OVERCOMING REGULATORY T CELL SUPPRESSION THROUGH EX VIVO PRIMING OF NATURAL KILLER CELLS FOR CANCER IMMUNOTHERAPY

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Declaration

I Fahim Ghourbandi confirm that the work presented in this thesis is my own. Where information has been derived from other sources. I confirm that this has been indicated in the thesis.

For my Wife

Abstract

Background: The tumour microenvironment (TME) promotes both immune evasion and cancer progression. TME is an essential component of promoting invasion, metastasis, as well as the ability to resist therapeutic interventions. One of the key aspects of this environment that supports the growth of cancerous cells is the recruitment of immunosuppressive cells such as the T regulatory cell. These immunosuppressive cells, Treg, often impairs the NK cell's function, which normally recognises cancer cells. This project has found a way to improve the NK cell function by tumour-priming NK, which enhanced anti-tumour functions for clinical use. The aim of this study is to investigate the relationship between NKs and Tregs in cancer cells. In particle to examine if by tumour-priming, NK can overcome the suppression of Treg in vitro. NK and Treg cells were isolated from leukocyte cone and Treg cells were expanded, and suppression was performed. NK cells were prime with cytokine, IL2 and CTV-1 cell line and co-incubated with K562, Raji and OVCAR3 cancer cell line, with and without Treg. These were followed by 4h of cytotoxicity assays and were read in flowcytometry or xCELLigence. The Treg cells after the expansion were found to be 90% suppressive at ratio of 1:2 (E:T). The cytotoxicity assays that were carried for 4h, showed a reduction in the NK cells cytotoxicity, with the addition of Treg cells, as against the NK cells with 3 different cell line K562, Raji and OVCAR3. In case of OVCAR3, at 4h of cytotoxicity assay, the Treg cells completely inhibited NK cell cytolysis. As for all the targeted cell lines, when the NK cells were primed with IL2, there was an increased in cytotoxicity, even with the addition of Treg cells. The TpNK cells cytotoxicity were not much higher than the NK cells; however, it manages to completely inhibit the Treg suppression. The NK cells, by themselves are not capable of inhibiting the suppression of Treg cells, but with additional treatments, like with IL2 or CTV-1, their resistance to Treg influence increases. TpNK cell manages to completely inhibits the Treg suppression.

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Abbreviation

A

acute myeloid leukaemia
ML-2 19
American-Type Culture Collection
(ATCC) 23
antibody-dependent cellular
cytotoxicity
(ADCC) 13
antigen presenting cells
(APC) 17

C

central SMAC
cSMAC 14
Complete media
(CM) 23

D

DNAX accessory molecule-1
DNAM-1 15

E

Enteropathy X-linked
(IPEX) 16
extracellular vesicles
(EVs) 14

F

forkhead family transcription factor
(FoxP3) 16

H

Hanks' Balanced Salt Solution
(HBSS) 24
Hematopoietic stem cells
(HSC)
13
human Burkitt’s lymphoma
Raji 23
human myelogenous leukemia cell
line
K562 23
Human Ovarian Cancer Cell Line
OVCAR3 24

I

inducible Treg
(iTregs) 17
interferon γ
(IFN-γ) 13

K

killer cell immunoglobulin like
receptor

W

2 *Background*

2.1 General

The tumour microenvironment (TME) promotes both immune evasion and cancer progression. TME is an essential component of promoting invasion, metastasis, as well as the ability to resist therapeutic interventions (Xiong et al., 2017). The key aspect of this environment supports the growth of cancerous cells, such as the: (i) evasion of growth suppressors and avoidance of senescence, as well as apoptosis; (Zeng et al., 2018) (ii) promotion of blood vessel formation, thereby helping cancerous cells to travel around the body through the lymph or blood; (Martin et al., 2013) and (iii) recruitment of immunosuppressive cells (Su et al., 2017), such has the T regulatory cell (Treg cell), tumour-associated macrophages (TAM), and myeloid-derived suppressor cells (MDSC) (Hanahan and Weinberg, 2011). The latter functions to neutralize the body's capability to mount a successful immune response. For example, in one of the recent developments, it has been discovered that natural killer (NK) cells are clearly lacking tumour infiltrates, which has been attributed to the presence of Tregs in the TME (Whiteside, 2008).

2.2 NK cells and the TME

The first discovery of NKs cell was in 1975, in mice, where the researchers detected unique sub-population of lymphocytes, which were capable of killing tumour cells without pre-sensitization (Waldhauer and Steinle, 2008). This led to the model of 'missing self' and 'induced self' (Cruz-Muñoz *et al.*, 2019). When compared with T-cell and B-cell which recognized foreign antigen, NK cells are 'self-centred' and detected self-molecules, which interacts with major histocompatibility complex class I (MHC-I) (Parham, 2000), which are presented in surfaces of autologous cells. It was Karee and colleagues who first noticed that NK cells reject tumour cells, which are deficient in expressing major histocompatibility complex (MHC) class I molecules (Ljunggren and Kärre, 1985, 1990; Kärre *et al.*, 1986).

NKs are part of the innate immune and have been defined as $CD3^-$ $CD56^+$ (Kwong et al., 1997). It is noteworthy that there are two subpopulations $CD3^-CD56^{brigth}$ and $CD3^-CD56^{dim}$ (Tarazona et al., 2002). Furthermore, NK cells are within 5-15% of

the total population of all the circulating lymphocytes, (Baginska et al., 2013; Waldhauer and Steinle, 2008). Around 10% of NK cells are $CD56^{dim}$ and $CD16^{low}$, and displayed a low antibody-dependent cellular cytotoxicity (ADCC) and mediated cytotoxicity (Farag and Caligiuri, 2006). ADCC is a process of cell mediated immune defence, where an effector cell, like T-cell or B-cell, actively lysis a target cell. On the surface of a target cell, specific antibodies are bounded, which are recognized by effector cells, like NK cell (CD16). This cross linking of CD16 triggers the degranulation into lytic synapse (see below), and the target cells die by apoptosis (Wang *et al.*, 2015; Yeap *et al.*, 2016). They are derived from $CD34^{+}$ hematopoietic stem cells (HSC), which have the ability to self-renew or produce mature blood cells, such as erythrocytes, leukocytes, platelets, and lymphocytes. Morphologically, NKs are large granular cells, and they phenotypically express CD56 (neural cell adhesion molecule, NCAM, homophilic binding glycoprotein), and CD16 a receptor that binds to the Fc portion of IgG. The remainder of NK cells (90%) are $CD56^{bright}$ and $CD16^{high}$. These cells secrete high levels of cytokines, tumor necrosis factor-α (TNF-α) and interferon γ (IFN-γ), IL-5, IL-10, IL-13, the growth factor GM-CSF, and the chemokines MIP-1α, MIP-1β, IL-8, and RANTES (Cho and Campana, 2009).

In humans, the main and widely investigated NK activating receptors are DNAM-1, NKG2D, NKp80, KIR (killer cell immunoglobulin like receptor), and Natural Cytotoxicity Receptors (NCR), which are NKp46, NKp30, NKp44, (Diefenbach *et al.*, 2001; Moretta *et al.*, 2001; Pesce *et al.*, 2015). Once engaged, the activating receptors induced the NK cell cytotoxicity (Yoon, Kim, and Choi, 2015) by releasing granules, such as Perforin and Granzyme (Paul and Lal, 2017). During infection or carcinogenesis, the level of NKG2D ligands, such as MICA, RAETI and the NKP30 ligand B7H6 are upregulated (Molfetta et al., 2017). Once the NK cell recognizes these ligands, using the aforementioned receptors or CD16, an immunological synapse is formed at the contact point. This leads to the recognition of actin cytoskeleton and the formation of a F-actin ring around the pSMAC (peripheral supramolecular activation cluster). These contain adhesion molecules (CD11a/CD18, LFA-1lymphocyte function-associated antigen 1), and within, there

is a presence of cSMAC (central SMAC). This is where the exocytosis of secretory lysosomes (also known as Lytic granules) is released in the direction of the target cell. This causes the polarization of MTOC (microtubule-organizing center) (Huse, 2012) and secretory lysosomes, towards the lytic synapse (Krzewski and Coligan, 2012). The secretory lysosomes are likely to move along the microtubules to lytic synapse, and then get fused with the cell membrane (Mace *et al.*, 2014). The cytotoxic content is later released towards the target cell membrane (Topham and Hewitt, 2009). Another described mechanism is that NK cells kill targets by releasing or secreting extracellular vesicles (NK-EVs), which contain typical NK markers (e.g., CD56) and killer proteins (e.g., perforin, granzyme A & B, granulysin and FasL) (Wu et al., 2019). This study was performed using healthy human samples, and it was discovered that NK-EV could kill solid tumours in the absence of cells (Wu *et al.*, 2019).

Within the cytoplasm of NK cells, there are high concentrations of cytolytic granules, and these lytic vesicles contain many cytolytic proteins, such as perforin and granzyme, as previously mentioned. These proteins are designed to induce cell death upon release (Cooper et al., 2001). During the signals from both activating and inhibitory receptors on the surface, the NK cells released these granules at the immunological synapse, which triggered the death of target cells (Moretta et al., 2002). Covering the membrane of these cytolytic granules, there are lysosomal-associated membranes protein-1 (LAMP-1 or CD107a, CD107b) (Krzewski et al., 2013). About 50% of proteins in the lysosomal membrane are from LAMP family (Andrejewski et al., 1999). LAMP has a short cytosolic tail, which interacts with trans-golgi mediators. Furthermore, it involves sorting and targeting proteins to the lysosomal pathway (Winchester, 2001). CD107a degranulation are used as a marker for NK cell functional activity, using flow cytometry. When the target cell line (K562) interacted with NK cell, the CD107a is considerably upregulated (Alter et al., 2004).

NKG2D is the main activating NK receptor, and the engagement of NKG2D by NKG2D-L, on the surface of a tumour, is sufficient to activate NK cell cytotoxicity, and to control the growth of tumours in animal models (Diefenbach et al., 2001;

Molfetta et al., 2017). Tumours which are at an advanced level, have a high level of NKG2D-L (Sheppard et al., 2018). There are two sub populations of NKG2D-L, MICA and MICB (part of MHC I chain-related family) and ULBP1-6. The shedding of these ligands is a major strategy that tumours use to evade NK cell cytotoxicity (Bauer et al., 1999). Another way through which tumour cell could evade NK cell mediated killing is by down regulating NKG2D ligand, by releasing transforming growth factor-β (TGF-Beta), which are immunosuppressive cytokines. Cytokines are secreted during the tumour growth, or by regulatory immune cells; for example, the Tregs (Lee *et al.*, 2004).

DNAM-1(DNAX accessory molecule-1) receptor plays a critical role in NK cell mediated recognition of various types of tumours cells, including ovarian carcinoma. The ligand for DNAM-1 is CD112 (Nectin-2) and poliovirus receptor (CD155, PVR), which are found on epithelial, endothelial, and antigen presenting cells. The DNAM-1 receptor triggers NK cell-mediated killing of tumour cells expressing CD155 and CD112 (Bottino et al., 2003). Tumours can evade this by lowering the expression of CD112/CD155, thereby resulting in the failure of NK cell killing (Kearney et al., 2016).

Pesce *et al*, 2015 identified B7-H6 (NCR3LG1) as a cell surface ligand of NKp30. B7-H6 ligands are found in lymphoma, leukaemia, melanoma, and carcinoma, and they are also present in soluble form. They are found in patients with ovarian cancer, where more than 50% had an impaired NKp30 receptor (Pesce et al., 2015; Schlecker et al., 2014). This could be due to B7-H6 ligand on the surface of the tumour cell, and as a soluble form. In a soluble form, B7-H6 could be bounded to NKp30, and this could prevent the activation of NKp30 mediated NK cell triggering (Pesce et al., 2015; Schlecker et al., 2014).

2.3 The role of regulatory T-cells (Tregs) in the TME

Sakaguchi et al. (1995) discovered a subgroup of $CD4^+$ T cells, found in a mouse, which could regulate immune responses. They were named regulatory T cells.

During the development of T and B cells, the cells go through a process known as central tolerance, where the self-reactive T and B cells are eliminated. If self-reactive T and B cells escaped, it is then managed by peripheral tolerance, in order not to cause any autoimmune disease (Xing and Hogquist, 2012). Another breakthrough was made in 2003, with the discovery of FoxP3—a forkhead family transcription factor in mice. It was an important governor in the development and function of Treg cells (Khattri et al., 2003; Hori et al., 2003). The importance of Treg cell became clearer, as a result of mutation in FoxP3, which eventually developed a disease called Immune dysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome (Halabi-Tawil et al., 2009). This disease caused an enlargement of secondary lymphoid organs, insulin-dependent diabetes, eczema, food allergies and concomitant infection (Halabi-Tawil *et al.*, 2009). This showed how vital a cell Tregs were, in the regulation of peripheral tolerance and inhibiting immune response (Gambineri et al., 2003).

Treg cells are developed at the thymus— the first stage of the T cell development, and they are double negative stage, where all T cells are $CD4^{-}CD8^{-}$ TCR^{-} (T cell receptor) (Caramalho et al., 2015). Each cell will rearrange its TCR-α genes to form an exclusive functional molecule, which is tested against self MHC (Owen et al., 2019). When the signal is received, T cells proliferate and express both $CD4^{+}$ and $CD8^{+}$ cells, and this stage is called double positive (DP). The TCR-α undergoes gene rearrangement to produce TCR-αβ. The DP T cell interacted with self-MHC I and II cells, which as a weak interaction will die, and cells with appropriate affinity will survive (Engel et al., 2013). The cell then migrates to medulla region, where Negative selection occurs. The T cells are presented antigen, by antigen presenting cells (APC), if they interact too strongly, they undergo apoptosis (Santamaria et al., 2021). The cells then downregulate either the CD4 or CD8 receptor, which will result to naïve CD4 (Tconv) or CD8 single positive cells, where it leaves the thymus and goes into periphery (Maggi et al., 2005).

Naïve Treg (nTreg) are created in the thymus, for the nTreg to develop, there has to be an interaction between the high affinity TCR and CD28, STAT5 (Signal transducer and activation of transcription 5). The co-stimulation between

TCR:CD28, leads to high expression of IL2R α chain (CD25) (Ohkura and Sakaguchi, 2020). In response to STAT5 activation, FoxP3 is expressed as, $CD4^{+}CD25highFoxP3^{-}$. nTreg do not produce IL2, but rely on Tconv cell to secrete IL2 and mice deficient in IL2 or CD25 have low levels of FoxP3 and Treg in thymus and peripheral (Workman et al., 2009).

The inducible Treg (iTregs) develops in the peripheral. The nTreg encounters weak antigen in the peripheral over a long period of time, while FoxP3 is upregulated by interaction of soluble molecule IL4, IL10 and TGF-β. The FoxP3 expression induces other Treg molecule CD127, CTLA-4 and GITR(Workman et al., 2009; Engel et al., 2013). Other regulatory cells are $CD4^{-}$ $CD8^{-}$ and $CD8^{+}CD28^{-}$ T cells (Karagöz et al., 2010).

