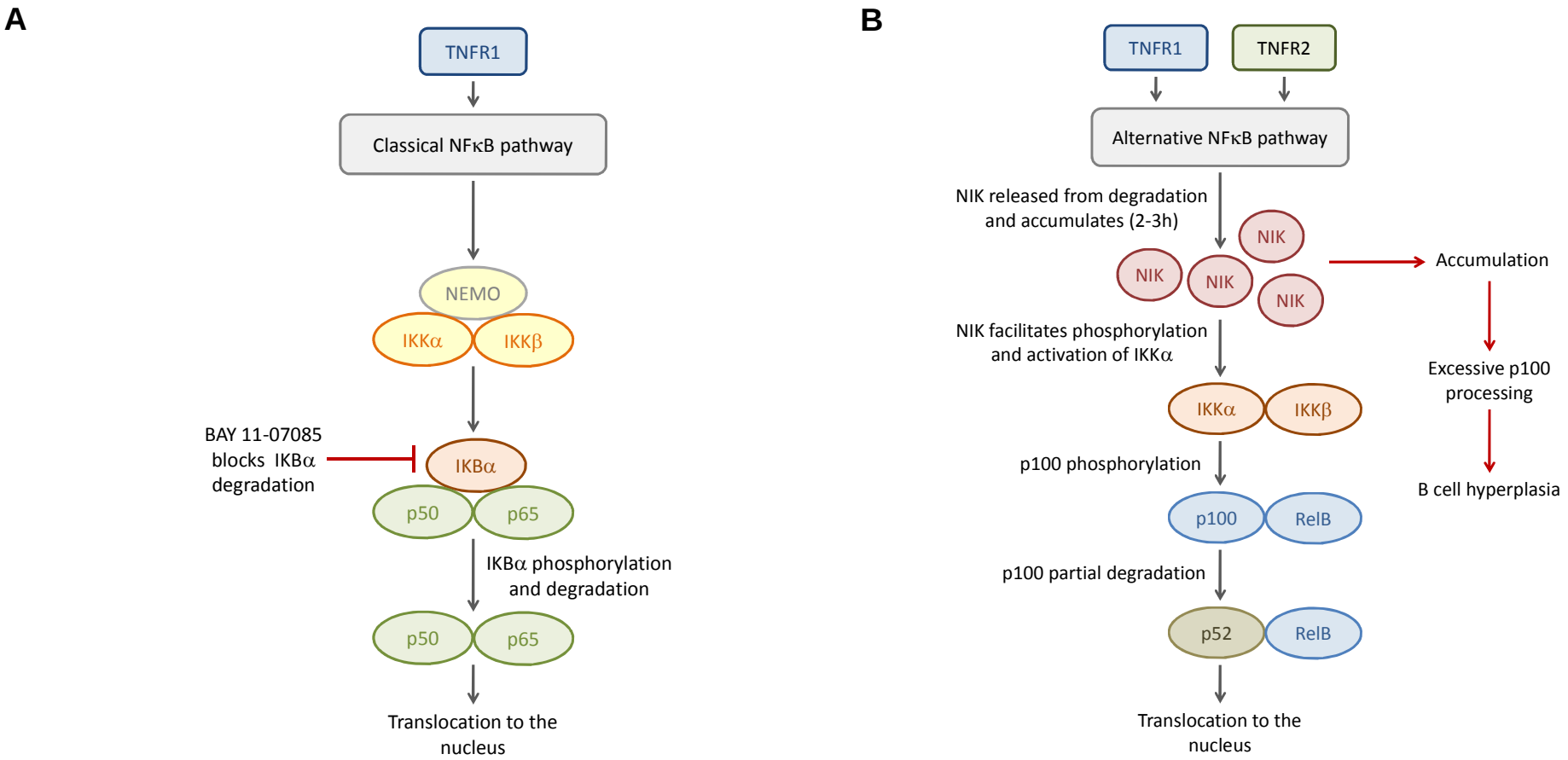


**Figure 40: Summary of the classical and alternative NFκB pathways.** *(A) TNFR1 but not TNFR2 activates the classical p65 NFκB pathway in moDC and this effect can be blocked by the use of Bay 11-07085 which prevents the degradation of IKBα and therefore blocks the subsequent activation and translocation of p65. (B) Both TNFR1- and TNFR2-mediated signalling activates the alternative NFκB pathway in moDC. This involves the accumulation of NIK (an excess of which is associated with B cell hyperplasia), phosphorylation of p100 and subsequent p100 processing to form p52 which then translocates to the nucleus.*

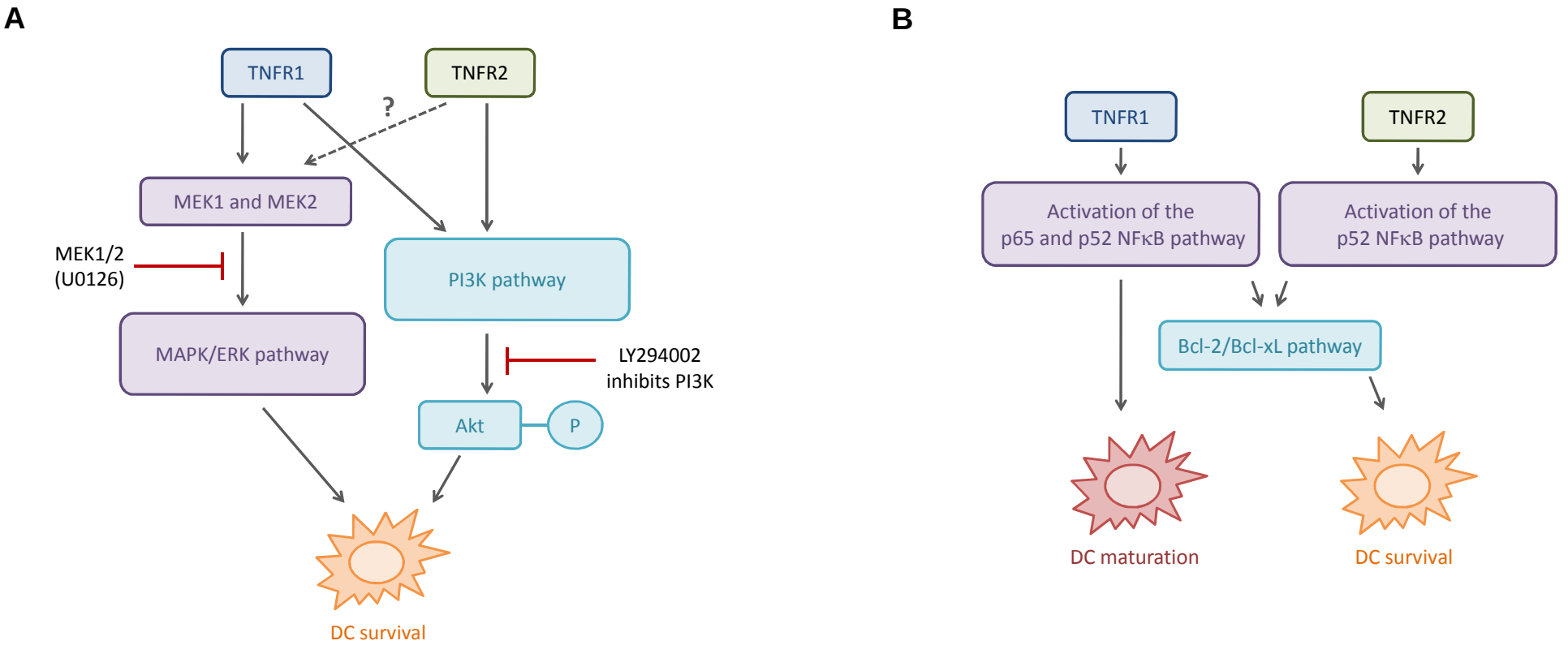


**Figure 41: Summary of signalling pathway inhibitor studies and NFκB activation studies in moDC.** *(A) Small molecule inhibitors were used to block the PI3K and the MAPK/ERK pathways (U0126 and LY294002, respectively). U0126 inhibits MEK1 and MEK2 kinase activity whereas LY294002 inhibits the phosphorylation of Akt. TNFR1 activates the MAPK/ERK pathway and the PI3K pathway. TNFR2 activates the MAPK/ERK pathway and potentially activates the PI3K pathway. Data summarised from Figure 39 (C and D). (B) TNFR1 activates both the p65 and p52 NFκB pathways whereas TNFR2 only activates the p52 NFκB pathway. Only TNFR1 leads to moDC maturation. Both TNFR1 and TNFR2 lead to moDC survival via the Bcl-2/Bcl-xL pathway. Data summarised from Figure 12, Figure 15 and Figure 38.*

# 5. *Ex vivo* analysis of myeloid DC

## *5.1. Introduction*

As discussed in the previous two chapters, the capacity of DC to regulate adaptive immunity is controlled by both their maturation state and their life span. DC are heterogeneous and the phenotype of different DC subsets plays an important role in T cell activation, polarisation and the induction of tolerance (Collin et al. 2013). DC can be divided into subsets based on location and surface phenotype (chapter 1.1.1; Figure 1). In humans two of the major subsets are myeloid DC ($CD11c^+CD1c^+$ or $CD11c^+CD141^+$) and plasmacytoid (p)DC ($CD123^+$BDCA-$2^+$BDCA-$4^+$), which exert different functions within the immune system. For example unlike the majority of DC, pDC are less efficient at antigen presentation, have lower MHC II expression and are B220/$CD45R^+$ (CD45R (including the B220 isoform) is the receptor for CD45, a lymphocyte common antigen, expressed on all leukocytes and is essential for T cell activation via the TCR). In addition, the lifespan of different DC populations varies along with their expression of apoptosis signalling molecules, with myeloid DC having a shorter lifespan than pDC (Kamath et al. 2002; Chen et al. 2007).

MoDC are regarded as a model for inflammatory DC i.e. DC derived from monocytes under inflammatory conditions (as reviewed in mice (Shortman & Naik 2007) and humans (Segura & Amigorena 2013)). Inflammatory DC are not normally found in healthy tissue but thought to be derived from monocytes during cases of chronic inflammation such as RA. Therefore, moDC do not necessarily represent DC from a healthy, steady-state environment but may reflect inflammatory conditions. $CD11c^+$/$CD1c^+$ myeloid DC isolated directly from peripheral blood (hereafter referred to as blood myeloid DC), have been used in this study in order to examine whether the TNF-mediated response of steady state DC differs to that of inflammatory/moDC. As previous research suggests that *in vivo* monocyte-derived DC pay little contribution to steady state DC I investigated blood myeloid DC isolated from healthy donors. In addition, I obtained 2 synovial fluid (SF) samples from RA patients in order to determine whether DC from a chronic inflammatory environment would have a phenotype comparable to the moDC inflammatory model or the steady state blood myeloid DC.

### *5.1.1. Specific aims*

1) Determine the involvement of TNFR-mediated signalling in the maturation of blood myeloid DC.
2) Investigate the individual roles of TNFR1- and TNFR2-mediated signalling in blood myeloid DC survival.
3) Determine the effect of TNFR1 and TNFR2 on the maturation and survival of myeloid DC isolated from synovial fluid.

### *5.1.2. Experimental Approach*

In order to further dissect the role of the two TNFR in regulating the function of human DC, freshly isolated steady-state blood myeloid DC were used. Blood myeloid DC were separated from PBMC by immuno-magnetic negative selection. Immediately after purification, blood myeloid DC were treated with sTNF, $CysTNF_{wt}$ or the TNFR-selective variants $CysTNF_{R1}$ and $CysTNF_{R2}$ plus MAb 80M2 (as done previously with moDC). As blood myeloid DC are present at extremely low concentrations in peripheral blood (0.6-1.8% of PBMC), flow cytometry was used to analyse the expression of surface markers characteristic of DC maturation and intracellular active caspase-3, which indicates cell death. The purity of the blood myeloid DC was routinely analysed (blood myeloid DC were defined as $CD11c^+/CD1c^+$) and populations with a purity of ≤ 70% were not used in future experiments. Whole PBMC were analysed by flow cytometry and pDC were defined as $DAPI^-/CD45^+/CD19^-/CD20^-/CD3^-/CD56^-$ and HLA-$DR^+/CD1c^-/CD123^+$.

Synovial fluid samples were obtained from patients with chronic arthritis and myeloid DC were again enriched using negative selection. SF myeloid DC were used as they are DC from a chronic inflammatory environment and therefore may be similar to ‘inflammatory’ moDC and behave in a different manner to ‘steady-state’ blood myeloid DC.

## *5.2. Results*

### *5.2.1. Purity and characterisation of blood myeloid DC*

PBMC were analysed for the surface expression of lineage markers and were comprised of; 13% $CD14^+$ for monocytes (as used in moDC experiments), 62% $CD3^+$ for T cells, 9% $CD19^+$ for B cells, 3% $CD1c^+$ indicates myeloid DC but is also expressed on subpopulations of B cells, 22% $CD11c^+$ for all myeloid cells (including DC and monocytes) and 2% $CD1c^+/CD11c^+$ for myeloid DC (Figure 42). The percentages of each cell type are within the expected range for PBMC except T cells which are slightly lower than expected in this donor; 10-30% monocytes, 70-80% $CD3^+$ T cells, 5-20% B cells, 5-20% NK cells and 1-2% DC. My results show that PBMC contain clear populations of each of these cell types some of which can also be identified by size (using FSC) and granularity (using SSC) (Figure 42). Blood myeloid DC ($CD1c^+/CD11c^+$) were enriched by negative selection (Figure 43) resulting generally in a population of 75-90% purity. On average 245 x $10^6$ PBMC were used resulting in an average of 1.05 x $10^6$ blood myeloid DC (0.4% yield). As PBMC are generally comprised of only 0.6-2% DC this shows around a 66-fold enrichment.

As the blood myeloid DC were generally 75-85% pure, the expression of T and B cell lineage surface markers was also analysed which shows ~3% T cell and ~1% B cell contamination (Figure 44). This is important to take into account as for example, the maturation of human DC can also be negatively regulated by $CD19^+$ B cells (Morva et al. 2012). Although other contaminating cells were not analysed they may include monocytes and NK cells. For all future experiments these contaminating cells were gated out during analysis, however their effect on the survival and maturation of blood myeloid DC during culture could not be evaluated at this stage.

I previously confirmed that both TNFR1 and TNFR2 are expressed on moDC (chapter 3.4.3, Figure 9). Flow cytometry was used to detect TNFR expression on the surface of peripheral blood myeloid cells (Figure 45). Both TNFR1 and TNFR2 are expressed on blood myeloid DC ($CD1c^+/CD11c^+$) following their enrichment from PBMC as described previously (Figure 45 (A)). Whole PBMC were also analysed for by flow cytometry and pDC were defined by surface marker expression (defined within PBMC as $DAPI^-/CD19^-/CD20^-/CD3^-/CD56^-/CD1c^-$ and $CD45^+/HLA\text{-}DR^+/CD123^+$) Figure 45 (B). Briefly, dead cells were excluded by DAPI positivity (a viability dye) and live leukocytes were identified as $CD45^+$ cells (lymphocyte common antigen). B cells ($CD19^+$ or $CD20^+$), T cells ($CD3^+$) and NK ($CD56^+$) cells were also excluded. Following this gating strategy, pDC were identified as $HLA\text{-}DR^+/CD1c^-/CD123^+$, and showed expression of both TNFR1 and TNFR2 (Figure 45 (B)). TNFR1 and TNFR2 were detected using H398 and

22221 (both $IgG_{2A}$ antibodies) respectively, and the same biotin and streptavidin conjugated antibodies. As different primary antibodies are used to detect each receptor it is difficult to accurately compare their relative amounts, however as both primary antibodies are $IgG_{2A}$ and saturating antibody concentrations were added the relative amounts of TNFR1 and TNFR2 can be generally compared. Results therefore show similar levels of TNFR1 and TNFR2 expression in blood myeloid DC, but higher levels of TNFR2 than TNFR1 expression in pDC. The number of donors and the MFI of TNFR1 and TNFR2 expression on each subset of DC is shown in Table 3.

| | Immature moDC | Blood myeloid DC | pDC |
|---|---|---|---|
| **TNFR1** | 2735 ± 610.9 n=3 | 3167 ± 1137 n=5 | 411.5 ± 316 n=5 |
| **TNFR2** | 8115 ± 1867 n=3 | 660 ± 651.5 n=5 | 2899 ± 1620 n=5 |

*Table 3: TNFR expression on moDC, $CD1c^+$ blood myeloid DC and $CD123^+$ pDC.*

### *5.2.2. TNFR1 and TNFR2 enhance the maturation of blood myeloid DC*

As shown in chapter 3.4.6 the maturation of DC can be characterised by an increase in the expression of surface molecules such as the co-stimulatory molecule CD86. Myeloid DC are CD1c positive therefore any CD1c negative cells were excluded, although there may still be a low amount of $CD1c^+$ B cell contamination (probably around 1%). The maturation markers CD83 and CD86 within the CD1c positive/caspase-3 negative populations were analysed (thereby excluding most of the dead or dying cells). Results show that within 24 hours of stimulation both TNFR1- or TNFR2-mediated signalling induced the expression of HLA-DR, CD83 and CD86 (Figure 46 (A)). Additionally, the expression of PD-L1 and ICAM-1 was increased by $CysTNF_{wt}$ which can activate both TNFR1 and TNFR2 (Figure 46 (B)). Time course analysis at 24 h and 48 h revealed that the enhanced expression of CD83 and CD86 following TNFR1- or TNFR2-mediated signalling is significant after 24 h (Figure 47 (A and B), respectively). Although sTNF, $CysTNF_{wt}$, $CysTNF_{R1}$ and $CysTNF_{R2}$ plus 80M2 still induce significant effects after 48 h, CD83 expression is much weaker (according to the MFI) than at 24 h and $CysTNF_{R2}$ plus 80M2 no longer has a significant effect on CD86 (Figure 47 (C and D)). Data in Figure 47 is summarised in Table 4. The difference between sTNF/$CysTNF_{wt}$ and the TNFR-selective ligands may be due to differences in the half life of the ligand/TNFR complexes and/or in their capability to induce TNFR internalisation, which might be affected by ligand oligomerisation (all TNF ligands except sTNF contain a free Cystein residue at their N-terminus) and point

mutations ($CysTNF_{R1}$ and $CysTNF_{R2}$, but not sTNF and $CysTNF_{wt}$ contain two point mutations per TNF monomer) in the ligands, whereas the difference between 24 h and 48 h may be due to an increase in the amount of dying cells. Figure 48 (A-D) shows that $CysTNF_{R2}$ alone and 80M2 alone have no effect on CD83 (A and C) or CD86 (B and D) expression at 24 h (A and B) or 48 h (B and D). In addition, LPS has a similar effect on CD83 as $CysTNF_{wt}$, and a greater effect on CD86 than any of the ligands.

| | CD83 24 h n=6 | CD83 48 h n=7 | CD86 24 h n=4 | CD86 48 h n=4 |
|---|---|---|---|---|
| **Unstimulated** | 86.7 ± 12.8 | 198.3 ± 18.8 | 175.1 ± 13.1 | 235 ± 25.6 |
| **sTNF** | 2485 ± 450.7 | 1324 ± 311.9 | 673.4 ± 54.2 | 776.3 ± 171.7 |
| **$CysTNF_{wt}$** | 3200 ± 396 | 2251 ± 423.3 | 823.4 ± 48.4 | 1879 ± 305.7 |
| **$CysTNF_{R1}$** | 1045 ± 322 | 404 ± 19.5 | 534.7 ± 47.9 | 299.3 ± 28.4 |
| **$CysTNF_{R2}$ + 80M2** | 509.7 ± 159.6 | 318.5 ± 23.9 | 413 ± 40.8 | 344.3 ± 49.6 |

*Table 4: CD83 and CD86 expression on blood myeloid DC following TNFR stimulation.*

To investigate whether the increase in maturation marker expression is mediated through TNFR1, TNFR2 or through co-operation between the two receptors (e.g. the production of mTNF via TNFR1-signalling may activate TNFR2 or TNFR2-mediated sTNF production may activate TNFR1), I used TNFR-specific antagonistic antibodies (as also used in chapter 4.4.2). Freshly isolated blood myeloid DC were treated with antagonistic TNFR1- or TNFR2-specific antibodies (10 μg/ml H398 or 22221, respectively) for 30 min prior to TNFR-stimulation. Firstly, I confirmed that neither of the TNFR antagonists had any effect on blood myeloid DC survival when used alone as compared to unstimulated cells (Figure 49). The anti-TNFR1 antibody completely blocked the $CysTNF_{R1}$-mediated survival of blood myeloid DC but did not affect the response to $CysTNF_{R2}$ + 80M2, indicating that TNFR2 acts in a TNFR1-independent manner (Figure 49). Alternatively, the anti-TNFR2 antibody inhibited the response to $CysTNF_{R2}$ plus MAb 80M2 but had no effect on the TNFR1-mediated response (Figure 49). It also confirmed that sTNF acts through TNFR1 as the pro-maturation effect of sTNF was blocked by anti-TNFR1 but not anti-TNFR2, whereas the effect of $CysTNF_{wt}$ was only partially blocked by antagonising either TNFR1 or TNFR2, indicating that mTNF enhances blood myeloid DC survival through both TNFR1- and TNFR2-mediated signalling. Results shown in Figure 49 are divided into corresponding donors and shown in Appendix B.

