University of Southampton

Faculty of Medicine

Clinical and Experimental Sciences

The role of Natural Killer cell Killer Immunoglobulin-like Receptors in the development and treatment of hepatocellular carcinoma

by

Dr Jonathan Coad

Thesis for the degree of Doctor of Medicine

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Abstract

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Background and aims

Hepatocellular carcinoma (HCC) is the fourth most common cause of cancer-related death worldwide, and incidence is increasing related to increasing prevalence of chronic liver disease. Survival is poor, and systemic treatments are limited. Immunotherapies are increasingly important in the treatment of many cancer types. NK cells are innate lymphocytes with an important role in tumour surveillance, and are an attractive target for immunotherapy. Their function is regulated the balance of activating and inhibitory receptors, an important component of which is the killer immunoglobulin-like receptor (KIR) family. KIR genotype has been associated with the outcome of various diseases. The activating KIR2DS2 receptor recognises flavivirus peptides in the context of HLA-C. This is a potential therapeutic target. Exosomes are extracellular vesicles with a role in cellular communication, which are a potential vehicle for cancer immunotherapies. I aimed to investigate the relationship between KIR genotype and hepatocellular carcinoma development in patients with hepatitis C. I then aimed to produce exosomes expressing HLA-C and the Dengue virus peptide DP1, and examine their potential to activate NK cells, and enhance killing cancer cell lines.

Results

In a cohort of 169 patients with hepatitis C, no patients with the KIR2DS3 gene developed HCC (p=0.04). Increasing age was significantly associated with the development of cirrhosis (p=0.007) and HCC(p=0.02). Exosomes were successfully purified from a 721:221

cell line expressing HLA-C + DP1. This was confirmed with electron microscopy, nanoparticle tracking analysis and Western blotting. In-vitro co-culture of exosomes with peripheral blood mononuclear cells (PBMCs) from KIR2DS2 positive donors did not produce KIR2DS2 positive NK cell expansion, increased degranulation, or produce enhanced killing of HCC cell lines. Injection of DP1 exosomes into a KIR transgenic mouse model produced significantly increased total NK frequency ($p=0.014$), and increased frequency of mature CD27-/CD11b+ NK cells compared to controls. This effect was not peptide-specific. KIR2DS2 positive NK cells exhibited significantly greater frequency of the liver residence and memory marker CXCR6 and activation marker CD69 than KIR2DS2 negative NK cells in C01 control and DP1 exosome injected mice. A heterotopic syngeneic murine cancer model using the RMA-S murine lymphoma cell line did not find any difference in survival, tumour volume, or NK cell tumour infiltration in DP1 exosome injected mice versus controls.

Conclusions

In this cohort of patients with hepatitis C from the UK, KIR2DS3 positivity was significantly associated with reduced incidence of HCC. In-vitro, DP1 exosomes did not impact NK cell proliferation or function. In a mouse model DP1 exosomes were significantly associated with increased NK cell frequency and maturity, which were not peptide specific. A tumour model did not demonstrate any reduction in tumour growth in exosome injected mice.

Table of Contents

Table of Tables

Table of Figures

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Definitions and Abbreviations

HCC – Hepatocellular carcinoma

HBV – Hepatitis B virus

HCV – Hepatitis C virus

NAFLD – Non-alcoholic fatty liver disease

BCLC – Barcelona clinic liver cancer

DAA – Direct acting antiviral

MAMPS - Microbe-associated molecular patterns

DAMPS - Damage-associated molecular patterns

NK – Natural killer cell

NKT – Natural killer T-cell

HSC – Hepatic stellate cell

MDSC - Myeloid-derived suppressor cell

Treg – Regulatory T-cell

IL - Interleukin

TGFβ - Transforming growth factor beta

VEGF - Vascular endothelial growth factor

TAMS - Tumour associated macrophages

CTLA-4 - Cytotoxic T-lymphocyte antigen 4

TERT - Telomerase reverse transcriptase

PDGFRβ - Platelet derived growth factor receptor β

MAPK1 - Mitogen activated protein kinase 1

MLL4 - mixed-lineage leukemia 4

HbxAg - Hepatitis B X antigen

HBsAg - Hepatitis B surface antigen

HBeAg - Hepatitis B e antigen

HBcAg - Hepatitis B core antigen

TRAIL - TNF-related apoptosis-inducing ligand

IFN-γ – Interferon gamma

BIM - Bcl2-interacting mediator

Breg – Regulatory B-cell

RNA - Ribonucleic acid

DNA - Deoxyribonucleic acid

EMT - Epithelial mesenchymal transition

ROS – Reactive oxygen species

WHO – World Health Organisation

STAT - Signal transducer and activator of transcription

TNF-α – Tumour necrosis factor alpha

NASH - Non-Alcoholic Steatohepatitis

MWA – Microwave ablation

RFA – Radiofrequency ablation

TACE – Transarterial chemoembolisation

RCT – Randomised controlled trial

VEGFR2 - Vascular endothelial growth factor receptor 2

TMB - Tumour mutational burden

PD-1 - Programmed cell death 1

FDA – Food and drug administration

TCR – T-cell receptor

CAR – Chimeric antigen receptor

CIK – Cytokine-induced killer cells

GPC3 - Glypican-3

BM – Bone marrow

SLT - Secondary lymphoid tissues

MALT - Mucosal associated lymphoid tissues

GM-CSF - Granulocyte macrophage-colony stimulating factor

CCL – Chemokine ligand

ADCC - Antibody-dependent cellular cytotoxicity

LAMP-1 - Lysosomal-associated membrane protein 1

ITIM - Immunoreceptor tyrosine-based inhibition motif

KIR - Killer-cell immunoglobulin-like receptors

ITAM - Immunoreceptor tyrosine-based activation motif

AML – Acute myeloid leukaemia

LMPP - Lymphomyeloid-primed multipotential progenitor

CLP - Common lymphoid progenitors

ILP - Innate lymphoid progenitors

NKP - NK cell progenitor

MCMV - Murine cytomegalovirus

HSV – Herpes simplex virus

FasL – Fas lignad

MICA - MHC class I-related chain A

LAK - lymphocyte-activated killer

mAb – Monoclonal antibody

BiKEs - Bi-specific killer engagers

TriKEs - Tri-specific killer engagers

EV – Extracellular vesicle

miR - micro RNA

cGAS-STING - Cyclic GMP-AMP synthase stimulator of interferon genes

DEX - Dendritic cell-derived exosomes

PFS - Progression free survival

siRNA - Small interference RNA

RPMI - Roswell Park Memorial Institute growth medium

FBS – Foetal bovine serum

PBS – Phosphate buffered saline

DMSO - Dimethyl sulfoxide

PBMC - Peripheral blood mononuclear cells

DMEM - Dulbecco's Modified Eagle Medium

ESCRT - Endosomal sorting complexes required for transport

DEN - Diethylnitrosamine

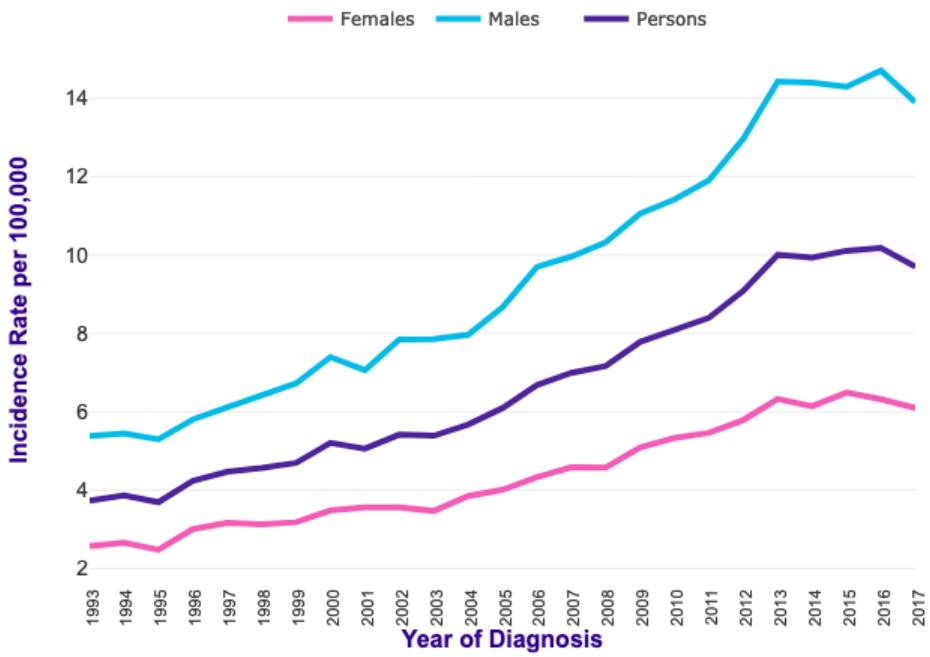
Chapter 1 Introduction

1.1 Hepatocellular Carcinoma

1.1.1 Epidemiology

Hepatocellular carcinoma (HCC) is the most common primary liver cancer(1), the fourth most common cause of cancer-related death worldwide, and the leading cause of cancer mortality in parts of the developing world(2). Its development is closely associated with chronic liver disease, and incidence reflects global variation in aetiological factors. The greatest burden of disease occurs in low or middle-resource countries, particularly in Asia and sub-Saharan Africa, accounting for approximately 80% of cases, predominantly related to endemic chronic hepatitis B (HBV) infection, and aflatoxin B1 exposure. In Europe and the USA, the predominant risk factors are excess alcohol consumption, hepatitis C (HCV) infection, and non-alcoholic fatty liver disease (NALFD)(3). As a consequence of this heterogeneity, incidence and mortality vary. Globally, incidence is rising. In low to middle-income countries this is related to population growth and ageing, and there has been a modest reduction in overall age-standardised incidence. Higher income countries have experienced sharp rises in the incidence of HCC; in the UK between 2004-2006 and 2014-2016 incidence has risen 60%, and 162% between 1993-1995 and 2014-2016(4)(See figure 1.1). Incidence in the USA tripled between 1975 and 2005(5). Similarly, age of onset varies geographically, and according to the aetiology and ethnicity. In Europe, Japan, and North America mean age of onset is >60 years of age, while evidence suggests median onset in sub-Saharan Africa may be 45 overall, and 32.5-37.5 years old for those with HBV(2). The global burden of disease is high, and rising, as the second leading cancer-related cause of lost life years, increasing 4.6% between 2005 and 2015(2).

Figure 1.1 HCC incidence in the UK(4)



Survival following a diagnosis of HCC is poor. In the UK overall 1 year survival is 36%, and 5 year survival 12%, however there is significant variability in treatment and survival according to extent of disease, and underlying liver function. Cases may be graded according to the Barcelona Clinic Liver Cancer (BCLC) staging system (see figure 1.2). Stage 0 includes those with limited disease and good liver function, and confers the best prognosis, with a 70-90% 5 year survival, and the possibility of curative therapy including liver resection and transplantation. In contrast stages C and D, with best available treatment, have a median survival of 6-11 months, and less than 4 months respectively(6). Greater than 50% of patients in Europe, North America, China, and South Korea first present with advanced (BCLC C) or terminal (BCLC D) stage disease(7). In lower-resource contexts, HCC survival is worse still, estimated to be approximately 2.5 months in sub-Saharan Africa, with 95% of patients presenting with advanced disease(2).

Figure 1.2 Barcelona Clinic Liver Cancer staging criteria(8)

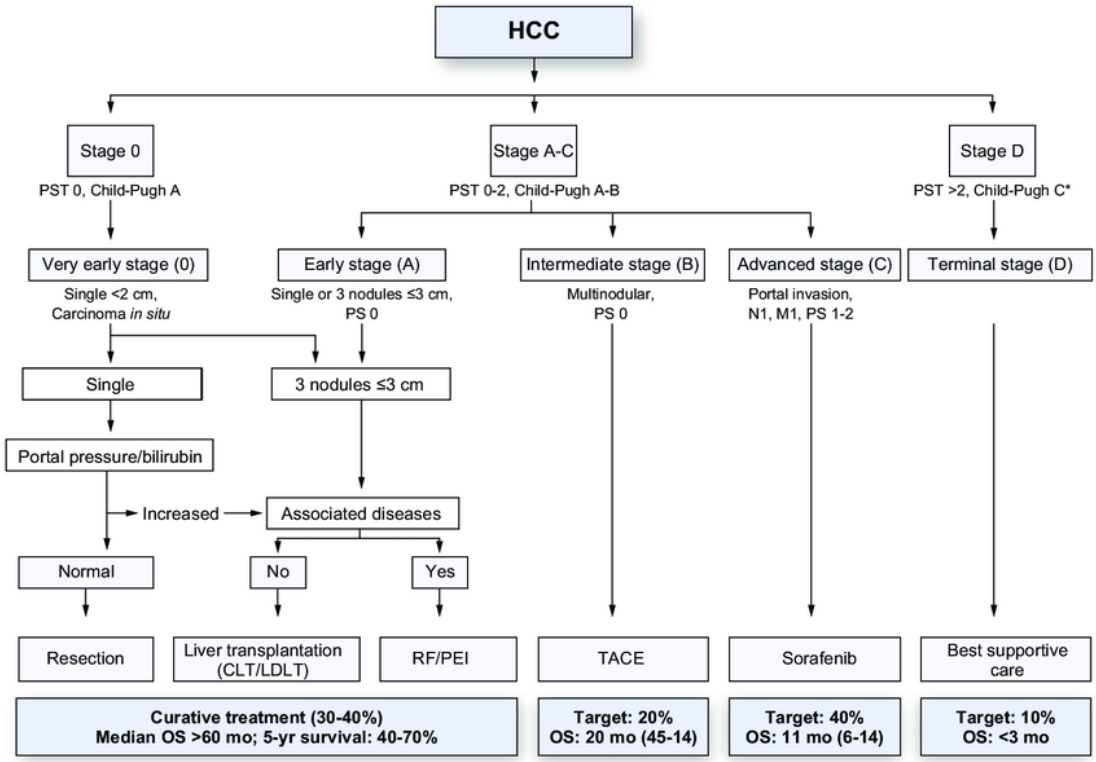


Fig. 1.2. Abbreviations: PST: Performance Status. CLT: Cadaveric Liver Transplant. LDLT: Living Donor Liver Transplant. RF: Radiofrequency Ablation. PEI: Percutaneous Ethanol Injection. TACE: Trans-Arterial Chemo-Embolisation. OS: Overall Survival.

Projections suggest that incidence of hepatocellular carcinoma is likely to continue to rise, reflecting the increasing burden of chronic liver disease. Widespread HBV vaccination has reduced the incidence of new chronic HBV infections(9), and development of direct-acting antiviral (DAA) drugs for HCV may reduce incidence of HCC secondary to viral hepatitis in the long-term, though it may rise for years to come. In Europe cancers secondary to NAFLD, related to rising obesity and metabolic syndrome, and alcohol-related liver disease are likely to continue to rise, leading to a predicted net increase in incidence of 142% between 2005 and 2030 in the UK, and 196-219% in the USA(10).

1.1.2 Pathogenesis of hepatocellular carcinoma

HCC is a primary liver cancer, distinct from other liver neoplasms, which include cholangiocarcinoma, hepatoblastoma, cystadenocarcinoma, haemangiosarcoma, and epithelial haemangioendothelioma(11). Pathogenesis is multi-factorial, related to a variety of infectious, metabolic, environmental, and socio-economic factors.

HCC is associated with inflammation, fibrosis and cirrhosis, and is a multi-step process, progressing through a sequence of liver injury, fibrosis and cirrhosis, dysplasia and malignancy, during which molecular and cellular alterations accumulate to promote its development. The liver has a unique capability to regenerate and repair following damage, as pre-existing hepatocytes proliferate to replace damaged tissue. In chronic liver disease necroinflammation (the inflammatory response to cell death) occurs, in which constant cell damage, compensatory regeneration and a consequent altered immune response leads to fibrosis, cirrhosis, and hepatocaricinogenesis. Cycles of necroinflammation and proliferation lead to genetic instability, and accumulation of mutations resulting in dysregulation of key oncogenes such as p53, β-catenin, E-cadherin and COX-2. Cell turnover promotes telomere shortening and chromosomal instability. Altered cell survival, senescence and proliferation signalling promotes tumour formation. The cellular and molecular drivers of hepatocarcinogenesis vary significantly according to the underlying aetiology(11-13).

1.1.3 Immunology of hepatocellular carcinoma

The liver has a uniquely tolerant immune environment. Via the portal circulation the liver receives a wide spectrum of microbes, microbe-associated molecular patterns (MAMPS), and damage-associated molecular patterns (DAMPS), which must be detected and cleared. Reflecting this, the liver has an array of innate and adaptive immune cells, including high concentrations of macrophages (Kupfer cells), natural killer (NK) cells, NKT cells, and T-cells, which can respond to pathogens, and damaged or infected cells(14). The liver must maintain an immunotolerant state to prevent immune responses from damaging the liver, and does so through a complex network of interactions between liver resident cells and a milieu of pro-inflammatory and anti-

inflammatory cytokines. Deregulation of this state in liver disease may lead to sustained inflammatory signalling, necroinflammation, autoimmunity and tumour development(15).

Tissue injury attracts neutrophils and macrophages, eliminating necrotic or apoptotic cells, pathogens, and debris. In a compensatory anti-inflammatory response, hepatic stellate cells (HSCs) and myofibroblasts release hepatocyte growth factor and induce myeloid-derived suppressor cells (MDSCs) and regulatory T lymphocytes (Tregs), which suppress effector cells preventing autoimmune tissue damage, and play a role in tissue healing. In HCC development dysregulation of this process may promote tumour growth; MDSCs exert pro-tumour effects through suppression of T cell and NK cell activity through transforming growth factor beta (TGFβ) and interleukin (IL)-10 production, and promote tumour angiogenesis through vascular endothelial growth factor (VEGF) production. M2 macrophages are produced, which produce anti-inflammatory and immunosuppressive cytokines and stimulate cell proliferation, tissue regeneration, angiogenesis, and formation of extra-cellular matrix, promoting tumour growth. These tumour associated macrophages (TAMS) interact with MDSCs, further stimulating M2 macrophage polarisation and Treg expansion. Under normal circumstances Tregs limit autoimmunity, but in the context of HCC contribute to the immunosuppressive microenvironment through expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4), and production of immunosuppressive molecules such as TGFβ and IL-10. Due to dysregulated tolerance-inducing inhibitory signalling, and immunosuppressive wound healing responses, a phenotype of exhausted T-cells is produced, reducing anti-tumour responses(16).

1.1.4 Hepatitis B infection

HBV is the most common cause of HCC globally, responsible for 54% of cases(12). Approximately 2 billion individuals worldwide have been infected with HBV and have developed antibodies to the virus, and 257 million people were living with chronic infection in 2015, corresponding to 3.5% of the global population(9). HBV is responsible for 320,000 deaths annually, 20-30% of which are attributable to HCC(11). Common modes of infection include vertical transmission from mother to child, and sexual contact(9). HBV can lead to HCC formation in the absence of cirrhosis, with concomitant implications for HCC surveillance.

HBV is a non-cytopathic, hepatotropic, partially double-stranded DNA virus, which directly and indirectly promotes carcinogenesis(11). HBV DNA integration into the human genome, may disrupt endogenous tumour suppressors and regulatory genes, or promote the activity of proto-oncogenes, resulting in enhanced tumour survival and proliferation, and reduced apoptosis(13). HBV integration has been associated with microdeletions and mutations in genes such as telomerase reverse transcriptase (TERT), platelet derived growth factor receptor β (PDGFRβ), mitogen activated protein kinase 1 (MAPK1), mixed-lineage leukemia 4 (MLL4), and others. HBV transcription has been demonstrated to alter expression of growth control genes, such as P-1, AP-2, c-EBP, ATF/CREB, and NFk-B(11, 17, 18). HBxAg may bind and inactivate p53, and p53 mutations may occur compromising senescence, DNA repair, and regulation of cellular proliferation(17). Viral-endoplasmic reticulum interactions produce oxidative stress, which causes mutations through the generation of free radicals, and stellate cells activation through growth and survival signalling pathways(18).

In acute infection clearance of HBV is related to potent antiviral responses from NK cells, NKT cells, CD4+ and CD8+ T cells. B cells are stimulated to produce antibodies to surface antigen (HBsAg), e antigen (HBeAg) and core antigen (HBcAg). The loss of immune tolerance during chronic infection leads to anti-HBV CD8+ responses inducing hepatocyte damage and necroinflamation. HBV specific CD8+ cells become increasingly exhausted in chronic infection due to high viral load and immunosuppressive cytokines such as IL-10 and TGF-β. These cells exhibit reduced effector functions through reduced interferon-γ (IFN-γ), perforin and granzyme production, and undergo increased apoptosis due to upregulation of TRAIL-2 and BIM, reducing effective tumour surveillance. Despite this these cells maintain a partially activated status which contributes to ongoing hepatocyte injury. T cell exhaustion is characterised by upregulation of inhibitory receptors such as programmed cell death 1 (PD-1) and CTLA-4, the presence of which has been correlated with poor outcomes in HCC. High viral load is correlated with increased frequency of circulating Tregs due to the interaction of HBsAg and MDSCs and HSCs. Tregs weaken tumour surveillance through suppression of effector cells via secretion of immunosuppressive cytokines, cell killing, and induction of apoptosis. B cells may differentiate into B regulatory cells (Bregs), which like Tregs, promote an immunosuppressive environment to prevent tissue damage, reducing tumour surveillance further. (18, 19).

1.1.5 Hepatitis C infection

The WHO estimate that in 2015 71 million individuals were living with chronic HCV, and that incidence was rising, with 1.75 million new infections exceeding the number of individuals cured (843,000), or dying with chronic HCV (399,000)(9). Approximately 20% of individuals with chronic HCV develop cirrhosis, and 2.5% develop HCC(11). Infection is commonly spread through unsafe healthcare procedures and injected drug use(9).

HCV is a non-cytopathic, hepatotropic RNA virus of the *flaviviridae* family. In contrast to HBV, HCV is more likely to develop into chronic infection (occurring in 60-80% of cases), and promote cirrhosis(11). Unlike HBV it does not integrate into host DNA. Hepatocarcinogenesis occurs due to viral-induced factors, and the host immunological response. HCV replication causes endoplasmic reticulum stress. Viral proteins may directly drive lipogenesis, promote oxidative stress, and act on cell signalling pathways (such as MAPK and epithelial mesenchymal transition (EMT)), prevent cell apoptosis, and inactivate tumour suppressor genes (such as retinoblastoma protein and p53). HCV may evade immune surveillance by direct inhibition of Fas-mediated apoptosis, promoting cell proliferation. Generation of reactive oxygen species (ROS) and promotion of oxidative stress contributes to genomic instability, and the accumulation of mutations(20, 21).

Innate response to HCV infection is characterised by interferon, NK cell and NKT responses(22), with delayed T-cell responses compared to HBV. In the acute phase NK cells show an activated phenotype with enhanced IFN-γ production. KIR2DL3-HLAC1 has been associated with HCV clearance (see section 1.2.3). In common with HBV, production of specific CD8+ and CD4+ T cells are correlated with resolution of infection, however these responses may be abolished by escape mutations. In chronic infection, necroinflammation and T-cell exhaustion occurs as with HBV, contributing to disregulated immunity, fibrosis, cirrhosis and HCC development (20, 23).

1.1.6 Alcohol-related liver disease

The WHO have identified harmful alcohol consumption as a leading risk factor for ill health worldwide(24). Excess alcohol consumption is estimated to be responsible for 60-80% of liver-related deaths in high-income countries(25). Globally, alcohol-related liver disease accounts for 15-30% of HCC(26).

The relationship between alcohol consumption and HCC is complex and incompletely understood. Alcohol is directly carcinogenic, and indirectly promotes HCC by the development of cirrhosis(11). Direct toxicity and oxidative stress produced by alcohol consumption and metabolism may produce inflammation, stellate cell activation, cell proliferation, fibrosis and cirrhosis. Alcohol induced oxidative stress may also influence HCC-related signalling pathways, such as reduced STAT-1 directed activation of IFN-γ signalling, leading to hepatocyte damage. CYP2E1-dependent metabolism of alcohol produces ROS, which leads to generation of lipid peroxidation products, which may produce p53 mutations. Furthermore, ROS production has been shown to disrupt mediators of angiogenesis and tumour regulation(26). Alcohol may disrupt DNA methylation, producing altered gene expression. Acetaldehyde produced by metabolism of ethanol is thought to promote DNA mutation, and interrupt DNA repair.

Alcohol consumption, and the resultant liver damage produces a dysregulated immune response. Alcohol consumption increases intestinal absorption of iron, leading to hepatic iron overload, and increased gut permeability to bacterial endotoxins (lipopolysaccharides) and DAMPs. These activate Kupfer cells, triggering a chain of pro-inflammatory cytokines including Tumour Necrosis Factor-α (TNF-α) and IL-17 (27). Alcohol consumption stimulates complement activation, which further stimulates Kupfer cells and cytokine release. NK cell number and function has been shown to be impaired in the peripheral blood of patients with alcohol-related cirrhosis, impairing their anti-fibrotic function(26). Decreased and hypo-functional circulating T cells, and B cells and dendritic cells have been demonstrated in patients with alcohol-related liver disease, resulting in failure to clear pro-inflammatory cellular debris derived from microbes and necrotic hepatocytes(28).

1.1.7 Non-alcoholic fatty liver disease

NAFLD is the most common cause of chronic liver disease worldwide, and prevalence is increasing, associated with increasing prevalence of obesity and metabolic syndrome. Estimates of global prevalence of hepatic steatosis are approximately 25-32%. It is anticipated that 10-30% of patients with NAFLD will develop the potentially progressive Non-Alcoholic Steatohepatitis (NASH). NAFLD accounts for 75% of chronic liver disease, and 14.1% of HCC in the USA(29, 30).

In common with other aetiologies, HCC in NAFLD is related to a step-wise process involving recurrent cycles of hepatocyte damage, inflammation, proliferation, regeneration, fibrosis and cirrhosis(31).

Obesity produces a state of low-grade chronic inflammation. Mouse models of NASH exhibit CD8+ activation and CD4+ loss. Adipose tissue expansion promotes release of pro-inflammatory cytokines such as TNF and IL-6, leading to the activation of pro-oncogenic, anti-apoptotic, and proliferative pathways. Adiposity reduces expression of anti-inflammatory adipokines such as adiponectin, reducing suppression of inflammatory signalling in Kupfer cells, and reducing inhibition of the mTOR oncogenic pathway, while increasing pro-inflammatory adipokines such as leptin, which exert pro-fibrotic effects via activation of Kupfer cells and stellate cells. Deposition of lipid within non-adipose tissues may interfere with cell signalling pathways and alter regulation of gene transcription. Fatty acid oxidation may promote oxidative stress. Adipose expansion, and inflammation produces insulin resistance, and hyperinsulinaemia, which has been linked to tumour development(12, 31).

1.1.8 Aflatoxin

Aflatoxin is a mycotoxin which may contaminate inappropriately processed staple cereals and seeds. It is widespread in areas with high HCC incidence such as sub-Saharan Africa, and exposure is thought to account in part for early onset of HCC(11). Between 25,200 and 155,000 HCC per year may be attributable to exposure (4.6–28.2%)(32). Aflatoxin B1, produced by Aspergillus, causes a mutation of the p53 tumour suppressor gene, a mutation associated with HCC(11). There

are proposed synergisms between HBV infection, and aflatoxin exposure; chronic HBV may induce cytochrome p450s that mobilise aflatoxin to a mutagenic form, and HBV may increase the probability of p53 mutations(2).

1.1.9 Management of hepatocellular carcinoma

The management of HCC is dictated by a variety of factors, including the extent of HCC, and the underlying liver function and co-morbidity. The poor prognosis of HCC is related to its resistance to existing anti-cancer therapies, the limitations of screening for early and resectable disease, and the limitations on therapy imposed by the underlying liver disease(11). The BCLC criteria are commonly used as a framework for management decisions (see Figure 1.2)(8).

Treatment with a curative intent is possible in those meeting BCLC 0 or A criteria. This includes resection, transplant, and ablation. Suitability for resection depends on the size and location of the tumour, underlying liver function and presence of portal hypertension, and patient suitability for surgery. It is a potentially curative intervention, but 50-70% of patients develop recurrent HCC within 5 years, with 5-year survival reported between 40-70% (2, 33, 34).

Liver transplant confers the highest survival benefit, addressing both the HCC and the underlying liver disease, which may be functionally impaired, and at risk of further HCC due to the continued presence of the underlying liver disease. HCC is the most common indication for first elective liver transplantation in the UK, accounting for 25% of cases(35). The Milan criteria form the framework for assessing acceptable tumour burden for transplant, requiring a single lesion ≤ 5 cm, or up to three separate lesions, none larger than 3 cm, with no evidence of gross vascular invasion and no regional nodal or distant metastases. Patients who meet these criteria and receive a transplant have a reported 85% 4 year survival(36, 37). Expanded guidelines are in use which include the use of loco-regional therapies to downstage HCC prior to transplant(2). Despite favourable outcomes, patient suitability for transplant and organ shortage limit those who benefit.

Ablation uses percutaneous locoregional application of heat to HCC, using microwave (MWA) or radio-frequency (RFA) ablation. Survival benefit compared to resection is controversial, and has

been reported as comparable or inferior to resection(38, 39). It is frequently offered to patients with limited BCLC 0 or A HCC who are otherwise not fit to undergo resection.

The recommended management for patients with intermediate-stage tumours is trans-arterial chemoembolisation (TACE). This utilises intra-arterial administration of cytotoxic chemotherapy in conjunction with embolisation of tumour-feeding vessels, resulting in a cytotoxic and ischaemic effect(40). Systematic review of 101 studies estimated an objective response to treatment in 52.5%(41), randomised controlled trials have demonstrated a survival benefit compared to best supportive care(42, 43).

Conventional systemic cytotoxic therapies have proven ineffective in HCC due to lack of anti-tumour response, or toxicity in the context of cirrhosis. Sorafenib, a multikinase inhibitor, is the current standard of care for advanced HCC not amenable to loco-regional therapy, but with adequate liver function. This class of medications inhibit tumour angiogenesis and cell proliferation (41). RCT has demonstrated a modest survival benefit, from a median of 7.9 months with placebo to 10.7 months with Sorafenib(44). Regorafenib and Levatinib are newer multi-kinase inhibitors, which have survival benefits in RCTs, though these continue to be modest. The RESORCE trial demonstrated an improvement in survival from 7.8 months to 10.6 months in those treated with Regorafenib compared with placebo in patients previously treated with Sorafenib.(45) In the REFLECT trial, Levatinib was found to be non-inferior to Sorafenib (survival 13.6 months with Levatinib compared to 12.3 months with Sorafenib)(46). Ramucirumab, a VEGFR2 antagonist, improved survival in the REACH-2 study in patients previously treated with Sorafenib to 8.5 months from 7.3 months with placebo(47). Cabozantinib, an oral tyrosine kinase inhibitor, improved survival compared to placebo in the CELESTIAL trial (median survival 10.2 months compared to 8.0 months), but grade 3 or 4 adverse events were reported in 68% of patients in the treatment group(48).

Immunotherapy is a rapidly growing field of cancer therapy, notably checkpoint inhibitors have become the standard of care for a variety of cancers. These target negative regulators of T-cells, so enhancing anti-tumour T-cell responses. Response to checkpoint inhibition varies among tumour types and individuals. This has been correlated with tumour mutational burden (TMB), thought to correspond to increased expression of immunogenic neoantigens, elevated numbers of tumour-infiltrating lymphocytes, and checkpoint expression. Resistance to checkpoint

inhibition may develop(49). The programmed cell death protein 1 (PD-1) inhibitor, Nivolumab, has been approved for treatment of hepatocellular carcinoma by the United States Food and Drug Administration (FDA), and incorporated into AASLD guidelines as a second line therapy for HCC(50). The CHECKMATE 040 phase I/II trial reported a 20% objective response rate, though 96% of patients discontinued therapy, 88% of which were due to disease progression(51). Phase III trials are in progress, though initial data from CHECKMATE 459 has so far failed to demonstrate a survival benefit compared to Sorafenib(52). The KEYNOTE 240 phase III trial of Pembrolizumab, another PD-1 inhibitor, did not demonstrate a significant survival benefit versus placebo(53). PD-1 blockade has been proposed as a combination therapy with loco-regional and systemic therapies, including other checkpoint inhibitors, including Cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) inhibitors. The HIMALAYA phase III trial is currently examining the combination of CTLA-4 Tremelimumab and PD-L1 inhibitor Durvalumab(54). The combination of the PD-1 Atezolizumab and anti-VEGF Bevacizumab has recently entered mainstream clinical use, having demonstrated significant benefit vs Sorafenib in 6 month progression-free survival (84.8% vs 72.2%) and overall survival (54.5% vs 37.2%) in the IMbrave 150 trial(55). Other treatments currently in early clinical trials aiming to enhance T-cell responses include Transforming Growth Factor-β inhibitors, T-cell immunoglobulin and mucin-domain containing-3 inhibitors, and Lymphocyte activation gene 3 inhibitors(50).

Other immunotherapeutic strategies are in development. Adoptive transfer of autologous lymphocytes cultured in-vitro have been studied in other cancer types for many years, including haematological malignancies, and metastatic melanoma. T cell receptor (TCR)-engineered T cells, and chimeric antigen receptor (CAR) T cells are undergoing phase I/II clinical trials in patients with HCC(56). In phase I trials of infusion of GP3 CAR-T cells following lymphodepletion, disease response was observed in 2 of 13 patients, though adverse events included cytokine release syndrome in 9 of 13 patients(57). Injection of autologous cytokine-induced killer cells (CIK: CD3+/CD56+ and CD3+/CD56- T cells and CD3-/CD56+ natural killer cells) has been investigated as an immunotherapy for HCC in multiple clinical trials. A study of 230 patients treated with CIK as an adjunct to resection or ablation found a significant recurrence free survival benefit (44 months in treatment group vs 30 months in control group)(58). Systematic review and meta-analysis of 12 trials found that adjuvant CIK improved progression-free and recurrence-free survival(59). Oncolytic viruses aim to preferentially target and replicate in cancer cells, producing tumour lysis, and enhancing NK anti-tumour immune responses. Multiple viruses have been trialled in pre-clinical, and phase I studies(60). A phase 3 trial comparing Pexa-Vec (JX-594) and sorafenib vs sorafenib alone has been halted following interim futility analysis, which suggested that the trial

was unlikely to demonstrate a clinical benefit(61). Peptide vaccines aim to generate an immune response to HCC. Glypican-3 (GPC3) has been identified as a potential target, due to its over expression in the majority of HCC. A GPC3-derived peptide vaccine was successful in inducing production of peptide-specific cytotoxic

T-lymphocytes in phase I trials, and increased 1-year survival in patients with GPC3 positive tumours when given as an adjunct to RFA in phase II trials(62).

1.2 Natural Killer Cells

1.2.1 Natural killer cell definition and function

Natural killer (NK) cells are large granular lymphocytes of the innate immune system, which have an important role in antimicrobial and antitumour immunity. They constitute 5-15% of peripheral blood lymphocytes, and are also present in abundance in the bone marrow (BM), liver, uterus, spleen, lung, secondary lymphoid tissues (SLT), mucosal associated lymphoid tissues (MALT), and the thymus. They are defined phenotypically by expression of CD56, and absence of CD3, and are named for their ability to detect and kill pathogens and tumours without antigen-specific recognition. In addition, they are able to secrete cytokines and chemokines, and have a role in recruiting, augmenting, and regulating other immune cell types. NK cells are able to directly kill by exocytosis of cytolytic granules adjacent to target cells. Perforin forms pores in the target cell, facilitating entry of granzymes into the cytoplasm, which initiate a sequence of signalling which instigates cell apoptosis. During degranulation CD107a (LAMP-1) appears on the cell surface. NK cells are also able to induce killing via antibody-dependent cellular cytotoxicity (ADCC), initiated by interaction of the IgG-Fc receptor CD16, and IgG coated target cells. Furthermore, NK may induce cytolysis through release of tumour necrosis factor (TNF)-α and interferon (IFN)-γ (63-65). They perform an important role in mediating the adaptive immune system, and may act as primary initiators of the immune response through release of immunoregulatory cytokines such as interleukin(IL)-5, IL-10, IL-13, granulocyte macrophage-colony stimulating factor (GM-CSF), and chemokines such as chemokine ligand (CCL)-3, CCL4, CCL5 and IL-8(66).

