Immunological mechanisms of tumour progression

The rationale for natural killer cell based immunotherapy for glioblastoma

Justyna Kmiecik

Dissertation for the degree philosophiae doctor (PhD)

at the University of Bergen

2013

Scientific environment

This study was carried out in the Brain Tumour Immunology and Therapy Group – an integral part of the Translational Cancer Research Group at the Department of Biomedicine, University of Bergen, Norway. Part of the experimental work was performed at the Laboratoire d'Immunogénétique-Allergologie CRP-Santé, Luxembourg.

Norwegian Cancer Society funded my PhD fellowship and research stay in Luxembourg. The Bergen Medical Research Foundation and L. Meltzers Høyskolefond supported my research.

Acknowledgements

I would like to thank my main supervisor Dr Martha Chekenya Enger for her great supervision. You were always very helpful and supportive. I appreciate a lot your kindness, patience and good spirit that motivated me and inspired a lot.

I am also grateful to my co-supervisor Dr Jacques Zimmer for his support, advice and for enabling me to perform part of my work in his group at the Laboratoire d'Immunogénétique-Allergologie CRP-Santé, Luxembourg.

I would like to thank my internal co-supervisor Prof. Rolf Bjerkvig for his kindness and support.

I would like to express my gratitude for all the help and support I received from my former and present colleagues at the Brain Tumour Immunology and Therapy Group and the Translational Cancer Research Group. I especially appreciate the assistance of the technical personnel: Tove, Bodil, Ingrid and Halala. I would like to thank Per Øyvind Enger and Andrea Grass Navarro for their kindness and fruitful collaboration. Special thanks go to Monika, Neža, Kasia, Lina, Ercan, Krishna, Heidi, Janice, Hrvoje, Per Øystein and Jian for their warm hearts and to Marek for his friendship and for making me laugh so much.

I am grateful to my colleagues at the Laboratoire d'Immunogénétique-Allergologie CRP-Santé in Luxembourg for a warm welcome and kindness. I would like to thank René Brons for his helpful assistance, patience and kind support. Special thanks go to Aurélie Poli for her help, teaching and all fun we had together and to Marwan Sleiman for his great friendship.

I would like to thank all the patients and the voluntary blood donors that agreed to participate in this study for their generous gift enabling my research. I would like to acknowledge the Department of Neurosurgery, Haukeland University Hospital for their coordinating and participating in the collection of patients' blood and tumour tissue for the biobank.

Finally, I would like to thank all my dear friends and family for their love, patience and support.

Bergen, October 2013

Justyna Kmiecik

List of abbreviations

7-AAD - 7-amino actinomycin D

ADCC - antibody dependent cellular cytotoxicity

ADP - adenosine diphosphate

AML - acute myeloid leukemia

APC - antigen presenting cell

ATP - adenosine triphosphate

BBB - blood brain barrier

BCSFB - blood – cerebrospinal fluid barrier

BP - band pass (filter)

CD – cluster of differentiation

CDKN2A - cyclin-dependent kinase inhibitor 2A

CFSE - carboxy-fluorescein diacetate succinimidyl ester

CNS – central nervous system

CpG-ODN - CpG-oligodeoxynucleotides

CSF - cerebrospinal fluid

CT - computerised tomography

CTL – cytotoxic T lymphocyte

CTLA-4 – cytotoxic T lymphocyte antigen

DC - dendritic cell

DNA – deoxyribonucleic acid

DSB - double-strand break

EC - endothelial cell

ECM - extracellular matrix

EGFR - epidermal growth factor receptor

FGF - fibroblast growth factor

FLAIR - fluid-attenuated inversion recovery

FSC - forward scatter

GBM - glioblastoma

G-CSF - granulocyte colony-stimulating factor

GFAP - glial fibrilliary acidic protein

GFP - green fluorescent protein

GM-CSF - granulocyte macrophage colony-stimulating factor

GMP - good manufacturing practise

GvHD - Graft *vs.* Host Disease

GvL - Graft versus Leukemia (effect)

HA - haemagglutinin

HCMV - human cytomegalovirus

HLA - human leucocyte antigen

HN - HA-neuraminidase

HSCT - haematopoietic stem cell transfer

Idd - insulin dependent diabetes

IDH - isocitrate dehydrogenase

IDO - indoleamine-2,3-dioxygenase

IFNγ - interferon gamma

Ig - immunoglobulin

IL – interleukin

ILT - immunoglobulin-like transcript

ITAM - immunoreceptor tyrosine-based activation motif

ITIM - immunoreceptor tyrosine-based inhibitory motifs

KIR - killer immunoglobulin-like receptor

LAK cells - lymphokine activated killer cells

LP - long pass (filter)

MAPK - mitogen-activated protein kinase

MCP-1 - monocyte chemotactic protein – 1

MFI - mean fluorescence intensity

MGMT - O6-methyguanin DNA methyltransferase

MHC - major histocompatibility complex

MICA - MHC class I – related chains A

MICB - MHC class I – related chains B

MIP1-α - macrophage inflammatory protein 1-α

MIP1-β - macrophage inflammatory protein 1-β

MMP - matrix metalloproteinase

MRI - magnetic resonance imaging

MTIC - 3-methyl-(triazen-1-yl)imidazole-4-carboxamide

NCR - natural cytotoxicity receptors

NK cell - natural killer cell

NKG2 - natural killer group 2

NOD/SCID – non-obese diabetic/severe combined immunodeficiency

PBMCs - peripheral blood mononuclear cells

PDGFR - platelet derived growth factor receptor

PET - positron emission tomography

PGE2 - prostaglandin E2

PI3K - phosphatidyl inositol 3 kinase

PIP2 - phosphatidylinositol -3,4 diphosphate

PIP3 - phosphatidylinositol -3,4,5 trisphosphate

PMV - perivascular macrophages

Prkdc - protein kinase, DNA activated catalytic polypeptide

PSMA - prostate-specific membrane antigen

PTEN - phosphatase tensin homolog

PTK - protein tyrosine kinase

RANTES - regulated on activation normal T cell expressed and secreted

RB – retinoblastoma

RFP - red fluorescent protein

SAS - subarachnoid space

SP - short pass (filter)

SSC - side scatter

TGFβ - transforming growth factor-beta

T_h cell - T helper cell

TLR - toll-like receptor

TMZ - temozolomide™

TNF - tumour necrosis factor

TRAIL - TNF-related apoptosis inducing ligand

T_{regs} – T regulatory cells

ULBP - UL16 binding proteins

VEGF - vascular endothelial growth factor

WHO – World Health Organisation

Abstract

Glioblastoma (GBM) is the most frequent and malignant brain tumour, where the patients' median survival after diagnosis is only 14.6 months. Therefore, concerted research is required to develop novel treatments that will improve patients' outcome. Immunotherapy is one of the promising strategies for novel treatment. However, GBM develops multiple mechanisms of immune suppression and escape from immune surveillance. Moreover, steroids, chemotherapy and radiotherapy render GBM patients immunocompromised. These aspects need to be taken into consideration prior to development of immunotherapy tailored to GBM patients.

Natural killer (NK) cells are large, granular lymphocytes that are able to recognise and kill tumour cells and virus – infected cells without prior sensitization and co-stimulation. Therefore, they might be one of the most potent effectors for use in an immunotherapy. However, there is currently a great paucity of studies investigating their potential as therapeutic agents for GBM.

This study confirmed the prognostic significance of elevated T cell infiltration into the tumour in GBM patients. However, NK cells represented a minor population in the tumour microenvironment. Several mechanisms of the tumour's escape from immune attack were identified, including the induction of a novel population of $CD8^+CD28^-$ $Foxp3^+$ regulatory T cells and expression of CD73 and CD39 ectonucleotidases. Tumour cells expressed classical HLA class I molecules that are ligands for inhibitory killer immunoglobulin-like receptors (KIRs) mediating NK cells tolerance towards self cells. Tumour infiltrating macrophages/microglia displayed phenotypic features that were indicative of their tolerisation by the $CD8^+CD28^-Foxp3^+$ regulatory T cells. These features include: (1) down-regulated expression of CD40, CD80 and CD86 co-stimulatory molecules and (2) up-regulated expression of immunoglobulin-like transcripts 2, 3, 4 (ILT2,3,4). In the systemic circulation, decreased T helper (T_h) cells, up-regulated expression of the inhibitory receptor CTLA-4 on the T_h cells and increased plasma concentration of IL-10 were identified in GBM patients' blood

compared to healthy controls. Despite these integrated mechanisms of tolerance and immunological escape, a proportion of the tumour cells might be susceptible to immune cell – mediated cytotoxicity due to their expression of Fas ligand, and MICA stress – induced ligand for NK cell NKG2D activating receptor.

Thus, taking into consideration the GBM patients' local and systemic immune suppression and escape, we hypothesised that the use of allogeneic NK cells in a KIR receptor – HLA ligand mismatch setting might be an amenable strategy against GBM cells. We demonstrated that allogeneic, *ex vivo* cultured NK cells efficiently lysed GBM cells *in vitro* and *in vivo*. However, the NK cell efficacy was donor – dependent. The presence of KIR2DS2 and KIR2DS4 genes in donors' NK cells correlated with increased cytotoxicity *in vitro* and this effect was partially independent of the inhibitory KIR genes repertoire. Intracranial injection of a single dose of 10^6 allogeneic *ex vivo* cultured NK cells improved the survival of GBM – bearing mice compared to controls. NK cells obtained from donor possessing KIR2DS2 and KIR2DS4 genes were more effective than NK cells missing these genes and manifested in prolonged animals' median survival, increased tumour cell apoptosis, decreased proliferation and diminished angiogenesis. In contrast, treatment with double doses of 10^6 NK cells from each donor did not result in improved survival. We observed increased recruitment of macrophages into the brain parenchyma of all NK cell treated animals compared to controls. However, the animals receiving single dose of NK cells exhibited higher proportions of F4/80+ macrophages that expressed the IL-7 receptor (CD127), while higher proportions of microglia expressed CD40 co-stimulatory molecules compared to control group. The tumour cells up-regulated the expression of nestin and HLA-ABC after single dose NK cell treatment as a response to inflammation.

In summary, this work provides the rationale for using allogeneic NK cells against GBM and indicates possible targets for adjuvant immunotherapy, such as the CTLA-4 inhibitory receptor and the targets of $CD8^+CD28^-Foxp3^+$ regulatory T cells – induced immunotolerisation. Moreover, the results of the effect of KIR2DS2 and KIR2DS4 in NK cell – mediated cytotoxicity against GBM provide a novel insight into tumour

immunology. However, further research is required to evaluate the safety and efficacy of allogeneic NK cell – based therapy and to confirm the role of activating KIR receptors in determining the potency of NK cells against solid tumours.

List of publications

Paper I

Kmiecik J., Poli A., Brons N.H.C., Waha A., Eide, G.E., Enger P.Ø., Zimmer J. and Chekenya M.

Elevated CD3+ and CD8+ tumor-infiltrating immune cells correlates with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor microenvironment and at the systemic level.

In press, *Journal of Neuro-Immunology*

Paper II

Kmiecik J., Zimmer J. and Chekenya M.

Natural Killer cells in intracranial neoplasms: presence and therapeutic efficacy against brain tumours.

Revised version submitted, *Journal of Neuro-Oncology*

Paper III

Gras Navarro A.[*], **Kmiecik J.**[*], Zelkowski M., Leiss L., Zimmer J. and Chekenya M.

Activated NK cells are potent effectors against glioblastoma cells due to activating KIR-HLA ligand interactions *in vitro*

Under revision, *Immunology*

[*]contributed equally

Paper IV

Kmiecik J., Grass Navarro A., Enger P.Ø., Zimmer J. and Chekenya M.

Purified and activated allogeneic NK cells efficiently kill human glioblastoma *in vivo*

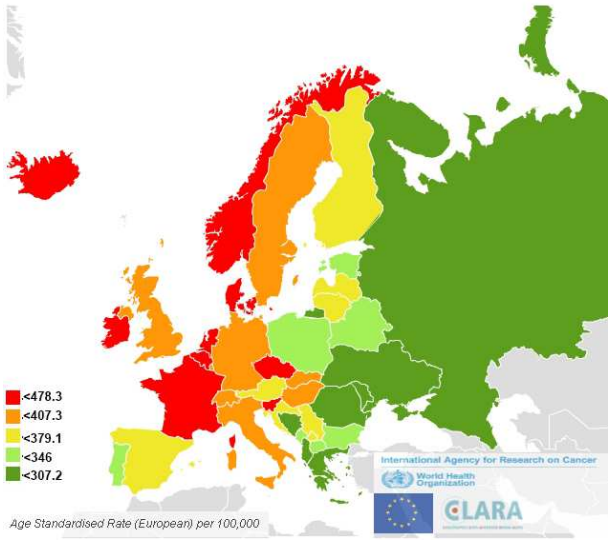
Manuscript

Contents

1. Introduction

Cancer is a disease affecting 12.7 million people worldwide every year [4]. Despite concerted efforts towards developing novel, more effective therapies, the mortality is still very high, with 7.6 million deaths every year [4]. According to the Global Burden of Disease, Injuries and Risk Factors Study 2010 (GBD 2010), cancer was the major cause of death globally in 2010, accounting for 15.1% of all deaths worldwide [5]. The estimated cancer incidence in Europe in 2012 was 355.7 new reported cases per 100000, with the highest incidence in Northern and Western European countries (Fig.1) [6]. Within all European countries, Norway has the second highest estimated cancer incidence of 451.2 per 100000 reported in 2012 [1, 6]. Moreover, the general cancer incidence in the western world has been steadily increasing the last decades, mainly due to life style choices such as tobacco smoking, obesity, but also due to the extended life expectancy and improved diagnostics.

Figure 1. Estimated incidence of cancer from all sites except non-melanoma skin

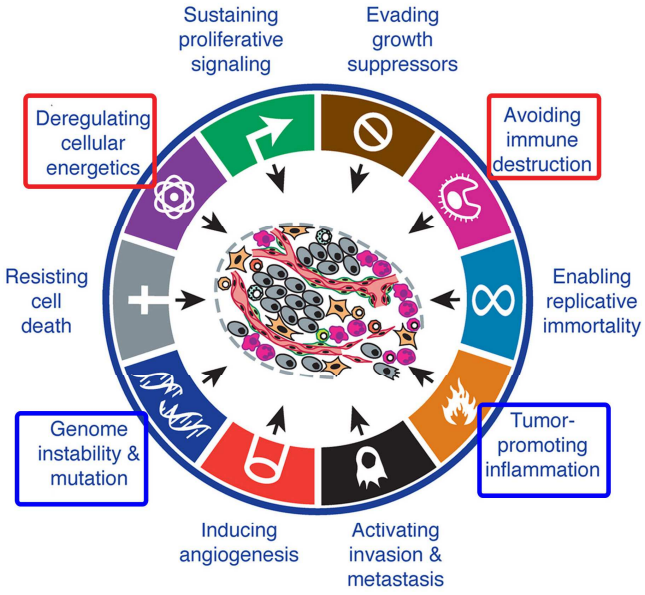


New reported incidences for both sexes in Europe, 2012. Demographics obtained from European Cancer Observatory: Cancer Incidence, Mortality, Prevalence and Survival in Europe. Version 1.0 (September 2012) European Network of Cancer Registries, International Agency for Research on Cancer. Available from http://eco.iarc.fr. [1]

1.1 The hallmarks of cancer

Cancer is a group of diseases that vary histologically depending on the putative cell of origin. Moreover, cancer is no longer considered as a homogenous mass of proliferating malignant cells, but rather, it is now viewed as heterogeneous tumour tissues that actively interact with their surrounding and permeating microenvironment. However, there are several features that are common for all cancers, described by Hanahan and Weinberg as the hallmarks of cancers (Fig.2) [7, 8].

Figure 2. Hallmarks of cancer. This figure summarizes characteristic features common for all cancers. Red and blue frames indicate emerging and enabling hallmarks, respectively. Figure obtained and modified from [7]. Reprinted with permission.



Cancer is characterised by uncontrolled growth and unlimited cell division due to acquired ability of sustaining autocrine proliferative signals, avoiding growth suppressing mechanisms and avoiding cell death (Fig. 2) [8]. These three hallmarks are

the result of a series of gain of function mutations affecting proto-oncogenes and loss of function mutations affecting tumour suppressor genes. All healthy cells possess multiple mechanisms of DNA repair and sensors of DNA damage that either remove the mutation or trigger the programmed cell death to prevent cells with altered DNA from proliferating. However, if this system is affected, cells accumulate mutations with each division. Therefore, genomic instability has been proposed as a feature enabling malignant transformation (Fig. 2) [7]. Moreover, cancer cells acquire unlimited replicative potential due to restored activity of telomerase [8]. In order to maintain the supply of nutrients and metabolic waste removal, tumour cells induce angiogenesis, a process of new blood vessel formation from the sprouting of existing vasculature [8]. Rapid cell growth and proliferation of tumour cells frequently result in reprogramming the metabolism, therefore this feature has been proposed as one of the new emerging hallmarks (Fig. 2) [7]. Eventually, malignant cells are able to invade blood or lymphatic vessels and migrate to distant parts of the body to give rise to new tumours in a process called metastasis [8]. Recently the immune system has been postulated to play an important role in tumour progression. On the one hand, immune cells are able to recognise and eliminate transformed cells thus preventing tumour development as well as protecting from metastasis. Tumour cells have been shown to develop multiple mechanisms to escape immune defence, therefore this has been proposed as a new emerging hallmark of cancer (Fig. 2) [7]. However, chronic inflammation resulting from immune response has been shown to promote tumour growth due to release of growth, survival and pro-angiogenic factors. Thus, the inflammation has been proposed as a feature enabling tumour progression (Fig. 2) [7].

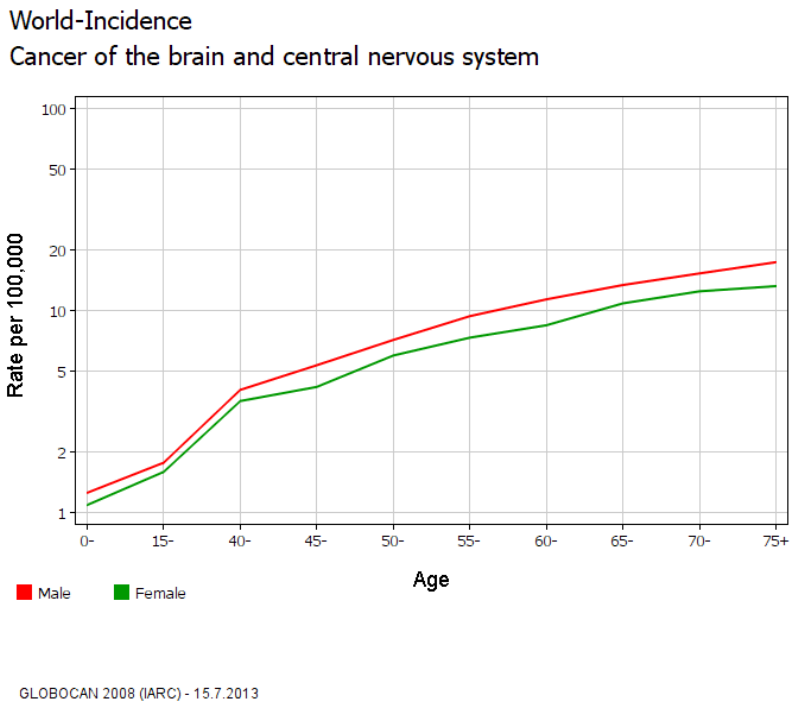
All the above described hallmarks of cancer not only represent features common for all cancers, but also indicate key therapeutic targets, such as, for example, inhibiting the vascular endothelial growth factor (VEGF) signalling to target angiogenesis. Nevertheless, the main challenges in treating cancer are: (1) to remove all abnormal cells within the heterogeneous tumour (2) to overcome the tumour's multiple mechanisms of avoiding cell death and (3) to selectively target tumour cells without damaging the normal tissue. In addition, the malignant cells often develop multiple

mechanisms of drug-resistance. Therefore, multiple or non-selective treatment strategies might be required to eliminate all cancer cells. However, such approaches result in side effects, as they inevitably affect also the normal, healthy cells.