I also determined cytokine production as an indicator of DC maturation, as done previously with moDC (chapter 3.4.6). Supernatants from cell cultures were analysed for production of IL-6 and IL-1β (Figure 50), which are both known mediators of inflammation. None of the TNF ligands resulted in the production of either IL-6 or IL-1β within 24 h of blood myeloid DC stimulation. Alternatively, LPS-matured blood myeloid produced IL-6 but not IL-1β. Therefore, there is no positive control for DC production of IL-1β, but as the standard for IL-1β was easily detected, I can exclude a technical problem with the ELISA. It is possible that an ELISA is not sensitive enough to detect low levels of cytokine production by the blood myeloid DC.

Results therefore show that in blood myeloid DC both TNFR1 and TNFR2 can enhance the expression of maturation markers but did induce a cytokine response. In line with these results, TNF-stimulation of moDC was also not sufficient to induce a cytokine response (chapter 3.4.6; Figure 25).

### *5.2.3. TNFR2- but not TNFR1-mediated signalling protects blood myeloid DC from cell death*

I have shown in the previous chapter (4.4.1) that moDC undergo cell death within 48 hours of culture after removal of IL-4 and GM-CSF from the moDC cultures, and that the addition of TNF can protect the moDC from cell death. To investigate whether blood myeloid DC die after cytokine withdrawal within a similar time period to moDC, blood myeloid DC were left unstimulated or were treated with sTNF, $CysTNF_{wt}$, $CysTNF_{R1}$ or $CysTNF_{R2}$ plus 80M2 for 24 h and 48 h. As previously, the processed form of caspase-3 was used to assess cell death by flow cytometry and was measured within the CD1c positive population. Figure 51 shows that culture without the addition of cytokines results in the death of 36% of blood myeloid DC within 24 h, which is slightly increased to 38% at 48 h. Furthermore, this cell death can be reduced by the addition of $CysTNF_{wt}$ to 13% at 24 h and 19% at 48 h, and to a lesser extent by sTNF to 28% at 24 h and 33% at 48 h (Figure 51). Cell death can also be reduced by the addition of $CysTNF_{R2}$ plus 80M2 to 24% at 24 h and 25% at 48 h, however, cell death was not reduced by $CysTNF_{R1}$ (38% at 24 h and 39% at 48 h) (Figure 51). As the reduction in blood myeloid cell death was more pronounced at 24 h and the effect was no longer significant art 48 h, the 24 h time point was used in all future experiments.

To study the regulation of blood myeloid DC survival by the individual TNFR, blood myeloid DC were treated with sTNF, $CysTNF_{wt}$, $CysTNF_{R1}$ or $CysTNF_{R2}$ plus 80M2, both with and without TNFR-specific antagonistic antibodies. Stimulation with sTNF and $CysTNF_{wt}$ significantly protected blood myeloid DC from cell death (from 38% active caspase-$3^+$ unstimulated cells to 27% with sTNF or 15% with $CysTNF_{wt}$, (Figure

52 (black circles) and Appendix C1). When compared to that of moDC, the $CysTNF_{wt}$ response is similar but the sTNF response is reduced (from 60% active caspase-$3^+$ in unstimulated moDC to 16% with sTNF or 14% with $CysTNF_{wt}$, chapter 4.4.1 Figure 29). Interestingly, $CysTNF_{R2}$ plus 80M2 significantly protected blood myeloid DC from cytokine deprivation-induced cell death (as shown by the reduction in active caspase-3 from 38% in unstimulated cells to 26% with $CysTNF_{R2}$ plus 80M2), whereas $CysTNF_{R1}$ did not have any effect (Figure 52 (black circles) and Appendix C1). This is in contrast to moDC as both TNFR1- and TNFR2-selective stimulation enhanced survival of moDC (from 60% active caspase-$3^+$ in unstimulated moDC to 35% with $CysTNF_{R1}$ or 44% with $CysTNF_{R2}$ + 80M2, chapter 4.4.1; Figure 29). This suggests that TNFR1 may play a role in DC survival under inflammatory conditions but not in steady state conditions, whereas TNFR2 promotes a similar response on DC survival under both conditions but is more pronounced in steady state conditions.

To identify whether the enhancement of blood myeloid DC survival occurred directly through TNFR2 or whether TNFR1 was also necessary I used TNFR-specific antagonistic antibodies. As in previous experiments I confirmed that neither of the TNFR antagonists alone had any effect on blood myeloid DC survival (Figure 52) indicating that endogenously expressed TNF does not affect the survival of these cells. The pro-survival effect of sTNF was blocked by anti-TNFR1 but not anti-TNFR2 indicating that sTNF acts primarily through TNFR1 in blood myeloid DC. Alternatively the pro-survival effect of $CysTNF_{wt}$ was only partially blocked by either TNFR1 or TNFR2 antagonism indicating that mTNF enhances blood myeloid DC survival through both TNFR1- and TNFR2-mediated signalling (Figure 52). The anti-TNFR2 antibody completely blocked survival mediated by $CysTNF_{R2}$ plus MAb 80M2 whereas the anti-TNFR1 antibody had no effect indicating that TNFR2 acts in a TNFR1-independent manner (Figure 52). Results therefore show that TNFR2- but not TNFR1-selective signalling resulted in enhanced survival in blood myeloid DC. Results in Figure 52 are divided according to corresponding donors in Appendix C2 and C3.

### *5.2.4. Myeloid DC isolation from synovial fluid*

As described previously, moDC are thought to represent an inflammatory cell type, whereas blood myeloid DC represent steady state conditions. In order to investigate this further, I compared these cell types with myeloid DC present during chronic inflammation to identify whether these behave more like moDC. To do this, synovial fluid samples were obtained from two RA patients. PBMC were separated and myeloid DC were enriched as described previously in this chapter. The purity of SF myeloid DC was determined by CD1c and CD11c expression as determined by flow cytometry and

both of the RA patient samples show ~43% purity (Figure 53). Any potential effect of contaminating cells on the maturation and/or survival of myeloid DC cannot be excluded at this time.

The maturation markers CD83, CD86 and HLA-DR within the CD1c positive and caspase-3 negative cell populations were analysed after 24 h in culture with TNF or the TNF ligands. Results in Figure 54 (A) show that sTNF has no effect on maturation whereas there is a slight increase in CD83 and CD86 expression with the addition of $CysTNF_{wt}$. Selective stimulation of TNFR1 or TNFR2 has no effect on maturation of myeloid DC in either of the samples (Figure 54 (A and B)). However the high expression of CD83 and CD86 in unstimulated cells suggests that these cells have, to some extent, already matured. All of the cells are HLA-DR positive which is to be expected as all myeloid DC express HLA-DR. As they originate from the synovial fluid of RA patients it is not unexpected that they will already be mature prior to TNF treatment due to the chronic inflammatory environment in the RA joint. This is confirmed by other groups who showed that synovial DC are more mature (in terms of activation markers and cytokine production) than DC from peripheral blood (Page et al. 2002; Radstake et al. 2004). An ELISA was also conducted on supernatants from the TNF-treated myeloid DC used in (Figure 54(A)) but no IL-6 or IL-1$\beta$ was detected (data not shown). This may indicate that the SF myeloid DC are not fully mature however, it is also possible that the DC are exhausted/paralysed which has previously been described in relation to IL-12 and LPS in human moDC (Langenkamp et al. 2000) and murine DC (Reis e Sousa et al. 1999).

Myeloid DC isolated from the synovial fluid of the two RA patients used in Figure 54 were also analysed for the expression of intracellular active caspase-3 to indicate the level of cell death (Figure 55). After 24 h unstimulated cells show 49% and 26% (donor A and donor B, respectively) active caspase-3 positive cells (Figure 55). This is reduced by $CysTNF_{wt}$ (49% active caspase-$3^+$ cells to 23% in donor A) and to a lesser degree by sTNF (49% active caspase-$3^+$ cells to 31% in donor A). Additionally, $CysTNF_{R1}$ had no effect in cells from either donor whereas $CysTNF_{R2}$ + 80M2 enhanced myeloid DC survival from 49% active caspase-$3^+$ cells to 29% in donor A and 26% to 19% in donor B (Figure 55 (A-C)). These data are in contrast to results in moDC where both TNFR1- and TNFR2-mediated signalling resulted in enhanced survival (chapter 4.4.1; Figure 29), but support results in blood myeloid DC where TNFR2- but not TNFR1-mediated signalling enhanced survival (Figure 52).

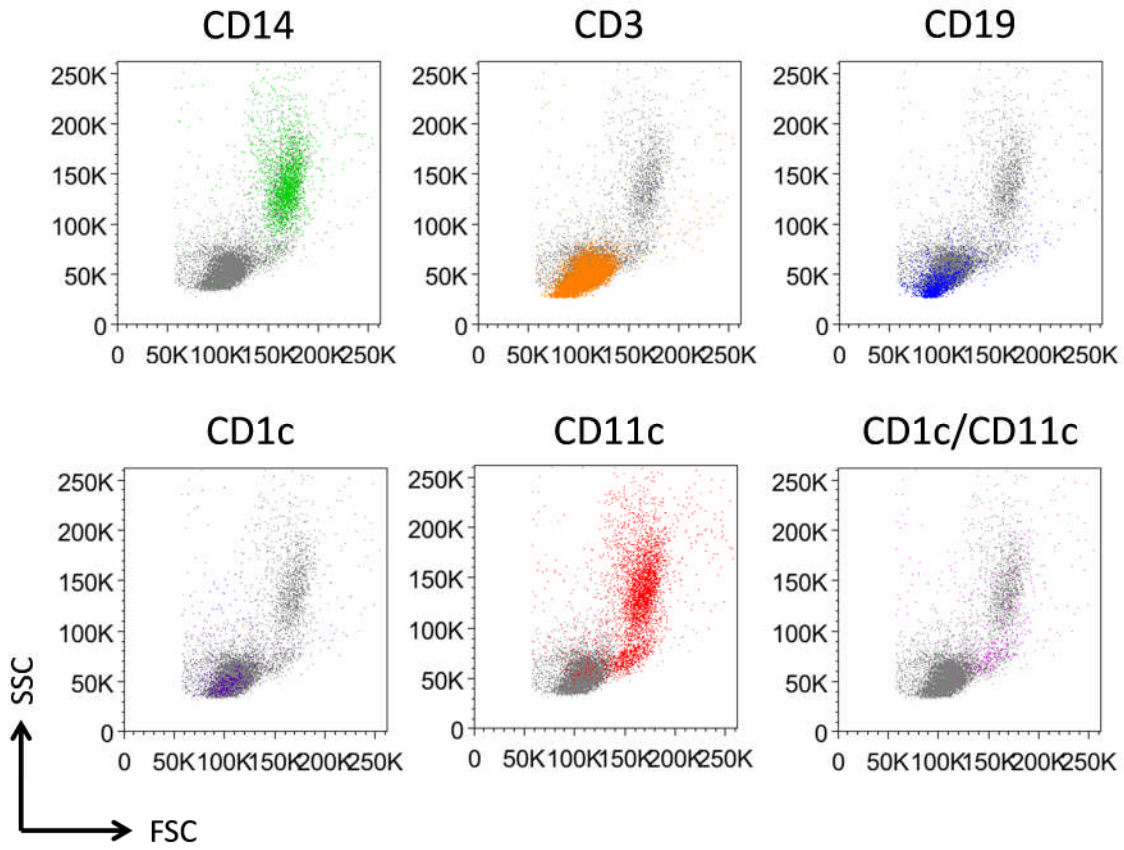


**Figure 42: Analysis of size/granularity of distinct cell populations within human PBMC.** *PBMC were isolated from peripheral whole blood and analysed by flow cytometry. Cells positive for the cell surface markers indicated were back-gated using FlowJo analysis software resulting in the visualisation of monocytes (CD14, green), T cells (CD3, orange), B cells (CD19, blue), myeloid DC and some B cells (CD1c, purple), myeloid cells (CD11c, red) and myeloid DC (CD1c/CD11c, pink) within the PBMC population (grey). Results show side scatter (y axis, representing granularity) versus forward scatter (x axis, indicating size) and represent at least three independent experiments with different donors.*

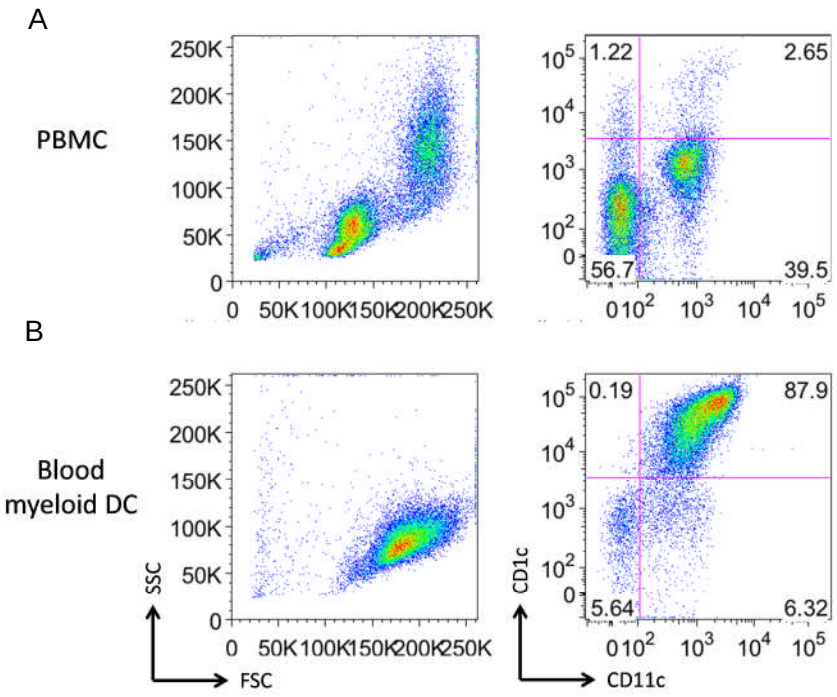


**Figure 43: Enrichment and purity of freshly isolated blood myeloid DC.** *PBMC (A) were isolated from peripheral whole blood or blood myeloid DC (B) were separated by negative selection (EasySep human myeloid DC enrichment kit; StemCell). The left column shows size/granularity, whereas the right column shows the purity of blood myeloid DC as determined by CD1c and CD11c expression after the exclusion of dead cells analysed by flow cytometry. Results represent at least 6 independent experiments with different donors.*

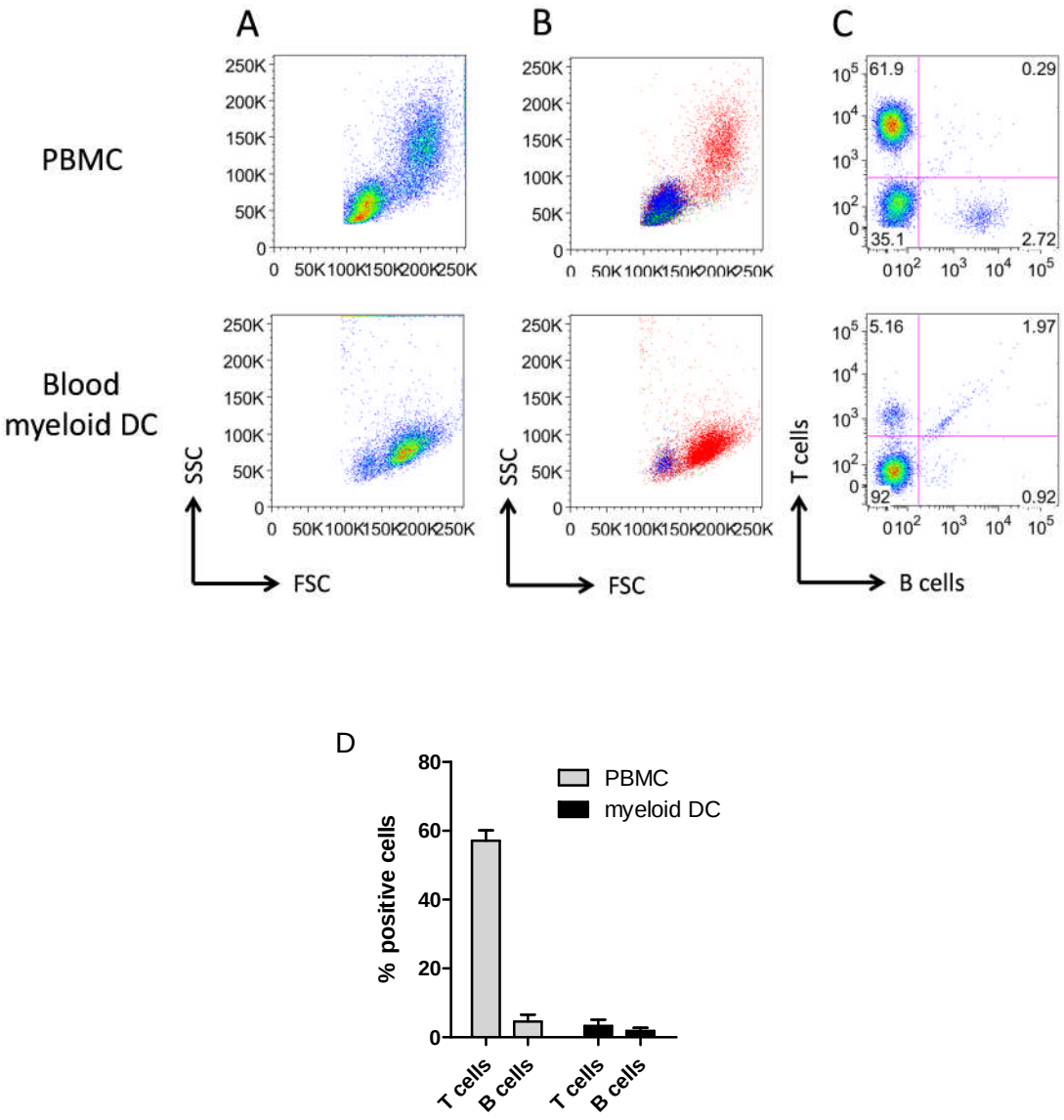


**Figure 44: Identification of cells contaminating the blood myeloid DC fraction.** *PBMC were isolated from peripheral whole blood and blood myeloid DC were enriched by negative selection (EasySep human myeloid DC enrichment kit; StemCell). Columns A and B show SSC (y axis, representing granularity) versus FSC (x axis, indicating size), column B indicates T cells (CD3, blue) and B cells (CD19, green) analysed within the PBMC or blood myeloid DC populations (red). Column (C) and histogram (D) show the percentage of B and T cells. Results represent (A-C) one and (D) three independent experiments with different donors.*

