Adenovirus vectors for manipulating human immune cells

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1 Preface

1.1 Acknowledgements

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1.2 Abstract

Natural Killer (NK) cell mediated immune surveillance is important in preventing and controlling malignancies. However, tumours ultimately evade NK cells, enhancing their survival and progression. The immunosuppressive cytokine TGF-β is an established, potent inhibitor of NK cell mediated anti-tumour immunity. Genetically manipulating NK cells to resist the actions of TGF-β is a potential route by which to enhance NK cell-mediated immunotherapy. However, NK cells are notoriously difficult to manipulate with conventional viral vectors or transfection techniques and alternative methodologies are required to achieve this. I have explored the ability of several virus vectors to transduce primary human NK cells, with a chimaeric adenovirus (Ad) vector proving the most promising. Replacing the Ad5 fibre with that from Ad35 (forming Ad5f35) generated a vector capable of efficient transduction of primary human NK cells and the NK cell lines, YT, NKL and NK92. Ad5F35 utilises CD46 as an entry receptor and NK cell transduction by Ad5f35 was CD46 dependent.

The Ad5f35 vector provides a route to genetically manipulate NK cells. Transfection experiments in non-lymphoid cells showed that expression of a dominant negative TGF-β receptor II or inhibitory SMADs (SMAD7) inhibit the TGF-β signalling pathway. Using recombination-based methods in *E.coli*, an Ad5f35 vector was constructed to deliver the dominant negative TGF-β receptor II into mammalian cells. High expression and inhibitory activity was achieved in non-lymphoid cells, but expression in NK cells was low and activity reduced. Nevertheless, the Ad5f35 system clearly has potential for future applications in NK cells, including the development of NK cell based cellular therapies.

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1.6 Abbreviations

°C	Degrees Celsius
AAV	Adenovirus-Associated Virus
Ad(s)	Adenovirus(es)
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADP	Adenovirus Death Protein
AKT	Protein kinase B
AP1	Activator protein 1
AP2	Activator protein 2
APC	Antigen presenting cell
ApC-Cy7	Allophycocyanin-Cyanine 7
APS	Ammonium persulfate
ATCC	American Type Culture Collection
ATP	Adenine Triphosphate
BAC(s)	Bacterial Artificial Chromosome(s)
BSA	Bovine serum albumin
CAR	Coxsackie B and Adenvirus Receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
CRISPR/Cas9	Clustered Regularly Interspaced Short Palindromic Repeats--associated protein-9 nuclease
CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4 ; CD152
DAI	DNA-dependent activator of interferon regulatory factor
DAP10	DNAX activation protein of 10kDa
DAP12	DNAX activation protein of 12kDa
DBD	DNA-binding domain
DBP	DNA-binding protein
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DNAM-1	DNAX accessory molecule-1, CD226
DNRII	Dominant negative transforming growth factor beta receptor II
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green-Fluorescent Protein
ECACC	European Collection of Authentic Cell Cultures
eIF-4	Eukaryotic initiation factor 4
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	Foetal bovine serum
FcRs	Constant region receptors
FCS	Foetal calf serum

FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GFP	Green-fluorescent protein
GFU	Green-fluorescent units
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hip1	Huntingtin-interacting protein 1
HLA	Human leukocyte antigen
HSCs	Hematopoietic stem cells
HSP	Heat shock protein
HSPGs	Heparan sulphate proteoglycans
HSV	Herpes Simplex Virus
ICAM1	Intercellular adhesion molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IFNγ	Interferon gamma
IFNα	Interferon alpha
IFNβ	Interferon beta
Ig	Immunoglobulin
IL	Interleukin
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITR	Inverted Terminal Repeats
JAK	Janus-associated kinases
JNK	Jun N-terminal Kinase
kDa	Kilodalton
KIR	killer cell immunoglobulin receptor
LAK	Lymphokine-activated killer
LAMP-1	Lysosomal-associated membrane protein 1; CD107a
M	Molar
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid-derived suppressor cell
mg	Milligram
MH1	MAD homology 1
MH2	MAD homology 2
MHC	Major histocompatibility complex
MIC A	MHC class I polypeptide-related sequence A
MIC B	MHC class I polypeptide-related sequence B
mL	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
mRNA	Messenger RNA
MTOC	Microtubule organising centre
mTOR	Mammalian target of rapamycin

MV	Measles virus
MVA	Modified vaccinia virus
n.s	Not significant
NCR	Natural cytotoxicity receptor
NF	Nuclear Factor
ng	Nanogram
NICE	National Institute for Health and Care Excellence
NK	Natural killer
NKG2D	natural-killer group 2, member D
NKp30	Natural cytotoxicity triggering receptor 3; NCR3, CD337
NKp44	Natural cytotoxicity triggering receptor 1; NCR2, CD336
NKp46	Natural cytotoxicity triggering receptor 1; NCR1, CD335
NLR	NOD-Like Receptor
NSCLC	Non-small cell lung cancer
NTD	N-terminal transactivation domain
oHSV	Oncolytic Herpes simplex virus
ORFs	Open-reading frame
OS	Overall survival
PAK-1	p21-activated kinase
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Solution
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PD-1	Programmed cell death protein 1; CD279
PE	R-Phycoerythrin
PFU	Plaque Forming Unit
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol-3,4-bisphosphate
PKA	Protein Kinase A
PKR	Protein Kinase R
Pol	Polymerase
PRR	Pattern-recognition receptor
PVDF	Polyvinylidene difluoride
RB	Retinoblastoma
RGD	Arganine-Glycine-Aspartic Acid
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
s.d	Standard deviation
SDS	Sodium Dodecyl Sulphate
SHP1	Src homology region 2 domain-containing phosphatase-1
SHP2	Src homology region 2 domain-containing phosphatase-2
SMAD	homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the *Caenorhabditis elegans* protein Sma
SP	Serine Protease

SSC	Side scatter
STAT	Signal transducer and activator of transcription
SYK	Spleen tyrosine kinase
TAA	Tumour associated antigen
TCR	T-cell receptor
tdnTGFβRII	Truncated Dominant Negative Transforming growth factor beta receptor
TGF-β	Transforming growth factor beta
TGFβR	Transforming growth factor beta receptor
TLR	Toll-Like Receptor
TNF	Tumour necrosis factor
TP	Terminal Protein
TPL	tripartite leader sequence.
TRAIL	TNF-related apoptosis-inducing ligand
TRAPP	Transformation/transcription domain-associated protein
Tregs	Regulatory T cells
ULBP1	UL16 binding protein 1
UTR	Untranslated Region
v/v	Volume per volume
VEGF	Vascular endothelial growth factor
w/v	Weight per volume
WASp	Wiskott–Aldrich Syndrome protein
WHO	World Health Organisation
wt	Wild type
ZAP70	Zeta-chain-associated protein kinase 70
μl	Microlitre
μM	Micromolar

2   Introduction

2.1   Immunosurveillance of cancer

The concept that the immune system can recognise and eradicate cancer cells was controversial for many years. This controversy stemmed largely from experiments performed using mouse strains defective in immune components (e.g. the reduced number of T cells in *nude* mice); these strains showed little difference to wild type mice in their responses to tumours. However, it was later realised that these mouse strains retained immune activity and were not as defective as originally believed. It was the development of mouse gene knock-out technology that provided the first clear evidence of the importance of the immune system in controlling tumours; targeted deletion of specific genes allowed the role of individual genes, cells and components of the immune system to be tested in an unequivocal manner (1). The role of the immune system in controlling cancer is now widely accepted (2–4) and the ability of a tumour to evade immunity is recognised as a hallmark of cancer (5).

Generating an adaptive immune response toward tumours is largely dependent upon recognising cancers antigens, which first relies upon the innate immune activation that leads to the priming of the adaptive immune cells with cancer antigens (6). Natural killer (NK) cells, a member of the innate immune system, initially recognise and kill transformed cells, this leads to tumour cell death and subsequent processing of their cellular fragments by antigen presenting cells (7), such as dendritic cells. Activated dendritic cells present the cancer antigens to T and B cells. In turn, this leads to a tumour specific immune response from T and B cells, by secretion of cytokines to further promote activation of both innate and adaptive immunity, producing antibodies and cytotoxic T cells (7). Together, the response from both innate and adaptive elements of the immune system leads to the elimination of tumour cells. However, the immune system's ability to eliminate emerging tumours can become compromised, with surviving tumours evolving with mutations that can evade an immune response, a process known as immunoediting (8). The current understanding of the immune system within the tumour microenvironment is now translating into new therapeutic strategies that exploit the immune system to eradicate cancer cells.

2.1.1 Immunogenicity of tumours

Speculation that the immune system had a role in monitoring cells for tumorigenesis was outlined in 1957 by Burnet (9), and together with Lewis Thomas (10), immunosurveilance was first articulated. Although, the concept was initially controversial, it is now accepted that transformed cells are under constant control of the immune system. Congenital defects of the immune system or acquired immunodeficiency in humans are often implicated in higher risks of tumour development (11), highlighting the importance of the immune system in this context. In particular, mice with deficiencies of CD8 cytotoxic T cell or NK cell associated proteins are susceptible to the development of tumours (12–14), which supports the argument that both innate and adaptive elements of the immune system are important in tumour immunosurveillance. Furthermore, clinical studies found that tumour biopsies with infiltrated CD8 T and NK cells have a better prognosis in patients, for example in ovarian, colon and gastric cancers (15). This study identified the importance of the immune system in tumour immunosurveillance, and as such, the number of transformed cells that never form detectable tumour is unknown. Consequently, tumours that arise as a clinically detectable entity have evolved mechanisms to evade the immune system, which has recently been described as a hallmark in cancer development (5).

The concept of immunosurveillance feeds into the notion of immunoediting, where emerging tumours that escape immunosurveillance control the characteristics and progression of cancer. It has been proposed that this process is composed of three phases: elimination, equilibrium and escape, which has recently been reviewed (16,17). The elimination phase is the initial phase in which tumour immunosurveillance is active and, if successful, the tumour cells are eliminated (4). The equilibrium stage is the phase where some tumour cells survive the elimination phase due to genetic variations that allow them to escape immune cell destruction, this is a sign of selection and tumour evolution. These surviving tumour cells may remain in a functional state of dormancy, in which the adaptive immune system prevents excessive tumour outgrowth, resulting in a stage of equilibrium. This phase was evident in one study where T cells primed with tumour antigen arrested pancreatic tumour growth in mice by inducing an interferon (IFN) and tumour necrosis factor (TNF) response (18). However, this stage allows the tumour cells to accumulate and shapes the immunogenicity of tumour cells. The final stage of immunoediting is

escape, this phase is an accumulation of genetic changes that results in an altered response in reducing immune recognition and cytotoxicity and/or increased immunosuppressive effects. For example, a well-established change in this phase is the loss of major histocompatibility complex (MHC) class I proteins, which prevents the presentation of tumour associated antigens to T cells (19). Tumours also establish an immunosuppressive microenvironment by producing cytokines, such as TGF-β (20) and enzymes such as indoleamine 2,3-dioxygenase (IDO) (21) which downregulate the immune response. Thus, the stages of immunoediting are critical in determining the course of tumour progression. Importantly, immune recognition does not necessarily result in tumour eradication. A major factor in determining the immune system response to a tumour is the accumulation of mutations over a period of time; this genetic instability underlies many of the hallmarks of cancer (22).

2.2 Natural killer cells

Natural killer cells were discovered in 1975 by their innate ability to lyse tumour cells (23,24). They constitute ~10% of lymphocytes in human peripheral blood and are derived from $CD34^+$ haematopoietic progenitor cells (HPCs). Human NK cells are characteristically defined as $CD56^+CD3^-$ cells; CD56 is also expressed on NK-like T (NKT) cells but these cells are distinctively $CD3^+$. The activating receptor NKp46 (encoded by the NCR1 gene) is also used to discriminate NK cells. In both humans and mice, NK cells participate in the early control against viral infection and tumour immunosurveilance (25,26). Unlike T and B cells, NK cells do not express antigen specific receptors, but instead have innate reactivity determined by a combination of activating and inhibitory receptors (27). Their ability to distinguish tumour cells from healthy cells is regulated through the balance of signals transduced via this repertoire of receptors. These receptors engage with MHC Class I, MHC Class I-like molecules and many other ligands to determine the NK cell response (4). Critically, NK cell mediated killing of healthy cells is prevented through the engagement of inhibitory receptors, which include Killer Immunoglobulin-like Receptors (KIRs) in humans or Lectin-like Ly49 in mice that recognise 'self'-MHC Class I molecules (28). The 'loss of self' MHC Class I molecules is observed in some virus-infected and transformed cells for the purpose of evading a response from CD8 T cells, therefore these MHC class I deficient targets become susceptible to NK cell killing. This process is known as the 'missing self' hypothesis (29). Thus, NK cells have been shown to kill pre-malignant cells and contribute to cancer immunosurveillance (Figure 2-1). Tumours depleted of MHC Class I, or those which upregulate activating ligands are rejected by NK cells, and enhancing NK cell activity using cytokines result in tumour elimination in mice (30–32). Other studies have shown that NK cell depletion in mice lead to more aggressive and metastatic tumour growth (13,33). Similarly in humans, a study assessed NK cell activity over a 11 year period and found that low NK cell activity was associated with increased cancer risk (34). Collectively, these studies highlight the importance of NK cells in cancer immunosurveillance.
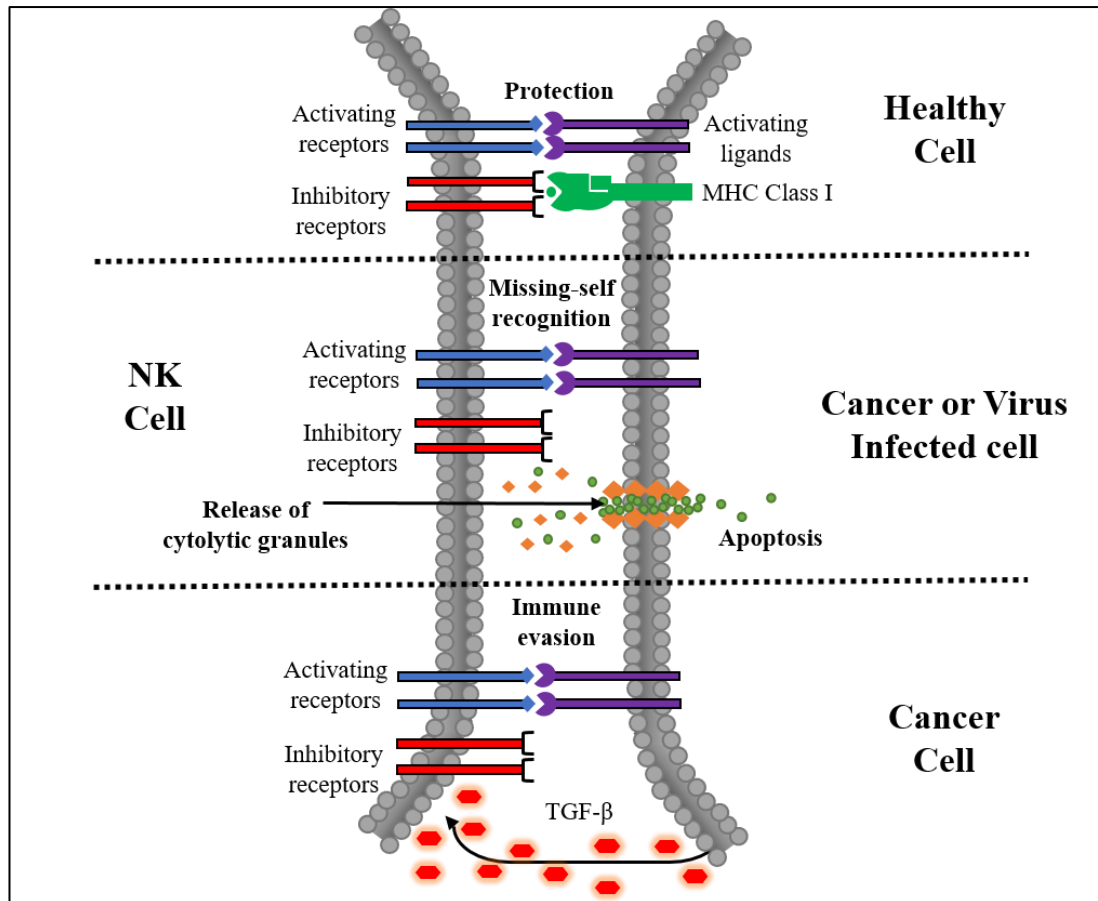
Figure 2-1 An overview of the mechanisms of target recognition by NK cells.

NK cells are regulated by the balance of signals transduced through their activating and inhibitory receptors. In healthy cells, the transduction of inhibitory signals are induced by the expression of MHC Class I ligands. Upon transformation or infection by virus (e.g. Adenovirus serotype 12), the expression of MHC Class I ligands are lost, thereby evading a T cell response. Stress pathways in abnormal cells also upregulates the expression of activating ligands (e.g. DNA damage induces expression of NKG2D ligands). When MHC class I levels are low and activating ligands are high the balance of signals transduced in NK cells will shift towards NK cell activation. Importantly, cancer cells exploit mechanisms to evade NK cell activation, e.g. by secreting immunosuppressive cytokines, such as TGF-β. Cancer cells might also upregulate inhibitory ligands and down-regulate activatory ligands to evade NK cell detection.

2.2.1 Receptors controlling natural killer cell activation

In humans, several NK cell receptors have been identified as important in tumour recognition; these include NKp46, NKp30, NKp44, DNAM1 and NKG2D (35), as well as others (Table 2-1). Upon activation, they trigger cytolytic activity, and the secretion of cytokines such as TNF-α and IFN-γ. Several NK cell activating receptors (except NKG2D and DNAM-1) signal through immunoreceptor tyrosine activating motifs (ITAMs) in the form of an associated molecule, such as disulphide linked homodimers of CD3ζ (36,37). These include the Natural Killer cytotoxicity receptors (NCRs): NKp30, NKp44 and NKp46 which have a key role in triggering activation through the binding of virus and tumour ligands. Upon activation, effector proteins are recruited to the phosphorylated tyrosine residue of the receptor to initiate the signalling cascade, this includes spleen tyrosine kinases (syk) and zeta chain associated protein kinase 70 (ZAP70) (Figure 2-2). The subsequent signalling pathways leads to NK cell cytotoxic granule exocytosis (degranulation) and the transcription of cytokine and chemokine genes. In contrast, the activating receptor, NKG2D, uses DAP-10 or -12, the former signals through an ITAM, whereas the latter binds to Grb2 and p85 and signals through phosphatidylinositol-3 kinase (PI3K) (38,39). The end result differs, with DAP-10 signalling resulting in cytotoxicity and DAP-12 signalling resulting in cytotoxicity and cytokine secretion (40,41). The receptor NKG2D plays a key role in recognising tumour cells as shown in a study in mice lacking NKG2D, which are more susceptible to tumours (12). The NKG2D molecule recognises multiple ligands that are induced on target cells by cell stress pathways, such as DNA damage or sustained proliferation (42). When tumour cells express the NKG2D ligands MICA and MICB and members of the ULBP family, they are recognised by NKG2D and favour NK cell activation. However, whether NK cell activating receptors trigger NK cell activity depends on the counter signals delivered by the inhibitory receptors (43).

Other ligands that bind to activating receptors are described in Table 2-1. The potency of activation might also differ between receptors, for example CD16 signalling through FCγR will activate NK cells, however NKp30 and Nkp46 signalling via FcγR requires co-activation with other activating receptors (44,45). In contrast, inhibitory receptors possess an immunoreceptor tyrosine inhibitory motif (ITIM) domain in their cytoplasmic portion, that upon phosphorylation recruit and activate SHP-1 and -2, Src homology 2 domain containing phosphatases, to prevent NK cell activation (Figure

2-2). Receptors that possess an ITIM domain include inhibitory KIRs which recognise cells expressing MHC class I molecules (Table 2-1) (44). However, NK cell activation can still occur if there is a sufficient amount of stimuli from activating ligands. This is indicative of a threshold in NK activation for efficient detection of target cells, however it also provides an opportunity for tumours to develop cell surface phenotypes to evade NK cell activation (46).

Table 2-1 Major NK cell receptors and ligands

Adapted from (47)

Inhibitory Receptors	Ligands	
KIR2DL1 (CD158a)	Group 2 HLA-C	
KIR2DL2/3 (CD158b1/2)	Group 1 HLA-C (In addition to: HLA-C and some HLA-B from group 2)	
KIR3DL1 (CD158e1)	HLA-Bw4	
NKG2A (CD159A)/CD94	HLA-E	
ILT family	HLA-G	
KLRG1	E and N-cadherin	
NKR-P1A (CD161)	LLT1	
NKR-P1B, NKR-P1D	Ocil/Clr-b	
PD-1	PD-L1/2	
Activating Receptors	**ITAM molecule**	**Ligands**
NKp46 (NCR1; CD335)	CD3ζ	HSPG, heparin
NKp30 (NCR3; CD337)	CD3ζ	B7-H6, BAT3, HSPG
FcγRIIIA (CD16)	CD3ζ/FcγR	Fc of human IgG immune complexes
NKG2D (CD314)	DAP12/DAP10	Human: MICA/B, ULBP1/2/3/4/5/6
KIR2DS1 (CD158h)	DAP12	HLA-C2
DNAM-1 (CD226)	Unknown mechanism	Nectin-2, PVR
NKG2C (CD159C)	DAP12/CD94	HLA-E
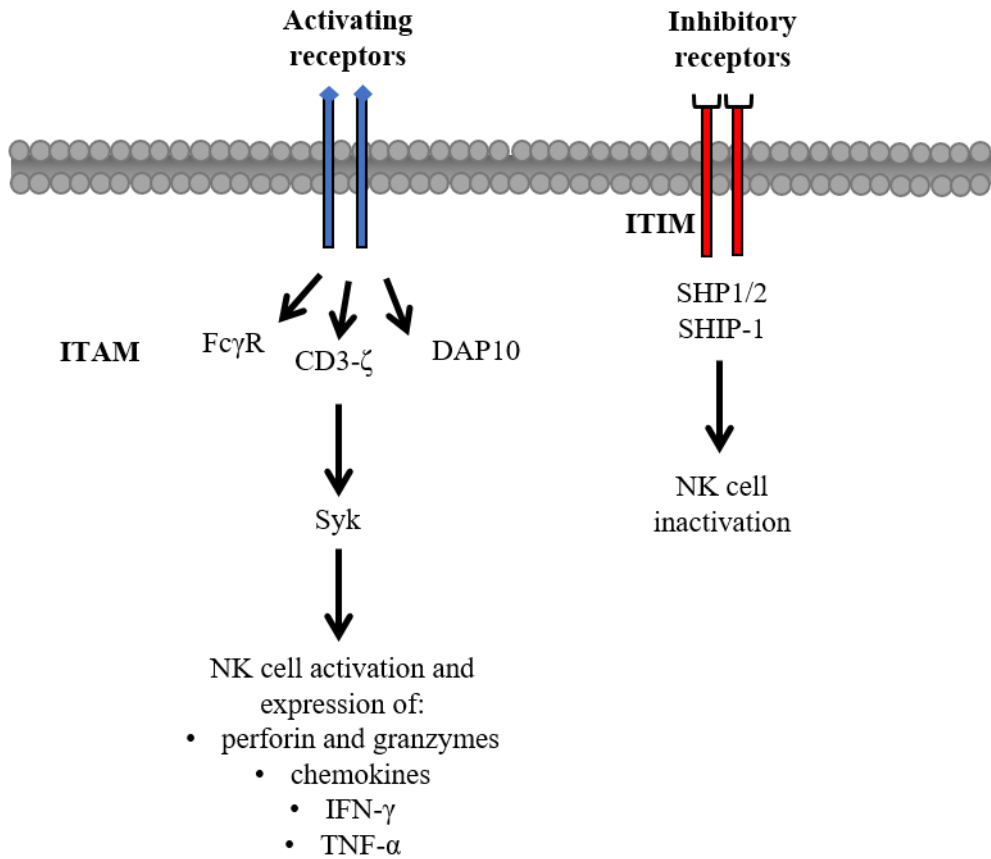
Figure 2-2 Regulation of NK cell response by activating and inhibitory receptors.

Upon engagement of activating receptor, the tyrosine residue(s) in the adaptor protein ITAM motif are phosphorylated. Phosphorylated ITAMs recruit the kinase Syk, which leads to further downstream signals and subsequent NK cell activation and gene expression. The engagement of inhibitory receptors leads to the phosphorylation of their cytoplasmic ITIM tyrosine and the recruitment and activation of the phosphatase SHP-1. In a simple model, SHP-1 dephosphorylates signalling intermediates downstream of the activating receptors, thereby preventing activation. However, there is little consensus on the precise mechanism.

2.2.2   Natural killer cells and cytokines

The balance of inhibitory and activating signals that are transduced in NK cells determines their response to target cells. In the event that activating signals are transduced, the signalling cascade results in degranulation of cytotoxic proteins and transcription of cytokine and chemokine genes. Hence, NK cells are major producers of pro-inflammatory cytokines such as IFN-γ, TNF-α and GM-CSF, as well as immunosuppressive cytokines such as IL-10 (35). Cytokines play a critical role in regulating an immune response in maintaining homeostasis, differentiation and tolerance. Principally, NK cells are potent producers of IFN-γ which directly modulates a response from the adaptive immune system and have anti-proliferative effects on transformed cells (48,49). Bystander cells are protected from NK cell mediated cytotoxicity due to IFN-γ mediated upregulation of MHC class I expression and antigen processing that protect healthy cells from immune cytotoxicity (50). NK cells also secrete TNF-α which also plays a role in the anti-tumour immune response, for example by allowing extravasation of immune cells into tissues and by driving dendritic cell maturation (51). As well as being regulated by their repertoire of activating and inhibitory receptors NK cells also are also regulated by many cytokines, including IL-2, IL-12, IL-15, IL-18, IL-21 and type I and type II IFN (52). Under physiological conditions, activated T cells produce IL-2 and this activates NK cells (53). The IL-2 signal cascade is mediated through Jak1/3 and STAT3/5 in NK cells, which results in increased IFN-γ secretion, cytotoxicity and promotes both survival and proliferation (54,55). Similar to IL-2, IL-15 signals through Jak1/3 and STAT1/3/5 to induce NK cell activation and proliferation (56). The metabolic regulator, mTOR has recently been identified as a participating component of IL-15 signalling (57). The cytokine IL-15 is unusual in that it is presented to NK cells by other cells (rather than being secreted). Under physiological conditions, IL-15 is *trans*-presented by activated macrophages and dendritic cells to the IL-15 receptor on NK cells which activates mTOR and the tyrosine kinases JAK1/3 that co-activate the transcription factor STAT5; activated STAT5 induces expression of genes that regulate proliferation and cytotoxicity (53). A number of studies have been carried out to synthetically enhance NK cells in patients (and mice) using cytokine treatment, resulting in improved anti-tumour immunity (58,59).

2.2.3 Mechanism of natural killer induced cell death

NK cells store cytotoxic proteins (granzymes and perforin) within acidic secretory lysosomes (60). These lytic granules are exocytosed upon triggering by target cells, this causes release of a group of proteases (granzymes) that induce apoptosis on target cells (61,62). This process is first initiated upon target cell recognition via NK cell receptors, NK cells form an immunological synapse that is catalysed by the interactions of their adhesion molecules, such as intracellular adhesion molecule 1 (ICAM1). This synapse forms a point of contact of NK cell receptor signalling with the target cell which becomes the focal point of where the lytic granules are exocytosed (63). The polarisation of actin and cytoskeletal rearrangement to the lytic synapse site is also critical for exocytosis of the lytic proteins. Patients with the immune cell disorder Wiskott-Aldrich syndrome have a mutation in a gene encoding WASp which is fundamental for the rearrangement of actin around the lytic synapse, and these individuals have a reduced ability to kill target cells (64). Once the lytic synapse has formed the lytic granules polarise towards the site of exocytosis, which is co-ordinated by the arrangement of the actin cytoskeleton and proteins that facilitate its transport. Patients with mutations in the cargo adapter, AP-3, which has a role in protein sorting targeted to lysosomes and clathrin recruitment, correspond to reduced cytotoxicity, suggesting that transportation is also critical in the exocytosis of lytic granules (65). When the lytic granules have been transported to the lytic synapse site, the lysosome anchors and fuses to the plasma membrane. This process is catalysed by N-ethylmaleimide-sensitive factor protein receptors which are anchored to the membrane and form a helical bundle that initiates fusion (66). This fusion releases perforin and granzymes at a neutral pH that results in their activation upon the target cells that is within close proximity (Figure 2-3).

The neutral pH environment and increase in calcium in the extracellular environment promotes perforin activation and binding to the membrane of target cells. Perforin forms a multimeric pore, which has been shown by electron microscopy (67). The assembly of the perforin pore facilitates the transport of granzymes into the target cells (67). Granzymes are a family of serine proteases, 11 have been described in mice and five in humans (granzyme A, B, H, K and M). The most abundant are granzymes A and B; granzymes A has trypsin-like activity and granzyme B is an aspase with similar specificity to caspases. Upon cell entry, granzymes activate apoptosis associated substrates (including caspases) to initiate programmed cell death (68,69).

Granzyme B also plays a critical role in triggering apoptopic cell death and is known to cleave the Bcl-2 family member Bid, a pro-apoptopic protein, which leads to the permeabilisation of the mitochondrial membrane to allow the escape of cytochrome C for the assembly of the apoptosome and the subsequent initiation of apoptosis (70). Overexpression of Bcl-2 proteins has been shown to have a protective effect against granzyme B induced apoptosis (71), showing that cells can overcome the apoptotic induced signals. NK cells are also able to use death receptors such as Fas, TNF-related apoptosis induced ligand (TRAIL) and other related pathways that induce apoptosis in the target cell (72). They also have the ability to detect antibody-coated cells through their FcγRIIIA (CD16) cell surface receptor, which mediates antibody-dependent cell cytotoxicity (ADCC) (28). Importantly, NK cell mediated ADCC is one mechanism by which therapeutic antibodies such as Herceptin (for breast cancer) exert their effects.

2.2.3.1 Death receptor induced cell death

The expression of TNF family molecules by NK cells plays an important role in mediating apoptosis, as well as regulating the immune system. TRAIL (also known as TNF-related apoptosis-inducing ligand) is a cytokine that belongs to the TNF family of cytokines and binds to TRAIL receptors (TRAIL-R and TRAIL-R2) to induce apoptotic signals (73). TRAIL is highly expressed on NK cells when stimulated with activating cytokines, such as IL-2, IL-15 and IFNs; NK cells mediate cell death on TRAIL sensitive tumour targets, one study demonstrating this in a metastatic liver model in mice (74). A number of TRAIL receptors do not transduce apoptotic signals and are considered to have a protective feedback role to regulate TRAIL mediated apoptosis (the so-called decoy receptors). In addition, NK cells can upregulate the expression of death receptors on targets and enhance their susceptibility to death ligands. For example, NK cells express FasL and can induce Fas expression on tumour targets via IFN-γ secretion, which leads to Fas induced cell death (75). This has been demonstrated in mice, showing that tumour metastasis is prevented through FasL expression on NK cells (76). Cell death is induced via the proteolytic activation of caspases which initiates the caspase cascade to induce apoptosis (Figure 2-3).

2.2.3.2 Antibody dependent cellular cytotoxicity (ADCC)

NK cells also expresses FcγRIIIA (CD16) receptors which binds to the Fc portion of human immunoglobulins (77). Upon binding to the receptor, the ITAM motif in the

CD3ζ and FcγR chains are phosphorylated, which generates an activating signaling cascade through PI3K, NF-κb and ERK pathways. This leads to a pro-inflammatory cytokine response and NK cell degranulation to kill targets, as described previously. ADCC can mediate anti-tumor activity through recognizing tumor specific antibodies, associated with CD16 receptor expression (Figure 2-3). Therapeutic monoclonal antibodies targeting tumor antigens are a current clinical tool to mediate an anti-tumor immune response.
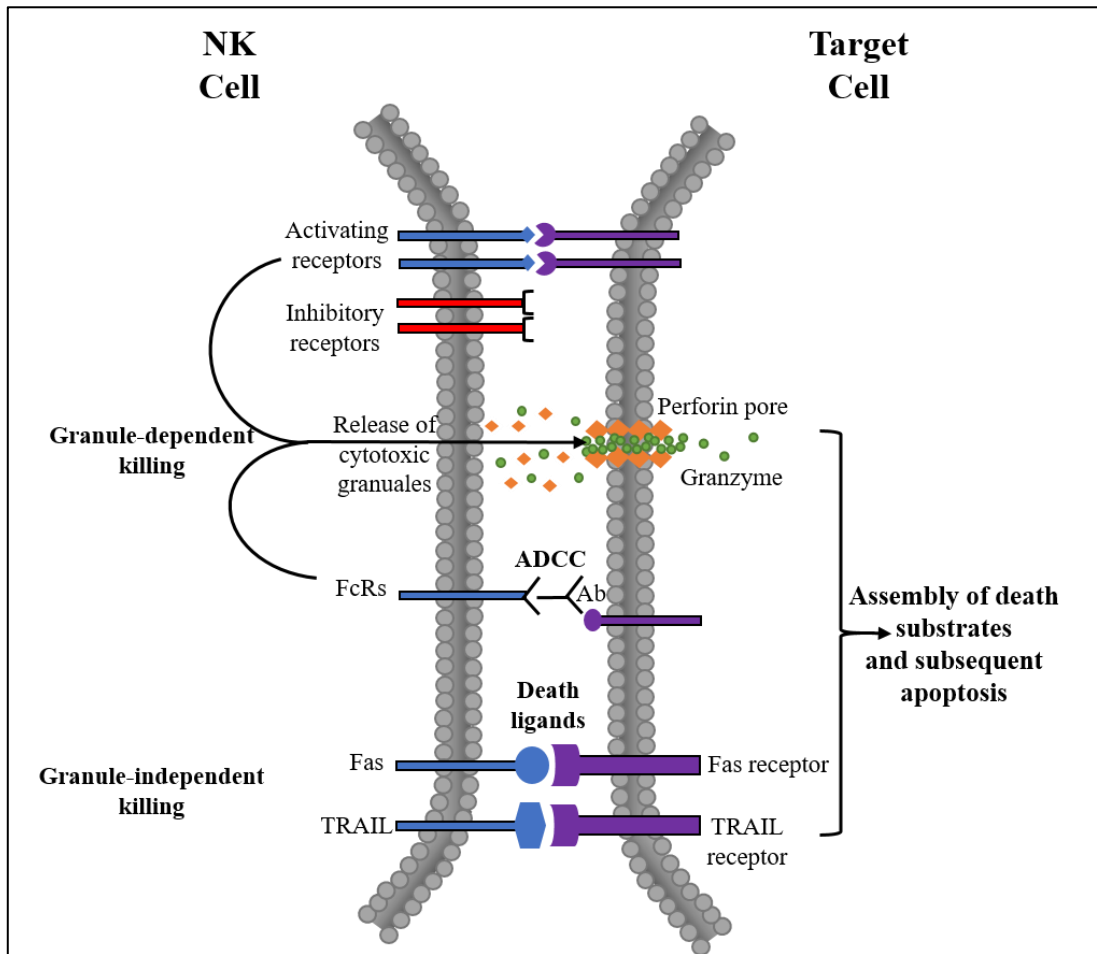
Figure 2-3 Mechanisms of NK cell killing

NK cell mediate cytotoxicity towards target cells by granule-dependent killing or granule independent killing. Granule-dependent killing is induced by signals transduced by activating receptors or through FcRs (CD16). Upon stimulation cytotoxic granules, containing perforin and granzyme, are released by exocytosis which leads to the cell death of target cell. Granule-independent killing is mediated by death ligands (e.g. FAS and TRAIL) which induces apoptotic signals on the target cell.

2.2.4 Tumour evasion of NK cells

As described previously (in section 2.1.1), evolving tumour cells evade multiple components of the immune system, including NK cells, either directly or by co-opting the properties of the tumour microenvironment. Tumour cells are known to up-regulate inhibitory signals and down-regulate activating signals of the immune system (78). For example, tumour cell shedding of stress induced NKG2D ligands, such as MICA/B and ULBP, lead to high levels of these proteins in patients' serum with haematopoietic malignancies (79–81) and colorectal cancer (82). These soluble ligands bind to NKG2D and cause it to internalise, generating NK cells with reduced cell surface NKG2D and a lowered capacity to detect the tumour (82).

Moreover, various cytokines/enzymes produced by either tumour cells or other cells (e.g. T_{regs}, dendritic cells) modulate NK cells function. These include IDO which is normally associated with dendritic cells for suppressing a response from immune cells and upregulated in the tumour microenvironment to inhibit NK and T cell activity through the catabolism of tryptophan which interferes with the IL-2 induced upregulation of NK activating receptors (e.g. NKG2D) (83). Regulatory T cells (T_{regs}) are known to play a role in this mechanism by inducing antigen presenting cells to express IDO.

Notably, a component of the cyclooxygenase (COX) pathway, prostaglandin E2 (PGE_2) is also up regulated in the tumour microenvironment and has been shown to suppress activated NK cells by inhibiting IFN-γ production and down regulating activatory receptors (84,85). Ultimately, this neutralises NK cell activity and the anti-tumour response. PGE_2 has also been shown to disrupt dendritic cell development which leads to their dysfunction (86). Indeed, tumour cells can suppress NK cells by exploiting proinflammatory or immunosuppressive molecules. This also includes transforming growth factor beta (TGF-β), an immunosuppressive cytokine that has effect on NK cell inhibition, which is discussed in the following section (2.3).

2.3   Transforming Growth Factor (TGF)-β

TGF-β belong to a diverse family of proteins, known as the TGF-β superfamily. They have broad roles in proliferation, differentiation, death, cytoskeletal organisation, adhesion, migration and development (87). There are two main groups within the superfamily, one belonging to TGF-β and activin members, and the second belonging to the bone morphogenetic proteins (BMPs) which have been divided according to their sequence similarities (88). In mammals, there are three isoforms of TGF-β (1-3) that predominantly effect cell growth, differentiation and apoptosis (89). These ligands form dimers, which is essential for receptor activation. Activated immune cells secrete TGF-β to downregulate proliferation and inflammatory cytokine production. This acts as a feedback inhibitory mechanism, preventing self-reactivity (90). Secreted TGF-β1-3 is covalently linked to the latency association protein (LAP) and remains inactive until it dissociated from the LAP complex to bind to its receptors and initiate signalling (91).

2.3.1   TGF-β signalling

The TGF-β receptors (TGFβR) are expressed on the plasma membrane of many cell types, including all cells of the immune system. They are composed of an extracellular domain that binds to TGF-β and an intracellular cytoplasmic domain, which contains serine and threonine protein kinases activity. Initially, TGF-β1/3 associates with TGFβR2 which activates the kinase domain in the cytoplasmic region, leading to the phosphorylation and activation of the juxtamembrane site on TGFβR1 (92,93). This forms a tetrameric receptor complex composed of two TGFβRI chains and two TGFβRII chains (93). The activation of TGFβRI initiates the TGF-β signalling cascade with the help of membrane anchor proteins, (known as SARA and Hrs/Hrg), via the canonical SMAD pathway or the non-canonical pathway, which utilises PI3K, AKT and JNK molecules (94). There is also a third receptor, TGFβR3, which associates with TGF-β2 and functions as an additional method for the transfer of ligand to TGFβR2 (95).

2.3.1.1   SMAD proteins

Notably, TGF-β signalling predominantly acts through the canonical SMAD (meaning homologies to Mothers Against Decapentaplegic 'MAD' and the *Caenorhabditis elegans,* SMA) family of genes) pathways. The role of SMAD proteins have been extensively reviewed and the proteins categorised into three groups

(93,96,97). One group is responsible for interacting with the receptors and acting as mediators of receptor activation, these are referred to as receptor regulated SMADs and includes SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8. The second group is referred to as inhibitory SMADs which inhibit the receptor SMADs from effectively transducing TGF-β signalling; these include SMAD6 and SMAD7. The third group are referred to as the common SMADs, these co-bind with the receptor SMADs to generate transcription factors for effective TGF-β signalling and includes SMAD4 (Figure 2-4).

The SMADs are well characterised, having a highly conserved *N*-terminal, MH1 domain and *C*-terminal, MH2 domain (MAD homology domain). The MH1 domain is involved in DNA binding and the MH2 domain interacts with other proteins (98,99). For SMAD2 and SMAD3, the MH2 domain interacts with TGFβRI, which is initially recruited by membrane anchor proteins, and the SXS domain (a serine rich region) in the MH2 region is phosphorylated (94,99). Following this, SMAD2 and SMAD3 homodimerise and are imported into the nucleus (100). This complex then associates with SMAD4 and binds to the DNA through their MH1 domain (101). In particular, SMAD3 and 4 have been shown to interact with the conserved SMAD binding element (102). They also associate with transcription factors that might be activating (including histone deacetylases) (Figure 2-4).

Unlike the receptor and common SMADS, the inhibitory SMADs (SMAD6 and SMAD7) lack a SXS domain on their MH1 domain, whereas their MH2 domain is conserved (103). This allows inhibitory SMADs to bind to type I receptors without inducing signalling, effectively inhibiting the pathway. A key inhibitor of TGF-β signalling is SMAD7, which was first discovered to block receptor SMAD activation by binding to TGFβR1 (104). SMAD7 has also been shown to recruit SMURF1, SMURF2 and NEDD4L, E3 ubiquitin ligases, to mediate ubiquitin-dependent degradation of the TGF-β receptor complex by the proteasome (105). The Smad7 and TGFβRI complex is regulated by the salt-inducible kinase, SIK1, which is also a transcriptional target of TGF-β signalling, hence it functions as a negative feedback mechanism by cooperating with ubiquitin ligases (106). In contrast, heat-shock protein -90kDa has been shown to inhibit the interactions between Smad7 and TGFβRI by binding to the TGFβRs (107). Lastly, SMAD6 is regarded to be specific to inhibits bone morphogenetic signalling only (binds to SMAD4). However, there is

some evidence showing its association with TGFβRI, the results of this study also show a reduction in SMAD2 phosphorylation (108).
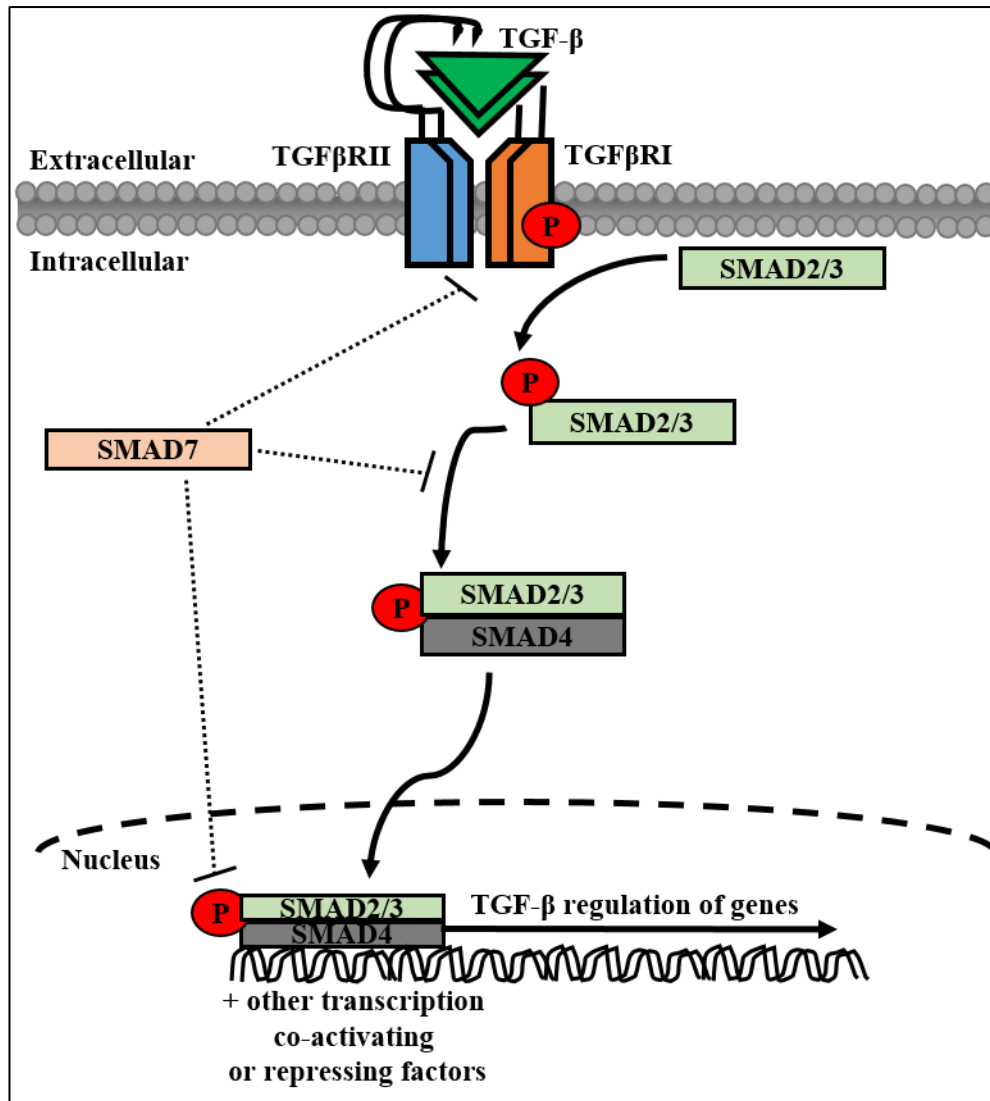
Figure 2-4 Regulation of TGF-β signalling by the SMAD dependent pathway.

Active TGF-β binds to and phosphorylates TGFβRII which in turn leads to the phosphorylation and activation of TGFβRI. Phosphorylated TGFβRI activates the receptor SMADs to form a complex with SMAD4. This complex translocates into the nucleus to bind to transcription factors regulating TGF-β gene expression. This include the upregulation of SMAD7 gene transcription. SMAD7 functions as an inhibitory SMAD that binds and degrades TGFβRI, which leads to blockade of the receptor SMAD activation and downregulation of TGF-β driven gene expression.

2.3.2 TGF-β in the tumour microenvironment

TGF-β is important in many physiological processes that include inflammation, immunosuppression, regulation of the extracellular matrix and proliferation (97,109). The inhibition of cell proliferation by TGF-β affects many cell types, including immune cells (20). TGF-β has been shown to mediate cell cycle arrest at the G1 phase by activating cell cycle inhibitors, such as CDKN1A and CDKN2A in epithelial cells (110). It has also been shown to repress the transcription factor *MYC,* a well-characterised transcription factor that regulates genes that are important in cell proliferation (111). In tumours, TGF-β growth-inhibitory effects are often negated, for example, through the downregulating of receptor expression (112). However, tumour cells can continue to express TGF-β; this results in loss of control of tumour cell proliferation but allows, pro-tumourogenic activity to continue. A common characteristic of tumour promoting activity of TGF-β is the induced transition of epithelial to mesenchymal cells which results in reduced cell to cell adhesion, such as the loss of E-cadherin (113). This has consequences in enhancing cellular migration and enabling metastasis, and has contributed to cancer progression in patients with breast and skin cancer (114). The production of TGF-β by tumours has widespread prominent effects in suppressing immunosurveillance by inhibiting immune cells, including NK cells, CD8 T cells, CD4 T cells, T_{regs}, B cells, monocytes and neutrophils (115). Studies have shown that TGF-β suppresses T cell proliferation and induces apoptosis on B cells (116,117). For NK cells, TGF-β inhibits activation through down regulating production of IFN-γ and reducing expression of cytotoxic components and activating receptors, thereby decreasing NK cell activation and effector function (118,119). It is thought that SMADs repress the expression of genes encoding NK activating receptors, however the molecular mechanism is not clear. A study has shown that TGF-β opposes the activation of mTOR in response to IL-15/IL-2, suggesting one mechanism in inhibiting the upregulation of activation signals (120). The repression of the transcription factor T-bet by SMAD has also been shown to inhibit the IFN-γ response (121). However, the genes that are upregulated by TGF-β and SMADs in NK cells remain unidentified. Importantly, TGF-β can also induce T_{reg} cell differentiation and induce the secretion of immunosuppressive cytokines such as IL-10, TGF-β itself and the expression of cytotoxic T lymphocyte antigen 4 (CTLA4) (122). Thus, TGF-β inhibits effector cells but promotes suppressive activity, all of which correlate with poor survival in breast, ovarian and adenocarcinoma cancer

patients (123–125). A knockdown of TGF-β using RNAi in a glioma cell line prevented NKG2D down regulation (126), and inhibiting TGF-β receptor signalling has been shown to restore the expression of NK activating receptors and IFN-γ synthesis (118), demonstrating that targeting of the TGF-β pathway is a therapeutic route for restoring NK cell anti-tumour immunity.

2.3.3 Therapeutic targets of TGF-β signalling pathway

TGF-β is a powerful immunosuppressive cytokine whose activity influences tumour growth (127–129). Hence, TGF-β plays an important role in the development of many solid tumours. This has fed into developing agents against TGF-β as a potential therapeutic approach. Strategies have been developed to combat the TGF-β immunosuppressive effects, including synthetic inhibitors, TGF-β neutralising antibodies, TGFβR kinase inhibitors and soluble forms of TGFβ receptors (109). The current challenge associated with synthetic inhibitors that are currently in the clinical trial stages is specificity in targeting the tumour promoting aspects of TGF-β alone. Several, monoclonal antibodies sequestering excess TGF-β have progressed through clinical studies, such as lerdelomimab, metelimumab and LY2382770 and several others have been described, however, many were discontinued due to ineffective outcomes on efficacy and inconvenient mode of delivery (109). Notably, mice that are TGF-β1 deficient display adverse effects, due to the essential role TGF-β has in regulating an immune response and maintaining homeostasis (130,131). However, disrupting TGF-β receptor expression on specific localised cells does not induce wide spread cytopathic effects (132). Hence, generating an immune cell that is insensitive to TGF-β is an appealing immunotherapeutic approach to target tumour cells that are secreting TGF-β and resisting immunosurveillance. NK cells expressing a truncated form of TGFβRII encouraged NK cell maturation in a mouse model (133). The expression of dominant negative TGFβRII in human NK cells from cord blood cultured *in vitro* showed that they retained their phenotype and cytolytic function in the presence of TGF-β (134). These studies support the rationale to evaluate immunotherapeutic strategies against TGF-β signalling, however only synthetic inhibitors or antibodies are being tested in current clinical trials. Interestingly, a vaccine has been developed (Vigil^TM) to express the protein GMCSF (Granulocyte macrophage colony stimulating factor), which stimulates the recruitment of immune cells, as well as an shRNA for furin, which is important for the expression of TGF-β1/2; used together, stimulates migration of immune cells (135). The early phase

clinical trial using this vaccine is ongoing and is being used to treat patients with Ewing's sarcoma, non-small cell lung cancer and liver cancer (NCT01061840).

2.4 Cancer immunotherapies

According to the World Health Organisation, cancer is the second leading cause of death in developed countries and is mainly associated with lifestyle and the ageing of the population (136). Cancer survival has improved with early diagnosis, improved screening systems and ongoing research in developing targeted therapies (137–140). The most effective treatment for solid cancers is surgery of the primary tumour and associated lymphatics. In contrast, chemotherapy and radiotherapy alone only kill a portion of cells per treatment. Combining therapy and surgery has reduced the mortality that is associated with surgery alone, where micrometastasis or relapse from residual tumour cells can occur. These chemotherapeutic agents are largely DNA-damaging agents that rapidly kill dividing cells. However the genetic instability of tumours and the selection placed on them by drug treatment carries a risk of inducing drug resistant cells, diminishing the efficacy of therapy and advancing tumour development (141). Studies in the last few decades have uncovered the hallmarks of cancer and their development. This has led to a better understanding of their signalling, proliferation capacity and requirements in their microenvironment (5). The understanding of these alterations has led to ongoing research in targeted therapeutic strategies. These developments, in combination with the emerging role of the immune system in cancer immunosurveillance, has contributed to immunotherapeutic strategies by directing tumour immune-cytotoxicity or stimulating the immune system to eliminate tumours, which has the potential for high-specificity (142).

