Activated NK cells are potent effectors against glioblastoma cells due to activating KIR2DS2 and KIR2DS4 - HLA ligand interactions

– *In vitro* study

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2. Summary

Background: Glioblastoma (GBM) is the most frequent and malignant brain tumour, where patients have a median survival of only 14,6 months after diagnosis, thus novel, effective therapies are required. Natural Killer (NK) cells were investigated as effectors for adoptive immunotherapy against GBM due to their selective cytotoxicity against tumour cells. This selectivity is provided by Killer Immunoglobulin-like receptors (KIRs) that recognize self-Human Leukocyte Antigens (HLA) as their ligands, thus inhibiting their autoreactivity. However, tumour or stressed cells that lack some HLA ligands become potentially susceptible to NK cell cytotoxicity. We hypothesized that (1) purified NK cells are more effective than Lymphokine Activated Killer (LAKs) cells against GBM and that (2) KIR-HLA-ligand mismatch might determine the killing potency of NK cells against solid tumours, such as GBM.

Methods: Multiparametric (13 parameters) flow cytometry was used to phenotype the immune cells present in the peripheral blood of (n=8) normal healthy donors and NK cells were purified by magnetic beads cell sorting. Furthermore NK cells were characterised in detail using a panel of markers, including CD56 and CD16. Standardized *in vitro* culture methods were employed to expand and activate the NK cells, as well as generate LAK cells. Flow cytometry based *in vitro* cytotoxicity assays were used to compare killing potential of purified, resting or activated NK cells to LAK cells derived from the same healthy donors, in different effector: target (E: T) ratios against patient-derived GBM cell lines (n=3) maintained in stem cell medium. The GBM cells were phenotyped for the expression of stress ligands MICA/B, ULPB -1,-2,-3, as well as HLA-A,-B,-C; HLA-G and HLA-E. Multicolour flow cytometry was used to characterize the effector populations involved in each cytotoxicity and their expression of activating and inhibitory KIR receptors. KIR genotyping of donors was performed by sequence specific primers polymerase chain reaction (SSP-PCR) and HLA-genotyping of the GBM cells was performed by sequence specific oligonucleotide hybridisations (SSOP) to determine the degree of KIR receptor-HLA ligand mismatch. *In vitro* cytotoxicity assays after blocking activating receptors were also performed in order to determine the critical receptor for the NK cells cytotoxicity. The leukaemia K562 cell line was used as a positive control for the cytotoxicity assays.

Results: Resting NK cells major subpopulation was $CD56^{dim}CD16^{+}$ while the major subpopulation shifted in the activated NK cells to become $CD56^{bright}CD16^{+}$. LAK cells' major subpopulation was T cells (72±12,5%, where $CD8^{+}$ cytotoxic T cells [CTL] comprised 47,44±11,9% and $CD4^{+}$ helper T [Th]) cells were 28±13%) in addition to a minor NK cell population (18,1±6,4%) that was predominantly $CD56^{bright}CD16^{dim/+}$. After culture, NK cells up-regulated activating receptors NKp46 and NKG2D that were not abundantly expressed by the resting NK cells under steady state. Cytotoxicity assays demonstrated donor and dose-dependent efficacy of NK cells against GBM cells, as well as varying potency of the effector cells from the same donor against the different GBM cells. Activated NK cells were more cytotoxic than resting NK cells and LAK cells against the same GBM and at similar E: T ratios. KIR-HLA ligand mismatch determined the greater cytotoxic potential of resting NK cells compared to activated NK cells. Ligation of activating KIR2DS2 and KIR2DS4 to their cognate ligands was identified for the first time to our knowledge, to determine greater potency of activated NK cells against GBM cells. Blocking antibodies against the activating receptors KIR2DS2, KIR2DS4 and NKG2D abrogated the cytotoxic potency proving the importance of these receptor-ligand interactions in NK cytotoxicity.

Conclusions: Activated NK cells are more potent therapeutic effectors against GBM compared to LAK cells. NK cells exhibit donor-dependent efficacy against different patient-derived GBMs due to the distinct KIR -HLA ligand mismatch between resting NK cells and GBM cells. However, for activated NK cells the ligation of activating KIR receptors to their cognate HLA ligands might be more important.

3. Abbreviations

Ab	antibody
ACT	Adoptive cell-transfer
ADCC	Antibody-dependent cellular cytotoxicity
APC	antigen presenting cell
BBB	blood brain barrier
BSA	Bovine Serum Albumin
CBTRUS	central brain tumour registry of the United States
CFSE	5(6)-Carboxyfluorescein Diacetate N-succinimidyl ester
CML	chronic myelogenous leukaemia
CNS	central nervous system
CSC	cancer stem cell
CTL	cytotoxic T-lymphocytes
DC	dendritic cells
DNA	deoxyribonucleic acid
EC	endothelial cell
FB	Fluorescent Activating Cell Sorting buffer
Fc	crystallisable fraction
Fcγ RIII	Fc gamma receptor type III
FLAIR	fluid-attenuated inversion recovery
FSC	forward scatter
GBM	glioblastoma
GSC	glioblastoma stem cells
GFAP	glial fibrillary acidic protein
GMP	good manufacturing practice
GvHD	Graft *versus* host disease
HLA	human leukocyte antigen (= MHC)
Ig	immunoglobulin
INF-γ	interferon gamma
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
KIR	killer immunoglobulin-like receptors
LAK	Lymphokine activated killer cells

LOH	loss of heterozygosity
MGMT	O^6-methyl guanine DNA methyl transferase
MHC	histocompatibility complex
MIC-A/-B	MHC class-I-chain-related protein A/B
MTIC	3-methyl-(triazen-1-yl) imidazole-4-carboxamide
MRI	magnetic resonance imaging
NB	Neural Basal® medium
N-CAM	neural cell adhesion molecule
NK cells	natural killer cells
NKG2D	natural killer group 2, member D (= CD314)
NKp46	natural killer cell p46-related protein
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR-SSOP	sequence-specific oligonucleotide probes polymerase chain reaction
PCR-SSP	sequence-specific primer polymerase chain reaction
PI(3)K	phosphatidyl inositol 3,4,5 bisphosphate kinase
PMN	polymorphonuclear cells
PS	Penicillin-Streptomycin antibiotics
PTEN	phosphatase and tensin homologue
RAS	rat sarcoma
RB	retinoblastoma
RPMI-1640	Roswell Park Memorial Institute medium variety
RTK	receptor tyrosine kinase
SEM	Standard error of the mean
SC	stem cell/stem cell medium
SSC	side scatter
SSEA-1	stage-specific embryonic antigen-1
TCR	T-cell antigen receptor
Th	T helper cells
TMZ	temozolomide
TNF-α	tumour necrosis factor alpha
WHO	world health organization

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6. Introduction

6.1 Cancer

The global burden of cancer is currently 12,7 million new reported cases with an estimated 7,6 million deaths and is projected to increase to 21,4 million with associated 13,2 million deaths by 2030 due to population growth and aging [1]. In Europe, 51644 new brain cancer cases were reported in 2009 and the incidence is projected to increase to 55477 by 2020 [2]. In Norway, 961 tumours of the brain and central nervous system (CNS) were diagnosed in 2009, amounting to 3,5% of all new cases [3]. The costs associated with new cancer cases in 2009 were estimated to US$286 billion [2], making cancer a major health and economic issue for the society at large.

Cancer is not a single disease but is considered a group of diseases, characterised by uncontrolled growth, apoptosis resistance and spread of malignant cells to distant sites. Over 100 different cancer types have been classified. Even though defined molecular pathways may differ amongst the various cancer types, the same fundamental pathways are deregulated through dominant loss of function mutations in tumour suppressor genes, activation of gain of function mutations in proto-oncogenes and unlimited replicative potential through telomere lengthening. Six defining hallmarks comprising common biological capabilities acquired by most, if not all, human tumours were proposed [4, 5]. The six Hallmarks of cancer comprise independence of paracrine growth factors, evasion of anti-growth signals and apoptosis, unlimited replicative potential, ability to form new blood vessels by angiogenesis, and to invade surrounding tissues and metastasise. Recently, two emerging hallmarks of cancer were proposed, namely reprogramming of energy metabolism and evading immune destruction [4]. Because neither capability is yet generalized and fully validated, they are termed emerging hallmarks. Additionally, they proposed two new enabling hallmarks of tumour-promoting inflammation and genome instability and mutation [4]. These enabling hallmarks facilitate the acquisition of both core and emerging hallmarks.

6.1.1 Brain cancer

Among all the cancers worldwide, primary brain tumours account for 2% of all the primary cancers [6]. Despite this low incidence compared to the other types of cancer, they remain

some of the most aggressive cancers with very poor prognosis. Primary brain tumours are classified by The World Health Organization (WHO) classification system based on their morphological and phenotypical similarities to the presumed cell of origin. Thus, the subgroup of tumours with neuroepithelium (glia) as origin cells is called gliomas. This group is further sub-divided based on the predominant type of cells. Astrocytomas are thought to arise from astrocytes and are the most frequent gliomas making up 60% of all primary brain tumours. The WHO classification system further grades the astrocytomas into 4 subgroups of increasing malignancy, depending on the presence of histological criteria such as nuclear atypia, anaplasia, mitotic activity, necrosis and microvascular proliferations (Table 1). Tumours belonging to Grade I are the most benign astrocytomas characterised by lack of all of the above criteria. Grades II and III may exhibit 2 or more of these criteria, respectively, but both grades do not show microvascular proliferations and necrosis. Grade IV is reserved for glioblastoma (GBM), the most malignant, and characterised by the presence of necrosis and or microvascular proliferations, in addition to several of the other criteria. The grading system has also prognostic relevance as Grade I pilocytic astrocytomas can be cured by surgical resection alone, while grade IV GBM has the poorest prognosis of all the tumours [7].

Table 1: WHO classification and grading system for astrocytomas.

	WHO Grading	**I**	**II**	**III**	**IV**
	Type of astrocytoma	Pilocytic astrocytoma	Diffuse astrocytoma	Anaplastic astrocytoma	Glioblastoma
Histological criteria	Nuclear atypia		●	●	●
	Infiltrative		●	●	●
	Anaplasia			●	●
	Mitotic activity			●	●
	Necrosis				●
	Microvascular proliferation				●

Represents the grading of astrocytomas by WHO system classification based on the histological criteria. (●) Symbolizes the criteria each type of astrocytoma presents. (Table adapted from [7] and [8])

6.1.1.1 Glioblastoma

6.1.1.1.1 Symptoms and diagnosis

Symptoms of a brain tumour are characteristically non-specific neurological symptoms, such as early morning headaches, nausea and vomiting, visual and cognitive disturbances and or paresis. Epileptic seizures are perhaps the most distinguishing sign. The type of focal symptoms depends also on the location of the tumour and are generally the result of increased intracranial pressure [9, 10]. The localization of the GBM can vary within the brain and spinal cord but most of them are confined within the four lobes of the supratentorial compartments in adults [9].

After physical examination, neuroimaging techniques are used for accurate diagnosis and follow-up of patients with gliomas. The imaging techniques include magnetic resonance imaging (MRI) with T1-weighted spin-echo (SE) sequence with and without administration of contrast agent gadolinium, and T2 fluid-attenuated inversion recovery (FLAIR). Brain tumours are generally revealed as poorly marginalized hypo-signal on T1-weighted and hyper-signal on T2 weighted sequences or FLAIR. On MRI, GBM often appears as ring-enhancing lesions, however, as this image could resemble other diseases such as metastasis or abscess, the definitive diagnosis requires a stereotactic needle biopsy or a craniotomy with tumour resection and further pathologic confirmation [9].

6.1.1.1.2 Incidence

Astrocytic gliomas account for 60% of the total 2% of incidence of the primary brain tumours [6], while Glioblastoma (GBM) accounts 60% in Europe [6] and 54% in USA [11] of all the astrocytic gliomas (Figure 1).

Figure 1: Distribution of primary brain and CNS Gliomas by histology subtypes in USA 2005-2009 (n=90.828).

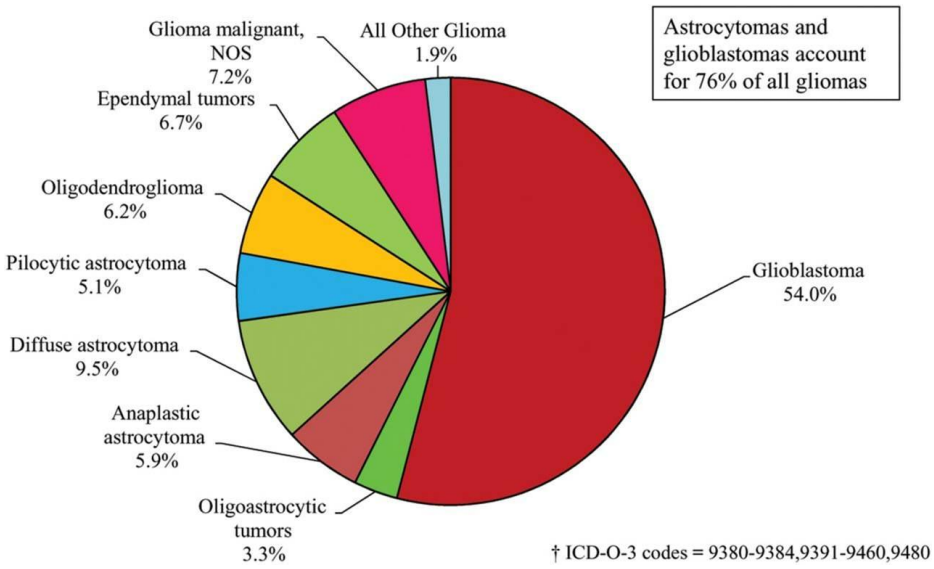


Figure taken with the permission from [11].

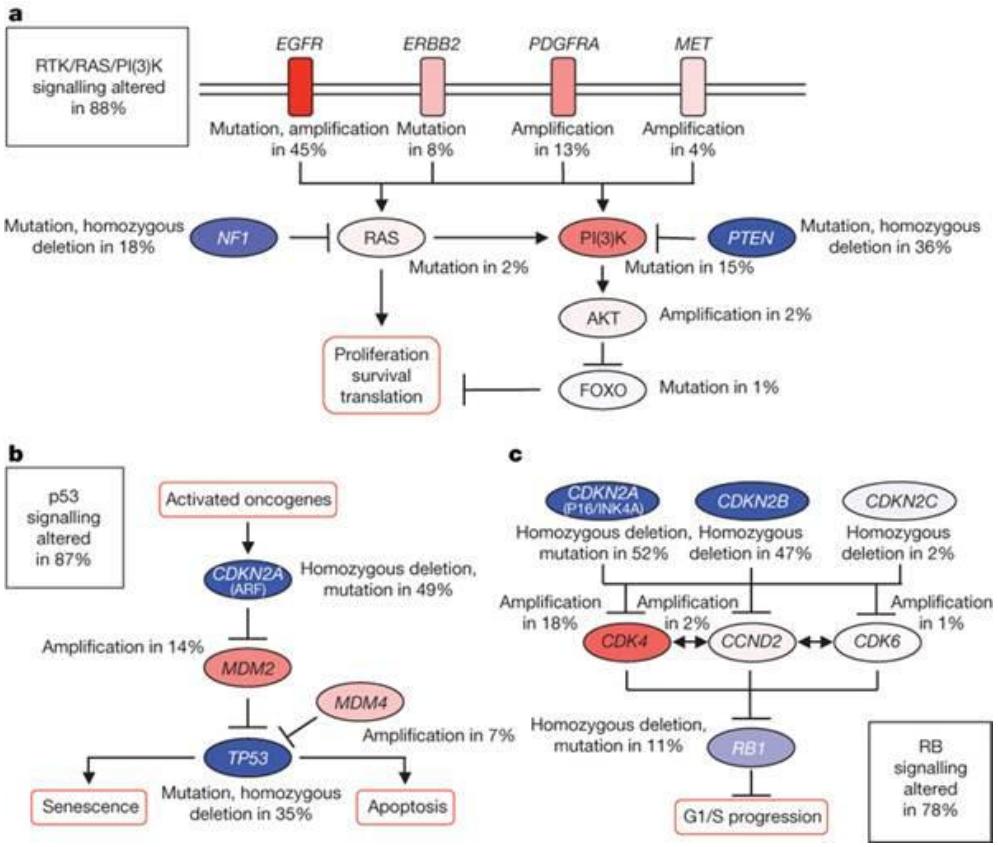
The incidence for GBM among young people is very low as it preferentially afflicts adults with a peak incidence between 45 and 70 years old people and with a median age at diagnosis of 59 years (median for all the primary brain and CNS tumours) [10, 11]. Gliomas develop most frequently in males, with an incidence rate of 7,16 male and 5,06 woman per 100.000 person [11].

6.1.1.1.3 Genetic alterations

GBM is characterised by genetic instability and apoptosis resistance [12]. Genetically, GBM is a very heterogeneous tumour, where the cells contain a range of genetic alterations that produce dysfunction and alter their normal behaviour. The most frequently altered signalling pathways include the receptor tyrosine kinase (RTK)/rat sarcoma (RAS)/phosphatidyl inositol 3,4,5 bisphosphate kinase (PI(3)K) signalling, p53 and retinoblastoma (RB) tumour suppressor genes, which are altered in 88%, 87% and 78% of the cases, respectively [13]. Within these pathway alterations, loss of heterozygosity (LOH) on chromosome 10 targeting the phosphatase and tensin homologue (PTEN) gene [13, 14] and the amplification of EGFR gene (oncogenic event) on chromosome 7 are the most common in the RTK/RAS/PI(3)K pathway (Figure 2, a). LOH of chromosome 9 deleting the cyclin-dependent kinase inhibitor

2A (CDKN2A) and LOH in chromosome 17 targeting TP53 are the most frequent in the p53 pathway (Figure 2, b). LOH on chromosome 9 deleting CDKN2A or CDKN2B are the most common in the RB pathway (Figure 2, c) [13]. Together these genetic alterations contribute to the hallmarks of GBM including unregulated proliferation, apoptosis resistance, increased survival signalling and tumour cell migration and invasion.

Figure 2: Frequency of the genetic alterations observed in GBM in three critical pathways.



(a) RTK/RAS/PI(3)K, (b) p53 and (c) RB signalling pathway's components and primary sequence alterations are represented. Red indicates activating genetic alterations. Blue indicates inactivating alterations. Darker colours correspond to higher percentage of alteration. The nature of the alteration and the percentage of tumours affected are indicated for each altered component. Percentages in the white boxes refer to total percentage of glioblastomas with alterations in at least one component of the pathway. G. Figure taken with permission from [13].

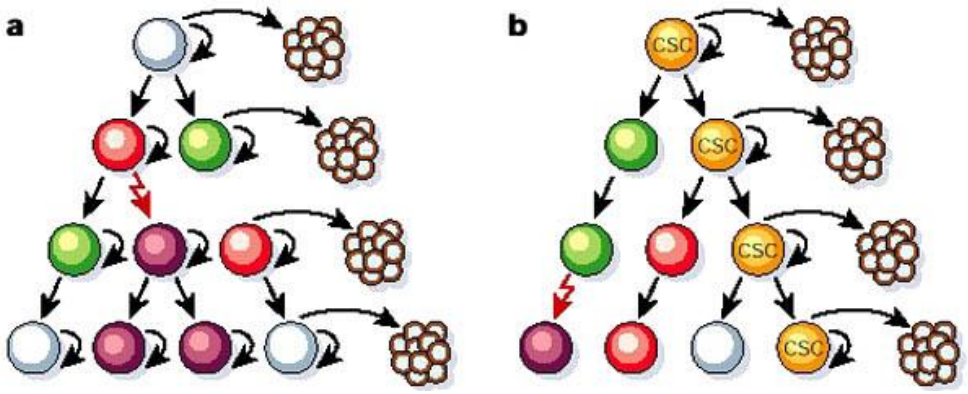
These pathways are crucial for the progression and survival of the cells, therefore, their disruption, modification or alteration leads to a dramatic change or dysfunction of the normal behaviour of the cells.

6.1.1.1.4 Cancer stem cells and Glioma stem-like cells

It has been proposed that several cancer types arise from distinct but minor population of cells amongst the heterogeneous tumour mass. This *so-called* cancer stem cell (CSC) population is highly tumourigenic and, like the normal stem cells (SC) present in the tissues of the body, is capable of regenerating all the cell types of the original tumour through self-renewal and asymmetric cell division [15, 16].

Therefore, two hypothesis of cancer development are established. (1) The clonal evolution hypothesis states that almost all the cells in a tumour are capable of extensive proliferation and deriving new tumours (Figure 3, a). On the other hand, (2) the CSCs hypothesis points that only few cells of the whole heterogeneous cancer population, CSCs, have the capacity of sustain tumour growth (Figure 3, b) [17, 18]. The CSCs hypothesis changed the general view of how cancer should be treated, in that it implied that therapies should target the minor tumourigenic population capable of regenerating the entire tumour at recurrence. By implication, the traditional therapies focused on eradicating the cells of the tumour bulk may be targeting the wrong cells [15, 16].

Figure 3: Hypothesis of heterogeneity in solid cancer tumours.



(a) Clonal evolution hypothesis. Most of the heterogenic tumour cells have the ability to proliferate and give rise to new tumours. (b) CSCs hypothesis. Only CSCs (yellow) have the ability to proliferate and form new tumours. Figure taken with permission from [17].

6.1.1.1.4 Glioma stem-like markers

Based on the clinical implication of the CSC hypothesis and on the evidence of heterogeneity in the ability of cancer cells to self-renew and differentiate in other cancer types, such as leukaemia and breast cancer, the markers that define cells with CSC features were investigated in our GBMs.

CD133 is a transmembrane glycoprotein whose function is yet unknown. However, since neural stem cells (NSCs) are characterised by the expression of CD133 and nestin, when Singh et al. identified a subpopulation of GBM cells that expressed CD133, these cells were subsequently appointed glioblastoma stem cells (GSCs) [19, 20]. They claimed that only this subpopulation was tumourigenic in transplantation models and could recapitulate all the cellular heterogeneity of the original cancer. However, this hypothesis was refuted by several groups that demonstrated that also $CD133^-$ cells were tumourigenic [21].

Nestin is an intermediate filament (IF) protein class IV expressed by all the cells in the brain during development [22]. In the adult brain, nestin is expressed by NSCs and tumour cells [23, 24]. Nestin protein is able to make polymers with other IF class III, such as **vimentin** [24, 25]. Nestin and vimentin IF expression has been reported to be co-expressed together with CD133 in GBM [26].

Glial fibrillary acidic protein (**GFAP**) is also an IF class III that is expressed upon differentiation of NSCs [24, 25]. All class III IF are involved in the structure and function of the cytoskeleton. GFAP up-regulation has been reported as a feature of reactive astrocytes, so it is commonly used as a marker for astrocytes [23, 27]. Nestin, vimentin and GFAP are co-expressed by reactive astrocytes [27].

GT3 gangliosides, are recognized by the ganglioside monoclonal antibody, commonly known as **A2B5.** As in the case of CD133, cells containing A2B5 ligand were thought to be a GSC candidate. Figarella-Branger et al. demonstrated that it was not the case, but A2B5 positive cells are in an early differentiation state from GSC. These cells are thought to be important for the initiation and malignant progression of GBM [28].

CD15 (3-fucosyl-N-acetyl-lactosamine), also known as SSEA-1 (stage-specific embryonic antigen-1), is a glycoprotein that has been reported as enriched protein in cells derived from GSC cells but that are CD133 negative [29].

6.1.1.1.5 CNS: BBB blood brain barrier - immune-privileged environment -

It is well established that the CNS is "isolated" from the peripheral circulation by the blood brain barrier (BBB). The BBB is a physical, anatomical barrier that is responsible for maintaining the particular microenvironment inside the CNS. The BBB prevents the entrance of certain soluble molecules and cells to the CNS.

Anatomically, the BBB is located at the cerebral microvascular endothelium and consists of endothelial cells, basal lamina, extracellular matrix, pericytes and astrocytes. The lumen of the capillaries in the BBB are circumscribed by single endothelial cells (ECs) that lack transendothelial fenestrations and are adhered to each other by tight junctions and adherent junctions. ECs also lack the capacity for pinocytosis and these features make them impermeable to passively diffused substances [30]. Astrocytes support the BBB by their end-feet together with the high-resistance tight junctions formation [31]. Pericytes have been reported as regulators of the BBB and integrators of endothelial and astrocyte functions [32] while neurons, in the brain parenchyma, provide the neurotransmitters and metabolites required for the function of the nervous tissue [30].

This barrier regulates the access of different molecules and cells depending on various parameters such as size, molecular weight and charge. This tight regulation allows the brain to have a local microenvironment with distinct conditions from the ones present in the peripheral blood. The brain was thus long considered an immune privileged organ due to the presence of the restrictive BBB, lack of lymphatic drainage and of professional antigen presenting cells (APCs). However, recent investigations demonstrated central memory T cells in the cerebral spinal fluid and myeloid cells in the meninges and choroid plexus, as well as perivascular macrophages and microglia with crucial APC functions [33]. This paradigm shift has generated enthusiasm for the potential for immunotherapy in GBM management.

6.1.1.1.6 Current Therapy

Without therapy, GBM patients die within 3 months, while those maximally treated with the current therapy have median survival of only 14.6 months and less than 5% of GBM patients have a median survival of 5 years [34]. The current therapy for the GBM is a combination-therapy, which involves debulking surgery, radiotherapy and concomitant temozolomide chemotherapy.

The aim of the surgery is to extract most of the tumour bulk and to take as little as possible of the normal healthy brain tissue without affecting the patient´s cognitive and neurological function. Due to the infiltrative nature of the GBM and the importance of the function of the organ the tumour is in, the complete resection of the tumour cells is often impossible [9].

The radiotherapy is employed after the surgery to eliminate all remaining tumour cells after the resection. The radiotherapy is used for killing the cancer cells because of its ability to exert damage to the DNA of the dividing cells. The standard radiotherapy regimen for the GBM is a total dose of 60Gy given in 2Gy daily fractions for 6 weeks. This fractionated radiotherapy allows normal cells of the brain to repair their DNA, whereas tumour cells will accumulate more DNA lesions that will eventually lead to apoptosis [9].

Temozolomide (TMZ) is the standard chemotherapy used as a therapy for GBM since 2005 [35]. TMZ (Temodar, Temodal, Temcad) is an oral prodrug that converts to 3-methyl-(triazen-1-yl) imidazole-4-carboxamide (MTIC) that has the ability to methylate guanine residues of the DNA sequences at the O6 position creating toxic DNA adducts leading the cells to apoptotic cell death [36]. TMZ is administered together with radiotherapy in order to induce maximal DNA damage to the tumour cells undergoing division. However, response to the TMZ-radiotherapy is dependent on the presence or not of the O^6-methyl guanine DNA methyl transferase (MGMT), a DNA repair enzyme. MGMT normally repairs the DNA adducts caused by TMZ, but when its promoter is silenced by methylation, the cancer cells are unable to repair the DNA damage and are thus rendered sensitive to the clastogenic effects of the TMZ- radiotherapy treatment. MGMT is thus a prognostic as well as predictive factor for response to TMZ treatment in GBM patients [36]. The feature that makes TMZ suitable for treatment of brain cancer is its competence for crossing the BBB because of its small molecular weight [36-38].

Most GBM patients experience tumour recurrence within a median of 6,9 months post treatment [39]. In addition, the poor overall survival of GBM patients indicates that these therapies are not sufficiently disrupting the key mechanisms that drive the tumour. These findings emphasize the need for continued search for novel and effective therapies.

6.1.1.1.7 Immunotherapies of glioblastoma

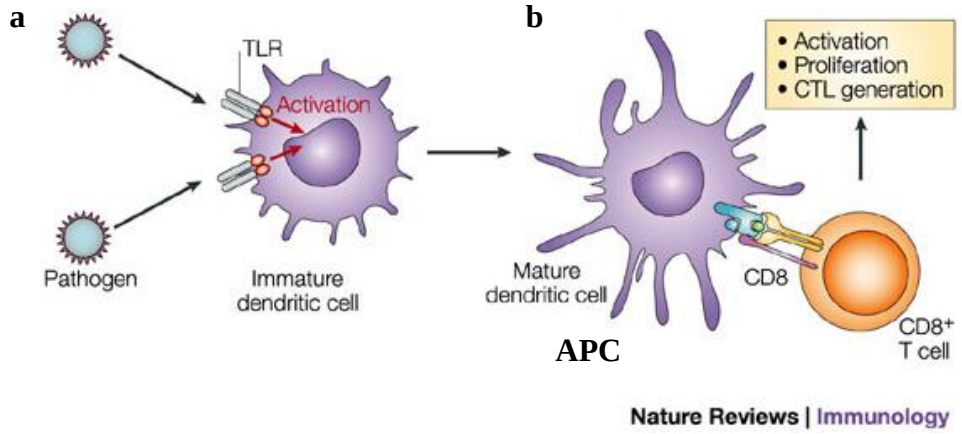
Great research effort is being invested into improved diagnosis, identifying novel therapeutics and overall management of GBM patients. There are several treatment strategies such as novel adjuvant chemotherapy, small molecule targeted therapies, gene therapy, anti-angiogenic therapies and various immunotherapy approaches under investigation [8]. Adoptive cell-transfer with cytotoxic T-lymphocytes and dendritic cell vaccines have been the most frequently evaluated cellular immunotherapies in clinical trials for GBM patients.

6.1.1.1.7.1 Dendritic cell (DC) vaccines

Dendritic cell (DC) vaccines consist of antigen presenting cells (APCs) pulsed with GBM specific antigens. They are generated *ex vivo* for the purpose of antigen presentation to autologous cytotoxic T-lymphocytes (CTLs, $CD3^{+}CD8^{+}$) *in vivo*. The CTLs subsequently attack GBM cells expressing these antigens in situ [40].