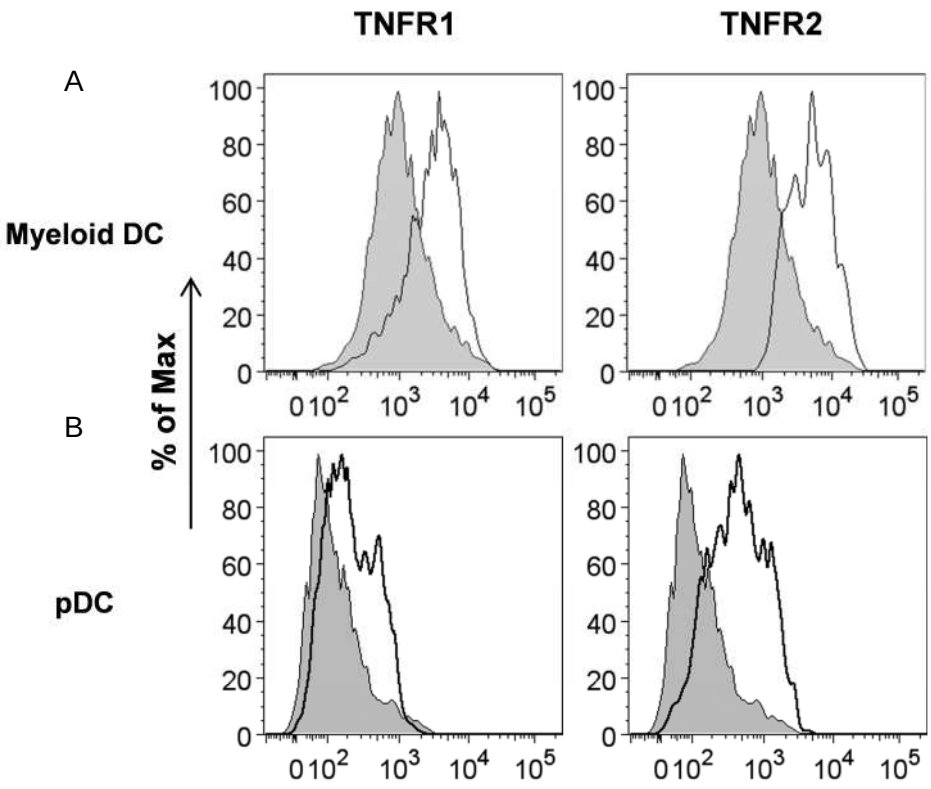


**Figure 45: Expression of TNFR1 and TNFR2 on the cell surface of myeloid cells.** *Surface expression of TNFR1 and TNFR2 on different cell populations within PBMC. (A) Freshly isolated CD1c$^+$/CD11c$^+$ blood myeloid DC (EasySep human myeloid DC enrichment kit; StemCell) and (B) plasmacytoid DC (defined within PBMC as DAPI$^-$/CD19$^-$/CD20$^-$/CD3$^-$/CD56$^-$/CD1c$^-$ and CD45$^+$/HLA-DR$^+$/CD123$^+$) were analysed by flow cytometry. TNFR were detected using a biotin/streptavidin-based amplification protocol. TNFR1-specific antibodies (H398; left, black line) or TNFR2-specific antibodies (22221; right, black line) are compared with an IgG$_{2A}$ isotype control (grey shaded). Data are representative of at least five independent donors.*

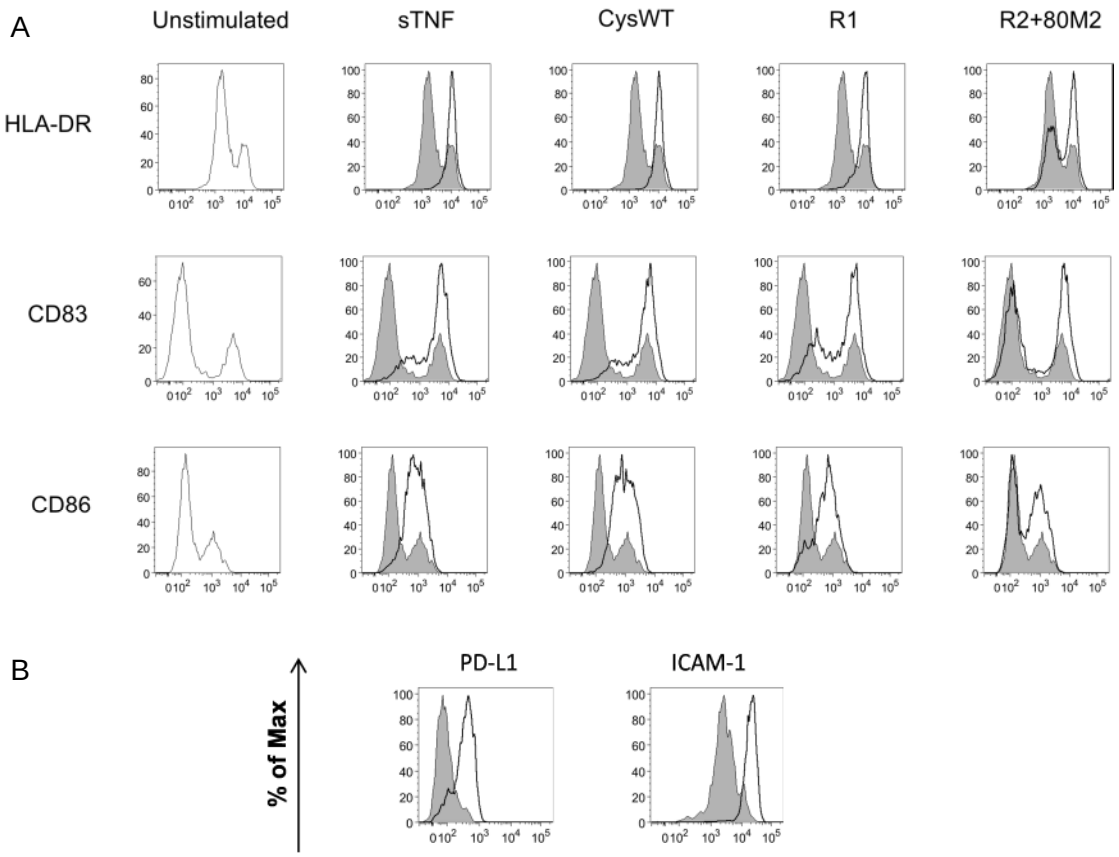


**Figure 46: TNFR1- and TNFR2-mediated signalling results in enhanced expression of maturation markers in blood myeloid DC.** *Freshly isolated blood myeloid DC were treated for 24 h as indicated with (A) sTNF, CysTNF$_{wt}$ or TNFR-selective ligands (50 ng/ml each) +/- MAb 80M2 (2 μg/ml) or (B) CysTNF$_{wt}$ (50 ng/ml). The expression of (A) CD83, CD86, HLA-DR and (B) PD-L1 and ICAM-1 was determined by flow cytometry. Unstimulated cells (grey shaded) were compared to TNF-stimulated cells (black line). Results represent (A) from 3 to 7 independent donors or (B) one donor.*

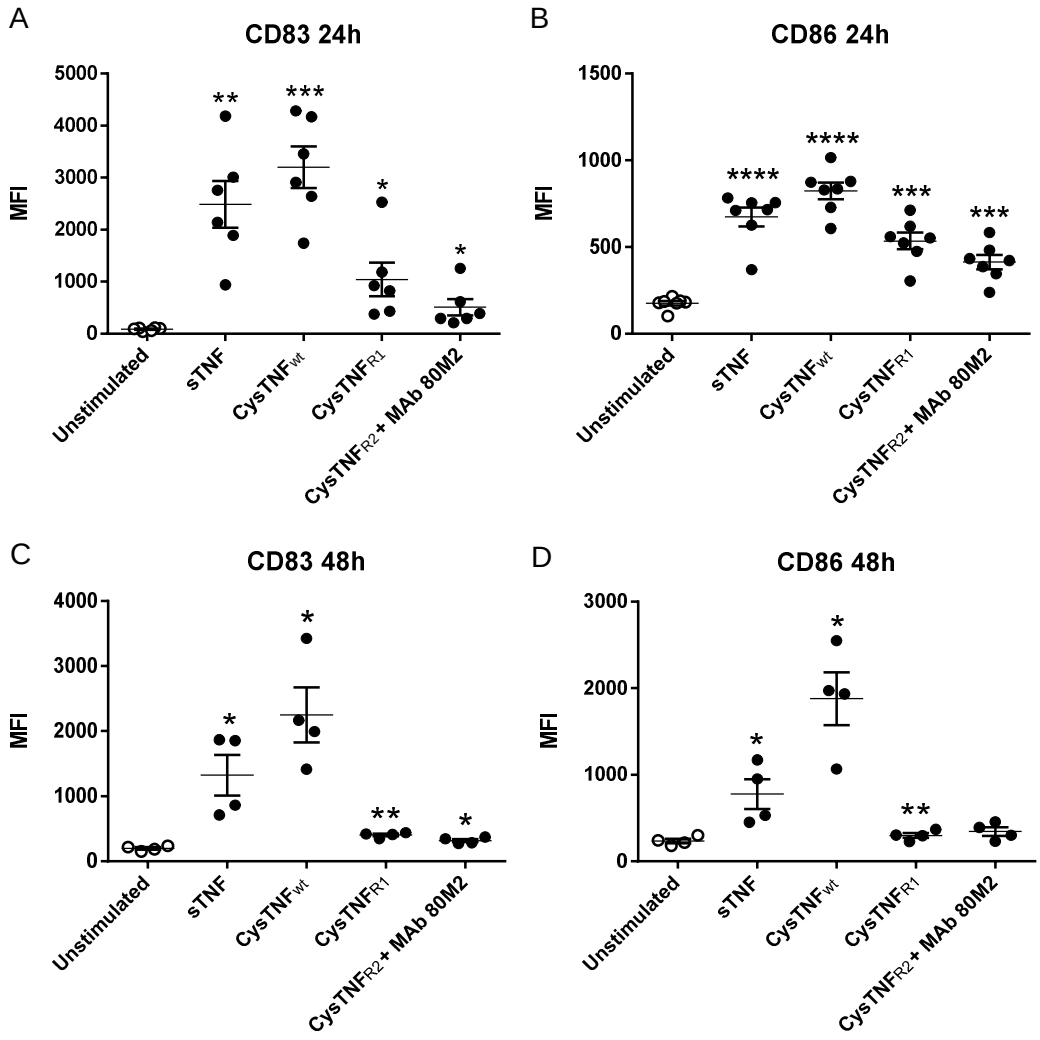


**Figure 47: TNFR1- and TNFR2-mediated signalling in blood myeloid DC enhanced the expression of maturation associated markers after 24 and 48 h.** *Freshly isolated blood myeloid DC were treated as indicated with sTNF, CysTNF$_{wt}$ or TNFR-selective ligands (50 ng/ml each) +/- MAb 80M2 (2 μg/ml) for 24 h (A, B) or 48 h (C, D). The expression of CD83 (A, C) and CD86 (B, D) was determined by flow cytometry. (A-D) The mean and SEM of the MFI (median fluorescence intensity) from 6 (A), 7 (B) or 4 (C, D) independent donors are shown. Statistical differences were determined in relation to unstimulated blood myeloid DC using a student's t test; *P≤0.05 **P≤0.01 ***P≤0.001 ****P≤0.0001.*

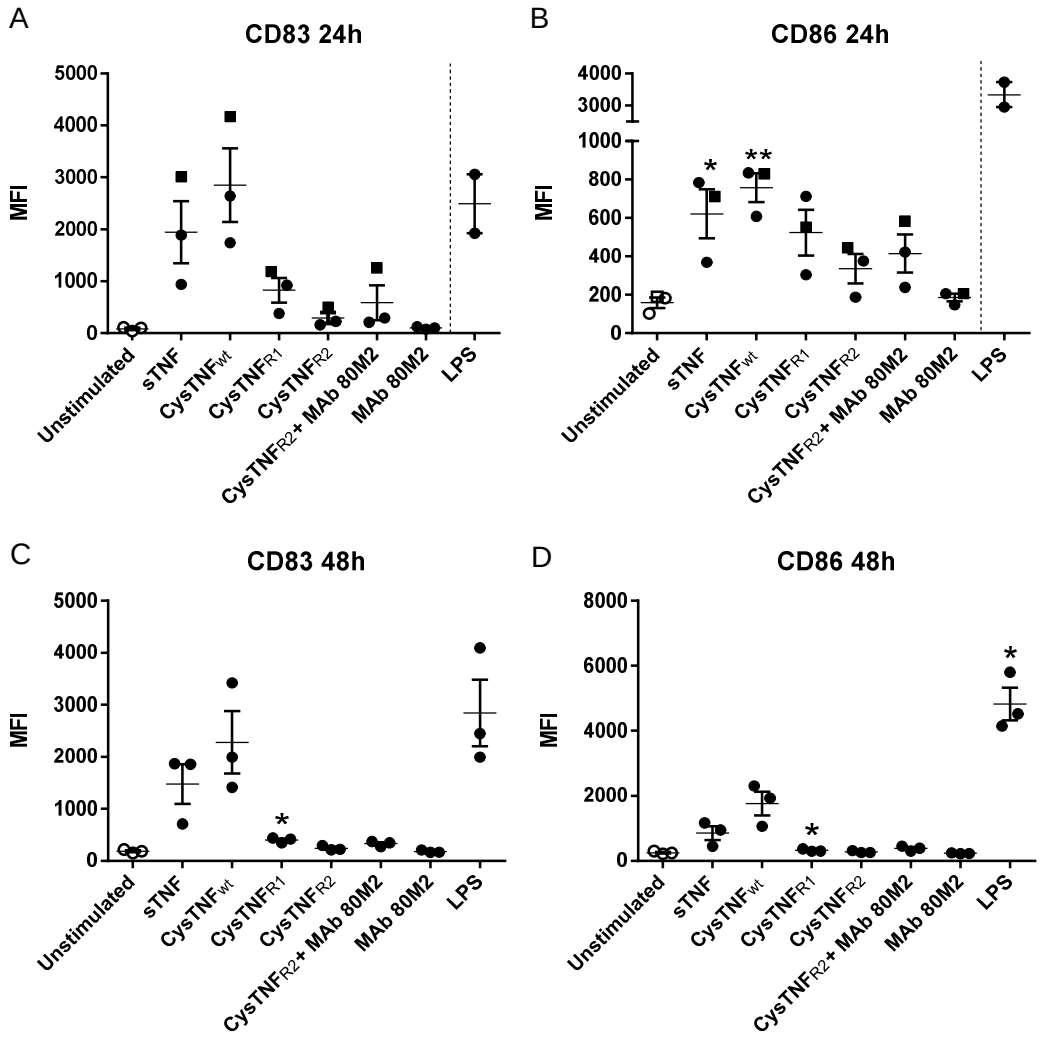


**Figure 48: TNFR1- and TNFR2-mediated blood myeloid DC maturation after 24 h and 48 h of stimulation.** *Freshly isolated blood myeloid DC were treated as indicated with LPS (100 ng/ml), sTNF, CysTNF$_{wt}$ or TNFR-selective ligands (50 ng/ml each) +/- MAb 80M2 (2 μg/ml) for 24 h (A, B) or 48 h (C, D). The expression of CD83 (A, C) and CD86 (B, D) was determined by flow cytometry. The mean and SEM of the MFI (median fluorescence intensity) from 3 (A-D) independent donors are shown except LPS (A, B) which shows 2 independent donors (separated by a dashed line). (A, B) The donor lacking LPS stimulation is indicated by a black square. Statistical differences were determined in relation to unstimulated blood myeloid DC using a student's t test; *P≤0.05 **P≤0.01 ***P≤0.001.*

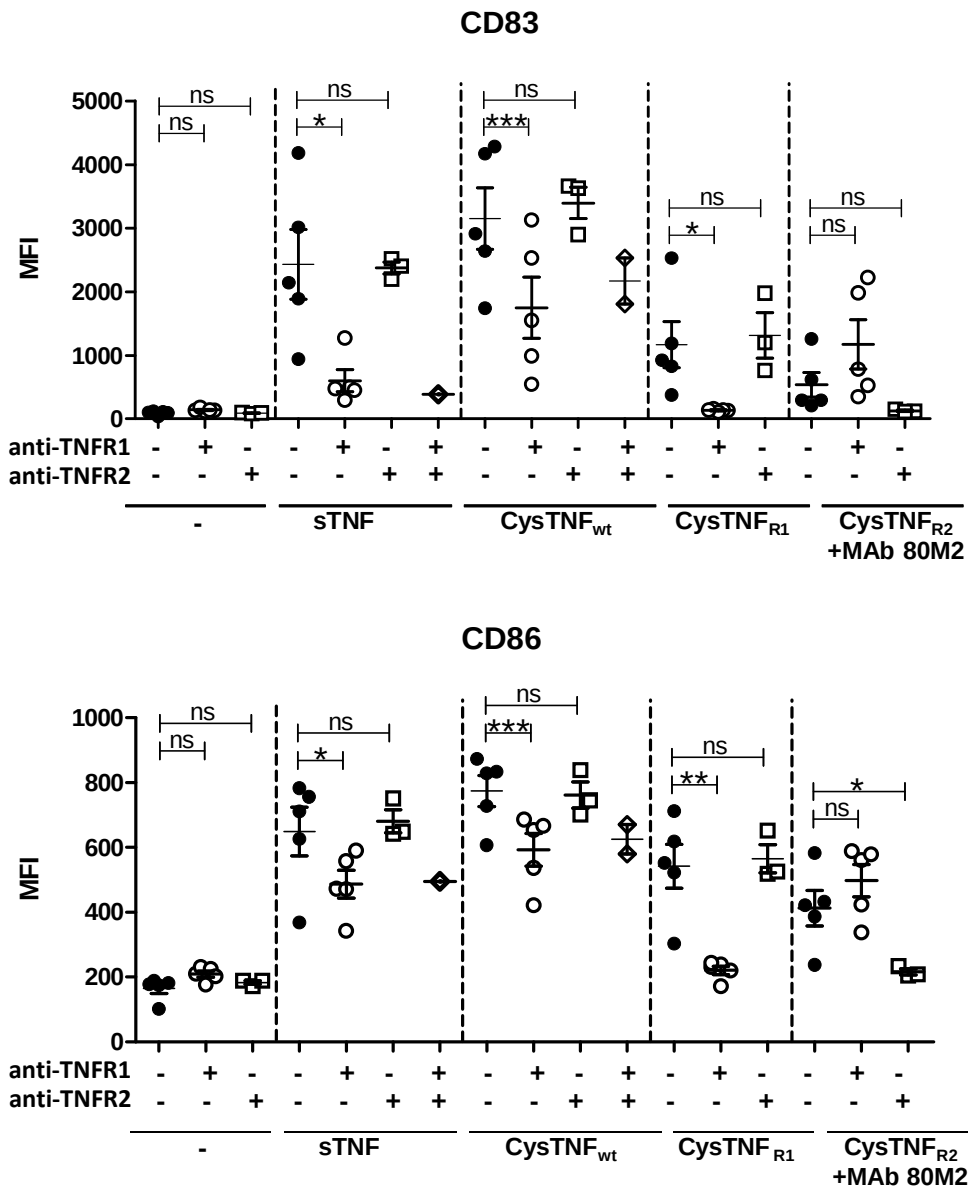


**Figure 49: TNFR-antagonism blocks TNFR-mediated enhancement of maturation marker expression induced by the corresponding TNFR but does not affect signalling via the other receptor.** *Blood myeloid DC were left untreated or were treated with antagonistic TNFR1- or TNFR2-specific antibodies for 30 min prior to stimulation with 50 ng/ml sTNF, CysTNF$_{wt}$, CysTNF$_{R1}$ or CysTNF$_{R2}$ + MAb 80M2 for 24 h. The expression of CD83 (top) and CD86 (bottom) was determined by flow cytometry. The mean and SEM of the MFI (median fluorescence intensity) are shown. Data from Figure 47 (A, B) is included. Statistical differences were determined in relation to unstimulated blood myeloid DC using a student's t test; *P≤0.05 **P≤0.01 ***P≤0.001.*