1.2 Brain tumours

Primary brain tumours are neoplasms originating from intracranial, neuroepithelial tissues and they account for approximately 1,4% of all cancers [9]. The incidence of brain and central nervous system (CNS) tumours increases with age and is higher in males than females (Fig. 3) [4].

Figure 3. Age-specific incidence of brain and CNS cancer in males and females. In each age group the incidence rate of brain and CNS cancer is higher in males (red line) than females (green line). Data obtained from GLOBOCAN 2008 v2.0 [4]. Available from: http://globocan.iarc.fr



To date, the only known risk factor related to brain tumours is an early exposure to ionising radiation at therapeutic doses in childhood [9, 10]. Incidence of astrocytic gliomas is increased in children with acute lymphocytic leukemia who have had prior brain radiotherapy. Also several heritable genetic disorders, like tuberous sclerosis, neurofibromatosis and nevoid basal cell carcinoma syndrome have been linked to predisposition to brain tumours [9], however, only about 5% of primary brain tumors have known hereditary factors. On the other hand, it has been shown that diseases such as asthma and allergies are related to decreased risk of developing brain tumour [10].

1.2.1 Classification of brain tumours

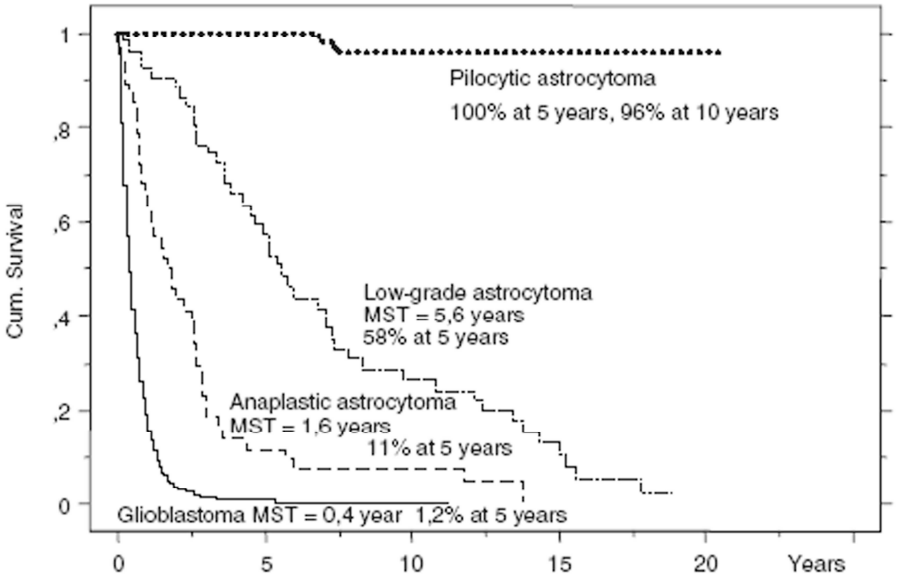
Brain tumours are classified according to a system established by the World Health Organisation (WHO) where the tumours are categorised by histological features depending on their resemblance to the putative cell of origin [11]. For example, tumours exhibiting morphological and histological features of astrocytes, such as expression of glial fibrilliary acidic protein (GFAP), are classified as astrocytic tumours [11].

The WHO classification system also assigns each tumour type a grade of malignancy that is based on histopathological and molecular characteristics (Table 1). The grading also reflects the patients' prognosis and has implications for their clinical management. Grade I corresponds to benign tumours with better prognosis, grade II and III tumours display features of malignant progression, while grade IV tumours are the most malignant and exhibit the poorest prognosis (Fig. 4) [11]. In addition to nuclear atypia and mitotic activity observed in lower grade tumours, the presence of microvascular proliferation and/or pseudopalisading necrosis is required to classify the tumour as grade IV (Table 1) [11, 12].

Table 1. The histological criteria for WHO grading of astrocytic tumours. Prepared based on [11].

Grade of malignancy	Histological designation	Morphological criteria
I	Pilocytic astrocytoma	None
II	Diffuse astrocytoma	Nuclear atypia
III	Anaplastic astrocytoma	Nuclear atypia and mitotic activity
IV	Glioblastoma	Nuclear atypia, mitotic activity, microvascular (endothelial) proliferation and/or necrosis

Figure 4. Comparison of survival of patients with astrocytic brain tumours of different grades. Pilocytic astrocytoma (grade I), low grade astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma (grade IV). Cum. – cumulative, MST – mean survival time. Reprinted with permission. Published in [13].



1.2.2 Glioblastoma

GBM is the most malignant brain tumour (grade IV according to WHO) [11]. It accounts for 12-15% of all intracranial tumours and for 54% of all gliomas. GBM is classified as an astrocytic tumour as it is composed mostly of neoplastic astrocytes. However, it is a very heterogeneous tumour and several histological variants have been distinguished: glioblastoma with oligodendroglioma component accounting for 12% of all GBMs, giant cell glioblastoma (5% of all GBMs), small cell glioblastoma and gliosarcoma (2% of all GBMs). GBMs may occur in the subcortical white matter of the cerebral hemispheres in all lobes of the brain [11]. They mostly afflict adults, and more frequently men than women [11]**.** GBM can result from successive malignant progression of lower grade astrocytic tumours (secondary GBM), but approximately 85% of GBMs occur as *de novo* tumours (primary GBM) without a prior evidence of precursor lesion [11, 14]. The primary GBMs are more common in older patients, while secondary GBMs usually develop in younger patients [11, 14].

1.2.2.1 Symptoms

There are no specific neurological symptoms associated with GBM. However, focal symptoms may arise, mostly as a result of elevated intracranial pressure [11] and these include epileptic seizures, early morning headache, nausea and vomiting [11, 15]. However, both intracerebral and extracerebral tumours may cause epileptic seizures. Moreover, differences in seizure frequencies exist between tumours of the same histological type indicating that tumour related epilepsy may derive its aetiology from several mechanisms, including tumour histological type, grade of malignancy [16], location, as well as, treatment modality [17], environmental and functional changes involving pH, neurotransmitters, such as glutamate [18], vascular and metabolic changes [19]. Other symptoms related to the location of the tumour may become apparent, such as personality changes (frontal cortex), visual disturbances (occipital lobe) and hemiparaplegia (motor cortex of the parietal lobe).

1.2.2.2 Diagnosis

GBM is diagnosed by a combination of methods including neurological examination, neuroimaging involving computerised tomography (CT), magnetic resonance imaging (MRI) or positron emission tomography (PET) techniques, and surgical or needle biopsy. On T1-weighted MRI with contrast, GBM manifests as a hypo-intense lesion with contrast-enhanced ring structure. The hypo-intense region represents central areas of tissue necrosis, while the enhancing ring represents the cellularised regions vascularised by permeable vessels that permit leakage of contrast agent and thus detection of the tumour [11]. On T2-weighted and fluid-attenuated inversion recovery (FLAIR) images, the lesion appears hyper-intense. The GBM also tends to exhibit more peritumoural oedema. CT scans usually visualize ring-like contrast enhancement around a dark hypo-dense necrotic area [11]. The regional glucose consumption observed on PET scans corresponds to cellularity and is correlated with reduced survival [11]. On magnetic resonance spectroscopy gliomas exhibit reduced *N*-acetyl aspartate, increased choline, and decreased creatine levels [20]. Additionally, a lactate peak is a frequent occurrence in higher grade tumours [20].

However, the final diagnosis is based on histopathological examination of the haematoxylin and eosin stained tumour tissue obtained by stereotaxic needle biopsy or craniotomy followed by tumour resection. Typical histological features of GBM are: (1) cellular heterogeneity, (2) the presence of large, multinucleated tumour cells, (3) microvascular proliferation that manifests as multiple layers of endothelial cells, or "glomerular tufts" often located close to areas of necrosis and (4) pseudopalisading structures comprised of central necrotic area surrounded by apoptotic tumour cells [11].

1.2.2.3 Molecular features of glioblastoma

Several genetic and epigenetic aberrations characterise GBM. These aberrations result in the altered regulation of genes modulating the growth and survival pathways, as well as, the cells' evasion from apoptosis. The most common alterations are: (1) p53 loss or mutation [9, 11] (2) epidermal growth factor receptor (EGFR) amplification or

mutation [9, 11] (3) deletion or mutation of phosphatase tensin homolog (PTEN) gene [9, 11] (4) MDM2 gene amplification or overexpression [9, 11] (5) platelet derived growth factor receptor (PDGFR) overexpression or amplification [9, 11] (6) cyclin-dependent kinase inhibitor 2A (CDKN2A) gene alterations [9, 11] (7) isocitrate dehydrogenase 1 and/or 2 (IDH1 and IDH2) mutation [21].

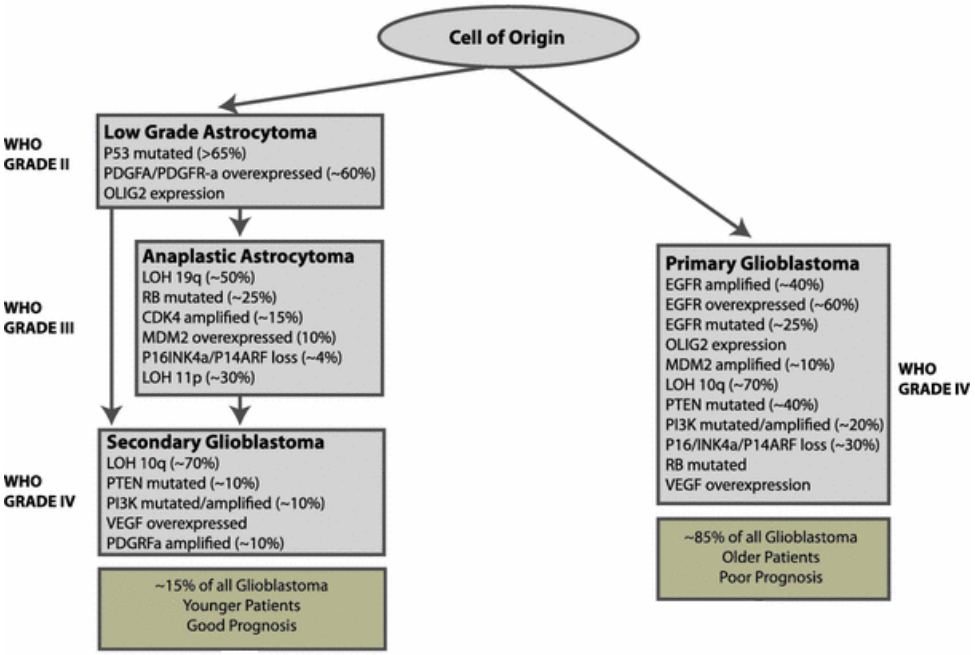
1.2.2.3.1 Primary vs. secondary GBM

Numerous studies demonstrated that *de novo* GBMs harbour different and usually mutually exclusive genetic alterations from secondary GBMs that arise from successive malignant progression of lower grade gliomas (Fig. 5). However, there is a great deal of redundancy and mutations often target alternative genes of the same signalling pathways [9, 11].

Primary GBMs display frequent EGFR amplification, overexpression and/or mutation [9, 11], Fig 5. Mutation of the EGFR gene most often results in expression of a receptor with ligand-independent, constitutive activity (EGFRvIII) [9, 11]. EGFR signalling regulates growth signals, thus its amplification or increased activity leads to uncontrolled progression through the cell cycle and ultimately, tumour growth. Amplification or overexpression of MDM2 gene is common in *de novo* GBMs and results in blocking the activity of p53 protein [9, 11], Fig 5. P53 signalling pathway induces apoptosis of the cell with DNA damage, therefore alterations in this control point lead to continued proliferation and growth of transformed cells harbouring multiple mutations. However, the p53 gene is usually not directly altered in *de novo* GBMs [9, 11], Fig 5. Loss of p16 expression results from alterations in CDKN2A gene and is also more frequently altered in primary than secondary GBM [9, 11]. Lack of p16 protein affects retinoblastoma (RB) signalling pathway that regulates cell cycle at the G1/S checkpoint, allowing the tumour cells' uncontrolled transition to S phase [9, 11]. The alteration of PTEN tumour suppressor gene is commonly reported in primary GBM [9, 11]. PTEN is a phosphatase and functions as a tumour suppressor that negatively regulates phosphatidyl inositol 3 kinase (PI3K) activity by dephosphorylating phosphatidylinositol -3,4,5 trisphosphate (PIP3) to

phosphatidylinositol -3,4 diphosphate (PIP2) and thereby terminating PI3K signaling [22]. Mutations of the *PTEN* gene in GBMs result in elevated levels of PIP3, through which PI3K hyperphosphorylates PDK1/AKT resulting in enhanced survival and proliferation of the tumour cells [23].

Figure 5. Molecular features of primary *vs.* secondary GBM. Secondary GBM results from malignant progression of low grade astrocytoma characterised by frequent mutation of P53 and PDGF/PDGFR overexpression. Loss of heterozygosity (LOH) on chromosomes 11p and 19q and RB mutation is common for anaplastic astrocytoma (grade III). LOH on chromosome 10q and VEGF overexpression leads to progression to GBM (grade IV). In contrast to secondary GBM, the most frequent aberrations observed in primary GBM are EGFR overexpression, amplification and mutation, LOH on 10q chromosome, PTEN mutation and p16 loss. Obtained and modified from [14]. Reprinted with permission.



Contrary to *de novo* GBM, in the secondary GBM the EGFR and MDM2 gene alterations are much less frequent, while p53 is commonly deleted or inactivated [9, 11], Fig 5. Typical for secondary GBMs, is also the over-expression of PDGFR that

results in increased tumour cell proliferation [9, 11]. The loss of RB function is frequently observed in secondary GBM [9, 11]. The PTEN gene alterations also occur, albeit, less frequently than in primary GBM [9, 11]. The recently identified glioma specific mutation, IDH1/2 mutation is more common for secondary than primary GBM and has been proposed as a marker to distinguish the GBM subtypes [21]. The presence of mutated IDH1/2 is associated with prolonged patients' survival, however, the role of the wild-type and mutant protein in tumour progression is not fully understood yet [21]. Taken together, primary and secondary GBMs display different patterns of genomic alterations reflecting two different mechanisms of GBM formation: (1) spontaneous growth of high grade tumour and (2) malignant progression through lower grade tumours.

1.2.2.4 Biological behaviour of GBM

The biology of GBM fully resembles the characteristic features of malignant tumours, such as aggressive growth, angiogenesis and invasion to surrounding tissue. However, GBM rarely metastasises outside the CNS.

1.2.2.4.1 Proliferation

GBM is a highly proliferative tumour as indicated by the presence of mitotic figures on histological examination [11]. The proliferative activity can also be measured by immunohistochemistry staining with antibody against Ki67 protein, that is expressed by actively proliferating cells but absent in resting cells. High Ki67 labelling index is associated with aggressive growth and it increases with the tumour grade [11]. GBM is characterised by high Ki67 labelling index of 15-20% compared to lower grade brain tumours (5-10% in anaplastic astrocytoma grade III and less than 4% in astrocytomas grade II) [11].

1.2.2.4.2 Angiogenesis

Growing tumours require nutrients, oxygen supply, and metabolic waste removal carried out by the blood circulation. However, with increasing size, the existing vasculature is insufficient and formation of new blood vessels is activated [24].

Angiogenesis is one of the histological hallmarks of GBM [11]. It is triggered by up-regulating pro-angiogenic signals induced both directly by tumour cells and also by stromal cells of the tumour microenvironment as a result of hypoxia occurring in the tumour areas with insufficient oxygen supply [9, 25]. The best characterised pro-angiogenic factors associated with glioma are: (1) vascular endothelial growth factor (VEGF) [26, 27] produced by tumour, stromal and inflammatory cells and VEGF receptors over-expressed on glioma cells, (2) fibroblast growth factor (FGF) and FGF receptors up-regulated on glioma and endothelial cells [24] and (3) extracellular matrix (ECM) remodelling by matrix metalloproteinases (MMPs) [9, 25]. The above mentioned growth factors induce the angiogenic switch [28] that stimulates the proliferation of endothelial cells and their remodelling of ECM facilitates their migration and also release and activation of more pro-angiogenic factors [9, 25]. However, tumour-induced angiogenesis results in forming disorganised and leaky blood vessels as the endothelial cells associated with the tumour vasculature do not form proper tight junctions and tumour capillaries are often not fully covered with astrocytic endfeet [9].

1.2.2.4.3 Diffuse Invasion

The infiltration of the tumour cells into surrounding tissue is a common feature of GBM. Glioma cells frequently migrate along white matter tracts and through the corpus callosum into the contralateral hemisphere forming the *so-called* "butterfly glioma" [11]. GBM cells often invade also other parts of the brain and form new lesions [11]. The highly invasive nature of GBM also results in the occurrence of recurrent tumours arising from neoplastic cells remaining in adjacent tissue after the tumour resection. However, invasion into the subarachnoidal space, infiltration into the blood vessels and subsequent metastases outside the brain occur very rarely [11].

1.2.2.5 Current standard treatment of GBM

Routinely, therapy is initiated with administration of steroids, such as dexamethasone (decadron) 4 mg pre-operatively the morning prior to surgery to reduce oedema and inflammation. However, if the patient presents with oedema, decadron may be

administered for the clinical management but it is tailored for each patient and assessed on a regular basis due to potential side effects. Furthermore anticonvulsant medication may be administered for the management of epileptic seizures when required. The current standard treatment for GBM patients includes surgery followed by concomitant chemo- and radiotherapy. The efficiency of surgical resection depends on the location of the tumour, however, the principle is to remove as much of the tumour bulk and associated tissue as possible without affecting the brain functionality [15]. Nevertheless, due to the highly invasive and infiltrative nature of GBM, residual tumour cells are inevitable, both in the adjacent resection margins and very often in the distant brain parenchyma. Therefore, 3-6 weeks post subtotal-surgery patients are administered concurrent ionising radiotherapy with chemotherapy depending on their performance status measured by the Karnofsky score [29]. External beam radiation is administered as fractionated doses of 2Gy daily, 5 days a week for 6 weeks, achieving a total 60Gy dose [30]. The major aim of this treatment is to induce double-strand break (DSBs), that is the most toxic form of DNA damage for the cell [9]. The DNA damage results in apoptosis or cell cycle arrest [9]. The chemotherapy is given during the course of radiotherapy in order to induce maximal DNA damage [30]. To date, temozolomide™ (TMZ) is the most efficient drug for GBM patients [31]. The standard protocol of TMZ administration is 75mg per square meter of body-surface area (mg/m^2) daily, 7 days per week during the course of radiotherapy [31]. Four weeks after the chemotherapy is completed, patients are given up to 6 cycles of adjuvant TMZ treatment at a dose of 150-200 mg/m^2 daily for 5 days every 28-day cycle [31]. TMZ is an orally administered pro-drug that is metabolised to 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC) [30]. MTIC induces DNA damage by methylation at different positions, however, N7-guanine and N3-adenine being most frequent, while methylation of O6-guanine is the most significant for the anti-tumour effect of TMZ [30]. An important predictive factor for patients' clinical response to TMZ is the methylation status of O6-methylguanin DNA methyltransferase (MGMT) gene promoter [30]. MGMT is an enzyme broadly expressed by normal and neoplastic tissues, however, the promoter of the MGMT gene is often hypermethylated in tumour

tissue [30]. MGMT repairs O6-guanine methylation induced by TMZ, thus silencing its gene expression is associated with better efficiency of TMZ treatment [30].