The procedure to obtain these APCs consists of culturing bone marrow-derived DC obtained from peripheral mononuclear blood cells (PBMC) together with GBM antigens obtained from GBM's messenger ribonucleic acid (mRNA), fixed GBM cells or isolated GBM antigens [41]. This co-culturing promotes the internalization of the antigen by the DC which process it and further present it by their Human Leukocyte Antigen (HLA), otherwise known as the major histocompatibility complex (MHC) class I and II (Figure 4, a) [40]. Once generated, the APC are administered to the patient and upon the binding of their HLA- bound antigen to T cells' receptor (Figure 4, b) and the secretion of cytokines by the APC to recruit other cells, T cells become cytotoxic against the GBM cells. Hence, these CTLs would recognize and attack specifically the GBM cells expressing the particular antigens [40, 42].

Figure 4: APC and CTL formation.



(a) Shows the APC formation by the interaction of the DC with antigens. (B) Represents the interaction of the APC with T cells via MHC class I – TCR recognition and the consequent CTL formation. Figure taken with permission from [43].

Based on www.clinicaltrials.gov, there are 11 trials currently in progress in various phases of development using DC vaccines for patient with GBM.

6.1.1.1.7.2 Adoptive cell-transfer (ACT) with cytotoxic T-lymphocytes (CTLs)

ACT is the process where immune cells are passively infused into a new recipient host or organ site with the goal of transferring their immunological function and characteristics into the new host.

CTLs are T cells with cytotoxic functions. Each CTL has a unique T-cell antigen receptor (TCR) on the surface with specificity for a singular target peptide [40, 44]. TCR recognizes its specific antigen presented by HLA class I on the surface of the target cells, so the binding of TCR to its specific HLA-antigen triggers the cytotoxic function of CTL that results in the lysis of the targeted cell. Moreover, activation of the CTL causes the secretion of inflammatory cytokines that further recruit other necessary immune cells to create an inflammatory environment for the subsequent immune response.

In establishing adoptive cell transfer (ACT) strategies for GBM treatment, GBM tumour-reactive lymphocytes (tumour-CTLs) that recognize specially tumour antigens are required

[44]. For this purpose, tumour-CTLs can be directly isolated from patient biopsies or T cells can be cultured in different conditions in order to obtain the tumour-CTLs [45].

6.2 Immune cells in the peripheral blood

6.2.1 Peripheral blood cells

In the human blood we can distinguish different types of cells with different features and functions. Blood cells can be divided in two big groups of cells, red blood cells/erythrocytes and leukocytes or white blood cells (WBCs). Erythrocytes have the function to bring oxygen to the body's tissues while the leukocytes have an immunologic function [46].

White blood cells are composed of 3 main types of cells (1) lymphocytes, (2) monocytes and (3) granulocytes. Lymphocytes have the unique capability of recognizing and distinguish specific foreign antigens from self-antigens and mediate immune responses.

Lymphocytes subsets are T cells, B cells and NK cells. T cells are the mediators of the cellular adaptive immunity and can be further divided into 3 subtypes: CTL ($CD8^+$) cells which have cytotoxic functions against target cells, Th ($CD4^+$) cells involved in immune responses against different pathogens and regulatory T cells (Treg, $CD4^+CD25^+$) which regulate the magnitude of the immune responses. B cells are the lymphocytes capable of producing antibodies, activate and regulate T cells. NK cells, which are explained in detail in section 6.6.2, are mediators of anti-viral and anti-tumour immunity [46-48].

Monocytes *so-called* macrophages in tissues are able to recognize and eliminate certain bacterial pathogens, and are involved in the activation of T cells and are in charge of eliminating the debris and dead cells from the blood [47].

Granulocytes are also divided in 3 subtypes. (1) Neutrophils, which are phagocytic cells able to eliminate foreign organisms, (2) basophils, whose function is still poorly understood but are thought to be involved in allergic inflammations and (3) eosinophils which have a role in the immune regulations and are also able to degranulate their granule content against foreign organisms [46, 47].

Leukocytes account for 4-11x10^9cells/l of blood and their number depends on the age, weight, health status and gender of the person they have been taken from, as well as on the method of numeration, e.g. whether by flow cytometry or light microscopy (Table 2) [46, 47].

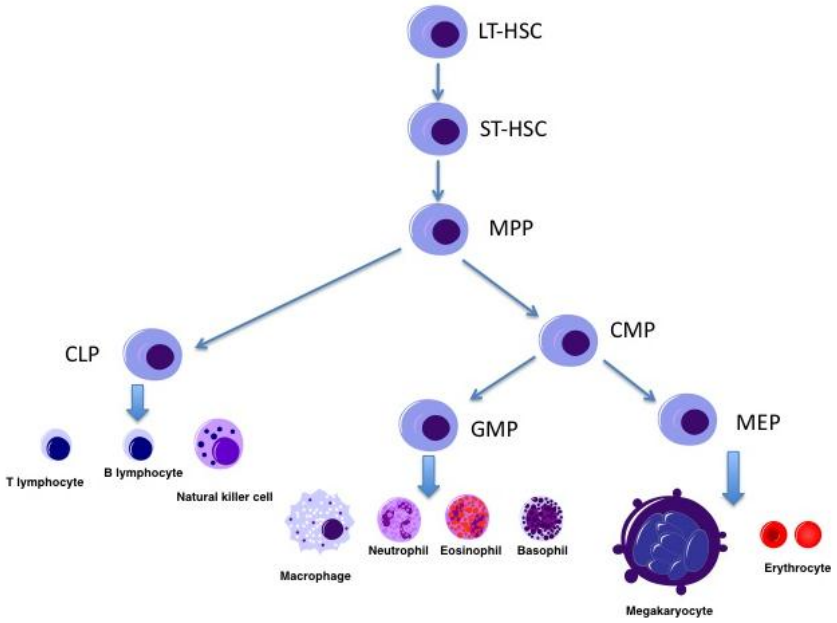
Table 2: Proportions of peripheral blood cells in blood.

		% of cells
Lymphocytes	B cells	2-6%
	T cells	17-34%
	NK cells	2%
	Total lymphocytes	**20-40%**
Monocytes		2-10%
Granulocytes	Neutrophils or PMNs	50-70%
	Eosinophils	1-5%
	Basophils	0,5-1,5%
Total leukocytes or WBC		**100%**

Table adapted from [46] and [47].

All the blood cells have a common major progenitor, haematopoietic stem cell (HSC) whose differentiation starts in the bone marrow. Upon HSC differentiation, multipotent progenitor haematopoietic stem cell (MPP) is generated and upon asymmetrical division, gives rise to the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP) that gives rise lymphoid and myeloid lineages, respectively. Lymphoid lineage provides the lymphocytes (T-, B- and NK cells) of the blood, while the myeloid lineage leads to Granulocyte-monocyte progenitor (GMP) and Megakaryocyte-erythrocyte progenitor (MEP) that will provide granulocytes and erythrocyte of the blood, respectively (Figure 5) [46, 47].

Figure 5: Hematopoietic lineage differentiation.



Lineage differentiation from a common haematopoietic stem cell (HSC) to the different cell types in the blood. Figure taken from [49] with permission.

In order to isolate all the different types of cells from the blood, the blood is centrifuged with Ficoll®, which displaces the cells according to their density. This procedure gives plasma, peripheral blood mononuclear cells (PBMCs) and erythrocytes in different layers. PBMCs are cells with round nuclei and consist of lymphocytes and monocytes.

6.2.1.1 Lymphokine activated killer (LAK) cells

Lymphokine activated killer cells are activated lymphocytes generated upon *in vitro* culture of PBMCs with IL-2 and IL-15 cytokines. LAKs contain variable, donor dependent numbers of CTLs and NK cells and have demonstrated cytotoxic capacity against cancer [50, 51]. Several on-going clinical trials using LAKs are registered on clinicaltrials.gov.

6.2.2 Natural Killer cells (NK cells)

6.2.2.1 General characteristics

Natural killer cells are a subset of lymphocyte cells with the principal function of detecting and killing viral-infected and tumour cells. NK cells are large granular lymphocytes derived from bone marrow precursors and have cytoplasmic granules. They represent around 2% of all the leukocytes in the blood (Table 2) [47, 48].

Some of the most typical features of NK cells are that (1) they secrete cytokines, predominantly interferon gamma (INF-γ) and tumour necrosis factor alpha (TNF-α), (2) they express a repertoire of activating and inhibitory receptors (3) they lack expression of HLA-class I ligands but (4) express killer immunoglobulin-like receptors (KIRs) that recognize MHC class I motifs [52].

6.2.2.2 Phenotype

NK cells are defined, like the other lymphocytes, by the expression of particular molecules on their surface. NK cells do not express cluster of differentiation 14 (CD14) and CD3, markers of granulocytes and T cells, respectively. Hence NK cells are characterized by the following phenotype: $CD14^-$, $CD3^-$, $CD56^+$ and $CD16^{-/+}$.

CD56 and CD16 are characteristic markers for NK cells, which are variably expressed depending on their state of activation. In steady states approximately 90% of the NK cells express CD56 in low levels ($CD56^{dim}$) and CD16 at a higher density ($CD16^+$) while the other 10% represents $CD56^{bright}CD16^{dim/-}$ [53, 54].

CD56, also known as neural cell adhesion molecule (N-CAM) is a membrane glycoprotein of the Immunoglobulin (Ig) superfamily involved in cell-cell adhesive interactions. CD56 is expressed by neural and muscle tissues as well as NK cells [55, 56]. CD16 is a crystallisable fraction (Fc) gamma receptor type III (low affinity Fc*γ* RIII) that recognizes the Fc domain (constant region of antibodies) of IgG and acts as a signal-transducing molecule [57]. CD16 is the receptor that triggers the antibody-dependent cell-mediated cytotoxicity (ADCC, detailed in chapter 1.1.1.4.2) and is also expressed by neutrophils and macrophages [58]. The crosslinking of CD16 with its ligand entail CD16 interaction with FcεRIγ or CD3ζ adaptor

protein, which have Immunoreceptor tyrosine-based activating motifs (ITAM) and upon phosphorylation, triggers the signal cascade for the natural cytotoxicity [59].

In steady states, NK cells expressing $CD56^{dim}$ contain more cytolytic granules and higher amount of their content and present more cytotoxic activity while $CD56^{bright}$ are less cytotoxic but have been reported as the most efficient cytokine producers [53, 54]. NK cells expressing CD16 are therefore, more efficient in ADCC.

6.2.2.3 NK cells receptors

NK cells express a variable repertoire of receptors on their surface. Activating and inhibitory receptors are both represented on the NK cells' surface. NKG2D, NKp46, CD94/NKG2A and KIRs are the most characteristic and representative of them.

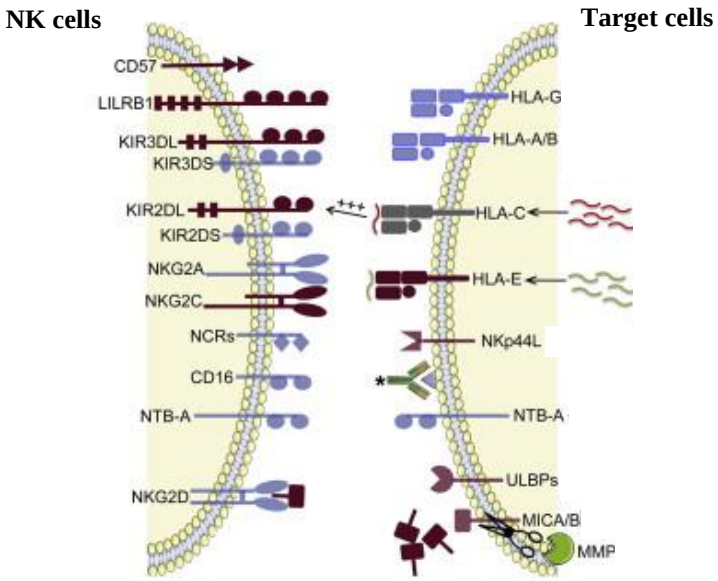
NKG2D (Natural killer group 2, member D) or CD314 is an disulphide-linked type II transmembrane homodimeric receptor [60] that recognizes molecules highly expressed by transformed or virus-infected cells such as MHC class-I-chain-related protein A (MIC-A), MIC-B and UL16-binding proteins (ULBPs, Figure 6) [61-63]. Upon ligation of NKG2D to its ligands, NKG2D interacts with DAP-10, which recruits PI(3)K and triggers activating signals transmission downstream that leads to NK cell activation [59]. (Appendix I, Table 13).

NKp46 (Natural killer cell p46-related protein), also known as NCR1 (Natural cytotoxicity triggering receptor 1) is a disulphide-linked homodimer that can associate FcεRIγ and CD3ζ in homo and heterodimer construction adaptor proteins (Figure 6). The crosslinking of NKp46 with its ligand entail NKp46 interaction with its adaptor protein triggers the signal cascade for the natural cytotoxicity in a similar way as CD16 [59, 64, 65]. The ligands for NKp46 are at present not known, however, experiments blocking NKp46 with antibodies abrogates NK cell cytotoxicity against target cells [66], indicating indirectly that ligation of NKp46 to its cognate ligands triggers NK cell cytotoxicity (Appendix I, Table 13).

CD94/NKG2s are receptors of the C-type lectin superfamily that recognize the non-classical HLA-E molecules and upon its recognition generate the corresponding signal cascade [67]. CD94 makes disulfide-bonded heterodimers with the NKG2 glycoproteins family members (NKG2-A, -C, -E) and seems to have chaperone function that transports the NKG2 proteins to the membrane (Figure 6, Appendix I Table 13) [67, 68]. NKG2A have 2 Immunoreceptor tyrosine-based inhibitory motifs (ITIM) that confer the inhibitory function. Upon recognition

of HLA-E, NKG2A associates with Src homology containing tyrosine phosphatase (SHP) triggering a cascade of inhibitory signalling [69].

Figure 6: Schematic of some NK cell receptors and their cognate ligands expressed on the target cells.



Schematic of a NK cell (left) illustrating some of the receptors typically present on the cell surface. Schematic of a target cell (right), (e.g. tumour cell) illustrating the cognate ligands for the NK cell receptors represented on the membrane. Figure taken with permission and adapted from [70].

6.2.2.3.1 KIR receptors

KIRs (Killer immunoglobulin-like receptors) are monomeric glycoproteins of the Ig superfamily that recognize HLA class I and are expressed by NK and a small portion of T cells. KIRs recognize groups of HLA class I molecules that are determined by the particular amino acids at the c-terminal portion of the HLA class I α1 helix. KIRs are extremely polymorphic in terms of gene numbers, alleles present and differ in affinities for their HLA class I ligands [68, 71, 72].

KIRs are divided in two subfamilies: KIR2D, which have 2 Ig-like domains and KIR3D with 3 Ig-like domains [68]. KIRs can also be classified as activating or inhibitory, depending on whether they induce activating or inhibitory signals to the cell upon ligation to their HLA ligand. Inhibitory KIRs present long cytoplasmic domains (KIR2D**L** or KIR3D**L**) with 2 ITIM sequences. Upon crosslinking KIR-ligand ITIMs autophosphorylate and recruit Src

homology containing tyrosine phosphatase (SHP-1 and -2) adapter proteins. SHP upon binding Vav-1 triggers inhibitory signal cascade [62, 69].

The activating KIRs are characterized by short cytoplasmic domains (KIR2D**S** or KIR3D**S**) with no ITIM motifs but with a charged amino acid in the transmembrane domain [68]. Activating KIRs interact with DAP12 protein, which contains ITAM. Upon the crosslinking of activating KIR to DAP12, DAP12 is autophosphorylated and binds ZAP-70 or Syk tyrosine kinases (TK), therefore, a cascade of activation of proteins downstream is initiated with the purpose of activating the NK cell for the target cell killing [59, 73].

Both activating and inhibitory signals that NK cells receive from its receptors are implemented to the common pathway of GTPase-activating protein 1 (Rac)/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)/p21-activated kinase (PAK)/ mitogen-activated or extracellular signal-regulated protein kinase (MEK) [62].

KIR2DL1 and KIR2DS1 recognise HLA-C with a Lys80 residue (HLA-C group 2 specificity) whereas KIR2DL2 and KIR2DL3 allelic forms recognize the HLA-C1 group with Asn80 residues [74]. However, others have demonstrated interaction between KIR2DL2 and KIR2DL3 with HLA-C2 allotypes [75]. Inhibitory KIR3DL1 interacts with Bw4-containing HLA-B alleles and KIR3DL2 recognises HLA-A3 and HLA-A11 allotypes [71] (Figure 6, Appendix I, Table 13).

Concerning the KIR activating receptors, although KIR2DS2 was first thought to bind the same HLA-C as KIR2DL2, Eric Vivier et al. refuted this hypothesis [76], therefore KIR2DS2 ligands remain unknown. KIR2DS4 activating KIR recognizes Cw4-containing HLA-C [77] (Figure 6, Appendix I, Table 8).

6.2.2.3.2 NK cell activation and "Missing-self" hypothesis

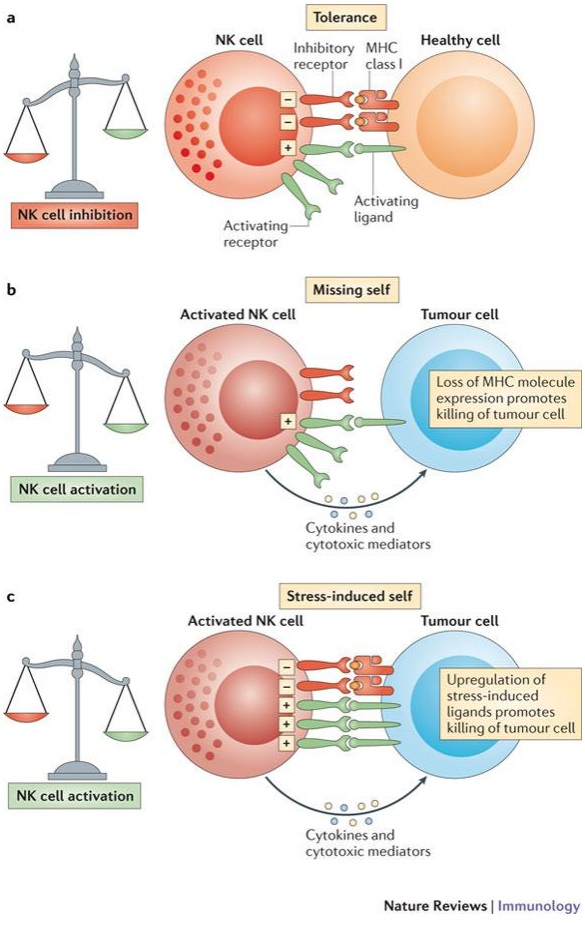
NK cells recognise and kill tumour or virus infected cells lacking class I- HLA ligands recognised by KIRs (6), through the "missing-self" hypothesis (7) or KIR-HLA mismatch.

NK cells are capable of distinguishing self from non-self-cells due to the interaction between their KIR receptors and their corresponding ligands on the target cell. The NK cells of our body interact by their KIR with the HLA class I molecules that all our body cells express on their surface. The interaction of inhibitory KIR to their cognate -HLA ligands transmits

inhibitory signals to the NK cell meaning "this is a self-cell, don't kill it". When the NK cells interact with a cancer or virus infected cell, which express differently HLA class I molecules than the healthy self-cells (either by not expressing HLA ligands for the KIR receptors or express different HLA allele repertoire than the self-cells), the KIRs do not interact with them so the inhibitory signal is not sent to the cell, meaning "this is not a self-cell because it has **missing self**-markers". This lack of the inhibitory signal together with the activating signals the cell may receive activates NK cell and licences it to kill the target cell (Figure 7, b) [69, 78-80].

As mentioned above, NK cells also interact with the target cells by their activating receptors. The activating receptors (such as NKG2D and NKp46) recognize stress ligands and send activating signals to the NK cell. When NK cells interact with a target cell, they receive both activating and inhibitory signals. Thus the overall balance between activating and inhibitory signals determines whether the NK cell will remain anergic or become cytotoxic.

Figure 7: Tolerance, missing-self or mismatch hypothesis and stress-induced self.



(a) When the balance between inhibitory and activating receptor-ligand is positive for the inhibitory KIR-HLA interactions, inhibitory signals are sent to the NK cell, so the target cell is not killed by the NK cell. This is the typical situation for the healthy self-cells, and is also known as tolerance.

(b) If the balance between inhibitory and activating receptor-ligand is positive for the activating receptor-ligand interactions, this interaction will send activating signals to the NK cell and the target cell will be killed. This is the case of the tumour and virus-infected cells and is called "Missing self".

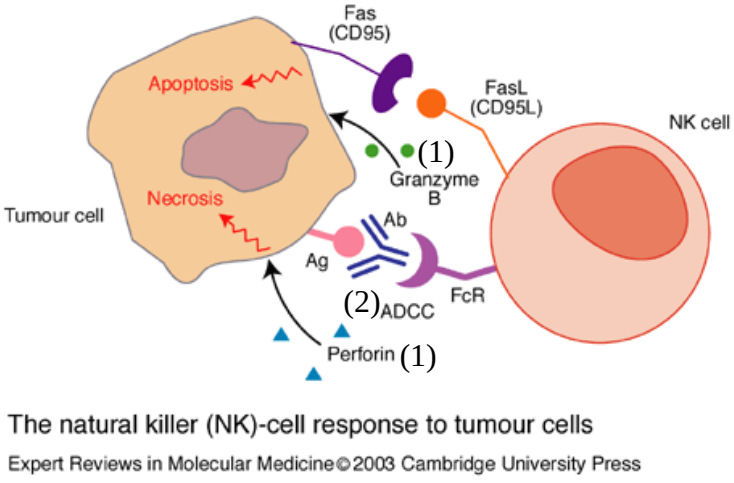
(c) In the case that both activating and inhibitory receptors have bound its ligand but the activating receptors have bound large amount of stress ligands, the balance turns to positive for the activating signal. This is called stress-induced self because even though the cells recognize self HLA molecules and send inhibitory signal, strong activating signal is also received indicating that something is wrong with the target cell. This is the situation for the tumour cells.

KIRs are highly polymorphic, in terms of gene numbers and alleles, and due to the variability of affinities for their HLA class I ligands [71, 81]. The fact that NK receptors and their HLA class I ligands are not genetically linked, in some situations, a receptor for self-HLA class I ligand might not be expressed by autologous NK cells. This fact enhances complexity to the KIR-HLA ligand recognition [82].

6.2.2.4 Cytotoxicity function

NK cells kill their target cells by secreting the contents of the cytotoxic lysosomes in a response of two situations; (1) positive balance in the activating/inhibitory signal obtained by the surface receptors (Lysosomes secretion cytotoxicity, Figure 8 and chapter 6.2.2.4.1) and (2) recognition of the specific antibodies that bind the antigen of their target cells (antibody-dependent cell-mediated cytotoxicity, Figure 8 and chapter 6.2.2.4.2). These processes are summarised in the schematic below.

Figure 8: Cytotoxic functions of NK cells against tumour cells.



(1) Lysosomes secretion cytotoxicity. Granszyme B and perforin are most of the content of NK cells lysosomes and are the mediators of the tumour cell cytolysis. (2) Antibody-dependent cell-mediated cytotoxicity (ADCC). Fc receptor (CD16) is represented recognizing the Ab linked to its antigen in the surface of the tumour cell. Fas ligand (FasL) and Fas, which is an apoptotic receptor, are also represented [83].

6.2.2.4.1 Lysosomes secretion cytotoxicity

NK cells contain lysosome organelles in the cytoplasm, in which various enzymes and proteases are contained, they make them be capable of an immediate non-antigen specific response, without transcription, translation or cell proliferation requirements [69, 84]. Upon receiving activating signals from their surface receptor-ligands interactions, NK cells release the contents of their lysosomes inducing the apoptosis of the target cells.

NK lysosomes contain cytotoxic proteins such as perforin and granzymes (Figure 8, 1). Due to the lytic power of those proteins, signalling resulting in their release is very tightly regulated. Firstly, NK cells contact the target cells by cell-cell associations. Although little is known about the mechanisms of this association, studies indicate that selectin family members and CD2 may be involved [85]. Once the target cell is attached to the NK cells, the latter receives initial intracellular activating signalling and an activated-lytic synapse is formed at the connection point. Integrin family adhesion molecules such as lymphocyte function associated antigen-1 (LFA-1) and macrophage-antigen-1 (MAC-1) have been shown as mediators of stronger adhesion in the synapse formation. Further activating signalling attends this adhesion, rearrangements of actin cytoskeleton take place and MTOC (microtubule-organizing center) and lysosomes lead to the synapse point [86]. Lysosome must fuse with the membrane of the target cell membrane to be able to release their content on the NK-target cell interface and SNARE (N-ethylmaleimide-sensitive factor) or equivalent protein may be involved in that membrane fusion-procedure [85]. Unfortunately, there is no conclusive evidence yet of that protein's interaction on NK cell lysosomes secretion and subsequent cytotoxicity [85].

NK lysosomes contain two different types of proteins: pore-forming proteins and proteases. Perforin belongs to the first group and polymerizes and inserts itself into the target cell membrane making a pore by creating transmembrane tubules. This pore made by the perforin partly disturbs the osmotic state of the cell and allows proteases access into the cell to mediate cell death. The main proteases the NK lysosomes contain are granzymes, which remain inactivated in the lysosomes because of the acidic pH. The major granzymes in terms of amount are Granzyme B and A, but Granzyme B is the most potent one. Their main function is to induce apoptosis of the cell [87]. Granzyme B achieves this purpose by attaching to caspase-3 and some of its substrates leading to DNA fragmentation and apoptosis [88].

6.2.2.4.2 Antibody-dependent cell-mediated cytotoxicity (ADCC)

Although ADCC is a cytotoxicity process that can be carried out by NK cells, neutrophils and macrophages also induce it. ADCC is a process that requires the interaction of 3 molecules, (1) an antigen on the target cell that is recognized by (2) an antigen-specific antibody (Ab) of a suitable isotype and (3) the FcR present on the cytotoxic cell. The FcR on NK cells is CD16 (low affinity Fcγ RIII) [57]. The interaction between NK cells and the target cancer cell via

the Fc constant domain of the Ab and CD16 (Figure 8, 2), respectively, mediates the activation of the NK cell, induce its pro-inflammatory cytokines release and triggers NK cell cytotoxic function [89]. The releasing of INF-γ, tumour necrosis factor-α (TNF-α) and other chemokines potentiates the recruitment of other immune cells, thus promoting a pro-inflammatory immune environment, the release of the lysosomal content and, ultimately, lysis of the target cell [84].

7. Hypothesis and Aims

The present thesis represents initial studies of the potential of allogeneic NK cells as a therapeutic agent against GBM before taking this investigation into *in vivo* experiments. Therefore, this project was built to achieve the following hypotheses and aims:

7.1 Hypothesis

We hypothesized that:

1. Purified human NK cells can be successfully expanded as opposed to LAK cells from the same healthy donor.
2. Purified NK cells from healthy donors can be activated in culture to be more cytotoxic than LAK cells against GBM cells *in vitro*.
3. KIR-HLA ligand interactions may mediate NK cells' cytotoxic potency against GBM cells.

7.2 Aims

1. Characterise major population of NK cells in peripheral blood of healthy donors and the impact of cell culture on expression of phenotypic markers.
2. Compare purified NK and LAK cells for the ability of expansion using standard methodology.
3. Investigate the cytotoxicity potency of NK cells *vs*. LAK cells from healthy donors against GBM stem like cells.
4. Investigate the role of KIR-HLA interactions in determining cytotoxic efficacy of resting *vs.* activated NK cells.
5. Identify the KIR-HLA ligand interactions most critical in determining NK cytotoxic potency against GBM.

8. Materials and methods

8.1 Blood donors and extraction

Eight healthy donors were enrolled into the study and gave their informed consent to their participation. The study was approved by the REKvest ethical board. 25-40ml of blood was extracted by a trained phlebotomist at the same time of the day, between the hours 10am-14pm (Table 3). For the extraction, butterfly cannulas (green, needle gauge with adapter and holder Vaccuette® (Ref: G450085, Greiner bio-one, Germany) were used. The blood was collected in Vaccuette® blood collection tubes (9ml K3EDTA tubes, Ref: 455036, Greiner bio-one) and stored overnight at 4°C in the fridge (for less than 24h).

Table 3: Donor demographics, time and volume blood extraction.

Donor number	Age (years)	Gender	Origin	Volume of blood	Time of extraction
1	27	Female	Polish	40ml	11-12h
2	26	Female	Iranian	25ml	13-14h
3	26	Male	Turkish	30ml	10-11h
4	39	Male	Norwegian	30ml	11-12h
5	26	Female	Polish	36ml	12-13h
6	32	Male	Estonian	36ml/27ml	10-11h/ 13-14h
7	26	Male	Spanish	36ml	12-12:30h/ 11:30h
8	28	Male	Iranian	36ml	11-12h

8.2 GBM cell lines

We established GBM cell lines from patients' biopsies obtained from routine craniotomy surgery performed at the Neurosurgical Department at Haukeland University Hospital

(Bergen, Norway). The collection of biopsy material into the GBM biobank was approved by the REKvest regional ethical board. The GBM cells were used as target cells in the cytotoxic assays. P3, 2010-046, 2010-062, 2012-020, 2010-20-I and 2012-018 were established from GBM patients' biopsies and the K562 cell line (ATCC, Sweden) from the chronic myeloid leukaemia patient. K562 cells lack MHC class 1 molecules and are highly susceptible to NK cell lysis [90]. Hence these cells were used as a positive control in the cytotoxic assays.