Tregs suppressed cells of the immune system, such as NK, NKT (Natural killer T-cell), which has $CD3^{-}$, $CD56^{+}$ cells (Tomchuck, Leung, and Dallas 2015), B cells, $CD4^{+}CD25^{-}$ conventional T cells (Tconv), Dendric cells (DCs), and $CD8^{+}$ T cells (Chen et al., 2005; Rueda, Jackson, and Chougnet, 2016). The way these cells work *in vivo* is still unknown; however, several mechanisms have been published including the expression of a high level of IL2 receptor (CD25), which competes for IL2 (Schmitt and Williams, 2013). The expression of inhibitory molecules, such as CTLA-4 (Read, Malmström, and Powrie, 2000), removal of CD80, CD86, and MHC molecules present on APCs, such as DCs, thereby limiting the contact between T-cell and APCs (Gu, Zhao, and Song, 2020). Tregs also release anti-inflammatory cytokines, like TGF-β, IL-10 and IL-35, to suppress cells (Schmidt, Oberle, and Krammer, 2012), as well as granzymes and perforin, which are also released (Romano et al., 2019). As previously mentioned, perforins are a group of molecules which forms a pore in cell membrane of target cell (Osińska, Popko and Demkow, 2014). Granzymes are released by the NK cell or T cytotoxic cell, and they are serine protease, which is responsible for apoptosis of target cell. Once the pore is formed by Perforin, the granzyme enters the target cell, thereafter activating caspases (especially caspase-3), where many substrates are cleaves, and this includes caspase-activated DNase, to execute cell death (Voskoboinik, Whisstock and Trapani, 2015). Tregs have been shown to release extracellular vesicles (EV),

such as exosomes (Smyth et al., 2013), which deliver suppressive molecules, such as miRNAs species into the target cells (Tung et al., 2020).

Not many studies have investigated how Treg cells modify NK cell function; however, circulating Tregs from healthy donors and cancer patients have been shown to down-regulate the expression of NKG2D, whilst membrane bound transforming growth factor beta (TGF-β) suppressed NK cell activity (Zimmer, Andrès, and Hentges, 2008).

One of the key mediators of immunosuppression in TME is the regulatory T cells (Tregs). Furthermore, with studies done on mice, the levels of Tregs were reduced in cancer (leukaemia, melanoma), and enhanced immune function, leading to tumour clearance, as observed (Shimizu, Yamazaki, and Sakaguchi, 1999). It becomes vital to consider Tregs when designing an immunotherapy strategy for cancer, since the effectiveness of the therapy is hindered by the presence of Tregs in TME. For example, it has been demonstrated in a NK clinical trial that despite the administration of donor NK cells to patients, this treatment failed due to the high levels of Tregs found in patients (Geller et al., 2011).

2.4 IL2 therapy

In 1984, IL2 was used for the first time as a therapy for the treatment of cancer on a 33-year-old woman (Rosenberg, 2014). The woman had metastatic melanoma and had undergone many treatments before she was infused with IL2. After one month, her tumours were examined, and it was discovered that the tumours were shrinking, and in a couple of months, the cancer cells were gone. That was the first patient evidence that IL2 treatment could help to destroy an invasive, vascularized cancer (Rosenberg, 2014). IL2 (15.5kDa) is a cytokine primarily secreted by $CD4^+$ T cells when stimulated by antigen—NK cells and $CD8^+$ T cells and DCs (Wang et al., 2018). An *in vitro* study that was conducted by Lehmann *et al.* (2001), investigated the effect of IL2 on NK cell perforin. They compared the K562 cell (sensitive to NK cell cytotoxicity) and the resistant cell line ML-2 (acute myeloid leukaemia) and found that the K562 cells were killed by NK cell granules, but the ML-2 cell did not. By co-incubating NK with IL2, the NK cells become activated and are

called, Lymphokine Activated Killer cells (LAK). This activation increases the percentage of cytotoxicity of a range of tumour cell lines (Yu et al., 2000; Sabry et al., 2011). When the NK cells were activated by IL2, there was an increase in ML-2 cell lysis. This was due to improved binding of perforin to the target cell (Lehmann, Zeis and Uharek, 2001). With the addition of LAK, the expression of CD107a increased in parallel with the cytotoxicity. The expression of CD107a could be a functional marker for NK cell's cytotoxic activity (Aktas et al., 2009).

The disadvantage of IL2 it is that it has to be infused in a high dose, which is very toxic and costly, and this limits its usage in large populations (Jiang et al., 2016). IL2 can also be used for Treg expansion, and the concentration used for IL2 is 1,000 U/ml (Marín Morales et al., 2019), and the concentration to activate NK cells are 100 IU/ml (Sabry et al., 2011).

2.5 Tumour priming (TpNK)

The CD69 receptor found on a NK cell was among the first receptor studied. CD69 is expressed on the surface of T-cell, B cell and NK cells. Once the CD69 receptor is activated, they act as a costimulatory molecule leading to T cell activation, proliferation, secretion or cytotoxicity (Ziegler et al., 1994). CD69 are used as an activation marker for T cell and NK cells (BORREGO et al., 1999). North et al. (2007) investigated a 'Two-Stage Process in Resting NK cell Activation'. Resting NK cells first need to be primed, and at the 2nd stage become triggered. To generate tumour-primed NK (TpNK) NK cells are co-incubated with a clinical grade leukemic cell line either CTV-1, and were able to lysis cell, Raji. When the NK cells are primed by CTV-1 cell, there is an upregulation of CD69 and tumour, which expressed CD69 ligands are lysis. This is an effective therapeutic strategy, as it overcomes the tumour immune evasion, and has consequently led to a clinical trial (Fehniger et al., 2018; Kottaridis et al., 2015). The published result of the phase 1 trial where TpNK was used to treat 7 patients with AML showed that this cellular therapy was initially efficacious, with no serious side effect observed. The advantages of TpNK are that they are not cytotoxic to autologous/ allogeneic leukocytes, (Kottaridis et al. ,2015); thereby reducing the risk of adverse effects. Despite this, very little is known about the interaction of TpNK cells and the other

immune cells in TME, including Tregs. An understanding of whether Tregs inhibits the TpNK cytotoxic function against cancer cell, or whether TpNK cells are resistant to Treg suppression is warranted. This forms the main part of this MRes project.

2.6 Hypothesis

Treg cells will suppress the NK cell killing of the cancer cell. However, by priming the NK cell with IL2 or CTV-1 cell line, the suppressive activity of Treg cell can be inhibited. If primed NK cells are unable to inhibit Treg suppression, then NK cell will be co-primed with both IL2 and CTV-1 cell line.

2.7 Aims:

The aim of this study is to investigate the relationship between NKs and Tregs, in the killing of cancer cells.

Aim 1:

Tregs cells are first extracted and expanded in the lab, and the suppression of Treg are tested. NK cells are 'activated' by incubating NK cells with cancer cells, so that they recognise them as an external, cancerous cell. A defined number of cancer cells are then added to the 'activated' NK cells, and the number of dead cancer cells (i.e. cells killed by the activated NKs) is counted. The next step is to add Treg cells into this mixture and count to see if there's further cancer cell death. If by adding Treg a low count of cancer is not observed, this shows that Treg has inhibited the function of NK cell killing.

Aim 2:

To investigate how to prevent Treg cells suppression on NK killing of the cancer cell. NK cells are primed with IL2 (LAK) and co-incubated with a defined number of cancer cells. The number of dead cancer cells are counted, which are killed by primed IL2 NK cells. Next to add Treg into the mix and see if the LAK has been inhibited. If a high cell count is observed then this shows Treg suppression has been inhibited.

Aim 3:

To investigate if TpNK cells can inhibit Treg cells suppression of NK cells killing. NK cells are activated by CTV-1 cell and co-incubated with a defined number of cancer cells. The number of dead cancer cells are counted by TpNK cell killing. Then Treg cells are added into the mix and if we observe high cell count, this tells us that Treg suppression are inhibited.

2.8 Objective:

To achieve the aims of the project, the following objectives were undertaken:

- Designing a protocol for Treg isolation and validation
- NK isolation and validation
- Creating LAK NK cell and assessing whether Treg inhibited the function of LAK NK cell
- Creating TpNK cells and assessing whether Tregs inhibit the function of TpNK cells

2.9 Justification:

This study seeks to investigate how to prevent Treg cells' suppression on NK killing of cancer cells. The importance of this research is to further deepen the knowledge of the fight against cancer. A cell-based therapy has been developed to kill off cancer cells in the lab. Very little is known about the interaction of TpNK cells and other immune cells in TME, including Tregs. An understanding of whether Tregs inhibits the TpNK cytotoxic function against cancer cell or whether TpNK cells are resistance to Treg suppression is warranted and forms the main part of this project.

3 *Methods*

3.1 Preparation of Cell Culture Media

Complete media (CM) was made by supplementing RPMI 1640 Medium (Roswell Park Memorial Institute 1640 Medium), which contains GlutaMAX, with heat inactivated Fetal calf serum (FCS, 10%) (Life Technology Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF. Cat:A476681) and antibiotic-antimycotic (1%) (GibcoTM Bishop Meadow Road, Loughborough, LE11 5RG. Cat:15240062). Moreover, the Treg culture media was X-VIVO 15 Medium (Lonza, Cat:ZBE02-060F) supplemented with 5% Human AB serum (Sigma, The Old Brickyard, New Rd, Gillingham Dorset, SP8 4XT. Cat:MFCD00165829), 100nM of Rapamycin (Generon 11, Progress Business Centre, Whittle Parkway, SL1 6DQ Slough - United Kingdom, Cat:A8167-24MG) and 1000 U/mL of human recombinant IL2 (Miltenyi Biotec, Almac House, Church Lane, Bisley, Woking, Surrey, GU24 9DR. Cat:130-097-743).

3.2 Thawing of frozen cells

Cryovials containing cells were removed from liquid nitrogen and transferred to dry ice. To thaw the cells, the cryovials were placed into a water bath at a temperature of 37ºC, until ice was left in the vial. Thereafter, the vial was sprayed with 70% of ethanol, and transferred to the biological safety cabinet. Using a Pasteur pipette, cells were taken out and added drop-by-drop into a 50ml falcon tube containing 10ml of CM. Cells were left for 5mins in the CM, before being centrifuged for 5mins at 300g. The supernatant was discarded, and the cell pellet was re-suspended in CM. Cells were counted and cultured at concentration of 0.5 x10^6 cells/ml.

3.3 Feeding Cell culture

K562 cell line (human myelogenous leukaemia cell line), was obtained from the American-Type Culture Collection (ATCC Corporate Office, 10801 University Blvd., Manassas, VA 20110, US). The Raji (human Burkitt's lymphoma), was obtained from the American-Type Culture Collection (ATCC, CCL-86). Both were

cultured using CM and were grown at 37ºC using 5% CO_2 humidified atmosphere. Furthermore, the culture was maintained by the replacement of fresh medium every 2 to 3 days. At the end of the culture, cells were resuspended at a final cells concentration of $0.5x10^6$/ml. OVCAR3 cell line (Human Ovarian Cancer Cell Line), obtained from ATCC were grown at 37ºC using 5% CO_2 and every 2 to 3 days the cells were detached using Tryple (Cat:12605-010, Gibco), washed with PBS (Phosphate buffered saline, with calcium and magnesium, Sigma, Cat: D8537-500ml) and re-seeded at the concentration of $0.5x10^6$/ml.

3.4 Freezing down cells

Freezing media were made by adding 20% of DMSO (WAK-Chemie Medical GmbH, Siemensstr. 9 D-61449 Steinbach/Ts.Germany, Cat: WAK-DMSO-50) to 80% of FCS. The freezing media is kept cold by placing it in a ice box. To freeze cells, cells were washed with Hanks' Balanced Salt Solution (HBSS, Gibco) at 300g for 5min/ RT. The supernatant was discarded, and cells pellet were re-suspended in 500ul of CM. Next, 500ul of freezing medium was added to the cells, and the cells were transferred to the cryovial. In addition, the cryovial was quickly placed into a Mr. Frosty, which was stored at -80ºC overnight. The following day, the cryovial was then transferred into liquid nitrogen for storage.

3.5 PBMCs isolation (see Figure 1)

Leukocyte cones were supplied by the NHS Blood and Transplant service (NC2020.15). The peripheral blood mononuclear cells (PBMCs) were separated using density gradient centrifugation. Firstly, the blood was diluted with equal amounts of HBSS and slowly layered onto the density separation medium, Lympholyte-H (Cedarlane, Ontario Canada). This was done to achieve a distinct interface between the medium and the cell suspension. After centrifuging (800g for 20 min, with the slowest brake settings) (all centrifuging were done at room temperature unless stated otherwise), a well-defined lymphocyte layer was seen at the interface. The layer was carefully collected using a Pasteur pipette, and transferred into a new centrifuge tube. Cells were washed with HBSS and centrifuged at 800g for 10 mins Thereafter, the supernatant was discarded, and the cell pellet was suspended in the CM.

3.6 Treg isolation

To establish a protocol for Treg isolation, two previously published but different isolation methods were tested (Boneschansker et al. 2016; Fraser et al. 2018).

Technique 1: Two step isolation of Treg cell (Stem Cell Technology and Miltenyi Biotec kits). Treg cells were isolated using a two-step bead selection process. Firstly, a negative bead selection was used to isolate $CD4^+$ cells, using RosetteSep Human $CD4^+$ T cell Enrichment cocktail. See Figure 1.

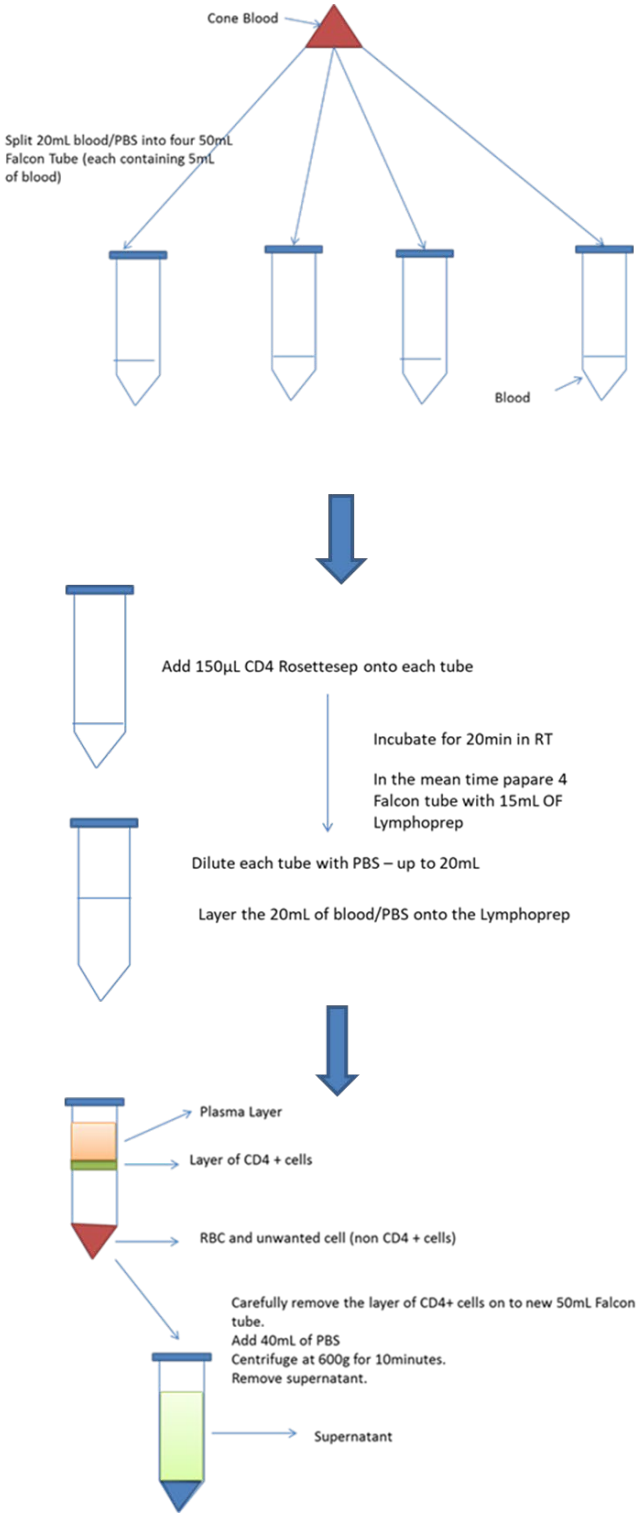

Figure 1: Schematic diagrams showing the initial steps involved in $CD4^+$ isolation

The schematic representation of $CD4^+$ isolation involves the following: 10ml of PBS was mixed with 10ml of blood cone. The mixture was placed into four 15ml tubes, with each containing 5mL of blood/PBS mixture. 150 µL CD4 RosetteSep was added to each of the tube, and the cells were incubated for 20 minutes. 15mL of PBS was then added onto each tube and mixture layered on top of lympholyte prep, before being centrifuged at 600-800g for 20 minutes. The layer below the plasma was removed into a 50mL tube, while the PBS was added, and the cells were isolated following the centrifuge at 600g for 10 minutes.

