Controlling the activation and inhibition of human natural killer cells

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Abstract

Natural Killer (NK) cells are innate lymphocytes that play an essential role in host defence against viral infection and tumours. The activation and function of NK cells is regulated by cytokines, in particular IL-15, which promotes their differentiation, survival and activation. IL-10 is a potent immunosuppressive cytokine; however, some studies report immunostimulatory activity towards NK cells. The aim of this study was to investigate the immunomodulatory effects of IL-10 on NK cell activity. Using an *in vitro* system of primary human NK cells, the effect of IL-10 stimulation on key NK cell activating pathways, such as cytotoxicity, receptor expression and proliferation was explored. IL-10 stimulation of NK cells enhanced granzyme B expression and NK cell cytotoxicity in a STAT3-dependent manner. Unlike the prototypical NK cell activator, IL-15, IL-10 stimulation did not alter the expression of NK cell activating receptors, induce proliferation or activate the mTOR pathway. The immunosuppressive cytokine TGF-β inhibits IL-15-mediated NK cell activation by inhibiting mTOR signalling. Interestingly, although this study does not show IL-10 activation of mTOR signalling, IL-10 mediated NK cell activation was susceptible to TGF-β-mediated inhibition. Moreover, this TGF-β mediated inhibition was independent of STAT3 signalling, suggesting the involvement of other, as yet undefined pathways. This study highlights distinct pathways of NK cell activation by IL-15 and IL-10, and defines an IL-10 mediated, mTOR-independent, activation pathway. Observations in this study suggest that the complex mechanisms leading to activation of NK cells can be induced independently of one another, providing further insight into immune cell regulation.

List of abbreviations

2-NBDG	2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxyglucose
ADCC	Antibody dependent cellular cytotoxicity
anti-CCP	Anti-cyclic citrullinated peptide
APC	Allocophycyanin
APC	Antigen presenting cell
BCR	B-cell receptor
BSA	Bovine serum albumin
CLP	Common lymphoid progenitor
CLR	C-type lectin receptor
CML	Chronic myelogenous leukaemia
CMV	Cytomegalovirus
cSMAC	Central supramolecular activation cluster
Ct	Cycle threshold
CTL	Cytotoxic T lymphocyte
CTV	Cell tracker violet
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
EBV	Epstein-Barr virus
ELISA	Enzyme linked immunosorbent assay
ETC	Electron transport chain
FBS	Fetal bovine serum
Fc	Constant region
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
GZMB	Granzyme B
HLA	Human leukocyte antigen
HMCV	Human cytomegalovirus
HSC	Haematopoietic stem cell
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
ILC	Innate lymphoid cell
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine based activation motifs
ITIM	Immunoreceptor tyrosine inhibition motifs
ITSM	Immunoreceptor tyrosine-based switch motifs
JAK	Janus kinase
KIR	Killer-Ig like receptor
LAMP-1	Lysosome-associate membrane protein 1
LCMV	Lymphocytic choriomeningitis virus
LN	Lymph node
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MFI	Median fluorescent intensity
MTOC	Microtubule organisation centre
mTOR	Mammalian target of rapamycin
NCR	Natural cytotoxicity receptor
NF-KB	Nuclear factor kappa-light chain enhancer of activated B cells
NK	Natural killer
NLR	NOD-like receptor
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PID	Primary immunodeficiency
PRR	Pattern recognition receptor
pSMAC	Peripheral supramolecular activation cluster
RA	Rheumatoid arthritis
RB	Retinoblastoma
RF	Rheumatoid factor
ROS	Reactive oxygen species

RPMI	Roswell park memorial
RT	Room temperature
RT-PCR	Real-time PCR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHP-1	SH2-domain-containing protein tyrosine phosphatase 1
SLO	Secondary lymphoid organ
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
TGF-β	Transforming growth factor beta
TH1	T helper 1
TH2	T helper 2
TIGIT	T cell immunoreceptor with immunoglobulin and ITIM domains
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF related apoptosis-inducing ligand
Tregs	Regulatory T cell
VZV	Varicella zoster virus
WT	Wild type

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Chapter 1

Introduction

1.1 The immune system – an overview

The immune system is a highly complex network that protects the host from infection and disease. Defence against infection comprises physical barriers (e.g. epithelial cell barriers, ciliated cells and mucus layers) as well as cells and proteins of the immune system, including soluble proteins (e.g. complement proteins, antibodies and defensins) and cellular responses (Chaplin, 2010, Turvey and Broide, 2010).

Immune responses are divided into two arms, innate immunity, providing a first line of defence, and adaptive immunity that provides an antigen specific response. A key defining factor between these two immune arms is the expression of specific antigen receptors. Innate immune function is regulated by the expression of germline encoded receptors, whereas adaptive immune responses require the expression of antigen-specific receptors that have undergone somatic rearrangement during development (hence the term, adaptive) (Bonilla and Oettgen, 2010).

Haematopoietic stem cells (HSCs) are the precursors of all immune cells, giving rise to two distinct lineages of cells, labelled as myeloid and lymphoid cells. Generally speaking, myeloid cells make up the innate immune system and lymphoid cells make up the adaptive immune system, with T and B lymphocytes expressing antigen-specific receptors, namely T-cell receptors (TCR) and B-cell receptors (BCR), respectively. The exception to this being

natural killer (NK) lymphocytes and the related, recently described innate lymphoid cells (ILC). Although these cells are derived from common lymphoid progenitor (CLP) cells, they do not express antigen-specific receptors and instead act as part of the innate immune response (Vivier et al., 2018).

Innate and adaptive immunity do not work in isolation but instead collaborate extensively to provide the appropriate immune response. Immune regulation is achieved by a balance of mechanisms that are used to induce an immune response, but also ones to limit that response. This tight regulation is essential to prevent uncontrolled inflammation that can lead to tissue damage and autoinflammatory or autoimmune diseases.

1.1.1 The innate immune system

The innate immune system acts as a first line of defence, providing rapid responses to control infection whilst the adaptive immune response develops. Innate immune cells express a variety of germline encoded pattern recognition receptors (PRR) to detect invading pathogens. Four classes of PRRs are found in humans, the transmembrane receptors toll-like receptors (TLR) and C-type lectin receptors (CLR), and the cytoplasmic receptors NOD-like receptors (NLR) and retinoic acid-inducible gene I like receptors (RLR). Each of the PRRs recognise distinct structural motifs expressed by pathogens (pathogen-associated molecular patterns [PAMP]) or released from damaged cells (damage-associated molecular patterns [DAMP]) (Takeuchi and Akira, 2010). Binding to these receptors stimulates an inflammatory response to control the infection, but also serves to initiate an adaptive immune response.

A key player in the initiation of the adaptive immune response are dendritic cells (DC). DCs are professional antigen presenting cells (APC) that detect pathogens, process their antigens and load them onto major histocompatibility complex molecules (MHC) that are then presented to T cells to induce an antigen specific response. DCs are innate phagocytes that process intracellular and exogenous antigens and present them on MHC I and MHC II molecules, respectively. Exposure to inflammatory cytokines (such as at sites of infection) induce the differentiation and maturation of DCs. Subsequently, DCs upregulate chemokine receptors that enables them to migrate to lymph nodes where they interact with T cells. In addition to TCR/MHC binding, DCs also express co-stimulatory molecules, such as CD80 and CD86, that are required for T cell differentiation and activation. This interaction is a key factor in priming adaptive immune response, therefore, DCs are positioned at the interface of innate and adaptive immunity (Lee and Iwasaki, 2007).

1.1.2 The adaptive immune system

The adaptive immune system is comprised of B and T lymphocytes (B cells and T cells) which mediate humoral and cell-mediated immunity, respectively. Both cell types express individual antigen receptors (BCR and TCR, respectively) that undergo somatic rearrangement during development, resulting in a highly diverse repertoire of receptors greatly enhancing the specificity of antigen recognition (Bonilla and Oettgen, 2010). B cells secrete antibody to affect humoral immunity. This includes the neutralisation of toxins, the coating of pathogens or infected cells for recognition by cells bearing Fc receptors (FCRs) (opsonisation) and the fixation of the complement cascade

(Hoffman et al., 2016). Mature T cells are generally grouped based on their expression of CD4 and CD8; CD4+ T cells being T helper cells and CD8+ T cells being cytotoxic T cells (CTLs). Once activated, CTLs mediate the killing of target cells whereas T helper cells differentiate into either Th1 or Th2 cells that have distinct differences in cytokine production (Andersen et al., 2006). The major effectors of a Th1 response are interferon-gamma (IFN-γ) and tumour necrosis factor (TNF), which promote T cell mediated immunity, whereas interleukin (IL)-4, IL-5, IL-6, IL-9, IL-10 and IL-13 are all Th2 cytokines that promote humoral responses (Romagnani, 2000). However, there is extensive crosstalk between these systems that ensure efficient immune protection.

1.2 Natural Killer cells

NK cells are a critical component of the innate immune system that play an essential role in the defence against transformed and virally infected cells. Accordingly, patients with NK cell deficiencies have increased susceptibility to viral infections and cancers. Patients with NK cell deficiencies are particularly susceptible to herpesvirus infection, importantly, several deaths have been reported in these patients as a result of severe infections with varicella zoster virus (VZV) infection, cytomegalovirus (CMV) infection and Epstein-Barr virus (EBV). Increased incidences of virus related cancers and leukaemias are also reported in this population. Furthermore, impaired NK function contributes to the pathogenesis of several primary immunodeficiencies (PID) (Orange, 2013, Mace and Orange, 2016).

NK cells cells were first identified as cells that displayed "natural cytotoxic reactivity" against a variety of tumours. These spontaneous killer cells had the morphology of small lymphocytes but lacked both T and B lymphocyte markers, leading to the classification of a third lymphocyte (B. et al., 1975a, B. et al., 1975b, R. et al., 1975). As well as lacking T and B cell antigen receptors, human NK cells are identified by the surface expression of CD56 in combination with the absence of CD3 ($CD56^{+}$ $CD3^{neg}$). The expression of CD56 is used to identify the two main subgroups of human NK cells, $CD56^{bright}$ and $CD56^{dim}$. Many reports suggest CD56 expression is linked to differentiation, with the $CD56^{bright}$ cells being a precursor to the $CD56^{dim}$, but this has been contested and the developmental relationship between these subgroups remains unclear (Domaica et al., 2012, Michel et al., 2016, Collins et al., 2019). Despite disputed stages of differentiation, $CD56^{bright}$ and $CD56^{dim}$ populations are both present in secondary lymph organs (SLO) and the periphery and appear to have different roles. $CD56^{dim}$ account for approximately 90 % of circulating NK cells, are highly cytotoxic and produce relatively low levels of cytokine, whereas $CD56^{bright}$ account for approximately 10% of circulating NK cells, are less cytotoxic but produce an abundance of cytokines. In contrast to the circulating population, approximately 90% of the NK cells in SLO are $CD56^{bright}$ and 10% are $CD56^{dim}$ (Caligiuri, 2008a) (Poli et al., 2009). The ability of $CD56^{bright}$ NK cells to make large amounts of IFN-γ and traffic to the lymph nodes is believed to be a mechanism by which NK cells promote Th1 responses and enhance T cell mediated immunity (Martín-Fontecha et al., 2004). The distinct roles of these subsets are apparent in resting NK cells and are accompanied by differential expression of receptors

and cytolytic proteins; however, upon activation, CD56^{bright} cells also acquire cytotoxicity and display anti-tumour activity (Michel et al., 2016, Wagner et al., 2017).

1.2.1 NK cell receptors

NK cells show some similarity to CTLs in their ability to kill target cells via granule mediated cytotoxicity and to secrete pro-inflammatory cytokines, such as IFN-γ (Paust et al., 2010). In contrast to antigen specific T cell activation, NK cells are regulated by a multitude of germline encoded activating and inhibiting receptors (Lanier, 2005). Our current understanding of NK cell receptors stems from the ‘missing self-hypothesis’. Based on the discovery that MHC deficient murine lymphoma cell lines were susceptible to NK cell mediated apoptosis, Karre et al. proposed that NK cells could discriminate between ‘self’ and ‘missing-self’ cells on the basis of MHC expression (Ljunggren and Kärre, 1990, Kärre et al., 1986). Since then, a plethora of NK cell activating and inhibiting receptors have been discovered (Pegram et al., 2011). Ultimately, NK cell function is determined by the balance of signals received through these receptors (Culley et al., 2009) (Figure 1).

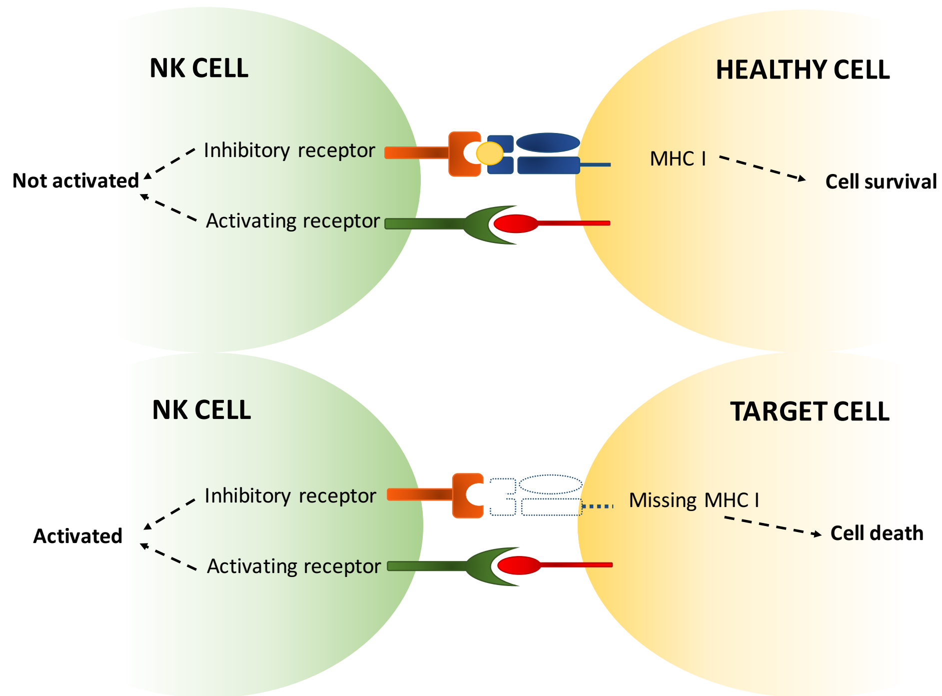


Figure 1: NK cell activation.

The activation of NK cells is governed by an array of activating and inhibitory receptors. The integration of signals through these activating and inhibitory receptors determines the activation and subsequent functions of NK cells. Figure adapted from (Lanier, 2005).

MHC: major histocompatibility complex; NK: natural killer.

1.2.1.1 NK cell inhibitory receptors

As cells of the innate immune system, NK cells respond rapidly in response to target recognition. Therefore, the activation of NK cells is tightly regulated by the expression of inhibitory receptors. Under steady state conditions, inhibitory receptor signalling prevents NK cells from eliciting unsolicited cytotoxicity, thereby protecting healthy cells and preventing autoimmune activity. The expression of inhibitory receptors is essential to educate NK cells (discussed in section 1.2.2) and allow for appropriate target recognition (Höglund and Brodin, 2010). The majority of inhibitory receptors recognise healthy 'self' cells through the expression of MHC class I molecules that are expressed by most healthy cells (Shifrin et al., 2014), notable exceptions being erythrocytes and neurons. Killer Ig-like receptors (KIRs) and the CD94/NKG2A heterodimer inhibit NK cell activation through the recognition of classical MHC I molecules (human leukocyte antigen [HLA]-A, B and C) and non-classical MHC I molecules (HLA-E), respectively (Table 1) (Braud et al., 1998, Lanier, 2005, Moretta and Moretta, 2004). Inhibitory KIRs and CD94/NKG2A share the common feature of an immunoreceptor tyrosine inhibition motifs (ITIMs) located in the cytoplasmic tail. Ligand binding induces the phosphorylation of ITIMs, which in turn phosphorylates SH2-domain-containing protein tyrosine phosphatase 1 (SHP-1) and blocks activation signals (Pegram et al., 2011). Additionally, NK cell inhibitory receptors that do not recognise MHC molecules have been identified. CD96 and T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), have both been shown to inhibit NK cell function through binding to nectin and nectin-like proteins (Martinet and Smyth, 2015). Although ITIMs have been identified

in the cytoplasmic tails of these receptors, less is known about the signalling mechanisms (Martinet and Smyth, 2015).

Expression of MHC I molecules provides protection from NK cells, conversely, T cells recognise antigens presented on MHC molecules. In an attempt to evade T cell recognition, transformed and virally infected cells frequently downregulate expression of MHC I (Bukur et al., 2012). For example, herpesviruses encode multiple proteins to inhibit antigen presentation by MHC class I (Hewitt, 2003, Lanier, 2008). In line with the missing self-hypothesis, this reduction or absence of MHC I expression would render them susceptible to NK cell mediated apoptosis (Ljunggren and Kärre, 1990).

1.2.1.2 Activating Receptors

NK cell recognition of target cells requires the engagement of activating receptors located on the plasma membrane of NK cells. Tumour cells and virally infected cells express a multitude of proteins in response to cellular stress that bind these receptors and subsequently activate NK cells (Chan et al., 2014). Multiple activating NK cell receptors have been identified in humans, including 2B4, Natural killer group 2 member D (NKG2D), DNAX accessory molecule 1 (DNAM1), and the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 (Chester et al., 2015, Pegram et al., 2011) (Table 1). Additionally, activating isoforms of KIRs, that also recognise MHC molecules, are also expressed by NK cells. Similar to the cytoplasmic ITIMs located on inhibitory receptors, most activating receptors signal via

immunoreceptor tyrosine based activation motifs (ITAMs); however, activating receptors do not encode these regions but rather associate with adaptor proteins that contain an ITAM (Moretta and Moretta, 2004). TCRζ, FcRγ and DAP-12 are ITAM bearing adaptor proteins that form complexes (homo/heterodimers) and associate with the cytoplasmic regions of specific receptors to transduce NK cell activating receptor signalling. Some activating receptors induce downstream signalling independently of ITAMs, for example, NKG2D associates with the transmembrane molecule DAP10 and 2B4 contains an immunoreceptor tyrosine-based switch motif (ITSM) (Pegram et al., 2011, Long et al., 2013).

In addition to activating receptors that detect target cell expressed ligands, NK cells also express CD16 (FcγRIII), a low affinity receptor for IgG; antibody dependent cellular cytotoxicity (ADCC) is triggered when CD16 binds to the Fc regions of clustered IgG antibodies on opsonised targets to induce cell cytotoxicity (Bryceson et al., 2006). CD16 signalling is also important in the context of therapeutic antibodies. For example, NK cell mediated ADCC is known to contribute to the actions of monoclonal antibodies used in the treatment of some cancers (Ochoa et al., 2017). NK cells do not possess a dominant activation receptor (e.g. analogous to the TCR or BCR), and subsequently require synergistic co-signalling through multiple receptors to achieve activation. With the exception of CD16 (which mediates ADCC rather than ‘natural killing’), engagement of no single receptor has been found to induce degranulation and so all are described to be ‘co-activation’ receptors (Long et al., 2013). Moreover, the balance of NK cell activating/inhibitory signals is further regulated by a ‘threshold’ of MHC I expression, meaning that

small changes in the expression of ligands can drastically alter NK cell responses. For instance, tumour cell expression of MHC I below the threshold results in NK cell activation, whereas tumour cells with MHC I expression above this threshold did not activate NK cells (Holmes et al., 2011).

NK cell receptors	Ligand	Activating/Inhibitiory
Natural cytotoxicity receptors (NCRs)		
NKp30	B7-H6/BAT3/BAG6	Activating
NKp44	Viral HA	Activating
NKp46	Viral HA, HSPG	Activating
NKp80	AICL	Activating
DNAM1	CD112 or CD155	Activating
NKG2D	MICA/B, ULBP 1-4	Activating
2B4	CD48	Activating
Killer Ig-like receptors (KIRs)		
KIR2DL4	MHC I (HLA-A, B, C)	Activating
KIR2DS1		Activating
KIR2DS2		Activating
KIR2DS3		Activating
KIR2DS4		Activating
KIR2DS5		Activating
KIR2DL1	MHC I (HLA-A, B, C)	Inhibitory
KIR2DL2/3		Inhibitory
KIR2DL5		Inhibitory
KIR3DL1		Inhibitory
KIR3DL2		Inhibitory
CD94/NKG2A	MHC I (HLA-E)	Inhibitory
TIGIT	CD155	Inhibitory
CD96	CD155	Inhibitory

Table 1: NK cell receptors

Activatory and inhibitory NK cell receptors and their reported ligands. Table adapted from (Long et al., 2013, Pegram et al., 2011, Martinet and Smyth, 2015).

AICL: activation-induced C-type lectin; BAG6: BCL2-associated athanogene 6; BAT-3; HLA-B-associated transcript 3; DNAM1: DNAX accessory molecule 1; HA: hemagglutinin; HLA: human leukocyte antigen; HSPG: heparin sulphate proteoglycan; MICA/B: MHC class 1 chain related protein A/B; MHC: major histocompatibility complex; NKG2D: Natural killer group 2 member D; TIGIT: T cell immunoreceptor with immunoglobulin and ITIM domains; ULBP: UL16 binding protein.

1.2.2 NK cell education

The expression of NK cell receptors is highly heterogeneous and results in many phenotypically distinct mature NK cells, including NK cells that are hyporesponsive. Mostly, NK cells express one KIR providing efficient detection of cells that lose the corresponding MHC class I ligand (e.g. as a result of natural selection by T cells). However, a small population of NK cells do not express KIRs, or express KIRs that do not have a corresponding MHC ligand. NK cells lacking KIRs (or lacking engageable KIRs) are potentially dangerous, as they cannot be inhibited by MHC class I on healthy cells; these NK cells are rendered hyporesponsive (Elliott and Yokoyama, 2011, Höglund and Brodin, 2010, Anfossi et al., 2006). This hyporesponsive state is a direct result of being ‘uneducated’. NK cell education is the term used to explain the paradoxical hypothesis that NK cells require inhibitory signalling to achieve effector functions and self-tolerance. During development, engagement of inhibitory KIRs educates the NK cells and is an important step to gain effector functions. The exact mechanism of how NK cells become educated is a debated topic and there are currently three proposed models. The ‘arming’ model proposes that NK cells become armed through the ligation of KIRS and subsequent signalling via the inhibitory signalling pathway. The ‘disarming’ model proposes that NK cells are readily reactive and require inhibitory KIR engagement to prevent sustained activation, failure to engage inhibitory receptors ‘disarms’ the NK cells by activation-induced anergy. Lastly, the ‘rheostat’ model proposes that NK cell reactivity is determined by the expression of KIRs and the affinity and availability of ligands, with higher expression of inhibitory KIRs resulting in better education of NK cells (Orr and

Lanier, 2010, Boudreau and Hsu, 2018). More recently, a role for NKG2A and the non-MHC-I receptor in NK cell education has emerged, with co-expression of receptors resulting in better responsiveness of NK cells (He and Tian, 2017). Unlike clonal deletion of autoreactive T-cells, uneducated NK cells remain in circulation in a hyporesponsive state. These uneducated NK cells can subsequently be activated by pro-inflammatory cytokines (Holmes et al., 2014). While educated NK cells are essential to recognise target MHC I deficient cells, the activation of uneducated NK cells is beneficial against some infections and tumours (Orr et al., 2010, Tu et al., 2016). Cytokine activation of these KIR negative, hyporesponsive NK cells enables them to attack target cells without being restricted by MHC I expression. However, these activated, uneducated NK cells are also a potential source of tissue damage, as they might attack MHC class I expressing healthy cells.

1.3 Mechanisms of NK cell cytotoxicity

The primary killing mechanism of NK cells is the exocytosis of lytic granules (degranulation) across an immunological synapse (IS), at the interface of the NK and the target cell (Figure 2). Upon target recognition and NK cell activation, ligation of integrins such as LFA-1 initiates the formation of the highly organised IS. Integrins are localised to the peripheral supramolecular activation cluster (pSMAC), whilst activating NK cell receptors localise to the central supramolecular activation cluster (cSMAC). Integrin signalling results in the reorganisation of F-actin and the polarisation of the microtubule organisation centre (MTOC) towards the IS. Lytic granules are then able to move along the microtubules and translocate to the MTOC. Once located at

the immunological synapse, the lytic granules navigate through the dense actin network and fuse with the plasma membrane to release their contents across the synapse; a regulated exocytic mechanism (Krzewski and Coligan, 2012, Mace et al., 2014). Perforin is essential to enable entry of granzymes into the target cell. Perforin monomers oligomerises on the surface of the target cell and create pores through which the granzymes can diffuse (Lopez et al., 2013) (Figure 2). Five granzymes have been identified in humans (A, B, H, K and M), of which granzyme B is the most abundant and well-studied. The granzymes are a family of serine proteases and, in the case of granzyme B, the specificity resembles that of caspases, cleaving on the *C*-terminal side of aspartate residues (Chowdhury and Lieberman, 2008). Granzyme B, a potent pro-apoptotic enzyme, induces apoptosis through caspase-dependent and caspase-independent pathways. The primary mechanism of human granzyme B mediated cell death is through cleavage of the pro-apoptotic protein BID, resulting in mitochondrial disruption, cytochrome *c* release and subsequent apoptosis (Pinkoski et al., 2001, Cullen and Martin, 2008). Additionally, granzyme B can directly cleave caspases and caspase substrates, resulting in rapid apoptosis of the target cell (e.g. within minutes) (Scott et al., 2010, Voskoboinik et al., 2015, Martinvalet, 2019). Recent studies have also described a role for reactive oxygen species (ROS) in granzyme B induced apoptosis (Jacquemin et al., 2015, Chiusolo et al., 2017). Granzyme B was shown to enter intact mitochondria via transport proteins and cleave subunits in the electron transport chain (ETC) complex I that resulted in impaired mitochondrial respiration and rapid ROS production. Moreover, inhibiting entry

to the mitochondria severely reduced the cytotoxic activity of granzyme B (Chiusolo et al., 2017, Jacquemin et al., 2015).

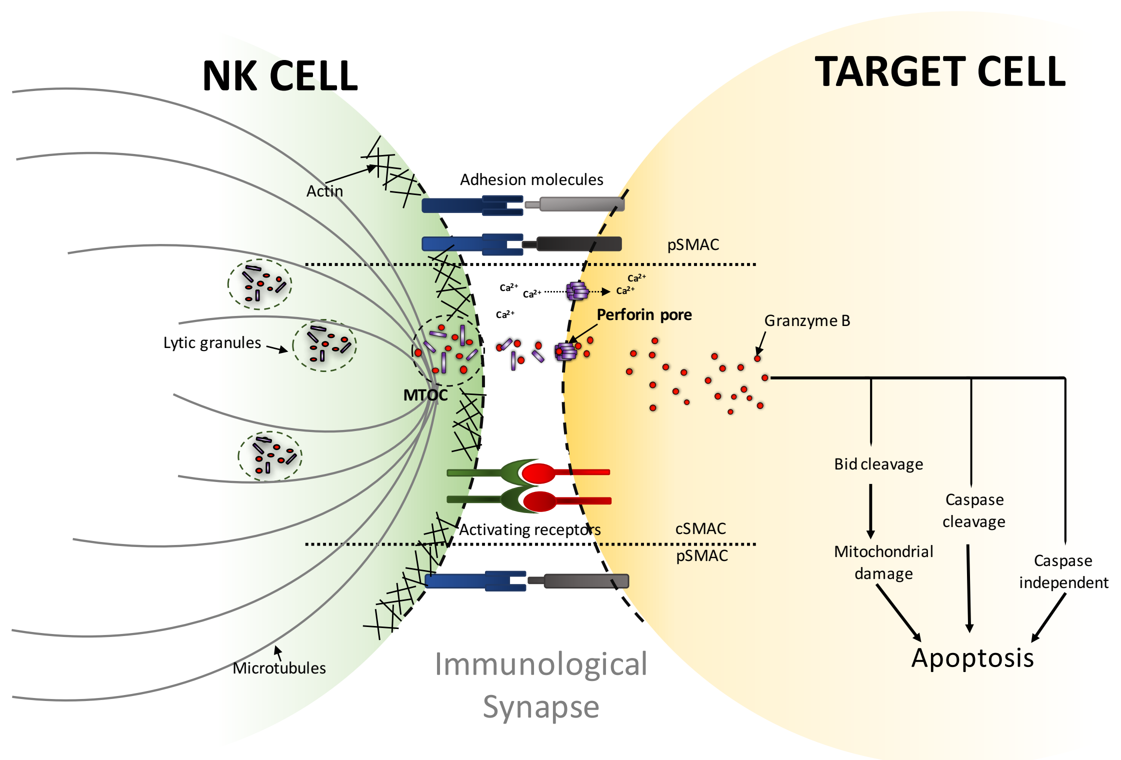


Figure 2: Activatory immunological synapse

Schematic overview of the immunological synapse between NK cell and target cells during degranulation. Upon activation, NK cells undergo cytoskeletal rearrangement that allows the secretion of lytic granules across the immunological synapse in a polarised manner. Once secreted, granzymes enter the target cells through perforin formed pores and induce apoptosis through several different pathways. Figure adapted from (Krzewski and Coligan, 2012, Smyth et al., 2005).

cSMAC: central supramolecular activation cluster; MTOC: microtubule organisation centre; NK: natural killer; pSMAC: peripheral supramolecular activation cluster.

1.4 Lytic granules

The lytic granules are specialised secretory lysosomes located within the NK cell cytoplasm that contain the major cytotoxic proteins perforin and granzymes. Due to the highly cytotoxic activity of the contents, the organisation and environment within the lytic granules inhibits the activity of the proteins to provide safe storage of the potentially lethal molecules. For instance, proteoglycans associate with perforin and granzymes in the core of the lytic granules to prevent diffusion of the proteins into the cell. Additionally, the luminal environment of the lytic granules is acidic (pH5.5) to inhibit the proteins that are optimally active at neutral pH (Anthony et al., 2010). Other proteins located in the lytic granules include cathepsins to proteolytically activate granzyme and perforin, and the pro-inflammatory molecule granulysin (Krzewski and Coligan, 2012). Lysosome-associated membrane protein 1 (LAMP-1/CD107a) is the most abundant protein located on the lytic granule membrane, and is therefore used as a marker of degranulation. The expression of LAMP-1 appears to be important in perforin recruitment to lytic granules and granule motility. LAMP-1 deficient cells display reduced perforin in the lytic granules, reduced motility of granules and ultimately reduced cytotoxicity (Krzewski et al., 2013).

In addition to granule dependent cell death, NK cells can induce target cell death via death receptor pathways. The NK cell expression of TNF related apoptosis-inducing ligand (TRAIL) and FAS ligand (FasL) mediate cell death of target cells that express the cognate receptors, ligation of these receptors recruit death domains and subsequently induces apoptosis via the caspase cascade (Kayagaki et al., 1999, Arase et al., 1995, Wallin et al., 2003).

1.5 Pro-inflammatory cytokine secretion

The second effector function of NK cells is the secretion of the pro-inflammatory cytokines such as IFN-γ, tumour necrosis factor (TNF) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Fehniger et al., 1999). In addition to activating receptor ligation, NK cell cytokine production can be induced through cytokine receptor stimulation. To enable rapid production of cytokines, NK cells constitutively express receptors for cytokines such as IL-12, IL-15 and IL-18 (Fehniger et al., 1999, Vivier et al., 2011, Holmes et al., 2014). These NK cell-derived cytokines have several immunomodulatory functions that contribute towards pathogen clearance and activation of the adaptive immune response.

1.5.1 infection

Upon pathogen detection, immune cells secrete a plethora of inflammatory cytokines that can induce NK cell cytokine secretion. For instance, the most abundantly expressed cytokines in response to viral infections are type 1 IFNs (IFN-α/IFN-β). Type 1 IFNs activate NK cells to secrete IFN-γ that helps to resolve viral infection (Christine A. Biron et al., 1999) (Mack et al., 2011, Martinez et al., 2008). Other cytokines such as IL-12 and IL-15 are also important mediators of NK cell function in viral infections. Type 1 IFNs induce the expression of IL-15 which in turn promotes NK cell survival, whereas IL-12 is a potent inducer of IFN-γ (Nguyen et al., 2002, Baranek et al., 2012, Orange and Biron, 1996). Furthermore, viruses can directly induce IFN-γ and

TNF-α from NK cells through TLR stimulation (Adib-Conquy et al., 2014) (Sivori et al., 2004).

1.5.2 Adaptive immune response

IFN-γ is essential to help prime adaptive immune responses, in particular, NK cell-derived IFN-γ is essential for Th1 polarisation. In lymph nodes, activated NK cells produce IFN-γ, which in turn stimulated the production of IL-12 from DCs that promotes Th1 polarisation (Martín-Fontecha et al., 2004). At sites of inflammation, NK cell-derived IFN-γ enhances the expression of MHC class I and II and promotes the differentiation of monocytes into DCs (Goldszmid et al., 2012). Moreover, DCs interact with NK cells via surface receptors, to induce the secretion of IFN-γ and TNF-α and promote DC maturation (Vitale et al., 2005).

1.5.3 Tumour immune surveillance

NK cell-derived cytokines also have an important function in tumour immune surveillance. As tumour cells are 'self' cells, they do not express PAMPs and thus do not trigger DCs via PRRs. However, NK cells identify tumour cells via the integration of signals through activating and inhibitory receptors. In response to activating receptor ligation, NK cells produce IFN-γ, TNF, GM-CSF and TNFSF14 that enhance anti-tumour activity (Holmes et al., 2014). GM-CSF promotes the maturation of local monocytes into DCs, whereas IFN-γ, TNF and TNFSF14 enhance antigen presentation and favour the maturation and activation of DCs. Subsequently, activated DCs express IL-15 which enhances NK cell survival and activation. Thus, through their ability to

identify “altered self” and produce DC activating cytokines, NK cells provide a surveillance function against self-cells that lack PAMPs (Holmes et al., 2014).

1.5.4 NK-DC crosstalk

NK-DC interactions are important mediators of the immune response, reciprocally activating one another at sites of inflammation and in the LN (Thomas and Yang, 2016). As previously mentioned, DCs activate NK cells which subsequently promote the maturation and activation of DCs (Ferlazzo and Morandi, 2014). This activation is dependent on the secretion of soluble inflammatory mediators, such as DC-derived IL-12 and NK cell-derived IFN-γ, and also cell-to-cell contact (Gerosa et al., 2002, Piccioli et al., 2002). In fact, NK-DC conjugates enable the polarised secretion of IL-12 across a synapse towards NK cells to induce IFN-γ expression (Borg et al., 2004). Additionally, NK-DC conjugation enables NK cells to recognise and kill immature DCs (through low MHC I expression), thereby selecting mature DCs that will subsequently provide better T cell activation (Morandi et al., 2012).

1.5.5. Interferon gamma regulation

NK cell-derived IFN-γ is finely tuned and dependent on the stimulus. Studies have shown that different combinations of cytokines induce IFN-γ production to different extents, for example, IL-12 and IL-18 are both potent inducers of IFN-γ and can act synergistically with other cytokines to enhance IFN-γ secretion (Nielsen et al., 2016, Freeman, 2015). IL-18 has also been shown to synergise with type 1 IFNs during viral infections ((Matikainen et al., 2001),

whilst IL-21 and IL-18 have been shown to enhance IL-15 mediated expression of IFN-γ (Strengell et al., 2003).

1.5.6 Cytokine secretion

Secretion of the pro-inflammatory cytokines IFN-γ and TNF is distinct from perforin secretion. Unlike the polarised release of perforin across a synapse towards a target cell, IFN-γ and TNF are secreted from the entire cell surface in a non-polarised manner via the conventional secretory pathway (Reefman et al., 2010).

1.6 IL-15 regulated NK cell activity

In addition to receptor ligation, NK cell activity is regulated by cytokines. Pathogens induce a plethora of immunomodulatory cytokine secretion which then modulate immune cell functions such as proliferation and activation. In particular, IL-15 is central to NK cell development and function; stimulating the differentiation, survival and activation of NK cells (Huntington, 2014).

1.6.1 Development

Studies in mouse models highlight the critical role of IL-15 in NK cell development. Bone marrow stromal cell-derived IL-15 is essential to promote the differentiation of $CD34^+$ progenitor cells into NK cells (Mrózek E, 1996), and mice that are deficient in IL-15 ($IL\text{-}15^{-/-}$) or the IL-15R ($IL\text{-}15R^{-/-}$) have profoundly reduced NK cell numbers. In $IL\text{-}15^{-/-}$ mice, the addition of

exogenous IL-15 can reverse NK cell deficiency, underlining the important role of IL-15 in NK cell expansion (Lodolce et al., 1998, Kennedy et al., 2000). Moreover, *IL-15R*$^{-/-}$ mice have significantly smaller lymph nodes than wild-type (WT) mice, caused by reduced homing to the lymph node, and reduced proliferation of cells in the lymph nodes (Lodolce et al., 1998).

1.6.2 Survival

The survival of NK cells is also mediated by IL-15. IL-15 supports the survival of NK cells by enhancing the expression of anti-apoptotic proteins (e.g. Bcl-2, Mcl-1) (Cooper et al., 2002, Ranson et al., 2003) and inhibiting the expression of pro-apoptotic proteins (e.g. Bim, Noxa, Puma) (Huntington et al., 2007, Inoue et al., 2010). This was first demonstrated by Cooper et al. using IL-15 knockout mouse models. NK cells that had been adoptively transferred into *IL-15*$^{-/-}$ failed to survive beyond 5 days, whereas NK cells transferred into *IL-15*$^{+/-}$ or WT mice could still be detected at this time-point. The anti-apoptotic protein Bcl-2 was identified as a key regulator of this survival, accordingly, adoptively transferred cells from mice overexpressing Bcl-2 could survive in *IL-15*$^{-/-}$ mice (Cooper et al., 2002). In addition to Bcl-2, IL-15 induced expression of the anti-apoptotic protein Mcl-1 has also been identified as a key regulator of NK cell survival (Huntington et al., 2007, Sathe et al., 2014). Mcl-1 has also been implicated in the development and differentiation of NK cells. Specific deletion of Mcl-1 in murine NK cells resulted in the complete loss of mature NK cells with only a minor population of immature NK cell remaining (Sathe et al., 2014).

Aside from anti-apoptotic proteins, IL-15 regulation of pro-apoptotic proteins also contributes to NK cell survival. IL-15 downregulates expression of the pro-apoptotic protein Bim through multiple different mechanisms. Firstly, IL-15 stimulation of the Erk1/2 pathway targets Bim for proteosomal degradation, secondly; IL-15 suppresses the transcription of Bim in a phosphoinositide 3-kinase (PI3K) dependent manner (Huntington et al., 2007).

1.6.3 Activation

IL-15 is the prototypical activator of NK cells and, as such, mediates multiple mechanisms that contribute to the robust activation of NK cells. Key activation mechanisms such as proliferation, cytotoxicity and cytokine production are all induced in response to IL-15 stimulation (Carson et al., 1994, Dunne et al., 2001, Holmes et al., 2014, Anton et al., 2015, Wagner et al., 2017). Furthermore, the expression of activating receptors such as NKG2D, DNAM1, NKp46 and NKp30 are all upregulated in response to IL-15 (Wilson et al., 2011, de Rham et al., 2007). As previously mentioned, activated DCs are a key source of IL-15 and therefore potent activators of NK cell effector function (Nguyen et al., 2002, Baranek et al., 2012).

IL-15 signals through a member of the common γ-chain family of cytokine receptors. Receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 all utilise the common γ-chain (CD132) as the signalling chain of their receptor complex. The IL-15 receptor is a heterotrimeric receptor comprised of the ligand binding domain (IL-15Rα), the IL-2Rβ (CD122) and CD132, the latter two components

of which it shares with the IL-2 receptor (Mishra et al., 2014, Lodolce et al., 2002). IL-15 initiates signalling through trans-presentation of soluble or membrane bound IL-15/IL-15Rα complexes to the CD122/CD132 complex (Lucas et al., 2007, Stonier and Schluns, 2010, Huntington et al., 2009, Zanoni et al., 2013). Cis-presentation of IL-15 has also been identified in NK cells. Unlike trans-presentation that requires cell-to-cell contact, cis-presentation requires the NK cell derived IL-15/IL-15Rα complex to be displayed on the cell surface where it can interact with the CD122/CD132 complex (Zanoni et al., 2013, Stonier and Schluns, 2010). While IL-15 can bind the CD122/CD132 in the absence of IL-15Rα, it has much higher affinity for IL-15Rα (Carson et al., 1994, Guo et al., 2017).

Like many cytokines, IL-15 utilises the Janus Kinase (JAK)- signal transducer and activator of transcription (STAT) signalling pathways to transduce signalling, specifically through the phosphorylation and activation of JAK1/3, STAT5 and STAT3 (Johnston et al., 1995, Lodolce et al., 2002). Additionally, IL-15 has also been shown to utilise other signalling pathways downstream of IL-15 receptor binding, including the PI3K and mitogen-activated protein kinase (MAPK) pathways (Wagner et al., 2017). Through these pathway, IL-15 regulates gene expression of anti-apoptotic and pro-inflammatory mediators that enhance the activation and survival of NK cells (Mishra et al., 2014).

Recently, IL-15 has been shown to regulate the immunometabolism of NK cells via the mammalian target of rapamycin (mTOR) pathway. Several studies have suggested that IL-15 stimulation induces a metabolic switch that increases the rate of glycolysis and oxidative phosphorylation (OXPHOS) that

is regulated by activation of the mTOR pathway (Nandagopal et al., 2014, Marçais et al., 2014). In particular, mTOR activation and the subsequent metabolic changes have been shown to enhance IFN-γ production, proliferation and anti-tumor activity (Donnelly et al., 2014, Nandagopal et al., 2014, Marçais et al., 2014).

1.7 TGF-β regulated NK cell activity

Transforming growth factor-beta (TGF-β) can impact the activity of most cell types; hence, it plays a role in several physiological and immunological processes (David and Massagué, 2018). In immunological processes, TGF-β is a prominent immunosuppressive cytokine that regulates inflammation via multiple mechanisms. For example, TGF-β mediated the differentiation of T cell subsets by supressing the differentiation of T helper cells and promoting the differentiation of T regulatory cells (Tregs) (Sanjabi et al., 2017, Yoshimura et al., 2010).

The canonical signalling pathway for TGF-β requires the phosphorylation and activation of downstream Smad proteins. Briefly, TGF-β binds to a heterotrimeric receptor comprised of one TGF-β receptor 1 (TGFBR1) chain and two TGFBR2 chains. Ligation of the receptor induces the phosphorylation of receptor-regulated Smads (R-Smads) 2 and 3, followed by the binding of cytoplasmic Smad 4 to form a transcriptional complex which translocates to the nucleus and binds hundreds of different loci throughout the genome (Heldin and Moustakas, 2016, Batlle and Massagué, 2019). This transcriptional complex binds DNA in combination with other co-

transcription factors (co-activators or co-repressors), the availability of which helps determine context specific responses (Mullen et al., 2011). Inhibitory Smads, Smad 6 and 7, are also regulated by TGF-β and provide a negative feedback loop by regulating TGFBR degradation (Batlle and Massagué, 2019). Additionally, MAPK and PI3K signalling has also been identified downstream of TGF-β signalling (Lee et al., 2007, Heldin and Moustakas, 2016); however, these Smad-independent pathways are less well-defined.