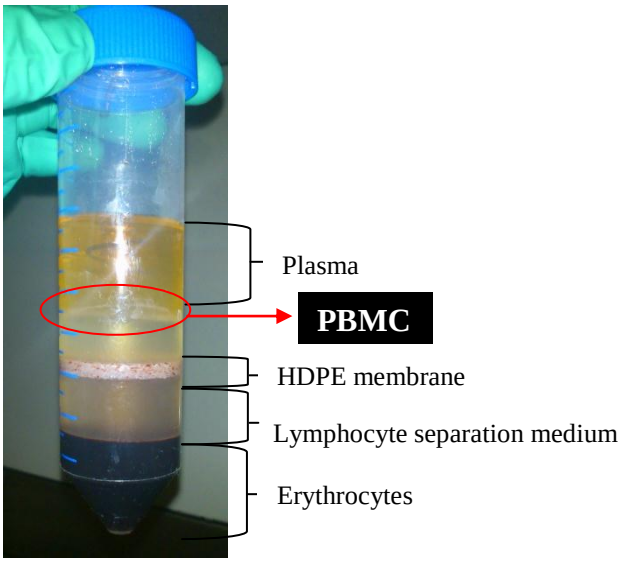
8.3 Cell isolation

8.3.1 Peripheral blood mononuclear cells (PBMCs) isolation

PBMC isolation was performed in order to obtain immune cells from blood for subsequent studies, such as antigenic phenotyping, natural killer cells (NK cells) isolation and culture of PBMCs to obtain lymphokine activated killer cells (LAKs). PBMCs were isolated from the overnight-stored donor blood by using density gradient centrifugation method. Leucosep® (50ml tubes with an high density polyethylene membrane HDPE, Cat n°: 227290, Greiner bio-one) were used and 15ml of lymphocyte separation medium (Cat n°: 17-829E, Lonza, Lysaker, Norway) was added and centrifuged for 5min at 400 rpm (Biofuge stratus, Heraeus instruments) at room temperature. After this, the lymphocyte medium collected below the membrane. Then, 20-25ml of 1:1 blood/phosphate buffered saline (PBS) was added on the membrane and centrifuged for 15min at 400rpm in room temperature.

Lymphocyte separation medium is composed by a mixture of Ficoll® and sodium diatrizoate with adjusted density to 1,077 g/ml. Ficoll® is a hydrophilic polymer used to create a density gradient of the blood cells in the sample, after centrifugation. PBMCs are distributed in that gradient as a white band above the HDPE membrane (Figure 9).

Figure 9: Leucosep® with Lymphocyte separation medium and blood dilution after centrifugation.



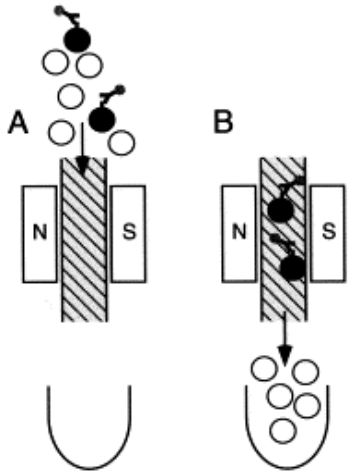
Blood cells distributed in different layers by density gradient. PBMCs are the white band situated between the membrane and the plasma band.

8.3.2 Natural killer (NK) cells isolation

NK cell isolation was performed in order to obtain pure NK cells for the cytotoxicity studies. NK cells were isolated from the healthy donors' PBMCs by using a negative selection procedure on magnetic column (NK Cell Isolation Kit, order no: 130-092-657, Miltenyi Biotech, Germany).

Briefly, to eliminate of cell clumps, PBMCs, were passed through a 30 µm pre-separation filter (Order nº: 130-041-407, Miltenyi Biotech). Then the cells were ready to be labelled according to the manufactures' protocol; Biotin-Ab cocktail, containing Biotin conjugated to Ab against lymphocytes but NK cells lineage-specific antigens, was added to the cells and incubated for 13 min. Thereafter, Microbeads Cocktail, containing beads conjugated to monoclonal Ab, was added and incubated 15min. After one washing with 2 ml. of MACS Buffer (MB) the cells were passed through the magnetic column (LS Column, Order no: 130-042-401, Miltenyi Biotech) (Figure 10), followed by 5 times MB column washes.

Figure 10: Schematic drawing of a negative selection procedure by magnetic beads column, denoted north (N) and south (S).



(A) Shows the start point with a mixture of cells (PBMCs), the not relevant (in black) are labeled with magnetic beads conjugated to monoclonal antibodies (Abs). The cells of interest (in white), NK cells, are not labeled. (B) Represents the attachment of bead-Abs labeled cells to the magnetic field and the collection of the isolated pure, "untouched" NK cells. Figure taken with permission from [91].

8.4 Cell culturing

8.4.1 PBMCs culturing

In order to obtain lymphokine activated killer (LAK) cells, PBMCs were cultured after the isolation for one week in RPMI-1640-Glutamax medium (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 10% v/v Fetal Cow Serum (FCS, Cat nº: A4503, Sigma-Aldrich), 5% v/v Penicillin-Streptomycin antibiotics (PS, Cat nº: DE17603E, BioWhittacker Inc., Walkersville, Maryland, USA), 1mM Hepes with 0,85% NaCl (Cat n°: 17-829E, Lonza), 100U/ml IL-2 (Cat nº: 202-IL-050, R&D System, UK) and 50ng/ml IL-15 (Cat nº: 247-IL-025, R&D System). The cells were cultured in the concentration of $1x10^6$cells/ml in 12 or 6 well-plates (Thermo Scientific Nunclon®, Waltham, Massachusetts, USA), depending on the total amount, and were incubated at 37°C and 5% of CO_2 (Steri-Cycle CO_2 incubator, Hepa Class 100, Thermo electron corporation). 0,5 ml of the same supplemented medium was added each 2-3 days.

8.4.2 NK cells culturing

In order to activate and expand the purified NK cells, they were cultured after the isolation for two weeks with sterile good manufacturing practice (GMP) serum free Stem Cell medium

(SC, Cat nº: 20802-0100, CellGenix, Freiburg, Germany) supplemented with 10% v/v FCS, 500U/ml IL-2 and proliferation beads of NK Cell Activation/Expansion Kit (Cat nº: 130-094-483, Miltenyi Biotech) according to the protocol. The activation beads consist of anti-NKp46 and anti-CD2 antibodies conjugated to microbeads. The cells were cultured in a concentration of $1x10^6$cells/ml in 6 well-plates and were incubated at 37°C and 5% of CO_2. 0,5 ml of the supplemented medium without the activation beads was added each 2-3 days and the cells were passaged when they became too dense.

8.4.3 K562 culturing

K562 is a cell line that we used as a positive control in the cytotoxicity assays. It was cultured in suspension with RPMI-1640-Glutamax medium supplemented with 10% v/v FCS, 5% v/v PS, 1mM Hepes with 0,85% NaCl. The cells were cultured at a concentration of $0,2x10^6$cells/ml in 75 cm^2 Nunc® Easy flasks (with Nunclon Δ, Cat nº: 156499, Thermo Fischer Scientific) and incubated at 37°C and 5% of CO_2. The cells were passaged when they became too dense and only cells in the exponential phase were used for the assays.

8.4.4 Patient-derived GBM cells culturing

P3, 2010-046, 2010-062, 2012-020, 2010-20-I and 2012-018 GBM cells were cultured with NeuroBasal® medium (NB, Cat nº: 21103-049, Invitrogen Hämeenlinna, Finland) supplemented with 1% v/v GlutaMAX® (Cat nº: 35050-061, Invitrogen), 2% v/v B-27® (Supplement Minus Vitamin A, Cat nº: 12587-010, Invitrogen), 5% v/v PS, 20% v/v recombinant human epidermal growth factor (EGF, Cat nº: AF-100-15, PeproTechc SE, Stockholm) and 20% v/v recombinant human basic fibroblast growth factor (FGF, Cat nº: 100-18B, PeproTech SE, Stockholm) to be used in cytotoxicity assays. The cells were cultured in 25 cm^2 Nunc® Easy flasks (with Nunclon Δ, Cat nº: 156367, Thermo Fischer Scientific) and incubated at 37°C and 5% of CO_2. They were passaged when they became dense and only cells in the exponential phase and within passages 1-25 were used for the assays.

Table 4: GBM patient biopsies information of the GBM cells in culture.

Patient ID	Cancer type	Gender	Age
P3	GBM	Male	64
2012-018	GBM Gliosarcoma	Male	81
2010-20-I	GBM	Male	51
2010-046	GBM	Female	61
2010-062	GBM	Male	66

8.4.4.1 P3 and 2011-20-I

P3 and 2011-20-I are GBM xenograft cell lines that have been serially passaged *in vivo* in nude rats (Table 4). P3 were carefully grown as monolayers in 25 cm^2 flasks while 2011-20-I grown as partial suspension and monolayers. 24 mm^2 cell scrapers (TPP Tissue Cell Scraper, Cat nº: 99002, MidSci, St. Louis, Missouri, USA) were used to detach the cells from the substratum during the passaging.

8.4.4.2 2010-046, 2010-062, 2012-020 and 2012-018

These are GBM primary culture lines that are derived directly from patients' tumour material.

2010-046 and 2010-062 GBM cells (Table 4) were grown in flasks as monolayers, while 2012-020 cells were grown half attached half in suspension and 2012-018 cells (Table 4) were grown as both monolayers and spheres, both attached to the substratum. In all the four cases 24 mm^2 scrapers were used to detach the cells during de passaging.

8.5 Cell counting

In order to calculate the concentration of cells present in the blood samples, the cells were counted by using Bürker counting chamber. The Bürker chamber is a standard cell counting method that consists of one chamber with two rafters to add samples of cells to be counted. Each rafter has an engraving of nine big squares of 1 mm^2 area encoded in 3 parallel lines

sides each (Figure 11, A). The chamber covered with cover slip is 0,1 mm deep. Therefore, each of the 9 squares has a 0,1mm^3 of capacity.

For our studies it was important to count only viable cells, so Trypan blue solution (0,4% in 0,81% Sodium Chloride and 0,06% potassium phosphate, dibasic; Cat n°: 72-75-1, Sigma-Aldrich) was used in 1:1 concentration to be able to distinguish the dead and live cells. Trypan blue is a dye that is not permeable to the membrane of live cells but diffuses freely into dead cells because of the disruption of those cells' membrane. After Trypan blue staining the live cells are distinguished as round, phase bright and unstained while the dead cells typically have irregular shape, are phase dim and retain the trypan blue colour (Figure 11, B, arrow).

To proceed to the counting 10µl of cells suspension was mixed with 10µl of Trypan blue in an eppendorf® tube (0,5ml, Cat nº: 0030 121.023, Eppendorf) and 10µl aliquot of the mix was added to the space between the chamber and the cover slip. Cells within the 1 mm^2 and the ones lying on the upper and left sides were counted using 10x objective of Microscope (Olympus CKX31), (Figure 11, B). It is important to point out that at least three out of nine squares of 1 mm^2 area were counted to ensure accuracy.

To calculate the number of cells present in the sample, the equation below was used,

$$\textbf{Number of cells} = n*d*V*10^4/s$$

Where:

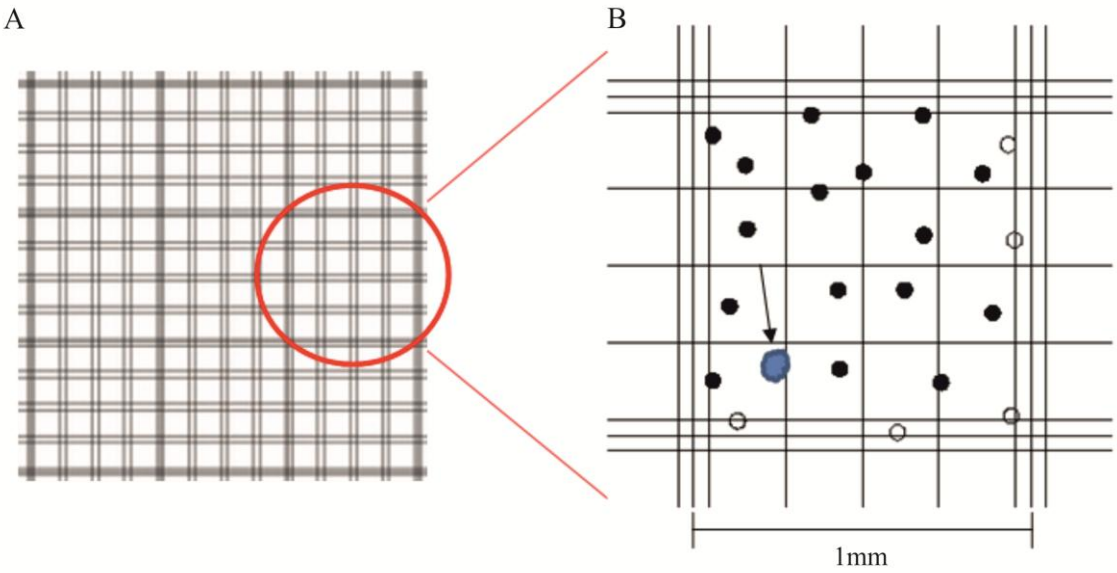
n: number of cells in all the 1 mm^2 counted squares.

V: total volume of the cell suspension in millilitres.

s: number of 1 mm^2 area counted squares (normally s=3).

d: dilution of the cell suspension sample due to addition of TrypanBlue (normally d=2).

Figure 11: Bürker chamber details.



(A) Detail of the nine 1 mm^2 area squares encoded in 3 parallel lines sides each. (B) Detail of one 1 mm^2 area square. Black dots represent viable cells in the right area to be counted. White dots represent cells which are not in the counting area because they lie on the bottom edge and the right side. The arrow points to a blue irregular shaped, phase dark/dim dead cells after the Trypan blue staining. Dead cells are excluded of the counting. Figures taken from [92, 93].

8.6 Cell staining

In order to characterize the phenotype of the PBMCs, NK and GBM cells used in this study, fluorochrome conjugated (Fl-conjugated) primary Ab, Fl-conjugated secondary Ab and intracellular cell staining were required to detect the expression of different markers by flow cytometry.

8.6.1 Fluorochrome-conjugated primary Ab staining

Antibodies (Ab) against all the markers of interest were, conjugated to different fluorochromes and the majority was used to detect surface markers of the different cells within the sample. The cell staining was done 0,5-1x10^6 cells in FACS tubes (5ml 12x75mm. Polystyrene round-bottom tubes, Ref: 352052, BD Biosciences Falcon®). Cells were incubated with the relevant Abs at previously optimised concentrations (see table?) for 20min at 4°C protected from light. After a couple of washing of the samples with FACS buffer (FB),

1 µl Live/Dead® Fixable Near-IR Dead Cell Stain Kit (L/D, Cat nº: L10119, Invitrogen) was added to distinguish the fluorescent signal emanating from the dead cells and the specific bonafide staining due to the binding of Ab-conjugated fluorochromes to the right epitopes. Live/Dead® Kit contains a fluorescent dye that binds to free amines both in the surface and inside of the cells. Due to the compromised state of the dead cells membrane, they are stained both on the surface and inside the cell, thus, dead cells are brightly stained than the alive cells, which are only stained on the surface. Staining in the tubes was undertaken in a final volume of 100µl of FB (Table 4).

Due to the lack of available specific Abs against certain molecular markers, such as KIR3DL2 and KIR2DL2, Abs against two of the subtypes of those markers were needed to be used. Therefore, anti-KIR3DL2/1 along with anti-KIR3DL1 and anti-KIR2DL2/3 coupled with anti-KIR2DL3 were applied. In the staining tubes that contain those combined Abs, previous incubation of the sample with only anti-KIR3DL1or anti-KIR2DL3 Abs were required in order to occupy specifically their epitopes. Thus, in a second incubation with all Fl-conjugated Abs, anti-KIR3DL2/1 or anti-KIR2DL2/3 were added to attach to the KIR3DL or KIR2DL left by the common Abs against two subtypes (mostly KIR3DL2 or KIR2DL2, respectively).

Control and compensation samples were needed in addition to the experimental samples. Three control tubes were included: one with only cells and no Ab added, to determine the auto-fluorescence of the unstained sample; another with isotype control Abs, to be able to check any unspecific binding of the isotype Ab to the samples; and the third one with isotype control Abs and some Fl-Abs, to detect any signal interference between isotype Abs and the Fl-Abs. The compensation tubes were needed for calibrating the flow cytometer settings before acquiring and data analyzing and to determine the spectral between detection channels (compensation tubes use is further explained in detail in *8.9.1.3* chapter).

Table 5: Example of NK cells and PBMCs staining frame.

	Sample	V450	V500	BrillViol570	FITC	PE	PE-Cy5	PE-Cy7	APC	Alexa700	APC-Cy7
1	PBMC										
2	PBMC	IgG1			IgG2b						
3	PBMC	CD56									
4	PBMC		CD3								
5	PBMC			CD4							
6	PBMC				CD16						
7	PBMC					NKG2D					
8	PBMC						CD14				
9	PBMC							CD8			
10	PBMC & beads								NKp46		
11	PBMC & beads									NKG2A	
12	PBMC										L/D
13	PBMC	CD56	CD3	CD4	CD16		CD14	CD8		IgG2a	L/D
14	PBMC donor	CD56	CD3	CD4	CD16	NKG2D	CD14	CD8	NKp46	NKG2A	L/D
15	PBMC donor	CD56	CD3	CD4	CD16	NKG2D	CD14	CD8		NKG2A	L/D
16	NK donor	CD56	CD3	CD4	CD16	NKG2D	CD14	CD8	NKp46	NKG2A	L/D
17	NK donor	CD56	CD3	CD4	CD16	KIR3DL2/1	CD14	CD8	KIR2DL4	KIR3DL1	L/D
18	NK donor	CD56	CD3	CD4	CD16	KIR2DL2/3	CD14	CD8	KIR2DL3	KIR2DL1	L/D
19	non-NK	CD56	CD3	CD4	CD16		CD14	CD8			L/D

Columns represent the different channels selected for the detection of the used fluorochromes. Rows represent different sample tubes. Tubes 1-2 and 13 are controls and un-specificity-binding checking tubes. Tubes 3-12 are compensation tubes used do compensate the signal in the machine. Tubes 14-19 are the tubes of study. In tubes 17 and 18 KIR3DL1 and KIR2DL1, respectively, are highlighted in purple due to fact that they needed to be added before the whole staining.

8.6.2 Fluorochrome-conjugated secondary Ab staining

For the GBM cell phenotyping sequential staining with primary Abs, followed by secondary Fl-conjugated was required. The secondary Ab staining was done as a second step to the full primary Ab staining. 0,5-1x10^6 cells were placed in FACS tubes after couple of washing steps with FB. The primary Ab was added in a final volume of 100µl of FB and incubated for 20min at 4°C protected from light. This incubation was followed by a washing step and incubation with the secondary Ab under the same conditions as with the primary Ab.

8.6.3 Cell fixation and Intracellular Ab staining

In order to stain intracellular antigens, after the surface staining, GBM cells were fixed and permeabilized using Cytofix/Cytoperm® Fixation/Permeabilization Solution Kit (Cat nº: 554714, BD Biosciences, Trondheim, Norway), through incubation for 20min at 4°C protected from light with 200µl of Cytofix followed by a washing procedure (centrifugation,

900rpm/10 min) with Cytoperm buffer. The washing was followed by the incubation with intracellular Fl-conjugated secondary Ab under the same conditions as with the primary Abs incubations.

8.7 Antibodies

8.7.1 Antibodies for phenotyping assays

8.7.1.1 NK and PBMC cells

Various commercially available primary Abs conjugated to different fluorochromes and the anti-human Fl-conjugated secondary Abs as well as isotype control Ab were chosen for our NK, PBMC and GBM cells staining and flow cytometry phenotyping. These are named in the following tables.

Table 6: Antibodies for flow cytometry phenotyping assays of NK and PBMC cells

Anti-X antibody	Fluorochrome conjugated to	Stock concentration (µg/ml)	Dilution	Source species and clone	Catalogue number	Supplier
CD56	Violet450 (V450)	-	1:20	IgG1, clone B159	560360	BD Biosciences (Trondheim, Norway)
CD3	Violet500 (V500)	-	1:20	IgG1, clone SP34-2	560770	BD Biosciences
CD16	Fluorescein isothiocyanate (FITC)	-	1:5	IgG1, clone 3G8	555406	BD Biosciences
CD8	Tandem Phycoerythrin-Cyanine7 (PE-Cy7)	-	1:20	IgG1, clone RPA-T8	557746	BD Biosciences
NKG2D	Phycoerythrin (PE)	25	1:10	IgG1, clone 149810	FAB139P	R&D Systems (UK)
NKp46	Allophycocyanin (APC)	10	1:5	IgG2b, clone 195314	FAB1850A	R&D Systems
NKG2A	Alexa700®	100	1:20	IgG2a, clone 131411	FAB1059N	R&D Systems

KIR2DL4	APC	25	1:10	IgG2a, clone 181703	FAB2238A	R&D Systems
KIR2DL1	Alexa700®	20	1:100	IgG1, clone 143211	FAB1844N	R&D Systems
KIR2DL3	APC	25	1:10	IgG2a, clone 181701	FAB2014A	R&D Systems
KIR2DS4	-	500	1:25	IgG2a, clone 179315	MAB1847	R&D Systems
CD4	BrillViol570®	-	1:20	IgG1, clone RPA-T4	300533	BioLegend (Oslo, Norway)
KIR3DL1	Alexa700®	500	1:20	IgG1, clone DX9	312712	BioLegend
KIR3DL1/2	PE	100	1:10	IgG1, clone 5133	130-095-205	Miltenyi Biotec (Scheelavägen, Sweden)
KIR2DL2/3	PE	100	1:10	IgG2a, clone DX27	130-092-618	Miltenyi Biotec
CD14	Tandem Phycoerythrin-Cyanine647 (PE-Cy647)	-	1:20	IgG1, MEM-15	21279147	ImmunoTools (Friesoythe, Germany)
KIR2DS2	-	960	1:800	IgG, clone	ab101524	Abcam (UK)

(-) In stock concentration column represents that the information was not provided by the supplier.

Table 7: Primary antibodies for flow cytometry phenotyping assays of GBM cells

Anti-X antibody	Fluorochrome conjugated to	Stock concentration (µg/ml)	Dilution	Source species and clone	Catalogue number	Supplier
nestin	Phencyclidine-Cyanine5.5 tandem (PCP-Cy5.5)	-	1:20	IgG1, clone 25/NESTIN	561231	BD Biosciences (Trondheim, Norway)
CD15	PE-Cy7	-	1:20	IgM, clone HI98	560827	BD Biosciences
HLA-ABC	APC	-	1:5	IgG1, clone G46-2,6	562006	BD Biosciences
vimentin	PE	-	1:20	IgG1, clone RV202	562337	BD Biosciences
HLA-DR,DP,DQ	FITC	-	1:5	IgG2a, clone Tu39	562008	BD Biosciences
MICB	APC	10	1:40	IgG2b, clone 236511	FAB1599A	R&D Systems (UK)
MICA	AlexaFluor488	100	1:40	IgG2B, clone 159227	FAB1300G	R&D Systems
ULBP-1	-	25	1:10	IgG2a, clone 170818	MAB1380	R&D Systems
ULBP-2/5/6	-	25	1:10	IgG2a, clone 166510	MAB1298	R&D Systems
ULBP-3	-	25	1:10	IgG2a, clone 166510	MAB1517	R&D Systems
A2B5	PE	100	1:10	IgM, clone 105-HB29	130-093-581	Miltenyi Biotec (Scheelavägen, Sweden)
CD133	APC	50	1:10	IgG1, clone AC133	130-090-826	Miltenyi Biotec
GFAP	FITC	500	1:25	IgG1, clone GA5	53-9892	eBioscience (Ireland, UK)
HLA-E	PE	100	1:20	IgG1, clone 3D12HLA-E	12-9953	eBioscience
CD31	PE-Cy7	12	1:20	IgG1, WM-59	25-0319	eBioscience
HLA-G	PE	50	1:20	IgG2a, clone 87G	12-9957	eBioscience

(-) In stock concentration column represents that the information was not provided by the supplier.

Table 8: Isotype antibodies for flow cytometry phenotyping assays

Isotype antibody type	Fluorochrome conjugated to	Stock concentration (µg/ml)	Dilution	Source species and clone	Catalogue number	Supplier
IgG1	V450	100	1:20	clone MOPC-21	560373	BD Biosciences (Trondheim, Norway)
IgM	PE-Cy7®	200	1:20	clone G155-228	560856	BD Biosciences
IgG2a	Alexa700®	50	1:20	clone 20102	IC003N	R&D Systems (UK)
IgG1	PE	-	1:20	clone PPV-06	21275514	ImmunoTools (Friesoythe, Germany)
IgG2b	FITC	-	1:20	clone PLRV219	21275533	ImmunoTools
IgG2a	FITC	-	1:20	clone PPV-04	21275523	ImmunoTools

(-) In stock concentration column represents that the information was not provided by the supplier.

Table 9: Secondary antibodies for flow cytometry assays

Anti-X antibody	Fluorochrome conjugated to	Stock concentration (µg/ml)	Dilution	Catalogue number	Supplier
Fragment of goat anti-mouse IgG	PacOrange	2000	1:40	P31585	Invitrogen (Hämeenlinna, Finland).
Goat polyclonal anti-rabbit IgG	AlexaFluor647®	2000	1:100	Ab150079	Abcam (UK)

Table 10: Antibodies for flow cytometry NK cells cytotoxicity assays with blocking antibodies

Anti-X antibody	Stock concentration (µg/ml)	Dilution	Source species and clone	Catalogue number	Supplier
KIR2DS4	500	1:25	IgG2a, clone 179315	MAB1847	R&D Systems (UK)
NKG2D	500	1:25	IgG1, clone 149810	MAB139	R&D Systems
KIR2DS2	960	1:800	IgG, clone	Ab101524	Abcam (UK)

Table 11: Isotype antibodies for flow cytometry NK cells cytotoxicity assays with blocking antibodies

Isotype Antibody type	Stock concentration (µg/ml)	Dilution	Source species and clone	Catalogue number	Supplier
IgG2a	500	1:25	clone 20102	MAB003	R&D Systems (UK)
IgG1	500	1:25	clone 11711	MAB002	R&D Systems
IgG	500	1:4000	Rabbit, clone	Ab37415	Abcam (UK)

8.8 Cytotoxicity assays

8.8.1 Cytotoxicity assays of LAK cells and NK cells against GBM target cells

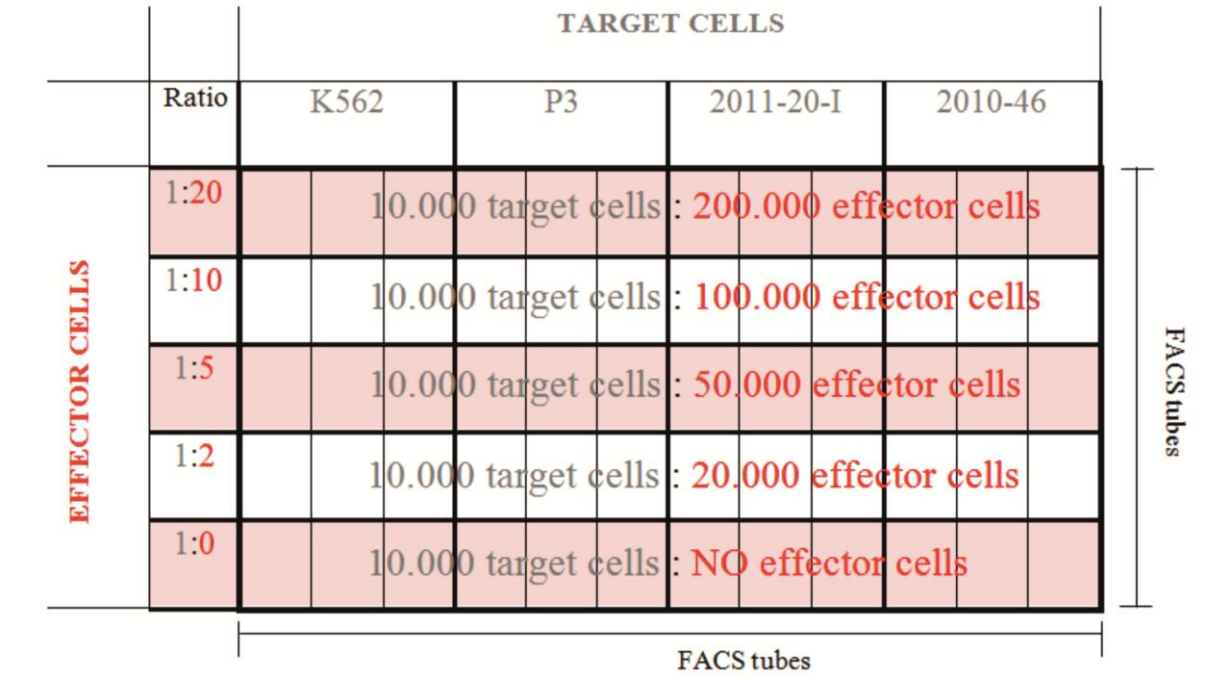
In order to investigate the ability of LAK cells and NK cells from each donor to kill different patient-derived GBM cells, cytotoxicity assays were performed. LAK cells and NK cells were the **effector** cells while GBMs were the **target** cells for the killing.

Cytotoxicity assay consists of co-culturing both effector and target cells in different ratios to identify the dose efficacy. Effector: target ratios in the range between 1:2 to 1:30 were performed depending on the amount of cells available from each donor and patient-derived GBM for the experiment. In order to detect and normalize for the values of spontaneously dead target cells without any effect from the effector cells, negative control sample was included. The negative control tubes consisted of monoculture of $1x10^4$ target cells. Furthermore, three replicates of each condition of study were performed to optimize and obtain statistical variation of the data.

To be able to distinguish the target from the effector cells, target cells were labelled with 5(6)-Carboxyfluorescein Diacetate N-succinimidyl ester (CFSE, Cat nº: 21888-25MG-F, Sigma-Aldrich), which is an intracellular fluorescent dye. The staining procedure was 1ml of 5mM CFSE per $2x10^6$ target cells and they were incubated during 20min at 37°C protected from light and briefly mixed every 5 min. Once they were stained they were resuspended as $1x10^4$cells/100µl RPMI-1640-Glutamax complete medium and added to the corresponding tubes (see Table 11, columns).