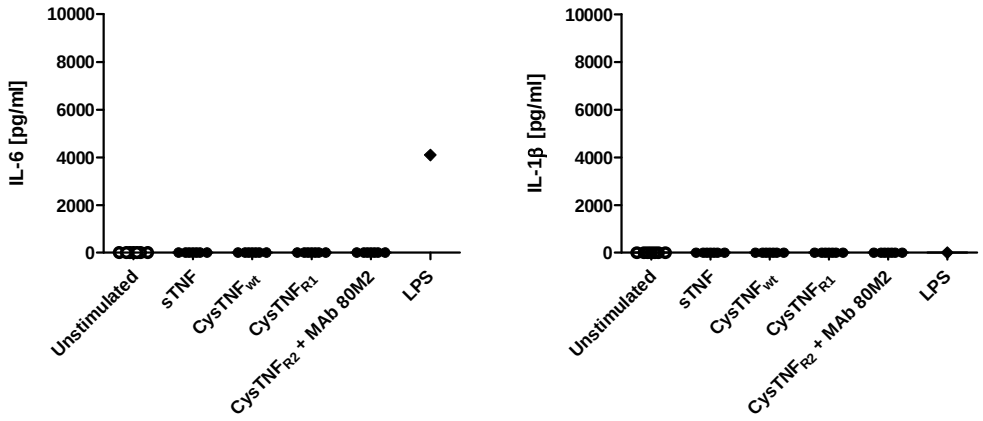


**Figure 50: Blood myeloid DC do not produce IL-6 or IL1β in response to TNF.** *Blood myeloid DC were treated as indicated with LPS (100 ng/ml), sTNF, $CysTNF_{wt}$ or TNFR-selective ligands (50 ng/ml) +/- MAb 80M2 (2 μg/ml) for 24 h. Supernatants were harvested and cytokine concentrations (IL-6 and IL-1β) were determined by sandwich ELISA. Results represent 7 independent donors except LPS which shows one donor only.*

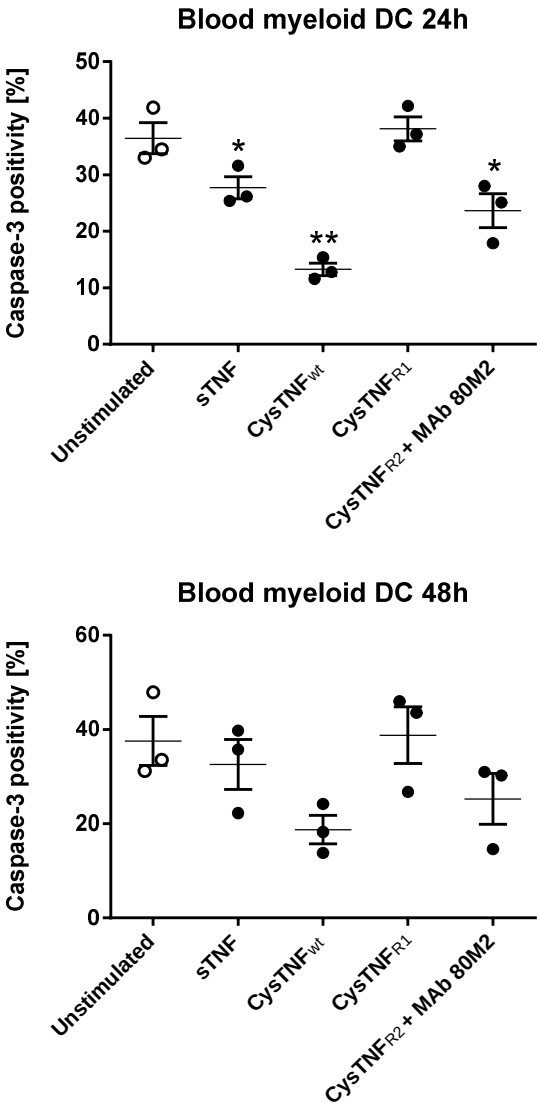


**Figure 51: Both sTNF and CysTNFwt protect blood myeloid DC from cytokine deprivation-induced cell death.** *Blood myeloid DC were cultured in the presence or absence of sTNF or CysTNF$_{wt}$ (50 ng/ml) for 24 or 48 h. The induction of cell death was assessed by the presence of intracellular active caspase-3 determined by flow cytometry. The percentages of cells gated positive for active caspase-3 are indicated. Results represent 3 or 4 independent experiments using different donors.*

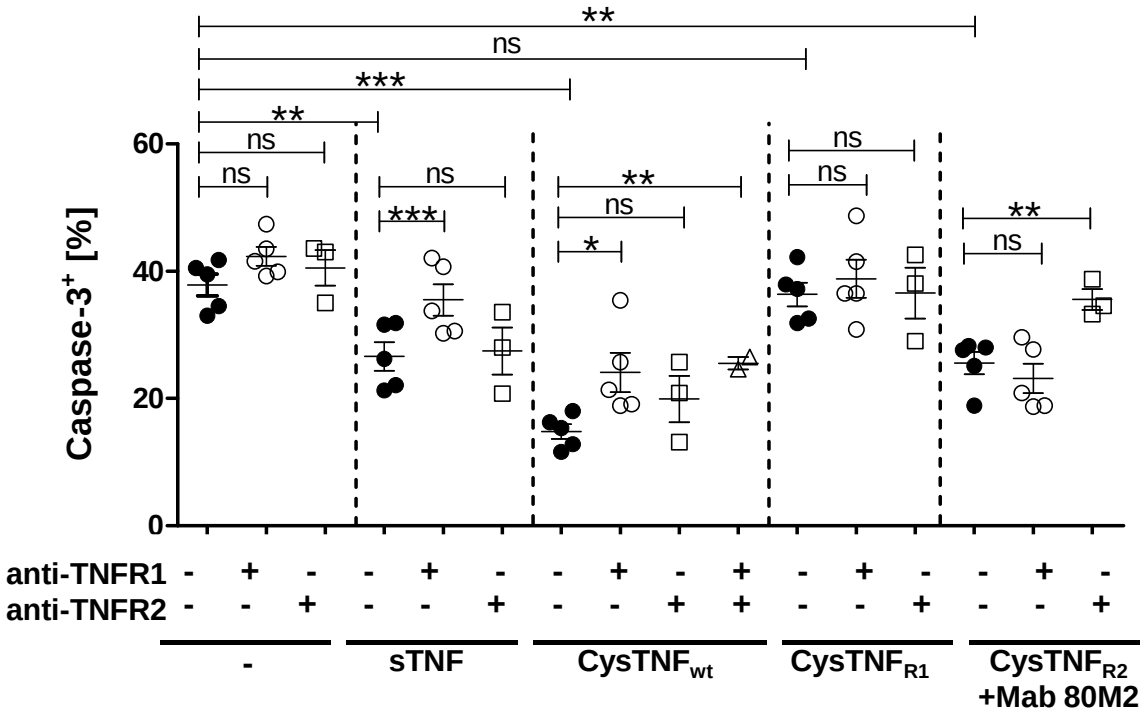


**Figure 52: TNFR2- but not TNFR1-mediated signalling protects blood myeloid DC from cytokine deprivation-induced cell death.** *Blood myeloid DC were cultured in the presence or absence of 10 μg/ml antagonistic TNFR1- or TNFR2-specific antibodies (H398 or 22221 respectively) for 30 min prior to stimulation with 50 ng/ml sTNF, CysTNF$_{R1}$ or CysTNF$_{R2}$ + MAb 80M2 for 24 h. Cell viability was assessed by the presence of intracellular active caspase-3 determined by flow cytometry. The percentages of cells gated positive for caspase-3 are indicated. The mean and SEM are shown for a minimum of 3 independent experiments with different donors. Statistical differences were determined using a student's t test; *P≤0.05 **P≤0.01 ***P≤0.001.*

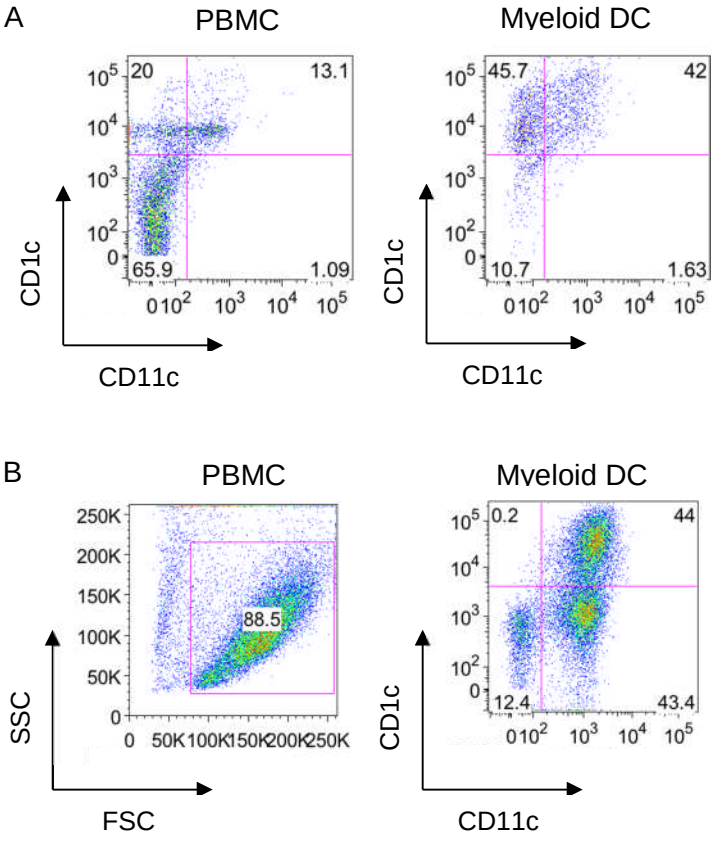


**Figure 53: Purity of myeloid DC isolated from the synovial fluid of two RA patients.** *PBMC were isolated from the synovial fluid of two RA patients (A and B) and blood myeloid DC were enriched by negative selection (EasySep human myeloid DC enrichment kit; StemCell). Purity of myeloid DC was determined by CD1c and CD11c expression. (A and B) show two independent RA patient samples.*

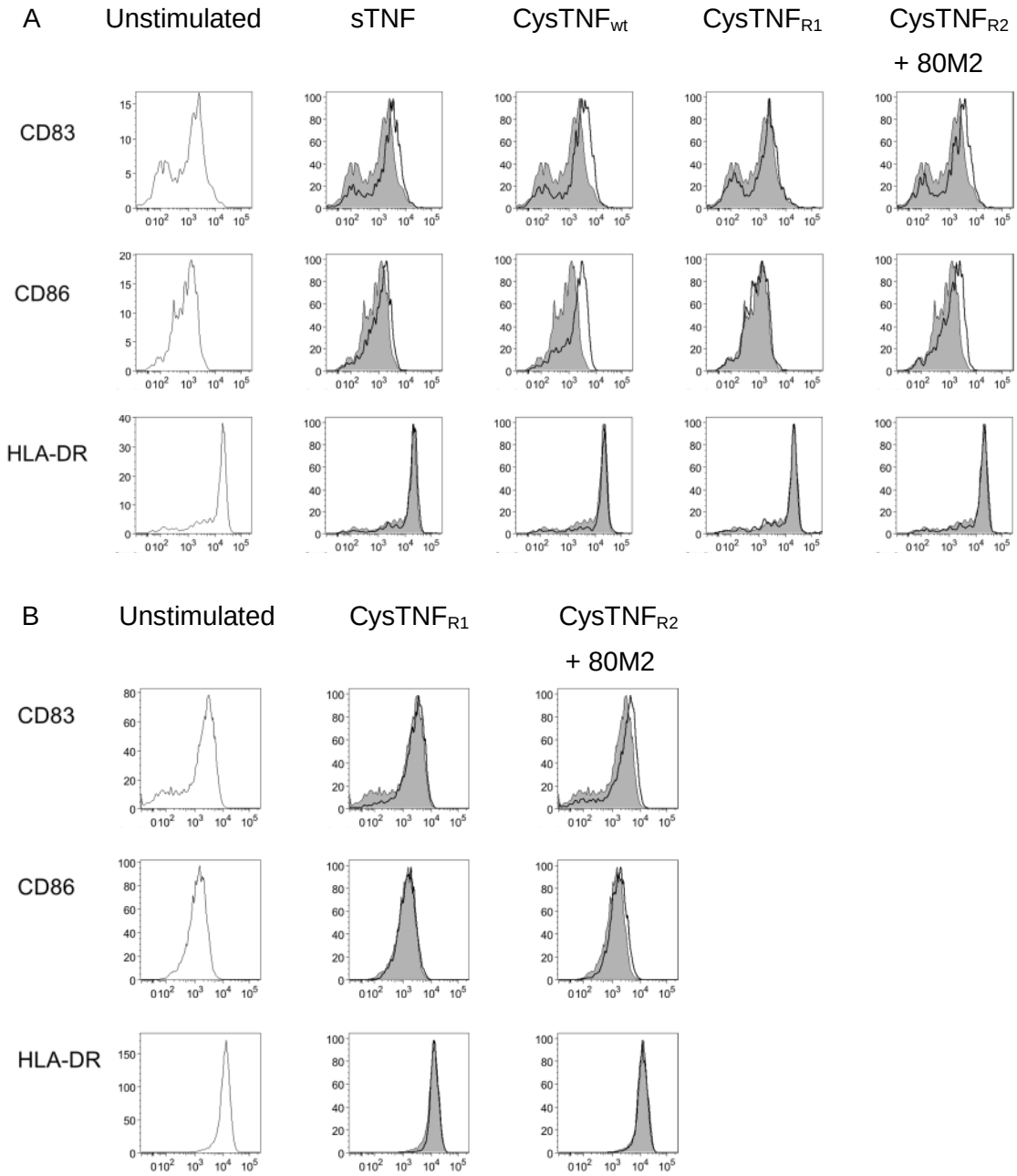


**Figure 54: TNF does not enhance expression of maturation markers on myeloid DC isolated from synovial fluid.** *Freshly isolated synovial fluid myeloid DC (purities analysed in Figure 53) were cultured in the presence or absence of sTNF, CysTNF$_{R1}$ or CysTNF$_{R2}$ + MAb 80M2 (50 ng/ml) for 24 h. CD83, CD86 and HLA-DR expression (black line) was compared to unstimulated cells (grey shaded) and determined by flow cytometry. (A) and (B) show cells isolated from the synovial fluid of two independent RA patients.*

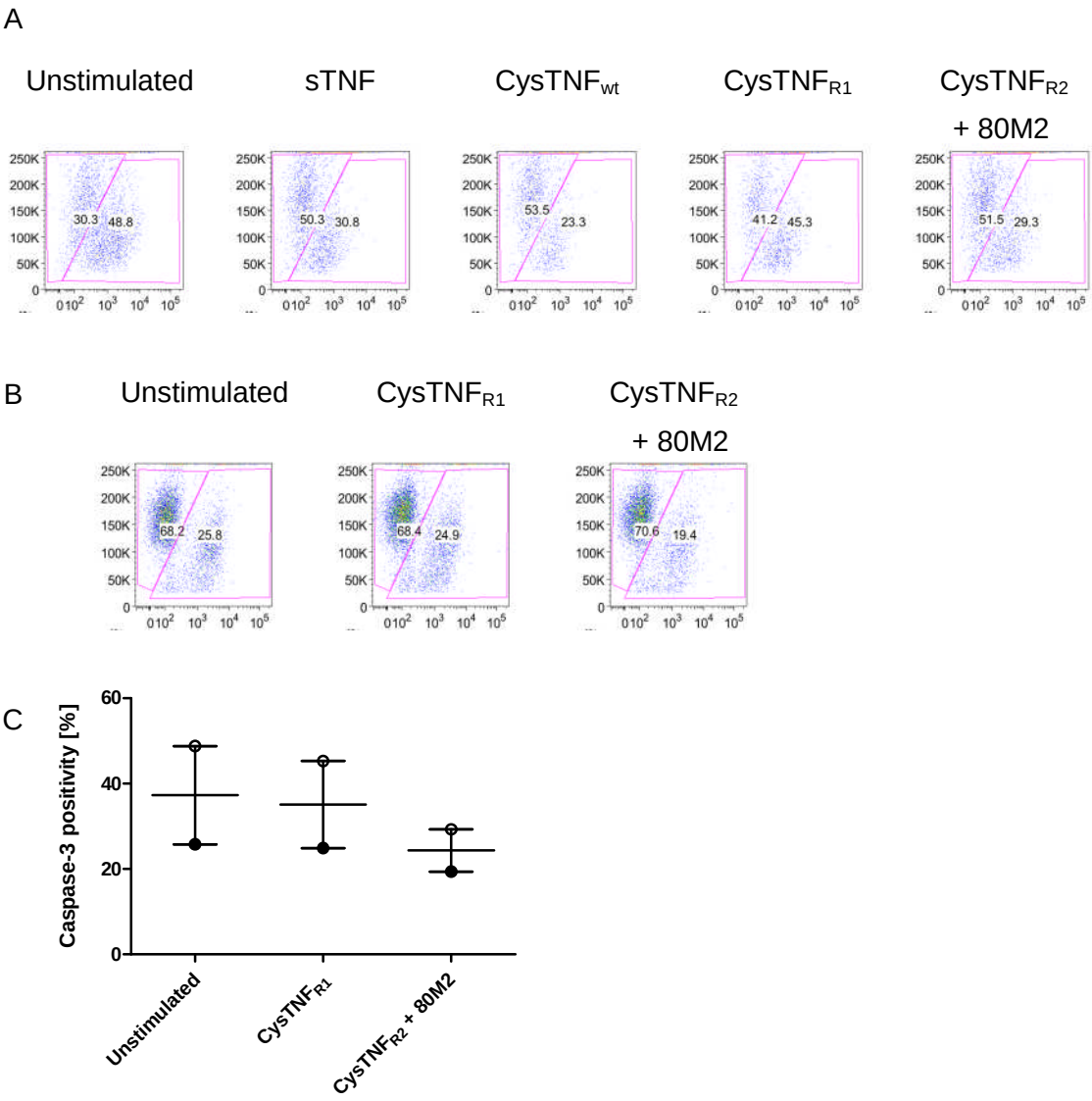


**Figure 55: TNFR2 but not TNFR1 enhances the survival of myeloid DC isolated from the synovial fluid of RA patients.** *PBMC from SF of two RA patients (A and B, as also shown in Figure 53 and Figure 54) were isolated and enriched for myeloid DC. Myeloid DC were cultured in the presence or absence of 50 ng/ml of sTNF, CysTNF$_{R1}$ or CysTNF$_{R2}$ + MAb 80M2 (2 μg/ml) for 24 h. Intracellular active caspase-3 levels within CD1c positive populations were determined by flow cytometry. (A) and (B) show two independent donors which are both shown in (C) Donor A; white circle and donor B; black circle.*

### *5.3. Discussion*

The aims of this chapter were to investigate the expression of TNFR1 and TNFR2 on human blood myeloid DC and to explore the effect of TNFR signalling on their maturation and survival. I have shown that blood myeloid DC do express TNFR1 and TNFR2 and that the downstream effects of each receptor are similar in blood myeloid DC and moDC. However, blood myeloid DC appear more sensitised to TNFR-mediated maturation when compared to moDC. My data show that TNFR1- and to a lesser extent TNFR2-mediated signalling results in blood myeloid DC phenotypical maturation as defined by enhanced expression of maturation markers CD83 and CD86. However, TNF-induced signalling alone (when compared to the TLR ligand LPS) is not sufficient to induce full maturation as shown by a lack of IL-6 production by blood myeloid DC. I also show data demonstrating that blood myeloid DC show enhanced by TNFR2- but suppressed TNFR1-mediated survival when compared to moDC, thereby indicating that blood myeloid DC respond differently (or show a shift in sensitivity) to TNFR-mediated signalling in comparison to moDC (Table 5).

| | DC Maturation | | | | DC Survival | | | |
|---|---|---|---|---|---|---|---|---|
| | sTNF | $CysTNF_{wt}$ | $CysTNF_{R1}$ | $CysTNF_{R2}$ + 80M2 | sTNF | $CysTNF_{wt}$ | $CysTNF_{R1}$ | $CysTNF_{R2}$ + 80M2 |
| **moDC** | ++ | ++ | + | - | ++++ | ++++ | ++ | + |
| **Blood Myeloid DC** | +++ | ++++ | ++ | + | ++ | +++ | - | ++ |

*Table 5: TNFR-mediated maturation and survival of moDC and blood myeloid DC.*

In addition, I analysed myeloid DC from the synovial fluid of RA patients to represent chronic inflammatory conditions and allow comparison with 'inflammatory' moDC and steady state blood myeloid DC. Only the use of $CysTNF_{wt}$ resulted in an increase in the expression of surface maturation markers in SF myeloid DC, although unstimulated cells showed high expression of CD83 and CD86, suggesting that the inflammatory environment of the RA joint had already resulted in some degree of maturation prior to SF myeloid DC isolation. Furthermore, the survival of SF myeloid DC was enhanced via TNFR2- mediated signalling. These data may, at least in part, underline the differences in the functional effects mediated by the two receptors demonstrating their distinct roles in regulating DC maturation and survival during both inflammatory and steady state environments.