The role of TGF-β in tumour progression is not straightforward, with studies detailing tumour suppressive and tumour promoting actions of TGF-β (Honjo et al., 2007, Liang et al., 2016). Generally, TGF-β supresses tumour progression through several mechanisms, including triggering apoptosis in cells harbouring oncogenic mutation and inhibiting the proliferation of cells through the upregulation of cell cycle inhibitors (Batlle and Massagué, 2019). However, TGF-β is a potent inhibitor of T cell and NK cell effector functions which can indirectly promote tumour progression (Sanjabi et al., 2017, Yoshimura et al., 2010). Tumour cells themselves frequently become refractory to the action of TGF-β, e.g. via mutations in the receptor or in signalling components. Thus, in a TGF-β rich tumour microenvironment, the tumour maybe unaffected but immunity will be impaired (Seoane and Gomis, 2017).

In NK cells, TGF-β signalling has been shown to inhibit several activation mechanisms, including cytokine induced NK cell proliferation, cytokine secretion and cytotoxicity (Bellone et al., 1995, Viel et al., 2016, Yu et al., 2006, Zaiatz-Bittencourt et al., 2018). Furthermore, TGF-β inhibits IL-15 mediated upregulation of activating receptors such as NKG2D, DNAM1,

NKp30 and NKp46 (Wilson et al., 2011, Crane et al., 2009). However, the molecular mechanisms that mediate TGF-β inhibition of NK cell activation are largely unknown. While some studies highlight the role of canonical TGF-β in NK cell inhibition (Trotta et al., 2008, Yu et al., 2006), other mechanisms have also been suggested. In particular, one study recently identified mTOR as a key target of TGF-β inhibition. In this study, TGF-β and the mTOR inhibitor, rapamycin, were shown to have comparable effects. In IL-15 stimulated NK cells, TGF-β inhibited the proliferation, metabolism and cytotoxicity of NK cells in an mTOR dependent manner. In response to TGF-β, NK cells had reduced expression of cytolytic proteins coupled with reduced ability to kill target cells. Moreover, *Tgfbr2-/-* NK cells had increased phosphorylation of S6 (downstream of mTOR), but no difference in STAT5 phosphorylation when compared to WT cells, suggesting that mTOR rather STAT5 is a key target of TGF-β (Viel et al., 2016).

In contrast to this, a recent study reported that canonical TGF-β signalling mediates inhibition of NK cell function independently of mTOR inhibition (Zaiatz-Bittencourt et al., 2018). As described, the signalling pathways utilised by TGF-β have not been fully elucidated; therefore, further investigation into TGF-β-NK cell interactions are needed.

1.8 Interleukin 10

Primarily described as an immunosuppressive cytokine, interleukin-10 (IL-10) is a key regulator of immunity. Originally named ‘cytokine synthesis inhibitory factor’, IL-10 was first identified as a factor secreted from Th2 clones that

could inhibit the production of cytokines from Th1 clones (Fiorentino et al., 1989). In many cell types, IL-10 is a potent immunosuppressor and works to limit the immune response and prevent autoimmune or inflammatory pathologies (Couper et al., 2008) (see section 1.8.4). However, increasingly, the immunosuppressive designation of IL-10 is being challenged. A growing number of studies detail immunostimulatory activities of IL-10 in a range of different cell types, leading to the acknowledgement that IL-10 is in fact a pleiotropic cytokine with multiple functions (Mocellin et al., 2003) (see section 1.8.5).

1.8.1 Sources of IL-10

Although T cells are still considered to be a main source of IL-10, it is now widely accepted that IL-10 is produced from almost all leukocytes. IL-10 production is regulated in a cell-type dependent manner, and cell-specific secretion of IL-10 mediates different environments (Saraiva and O'Garra, 2010). For instance, IL-10 plays a pivotal role in gut homeostasis (Zhou and Sonnenberg, 2018) and a specific role for T cell-derived IL-10 has been highlighted. Conditional deletion of *Il-10* in murine $CD4^+$ T cells resulted in intestinal inflammation leading to the development of inflammatory bowel disease (IBD) (Roers et al., 2004). In contrast, myeloid-derived IL-10, but not T cell-derived IL-10 was shown to be important in the context of lipopolysaccharide (LPS) infection. In this model, selective inactivation of *Il-10* in monocytes and neutrophils increased the sensitivity to LPS infection and enhanced leukocyte infiltration, whereas mice with *Il-10* deficient T cells had a similar response to WT mice (Siewe et al., 2006).

1.8.2 IL-10 in Disease

The central role of IL-10 in immunity dictates that aberrant expression or activity of IL-10 will impact upon many immunological processes. As such, IL-10 is known to contribute to the pathogenesis of several autoimmune and autoinflammatory diseases such IBD, psoriasis and rheumatoid arthritis (Ouyang, 2011, Trifunović et al., 2015). The non-redundant, anti-inflammatory role of IL-10 in IBD is emphasised in knockout mouse models. Animals deficient in IL-10 (*Il-10*$^{-/-}$) have exaggerated responses to gut microbiota and uncontrolled intestinal inflammation (Kühn et al., 1993). In humans, loss of function mutations in IL-10 or IL-10R are associated with very early onset IBD, characterised by severe IBD symptoms, and infantile IBD (Kotlarz et al., 2012, Zhu et al., 2017, Lee et al., 2014). Dysregulated expression of IL-10 is also a factor in the pathology of psoriasis. Patients with psoriasis have reduced levels of IL-10 (Al-Robaee et al., 2008, Asadullah et al., 1998), often in combination with elevated levels of pro-inflammatory cytokines such as IL-8, IL-12 and IL-19 (Hofmann et al., 2012). The risk of psoriasis can also correlate with genetic alteration of IL-10, specifically polymorphisms in the IL-10 promoter region (Karam et al., 2014). In contrast to diseases characterised by IL-10 deficiency, patients with rheumatoid arthritis have elevated levels of serum and synovial fluid IL-10. In these patients, the pleiotropic role of IL-10 is evident, with IL-10 supressing the expression of pro-inflammatory cytokines whilst promoting the secretion of autoantibodies. Accordingly, the overexpression of IL-10 is associated with expression of rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) autoantibodies (Hernández-Bello et al., 2017).

1.8.3 IL-10 Signalling

IL-10 exerts its biological effects through extracellular binding of cell surface receptors IL-10R1(α) and IL-10R2(β). Upon receptor engagement, the IL-10R transduces signals through multiple pathways, of which the JAK-STAT pathway is the most well-characterised (Verma et al., 2016). The IL-10 receptors form a tetrameric complex consisting of two IL-10R1 and two IL-10R2 chains (Figure 3) (Carey et al., 2012). Each receptor has distinct functions based on their affinity for IL-10. IL-10R1 has greater affinity and is the specific ligand binding chain, whereas IL-10R2 has lower affinity and acts as an accessory chain (Walter, 2014). IL-10R2 does not physically bind to IL-10 but instead associates with the IL-10/IL-10R1 complex and activates the signalling cascade. IL-10R2 does not affect the affinity of IL-10R1/IL-10 binding, but expression of both receptors is essential to activate the JAK/STAT signal transduction pathway (Ding et al., 2001). Upon receptor/ligand binding, JAK1 (Janus Kinase 1) and Tyk2 (Tyrosine kinase 2) phosphorylate themselves and trans-phosphorylate tyrosine resides on IL-10R1 which acts as a docking site to recruit STAT3. Following this, STAT3 is phosphorylated, resulting in the homo/hetero-dimerisation of STAT3 proteins, translocation to the nucleus and subsequent regulation of gene expression (Glocker et al., 2011) (Figure 3). As mentioned, multiple pathways have been identified in IL-10 signalling (e.g. PI3K/Akt signalling regulates the expression of a subset of IL-10 inducible genes in macrophages (Antoniv and Ivashkiv, 2011), detailing a complex network of pathways that are yet to be fully elucidated (Verma et al., 2016).

1.8.4 Anti-inflammatory activity of IL-10

The anti-inflammatory effect of IL-10 have been extensively studied and a number of IL-10 mediated effects have been identified (Akdis and Blaser, 2001, Hutchins et al., 2013, Couper et al., 2008). Perhaps the most well-known function of IL-10 is the suppression of monocyte/macrophage activity. For example, IL-10 suppresses the production of pro-inflammatory cytokines from activated macrophages (Fiorentino et al., 1991, Oswald et al., 1992), and inhibits antigen presentation in monocytes/macrophages via the downregulation of MHC II expression. Additionally, IL-10 induces the expression of Membrane-associated RING-CH protein I (MARCH-I) that ubiquitinates MHC II leading to its lysosomal degradation, a mode of action that indirectly inhibits T cell activation (Mittal and Roche, 2015). Furthermore, IL-10 has been shown to negatively regulate NF-κB activity. IL-10 induced STAT3 positively regulates the transcription of several anti-inflammatory response (AIR) factors, such as BCL3 and ETV3, which suppress NK-κB mediated gene expression (Hutchins et al., 2013). IL-10 also induces the expression of microRNAs that negatively regulative APC activity by supressing TLR signalling (Curtale et al., 2013).

Whilst IL-10 indirectly inhibits NK cell and T cell activity through APC-dependent mechanisms (Couper et al., 2008), direct inhibition of NK cell and T cell activity has also been reported. In a murine model of retroviral infection, neutralisation of Treg derived IL-10 was shown to enhance NK cell proliferation and activity independent of effects on APC activation, suggesting IL-10 directly inhibits NK cells in this model (Littwitz-Salomon et al., 2018).

Conversely, *in vitro* analysis of isolated murine NK cells suggests that IL-10 has limited direct effect on NK cell proliferation and cytotoxicity (Brady et al., 2010). In a murine model of lymphocytic choriomeningitis virus (LCMV) infection, IL-10 was shown to inhibit the expansion of $CD4^+$ but not $CD8^+$ memory T cells (Brooks et al., 2010); however, IL-10 was shown to directly inhibit the expansion of $CD8^+$ memory T cells in a murine model of *Listeria monocytogenes* infection (Biswas et al., 2007). Additionally, IL-10 has also been shown to inhibit $CD8^+$ T cell responses by decreasing antigen specificity, thereby increasing the threshold for activation, through enhancing branching of cell surface glycoproteins (Smith et al., 2018b).

Further evidence to support the anti-inflammatory role of IL-10 can be demonstrated by viral infections that manipulate IL-10 to benefit pathogen survival. Viruses can suppress immune activation by upregulating the expression of cellular IL-10. Furthermore, HCMV (Human cytomegalovirus) and EBV encode orthologues of IL-10 (vIL-10) which have been shown to exhibit comparable biological functions to human IL-10 (Ouyang et al., 2014), providing a virus-encoded strategy to evade immunity and favour viral replication.

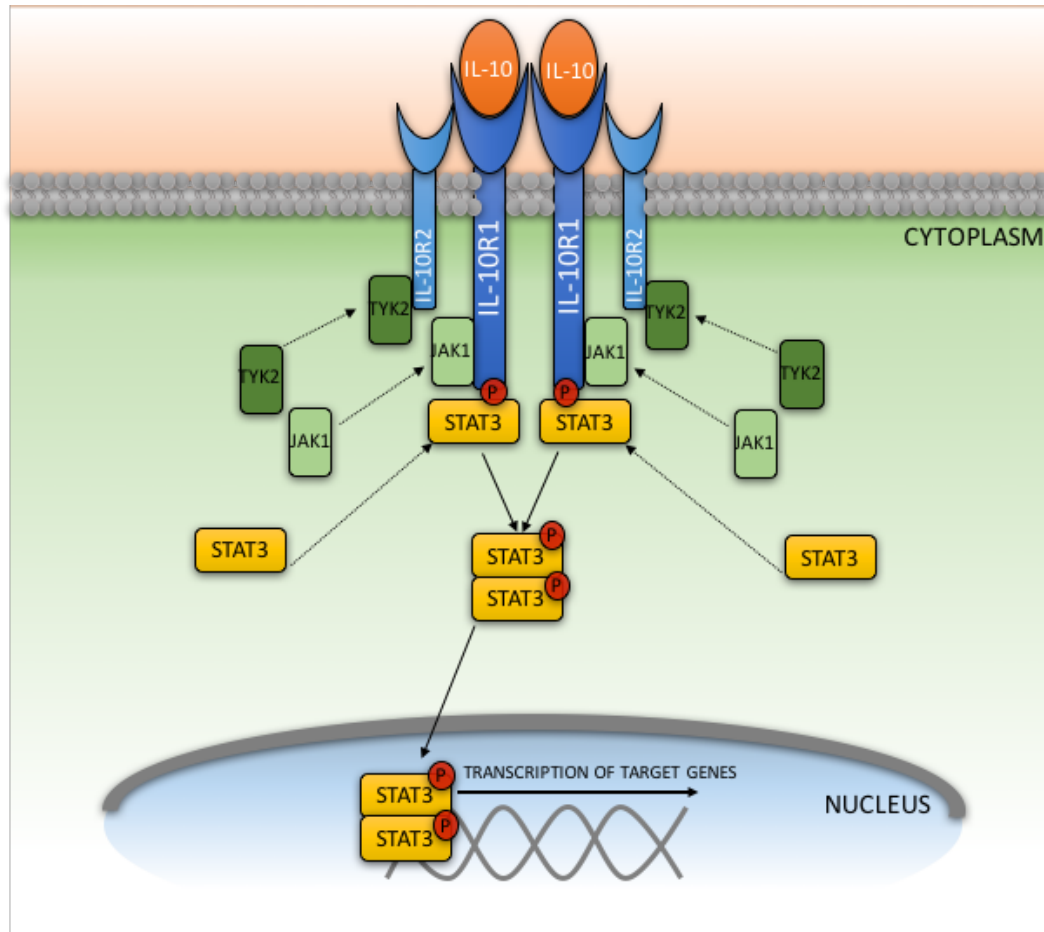


Figure 3: IL-10-STAT3 signalling pathway

IL-10 mediates the transcription of genes through activation of the STAT3 pathway. IL-10 binds to the IL-10R, causing the downstream phosphorylation and modernisation of STAT3 molecules. Subsequently, these dimers translocate to the nucleus where they regulate the expression of STAT3 responsive genes. Figure adapted from (Glocker et al., 2011).

IL: interleukin; JAK: Janus kinase; P: phosphate; R1: receptor 1; R2: receptor 2; STAT: signal transducer and activator of transcription; TYK2: tyrosine kinase 2.

1.8.5 Pro-Inflammatory activity of IL-10

Undoubtedly, IL-10 is an immunosuppressive cytokine in a multitude of cell types and environments. However, there is an increasing number of studies that report immunostimulatory functions of IL-10. Immunostimulatory activities have been demonstrated in multiple cell types, including B cells, mast cells, T cells and NK cells.

In B cells, stimulation of TLR9, CD40 and IL-4R induces the expression of IL-10. This autocrine IL-10 then promotes the differentiation of B cells into IgM and IgG secreting plasmablasts, therefore promoting humoral immune responses (Heine et al., 2014). As with many cytokines, the effects of IL-10 on B cell activation is thought to be context dependent, i.e pre or post-activation (Itoh and Hirohata, 1995).

Similarly, activatory effects have been identified in mast cells (Qayum et al., 2016). In a mouse model of food allergy, IL-10 played an essential role in the proliferation and activation of mast cells, including the upregulation of mast cell-derived cytokines and the expression of FcεRI that mediated IgE responses (Polukort et al., 2016).

IL-10 mediated activation has also been noted in the context of the tumour microenvironment. In a mouse model of pre-established tumours, intra-tumoural IL-10 treatment resulted in infiltration of CTLs, increased intra-tumoural expression of MHC molecules and elevated levels of IFN-γ. Moreover, infiltrating CTLs displayed a 3-4-fold increase in IFN-γ and granzyme B secretion and greater expression of IL-10R1 when compared to peripheral T cells (Mumm et al., 2011). IL-10 has also been shown to induce

the activation and proliferation of tumour resident CTLs, leading to tumour rejection (Emmerich et al., 2012).

Many studies report that IL-10 activates NK cells, but the molecular mechanisms that regulate this are largely unknown. Several studies have investigated the impact of IL-10 on cytokine production, cytotoxicity and proliferation of NK cells. In the presence of IL-2, IL-10 enhanced the production of IFN-γ, TNF and GM-CSF (Carson et al., 1995). Similarly, IL-10 could enhance IFN-γ production in combination with IL-18, but not IL-12 (Cai et al., 1999). These studies also report that IL-10 alone was not sufficient to induce the production of these cytokines. IL-10 also enhanced NK cell cytotoxicity, alone or in combination with other activating cytokines. For instance, combinations of IL-2 and IL-10 or IL-15 and IL-10 enhance cytotoxicity resulting in increased lysis of target cells; an effect that could be inhibited by IL-10 neutralisation (Carson et al., 1995, Cai et al., 1999, Park et al., 2011). Additionally, IL-10 alone did not induce NK cell proliferation but could potentiate the proliferative effects of other cytokines (Cai et al., 1999). One particular study reported contradictory results from *in vitro* and *in vivo* models. *In vivo*, post-LPS administration of IL-10 increased serum levels of granzyme B, IFN-γ and IFN-γ-dependent chemokines CXCL10 and CXCL9. However, in *in vitro* models, these stimulatory effects were not observed and IL-10 inhibited the production of IFN-γ (Lauw et al., 2000). Data in these studies are generated from several different NK cell sources, including NK cells within whole blood, isolated human NK cells, NK cells differentiated from $CD34^+$ HSCs, murine NK cells and the NK-like cell line NK-92, which may impact the observations detailed here (Cai et al., 1999, Carson et al., 1995,

Park et al., 2011, Lauw et al., 2000). With increasing amounts of evidence supporting a pro-inflammatory role, particularly in the context of NK cells, it is important to investigate these mechanisms further.

1.9 Aim

The overall aim of this thesis is to improve our understanding of NK cell activity, and how this is controlled by cytokines. The goal of this study is to explore the immunomodulatory effects of IL-10 on NK cells. As described, existing literature regarding the function of IL-10 is somewhat ambiguous; with data generated from multiple sources of NK cell-like lines, animal models and primary cells. Although generally described as immunosuppressive, a number of studies have highlighted the immunostimulatory function of IL-10 in multiple cell types, including NK cells. In light of this, I aim to resolve the contradictory findings of IL-10 function using human primary NK cells.

To do this, an *in vitro* system was used to culture human primary NK cells in the presence of IL-10 and subsequent downstream assays were performed to assess IL-10-induced activity. In particular, the signalling pathways and resulting activatory/inhibitory impact of IL-10 stimulation was investigated. Additionally, IL-10-induced NK cell responses were compared to IL-15- and TGF-β-induced NK cell responses, as these are known to potently activate and inhibit NK cell functions, respectively.

Chapter 2

Materials and methods

2.1 Cell Culture

2.1.1 Peripheral Blood Mononuclear Cell Isolation

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donor apheresis cones (obtained from NHS blood and transplant, Leeds) by density gradient centrifugation using Lymphoprep™ (Axis-Shield). Blood was diluted 1:6 or 1:11 with Phosphate Buffered Saline (PBS), 30 mL was then carefully layered onto 15 mL of room temperature (RT) Lymphoprep in a 50 mL centrifuge tube and centrifuged at 800 *x g* for 20 minutes at RT with no brake (all centrifugations were carried out using an Eppendorf 5810). The PBMC interface (Figure 4) was then transferred to a new 50 mL centrifuge tube by pastette, resuspended in PBS and centrifuged at 220 *x g* for 15 minutes (brake on). The supernatant was aspirated and the PBMCs were resuspended in PBS, cell number was determined by counting with a haemocytometer before centrifuging at 300 *x g* for 5 minutes. The supernatant was discarded and the PBMCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) at a density of 2×10^6/mL; cells were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO_2) using a CO_2 incubator (Sanyo).

2.1.2 Natural Killer cell isolation

NK cells were purified from isolated PBMCs (described in section 2.1.1) using a negative selection method which provides a highly pure population of untouched NK cells (routinely >95% when tested) by depletion of magnetically labelled non-NK cells (Figure 5). Human NK Cell Isolation Kit and LS columns (Miltenyi Biotec) were used according to manufacturer's instructions. 1×10^7 PBMCs are used per column to obtain an approximate yield of 1×10^6 NK cells. NK cells were isolated directly after PBMC isolation or after overnight incubation. PBMCs were centrifuged at 300 *x g* for 10 minutes (4˚C) and resuspended in 440 µL of ice-cold MACS buffer (10% bovine serum albumin (BSA) and 0.5M EDTA in PBS) and 110 µL of a cocktail of biotin-conjugated antibodies against non-NK cell specific antigens. After incubation at 4°C for 5 minutes, 330 µL of ice-cold MACS buffer containing 220 µL of anti-biotin magnetic beads was added and incubated at 4°C for a further 10 minutes. LS columns were mounted onto a MACS magnetic stand and equilibrated with 3 mL MACS buffer, the cell suspension was passed through the columns and flushed with a further 9 mL of ice-cold MACS buffer. Labelled non-NK cells were retained in the LS column and the eluted untouched NK cells collected in a 15-mL centrifuge tube. NK cells were pelleted (300 *x g* for 10 minutes) and cultured at 1×10^6/mL in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) and 10% human AB serum (Gemini Bio-Products) at 37°C, 5% CO_2.

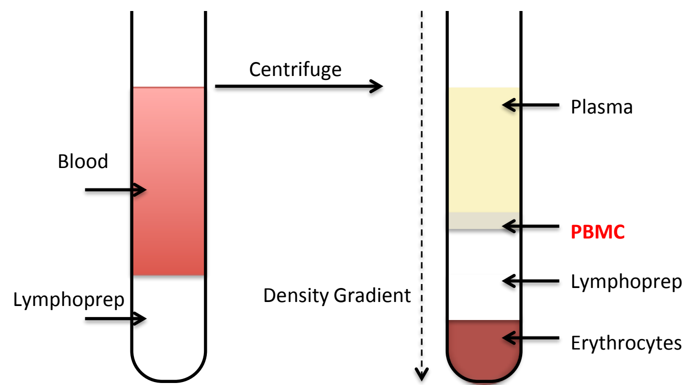


Figure 4: Lymphoprep density gradient centrifugation

Blood /PBS is layered onto Lymphoprep, during centrifugation high-density erythrocytes positon at the bottom of the tubes whilst the PBMC layer is positioned at the interface of the Lymphoprep and the plasma layer.

PBMC: peripheral blood mononuclear cell

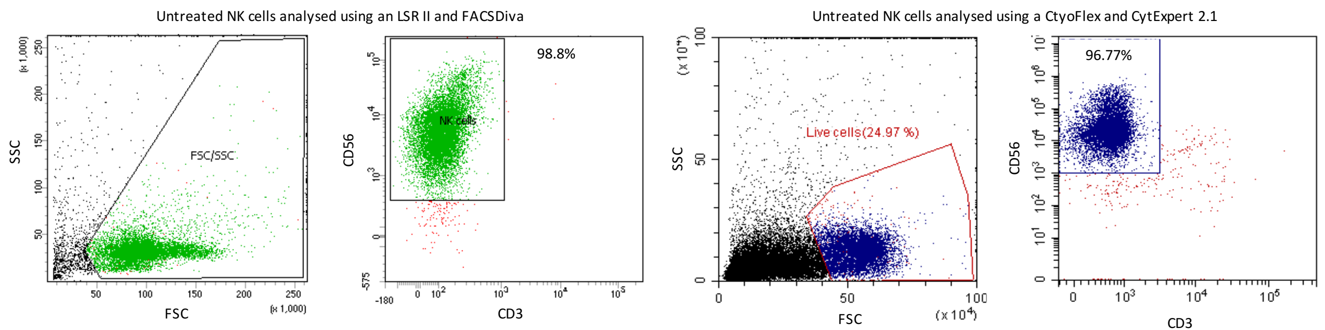


Figure 5: Examples of NK cell isolation from PBMCs

NK cells were isolated from PBMCs as described in section 2.1.2 and incubated for 48 hours (as untreated controls). Cells were then stained with anti-CD56 and anti-CD3 antibodies and analysed by flow cytometry (as described in section 2.2 and 2.2.1).

2.1.3 Cell Stimulation

PBMCs or NK cells were cultured as described in section 2.1.1 and 2.1.2, respectively. Cells were transferred to appropriately sized cell-culture plates and stimulated with purified cytokines and reagents at the concentrations listed in Table 2.

Cytokine/Reagent	Working Concentration	Company
IL-10	50 ng/mL	Miltenyi Biotec
IL-15	20 ng/mL	Miltenyi Biotec
IL-12	1 ng/mL	R&D systems
IL-18	5 ng/mL	R&D systems
IL-21	50 ng/mL	Miltenyi Biotec
IL-1β	10 ng/mL	R& D systems
LPS (E. Coli k12)	100 ng/mL	Invivogen Ultrapure
TGF-β	5 ng/mL	Miltenyi Biotec
c188-9	1, 5, 10 µM	Merck Millipore
Palbociclib (PD-0332991)	1 µM	Sigma-Aldrich

Table 2: Cytokines/reagents used to stimulate NK cells

2.1.4 Cell Lines

K562

The K562 cell line is derived from a patient with chronic myelogenous leukaemia (CML) (Lozzio, 1975). It is a non-adherent cell line that is a well validated *in-vitro* target for NK cells (Zarcone et al., 1987). Cells were cultured in RPMI-1640 media (Sigma-Aldrich) and 10% FBS and kept at a density of 10^5-10^6 cells/mL at 37°C, 5% CO_2.

SKOV3

The SKOV3 cell line is an adherent cell line derived from a patient with ovarian adenocarcinoma. Cells were kindly provided by Dr Victoria Jennings, University of Leeds, and cultured in DMEM (Sigma-Aldrich) and 10% FBS at 37°C, 5% CO_2. Before using cells in assays, culture medium was removed and cells were gently washed by adding PBS to the flask. The PBS was then discarded and cells were harvested by adding 2 mL of 1X trypsin (Sigma-Aldrich) and incubating at 37˚C for approximately 5 minutes, cell detachment was confirmed by microscope observation. Cells were then resuspended in culture medium and centrifuged at 300 *x g* for 5 minutes. Cells were resuspended in culture medium at the appropriate density and transferred into new cell-culture plates to be used in subsequent assays.

2.2 Flow Cytometry

Flow Cytometry was performed using either an LSRII flow cytometer (BD Biosciences) and data was analysed using FACSDiva software (BD biosciences), or using the Cytoflex (Beckman Coulter) with analysis software CytExpert 2.1. Where appropriate, isotype controls were included to assess background staining. Gates for positive staining were set at 2% based on the isotype staining. Single fluorophore controls were used for compensation where needed. Antibodies, reagents and isotypes used for flow cytometry can be found in tables 3-5.

2.2.1 Surface Staining

Cells were harvested into 5 mL round bottom polystyrene tubes (FACS tubes) and washed twice in FACS buffer (0.5% BSA and 0.09% sodium azide in PBS) (300 *x g* for 5 minutes). Cells were then resuspended at approximately 1 x 10^6 cells/100 µL in FACS buffer containing relevant antibodies and incubated for 30 minutes at 4°C protected from light. Cells were then washed twice (300 *x g* for 5 minutes) and resuspended in 300 µL FACS buffer before flow cytometric analysis.

2.2.2 Intracellular Staining

Cells were transferred into 15 mL centrifuge tubes and fixed using Cytofix fixation buffer (BD Biosciences). Pre-warmed (37°C) Cytofix was added in equal volume to the cell suspension and incubated at 37°C for 10 minutes in a water bath. Cells were centrifuged at 600 *x g* for 6 minutes, the supernatant

was discarded, and the pellet was resuspended in 1 mL of cold Perm Buffer III (BD Biosciences) and incubated on ice for 30 minutes. Cells were then transferred to FACS tubes and washed three times in stain buffer (2% FCS and 0.09% sodium azide in PBS) (600 *x g* for 6 minutes) before being resuspended in stain buffer (approximately 1 x 10^6/100 µL) containing relevant antibodies or isotype controls for 1 hour at RT, protected from light. Cells were then washed twice (600 *x g* for 6 minutes) and resuspended in 300 µL stain buffer before flow cytometric analysis.

2.2.3 Intracellular cytokine staining

GolgiStop (BD Biosciences) and GolgiPlug (BD Biosciences) were added to NK cells at 1:1500 and 1:1000, respectively and incubated at 37˚C for 5 hours. Cells were then transferred to FACS tubes, and washed in FACS buffer at 300 *x g* for 5 minutes. Cells were resuspended in 100 µL FACS buffer and stained with anti-CD3 and anti-CD56 antibodies for 30 minutes at 4˚C, protected from light. Cells were then washed twice in FACS buffer at 300 *x g* for 5 minutes before resuspending in 100 µL FACS buffer and 100 µL intracellular fix buffer (eBioscience) and incubating at RT for 30 minutes, protected from light. Cells were then washed twice in intracellular permeabilisation buffer (eBioscience) (diluted 1:10 with PBS fresh before use) (300 *x g* for 5 minutes) and resuspended in 50 µL intracellular permeabilisation buffer and 10 µL anti-IFN-γ antibody and incubated at RT for 30 minutes, protected from light. Cells were washed again in intracellular permeabilisation buffer at 600 *x g* for 5 minutes and resuspended in 300 µL FACS buffer before being analysed by flow cytometry.

2.3 Cytotoxicity Assays

NK cells were stimulated with cytokines (previously described) for 48 hours prior to being used in these assays. K562 target cells (10^6/mL) were labelled with a fluorescent dye before being co-cultured with NK cells. K562 cells were labelled in media (RPMI 10% FCS) containing either 2 µM CellTracker™ violet BMQC (2,3,6,7-tetrahydro-9-bromomethyl-1H,5H-quinolizino(9,1-gh)coumarin) dye (CTV) (Invitrogen) or 0.4 µM CellTracker™ green CMFDA (5-chloromethylfluorescein diacetate) dye (Invitrogen), and incubated at 37˚C for 30-45 minutes. Labelled target cells were washed twice in media and resuspended in fresh media at 10^6/mL, then transferred into 96-well round bottomed cell-culture plates. NK cells (effectors) were then added at effector to target (E: T) ratios of 1:1, 2:1, and 5:1, and incubated for 4 hours at 37°C, 5% CO_2. After incubation, the cells were pelleted (300 *x g* for 5 minutes), washed in PBS (300 *x g* for 5 minutes) and resuspended in 50 µL Zombie NIR™ (Biolegend) (1:1000 with PBS) and incubated on ice, protected from light for 20 minutes. Finally, the cells were pelleted (300 *x g* for 5 minutes), washed in PBS (300 *x g* for 5 minutes) and resuspended in 150 µL FACS buffer before being analysed on a Cytoflex cytometer (Beckman Coulter). Control wells included target cells alone and NK cells alone stained with Zombie NIR™ to measure spontaneous cell death.

2.4 Degranulation assays

NK cells and targets were prepared as in section 2.1.2 and effectors and targets were co-cultured at 1:1 in 96-well round bottomed cell-culture plates.

After 1 hour incubation at 37˚C, an anti-CD107a (1:40) antibody and GolgiStop (BD Biosciences) (1:1000) were added. The plate was then incubated at 37˚C for a further 4 hours. Cells were then pelleted and washed in PBS (300 *x g* for 5 minutes), before being resuspended in 150 µl FACS buffer and analysed on a the Cytoflex cytometer (Beckman Coulter). Control wells included target cells alone and NK cells alone stained with anti-CD107a to measure spontaneous degranulation events.

2.5 Glucose fluxing analysis

NK cells were stimulated for 48 hours prior to being used in this assay. NK cells were washed out of NK cell media (300 *x g* 5 minutes), resuspended in pre-warmed glucose free RPMI (Sigma-Aldrich) supplemented with 10% FCS and incubated at 37˚C. After 15 minutes, 50 µM 2-NBDG (2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxyglucose) (Invitrogen) was added and the cells were then further incubated at 37˚C for 60 mins. Cells were then transferred to centrifuge tubes (on ice) and washed twice in ice cold PBS (300 *x g* 5 minutes). The cells were then resuspended in 500 µL PBS, transferred to FACS tubes and analysed by flow cytometry.

2.6 Cell Cycle Analysis (Propidium Iodide)

Isolated NK cells were treated with cytokines and incubated at 37°C for 96 hours. NK cells were then harvested into FACS tubes and washed twice in PBS (300 x *g* for 5 minutes). After washing, cells were fixed in ice-cold 70% ethanol by adding the ethanol dropwise to the cells whilst being gently

vortexed (1 x 10^6 cells/mL). Cells were then incubated on ice for 30 minutes and stored at -20°C overnight. Cells were then washed in stain buffer (600 x *g* for 5 minutes) three times before adding 50 µl of 100µg/mL RNAse A (Qiagen) directly to the cell pellet. Cells were then resuspended in 300 µL stain buffer containing 20 µL/10^6 cells propidium iodide (PI) (Life technologies). Cells were incubated for 30 minutes at RT, protected from light, then analysed with the LSRII flow cytometer.

Target	Fluorophore	Manufacturer	Clone	Volume
CD3	BV421	BD horizon	SK7	2/100
	PerCP	BD horizon	SK7	2/100
CD56	APC	Miltenyi Biotec	AF12-7H3	2/100
	PE CY 5	BD Pharmingen	B159	2/100
	PE-VIO770	Miltenyi Biotec	REA196	2/100
CD69	FITC	Miltenyi Biotec	FN50	5/100
	FITC	Biolegend	FN50	5/100
	FITC	Biolegend	FN50	5/100
CD314 (NKG2D)	PE	BD Pharmingen	1D11	5/100
	PE	Miltenyi Biotec	BAT221	5/100
CD335 (NKp46)	APC	BD Pharmingen	9E2/NKp46	5/100
CD337 (NKp30)	PE	BD Pharmingen	p30-15	5/100
CD226 (DNAM1)	PE	Miltenyi Biotec	DX11	5/100
	PE	BD Pharmingen	DX11	5/100
CD107a	FITC	Miltenyi Biotec	H4A3	5/100
Granzyme b	PE	Miltenyi Biotec	REA226	3/100
IFN-γ	FITC	Miltenyi Biotec	REA600	10/60
CD210 (IL-10R)	PE	Miltenyi Biotec	REA239	10/100

Table 3: Antibodies used for flow cytometry

APC (Allocophycyanin), FITC (Fluorescein isothiocyanate), PE (phycoerythrin) and BV-421(brilliant violet -421).

Isotype	Subclass	Manufacturer	Clone
APC	mouse IgG1k	Miltenyi Biotec	IS5-21F5
APC	mouse IgG1k	BD Pharmingen	MOPC-21
BV421	mouse IgG1k	BD Horizon	X40
BV421	mouse IgG1k	Biolegend	MOPC-21
PE	mouse IgG1k	BD Pharmingen	MOPC-21
PE	mouse IgG1k	Miltenyi Biotec	IS5-21F5
FITC	mouse IgG1k	BD Pharmingen	MOPC-21
FITC	mouse IgG1k	Miltenyi Biotec	IS5-21F5
PerCP-Cy5.5	mouse IgG1k	BD Pharmingen	MOPC-21
PerCP-Cy5.5	mouse IgG2a, k	BD Phosflow	MOPC-173
PEVIO770	mouse IgG1k	Miltenyi Biotec	IS5-21F5

Table 4: Isotypes used for flow cytometry

Reagent	Activity	Company	Dilution
Zombie NIR™	Dead cell discriminator	Biolegend	1 in 1000
Propidium iodide (PI)	DNA stain	Life Technologies	1 in 15
CellTracker™ green	Fluorescent dye	Invitrogen	1 in 5000
CellTracker™ violet	Fluorescent dye	Invitrogen	1 in 1000
2-NBDG	Fluorescent glucose analogue	Invitrogen	50 μM

Table 5: Reagents used for flow cytometry

Zombie is a dead cell discriminator that binds to the N-terminus of proteins. Propidium Iodide is a DNA intercalating agent used to analyse DNA content. Cell tracker green/violet is a fluorescent dye used to label cells.

2.7 Western Blotting

2.7.1 Cell Lysate Preparation

Cells were harvested and washed in ice-cold PBS (300 *x g* for 5 minutes) before being lysed with ice-cold RIPA buffer (150mM NaCl, 10mM Tris pH 7.2, 0.1% SDS, 0.1% Triton-X, 1% deoxycholic acid, 5mM EDTA) containing cOmplete™ Mini EDTA-free protease inhibitor cocktail (Roche) and PhosStop (Roche) phosphatase inhibitor (1 x 10^6 cells/50 µL). Lysates were incubated on ice for ~30 minutes and stored at -20°C until used. On the day of use, lysates were thawed and 5X Laemmli buffer (65.8mM Tris pH6.8, 26.3% glycerol, 2.1% sodium dodecyl sulphate, 0.01% bromophenol blue, 1% β-mercaptoethanol) was added to lysates followed by heating at 95°C for 10-20 minutes. Cell lysates contained the same concentration of cells in each sample, e.g. 1 x 10^6/ 60 µL.

2.7.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

TRIS Glycine gels were used throughout with the appropriate percentage gel chosen based on the size of the protein to be detected. Resolving gel: 10%,12% or 15% acrylamide, 0.375M Tris pH8.8, 0.1%S DS, 0.1% APS, 0.001% TEMED. Stacking gel: 6% acrylamide, 0.140M Tris pH6.8, 0.1% SDS, 0.1% Aps, 0.001% TEMED. The gel cassette was placed into an electrophoresis tank and submerged in running buffer (25mM Tris, 192mM glycine, 0.1% SDS). Equal volumes of cell lysates for each treatment were loaded into individual sample wells and a molecular weight marker (SeeBlue

PLus2, Thermo Fisher Scientific) was loaded into a separate sample well; a potential voltage difference of 120V was applied for ~1 hour 30 minutes.

2.7.3 Western transfer

The proteins were transferred from the gel to a Hybond-P polyvinylidene fluoride (PVDF) membrane (GE Healthcare Life Sciences) using a semi-dry transfer machine (BioRad). The membrane was activated in methanol for ~30 seconds, the membrane and the gel were then sandwiched between transfer buffer (25mM Tris, 192mM glycine, 20% methanol) soaked filter papers. A potential voltage difference of 15V was applied for 1 hour.

2.7.4 Antibody Staining

Membranes were incubated in blocking buffer (1X Tris buffered saline [25 mM Tris base, 134 mM sodium chloride, pH 7.5] with 0.1% Tween-20 and 5% BSA) for 1 hour at RT or overnight at 4˚C, with continuous agitation. The blocking buffer was discarded and replaced with primary antibody (diluted in blocking buffer) and incubated overnight at 4°C with continuous agitation. The primary antibody was removed and the membrane washed in 1X Tris buffered saline with 0.1% Tween (TBST) for 5 minutes, repeated twice. The secondary antibody (diluted in blocking buffer) was then added and incubated at RT for 1 hour, with continuous agitation. The secondary antibody was removed and the membrane washed (as above) before pipetting enhanced chemiluminescence solution (Amersham™ ECL Prime [GE Healthcare Life Sciences] or Clarity™ western ECL substrate [Bio Rad]) over the membrane

for ~ 1 minute. The membrane was then sealed in transparent plastic, transferred to a light tight X-ray film cassette and exposed onto ECL Hyperfilm (GE Healthcare) in a dark room. The film was then developed using a Konica SRX-101A tabletop X-ray Film Processor (Konica). Exposure times vary depending on the strength of signal

Antibody	Host species	Manufacturer	dilution
pStat3^{y705}	Mouse	Cell signalling technologies	1 in 2000
Stat3	Mouse	Cell signalling technologies	1 in 1000
β-Actin	Mouse	Sigma-Aldrich	1 in 1000
pS6 $^{s240/244}$	Rabbit	Cell signalling technologies	1 in 1000
Puromycin	Mouse	Merck	1 in 10,000
Granzyme b	Mouse	BD Pharmingen	1 in 1000
Anti-Rabbit HRP	Goat	Cell Signalling Technologies	1 in 5000
Anti-Mouse HRP	Horse	Cell Signalling Technologies	1 in 10,000

Table 6: Antibodies used for Western Blotting

2.8 Proteome Profiler Human Phospho-Kinase Array Kit (R&D systems)

2.8.1 Sample preparation

1.5 x 10^7 isolated NK cells were stimulated with cytokines for 60 minutes. Cells were transferred to centrifuge tubes, centrifuged at 300 *x g* for 5 minutes, washed in PBS at 300 *x g* for 5 minutes and finally resuspended in 400 µL "lysis buffer 6" (3.75 x 10^7/mL) and transferred to microcentrifuge tubes. Lysates were incubated at 4˚C for 30 minutes with constant agitation then centrifuged at 14,000 *x g* for 5 minutes. Supernatants were transferred to a clean microcentrifuge tubes and stored at -80˚C until used.

2.8.2 Assay procedure

334 µL of lysate were diluted to a final volume of 2 mL with array buffer 1. The assay was performed following the manufacturer's instructions. Multiple exposure times (1 minute, 3 minutes, 5 minutes, 10 minutes and 60 minutes) using a light tight X-ray film cassette and ECL Hyperfilm (GE Healthcare) were developed in a dark room using a Konica SRX-101A tabletop X-ray Film Processor (Konica). Analysis was performed using "protein array analyzer" plugin for ImageJ.

2.9 Enzyme Linked Immunosorbent Assay

Human tumour necrosis factor (TNF) ELISA set (BDOptEIA, BD Bioscience) was used to quantify levels of secreted TNF in cell culture supernatants according to the manufacturer's instructions. PBMCs were isolated and stimulated as previously described before harvesting the supernatants,

PBMCs were transferred to a falcon tube and centrifuged for 5 mins at 300 x *g*, the supernatants were carefully pipetted into Eppendorf tubes and frozen at -20°C. In brief, 96 well plates (Nunc™ MicroWell™ 96-Well Microplates) were coated with 1:250 dilution of Anti-human TNF in 0.1M $NaHCO_3$ pH8.2 (capture antibody: coating buffer solution) and incubated overnight at 4°C. The solution was discarded and the plates were washed three times with ELISA wash buffer (PBS + 0.05% tween) and blocked with ELISA assay diluent (PBS + 10% FBS pH7) at RT for 1 hour. The assay diluent was discarded and the plates were washed again with ELISA wash buffer three times. Recombinant human TNF standards of known concentrations (500 pg/mL – 0 pg/mL) were prepared and loaded into the wells in triplicate. The samples were then loaded into the wells in triplicate and incubated at RT for 2 hours in a sealed plate. The standards and samples were discarded and the plate was washed five times in ELISA wash buffer. Detection antibody (1:250 biotinylated anti-human TNF in ELISA assay diluent) and enzyme reagent (1:250 streptavidin-horseradish peroxidase conjugate in detection antibody) was added and incubated in a sealed plate at RT for 1 hour. The solution was discarded and the plates was washed 7 times in ELISA wash buffer before adding the substrate solution (Tetramethylbenzidine (TMB) and Hydrogen Peroxide) (BD Pharmingen™) and incubating at RT for 30 minutes, protected from light. The reaction was stopped by adding 50 µL 2N H_2SO_4 and the plate was read at 450nm using a Multiskan EX plate reader (Thermo Fisher).