This was followed by positive selection for $CD25^+$ cells, using human CD25 MicroBeads II and LS Columns. An average of 287 x 10^6 cells $CD4^+$ cells (Tconv) cells were isolated. Furthermore, an average of 15 x 10^6 Treg cells were isolated. The $CD4^+$ Tconv cell fraction (approx.126 x 10^6 cells) was cryopreserved and used for suppression assays. The isolated Treg cells were re-suspended at a concentration of 1x10^6cells/mL and seeded into a 24-well plate and cultured with 100nM Rapamycin in X-VIVO 15 Medium that had been supplemented with 5% Human Sera (T cells expansion medium) and T cell activator (Dynabeads™ Human T-Activator CD3/CD28, Life Technology). On day 2, post-stimulation half of the media (500 µL) were removed and replaced with fresh media, which contained 1000 U/mL of human recombinant IL2 (R&D system United Kingdom, 19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB) and 100nM Rapamycin (ThermoFisher™, 151 Brook Drive, Milton Park, Abingdon, GB-OXF OX14 4SD).

Technique 2: EasySep Treg isolation. Tregs were isolated using EasySep™ Human $CD4^+CD127^{low}CD25^+$ Regulatory T Cell Isolation. The isolated PBMC cells were placed into a 5mL polystyrene round-bottom tube (STEMCELL Technologies, building 7100, Cambridge Research Park, Beach Drive, Waterbeach, Cambridge, UK, CB25 9TL, Cat: 38007). 50ul/ml of anti CD25 Ab cocktail (positive selection) was added to the sample at a room temperature for 5min. Next, 30ul/ml of Releasable Rapid Spheres were also added to the sample after 5min, and finally 50ul/ml of $CD4^+$ T Cell Enrichment Cocktail was added. Moreover, after 5min the sample was placed on the magnet (STEMCELL Technologies, EasySepTM Cat:

18000) and left for 10min. Thereafter, the non-attached cells were transferred into a new 5mL tube. Approximately $1x10^6$ cells were isolated. The Tregs after isolation were cultured at $1x10^6$cells/ml in 96 well plate (100µl per well) in T cells expansion medium that had been supplemented with 500 IU/ml of IL2, 25ul of T cell activator (ImmunoCultTM-XF anti-CD3/CD28/CD2 beads, Stem Cell Technology), and Rapamycin 100nM.

3.7 Treg expansion and validation (see Figure 2)

The isolated Treg cells were cultured using Rapamycin (100nM) in X-VIVO 15 Medium, which had been supplemented with T-cell expansion media and T-cell activator (Gibco™ Dynabeads™ Human T-Activator CD3/CD28). Cells were re-suspended at a concentration of $1x10^6$cells/mL and seeded in a 24-well plate. On day 1 post-stimulation, half of the media (500 µL) was removed and 500 µL fresh media was added. Moreover, 1000 U/mL human recombinant IL2 was added, while rapamycin (100nM) was replenished. On day 4 and day 6, the cells were counted (data not shown). On day 9 or 10, the activation beads were removed using a magnet, and the cells 'rested' in IL2 (1000 U/mL) containing media, until day16.

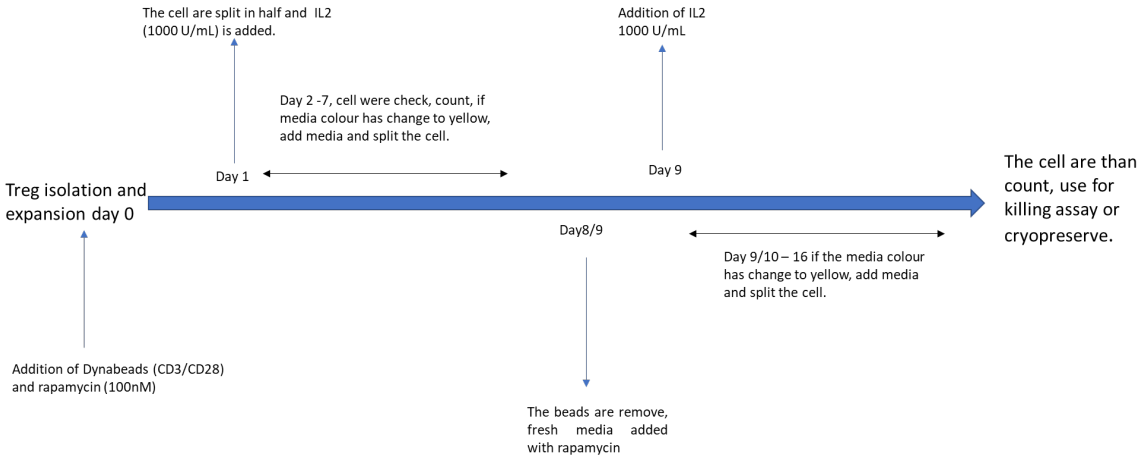

Figure 2: Schematic representation of the timeline for the expansion of Treg cells

Treg cells were isolated on day 0 using either Stem cell EasySep or Miltenyi Biotec/Gibco from whole Blood or PBMC. The cells were seeded at 1x10^6 in X-vivo 15 media, which had been supplemented with 5% of Human Sera, 100nM of Rapamycin, and anti-CD3/CD28 beads. On day 1, IL2 was added at a concentration of 1000 U/mL. At every 2 to 3 days, the cell media was replenished with fresh media. Furthermore, beads were removed at day 9/10, and cells were cultured until day 16. A suppression assay was carried out at the end of the harvest, with the ultimate goal of testing the functionality of the cells.

3.8 Labelling Tregs with PKH67

The labelling kit that was used to stain Treg cells was from Sigma (PKH67GL), which contained Diluent C and PKH67 solution. Treg cells were washed with HBSS and centrifuged at 300g for 7min. The supernatant was removed, and the cell pellet was re-suspended in 500ul of Diluent C. In the meantime, the dye solution was prepared using the following procedure: 4ul of PKH67 was added to 500ul of Diluent C, and both thoroughly mixed. Thereafter, the cells were transferred to the dye mixture, where they were gently mixed. After 3mins of incubation (in the dark), the reaction was stopped by adding 1ml of CM, which contained at least 1% of proteins. After 1min, incubated cells were centrifuged at 300g for 7min. Next, the supernatant was removed, and 10ml of CM was added. The cells were transferred into a new tube and centrifuged again. The supernatant was discarded, and the cells were re-suspended in fresh CM.

3.9 Labelling cells with CellTrace-Violet (CTV)

CTV stock solution (stock conc. 5mM) was prepared by adding and thoroughly mixing 20ul of DMSO into a vial of CellTrace Violet reagent (ThermoFisher). Cells were washed using PBS (300g, 5min, RT), and thereafter re-suspended in PBS to obtain a final concentration of 1x10^6 cell/ml. In addition, 1ul of CTV stock solution was added to each 1ml of cells suspension. The cells were incubated for 20 min at 37^{o}C, protected from light, and mixed every 5min to ensure uniform labelling. After the incubation step, excess CM (5 times the original staining volume) was added,

and the cells were left for 5min. Cells were then pelleted by centrifugation, re-suspended in pre-warmed CM, and left for at least 10min in the incubator. This was to allow the CTV reagent to undergo acetate hydrolysis.

3.10 Treg Suppression Assay

Treg suppression assays were carried out at the end of the expansion period. Suppression assays were set up according to Collison et al. (2011). In summary, thawed Tconv cells were labelled with 5uM CTV, while Treg cells were labelled with 4uM of PKH67. Both cell types were co-cultured in 96-U bottom plate for 5 days at various ratios, in the presence of anti-CD3/CD28 coated beads (Life Technology Dynabeads™), at the ratio of 1:42 (beads:Tconv). On the last day of co-culture, the dye dilution (proliferation of Tconv) was measured using Novocyte3000 Flow Cytometer and analysed via NovoExpress. The gating strategy is shown in Figure 3A and B, as well as an example showing that 90.17% of Tconv activated with beads over 5 days proliferated (Figure 3A). Furthermore, according to Figure 3B, with the addition of Treg, the proliferation of Tconv cells were inhibited to only 27.72%.

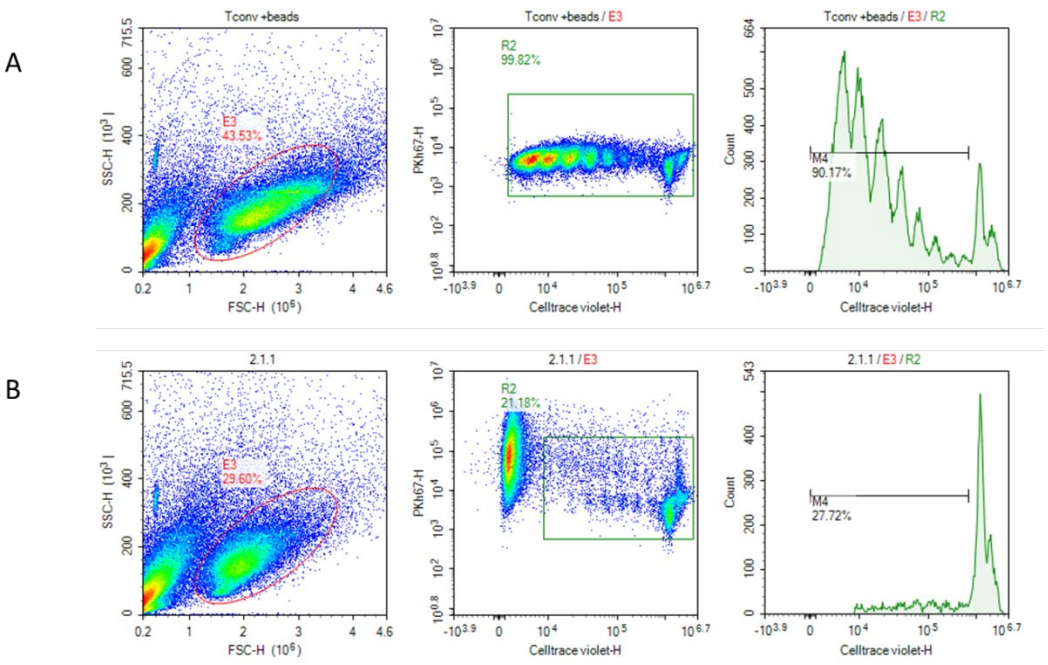

Figure 3: Gating strategy for the Suppression assay

The Analysis of suppression assay was performed by selecting FSC and SSC, while the lymphocytes were gated (red gate, left hand plot). Next, the CTV labelled Tconv were gated (green box), middle plot. Histograms were selected to show the CTV dilution (right hand plots). (A) Tconv cells were labelled with CTV and incubated with the addition of beads (ratio of beads:Tconv 1:42). The histogram showed many peaks, which confirmed that division had occurred in the sample, and the range gate (M4) shows the percentage of CTV dilution. (B) Treg cells were labelled with pKH67 and incubated with addition of beads before adding to CTV labelled Tconv at ratio 2:1, in the presence of beads. CTV+ pKH67- cells were gated (green gate) and the Tconv proliferation was shown as a histogram, with M4 showing the percentage of CTV dilution.

3.11 NK cell isolation

As described above, PBMC were isolated from leukocyte cones, using density gradient centrifugation. NK cells were isolated using negative bead selection (EasySep™ Human NK Cell Enrichment Kit; Stem Cell Technologies) and an EasySep magnet, following manufacture's protocols. PBMCs were counted, washed, and re-suspended at the final concentration $50x10^6$/ml in EasySep Buffer. These cells were transferred into a round bottom tube containing 5 mL of polystyrene and 50ul of Enrichment Cocktail per ml of sample was added. The sample was mixed and incubated for 10min RT. Next magnetic beads (after vortexing), 100ul per ml of sample, were added to the cells and left for 5min RT and after incubation, EasySep Buffer was added. The EasySep Magnet was chosen for volumes up to 2.5ml whilst the BigEasy magnet was used for volumes up to 5ml. Before placing on the appropriate magnet, cells were mixed and then placed in the magnet for 2.5 min and non-adherent cells were collected. The yield of NK cells isolated was calculated using Equation 1 below and NK cells were assessed by phenotyping pre- and post-isolated fraction by flow cytometry using antibodies that recognise proteins on NK cells (see 1.13).

Equation 1: Calculation of yield

$$A: Number\ of\ NK\ in\ PBMCs = \frac{Total\ number\ of\ PBMCs * \%of\ NK\ in\ PBMCs}{100\%}$$

$$B: Number\ of\ \ NK\ cells = \frac{Total\ numbers\ of\ cells\ after\ sort * \%of\ NKcells}{100\%}$$

$$Yield\ of\ sort = \frac{A}{B} * 100\%$$

3.12 Flow cytometry Compensation Staining

To perform compensation UltraComp eBeads™ (Invitrogen™ 01-2222-42) was used. Firstly, FACS tubes were labelled for each antibody conjugate that was used in the experiment. The compensation beads were mixed by inverting and 1 drop of beads was placed in each tube. Furthermore, 5ul of antibody conjugate was added to the appropriate tube. After mixing, the tubes were incubated for 15min at 2-8^{0}C in the dark. Then, to each tube, 2ml of Staining Buffer (PBS+2%FBS) was added, and tubes were washed by centrifuging (300g, 5min). The supernatant was removed and 200ul of Staining Buffer was added to the tubes. Compensation was performed using automated settings on NovoCyte Flow Cytometer.