NK cell function is regulated by a balance of inhibitory and activating signals which determine the outcome of interactions with other cells, and regulates initiation of the cytolytic process to avoid tissue damage. Activating receptors (such as KIR 2DS2, NKG2D) recognise ligands on infected, stressed, or abnormal cells, while inhibitory receptors (such as KIR 2DL1, NKG2A) recognise normal cells (see figure 1.3). Inhibitory receptors predominantly recognise MHC class I, which are cell surface proteins expressed by almost all healthy cells, but are lost in many stressed or infected cells (the 'missing-self' hypothesis). Loss of self-MHC I, reduces inhibitory signalling, and simultaneously NK cells are likely to receive activating signals from interaction with the abnormal cell, resulting in a tipping of the balance toward activation, and killing of the cell. Inhibitory receptors bear the immunoreceptor tyrosine-based inhibition motif (ITIM) on their cytoplasmic tails, which blocks activating receptor pathways, hence inhibitory signalling dominates over activation(65, 67). Reactivity and sensitivity to inhibition may be altered by NK cell education; the capacity to sense a downregulation in a particular MHC class I molecule. This requires recognition of self-MHC class I by inhibitory killer-cell immunoglobulin-like receptors (KIR). Educated NK cells have lower thresholds for activation against target cells with reduced self-MHC expression, but preserve tolerance to self to avoid autoimmunity. Conversely, uneducated NK lack self-MHC class I inhibitory receptors, and so are insensitive to MHC class I inhibition, but have a higher threshold for activation from activating receptors(68). Activating receptors recognise a broad and heterogenous array of ligands, some of which may be encountered on healthy cells. Many activating receptors share the immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic tails, which produce signals that promote target cell killing, and cytokine release. Activation may occur due to the interaction of specific receptors (such as NKG2D), which may overcome the inhibitory signalling of self-MHC class I, allowing cytotoxic activity against MHC-1 sufficient tumours ('altered-self')(69).

The importance of NK cells in tumour surveillance is difficult to study in isolation, but evidence suggests that they are a significant contributor. There is no known inherited disorder which exclusively causes selective NK deficiency(70), though several studies have reported a higher incidence of cancers in individuals with genetic conditions which produce impairment in NK function, such as Chédiak–Higashi syndrome, and MCM4 mutation(71). Decreased NK cell function has been demonstrated in patients with various cancer types, indicating that tumours may develop mechanisms of escaping NK surveillance during development. Mouse models of NK depletion have shown greater tumour growth and metastasis(72). Ruggeri et al. demonstrated that the presence of KIR mismatched alloreactive NK cells in hematopoietic cell transplantation for acute myeloid lukemia (AML) improved relapse and event-free survival(73).

Table 1.1 Activating and inhibitory NK receptors and their ligands(74).

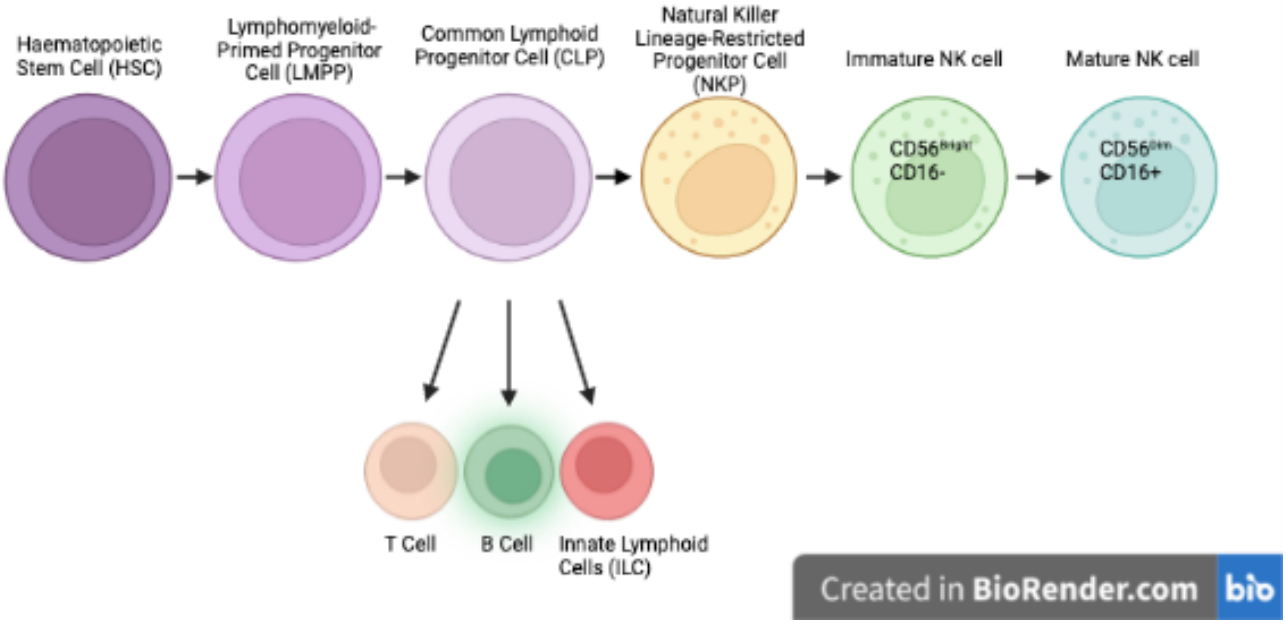
Receptor	Ligand(s)	Expression	Function
Natural cytotoxicity receptors			
NKp46	Unknown	Resting and activated NK cells	Stimulatory
NKp30	Unknown	Resting and activated NK cells	Stimulatory
NKp44	Unknown	Activated NK cells	Stimulatory
NKp80	Unknown	Resting and activated NK cells, CD3+/CD56+ T cells	Co-stimulatory
Killer immunoglobulin-like receptors (KIR)			
KIR2DL1	HLA-Cw2,4,5,6	NK, T cells	Inhibitory
KIR2DL2	HLA-Cw1,3,7,8	NK, T cells	Inhibitory
KIR3DL1	HLA-Bw4	NK, T cells	Inhibitory
KIR3DL3	HLA-A3-A11	NK, T cells	Inhibitory
KIR2DS1	HLA-Cw2,4,5,6	NK, T cells	Stimulatory
KIR2DS2	HLA-Cw1,3,7,8	NK, T cells	Stimulatory
KIR2DS4	Unknown	NK, T cells	Stimulatory
NKG2 family			
CD94/NKG2A	HLA-E	NK, CTL	Inhibitory
CD94/NKG2C	HLA-E	NK, CTL	Stimulatory
CD94/NKG2E	HLA-E	NK, CTL	Stimulatory
NKG2D	MICA, MICB, ULBPs	NK, gamma delta T, CD8+ T cells	Stimulatory
CD2 family			
2B4 (CD244)	CD48	NK, CD8+ T cells, monocytes	Co-stimulatory

Receptor	Ligand(s)	Expression	Function
NTB-A	Homophilic	NK, T cells, B cells	Co-stimulatory
DNAM-1 (CD226)	PVR (CD155), Nectin-2 (CD112)	NK, T cells, B cells	Co-stimulatory
CS1 (CRACC)	Homophilic	CTL, activated B cells, NK cells, mature DCs	Co-stimulatory

1.2.2 NK cell development and maturation

NK cells are derived from CD34+ hematopoietic stem cells (HSC), a subset of which differentiate into lymphomyeloid-primed multipotential progenitor (LMPP), which transition into common lymphoid progenitors (CLP) and innate lymphoid progenitors (ILP), which in turn give rise to early NK cell progenitors (NKP)(75)(see figure 1.4). NK cells were thought develop exclusively in bone marrow, but more recent evidence suggests that they may develop and mature in SLTs, such as lymph nodes, and the spleen. In mice, functional maturation is reflected in expression of CD27 and CD11b, progressing from the least mature CD27-/CD11b-, to CD27+/CD11b-, then CD27+/CD11b+, and finally CD27-/CD11b+, which are thought to be most mature. Double positive cells have been demonstrated to have greater effector functions than CD27-/CD11b+ cells, which have high cytolytic function. KLRG1, an inhibitory receptor, is further marker of terminal maturation(69, 76). Analogous to CD27/CD11b in mice, in humans maturity is broadly divided into cells which are $CD56^{bright}CD16-$, and those which are $CD56^{dim}CD16+$. $CD56^{bright}CD16-$ are considered less mature and show greater ability to secrete cytokines, including TNF-α, TNF-β, and GM-CSF, serving in an immunomodulatory role. The $CD56^{dim}CD16+$ phenotype is associated with greater maturity, and greater natural and antibody-dependent cytotoxicity in response to stimulation with IL-12, IL-15, and IL-18(63). $CD56^{dim}$ NK cells may also produce IFN-γ in response to stimulation with IL-2 and/or IL-15(77). $CD56^{dim}CD16+$ cells constitute the majority of NK in the peripheral circulation (>90%), whereas $CD56^{bright}CD16-$ primarily reside in SLTs(69).

Figure 1.3 Stages of NK cell development(69)



1.2.3 Killer-cell immunoglobulin-like receptors

KIRs are a family of inhibitory and activating NK cell receptors, encoded by a region of chromosome 19 called the leucocyte receptor complex. They bind to Human Leukocyte antigen in conjunction with a bound peptide. KIR exhibit significant genetic polymorphism, generating diverse receptor repertoires, with more than 2,000 variants identified, which exhibit variability in function(78). It is generally accepted that fifteen KIR genes, and 2 KIR pseudogenes have been described, though this is inconsistently reported as the pairs KIR2DS1 and 3DL1, and KIR2DL2 and 2DL3 are allelic. Nomenclature is based on protein structure; 'D 'and its preceding number refers to the number of immunoglobulin-like domains in the molecule, 'L' to the presence of a long cytoplasmic tail and inhibitory signalling, and 'S 'to a short cytoplasmic tail and activatory signalling(79). The exception to this is KIR2DL4, which produces an activating signal. KIR haplotypes are divided into 2 major groups; A with predominantly inhibitory composition, and B with a greater variety of activating genes (80).

KIR genotype has been associated with clinical outcome in a variety of diseases. Resolution of, and protection from HCV infection has been associated with KIR2DL3 and its ligand HLA-C1. KIR2DS4,

another component of the A haplotype has been associated with protection from chronic HCV, and conversely B haplotype has been associated with poorer outcomes(80). These findings have been reproduced in HBV, with reduced A haplotype frequency observed in chronic infection, implying a protective effect(81). It has been hypothesised that the protective advantage conferred by inhibitory genes may be related to capacity for NK education. KIRs have been shown to contribute to numerous autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, and type-1 diabetes. Autoimmune hepatitis is significantly correlated with KIR2DS1, KIR3DL1/HLA-B Bw4-80Ile and KIR2DL3/HLA-C, and there are possible protective associations with KIR2DL1/HLA-C2 and KIR3DL1/HLA-B Bw4(82, 83).

Associations have been found between KIR genotype and outcomes of malignancy. Breast cancer has been associated with KIR2DS1 and KIR2DL2/HLA-C1 in separate studies(84). Gastric cancer has been associated with haplotype B(85), and neuroblastoma with KIR2DL2 and KIR2DS2(86). No association has been demonstrated in multiple malignancies including lymphoma, colorectal cancer and melanoma. Conflicting associations have been observed with development and outcome of acute lymphoblastic leukaemia (ALL) over multiple studies. It has been hypothesised that these contradictory results may reflect ethnic variabilities(79). There have been few studies examining the relationship between KIR genotype and HCC development. A study of 170 Japanese patients undergoing hepatectomy found that recurrence was significantly lower in patients with 3 of KIR2DL1-C2, KIR2DL2-C1, KIR3DL1-BW4, or KIR3DL2-A3 than those with 2 or less over a period of 5.4 years(87). A recent cohort of Japanese patients with hepatitis B suggested an association between HCC and KIR2DL3/HLA-Bw4. A cohort of Italian patients with hepatitis C found that reduced KIR3DS1+HLA-Bw4 frequency was associated with development HCC(88).

1.2.4 KIR 2DS2

KIR2DS2 is an activating receptor, which has been associated with outcomes in a variety of viral and malignant conditions. In common with other KIR, prevalence varies greatly amongst populations, presumably reflecting local selection pressures. In the UK approximately 50% of the population have the KIR2DS2 gene, with some regional and ethnic variation; 44% of individuals tested in Oxfordshire were KIR2DS2 positive compared to 55% in the West Midlands(89). Studying function of KIR2DS2 has been hampered by the lack of specific antibodies, and until recently lack of known ligands. Recent study has ascertained that KIR2DS2 recognises highly conserved

flaviviral peptides bound to HLA-C. Flaviviruses include HCV, Dengue, Yellow Fever and Japanese Encephalitis(90). This is consistent with the finding that KIR2DS2 is protective of HCV infection. Positivity has been further associated with reduced risk of relapse and improved survival following cord blood transplantation in patients with leukaemia and lymphoma, and improved survival in lung, breast and colorectal cancers(91-93).

Antibody detection of KIR2DS2 has been challenging, as the 1F12 antibody binds KIR2DS2 and KIR2DL3, so can only detect KIR2DS2 in a 2DS2/2DL2 homozygous genotype, present in 10% of the caucasian population. A KIR2SDS2 high (but not exclusive) population may be defined as KIR2DL3-/CD158b+(94).

Due to the identification of ligands to KIR2DS2, and its clinical importance, it is an attractive therapeutic target for cancer immunotherapy, as NK cells may potentially be activated by presentation of flavivirus peptides, enhancing anti-tumour responses.

1.2.5 NK cells in the liver

The liver contains a high frequency of NK relative to the spleen, peripheral blood and bone marrow, accounting for up to 50% of intrahepatic lymphocytes(77). Liver-resident NK are phenotypically and functionally distinct from those found in other tissues. In mice, liver NK express low levels of markers associated with maturation, predominantly expressing the $CD49a^{+}/DX5^{-}$ phenotype, in contrast to the $CD49a^{-}$/DX5+ phenotype found in circulating NK, and decreased expression of CD11b, Ly49, CD43, and KLRG1(77). Recent evidence suggests that murine liver NK may develop from separate ILP lineages, and have a separate transcriptional profile to conventional NK; $T\text{-}bet^{+}/Eomes^{-}$ NK are enriched in the liver, whereas conventional NK are predominantly $T\text{-}bet^{-}/Eomes^{+}$(95). In humans 50% of liver NK are $CD56^{bright}$, which predominantly reside in the liver sinuoids, and express the activation and residence marker CD69, and chemokine receptors CXCR6 and CCR5. However, this does not define liver residence, as $CD56^{bright}$, CXCR6+ NK are found in both the liver and circulation. The $T\text{-}bet^{-}/Eomes^{+}$, $CD56^{bright}$, CXCR6+ phenotype is thought to represent human liver resident NK cells. Studies of HLA variance in liver transplant patients suggest that this phenotype of liver resident NK cells do not leave the liver, and donor liver NK persist following transplant(96).

Liver resident NK have been shown to exhibit 'memory-like' traits, exerting antigen-specific responses to previously sensitised viruses or haptens in a mechanism independent of T and B cells(97). This was first observed in a hapten induced contact hypersensitivity model in T and B cell deficient mice(98), and has since been demonstrated in viruses such as murine cytomegalovirus (MCMV), herpes simplex virus (HSV), and influenza(99). In humans, expansion of NKG2C+ NK cells has been demonstrated in patients previously infected with cytomegalovirus in response to recipient cytomegalovirus antigen(100). Memory-like functionality has been demonstrated in cytokine stimulated cells; NK cells pre-activated with IL-12/15/18 show enhanced IFN-γ production when restimulated or exposed to target cells, suggesting that memory can be generated by exposure to an inflammatory milieu. Furthermore, memory-like NK may be primed by contact with tumour cells, producing subsequent enhanced anti-tumour activity(97, 101, 102).

There is evidence to suggest that liver NK may play multiple roles, promoting both immune tolerance and immunogenicity in different contexts or when exposed to different stimuli, and interacting with other liver resident cells to moderate hepatic regeneration following damage. Liver resident NK may have higher cytotoxic potential than circulating NK, contain greater levels of perforin and granzymes, and express higher levels of TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL), reflecting the need to respond to the variety of pathogens encountered via the portal circulation. Hepatic NK may promote immune tolerance through inhibition of dendritic cell function via production of transforming growth factor beta (TGF-β) and IL-10, which in turn promotes T-reg expansion. Anti-fibrotic effects may occur through killing of activated HSCs. Production of chemokines such as CXCL7, CXCL2 and CCL5 have a role in recruitment and differentiation of mesenchymal stem cells in hepatic regeneration(77, 103).

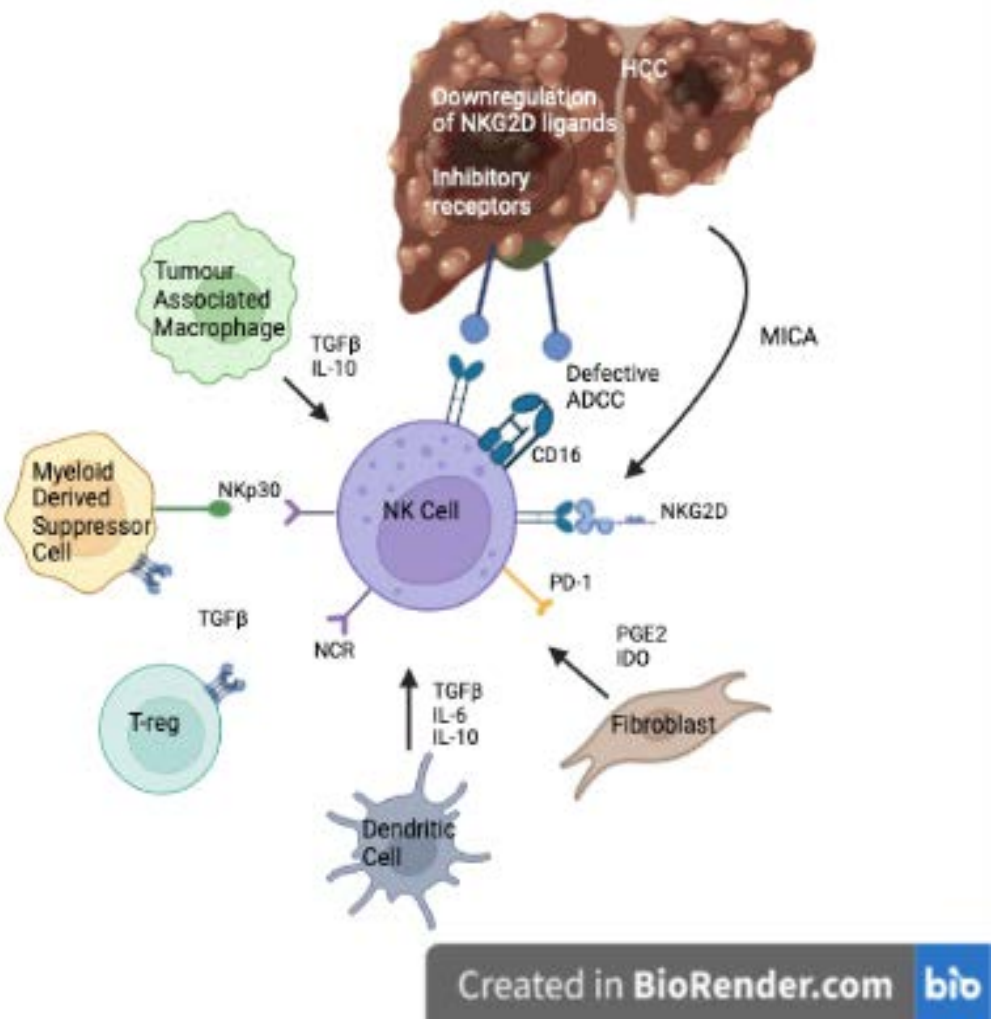
1.2.6 NK cell dysfunction in hepatocellular carcinoma

Numerous studies suggest that NK cell depletion and dysfunction may play an important role in hepatocarcinogenesis. A study of 110 patients with HCC demonstrated reduced peripheral NK frequency versus healthy controls, and a marked reduction in $CD56^{dim}CD16+$ cells, with a resultant increased ratio of $CD56^{bright}$:$CD56^{dim}$, and decreased IFN-γ production, CD107a expression, and cytotoxicity. Analysis of surgical resection specimens and explants revealed reduced

intratumoural NK and decreased cytolytic activity compared to those found in non-tumour hepatic tissue(104). In a cohort of 294 patients undergoing liver resection or transplant, NK were significantly depleted in intratumoural tissue versus chronic hepatitis and healthy controls (marginal tissue from haemangioma resection), and increasing density of NK in resected HCC was found to predict greater survival and slower progression. Depletion in intratumoral NK was found to be predominantly due to reductions in $CD56^{bright}CD16-$ frequency, with an associated decrease in effector functions. Analysis of intratumoural and peripheral NK suggests that tumour-activated monocyte-mediated activation in the peritumoral stroma was associated with NK exhaustion and apoptosis in the intratumoral region(105). Several mechanisms of NK suppression have been proposed, though this is incompletely understood (see Figure 1.5). Recent studies have expanded on the interactions between NK and other actors in the HCC microenvironment, demonstrating several immunosuppressive mechanisms including expansion of T-regs, inhibitory signalling from HCC derived MDSCs(106), HCC-associated fibroblasts, TAMS, and defective dendritic cells(107-109).

A further possible mechanism of HCC immune escape is defective tumour recognition by NK cell receptors. Downregulation of the activating receptor NKG2D has been shown in intratumoural liver resident NK, with impaired cytolytic potential(110), and reducing NKG2D in peripheral NK correlates with risk of developing HCC in patients undergoing treatment for HCV(111). A proposed mechanism for this observation is release of the soluble form of the NKG2D ligand MHC class I-related chain A (MICA) by a variety of cancers including HCC, which inhibits cell surface expression of NKG2D(112). Dysregulation of microRNAs has been implicated in various aspects of NK dysfunction, including receptor downregulation and dysfunction, interruption of signalling pathways and suppression of cytolysis(113).

Figure 1.4 NK cell dysfunction in tumour microenvironment of hepatocellular carcinoma(109)



1.2.7 NK cell cancer immunotherapy

Due to their role in tumour surveillance, the unique immunology of the liver, and their dysfunction in HCC, NK cells are an attractive target for immunotherapy. No therapies are currently in mainstream use, though several strategies are under development.

Infusion of autologous NK cells has been trialled in different forms in pre-clinical and clinical contexts. The first of these to undergo clinical trials was lymphocyte-activated killer (LAK) cells, consisting of IL-2 activated NK, NKT and T-cells. Case reports and series of use in HCC suggested potential, but durable benefit was not established, and toxicity related to IL-2 limited utility. CIK infusion has demonstrated benefit in RCT and meta-analysis, and is discussed in section 1.1.9. Allogenic NK cell infusion has been investigated as an adjuvant to resection or transplantation, to take advantage of KIR/HLA mismatch. Retrospective data has suggested a survival benefit in combination with irreversible electroporation in those with advanced HCC(114). Infusion of allogenic donor liver NK following orthotopic liver transplant has undergone phase I and II trials to attempt to harness the capabilities of liver NK(115).

Monoclonal antibodies (mAb) may be directed against inhibitory NK receptors to boost anti tumour immunity. Anti-PD1 immune checkpoint blockade primarily aims to release T-cell inhibition, however a PD-1+ phenotype of mature NK has been identified, and inhibition may recover NK cytotoxicity(116). Likewise, CTLA-4 is expressed on a population of tumour infiltrating NK. Lirulimab, an anti-KIR 2DL1/2/3 mAb, is the first mAb aiming to specifically block NK inhibition. However, clinical trials in haematological malignancy have been disappointing, as phase 2 trials of monotherapy for AML were terminated due to early relapse(117), and subsequent data has suggested that KIR blockade may have rendered NK hyporesponsive through loss of KIR2D expression via trogocytosis(118), and interrupted NK education(119). Monalizumab, a mAb to NKG2A found on NK and a subset of activated CD8+ T-cells, has completed phase II, and has phase III trials planned for treatment of head and neck cancer(120). A variety of mAb targeting NK receptors are in pre-clinical and early clinical development (see figure 1.5).

Table 1.2 Checkpoint inhibitors influencing NK function (121)

Targeted receptor	Name	Status	Disease
PD-1	Nivolumab, Pembrolizumab, others	Widespread clinical use	Numerous
CTLA-4	Ipilimumab, Tremelimumab, others	Widespread clinical use	Numerous
KIRDL1/2/3	Lirulimab	Phase I, phase II discontinued	AML, CLL, multiple myeloma
NKG2A	Monalizumab	Phase I/II. Phase III planned	Haematological, gynaecological, lung malignancies
TGIT	Tiragolumab	Phase I/II	Solid malignancies, lung malignancies
LAG-3	Relatlimab	Phase I/II	Haematological, solid malignancies, GI malignancy, Glioblastoma
TIM3	Cobolimab	Phase I/II	Melanoma, Solid and Haematological malignancies
CD200	Samalizumab	Phase I	CLL. Solid malignancies
CD47	Magrolimab	Phase I/II	Haematological, solid malignancies, colorectal cancer
B7-H3	Enoblituzumab	Phase II/III	Prostate cancer

Another strategy to enhance NK cytotoxicity is generation of bi- and tri-specific killer engagers (BiKEs and TriKEs) to enhance ADCC by directly binding target cells with CD16. Pre-clinical studies have shown promising results, TriKEs including IL-15 have produced enhanced NK proliferation and persistence versus BiKEs. CD16/IL-15/CD33 TriKE is currently undergoing phase I/II trials for treatment of high-risk haematological malignancy(122-124).

CAR-T have been trialled in patients with haematological malignancies and HCC (see section 1.1.9). CAR-NK are engineered to express activating receptors to specific tumour antigens, including CD19, CD7 and CD33 targeted at lymphoma and leukaemia, and HER2 targeted at breast cancer, glioblastoma multiforme, and renal cell carcinoma. Initial clinical studies have yielded promising results; infusion of cord blood derived, anti-CD19 CAR-NK cells following lymphodepeletion produced a response in 8 of 11 patients with haematological malignancy, with no episodes of serious toxicity(125, 126).

1.3 Exosomes

1.3.1 Introduction

The identification of ligands for KIR2DS2 presents a possible therapeutic target. Exosomes are vesicles which have been proposed as potential vehicles for immunotherapy, and so I chose to investigate exosomes as a means of presenting these ligands to activate NK cells.

1.3.2 Exosome definition and function

Exosomes are extracellular vesicles (EVs) of endosomal origin, secreted by almost all cells under normal physiological conditions. They range from approximately 40-160nm in size, and are formed from sequential invagination of the cell plasma membrane and secretion by exocytosis of multivesicular endosomes. They exhibit significant heterogeneity in size, content and function, potentially containing membrane proteins, cytosolic and nuclear proteins, extra-cellular matrix proteins, metabolites, micro RNA (miR), RNA, and DNA. Their precise function is not completely understood, and remains a topic of debate. It had initially been proposed that exosome production is a mechanism of discarding cellular waste, however recent developments have elucidated their role in cell to cell communication(127, 128).

Exosomes may have a complex architecture, and under normal circumstances their proteomes reflect those of their parent cells. They are typically rich in tetraspanins (CD9, CD63, CD81, CD82), which are involved in cell penetration, heat shock proteins (HSP70, HSP90), and microvesicle formation proteins involved in exosome release (Alix, TSG101). Once released, cell interactions occur between cell receptors and exosomal surface-bound ligands, via direct fusion of cell membranes, macro-pinocytosis, or endocytosis. If internalised they may then deliver molecules which produce receptor activation, or deliver proteins, lipids, and genetic material, which may produce reprogramming of target cells. It is not known whether different methods of interaction produce altered localisation, degradation, or function of exosome constituents. There is some evidence to suggest that production and uptake of exosomes may vary with cellular stress or inflammation(127, 128).

Recent experiments have suggested a potential role in regulation and co-ordination of responses to infection and cancer. During infection or tumour growth exosomes may have a role in presentation and transfer of antigenic peptides, and delivery of cyclic GMP-AMP synthase stimulator of interferon genes (cGAS-STING) signalling, which induces inflammatory genes, and promotes IFN production. Exosomes from antigen-presenting cells carry MHC II with antigenic and co-stimulatory peptides to T-cells, inducing activation. Conversely, bacteria and parasite-derived exosomes may be involved in propagating infection, and enhancing parasite survival. It has been hypothesised that viruses may utilise exosomes as a vehicle to spread virus and viral components. Tumour-derived exosomes are thought to have a role in cancer growth, metastasis, paraneoplastic events, and resistance to therapy. Expression of PDL-1 and FasL on melanoma derived exosomes has been found to suppress T-cell function in vivo, and block dendritic cell maturation and migration. PDL-1+ exosomes promote tumour growth in mice(129, 130). Uptake of exosomal tumour DNA by neutrophils may result in tissue factor and IL-8 production, leading to inflammation and thrombosis. Exosomal miR may initiate oncogenic mutation, and has been implicated in downregulation of dendritic cell function and promotion of M2 macrophage polarisation, contributing to an immunosupressive tumour microenvironment, and reduced anti-tumour immunity(127, 128, 131).

In view of the ubiquity and content of exosomes, they have been proposed as potential diagnostic and prognostic tools. Elevated exosomal GPC has been associated with pancreatic cancer, and miR signatures have been associated with a variety of cancer types, including oesophageal, colon, pancreatic and breast cancer. Assessing multiple miR clusters, and exosome protein content may enable more accurate and focussed diagnosis(128, 132).

1.3.3 Therapeutic use of exosomes against cancer

The ability of exosomes to deliver ligands, and to enter cells to deliver a functional cargo without the toxicity associated with cell therapy makes them potential therapeutic agents.

Cytotoxic chemotherapy may be loaded into exosomes by electroporation and/or sonication in a suspension of the desired drug. Direct uptake of exosomes may increase dose to targeted cells,

while minimising systemic toxicity. Macrophage-derived exosomes loaded with paclitaxcel have been found to preferentially accumulate within cancerous cells in a murine lung cancer model, and doxorubicin loaded dendritic cell exosomes engineered with an integrin-specific peptide to promote targeting localised to diseased cells in a murine breast cancer model(133, 134).

Dendritic cell-derived exosomes (DEX) express functional MHC complexes, antigens, and other co-stimulatory molecules. DEX have undergone multiple phase I, and a recent phase II trial of IFN-γ DEX as maintenance following chemotherapy for non-small cell lung cancer. This has demonstrated the feasibility and tolerability of the technique, but failed to meet the primary clinical endpoint of progression free survival (PFS) in 50% of patients at 4 months, reaching 32% PFS, with no objective tumour response. Expected induction of antigen-specific T-cell responses failed to occur, but NKp30-dependent NK cell activation was demonstrated(135, 136).

Exosomes may be used to alter gene expression in recipient cells. Micro RNA content can be engineered by transfecting parent cells, and purifying exosomes from culture supernatant. This enables delivery of miR to target cells, and protects them from circulating ribonucleases. Engineering of surface peptides can enhance targeting Successful delivery of exosomal miR to EGFR has been achieved in a xenograft murine breast cancer model, and in a rat model of glioma, where exosome therapy led to a significant reduction in tumour growth(137, 138). A phase I clinical trial is currently in progress of mesenchymal stem cell-derived exosomes loaded with small interference RNA (siRNA) against KrasG12D in patients with metastatic pancreatic cancer with KrasG12D mutation(128).

1.3.4 Exosome isolation

There has been a rapid increase in the study of exosomes from a broad array of cell types and bodily fluids, and several isolation and characterization methods have been described. However, composition of isolates may vary according to extraction method, and the extracellular milieu may include a range of extracellular vesicles, and extracellular RNA-protein complexes, which may remain in isolates and influence experimental results. Consequently the International Society for Extracellular Vesicles have published minimum experimental requirements for the definition of extracellular vesicles. These propose analysing the general protein content of isolates compared

with the secreting cells, demonstrating enrichment of exosome markers, and the absence intracellular proteins. This is performed by Western blotting, mass spectroscopy, or flow cytometry. Single vesicles are characterised using 2 methods: Heterogeneity in size of isolated vesicles is assesed through nanoparticle tracking analysis, dynamic light scattering, or resistive pulse sensing. Close-up and wide field electron microscopy or atomic force microscopy should be used to demonstrate membrane-bound vesicles(139). Due to the evolving nature of this field, a database has been created to record methodological specifications used in research and aid standardisation and quality assessment(140).

The most common method of isolation is differential ultracentrifugation. Samples are sequentially centrifuged; at 500G to pellet cultured cells, 1000G for cellular debris, 10,000G for apoptotic bodies and microparticles, before ultracentrifuging at 100,000G to pellet exosomes. These steps are time-consuming, impractical for small samples, and due to variability or overlap in particle size cannot produce entirely pure exosome preparations. However, relatively large sample volumes can be processed, and RNA and protein composition of extracted exosomes is not significantly affected. A refinement of this protocol is density gradient centrifugation, which uses a gradient medium within ultracentrifuge tubes before ultracentrifuging at 210,000G for 16hrs. This results in greater exosome purity, at the expense of lower yield, greater expense and complexity, and longer extraction. Various exosome extraction kits are available. Immunomagnetic beads coated with antibodies against exosome receptor molecules such as CD9, CD63 and CD81 can be used to extract specific populations of exosomes, however with low yield, and the potential for impaired function of extracted exosomes. Compound precipitation methods capture vesicles according to size (commonly 60-180nm diameter), and require only basic equipment. However, this technique requires expensive reagents, and cannot discriminate exosomes from contaminants such as microvesicles and protein complexes. Size-exclusion chromatography separates vesicles by size by filtration through a gel, which may produce exosomes with relatively low isolation times, but also cannot discriminate exosomes from particles of a similar size (127, 141-143). This is an evolving field, so further developments are anticipated.

1.4 Summary

The incidence of hepatocellular carcinoma is rising, reflecting increasing prevalence of chronic liver disease. Prognosis is poor and systemic therapies are currently limited. In common with other cancer types, immunotherapies have recently entered clinical practice, and are under development. NK cells are innate lymphocytes, which play an important role in tumour surveillance. The liver contains a high frequency of NK cells compared to other organs, and liver-resident NK have unique phenotypic and functional characteristics. Hepatocellular carcinoma develops in the context of an immunosuppressive microenvironment which produces NK dysfunction. NK cell function is regulated by the balance of activating and inhibitory receptors, an important constituent of which is the KIR family. Flavivirus peptides bound to HLA-C have recently been identified as ligands for the activating KIR2DS2 receptor, which may be a potential therapeutic pathway. Exosomes are extracellular vesicles which have been proposed as vehicles for immunotherapy. Exosomes produced from cells expressing these ligands for KIR2DS2 may enhance NK cell activation, and so enhance killing of cancer cells.

1.5 Hypothesis and aims

1.5.1 Hypothesis

1. KIR2DS2 positivity reduces risk of developing hepatocellular carcinoma.

2. KIR2DS2 can be activated by exosomes expressing flavivirus peptides bound to HLA-C, to producing enhanced killing of cancer cells.

1.5.2 Aims

1. I will examine the relationship between KIR genotype and development of HCC in a cohort of patients with hepatitis C.

2. I aim to isolate exosomes from a cell line transfected to express HLA-C with the DP1 flavivirus peptide.

3. I aim to examine the phenotypic and functional effect of these exosomes on NK cells. This will be achieved via in-vitro and in-vivo experiments examining NK cell proliferation and phenotype, NK cell degranulation, and killing of hepatocellular carcinoma cell lines.

Chapter 2 Materials and methods

2.1 Retrospective data collection

A retrospective study was conducted examining the relationship between NK KIR genotype, and hepatocellular carcinoma occurrence, stage, and survival. Data were collected from a cohort of patients KIR genotyped as a part of a previous study. Patients with hepatitis C had been recruited and the presence or absence of ten KIR genes (KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1) determined by PCR. The cohort consisted of 169 patients referred to the hepatology clinic University Hospital Southampton NHS Trust, UK. Electronic medical records were accessed, and data collected for hepatocellular carcinoma incidence, Barcelona Clinic Liver Cancer stage, treatment received, comorbidites, and survival.

2.2 721.221 cell culture

MHC class 1 deficient 721.221 lymphoblastoid EBV-B cells, and 721.221 cells transduced with pIB2 constructs to express HLA C*0102 alone or -IVDLMCHATF (C*0102-DP1) were provided by colleagues. Cell culture was performed by myself. Cells were cultured in R10 medium (RPMI 1640 with 10% heat inactivated foetal bovine serum (FBS, Sigma) and 1% penicillin + streptomycin (Life technologies)) selected with blasticidine. They were routinely tested for mycoplasma contamination. Cells were routinely checked for HLA-C expression by flow cytometry following staining with DT-9 antibody. Cells were kept at 37ºC, 5%CO_2, in a humidified atmosphere. Cells were passaged and re-seeded in fresh media at standardised intervals.