To quantify human interferon-gamma (IFN-γ) ELISA, Human IFNγ ELISA set (BDOptEIA, BD Bioscience) was used according to the manufacturers protocol. For the IFN-γ ELISA, the protocol followed was the

same (as above) with minor changes. The pH of the coating buffer was changed to pH9.5, and antibodies and recombinant standards were Anti-human IFN-γ. Supernatants from PBMCs and NK cells were harvested after cytokine stimulation and frozen at -20˚C until used.

2.10 Realtime PCR

2.10.1 RNA extraction

RNA was extracted from Isolated NK cells using RNeasy plus kit (Qiagen) according to the manufacturer’s instructions. The cells were harvested into centrifuge tubes and pelleted at 300 *x g* for 5 minutes and washed in PBS at 300 *x g* for 5 minutes. The cells were then lysed in the supplied lysis buffer (RLT buffer) and 1 volume of ethanol was added to the lysate. The lysate was then transferred to a spin column and centrifuged followed by sequential centrifuging steps with the supplied wash buffers (as in the protocol). 30 µL of RNase-free water was then added directly to the spin column membrane and centrifuged to elute the RNA. 1 µL of the RNA was used to determine the RNA concentration and purity was determined using a Nanodrop Spectrophotometer (Thermo Fisher Scientific), eluted RNA was frozen at -80°C. RNA was considered ‘pure’ enough to use when the ratio of the UV absorbance at 260nm/280nm was between 1.7 and 2.1.

2.10.2 cDNA synthesis

50-100 ng total RNA was reverse transcribed using SuperScript™III (Invitrogen) according to the manufacturers protocol. 1 µL of Random Primer

Mix (New England Biolabs) and 1 µL of dNTPs (Invitrogen) were combined with RNA/water to a final volume of 13 µL per reaction. Each reaction was heated at 65°C for 5 minutes before placing on ice for >1 minute. 4 µL of 5x first-strand buffer (Invitrogen), 1 µL of 0.1M DTT (Invitrogen), 1 µL SuperScript™III and 1 µL RNAse-OUT™ (Invitrogen) was added to each reaction (final volume 20 µl) before transferring into PCR tubes and placing in a thermocycler (Applied Biosciences). Reactions were then incubated at 25°C for 5 minutes, 50°C for 60 minutes then 70°C for 15 minutes. cDNA was frozen at -80°C until used.

2.10.3 Realtime PCR/TaqMan

Real-Time PCR (RT-PCR) was performed using QuantStudio® 5 (Applied Biosystems) and prepared in microAmp fast optical 96 well reaction plates (Applied Biosystems). Each 20 µL reaction contains 10 µL TaqMan® universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems), 8 µL nuclease-free water, 1 µL cDNA and 1 µL specific primers (Table 7). Reactions were performed in triplicate using the following conditions: 95˚C for 10 minutes, followed 40 cycles of 95˚C (15 seconds) and 60˚C (1 minute). Cycle threshold (Ct) values were automatically generated and expression was analysed using the $\Delta\Delta$Ct method.

Gene	Supplier	Assay ID	Dye	Amplicon length
18s	Thermo Scientific	Hs03003631_g1	FAM-MGB	69
GZMB	Thermo Scientific	Hs00188051_m1	FAM-MGB	114
IFNG	Thermo Scientific	Hs00989291_m1	FAM-MGB	73

Table 7: TAQMAN probes

2.11 Statistical analysis

All statistical analyses were performed using Prism7 GraphPad software. Unless otherwise stated, data was analysed using repeated measures two-way ANOVA with Tukey's correction for multiple comparisons. In all graphs, data are represented as individual donors with standard error of the mean (SEM) error bars; within individual graphs, coloured data points correspond to the same donor across treatments.

2.12 Ethical Statement

PBMCs and NK cells were isolated from NC24 leukocyte cones, a waste product of platelet donations classed as 'donated material surplus to clinical requirement or unsuitable for therapeutic use' and issued by the NHS blood and transplant service under 'non-clinical issue for research purposes'.

Chapter 3

Modulation of natural killer cell effector function by interleukin 10

3.1 Introduction

Interleukin 10 (IL-10) is most frequently described as an anti-inflammatory cytokine due to its ability to suppress pro-inflammatory cytokine production and inhibit antigen presentation (Couper et al., 2008). Aberrant IL-10 expression is associated with a variety of autoimmune and inflammatory diseases such as inflammatory bowel disease (IBD), rheumatoid arthritis and psoriasis; however, the activity of IL-10 in these diseases is complex. Reduced IL-10 levels are characteristic of inflammatory diseases such as psoriasis (Al-Robaee et al., 2008), but paradoxically, patients with rheumatoid arthritis show overexpression of IL-10 and elevated serum IL-10 is associated with disease severity (Hernández-Bello et al., 2017). IL-10 is a good candidate to be targeted therapeutically as it contributes to the pathology of a number of diseases. However, IL-10 therapy has shown limited success to date; whilst recombinant IL-10 therapy has shown efficacy in psoriasis (Asadullah et al., 1998), use in IBD has failed to show any beneficial effects in humans (Marlow et al., 2013), with one study reporting increased systemic inflammation (Tilg et al., 2002). This dichotomy can also be seen in the context of the tumour microenvironment. Immunosuppressive cytokines such as IL-10 are prevalent in many tumours and are usually reported to promote tumour growth and suppress immunity (Sharma et al., 1999, García-Hernández et al., 2002, Vahl

et al., 2017); however, some studies report beneficial effects of IL-10 treatment in cancer (Oft, 2014, Mumm et al., 2011, Tanikawa et al., 2012, Fujii et al., 2001, Emmerich et al., 2012). It is clear that the role of IL-10 in autoimmune/inflammatory diseases and cancer is both controversial and complex.

Although it is predominantly described as immunosuppressive, IL-10 can have pro-inflammatory properties in a variety of different cell types, including NK cells. Previous data regarding the role of IL-10 on NK cell biology is somewhat ambiguous, with data varying between human NK cells, murine NK cells and NK-like cell lines. Whilst the use of cell lines is invaluable for research, cell lines such as NK-92 require the presence of IL-2 for survival. As IL-2 is a potent activating cytokine for both T cells and NK cells (Wang et al., 1999), these cells are already primed/activated and are therefore not a good candidate for studying the activation potential of cytokines. Additionally, there are several immunological differences between human and murine models that may impact the effect of cytokine stimulation, such as receptor structure and expression (Mestas and Hughes, 2004). Data from human NK cells seem to support an immunostimulatory role of IL-10 in NK cells; however, previous studies detail varied responses to IL-10 in combination with different cytokines, along with contradictions from *in vivo* and *in vitro* studies. Experiments detailed in this chapter aim to confirm the activity of IL-10 on human primary NK cells, negating problems with cell lines or inter-species variation, and investigate the mechanisms that mediate these. Here I have explored how IL-10 effects the main NK cell activities, cytotoxicity and cytokine production.

3.2 IL-10 inhibits production of pro-inflammatory cytokines from PBMC

For the assays described I have used a commercial source of IL-10 (Table 2). To verify the activity of IL-10, I used a well-established assay in which Lipopolysaccharide (LPS)-induced TNFα production from macrophages is suppressed by IL-10. The outer wall of gram negative bacteria (such as *E.coli* and *Salmonella*) contains LPS (also known as endotoxin), a potent activator of innate immunity and ligand for the TLR4-MD2 complex, expressed on monocytes, macrophage and DC (Guha and Mackman, 2001, Kaisho et al., 2001). Engagement of LPS by TLR4-MD2 and the co-receptor CD14 induces a signalling cascade resulting in the release of NF-κB from IκB and subsequent translocation of NF-κB to the nucleus, where it induces transcription of target genes, including TNFα (Lu et al., 2008). The anti-inflammatory action of IL-10 on monocytes/macrophages includes the ability to suppress LPS-mediated induction of TNFα release, which can be used to determine IL-10 specific activity. PBMCs were cultured in the presence of LPS ± IL-10 for 16 hours before harvesting the supernatants for analysis. The results shown in Figure 6A and B indicate that TNFα production was strongly induced by LPS stimulation and that IL-10 reduced TNFα production in a dose-dependent manner (Figure 6B). The recombinant source of IL-10 therefore effectively inhibits LPS-induced TNFα production from PBMCs and importantly, validated the activity of the IL-10 used in this work.

These data demonstrate that IL-10 has potent immunosuppressive actions on PBMCs in a mixed population. However, this assay does not delineate the activity of IL-10 on individual cell types. In mixed cell populations such as PBMCs, cells respond to both direct cytokine stimulation, and

indirectly through secondary secreted molecules or via cell-to-cell contacts. In order to determine whether NK cells are directly modulated by IL-10, subsequent assays were performed using isolated NK cells. Although this type of analysis may not account for the complexity of an immune response with multiple interacting cell types, the direct effect of IL-10 on NK cells and the molecular mechanisms of action will be interrogated.

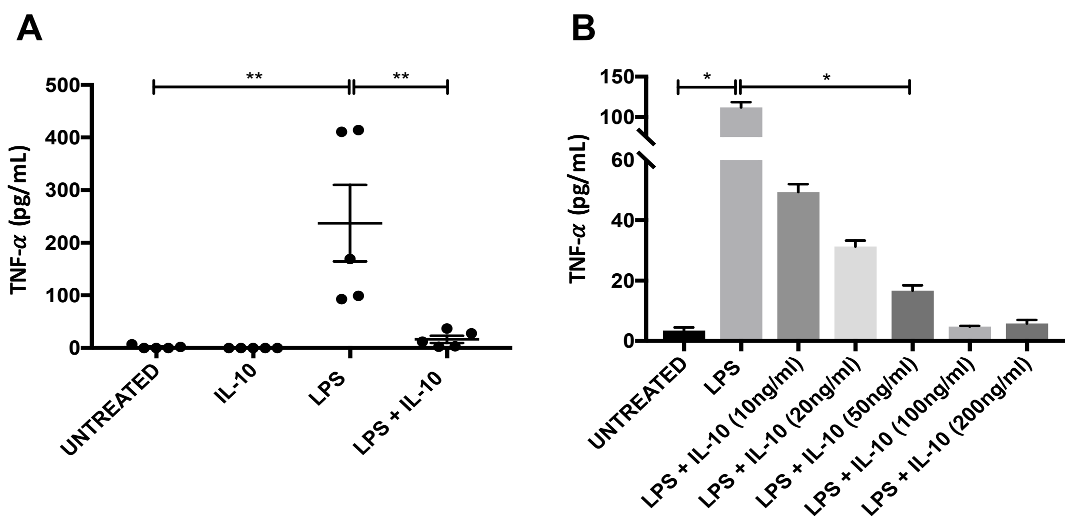


Figure 6: TNF-α production is inhibited by IL-10

PBMC's were stimulated with 100 ng/mL LPS ± 20 ng/mL IL-10 for 16 hours; TNF-α production was determined using ELISAs. (A) Graphs show mean concentration of TNF-α; each dot represents the mean of a triplicate measurement from a single donor (n=5), and (B) mean of triplicate measurements from one experiment (n=1), *P=<0.05, **P=<0.01 determined using a one-way ANOVA with Tukey's multiple comparison test.

3.3 NK cells express a functional IL-10 receptor

IL-10 signals via the IL-10 receptor, IL-10R (also known as CD210). To determine IL-10R expression on NK cells and their ability to signal via IL-10, NK cells were identified within a mixed population of PBMCs and IL10R expression was interrogated using flow cytometry (Figure 7A and B). Lymphocytes were identified and gated on based on their forward scatter (FSC) and side scatter (SSC) parameters (measures of cell size and granularity, respectively) and NK cells were identified within this gate as $CD56^{+}CD3^{neg}$ cells; expression of IL-10R was then determined (Figure 7C-E). Analysis showed that cell surface expression of IL-10R was variable between donors, however, all donors demonstrated a high percentage of NK cells that were positive for IL-10R (>60%; Figure 7D). IL-15 is the prototypical NK cell activating cytokine, and has been shown to alter NK cell surface expression of a multiple receptors (Wilson et al., 2011). However, stimulation with either 50 ng/mL IL-10 or 20 ng/mL IL-15 did not significantly alter the percentage of NK cells expressing IL-10R (Figure 7D) or the density of IL-10R expression (Figure 7E). These data indicate that NK cells have the potential to be regulated by IL-10.

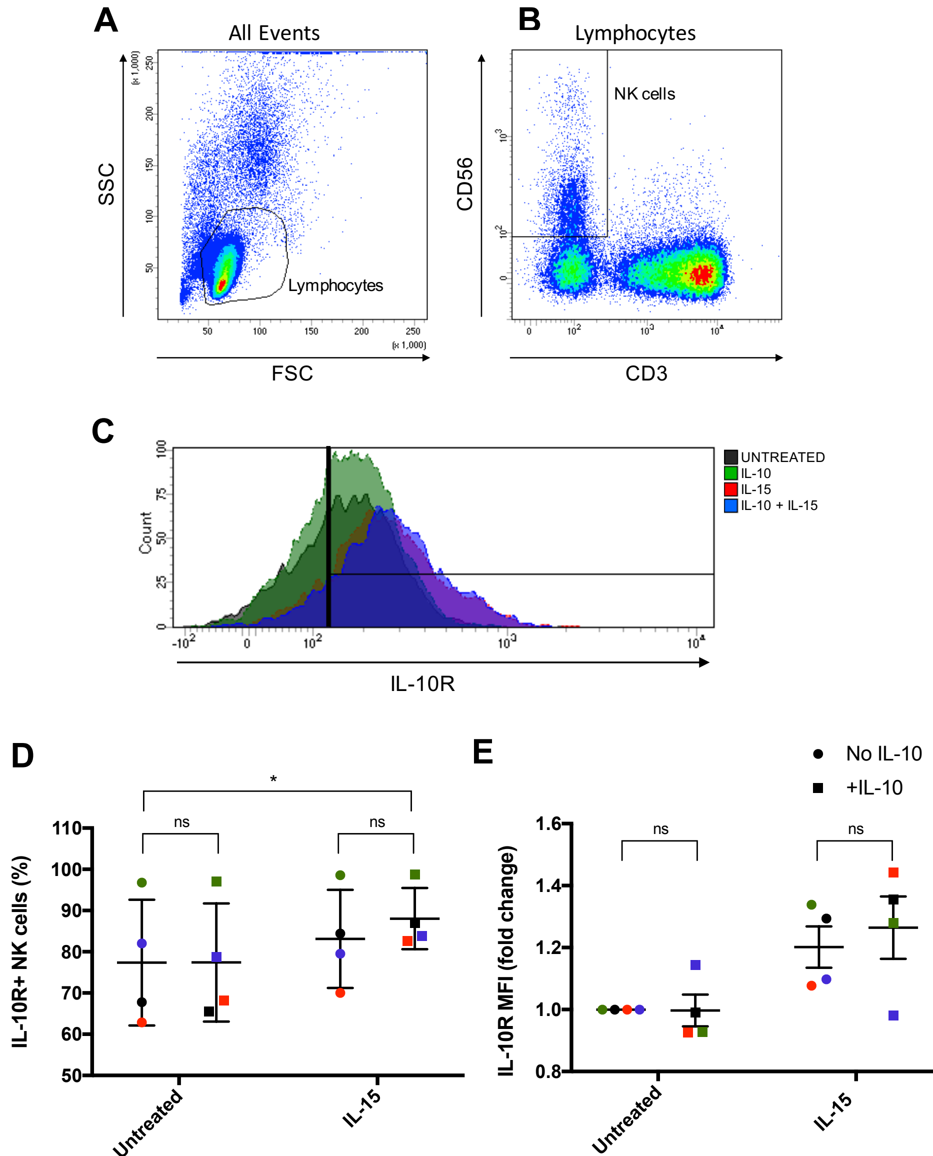


Figure 7: NK cells express the IL-10 receptor

NK cells were evaluated for IL-10R expression by flow cytometry after being stimulated with 50 ng/mL IL-10 or 20 ng/mL for 48 hours. (A) Lymphocytes were identified from the PBMC population based on FSC and SSC gating. (B) Subsequently, NK cells were positively identified by CD56^{+} and CD3^{neg} expression; defined as the CD56^{+} CD3^{neg} population. (C) NK cells were further analysed for IL-10R expression. Representative histograms show IL-10R expression to the right of the isotype bar (black line) set at 2% of the isotype control. (D) Graphs show the percentage of NK cells positive for IL-10R expression, and (E) the median fluorescent intensity (MFI) of expression (fold change relative to untreated cells) (n=4). * P=<0.05, ns = not significant.

3.4 NK cells treated with IL-10 activate the JAK-STAT pathway

The most intensively studied and frequently reported IL-10 signalling pathway is the JAK1/STAT3 pathway (Qayum et al., 2016, Niemand et al., 2003, Rodríguez-Bayona et al., 2013). IL-10/IL10R interactions induce JAK1 and TYK2 activity that subsequently induce the downstream phosphorylation of STAT3 at tyrosine residue 705 (pSTAT3^{Y705}) (Glocker et al., 2011) (Figure 3). To confirm signalling via IL-10R in NK cells, phosphorylation of STAT3^{Y705} was analysed. Isolated NK cells were treated with increasing concentrations of IL-10 for 30 minutes, and the expression of phospho-STAT3^{Y705} was determined by western blotting. IL-10 stimulation (0.5 – 500 ng/mL) induced the phosphorylation of STAT3 in a dose dependent manner (Figure 8A), whilst total STAT3 expression remained unchanged by the addition of IL-10. Stimulation with 50 ng/mL for 30 minutes resulted in detectable pSTAT3^{Y705} expression that was maintained at 48 hours (Figure 8B); these results confirm that IL-10 induces activation of the STAT3 signalling pathway in NK cells.

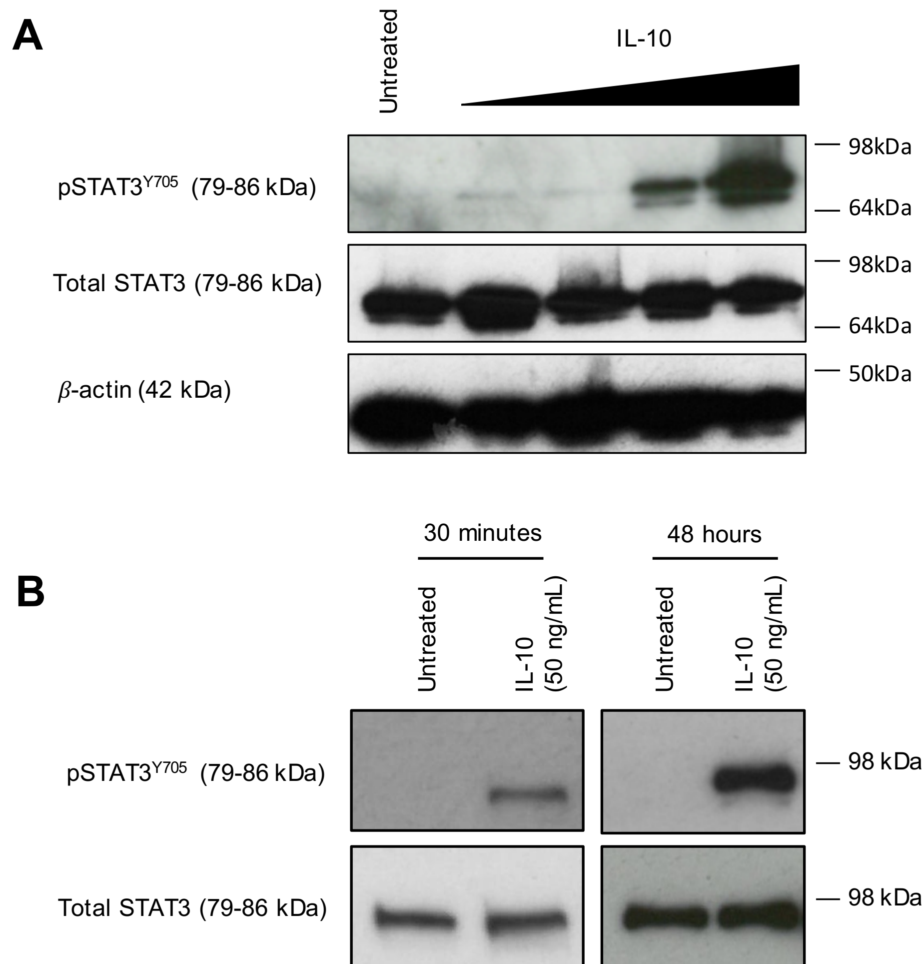


Figure 8: IL-10 signals via STAT3 in NK cells

(A) Isolated NK cells were stimulated with increasing concentrations of IL-10: 0.5 ng/mL, 5 ng/mL, 50 ng/mL and 500 ng/mL, for 30 minutes prior to lysis and analysis by western blot for $pSTAT3^{Y705}$ expression. Total STAT3 and β-actin were probed for as loading controls (n=1). (B) Isolated NK cells were either untreated or stimulated with 50 ng/mL IL-10 for 30 minutes or 48 hours prior to lysis and western blot analysis for $pSTAT3^{Y705}$ expression (total STAT3 was probed for as a loading control) (representative of n=3 [30 minute stimulation] and n=2 [48 hour stimulation]).

3.5 IL-10 upregulates Granzyme B expression in NK cells

Unlike T cells, which require antigen stimulation to trigger the synthesis of cytotoxic components, NK cells constitutively express cytolytic granules. This enables a rapid response against infected cells and tumours, providing killer cell activity whilst T cells become armed. The cytotoxic granules of NK cells contain the five granzyme molecules, A, B, H, K and M and perforin, a pore forming molecule that allows granzymes to enter the target cell (Ewen et al., 2012, Lopez et al., 2013). Granzyme B is the most abundant and intensively studied granzyme molecule and plays a key role in the induction of target cell apoptosis (Chowdhury and Lieberman, 2008). Granzyme B is constitutively expressed by NK cells (Kai et al., 2005) and further induced upon NK cell activation by target cells or activating cytokines such as IL-2, IL-12, IL-15 and IL-18 (Liu et al., 2002). To determine the effect of IL-10 on primary NK cell cytolytic functions, granzyme B expression was analysed at the mRNA and protein level. NK cells were stimulated with IL-15 (as a positive control) or IL-10 for 48 hours and analysed by western blot and flow cytometry. Both methods detected granzyme B expression in unstimulated NK cells, and this was upregulated by IL-15 (Figure 9) as reported previously (Wilson et al., 2011). In these assays, IL-10 also upregulated granzyme B expression at both the mRNA and protein level. Western blot analysis showed IL-10 upregulated granzyme B protein levels comparable to that induced by IL-15 (Figure 9A and C). Flow cytometry confirmed the upregulation of expression of granzyme B although the extent of upregulation was reduced compared to that seen by western blotting (Figure 9B). All assays consistently demonstrated an increase in granzyme B expression in response to IL-10. The induction of

granzyme B protein by IL-10 was mirrored by an increase in GZMB mRNA following IL-10 treatment (Figure 9D). Furthermore, mRNA and western blotting show that the combination of IL-10 and IL-15 induced greater levels of granzyme B than either cytokine alone (Figure 9A, C and D).

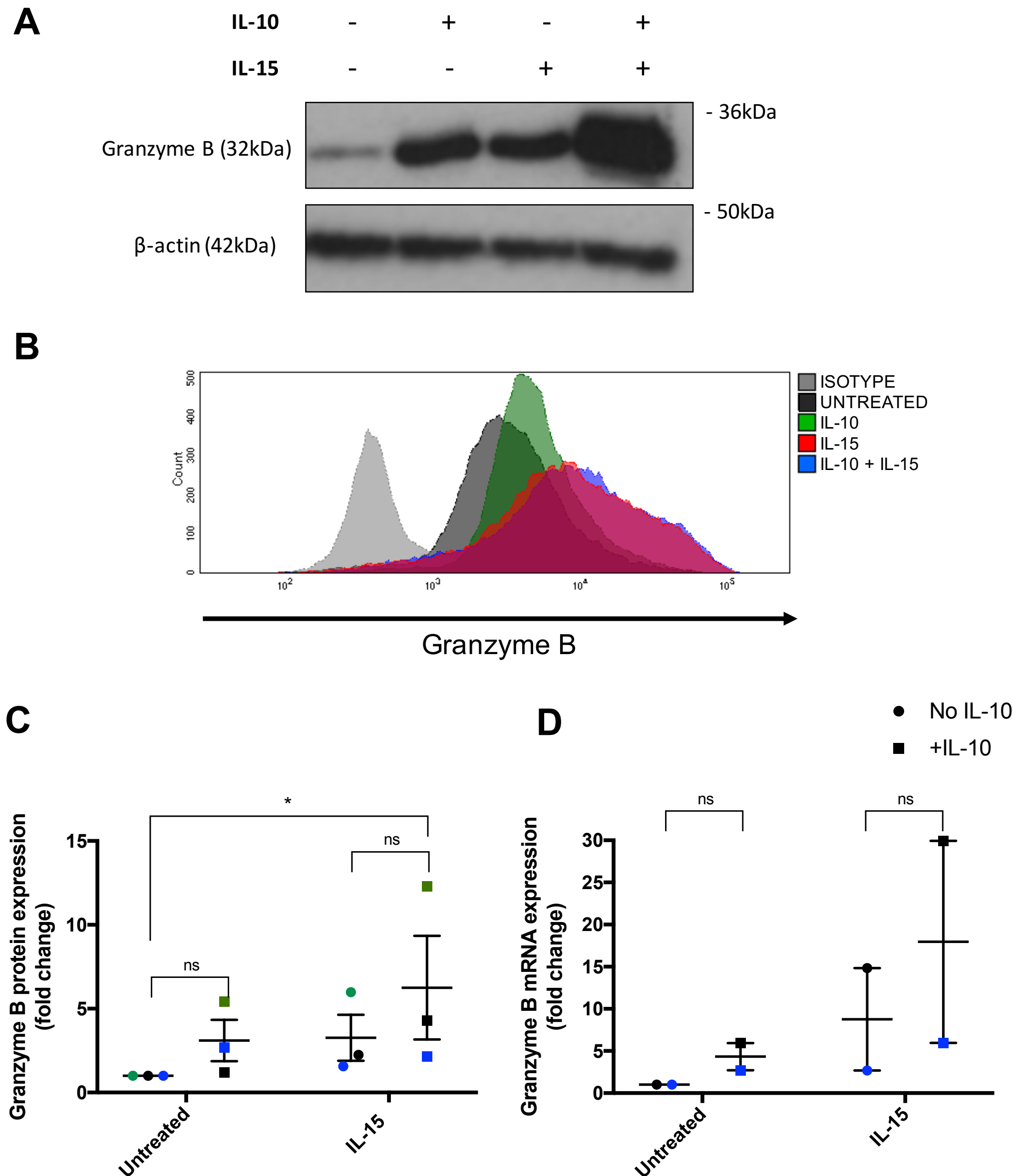


Figure 9: Granzyme B expression in cytokine stimulated NK cells

NK cells were stimulated with 50 ng/mL IL-10, 20 ng/mL IL-15 or both for 48 hours before analysis by (A) western blot with β-actin probed for as a loading control (representative of n=4) or (B) intracellular flow cytometry (n=1); grey histogram = isotype, black = untreated, green = IL-10, red = IL-15 and blue = IL-10 + IL-15. (C) Densitometry analysis was performed using ImageJ on 3 individual experiments, β-actin was used as a loading control and values are expressed as a fold change from untreated (n=3). (D). qRT-PCR for granzyme B mRNA transcripts was performed on cDNA synthesised from RNA isolated from NK cells (n=2). * P=<0.05, ns = not significant.

3.6 IL-10 enhances NK cells degranulation

Granzyme B expression is an indicator of cytolytic potential, however the observed increased in granzyme B expression does not illuminate whether it is the number of granules, or the content of the granules that is increased. Recent studies concerning the kinetics of NK cell degranulation have found that the size and content of the lytic granules influences the efficiency of target cell death. Whilst the NK cell lines YTS and NK92 contained similar numbers of lytic granules, YTS induced target cell death faster than NK92, an observation that was attributed to the larger granule size containing more effector molecules (Gwalani and Orange, 2018). The fact that IL-10 and IL-15 increase granzyme B expression suggests that they either have more granules, enabling them to kill more target cells, or increased granule content to improve target cell death efficiency. In order for increased granzyme B expression to contribute to cytolytic activity, NK cells must first exocytose the lytic granules across an immunological synapse. Measuring the level of granule exocytosis provides a more detailed picture of NK cell responses than measuring target cell death alone. Degranulation can be measured with a flow cytometric assay that analyses the expression of CD107a (LAMP-1) on the NK cell surface. CD107a is a granule membrane associated protein that is transiently displayed on the cell surface during degranulation, allowing for the detection of NK cells that have degranulated (Aktas et al., 2009).

NK cells were stimulated with IL-10 or IL-15 for 48 hours prior to being co-cultured with target cells in the presence of an intracellular protein transport inhibitor (GolgiStop) to prevent recycling of the lysosomal proteins from the NK cell surface. NK cells were analysed for cell surface expression of

CD107a, NK cells cultured alone ± cytokines were used as a control to measure the basal expression level of CD107a on the cell surface. Two target cell lines were used, K562 and SKOV3. K562 (derived from a myeloid leukaemia) is a well-established NK target cell line that expresses low levels of MHC I and therefore provides a strong activating stimulus to NK cells (Lozzio, 1975). SKOV3 is an ovarian cancer cell line shown to be less sensitive to NK cell lysis (Kruschinski et al., 2008). In the presence of K562, 48% of unstimulated NK cells degranulated, compared to only 5% in the presence of SKOV3 (Figure 10B and C). As expected, regardless of target cell, IL-15 stimulated NK cells degranulated more readily, with 68% of NK cells expressing CD107a in the presence of K562 (1.4-fold increase) and 18% in the presence of SKOV3 (3.6-fold increase). Interestingly, IL-10 stimulation also induced NK degranulation towards both targets, with 61% against K562 (1.25-fold increase) and 13% against SKOV3 (2.6-fold increase) (Figure 10B and C). The simultaneous addition of both IL-10 and IL-15 stimulation did not increase degranulation above IL-15 alone when co-cultured with K562 (67%) but did show an additive effect against the SKOV3 target (29%) (Figure 10B and C). When comparing untreated NK cells with IL-10 stimulated NK cell degranulation, a greater percentage of IL-10 stimulated NK cells degranulated towards target cells (K562 [p=0.0001]); SKOV3 [p=0.0551]) (Figure 10D and E).

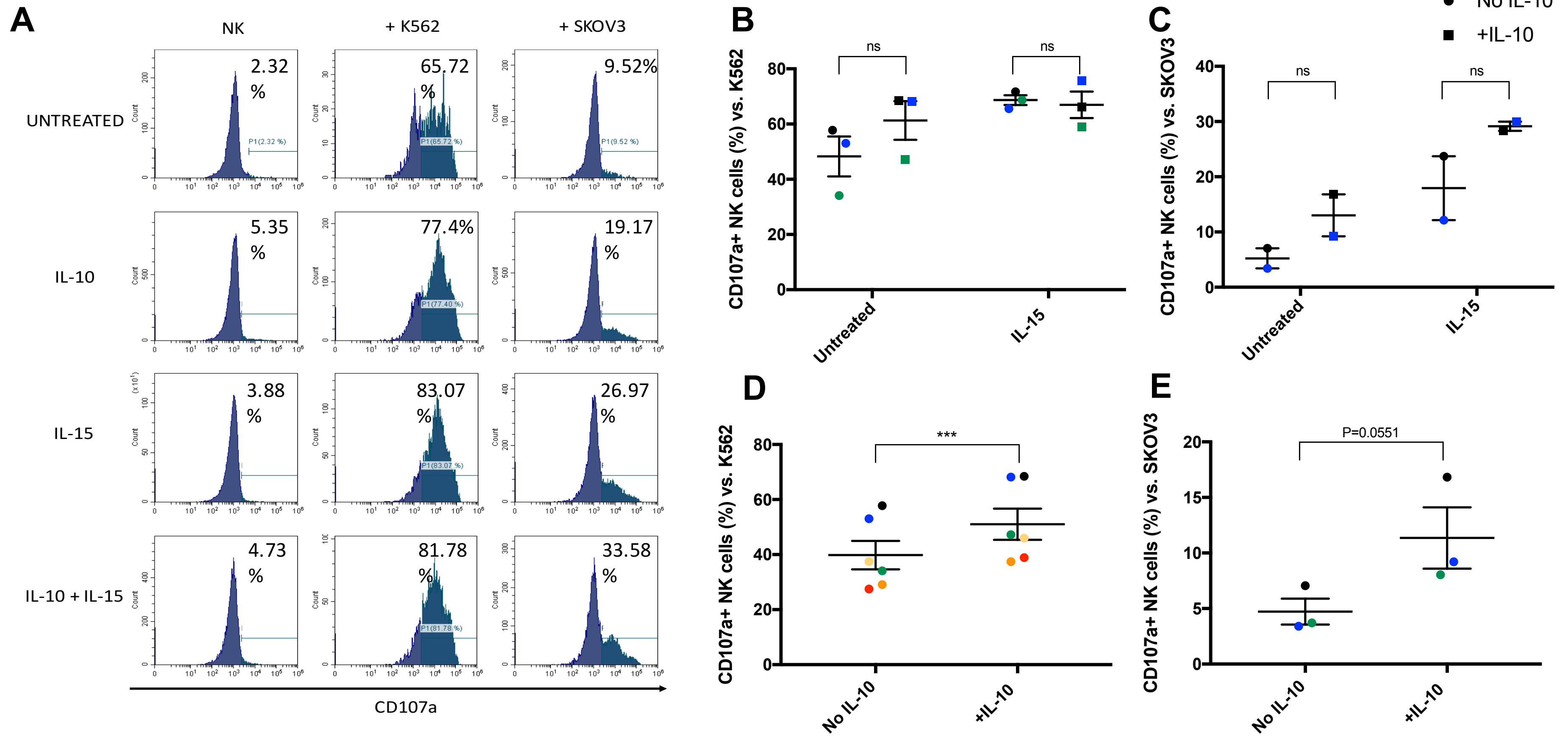


Figure 10: NK cell degranulation

NK cells were stimulated with 50 ng/mL IL-10, 20 ng/mL or both for 48 hours before being co-cultured with cell tracker violet (CTV) labelled K562 or SKOV3 target cells at 1:1 (E:T). Co-cultures were analysed by flow cytometry, CTV negative (CTV^{neg}) NK cells were gated on and analysed for expression of CD107a. (A) Representative histograms from one donor. (B) Graphs show mean % CD107a+ NK cells against K562 (n=3) or (C) SKOV3 (n=2). (D) Graphs show CD107a+ untreated NK cells vs. NK cells stimulated with IL-10 against K562 (n=8) or (E) SKOV3 (n=3) (data shown in D and E are from the untreated and IL-10 treatment groups in several assays in this thesis to increase the n number).*** P=<0.001, determined using a paired t-test (Figure 10 D and E).

3.7 IL-10 stimulated NK cells induce target cell death

In similar co-culture assays, target cell death can be also be measured to determine NK cell cytolytic activity. NK cells were stimulated, as in the degranulation assays, prior to being co-cultured at effector to target ratios (E:T) of 1:1 or 5:1 with K562 target cells. After incubation, cell death was determined with the use of a dead cell discriminator (Zombie NIR™) and analysed by flow cytometry. Both IL-10 and IL-15 stimulated NK cells induced more target cell death than untreated NK cells. IL-10 stimulation resulted in 2.2 - 2.5-fold increase in cell death and IL-15 stimulation resulted in 3.4 - 5-fold increase at E:T 1:1 and 5:1, respectively (Figure 11C and D). The combination of IL-10 and IL-15 stimulation did not enhance target cell death more than IL-15 alone at the lower E:T ratio; however, the data suggests an additive effect at the higher E:T ratio resulting in 10% more cell death than IL-15 alone (Figure 11D). When comparing target cell death induced by untreated NK cells vs. IL-10 stimulated NK cells, IL-10 stimulated NK cells induced a greater percentage of target cell death (p=0.0004) (Figure 11E).

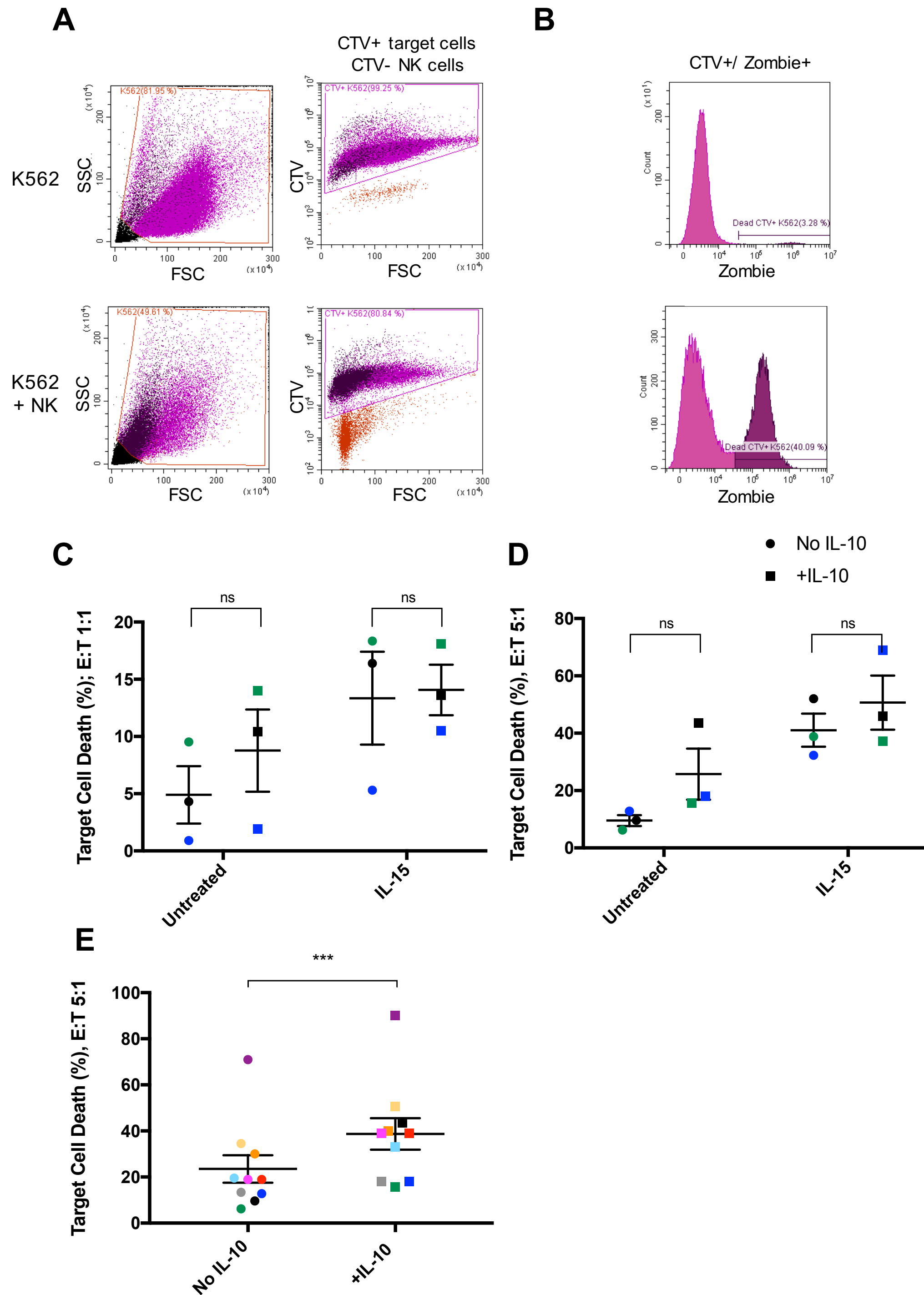


Figure 11: NK cell induced target cell death

NK cells were treated for 48 hours with 50 ng/mL IL-10, 20 ng/mL IL-15 or both prior to being co-cultured with CTV labelled K562 target cells at 1:1 and 5:1 E:T. After incubation, the co-culture was stained with a dead cell discriminator (Zombie NIR™) and analysed by flow cytometry to measure target cell death. (A) Representative gating strategy (1:1); target cells were identified as CTV+ and effectors as CTV^{neg} (top panel). The target cells were gated on and cell death was determined by Zombie

NIR™ fluorescence (bottom panel). (B) Representative histograms of from one donor of target cells co-cultured with IL-15 stimulated NK cells (gated as in panel A). (C) Graphs show mean target cell death at 1:1 and (D) 5:1 (n=3). (E) Graphs show mean target cell death with untreated NK cells vs. NK cells stimulated with IL-10 (n=10) (data shown in these graphs are from the untreated and IL-10 treatment groups in several assays in this thesis to increase the n number). *** P=<0.001, determined using a paired t-test (Figure 11E).

3.8 IL-10 does not enhance IFN-γ secretion

As well as having potent cytotoxic activity, activated NK cells produce pro-inflammatory cytokines such as IFN-γ (Caligiuri, 2008b), allowing activated NK cells to interact with other cell types and regulate immune responses. NK derived IFN-γ is essential for priming of Th1 cell responses in lymph nodes (Martín-Fontecha et al., 2004) and for the differentiation of monocytes into macrophages and DCs at sites of infection (Goldszmid et al., 2012). To further investigate the functional consequences of IL-10 signalling, IFN-γ production was analysed. PBMCs were stimulated with IL-15 ± IL-10 or IL-1β ± IL-10 for 48 hours and supernatants analysed by ELISA for IFN-γ. Treatment with IL-1β or IL-15 induced similar levels of IFN-γ (Figure 12A); however, combination treatments with IL-10 showed diverse effects. In combination with IL-1β, IL-10 displayed anti-inflammatory activity by inhibiting the production of IFN-γ, but interestingly, IL-10 did not inhibit IL-15 induced IFN-γ production (Figure 12A). Using PBMCs and ELISA assays, it is not possible determine which cell type IL-1β is stimulating, or the source of IFN-γ. Many immune cells are capable of producing IFN-γ; however, the predominant innate immune cell source is NK cells (Thäle and Kiderlen, 2005, Abboud et al., 2016, Uemura et al., 2009). IL-1β is a potent activator of the NF-κB pathway which induces the secretion of many pro-inflammatory cytokines, including the cytokine IL-12 (formerly known as NK cell stimulating factor) (Kobayashi et al., 1989). Additionally, IL-1β requires co-stimulation with IL-12 for the production of IFN-γ in NK cells (Cooper et al., 2001). Therefore, in the experimental model used here, IL-1β is most likely stimulating IL-12 (or IL-18) secretion from mononuclear cells that further stimulates IFN-γ secretion from NK cells. Presumably, IL-10 inhibits IL-

12 release from monocytes and hence indirectly reduces IFN-γ production by NK cells. This demonstrates the classical IL-10 activity, suppressing the synthesis of pro-inflammatory cytokines from monocytes. Unexpectedly, the addition of IL-10 to IL-15 stimulated PBMCs did not suppress IFN-γ secretion. Unlike the marked reduction observed in the IL-1β/IL-10 stimulation, the presence of IL-10 had no effect on the ability of IL-15 to induce IFN-γ secretion (Figure 12A). IL-15 directly activates NK cells, suggesting that IL-10 does not directly inhibit NK cells. Although IL-10 inhibits IFN-γ secretion after IL-1β stimulation, this is presumably indirectly by suppressing the production of other pro-inflammatory cytokines from other cell types. The presence of IL-10 in these assays demonstrate the pleiotropic effects of IL-10 in response to different stimuli and presumably on different cell types. To investigate this further, isolated NK cells were stimulated with IL-12 + IL-18 (potent inducers of IFN-γ (Chaix et al., 2008)) ± IL-10 for 48 hours before analysing the supernatant for IFN-γ; IL-12 + IL-18 stimulation induced an abundance of IFN-γ from NK cells that was not inhibited by the presence of IL-10 (Figure 12B), confirming that IL-10 does not directly inhibit IFN-γ production from NK cells. Similar to PBMCs stimulated with IL-10, isolated NK cells stimulated with IL-10 did not secrete IFN-γ (Figure 12B). To further understand the role IL-10 plays in IFN-γ production, I analysed the expression of mRNA from isolated NK cells using qRT-PCR. Isolated NK cells were stimulated with IL-10 or IL-15 for 48 hours. In contrast to IFN-γ protein, IL-10 stimulation upregulated IFN-γ mRNA, although to a much lower extent than IL-15 (Figure 12C).