1.2.2.6 Prognostic and predictive factors

Prognostic factors are patient's characteristics that are associated with their prognosis independently of the treatment received. Predictive factors are features associated with patient's response to specific treatment.

Important established prognostic factors in GBM are patient's age at diagnosis, patient's performance status measured by the Karnofsky scale and neurological function [32]. Younger patients (<50 years) and those with better performance status and intact neurological functions tend to survive longer [32]. Moreover, radical tumour resection is also associated with better prognosis [32]. Prolonged survival was also observed in GBM patients with IDH1 mutation compared to those without this mutation [21]. As mentioned above, the methylation status of MGMT gene promoter predicts clinical response to TMZ treatment, however, it has also been demonstrated to be a prognostic factor [33]. However, all GBM patients receive the standard treatment including TMZ regardless of the methylation status of MGMT gene promoter.

1.3 Brain immunology

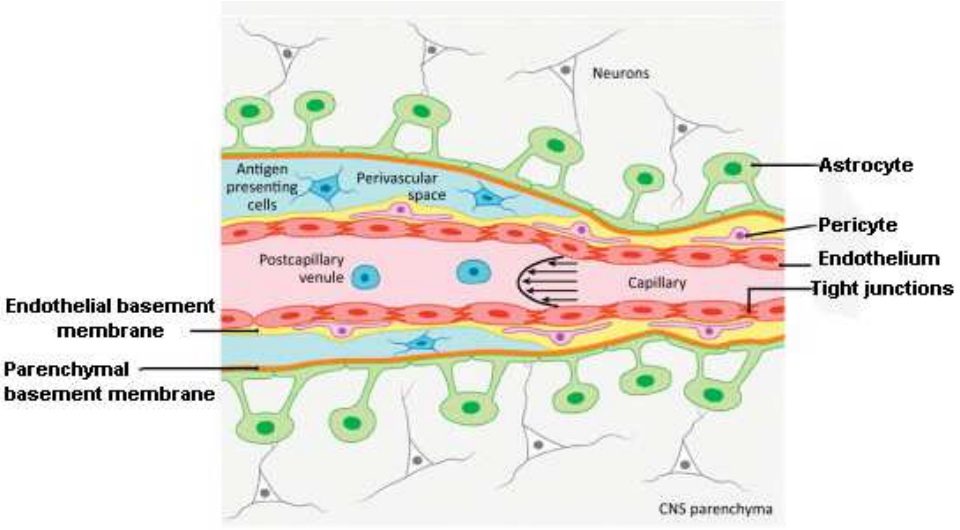
For a long time, the brain was considered as an immune-privileged organ and several lines of observations supported this dogma. The lack of immune surveillance was postulated due to the presence of the blood brain barrier (BBB), lack of lymphatic drainage, low levels of major histocompatibility complex (MHC) expression required for antigen presentation and low numbers of professional antigen presenting cells (APCs). On the other hand, occurrence of autoimmune diseases and inflammation within the brain suggests that the brain is not fully protected from the immune system. Indeed, it is established that activated immune cells are able to traverse the BBB [3] and enter the brain with cerebrospinal fluid (CSF) circulation [34]. Moreover, microglia have been shown to express MHC class II and perform immune functions

similar to macrophages [35]. Therefore, microglia are considered the brain resident antigen presenting cells (APCs). In addition, the central nervous system (CNS) antigens can be transported to the deep cervical lymph nodes with CSF flux via the nasal mucosa and afferent lymphatics [34]. The fact that the immune system has access to the brain and furthermore, that the BBB is variably disrupted at the brain tumour site, implies that anti-tumour immune responses can potentially occur in brain cancers. This perspective opens the possibility of developing an immunotherapy for brain tumour patients.

1.3.1 Blood – brain barrier and blood – cerebrospinal fluid barrier

The BBB is a physical barrier between the brain and peripheral blood circulation. The physiological functions of the BBB are (1) to protect the brain against infectious and toxic agents that could potentially enter the brain via blood circulation (2) to regulate the supply of nutrients and metabolic waste removal and (3) to maintain homeostasis [9]. The BBB is composed of endothelial cells (ECs) at the lumen of blood vessels, perivascular pericytes covered with basal lamina and further supported by astrocytes' end-feet (Fig. 6) [9].

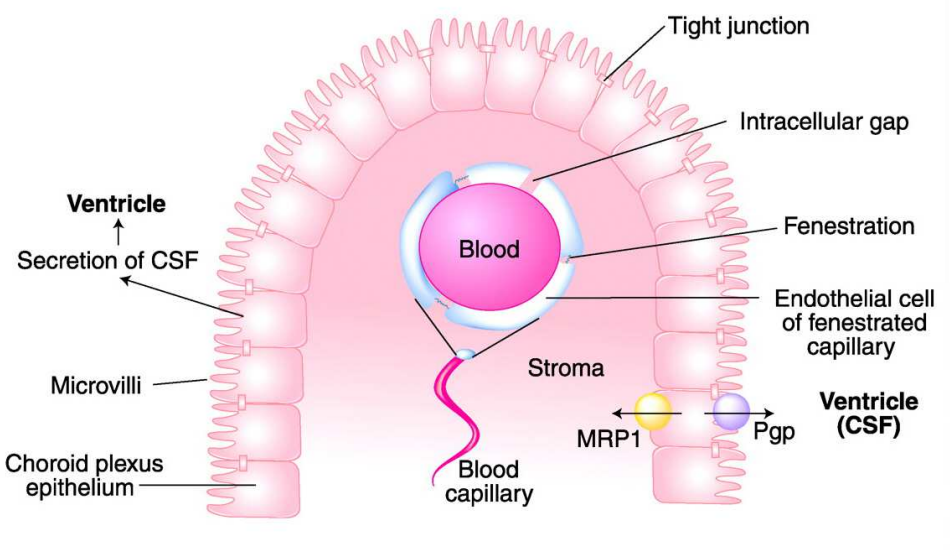
Figure 6. The structure of blood – brain barrier (BBB). The BBB is formed by endothelial cells connected by tight junctions and covered with basement membrane. Pericytes and astrocyte endfeet further support the BBB structure. Reprinted with permission. Published in [36].



The ECs of brain capillaries are continuously connected to each other by tight junctions, they lack fenestrae and have limited vesicles for pinocytosis (Fig. 6). These features mainly contribute to reduced permeability of the cerebral vasculature [9]. Nutrient uptake is carried out by selective molecular carriers and the ion passage is maintained by ion transporters [9]. Pericytes can further support the BBB function by their capability to phagocytose the molecules that manage to cross the endothelial wall [9]. The basal lamina is composed of collagen, fibronectin and heparin sulphate proteoglycans produced by ECs and astrocytes [9]. The extracellular matrix components that are unique for brain vasculature are laminin 1 and 2 [9]. Astrocyte end-feet cover the surface of brain blood vessels and capillaries, maintaining the high electrical resistance tight junctions in ECs (Fig. 6) [9]. Perivascular macrophages (PMV) also play important roles in the regulation of permeability of the brain microvessels and at the boundary of arterioles and capillaries, but they are absent in brain capillaries [9]. They are able to phagocytose pathogens and macromolecules and they contribute to the regulation of cellular trafficking across the BBB [9]. In contrast

to the BBB, the blood – cerebrospinal fluid barrier (BCSFB) is based on the tight junctions formed by choroid plexus epithelium while the endothelial cells of BCSFB lack the tight junctions (Fig. 7) [37]. Due to the fenestrated endothelium as well as absence of astrocyte end-feet and pericytes, the BCSFB is more permeable than the BBB (Fig. 7).

Figure 7. The structure of blood – cerebrospinal fluid barrier (BCSFB). The BCSFB is maintained by tight junctions connecting the epithelial cells of choroid plexus. The endothelial cells forming the vessel wall of choroid plexus capillaries do not form tight junctions and are fenestrated. The transport across the BCSFB is maintained by molecular transporters such as multidrug resistance-associated protein 1 (MRP1) and P-glycoprotein (Pgp). Reprinted with permission. Published in [38].

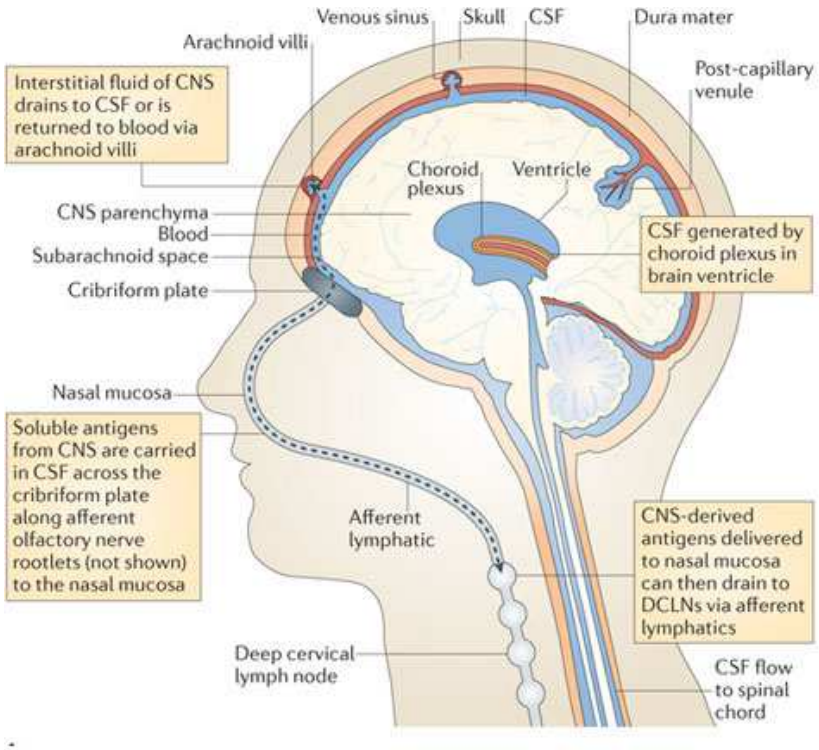


1.3.2 Immune surveillance in the central nervous system

Despite the presence of the BBB and BCSFB, under physiological conditions the CNS is under constant immune surveillance and active immune response can be triggered in various pathological conditions like infection, injury, as well as autoimmune disease. Even though there is no lymphatic drainage in the CNS, the CSF produced in the choroid plexus and circulating within the subarachnoid space (SAS) might be considered as an equivalent of lymph [34]. The CSF is reabsorbed at the arachnoid villi

into the venous blood, however, CSF draining along the cranial and spinal nerves can also reach the lymph nodes, e.g. deep cervical lymph node (Fig. 8) [34].

Figure 8. CSF circulation in the CNS and drainage to deep cervical lymph node. CSF is produced by the choroid plexus in the ventricle, circulates within subarachnoid space (SAS) and most of it is reabsorbed to blood via arachnoid villi. However, part of the CFS can carry the CNS-derived antigens into the deep cervical lymph node via afferent lymphatics along olfactory nerve. Reprinted with permission. Published in [34].

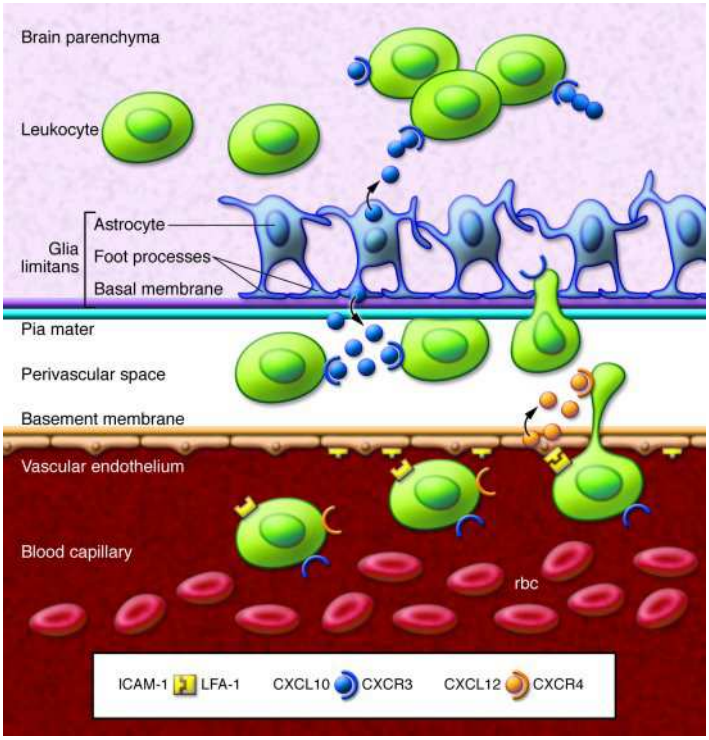


Therefore, CSF is a carrier of CNS – derived soluble antigens that enter the CSF circulation with the interstitial fluid from brain parenchyma [34]. Once entered the CSF, these antigens can be sampled by meningeal and choroid plexus macrophages or delivered to lymph nodes [34]. Moreover, CNS is patrolled by peripheral immune cells that enter the CSF via the choroid plexus [34]. The major cellular component (more than 90% of all cells) of CSF is memory T cells, predominantly $CD4^+$ T cells [34, 39].

Other immune cells detected in the CSF of healthy brain are B cells, NK cells, dendritic cells (DCs), monocytes and naïve T cells [34, 39].

Under normal physiological conditions the only immune cells present in the brain parenchyma are microglia [34]. However, it has been demonstrated that T cells and B cells activated upon various pathological conditions are able to cross the BBB and access the brain directly from the blood vessels and capillaries (Fig. 9) [3, 34].

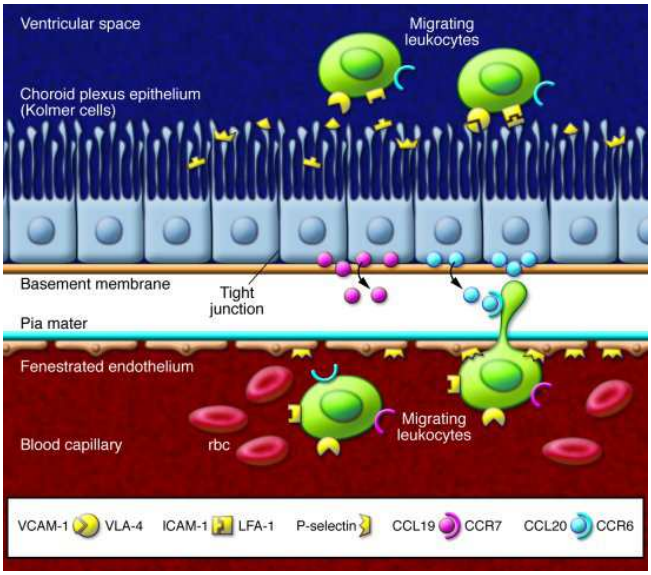
Figure 9. Leukocyte trafficking across the BBB.



The attachment of rolling activated leukocytes is mediated by interactions of adhesion molecules (ICAM-1) and integrins (LFA-1). Further signalling with adhesion molecules, integrins and selectins is required for diapedesis into perivascular space (e.g. interaction of CXCR4 with CXCL12). Migration across the glia limitans is facilitated by further positive signals from CNS parenchyma, including chemokines. However, high concentration of CXCL10 causes retention of immune cells within the perivascular space. Reprinted with permission. Published in [3]

The immune cell trafficking across both BBB and BCSFB is initiated by rolling along the vessel wall, adhesion and diapedesis across the vascular endothelium and is followed by migration across the glia limitans (basal membrane and astrocyte end-feet) into the brain parenchyma (Fig. 9). Alternatively, the cells may enter the CSF across the choroid plexus epithelium (Fig. 10) [3]. These processes require expression of several adhesion molecules, integrins, selectins, chemokines and their receptors [3]. However, the exact molecular mechanism of immune cell trafficking in the CNS has not yet been fully resolved.

Figure 10. Leukocyte trafficking across the BCSFB.



Activated leukocytes undergo rolling and adhesion to the vessel wall mediated by integrins and adhesion molecules. After crossing the fenestrated endothelium, they migrate to choroid plexus epithelium attracted by chemokines (CCL19, CCL20) expressed on the basolateral side of the epithelial cells and enter the CSF in the ventricle. Reprinted with permission. Published in [3]

Microglia are the only resident immune cells within the brain parenchyma. They are cells of myeloid origin that, besides supporting neurons' functionality, perform also functions similar to tissue specific macrophages [35, 40, 41]. They are able to phagocyte the debris and dead cells occurring in the CNS [40]. They constitutively express MHC class II molecules and they perform constant surveillance of the extracellular space, thus they can sample and present antigens [40]. Upon activation, they can also secrete cytokines such as tumour necrosis factor (TNF), interferon

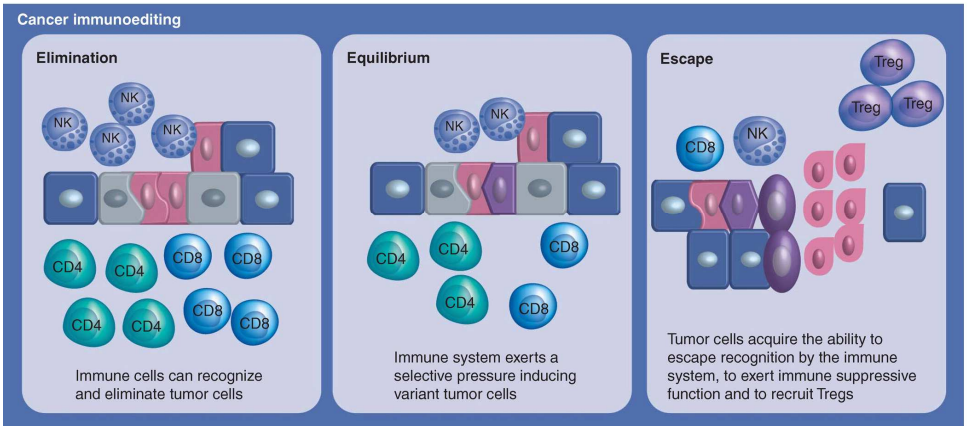
gamma (IFNγ), interleukin (IL)-1 and chemokines to recruit other immune cells to the site of infection or injury [35]. Moreover, microglia are also able to respond to certain infections, for example they can recognise bacterial lipopolysaccharide [35]. Therefore they serve as a first line immune defence in the CNS. Astrocytes and other glial cells have also been shown to contribute to brain immunity by producing various cytokines and chemokines [40].

1.3.3 The immune status of glioblastoma patients

As described above, under physiological conditions parenchymal microglia, meningeal, choroid plexus and perivascular macrophages and T cells circulating in the CSF patrol the CNS. Thus, the anti-tumour immune response can be potentially triggered in the brain cancer, including GBM patients. On the other hand, GBM develops multiple mechanisms of immune escape. Moreover, the standard treatment can also contribute to GBM patients' immune suppression. Novel adjuvant immunotherapies hold a promise of improving the patients' outcome by stimulating the anti-tumour immune response and/or targeting tumour immune escape and immunosuppressive mechanisms. However, for developing immune-based treatment it is of great importance to investigate the immune status of GBM patients.

An interesting concept of cancer immunoediting emerged recently [42]. In this concept, the interactions of immune system with tumour can be divided into three major steps (Fig. 11). At the early stage of cancer development, the immune system eliminates susceptible tumour cells (elimination) that leads to selection of immune-resistant cells (equilibrium) and further to develop immune escape and immunosuppressive mechanism (escape) [42].

Figure 11. The concept of cancer immunoediting. The immune cells eliminate susceptible tumour cells (elimination) that leads to selection of immune-resistant cells (equilibrium) that further develop immune escape and immunosuppressive mechanisms (escape). Reprinted with permission. Published in [43].