### *5.3.1. Analysis of blood myeloid DC populations*

Following enrichment by negative selection, the population of blood myeloid DC ($CD1c^+/CD11c^+$) was 75-90% pure and also contained ~3% T cell and ~1% B cell contamination plus other unknown cells (potentially including monocytes and NK cells). These cells may exert effects on the blood myeloid DC independently to the exogenously added TNF, for example, TNF-producing cells may be present. Also, the maturation of human DC can be negatively regulated by $CD19^+$ B cells (Morva et al. 2012), although my data show an increase in maturation making B cell-mediated suppression unlikely. The effect of contaminating cells on the blood myeloid DC during 24 h culture could not be evaluated at this stage, but might have affected the interpretation of this study. To prevent any effects of contamination with other cell populations, in future experiments DC subsets could be sorted using multi-colour flow sorting prior to TNFR-stimulation and analysis.

I have shown that both TNFR1 and TNFR2 are expressed on freshly isolated human blood myeloid DC and plasmacytoid DC. As with moDC, TNFR expression levels varied between each donor, a difference which may account for donor to donor variability in other assays. However, as the TNFR expression was not analysed for each donor in parallel to the maturation and survival assays, it is not possible to correlate TNFR expression with the TNF response.

### *5.3.2. TNF-mediated maturation of blood myeloid DC*

As discussed in the previous chapter (3.5.3), DC maturation in mouse models has been shown to require TNFR1-mediated signalling in response to infection (Ding et al. 2011; Sundquist & Wick 2005), whereas TNFR2 was not required (Ding et al. 2011) or was not investigated (Sundquist & Wick 2005). My data show that in blood myeloid DC TNFR1- and to a lesser extent TNFR2-mediated signalling significantly increased the expression of surface markers involved in the co-stimulation and activation of T cells. The enhanced phenotypical maturation induced by $CysTNF_{R1}$ and $CysTNF_{R2}$ + 80M2 was weak compared to that of sTNF or $CysTNF_{wt}$, and was diminished by 48 h, potentially due to higher levels of cell death. As to be expected in steady state conditions, the unstimulated blood myeloid DC had consistently low CD83 and CD86 suggesting that they were in an immature state within the blood prior to isolation.

Both $CysTNF_{R1}$ and $CysTNF_{R2}$ + 80M2 were less effective at enhancing the phenotypical maturation of blood myeloid DC than sTNF or $CysTNF_{wt}$. Again, as discussed in the previous chapter (3.5.3), the reason for this remains unclear, although it is interesting that in this case $CysTNF_{wt}$ has a greater effect than sTNF which suggests that $CysTNF_{wt}$ is simultaneously acting through a combination of both TNFR1

and TNFR2 potentially leading to an additive effect. As with moDC (chapter 3.5.3), the phenotypical maturation induced by TNFR-stimulation was not sufficient to induce full blood myeloid DC maturation shown by the lack of cytokine production and again is consistent with previous work showing that full maturation of DC requires activation through a pathogen recognition receptor (PRR) (Spörri & Reis E Sousa 2005; Roelofs et al. 2005).

Furthermore, the blocking effect of the TNFR1-specific antagonistic antibody H398 was complete for $CysTNF_{R1}$, almost complete for sTNF but was not complete for $CysTNF_{wt}$, suggesting that $CysTNF_{wt}$ does in fact act via both TNFR1 and TNFR2. However, the combination of both TNFR1- and TNFR2-specific antagonistic antibodies also did not fully inhibit $CysTNF_{wt}$, this may be due to strong avidity of the pre-oligomerised ligand or due to the lack of donors for this condition. The TNFR2-specific antagonistic antibody 22221 fully blocked the $CysTNF_{R2}$ + 80M2 mediated enhancement of CD86 expression and appeared to fully block enhancement of CD83 expression although CD83 expression was so low that this result turned out not to be significant. Further analysis of blood myeloid DC maturation would include investigation of their functional ability to activate naïve T cells using an MLR as was done with moDC, although the small numbers of blood myeloid DC would make this more challenging and require further optimisation.

### *5.3.3. TNFR-mediated blood myeloid DC survival*

My results show that stimulation with $CysTNF_{wt}$, $CysTNF_{R2}$ + 80M2 and to a lesser extent sTNF, protected blood myeloid DC from cell death. This is similar to the response of moDC except that the moDC response to sTNF was comparable to $CysTNF_{wt}$. Interestingly, $CysTNF_{R1}$ did not have any effect on blood myeloid DC survival even though there was an effect with sTNF which is known to acts primarily through TNFR1 (Grell et al. 1995; Richter et al. 2012; Krippner-Heidenreich et al. 2002). In order to determine if the effect of sTNF was indeed mediated via TNFR1 I used TNFR-specific antagonistic antibodies. In line with my previous results using moDC, the effect of sTNF on myeloid blood DC was fully blocked by antagonising TNFR1 but not TNFR2 demonstrating that the effects of sTNF are mediated via TNFR1 alone. As discussed previously (chapter 3.5.3), the reason for this difference between the effects of sTNF and $CysTNF_{R1}$ remains unclear but may be due to the effects of the point mutations in $CysTNF_{R1}$ which give the ligand its receptor-selectivity. In addition, the use of a TNFR2-specific antagonist (22221) fully inhibited the effect of $CysTNF_{R2}$ + 80M2 whereas the TNFR1-specific antagonist (H398) had no effect thereby confirming that TNFR2 enhances blood myeloid DC survival independently of TNFR1. As seen with moDC, the pro-survival effect of $CysTNF_{wt}$ was not fully blocked when

antagonising either TNFR which may be due to the strong avidity effect of the pre-oligomerised ligand, which is difficult to antagonise using anti-TNFR antibodies.

### *5.3.4. Myeloid DC freshly isolated from synovial fluid*

I also obtained two synovial fluid (SF) samples from RA patients in order to determine whether DC from a chronic inflammatory environment would have a phenotype comparable to the moDC inflammatory model or the steady state blood myeloid DC. Myeloid DC obtained from SF were around 43% pure (i.e. $CD1c^+/CD11c^+$) which is much lower than blood myeloid DC. This may be due to the enrichment kit used (EasySep human myeloid DC enrichment kit; StemCell) which is not optimized for use on SF. As the purity of myeloid DC populations from SF was low and the sample size was small I cannot draw accurate conclusions from the TNFR-mediated signalling data as the contaminating cells may act on the myeloid DC during the 24 h culture and there are not enough samples to determine significance. It is unknown what cell types represent the other ~57% of the samples but it potentially includes T cells, B cells, NK cells, monocytes and macrophages. In a proteoglycan induced arthritis (PGIA) mouse model SF was shown to contain myeloid-derived suppressor cells which may suppress DC maturation (Egelston et al. 2012). To further investigate this line of research DC would be analysed following multi-colour flow sorting prior to analysis, however this is beyond the scope of this project.

Selective stimulation of TNFR1 or TNFR2 did not have any effect on the maturation of myeloid DC in either of the RA SF samples (Figure 54). Other research has shown that myeloid DC from rheumatoid SF display a semi-mature phenotype which resembles that of moDC that have been generated *in vitro* (Thomas et al. 1999). RA SF myeloid DC also show higher expression of CD40, CD80, CD83 and CD86 compared to those from peripheral blood but no significant differences in cytokine secretion were detected (Jongbloed et al. 2006; Moret et al. 2013), also suggesting a semi-mature phenotype. This is in agreement with my results showing that surface maturation marker expression is high in freshly isolated SF myeloid DC and that no cytokine secretion could be detected in blood myeloid DC or SF myeloid DC following TNFR stimulation. As the baseline CD83 and CD86 expression was high, the lack of response to TNF may be because the myeloid DC had already matured within the inflammatory environment of the RA joint. Additionally, TNF may be produced by contaminating monocytes/macrophages which could enhance maturation and affect my results. In order to determine if this is the case and that maturation marker expression is already at its maximum, LPS should be used as a positive control. In addition, the expression of TNFR1 and TNFR2 could be investigated prior to TNFR-stimulation as receptor

downregulation in response to maturation may partially explain the lack of response to TNF.

My results also show that the survival of myeloid DC isolated from the synovial fluid of the two RA patients was enhanced by stimulation with sTNF, $CysTNF_{wt}$ and $CysTNF_{R2}$ + 80M2, whereas $CysTNF_{R1}$ did not have any effect. This is in line with my data in blood myeloid DC and in contrast to moDC (chapter 4.4.1; Figure 29), where both TNFR1 and TNFR2 enhanced survival. Segura et al. describe human inflammatory DC as a distinct population identified in SF samples from RA joints which shares gene signatures with *in vitro* moDC and inflammatory macrophages, suggesting they are derived from monocytes and are the *in vivo* equivalents of moDC (Segura, Touzot, et al. 2013). Inflammatory DC also displayed features from both conventional DC and inflammatory macrophages and were involved in induction and maintenance of Th17 responses (Segura, Touzot, et al. 2013). This is partially in contrast to my data as the survival of SF myeloid DC in response to TNFR-selective stimulation was similar to that of blood myeloid DC, but differed to that of moDC. This suggests that with regard to TNFR-responsivity, inflammatory DC isolated from the SF of RA patients represent blood DC more than inflammatory moDC. However, more patients would be needed to accurately draw such conclusions as statistical significance could not be determined at this time due to a lack of SF donors.

### *5.3.5. Conclusion*

My results show that the phenotypical maturation of human DC is most potently regulated by TNFR1-mediated signalling as in moDC TNFR2-mediated signalling has no effect and in myeloid blood DC activation of TNFR2 is much less effective in enhancing the expression of surface maturation markers when compared to TNFR1. Alternatively, human DC survival can be significantly enhanced by either TNFR in moDC but mainly via TNFR2 in myeloid blood DC and SF myeloid DC. Therefore, my data show that moDC and blood myeloid DC exert different sensitivities towards TNFR1- and TNFR2-mediated DC maturation and survival. Whereas in blood myeloid DC maturation is generally enhanced by TNFR1- or TNFR2-signalling, their survival seems to be promoted by TNFR2-, but not TNFR1-signalling when compared to moDC/inflammatory DC.

# 6. General discussion

The individual roles of TNFR1- and TNFR2-mediated signalling in human DC have never been studied in detail. This study is the first to make use of TNFR-selective ligands to investigate and dissect the roles of TNFR1 and TNFR2 in regulating the maturation and survival of human DC while comparing 'inflammatory' moDC, SF-derived myeloid DC and steady-state blood myeloid DC. My research was driven by the aims to;

1. Determine the expression of TNFR1 and TNFR2 on human DC subsets
2. Determine the competency of each TNFR for the activation of NFκB
3. Determine the involvement of TNFR1 and TNFR2 in DC maturation and survival
4. Identify signalling molecules/pathways involved in TNFR1- and TNFR2-mediated regulation of DC survival
5. Compare the responses of moDC, blood myeloid DC and myeloid DC isolated from RA patient synovial fluid

The major findings of this study are briefly summarised in Figure 56 and more detailed results from moDC are summarised in chapter 4.4.4; Figure 41 (B). In moDC, TNFR1-selective, but not TNFR2-selective stimulation resulted in activation of the classical NFκB pathway, increased expression of DC maturation markers CD83 and CD86 and enhanced T cell stimulatory capacity, whereas both TNFR1 and TNFR2 activated the alternative p100/p52 NFκB pathway and enhanced moDC survival. This demonstrates that innate signals can promote DC survival in the absence of DC maturation. Accordingly, the p65 NFκB pathway was involved in the pro-survival effect of TNFR1 whereas the Bcl-2/Bcl-xL pathway was essential to survival mediated by both TNFR1 and TNFR2 thus identifying both differences and overlap in TNFR-mediated signalling. In contrast, in myeloid blood DC, phenotypical maturation was most potently regulated by TNFR1, whereas TNFR2 was superior in protecting DC from cell death. Selective stimulation of TNFR1 or TNFR2 did not have any effect on the maturation of myeloid DC isolated from the SF of RA patients as cells already displayed a mature phenotype, however TNFR2-mediated signalling enhanced SF-derived myeloid DC survival, whereas TNFR1-mediated signalling only enhanced survival via sTNF (but not $CysTNF_{R1}$). Therefore my data shows that TNF promotes DC maturation and survival through distinct signalling pathways and that moDC and myeloid DC have different sensitivities towards TNFR1- and TNFR2-mediated signalling.

Although my data show differences between the responses of moDC and myeloid DC to TNFR1- and TNFR2-selective stimulation, these differences may not be as clear in

an *in vivo* setting. The concentration of TNF *in vivo* is difficult to assess as local concentrations can become high in small intercellular spaces although they may not reach the saturating concentration of 50 ng/ml used in these experiments. The effects mediated by each TNFR in different cell types may ultimately involve differences in sensitivity which could alter the balance between maturation, survival and apoptosis. Moreover, the ratio of TNFR1 and TNFR2 co-expression may be important in shifting the balance between cellular survival and apoptosis. Therefore, it remains unknown if steady state DC and inflammatory DC would respond differently to TNF *in vivo*.

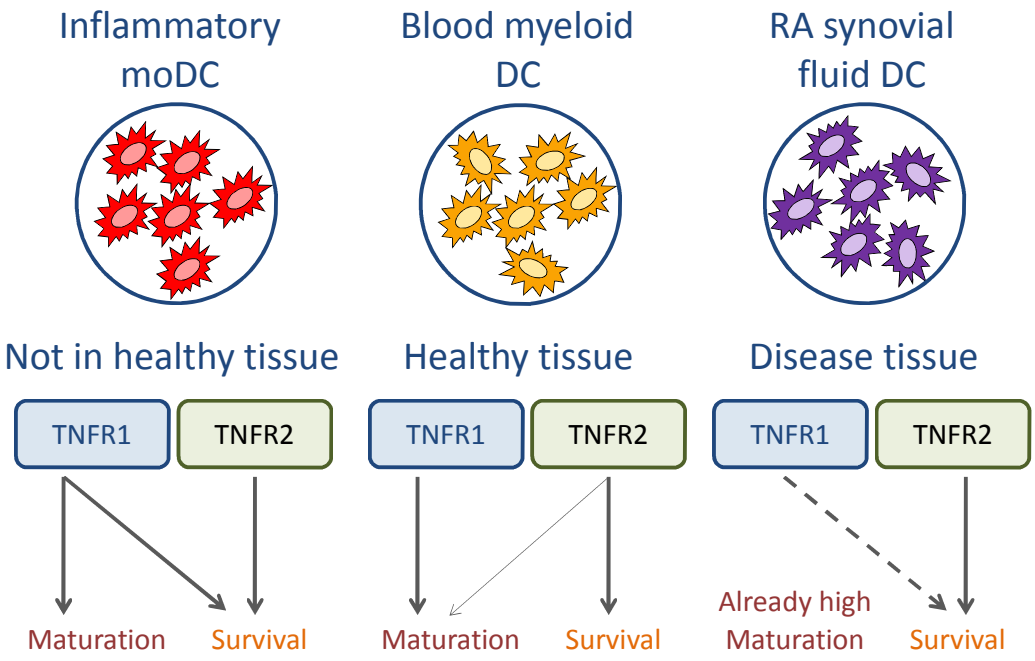


**Figure 56: Summary of TNFR-mediated maturation and survival in DC subtypes.** *TNFR1 enhanced moDC maturation and survival whereas TNFR2 only enhanced survival. TNFR1 and to a lesser extent TNFR2 enhanced blood myeloid DC maturation but only TNFR2 enhanced survival. Myeloid DC isolated from synovial fluid already showed enhanced maturation but survival was mainly enhanced by TNFR2.*

### *6.1. A comparison of DC subtypes*

It has been known for a number of years that monocytes can differentiate into DC *in vitro* (Sallusto & Lanzavecchia 1994). In this thesis moDC were used as a model for inflammatory DC which are not normally found in healthy tissue but are thought to differentiate from monocytes during cases of chronic inflammation such as RA (as reviewed in (Shortman & Naik 2007; Segura, Durand, et al. 2013)). Previously, murine models have demonstrated that monocytes develop into moDC *in vivo* during infections with *Leishmania major* (León et al. 2007) or *Listeria monocytogenes* (Serbina et al. 2003) and in models of inflammatory disease such as RA (Campbell et al. 2011). Also,

monocytes from the Ly6C$^+$ inflammatory mouse model do not produce DC in lymphoid organs when transferred into a non-irradiated mouse, but do produce DC when transferred into mice subjected to GM-CSF-dependent inflammation (Naik et al. 2006). MoDC which develop in mice have also been analysed in detail and were shown to be DC in terms of motility, location (proximity to T cell areas), phenotype and function (T cell stimulatory capacity) (Cheong et al. 2010).

Inflammatory DC have been identified in humans using transcriptomic analysis as a distinct subset which differs from other DC and from inflammatory macrophages (Segura, Touzot, et al. 2013). These human inflammatory DC are proposed to be monocyte-derived, are present in inflammatory environments including synovial fluid from RA patients and are involved in the Th17 response (Segura, Touzot, et al. 2013). As previous research suggests that monocyte-derived DC pay little contribution to steady state DC I also investigated blood myeloid DC isolated from healthy donors. I also obtained two SF samples from RA patients in order to determine whether DC from a chronic inflammatory environment would have a phenotype comparable to the moDC inflammatory model or the steady state blood myeloid DC.

Other research shows that myeloid DC from rheumatoid SF display a semi-mature phenotype which resembles that of moDC which have been generated *in vitro* (Thomas et al. 1999) and show with higher expression of CD40, CD80, CD83 and CD86 compared to those from RA peripheral blood (Jongbloed et al. 2006). This is in agreement with my initial results showing that surface maturation marker expression is high in unstimulated DC. As the myeloid DC populations from SF were only ~43% pure and the sample size was low I cannot draw accurate conclusions from this data. It is unknown what cell types represent the other ~57% of the samples, for example in a proteoglycan induced arthritis (PGIA) mouse model SF was shown to contain myeloid-derived suppressor cells which may suppress DC maturation (Egelston et al. 2012). For future analysis DC subsets would be sorted using multi-colour flow sorting however this is beyond the scope of this project.

DC precursors in peripheral blood can differentiate after entry to rheumatoid synovial tissue (Pettit et al. 2000) and SF from both RA and PsA patients have been shown to contain both myeloid DC and pDC (Jongbloed et al. 2006). It has been suggested that myeloid DC contribute to RA disease activity (Richez et al. 2009). Data has shown that DC progenitors and growth factors are present in RA but not OA synovial fluid therefore potentially play a role in RA disease progression and also that cell-free RA SF can induce DC maturation (Santiago-schwarz et al. 2001). In addition, it has also been proposed that TNF may negatively regulate CD14-derived DC differentiation *in vivo* as enhanced numbers of CD14-derived DC correlates with enhanced levels of soluble

TNFR1 which acts as a TNF antagonist, thus suggesting that TNF inhibits the development of CD14-derived DC (Santiago-schwarz et al. 2001). This is in contrast to my data which shows that TNF-mediated signalling enhanced CD14-derived moDC and myeloid DC survival, thereby reiterating the point that more work is needed to differentiate between effects mediated by TNFR1 and TNFR2 in RA.

### *6.2. TNFR-mediated activation of the NFκB pathway in DC*

Regulation of the classical and alternative NFκB pathways is a crucial factor in the balance between inflammation and tolerance. NFκB signalling is widely implicated in inflammatory diseases, both in pro- and anti-inflammatory processes (Bonizzi & Karin 2004). My data showed that TNFR1- but not TNFR2-mediated signalling activated the classical NFκB pathway whereas both TNFR1 and TNFR2 activated the alternative NFκB pathway in moDC. My findings that the classical and alternative NFκB signalling pathways in moDC are differentially activated by TNFR1 and TNFR2 may partially explain the opposing functions of TNF in disease as these pathways can mediate a wide range of effects. The lack of activation of p65 via TNFR2 seen in my data may be cell-type specific as other research has shown that the classical NFκB pathway is activated by TNFR2, for example in mouse neurones which lack TNFR1 (Marchetti et al. 2004). Overlapping mechanisms of NFκB pathway activation by different molecules within the pathway are shown in Figure 57 and described in the following paragraphs.