2.3 Isolation of extracellular vesicles from 721.221 cells

721.221 / 721.221-HLA C*0102 / 721.221-HLA C*0102-DP1 cells were cultured in T175 flasks in 50mls exosome-depleted R10 medium (produced with exosome deplete FBS - the supernatant of centrifugation FBS at 100,000g for 16hrs), lying flat. Cells were counted using an improved Neubauer haemotocytometer, using 10µL of media diluted with 10µL of trypan blue. Exosome extraction was carried out once a concentration of $1x10^6$ cells per millilitre was achieved. The medium was then transferred to 50mL Falcon tubes, and centrifuged at 500g for 5mins to pellet floating cells, followed by 2000g for 10mins to pellet debris. The supernatant was then transferred to fresh 50mL Falcon tubes and centrifuged at 10,000g for 30mins to pellet apoptotic bodies and 1µm particles. The ultracentrifuge rota was pre-chilled at 4ºC. Supernatant was then transferred to ultracentrifuge tubes (Beckman Coulter; each 50mls Falcon divided between 3 tubes) which had been marked on the radial aspect, and balanced to within 0.1g, and then ultracentrifuged at 100,000g for 75mins at 4ºC to pellet extracellular vesicles. Supernatant was then aspirated from the tubes and discarded, leaving 1ml at the bottom of the tube. The pellet (not visible) was then resuspended, and pooled into fewer centrifuge tubes (3 ultracentrifuge tubes pooled into 1). 2µL DIO lipophilic dye (Invitrogen) was added per tube, and incubated at 37ºC for 22mins. Tubes were then filled to capacity with PBS and balanced, and ultracentrifuged again at 100,000g for 75mins at 4ºC. All supernatant was then aspirated, and the dyed pellet resuspended from the radial aspect of the tube in PBS (endotoxin free PBS for in-vivo experiments), or ultrapure water for electron microscopy. Sample protein was estimated using a BCA protein assay on a NanoDrop™ One Spectrophotometer (Thermo Fisher). Samples were stored at 4ºC prior to use for up to 7 days. Exosome isolation was carried out by myself.

2.4 Transmission electron microscopy

Transmission electron microscopy (TEM) was carried out at with a Hitachi HT7700 Transmission Electron Microscope at the Biomedical Imaging Unit in Southampton General Hospital with negative staining. This was performed by myself under the supervision of staff at the Biomedical Imaging unit.

2.5 Nanoparticle tracking analysis

Nanoparticle tracking analysis was carried out using a NanoSight™ instrument at a 1:5000 dilution at the Institute of Cancer Research, University of Ghent, Belgium, by Joeri Tulkens.

2.6 Western blotting

Isolated exosomes and parent cells were lysed using sonication. Protein content was determined by Bradford protein assay. Stacking gels were prepared and overlaid with separating gel and allowed to set. 50µg of sample was added per well with 5µL running buffer, and volume equalised with ultrapure water. These were then placed in an electroporator with running buffer, and samples added to wells. The electroporator was then connected and run at 60V-140V for approximately 1 hour. The gel was then removed and sandwiched as follows: sponge, 3 filter papers (wet with transfer buffer), gel, polyvinylidene fluoride (PDVF) blotting membrane (wet with methanol), 3 filter papers (wet with transfer buffer). This was placed in transfer buffer and transferred for 90 minutes. The membrane was then blocked in 5% milk in TBST for 1 hour. Antibodies were then added in 5% BSA and placed overnight on a shaker. The membrane was then washed in TBST 3 times, secondary antibodies were added in 5% milk in TBST, and it was then washed 3 further times in TBST. The membrane was then incubated for 2 minutes in ECL (Thermo Fisher), before developing onto film in a dark room(144). Western blotting was performed by myself.

2.7 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers. Approximately 50mls of blood was drawn from each volunteer, using a Vacutainer (BD), into EDTA tubes. Blood was diluted 1:1 with PBS. 15mL of Ficoll density gradient was pipetted into 50mL Falcon tubes, and 35mL blood/PBS layered on top. This was then centrifuged at 2,000rpm for 30mins at 20ºC with no break or acceleration. PBMCs were then harvested with a Pasteur pipette, and washed & centrifuged 3 times in PBS (first wash in 50ml PBS 1800rpm for 10mins, second wash in 25ml PBS 1500rpm 10mins, third wash 10ml PBS 1200rpm 5mins). Cells were

resuspended in PBS, and 10µL were then diluted in 10µL trypan blue, and counted using an improved Neubauer haemotocytometer. Cells were centrifuged at 1,200rpm for 5 mins, and resuspended in FBS 10% dimethylsulphoxide (DMSO), and frozen at -80ºC for 24hours, prior to storage in liquid nitrogen. Cells were thawed, washed, and cultured overnight in R10 media prior to use. Venesection, cell isolation and all experiments using these cells were performed and analysed by myself.

2.8 Human NK cell surface staining for flow cytometry

Cells were washed with 150µL FACS washing buffer (1% Bovine Serum Albumin (BSA), 0.1% Sodium Azide in PBS) and centrifuged at 1200rpm for 5mins. Supernatant was discarded, and cells resuspended in 100µL blocking buffer (FACS washing buffer + 10% human serum), and incubated at 4ºC for 30mins. Cells were then washed with 150µL FACS washing buffer and centrifuged at 1200rpm for 5mins twice, prior to staining with 50µL surface antibody solution, and incubated at 4ºC for 30mins in darkness. Antibody panels are shown in table 2.1. Cells were washed twice more, and resuspended in 200µL FACS washing buffer, or fixing buffer (FACS washing buffer, 1% paraformaldehyde) if analysis not being performed immediately. Analysis was performed using a 2 laser Accuri C6 flow cytometer (BD), or a 3 laser FACS Aria (BD) flow cytometer. Cytometers were calibrated according to guidelines prior to use, and compensation was calculated using single staining. Gates were set using single staining controls. Data were analysed using FlowJo version 10.6.1 (Beckton Dickinson).

Table 2.1: Antibodies used for human NK cell surface staining

Antibody	Flurochrome	Purpose	Concentration	Company	Item Code
CD3	PerCP	NK cell identification	1:200	Biolegend	300428
CD56	PE-Cy7	NK cell identification	1:25	Biolegend	318318
CD158b	PE	2DS2 NK cell identification	1:25	BD	559785
KIR 2DL3	FITC	2DS2 NK cell identification	1:10	Miltenyi	130-100-125
CD107a	eFlour 660	Activation	1:350	Invitrogen	50-1079-42

2.9 Human NK cell proliferation assay

Thawed PBMC were counted, and $0.25x10^6$ cells per well seeded in 96 well U-bottom plate in 200µL R10 with varying concentrations of IL-15. These were then co-cultured with exosomes derived from 721.221 / 721.221-HLA C*0102 / 721.221-HLA C*0102-DP1 cells. Media and IL-15 was changed on day 3, and day 5 if incubated for 7 days. For analysis cells were stained with PE-CD158b, FITC-KIR2DL3, PE-Cy7-CD56, BV510-CD3, PerCP-NKG2A, allowing for identification of total NK cells, and a 2DS2 positive-high population of NK cells. Cells were acquired on a FACS Aria cytometer.

2.10 Human NK CD107a functional assay

A CD107a functional assay was performed, either with exosome stimulated PBMCs alone, or with exosome stimulated PBMCs with a PLC HCC tumour cell line target. PBMC were thawed and $0.25x10^6$ per well seeded in a 96 well U-bottom plate in 200µL R10 + 1ng/mL IL-15 overnight. Varying concentrations of exosomes suspended in PBS were then added, and co-cultured for between 4 and 48 hours. If PLC tumour model targets were used, $0.6x10^5$ cells per well were seeded in a 96 well U-bottom plate the day prior to analysis. Effector PBMCs were centrifuged and stained with APC-CD107a antibody in 50µL R10 medium. If tumour model targets were used effector cells were then resuspended and added to the plate containing the PLC targets. No target, no CD107a, and single staining controls were included. The cells were then incubated at 37ºC for 1 hour. Following this, 6µg/mL GolgiStop diluted in R10 was added to each well, and incubated at 37ºC for a further 3 hours. Cells were then resuspended, and transferred to a 96 well V-bottom plate, washed with FACS washing buffer, and centrifuged at 1500rpm for 5 mins prior to staining with PE-CD158b, FITC-KIR2DL3, PE-Cy7-CD56, BV510-CD3, PerCP-NKG2A. Cells were acquired on a FACS Aria cytometer.

2.11 Human hepatocellular carcinoma (HCC) cell lines

HCC cell lines were provided by colleagues, and cultured in R10 or D10 (Dulbecco's Modified Eagle's Medium (DMEM) with 10% heat inactivated FBS (Sigma) and 1% penicillin + streptomycin (Life technologies)). All cell lines used were adherent. When passaging, media was discarded, and the adherent cells washed with 10mL PBS, then incubated at 37ºC with Trypsin (1.5mL for a T25 flask; Sigma) for 3 minutes. Following this they were washed in PBS and centrifuged at 1200rpm for 5 mins prior to seeding. They were routinely tested for mycoplasma contamination. Details of cell lines and media used can be found in table 2.2.

Table 2.2. Human HCC cell lines

Cell line	Origin	Culture medium
Hep G2	15 year old Caucasian male. Hepatoblastoma.	D10
PLC	24 year old African male.	D10
SNU 387	41 year old Asian female. Hepatitis B.	R10
SNU 398	42 year old Asian male. Hepatitis B.	R10
SNU 423	40 year old Asian male. Hepatitis B.	R10
SNU 475	43 year old Asian male. Hepatitis B.	R10

2.12 Hepatocellular carcinoma cell line killing assay

This experiment measured killing of HCC cells by exosome stimulated PBMCs. PBMC effectors were co-cultured with exosomes in R10 medium + 2ng/mL IL-15 for 48 hours. Target cells as table 2.2 were counted, and $0.6x10^5$ per well were seeded in a 96 well U-bottom plate overnight. Targets were stained with 1µM CellTrace far red DDAO-SE (Thermo Fisher), and incubated at 37ºC for 20mins in the dark, then centrifuged at 1500rpm for 5mins and washed in fresh media twice. Pellets were then resuspended in R10, added to target PLC cells, and incubated at 37ºC for a further 4 hours. Following co-incubation cells were centrifuged at 1500rpm for 5 mins and washed, then stained with Live/Dead (Thermo fisher; vial A suspended in 50µL vial B (DMSO), diluted 1:1000 in PBS, 100µL per well), and incubated in the dark for 30 mins at room temperature. The plate was then centrifuged at 1500rpm for 5 mins, and washed with PBS twice, then resuspended in 100µL PBS + 1% PFA for analysis on an Accuri C6 cytometer.

2.13 Mouse models

Mice were bred in a closed research facility, with pathogen-free individually ventilated cages at the University of Southampton Pre-Clinical Unit (PCU), with approval by the University of Southampton ethics committee. Experiments were conducted under UK home office project licences (PPL) P4D9C89EA and PP2322487, and personal licences (PIL) IF2588E81, ID8D30212, and I96F6BEAA. Homozygote KIR transgenic mice expressing a complete human KIR B haplotype, on a C57BL6 background MHC class I–deficient Kb-/- Db-/- were used. These were originally provided by J Van Bergen(145). Mice were used in experiments aged between 8 and 14 weeks, and were randomly assigned to experimental groups prior to experiments.

Mice were injected with exosomes suspended in endotoxin-free PBS via tail veins, following a period of warming. For tumour experiments, an RMA-S murine lymphoma cell line was used, as the mouse model did not accommodate a hepatocellular carcinoma cell line. The left flank of the mouse was shaved, and $1x10^6$ cells injected subcutaneously. Once tumour was visible, mice were checked daily. Tumour dimensions were measured with callipers, and weight recorded. The measurer was blinded to the experimental group of the mouse by an assistant who held mouse identifiable data, and recorded results.

At the conclusion of experiments, or at the predetermined humane endpoint, mice were euthanised using a rising concentration of carbon dioxide (as schedule 1 of the Animals (Scientific Procedures) Act), and the spleen, liver, and tumour (if present) extracted for analysis. Injection and routine checks were performed by PCU staff. Tumour measurement, welfare checks, sacrifice, dissection, experimental protocols and analysis was performed by myself.

2.14 Isolation of murine splenocytes

Following sacrifice, spleens were dissected and kept in sterile PBS. For extraction of splenocytes they were placed in a 70uM cell strainer (GBO) with 10mL RPMI medium in a petri dish, and pressed through the strainer with the plunger of a 10mL disposable syringe (BD). The cell suspension was then transferred to a 50mL Falcon tube, and the petri dish rinsed with RPMI, and

added to falcon to fill up to 50mL. The Falcon was then centrifuged at 1800rpm for 5mins, and the supernatant discarded. 2mL per spleen of ACK lysing buffer (Thermo Fisher) was then added for 5mins, keeping under suspension. This was then neutralised with 50mL RPMI, and centrifuged agin at 1800rpm for 5 mins. The supernatant was discarded, and the cells resuspended in R10 + β-mercaptoethanol. Cells were then counted, and either seeded for use, or centrifuged and resuspended in FBS 10%DMSO, for freezing at -80ºC prior to transfer to liquid nitrogen.

2.15 Isolation of murine liver lymphocytes

Following sacrifice, livers were dissected and kept in sterile PBS. They were then cut into small pieces in a 60mm petri dish with scissors, and placed in a 70uM cell strainer with 3mL PBS, and pressed through the cell strainer with the plunger of a 10mL disposable syringe. The suspension was then filtered through another 70uM cell strainer into a 50mL Falcon tube. The petri dish was washed with 10mL PBS, and filtered through the cell strainer into the same tube. 10mL of Percoll density gradient media (Sigma) kept at 4ºC, and 300µL (300i.u) Heparin were added per tube. This was then centrifuged for 20 mins at 2,000rpm with break off. The parenchymal cells and supernatant was then discarded, and the pellet washed in PBS, and transferred to a fresh tube, before centrifuging again at 1,500rpm for 5 mins. The supernatant was discarded, and the pellet resuspended in 5mL ACK lysing buffer for 5 mins, keeping under suspension. This was then neutralised with 25mL of RPMI, and centrifuged for 5 mins at 1,500rpm. Cells were then resuspended in R10 + β-mercaptoethanol, and counted for staining and analysis.

2.16 Murine NK cell surface staining for flow cytometry

Cells were counted, and seeded in a 96 well V-bottom plate. Cells were centrifuged at 1,500rpm for 5 mins, and blocked with TruStain fcX (Biolegend; 2uL block per 10^6 cells in 100uL PBS), and incubated for 10 mins on ice. Following this they were washed with FACS wash, and centrifuged at 1,500rpm for 5 mins, and stained with 50µL of surface antibody cocktail (see table 2.3 for antibody panel), and incubated at 4ºC for 30mins. The 1F12-FITC antibody used to selectively stain KIR2DS2 was provided by Dr P. Rettman(146). After staining cells were washed with 150µL of FACS wash, and centrifuged at 1,500rpm for 5 mins. They were then resuspended in 200µL of FACS wash for analysis on a FACS Aria II cytometer. Cytometers were calibrated according to

guidelines prior to use, and compensation was calculated using single staining. Gates were set using single staining controls. Data were analysed using FlowJo version 10.6.1.

Table 2.3: Antibodies used for murine NK cell surface staining

Antibody	Flurochrome	Purpose	Concentration	Company	Item Code
CD3	PE	NK cell identification	1:200	Biolegend	100308
NK 1.1	BV421	NK cell identification	1:50	Biolegend	108741
1F12	FITC	2DS2 NK cell identification	1:25	Provided by Dr P. Rettman	N/A
KLRG1	PECy7	Maturity	1:200	Biolegend	138416
CXCR6	APC	Liver residence / memory	1:50	Biolegend	151106
CD49a	PerCP-Cy5.5	Memory	1:50	BD	564862
CD69	PerCP	Activation	1:50	Biolegend	104507
CD107a	AF647	Activation	1:50	Biolegend	121610
CD11b	APC-Cy7	Maturity	1:200	Biolegend	101226
CD27	BV510	Maturity	1:50	Biolegend	124229

2.17 CD107a assay from exosome exposed murine splenocytes

To measure NK cell degranulation, CD107a expression was measured. Following isolation of splenocytes, cells were counted, and $3x10^5$ cells seeded per well in a 96 well U-bottom plate in 50µL R10 + β-mercaptoethanol. PLC target cells were counted and $0.6x10^5$ (effector:target ratio of 5:1) seeded in a separate 96 well U-bottom plate in 50µL D10 medium, and cultured overnight. On day 2 the splenocyte plate was centrifuged at 1500rpm for 5 mins, and the supernatant discarded. Splenocytes were then resuspended in 50µL of CD107a-AF647 antibody in R10 media (1:50 concentration), added to the PLC target plate, and incubated for 1 hour at 37ºC. Following this 7µL of GolgiStop solution (1:100 concentration, diluted in R10) was added, the wells resuspended, and cultured for a further 4 hours at 37ºC. After incubation the cells were transferred into a 96 well V-bottom plate, washed in washing buffer, and centrifuged at 1,500rpm for 5 mins. Cells were then centrifuged at 1,500rpm for 5 mins, and blocked with TruStain fcX, and incubated for 10 mins on ice. They were then washed, centrifuged, and resuspended in 50µL of an antibody cocktail of CD3-PE, NK1.1-BV421, 1F12-FITC, before being incubated at 4ºC for 30 mins in the dark. They were then washed in washing buffer and centrifuged at 1,500rpm for 5 mins twice, after which they were resuspended in PBS 1% PFA for acquisition on a FACS Aria II cytometer. No target, no CD107a, and single staining controls were included.

2.18 Histology from RMA-S murine lymphoma cell line in exosome vaccinated mice

Following sacrifice tumours were removed and embedded in paraffin. Paraffin sections were then cut to 5µm thickness with a microtome. Cut sections were removed from the microtome and placed in a 45ºC water bath for 30-60 seconds, before being placed on a slide and dried overnight in a drying oven at 37ºC. Slides were then de-waxed with Tissue-Clear (2x10 minutes), followed by washing with ethanol twice, and 70% ethanol once. 0.5% hydrogen peroxide in methanol was then added, followed by washing with TBS 3 times. Slides were then placed in tubs with 330µL of 0.01M citrate buffer and microwaved at 50% power for 25 minutes. Slides were then washed in cold water for 2 minutes, and washed in TBS for 2 x 5 minutes. Avidin solution was then applied for 30 minutes, washed 3 times in TBS, then Biotin solution applied for 30 minutes, washed again 3 times in TBS, and blocking buffer applied for 30 minutes. 200µL of 1:200 Anti-NCR1 antibody (Abcam ab214468) was applied and incubated overnight at 4ºC. The next day slides were washed in TBS 3 times, and 1:800 biotinylated goat anti-rabbit secondary antibody added for 30 minutes.

After washing 3 times in TBS a solution of TBS, Avidin and Biotin at 75:1:1 dilution was added for 30 minutes, and washed 3 times in TBS. DAB substrate was then applied for 5 mins and washed 3 times in TBS. Slides were then washed in ethanol 3 times, followed by Tissue Clear for 3 minutes, after which cover slips were applied. Sacrifice and dissection was performed by myself, histology was performed by, or under the supervision of staff at the University of Southampton.

2.19 Statistical analysis

Graphs and statistical analysis for experimental data were performed on GraphPad Prism version 8. Retrospective clinical data were analysed using SPSS statistics version 26 (IBM).

2.20 Ethical approval

Ethical approval was granted by the Research Ethics Committee for Wales, REC reference: 13/WA/0329.

Chapter 3 Effect of killer immunoglobulin-like receptor genotype on cirrhosis and HCC development in patients with Hepatitis C

3.1 Introduction

In this chapter I will address my first research aim by investigating the relationship between KIR genotype and development of HCC in a cohort of patients with hepatitis C. A summary of the relevant literature can be found in chapter 1, 1.1 and 1.2. Methods can be found in chapter 2, 2.1.

As discussed in Chapter 1, NK activity is modulated by inhibitory and activating receptors. Killer immunoglobulin-like receptors (KIR) are a family of receptors, the composition of which varies significantly between individuals. The nomenclature describes protein structure, which dictates the signalling produced by receptor activation; 'L' refers to a long cytoplasmic tail and inhibitory signalling, and 'S' a short cytoplasmic tail and activatory signalling. The exception is KIR2DL4 which is activatory. KIR genotype may be grouped into haplotype A (predominantly activating genes) and haplotype B (predominantly inhibitory genes)[147]. Variability in KIR genotype has been investigated a risk factor for a variety of infectious, malignant, and autoimmune diseases[80,85,87].

Hepatitis C is a leading cause of hepatocellular carcinoma worldwide. A variety of KIR genes have been associated with outcomes of infection in various contexts and ethnicities. KIR2DS3 positivity and KIR2DL2 absence have been associated with development of chronic infection in studies including Italian and Spainish patients [88,148]. KIR2DS4/KIR2DS1/KIR2DL1 rs35440472-A and HLA-C rs1130838-A were identified as conferring greater susceptibility to HCV infection in a Chinese cohort[149]. Resolution of infection was shown to be influenced by KIR2DL3-HLA-C1 in a study of patients from the UK and USA[150].

3.2 Study design

One hundred and sixty-nine patients referred to hepatology clinic with hepatitis C at a single large UK centre were recruited and genotyped for the presence or absence of KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5 and KIR3DS1 by PCR. Participants were followed up routinely in a viral hepatitis or general hepatology clinic. Patients with cirrhosis underwent 6 monthly HCC surveillance with abdominal ultrasound. Retrospective data were collected for patient demographics, co-morbidities, risk factors for liver disease (alcohol misuse, diabetes and HBV infection) and clinical outcomes including HCV genotype, HCV clearance, presence of cirrhosis, HCC, and HCC treatment, and survival over a follow-up period of greater than 10 years.

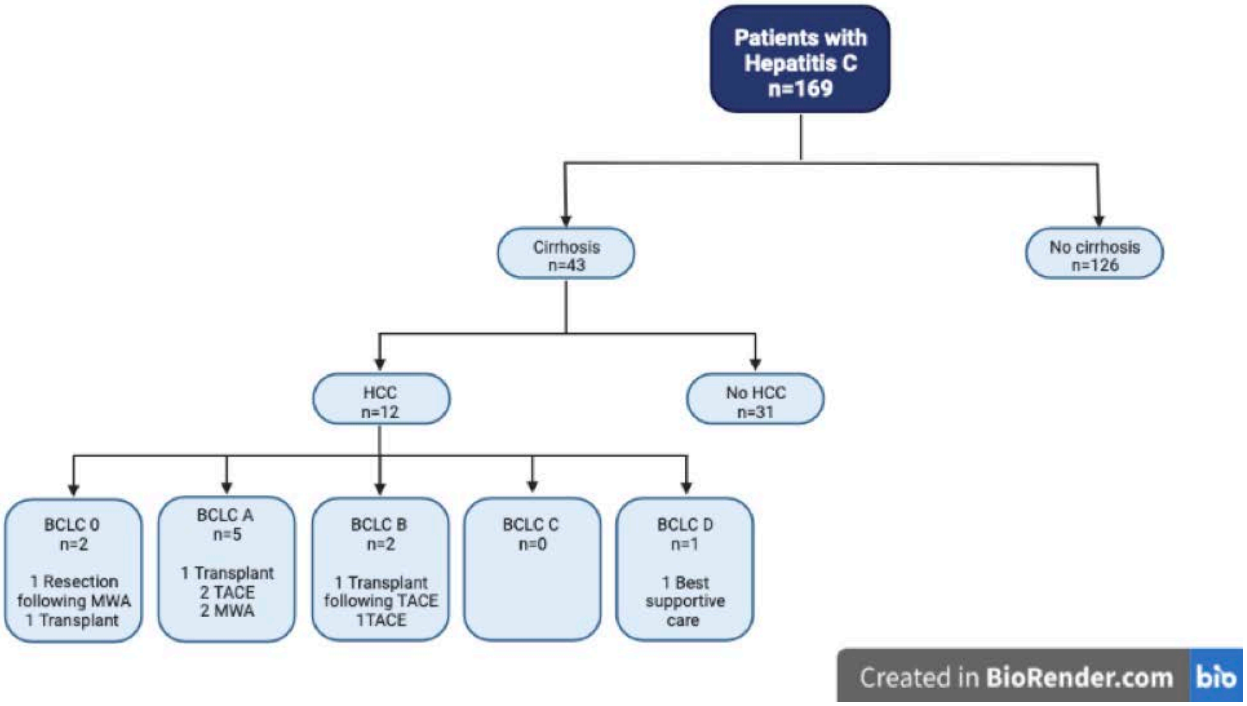
Table 3.1 Characteristics of study population

Diagnosis	N (%)	Median age (IQR)	Male/Female (%Male)	Alcohol misuse (%)	Diabetes (%)	HBV (%)
No cirrhosis	126 (75%)	61 (52.5-67)	87/39 (69%)	5 (4.0%)	2 (1.6%)	1 (0.8%)
Cirrhosis	43 (25%)	65 (60.8-69.3)	34/9 (79%)	4 (9.3%)	2 (4.7%)	1 (2.3%)
HCC	12 (7%)	65 (61-69)	11/1 (92%)	0	1 (9.1%)	0

The study population in all diagnosis groups was predominantly male. Median age was similar between groups. 43 patients (25%) in the cohort had a diagnosis of cirrhosis. 12 patients (7%) were diagnosed with HCC, all of whom had a diagnosis cirrhosis. At diagnosis 2 (17%) were BCLC stage 0 at diagnosis, 5 BCLC A (42%), 2 BCLC B (17%), 1 BCLC D (8%). 3 patients received a liver transplant, 1 underwent surgical resection, and 3 microwave ablation. 4 patients received TACE, 1 patient received Sorafenib, and 1 patient received palliative care only. Therefore, this group contained a greater proportion of patients with HCC presenting with limited disease than might

be expected compared to national data. Other major risk factors for liver disease including alcohol misuse, diabetes, and hepatitis B were uncommon, and it is therefore possible that they reflect a less co-morbid population, or these factors may have been underreported in patient records. Insufficient data were available to calculate further risk factors for HCC such as Child-Pugh-Turcotte score.

Figure 3.1 Patient outcomes



3.3 Statistical analysis

The distribution of KIR genes within the study population can be seen in figure 3.2. No patients expressing KIR2DS3 developed HCC (p=0.02), and the presence of KIR2DS4 was associated with reduced risk of HCC diagnosis (p=0.04, OR=0.25). There was an apparent trend towards KIR2DL2 or KIR2DS2 presence and reduced risk of cirrhosis, neither of which reached significance. BB haplotype was relatively uncommon in this cohort, with AB the most common haplotype in all diagnosis groups. AB haplotype was associated with reduced risk of cirrhosis (OR=0.46, p=0.03), and BB haplotype with increased risk of HCC (OR=4.42, p=0.03).

Table 3.2 Frequency of KIR genes in patients with and without a diagnosis of cirrhosis and HCC.

Gene	No cirrhosis (N=126)	Cirrhosis (N=43)			HCC (N=12)		
	N (%)	N (%)	Chi-squared (p)	OR (95% CI)	N (%)	Chi-squared (p)	OR (95% CI)
KIR2DL1	124 (98%)	41 (95%)	1.30 (0.25)	0.33 (0.05-2.42)	12 (100%)	0.31 (0.58)	N/A
KIR2DL2	74 (59%)	18 (42%)	3.71 (0.06)	0.51 (0.25-1.02)	6 (50%)	0.10 (0.75)	0.83 (0.26-2.67)
KIR2DL3	111 (88%)	37 (86%)	0.12 (0.73)	0.83 (0.30-2.31)	10 (83%)	0.21 (0.64)	0.69 (0.14-3.83)
KIR3DL1	118 (94%)	38 (88%)	1.26 (0.26)	0.52 (0.16-1.70)	10 (83%)	1.47 (0.23)	0.38 (0.73-1.94)
KIR2DS1	52 (41%)	15 (35%)	0.55 (0.46)	0.76 (0.37-1.57)	4 (33%)	0.22 (0.64)	0.75 (0.22-2.58)
KIR2DS2	77 (61%)	19 (44%)	3.74 (0.053)	0.50 (0.25-1.02)	6 (50%)	0.24 (0.62)	0.74 (0.23-2.41)
KIR2DS3	39 (31%)	9 (21%)	1.58 (0.21)	0.59 (0.26-1.35)	0	5.12 (0.02)	N/A
KIR2DS4	117 (93%)	37 (86%)	1.84 (0.18)	0.47 (0.16-1.42)	9 (75%)	4.15 (0.04)	0.25 (0.06-1.04)
KIR2DS5	43 (34%)	12 (28%)	0.57 (0.45)	0.75 (0.35-1.60)	4 (33%)	0.04 (0.95)	1.04 (0.30-3.61)
KIR3DS1	48 (38%)	14 (33%)	0.42 (0.52)	0.78 (0.38-1.63)	4 (33%)	0.06 (0.80)	0.85 (0.25-2.96)
AA	32 (25%)	17 (40%)	3.11 (0.08)	1.92 (0.92-3.99)	4 (33%)	0.12 (0.73)	1.24 (0.36-4.34)
BB	9 (7%)	5 (12%)	0.85 (0.36)	1.71 (0.54-5.42)	3 (25%)	4.75 (0.03)	4.42 (1.05-18.73)
AB	85 (67%)	21 (49%)	4.76 (0.03)	0.46 (0.23-0.93)	5 (42%)	2.45 (0.12)	0.40 (0.12-1.31)

Binary logistic regression (figure 3.3) was carried out. Increasing age was seen to significantly increase risk of cirrhosis (p=0.004, OR=3.343). All other variables were not significantly associated with development of cirrhosis or HCC, though female gender approached a significant reduction in risk of cirrhosis (p=0.056, OR=0.366).

Table 3.3 Binary logistic regression - KIR genes associated with cirrhosis

Variables in the equation	Beta	S.E	Sig.	OR	95% CI for OR	
					Lower	Upper
KIR2DL1	-2.589	1.553	0.095	0.075	0.004	1.576
KIR2DL2	-0.738	1.329	0.579	0.478	0.035	6.464
KIR2DL3	0.562	0.837	0.502	1.755	0.340	9.044
KIR3DL1	-2.477	1.533	0.106	0.084	0.004	1.696
KIR2DS1	-20.167	19841.488	0.999	0.000	0.000	
KIR2DS2	0.302	1.514	0.842	1.353	0.070	26.310
KIR2DS3	0.192	0.652	0.769	1.211	0.337	4.348
KIR2DS4	1.539	1.791	0.390	4.658	0.139	155.872
KIR2DS5	-0.300	1.108	0.786	0.741	0.084	6.498
KIR3DS1	20.125	19841.495	0.999	549506234	0.000	
AA	0.189	0.912	0.835	1.209	0.202	7.223
BB	-1.539	1.791	0.390	0.215	0.006	7.185
AB	-0.189	0.912	0.835	0.827	0.138	4.945
Age	1.207	0.419	0.004	3.343	1.470	7.604
HCV Clearance	-0.109	0.460	0.812	0.896	0.364	2.210
Diabetes	1.536	1.308	0.240	4.648	0.358	60.298
Alcohol excess	-0.439	1.065	0.680	0.645	0.080	5.200
Gender	-1.006	0.527	0.056	0.366	0.130	1.028
Constant	-0.379	2.407	0.875	0.684		

Table 3.4 Binary logistic regression - KIR genes associated with HCC

Variables in the equation	Beta	S.E	Sig.	OR	95% CI for OR	
					Lower	Upper
KIR2DL1	21.425	19240.408	0.999	2.017E+09	0.000	
KIR2DL2	19.031	17759.459	0.999	184098391	0.000	
KIR2DL3	-1.879	1.773	0.289	0.153	0.005	4.937
KIR3DL1	-14.435	2086.172	0.994	0.000	0.000	
KIR2DS1	-18.377	18459.960	0.999	0.000	0.000	
KIR2DS2	-17.316	17759.459	0.999	0.000	0.000	
KIR2DS3	-49.433	5584.728	0.993	0.000	0.000	
KIR2DS4	-16.154	2089.534	0.994	0.000	0.000	
KIR2DS5	-0.390	9930.435	1.000	0.677	0.000	
KIR3DS1	19.119	19.119	0.999	201125240	0.000	
AA	2.054	2.054	0.342	7.798	0.113	540.454
BB	14.100	2089.533	0.995	1328801.92	0.000	
AB	-2.054	2.163	0.342	0.128	0.000	
Age	28.152	2952.632	0.992	1.684E+12	0.000	
HCV Clearance	-1.387	0.871	0.111	0.250	0.045	1.377
Diabetes	-0.260	1.594	0.871	0.771	0.034	17.541
Alcohol excess	-46.558	11358.170	0.997	0.000	0.000	
Gender	-29.200	2952.633	0.992	0.000	0.000	
Constant	-117.919	21891.049	0.996	0.000		

Including multiple variables in a model has the advantage of considering multiple factors which may impact the dependent variable. However, it may also have an adverse impact on the accuracy of a model. Including multiple predictors reduces statistical power, particularly with a limited sample size, so may over or underestimate associations. Overfitting may occur, where a model fits anomalies which are a random product of the study population rather than relevant factors. Therefore, variable selection was necessary to reveal the most significant factors to be included. Stepwise regression was performed (for summary see figure 3.3 and 3.4). Full details of stepwise regression can be found in appendix A.

Table 3.5 Stepwise regression - KIR genes associated with cirrhosis

Included variables

Model	Variable	Beta	Std. Error	Standardised Beta	t	Sig.	95% CI for Beta	
							Lower	Upper
1	Constant	-0.343	0.221		-1.554	0.122	-0.780	0.093
	Age	0.166	0.061	0.222	2.736	0.007	0.046	0.286

Increasing age was associated with increased risk of cirrhosis and HCC. It is possible that this reflects duration of HCV infection, though it was not possible to accurately establish the date of HCV diagnosis in this dataset. No KIR genes or risk factors were significantly associated with development of cirrhosis.

Table 3.6 Stepwise regression - KIR genes associated with HCC

Included variables

Model	Variable	Beta	Std Error	Standardised Beta	t	Sig.	95% CI for Beta	
							Lower	Upper
1	Constant	-0.253	-0.135		-1.881	0.062	-0.520	0.013
	Age	0.092	0.037	0.202	2.472	0.015	0.018	0.165
2	Constant	-0.206	0.135		-1.523	0.130	-0.473	0.061
	Age	0.086	0.037	0.190	2.346	0.020	0.014	0.159
	KIR2DS3	-0.098	0.047	-0.168	-2.075	0.040	-0.191	-0.005

Stepwise selection identified age and KIR2DS3 as the most significant factors associated with development of HCC. The model produced was statistically significant (p=0.006). As with cirrhosis, increasing age conferred an increased risk of HCC (Beta=0.086, p=0.020). KIR2DS3 was associated with a reduced risk (Beta=-0.098, p=0.040). No other factors approached significance.

3.4 Discussion

Increasing age conferred an increased risk of both cirrhosis and HCC. Increasing age is an established risk factor for cirrhosis and HCC development in patients with HCV, with multiple studies corroborating this finding. While this may reflect duration of infection, evidence suggests that progression of fibrosis is greater in older patients, and that they are at greater risk of hepatocarcinogenesis following viral eradication[151-153].

In this cohort presence of KIR2DS3 frequency was significantly reduced in patients who developed HCC. No significant association was found between KIR genotype and development of cirrhosis. This data contrasts with a study by Joshita et al. of 280 Japanese patients with hepatitis B in which KIR2DS3 frequency was significantly higher in patients with HCC[154]. Current literature suggests a strong relationship between KIR2DS3 and outcome of viral hepatitis, having been associated

with failure to clear hepatitis B and C in multiple studies across a variety of populations [88, 155-157]. In other contexts KIR2DS3 has been correlated with reduced risk of acute myeloid leukaemia[158], increased frequency in patients with autoimmune conditions such as ulcerative colitis and SLE, and in conjunction with HLA-C, increased risk of basal cell and squamous cell skin carcinomas[156].

The mechanism for the relationship between KIR2DS3 and these clinical outcomes is unclear. No ligands for KIR2DS3 have yet been identified, and so function is difficult to study. The KIR2DS3 protein is thought to largely reside intracellularly, with greatly reduced surface expression compared to other KIR. Consequently it has previously been hypothesised that the molecule no longer serves a functional purpose, or alternatively that the surface expression is dependent on the presence of other KIR, or may vary depending on the state of the NK cell[159]. It has been suggested that the apparent clinical relevance of KIR2DS3 may suggest that it is a constituent of a haplotype which is associated with these clinical outcomes[156].

Other recent studies have suggested alternative KIR associations with HCC development. Umemura et al. found that HLA-Bw4 and the KIR3DL1+HLA-Bw4 pair were significantly associated with a HCC development in a cohort of 211 Japanese patients with HCV associated cirrhosis[160]. Interestingly, Joshita et al observed reduced KIR3DL1+HLA-Bw4 frequency in patients free from nucleoside treatment compared to patients requiring nucleoside treatment for hepatitis B[154]. De Re et al found a significant association between reduced KIR3DS1+HLA-Bw4 frequency and increased incidence of HCC[88].

Incidence of HCC in HCV infected individuals with cirrhosis has been reported as 1-4% per annum[161]. Incidence in this cohort was consistent with this finding, and consequently included relatively few cases of HCC, and may be underpowered to demonstrate associations with KIR genotype, in particular it may be insufficiently powered to demonstrate any effect KIR2DS2 positivity may have on risk of HCC development. Large datasets may be required to fully clarify the role of KIR genotype, and reveal the impact of the multiple possible combinations of KIR genotype on risk of cirrhosis and HCC.