This concept seems to fit also GBM and potentially has implications for the efficiency of immunotherapies [43]. For example, GBM treated with targeted therapies based on single tumour associated antigen, like anti-VEGF or anti-EGFRvIII treatments, after initial regression due to the clearance of treatment-susceptible cells (elimination) may relapse due to selection of resistant cells (equilibrium) and develop alternative signalling pathways and/or resistance to immune mediated killing (escape) in case of immune-stimulating therapies, like therapeutic antibodies and vaccines [43].

1.3.3.1 Immune cell populations infiltrating the brain tumours

1.3.3.1.1 Macrophages/microglia

Brain resident microglia together with infiltrating macrophages are the major immune cell population within GBM microenvironment and comprise approximately one third of all cells in the tumour tissue [44, 45]. There is no specific marker that could be used to distinguish microglia from macrophages histologically but most flow cytometry based studies define microglia and macrophages as $CD11b^{+}CD45^{low}$ and $CD11b^{+}CD45^{high}$ populations, respectively [46]. Both microglia and macrophages can represent two functionally different activation states depending upon exposure to

specific stimuli and environmental conditions. Stimulation with pro-inflammatory cytokines such as IFNγ, TNF and recognition of pathogens induce the *so-called* M1 phenotype (classically activated), while anti-inflammatory cytokines like IL-4, IL-13 and IL-10 induce M2 type (alternatively activated) macrophages/microglia [47, 48]. The M1 type macrophages/microglia are important for removal of pathogens, antigen presentation and stimulation of T cell responses [47, 48]. They up-regulate co-stimulatory molecules CD80 and CD86, MHC class II and toll-like receptors 2 and 4 (TLR2 and TLR4) and produce pro-inflammatory cytokines such as IFNγ, TNFα, IL-12 and IL-23 [47, 48].

On the other hand, M2 type macrophages/microglia have less antigen presenting capacity and are involved in tissue repair and immune modulation by producing anti-inflammatory cytokines like IL-10 and TGFβ [47, 48]. Phenotypically, they are characterised by up-regulation of mannose receptor (CD206) and scavenger receptors CD163 and CD204 and decreased expression of co-stimulatory molecules CD80 and CD86 [47, 48]. The polarization of macrophages/microglia into M1 and M2 type is considered to have implications in various pathological conditions, including brain tumours [47]. It is believed that M1 type macrophages/microglia can potentially mediate anti-tumour activity, while M2 type macrophages/microglia have been demonstrated to support glioma progression [45, 46]. The tumour-supporting M2 type macrophages/microglia seem to be the predominating immune cell population within the tumour microenvironment as a result of glioma – mediated recruitment and immunomodulation [46].

1.3.3.1.2 Lymphocytes

A number of studies demonstrated T cell infiltration of both $CD4^+$ and $CD8^+$ phenotype in GBM [49-53]. The degree of T cell infiltration and their distribution within the tumour tissue varied between patients [50-52]. Tumour infiltrating T cells were usually grouped around blood vessels, necrotic areas, with single cells scattered in the tumour parenchyma [50, 51]. Most of the recent investigations reported that the increased number of T cells within the tumour microenvironment correlates with

longer survival of GBM patients [49, 50, 53, 54]. Several studies evaluated the functional status of tumour infiltrating T cells. Some of them demonstrated that T cells isolated from GBM biopsies and cultured *ex-vivo* were capable of lysing autologous tumour cells [55, 56]. However, such experimental conditions do not fully reflect the *in situ* situation, as T cells might be activated upon *ex-vivo* culture conditions. One study showed that tumour infiltrating T cells express granzyme B and establish immunological synapses upon contact with GBM cells. This suggested that T cell – mediated anti-tumour immune response occurs in GBM [57].

Detailed phenotyping of tumour infiltrating lymphocytes revealed the presence of T regulatory cells (T_{regs}) of $CD4^{+}CD25^{high}Foxp3^{+}$ phenotype within the tumour microenvironment. T_{regs} are implicated in T cell suppression [58], however, there is currently no consensus regarding their prognostic significance in GBM patients [59, 60].

Several studies investigated B cell [51, 61] and NK cell [51, 57, 61, 62] infiltration in GBM, but those lymphocyte subsets were either absent or constituted minor immune cell populations within the tumour microenvironment. However, as discussed in Paper II, the methodology used in those studies was not sufficient for specific NK cell detection.

1.3.3.2 Glioma – induced immunosuppression

Numerous observations indicate that GBM patients are immunosuppressed both locally at the tumour site, and at the systemic level. It has been postulated that this immunosuppression is, at least, partially induced by the cancer cells [63]. In addition to lymphopenia, systemic immunosuppression of GBM patients manifests also in impaired function of peripheral lymphocytes, such as decreased proliferation in response to various mitogens, cytokine production [63] and cytotoxic activity [64]. Peripheral blood monocytes down-regulate the expression of MHC class II molecules, resulting in their reduced ability of antigen presentation [63]. Moreover, the cytokine balance in GBM patients is shifted in favour of anti-inflammatory and immune-

modulating T helper 2-type responses [63] that are characterised by stimulation of the humoral response (e.g. B cell activation and antibody production) and inhibition of the pro-inflammatory T_h1 response [65]. Glioma is also believed to preferentially recruit and/or induce immune cell subsets of tumour-supporting, immune-tolerizing and immune suppressive properties, such as M2 type macrophages and microglia or T_{regs} [46, 60]. The best known immunosuppressive factors secreted by glioma are transforming growth factor-beta (TGFβ), prostaglandin E2 (PGE2) and IL-10 [66]. TGFβ suppresses T cell activation and proliferation and CTLs' function by down-regulating the expression of FasL, and the production of granzymes, perforin and IFNγ [66]. Glioma – derived TGFβ has been also shown to suppress NK cells by down-regulating the expression of the NK cell activating receptor NKG2D [67]. PGE2 mediates decreased secretion of T_h1 type cytokines and increased production of T_h2 type cytokines and it inhibits anti-tumour activity of T cells and NK cells and enhances activity of T_{regs} [66]. IL-10 is a T_h2 type cytokine that down-regulates T cell proliferation and activation and MHC class II expression on APCs [66].

1.3.3.3 The impact of treatment on the immune system of GBM patients

The GBM patients' immunosuppression is not only a result of glioma – derived factors, but also a side effect of chemo- and radiotherapy. It has been observed that brain tumour patients treated with steroids are immunocompromised and at increased risk of infection [68, 69]. Temozolomide has also been demonstrated to induce immunosuppression of GBM patients that is associated with reduced lymphocyte count, reduced numbers of $CD3^+$, $CD4^+$, $CD8^+CD56^+$ T cells, NK cells, B cells and an increased proportion of $CD4^+CD25^+Foxp3^+$ T_{regs} [70].

1.4 Natural Killer cells

Natural killer (NK) cells are large granular lymphocytes of the innate immune system that account for approximately 5-15% of the lymphocytes in peripheral blood [65]. They originate from a common lymphoid progenitor derived from haematopoietic stem

cells, therefore, they belong to the lymphocyte family [65]. NK cells are larger in size and are more granular than T cells and B cells [65]. They play important roles in viral infections and anti-tumour immunity [65, 71]. Due to their ability to recognise and directly lyse the virus-infected or transformed cells without prior sensitisation or co-stimulation, NK cells are considered as components of the innate immune system [71]. They do not express T cell receptors [71] thus, they are phenotypically characterised as CD3 negative CD56 positive lymphocytes. NKp46 receptor expression is also commonly used as a marker of NK cells [72]. NK cells can be subdivided into two functionally distinct subsets based on the expression level of CD56 [73]. In the peripheral blood, under steady state conditions, the main population are $CD56^{dim}$ NK cells that are considered more cytotoxic [73]. The minor subset of $CD56^{bright}$ NK cells are more involved in cytokine and chemokine secretion [73]. Most NK cells also express FcγRIIIA receptor (CD16) that in combination with the CD56 expression level may serve for further sub-classification of NK cells [74].

1.4.1 Target cell recognition

NK cells express a variety of receptors on their surface that allows them to distinguish normal cells from transformed or virus infected cells. Upon binding their ligands, these receptors transduce activating or inhibitory signals and the overall balance determines whether the NK cell will recognise an encountered cell as a target to kill or will remain tolerant.

1.4.1.1 Killer Immunoglobulin-like Receptors

Killer Immunoglobulin-like Receptors (KIR) belong to the immunoglobulin (Ig) superfamily and recognise MHC class I motifs expressed on all nucleated self-cells. The nomenclature is based on their structure that determines also their activating or inhibitory function. KIRs contain two or three Ig-like domains in their extracellular part (KIR2D and KIR3D, respectively), a transmembrane domain and a long-tailed (KIR2DL and KIR3DL inhibitory receptors) or short-tailed (KIR2DS and KIR3DS activating receptors) cytoplasmic domain [75]. The long-tailed cytoplasmic domains

possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that are critical for transducing inhibitory signals [75]. The short-tailed cytoplasmic domain mediates activating signals through interaction with DAP12 adaptor protein containing immunoreceptor tyrosine-based activation motif (ITAM) [75]. The KIRs recognise different allelic variants of human leucocyte antigen-A (HLA-A), HLA-B and HLA-C molecules (Table 2). KIR2DL1, KIR2DS1 and KIR2DS4 interact with HLA-C2 group containing Lysine residues at position 80 in the α1-domain of the HLA heavy chain [76]. KIR2DL2, KIR2DL3 and KIR2DS3 bind HLA-C1 group with Asparagine at position 80 in the α1-domain of the HLA heavy chain [76, 77]. KIR3DL1 recognises HLA molecules with Bw4 epitope, while KIR3DL2 ligands are HLA-A3 and HLA-A11 [76, 78, 79]. Ligands for KIR3DL3, KIR2DL5, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1 remain unknown [76].

Table 2. KIR receptors and their HLA ligands. Prepared based on published literature [76].

	KIR receptor	**HLA ligand**
Inhibitory receptors	KIR2DL1	HLA-C2 group
	KIR2DL2	HLA-C1 group
	KIR2DL3	HLA-C1 group
	KIR2DL4∞	HLA-G
	KIR2DL5	unknown
	KIR3DL1	HLA-Bw4
	KIR3DL2	HLA-A3, -A11
	KIR3DL3	unknown
Activating receptors	KIR2DS1	HLA-C2
	KIR2DS2	unknown
	KIR2DS3	unknown
	KIR2DS4	HLA-C: C*0501, C*1601, C*0202
	KIR2DS5	unknown
	KIR3DS1	unknown

∞ can mediate also activating signals

Exceptional among KIR receptors is KIR2DL4. In contrast to other KIRs, it recognises a non-classical HLA ligand, HLA-G and it is constitutively expressed by all NK cells [80]. Even though it contains a long-tailed cytoplasmic domain with ITIM, KIR2DL4

can mediate both inhibitory and activating signal [80]. Although some activating KIR recognise the same HLA motifs as their inhibitory homologues (for example KIR2DS1 and KIR2DL1), they are considered to bind the ligands with lower affinity [81]. Due to high polymorphism of KIR genes, NK cells from different individuals express different repertoires of KIR receptors. However, based on the genotype, two main haplotype groups can be distinguished [82]. The haplotype A contains KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2, KIR3DL3 and KIR2DS4, while the haplotype B has been defined as containing more than one activating KIR gene [82]. Inhibitory KIRs are essential for distinguishing self cells from non-self cells. The function of activating KIRs has not been fully understood yet.

1.4.1.2 Natural cytotoxicity receptors

Natural cytotoxicity receptors (NCR): NKp30, NKp44 and NKp46 belong to the Ig superfamily and are essential for NK cell activation. NCR-mediated activating signalling requires an association of the receptor with an adaptor protein containing ITAM [83]. NKp30 is constitutively expressed on NK cells and among other ligands, it recognises tumour antigens B7-H6 (expressed on leukemias, lymphomas, carcinomas, melanomas [84]) and BAG6 [83]. NKp44 is expressed only on activated NK cells and plays an important role in immune responses to viral and bacterial infections [83]. NKp46 is considered as the most reliable marker of NK cells. It binds viral haemagglutinin (HA) and HA-neuraminidase (HN) and tumour-associated ligands [83]. Although NCRs are in general considered as activating receptors, in some cases binding their ligands inhibits the activating signal transduction. For example, binding the pp65 protein expressed on human cytomegalovirus (HCMV) infected cells to NKp30 disrupts the interaction of NKp30 with the adaptor protein CD3ζ that is essential for transducing the activating signal [83]. Although not all the ligands for NCRs have been identified, their expression on tumour and infected cells has been demonstrated by using fusion proteins [85] and the role of the NCRs has been proven in cytotoxicity experiments by blocking the receptor-ligand interactions [85, 86].

1.4.1.3 Natural killer group 2 receptors

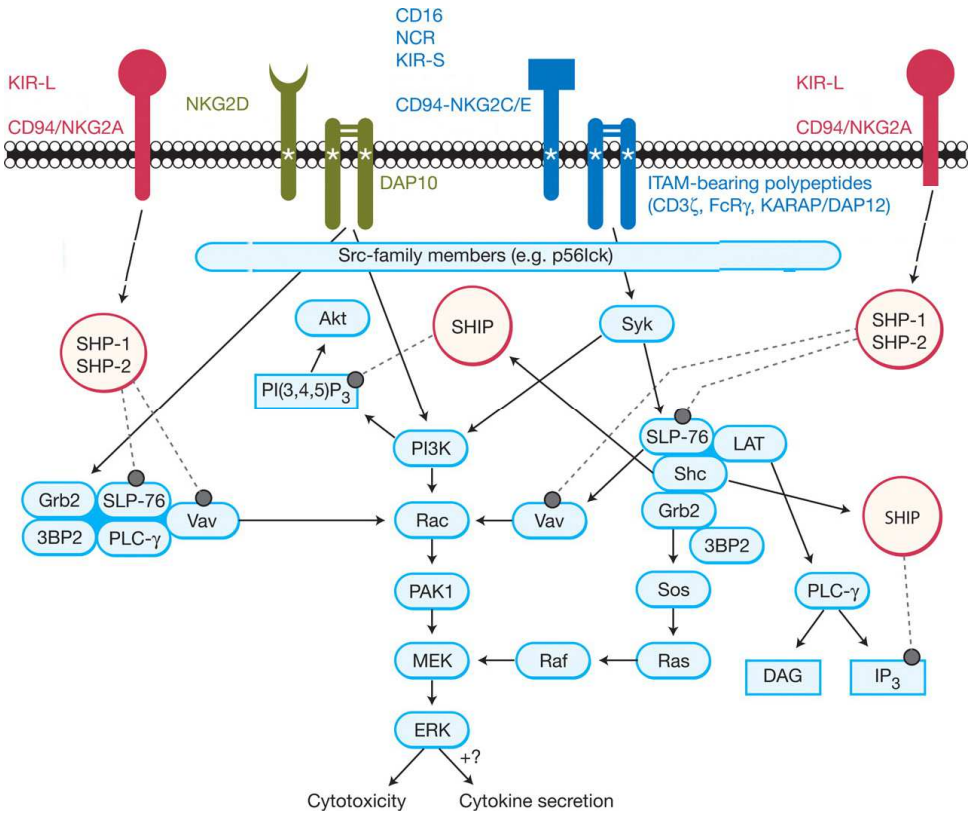
Natural killer group 2 (NKG2) receptors belong to the C-type lectin-like receptor superfamily expressed on the majority of NK cells and a proportion of cytotoxic T lymphocytes (CTLs) [87]. All of them, except NKG2D, form heterodimer complexes with CD94 and recognise the non-classical MHC class I molecule, HLA-E [87]. NKG2A and NKG2B have two ITIMs in their cytoplasmic domains and transduce inhibitory signals [87]. NKG2C, NKG2E and NKG2H lack ITIMs and interact with the DAP12 adaptor protein containing ITAM, and thus mediate activating signal [87]. NKG2E binds HLA-E with the same affinity as NKG2A, while NKG2C binds the ligand with much lower affinity [87]. NKG2 receptors play a role in the regulation of NK cell function. The interaction of NKG2A with HLA-E has also been postulated as a mechanism of modulating the adaptive immunity [88]. NKG2D differs from the other members of the NKG2 group. It is expressed by the majority of NK cells and CTLs as homodimer in association with adaptor protein DAP10 [89]. NKG2D is an activating receptor and recognizes a variety of ligands expressed on transformed, infected or stressed cells. These ligands are MHC class I – related chains A and B (MICA and MICB) and UL16 binding proteins (ULBPs) [90] and under physiological conditions, they are not expressed by normal, healthy cells, or are expressed at very low levels. Therefore, NKG2D, in addition to NCRs, is essential in recognising transformed and infected cells expressing normal levels of MHC class I molecules.

1.4.1.4 NK cell signalling pathway

The interaction of NK cell activating receptors with ITAM bearing adaptor proteins (DAP12, CD3ζ, FcRγ) initiates a signalling cascade resulting in cytokine release and/or secretion of lytic granules. First, ITAM proteins are phosphorylated by the Src family of protein tyrosine kinases (PTKs) that results in activation of the Syk family of PTKs (Fig. 12) [91, 92]. This in turn, leads to activation of NK cell cytotoxicity pathway involving the phosphatidylinositol 3-kinase (PI3K) – Akt pathway and the mitogen-activated protein kinase (MAPK) pathway (Fig. 12) [91, 92]. The NKG2D receptor associated with DAP10 induces similar signalling cascade, however, DAP10-

mediated NK cell activation is independent from the Syk family of PTK [91, 92]. Another major difference is that NKG2D-mediated activation alone is not sufficient to trigger cytokine release, while ITAM-induced signalling results in both cytotoxicity and cytokine secretion [91].

Figure 12. NK cell signalling pathways. Activating receptors (blue and green) associate with adaptor proteins (blue and green) and initiate signalling cascade (arrows) leading to secretion of cytolytic granules and cytokines. The inhibitory receptors (red) activate phosphatases SHP-1 and SHP-2 that inhibit the activating signal (dashed lines) by dephosphorylation of various signal transducers. Reprinted with permission. Published in [91].



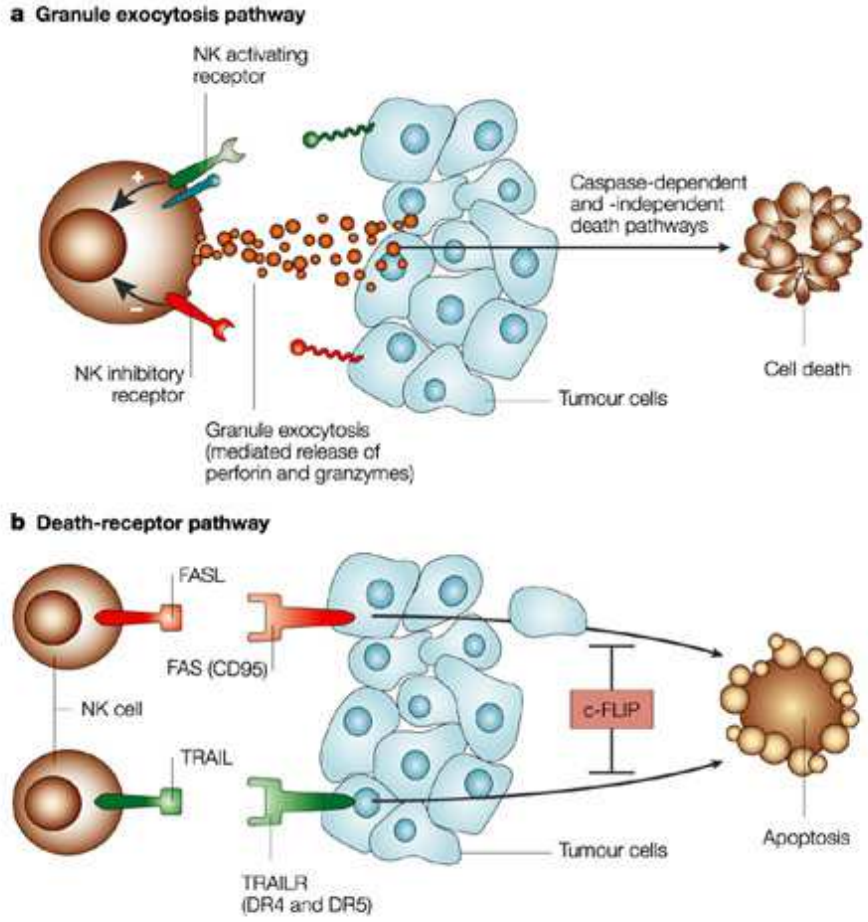
On the other hand, binding of ligands to NK cell inhibitory receptors bearing ITIM domains results in tyrosine phosphorylation of ITIMs and recruitment of protein tyrosine phosphatases SHP-1 and SHP-2 (Fig. 12) [91]. They inhibit NK cell activation by dephosphorylating proteins involved in down-stream ITAM- and DAP10-mediated

signalling, like Vav and SLP-76 proteins (Fig. 12) [91, 92]. Therefore, the overall balance between phosphorylation induced by activating signals and dephosphorylation of the same substrates initiated by inhibitory signals determines the triggering of NK cell cytotoxicity and cytokine release.