Approaches in cancer immunotherapy include the use of monoclonal antibodies, and cellular therapies (142). Successful monoclonal antibody treatments include trastuzumab which targets Her-2 (143,144). trastuzumab blocks interaction of the HER2 receptor with growth factors (e.g. epidermal growth factor-like ligands) by binding to the extracellular domain of HER-2, it also induces NK cell mediated cell death via ADCC, leading to improved survival in breast cancer patients (145). Monoclonal antibodies are also used to target immune inhibitory checkpoints, for example, ipilimunab is an anti-CTLA4 antibody which would normally regulate the amplitude of T cell activation (146) and pidilizumab which targets programmed cell death- 1 (PD-1), an inhibitor of T cell activation (147). However malignant cells develop mechanisms to evade immunosurveillance, and resistance to new compounds can emerge. The continuous investigation of cancer antigens and immune-regulatory

targets identifies more candidates for the development of monoclonal antibodies and vaccines. An alternative approach that might overcome these limitations is to use cellular therapies to control and eradicate tumour growth. The developments in cancer immunotherapy have encouraged investigations into enhancing specific immune cell activity in patients with malignancies.

2.4.1 NK cellular therapies

Since their discovery, NK cells have been identified as key players in the anti-tumour immune response. As such, NK cells hold great promise for immunotherapy. Several strategies for NK cell based therapy have been proposed, which include the use of NK cells derived from the patients' blood (autologous), or from healthy donors (allogenic) (148–150).

2.4.1.1 Allogeneic derived NK cells

Allogeneic, primary NK cells have had some success, demonstrating heightened cytotoxicity towards malignancies in non-small lung cancer (151), however one of the main problems with using these cells is rejection due to MHC mismatch. It has been suggested that using uneducated NK cells that might be educated by patients MHC to improve tolerance might overcome this issue (149). NK cell education was first discovered when NK cells expressing inhibitory receptors engaged with endogenous MHC molecules to establish self-tolerance and was termed 'NK cell licensing' (152). Alternatively, the use of NK cell lines such as NKL and NK-92MI holds promise due to their lack of MHC ligand expression. In particular, the cytotoxicity and characterisation of NKL have been well documented (153), one study showing greater cytotoxicity compared to NK-92MI (154). Using cell lines as a source for NK adoptive immunotherapy has a distinct advantage compared to autologous NK cells in that they are easily maintained and expanded *in vitro* for large scale production. However, their use in clinical trials has been restricted to the treatment of renal cell cancer and malignant melanoma (155). However, they must be used with caution due to their leukemic origins, which is a distinct disadvantage. Further, investigation required to render NK cell lines incapable of proliferation without weakening their activity; irradiation being an effective measure for NK-92 (156).

2.4.1.2 Autologous derived NK cells

Use of autologous NK cells would be favourable, having minimal toxicity and high viability *in vivo*, however administering cytokines systemically to activate NK cells displayed poor anti-tumour efficacy and posed a significant risk with some patients developing toxic shock syndrome, a side effect from administrating a high dose of IL-2 (157). However, administrating a lower dose of IL-2 showed an improved response against renal cell carcinoma (158). Due to the poor outcome of indirectly enhancing NK cell mediated anti-tumour immunity with cytokines, current investigations focus

on transferring activated and modified autologous NK cells. Using *ex vivo* expanded and activated autologous NK cells has resulted in no adverse side effects, but has also shown little clinical impact in patients with metastatic melanoma and renal cell carcinomas (159). NK cells are derived from CD34+ hematopoietic stem cells (HSCs) in the bone marrow. These HSCs can also be found in umbilical cord blood and both bone marrow and cord blood provide a rich source of HSCs and an alternative method to generate autologous NK cells compared to PBMC-derived NK cells. This has been accomplished using a cell separation method enriching for CD34+ cells, followed by *ex vivo* expansion and differentiation into CD56+ NK cells (160). Cord blood-derived NK cells have been shown to express cytotoxic receptors, secrete IFN and target melanoma and haematological malignancy cell lines effectively (160,161). The NK cells that were differentiated from bone marrow derived HSCs were shown to be more effective in mediating an anti-tumour response compared to cord blood NK cells (162). However, maintaining NK cells in an induced active state through cytokine stimulation can cause cell exhaustion, eventually rendering the cell incapable of targeting tumour cells (163). A promising method is the use of induced pluripotent stem cells (IPSC), however an efficient protocol that generates NK cells consistently from human IPSC has yet to be established. A study has demonstrated the use of human peripheral blood derived IPSCs as a promising direction for NK cell generation on a large scale, which can be applied in both autologous and allogenic settings, however varying quantities of NK cell production were found (164). Preclinical models (where relatively small numbers of NK cells were generated from IPSC) have shown that NK cells differentiated from IPSC are functional (165) and that IPSC derived NK cells can be generated carrying chimaeric antigen receptors (CAR) that confer anti-tumour activity (166). In addition, the minimum NK cell expansion required for a clinical effect is unknown and unpredictable for the treatment of solid tumours due to the local immunosuppressive microenvironment. One other promising method in NK cell-based immunotherapy is engineering NK cells that enables them to be directed towards the tumour site, which can be accomplished by generating chimeric antigen receptors (to specifically target the tumour cells) and overexpressing a chemokine receptor (to localise the NK cells to the tumour (167,168). Other targets of NK cell genetic engineering include inhibiting the signalling induced by immunosuppressive cytokines generated from the tumour microenvironment, for example TGF-β (134). The use of genetically modified *ex vivo* expanded autologous

NK cells is a promising route by which to overcome the inhibitory effect of the tumour microenvironment (Figure 2-5).
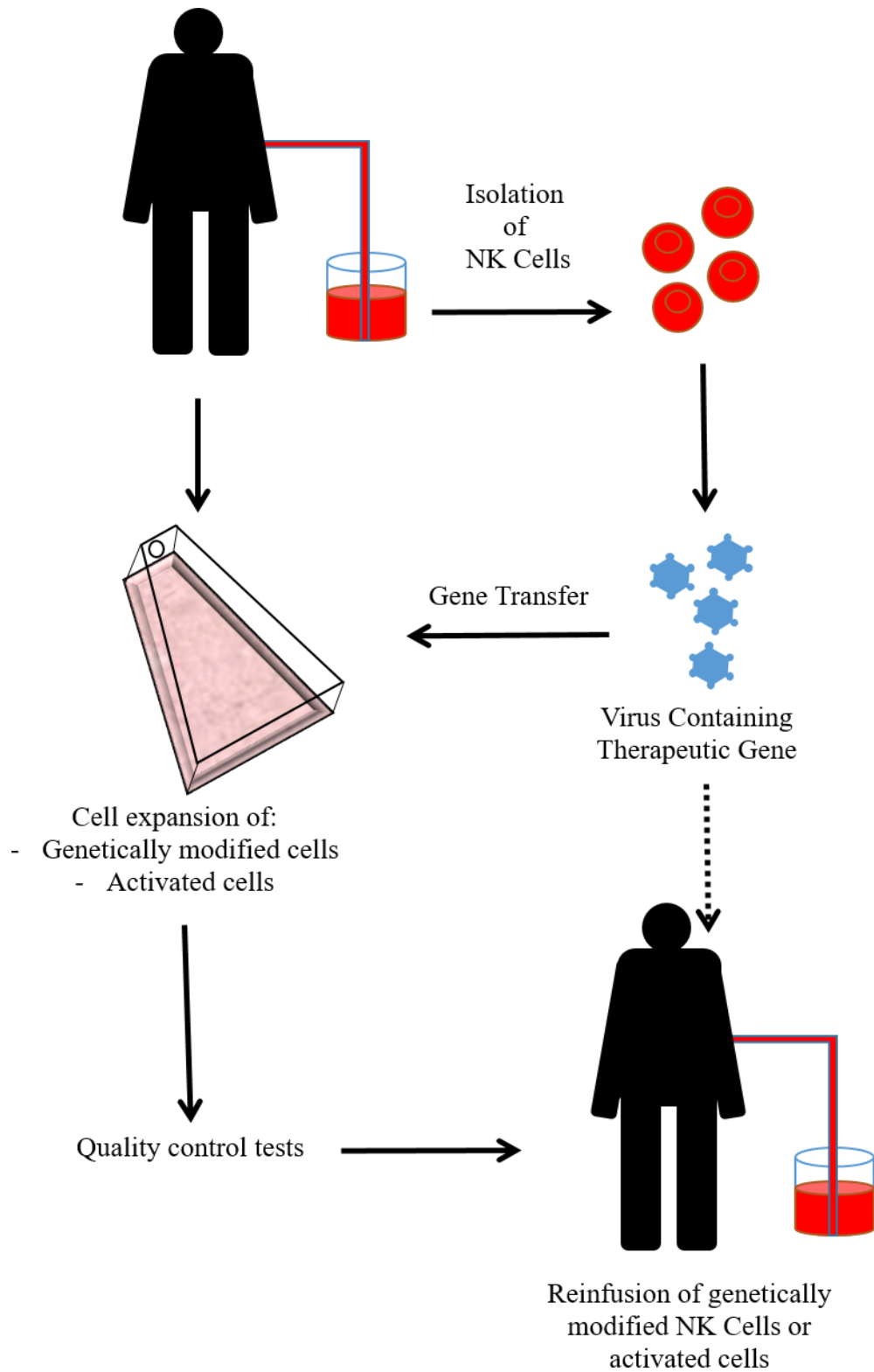
Figure 2-5 Model for autologous NK cell immunotherapy.

Peripheral blood is taken from the patient and NK cells isolated by negative selection. Cells might be activated for expansion and activation and/or genetically modified to express a therapeutic gene.

2.5 Genetic modification of NK cells

The limitation with genetically modified, *ex vivo* expanded autologous NK cells is the lack of an efficient gene delivery system. Commonly used gene delivery systems are known to be ineffective (or inconsistent) for use with NK cells and are associated with poor NK cell survival. Hence, the success of NK cell genetic manipulation is limited and is likely due to the innate immune properties associated with NK cells. NK cells have evolved to seek out and operate within infected tissue and may harbour strong intrinsic anti-viral mechanisms to allow them to do this. In support of this, a study has shown that inhibiting intracellular anti-viral receptors, such as toll like receptors, in NK cells can enhance transduction efficiency (169). Despite this, a small number of groups have published results of NK genetic manipulation, using DNA transfection and viral based gene delivery systems (as reviewed (150)).

2.5.1 Transfection systems

Delivering DNA into NK cells by transfection has mainly been accomplished by electroporation (150,170). Electroporation is a technique whereby genetic material is delivered into cells by increasing the permeability of the cell membrane through administering a time and frequency controlled electrical pulse. Studies have shown efficient transgene expression using electroporation in up to 40% in primary NK cells (171) and up to 70% in NK cell lines (172), with high cell viability. In addition, one study showed that transfecting mRNA (as opposed to cDNA) increased transgene expression, with transfection efficiencies up to 90% in NK cells (173). This technique does not require dividing cells (unlike most viral vector systems). However, RNA transfection only provides transient transgene expression. Use of genome engineering systems, such as CRISPR/Cas9, would allow integration and modification of the genome and this could be accomplished via transient transfection via electroporation of DNA (or mRNA). However, this has not yet been accomplished in NK cells.

2.5.2 Virus systems

The majority of virus mediated transduction on NK cells use lentiviral or retroviral vector systems (150), however vaccinia virus and adenovirus vectors have also been used (174,175).

2.5.2.1 Retroviral vectors

Retroviral vectors have several beneficial properties, such as genetic stability and high transgene expression directed by a retroviral promoter. Retroviruses encode three major proteins: Gag, Pol and Env. Gag, which is also known as group antigens, is a polyprotein processed during maturation into matrix protein, capsid protein, spacer peptides and nucleocapsid proteins that forms the viral core structure. Env is the envelope protein and Pol is the reverse transcriptase. Retrovirus tropism is dependent upon the type of envelope proteins and cellular receptor recognition. Virus-cell recognition leads to entry by either direct fusion to the cell membrane or low pH induced endosomal fusion, which leads to the injection of the nucleoprotein core. Viral RNA is subsequently converted into cDNA by the viral reverse transcriptase, which is an RNA and DNA-dependent DNA polymerase. Retroviruses utilise an integrase that is encoded by the pol gene to integrate the viral cDNA into the infected cell genome. A number of studies have shown successful transduction of NK cell lines and isolated primary NK cells, however the transduction efficiencies of 10-62% are highly variable between studies (134,167,176–179). These studies also omit measurements of cell viability, which could be having an impact as a result of the cellular DNA damage response and its association with triggering apoptosis (180). Despite this, retroviral transduction is not considered to alter the phenotype of NK cells (134,179). Notably, retroviral transduction is limited to dividing cells, therefore NK cells that have not been stimulated with cytokines will not be susceptible to retroviral transduction. Lentiviruses (a sub-group of retroviruses whose prototype is HIV) are capable of infecting both replicating and cells in replicative senescence, however higher transduction efficiency has been observed in cytokine-stimulated NK cells (172). Similar to retroviral transduction, lentiviral transduction efficiency of NK cells and cell lines is highly variable, with quoted efficiencies between 8-73% (172,181,182). Similar to retroviral studies, the viability of cells post-transduction were not reported. A common feature observed amongst these studies is the mostly low transduction rates and the requirement to enrich cells expressing the transgene, possibly through multiple rounds of transduction. Further, the difficulty in reproducing high transduction rates for NK cells is also concerning. Hence, studies of efficient and reproducible approaches for gene delivery to NK cells are ongoing.

2.5.2.2 Other viral vectors

The envelope proteins of lentiviruses can be manipulated to alter their tropism (a procedure known as pseudo-typing). Pseudo-typing the lentivirus envelope with measles virus glycoproteins H and F allows for the lentivirus to target CD46 expressing cells (183,184). The measles virus (MV) is a member of the paramyxoviruses. It primarily utilises signalling lymphocyte activating molecule (SLAM) as a receptor for cell attachment, however vaccine strains also utilises CD46 molecule for virus entry and CD46 is expressed on all nucleated cells (185). Although, measles virus mediated transduction on NK or T cell have not been reported, utilising lentivirus pseudo-typed with the MV glycoproteins on their envelope provides efficient transduction of primary T cells and cell lines at ~60% (183,184). This system (or measles virus itself) could potentially be used in NK cells.

Other virus systems have been tested for their ability to transduce NK cells, such as the vaccinia virus and adenovirus. Vaccinia virus has historically been used as a live vaccine against smallpox (186). It contains a large double stranded DNA genome of ~190kb in size that encodes ~250 proteins (187). A number of replication deficient strains have been developed for clinical investigations, including modified vaccinia Ankara (MVA), Copenhagen strain (NYVAC), avipoxvirus and orthopoxvirus. Due to its capacity to accommodate up to 30kb of transgene, strategies have been developed to utilise the vaccinia virus as a gene delivery vector. Recombinant viruses are constructed using homologous recombination or transient dominant selection (188,189). Fluorescent marker genes, such as green fluorescence protein (GFP) and yellow fluorescent protein (YFP) have been inserted into vaccinia strains to study the morphology of infected cells (190,191). These reporter expressing vaccinia viruses have also been used to detect which organs are targeted for virus replication (190). Clinically, vaccinia virus vectors have also been utilised as an oncolytic therapy for the treatment of cancer (192). The growing interest in immunotherapy has also shown vaccinia virus' ability to infect NK cells. Recombinant vaccinia virus expressing dominant negative SHP-1 in NK-92 has been shown, however the transduction efficiency was not described (193). Other studies have also used recombinant vaccinia vectors to express proteins that attempt to inhibit the inhibitory response on NK cells, such as mutated KIR, Syk and dominant negative SHIP (193–196). Studies using vaccinia virus vectors in NK cells are very limited, which might be due to the short term gene expression or the low efficiency at transduction.

In addition, replication defective adenoviruses have been shown to transduce dendritic, B, T and NK cells efficiently (175,197). NK cells were demonstrated to be transduced up to 60%, while cell lines NK-92 and YT were transduced up to 80%. Furthermore, the proliferative function of NK cells was retained post-transduction (175). The adenovirus vector used to demonstrate gene transfer was derived from adenovirus serotype 5 which had been modified to broaden its tropism to CD46 expressing cells. The protein responsible for cell attachment originates from adenovirus serotype 35 which recognises CD46 (198). This system has potential as a tool for the delivery of genes into NK cells, however to date, only reporter genes have been transferred.

2.5.3 Overview of gene targets

Transfection and viral vectors have been utilised to deliver genes into NK cells with the aim of manipulating the anti-tumour response. The main targets of interest have focused on enhancing cytotoxicity, migration capacity and cytokine production, thereby improving anti-tumour activity (Table 2-2). Several pre-clinical investigations have focused on utilising chimeric antigen receptors (CARs) to enhance anti-tumour cytotoxicity. CARs are artificial receptors which contain an intracellular domain to transduce activating signals (which include CD3-ζ or Fc receptor γ chains) (199,200). The extracellular domain is a single chain variable antibody fragment that recognises the antigen of interest, and upon binding will activate NK cells. For NK cells, CARs have been generated to target CD19 and CD20 (on B cells) and HER2 (for breast cancer), as described in (Table 2-2) and further reviewed by Glienke (201). The pre-clinical studies identifying antigen associated targets, such as CD19 specific CARs against B cell malignancies, and CARs targeting HER2 on breast cancer cells have been shown to direct NK anti-tumour cytotoxicity (202,203). Another strategy to improve anti-tumour cytotoxicity is to introduce genes to render NK cell insensitive to suppressive cytokines, such as TGF-β. Two studies using NK-92 and NK cells from cord blood respectively, expressed a dominant negative TGFβ receptor II; these studies showed that their cytotoxicity is preserved in the presence of TGF-β (134,204). The adoptive transfer of the TGF-β insensitive NK-92 cells into mice with lung cancer improved survival rates and increased IFN-γ levels. Other strategies of NK manipulation involve promoting the activation and expansion of NK cells by cytokine stimulation. In particular, IL-2 and IL-15 expressing NK cells improves the anti-tumour response (176,205). Hence, introducing genes encoding cytokines to improve

activation and expansion is promising in consideration that systemically activated NK cells displayed poor anti-tumour efficacy and posed a significant risk with some patients developing toxic shock syndrome (158). Under physiological conditions a potentially limiting factor is the migration capacity of adoptively transferred NK cells to tumour sites. A study has also shown that *ex vivo* expanded NK cells have reduced migration capabilities (206). However, expressing chemokine receptors on NK cells has shown to be effective in improving migration (207), and tumour infiltration by NK cells is associated with a good prognosis (208). Studies investigating genes that modify NK cell migration are limited, however expressing chemokine receptors is a strategy that may improve NK infiltration of tumours.

In conclusion, a number of studies have reported the use of gene delivery tools to manipulate NK cells to enhance their anti-tumour activity. One challenging aspect is the off-target risks associated with genetically engineering or stimulating NK cells *in vivo,* such as inducing cytokine release syndrome when overstimulating with cytokines or inducing an autoimmune response when desensitising immunosuppressive cytokines. A clinical study using T cells expressing a CAR specific to HER2 displayed safety concerns, with one patient developing respiratory failure, however this might likely be resolved using ultra-low doses showing that a number of factors play a role in the efficacy of the adoptive transfer of genetically enhanced immune cells (209,210). For NK cells, reports have mainly focused on engineering NK cells to enhance their anti-tumour response and it is likely that a combination of genes enhancing stimulation, cytotoxicity and migration will be the best option in generating an efficient anti-tumour response.

Table 2-2 Studies on the manipulation of NK cells

	Gene Expressed	Tumour target (*in vitro)*	Cell Type	Mode of delivery	Ref
Cytokine production	IL-2	Liver metastases (mouse model)	NK-92 and YT	Retrovirus	(176)
	Membrane bound IL-15 encoding a CD8α transmembrane domain	B cell malignancies	Primary NK cells	Retrovirus	(178)
	IL-15	Breast carcinoma cells	NK-92 and NKL	Lentivirus	(205)
Migration	CXCR2	Renal cell carcinoma	Primary NK cells and Jurkhat	Retrovirus	(207)
	CCR7 mRNA	Lymph node-associated chemokine CCL19	Primary NK cells	Electroporation	(168)
NK Cytotoxicity	CAR: CD19 receptor harbouring CD28-CD3ζ	B cell malignancies	NK-92	Lentivirus	(203)
	CAR: anti-CD19-BB-ζ	B cell malignancies	Primary NK cells	Electroporation	(171)
	Dominant negative TGF-β receptor	Glioblastoma cells	Cord blood NK cells	Retrovirus	(134)
	Dominant negative TGF-β receptor	Lung metastasis (mouse model)	NK-92	Amaxa nucleofection	(204)
	CAR: ErbB2 (HER2) antigen harbouring CD28-CD3ζ	Breast carcinoma	NK-92	Lentivirus	(202)
	CD16 mRNA	K562s	Primary NK cells	Electroporation	(168)

2.6 Human adenoviruses

Adenoviruses (Ad) are known to infect a wide range of cells over different species. In humans, adenoviruses were isolated from adenoids in 1953 by Rowe at al after identifying a degeneration of cells that were harvested from adenoidal tissue from children (211) and they were officially termed 'Adenovirus' in 1956 (212). There are currently 85 human adenovirus types classified according to the 'Adenovirus Working Group' (213) and they are categorised into seven species: A, B (B1, and B2), C, D, E, F and G, according to their genomic content, protein size and immunological criteria (214,215). The associated diseases of adenovirus infections vary amongst types but are usually mild and common in young children. For example, types from species D manifest keratoconjunctivitis, whereas gastrointestinal infections are common amongst species A, F and G, and respiratory infections are common in species C and B1 (216,217). Although not common amongst adults, a level of immunity might limit the use of adenovirus-based vectors. However, adenovirus-based vectors have been used in several clinical investigations, mostly in gene therapy and oncolytic applications due to their ability to infect a broad range of cells (218,219). The majority of investigations have utilised Ad5 or Ad2 as a vector and have a good biosafety record as they do not integrate within the host genome, i.e. reducing the likelihood that they will induce malignant transformation. However, Ad5 and Ad12 have been shown to transform rodent cells *in vitro* (220). Their clinical applications is discussed further in section 2.6.6.

Structurally, adenoviruses are non–enveloped icosahedral viruses that harbour a linear double-stranded (ds) DNA genome of 30-38kb (221). The outer capsid consists of 240 copies of homo-trimeric hexon proteins which from the facets of the icosahedral particle and homo-pentameric penton bases at the 12 vertices form which project trimeric fibre proteins, which largely determine virus tropism. Further description of their structural characterisation is described in in section 2.6.4.
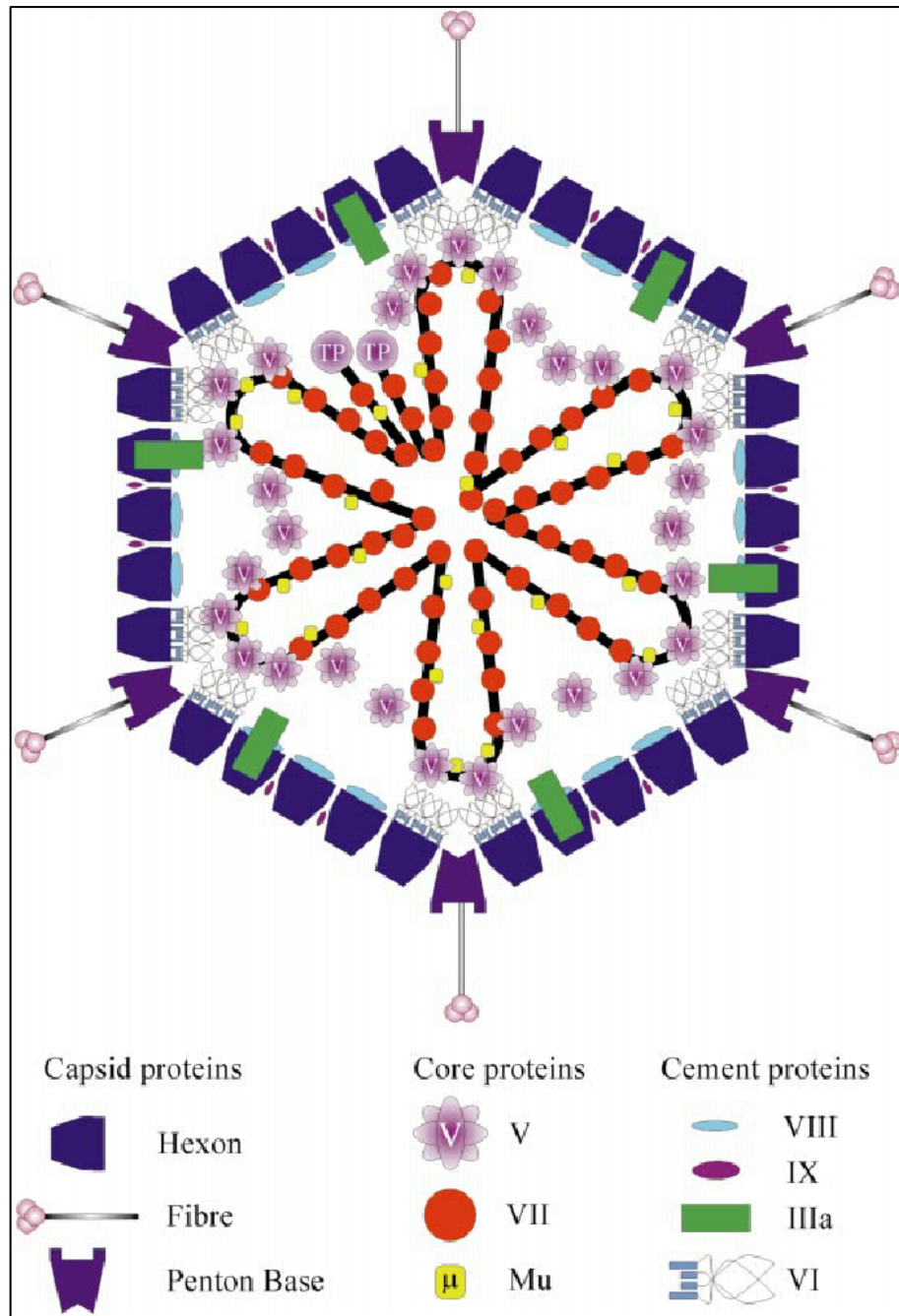
Figure 2-6 A diagrammatic representation of the structure of human adenovirus 5 and its capsid proteins.

A representation of the Adenovirus structure, taken from Russell (222). In brief, adenoviruses are non–enveloped icosahedral viruses comprising 250 trimeric hexon capsomers that form 20 triangular facets (221). The 12 vertex capsomers are comprised of single pentameric penton base proteins from which project trimeric fibre proteins which are involved in host cell recognition and the initiation of cell entry (221). Other minor proteins make up the remaining components of the capsid and include proteins VI, VIII, and IX and structural proteins associated with the genome such as protein V, VII and Mu.

2.6.1 Cell attachment

Adenovirus (Ad) tropism is dependent on the expression of cellular attachment molecules or receptors. The fibre shaft and globular knob domain vary in length between Ad types and bind to different receptors (223). The N-terminus of the fibre consists of a motif (FNPVYPY) that binds to the interface of the penton monomer (224,225). The length of the shaft varies amongst types, with the shaft core having differences in the number of sequence repeats that intertwine to form a triple β-spiral (226) (Figure 2-7). For example, there are 22 repeating units in Ad5 and Ad2, whereas there are five in Ad35 (225–227). A repeating unit is a sequence that encodes two connected β elements that are entwined with a β-turn that forms a loop that composes the shaft domain. The number of residues within the fibre shaft determines the flexibility of the fibre and binding to cellular receptors. The binding of the fibre to cellular receptors is determined by the C-terminal globular domain, as shown in Figure 2-7.

The Coxsackie and Adenovirus receptor (CAR) is the best characterised cell-surface molecule for adenovirus cell attachment and is mainly expressed on epithelial cells. Ad5 and Ad2 are well characterised types that utilise CAR, however many other Ad types do not interact with CAR due to structural features of the fibre protein. For example, the species B type Ad35 attaches to CD46 (described further in section 2.6.1.2), Ad3 utilizes desmoglein-2 and Ad11 can utilize both for cell attachment (Table 2-3) (228). Following interaction between the fibre knob and the cellular receptor, the penton base binds to cell surface α_v integrins for cell entry. At this stage, interaction of α_v integrin with the conserved arginine-glycine-aspartate (RGD) motif of the penton base is important in stimulating membrane changes to allow entry. However, the binding of integrins to RGD on penton bases is not critical, as mutations in this region only slows entry (229). Overall, cell entry is a two stage process where the major function of the fibre protein is to determine the tropism of the virus through cell-surface molecule recognition, which allows the virus to be held at close proximity for interaction between integrin molecules and the penton base to initiate entry. As cell recognition varies between types their pathways of cell entry also differs, for example Ad5 enters via clathrin-mediated endocytosis whereas Ad35 enters via micropinocytosis.
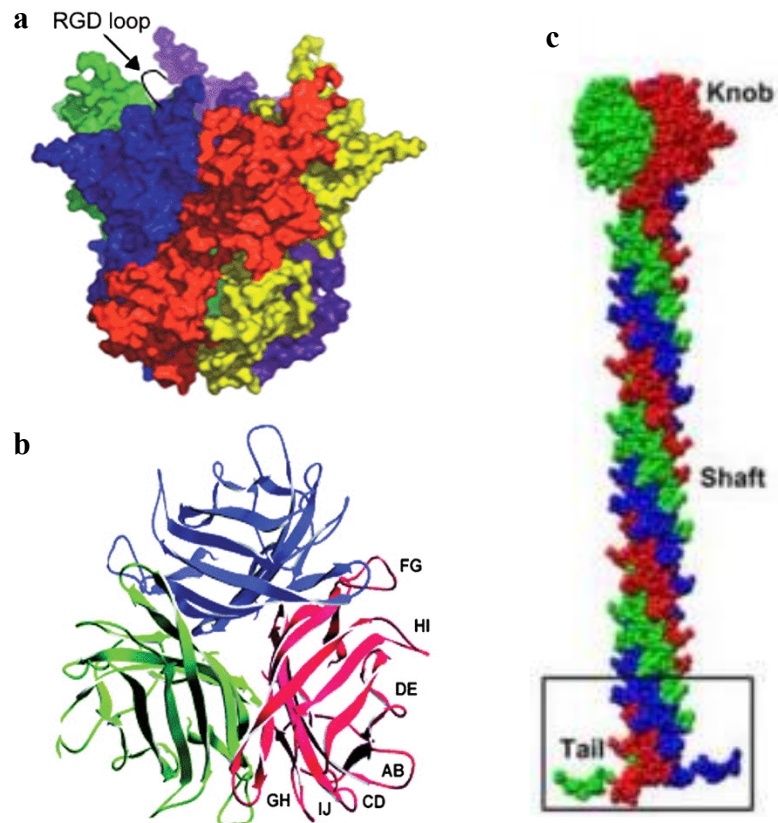
Figure 2-7 A representation of the fibre.
A) An image construction of Ad5 pentameric base showing the RGD loop. B) A ribbon structure representation of the fibre globular knob domain of Ad35, variations in amino acid numbers vary amongst other types. a and b figures were taken from Russell et al (217). C) A complete model of the fibre as represented by Ad5, showing the globular knob C-terminal, a long fibre shaft and N-terminal tail composed of 3 subunits. Taken from Liu et al (227).

2.6.1.1   The Coxsackie Adenovirus Receptor (CAR)

CAR is a transmembrane adhesion molecule that functions as the primary attachment protein for the entry of most adenovirus types as well as species B coxsackieviruses (230). CAR is expressed on epithelial cells and expression has been detected in organs, such as the brain, heart, lungs and skeletal muscle. CAR permits the attachment of adenoviruses of species A, C–F (Table 2-3). Ad5 and Ad2 from adenovirus species C are well-characterised types that use CAR. Adenovirus-mediated transduction of cultured cells has been demonstrated to be directly dependent on the expression of CAR (231). Structural studies of the Ad5 fibre knob interaction with CAR have shown that the interaction occurs on the CAR N-terminal domain and on the outer surface of the trimeric fibre knob (232). However, the Ad5 and the Ad2 fibre and knob proteins are also capable of interacting with cell surface heparan sulphate glycosaminoglycans (HSPGs), which are glycosylated carbohydrates that mediate CAR-independent attachment (233). Coagulation factor X (FX) has also been shown to mediate Ad5 transduction by acting as a bridge between HSPGs on target cells and the capsid protein, hexon. However in cells (such as lymphocytes) with small quantities of HSPGs on their surface, Ad5-mediated transduction is reduced in the presence of FX (234). In addition, Ad5 has also been shown to attach to vascular adhesion molecule-1, which is highly expressed on epithelial cells (Table 2-3).

2.6.1.2   CD46

Serotypes from species B adenoviruses, Ad11, Ad3 and Ad35, attach to CD46 (also known as membrane cofactor protein), a complement regulatory protein expressed as a transmembrane glycoprotein on all nucleated human cells. Receptors for other Ad types are shown in Table 2-3. CD46 functions as a receptor for other viruses and bacteria, such as measles virus, human herpesvirus 6 and *Streptococcus pyogenes* (235–237). CD46 functions as a receptor for complement components (C3b and C4b) to protect the cell from damage by the complement pathway (238). Upon activation, complement mediates opsonisation and lysis of cells without discriminating between self-cells and pathogenic cells. Hence, CD46 functions as a protective mechanism against complement mediated lysis. The structure of CD46 consists of four cysteine-rich short consensus repeats, a hydrophobic transmembrane region and a cytoplasmic domain. The first two short consensus repeats have been shown to be important for the interaction between the fibre knob domain of Ad35 and CD46 (239).

2.6.1.3 Other receptors

Types from species D adenoviruses, Ad8 and Ad37, have also been shown to utilise sialic acid, a component of the glycoproteins and glycolipids (240,241). Ad37 can also bind to CD46 (Table 2-3) (242). The residues that are considered responsible for binding to receptors are conserved in the fibres amongst types, however the receptor sites of binding are not clearly defined.

Table 2-3 Cell surface attachment molecules utilised by human Adenoviruses

Species	Type	Receptor
A	Ad12, Ad18, Ad31	CAR
C	Ad2, Ad5, Ad31	
D	Ad15, Ad19	
E	Ad4	
F	Ad41	
B1	Ad3, Ad16, Ad21, Ad50	CD46
B2	Ad35, Ad11, Ad14	
D	Ad37	
B1	Ad11	Desmoglein-2
B2	Ad3, Ad7, Ad14	
C	Ad2, Ad5	Heparan sulphate proteoglycans
D	Ad8, Ad19, Ad37	Sialylated glycoproteins
C	Ad5	V-CAM-1

*An evaluated summary of reviewed adenovirus receptors (215,216,243)

2.6.2 Cell entry

Following interaction with cell-surface attachment molecules, adenoviruses are internalised, in general, by endocytosis, but the mechanisms vary between types, which may be internalised by coated or coat-independent endocytosis (244). Ad5 and Ad2 enter via clathrin mediated endocytosis (244). Clathrin-mediated endocytosis occurs at the plasma membrane where the formation of a clathrin coat emerges between the attachment molecules and cytosolic adaptor proteins which reconfigures the membrane to produce a vesicle. In contrast, Ad35 and Ad3 enter via macropinocytosis triggered by integrins (245). The mechanism of macropinocytosis involves ruffling protrusion of the plasma membrane that fuses to form a cavity that engulfs extracellular material. The process is controlled through the activation of PI3K which is considered to be activated by signalling through integrins (246). The virus is then transported into the cytoplasm via endosomes. Adenovirus-mediated endocytosis is initiated with the binding of integrins which triggers a signalling cascade to regulate vesicle trafficking (247).

Upon entry, the virus is trafficked within the endosomal compartment before escaping this acidic environment into the cytoplasm (244). Ad5 and Ad2 are known to escape during the early stage endosome of development. The pathway of escape mediated by macropinosomes is less defined, however adenoviruses in these vesicles are thought to escape at a later endosome stage. One factor that plays a role in endosomal escape is the low pH which leads to conformational changes of the virus (248). Partial uncoating of the capsid exterior allows the release of pVI which has been shown to rupture the endosomal membrane, thus allowing the virus to escape into the cytoplasm (249). Upon cytoplasmic release, the adenovirus is trafficked to the nuclear pore complex by microtubules via dynein-dependent transport where it enters the nucleus (250,251). The docking of the virion to the nuclear pore complex is mediated by the interaction of the Ad capsid protein IX with kinesin which facilitates the release of the adenovirus genome into the nucleus where it begins transcription and replication (252).

2.6.3 Genome organisation and replication

The adenovirus genome is composed of 30-38kb linear double-stranded DNA that encodes approximately 45 proteins. The ends of the Ad genome contain inverted terminal repeats and the 5' ends are covalently attached to the terminal protein. An

origin of replication is located within the inverted terminal repeats, where DNA synthesis is initiated by the addition of a dCMP residue to the precursor of the terminal protein (pTP), which acts a primer for DNA synthesis (253). This leads to the dissociation of the adenovirus DNA polymerase from the terminal protein to allow polymerisation of the replicating DNA (254). The cellular transcription factors, NFI and OCT-1 enhance DNA replication and bind to the auxiliary region within the terminal repeats, dissociating from the DNA when the strands become single stranded at the replication fork site (255,256). The adenovirus DNA binding protein (DBP) is essential during elongation, unwinding the dsDNA template and enhancing Ad polymerase processing by removing secondary structures (257,258). The collective function of pTP, polymerase, DBP and transcription factors initiate the process of replication (259). Replication is terminated after the precursor terminal protein is cleaved by a viral protease (260).

The genome is composed of immediate early (E1A), early (E1B, E2A, E2B, E3, E4) and late transcription units that coordinate viral replication and assembly within the host cell (Figure 2-8) (261,262). The immediate early and early genes are critical. In particular the expression of the E1A gene allows expression of the other early genes and induces mitogenic activity in the infected cell (263). The E1A proteins accomplish this by binding to key regulators of the cell cycle. In particular, E1A proteins associate with the retinoblastoma tumour suppressor protein (pRb) and associated proteins (such as p130 and p107), which function as a regulator of the G1 phase of the cell cycle (264,265). One group of proteins with which pRB interacts is the E2F family of transcription factors that regulate the expression of genes responsible for cell cycle progression into the S phase of the cell cycle. This G1 to S phase transition is regulated by mitogenic stimulation (e.g. by a growth factor), resulting in the phosphorylation of pRb (by cyclin dependent kinases) which triggers the dissociation of E2F and subsequent transcriptional activation of E2F target genes. These include genes encoding products important in nucleotide metabolism, DNA synthesis and cyclin regulators (266). E1A has been shown to inactivate pRb/E2F complexes by direct binding, thus allowing the release and activation of E2F to induce mitogenic activity (267). E1A has also been shown to modulate chromatin remodelling factors, such as histone acetyltransferase and histone deacetylase to promote accessibility of the chromatin for the transcriptional complexes. This combination of effects induces DNA synthesis and cell proliferation. However, adenovirus infection activates tumour

protein 53 (p53). The activation of p53 is triggered in cells undergoing various forms of cellular stress (e.g. DNA damage) and results in growth inhibition or apoptosis (268,269). One of the effects of p53 on the cell cycle includes the upregulation of p21, which binds to and inhibits cyclin dependent kinases (270,271). This leads to the accumulation of phosphorylated pRb bound E2F inactive complexes and the arrest of the cell cycle (266). E1A-mediated E2F activation also leads to MDM2 dysregulation, which is associated with increased levels of p53 (272,273). However, adenovirus overcomes the negative effects of p53 on the cell cycle; E1B-55kDa protein blocks p53 induced cell cycle inhibition and apoptosis by binding to p53 (274), hence the E1 proteins work collaboratively to allow efficient replication of the virus. E1B-55kDa is also known to form a complex with E4-ORF6 with p53, which leads to ubiquitination and degradation of p53 (216). Furthermore, E1B-55kDa is also required for transport of mRNA into the cytoplasm for protein synthesis (275). Other early gene units such as E2 mediate DNA replication, E3 proteins mediate the host cell immune responses and E4 proteins alter cell signalling (217,276). In contrast, the late transcriptional units encode proteins that include structural and packaging proteins which leads to the assembly of the virus.

The early gene units encode proteins that alter the host innate immune responses, in particular proteins encoded by E1 and E3. Specifically, E1A blocks transcription of type I IFN and chemokine induced gene transcription (277,278). E3 proteins provide resistance against CD8 T cell killing by binding to MHC Class I molecules in the endoplasmic reticulum, thus preventing the trafficking of the adenovirus associated antigens to the cell surface (279). E3 can also form a receptor internalisation degradation complex which downregulates the expression of death receptor ligands such as TRAIL and FAS, reducing NK cell and CD8 T cell mediated killing (280). However, adenovirus infection activates the host innate immune response through other pathways and induces inflammation related cytokines, which is discussed below in Section 2.6.5.
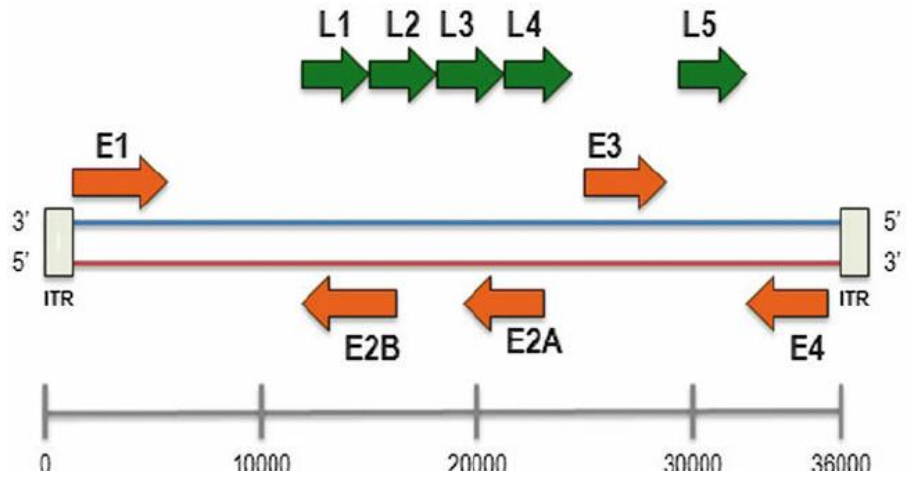
Figure 2-8 Transcriptional map and genome organisation of Ad5.

A general schematic model representing the organisation of the adenovirus genome. The orange arrows represent early transcription regions (E1-E4) and the green arrow represent the late transcripts (L1-L5) (281).

2.6.4 Virus assembly

The translation of viral mRNA occurs in the cytoplasm over two phases; early and late. The translation of early phase viral mRNA transcripts occurs by ribosomal shunting, which is a cap-dependent process that is regulated by the 5' cap and 3'poly-A tail on early phase transcripts (282). The early phase mRNA transcripts synthesise proteins that are regulatory. In contrast, the late Ad mRNA possess a 200 nucleotide 5' non-coding region, also known as a tripartite leader sequence (TLS). The tripartite leader region is required for the translation of later viral mRNA by initiating ribosome shunting (282), and the mRNA transcripts synthesise structural virus proteins that package the genetic material. The translation of late phase transcripts is also associated with the inhibition of cellular protein synthesis.

Following translation, viral proteins are transported to the nucleus for the final stage of the adenovirus infectious cycle; the assembly of virions. This stage is made up of two steps: assembling empty capsids and encapsidation. Empty capsids are assembled to form the capsid region and inner core. The viral capsid consists of major components (hexon, penton, fibre) and minor components (IIIa, VI, VIII and IX). Other virus proteins (V, VII and mu) associate with the DNA forming a virus core. The hexon is the most abundant viral protein, consisting of 240 hexon capsomers and associating with pentons at the 12 vertices and the penton base associates with a trimer of the fibre (221). The minor viral component, protein VI, links the capsid to the nuclear protein core. The nuclear protein core protein IIIa occupies a position under the vertices and has a role in signalling for vertex and genome release during uncoating. pVIII occupies an area between pIIIa and the hexon bases and its role is less clear, however it is considered to be involved with genome packaging due to its association with a packaging motor (IVa2). In contract, polypeptide IX is located on the outer part of the capsid between hexon trimers and its main role is stabilising the capsid. Adenovirus encapsidation is not fully understood, however two viral transcripts and protein are associated with this process: L1, L4 and IVa2 respectively. The IVa2 protein directly binds to the A-repeat sequence on the packaging domain in the left hand ITR and mutant forms have confirmed it is essential for virus assembly (283). The L4 and L1 protein was also found to associate with the packaging sequence, with L4 additionally showing an association with IVa2 *in vitro* (284).

Following virion assembly, maturation is required for virions to become infective. During this stage the adenovirus-encoded cysteine protease processes several viral

proteins by proteolytic cleavage at a specific motif site (MLCGCG or MLCGGC) (260). Two cofactors are also required for enhanced protease activity on precursor proteins: a peptide released from pVII and viral DNA. This process renders the virion infectious. In species C adenoviruses the adenovirus death protein (ADP) promotes the final stage of the infectious cycle by promoting cell lysis and the release of virions (285). It is encoded by E3, an early region of the genome but expressed more in the late stages of infection by the major late promoter. The molecular mechanism by which ADP operates is not clear, however it has been shown to localise at the nuclear membrane and Golgi (286).

2.6.5 Innate immune response to adenovirus

In clinical studies, administration of adenovirus or adenovirus vectors is associated with an inflammatory response and the activation of immune cells. A well-known case study using an E1 deleted adenovirus construct containing a therapeutic gene for the treatment of ornithine transcarbamylase resulted in mortality in one patient due to an immune response toward the vector (287). Hence, understanding the interaction between adenovirus transduction and this immune response is fundamental for effective use as a gene therapy vector (288,289). Several key steps have been identified as inducing the innate response to adenovirus, including viral attachment and endosomal escape (289). Characteristically, adenovirus infection leads to a proinflammatory cytokine and chemokine response, as well as the activation of complement. Consequently, the activation of the immune system is associated with reduced efficacy in gene transfer (289).

One of the earliest steps of adenovirus infection occurs at the binding of the fibre to cellular receptors. The interaction of fibre with CAR has been shown to activate PI3K, JNK and MAPK which leads to NF-kB activation and the upregulation of inflammatory cytokines (290–292). Furthermore, the binding of the penton base RGD motif to integrins has also been shown to induce NF-kB activation (292). Following adenovirus escape into the cytoplasm, detection can be as a result of recognition from DNA dependent activator of IFN regulatory factors (DAI) and nucleotide oligomerization domain like receptors (NLR) (293). The binding of NLRs in the cytoplasm leads to the assembly of the inflammasome (294). Specifically, the NLR family members NLRP1, NLRP3 and NLRPC4 assemble into inflammasome complexes which subsequently leads to the proteolytic cleavage of pro-IL1β and pro-IL-18 to their respective mature forms (294,295). IL-1β binding to the IL-1 receptor

leads to activation of NF-kB and the release of chemokines (296), whereas IL-18 induces IFN-γ secretion and upregulates T cells (297). The DAI becomes activated upon recognition of adenovirus DNA in the cytoplasm, which leads to the activation of IRF3/7 and TBK1/IKK1 and the upregulation of an IFN and pro-inflammatory response (293).

Upon internalisation of the virus, Toll-like receptors (TLR) in the endosome can detect adenovirus double stranded DNA. TLRs are pattern recognition receptors (PRRs) that recognise pathogen associated molecular pathogens (PAMPs). Adenoviruses utilising both CAR and CD46 induce TLR9 mediated activation (298). TLR9 is expressed on both the plasma membrane and intracellular membranes, in particular the inner compartment of the endosome membrane, which recognises adenoviral CpG-rich DNA and upon activation induces an interferon response via a MyD88-dependent pathway (298). The activation of the MyD88 pathway by TLR9 leads to the activation of transcription factors NFkB and AP-1 which are responsible for the transcription of proinflammatory genes (e.g. IL-6, IL-12 and TNF) (299). TLR9 is mainly expressed in dendritic cells, B cells, with low expression in NK cells, which suggests one reason why the interferon response to adenovirus infection also varies amongst cell types (300,301). There are three types of IFN; type I IFNs have 13 subtypes and are responsive towards viral infection. The type II IFNs are secreted by lymphocytes in response to adaptive immune cell activation. Much less is known about the type III IFNs. The pathway responsible for inducing an interferon response in adenovirus infection of NK cells is not clear, however it has been reported that NK cells respond to adenovirus dsRNA by inducing an IFNα/β response (302,303). In dendritic cells, adenovirus mediated binding of TLR9 leads to the secretion of IFN-α (304,305). This pathway is mediated by MyD88 which also recruits the IL-1 receptor associated kinase 1 and TNF receptor associated factor-6 which together forms a complex that activate MAP kinases and interferon regulatory factor-7. The phosphorylation of the interferon regulatory factor-7 signalling complex induces a type I IFN response (304,305). The expression of type I IFNs lead to the activation of JAK kinases and the phosphorylation and signalling of STAT. STAT1 and STAT2 interacts with IRF9 to form ISGF3 which leads to the transcription of IFN-stimulated genes (306). Consequently, the activation of the host innate immune system leads to virus elimination, hence reducing the efficacy of gene transfer (288,289).

2.6.6 Clinical applications

Adenoviruses have been used in both gene therapy and oncolytic virus clinical trials. Adenoviral based vectors do not integrate within the host genome, reducing the likelihood that they will induce malignant transformation and cancer in human cells. However, several Adenoviruses (including Ad5 and Ad12) have been shown to transform rodent cells *in vitro* (220). Adenovirus vectors used in gene therapy studies are replication deficient, but retain their packaging signals, minimising viral toxicity. In particular, clinical studies have used adenovirus to deliver the therapeutic gene CFTR for the treatment of cystic fibrosis, however participants manifested inflammatory responses to treatment (307), demonstrating that rapid clearance of the virus by the immune system is a limitation of their use. Although clinically developed vectors are replicative-defective, they can still trigger an immune response (308), resulting in the loss of the therapeutic gene (for example, via the detection of viral DNA and RNA species by TLRs). However, adenovirus delivery of genes and genome engineering systems of cells has been shown to result in prolonged transgene expression (309). The relative small size of the Ad genome (compared to pox or herpes virus) means that only relatively small transgenes can be delivered by Ad based vectors and non-essential viral DNA needs to be removed to accommodate larger genes. Indeed the deletion of early region genes in replication defective vectors does provide capacity for larger transgenes.

Replication competent adenovirus vectors have been studied for oncolytic viral therapy (310). Many human cancers harbour mutations in *TP53 (*encoding p53) and lose pRb expression (either by mutation or gene silencing). Loss of activity of these key tumour suppressor functions favours dysregulated proliferation (5). The inactivation of these pathways also provides for efficient adenovirus replication (Section 2.6.3). Indeed, adenovirus E1A binds to pRb and prevents E2F inactivation, favouring cell cycle progression (267). In addition, the E1B-55K binds to p53 (stabilised via E1A activity) and converts it from a transcriptional activator to a powerful repressor, thereby bypassing p53 mediated checkpoints that normally restrict cell division (311). Adenoviruses with mutations in the early gene units which are responsible for inactivating these pathways are unable to replicate efficiently in normal cells (i.e. they cannot bypass pRb and p53 control) but retain the ability to replicate in cancer cells where these pathways are already dysregulated. Hence, mutated adenoviruses can selectively target cancer cells with aberrant cell cycle

regulation, endowing these adenoviruses with oncolytic virus activity (310). The Adenoviruses ONYX-015 is a well-studied oncolytic virus in which the p53 binding site on E1B-55kDa has been deleted (218,312). In principle, cells with inactive p53 pathways will be preferentially targeted and this agent was shown to be effective in patients when combined with chemotherapy (312). Further studies have investigated mutation in the E1A gene, for example, the KD1 oncolytic vector replicates and kills lung adenocarcinoma cells (313). However, this vector also includes a surfactant protein B promoter which drives the transcription of lung-specific genes and enhanced tissue specificity. It is likely that additional modifications to enhance tissue specificity will effectively target tumours in addition to the intrinsic oncolytic characteristics.