3.13 NK and Treg cell Phenotyping

Samples of interest were placed into FACS tubes and washed by adding 2ml of Staining Buffer (300g, 5min). The cell pellet was re-suspended in 100ul of Staining Buffer and mAbs were added to the samples, according to manufacturer's protocol. For NK phenotyping, the following mAbs were used: CD3(BV652: Clone SK7, BD Biosciences) and 5ul of CD56 (APC-Cy7: Clone HCD56, Biolegend). Samples were incubated for 15min at 2-8^{0}C in the dark. After the incubation time, 2ml of Staining Buffer was added, and tubes were centrifuged at 300g for 5min. Thereafter, the supernatant was removed and 200ul of staining buffer was added.

The samples were analyzed on Novocyte Flow cytometer. Analysis of NK cells were performed by selecting FSC and SSC, next the $CD3^{-}$ $CD56^{+}$ cells were gated, and the unstained sample were used as a control.

For Treg phenotyping, at the end of culture, Treg were stained with the following antibodies: anti-CD4 PerCp-Cy5.5 (OKT4 clone, Life Technology), anti-CD25 PE (4E3 clone, Miltenyi Biotec), and anti-FoxP3 (PCH101clone, Life Technology). The expression of these markers was assessed by flow cytometry using the Novocyte 3000 (Agilent Technologies LDA UK Limited, 5500 Lakeside, Cheadle Royal Business Park Stockport, Cheshire SK8 3GR). The results were analysed using NovoExpress software. The mAbs were used for extracellular staining: CD4 (Anti-Hu, Invitrogen) and CD25 (4E3, Miltenyi Biotec). Then a staining for the transcription factor FoxP3, which was expressed by Tregs using a kit from Invitrogen eBioscience™ Fixation/Perm Diluent (REF: 00-5223-56). Briefly, the media Fix/perm (1ml Fixation Diluent and 3ml of Permeabilization Diluent) and Perm Buffer (1ml of Permeabilization buffer and 9ml of Deionized water) were firstly prepared. After performing surface staining, samples were washed and 1ml of fix/perm was added to the tubes. Samples were incubated between 30-60 minutes in RT or at 2-8^{0}C. After incubation time, 2ml of perm buffer was added to the samples, and tubes were centrifuged between 400g – 600g for 5mins. Then, the supernatant was discarded and FoxP3 mAb was added (FoxP3 PE Antibody, anti-mouse, REAfinity™ clone:REA788). Furthermore, samples were incubated for 30min in the dark, RT. Next, 2ml of perm buffer was added and the tubes were centrifuged at 600g for 5 min twice. After washing, the supernatant was removed and cells were re-suspended in 200ul of Staining Buffer, and samples were analysed on Novocyte Flow cytometer.

3.14 NK cell cytotoxicity assay

To test NK cytotoxicity function, a 4-hour killing assay was performed using K562 as target cells; which are sensitive to NK cells due to their lack of MHC class I expression (Nishimur et al., 1994). The K562 cells were washed with HBSS, then labelled with CTV and a total of 100µl of media containing $1x10^{5}$ CTV -labelled target cells were added to 400µl medium containing $5x10^{5}$ NK cells (5:1, E: T

ratio). Cells were incubated for 4 hours at 37^0C and 5% CO_2. To assess cell death, cultured cells were re-suspended in a solution of TO-PRO3 iodide (Invitrogen, Cat: 642/661), just before the analysis on Flow Cytometer. Background cell death was determined from the spontaneous lysis (death) of target cells that were incubated alone. All calculations were based on the absolute count of target cells in each experimental sample. To calculate % of lysis, the equation below was used.

Equation 2: Calculation for cytotoxicity

The calculation of cytotoxicity is based on absolute count of target cell in experimental samples. Absolute count if taken from the number of live cells multiplied by the total volume of sample.

$$0h - 4h = A$$

$$\frac{A\ X\ 100}{4h} = B$$

$$\%\ of\ lysis = 100 - B = C$$

$$\%\ of\ specfic\ lysis = C - B$$

3.15 Generation of TpNK cells

Cryopreserved NK cells were thawed and were placed in CM for 2 hours at 37^0C. CTV-1 cell lines were mitomycin-C pre-treated, in order to stop the growth of the cell. Both cell viability and numbers were checked by flow cytometry using TO-PRO as a viability dye. If NK cells viability were 45% and above, they were re-suspended at $1x10^6$/ml, and incubated 16h under the following conditions: alone or with addition of CTV-1 -to generate TpNK (ratio NK: CTV-1 1:2). The next day cells were washed and used in killing assay (at the ratio of 1:5 effector to target), as described in 3.14.

3.16 Assessment of Treg effect on NK cell function

NK cells co-incubated with CTV-1 (TpNK) were then co-cultured in the presence or absence of Tregs (NK:Tregs ratio 1:1) and with various doses of IL2 (100IU, 200IU, and 400IU). In addition, a killing assay was performed against K562, Raji and OVCAR3, to check cytotoxicity of TpNK cells, following exposure to Tregs. This was done in triplicate or duplicate, depending on the number of NK cells isolated.

3.17 Degranulation Assay

The killing assay and degranulation assay were performed co-currently (for killing assay, see NK Cell cytotoxicity assay above), with appropriate control tubes for each condition. On the first day of the experiment, NK cells were incubated alone, with or without Treg; with the presence or absence of IL2 (different doses). The next day samples were washed and CTV labelled K562 were added to each condition (ratio NK:Target 5:1) for 4hours. 5ul of anti-CD107a mAb (VioBright 515, clone:REA792, Miltenyi) was added and after one hour of incubation at 37^{o}C, 5ul of monensin (ThermoFisher, Cat:00-4505-51) was added to each sample for 3 hours. After the incubation time, extracellular staining was performed: cells were washed with staining buffer and labelled with anti-CD3 (BV652: Clone SK7, BD biosciences) and anti-CD56 mAbs (APC-Cy7: Clone HCD56, Biolegend). After 15min of staining, samples were washed and re-suspended in staining buffer. The degranulation of NK cells was assessed simultaneously with cytotoxicity of NK by addition of TO-PRO. The gating strategy used is shown in Figure 4.

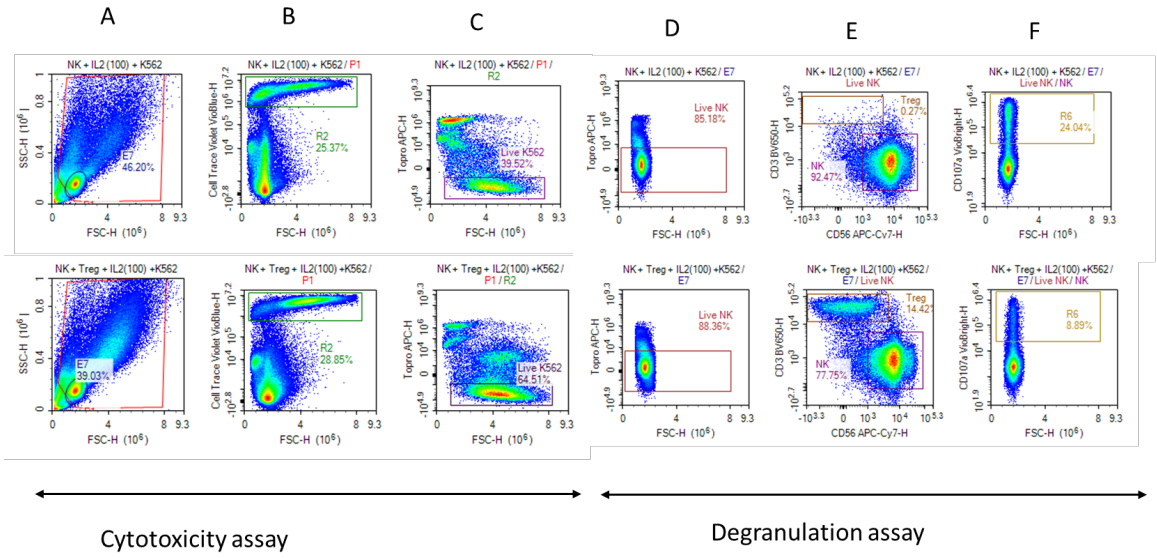

Figure 4: Gating strategy for NK cytotoxicity and degranulation

The NK killing assay and CD107a measurement were performed for over 4 hour and co-currently. A, B, and C showed the gating strategy for NK cell killing. (A) Showed plot FSC vs. SSC with debris exclusion, while (B) showed K562 cells which have been labelled with CTV, and are gated (R2); hence, 10,000 events were collected, for consistency. (C) The Live cells (negative for ToPRO) were gated from R2, while D, E and F showed the gating strategy for degranulation. (D) Live NK cell was gated from E7 (A) and in (E), where Treg and NK cell were gated. (F)The percentage of CD107a, is expressed on NK cells

3.18 Killing assay performed on xCELLigence, using OVCAR3 as target cell line.

5000 OVCAR3 cell, (volume 50ul in culture media) were seeded into a 16 well plate (Agilent E-Plate Cat: 6565382001). The isolated NK cells were incubated alone, or with IL2, or in the presence of CTV-1, either with or without Treg cells. The following day, each condition were added onto 16 well plates (in triplicate), (where the adherent OVCAR3 cells has expanded) at ratio 5:1 (effecter : target). xCELLigence (RTCA: Real Time Cell Analysis) was used to measure the killing data continuously over a 3day period, recording the data every 15min.

3.19 Statistical analysis

The statistical analysis was performed using GraphPad Prism version 8.00 (GraphPad Software, San Diego, California, USA). When two groups are compared, an unpaired t-test was applied. However, when there are three or more groups being compared, a one-way ANOVA was used. * = $p>0.05$

4 *Results*

4.1 Two step bead isolation of Tregs generated better purity and yield compared to Stem cell Kit

In order to isolate and expand Tregs from PBMCs, two previously published methods, StemCell EasySep (Zizzari et al., 2015) and a Two Step bead isolation protocol (Fraser et al., 2018) (reagent were from Miltenyi) were tested. As expected, both methods isolated $CD4^+25^+Foxp3^+$ / $CD4^+25^+CD127^{low}$Treg cells (Figure 5 A-G) with the Stem Cell EasySep method, giving a high purity of $CD4^+$ $CD25^+$cells (86.93%, Figure 5B). However, only 22.3% of these cells were $Foxp3^+$ (Figure 5C), and 88.86% were $CD127^{low}$ (Figure 5D). In comparison, the Two Step protocol gave a lower $CD4^+$ $CD25^+$purity of 77% (Figure 5B); however, 76.26% of these were $Foxp3^+$ (Figure 5C), while 97.19% expressed $CD127^{low}$ (Figure 5D). It is important to state that the flow cytometry data in Figure 3 (A-D) is from one donor. Figure 5E, shows the purity of isolation according to $CD4^+CD25^+Foxp3^+$ and there were significant differences between the two methods. The Two Step method showed up to 90% purity compare to StemCell, where the highest purity was below 60%. Figure 5F shows the purity of Treg isolation according to $CD4^+25^+CD127^{low}$ and there was a significant difference between the two methods. The lowest percentage for the Two Step method was 50% and 10% for the StemCell Treg isolation. For the purity of Treg cell after isolation the Two Step method showed a better result compare to StemCell Treg isolation. Figure 5G, shows the percentage of yield obtained after the Treg isolation for both methods and there were no significant differences. However, on average the percentage of yield for the Two Step method is higher compare to StemCell Kit. The highest yield obtained from the Two Step method was 70% and 40% for StemCell Treg isolation.

Additionally (data not shown), differences were seen in the number of $CD4^+$ $CD25^+$cells that were isolated using these methods. Around 2-4 x 10^6 $CD4^+$

$CD25^{+}$cells were obtained using the Two Step protocol, whereas fewer than a million cells were obtained using the EasySep isolation. Unfortunately, cells isolated from the latter did not expand to sufficient numbers, whereas cells isolated from the Two Step isolation expanded well. After expansion an average of 30 $x10^{6}$ cells, with a purity of 90% $CD4^{+}25^{+}Foxp3^{+}$ Treg cells were obtained at the end of harvest. Based on these findings, the Two Step method of isolation was used throughout the project, to isolate $CD4^{+}25^{+}Foxp3^{+}$ cells.

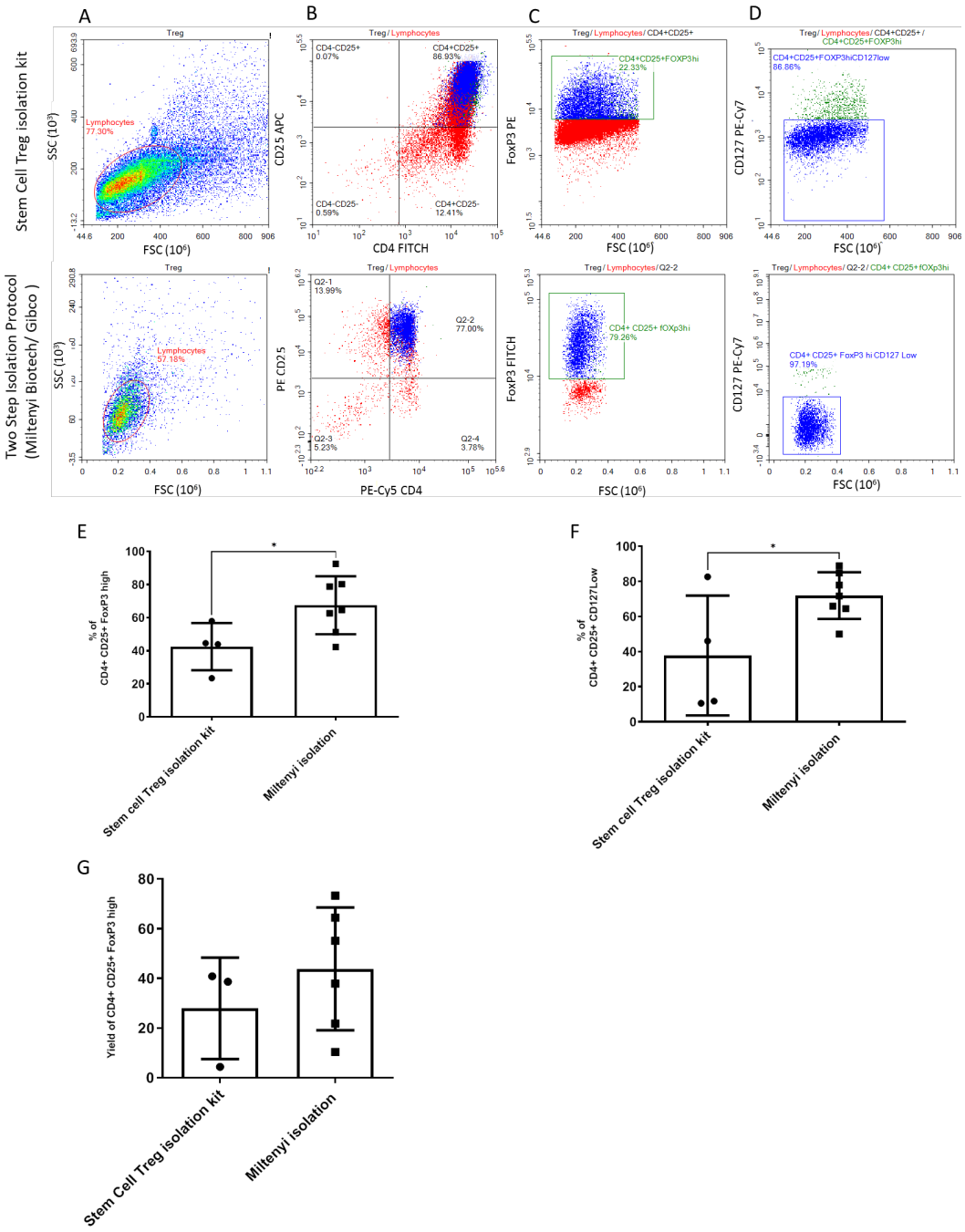

Figure 5: Comparison of Isolation Strategies, Stem cell Kit and Two Step Isolation Protocol