The effector cells were also resuspended in 100µl of the same medium in different concentrations depending on the effector ratios required for each condition (Table 11, rows). The cells were cultured in a final volume of 200µl of the complete medium for 4h at 37°C and 5% of CO_2 in FACS tubes.

Table 12: An example of a diagram of cytotoxicity assay performing.



Each individual square inside the big square delimited by bold black line represents one sample. All the samples contained $1x10^4$ of the corresponding target cells depending on the column they were in and $20x10^4$, $10x10^4$, $50x10^4$, $2x10^4$ or 0 effector cells depending on the row. It is illustrated how 3 replicas of each condition were performed.

After 4h of incubation and the washing procedure (centrifugation 900rpm/10min), the samples were stained, 1 min before being acquired by the cytometer, with 1µl of 1mM TO-PRO3®-3 iodide in DMSO (TO-PRO, Cat nº: T3605, Invitrogen) in order to detect the dead cells. TO-PRO is a blue DNA intercalating dye that, due to the disruption of the membrane of the dead cells, is able to penetrate to the nuclei and stain their DNA.

Finally, the data was acquired on the BD LSR Fortessa cytometer (BD Biosciences) and analysed with use of BD FACSDiva software (BD Biosciences). With the data from BD FACSDiva some calculation were carried out by Microsoft Excel (Windows) to get the cytotoxicity effect of each donor's effector cells.

% lysis = (D-SD)*100

Where:

% lysis: % of target cells killed by the effector cells.

D: number of dead target cells under co-culture conditions with the effector cells (Figure 16, B).

SD: number of dead target cells under monoculture of target cells conditions (Figure 16, A).

8.8.2 Cytotoxicity of NK cells with blocking antibodies

In order to mechanistically prove the involvement of the KIR2DS2 and KIR2DS4 activating receptors we found to be relevant in NK cytotoxicity, we performed cytotoxicity assays against patient-derived GBM cells (n=2) using donors' (n=4) NK cells with or without blocked activating receptors.

These cytotoxicity assays with blocking antibodies were performed in the same way as the simple cytotoxicity assays but with addition of blocking Abs. Cytotoxicity assays with single blocking of NKG2D, KIR2DS2 or KIR2DS4 receptors were performed as well as double and triple blockings. No blocking Ab and Isotype control Abs conditions were used as controls to ensure that the results were due to a specific blocking. Target: Effector ratios in the range between 1:5 and 1:20 were performed and three replicates of each condition of study were performed to obtain statistical significance of the data. NKG2D, KIR2DS2 or KIR2DS4 receptors were phenotyped in order to determine their expression level in each donor.

8.9 Flow cytometry

Flow cytometry is a biophysical technique used for analysing different parameters of individual cells in heterogeneous samples. A flow cytometer is required for these measurements, and in this study a BD LSRFortessa® cytometer (BD Biosciences) was used.

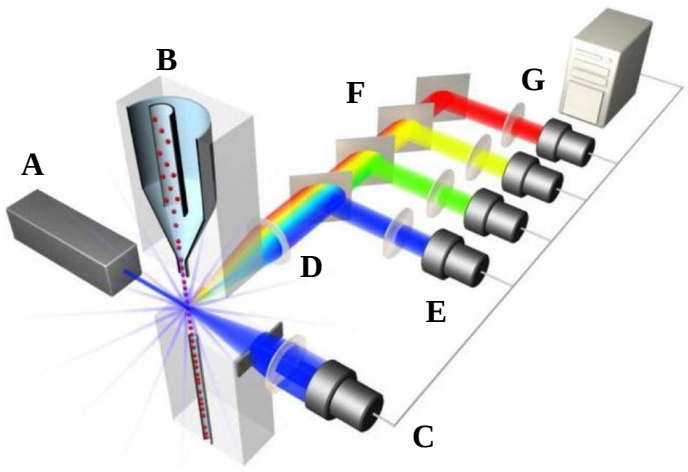
Flow cytometer is a machine that accomplishes the measurements by passing the cells in a soluble sample one by one through a laser beam. The signal emitted by the cells is processed by the different parts of the system, detected by the different detectors and treated and

analysed statistically by the software, in this case BD FACSDiva® Software 6.0 (BD Biosciences) [94, 95].

Importantly, flow cytometer utilizes 3 types of detectors (Figure 12) depending on the processing of the signal it receives:

- Forward Scatter (FSC) detector: Detects the light scattered forward by the cell while passing through the laser beam. It provides data proportional to the cell size.
- Side Scatter (SSC) detector: Detects the light dispersed in all the directions by the granules that cells have in the cytosol. It provides values proportional to the complexity/granularity of the cells.
- Fluorescence detectors/channels: Each channel detects the specific wavelength of the fluorescence emitted by the sample after passing through a serial of mirrors and filters.

Figure 12: Diagram of a Flow cytometer.



A) Laser beam

B) Fluidic System

C) FSC detector.

D) SSC detector.

E) Fluorescence detectors

F) Mirrors system

G) Optical Filter system

Figure taken from [96].

8.9.1 Phenotyping assays

8.9.1.1 Frame setting

Since the main purpose of employing flow cytometry was to determine the expression of multiple markers/antigens of the cells in multiple samples, multi-parametric flow cytometry assays were performed. Thus, well-defined frames of the different Fl-conjugated Ab used in each sample-tube were required. As the different filters of the cytometer detect the emission

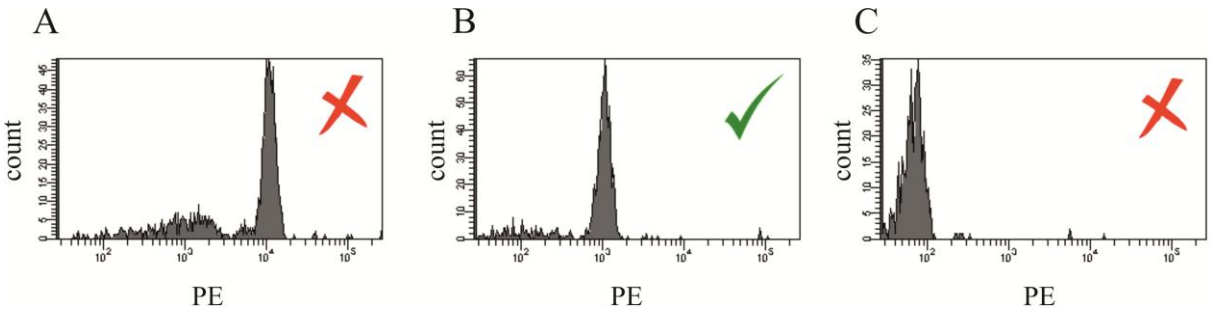
light within different wavelengths, Fl-conjugated Abs used in the same tube needed to have separate emission spectra and to be detected by different detectors.

Despite making correct choices within the Fl-conjugated Ab repertoire, in multi-parametric flow cytometry assays spectral overlapping is inevitable. Spectra overlap is the detection of various emission spectra by one filter or the sharing of emission spectra area by different Fluorochromes. The spectral overlap can be corrected by compensation (The compensation procedure is explained in detail in chapter *8.9.1.3*).

8.9.1.2 Machine setting

It is very important to properly set-up the cytometer in order to get satisfactory data from the samples. For the acquisition, it is crucial to set the voltages of the emitted fluorescence signals within the correct range of fluorescence intensity in order to avoid signal loss and obtain optimal data. Therefore, compensation tubes (containing cells stained with single Fl-conjugated Abs or dyes) are used to adjust the voltage settings (Figure 13).

Figure 13: Histograms of the machine setting procedure.



(A) The signal obtained has too high voltage. (B) Correct voltage for the signal –no signal is lost. (C) The signal obtained has too low voltage.

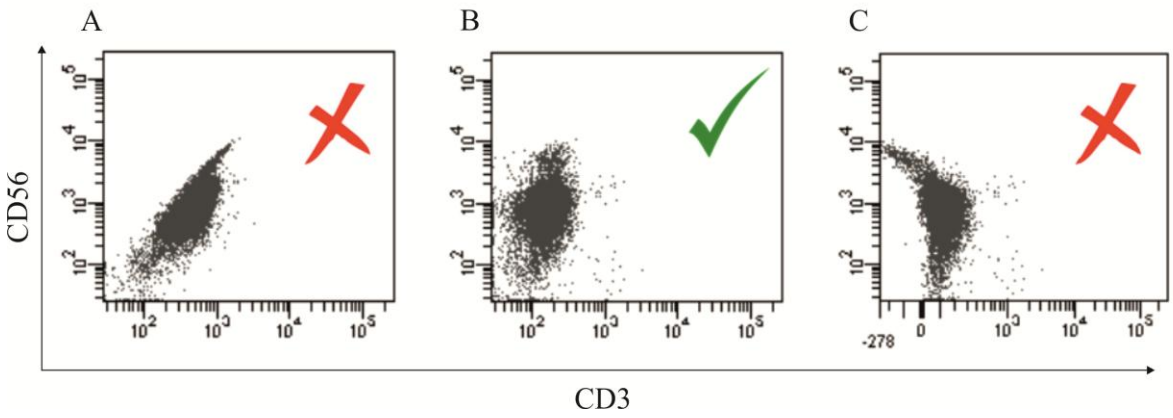
8.9.1.3 Compensation

Before starting with the analyses, it is necessary to compensate the signals obtained from the different channels. This means to correct the spectral overlap by excluding the signal caused by the neighbouring fluorochromes.

For the compensation, single Fl-conjugated Ab stained samples are required. From the data of the compensation tubes, the signal detected by the other filters that are not the corresponding

one for the specific Fl-conjugated Ab is subtracted from these filters (Figure 14). This subtracted signal occurs because of the broadness of the emission peak of the Fl-conjugated Ab that overlaps with other filters with closer wavelength detection.

Figure 14: Compensation procedure example on BD Horizon V450 channel against BD Horizon V500 channel.



(A) Non-compensated signal. (B) Well-compensated signal. (C) Over-compensated signal.

8.9.1.4 Analyses

In order to remove the background and debris that could interfere with data and give false florescence signals, a proper gating of the data is required. Therefore, there are four dot plots and gating procedures that are required before starting the analysing:

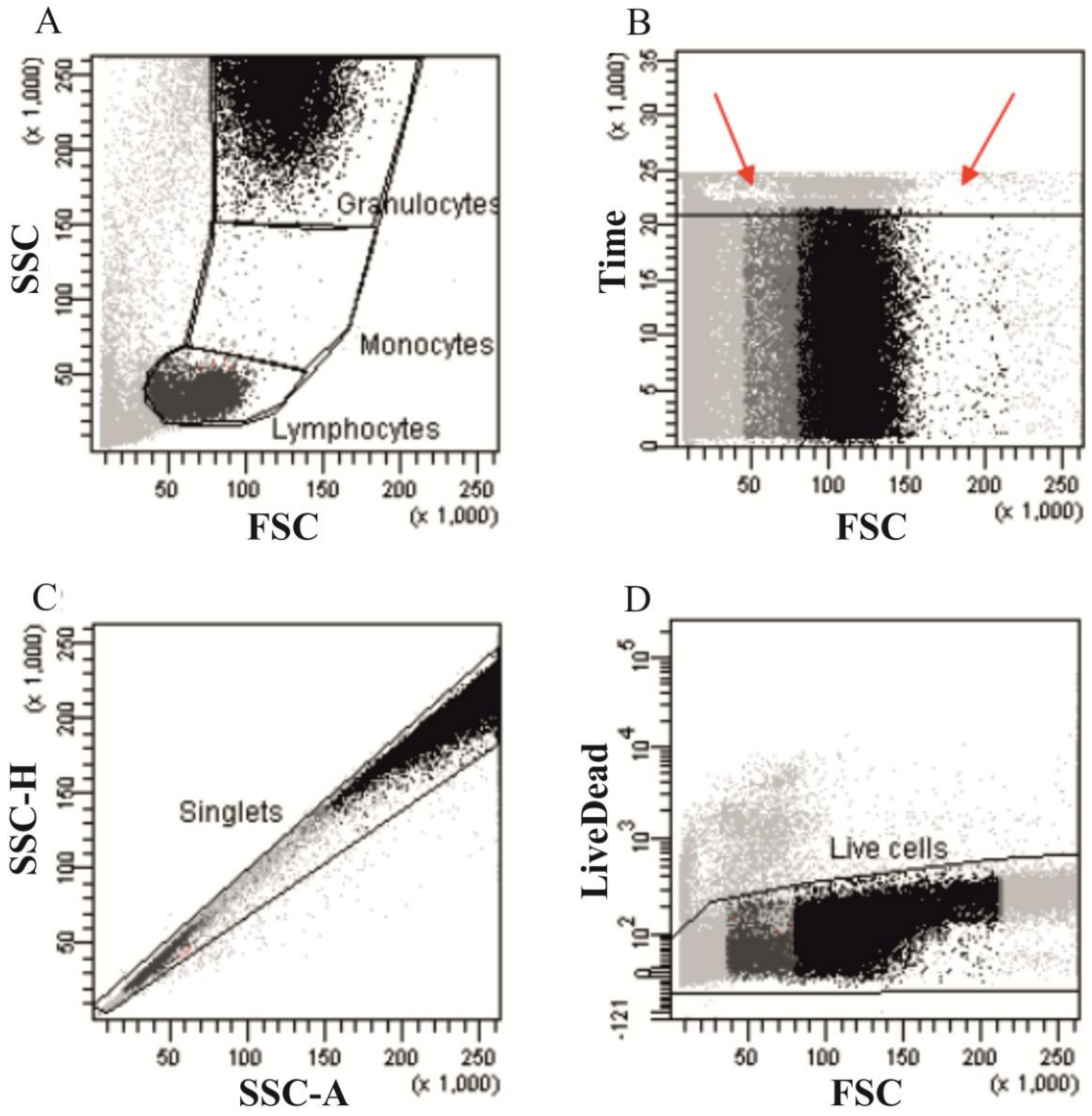
1. FSC/SSC (Figure 15, A) is required in order to gate the cell populations in the sample based on correct size and avoid the debris –corresponding to the very small fragmented particles.

2. FSC/Time (Figure 15, B) was used to remove the data acquired when the cytometer was not appropriately set-up –corresponding to white bands without plotted cells (Figure 15, B, arrows).

3. SSC-A/SSC-H (Figure 15, C) was needed to include only data from single cells and to avoid the doublets and clumps of cells that may give false fluorescence percentages.

4. FSC/Live Dead stain (Figure 15, D) was used to ensure that the data analysed was from live cells –dead cells may give auto-fluorescence for certain wavelength detectors and bind Abs unspecifically.

Figure 15: Dot plots and gating procedures required before analysing of phenotyping data.



(A) FSC/SSC: To gate the cells of interest -lymphocytes. (B) FSC/Time: Data acquired correctly gated. The arrows correspond to data acquired when cytometer was not appropriately set-up (C) SSC-A/SSC-H: The gate corresponds to single cells. (D) FSC/Live Dead stain: live cells (Live Dead$^-$) are gated.

8.9.2 Cytotoxicity assays

8.9.2.1 Machine setting

As it is for the cellular phenotyping, it was very important to properly calibrate the cytometer in order to obtain accurate data from the samples. Therefore, the voltage settings were adjusted to obtain optimal signal.

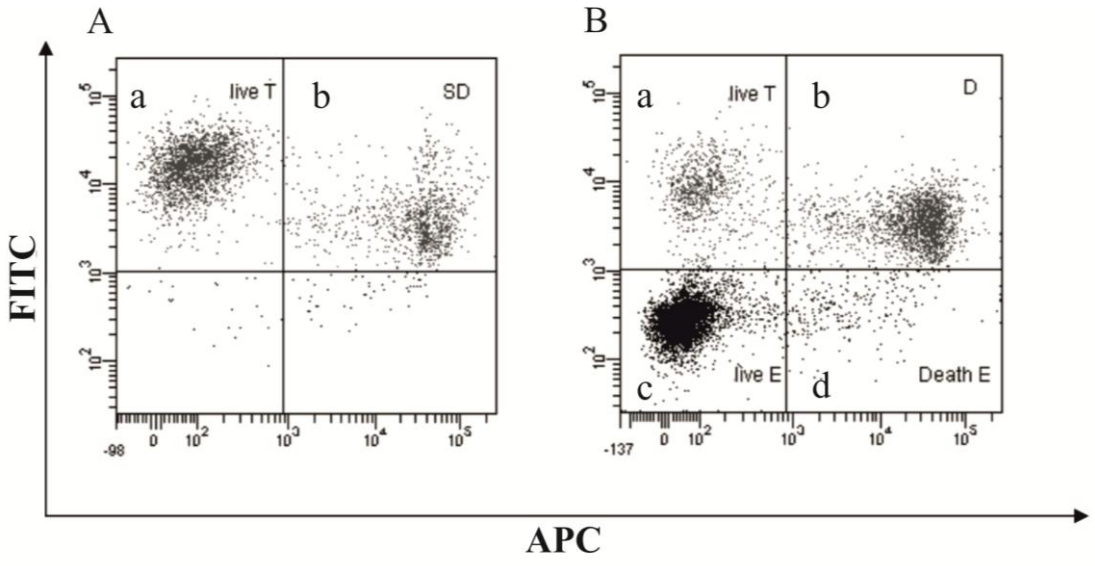
However, in the cytotoxicity assays, it is not needed to use compensation control samples (with single dye) because there is no significant spectral overlap between signals emitted by the dyes used in the assay.

8.9.2.2 Analyses

Similar to the phenotyping assays, dot plots and gating are required before starting the analysis, but in this case, only FSC/SSC (Figure 16, A), FSC/Time (Figure 16, B) and SSC-A/SSC-H (Figure 16, C) are required. FSC/Live Dead was not used as a "cleaning" procedure as previously in the phenotyping assays because the cell death was a parameter that was a variable and expected result of our experiments. Cell death was thus recorded as data.

For the cytotoxicity assays, TO-PRO3, that has an excitation of maximum 642nm wavelength, was used as a death indicator, while CFSE with a 521 nm maximum excitation wavelength was used to distinguish effector and target cells in the plot. The wide difference in the maximum excitation wavelength makes them suitable to be used together in flow cytometry detection. APC and FITC channels were used in order to detect the death and target-stained signals, respectively, from the samples (Figure 16).

Figure 16: Dot plots and gating required for analysing cytotoxicity assays.



(A) FITC/APC dot plot of a monoculture of target cells sample. (Aa) Live target cells. (Ab) SD: Dead target cells. (B) FITC/APC dot plot of a co-culture sample. (Ba) Live target cells. (Bb) D: Dead target cells. (Bc) Live effector cells. (Bd) Dead effector cells.

8.10 DNA isolation and Genotyping

The GBM and NK cell DNA extraction and genotyping procedures explained below were carried out with the assistance of colleagues in the research group and ProImmune LTD Company, respectively. The data from these experiments were required to obtain mechanistic insights into the differential cytotoxicity findings of the project.

8.10.1 DNA isolation

To obtain DNA for the genotyping, the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA) was used to isolate DNA from $1x10^6$ GBM cells and 200µl of whole donors' blood. The first step of the isolation was different for GBM and blood sample. In the case of the blood, this was mixed with proteinase K, PBS and RNase A incubating for 2min, while GBM cells needed to be centrifuged for 5min at 300g, to take out the supernatant, and then they were resuspended with PBS and proteinase K overnight. RNase A was added the day after and incubated for 2min. Thereafter, the procedure was the same for both samples but the amounts were added in a 2 fold for the blood sample (according with the manufacturer's protocol). Lysis buffer was added and incubated for 10min at 56ºC. Afterwards pure ethanol was added and the sample was placed in the DNeasy Mini Spin column to proceed to centrifuge the samples 4 times in a row. The first, second and fourth centrifugations were for 1min at 8000rpm while the third one was at 14000rpm for 3min, all 4 at room temperature and in all the centrifugations but the last one, the flow-through was discarded. Before the second centrifugation ethanol based wash buffer was added, ethanol based wash buffer of higher concentration was added before the third and before the last one elution buffer was added and incubated for 1min to get the DNA out of the column. The last centrifugation was repeated to get as much of sample as possible.

Myself, Justyna Kmiecik and Mateusz Zelkowski isolated the DNA from the samples for these experiments.

8.10.2 Genotyping

8.10.2.1 GBM Human Leukocyte Antigen (HLA) genotyping by PCR-SSOPs

The 2µg DNA at concentration 20µg/ml from GBM cells was sent to ProImmune LTD (Oxford, UK) who performed sequence specific oligonucleotide probes polymerase chain reaction (PCR-SSOPs) in order to determine the presence of HLA ligands.

PCR-SSOP is a PCR based method that consists of the use of sequence specific oligonucleotide probes (OP) conjugated to fluorescent microspheres that bind to the different HLA genes. PCR is thereafter performed to amplify the PCR gene products. The resulting sample from the PCR amplification is then incubated with a panel of OP, which binds the genes of interest, if this is the case. Therefore the presence of the genes is detected by Luminex® as fluorescent signals from the OP bound to the paired DNA sequences.

8.10.2.2 Donors' KIR receptors genotyping by PCR-SSPs

The DNA from Donors' blood was genotyped for killer immunoglobulin like receptors (KIR) genes by a KIR-typing Kit (Miltenyi Biotech, Gladbach, Germany) that is a Sequence-specific primer Polymerase Chain Reaction (PCR-SSPs) based Kit. Justyna Kmiecik and Mateusz Zelkowski carried out this procedure and I assisted in the data interpretation.

PCR-SSPs is a PCR based method that consists of the use of DNA primers for specific sequences of the region of interest. This is a very accurate procedure that can discern single-nucleotide polymorphisms. In our case the primers were specific, non-overlapping sequences of the 15 different human KIR receptor known genes and 2 pseudogenes (KIR2DS4del, with a deletion, and KIR2DS4ins, with an insertion). The presence of the gene of interest is detected as PCR amplification product by electrophoresis on an agarose gel.

The PCR was performed in a MJ Research PTC-200 Peltier Thermal Cycler by using 2-3µg (25µl of 75-125µg/l) of donors' DNA per each sample with a buffer containing Taq DNA-Polymerase. The samples established for the PCR are: (1) 17 samples for the different KIRs typing containing a positive internal control primer which leads a 400bp band in each sample; (2) 1 sample with the 100bp DNA ladder (500µl/ml, New England BioLabs, Ipswich, England) to define the sizes; (3) 1 positive control for the PCR with the positive internal control primer, will only leads the 400bp band; and (4) negative control with no DNA. The

amplification by PCR consisted in a single cycle at 95ºC for 60s. and 28 cycles of 94ºC for 20s., 63ºC for 20s. and 72ºC for 90s. After the cycling, the samples were disposed in a 2% agarose gel (Nusieve® 3:1 Agarose, Lonza) containing Ethidium Bromide (EtBr, 10mg/ml in H_2O, Sigma-Aldrich), 1µl per 25ml of gel, to be run in an electrophoresis for 40min. at 100V in a BioRad with Horitzontal Gel Elecrophoresis System (Horitzon®58, Life Technologies). After the electrophoresis, the gel needs to be developed in an UV light chamber with camera (Fujifilm) to detect the fluorescence from the intercalated EtBr in the DNA.

8.11 Statistics

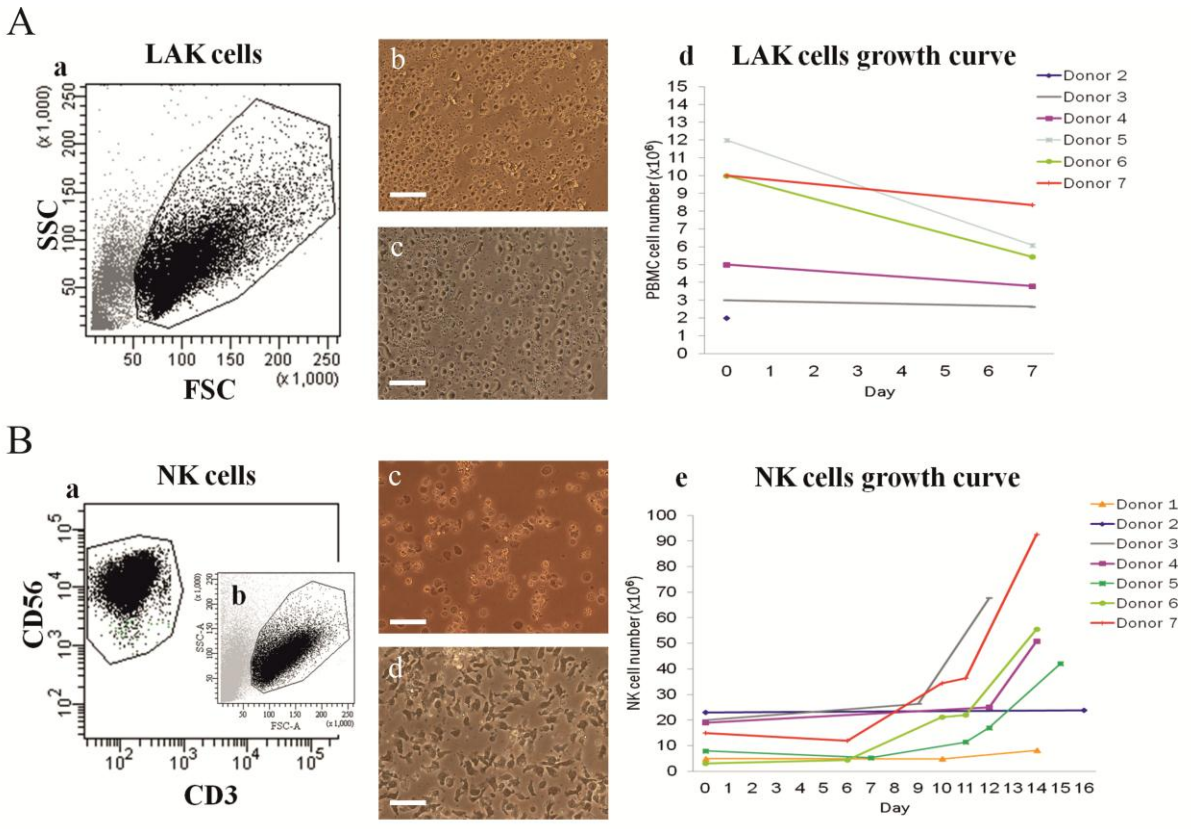
When comparing more than two variables and categories, we used One-Way Analysis of Variance (ANOVA) (with Bonferonni posthoc test). When comparing two groups with one variable, two-tailed, unpaired T-tests were performed. In all analyses probability values less or equal to 0.05 were considered significant. Descriptive statistics are reported as mean ± standard error of the mean (SEM). All statistical analyses were performed with the Prism statistical software, version 5.0 (GraphPad, La Jolla, CA, USA).

9. Results

9.1 NK and LAK cells' biological characteristics in culture.

PBMC and NK cells were propagated in culture after isolation in order to activate and expand their numbers. PBMCs were cultured in RPMI complete medium for 7 days while NK cells were maintained in SC supplemented medium for 14 days. As a result, PBMCs became lymphokine activated killer cells (LAKs) [51]. Moreover, these LAK cells (Figure 17, Ab and Ac) were revealed on flow cytometric forward and side scatter dot plots as heterogeneous populations by their size and granularity (Figure 17, Aa). The LAK cells did not proliferate in culture and subsequently became reduced in number during the culturing period of 1 week (Figure 17, Ad). In contrast, purified NK cells attained activated phenotypes compared to their resting states. NK cells (defined as $CD3^{-}CD56^{+}$ large lymphocytes, Figure 17, Ba) changed their shape from a rounded phase bright morphology under steady state (Figure 17, Bc), to a ramified phase dark morphology after the culturing (Figure 17, Bd). Donor-dependent variability was observed. NK cells remained viable and were expandable to 6-9 fold (Figure 17, Be).

Figure 17: LAK and NK cells biological characteristics in culture.

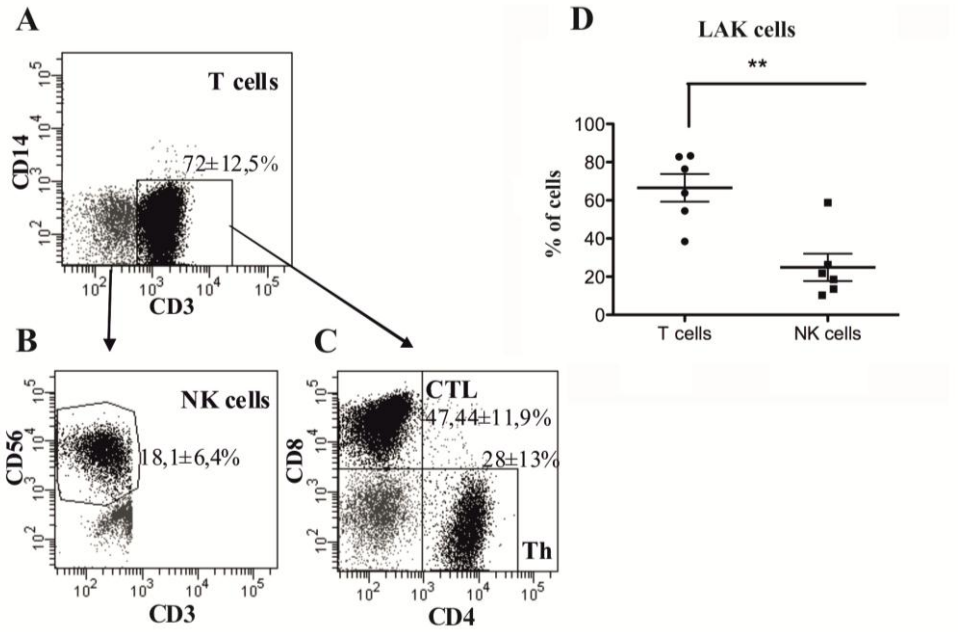


(A) LAK cells. (Aa) LAK cells represented in a FSC/SSC dot plot. (Ab) LAK cells after isolation (0 days in culture). (Ac) Phase contrast images of LAK cells after 7 days in culture. (Ad) Line graph shows the proliferation of LAK cells from each donor over the course of 7 days in culture. (B) NK cells. (Ba) NK cells in a CD56/CD3 dot plot. (Bb) NK cells represented in a forward and side scatter dot plot. (Bc) NK cells after isolation (0 days in culture) showing rounded phase bright cells. (Bd) NK cells showing phase dark, ramified morphology after 14 days in culture. (Be) Line graph shows the exponential proliferation of purified NK cells from each donor during 14 days in culture. Magnification 200X, scale bars represent 100µm.