Replication defective adenovirus vectors have deletions in the early gene units, which are critical for virus replication. The first generation Ad vectors include the deletion of E1A, E1B and E3 (314). The deletion of these genes allow for the insertion of a therapeutic gene. This allows for the generation of a vector in gene therapy applications. Chimeric vectors have been generated to modify the tropism of adenovirus serotypes. As Ad5 and Ad2 are the most well characterised and used in clinical investigations, CAR is the primary receptor for targeted cells. However, CAR is often downregulated on cancer cells and expressed at very low or undetectable levels on lymphocytes (234,315). Hence, generating a chimeric virus for CAR-independent cell recognition provides flexibility in vector tropism. Cell recognition and entry is paramount for the manipulation of NK cells for immune-cellular therapies. However, Ad5 cannot infect lymphocytes due to the absence of CAR (316). The chimeric vector, Ad5f35, is structurally similar to Ad5 but has had the Ad5 fibre shaft and knob domain replaced with that of Ad35 (198), and consequently uses CD46 for cell attachment and has the ability to transduce NK cells (175). Other chimeric adenoviruses that target CD46 expressing cells include Ad5f11, Ad5f35 and Ad5f7 (317–319). Other vectors include second and third generation adenovirus vectors which have further deletions of E2 and E4 (314). Despite using replication defective vectors, immune responses to these vectors are still observed, likely from the retained adenovirus proteins in the vector. One approach to overcome this was to generate a vector with all of the coding genes deleted; this has been termed a "gutless" vector (320). These vectors have a reduced capacity to induce an immune or inflammatory response due to the lack of adenovirus expression, however their efficacy in gene transfer and stability in replication is reduced. The concept of using adenovirus to

deliver genes *ex vivo* is one strategy to overcome some of these limitations, which has been demonstrated in primary epithelial cells (321). This strategy also allows for multiple gene targets with additional adenovirus vectors.

2.7   Aims of the project

The potency of the NK cell anti-tumour response makes these cells attractive agents for cancer immunotherapy. There is however a need to improve the tools to enhance NK cell migration to tumours and to allow them to function more efficiently in the immunosuppressive tumour microenvironment. This can be accomplished by *ex vivo* manipulation of primary NK cells. However, this is still limited by the inefficient tools currently available for gene transfer. Current vehicles or methods are also difficult to reproduce consistently, or are not appropriate for clinical use.

The principal aims of this work are;

- To identify viral vectors suitable for gene delivery to primary human NK cells
- To use this vector system to express a therapeutic gene in primary NK cells to enhance their activity in the tumour microenvironment.

This will provide a novel strategy by which to engineer NK cells from patients to make them more potent anti-cancer agents.

3   Materials and Methods

3.1   Materials

3.1.1   Chemicals

General chemicals were 'Molecular Biology' grade and were purchased from Sigma-Aldrich (Poole, UK), unless otherwise stated.

3.1.2   Buffers

10X TBE: 0.89M Tris Base; 0.85M Boric Acid; 40ml 0.5M EDTA (pH 8.0). Dissolved in 1L ddH$_2$0

10X Orange G: 5g Ficoll 400; 0.08mM Orange G; 2ml 1M Tris-HCl (pH 7.4); 4ml 1M EDTA (pH8.0). Dissolved in 20ml ddH$_2$0.

1x Loading buffer (DNA): 10% (v/v) glycerol, 0.025% (w/v) bromophenol blue in 1xTBE.

PBS: Tablets purchased from Oxoid Ltd (Hampshire, UK), dissolved in ddH$_2$0 (1 tablet per 100 ml includes 140 mM NaCl, 10 mM phosphate buffer, and 3 mM KCl at pH 7.4) and autoclaved before use.

RIPA: 10mM Tris-HCl (pH 8); 1mM EDTA; 0.5mM EGTA; 140mM NaCl; 1 % Triton X-100; 0.1 % sodium deoxycholate; 0.1 % v/v SDS. Dissolved in dH20.

2x Loading buffer (protein): 100mM Tris HCl (pH 6-8); 4% (w/v) SDS; 0.2% (w/v) bromophenol blue; 20% (v/v) glycerol; 10% β-mercaptoethanol. Dissolved in dH20.

Running buffer: 25mM Tris base; 250mM glycine; 0.1% (w/v) SDS. Dissolved in ddH$_2$0.

Transfer buffer: 12mM Tris base; 96mM glycine; 20% (v/v) methanol. Dissolved in ddH$_2$0.

TBST: 25mM Tris base; 134mM sodium chloride (pH 7.5); 0.01% (v/v) Tween-20. Dissolved in ddH$_2$0.

FACS buffer: 0.5% (v/v) FCS; 0.5% (w/v) sodium azide in PBS.

MACS buffer: 0.5% BSA; 2mM EDTA (pH 8.0) in PBS.

M9 salts (1X): 40mM Na2HPO^4; 0.2M KH2PO4; 14mM NH4Cl; 8mM NaCl. Dissolved in 1L ddH$_2$0 and autoclaved before use.

LB medium: 10g/l Tryptone; 5g/l Yeast Extract;10g/l NaCl. Dissolved in ddH$_2$0 and autoclaved before use.

Half-salt LB media: 10g/l Tryptone; 5g/l Yeast Extract; 5g/l NaCl. Dissolved in ddH$_2$0 and autoclaved before use.

Recombineering selection cassette plates: 10g/l tryptone; 5g/l Yeast extract; 50g/l Sucrose; 12.5μg/ml Chloramphenicol; 80μg/ml X-gal; 0.2mM IPTG; 15g/l agar. Dissolved in ddH$_2$0 and autoclaved before use.

Other plates: Initially the medium was sterilised by autoclaving and supplemented with the appropriate antibiotic when the medium was cooled.

Ampicillin plates: 10g/l tryptone; 10g/l NaCl; 5g/l yeast extract; 15g/l agar; 50μg/ml ampicillin. Dissolved in ddH$_2$0.

Chloramphenicol plates: 10g/l tryptone; 10g/l NaCl; 5g/l yeast extract; 15g/l agar; 12.5μg/ml chloramphenicol. Dissolved in ddH$_2$0.

Recombineering plates: 10g/l tryptone; 10g/l NaCl; 5g/l yeast extract; 15g/l agar; 12.5μg/ml chloramphenicol, 0.1mg/ml X-gal, 20mg/ml IPTG. Dissolved in ddH$_2$0.

3.1.3 Primers

Table 3-1 Primers

All primers were purchased from Sigma Aldrich.

Primers used to synthesis dominant negative TGFβRII for recombineering	
dnTGFR2_Adz_F	5'-AACCGTCAGATCGCCTGGAGACGCCATCCA CGCTGTTTTGACCTCCATAGAAGACACCGGGA CCGATCCAGCCTGGATCCGTCTGCCATGGGTC GGGGGCTGCT-3'
Flag_Adz_R	3'-GGCGTGACACGTTTATTGAGTAGGATTACA GAGTATAACATAGAGTATAATATAGAGTATA CAATAGTGACGTGGGATCCCTTGTCGTCATCG TCTTTGTAGTC-5'
Primers used in colony PCR and RT-PCR for dominant negative TGFβRII	
TGFBR2 small R	3'-GGAGAAGCAGCATCTTCC-5'
TGFBR2 small F	5'-GGCTGCTCAGGGGCCTGT-3'
Primers used to detect recombinants for sequencing	
AdZ Insert Sequencing F	5'-AATGTCGTAACAAGTCCG-3'
AdZ Insert Sequencing R	3'-ACCTGATGGTGATAAGAAG-5'
TGFBR2 small R	3'-GGAGAAGCAGCATCTTCC-5'
TGFBR2 small F	5'-GGCTGCTCAGGGGCCTGT-3'
Primers used to detect E1A	
E1a exon 1 F	5'-CGGTGTACACAGGAAGTGACAA-3'
E1a exon 1 R	3'-GCCGTCACGTCTAAATCATACAG-5'
Primers used in CRISPR study	
B2M Exon 1 F	5'-GTCCCTCTCTCTAACCTGGC-3'
B2M Exon 1 R	3'-ACTTGGAGAAGGGAAGTCAC-5'

3.1.4 Antibodies

Table 3-2 Western blotting antibodies

Western Blotting Antibodies				
Primary antibodies	**Company**	**Catalogue number**	**Stock concentration**	**Dilution**
Anti-MADH7	Abcam (Cambridge, UK)	ab190987	0.3mg/ml	1/1000
Anti-SMAD6	Abcam	ab63713	0.2mg/ml	1/1000
Anti- TGFβ Receptor II antibody	Abcam	ab186838	1.68mg/ml	1/500
Anti-B-actin	Sigma	A1978	x	1/10000
Secondary antibodies	**Company**	**Catalogue number**	**Stock concentration**	**Dilution**
Anti-Rabbit IgG HRP	Sigma	AG154	x	1/10000
Anti-Mouse IgG HRP	Sigma	A6782	x	1/10000

Table 3-3 Flow cytometry antibodies

Flow cytometry antibodies	Product Code	Lot Number	Company	Dilution	Final concentration	Isotype
NK Cells						
CD56-PE	130-090-755	5.15E+09	Miltenyi biotec (Surrey, UK)	10µl per 100µl 10^7	0.22µg/10ul	IgG1
CD3-BV421	562426	5065838	BD Biosciences (Oxford, UK)	5µl per 100µl 10^6	0.25µg/5ul	IgG1, k
CD56-APC	130-090-843	5.15E+09	Miltenyi biotec	10µl per 100µl 10^7	0.0825µg/10ul	IgG1
CD3-PerCP	130-094-905	x	Miltenyi biotec	10µl per 100ul 10^7	x	IgG1
CD56-PeVio770	130-100-673	57170809221	Miltenyi biotec	10µl per 100ul 10^7	x	IgG1
T Cells						
CD3-BV421	562426	6065838	BD Biosciences	5µl per 100µl 10^6	0.25µg/5ul	IgG1, k
CD8-APC	555369	4269814	BD Biosciences	20ul per 100ul 10^6	0.06µg/20ul	IgG1, k
CD4-PeVio770	130-100-452	5.15E+09	Miltenyi biotec	10µl per 100µl 10^7	0.22µg/10ul	IgG1
B Cells						
CD20-BUV395	563782	4255845	BD Biosciences	5µl per 100µl 10^6	0.5µg/5ul	IgG2b, k
Dead Cell discriminator						
Zombie NIR™ APC-Cy7	423106	B182975	Biolegend (London, UK)	5µl of 200x Diluted Zombie NIR™ dye per 100ul 1.0 x 10^6 cells in PBS		
CD46 Staining						
CD46-FITC	555949	4059963	BD	20µl per 100µl 10^7	1.0µg/20ul	IgG$_{2a}$, κ
NK cell receptors						
CD16-FITC	555406	3351672	BD Biosciences	20µl per 100µl 10^6	2.0µg/20ul	IgG1, k
NKG2D-PE	557940	4175890	BD Biosciences	20µl per 100µl 10^6	0.125µg/20ul	IgG1, k
DNAM-1-PE	130-092-476	5.15E+09	Miltenyi biotec	10µl per 100µl 10^7	0.33µg/10ul	IgG1
NKp30-PE	120-003-054	5.07E+09	Miltenyi biotec	10µl per 100µl 10^7	0.33µg/10ul	IgG1
NKp44-PE	558563	4038699	BD Biosciences	20µl per 100µl 10^6	0.125µg/20ul	IgG1
NKp46-APC	558051	4119779	BD Biosciences	20µl per 100µl 10^6	0.5µg/20ul	IgG1
CD69-FITC	130-098-901	5160706378	Miltenyi biotec	10µl per 100µl 10^7	x	IgG1
HeLa Characterisation						
MHC Class I - APC	311410	B147481	Biolegend	5µl per 100µl 10^6	0.01µg/5ul	IgG$_{2a}$, κ
B2M-PE	316306	B169085	Biolegend	5µl per 100µl 10^6	0.025µg/5ul	IgG1
Intracellular						
Hexon Clone: 2Hx2	Antibody made by Eric Blair, University of Leeds. Cell line HB-8117 (ATCC)					
Alexa Fluor 647 Donkey Anti-mouse	A21447	1841382	Invitrogen (UK)	x	0.2µg/1µl	x
SMAD2/3-P (clone 072-670)	562686	7096912	BD Biosciences	x	x	IgG1
Cell tracking						
Cell tracker violet-BMQC	C10094	x	Invitrogen	1/10,000	2uM	x
Cell tracker green-CMFDA	C2925	x	Invitrogen	1/10,000	2uM	x
NK functional assays						
CD107a-PE	555801	6083963	BD Biosciences	1µl per 100ul 2^5	x	IgG1
GolgiSTOP	51-209K2	5148620	BD Biosciences	1/1000	x	x
CD46 blocking						
CD46	H294	H294	Santa Cruz (USA)	x	1ug/250µl	Rabbit IgG

3.1.5 Cytokines

Table 3-4 Cytokines

Cytokine	Company	Catalogue number	Working Concentration
Human IL-2, premium grade	Miltenyi biotec	130-097-744	50-500IU/mL
Human IL-15, premium grade	Miltenyi biotec	130-095-764	20ng/mL
Human TGF-β1, premium grade	Miltenyi biotec	130-095-067	5-10ng/mL

3.1.6 Cell lines

Table 3-5 Cell lines

Cell line	Medium	Derivation
911	Dulbecco's Modified Eagle's Medium (DMEM) and 10% Fetal bovine serum.	Primary human embryonic retinoblasts that contain integrated Ad5 E1A and E1B genes. Source: R. Hoeben, University of Leiden, Netherlands.
HeLa	DMEM and 10% Fetal calf serum (FCS).	Human cell line derived from a cervical adenocarcinoma harbouring Human Papillomavirus 18. Source: European Collection of Authentic Cell Cultures (ECACC).
A549	DMEM and 10% FCS	Human line epithelial cells derived from lung carcinoma tissue. Source: ECACC.
SKOV-3	Roswell Park Memorial Institute, 1630 series (RPMI-1640), 10% FCS	Human cell line derived from a patient with ovary adenocarcinoma. Source: Cancer Research UK Cell Services.
K562	RPMI-1640, 10% FCS	Human erythroleukemia cell line isolated from a patient with chronic myelogenous leukaemia. Source: ECACC.
YT	RPMI-1640, 10% FCS	An NK-like lymphoid cell lines. Source: G. B. Cohen, Harvest University, USA.
NKL	RPMI-1640, 10% FCS	Isolated from a patient with CD3-CD16+CD56+ large granular lymphocyte (LGL) leukaemia. This cell line is strictly dependent on IL-2 for sustained growth [93]. Source: Professor Salim Khakoo, University of Southampton.
NK92MI	Minimum essential medium (MEM), 2mM L Glutamine, 0.2mM inositol, 0.02mM Folic acid, 0.1mM 2-mercaptoethanol, 12.5% Horse serum, 12.5% FCS	NK cell line isolated from a non-Hodgkin's Lymphoma patient. This cell line shows IL-2 independent growth due to integration of IL-2 gene via a retroviral vector. Source: American Type Culture Collection (ATCC).
HEK293T	DMEM and 10% FCS	A highly transfectable derivative of human embryonic kidney 293 cells. Source: G. E. Blair, University of Leeds.
HaCaT	DMEM and 10% FCS	In vitro spontaneously transformed keratinocytes from histologically normal skin. Source Miriam Wittmann, University of Leeds.
Huh7	DMEM and 10% FCS	A well differentiated hepatocyte derived cellular carcinoma cell line that was originally taken from a liver tumour in a 57-year-old Japanese male in 1982. Source: Mark Harris, University of Leeds.
PBMCs	RPMI-1640, 10% FCS	PBMCs and primary NK cells were provided by NHS blood and transplant donations and cell isolation was undertaken on the same day.
Primary NK cells	DMEM and 10% Human serum.	

3.1.7 Plasmids

Table 3-6 Luciferase plasmids

Luciferase plasmids	Antibiotic resistance	Size	Company	Catalogue number
LNCX TbetaRII DN	amp	600bp insert	Addgene (Cambridge, UK)	12640
pCMV5-Smad7-HA	amp	4700bp insert	Addgene	11733
Flag-Smad6	amp	4100bp insert	Addgene	14961
p3TP-lux	amp	x	Addgene	11767
pRL-TK	amp	x	Promega (UK)	E2241

Table 3-7 CRISPR plasmids

CRISPR plasmids	Antibiotic	Promega backbone	gRNA Name	gRNA target sequence	Genomic Location of B2M
pD1301-AD:155576	amp	DNA 2.0	B2M 118447	TTTGACTTTCC ATTCTCTGC	Exon 2
pD1301-AD:155575	amp	DNA 2.0	B2M 118445	TGGAGTACCT GAGGAATATC	Intronic Sequence and Exon 2
pD1301-AD:155574	amp	DNA 2.0	B2M 118443	ACTCACGCTG GATAGCCTCC	Exon 1 and Intronic Sequence
pD1301-AD:155573	amp	DNA 2.0	B2M 118441	CTCGCGCTACT CTCTCTTTC	Exon 1
pD1301-AD:155572	amp	DNA 2.0	B2M 118439	GGCCACGGAG CGAGACATCT	5'UTR and Exon 1

3.1.8 Viruses

Table 3-8 Viruses

Virus	Description	Stock concentrations
Ad3-EGFP	A replication-deficient Ad3 virus with a CMV driven EGFP transgene. (Dr Silvio Hemmi, University of Zurich)	4.58×10^9 FFU/ml
Ad5-EGFP	A replication-deficient Ad5 virus with a CMV driven EGFP transgene. (Professor Aviva Tolkofsky, University of Cambridge)	4.96×10^9 FFU/ml
Ad5F11-EGFP	E1-and-E3-deleted Ad5 virus with the Ad5 fibre replaced with the Ad11 fibre that expresses EGFP under the control of a CMV-promoter. (Prof M Tagawa, Chiba, Japan)	1.062×10^{10} FFU/ml
Ad5F35-EGFP	E1-and-E3-deleted Ad5 virus with the Ad5 fibre replaced with the Ad35 fibre that expresses EGFP under the control of a CMV-promoter. (Prof M Tagawa, Chiba, Japan)	8.2×10^{10} FFU/ml
Ad5f35-CFP	E1-and-E3-deleted Ad5 virus with the Ad5 fibre replaced with the Ad35 fibre that expresses CFP in place of the E3 gene. (James Findlay, University of Leeds)	6×10^6 CFU/ml
Ad5f35-CFP-tdnTGFβRII.1	E1-and-E3-deleted Ad5 virus with the Ad5 fibre replaced with the Ad35 fibre that expresses the dominant negative TGFβ receptor II under the control of a CMV-promoter. CFP is also expressed in place of the E3 gene.	1.4×10^7 CFU/ml and 2.1×10^7 CFU/ml
Ad5f35-CFP-tdnTGFβRII.2	E1-and-E3-deleted Ad5 virus with the Ad5 fibre replaced with the Ad35 fibre that expresses the dominant negative TGFβ receptor II and a mixed population of flag under the control of a CMV-promoter. CFP is also expressed in place of the E3 gene.	3×10^7 CFU/ml
MV-GFP	Edmonton strain of measles virus with the incorporation of GFP. (Dr Fiona Errington-Mais, University of Leeds)	1×10^7/ml
MVA-GFP	Modified Vaccinia Virus Ankara stain of the pox viruses with the incorporation of GFP. (Jenner Institute, University of Oxford).	1×10^8 PFU/ml

3.2 Methods

3.2.1 Cell culture

All cell lines were grown in a Sanyo 37°C humidified incubator with 5% CO_2 and passaged every 2-4 days in $25cm^2$, $75cm^2$ or $150cm^2$ flasks (Corning Life Sciences) using standard aseptic techniques in Nuaire Class II Microbiological Safety Cabinets. 50 ml or 15 ml sterile polypropylene tubes (BD Falcon) were used for harvesting and washing cells. Cells were plated in 6, 12, 24, 48 and 96 well plates (Corning Life Sciences) for assays as indicated. The cell culture media: DMEM with 4500mg glucose/L, 110mg sodium pyruvate, 2mM L-glutamine and RPMI-1640 with L-glutamine and sodium bicarbonate and Minimum Essential Medium Eagle Alpha Modification, with sodium bicarbonate, without L-glutamine, ribonucleosides and deoxyribonucleosides were all purchased from Sigma-Aldrich. Unless otherwise indicated all media were supplemented with foetal calf serum (FCS) (PAA Cell Culture Company) and where indicated, 100 μg/mL penicillin/streptomycin solutions (HyClone™, distributed by GE healthcare life sciences, Buckinghamshire, UK). FCS was heat inactivated prior to use at 56°C for 20mins. All media were filter sterilised by using a bottle-top vacuum filter system (Corning Life Sciences) before use.

3.2.1.1 Adherent Cell lines

During passage, HeLa, A549, HaCaT, 911 and HEK293 cell lines were washed with PBS, detached with 1x trypsin-EDTA for 10mins and fresh medium added before cells passaged at a dilution ratio of 1:4.

3.2.1.2 Suspension Cells

NKL, YT and K562 were maintained in RPMI-1640 media and 10% FCS. NKL were stimulated with 100IU/mL IL-2 (3.1.5). NK-92MI was maintained in Minimum Essential *Medium* (Sigma Aldrich), 2mM L glutamine, 0.2mM inositol, 0.02mM folic acid, 1mM mecaptoethanol, 12.5% horse serum. NK cell lines and K562 were passaged at a dilution ratio of 1:3 and 1:4 respectively.

3.2.1.3 Cryopreservation

Cells were harvested and pelleted by centrifugation at 300xg for 5min. The cell pellets were re-suspended in (90% (v/v) FCS; 10% (v/v) dimethyl sulphoxide (DMSO, sigma)) and stored in 1 ml cryovials (Nunc®, Fisher Scientific, Hampshire). Cryovials were stored at −80˚C in a Mr Frosty freezing container which freezes cells at a rate of -1˚C per min, before being transferred to liquid nitrogen within one month. Cells were thawed quickly in a 37˚C water bath, re-suspended in growth medium and placed into a culture flask.

3.2.1.4 Isolation of human PBMC using density gradient separation

PBMCs were isolated from healthy individuals received from NHS blood and transplant leucocyte cone donations. The blood was retrieved from the cone by using a syringe containing an equal volume of PBS to flush out the cells, 30ml of diluted sample was then layered on top of 15ml of lymphoprep (Axis-Shield, Oslow, Norway). PBMCs were separated by density gradient separation at 800xg for 20 min at RT, no brake. The PBMC fraction between the plasma and ficoll fraction was removed using a pasteur pipette (Figure 3-1). PBMCs were washed with PBS and centrifuged at 220xg for 15 minutes, RT, to remove platelets, before counting using a haemocytometer. PBMCs were re-suspended in RPMI supplemented with 10% FCS at $2x10^6$ cells/ml or re-suspended in cold MACS buffer (0.5% w/v bovine serum albumin (BSA) and 2mM sodium EDTA in PBS) for NK cell isolation.

Plasma layer containing platelets, cytokines, hormones and electrolytes

Layer containing lymphocytes and monocytes

Layer containing erythrocytes, eosinophils and neutrophils

Cell density of <1.077g/ml
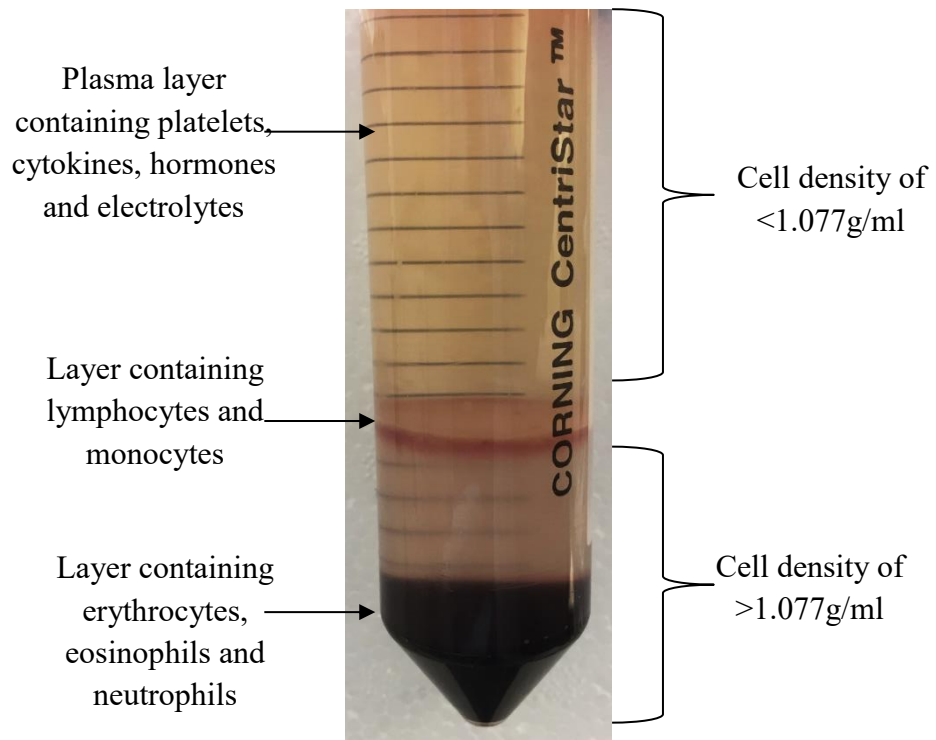
Cell density of >1.077g/ml

Figure 3-1 PBMC isolation using lymphoprep

The isolation of PBMCs is carried out by density gradient centrifugation in combination with a specialised separation medium i.e. lymphoprep solution. Lymphoprep has a density of 1.077g/ml which pushes lighter cells, such as mononuclear cells above the solution while separating the higher density cells (e.g. red blood cells) to the bottom of the tube.

3.2.1.5 Primary Cells

PBMCs were maintained in RPMI-1640 media, supplemented with 10% FCS and stimulated with 50IU/mL IL-15 (Miltenyi). Primary NK cells were maintained in DMEM, supplemented with 10% human AB serum and stimulated with 50-100IU/mL IL-2 (Miltenyi), unless otherwise stated.

3.2.1.6 NK cell isolation by magnetic cell sorting

NK cell isolation was carried out according to manufacturer's instructions. Briefly, 1.1×10^8 PBMCs were resuspended in 440μl MACS buffer and 110μl of biotinylated antibodies and incubated at 4°C for 5 mins. PBMCs were then resuspended in a further 330μl MACS buffer and 220μl of magnetic beads and incubated at 4°C for 10 mins. 15ml of MACS buffer was added to dilute out the excess beads and antibodies, and the solution was centrifuged 300xg for 10 mins. The pellet was resuspended in 500μl of cold MACS buffer. During centrifugation a LS column was placed on a magnet containing LS column stand, equilibrated with 3ml of cold MACS buffer, the cell suspension was then passed through the LS separation column. Labelled cells were retained within the column, allowing unlabelled NK cells to pass through. The column was washed three times with 3ml of MACS buffer. The flow through containing isolated NK cells were pooled and resuspended at 1×10^6/ml in filtered DMEM media supplemented with 10% human AB serum and 10% FCS.

3.2.2 Transfections

3.2.2.1 Bacterial artificial chromosome (BAC)

911 cells were seeded at a density of 1.5×10^6 in a T25 flask in 3ml of media the day before transfection. On the day of transfection cells were checked for 80-90% confluency. Approximately 5µg of BAC construct was diluted in a total volume of 500µl of OptiMEM (Invitrogen), and 20µl lipofectamine 2000 (Invitrogen) was diluted in 480µl OptiMEM and incubated at RT for 5min before both solutions were mixed and incubated at RT for 20min. Cells were washed in PBS and supplemented with 2ml of optimem. The transfection mix was added to flasks and mixed by gently rocking. Cells were incubated at 37°C for 6hrs before replacing with DMEM containing 10% FCS. Cells were harvested 7-10 days post-transfection and stored at -80°C as lysates.

3.2.2.2 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

HeLa cells were seeded at an optimised density of 1×10^5 in a 6 well plate in a volume of 3ml media the day before transfection. For optimising transfection, PEI (Polysciences Inc, Germany) transfection reagent was used. Briefly, 2µg of CRISPR plasmid DNA or plasmid-EGFP positive control or PBS negative control was mixed with 8µg PEI in 100µl DMEM serum free media and left at room temperature for 5min before adding dropwise to the cells. Cells were incubated at 37°C 5% CO_2 for 24 hours post-transfection.

3.2.2.3 Luciferase assay

To determine inhibitory function of selected genes, HaCaT cells were seeded in a 24 well plate at a density of 5×10^4 cells per well one day prior to transfection. In each transfection the luciferase gene expression was dependent on a TGFβ promotor response element, p3TP-lux. Each transfection was controlled for with the co transfection of the Renilla construct, pRL-TK Vector. The activity of the TGFβ promotor was determined in the presence of TGFβ stimulation, and in the presence of plasmid vectors expressing Smad6, Smad7 and dominant negative TGFβ receptor II (Table 3-6 Luciferase plasmids). Briefly, 0.3µg of p3TP-lux and pRL-TK was used, either together or in combination with 1µg Smad6, Smad7 or dominant negative TGFβ receptor II expressing plasmids. Plasmid were mixed with 5-6µg PEI in 100µl DMEM serum free media and left at room temperature for 5min before adding dropwise to the cells. After 6hrs the cells were washed and complete medium was added incubated at

$37°C$ 5% CO_2. At 24hrs post-transfection cells were treated with TGF-β, as specified and the cells analysed for luciferase output at 48hrs post-transfection. The Dual-luciferase reporter assay system (Promega) was used to detect output according to manufacturer's protocols.

3.2.3   Virus transductions

3.2.3.1   Adherent cell line A549

Cells were seeded one day prior to transduction. The following day the medium was removed and the cells washed with PBS and replenished with DMEM serum free media. Variable amounts of virus was added and the cells were incubated for 1 hour at $37°C$. After 1 hour, complete media was added and the cells incubated for a further 24 hours or longer as required.

3.2.3.2   Lymphoid cell lines and primary cells

Primary NK cells were pre-activated with IL-2 the previous day. All cells were pelleted and washed with PBS and seeded at a density of 2.5×10^5 in 125µl in a 24 well plate. Approximately 500µl of DMEM serum free media and variable amounts of virus (MVA-GFP, MV-GFP and Ad viruses, see Table 3-8) added, mixed and transferred to a 24 well plate. The cells were incubated for 1hr. After this incubation period 125µl of complete media was added and the cells were incubated for 24-48hrs and analysed at 24-48hrs post-transduction by flow cytometry.

Transduction was further optimised to include a centrifugation step, post-virus addition. For experiments in Chapter 5 (unless stated otherwise), cells were washed in PBS, seeded at $0.3-2.5 \times 10^5$ at 125µl in a 24 well plate. Variable amounts of adenovirus was added (as stated) and centrifuged at 1,600xg for 45min at RT. The cells were cultured for 3hrs and activated with IL-2 and 125µl of complete medium. EGFP/CFP expression was determined by flow cytometry between 1-7 days post-transduction.

3.2.4   CD46 Blocking

A549 cells were seeded at 2×10^5 in a 6 well plate the day before transduction. Primary NK cells were isolated the day before transduction. All cells were washed in PBS and resuspended in serum free media. Cells were either treated with a monoclonal CD46 antibody (see Table 3-3 Flow cytometry antibodies) or a rabbit IgG (cell signalling, The Netherlands). Ad5f35-EGFP was immediately added to cells and the subsequent

virus transduction protocol followed 3.2.3. Control samples also include a combination of untransduced and transduced cells, with and without the combination of antibodies. All cells were analysed 24hrs post-transduction by flow cytometry.

3.2.5 Flow cytometry

All FACS acquisition was carried out using a LSRII flow cytometer (BD) and analysis on FACSDiva™ software (BD).

3.2.5.1 Cell surface staining

Approximately $0.03\text{-}1x10^6$ cells were harvested into FACS tubes (BD), washed with 1 ml PBS and pelleted by centrifugation at 300xg for 5mins. Cells were re-suspended in 100µl FACS buffer containing fluorescently conjugated antibody (see Table 3-3 Flow cytometry antibodies) and incubated on ice for 20 mins. The cells were washed in 3ml FACS buffer and centrifuged, then re-suspended in 250µl FACS buffer. In experiments involving transduced cells, cells were resuspended in 250µl 1% PFA (1% (w/v) paraformaldehyde in PBS) and incubated at RT for 10min. Approximately 10,000 events per sample was acquired.

3.2.5.2 EGFP/GFP expression

EGFP expression was determined post-transduction with Ad, MV or MVA in A549, NK cell lines and primary NK cells. Approximately $2.5x10^5$ cells were harvested into FACS tubes, washed with 1 ml PBS and pelleted by centrifugation. Cells were re-suspended in 100µl FACS buffer and kept on ice. A549 and NK cell lines were stained for cell viability alone. Primary NK cells were incubated with CD56 and CD3 antibody on ice for 20 mins. The cells were then washed by the addition of 3ml PBS and centrifuged and stained for cell viability. Cells were fixed with 250µl 1% PFA and stored at 4ºC prior to acquisition. Approximately 10,000 events per sample was acquired. In analysis viable cells were gated for cell populations. Primary NK cells were gated for CD56 and the lack of CD3 expression (Figure 3-2). EGFP excitation was measured using the 488nm laser. In the no virus negative control a second gate was set in the GFP channel at 2% of the cell population, cells falling within this gate represented EGFP/GFP positive cells.

3.2.5.3 CFP expression

CFP expression was determined in cells transduced with Ad5f35-CFP in A549, NK cell lines and primary NK cells. Approximately $2.5x10^5$ cells were harvested into

FACS tubes, washed with 1 ml PBS and pelleted by centrifugation at 300xg for 5mins. The cell pellet were re-suspended in 100µl FACS buffer and kept on ice. A549 and NK cell lines were stained for cell viability alone. Primary NK cells were incubated with CD56 and CD3 antibody on ice for 20 minutes. The cells were then washed by the addition of 3ml PBS and centrifuged and stained for cell viability. Cells were fixed with 250µl 1% PFA and stored at 4ºC prior to acquisition. Approximately 10,000 events per sample was acquired. In analysis viable cells were gated for cell populations. Primary NK cells were gated for CD56 and the lack of CD3 expression (Figure 3-2). CFP excitation was measured using a 405nm laser. In the negative control (no virus) a second gate was set to include 2% of cells with EGFP/GFP background emission to determine the CFP fluorescence in transfected cells.

3.2.5.4   DasherGFP expression on CRISPR transfections

DasherGFP/EGFP expression was determined by flow cytometry at 24 and 48 hours post-transfection. Approximately $1x10^5$ cells were harvested and centrifuged, re-suspended in 250µl FACS buffer and kept on ice. Approximately 10,000 events per sample was acquired and analysis of DasherGFP excitation was measured using a blue laser. In the negative control (no plasmid) a second gate was set to include 2% of cells with DasherEGFP background emission to determine the GFP fluorescence in transfected cells.

3.2.5.5   Cell viability

Cell viability was determined using Live/Dead discrimination by zombie (Biolegend) staining which reacts with the primary amine group on proteins. Dead cells have an impaired cell membrane, increasing the zombie dye to bind to cytoplasmic proteins and producing a brighter fluorescent output compared to viable cells. Cells were harvested into FACS tubes, washed with 1 ml PBS and pelleted by centrifugation. Cells were then re-suspended in 100µl of 1/200 dilution of Zombie NIR (excited by the 633nm red laser, which emits into the APC-Cy7 channel). Cells were stained for 30 mins at RT in the dark, washed once with 2 ml PBS and pelleted, before being washed with 2 ml PBS/1% (v/v) FCS and fixed with 250µl 1% PFA. Where indicated, cell viability staining was carried out on cells that were pre-stained with fluorescently-conjugated antibodies for cell surface antigens. Prior to acquisition cells were stored at 4ºC.
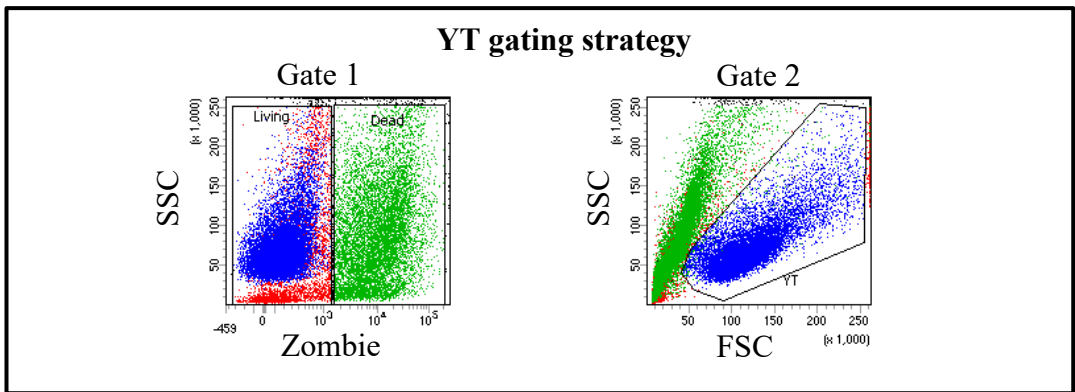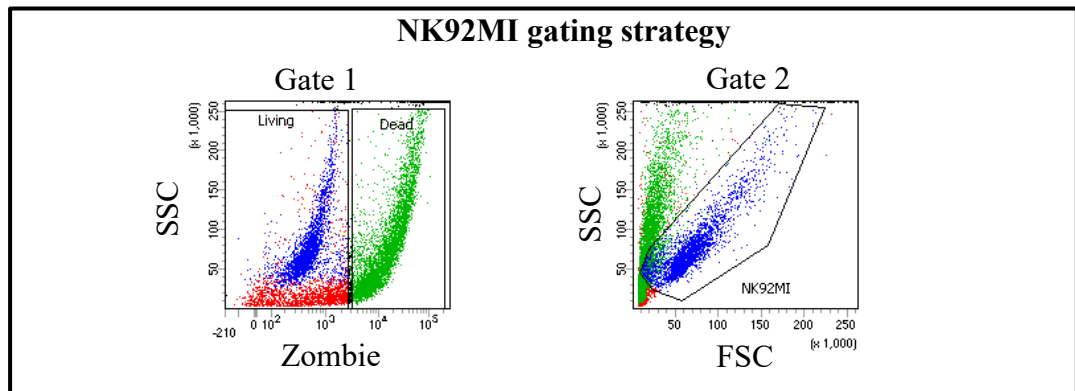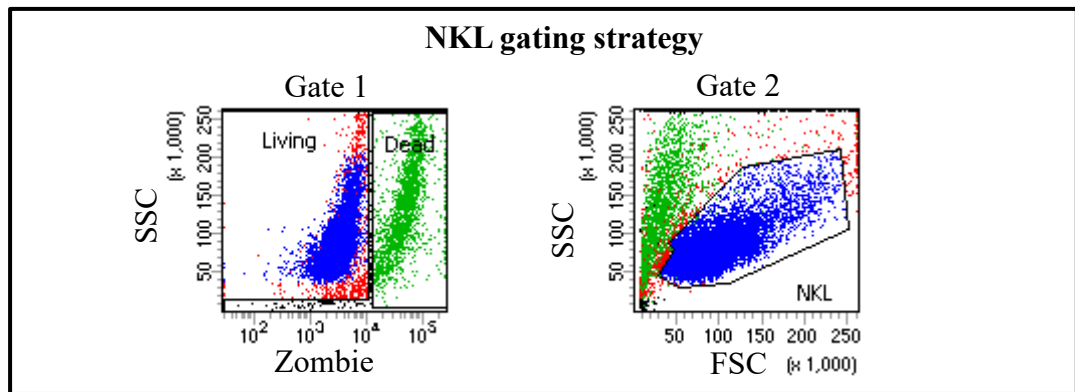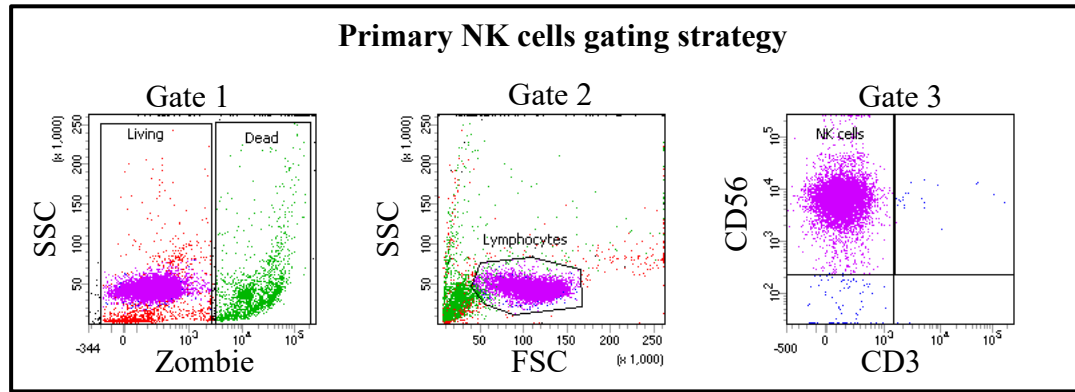
3.2.5.6 Hexon Staining

All cells were harvested and washed in PBS. Cells were resuspended in 200µl of PBS and split between two 1.5ml Eppendorf tubes, before centrifuged and fixing in 100µl of 1% PFA, and incubated for 20min at RT. The cells were subsequently centrifuged and the permeabilised by resuspending in 100µl 1% Triton X-100 in PBS, and incubated for 5min at RT. Following centrifugation, permeabilised cells were resuspended in 10% normal goat serum (vector labs) in PBS and incubated for 10min at RT. Cells were centrifuged and the pellets were either resuspended in mouse monoclonal antibody (clone 2Hx2) at a dilution of 1:1000 or resuspended in the appropriate isotype (Sigma M5409) in 1% NGS, 0.1% Triton X-100 in PBS, and incubated for 1hr at RT. The cells were centrifuged and the pellet was resuspended in 50µl of Alexa 647-conjugated goat anti-mouse immunoglobulin (Invitrogen) diluted in 1% NGS, 0.1% Triton X-100 in PBS. Cells were washed and resuspended for analysis by flow cytometry.

3.2.5.7 SMAD2/3 Staining

Cells were pre-treated with cytokines; for TGF-β, cells were treated 30min prior to analysis. Cells were harvested and fixed with an equal volume of pre-warmed cytofix buffer (BD) and incubated at 37°C for 10min in a water bath. Cells were centrifuged and the supernatant discarded. The cells were permeabilised with the addition of 1ml of ice-chilled perm buffer III (BD). Cells were mixed and incubated for 30min. Cells were centrifuged and resuspended in FACS buffer containing SMAD2/3-P -PE (BD) or IgG isotype control and incubated in the dark for 50min. Cells were washed in 3ml FACS buffer and resuspended in 250µl FACS before acquisition.

3.2.5.8 Gating strategies

Primary NK cells gating strategy

Gate 1 · Gate 2 · Gate 3

NKL gating strategy

Gate 1 · Gate 2

NK92MI gating strategy
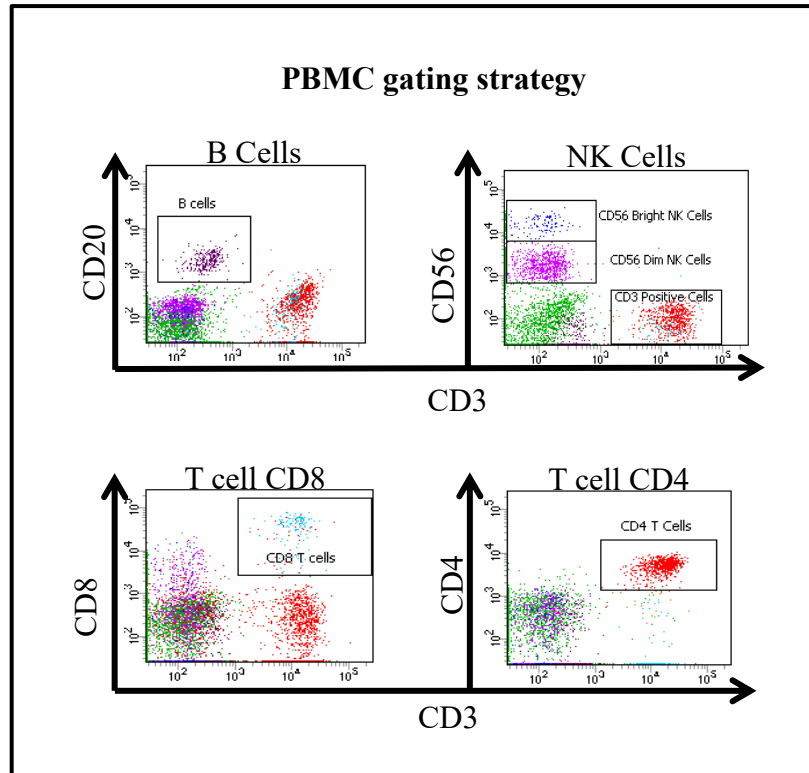
Gate 1 · Gate 2

YT gating strategy

Gate 1 · Gate 2

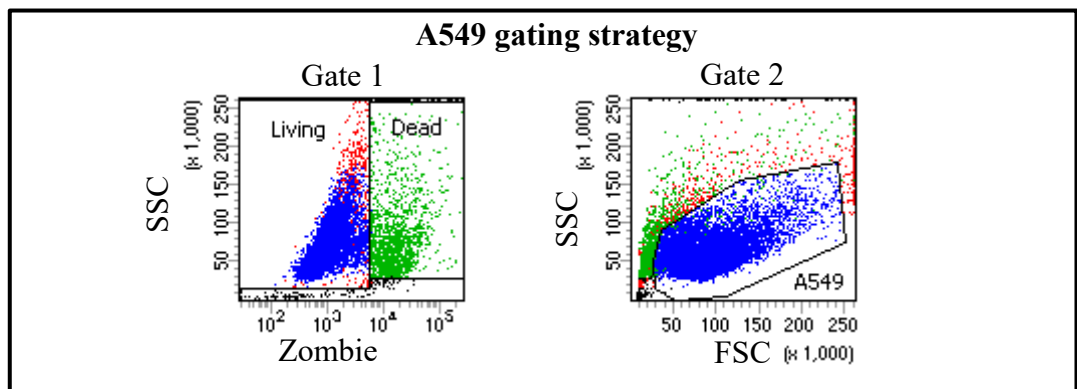**Figure 3-2 Gating strategy PBMCs, NK cells and NK cell lines.**

Figure 3-3 Gating strategy for A549 cells.

3.2.6 Molecular techniques

3.2.6.1 PCR

Table 3-9 PCR cycle setup

PCR used in CRISPR study	
PCR Reaction	**PCR Cycle**
5X HF Buffer (with MgCl2) 5 µl	1) Initial step 98 °C 30s
10mM dNTPs 0.5 µl	2) Denaturation step 98 °C 10s
10mM Forward primer 1.25 µl	3) Annealing step 57 °C 30s
10mM Reverse primer 1.25 µl	4) Elongation step 72 °C 30s
DMSO 0.75 µl	5) Final elongation step 72 °C 10 min
1 unit/25µl reaction Phusion DNA polymerase 0.25 µl	Steps 2 – 4 repeated 30 cycles
20ug/ml DNA 5 µl	
ddH20 11 µl	
Total volume 25 µl	
PCR used to generate DNTGFβRII insert	
HiFi PCR reaction	**PCR cycle:**
PCR reaction	1) Initial 94˚C for 2 minutes
1 x Expand HiFi Reaction Buffer 1 (with MgCl2) 2.5µl	2) Denaturation step 94˚C for 15 seconds
0.2 mM dNTPs 1µl (Invitrogen)	3) Annealing step 55-65˚C for 30 seconds
10mM Forward primer 2µl	4) Elongation step 72˚C for 1 minute / kb
10mM Reverse primer 2µl	5) Denaturation step 94˚C for 15 seconds
DNA 50ng 3µl	6) Annealing step55-65˚C for 30 seconds
1.25 units HiFi DNA polymerase 0.2µl	7) Elongation step 72˚C for 1 minute / kb (+ 5 seconds/cycle)
ddH20 14.3µl	8) Final elongation step 72˚C for 7 minutes
Total volume 25 µl	Steps 2 – 4 repeated 10 times, steps 5 – 7 repeated 20 cycles
PCR used for colony PCR and generation of sequencing template for DNTGFβRII	
Taq PCR reaction	**PCR cycle:**
PCR reaction	1) 98˚C for 2 minutes
1 x Taq Buffer (with MgCl2) 2.5µl	2) Denaturation step 98˚C for 15 seconds
10mM dNTPs 0.1µl	3) Annealing step 55˚C for 30 seconds
10mM Forward primer 2µl	4) Elongation step 68˚C for 1 minute / kb
10mM Reverse primer 2µl	5) Final elongation step 68˚C for 10 minutes
0.625 U Taq DNA polymerase 0.125µl	Steps 2 - 4 repeated 35 cycles
DNA (5µl colony mixture or 50ng BAC template)	
ddH20 (variable)	
Total volume 25µl	

3.2.6.1.1 E1A PCR

PCR was carried out according to the Taq polymerase (NEB, Hitchin, UK) manufacturer's protocols for amplifying *E1a* exon one.

3.2.6.1.2 PCR Generating DNTGFβRII

A gradient PCR was carried out according to the HiFi polymerase (Roche, Burgess Hill, UK) manufacturer's protocols for amplifying DNTGFβRII.

3.2.6.1.3 Colony PCR and PCR to generate template for sanger sequencing of DNTGFβRII

For colony PCR, one colony was picked and vortexed in 50µl of LB medium without antibiotic. 5µl of the resuspended colony was used for DNA template and PCR was carried out using Taq polymerase. For sequencing, a PCR was performed using Taq DNA polymerase to amplify DNTGFβRII and the flanking adenovirus sequence. The amplified product was extracted from agarose gel and used for sequencing.

3.2.6.1.4 CRISPR study

PCR was optimised and performed using Phusion DNA polymerase (NEB) for amplifying *B2M* exon one for Sanger sequencing.

3.2.6.2 BAC Electroporation

SW102 cells were grown in 5 ml half-salt LB medium (supplemented with 12.5mg/ml chloramphenicol) in a 32˚C shaking incubator overnight. A 0.5 ml aliquot of this culture was used to inoculate 25ml half-salt LB medium (supplemented with 12.5mg/ml chloramphenicol) and incubated in a 32˚C shaking incubator until the OD600 was 0.6. The cells were transferred to a 50ml flacon tube and incubated in a 42˚C water bath for 15min with frequent mixing by inverting to induce recombination genes. The cells were transferred to ice for 15 min and centrifuged for 5 minutes at 4,000 rpm at 0˚C. The supernatant was removed and cells were resuspended in 1ml ice-cold ddH$_2$O by gentle swirling. A further 19 ml of ice-cold ddH$_2$O was then added and the cells centrifuged for 5 min at 4,000 rpm at 0˚C. The supernatant was removed and the washing process repeated. After this centrifugation, the supernatant was removed and the cells were resuspended in the residual H2O. Approximately 25µl of cells and >10ng of PCR product were transferred to pre-chilled 0.2 cm electroporation cuvettes and electroporated in a Biorad Gene Pulsar X cell electroporater at 2.5kV

with a time constant of approximately 5ms. The cells were recovered in 1ml half-salt LB for 4hrs in a 32˚C shaking incubator. The cells were then washed and resuspended in 1ml M9 salts before being plated on recombineering plates and incubated at 32˚C for 2-5 days (Figure 3-4).

3.2.6.3 BAC isolation

Single colonies of *E. coli* SW102 strain containing the BAC were picked and grown overnight in 5ml half-salt LB medium supplemented with 12.5μg/ml chloramphenicol in a 32˚C shaking incubator. Cells were then transferred to a 50ml suspension in a conical flask and incubated on a shaker overnight at 32°C. BAC was purified with Qiagen Maxi prep kit (Qiagen, Crawley, UK). Protocols were followed according to manufacturer's instructions. Briefly 50ml of culture was pelleted and suspended in buffer and lysed with buffer. The supernatant was loaded into a spin column and washed several times. The DNA was eluted in 150µl and the quantity of DNA was measured on a NanoDrop (NanoDrop™).
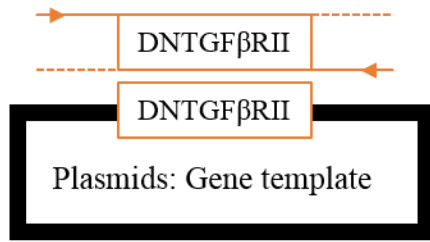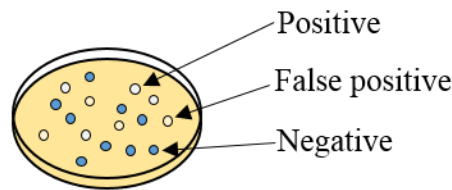
1. Generation of DNTGFβRII insert with flanking adenovirus sequence using long primers with adenovirus sequence to generate homology in preparation of recombineering
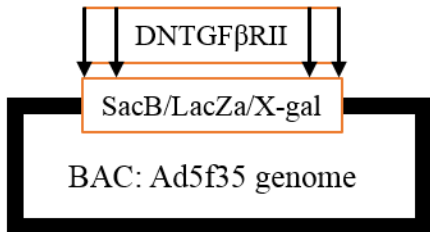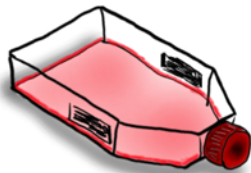
2. Recombineering

2. Screening for successful recombinants (1 week)

Positive

False positive

Negative

3. Propagation and isolation of E. Coli with BAC containing DNTGFβRII (3 days)

4. Purified BAC transfected into helper cells (14 days). Cell lysate used to propagate virus in more cells on a large scale (30xT175) in preparation of isolation (1 month)

Figure 3-4 Construction of BAC containing DNTGFβRII.