Receptor interacting protein (RIP) kinases are crucial regulators of cell survival which are capable of activating NFκB and are expressed in moDC both with and without TNFR1-stimulation (Figure 18). Generally, TNFR1-mediated signalling recruits RIP1 (also known as RIPK1) and TRAF2 leading to classical NFκB pathway activation and increased expression of anti-apoptotic genes, resulting in cell survival. However, opposing data suggests that RIP1 is not essential for the activation of classical NFκB signalling by TNFR1 as TNFR1 stimulation in $RIP1^{-/-}$ MEF results in classical NFκB activation (including p65 translocation) and enhanced survival suggesting a death-inducing role for RIP1 in these cells (Wong et al. 2010). Alternatively, TNFR1-mediated activation of the alternative NFκB pathway is suppressed by the adapter kinase RIP1 through the inhibition of TNF-mediated TRAF2 and cIAP1 degradation (Kim et al. 2011; Gentle et al. 2011). In addition, when classical NFκB signalling is low, TNFR1 can mediate a pro-apoptotic signal via activation of the caspase cascade where caspase-8 may mediate the cleavage of RIP1 blocking further anti-apoptotic signals (Festjens et al. 2007). One reason for the apparent lack of TNF-mediated alternative NFκB activation in some cell types may be due to its negative regulation by RIP1 which stabilises TRAF2, decreasing its degradation and therefore preventing NIK stabilisation

(Kim et al. 2011; Gentle et al. 2011). In accordance, TNF can induce the alternative NFκB pathway in cells deficient of RIP1 (Kim et al. 2011; Gentle et al. 2011). However, in contrast my results show that in immature moDC RIP1 is present and the alternative NFκB pathway is active thus RIP1 does not suppress the alternative pathway in moDC.

The alternative NFκB pathway is activated by several TNFR family members including LTβ receptor (Dejardin et al. 2002) and CD40 (Coope et al. 2002). Activation of the alternative NFκB pathway is usually triggered by non-inflammatory stimuli, and resulting signals inhibit pro-inflammatory cytokines and play a role in T cell tolerance (Zhu & Fu 2010). This could potentially result in a negative feedback mechanism where the pro-inflammatory effect of sTNF is diminished. However, the alternative pathway can also mediate a pro-inflammatory response depending on the original stimuli. For example, CD40-mediated activation of the alternative NFκB pathway induced pro-inflammatory IL-12 cytokine production in LPS-matured murine myeloid DC (Yanagawa & Onoé 2006). Furthermore, defects in the alternative NFκB pathway have been shown to result in defects in immune development (Caamaño et al. 1998; Franzoso et al. 1998) and lead to autoimmunity (Cheema et al. 2001). Anti-inflammatory IDO production has been shown to require alternative NFκB signalling in DC and selective activation of alternative NFκB signalling resulted in non-inflammatory DC that suppress T cell activity and promote T cells with regulatory properties (Tas et al. 2007). These studies and my data suggest that TNFR2-stimulated DC may produce anti-inflammatory signals via the alternative NFκB pathway. This could be further investigated by identifying the expression/secretion of anti-inflammatory molecules (e.g. soluble TNFR).

NIK is another signalling molecule involved in the activation of the alternative NFκB pathway. After receptor activation NIK is released from its constitutive degradation and accumulates before phosphorylating IKK molecules, leading to the partial degradation of p100 and release of p52. The p52 molecule can then form a complex with RelB and translocate to the nucleus. The longer time frame taken to activate the alternative NFκB pathway compared to the classical NFκB pathway may involve the cleavage of p100 which is a slower process than that of p65-phosphorylation (Naude et al. 2011) and also the time taken for NIK to accumulate.

The alternative NFκB pathway is important in DC maturation and function as the deletion of NIK (which controls RelB) specifically in mouse DC results in deficient Th1 and Th17 responses (Hofmann et al. 2011). In addition, a mouse model of inflammatory arthritis showed that mice lacking functional NIK were resistant to antigen induced arthritis (Aya et al. 2005). Furthermore, the Aly (alymphoplasia) mouse model

contains a spontaneous point mutation in the NIK gene which prevents its interaction with IKK complexes. This Aly model has been used to show that a defect in NIK prevents activation of the alternative NFκB pathway in DC in response to CD40 and impairs the ability of the DC to cross-prime $CD8^+$ T cells (Lind et al. 2008). It has also been used to show that a defect in NIK decreased DC-mediated T cell differentiation into regulatory T cells (Tamura et al. 2006). With regard to the classical NFκB pathway, in the Aly mouse model NIK is required for classical NFκB signalling downstream of CD40 in B cells but not in DC (Garceau et al. 2000) and in $NIK^{-/-}$ mice, NIK is not required for TNF-mediated activation of the classical NFκB pathway (Yin et al. 2001). This indicates differences in the activation of classical NFκB signalling in different cell types.

It has been shown that CD40 ligation activated the alternative NFκB pathway and induced IL-12 cytokine production in LPS-matured murine myeloid DC and alternative NFκB pathway activation was abolished in NIK-mutated mature DC (Yanagawa & Onoé 2006). This shows that CD40 ligation is a potent enough signal to induce DC-mediated cytokine production (unlike TNF), and also that NIK is important in the activation of the alternative NFκB pathway. In mouse B cell lines, CD40 and TNFR2 activate NFκB signalling during B cell activation via different mechanisms (Munroe & Bishop 2004). The CD40-mediated response was shown to activate NFκB-mediated transcription 3 times greater than the TNFR2-mediated response, although the CD40-mediated response was reduced by 30% by blocking TNFR2 suggesting an overlapping mechanism. CD40 was shown to activate the classical NFκB pathway and to a lesser extent than the alternative NFκB pathway. Alternatively, and in line with my data, TNFR2 was shown to exclusively activate the alternative NFκB pathway (Munroe & Bishop 2004).

The Rel homology domain of p100 is most commonly associated with RelB. During alternative NFκB signalling RelB forms complexes with p52 and translocates to the nucleus and induces the transcription of a range of different genes. RelB deficient mice have defects in DC cross-priming (Castiglioni et al. 2002) indicating its importance in the immune response. RelB entering the nucleus in response to TNF cannot bind to DNA in MEF as TNF promotes its association with p65 (Jacque et al. 2005). TNF-induced phosphorylation of p65 at serine-276 is required for p65/RelB complex formation and in the absence of ser-276 phosphorylation, TNF stimulation leads to a strong increase in RelB controlled genes such as Bcl-xL (Jacque et al. 2005). Therefore, p65 plays a role in reducing RelB activity in response to TNF and shows one mechanism of cross-talk between the two NFκB pathways (Figure 57). In addition to TNFR1, other receptors show similar cross-talk between NFκB pathways. For

example, Dectin-1, an innate receptor involved in antifungal immunity on human DC can activate both the classical p65 and alternative RelB NFκB subunits (Gringhuis et al. 2009). The suppression of the alternative NFκB pathway by heterodimerisation of RelB with p65 has been shown to induce cytokines involved in T cell differentiation such as IL-12p40 (Gringhuis et al. 2009). Therefore mechanisms which sequester RelB into inactive p65-RelB dimers may prevent anti-inflammatory signals and increase pro-inflammatory T cell responses.

Differences in expression of sTNF and mTNF may also play a role in the activation of NFκB as sTNF only strongly activates TNFR1 but mTNF has the capacity to activate both TNFR. For example, it has been demonstrated in cell lines and in primary T cells that only mTNF not sTNF stimulates alternative NFκB signalling via TNFR2 whereas sTNF and TNFR1 were not able to induce p52 activation (Rauert et al. 2010). This is partially in contrast to my data as in moDC both TNFR1 and TNFR2 activated the alternative NFκB pathway. Data therefore suggest that the effects of TNFR1 and TNFR2 differ between DC and T cell populations. Also, the differences between signalling induced by mTNF and sTNF can affect the induction of immune responses. For example, it has been shown that mTNF and its interaction with TNFR2 is essential for the induction of antigen-specific $T_{reg}$ cells by tolerogenic (vitamin D3-modulated) DC (Kleijwegt et al. 2010). In addition, transgenic mice which express non-cleavable mTNF in endothelial cells were protected from immune-mediated Concanavalin A-induced acute hepatitis (Willuweit et al. 2001) which may suggest a role for TNFR2 in these protective effects.

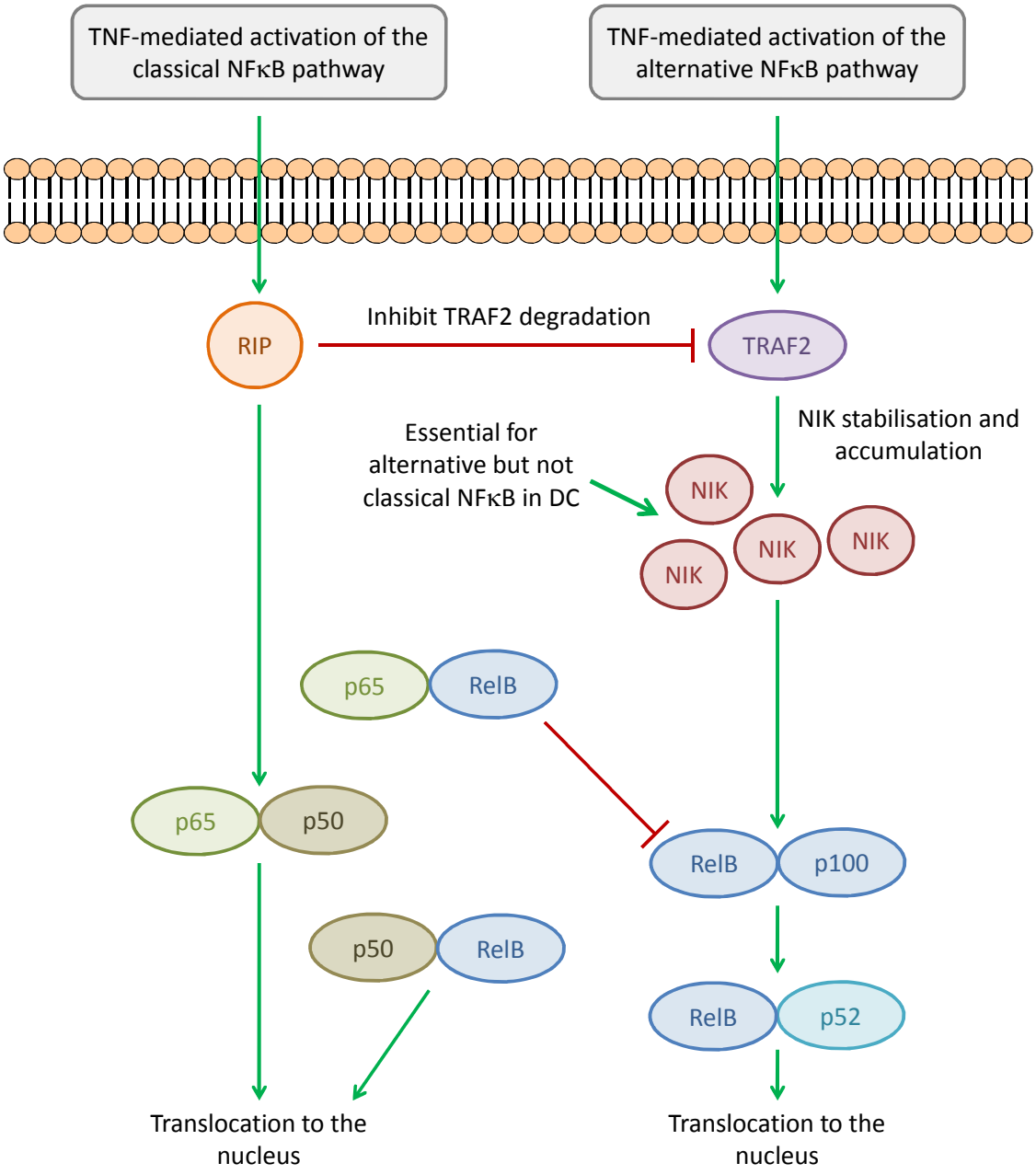


**Figure 57: RIP, NIK and RelB control NFκB activation in response to TNF-mediated signalling: a summary of previous studies.** *RIP can mediate the activation of the classical NFκB pathway, although it may not be essential for TNFR1-mediated signalling. RIP activation can inhibit TRAF2 degradation and thereby inhibit the alternative NFκB pathway. NIK is essential for activation of the alternative but not the classical NFκB pathway in DC. The RelB dimerisation partner may determine whether the classical or alternative NFκB pathway is activated. Adapted from (Kim et al. 2011; Gentle et al. 2011; Wong et al. 2010; Hofmann et al. 2011; Lind et al. 2008; Garceau et al. 2000; Jacque et al. 2005).*

### *6.3. DC survival mediated by TNF and other members of the TNF superfamily*

The survival of DC is a critical parameter in the regulation of adaptive immune responses and is regulated by several TNF superfamily members. The TNF superfamily contains 19 members, which signal through 29 receptors (Aggarwal 2003), several of which can cross-react and therefore contribute to the regulation of a vast range of cellular effects. For example, in DC the binding of CD40L to its cognate receptor can lead to an upregulation of TNF (Caux et al. 1994) which subsequently may lead to the activation of TNFR1. Also, murine BM-derived DC overexpressing CD40L, RANKL or 4-1BB were shown to have significantly enhanced survival compared to control DC (Yurkovetsky et al. 2006).

Receptor activator of NFκB ligand (RANKL) is expressed on activated T cells and has two receptors; RANK and osteoprotegerin (OPG) both of which are expressed by DC. OPG expression is upregulated in human moDC maturation induced by TNF, RANKL or LPS (Schoppet et al. 2007). Knock out of OPG in BM-derived murine DC resulted in enhanced survival and production of TNF, IL-12p40 and IL-23 in response to LPS (Chino et al. 2009). Therefore the RANKL-RANK interaction promotes DC survival and the interaction is limited by OPG. As well as enhancing DC survival, RANKL can also induce functional (but not total) maturation of human moDC (Schiano de Colella et al. 2008).

TNF-related activation-induced cytokine (TRANCE) is expressed by activated T cells and is a DC survival factor which upregulates the expression of the anti-apoptotic molecule Bcl-xL in murine BM-derived DC and inhibits apoptosis in both murine BM-derived DC and human moDC (Wong et al. 1997). This suggests a possible overlapping mechanism of survival with TNF as my data show that both TNFR1- and TNFR2-mediated signalling enhanced the expression of Bcl-xL in human moDC. Similarly to CD40L, TRANCE can enhance DC survival and induce the expression of proinflammatory cytokines such as IL-6 (Josien et al. 1999). Although unlike CD40L, the TRANCE receptor is mainly expressed on mature DC (and not immature DC), therefore does not appear to be important in DC maturation (Wong et al. 1997). Furthermore, TRANCE can co-operate with TNF or CD40L to enhance splenic and BM-derived DC survival to a greater extent than either ligand alone (Josien et al. 1999) thereby increasing T cell-mediated immune responses. TRANCE can also be expressed on osteoblasts and induces osteoclast activation leading to bone resorption. TRANCE activity can be inhibited by OPG (Fuller et al. 1998) which in turn can be neutralised by TRAIL (Emery et al. 1998) illustrating multiple mechanisms involved in the regulation of biological processes such as cell activation or apoptosis.

4-1BB (CD137) is a costimulatory molecule expressed on T cells but it is also expressed on DC along with its ligand 4-1BBL. Knock out mice have indicated that 4-1BB is induced during DC maturation (but is not necessary for maturation itself) and functions as a DC survival factor both *in vitro* and *in vivo* (Choi et al. 2010). In murine BM-derived DC, 4-1BB activation increased the expression of DC maturation markers CD80 and CD86, increased the secretion of IL-6 and IL-12 and increased DC survival due to increased levels of Bcl-2 and Bcl-xL (Kuang et al. 2012). Alternatively, using knock out mice, a lack of 4-1BB resulted in reduced expression of antiapoptotic molecules Bcl-2 and Bcl-xL (Choi et al. 2010). This is similar to enhanced Bcl-xL expression in DC mediated by TRANCE (Wong et al. 1997), and TNFR1 and TNFR2 (my data). The cross-linking of 4-1BBL on human moDC has been shown to induce maturation by means of autocrine signalling via the release of TNF as the neutralisation of TNF inhibited the increase in CD83 expression (Lippert et al. 2008). Furthermore, neutralisation of autocrine TNF during DC maturation can impair the survival of DC and enhance co-stimulatory molecule expression and T cell stimulatory capacity (Baldwin et al. 2010; van Lieshout et al. 2005; Lehner et al. 2012).

Data from the literature and my own studies therefore show that the role of TNF and other TNF superfamily members in mediating DC survival is complex and overlapping with the potential for multiple survival inducing signals to interact with each other.

### *6.4. The therapeutic potential of TNFR2-specific activation*

The regulation of DC survival is important in determining DC function. My data demonstrate that TNFR1 and TNFR2 promote DC maturation and survival through distinct and independent signalling pathways, as signals enhancing survival do not necessarily result in enhanced maturation. In particular, results showing that TNFR2 enhances moDC survival without inducing maturation represent a novel finding in the regulation of DC lifespan. This result is of interest as specific activation of TNFR2 has recently been suggested as a new immunotherapeutic strategy to selectively destroy autoreactive T cells (Faustman & Davis 2013) and to stimulate the expansion of regulatory T cells in inflammatory and autoimmune diseases due to the restricted expression of TNFR2 compared to TNFR1.

TNFR2, as opposed to TNFR1, has been shown to play a protective role in several diseases including autoimmune disorders (Ban et al. 2008), neurodegenerative disorders (Fontaine et al. 2002) and heart disease (Monden et al. 2007). Furthermore, polymorphisms in the TNFR2 gene have been associated with susceptibility to RA (Hussein et al. 2011) and although the functional effect is unknown it has been shown that the polymorphism in TNFR2 results in a significantly lower ability to induce TNFR2-

mediated NFκB signalling. The effect of TNFR2-mediated signalling was first shown using a mouse model with homozygous deficiencies in either TNFR1 or TNFR2, and revealed that TNFR2-stimulation can induce cell death in mature T cells (Zheng et al. 1995). More recently, the specific activation of TNFR2 has been proposed as a novel approach to kill autoreactive $CD8^+$ T cells in autoimmune diseases (Kodama et al. 2005; Faustman & Davis 2010). It has been shown that a TNFR2 agonist selectively killed autoreactive T cells but not healthy T cells in isolated human blood from type I diabetes patients (Ban et al. 2008). Also, a proof-of-concept randomized, controlled clinical trial has shown that BCG (the mycobacterium bovis bacillus Calmette-Guerin) induced TNF and killed insulin-specific autoreactive T cells in the circulation of type I diabetes patients (Faustman et al. 2012), which showed low toxicity although it is not specific to TNFR2.

As my data indicates that TNFR2-signalling enhances the lifespan of DC, it is vital that this is taken into account when designing therapeutic strategies involving agonistic TNFR2 ligands. For example, the unwanted side-effect of specifically activating TNFR2 and prolonging the lifespan of DC could be beneficial and support tolerance, although it could also be counterproductive and lead to a break in self-tolerance giving rise to autoimmunity (Wang et al. 1999; Chen et al. 2006). In line with this, the upregulation of TNFR2 has been associated with enhancement of autoimmune diseases such as Crohn's disease (Holtmann et al. 2002) and it has been shown in a transgenic mouse model that high levels of TNFR expression results in severe inflammatory diseases and systemic toxicity regardless of TNF levels (Douni & Kollias 1998). However, development of a therapeutic TNFR2 agonist may prove useful in the *ex vivo* generation of tolerogenic DC-based immunotherapies which would benefit from prolonged survival without the induction of maturation. In contrast there are also data suggesting that TNFR2 can mediate protective effects in arthritis, for example, using knock out mice the absence of TNFR1 has been shown to suppress induced arthritis whereas the absence of TNFR2 increased arthritis development (Blüml et al. 2010). This suggests that TNFR2 may be protective or detrimental depending on the cell type it is expressed on, thus demonstrating the importance of investigating TNFR function on specific cell types.