The plots showed the gating strategy of Treg cells for the two methods Stem cell Kit and Two Step Isolation Protocol. After the isolation of Treg, the cells

were stained with antibodies, such as CD4[FITC], CD25[APC], FoxP3[PE], CD127[PE-Cy7] and the following: (A) all the lymphocytes were gated, (B) Quadrant gate was selected to identify the $CD4^{+}$, and $CD25^{+}$ cells, (C) rectangular gate showed an expression of FoxP3, and (D) The Tregs cells had $CD127^{Low}$, which was gated. After the Isolation of Treg from Cone for both methods, the percentage of $CD4^{+}CD25^{+}Foxp3^{+}$ and $CD4^{+}CD25^{+}CD127^{low}$(E,F), as well as the yield (G) were taken from $CD4^{+}CD25^{+}FOXP3^{+high}$. The Stem cell Kit purity is n = 4, yield n = 3, while the Two Step Isolation (Miltenyi) purity is n= 7, yield n = 6. In all the experiment, the data present had a mean ± SD, and it was analysed using unpaired t-test with p < 0.05.*

4.2 Isolated Tregs suppressed T cell proliferation

Having established a protocol for Treg isolation and expansion, next the suppressive activity of cultured Tregs was assessed, using a previously published Tconv suppression assay (Collison and Vignali 2011; Fraser et al., 2018b). The suppressive capacity of Tregs isolated and expanded from four healthy donors was assessed. Tconv cells were activated with anti-CD3/CD28 coated beads and 90% of the cells proliferated as observed by the dilution of CTV (Method Section 3.9). In comparison, the addition of Tregs at a 2:1 ratio inhibited this with only 27% of Tconv proliferating, in the presence of these cells (Figure 6). By titrating the number of Tregs to Tconv, the potency of the Tregs was measured by calculating the percentage of suppression according to the formula shown in the Materials section. As expected, it was found from calculating percentage of suppression, that decreasing the number of Tregs led to less suppression and an increase in Tconv proliferation (Figure 6 A and B). also It was also observed that the Tregs isolated from each individual cone had different suppressive capacities, some were highly suppressive, (Figure 6A) inhibiting Tconv proliferation even at a 1:16 Treg:Tconv ratio, whilst some donors Tregs were less suppressive giving only 30% suppression at a 1:4 ratio (Figure 6B). In conclusion, the Tregs isolated and expanded *in vivo* with IL2 and

anti-CD3/28 beads were capable of suppressed $CD4^{+}25^{-}$ T cell proliferation. For future work on NK cells, a ratio of 1:2 (Tregs:NK) was used to ensure that the suppressive effect of Tregs was measured.

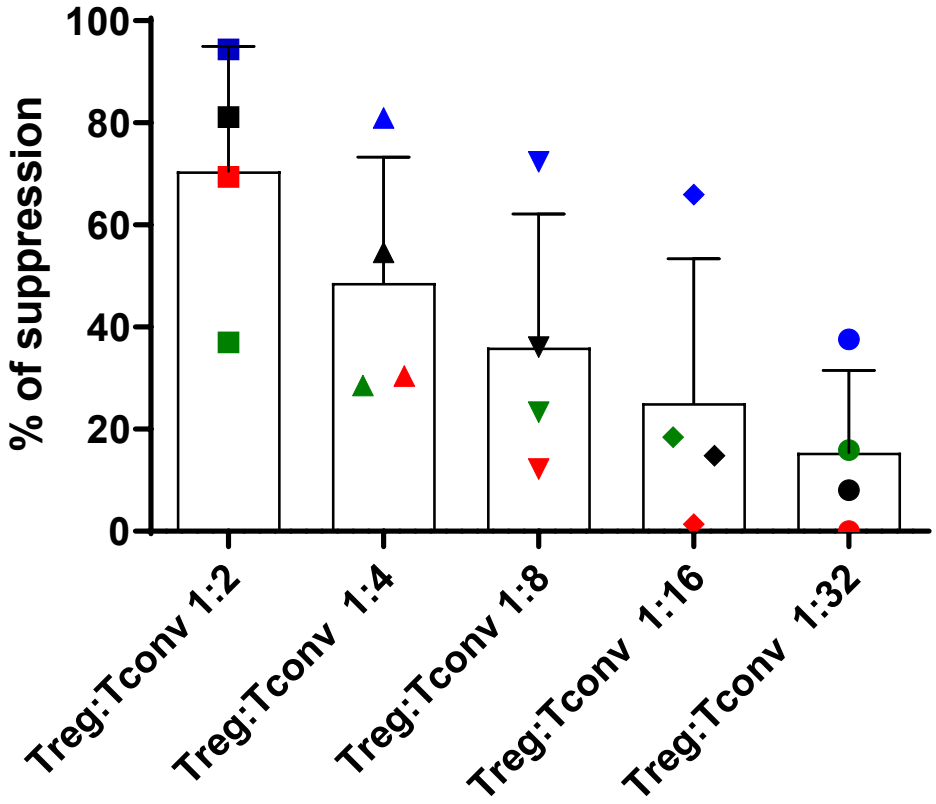

Figure 6: Treg suppression increased as the concentration of Tconv decreased.

Tconv cells were labelled with 5uM CTV while the Treg cells were labelled with 4uM of PKH67. The suppression assay was done over 5 days and the expansion of Tconv, was read by flow cytometry, which was followed by calculating the percentage of suppression. The graph presents suppression of Tconv proliferation for different ratios of Tconv: Treg cells 2:1, 4:1, 8:1, 16:1 and 32:1 represents T regs the pooled data from 4 donors.

4.3 Isolated NK cells using StemCell NK isolation kit showed 90% purity

To isolate NK cells from PBMCs, a negative bead isolation protocol was performed, using a NK Enrichment Kit. After isolation, NK purity was assessed by immunophenotyping using the surface markers: CD3 (VioBlue) and CD56 (APC-Cy7), as NK cells were described as $CD56^{+}CD3^{-}$ (Molfetta et al., 2017). In Figure 7A-C about 20% of PBMCs were $CD56^{+}CD3^{-}$, but after isolation, the purity was 90% of $CD56^{+}$ $CD3^{-}$ (Figure 7D). Figure 7E shows the yield, which is defined as the number of NK cells isolated as a percentage of the total number of NK cells in the starting population. Next isolated NK cells were tested in killing assays, using three different cell lines as targets.

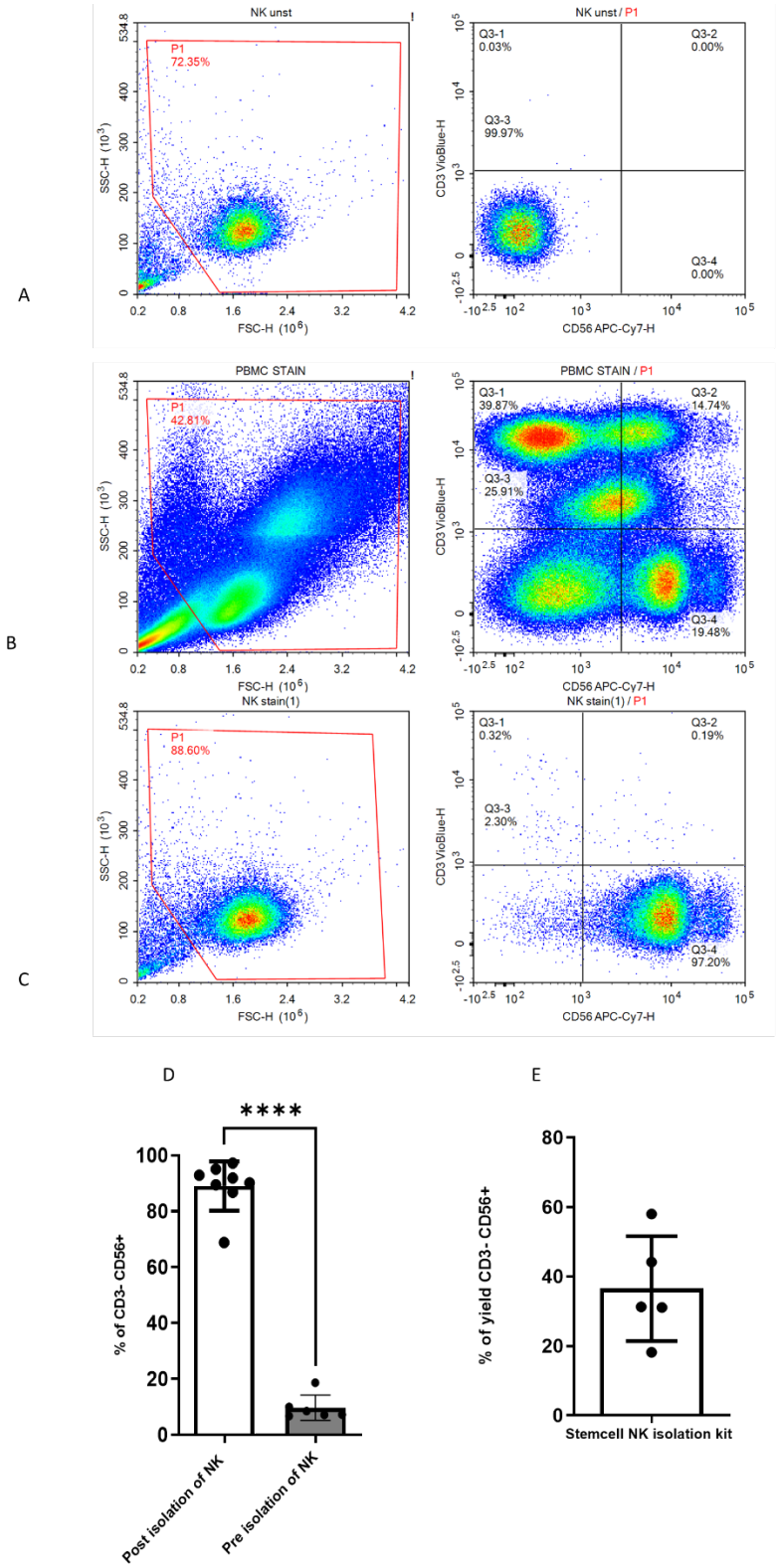

Figure 7: NK cell isolation using the StemCell positive selection kit

*NK cells were isolated using a Stemcell NK kit, which is a negative bead selection method from the PBMCs. The isolated NK were stained with CD3 [VioBlue] and CD56 [APC Cy-7], while the flow data is shown for NK isolated from one donor. The unstained sample (A) was used as controlled, and the NK cells were gated for $CD56^+$ $CD3^-$ (B-C). (D) The NK purity was measured as $CD56^+CD3^-$ (number of donors n= 8 for post isolation, and n = 6 for pre isolation) (E) Cumulative data of 5 independent experiment data showing the percentage of yield for $CD56^+$ $CD3^-$. In all the experiment, the data are presented as mean ± SD and analysed using unpaired t-test with p**** < 0.0001.*

4.4 Isolated NK cells can kill K562, Raji and OVCAR3 targets but in the presence of autologous Tregs, the cytotoxicity is inhibited.

To confirm the cytotoxicity of isolated NK cells, a flow cytometry based killing assay was used, using K562 and Raji cell lines as target cells. K562 is an erythroleukemia cell line which is sensitive to NK cell killing, due to the lack of MHC I & II on these cells (Nishimur et al., 1994). Raji is a Burkitt's lymphoma cell line and is relatively resistant to NK-mediated killing (Hasenkamp et al., 2006). Additionally, an adherent, ovarian cancer cell line, OVCAR3 was also chosen as a target cell. In contrast to the suspension cultured target cells mentioned above, analysis of killing of OVCAR3 was performed using the xCELLigence, and the data was presented in real time for over 4 hours (see method 3.18).

As expected, the isolated NK cells killed the K562 cell line, as indicated in Figure 8A, with NK cells isolated from 3 out of 4 donors showing a similar percent of cytotoxicity (15–40%). Whereas one donor (Green symbol) managed to kill 60% of the target cells. On addition of autologous Tregs, the percentage of cytotoxicity became reduced for all the four donors. However, for one donor (grey symbol), their Treg completely inhibited the killing.

In contrast to expectation, NK cells from all four donors killed Raji cells in the flow cytometric assay, with 40% or more killing observed, with one donor (Blue symbol) showing more than 60% lysis (Figure 8B). This could likely be due to transient NK cell activation during transit on the blood filters. Despite this high level of innate killing of Raji, the addition of autologous Tregs decreased the percentage of cytotoxicity in all cases, and one donor (Green symbol) showed a significant reduction when compared with the other two donors.

NK cells from a single donor were tested against OVCAR3 cells, as depicted in Figure 8C. The percentage of cytotoxicity by freshly isolated NK cells was 30%, but with the addition of Treg to the cultures for 4 hours, all OVCAR3 killing was suppressed. Although the data shown represents a single donor, it allows us to conclude that the bead isolated NK cells and could kill up to three different cell lines, and that the killing can be inhibited by Tregs.