As a single-centre, retrospective study this dataset has inherent vulnerabilities. Data collected may be influenced by local factors such as ethnic variation in KIR genotype which may limit applicability to wider contexts[78]. Furthermore, many of these patients were recruited before the widespread introduction of direct acting antivirals, which has greatly improved viral eradication, and consequently reduced risk of HCC development[162]. This study relied on clinical record-keeping which was not always complete, which is likely to contribute to unexpectedly low incidence of co-factors for liver disease such as alcohol consumption or diabetes. Patient engagement and location was difficult to establish, which may have led to inclusion of patients no longer under follow-up or no longer living in the hospital catchment area. These observations could also reflect a bias in the cohort, as recruiting patients from a clinic population may exclude patients who are not engaged with services or hard to reach. Most chronic HCV infections in the UK are associated with injected drug use, and it is recognised that there are barriers to the testing and treatment of people who inject drugs[163]. The low incidence of co-morbidity, and higher incidence of BCLC 0 and A HCC in this cohort may reflect a biased population towards an engaged clinic population, limiting the generalisability of these data.

Chapter 4 Exosome purification and in-vitro cell culture

4.1 Introduction

In the previous chapter I studied the relationship between NK cell KIR genotype and development of cirrhosis and HCC in a cohort of patients with HCV. In this chapter I will examine whether NK cells can be activated via the KIR2DS2 receptor by a viral peptide expressed on an exosome as a potential therapeutic pathway. KIR2DS2 was selected as it is an activating receptor, for which ligands have recently been identified.

To achieve this, exosomes were isolated from a cultured cell line expressing ligands to KIR2DS2, and the used these in in-vitro experiments. The aim of these experiments was to confirm the purification of exosomes derived from 721.221 cells, including those expressing the DP1 flavivirus peptide, and examine whether they influence the function of human KIR2DS2 positive NK cells, assessed by measuring cell frequency, degranulation, and ability to kill hepatocellular carcinoma cell lines in-vitro. For detailed methodology for this chapter, see chapter 2, sections 2.2-2.13.

There are multiple potential methods of isolating exosomes from cell culture, including charge neutralization precipitation, gel-filtration, and affinity purification, which use commercially available kits. Differential ultracentrifugation was chosen, as it allows a high yield to be obtained from a relatively large volume of cell culture, evidence suggests that functional properties are preserved, and appropriate ultracentrifuges were available(164).

The International Society for Extracellular Vesicles has published standards for characterisation of exosomes, and validating their isolation(139). Heterogeneity of individual vesicles should be characterised by 2 methods. Size distribution may be assessed by nanoparticle-tracking analysis, dynamic light scattering, or resistive pulse sensing, demonstrating heterogeneity of vesicle size, and that isolates do not contain cellular contaminants. As this does not discriminate extracellular vesicles from contaminant particles of a similar size, electron microscopy should be used to demonstrate individual membrane-bound vesicles in close up, and a wide-field view of multiple vesicles to indicate heterogeneity. A general overview of protein composition must be provided, including assessment of multiple (3 or more) proteins expected to be found in exosome preparations, and proteins expected to be absent. This is typically achieved by Western blotting, flow cytometry, or mass spectroscopy. In functional experiments, a dose-function relationship should be examined.

4.2 Exosome purification

Exosome isolation was performed by differential ultracentrigation of cultured 721.221 (221), 721.221-HLA C*0102 (C01), 721.221-HLA C*0102-DP1 (DP1) cells. This process involves sequential pelleting and removal of contaminants by centrifugation in defined steps. First cells are centrifuged at 500g to remove the cultured cells, and the pellet discarded or retained for use in western blotting. The supernatant is then centrifuged again at 2000g to remove cellular debris. The supernatant is then transferred to an ultracentrifuge at 10,000g to remove apoptotic bodies and microparticles. This supernatant is then centrifuged at 100,000g for 70 minutes twice. After the first of these most of the supernatant is discarded, the remnant pooled and a dye added to allow concentration of the isolated vesicles into a visible pellet which can be harvested.

Figure 4.1 Transmission electron microscopy of purified exosomes

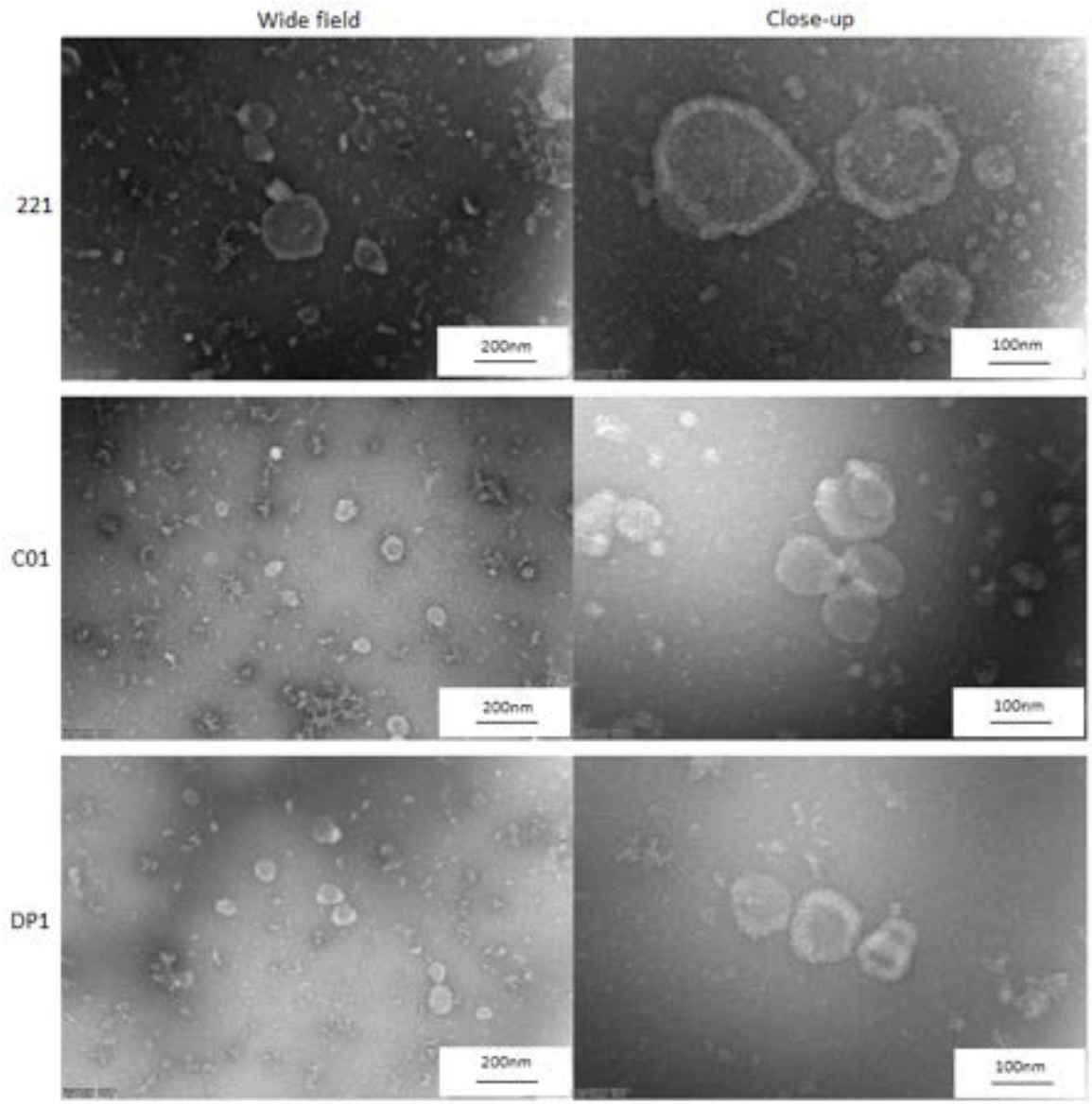


Fig 4.1 Wide field (100000x magnification), and close-up (200000x magnification) electron micrograph of purified exosomes from 221, C01, DP1 cells.

Imaging of individual vesicles was performed by transmission electron microscopy (TEM) with negative staining(see Figure 4.1), showing circular membrane-bound vesicles in all preparations in close up (200000x magnification). Wide field view (100000x magnification) demonstrates multiple homogenous membrane bound vesicles, approximately 50-200nm in size. Some debris is visible in the images, which being smaller than the vesicles is retained by the sequential ultracentrifugation process.

Nanoparticle tracking analysis, performed on a NanoSight instrument with samples diluted in water demonstrated particles with a modal size of 131.8 +/- 4.8 nm for the 221 preparation, 150.1 +/- 12.4 nm for the C01 preparation, and 108.3 +/- 6.8 nm for the DP1 preparation. Concentration was found to be $5.57x10^8$ +/- $2.08x10^7$particles/ml at 1:5000 dilution for 221, $7.33x10^8$ +/- $6.33x10^7$ particles/ml at 1:1000 dilution for C01, and $2.74x10^8$ +/- $3.41x10^7$ particles/ml at 1:5000 for DP1. As demonstrated in Figure 4.2, size distribution was consistent with isolation of extracellular vesicles, with no large particles present to indicate contamination. In conjunction with TEM images, this confirms preparations of membrane bound vesicles of a size distribution consistent with extracellular vesicle isolation.

Figure 4.2 Nanoparticle tracking analysis of purified exosomes

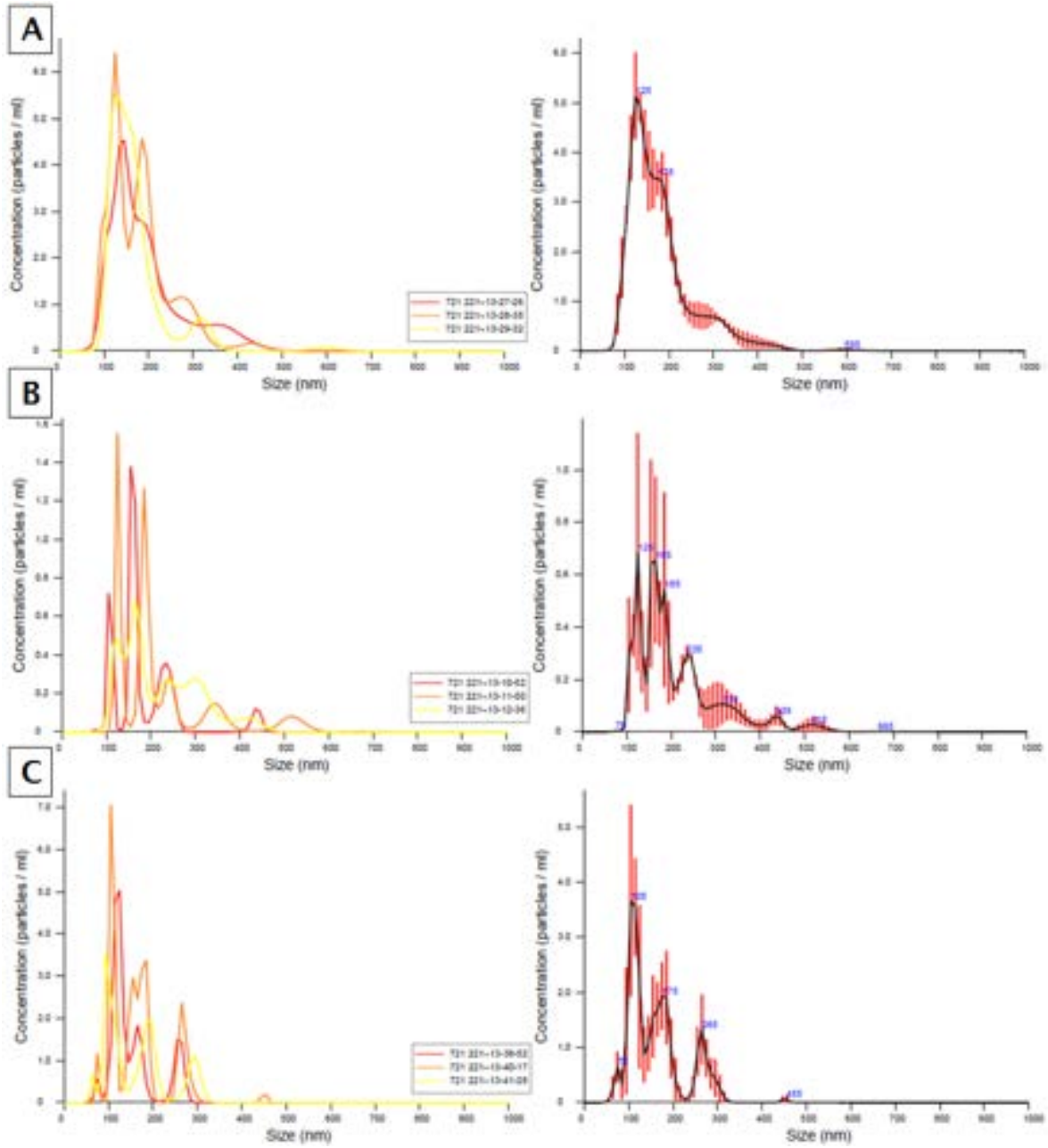


Fig 4.2 Nanoparticle tracking analysis of exosomes isolated from 221 (A), C01 (B), DP1 (C) cells, demonstrating size distribution of extracted vesicles.

Western blotting was used to assess protein content of exosome preparations. 3 markers which would be expected to be enriched in exosomes were used; Alix, CD63, and CD81. Alix is a protein associated with the endosomal sorting complexes required for transport (ESCRT) mechanism, which regulates extracellular vesicle sorting and release(165). CD63 and CD81 are tetraspanins, transmembrane proteins which are abundant in extracellular vesicles. GM 130 is a golgi marker which should not be present in exosome preparations, and would indicate probable contamination. All are commonly used in exosome characterisation. (166). W6/32, an MHC-I marker was included, as 721.221 cells are MHC-I deplete, and C01 and DP1 cells are 721.221 cells transduced to include HLA*C0102 or HLA*C0102-DP1 constructs, giving an indication if MHC-I expression has persisted during exosome isolation. Actin was used as an equal loading control.

Figure 4.3 Comparison of protein composition of purified exosomes and 721.221 parent cells

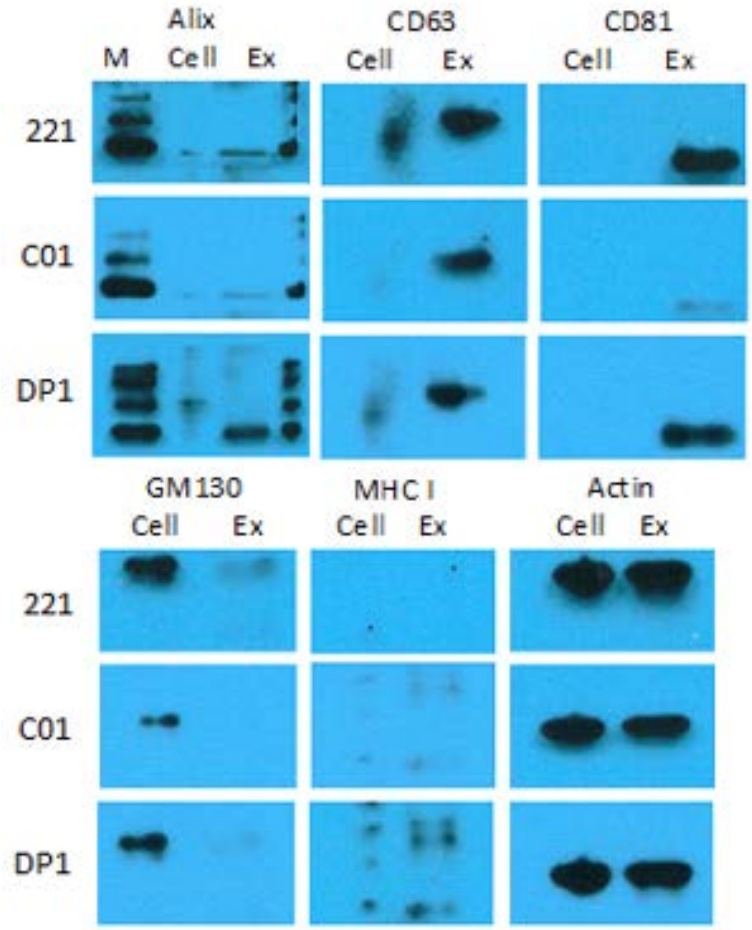


Fig 4.3 Western blot of parent cell (Cell) and exosome (Ex) protein composition.

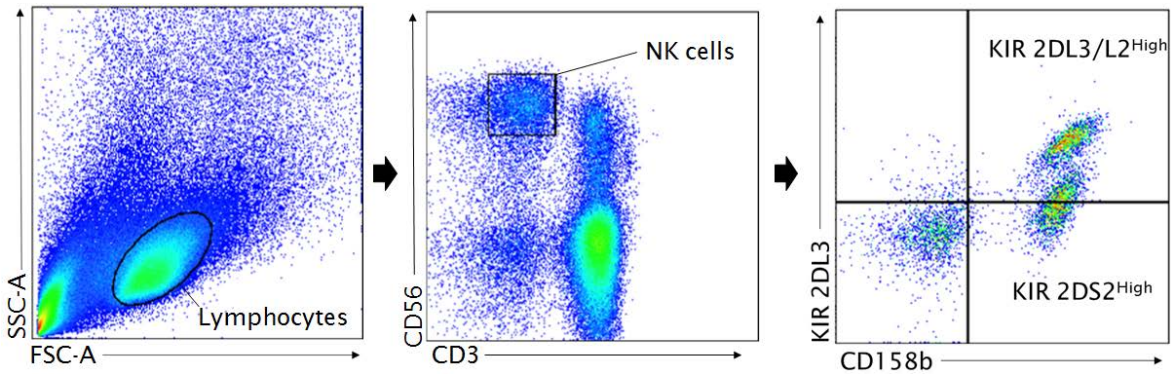
Figure 4.3 demonstrates enriched Alix, CD63, and CD81 in all exosome preparations compared to the parent cell pellet. GM 130 is enriched in the parent cell pellet compared to exosome preparations. MHC-I appears enriched in C01 and DP1 exosome preparations and cell pellets compared to 221, suggesting the presence of MHC-I in C01, and DP1 exosomes, as in their parent cells. These findings are consistent with the expected composition of exosomes, and together with the results of nanoparticle tracking analysis, and electron microscopy, satisfy criteria to identify isolation of extracellular vesicles.

4.3 NK and KIR 2DS2 positive NK cell proliferation did not occur following exosome stimulation of PBMC in vitro

To study the whether DP1 exosomes activate KIR2DS2 positive NK cells, initial experiments aimed to examine whether culturing exosomes with peripheral blood mononuclear cells (PBMCs) from KIR2DS2 positive donors produced a change in KIR2DS2 NK cell frequency.

Healthy volunteers were screened for KIR2DS2 positivity using the flow cytometry panel in Figure 4.4. There are a lack of antibodies able to discriminate between KIR2DS2 and the inhibitory KIRS 2DL2 and 2DL3. The KIR2DL3-/CD158b+ population demonstrated has been identified as a method of identifying a KIR2DS2 high population(167). Once an individual was identified as KIR2DS2 positive, they were consented for further donation, and PBMC were extracted as per the protocol in chapter 2.4, frozen, and stored in liquid nitrogen prior to use. KIR2DS2 negative donors were processed and frozen as required for use as controls.

Figure 4.4 Representative flow cytometry plots for NK and 2DS2 identification



Initial experiments trialled a range of concentrations of interleukin-15 (IL-15), and a range of concentrations of 221, C01, and DP1 exosomes in 3 donors in order to establish optimum experimental conditions. IL-15 was chosen as it is the predominant cytokine required for NK cell survival, development, proliferation, metabolism and function, though protocols to expand large numbers of cells can also include supra-physiological levels of a variety of cytokines including IL-2, IL-12, and IL-18(168, 169). Concentrations of 1ng/ml, 2ng/ml, and 5ng/ml IL-15 were trialled, with exosome concentrations of 5mcg/ml, 10mcg/ml, and 20mcg/ml. Analysis was performed on day 5, with change of media and cytokines on day 3 (see Figure 4.5).

Figure 4.5 Co-culture of PBMC and exosomes with varying concentrations of IL-15

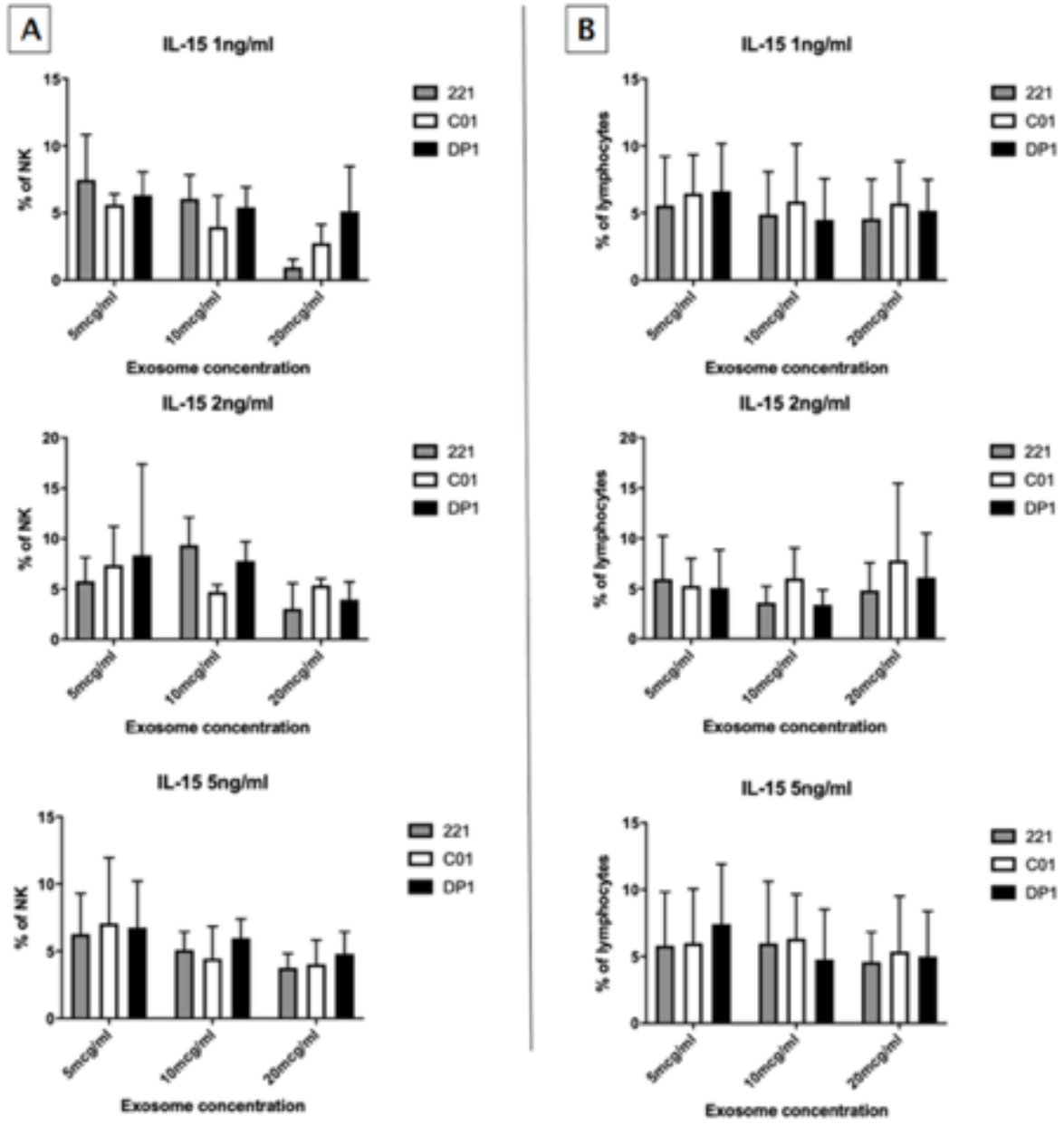


Fig 4.5. Mean frequency of NK cells (A), and 2DS2 positive NK cells (B) in PBMC of 3 2DS2 positive donors co-cultured with varying concentrations of 221, C01, and DP1 exosomes in R10 plus 1ng/ml, 2ng/ml, or 5ng/ml IL-15. Analysis on day 5.

No significant differences in NK or 2DS2 positive NK frequency were found between exosome or IL-15 groups, and no consistent trends were observed. Seven day incubation was trialled, with change of media and cytokines on day 3 and 5, though excessive cell death prevented adequate identification of NK on flow cytometry. 7 day incubation was repeated including IL-2 in addition to IL-15, though again cell death prevented analysis.

This experiment was repeated with 5 KIR2DS2 positive donors, and a KIR2DS2 negative donor as a control in order to examine peptide specificity. Analysis was performed on day 5. Due to the difficulty producing sufficient quantities of exosome to investigate a large number of variables, 2ng/ml IL-15 concentration was selected following literature review and advice from colleagues. Exosome concentrations were maintained at range from 5mcg/ml-20mcg/ml as in the previous experiment.

Figure 4.6 NK cell frequency in PBMC stimulated with exosomes

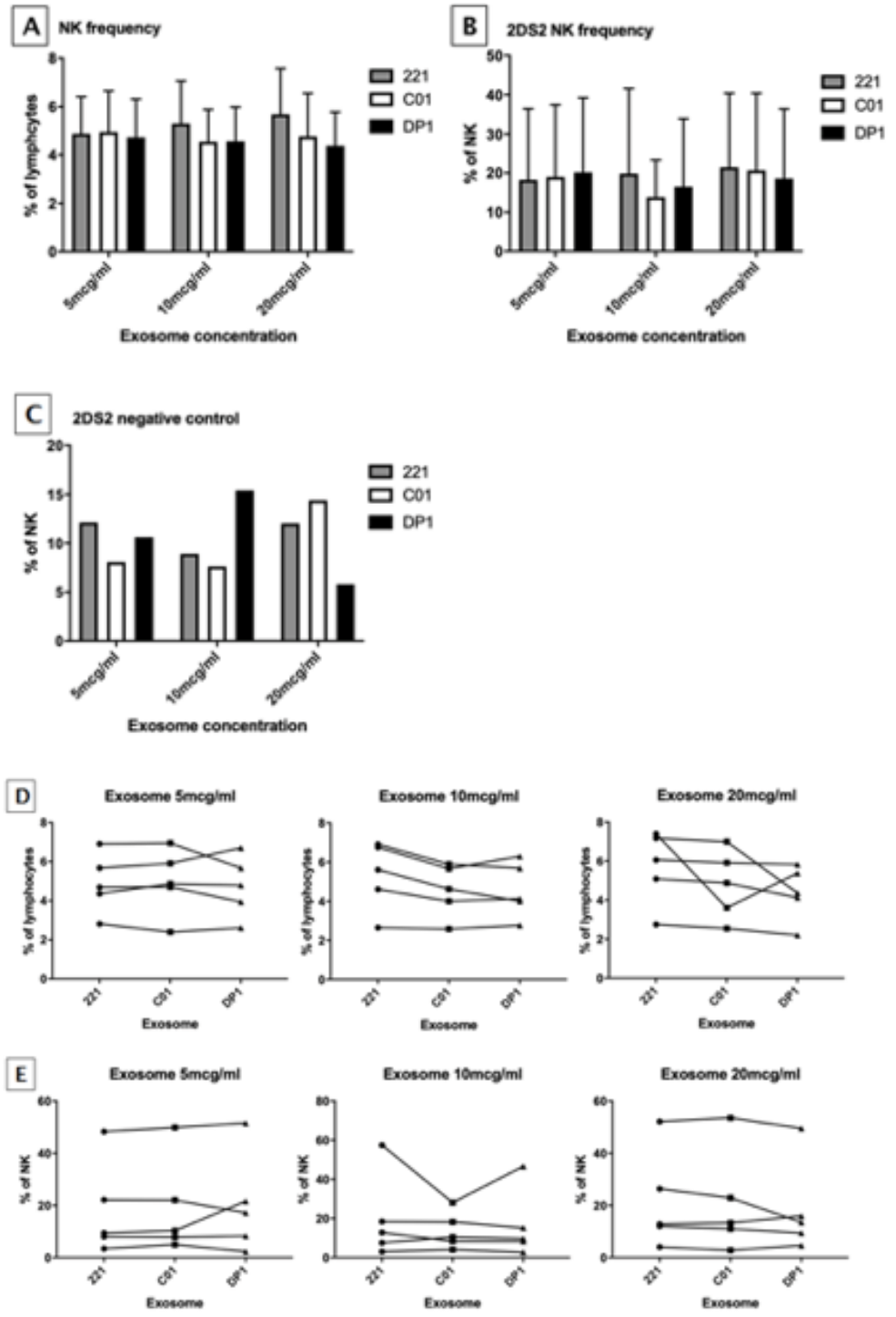


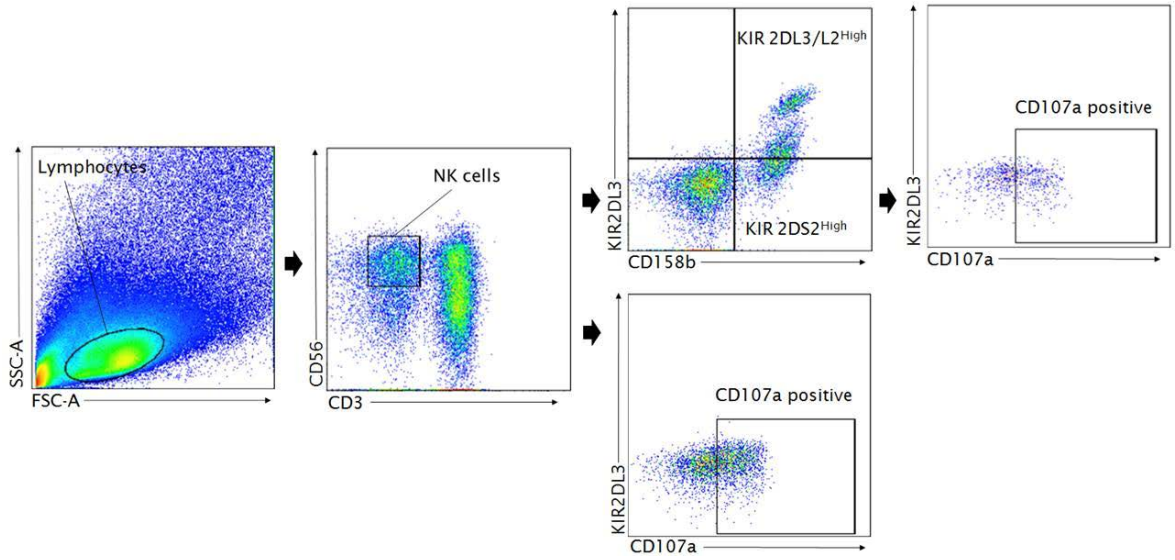
Fig 4.6. Mean frequency and standard deviation of NK cells (A), and 2DS2 positive NK cells(B) in PBMC of 5 2DS2 positive donors co-cultured with varying concentrations of exosomes. Frequency of NK cells in PBMC of 2DS2 negative donor following co-culture with exosomes (C). Trends in individual donors for total NK cell frequency (D), and KIR2DS2 positive NK cells (E) at different concentrations of exosome.

In continuity with preliminary data, no significant differences or trends were found in total NK cell or 2DS2 positive NK cell frequency between treatment groups in KIR2DS2 positive donors. Figure 4.6 (A) and (B) demonstrate pooled mean frequencies of NK and KIR2DS2 positive NK respectively. (D) and (E) display data from individual donors to highlight any trends. This experiment suggests that co-culture of exosomes and PBMCs does not produce proliferation. However, due to limitations on exosome production, and availability of KIR2DS2 positive donors, a limited range of conditions could be investigated, and further optimisation may have been required for proliferation to occur, such as a greater range of IL-15 concentrations, or other cytokines such as IL-2, IL-12, and IL-18.

4.4 Exosome stimulation did not enhance degranulation of NK cells against an HCC cell line in vitro

In order to assess the whether DP1 exosomes influence the functional activity of KIR2DS2 positive NK cells, a CD107a (LAMP-1) assay was performed. As discussed in chapter 1, CD107a is expressed on the surface of NK cells during degranulation following initiation of target cell killing. CD107a expression was determined by flow cytometry as Figure 4.7, gating within total NK, and KIR2D2 high populations.

Figure 4.7 Representative flow cytometry plots for NK and 2DS2 NK CD107a comparison



PBMCs were cultured with varying concentrations of exosomes in R10 with 2ng/ml IL-15 for between 4 and 48 hours, before use. Initial experiments incorrectly analysed CD107a expression following co-culture of exosomes and PBMC without a target cell to stimulate degranulation, yielding low levels of CD107a expression. A target cell is required for the effector cell (the NK cell) to identify and induce degranulation, and so subsequent experiments utilised the PLC human HCC cell line as target cells. Preliminary experiments found no trend between 221, C01, or DP1 exosomes after 4 hours or 24 hours of co-culture with PBMC, or with 5mcg/ml, 10mcg/ml, or 20mcg/ml of exosome. Therefore, co-culture was extended to 48hours, with 10mcg/ml, 20mcg/ml, and 50mcg/ml exosome.

As in section 4.2, isolation of exosomes, and limited KIR2DS2 positive donors limited the range of conditions which could be explored. In order to conserve these resources, preliminary experiments trialled effector:target (E:T) ratios of 5:1 and 10:1 (see figure 4.8) with PBMC cultured with 20mcg/ml of C01 and DP1 exosomes for 48hrs in 3 2DS2 positive donors, and 1 2DS2 negative donor.

Figure 4.8 Total NK cell and 2DS2 positive NK cell CD107a expression with PLC HCC cell line target cells at 5:1 and 10:1 effector:target ratio.

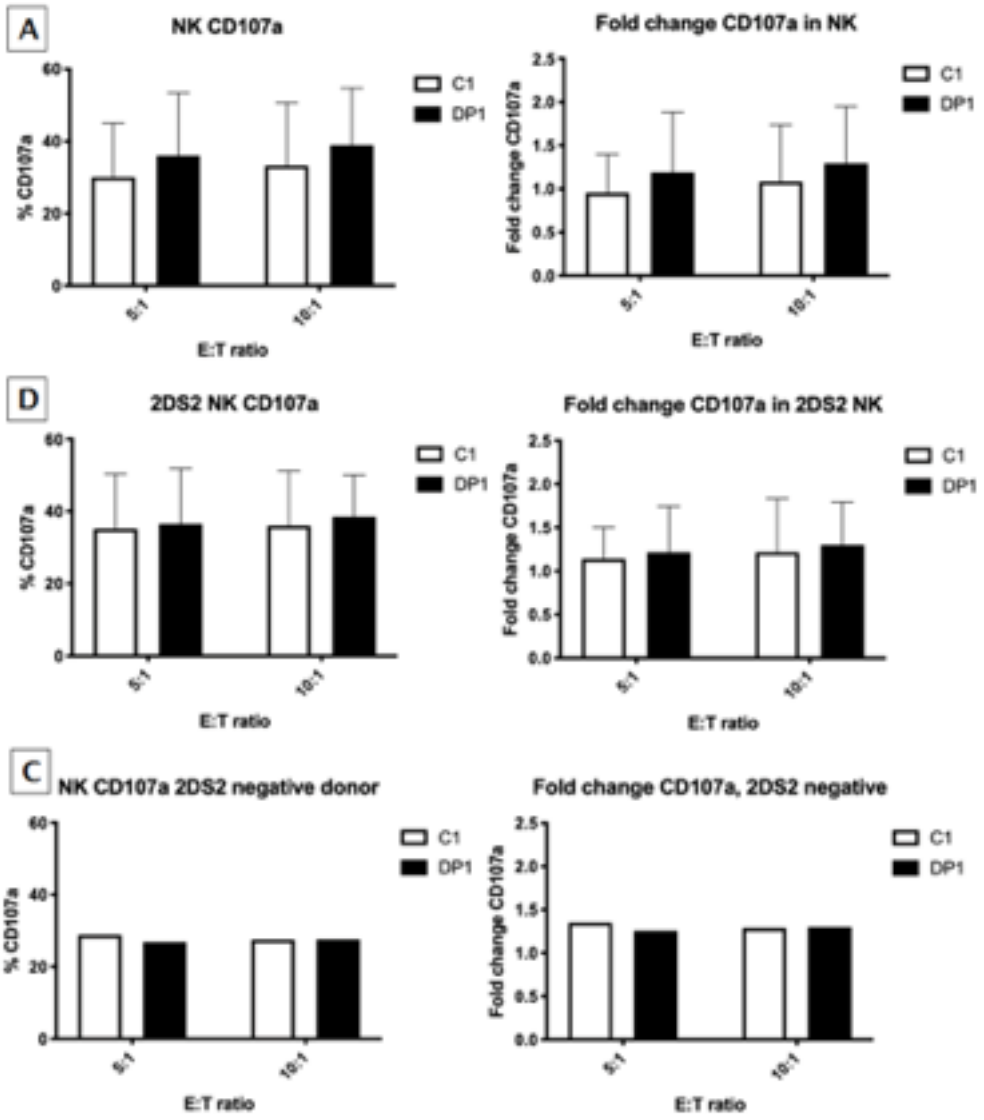


Fig 4.8. Mean total NK cell CD107a expression in 3 2DS2 positive donors(A), 2DS2 positive NK cell CD107a expression in 3 donors(B), Total NK cell CD107a expression in 1 2DS2 negative donor (C). Fold change in total NK cell CD107a expression in 3 2DS2 positive donors (D), Fold change in 2DS2 positive NK cell CD107a expression in 3 donors (E), fold change in total NK cell CD107a expression in 1 2DS2 negative donor (F).