1.4.2 Direct cellular cytotoxicity

Upon recognition of target cells, if the activating signals override the inhibitory signals, NK cells are triggered to perform their cytotoxic functions. The two major mechanisms that mediate NK cell – dependent killing are: (1) secretion of cytotoxic granules (2) inducing apoptosis via death receptors (Fig. 13) [93]. NK cells constitutively express perforin and granzymes that are encapsulated in lytic granules in the cytoplasm [94]. Upon activation, NK cells form an immunological synapse with the target cell and rearrange their cytoskeleton to facilitate trafficking of the lysosomes towards the site of the synapse [94]. Exocytosis of the lysosomes results in release of the lytic granules towards the target cell [94]. Perforin forms pores in the cytoplasmic membrane of the target cells and enables the entry of granzymes into the cytosol [94]. Granzymes are serine proteases that are capable of inducing both classical apoptosis and caspase-independent cell death [95]. Granzyme A and Granzyme B are the most abundantly expressed and best described among granzymes. Granzyme B cleaves caspases and their substrates, while Granzyme A induces DNA damage by single-stranded nicks [95]. Multiple mechanisms of inducing cell death by granzymes enable the elimination of apoptosis-resistant cells.

Figure 13. Mechanisms of NK cell – mediated cytotoxicity. (a) secretion of cytotoxic granules and (b) apoptosis induced by interactions of death receptors with their ligands. Reprinted with permission. Published in [93].



NK cells express also ligands for death receptors – Fas Ligand (CD95L, CD178), tumour necrosis factor (TNF) and TNF-related apoptosis inducing ligand (TRAIL) [93]. Binding ligands to death receptors - Fas (CD95) or TNF receptor - leads to apoptosis of the receptor-bearing cell (Fig. 13) [93]. Therefore, efficiency of Fas Ligand- or TNF- mediated NK cell cytotoxicity is dependent on the level of death receptors expressed on target cells and their inherent susceptibility or resistance to apoptosis.

1.4.3 Antibody dependent cellular cytotoxicity

NK cells are able to recognize and kill cells coated with antibodies through a process called antibody dependent cellular cytotoxicity (ADCC). It is initiated by binding of the Fc part of antibody to the FcγRIIIA (CD16) receptor expressed on the majority of NK cells. CD16 recognises antibodies of IgG class. Upon binding its ligand, CD16 activates NK cell - mediated cytotoxicity and cytokine secretion [91]. CD16 interacts with ITAM-bearing adaptor proteins CD3ζ and FcRγ transducing activating signals in a manner similar to the other NK cell activating receptors [91]. This process plays an important role in enhancing the adaptive immune response.

1.4.4 Cytokine and chemokine secretion

NK cells secrete a variety of cytokines, pro-inflammatory such as IFNγ, anti-inflammatory, including IL-5, IL-10, IL-13 and growth factors, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) [71, 93]. Among them, IFNγ has attracted most attention due to its multiple immune-stimulatory properties. IFNγ is a pro-inflammatory cytokine that promotes T-helper 1- (T_h1) type responses and stimulates effector lymphocytes – NK cells and CTLs [93]. Moreover, IFNγ also activates the innate immune cells, for example by up-regulating MHC class I and II and expression of co-stimulatory molecules on macrophages [93]. NK cells also mediate the recruitment of other immune cells into the site of inflammation by secreting various chemokines, such as monocyte chemotactic protein – 1 (MCP-1, CCL2), macrophage inflammatory protein 1-α (MIP1-α, CCL3), macrophage inflammatory protein 1-β (MIP1-β, CCL4) and regulated on activation normal T cell expressed and secreted (RANTES, CCL5) [71].

1.4.5 Tolerance to normal cells

The tolerance to self, healthy cells is maintained mainly by two factors occurring at the same time: (1) interactions of inhibitory KIR receptors expressed on NK cells and their ligands, HLA class I molecules expressed on each somatic cell, and (2) lack of or very

low expression of pathogen-specific or stress-induced antigens that are ligands for activating NK cell receptors (Fig. 14). However, KIR receptors are inherited independently from HLA molecules and due to high polymorphism of both KIR and HLA genes, it may potentially occur that some NK cells express receptors that do not recognize self-ligands and these NK cells may be autoreactive to normal cells [96]. However, these NK cells remain hyporesponsive and do not cause autoimmunity. Yet, they can be activated upon infection or inflammation by cytokines like IL-2 and IL-12 [96]. The mechanism mediating tolerance of NK cells missing inhibitory KIRs for self MHC class I ligands has not been fully understood yet. So far, there is no evidence of negative or positive selection of NK cells taking place in the bone marrow during NK cell development or in the periphery [96]. One hypothesis postulates that to become functional effector cells, NK cells require "licensing" that is mediated by interaction of inhibitory KIR with its specific ligand [96]. The opposite hypothesis suggests that fully functional NK cells become anergic under chronic stimulation in the absence of inhibitory signals mediated by inhibitory KIRs [96]. Another model proposed that NK cell potency is determined by the number of inhibitory KIRs expressed and their affinity to self ligands [96]. The interaction of NKG2A receptor with HLA-E plays also an important role in NK cell regulation and self-tolerance. However, further studies are needed to fully resolve the mechanism of NK cell self-tolerance.

1.4.6 NK cells in tumour surveillance and anti-cancer therapy

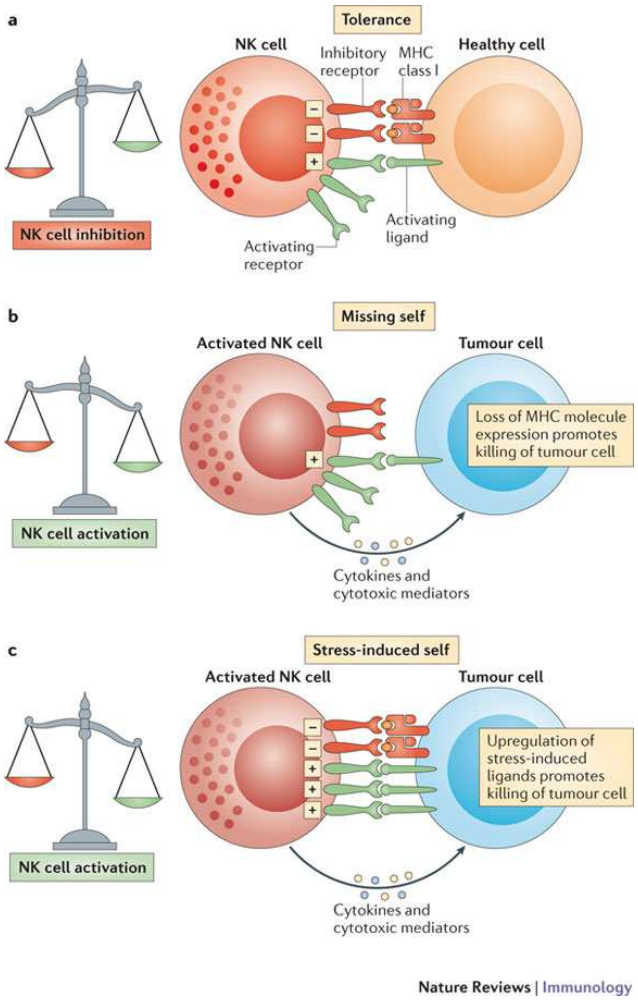
Due to their above described physiological functions, NK cells play an important role in anti-cancer immunity. In various types of cancers NK cells have been demonstrated to perform surveillance and contribute to the anti-tumour immune response [93, 97]. In haematological malignancies, haploidentical allogeneic NK cells have been shown to mediate Graft versus Leukemia (GvL) effect and protect patients receiving haematopoietic stem cell transfer (HSCT) against Graft *vs.* Host Disease (GvHD) [98]. Moreover, numerous studies reported prognostic significance of endogenous NK cell tumour infiltration and activity in various solid tumours [99-103]. NK cells are also considered to play an important role in controlling metastasis [101]. The anti-tumour

response mediated by endogenous NK cells occurs due to the "missing self" and/or "induced self" mechanisms, while in allogeneic settings the KIR-HLA mismatch is considered to play an essential role.

1.4.6.1 "Missing self" and "induced self" hypothesis

As mentioned above, NK cells discriminate between self and non-self cells by recognising HLA class I molecules expressed on all healthy somatic cells. In the absence of ligands for activating receptors, binding of HLA to inhibitory KIR receptors keeps NK cells tolerant. Tumour cells often down-regulate the expression of HLA in order to avoid destruction from CTLs. However, such cells are recognised by NK cells as non-self due to the lack of HLA expression (Fig 14). On the other hand, some transformed cells express HLA at normal levels. Those cells can be eliminated due to the "induced self" mechanism (Fig. 14) [71, 93, 104]. Tumour cells frequently over-express on their surface stress-induced molecules that are recognised by the NK cell activating receptor NKG2D, thus the activating signal overrides the inhibitory signal mediated by the KIR-HLA interaction.

Figure 14. NK cell tolerance of normal cells and recognition of tumour cells via "missing self" and "induced self" mechanisms.



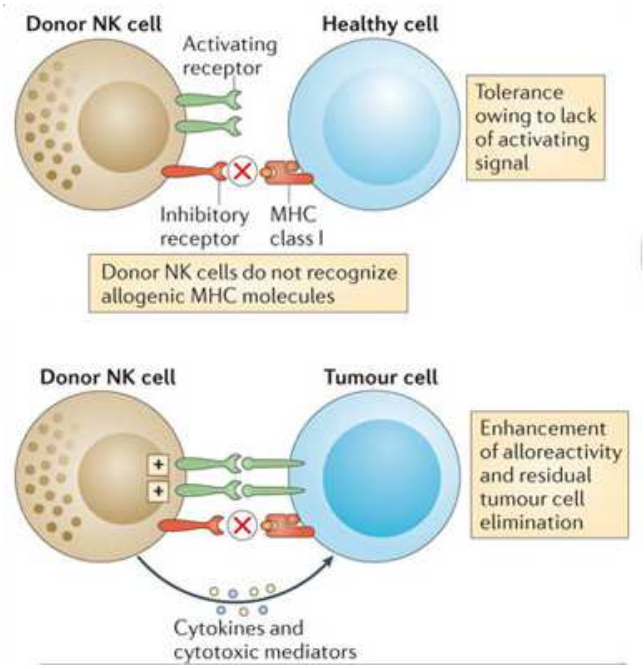
(a) Healthy cells express MHC class I molecules recognised by inhibitory KIRs expressed on NK cells and do not express activating ligands for NK cell at sufficient level, thus NK cells remain tolerant. (b) NK cells recognise tumour cells that down-regulate MHC class I expression due to "missing self" mechanism. (c) Tumour cells often up-regulate stress-induced ligands of NK cell activating receptors. Thus, despite expression of MHC class I molecules, the activating signals override the inhibition mediated by inhibitory KIRs. Reprinted with permission. Published in [2].

1.4.6.2 The KIR – HLA mismatch

The term "KIR - HLA mismatch" refers to an approach, so far, mainly in HSCT for leukaemia patients [98]. The genes encoding HLA molecules are very polymorphic and different individuals express different allelic variants of HLA. This is one of the

essential issues to control for in the transplantation. In order to reduce the risk and severity of potential GvHD, the cells for transplantation are obtained from donor with maximal HLA-match. The first choice of donor is a sibling and the next possibility is to find a HLA-matched unrelated donor [105]. NK cells from different individuals also express different repertoires of KIR receptors. Therefore, it is possible to find a donor with NK cell subsets that do not recognise some of the recipients' HLA molecules. Thus, these NK cells are potentially highly cytotoxic for that individuals' cancer cells (Fig. 15). Normal recipients' cells would be spared from allogeneic NK cell-mediated killing due to the absence of stress ligands for the activating NK cell receptors (Fig. 15).

Figure 15. The KIR receptor – HLA mismatch hypothesis.



Donor's NK cells expressing KIR receptors that do not recognise recipient's MHC class I molecules are activated by activating ligands expressed on tumour cells and kill the tumour cells due to the absence of inhibitory signals. Healthy cells are spared due to the lack of activating ligands. Reprinted with permission. Published in [2].

1.4.6.3 NK cells in immune-stimulatory anti-cancer therapies.

One of the promising anti-cancer therapeutic strategies is to stimulate potent anti-tumour immune responses. Several experimental therapies have been demonstrated to augment the capacity of endogenous NK cells to kill the tumour cells. An example of such effect can be targeting indoleamine-2,3-dioxygenase (IDO) in cervical cancer [106]. This enzyme is involved in tryptophan degradation thus expression of IDO on tumour cells is considered as one of the immunosuppressive mechanisms via tryptophan deficiency affecting T cells [106]. The study of Sato et al. demonstrated that down-regulation of IDO induced NK cell accumulation at the tumour site and inhibited the tumour growth [106]. Harnessing NK cell – mediated cytotoxicity with use of tumour specific antibodies to induce ADCC is a commonly investigated strategy, as shown with trastuzumab and cetuximab (humanised anti-EGFR mAbs) for treatment of breast cancer [107, 108]. An interesting approach is the use of immunoligands. These are fusion proteins that recognize tumour – specific antigen and contain ligands for the NKG2D activating receptor. Several immunoligands have been already tested *in vitro* and *in vivo*, like for example immunoligand targeting prostate-specific membrane antigen (PSMA) in prostate cancer [109] and CD138 on multiple myeloma cells [110]. A number of studies investigated NK cell – stimulating therapies against glioma. For example, Avril et al. reported that NK cells in combination with cetuximab efficiently kill GBM cells *in vitro* via ADCC [111]. The work of Alizadeh et al. demonstrated that therapy with CpG-oligodeoxynucleotides (CpG-ODN), a ligand for toll-like receptor 9 (TLR9), stimulated the host NK cell - mediated immune responses *in vivo* and induced resistance to tumour re-challenge [112]. NK cells functionality involves also interaction with other components of the immune system, like for example cross-talk with DCs [113]. Therefore DCs vaccinations may potentially stimulate both T cells and NK cells. Indeed, Pellegatta et al. observed increased frequency of circulating NK cells and increased NK cell-mediated IFNγ production in a subset of patients receiving DC vaccines [114]. Moreover, this effect was associated with improved patients' survival. On the other hand, some experimental

therapies, like virotherapies, might be negatively influenced by endogenous NK cells, due to their ability to recognise virus-infected cells [115].

1.4.6.4 Clinical trials investigating NK cell activity against GBM.

One of the broadly investigated cellular therapies against GBM is the adoptive transfer of immune cells. In this approach patients own immune cell are isolated from peripheral blood, cultured and/or modified *ex vivo* and transferred back to the patient systemically or directly to the tumour site. To date, several clinical trials utilized lymphokine activated killer (LAK) cells combined with IL-2 injections [116] and one trial used NK cells obtained from patients' peripheral blood lymphocytes by selective *ex vivo* expansion [117]. LAK cells are a mixture of effector cells, mainly CTLs and NK cells, derived from peripheral blood mononuclear cells (PBMCs) by culturing under specific conditions. The composition of LAK cells depends on the donor and culture conditions. Clinical trials using autologous LAK cells combined with IL-2 injections published so far reported partial efficacy and moderate toxicity [116]. Ishikawa et al. tested pure NK cell – based therapy against recurrent malignant glioma and reported tumour regression measured by MRI in 4 of 9 patients [117]. These attempts show the potential of NK cells as effectors against GBM, however, further studies are required to reveal the exact mechanism of action and in the case of LAK cells – based therapies, the cellular population involved should be characterised in detail.

2. Methodological considerations

2.1 Multiparametric flow cytometry

Flow cytometry is a fluorescence – based, powerful analytic tool with multiple applications, such as cell cycle analysis, phenotyping and quantitative functional assays like proliferation and cytotoxicity assays. This technique enables also sorting of viable cells of certain properties from the suspension containing mixed cell populations. In principle, single cells pass one by one through a laser beam and the light signal scattered or emitted by the cell is detected, amplified and recorded on a computer.

2.1.1 Flow cytometry – based phenotyping

Flow cytometry can be used to detect the expression of molecules of interest on the cells in the studied sample. The cells are labelled with specific primary antibodies and then incubated with secondary antibodies conjugated to fluorochromes – molecules that emit light within specific wavelength range when excited by a laser. Currently, there are many specific fluorochrome – conjugated antibodies available on the market so that direct staining can be performed. Then the data are acquired by the flow cytometer and can be further analysed using dedicated software. The essential data obtained include the number of events recorded and mean fluorescence intensity (MFI). Depending on the capacity of the flow cytometer (number of lasers, detectors and optic filters available), up to 16 parameters can be detected simultaneously. The basic parameters are forward scatter (FSC) and side scatter (SSC). The FSC signal is proportional to the size of the cell and SSC signal reflects the cell's complexity and granularity.

Depending on the quality of the sample analysed and the experimental procedure, analysed cell suspensions may contain considerable amounts of dead cells, debris and doublets (two cells that clumped together) that need to be excluded from analysis.

Doublets can be distinguished from singlets for example on the SSC-A vs. SSC-H plot. Debris are usually easy to exclude based on the FSC *vs.* SSC plot, where they form a separate population of smallest signal intensity (small size and minimal complexity).

In order to separate viable cells from dead cells, the sample has to be incubated with a specific dye that stains dead cells. It may be, for example, a DNA dye that does not cross the intact membrane of viable cells, but can enter the dead cell via a compromised membrane. Then events of high fluorescence intensity will represent dead cells, while events of minimal (the same, as unstained cells) will represent viable cells. There are many commercially available dyes of different properties in terms of excitation and emission spectrum and some of them are designed for specific experimental conditions, like staining fixed and permeabilized cells.

Simultaneous analysis of many fluorescence parameters enables distinction of several phenotypically different cellular populations and subsets and investigating co-expression of different molecules within a complex sample. However, it is a very complex task due to the spectral overlap between emission spectra of the fluorochromes used in the analysis. Therefore, the experiment should be carefully designed to reach the balance between numbers of markers needed to be analysed simultaneously and the predicted complexity of data analysis. If possible, fluorochromes with minimal spectral overlap should be chosen for use within the same sample. Also, samples stained with only one dye or fluorochrome-conjugated antibody should be recorded in order to calculate the compensation. The compensation is a proportion of signal assigned to specific detector that is overlapping with signal detected by other detector(s) and should be subtracted. A proper choice of optic filters may also help in obtaining optimal measurement conditions. Long pass filters (LP), band pass filters (BP) and short pass filters (SP) limit the detected emission signal to specific wavelength range.