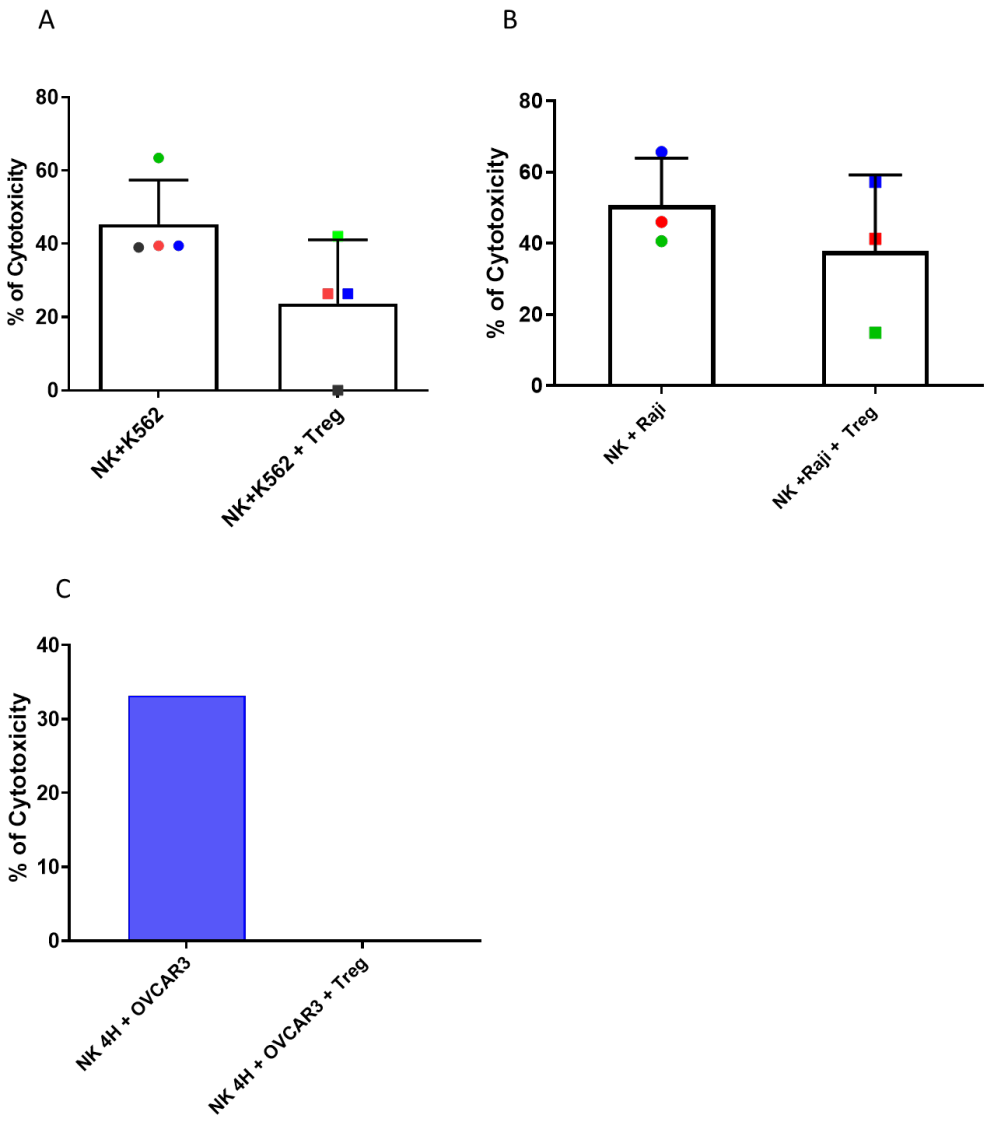

Figure 8: NK cell killing of K562, Raji and OVCAR3 target cells were inhibited by Treg

The isolated NK cells were co-incubated for 4 hours with K562, Raji, and 72 hours with OVCAR3 cell was at a 5:1 effector: target ratio, with or without Treg. (A) Cumulative data of 4 donors, showing the percentage of the cytotoxicity *for K562 cell line, and the Treg suppression of NK cell. (B) Cumulative data of 3 donors data, showing the percentage of the cytotoxicity for Raji cell line, and the Treg suppression of NK cell. The 4 donors for A and 3 donors C are different. (C) One experiment is shown, the percentage of the*

cytotoxicity for OVCAR3 cell line. Please see Appendix B and C for example of the gating strategy for cytotoxicity assay.

4.5 IL2 primed NK cells increased the cytotoxicity, and this is reduced with the addition of Tregs

In order to assess whether Treg can inhibit LAK cells, NK cells were co-incubated with IL2 (100IU) for 16hours to induce LAK cells. The NK cells from all three donors killed K562 cells and this increased after NK cells were treated with IL2, regardless of the concentration used (Figure 9A). The percentage of cytotoxicity of K562 cells (by resting) and LAK cells was high. Resting NK cells killed 40% of the K562 cells in this killing assay; however, this was increased to around 60% after the NKs were exposed to 100IU, 200IU or 400IU of IL2. In the literature (Hood *et al.*, 2019), 100IU of IL2 is commonly used to induce LAK, however this study wanted to assess if increasing the IL2 concentration to 200IU and 400IU would have any effect in the ability of Tregs to suppress LAK cells. There was no significant difference between 200IU and 400IU of IL2, as indicated in Figure 9A and B. In Figure 9C, NK cells were primed by 100IU of IL2, and the target cells were Raji. The percentage of cytotoxicity for LAK was between 70-80% for all 3 donors. With the addition of Treg, one donor (red symbols), the percentage of cytotoxicity was reduced by 10%. NK cells from the two donors (blue and green) with the addition of Treg with LAK, showed a minor reduction in percentages of cytotoxicity. Figure 9D, OVCAR3 are used as a target cell line and NK cells were primed by IL2 at 100IU. The data shown is for only one donor. The NK cell alone showed 30% of cytotoxicity, and with the addition of Treg, no cytolysis occurred. With LAK, the percentage of cytotoxicity was 90% and with the addition of Treg, the cytotoxicity was reduced to 70%.

In conclusion, LAKs showed an increase in the killing for all three different cell lines.

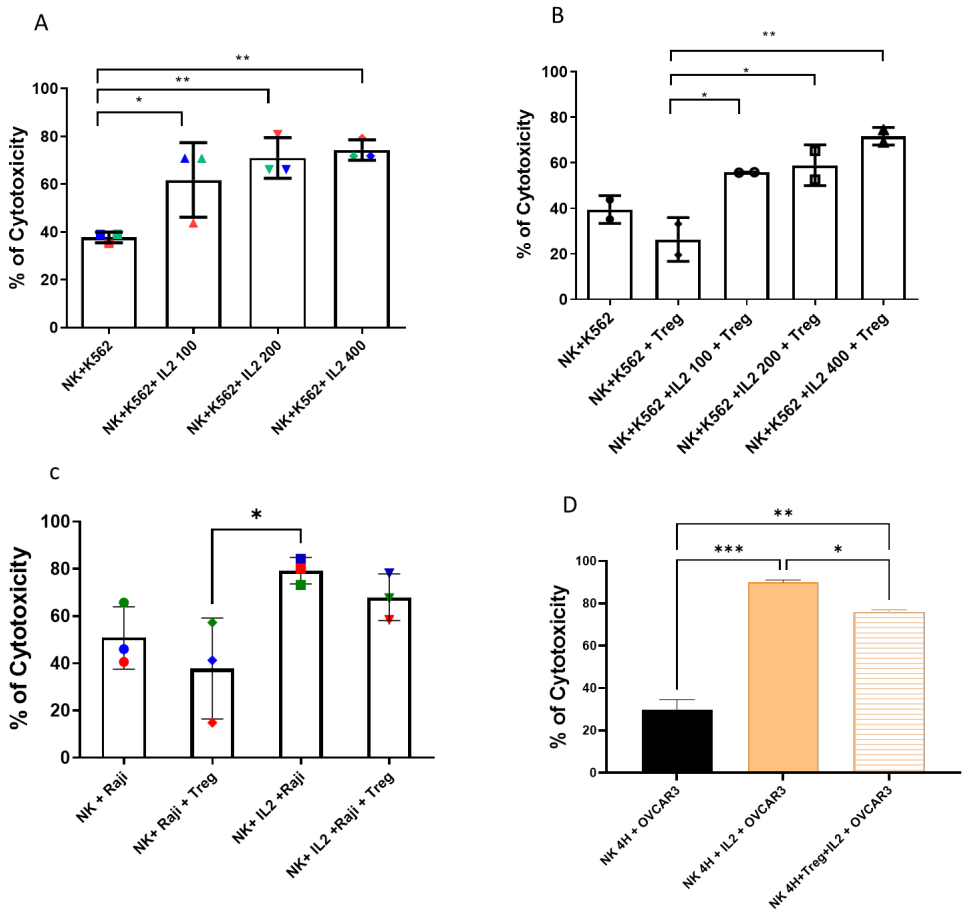

Figure 9: IL2 primed NK cell increased the NK cell killing and overcame the Treg supperssion.

IL2 primed NK cell or untreated NKs were co-incubated for 4 hours with K562, Raji, or OVCAR3 cell at a 5:1 effector: target ratio and the percentage of cytotoxicity are shown. (A) Cumulative data of 3 donor showing the percentage of cytotoxicity for K562. (B). Cumulative data of 2 donors showing the percentage of cytotoxicity for K562. (C) Cumulative data of 3 donors showing the percentage of cytotoxicity for Raji cell line. The 3 donors for A and 3 donors for C are different. (D) One experiment was performed using OVCAR3 cell line. In all the experiment, the data presented as mean ± SD and analysed using one-way ANOVA with p < 0.05. p** <0.01 p***<0.001 respectively. Please see*

Appendix B and C for example of the gating strategy for A,B and C cytotoxicity assay.

4.6 NK killing of OVCAR3 increased with time but was suppressed by Tregs, which was reversed with the addition of IL2

To assess whether increasing the time exposure to IL2 from 4h to 48h had any effect on LAK killing of OVCAR3 and whether Treg suppression also occurred with prolonged exposure, the NK cells were seeded in 16 well plates (5000 cells per well) and co-incubated with IL2, with and without Tregs. On the following day, the NK cells were added to the OVCAR3 in wells of the xCELLigence plate. The cell lysis was measured using xCELLigence, and the data given in real time over a 48h period. The cytotoxicity was read at 4, 8 16, 24 and 48h respectively, and the data is represented in Figure 10 A-D. It was observed that in the presence of NKs, the percentage killing of OVCAR3 increased over time, as indicated in Figure 10A, which was inhibited by Tregs at all times points (Figure 10B). In line with what was observed with the non-adherent cells, IL2 treatment of NK cells increased their killing abilities, which was seen as early as 4hr, thereby reaching 100% at 16h (Figure 10C). As depicted in Figure 10, IL2 primed NK cells were susceptible to Treg suppression at early time points (4hours); however, after 24h of co-culture, this was lost.

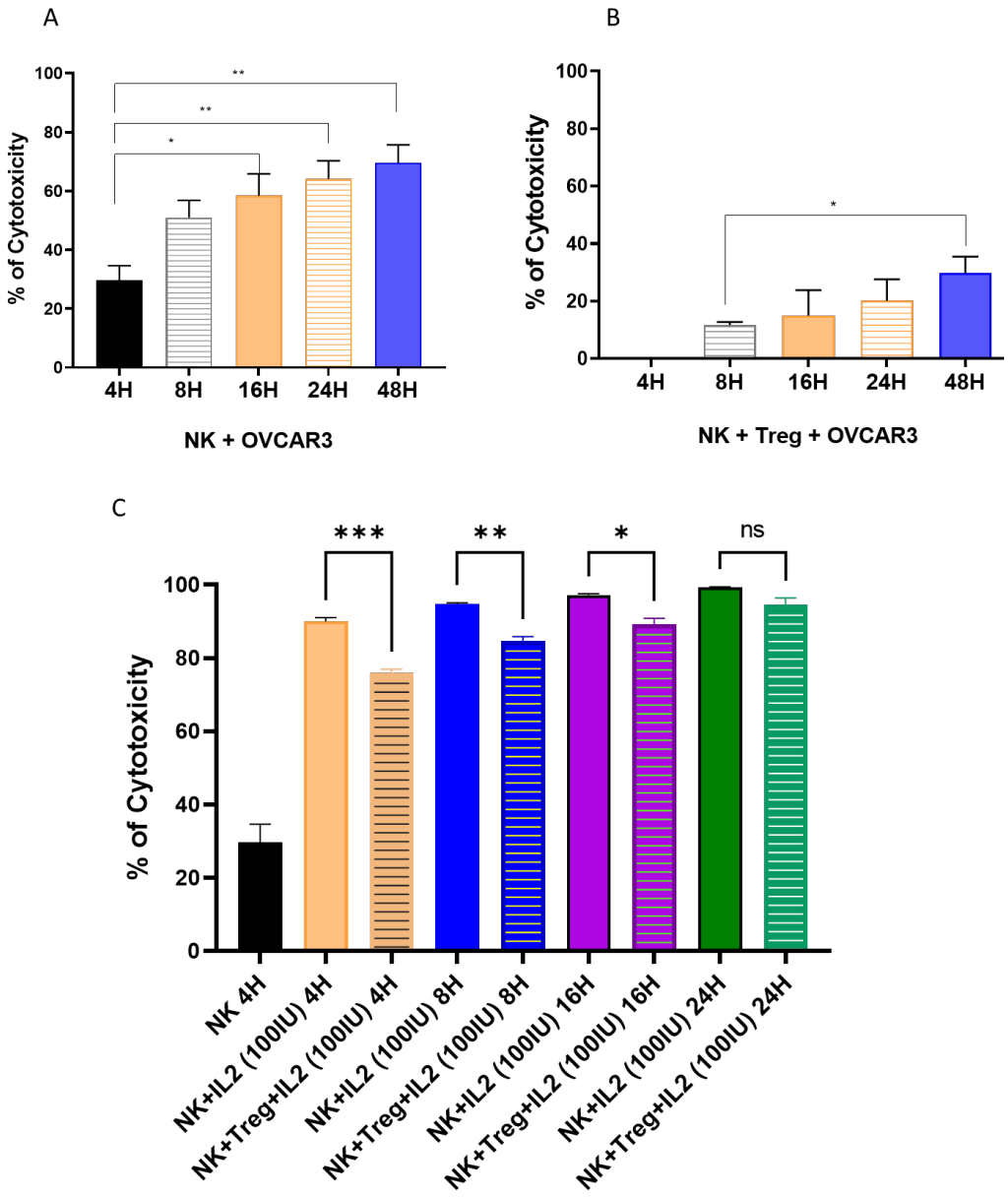

Figure 10: LAK inhibits the Treg suppression over time.

The target cell OVCAR3 is seeded at 5000 cells per well in triplicate, using a 16 well plate, while the naive NK cells were co-incubated with IL2, with or without Treg at day 1. At day 2, each assay condition is added onto the target cell at a ratio of 5:1 (effector to target), while the real time cell lysis is recorded every 15min by xCELLigence. The data above are shown for 4, 8 16, 24 and

48h respectively. (A). (B) (C)). The data are presented as mean ± SD and analysed using one-way ANOVA with p < 0.05, p** <0.01, p***<0.001.*

4.7 CD107a degranulation shows no relationship between prime NK cell with and without Treg

An alternative assay to measure NK-mediated direct target cell lysis is the enumeration of $CD107a^+$ NK cells pre and post exposure to tumour target cells. CD107a is expressed at the time of NK cell degranulation and release of lytic vesicles (Aktas et al., 2009), as described in the introduction. In order to find out more about the nature of Treg suppression of NK function, the NK cell expression of CD107a as a degranulation assay was done co-currently with killing assay. Figure 11A, showed that for one donor (Blue symbols) the addition of Tregs to the co-culture reduced the percentage of CD107a expression from 15% to 5%. This confirmed what had been observed in Figure 8A and B, whereby the addition of Treg reduced NK function. However, the addition of Treg also inhibited CD107a degranulation for two donors (green and black symbols) although this was not as drastic.

Next, NK degranulation was assessed by the presence of IL2 at different concentrations (100IU, 200IU, 400IU). In Figure 11B, the CD107a degranulation was increased when IL2 was added to the condition, for 1 donor (Blue symbols), with expression of 25%. For the other two donors not much difference can be seen. With the addition of Treg, only 1 donor (Blue symbol) showed a reduction in CD107a degranulation by 10%.