It is possible that IL-10 upregulates IFN-γ mRNA without upregulating its protein product, and that further signals may be required to induce

translation. It is therefore important to determine whether NK cells are producing IFN-γ protein in response to IL-10, consequently, another method of protein detection was employed to confirm the previous results. To this end, intracellular cytokine staining of IFN-γ was performed and analysed using flow cytometry.

NK cells were stimulated with IL-10 or IL-15 for 48 hours, Golgi stop was then added to the culture for 4 hours before cells were fixed, permeabilised and analysed for IFN-γ expression. Only a very low percentage of unstimulated NK cells expressed IFN-γ and this was enhanced by IL-15 stimulation. However, IL-10 stimulation did not upregulate IFN-γ expression compared to the untreated control (Figure 13A and B). In agreement with the ELISA, IL-10 did not inhibit IL-15 mediated IFN-γ upregulation (Figure 13A and B). To understand whether a second signal was required to translate the IFN-γ mRNA into protein, NK cells were pre-stimulated with IL-10 or IL-15 before being co-cultured with K562 and analysed for IFN-γ expression as described above (Figure 13A and C). The presence of K562 increased the percentage of IFN-γ^+ NK cells, but stimulation with IL-10 did not enhance this when compared to the untreated control. IL-15 stimulation greatly enhanced the expression of IFN-γ and, as with NK cells alone, IL-10 did not inhibit IL-15 induced IFN-γ expression (Figure 13A and C). Although IL-10 upregulated IFN-γ mRNA, it was not possible to detect upregulated protein expression or secretion from NK cells. However, it should also be noted that whilst IL-10 did not enhance IFN-γ production, IL-10 did not inhibit the expression or secretion of IFN-γ from IL-15 stimulated NK cells.

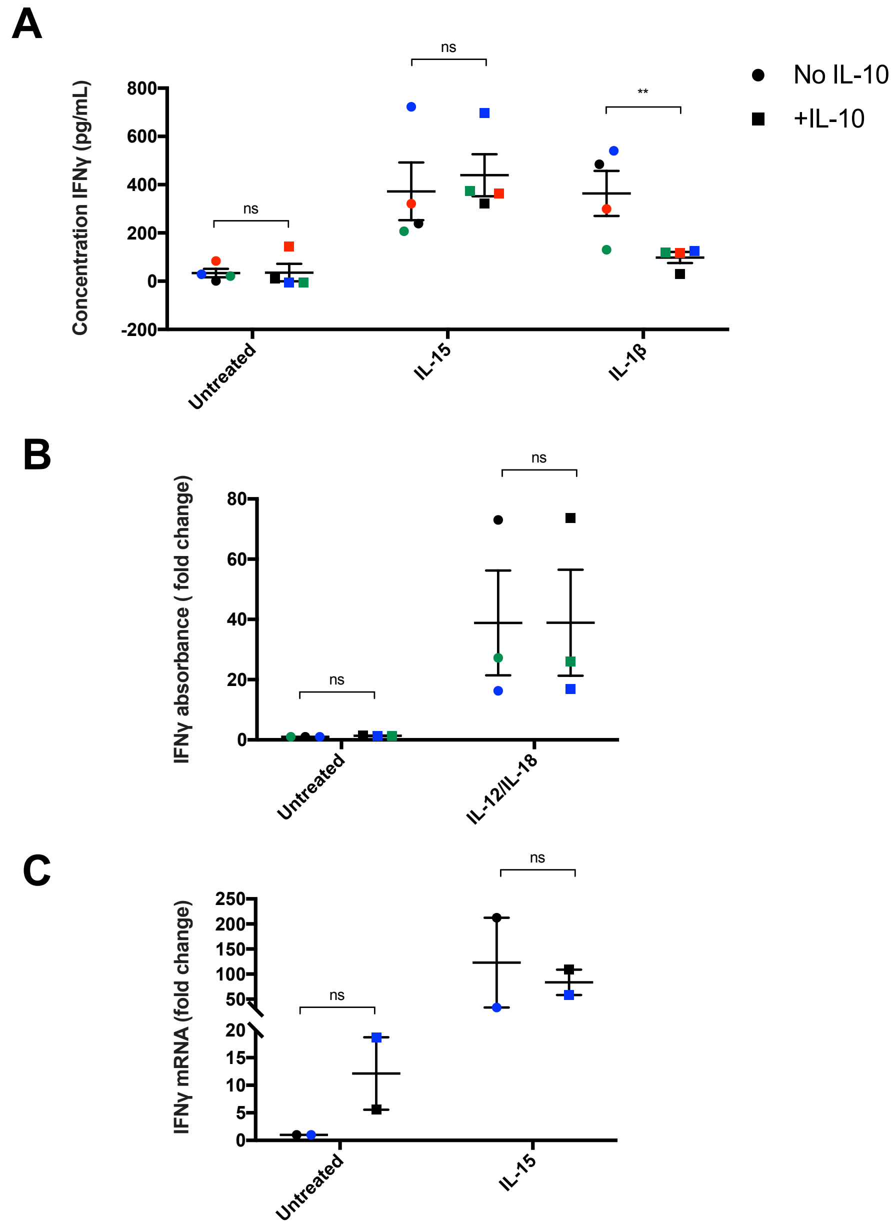


Figure 12: NK cell IFN-γ production is not inhibited by IL-10

(A) PBMCs were stimulated with IL-15 (20 ng/mL) +/- IL-10 (50 ng/mL), IL-1β (10 ng/mL) ± IL-10 for 48 hours prior to being analysed by ELISA for IFN-γ (n=4). (B) NK cells were stimulated with IL-12 (1 ng/mL) and IL-18 (5 ng/mL) ± IL-10 (50 ng/mL) for 48 hours prior to be analysed by ELISA for IFN-γ (n=3). Graph shows fold change from untreated control. (C) cDNA was synthesized from NK cells that were stimulated with IL-15 (20 ng/mL), IL-10 (50 ng/mL) or both for 48 hours and analysed by qRT-PCR for IFN-γ mRNA transcripts (n=2). Values were normalised to housekeeping control gene 18S and are expressed as fold from an untreated control. ns = not significant.

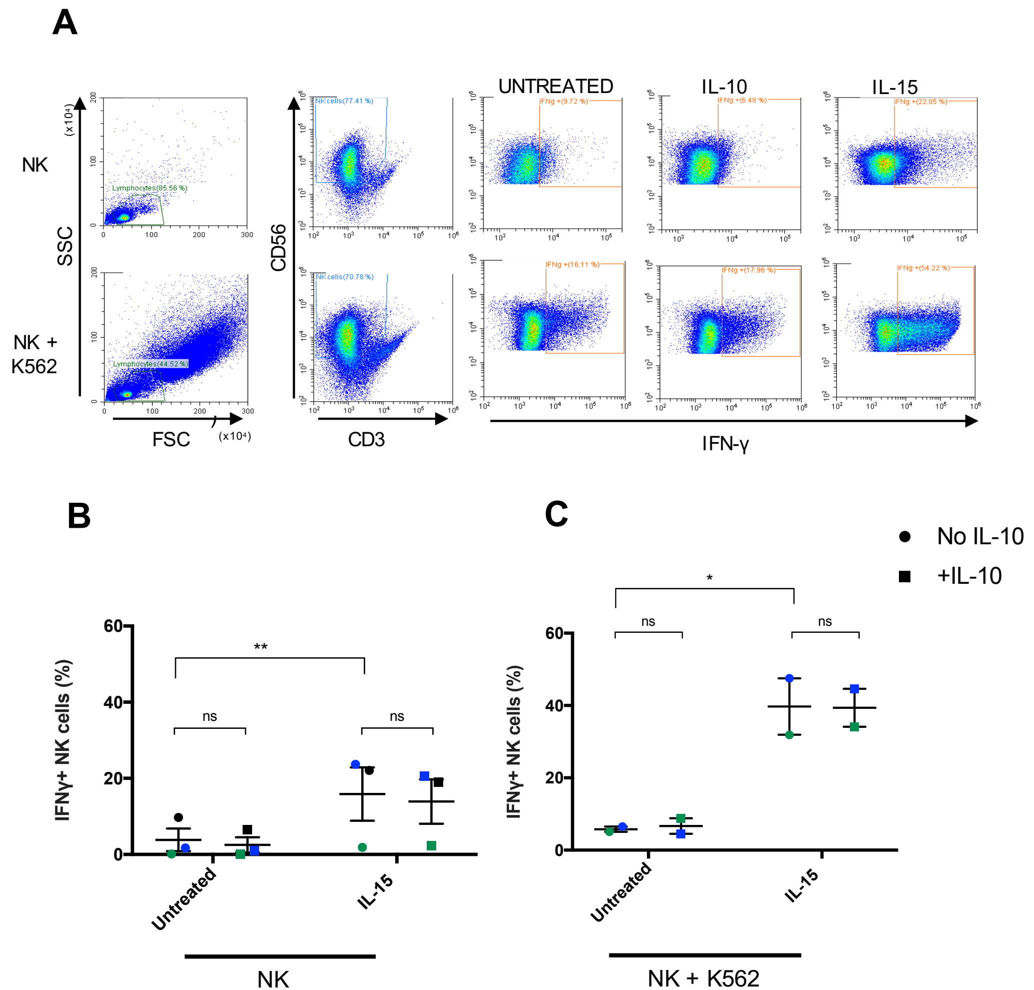


Figure 13: NK cell IFN-γ secretion is not enhanced by IL-10

NK cells were stimulated with IL-15 (20 ng/mL), IL-10 (50 ng/mL) or both for 48 hours prior to being co-cultured 1:1 with K562 for 5 hours. The co-cultures were then fixed and permeabilised before being stained with anti-IFN-γ and analysed by intracellular flow cytometry. (A) Representative flow cytometry plots. (B) Graph shows percentage of IFN-γ^{+} NK cells in the absence of K562 (n=3), and (C) and IFN-γ^{+} NK cells in the presence of K562 (n=2). *P=<0.05, **P=<0.01, ns = not significant.

3.9 Discussion

The pleiotropic effects of IL-10 have previously been observed across many cell types, including NK cells. Various studies detail pro-inflammatory effects of IL-10 on NK cells, including enhanced cytotoxicity, increased IFN-γ production and the ability to potentiate production of other pro-inflammatory cytokines (Carson et al., 1995, Lauw et al., 2000, Cai et al., 1999). However, IL-10 has also been shown to have inhibitory effects towards NK cells; Scott et al. reported that IL-10 neutralisation enhanced NK cell activation in a murine model of bacterial infection (Scott et al., 2006). Investigations into IL-10 mediated NK cell activity have revealed conflicting data, this could be in part down to the source of NK cells (NK-like cell lines vs. primary cells) distinct activities in different models (tumour co-cultures, bacterial infections) and the differences between *in vivo* and *in vitro* systems.

This study aimed to uncover how IL-10 influences primary human NK cells. IL-10 receptor expression is crucial for IL-10 to exert its effect on cells and I have confirmed that it is expressed by human NK cells. IL-10 receptor expression was initially characterised on NK cells by Carson et al.; this study showed that NK cells constitutively expressed the IL-10R at low density and this was not altered by IL-2 activation, despite IL-2 treatment resulting in downregulation of IL-10R mRNA (Carson et al., 1995). Here I demonstrate cell surface expression of IL-10R, which was not significantly altered by either IL-10 or IL-15 stimulation.

The JAK/STAT signalling pathways are of major importance in cytokine mediated cellular activity, with many cytokines activating multiple STATs to achieve different cellular responses. IL-10 is predominantly reported to

activate the STAT3 pathway and that has been confirmed in variety of immune cells. Here I have shown that IL-10 stimulation of NK cells induces phosphorylation of STAT3 in a dose dependent manner, subsequently resulting in enhanced cytotoxicity. It is still unclear whether this is the only mechanism of IL-10 activity, or indeed the mechanism responsible for this enhanced NK cell activity. Whether STAT3 signalling results in NK cell activation or inhibition is debated. Gotthardt et al. show that in a STAT3$^{-/-}$ mouse model, NK cell development and maturation are unaltered and tumour surveillance is enhanced (Gotthardt et al., 2014). STAT3 depletion resulted in increased granzyme B, perforin and DNAM1 expression that contributed to an increase in *in vitro* target cell killing of the melanoma cell line B16F10, and prolonged *in vivo* survival in a *v-abl*$^{+}$ leukaemia model. This study demonstrates an immunosuppressive role for STAT3 in NK cells; however, increased signalling via STAT4 and STAT5 in the absence of STAT3 was also observed (Gotthardt et al., 2014). Whilst IL-2/IL-15 and IL-12 are known to signal via STAT5 and STAT4, respectively, these activating cytokines are also known to mediate their effects via STAT3 (Johnston et al., 1995, Jacobson et al., 1995). The deficiency of STAT3 could be compensated for by other STAT pathways resulting in stronger signalling, the enhanced cytotoxicity demonstrated here could be in part due to higher activity of other STAT proteins compensating for the loss of STAT3. In contrast to this, Zhu et al. show that the expression of a key NK cell activating receptor, NKG2D, was driven by STAT3. NKG2D expression is transcriptionally controlled by STAT3, due to a STAT3 binding site in the NKG2D promoter, and that binding is enhanced by treatment with IL-10 or IL-21. Cell surface expression and mRNA

levels of NKG2D were upregulated by STAT3 activation and could be blocked with small molecule inhibitors of STAT3 (Zhu et al., 2014). In addition, this study also observes enhanced degranulation of NK cells with activated STAT3 that was reduced in the presence of STAT3 inhibitors. Reduced NKG2D expression was also confirmed in NK cells isolated from haematopoietic STAT3$^{-/-}$ mice compared to WT, and the ability of STAT3 activating cytokines to increase expression of NKG2D was blunted in hyper-IgE syndrome patient samples with dominant negative mutations in STAT3 (Zhu et al., 2014). These studies show clear yet distinct roles of STAT3 in NK cell activity. This is not unique to NK cells, STAT3 plays multiple roles dependent on cell type and stimulus. STAT3 mediates both IL-6 and IL-10 signal transduction to provide pro and anti-inflammatory responses respectively in monocytes (Niemand et al., 2003), highlighting that this transcription factor is central to many cellular activities. It is possible that STAT3 mediates multiple responses in NK cells, as it does in monocytes (Niemand et al., 2003). Although knockout models provide great insight into the role of STAT3, it would be useful to investigate its role in response to different stimuli and understand how one transcription factor mediates such diverse outcomes.

The connection between IL-10 and granzyme B expression has been investigated in other studies. As with much of the data concerning IL-10 activity, these reports show cell type or environment specific activity. Murine CD8^{+} T cells isolated from IL-10 treated tumours were shown to have highly upregulated granzyme mRNA expression and the same was observed in human CD8^{+} T cells stimulated *in vitro* with IL-10 (Mumm et al., 2011). Conversely, in the context of hepatocellular carcinoma (HCC), the frequency

of granzyme B^+ $CD4^+$ T cells were found to negatively correlate with IL-10 expressing B cells (Bregs). Moreover, when cultured together *in vitro,* Bregs were found to suppress granzyme B expression in $CD4^+$ T cells (Xue et al., 2016). *In vitro* stimulation of BCR-engaged B cells with IL-10 in combination with IL-4 was found to enhance granzyme B expression in B cells (Hagn et al., 2009), and IL-10R blockade reduced the number of granzyme B^+ NK cells from the lungs and spleen of an MCMV infection model (Stacey et al., 2011). These studies exemplify the range of cell types and systems used to investigate this relationship, highlight the varied responses, and why it is important to assess the effect of IL-10 on granzyme B expression in primary human NK cells. The results in this chapter clearly show increased granzyme B expression at the mRNA and protein level in response to IL-10. Regardless of donor variability, western blotting and FACS analysis showed upregulated granzyme B protein expression, and western blotting and qRT-PCR data indicate an additive effect of IL-10 and IL-15 in combination. It is possible that the combination of cytokines triggers distinct signalling pathways via STAT3 and STAT5 to induce granzyme B expression, or perhaps both result in strong STAT3 signalling or STAT3/STAT5 heterodimers to achieve this. The concept of cytokine synergy is well-known and many cytokines work cooperatively to determine cell fate/activity. Combinations of cytokines have also been shown to enhance other function in NK cells, such as proliferation and IFN-γ production (Cai et al., 1999, French et al., 2006). It is interesting to see in this study, that IL-10 does not inhibit IL-15 induction of granzyme B, further supporting a pro-inflammatory role of IL-10.

IL-10 stimulation of NK cells also resulted in enhanced degranulation. Interestingly, the combination of cytokines did not indicate an additive effect when co-cultured with K562, showing similar levels of degranulation to IL-15 stimulated cells. However, co-culture with SKOV3 targets showed enhanced levels of degranulation following IL-10 treatment. Both K562 and IL-15 are potent NK cell activators, in combination these maximally stimulate the NK cells and the addition of IL-10 is negated. The weaker stimulation from SKOV3 targets allows for the additive effect of other activating stimuli, such as IL-10, to contribute to overall degranulation. This raises important considerations for the environments in which IL-10 could exert pro-inflammatory effects. Nevertheless, IL-10 was able to prime NK cells for better degranulation upon target contact, whether this is achieved through the same mechanisms as IL-15 remains to be explored.

Increasing the E:T ratio increased target cell death as expected, as more NK cells are able to form synapses with the target cells, and this was seen in all conditions. However, less target cell death was observed with IL-10 stimulated NK cells than IL-15 stimulated NK cells regardless of E:T ratio. Given that IL-10 increased granzyme B expression and degranulation in a similar manner to IL-15, I expected target cell death to be similar in both conditions. This suggests that IL-15 induces more degranulation than IL-10. Although we can detect degranulation, this method does not provide information on the serial killing ability of these cells. It is possible that IL-15 stimulated NK cells are able to degranulate quicker, or are more capable of forming successive synapses than IL-10 stimulated NK cells.

The role IL-10 plays in the control of IFN-γ remains elusive. IFN-γ expression and secretion is tightly controlled by an array of epigenetic, transcriptional and post-transcriptional mechanisms (Mah and Cooper, 2016); NK cells have a constantly accessible IFNG locus and low-level transcription of IFN-γ mRNA (Stetson et al., 2003), presumably to allow for rapid induction of effector functions. Upregulation of IFN-γ expression is achieved by NKR ligation, antibody recognition by CD16 or cytokine stimulation; each of which activate distinct transcription factors including MAPK, STATs and NF-κB, that can bind promoter and enhancer regions to promote IFN-γ transcription (Mah and Cooper, 2016). NK cell recognition of K562 target cells is dependent on the engagement of activatory NKRs which induce IFN-γ production. Keppel et al. showed that the mechanisms of IFN-γ production are dependent on the nature of the stimulus. Whilst both cytokine and NKR stimulation resulted in increased protein production, IL-12/IL-18 stimulation highly upregulated IFN-γ mRNA whereas only modest changes were seen with NKR stimulation, indicating that the transcription and translation of IFN-γ are independently controlled. This suggests that IFN-γ production by cytokine stimulation is transcriptionally controlled, whereas NKR stimulation was post-transcriptionally controlled (Keppel et al., 2015). Here I have shown that NKR engagement (by addition of K562 target cells) upregulates IFN-γ protein in the absence of cytokine stimulation and this was enhanced in IL-15 stimulated NK cells. IL-15 stimulation enhanced IFN-γ protein regardless of NKR engagement, indicating that IL-15 regulates both transcriptional and translation pathways. IL-10 stimulation induces mRNA but not protein

expression, suggesting that IL-10 does not activate pathways required for IFN-γ translation.

More recently, the metabolic requirements of NK cell activation have been investigated (Mah et al., 2017, Keppel et al., 2015, Keating et al., 2016, Donnelly et al., 2014). Blocking oxidative phosphorylation (OXPHOS) inhibited IFN-γ production from NKR stimulated NK cells, identifying respiration as essential to induce IFN-γ production after NKR stimulation. This inhibition was not observed in NK cells stimulated with IL-12/IL-18, revealing OXPHOS independent IFN-γ production. Interestingly, stimulation with IL-12/IL-15 was partially impaired by OXPHOS blockade, suggesting this signal is weaker than IL-12/IL-18. However, prolonged high dose IL-15 treatment could overcome these metabolic requirements (Keppel et al., 2015). Furthermore, IL-2 and IL-12/IL-15 stimulation has been shown to metabolically reprogram NK cells by upregulating nutrient receptors (CD71, CD98 and GLUT1) and the metabolic regulator mTOR. This reprogramming increases the rate of glycolysis and OXPHOS, allowing for biosynthetic demands such as IFN-γ production (Keating et al., 2016, Donnelly et al., 2014). Cellular metabolism has also been shown to regulate cytokine production through levels of GAPDH. Whilst the primary role of GAPDH is in glycolysis, it has also been shown to repress IFN-γ translation by binding AU rich elements (AREs) in the 3'UTR. The metabolic switch from OXPHOS to aerobic glycolysis that is observed in activated T cells, increases the requirement for GAPDH in glycolysis, therefore relieving GAPDH mediated repression (Chang et al., 2013). IL-15 increases the rate of glycolysis in NK cells (Keating et al., 2016) and therefore recruits GADPH into the glycolytic

pathway, allowing IFN-γ to be translated. It is possible that IL-10 does not stimulate glycolysis, therefore GAPDH remains bound to IFNG mRNA and represses IFN-γ translation. Aside from metabolism, a possible reason for the lack of protein production induced by IL-10 could be explained by unstable mRNA transcripts. Many inflammatory mediators have unstable mRNAs due to the presence of AREs in the 3'UTR. AREs have been shown to promote mRNA degradation through interactions with multiple proteins, providing another level of regulation for inflammatory responses (Stumpo et al., 2010). IL-12 and IL-18 stimulation has been shown to stabilise IFN-γ mRNA by via MAPK p38, and IFN-γ protein production was greatly reduced by p38 inhibition (Mavropoulos et al., 2005).

It should be noted that although many of these experiments were performed with isolated NK cells, the nature of negative cell selection does not ensure 100% pure NK cells. Therefore, a small percentage of cells in these assays are non-NK PBMCs (T cells, B cells or monocytes). As these can respond to IL-10 and interact with NK cells (either through cell interactions or cytokines), the number of non-NK cells in culture may impact NK cell responses. For instance, IL-10 treatment will inhibit the production of pro-inflammatory cytokines from monocytes but IL-15 will not, meaning a high percentage of monocytes in the assays could lead to reduced NK cell activation in the IL-10 stimulated groups through indirect monocyte-mediated mechanisms. These interactions must be considered when interpreting data from these assays; however, as the number of non-NK cells in these assays is minimal, these effects will also be minimal.

The aim of the work in this chapter was to confirm the signalling pathway used by IL-10 in NK cells, and investigate how this signalling effects NK cell function. The data presented confirms that IL-10 signals via STAT3, and that IL-10 enhances NK cell cytotoxicity, but does not upregulate IFN-γ protein production. Taken together, this data provides strong evidence of a pro-inflammatory effect of IL-10 on NK cells. The next chapter of this study investigates how IL-10 affects other NK cells activation mechanisms such as proliferation, activating receptor expression and metabolism.

Chapter 4

IL-10 and NK cell activation pathways

4.1 Introduction

Activation is an umbrella term used to describe a variety of cellular changes that distinguish between unstimulated and stimulated cells. In addition to enhanced cytotoxicity, activated lymphocytes can be characterised by altered expression of activation receptors (Sanchez-Correa et al., 2017, de Rham et al., 2007, Vendrame et al., 2017) and metabolic changes (Donnelly et al., 2014). The previous chapter showed that IL-10 was capable of enhancing NK cell cytotoxicity but not cytokine secretion, highlighting the dichotomous activity of IL-10. The experiments in this chapter aim to understand whether IL-10 regulates the other mechanisms involved in NK cell activation.

4.2 IL-10 does not mediate cellular changes associated with early activation

Lymphocytes in culture grow as single cells in suspension. Activating signals induce conformational changes of integrins expressed on the cell surface, increasing the avidity of the receptors (Abram and Lowell, 2009) and causing the formation of cellular aggregates. To determine the effect of IL-10 on the ability of NK cells to form aggregates, isolated NK cells were cultured in the presence of IL-15, IL-10 or a combination of both for 48 hours and analysed by light microscopy. Stimulation of NK cells with IL-15 induced visible clumping of the NK cells and the formation of aggregates (Figure 14A, bottom

left panel); however, IL-10 stimulation of NK cells did not induce the formation of cellular aggregates (Figure 14A, top right panel). Those cells cultured with a combination of IL-15 and IL-10 show comparable morphology to those that have been cultured with IL-15 alone, suggesting that IL-10 does not inhibit the formation of these aggregates (Figure 14A, bottom right panel).

Additionally, lymphocyte activation can also be determined by measuring the cell surface expression of CD69, a well-established marker of lymphocyte activation (Cibrián and Sánchez-Madrid, 2017). PBMCs were stimulated as before with IL-15, IL-10 or a combination of both for 48 hours and analysed by flow cytometry. NK cells were gated based on lymphocyte size (FSC/SSC plots) and identified as $CD56^{+}$ $CD3^{neg}$ as in Figure 7A. A small percentage of resting NK cells expressed CD69 (11%) and this varied greatly depending on the donor. Stimulation with IL-15 consistently upregulated expression of CD69 in the vast majority NK cells (88% in Figure 14B), whereas IL-10 stimulation did not increase CD69 expression (Figure 14B and C). Stimulation with the combination of IL-10 and IL-15 showed similar CD69 expression to IL-15 stimulated NK cells (90%), indicating that IL-10 did not inhibit the expression of CD69. These measurements of early lymphocyte activation suggest that IL-10 does not activate NK cells through the same mechanisms as IL-15.

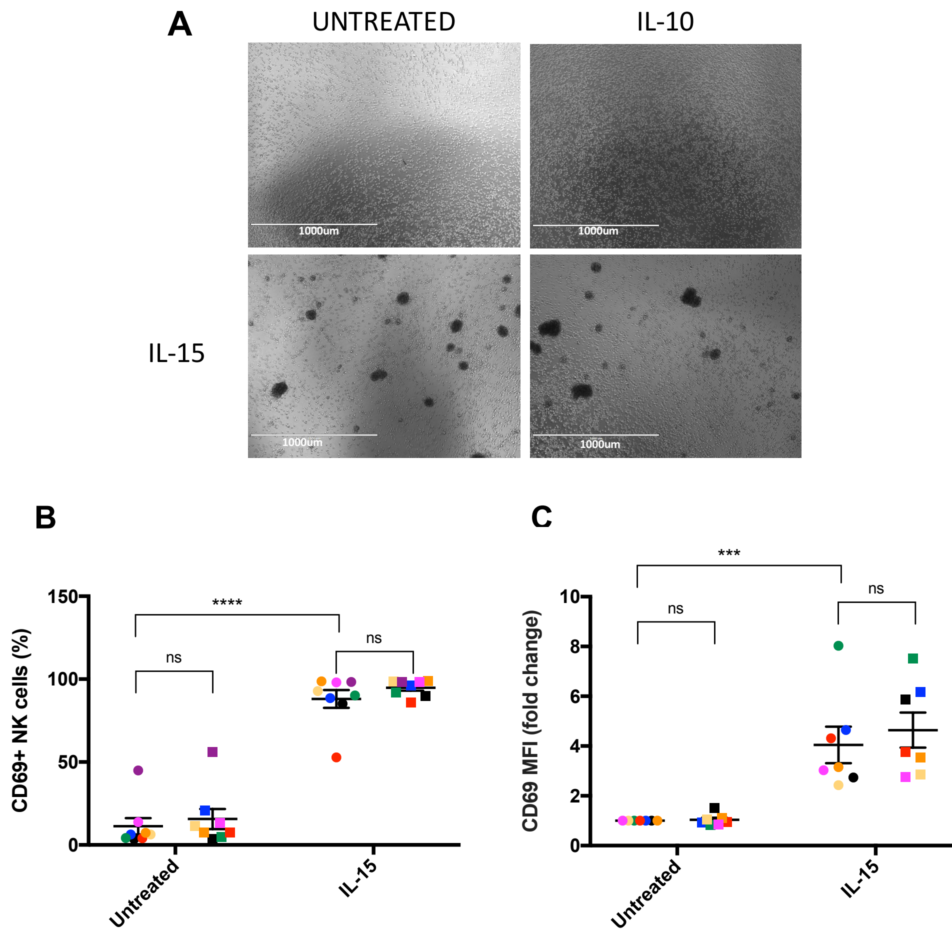


Figure 14: IL-10 does not induce NK cell activation markers

(A) Isolated NK cells were cultured alone, with IL-10 (50 ng/mL), IL-15 (20 ng/mL) or both for 48 hours before being analysed by light microscopy. (B) PBMCs were cultured with IL-10 (50 ng/mL), IL-15 (20 ng/mL) or both for 48 hours prior to analysis of CD69 expression with flow cytometry. Graphs show percentage of NK cells positive for CD69 expression and (C) MFI (fold change from untreated), n=8. ***P=<0.001, ****P=<0.0001, ns = not significant.

4.3 IL-10 does not upregulate expression of key NK cell activating receptors

In addition to CD69, the complex NK cell receptor repertoire can be altered with activating stimuli. Cytokine stimulation plays a key role in augmenting NK cell receptor expression and upregulating activating receptors (Vendrame et al., 2017). To investigate whether IL-10 induced changes in NK cell receptor expression, PBMCs were stimulated with IL-15, IL-10 or a combination of both for 48 hours and analysed for the expression of the key activating receptors NKG2D, DNAM-1, NKp30 and NKp46. NKG2D and NKp46 were expressed on a high percentage of resting NK cells (85% and 86%, respectively) and the proportion of expressing cells remained unchanged in all conditions (Figure 15A). However, IL-15 stimulation increased the expression of DNAM-1 and NKP30 as well as the density of NKG2D expression (Figure 15A and B). In contrast, treatment with IL-10 did not increase the expression of NKG2D, DNAM1, NKp30 or NKp46 (Figure 15A and B) and their expression on NK cells stimulated with a combination of IL-10 + IL-15 was similar to that of IL-15 stimulated NK cells (Figure 15A and B). Multiple cellular populations within PBMCs express IL-10R and are capable of responding to IL-10. Stimulating PBMCs with IL-10 induces cellular interactions and the secretion of immunomodulatory molecules. Therefore, phenotyping NK cells from within this environment may not reveal how IL-10 affects NK cells, but rather the effects of these cellular interactions. To understand the direct effect of IL-10 on NK cells, the above phenotyping assays were repeated in isolated NK cells. IL-10 did not significantly upregulate the expression of CD69, NKG2D, DNAM-1, NKp30 or NKp46, inhibit IL-15 induced expression of receptors, or reduce

basal expression of any of the receptors investigated (Figure 16A-C). The exception to this is the density of NKp46 expression, which is significantly upregulated by IL-10 (P=<0.01). Additionally, a trend towards upregulation of NKG2D is also observed in IL-10 stimulated NK cells, but this does not reach statistical significance in this study (P=0.0835) (Figure 16C).

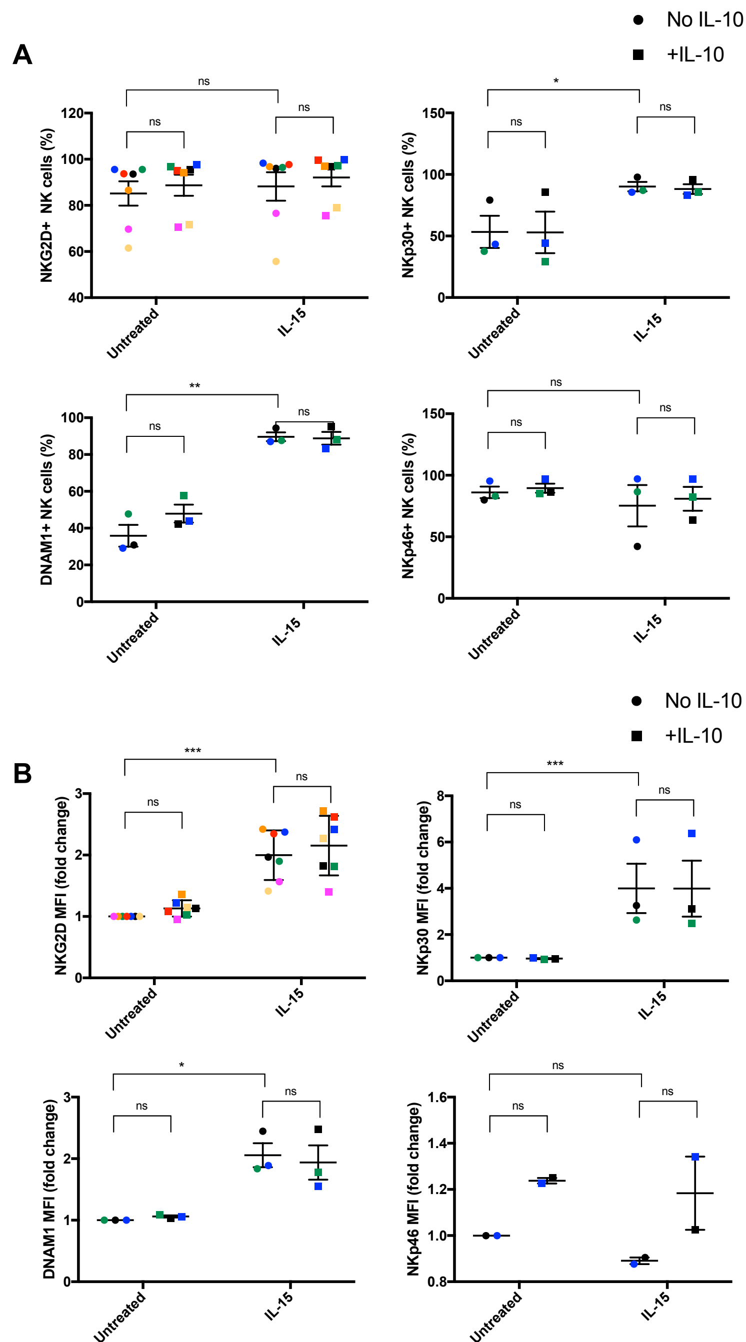


Figure 15: IL-10 does not upregulate NK cell receptor expression (in PBMCs)

PBMCs were cultured with IL-10 (50 ng/mL), IL-15 (20 ng/mL) or both for 48 hours prior to flow cytometry analysis for receptor expression. NK cells were identified as $CD56^{+}$ and $CD3^{neg}$, and the expression of the receptors on NK cells was measured. (A) Graphs show percentage of NK cells positive for receptor expression and (B) the MFI (fold change from untreated) for NKG2D (n=7), DNAM1 (n=3), NKp30 (n=3), and NKp46 (n=3). *P=<0.05, **P=<0.01, ***P=<0.001, ns = not significant.

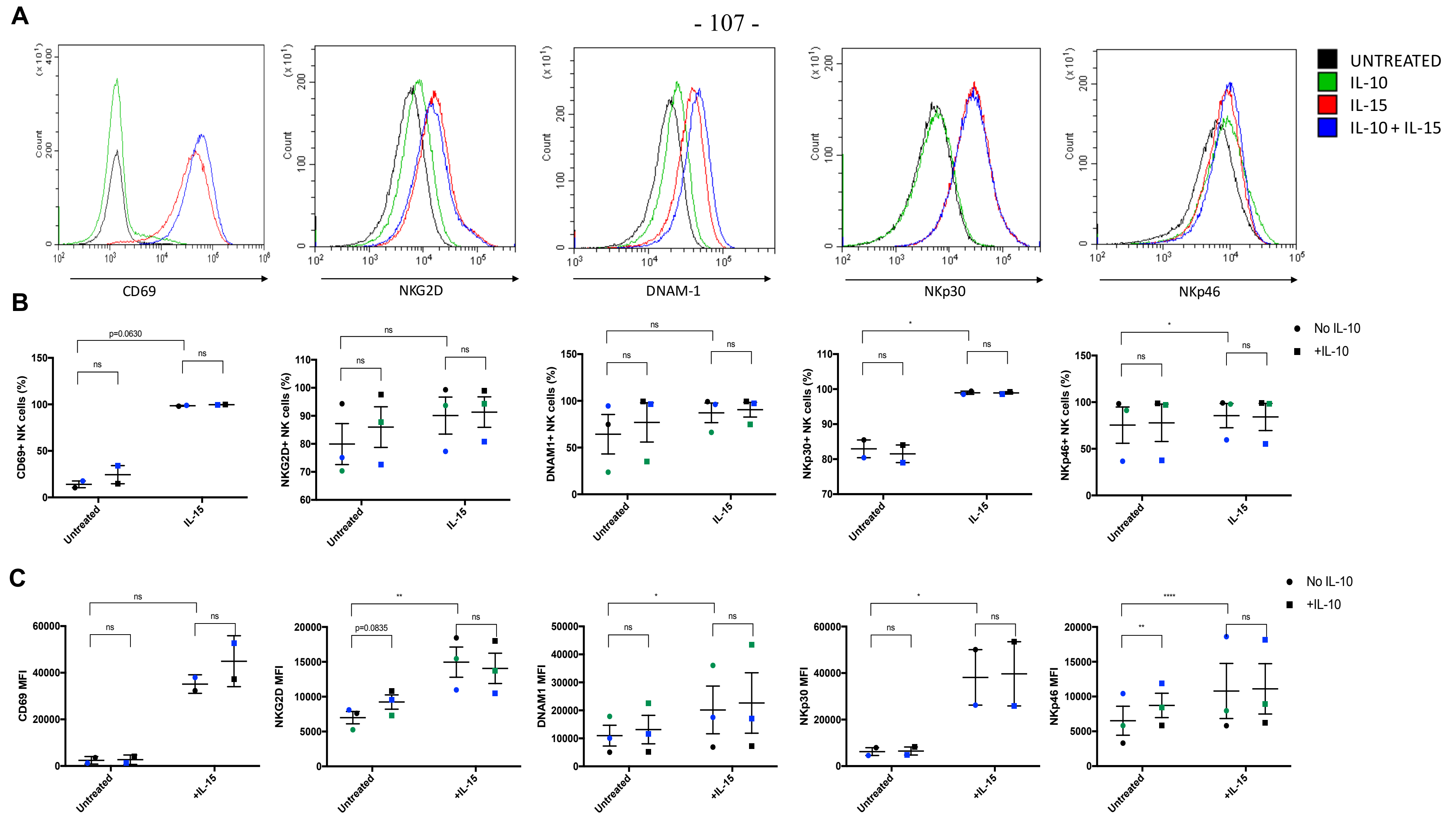


Figure 16: IL-10 does not upregulate NK cell receptor expression

Isolated NK cells were cultured with 50 ng/mL IL-10, 20 ng/mL IL-15 or both for 48 hours, receptor expression was measured by flow cytometry. (A) Example histograms from 1 donor, black = untreated, green = IL-10, red = IL-15 and blue = IL-10 + IL-15. (B) Graphs show percentage of NK cells positive for receptor expression and (C) MFI of receptor expression for CD69 (n=2), NKG2D (n=3), DNAM1 (n=3), NKp30 (n=2), and NKp46 (n=3). *P=<0.05, **P=<0.01, ***P=<0.001, ****P=<0.0001, ns = not significant

4.4 IL-10 does not induce proliferation

Resting NK cells are considered to be quiescent as they do not proliferate and display a gene signature that maintains the quiescent state (Dybkaer et al., 2007). A variety of cytokines can induce the proliferation of NK cells, including common γ-chain cytokines IL-15 and IL-2, but also IL-12 and IL-18 (Wang et al., 1999, Carson et al., 1994, Lauwerys et al., 1999). Proliferation is characteristic of activated lymphocytes in inflammatory environments, therefore I investigated the effect of IL-10 on the proliferative properties of NK cells. Isolated NK cells were cultured in the presence of cytokines for 96 hours, DNA content and cell cycle stage was determined by flow cytometry using the fluorescent DNA intercalating agent, propidium Iodide (PI). A gating strategy to ensure only single cells were analysed was applied based on the cell height and area (Figure 17A). Untreated NK cells remained quiescent in G0/G1, indicated by a single peak of PI+ cells. IL-15 stimulation induced 28% of cells to enter S/G2 stage of the cell cycle, indicated by increased PI staining in a proportion of cells, identified in the S/G2 gate (Figure 17B and C). Interestingly, IL-10 stimulation did not induce cell cycle entry and all NK cells remained in the G0/G1 stage (Figure 17B and C), similar to resting NK. These data indicate that IL-10 does not provide a mitogenic signal to NK cells. I have previously shown that 50 ng/mL IL-10 was sufficient to induce phosphorylation of STAT3 and subsequently enhance cytotoxicity (Figure 7). It is possible however; that IL-10 is a much weaker activation signal than IL-15, and that increased concentrations of IL-10 could influence proliferation. To test this, IL-10 and IL-15 were titrated onto NK cells and proliferation assessed as before. Low concentrations of IL-15 (0.2 – 2 ng/mL) did not induce cell cycle entry,

whereas the working concentration of 20 ng/mL and the higher concentration of 200 ng/mL induced similar percentages of cells to proliferate, 19.2% and 22.06%, respectively. However, neither 50 ng/mL or a high concentration of 500 ng/mL of IL-10 induced NK cell proliferation (Figure 17D). To determine if IL-10 has an inhibitory effect on proliferation, NK cells were stimulated with typical pro-proliferative cytokines: IL-15, IL-2, or IL-12 + IL-18 in the presence of 50 ng/mL IL-10. All cytokines induced NK cell proliferation which was neither enhanced or inhibited by IL-10 (Figure 17E).