The role of TNFR2-mediated enhancement of DC survival in the absence of maturation is presently uncertain. Previous work has demonstrated that the regulation of DC survival in itself is important in determining DC function. For example, in mice, defective DC apoptosis leads to autoimmunity (Chen et al. 2006) and in humans, mutations in caspase-10 have been shown to underlie defects in death receptor-induced DC apoptosis in autoimmune lymphoproliferative syndrome (APLS) type I (Wang et al. 1999). Fms-like tyrosine kinase 3 (FLT3) is a cytokine receptor involved in

cell survival, proliferation and differentiation. Inhibition of FLT signalling can induce apoptosis in mouse and human DC and thereby downregulate autoimmune responses. In addition, targeted FLT3 inhibition improved disease in models for MS and EAE therefore showing potential for development into therapies for autoimmune diseases (Whartenby et al. 2005). Thus, the prevention of DC apoptosis favours autoimmunity. As TNFR2-selective stimulation enhanced DC survival, data therefore support a possible role of TNFR2 in assisting immune and/or inflammatory responses with the potential to also enhance autoimmune responses.

Impaired apoptosis is not only associated with autoimmunity but is critical in cancer development and forms a barrier to effective treatment. BH3-only proteins are Bcl-2 family members and are essential initiators of programmed cell death. BH3-only mimetics have the potential for development of novel anti-cancer drugs (Plötz & Eberle 2014; Karst & Li 2007; Vaillant et al. 2013; Adams & Cory 2007). It has been shown *in vivo* with regards to cancer therapy, that a TNFR2 agonist in non-human primates was non-toxic in a dose-dependent manner, unlike TNF which showed systemic toxicity (Welborn et al. 1996). DC have also been studied in relation to cancer immunotherapy and the development of DC-based vaccines (Palucka et al. 2007; Palucka et al. 2011). The effectiveness of these vaccines would also depend on the survival potential of the DC.

### *6.5. Conclusions*

In conclusion my data show that DC maturation and survival are differentially and independently regulated through TNFR1 and TNFR2. This study is novel as the individual roles of TNFR1- and TNFR2-mediated signalling in human DC have never been investigated. As my study is the first to make use of TNFR-selective ligands to investigate and dissect the roles of TNFR1 and TNFR2, my data represent an important step forward in the TNF-mediated regulation of human DC maturation and survival. Previous studies have been performed using knock-out mice however, as my data shows, the TNFR1- and TNFR2-mediated signalling pathways overlap, which may easily result in misinterpretations of TNFR knock out models. In particular, my data comparing ‘inflammatory’ moDC, steady-state blood myeloid DC and SF-derived myeloid DC suggests that differences between TNFR-mediated signalling pathways are not black and white and may be due to differences in receptor expression and sensitivity which can then alter the balance between receptor activation, cell maturation and apoptosis.

### *6.6. Strengths and weaknesses*

The strengths of this study include the use of TNFR-selective ligands to dissect the roles of TNFR1 and TNFR2 which has not previously been done in DC. Also the use of DC from different conditions (i.e. inflammatory versus steady state) allows data to be analysed in the context of human disease. The main weakness of this study is that moDC may not represent the majority of DC *in vivo* while the blood myeloid DC populations were not 100% pure. Also there is a lack of data regarding RA SF myeloid DC as only two samples were available. Methods to overcome these weaknesses are discussed in the following section (6.7). The use of moDC is of benefit to this project as large numbers of cells can be generated from each donor, however, there are still a number of difficulties associated with their use. For example, when LRS cones are collected from the blood donor centre the cells are at a very high density which may cause a stress response in some cells. It is therefore important that the cells were donated on the day of collection (i.e. not left from a donation given the previous day) and that the cone was processed as soon as possible. Also, factors during culture such as slamming the incubator door may cause activation of the moDC thus affecting results. The use of cell lines to such as MUTZ-3 as an in vitro model also has weaknesses as MUTZ-3 DC also require differentiation via the addition of cytokines as with moDC (Masterson et al. 2002), are heterogeneous (cells include a proliferating pool, an intermediate stage and non-proliferating DC precursor pool), and DC bypass the immature stage (Santegoets et al. 2008) making them unsuitable for this study. MUTZ-3 DC have also been shown to more closely resemble moDC than *ex vivo* primary DC (Lundberg et al. 2013).

When blocking the individual TNFR (i.e. the use of antagonistic antibodies) or using small molecule inhibitors, the blocking was not necessarily complete and there was high donor variability. I have postulated that the difference in the response to TNF between donors may be due to an initial difference in TNFR expression. Data would therefore benefit from the analysis of TNFR expression alongside the DC survival assays. In addition, experiments could be repeated in triplicate using parallel wells from the same donor to determine inter-experiment variability.

### *6.7. Future work*

For future work DC subsets (including pDC) would need to be isolated using multi-colour fluorescence activated cell sorting in order to give pure populations, although this process may also result in problems with enhanced DC activation through potential contamination of the cell sorter with endotoxins. Also, more donors are needed in order to accurately analyse DC from the SF of RA patients and to determine the statistical significance. In my results, the blocking effect of TNFR1 and TNFR2 antagonistic antibodies was superior to siRNA as they did not affect moDC maturation; however the blocking effect was still not complete in some cases presumably due to the weak agonistic activity of the antibodies. Alternatively, a Fab fragment could be used to specifically block the individual TNFR which compared to the bivalent H398 or $Fab_2$ fragment has no agonistic activity, however due to its monovalence high amounts would be required to obtain full blocking. In addition, the analysis of TNFR-specific downstream signalling the NFκB pathway analysis could be expanded along with the investigation of other TNF-activated pathways such as JNK. One potential reason for the high variation between donors may be due to the differences in TNFR expression therefore further studies would benefit from the analysis on TNFR expression in parallel to functional assays. Additionally, it would be interesting to investigate whether TNFR1 and/or TNFR2 have any synergistic effects with other DC survival signals including other members of the TNFR superfamily (as discussed in chapter 6.3). Furthermore, it would be interesting to exploring the effects of survival, maturation and T-cell activating potential following TNFR1 and/or TNFR2 stimulation in freshly isolated DC subsets (pDC and myeloid DC) from tissues (*ex vivo*) as well as DC from RA patients.

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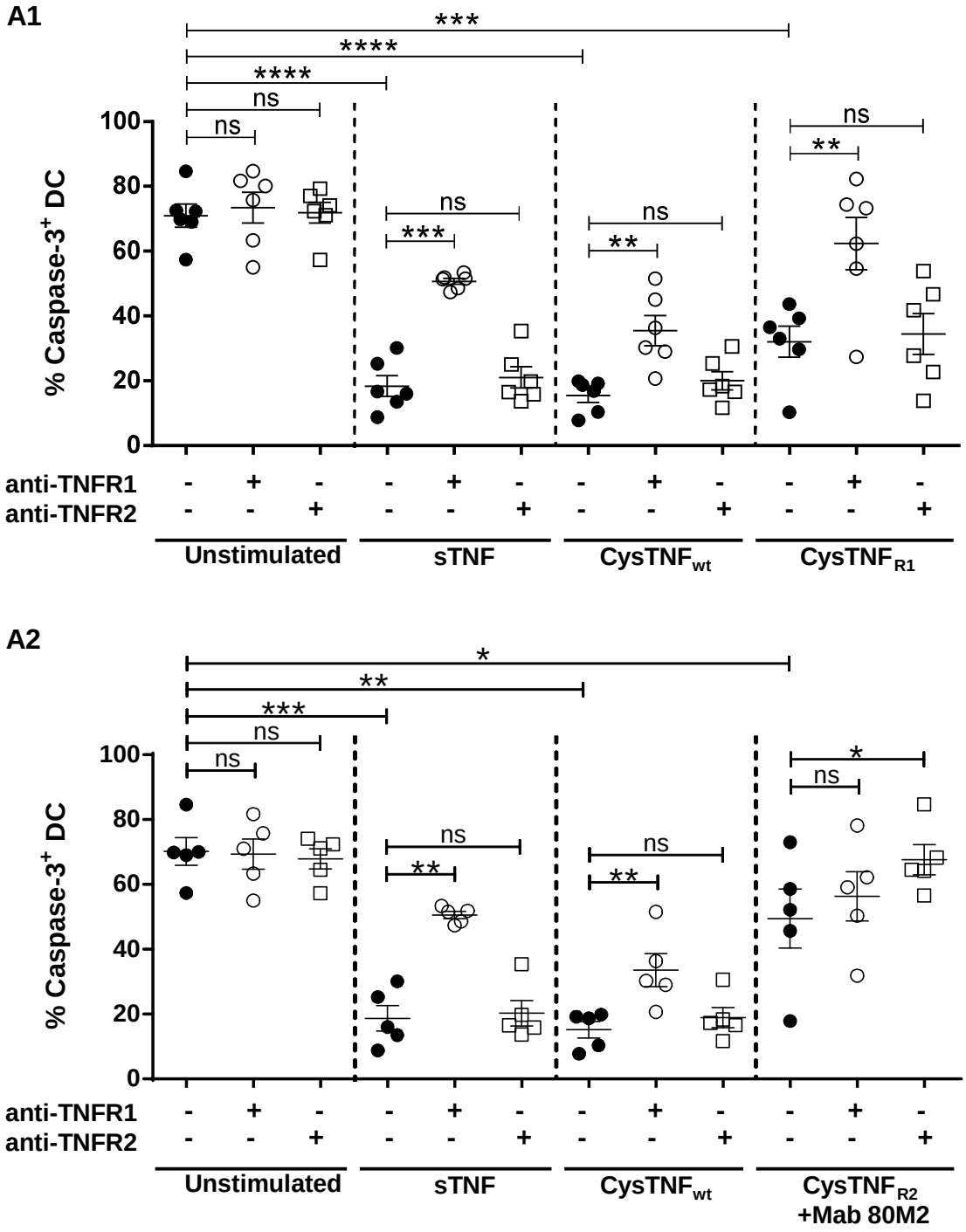
# 8. Appendices

***Appendix A: Additional figures pertaining to chapter 4.4.2.***

Data from Figure 33 “TNFR1-antagonism blocks TNFR1-mediated but not TNFR2-mediated protection from cell death and vice versa” (in moDC) divided into comparable donors.



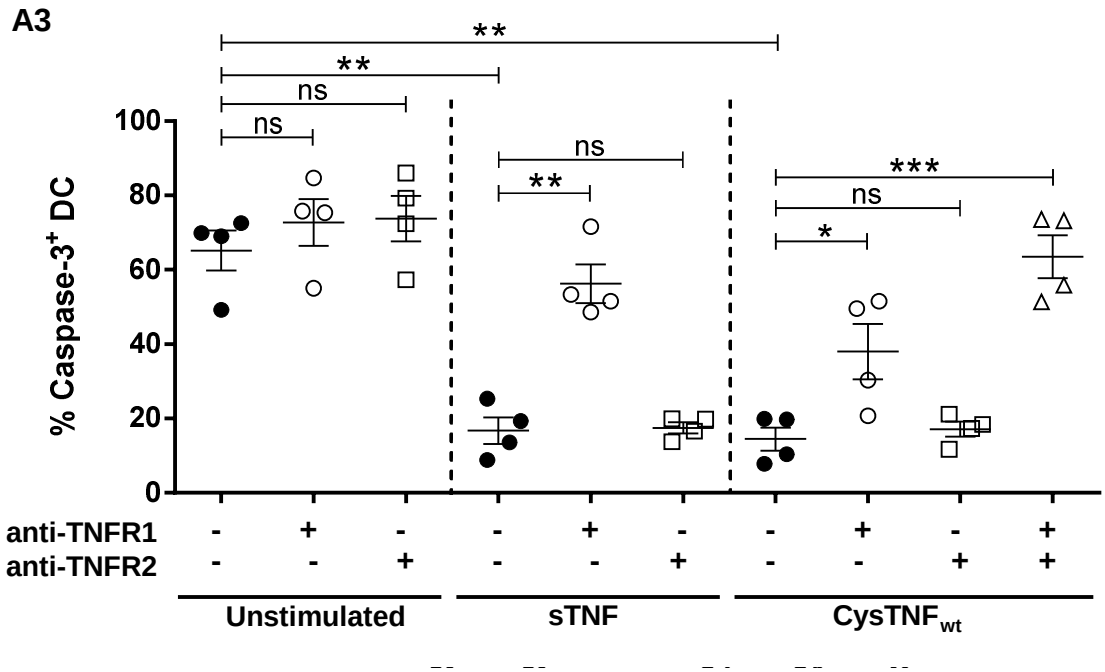
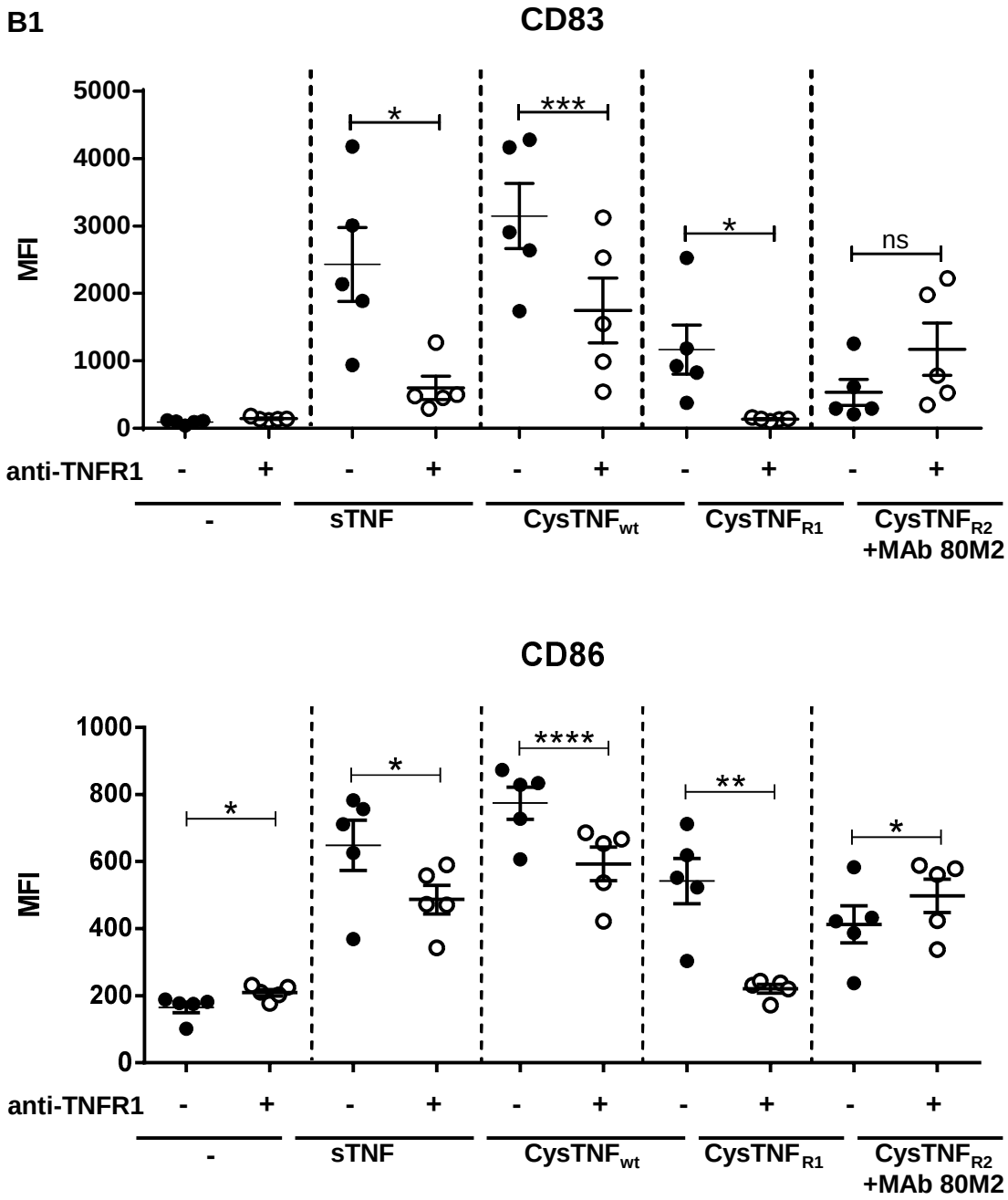
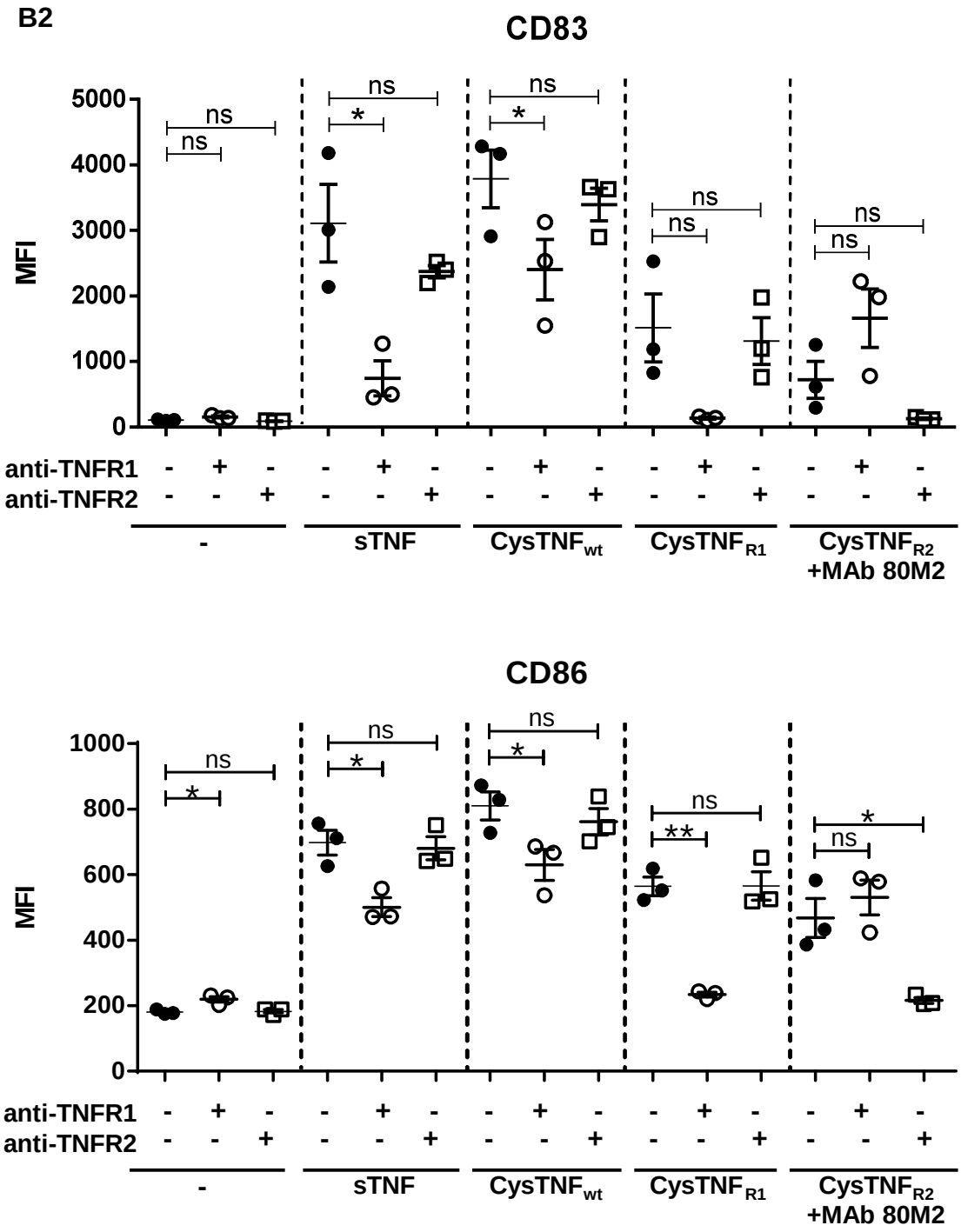



**Figure A: TNFR1-antagonism blocks TNFR1-mediated but not TNFR2-mediated protection from cell death and vice versa in moDC.** *Immature moDC were left untreated or were treated with antagonistic TNFR1- or TNFR2-specific antagonistic antibodies (clone H398 or 22221 respectively) for 30 min prior to stimulation with sTNF (A1-3), CysTNF$_{wt}$ (A1-3), CysTNF$_{R1}$ (A1) or CysTNF$_{R2}$ (A2) (all at 50 ng/ml) +/- MAb 80M2 for 48 h. Cell viability was assessed by the presence of active caspase-3 determined by flow cytometry. The percentages of cells gated positive for active caspase-3 are indicated. Results represent 6 (A1), 5 (A2) or 4 (A3) independent experiments with different donors. The mean +/- SEM are shown and statistical differences were determined in relation to unstimulated (immature) moDC using a student's t test; *P≤0.05 **P<0.01 ***P≤0.001 ****P≤0.0001.*

***Appendix B: Additional figures pertaining to chapter 5.2.2.***

Data from Figure 49 “TNFR-antagonism blocks TNFR-mediated enhancement of maturation marker expression induced by the corresponding TNFR but does not affect signalling via the other receptor” (in blood myeloid DC) divided into comparable donors.