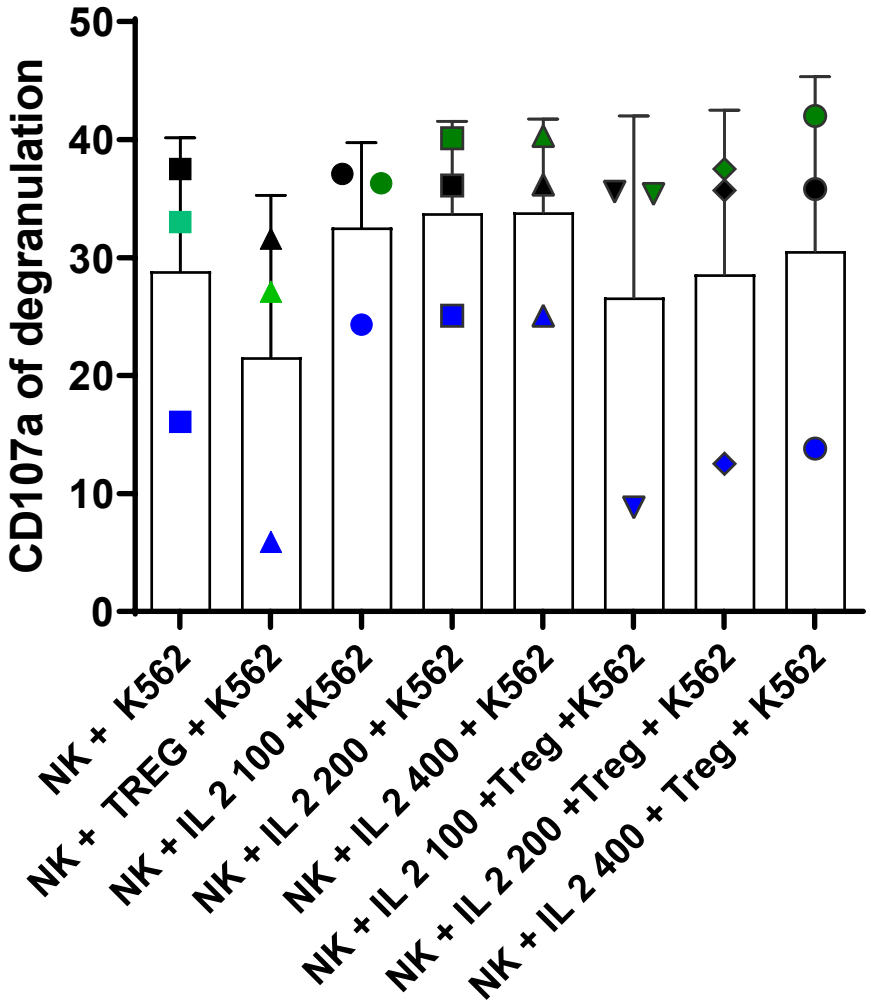

Figure 11: CD107a degranulation of NK cells

The isolated NK cells alone or co-incubated overnight with IL2 or Treg or both. The following day, the target cell lines K562 are added to the mix (ratio of 5:1 effector to target) for 4 hours. This was followed by phenotype for CD3, CD56, and CD107a respectively, which analysed by flow cytometry (for further details, please see the method section 3.17). Cumulative data of 3 donors showing the percentage of CD107a degranulation. All 3 donors represented by blue, green and black colour are same across. Please see Appendix B, for example of gating strategy for CD107a assay.

4.8 TpNK treatment of NK cells inhibited the Treg suppression

In order to overcome the suppression by Treg cells, NK cells were treated with CTV-1, as described previously (Sabry *et al.*, 2019). This NK:CTV-1 co-culture induced NK cell activation akin to IL2 priming but in the absence of IL2. These CTV-1 exposed NK cells were termed "Tumour-primed NK cells" – TpNK(Sabry *et al.*, 2019. This study used Raji (Figure 12A) and OVCAR3 (Figure 12B) as the target cell line to assess if TpNK cells were resistant to Treg suppression. As indicated in Figure 12A, the addition of Tregs had no effect in the percentage of cytotoxicity induced by TpNK for all 3 donors tested. The TpNK cells killed Raji targets in the presence of Tregs, suggesting that TpNK cells were resistant to Treg suppression. In Figure 12B, lysis of OVCAR3 cell line by TpNK was evident at each time point of co-culture, with an increase percentage in cytotoxicity observed with time. The addition of Tregs however inhibited this at early time points, 4 and 8h, however Treg suppression was overcome with increased exposure to TpNK, and at 16h, 24h, and 48h, TpNK inhibited the Treg suppression seen at early time points.

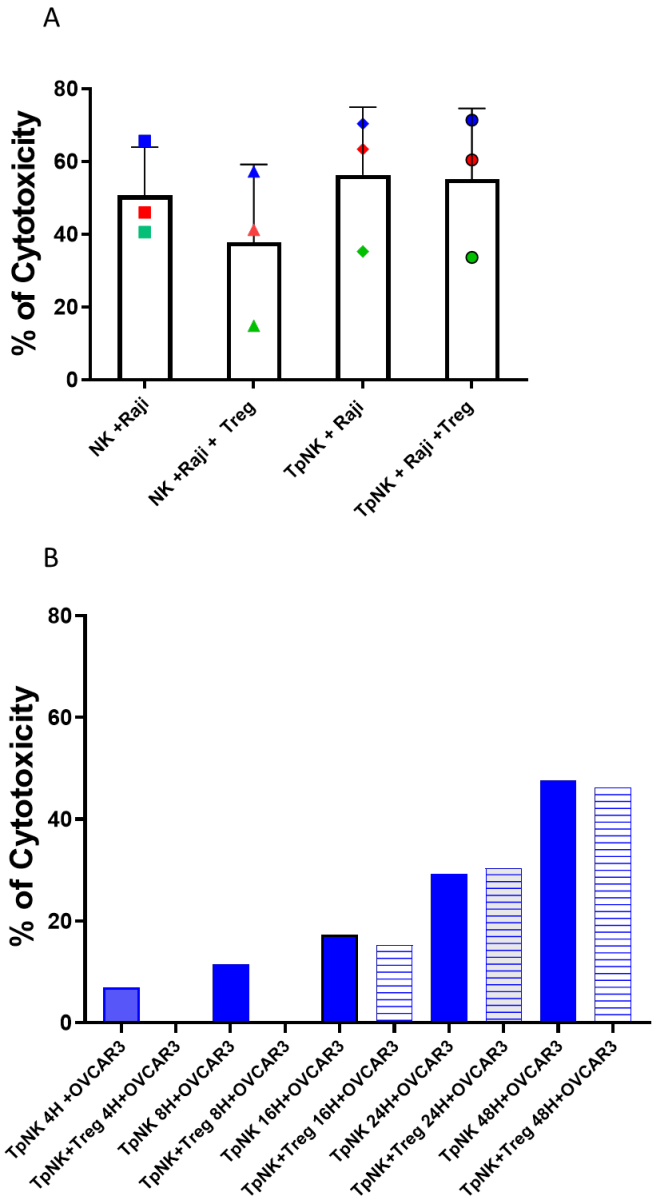

Figure 12:TpNK are resistant to Treg suppression

The isolated NK cells are co-incubated with CTV-1, with or without Treg. Each condition was incubated overnight (the NK: CTV-1 were at the ratio of 1:2) and TpNK were co-incubated for 4 hours with target cell line at a 5:1 effector: target ratio and the percentage of Cytotoxicity for each donor is shown. (A) The target cell line was Raji, and each colour dot represents one

donor (n=3) (B) The target cell line were OVCAR3, and only one donor is shown. The graph shows the % of Cytotoxicity for TpNK and TpNK:Treg at different time point and the non-fill bar are at 4h and 8h, represent no % of specific cytolysis. The mean % Cytotoxicity +/- SD is represented as a bar chart. Please see Appendix C, for example of gating strategy for Cytotoxicity assay Raji cell line.

5 *Discussion*

The aim of this project was to determine if Treg cell could suppress NK cell cytolysis, and by priming the NK cells through the use of IL2(LAK) and CTV-1 cell line (TpNK), which inhibited the Treg suppression. By examining the effect of the interactions between NK and Treg cells, a better understanding emerged, which could be applied towards NK immunotherapy in cancer patients. During this project, Treg cells were able to be isolated, expanded, and suppressed up to 90%. The cytotoxicity assays that were carried out showed a reduction in the NK cells cytotoxicity, with the addition of Treg cells, as against the 3 different cell line K562, Raji and OVCAR3**.** In case of OVCAR3, at 4h of cytotoxicity assay, the Treg cells completely inhibited NK cell cytolysis. As for all the targeted cell lines, when the NK cells were primed with IL2, there was an increase in cytotoxicity, even with the addition of Treg cells. The TpNK cells cytotoxicity were not much higher than the NK cells; however, it manages to completely inhibit the Treg suppression. CD107a were also studied in order to explain the reason why the Treg cell manages to suppress the cytotoxicity of NK cell.

Two protocols for Treg isolation, expansion, and suppression had been optimised; where each of the expansions took around 3-4 weeks, and a significant amount of Treg was obtained. The main part of the optimisations were to add Rapamycin to the medium, as rapamycin has been described as immunosuppressive drug. This had been known for years, but without the knowledge of its mechanism of action (Morelon et al., 2001). This was discovered by (Strauss et al., 2009), that rapamycin selectively promotes Treg's growth, and it inhibits the expansion of $CD4^{+}CD25^{-}$ conventional T cells, thereby resulting into apoptosis. During the first week of expansion, there was limited growth attributed to the Treg; while in the second week, there was proliferation, although it started slowly. In the third week,

the growth was exponential, which was in agreement with the findings of (Strauss et al., 2009).

The suppression activity of Treg cells were measured by proliferation assay, with Tconv ($CD4^{+}CD25^{-}$) as the target cell, while Treg cells was an effector. The Treg cell that showed suppression were from the ratio of effector to target cell; i.e. (E:T) 1:1 to 1:16. This finding was similar to what was expected (Mercadante and Lorenz, 2017). Even after a long expansion in vitro, not only did Treg maintain its suppression, even at 1:8, it managed to suppress Tconv cells.

Numerous findings found that Treg cells are capable of suppressing NK cell cytotoxicity. The mechanism by which this occurred are through the membrane bound TGF-β on Treg, which suppresses NKG2D-mediated killing (Trzonkowski et al., 2004; Ghiringhelli et al., 2005; Smyth et al., 2006). we were able to isolate he majority of studies conducted on the NK-Treg cells interaction were done using mice, and it was just two papers that were published on humans. Ghiringhelli et al. (2005) is the leading published paper, as comprehensive studies were reflected therein. For instance, the authors examined the NK-Treg interaction in a healthy donor and cancer patient, in vitro and in vivo mouse model. They investigated different cytokine conditions to overcome the suppressive activity of Treg. In addition, TGF-β was studied using formaldehyde-fixation of Tregs. Trzonkowski *et al.*, (2004) studied human NK cells, where the researchers did not isolate the NK cells, and had incubated the PBMC NK cell with the Treg cell for 40h. This was done with the addition of vaccine preincubated monocytes, which mimic Antigen presenting cell activation. These findings are not in direct contradicting with this study's findings. Ghiringhelli *et al*. discovered that the resting Tregs are capable of suppression; however, this was unexpected. Treg cells require T-cell receptor (TCR) stimulation for its regulatory function (Ralainirina et al., 2006). Furthermore, Smyth et al. (2006) found that the resting Treg in murine were not suppressive; while Ghiringhelli et al. submitted that the suppression of resting Tregs were never replicated. However, to perform suppression assay, CD3/CD28 beads are required to stimulate the proliferation of Tconv cells. Resting Tregs are

not well studied; however, one study conducted by Hagness et al., (2012) examined this gap, where Treg cells were formaldehyde-fixed, this would evade the TCR stimulation during the suppression assay. This will eventually be helpful in comparing the resting and the pre-active Treg cells, which led to the discovery that about 54% of the donors were suppressive for resting Treg, and the TCR stimulation of Treg cells were all suppressive (Hagness *et al.*, 2012).

Further difficulty in the assessment of the Treg function are in isolation, as it has been nearly impossible to isolate Treg without any contamination, but it's possible with FACS sorting, which this study did not have. Treg and Tconv cells have opposite inflammatory functions; however, they were both stimulated identically. Hence, isolated or stimulated pure Tregs are problematic. However, this was reduced by adding rapamycin, where the mTOR pathway was inhibited, and Treg cells were selectivity expanded with fewer contaminates (Battaglia et al., 2005).
Phenotypical Tregs are mainly defined by their expression of FoxP3, which could be used on the isolated Treg population, because of the transcription factor. However, FACS sorting can be used for this. If Treg is still isolated, the Tconv cells are known to upregulate FoxP3 during activation (Schmidt et al., 2012). The CD25 expression are another marker which could be used, but during inflammatory response, paracrine and autocrine IL-2, and T-cell upregulate CD25 (Fazekas De St. Groth, Smith, and Higgins, 2004). However, CD25 marker did not correlated highly with the expression of FoxP3 (Liu et al., 2006).
There are several other markers which were expressed constantly in Treg cell, such as CTLA-4, ICOS, GITR, and OX40, but were not limited. During T-cell activation, these markers were upregulated (Liu *et al.*, 2006; Xiao *et al.*, 2012). Additional surface markers like CD39 and CD79 were also purposed, which were responsible for direct suppression via ATP hydrolysis. Of these two markers, CD39 is more interesting, as it correlated with suppression activity and FoxP3 expression (Gu et al., 2017; Deaglio et al., 2007; Mandapathil et al., 2010). They are more highly expressed in human Tregs than Tconv cells (Mandapathil et al., 2010). The main marker in recent years purposed for Treg are Low expression of CD127. This was because it has a direct invert correlation with FoxP3 expression

(Liu et al., 2006). The isolation of Tregs in this thesis were based on CD25, high FoxP3 and low CD127 marker, which resulted in a high purity of Treg, and were able to become suppressive. The results of this study showed around 90% suppression at ratios of 1:2, but by increasing the target cell, which showed an increase in the proliferation and overcoming of suppression.

During this project, it was noticed that the untreated NK cells which were isolated from leukocyte cone shows cytotoxicity of 30 to 60% for K562, Raji and OVCAR3. This could be that the cones were already activated during the process to concentrate the leukocyte into a small volume. In the lab, it was noticed that the activating marker CD69, which triggers NK-cell-mediated cytolytic activity (BORREGO et al., 1999) are above 60% (unpublished observations), whereas normally it's between 2.5-5% (Bornego et al., 1993; Benlahrech et al., 2009). With the addition of Treg, the NK cytotoxicity had reduced, which is in correlation with the literatures (Trzonkowski et al., 2004; Ghiringhelli et al., 2005).

In order to increase the NK cytotoxicity, it was primed with IL-2, which had resulted in an increased NK cell cytotoxicity against K562, Raji and OVCAR3. The increase in cytotoxicity was an agreement with the findings of (North et al., 2007; Sabry et al., 2011). This could be due to the activation of the NK cells via MAPK 1/ extracellular signal-related protein kinase (Yu et al., 2000). The hypothesis was that NK and Treg cells would compete for IL-2. Hence, that was why Treg suppression was seen with IL-2 at the concentration of 100IU, and not so much in 200IU and 400IU.

The group's previous findings showed that priming the NK cell with a tumour cell (CTV-1), the interaction of CD2 and CD15 is critical for creating TpNK cells (Sabry et al., 2011). In theory, Lak and TpNK cells should show a similar level of cytotoxicity against cancer cell lines. However, these results for TpNK not only showed far less cytotoxicity, but very similar to a resting NK cell. The hypothesis for this was that because CTV-1 is a cancer cell, it could have been muted. The consequences of this would have been the downregulation of CD15, which was

required to prime NK cells. Anther hypothesis would be that during the overnight incubation, the NK cells were trying to kill off CTV-1 cells, and were already very exhausted before the cytotoxicity assay. The final hypothesis is that some NK cells are already attached to the CTV-1 cell, and cannot contribute in the cytotoxicity assay. With the addition of Treg cells, there was no reduction in TpNK cell cytotoxicity of Raji cell.