2.1.2 Advantages and limitations

The main advantage of flow cytometry is the possibility of measuring the expression of several molecules simultaneously. Certain cellular subsets cannot be identified within heterogeneous samples with use of only one or even two phenotypic markers. An example may be detection of T_{regs} in the sample containing all types of leukocytes. With flow cytometry it is also easy to obtain quantitative data, like the proportion of cells expressing a particular molecule (number of positive events) or the level of expression (MFI). However, the fluorescence intensity depends on the voltage settings that are usually set up each time the cytometer is turned on and thus might be different each time. Therefore, MFI data obtained from two independent measurements are often not comparable. Multiparametric analysis also often saves time and resources.

Even though this method offers many benefits, several limitations should be taken into consideration. Analysing solid tissue with flow cytometry requires tissue disintegration to obtain single-cell suspensions, thus it does not provide information about the initial tissue structure. Thus, such investigation should be supported with histological methods. Analysis of single cell suspensions alone also does not provide direct evidence of cell-cell interactions. Other limitations are related to the specificity of antibodies and stability of fluorochrome conjugates.

2.1.3 Flow cytometry – based cytotoxicity assays

Flow cytometry can be also used to measure the % of lysis in the cytotoxicity assays. To distinguish target from effector cells, one population is labelled with a dye, e.g. carboxy-fluorescein diacetate succinimidyl ester (CFSE) before co-culture. After the co-culture of target cells with effector cells, another dye is applied to enable the distinction between dead and viable cells, for example 7-amino actinomycin D (7-AAD). Finally, the numbers of viable and dead target cells are measured by flow cytometer and % of lysis is calculated. The advantage of this method over the standard ^{51}Cr release assay is that it is more sensitive and does not require radioactive labelling [118].

2.2 Relevant in vivo model

The choice of an experimental animal model for immunotherapy studies represents a great challenge and is often a point of debate. To study brain cancer, several rodent models can be used: (1) syngeneic animal models, e.g. GL26 glioma in C57BL/6 mice and CNS-1 in Lewis rats (2) xenograft models, e.g. U251 cells in Balb/c mice and U251 cells in nude rats. The advantage of syngeneic models is that the tumour develops in the immune competent animal so that the model reflects interactions of tumour with the host immune system. However, xenograft models created by implanting human tumour cells into immunodeficient animals are better to use for pre-clinical investigation of therapies targeting human molecules, like VEGF or EGFR. On the other hand, for studying immunotherapies, syngeneic models are usually used. Recently, xenograft models implanted with patient-derived tumours have been developed [119, 120]. They are considered to better reflect the initial patient's tumour biology and accurately represent the impact of their genomic characteristics.

2.2.1 Non-obese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice

NOD/SCID mouse model was created for the needs of haematological research [121]. NOD/SCID mice are a result of crossing C.B-17-*scid* mice lacking functional T cells and B cells with NOD/Lt strain characterised by functional NK cell deficiency, defective APCs and lack of circulating complement [121]. The C.B-17-*scid* mice harbour a mutation on the gene "protein kinase, DNA activated catalytic polypeptide" (*Prkdc*) encoding a catalytic subunit of DNA-dependent protein kinase [122]. Lack of the functional *Prkdc* gene results in their inability to express rearranged antigen receptors leading to dysfunction in adaptive immunity [122]. The NOD mice spontaneously develop autoimmune (type 1) diabetes due to the defect in insulin dependent diabetes (*Idd*) gene loci [123]. These gene loci are associated with various immune functions and their dysfunction result in impaired innate immune system. For example, the *Idd1* locus is related to MHC class I and II genes, the genes on *Idd3* locus

are considered to encode cytokines IL-2 and IL-21 and aberration in the *Ctla-4* gene on *Idd5.1* locus results in defects in immune regulation mediated by CTLA-4 receptor expressed on T_h cells [123]. The NOD/SCID mice display following immune features: (1) T cell and B cell deficiency, (2) absence of C5 complement, (3) decreased NK cell activity, (4) defective IL-1 secretion by macrophages and (5) impaired expression of cytokine receptors and protein kinase C on macrophages [121]. Above mentioned immune defects enable efficient engraftment of xenotransplants, also human cells [121]. This model and its humanized version have been used to study the human hematopoietic stem and progenitor cells engraftment and mobilization [124] and HIV infection [125]. Moreover, these mice and NOD/SCID mice transfected with green or red fluorescent proteins (GFP or RFP, respectively) have been also used to study GBM [126, 127].

2.2.2 The rationale of using NOD/SCID mice in studying tumour – NK cells interaction

The purpose of our study was to investigate the role of KIR – HLA interactions in allogeneic NK cell – mediated cytotoxicity against GBM solid tumour *in vivo*. As we were interested especially in investigating the impact of KIR2DS2 and KIR2DS4 receptors, we decided to use human NK cells against human GBM. Therefore, we conducted the *in vivo* experiments in immune deficient NOD/SCID mice xenografted with patient-derived and *in vivo* passaged P3 GBM, a model established in our laboratory. Moreover, the endogenous NK cells in the NOD/SCID mice are not functional, thus they should have no impact on the experiments. Another advantage of using this model is that xenograft models transplanted with patients' tumour are considered to resemble the biological and genetic features of the initial tumour more than syngeneic models. However, the critical point of using immune deficient animals is the absence of the host immune system interactions with the tumour. Even though the GBM patients are severely immune compromised due to the glioma – derived factors and treatment they receive, several immune aspects have been proven to play an important role in tumour progression, anti-tumour immunity and outcome of

experimental therapies, like for example the presence of T_{regs}. These aspects cannot be addressed in the immune deficient animals.

3. Hypotheses and Aims of the thesis

Paper I: Elevated CD3+ and CD8+ tumor-infiltrating immune cells correlate with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor microenvironment and at the systemic level

Hypothesis: GBM patients may be more immunosuppressed at the systemic level compared to healthy, age and gender matched controls

The aims of the study were to:

I. Characterise the immune status of GBM patients both within the tumour microenvironment and in the peripheral blood compared with age and gender matched healthy controls.

II. Identify the integrated mechanisms of tumour immunological escape and tolerance to destruction by the immune system.

III. Identify potential therapeutic targets

Paper II: Natural Killer cells in intracranial neoplasms: presence and therapeutic efficacy against brain tumours

The aims of this study were to:

I. Critically review and discuss the literature investigating NK cell infiltration into brain tumours. The motivation was to summarise what is currently known about the numbers of NK cells infiltrating brain tumours, their phenotype, function and reported clinical trials investigating the efficacy of NK cells against brain tumours. The findings emphasised the need for further research on this topic, with greater use of state-of-the-art methodologies.

Paper III: Activated NK cells are potent effectors against glioblastoma cells due to activating KIR-HLA ligand interactions *in vitro*

Hypothesis:

I. KIR – HLA ligand mismatch might determine the NK cell cytotoxic potency against GBM cells *in vitro*

II. Purified NK cells may be more cytotoxic effectors against GBM cells compared to lymphokine activated killer (LAK) cells

The aims of this study were to:

I. Identify the particular KIR-HLA ligand interactions that determine NK cell potency against GBM cells

II. Compare and determine the cytotoxic potency of allogeneic pure NK cells *versus* LAK cells obtained from the same donors against patient-derived GBM cells *in vitro*.

Paper IV: Purified and activated allogeneic NK cells efficiently kill human glioblastoma *in vivo*

Hypothesis:

I. Activating KIR2DS2 and KIR2DS4 – HLA ligand interactions might determine greater cytotoxic efficiency of NK cells against GBM *in vivo* compared inhibitory KIR-HLA ligand mismatch

II. Intratumoural adoptive NK cell transfer is tolerable

The aims of this study were to:

I. Investigate the cytotoxic potency of NK cells bearing KIR2DS2 and KIR2DS4 receptors *in vivo* measuring as endpoints tumour growth and survival. The overall goal was to unravel the biological mechanisms of human purified NK cell – mediated cytotoxicity against patient – derived GBM *in vivo*.

II: Identify the host cellular subtypes mediating the therapeutic efficacy

III: Investigate the therapeutic efficacy of one versus two doses of intracranially injected NK cells

4. Summary of results

Paper I

The immunohistological analysis of 65 human GBM specimens revealed that the immune infiltration of $CD3^+$, $CD8^+$ and $CD4^+$ immune cells varied between the patients. The increased numbers of $CD3^+$ cells correlated with longer survival of the GBM patients independently of the other established prognostic factors. We observed decreased T_h cell proportion, increased expression of CTLA-4 (CD152) inhibitory receptor on T_h cells and increased plasma concentration of IL-10 in GBM patients' peripheral blood compared to healthy controls. There was no difference in proportions of $CD4^+CD25^{high}Foxp3^+$ T_{regs} between the patients' and controls' peripheral blood. Tumour infiltrating CTLs and T_h cells downregulated the expression of CD28 stimulatory receptor and L-selectin CD62L. Moreover, tumour infiltrating CTLs displayed decreased expression of CD56. Within the patients' GBM biopsies we detected the presence of $CD8^+CD28^-Foxp3^+$ T_{regs}, previously not described in gliomas, while $CD4^+CD25^{high}Foxp3^+$ T_{regs} were absent. We also detected small populations of NK cells and B cells. We observed substantial proportions of macrophages/microglia within the tumour microenvironment that expressed inhibitory receptors immunoglobulin like transcript 2, 3 and 4 (ILT2, ILT3 and ILT4) as well as HLA-G and HLA-E, while small proportions expressed co-stimulatory molecules CD40, CD80 and CD86. Almost all of the tumour cells expressed classical MHC class I molecules HLA-A,B,C, while only minimal proportions expressed non-classical HLA-E and HLA-G. MICA and Fas were expressed on 24.53% and 21.54% of tumour cells, respectively. All tumour cells were FasL negative. Interestingly, a proportion of tumour cells expressed an ectonucleotidase CD73, while the expression of CD39 ectonucleotidase was restricted to immune cells. Taken together, these findings suggest, that despite multiple escape mechanisms, at least a proportion of tumour cells might be susceptible to immune cell – mediated clearance as indicated by positive correlation of increased immune cells infiltration with prolonged patients' survival.

However, some immune cell subsets might mediate tumour – driven immunosuppression, thus supporting the tumour development.

Paper II

There is currently limited knowledge available regarding the role of NK cells in the immune surveillance of brain tumours. Given the potentially important role that NK cells may exert in controlling brain tumours and the fact that various therapies used for brain tumor patients may influence the efficacy of these cells, a critical review of the literature was undertaken. The literature investigating the degree of NK cell infiltration and functionality in the brain tumours revealed a great variation in the conception of the role of NK cells, and a remarkable paucity of research within this topic. Moreover, most of the studies were conducted before the development of novel methods enabling specific detection or isolation of NK cells. On the other hand, increasing numbers of publications demonstrate the anti-glioma potential of NK cells *in vitro* and *in vivo.* This is demonstrated directly using NK cells as therapeutic effectors or indirectly by characterising their mechanisms of modulating the therapeutic efficacy of various experimental treatments.

Paper III

The freshly isolated (resting) NK cells were predominantly $CD56^{dim}CD16^{+}$ in contrast to *ex vivo* cultured (activated) NK cells that were mostly $CD56^{bright}CD16^{+}$. There was no difference in expression of NK cell receptors: NKp46, NKG2A, NKG2D, KIR2DL1, KIR2DL2/3, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2/1 between the resting and activated NK cells. The major cell population within the LAK cell were T cells (72 ±12%) followed by NK cells (18 ±6%). Patient-derived GBM cells were phenotyped using flow cytometry. Almost all P3 and 2010-20-I cells (82.4% and 98,9%, respectively) and half of 2012-018 cells (50,3%) expressed HLA-ABC. The expression of the non-classical HLA-E and HLA-G, and of the tumour stress ligands MICA, MICB and ULBPs was none or minimal. On the genomic level, 2012-018 cells possessed genes of known HLA-ligands for all KIRs, while P3 and 2010-20-I cells

missed the genes of HLA-ligands for KIR3DL2. NK cell donors displayed different KIR genes repertoire. Allogeneic activated NK cells killed patient – derived GBM cells (n=3) more efficiently than resting NK cells and LAK cells obtained from the same donors (n=8) *in vitro*. The NK cell potency was donor- and dose – dependent. The inhibitory KIR gene repertoire did not directly correlate with NK cell cytotoxic efficiency against the same GBM cells. In the majority of cases, the most efficient NK cells possessed activating receptors KIR2DS2 and/or KIR2DS4 genes. However, the expression of KIR2DS2 and KIR2DS4 receptors on NK cells was low or moderate. Blocking the KIR2DS2 and/or KIR2DS4 receptors with blocking antibodies minimally abrogated the NK cell-mediated lysis in 2 out of 4 donors against P3 and 2012-018 GBM cells. Blocking those receptors in combination with blocking NKG2D receptor resulted in additive cytotoxic effects. Taken together, these results suggest that KIR2DS2 and KIR2DS4 activating NK cell receptors may contribute to increased anti-tumour effect mediated by allogeneic NK cells *in vitro*, partially independently of inhibitory KIR – HLA ligand mismatch.

Paper IV

Due to the findings of the potential role of KIR2DS2 and KIR2DS4 in determining cytotoxic potency of NK cells against GBM reported in paper III, we embarked on investigating the hypothesis that KIR2DS2 and KIR2DS4 activating receptors determined greater cytotoxic potency compared to inhibitory KIR-HLA ligand mismatch of purified NK cells against solid GBM *in vivo. In vivo* study resembles patients' condition more accurately than *in vitro* experiments and enables the evaluation of the impact of NK cell efficiency on tumour growth parameters and animal survival, as well as by assessment of the possible toxic effects. Purified, *ex vivo* expanded NK cells obtained from 2 healthy donors with different KIR gene repertoires were used for intracranial injection into P3 GBM bearing mice and for *in vitro* cytotoxicity assays against P3 GBM cells maintained in culture for 2 and 3 weeks. The two donors displayed different cytotoxic potential against patient – derived GBM cells *in vitro*. Donor A lacked genes encoding KIR2DL2, KIR3DL1 inhibitory receptors

indicating greater inhibitory KIR-HLA ligand mismatch. This situation reduced the inhibitory signals to NK cells allowing penetrance of activating signals, however, this donor lacked the KIR2DS2, KIR2DS4 activating receptors. In contrast, donor B possessed activating KIR2DS2 and KIR2DS4 and all inhibitory KIR genes matched to their cognate HLA ligands indicating the presence of strong inhibitory signals. In addition, this donor lacked activating KIR2DS1 and KIR2DS5 genes. These two donors allowed us to investigate the contribution of (1) inhibitory KIR-HLA ligand mismatch in the absence of KIR2DS2 and KIR2DS4 and (2) the contribution of KIR2DS2 and KIR2DS4 activating receptor-ligand interactions in the absence of inhibitory KIR-HLA ligand mismatch. After 2 weeks culture, donor B's NK cells were more cytotoxic than donor A's NK cells against the same GBM target cells *in vitro*. However, intracranial injection of a single dose of 10^6 NK cells from each donor improved the survival of human GBM – bearing NOD-SCID mice compared to the control group. However, treatment with NK cells from donor B resulted in better outcome of animals as demonstrated by prolonged animal survival, reduced proliferation, increased apoptosis of the tumour cells and reduced angiogenesis within the tumour tissue. On the other hand, treatment with double dose of 10^6 NK cells did not improve animal survival compared to controls. Using flow cytometry phenotyping we demonstrated increased recruitment of macrophages into the brain of all treated animals compared to controls. However, a higher proportion of tumour-infiltrating macrophages of single dose treated mice expressed F4/80, IL-7 receptor (CD127), while increased proportions of microglia expressed CD40 compared to the macrophages and microglia in double dose treated mice and controls. We also observed up-regulated expression of nestin and HLA-ABC on the tumour cells in single dose treated animals compared to double dose treated and control groups. Taken together, these results may confirm the role of KIR2DS2 and/or KIR2DS4 in NK cell – mediated cytotoxicity against GBM *in vivo*. Moreover, these findings suggest that treatment with NK cells results in recruitment and stimulation of endogenous immune cells, like macrophages. However, an increased dose of NK cells might cause adverse effects.

5. Discussion

Immunotherapy is considered to be a promising novel treatment for GBM patients [128]. Several strategies of cellular therapy have already been tested including adoptive T cell [129], LAK cell [116] or NK cell transfer [117] and dendritic cell vaccination [114, 130]. However, it is of great importance to resolve the patients' immune status and potential mechanisms of tumour-induced immunosuppression and immune escape, as it may influence the success of the experimental immune-based treatment. A number of studies investigated glioma-induced immunosuppression, as reviewed in Dix et al. and Gomez et al. [63, 66] Our research (paper I) confirmed the prognostic significance of immune infiltration in GBM reported by other groups [49, 50, 53, 54]. We found that increased CD3 positive T cell infiltration was associated with improved outcomes independent of age at diagnosis and anti-tumour treatment. Moreover, increased CD8 positive cell infiltration had a modest association with patient survival. The observation that *MGMT* promoter methylation was not significantly correlated with patient survival might be explained by the relatively small sample size and that we analyzed samples from only one clinical center. Future work will be required to validate these findings with larger patient cohorts obtained from multicenter clinics. Moreover, we outlined novel interconnected mechanisms of GBM's evasion from the immune system and indicated possible therapeutic targets. Systemically, we reported increased expression of the inhibitory receptor CLTA-4 on T_h cells. The diminution of CTLA-4 expression seems to be a predictive survival factor following DC vaccination [131]. This upregulated CTLA-4 might be a potent therapeutic target with ipilimumab as demonstrated for patients with melanoma [132, 133]. However, in contrast to other reports [134, 135], we found no significant difference in the proportions of $CD4^{+}CD25^{high}Foxp3^{+}$ T regulatory cells between the patients' and donors' peripheral blood. As Fecci et al. demonstrated that the increased Treg fraction was patient dependent, and as we tested a small sample size it could explain why we did not obtain similar results [135].

We detected the presence of $CD8^+CD28^-Foxp3^+$ T_{regs} in 60% of the tested GBM biopsies. This is a novel finding and may explain the modest correlation of $CD8^+$ infiltrates with patients' survival. This T_{reg} subpopulation is considered to be induced by cancer at the tumour site and it induces IL-10 - mediated immunotolerization of antigen presenting cells by down-regulating the expression of co-stimulatory molecules CD40, CD80 and CD86, and up-regulating the expression of inhibitory receptors ILT2,3,4 [136, 137]. Thus we phenotyped the tumour infiltrating macrophages/microglia and we could demonstrate the presence of all above mentioned features of $CD8^+CD28^-Foxp3^+$ T_{regs} – induced immunotolerization. Analysis of the plasma further demonstrated elevated levels of IL-10 in the patients' plasma compared to controls. We observed a substantial expression of CD39 and CD73 ectonucleotidases within the tumour microenvironment. These enzymes catalyse the degradation of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine that at elevated extracellular level suppresses T cells [138, 139]. Interestingly, CD73 was expressed almost exclusively on tumour cells while CD39 expression was restricted to tumour infiltrating immune cells, suggesting that GBM might dynamically adapt to its immune microenvironment. Down-regulation of the expression of classical MHC class I molecules in order to avoid CTLs – mediated cytotoxicity and expression of the non-classical MHC class I molecules, HLA-E and HLA-G, to escape NK cell – mediated lysis are frequently observed immune escape mechanisms in cancer [140-142]. However, inverse results were obtained in our study. Almost all the tumour cells within GBM biopsies expressed HLA-ABC that probably protects them from endogenous NK cells, while they were negative for HLA-E and HLA-G. On the other hand, a proportion of tumour cells might be susceptible to anti-tumour immune responses due to the expression of Fas receptor that, upon binding its ligand expressed on NK cells and CTLs induces cell apoptosis. The tumour cells also expressed MICA that is a ligand for the NKG2D activating receptor that is expressed on NK cells and CTLs. However, the limitation of this study is the small number of patients, especially those available for flow cytometry – based phenotyping. Moreover,

the conclusions made based on the cells' phenotype would need to be supported by functional data to be more robust.