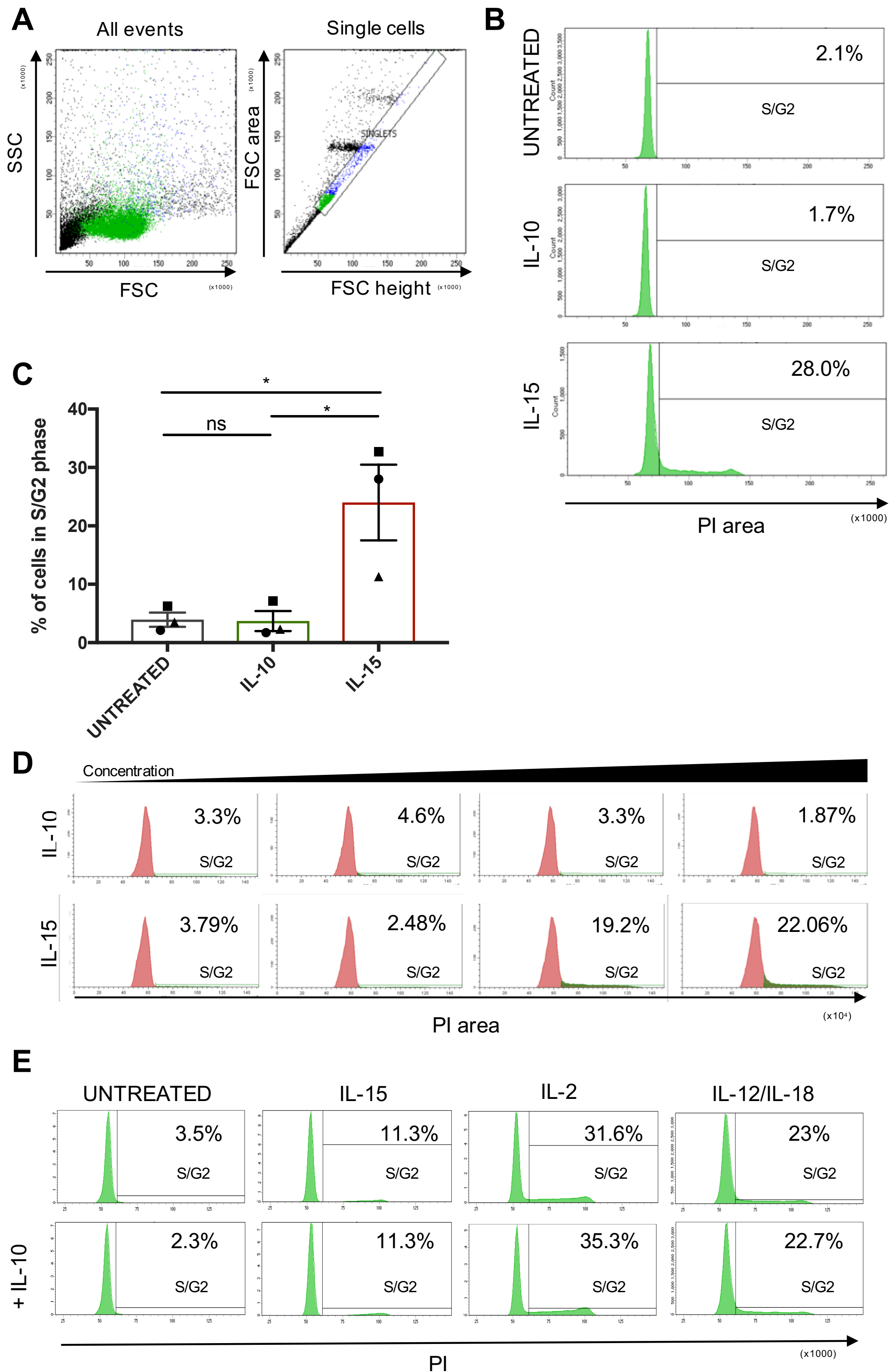


Figure 17: IL-10 does not drive proliferation

(A) Isolated NK cells were cultured with 50 ng/mL IL-10, 20 ng/mL IL-15 or both for ~96 hours before being fixed in ethanol and stained with PI and analysed by flow

cytometry. Flow cytometry gating strategy, events were collected on a linear scale and area vs. height was used to determine single cells. Single cells were then analysed further for PI fluorescence to determine DNA content/cell cycle stage. (B) Representative histograms of PI staining. S/G2 gating bar was set at 2% in the untreated control cells, cells in this gate are considered to be in S/G2 phase. (C) Percentage of cells that are in S/G2 phase (n=3), each shape represents a different donor (n=3). (D) Isolated NK cells were stimulated with increasing concentrations of IL-10 (0.5, 5, 50 and 500 ng/mL) or IL-15 (0.2, 2, 20 and 200 ng/mL) for 96 hours before being fixed in ethanol and stained with PI and analysed by flow cytometry (n=1). (E) Isolated NK cells were stimulated with either 10 ng/mL IL-15, 20 ng/mL IL-2 or 10 ng/mL IL-12 + 100 ng/mL IL-18 with or without 50 ng/mL IL-10 (n=1). *P=<0.05; ns = not significant, determined using a repeated measures one-way ANOVA with Tukey's multiple comparison test.

4.5 IL-10 does not alter growth, but does increase protein synthesis

Activation of cells is intrinsically linked to cellular changes such as increased protein production and cellular growth. As such, activated lymphocytes undergo metabolic reprogramming to facilitate these increased biosynthetic demands (Loftus and Finlay, 2016). These cellular changes are often co-incident with proliferation, for example, proliferating cells must increase in size before division to produce daughter cells of the appropriate size. However, my current data suggests that IL-10 may activate NK cells in a different manner to IL-2 and IL-15, inducing increased killing capacity in the absence of proliferation. To further understand this, I determined the influence of IL-10 on protein content and cell size as a surrogate for increased metabolic activity of NK cells. In order to fulfil the increased demands of activation, activated NK cells increase the rate of protein synthesis. To measure protein synthesis, I used a puromycin incorporation assay. The structure of puromycin is similar to that of aminoacyl-transfer RNA, and as such can be incorporated into *de novo* proteins whilst in the elongation phase (Schmidt et al., 2009). Therefore, the incorporation of puromycin is used to detect newly synthesized proteins. To investigate whether IL-10 induces protein synthesis, I stimulated NK cells with IL-10 or IL-15 for 24 hours prior to adding puromycin into the culture. The cells were then lysed and analysed by western blotting for the presence of puromycin (using an anti-puromycin antibody). Protein synthesis was detected in all conditions, including the cells not treated with cytokine. Importantly, puromycin incorporation was blocked by the protein synthesis inhibitor cycloheximide (CHX). Increased protein synthesis was detected in

both IL-10 and IL-15 stimulated NK cells; however, IL-15 stimulation induced more protein synthesis (Figure 18A and B). This is unsurprising as previous data shows that IL-15 is a strong activator (Figures 9-17), inducing proliferation and upregulating the expression of activating receptors and IFN-γ. These data indicate that IL-10 induces protein synthesis in NK cells, but not to the extent seen in IL-15 activated NK cells.

To measure cell growth i.e. an increase in the physical size of a cell, I determined the FSC and SSC of NK cells by flow cytometry. These measurements provide a relative indication of size (FSC) and granularity (SSC) of cells. Isolated NK cells were stimulated for 48 hours with IL-10, IL-15 or a combination before analysis by flow cytometry. Plotting the FSC and SSC values in histograms allows direct comparison of cytokine treated NK cells to untreated control cells. IL-15 stimulation induced an increase in both size and granularity of the NK cells, indicated by a shift of the histogram peaks to the right (Figure 19). IL-10 stimulation did not induce any measurable changes in size or granularity (Figure 19). Given the increased protein synthesis demonstrated in IL-10 treated NK cells, we might expect an increase in metabolic activity and therefore size. Again, similar to the proliferation data, the presence of IL-10 did not inhibit IL-15 induced growth (Figure 19).

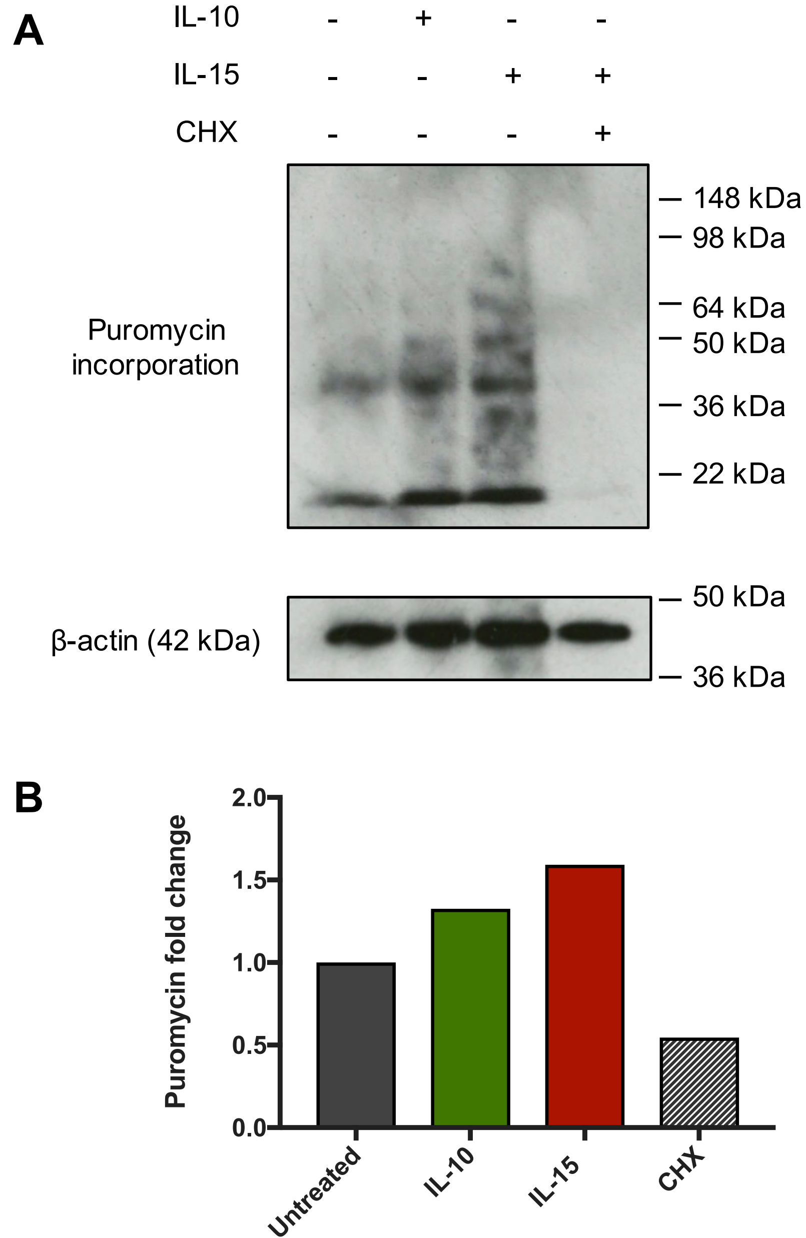


Figure 18: Puromycin incorporation is increased by IL-10 stimulation

(A) Isolated NK cells were stimulated with 50 ng/mL IL-10 or 20 ng/mL IL-15 for 24 hours, 1 µg/mL puromycin was then added to the cultures and incubated for 10 mins. CHX (30 µg/mL) was used as a negative control for the assay; added into IL-15 stimulated NK cells to inhibit protein synthesis. Cells were then lysed and analysed by western blotting for puromycin expression (n=1). (B) Quantification of the western blot using β-actin as a loading control, performed using ImageJ software (n=1).

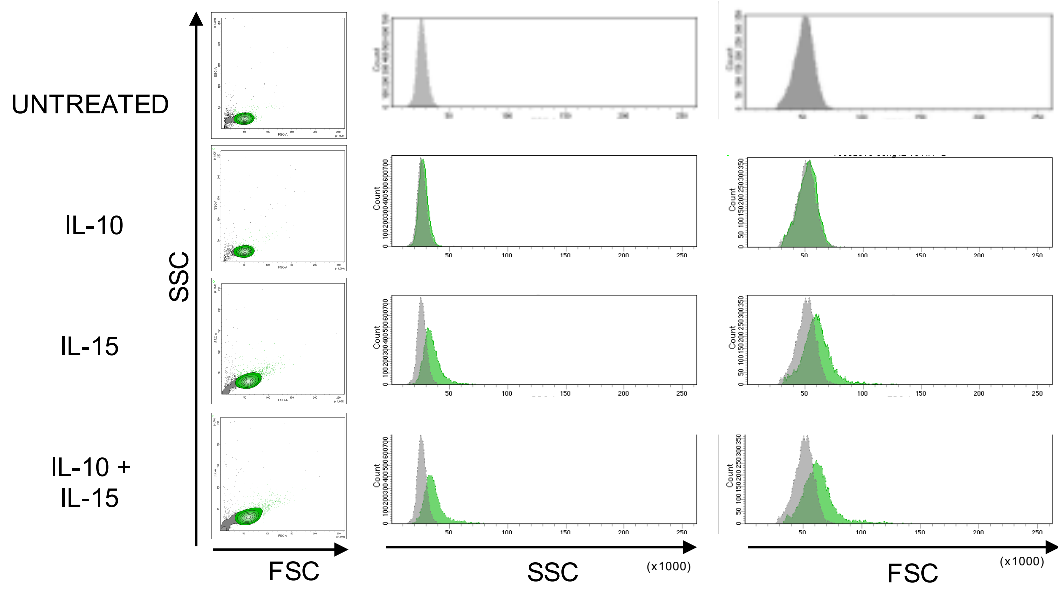


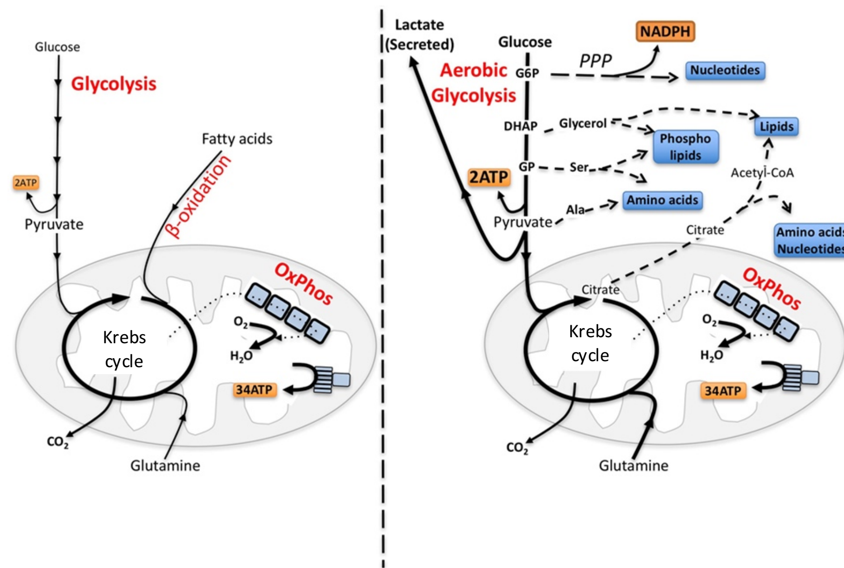
Figure 19: IL-10 does not induce NK cell growth

Isolated NK cells were cultured with 50 ng/mL IL-10, 20 ng/mL IL-15 or both for 48 hours prior to being analysed by flow cytometry. Images show contour plots, SSC histograms and FSC histograms of NK cells (green). Grey peaks in histograms indicate untreated NK cells.

4.6 IL-10 does not increase glucose uptake

Increased metabolic activity is essential to facilitate cell growth and proliferation of activated lymphocytes. Quiescent lymphocytes use glycolysis and OXPHOS to maintain homeostatic levels of ATP, whereas activated lymphocytes increase the glycolytic flux (rate of glycolysis). This increase is a very inefficient way to generate ATP and much of the pyruvate is converted into lactate and excreted from the cell, however, it enables the metabolic intermediates to be diverted into biosynthetic pathways to generate material such as nucleotides and lipids to support growth and proliferation (Figure 20A) (Loftus and Finlay, 2016). This altered metabolism is known as aerobic glycolysis (the Warburg effect) and can be measured by an increased uptake of glucose. Increased glucose uptake is indicative of the increased glycolytic flux which is characteristic of this metabolic shift. To confirm that IL-10 treated NK cells do not have increased metabolic activity as indicated by cell growth measurements, I have used a glycolytic flux measurement as a surrogate for cellular metabolism. In order to investigate how the metabolism of cytokine stimulated NK cells differed from untreated NK cells, I used a fluorescent glucose analogue, 2-deoxyglucose analog 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxyglucose (2-NBDG), to determine glucose uptake in NK cells. NK cells were cultured for 48 hours in the presence of IL-10 or IL-15, the media was then removed and replaced with glucose-free media and 2-NBDG (section 2.5). Cells that had not been incubated with 2-NBDG were used as a negative control (grey peaks). All conditions including the untreated control displayed bimodal peaks for 2-NBDG fluorescence, which suggests there could be populations with differing metabolic activity. IL-15 stimulation

resulted in increased glucose uptake in both populations (red peak), whilst IL-10 stimulated NK cells (green peak) showed similar glucose uptake to untreated NK cells (black peak). Unexpectedly, cells stimulated with both IL-10 and IL-15 exhibited slightly increased glucose uptake (blue peak) in comparison to IL-15, suggesting IL-10 may enhance IL-15 induced glucose uptake (Figure 20B). As with each of the other IL-15 induced activation mechanisms discussed, IL-10 does not inhibit glucose uptake. These data indicate that IL-10 mediated NK cell cytotoxicity may have different metabolic requirements to IL-15 activation, and is independent of cell growth. These data should be interpreted with caution as recent data suggests that 2-NBDG may not be a reliable reporter of glucose uptake (discussed in section 4.7).



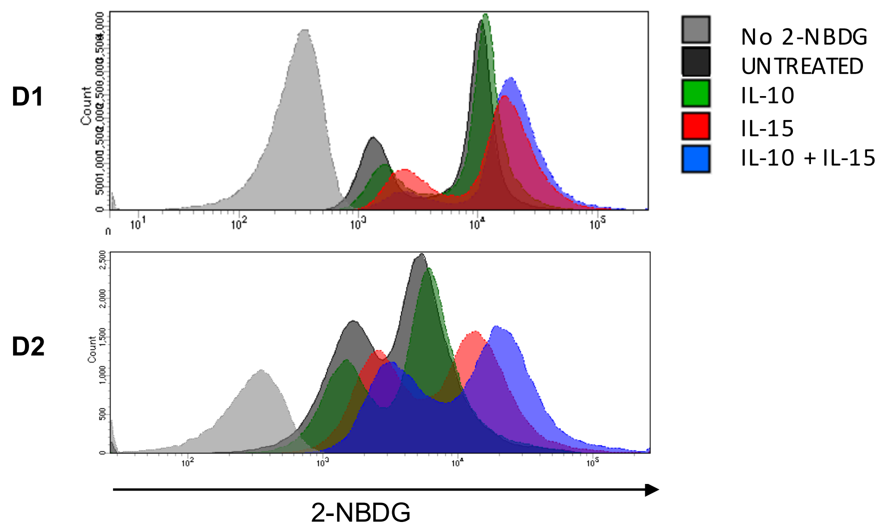


Figure 20: Glucose fluxing is not enhanced by IL-10

(A) Schematic diagrams of glycolysis and aerobic glycolysis (Loftus and Finlay, 2016). (B) Isolated NK cells were stimulated with 50 ng/mL IL-10, 20 ng/mL IL-15 or both for 48 hours; cells were placed in glucose free media for 1 hour before adding the glucose analogue 2-NBDG and then analysed by flow cytometry. Grey peak = NK cells without 2-NBDG, black = untreated NK cells, green = IL-10 stimulated NK cells, red = IL-15 stimulated NK cells, blue = IL-10 + IL-15 stimulated NK cells. D1 = donor 1, D2 = donor 2.

4.7 IL-10 does not induce mTOR signalling

I have shown that IL-10 does not provide a growth/proliferation signal to NK cells, however, it does increase protein content and appears to enhance IL-15 induced glucose uptake (Figure 18A and B, Figure 20B). To further investigate the effects of IL-10 on metabolism, I investigated the expression of a key metabolic regulator, mammalian target of rapamycin (mTOR). Recent studies have identified mTOR as a key regulator of the metabolic shift in activated lymphocytes, and it is reported to be activated in NK cells by IL-2, IL-12, IL-15 and IL-18 (Marçais et al., 2014, Donnelly et al., 2014). Considering that IL-10 did not increase mechanisms reported to be downstream of mTOR activation such as cell growth or proliferation, it is unlikely that IL-10 induces mTOR activation. To confirm this hypothesis, I investigated the phosphorylation of proteins in the mTOR pathway in response to IL-10 stimulation, specifically phospho-Akt^{T308} (p-Akt) and phospho-S6$^{S240/244}$ (p-S6). Isolated NK cells were stimulated with IL-10 or IL-15 for 45 minutes before analysis by intracellular flow cytometry for p-Akt expression (Figure 21 A and B), or 48 hours before western blotting to determine p-S6 expression (Figure 21C). As expected, IL-15 stimulation induced the phosphorylation of both Akt and S6, indicating that IL-15 activates the mTOR pathway as previously described (Marçais et al., 2014). Stimulation with IL-10 did not increase expression of p-Akt or p-S6 and therefore did not activate the mTOR pathway, correlating with the lack of cell growth and proliferation.

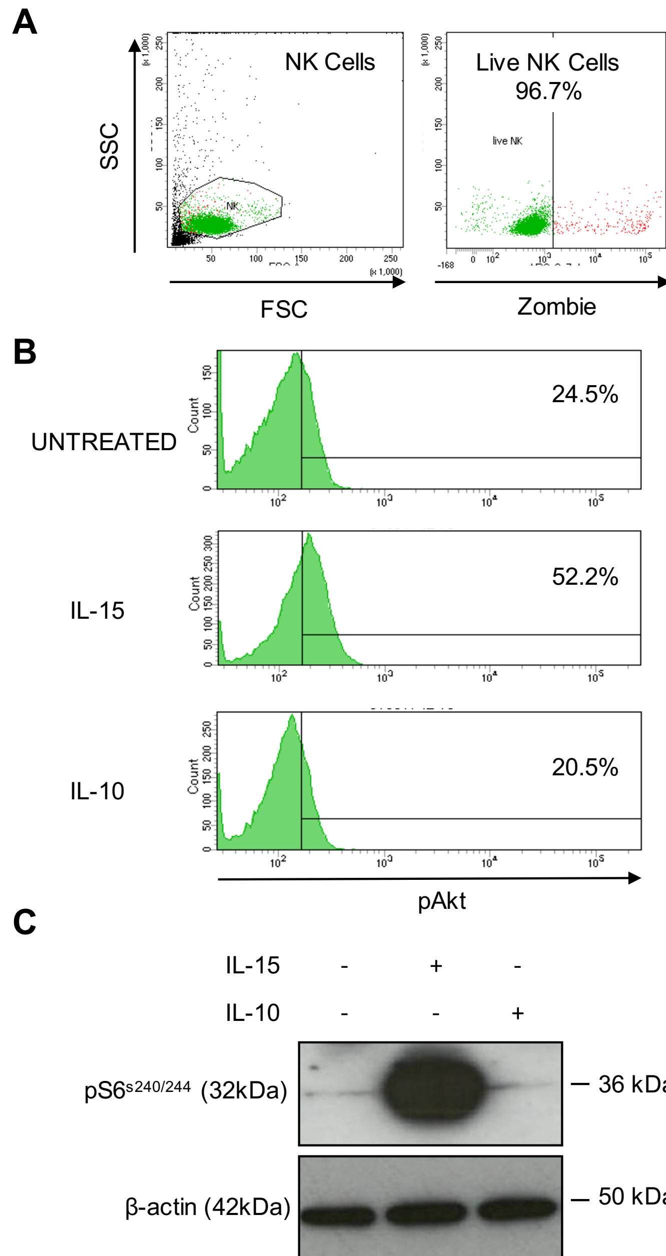


Figure 21: IL-10 does not induce phosphorylation of Akt or S6

Isolated NK cells were stimulated with 50 ng/mL IL-10 or 20 ng/mL IL-15 for 45 minutes before being analysed by intracellular flow cytometry. (A) NK cells were gated on based on FSC and SSC and further selected for live cells based on negative staining with a dead cell discriminator (Zombie NIR™). (B) Intracellular staining using an anti-p-Akt antibody, positive staining was determined by use of an isotype control antibody and setting the gate at 2% positive (black vertical line) (n=1). (C) NK cells were stimulated with 50 ng/mL IL-10 or 20 ng/mL IL-15 for 48 hours before being lysed and analysed by western blotting for phosphorylated S6 expression (pS6); β-actin was used as a loading control (representative of n=2).

4.8 Discussion

The activation of lymphocytes is an overarching phenomenon that encompasses many cellular changes. Metabolic reprogramming of cells has been identified as a key step that facilitates many of these processes, enabling cells to cope with the demands of rapid proliferation and synthesis of immunomodulatory molecules (Donnelly et al., 2014). In this chapter, I have investigated cellular changes associated with activation in isolation to try to understand the unique effects of IL-10 on NK cells. IL-15 is a potent NK cell activator and induces changes to each of the activation mechanisms investigated: cytotoxicity, cytokine production, activating receptor expression and proliferation. Conversely, I have shown that IL-10 upregulates cytotoxicity independently of other activating mechanisms, suggesting that IL-10 regulates independent/divergent pathways that induce only some of the cellular changes associated with activation. Moreover, IL-10 mediated cytotoxicity was independent of the enhanced metabolic requirements of IL-15 mediated activation. Indeed, the mTOR pathway was not activated in response to IL-10, leading to the proposed mTOR independent activation of NK cells.

As previously discussed, the expression of NK activating receptors is a key determinant in cellular responses. Many studies have previously shown that the expression of both activating and inhibitory receptors can be altered by cytokine stimulation (Vendrame et al., 2017, de Rham et al., 2007, Wilson et al., 2011). Despite enhancing cytotoxicity, IL-10 does not significantly alter the expression of key activating NK cell receptors NKG2D, NKp30, or DNAM1. This was surprisingly given that previous studies have shown NKG2D

expression to be transcriptionally controlled in a STAT3 dependent manner (Zhu et al., 2014). This study detailed that upon stimulation with 50 ng/mL IL-10, NKG2D expression was upregulated 1.6-fold and peaked at 48 hours post treatment. In contrast to this, the data presented in this study does not show enhanced NKG2D with IL-10 stimulation. Interestingly, a trend towards upregulation of NKG2D and significant upregulation of NKp46 was observed when isolated NK cells were stimulated with IL-10 (1.3-fold and 1.4-fold, respectively). To gain a better understanding of how IL-10 alters NKR expression, further experiments to increase the number of donors would be needed to perform accurate statistical analysis.

The CD69 molecule is not, to the best of our knowledge, involved in target cell recognition. However, it is rapidly upregulated on activated lymphocytes and is commonly used as a marker of activation. The exact function of CD69 has not been fully elucidated, but a role for CD69 in the migration and metabolism of lymphocytes has been identified through protein-protein interactions with sphingosine-1-phosphate receptor 1 (S1P1) and the amino-acid transporter complex (LAT-1/CD98), respectively (Cibrián and Sánchez-Madrid, 2017). Immune surveillance requires the continuous circulation of lymphocytes through lymphoid organs and egress of lymphocytes from lymph nodes (LN) is mediated by gradients of the chemoattractant sphingosine 1-phosphate (S1P). High concentrations in the blood/lymph, secreted by endothelial cells, and low concentrations in the lymph nodes direct the migration of lymphocytes through binding of S1P-receptors (S1PRs) (Rivera et al., 2008). These interactions can be transiently disrupted by upregulation of CD69 in activated lymphocytes. CD69 forms

lateral protein-protein complexes with S1PR, resulting in the downmodulation of S1PRs and the retention of lymphocytes in the LN (Shiow et al., 2006). Similarly, CD69 has also been shown to interact with LAT-1/CD98 on activated T cells. This interaction has been reported to stabilize the cell surface expression of LAT-1/CD98, thereby enhancing amino-acid uptake enabling greater signalling through mTOR (Cibrián and Sánchez-Madrid, 2017). In this study, CD69 was not upregulated in response to IL-10, which is perhaps unsurprising. Activated NK cells producing IFN-γ migrate to lymph nodes and aid cytotoxic T cell priming (Martín-Fontecha et al., 2004). The data presented in Chapter 3 demonstrated that IL-10 does not induce IFN-γ production from NK cells, therefore retaining IL-10 activated NK cells in the LN would not be useful. Furthermore, as IL-10 stimulated NK cells did not induce mTOR activity, increased LAT-1/CD98 activity would not be beneficial.

Many studies report differential roles of IL-10 in proliferation, which differ between cell types, combinations of cytokines and the activation status of cells. For instance, in intestinal T cell populations, the nature of the stimulus was observed to be the defining factor in whether IL-10 was anti- or pro-proliferative. IL-10 inhibited the proliferation of phytohaemagglutinin (PHA) activated T cells, but enhanced the proliferation of IL-2 activated T cells (Ebert, 2000). Similarly, Groux et al. observed that IL-10 acted as a co-factor with IL-2 to enhance CD8 T cell proliferation, but was unable to induce T cell proliferation in isolation. Moreover, IL-10 inhibited proliferation when T cells were stimulated with APCs, but not via CD3 activation, suggesting that the inhibition was indirect and due to the inhibition of APC function (Groux et al., 1998). Using a murine macrophage model, IL-10 was revealed to inhibit

proliferation by arresting cells in G1, and that inhibition was not due to IL-10 induced secretion of auto/paracrine anti-inflammatory mediators (O'Farrell et al., 1998). Supporting this, gene profile analysis performed in IL-10 stimulated NK cells identified multiple upregulated genes associated with cell-cycle-suppression such as P21 and RB (Mocellin et al., 2004). p21 and pRb both play a role in regulating cell-cycle progression. p21 is a cyclin-dependent kinase (CDK) inhibitor that prevents cell cycle progression at multiple points by binding to cyclin/CDK complexes (D/CDK4/6 in G1 phase, cyclin E/CDK2 at G1/S phase and A/CDK1,2 through S phase) (Karimian et al., 2016) whereas pRb inhibits cell cycle progression at the ‘restriction point’ in G1 phase of the cell cycle. Hypophosphorylated pRb prevents the transcription of genes necessary for progression into S phase by binding E2F transcription factors. Mitogenic signals are required to inactivate pRb, which requires hyperphosphorylation mediated by cyclin/CDK complexes (Giacinti and Giordano, 2006).

In contrast to IL-10 acting in a supporting role, IL-10 has been shown to induce proliferation to a greater extent than both IL-18 and IL-12 in bone-marrow-derived murine NK cells, and displays selective additive proliferative effects in combination with IL-18, but not IL-12 (Cai et al., 1999). The data regarding the effect of IL-10 on proliferation is somewhat contradictory and it appears that IL-10 can play dual roles. Here, I attempted to uncover how IL-10 affected the proliferation of human primary NK cells, in isolation and in combination with other activating cytokines. IL-10 alone did not induce proliferation of NK cells at any concentration used (5-500 ng/mL), or enhance the proliferative effects of IL-15 or IL-12/IL-18, and only a modest increase

was observed in combination with IL-2 (n=1). Additionally, IL-10 did not inhibit the proliferative effects of IL-15, IL-2 or IL-12/IL-18.

In addition to proliferation, IL-10 did not induce cell growth but did increase protein synthesis. Preliminary data suggests that protein synthesis was modestly increased in comparison to the increase observed with IL-15, reflective of the number of cellular changes IL-15 has been shown to induce. To support these cellular changes, IL-15 signals for a metabolic switch, enabling activated lymphocytes to fulfil energetic demands. In line with other studies, this is demonstrated by increased glucose uptake and mTOR activation. Using an *in vivo* model, poly I:C activated NK cells were found to be larger, have a higher level of glucose fluxing, and increased expression of nutrient transporters in comparison to untreated NK cells, all of which were impaired in the presence of the mTOR inhibitor, rapamycin. Additionally, *ex-vivo* stimulation of NK cells with IL-2/IL-12 upregulated levels of glycolysis and OXPHOS, however, only glycolysis was mTOR-dependent (Donnelly et al., 2014). Similarly, Marçias et al. also observed mTOR dependent cell growth, increased aerobic glycolysis and increased OXPHOS of NK cells in response to IL-15. mTOR deficient murine NK cells had reduced expression of IL-15R and dramatically reduced proliferation during development stages. Additionally, mTOR deficient NK cells exhibited impaired growth and glucose uptake when challenged with poly I:C. Interestingly, this study also observed that poly I:C stimulation of mTOR deficient cells had reduced granzyme B expression, but IFN-γ production was unaffected in comparison to WT cells (Marçais et al., 2014). I have shown that IL-10 does not induce mTOR signalling and consequently does not increase proliferation. Moreover, this

finding supports a rationale for the lack of IFN-γ production by IL-10 stimulation that is consistent with current literature (Mah and Cooper, 2016, Keppel et al., 2015, Keating et al., 2016, Donnelly et al., 2014). Although preliminary data in Figure 20 suggest that IL-10 does not impact glucose uptake in NK cells, this must be interpreted with caution. In a recent study, Sinclair et al. observed that 2-NBDG uptake correlated well with relative glucose transporter expression and metabolically active $CD8^+$ T cells; however, 2-NBDG uptake was also observed in non-metabolically active $CD4^+$/$CD8^+$ double positive thymocytes. Through interrogation of proteomic data sets, it was shown that murine T cells did not express several glucose transporters and co-transporters, including Glut 2 (a validated target of 2-NBDG), and that 2-NBDG did not follow simple glucose transporter principles as uptake was not blocked by inhibition of glucose transporters or competitively inhibited by substrates. These data indicate that 2-NBDG uptake alone may not be a reliable reporter of glucose uptake or glycolytic activity. (Sinclair et al., 2020).

These data show that IL-10 stimulated NK cells do not undergo the same process of cell growth and proliferation that IL-15 treated cells do, and therefore do not need to undertake the same levels of biosynthetic activity associated with IL-15 treatment. However, IL-10 did induce JAK/STAT signalling, upregulate granzyme B expression and enhance cytotoxicity (Chapter 3). The ability to induce some mechanisms of activation and not others suggests that each of these mechanisms are governed by independent signalling pathways. Investigations in the next chapter will focus on identifying key pathways required for IL-10 induced activation of NK cells.

Chapter 5

Distinct modes of NK cell activation by IL-10 and IL-15

5.1 Introduction

Previous chapters highlighted the diversity of IL-15 and IL-10 induced NK cell activation, the former utilising multiple mechanisms to induce robust activation, and the latter selectively enhancing just a fraction of these mechanisms. It is well-known that STAT3 plays a key role in IL-10 signalling (Verma et al., 2016, O'Farrell et al., 1998, Niemand et al., 2003), and activation of this pathway has been confirmed in primary human NK cells (Figure 8). Experiments performed in this chapter aimed to further understand how STAT3 signalling determines NK cell activity and which, if any, other pathways are involved. Using a combination of selective and non-selective inhibitors, this chapter investigates the STAT3-dependancy of IL-10 mediated cytotoxicity.

5.2 IL-10 induced activation differs from IL-15 induced activation

IL-10 mediates only a fraction of NK cell functions compared to IL-15. One possibility is that IL-10 is simply a weak NK cell stimulus compared to IL-15. I previously demonstrated that IL-15 induced NK cell proliferation at concentrations as low as 10 ng/mL, whilst IL-10 did not induce proliferation at low or high concentrations (0.5-500 ng/mL) (Figure 18). To further investigate the ‘strength’ of IL-10 compared to IL-15 stimulation, I analysed the surface expression of CD69 and intracellular granzyme B, proteins that I have

previously shown to be unaffected and upregulated by IL-10 stimulation, respectively (Figures 8, 13B and C).

NK cells were stimulated with increasing concentrations of IL-10 (0.5, 5, 50 and 500 ng/mL) or IL-15 (0.2, 2, 20 and 200 ng/mL) for 48 hours before being analysed by flow cytometry. As expected, the majority (>97%) of untreated NK cells were positive for granzyme B expression (Figure 22A); therefore, changes in MFI were used to measure changes in expression. In agreement with the previous data (Figure 9), both IL-15 and IL-10 upregulated the expression of granzyme B. Granzyme B expression in IL-15 stimulated cells peaked at 20 ng/mL (2.1-fold-increase), whereas the highest concentration of IL-10 (500 ng/mL) resulted in the highest expression of granzyme B, 1.5-fold-increase compared to 1.3-fold-inrease with 50 ng/mL (Figure 22B).

IL-15 induced CD69 expression at all concentrations; however, IL-10 did not induce CD69 expression even at the highest concentration of 500 ng/mL (Figure 22C). This selective upregulation of granzyme B but not CD69 suggests that IL-10 is not weakly activating the same pathways as IL-15, but rather activating a subset of, or different pathways to achieve NK cell activation.

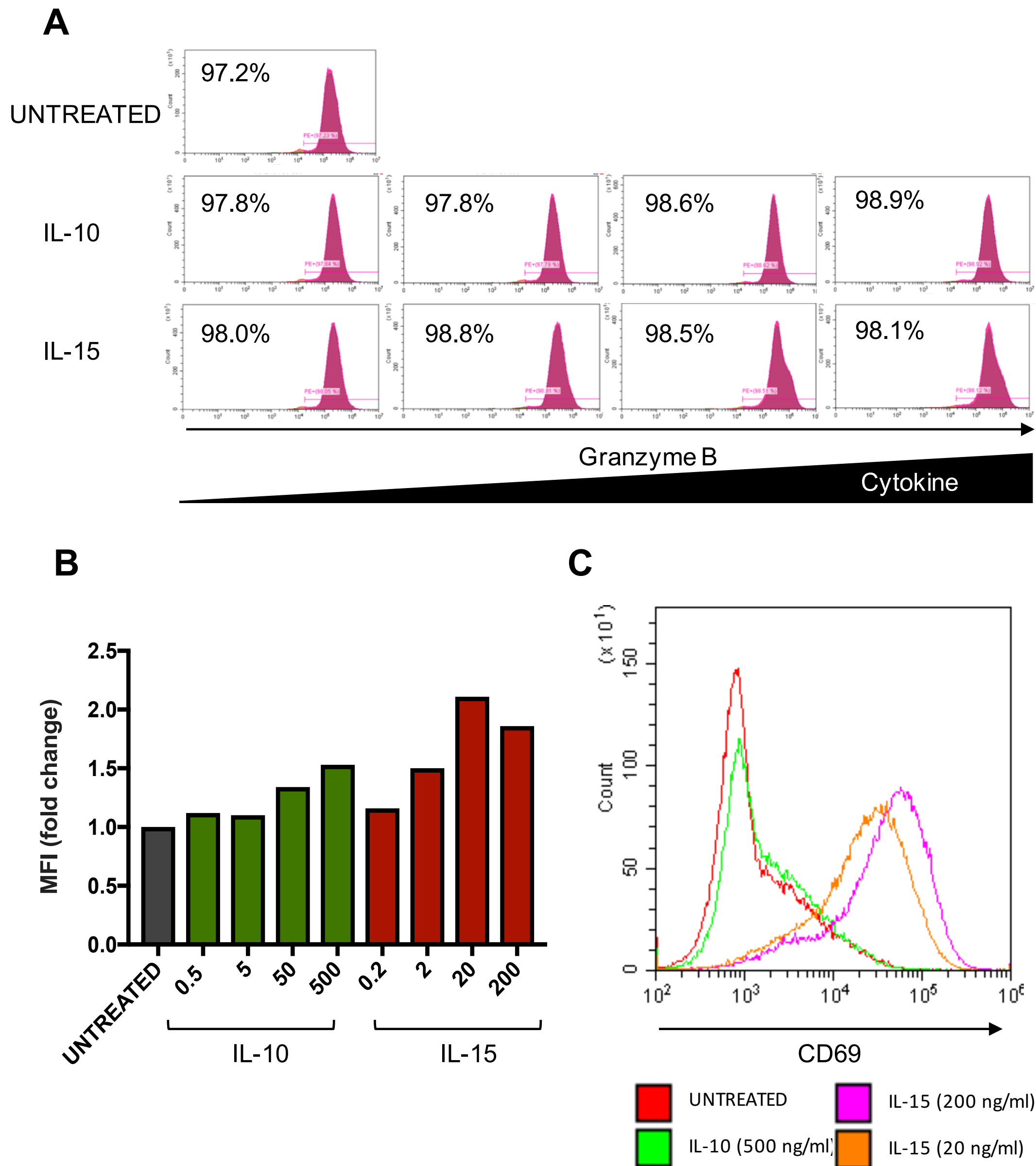


Figure 22: High concentration of IL-10 does not induce CD69, but does increase granzyme B

(A) NK cells were cultured with increasing concentrations of IL-10 (0.5, 5, 50 and 500 ng/mL) or IL-15 (0.2, 2, 20 and 200 ng/mL) for 48 hours prior to being analysed by flow cytometry for cell surface expression of CD69 (n=1) and (B) intracellular expression of granzyme B (n=1). (C) Histograms of CD69 expression, positive gating was set on an appropriate isotype at 2% (n=1).

5.3 IL-10 induced NK activation is susceptible to inhibition by TGF-β

IL-15 stimulates robust activation of NK cells through the activation of multiple pathways, resulting in enhanced cytotoxicity, receptor expression, cytokine production and proliferation (Figures 9-17) (Wilson et al., 2011, Carson et al., 1994, Gosselin et al., 1999). A key component of this activation is the increased metabolic capacity that is orchestrated via the mTOR pathway, shown to be required for optimum NK cell activation (Marçais et al., 2014, Donnelly et al., 2014, Keating et al., 2016). TGF-β, a potent inhibitor of IL-15 stimulation, has been shown to inhibit IL-15 induced NK cell activity via mTOR inhibition (Viel et al., 2016). I have previously shown that IL-10 does not induce mTOR signalling in NK cells, I therefore hypothesised that IL-10 stimulated NK cells would be refractory to TGF-β inhibition; such activity might have important implications for immunotherapy. To test this hypothesis, NK cells were cultured with IL-10 or IL-15 +/- TGF-β for 48 hours prior to analysing cytotoxicity and degranulation (as described previously in chapter 2, 3). As expected, TGF-β inhibited IL-15 induced NK cell-mediated killing of K562 cells at both E:T ratios tested and, contrary to my hypothesis, TGF-β also inhibited the cytotoxicity of IL-10 activated NK cells (Figure 23A and B). Similar results were obtained when assaying NK cell degranulation in response to K562 and SKOV3 target cells (Figure 23C). In the presence of TGF-β, target cell death was reduced by 34% at E:T 1:1 and 69.5% at E:T 5:1 (Figure 23A and B). Degranulation was also reduced by 31.1% against K562 and 58.8% against SKOV3 (n=1) (Figure 23C). These preliminary data indicate that TGF-β is inhibiting IL-10 independently of its effects on mTOR (as mTOR is not activated by IL-10; Figure 21). Therefore, I investigated whether TGF-β

inhibited the STAT3 signalling pathway. Isolated NK cells were cultured with IL-10 +/- TGF-β for 48 hours before being lysed and analysed by western blotting for expression of $pSTAT3^{Y705}$. Surprisingly, TGF-β did not inhibit IL-10 induced $STAT3^{Y705}$ phosphorylation; however, granzyme B expression was reduced to basal levels in the presence of TGF-β (Figure 23D). This suggests that TGF-β inhibits IL-10 induced NK cell activation independently of mTOR and STAT3 phosphorylation, and that IL-10 is potentially stimulating additional pathways to induce NK cell activation.