In this project, CD107a were used as a functional marker for NK cell activation, which was purposed by (Penack et al., 2005). The hypothesis was that in the presence of target cell line K562, the CD107a expression should increase, and with the addition of Treg, this should decrease. The results showed this for one donor, whereas two other donors showed a minor decrease in CD107a expression when Tregs were added in the mix. The expression of CD107a in presence of target cells were in line with the findings of (Penack et al., 2005). NK cell has a different subunit, where 90% of circulating NK in the body are $CD56^{dim}CD16^{+}$ and 10% $CD56^{bright}CD16^{dim/-}$ (Fehniger et al., 2003). Penack et al. discovered another subunit of NK cells—$CD56^{dim}CD16^{-}$, which are mostly responsible for cytotoxicity against the target cell line. This project did not have enough time to carry out an experiment to examine $CD56^{dim}CD16^{-}$.

In the end, the method that had the best results was the treatment of NK culture with a replication incompetent tumour cell line - CTV-1 - which strengthened the NK cells to the point where no change was seen in the cytotoxic activity after Treg introduction into the system.

To apply this knowledge into a clinical trial setting, first the patient NK cell has to be isolated and phenotyped for a different subunit. This is followed by the activation of NK cells through co-incubating with CTV-1 cell line and cytotoxicity assay performed on the final product. It is important to state that this would be intravenously infused into the patient.

6 *Conclusion*

By analysing the results, it is clear that the Treg cells played an important role in the development of tumours, as it inhibits the cytotoxic activities of the NK cells in the TEM environment. Even though NK cultures could be successful in killing cancerous cells, the introduction of Treg cells into the system partially inhibits their cytotoxic capacity, or even completely blocks it.

The NK cells by themselves are not capable of inhibiting the suppression of Treg cells, but with additional treatments, such as IL2 or CTV-1, their resistance to Treg influence increases. Thus, gaining more cytotoxicity/cytolysis capacity, and more effectiveness in removing the affected parts. Nevertheless, to have a better statistical perception of NK and Treg characteristics, it is necessary to conduct a study with a bigger group, with varying physical characteristics, such as age and sex, in order to understand the different immunological responses that appeared in the gathered data, and to create an effective treatment methodology for all the different responses that might exist.

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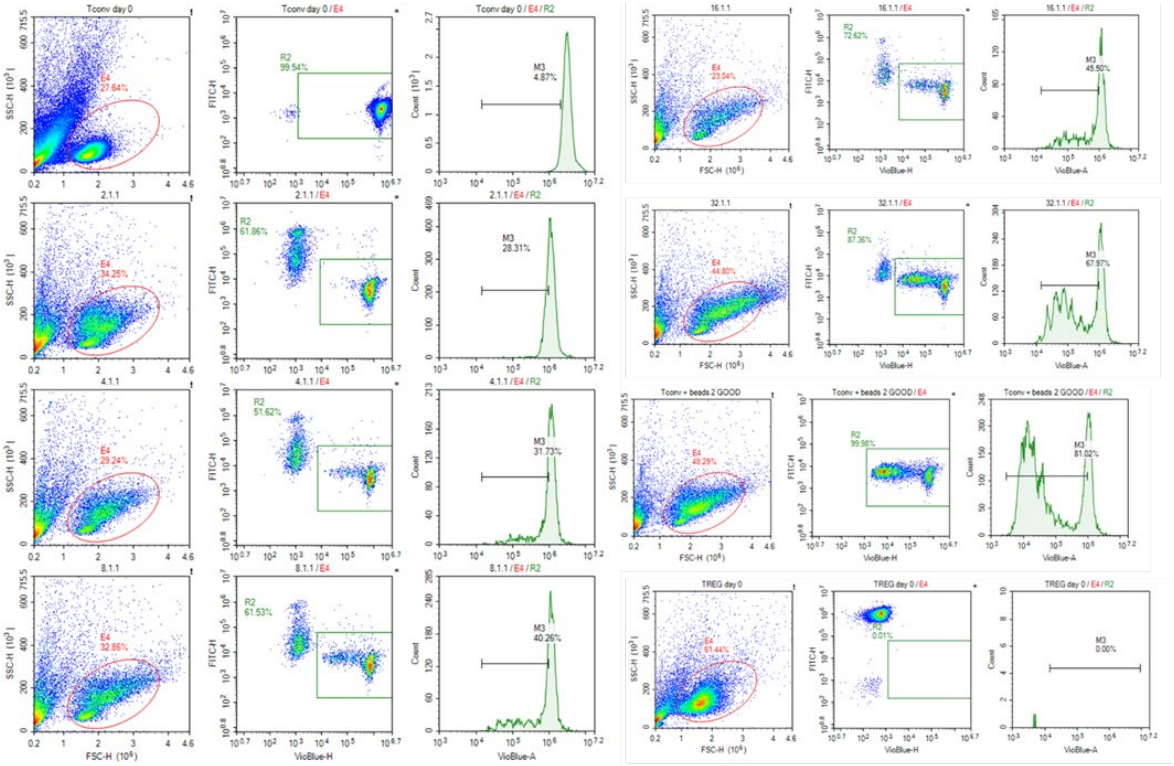
8 *Appendix*

8.1 Appendix A, one example of suppression assay

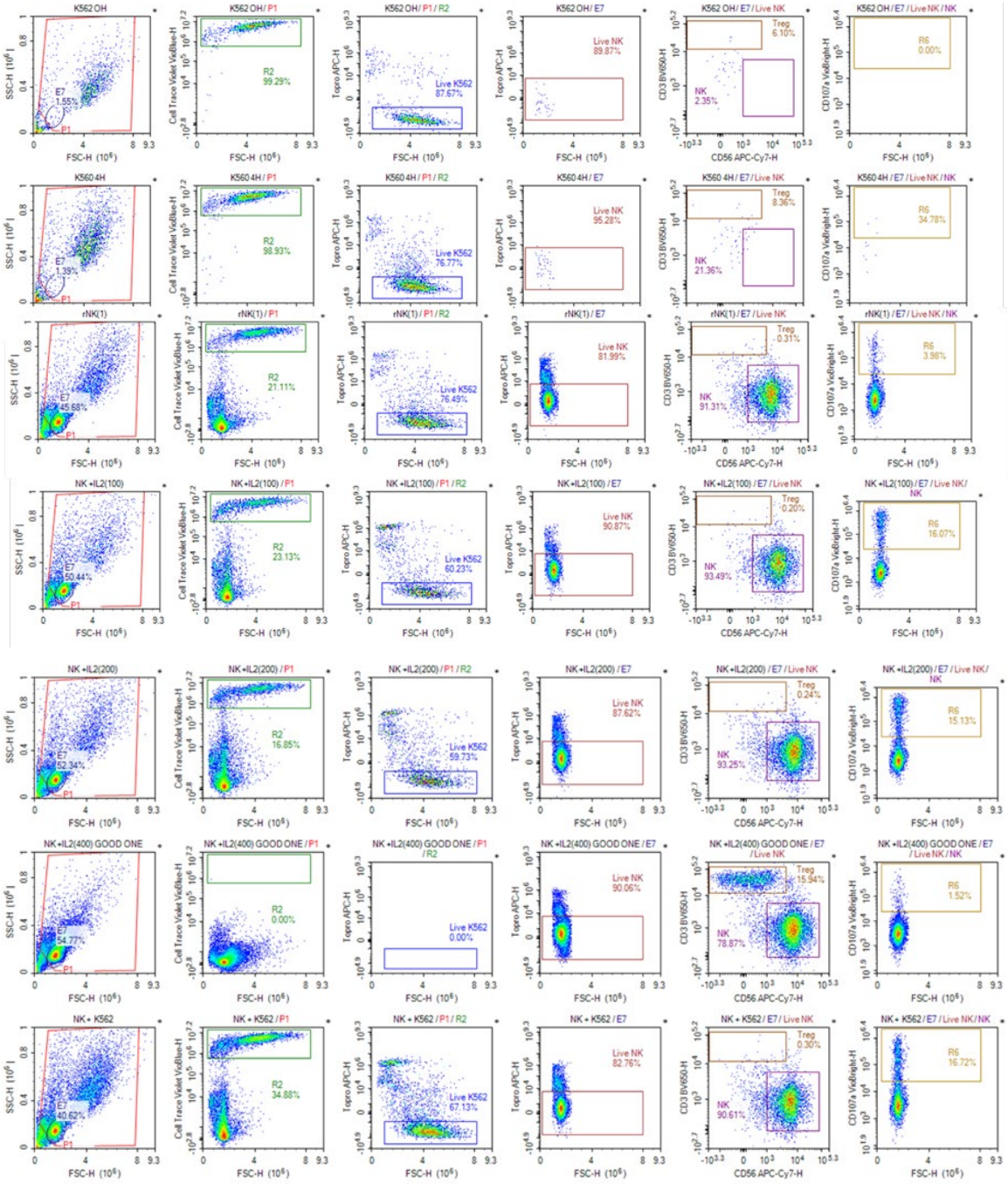
8.2 Appendix B, one example of cytotoxicity assay, K562 and CD107a assay.

8.3 Appendix C, one example of cytotoxicity assay for Raji cell line.

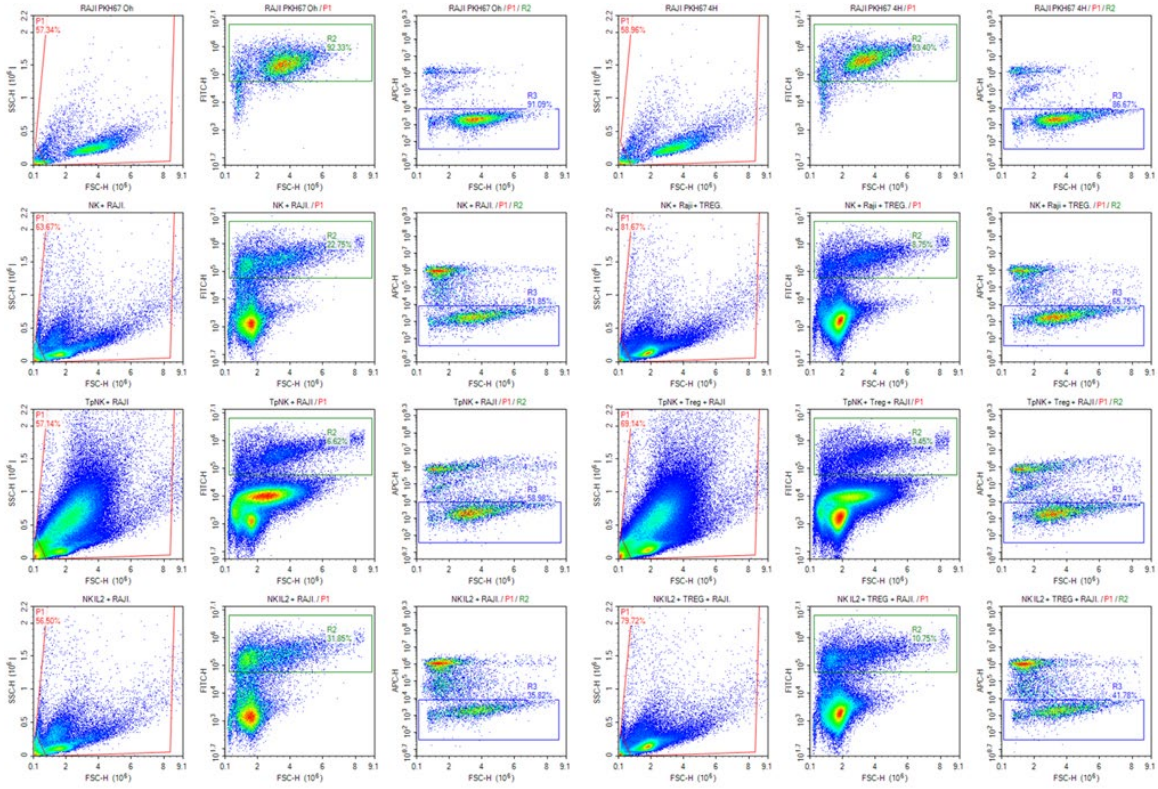
Appendix A, example of gating strategy of Treg suppression assay



Appendix B, an example of gating strategy for cytotoxicity assay, K562 cell line and CD107a assay.



Appendix C, an example of gating strategy for cytotoxicity assay for Raji cell line.



UCL Biobank Ethical Review Committee - Royal Free London NHS Foundation Trust (RFL B-ERC)

RFL Biobank, Royal Free London,
NHS Foundation Trust Rowland Hill Street,
London, NW3 2PF

15th May 2020

Prof Mark Lowdell,
CCCGT
Royal Free Hospital
London, NW3 2QG
United Kingdom

Project Title: Investigation of autologous and allogenic immune response to acute myeloid leukaemia and myelodysplastic syndrome

Reference number: NC2020.15

Dear Professor Lowdell,

I am writing to inform you that your application to RFL B-ERC has been approved as part of the Royal Free London (RFL) NHS Foundation Trust Biobank (National Research Ethics Service, NRES, approved Research Tissue Bank). Your application was approved with the following conditions:

- **Ensure that you have appropriate R&D research permission for your project at sites where research is being carried out. The biobank now has a process agreed with R&D for getting such approvals and we are happy to work with you on this.**
- **Ensure that all staff consenting participants have been appropriately trained in taking consent.**
- **Maintain records of samples being collected, ensuring GDPR compliance in accordance with your institution's regulations.**

Please find enclosed a copy of the NRES ethics approval letter for the RFL Biobank for your records. The current duration of the RFL Biobank ethical approval is until 5th October 2021 in line with the duration of the Research Tissue Bank approval. Individual project ethics is approved for 3 years from the date of this letter. This approval can be renewed for a further period in the future.

We will be asking you to complete an annual return giving information on the number of patients consented and samples taken. We may at any time request to audit your project to ensure compliance with the necessary regulatory and governance requirements.

Thank you for your application to the Biobank and if you have any questions, please do not hesitate to contact Amir Gander (a.gander@ucl.ac.uk).

Yours sincerely,

pp Dr Chris Streather
Chair RFL B-ERC
RFL Group Chief Medical Director

pp Mark Lowdell
RFL Biobank Director and DI for the HTA.

UCL Biobank Ethical Review Committee - Royal Free London NHS Foundation Trust (RFL B-ERC)

RFL Biobank,
Royal Free London,
NHS Foundation Trust
Rowland Hill Street,
London, NW3 2PF

15th May 2020

Prof Mark Lowdell,
CCCGT
Royal Free Hospital
London, NW3 2QG
United Kingdom

Project Title: Study of immune response to myelodysplastic syndrome cancer stem cells
Reference number: NC2020.19

Dear Professor Lowdell,

I am writing to inform you that your application to RFL B-ERC has been approved as part of the Royal Free London (RFL) NHS Foundation Trust Biobank (National Research Ethics Service, NRES, approved Research Tissue Bank). Your application was approved with the following conditions:

- **Ensure that you have appropriate R&D research permission for your project at sites where research is being carried out. The biobank now has a process agreed with R&D for getting such approvals and we are happy to work with you on this.**
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