9.2 Subpopulations of LAK cells.

We hypothesized that LAK cells were constituted of heterogeneous cell subtypes. Thus, samples of LAK cells from each donor were phenotyped by flow cytometry in order to identify these subpopulations. All the donors' LAK cells were predominantly composed of T cells (defined as $CD14^{-}CD3^{+}$, Figure 18, A), of which both CTL ($CD8^{+}CD3+$) and Th ($CD4^{+}CD3+$) were present (Figure 18, C), and NK cells ($CD14^{-}CD3^{-}CD56^{+}$, Figure 18, B). LAK cells' major subpopulation was T cells with 72±12,5% of cells (Figure 18, D; 47,44±11,9% of those were CTL and 28±13% were Th). In addition, LAK cells contained a minor NK cell population (18,1±6,4% of all cells, Figure 18, D) that was predominantly $CD56^{bright}CD16^{dim}$.

Figure 18: LAK cells subpopulations.



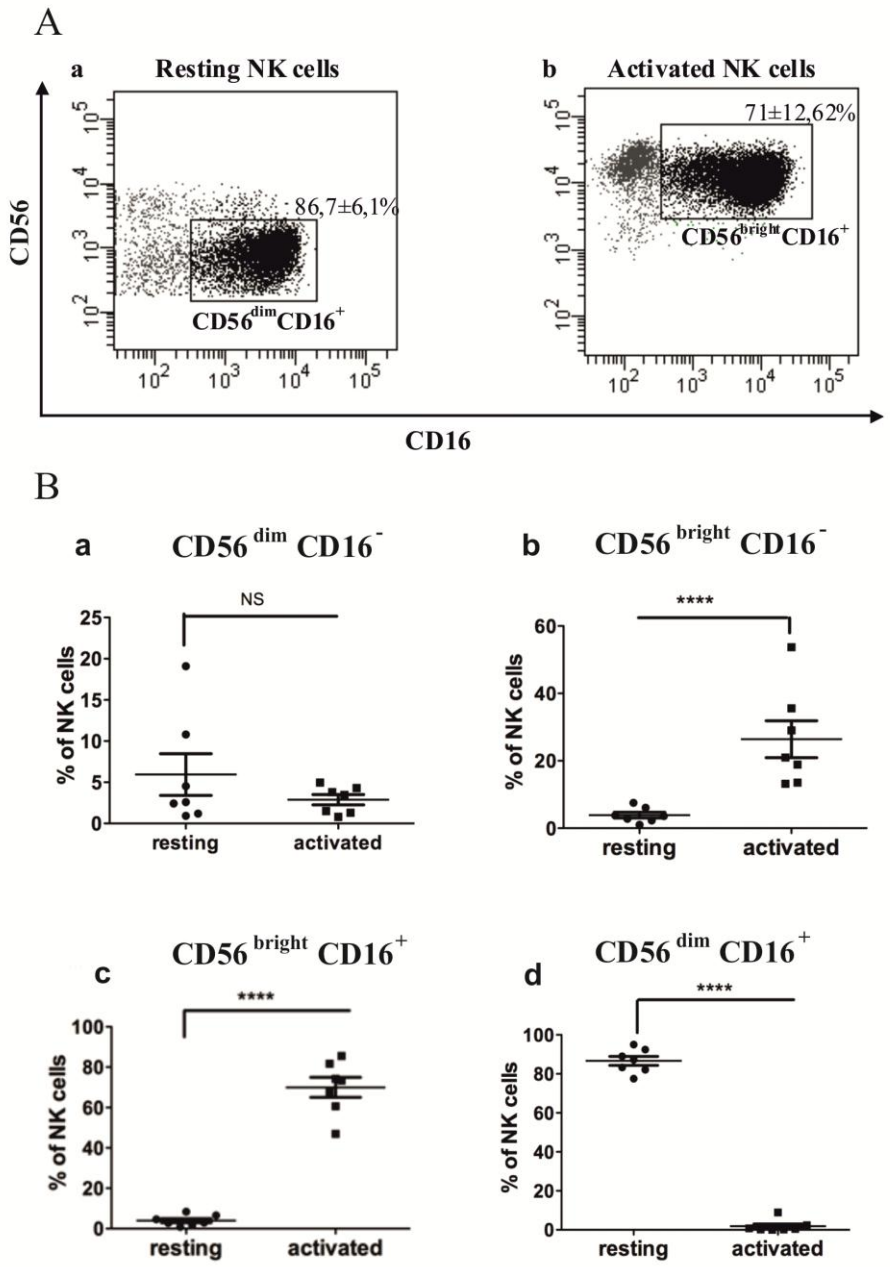
(A) T cells population. Dot plot shows T cells gated as $CD3^+CD14^-$. (B) NK cells population. Dot plot shows NK cells gated as $CD3^-CD56^+$. (C) T cells subpopulations. Dot plot shows CTL gated as $CD8^+$ and Th as $CD4^+$. (A, B, C) Data represents data represents mean±SEM, n=7. (D) Graph shows percentages of T and NK cells in the LAK cells of each donor. ** Two-tailed T-test p= 0.002, n=7.

9.3 NK cells' major subpopulation changed after culture

In order to investigate the effect of culture on NK cell differentiation, flow cytometry based phenotyping analyses of NK cell samples from each donor, before and after being in culture, (resting and activated NK cells, respectively) were performed. We aimed to identify the NK cell subpopulations, receptor expression and their maturation status. Under steady states, NK cells can be divided into four subpopulations based on their expression of CD56 and CD16 receptors: $CD56^{bright}CD16^-$, $CD56^{bright}CD16^+$ (Figure 19, Ab), $CD56^{dim}CD16^-$ and $CD56^{dim}CD16^+$ (Figure 19, Aa) [53, 54].

The major subpopulation of resting NK cells was $CD56^{dim}CD16^+$ (86,7±6,1%, Two-way ANOVA $T_{20.26}$, df=6 p=0.00001; Figure 19, Aa, Bd) while after activation in culture the major subpopulation turned out to be $CD56^{bright}CD16^+$ NK cells (71±12,62%, Two-way ANOVA, $T_{15.77}$, df=6; p=0.00001; Figure 19, Ab, Bc). There was no significant change in the $CD56^{dim}CD16^-$ NK subpopulations based on culture conditions (Two-way ANOVA with Bonferroni posthoc multiple comparisons test, $T_{0.732}$, df=6; p>0.05; Figure 19, Ba) but after activation in culture, the $CD56^{bright}CD16^-$ NK cell subpopulations were significantly enriched (Two-way ANOVA, $T_{5.83}$, df=6; p=0.00001; Figure 19, Bb).

Figure 19: Major subpopulations of resting and activated NK cells.



(A) Dot plot shows CD56 *versus* CD16 expression of resting NK cells. The major subpopulation in resting NK cells is gated: $CD56^{dim}CD16^{+}$. (B) Dot plot shows CD56 *versus* CD16 expression of activated NK cells. Activated NK cells' major subpopulation is gated: $CD56^{bright}CD16^{+}$. (Ba) Percentages of $CD56^{dim}CD16^{-}$ subpopulations in the NK cells of each donor before and after being in culture. (Bb) Percentages of $CD56^{bright}CD16^{-}$ subpopulations in the NK cells of each donor before and after being in culture. (Bc) Percentages of $CD56^{bright}CD16^{+}$ subpopulations in the NK cells of each donor before and after culture. (Bd) Percentages of $CD56^{dim}CD16^{+}$ subpopulations in the NK cells of each donor before and after being in culture. Two-Way ANOVA with Bonferroni multiple comparisons Test, n=7. Data represent mean ±SEM.

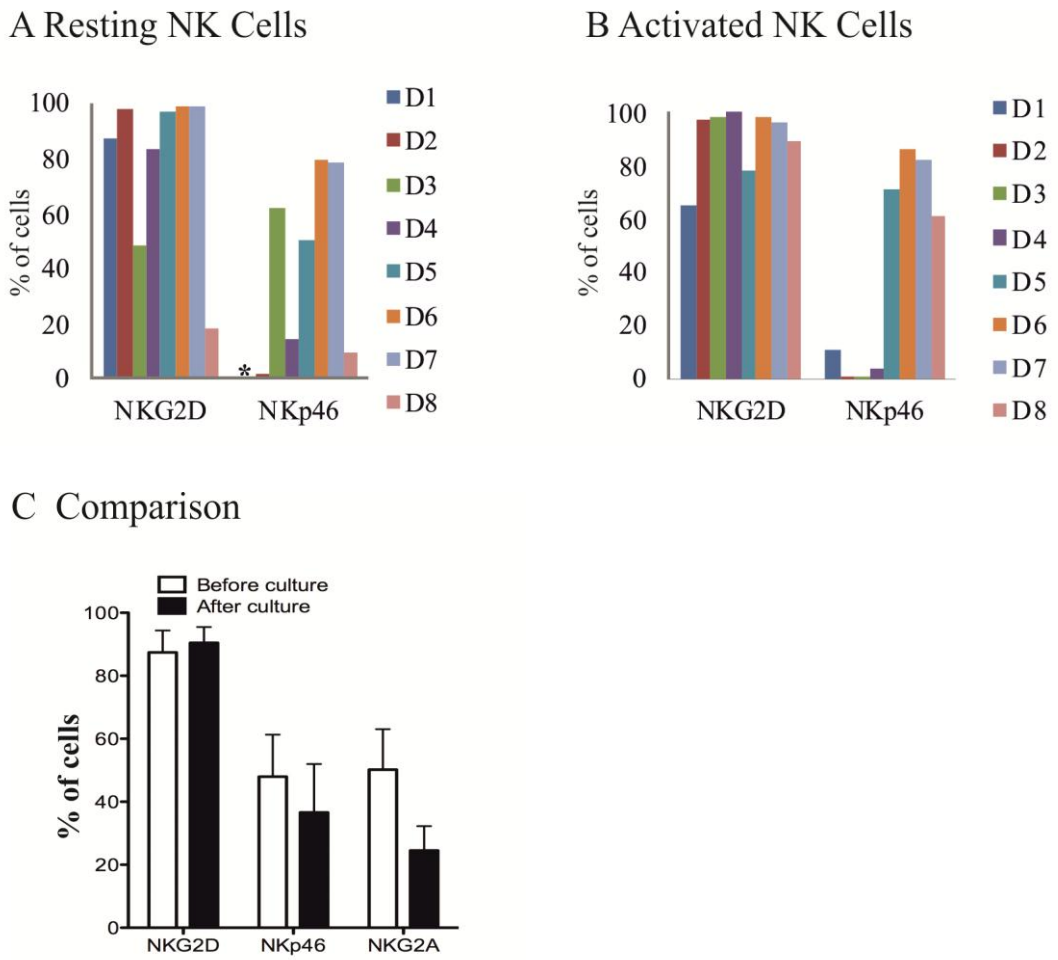
9.4 Dynamic modulation of activating and inhibitory NK cell receptors expression upon culture

9.4.1 Activating and cytotoxic receptors

To investigate whether the differing major subpopulations from resting and activated NK cells were associated with prevalence of activating or inhibitory receptors expressed on the surface, flow cytometry phenotyping analyses were performed. All the receptors showed a donor-dependent variability at steady states and dynamic expression in response to the culture conditions.

The expression of NKG2D and NKp46 in resting and activated states showed donor-dependent variability. Generally NKG2D was highly expressed in both, resting and activated states. Interestingly, donors 3, 4, 5 and 7 with fewer NK cells expressing activating NKG2D receptors during the resting or activated states, up-regulated NKp46 natural cytotoxicity receptors. For example, D3 had fewer NK cells that expressed NKG2D (48,2%) compared to 62,1% that expressed NKp46 in the resting state (Figure 20, A) NKG2D was up-regulated (98,1%) for this donor while NKp46 was radically down-regulated (1,3%) in the activated NK cells (Figure 20, B) compared to resting states. In contrast, 97% of D5 resting NK cells expressed NKG2D while 50,5% expressed NKp46 (Figure 20, A). The number of NK cells expressing NKG2D was down-regulated (78%) for D5 while those expressing NKp46 were up-regulated to 71,1% after culture (Figure 20, B). These results might indicate that the expression of activating NKG2D receptor equilibrates to the expression of other cytotoxicity receptors, such as NKp46.

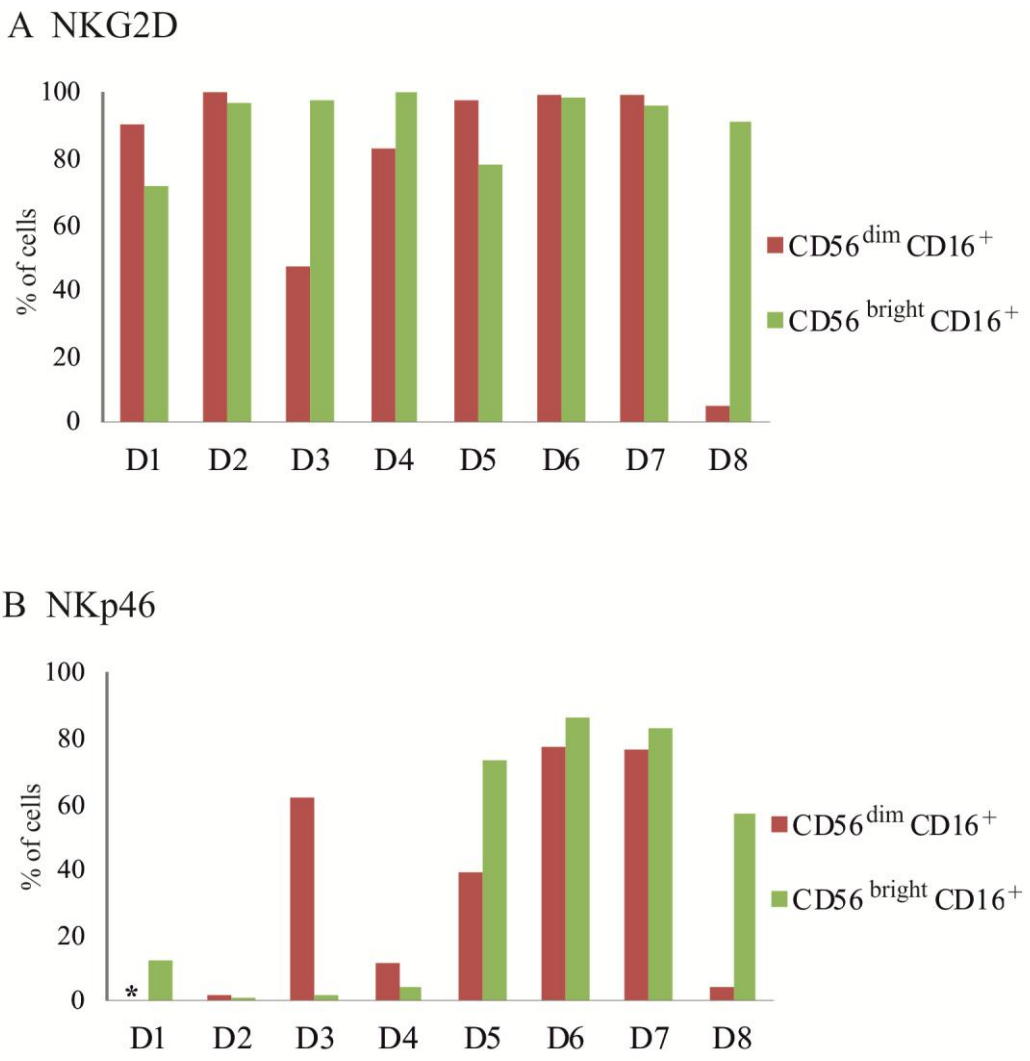
Figure 20: Resting and activated NK cells activating receptors expression (% of cells).



(A) Bar graph shows resting NK cells activating receptors expression of each donor. The asterisk (*) points that NKp46 was not phenotyped for resting NK cells from D1. (B) Bar graph shows activated NK cells activating receptors expression of each donor. (C) Comparison of inhibitory (before culture) and activated (after culture) NK cell expression of NKG2D, NKp46 and NKG2A.

The expression of NKG2D and NKp46 in the major subpopulations of both resting and activated states showed donor-dependent variability. Major subpopulations of both resting and activated NK cells had mostly high number of cells expressing NKG2D. NKp46 expression in the major subpopulations of both resting and activated states did not showed any clear tendency (Figure 21).

Figure 21: Resting and activated NK cells major subpopulations activating receptors expression (% of cells).



(A) Bar graph shows NKG2D expression of the major subpopulations of resting and activated NK cells, $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{+}$, respectively, of each donor. (B) Bar graph shows NKp46 expression of the major subpopulation of resting and activated NK cells of each donor. The asterisk (*) points that NKp46 was not phenotyped for resting NK cells from D1.

9.4.2 Inhibitory receptors

The expression of NKG2A inhibitory receptor by resting NK cells was greater than 65% of cells expressing it in 4 out of 7 donors while it was less than 31% in the other 3 donors. For D4, D6, D7 and D8 it was 77,3%; 90,5%; 93,4% and 69% respectively, while it was 16,3%; 30,5% and 23,7% for D2, D3 and D5, respectively (Figure 22, A). Upon activation, 6 out of 7

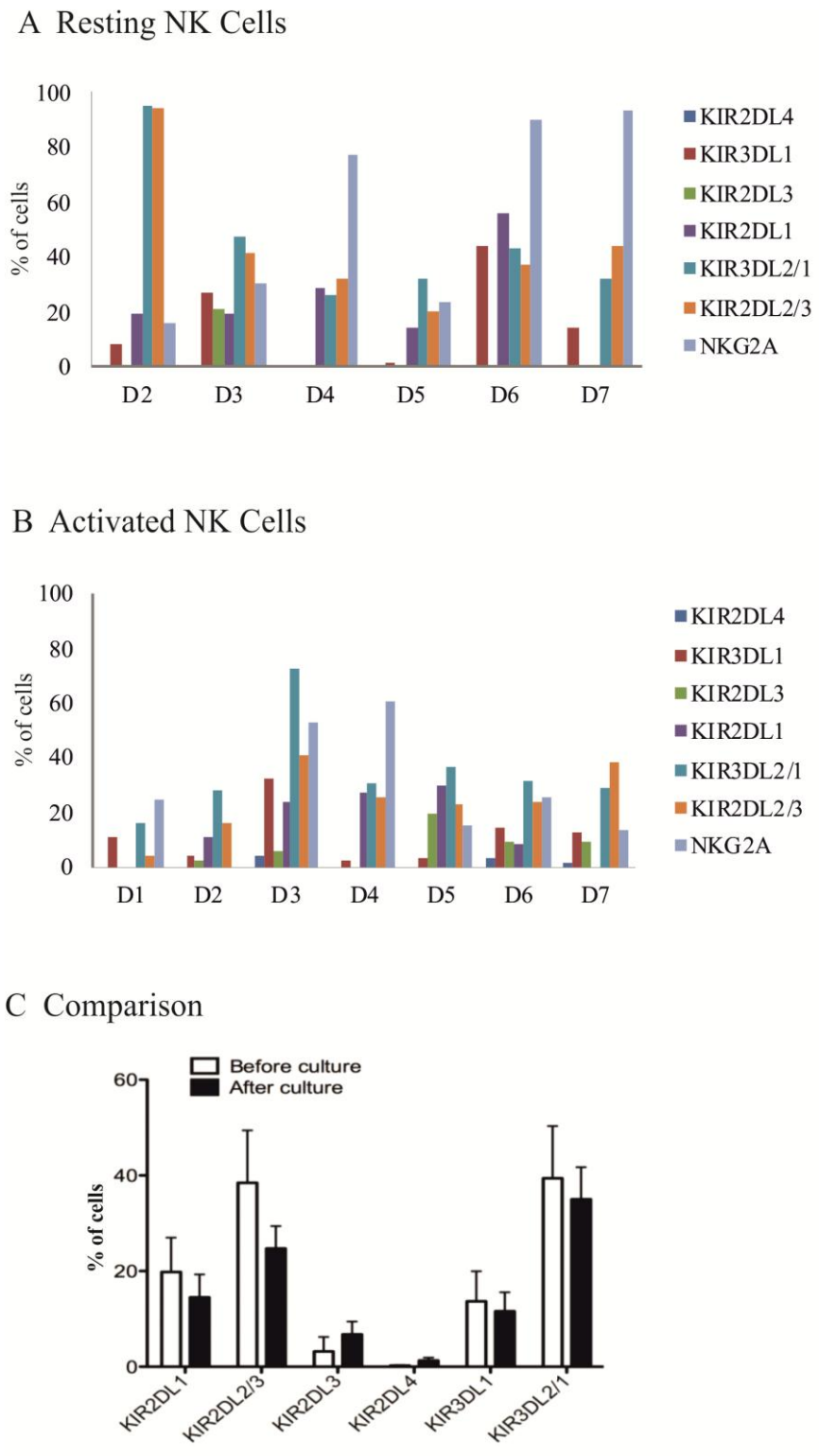
donors down-regulated it and only NK cells from D3 up-regulated NKG2A to 52,7% after culture (Figure 22, B). Taken together, there was no significant difference ($p>0.05$) in the % cells expressing these receptors before and after culture (Figure 20, C).

We investigated the expression of the inhibitory KIR receptors on resting compared to activated NK cells, (Figure 22, A and B). As expected, donor-dependent variability in the expression of the inhibitory KIR receptors was observed. However, in the majority of donors, less than 50% of their NK cells expressed KIR receptors on the surface, with the exception of D2, where 94,6% and 95,2%, of resting NK cells expressed KIR2DL2/3 and KIR3DL2/1, respectively, and 56,1% of D6's resting NK cells expressed KIR2DL1 (Figure 22, A). In addition, expression of KIR2DL4 was negligible in all donors' regardless of their activation status (Figure 22, B).

The expression of inhibitory KIR receptors was modulated in different ways depending on the donor and activation status. For example resting NK cells (Figure 22, A) from D2 expressing inhibitory KIR receptors were: 8,7 % KIR3DL1, 0,6% KIR2DL3, 19,1% KIR2DL1, 95,2% KIR3DL2 and 94,6% KIR2DL2 while activated NK cells (Figure 22, B) down-regulated KIR3DL1 (4,2%), KIR2DL1 (10,9%), KIR3DL2/1 (28,4%) and KIR2DL2/3 (16,5%) and up-regulated KIR2DL3 (2,6%). On the other hand, in D3 the percentage of resting NK cells (Figure 22, Ab) expressing each of the KIRs were 26,6% KIR3DL1, 21,3% KIR2DL3, 19,7% KIR2DL1, 47,4% KIR3DL2/1 and 41,2% KIR2DL2/3 while activated NK cells (Figure 22, Bb) down-regulated KIR2DL3 expression to 5,8% and up-regulated KIR2DL4 (4,1%), KIR3DL1 (32,8%), KIR2DL1 (24%), KIR3DL2/1 (72,5%) and KIR2DL2/3 (38%).

Taken together, there was no significant difference ($p>0.05$) in the expression of the KIR receptors comparing resting and activated NK cells (Figure 22, C).

Figure 22: Resting and activated NK cells inhibitory receptors expression (% of cells).



(A) Bar graph shows inhibitory receptors expression on resting NK cells of each donor. (B) Bar graph shows inhibitory receptors expression on activating NK cells of each donor. (C) Comparison of inhibitory KIR receptors average expression in resting and activated NK cells.

9.5 LAK cells receptors expression

Next we investigated the impact of culture on receptor expression within the LAK cell subpopulations and aimed to determine if similar receptors were expressed as in the purified cells. There was a donor-dependent variability in the expression of receptors and in response to the culture conditions.

9.5.1 Receptors expression

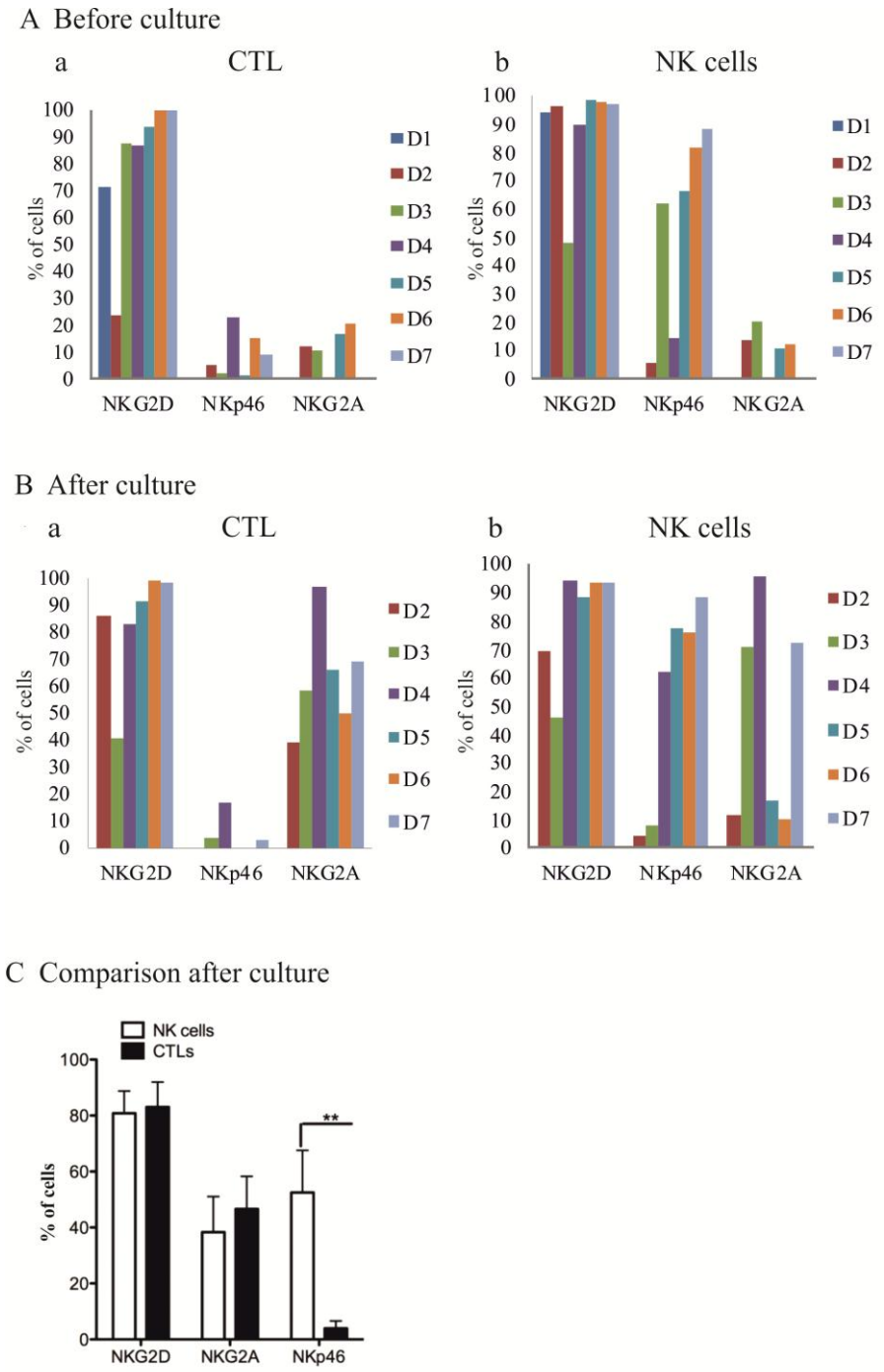
Interestingly, within the LAK cells from donors 2, 4, 5, 6 and 7, the CTL subpopulations up-regulated NKG2D after being in culture (Figure 23, Aa and Ba), while the NK cell subpopulations highly expressed it regardless of culture conditions, with the exception of D2 that moderately down-regulated NKG2D after culture (Figure 23, Ab and Bb).

Importantly, the NKp46 natural cytotoxicity receptor was expressed in the major number of NK cells than CTL. All the donors' NK populations expressed NKp46, where 4 out of 7 donors had more than 60% of cells expressing it after being in culture (Figure 23, Bb) while all 7 donors' CTL populations had less than 10% of CTL expressing it after culture. Six out of those 7 donors' CTL had less than 3% of cells expressing NKp46 (Figure 23, Ba) with no difference between the expression in both resting and activate state.

The expression of NKG2A inhibitory receptor was similar in the CTL and NK cell components of LAK cells, both before and after culture. CTL cells from donors D2, D3, D5 and D6 had 4,2%, 9,5%, 8,8% and 14,9% of cells expressing NKG2A, respectively (Figure 23, Aa), while NK cells from corresponding donors expressing it were 13,8%, 20,3%, 11% and 12%, respectively, (Figure 23, Ab), before culture. D4 and D7 did not express NKG2A before culture for any of both populations. However, all the donors' CTLs highly up-regulate NKG2A expression, being 30,1%; 58,3%; 96,9%; 65,8%; 49,7% and 68,8% from D2 to D7, respectively (Figure 23, Ba), while 4 out of 6 donors' NK cells up-regulate it and the other 2 down-regulate it. NK cells expressing NKG2A after culture were 70,7%; 95,9%; 16,4% 72,5% of cells from D3, D4, D5 and D7, respectively, which up-regulated its expression and 11,6% of D2 and 10,4% of D6, that down-regulate NKG2A expression (Figure 23, Bb).

Collectively, the only receptor that was significantly differentially expressed between the NK cell and CTL subgroups of LAK cells was NKp46 (Two-Way ANOVA, $T_{3.82}$, df=1; p=0.001, Figure 23, C). This is consistent with this receptor being a distinguishing marker for NK cells.

Figure 23: LAK cells' CTL and NK cells receptors expression.