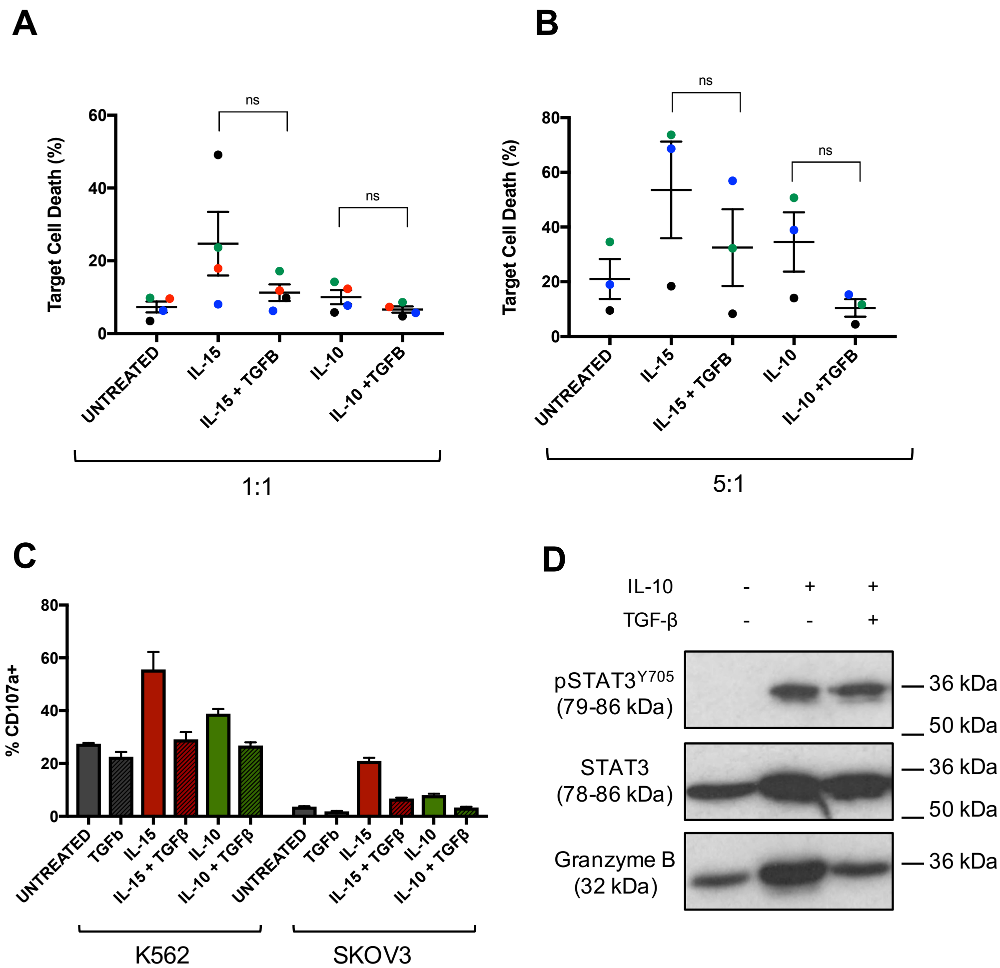


Figure 23: TGF-β inhibits IL-10 induced NK cell cytotoxicity

NK cells were treated for 48 hours with 50 ng/mL IL-10 or 20 ng/mL +/- 5 ng/mL TGF-β. NK cells were then co-cultured with CTV labelled K562 target cells at 1:1 (n=4) and (B) 5:1 (n=3) E:T, dead cells were identified using Zombie NIR™. (A) Graphs show mean target cell death at 1:1 (n=4) and (B) 5:1 (n=3) and were analysed using repeated measures one-way ANOVA with Tukey's multiple comparison test. (C) NK cells were treated and co-cultured with CTV labelled K562 target cells (as above) prior to being analysed by flow cytometry for CD107a expression. Graph shows mean expression from triplicates (n=1). (D) NK cells were stimulated with 50 ng/mL IL-10 +/- 5 ng/mL TGF-β for 48 hours prior to being lysed and analysed by western blotting (n=1). ns = not significant.

5.4 Potential pathways activated by IL-10

The Jak1/STAT3 pathway is considered to be the central mediator of IL-10 function; however, activation of others pathways such as PI3K has also been reported in monocytes and promyeloid cells (Verma et al., 2016, Zhou et al., 2001, Crawley et al., 1996). In order to investigate whether other signalling pathways were activated in NK cells, the phosphorylation of key immune signalling proteins was investigated. Isolated NK cells were either unstimulated, or stimulated with IL-10 for 30 minutes prior to being lysed and analysed using a membrane-based sandwich immunoassay (Figure 24A) (see section 2.8). Despite previously showing highly increased pSTAT3^{Y705} levels through conventional western blotting methods (Chapter 3 Figure 8), the phospho-kinase array revealed only modest upregulation with IL-10 stimulation (~1.4-fold increase) (Figure 24C). In fact, all proteins showed only small changes in their phosphorylation levels with IL-10 stimulation compared to untreated NK cells, with the highest increase seen in pSTAT5b^{Y699}. STAT5 is not reported to be IL-10 activated (Lai et al., 1996), some studies show that IL-10 signalling is able to induce the dimerisation of STAT3/STAT5 complexes, but this has only been shown in a cell restricted manner (Weber-Nordt et al., 1996, Jens et al., 1996). This results from this assay are inconclusive, therefore, further investigation into IL-10 activated signalling pathways in NK cells is required.

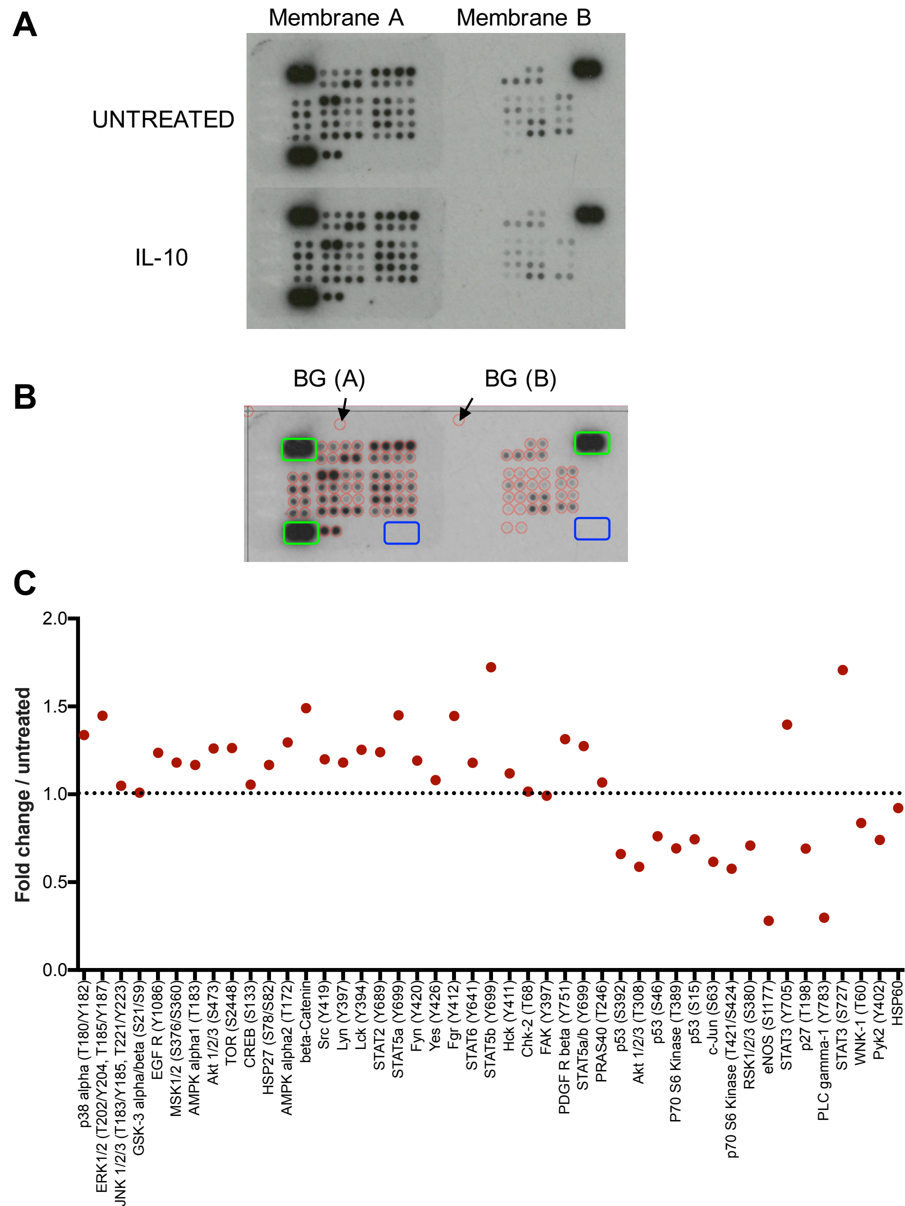


Figure 24: Proteome Profiler

(A) Isolated NK cells were either untreated or stimulated with 50 ng/mL IL-10 for 30 minutes, cell lysates were subsequently washed over the phospho-kinase array membranes. (B) ImageJ plugin protein array analyser was used to analyse the blots. Red circles define area to be measured. BG = Background, green boxes are membrane alignment dots, blue boxes are negative controls. (C) Phospho-protein expression is displayed as fold change from the corresponding protein in the untreated control, the dotted line indicates normalised untreated value.

5.5 STAT3 phosphorylation is required for IL-10 mediated cytotoxicity

As previously shown, TGF-β did not inhibit phosphorylation of STAT3 but did inhibit granzyme B upregulation (Figure 23D). To further investigate the mechanisms by which STAT3 mediates NK cell activation, I evaluated the effects of STAT3 inhibition on IL-10 induced NK cell cytotoxicity using granzyme B expression and cytotoxic activity as readouts. To inhibit STAT3 mediated signalling, a small-molecule inhibitor of STAT3 was used; c188-9. This inhibitor blocks ligand-induced STAT3 phosphorylation and dimerisation by targeting the phosphotyrosine peptide binding site within the SH2 domain (Redell et al., 2011). NK cells were stimulated with IL-10 in the presence of c188-9 for 48 hours before analysing the expression of granzyme B, or being co-cultured with K562 target cells and analysing target cell death. Increasing concentrations of c188-9 (1 μM, 5 μM and 10 μM) reduced the expression of pSTAT3 in a dose-dependent manner whilst the expression of total STAT3 remained constant, confirming the specificity of phosphorylation inhibition. In the presence of c188-9, granzyme B expression was also reduced in a dose-dependent manner (Figure 25A), confirming a role for STAT3 in the IL-10 mediated regulation of granzyme B.

Furthermore, preliminary results from NK cell: target cell co-cultures also show that, in the presence of c188-9, target cell death was reduced in a dose-dependent manner (Figure 25B). This finding further supports a role of STAT3 signalling in IL-10 mediated NK cell cytotoxicity.

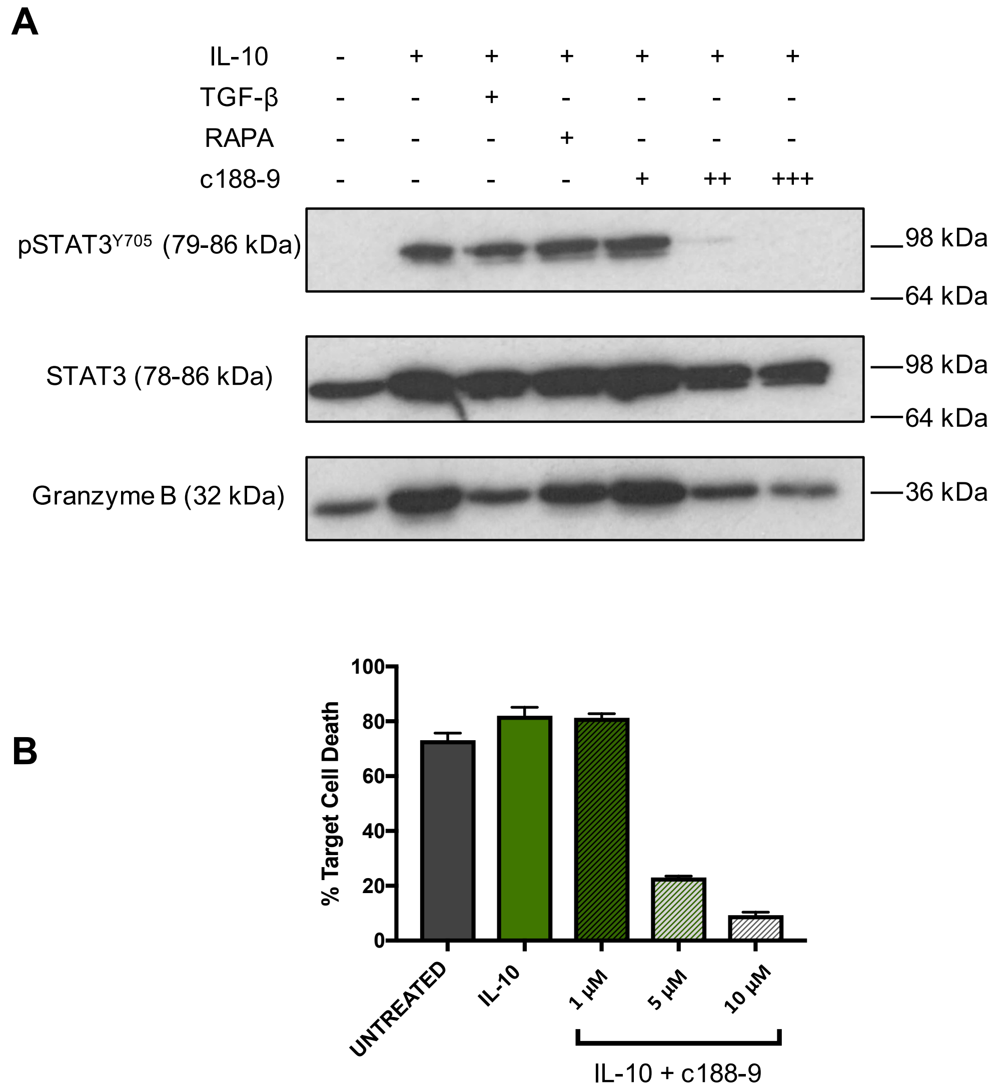


Figure 25: STAT3 inhibition reduces granzyme B and target cell death

(A) NK cells were stimulated with 50 ng/mL IL-10 + increasing concentrations of C188-9 (1, 5, 10 µM) for 48 hours prior to being lysed and analysed by western blotting (also see Figure 23D) (STAT3 was used as a loading control and to confirm phosphorylation inhibition) (n=1), or (B) co-culturing with CTV labelled K562 target cells at 5:1 E: T. Target cell death was measured using Zombie NIR™. Graphs shows mean target cell death from triplicates (n=1).

5.6 IL-21 and IL-10 activate NK cells in a similar fashion

To explore the role of STAT3 in NK cell activation further, I employed another STAT3 activating cytokine, IL-21. I investigated pathways that I have previously shown to be regulated by IL-10 treatment (granzyme B expression, cytotoxicity and degranulation). Treatment with IL-21 alone, or in combination with other cytokines, has been shown to activate NK cells via STAT3 (Li et al., 2015, Skak et al., 2008, de Rham et al., 2007, Eriksen et al., 2009, Strengell et al., 2003) and enhance cytotoxicity (Bhatt et al., 2015). Therefore, I hypothesised that IL-10 and IL-21 would induced the same degree of activation in NK cells. Firstly, to confirm that IL-21 signalled via STAT3, NK cells were stimulated with 50 ng/mL IL-21 and the expression of phosphorylated STAT3 was determined by western blot analysis (Figure 26A). The results show that IL-21 induced STAT3 phosphorylation to a lesser extent than IL-10, but in combination with IL-15, phosphorylation of pSTAT3 expression was comparable to IL-10 stimulation. Granzyme B expression was upregulated in response to both IL-21 and IL-10 to a similar degree, but to a lower extent than IL-15 (Figure 26A). Unlike IL-10, these results do not show an additive effect of IL-21 in combination with IL-15; however, this is only from one donor and further investigations are required to confirm this. Similar to the previous observation in IL-10 stimulated NK cells (Figure 26A), the presence of TGF-β did not inhibit IL-21 induced STAT3 phosphorylation, but did reduce the expression of granzyme B (Figure 26A).

To investigate the effect of IL-21 on cytotoxicity, isolated NK cells were stimulated with IL-10, IL-15 or IL-21 for 48 hours prior to being co-cultured with K562 target cells. Target cell death and NK cell degranulation was then

analysed using flow cytometry (as detailed in Figures 10 and 11). IL-21 showed similar activity to IL-10, increasing degranulation and target cell death. Compared to the untreated control, IL-21 increased target cell death by 1.87-fold (Figure 26B) and degranulation by 1.26-fold, whilst IL-10 increased target cell death and degranulation by 1.5-fold and 1.23-fold, respectively (Figure 26C). Similar to previous results, IL-15 induced higher levels of degranulation and target cell death than IL-21 or IL-10, indicating that these cytokines are less potent activators of NK cells. To further compare the similarities of IL-21 and IL-10, I evaluated the expression of CD69, as previous results show that this is not regulated by IL-10 stimulation, hence it is unlikely to be downstream of STAT3. NK cells were stimulated with IL-21, IL-10 or IL-15 for 48 hours prior to be analysed by flow cytometry for surface expression of CD69. These results show that the expression of CD69 remained unchanged in IL-21 stimulated NK cells (Figure 26D). Taken together, these data suggest that IL-21 and IL-10 activate NK cells in a similar STAT3 dependent manner.

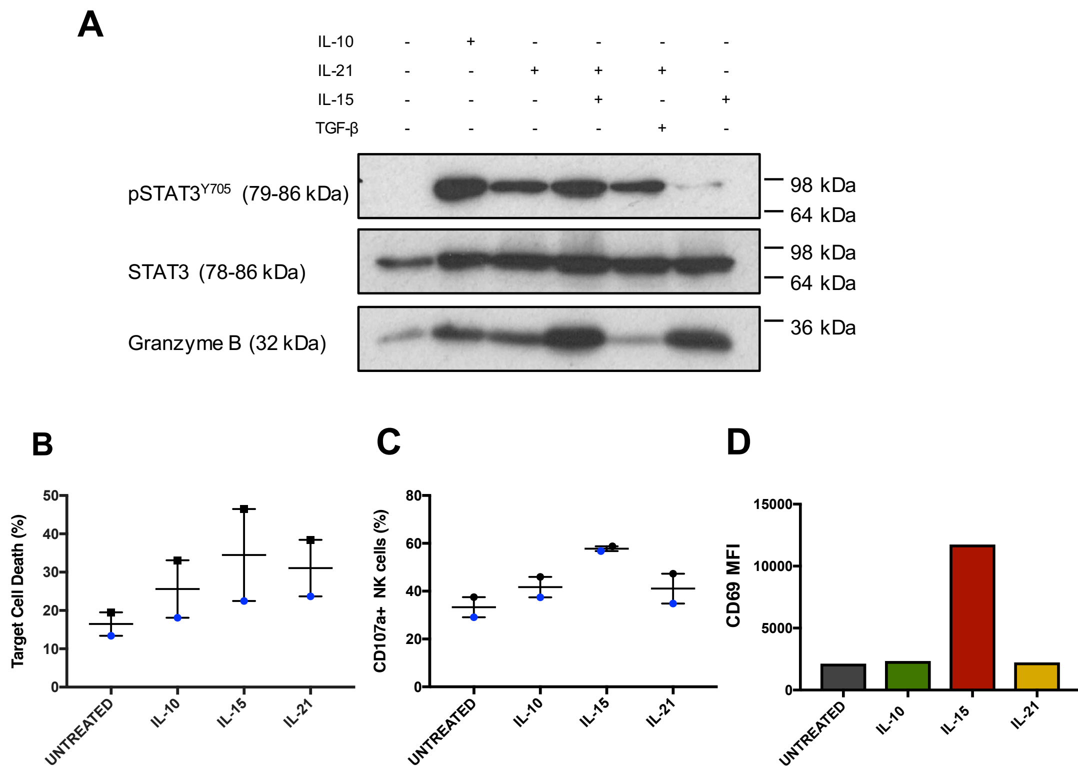


Figure 26: IL-21 induces NK cell cytotoxicity

(A) NK cells were stimulated with combinations of 50 ng/mL IL-10, 20 ng/mL IL-15, 50 ng/mL IL-21 and 5 ng/mL TGF-β for 48 hours prior to being lysed and analysed by western blotting (n=1). (B) NK cells were treated for 48 hours with 50 ng/mL IL-10, 20 ng/mL IL-15 or 50 ng/mL IL-21prior to being co-cultured with CTV labelled K562 target cells at 5:1 E:T, dead cells were identified using Zombie NIR™. Graphs shows mean target cell death (n=2). (C) NK cells were treated and co-cultured with CTV labelled K562 target cells (as above), CD107a expression was analysed by flow cytometry (n=2). (D) NK cells were treated for 48 hours with 50 ng/mL IL-10, 20 ng/mL IL-15 or 50 ng/mL IL-21, prior to being analysed by flow cytometry for CD69 expression (n=1).

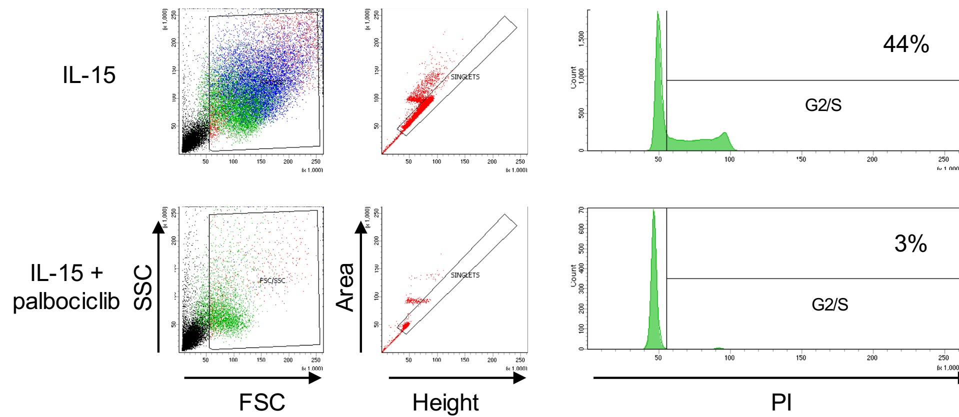
5.7 NK cell cytotoxicity is independent of proliferation

It is unclear whether STAT3 is the sole pathway that mediates IL-10 activation of NK cells, but efforts to identify further pathways (such as the use of the phosphoproteome screen in Figure 24) were inconclusive.

The data presented here show that IL-10 induces NK cell cytotoxicity independently of other mechanisms, such as proliferation. In contrast, IL-15 stimulates both proliferation and cytotoxicity very strongly. I therefore investigated the contribution of NK cell proliferation to cytotoxicity by specifically inhibiting cell cycle progression. To do this, isolated NK cells were cultured with IL-15 +/- palbociclib, a CDK4/6 inhibitor that stalls cells in the G1/S phase; these NK cells were then assayed for their cytotoxicity activity (as previously described in section 2.3). To confirm that palbociclib inhibited proliferation in NK cells, palbociclib was added into cytokine stimulated cell cultures for 96 hours prior to analysing the DNA content and cell cycle stage with PI staining (as previously described in section 2.6). As expected, IL-15 induced NK cell proliferation (44% of cells in G2/S phases) and this was completely inhibited in presence of palbociclib (3% of cells in G2/S phases) (Figure 27A). As found in previous cytotoxicity assays, using either IL-15 or IL-10 stimulated NK cells resulted in more target cell death compared to untreated NK cells (Figure 27B). As expected, palbociclib did not inhibit IL-10 induced cytotoxicity (E:T 1:1 13.2% vs 12.89%; E:T 5:1 43.2% vs 39.7% for IL-10 and IL-10 + palbociclib, respectively). Interestingly, despite profoundly inhibiting proliferation, the presence of palbociclib did not inhibit the cytotoxicity of IL-15 stimulated NK cells (E:T 1:1 20.5% vs 19.4%; E:T 5:1 65.8% vs 56.5% for IL-15 and IL-15 + palbociclib, respectively) (Figure 27B).

This suggests that IL-15 induced proliferation and cytotoxicity are governed independently, and proliferation is not essential for IL-15 mediated cytotoxicity.

A



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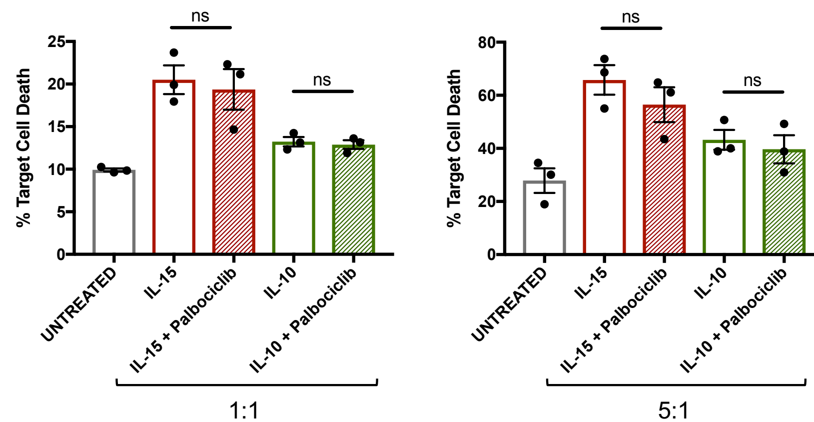

Figure 27: Proliferation is not essential for IL-15 induced cytotoxicity

(A) Isolated NK cells were cultured with 20 ng/mL IL-15 +/- 1 µM palbociclib for 96 hours before being analysed for cell cycle phase using PI staining and flow cytometry. Flow cytometry gating strategy; events were collected on a linear scale and area vs. height was used to determine single cells. Single cells were then analysed further for PI fluorescence. Histograms of PI staining; S/G2 gating bar was set at 2% in the untreated control cells, cells in this gate are considered to be in S/G2 phase (representative of n=3). (B) NK cells were treated for 48 hours with 50 ng/mL IL-10 or 20 ng/mL IL-15 +/- 1 µM palbociclib. NK cells were then co-cultured with CTV labelled K562 target cells at 1:1 and 5:1 E:T; dead cells were identified with Zombie NIR™ using flow cytometry. Graphs shows mean target cell death (n=3); ns = not significant, determined using a repeated measures one-way ANOVA with Tukey's multiple comparison test.

5.8 Discussion

Previous chapters have highlighted several differences between IL-15 and IL-10 induced NK cell activation. In this chapter, I aimed to further understand these differences by investigating the STAT3 dependency of IL-10 induced activation. NK cells are activated by several different cytokines, each of which induce unique combinations of cytokine secretion, protein synthesis and cytotoxicity (Lauwerys et al., 2000, Fehniger et al., 1999, Nguyen et al., 2002, Meghnem et al., 2017, Wang et al., 2014, Ferlazzo et al., 2004). Furthermore, combinations of cytokines can act synergistically to enhance NK cells activity (Strengell et al., 2003, Lauwerys et al., 1999). Experiments performed in this chapter confirm that IL-10 activation is not a weaker form of IL-15 activation but is in fact distinct from IL-15 activation. Several cytokines preferentially enhance specific activatory mechanisms; for instance, IL-12 and IL-18 enhance IFN-γ expression to greater extent than IL-15 (Fehniger et al., 1999) and IL-15 induces greater proliferation than IL-12 (Nguyen et al., 2002). Even IL-2 and IL-15, cytokines that share receptor components, have numerous differences in their downstream effects. Wang et al. identified 374 differentially expressed genes that resulted in enhanced proliferation capacity of IL-15 stimulated NK cells and greater cytotoxic potential of IL-2 stimulated NK cells (Wang et al., 2014). Here I have shown that IL-10 activates only a subset of activatory mechanisms in comparison to IL-15 stimulated NK cells. Concentrations as high as 500 ng/mL of IL-10 were not capable of inducing CD69 expression or proliferation, both of which are hallmarks of IL-15 activation. However, higher granzyme B expression was induced with 500

ng/mL than 50 ng/mL IL-10, highlighting the ability of IL-10 to further enhance the cytotoxicity potential but, not proliferation.

As previously shown, IL-10 does not activate the mTOR pathway in NK cells (Figure 21), therefore it was surprising to find that IL-10 induced activation was susceptible to inhibition by TGF-β. It has long been known that TGF-β is a potent inhibitor of NK cell functions. Reduced expression of activating receptors, chemokine receptors, IFN-γ secretion, cytolytic activity and ADCC in response to TGF-β have all been reported (Wilson et al., 2011, Regis et al., 2017, Trotta et al., 2008). Moreover, inhibiting TGF-β has been shown to restore NK cell cytotoxicity in numerous tumour models (Otegbeye et al., 2018, Wilson et al., 2011). Multiple pathways have been identified to play a role in this inhibition, including the canonical TGF-β pathway and the mTOR pathway, but despite the obvious importance of TGF-β in NK cell regulation, the mechanisms that control this remain unclear. TGF-β was shown to inhibit CD16 mediated IFN-γ secretion from murine NK cells via a mechanism involving Smad3. The presence of TGF-β did not inhibit the phosphorylation of STATs, ERK or p38, but did suppress the expression of T-BET, a positive IFN-γ regulator. Over expression of Smad3 could mimic TGF-β effects, but interestingly, TGF-β exhibited inhibitory effects in *smad3*$^{-/-}$ cells, indicating that TGF-β mediated inhibition is not solely Smad3-dependent. Moreover, TGF-β was able to inhibit the expression of IFN-γ in *Tbx21*$^{-/-}$ cells (lacking T-bet), suggesting that TGF-β inhibits multiple pathways (Trotta et al., 2008). In addition to multiple pathways, the activity of TGF-β was also shown to be modulated by kinetics. Whilst at shorter time-points TGF-β did not inhibit ADCC, the presence of TGF-β in longer cultures of NK cells (4 days) did inhibit

ADCC via downregulation of granzyme A and B proteins (Trotta et al., 2008). Another pathway that has been proposed to regulate TGF-β mediated inhibition is the mTOR pathway (Viel et al., 2016). In IL-15 stimulated murine NK cells, TGF-β displayed similar activities to that of the mTOR specific inhibitor, rapamycin. TGF-β reduced metabolic activity, inhibited the phosphorylation of mTOR associated proteins S6, 4EBP1 and Akt, reduced the expression of cytolytic proteins and inhibited degranulation in the presence of target cells. This study also highlighted similarities in protein expression between *mTor*$^{-/-}$ cells and cells with constitutive TGF-β signalling, further supporting the hypothesis that TGF-β inhibits the mTOR pathway (Viel et al., 2016). In contrast to this, a recent study in human primary NK cells showed distinct actions of TGF-β and rapamycin at different time-points (Zaiatz-Bittencourt et al., 2018). In accordance with the previous study by Viel et al., TGF-β significantly decreased the rate of OXPHOS and CD71 expression in IL-2 or IL-12/IL-15 stimulated NK cells; however, the dependency on mTOR inhibition to achieve this differed between time-points. At shorter time points of 30 minutes and 1 hour, the presence of TGF-β did not inhibit the activity of mTOR (measured by phosphorylation of S6 and 4EBP1) in IL-2 or IL-12/IL-15 stimulated NK cells. Interestingly, TGF-β did inhibit mTOR activity at later time-points (5 days), indicating that different pathways were targeted by TGF-β at specific time-points. Furthermore, the addition of a TGF-β receptor inhibitor to IL-2 + TGF-β stimulated NK cells restored functional and metabolic responses, suggesting that early TGF-β mediated inhibition is driven by the canonical TGF-β signalling pathway (Zaiatz-Bittencourt et al., 2018).

Multiple studies agree that TGF-β does not inhibit the phosphorylation of STAT proteins (Trotta et al., 2008, Viel et al., 2016, Sudarshan et al., 1999). In IL-15 stimulated NK cells, pSTAT5 expression remained unaltered in the presence of TGF-β or an anti-TGF-β antibody (Viel et al., 2016). In IL-12 and IL-2 activated NK cells and T cells, the presence of TGF-β did not inhibit the expression or phosphorylation or nuclear translocation of STAT4 and STAT5 proteins, respectively (Sudarshan et al., 1999). In this study, I have shown that TGF-β inhibits IL-10 mediated NK cell cytotoxicity in an mTOR and pSTAT3 independent manner, but the mechanism of this inhibition remains unknown. The fact that granzyme B expression is reduced in a STAT3 independent manner by TGF-β suggests that other pathways may also be contributing to this upregulation, or that TGF-β inhibits STAT3 signalling further downstream. It is possible that TGF-β inhibits IL-10 via the canonical (Smad2/3 dependent) pathway at a point further downstream than the phosphorylation of STAT3. Cross-talk between Smad3 and STAT3 has been identified in several cell types and biological processes such as tumourigenesis, epithelial-mesenchymal-transition and T-cell differentiation (Tang et al., 2017, Itoh et al., 2017). Multiple direct and indirect mechanisms of reciprocal STAT3 and Smad3 negative regulation have been reported, including epigenetic modifications, inhibition of transcription factors and direct DNA binding (Itoh et al., 2017). In the context of T-cell differentiation, STAT3 supresses Smad3 induced FoxP3 expression by binding the FoxP3 promoter region, but works as a co-activator with phosphorylated Smad3 to regulate RoRγt expression (Itoh et al., 2017). The interplay between STAT3 and Smads is highly complex and context-dependent, therefore, elucidating the

mechanism of TGF-β mediated inhibition in IL-10 activated NK cells will be challenging.

Irrespective of TGF-β, experiments in this chapter show that STAT3 inhibition suppresses IL-10 induced granzyme B expression and NK cell cytotoxicity. To investigate whether STAT3 activation mediated the same NK cell responses from another stimulus, I employed another NK cell activating cytokine known to signal via STAT3, IL-21 (Li et al., 2015, Denman et al., 2012, Skak et al., 2008). IL-21 enhanced granzyme B expression and degranulation in a similar fashion to IL-10, suggesting that IL-21 and IL-10 are less potent than IL-15. Moreover, IL-21 mediated some activation mechanisms independently of others, i.e IL-21 enhanced cytotoxicity, but not CD69 expression.

In contrast to this, several studies report that IL-21 modulates the expression of cell surface receptors including CD69 (Bhatt et al., 2015, Skak et al., 2008) and demonstrates pro-proliferative effects of IL-21 towards NK cells (Zeng et al., 2007, Skak et al., 2008, Denman et al., 2012). However, species and cell types used in these studies differ and can provide conflicting evidence with regards to the role of IL-21. For instance, IL-21 induced proliferation in human NK cells, but not murine NK cells (Brady et al., 2004) and two independent studies performed on human NK cells showed both upregulation and down regulation of NKG2D expression (Zhu et al., 2014, Burgess et al., 2006). Importantly, in human NK cells, the MAPK and PI3K pathways were found to mediate IL-21 induced NK cell proliferation, with inhibition of either pathways suppressing proliferation (Zeng et al., 2007). As these pathways are not known to be activated by IL-10 in NK cells, this

suggests that STAT3 is responsible for IL-21 and IL-10 induced cytotoxicity, and that these mechanisms are independently regulated. Additionally, IL-21 has been shown to activate STAT1 and STAT5 pathways (to a weaker extent) that could potentially impact other activation mechanisms (Wan et al., 2015).

Whether other additional pathways are required to induce IL-10 mediated NK cytotoxicity remains unknown. Although steps have been taken in this study to try and determine other signalling pathways that may be utilised by IL-10, this study was not able to define these. Although STAT3 is the most prominent IL-10 activated pathway, (Verma et al., 2016) the potential involvement of other pathways at play in NK cells cannot be overlooked. The phosphorylated kinases 'Proteome profiler' was chosen as it allows for the simultaneous identification of 43 phospho kinases and 2 related total proteins, providing a time and cost-effective method to analyse multiple signalling pathways within one sample and eliminate donor dependent differences. However, the method used here did not indicate the involvement of other pathways in NK cells. Whilst there are benefits to using this approach, such as the ability to investigate multiple proteins in the same sample, there are also many limitations. The design of the membrane only allows for one time-point to be compared. For this assay, cells were stimulated for 30 minutes as STAT3 has been shown to be phosphorylated at this time-point; however, this may not be optimal for all proteins investigated therefore may be more favourable to some proteins. Furthermore, this assay also requires a large amount of protein to overcome the sensitivity threshold and detect phosphorylation. Obtaining such large amounts of protein from primary lymphocytes acquired from a single donor can be difficult and costly. This

approach has led to inconclusive results, showing only marginal differences in kinase activity between untreated and IL-10 treated NK cells. Cytokines are known to activate multiple pathways including JAK/STAT, PI3K, and MAPK pathways (Ali et al., 2015, Nandagopal et al., 2014, Mishra et al., 2014), and the phosphorylation of so many kinases is not surprising. Interestingly, in this study I have shown that distinct pathways may be responsible for specific activation mechanisms. Blocking proliferation in IL-15 stimulated NK cells did not inhibit cytotoxicity, suggesting that the pathways mediating these responses are activated independently and are not associated. Similarly, in an IL-10 stimulated monocyte cell line, inhibition of the PI3K-mTOR pathway suppressed proliferation, but not anti-inflammatory effects (Crawley et al., 1996). Simplistically, if one pathway regulates one activation mechanism, then it is possible that IL-10 does not activate several pathways in NK cells as it only enhances a subset of activation mechanisms. Nevertheless, cytokine signalling pathways are much more complex and further investigation of the pathways involved in IL-10 signalling is required to understand whether the pro-inflammatory effects of IL-10 are mediated solely via the STAT3 pathway.

Chapter 6

Summary and conclusion

6.1 Summary

Primarily described as an anti-inflammatory cytokine (Couper et al., 2008, Verma et al., 2016), the pleiotropic activity of IL-10 is becoming clear in several cell types (Qayum et al., 2016, Polukort et al., 2016, Heine et al., 2014, Smith et al., 2018a, Mocellin et al., 2005). IL-10 has been shown to activate NK cells (and other immune cells) in several studies. However, many of these studies have been performed using NK cell-like cell lines that require cytokine stimulation to enable their proliferation and survival in long-term culture. In this study, I have investigated the potential of IL-10 to activate human primary NK cells.

In this study, I have shown that IL-10 induces the phosphorylation of STAT3 in NK cells and subsequently enhances cytotoxicity. IL-10 stimulation upregulated granzyme B expression, NK cell degranulation and NK cell mediated target cell death. Additionally, I showed that IL-10 has an additive effect when used in combination with IL-15, inducing greater expression of granzyme B. Unlike IL-15, IL-10 did not induce the expression of lymphocyte activation marker CD69, or significantly alter the expression of key NK cell activating receptors NKG2D, DNAM1 or NKp30.

IL-10 stimulation did not alter the production of IFN-γ; however, IL-10 did increase the mRNA expression, suggesting that additional pathways are required to translate this into protein. Whether this secondary process is mRNA stabilisation through other signalling pathways, or changes in cellular

metabolism is undetermined. Recent studies highlight the importance of metabolic reprogramming in immune cells to enable optimal production of IFN-γ (Chang et al., 2013, Keating et al., 2016). Preliminary observations in this study revealed that IL-10 stimulation of NK cells did not result in metabolic changes, as determined by glucose uptake and cell growth, suggesting that this metabolic switch could be the secondary mechanism required for IFN-γ production. As mentioned previously, data regarding glucose uptake in this study should be interpreted cautiously given the recent data indicating that 2-NBDG staining alone may not be a reliable indicator of glycolytic activity (Sinclair et al., 2020). Another possibility is the that IL-10 does not activate proteins that are required for mRNA stabilization. Typical inducers of IFN-γ, such as IL-12 and IL-18, stabilise IFN-γ mRNA through MAPK activation (Mavropoulos et al., 2005). This study was unable to define additional NK cell pathways regulated by IL-10; therefore, whether IL-10 activates pathways required for mRNA stabilisation remains to be determined. Additionally, IL-10 has been shown to negatively regulate cytokine expression in macrophages by inducing the expression of tristetraprolin (TTP), a zinc finger binding protein that regulates mRNA expression by binding AU rich elements in mRNA and promoting degradation (Fu and Blackshear, 2016, Stumpo et al., 2010), highlighting another level of regulation. Further investigation into IL-10 mediated cytokine production is required to understand the mechanisms that regulate this.

Activation of NK cells with IL-10 occurred in a STAT3-dependent manner. Inhibition of STAT3 signalling, using a small molecule inhibitor that directly binds to STAT3 phosphorylation sites, completely abrogated IL-10

mediated enhancement of cytotoxicity. Interestingly, IL-10 mediated activation was susceptible to TGF-β inhibition in a STAT3-independent way; TGF-β did not inhibit the phosphorylation of STAT3 but did reduce the expression of granzyme B to basal levels and suppress the cytotoxicity of NK cells. Whether TGF-β prevents STAT signalling further downstream of pSTAT3 is yet to be defined. It is possible that TGF-β could inhibit the transcription factor activity of STAT3. Further investigations using a chromatin immunoprecipitation-sequencing (ChIP-seq) approach would reveal if TGF-β inhibits the DNA-binding of STAT3. Several Smad-dependent and Smad-independent pathways are downstream of TGF-β signalling (Papageorgis and Stylianopoulos, 2015); it would be interesting to explore whether transcription factors and coactivators/corepressors downstream of these pathways block DNA binding through shared binding sites, or bind promoter regions of STAT3 regulated genes. Of course, it is also possible that TGF-β indirectly inhibits IL-10 induced cytotoxicity through the expression of TGF-β mediated genes (Figure 28), for example by inducing the expression of transcriptional repressors or antagonists of IL-10 signalling. As previously mentioned, the potential cross-talk of Smads and STAT3 highlight the complexity of these signalling events and elucidating this interplay will be challenging.

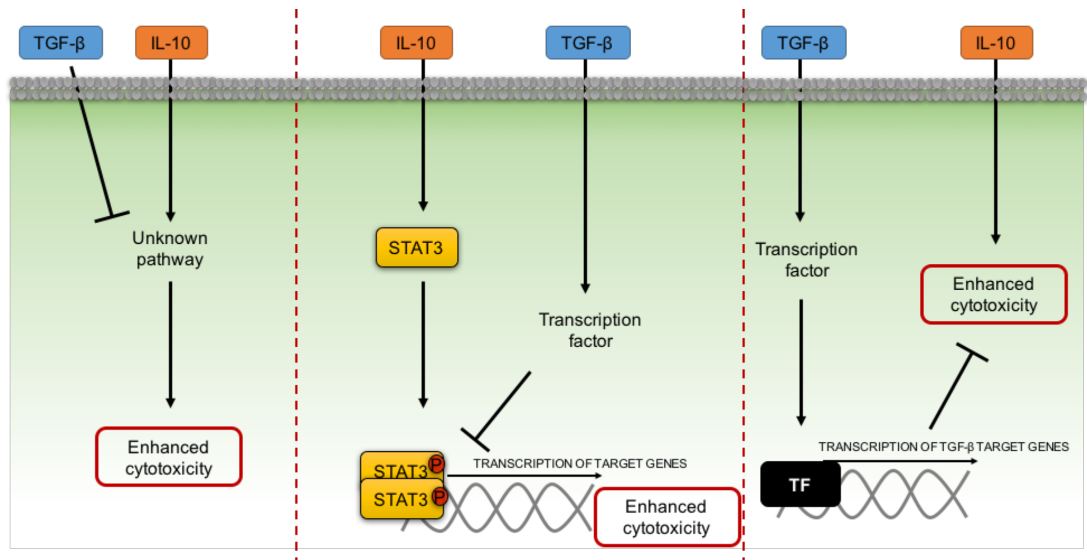

Figure 28: Possible mechanisms of TGF-β mediated inhibition of IL-10 activated NK cells

TGF-β mediated inhibition of IL-10 mediated NK cell activation remains unknown. This schematic details possible mechanisms by which TGF-β potentially inhibits IL-10; through the inhibition of unidentified pathways, transcriptional repression of STAT3 mediated genes/STAT3 binding, and through indirect mechanisms as a result of TGF-β induced gene expression.