The beneficial effect of immune cell infiltration into the tumour site and potential susceptibility of some GBM cells to immune – mediated destruction provides a rationale for developing immunotherapy for GBM. One of the promising strategies is the adoptive transfer of cytotoxic lymphocytes: CTLs and/or NK cells. However, as reviewed in paper II, little is known about NK cell infiltration into the brain tumours, their functionality and prognostic significance. In spite of that, emerging functional studies *in vitro* and *in vivo* demonstrate the anti-tumour potential of NK cells against GBM. Moreover, a number of clinical trials tested the treatment of GBM patients with autologous LAK cells [116] that are composed of a mixture of effector cells (mostly CTLs, T_h cells and NK cells) derived from patients' peripheral blood lymphocytes. One trial used pure autologous NK cells [117]. The use of autologous LAK cells or NK cells demonstrated partial efficacy and moderate adverse effects (mostly oedema, self-limiting fever, and headache) [116, 117].

Based on our finding that GBM cells express classical MHC class I molecules recognised by inhibitory KIR receptors of endogenous NK cells, we hypothesised that donor derived, allogeneic NK cells might be more effective against GBM than autologous cells. Allogeneic NK cells have been demonstrated to mediate graft vs. leukemia effect (GvL) in leukemia patients receiving hematopoietic stem cell transplantation (HSCT) due to the receptor –ligand mismatch that occurs between KIR receptors expressed on donor's NK cells and MHC class I ligands expressed on the recipients' cells [98]. In our study (paper III) we investigated *in vitro* if the same mechanism might determine NK cell potency against cancer cells derived from patients' GBM that is a solid tumour. First, we demonstrated that purified and *ex vivo* cultured (activated) NK cells were phenotypically $CD56^{bright}CD16^{bright}$ and were more cytotoxic than freshly isolated (resting) NK cells that were of predominantly $CD56^{dim}$ $CD16^{+}$ subtypes or LAK cells obtained from the same donor. Furthermore, we observed that NK cells obtained from donors possessing activating receptors'

KIR2DS2 and KIR2DS4 genes were more efficient against GBM cells. Interestingly, this donor-dependency was at least, partially independent from the degree of inhibitory KIR – HLA mismatch. However, the ligands for several KIRs, remain unknown, thus it was not possible to assess to potential role of those receptors in the efficiency of NK cell – mediated killing of GBM cells. Also the ligands for KIR2DS2 have not been identified yet, therefore, the role of this receptor in our study remains speculative. The strong point of our study is the use of GBM patients' derived material instead of established cell lines, as it resembles the original tumour's heterogeneity and tumour cells' properties. However, it would be of great advantage to obtain low-passaged GBM cells from more patients with different HLA class I allelic variants. Another limiting factor was that KIR2DS2 and KIR2DS4 receptors were expressed on less than half of the NK cells within each donor positive for KIR2DS2 and KIR2DS4 genes. This may at least partially explain lack of the robust effect when blocking antibodies were used in cytotoxicity assays. It would be of potential interest to develop a method enabling purification of KIR2DS2 and KIR2DS4 positive NK cells and then compare their potency with NK cells missing those receptors.

The results obtained with *in vitro* experiments (paper III) were confirmed *in vivo*, when we treated human GBM – bearing mice with human NK cells obtained from two donors with different KIR gene repertoire (paper IV). Although the benefit in survival was not statistically significant when comparing animals treated with single doses of donor A's and donor B's NK cells, we observed greater biological effect within mice receiving donor B's NK cells, that possessed all inhibitory KIR genes and activating KIR2DS2 and KIR2DS4 genes, in contrast to donor A that lacked KIR2DS2, KIR2DS4, KIR2DL2, KIR3DL1. Treatment with single dose of 10^6 NK cells of each donor resulted in significantly prolonged animals' survival compared with controls, while double doses of 10^6 NK cells with one week interval appeared to be intolerable. The critical point of the assessment of treatment efficiency in our study might be that the primary end points did not include radiological response. However, the evaluation of clinical response with use of MRI imaging has been questioned lately, as approximately 20-30% of patients display pseudoprogression resulting from local

treatment and/or radiation-induced necrosis [143]. Another issue could be the use of immunodeficient animals, thus it does not fully address all the possible interactions between the host's and the grafted immune cells that may have a substantial impact on the treatment efficacy and overall outcome. Interestingly, we observed increased recruitment of endogenous microglia and macrophages in all treated animals compared to controls, but in mice treated with single dose NK cells the greater proportion of microglia expressed IL-7 receptor (CD127) that has been associated with inflammation [144, 145]. Mice treated with single dose NK cells also had larger proportions of macrophages that expressed CD40 receptors that co-stimulate NK cells via interaction with its ligand, CD154 [146]. Moreover, phenotyping of the tumour cells showed that GBM cells from animals treated with single dose NK cells up-regulated the expression of HLA-ABC and intermediate filament nestin that could be related to on-going inflammation [147-149]. A recent study conducted in our group demonstrated that the therapeutic effect of NK cells *in vivo* is mediated by IFNγ secreted by NK cells that induces inflammatory immune response and transformation of microglia from tumour-supportive M2 type into anti-tumour M1 type [150]. We speculate similar mechanism may contribute both to beneficial effect of single dose treatment and toxic effect of double dose treatments, however, further studies are required to confirm this hypothesis.

Activating KIR receptors often share the same ligands with their inhibitory homologues, however, they bind HLA molecules with lower affinity and their physiological function remains elusive [81]. Thus, most studies investigating the role of KIRs in the context of cancer were focused mainly on the inhibitory KIRs – HLA ligands interactions. Nevertheless, our *in vitro* (paper III) and *in vivo* (paper IV) studies suggest that the activating KIRs, especially KIR2DS2 and KIR2DS4, may be also important for the efficiency of NK cell-mediated anti-tumour effect. Moreover, in the perspective of the development of allogeneic NK cell-based therapy, the repertoire of activating KIR genes might help to identify most suitable NK cell donors. However, further research is needed for better understanding the role of these receptors and underlying mechanisms. Furthermore, more pre-clinical studies are required to

evaluate the effectiveness and safety of intracranial transfer of allogeneic NK cells as GBM treatment.

6. Concluding remarks

In summary, the findings reported in paper I confirm the prognostic significance of T cell infiltration in GBM and suggest that a proportion of tumour cells might be susceptible to anti-tumour immune response due to Fas and MICA expression. These observations provide a rationale for further efforts to develop immunotherapy for GBM patients. One of the potential targets would be the inhibitory receptor CTLA-4 overexpressed in the peripheral Th cells of GBM patients. The most potent cellular effectors would be CTLs and NK cells. However, we showed that the tumour microenvironment is highly immunosuppressive towards T cells as manifested by the expression of CD39 and CD73 ectonucleotidases. Moreover, we suggest that $CD8^{+}CD28^{-}Foxp3^{+}$ T_{regs} suppress tumour infiltrating APCs that are required for T cell stimulation. Therefore, NK cells seemed to be better candidates for cellular therapy against GBM due to their high capacity of recognising and killing the tumour cells without prior sensitization or co-stimulation.

The review of literature (paper II) revealed a paucity of studies investigating NK cells in the context of brain tumour. Especially, reports describing tumour infiltrating NK cells need to be revised pending use of state-of-the art methods. However, recent functional studies demonstrated that employing NK cells holds a promising strategy against GBM.

As we demonstrated that GBM cells highly express classical MHC class I molecules that may protect them from NK cell mediated lysis, we hypothesised, that allogeneic NK cells with KIR – HLA mismatch would be more efficient than autologous NK cells. On the contrary, our *in vitro* and *in vivo* studies demonstrated that donor-dependent cytotoxicity of NK cells against GBM might be mediated by activating KIR – HLA interactions. This effect was partially independent of the inhibitory KIR – HLA mismatch. Particularly, the presence of KIR2DS2 and KIR2DS4 genes seemed to correlate with increased cytotoxicity *in vitro*, prolonged survival and better outcome *in vivo*. However, the efficacy of NK cell based cellular therapy should be further

evaluated *in vivo* and the safety and tolerable dose should be carefully assessed, as increased dose may result in toxic effect we could observe in our *in vivo* study. This future avenue requires carefully designed animal models however, that can accommodate engraftment of biologically relevant, patient derived tumours and adoptive transfer of human allogeneic NK cells. Nevertheless, our research demonstrates a great potential to be harnessed in using activated NK cells against GBM.

Summarizing, this research adds novel findings within brain tumour immunology field, like the presence of $CD8^{+}CD28^{-}Foxp3^{+}$ T_{regs} within the tumour environment. The results of this study provide also a rationale for developing an immunotherapy against GBM, especially with use of pure and activated NK cells – an approach that has been poorly explored so far, with only two in vitro studies [86, 111] and one clinical trial [117]. Moreover, the suggested role of activating KIR receptors in NK cell – mediated cytotoxicity against GBM provides novel insight into anti-tumour immunity, as those receptors might have been unappreciated so far.

7. Future perspectives

The research findings described above provide the basis and a solid rationale for further research within the brain tumour immunology field.

The presence of $CD8^+CD28^-Foxp3^+$ T_{regs} in the GBM tumour microenvironment and their potential role in GBM-mediated immunosuppression deserves further functional study to answer several questions: (1) what factors mediate the differentiation of T cells into $CD8^+CD28^-Foxp3^+$ T_{regs}, (2) does this population contribute to APCs inhibition via mechanisms described before and (3) can any point of this mechanism serve as a target for adjuvant therapy? The potential of targeting CTLA-4 in GBM patients should also be explored.

A growing amount of data is accruing regarding the phenotype of T cells, macrophages and microglia associated with brain tumour and these immune cell populations are continuously investigated in terms of their functionality and prognostic significance. However, little is known about the role of NK cells in brain tumour immunity. As emerging studies demonstrate the potential of harnessing NK cells' cytotoxic potential against brain tumours both by adoptive LAK or NK cell transfer as well as via the immune stimulatory treatment, it is of great importance to investigate the NK cell status of GBM patients: (1) to determine whether they infiltrate the brain tumour (2) to investigate their prognostic significance in brain tumour patients (3) to study the functionality of GBM patients' peripheral NK cells.

Our *in vitro* and *in vivo* studies investigating the role of KIR – HLA interactions in determining NK cell potency against GBM demonstrated the potential of NK cells as anti-GBM agent and added a novel insight in the fields of NK cell biology and brain tumour immunotherapy that should be further explored. Intense research aimed at resolving the detailed mechanism of NK cell – mediated anti-tumour effect in the context of GBM may result in the development of potent adjuvant immunotherapy for GBM patients. However, translating these promising results to the clinic needs careful

pre-clinical evaluation of safety and efficacy in the context of competent host immune system and developing optimal good manufacturing practise (GMP) methods for obtaining clinical grade, functional NK cells in high abundance. The latter has been the rate-limiting step in translating NK cell based treatments to the clinic. Confirming the role of activating KIR2DS2 and KIR2DS4 receptors in the efficiency of NK cell – mediated cytotoxicity is paramount as this may prove to be a biomarker for identifying the most potent NK cell donors and amenable GBM patient recipients with the corresponding ligands. Moreover, several other aspects need to be considered in developing allogeneic NK cell – based treatment: (1) route of administration: systemic or intratumoural (2) patients' immunosuppressive pre-conditioning (3) haploidentical matching of donors and recipients. Miller et al. demonstrated that systemically injected allogeneic NK cells obtained from haploidentical related donor can expand *in vivo* in acute myeloid leukemia (AML) patients and this treatment was well tolerated [151]. However, successful treatment with allogeneic NK cells administered systemically required immunosuppressive pre-conditioning in order to avoid host immune system reaction. Thus, injection of NK cells directly into the tumour site should be considered as an alternative for solid tumours, especially for brain cancer patients, as despite disrupted BBB, the access of immune cells into the tumour is limited. Furthermore, additional immunosuppressive preparation to facilitate successful allogeneic NK cell transfer should take into consideration the fact, that GBM patients are already immunocompromised due to the glioma-driven immunosuppression and treatment received (steroids, chemotherapy and TMZ). Finally, development of allogeneic NK cell – based therapy for brain tumour may require trans-disciplinary collaboration of specialists within the fields of neuro-oncology, haematology and transplantation.

REFERENCES

1. Steliarova-Foucher E, O.C.M., Ferlay J, Masuyer E, Forman D, Comber H, Bray F. *European Cancer Observatory: Cancer Incidence, Mortality, Prevalence and Survival in Europe. Version 1.0*. 2012.
2. Vivier, E., et al., *Targeting natural killer cells and natural killer T cells in cancer.* Nat Rev Immunol, 2012. **12**(4): p. 239-52.
3. Wilson, E.H., W. Weninger, and C.A. Hunter, *Trafficking of immune cells in the central nervous system.* J Clin Invest, 2010. **120**(5): p. 1368-79.
4. Ferlay J, S.H., Bray F, Forman D, Mathers C and Parkin DM, *GLOBOCAN 2008 v2.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]*. 2010, International Agency for Research on Cancer: Lyon, France.
5. Lozano, R., et al., *Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010.* Lancet, 2012. **380**(9859): p. 2095-128.
6. Ferlay, J., et al., *Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012.* Eur J Cancer, 2013. **49**(6): p. 1374-403.
7. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
8. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
9. *Brain Tumors*. 2005, New Jersey: Humana Press.
10. McKinney, P.A., *Brain tumours: incidence, survival, and aetiology.* J Neurol Neurosurg Psychiatry, 2004. **75 Suppl 2**: p. ii12-7.
11. *Pathology and Genetics of Tumours of the Nervous System*. 2000, Lyon: IARC.
12. Louis, D.N., et al., *The 2007 WHO classification of tumours of the central nervous system.* Acta neuropathologica, 2007. **114**(2): p. 97-109.
13. Ohgaki, H., *Epidemiology of brain tumors.* Methods Mol Biol, 2009. **472**: p. 323-42.
14. Agnihotri, S., et al., *Glioblastoma, a brief review of history, molecular genetics, animal models and novel therapeutic strategies.* Arch Immunol Ther Exp (Warsz), 2013. **61**(1): p. 25-41.
15. Rees, J.H., *Diagnosis and treatment in neuro-oncology: an oncological perspective.* Br J Radiol, 2011. **84 Spec No 2**: p. S82-9.
16. van Breemen, M.S., E.B. Wilms, and C.J. Vecht, *Epilepsy in patients with brain tumours: epidemiology, mechanisms, and management.* Lancet Neurol, 2007. **6**(5): p. 421-30.
17. Brada, M., et al., *Phase II study of primary temozolomide chemotherapy in patients with WHO grade II gliomas.* Ann Oncol, 2003. **14**(12): p. 1715-21.
18. de Groot, J. and H. Sontheimer, *Glutamate and the biology of gliomas.* Glia, 2011. **59**(8): p. 1181-9.
19. de Groot, M., et al., *Epilepsy in patients with a brain tumour: focal epilepsy requires focused treatment.* Brain, 2012. **135**(Pt 4): p. 1002-16.
20. Tien, R.D., et al., *Single-voxel proton brain spectroscopy exam (PROBE/SV) in patients with primary brain tumors.* AJR Am J Roentgenol, 1996. **167**(1): p. 201-9.
21. Kloosterhof, N.K., et al., *Isocitrate dehydrogenase-1 mutations: a fundamentally new understanding of diffuse glioma?* Lancet Oncol, 2011. **12**(1): p. 83-91.

22. Maehama, T. and J.E. Dixon, *The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate.* J Biol Chem, 1998. **273**(22): p. 13375-8.
23. Krakstad, C. and M. Chekenya, *Survival signalling and apoptosis resistance in glioblastomas: opportunities for targeted therapeutics.* Mol Cancer, 2010. **9**: p. 135.
24. Folkman, J., *Tumor Angiogenesis: Therapeutic Implications.* New England Journal of Medicine, 1971. **285**(21): p. 1182-1186.
25. Tate, M.C. and M.K. Aghi, *Biology of angiogenesis and invasion in glioma.* Neurotherapeutics, 2009. **6**(3): p. 447-57.
26. Connolly, D.T., et al., *Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis.* The Journal of Clinical Investigation, 1989. **84**(5): p. 1470-1478.
27. Leung, D.W., et al., *Vascular Endothelial Growth Factor Is a Secreted Angiogenic Mitogen.* Science, 1989. **246**(4935): p. 1306.
28. Hanahan, D. and J. Folkman, *Patterns and Emerging Mechanisms of the Angiogenic Switch during Tumorigenesis.* Cell, 1996. **86**(3): p. 353-364.
29. Karnofsky, D.A., et al., *The use of the nitrogen mustards in the palliative treatment of carcinoma. With particular reference to bronchogenic carcinoma.* Cancer, 1948. **1**(4): p. 634-656.
30. Johnson, D.R. and S.M. Chang, *Recent medical management of glioblastoma.* Adv Exp Med Biol, 2012. **746**: p. 26-40.
31. Stupp, R., et al., *Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma.* The New England journal of medicine, 2005. **352**(10): p. 987-96.
32. Stupp, R., et al., *High-grade malignant glioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Ann Oncol, 2010. **21 Suppl 5**: p. v190-3.
33. Hegi, M.E., et al., *MGMT gene silencing and benefit from temozolomide in glioblastoma.* N Engl J Med, 2005. **352**(10): p. 997-1003.
34. Ransohoff, R.M. and B. Engelhardt, *The anatomical and cellular basis of immune surveillance in the central nervous system.* Nat Rev Immunol, 2012. **12**(9): p. 623-35.
35. Ousman, S.S. and P. Kubes, *Immune surveillance in the central nervous system.* Nat Neurosci, 2012. **15**(8): p. 1096-101.
36. Engelhardt, B. and R.M. Ransohoff, *Capture, crawl, cross: the T cell code to breach the blood-brain barriers.* Trends Immunol, 2012. **33**(12): p. 579-89.
37. Redzic, Z., *Molecular biology of the blood-brain and the blood-cerebrospinal fluid barriers: similarities and differences.* Fluids Barriers CNS, 2011. **8**(1): p. 3.
38. Deeken, J.F. and W. Loscher, *The blood-brain barrier and cancer: transporters, treatment, and Trojan horses.* Clin Cancer Res, 2007. **13**(6): p. 1663-74.
39. de Graaf, M.T., et al., *Central memory CD4+ T cells dominate the normal cerebrospinal fluid.* Cytometry B Clin Cytom, 2011. **80**(1): p. 43-50.
40. Ransohoff, R.M. and M.A. Brown, *Innate immunity in the central nervous system.* J Clin Invest, 2012. **122**(4): p. 1164-71.
41. Ransohoff, R.M. and A.E. Cardona, *The myeloid cells of the central nervous system parenchyma.* Nature, 2010. **468**(7321): p. 253-62.
42. Kim, R., M. Emi, and K. Tanabe, *Cancer immunoediting from immune surveillance to immune escape.* Immunology, 2007. **121**(1): p. 1-14.
43. Pellegatta, S., L. Cuppini, and G. Finocchiaro, *Brain cancer immunoediting: novel examples provided by immunotherapy of malignant gliomas.* Expert Rev Anticancer Ther, 2011. **11**(11): p. 1759-74.