(A) Graphs of expressions before culture. (Aa) Bar graph shows expression of NKG2D, NKp46 and NKG2A on CTLs of each donor before culture. (Ab) Bar graph shows expression of NKG2D, NKp46 and NKG2A on NK cells of each donor before culture. (B) Graphs of expressions after culture. (Ba) Bar graph shows expression of NKG2D, NKp46 and NKG2A on CTLs of each donor after culture. (Bb) Bar graph shows expression of NKG2D, NKp46 and NKG2A on NK cells of each donor after culture. (C) Bar graph shows a comparison of the average of the number of CTL and NK cells expressing each receptor after culture from all the donors.

9.5.1.1 KIR receptors expression

We hypothesised that the CTL and NK cellular subgroups within the LAK cells might differentially express the killer immunoglobulin-like receptors (KIRs). Thus, we investigated their expression.

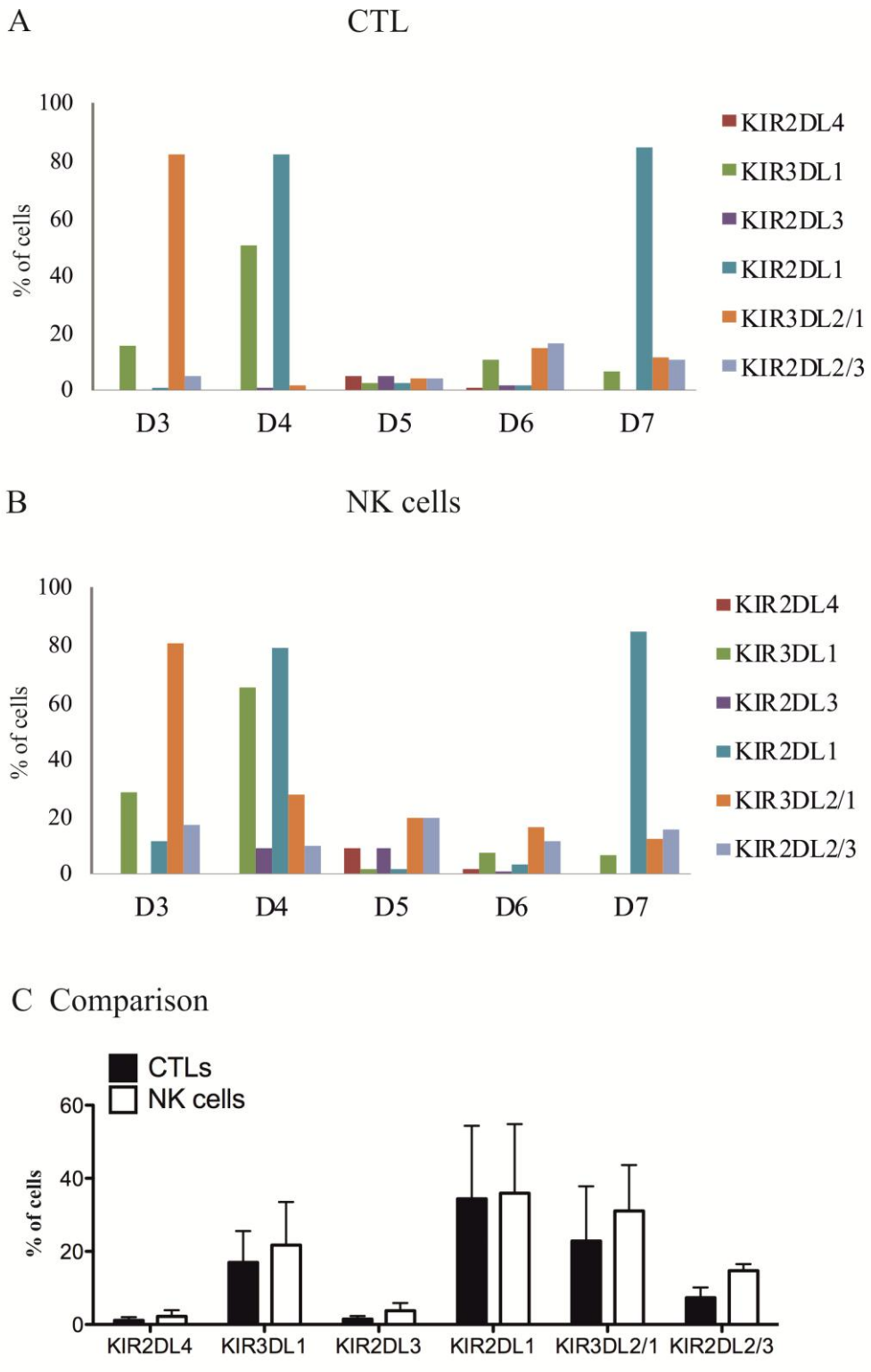
Interestingly, most of the KIR receptors examined were not highly expressed. Both CTLs and NK cells in some donors expressed KIR2DL4, KIR3DL1 KIR2DL3, KIR2DL1, KIR2DL2/1 and KIR2DL2/3, albeit in reduced percentage of cells (between 3,5% and 29%). The percentages of cells expressing those KIRs were mostly higher in NK than in CTL cells. Hence, as examples D3 expressed KIR3DL1 in 14,1% of CTLs and 28,5% of NK cells and D4 expressed KIR3DL2/1 in 4,4% of CTLs and 27,5% of NK cells (Figure 24, A and B).

Despite this low percentage of cells expressing KIRs, some KIR receptors in donors 3, 4 and 7 were expressed by more than 50% of cells and with differences between CTL and NK cells. D3 had 82,1% of CTLs and 80,3% of NK cells expressing KIR3DL2/1, D4 had 50,3% and 82% of CTLs and 65,2% and 79% of NK cells expressing KIR3DL1 and KIR2DL1 respectively, and D7 had 84,7% of CTLs and 84,9% of NK cells expressing KIR2DL1 (Figure 24, A and B).

Although the % of NK and CTLs cellular subgroups within the LAK cells that expressed KIRs varied in the individual donors, there were no significant differences between these cell types, $p>0.05$ (Figure 24, C). This is consistent with the fact that these are germline-encoded receptors.

Taken together, KIRs expression within LAKs was donor dependent and no significant difference was found when comparing LAK subpopulations. However KIR3DL1, KIR3DL2 and KIR2DL1 were the only KIRs expressed by a large number of cells in some of the donors.

Figure 24: LAK cells' CTL and NK cells inhibitory KIR receptors expression.



(A) Bar graph shows CTL inhibitory KIR receptors expression after culture of PBMCs of each donor. (B) Bar graph shows NK cells inhibitory KIR receptors expression after culture of PBMCs of each donor. (C) Bar graph shows a comparison of the average of the number of CTL and NK cells expressing each KIR receptor from all the donors' LAK cells.

9.6 GBM cells markers and ligands expression

To functionally investigate the cytotoxic efficacy of LAK cells compared to purified NK cells, we established GBM primary cultures from patient GBM biopsies obtained from surgical resections at Haukeland University Hospital. Several studies have demonstrated that GBM cells propagated in serum free stem cell medium as "stem-like" cells are more susceptible to NK cell lysis compared to those cultured under serum containing conditions. Stem cell-like cells are characterised by expression of markers of immature cell types. Therefore, to verify that our primary GBM cultures fulfilled the criteria for stem-like cells, we phenotyped them for various markers of immature cell types.

This stem-like expression was verified with the phenotyping of the stem-like markers expression in standard cell lines (U87 and U373) propagated in serum containing medium (Appendix I, Table 14).

9.6.1 CNS cell differentiation markers

Glial fibrillary acidic protein (GFAP), nestin and vimentin are intermediate filaments (IF) expressed at various differentiation states of neural stem cells (NSC). They can be co-expressed in a cell and their expression has been reported in GBMs [23-27].

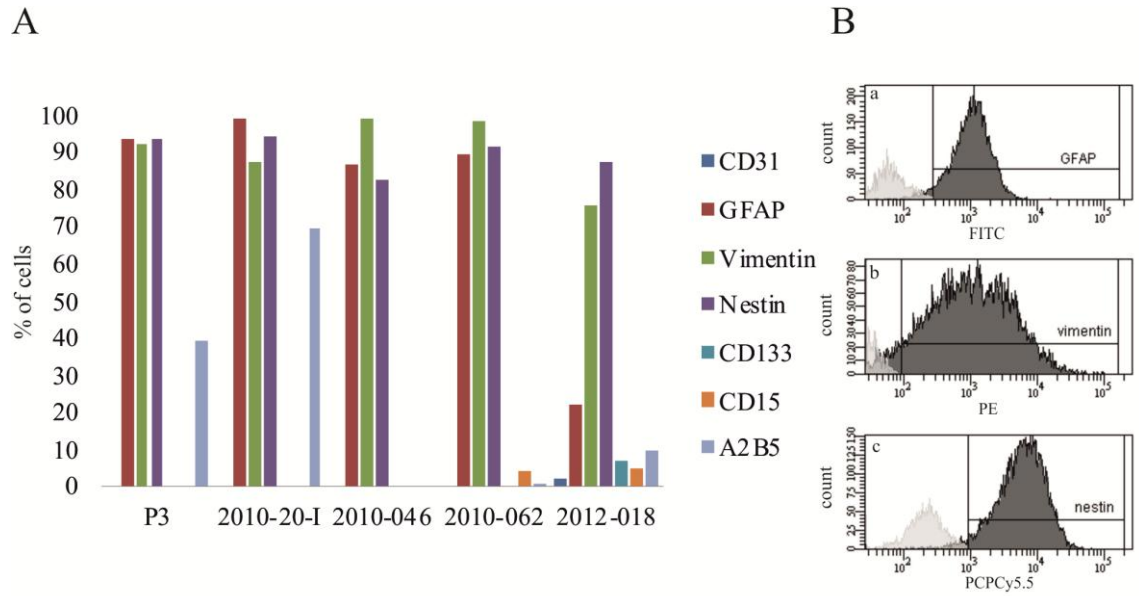
As expected, we confirmed high expression of nestin, vimentin and GFAP in all the patient derived-GBM we used (Figure 25 and Appendix I, table 14). However, fewer 2012-018 gliosarcoma cells expressed GFAP (21,8%, Figure 25 and Appendix I, Table 14). 89,6±4,96% of cells expressed nestin, 90,28±9,63% of cells expressed vimentin and 91,9±5,36% of cells expressed GFAP (2012-018 gliosarcoma cells expressing GFAP is not included in this average).

A2B5 is an antibody that recognises several GT3 gangliosides, expressed in early differentiation state of GSC (Glioma stem cells). A2B5 antigens were expressed by 39,1% of P3 cells, 69,1% of 2010-20-I cells and 9,6% of 2012-018 cells (Figure 25 and Appendix I, Table 14). None of the cell lines but 2012-018 (6,8% of cells) expressed CD133, a surface glycoprotein expressed on NSC and a marker for cancer stem cells (Figure 25 and Appendix I, Table 14). CD15 is a glycoprotein, which enriched expression has been reported in GBM [29]. None of the GBMs expressed it significantly, P3 and 2010-20-I did not expressed it at

all while 3,8% of cells of 2010-062 and 4,9% of 2012-018 expressed it (Figure 25 and Appendix I, Table 14).

As negative control, we stained the GBM cells for irrelevant marker for endothelial cells and immune cells CD31 [97]. As expected none of the cells expressed it significantly, CD31 was expressed by only 1,8% of 2012-018 GBM cells consistent with the fact that gliosarcoma component has vascular origin [10] (Figure 25 and Appendix I, Table 14).

Figure 25: Different GBMs' CNS cell differentiation markers expression.



FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, PCPCy5.5: Phencyclidine-Cyanine5.5 tandem fluorochrome

(A) Bar graph shows the percentage of cells of the different GBMs expressing each CNS cell differentiation marker. CD31 is an epithelial marker used as a control. (B) Histograms show expression of the 3 intermediated filaments in P3, as an example of positive expression gating. Light grey represents the signal of the negative control for this GBM cell line in each channel of detection while dark grey represents its signal obtained after staining. (Ba) Signal obtained for P3 stained with anti-GFAP-FITC. (Bb) Signal obtained for P3 stained with anti-vimentin-PE. (Bc) Signal obtained for P3 stained with anti-nestin-PCPCy5.5.

9.6.2 GBM expression of ligands for NK cells receptors

Since NK cell cytotoxicity against cancer cells is mediated via the overall balance of activating *versus* inhibitory signals transmitted via ligation of the receptors on NK cells to

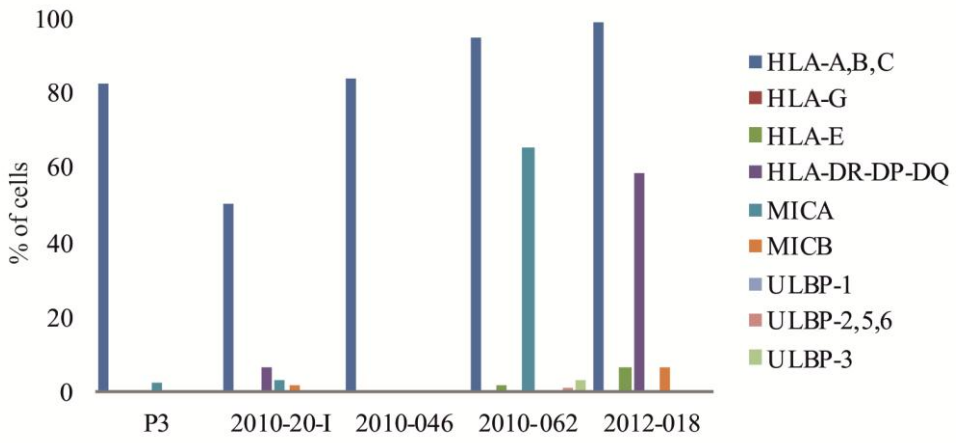
their cognate ligands on the target cancer cells, we aimed to establish the expression levels of the various ligands for NK cell receptors by the GBM cells.

Human leukocyte antigen A, -B, and -C (HLA-A,B,C) that are ligands for the inhibitory KIR receptors were expressed by the majority of GBM cell lines with 89,98±8,09% of the cells expressed these ligands in 4 out of 5 cell lines, while the other cell line, 2010-20-I, had 50,3% of GBM cells expressing HLA A,B,C (Figure 26 and Appendix I, Table 14).

While 4 out of 5 cell lines did not express any of the HLA class II molecules, 58,3% of 2012-018 cells expressed HLA-DR-DP-DQ, ligand for the TCR of Th cells (Figure 26 and Appendix I, Table 14). None of the GBM cell lines expressed the non-classical class I HLA-G that is the ligand for KIR2DL4 and only 6,4% and 1,7% of 2012-018 and 2010-062 cells, respectively, expressed HLA-E, ligand for CD94/NKG2A inhibitory receptor (Figure 26 and Appendix I, Table 14).

MIC-A, MIC-B, ULBP-1, ULBP-2,5,6 and ULBP-3 that are known ligands for the activating receptor NKG2D were expressed in very small proportion of cells, with only one exception, 2010-062, where 65,4% of the GBM cells expressed MIC-A (Figure 26 and Appendix I, Table 14). MIC-A was also expressed by very few cells of P3 and 2010-20-I, 2,5 and 3,2%, respectively. MIC-B was expressed by only 6,4 and 1,4% of cells of 2012-018 and 2010-20-I, respectively, while only 1,2 and 2,9% of 2010-062 GBM cells expressed, respectively, ULBP-2,5,6 and ULBP-3 NKG2D ligands (Figure 26 and Appendix I, Table 14).

Figure 26: Different GBMs' ligands expression.



HLA: Human Leukocyte Antigen, MICA/B: MHC class-I-chain-related protein A/B, ULBP: UL16 binding protein.

Bar graph shows the percentage of cells expressing each ligand for the NK cell receptor in the different GBM cells.

9.7 Cytotoxicity assays

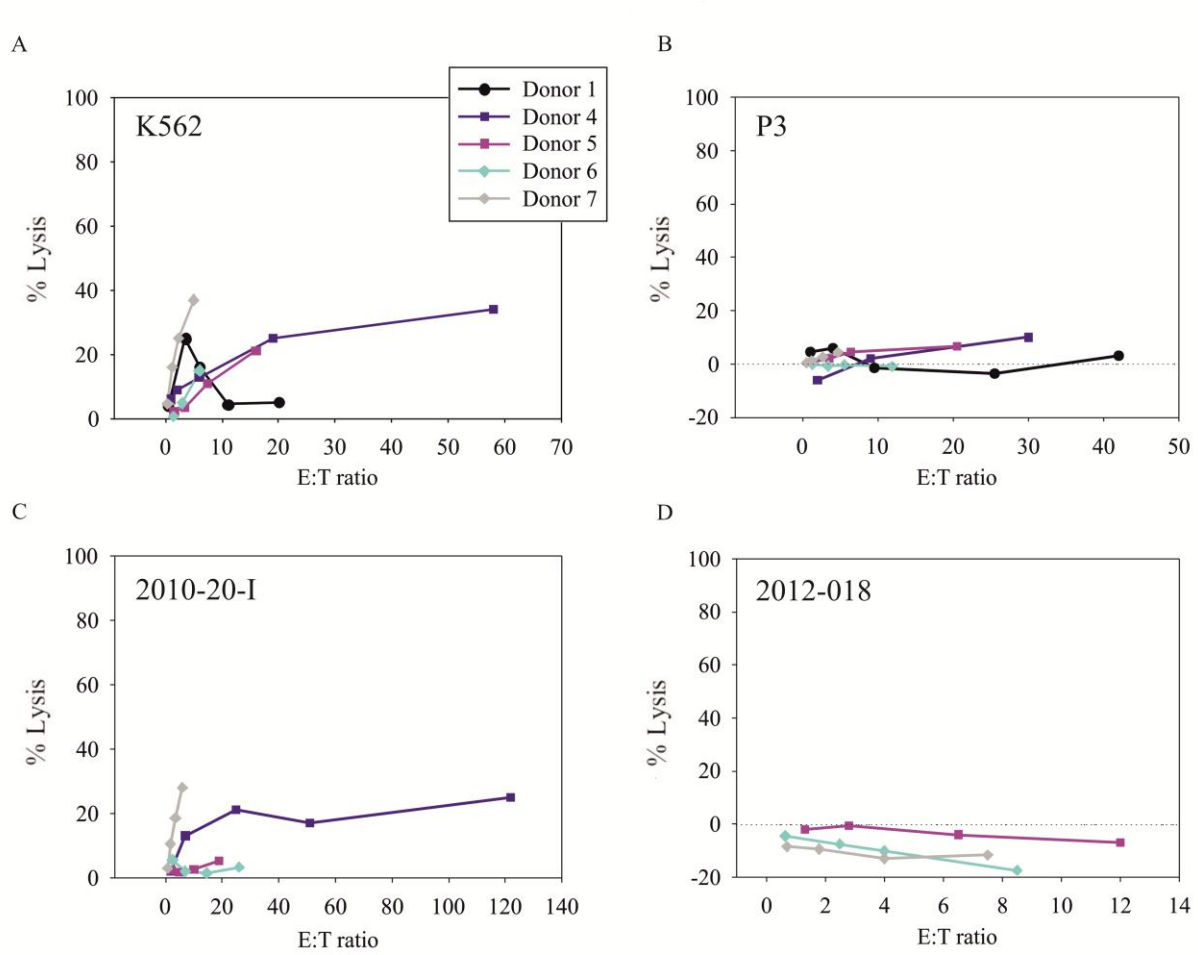
Since adoptive cellular immunotherapy preclinical and clinical trials for GBM has predominantly administered LAK cells [98], we hypothesised that NK cells might be more potent effectors against GBM. Thus, we investigated the cytotoxicity efficacy of LAK cells compared to NK cells against patient-derived GBM cells *in vitro*. We also aimed to determine the cytotoxic efficacy of resting NK cells compared with activated NK cells. Thus cytotoxicity assays using LAK cells, resting or activated NK from the same donor in a cohort of (n=8) donors against GBM cells (n=5) were performed. P3, 2010-046, 2010-062, 2010-20-I and 2012-018 GBM cell lines were used for this purpose (Table 4 in 8.4.4 section). Cytotoxicity assays with the same effector cells were performed against U87 and U373 cells grown in serum containing medium (Appendix II, Figure 35).

9.7.1 Resting NK cells cytotoxic effect

We hypothesised that NK cells required to be activated to have an efficient cytolytic effect against cancer cells. Therefore, we performed cytotoxicity assays of resting NK cells against the GBMs and K562.

While purified resting NK cells exhibited donor-dependent cytotoxicity against the control cell line, K562, and 2010-20-I GBM cells (Figure 27, A and C, respectively), none of the donors' NK cells efficiently killed P3 and 2012-018 cell lines (Figure 27, B and D, respectively). Importantly, only D4 and D7 derived NK cells demonstrated moderate cytotoxicity effect against both control (Figure 27, A) and 2010-20-I (Figure 27, C) cell lines and D5 also demonstrated moderate cytotoxicity effect only against the control cell line (Figure 27, A). These experiments confirmed our hypothesis showing low or none cytolytic effectiveness of resting NK cells against GBM.

Figure 27: *In vitro* cytotoxicity effect of resting NK cells from different donors against GBM cells.



(A) Resting NK cells cytotoxicity effect against K562. (B) Resting NK cells cytotoxicity effect against P3. (C) Resting NK cells cytotoxicity effect against 2010-20-I. (D) Resting NK cells cytotoxicity effect against 2012-018.

9.7.2 Activated NK cells cytotoxic effect

After demonstrating that resting NK cells are not an effective cytolytic tool against GBM, we to further investigates the hypothesis that NK cells required to be activated to effectively kill cancer cell. Thus, cytotoxicity assays of activated NK cells against the GBMs and K562 cells were executed. We also hypothesised that GBM cells lacking ligands for the inhibitory KIRs present in the donor (inhibitory **KIR receptor-HLA ligand mismatch**) or presenting ligands for the activating donors (activating **KIR receptor-HLA ligand** match) would be killed more efficiently by the NK cells of that donor. To investigate whether the KIR receptor-HLA ligand match/mismatch mediate NK cells' cytotoxic potency against GBM, we examined KIR

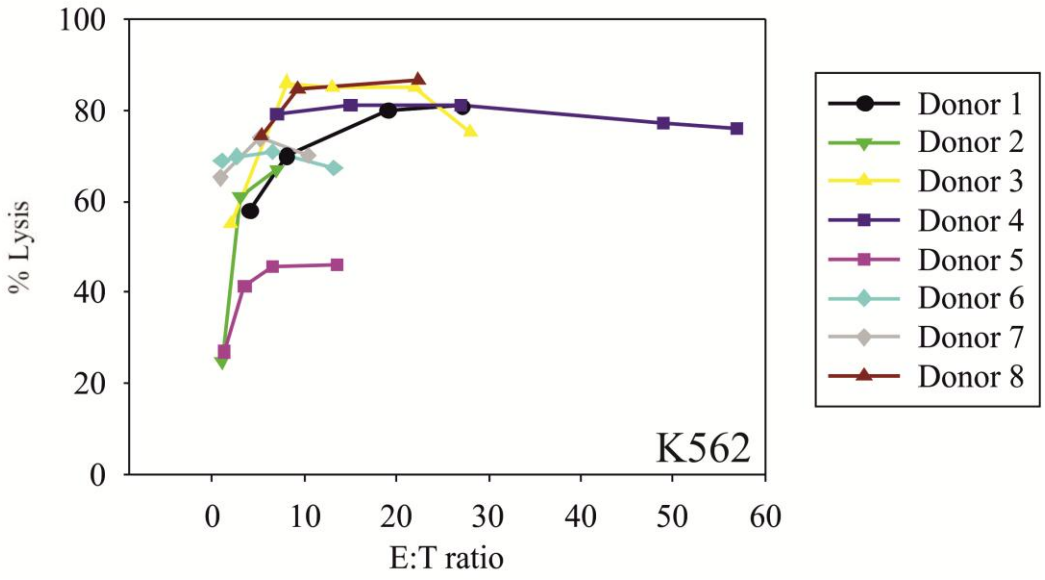
receptor and HLA ligand genotype and established tables with the mismatch relation between all the donors with the different patient-derived GBM we used.

9.7.2.1 NK cells cytotoxicity against K562

K562 is a chronic myelogenous leukaemia (CML) cell line with a Philadelphia chromosome, no presence of EBV and herpes-like virus and lack of HLA expression [90]. Since HLA are the major ligands responsible for the inhibitory signalling of NK cells and K562 cells lack them, NK cells easily kill K562 cells. Therefore K562 cells have been established as a positive control for the NK cells cytotoxicity. With the same purpose, cytotoxicity assays of activated NK cells derived from all the donors against K562 cells were performed.

All the donors' activated NK cells exhibited donor-dependent cytotoxic efficacy against K562, where all the effectors lysed greater than 60% of the CML cells at E:T ratios over 5 with the exception of D5's activated NK cells which lysed 40% of K562 cells (Figure 28). Donors' D3, D4 and D8 activated NK cells were the most cytotoxic against K562 control cell line with 80% lysed cells (Figure 28). The high cytotoxic effect that the donors showed indicates that all the donors had the ability of being cytotoxic.

Figure 28: *In vitro* cytotoxicity effect of activated NK cells from different donors against K562 cells.



9.7.2.2 NK cells cytotoxicity against P3

We observed that activated NK cells from D2, D3, D5, D6, D7 and D8 effectively lysed 35-55% P3 GBM cells at E:T ratios under 10 (Figure 29, B), with the exception of activated NK cells from D1 and D4, that were the least cytotoxic (between 15 and 20% lysis at E:T ratio 5, Figure 29, B). In addition, D2 and D5 NK cells had intermediate cytotoxicity of approximately 35% lysis at E:T ratio 5 (Figure 29, B).

To study the hypothesis of the KIR receptor-HLA ligand mismatch as mediator of the NK cells' cytotoxic potency against GBM, KIR-HLA mismatch pattern was correlated to the cytotoxicity results (Figure 29, A and B). No correlation between inhibitory KIR-HLA ligand mismatch and cytotoxicity efficacy was observed.

All the donors had mismatch for KIR3DL2 and KIR2DL4 where the P3 GBM cells lacked their cognate ligands A3/A11 and HLA-G, respectively (Table 29, A). Out of the donors that had greater cytotoxicity against P3 (Figure 29, B), which are D3, D6 and D7, only D3 did not have additional mismatch patterns besides those already named above (Table 29, A). D6 lacked KIR2DL5 and D7 lacked KIR2DL1, KIR2DL3 and KIR2DL5 (Table 29, A). Despite those differences in the inhibitory mismatch in these 3 donors, some similarities were found in terms of activating KIR receptor matches. All 3 highly cytotoxic donors had KIR2DS4 and KIR2DS2 but lacked of KIR2DS1 and KIR2DS5. D3's effectors had KIR2DS3 genes in addition (Figure 29, A). Interestingly, D2 and D5, that exhibited intermediate cytotoxic efficacy, showed presence of KIR2DS4 and lack of KIR2DS2 and KIR2DL2 genes. D1's and D4's effectors that had lowest cytotoxicity, also lacked KIR2DS2 and KIR2DL2 but showed presence of KIR2DS1 genes. D4's effectors also had KIR2DS3 in the genome (Figure 29, A). On the other hand D8, which had match in all the KIR receptors with known ligands and presented also the genes for KIR2DS1, KIR2DS4, KIR2DS2 and KIR2DS3, had also lowest cytotoxicity (Figure 29, A and B).

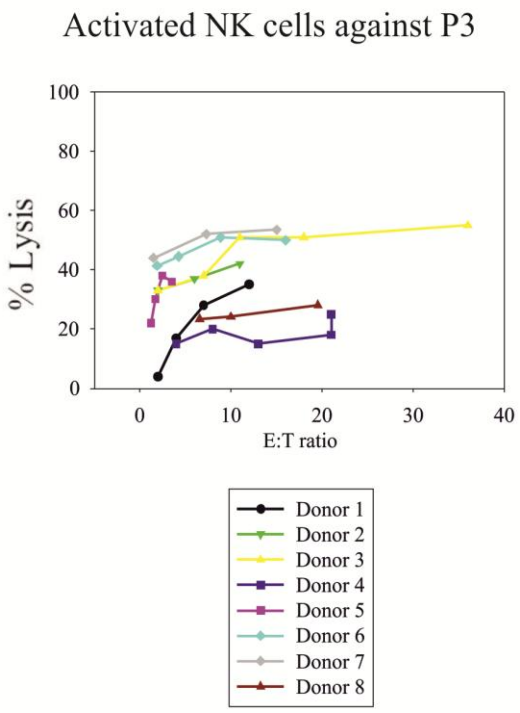
Taken together, we concluded that an important role of the activating receptors KIR2DS2 and KIR2DS4 might determine potency of activated NK cells against P3 GBM. Although the ligands for KIR2DS2 are unknown to the moment, 3 out of 4 donors that have its genes had higher cytotoxicity, what may indicate that P3 probably express the cognate ligand for that KIR.

Figure 29: *In vitro* cytotoxicity effect of activated NK cells against P3 cells and KIR receptors and cognate HLA ligands genotyping table.

A

KIR Receptors		P3 HLA Ligands	Donors							
			1	2	3	4	5	6	7	8
Inhibitory	KIR2DL1	C*05								
	KIR2DL2	C*0501								
		C*01								
	KIR2DL3	C*0501								
		C*01								
	KIR3DL1	B*4402								
		B*2705								
	KIR3DL2	A*03⁻/ A*11⁻								
	KIR2DL4	HLA-G⁻								
	KIR3DL3	Unknown								
	KIR2DL5	Unknown								
Activating	KIR2DS1	C*05								
	KIR2DS2	unknown								
	KIR2DS4	C*0501								
	KIR2DS3	C*01								
	KIR2DS5	unknown								
	KIR3DS1	unknown								

B



KIR: killer immunoglobulin-like receptor; HLA: Human Leukocyte Antigen

(A) KIR-HLA genotyping mismatch of each donor with P3. Red represents match (presence of both KIR gene on the effector cells and cognate HLA ligand gene on the tumour cells), green represents mismatch (lack of KIR gene on effector cells and/or HLA ligand gene on the tumour cells) and white represents unknown match/mismatch. Only HLA ligands present in the GBMs of this study are represented in the tables. (⁻) Superscript in HLA ligands indicates lack of gene for that particular ligand in the GBM. (B) *In vitro* cytotoxicity effect of activated NK cells from different donors against P3 cells.