Results from this study clearly demonstrate that IL-10 and IL-15 mediate distinct mechanisms of activation. IL-15 is a potent inducer of several effector pathways such as cytotoxicity, cytokine secretion, activating receptor expression and proliferation, whereas IL-10 selectively enhances cytotoxicity. In IL-15 stimulated NK cells, blocking entry to the cell cycle using a CDK4/6 inhibitor, and therefore blocking proliferation, did not inhibit the IL-15-induced cytotoxicity of NK cells. This indicates that these activation pathways can be regulated independently of one another, and that different signalling pathways may be regulated individual mechanisms.

Independent regulation of these mechanisms may be important when considering the environments that are likely to contain these cytokines. IL-15 provides a strong survival and proliferation signal to NK cells, primarily from DCs in the LN and in response to infection (Ferlazzo et al., 2004, Wagstaffe et al., 2018, Wu et al., 2017, Lucas et al., 2007). However, prolonged activation with IL-15 is associated with NK cell exhaustion, resulting in the expression of checkpoint inhibitors LAG3 and PD-1 and genome-wide epigenetic changes that rendered them irresponsive to target cells (Merino et al., 2019). Furthermore, limiting the strength of IL-15 by inhibiting mTOR signalling was shown to rescue NK cell function, highlighting the role of mTOR in driving NK cell exhaustion (Felices et al., 2018).

A possible benefit of the ‘restricted’ activation IL-10 induces is that it does not activate mTOR signalling or proliferation, therefore may be beneficial in environments that have limited nutrients, such as the tumour microenvironment (TME). Increasing metabolism to meet the biosynthetic demands of growth and proliferation requires an increased influx of nutrients.

Multiple cellular populations, including tumour cells and stromal cells, are competing for these and impaired immune cell activity in the TME is, at least partially, a result of nutrient competition. IL-10 is present in the tumour microenvironment and is therefore a potential site of interaction with NK cells. In this context, IL-10 could provide a mechanism of NK cell activation that is independent of increased nutrient uptake, and therefore not susceptible to inhibition by lack of nutrients. Indeed, others have shown anti-tumour activity of IL-10. In animal models, IL-10 has been shown to enhance tumour rejection by activating tumour-resident T cells, and T cells isolated from IL-10 treated tumours exhibit increased expression of granzyme B and IFN-γ (Mumm et al., 2011, Emmerich et al., 2012). In a clinical trial evaluating the use of pegylated IL-10 in solid tumours, systemically administered IL-10 increased serum levels of pro-inflammatory cytokines IFN-γ and IL-18 whilst decreasing TGF-β (Naing et al., 2016). Furthermore, tumours being treated with mTOR inhibitors would be expected to have reduced T cell activity and IL-15 mediated NK cell activity. However, IL-10 (or IL-21) mediated NK cell activity might be retained under these conditions.

IL-10 clearly plays an important role in the pathogenesis of many autoimmune and autoinflammatory diseases, but whether this role is solely immunosuppressive is up for debate. The pleiotropic activity of IL-10 is exemplified in rheumatoid arthritis, where overexpression of IL-10 contributes to immunosuppression but also enhances humoral immunity (Hernández-Bello et al., 2017). Furthermore, the use of recombinant IL-10 to treat IBD has yielded poor results (Marlow et al., 2013), and in some cases, has enhanced inflammation (Tilg et al., 2002). IL-10, in this study and others, has shown

stimulating effects towards several immune cell populations and could therefore contribute to inflammation in disease, and promote anti-tumour immunity. However, whether the activity of IL-10 is anti-inflammatory or pro-inflammatory is likely to be context and cell type-dependent. For example, although IL-10 activates NK cells when studied in isolation, the overall effect of IL-10 within the complex mixture of cells within the tumour microenvironment might be different, with IL-10 potentially inhibiting TNF release from monocytes and macrophage and reducing the overall pro-inflammatory nature of the tumour microenvironment. Teasing apart such complex interactions will likely require the use of tumour-bearing IL-10R conditional knockout mouse models (e.g. using a floxed IL10R gene in conjunction with cell type expression of Cre recombinase).

6.2 Future work

Firstly, it must be noted that many of the experiments in this study do not show statistical significance, but rather ‘trends’ in the data. As this study investigates the cellular responses of individual human donor samples, rather than genetically identical cell lines or murine samples, the magnitude of variation between the samples can greatly impact the statistical significance of experiments. For instance, cells from healthy individuals will produce cytokines in response to certain stimuli, but the level of response may be higher/lower in one donor versus another (e.g. due to polymorphisms in genes encoding components of particular pathways). Therefore, it is important to increase the number of donors, repeat the assays and collect more data that can bolster the trends observed in this study. Moreover, further donors will

increase the power of the statistical tests, resulting in more robust, reliable data.

Following on from the data presented in this study, identifying other pathways activated by IL-10 in NK cells would further our understanding of how this pleiotropic cytokine regulates NK cell activity, and help decipher the differences between IL-10 and IL-15 stimulation. Approaches such as phosphoproteomics have greater sensitivity than the proteome profiler used in this study and could therefore provide a more definitive list of pathways utilised by IL-10 to selectively enhance NK cell activities. Additionally, further research is required to understand the metabolic activity of NK cells following IL-10 stimulation. This study suggests that this is a key differentiator of IL-15- and IL-10-induced activation; therefore, techniques such as Seahorse analysis (measuring oxygen consumption rate [OCR] and extracellular acidification rate [ECAR]) could be employed to delineate metabolic changes induced by IL-10. This would be complemented by further analysis of proteins in the mTOR pathway. Lastly, it would be interesting to build upon the observation that TGF-β inhibited IL-10 induced NK cell activity independent of pSTAT3 inhibition. In addition to identifying other IL-10 activated pathways that may be targeted by TGF-β, comparing DNA-binding regions of pSTAT3 with TGF-β-induced transcription factors (using ChIP-seq) would provide more information about this interaction.

6.3 Conclusion

In conclusion, the data presented from this study indicates that IL-10 activates human primary NK cells in a mechanism that is distinct from IL-15. IL-10 stimulation of NK cells results in STAT3-dependent upregulation of granzyme B expression and cytotoxicity. Unlike IL-15 stimulation that induces several activation mechanisms, such as proliferation, NK cell receptor expression, cytokine production and cytotoxicity, IL-10 stimulation selectively regulates a limited number of activation mechanisms. This selectivity may be useful in environments with limited nutrients, further investigation is required to understand the full extent of IL-10 mediated NK cell activation.

References

ABBOUD, G., TAHILIANI, V., DESAI, P., VARKOLY, K., DRIVER, J., HUTCHINSON, T. E. & SALEK-ARDAKANI, S. 2016. Natural Killer Cells and Innate Interferon Gamma Participate in the Host Defense against Respiratory Vaccinia Virus Infection. *Journal of Virology,* 90**,** 129-141.

ABRAM, C. L. & LOWELL, C. A. 2009. The ins and outs of leukocyte integrin signaling. *Annual review of immunology,* 27**,** 339-362.

ADIB-CONQUY, M., SCOTT-ALGARA, D., CAVAILLON, J.-M. & SOUZA-FONSECA-GUIMARAES, F. 2014. TLR-mediated activation of NK cells and their role in bacterial/viral immune responses in mammals. *Immunology & Cell Biology,* 92**,** 256-262.

AKDIS, C. A. & BLASER, K. 2001. Mechanisms of interleukin-10-mediated immune suppression. *Immunology,* 103**,** 131-136.

AKTAS, E., KUCUKSEZER, U. C., BILGIC, S., ERTEN, G. & DENIZ, G. 2009. Relationship between CD107a expression and cytotoxic activity. *Cellular Immunology,* 254**,** 149-154.

AL-ROBAEE, A. A., AL-ZOLIBANI, A. A., AL-SHOBILI, H. A., KAZAMEL, A. & SETTIN, A. 2008. IL-10 Implications in Psoriasis. *International Journal of Health Sciences,* 2**,** 53-58.

ALI, A. K., NANDAGOPAL, N. & LEE, S.-H. 2015. IL-15–PI3K–AKT–mTOR: A Critical Pathway in the Life Journey of Natural Killer Cells. *Frontiers in Immunology,* 6**,** 355.

ANDERSEN, M. H., SCHRAMA, D., THOR STRATEN, P. & BECKER, J. C. 2006. Cytotoxic T Cells. *Journal of Investigative Dermatology,* 126**,** 32-41.

ANFOSSI, N., ANDRÉ, P., GUIA, S., FALK, C. S., ROETYNCK, S., STEWART, C. A., BRESO, V., FRASSATI, C., REVIRON, D., MIDDLETON, D., ROMAGNÉ, F., UGOLINI, S. & VIVIER, E. 2006. Human NK Cell Education by Inhibitory Receptors for MHC Class I. *Immunity,* 25**,** 331-342.

ANTHONY, D. A., ANDREWS, D. M., WATT, S. V., TRAPANI, J. A. & SMYTH, M. J. 2010. Functional dissection of the granzyme family: cell death and inflammation. *Immunological Reviews,* 235**,** 73-92.

ANTON, O. M., VIELKIND, S., PETERSON, M. E., TAGAYA, Y. & LONG, E. O. 2015. NK Cell Proliferation Induced by IL-15 Transpresentation Is Negatively Regulated by Inhibitory Receptors. *Journal of immunology (Baltimore, Md. : 1950),* 195**,** 4810-4821.

ANTONIV, T. T. & IVASHKIV, L. B. 2011. Interleukin-10-induced gene expression and suppressive function are selectively modulated by the PI3K-Akt-GSK3 pathway. *Immunology,* 132**,** 567-577.

ARASE, H., ARASE, N. & SAITO, T. 1995. Fas-mediated cytotoxicity by freshly isolated natural killer cells. *The Journal of Experimental Medicine,* 181**,** 1235-1238.

ASADULLAH, K., STERRY, W., STEPHANEK, K., JASULAITIS, D., LEUPOLD, M., AUDRING, H., VOLK, H. D. & DÖCKE, W. D. 1998. IL-10 is a key cytokine in psoriasis. Proof of principle by IL-10 therapy: a new therapeutic approach. *Journal of Clinical Investigation,* 101**,** 783-794.

B., H. R., E., N. M. & H., L. D. 1975a. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. *International Journal of Cancer,* 16**,** 216-229.

B., H. R., E., N. M., T., H. H. & H., L. D. 1975b. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *International Journal of Cancer,* 16**,** 230-239.

BARANEK, T., VU MANH, T.-P., ALEXANDRE, Y., MAQBOOL, MUHAMMAD A., CABEZA, JOAQUIN Z., TOMASELLO, E., CROZAT, K., BESSOU, G., ZUCCHINI, N., ROBBINS, SCOTT H., VIVIER, E., KALINKE, U., FERRIER, P. & DALOD, M. 2012. Differential Responses of Immune Cells to Type I Interferon Contribute to Host Resistance to Viral Infection. *Cell Host & Microbe,* 12**,** 571-584.

BATLLE, E. & MASSAGUÉ, J. 2019. Transforming Growth Factor-β Signaling in Immunity and Cancer. *Immunity,* 50**,** 924-940.

BELLONE, G., ASTE-AMEZAGA, M., TRINCHIERI, G. & RODECK, U. 1995. Regulation of NK cell functions by TGF-beta 1. *The Journal of Immunology,* 155**,** 1066-1073.

BHATT, S., MATTHEWS, J., PARVIN, S., SAROSIEK, K. A., ZHAO, D., JIANG, X., ISIK, E., LETAI, A. & LOSSOS, I. S. 2015. Direct and immune-mediated cytotoxicity of interleukin-21 contributes to antitumor effects in mantle cell lymphoma. *Blood,* 126**,** 1555-1564.

BISWAS, P. S., PEDICORD, V., PLOSS, A., MENET, E., LEINER, I. & PAMER, E. G. 2007. Pathogen-Specific CD8 T Cell Responses Are Directly Inhibited by IL-10. *The Journal of Immunology,* 179**,** 4520-4528.

BONILLA, F. A. & OETTGEN, H. C. 2010. Adaptive immunity. *Journal of Allergy and Clinical Immunology,* 125**,** S33-S40.

BORG, C., JALIL, A., LADERACH, D., MARUYAMA, K., WAKASUGI, H., CHARRIER, S., RYFFEL, B., CAMBI, A., FIGDOR, C., VAINCHENKER, W., GALY, A., CAIGNARD, A. & ZITVOGEL, L. 2004. NK cell activation by dendritic cells (DCs) requires the formation of a synapse leading to IL-12 polarization in DCs. *Blood,* 104**,** 3267-3275.

BOUDREAU, J. E. & HSU, K. C. 2018. Natural Killer Cell Education and the Response to Infection and Cancer Therapy: Stay Tuned. *Trends in Immunology,* 39**,** 222-239.

BRADY, J., CAROTTA, S., THONG, R. P. L., CHAN, C. J., HAYAKAWA, Y., SMYTH, M. J. & NUTT, S. L. 2010. The Interactions of Multiple Cytokines Control NK Cell Maturation. *The Journal of Immunology,* 185**,** 6679-6688.

BRADY, J., HAYAKAWA, Y., SMYTH, M. J. & NUTT, S. L. 2004. IL-21 Induces the Functional Maturation of Murine NK Cells. *The Journal of Immunology,* 172**,** 2048-2058.

BRAUD, V. M., ALLAN, D. S. J., O'CALLAGHAN, C. A., SÖDERSTRÖM, K., D'ANDREA, A., OGG, G. S., LAZETIC, S., YOUNG, N. T., BELL, J. I., PHILLIPS, J. H., LANIER, L. L. & MCMICHAEL, A. J. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature,* 391**,** 795-799.

BROOKS, D. G., WALSH, K. B., ELSAESSER, H. & OLDSTONE, M. B. A. 2010. IL-10 directly suppresses CD4 but not CD8 T cell effector and memory responses

following acute viral infection. *Proceedings of the National Academy of Sciences,* 107, 3018-3023.

BRYCESON, Y. T., MARCH, M. E., LJUNGGREN, H.-G. & LONG, E. O. 2006. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood,* 107, 159-166.

BUKUR, J., JASINSKI, S. & SELIGER, B. 2012. The role of classical and non-classical HLA class I antigens in human tumors. *Seminars in Cancer Biology,* 22, 350-358.

BURGESS, S. J., MARUSINA, A. I., PATHMANATHAN, I., BORREGO, F. & COLIGAN, J. E. 2006. IL-21 Down-Regulates NKG2D/DAP10 Expression on Human NK and CD8⁺ T Cells. *The Journal of Immunology,* 176, 1490-1497.

CAI, G., KASTELEIN, R. A. & HUNTER, C. A. 1999. IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-γ when combined with IL-18. *European Journal of Immunology,* 29, 2658-2665.

CALIGIURI, M., A. 2008a. Human Natural Killer Cells

. *Blood,* 112, 461-469.

CALIGIURI, M. A. 2008b. Human natural killer cells. *Blood,* 112, 461-469.

CAREY, A. J., TAN, C. K. & ULETT, G. C. 2012. Infection-induced IL-10 and JAK-STAT: A review of the molecular circuitry controlling immune hyperactivity in response to pathogenic microbes. *JAK-STAT,* 1, 159-167.

CARSON, W. E., GIRI, J. G., LINDEMANN, M. J., LINETT, M. L., AHDIEH, M., PAXTON, R., ANDERSON, D., EISENMANN, J., GRABSTEIN, K. & CALIGIURI, M. A. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *The Journal of Experimental Medicine,* 180, 1395-1403.

CARSON, W. E., LINDEMANN, M. J., BAIOCCHI, R., LINETT, M., TAN, J. C., CHOU, C. C., NARULA, S. & CALIGIURI, M. A. 1995. The functional characterization of interleukin-10 receptor expression on human natural killer cells. *Blood,* 85, 3577-3585.

CHAIX, J., TESSMER, M. S., HOEBE, K., FUSÉRI, N., RYFFEL, B., DALOD, M., ALEXOPOULOU, L., BEUTLER, B., BROSSAY, L., VIVIER, E. & WALZER, T. 2008. Cutting edge: Priming of NK cells by IL-18. *Journal of immunology (Baltimore, Md. : 1950),* 181, 1627-1631.

CHAN, C. J., SMYTH, M. J. & MARTINET, L. 2014. Molecular mechanisms of natural killer cell activation in response to cellular stress. *Cell death and differentiation,* 21, 5-14.

CHANG, C.-H., CURTIS, J. D., MAGGI, L. B., JR., FAUBERT, B., VILLARINO, A. V., O'SULLIVAN, D., HUANG, S. C.-C., VAN DER WINDT, G. J. W., BLAGIH, J., QIU, J., WEBER, J. D., PEARCE, E. J., JONES, R. G. & PEARCE, E. L. 2013. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell,* 153, 1239-1251.

CHAPLIN, D. D. 2010. Overview of the immune response. *The Journal of allergy and clinical immunology,* 125, S3-S23.

CHESTER, C., FRITSCH, K. & KOHRT, H. E. 2015. Natural killer cell immunomodulation: targeting activating, inhibitory, and co-stimulatory receptor signaling for cancer immunotherapy. *Frontiers in Immunology,* 6.

CHIUSOLO, V., JACQUEMIN, G., YONCA BASSOY, E., VINET, L., LIGUORI, L., WALCH, M., KOZJAK-PAVLOVIC, V. & MARTINVALET, D. 2017. Granzyme B enters the mitochondria in a Sam50-, Tim22- and mtHsp70-dependent manner to induce apoptosis. *Cell Death & Differentiation,* 24**,** 747-758.

CHOWDHURY, D. & LIEBERMAN, J. 2008. Death by a Thousand Cuts: Granzyme Pathways of Programmed Cell Death. *Annual review of immunology,* 26**,** 389-420.

CHRISTINE A. BIRON, KHUONG B. NGUYEN, GARY C. PIEN, LESLIE P. COUSENS, A. & SALAZAR-MATHER, T. P. 1999. NATURAL KILLER CELLS IN ANTIVIRAL DEFENSE: Function and Regulation by Innate Cytokines. *Annual Review of Immunology,* 17**,** 189-220.

CIBRIÁN, D. & SÁNCHEZ-MADRID, F. 2017. CD69: from activation marker to metabolic gatekeeper. *European Journal of Immunology,* 47**,** 946-953.

COLLINS, P. L., CELLA, M., PORTER, S. I., LI, S., GUREWITZ, G. L., HONG, H. S., JOHNSON, R. P., OLTZ, E. M. & COLONNA, M. 2019. Gene Regulatory Programs Conferring Phenotypic Identities to Human NK Cells. *Cell,* 176**,** 348-360.e12.

COOPER, M. A., BUSH, J. E., FEHNIGER, T. A., VANDEUSEN, J. B., WAITE, R. E., LIU, Y., AGUILA, H. L. & CALIGIURI, M. A. 2002. In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. *Blood,* 100**,** 3633-3638.

COOPER, M. A., FEHNIGER, T. A., PONNAPPAN, A., MEHTA, V., WEWERS, M. D. & CALIGIURI, M. A. 2001. Interleukin-1β costimulates interferon-γ production by human natural killer cells. *European Journal of Immunology,* 31**,** 792-801.

COUPER, K. N., BLOUNT, D. G. & RILEY, E. M. 2008. IL-10: The Master Regulator of Immunity to Infection. *The Journal of Immunology,* 180**,** 5771-5777.

CRANE, C. A., HAN, S. J., BARRY, J. J., AHN, B. J., LANIER, L. L. & PARSA, A. T. 2009. TGF-β downregulates the activating receptor NKG2D on NK cells and CD8+ T cells in glioma patients. *Neuro-Oncology,* 12**,** 7-13.

CRAWLEY, J. B., WILLIAMS, L. M., MANDER, T., BRENNAN, F. M. & FOXWELL, B. M. J. 1996. Interleukin-10 Stimulation of Phosphatidylinositol 3-Kinase and p70 S6 Kinase Is Required for the Proliferative but Not the Antiinflammatory Effects of the Cytokine. *Journal of Biological Chemistry,* 271**,** 16357-16362.

CULLEN, S. P. & MARTIN, S. J. 2008. Mechanisms of granule-dependent killing. *Cell Death & Differentiation,* 15**,** 251-262.

CULLEY, F. J., JOHNSON, M., EVANS, J. H., KUMAR, S., CRILLY, R., CASASBUENAS, J., SCHNYDER, T., MEHRABI, M., DEONARAIN, M. P., USHAKOV, D. S., BRAUD, V., ROTH, G., BROCK, R., KÖHLER, K. & DAVIS, D. M. 2009. Natural Killer Cell Signal Integration Balances Synapse Symmetry and Migration. *PLoS Biol,* 7**,** e1000159.

CURTALE, G., MIROLO, M., RENZI, T. A., ROSSATO, M., BAZZONI, F. & LOCATI, M. 2013. Negative regulation of Toll-like receptor 4 signaling by IL-10–dependent microRNA-146b. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 11499-11504.

DAVID, C. J. & MASSAGUÉ, J. 2018. Contextual determinants of TGFβ action in development, immunity and cancer. *Nature Reviews Molecular Cell Biology,* 19**,** 419-435.

DE RHAM, C., FERRARI-LACRAZ, S., JENDLY, S., SCHNEITER, G., DAYER, J.-M. & VILLARD, J. 2007. The proinflammatory cytokines IL-2, IL-15 and IL-21 modulate the repertoire of mature human natural killer cell receptors. *Arthritis Research & Therapy,* 9, R125-R125.

DENMAN, C. J., SENYUKOV, V. V., SOMANCHI, S. S., PHATARPEKAR, P. V., KOPP, L. M., JOHNSON, J. L., SINGH, H., HURTON, L., MAITI, S. N., HULS, M. H., CHAMPLIN, R. E., COOPER, L. J. & LEE, D. A. 2012. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS One,* 7, e30264.

DING, Y., QIN, L., ZAMARIN, D., KOTENKO, S. V., PESTKA, S., MOORE, K. W. & BROMBERG, J. S. 2001. Differential IL-10R1 Expression Plays a Critical Role in IL-10-Mediated Immune Regulation. *The Journal of Immunology,* 167, 6884-6892.

DOMAICA, C. I., FUERTES, M. B., URIARTE, I., GIRART, M. V., SARDAÑONS, J., COMAS, D. I., DI GIOVANNI, D., GAILLARD, M. I., BEZRODNIK, L. & ZWIRNER, N. W. 2012. Human Natural Killer Cell Maturation Defect Supports In Vivo CD56bright to CD56dim Lineage Development. *PLOS ONE,* 7, e51677.

DONNELLY, R. P., LOFTUS, R. M., KEATING, S. E., LIOU, K. T., BIRON, C. A., GARDINER, C. M. & FINLAY, D. K. 2014. mTORC1-Dependent Metabolic Reprogramming Is a Prerequisite for NK Cell Effector Function. *The Journal of Immunology,* 193, 4477-4484.

DUNNE, J., LYNCH, S., O'FARRELLY, C., TODRYK, S., HEGARTY, J. E., FEIGHERY, C. & DOHERTY, D. G. 2001. Selective Expansion and Partial Activation of Human NK Cells and NK Receptor-Positive T Cells by IL-2 and IL-15. *The Journal of Immunology,* 167, 3129-3138.

DYBKAER, K., IQBAL, J., ZHOU, G., GENG, H., XIAO, L., SCHMITZ, A., D'AMORE, F. & CHAN, W. C. 2007. Genome wide transcriptional analysis of resting and IL2 activated human natural killer cells: gene expression signatures indicative of novel molecular signaling pathways. *BMC Genomics,* 8, 230.

EBERT, E. C. 2000. IL-10 enhances IL-2-induced proliferation and cytotoxicity by human intestinal lymphocytes. *Clinical and experimental immunology,* 119, 426-432.

ELLIOTT, J. M. & YOKOYAMA, W. M. 2011. Unifying concepts of MHC-dependent natural killer cell education. *Trends in Immunology,* 32, 364-372.

EMMERICH, J., MUMM, J. B., CHAN, I. H., LAFACE, D., TRUONG, H., MCCLANAHAN, T., GORMAN, D. M. & OFT, M. 2012. IL-10 Directly Activates and Expands Tumor-Resident CD8⁺ T Cells without De Novo Infiltration from Secondary Lymphoid Organs. *Cancer Research,* 72, 3570-3581.

ERIKSEN, K. W., SØNDERGAARD, H., WOETMANN, A., KREJSGAARD, T., SKAK, K., GEISLER, C., WASIK, M. A. & ØDUM, N. 2009. The combination of IL-21 and IFN-α boosts STAT3 activation, cytotoxicity and experimental tumor therapy. *Molecular Immunology,* 46, 812-820.

EWEN, C. L., KANE, K. P. & BLEACKLEY, R. C. 2012. A quarter century of granzymes. *Cell Death and Differentiation,* 19, 28-35.

FEHNIGER, T. A., SHAH, M. H., TURNER, M. J., VANDEUSEN, J. B., WHITMAN, S. P., COOPER, M. A., SUZUKI, K., WECHSER, M., GOODSAID, F. & CALIGIURI, M. A.

1999. Differential Cytokine and Chemokine Gene Expression by Human NK Cells Following Activation with IL-18 or IL-15 in Combination with IL-12: Implications for the Innate Immune Response. *The Journal of Immunology,* 162, 4511-4520.

FELICES, M., LENVIK, A. J., MCELMURRY, R., CHU, S., HINDERLIE, P., BENDZICK, L., GELLER, M. A., TOLAR, J., BLAZAR, B. R. & MILLER, J. S. 2018. Continuous treatment with IL-15 exhausts human NK cells via a metabolic defect. *JCI insight,* 3, e96219.

FERLAZZO, G. & MORANDI, B. 2014. Cross-Talks between Natural Killer Cells and Distinct Subsets of Dendritic Cells. *Frontiers in immunology,* 5, 159-159.

FERLAZZO, G., PACK, M., THOMAS, D., PALUDAN, C., SCHMID, D., STROWIG, T., BOUGRAS, G., MULLER, W. A., MORETTA, L. & MÜNZ, C. 2004. Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. *Proceedings of the National Academy of Sciences of the United States of America,* 101, 16606-16611.

FIORENTINO, D., BOND, M. & MOSMANN, T. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *The Journal of Experimental Medicine,* 170, 2081-2095.

FIORENTINO, D. F., ZLOTNIK, A., MOSMANN, T. R., HOWARD, M. & O'GARRA, A. 1991. IL-10 inhibits cytokine production by activated macrophages. *The Journal of Immunology,* 147, 3815-3822.

FREEMAN, E. B., RAUÉ, H.P, HILL, A.B, SLIFK, M.K 2015. Cytokine-mediated activation of NK cells during viral infection. *Journal of Virology*.

FRENCH, A. R., HOLROYD, E. B., YANG, L., KIM, S. & YOKOYAMA, W. M. 2006. IL-18 acts synergistically with IL-15 in stimulating natural killer cell proliferation. *Cytokine,* 35, 229-234.

FU, M. & BLACKSHEAR, P. J. 2016. RNA-binding proteins in immune regulation: a focus on CCCH zinc finger proteins. *Nature Reviews Immunology,* 17, 130.

FUJII, S.-I., SHIMIZU, K., SHIMIZU, T. & LOTZE, M. T. 2001. Interleukin-10 promotes the maintenance of antitumor CD8⁺ T-cell effector function in situ. *Blood,* 98, 2143-2151.

GARCÍA-HERNÁNDEZ, M. L., HERNÁNDEZ-PANDO, R., GARIGLIO, P. & BERUMEN, J. 2002. Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumour and vascular cell proliferation. *Immunology,* 105, 231-243.

GEROSA, F., BALDANI-GUERRA, B., NISII, C., MARCHESINI, V., CARRA, G. & TRINCHIERI, G. 2002. Reciprocal activating interaction between natural killer cells and dendritic cells. *The Journal of experimental medicine,* 195, 327-333.

GIACINTI, C. & GIORDANO, A. 2006. RB and cell cycle progression. *Oncogene,* 25, 5220-5227.

GLOCKER, E.-O., KOTLARZ, D., KLEIN, C., SHAH, N. & GRIMBACHER, B. 2011. IL-10 and IL-10 receptor defects in humans. *Annals of the New York Academy of Sciences,* 1246, 102-107.

GOLDSZMID, ROMINA S., CASPAR, P., RIVOLLIER, A., WHITE, S., DZUTSEV, A., HIENY, S., KELSALL, B., TRINCHIERI, G. & SHER, A. 2012. NK Cell-Derived Interferon-

γ Orchestrates Cellular Dynamics and the Differentiation of Monocytes into Dendritic Cells at the Site of Infection. *Immunity,* 36**,** 1047-1059.

GOSSELIN, J., TOMOÏU, A., GALLO, R. C. & FLAMAND, L. 1999. Interleukin-15 as an Activator of Natural Killer Cell-Mediated Antiviral Response. *Blood,* 94**,** 4210-4219.

GOTTHARDT, D., PUTZ, E. M., STRAKA, E., KUDWEIS, P., BIAGGIO, M., POLI, V., STROBL, B., MÜLLER, M. & SEXL, V. 2014. Loss of STAT3 in murine NK cells enhances NK cell–dependent tumor surveillance. *Blood,* 124**,** 2370-2379.

GROUX, H., BIGLER, M., DE VRIES, J. E. & RONCAROLO, M.-G. 1998. Inhibitory and Stimulatory Effects of IL-10 on Human CD8+ T Cells. *The Journal of Immunology,* 160**,** 3188-3193.

GUHA, M. & MACKMAN, N. 2001. LPS induction of gene expression in human monocytes. *Cellular Signalling,* 13**,** 85-94.

GUO, Y., LUAN, L., PATIL, N. K. & SHERWOOD, E. R. 2017. Immunobiology of the IL-15/IL-15Rα complex as an antitumor and antiviral agent. *Cytokine & growth factor reviews,* 38**,** 10-21.

GWALANI, L. A. & ORANGE, J. S. 2018. Single Degranulations in NK Cells Can Mediate Target Cell Killing. *The Journal of Immunology,* 200**,** 3231-3243.

HAGN, M., SCHWESINGER, E., EBEL, V., SONTHEIMER, K., MAIER, J., BEYER, T., SYROVETS, T., LAUMONNIER, Y., FABRICIUS, D., SIMMET, T. & JAHRSDÖRFER, B. 2009. Human B Cells Secrete Granzyme B When Recognizing Viral Antigens in the Context of the Acute Phase Cytokine IL-21. *The Journal of Immunology,* 183**,** 1838-1845.

HE, Y. & TIAN, Z. 2017. NK cell education via nonclassical MHC and non-MHC ligands. *Cellular & molecular immunology,* 14**,** 321-330.

HEINE, G., DROZDENKO, G., GRÜN, J. R., CHANG, H.-D., RADBRUCH, A. & WORM, M. 2014. Autocrine IL-10 promotes human B-cell differentiation into IgM- or IgG-secreting plasmablasts. *European Journal of Immunology,* 44**,** 1615-1621.

HELDIN, C.-H. & MOUSTAKAS, A. 2016. Signaling Receptors for TGF-β Family Members. *Cold Spring Harbor Perspectives in Biology,* 8.

HERNÁNDEZ-BELLO, J., OREGÓN-ROMERO, E., VÁZQUEZ-VILLAMAR, M., GARCÍA-ARELLANO, S., VALLE, Y., PADILLA-GUTIÉRREZ, J. R., ROMÁN-FERNÁNDEZ, I. V., PALAFOX-SÁNCHEZ, C. A., MARTÍNEZ-BONILLA, G. E. & MUÑOZ-VALLE, J. F. 2017. Aberrant expression of interleukin-10 in rheumatoid arthritis: Relationship with IL10 haplotypes and autoantibodies. *Cytokine,* 95**,** 88-96.

HEWITT, E. W. 2003. The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology,* 110**,** 163-169.

HOFFMAN, W., LAKKIS, F. G. & CHALASANI, G. 2016. B Cells, Antibodies, and More. *Clinical journal of the American Society of Nephrology : CJASN,* 11**,** 137-154.

HOFMANN, S. R., RÖSEN-WOLFF, A., TSOKOS, G. C. & HEDRICH, C. M. 2012. Biological properties and regulation of IL-10 related cytokines and their contribution to autoimmune disease and tissue injury. *Clinical Immunology,* 143**,** 116-127.

HÖGLUND, P. & BRODIN, P. 2010. Current perspectives of natural killer cell education by MHC class I molecules. *Nature Reviews Immunology,* 10**,** 724-734.

HOLMES, T. D., EL-SHERBINY, Y. M., DAVISON, A., CLOUGH, S. L., BLAIR, G. E. & COOK, G. P. 2011. A Human NK Cell Activation/Inhibition Threshold Allows Small Changes in the Target Cell Surface Phenotype To Dramatically Alter Susceptibility to NK Cells. *The Journal of Immunology,* 186**,** 1538-1545.

HOLMES, T. D., WILSON, E. B., BLACK, E. V. I., BENEST, A. V., VAZ, C., TAN, B., TANAVDE, V. M. & COOK, G. P. 2014. Licensed human natural killer cells aid dendritic cell maturation via TNFSF14/LIGHT. *Proceedings of the National Academy of Sciences,* 111**,** E5688-E5696.

HONJO, Y., BIAN, Y., KAWAKAM, K., MOLINOLO, A., LONGENECKER, G., BOPPANA, R., LARSSON, J., KARLSSON, S., GUTKIND, J. S., PURI, R. K. & KULKARNI, A. B. 2007. TGFβ Receptor I Conditional Knockout Mice Develop Spontaneous Squamous Cell Carcinoma. *Cell Cycle,* 6**,** 1360-1366.

HUNTINGTON, N. D. 2014. The unconventional expression of IL-15 and its role in NK cell homeostasis. *Immunology & Cell Biology,* 92**,** 210-213.

HUNTINGTON, N. D., LEGRAND, N., ALVES, N. L., JARON, B., WEIJER, K., PLET, A., CORCUFF, E., MORTIER, E., JACQUES, Y., SPITS, H. & DI SANTO, J. P. 2009. IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. *The Journal of Experimental Medicine,* 206**,** 25-34.

HUNTINGTON, N. D., PUTHALAKATH, H., GUNN, P., NAIK, E., MICHALAK, E. M., SMYTH, M. J., TABARIAS, H., DEGLI-ESPOSTI, M. A., DEWSON, G., WILLIS, S. N., MOTOYAMA, N., HUANG, D. C. S., NUTT, S. L., TARLINTON, D. M. & STRASSER, A. 2007. Interleukin 15–mediated survival of natural killer cells is determined by interactions among Bim, Noxa and Mcl-1. *Nature Immunology,* 8**,** 856-863.

HUTCHINS, A. P., DIEZ, D. & MIRANDA-SAAVEDRA, D. 2013. The IL-10/STAT3-mediated anti-inflammatory response: recent developments and future challenges. *Briefings in Functional Genomics,* 12**,** 489-498.

INOUE, S., UNSINGER, J., DAVIS, C. G., MUENZER, J. T., FERGUSON, T. A., CHANG, K., OSBORNE, D. F., CLARK, A. T., COOPERSMITH, C. M., MCDUNN, J. E. & HOTCHKISS, R. S. 2010. IL-15 Prevents Apoptosis, Reverses Innate and Adaptive Immune Dysfunction, and Improves Survival in Sepsis. *The Journal of Immunology,* 184**,** 1401-1409.

ITOH, K. & HIROHATA, S. 1995. The role of IL-10 in human B cell activation, proliferation, and differentiation. *The Journal of Immunology,* 154**,** 4341-4350.

ITOH, Y., SAITOH, M. & MIYAZAWA, K. 2017. Smad3–STAT3 crosstalk in pathophysiological contexts. *Acta Biochimica et Biophysica Sinica,* 50**,** 82-90.

JACOBSON, N. G., SZABO, S. J., WEBER-NORDT, R. M., ZHONG, Z., SCHREIBER, R. D., DARNELL, J. E. & MURPHY, K. M. 1995. Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4. *The Journal of Experimental Medicine,* 181**,** 1755-1762.

JACQUEMIN, G., MARGIOTTA, D., KASAHARA, A., BASSOY, E. Y., WALCH, M., THIERY, J., LIEBERMAN, J. & MARTINVALET, D. 2015. Granzyme B-induced mitochondrial ROS are required for apoptosis. *Cell death and differentiation,* 22**,** 862-874.

JENS, W., FABRICE, G., BERND, G., JUERGEN, F., ROLAND, M. & RENATE, M. W.-N. 1996. IL-10 induces DNA binding activity of three STAT proteins (Stat1, Stat3, and Stat5) and their distinct combinatorial assembly in the promoters of selected genes. *FEBS Letters,* 394, 365-370.

JOHNSTON, J. A., BACON, C. M., FINBLOOM, D. S., REES, R. C., KAPLAN, D., SHIBUYA, K., ORTALDO, J. R., GUPTA, S., CHEN, Y. Q. & GIRI, J. D. 1995. Tyrosine phosphorylation and activation of STAT5, STAT3, and Janus kinases by interleukins 2 and 15. *Proceedings of the National Academy of Sciences of the United States of America,* 92, 8705-8709.

KAI, B., MICHAEL, K., BRITTA, B., CHRISTIAN, V. J. & WERNER, L. 2005. Differential expression of human granzymes A, B, and K in natural killer cells and during CD8+ T cell differentiation in peripheral blood. *European Journal of Immunology,* 35, 2608-2616.

KAISHO, T., TAKEUCHI, O., KAWAI, T., HOSHINO, K. & AKIRA, S. 2001. Endotoxin-Induced Maturation of MyD88-Deficient Dendritic Cells. *The Journal of Immunology,* 166, 5688-5694.

KARAM, R. A., ZIDAN, H. E. & KHATER, M. H. 2014. Polymorphisms in the TNF-α and IL-10 gene promoters and risk of psoriasis and correlation with disease severity. *Cytokine,* 66, 101-105.

KARIMIAN, A., AHMADI, Y. & YOUSEFI, B. 2016. Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage. *DNA Repair,* 42, 63-71.

KÄRRE, K., LJUNGGREN, H. G., PIONTEK, G. & KIESSLING, R. 1986. Selective rejection of H–2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature,* 319, 675-678.

KAYAGAKI, N., YAMAGUCHI, N., NAKAYAMA, M., TAKEDA, K., AKIBA, H., TSUTSUI, H., OKAMURA, H., NAKANISHI, K., OKUMURA, K. & YAGITA, H. 1999. Expression and Function of TNF-Related Apoptosis-Inducing Ligand on Murine Activated NK Cells. *The Journal of Immunology,* 163, 1906-1913.

KEATING, S. E., ZAIATZ-BITTENCOURT, V., LOFTUS, R. M., KEANE, C., BRENNAN, K., FINLAY, D. K. & GARDINER, C. M. 2016. Metabolic Reprogramming Supports IFN-γ Production by CD56^{bright} NK Cells. *The Journal of Immunology,* 196, 2552-2560.

KENNEDY, M. K., GLACCUM, M., BROWN, S. N., BUTZ, E. A., VINEY, J. L., EMBERS, M., MATSUKI, N., CHARRIER, K., SEDGER, L., WILLIS, C. R., BRASEL, K., MORRISSEY, P. J., STOCKING, K., SCHUH, J. C. L., JOYCE, S. & PESCHON, J. J. 2000. Reversible Defects in Natural Killer and Memory Cd8 T Cell Lineages in Interleukin 15–Deficient Mice. *The Journal of Experimental Medicine,* 191, 771-780.

KEPPEL, M. P., SAUCIER, N., MAH, A. Y., VOGEL, T. P. & COOPER, M. A. 2015. Activation-Specific Metabolic Requirements for NK Cell IFN-γ Production. *The Journal of Immunology,* 194, 1954-1962.

KOBAYASHI, M., FITZ, L., RYAN, M., HEWICK, R., CLARK, S., CHAN, S., LOUDON, R., SHERMAN, F., PERUSSIA, B. & TRINCHIERIT, G. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *The Journal of Experimental Medicine,* 170, 827-845.

KOTLARZ, D., BEIER, R., MURUGAN, D., DIESTELHORST, J., JENSEN, O., BOZTUG, K., PFEIFER, D., KREIPE, H., PFISTER, E. D., BAUMANN, U., PUCHALKA, J., BOHNE, J., EGRITAS, O., DALGIC, B., KOLHO, K. L., SAUERBREY, A., BUDERUS, S., GÜNGÖR, T., ENNINGER, A., KODA, Y. K. L., GUARISO, G., WEISS, B., CORBACIOGLU, S., SOCHA, P., USLU, N., METIN, A., WAHBEH, G. T., HUSAIN, K., RAMADAN, D., AL–HERZ, W., GRIMBACHER, B., SAUER, M., SYKORA, K. W., KOLETZKO, S. & KLEIN, C. 2012. Loss of Interleukin-10 Signaling and Infantile Inflammatory Bowel Disease: Implications for Diagnosis and Therapy. *Gastroenterology,* 143**,** 347-355.

KRUSCHINSKI, A., MOOSMANN, A., POSCHKE, I., NORELL, H., CHMIELEWSKI, M., SELIGER, B., KIESSLING, R., BLANKENSTEIN, T., ABKEN, H. & CHARO, J. 2008. Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 17481-17486.

KRZEWSKI, K. & COLIGAN, J. 2012. Human NK cell lytic granules and regulation of their exocytosis. *Frontiers in Immunology,* 3.

KRZEWSKI, K., GIL-KRZEWSKA, A., NGUYEN, V., PERUZZI, G. & COLIGAN, J. E. 2013. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood,* 121**,** 4672-4683.

KÜHN, R., LÖHLER, J., RENNICK, D., RAJEWSKY, K. & MÜLLER, W. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell,* 75**,** 263-274.

LAI, C.-F., RIPPERGER, J., MORELLA, K. K., JURLANDER, J., HAWLEY, T. S., CARSON, W. E., KORDULA, T., CALIGIURI, M. A., HAWLEY, R. G., FEY, G. H. & BAUMANN, H. 1996. Receptors for Interleukin (IL)-10 and IL-6-type Cytokines Use Similar Signaling Mechanisms for Inducing Transcription through IL-6 Response Elements. *Journal of Biological Chemistry,* 271**,** 13968-13975.

LANIER, L. L. 2005. NK CELL RECOGNITION. *Annual Review of Immunology,* 23**,** 225-274.

LANIER, L. L. 2008. Evolutionary struggles between NK cells and viruses. *Nature Reviews Immunology,* 8**,** 259-268.