A slight non-significant trend towards enhanced cytotoxicity in the DP1 group in total NK cells and 2DS2 positive NK cells was observed (see figure 4.8). This trend was seen at both 5:1 and 10:1 E:T ratios, with no difference observed between them. As an E:T ratio of 5:1 requires fewer effector cells (fewer PBMCs), and so utilised limited KIR2DS2 positive PBMCs more efficiently, this condition was selected for subsequent experiments.

A CD107a assay was then performed in 5 2DS2 positive donors, and 2 2DS2 negative controls. PBMC effectors were stimulated with 10mcg/ml, 20mcg/ml or 50mcg/ml exosomes for 48 hours prior to exposure to PLC HCC targets (see figures 4.9 and 4.10).

Figure 4.9 Total NK cell and 2DS2 positive NK cell CD107a expression with PLC HCC cell line target cells at 5:1 effector:target ratio.

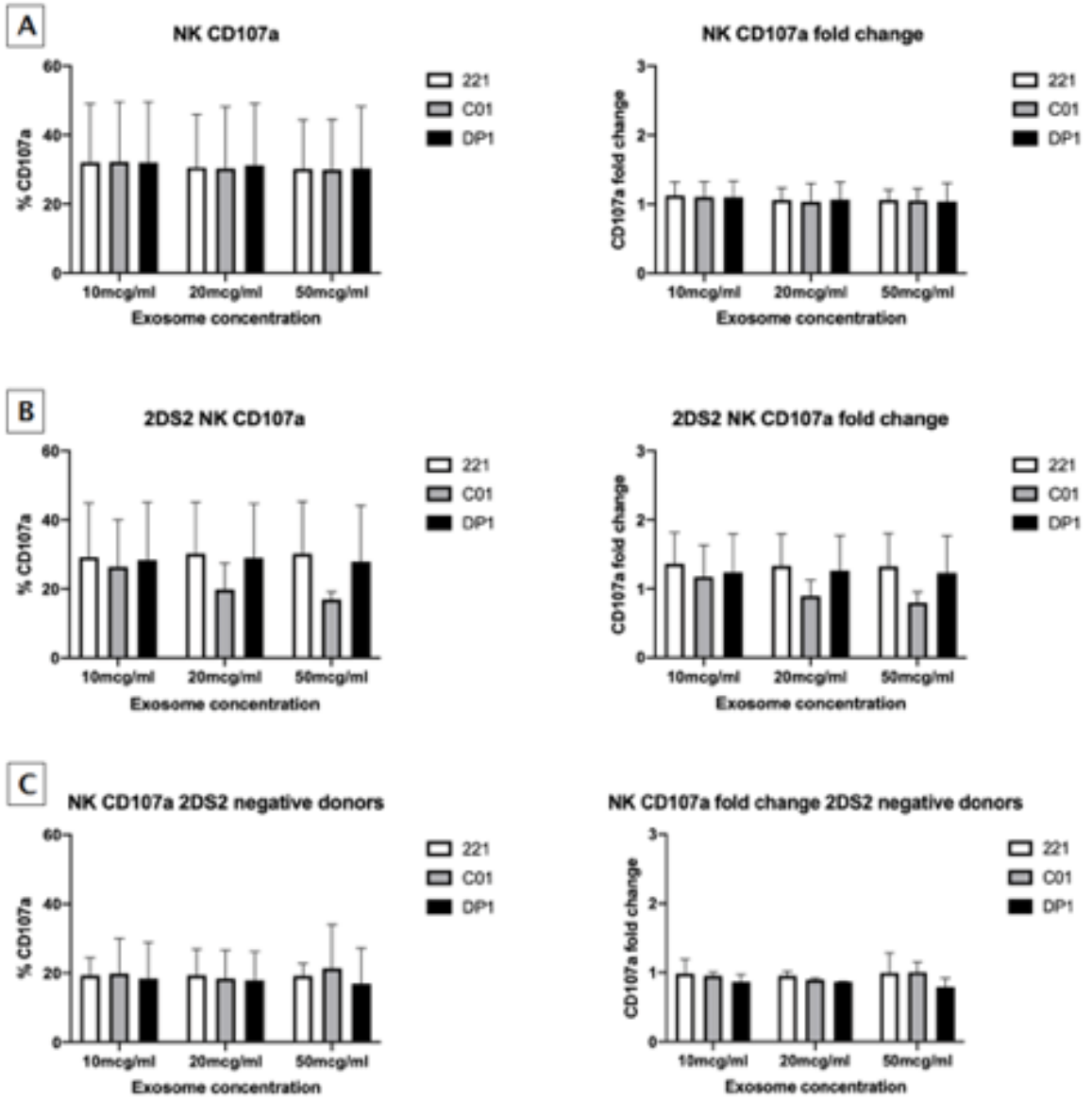


Figure 4.9. Mean NK cell CD107a expression in exosome stimulated PBMC, absolute and fold change in 5 2DS2 positive donors (A). 2DS2 positive NK cell CD107a absolute and fold change in 5 donors (B). NK cell CD107a expression in exosome stimulated PBMC in 2 2DS2 negative donors, absolute and fold change (C).

No significant difference in CD107a expression in total NK cells or KIR2DS2 positive NK cells was found between exosome type, or exosome concentration groups. No trend towards enhanced cytotoxicity was seen in the DP1 group, though a trend towards reduced cytotoxicity in KIR2DS2 positive NK cells cultured with C01 exosomes is suggested at exosome concentrations of 20mcg/ml and 50mcg/ml in Figure 4.9 (B).

Figure 4.10 Individual trends in CD107a fold change

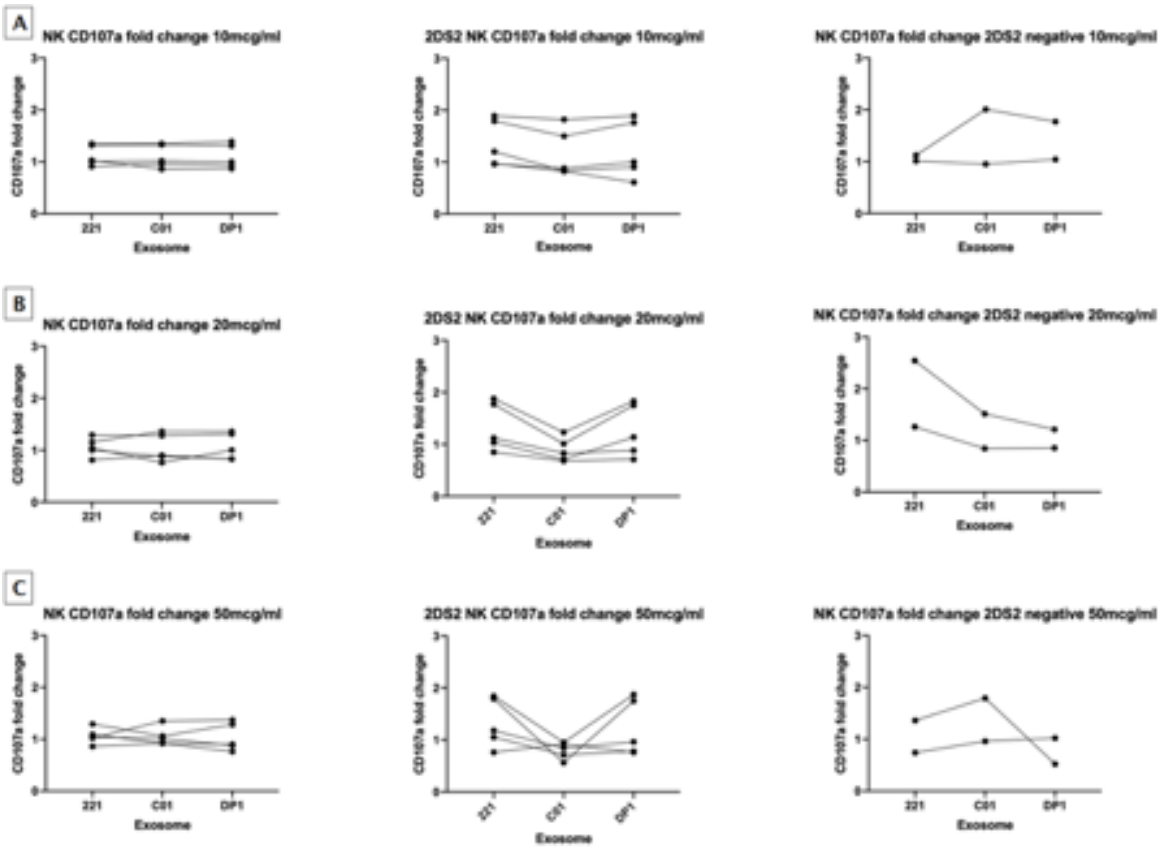


Figure 4.10. Individual trends in fold change of NK, 2DS2 NK, and 2DS2 negative CD107a expression in exosome stimulated PBMC with 10mcg/ml exosome(A), 20mcg/ml exosome(B), and 50mcg/ml(C).

Figure 4.10 shows CD107a fold change for individual donors to highlight any individual trends. This shows that little discernable variation in NK cell CD107a occurred in individual donor PBMC when exposed to 221, C01, or DP1 exosomes. The trend towards reduced cytotoxicity in KIR2DS2 positive NK cells exposed to C01 exosomes at 20mcg/ml and 50mcg/ml can be seen to occur predominantly in only 2 of the 5 donors.

This data suggests that DP1 exosomes do not enhance KIR2DS2 positive NK cell function assessed by CD107a expression under these conditions. As discussed, a limited amount of optimisation of this experiment was possible due to limited KIR2DS2 positive PBMCs, and the difficultly of producing exosomes, and it is possible that either these conditions may may not support NK cell homeostasis sufficiently to allow normal functionality, or other E:T ratios may be optimal. An alternative method of assessing cytotoxicity is trialled below.

4.5 DP1 exosome stimulated PBMC did not exhibit enhanced killing of HCC cell lines compared to 221 or C1 stimulated PBMC.

A killing assay was performed to investigate whether PBMC exhibited enhanced killing of human HCC cell lines following stimulation with exosomes. A panel of 6 HCC cell lines was selected, as the aetiology and characteristics of individual cell lines vary (see chapter 2, table 2.2 for details of cell lines and media). Effector PBMCs from 7 KIR2DS2 positive and 2 KIR2DS2 negative donors were co-cultured with 221, C01, of DP1 exosomes for 48 hours prior to use. Cell line targets were labelled with Cell Trace prior to incubation with PBMCs, after which cells were stained for Live/Dead, allowing identification of target cells killed by PBMC, using the flow cytometry panel in figure 3.11. As in previous experiments, in order to conserve exosomes and PBMCs, Effector:Target ratios were trialled at 5:1 and 10:1 in the first 4 donors.

Figure 4.11 Representative flow cytometry plots for comparison of exosome stimulated PBMC killing of HCC cell lines.

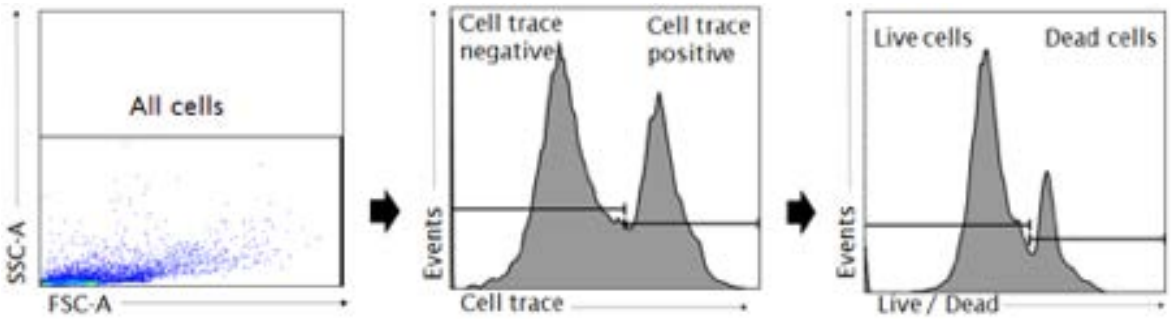


Figure 4.12 Killing of HCC cell lines by exosome stimulated PBMC - sample individual donor.

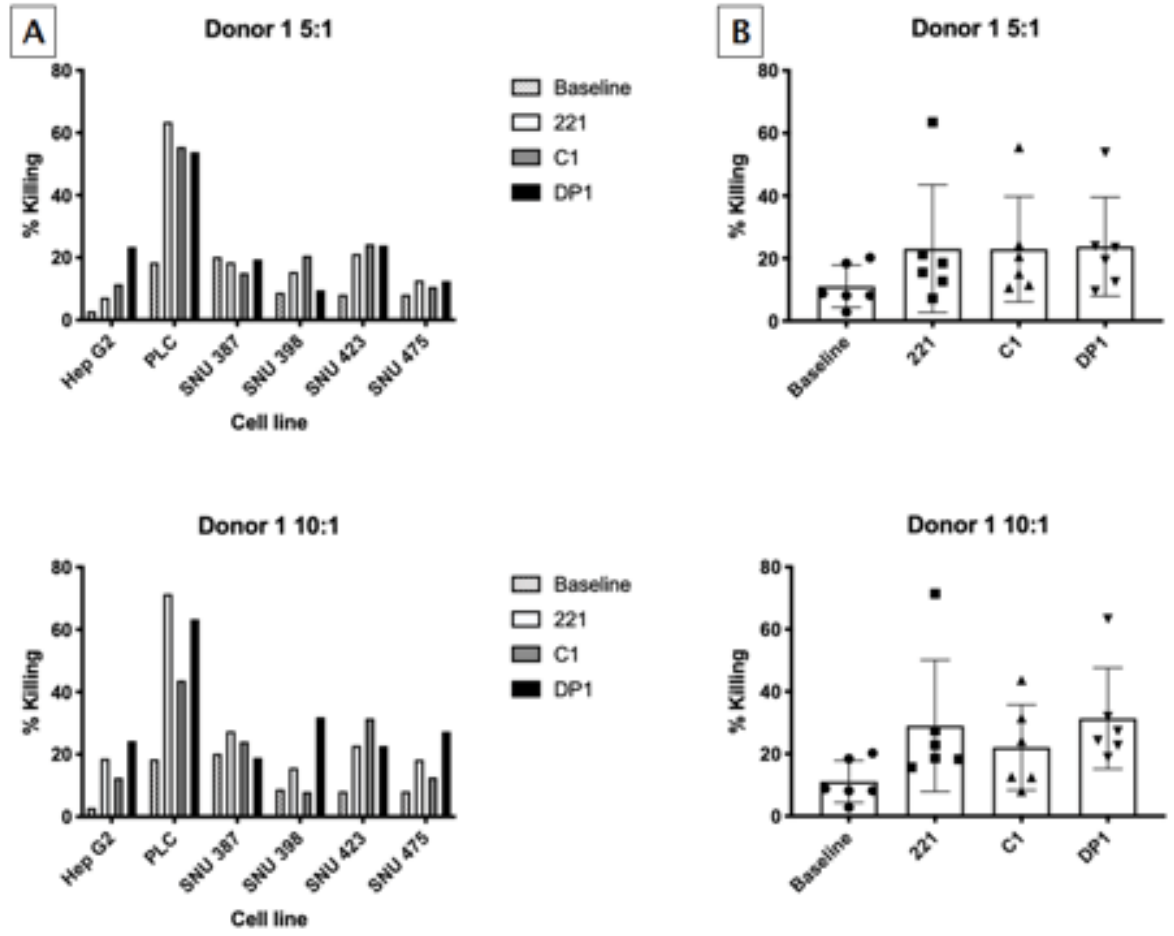
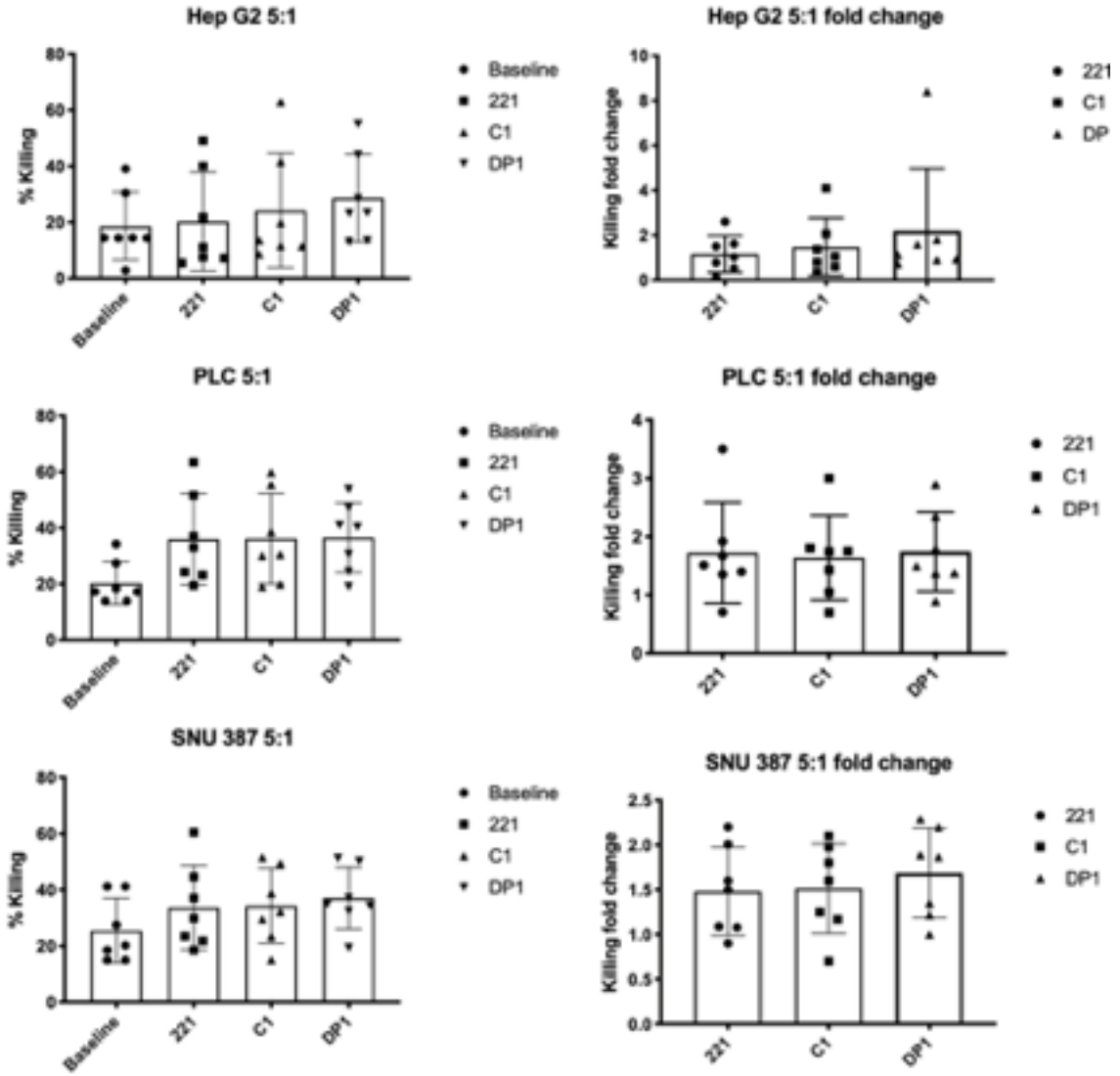


Figure 4.12. Killing of HCC cells lines by exosome stimulated PBMC in KIR2DS2 positive donors. Killing of individual cell lines by donor (A), pooled killing of all cell lines by exosome type (B). For all individual donors see appendix B.

No difference in individual cell lines, individual donor pooled results, or overall pooled results were observed between 5:1 and 10:1 E:T ratios. Therefore, as in previous experiments, an E:T ratio of 5:1 was selected for the remaining donors. As shown in figure 4.12 and appendix B, no clear trends can be observed within cells lines in individual donors, though killing of different cell lines appears to vary significantly between donors. In donor 7 and 2DS2 negative donor 2, mean PBMC killing did not significantly exceed baseline, which may indicate an experimental error such as death of PBMCs following thawing, and consequent low cytotoxicity. These observations appear to be related to the time experiments were conducted. Experiments for donor 2 and 3 were run concurrently, donor 5, donor 6 and 2DS2 negative donor 1 were run concurrently, and donor 7 and 2DS2 negative donor 2 were run concurrently. It can be seen that patterns of killing are similar within these groups. This suggests that variability in cell line killing may be attributable

to experimental conditions, and may reflect the condition of cultured cell lines. While thawing and culture conditions followed a consistent protocol throughout experiments, these data suggest that cell lines behaved differently at different times, suggesting possible variability in cell health and viability.

Figure 4.13. Pooled data from all donors at 5:1 E:T ratio for individual HCC cell lines.



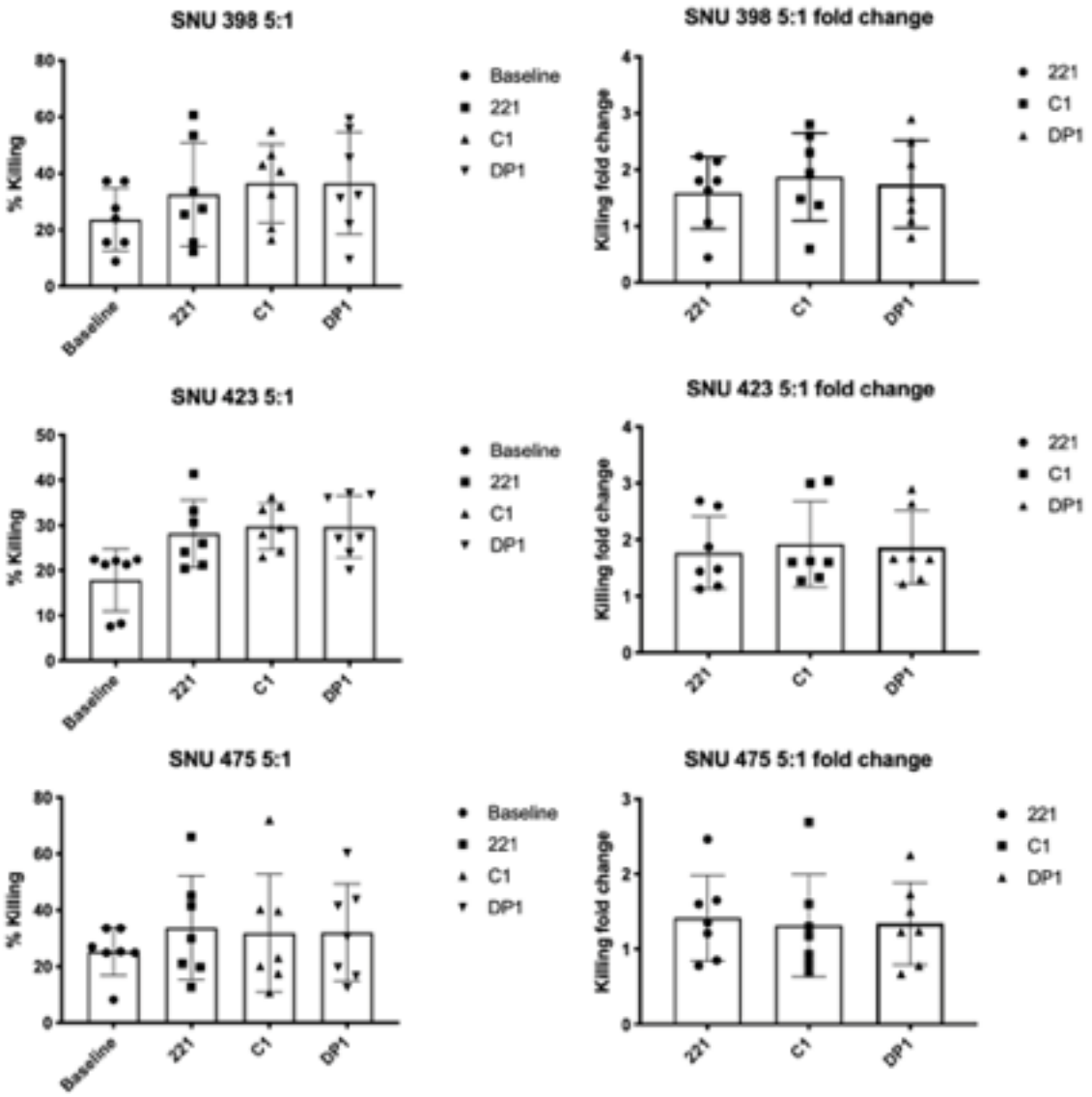


Fig 4.13. Pooled killing and killing fold change by cell line at 5:1 E:T ratio for 7 KIR2DS2 positive donors.

Pooled data for individual cell lines (see figure 4.13) did not demonstrate any significant difference between 221, C01, or DP1 exosomes in any cell line when analysed as absolute or fold change.

Figure 4.14 Killing of HCC cell lines by exosome stimulated PBMC - pooled data, 5:1 E:T ratio.

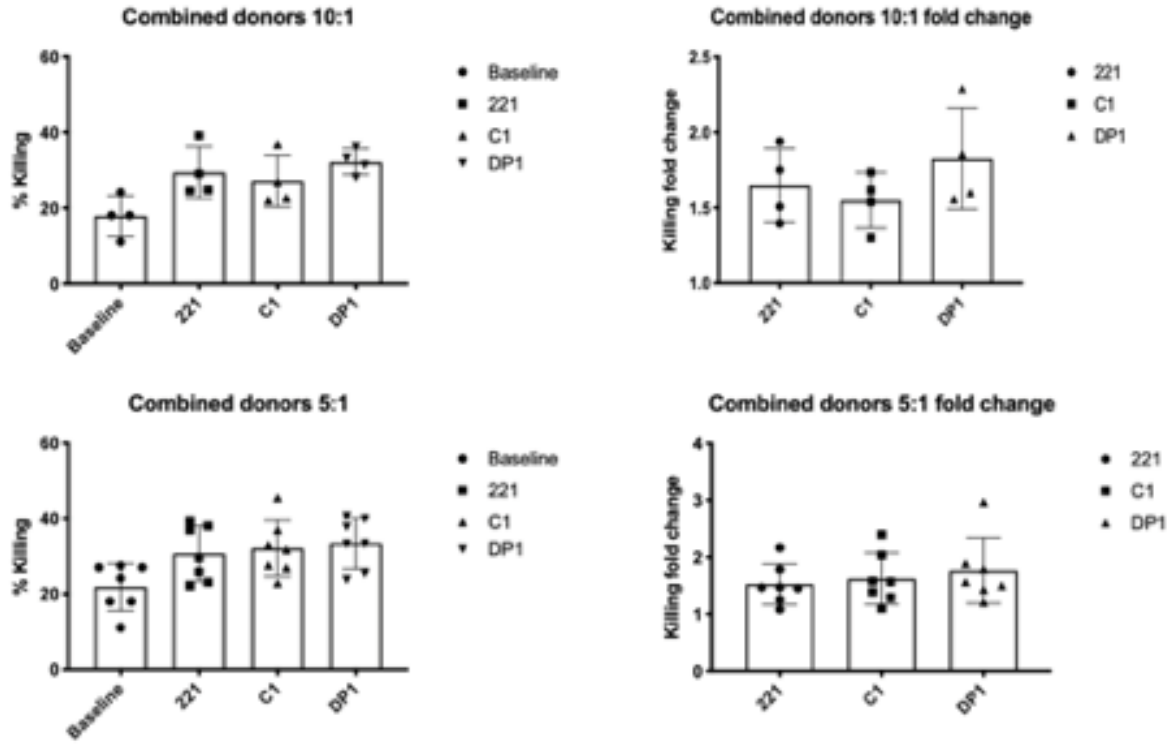


Fig 4.14. Pooled mean killing and killing fold change for all KIR2DS2 positive donors at E:T ratios of 5:1 and 10:1.

Overall pooled data (see figure 4.14) shows consistent killing across 221, C1 and DP1 groups. Mean killing fold change appears greater in the DP1 group at 10:1 E:T ratio, however this is contingent on one value, and is non-significant.

This experiment demonstrated that exosome stimulation did not enhance the cytotoxicity of PBMC under these experimental conditions. As with previous experiments, results may reflect chosen conditions, and optimisation may influence results. In particular, patterns of HCC cell line killing appeared to vary temporally, corresponding to when the experiment was performed, suggesting that the condition of cells in culture varied between experiments, which may influence results.

4.6 Discussion

Differential ultracentrifugation of cultured 721.221, C01, and DP1 cells successfully isolated exosomes, demonstrated by the presence of membrane bound, appropriately sized vesicles on electron microscopy and nanoparticle tracking analysis, and the presence of enriched exosome markers on Western blotting. No evidence of contamination was seen in preparations. However, no significant phenotypic or functional effects on KIR2DS2 NK cells, total NK cells, or PBMCs were observed in a range of experiments aiming to investigate their impact on cell proliferation, degranulation, and killing of hepatocellular carcinoma cell lines.

PBMC were utilised in these experiments, which aimed to measure an effect which would be produced by KIR2DS2 positive NK, which are a small proportion of cells present in PBMC. Therefore, any degranulation or killing related to exosome stimulation of KIR2DS2 positive NK may be not be visible, and a possible evolution of this protocol may be to use purified NK cells. This was not initially chosen as this project aims to investigate the utility of DP1 exosomes as a potential immunotherapy, and an effect only visible in a population of purified NK, and not in PBMC may not be clinically relevant.

These in-vitro experiments allowed relatively simple, reproducible, and timely investigation of the research question. However, there are some significant caveats which must be considered when designing and interpreting them. Normal homeostatic processes may be interrupted by cell culture, and so experimental conditions must be optimised to facilitate the processes which are being investigated. Variables include, but are not limited to, concentrations of cells, exosomes, cytokines and media, the length of incubation and co-culture, and the method of analysis. There are therefore a very large variety of potential conditions, which may not be practically investigated fully, and which may or may not offer a generalisable reflection of physiological conditions. For example, in these experiments IL-15 was included in culture media due to its essential role in NK cell homeostasis. This may have been insufficient to support NK function, and protocols for proliferation of NK include a wider array of cytokines as discussed in 4.3. However, supra-physiological concentrations may influence NK cell function to the extent that abnormal, and physiologically unreproducible effects are observed. A method of ameliorating some of these considerations is to investigate these interactions in-vivo, within a complex organism where these homeostatic processes are preserved to a greater extent, which will be addressed in chapter 5.

Chapter 5 Murine NK cell phenotype and function following exosome injection, and a murine tumour model

5.1 Introduction

The previous chapter focussed on exosome isolation and characterisation, and in-vitro experiments to examine the whether DP1 exosomes expressing a flavivirus peptide in the context of HLC A produce activation of KIR2DS2 positive NK cells. This chapter focusses on in-vivo experiments, using a mouse model to investigate this further, examining NK cell phenotype, markers of NK activation and degranulation, and survival and tumour growth in a heterotopic syngeneic tumour model following exosome injection. Full methods for this chapter can be found in chapter 2, 2.13-2.18.

Mouse models are the most common model organism for preclinical studies, playing a crucial role in elucidating the mechanisms by which cancer develops, and in development of therapies. Mice have several advantages as a model animal, including ubiquity, size, and a similar genome to humans with a wide variety of available genetic modifications, and established and validated methods of simulating disease. They preserve the complex homeostatic and immunological processes of a complex organism, which in-vitro experiments may lack. In-vivo testing is an essential pre-clinical step in drug development, providing data on efficacy and toxicity of therapies, and mouse models are the most common method of achieving this. Despite these advantages, mouse models have numerous limitations, and do not provide a complete simulation of human disease, or necessarily predict outcomes in humans.

Hepatocellular carcinoma develops in the context of chronic liver disease, and in a complex microenvironment which is the product of the liver's unique immunology, and the underlying disease, involving complex genetic and epigenetic alterations (see chapter 1). Consequently it is not currently possible to fully recreate these conditions in a mouse, but instead a variety of solutions have been developed to investigate specific aspects of HCC development and treatment. Genetic manipulation of oncogenes or tumour suppressor genes (such as liver specific p53

knockout or over-expression of MYC) enables study of hepatocarcinogenesis, but may have unreliable incidence, or long latency periods prior to development of HCC, affecting suitability for therapeutic studies. Intra-peritoneal injection of carcinogens such as Diethylnitrosamine (DEN) may be used to induce carcinogenesis. The most common induction method is injection of human or murine cancer cell lines into subcutaneous tissue (a heterotopic model), or into the liver (orthotopic model). Orthotopic models have the advantage of tumour growth occurring within the liver, with concomitant benefits of simulating the liver microenvironment, but are more technically demanding, more invasive, and more difficult to monitor than subcutaneous tumours which can be directly measured. Injected cell lines may be xenografts, injection of human cell lines, which may be preferred in order to reflect a human disease process, though an immunodeficient mouse must be used to prevent rejection. Syngeneic models utilise murine cell lines, which may not represent human disease as accurately, but grow in immunocompetent mice. Immunologically humanised mice have been developed in immunodeficient mice via transplantation of haematopoietic stem cells, to enable immunotherapy of xenograft cell lines to be studied, though this is complex, and engraftment may be difficult to establish(170).

A limitation of these HCC induction methods is that they do not include a representation of liver disease, fibrosis, and cirrhosis. Intra-peritoneal injection or oral gavage of carbon tetrachloride is widely used to promote hepatic fibrosis, and may be combined with other methods to produce HCC with cirrhosis. Specific aetiologies of cirrhosis can be simulated. Alcohol-induced liver disease protocols include alcohol containing feeding regimes, which are limited by mouse aversion to alcohol, and surgical alcohol pumps may be used. Features of fatty liver disease can be induced through diet or genetic modification, though not all models produce progressive liver disease and accompanying metabolic syndrome. For example db/db or ob/ob mice either have a leptin receptor mutation, or lack functional leptin respectively, and produce a phenotype representative of metabolic syndrome, but require additional stimuli to produce hepatic fibrosis. Hepatitis C infection can be simulated through transgenic expression of envelope genes E1 and E2, and Hepatitis B through transgenic expression of HBV large envelope polypeptide, producing accumulation of HBV surface antigen and HBx protein, producing chronic hepatitis, and HCC in approximately 12 months.

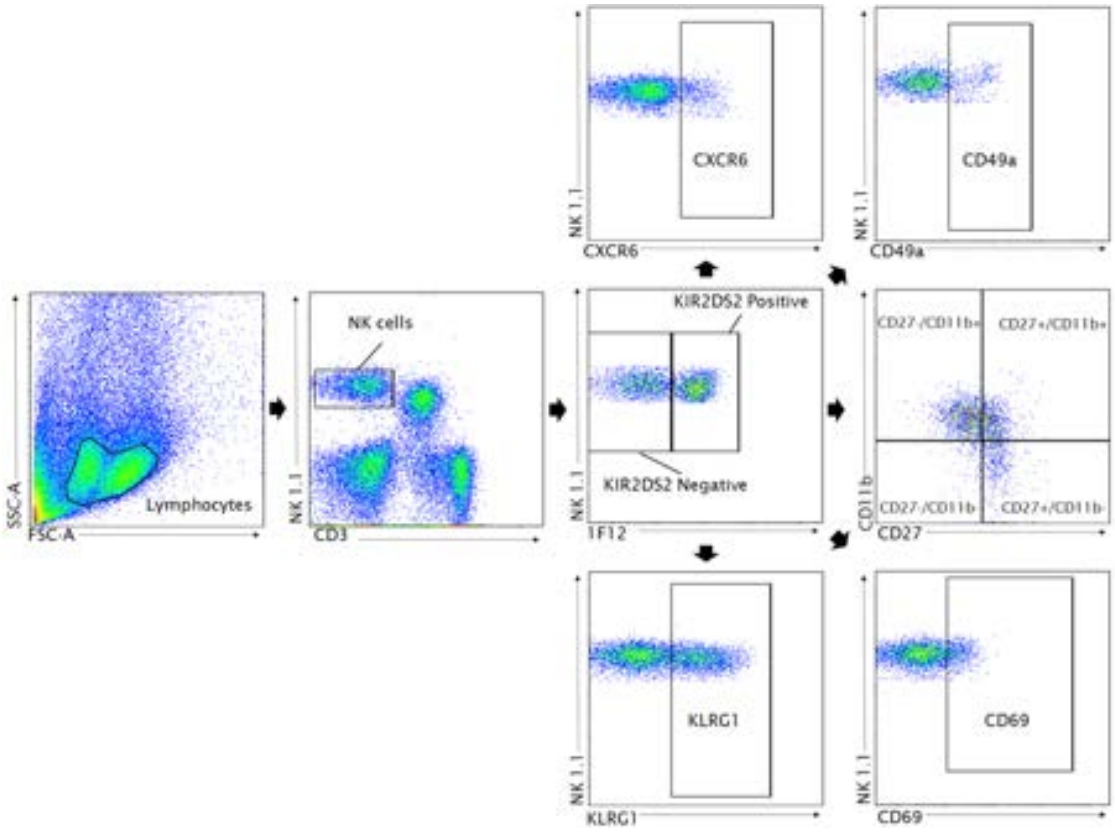
In this study I am examining the phenotypic and functional effects of exosomes on NK cells as a potential therapy for hepatocellular carcinoma, particularly focussing on exosomes expressing flavivirus peptides, targeting the activating KIR 2DS2 receptor. As discussed in chapter 1, mice lack

KIRs, instead having the Ly49 family of receptors, which are functionally analogous to human KIR, but structurally distinct. Therefore, a KIR transgenic mouse, genetically modified to express human KIR on murine NK cells, on a C57BL6 background was used in all experiments(171, 172).