B2
CD83
MFI
5000
4000
3000
2000
1000
0
ns
*
anti-TNFR1
anti-TNFR2
-
sTNF
CysTNF$_{wt}$
CysTNF$_{R1}$
CysTNF$_{R2}$
+MAb 80M2
CD86
1000
800
600
400
200
**

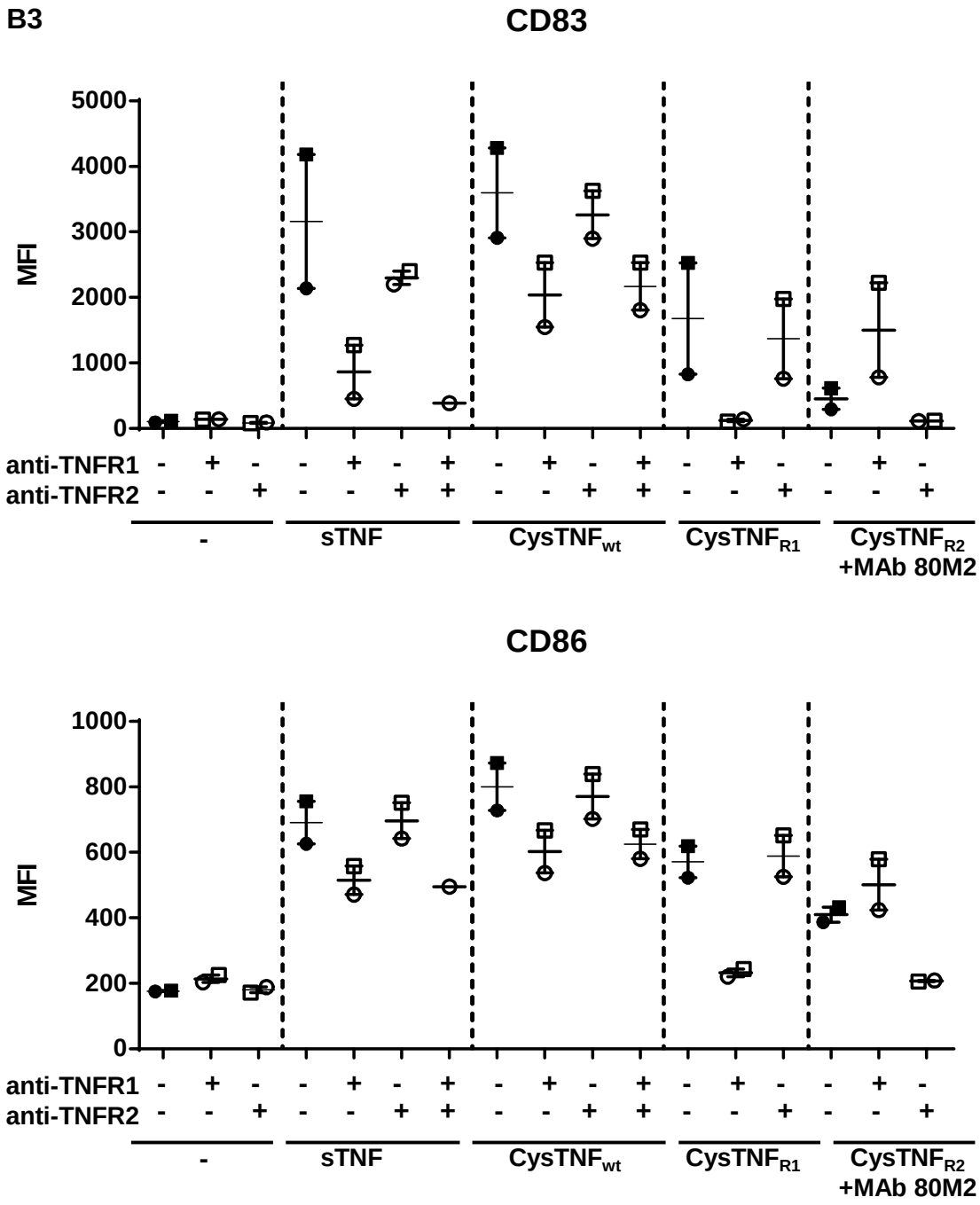
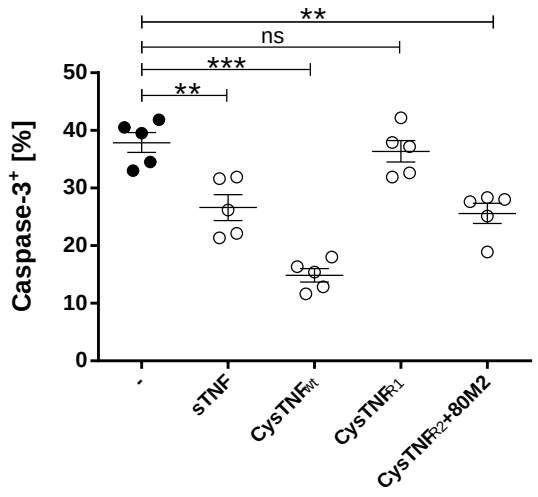



**Figure B: TNFR-antagonism blocks TNFR-mediated enhancement of maturation marker expression induced by the corresponding TNFR but does not affect signalling via the other receptor.** *Blood myeloid DC were left untreated or were treated with antagonistic TNFR1- (B1-3) or TNFR2-specific (B2-3) antibodies for 30 min prior to stimulation with 50 ng/ml sTNF, CysTNF$_{wt}$, CysTNF$_{R1}$ or CysTNF$_{R2}$ + MAb 80M2 for 24 h. The expression of CD83 (top) and CD86 (bottom) was determined by flow cytometry. The mean and SEM of the MFI (median fluorescence intensity) from 5 (B1), 3 (B2) or 2 (B3) independent donors are shown. Statistical differences were determined in relation to unstimulated blood myeloid DC using a student's t test; *P≤0.05 **P≤0.01 ***P≤0.001.*

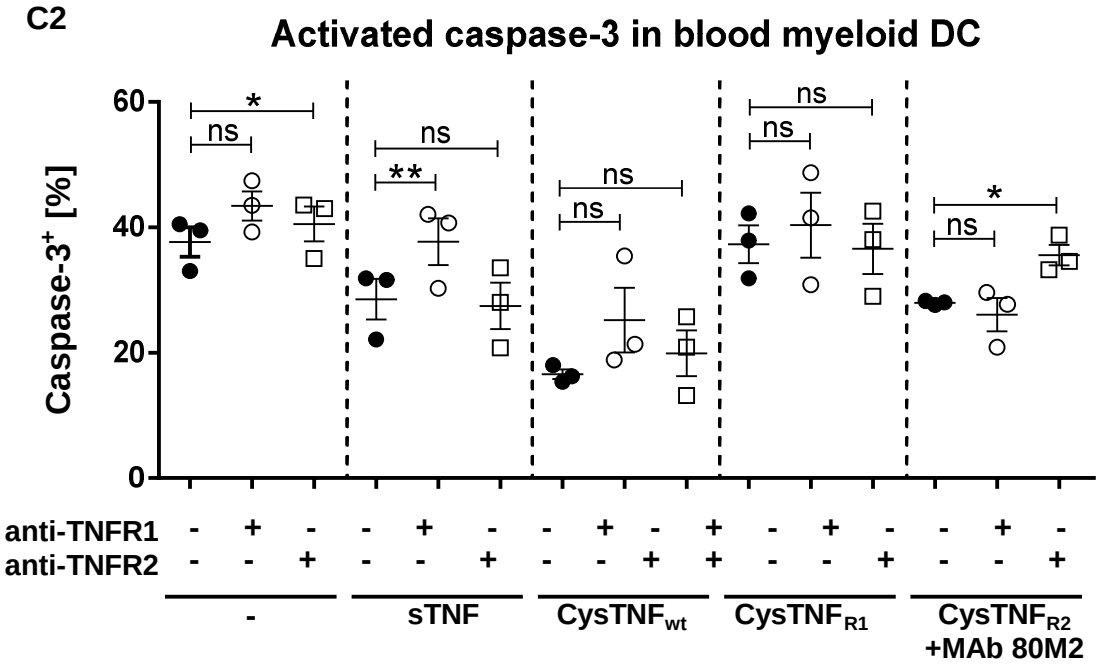
***Appendix C: Additional figures pertaining to chapter 5.2.3.***

Data from Figure 52 “TNFR2- but not TNFR1-mediated signalling protects blood myeloid DC from cytokine deprivation-induced cell death” divided into comparable donors.

**C1**




**C2**



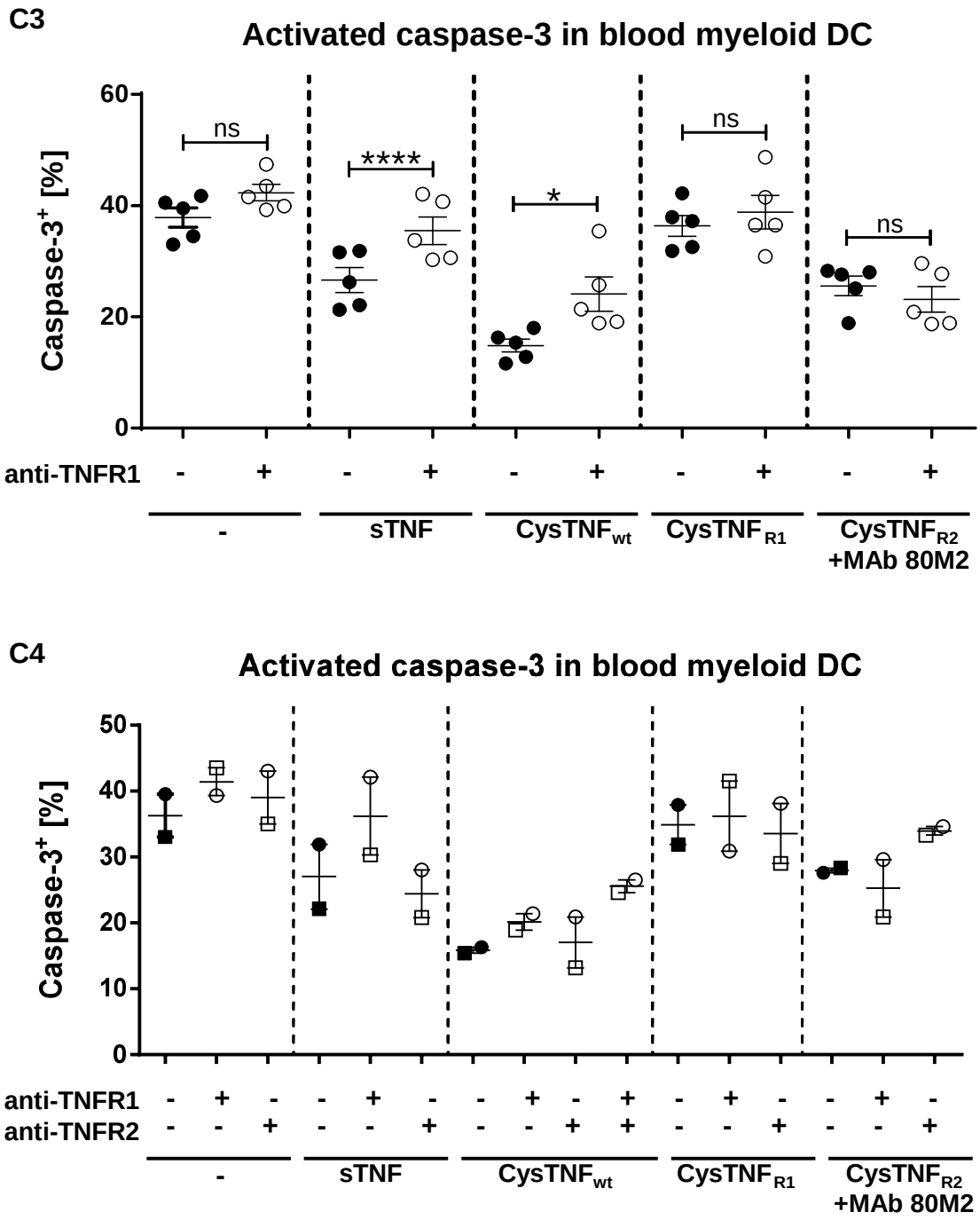



**Figure C: TNFR2- but not TNFR1-mediated signalling protects blood myeloid DC from cytokine deprivation-induced cell death.** *Blood myeloid DC were cultured in the absence (C1-4) or presence (C2-4) of 10 μg/ml antagonistic TNFR1- or TNFR2-specific antibodies (H398 or 22221 respectively) for 30 min prior to stimulation with 50 ng/ml sTNF, CysTNF$_{R1}$ or CysTNF$_{R2}$ + MAb 80M2 for 24 h. Cell viability was assessed by the presence of intracellular active caspase-3 determined by flow cytometry. The percentages of cells gated positive for caspase-3 are indicated. The mean and SEM are shown 5 (C1, C3), 3 (C2) or 2 (C4) independent experiments with different donors. Statistical differences were determined using a student's t test; *P≤0.05 **P≤0.01 ***P≤0.001 ****P≤0.0001.*

***Appendix D: HLA-DR positive cells enriched from the SF of a juvenile idiopathic arthritis (JIA) patient.***

In addition to myeloid cells from two RA patients, one JIA SF sample was obtained. PBMC were separated and enriched for myeloid DC as previously, however, during the sample purity check no cells were CD1c+ suggesting that there were no myeloid DC present (although it is also possible that CD1c was absent an error as there was no positive control). Data presented below shows HLA-DR+ cells which therefore could not be compared to the RA samples discussed in this thesis. The maturation markers CD83 and CD86 within the HLA-DR positive and caspase-3 negative cell populations were analysed after 24 h in culture with $CysTNF_{R1}$ or $CysTNF_{R2}$ plus 80M2. Results show that stimulation of TNFR2 but not TNFR1 results in an increase in the expression of CD83 and CD86. The same cells were simultaneously analysed for the expression of intracellular active caspase-3 to indicate the level of cell death. After 24 h 41% of the unstimulated cells were caspase-3 positive, this is reduced by $CysTNF_{R2}$ plus 80M2 to 23% but remains at 42% when stimulated by $CysTNF_{R1}$.

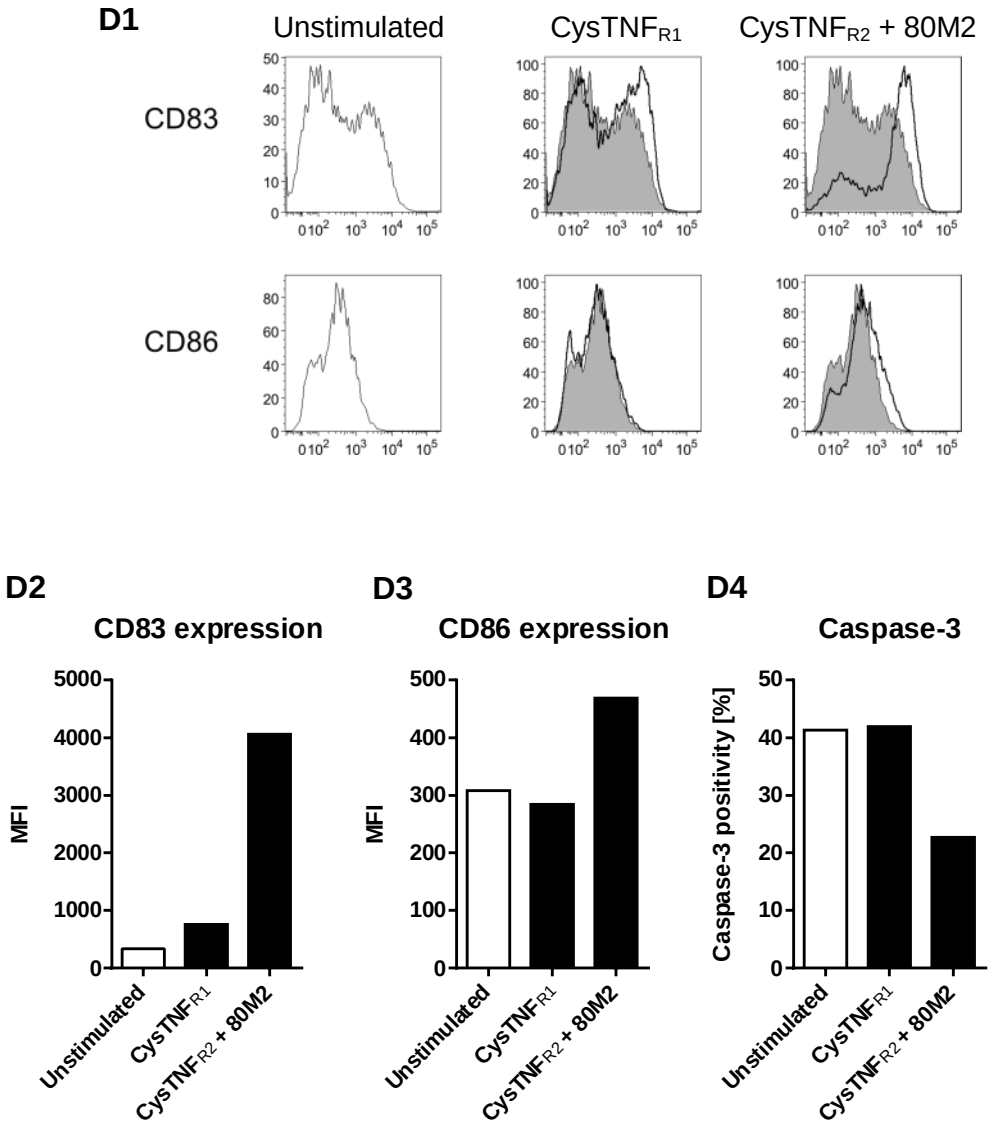



**Figure D: TNFR2- but not TNFR1-stimulation enhances the expression of surface markers CD83 and CD86 and reduces the activation of caspase-3 in HLA-DR positive cells isolated from JIA synovial fluid.** *Freshly isolated synovial fluid cells were cultured in the presence or absence of CysTNF$_{R1}$ or CysTNF$_{R2}$ (50 ng/ml) +/- MAb 80M2 for 24 h. CD83 (D1, top and D2) and CD86 (D1, bottom and D3) expression was determined by flow cytometry. (D1) Expression of CD83 and CD86 following TNFR-stimulation (black line) are shown in comparison to unstimulated cells (grey shaded) and (D2-3) the median fluorescent intensity (MFI) is shown.(D4) The induction of cell death was assessed by the presence of intracellular active caspase-3 determined by flow cytometry and the percentage of cells gated positive for active caspase-3 are indicated. (D1-4) Results represent one donor.*

***Appendix E: Published data pertaining to this thesis.***

Maney, N.J. et al., 2014. Dendritic Cell Maturation and Survival are Differentially Regulated by TNFR1 and TNFR2. *Journal of Immunology.*