9.7.2.3 NK cells cytotoxicity against 2010-20-I

To test the hypothesis that KIR2DS2 and KIR2DS4 determine the cytotoxic potency of NK cells against other GBM cells, we investigated the association of these activating receptors with NK cytotoxic potency against 2010-20-I GBM cells. Only activated NK cells from donors D3, D4 and D7 were efficient against the 2010-20-I GBM cells, with 60% cell lysis at E:T ratio 10 (Figure 30, B). D5 and D6 had very low cytotoxicity (around 10% cell lysis in E:T ratios near 10). D1's effectors were not able to lyse 2010-20-I GBM cells at all (Figure 30, B).

When examining the KIR and their cognate ligand mismatch, no correlation between inhibitory receptors' mismatch pattern and cytotoxicity efficacy was observed. All the donors had mismatch for KIR3DL2 and KIR2DL4 genes where the 2010-20-I GBM cells lacked their cognate ligands A3/A11 and HLA-G genes, respectively (Figure 30, A).

D3, D4 and D7's effectors that had greater cytotoxicity against 2010-20-I, had different mismatch patterns, D3 had no mismatch, D4 lacked KIR2DL2 and KIR3DL1, and D7 lacked KIR2DL1, KIR2DL3 and KIR2DL5 (Table 30, A). Despite these differences in the inhibitory receptor mismatch, similarities were discovered in terms of activating receptor matches. D3 and D7's effectors had KIR2DS2 and KIR2DS4 genes but lacked of KIR2DS1 and KIR2DS5, while D4's effectors had KIR2DS1 but lacked KIR2DS2 and KIR2DS4. D3 and D4's effectors also had KIR2DS3 (Table 30, A).

D5 and D6's effectors exhibited intermediate cytotoxic efficacy (Table 30, B). D5 had KIR2DS1 gene while lacked for the gene of the inhibitory KIR2DL2 and D6 did not have any extra inhibitory mismatch but had the genes of the activating KIR2DS2. Both donors also had KIR2DS4 genes (Figure 30, A). D1's effectors had the same inhibitory KIR receptor mismatch and activating KIR receptor-HLA ligand matches as D5's effectors with the exception that D1 also lacked KIR2DL5. For D5's effectors, it was not known the mismatch state of KIR2DS5 and KIR3DS1, because their ligands have not been discovered yet, while they were lacking in D1 (Figure 30, A).

To summarize, highly cytotoxic NK cells derived from donors D3 and D7 possess KIR2DS2 and KIR2DS4 genes. D4's NK cells that are also highly cytotoxic to 2010-20-I do not have KIR2DS2 and KIR2DS4 genes, but have mismatch in inhibitory KIR2DL2 and KIR3DL1, where donors D3 and D7 do not have these KIR-HLA mismatches. Donor D4 had also additional activating KIR through the presence of KIR2DS1 and KIR2DS3. The potency of D3 and D7 may be determined by KIR2DS2 and KIR2DS4 activating KIR-HLA ligand matches, whereas activating signals obtained from KIR2DS1, KIR2DS3, KIR2DS5 and KIR3DS1 might cause the cytotoxic potency of D4's NK cells.

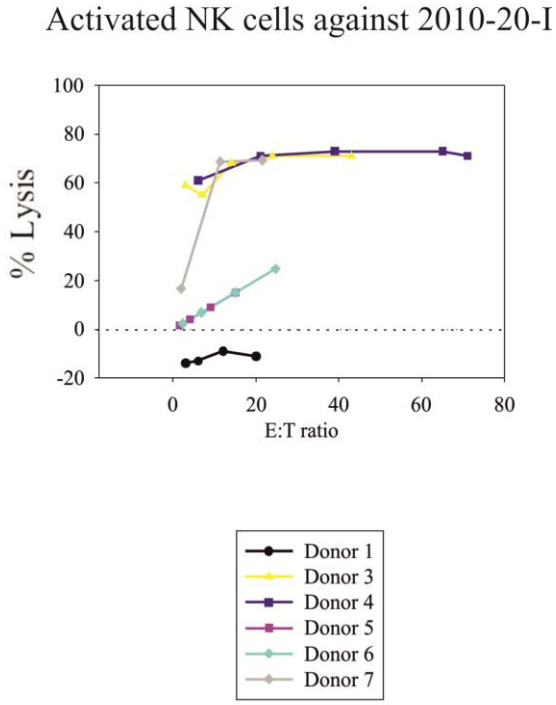
Overall, these data support the dominance of activating signals provided by KIR2DS2 and KIR2DS4 in determining cytotoxic potency of activated NK cells against GBM cells, whose loss could be compensated for through presence of numerous other activating KIRs.

Figure 30: *In vitro* cytotoxicity effect of activated NK cells against 2010-20-I cells and KIR receptors and cognate HLA ligands genotyping table.

A

KIR Receptors		2010-20-I HLA Ligands	Donors						
			1	2	3	4	5	6	7
Inhibitory	KIR2DL1	C*05							
	KIR2DL2	C*0501							
		C*1601							
	KIR2DL3	C*0501							
		C*1601							
	KIR3DL1	B*4402							
		B*4403							
	KIR3DL2	A*03⁻/ A*11⁻							
	KIR2DL4	HLA-G⁻							
	KIR3DL3	unknown							
	KIR2DL5	unknown							
Activating	KIR2DS1	C*05							
	KIR2DS2	C*1601							
	KIR2DS4	C*0501							
		C*1601							
	KIR2DS3	C*1601							
	KIR2DS5	unknown							
	KIR3DS1	unknown							

B



KIR: killer immunoglobulin-like receptor; HLA: Human Leukocyte Antigen

(A) KIR-HLA genotyping mismatch of each donor with 2010-20-I. Red represents match (presence of both KIR gene on the effector cells and cognate HLA ligand gene on the tumour cells), green colour represents mismatch (lack of KIR gene on effector cells and/or HLA ligand gene on the tumour cells) and white colour represents unknown match/mismatch. Only HLA ligands present in the GBMs of this study are represented in the tables. (⁻) Superscript in HLA ligands indicates lack of gene for of that particular ligand in the GBM. (B) *In vitro* cytotoxicity effect of activated NK cells from different donors against 2010-20-I cells.

9.7.2.4 NK cells cytotoxicity against 2012-018

To investigate the robustness of the hypothesis that KIR2DS2 and KIR2DS4 determine NK cells cytotoxic potency against GBM, we aimed to establish this association for 2012-018 GBM cells with gliosarcoma differentiation that expressed high levels of HLA-DR,DQ, DP in addition to HLA-A,B and C.

Activated NK cells from donors D6, D7 and D8 exhibited intermediate cytotoxicity against 2012-018 gliosarcoma differentiated GBM cells in the range of 20-25% lysis at E:T ratio 10, while D5's and D3's NK cells were unable to kill the 2012-018 cells (Figure 31, B).

In terms of inhibitory mismatch, all 5 donors had inhibitory KIR-HLA mismatch for KIR2DL4 where the 2012-018 GBM cells lacked their cognate ligand HLA-G (Figure 31, A). In addition, D5's effectors lacked KIR2DL2, D6 and D8 lacked KIR2DL5 and D7 lacked KIR2DL1, KIR2DL3 and KIR2DL5 (Figure 31, A). Thus, no association between inhibitory mismatch pattern and cytotoxicity efficacy was observed.

Importantly, when examining the activating KIR receptor-HLA ligand matches, D3, D6, D7 and D8 derived NK cells may have KIR2DS2 gene while D5's effectors did not have this and all of them had KIR2DS4 gene (Figure 31, A). Moreover, KIR2DS1 gene was present in D3, D5 and D8's effectors while KIR2DS3 was matched in D3 and D8 and was not present in the genome of the other donors (Figure 31, A).

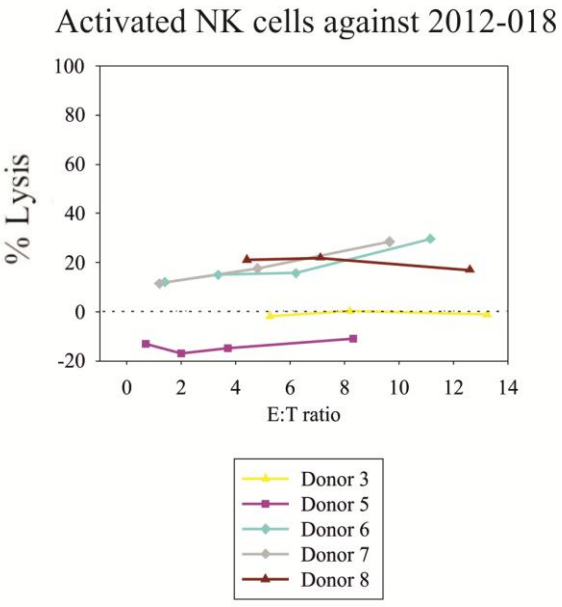
Taken together, the data indicate that KIR2DS2 may contribute to the cytotoxic potency in addition to KIR2DS4.

Figure 31: *In vitro* cytotoxicity effect of activated NK cells against 2012-018 cells and KIR receptors and cognate HLA ligands genotyping table.

A

	KIR Receptors	2012-018 HLA Ligands	Donors							
			1	2	3	4	5	6	7	8
Inhibitory	KIR2DL1	C*04								
	KIR2DL2	C*07								
	KIR2DL3	C*07								
	KIR3DL1	B*3701								
	KIR3DL2	A*03								
	KIR2DL4	HLA-G								
	KIR3DL3	unknown								
	KIR2DL5	unknown								
Activating	KIR2DS1	C*04								
	KIR2DS2	unknown								
	KIR2DS4	C*04								
	KIR2DS3	C*07								
	KIR2DS5	unknown								
	KIR3DS1	unknown								

B



KIR: killer immunoglobulin-like receptor; HLA: Human Leukocyte Antigen

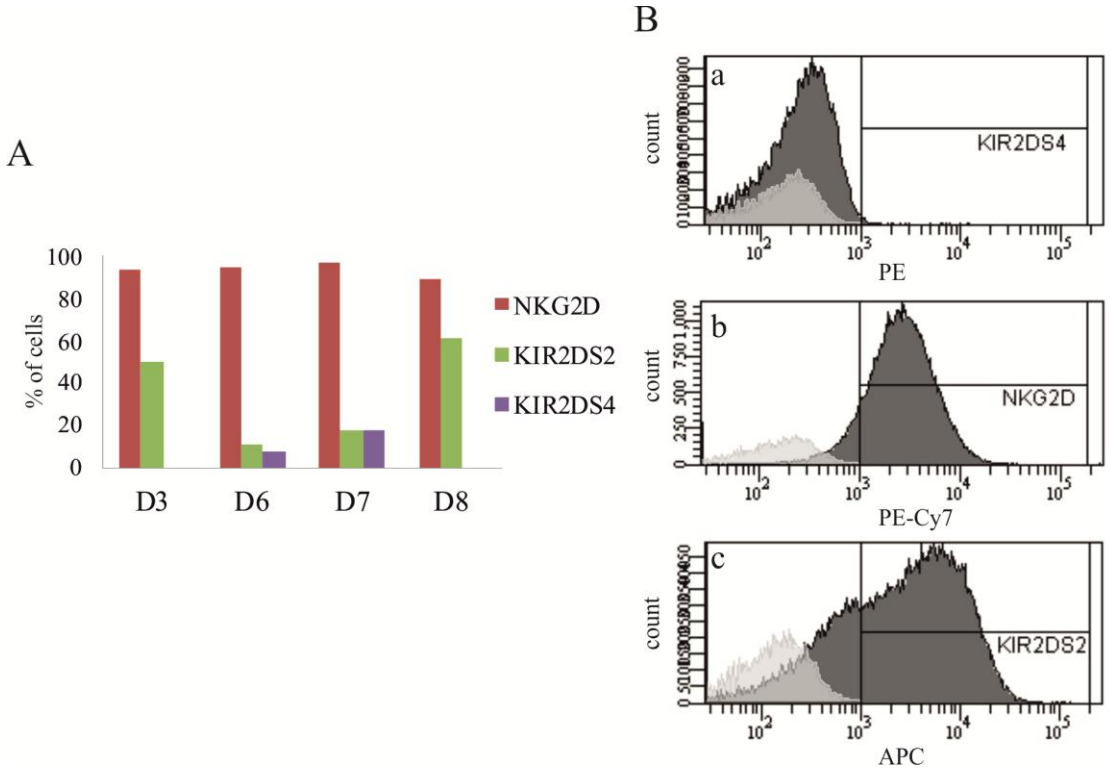
(A) KIR-HLA genotyping mismatch of each donor with 2012-018. Red represents match (presence of both KIR gene on the effector cells and cognate HLA ligand gene on the tumour cells), green colour represents mismatch (lack of KIR gene on effector cells and/or HLA ligand gene on the tumour cells) and white colour represents unknown match/mismatch. Only HLA ligands present in the GBMs of this study are represented in the tables. (¯) Superscript in HLA ligands indicates lack of gene for that particular ligand in the GBM. (B) *In vitro* cytotoxicity effect of activated NK cells from different donors against 2012-018 cells.

9.7.2.5 NK cells cytotoxicity with blocking antibodies

To provide experimental proof that KIR2DS2 and KIR2DS4 determine NK cell cytotoxic potency, we aimed to functionally block the interaction with their HLA-cognate ligand and investigate the effect on their cytotoxic efficacy. NKG2D was also blocked as positive control.

To confirm the presence of those receptors in the NK cells surface, their expression was evaluated by flow cytometry phenotyping. NKG2D was highly expressed by all the 4 donors (93,58±3,43%, Figure 32, A) while KIR2DS2 and KIR2DS4 were differently expressed by the 4 donors. Interestingly, D3 and D8 that had more cells expressing KIR2DS2 (49,5% and 61,2%, Figure 32, A) than the other donors, did not expressed KIR2DS4. In addition, D6 and D7, which expressed KIR2DS2 in less number of cells than the other donors (11,5% and 18,2%, respectively, Figure 32, A), did also express KIR2DS4, 7,4% and 18% of cells express it, respectively.

Figure 32: Phenotype profile of the donors used in the NK cells cytotoxicity with blocking antibodies.



PE: Phycoerythrin, PE-Cy7: Phycoerythrin-Cyanine7 tandem fluorochrome, APC: Allophycocyanin

(A) Bar graph shows the percentage of D3, D5, D6 and D7's activated NK cells expressing NKG2D, KIR2DS2 and KIR2DS4 activating receptors on the surface. (B) Histograms show expression of the 3 receptors, blocked in the experiment, for donor 8 as an example of positive expression gating. Light grey represents the signal of the negative control for this donor in each channel of detection while dark grey represents its signal obtained after staining. (Ba) Signal obtained for NK cells stained with anti-KIR2DS4-PE. (Bb) Signal obtained for NK cells stained with anti-NKG2D-PE-Cy7. (Bc) Signal obtained for NK cells stained with anti-KIR2DS2 Ab together with a secondary APC-Ab.

To prove the importance role of KIR2DS2 and KIR2DS4 receptors, cytotoxicity assays with activated NK cells against P3, 2012-018 GBMs and K562 and blocking antibodies were performed. Blocking Ab against KIR2DS2, KIR2DS4 and NKG2D, as a control, were used.

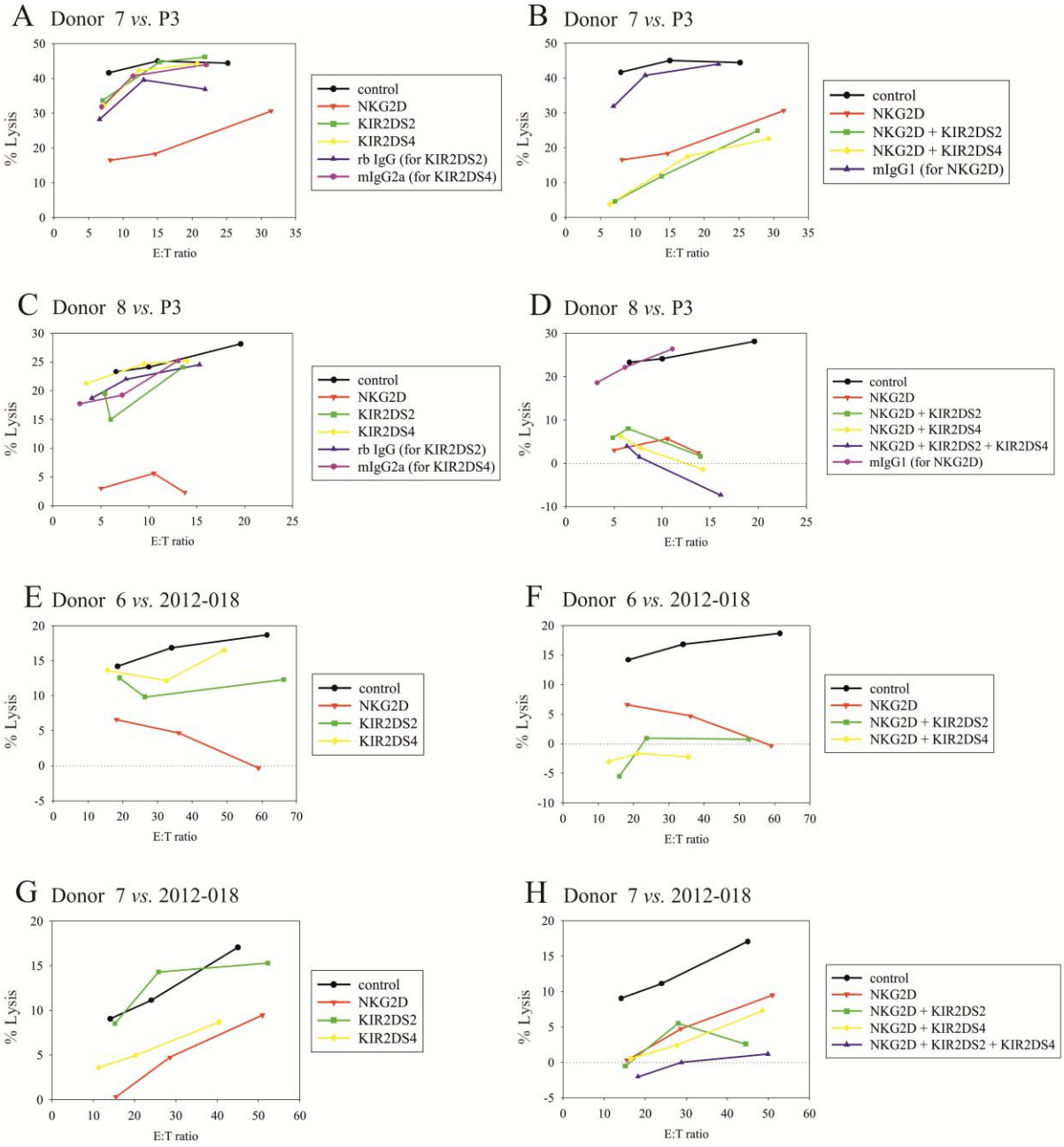
The cytotoxicity effect of NK cells from D7 against P3 diminished at low E:T ratios when blocking KIR2DS2 and KIR2DS4 were present (Figure 33, A). When those blocking Ab were present together with anti-NKG2D the cytotoxicity potency was highly reduced, also compared with NKG2D blocking alone, that had intermediate effect (Figure 33, B). The low expression of KIR2DS2 and moderate in KIR2DS4 showed by the phenotyping assays (Figure 32, A) may explain the difference observed with both blocking Ab.

Cytotoxicity assays against the same cell line were also performed with D8 NK cells as effector, in that case no diminution of cytotoxicity was observed when blocking KIR2DS4 while the results were similar as for D7 when KIR2DS2 was blocked (Figure 33, C). Less cytotoxicity was also observed when NKG2D was blocked and/or the blocking Abs were combined (Figure 33, D). This different cytotoxicity inhibition observed can also be linked to the blocked receptors expression level (Figure 32, A). In addition, as control, cytotoxicity experiments were established with isotype controls for the receptors in study and, as expected, all presented similar cytotoxicity as the controls (Figure 33, A, B, C and D). However, same experiments were performed for D3 and D6 NK cells but the blocking of KIR2DS2 and/or KIR2DS4 did not result in abrogation of cytotoxicity (data not shown).

The same cytotoxicity experiments were performed against 2012-018 due to presenting different phenotype. When KIR2DS2 or KIR2DS4 receptors of D6 NK cells were blocked the lysis of 2012-018 cells decreased, but less than in the blocking the NKG2D receptor case (Figure 33, E). The combination of blocking Ab resulted in additive abrogation, as in the P3 case (Figure 33, F). However, the cytotoxic decrease with the blocking of KIR2DS2 and KIR2DS4 was not as robust as in the case of blocked NKG2D, what might be partially explained again with the low and moderate expression levels of KIR2DS2 and KIR2DS4, respectively, observed (Figure 32, A).

In the case of D7 cytotoxicity assays against 2012-018, no diminishing of cytolysis was observed when KIR2DS2 was blocked alone (Figure 33, G). However, when KIR2DS2 was blocked with NKG2D or in combination of both NKG2D and KIR2DS2, 2012-018 cells were less killed (Figure 33, H). Same experiments against 2012-012 were performed with D3 and D8, but no effect was observed when blocking for KIR2DS2 and/or KIR2DS4 (data not shown).

Figure 33: Cytotoxicity assays with blocking antibodies against NKG2D, KIR2DS2 and KIR2DS4.



rbIgG: rabbit Immunoglobulin G; mIg: mouse Immunoglobulin

(A, B) Donor's 7 activated NK cells cytotoxicity effect against P3 with addition of blocking Ab alone or combined or isotype control Ab. (C, D) Donor's 8 activated NK cells cytotoxicity effect against P3 with addition of blocking Ab alone or combined or isotype control Ab. (E, F) Donor's 6 activated NK cells cytotoxicity effect against 2012-018 with addition of blocking Ab alone or combined. (G, H) Donor's 7 activated NK cells cytotoxicity effect against 2012-018 with addition of blocking Ab alone or combined.

9.7.3 NK cells *versus* LAK cells cytotoxicity

Given that LAK cells are predominantly used as effector cells in GBM immunotherapy clinical trials [98], we aimed to investigate whether the LAK cells were more cytotoxic against GBM cells compared to purified activated NK cells derived from the same donor.

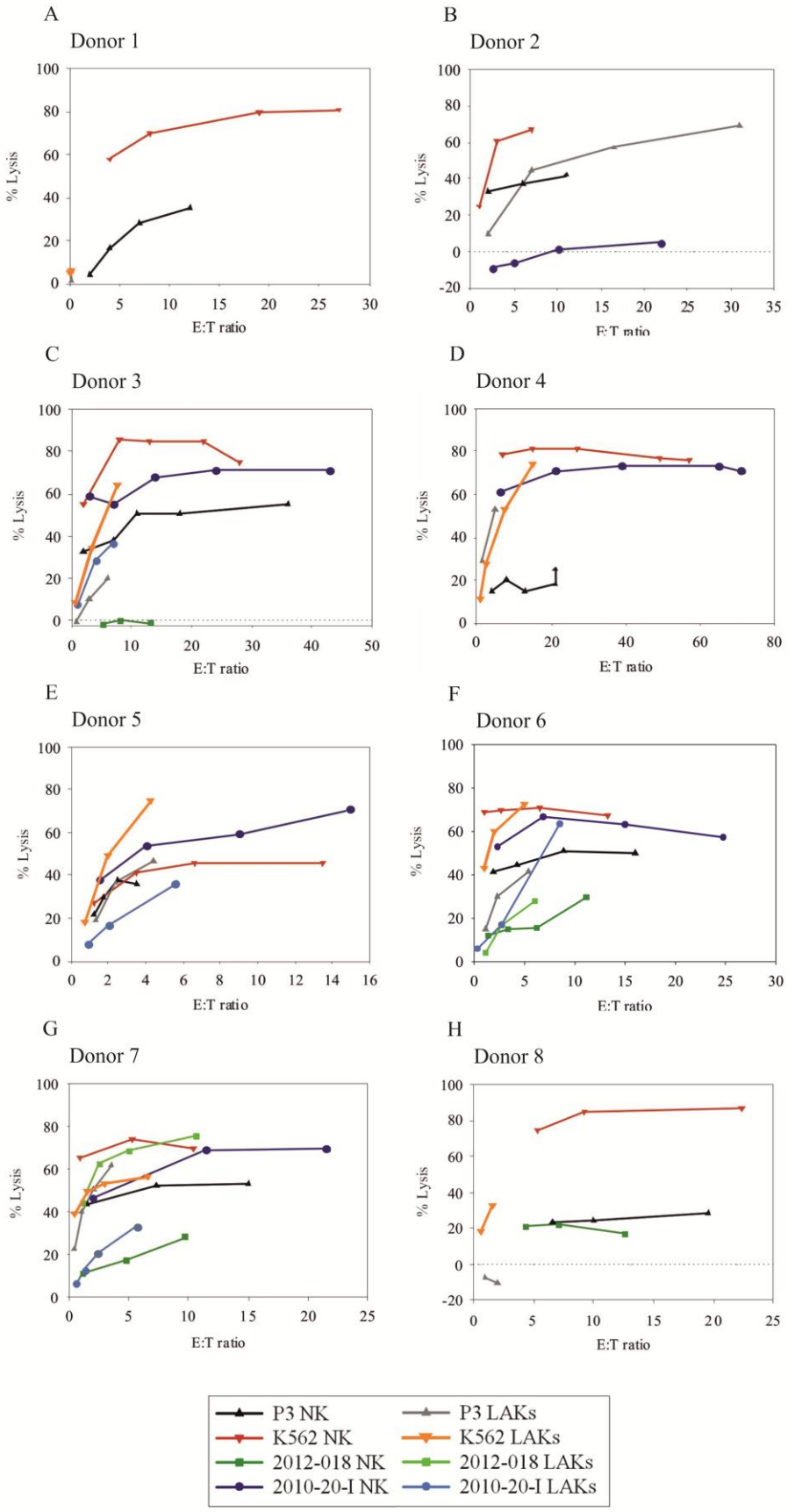
The NK cell susceptible K562 cell line was killed more efficiently by the NK cells from donors D1, D2, D3, D4, D7 and D8 than by LAK cells derived from the same donors (Figure 34, A, B, C, D, G and H, respectively). Contrary, D5's LAK cells killed K562 more efficiently than its purified NK cells (Figure 34, E) whilst D6's NK and LAK cells were equally cytotoxic against K562 (Figure 34, F).

D1, D2, D3, D6 and D8 NK cells were more cytotoxic than LAK cells against P3 GBM (Figure 34, A, B, C and F, respectively) while D5 showed no difference in cytotoxic efficacy between both effector cells (Figure 34, E). Nevertheless, D4 and D7's LAK cells demonstrated to be more efficient than their NK cells against P3 (Figure 34, D and G, respectively). Interestingly, when evaluating the cytotoxicity effect against 2010-20-I GBM cells, D3, D5, D6 and D7's NK cells were more efficient than their LAK cells (Figure 34, C, E, F and G, respectively) and no donors' LAK cells exhibited higher cytotoxicity potency than corresponding NK cells against 2010-20-I.

Furthermore, the cytotoxic efficacy against 2012-018 GBM cells was investigated for 2 donors and both D6 and D7's LAK cells exhibited better potency than their corresponding NK cells (Figure 34, F and G, respectively). In the case of D6's NK and LAK cells, the cytotoxicity levels were similar but for D7 LAK cells were highly potent than NK cells with % lysed cells greater than 60% at E:T ratio 10.

Taken together this shows a tendency of NK cells to be more cytotoxic against the control cell line K562, P3 and 2010-20-I GBM cells than LAK cells. Nevertheless, it was not the case against 2012-018 were LAK cells were more effective.

Figure 34: *In vitro* cytotoxicity effect of LAK cells and activated NK cells from 7 different donors against GBM cells.



(A) Donor's 1 LAK cells and activated NK cells cytotoxicity effect against P3 and K562. (B) Donor's 2 LAK cells and activated NK cells cytotoxicity effect against P3, K562 and 2010-20-I. (C) Donor's 3 LAK cells and activated NK cells cytotoxicity effect against P3, K562, 2010-20-I and 2012-018. (D) Donor's 4 LAK cells and activated NK cells cytotoxicity effect against P3, K562 and 2010-20-I. (E) Donor's 5 LAK cells and activated NK cells cytotoxicity effect against P3, K562 and 2010-20-I. (F) Donor's 6 LAK cells and activated NK cells cytotoxicity effect against P3, K562, 2010-20-I and 2012-018. (G) Donor's 7 LAK cells and activated NK cells cytotoxicity effect against P3, K562, 2010-20-I and 2012-018. (H) Donor's 8 LAK cells and activated NK cells cytotoxicity effect against P3, K562 and 2012-018.

10. Discussion

GBM is the most frequent and aggressive brain tumour where despite multimodal therapies, the patients have very poor prognosis of 14,6 months median survival [34]. GBM tumour induces anti-inflammatory microenvironment by secretion of immunosuppressive factors and expression of inhibitory molecules for the immune response. Therefore, the immune cell subpopulations surrounding the tumour are reduced and an immune suppressive microenvironment is created [99]. Thus, novel immunotherapies with adoptively transferred cells capable of switching the microenvironment to a pro-inflammatory one may contribute to re-establishing an immune response against the GBM, resulting ultimately, in its elimination.