5.2 Phenotype of liver and spleen NK cells from exosome injected KIR transgenic mice

In the first two experiments 9 mice were randomly assigned to equal groups receiving a single dose of 221, C01, or DP1 exosomes in endotoxin-free PBS via tail vein injection. Dosage was determined by BCA protein estimation of exosome preparations, receiving 60mcg per mouse in 100ul endotoxin free PBS. This initial dose was chosen based on the quantity of exosome which could be reliably produced, limited by ultracentrifuge capacity. Mice were monitored for signs of toxicity until culling on day 7, after which livers and spleens were processed, and hepatic lymphocytes and splenocytes analysed via flow cytometry. This experiment was then repeated with a further 9 mice. Full methods can be found in chapter 2.10 to 2.15. Phenotype was assessed using the panel in figure 5.1.

Figure 5.1 Representative flow cytometry plots for murine phenotype.



This panel identifies KIR2DS2 via the 1F12 antibody. CXCR6, and CD49a are associated with liver residence and adaptive, memory-like characteristics. CD27/CD11b defines NK cell maturity, with CD27-/CD11b- representing the least mature, progressing to CD27+/CD11b-, CD27+/CD11b+, and CD27-/CD11b+ representing the most mature NK cells. KLRG1 is up-regulated in activated NK cells. CD69 is a marker of both liver residence and activation(69).

No toxicity was observed, so a dose escalation experiment was conducted, during which 6 mice randomly assigned to C01 or DP1 groups received 2 doses of 60mcg on day 0 and day 7, and a further 6 mice received 2 doses of 120mcg on day 0 and day 7. 221 was excluded due to the large quantity of exosomes required for higher doses, and limited ultracentrifuge capacity. Again, no toxicity was observed, and all mice were sacrificed on day 14, and liver lymphocytes and splenocytes analysed.

Figure 5.2. Hepatic NK cell frequency was significantly increased in DP1 injected mice

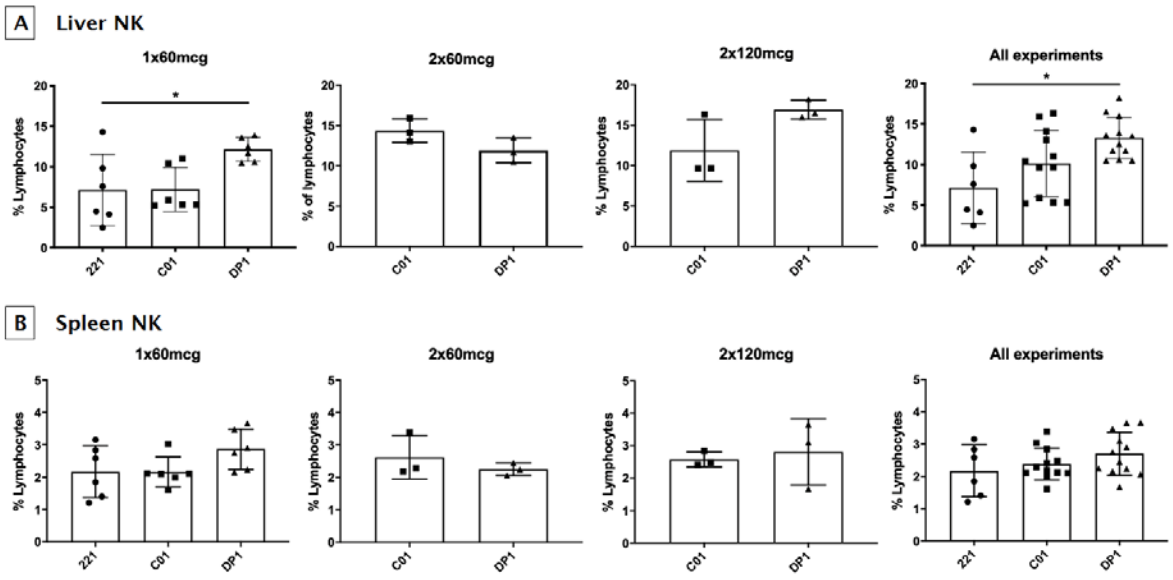


Fig 5.2.Mean and SD liver (A) and spleen (B) NK cell frequency at various dosing strategies, and pooled data for all experiments. Statistical analysis with Kruskal-Wallis test where 3 groups, and Mann Whitney test where 2 groups.

Liver NK cell frequency was significantly greater in DP1 groups compared to C01 and 221 groups in the 1x60mcg experiment (p=0.024), and in pooled data of all experiments (p=0.014). The 2x120mcg experiment shows a trend consistent with this finding, though within the 2x60mcg experiment mean NK frequency was greater in the C01 group. The trends within splenic NK cells follow a the same pattern of higher mean expression in DP1 groups in 1x60mcg, 2x120mcg, and overall, but to a lesser degree, with no statistically significant difference found.

Figure 5.3 Liver and spleen KIR2DS2 positive NK cell frequency was not significantly different across exosome groups.

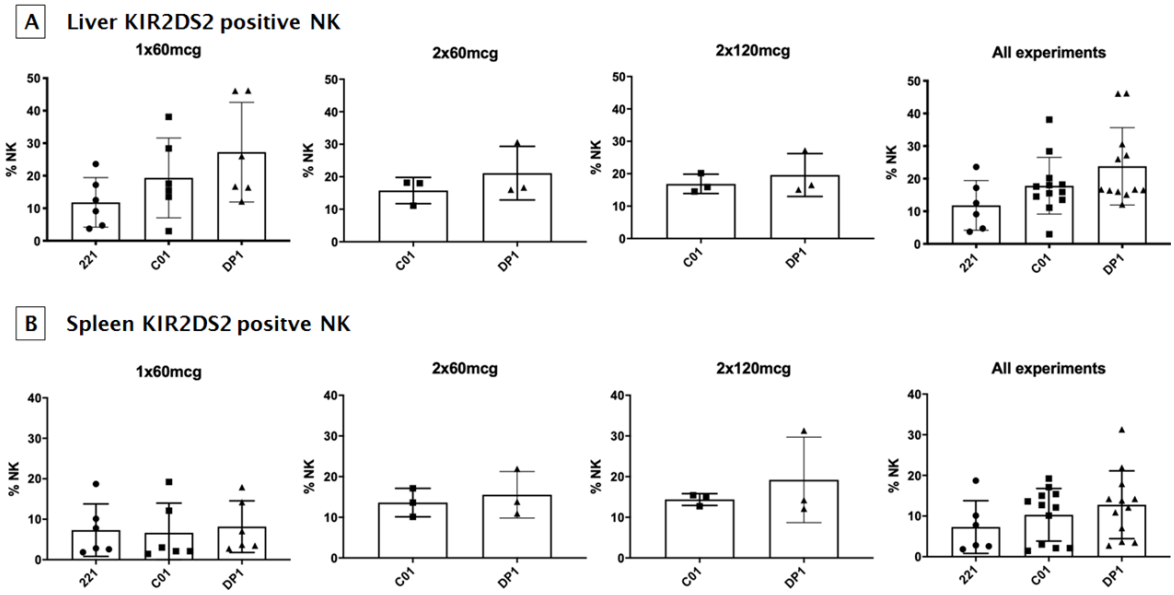


Fig 5.3 Mean and SD liver (A) and spleen (B) 2DS2 positive NK cell frequency at various dosing strategies, and pooled data for all experiments. Statistical analysis with Kruskal-Wallis test where 3 groups, and Mann Whitney test where 2 groups.

A non-significant trend can be seen in liver KIR2DS2 positive NK towards higher frequency in the DP1 treatment group in the liver, seen in all dosing strategies and in pooled data. However, this is dependent on few individuals; in pooled data 7/12 individuals in the DP1 group are clustered between 12-18% KIR2DS2 positive NK, in common with 8/12 individuals in the C01 group. The trend is produced by 4 individuals exceeding 26% KIR2DS2 frequency. This is mirrored in the spleen, where DP1 has the highest mean frequency in overall data, but is contingent on 2 individuals. No conditions meet statistical significance. Taken with the total liver NK increase seen in figure 4.2, this suggests that injection with DP1 exosomes may have stimulated proliferation of liver NK to a greater extent than C01 or 221 exosomes, but this effect may not be entirely explained by the specific interaction of the DP1 peptide and KIR2DS2, raising the possibility that the peptide may interact with other NK receptors or possibly antigen presenting cells which may indirectly stimulate NK.

Figure 5.4 Liver and spleen CD49a was not significantly different between exosome groups after a single dose.

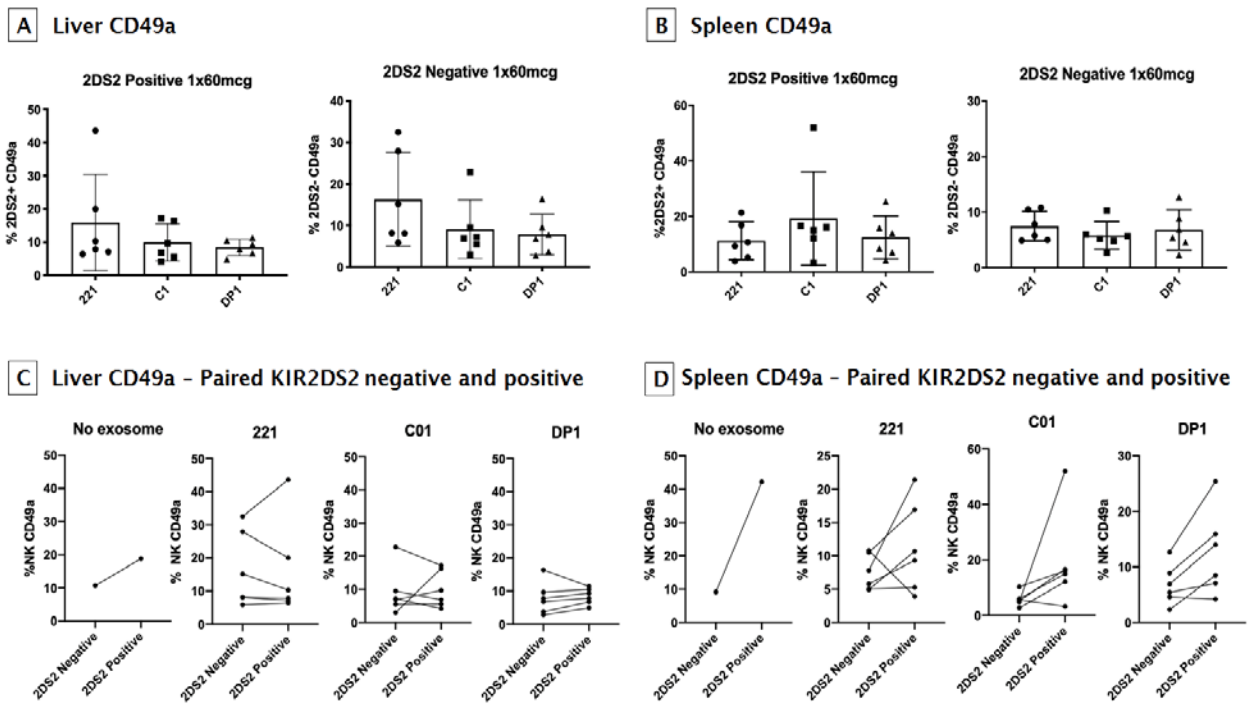
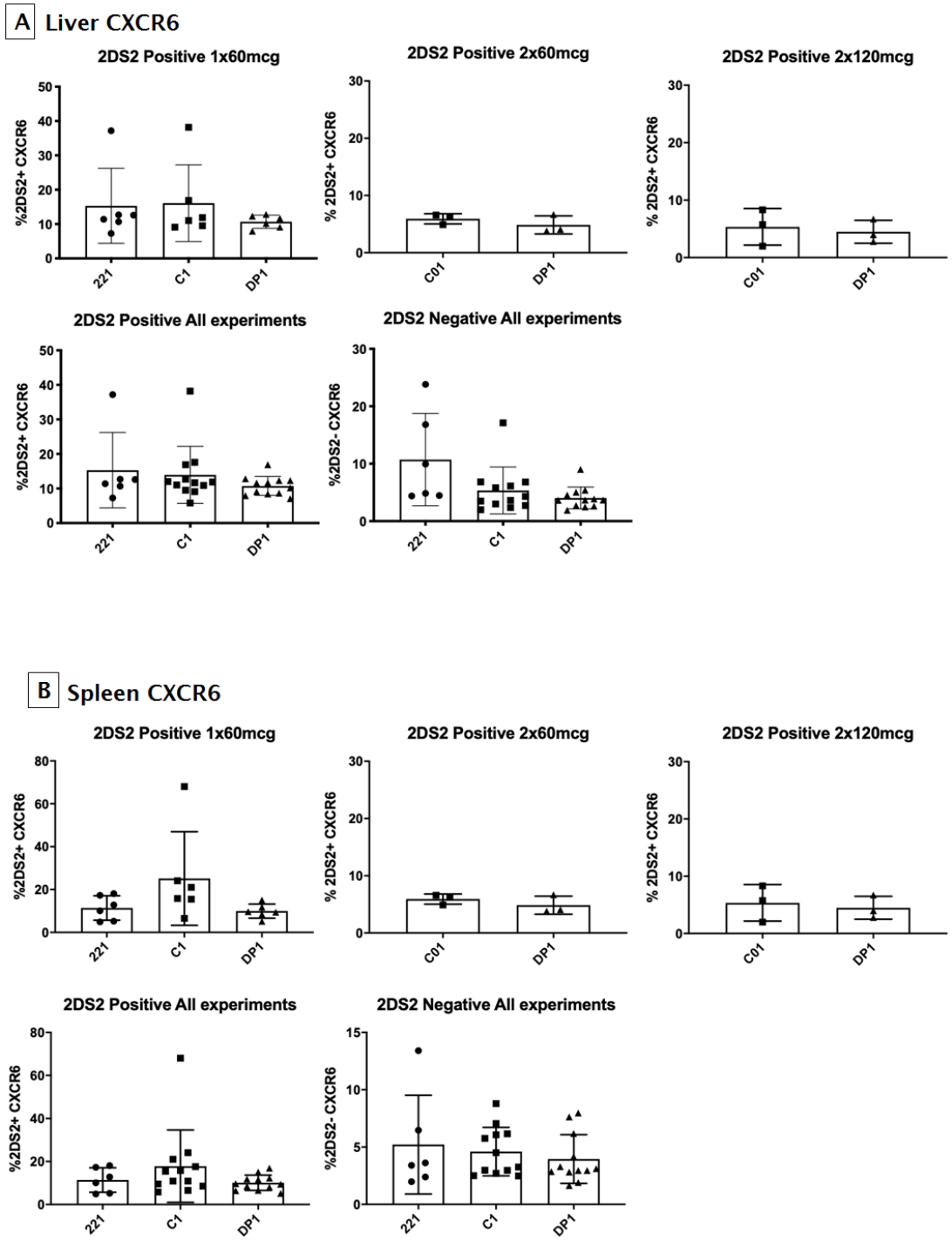


Fig 5.4 Mean and SD liver (A), and spleen (B) CD49a expression in KIR2DS2 positive and negative NK cells. Paired analysis of CD49a expression in KIR2DS2 positive and negative NK cells in liver (C) and spleen (D). Statistics by Kruskal Wallis test in (A) and (B), and Wilcoxon matched-pairs signed rank test in (C) and (D).

CD49a was included in the flow cytometry panel only in the 1x60mcg initial experiments. As it gave no positive data it was removed from the panel in order to accommodate CD69 in subsequent experiments. No significant differences in expression were observed between exosome groups in KIR2DS2 positive or negative NK cells. Paired analysis showed a trend towards greater CD49a expression in KIR2DS2 positive spleen NK cells vs KIR2DS2 negative NK cells, though this did not achieve significance, and is seen in all exosome groups, and in a single KIR transgenic mouse not injected with exosomes. This may suggest that KIR2DS2 positive NK cells in the spleen may have inherently greater CD49a expression than the 2DS2 negative NK cells, and this trend may not be related to injection of exosomes.

Figure 5.5 Liver and spleen CXCR6 was not significantly different between exosome groups in any of the dose ranges. CXCR6 expression was significantly greater in KIR2DS2 positive NK cells vs negative NK cells.



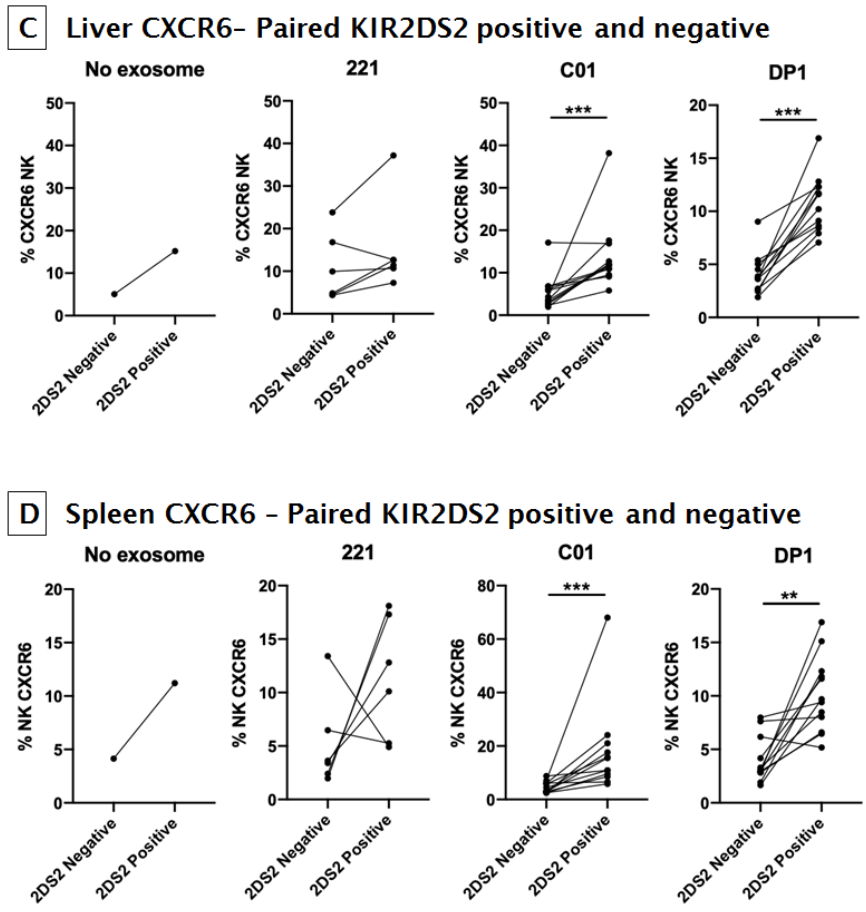
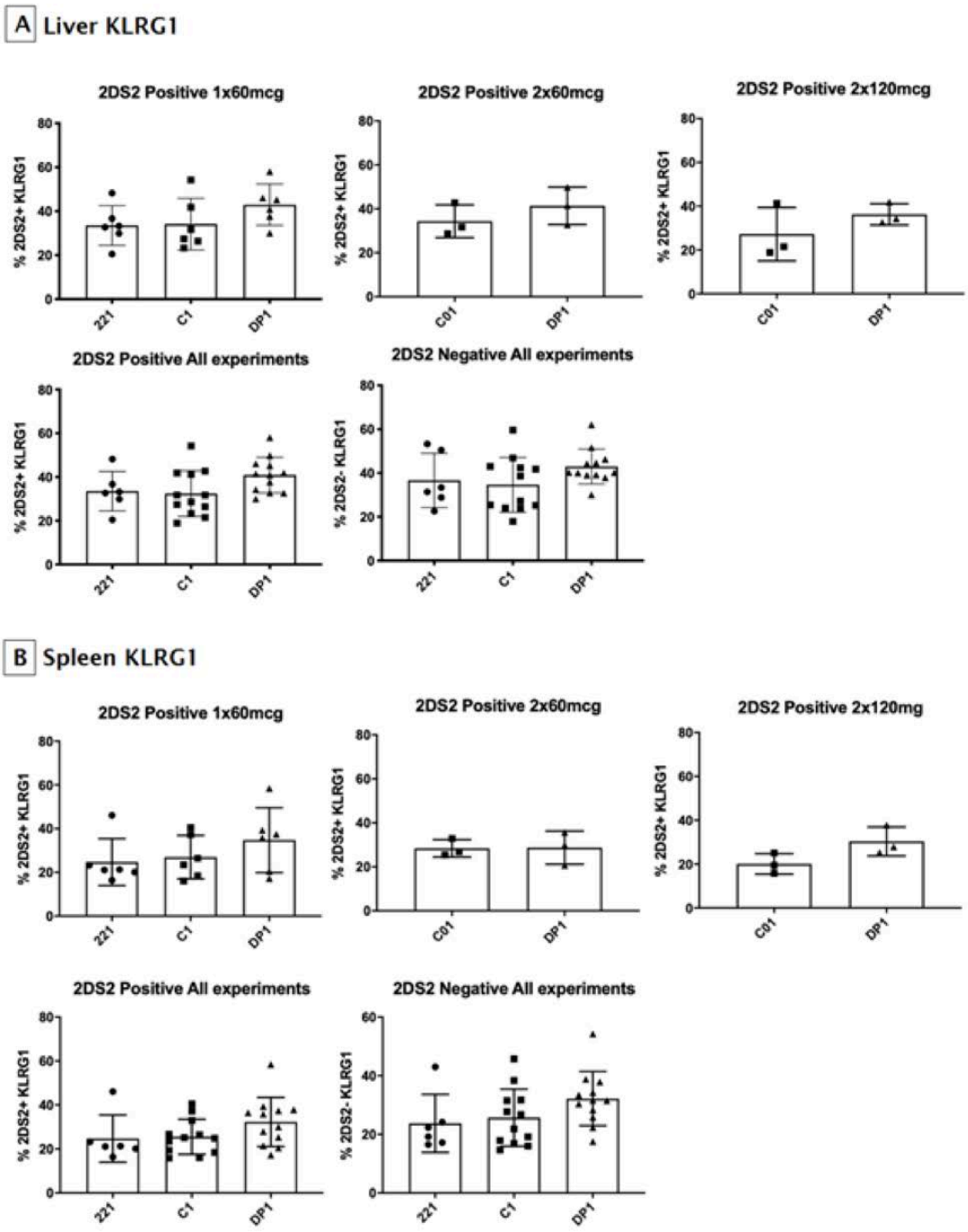


Fig 5.5 Fig 4.4 Mean and SD liver (A), and spleen (B) CXCR6 expression in KIR2DS2 positive and negative NK cells. Paired analysis of CXCR6 expression in KIR2DS2 positive and negative NK cells in liver (C) and spleen (D). Statistics by Kruskal Wallis test where 3 groups in (A) and (B), Mann Whitney test where 2 groups, and Wilcoxon matched-pairs signed rank test in (C) and (D).

No significant difference was observed in CXCR6 frequency between exosome groups in livers or spleens throughout all dosing strategies. However, as seen in figure 4.5 (C) and (D), paired analysis demonstrated significantly greater CXCR6 expression in KIR2DS2 positive vs negative NK cells in C01 and DP1 groups in liver and spleens. A similar trend can be seen in 4 of 6 individuals in the 221 group, and in no exosome which did not reach significance. More data are required to interpret these findings. C01 and DP1 groups are larger than either no exosome or 221. If under equal conditions they reached significance this would suggest, as with CD49a, that the baseline phenotype of KIR2DS2 positive NK cells includes greater CXCR6 expression than KIR2DS2 negative NK cells. Alternatively, if with more data, 221 and no exosome groups did not show a significant difference, this would indicate that C01 and DP1 exosomes may induce CXCR6 expression in KIR2DS2 positive cells.

Figure 5.6 KLRG1 expression was not significantly different between any exosome groups, or between KIR2DS2 negative and positive NK cells.



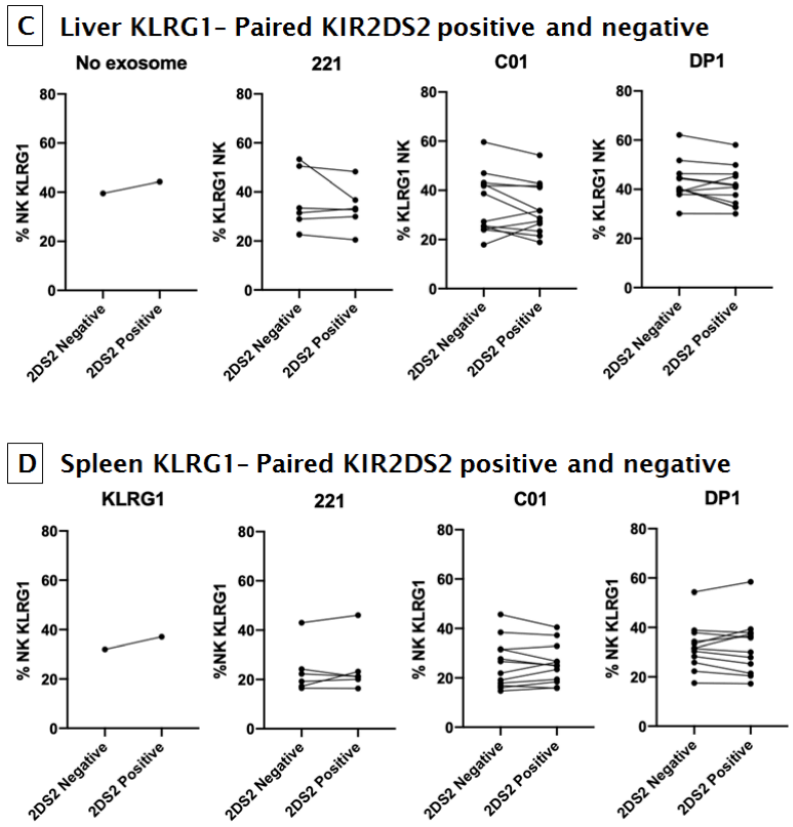


Fig 5.6 Mean and SD liver (A), and spleen (B) KLRG1 expression in KIR2DS2 positive and negative NK cells. Paired analysis of KLRG1 expression in KIR2DS2 positive and negative NK cells in liver (C) and spleen (D). Statistics by Kruskal Wallis test where 3 groups in (A) and (B), Mann Whitney test where 2 groups, and Wilcoxon matched-pairs signed rank test in (C) and (D).

There was no statistically significant difference in KLRG1 expression between exosome groups, though as seen in figure 4.6 (A) and (B), mean expression was greatest in the DP1 group in all experiments in the liver and spleen in KIR2DS2 positive and negative NK. This raises the possibility that further optimisation of the experiment may produce a significant result, such as a higher dose, more doses, or a longer interval between dosing and analysis. However, the presence of this trend in KIR2DS2 negative NK suggests that this may not be due to the interaction of the DP1 flavivirus peptide with the KIR2DS2 receptor, and again suggests that there may be interactions between DP1 exosomes and other receptors or cells. Exosomes are likely to present other antigens which may provoke NK cell responses, however, these are equally likely to be expressed by C01 and 221 exosomes, so this pattern of results suggests an effect provoked by the DP1 peptide, but in a manner not specific to KIR2DS2. There was no discernible difference between KIR2DS2 positive and negative NK seen on paired analysis.

Figure 5.7 Liver and Spleen CD69 did not vary between exosome groups, but is greater in KIR2DS2 positive than negative NK cells.

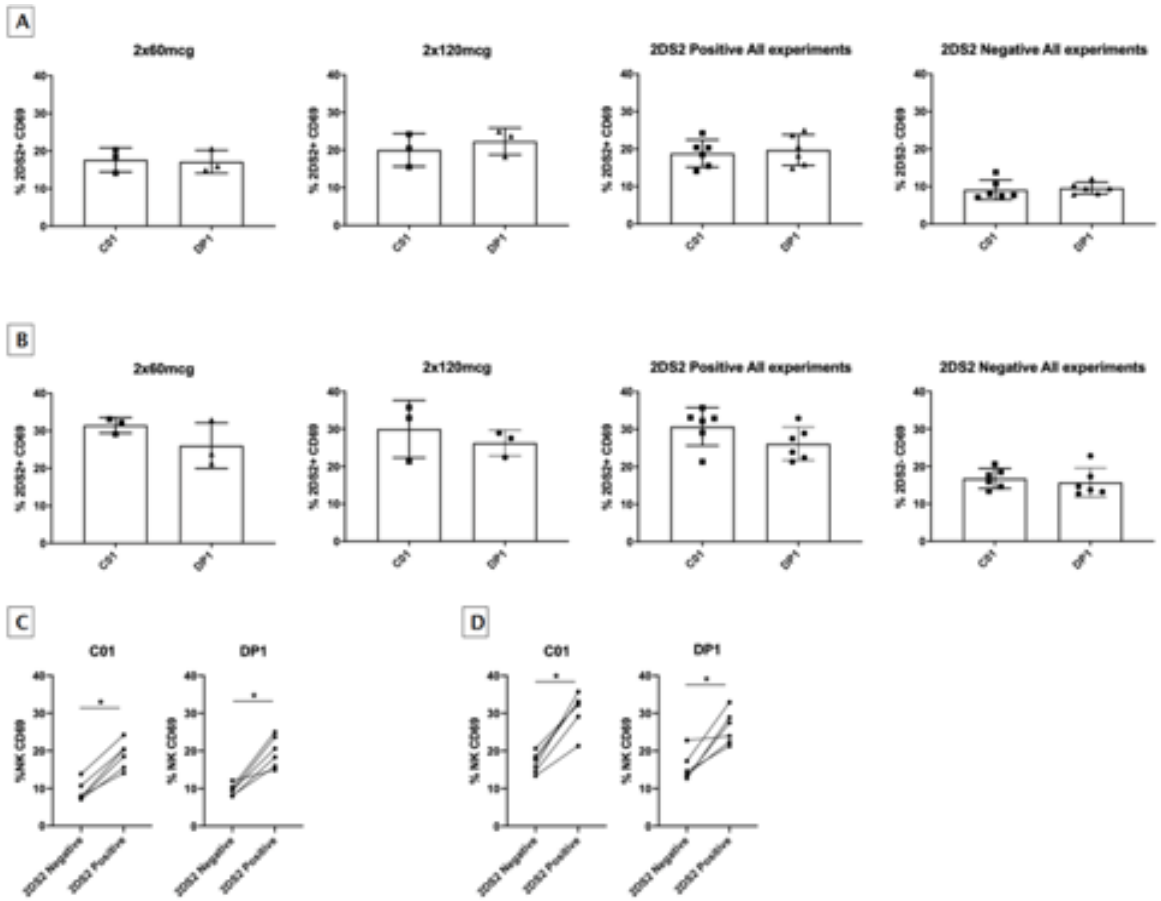
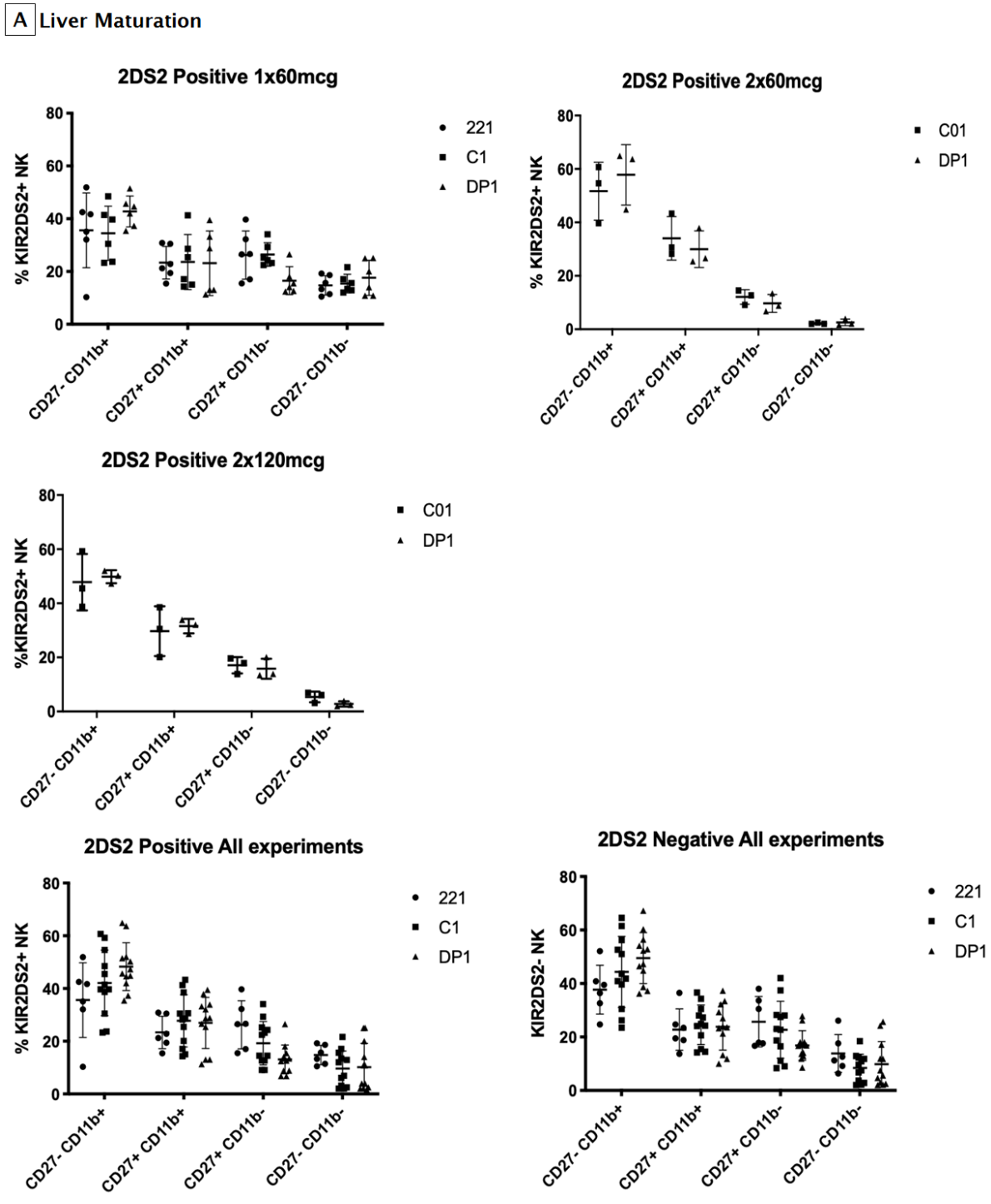


Fig 5.7 Mean and SD liver (A), and spleen (B) CD69 expression in KIR2DS2 positive and negative NK cells. Paired analysis of CD69 expression in KIR2DS2 positive and negative NK in liver (C) and spleen (D). Statistical analysis by Mann Whitney test (A) and (B), and Wilcoxon matched-pairs signed rank test in (C) and (D).

Following the first two single-dose experiments CD69 was included in the analysis in the place of CD49a to provide a marker of NK activation, as previous experiments had not demonstrated evidence of enhanced NK cell memory markers. Therefore, data were obtained for the 2x60mcg and 2x120mcg doses. No difference was seen between 60mcg and 120mcg at either dose. Expression in KIR2DS2 positive NK cells was significantly greater than KIR2DS2 negative NK cells in both C01 and DP1 groups. This therefore raises the same possibilities as the KLRG1 paired analysis; that non-peptide specific exosome stimulation is possible, but more data is required to draw firm conclusions as this may instead reflect the inherent phenotype of KIR2DS2 positive NK cells.

Figure 5.8. Liver maturation. CD27-/CD11b mature NK cell frequency is significantly greater in DP1 in KIR2DS2 positive and negative NK cells.