The gene fragment was synthesised by PCR with flanking adenovirus sequence. This allows for successful recombination into the virus genome contained in the BAC of *E. coli*. The gene 'recombineers' adjacent to the CMV promotor and replaces a cassette of genes that encode selection for LacZa and sucrose sensitivity. Positive selection is determined by screening white colonies for the presence of the gene by PCR; which subsequently leads to propagation and purification of the virus.

3.2.6.4 Plasmid Isolation

1.1.1.1.1 CRISPR plasmids

The five CRISPR-Cas9-B2M-DasherGFP constructs were provided by Adgene Horizons as part of the free reagents programme (see Table 3-7 CRISPR plasmids). Plasmid DNA was bulked up by transformation into DH5α competent *E.coli* bacteria (NEB), cultured and purified using Qiagen plusmidiprep kits. Bacteria were transformed as follows: 1µg of plasmid DNA and 50µl of competent *E.Coli* were mixed in a 1.5ml Eppendorf tube and incubated on ice 20 mins, heat shocked for 45s in a 42°C water bath, followed by a further 2 mins on ice before the addition of 250µl of LB Broth and incubated in a 37°C shaker (200 rpm) for 1hr. 100µl of transformed bacteria were spread on agar plates containing 50µg/ml ampicillin and placed in an incubator at 37°C overnight. Single colonies were picked and transferred to a 3ml suspension of LB medium containing 50µg/ml ampicillin and incubated on a shaker (200rpm) at 37°C for 8 hours before being transferred to a 30ml suspension a conical flask and incubated on a shaker overnight at 37°C. Plasmid was purified with Qiagen Midi prep kit, as described in (3.2.6.4.1).

3.2.6.4.1 Luciferase plasmids

DNA (Table 3-6 Luciferase plasmids) was purified from expanded single cell colonies with Qiagen Midi prep kit. Protocols were followed according to manufacturer's instructions. Briefly 30ml of transformed culture was pelleted and suspended in buffer and lysed with buffer. The supernatant was loaded into a spin column and washed several times. The DNA was eluted in 200µl and the quantity of DNA was measured on a NanoDrop (NanoDrop™).

3.2.6.5 Restriction endonuclease digestion

Purified BAC DNA was incubated with 1unit of BamHI-HF restriction endonuclease and 1x cut smart buffer (NEB). Reactions were incubated at 37˚C for 90min before separation by gel electrophoresis.

3.2.6.6 Gel electrophoresis

Gel electrophoresis was used to resolve PCR products, DNA digests or plasmids/BACs. A 0.8 to 2% (w/v) agarose gel was made by dissolving agarose (Bioline, London, UK) in 1x buffer TBE. In Chapter 6, in the CRISPR section, 0.5ug/ml ethidium bromide (Alfa Aesar) was used to visualise the gel. For

DNTGFβRII related experiments, the DNA was loaded onto the gel with Orange-G loading buffer (at 1X final concentration) and SYBR green (1/10 000 dilution of stock) in all samples. Along with 2-log DNA ladder (NEB). All gels were placed in an electrophoresis tank filled with 1X TBE buffer and electrophoresis performed under constant voltage, 120V for small gels and 150V for large gels. For CRISPR related experiments 1 kb DNA ladder or 100bp DNA ladder (NEB) was used. All Gels were imaged on BioRad imaging Fujifilm FLA-5000.

3.2.6.7 Gel extraction of PCR products

PCR products were isolated from an agarose gel and purified following manufacturer's instructions from the Qiagen Gel Extraction Kit. Briefly, the DNA fragment on the agarose gel was visualised under a UV-light box and cut out using a scalpel. The agarose was dissolved in Buffer QG, loaded on to a QIAprep spin column, washed in Buffer PE and the DNA eluted in 30µl Buffer EB.

3.2.6.8 Sanger Sequencing

Sanger sequencing of *B2M* exon 1 from HeLa cell line manipulated with CRISPR was carried out by Eurofins genomics, Germany. The following was sent in a SmartSeq pre-labelled bar code tube: 15µl of 1ng/µl PCR products, 10pmol/µl of 2µl forward and reverse oligonucleotides each. Sanger Sequencing of the TGF-β receptor II gene was carried out by GATC (Germany). Pre-labelled coded tubes containing 20µl of 120ng of BAC DNA and a matching labelled tubes containing 10pmol/µl of 2µl forward and reverse oligonucleotides.

3.2.7 Western blotting

3.2.7.1 Sample preparation

NK cells were either pre-treated with cytokines or transduced with Adenovirus, as specified. A549 were pre-transduced with adenovirus. All cells were counted and equal numbers of cells were harvested, washed in ice cold PBS and centrifuged at 4°C. The supernatant was discarded and the cells were re-suspended in 50µL RIPA buffer supplemented with protease inhibitor (Roche, Basel, Switzerland) and phosphatase inhibitor (Roche) according to the manufacturer's guidelines. Cells were incubated on ice for 10 mins. Where indicated, the supernatant of adenovirus transduced A549 cells were harvested and concentrated by centrifugation. All samples had loading buffer added at equal volumes and heated to 95°C for 5-15 min to denature proteins before being used immediately or frozen at -20°C.

3.2.7.1.1   Cell membrane biotinylation

A549 cells were pre-transduced with virus, where indicated in a 6 well-plate. Cells were washed in PBS three times and incubated for 45min on ice with gently shaking in 125µl biotin (2mM $MgCl_2$, 2mM $CaCl_2$, 0.3mg/ml biotin (NHS-LC-LC-Biotin, Thermo Fisher). Cells were washed in TBS, followed by PBS. Cells were lysed in RIPA buffer on ice for 1hr, harvested and centrifuged. The cell lysate was incubated in 40µl of streptavidin beads (Pierce Streptavidin Agarose, Thermo Fisher) on a rotating wheel at 4^0C for 3hrs and washed in PBS. Cell lysates were centrifuged and resuspended in loading buffer and heated to 95°C for 5-15 min to denature proteins.

3.2.7.2   Western blotting

Samples were separated on a 15% poly-acrylamide gel, prepared from: ddH_20; 30% acrylamide mix; 10% (w/v) SDS; 1.5 M Tris, pH 8.8 (running gel); 1M Tris, pH 6.8 (stacking gel); 10% (w/v) ammonium persulphate (APS) and tetramethylethylenediamine (TEMED). The running gel was immediately poured into a gel cassette and layered with 100% isopropanol to remove air bubbles and prevent it from drying out. Once set, the isopropanol was removed and the stacking gel was added to the cassette and the comb inserted. 18µl of each sample was loaded along with 3µl of SeeBlue®Plus prestained standard protein molecular weight marker (Invitrogen) to verify protein size. Gels were run in a cell tank, containing running buffer for approximately 90 mins at 140V until the loading dye had run to the bottom of the gel. The gels were then removed from the cassettes and briefly washed in transfer buffer. The gel was sandwiching between a sheet of polyvinylidene difluoride (PVDF), which had been pre-activated in methanol, and two pieces of Whatman 3 MM Chromatography paper that had been pre-soaked in transfer buffer. The proteins were transferred at 15 V for 60min using a semi-dry transfer apparatus (Bio-Rad, Hampstead, UK). The PVDF membrane was briefly washed in PBS and then incubated in blocking buffer; 5% milk in TBST for 45-60min at RT.

3.2.8   Probing and development

Primary antibodies were added to blocking buffer and incubated for 1hr at RT or overnight at 4°C on a shaker, as specified. The membrane was washed for 5 min 3 times with TBST to remove the excess primary antibodies. Secondary antibodies were added in blocking buffer and incubated for 1hr at RT on a shaker. The membrane was washed again for 5 mins 3 times with TBST to remove the excess secondary

antibodies. PVDF membranes were developed and analysed using a chemiluminescence reagent (GE Healthcare, UK), a light proof cassette, CL-XPosure film (Thermo-Fisher Scientific) and a Konica SRX-101A Tabletop X-ray Film Processor (Konica, UK). The expression of β-actin was used to assess the equal loading of protein samples.

3.2.9 Adenovirus propagation and isolation

All adenovirus vectors were propagated in 911 cells. The lysate from 3.2.2.1 was subjected to three cycles of freeze thawing and sonication (in a bath-type sonicator) and used to infect 3 x T175 flasks. Following 3-5 days post-infection, the cells and supernatant were harvested, lysed and used to infected 30-40 x T175 flasks (Figure 3-4). The cell lysates were centrifuged and the pellets pooled resuspended in a total volume of 10 ml 0.1M Tris-HCl (pH 8.0). The cell lysates were incubated with 1 ml 5% sodium deoxycholate for 30 minutes at room temperature until viscous, followed by incubation with 100µl 2M $MgCl_2$ and 0.5mg DNase I for 30 minutes at 37°C to digest DNA. The cellular debris was pelleted by centrifugation at 3000 rpm for 15 minutes at 4°C and the supernatant containing virus was harvested. The supernatant was layered on top of a CsCl gradient in Beckman Ultra-Clear Centrifuge Tubes (14x95mm, 344060). The gradient consisted of a lower phase 1ml 1.5d CsCl, middle phase 2.5ml 1.35d CsCl and upper phase 2.5ml 1.25d CsCl. The tubes were centrifuged at 35,000xg for 1hr at 10°C in a SW40 rotor in a Beckman L5-50B centrifuge machine with minimal acceleration and brake. The lowest band containing virus was removed using a sterile Pasteur pipette and diluted with 500µl 0.1M Tris-HCl (pH 8.0). This was layered on top of 2.5ml 1.35d CsCl in a Beckman Ultra-Clear Centrifuge Tube (13 mm x 55 mm). This tube was centrifuged at 35,000xg for 12hrs at 4°C in a SW55 rotor in a Beckman L5-50B centrifuge machine with minimal acceleration and no brake (Figure 3-5). The virus band was removed using a sterile Pasteur pipette and dialysed against 10mM HEPES-KOH (pH 8.0) in Slide-a-Lyser dialysis cassettes 7000 MWCO at 4°C for 8 hours. Glycerol was added to 10% (v/v) and the virus divided into 40µl aliquots and stored at -80°C. To determine the number of CFP expressing virus particles, A 24-well plate of A549 cells were transfected with various dilutions. At 24 hours post-transduction cells were analysed for the expression of CFP by flow cytometry. A gate for CFP-positive cells was set up on untransfected cells. A virus dilution which infected around 50% of cells was used to calculate CFP focus forming units (CFU) per ml. The percentage of CFP positive cells was

multiplied by the number of cells in a well and then multiplied by the dilution factor. The result was presented in CFU/ml.
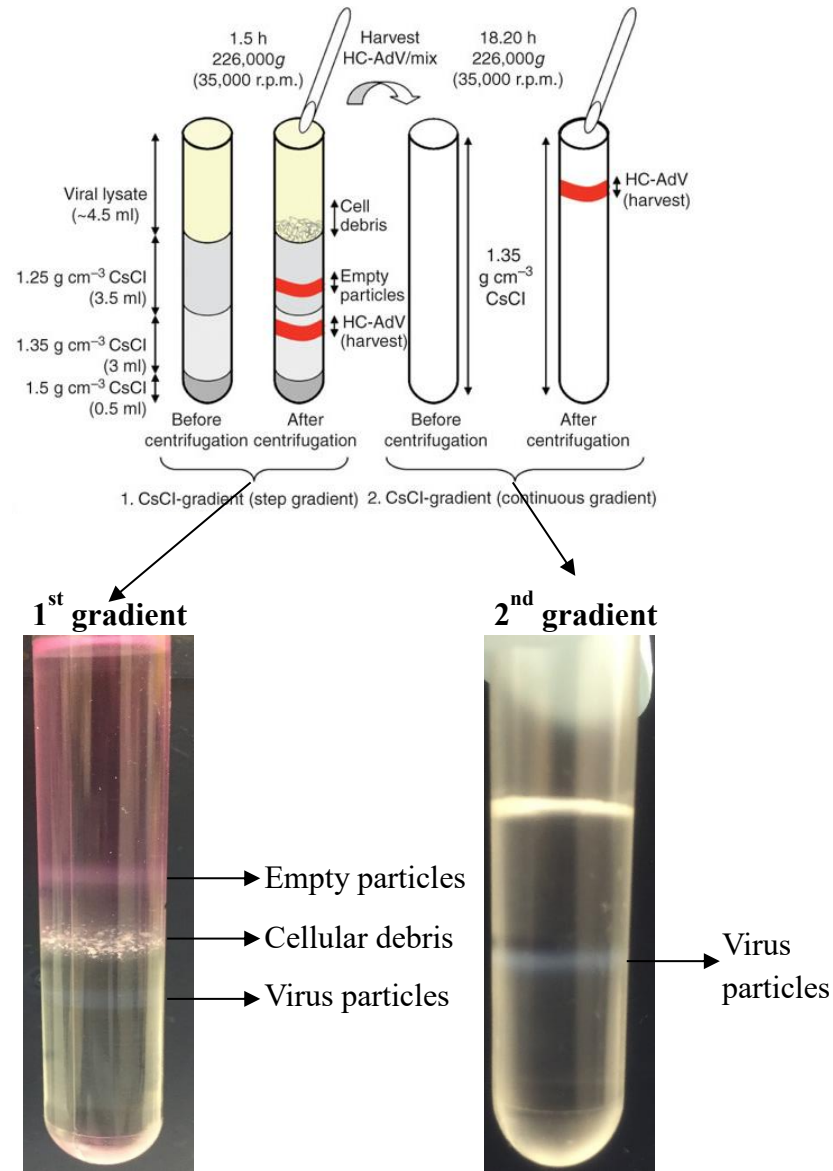

Figure 3-5 CsCl purification of adenovirus.

Adapted from Jager (322).

3.2.10 NK cell functional assays

3.2.10.1 CD107a degranulation assay

NK cell degranulation was determined by cell surface expression of CD107a. NK cells (effectors) and K562 target tumour cells were harvested and re-suspended at $1x10^6$/ml and co-cultured in a 96 U bottom plate at a ratio of 1:1 and 1:3 effector cells (E) to target cells (T) in a total volume of 200μl, with a total cell count of $2x10^5$. All samples were conducted in triplicate. After 1hr of incubation at 37°C, golgistop (BD Biosciences, Oxford, UK) was added at a concentration of 1:1000 to inhibit the internal recycling of CD107a from the surface. The cells were incubated for 5hrs at 37°C. Following this, the 0hr control target and effector cells were mixed and all samples were pelleted. They were immediately stained with CD107a-PE (BD Biosciences) and re-suspended in PBS before acquisition. The forward and side scatter parameters were used to gate on the cell population. A second gate was set to select on isotype control stained cells at 2%. Approximately 5000-10,000 events were collected for each sample. The mean of each triplicate was calculated and the 0hr percentage was subtracted from the 5hr percentage to establish the percentage of primary NK cell degranulation.

3.2.10.2 Cytotoxic assay

The K562 target cells were harvested and resuspended in media containing a cell tracker dye at a concentration of $1x10^6$/mL and incubated for 1hr at 37°C. Once stained, the cells were washed with PBS and resuspended in media. Pre-activated primary NK cells were pelleted by centrifugation and resuspended in NK cell media accordingly to E:T ratio ranging from 1:1 to 3:1. Effectors and target cells were co-cultured with a total cell count of $2x10^5$ cells per well in a total volume of 200μl per l reaction in a U-bottomed 96-well plate for four hours at 37°C 5% CO_2. All samples were conducted in triplicate. A 0hr control was set up alongside each reaction that included the same number of effector and target cells and cell viability staining was immediately carried out to determine background levels of death. After four hours the cells were harvested, centrifuged and supernatant removed. Cell viability was determined, as described 3.2.5.5. Staining controls include target cells negative for cell tracker dye and NK cell markers (CD56 and CD3) and isotype matched controls to differentiate effector cells from target cells. After staining, the cells were washed with chilled FACS buffer and resuspended in FACS buffer before acquisition.

Approximately 5000-10,000 events were collected for each sample. The mean of each triplicate was calculated and the 0hr percentage was subtracted from the 5hr percentage to establish the percentage of primary NK cell degranulation.

3.2.11 Statistical analysis

Statistical analyses were performed using ratio paired t-tests. Experimental data are presented as the mean and/or $\pm$ standard error of the mean. A P-value >0.05 was considered not significant, data generated with a p value of <0.05 were given * or $p<0.001$ **. Statistical analysis was performed using GraphPad Prism software, version 7.0.

4   Assessing the ability of viral vectors to transduce Natural Killer cells *in vitro*

4.1 Introduction

Genetic engineering of human NK cells offer great potential for the NK cell based immunotherapy of cancer. Unfortunately, commonly used viral vectors that transduce other cell types do not typically transduce NK cells and this has greatly limited the development of NK cell based therapies. Transient expression of genes by transfection has shown some success, in particular, nucleofection, an electroporation-based system has achieved efficient primary NK cell transduction (323). However the viability, phenotype and proliferative capacity of NK cells post-transfection has yet to be described. In contrast, studies using retroviral and lentiviral vectors to transduce NK cells have been more comprehensive, demonstrating transduction efficiency and transgene expression on viral transduced NK cells (177,182,324). The use of lentiviral vectors has been investigated, with studies demonstrating transduction of primary murine and human NK cells with efficiencies of 15-40% (172,325). The efficiencies are coupled with stable transgene expression following transduction and offer the opportunity to manipulate the NK cells phenotype. However, cell viability analysis is often omitted in these studies (172,182,324,325), and repeated rounds of transduction are often required that inevitably reduces cell viability. There are also concerns of insertion mutagenesis following lentiviral vector integration that may limit their use (326–328). Alternative viral delivery systems, such as adenoviral and vaccinia virus vectors have also been used to transduce NK cell, however, again studies are limited (175,193). Generally, replication defective adenoviruses do not integrate within the human host genome, so cell transformation events are possible but rare (329,330), making adenovirus vectors much safer than other viral vectors. The chimeric adenovirus (Ad5f35) induces transduction efficiencies of 30-60% in primary NK cells and lymphoid cell lines (175,315), and therefore provides the opportunity to deliver genes into NK cells. Adenoviral transduction surpasses the efficiencies of other viral and transfection systems.

Herein I undertook a comprehensive comparison of viral vector delivery systems to determine the optimal conditions that retain NK cell viability following high efficiency transduction.

4.2 Results

4.2.1 CD46 receptor expression on lymphoid cell lines and primary cells

The complement regulatory molecule, CD46, serves as a receptor for viruses such as human herpesvirus 6, adenoviruses (e.g. Ad35 and Ad11) and the Edmonston measles virus (MV) strain (215,237,331,332). These viruses have a tropism for many cell types consistent with the expression of CD46. As a first step towards assessing the ability of candidate viral vectors to infect NK cells, the cell-surface expression of CD46 was analysed on a panel of human NK cell lines (NKL, YT and NK92MI), PBMCs and primary NK cells (isolated from PBMC) using flow cytometry (Figure 4-1a). As a control in viral transduction, A549 cells, a lung epithelial cell line, were also assessed for CD46 expression; CD46 was expressed on 96% of A549 cells. In addition, CD46 was expressed on over 98% of primary NK cells, PBMCs, YT and NKL cell lines and 52% on NK-92MI (Figure 4-1b), consistent with previous findings (333). The number of CD46 molecules expressed on different cells could be assessed using Fc receptor-coated beads with known antibody binding capacity; fluorescence of antibodies bound to these beads with defined numbers of Fc molecules is then compared to the fluorescence of cells labelled with the same fluorochrome-conjugated antibody binding via the antigen binding site. This approach was previously used to quantify CD38 expression on cells (334). Using conventional flow cytometry, CD46 was expressed on NK cells suggesting that Ad35, Ad11 and MV have the potential to transduce these cells.
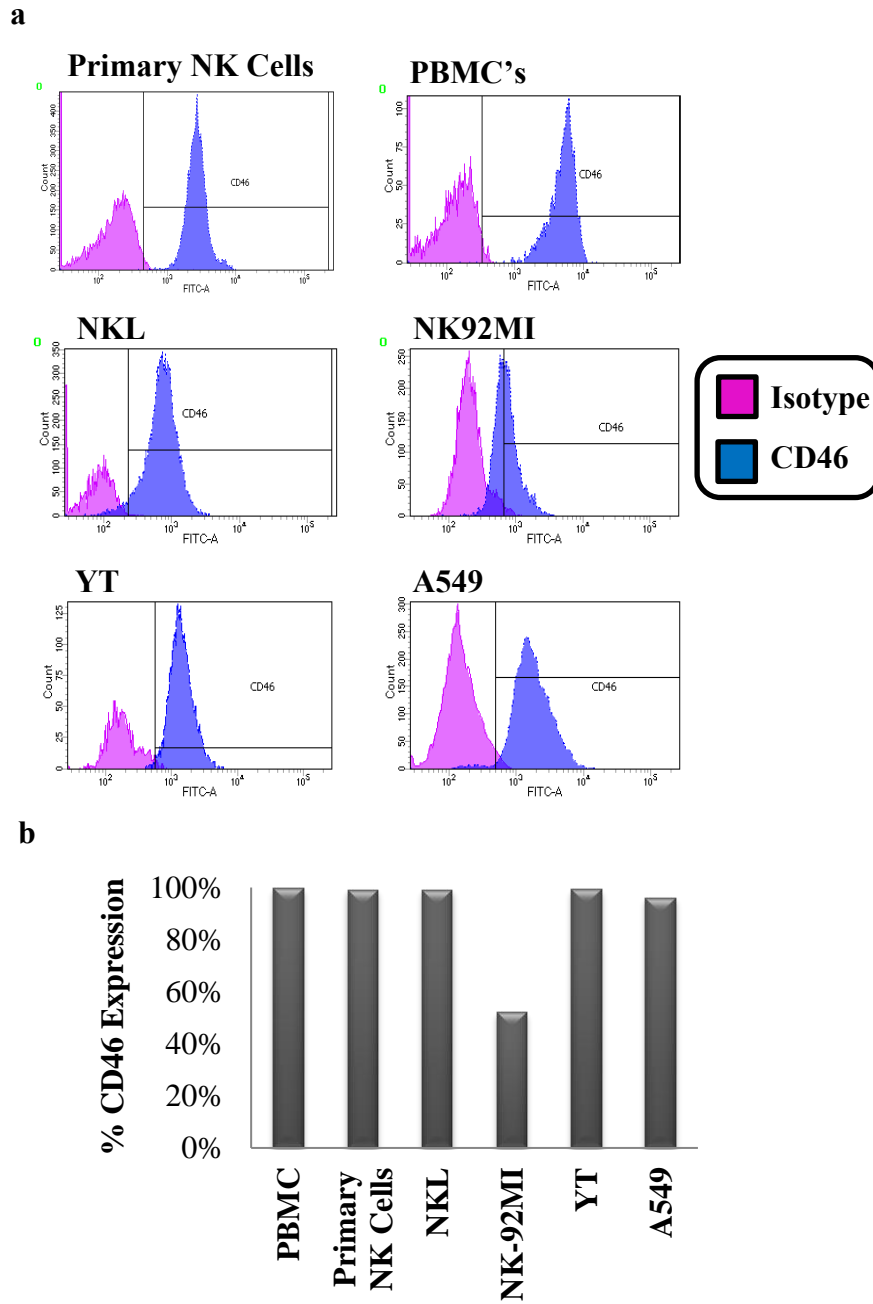
**Figure 4-1 CD46 expression on primary cells and NK cell lines.**

A) Isolated Primary NK cells, PBMCs and the NK cell lines, NKL, NK92MI and YT were examined for surface expression of CD46 by flow cytometry. Histograms indicate cells stained with an isotype control antibody (pink) or cells stained with CD46-FITC antibody (blue). B) The collective percentages of CD46 expressing cells from (A) represented in a bar graph (n=1).

4.2.2 Measles virus mediated transduction of natural killer cells

Measles virus (MV) binds to CD150, which is also known as signalling lymphocytic activation molecule (SLAM) and is commonly expressed on lymphocytes (332), with the Edmonston strain having acquired additional tropism for CD46 (335). The CD46 molecule binds to the fusion and hemagglutinin structural proteins of MV that induce virus cell entry (336). The expression of CD46 on NK cells (Figure 4-1) suggest that they might be susceptible to MV transduction. In support of this, a study has demonstrated lymphocyte transduction using a lentivirus encoding MV glycoproteins that conserved its tropism through CD46 and CD150 receptors (183). An Edmonston strain of measles virus containing GFP (337) was used to test transduction of IL-2 activated cells and compared to transduction of the lung epithelial cell line, A549. Transduction efficiency was determined by flow cytometry, analysing GFP expression in live cells compared to an untransduced control (see methods section 3.2.3). A549 cells were transduced with 50 to 100 MOI and analysed at 24hr intervals up to 72hrs; transduction of A549 was at <4% (Figure 4-2a). Previous findings reported high transduction of B and T cells with 10 MOI using a lentivirus encoding measles virus tropism (183), however the transference of MOI between different viruses do not usually correlate, even if they use CD46. In NK cell lines, NK92MI (Figure 4-2b) and NKL cells (Figure 4-2c) transduction of <8% was observed with 10 MOI. Similar findings were observed using NK cells with only <4% transduced at 24hrs (Figure 4-2d-e). Cell viability remained consistently high, both transduced and untransduced controls with all cell lines. The low transduction efficiency seen in all of these cell lines was in contrast to that observed in melanoma cell lines using the same virus stock (338,339). Due to low transduction of NK cells, MV would be a challenging vector to use in gene transfer studies on NK cells. I therefore decided to test other viruses as suitable vectors for NK cell gene transfer.
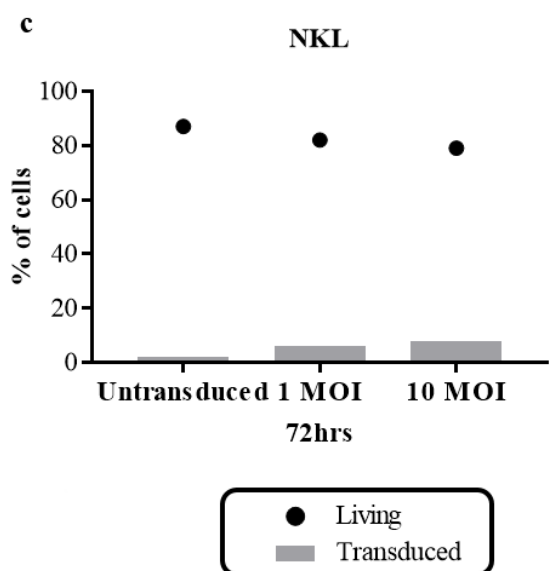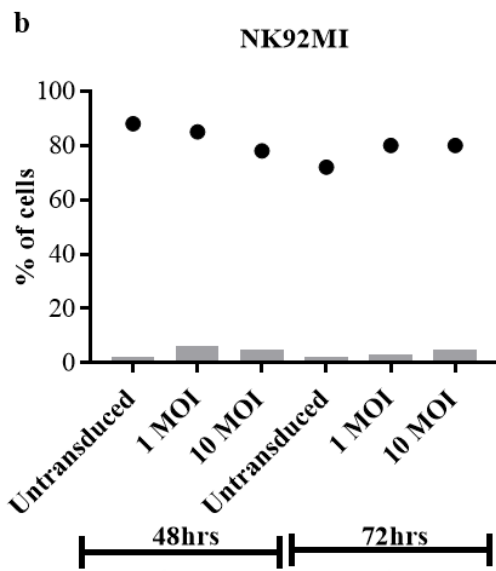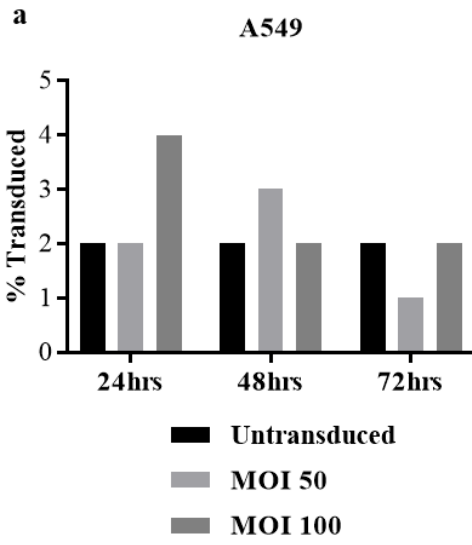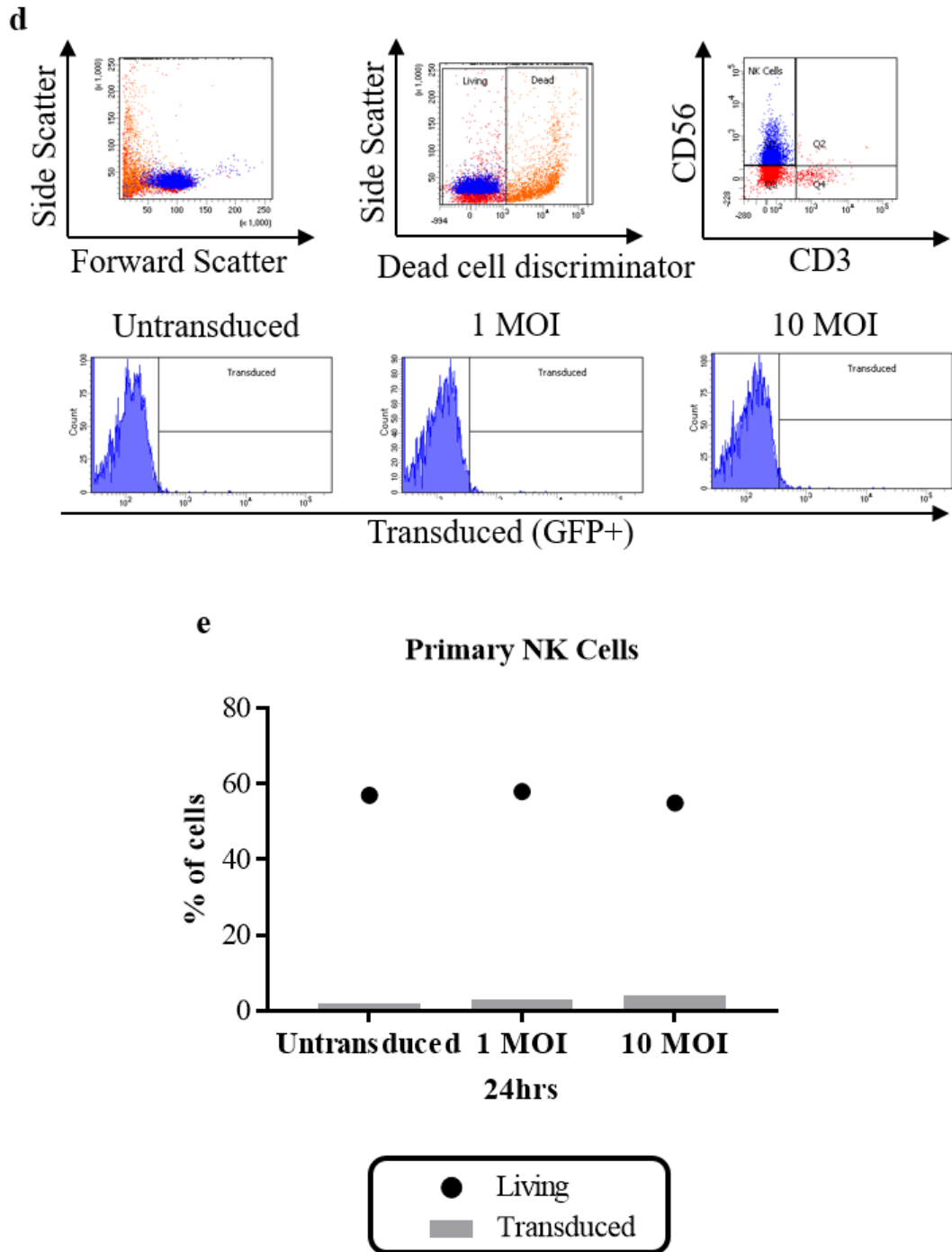
Figure 4-2 Transduction of primary NK cells and cell lines using the measles virus.

Flow cytometry analysis of measles virus transduction in A549s, NK92-MI, NKL and primary NK cells MV-GFP vectors. A) A549s and B) NK92s were culture for 24, 48 and 72hrs at different multiplicities of infection (MOI). C) NKLs were cultured for 72hrs and D) primary NK cells were cultured for 24hrs. After each time point the cells were analysed for GFP expression by flow cytometry. Results are summarised from the percentage of GFP^{+} cells in histograms by flow cytometry.

4.2.3 Vaccinia virus mediated transduction of natural killer cells.

Attenuated strains of vaccinia virus (such as MVA) have broad uses in gene transfer studies and this virus has an excellent biosafety record due to its inability to replicate in human cells (340). The cellular receptor responsible for virus cell entry is unknown, however virion protein A21 is important in cell binding and entry (341). Previously, studies have described vaccinia virus mediated gene transfer into the NK cell line NK92 (193,342) providing a precedent to study primary NK cell mediated transduction using vaccinia virus. I chose to use modified vaccinia virus ankara (MVA) containing GFP to investigate NK cell transduction, again using IL-2 activated NK cells. This particular strain is an attenuated form of vaccinia virus, and it has frequently been used as a vector to express recombinant proteins in vaccine systems (343,344). All cells were analysed by flow cytometry using a gating strategy previously described (Section 3.2.5). Initially, MVA-GFP transduction was analysed in the A549 cell line; at 30 MOI, 91% of A549 cells were transduced and 98% of cells were viable 24hrs post-transduction (Figure 4-3a). For the NK cell line YT, cells were transduced and analysed at 24hrs and 48hrs post-transduction, with optimal transduction of 80% at 20 MOI after 48hrs (Figure 4-3b). However, YT cells showed poor viability even in the absence of transduction and this decreased further upon transduction (Figure 4-3b). Primary NK cell transduction was then determined in 3 donors. The viability of untransduced primary NK cells was high (~80%). Transduction efficiencies of between 25-39% (n = 3 donors) at 20 MOI was observed. However, this useful level of transduction was offset by the poor viability of NK cells post-transduction; viability reduced from 80-85% in untransduced cells to 11-24% (at 20 MOI) in transduced NK cells (Figure 4-3c). The reduced percentage of living cells in transduced NK cells suggests that MVA has cytopathic effects, however, this virus is not known to replicate in human cells (340). Here, cytotoxicity was only observed in NK cells and not in A549, therefore the cytopathic effects are likely caused by an innate response through an induced interferon and apoptotic reaction in transfected NK cells.

The MVA strain used was produced after >500 passages from vaccinia virus in chicken fibroblast cells, and varies from the original isolate by loss of 15% of the virus genome and likely the loss of the genes responsible for reduced modulation in a cellular response (345,346). Although NK cell transduction efficiency by MVA is high, low viability restricts the use of MVA-GFP as a viable vector for gene delivery in NK cells. Further investigations using other vaccinia strains, or making modifications to the genome would be required to resolve these cytopathic effects. Instead, I chose to evaluate a third viral vector type based on adenovirus, an established vector in gene therapy clinical trials (347), warranting further investigation as an agent for transduction of primary NK cells.

a

A 549

b

Y T

Transduced
■ Living

■ 24hrs Transduced ▲ 24hrs Living
■ 48hrs Transduced ● 48hrs Living

c

d

Primary NK Cells

● Donor 1 Living
■ Donor 2 Living
▲ Donor 3 Living
Donor 1 Transduced
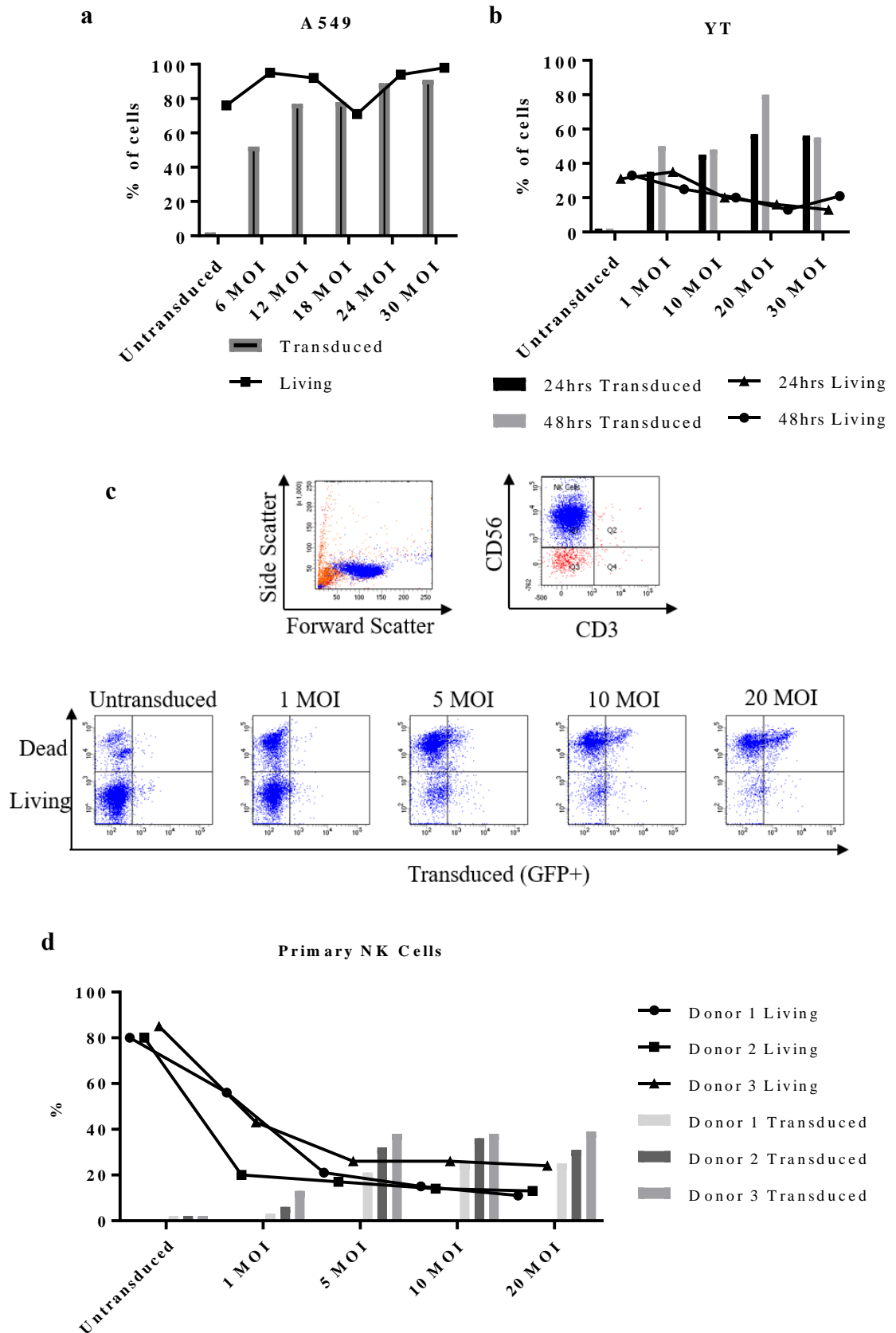Donor 2 Transduced
Donor 3 Transduced

Figure 4-3 MVA-GFP transduced and induces cells death in primary NK cells.

A549s, primary NK cells and YT cells were transduced with MVA-GFP vectors ((see methods section 3.2.3) and transduction efficiency and cell viability were analysed by flow cytometry. Increasing amounts of MOI was used to transduce cells and at 24hrs
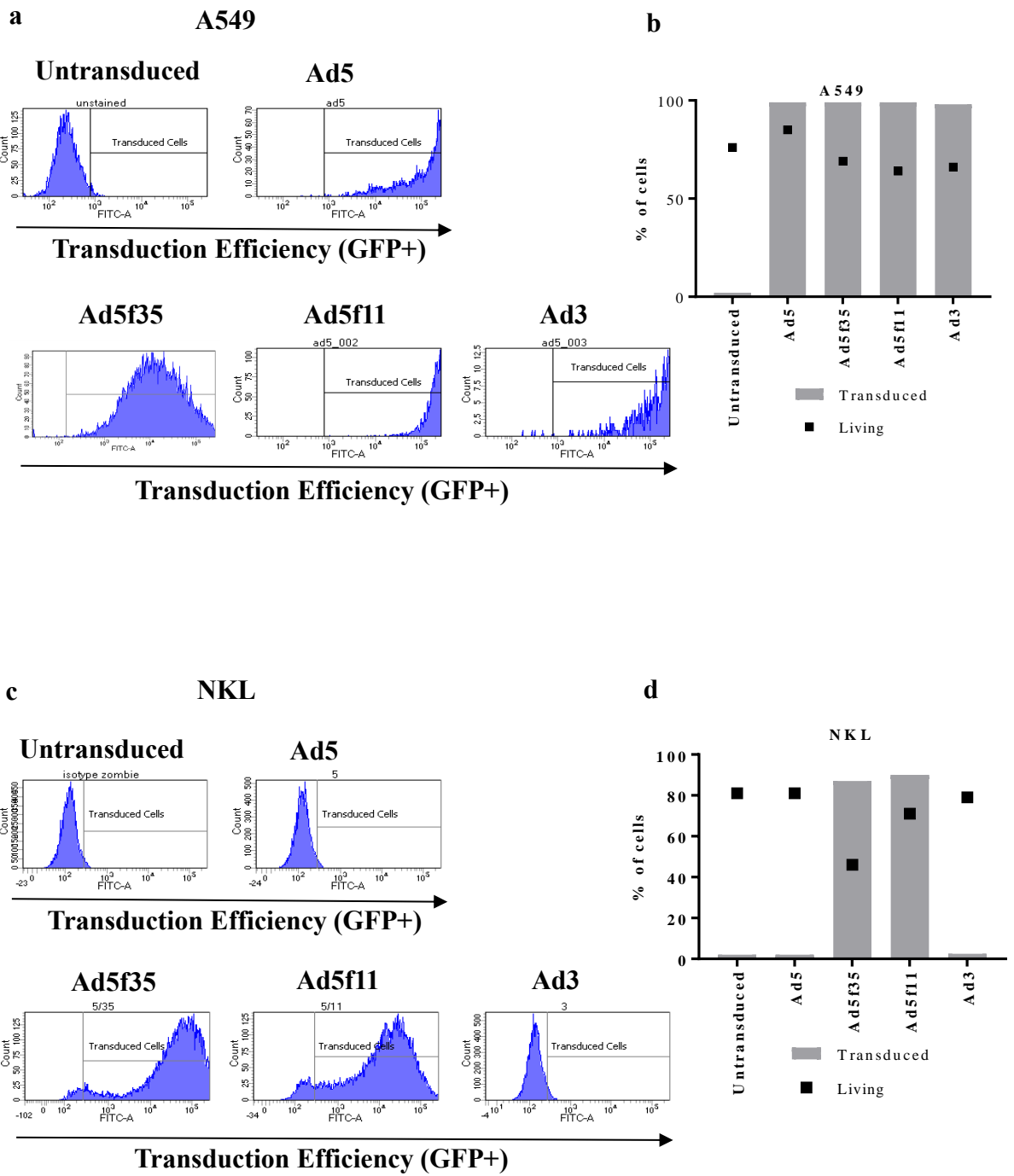
cells were analysed for GFP expression by flow cytometry, or at 48hrs in YT cells. Results are the summary of percentage GFP$^+$ cells in histograms by flow cytometry. Cell viability was analysed for Zombie^{neg} expression by flow cytometry. A) The results for A549, B) YT and C) Primary NK cells were summarised.

4.2.4 Adenovirus mediated transduction of natural killer cells

Modification of adenovirus Ad5, replacing the fibre shaft and knob domain with that from Ad35 or Ad11 allows the newly generated chimeric viruses (Ad5f35 and Ad5f11 respectively) to utilise CD46 for virus entry (instead of CAR) and has been shown to allow NK cell transduction (175,315). Before testing the transduction of NK cells I compared a series of GFP-encoding chimeric vectors for their ability to transduce A549 cells, providing a positive control in their utilisation of the different vectors. I compared transduction of the parental vector, Ad5-GFP (MOI 50), with that of the chimaeric vectors Ad5f35-EGFP (MOI 820) and Ad5f11-EGFP (106 MOI). Alongside these vectors I also tested Ad3-EGFP (MOI 45), which like Ad5f35 is a species group B adenovirus, however this Ad3 based vector uses desmoglein for cell attachment (243). A variation in MOI between vectors was used due to variations in virus titre during these preliminary investigations. Transduction efficiency was determined by GFP expression (using flow cytometry) at 24hrs (Figure 4-4a-b). The data show that all four of these Ad-based vectors transduced >98% of A549 cells with cell viability of >66%. Cell viability was reduced in transduced cells indicating that either transduction or virus present in the media have a cytotoxic effect on the cells.

The ability of the adenoviral vectors to transduce NKL cells by flow cytometry was then determined (Figure 4-4c). As expected, there was minimal transduction of NKL with the parental Ad5-EGFP (MOI 20) and Ad3-EGFP (MOI 18) (<2.5%), both of which are known to utilise CAR and desmoglein respectively for cell attachment; the expression of these receptors are not detected on NK cells. However, NKL were highly transduced using Ad5f35-EGFP (MOI 328) and Ad5f11-EGFP (MOI 42) at 90% and 87% respectively (Figure 4-4d). These viruses utilise CD46 which is expressed on NK cells (Figure 4-1). Cell viability decreased from 81% in untransduced cells to 46% in Ad5f35 transduced cells, indicating a cytotoxic effect (Figure 4-4.d), similar to the observations in A549 cells. Despite this cytotoxicity, the ability to transduce the NK cell line NKL at high efficiency with Ad5f35 and Ad5f11 suggested that these vectors might prove useful in the transduction of primary NK cells.

The next step was to analyse adenovirus mediated transduction of primary NK cells that were purified from PBMC. Transduction using Ads vector was determined by flow cytometry using a gating strategy previously described (Section 3.2.5) (Figure 4-4e). At 24hrs post-transduction, minimal NK cell transduction was observed and was at its highest using Ad5f35-EGFP at 7% (Figure 4-4f). At 48hrs post-transduction using Ad5f35-EGFP, 63% of cells were transduced in one donor. However, transduction efficiency was inconsistent between 3 donors suggesting that the conditions required optimisation (Figure 4-4g). In contrast to NKL the viability of primary NK cells remained acceptable (>60%) and did not differ from that of untransduced controls. These results are not in agreement with one study using a modified Ad virus which reduced T cell viability by 75% at 72 hours, and an Ad-WT (Wild Type) was 1.8 fold less than this (348), suggesting that a modified Adenovirus that boosts cell entry increases toxicity. Surprisingly, Ad transduction has a critical effect on cell viability in NKL and has not been described in other cell lines such as NK-92MI, Jurkat and YT (149), indicating a cytopathic effect on transduced cells. Ad5f35 and Ad5f11 mediated transduction utilises CD46 for cell attachment, whereas Ad5 and Ad3 utilises CAR and Desmoglein respectively (315) which are absent from NK cells. This suggests that adenovirus cell attachment is critical in cell entry.

a   A549

Untransduced   Ad5

Transduction Efficiency (GFP+)

Ad5f35   Ad5f11   Ad3

Transduction Efficiency (GFP+)

b

c   NKL

Untransduced   Ad5

Transduction Efficiency (GFP+)

Ad5f35   Ad5f11   Ad3

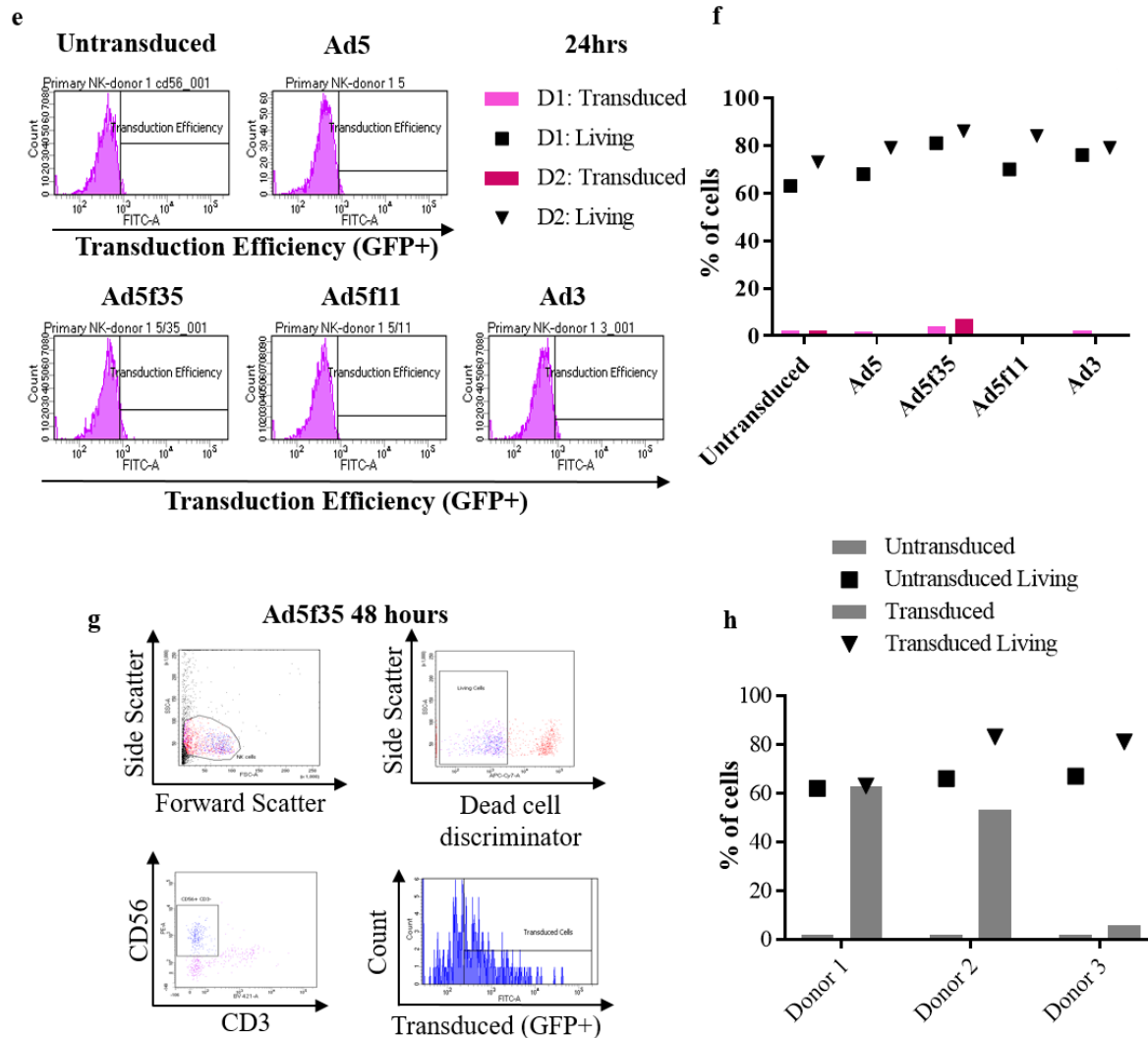Transduction Efficiency (GFP+)

d

Figure 4-4 Fibre modified Ad5 adenoviruses efficiently transduces NKL cell line, A549 cells and requires optimisation in primary NK cells.