Immunotherapy clinical trials in patients with GBM have been predominantly administered using LAK cells generated by culturing PBMCs *ex vivo* [50, 51, 98]. LAKs are a mixture of activated lymphocytes composed of a major population of T cells and a minor population of NK cells. Moreover, LAK cells may create a subpopulation of regulatory T cells, which has been reported to present inhibitory functions against NK cells [100]. In addiction T cells may contribute to potentially fatal Graft *versus* host disease (GvHD) [101] while NK cells do not induce it [102]. Currently, there is several ongoing investigations using purified NK cells as immunotherapy effectors against GBM and 6 clinical trials in different phases are registered on www.clinicaltrials.gov [98]. Therefore the goal of our study was to investigate the cytotoxic efficacy of NK cells against glioblastoma. We hypothesised that NK cells capacity to kill GBM stem-like cells may be determined by the KIR-HLA ligand mismatch hypothesis. Thus, we compared the cytotoxic potency of purified resting NK cells, activated NK cells and LAK cells from the same healthy donor against the GBM stem-like cells.

10.1 NK and LAK cells' biological characteristics in culture.

As NK cells comprise only 5-20% of peripheral blood lymphocytes it may be difficult to obtain adequate numbers for NK-mediated immunotherapy. Moreover freshly isolated NK cells are typically in a resting state and require subsequent activation for efficient target engagement. Protocols for their expansion are thus required.

We demonstrated that NK cells could be expanded 6-9 fold in the presence of IL-2 and antibodies to NKp46 and CD2 coupled to microbeads. However, for clinical purposes this

technique has two very important weaknesses, (1) 6-9 fold give us a very low number of cells not optimal for clinical applications and (2) this is an expanding method by using Miltenyi expanding kit, which requires the ligation of NK cells to microbeads to get activated. These beads would require to be removed before a clinical treatment, a procedure extremely difficult to perform without affecting the viability of the cells. These weaknesses make this method, although efficient, not very practical for clinical application. Hence, other well defined techniques, such as expansion from umbilical cord blood $CD34^+$ cells or co-cultivation with irradiated human Jurkat T-lymphoblast line (KL-1) cells (*ex vivo* expansion of NK with irradiated tumour) that has been recently established, would be needed. In the first one, Dolstra et al. obtain 1000-fold NK cell expansion with near 100% of purity by a protocol with 2 consecutive differing culturing conditions of $CD34^+$ umbilical cord cells in one month [103]. In contrast, Lim et al. co-cultured PBMC cells from blood with irradiated KL-1 cell line sensitive for NK cytotoxicity which stimulated the NK cells to expand to 100-fold with 90% purity in 15 days [104]. Although there were effective expansions, where they obtained NK cells expressing different KIRs, activating receptors, such NKG2D, and $CD56^{bright}$ marker, both techniques had small subsets of $CD16^+$ NK cells. Based on our results, it is necessary to have higher $CD16^+$ NK cell subsets for potent cytotoxicity effect and CD16 expression would also be useful for inducing ADCC in combination therapies with monoclonal antibodies.

LAK cells could not be expanded efficiently in the presence of IL-2 and IL-15, despite the fact that they were cultured following the current protocol that is used for clinical application [105]. This feature represents a rate-limiting step for their successful application, as one would need to start the culture with a lot of cells from the donors to obtain an adequate number of cells for the treatment. Owing to the patients' often weak general condition, autologous transfer of patient own cells at high doses might be difficult to achieve.

10.2 Subpopulations of LAK cells.

As reported in previous studies that used LAK cells as immunotherapy effectors [50, 51], we observed a mixed phenotype of the LAK cells. While NK cells were greater than 96% pure after culture, LAK cells consisted of predominantly T-lymphocytes, the majority of which were CTLs, followed by Th cells. The NK cell fraction in LAK cells was minor and

represented approximately 18% of cells. Interestingly, these NK cell subsets within the LAK cells exhibited $CD56^{bright}CD16^{dim}$ major phenotype, the cytokine responsive phenotype previously reported to be more prone to IFNγ production [106]. This subpopulation of NK cells was the same as the one observed in the resting NK cells, which did not have potent efficacy of killing GBM cells.

We showed that LAK cells contain a mixture of T cells and NK cells with a donor dependent composition. As an example we obtained much higher proportion of NK cells in D7' LAK cells than the average and greater cytotoxicity of those against 2012-018. Likely, that was due to larger NK cell population they had. This donor variability in the composition of LAKs and the unpredictability of the therapeutic aspects it may have, might pose challenges to standardising LAK-based treatments in clinical trials. However, it has also been reported donor variability in LAKs composition in one study with cytotoxicity assays against cells infected with Toxoplasma-Gondi [107] but few of the LAK cell studies do phenotype characterisation and quantify both NK cells and CTLs subsets from LAKs like we did in the present study.

10.3 NK cells' major subpopulation changed after culture

As Caligiuri et al. and Zimmer et al. reported, we also obtained a $CD56^{dim}$ $CD16^{+}$ predominant subpopulation of resting NK cells. After activation in culture, as expected, the $CD56^{bright}CD16^{+}$ NK cell subpopulations were significantly enriched and became the major subpopulation [53, 54]. This switch upon culturing to a $CD56^{bright}$ is a well-known behaviour of the NK cells cultured by IL-2, in addition this phenotypic switch augments NK cells cytotoxicity activity [53].

According to Caligiuri et al. and Zimmer et al. findings, $CD56^{dim}CD16^{+}$ NK cells (corresponding to our resting NK cells) contain the major number and content of cytolytic granules while $CD56^{bright}CD16^{+}$ (corresponding to our activated NK cells) are more efficient in cytokine production and with higher ADCC efficiency [53, 54]. Our findings validate and confirm the studies from these independent laboratories.

10.4 NK cells *versus* LAK cells cytotoxicity

Since several trials with adoptive cellular immunotherapy against GBM are accomplished by LAK cells [98] and the major cytotoxic effectors within the LAK cells might be NK cells [107] we hypothesised that NK cells might be more potent effectors against GBM. Thus, we studied the cytotoxicity efficacy of LAK cells compared to NK cells against patient-derived GBM cells *in vitro*.

Although there was a general trend for NK cells being more potent than LAK cells against the GBM stem-like cells, there was a donor dependent cytotoxic efficacy for LAK cells against GBMs. In our study we compared cytotoxicity potency of LAK cells with a $CD56^{bright}CD16^{dim}$ NK subset with purified NK cells that were predominantly $CD56^{bright}CD16^{+}$. Their obvious difference was the expression of CD16 molecules in the surface, which confines $CD16^{+}$ higher ADCC efficiency than $CD16^{dim}$ [53, 54], but this cytotoxicity function is not relevant in *in vitro* assays due to the lack of antibodies to recognize the Fc portion by CD16. In addition, the distinguishing marker for NK cells, NKp46, was significantly expressed by NK cells than CTLs, what reflects the NK cell lineage.

Despite the fact that cytotoxic efficacy of LAK cells is likely a result of both NK and CTLs derived mechanisms, Subauste et al. experiments showed better cytotoxicity effects with donors' LAK cells enriched with NK cell subsets [107], tempting one to presume that NK cells may be the major mediators of the cytotoxicity within LAK cells populations. We found that D7 LAK cells, as an exception, were composed of greater NK cell numbers compared to CTLs and they were be more potent against P3 and 2012-018 GBM cells than the other LAK cells and even the purified NK cells.

Taken together, the findings support our hypothesis and indicate that NK cells are a better option as immunotherapy effectors against GBM than LAK cells.

10.5 Dynamic modulation of activating and inhibitory NK cell receptors expression upon culture

10.5.1 Activating and cytotoxicity receptors

In our study, there was no significant difference in density levels of NKp46 and NKG2D between the resting and activated NK cells. The expression of NKG2D and NKp46 in the major subpopulations of both resting and activated states showed donor-dependent variability. While major subpopulations of both resting and activated NK cells had mostly high number of cells expressing NKG2D, they had, generally, less cells expressing NKp46. However, the results suggested that the expression of activating NKG2D receptor equilibrates to the expression of other cytotoxicity receptors, such as NKp46. This compensation hypothesis has also been established in the case of (1) NKG2D and DNAM-1 in mice [108] by Gilfillan et al. and compensation of activating receptors may also be the case in the very recent publication of Sheppard et al., where they demonstrated that (2) double mutant mice of NKp46 and NKG2D accomplish NKG2D and NKp46 related functions [109]. Hence, since our compensation hypothesis between NKG2D and NKp46 has not been described previously, it may indeed be a novel funding. However, further studies with larger number of donors are required to confirm this tendency.

10.5.2 Inhibitory receptors

The expression of NKG2A inhibitory receptor by resting NK cells showed donor-dependent variability but most of the donors down-regulated the number of cells expressing NKG2A after the culture. Although no evidence of direct NKG2A down-regulation has been reported previously, $NKG2A^{-}/Fas^{+}$ NK populations were found by Son et al. in new-onset psoriasis patients and were defined as an activated pro-inflammatory phenotype [110]. Despite this NKG2A down-regulation observation, there was no significant difference ($p>0.05$) in the % cells expressing these receptors before and after culture. Nevertheless, NKG2A did not represent a relevant receptor in our study due to none of the tumour cells included in our research expressed its ligand, the non-classical HLA-E.

Donor-dependent variability in the expression of the inhibitory KIR receptors was observed, this is consistent with the fact KIR have different haplotypes, with a large variability of genes in each one, and allelic polymorphism [71, 72]. However, most of the donors had low number

of NK cells (<50%) expressing inhibitory KIRs and their expression was modulated in different ways and with no statistical significance depending on the donor and activation status.

10.6 LAK cells receptors expression

LAK cells' subpopulations receptor expression was investigated in order to determine the similarities between LAK cells and purified NK cells that could explain the cytotoxicity. As in the case of pure NK cells, donor-dependent variability in the expression of receptors and in response to the culture conditions was observed, consistent with the published literature [68].

Despite the fact that there were no significant differences in the expression of activating NKG2D or NKG2A inhibitory receptors between NK cells and CTLs fractions of the LAK cells, NK cells were denoted by elevated NKp46 expression compared to the CTL subpopulation. This is consistent with the notion that NKp46 is the most specific marker for NK cells across mammalian species [111] although discreet subsets of T cells weakly express it.

Mingari et al. reported in their study that KIRs are also expressed by a small fraction of T cells [112]. Indeed, expression of KIR has been described on both CD4 T cells, in the context of cytomegalovirus infected cells [113], and on CD8 T cells [114], in which they found different expression of the KIRs in CTLs than in NK cells. Therefore we hypothesised that the CTL and NK cellular subgroups within the LAK cells might differentially express the KIRs and thus impact on their differential cytotoxic potency against GBM. In our experiments, KIRs were not-highly expressed in general terms in LAK cells and, surprisingly, no significant difference in their expression on CTLs and NK cells was observed in our study.

10.7 GBM cells markers and ligands expression

We investigated the phenotypic features of the GBM primary cultures we established from patient GBM biopsies obtained from surgical resections at Haukeland University Hospital. As reported previously, the expression of stem-like markers on GBM cells affects the cytotoxicity of NK cells against GBM cells [66, 115]. Therefore, the GBM cell lines were

phenotyped for various markers of immature cell types and for the activating and inhibitory ligands for NK cell receptors. The same phenotyping was established for the standard cell lines U87 and U373 propagated in serum containing medium for comparison (Appendix II, Figure 14).

10.7.1 CNS cell differentiation markers and stemness

Glial fibrillary acidic protein (GFAP), nestin and vimentin were highly expressed by most of the GBMs and it is consistent with previous reports [10, 23, 26]. They are all intermediate filaments (IF) expressed at various differentiation states from neuronal stem cells (NSC) and can be co-expressed in the same cell to make hetero-filaments that include sometimes 2 of those [23-27]. However, only low expression of GFAP was demonstrated in 2012-018 GBM cells and this is consistent with the fact that this is a gliosarcoma differentiated GBM. This GBM histological type is denoted by both neuroepithelial and mesodermal features consistent with its organisation from vessels, hence the name gliosarcoma.

A2B5 is an antibody that recognises several GT3 gangliosides, while CD133 and CD15 have been speculated to identify GSCs by some authors [26, 28, 29]. Despite the fact that the cancer stem cell hypothesis has been refuted for glioblastoma, these markers are albeit expressed, and may represent the cellular heterogeneity of GBM rather than their stemness or states of CSC origins. Our results were consistent with the reports of seldom expression of CD133 on the GBM cells. While A2B5 was expressed in cells of 3 out 5 of the GBMs, only 6,8% of the cells of 2012-018 expressed CD133 and only 3,8% and 4,9% of 2010-062 and 2012-018 cells, respectively, expressed CD15.

Other markers of “stemness” such as SOX2, Ki-62 and neuron glial-2 (NG2) have also investigated in other studies [66, 115]. Inclusion of these markers would complete our study of characterisation of the stemness state of our GBM cultures. However, we unable to efficiently optimise the methods of assessing these markers, thus they were not included in our results.

10.7.2 GBM expression of ligands for NK cell receptors

There have been only two publications in the last 3 years utilizing purified NK cells to target glioma cells propagated *in vitro* as stem-like cells [66, 115]. Castriconi et al. investigated the

susceptibility of GBM stem-like cells to resting or IL-2 long-term cultured NK cells. They reported that GBM cells expressed levels of HLA class I that were not sufficient to protect against NK mediated lysis [66]. However, we also report expression of HLA A,-B,-C in all the GBMs and as in Castriconi et al. experience, they might be expressed in a not protective density for the NK cytotoxicity.

Furthermore, Castriconi et al. concluded that the mechanisms were mediated by combined action of NK natural cytotoxicity receptors (NCRs) NKp46 and DNAM-1 [66]. DNAM-1, together with NKp30 and NKp44, were not included in our phenotyping experiments; therefore, we cannot presume their contribution in the NK cell cytotoxicity against the GBM cells.

None of our patient-derived GBM primary cultures expressed the non-classical class I HLA-G and only few cells of 2012-018 and 2010-062 GBMs expressed HLA-E, both ligands for CD94/NKG2A inhibitory receptor. None of the HLA class II molecules were expressed by any of the GBMs, with the exception of 2012-018 GBM that had more than 50% of the cells expressing HLA-DR-DP-DQ. As this is a ligand for the TCR of Th cells, this may be the reason why 2012-018 cells are killed more efficiently by the LAK cells from some donors than by their NK cells.

NKG2D receptor ligands, MIC-A, MIC-B, ULBP-1, ULBP-2,5,6 and ULBP-3 were not highly expressed by any of the GBM cells. 2010-062 cells, that highly expressed MIC-A, were an exception, but, unfortunately, no comparable cytotoxicity assay was established with this cell line. MIC-A was also expressed by 2,5% of P3 and 3,2% of 2010-20-I cells. MIC-B was expressed by 6,4% of 2012-018 cells and 1,4% of 2010-20-I cells. And only 1,2% and 2,9% of 2010-062 GBM cells expressed ULBP-2,5,6 and ULBP-3, respectively. Despite their low levels of expression, they nevertheless contribute to NK cell cytotoxicity since blocking of NKG2D abrogates the killing efficacy. However, the low expression of ULBPs is consistent with reports from other studies [66].

10.8 Cytotoxicity assays

Since we confirmed that purified NK cells kill efficiently GBMs compared to LAK cells generated from the same healthy donor, we aimed to determine the cytotoxic efficacy of resting NK cells compared to activated NK cells.

10.8.1 Resting NK cells cytotoxic effect

Our results showed a donor-dependent moderate cytotoxic effect of purified resting NK cells against K562 CML Leukaemia cell line, consistent with previous reports [66], and against 2010-20-I GBM, but none had the ability to kill efficiently p3 and 2012-018 GBM cells. Based on the results obtained from the genotyping of 2010-20-1 GBM cells, the ability of resting NK cells to kill this GBM might have been mediated by reduced inhibitory signals as a result of 3 inhibitory KIR-HLA ligand mismatch and increased activation signals. In addition, the 2010-20-I cell line had lower expression levels of HLA-A,B,C than the other ones (50,3% of cells expressing it compared to 89,98±8,09% of the other GBMs), a fact that may result in less interaction with inhibitory KIRs, leading to penetration of more activating signals and subsequent cytolysis.

It has been reported that NK cells in steady states express $CD56^{dim}$, contain more cytolytic granules, higher amount of their content and present more cytotoxic activity while $CD16^{+}$ are more efficient in ADCC [53, 54]. Due to this fact and as the major subpopulation of resting NK was $CD56^{dim}CD16^{+}$, higher cytotoxic effect of the resting NK was expected. However, the low cytotoxicity efficacy may be because of the fact that under the *in vitro* conditions, ADCC does not happen, as explained above. Furthermore, the fact that $CD56^{dim}$ containing more cytolytic granules may not necessarily be related to their efficiency to secrete their content.

10.8.2 Activated NK cells cytotoxic effect.

10.8.2.1 NK cells cytotoxicity against K562

Since the cytotoxicity assays against K562 were established as a positive control due to the susceptibility of this cell line to the cytotoxicity effect of NK cells [66], high cytotoxicity effect was expected. Correspondingly, all our studied donors effectively killed K562 cells. Unexpected, however, was the donor-dependent effect, which may be explained because of

the donors' different expression of the other receptors mediating the cytotoxicity against K562, such as NKG2D that recognize the stress ligands and NKp46 whose ligands are as yet unknown.

10.8.2.2 NK cells cytotoxicity against patient-derived GBMs

Aware that resting NK cells were not able to kill efficiently GBMs, cytotoxicity assays of activated NK cells against GBMs were performed to investigate the hypothesis that activated NK cells are more proficient in killing GBMs than resting NK cells and also LAK cells.

Our cytotoxicity assays suggested that the activating receptors KIR2DS2 and KIR2DS4 might determine cytolytic potency of activated NK cells against P3, 2010-20-I and 2012-018 GBMs. The donor NK cells with the highest cytotoxicity against P3 had KIR2DS2 and KIR2DS4 genes while the NK cells from the donors with intermediate cytotoxicity efficacy had only one of the activating receptors, KIR2DS2, and lacked KIR2DS4. Finally the two donors with the lowest cytotoxic efficiency lacked KIR2DS2 while only one had KIR2DS4 genes. This data may also suggest that KIR2DS2 is the most relevant activating KIR of the two, although their ligands are at present unknown. Moreover, 2 out of 3 donors with high cytotoxic efficient NK cells against 2010-20-I possessed both genes, KIR2DS2 and KIR2DS4, while donors with intermediate cytotoxic potency had only KIR2DS4 genes. The other donor with high cytotoxic effect, despite not having both KIR2DS2 and KIR2DS4 receptors, may have compensated for this lacking with the possession of numerous additional activating receptors.

Cytotoxicity assays against 2012-018 gliosarcoma was established because this is a GBM with gliosarcoma differentiation that expressed high levels of HLA-DR, DQ, DP in addition to HLA-A, B and C and which exhibited different mismatch pattern than the other GBMs. Importantly, despite all the donors included in the assays against 2012-018 had KIR2DS4 match and KIR2DS2 gene, there was no high cytotoxicity effect against 2012-018. Most likely the high levels of HLA-DR, DQ, DP award protection against efficient killing by NK cells.

10.8.2.3 NK cells cytotoxicity with blocking antibodies

To confirm our hypothesis that KIR2DS2 and KIR2DS4 determine NK cell cytotoxic potency, we aimed to functionally block their interaction with the HLA-cognate ligand to determine whether it affects their cytotoxic efficacy or not. NKG2D was also blocked as positive control due to be an activating receptor highly expressed in NK cells, as we showed, and has as ligands various stressed molecules [61].

Activating receptors KIR2DS2 and/or KIR2DS4 showed an important role in determining potency of activated NK cells against all GBM cells P3, 2010-20-1 and 2012-018, where regardless of the number of inhibitory KIRs mismatched with their cognate HLA ligands, the presence of KIR2DS2 and KIR2DS4 was associated with greater NK cytotoxic potency. Likewise, reduced NK cytotoxic potency due to the loss of KIR2DS4 could probably be compensated by the presence of other activating KIR. Blocking KIR2DS2 and KIR2DS4 receptors resulted in decreased cytotoxicity of NK cells against both P3 and 2012-018 cells in 2 of 4 donors, partially confirming our hypothesis. However, those receptors are expressed only on a small proportion of cultured NK cells and this might explain why we did not observe as robust lysis abrogation as when NKG2D receptor was blocked. Unfortunately, ligands for KIR2DS2, KIR2DS5, KIR3DS1 and for KIR3DL3 and KIR2DL5 are presently unknown. Thus, the contribution of these receptors to the cytotoxic potency of NK cells against GBM cells could not be assessed. Another caveat to this study is the small size of tumours analysed. Although we found a dominant effect of KIR2DS2 and KIR2DS4 in determining cytotoxic potency against P3 and 2012-018 GBM cells, and that KIR2DS1 determined low cytotoxic efficiency of NK cells against P3, 2012-018, 2010-20-1, further studies with more tumours are required to confirm these findings.

11. Conclusion

Upon 14 days of culturing with the presence of IL-2, NKp46 and CD2 mAb conjugated beads, NK cells became $CD56^{bright}CD16^{+}$ and were highly cytotoxic against GBM tumour cells, while resting NK cells with a $CD56^{dim}CD16^{+}$ phenotype, did not kill GBMs efficiently. Furthermore, activated NK cells tended to be more effective against GBM cells than LAK cells, that were composed of a mixture of CTL and NK cells.

Despite the fact that ligands for many of the activating KIRs (such as KIR2DS2, KIR3DL3, KIR2DL5) are presently unknown and hamper the fully exploration of the KIR involvement of in the cytotoxic potency of NK cells against GBM cells, our study demonstrated an important role of the activating receptors KIR2DS2 and/or KIR2DS4 in commanding the potency of activated NK cells against P3, 2010-20-1 and 2012-018. The presence of those KIRs were associated with higher efficient cytotoxicity, despite inhibitory KIRs mismatches were present. In the case of loss of KIR2DS4, the cytotoxicity potency may be compensated by the presence of other several activating KIRs. These findings are highly novel and KIR2DS2 and KIR2DS4 may represent biomarkers for identifying the donors with the most potent NK cells for allogeneic NK cell immunotherapy.

Purified NK cells were more efficiently expandable compared to LAK cells.

12. Future perspectives

The present study has opened a new perspective in the potential of NK cell cytotoxicity against solid cancer. Our findings on the important involvement of activated KIRs in the NK cell mediated cytotoxicity should be reproduced after inclusion of larger numbers of donors and patient-derived GBM cells to consolidate the implication of activating KIRs for the NK cell potency. It will be imperative in the future to perform combination treatments *in vitro* and *in vivo* in animal models, such as blocking other KIR receptors to identify biomarkers for NK cell potency against GBM. Combination adjuvant therapies, such as proteasome inhibitor bortezomib, and antibodies against gangliosides, NG2, or epidermal growth factor receptors that are established tumour antigens that drive malignant progression could be combined with potent NK cells for maximal tumour control.

Furthermore, there currently exist some controversies regarding the identified KIRs ligands expressed on target cells, where many are simply unknown. In the future when these disparities are resolved there will be greater potential in identifying further receptor- ligand interaction that mediate NK cytotoxicity potency. Their investigation and discovery, together with the development of specific antibodies for their further study and application in research is key to in depth investigation into the complex mechanism of the HLA-ligand mismatch hypothesis.

Our group is currently performing *in vivo* experiments where P3 tumour spheres have been implanted in mouse brains and different HLA-ligand mismatched donors' NK cells were injected as adoptively transferred immunotherapy. Despite the fact that the results have not yet been fully analysed, preliminary data indicate that this is a very promising therapeutic approach and will provide further information not attainable from *in vitro* experiments. Therefore, similar experiments with other HLA-ligand mismatched donors' and with the combination of adjuvant therapies would also contribute valuable knowledge on the therapeutic potential and possible side effects of NK cell based immunotherapy for solid tumours.

Appendix I

Table 13: NK cells receptors

Receptor	Activating (+)/ Inhibitory (-)	Ligand/s
NKG2D	+	MICA, MICB, ULBPs
NKp46	+	Unknown
CD94/NKG2A	-	HLA-E
KIR2DL/KIR3DL	-	HLA class I
KIR2DL1	-	HLA-C2
KIR2DL2	-	HLA-C1,-C2
KIR2DL3	-	HLA-C1,-C2
KIR2DL4	-	HLA-G
KIR3DL1	-	Bw4-HLA-B
KIR3DL2	-	HLA-A3,-A11
KIR2DS/KIR3DS	+	HLA class I
KIR2DS1	+	HLA-C2
KIR2DS2	+	Unknown
KIR2DS3	+	HLA-C
KIR2DS4	+	HLA-C

Table representing NK cell receptors name, whether if they have activating (+) or inhibitory (-) function and their cognate ligands names (if they are known). Table adapted from [61-63, 68, 71, 74-77].

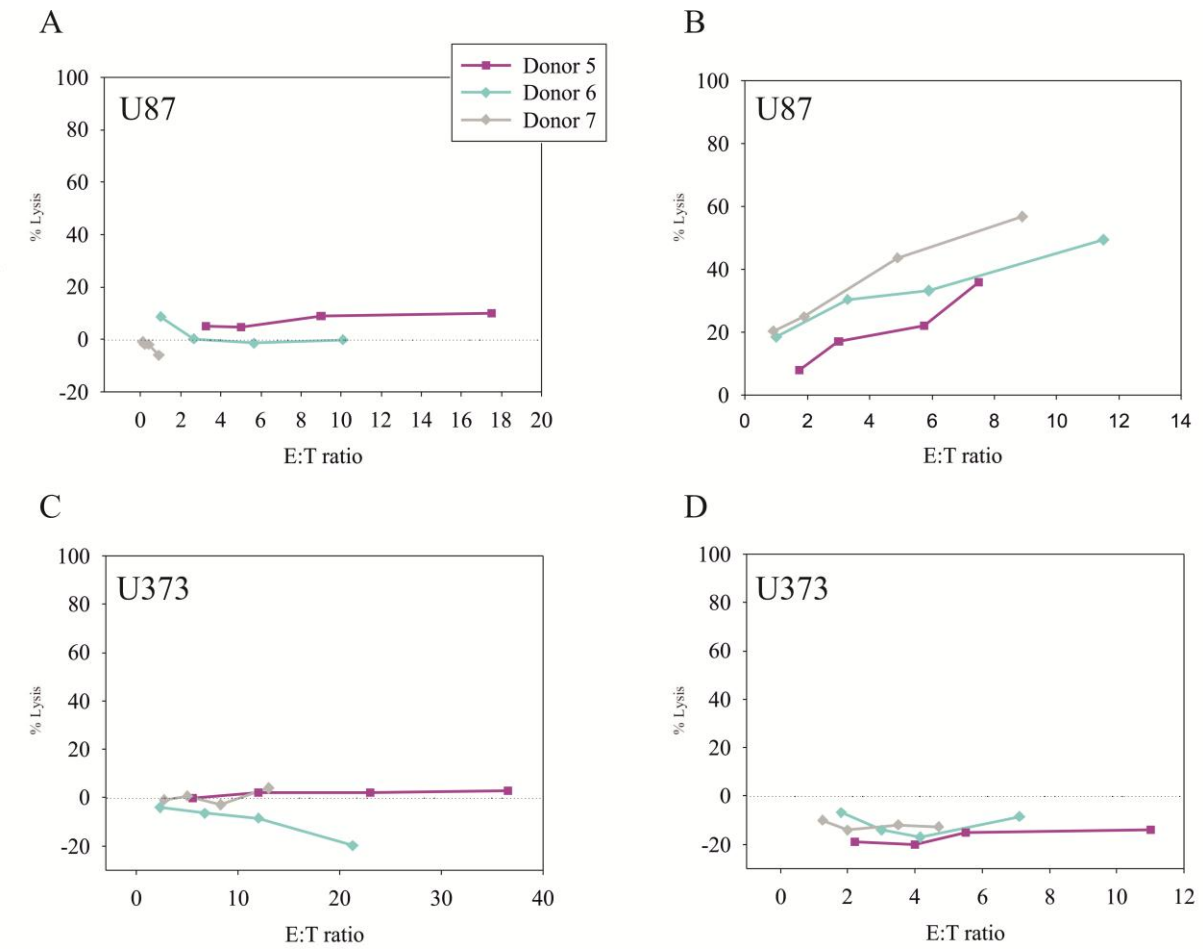
Table 14: GBM stem-like markers expression

	P3	2010-20-I	2010-046	2010-062	2012-018	U87	U373
GFAP	93	98,9	86,6	89,1	21,8	5,1	1,6
Vimentin	92,3	87	98,8	98	75,3	86,7	39,9
Nestin	93,3	94,2	82,2	91,2	87,1	93,9	57,25
CD31	0	0	0	0	1,8	1,3	5,4
CD15	0	0,1	-	3,8	4,9	5,7	0,6
CD133	0	0	-	-	6,8	0,6	1,7
A2B5	39,1	69,1	-	0,4	9,6	4,7	9,6
HLA-A,B,C	82,4	50,3	83,9	94,7	98,9	89,1	85,2
HLA-G	0,2	0,2	-	0,1	0	7,4	5,2
HLA-E	0,2	0,1	-	1,7	6,4	0,8	40,8
HLA-DR-DP-DQ	0	6,7	-	0,6	58,3	0,9	0
MICA	2,5	3,2	-	65,4	0	0	0
MICB	0,1	1,4	-	0,1	6,3	7,9	0,6
ULBP-1	0,1	0,1	0,1	0,5	0,4	0,3	0
ULBP-2,5,6	0,2	0,4	0,2	1,2	0,5	0,2	0,3
ULBP-3	0,1	0,4	0,1	2,9	0,6	0,1	0,1

Table representing the percentage of cells of the different GBMs expressing each CNS cell differentiation marker, each ligand for the NK cell receptor and CD31, an epithelial marker used as a control.

Appendix II

Figure 35: *In vitro* cytotoxicity effect of resting and activated NK cells from different donors against standard cell lines U87 and U373.



(A) *In vitro* cytotoxicity effect of resting NK cells from different donors against U87 cells. (B) *In vitro* cytotoxicity effect of activating NK cells from different donors against U87 cells. (C) *In vitro* cytotoxicity effect of resting NK cells from different donors against U373 cells. (D) *In vitro* cytotoxicity effect of activating NK cells from different donors against U373 cells.

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