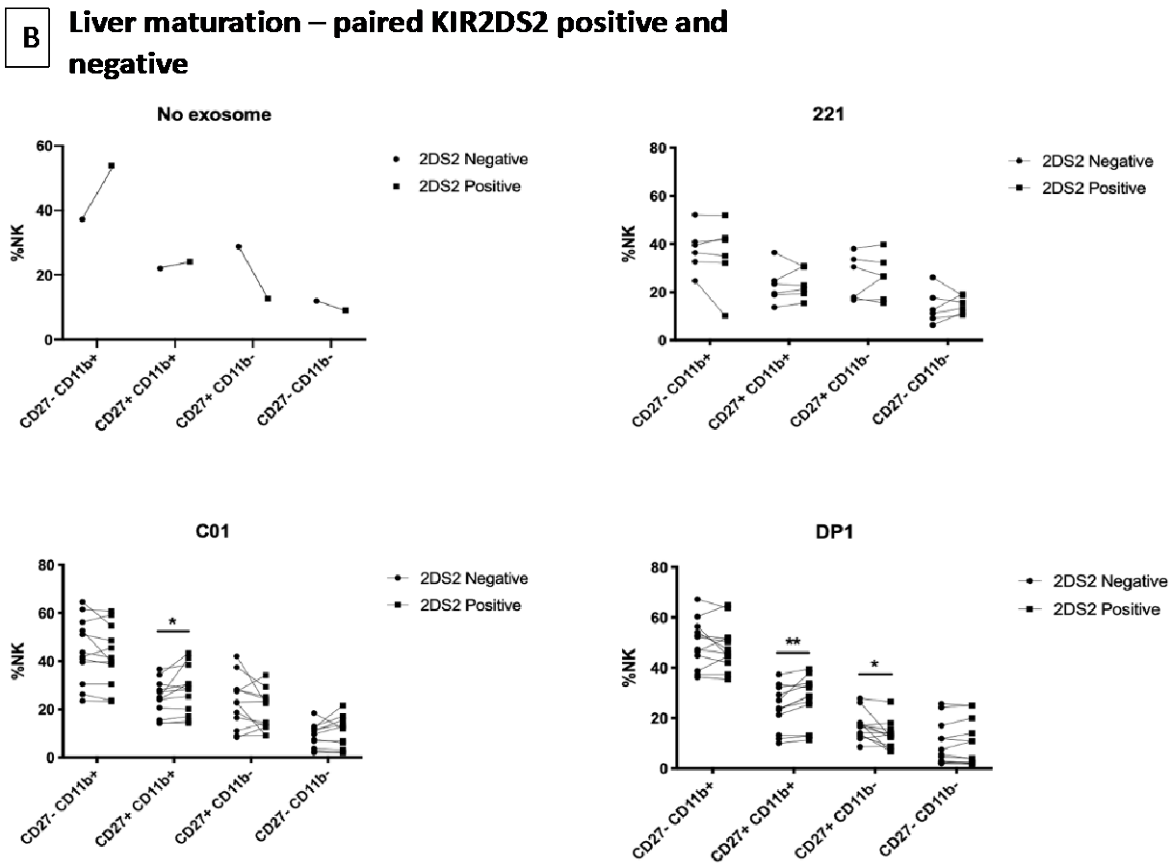
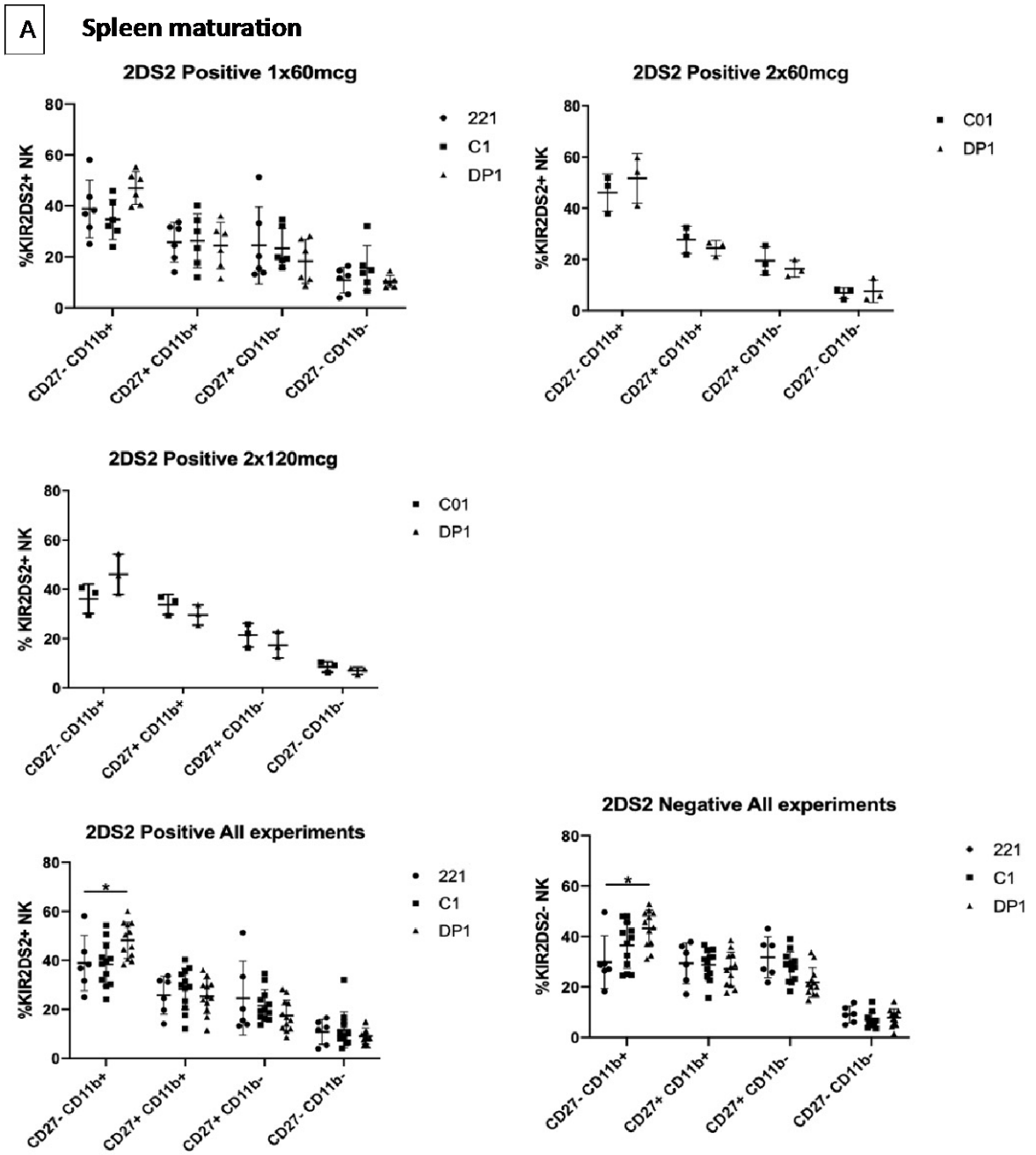


Figure 5.8 Mean and SD liver maturity in KIR2DS2 positive and negative NK cells (A). CD27-/CD11b+ most mature, CD27-/CD11b- least mature. Paired analysis of maturity in KIR2DS2 positive and negative NK (B). Statistics using Kruskal Wallis test where 3 groups in (A), Mann Whitney test where 2 groups, and Wilcoxon matched-pairs signed rank test in (B).

Figure 5.9. Spleen maturation. CD27-/CD11b mature NK cell frequency is significantly greater in DP1 in KIR2DS2 positive and negative NK cells.



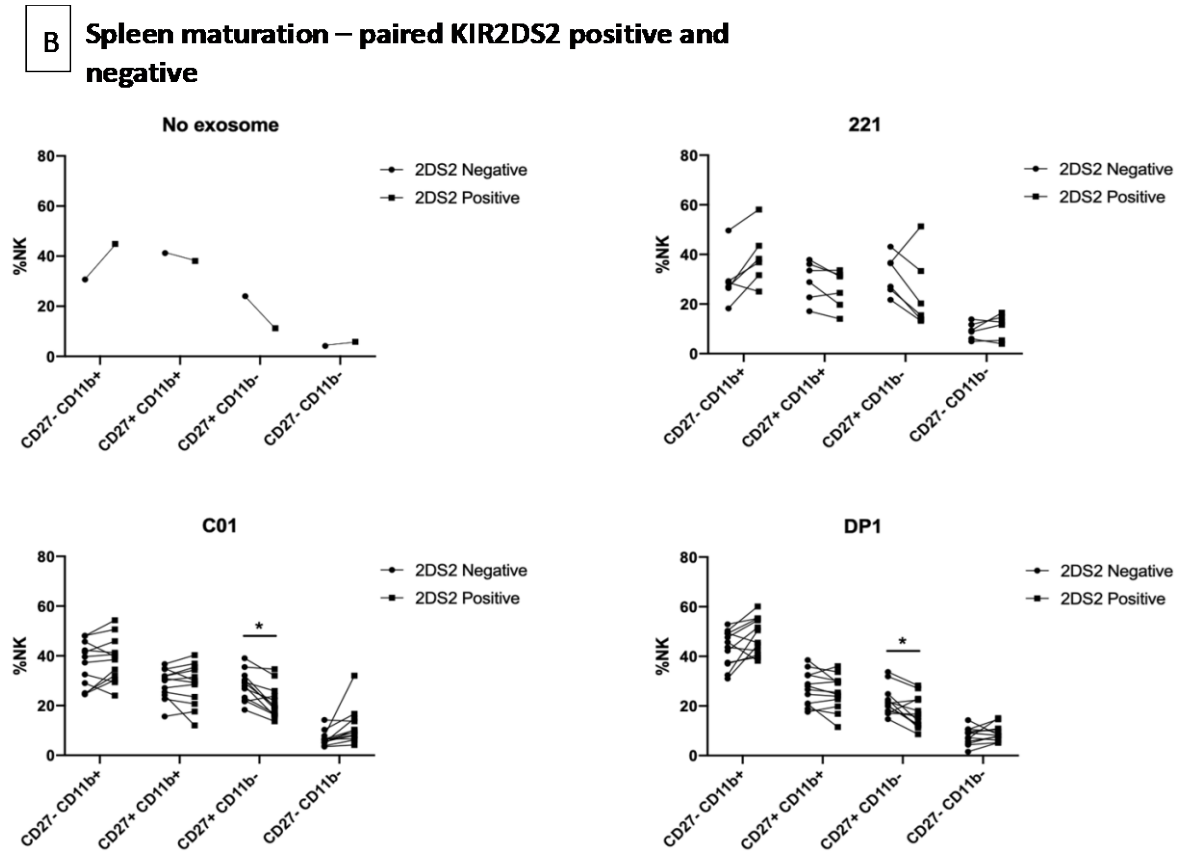


Figure 5.9 Mean and SD splenocyte maturity in KIR2DS2 positive and negative NK cells (A). CD27-/CD11b+ most mature, CD27-/CD11b- least mature. Paired analysis of maturity in KIR2DS2 positive and negative NK (B). Statistics using Kruskal Wallis test where 3 groups in (A), Mann Whitney test where 2 groups, and Wilcoxon matched-pairs signed rank test in (B).

CD27-/CD11b+ mature NK cells were significantly enriched in DP1 vs C01 or 221 splenocytes in KIR2DS2 positive pooled data (p=0.03), and KIR2DS2 negative data (p=0.03). A similar trend is seen in all liver and spleen experiments, with a further non-significant trend towards reduced frequency of less mature CD27+/CD11b- NK cells (see figure 4.8 and 4.9 (A) and (B)), suggesting a shift from less mature to more mature NK cells in DP1 injected mice. As discussed above, the presence of this effect in 2DS2 positive and negative populations suggests an effect specific to the DP1 exosome, but not specific to the KIR2DS2 receptor. Paired analysis shows significantly greater CD27+/CD11+ expression in KIR2DS2 positive NK cells vs negative in C01 and DP1, and significantly lower expression of CD27+/CD11b- in positive vs negative in splenocytes. The trend from the un-injected mouse suggests that this may also be due to the pre-existing phenotype of KIR2DS2 positive cells.

5.3 CD107a functional assay of murine splenocytes from exosome injected mice with PLC HCC targets.

Following phenotypic analysis of the dose expansion experiments (2x60mcg and 2x120mcg groups), splenocytes from the C01 and DP1 mice were cultured overnight, and a CD107a functional assay with PLC HCC cell line targets conducted the following day, assessing degranulation of NK cells in response to a cancer cell line. A 5:1 effector:target ratio was used. Due to technical issues with the cytometer, 2 samples were lost, a C01 2x60mcg vs PLC sample, and a C01 2x120mcg no target sample. Evidence of this can be seen in the gating strategy, figure 4.9, where problems with the stream have affected the NK 1.1/CD3 plot.

Figure 5.10 Representative flow cytometry gating for CD107a assay

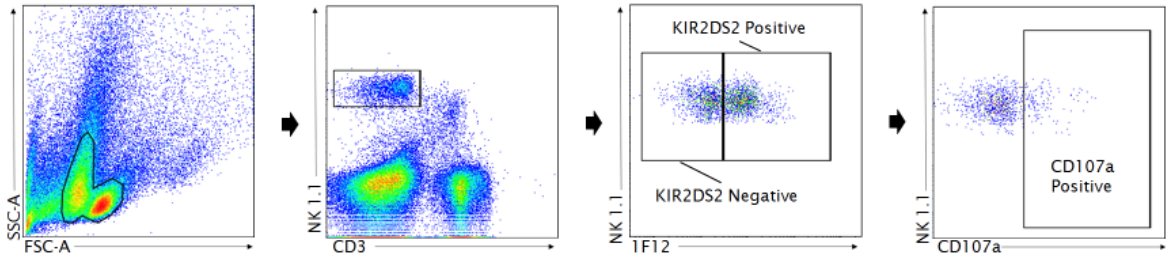


Figure 5.11 Degranulation was not significantly different between C01 and DP1 exosome groups, or between KIRDS2 positive and negative NK.

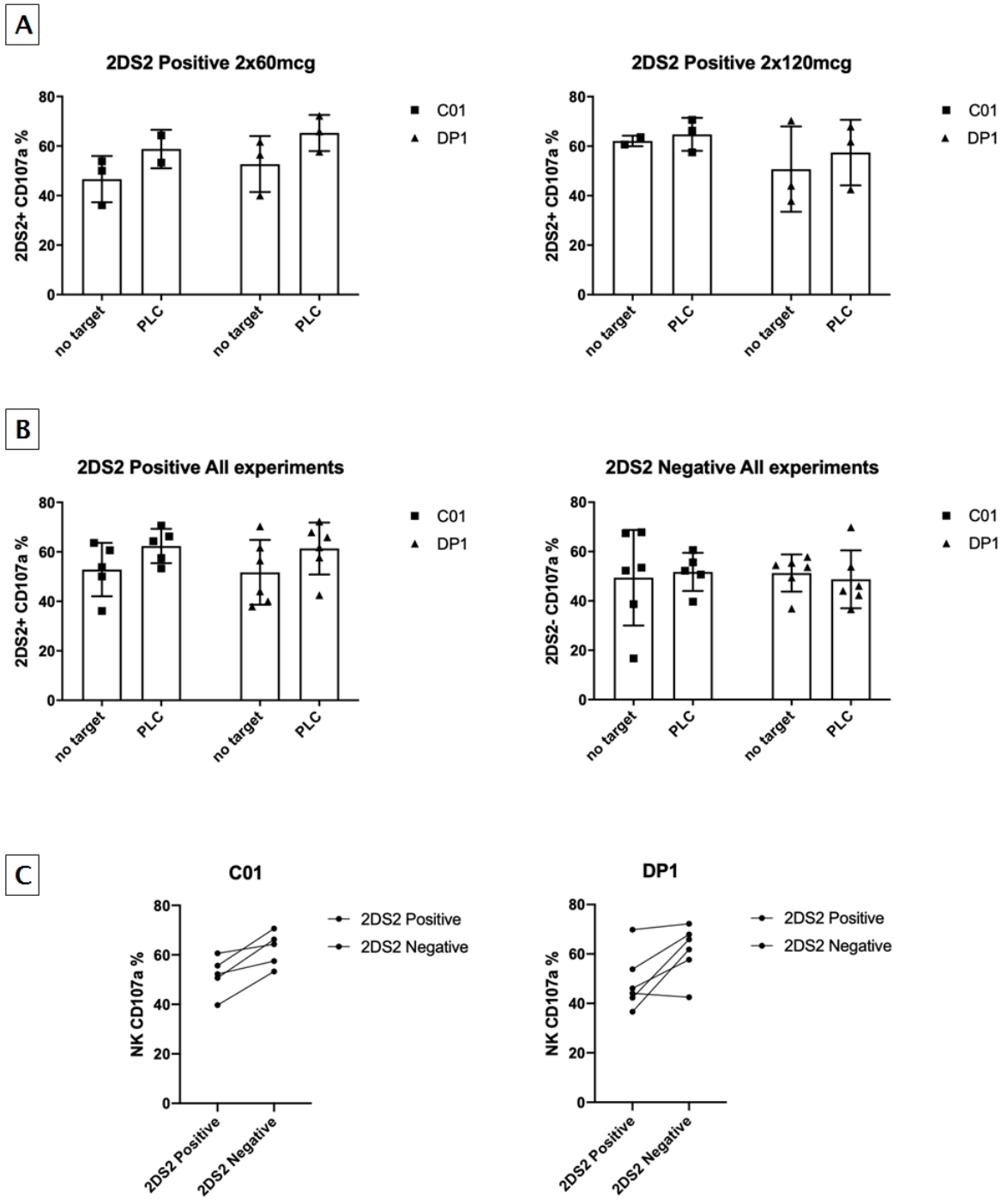


Fig 5.11 CD107a expression in KIR2DS2 positive NK cells at 2x60mcg and 2x120mcg doses of C01 and DP1 exosome (A), pooled CD107a expression for both experiments in KIR2DS2 positive and negative NK cells (B), paired analysis of CD107a expression in KIR2DS2 positive vs negative NK cells in C01 and DP1 groups (C). Statistical analysis by Mann Whitney test (A) and (B), and Wilcoxon matched-pairs signed rank test in (C) and (D).

No difference was seen in CD107a expression between C01 and DP1, in KIR2DS2 positive and negative NK cells, and no dose-dependent response was observed. There was a non-significant trend towards enhanced cytotoxicity in KIR2DS2 positive vs negative NK cells. In all conditions, no target CD107a expression was high; in KIR2DS2 negative overall, and 2x120mcg KIR2DS2 positive C01 conditions, the baseline cytotoxicity was equal or greater than those with PLC targets (figure 4.10 (A) and (B)). This may indicate a problem with the experiment protocol, or indicate poor condition of the cultured splenocytes.

5.4 Murine tumour immunotherapy model

The final experiment of this chapter is a murine tumour model investigating whether DP1 exosome injection influenced tumour growth, time taken to reach the humane endpoint (survival), and NK cell invasion of tumours seen on histology. Only one tumour experiment was conducted due to increased clinical commitments and lab closures during the COVID-19 pandemic.

As a KIR transgenic (KIR Tg) immunocompetent mouse was required, this restricted tumour selection to syngeneic models; murine rather than human cell lines. Adoptive transfer of NK cells from a KIR transgenic to an immunodeficient mouse model would allow study of human cell lines, but would require a larger number of mice, so a syngeneic model was selected in accordance with the principles of 'Replace, Reduce, Refine'(173). A heterotopic model was selected to allow tumour growth to be measured, as subcutaneous tumour growth can easily be measured with callipers, rather than orthotopic injection of a cell line into the liver, which is technically challenging, invasive, and the growth of which requires imaging to characterise. This therefore provided a mouse model which enabled the study of activation of NK cells via the KIR2DS2 receptor, and the impact of this on tumour growth, but it did not model the disease processes or tumour microenvironment associated with HCC formation. Preliminary experiments conducted by a colleague established that the available murine HCC cell lines would not grow in KIR Tg mice, though the B16 melanoma cell line, and RMA-S lymphoma cell line did. RMA-S was chosen as it is known to be NK sensitive(174), it was possible to grow a discrete, solid tumour more amenable to measurement with calipers and histology, and grew less rapidly than B16, facilitating a longer experiment. For this first tumour experiment, two groups of 4 mice were injected via tail vein with either endotoxin-free PBS or DP1 exosomes in PBS. An exosome control was not used to establish

if any effect from exosome injection could be observed in the first instance. Mice were randomly assigned to groups and injected with $1x10^6$ RMA-S cells subcutaneously, and 100mcg of DP1 exosome or 100ul PBS on day 0, then had a further injection of exosome or PBS on day 7. Once tumours were seen they were measured daily, with the measurer blinded to the group to which the mouse belonged.

Figure 5.12 Survival plot for PBS and DP1 groups

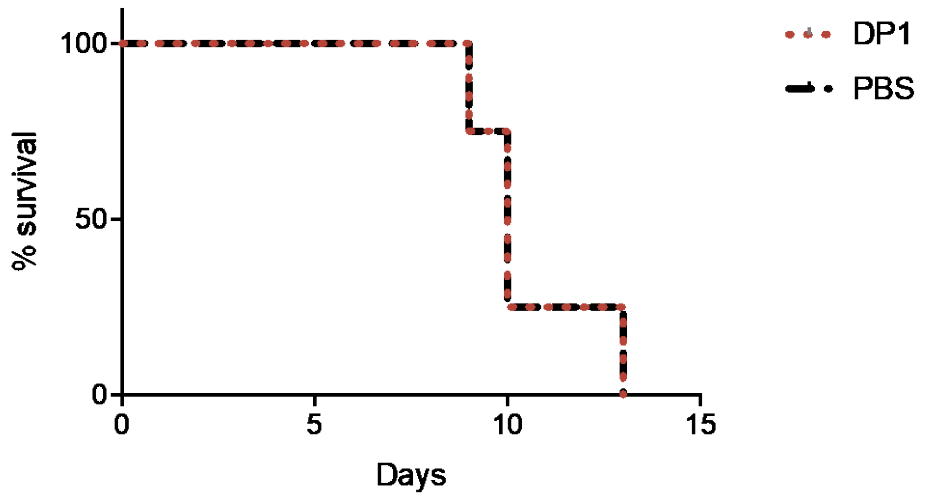


Fig 5.12 % Survival of KIR Tg mice with RMA-S tumours in DP1 exosome and PBS groups.

Mice were culled after reaching the study protocol humane endpoint. PBS and DP1 groups met endpoints equally (see figure 4.11). However a new project licence was introduced prior to the experiment, mandating a humane endpoint of a tumour 15mm length in any axis, rather than 15mmx15mm as planned prior to this. Some mice with low tumour volume, but meeting project licence criteria were therefore culled, where under the planned protocol the experiment would have continued.

Figure 5.13 RMA-S tumour volume in PBS and DP1 groups

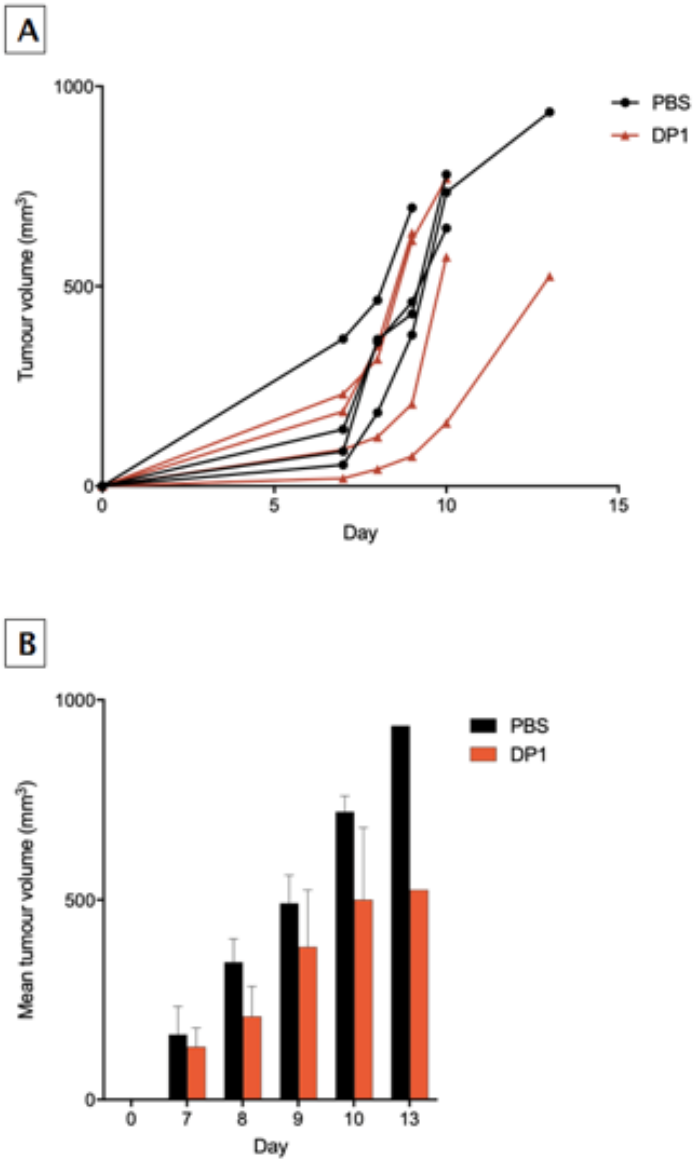


Fig 5.13 RMA-S tumour volumes for individual mice (A), and mean and SD tumour volume for all mice in PBS and DP1 groups (B).

As shown in figure 5.12, tumours were seen from day 7, and grew rapidly following identification. Mice were culled on days 9,10, and 13. There are no significant differences in tumour volume between PBS and DP1 groups at any time points. As shown in figure 4.12 (A), a non-significant trend towards lower mean tumour volume was seen in the DP1 group due to the survival of a DP1 injected individual with low tumour volume, and a PBS individual with high tumour volume. Otherwise all mice are clustered tightly together irrespective of group, and culled on day 9 or 10.

Figure 5.14 Histology of heterotopic murine RMA-S tumours

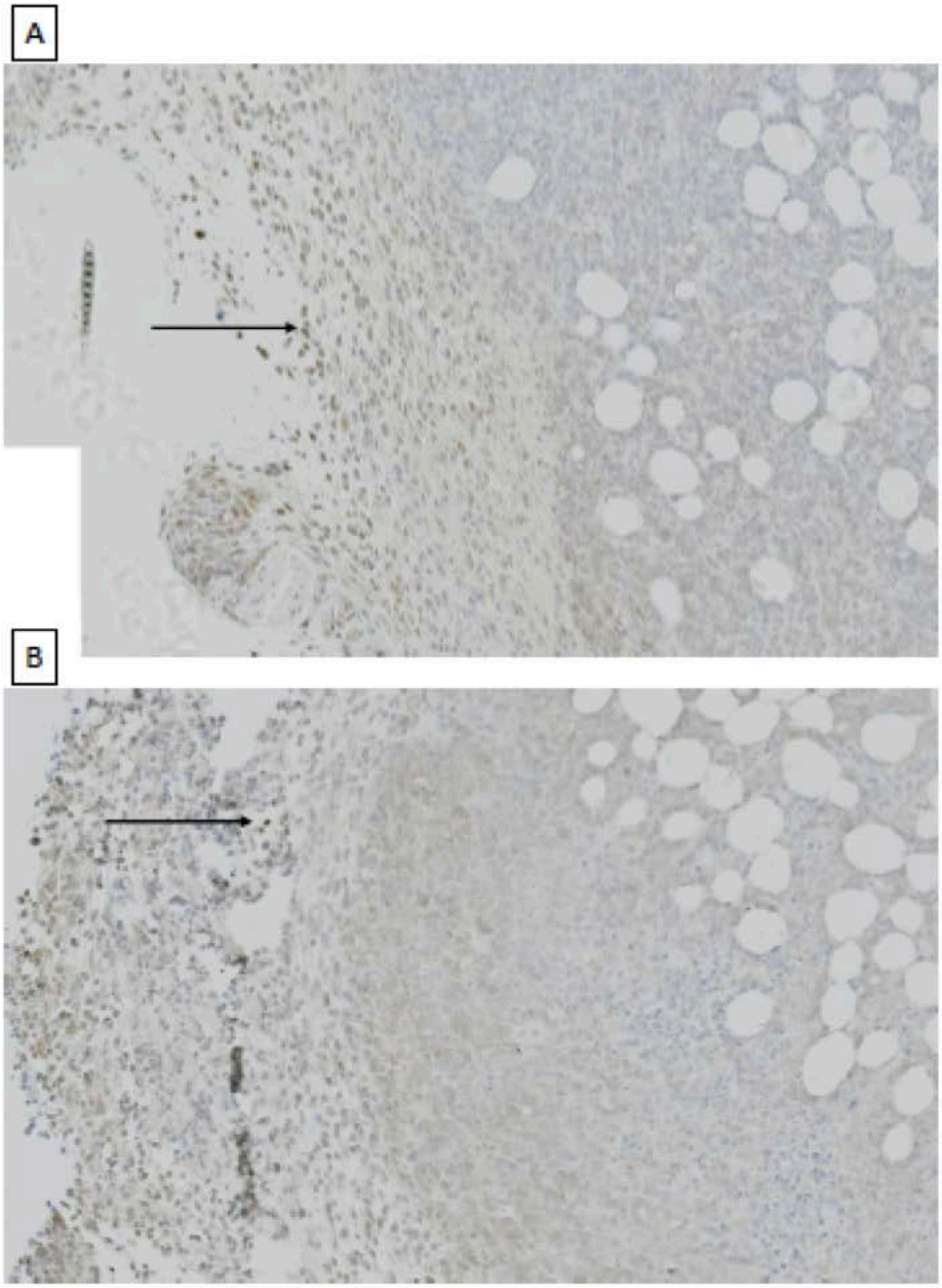
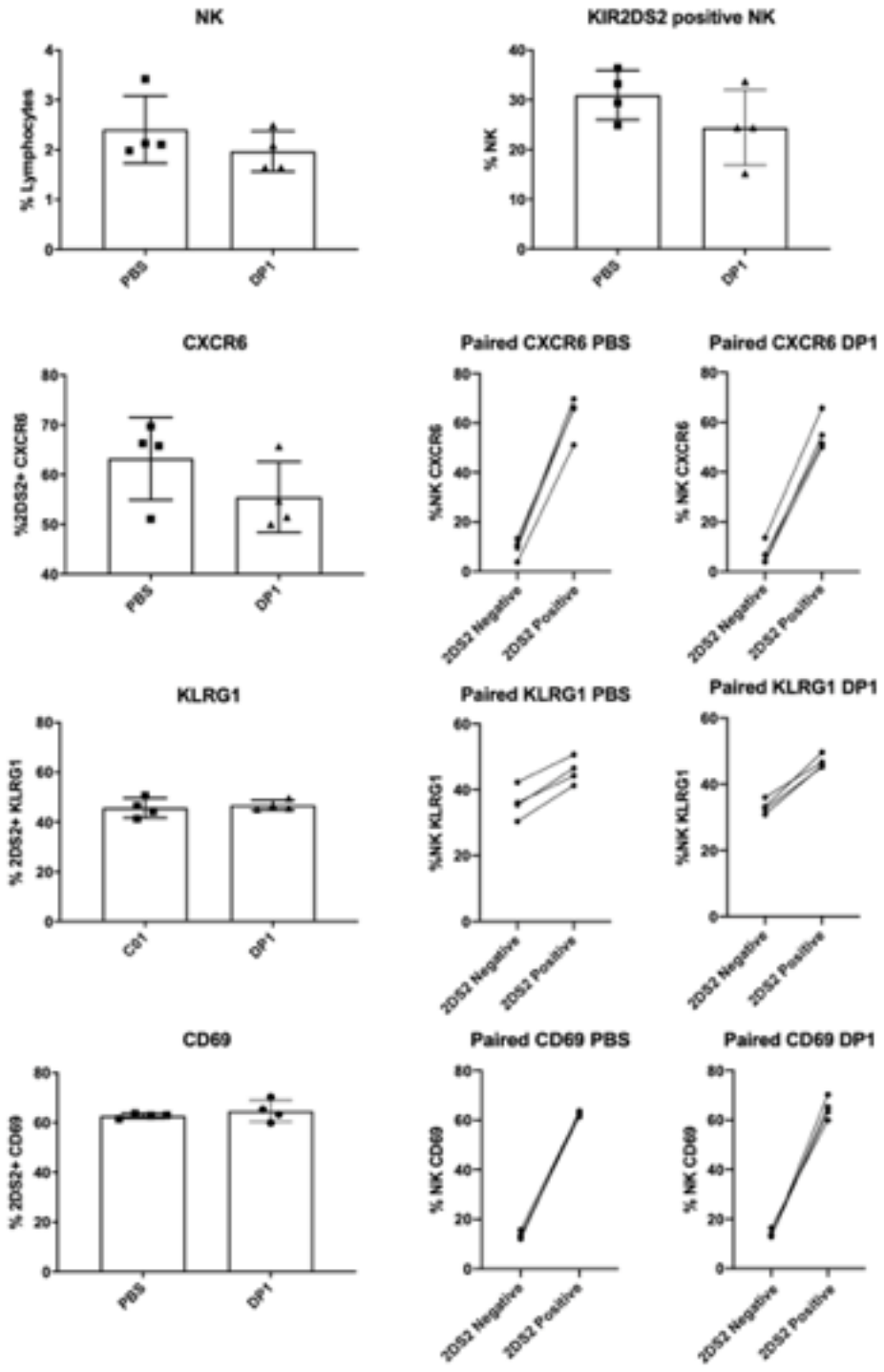


Fig 5.14 Histology of RMA-S tumour for mouse in PBS group (A), and DP1 group (B). NK cells stained with NKP46 (highlighted with arrow).

Few NK cells were observed on the periphery of RMA-S tumours on histology, with no observable concentrations of intra-tumoural NK cells seen in both PBS and DP1 groups.

Figure 5.15 Phenotype of spleen NK from PBS and DP1 injected mice with RMA-S tumour



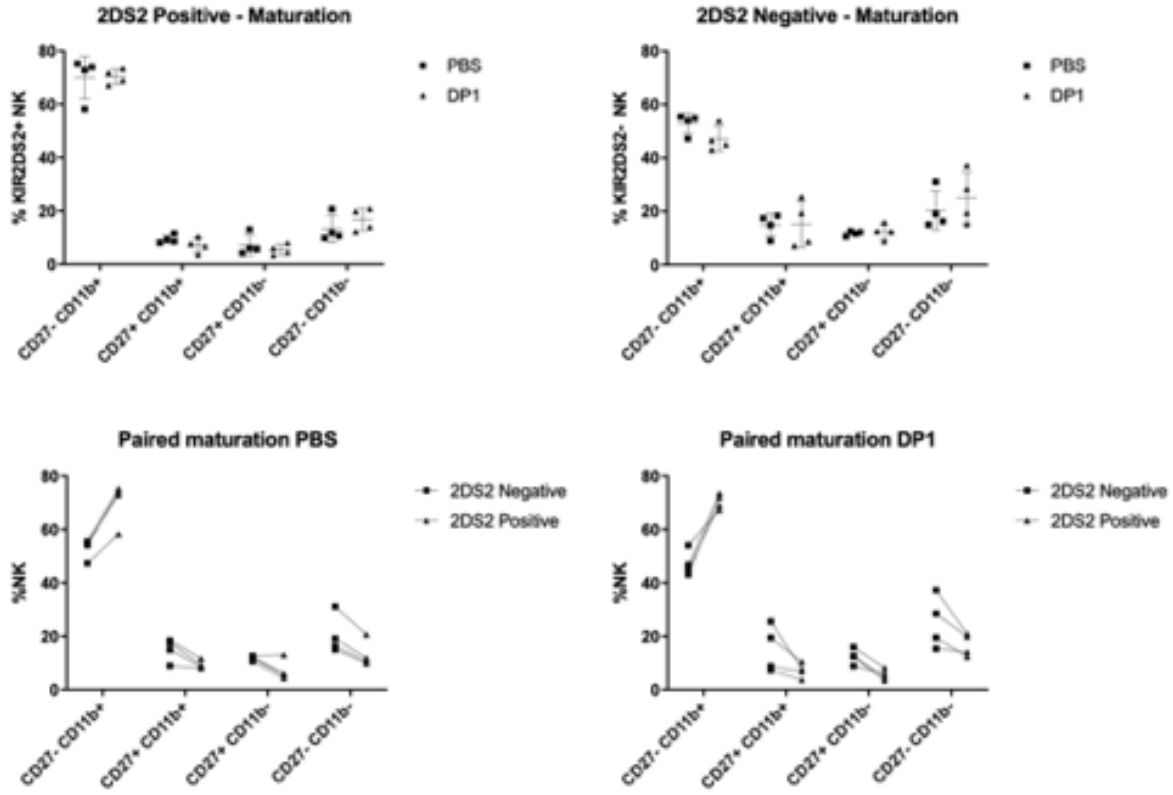


Fig 5.15 Phenotype of splenic NK cells from mice with RMA-S tumour injected with PBS or 2x120mcg DP1 exosomes. Statistical analysis by Mann Whitney test for PBS vs DP1, and Wilcoxon matched-pairs signed rank test for paired analyses.

Analysis of the phenotype of spleen NK cells from the tumour model does not show any significant differences between PBS and DP1 groups. However, the paired analysis adds data to the hypothesis that the pre-existing phenotype of KIR2DS2 positive NK is different to KIR2DS2 negative NK, exhibiting greater expression of markers indicative of memory, maturation, liver residence, and activation. This is shown in trends towards greater CXCR6, CD69, and KLRG1 in KIR2DS2 positive NK in both PBS and DP1 injected mice. CD27/CD11b expression demonstrates a consistent trend in PBS and DP1 mice towards a greater frequency of highly mature CD27-/CD11b+ cells, and fewer less mature NK cells in KIR2DS2 positive cells.