Cells were transduced with either Ad5, Ad5f35, Ad5f11 and either Ad3 (see methods section 3.2.3) and transduction efficiency was assessed at either 24 or 48hrs by flow cytometry. A) A549 were transduced with all chimeric adenoviruses and analysed at 24hrs post-transduction by flow cytometry. B) The transduction efficiency that was analysed by GFP expression within the living population of cells in A) was summarised. C) NKL cells were transduced with all chimeric adenoviruses and analysed for GFP expression by flow cytometry within the living population of cells at 24hrs. D) The flow cytometry plots of transduced cells and living cells were summarised from C). E) The flow cytometry plots of primary NK cells from one donor that was transduced with all chimeric adenovirus and analysed by gating on $CD56^+CD3^-$ living cells and analysed for EGFP expression at 24hrs. F) The summary from E) from 2 donors at 24hrs. G) Representative flow cytometry analysis from one healthy donor transduced with Ad5f35-EGFP at 48hrs. H) The summary of Ad5f35-EGFP mediated transduction of primary NK cells in 3 donors at 48hrs.

4.3 Discussion

Generally, viral transduction is associated with higher degrees of transgene delivery in NK cells compared to transfection. Typically, most studies of viral transduction of NK cells have used lentiviral vectors which have been utilised with success in restoring tumour reactivity in NK cells (324). More recently, studies have described the genetic modification using tumour specific chimeric antigen receptors (349,350). Similar to other studies using lentiviruses, they observed a transduction efficiency of <20%, with one study showed that the NK cells were fully functional (349). Using a lentivirus vector to deliver genes into primary NK cells is a promising option, and one which could also deliver shRNA. The disadvantage of using a lentivirus for gene delivery clinically is concerns with biosafety, such as insertion mutagenesis from vector integration and viral associated cell death which may cause limitations in its application (326–328). Here, MV-GFP, Ad5f35-EGFP and MVA-GFP were assessed for compatibility in transducing primary NK cells. The data identified Ad5f35-EGFP and MVA-GFP as promising viral vector candidates in NK gene delivery. The data showed that Ad5f35-EGFP transduces lymphoid cell lines and primary NK cells at greater levels compared to other delivery systems analysed. These data are reinforced by other studies that describe the ability of Ad5f35 transduction in NK cells, T cells and B cells (175,315,351). Despite this, I observed lower primary NK cell transduction efficiencies than published, and variability between donors. Observations of cell death also require resolving. This issue might be caused by the virus replicating and requires further investigation. Regardless, Adenovirus have an excellent biosafety record with notably no documentation of integrating within the host genome of human cells. Ad5 in particularly has been extensively studied as a tool for the infection and manipulation of cells, and therefore has the potential to be utilised as a tool for the purpose of immunotherapeutic strategies in NK cells.

Surprisingly, MV-GFP transduction of NK cells was shown to be substantially low compared to Ad5f35-EGFP considering both viruses use CD46 as the primary receptor for cell binding and entry (331,332). CD46 is often upregulated on human malignancies, presumably as a defence against complement mediated lysis and reported as the oncolytic mechanism in MV (352). The importance of CD46 expression for MV transduction is reinforced by a study showing correlation of MV cell entry efficiency in cell lines with varying amounts of CD46 expression (353).

Here low MV-GFP transduction in CD46 expressing cells was observed however, a study using a lentivirus vector encoding MV glycoproteins has been successful in mediating transduction of B and T cells (183,354). It is likely that a higher MOI was required to increase the capacity for MV-GFP transduction; however further investigations were not pursued. Evidently, MV-GFP was not a leading viral vector candidate due to higher transduction of NK cells by Ad5f35-EGFP and MVA-GFP.

MVA-GFP is a well characterised viral vector for transgene delivery and has been successful in transducing NK cell lines, however there have been no recent studies in primary NK cell transgene delivery using this vector (>10years) (193,342). Here, MVA-GFP was shown to be compatible in primary NK cells transduction. Typically, wild-type vaccinia induces cytopathic effects upon infection, however modified vaccinia virus is considered to be non-replicative in human cells. Despite this, MVA-GFP induced NK cell death. MVA-GFP have 15% less genomic content compared to wild type which renders the virus non-replicating and the cause of their loss in immunomodulatory functions that likely results in induced cell death of transduced cells (345,346). Similar findings in MVA infected dendritic cells have been observed and suggested to be responsible for apoptosis of infected cells (355). Consequently, the disadvantage of using MVA supersedes its ability to transduce primary NK cells effectively.

Compared to viral transduction, transfection of NK cells is independent of receptor expression. Transfection techniques also have an advantage in biosafety as it does not involve the use of viral vectors. However, efficacy of transfection is low compared to virus transduction. Viral transduction might be higher compared to transfection due to utilising receptors to gain cell entry, and they also have an innate ability to escape and survive the acidic environment of the endosome upon endocytosis. Regardless, success of NK cell transduction is limited and likely due to the innate properties associated with NK cells. Speculatively, viruses that have gained cell entry might have direct contact with NK cytolytic granules containing granzymes that could potentially hinder the viral cell entry pathway. Apoptosis of transduced NK cells might also be triggered upon viral recognition through their pattern recognition receptors (356). Hence, the inherent characteristics of NK cells is the probable cause of poor efficacy in viral and transfection techniques of gene delivery. Despite this, viral vectors do provide an option in gene delivery. Generally, viral transduction is associated with cell death, however enrichment of NK cells post-transduction might overcome this

issue. Here Ad5f35-EGFP transduction of NK cells has been shown to have an advantage in NKL cell transduction and higher viability compared to MV-GFP and MVA-GFP. Provided that adenovirus transduction efficiency is optimised in primary NK cells, the delivery method using Ad5f35-EGFP is plausible. Achieving a viable approach in NK cell genetic manipulation would contribute to the direction of NK immunotherapeutic strategies. In conclusion, this chapter shows that transduction efficiency is inconsistent in primary human NK cells, however transduction is consistently high in cell lines. The next chapter will investigate approaches to resolve these variabilities between donors, and enhance NK cell transduction using Ad5f35-EGFP.

5 Enhanced transduction into natural killer cells with the chimeric adenoviral vector Ad5f35

5.1 Introduction

Adenoviruses are well-characterised, with Ad5 being the most commonly used vector in gene therapy, not least because of the high titres possible and their low pathogenicity. Ad35, a subgroup B adenovirus, utilises CD46, a cell surface molecule that is highly expressed on NK cells, as its natural receptor. Replacement of the Ad5 fibre and knob domain with that of Ad35 confers binding and entry into NK cells, as demonstrated in the previous chapter; Ad5f35 was shown to be compatible for primary NK cell transduction, with high transduction in NKL in the previous chapter. However, transduction and cell viability of primary NK cells were variable between donors, thus enhancing transduction efficiency for optimal levels of transgene delivery is fundamental in establishing a superior methodology compared to other viral gene transfer methods. Studying the phenotype and viability of post-transduced NK cells clearly requires further investigation to validate its use as a therapeutic vector for gene delivery. Hence, this chapter focuses on optimising the conditions to produce consistently high Ad5f35 mediated transduction on NK cells.

5.2   Results

5.2.1   Optimisation of Ad5f35-EGFP mediated transduction of NK cells

A previous study described enhanced lentivirus gene transfer into primary NK cells when using a high concentration of IL-2 and polybrene (172). Polybrene, a polycation, is known to enhance viral transduction by increasing virus-cell interaction by neutralising potentially repulsive negative charges on the cell surface (e.g. from sialic acid) (357). IL-2 is a cytokine that regulates NK cell activation through Jak1/3 and STAT3/5 signalling to induce IFN-γ secretion, cytotoxicity and promotes survival and proliferation (54,55). Hence, a high concentration of IL-2 will enhance survival post-virus transduction (172). Centrifuging NK cells has also been shown to enhance lentiviral transduction (172), and adenovirus-mediated transduction of dendritic cells (358). To determine if these conditions allow for the efficient adenovirus-mediated transduction of NK cells, purified NK cells were transfected with 320 MOI Ad5f35-EGFP in combination with 100 IU IL-2, 500 IU IL-2 and 5μg polybrene. All cells were then centrifuged at 1800g for 45 minutes (as described in section 3.2.3). All experiments were analysed by flow cytometry; using a gating strategy described in section 3.2.5, and detecting transduction efficiency by the expression of EGFP emission within the FITC channel. Centrifugation was not utilised in virus transduction in the previous chapter, in this section centrifugation enhanced Ad5f35-EGFP transduction (Figure 5-1). The addition of five-fold more IL-2 increased transduction from 57% to 64% in one donor, while the polybrene additive reduced transduction to 37% and cell viability to 31%, compared to 62% in the untransduced control. This is likely due to the toxic effects of polybrene, reducing cell viability and transduction efficiency. Overall, viability in transduced cells with all conditions was reduced but highest when treated with 500 IU IL-2 at 51% (Figure 5-1a). The preliminary data in this donor shows that centrifugation enhanced adenovirus transduction when compared to cells that were not centrifuged (Chapter 4). However, a detailed controlled comparison of the effect of centrifugation was not made (e.g. transduction of NK cells from a single donor, with and without centrifugation). Such an experiment would have formally clarified the effect of centrifugation on transduction. Little is known about the mechanism responsible for enhancing viral transduction by centrifugation, however one study has hypothesised that centrifugal forces increase the interactions between adenovirus and cellular receptors (358). In order for this approach to be useful cell viability is important, and a high concentration

of IL-2 increased the percentage of living cells post-transduction. Hence, combining centrifugal force and a high concentration of IL-2 consistently aided good levels of Ad5f35-EGFP mediated transduction of NK cells.

The optimal conditions for Ad5f35-EGFP transduction that were determined in Figure 5-1.a was applied to increasing amount of virus to NK cells to distinguish optimal MOI for efficient virus transduction. Flow cytometry analysis determined EGFP expression at 24hrs post-transduction by flow cytometry (Figure 5-1b). NK cells were optimally transduced at 150 MOI at 67%, with cell viability at 42% compare to 77% in the untransduced control (Figure 5-1c). Transduction was also confirmed through detecting EGFP expression in cells by immunofluorescence microscopy (EVOS® FL Cell Imaging System) (appendix section 9.1). These conditions were used to analyse transduction efficiency in NKL and NK92MI cell lines. NKL were optimally transduced at 300 MOI at 79%, however cell viability was reduced from 40% in the untransduced control to 10% (Figure 5-1d). Notably, the Ad5f35-EGFP vector used in Figure 5-1d was found to have a contamination of replicating virions with constructs retaining E1A/B, an issue that affects cell viability and discussed later in section 5.2.3. In contrast, NK92MI cell viability retained at 89%. However NK92MI were only transduced at 25% with 400 MOI (Figure 5-1e) and likely due to lower levels of CD46 expression (Figure 4-1), which has presumably restricted viral cell attachment. In both NK cell lines, transduction was also confirmed by detecting EGFP expression in cells by immunofluorescence microscopy (appendix section 9.2), where cells appear to clump more so compared to the untransduced controls.

These findings suggest that NK cell lines were highly transduced using an MOI of between 150-300, in combination with centrifugal force and a high concentration of IL-2. However, high transduction efficiency in primary NK cells and cell lines was coupled with reducing cell viability indicating cytotoxic effects of viral transduction.

To conclude investigations on Ad5f35-EGFP transduction, optimised conditions were utilised in isolated PBMCs and purified NK cells. Firstly, PBMCs were transduced (section 3.2.3) and transduction efficiency in primary NK cells, T cells and B cells were determined by analysis of EGFP expression in cells stained with characteristic markers at 24hrs post-transduction (section 3.2.5). A transduction efficiency of 23-48% was observed in the NK population of PBMCs (n=3), less than as previously observed in isolated primary NK cells (Figure 5-1c). Analysis of T and B cells indicate

that they are also capable of Ad5f35-EGFP mediated-transduction, with the highest observed percentage of transduction of 37% and 43% respectively (Figure 5-1f). Similarly, other studies have shown Ad5f35-EGFP T cell transduction efficiency at 45% at an MOI of 1000 (175) and 20-45% in B cells using Ad5f35-EYFP at an MOI of 500 (359). These data suggest that Ad5f35-EGFP transduction of PBMCs is a useful vector not only for the transduction of primary NK cells, but also of B and T cells.

Transduction efficiency was then determined in purified primary NK cells of 6 healthy donors to determine the reproducibility of these conditions. Transduction efficiency was determined by flow cytometry, selecting viable cells that are CD56+CD3- and analysing EGFP expression 24hrs post-transduction. NK transduction efficiency varied between 6-67%, with a mean of 39%, results not dissimilar to those observed in transduced NK cells within a population of PBMCs. Cell viability was retained when compared to the untransduced controls indicating that a replicative defective vector and transduction event is not inducing cell apoptosis at 24hrs (Figure 5-1g). Importantly, high transduction efficiency (38-67%) was observed in four of the six donors. Whether this variability in transduction between donors was due to CD46 density was not determined. However, this could be remedied by using a large panel of NK cell donors and determining cell surface CD46 expression (by flow cytometry) and Ad5f35-EGFP transduction and assessing their correlation. Interestingly, others have reported that the transduction efficiency of CHO cells transfected with different levels of CD46 showed a correlation between CD46 density and transduction efficiency. However, in the same study, there was no correlation between transduction efficiency and CD46 expression density when compared between different tumour cell lines (360). Nevertheless, the results indicate variability in transduction efficiency between donors, and has been observed elsewhere (359). These results show that Ad5f35-EGFP is a useful tool for gene transfer into primary NK cells and hence offers promise as a therapeutic options for gene delivery. However, successful exploitation in cancer immunotherapy requires that the transduced NK cells retain the ability to express a transgene for several days post-transduction and that transduction does not alter the key anti-tumour functions of key anti-tumour functions of NK cells. It was therefore essential to evaluate the functional activity of NK cells transduced with Ad5f35.
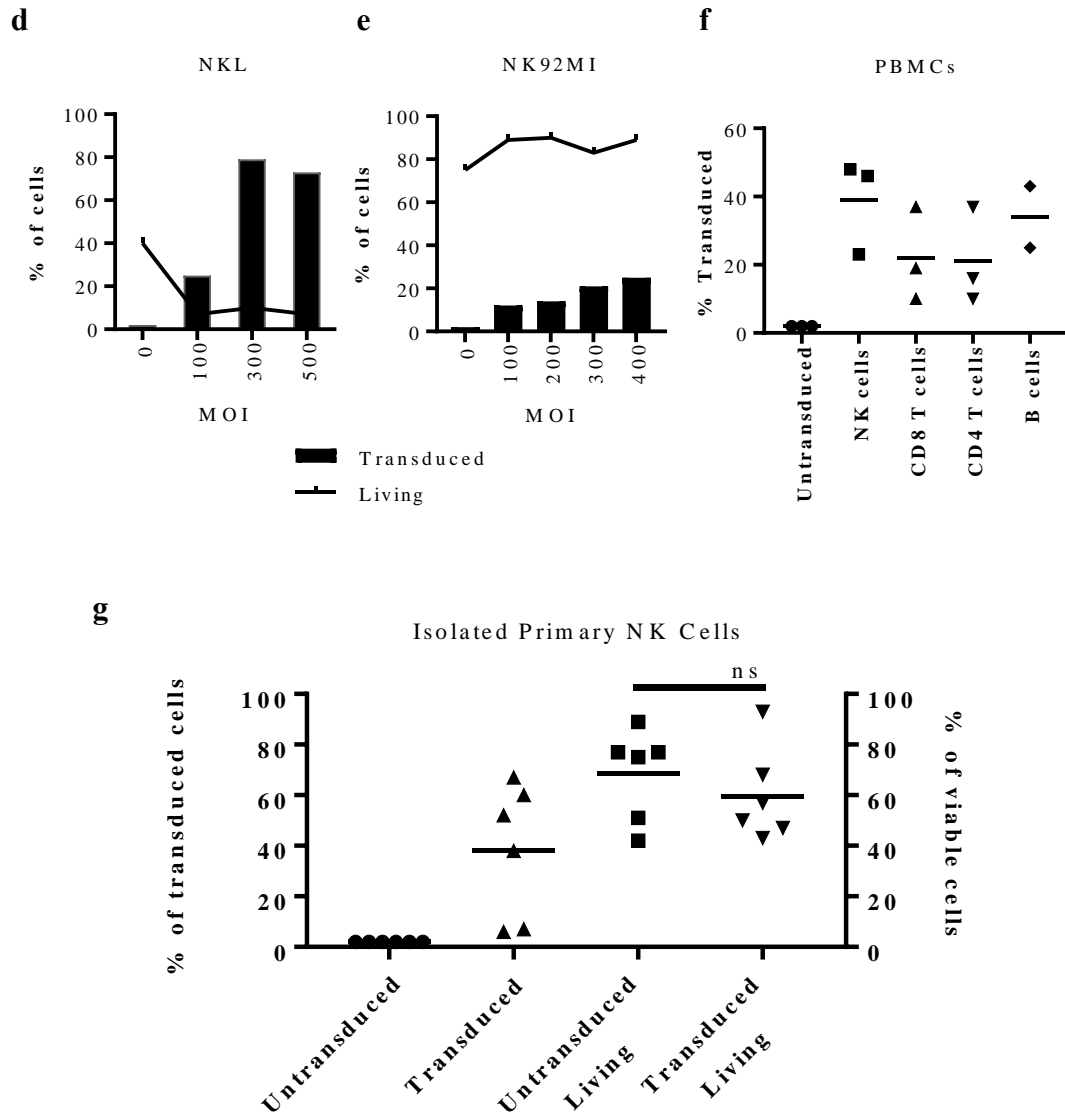
a

b

Transduction Efficiency (GFP+)

Transduction Efficiency (GFP+)

Transduction Efficiency (GFP+)

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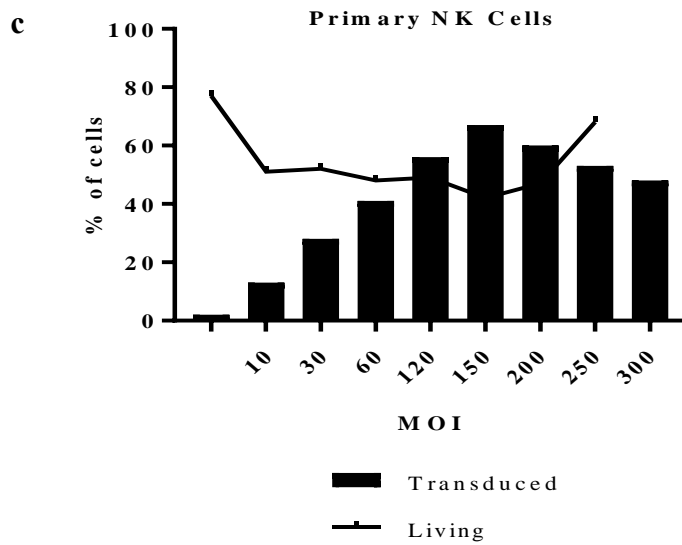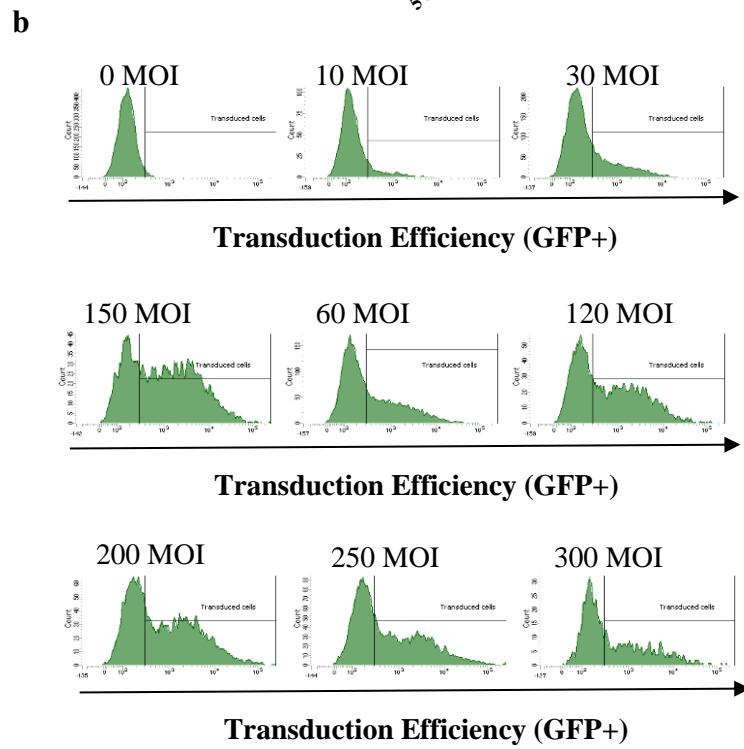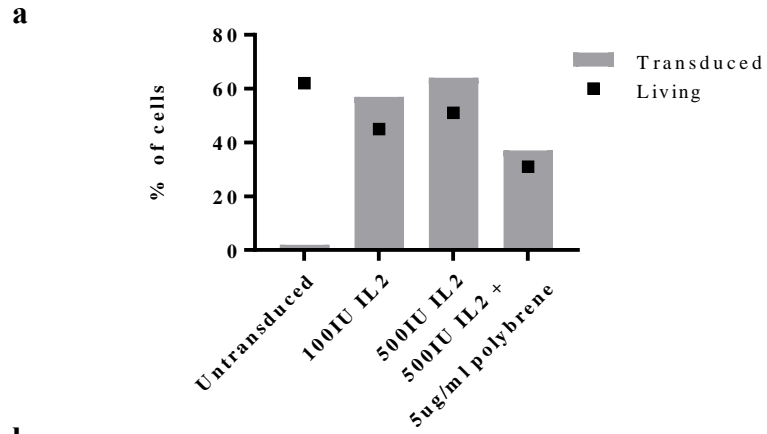
Figure 5-1 Enhancement of Ad5f35-EGFP transduction by centrifugal forces and IL-2

All experiments were analysed at 24hrs post-transduction. A) Primary NK cells were seeded at $2.5x10^5$ in a 24 well plate in 125μl of serum free media in the presence of 320 MOI Ad5f35-EGFP and 100 IU IL-2, 500 IU IL-2 or in combination with 5ug/ml polybrene and centrifuged for 45 min at 1800g, cultured for 3 hours and125μl of media containing serum was added before analysis at 24hrs post-transduction. The graph shows a summary of the percentage of transduced cells (n=1). B) Primary NK cells were left untransduced or transduced at an MOI of (10-300) and transduction efficiently and analysed by GFP expression using flow cytometry (n=1). C) Summary from flow cytometry analysis from B). Collective percentages of cell viability and transduced cells for D) NKL at MOI 0-500 and E) NK92MI at MOI 0-400. F) The collective percentages of transduced PBMCs with Ad5f35-EGFP MOI 1000 (n=3). G) Isolated primary NK cells transduction with Ad5f35-EGFP MOI 1000 (n=6). The results shown the mean percentage of transduced cells. A t-test was performed on cell viability between untransduced and transduced cells. Statistical analysis is statistically significant at P<0.05.

5.2.2   Time course of transgene expression

To determine the time period of gene expression delivered by Ad5f35 on NK cells, EGFP expression was analysed over a 7 day period post-transduction in isolated primary NK cells and in the NK cell lines; NKL and YTs. All cells were transduced (methods section 3.2.3) and re-suspended in fresh culture medium and IL-2 every 2 days. Flow cytometry determined transduction efficiency by analysing EGFP expression within the living population of cells that were $CD56^+CD3^-$; as described in section 3.2.5and Figure 5-2a. Transgene expression was highest at 24hr post-transduction (Day 1) at 67% and 78% in primary NK cells and NKL respectively. However, transduction was highest in YT cells at 48hrs post-transduction (Day 2) at 95%. Adenovirus vectors are engineered to not replicate and they do not integrate into the host cell genome, hence the expression of EGFP was transient, such that the percentage of cells expressing EGFP declines rapidly from 72hrs post-transduction (3 days) in all cell types, until levels were close to baseline at 7 days (<5%) (Figure 5-2b). Notably, cell viability declined in primary NK cells from 43% at day 1 to 20% at day 7 in transduced cells compared to 51% at day 1 to 7% at day 7 in the untransduced control despite frequent treatment of IL-2. Similar findings were observed in NKL where from day 1 transduced cell viability was reduced to <5% compared to the untransduced control at >47% from days 1-6. Cell viability of YT cells also declined from day 2 with viability at 2% in transduced cells compared to 62% in untransduced cells on day 3 (Figure 5-2c). Importantly, the results show that transgene expression in primary NK cells is maximal around 24-48hr post-transduction. Furthermore, the adenoviral induced cell death is a recurring observation that instigates further investigation in its capability as a vector for gene delivery in NK cells.

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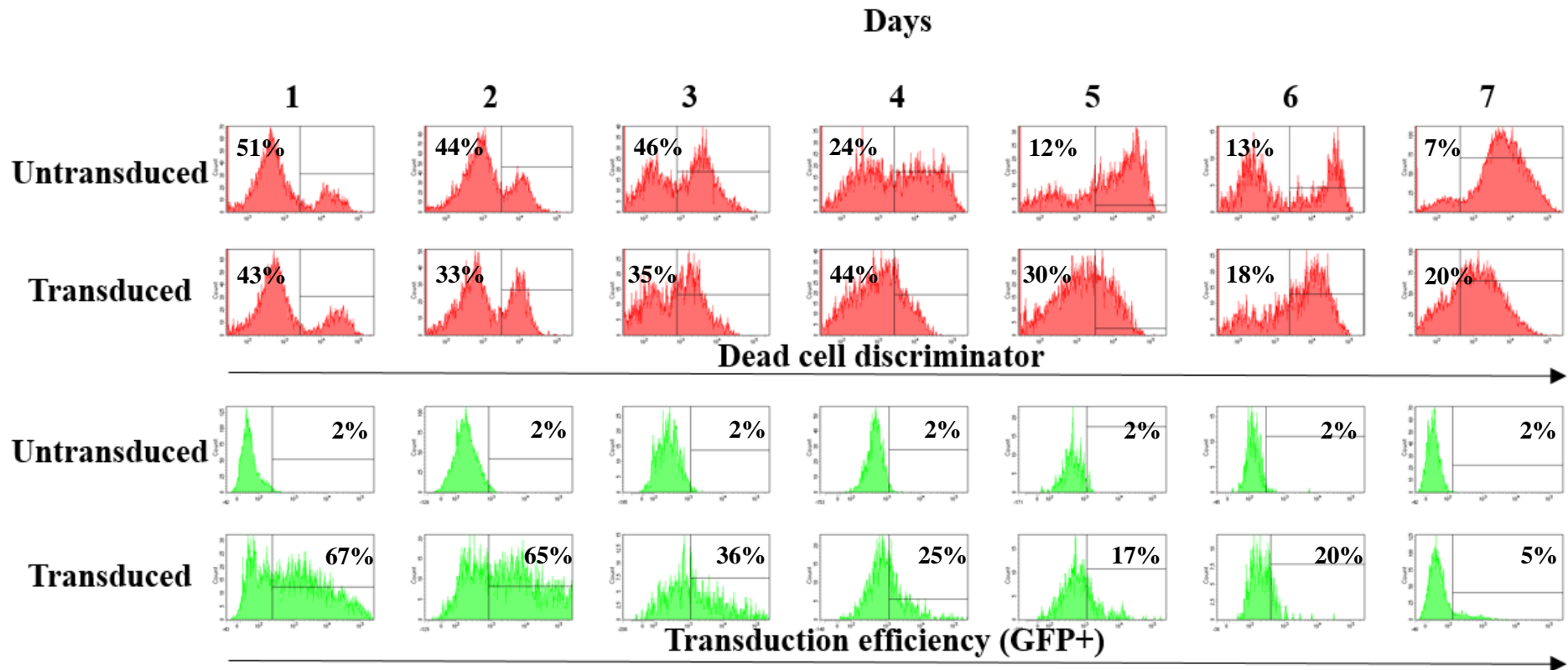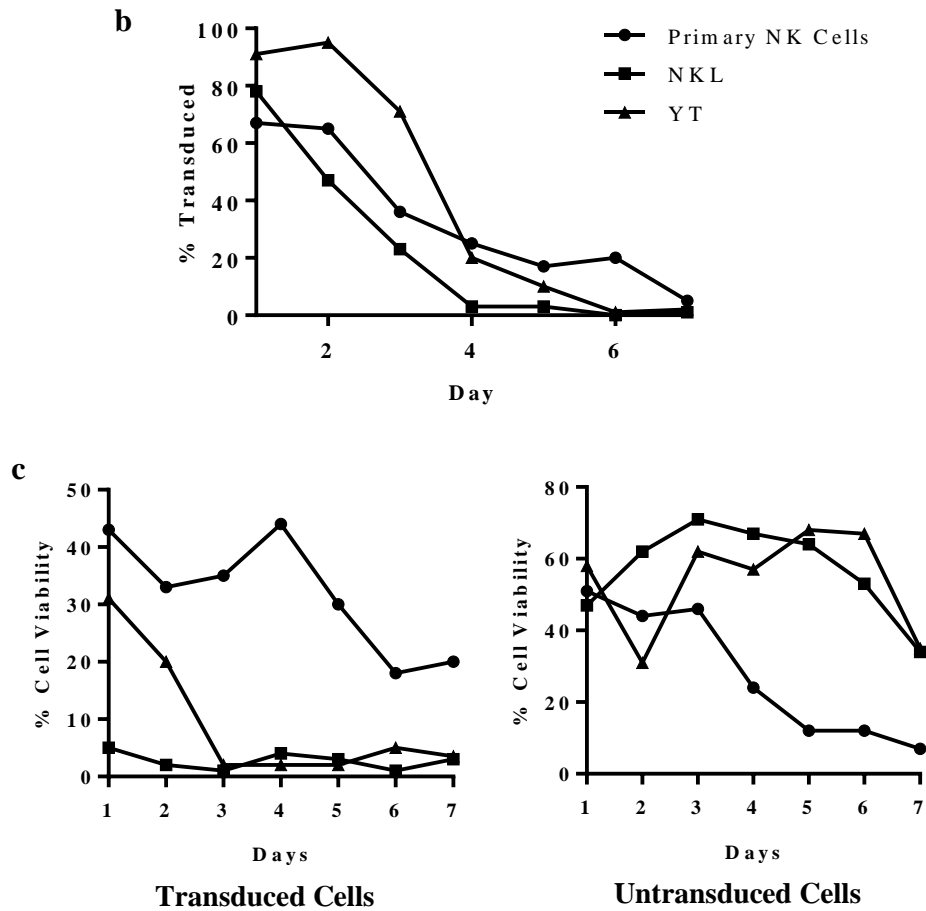
Days

Figure 5-2 EGFP expression declines over 7 day period post-adenovirus transduction.

EGFP expression following Ad5f35-EGFP transduction of primary NK cells, NKL and YT cells over 7 days. A) Representative flow cytometry data from transduced purified NK cells. B) Purified NK cell, YT and NKL were transduced with the Ad5f35-EGFP at an MOI of 300 MOI and transduction frequencies were analysed daily for 7 days by flow cytometry. C) The percentage of cell viability was analysed daily by flow cytometry in comparison to untransduced controls.

5.2.3 Virus Induced cell death

Wild type Ad5 replicates under control of the immediate early genes. For therapeutic purposes (including safety), genomic modifications in replication defective derivatives include the deletion of the E1A/B region, interfering with the virus life cycle and its ability to replicate (361). During the process of virus expansion in 911 cells (the producer cell line), *trans*-complementation of E1 gene region is provided to overcome the absence of the early region in the vector and allow viral production (362). However, sequence homology between the E1 region inserted into the 911 cells and the adenovirus vectors is known to allow a recombination event to take place in which the E1 region is recaptured by the vector, this recombination is responsible for contaminating replication-deficient adenovirus vectors with replication competent adenovirus (363). Due to previous observations of reduced cell viability in post-transduced NK cells, replication competent adenovirus contamination was determined in Ad5f35-EGFP as a cause for these effects. First, I looked for evidence of E1A gene sequences in my vectors using PCR; a PCR product of E1A was synthesised using Ad5f35-WT, Ad5-WT, Ad5-EGFP and Ad5f35-EGFP virus stocks as templates (see section 3.1.8 for further details), therefore contamination with replicative competent adenoviruses is likely (Figure 5-3a). The expected size of the PCR product is 653bp, however the size of the PCR products varied between templates, suggesting several distinct recombination events had occurred in these stocks. To conclusively determine if replication is occurring, the expression of hexon, a late virus protein expressed post-virus replication, was assessed by flow cytometry, as represented in Figure 5-3b showing hexon expression in transduced A549 cells. To determine the extent of this problem a new stock of Ad5f35-EGFP (Ad5f35-E1a[neg]) was analysed in transfected A549 cells and was found to have minimal hexon expression (2%), as shown in flow cytometry data in Figure 5-3b. Using the two stocks of Ad5f35 (which were propagated independently), hexon expression was assessed in A549, NKL and primary NK cells transfected cells were assessed (Figure 5-3c). The new virus stock, which was determined to be replication incompetent was then analysed for its effects on cell viability in transduced cells. There was no loss of viability in A549, primary NK cells or YT cells, however cell viability declined from 41% to 20% in NKL (Figure 5-3d). It is possible that this might be caused by the loss of an anti-apoptotic function in virus lacking the E1 region and that NKL cells might be particularly susceptible to this death pathway. However, the absence of hexon (a surrogate for viral

replication) was deemed to be more important, as replicating virus is a cause for concern for therapeutic purposes.

The use of the new Ad5f35-EGFP stock resolved the issue of contamination with E1 containing recombinants. However this stock must also of course be propagated using 911 cells (or a similar helper line) and the potential exists for recombination to occur at any time propagation is undertaken. Thus, the potential for introducing replication-competent contaminates should always be considered when using this type of vector. A helper cell line has been developed to overcome this obstacle, known as PER.C6 (364), presenting a solution to the problem. However, use of this cell line is prohibitively expensive and it has not been made available for academic research.
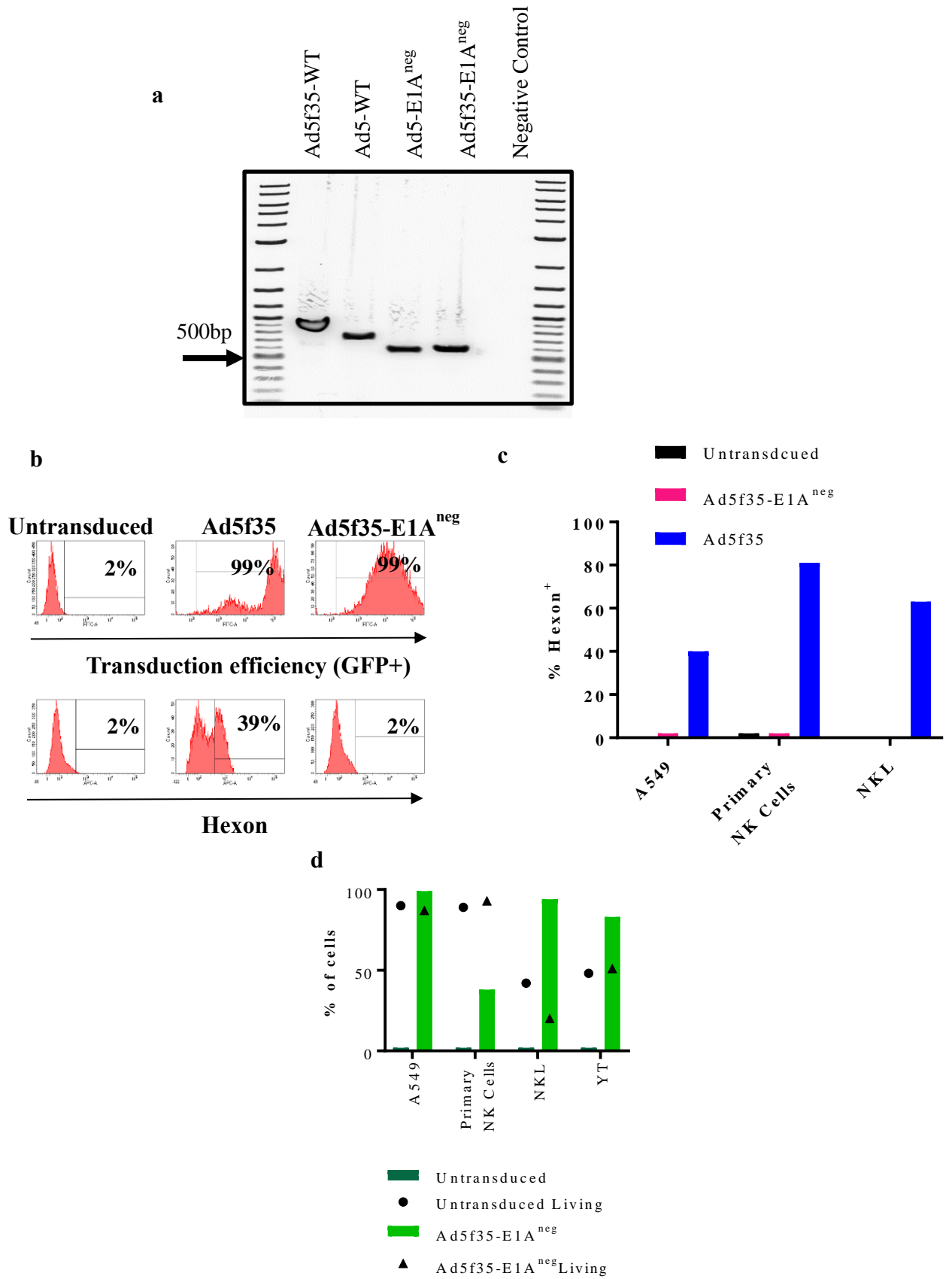
Figure 5-3 Replicating competent adenovirus contamination.

A) PCR of E1A region in replicative incompetent chimaeric adenovirus and as a positive control; replicative competent Ad5-WT and Ad5f35-EGFP. The expected

fragment size in E1A region sequence is 653bp and shown to be at the expected size for Ad5f35-WT and Ad5-WT. PCR of Ad5-E1A[neg] and Ad5f35-E1A[neg] show a fragment size of 500-600bp. B) An example of hexon expression analysis by flow cytometry from two different stocks of Ad5f35-EGFP; the FACS plots represent one experiment in A549 cells at 48hrs post-transduction. C) A summary of hexon expression from two different stocks of Ad5f35 in transduced A549, NKL and primary NK cells. D) Transduction and cell viability was analysed by flow cytometry 48hrs post-transduction using replicative incompetent Ad5f35-EGFP in A549, primary NK cells, NKL and YT cells.

### 5.2.4 Functional analysis of transduced primary NK cells

The ability of Ad5f35-EGFP to transduce primary NK cells provides a new avenue for gene transfer, and potentially a new perspective in NK cell-based adoptive immunotherapy strategies. In order to understand whether Ad5f35-EGFP transduction affects NK activation and function, NK cell' responses to a tumour target were analysed, using functional assays (e.g. cytotoxicity and degranulation), as described in section 3.2.10.1 - 3.2.10.2 and analysed by flow cytometry using the gating strategy described in Figure 5-4a. In brief, NK cells from four donors were transduced and analysed at 48hrs post-transduction by flow cytometry; cells were transduced between 7 and 25% (Figure 5-4b). Initially, NK cytotoxicity towards K562 (tumor target) was investigated to identify impairments in protein function or expression (e.g. granzyme and perforin). However, this assay includes the cytotoxicity induced from the total population of cells post-adenovirus transduction; thus a mixed population of untransduced and transduced cells were included. The tumour target, K562 cells were identified by flow cytometry in the forward and side scatter and were distinguished from NK cells by a cell tracker dye. After 5hrs of co-culture, K562s were stained with a dead cell discriminator as described in Figure 5-4a. The results show that cells with up to 25% transduced cells have a higher fold difference of cytotoxicity compared to that of untransduced controls; with a fold difference of +0.7 and +4.7 at a 1:1 and 3:1 effector: target ratio respectively. A t-test indicated that this difference was not significant (Figure 5-4c). The limiting factor to this assay is the inability to differentiate cytotoxicity mediated by transduced cells alone from the untransduced population.

The limitation of the cytotoxicity assays can be overcome by analysing the ability of NK cells to degranulate in response to K562. Upon activation, NK cells rapidly release cytolytic granules. Lining the luminal membrane of these granules is lysosomal associated membrane protein-1 (LAMP-1), also known as CD107a. The granule fusion to the cellular membrane upon exocytosis externalises CD107a to the cell surface, thus allowing for the assessment of degranulation; this provides a quantitative measurement of NK cell response to tumour targets (365). Upon degranulation, the content of the granules induce perforin-mediated damage on the target cells whereby granzyme family members (Granzyme A and B) translocate into the target cell and initiate the apoptotic cascade by cleaving numerous substrates (e.g. caspases, Bid, Ku70 and lamins) (366). NK cells were identified by their forward and side scatter

and having no cell tracker dye compared to that of the targets (Figure 5-4a). The expression of CD107a was determined in NK cells without target to establish the basal level of surface expression. It is clear that there was donor variability in their capacity to degranulate, however there was no significant difference in degranulation compared to their respective untransduced controls. The mean values being a +0.1 fold difference in degranulation compared to that of the untransduced control at an effector: target ratio of 3:1 (Figure 5-4d).

These findings reveal that NK cells retain their capacity to degranulate post-transduction as demonstrated by their ability to recognise targets and respond by releasing cytotoxic granules and inducing apoptosis in target cells. It is reasonable to assume the induction of CD107a on the surface of NK cells and target cell lysis correlate, and a previous study has shown that these events correlate (367). However, the absence of cytolytic proteins (such as granzymes or perforin) are known to impair the lytic function of NK cells (368). Despite, the limitations of the cytotoxic assay in differentiating the killing ability from untransduced and transduced populations, the level of killing was not reduced compared to experiments using untransduced cells. The cumulative data, suggests that adenovirus mediated transduction of primary NK cells does not significantly affect activation and functional activity with regards to degranulation. Overall, the ability of NK cells to retain function at 48hrs post-adenovirus transduction is a supportive prospect in the use of this viral vector as a strategy for gene transfer and NK cell-based adoptive immunotherapy.
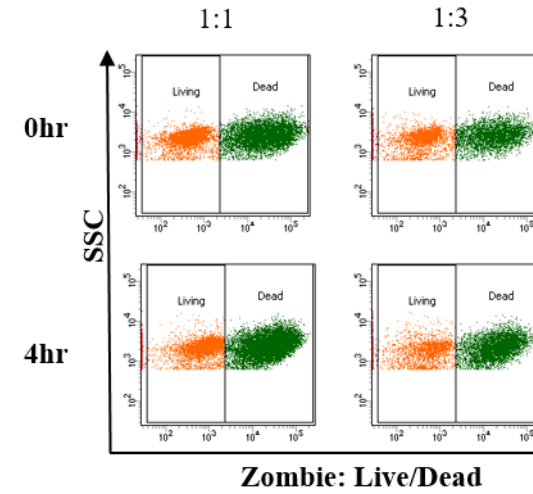
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c

d
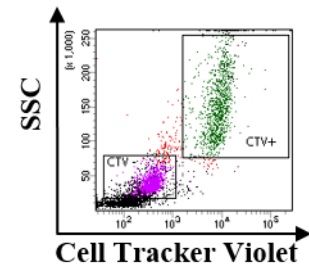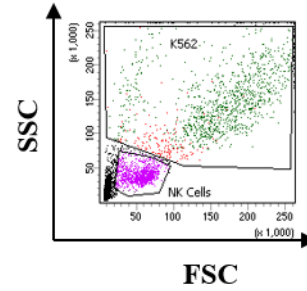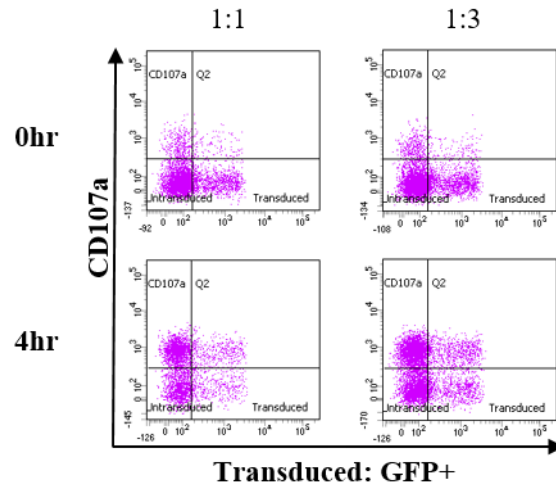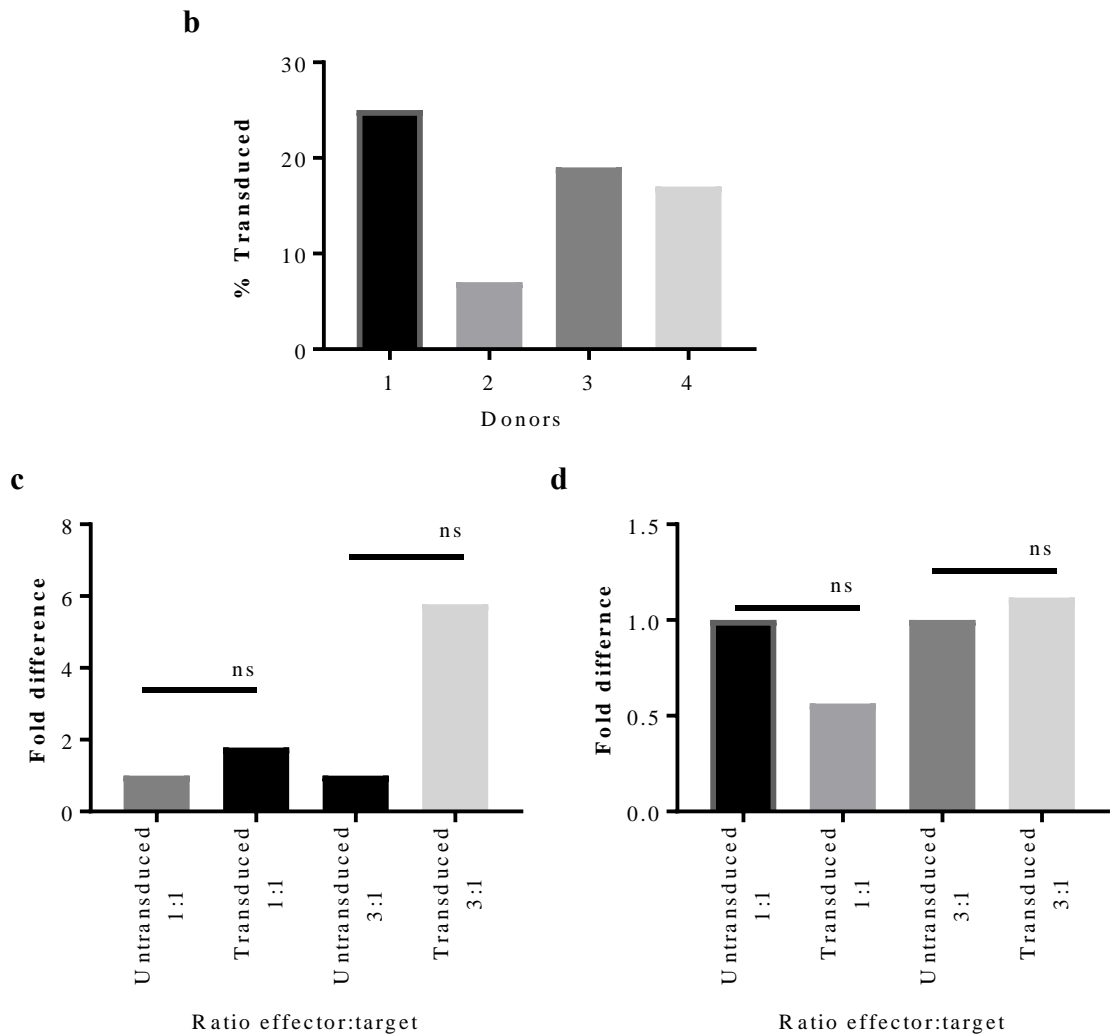
Figure 5-4 Ad5f35-EGFP mediated transduction of NK cells do not affect functional properties.

Degranulation and cytotoxic assays were performed on isolated NK cells that were either untransduced or transduced 48 hours prior with 1000 MOI Ad5f35-EGFP. K562s were used a tumour target and co-incubated for 4 hours at a ratio of 1:1 or 3:1, NK:K562 cells. The experiments was repeated from 4 healthy donors. A) The gating strategy and flow cytometric analysis used for degranulation and cytototoxic assays of untransduced and transduced NK cells. Degranulation was quantified using cell surface expression of CD107a on NK cells and cytotoxicity was assessed using a dead cell discriminator on K562. The percentage of untransduced controls were arbitrarily set to a fold account of 1, which the fold difference of transduced samples were determined. B) Percentage of Ad5f35-EGFP transduced NK cells with 1000 MOI. C) Summary of degranulation assays. D) Summary of cytotoxicity assay. Statistical analysis was determined using a ratio paired T test conducted between untransduced and transduced cells P<0.05; calculated p values are not significant.

5.2.5 CD46 is a key molecule in Ad5f35 transduction

A previous study demonstrated that Ad5 transduction efficiency is correlated to virus receptor expression (CAR) on cells (369). The importance of CD46 expression for Ad5f35-GFP cell binding and entry was determined using an anti-CD46 antibody. A549 cells were transfected with 0.3 MOI Ad5f35-EGFP in the presence of a CD46 antibody, as described in (Section 3.2.4). Transduction efficiency was determined at 24 hours, analysing EGFP expression by flow cytometry (Figure 5-5a). The result of 3 experiments shows a reduction of 41% in the mean value of transduced cells in the presence of a CD46 antibody (Figure 5-5b). The results suggest that Ad5f35-EGFP mediated transduction of A549 cells is partially dependent on CD46 expression, as shown previously (242). The importance of CD46 for Ad5f35 entry into NK cells was then determined. Purified NK cells were transduced with 500 MOI Ad5f35-EGFP in the presence of a CD46 antibody and EGFP expression was analysed 24hrs post-transduction by fluorescent microscopy (Figure 5-5c) and quantified by flow cytometry at 24hrs post-transduction. The result of 3 experiments shows a reduction of 33% in the mean value of transduced cells in the presence of a CD46 antibody, suggesting that Ad5f35 entry into NK cells is also partially dependent upon CD46 receptor expression (Figure 5-5d). However, this may have been reduced further if a higher concentration of antibody was used. Other factors that might be important in Ad5f35 transduction is cell membrane fluidity which has been suggested to influence endocytosis of the Ad5f35 virus in a T cell line (197). The $\alpha V\beta_3$ and $\alpha V\beta_5$ integrin is also described as a key molecule for Ad5 binding and entry through interactions of the viral penton base which triggers internalisation of the virus following receptor attachment (197,370).

a

1   **2**   **3**

Transduced
CD46
Blocking

Transduced

Transduction Efficiency (GFP+)

b

A549

Ad5f35

c

Untransduced   Transduced:IgG   Transduced   Transduced:CD46

d

Primary NK Cells
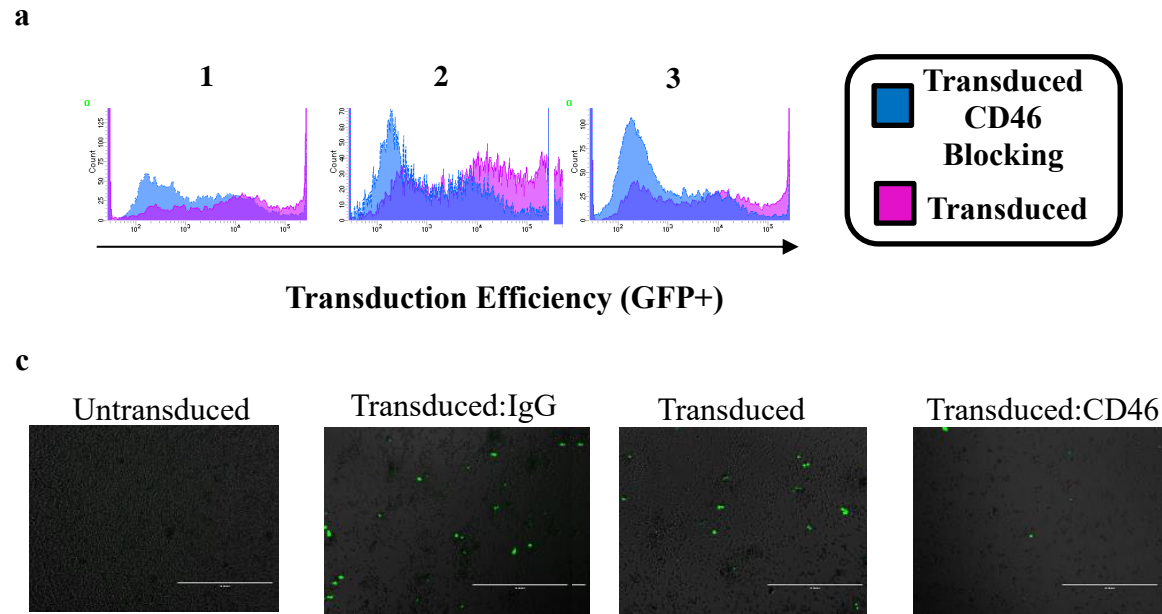
Ad5f35

Figure 5-5 CD46 expression is not the only factor affecting Ad5f35 transfection of A549 cells.