44. Giometto, B., et al., *Immune infiltrates and cytokines in gliomas.* Acta Neurochir (Wien), 1996. **138**(1): p. 50-6.
45. Li, W. and M.B. Graeber, *The molecular profile of microglia under the influence of glioma.* Neuro Oncol, 2012. **14**(8): p. 958-78.
46. Watters, J.J., J.M. Schartner, and B. Badie, *Microglia function in brain tumors.* J Neurosci Res, 2005. **81**(3): p. 447-55.
47. Kofler, J. and C.A. Wiley, *Microglia: key innate immune cells of the brain.* Toxicol Pathol, 2011. **39**(1): p. 103-14.
48. Durafourt, B.A., et al., *Comparison of polarization properties of human adult microglia and blood-derived macrophages.* Glia, 2012. **60**(5): p. 717-27.
49. Yang, I., et al., *CD8+ T-cell infiltrate in newly diagnosed glioblastoma is associated with long-term survival.* J Clin Neurosci, 2010. **17**(11): p. 1381-5.
50. Kim, Y.H., et al., *Tumour-infiltrating T-cell subpopulations in glioblastomas.* Br J Neurosurg, 2012. **26**(1): p. 21-7.
51. Rossi, M.L., et al., *Immunohistological study of mononuclear cell infiltrate in malignant gliomas.* Acta Neuropathol, 1987. **74**(3): p. 269-77.
52. Waziri, A., et al., *Preferential in situ CD4+CD56+ T cell activation and expansion within human glioblastoma.* J Immunol, 2008. **180**(11): p. 7673-80.
53. Lohr, J., et al., *Effector T-cell infiltration positively impacts survival of glioblastoma patients and is impaired by tumor-derived TGF-beta.* Clin Cancer Res, 2011. **17**(13): p. 4296-308.
54. Dunn, G.P., I.F. Dunn, and W.T. Curry, *Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human glioma.* Cancer Immun, 2007. **7**: p. 12.
55. Kuppner, M.C., M.F. Hamou, and N. de Tribolet, *Immunohistological and functional analyses of lymphoid infiltrates in human glioblastomas.* Cancer Res, 1988. **48**(23): p. 6926-32.
56. Sawamura, Y., et al., *Antitumor activity and surface phenotypes of human glioma-infiltrating lymphocytes after in vitro expansion in the presence of interleukin 2.* Cancer Res, 1989. **49**(7): p. 1843-9.
57. Barcia, C., Jr., et al., *Infiltrating CTLs in human glioblastoma establish immunological synapses with tumorigenic cells.* Am J Pathol, 2009. **175**(2): p. 786-98.
58. Fecci, P.E., et al., *Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma.* Cancer research, 2006. **66**(6): p. 3294-302.
59. Heimberger, A.B., et al., *Incidence and prognostic impact of FoxP3+ regulatory T cells in human gliomas.* Clinical cancer research : an official journal of the American Association for Cancer Research, 2008. **14**(16): p. 5166-72.
60. Jacobs, J.F., et al., *Prognostic significance and mechanism of Treg infiltration in human brain tumors.* Journal of neuroimmunology, 2010. **225**(1-2): p. 195-9.
61. Stevens, A., I. Kloter, and W. Roggendorf, *Inflammatory infiltrates and natural killer cell presence in human brain tumors.* Cancer, 1988. **61**(4): p. 738-43.
62. Yang, I., et al., *Immune cell infiltrate differences in pilocytic astrocytoma and glioblastoma: evidence of distinct immunological microenvironments that reflect tumor biology.* J Neurosurg, 2011. **115**(3): p. 505-11.
63. Dix, A.R., et al., *Immune defects observed in patients with primary malignant brain tumors.* J Neuroimmunol, 1999. **100**(1-2): p. 216-32.
64. Servadei, F., et al., *Particular features of cell-mediated immunity in patients with anaplastic gliomas. A comparison with kidney and bladder cancer patients.* J Neurooncol, 1983. **1**(4): p. 327-32.

65. Roitt, I., J. Brostoff, and D. Male, *Immunology*. 1998, London: Mosby International Ltd.
66. Gomez, G.G. and C.A. Kruse, *Mechanisms of malignant glioma immune resistance and sources of immunosuppression.* Gene Ther Mol Biol, 2006. **10**(A): p. 133-146.
67. Crane, C.A., et al., *TGF-beta downregulates the activating receptor NKG2D on NK cells and CD8+ T cells in glioma patients.* Neuro Oncol, 2010. **12**(1): p. 7-13.
68. Dietrich, J., et al., *Corticosteroids in brain cancer patients: benefits and pitfalls.* Expert Rev Clin Pharmacol, 2011. **4**(2): p. 233-42.
69. Hughes, M.A., et al., *Primary brain tumors treated with steroids and radiotherapy: low CD4 counts and risk of infection.* Int J Radiat Oncol Biol Phys, 2005. **62**(5): p. 1423-6.
70. Fadul, C.E., et al., *Immune modulation effects of concomitant temozolomide and radiation therapy on peripheral blood mononuclear cells in patients with glioblastoma multiforme.* Neuro Oncol, 2011. **13**(4): p. 393-400.
71. Vivier, E., et al., *Innate or adaptive immunity? The example of natural killer cells.* Science, 2011. **331**(6013): p. 44-9.
72. Walzer, T., et al., *Natural killer cells: from CD3(-)NKp46(+) to post-genomics meta-analyses.* Curr Opin Immunol, 2007. **19**(3): p. 365-72.
73. Cooper, M.A., T.A. Fehniger, and M.A. Caligiuri, *The biology of human natural killer-cell subsets.* Trends Immunol, 2001. **22**(11): p. 633-40.
74. Fan, Y.Y., B.Y. Yang, and C.Y. Wu, *Phenotypically and functionally distinct subsets of natural killer cells in human PBMCs.* Cell Biol Int, 2008. **32**(2): p. 188-97.
75. Campbell, K.S. and A.K. Purdy, *Structure/function of human killer cell immunoglobulin-like receptors: lessons from polymorphisms, evolution, crystal structures and mutations.* Immunology, 2011. **132**(3): p. 315-25.
76. Thielens, A., E. Vivier, and F. Romagne, *NK cell MHC class I specific receptors (KIR): from biology to clinical intervention.* Curr Opin Immunol, 2012. **24**(2): p. 239-45.
77. Dohring, C. and M. Colonna, *Human natural killer cell inhibitory receptors bind to HLA class I molecules.* Eur J Immunol, 1996. **26**(2): p. 365-9.
78. Dohring, C., et al., *A human killer inhibitory receptor specific for HLA-A1,2.* J Immunol, 1996. **156**(9): p. 3098-101.
79. Colonna, M., *Specificity and function of immunoglobulin superfamily NK cell inhibitory and stimulatory receptors.* Immunol Rev, 1997. **155**: p. 127-33.
80. Rajagopalan, S., *Endosomal signaling and a novel pathway defined by the natural killer receptor KIR2DL4 (CD158d).* Traffic, 2010. **11**(11): p. 1381-90.
81. Lopez-Botet, M., et al., *Paired inhibitory and triggering NK cell receptors for HLA class I molecules.* Hum Immunol, 2000. **61**(1): p. 7-17.
82. Hsu, K.C., et al., *The killer cell immunoglobulin-like receptor (KIR) genomic region: gene-order, haplotypes and allelic polymorphism.* Immunol Rev, 2002. **190**: p. 40-52.
83. Koch, J., et al., *Activating natural cytotoxicity receptors of natural killer cells in cancer and infection.* Trends Immunol, 2013. **34**(4): p. 182-91.
84. Brandt, C.S., et al., *The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans.* J Exp Med, 2009. **206**(7): p. 1495-503.
85. Wu, A., et al., *Expression of MHC I and NK ligands on human CD133+ glioma cells: possible targets of immunotherapy.* J Neurooncol, 2007. **83**(2): p. 121-31.
86. Castriconi, R., et al., *NK cells recognize and kill human glioblastoma cells with stem cell-like properties.* J Immunol, 2009. **182**(6): p. 3530-9.

87. Borrego, F., et al., *The CD94/NKG2 family of receptors: from molecules and cells to clinical relevance.* Immunol Res, 2006. **35**(3): p. 263-78.
88. Takao, S., et al., *The rapid induction of HLA-E is essential for the survival of antigen-activated naive CD4 T cells from attack by NK cells.* J Immunol, 2010. **185**(10): p. 6031-40.
89. Moretta, A., et al., *Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis.* Annu Rev Immunol, 2001. **19**: p. 197-223.
90. Bottino, C., et al., *Cellular ligands of activating NK receptors.* Trends Immunol, 2005. **26**(4): p. 221-6.
91. Vivier, E., J.A. Nunes, and F. Vely, *Natural killer cell signaling pathways.* Science, 2004. **306**(5701): p. 1517-9.
92. Chiesa, S., et al., *Coordination of activating and inhibitory signals in natural killer cells.* Mol Immunol, 2005. **42**(4): p. 477-84.
93. Smyth, M.J., et al., *New aspects of natural-killer-cell surveillance and therapy of cancer.* Nat Rev Cancer, 2002. **2**(11): p. 850-61.
94. Topham, N.J. and E.W. Hewitt, *Natural killer cell cytotoxicity: how do they pull the trigger?* Immunology, 2009. **128**(1): p. 7-15.
95. Lieberman, J., *The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal.* Nat Rev Immunol, 2003. **3**(5): p. 361-70.
96. Orr, M.T. and L.L. Lanier, *Natural killer cell education and tolerance.* Cell, 2010. **142**(6): p. 847-56.
97. Waldhauer, I. and A. Steinle, *NK cells and cancer immunosurveillance.* Oncogene, 2008. **27**(45): p. 5932-43.
98. Ruggeri, L., et al., *Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants.* Science, 2002. **295**(5562): p. 2097-100.
99. Hsia, J.Y., et al., *Prognostic significance of intratumoral natural killer cells in primary resected esophageal squamous cell carcinoma.* Chang Gung Med J, 2005. **28**(5): p. 335-40.
100. Ishigami, S., et al., *Prognostic value of intratumoral natural killer cells in gastric carcinoma.* Cancer, 2000. **88**(3): p. 577-83.
101. Kondo, E., et al., *Preoperative natural killer cell activity as a prognostic factor for distant metastasis following surgery for colon cancer.* Dig Surg, 2003. **20**(5): p. 445-51.
102. Plonquet, A., et al., *Peripheral blood natural killer cell count is associated with clinical outcome in patients with aaIPI 2-3 diffuse large B-cell lymphoma.* Ann Oncol, 2007. **18**(7): p. 1209-15.
103. Villegas, F.R., et al., *Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer.* Lung Cancer, 2002. **35**(1): p. 23-8.
104. Ljunggren, H.G. and K.J. Malmberg, *Prospects for the use of NK cells in immunotherapy of human cancer.* Nat Rev Immunol, 2007. **7**(5): p. 329-39.
105. Gyurkocza, B., A. Rezvani, and R.F. Storb, *Allogeneic hematopoietic cell transplantation: the state of the art.* Expert Rev Hematol, 2010. **3**(3): p. 285-99.
106. Sato, N., et al., *Downregulation of indoleamine-2,3-dioxygenase in cervical cancer cells suppresses tumor growth by promoting natural killer cell accumulation.* Oncol Rep, 2012. **28**(5): p. 1574-8.
107. Roberti, M.P., et al., *IL-2- or IL-15-activated NK cells enhance Cetuximab-mediated activity against triple-negative breast cancer in xenografts and in breast cancer patients.* Breast Cancer Res Treat, 2012. **136**(3): p. 659-71.

108. Kohrt, H.E., et al., *Stimulation of natural killer cells with a CD137-specific antibody enhances trastuzumab efficacy in xenotransplant models of breast cancer.* J Clin Invest, 2012. **122**(3): p. 1066-75.
109. Jachimowicz, R.D., et al., *Induction of in vitro and in vivo NK cell cytotoxicity using high-avidity immunoligands targeting prostate-specific membrane antigen in prostate carcinoma.* Mol Cancer Ther, 2011. **10**(6): p. 1036-45.
110. von Strandmann, E.P., et al., *A novel bispecific protein (ULBP2-BB4) targeting the NKG2D receptor on natural killer (NK) cells and CD138 activates NK cells and has potent antitumor activity against human multiple myeloma in vitro and in vivo.* Blood, 2006. **107**(5): p. 1955-62.
111. Avril, T., et al., *Human glioblastoma stem-like cells are more sensitive to allogeneic NK and T cell-mediated killing compared with serum-cultured glioblastoma cells.* Brain Pathol, 2012. **22**(2): p. 159-74.
112. Alizadeh, D., et al., *Induction of anti-glioma natural killer cell response following multiple low-dose intracerebral CpG therapy.* Clin Cancer Res, 2010. **16**(13): p. 3399-408.
113. Marcenaro, E., et al., *NK/DC crosstalk in anti-viral response.* Adv Exp Med Biol, 2012. **946**: p. 295-308.
114. Pellegatta, S., et al., *The natural killer cell response and tumor debulking are associated with prolonged survival in recurrent glioblastoma patients receiving dendritic cells loaded with autologous tumor lysates.* Oncoimmunology, 2013. **2**(3): p. e23401.
115. Alvarez-Breckenridge, C.A., et al., *NK cells impede glioblastoma virotherapy through NKp30 and NKp46 natural cytotoxicity receptors.* Nat Med, 2012. **18**(12): p. 1827-34.
116. Ishikawa, E., et al., *Adoptive cell transfer therapy for malignant gliomas.* Adv Exp Med Biol, 2012. **746**: p. 109-20.
117. Ishikawa, E., et al., *Autologous natural killer cell therapy for human recurrent malignant glioma.* Anticancer Res, 2004. **24**(3b): p. 1861-71.
118. Shacklett, B.L., *Beyond 51Cr release: New methods for assessing HIV-1-specific CD8+ T cell responses in peripheral blood and mucosal tissues.* Clin Exp Immunol, 2002. **130**(2): p. 172-82.
119. Wang, J., et al., *A reproducible brain tumour model established from human glioblastoma biopsies.* BMC Cancer, 2009. **9**: p. 465.
120. Jin, K., et al., *Patient-derived human tumour tissue xenografts in immunodeficient mice: a systematic review.* Clin Transl Oncol, 2010. **12**(7): p. 473-80.
121. Shultz, L.D., et al., *Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice.* J Immunol, 1995. **154**(1): p. 180-91.
122. Greiner, D.L., R.A. Hesselton, and L.D. Shultz, *SCID mouse models of human stem cell engraftment.* Stem Cells, 1998. **16**(3): p. 166-77.
123. Driver, J.P., D.V. Serreze, and Y.G. Chen, *Mouse models for the study of autoimmune type 1 diabetes: a NOD to similarities and differences to human disease.* Semin Immunopathol, 2011. **33**(1): p. 67-87.
124. Herbert, K.E., et al., *The use of experimental murine models to assess novel agents of hematopoietic stem and progenitor cell mobilization.* Biol Blood Marrow Transplant, 2008. **14**(6): p. 603-21.
125. Zhang, B., Z. Duan, and Y. Zhao, *Mouse models with human immunity and their application in biomedical research.* J Cell Mol Med, 2009. **13**(6): p. 1043-58.

126. Wang, J., et al., *Targeting the NG2/CSPG4 proteoglycan retards tumour growth and angiogenesis in preclinical models of GBM and melanoma.* PLoS One, 2011. **6**(7): p. e23062.
127. Jacobsen, H.K., et al., *Establishment of a novel dsRed NOD/Scid mouse strain to investigate the host and tumor cell compartments.* Cancer Invest, 2013. **31**(4): p. 221-30.
128. Finocchiaro, G. and S. Pellegatta, *Immunotherapy for glioma: getting closer to the clinical arena?* Curr Opin Neurol, 2011. **24**(6): p. 641-7.
129. Vauleon, E., et al., *Overview of cellular immunotherapy for patients with glioblastoma.* Clin Dev Immunol, 2010. **2010**.
130. Yamanaka, R., *Dendritic-cell- and peptide-based vaccination strategies for glioma.* Neurosurg Rev, 2009. **32**(3): p. 265-73; discussion 273.
131. Fong, B., et al., *Monitoring of regulatory T cell frequencies and expression of CTLA-4 on T cells, before and after DC vaccination, can predict survival in GBM patients.* PLoS One, 2012. **7**(4): p. e32614.
132. Robert, C., et al., *Ipilimumab plus dacarbazine for previously untreated metastatic melanoma.* N Engl J Med, 2011. **364**(26): p. 2517-26.
133. Hodi, F.S., et al., *Improved survival with ipilimumab in patients with metastatic melanoma.* N Engl J Med, 2010. **363**(8): p. 711-23.
134. El Andaloussi, A. and M.S. Lesniak, *An increase in CD4+CD25+FOXP3+ regulatory T cells in tumor-infiltrating lymphocytes of human glioblastoma multiforme.* Neuro Oncol, 2006. **8**(3): p. 234-43.
135. Fecci, P.E., et al., *Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma.* Cancer Res, 2006. **66**(6): p. 3294-302.
136. Wang, R.F., *CD8+ regulatory T cells, their suppressive mechanisms, and regulation in cancer.* Human immunology, 2008. **69**(11): p. 811-4.
137. Chui, C.S. and D. Li, *Role of immunolglobulin-like transcript family receptors and their ligands in suppressor T-cell-induced dendritic cell tolerization.* Human immunology, 2009. **70**(9): p. 686-91.
138. Zhang, B., *CD73: a novel target for cancer immunotherapy.* Cancer research, 2010. **70**(16): p. 6407-11.
139. Hausler, S.F., et al., *Ectonucleotidases CD39 and CD73 on OvCA cells are potent adenosine-generating enzymes responsible for adenosine receptor 2A-dependent suppression of T cell function and NK cell cytotoxicity.* Cancer immunology, immunotherapy : CII, 2011. **60**(10): p. 1405-18.
140. Mittelbronn, M., et al., *Elevated HLA-E levels in human glioblastomas but not in grade I to III astrocytomas correlate with infiltrating CD8+ cells.* Journal of neuroimmunology, 2007. **189**(1-2): p. 50-8.
141. Agaugue, S., E.D. Carosella, and N. Rouas-Freiss, *Role of HLA-G in tumor escape through expansion of myeloid-derived suppressor cells and cytokinic balance in favor of Th2 versus Th1/Th17.* Blood, 2011. **117**(26): p. 7021-31.
142. Jung, Y.W., et al., *Correlation of human leukocyte antigen-G (HLA-G) expression and disease progression in epithelial ovarian cancer.* Reprod Sci, 2009. **16**(11): p. 1103-11.
143. van den Bent, M.J., et al., *End point assessment in gliomas: novel treatments limit usefulness of classical Macdonald's Criteria.* J Clin Oncol, 2009. **27**(18): p. 2905-8.
144. Fry, T.J. and C.L. Mackall, *Interleukin-7: from bench to clinic.* Blood, 2002. **99**(11): p. 3892-904.

145. Hartgring, S.A., et al., *Elevated expression of interleukin-7 receptor in inflamed joints mediates interleukin-7-induced immune activation in rheumatoid arthritis.* Arthritis Rheum, 2009. **60**(9): p. 2595-605.
146. Scott, M.J., et al., *CD40-CD154 interactions between macrophages and natural killer cells during sepsis are critical for macrophage activation and are not interferon gamma dependent.* Clin Exp Immunol, 2004. **137**(3): p. 469-77.
147. Ha, Y., et al., *Nestin and small heat shock protein expression on reactive astrocytes and endothelial cells in cerebral abscess.* Neurosci Res, 2002. **44**(2): p. 207-12.
148. Jin, J.K., et al., *Increased expression of the embryonic intermediate filament, nestin, in the brains of scrapie-infected mice.* Neurosci Lett, 2004. **367**(2): p. 254-8.
149. Reddy, P.G., et al., *Effect of recombinant fibroblast interferon and recombinant immune interferon on growth and the antigenic phenotype of multidrug-resistant human glioblastoma multiforme cells.* J Natl Cancer Inst, 1991. **83**(18): p. 1307-15.
150. Poli A, W.J., Domingues O, Brekke C, Yan T, Thorsen F, Planagumà J, McCormack E, Hentges F, Pedersen PH, Zimmer J, Enger PØ, and a Chekenya M, , *Targeting glioblastoma with NK-cells and mAb9.2.27 against NG2/CSPG4 diminishes tumor progression and prolongs surviva.* Cinical Cancer Research, 2013, *submitted*.
151. Miller, J.S., et al., *Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer.* Blood, 2005. **105**(8): p. 3051-7.