5.5 Discussion

Injection of exosomes into KIR Tg mice produced a range of findings, none of which can be clearly attributed to the interaction of the DP1 peptide and the KIR2DS2 receptor. Overall NK cell frequency was significantly greater in DP1 exosome injected mice compared to 221 of C01 groups. However, a corresponding significant rise in KIR2DS2 positive NK was not seen. The hypothesis of this project is that stimulation of KIR2DS2 by a flavivirus peptide may cause KIR2DS2 specific NK expansion, or alteration of the phenotype of KIR2DS2 positive cells. This therefore raises the possibility of NK cell proliferation in response to a non-specific stimulus by DP1 exosomes on another receptor or cell type. This pattern was seen in maturation markers KLRG1 and CD27/CD11b, where trends towards enhanced maturation were found in KIR2DS2 positive and negative NK, again suggesting stimulation by an alternative pathway to KIR2DS2. Exosomes may interact with many cell types, and may be taken up by other immune cells, producing unforeseen effects.

The other prominent finding in the murine phenotype was enhanced frequency of markers of maturation, memory, and activation in KIR2DS2 positive NK compared to KIR2DS2 negative NK. This was seen in a mouse which did not receive an injection, and mice injected with sterile PBS, and all types of exosome. This suggests that KIR2DS2 cells did not exhibit enhanced memory, maturation and function due to exosome stimulation, but have inherently enhanced frequency of CXCR6, CD69, and CD27-/CD11b+ than KIR2DS2 negative cells, and there were trends towards enhanced CD49a, KLRG1, and CD107a expression. This adds to the findings of a study which demonstrate that KIR2DS2 positive NK cells exhibit enhanced functional markers and a transcriptional profile consistent with enhanced cytotoxicity(175), and multiple studies correlating KIR2DS2 positivity with improved survival in a variety of cancers, which may indicate greater functional capacity(176-179).

An initial tumour model found no significant difference in survival or tumour volume. Histology from these tumours did not suggest significant tumour infiltrates of NK cells in either PBS or DP1 groups. A heterotopic, syngeneic model was selected to allow a model of cancer immunotherapy in a single mouse. This allowed practical measurement of tumour volume, however has some important caveats. This did not model hepatocellular carcinoma as the KIR transgenic mouse model did not accommodate an HCC cell line, so the murine RMA-S lymphoma cell line was used. A heterotopic model does not simulate the underlying disease processes and tumour microenvironment which influence the immunology of the disease studied. A human HCC cell line

could be studied in a mouse model, but this would require adoptive transfer of NK cells from an injected KIR transgenic mouse to an immunodeficient mouse injected with tumour cells. Strategies have been described which may model cirrhosis and specific liver diseases, however they are complex, and monitoring tumour development requires sacrifice or imaging(172).

Chapter 6 Final Discussion

6.1 Absence of NK2DS3 is associated with development of hepatocellular carcinoma

In this cohort of patients with hepatitis C referred to a single large UK hospital, absence of KIR2DS3 was significantly associated with development of hepatocellular carcinoma. There are few studies examining the relationship between KIR genotype and HCC, though current literature has suggested a conflicting range of associations. A Japanese cohort of patients with hepatitis B associated KIR2DS3 with greater risk of HCC(180), and a further Japanese cohort of patients with hepatitis C associated HCC with HLA-Bw4 and KIR3DL1/HLA-Bw4(181). A study including Italian patients established a link between absence of KIR3DS1/HLA-Bw4 and HCC(88). All of these studies are relatively small, and larger studies may be required to clarify this relationship. However, KIR are known to be highly diverse, and so the varying populations and ethnicities studied may account for these findings. Furthermore, various KIR genotypes have been correlated with outcomes of underlying aetiologies of liver disease; KIR2DL3/HLA-C with resolution and protection from hepatitis C(150), and A haplotype protective for hepatitis B and C(80,81). As HCC develops in the context of chronic liver disease, associations between KIR genotype and HCC might be expected to vary with aetiology.

As discussed in chapter 3, the mechanism for the relationship between KIR2DS3 and clinical outcomes is not clear. No ligands for KIR2DS3 have been identified. Cell surface expression of KIR2DS3 has been shown to be greatly reduced compared to other KIR, residing predominantly intracellularly. It has been hypothesised that KIR2DS3 is no longer functional, however a diverse variety of clinical associations have been established, including with outcomes of viral hepatitis, HIV, acute myeloid leukaemia and sarcoidosis (156,158,182,183). This suggests a significant role in NK cell function which has yet to be fully understood. Alternatively it has been hypothesised that KIR2DS3 in these contexts represents a haplotype which is relevant to the outcome. It is likely that more data will be required from larger studies to understand this more fully.

6.2 Targeting NK cell activation via KIR2DS2

NK cells are attractive targets for anti-cancer immunotherapy for hepatocellular carcinoma due to their ability to kill target cells without antigen-specific recognition or priming, the unique immunological landscape of the liver, and the microenvironment of HCC. NK killing is controlled by a balance of activating and inhibitory receptors, including the Killer Immunoglobulin-like Receptor (KIR) family. The activating KIR2DS2 receptor recognises ligands from flaviruses such as dengue and zika virus(90). A hypothesis of this project was that KIR2DS2 positive NK can be activated, and anti-cancer effects enhanced, by the DP1 dengue peptide expressed on exosomes derived from the 721.221 cell line.

In-vitro and in-vivo models of killing and degranulation in response to cancer cell lines did not demonstrate enhanced degranulation or cytotoxicity in DP1 exosome treatment groups, although further mouse tumour experiments were prevented by the COVID-19 pandemic. Mice injected with DP1 exosomes did exhibit significantly greater liver NK frequency, and greater frequency of mature CD27-/CD11b+ splenic KIR2DS2 positive NK vs controls. However, both of these findings cannot be attributed solely to peptide specific interaction with KIR2DS2, as KIR2DS2 positive NK frequency was not significantly enhanced, and maturity was equally enhanced in KIR2DS2 negative cells. This suggests another interaction may be partly responsible for these findings. Activation of immune cells by exosomes via MHC-peptide complexes has been reported in various cell types. Zitvogel et al demonstrated that exosomes expressing MHC-I loaded with tumour specific peptides produced priming of tumour-specific cytotoxic T lymphoctyes in 1998(184), and Théry et al demonstrated that activation of CD4+ T cells by peptide bearing dendritic cell exosomes was dependent on the presence of mature dendritic cells(185). This raises the possibility that the effects observed on NK frequency and maturity in DP1 vaccinated mice may not be due to direct effects on NK cells, but may be dependent on interactions with other immune cells. Furthermore, it raises the issue of selection of parent cell. The 721:221 lymphoblastoid cell line was used in this study, due to its MHC I deficiency. However, as effects have previously been demonstrated using dendritic cell exosomes, exosome origin may be factor in the response produced.

Paired analyses of KIR2DS2 positive vs negative NK found evidence of enhanced maturity, memory, and activation, demonstrated by significantly greater CXCR6, CD69, and CD27+/CD11b+

NK, and fewer CD27+/CD11b- NK. This was seen in the liver and spleen across treatment groups, including mice injected with PBS alone. This suggests that the KIR2DS2 positive NK cells had a different baseline phenotype to negative NK cells in KIR transgenic mice. KIR2DS2 positive NK have been shown to have enhanced CD69 and CD16 compared to KIR2DS2 negative patients in a glioblastoma animal model(186). KIR2DS2 is present in approximately 50% of the population in the UK, with greater frequency in central and Southern African, and South American populations(89). Positivity has been associated with improved outcomes in various viral infections and haematological malignancy following hematopoietic stem cell transplantation (90,178,187). It is possible that these protective effects are due to enhanced memory, maturity and activation in KIR2DS2 positive NK. Targeting this subset of NK with immunotherapies may therefore be advantageous.

The interaction between KIRD2DS2 and a flavivirus peptide occurs in the context of a variety of other activating and inhibitory signals acting upon NK cells. This is potentially a complex and dynamic process, with numerous variables dictating the outcome. For example, activating signals from KIR2DS2 may depend on binding with the peptide and parent cell simultaneously, in which case enhanced activation may be short lived. Not all NK activating and inhibitory receptors have equal influence over function, so the influence of KIR2DS2 may depend both on its relative dominance, and the co-existing signalling at that time. For example, it is not clear if it could overcome an overwhelmingly inhibitory milieu, which is particularly important in the tumour microenvironment of HCC, where NK activation may need to overcome the immunosuppressive environment in order to restore NK tumour surveillance.

6.3 In-vitro experimental protocols

A hypothesis of this thesis is that NK cells may be activated via the KIR2DS2 receptor by the DP1 peptide and HLA-C expressed on exosomes. The experimental protocols aimed to examine this in-vitro by assessing NK cell proliferation, degranulation, and killing of cancer cell lines. No significant differences were observed from in-vitro experiments. Cytokines are required for NK homeostasis; IL-15 is required for cell survival, proliferation, development and functionality, and so was included in culture media during experiments. Other cytokines known to positively influence NK cell function include IL-2, IL-12, IL-18, IL-21, and IFN-γ. IL-2 was trialled, and not found to impact proliferation in this context. Kits and protocols to induce proliferation of NK cells include supra-

physiological levels of these cytokines. Exposure to high concentrations of cytokines has been associated with phenotypic and functional changes(69,168,169). Experiment design is therefore a balance between providing enough cytokine stimulation to facilitate the homeostatic mechanisms of NK cells, and inducing affects which may be erroneously attributed to exosome stimulation. Further optimisation of protocols may be required, though this was limited by availability of donors for PBMC extraction, and the difficulty of exosome preparation.

In vitro experiments utilised PBMC from donors. This may preserve relevant interactions between cell types, and improve cell survival. However, approximately 5-20% of PBMC are NK cells, and so any effects observed in killing assays may be related to other effector cells, or NK cell killing may be obscured by that of other cells. Furthermore NK cell numbers may not remain consistent throughout experiments. Despite these drawbacks, I elected to use PBMC due to enhanced cell survival in culture, and it suiting the aims of this project. I aimed to examine DP1 exosomes as a potential immunotherapy. Any effect would therefore need to be relevant with normal NK cell frequency. Repeating experiments utilising purified NK cells may allow more detailed assessment of the functional impact of exosome stimulation in killing assays.

6.4 Exosomes as a vehicle for cancer immunotherapy

The study of exosomes as therapeutic agents is an evolving field, and no therapies are currently in widespread clinical use. The function of exosomes in health and disease is incompletely understood, but is thought to include a diverse role in cellular signalling and immune priming, and propagation of infection and metastasis of cancer. The ability to deliver functional cargos directly to target cells, including chemotherapeutic agents, ligands and genetic material, makes them an attractive vehicle for potential therapies. In this project I investigated the utility of exosomes derived from cells expressing a flavivirus peptide in the context of HLA C, which is recognised by the activating KIR2DS2 receptor on NK cells. Investigating exosomes in a functional capacity is challenging, and raises a number of methodological questions.

Exosomes are produced by nearly all cell types, and may be extracted from preparations of parent cells with a variety of techniques. While it is recognised that the method may influence purity, integrity, and function of isolates, there is no clear consensus on the superiority of one method in

all circumstances(188). This study demonstrated exosome purification by sequential ultracentrifugation at a maximum of 100,000G, selected for the capacity to process large volumes of culture medium, and so produce larger quantities of exosome with modest consumption of resources. Other methods available include kits based on precipiation, gel filtration, exclusion chromatography, or magnetic affinity purification. It has been reported that the ultracentrifugation technique may produce mechanical disruption of vesicles(189), though functional data from multiples studies suggest preserved functional capacity compared to other extraction methods(143, 190). There are however other variables that affect yield and contamination, such as rota selection and viscosity of the centrifuged fluid. A fixed angle rota was used in this instance, which is optimal for separation of particles, at the expense of a less compact pellet. This protocol can be augmented with a filtration step, but this was deemed unnecessary, as it would be likely to reduce yield, and few large particles were observed on nanoparticle tracking analysis. It is recognised that all of these methods are vulnerable to contamination from similarly sized particles, which may produce inaccurate functional data, and therefore techniques have been described to remove co-isolated contaminants, including fractionation of preparations using density gradients, and antibody capture, which could improve the purity of exosome preparations(142).

A further limitation of the technique I used in this study was that the presence of the proposed functional component, the DP1 peptide, was not confirmed in isolated exosomes. The proteomes of exosomes mirror those of their parent cells, and so cultured cells were monitored for expression of HLA-C via DT-9 staining using flow cytometry, and MHC I expression was assessed in exosomes via western blotting. Phenotypic changes can be seen in DP1vs control exosome vaccinated mice in NK frequency and maturity, suggesting that the peptide was preserved, as control exosomes were from identical parent cells with and without HLA C expression. Exosome protein content can be more fully characterised by mass spectrometry, and exosome surface proteome can be specifically described by digestion of surface proteins prior to liquid chromatography-tandem mass spectrometry(191,192). A further problem is the inherent heterogeneity of isolated vesicles. Diverse populations of vesicles have been identified, which may have differing functional effects. Vesicle secretion by cells may be altered by cellular stress produced by conditions such as hypoxia, culture medium, cell confluency and passage. Exosomes produced following stressors have been found to exhibit different microRNA profiles(142,193). It is therefore important that culture conditions are rigorously maintained, and ideally exosomes for functional experiments would be characterised and purified with every extraction. However, this may not be practical as functional experiments also require relatively large quantities of exosome,

and this would very greatly complicate and increase the cost of experiments. During my project I followed what is currently common practice, which is to characterise exosomes produced by parent cell and technique, and standardise culture methods and extraction thereafter. Validation of my extraction was successful within the limitations of the method used, which was able to determine that membrane bound vesicles of a consistent size, with a protein content consistent with exosome extraction was present. These techniques are destructive however, so cannot be used to validate every extraction, and cannot discriminate between similar sized contaminants, or vesicle subpopulations. As this is a developing field, it is likely that these techniques will evolve.

6.5 Future directions

6.5.1 Investigating the relationship between killer immunoglobulin-like receptor genotype and development of hepatocellular carcinoma

In order to better address this question, a greater number of patients with hepatocellular carcinoma are required to increase statistical power. A prospective cohort study would provide high-quality data, but given the prevalence of hepatocellular carcinoma and the long time it may take to develop, would require a large sample size and a long period of follow-up, making this expensive and impractical. A case-control study has the advantage of recruiting patients based on outcome (i.e., with a diagnosis of hepatocellular carcinoma), allowing a more robust assessment of the relationship between KIR genotype and HCC development in a more timely fashion (194). Recruiting different aetiologies of chronic liver disease would enable comparison between these groups, though would require a far larger sample size.

6.5.2 Activating natural killer cells via KIR2DS2

There are numerous possible refinements to my protocol, which may allow more detailed analysis of the data collected. As discussed in 6.4, a flaw in the protocol I used was the inability to confirm that parent cells and isolated exosomes retained expression of the DP1 peptide. This could be assessed through mass spectroscopy which would allow more detailed characterisation of isolated vesicles.

In vitro experiments were performed using PBMC, and so other effector cells may mask the cytotoxic effects of NK cells. Repeating experiments with purified NK cells would allow this to be investigated in more detail. Functional experiments could be further refined by using RNA

sequencing to establish whether pathways associated with NK cell cytotoxicity are upregulated following exosome exposure.

The appropriateness of further murine tumour models would need to be assessed in the context of the experiments above. A heterotopic, syngeneic murine tumour model was chosen as an initial experiment to assess tumour killing in-vivo, which did not accommodate an HCC cell line. In order to investigate the effect of exosome injection on killing of human HCC lines adoptive transfer from KIR transgenic mice to immunodeficient mice could be performed.

Appendix A Stepwise regression - Effect of killer immunoglobulin-like receptor genotype on cirrhosis and HCC development in patients with Hepatitis C

Stepwise regression - KIR genes associated with cirrhosis

Included variables

Model	Variable	Beta	Std. Error	Standardised Beta	t	Sig.	95% CI for Beta	
							Lower	Upper
1	Constant	-0.343	0.221		-1.554	0.122	-0.780	0.093
	Age	0.166	0.061	0.222	2.736	0.007	0.046	0.286

Excluded variables

Model	Variable	Beta	t	Sig.
1	KIR2DL1	-0.133	-1.651	0.101
	KIR2DL2	-0.092	-1.137	0.258
	KIR2DL3	-0.011	-0.130	0.896
	KIR3DL1	-0.110	-1.355	0.177
	KIR2DS1	-0.060	-0.731	0.466
	KIR2DS2	-0.087	-1.064	0.289
	KIR2DS3	-0.077	-0.939	0.349

	KIR2DS4	-0.045	-0.553	0.581
	KIR2DS5	-0.044	-0.530	0.597
	KIR3DS1	-0.024	-0.287	0.774
	AA	0.110	1.360	0.176
	BB	0.045	0.553	0.581
	AB	-0.129	-1.597	0.113
	Gender	-0.145	-1.790	0.076
	Alcohol	0.018	0.223	0.824
	Diabetes	0.101	1.244	0.216
	Hepatitis C clearance	0.003	0.039	0.969

Model Summary

Model	R	R Square	Adjusted R Square	Std Error of the Estimate	R Square Change	F Change	F Change Sig.
1	0.222	0.049	0.043	0.42703	0.049	7.483	0.007

ANOVA

Model		Sum of Squares	df	Mean Square	F	Sig.
1	**Regression**	1.365	1	1.365	7.483	0.007
	Residual	26.259	144	0.182		
	Total	27.623	145			

Stepwise regression - KIR genes associated with HCC

Included variables

Model	Variable	Beta	Std Error	Standardised Beta	t	Sig.	95% CI for Beta	
							Lower	Upper
1	Constant	-0.253	-0.135		-1.881	0.062	-0.520	0.013
	Age	0.092	0.037	0.202	2.472	0.015	0.018	0.165
2	Constant	-0.206	0.135		-1.523	0.130	-0.473	0.061
	Age	0.086	0.037	0.190	2.346	0.020	0.014	0.159
	KIR2DS3	-0.098	0.047	-0.168	-2.075	0.040	-0.191	-0.005

Excluded variables

Model	Variable	Beta	t	Sig.
1	KIR2DL1	0.045	0.553	0.581
	KIR2DL2	-0.016	-0.199	0.842
	KIR2DL3	-0.008	-0.99	0.921
	KIR3DL1	-0.107	-1.315	0.191
	KIR2DS1	-0.045	-0.541	0.589
	KIR2DS2	-0.029	-0.360	0.720
	KIR2DS3	-0.168	-2.075	0.040
	KIR2DS4	-0.099	-1.209	0.229
	KIR2DS5	-0.004	-0.043	0.966
	KIR3DS1	-0.026	-0.310	0.757
	AA	0.038	0.460	0.646
	BB	0.099	1.209	0.229
	AB	-0.093	-1.143	0.255

	Gender	-0.136	-1.681	0.095
	Alcohol	-0.063	-0.767	0.444
	Diabetes	0.116	1.430	0.155
	Hepatitis C clearance	-0.055	-0.670	0.504
2	**KIR2DL1**	0.043	0.526	0.115
	KIR2DL2	0.066	0.743	0.880
	KIR2DL3	-0.073	-0.840	0.508
	KIR3DL1	-0.128	-1.586	0.110
	KIR2DS1	0.013	0.152	0.845
	KIR2DS2	0.061	0.664	0.729
	KIR2DS4	-0.131	-1.610	0.704
	KIR2DS5	0.016	0.195	0.110
	KIR3DS1	0.030	0.347	0.532
	AA	-0.034	-0.381	0.150
	BB	0.131	1.610	0.528
	AB	-0.053	-0.626	0.223
	Gender	-0.118	-1.449	0.389
	Alcohol excess	-0.051	-0.633	0.528
	Diabetes	0.099	1.225	0.223
	HCV clearance	-0.070	-0.865	0.389

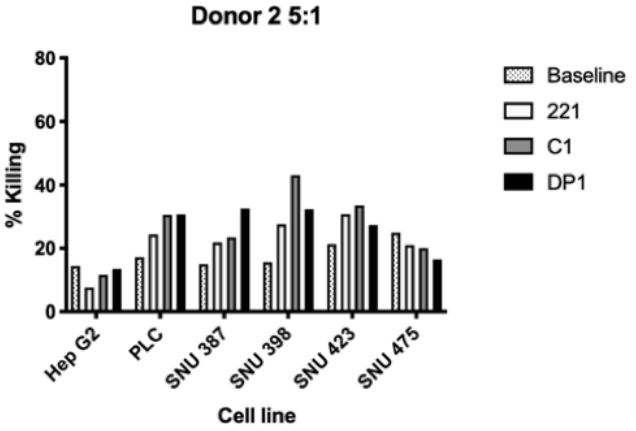
Model summary

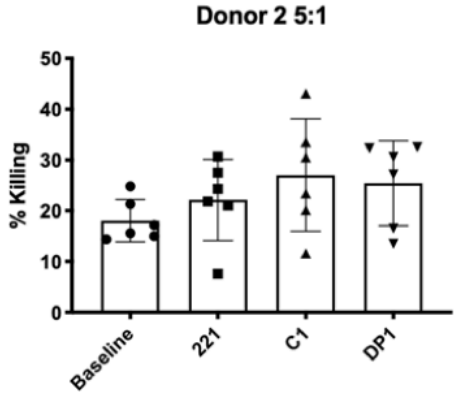
Model	R	R Square	Adjusted R Square	Std Error of the Estimate	R Square Change	F Change	F Change Sig.
1	0.202	0.041	0.034	0.26030	0.041	6.112	0.015
2	0.262	0.069	0.056	0.25737	0.028	4.305	0.040

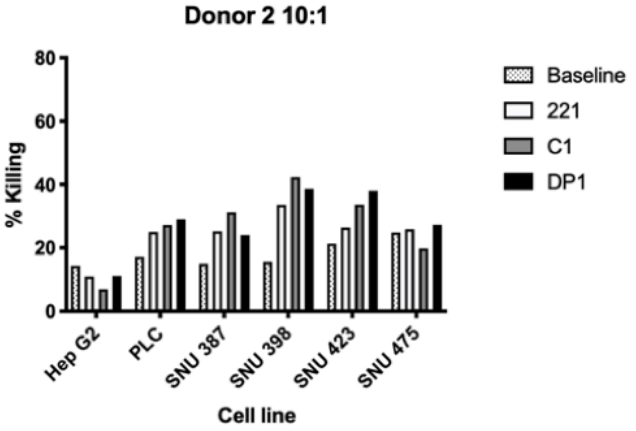
ANOVA

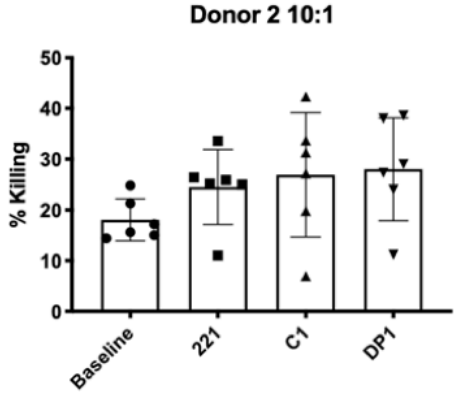
Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	0.414	1	0.414	6.112	0.015
	Residual	9.757	144	0.068		
	Total	10.171	145			
2	Regression	0.699	2	0.350	5.279	0.006
	Residual	9.472	143	0.066		
	Total	10.171	145			

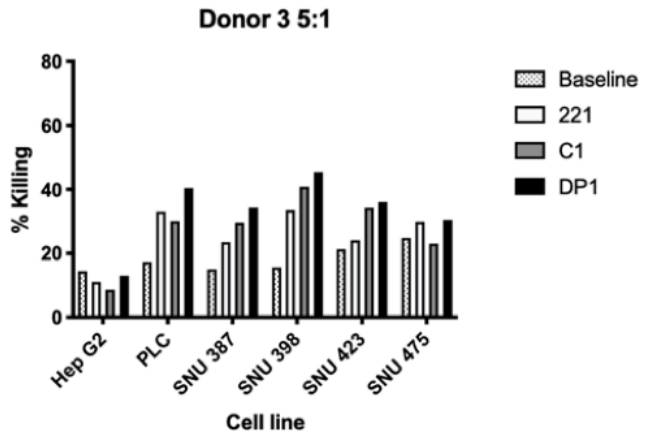
Appendix B Killing of HCC cell lines by exosome stimulated PBMC - individual donors

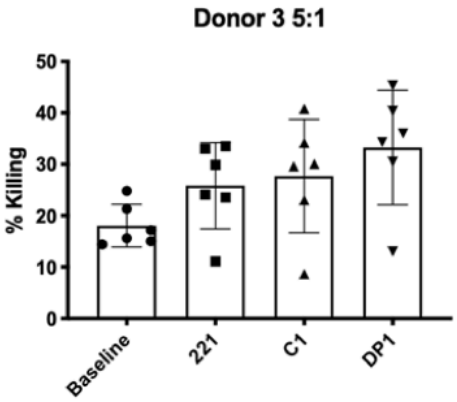









Donor 3 5:1
% Killing
Cell line
Hep G2
PLC
SNU 387
SNU 398
SNU 423
SNU 475
Baseline
221
C1
DP1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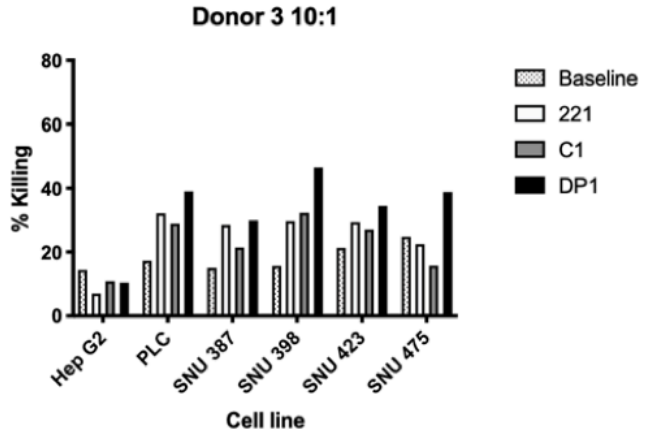
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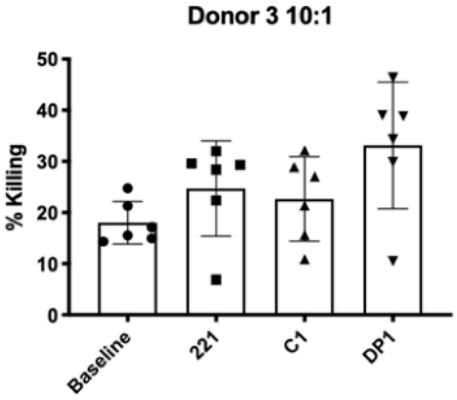
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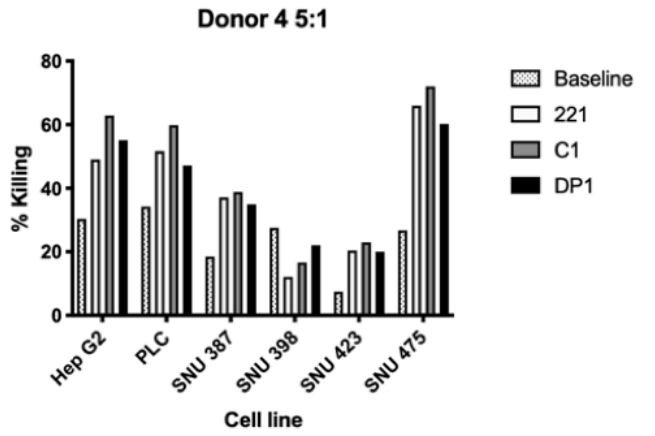
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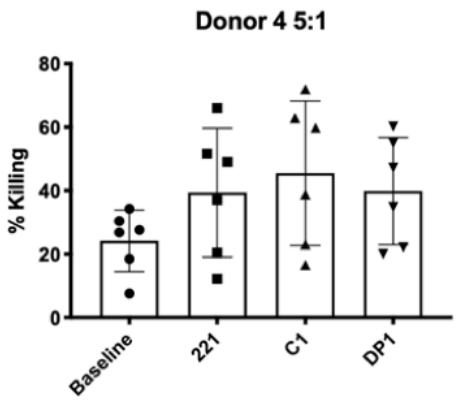
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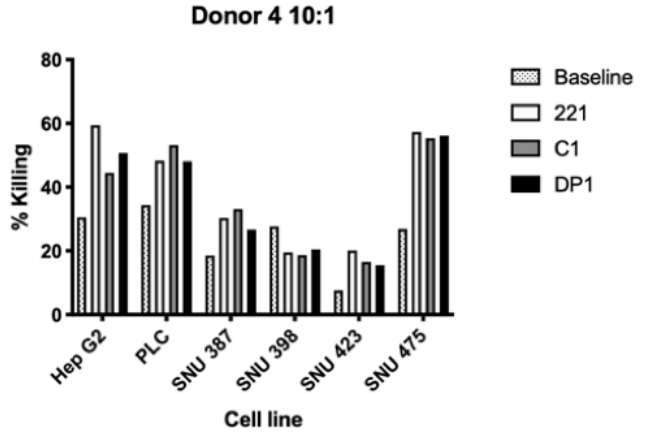
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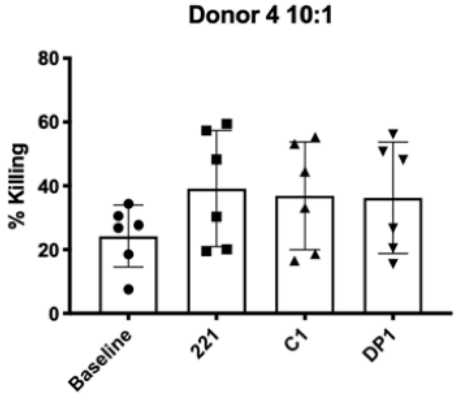
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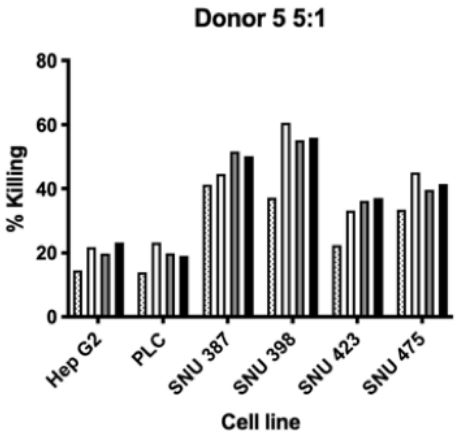
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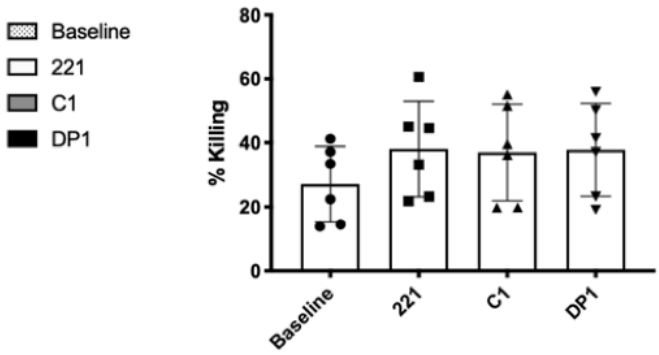
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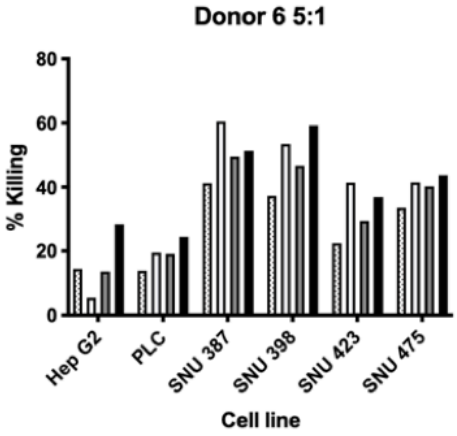
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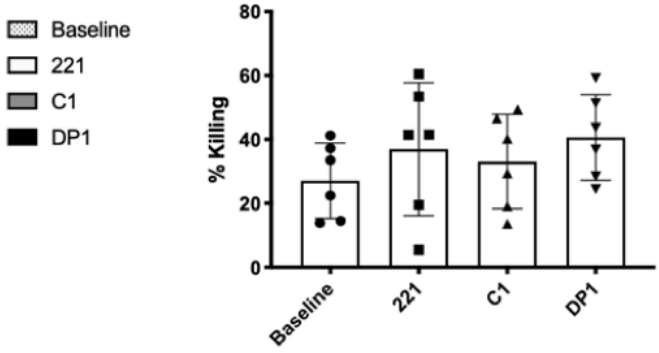
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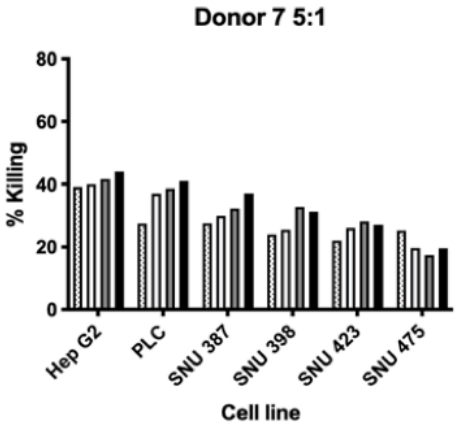
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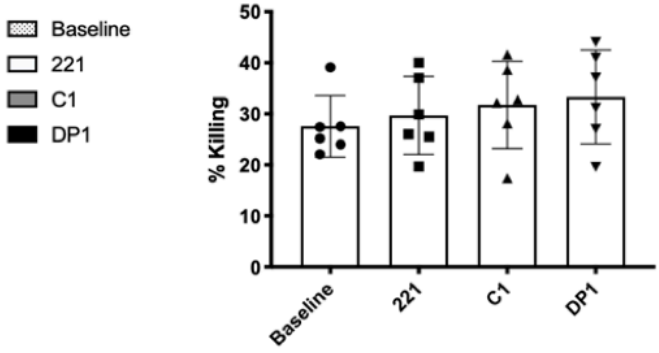
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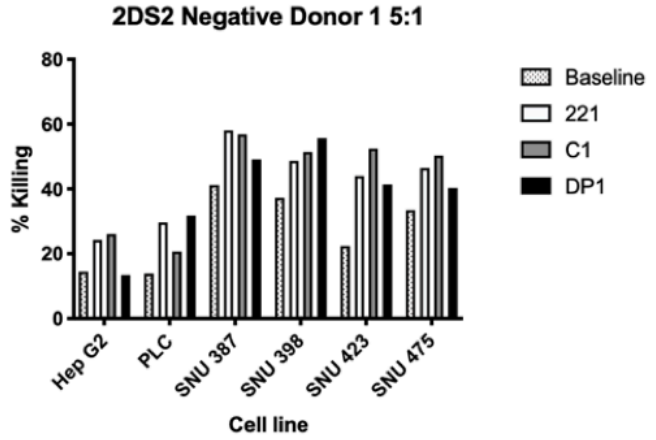
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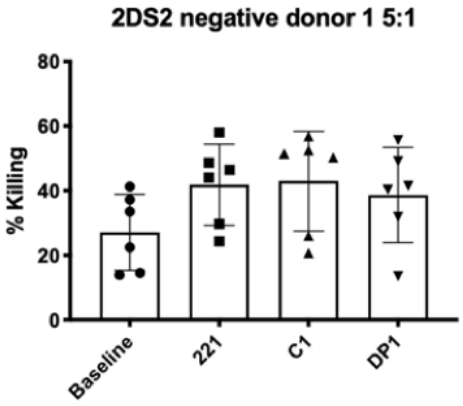
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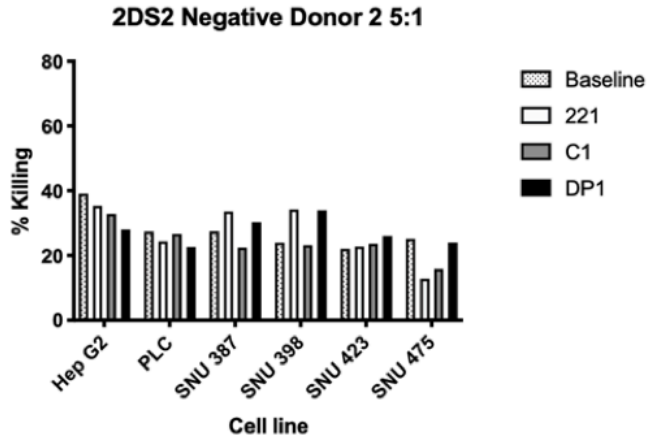
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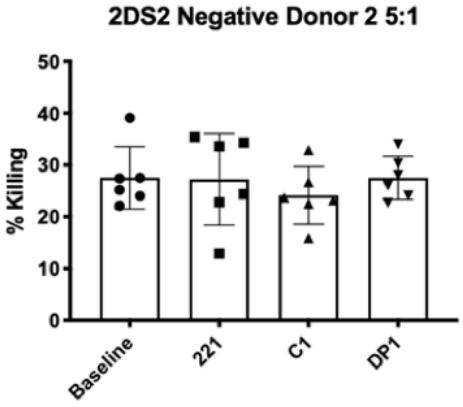
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List of References

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