A549 and purified NK cells were treated with IgG, CD46 polyclonal antibody or untreated in combination with either Ad5f35 or no virus. A) GFP-positive A549 cells was determined by flow cytometry and summarised in B), showing the mean of 3 experiments. C) GFP-positive purified NK cells was determined by fluorescent microscopy and summarised in D), showing the mean of 3 experiments.
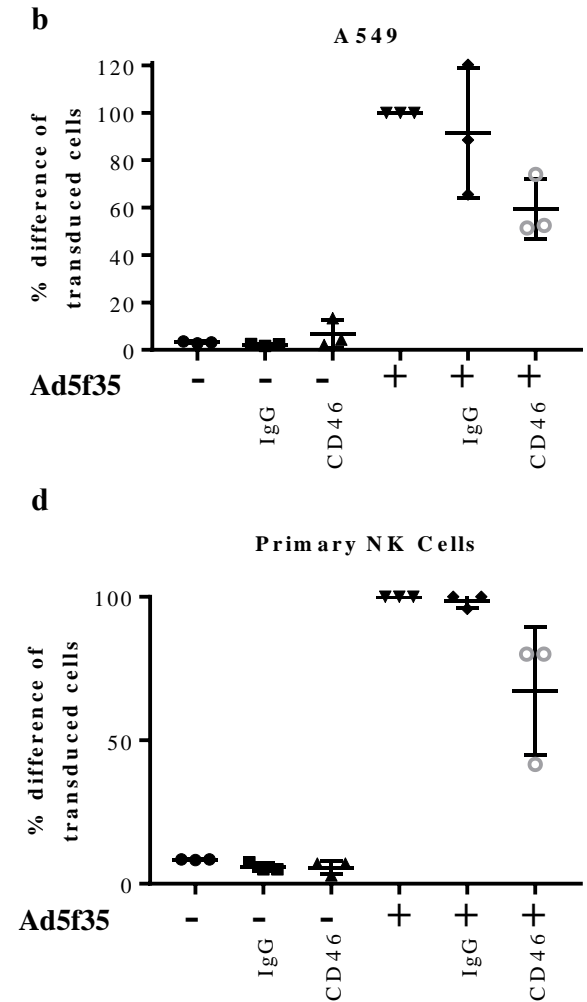
5.3   Discussion

Existing methods for gene transfer in NK cells are inefficient. Here I used the chimeric adenovirus, Ad5f35 to transduce human NK cells with an efficiency that surpasses lentiviral and other delivery systems described (172,182,323–325). These data is reinforced by other studies that describe the ability of Ad5f35 transduction in NK cells, T cells and B cells (175,315,351). To enhance Ad5f35 mediated transduction of NK cells the experimental conditions were evaluated, and using centrifugation (also referred to as spinoculation) showed that efficient transduction of primary NK cells was consistent. Centrifugal force is a well-established step in enhancing lentiviral transduction, however little is known about the mechanism. There is one study utilising centrifugation to enhance adenovirus mediated transduction of dendritic cells (358), however it is not normally used in adenoviral transduction experiments. Speculatively, centrifugation might force NK cells that are normally in suspension to temporarily adhere which might increase the interactions between Ad5f35 and the cellular receptor, CD46 and integrin. Prolonged centrifugal force might also affect the surface area or fluidity of the membrane, thus enhancing the prospect of the virus entering the cell through clathrin mediated endocytosis. Furthermore, a high concentration of 150-300 MOI was required to achieve optimal transduction at 67% in purified NK cells (contrasting with the <1 MOI routinely used to transduce A549 cells with the same virus stock). However, 150-300 MOI is not excessive in comparison to one study that investigated increasing MOI of Ad5f35 mediated transduction in NK cells, which used 1,000-10,000 MOI to achieve 50-60% transduction (175). My initial experiments found that IL-2 enhanced transduction efficiency, which is unsurprising as it likely prolonged cell viability of transduced cells. IL-2 induces NK activation, proliferation and survival through STAT5 signalling, which results in induced cell cycle and regulation of NK activating genes (371). In particular, the IL-2 mediated induction of the anti-apoptotic gene BCL2 is likely to enhance NK cell survival (372); hence a higher level of viable transduced cells are observed in IL-2 activated cells. Thus, these conditions has strengthened the prospect of using Ad5f35-EGFP as a tool for gene transfer in NK cells.

The comprehensive investigation on adenovirus mediated transduction of NK cells established issues in cell death and clumping of cells. NK cell clumping following adenovirus transduction was noted in my experiments. Clumping of cells has

previously been observed as a cytopathic effect of Ad5, and linked to the penton base. Removal of the RGD motif in the penton base can help overcome cell rounding and clumping; however this results in delayed cell entry (229,373). Whether NK cell clumping is deleterious is unclear. However, it might make delivery of transduced NK cells to patients less efficient. Cell death was also an issue in the early experiments and this was linked to the contamination of virus stocks with replication competent viruses via recombination during propagation in the producer cells (911 cells). The E1 region of the adenovirus genome is essential for the expression of early and late genes, and for virus replication (374); deletion of this region renders the virus replication deficient. The producer cell line, 911 has incorporated Ad5 sequence from 79 – 5789 (of Ad5), encoding the E1A and E1B genes to *trans*-complement early region function in vectors missing this region to enable virus expansion (375). The disadvantage of this system is having Ad vectors that are deleted from ~400 – 3500 (376) (i.e. wholly within the region inserted into 911 cells), allowing recombination between these sequences and generation of viruses with restored early region function, thereby contaminating virus stocks with replicating competent vectors. The E1A region consists of two exons, to confirm E1A presence in the virus stock, exon 1 was amplified by PCR and detected, but found to be at a different size to that of the wild type stock. Despite this discrepancy, the expression of hexon, a protein expressed late in virus replication as a result of E1A activity regions of virus replication supported the evidence that the virus was replicating. This has been a long-established problem in both viral production and clinical applications. However, stocks may still be used if the concentration of replicating virus particles are low, according to a report by the FDA (377). Attempts have been made to develop producer cell lines with varying amounts of adenovirus sequence designed to reduce the occurrence of recombination; however the reports differ in their efficacy (363). One such cell line, PER.C6, like 911 are derived from human embryonic retinoblast cells and have the E1A and E1B region incorporated from Ad5 genome sequence from positions 459 – 3510 (364). This cell line is likely the best designed cell line for the expansion of replication deficient adenovirus for clinical applications as it has the smallest fragment of Ad5 sequence incorporated, therefore restricting the levels of sequence homology and resulting recombination (378). However, due to the licensing costs of working with PERC.6, the 911 cell line is the preferred producer cell line for virus expansion in this study (and most others). Regardless of these issues the occurrence of recombination is low

in the first passages of adenovirus (362), and this was confirmed by testing and utilising different stock of virus. Overcoming this issue through limiting passages during virus expansion and testing for replication is the best approach for restricting this issue from recurring.

Evaluating the functional effects of Ad5f35 transduced purified NK cells was essential for using this system as a tool in manipulating NK anti-tumour activity. Investigating adenoviral vector-mediated gene delivery and response from the adaptive immune system has been demonstrated (379), however less is known concerning an NK cell response. One study has demonstrated NK cell induced activation from replication deficient adenovirus vectors in mice (302). This is unsurprising as NK cells have evolved to respond to viral infections and rapidly activated following infection, undergoing proliferation and producing IFNγ (380). NK cells have also been reported to respond to double stranded RNA produced during the adenovirus life cycle, inducing an IFNα/β response (303), however the pathway by which this occurs is unknown in NK cells (302,303). Nevertheless, these studies suggest that replication defective adenoviral vectors mediate NK activation, however the phenotype of Ad5f35 transduced NK cells have not been investigated (175). Furthermore, these studies do not distinguish the anti-viral response of the NK cell from functional changes that might occur as a result of transduction. Here I have demonstrated that NK cells have retained their ability to recognise, degranulate and kill tumour targets 48 hours post-transduction, a key desirable feature if these vectors are to be used in clinical applications. These limited reports on NK cells activation in response to adenovirus suggest that the effects of adenoviral mediated transduction of NK cells might enhance activation and improve their anti-tumour capacity. Other viral vectors, such as the vaccinia virus, has been shown to impair NK cell cytotoxicity (381,382).

Monitoring the expression of EGFP post-transduction in NK cells indicated that protein expression is best evaluated at 24 and 48 hours post-transduction, where subsequently cell viability is reduced. Retaining cell viability for longer periods permits observations of long-term transgene expression in a laboratory setting. There are a number of protocols currently in use to expand primary NK cells (383,384). Short-term NK expansion (~14 days) can be achieved by IL2 or IL15 treatment, which induces signals for survival, proliferation and activation. In comparison, feeder cells, such as K562 or Jurkat (a T cell line) have been used to achieve NK cell expansion for longer periods (385). The use of autologous PBMC has also been effectively

demonstrated in NK cell expansion, however not as effective as K562 cells (385). The disadvantage of these applications would be the risk of losing transgene expression due to the turnover of modified NK cells with NK cells that do not carry the transgene. Estimates of the lifespan of NK cells are variable, with some studies performed *in vivo* suggesting a half-life of 1-2 weeks in humans (386), and 7-10 days in mice (387), however transducing NK cells with adenovirus might have an impact on the kinetics of NK cells and therefore lifespan might vary. One report has used an anti-TNF antibody to block apoptosis in NK cells (388), which might provide a solution to NK cells retaining transgene expression for a longer period. Regardless of these reports, the results here show that the detection of EGFP rapidly declines from 3 days post-transduction. The short term viability and detection of transgene expression in NK cells might be observed as an advantage under a clinical perspective whereby genetically modified cells do not accumulate, therefore globally enhanced NK cells do not cause potential long-term problems, such as induced autoimmunity. A scenario in which autologous NK cells are maximally expanded ex vivo, then transduced and reinfused within ~24 hours might allow the maximum number of transduced cells to be delivered to a patient.

In conclusion, the results here describe an efficient method for transducing human NK cells using the chimeric adenovirus, Ad5f35. This method provides an alternative option to using lentiviral vectors in delivering and analysing short-term gene expression in primary NK cells. This vector's ability to transduce primary NK cells without altering their anti-tumour cytotoxicity is a significant asset and a promising tool in analysing pathways in NK cells or in clinical applications in immunotherapy of cancers. Many cancers exploit the immunosuppressive cytokine TGFβ to evade anti-tumour immunity in general and NK and cytotoxic T cells in particular (118,389). Engineering NK cells using Ad5f35 recombinants to resist the actions of this cytokine might generate a more potent anti-tumour response (134,204); this is addressed in the next chapter.

6 Engineering resistance to Transforming Growth Factor (TGF)-β

6.1 Introduction

Tumour progression is no longer solely viewed as an intrinsic property of the malignant cells. It is now recognised that multiple cell types (tumour cells, blood vessels, immune cells and stroma cells) contribute to the disease phenotype (5). The ability of the tumour to evade immunity is now recognised as a hallmark of cancer (5), complementing the concept and importance of tumour immunosurveillance (as described in Chapter 1). TGF-β, a cytokine known for its anti-proliferative affects plays a key role in tumour progression. Tumour cells themselves lose responsiveness to TGF-β, for example by acquiring mutations in the TGF-β signalling pathway (112). However, localised TGF-β can act upon the immune cells and inhibit both their proliferation and activation; one major target of TGF-β is NK cells (118,120,390). The addition of TGF-β to NK cells activated *in vitro* by IL-15 or IL-2 inhibits proliferation, cytotoxicity and interferon-γ secretion (118,120,390). Strategies are being investigated to block TGF-β tumour-mediated progression, thereby protecting immune cells from its immunosuppressive action. These include compounds and antibodies that target TGF-β and its receptors. The disadvantages of these strategies (that affect their efficiency) include their short half-life, specificity (especially for kinase inhibitors) and ineffective application when used alone (391). However, when used in combination with an adoptive transfer of activated immune cells (e.g. T cells), they have a greater effect (392). Thus, exploiting the use of autologous enhanced immune cells that can overcome TGF-β inhibition is a novel strategy in combating the effect of TGF-β induced tumour immunosuppression.

The disruption of TGF-β signalling in T cells or NK cells is known to improve anti-tumour immune responses (120,393), thus identifying the TGF-β pathway as a viable target by which to enhance anti-tumour immunity. Studies have utilised the expression of dominant negative TGFβRII in NK-92 and primary NK cells from cord blood and demonstrated that this reduces the action of TGF-β (134,204). Further, the NK-92 cell line engineered to express dominant negative TGFβRII was adoptively transferred into mice with lung cancer, which resulted in a higher survival rate and an increase in IFN-γ levels (204). These studies provide a foundation for the use of adoptive transfer of genetically engineered NK cells that are insensitive to TGF-β tumour immunosuppression. However, TGF-β exerts activity on many cell types and

blocking TGF-β activity can be deleterious; a dominant negative TGFβRII expressed in mice results in an increased risk and promotion of tumorigenesis in mammary and lung epithelial cells (394,395), raising concerns for enhanced outgrowth of tumours. However, this approach has yet to be described in human NK cells isolated from whole blood from either healthy donors or patients.

In this chapter, the assessment of inhibitors in the TGF-β pathway will be assessed and used in combination with Ad5f35 as a reliable method for gene transfer into primary NK cells.

6.2 Results

### 6.2.1 TGF-β modulates the expression of NK activating receptors and cytolytic activity

The NK cell receptors NKG2D, DNAM-1, NKp46 and NKp30 play an important role in recognising tumour targets (396–398). In addition, the Fc receptor CD16 is important in antibody-dependent cell-mediated cytotoxicity (ADCC) (399), this includes mediating NK cell activation in response to therapeutic antibodies such as Herceptin (400) and Rituximab (401). These NK cell activating receptors are known to be downregulated by the action of TGF-β (118,119), thereby reducing NK cell function responses to tumour cells. As the phenotypic effect TGF-β has on NK cells is well known, I first confirmed that TGF-β did indeed modulate the expression of these cell surface molecules using isolated primary NK cells purified from one donor. NK cells were either left unstimulated, or treated with IL-15 alone or in combination of IL-15 and TGF-β treatment, for 48hrs. IL-15 was used as it is a well-characterised cytokine that upregulates the expression of NK cell activation receptors through JAK and STAT signalling (56). The results show that the cell surface expression levels of NKp30 and DNAM-1 were induced by IL-15 (2-3 fold) but that this induction was blocked by a combination of IL-15 and TGF-β (Figure 6-1a and b). Expression of NKp46 was weakly induced by IL-15 (consistent with previous studies (118)) but expression was inhibited by TGF-β treatment. Expression of CD16 was enhanced in this one donor by IL-15 and TGF-β. Although just performed in a single donor, these results agree with findings from previous studies where human NK cells treated with TGF-β were shown to down regulate the expression of the activating receptors NKp30, NKG2D and DNAM-1 (118,119). These receptors play a key role in NK cell recognition of tumour cells (35) and their reduced expression was predicted to reduce NK cell-mediated killing of tumour targets, as shown in previous studies (118). Cytotoxicity assays were performed using the leukemic cell line K562 as a tumour target. Isolated NK cells (from two donors) were pre-treated with IL-15, alone or in combination with TGF-β for 48hrs prior to analysis. K562 cells were labelled with a cell tracker dye and then co-cultured with the cytokine-treated NK cells for 4hrs at a ratio of 1:1 and 1:3 respectively before targets were analysed by staining with a dead cell discriminator and assessed by flow cytometry (Figure 6-1c). The results show that NK cell mediated killing was reduced when NK cells were treated with IL-15 and

TGF-β compared to IL-15 alone (Figure 6-1d). A control in which natural killer cells were treated with TGF-β alone was not included in this experiment. However, it is well established that TGF-β antagonises the action of IL-15, inhibiting NK cell activation by this cytokine (118–120); treatment with TGF-β alone has little or no effect, at least *in vitro*. Nevertheless, these results confirm that TGF-β has potent immunosuppressive activity towards NK cells and represents a potential immunotherapeutic target.

a

b
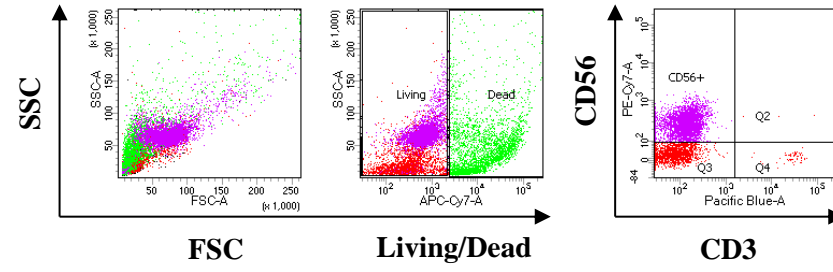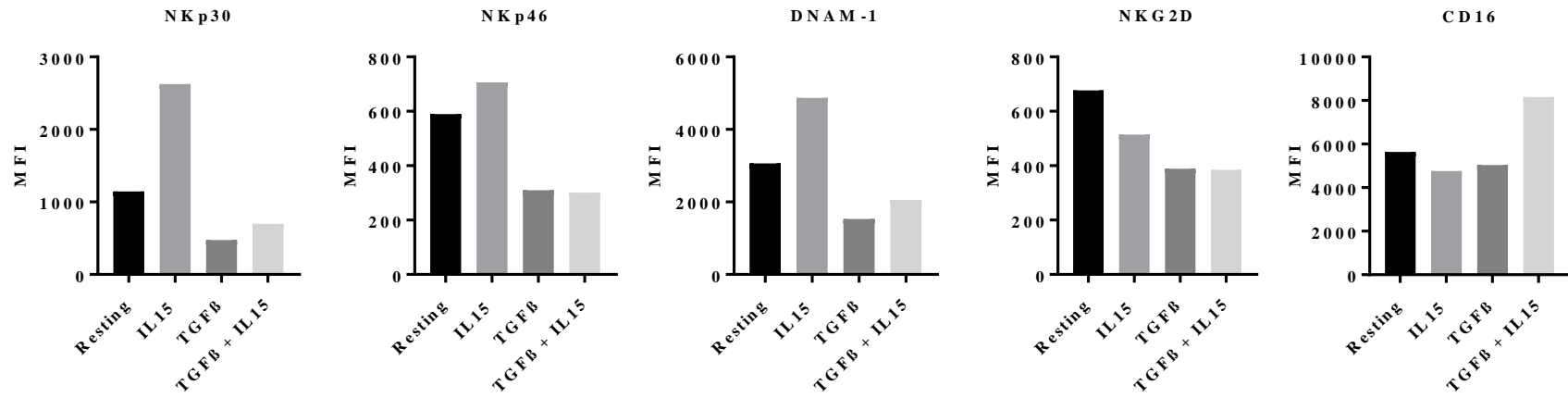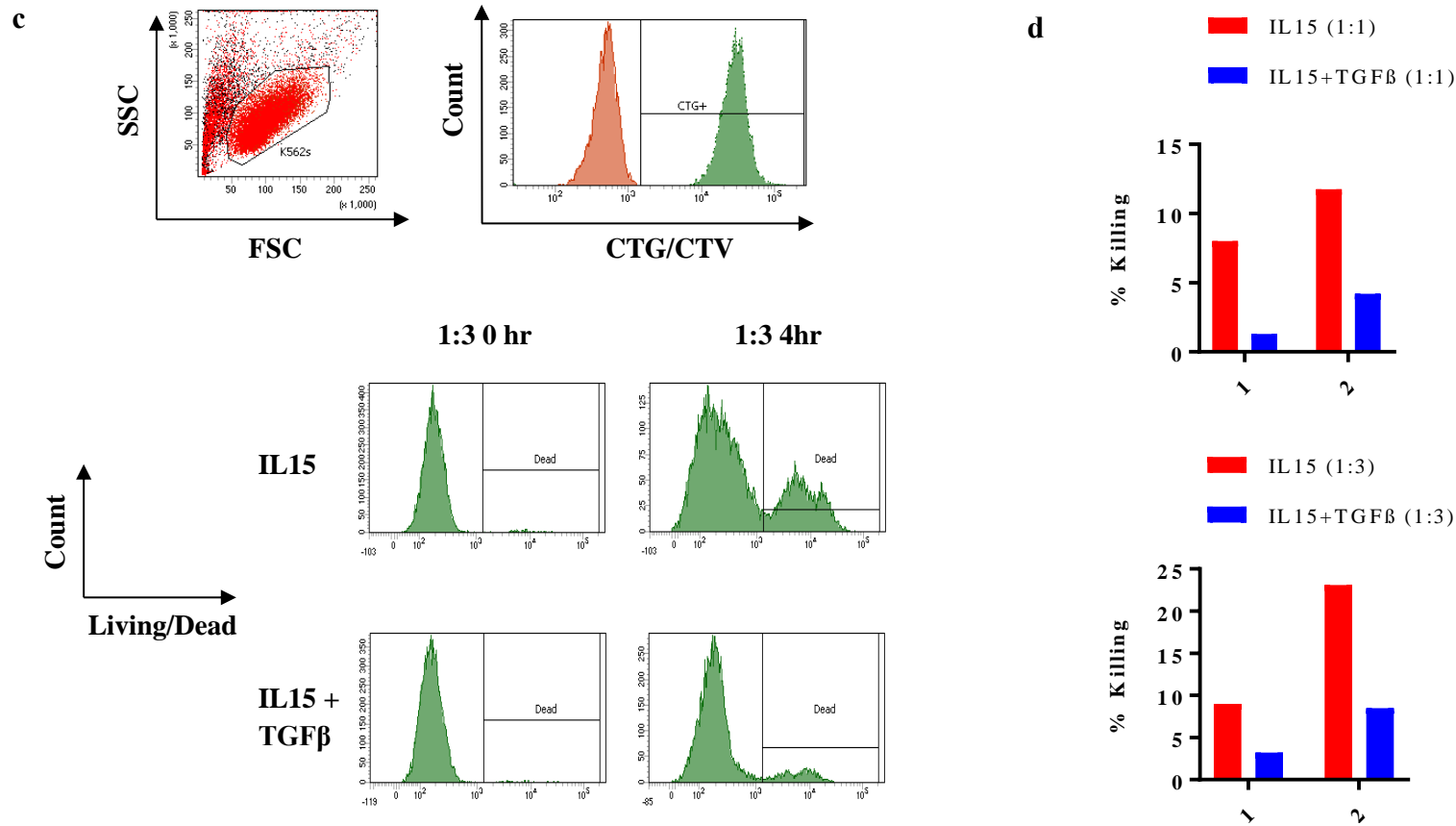
Figure 6-1 TGF-β antagonises IL-15 induced the expression of NK cell activation receptors and reduces cytotoxicity against K562 cells.

A) NK cells were unstimulated or stimulated with either 20 ng/ml IL-15 (15) or 5 ng/ml TGF-β or IL-15 plus 5 ng/ml TGF-β for 48hrs. Cell surface expression of NK cell activation receptors as indicated were analysed by flow cytometry using the representative gating strategy for NK cells. B) Summarised of (A) from one donor. C) A standard killing assay was performed against K562 tumour cell line at an Target: Effector ratio of 1:1 and 1:3 and analysed by flow cytometry using the representative gating strategy; K562s were stained with cell tracker green prior to co-culture with NK cells for 4hrs and stained with Zombie dye, a dead cell discriminator. D) The results from two donors were summarised, measuring the percentage of dead K562 cells.

6.2.2 TGF-β signalling in the presence of inhibitory molecules

The inhibitory effects of TGF-β on NK cells can be overcome by expressing dominant negative (dn) TGFβRII (134,204). This synthetic type II receptor lacks the cytoplasmic serine/threonine kinase domain, which stops the formation of the heteromeric complex and the phosphorylation by the type I receptor, therefore limits further intracellular signaling from TGF-β (134,204). TGF-β signalling induces at least two feedback inhibitory molecules, SMAD6 and SMAD7 (106). Ectopic expression of these molecules is also predicted to inhibit TGF-β signalling (22,23). Before attempting to manipulate these molecules, their expression was analysed. Primary NK cells were purified from PBMC from 3 donors and were treated with IL-2, TGF-β and a combination of both. IL-2, and not IL-15 was used to stimulate NK cells in this experiment as it was the main cytokine that was used to enhance NK cell survival during adenovirus mediated transduction of cells (see Chapter 4). At 48hrs post-treatment protein expression was assessed by Western blot, as shown in Figure 6-2a and described in the methods section 3.2.7. Quantification by densitometry from 3 donors was performed (Figure 6-2b-d). These results showed that the levels of TGFβRII, SMAD7 and SMAD6 remain consistent across all conditions. TGF-β has been shown to downregulate transcription of its receptors and upregulate SMAD7 in chondrocytes (402), however these results suggest that at 48hrs post-treatment, TGF-β does not affect expression of these molecules in NK cells. Nonetheless, these results show that SMAD6 and SMAD7 are expressed by NK cells.

I next used a reporter assay system, in an attempt to prove the inhibitory actions of dnTGFβRII, SMAD7 and SMAD6 on TGF-β signalling. This assay utilised HaCaT cells, immortalised keratinocytes that are readily transfectable and which respond to TGF-β (403). I used a reporter construct (3TP-Lux) which fuses the TGF-β responsive promoter from the human PAI-I gene to firefly luciferase; TGF-β signalling thus induces luciferase expression which is assayed using a luminescence-based enzyme assay (404). HaCaT cells were transfected with 3TP-lux along with pRL-TK-Renilla, which was used as a transfection control, as described in the methods section 3.2.2.3. At 24hrs post-transfection, cells were treated with TGF-β and at 48hrs post-transfection, cells lysates were analysed using a luminometer (Berthold Mithras). As expected, TGF-β exerted a dose dependent increase in luciferase activity until it reached a peak in the presence of 10-15ng/ml TGF-β and then reduces at 20ng/ml TGF-β (Figure 6-3a). Simultaneous addition of TGF-β with Galunisertib, a TGFβRI

inhibitor (405), prevented the TGF-β mediated induction of luciferase activity, even at a maximal concentration of 20ng/ml TGF-β (Figure 6-3a). This data shows that this assay can be used to measure TGF-β signalling in the presence of inhibitors and would therefore be useful in screening genes with inhibitory function in the TGF-β signalling pathway.

I investigated the effects of overexpressing the inhibitory SMADs and the dnTGFβRII on TGF-β signalling using the luciferase assay. Plasmids encoding dnTGFβRII, SMAD6 and HA-tagged SMAD7 were co-transfected with the PAI-I-luciferase and Renilla plasmids. After 48hrs, cell lysates were used in western blots, to confirm expression of the co-transfected molecule and then used in luciferase assays. The blots show that SMAD7 (detected with the HA-tag) and SMAD6 were expressed in HaCaT cells (Figure 6-3b). However, only a band size equivalent to endogenous TGFβRII was detected, and not the transfected dnTGFβRII (Figure 6-3b). This could be due to several factors, for example the specificity of the antibody used to detect dnTGFβRII, the efficiency of transfection of HaCaT cells or expression of the dnTGFβRII molecule in HaCaT cells. The ability to detect TGF-β inducible luciferase activity in HaCat (Figure 6-3a) suggests that transfection of HaCaT is efficient. Furthermore, transfected SMAD6 and SMAD7 were readily detected (Figure 6-3b). It seems likely that the dnTGFβRII construct is either inefficiently expressed or the antibody is not capable of detecting this species in HaCaT cells. A dnTGFβRII with an epitope tag (e.g. HA, as used to detect SMAD7) at the *N*-terminus of the mature extracellular domain would be a useful control to ensure expression of this molecule. Transfection of either dnTGFβRII or SMAD7, led to reduced TGF-β signalling as assayed using the luciferase reporter system, whereas no effect was seen when SMAD6 was expressed (Figure 6-3c). Statistically significant impairment of TGF-β signalling was observed only in the case of SMAD7 (p=0.035), whereas expression of dnTGFBRII did not exert statistically significant effects. However, dnTGFβRII is known to hinder TGF-β signalling in NK cells (134) and TGF-β signalling was markedly reduced in the luciferase assay. It is possible that TGF-β signalling within cell lines exhibit some variation, thus affecting the outcome of the luciferase assay. Supporting this, contrasting results have been published in over-expression of SMAD7 in a luciferase based assay in Mv1Lu (406) and COLO-357 p3TP-lux transfected cells (407). However, the results here identify dnTGFβRII and SMAD7 as candidates by which to inhibit TGF-β signalling. Notably, SMAD6 is traditionally known to inhibit the

receptor SMADs for BMP signalling, however a recent study has also shown that SMAD6 also inhibits TGF-β1 non-canonical signalling (408). The next step was to generate recombinant Ad5f35 encoding these molecules for delivery to NK cells.

a

b   **TGF-β Receptor II**
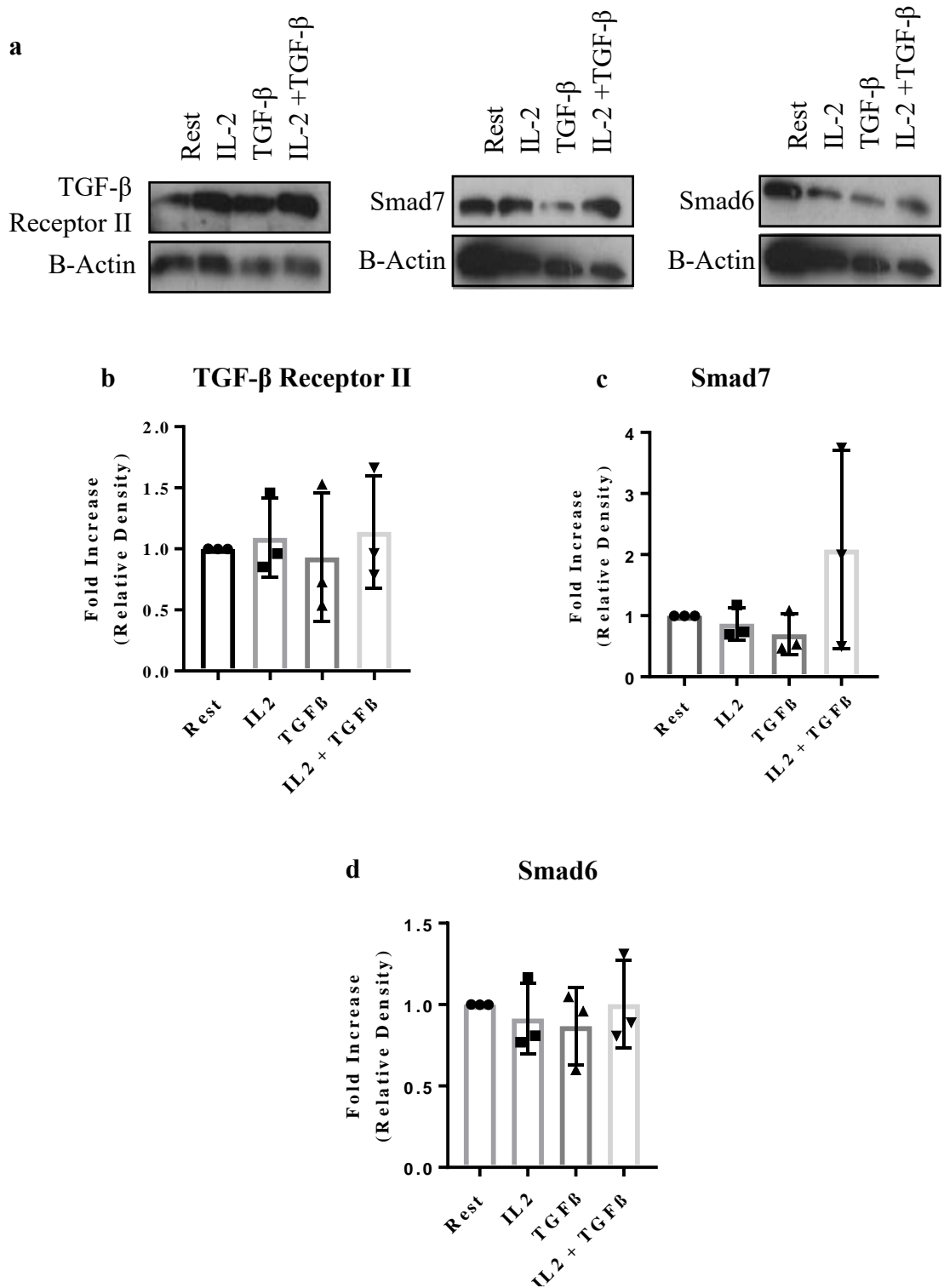
c   **Smad7**

d   **Smad6**

Figure 6-2 The levels of TGFβRII, SMAD7 and SMAD6 remain consistent in unstimulated and IL-2 and/or TGF-β stimulated NK cells.

A) Primary cells were treated with either 100IU/ml IL-2 and/or 5ng/ml TGF-β1 and cultured for 48hours before detecting by western blot as represented by one donor and (B) summarised by densitometry in 3 donors for the expression of TGF-β Receptor II, (C) Smad7 and (D) Smad6. The bands from all donors were analysed by densitometry and beta-actin was used as a protein control. The resting samples were arbitrarily set to 1 and the results are the mean and standard deviation from three donors.

a

b

c
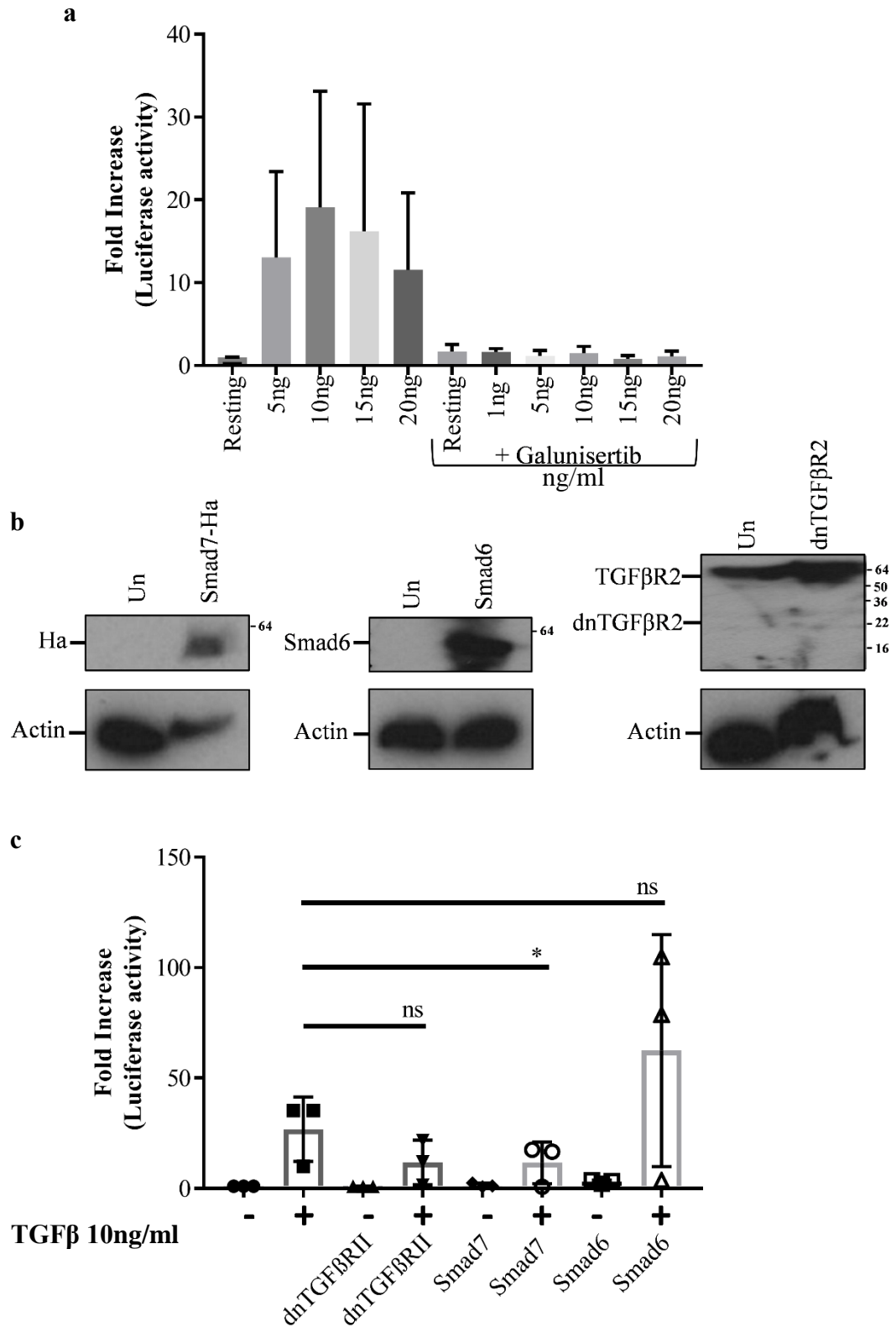
Figure 6-3 Dominant negative TGF-β receptor II and Smad7 is involved in inhibiting TGF-β signalling mediated by TGF-β1 in HaCaT cells.

A) HaCaT cells were co-transfected with 0.5μg p3TP-Lux reporter construct, along with 0.4ug pRL-TK as a control for transfection efficiency. At 24hrs post-transfection they were treated with either TGF-β1, 10μM Galunisertib or untreated. B) HaCaT cells were further co-transfected with either Smad6, Smad7 or dominant negative

TGF-β receptor II and a western blot was performed from one transfection to determine protein expression 48hrs post-transduction. C) The luciferase fold difference was determined in cells transfected with either Smad6, Smad7 or dominant negative TGF-β receptor II in cells treated with TGF-β1. The luciferase output is represented as fold increase, with the resting and unstimulated cells arbitrary set to 1. The graphs show the mean ± SD of three independent transfections conducted in triplicate. A t-test was performed as illustrated from the numerical figures depicted in (C); statistical analysis is statistically significant at $P<0.05$.

6.2.3   Generation of Ad5f35-CFP-dnTGFβRII

Expressing dnTGFβRII in cord blood NK cells has been shown to hinder inhibition from TGF-β and retain activation towards tumour targets (134). The findings from Figure 6-3 also suggest that expressing dnTGFβRII has the capacity to reduce TGF-β signalling, therefore this receptor was chosen for further investigation in isolated primary NK cells. Recombineering ("Recombination-mediated genetic engineering") was the system used to insert the dnTGFβRII gene into Ad5f35, which was initially developed using a replication-deficient Ad5 vector (409). This system uses a bacterial artificial chromosome (BAC) that contains the Ad5f35 genome maintained as a single copy bacmid in the *E.coli* SW102 strain (410)**.** The Ad5f35 genome used has the E3 region deleted and replaced with CFP under control of a CMV promoter, and the E1 region deleted and replaced with a selection cassette. This selection cassette includes three genes that encode ampicillin resistance, sensitivity to sucrose (*Sac*B) and *lac*Z (to produce blue colonies when provided with the chromogenic substrate, X-gal). A schematic diagram of this process is explained in the methods section, Figure 3-4.

The first step in recombineering was synthesising the dnTGFβRII fragment with flanking adenovirus sequence for homologous recombination events that replace the selection cassette (Figure 6-4a). For optimal specificity, primers were designed to add 80bp of Ad DNA homology flanking the dnTGFβRII gene. A gradient PCR was set up to identify the appropriate annealing temperature that was found to be optimal at temperatures between 55-64°C (see methods section 3.2.6.1.2). A PCR fragment was purified from the agarose gel in preparation for recombineering (Figure 6-4b). The purified dominant negative TGFβRII gene fragment was then electroporated into SW102 cells containing the Ad5f35 BAC.

Recombination was mediated by a modified λ prophage system that has enhanced efficiency in homologous recombination (411). This λ prophage system was modified by deletion of lytic genes and controlling the genes required for recombination. The left promoter (pL) is responsible for the expression of genes responsible for recombineering: *Gam, Exo* and *Beta*. The *Gam* gene encodes a protein that inhibits the host's nucleases which are involved in double-stranded break repair and would otherwise degrade the linear DNA of the incoming fragment. The *Exo* and *Beta* genes encode proteins that perform the recombination process. The *Exo* gene encodes a 5′-3′ dsDNA dependent-endonuclease that binds to the end of the introduced dsDNA and

degrades one strand to create a 3′ overhang. The *beta* gene encodes a protein that binds to the overhang to prevent degradation of the ssDNA and facilitates the interaction between homologous sequences. Expression of these recombination genes is controlled by the *cI857* gene, a temperature-sensitive mutant repressor from bacteriophage λ that is inactivated at 42°C. Inactivation of the repressor allows the expression of pL that results in the expression of the genes that facilitate recombination (see methods section 3.2.6.2 for further details). Putative recombinants were selected by plating electroporated cells on agar plates containing chloramphenicol, sucrose, IPTG and X-gal. The *LacZa* encoded selection catabolises X-gal to produce a blue phenotype in the presence of IPTG, therefore recombinant should only form a white colony (circled in green in Figure 6-4.c). However, as the dnTGFβRII gene replaces the selection cassette, recombinants should be the only colonies to grow in the presence of sucrose. However, non-recombinant (blue) colonies were observed on the sucrose plates, suggesting that some cells were insensitive to sucrose (circled in red in Figure 6-4c). To confirm recombinants, colony PCR was performed on white colonies using primers that were specific to the dnTGFβRII gene (methods section 3.2.6.1.3). As a positive control, the PCR was also performed on the dnTGFβRII plasmid. A PCR product was formed from 3 colonies; 11, 27 and 28 out of a total of 37 colonies screened (Figure 6-4d). This shows that the efficiency of recombination is approximately ~8 per 100 colonies. The three colonies were inoculated into larger cultures and the BAC isolated for further validation, as well as transfected into 911 cells for propagation of the recombinant virus.
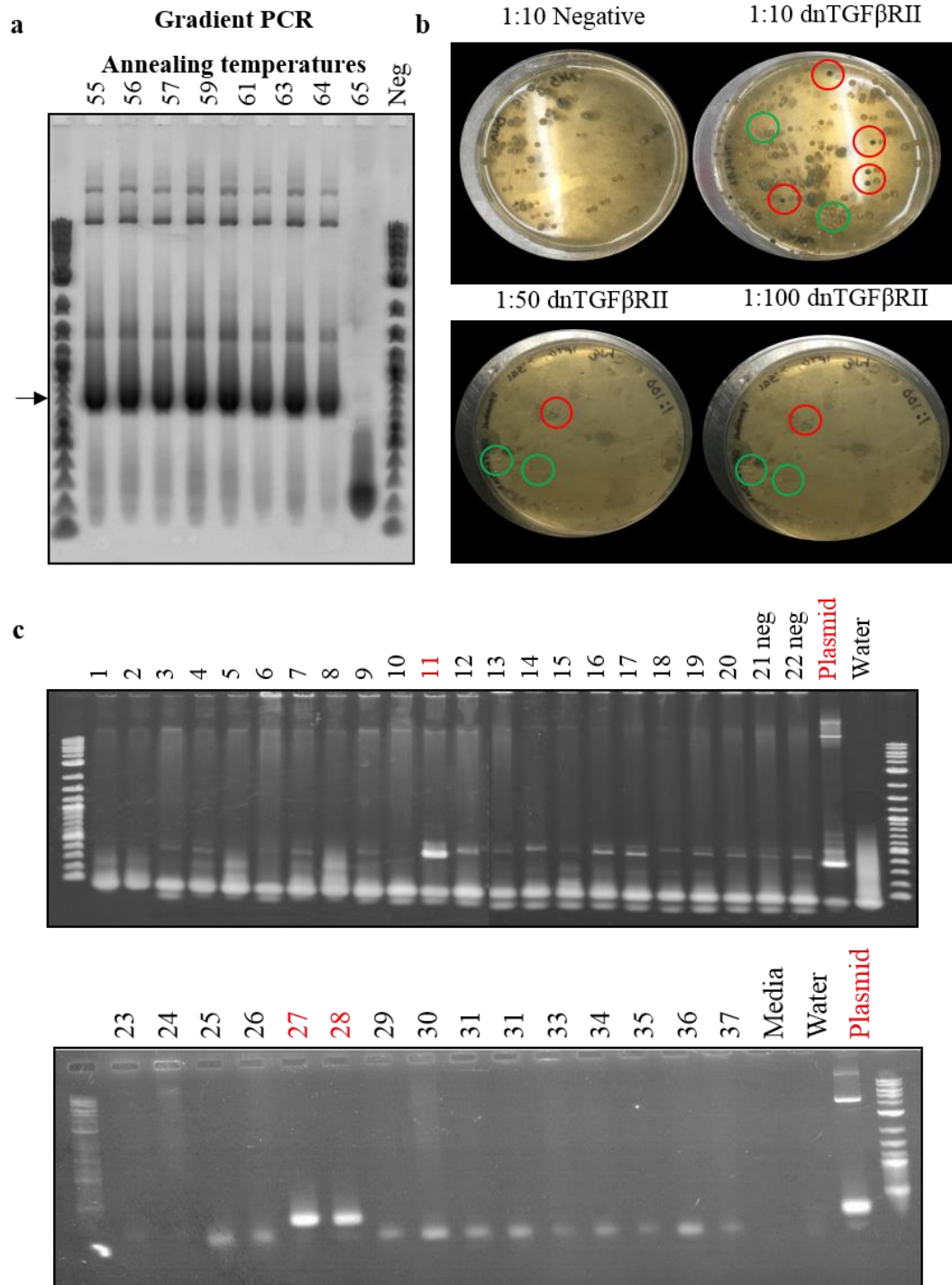
Figure 6-4 Generation of the DNRII in the BAC containing Ad5F35-CFP genome.

The AdZ system: the gene fragment with flanking adenovirus sequence is cloned into the BAC containing the Ad5f35 genome by a single recombination step that replaces the selectable markers and permits for identification of positive recombinants (circled in green). A) The dominant negative TGFβ receptor II gene was amplified using HiFi DNA polymerase from the pLNCX-TbetaRIIDN plasmid (Addgene). Included as a negative control (Neg) was a PCR performed with no template DNA. A gradient PCR with different annealing temperatures as indicated was separated by electrophoresis on a 0.8% agarose gel. The arrow marks the expected PCR product. The PCR product from the gradient PCR was separated by electrophoresis for gel extraction for recombineering. B) Competent SW102 cells were electroporated with 10ng of the

dominant negative TGFβ receptor II PCR product and recovered in 1ml LB in 32°C shaking incubator. After 1 h, the cells were washed in M9 salts and plated at different concentrations onto chloramphenicol, X-gal and IPTG selection plates. The plates were then incubated at 32˚C until colonies were present. C) Potential recombinants were identified as white colonies (circled in green) and were screened for the presence of the dnTGFβRII gene by colony PCR. PCR was performed using Taq DNA polymerase with primers specific for the dnTGFβRII gene. A sample of the products of each of the PCR reactions was separated by electrophoresis on a 0.8% agarose gel. The pLNCX-TbetaRIIDN plasmid was included as a positive control and no template was included as a negative control.

6.2.4 DNA sequence analysis of Ad5f35-CFP- dnTGFβRII

Following recombination, steps were made to confirm the dnTGFβRII gene insert was correctly cloned into the BAC at the selection cassette site. First, the BAC from the three constructs were isolated and incubated with the *Bam*HI restriction endonuclease. Recombinants appeared correct due to the loss of the 1.7 kb and 2.5kb bands encompassing the selection cassette (Figure 6-5a). Secondly, Sanger sequencing was performed on PCR products that amplified the insert (using primers from flanking adenovirus sequence) which confirmed that the dominant negative TGFβRII gene was located at the correct site. The construct from colony 28 showed a point mutation and a single nucleotide deletion in the dnTGFβRII sequence, therefore was not used in further investigation (data not shown). The construct from colony 11 displayed the same nucleotide deletion as colony 28. Analysis of this deletion shows a single adenosine base is deleted within a cluster of ten adenosine residues, which predictably results in a frameshift mutation (Figure 6-5b) (Complete sequence in appendix 9.3).

Lastly, sequencing from colony 27 shows a mixed sequence trace, the dominant trace is that of the expected sequence for dnTGFβRII and the minor sequence shows the same base deletion as colony 11 and colony 28 (Figure 6-5b) (Complete sequence in appendix 9.4). The frameshift mutation observed in colony 11 and 27 alters the reading frame and allows it to continue for a further 34 amino acids before a stop codon is encountered (Figure 6-5c). This generates a predicted protein product that is 161 amino acids in length (approximately 17kDa). Furthermore, the alteration to the open reading frame occurs on the *N*-terminal side of the predicted transmembrane domain of the wild type protein (Figure 6-5c). This suggests that this construct, here denoted truncated (t) dnTGFβRII would express a protein that would not be anchored to the plasma membrane and might result in the receptor being secreted.

Deletion of intracellular domains from TGFβ receptors results in mis-sorting of the molecules in polarised cells (412). The mixed population of sequences found in colony 27 will likely result in two different viruses expressing two different receptor variants, one being a truncated form of dominant negative TGFβRII (tdnTGFβRII ~21kDa) and the second being the expected dnTGFβRII (~33kDa).

Interestingly, the mutation present in tdnTGFβRII has been observed in colorectal cancer from Lynch syndrome patients, who have mutations in mismatch repair genes (112). The mutation is associated with reduced cell surface expression of TGFβRII

(413). This mutation predictably allows tumour cells to escape TGF-β growth control and aids in tumour progression. In this project, the deletion might be caused by one of two reasons: firstly, the initial generation of the fragment by PCR might have introduced the deletion through error of the polymerase or secondly, the error might have been caused during BAC replication when propagating the construct. The mixed population of sequences observed in colony 27 suggests that it is unlikely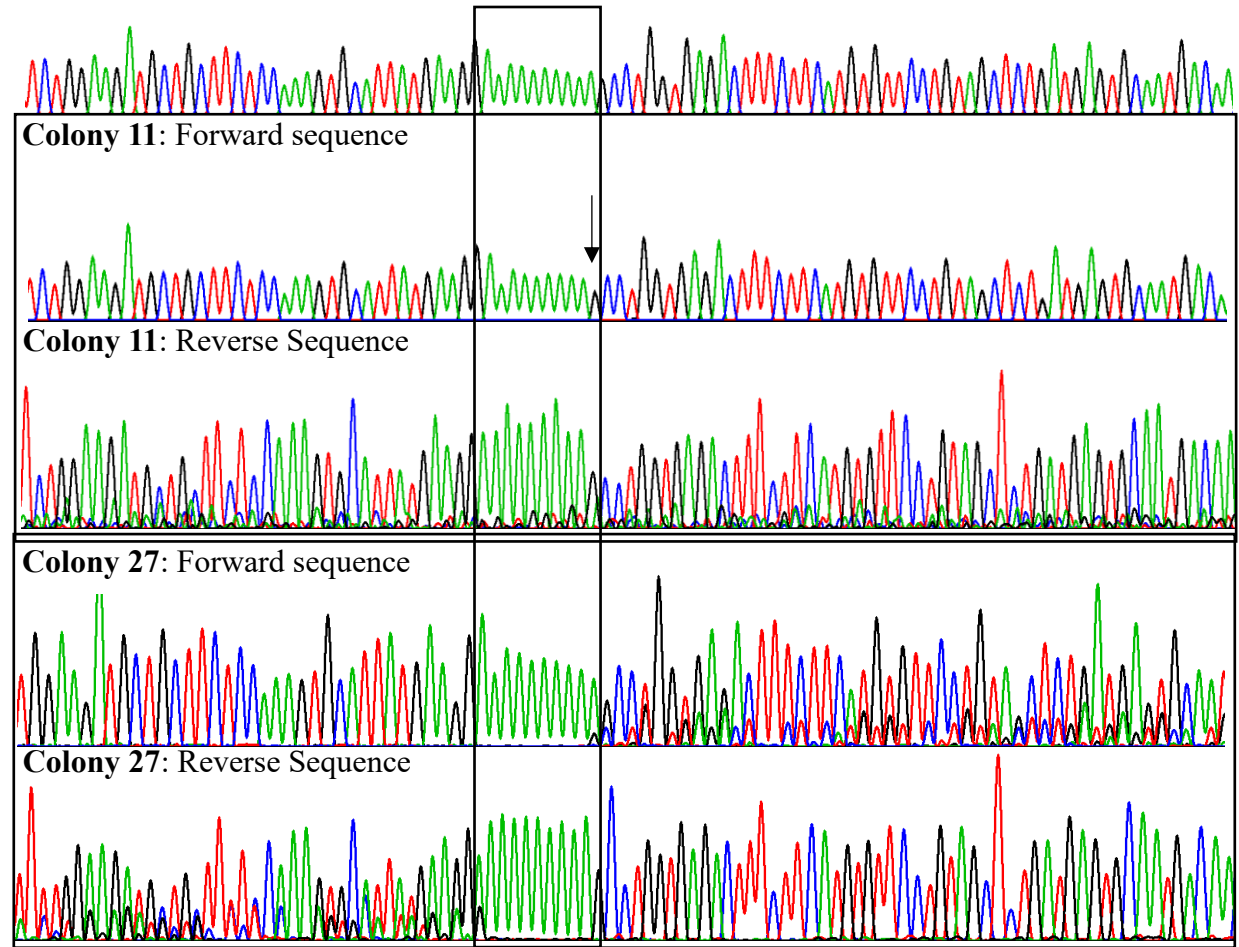 to be caused by the PCR that generated the insert. This would suggest that clustered adenosine repeats is problematic for polymerase based error during BAC replication in the *E. coli* strain used for BAC recombination. However, this system was not efficient in repairing the deletion which might be due to mutagenesis of the repair system or most likely the efficiency of recruiting the DNA repair system to the site of the deletion. The recurrence of the same mutation in all three colonies also suggests that this issue would not be resolved from repeating the recombination step. Therefore it was decided to investigate whether these constructs did encode a functional form of a dnTGFβRII.