LAUW, F. N., PAJKRT, D., HACK, C. E., KURIMOTO, M., VAN DEVENTER, S. J. H. & VAN DER POLL, T. 2000. Proinflammatory Effects of IL-10 During Human Endotoxemia. *The Journal of Immunology,* 165**,** 2783-2789.

LAUWERYS, B. R., GAROT, N., RENAULD, J.-C. & HOUSSIAU, F. A. 2000. Cytokine Production and Killer Activity of NK/T-NK Cells Derived with IL-2, IL-15, or the Combination of IL-12 and IL-18. *The Journal of Immunology,* 165**,** 1847-1853.

LAUWERYS, B. R., RENAULD, J.-C. & HOUSSIAU, F. A. 1999. SYNERGISTIC PROLIFERATION AND ACTIVATION OF NATURAL KILLER CELLS BY INTERLEUKIN 12 AND INTERLEUKIN 18. *Cytokine,* 11**,** 822-830.

LEE, C. H., HSU, P., NANAN, B., NANAN, R., WONG, M., GASKIN, K. J., LEONG, R. W., MURCHIE, R., MUISE, A. M. & STORMON, M. O. 2014. Novel de novo mutations of the interleukin-10 receptor gene lead to infantile onset inflammatory bowel disease. *Journal of Crohn's and Colitis,* 8**,** 1551-1556.

LEE, H. K. & IWASAKI, A. 2007. Innate control of adaptive immunity: Dendritic cells and beyond. *Seminars in Immunology,* 19**,** 48-55.

LEE, M. K., PARDOUX, C., HALL, M. C., LEE, P. S., WARBURTON, D., QING, J., SMITH, S. M. & DERYNCK, R. 2007. TGF-β activates Erk MAP kinase signalling through direct phosphorylation of ShcA. *The EMBO Journal,* 26, 3957-3967.

LI, Q., YE, L. J., REN, H. L., HUYAN, T., LI, J., SHI, J. L. & HUANG, Q. S. 2015. Multiple effects of IL-21 on human NK cells in ex vivo expansion. *Immunobiology,* 220, 876-88.

LIANG, Y., ZHU, F., ZHANG, H., CHEN, D., ZHANG, X., GAO, Q. & LI, Y. 2016. Conditional ablation of TGF-β signaling inhibits tumor progression and invasion in an induced mouse bladder cancer model. *Scientific Reports,* 6, 29479.

LITTWITZ-SALOMON, E., MALYSHKINA, A., SCHIMMER, S. & DITTMER, U. 2018. The Cytotoxic Activity of Natural Killer Cells Is Suppressed by IL-10+ Regulatory T Cells During Acute Retroviral Infection. *Frontiers in Immunology,* 9.

LIU, K., CATALFAMO, M., LI, Y., HENKART, P. A. & WENG, N.-P. 2002. IL-15 mimics T cell receptor crosslinking in the induction of cellular proliferation, gene expression, and cytotoxicity in CD8⁺ memory T cells. *Proceedings of the National Academy of Sciences,* 99, 6192-6197.

LJUNGGREN, H.-G. & KÄRRE, K. 1990. In search of the ‘missing self’: MHC molecules and NK cell recognition. *Immunology Today,* 11, 237-244.

LODOLCE, J. P., BOONE, D. L., CHAI, S., SWAIN, R. E., DASSOPOULOS, T., TRETTIN, S. & MA, A. 1998. IL-15 Receptor Maintains Lymphoid Homeostasis by Supporting Lymphocyte Homing and Proliferation. *Immunity,* 9, 669-676.

LODOLCE, J. P., BURKETT, P. R., KOKA, R. M., BOONE, D. L. & MA, A. 2002. Regulation of lymphoid homeostasis by interleukin-15. *Cytokine & Growth Factor Reviews,* 13, 429-439.

LOFTUS, R. M. & FINLAY, D. K. 2016. Immunometabolism: Cellular Metabolism Turns Immune Regulator. *Journal of Biological Chemistry,* 291, 1-10.

LONG, E. O., KIM, H. S., LIU, D., PETERSON, M. E. & RAJAGOPALAN, S. 2013. Controlling NK Cell Responses: Integration of Signals for Activation and Inhibition. *Annual review of immunology,* 31, 10.1146/annurev-immunol-020711-075005.

LOPEZ, J. A., SUSANTO, O., JENKINS, M. R., LUKOYANOVA, N., SUTTON, V. R., LAW, R. H. P., JOHNSTON, A., BIRD, C. H., BIRD, P. I., WHISSTOCK, J. C., TRAPANI, J. A., SAIBIL, H. R. & VOSKOBOINIK, I. 2013. Perforin forms transient pores on the target cell plasma membrane to facilitate rapid access of granzymes during killer cell attack. *Blood,* 121, 2659-2668.

LOZZIO, C. B., AND LOZZIO, B. B. 1975. Human Chronic myelogenous leukemia cell-line with positive philadelphia chromosome. *Blood,* 45, 321-334.

LU, Y.-C., YEH, W.-C. & OHASHI, P. S. 2008. LPS/TLR4 signal transduction pathway. *Cytokine,* 42, 145-151.

LUCAS, M., SCHACHTERLE, W., OBERLE, K., AICHELE, P. & DIEFENBACH, A. 2007. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity,* 26, 503-517.

MACE, E. M., DONGRE, P., HSU, H.-T., SINHA, P., JAMES, A. M., MANN, S. S., FORBES, L. R., WATKIN, L. B. & ORANGE, J. S. 2014. Cell biological steps and checkpoints in accessing NK cell cytotoxicity. *Immunology and Cell Biology,* 92, 245-255.

MACE, E. M. & ORANGE, J. S. 2016. Genetic Causes of Human NK Cell Deficiency and Their Effect on NK Cell Subsets. *Frontiers in immunology,* 7**,** 545-545.

MACK, E. A., KALLAL, L. E., DEMERS, D. A. & BIRON, C. A. 2011. Type 1 Interferon Induction of Natural Killer Cell Gamma Interferon Production for Defense during Lymphocytic Choriomeningitis Virus Infection. *mBio,* 2**,** e00169-11.

MAH, A. Y. & COOPER, M. A. 2016. Metabolic Regulation of Natural Killer Cell IFN-γ Production. *Critical reviews in immunology,* 36**,** 131-147.

MAH, A. Y., RASHIDI, A., KEPPEL, M. P., SAUCIER, N., MOORE, E. K., ALINGER, J. B., TRIPATHY, S. K., AGARWAL, S. K., JENG, E. K., WONG, H. C., MILLER, J. S., FEHNIGER, T. A., MACE, E. M., FRENCH, A. R. & COOPER, M. A. 2017. Glycolytic requirement for NK cell cytotoxicity and cytomegalovirus control. *JCI Insight,* 2**,** e95128.

MARÇAIS, A., CHERFILS-VICINI, J., VIANT, C., DEGOUVE, S., VIEL, S., FENIS, A., RABILLOUD, J., MAYOL, K., TAVARES, A., BIENVENU, J., GANGLOFF, Y.-G., GILSON, E., VIVIER, E. & WALZER, T. 2014. The metabolic checkpoint kinase mTOR is essential for IL-15 signaling during the development and activation of NK cells. *Nature Immunology,* 15**,** 749.

MARLOW, G. J., VAN GENT, D. & FERGUSON, L. R. 2013. Why interleukin-10 supplementation does not work in Crohn's disease patients. *World Journal of Gastroenterology : WJG,* 19**,** 3931-3941.

MARTÍN-FONTECHA, A., THOMSEN, L. L., BRETT, S., GERARD, C., LIPP, M., LANZAVECCHIA, A. & SALLUSTO, F. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-γ for TH1 priming. *Nature Immunology,* 5**,** 1260.

MARTINET, L. & SMYTH, M. J. 2015. Balancing natural killer cell activation through paired receptors. *Nature Reviews Immunology,* 15**,** 243.

MARTINEZ, J., HUANG, X. & YANG, Y. 2008. Direct Action of Type I IFN on NK Cells Is Required for Their Activation in Response to Vaccinia Viral Infection In Vivo. *The Journal of Immunology,* 180**,** 1592-1597.

MARTINVALET, D. 2019. Mitochondrial Entry of Cytotoxic Proteases: A New Insight into the Granzyme B Cell Death Pathway. *Oxidative Medicine and Cellular Longevity,* 2019**,** 13.

MATIKAINEN, S., PAANANEN, A., MIETTINEN, M., KURIMOTO, M., TIMONEN, T., JULKUNEN, I. & SARENEVA, T. 2001. IFN-α and IL-18 synergistically enhance IFN-γ production in human NK cells: differential regulation of Stat4 activation and IFN-γ gene expression by IFN-α and IL-12. *European Journal of Immunology,* 31**,** 2236-2245.

MAVROPOULOS, A., SULLY, G., COPE, A. P. & CLARK, A. R. 2005. Stabilization of IFN-γ mRNA by MAPK p38 in IL-12– and IL-18–stimulated human NK cells. *Blood,* 105**,** 282-288.

MEGHNEM, D., MORISSEAU, S., FRUTOSO, M., TRILLET, K., MAILLASSON, M., BARBIEUX, I., KHADDAGE, S., LERAY, I., HILDINGER, M., QUÉMÉNER, A., JACQUES, Y. & MORTIER, E. 2017. Cutting Edge: Differential Fine-Tuning of IL-2– and IL-15–Dependent Functions by Targeting Their Common IL-2/15Rβ/γc Receptor. *The Journal of Immunology,* 198**,** 4563-4568.

MERINO, A., ZHANG, B., DOUGHERTY, P., LUO, X., WANG, J., BLAZAR, B. R., MILLER, J. S. & CICHOCKI, F. 2019. Chronic stimulation drives human NK cell

dysfunction and epigenetic reprograming. *The Journal of Clinical Investigation,* 129**,** 3770-3785.
MESTAS, J. & HUGHES, C. C. W. 2004. Of Mice and Not Men: Differences between Mouse and Human Immunology. *The Journal of Immunology,* 172**,** 2731-2738.
MICHEL, T., POLI, A., CUAPIO, A., BRIQUEMONT, B., ISERENTANT, G., OLLERT, M. & ZIMMER, J. 2016. Human CD56^{bright} NK Cells: An Update. *The Journal of Immunology,* 196**,** 2923-2931.
MISHRA, A., SULLIVAN, L. & CALIGIURI, M. A. 2014. Molecular pathways: interleukin-15 signaling in health and in cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 20**,** 2044-2050.
MITTAL, S. K. & ROCHE, P. A. 2015. Suppression of antigen presentation by IL-10. *Current Opinion in Immunology,* 34**,** 22-27.
MOCELLIN, S., MARINCOLA, F. M. & YOUNG, H. A. 2005. Interleukin-10 and the immune response against cancer: a counterpoint. *Journal of Leukocyte Biology,* 78**,** 1043-1051.
MOCELLIN, S., PANELLI, M., WANG, E., ROSSI, C. R., PILATI, P., NITTI, D., LISE, M. & MARINCOLA, F. M. 2004

. IL-10 stimulatory effects on human NK cells explored by gene profile analysis. *Genes Immun,* 5**,** 621-630.
MOCELLIN, S., PANELLI, M. C., WANG, E., NAGORSEN, D. & MARINCOLA, F. M. 2003. The dual role of IL-10. *Trends in Immunology,* 24**,** 36-43.
MORANDI, B., MORTARA, L., CHIOSSONE, L., ACCOLLA, R. S., MINGARI, M. C., MORETTA, L., MORETTA, A. & FERLAZZO, G. 2012. Dendritic cell editing by activated natural killer cells results in a more protective cancer-specific immune response. *PloS one,* 7**,** e39170-e39170.
MORETTA, L. & MORETTA, A. 2004. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. *The EMBO Journal,* 23**,** 255-259.
MRÓZEK E, A. P., CALIGIURI MA 1996. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. *Blood,* 87**,** 2632-2640.
MULLEN, ALAN C., ORLANDO, DAVID A., NEWMAN, JAMIE J., LOVÉN, J., KUMAR, ROSHAN M., BILODEAU, S., REDDY, J., GUENTHER, MATTHEW G., DEKOTER, R. P. & YOUNG, RICHARD A. 2011. Master Transcription Factors Determine Cell-Type-Specific Responses to TGF-β Signaling. *Cell,* 147**,** 565-576.
MUMM, JOHN B., EMMERICH, J., ZHANG, X., CHAN, I., WU, L., MAUZE, S., BLAISDELL, S., BASHAM, B., DAI, J., GREIN, J., SHEPPARD, C., HONG, K., CUTLER, C., TURNER, S., LAFACE, D., KLEINSCHEK, M., JUDO, M., AYANOGLU, G., LANGOWSKI, J., GU, D., PAPORELLO, B., MURPHY, E., SRIRAM, V., NARAVULA, S., DESAI, B., MEDICHERLA, S., SEGHEZZI, W., MCCLANAHAN, T., CANNON-CARLSON, S., BEEBE, AMY M. & OFT, M. 2011. IL-10 Elicits IFNgamma-Dependent Tumor Immune Surveillance. *Cancer Cell,* 20**,** 781-796.
NAING, A., PAPADOPOULOS, K. P., AUTIO, K. A., OTT, P. A., PATEL, M. R., WONG, D. J., FALCHOOK, G. S., PANT, S., WHITESIDE, M., RASCO, D. R., MUMM, J. B.,

CHAN, I. H., BENDELL, J. C., BAUER, T. M., COLEN, R. R., HONG, D. S., VAN VLASSELAER, P., TANNIR, N. M., OFT, M. & INFANTE, J. R. 2016. Safety, Antitumor Activity, and Immune Activation of Pegylated Recombinant Human Interleukin-10 (AM0010) in Patients With Advanced Solid Tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology,* 34**,** 3562-3569.

NANDAGOPAL, N., ALI, A. K., KOMAL, A. K. & LEE, S.-H. 2014. The Critical Role of IL-15–PI3K–mTOR Pathway in Natural Killer Cell Effector Functions. *Frontiers in Immunology,* 5.

NGUYEN, K. B., SALAZAR-MATHER, T. P., DALOD, M. Y., VAN DEUSEN, J. B., WEI, X.-Q., LIEW, F. Y., CALIGIURI, M. A., DURBIN, J. E. & BIRON, C. A. 2002. Coordinated and Distinct Roles for IFN-αβ, IL-12, and IL-15 Regulation of NK Cell Responses to Viral Infection. *The Journal of Immunology,* 169**,** 4279-4287.

NIELSEN, C. M., WOLF, A.-S., GOODIER, M. R. & RILEY, E. M. 2016. Synergy between Common γ Chain Family Cytokines and IL-18 Potentiates Innate and Adaptive Pathways of NK Cell Activation. *Frontiers in Immunology,* 7.

NIEMAND, C., NIMMESGERN, A., HAAN, S., FISCHER, P., SCHAPER, F., ROSSAINT, R., HEINRICH, P. C. & MÜLLER-NEWEN, G. 2003. Activation of STAT3 by IL-6 and IL-10 in Primary Human Macrophages Is Differentially Modulated by Suppressor of Cytokine Signaling 3. *The Journal of Immunology,* 170**,** 3263-3272.

O'FARRELL, A. M., LIU, Y., MOORE, K. W. & MUI, A. L. 1998. IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways. *The EMBO journal,* 17**,** 1006-1018.

OCHOA, M. C., MINUTE, L., RODRIGUEZ, I., GARASA, S., PEREZ-RUIZ, E., INOGÉS, S., MELERO, I. & BERRAONDO, P. 2017. Antibody-dependent cell cytotoxicity: immunotherapy strategies enhancing effector NK cells. *Immunology & Cell Biology,* 95**,** 347-355.

OFT, M. 2014. IL-10: Master Switch from Tumor-Promoting Inflammation to Antitumor Immunity. *Cancer Immunology Research,* 2**,** 194-199.

ORANGE, J. S. 2013. Natural killer cell deficiency. *Journal of Allergy and Clinical Immunology,* 132**,** 515-525.

ORANGE, J. S. & BIRON, C. A. 1996. An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. *The Journal of Immunology,* 156**,** 1138-1142.

ORR, M. T. & LANIER, L. L. 2010. Natural Killer Cell Education and Tolerance. *Cell,* 142**,** 847-856.

ORR, M. T., MURPHY, W. J. & LANIER, L. L. 2010. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nature Immunology,* 11**,** 321-327.

OSWALD, I. P., WYNN, T. A., SHER, A. & JAMES, S. L. 1992. Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon

gamma-induced activation. *Proceedings of the National Academy of Sciences,* 89**,** 8676-8680.

OTEGBEYE, F., OJO, E., MORETON, S., MACKOWSKI, N., LEE, D. A., DE LIMA, M. & WALD, D. N. 2018. Inhibiting TGF-beta signaling preserves the function of highly activated, in vitro expanded natural killer cells in AML and colon cancer models. *PLOS ONE,* 13**,** e0191358.

OUYANG, P., RAKUS, K., VAN BEURDEN, S. J., WESTPHAL, A. H., DAVISON, A. J., GATHERER, D. & VANDERPLASSCHEN, A. F. 2014. IL-10 encoded by viruses: a remarkable example of independent acquisition of a cellular gene by viruses and its subsequent evolution in the viral genome. *Journal of General Virology,* 95**,** 245-262.

OUYANG, W., RUTZ, S., CRELLIN, N.K., VALDEZ, P.A., AND HYMOWITZ, S 2011. Regulation and Functions of the IL-10 family or cytokines in inflammation and disease. *Annual Review of Immunology,* 29**,** 71-109

.

PAPAGEORGIS, P. & STYLIANOPOULOS, T. 2015. Role of TGFβ in regulation of the tumor microenvironment and drug delivery (review). *International journal of oncology,* 46**,** 933-943.

PARK, J. Y., LEE, S. H., YOON, S. R., PARK, Y. J., JUNG, H., KIM, T. D. & CHOI, I. 2011. IL-15-induced IL-10 increases the cytolytic activity of human natural killer cells. *Mol Cells,* 32**,** 265-72.

PAUST, S., SENMAN, B. & VON ANDRIAN, U. H. 2010. Adaptive Immune Responses Mediated by Natural Killer Cells. *Immunological reviews,* 235**,** 286-296.

PEGRAM, H. J., ANDREWS, D. M., SMYTH, M. J., DARCY, P. K. & KERSHAW, M. H. 2011. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol,* 89**,** 216-224.

PICCIOLI, D., SBRANA, S., MELANDRI, E. & VALIANTE, N. M. 2002. Contact-dependent Stimulation and Inhibition of Dendritic Cells by Natural Killer Cells. *The Journal of Experimental Medicine,* 195**,** 335-341.

PINKOSKI, M. J., WATERHOUSE, N. J., HEIBEIN, J. A., WOLF, B. B., KUWANA, T., GOLDSTEIN, J. C., NEWMEYER, D. D., BLEACKLEY, R. C. & GREEN, D. R. 2001. Granzyme B-mediated Apoptosis Proceeds Predominantly through a Bcl-2-inhibitable Mitochondrial Pathway. *Journal of Biological Chemistry,* 276**,** 12060-12067.

POLI, A., MICHEL, T., THÉRÉSINE, M., ANDRÈS, E., HENTGES, F. & ZIMMER, J. 2009. CD56(bright) natural killer (NK) cells: an important NK cell subset. *Immunology,* 126**,** 458-465.

POLUKORT, S. H., ROVATTI, J., CARLSON, L., THOMPSON, C., SER-DOLANSKY, J., KINNEY, S. R. M., SCHNEIDER, S. S. & MATHIAS, C. B. 2016. IL-10 Enhances IgE-Mediated Mast Cell Responses and Is Essential for the Development of Experimental Food Allergy in IL-10-Deficient Mice. *Journal of immunology (Baltimore, Md. : 1950),* 196**,** 4865-4876.

QAYUM, A. A., PARANJAPE, A., ABEBAYEHU, D., KOLAWOLE, E. M., HAQUE, T. T., MCLEOD, J. J. A., SPENCE, A. J., CASLIN, H. L., TARUSELLI, M. T., CHUMANEVICH, A. P., BAKER, B., OSKERITZIAN, C. A. & RYAN, J. J. 2016. IL-10–Induced miR-155 Targets SOCS1 To Enhance IgE-Mediated Mast Cell Function. *The Journal of Immunology,* 196**,** 4457-4467.

R., K., EVA, K. & H., W. 1975. „Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *European Journal of Immunology,* 5**,** 112-117.

RANSON, T., VOSSHENRICH, C. A. J., CORCUFF, E., RICHARD, O., MÜLLER, W. & DI SANTO, J. P. 2003. IL-15 is an essential mediator of peripheral NK-cell homeostasis. *Blood,* 101**,** 4887-4893.

REDELL, M. S., RUIZ, M. J., ALONZO, T. A., GERBING, R. B. & TWEARDY, D. J. 2011. Stat3 signaling in acute myeloid leukemia: ligand-dependent and -independent activation and induction of apoptosis by a novel small-molecule Stat3 inhibitor. *Blood,* 117**,** 5701-5709.

REEFMAN, E., KAY, J. G., WOOD, S. M., OFFENHÄUSER, C., BROWN, D. L., ROY, S., STANLEY, A. C., LOW, P. C., MANDERSON, A. P. & STOW, J. L. 2010. Cytokine Secretion Is Distinct from Secretion of Cytotoxic Granules in NK Cells. *The Journal of Immunology,* 184**,** 4852-4862.

REGIS, S., CALIENDO, F., DONDERO, A., CASU, B., ROMANO, F., LOIACONO, F., MORETTA, A., BOTTINO, C. & CASTRICONI, R. 2017. TGF-β1 Downregulates the Expression of CX3CR1 by Inducing miR-27a-5p in Primary Human NK Cells. *Frontiers in Immunology,* 8.

RIVERA, J., PROIA, R. L. & OLIVERA, A. 2008. The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nature reviews. Immunology,* 8**,** 753-763.

RODRÍGUEZ-BAYONA, B., RAMOS-AMAYA, A., LÓPEZ-BLANCO, R., CAMPOS-CARO, A. & BRIEVA, J. A. 2013. STAT-3 Activation by Differential Cytokines Is Critical for Human In Vivo–Generated Plasma Cell Survival and Ig Secretion. *The Journal of Immunology,* 191**,** 4996-5004.

ROERS, A., SIEWE, L., STRITTMATTER, E., DECKERT, M., SCHLÜTER, D., STENZEL, W., GRUBER, A. D., KRIEG, T., RAJEWSKY, K. & MÜLLER, W. 2004. T Cell–specific Inactivation of the Interleukin 10 Gene in Mice Results in Enhanced T Cell Responses but Normal Innate Responses to Lipopolysaccharide or Skin Irritation. *The Journal of Experimental Medicine,* 200**,** 1289-1297.

ROMAGNANI, S. 2000. T-cell subsets (Th1 versus Th2). *Annals of Allergy, Asthma & Immunology,* 85**,** 21-18.

SANCHEZ-CORREA, B., BERGUA, J. M., PERA, A., CAMPOS, C., ARCOS, M. J., BAÑAS, H., DURAN, E., SOLANA, R. & TARAZONA, R. 2017. In Vitro Culture with Interleukin-15 Leads to Expression of Activating Receptors and Recovery of Natural Killer Cell Function in Acute Myeloid Leukemia Patients. *Frontiers in immunology,* 8**,** 931-931.

SANJABI, S., OH, S. A. & LI, M. O. 2017. Regulation of the Immune Response by TGF-β: From Conception to Autoimmunity and Infection. *Cold Spring Harbor Perspectives in Biology,* 9.

SARAIVA, M. & O'GARRA, A. 2010. The regulation of IL-10 production by immune cells. *Nat Rev Immunol,* 10**,** 170-181.

SATHE, P., DELCONTE, R. B., SOUZA-FONSECA-GUIMARAES, F., SEILLET, C., CHOPIN, M., VANDENBERG, C. J., RANKIN, L. C., MIELKE, L. A., VIKSTROM, I., KOLESNIK, T. B., NICHOLSON, S. E., VIVIER, E., SMYTH, M. J., NUTT, S. L., GLASER, S. P., STRASSER, A., BELZ, G. T., CAROTTA, S. & HUNTINGTON, N. D.

2014. Innate immunodeficiency following genetic ablation of Mcl1 in natural killer cells. *Nature Communications,* 5**,** 4539.

SCHMIDT, E. K., CLAVARINO, G., CEPPI, M. & PIERRE, P. 2009. SUnSET, a nonradioactive method to monitor protein synthesis. *Nature Methods,* 6**,** 275.

SCOTT, GINA B., BOWLES, PAUL A., WILSON, ERICA B., MEADE, JOSEPHINE L., LOW, BOON C., DAVISON, A., BLAIR, G. E. & COOK, GRAHAM P. 2010. Identification of the BCL2/adenovirus E1B-19K protein-interacting protein 2 (BNIP-2) as a granzyme B target during human natural killer cell-mediated killing. *Biochemical Journal,* 431**,** 423-431.

SCOTT, M. J., HOTH, J. J., TURINA, M., WOODS, D. R. & CHEADLE, W. G. 2006. Interleukin-10 suppresses natural killer cell but not natural killer T cell activation during bacterial infection. *Cytokine,* 33**,** 79-86.

SEOANE, J. & GOMIS, R. R. 2017. TGF-β Family Signaling in Tumor Suppression and Cancer Progression. *Cold Spring Harbor Perspectives in Biology,* 9.

SHARMA, S., STOLINA, M., LIN, Y., GARDNER, B., MILLER, P. W., KRONENBERG, M. & DUBINETT, S. M. 1999. T Cell-Derived IL-10 Promotes Lung Cancer Growth by Suppressing Both T Cell and APC Function. *The Journal of Immunology,* 163**,** 5020-5028.

SHIFRIN, N., RAULET, D. H. & ARDOLINO, M. 2014. NK cell self tolerance, responsiveness and missing self recognition. *Seminars in immunology,* 26**,** 138-144.

SHIOW, L. R., ROSEN, D. B., BRDIČKOVÁ, N., XU, Y., AN, J., LANIER, L. L., CYSTER, J. G. & MATLOUBIAN, M. 2006. CD69 acts downstream of interferon-α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature,* 440**,** 540-544.

SIEWE, L., BOLLATI–FOGOLIN, M., WICKENHAUSER, C., KRIEG, T., MÜLLER, W. & ROERS, A. 2006. Interleukin-10 derived from macrophages and/or neutrophils regulates the inflammatory response to LPS but not the response to CpG DNA. *European Journal of Immunology,* 36**,** 3248-3255.

SINCLAIR, L. V., BARTHELEMY, C. & CANTRELL, D. A. 2020. Single Cell Glucose Uptake Assays: A Cautionary Tale. *Immunometabolism,* 2**,** e200029-e200029.

SIVORI, S., FALCO, M., CHIESA, M. D., CARLOMAGNO, S., VITALE, M., MORETTA, L. & MORETTA, A. 2004. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: Induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 10116-10121.

SKAK, K., FREDERIKSEN, K. S. & LUNDSGAARD, D. 2008. Interleukin-21 activates human natural killer cells and modulates their surface receptor expression. *Immunology,* 123**,** 575-583.

SMITH, C.-J., ALLARD, D. E., WANG, Y., HOWARD, J. F., MONTGOMERY, S. A. & SU, M. A. 2018a. IL-10 Paradoxically Promotes Autoimmune Neuropathy through S1PR1-Dependent CD4⁺ T Cell Migration. *The Journal of Immunology,* 200**,** 1580-1592.

SMITH, L. K., BOUKHALED, G. M., CONDOTTA, S. A., MAZOUZ, S., GUTHMILLER, J. J., VIJAY, R., BUTLER, N. S., BRUNEAU, J., SHOUKRY, N. H., KRAWCZYK, C. M. &

RICHER, M. J. 2018b. Interleukin-10 Directly Inhibits CD8(+) T Cell Function by Enhancing N-Glycan Branching to Decrease Antigen Sensitivity. *Immunity,* 48**,** 299-312.e5.

SMYTH, M. J., CRETNEY, E., KELLY, J. M., WESTWOOD, J. A., STREET, S. E., YAGITA, H., TAKEDA, K., VAN DOMMELEN, S. L., DEGLI-ESPOSTI, M. A. & HAYAKAWA, Y. 2005. Activation of NK cell cytotoxicity. *Mol Immunol,* 42**,** 501-10.

STACEY, M. A., MARSDEN, M., WANG, E. C. Y., WILKINSON, G. W. G. & HUMPHREYS, I. R. 2011. IL-10 Restricts Activation-Induced Death of NK Cells during Acute Murine Cytomegalovirus Infection. *The Journal of Immunology,* 187**,** 2944-2952.

STETSON, D. B., MOHRS, M., REINHARDT, R. L., BARON, J. L., WANG, Z.-E., GAPIN, L., KRONENBERG, M. & LOCKSLEY, R. M. 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *The Journal of experimental medicine,* 198**,** 1069-1076.

STONIER, S. W. & SCHLUNS, K. S. 2010. Trans-presentation: a novel mechanism regulating IL-15 delivery and responses. *Immunology letters,* 127**,** 85-92.

STRENGELL, M., MATIKAINEN, S., SIREN, J., LEHTONEN, A., FOSTER, D., JULKUNEN, I. & SARENEVA, T. 2003. IL-21 in Synergy with IL-15 or IL-18 Enhances IFN- Production in Human NK and T Cells. *The Journal of Immunology,* 170**,** 5464-5469.

STUMPO, D. J., LAI, W. S. & BLACKSHEAR, P. J. 2010. Inflammation: cytokines and RNA-based regulation. *Wiley interdisciplinary reviews. RNA,* 1**,** 60-80.

SUDARSHAN, C., GALON, J., ZHOU, Y.-J. & O'SHEA, J. J. 1999. TGF-β Does Not Inhibit IL-12- and IL-2-Induced Activation of Janus Kinases and STATs. *The Journal of Immunology,* 162**,** 2974-2981.

TAKEUCHI, O. & AKIRA, S. 2010. Pattern Recognition Receptors and Inflammation. *Cell,* 140**,** 805-820.

TANG, L.-Y., HELLER, M., MENG, Z., YU, L.-R., TANG, Y., ZHOU, M. & ZHANG, Y. E. 2017. Transforming Growth Factor-β (TGF-β) Directly Activates the JAK1-STAT3 Axis to Induce Hepatic Fibrosis in Coordination with the SMAD Pathway. *Journal of Biological Chemistry,* 292**,** 4302-4312.

TANIKAWA, T., WILKE, C. M., KRYCZEK, I., CHEN, G. Y., KAO, J., NÚÑEZ, G. & ZOU, W. 2012. Interleukin-10 ablation promotes tumor development, growth, and metastasis. *Cancer research,* 72**,** 420-429.

THÄLE, C. & KIDERLEN, A. F. 2005. Sources of interferon-gamma (IFN-γ) in early immune response to Listeria monocytogenes. *Immunobiology,* 210**,** 673-683.

THOMAS, R. & YANG, X. 2016. NK-DC Crosstalk in Immunity to Microbial Infection. *Journal of immunology research,* 2016**,** 6374379-6374379.

TILG, H., VAN MONTFRANS, C., VAN DEN ENDE, A., KASER, A., VAN DEVENTER, S. J. H., SCHREIBER, S., GREGOR, M., LUDWICZEK, O., RUTGEERTS, P., GASCHE, C., KONINGSBERGER, J. C., ABREU, L., KUHN, I., COHARD, M., LEBEAUT, A., GRINT, P. & WEISS, G. 2002. Treatment of Crohn's disease with recombinant human interleukin 10 induces the proinflammatory cytokine interferon γ. *Gut,* 50**,** 191-195.

TRIFUNOVIĆ, J., MILLER, L., DEBELJAK, Ž. & HORVAT, V. 2015. Pathologic patterns of interleukin 10 expression--a review. *Biochemia medica,* 25**,** 36-48.

TROTTA, R., DAL COL, J., YU, J., CIARLARIELLO, D., THOMAS, B., ZHANG, X., ALLARD, J., 2ND, WEI, M., MAO, H., BYRD, J. C., PERROTTI, D. & CALIGIURI, M. A. 2008. TGF-beta utilizes SMAD3 to inhibit CD16-mediated IFN-gamma production and antibody-dependent cellular cytotoxicity in human NK cells. *Journal of immunology (Baltimore, Md. : 1950),* 181**,** 3784-3792.

TU, M. M., MAHMOUD, A. B. & MAKRIGIANNIS, A. P. 2016. Licensed and Unlicensed NK Cells: Differential Roles in Cancer and Viral Control. *Frontiers in immunology,* 7**,** 166-166.

TURVEY, S. E. & BROIDE, D. H. 2010. Innate immunity. *The Journal of allergy and clinical immunology,* 125**,** S24-S32.

UEMURA, A., TAKEHARA, T., MIYAGI, T., SUZUKI, T., TATSUMI, T., OHKAWA, K., KANTO, T., HIRAMATSU, N. & HAYASHI, N. 2009. Natural killer cell is a major producer of interferon γ that is critical for the IL-12-induced anti-tumor effect in mice. *Cancer Immunology, Immunotherapy,* 59**,** 453.

VAHL, J. M., FRIEDRICH, J., MITTLER, S., TRUMP, S., HEIM, L., KACHLER, K., BALABKO, L., FUHRICH, N., GEPPERT, C.-I., TRUFA, D. I., SOPEL, N., RIEKER, R., SIRBU, H. & FINOTTO, S. 2017. Interleukin-10-regulated tumour tolerance in non-small cell lung cancer. *British Journal Of Cancer,* 117**,** 1644.

VENDRAME, E., FUKUYAMA, J., STRAUSS-ALBEE, D. M., HOLMES, S. & BLISH, C. A. 2017. Mass Cytometry Analytical Approaches Reveal Cytokine-Induced Changes in Natural Killer Cells. *Cytometry Part B: Clinical Cytometry,* 92**,** 57-67.

VERMA, R., BALAKRISHNAN, L., SHARMA, K., KHAN, A. A., ADVANI, J., GOWDA, H., TRIPATHY, S. P., SUAR, M., PANDEY, A., GANDOTRA, S., PRASAD, T. S. K. & SHANKAR, S. 2016. A network map of Interleukin-10 signaling pathway. *Journal of Cell Communication and Signaling,* 10**,** 61-67.

VIEL, S., MARÇAIS, A., GUIMARAES, F. S.-F., LOFTUS, R., RABILLOUD, J., GRAU, M., DEGOUVE, S., DJEBALI, S., SANLAVILLE, A., CHARRIER, E., BIENVENU, J., MARIE, J. C., CAUX, C., MARVEL, J., TOWN, L., HUNTINGTON, N. D., BARTHOLIN, L., FINLAY, D., SMYTH, M. J. & WALZER, T. 2016. TGF-β inhibits the activation and functions of NK cells by repressing the mTOR pathway. *Science Signaling,* 9**,** ra19-ra19.

VITALE, M., CHIESA, M. D., CARLOMAGNO, S., PENDE, D., ARICÒ, M., MORETTA, L. & MORETTA, A. 2005. NK-dependent DC maturation is mediated by TNFα and IFNγ released upon engagement of the NKp30 triggering receptor. *Blood,* 106**,** 566-571.

VIVIER, E., ARTIS, D., COLONNA, M., DIEFENBACH, A., DI SANTO, J. P., EBERL, G., KOYASU, S., LOCKSLEY, R. M., MCKENZIE, A. N. J., MEBIUS, R. E., POWRIE, F. & SPITS, H. 2018. Innate Lymphoid Cells: 10 Years On. *Cell,* 174**,** 1054-1066.

VIVIER, E., RAULET, D. H., MORETTA, A., CALIGIURI, M. A., ZITVOGEL, L., LANIER, L. L., YOKOYAMA, W. M. & UGOLINI, S. 2011. Innate or adaptive immunity? The example of natural killer cells. *Science (New York, N.Y.),* 331**,** 44-49.

VOSKOBOINIK, I., WHISSTOCK, J. C. & TRAPANI, J. A. 2015. Perforin and granzymes: function, dysfunction and human pathology. *Nature Reviews Immunology,* 15**,** 388.

WAGNER, J. A., ROSARIO, M., ROMEE, R., BERRIEN-ELLIOTT, M. M., SCHNEIDER, S. E., LEONG, J. W., SULLIVAN, R. P., JEWELL, B. A., BECKER-HAPAK, M.,

SCHAPPE, T., ABDEL-LATIF, S., IRELAND, A. R., JAISHANKAR, D., KING, J. A., VIJ, R., CLEMENT, D., GOODRIDGE, J., MALMBERG, K.-J., WONG, H. C. & FEHNIGER, T. A. 2017. CD56bright NK cells exhibit potent antitumor responses following IL-15 priming. *The Journal of clinical investigation,* 127**,** 4042-4058.

WAGSTAFFE, H. R., NIELSEN, C. M., RILEY, E. M. & GOODIER, M. R. 2018. IL-15 Promotes Polyfunctional NK Cell Responses to Influenza by Boosting IL-12 Production. *Journal of immunology (Baltimore, Md. : 1950),* 200**,** 2738-2747.

WALLIN, R. P. A., SCREPANTI, V., MICHAËLSSON, J., GRANDIEN, A. & LJUNGGREN, H.-G. 2003. Regulation of perforin-independent NK cell-mediated cytotoxicity. *European Journal of Immunology,* 33**,** 2727-2735.

WALTER, M. R. 2014. The Molecular Basis of IL-10 Function: From Receptor Structure to the Onset of Signaling. *Current topics in microbiology and immunology,* 380**,** 191-212.

WAN, C.-K., ANDRASKI, A. B., SPOLSKI, R., LI, P., KAZEMIAN, M., OH, J., SAMSEL, L., SWANSON, P. A., MCGAVERN, D. B., SAMPAIO, E. P., FREEMAN, A. F., MILNER, J. D., HOLLAND, S. M. & LEONARD, W. J. 2015. Opposing roles of STAT1 and STAT3 in IL-21 function in CD4⁺ T cells. *Proceedings of the National Academy of Sciences,* 112**,** 9394-9399.

WANG, K. S., RITZ, J. & FRANK, D. A. 1999. IL-2 Induces STAT4 Activation in Primary NK Cells and NK Cell Lines, But Not in T Cells. *The Journal of Immunology,* 162**,** 299-304.

WANG, W., MENG, M., ZHANG, Y., WEI, C., XIE, Y., JIANG, L., WANG, C., YANG, F., TANG, W., JIN, X., CHEN, D., ZONG, J., HOU, Z. & LI, R. 2014. Global transcriptome-wide analysis of CIK cells identify distinct roles of IL-2 and IL-15 in acquisition of cytotoxic capacity against tumor. *BMC Medical Genomics,* 7**,** 49.

WEBER-NORDT, R. M., RILEY, J. K., GREENLUND, A. C., MOORE, K. W., DARNELL, J. E. & SCHREIBER, R. D. 1996. Stat3 Recruitment by Two Distinct Ligand-induced, Tyrosine-phosphorylated Docking Sites in the Interleukin-10 Receptor Intracellular Domain. *Journal of Biological Chemistry,* 271**,** 27954-27961.

WILSON, E. B., EL-JAWHARI, J. J., NEILSON, A. L., HALL, G. D., MELCHER, A. A., MEADE, J. L. & COOK, G. P. 2011. Human Tumour Immune Evasion via TGF-β Blocks NK Cell Activation but Not Survival Allowing Therapeutic Restoration of Anti-Tumour Activity. *PLOS ONE,* 6**,** e22842.

WU, Y., TIAN, Z. & WEI, H. 2017. Developmental and Functional Control of Natural Killer Cells by Cytokines. *Frontiers in Immunology,* 8.

XUE, H., LIN, F., TAN, H., ZHU, Z.-Q., ZHANG, Z.-Y. & ZHAO, L. 2016. Overrepresentation of IL-10-Expressing B Cells Suppresses Cytotoxic CD4(+) T Cell Activity in HBV-Induced Hepatocellular Carcinoma. *PLoS ONE,* 11**,** e0154815.

YOSHIMURA, A., WAKABAYASHI, Y. & MORI, T. 2010. Cellular and molecular basis for the regulation of inflammation by TGF-β. *The Journal of Biochemistry,* 147**,** 781-792.

YU, J., WEI, M., BECKNELL, B., TROTTA, R., LIU, S., BOYD, Z., JAUNG, M. S., BLASER, B. W., SUN, J., BENSON, D. M., JR., MAO, H., YOKOHAMA, A., BHATT, D.,

SHEN, L., DAVULURI, R., WEINSTEIN, M., MARCUCCI, G. & CALIGIURI, M. A. 2006. Pro- and Antiinflammatory Cytokine Signaling: Reciprocal Antagonism Regulates Interferon-gamma Production by Human Natural Killer Cells. *Immunity,* 24**,** 575-590.

ZAIATZ-BITTENCOURT, V., FINLAY, D. K. & GARDINER, C. M. 2018. Canonical TGF-β Signaling Pathway Represses Human NK Cell Metabolism. *The Journal of Immunology***,** ji1701461.

ZANONI, I., SPREAFICO, R., BODIO, C., DI GIOIA, M., CIGNI, C., BROGGI, A., GORLETTA, T., CACCIA, M., CHIRICO, G., SIRONI, L., COLLINI, M., COLOMBO, MARIO P., GARBI, N. & GRANUCCI, F. 2013. IL-15 cis Presentation Is Required for Optimal NK Cell Activation in Lipopolysaccharide-Mediated Inflammatory Conditions. *Cell Reports,* 4**,** 1235-1249.

ZARCONE, D., TILDEN, A. B., FRIEDMAN, H. M. & GROSSI, C. E. 1987. Human Leukemia-derived Cell Lines and Clones as Models for Mechanistic Analysis of Natural Killer Cell-mediated Cytotoxicity. *Cancer Research,* 47**,** 2674-2682.

ZENG, R., SPOLSKI, R., CASAS, E., ZHU, W., LEVY, D. E. & LEONARD, W. J. 2007. The molecular basis of IL-21–mediated proliferation. *Blood,* 109**,** 4135-4142.

ZHOU, J.-H., BROUSSARD, S. R., STRLE, K., FREUND, G. G., JOHNSON, R. W., DANTZER, R. & KELLEY, K. W. 2001. IL-10 Inhibits Apoptosis of Promyeloid Cells by Activating Insulin Receptor Substrate-2 and Phosphatidylinositol 3′-Kinase. *The Journal of Immunology,* 167**,** 4436-4442.

ZHOU, L. & SONNENBERG, G. F. 2018. Essential immunologic orchestrators of intestinal homeostasis. *Science Immunology,* 3**,** eaao1605.

ZHU, L., SHI, T., ZHONG, C., WANG, Y., CHANG, M. & LIU, X. 2017. IL-10 and IL-10 Receptor Mutations in Very Early Onset Inflammatory Bowel Disease. *Gastroenterology research,* 10**,** 65-69.

ZHU, S., PHATARPEKAR, P. V., DENMAN, C. J., SENYUKOV, V. V., SOMANCHI, S. S., NGUYEN-JACKSON, H. T., MACE, E. M., FREEMAN, A. F., WATOWICH, S. S., ORANGE, J. S., HOLLAND, S. M. & LEE, D. A. 2014. Transcription of the activating receptor NKG2D in natural killer cells is regulated by STAT3 tyrosine phosphorylation. *Blood,* 124**,** 403-411.