For simplicity, the virus constructs were termed tdnTGFβRII.1 for colony 11 and tdnTGFβRII.2 for colony 27. The BACs were transfected into 911 cells and the propagated virus was isolated and validated by electron microscopy, showing the presence of intact adenovirus particles (appendix section 9.5). A549 and 911 cells were transduced and transduction efficiency was determined by fluorescence microscopy at 48hrs post-transduction (Figure 6-6a). The problems experienced with recombination of early region genes back into the vector during propagation in 911 cells (Chapter 4) prompted analysis of replication by these recombinants. The expression of hexon protein (a late gene, requiring early region gene expression) was determined by flow cytometry in transduced cells (and untransduced controls) (Figure 6-6b). The 911 cells provide E1A *in trans* and are used as a producer cell line; transduction into these cells induced hexon expression whereas A549 cells lack E1A and no hexon expression was detected. This indicated that these preparations were not contaminated with replication-competent viruses generated during propagation in 911 cells.

c

Figure 6-5 Validation of the frame shift mutation in the DNRII insert.

A) BAC DNA from three isolated colonies and a control was isolated. Recombinants were tested by incubating the BACs with BamHI and were separated on a 0.8% agarose gel. Colonies were identified by the loss of the 1.7 and 2.5kb products (marked with arrows) that were present in the control. B) The BACs were sequenced and compared to the dnTGFβRII plasmid sequence control. A mutation is indicated in exon 3 showing a single adenosine base deletion on the reverse and forward strands and control sequence (shown with arrows). C) The base deletion results in a frame shift (shown in red) prompting a premature stop codon at the beginning of the transmembrane region (shown in bold).

Figure 6-6 Virus constructs are replication deficient.

A) A549 and 911 cells were transduced at MOI of one with tdnTGFβRII.1 and tdnTGFβRII.2. Transduction efficiency was determined by fluorescent microscopy 48hrs post-transduction. B) In addition, cells were assessed for transduction efficiency by flow cytometry by CFP expression. Virus replication was determined by hexon expression in untransduced and transduced controls.

6.2.5 The expression of dominant negative TGFβRII in A549 cells

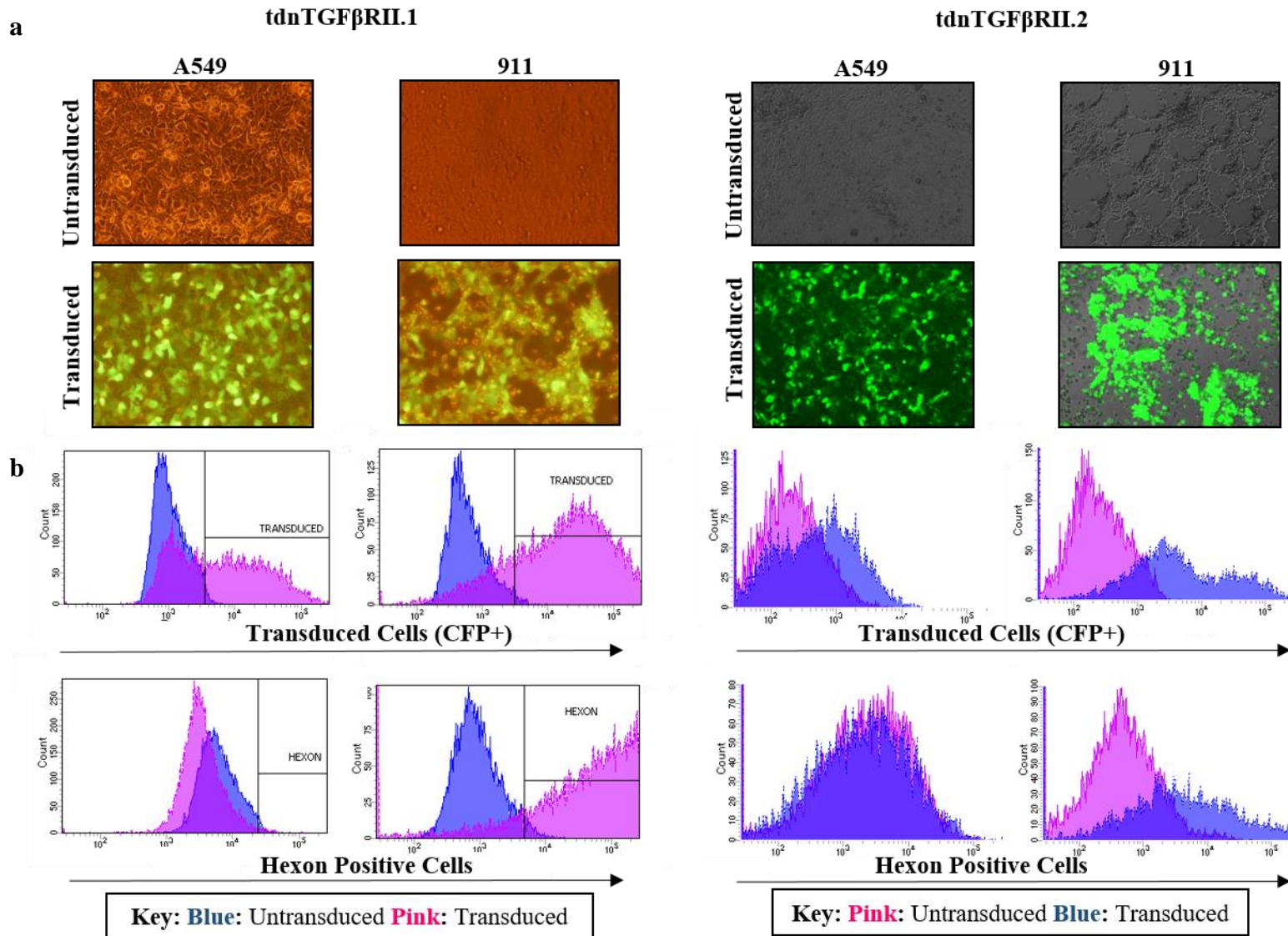The frameshift mutation observed in both Ad5f35 tdnTGFβRII.1 and tdnTGFβRII.2 predicts that the dnTGFβRII will be truncated due to a premature stop codon (Figure 6-5). This frameshift mutation might result in reduced transport and expression of the receptor at the cell surface, thus reducing its inhibitory function. Using the protein sequence, which was translated from the DNA analysis using 'ExPASY translate tool', the open reading frame was analysed in TmPred, a transmembrane prediction tool which evaluates the hydrophilicity and hydrophobicity of the protein sequence (414). The results generated a plot using a 'hydropathy scale' which identified the leader sequence (also known as the signal peptide) which prompts translocation into the endoplasmic reticulum (Figure 6-7a). The predicted polypeptide sequence of tdnTGFβRII shows a cryptic transmembrane site at the C-terminus (Figure 6-7a). Thus, it is plausible that the tdnTGFβRII receptor will be expressed at the plasma membrane, however this computational approach is speculative.

To further analyse the expression of the truncated form of dnTGFβRII at the plasma membrane, A549 cells were used to determine protein expression, as these cells are readily transduced with Ad5f35. A549 cells were transduced with Ad5f35 tdnTGFβRII.1 and tdnTGFβRII.2 and the cell surface expression of TGFβRII determined by flow cytometry. The expression of virus-encoded CFP was also determined and compared to untransduced cells at 48hrs post-transduction. Cells that were transduced with tdnTGFβRII.1 showed a mean 87-fold increase in TGFβRII surface expression, with 87% of cells CFP positive. In contrast, cells that were transduced with Ad5f35 tdnTGFβRII.2 showed a mean 10-fold increase in TGFβRII expression, with 57% CFP positive (Figure 6-7b). This is in agreement with a previous study showing that A549 express TGFβRII at high levels, especially compared to hepatocellular carcinoma cell line (HepG2) and a lung fibroblast cell line (HFL-1) (415). This study suggests that overexpression of TGFβRII in A549 has a direct effect on the expression of metalloproteases, which aid in tumour progression (415). Both constructs display the same mutation, however the mixed sequences shown in Ad5f35 tdnTGFβRII.2 will predictably express two types of dnTGFβRII. The first type being the aberrantly truncated form of dominant negative TGFβRII where the frameshift results in a premature stop codon close to the transmembrane region. The second being the expected dominant negative TGFβRII protein which is known to be functional and expressed on the cell surface (416). Therefore, the level of CFP will

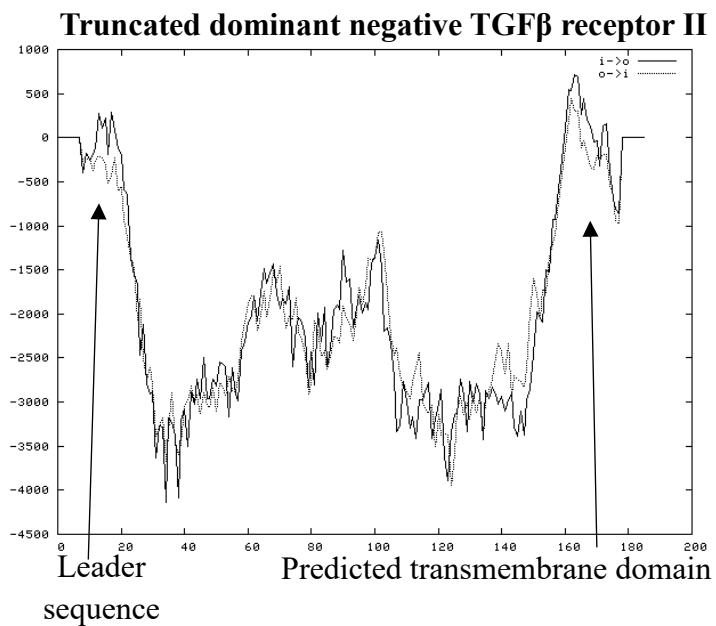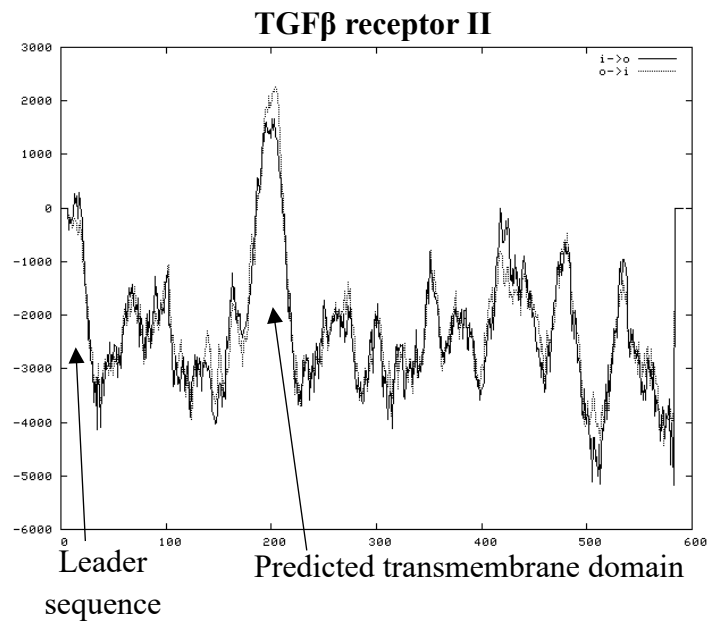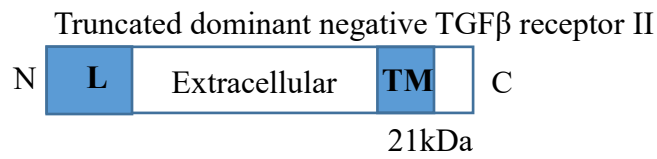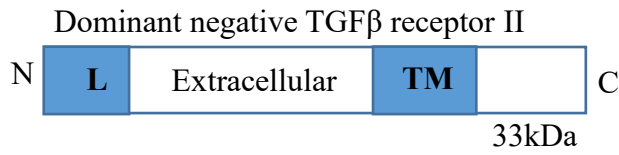remain consistent with both types, however the level of dnTGFβRII being analysed will vary.

To further investigate expression of TGFβRII on the cell membrane, western blot was used to detect dominant negative TGFβRII expression by A549 cells that were transduced with Ad5f35 tdnTGFβRII.1 and compared to untransduced cells at 48hrs.

Blotting of untransduced cells and cells transduced with the parental Ad5f35 vector (without the dominant negative receptor) identified a single species of TGFβRII at approximately 68kDa (Figure 6-7c). This corresponds to the endogenous TGFβRII molecule which (at 565 amino acids) has a predicted molecular weight of 65kDa. Importantly, TGFβRII is *N*-glycosylated and the extent of these modifications varies between cell types, with molecular weights in the range of 65-90 kDa reported (41). At 65 kDa, the species identified in A549 cells in Figure 6-7c is similar to the major species reported in A549 cells by others (41). At 301 amino acids, the dominant negative TGFβRII molecule has a predicted molecular weight of ~33 kDa (without glycosylation) and the truncated derivative (at 186 amino acids), ~21 kDa (calculated using the Compute pI/Ww tool at ExPASy). However, both the dominant negative and the truncated dominant negative molecules have an intact extracellular domain containing two predicted sites for *N*-linked glycosylation (Asn[70] and Asn[94]; ref 41) suggesting that the dn and tdnTGFβRII molecules will have a molecular weight greater than 33 or 21 kDa respectively. This *N*-linked glycosylation on the extracellular domain is essential for transportation to the cell surface and efficient ligand binding (417). The absence of a band clearly identified as the dn and tdnTGFβRII species and the complications of unpredictable changes in molecular weight due to at *N*-glycosylation suggest that the glycosylation should be investigated. Expression using cell lines defective in specific glycosylation steps or the treatment of lysates with different glycosidases would help to define the molecular weight of the core expressed polypeptide. In addition, immunoprecipitation of expressed material followed by limited protease digestion and mass spectrometry would aid definitive identification of the species interacting with the anti-TGFβRII antibody used in these experiments.

In summary, transduction of A549 cells with Ad5f35 tdnTGFβRII.1 demonstrated a substantial increase in TGFβRII expression and a variety of migrating species in the range of ~45-65 kDa (Figure 6-7c); this suggested that cells transduced with this virus

did express exogenous, virus-encoded TGFBRII derivative(s). Biotinylation of cell surface molecules followed by streptavidin pull down showed that the exogenous TGFβRII was expressed at the cell surface and this approach enriched for material ~65 kDa, with evidence of increased expression following transduction (Figure 6-7d). In addition, conditioned media was analysed from untransduced and transduced cells; both neat and concentrated supernatants showed evidence of released TGFβRII at ~65 kDa (Figure 6-7e).

Collectively, the computational analysis and protein analysis suggests that dnTGFβRII might be expressed on the plasma membrane. However, the discrepancies in the molecular weight of the exogenously expressed species means that it has not been possible to formally detect expression of dn or tdnTGFβRII. Additional analysis, such as the investigation of glycosylation and use of mass spectrometry to identify the species would help to resolve these issues.

a

TGFβ receptor II

N | L | Extracellular | TM | Intracellular kinase domain | C
65kDa

Dominant negative TGFβ receptor II

N | L | Extracellular | TM | | C
33kDa

Truncated dominant negative TGFβ receptor II

N | L | Extracellular | TM | | C
21kDa

TGFβ receptor II

Leader sequence

Predicted transmembrane domain

Truncated dominant negative TGFβ receptor II

Leader sequence

Predicted transmembrane domain

b

Figure 6-7 Detecting tdnTGFβRII expression in A549 cells at 48hrs post-transduction.

A549 cells were transduced with either Ad5f35 CFP, Ad5f35 tdnTGFβRII.1 or Ad5f35 tdnTGFβRII.2 and analysed for the expression of TGFβRII at 48hrs post-transduction. A) membrane. B) A549 cells were transduced with Ad5f35 tdnTGFβRII.1 or tdnTGFβRII.2 at 1 MOI and transduction efficiency was assessed by CFP emission and cell surface expression of TGFβRII was assessed by flow cytometry and compared to the untransduced control (n=3). C) A549 cells were transduced with Ad5f35 tdnTGFβRII.1 and total cell lysate (n=1) or (D) membrane (n=3) was isolated (Section 3.2.7.1.1) and the expression of TGFβRII was detected by western blot in either untransduced or transduced cells, as specified. E) The expression of tdnTGFβRII was analysed by western blot in the supernatant of A549 cells that were either untransduced or transduced with Ad5f35- tdnTGFβRII .1.

6.2.6 Truncated dominant negative TGFβRII reduces SMAD2/3 phosphorylation in A549 cells.

To determine the functional properties of the truncated form of dnTGFβRII, downstream TGF-β signalling was analysed. As described in detail in Chapter 2 (Figure 2-4), TGF-β binds to TGFβRII which *trans*-phosphorylates TGFβRI and leads to the phosphorylation of SMAD2 and SMAD3, which then translocate into the nucleus and form a trimeric complex with SMAD4 to target TGF-β responsive genes (418). The dnTGFβRII lacks the cytoplasmic serine/threonine kinase domain and is incapable of forming a heteromeric complex with the type I receptor, therefore limiting further intracellular signaling from TGF-β. To quantify the functional effect of tdnTGFβRII.1 and tdnTGFβRII.2 in TGF-β signalling, phosphorylated SMAD2/3 was quantified by intracellular flow cytometry (methods section 3.2.5.7) in A549 cells that were either untransduced or transduced, and either unstimulated or stimulated with TGF-β for 30 min prior to analysis by flow cytometry, as represented using tdnTGFβRII.1 (Figure 6-8a). A summary of the data (that includes tdnTGFβRII.2), suggests that there is no difference in SMAD2/3 phosphorylation in cells transduced with Ad5f35-CFP and Ad5f35-tdnTGFβRII.2. However there was a significant decrease in cells treated with TGF-β and transduced with Ad5f35-tdnTGFβRII.1, but not in comparison to cells that were untreated and untransduced (Figure 6-8b). Therefore, the ability of tdnTGFβRII.1 to sequester TGF-β away from endogenous, functional TGFβRII, is speculative. Notably, there is no differences between SMAD2/3 phosphorylation in TGF-β treated and untreated untransduced controls, which would suggest that A549s is also not a good cell line to model this assay.

Speculatively, Ad5f35-tdnTGFβRII.1 might also be producing a soluble form of the dnTGFβRII. To test this possibility, conditioned medium from A549 transduced cells with Ad5f35-tdnTGFβRII.1 was collected and used to test whether it could block TGF-β activity using the luciferase assay in HaCaT cells (described in Figure 6-3). As a positive control, an anti-TGFβ1 antibody was used. The results showed that the mean TGF-β induced luciferase expression was reduced when conditioned medium from tdnTGFβRII.1 (or the blocking antibody) was used, but this inhibition was not statistically significant (Figure 6-9). One possibility is that that the amount of exogenous TGF-β used saturates the tdnTGFβRII (and antibody) in the conditioned medium. One limiting factor of this assay was the use of fresh medium, as opposed

to using conditioned medium from untransduced cells as an additional control. Unfortunately, the concentration of tdnTGFβRII in the supernatant is unknown and represents a limiting factor. This factor could be overcome using an ELISA to quantitate soluble TGFβRII. However, the lack of information on the antibodies used in commercially available ELISAs and the identity of their epitopes questions whether this would be useful approach.

Overall, these observations suggest a soluble form as well as membrane expression of tdnTGFβRII is capable of inhibiting TGF-β signalling. The next step was to determine whether this reduced the effect of TGF-β on NK cells.
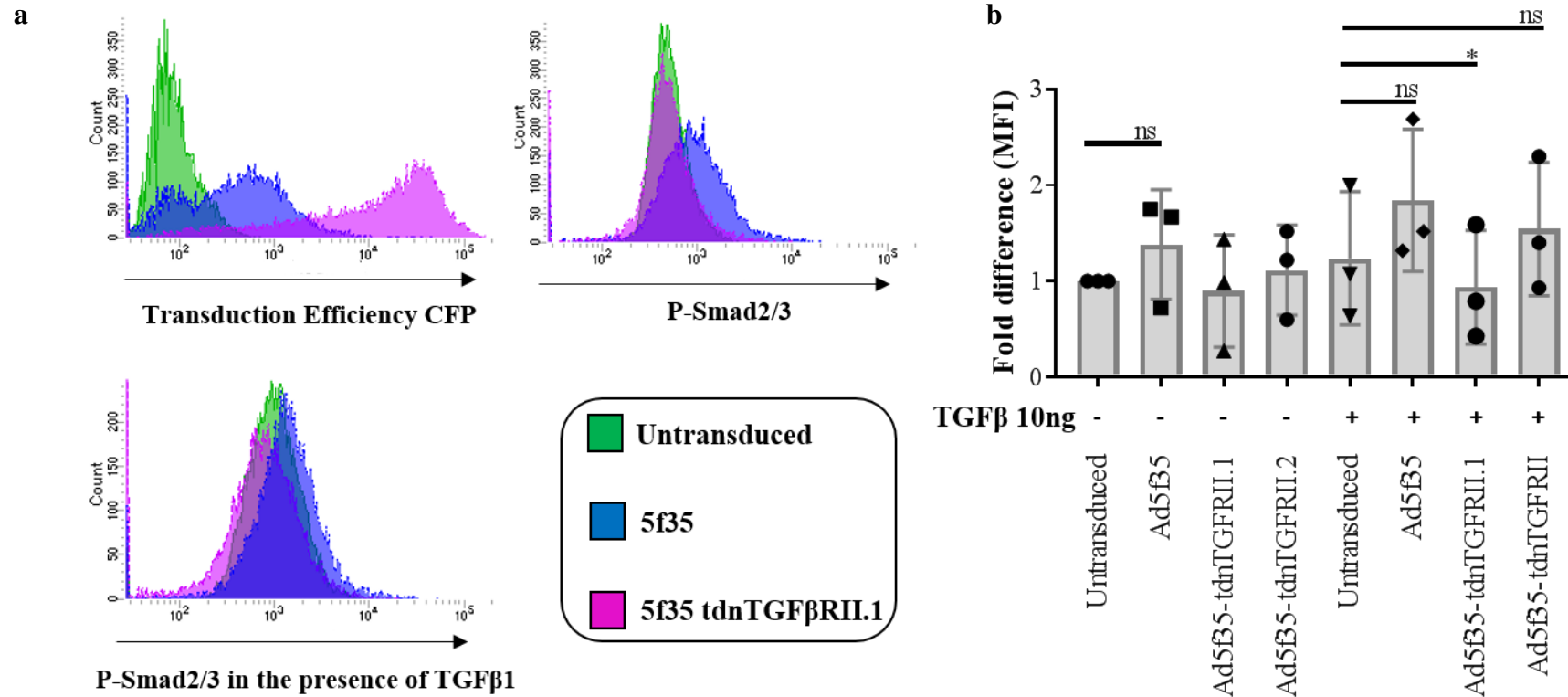
Figure 6-8 tdnTGFβRII reduces SMAD2/3 phosphorylation.

A) A549 cells were either untransduced or transduced with 1 MOI of Ad5f35-CFP, Ad5f35-tdnTGFβRII.1 or Ad5f35-tdnTGFβRII.2 and at 48hrs post-transduction cells were treated with 10ng/ml TGFβ for 30min before analysis . Cells were analysed for CFP expression to determine transduction efficiency, and phosphorylated SMAD2/3 was determined to measure TGFβ signalling by flow cytometry. B) The MFI values was used to determine the fold differences in phosphorylated SMAD2/3, in comparison to the untransduced and untreated cells. The results are the mean + SD of three independent experiments. Statistical analysis is statistically significant at P<0.05.
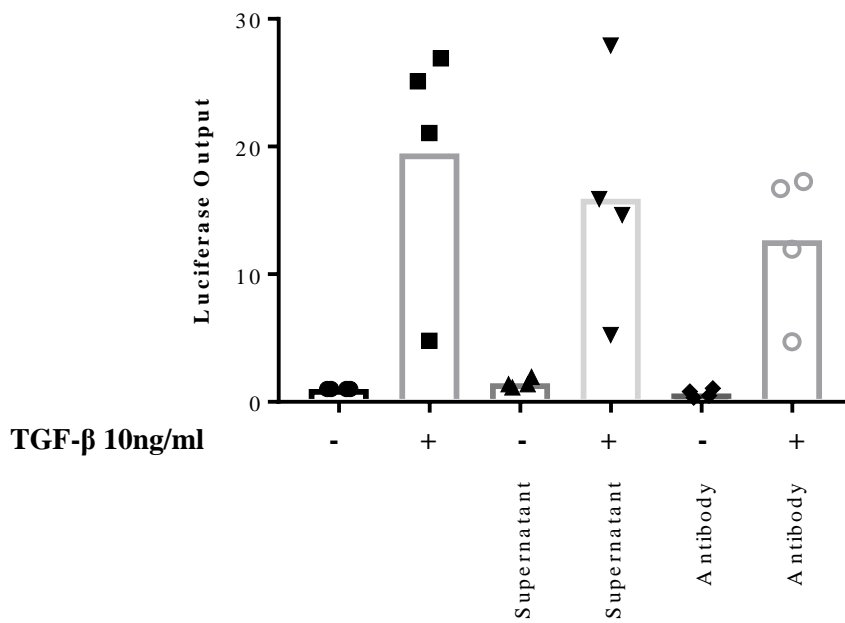
Figure 6-9 TGF-β signalling is reduced in HaCaT cells treated with supernatant containing dominant negative TGFβ receptor II.

HaCaT cells were co-transfected with 0.5 μg p3TP-Lux reporter construct and 0.4ug pRL-TK as a control for transfection efficiency. 24hrs following transfection, cells were treated with 10ng/ml TGF-β or with the addition of 1ug/ml of anti-TGFβ1 antibody or conditioned media containing tdnTGFβRII (from transduced A549 cells) diluted at 1:5. At 48hrs the luciferase output was measured. Value of samples from cells transfected with the constructs and left untreated was arbitrarily set to 1. The results are the mean of four independent transfections conducted in triplicate.

6.2.7 Ad5f35-tdnTGFβRII.1 and Ad5f35-tdnTGFβRII.2 mediated transduction of NK cells

The effects of a soluble form of dnTGFβRII was inconclusive for TGF-β induced SMAD2/3 phosphorylation in A549 cells (Figure 6-8). However, a soluble form of dnTGFβRII might modulate TGF-β signalling by binding to TGF-β, thereby preventing binding to endogenous TGFβRII at the cell surface. A study in cord blood derived NK cells has also shown that membrane bound dnTGFβRII reduces TGF-β signalling (134). Combining both a membrane bound and soluble version of dominant negative TGFβRII is predicted to produce highly effective inhibition of TGF-β signalling in NK cells. In this regard, the tdnTGFβRII.2 construct might prove useful as it contains both the intact dnTGFβRII and the secreted form. This construct (tdnTGFβRII.2) was analysed in transduced NK cells. Cells were assessed at 48hrs post-transduction by flow cytometry for TGFβRII surface expression and CFP expression (Figure 6-10a). A lower MOI of 0-20 was used to transduce cells as the total virus concentration was low; a higher MOI was not used due to the limited volume and concentration of virus and also because using a larger virus volume that is made up in 10% glycerol might have a toxic effect on the NK cells. The lower titre of this virus might be due to reducing virus passage during production in the 911 helper cell line in an attempt to eliminate the risk of contaminating stocks with replication-competent viruses (described in Chapter 4). The results obtained showed that NK cells expressing CFP were highest at a mean of 15% with a <0.2 mean fold increase of TGFβRII expression, which suggests that dnTGFβRII was not being highly expressed on the cell surface at 48hrs post-transduction (Figure 6-10b).

Studies have shown that the expression of activating receptors, such as NKp30, NKG2D and DNAM-1, are strongly downregulated by TGF-β (118,119), and this was confirmed in Figure 6-1. However, a previous study showed that NK cell activating receptors were not modulated by TGF-β in NK cells expressing dnTGFβRII (134). Therefore the effect of TGF-β was determined on the expression of activating receptors NKp30 and CD69 in NK cells transduced with Ad5f35-tdnTGFβRII.2. NK cells were isolated and transduced with Ad5f35-tdnTGFβRII.2 and treated with TGF-β 24hrs post-transduction at an MOI of 10; the expression of activating receptors NKp30 and CD69, was determined 48hrs post-transduction. Transduced cells were distinguished by CFP expression and receptor expression was analysed within this population and compared to cells that were untransduced (Figure 6-10a).

Interestingly, the data revealed an increase in receptor expression of transduced cells, irrespective of the addition of exogenous TGF-β. This might result from either the transduction process or from tdnTGFβRII.2 inhibiting the action of endogenous TGF-β present in the culture medium. However, these changes were not statistically significant (Figure 6-10b). Thus, the dnTGFβRII did not significantly affect the expression of NKp30 or CD69. However Ad5f35-tdnTGFβRII.2 had a general effect in the increase of receptor expression in NK cells.

This therefore led to the use of the tdnTGFβRII.1 construct, which despite producing secreted dominant negative TGFβRII, express the receptor at high levels and showed functional reduction in TGF-β signalling in HaCaT cells (Figure 6-8 and Figure 6-9). NK cells were transduced with Ad5f35-CFP and Ad5f35-tdnTGFβRII.1 at an MOI of 20 and analysed at 48 hours post transduction for CFP expression by flow cytometry. The results showed that cells were transduced at <6% (Figure 6-11a). The transduction efficiency is low for use in primary NK cells, again, this is likely due to the limited number of passages of the virus in the helper cell line during propagation. An alternative strategy was adopted. The conditioned medium from Ad5f35-tdnTGFβRII.1 transduced A549 cells was harvested and the protein in the supernatant was concentrated by centrifugation. This conditioned medium was incubated with primary NK cells that were activated with IL-15 and treated with TGF-β. The resultant TGF-β signalling was then quantified by SMAD2/3 phosphorylation, using intracellular flow cytometry. The results in Figure 6-11.b show that the SMAD2/3 phosphorylation was induced by TGF-β but there was relatively little effect of adding the conditioned media containing the dnTGFβRII. However, in the absence of conclusive evidence that soluble tdnTGFβRII is being expressed, these results must be considered preliminary. Further investigations to identify the soluble species, such as alternative anti-TGFβBRII antibodies, epitope tagging and/or the use of mass spectrometry and protein sequencing will help to resolve these issues.

Following the addition of conditioned medium, the expression of NK cell activating receptor, NKG2D, NKp30 and DNAM-1 (Figure 6-11c) was also tested. The IL-15 mediated increase in cell-surface expression of these molecules was significantly reduced by TGF-β. Furthermore, the dnTGFβRII significantly increased the expression of NKG2D in the presence of TGF-β compared to cells that were treated with IL-15 and TGF-β. The expression of NKp30 was also increased in the presence of the dnTGFβRII, though this did not reach statistical significance (Figure 6-11c).

The analysis of cell-surface receptor changes has higher sensitivity compared to measuring the phosphorylation of SMAD by intracellular flow cytometry due to the biology of the NK cell; where TGF-β directly exerts its functional suppression on NK cells receptor expression.

Co-culture of IL-15 activated NK cells with the ovarian cancer cell line SKOV-3 inhibits NK cell activity in a TGF-β dependent manner. The expression of NKG2D, NKp30 and DNAM-1 in NK cells can be enhanced in these co-cultures by inactivating TGF-β with a TGF-β antibody, or by using a TGF-β receptor signalling inhibitor (118). This system was used to analyse the effect of the truncated/dominant negative TGFβRII. The MFI values for each receptor were quantified and compared. However, similar to the results using recombinant TGFβ, the data did not show statistical significance (Figure 6-11c).

Overall, this data suggests that, due to the mutation in the dnTGFβRII gene, a premature stop codon in the transmembrane region has resulted in a truncated form of dominant negative TGFβRII that could potentially be secreted. The secreted dnTGFβRII may bind to TGF-β to reduce the amount of TGF-β binding to TGFβRII. This truncated form of dominant negative TGFβRII here has not been used to hinder TGF-β previously, however studies using dominant negative TGFβRII that are expressed on the membrane have been shown to be effective on NK cells in retaining their phenotype and cytolytic activity in the presence of TGF-β (134). Altogether, the adenovirus system is efficient at transducing NK cells, however it has the disadvantage that recombination with early region genes present in the propagating helper cell line can produce replication-competent virions that kill NK cells. This was avoided by reducing the number of passages of the virus in 911 cells, however this has the disadvantage of producing low titres and reduced efficiency in NK transduction. Nevertheless, work described in this chapter has demonstrated that the AdZ system can be used to express transgenes, and that despite the molecular challenges, the recombination system utilised has potential, with capacity for optimisation. Here, the dnTGFβRII system has not been successful in NK cells. Evidently, the mutations occurring in dominant negative TGFβRII has made the system difficult to use and quantify the effects of dnTGFβRII in NK and A549 cells. However, expressing other candidate proteins is a possibility, such as CRISPR due to the advantage of stable modification of the genome.

a

b
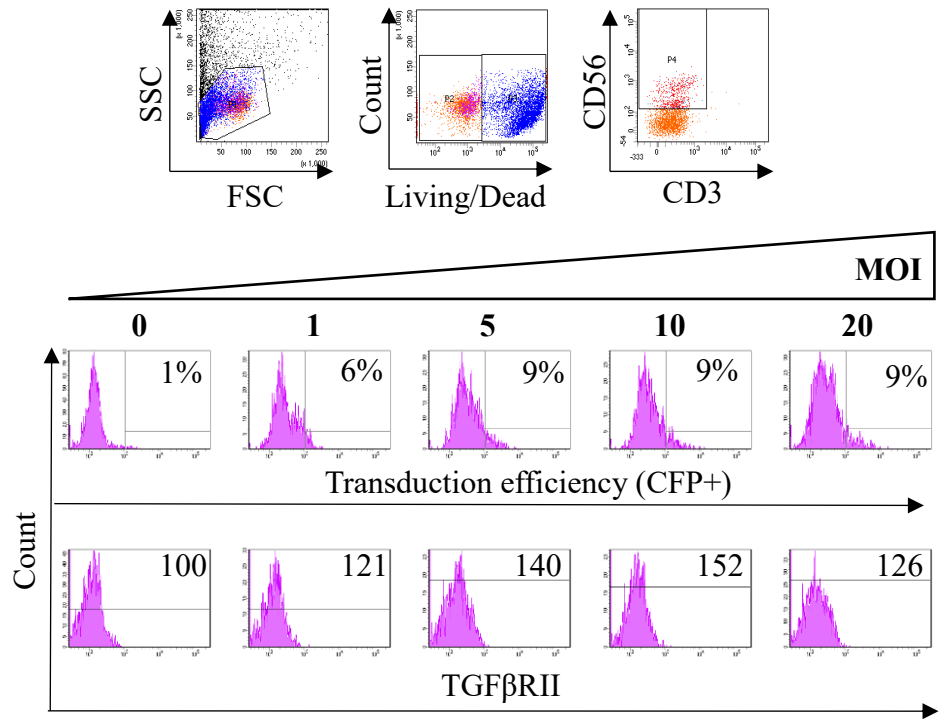
A d 5 f 3 5 - t d n T G F ß R II . 2
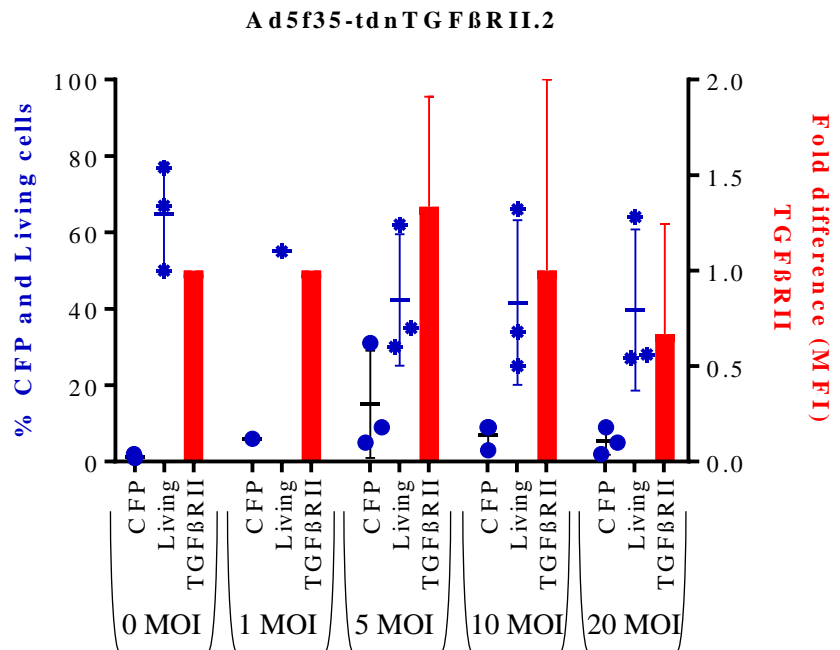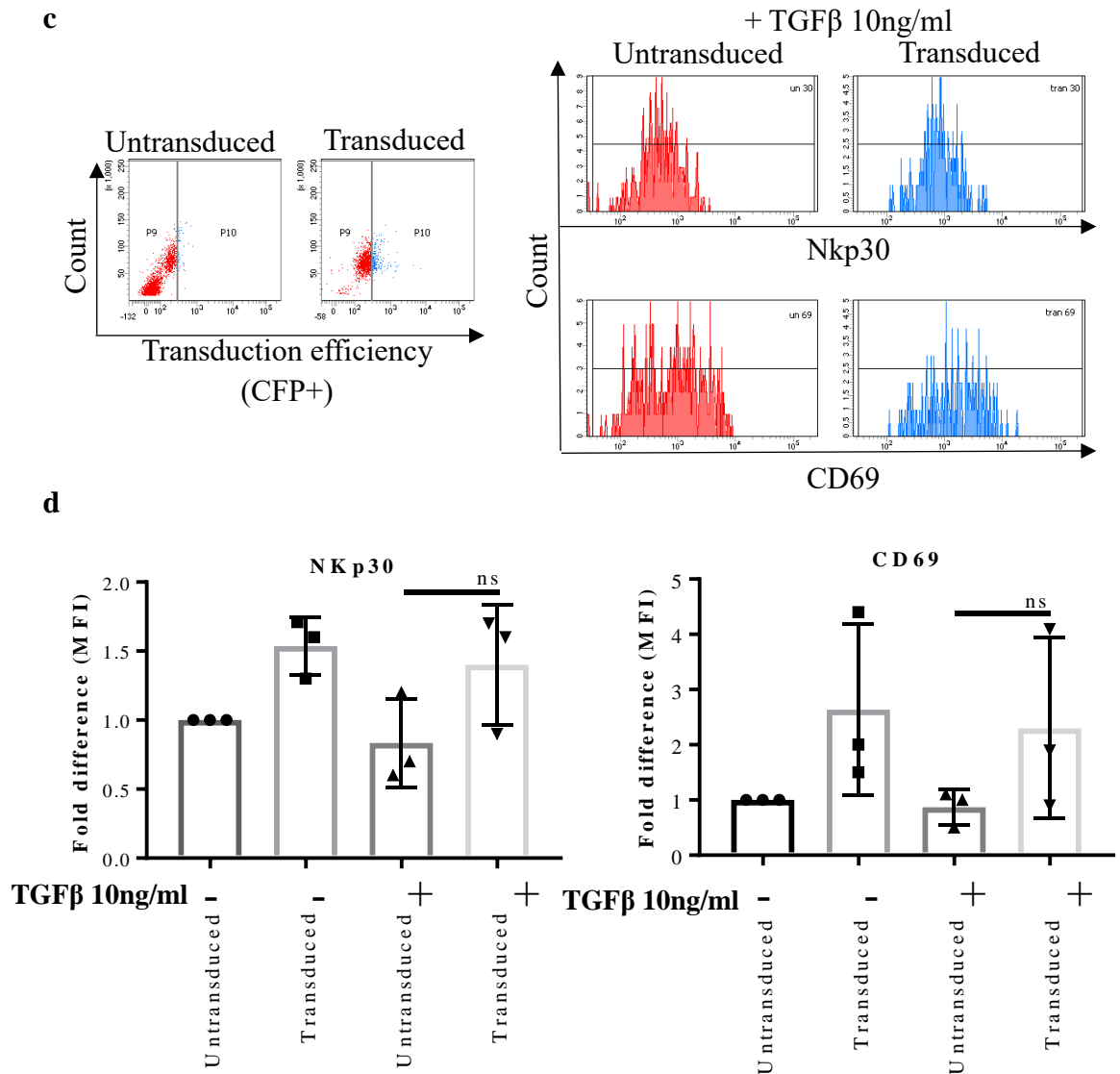
Figure 6-10 Ad5f35- tdnTGFβRII.2 mediated transduction of NK cells

A) NK cells were transduced with Ad5f35-DNRII.2 (1-20 MOI) and analysed by flow cytometry at 48hrs for the expression of dnTGFβRII (MFI) and CFP (%), gating on the living cell population. B) NK cells in up to 3 donors were transduced with Ad5f35-tdnTGFβRII.2 (1-20 MOI) and the expression of TGFβRII and CFP was summarised at 48hrs post-transduction. C) Transduced NK cells (CFP+) were further analysed for the expression of Nkp30 and CD69; with and without the treatment of TGF-β (48hrs) and compared to the untransduced controls. D) The summary of NK cells from 3 donors transduced with Ad5f35-tdnTGFβRII.2, analysing the expression of Nkp30 and CD69. The results are the mean + SD, statistically significance at $P<0.05$.
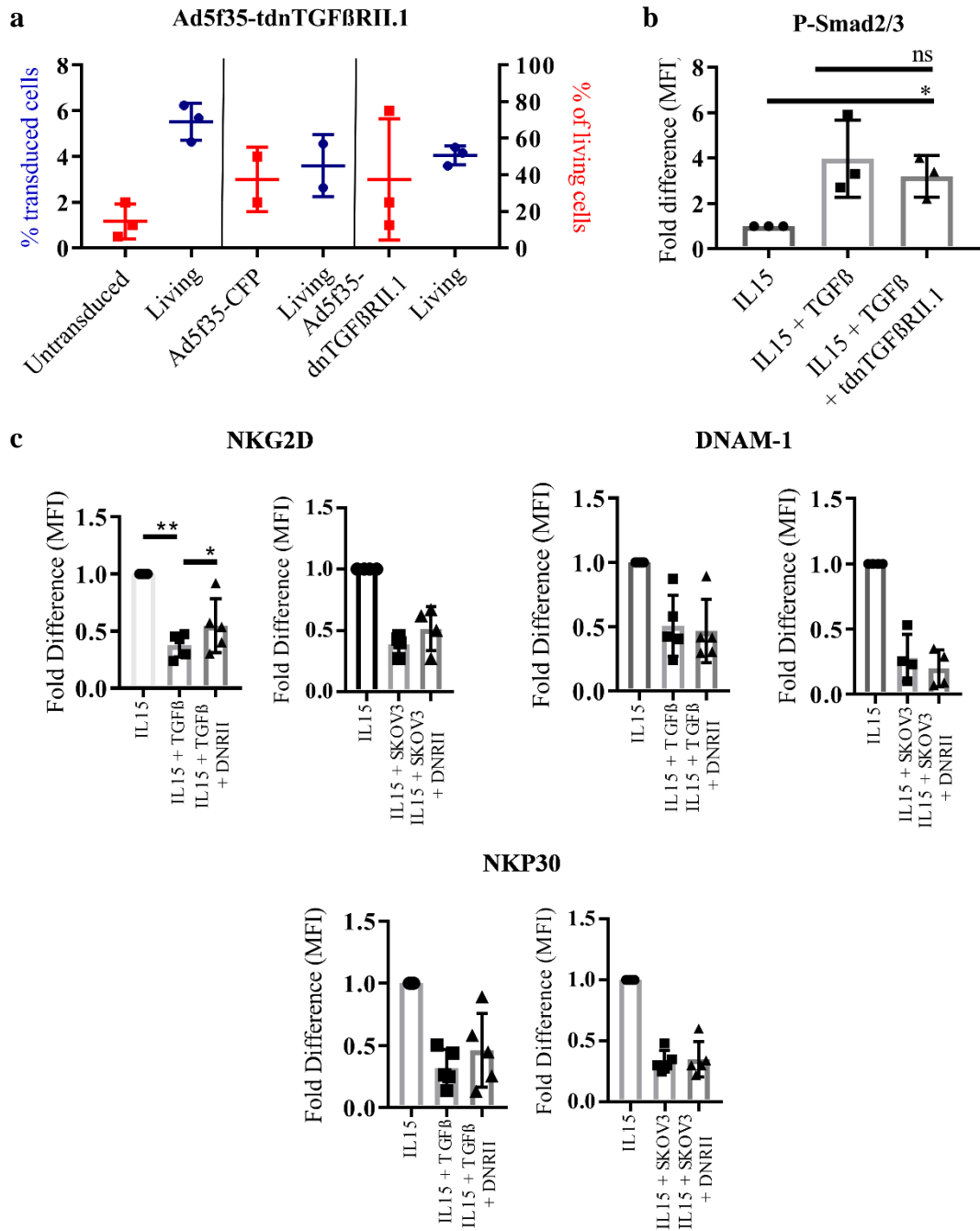
Figure 6-11 Soluble tdnTGFβRII treatment of NK cells.

A) NK cells were transduced with either Ad5f35-tdnTGFβRII.1 or Ad5f35-CFP (20 MOI) and CFP expression was analysed at 48hrs post-transduction and summarised. B) A549 cells were transduced with Ad5f35-tdnTGFβRII and analysed at 48hrs post-transduction for CFP expression. The medium was harvested in transduced samples and the quantity of tdnTGFβRII was concentrated and diluted at a ratio of 1:5 (a guideline used as described in Figure 6-9) and mixed with isolated NK cells that were treated with 20ng/ml IL-15 for 48hrs previously. Cells were either untreated or treated with 10ng TGFβ or in combination with the supernatant containing tdnTGFβRII for 20 minutes before cells were analysed by flow cytometry for phosphorylated SMAD2/3. C) Isolated NK cells were pre-treated with 20ng/ml IL-15 for 48hrs before the addition of either, 10ng TGF-β, TGF-β + medium (containing soluble tdnTGFβRII). Cells were also co-cultured with SKOV-3, either on its own or with

medium (containing soluble tdnTGFβRII). After 48hrs cells were analysed for the expression of NK receptors: NKG2D, NKp30 and DNAM-1 (n=5). The results are the mean showing statistically significance at P<0.05; no significance was found using medium containing soluble tdnTGFβRII.

6.2.8 Utilising CRISPR/Cas9 for stable genome modification

The CRISPR/Cas9 technology is a relatively new tool for genetic manipulation and is defined by its high specificity and low risk of off target effects. It was initially described as a genome engineering technology in 2013 and derived from bacterial type II CRISPR from *Streptococcus pyogenes* (419–421). Briefly, the CRISPR/Cas9 tool consists of two components, a synthetic RNA consisting of combined tracrRNA and crRNA, and secondly Cas9 (422) (Figure 6-12a). The gRNA targets the gene of interest for 'knock out' and the Protospacer Adjacent Motif (PAM) sequence is required adjacent to the sequence target for sufficient Cas9 activity which relies on RuvC-like and HNH-like nuclease domains (423). The system knocks out genes by Cas9-induced double stranded DNA breaks, resulting in host DNA repair by the cellular non-homologous end joining (NHEJ) pathway; this leads to insertions/deletions and disrupts expression or function (424). The incidence of off-target mutations that can occur due to Cas9 tolerance of up to 5 base pair mismatches within the gRNA region are relatively low. However, they are difficult to detect without whole exome sequencing analysis (425). This disadvantage can be overcome through the use of paired 'nickases' which creates single DNA nicks (426), and using web based CRISPR tools available to identify potential off target effects.

It was decided to utilise this system in HeLa cells to assess its efficiency in genetic modification and its potential use in the Ad5f35 system. HeLa cells were used as they are a widely used cell line for studying human cellular and molecular biology (427). Beta-2 microglobulin (B2M) was chosen as a proof-of-concept target for the CRISPR/Cas9 system as it can be analysed easily on the cell surface by flow cytometry. The *B2M* gene encodes the accessory chain for the MHC class I molecule, and is required for stable expression at the cell surface (428). Initially, MHC class I and B2M expression were confirmed on HeLa cells using flow cytometry (Figure 6-12.b). Five CRISPR/Cas9 plasmids, each containing a different gRNA targeting different sites in either exon 1 or exon 2 of the *B2M* gene were transfected into HeLa cells (3.1.7). Using more than one targeting gRNA increases the chances of a 'knock out' in comparison to only using one. Each vector also contained DasherGFP which is detected by flow cytometry. Initially one plasmid was chosen to optimise the transfection efficiency into HeLa cells using PEI. The ratio of PEI to plasmid was varied, and transfection efficiency of >50% was achieved with a ratio of 1:4 DNA: PEI (data not shown). Subsequently, all five CRISPR/Cas9 plasmids were transfected

into HeLa cells (methods section Table 3-7 CRISPR plasmids). Each construct was tested for its ability to efficiently knock out B2M by gating on the transfected cells using DasherGFP+ and assessing B2M expression (Figure 6-12c). DasherGFP positive cells display a decreased level of expression of B2M (Figure 6-12d). I found that flow cytometry provided certainty of a knock out effect with the reduced level of B2M expression at 48 hours post transfection. Single cells from the same transfection experiment were seeded into a 96 well plate for single cell colony formation and manually inspected for DasherGFP expressing single cells. Colonies of cells that were identified were then expanded. Many wells that appeared to contain single cells did not form colonies, and likely died. However, I identified a colony that was 16% positive for B2M expression (Figure 6-12e), as opposed to the WT of 99% (Figure 6-12b). This suggests that more than one cell had distributed into the well during isolation, one of which had been transfected and *B2M* mutated by the CRISPR/Cas9 system. However, these data suggested that the CRISPR/Cas9 system was working in HeLa cells.

In order to separate the *B2M* knock out cells, the expanded cells were sorted into three distinct populations of B2M expression being: positive, intermediate and negative (Figure 6-12e). Following expansion, the negative and intermediate sorted populations had 19-20% of B2M expression and almost absent expression of MHC Class I with 1.8-2.7% expression (Figure 6-12f). This clearly demonstrates knockdown of B2M and therefore MHC class I at the surface of the Hela cells by the CRISPR/Cas9 system.

To explore the genomic effects of the CRISPR/Cas9 system, Sanger sequencing was performed on the target region of *B2M* exon 1. The gRNA target is 20 base pairs in length and homologous to the 5'UTR sequence and start of the exon 1 sequence of *B2M*. WT cells displayed wild type sequence at the gRNA target site. However the negative and intermediate population have scrambled sequences at the locus where the gRNA targets (Figure 6-12g). This suggests that the CRISPR/Cas9 system efficiently targeted the locus and disrupted the expression of *B2M*. The sequencing observations show that multiple sequence variants are being processed, therefore the exact sequence deletion or mutation causing the disruption in expression is unclear. Further investigations suggest that the cause of the scrambled sequences might be due to the origin of the cells that may have been generated from multiple colonies affected by the CRISPR/Cas9 constructs, as opposed to a single cell. This could potentially

generate different mutations following NHEJ DNA repair mechanism that induces unpredictable mutations. An additional issue was the karyotype of HeLa cells that has recently been described as >2n, and recorded to have three copies of chromosome 15. *B2M* location is on chromosome 15q21.1 (www.ensembl.org), as a result the CRISPR/Cas9 complex is likely to have targeted the *B2M* locus on each chromosome and through NHEJ DNA repair induced distinct mutations at each allele resulting in multiple sequence variants. A study showing *B2M* 'knock out' using the CRISPR/Cas9 system in HEK293T cells (normal karyotype for chromosome 15) show clear deletions within the target site (429). This indicates that the karyotype is important for the analysis of mutations using the CRISPR/Cas9 system. The population of cells that have an intermediate expression of B2M may have resulted from one chromosome remaining intact to the WT sequence of *B2M*, however the MHC Class I expression is severely affected by this (Figure 6-12g). Despite the variations in the mutation and expression levels, the knock out was effective in HeLa cells and could be used to 'knock out' genes, such as TGFβRII in NK cells. Moreover, a study has recently described the 'knock out' of TGFβRII with CRISPR in primary NK cells using a DNA-free method, in which the CAS9 protein and guide RNA is delivered directly by electroporation; this has certainly encouraged the direction of NK manipulation for strategies in NK immunotherapy (430). Furthermore, similar methods have now been used to target NKp46 on primary human NK cells (431); this study utilised FACS to sort for cells lacking the target cell surface receptor, which proved to be a sensitive validation tool. Similarly here, the use of flow cytometry was used as a screening tool to identify and isolate cells lacking the target cell surface molecules.

a

Double Strand DNA Break
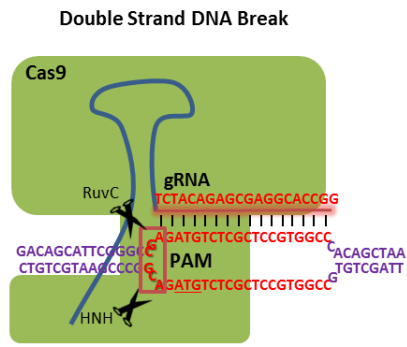
DNA Repair: Non-Homologous End Joining

b

B2M

MHC Class I

Isotype control

B2M or
MHC Class I
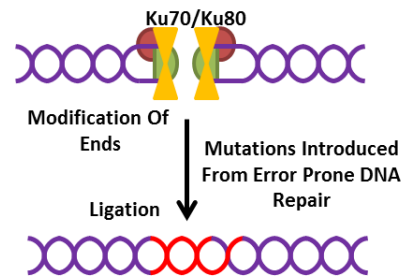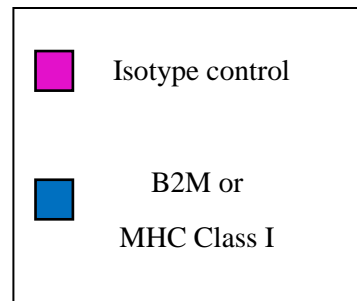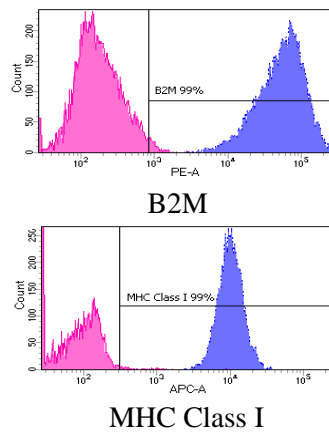
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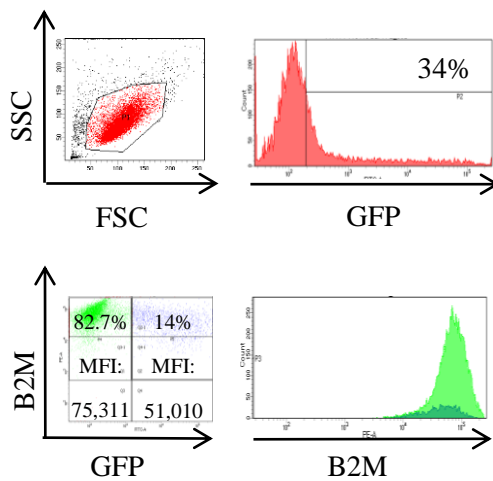
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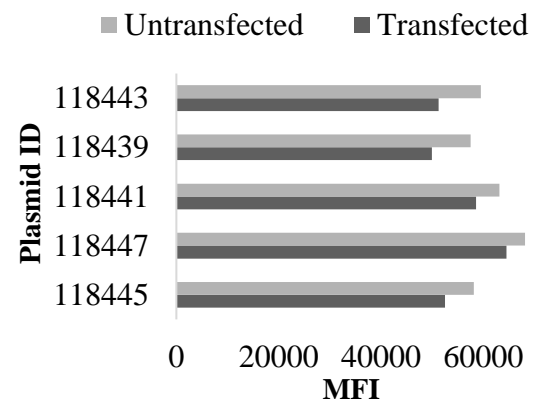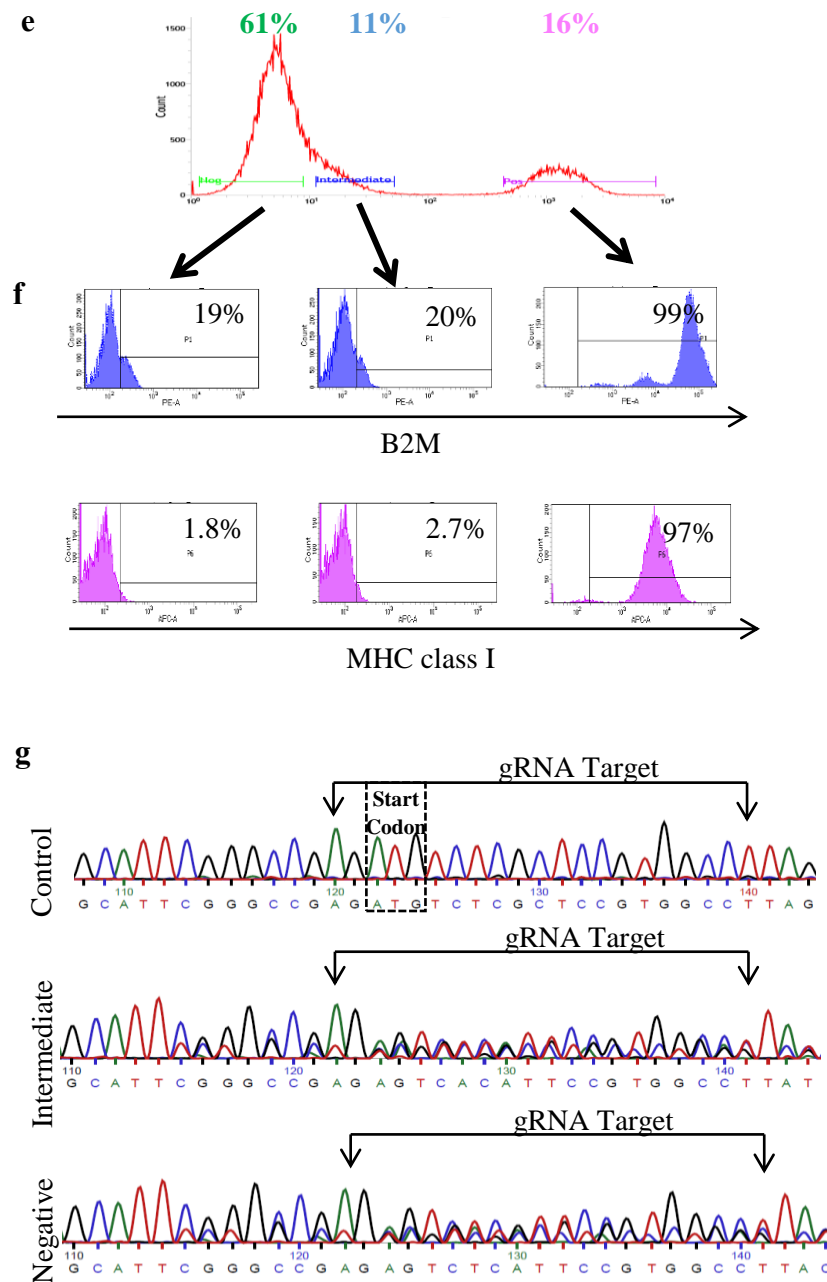
Figure 6-12 B2M knock out using CRISPR.

A) CRISPR has been developed by fusing crRNA and tracrRNA sequences to produce a synthetic chimeric single-guided RNA (sgRNA). The selected target sequence consists of a complementary 20 base pair DNA sequence to the crRNA or the chimeric sgRNA, followed by the trinucleotide (5'-NGG-3') protospacer adjacent motif (PAM) which is recognized by Cas9 itself and essential for cleavage. The Cas9 endonuclease is guided to a specific genomic sequence to generate double-strand break (DSB) in target DNA. The DSB is repaired by the error prone mechanism of NHEJ and induced errors in the DNA, often resulting in degenerative gene expression. B) Representative histograms indicate HeLa cells stained with isotype antibody staining (pink) and B2M and MHC Class I (blue). C) The CRISPR knock out efficiency was analysed by flow cytometry. Viable HeLa cells were gated on the FSC and SSC and transfection efficiency was analysed in the FITC channel. The levels of B2M knock out were analysed using a PE-conjugated monoclonal anti-B2M antibody. The representative

histogram shows transfected cells B2M expression (green), and un-transfected cells B2M expression (red). Gates were set at 98% B2M expression in wild type HeLa expression. D) The collective MFI values on B2M expression was compared in transfected (green) and un-transfected (red) cells from experiments using 5 plasmids with variable gRNA's targeting B2M. E) Cells were seeded as single cells in a 96 well plate. Colonies formed from single or multiple cells and were transferred to a larger container and bulked up for flow cytometry analysis. One population of cells derived from multiple surviving colonies producing two B2M expressing phenotypes as shows in the histogram and the cell population were sorted according to its B2M phenotype using the PE-conjugated monoclonal anti-B2M antibody. Three B2M variable phenotypes were targeted as either having negative, intermediate or wild type/positive expression. F) Proceeding cell sorting, the sorted cell populations were analysed by flow cytometry for B2M expression (blue) and MHC Class I expression (pink). G) The sorted cell populations were assessed for mutations by Sanger sequencing to determine specific mutation events.

6.3 Discussion

This project focused on the inhibitory effects of TGF-β on NK cells. Initially, TGF-β mediated inhibition was confirmed using primary NK cells, which showed inhibition of expression of NKp30, NKp46, DNAM-1 and NKG2D receptors, in agreement with previously published data (118). These receptors have an established role in the killing of tumour-targets by NK cells, as demonstrated by reduced cytotoxicity towards K562 target cells. However, TGF- β also inhibits the expression of the cytotoxic components (granzymes and perforin) and this also leads to reduced killing (4). These results highlight the importance of engineering NK cells to be resistant to TGF-β inhibition, particularly within the tumour microenvironment.

In this chapter, an adenovirus recombination system has been used to integrate dnTGFβRII into the Ad5f35 genome. This investigation encountered problems within the gene insert, with findings of a frame shift mutation that was probably introduced during BAC colony expansion. This mutation was predicted to introduce a stop codon close to the transmembrane region which affected expression and the ability to antagonise TGF-β signalling in NK cells. The lack of conclusive evidence for expression, both at the cell membrane and secreted, was also a limiting factor for the progress of this investigation. Further studies need to incorporate epitope tags, new antibodies, glycosylation studies and the use of protein sequencing as described in the results section in this chapter. The functional effect on NK cells were also not statistically significant. However, this mutation has been described recently in patients with colorectal cancer with a deficiency in DNA mismatch repair (112). This suggests that the described microsatellite stretch of adenine repeats in *TGFBR2* has a higher risk of mutation and, without an efficient repair mechanism in place, the mutation goes unchecked. Consequently, due to the molecular abnormalities, a true dnTGFβRII was not expressed. However, the truncated expression of dnTGFβRII might have resulted in a secreted form. A three dimensional structure of TGF-β3 (not TGF-β1) bound to TGFβRII has been determined (432). This structure suggests that the frameshift mutation in the extracellular domain introduced here is likely to severely reduce ligand binding.

Despite the molecular challenges on this study, a soluble form of the TGFβRII extracellular domain might have been expressed, which has also been described

elsewhere to interfere with TGF-β binding to TGFβRII (433). The extracellular domain, fused with the Fc portion of a murine IgG1 heavy chain, reduced metastasis and increased apoptosis of tumour cells in mice transplanted with breast cancer (433). The long term clinical risks of such a treatment might include effects on the immune system, with possibilities in developing autoimmune disease and fostering an environment for tumour development (130,131,434). However, a mouse breast cancer model showed that long-term exposure of this soluble receptor provided extended protection against metastasis and the occurrence of benign malignancies in the lung, pancreas and kidney (435). Together, these studies support the theory that engineering an NK cell to secrete a soluble receptor, as opposed to using an antibody to sequester TGF-β, will not only provide protection against tumour progression but enhance the ability of NK cells to activate and kill tumour targets. This has also been demonstrated in T cells that have been engineered with either cell surface dnTGFβRII, soluble TGFβRII, or a TGFβRII-Fc chimera, in a mouse melanoma tumour model (436). The results suggested that T cells expressing the cell surface dnTGFβRII was most effective, whereas the soluble receptors showed no enhancement of T cell anti-tumour activity (436). This suggests that direct cellular protection of cytotoxic lymphocytes against TGF-β is more powerful than approaches to sequester TGF-β using soluble molecules.

TGF-β blockade can also be achieved through small molecules that bind to the TGF-β receptors and was first described for a compound known as SB-431542, that binds to TGFβRI to inhibit the phosphorylation of SMAD2/3 (437). Here, I have used galunisertib, a compound that also binds to TGFβRI (438), which was demonstrated in HaCaT cells using the luciferase assay. There is also a possibility that compounds might cause global disruption in TGF-β mediated immune homeostasis, which might cause complications similar to those seen in mouse models (439). Therefore, specific TGF-β blockade in NK cells (or T cells) appears preferable. In mice, engineered T cells expressing dnTGFβRII showed no difference in cytolytic activity towards prostate carcinomas, however tumour regression and reduced TGF-β effects were observed when used in combination with a modified TCR (440). Importantly, expressing dnTGFβR in mouse T cells resulted in CD8^{+} T cell lymphoproliferative disease, presumably due to the loss of TGF-β mediated homeostasis of T cell proliferation; such effects must be taken into consideration if blocking TGF-β activity (441). Clearly, tumour antigen recognition is critical for T cell activation, however

NK cell recognition of tumour cells is antigen-independent but dependent upon adequate stimulation of activating receptors; therefore the efficacy of this system is likely to be different. More recently, a study expressing dnTGFβRII in *ex vivo* expanded cord blood NK cells by retroviral transduction maintained their ability to recognise and kill glioblastoma cells in the presence of TGF-β, which supports its efficacy (134). Clinically, the risks involved with introducing dnTGFβRII highlight the requirement for a strategy in cell targeting and controlling gene expression. Despite these risks, the considerable challenge for clinical applications remains the difficulty in delivering exogenous genes into primary NK cells.

A major deterrent to inhibiting the TGF-β signalling pathway is toxicity. As described previously, a soluble receptor expressed to sequester TGF-β has been shown to be effective in mouse models, however benign malignancies did form (433). Reviews of compounds that bind to cellular receptors have reported toxicities in mice (439) and one study showing no response in human clinical trials towards melanoma, speculatively due to low dose (442). Clinical trials are still in progress using small molecular inhibitors, and updated on clinicaltrials.gov website, the majority being sponsored by Eli Lilly and Company. Overall, these studies highlight the requirement in specificity and the main problem being the broad role that TGF-β plays, particularly on the immune system. Engineering a population of lymphocytes and in particular, NK cells, to become insensitive to TGF-β and maintain their ability to localise to the site of the tumour to kill the tumour cells is an approach that remains advantageous compared to antibodies and compounds. Here, the chosen gene, dnTGFβRII might not have been proficient at the molecular level using this adenovirus system, however, other genes, such as SMAD7 might have produced a different result. Therefore, further investigation using this adenovirus system with other gene candidates remains a promising approach.

It is well known that TGF-β signal transduction is enforced by the phosphorylation of SMAD2/3 which accumulates in the nucleus and mediates the transcriptional effects of TGF-β signalling; SMAD7 is an established inhibitor of this pathway (105). However, a recent study has shown that PPM1A is responsible for the de-phosphorylation of SMAD2/3 (443). PPM1A is a phosphatase that is able to cleave phosphorylated serine and threonine residues on the SXS motif phosphorylation sites on SMAD proteins (SMADs 1/5/8/2/3). This gene might also be considered as a candidate for overexpression in NK cells to reduce TGF-β signalling. PPM1A is also

only 1149bp in size, therefore it is small enough to incorporate into the Ad5f35 genome. An alternative candidate that was considered in this project was SMAD7, which was shown to reduce TGF-β signalling in HaCaT cells using a luciferase assay, measuring TGF-β through PAI-I induction by p3TP-lux transfected cells. This is in agreement with another study showing that over-expression of SMAD7 inhibits TGF-β mediated PAI-I induction in mink lung epithelial cells (Mv1Lu) (406). In contrast, another study showed that SMAD7 overexpression is incapable of inhibiting TGF-β activation in COLO-357 cells transfected with p3TP-lux (407). This highlights the issues that TGF-β signalling is distorted amongst cell lines and that cell type is important when establishing a screening assay for inhibitors of the TGF-β signalling pathway. In this study, the expression of dnTGFβRII reduced signalling although not significantly, which agrees with previous work in which truncated TGFβRI was expressed in Mv1Lu, a luciferase system, and did not inhibit TGF-β mediated PAI-I induction (444). Even so, the expression of a dnTGFβRII has been shown to block TGFβ signalling. An alternative approach using the luciferase assay would have been to measure the ability of TGF-β to activate a different promoter element, such as WWp-lux, which is under the control of the p21 promoter to measure the cell cycle. However, again, this construct has conflicting reports in SMAD7 overexpression when compared to measuring p3TP-lux (407). Nonetheless, it is accepted that SMAD7 prevents the interaction of SMAD2/3 with the TGF-β receptor complex, thus blocking TGF-β signalling. SMAD7 is also 1.3kb in size, therefore a suitable size to integrate into the Ad5f35 genome which has a predicted cargo capacity of 2-3kb. Overall, *PPM1A* and *SMAD7* are attractive as genes to overexpress in NK cells for future investigations.

A disadvantage of the adenovirus system in overexpressing genes in NK cells is that it is transient (i.e. the viral genome neither integrates into the host genome nor replicates in the host cell). Using systems that can modify the NK cell genome, such as CRISPR/Cas9 does offer the advantage of stable genomic alteration. In this study, the CRISPR/Cas9 system was used in HeLa cells to target *B2M,* a protein component of cell-surface MHC Class I molecules, to demonstrate its efficiency at knocking out genes within the genome. The CRISPR/Cas9 was optimally transfected and 1 of 5 gRNA targets succeeded in mutagenic activity within the *B2M* locus causing loss of MHC Class I expression on the cell surface of Hela cells. This left Hela cells susceptible to death by killing from activated primary NK cells and NKLs (data not

shown). These results highlighted the importance of the target cell karyotype, and the possible off-target effects this system may cause. However, to establish this system in NK cells, the Ad5f35 delivery system would be required to encode the *S. pyogenes* Cas9 (~4.5kb) and the single guide RNA (sgRNA) of 100-110bp in size. However, the adenovirus has a restrictive cargo size of 105% of its WT genome (~35kb) restricting genomic uptake capacity to ~2-3kb, therefore it presents an obstacle for packaging the CRISPR/Cas9 system. The alternative is using the Cas9 enzyme isolated from *Staphylococcus aureus* which is 1,000bp less in size, and has been successfully packaged into an adeno-associated virus, which is smaller than WT-Ad (445). Despite this obstacle, the *S. pyogenes* CRISPR/Cas9 tool has been used in adenoviruses successfully in mice, showing it is feasible (446,447). Together, this system could be combined with the Ad5f35 system to irreversibly knock out the expression of TGFβRII (or other molecules) in NK cells. However, recent studies utilised CRISPR to knock out TGFβRII and NKp46 in primary NK cells using electroporation; these studies highlight the direction of the system in use in NK cells, interestingly without the use of viral vectors or DNA-based transfection (430,431).

Clinically, the CRISPR/Cas9 system has generated controversy over its use in human embryos by fertility specialists (448). This concern is due to the off-target and long term effects of passing genetic modifications down through generations. Adding to this controversy, cells that are insensitive to TGF-β have previously been shown to form malignancies, therefore combining this system to this target is a concern. A solution to overcome this issue might be to include a 'suicide gene'. One well described suicide gene is herpes simplex virus thymidine kinase which phosphorylates ganciclovir, resulting in a toxic monophosphate form of the drug, thereby killing cells (449). Thymidine kinase can further phosphorylate ganciclovir into di- and triphosphate forms, the latter acts as a GTP analogue but inhibits DNA polymerase, resulting in apoptosis. A report also suggests that this suicide gene enhances NK cell killing activity *in vivo* (449)*,* which would suggest that unmodified NK cells will benefit from this modification. The gene insert size is also small enough to incorporate into the Ad5f35 genome at 1143bp, further supporting that this is a plausible safeguarding approach following genome modification *ex vivo.*

Overall, there are many options for the manipulation of NK cells. The advantage of using Ad5f35 to deliver the CRISPR/Cas9, as opposed to the transfer of therapeutic genes alone is overcoming the transient gene expression achieved using adenovirus.

The CRISPR/Cas9 system can permanently modify the genome and maintain genomic modification after proliferation, during which the adenoviral vectors will be lost. Therefore transduced primary NK cells will maintain the introduced modifications and increase the number of effective cells against malignancies. Consequently, this system has the potential to be utilised for effective cancer or other immunotherapeutic strategies in NK cells or other cells used in cellular therapy.

7 General discussion

Cancer is a major cause of death across the world and, despite many successes in therapy, treatment options for many cancers remain limited. For decades, the traditional forms of treatment include surgery, radiotherapy and chemotherapy. However, these treatments can be limiting in regards to specificity and often have severe side effects; this highlights the need for new strategies for effective management of this disease. Currently, immunotherapies are being investigated, as an alternative or in combination with traditional treatments, to improve patient outcome. There has been considerable interest in TGF-β blockade, however development is still in the early stages of clinical trials. The most promising, galunisertib, is a small molecule TGFβRI inhibitor, has been described as being safe for use in humans (450) and early stage clinical trials are promising, specifically for patients with glioblastomas, where results show an improved or stable disease state (451). Fundamentally, this approach requires the identification of tumours where TGF-β is implicated; solid tumours are more inclined to have a TGF-β tumour promoting microenvironment, however certain haematological malignancies such as chronic myeloid leukaemia are also implicated in this phenotype (452). Generally, the goal for TGF-β inhibitors in the clinic is to re-sensitize tumour cells to other therapeutic agents. In addition, combination therapy with enhanced or activated immune cells provides an alternative approach in immunotherapeutic strategies against TGF-β expressing tumours. In this area, pre-clinical investigations have looked at using synthetic inhibitors in combination with adoptive transfer of T cells in mice, which increased activation and infiltration of immune cells and reduced (or cured) all malignancies (392). However, many inhibitors have not progressed beyond early clinical trials due to side effects or poor efficacy (391). Hence, engineering activated immune cells that are TGF-β insensitive provides a dual approach for immunotherapies against TGF-β expressing tumours. In particular, improving the anti-tumour response by genetic manipulation to enhance tumour recognition and killing is one appealing approach. It is well known that the anti-tumour response of NK cells is important in cancer immunosurveillance, hence the need to develop tools to enhance this response in a clinical setting. Although this has not been tested in a clinical setting in humans, it has been accomplished by *ex vivo* manipulation of human

NK cells (134,168,171). However, this approach is limited by the tools available for efficient gene transfer. It is also difficult to reproduce effective methodologies; thus there is a demand for robust and efficient strategies by which to transduce NK cells for clinical use.

Here, virus vectors (adenovirus, measles and vaccinia) were tested for their ability to transduce human NK cells. An adenovirus vector system was pursued in further investigations and utilised to express a dominant negative TGF-β receptor (dnTGFβRII). The results (summarised below) highlight a novel strategy to use adenovirus as a vehicle to deliver genes to primary NK cells and increase the potency of NK cytotoxicity towards tumour targets.

7.1 Main results

- Initial experiments identified Ad5f35-EGFP and MVA-GFP as promising viral vector candidates for NK cell gene delivery. However, cell viability was better using Ad5f35-EGFP and this system was pursued in further investigations.

- Ad5f35-EGFP mediated transduction of primary NK cells was consistently efficient when using a "spinoculation-like" approach (similar to that used for lentiviral transduction). NK cell viability was maintained by treating cells with IL-2.

- Further investigations showed that CD46 is an important molecule for Ad5f35 transduction of human NK cells. The CD46 molecule functions as the initial point of contact for virus attachment on NK cells.

- Ad5f35 transduced NK cells retained their ability to degranulate towards tumour targets.

- TGF-β, a well-known immunosuppressive cytokine, was demonstrated to hinder NK cell activation and cytotoxicity by downregulating key NK cell activating receptors. This data provides rationale for targeting the pathway in order to enhance an NK cell anti-tumour response in the presence of TGF-β. Henceforth, dnTGFβRII was chosen as a suitable candidate for further investigations to inhibit TGF-β signalling.

- The AdZ system was utilised to insert dnTGFβRII into a BAC containing the genome of Ad5f35. A premature stop codon was introduced at the

transmembrane region due to a frame-shift deletion in an earlier region of the sequence. This caused truncation of the receptor and the disruption of the expression of the receptor on the plasma membrane. A western blot identified a protein species consistent with endogenous TGFβRII; this species was detected at the cell surface and in the supernatant of transduced A549 cells.

- The phenotype of primary NK cells transduced with Ad5f35-tdnTGFβRII.1 and tdnTGFβRII.2 was analysed in the presence of TGF-β, however no statistically-significant changes were detected.

- CRISPR/Cas9 was identified as an effective genome engineering system in HeLa cells and represents a potential avenue to pursue genetic manipulation in NK cells.

7.2 Summary and future work

It is widely established that NK cells are difficult to transfect with traditional techniques, and virus systems are equally challenging, demonstrating poor efficacy in gene delivery. This is thought to be due to the inherent characteristics of NK cells. The technical challenges associated with transduction hinders many approaches in gene delivery. Thus, a need to develop a strategy to effectively transduce or transfect NK cells. Here, I demonstrated that a chimaeric adenovirus vector transduced NK cells at high efficiency. The adenovirus used is an Ad5 derivative with the fibre replaced by that from Ad35 (generating Ad5f35) to expand the tropism of the virus to include the recognition of CD46, a molecule expressed on NK cells. The adenovirus vector has a well-described recombination system which can be used to genetically modify the Ad genome to deliver chosen genes; here the target was to block TGF-β induced inhibition of NK cells by expression of a dominant negative TGF-β receptor.

It is widely known that TGF-β has a global impact on immune cells, in particular its direct effect on suppressing cytolytic activation of T and NK cells, and its ability to induce apoptosis in T and B cells (118,120). Hence, it is common for solid tumours (e.g. breast, ovarian and colon cancers) to secrete TGF-β in order evade anti-tumour immunity (453–455). For TGF-β secreting breast cancers, the metastatic potential is higher in patients with an elevated level of TGF-β (453). This is due to the ability of TGF-β to downregulate an anti-tumour immune response and to induce epithelial to mesenchymal transition (EMT), which favours metastasis. Here, dnTGFβRII was

chosen as a tool to inhibit the TGF-β induced inhibition of NK cells. A previous study showed that dnTGFβRII reduced NK cell inhibition when expressed on cord blood NK cells (*ex vivo*) (134). For T cells, dnTGFβRII expression in mice resulted in attenuation of TGF-β signalling (456). However, after 3 months, mice exhibited inflammation and autoantibody secretion and displayed multiple symptoms of sickness (such as weight loss and diarrhoea) (456). Despite the risks of expressing dnTGFβRII on T cell *in vivo*, this study brings to the attention of a need to modulate expression.

Here, the cloning of the dnTGFβRII into the Ad5f35 vector resulted in a deletion and introduction of a premature stop codon just prior to the transmembrane domain, producing an alternative form of dnTGFβRII. Interestingly, this deletion is also observed in tumours with microsatellite instability, due to mutations in mismatch repair genes (413). In particular, this has been described in colon cancer and is thought to result in a truncated or kinase inactive mutant form, providing a protective mechanism against the anti-proliferative effects of TGF-β (413). Clearly, the repair mechanisms within *E. coli* are not as efficient when sequences are mis-processed, showing one limitation to using the AdZ system. As a result, no major differences were detected in NK cells responses to TGF-β when transduced with this alternative form of dnTGFβRII. Other studies have shown that dnTGFβRII can be expressed in NK cell lines or cord blood derived NK cells and retain their phenotype and cytolytic activity in the presence of TGF-β (134,204). However, human blood derived NK cells, the cells that would be used in the clinic, are yet to be efficiently modified using viral vectors. Evidently, the mutations in dnTGFβRII made this model difficult to use and quantify the effects in NK cells. Nevertheless, this project has demonstrated that human peripheral blood-derived NK cells are readily transduced by Ad5f35 and that the AdZ system can be used to express transgenes, in spite of the molecular challenges encountered here.

There is also a need to investigate other targets to inhibit TGF-β signalling in NK cells. One such target might include expressing SMAD7, a SMAD inhibitor, to inhibit the TGF-β signalling cascade. In addition, a phosphatase within the pathway might also prove to have an influential effect on TGF-β signalling; in particular PPM1A has been shown to dephosphorylate SMAD2/3 (443). However, the AdZ/BAC system is at a high risk of incorrectly processing long repetitive sequence repeats, hence the need for caution when selecting and cloning genes using the AdZ system.

In addition, a further disadvantage of the AdZ system is the potential to inadvertently produce replication competent virions. This is a well-documented problem in both viral production and clinical applications. However, small amounts of replicating virus is acceptable according to the FDA (377). Here, adenovirus vectors that lacked E1A and E1B (early region genes) were used, however, due to sequence homology between the E1 region inserted into the 911 cells and adenovirus vectors, a recombination event occurred in one of the stocks where the E1 region was recaptured by the vector, thus contaminating replication-deficient adenovirus vectors with replication competent adenovirus (363). This represents a limiting factor for this system, however the risks can be reduced by using a cell line such as PER.C6, which has reduced sequence homology (364). On this project, this was avoided by reducing the propagation time in 911 cells, however this resulted in the disadvantage of producing low titres, which might not be a problem when transducing some cell types. However, here it reduced the efficacy of NK transduction. Further use might include longer periods/multiple rounds of propagation, with frequent intervals of testing for replicating virus throughout propagation to avoid these limiting factors.

This project focused on overcoming TGF-β induced inhibition on NK cells, however future direction using the AdZ system opens possibilities of broadening genetic manipulation on NK cells. Here the possibility of introducing genome engineering systems, such as CRISPR/Cas9 holds potential for broadening manipulation. However, the capacity to integrate CRISPR/Cas9 into Ad5f35 is challenging, but not impossible (457). This area remains unexplored for the genetic manipulation of lymphocytes and, in the case of genetically enhancing NK cells, holds great promise for immunotherapeutic strategies in cancer patients.

In summary, this project identifies a strategy to use a chimaeric adenovirus as a vehicle to deliver genes to human primary NK cells *ex vivo*. Future applications using this strategy can include genetic manipulation of NK cells to promote NK cytotoxicity, migration or cytokine production in order to enhance anti-tumour immunity.

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9   Appendix

9.1   Fluorescent microscopy of Ad5F35-EGFP transduced NK cells.

Primary NK Cells
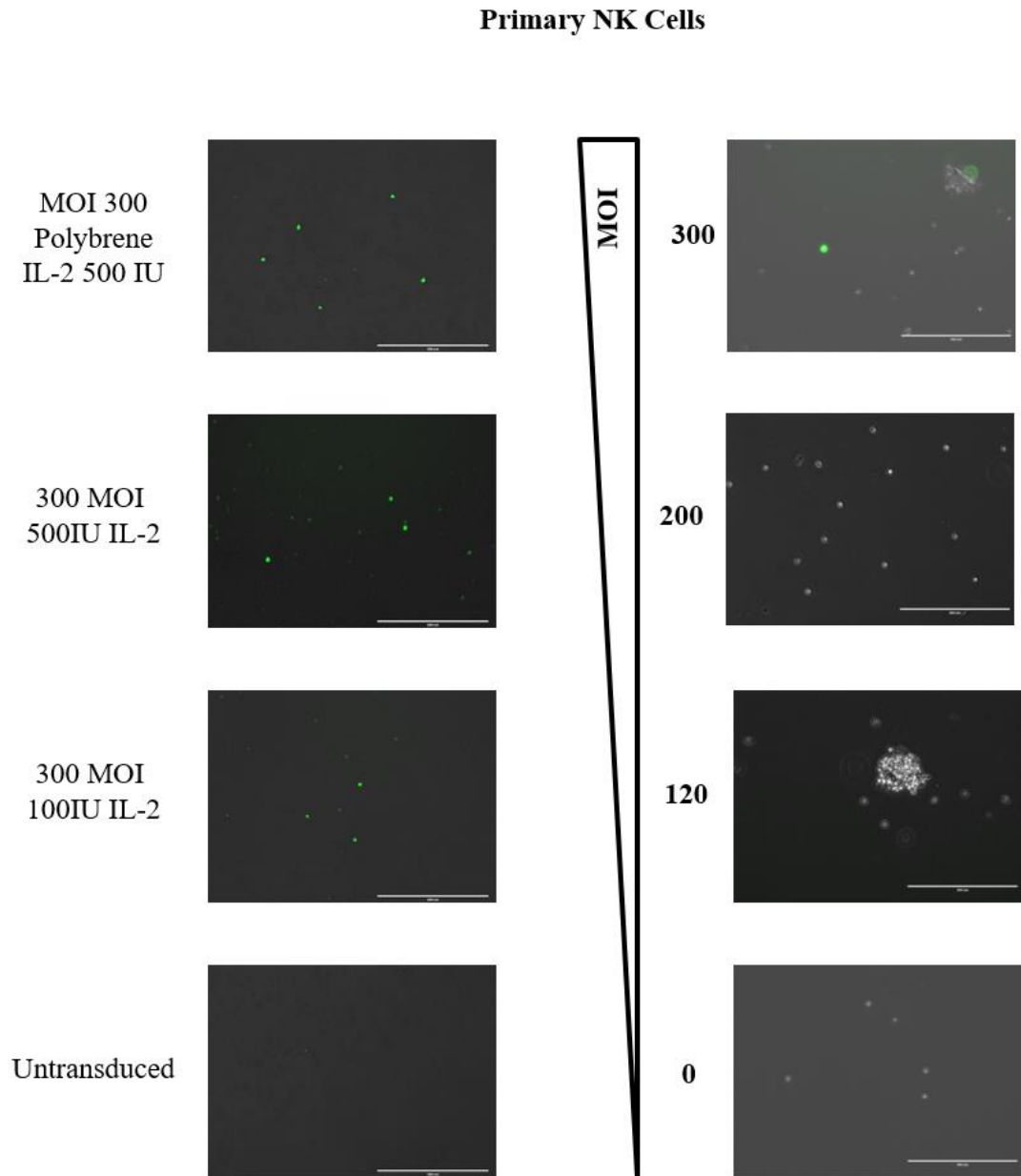
9.2 Fluorescent microscopy of Ad5F35-EGFP transduced NK cell lines.
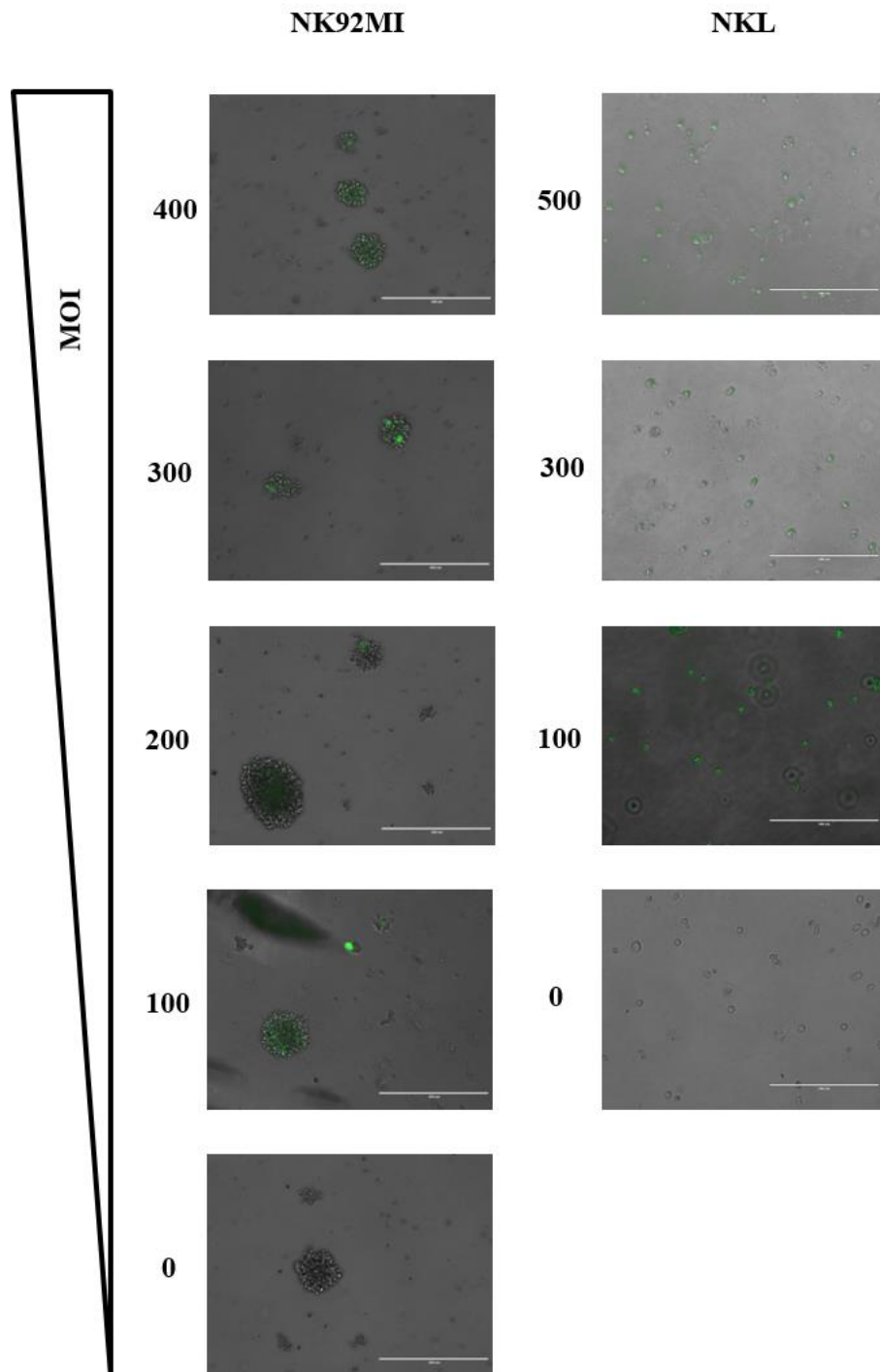
9.3 Sequence of Ad5F35-tdnTGFβRII.1

Forward

```
Expected        GGACGCGTATCGCCAGCACGATCCCACCGCACGTTCAGAAGTCGGTTAATAACGACATGA    315
Ad5f35-TGFBR2DN ----GCGTATCGCCAGCACGATCCCACCGCACGTTCAGAAGTCGGTTAATAACGACATGA

Expected        TAGTCACTGACAACAACGGTGCAGTCAAGTTTCCACAACTGTGTAAATTTTGTGATGTGA    375
Ad5f35-TGFBR2DN TAGTCACTGACAACAACGGTGCAGTCAAGTTTCCACAACTGTGTAAATTTTGTGATGTGA

Expected        GATTTTCCACCTGTGACAACCAGAAATCCTGCATGAGCAACTGCAGCATCACCTCCATCT    435
Ad5f35-TGFBR2DN GATTTTCCACCTGTGACAACCAGAAATCCTGCATGAGCAACTGCAGCATCACCTCCATCT

Expected        GTGAGAAGCCACAGGAAGTCTGTGTGGCTGTATGGAGAAAGAATGACGAGAACATAACAC    495
Ad5f35-TGFBR2DN GTGAGAAGCCACAGGAAGTCTGTGTGGCTGTATGGAGAAAGAATGACGAGAACATAACAC

Expected        TAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTATTCTGGAAGATGCTG    555
Ad5f35-TGFBR2DN TAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTATTCTGGAAGATGCTG

Expected        CTTCTCCAAAGTGCATTATGAAGGAAAAAAAAAAGCCTGGTGAGACTTTCTTCATGTGTT    615
Ad5f35-TGFBR2DN CTTCTCCAAAGTGCATTATGAAGGAAAAAAAAAGCCTGGTGAGACTTTCTTCATGTGTT

Expected        CCTGTAGCTCTGATGAGTGCAATGACAACATCATCTTCTCAGAAGAATATAACACCAGCA    675
Ad5f35-TGFBR2DN CCTGTAGCTCTGATGAGTGCAATGACAACATCATCTTCTCAGAAGAATATAACACCAGCA

Expected        ATCCTGACTTGTTGCTAGTCATATTTCAAGTGACAGGCATCAGCCTCCTGCCACCACTGG    735
Ad5f35-TGFBR2DN ATCCTGACTTGTTGCTAGTCATATTTCAAGTGACAGGCATCAGCCTCCTGCCACCACTGG

Expected        GAGTTGCCATATCTGTCATCATCATCTTCTACTGCTACCGCGTTGGATCCATGGACTACA    795
Ad5f35-TGFBR2DN GAGTTGCCATATCTGTCATCATCATCTTCTACTGCTACCGCGTTGGATCCATGGACTACA

Expected        AAGACGATGACGACAAGGGATCCCACGTCACTATTGTATACTCTATATTATACTCTATGT    855
Ad5f35-TGFBR2DN AAGACGATGACGACAAGGGATCCCACGTCACTATTGTATACTCTATATTATACTCTATGT

Expected        TATACTCTGTAATCCTACTCAATAAACGTGTCACGCCTGTAAAA                    899
Ad5f35-TGFBR2DN TATACTCTGTAATCCTACTCAATAAACGTGTCACGCCTGTGAAA
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Reverse

```
Expected        TAACAAGTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT    60
Ad5f35-TGFBR2DN TAACAAGTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT

Expected        AAGCAGAGCTCTCCCTATCAGTGATAGAGATCTCCCTATCAGTGATAGAGAGTTTAGTGA    120
Ad5f35-TGFBR2DN AAGCAGAGCTCTCCCTATCAGTGATAGAGATCTCCCTATCAGTGATAGAGAGTTTAGTGA

Expected        ACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGG    180
Ad5f35-TGFBR2DN ACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGG

Expected        ACCGATCCAGCCTGGATCCGTCTGCCATGGGTCGGGGGCTGCTCAGGGGCCTGTGGCCGC    240
Ad5f35-TGFBR2DN ACCGATCCAGCCTGGATCCGTCTGCCATGGGTCGGGGGCTGCTCAGGGGCCTGTGGCCGC

Expected        TGCACATCGTCCTGTGGACGCGTATCGCCAGCACGATCCCACCGCACGTTCAGAAGTCGG    300
Ad5f35-TGFBR2DN TGCACATCGTCCTGTGGACGCGTATCGCCAGCACGATCCCACCGCACGTTCAGAAGTCGG

Expected        TTAATAACGACATGATAGTCACTGACAACAACGGTGCAGTCAAGTTTCCACAACTGTGTA    360
Ad5f35-TGFBR2DN TTAATAACGACATGATAGTCACTGACAACAACGGTGCAGTCAAGTTTCCACAACTGTGTA

Expected        AATTTTGTGATGTGAGATTTTCCACCTGTGACAACCAGAAATCCTGCATGAGCAACTGCA    420
Ad5f35-TGFBR2DN AATTTTGTGATGTGAGATTTTCCACCTGTGACAACCAGAAATCCTGCATGAGCAACTGCA

Expected        GCATCACCTCCATCTGTGAGAAGCCACAGGAAGTCTGTGTGGCTGTATGGAGAAAGAATG    480
Ad5f35-TGFBR2DN GCATCACCTCCATCTGTGAGAAGCCACAGGAAGTCTGTGTGGCTGTATGGAGAAAGAATG

Expected        ACGAGAACATAACACTAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTA    540
Ad5f35-TGFBR2DN ACGAGAACATAACACTAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTA

Expected        TTCTGGAAGATGCTGCTTCTCCAAAGTGCATTATGAAGGAAAAAAAAAAACTGGTGAGACT    600
Ad5f35-TGFBR2DN TTCTGGAAGATGCTGCTTCTCCAAAGTGCATTATGAAGGAAAAAAAAAAGCCTGGTGAGAC
```

9.4 Sequence of Ad5F35-tdnTGFβRII.2

Forward

```
Expected         GGACGCGTATCGCCAGCACGATCCCACCGCACGTTCAGAAGTCGGTTAATAACGACATGA   315
Ad5f35-TGFBR2DN  ----GCGTATCGCCAGCACGATCCCACCGCACGTTCAGAAGTCGGTTAATAACGACATGA

Expected         TAGTCACTGACAACAACGGTGCAGTCAAGTTTCCACAACTGTGTAAATTTTGTGATGTGA   375
Ad5f35-TGFBR2DN  TAGTCACTGACAACAACGGTGCAGTCAAGTTTCCACAACTGTGTAAATTTTGTGATGTGA

Expected         GATTTTCCACCTGTGACAACCAGAAATCCTGCATGAGCAACTGCAGCATCACCTCCATCT   435
Ad5f35-TGFBR2DN  GATTTTCCACCTGTGACAACCAGAAATCCTGCATGAGCAACTGCAGCATCACCTCCATCT

Expected         GTGAGAAGCCACAGGAAGTCTGTGTGGCTGTATGGAGAAAGAATGACGAGAACATAACAC   495
Ad5f35-TGFBR2DN  GTGAGAAGCCACAGGAAGTCTGTGTGGCTGTATGGAGAAAGAATGACGAGAACATAACAC

Expected         TAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTATTCTGGAAGATGCTG   555
Ad5f35-TGFBR2DN  TAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTATTCTGGAAGATGCTG

Expected         CTTCTCCAAAGTGCATTATGAAGGAAAAAAAAAAGCCTGGTGAGACTTTCTTCATGTGTT   615
Ad5f35-TGFBR2DN  CTTCTCCAAAGTGCATTATGAAGGAAAAAAAAAA----SCRAMBLED-------------
```

Reverse

```
Expected         TAACAAGTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT   60
Ad5f35-TGFBR2DN  TAACAAGTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT

Expected         AAGCAGAGCTCTCCCTATCAGTGATAGAGATCTCCCTATCAGTGATAGAGAGTTTAGTGA   120
Ad5f35-TGFBR2DN  AAGCAGAGCTCTCCCTATCAGTGATAGAGATCTCCCTATCAGTGATAGAGAGTTTAGTGA

Expected         ACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGG   180
Ad5f35-TGFBR2DN  ACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGG

Expected         ACCGATCCAGCCTGGATCCGTCTGCCATGGGTCGGGGGCTGCTCAGGGGCCTGTGGCCGC   240
Ad5f35-TGFBR2DN  ACCGATCCAGCCTGGATCCGTCTGCCATGGGTCGGGGGCTGCTCAGGGGCCTGTGGCCGC

Expected         TGCACATCGTCCTGTGGACGCGTATCGCCAGCACGATCCCACCGCACGTTCAGAAGTCGG   300
Ad5f35-TGFBR2DN  TGCACATCGTCCTGTGGACGCGTATCGCCAGCACGATCCCACCGCACGTTCAGAAGTCGG

Expected         TTAATAACGACATGATAGTCACTGACAACAACGGTGCAGTCAAGTTTCCACAACTGTGTA   360
Ad5f35-TGFBR2DN  TTAATAACGACATGATAGTCACTGACAACAACGGTGCAGTCAAGTTTCCACAACTGTGTA

Expected         AATTTTGTGATGTGAGATTTTCCACCTGTGACAACCAGAAATCCTGCATGAGCAACTGCA   420
Ad5f35-TGFBR2DN  AATTTTGTGATGTGAGATTTTCCACCTGTGACAACCAGAAATCCTGCATGAGCAACTGCA

Expected         GCATCACCTCCATCTGTGAGAAGCCACAGGAAGTCTGTGTGGCTGTATGGAGAAAGAATG   480
Ad5f35-TGFBR2DN  GCATCACCTCCATCTGTGAGAAGCCACAGGAAGTCTGTGTGGCTGTATGGAGAAAGAATG

Expected         ACGAGAACATAACACTAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTA   540
Ad5f35-TGFBR2DN  ACGAGAACATAACACTAGAGACAGTTTGCCATGACCCCAAGCTCCCCTACCATGACTTTA

Expected         TTCTGGAAGATGCTGCTTCTCCAAAGTGCATTATGAAGGAAAAAAAAAAACTGGTGAGACT   600
Ad5f35-TGFBR2DN  TTCTGGAAGATGCTGCTTCTCCAAAGTGCATTATGAAGGAAAAAAAAAA-SCRAMBLED-
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9.5   Electron microscopy of Ad5F35-tdnTGFRII.1
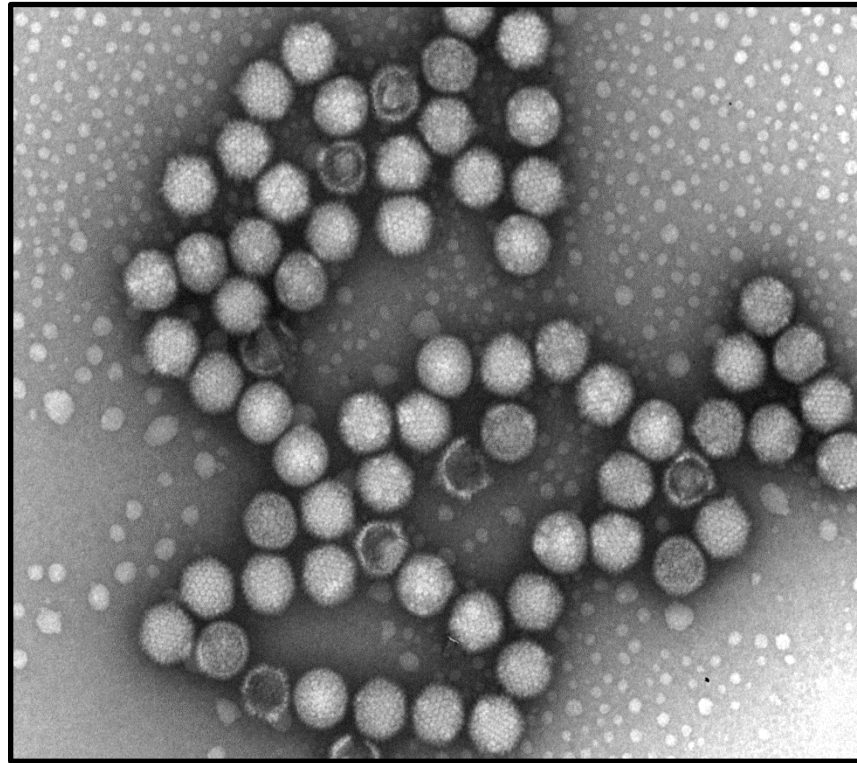
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Cal: 0.001033 µm/pix
10:07:44 07/11/2016

100 nm
Direct Mag: 20000x
Leeds University